

THE IDENTIFICATION OF NOVEL SUSCEPTIBILITY GENES INVOLVED IN ANXIETY DISORDERS

by

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Declaration

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Abstract

The etiology of anxiety disorders remains incompletely understood. Clear evidence for a genetic component has been proposed; however, there is also an increasing focus on environmental factors and the interaction between these and the genetic components that may mediate (anxiety) disorder pathogenesis.

No single gene or genetic component has been explicitly identified as being involved in the development of anxiety disorders. This is most likely due to a number of reasons, which include, for example, the heterogeneity of anxiety disorders, the contribution of environmental factors and methodological limitations (e.g. small sample size) of research studies. Until now, genetic association studies usually focused on one particular psychiatric disorder at a time. However, with the difficulty in identifying susceptibility genes and/or loci in heterogeneous disorders like obsessive-compulsive disorder and other conditions in the anxiety spectrum, it is perhaps timely to consider multivariate genetics and epidemiological studies in a number of disorders sharing a core characteristic – such as anxiety. In addition to genetic underpinnings, a number of environmental variables have also been identified as risk factors for pathological anxiety, including adverse life events like childhood physical and sexual abuse.

The hypothesis for this project is that a pre-existing genetic vulnerability (or genetic risk) interacts with the impact of adverse life events to result in the development of one or more anxiety disorder(s). Considering phenotypic overlap amongst the anxiety disorders, it is likely that diverse networks of genes and/ or interacting pathways are responsible for the phenotypic manifestations observed. Sprague Dawley rats exhibiting behaviours indicative of anxiety in the context of environmental stressors (maternal separation and restraint stress) were used as model for the identification of novel susceptibility genes for anxiety disorders in humans. The striatum has previously been implicated as a candidate in the brain architecture of anxiety pathogenicity, and is also a site exhibiting a high degree of synaptic plasticity. The synaptic plasticity pathway was investigated using the dorsal striatum of the rat brain and several genes were identified to be aberrantly expressed in “anxious” rats relative to controls (*Mmp9*, *Bdnf*, *Ntf4*, *Egr2*, *Egr4*, *Grm2* and *Arc*).

In humans, it was found that the severity of early adversity was significantly and positively associated with the presence of an anxiety disorder in adulthood. When the human homologues of the susceptibility candidate genes that were identified using the animal model were screened in a human cohort of patients with obsessive-compulsive disorder (OCD), panic disorder (PD) or social anxiety disorder (SAD) (relative to controls), five single nucleotide polymorphisms (SNPs) were found to be significantly associated with these conditions. Four of these SNPs were also found to significantly interact with the severity of childhood trauma. Haplotype analysis of variants within the identified susceptibility candidates revealed novel haplotype associations, four of which are located in the *MMP9* gene. Notably, this the first study to link these particular mutations in the *MMP9* gene with anxiety disorders and this finding is consistent with previous work suggesting that *MMP9* is involved in conditions like cardiovascular disease and cancer which have been associated with increased prevalence of anxiety disorders.

In conclusion, this project yielded important findings pertaining to the etiology of anxiety disorders. The use of a combined anxiety disorders cohort (OCD, PD and SAD) may suggest that the associations found here may hold true for anxiety disorders in general and not only for a particular clinically delineated condition. Childhood trauma was confirmed as an increased susceptibility risk for anxiety disorders. Also, this research contributed several novel susceptibility genes (*MMP9*, *EGR2*, *EGR4*, *NTF4*, and *ARC*), five significant SNP associations, four significant SNP-environment interactions and five haplotype associations (within *MMP9* and *BDNF*) as candidates for anxiety pathogenicity. The identified polymorphisms and haplotypes were demonstrated to be associated with susceptibility to anxiety disorders in a gene-environment correlation and gene-environment interaction.

Opsomming

Die oorsake van angssteurings word steeds nie volledig verstaan nie. Daar is duidelike bewyse vir 'n genetiese komponent, maar daar is ook toenemende fokus op omgewingsfaktore en die interaksie tussen hierdie omgewingsfaktore en genetiese komponente by angssteurings.

Geen enkele geen of genetiese komponent is al geïdentifiseer as dié wat betrokke is by die ontwikkeling van angssteurings nie. Dit is waarskynlik weens 'n aantal redes, wat byvoorbeeld, die heterogeneïteit van angssteurings, die bydrae van omgewingsfaktore en metodologiese beperkings (bv. klein steekproef) van die navorsingstudies, insluit. Verder het genetiese assosiasiestudies tot nou toe gewoonlik net op een spesifieke psigiatriese versteuring op 'n slag gefokus. Maar, gegewe die uitdaging om vatbaarheidsgene en / of loci in heterogene steurings soos obsessief – kompulsiewe steuring (OKV) en ander toestande op die angsspektrum te identifiseer, is dit tyd om genetiese en kliniese studies in 'n aantal steurings - met 'n oorvleuende kern-element soos angs -, gesamentlik te oorweeg. Bykomend tot die genetiese boustene, is 'n aantal omgewingsveranderlikes soos traumatiese lewenservarings tydens die kinderjare as risikofaktore vir patologiese angs geïdentifiseer.

Die hipotese vir hierdie projek is dat daar 'n interaksie tussen genetiese kwesbaarheid (of genetiese risiko) en traumatiese lewenservarings is en dat dit tot die ontwikkeling van 'n / veelvoudige angssteuring(s) kan lei. Inaggenome die fenotipiese oorvleueling tussen die angssteurings, is dit waarskynlik dat diverse netwerke van gene en / of interaktiewe geen-paare vir die manifestasie van hierdie toestande verantwoordelik is. Sprague Dawley-rotte met gedragswyses aanduidend van angs, in die konteks van omgewingstressore (d.i. skeiding van die ma-rot en bedwang-stres [*restraint stress*]), is as model gebruik vir die identifisering van nuwe vatbaarheidsgene vir angssteurings in mense. Die striatum is voorheen as 'n kandidaat in die brein-argitektuur van patologiese angs voorgehou, en is ook 'n plek met 'n hoë mate van sinaptiese plastisiteit. Die sinaptiese plastisiteit is ondersoek deur te fokus op die dorsale striatum van die rotbrein en daar is verskeie gene gevind wat anders is in “angstige” rotte in vergelyking met kontroles (*Mmp9, Bdnf, Ntf4, Egr2, Egr4, Grm2* en *Arc*).

In mense is daar gevind dat die ernstigheidsgraad van vroeë trauma beduidend en positief met die teenwoordigheid van 'n angssteuring tydens volwassenheid verband hou. Toe die menslike ekwivalente van die vatbaarheidsgene wat met die dieremodel geïdentifiseer is in 'n mens-kohort met obsessief-kompulsiewe steuring (OKS), panieksteuring (PS) en sosiale angssteuring (SAS) ondersoek is, is gevind dat daar 5 enkele nukleotied polimorfismes (ENPs) is wat met die toestande verband hou. Daar is ook gevind dat vier van hierdie ENPs beduidend verband hou met die ernstigheidsgraad van trauma tydens die kinderjare. Haplotipe analise van variante binne die geïdentifiseerde vatbaarheidsgene het op nuwe haplotipe assosiasies – waarvan 4 op die *MMP9*-geen geleë is – gedui. Hierdie is dus die eerste studie wat gevind het dat dié spesifieke mutasies van die *MMP9*-geen met angssteurings verband hou. Hierdie bevinding strook met vorige werk wat daarop dui dat die *MMP9*-geen by toestande soos kardiovaskulêre siekte en kanker wat ook met verhoogde voorkoms van angssteurings verband hou, betrokke is.

Ter afsluiting kan ons sê dat hierdie projek belangrike bevindinge oor die oorsake van angssteurings gemaak het. Die gebruik van 'n gekombineerde angssteurings-kohort (OKS, PS en SAS) kan moontlik suggereer dat die assosiasies wat ons hier gevind het, waar is vir alle angssteurings en nie net vir 'n spesifieke afgebakende toestand nie. Traumatisiese ervarings tydens die kinderjare is ook bevestig as 'n risiko vir die ontwikkeling van angssteurings. Hierdie navorsing het ook verskeie nuwe vatbaarheidsgene (*MMP9*, *EGR2*, *EGR4*, *NTF4*, en *ARC*), 5 beduidende ENP assosiasies, 4 beduidende ENP-omgewings-interaksies en 5 haplotipe assosiasies (by *MMP9* en *BDNF*) geïdentifiseer as moontlike kandidate wat 'n rol speel by die ontstaan van patologiese angs. Daar is ook gevind dat die geïdentifiseerde polimorfismes en haplotipes met vatbaarheid vir angssteurings in 'n geen-omgewing- korrelasie en geen-omgewing- interaksie verband hou.

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List of Abbreviations and Symbols

%	percentage
<	less than
>	greater than
µg/dl	micrograms per deciliter
µg/l	micrograms per liter
µM	micromolar
3'	3- prime end
5'	5- prime end
A	adenine
<i>ARC</i>	activity regulated cytoskeletal protein (<i>Homo sapiens</i>)
<i>Arc</i>	activity regulated cytoskeletal protein (<i>Rattus norvegicus</i>)
<i>BDNF</i>	brain-derived neurotrophic factor (<i>Homo sapiens</i>)
<i>Bdnf</i>	brain-derived neurotrophic factor (<i>Rattus norvegicus</i>)
bp	base pair
C	cytosine
CAMS	cell adhesion molecules
CBT	cognitive behavioural therapy
chr	chromosome
CI	confidence interval

CREB	cAMP response element binding
CTQ	childhood trauma questionnaire
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSM-IV	Diagnosics and Statistical Manual of Mental Disorders IV
DSM-5	Diagnosics and Statistical Manual of Mental Disorders 5
ECM	extra-cellular matrix
ECR	evolutionary conserved regions
<i>EGR2</i>	early growth response 2 (<i>Homo sapiens</i>)
<i>Egr2</i>	early growth response 2 (<i>Rattus norvegicus</i>)
<i>EGR4</i>	early growth response 4 (<i>Homo sapiens</i>)
<i>Egr4</i>	early growth response 4 (<i>Rattus norvegicus</i>)
EPM	elevated-plus maze
EtBr	ethidium bromide
Ex	exonic primer
F	forward primer
F1	first generation
F2	second generation
FST	forced swimming test
G	gigabases
g	grams
G	guanine

GABA	gamma-aminobutyric acid
GAD	Generalised anxiety disorder
<i>GRM2</i>	glutamate receptor 2 (<i>Homo sapiens</i>)
<i>Grm2</i>	glutamate receptor 2 (<i>Rattus norvegicus</i>)
GSAD	generalized subtype of SAD
GWAS	genome-wide association study
HPA	hypothalamus-pituitary-adrenal
hr	hour
HWE	Hardy Weinberg Equilibrium
IEG	immediate-early response gene
Indel	insertion/ deletion
K ₂ EDTA	di-potassium ethylenediamine tetraacetic acid
Kb	kilobasepair
LTD	long-term depression
LTP	long-term potentiation
M	molar
MAF	minor allele frequency
MAOI(s)	monoamine oxidase inhibitor(s)
MD	minimization/ denial score
MDD	major depressive disorder
mg	milligrams
MgCl ₂	magnesium chloride

mGluRs	metabotropic glutamate receptors
min	minutes
ml	millilitres
mM	millimolar
<i>MMP9</i>	matrix metalloproteinase 9 (<i>Homo sapiens</i>)
<i>Mmp9</i>	matrix metalloproteinase 9 (<i>Rattus norvegicus</i>)
mRNA	messenger ribonucleic acid
MS	maternal separation
MS-RS	combination maternal separation-restraint stress
NaCl	sodium chloride
ng	nanogram(s)
NGF	nerve growth factor
NGS	next generation sequencing
nMS	non-maternal separation
nRS	non-restraint stress
<i>NTF4</i>	neurotrophin 4 (<i>Homo sapiens</i>)
<i>Ntf4</i>	neurotrophin 4 (<i>Rattus norvegicus</i>)
<i>NTF5</i>	neurotrophin 5 (<i>Homo sapiens</i>)
°C	degrees Celcius
OCD	obsessive-compulsive disorder
OFT	open-field test
OR	odds ratio

<i>P</i>	p-value
PCR	polymerase chain reaction
PD	Panic disorder
pH	percentage hydrogen
PND	post-natal day
PTSD	post-traumatic stress disorder
QC	quality score
QTL	quantitative trait loci
R	reverse primer
rpm	revolutions per minute
RQI	RNA quality indicator
RS	restraint stress
SAD	social anxiety disorder
SANBS	South African National Blood Services
SB	sodium tetraborate decahydrate
sec	seconds
SI	social interaction
SMART	simple modular architecture research tool
SNP(s)	single nucleotide polymorphism(s)
SP	specific phobia
SSRI(s)	serotonin reuptake inhibitor(s)
T	thymine

T_{A1}	annealing temperature1
T_{A2}	annealing temperature2
<i>Taq</i>	<i>Thermus aquaticus</i>
TCA(s)	tricyclics
T_m	melting point
tNGS	targeted next generation sequencing
TrkA/B	tyrosine kinase receptor A/ B
U	units
UTR	untranslated region
v/v	volume per volume
w/v	weight per volume
WT	wild-type
x g	times gravity
Y-BOCS	Yale-Brown OCD severity scale
Zn ₂ CH ₂	Zinc-finger domain
β	bet
μg	micrograms
$\mu\text{g/ml}$	microgram per millilitre
μl	microlitre
χ^2	chi-squared

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Finally, Mary-Anne Fortune, the best grandmother the world could ever know. Who when alive, wanted nothing more than to see this dream realized for me and whom I now hope to honor with its realization.

“Worrying is carrying tomorrow's load with today's strength- carrying two days at once. It is moving into tomorrow ahead of time. Worrying doesn't empty tomorrow of its sorrow, it empties today of its strength.”

Corrie ten Boom

Introduction

Overview

Anxiety disorders are now recognized as being of the world's most debilitating conditions, associated with substantial morbidity and impaired quality of life. Twenty years ago it was estimated that around 2 – 3 % of the world's population may be affected by one or more of these conditions (Weissman et al., 1994). More recent statistics put the life-time prevalence for any anxiety disorder at 18.1% (United States) for adults and even higher at 25.1% for adolescents (13 – 18 years) (United States) (Kessler, 2005). Two years later a meta-analysis suggested an increased prevalence of 31% in the United States (Kessler et al., 2007). In South Africa the estimated prevalence for any anxiety disorder is 15.8% with a life-time prevalence of 30.1%, which are of the highest globally (Kessler et al., 2007). Understanding the pathophysiology of these conditions is therefore paramount. Major advances have recently been made in terms of the molecular etiology of these disorders, largely within the serotonin, corticotrophin and dopamine pathways. These strides emphasize the complex nature of these conditions. Although there is a clear genetic component, no single gene or genetic abnormality has been exclusively identified as being involved in the development and/ or progression of anxiety disorders (Hettema et al., 2005). Literature has also routed a large seating in the contribution of environmental stress and the interaction that genes and the environment (specifically trauma in anxiety disorders) may play in disorder pathogenesis (Hettema et al., 2005; Van Grootheest et al., 2007; Hovens et al., 2012; Norman et al., 2013).

This dissertation focused on identifying novel susceptibility genes in anxiety disorders, arguing that the use of an animal model of behaviours suggestive of anxiety could point to susceptibility candidates for anxiety disorders in humans. The overall objective of this study was to determine whether rats subjected to early-developmental stress and exhibiting anxiety-like behaviours would show differential gene expressions in a particular pathway, and then if these differentially expressed genes would hold true as candidates in humans using a case-control association study. This investigation may support use of an animal model of anxiety-like behaviours after early-developmental stress as an effective model for the identification of novel candidate genes in humans with anxiety disorders and with a history of severe

childhood trauma. Literature has previously implicated synaptic plasticity and the ability of the body to modify neuronal circuitry in response to an event, in the pathogenesis of anxiety (Citri and Malenka, 2008; McEwen et al., 2012). This was therefore the focal pathway chosen for this dissertation, and since the striatum (comprising the caudate nucleus, putamen and nucleus accumbens) is a major site of synaptic plasticity in the basal ganglia (Bolam et al., 2000; Gerfen, 2000; Gerdeman et al., 2003; Sturm et al., 2003; Haji et al., 2012), this was chosen as the focal brain region.

Chapter 2 focuses on the methodology employed to assess the aforementioned objectives of this study and the results are presented in Chapter 3. The findings and the conclusions in addition to study limitations can be found in Chapter 4.

Chapter 1

Literature Review

1. Literature Review

The central aim of Chapter 1 is to review the existing literature on the molecular standing of anxiety disorders, as well as environmental contributions in the form of trauma, providing an introduction to and motivation for subsequent chapters. Each anxiety disorder focused on in this dissertation will be briefly discussed in terms of neurobiology and pathogenicity and then the contribution of trauma to the pathogenesis of these disorders will be jointly evaluated. The use of animal models in elucidating the etiology of anxiety disorders in humans will also be addressed.

1.1. An introduction to anxiety disorders

Conditions characterized by pathological anxiety were initially classified under a broad term namely, anxiety neurosis (First et al., 1998), and were only separated into a number of conditions categorized as anxiety disorders much later on. This division was beneficial as it allowed for a more specific diagnosis, and ultimately more specific treatment. This disorder sub-categorization facilitated more homogenous research cohorts which are necessary for predicting clinical features associated with psychobiological mediating factors (Stein, 2004). A strong seating in literature supports the notion of genetic contributions to susceptibility for anxiety disorders (van Grootheest et al., 2005; Nugent et al., 2011; Papaleo et al., 2011; Schumacher et al., 2011; Sokolowska, 2013). In trying to identify possible common genetic traits associated with pathological anxiety, the grouping of patients with different anxiety disorders into a single cohort for comparison on a genetic level might provide better insight into common underlying causes of these conditions.

Large scale studies performed in the 1990s and early 2000s have shown that anxiety disorders are more common than originally thought and are debilitating and costly in both the developed and developing world (reviewed in Stein, 2004). Estimates by Kessler et al., in 2007 showed substantial increases in country specific prevalence compared to earlier work (Kessler, 2005; Kessler et al., 2007) Even with high prevalence, however, many cases still

go under-diagnosed or are mistreated, further contributing to suffering and the cost associated with the disorders. Studies have also shown that anxiety disorders precede the development of a variety of other disorders (e.g. depression and mood syndromes (Goes, 2012; Penninx and Comijs, 2012) and have a chronic course (Stein, 2004; Yonkers et al., 2000).

The etiology of anxiety disorders remains incompletely understood. A number of neurochemical systems and neurocircuitry have been implicated to date (reviewed by Stein, 2004 and Sokolowska, 2013). Initial work involved focus on the noradrenergic system, but with the advent of serotonin reuptake inhibitor efficacy in the treatment of anxiety disorders, focus was shifted to serotonin receptors and sub-receptors (Stein, 2004). Further consideration focusing on benzodiazepines highlighted the role of glutamate and GABA neurosystems in the development and progression of anxiety disorders (Kendell et al., 2005; Möhler, 2012). Molecular (such as that pertaining to serotonergic and dopaminergic systems) research provides promise regarding the elucidation of the roles these and other systems may play in the etiology of anxiety disorders. Molecular focus, however, should by no means negate any environment aspects that may be involved in disorder pathogenesis (Sánchez et al., 2001); for example, childhood trauma has been associated with the development of anxiety disorders in later life (Stein et al., 2008; Simon et al., 2009; Nugent et al., 2011).

1.2. Anxiety Disorders

The following is a brief discussion on the current literature pertaining to the neurobiology and pathogenicity of three anxiety disorders (Obsessive-Compulsive Disorder (OCD), Panic Disorder (PD) and Social Anxiety Disorder (SAD) – according to DSM-IV diagnostic criteria). A dataset with data from patients with a primary disorder of OCD, PD or SAD were available to us and these disorders were thus used as focal disorders in this study.

The DSM-IV categorizes several disorders together under the anxiety spectrum, namely generalized anxiety disorder (GAD), SAD, specific phobia (SP), post-traumatic stress disorder (PTSD) and OCD. Table 1.1 below gives a brief summary and characterization of each of the disorders mentioned and the three that will serve as focal point for this dissertation. The nature of each of these disorders and that which makes them distinct,

however, allows for significant comorbidity amongst the anxiety disorders as well as comorbidity for other mental illnesses such as depression and substance abuse (Egan et al., 2010). Anxiety and fear can be considered natural responses to possibly threatening situations. In anxiety disorders, however, these responses are either exaggerated or prolonged and in being so inhibit or disturb the daily life of an affected individual. Current treatment strategies involve behavioural (e.g. cognitive behavioural therapy) and/ or pharmacotherapy (e.g. benzodiazepines or serotonin reuptake inhibitors) options, but these present with addictive, tolerance build-up or inadequate efficacy in some individuals (Sokolowska, 2013). That said it is important to investigate and understand the molecular mechanisms underlying anxiety disorders, in order to contribute to the development of wider acting and more effective anxiolytics. Genetic studies offer an ideal target for considering molecular background as the identification of gene candidates can point directly to protein function and pathway.

Table 1.1: DSM-IV classifications of disorders under the anxiety spectrum

Disorder	Characterization	Treatment
OCD	Recurrent, intrusive obsessions and distressing thoughts, images or feelings	CBT, SSRIs
PD	Unexpected panic attacks of brief, sudden, intense periods of anxiety	CBT, SSRIs, TCAs, MAOIs, Benzodiazepines
SAD	Excessive fear of negative evaluation by others	CBT, SSRIs, MAOIs, benzodiazepines
PTSD	Subsequent to experiencing a traumatic event: afflicted individuals re-experience the trauma, avoidance behaviour, numbing of emotions	CBT, SSRIs, MAOIs, TCAs
GAD	Excessive and uncontrollable worries about life events	Benzodiazepines, antidepressants

**OCD: obsessive-compulsive disorder; PD: panic disorder; SAD: social anxiety disorder; PTSD: post-traumatic stress disorder; GAD: generalized anxiety disorder; CBT: cognitive*

behavioural therapy; SSRIs: selective serotonin reuptake inhibitors; MAOIs: monoamine oxidase inhibitors; TCAs: tricyclics

Anxiety disorders are complex and caused by a combination of both environment and genetic factors. Recent Genome Wide Association Studies (GWAS) into anxiety-related personality trait neuroticism and panic disorder have identified a large number of small effect-size common and rare variation in anxiety predisposition (Shifman et al., 2007; Otowa et al., 2009; Calboli et al., 2010; Terracciano et al., 2010). This infers that sample sizes of several thousand would be required to identify variation predisposing to anxiety disorder. Considering the practicality and feasibility of this, the consideration of gene-environment interaction and strict population stratification may be prudent in studies in which these sample numbers are not possible to reach (Sokolowska, 2013).

The neurobiology of anxiety disorders

The genetic basis for anxiety disorders has been explored within the literature and there are many proposals with regards to neuropathophysiology. Proposals for fear arousal and anxiety being organized in the locus coeruleus have been proposed. Neurons in this area may project to the paraventricular nucleus in the hypothalamus allowing for the activation of the HPA axis, triggering an anxiety response (Steimer, 2002; Egan et al., 2010). The locus coeruleus may also project into the amygdala, striatum, prefrontal cortex, bed nucleus of stria terminalis, hippocampus, periaqueductal gray, hypothalamus, and nucleus solitaries based on assumptions regarding differences found within the serotonergic, GABAergic, neurosteroid and HPA hormone axis systems (reviewed by Steimer, 2002; Meaney, 2010; Egan et al., 2010; Maschi et al., 2013).

With regards to the anxiety disorders included in this project: OCD is characterized by recurrent obsessions and compulsions in both an individual's personal and psychological life. They can result in severe distress and increasing risk for comorbidity, such as major depression (Stein, 2004; Hemmings et al., 2008; Egan et al., 2010). Obsessions may manifest as repetitive thoughts, ideas, and images which result in anxiety. Compulsions or urges

follow and are repetitive mental or motor activities that occur in order to neutralize anxiety (Stein, 2004; Abramowitz, 2012). Literature supports a familial link to OCD progression and/ or development; for example studies by Pauls et al., 1995, 1986 showed that first-degree relatives of probands with OCD had higher risk for clinical and subclinical OCD manifestation. Medical conditions such as Sydenham's chorea and the obsessive-compulsive symptoms observed have implicated the basal ganglia in OCD neuroanatomy. Cortical-striatal-thalamic-cortical pathway (Figure 1.1) has been implicated based on imaging studies investigating the clinical manifestations of OCD (Milad and Rauch, 2012). The orbitofrontal cortex, anterior cingulate cortex and the caudate nucleus have demonstrated hyperactivity in resting-state and this has been shown to be attenuated with exposure/ response prevention and serotonergic treatment therapies (Baxter et al., 1996). The aforementioned, amongst others, have led to a popularity for cortico-striatal models of the pathophysiology of OCD (Stein et al., 2010; Milad and Rauch, 2012).

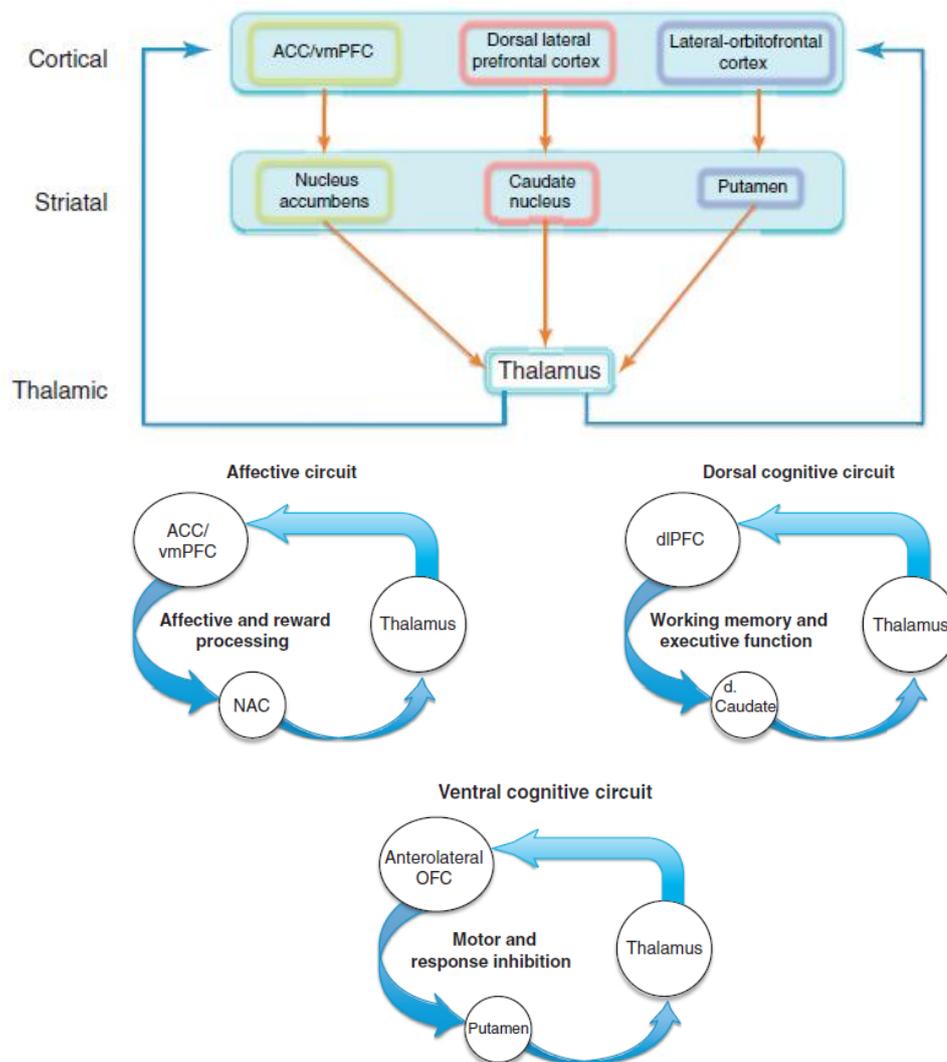


Figure 1.1: Schematic representation of the different components of the cortico-striato-thalamic-cortical pathways implicated in OCD psychopathology and the cortico-striatal loops involved (Milad and Rauch, 2012). ACC: anterior cingulate cortex, vmPFC: ventromedial prefrontal cortex, dlPFC: dorsolateral prefrontal cortex, NAC: nucleus accumbens, OFC: orbitofrontal cortex

PD is characterized by the tendency of a patient to misinterpret their own bodily sensations as signs of imminent physical or mental harm. In PD, individuals experience recurrent and unexpected panic attacks (Association, 2000; Stein, 2004; Egan et al., 2010). PD, as with OCD, can be quite debilitating and has major impact on the sufferer's quality of life. It has been shown to be associated with a decrease in physical and mental functioning and can be compounded with co-morbid chronic medical conditions and depression (Stein, 2004). The

disorder is often under-diagnosed (Sartorius et al., 1993; Steimer, 2002; Schumacher et al., 2011) and this may be attributed to its typical presentation as predominantly somatic symptoms such as chest pain or dizziness as oppose to psychologically based observations. Literature revolving specifically around PD, as with the other anxiety disorders, has implicated a number of systems to be involved in the pathogenesis of PD; namely, the brain monoaminergic systems (noradrenergic and serotonin systems), the GABA (γ -aminobutyric acid) system and respiratory and cardiovascular functions (Gorman, 2000; Stein, 2004; Schumacher et al., 2011). Neuroanatomical investigations into the pathogenesis of panic disorder have implicated several brain areas, similar to the aforementioned OCD neurobiology. The locus coeruleus, principal nucleus of noradrenergic systems within the brain, has also been implicated in the development of fear and anxiety in PD affliction (Redmond et al., 1976; Stein, 2004; Reif et al., 2013). Other repeat structures (structures also included in other anxiety disorders) of the central nervous system (CNS) that have been implicated include the striatum, thalamus, hypothalamus, hippocampus, anterior cingulated gyrus, peri-aqueductal gray area and the frontal cortex (Gorman, 2000; Stein, 2004).

SAD can be defined as a marked and persistent fear of one or more social or performance situations in which a person is exposed to unfamiliar people or to scrutiny by others (Association, 2000; Egan et al., 2010). The individual in question fears a behavioural manifestation that will result in humiliation or embarrassment and experiences anxiety as a result. This anxiety may manifest in the form of a panic attack. Despite this, the affected individual understands that this behaviour is excessive or unreasonable and therefore avoids these kinds of situations. The fear experienced in social situations can result in severe anxiety or marked distress and functional impairment (Stein, 2004). As with the other anxiety disorders, most SAD sufferers have one or more comorbidities such as depression, PD, agoraphobia, generalized anxiety disorder or substance abuse disorders (Schneier et al., 1992; Stein, 2004; Egan et al., 2010; Shirayama et al., 2013). Similar to some of the other anxiety disorders (e.g. OCD above), the first-degree relatives of patients with SAD have shown increased rates of SAD (Fyer AJ, 1993; Goes, 2012), suggesting a significant genetic (and environmental) contribution. Initial theories regarding the pathogenesis of SAD involved the presence of an overactive autonomic nervous system considering autonomic arousal when in social settings (Levin et al., 1993). However, when blood pressure and heart rate were monitored in SAD patients relative to controls no significant differences were

found, suggesting that SAD in this instance was limited to performance anxiety associated with increase autonomic reactivity. Literature then continues to implicate, as with OCD and PD, similar neurobiological systems such as dopaminergic transmission where patients with SAD showed a preferential response to monoamine oxidase inhibitors (MAOIs), and also decreased striatal dopamine D₂ receptor and dopamine transporter binding (Sidhu, Laruelle, & Vernier, 2003; D. J. Stein, 2004). Several other systems have also been implicated in SAD pathogenicity including serotonin and growth hormone abnormalities, as well as amygdala function in fear conditioning (Stein, 2004; Morreale et al., 2009), as with OCD and PD and anxiety disorders pathogenesis in general.

The striatum, plasticity and anxiety disorders

Considering the literature on the striatal involvement in anxiety disorders, the striatum has been used as a target source for real-time investigation into aberrant gene expression. The striatum is a prime area of high synaptic plasticity (Shin and Liberzon, 2009; Schilman et al., 2010; Guyer et al., 2012). Plasticity refers to the ability of the neural activity generated by an experience to modify neural circuit function which could ultimately modify thoughts, feelings and behaviour (Citri and Malenka, 2008). Synaptic plasticity refers to the ability of the brain to create persistent memory traces by the activity-dependent modification of the efficacy of a synaptic transmission at pre-existing synapses. Synaptic plasticity is believed to play a key role during early neural circuitry development and that major events (e.g. trauma) altering the regular plasticity mechanisms may contribute to neuropsychiatric disorders (Citri and Malenka, 2008). Identifying the mechanisms and genes responsible for changes at neuronal synapses could prove crucial to the understanding and/ or progression of anxiety disorders, and possibly other neurological disorders involving synaptic circuitry considering the cortico-striato-thalamic-cortical pathway seating for anxiety psychopathology (Milad and Rauch, 2012).

The striatum comprises dorsal (caudate nucleus and putamen) and ventral (nucleus accumbens and ventral portions of caudate and putamen) brain structures and is the primary input nucleus receiving excitatory impulses from the cortex and thalamus (Figure 1.1). It also receives innervations from midbrain dopamine neurons, and represents a major site of

synaptic plasticity in the basal ganglia (Bolam et al., 2000; Gerfen, 2000; Gerdeman et al., 2003; Milad and Rauch, 2012). The striatum is involved in basal ganglia circuitry by two parallel cortex-basal ganglia-thalamus-cortex loops diverging within the striatum, differentially modulated by dopamine. Considering these pathways, the high level of synaptic plasticity occurring in the striatum as well as the association of the striatum in the pathophysiology of OCD, SAD and PD mentioned above (Shin and Liberzon, 2009; Guyer et al., 2012; Milad and Rauch, 2012) the striatal brain region is a prime candidate for investigating the effects of gene-environment interactions in the development and/ or progression of anxiety disorders.

Pharmacotherapy and psychotherapy

A number of pharmacotherapies are available for the treatment of SAD which include, but are not limited to, SSRIs, benzodiazepines, MAOIs, β -adrenergic blockers and buspirone (Bouwer and Stein, 1998; Stein MB, 1998; Roy-Byrne et al., 2010). Current drug therapies, however, only work on a subset of those affected and it is estimated that approximately half of those affected by one or more anxiety disorders respond effectively to drug therapies. The goal in treatment is to reduce or completely eliminate avoidance, anticipatory anxiety, panic attacks etc. (depending on the anxiety disorder(s) in question) and to treat any co-morbidity which may be present. Psychotherapy schedules include exposure, cognitive restructuring, relaxation timing and social skills training; which are all facets of cognitive behavioural therapy (CBT). CBT is the most extensively researched and empirically supported of the psychotherapies utilized in the treatment of anxiety disorders (Stein, 2004; Olatunji et al., 2010). Clinical studies have shown efficacy for both cognitive-behavioural as well as pharmacological therapies; especially when implemented together (Heimberg, 2001; Olatunji et al., 2010).

Anxiety disorders are challenging conditions, not only for patients and their families, but also for clinicians. As mentioned above, there is clear overlap in the neurobiological pathways for independent disorder diagnoses as well as response to pharmacotherapeutic agents. Their possible early onset and chronic course make it all the more important to find more effective treatments and treatment strategies that benefit the patient and his/her significant others.

More recent treatments, such as serotonergic agents, have significantly contributed to the alleviation, if only partially, of symptoms improving a patient's quality of life. There is still, however, much research that needs to be done regarding further elucidation into the pathophysiology of anxiety disorders. The identification of novel pathways, genes or gene-environment interactions underpinning the development and/ or progression of the anxiety disorders are crucial to a better understanding of these disorders, and may ultimately lead to better treatment strategies and outcomes.

1.3. Etiology of Anxiety disorders: a gene-environment interaction

Trauma as a risk factor for developing an anxiety disorder has been well depicted within the literature. An example of environmental influence is the development of OCD after sexual assault, personal violence or road accident incidents (de Silva and Marks, 1999; Pitman, 1993; Sasson et al., 2005). These clinical and neurobiological observations are further backed by community studies indicating that exposure to a situation in which a person fears a serious injury or death is associated with increased susceptibility to PTSD, agoraphobia, SAD and OCD (Boudreaux et al., 1998; Jordan et al., 1991; Maes et al., 2000).

Early life stress (such as childhood trauma) is an established predictor of adverse outcome across the lifespan and can encompass psychiatric, behavioural and neurocognitive spheres (Gunnar and Quevedo, 2007; Danese et al., 2009; Irish et al., 2010; Nugent et al., 2011). Literature has also specified an elevated risk for internalizing disorders (such as anxiety) in individuals who have suffered early life stress (Meaney, 2010; Nugent et al., 2011; Maschi et al., 2013). Although early life stress and childhood trauma are well established risk factors for several psychiatric disorders (e.g. the anxiety disorders), they do not always result in dysfunction or impairment or the development of a psychiatric condition. This divergent outcome can be best explained by an interaction between the environment (the early life stress or childhood trauma) and genetic predisposition (Caspi et al., 2002; Lochner et al., 2002; Caspi and Moffitt, 2006; Nugent et al., 2011; Maschi et al., 2013). Gene-environment interaction can therefore be defined as the ability of genetic variables to interact with the environment (early-life trauma) and result in an anxiety disorder(s) psychopathology.

Figure 1.2 graphically depicts the gene-environment interaction. Under low environmental stress, functioning is considered normal; however, normal function can be impaired under conditions of high environmental stress (curved line). A high level of environmental stress

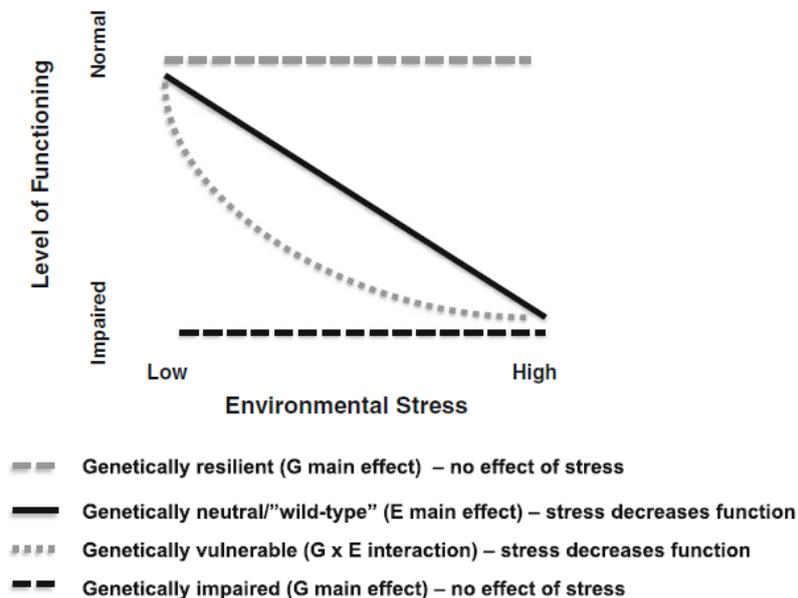


Figure 1.2: A depiction of gene-environment interaction (Nugent *et al.*, 2011) *level of functioning may refer to gene, social, cognitive etc.

alone (e.g. trauma) could impair functioning (solid line), however, the gene-environment interaction implies a genetically determined increase in susceptibility to the environmental influence and therefore a non-linear decline in normal functioning is expected (Nugent *et al.*, 2011). Genetically determined resilience (grey dashed line) or impairment (black dashed line) can be associated with normal functioning or impairment, respectively, independent of environmental contributions (Figure 1.2) and these also need to be considered within the gene-environment interplay.

A number of case studies have demonstrated how trauma may contribute to the development of anxiety disorders. Gene-environment interaction in early life stress has been characterized extensively for a number of promising target candidate genes and/ or pathways that are involved in the development and/ or progression of anxiety including the serotonin system, the HPA axis, neurotrophins, dopaminergic and GABA genes (Nugent *et al.*, 2011). A summary of these studies focusing on those that include anxiety disorders can be found in Table 1.2.

Childhood trauma can have persistent or intermittent mental effects including, but not limited to, psychiatric disorders (such as anxiety and depression), cognitive impairment, and maladaptive stress responses (Stessman et al., 2008; Gagnon and Hersen, 2000; Maschi et al., 2013). Other stressful life events may occur later on at different points during the life course and this could exacerbate existing subjective symptoms (Maschi, 2006). Research regarding the temporal effects of childhood trauma, especially concerning later life functioning, point to increased occurrence of minor psychological distress which often leads to more severe mental health problems like anxiety disorders and depression (Neal et al., 1995; Shmotkin and Litwin, 2009; Hovens et al., 2012; Maschi et al., 2013).

Studies exist which focus on epidemiological data regarding childhood trauma/ maltreatment and have shown increased risk for SAD in such cases (Nelson et al., 2002; Kuo et al., 2011). Further evidence regarding the association of childhood trauma with SAD looked at the generalized subtype (GSAD) for the prevalence of childhood trauma in a clinical population who are at sufficient severity to seek treatment (Simon et al., 2009). Trauma can be expanded beyond that of physical and sexual criteria and include emotional abuse and neglect as well (Mancini et al., 1995; Hovens et al., 2012; Maschi et al., 2013). When including emotional abuse and neglect as well as physical and sexual abuse, significant findings were identified for childhood trauma history in a GSAD cohort with a greater SAD symptom severity and poorer associated function (Simon et al., 2009). Simon and associates (2009) found that emotional neglect and abuse amongst their GSAD cohort was associated with an observed symptom severity and that multiple types of childhood maltreatment in a single individual had an additive effect. This is an important finding considering evidence for childhood maltreatment contributing to increased risk of anxiety, especially when coupled to genetic risk factors (Stein et al., 2008). Stein and associates (2008) demonstrated a classic example of genetic risk factors (specifically the serotonin transporter gene polymorphism) for anxiety in conjunction with childhood maltreatment where it can amplify anxiety phenomena such as anxiety sensitivity. The serotonergic involvement in anxiety disorder pathogenesis and exacerbation in the presence of childhood trauma was addressed in a review by Meaney (2010).

Table 1.2: Studies highlighting candidate genes and GxE interaction in early life stress (adapted from Nugent et al., 2011)

Author	Sample Gender(M, F); Ethnicity	Age of ELS	Age at outcome M (SD) or range	Type of ELS	ELS assessment	Outcome	Outcome Assessment	Major Findings
<i>5-HTTLPR: studies supporting increased risk associated with s/s'</i>								
Cicchetti et al., 2007	184, 155 209 AA, 79 EA, 43 H, 8 other	Childhood and adolescence	17 (1)	Neglect, physical or sexual abuse, emotional abuse	DHS records	Depressive symptoms: anxious, depressed and somatic symptoms	Clinical interview, self- report	Significant of sexual abuse x <i>5-HTTLPR</i> : sexually abused s/s genotype predicted increased depression/ anxiety Significant sexual abuse x <i>5-HTTLPR</i> x <i>MAOA</i> : sexually abused low <i>MAOA</i> activity, s carriers at greatest depression

Nobile et al., 2009	315, 292	Childhood and adolescence	12 (1)	“Family structure” (i.e., single vs. two-parent families)	Parent report	Affective problems	Parent-report questionnaire	rGE trend: excess s/s in single parent families
	E 592							Significant ELS x 5-HTTLPR: single-parent s’ carriers greatest affective problems
Stein et al., 2008	76, 171	Childhood and adolescence	19 (2)	Emotional or physical abuse	Retrospective self-report questionnaire	Anxiety sensitivity	Self-report questionnaire	Significant ELS x 5-HTTLPR: greatest anxiety sensitivity (especially physical sensitivity) in s/s or s’/s’ with emotional or physical abuse history
Xie et al.,	656, 596	Before age 13	40 (10)	Violent crime, sexual	Retrospective	PTSD	Semi-structured	Significant

2009	582 EA, 670 AA			abuse, physical neglect	abuse, interview	semi-structured interview	diagnosis	interview	ELS x HTTLPR: carriers likely develop PTSD	5- s' more to
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Studies supporting increased risk associated with l/l' alleles

Laucht et al., 2009	142, 167 309 E	Childhood	19	Family parent unwanted overcrowding, etc.)	adversity (low education, pregnancy, partum	Parent interview at 3 months post- partum	Depressive symptoms & diagnosis: anxiety diagnosis	Clinical & interview; self- report questionnaire	Significant ELS x HTTLPR: increased anxiety or depression diagnosis in youth with ELS and l'/l' (or l/l) genotype	5-
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Studies reporting no G x E effects

Araya et al., 2009	2,306,2,028 4,170 E	5 - 7	7	Maternal depression; life events	17 postnatal adverse	Maternal report	Emotionality symptoms	Parent-report questionnaire	No significant ELS x HTTLPR predicting emotional symptoms	5-
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Additional serotonin system candidate genes investigations of early life stress (ELS)

Nobile et al., 2009	315, 292 E 592	Childhood and adolescence	12 (1)	“Family structure” (i.e. single- vs. two-parent families)	Parent report	Affective problems	Parent-report questionnaire	Significant ELS x <i>TPH2</i> G-703T: single-parent G carriers greatest affective problems
Cicchetti et al., 2007	184, 155	Childhood and adolescence	17 (1)	Neglect, physical or sexual abuse, emotional abuse	DHS records	Depressive symptoms; anxious/depressed symptoms	Clinical interview, self-report	Significant <i>MAOA</i> x number of maltreatment subtypes: low activity variant at greatest risk for depression in 3 – 4 subtypes Significant sexual abuse x <i>5-HTTLPR</i> x <i>MAOA</i> : sexually abused low <i>MAOA</i> activity, s
	209 AA, 79 EA, 43 H, 8 other							

										carriers at greatest depression
Chipman et al., 2010	3,177, 3,294 6,471 AC	Childhood and adolescence	20 – 24; 40 – 44; 60 - 64	17 adversities ranging from maternal mental health concerns to abuse		Self-report questionnaire	Depression and anxiety symptoms	Self-report questionnaire	No significant interaction or main effects	
<i>HPA axis candidate gene investigations of early life stress (ELS)</i>										
Heim et al., 2009	424, 639	Childhood	18 – 77	Physical, emotional abuse	sexual,	Retrospective self-report questionnaire	Depressive and PTSD symptoms; cortisol response	Self-report questionnaire	Significant ELS x <i>CRHR1</i> on depressive symptoms in men: male abused carriers reported increased depressive symptoms	
	1,063 AA		18 - 45						Significant physical abuse x <i>CRHR1</i> on depressive symptoms: G/G	

	25, 53 NR								genotype associated with depressive symptoms Significant ELS x <i>CRHR1</i> in men: male abused rs110402 G evidenced increased cortisol response
Tyrka et al., 2009	51, 78 129 EA	Childhood	18 - 61	Emotional/sexual abuse; emotional neglect	physical/physical/	Retrospective self-report questionnaire	Cortisol response	Dexamethasone/corticotrophin-releasing (DEX/CRH) hormone test	Significant ELS x <i>CRHR1</i> : rs110402 & rs242924 G/G genotypes associated with increased cortisol response only if reporting moderate to

predict smaller gray matter in hippocampus and lateral prefrontal cortex which in turn predicts depressive symptoms

Haeffel et al., 2010	251, 222 3 years of age 3 or younger	Parenting practices and interaction styles	Ratings of parent supportiveness/ intrusiveness during standardized interaction task	Diagnoses of depression, anxiety, ODD; symptoms of internalizing, externalizing, anxious/ depressed	Diagnostic interview completed with parents; parent report questionnaire	Significant rGE between child DRD2 and parent behaviour during task Marginally significant intrusiveness x <i>DRD2 Taq1A: A2</i> homozygotes showed positive association
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									between parent intrusiveness and symptoms while A1 carriers showed a negative association
Evans et al., 2009	3,016, 5,838	5 - 7	7	Maternal depression, 17 life events	postnatal adverse	Maternal report	Emotionality symptoms	Parent report	No significant ELS x <i>COMT</i>
Nelson et al., 2009	259 NR	Childhood	“Adult”	Sexual abuse, emotional/physical partner maltreatment	physical	Retrospective self-report	PTSD	Interview	Significant ELS x <i>GABRAA2</i> predicting PTSD

**M: Male; F: Female; AA: African American; AsianA: Asian American; AC: Australian White/ Caucasian; BiR: Biracial; EA: European American/White/Caucasian; E: European; H: Hispanic; NZC: New Zealand White Caucasian; NR: not reported; rGE: gene-environment correlation*

Although childhood trauma from an emotional abuse and neglect aspect has not been well characterized to date, sexual and physical abuse can be found more readily amongst literature (Bryer et al., 1987; Mancini et al., 1995; Mulder et al., 1998; Nelson et al., 2002; Hovens et al., 2012; Maschi et al., 2013). Nelson and associates (2002) demonstrated an increased risk for many a disorder, including SAD and depression, using twin studies (childhood sexual abuse versus those without). Increased risk for SAD amongst sexual abuse cases, although specifically in women, was also reported in an Australian study (Dinwiddie et al., 2000). Simon and associates (2009) reported an incidence of 17% of childhood sexual abuse in their clinical GSAD sample cohort and found it was associated with a higher risk for disability when data was adjusted for age and gender. Simon and co-workers (2009) found a similar trend for increased risk in women over men in their GSAD childhood sexual abuse cases. Overall, data obtained using the Liebowitz Social Anxiety Scale (LSAS) and Childhood Trauma Questionnaire (CTQ) suggested increased illness severity in both men and women with childhood maltreatment (Simon et al., 2009) and this theory seems to hold true for other anxiety conditions (OCD and PD) and childhood adversities (Kessler et al., 1997).

Using the revised CTQ (Bernstein et al., 1994, 1997) OCD patients were found to have significantly higher incidence of childhood maltreatment compared to controls (Lochner et al., 2002). The OCD patients were found to score highly concerning emotional neglect implicating a possible common childhood trauma marker amongst the two disorders (trichotillomania and OCD). Although the use of an all-female cohort as well as the need to consider other environmental factors (such as socio-economic status, comorbidity and age) can be considered limitations, Lochner and colleagues (2002) have confirmed an association between childhood trauma and OCD. A higher prevalence of childhood trauma in patients with PD compared to healthy controls has also been reported (Stein, 2004; Lochner et al., 2010). Previous studies have specifically associated emotional abuse during childhood with increased risk for PD later in life (David et al., 1995; Young et al., 1997).

In summary, there is a strong association between early trauma and anxiety disorders. This literature has specifically highlighted early developmental stress and trauma in the development of OCD, SAD and PD. Furthermore, the literature indicates links between the molecular underpinnings and gene-environment interaction in the development and or progression of anxiety disorders (Maschi, 2006; Lochner et al., 2010; Meaney, 2010; Hovens

et al., 2012; Maschi et al., 2013). Specific types of trauma exposure have been associated with the development or exacerbation of specific anxiety disorders (Lochner et al., 2010; Hovens et al., 2012). Taking all of this into account, it is crucial that when considering the molecular underpinnings of anxiety disorders, one must also consider trauma history. The gene-environment interaction can therefore not be negated when investigating the molecular etiology of anxiety disorders.

1.4. The use of animal models in anxiety disorders research

It has been argued that animal models represent useful tools for investigating the pathophysiology of psychiatric conditions, particularly those that “mimic” the behavioural aspects of the disorders. Tests are often assessed based on face (looks like it would be a good test), test (accurately measures the desired trait, usually based on evidence to support the interpretations of test scores in psychological testing) and construct (the degree to which the test measures what it claims) validity. However, for an animal model to be effective, it needs to more or less mimic the condition of interest with regards to etiology and symptomatology (amongst other things) (Bloom and Kupfer, 1995). Anxiety is an evolutionary conserved response and can be measured in animal models (Stein and Bouwer, 1997; Nesse, 1998). At the same time animal models allow for the harvesting of brain tissue at any time-point. The key question to be answered, however, is, ‘Can we use an animal (rat or mouse specifically) to model aspects of human anxiety disorders?’ In complex psychiatric conditions, like the anxiety disorders, many of the cardinal features are based on subjective verbal report making the ‘ideal’ animal model completely unfeasible. This means that when looking at an animal model for anxiety disorders it is important to redefine the usual requirements and focus on approaches relying on the mimicking of specific signs, symptoms, behaviours or responses as oppose to trying to replicate the entire condition (Müller and Holsboer, 2006; Schmidt and Müller, 2006).

From a behavioural state anxiety is a response to signals of danger, however, physiologically it involves the activation of the hypothalamus-pituitary-adrenal (HPA) axis allowing for steroid secretion (Korte, 2001; Boyce and Ellis, 2005). The genes that code for the steroid hormones associated with the stress response are highly conserved across diverse species

including primates, rodents, reptiles and amphibians (Lovejoy and Balment, 1999; Lovejoy and Jahan, 2006). It has also been shown that the neural structure and functioning underlying a fear or anxiety response are similar across species (Elliot, 2008). In rodents, the approach-avoidance paradigm is what is monitored in order to assess for anxiety-like behaviours (Dell'Osso et al., 2003; Stein and Paulus, 2009) and these have been evaluated and validated with drugs that have been used to treat human variations of these disorders (Gordon and Hen, 2004). The most popular and well validated testing paradigms used to evaluate approach-avoidance behaviour include the open-field test (OFT) and elevated-plus maze (EPM) (Pellow et al., 1985; Lau et al., 2008; Chadman et al., 2009). As mentioned, although animal models prove useful in studying complex disorders such as anxiety disorders, they do only mimic certain aspects of disease and one cannot ignore the cognitive differences between rodent and humans. Nevertheless, the behavioural differences between stressed and unstressed rats may still point to potential candidate susceptibility genes in humans. A diagrammatic outline of the general approach for using an animal model to identify susceptibility candidate genes can be found in Figure 1.3 below.

Quantitative trait loci (QTL) mapping has been used to identify susceptibility candidates in rodents (Ramos et al., 1999; Zhu et al., 2012), with underlying notion that genes in the human homologue loci could be considered susceptibility genes for human phenotypes - in this case, anxiety disorders. Initially, QTL mapping was based on genome-wide marker usage within the F2 rodent generation, relative to anxiety-like behavioural assessment; but this showed relatively poor resolution. Present day models include, but are not limited to, genetic manipulation (when a specific gene target has been selected for and incorporated/ knocked-out), strain selection (capitalizing on the inherent ability of a particular strain to be high- or low-anxiety), bidirectional breeding (using inherent strains for comparison independent of an outside stimulus), and environmental innervations (the application of an external stimulus like a stressor to produce a behavioural response that may be monitored) (Mott and Flint, 2002; Yalcin et al., 2005; Valdar et al., 2006; Zhu et al., 2012). These may all be combined with pathway specific screening and/ or tissue specific expression for increased resolution or candidacy identification.

Rodents represent a good model system for evaluating human anxiety disorders as 1) the central nervous system is sufficiently developed enough to mimic aspects of human anxiety 2) there are multiple strains available and the full genomic sequence for most of these are easily accessible 3) there are well established techniques allowing for transgenic manipulation within rodents and 4) maintenance of the model is relatively cost-effective (Keane et al., 2011; Sokolowska, 2013).

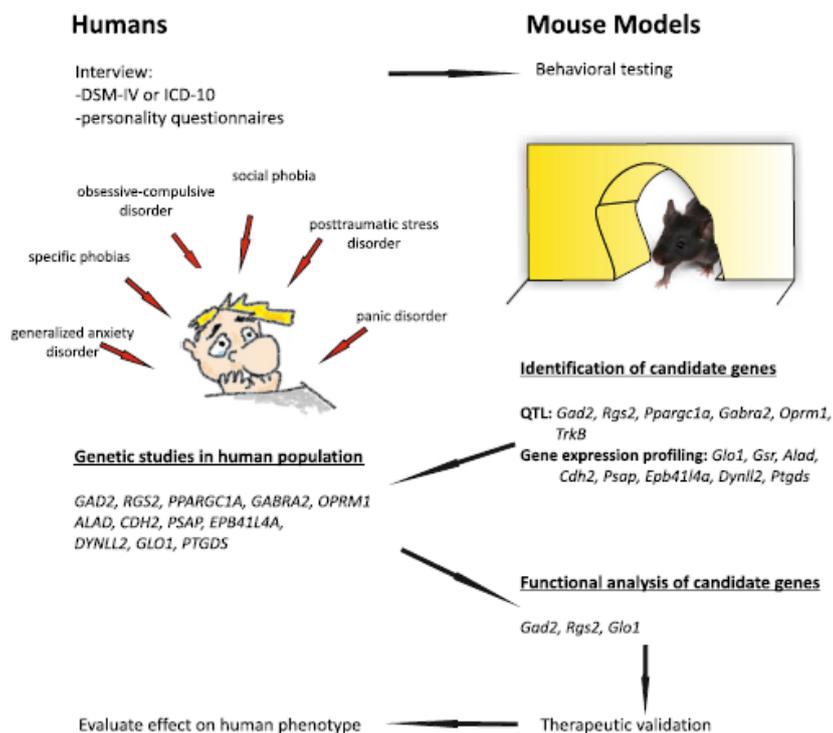


Figure 1.3: A strategy for cross-species (rodent-human) identification of susceptibility genes for anxiety disorders (Sokolowska, 2013)

Maternal separation (MS) is the most widely accepted model in rats for investigating the effects of early-life trauma and many studies have indicated its validity in humans (Andersen, 2003; Lehmann and Feldon, 2000; Roman, 2004; McEwen et al., 2012). Separating rat pups from their mothers during the postnatal period results in a range of physiological changes, the nature of which depend of the duration of separation, the specifics of the separation act itself as well as the environmental conditions under which the separation takes place (Lehmann and Feldon, 2000). The behavioural changes associated with MS has been known for past 50 years (Levine, 1957; Denenberg and Morton, 1964) where it was initially found that the

handling and or stroking of pups allowed them to develop differently to those not handled. Furthermore these short periods of MS were found to have profound long-term effects on certain hormones, resulting in alterations in corticosterone response, enhanced learning, enhanced attention abilities and decreased emotional activity (Arnold et al., 2009). Prolonged periods of MS then led to the development of animal models better mimicking behaviours useful for extrapolation to human depression and anxiety studies (Hall, 1998; Kuhn and Schanberg, 1998). Post-natal (PND) days 4 – 14 are found to be the most sensitive period (hypo-responsive period) for MS applications (Roman, 2004). Rats do not respond to mild-stressors during this time due to low levels of circulating glucocorticoids (Sapolsky and Meaney, 1986; Schmidt et al., 2005). The MS model can be performed with single occasion for a specific duration, or at multiple time periods during the hypo-responsive period. Common protocol for anxiety models dictate minimum separation periods of between 3 and 15 min all showing to be effective in producing anxiety-like behaviours during adulthood. Prolonged periods of separation (> 1hr) can also be applied. Shorter MS application periods have been shown to result in neurochemical, behavioural and hormonal changes that culminate in the rodent's ability to cope with and adapt to more stressful situations as adults, whereas interestingly the longer periods of application appear to result in contradictory behavioural observations (Anisman et al., 1998; Ladd et al., 1999; Anand and Scalzo, 2000; Lehmann and Feldon, 2000; Pryce and Feldon, 2003; Roman, 2004; Eiland and McEwen, 2012). Contradictory results regarding prolonged MS application has been painted predominantly as variation in experimental protocol between research facilities (individual or litter separation, temperature conditions, lighting, or handling periods) (Lehmann and Feldon, 2000; Pryce and Feldon, 2003); however literature supports the results from studies on early-life experiences modifying behaviour under shorter MS application times with replications in rats, mice and primates (Clausing et al., 2000; Romeo et al., 2003; Levine and Mody, 2003; Schmidt et al., 2004; Roman, 2004; O'Mahony et al., 2009; George et al., 2010; Eiland and McEwen, 2012).

Restraint stress (RS) is considered to be an acceptable model to be used to mimic a human adult stressor (Doremus-Fitzwater et al., 2009; Spear, 2000). During RS, rodents are placed in a clear plexi-glass container with a narrowing tailgate. This impedes their locomotive ability without hindering blood flow or inflicting pain. This particular stress is either applied to adolescence rodents (PND 28 – 42) or during adulthood (PND 42 +) (Doremus-Fitzwater

et al., 2009). Restraint stress can also be applied prenatally in the same fashion as above, but then applied to the pregnant mother rats during different stages of fetal development (Maccari and Morley-Fletcher, 2007; Morley-Fletcher et al., 2013). Repeated exposure to RS has been shown to increase anxiety-like behaviours in rodents (Gehlert et al., 2005; Sajdyk et al., 2006; Sevgi et al., 2006; Doremus-Fitzwater et al., 2009) pre- or postnatally, evaluated by validated testing [pharmacological testing with anxiolytic and anxiogenic compounds (Cooper and Hendrie, 1995; File and Seth, 2003)] paradigms, namely EPM, OFT and also social interaction (SI) testing. The use of RS in an animal model to mimic mild adulthood stress in humans has received some paucity amongst the literature. For example, although the EPM test is an established test for anxiety levels in rodents exposed to a postnatal stress application, it doesn't always show significant differences between cases and controls in application (Albonetti and Farabollini, 1992; Chadda and Devaud, 2005; Doremus-Fitzwater et al., 2009); however when the same rats are evaluated using an alternative testing paradigm (e.g. SI) RS rats show significantly higher anxiety compared to controls (Doremus-Fitzwater et al., 2009). These contradictions have been attributed, as with MS, to inter-laboratory protocol deviations; however the over-all accreditation on restraint stress is that it results in an increased level of anxiety-like behaviour when applied prenatally (Maccari and Morley-Fletcher, 2007; Morley-Fletcher et al., 2013) or postnatally (Doremus-Fitzwater et al., 2009; Wang et al., 2012).

Rats exposed to environmental stressors can, therefore, subsequently be assessed for anxiety-like behaviours using various well-established behaviour assessment paradigms (EPM, OFT, SI etc.). The use of short-term MS during early developmental stages has been repeatedly shown to allow for long-term behavioural, neurochemical and hormonal changes in rodents, and also that short application time MS better prepares for adulthood stressors (Roman, 2004; Eiland and McEwen, 2012). Literature indicates RS application to mimic adult human neurobehavioral anxiety features, and in conjunction with MS (relative to RS only independent controls) could provide insight into the mechanisms involved in the pathogenesis of anxiety disorders in humans with a trauma history, and subsequent trauma exposure. In this study the use of an inherently 'calm' rat strain (Sprague Dawley) was subjected to environmental interventions of MS and RS, separately and in combination, to investigate this for use as an effective model for anxiety for candidate susceptibility gene identification.

Arguably these findings may assist in the search for novel candidate genes and/ or pathways involved in anxiety disorders in humans.

In conclusion, there are genetic component(s) to the pathogenesis of anxiety disorders; however, this is not independent of interaction with environmental variables, such as early life trauma. Investigating genes that are differentially expressed in trauma-exposed animals with subsequent anxiety-like behaviours, could point to novel susceptibility genes for anxiety disorders in humans. It is hoped that this would ultimately allow for a better understanding of the molecular etiology of anxiety disorders. This dissertation therefore aims to use trauma-exposed rats exhibiting anxiety-like behaviour to identify target genes for anxiety pathology and that may also be subject to interaction with early-developmental trauma. Investigating childhood trauma in the human cohort as a variable possibly interacting with the genetic components could elucidate on gene-environment interactions that may be present. By doing so, it is hoped to significantly enhance the understanding of the molecular basis of anxiety disorders and provide researchers with novel anxiety susceptibility genes to test for association in populations. In a clinical context, the identification of novel susceptibility candidates for anxiety pathology could lead to better treatment strategies by yielding novel drug targets. Treatment strategies may also be better designed or altered with a clearer understanding of the gene-environment interaction concerning these gene targets. This knowledge is pivotal considering the literature content indicating childhood trauma to be a susceptibility risk factor in developing anxiety disorders.

Study objectives

This project aims to evaluate whether the expression of certain genes encoding proteins involved in synaptic plasticity would be altered in maternally separated and restraint stressed rats compared to non-stressed controls. Genes identified to be differentially expressed in stressed rats expressing anxiety-like behaviours may be considered plausible candidate genes (novel and/ or previously described) for increased susceptibility to anxiety disorders in humans.

The objectives of this study were as follows:-

1. To determine the effect of stress (maternal separation and restraint stress) on gene expression in the rat striatum, i.e. to see if there are differentially expressed genes in rats with a trauma history vs. those without.
2. To determine whether the identified differentially expressed genes are potential susceptibility genes for anxiety-related disorders using case-control association studies.
3. To determine whether genes that are differentially expressed in rats with a trauma history and with consequent anxiety-like behaviours point to susceptibility genes for anxiety disorders in humans for which trauma severity scores are known.

Chapter 2

Materials and Methods

2. Materials and Methods

The central aim of Chapter 2 is to discuss the methodology employed. Chapter 2 will cover all methodologies, including clinical assessment, animal paradigms and the extrapolation onto a human cohort.

Role of the incumbent

Clinical interviews, data collection, sampling and human DNA extractions were performed by trained clinicians and a laboratory technician. The animal work was performed by experts in the field at the University of Cape Town. The role of the incumbent of this study was therefore to analyze the behavioural data obtained, to perform the RNA extractions and to do the cDNA conversions for expression analyses. In addition, he executed these assays and analyzed the data obtained to subsequently identify susceptibility genes in accordance with the aims and objectives of this study. The incumbent tested for carry-over significance within a human cohort of anxiety disorders patients and controls by implementing targeted next generation sequencing, and analyzed extended genotyping data obtained for the human cohort. Gene-environment correlation, gene-environment interaction and haplotype analyses were performed and conclusions were subsequently drawn and discussed within this dissertation.

2.1. Ethics

The study has been approved by Health Research Ethics Committee of the Faculty of Medicine and Health Sciences, University of Stellenbosch (Project number: 99/013). The work with humans is conducted in accordance with the guidelines of the International Conference on Harmonization Good Clinical Practice Guidelines (ICH/GCP, 1996), The Declaration of Helsinki (Edinburgh 2000) and The Medical Research Council of South

Africa's guidelines (2002) on the ethical conduct of research studies in humans. All participants provided written and informed consent for the study. If under the age of 18 years, participants provided assent and their parents or guardians consent for their participation.

The work with the animals is part of a larger study for which ethics approval has been obtained from the University of Cape Town, Department of Human Biology. (None of the animal work for this project was performed at Stellenbosch University, but completed in its entirety under the supervision of Dr. Jacqueline Dimatelis and Prof Vivienne Russell.) (UCT ethics approval Rec Ref: 010/030)

2.2. Animal work

A total of 51 male Sprague Dawley rats were used. Sprague Dawley rats are an outbred breed of albino rats that have been used extensively in medical research for close to 100 years. Their particular use within this study can be attributed to their calmness and ease of handling (Rodeheaver et al., 1996). Male rats were used to minimize any gender bias that may be associated with X-linked traits.

The rats were divided into a set of four experimental groups and all testing paradigms were carried out in the Department of Human Biology at the University of Cape Town:

- Maternal Separation (MS) group: rats were subjected to MS from post-natal day (PND) 2-14 (i.e. days 2 – 14 after birth), after which they were kept under controlled conditions until they reached PND75, when they were sacrificed (decapitation).
- Restraint Stress (RS) group: rats were subjected to RS for five consecutive days during adulthood (PND65) and left to reach PND75 before decapitation.
- MS+RS group: MS rats underwent RS for 5 consecutive days during adulthood (PND65) and left to reach PND75 before decapitation.
- Control group: rats were not subjected to MS or RS and were kept under controlled conditions until PND75 when they were decapitated and used as a control group for comparison to the above trials.

2.2.1. Maternal Separation

On PND2, rat pups were removed from their home cages (to separate them from their mothers) for a period of 3 h/day for 14 days, between 09h00 and 12h00. During the separation period, the pups were housed under infrared lights at a constant temperature of 31 – 33 °C inside the cage, to prevent hypothermia. During the separation procedure, the dam was housed in a separate room under controlled conditions. After 3 hours, the pups were reunited with their mothers and normal housing was not disrupted until the pups were weaned at PND21. Control animals were not separated from their mothers.

2.2.2. Restraint stress

The RS rat group was repeatedly stressed from PND 65 for 5 consecutive days using restraint stress. Male rats are considered adults from PND 60 (Spear, 2000), and this is therefore considered indicative of a mild adulthood stress. Rats were placed in a Plexiglas restrainer with the tailgate adjusted to keep the rat well contained, but without impairing circulation to the limbs. This was performed for each rat in the group for 4h per day for 5 consecutive days.

2.2.3. Behavioural tests

Behavioural tests were carried out by Dr Jacqueline Dimatelis and Prof Vivienne Russell in the Department of Human Biology at the University of Cape Town. These were performed in order to assess, relative to a control group, whether the environmental interventions of MS, RS and MS-RS lead to increased anxiety-like behaviour by the different rat experimental groups. Data were made available to me in Excel format.

The Open Field Test involves exposing individual rats to an open 1 m² arena enclosed by 50 cm high walls to measure anxiety levels (time spent in middle zone) and locomotor activity (distance travelled). The distance travelled is indicative of a level of exploration within the arena. Longer distance-travelled values are associated with increased time spent around the periphery of the arena and considered indicative of increased levels of anxiety. Rats tend to stay along the peripheral walls where it's 'guarded' in higher anxiolytic states (Lau et al., 2008). Distances were measured by Dr Jacqueline Dimatelis using EthoVision video tracking software (in cm) at UCT.

The Elevated-Plus Maze (EPM) is a plus-shaped maze that is elevated 50 cm above the ground. The ends of the four arms are open, two of the arms have short 2 cm walls (open-arms) and two of the arms have high 50 cm walls (closed-arms). Individual rats were placed in the maze for 5 minute intervals for a singular occurrence per rat. Time spent in the open arms suggests decreased anxiety-like behaviour and time spent in closed arms suggests increased anxiety-like behaviour (Pellow et al., 1985; Schmidt and Müller, 2006). Times were again measured by Dr Jacqueline Dimatelis using EthoVision video tracking software (in seconds) at UCT.

The Forced Swimming test (FST) makes use of a transparent cylinder (19 cm in diameter and 40 cm in height), containing tap water (25°C, depth = 19 cm). Rats are habituated in water for 15 minutes and tests are carried out 24hrs after habituation. Rats are subjected to 5 min swim test periods within the cylinder. After the test (as well as after habituation), rats are dried under a lamp to prevent hypothermia. Tests were evaluated using an over-head camera, where higher immobility times are associated with increased levels of anxiety. Although FST is usually used as a measure of depression, this is often a comorbid condition in anxiety disorders and can be associated with increased levels of anxiety as well (Ramboz et al., 1998; Dulawa et al., 2004; Suvrathan et al., 2010). Data were recorded by Dr Jacqueline Dimatelis at UCT.

After the behavioural assessments, rats were decapitated and the striatum surgically excised for further analysis. Decapitation occurred on PND 75 and the dorsal and ventral striatal regions were surgically removed by a skilled technician in the Department of Human Biology at the UCT. Striatal regions were subsequently housed at the Division of Biomedical

Sciences in 1.5 ml cryotubes (ThermoScientific - Johannesburg, South Africa) containing RNAlater (Qiagen) and stored immediately at -80°C until needed.

2.2.4. Behavioural data analysis

Time-bin and movement data was collected and assessed using the automated EthoVision video tracking software platform. Locomotive information was measured in time-bins in order to control for the initial minute of handling and acclimatization of the rat to the testing arena. The final (6th) minute was also controlled for regarding retrieval of the rat from the testing arena. Statistical analyses were performed using Statistica 10 (StatSoft Inc., 2011). MS, RS and MS-RS and control groups were compared in order to assess deviation in behaviours as a result of the stressor(s) used. Statistical correlation of groups with regards to a time effect (the comparison of individual time-bin minutes with each other to control for animal handling and acclimatization factors) were evaluated by means of mixed model repeated measures ANOVA and group assessment by one-way ANOVA. In all cases, Fisher LSD post-hoc tests were performed.

2.3. Human work

Assessments were conducted and recorded by a clinician with expertise within the field. Blood was collected by a nurse and DNA extracted by a qualified laboratory technician using the methodology described in section 2.4 below.

2.3.1. Participants

The study cohort comprised 149 patients (89 OCD, 33 PD, and 29 SAD) and 181 control individuals of South African Caucasian descent. Patients of various ages (between 9 and 82 years; 34.8 ± 13.2) were recruited countrywide, with the majority being from the Western

Cape Province. Patient recruitment was implemented *via* media advertisements and recruitment calls to psychiatrists and psychologists, primary care practitioners, and advocacy groups. Patients had to meet the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (First et al., 1998) criteria for a primary diagnosis of their respective illnesses (OCD, PD or SAD) for study inclusion. Patients were included irrespective of baseline (not receiving treatment for primary psychiatric disorder) or receiving treatment for primary diagnosis. Patients with comorbidities for any of the primary disorders in question (e.g. PD with comorbid SAD) were excluded.

The controls were recruited countrywide from both university and non-university settings (e.g. from organizations like the SA National Blood Services [SANBS], Metropolitan, Old Mutual etc.). Possible participants were approached either directly (the case of people attending SANBS clinics) or indirectly, i.e. with notices and advertisements sent out by their respective Human Resources departments. Most controls did not undergo comprehensive diagnostic interviews, and can be taken as representative of the local general population.

2.3.2. Participant Interviews

Data, including age at interview, age of onset of their primary disorder, highest level of education, current employment status, and population group were obtained from all participants. The clinical diagnoses were made based on the Structured Clinical Interview for Diagnostics and Statistical Manual of Mental Disorders, Fourth Edition, Axis I Disorders-Patient Version (First et al., 1998; First and Gibbon, 2004).

2.3.3. Self-report questionnaires

The Childhood Trauma Questionnaire (CTQ) (Bernstein et al. 1994), a scale proven to be a valid and reliable measure of past traumatic experiences (Bernstein et al. 1997), was used as a self-report questionnaire to assess the nature and severity of childhood trauma. Notably, a subset of the total sample of patients ($n = 92$) and controls ($n = 194$) for whom we obtained a

blood sample for DNA extraction completed this self-report questionnaire. The CTQ items are divided into 5 subscales: emotional abuse, physical abuse, sexual abuse, emotional neglect and physical neglect. The CTQ also includes a 3-item minimization/denial (MD) scale, which is indicative of potential underreporting of maltreatment or idealizing the family of origin. The scoring of responses on the 3 items comprising the MD scale were dichotomized (“very often true” = 1, all other responses = 0) and summed. A total of 1 or greater suggests the possible underreporting of maltreatment (false negatives) (Bernstein and Fink 1998) whereas a score of 3 indicates extreme minimization or denial of maltreatment during childhood. MD-scores of 3 were taken into consideration as possible confounders in the analyses.

All controls were also required to complete a questionnaire pertaining to his/her personal demographic data.

2.4. DNA/ RNA Extraction

2.4.1. DNA/RNA extraction from rat striatal tissue

Simultaneous DNA/ RNA extraction was performed using the Qiagen AllPrep DNA/RNA/Protein extraction kit. Rat striatal samples stored in RNALater (Qiagen - Hilden, Germany) stabilizing reagent prior to extraction, were then removed and transferred to 2 ml FastPrep-24 lysing matrix tubes (MP Biomedicals), containing 600 µl of RLT PLUS buffer, for homogenization. Samples were homogenized at full speed for 20 sec in the FastPrep FP120 (Savant), cooled on ice for 1 min and then re-homogenized at full speed for a further 20 sec. The matrix tube was subsequently centrifuged for 3 min at 10 000 rpm, and the supernatant removed and transferred to an AllPrep DNA spin column placed in a 2 ml collection tube. DNA/RNA extraction was then carried out according the manufacturers’ instructions. Post extraction DNA and RNA were stored at -80°C until needed.

2.4.2. DNA extraction from human whole blood

Genomic DNA was extracted from whole-blood using venipuncture and a chloroform/isopropanol extraction method previously described (Meulenbelt et al., 1995). Blood samples (between 5 and 10 ml) were collected in spray-coated K₂EDTA BD Vacutainer[®] Venous Blood Collection tubes (New Jersey, USA), and stored at 4°C until extraction and extractions were performed within 5 days of collection.

2.5. DNA/ RNA quantification and qualification

DNA and RNA quantification and qualification was assessed using the Nanodrop (Delaware, USA). Concentrations were assessed at a maximum absorbance 260 nm (A_{260}) for DNA and 230 nm A_{230} for RNA. DNA sample purity was assessed using the A_{260}/A_{280} ratio, with a ratio > 1.8 showing adequate purity and minimal protein contamination.

RNA quality and quantity was further assessed using the Experion RNA StdSens RNA Analysis kit and Experion automated electrophoresis system (BioRad – Johannesburg, South Africa) running Experion Software System Operation and Data Analysis Tools V3.0.226.0. The analysis kit and RNA StdSens chips were used according to the manufacturers' protocol (BioRad) and digital RNA gel images, concentrations and RNA quality indicator (RQI) values assessed for RNA quality.

2.6. Gene expression analysis using the RT² Profiler[™] PCR Array

The RT² Profiler[™] Array (SABiosciences, a Qiagen Company), in question is a 384-well plate based system capable of assessing tissue-specific expression for 84 genes in the synaptic plasticity pathway for 4 independent samples. The plate also houses a series of internal controls including a genomic DNA control (to assess for genomic DNA contamination), non-template control (to assess for plate contamination in general) and positive controls (to assess for plate integrity).

2.6.1. cDNA synthesis

Complementary DNA (cDNA) synthesis was performed using the RT² First Strand Kit (Qiagen) to ensure optimal results for detection of reverse transcription controls contained in the RT² Profiler PCR Array. cDNA was prepared for use with the RT² Profiler PCR Array format E 384 (96 x 4) option and therefore the recommended 400 ng input RNA was used for cDNA conversion. cDNA conversion was performed according to the manufacturers' protocol.

2.6.2. QC Array

The RT² QC PCR Array is designed to assess the quality of RNA samples before gene expression analysis using any of the RT² Profiler PCR Array systems available. The QC Array was used according to the manufacturer's instructions. Twenty-four individual PCR component mixes were prepared containing 2.4 µl of the prepared cDNA for each respective rat striatal sample as well as 27.6 µl of RNase-free water. Dilutions (1/100) were prepared for the same twenty-four samples using the extracted RNA and not cDNA conversions to use in the genomic contamination control wells of the QC array plate. Samples and master mixes were aliquoted into the wells of the QC Array using the EpMotion 5070 automated liquid handler (Eppendorf – Hamburg, Germany) and the plate subsequently run on the 7900HT Fast Real-Time PCR Machine (Applied Biosystems – Carlsbad, California) according to the following protocol: 95°C for 10mins, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Analysis of PCR amplification was done using SDS software Version 2.4 (Applied Biosystems – Carlsbad, California).

2.6.3. Rat Synaptic Plasticity RT² Profiler PCR Array

The Rat Synaptic Plasticity PCR Array, PARN – 126Z (SABiosciences - Hilden, Germany) was used to assess differential expression in the synaptic plasticity pathway genes between the aforementioned four (MS, RS, MS-RS and Control) rat groups (Section 2.1). The genes included in the PCR array include, but are not limited to the following groupings: immediate-early response genes (IEGs), late response genes, long-term potentiation (LTP), long-term depression (LTP), cell adhesion, extracellular matrix and proteolytic processing, cAMP response element binding (CREB) factors, neuronal receptors and post-synaptic density. A detailed list of the genes included within these groupings can be found in Appendix II. Five housekeeping genes (*Rplp1*, *Hprt1*, *Rpl13A*, *Ldha* and *Actb*) were included for normalization of the expression data, and a rat genomic template control to determine whether the extracted RNA samples were contaminated with genomic contamination. Reverse transcription and positive amplification controls were also included. cDNA representative of a single rat specimen from each of the aforementioned groups was analyzed on a single array to address grouping-bias that may be introduced when running all control and all patient samples in a single run. Mastermixes were prepared according to the manufacturer's protocol using RT² SYBR Green ROX qPCR Mastermix (Qiagen - Hilden, Germany) and mastermixes were aliquoted into the wells of the QC Array using the EpMotion 5070 automated liquid handler (Eppendorf – Hamburg, Germany). Real-time PCR was performed using the 7900HT Fast Real-Time PCR Machine (Applied Biosystems – Carlsbad, California) running SDS software Version 2.4 (Carlsbad, California). Cycling conditions were as follows: 95°C for 1 min to allow for HotStart Taq Polymerase activation, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min for which fluorescence data capture was performed. A dissociation curve (machine default) was employed post amplification to verify PCR specificity.

2.6.4. RT² Expression Profile Analysis

Expression data obtained using the RT² Profiler PCR Array (SABiosciences, a Qiagen Company) were analyzed online at <http://www.sabiosciences.com/pcrarraydataanalysis.php> using the prescribed data analysis software. The software employs a $2^{-\Delta\Delta C_t}$ approach using all five of the included housekeeping genes in the analyses to provide fold-changes as well as *P*-

values relative to the control group. The software also analyses the internal genomic, reverse transcriptase and positive amplification controls for quality assessment.

2.7. Candidate susceptibility gene characterization

Characterization of candidate genes was done using a combination of a TagSNP approach using the online databases and MAFs, as well as the identification of inter-species (rat, mouse and human) DNA conservation. Next generation sequencing (NGS) was employed in cases where the current databases lacked sufficient information for informative SNP selection for the population group used in this study.

2.7.1. TagSNP identification

The susceptibility genes identified using the RT² Profiler™ array were used as input into the International HapMap Project database (www.hapmap.org), HapMap genome browser release #27 (Phase II & III – merged genotypes and frequencies) on Feb09, on NCBI B36 assembly, dbSNP b126. TagSNPs were identified by searching the susceptibility gene of interest in the aforementioned data source to find informative SNPs spread across the gene. The HapMap TagSNP data file was downloaded and TagSNPs confirmed using the Haploview software analysis program (www.broadinstitute.org/haploview/haploview). Minor allele frequencies (MAFs) of ≥ 0.2 and $R^2 \geq 0.8$ were used at cut-offs for SNP inclusion.

2.7.2. Core-domain region identification

Simple Modular Architecture Research Tool (SMART) software (<http://smart.embl-heidelberg.de/>), Swiss-Prot, SP-TrEMBL (<http://www.ebi.ac.uk/uniprot>), and Esembl

(www.esembl.org) databases were used to determine the domain structure of the proteins encoded by the identified candidate genes.

2.7.3. Inter-species conservation analysis

rVISTA software analysis tool (<http://r Vista.dcode.org/>) and ECR Browser (<http://ecr browser.dcode.org/>) were used to search for conserved genomic DNA regions of candidate genes between human and rat species.

2.7.4. Targeted next generation sequencing (NGS)

Targeted NGS was performed using a combination of whole-genome amplification (as opposed to a tagged-fragment approach). Successfully amplified genes were normalized (SeqTarget Normalization, Qiagen) to ensure even coverage during reading, then tagged and fragmented (NexteraXT, Qiagen) for use with Illumina's MiSeq platform. The NGS cohort comprised 12 patients (even numbers of OCD, PD and SAD) and 19 controls, in accordance with the run requirements for kit usage on the MiSeq Illumina experimental setup. CTQ total score means were calculated using Graphpad Prism 5.0 (*student's t-test*) and confirmed using the descriptive statistics add-on package for Excel (Microsoft Office 2010). Cases for inclusion were selected by choosing those with higher CTQ total scores in OCD, SAD and PD sub-populations so as to allow for even numbers of OCD, PD and SAD participants (mean CTQ_{tot}: 51.16). Controls were selected choosing those that were the same or closely related mean CTQ total score (mean CTQ_{tot}: 26.66) of the control group to represent the general population. Selected individuals were controlled for gender and age. An underlying genetic component associated with anxiety disorders in general is the target focus of this study, not a specific disorder. The small number of individuals able to be screened using NGS considering cost also required the merging of this individual disorders into one group to maximize statistical power.

2.7.4.1 Whole-Genes amplification

Whole gene amplification was carried out on *ARC*, *EGR2*, *EGR4*, *NTF4* and *GRM2*, considering the absence of sufficient HapMap database data to perform a TagSNP analyses. A total of 31 individuals (12 patients and 19 controls) were selected (criteria described in 2.7.4 above) for whole-gene amplification. DNA quality was assessed using the Nanodrop (Delaware, USA) as well as agarose gel electrophoresis to assess integrity. Age- and sex-matched patients and control individuals above the age of 18 years were selected for whole-gene PCR amplification.

The aforementioned genes were amplified using the primer sets specified in Table 2.1 in conjunction with QIAGEN LongRange PCR kit. Primers were designed using Integrated DNA Technologies (www.idtdna.com) and *in silico* specificity testing performed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR conditions were carried out according the manufacturer' instructions using either cycling conditions A or B with the annealing temperatures specified in Table 2.2.

Cycling Condition A

Initial denaturation at 93°C for 3 min, followed by 35 cycles of denaturation at 93°C for 15 sec, annealing at the specified annealing temperature for 30 sec, and extension at 68°C for 8 min. The reaction was then set to cool to 4°C until removed from the PCR machine for further use.

Cycling Condition B

Initial denaturation at 93°C for 3 min, followed by 10 cycles of denaturation at 93°C for 15 sec, annealing at the specified annealing temperature for 30 sec, and extension at 68°C for 10 min. This was followed by 28 cycles of 93°C for 15 sec, annealing at the specified annealing temperature for 30 sec, and extension at 68°C for 10 min + 20sec with every cycle. The reaction was then set to cool to 4°C until removed from the PCR machine for further use.

Table 2.1: Whole gene amplification primer specifications

Gene	Primer (5' – 3')	T _a (°C)	Product size (bp)	Cycling condition
<i>EGR2</i>	CGCGAGCGAGAACTTTCCCAAAT	58	8156	A
	CGTGCAAGAAGCACCCCTGAACATT			
<i>EGR4</i>	TTTGGTGACTTGGACCCTTGTCTT	58	6752	A
	TCAGGTGGCCCATGCCTAATAAGT			
<i>NTF4</i>	AAATAACTGGAGCGGCCTTGTGTG	62	7936	A
	AATCCTGCTTTGCAGTCGGAGGTA			
<i>ARC</i>	AGTTTGCTGAGCCAGCTCTCACAT	58	3269	A
	AAATGACAGCTTTCAGGCAGCACC			
<i>GRM2</i>	TTTGTGGCATGTCTTGTGTGAGC	62	7222	A/B
	ACAGCTAGCAGGTCTTGGTGTCAA			
<i>GRM2</i>	TGTGGACACTTGACACCAAGACCT	62	8328	A/B
	ATTCACCATGATGAGTGGCTCCCT			

Abbreviations: bp: base-pair, T_a: annealing temperature

Successful amplifications were tested using horizontal agarose gel electrophoresis on a 1% (w/v) agarose gel in 1X sodium tetraborate decahydrate (SB) buffer. Samples were electrophoresed for 1 hr at 120 V along with Hyperladder I (1 Kb ladder; Bioline – Cape Town, South Africa) for size comparison, and subsequently visualized using ultraviolet light transillumination using GeneSnap V 6.07 (SynGene). Successful amplifications were confirmed using semi-automated bi-directional sequencing analysis. Sequencing results were analyzed using ClustalW and BioEdit Sequence Alignment Editor software (Hall, 1999; Thompson et al., 1997).

Due to the sporadic GC-rich regions contained within the *ARC* gene, whole-gene optimization could not be successfully achieved. The QIAGEN SeqTarget Primer Select kit (Cat. 122001-ST00745787) was used in conjunction with QIAGEN LongRange PCR kit according to the manufacturers' protocol using PCR cycling condition B for amplification of the *ARC* gene. Successful amplification was assessed using horizontal gel electrophoresis as described above.

2.7.4.2 SeqTarget normalization

Long-range PCR products were purified by size exclusion and adsorbed onto a silica membrane for elution using the SeqTarget Normalization kit (Qiagen). Purification and normalization was performed according to the manufacturer's instructions for microtiter-plate centrifuge and vacuum manifold utilization.

2.7.4.3 Sample and library preparation

Sample and library preparation was performed using the Nextera XT DNA Sample preparation kit (Qiagen). Tagmentation (fragmenting and tagging) of normalized DNA was simultaneously performed by the Nextera XT transposome according to the manufacturer's recommendations.

PCR amplification and the incorporation of indexes (i7 and i5) and sequences required for cluster formation was performed by initially selecting the necessary indexes to insure sample distinction post run. Indexing i7 1 – 12 were selected and combined with i5 1 – 3 to ensure unique tags for all individual samples to be processed (Table 2.2). Samples were centrifuged at 280 x g for 1 min and subsequently amplified in a thermocycler under the following conditions: 72°C for 3 mins, 95°C for 30s, followed by 12 cycles of 95°C for 10s, 55°C for 30s and 72°C for 30s. A final incubation step at 72°C for 5 min was then followed by immediate hold at 10°C until further processing.

Table 2.2: Indexing (i7 and i5) combinations added to each sample as unique identifiers

i5	i7											
S501	N701	N702	N703	N704	N705	N706	N707	N708	N709	N710	N711	N712
i5	i7											
S502	N701	N702	N703	N704	N705	N706	N707	N708	N709	N710	N711	N712
i5	i7											
S503	N701	N702	N703	N704	N705	N706	N707					

Tagmented samples were clean-up post PCR using AMPure XP beads according to the manufacturers' protocol (Beckman Coulter - Kloof, South Africa).

Library pooling was performed by transferring 5 μ l of each to a clean 1.5 ml Eppendorf tube, and mixed by pipetting. A 1: 24 dilution of the library was prepared by transferring 24 μ l of the pooled library to a clean 1.5 ml Eppendorf tube containing 576 μ l of HT1. As a positive control, the diluted library was spiked by supplementing 180 μ l of it with 120 μ l of 10pmol PhiX. The diluted library was loaded onto a thawed MiSeq reagent cartridge into the 'Load Samples' reservoir and sequenced according to the Illumina MiSeq System User Guide instructions. The sample sheet required for sequencing was setup to allow for paired-end sequencing with 2 X 250 reads. Amplicon sequencing methodology was selected and amplicon manifolds were created for the 5 target genes relative to *Homo Sapiens* UCSC Hg19 reference sequence. Upon completion of the run, the MiSeq Reporter Software automatically aligns the amplified sequences for the specified target genes to the UCSC Hg19 reference sequence using the Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009). Genome Analysis Toolkit (GATK) (Broad Institute) was used for variant calling. GATK calls raw variants for each sample read, analyzes them against known variants (online databases such as NCBI and Ensembl), and applies a calibration procedure to compute false discovery rates for each variant. Details of the algorithms used for these calibrations can be found at <http://www.broadinstitute.org/gatk>.

Due to the possibility of identifying a large number of polymorphisms within the individuals screened using the aforementioned tNGS platform; the identified polymorphisms were

prioritized based on their possibility to be informative with regards to an anxiety disorder diagnosis. Considering the preliminary nature of tNGS in this study (novelty of this kind of approach within anxiety disorders) and absence of available comparative datasets, further analyses was limited to those polymorphisms identified in either the cases or control individuals only.

2.8. Genotyping

Genotyping for the *BDNF* and *MMP9* TagSNPs identified was performed using TaqMan[®] Probe assays (Applied Biosystems) according the manufacturers' instructions. Genotyping of the polymorphisms identified using tNGS (*ARC*, *ERG2*, *EGR4*, *GRM2* and *NTF4*) was performed by KBiosciences (Herts, UK) using KASP[™] genotyping technology. TagSNP analysis was utilized in order to maximize informative output and minimize cost considering the cost of genotyping analyses and the financial constraints associated with this study. tNGS was also only carried out in subset of the cohort due to the cost associated with this kind of analyses. Ideally a much larger, if not the entire cohort, would have been the preferred target for polymorphism assessment using tNGS.

2.9. Statistics / data analysis

2.9.1. Behavioural data

Experimental rat groups (i.e. MS, RS and MS+RS) were compared in terms of their behaviour, relative to unstressed controls. Statistical analyses were performed with assistance from Prof Martin Kidd (Head of the Statistical Consultation Center at Stellenbosch University) using Statistica 10 (StatSoft Inc., 2011). MS, RS and MS+RS groups were compared to controls in order to assess deviation in terms of distance travelled (OFT), time spent within open arms (EPM) and immobility times (FST). Statistical correlation of groups with regards to a time effect were evaluated by means of mixed model repeated measures

ANOVA and group assessment by one-way ANOVA. In all cases Fisher LSD post hoc tests were performed.

2.9.2. Clinical data, genotyping and association testing

One of the aims of the present study was to assess whether the human homologues to genes that were differentially expressed in anxious rats could be associated with a diagnosis of OCD, SAD or PD in humans.

Hardy Weinberg Equilibrium was assessed in case and control individuals using RStudio (Version 0.97) in conjunction with the R Genetics package. The same package was also utilized to assess for significant association between SNPs identified and anxiety in a case-control association fashion, adjusting for age and gender. The additive, dominant and recessive genetics models were evaluated and the best fit selected. *p*-values were ascertained using the effect size displayed for the most appropriate model by running ANOVA general linear models, adjusting for the aforementioned covariates. To test for an interaction between CTQ total score and a SNP in anxiety disorders, an interaction term for CTQ total score and the polymorphism in question (CTQtot*SNP) was included in the generalized linear model (still adjusting for gender). The results of this glm (including a SNP-CTQ total score interaction consideration) was assessed using ANOVA to identify significant variation between cases and controls. Odds ratios (ORs) and confidence intervals (CIs) were calculated in R Genetics based on the anova.glm testing performed and calculated by imputing the anova.glm effect size generated for the significant *p*-values observed.

Haplotype analyses were performed using Haploview 4.2, where D' was assessed based on the methodology described by Gabriel et al., 2002 for block selection. Haploblocks were designated based on 95% confidence bounds on D' indicating strong linkage disequilibrium (LD). This method ignores markers with $MAF < 0.05$. This criteria was adjusted for genes in which 95% confidence bounds were not met due to SNPs interrupting LD within a gene, but for which LD appeared maintained by high D' beyond the relevant SNP(s) inclusion. LD obtained in Haploview was validated using the LD function from the R statistical genetics

package in RStudio (Version 0.97). Identified haplotypes were confirmed using the Haplostats statistical package in RStudio (Version 0.97), which was also used to calculate ORs. To assess for possible environmental - haplotype associated contributions to affection status, cases were subdivided into the top 33rd (high CTQ total score) and bottom 33rd percentile (low CTQ total score) for comparison against controls. This was performed in Haploview 4.2 according to the same methodology discussed above. All statistically significant haplotypes identified were analyzed in RStudio using the Haplo.stats and Genetics library packages and implementation of a haplotype generalized linear model (haplo.glm). Gender was considered as a covariate during glm fitment. ORs and confidence intervals (CIs) were calculated in R Genetics based on the haplo.glm testing performed and calculated by imputing the haplo.glm effect size generated for the significant p -values observed.

Student's t-test in GraphPad Prism Version 5.00 was used to assess for a significant difference in the distribution of CTQ total scores between cases and controls, as well as for the individual subcomponents of the CTQ scale (emotional abuse, emotional neglect, physical abuse, physical neglect and sexual abuse). The subcomponents of the CTQ, although assessed for significance, were not used in conjunction with the RStudio R Genetics package to assess for a gene – environment interaction with the identified polymorphisms. This was considered to control for problems associated with multiple testing, increasing the chance for possible Type I error with the inclusion of multiple variables (the 5 sub-components of the CTQ scale) between a two-group (case-control) comparisons. Considering CTQ total score is an additive representation of its subcomponents, only CTQ total score was considered for gene – environment interaction assessments within RStudio. To test for this aforementioned interaction, *i.e.* the severity of childhood trauma (as measured by the CTQ total score) with a particular SNP, interaction assessments were incorporated in the additive, dominant or recessive models utilized in the Genetics program of RStudio (Version 0.97) (as discussed above). Generalized linear models (glms) were fit considering affection status (case or control), gender, CTQ total score, the relevant polymorphism and the interaction of that polymorphism with the CTQ total score in relation to affection status. A binomial family object was applied for glm testing. The analyses of deviance produced by the applied glms were assessed for a significant p -value using the ANOVA for glm in R Genetics. The resulting output generated p -value associations before adjusting for the specified covariates, as well as when adjusted for gender. Statistical representation of the

interaction of CTQ total score between cases and controls while considering the presence of a bi-allelic SNP were also produced based on ANOVA analyses of the best fit glm. ORs and CIs were calculated in R Genetics based on the glm genotype testing performed and calculated by imputing the glm effective size generated for the significant p -value observed. ORs were considered to be representative of increased (>1) or decreased (<1) susceptibility to present with anxiety disorder with CIs indicative of the possible range of this prediction based on the data utilized.

Chapter 3

Results

3. Results

3.1. Animal work

3.1.1. Behavioural data

Behavioural data was assessed using the EthoVision video tracking software platform and data was statistically assessed (Statistica 10) using time-bin interval outputs for each group (MS, RS, RS-MS) relative to the controls (nRS nMS) as discussed in Chapter 2 (Section 2.9.1). As noted previously, 8 (instead of 13) rats per an experimental group were used for the RT² ProfilerTM array due to budgetary constraints. The same statistical approach (as described in 2.7.1), however, was performed for the full cohort of each experimental group (n = 13) as well as the 8 utilized for the RT² ProfilerTM Array to ensure the trend observed remains constant.

3.1.1.1. Forced Swimming Test (FST)

Levene's test for homogeneity of variances yielded a significant p -value of 0.043 for immobility times. This indicates that any differences in immobility observed between the stressed groups relative to the controls was not due to chance alone. Rats subjected to restraint stress alone showed the lowest immobility times, when compared to the three other cohorts (Figures 3.1 and 3.2), suggesting lowest anxiety ($p < 0.001$ in all cases). Rats exposed to maternal separation showed the highest immobility times, assumed to be indicative of highest anxiety, comparatively. A higher observable level of immobility (and therefore anxiety) was expected for the combination MS+RS cohort compared to the MS cohort, however, their times were not significantly different from that of the MS group ($p = 0.73$ for all rats and $p = 0.53$ for those rats used in the RT² Profiler Array) (Figures 3.1 and

3.2). Considering the different stress-groups in their entirety (for observable trend, RS → MS → MS+RS) for a better statistical power, significantly different immobility times were observed between the RS group and controls ($p = 0.004$) and MS and MS+RS groups and the controls ($p < 0.001$ and $p = 0.001$, respectively), but not between MS and MS+RS groups (Table 3.1). The MS and MS+RS groups also differed significantly from the rats exposed to restraint stress only (Table 3.1).

Table 3.1: Fisher's LSD test for variability in immobility times between stressor-groups for the forced swimming (FST) test

GROUP	Immobility mean (sec)	+SD (sec)	nRS nMS (p-value)	RS nMS (p-value)	nRS MS (p-value)	RS MS (p-value)
nRS nMS	116.750	9.981	-	0.005	0.001	0.002
RS nMS	91.923	24.700	0.005	-	0.000	0.000
nRS MS	147.250	19.377	0.001	0.000	-	0.735
RS MS	144.384	25.382	0.002	0.000	0.735	-

*nRS nMS = controls; RS nMS = RS; nRS MS = MS; RS MS = RS-MS; SD = standard deviation

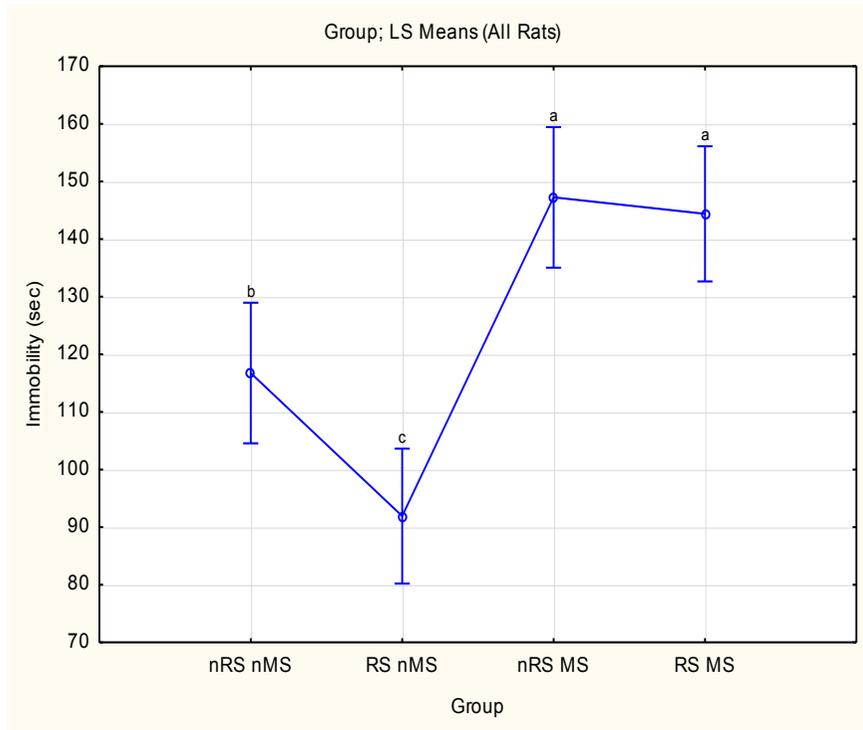
Immobility times for Forced Swimming (FST) Test

Figure 3.1: Least squares (LS) mean immobility values for restraint-stress (RS nMS), maternal-separation (nRS MS), combination restraint-maternal separation (RS MS) and controls (nRS nMS). Statistically significant differences are indicated by letters a, b and c with differing letters showing significant differences between those groups.

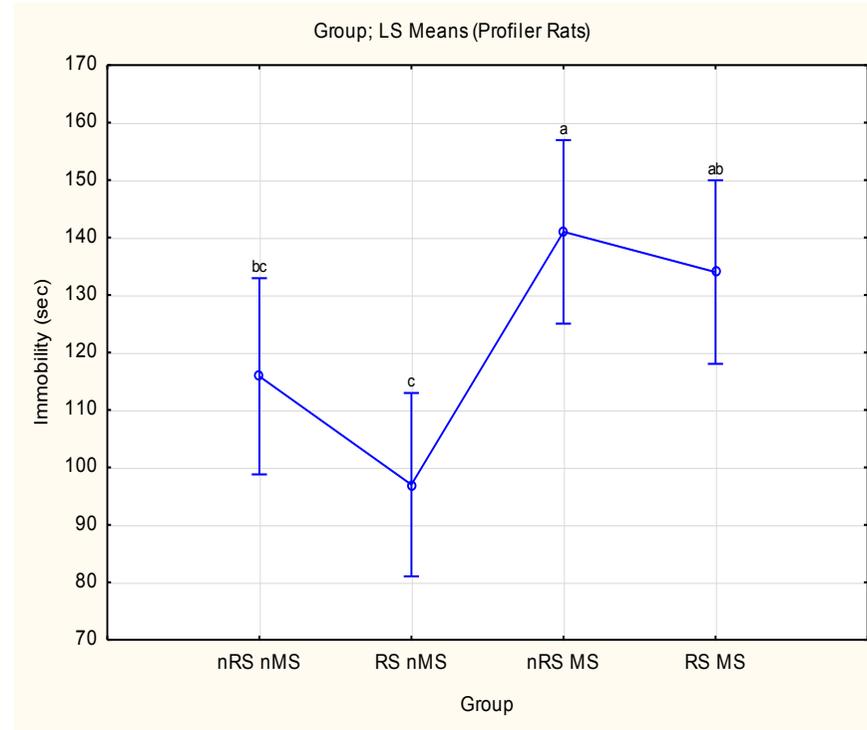
Immobility times for Forced Swimming (FST) Test

Figure 3.2: Least square (LS) mean immobility values for restraint-stress (RS nMS), maternal-separation (nRS MS), combination restraint-maternal separation (RS MS) and controls (nRS nMS). Statistical significances are indicated by letters a, b and c with differing letters showing significant differences between those groups.

3.1.1.2. Open-Field Test (OFT)

In the OFT test, a greater distance travelled can be indicative of an increased level of anxiety; as a greater distance needs to be travelled for rats to remain within the periphery of the experimental area (George et al., 2010). The periphery represents a more controlled space and time spent within the periphery is associated with increased stress and a reluctance to explore (move within the center zone with all sides exposed). The control group showed the lowest mean values for total distance travelled, indicative of a less anxious phenotype than rats exposed to an environmental stressor (Figure 3.3). Significant p values were found when comparing RS nMS rats to controls ($p < 0.001$) as well as combination RS MS ($p = 0.007$) (Table 3.2). nRS MS and RS MS rats were not statistically different from one another with regards to the total distance travelled ($p = 0.112$) but were shown to be significantly different from the controls and restraint stress groups in this regard (Table 3.2). As seen with the FST, profiler rats showed a similar trend for total distance travelled when compared to statistics representative of all rats used (Figure 3.3). In summary, from observations in the OFT, nRS MS and RS MS rats therefore showed the highest levels of anxiety-like behaviours (as measured by total distance travelled values), but were not found to be significantly different from each other. RS nMS showed the least anxious phenotype, however, this was still found to be significantly higher than the control group.

Table 3.2: Fisher's LSD test for variability in total distance travelled between stressor-groups for the open field test (OFT)

GROUP	TDT mean (cm)	+SD (cm)	nRS nMS (p -value)	RS nMS (p -value)	nRS MS (p -value)	RS MS (p -value)
nRS nMS	545.743	17.147		0.000	0.226	0.007
RS nMS	575.468	17.147	0.000		0.001	0.073

nRS MS	660.126	17.847	0.226	0.001	0.112
RS MS	614.722	17.147	0.007	0.073	0.112

*nRS nMS = controls; RS nMS = RS; nRS MS = MS; RS MS = RS-MS; TDT = total distance travelled

Assessment of the duration spent within the center zone indicated that the control group spent the most time in this area, compared to stress-applied groups and this was used as base-line anxiety (low anxiety) (Figure 3.5), and observed to be the lowest compared to the other groups. RS nMS rats showed the lowest mean time spent within the center zone compared to controls ($p = 0.028$) followed closely by the maternally separated rats.

Total distance travelled for Open Field Test (OFT)

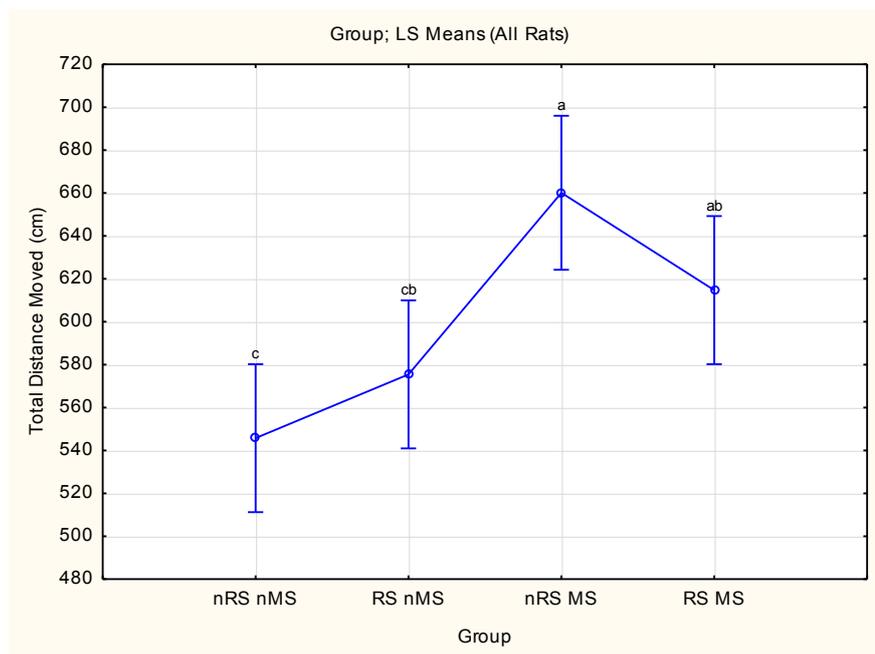


Figure 3.3: Least square (LS) mean total distance travelled values for restraint-stress (RS nMS), maternal-separation (nRS MS), combination restraint-maternal separation (RS MS) and controls (nRS nMS). Statistical significances are indicated by letters a, b and c with differing letters showing significant differences between those groups.

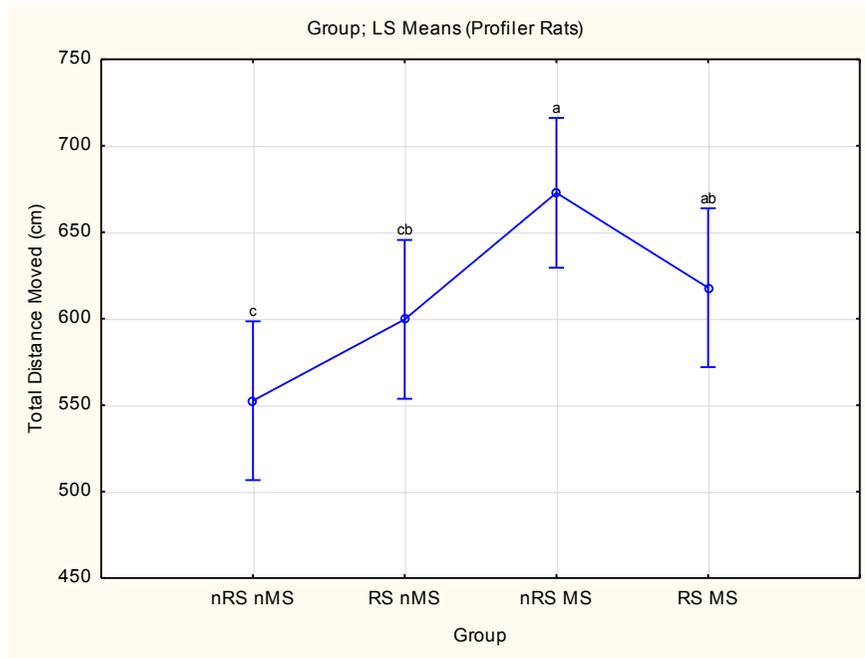
Total distance travelled times for Open Field Test (OFT)

Figure 3.4: Least square (LS) mean total distance travelled values for restraint-stress (RS nMS), maternal-separation (nRS MS), combination restraint-maternal separation (RS MS) and controls (nRS nMS). Statistical significances are indicated by letters a, b and c with differing letters showing significant differences between those groups.

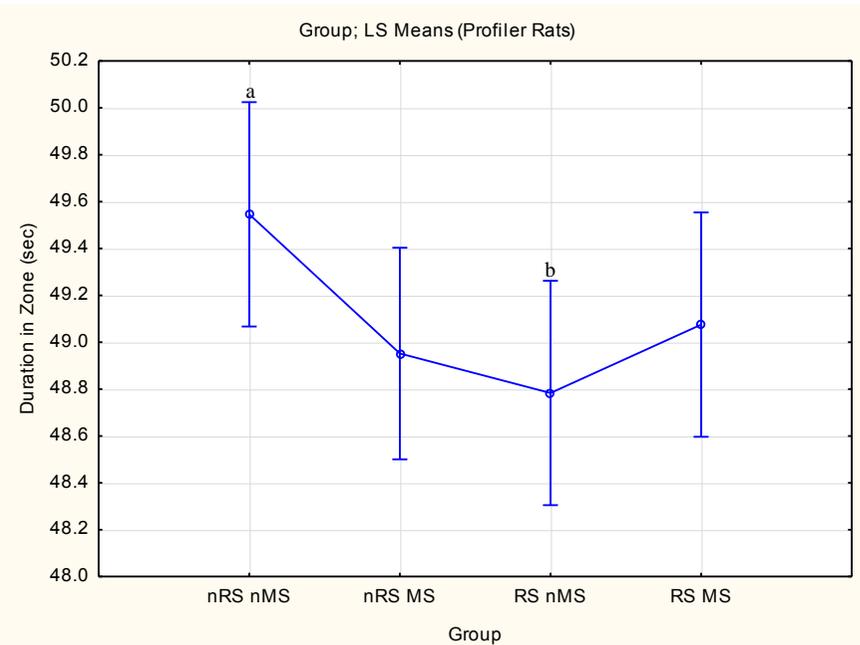
Duration within center zone for Open Field Test (OFT)

Figure 3.5: Duration within the center zone for restraint-stress (RS nMS), maternal-separation (nRS MS), combination restraint-maternal separation (RS MS) and controls (nRS nMS). Statistical significances are indicated by letters a and b, with differing letters showing significant differences between those groups.

*LS = least squares

3.1.1.3. Elevated-Plus Maze (EPM)

In the EPM test, increased time spent within the closed-arms is indicative of anxiety. When considering stressor groups and control rats together, there was an overall increase in preference for the closed arms (Figure 3.6) ($p < 0.001$).

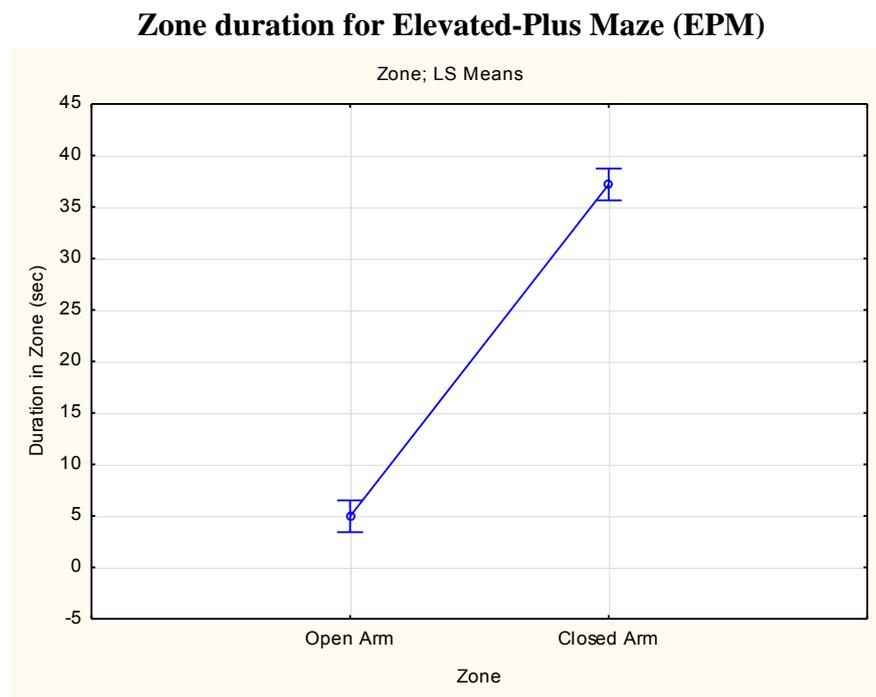


Figure 3.6: Least squares (LS) mean duration within closed and open arms for all rats.

3.2. RNA quantification and qualification

RNA was quantified and the integrity assessed using the Experion RNA StdSens RNA Analysis kit and Experion automated electrophoresis system (Biorad - Parklands, Johannesburg). An example of the digital RNA images indicating RNA quality can be seen below (Figure 3.7). RNA Quality Indicator (RQI) values of 7 or above were considered adequate for subsequent cDNA conversion (Appendix II) (Denisov et al., 2008).

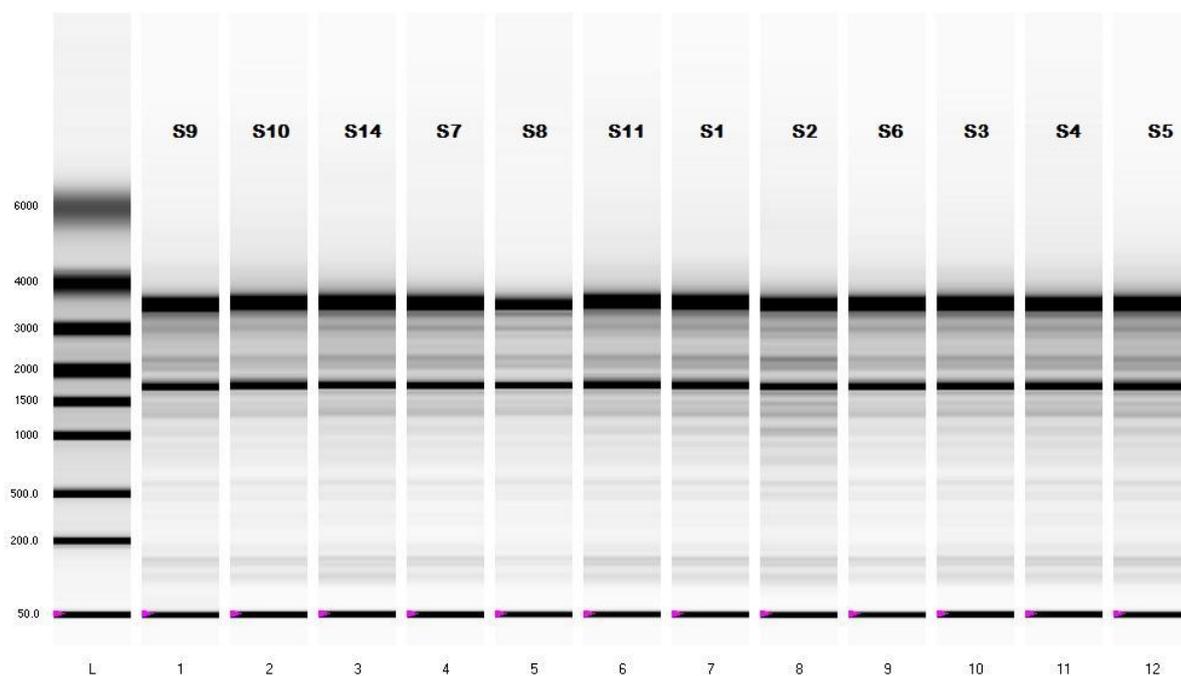


Figure 3.7: Digital gel image obtained using Experion machine (Biorad – Parklands, Johannesburg) indicating the quality of RNA extracted from rat striatal tissue samples. Experion RNA ladder used. “S(n)” refers to striatal sample and sample number.

3.3. RT² ProfilerTM Array, Synaptic Plasticity Pathway

cDNA synthesis was performed using the RT² First Strand Kit (Qiagen) to ensure optimal results when using the RT² Profiler Array. cDNA quality was assessed using the RT² QC Array according to the manufacturer's instructions. All samples passed quality control.

The expression of 84 genes involved in the synaptic plasticity pathway was assessed using the Rat Synaptic Plasticity PCR Array, PARN – 126Z (SABiosciences, Germany). A total of 8 rats for each group (*i.e.* RS nMS, nRS MS, RS MS and controls) were utilized in the PCR array analysis based on a combination of the best behavioural data assessment (*i.e.* rats that showed the highest immobility, total distance travelled and duration within closed arm scores in FST, OFT and EPM, respectively, relative to controls), RQI assessment values, and QC array scores (according the manufacturer's recommendations). Analysis was performed online at <http://www.sabiosciences.com/pcrarraydataanalysis.php> according to the manufacturer's instructions.

Several genes were found to be differentially expressed in the stress-exposed rats compared to the control rat group. Candidate susceptibility genes were subsequently selected on a basis of differential expression values greater than or equal to 1.7-fold (Table 3.3 and Figure 3.8) (Wurmbach et al., 2002; Pfaffl et al., 2003). These were *Bdnf*, *Mmp9*, *Arc*, *Grm2*, *Egr4*, *Egr2* and *Ntf4*.

Table 3.3: Fold-regulation representing differential expression of 1.7-fold or greater for RS nMS, nRS MS and RS MS rats, compared to controls

EXPERIMENT	GENE	FOLD +/-
nRS MS vs. nRS nMS	<i>Mmp9</i>	+ 1.80
	<i>Bdnf</i>	+ 1.97
	<i>Egr4</i>	+ 2.20
	<i>Arc</i>	+ 1.80
RS nMS vs. nRS nMS	<i>Bdnf</i>	+ 3.22

MS RS vs. nRS nMS	<i>Egr4</i>	+ 2.15
	<i>Arc</i>	+ 1.90
	<i>Ntf4</i>	+ 2.19
	<i>Bdnf</i>	+ 3.33
	<i>Egr2</i>	+ 2.04
	<i>Egr4</i>	+ 2.14
	<i>Arc</i>	+ 2.12
	<i>Ntf4</i>	+ 2.65
	<i>Grm2</i>	- 1.80

*(+) = up-regulation; (-) = down-regulation relative to the control (nRS nMS) group

Synaptic Plasticity Fold-Regulation

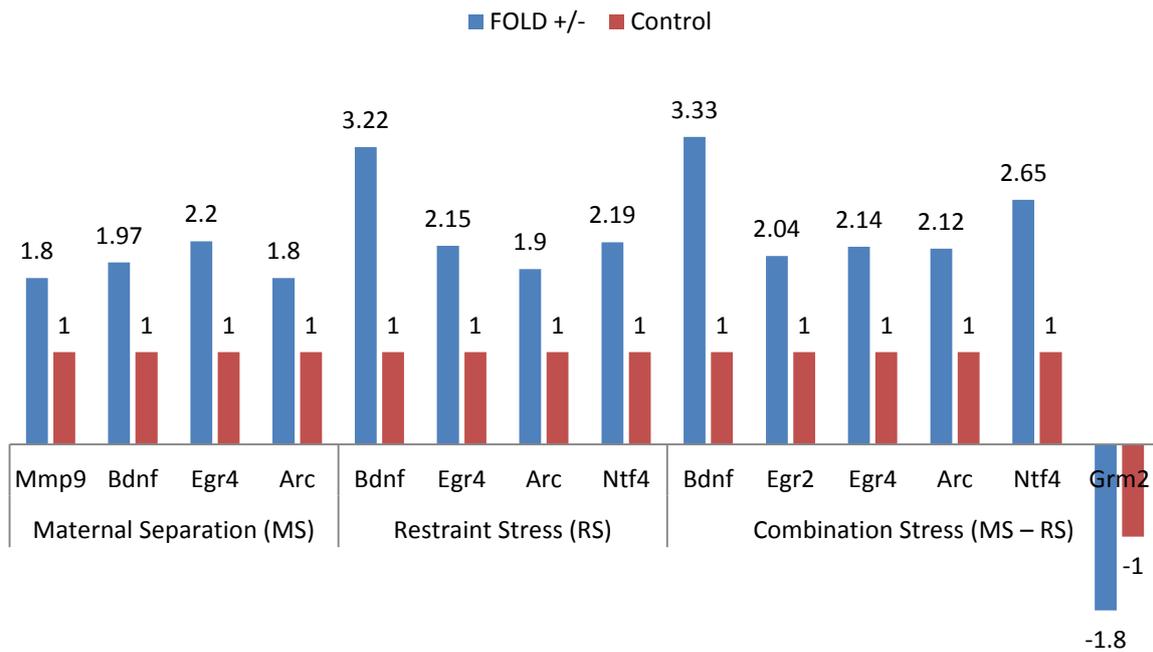


Figure 3.8: Graphical representation of fold-regulation increases and decreases in gene expression from the synaptic plasticity pathway, normalized to 1 (relative to control rats) (as depicted in Table 3.3). Fold-regulation values >1.7-fold were considered and are depicted.

3.4. Human Work

3.4.1. Clinical data

The human cohort comprised 92 cases (52 with a primary diagnosis of OCD, 18 with PD and 22 with SAD) and 194 healthy controls for which information regarding age, sex, comorbidity and childhood trauma was available. Ages at interview, as well as the ages of males and females, were similar between cases and control groups (Table 3.4). The mean total CTQ score (CTQtot) for patients (43.84 +/- 16.97) was significantly higher than controls (34.60 +/- 11.64) ($p < 0.001$, unpaired *t*-test; Welch's correction), suggesting that patients reported significantly higher levels of childhood trauma compared to controls (Table 3.4 and Figure 3.9). The control cohort was not interviewed and is representative of the general population (and matched with patients in terms of ethnicity), but as a convenience cohort. As an assumed representative of the general population, they were taken as a gross approximation of the general population with regards to the investigated conditions (OCD, SAD and PD). Comorbidity with major depressive disorder (MDD) was assessed within the patient group, but not controls. The same assumptions were therefore made for MDD amongst the control cohort, i.e. similar rates of MDD as the average population.

Table 3.4: Demographic and clinical characteristics of patient and control cohorts

Variable	Patients	Controls	<i>p</i> -value
	92	194	-
Male (n) (%)	33 (36)	83 (43)	-
Female (n) (%)	58 (64)	111 (57)	-
Age (yrs. +/- SD)	36.82 +/- 13.37	36.60 +/- 13.49	-
Comorbid MDD (n) (%)	65 (70)	NA	-
CTQea mean (SD)	10.31 +/- 5.54	7.38 +/- 3.32	< 0.001
CTQpa mean (SD)	7.73 +/- 3.90	6.68 +/- 2.83	0.014
CTQsa mean (SD)	7.63 +/- 5.06	5.78 +/- 2.60	< 0.001

CTQen mean (SD)	11.39 +- 5.14	8.2 +- 3.76	< 0.001
CTQpn mean (SD)	6.77 +- 2.87	6.55 +- 2.78	0.539
CTQtot mean (SD)	43.84 +- 16.97	34.60 +- 11.64	< 0.001

**CTQea* = emotional abuse; *CTQen* = emotional neglect; *CTQpa* = physical abuse; *CTQpn* = physical neglect; *CTQsa* = sexual abuse; *CTQtot* = total CTQ score; *MDD*: =major depressive disorder; *n* = number of; *NA* = not assessed; *SD* = standard deviation; *p*-values indicated statistically significant differences between patients and controls for the components of the CTQ scale

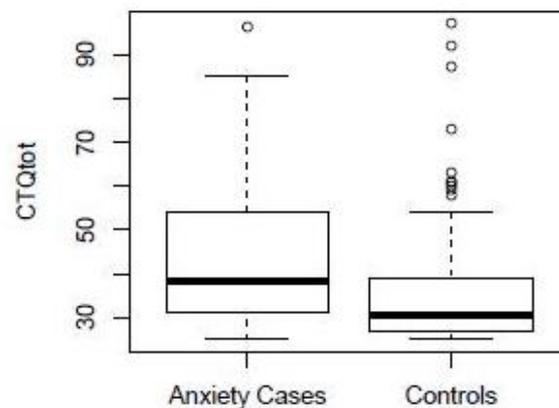


Figure 3.9: Box-plot representation of CTQtot score differences between cases and control cohorts. CTQtot scores were significantly different between cases and controls ($p < 0.001$)

3.4.1.1. CTQ scale interaction

CTQ scores of patients and controls were compared using Student's *t*-test. The groups differed significantly on all subscales except for physical neglect (Table 3.4). As noted above, total severity of childhood trauma was significantly worse in patients compared to controls ($p < 0.001$). Table 3.4 indicates variables considered as possible covariates for inclusion in subsequent statistical analyses (gene-environment correlation and interaction). As indicated, CTQ total score (a cumulative representation of the sub-components to be used

for consideration in further analyses) meets criteria for covariate inclusion (statistically significance in cases compared to controls).

3.4.2. TagSNP identification

Genes identified to be differentially expressed via the RT² Profiler Array were assessed using a TaqSNP approach. The HapMap project online database (www.hapmap.org), using HapMap genome browser release #27 (Phase II & III – merged genotypes and frequencies) on Feb09, on NCBI B36 assembly, dbSNP b126 was utilized to identify tagSNPs (MAFs of ≥ 0.2 and $R^2 \geq 0.8$ used at cut-offs) in the human homologues of *Bdnf* and *Mmp9* (Table 3.5), *BDNF* and *MMP9*. The CEU (Utah residents with ancestry from Northern and Western Europe) population was used in TagSNP identification and all the identified TagSNPs were selected for inclusion in this study. The HapMap database did not possess sufficient population-specific genotyping information for the remaining 5 gene homologues (*NTF4*, *ARC*, *GRM2*, *EGR2* and *EGR4*).

Table 3.5: TagSNPs selected for *BDNF* and *MMP9* screening based on CEU

GENE	TagSNPs	Other alleles Captured
<i>BDNF</i>	rs6265	None
	rs925948	None
	rs7103411	rs6484320
	rs11030104	rs16917237
	rs2049045	None
		rs11030108
	rs1401635	rs1013402
		rs7124442
		rs7127507
	rs10835210	rs7103873
		rs11030101
rs11030107	rs10835211	

		rs11030102
	rs11030109	None
	rs28383487	n/a
	rs2274755	None
	rs2236416	rs2274756
		rs3918261
	rs17576	rs3918249
	rs3918253	rs3918256
<i>MMP9</i>	rs1805088	None
	rs3787268	None
	rs3918242	n/a
	rs17577	n/a
	rs13925	n/a

* *MAFs* of ≥ 0.2 and $R^2 \geq 0.8$ used at cut-offs; *CEU*: Utah residents with Northern and Western European decent according to the HapMap database.

3.4.3. SMART assessment and inter-species conservation

The remaining 5 genes were subjected to SMART assessment to identify core domain regions in the proteins for which they encode. Core domains were identified in 4 of the 5 candidate proteins. The *ARC* gene was subjected to an inter-species conservation assessment after no core domains were identified for the *ARC* protein. A 301 bp (mouse) and 326 bp (rat) region involving the immediate 3'UTR promoter region, as well as the first exon, was found to be highly conserved when related to the *Homo sapiens* species (Table 3.6).

Table 3.6: Core domains and inter-species conservation sites in *EGR2*, *EGR4*, *NTF4*, *GRM2* and *ARC*

Gene	Domain	Amino acid	bp	Polymorphisms	Type
<i>EGR2</i>	Zn_C2H2	340 - 364	72	CM993142	coding unknown

				rs104894161	nonsynonymous
				rs147417827	nonsynonymous
				rs45602133	synonymous
	Zn_C2H2	370 - 392	66	CM004043	coding unknown
				rs104894160	nonsynonymous
	Zn_C2H2	398 - 420	66	rs104894159	nonsynonymous
				rs121434563	nonsynonymous
EGR4				rs145518054	synonymous
				rs147950764	synonymous
	Zn_C2H2	380 - 404	72	rs7558708	synonymous
				rs139071376	synonymous
				rs146218927	nonsynonymous
	Zn_C2H2	410 - 432	66	rs146026377	synonymous
	Zn_C2H2	438 - 460	66	rs149141742	nonsynonymous
				rs141733943	nonsynonymous
NTF4				rs149008630	synonymous coding
				rs145846156	nonsynonymous
				rs144842220	nonsynonymous
				rs62127897	synonymous
				rs34922845	frameshift
	NGF	89 - 202	339	rs140946623	nonsynonymous
				rs147294437	synonymous
				rs144346396	nonsynonymous
GRM2	Transmembrane	567 - 589	66	rs1054625	synonymous
	Transmembrane	602 - 624	66	None	-
	Transmembrane	634 - 651	51	rs150657954	synonymous
				rs61729081	nonsynonymous
	Transmembrane	678 - 700	66	rs138903693	nonsynonymous
				rs61729078	synonymous
				rs144440968	synonymous
	Transmembrane	728 - 747	57	rs148405091	synonymous
				rs149954328	synonymous
			rs145802255	nonsynonymous	

	Transmembrane	760 - 782	66	rs143499566	synonymous
	Transmembrane	797 - 819	66	rs149853208	nonsynonymous
Inter-species conservation analysis					
Gene	Species	Location		bp	
ARC	Conserved Region	chr8: 143692410 -			
	Mouse (<i>mm9</i>) - exon & UTR	143692710		301	
		(80.1% Identity)			
	Conserved Region	chr8: 143692410 -			
	Rat (<i>rn4</i>) - exon and UTR	143692735		326	
		(77.9% Identify)			

**chr8* = chromosome 8; *Zn_C2H2* = Zinc-finger binding domain; *NGF* = nerve growth factor; *UTR* = un-translated region

A graphical representation of the conserved regions identified for the *ARC* specified genomic region (chr8: 143692410 – 143695833) can be seen in Figure 3.10 (ECRbrowser V 2.0) (Ovcharenko et al., 2004). The figure highlights both core evolutionary conserved regions (core ECRs) (A), which comprised UTR and coding exonic regions; as well as the full ECR annotation for the gene (B).

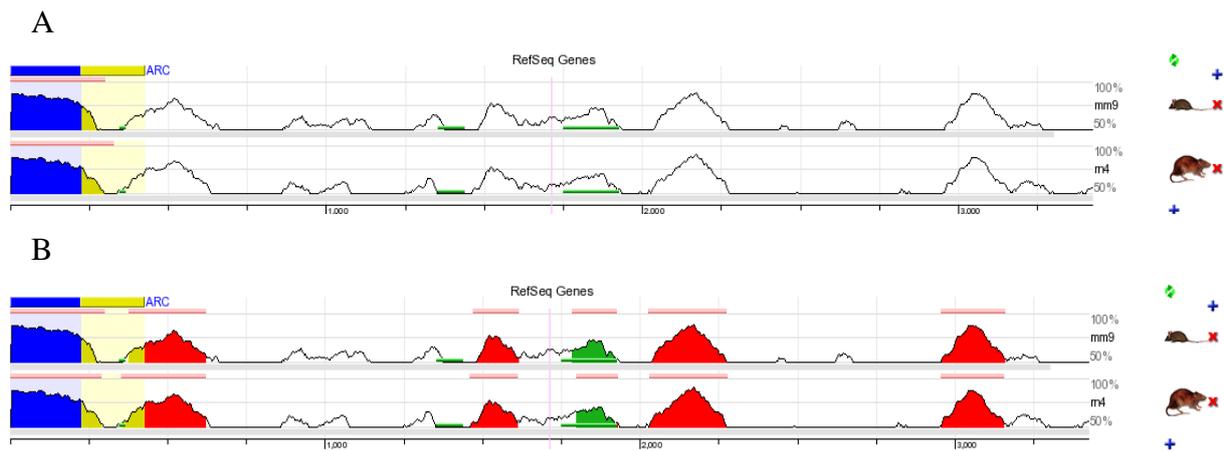


Figure 3.10: Graphical representation of sequencing conservation between human, rat and mouse species for *ARC* using rVista and ECRbrowser (<http://rvista.dcode.org> and <http://ecrbrowser.dcode.org>). The X-axis represents the genomic region and Y-axis the percentage sequence conservation for the right-margin indicated species, relative to *Homo sapiens*. A: core evolutionary conserved regions (ECRs) only. B: all ECRs. *Blue: coding exons; Yellow: UTRs; Red: intergenic regions; Green: transposons and simple repeats; Pink: ECRs

3.5. Next-generation sequencing (NGS)

Next-generation sequencing yielded 6.4 gigabases (G) total data, more than 75% of which had a Q-Score greater than 30 (error rate of 1 in 1000) (Table 3.8). Initial alignment of PhiX spike was at ~ 30% meaning the remaining ~70% of data pertained to the pooled library samples.

3.5.1. Variation

Variant calling was performed according to the default settings of the MiSeq Reporter 2.1 software (Illumina). MiSeq Reporter software v 2.1 utilizes algorithms which transform Phred-scaled [Phil Green's PHRED base-calling software where $\text{PhredScore} = -10\log(10C)$] genotype likelihoods into variation scores that GATK (Genome Analysis Toolkit) can use

internally. This allows for reliable and accurate quality (Q) scores (Quail et al., 2012). A total of 300 SNPs and 76 insertions/ deletions (indels) were identified across the five amplicons amongst the 12 patients and 19 controls (see Section 2.7.4) subjected to NGS. Of these, 103 SNPs and 9 indels passed all default filter criteria set by the MiSeq Reporter software (according to the manufacturer's recommendations and a minimum of 10X read depth per included SNP). The quality control and yields are presented in Table 3.7. Furthermore, 38 SNPs and 4 indels were found to be unique to either patient or control individuals only. These variants can be seen in Table 3.8 below. The frequency listed refers to the frequency of that variant at that particular coverage depth (referred to as 'Depth' in Table 3.8). Only 7 of the 42 (16.67%) variations identified were classified on the dbSNP and/ or alternative databases (Ensembl and 1000 Genomes). The remaining 83.33% of unique pass variation identified was therefore novel.

Table 3.7: MiSeq Analysis Software NGS paired-end run summary indicated total yield data and quality (Q30) of data produced

Lane	Yield Total (G)	Projected Total Yield (G)	Yield Perfect (G)	Yield <= 3 errors (G)	Aligned (%)	% Perfect [Num Cycles]	% <= 3 errors [Num Cycles]	Error Rate (%)	Intensity Cycle 1	% Intensity Cycle 20	% >= Q30
Read 1	3.1	3.1	0.6	0.9	29.98	67.2 [250]	91.7 [250]	1.17	121	140.1	75.3
Read 2	0.1	0.1	0.0	0.0	0.00	0.0 [7]	0.0 [7]	0.00	339	0.0	64.1
Read 3	0.1	0.1	0.0	0.0	0.00	0.0 [7]	0.0 [7]	0.00	93	0.0	66.7
Read 4	3.1	3.1	0.5	0.8	28.94	53.2 [250]	84.3 [250]	1.51	117	130.2	59.9
Total	6.4	6.4	1.1	1.6	29.46	60.3	88.1	1.34	168	135.1	67.5

*G = gigabases; Num = number

Table 3.8: Variation identified with NGS unique to either patient or control individuals only

Sample	Chromosome	Location	Type	Variation	Frequency	Depth	dbSNP	MAF current/dbSNP	Gene	Intron/ Exon/ UTR
Patient	chr19	49563625	SNP	T> <u>G</u>	0.22	54	novel		NTF4	3' UTR
Patient	chr19	49565418	SNP	G> <u>A</u>	0.52	250	rs77113200	0.04/0.12	NTF4	Intron 1
Patient	chr19	49565472	SNP	C> <u>I</u>	0.24	58	novel		NTF4	Intron 1
Patient	chr19	49565762	SNP	C> <u>I</u>	0.46	249	novel		NTF4	Intron 1
Patient	chr3	51741114	SNP	C> <u>G</u>	0.25	16	novel		GRM2	Exon 1 (NonCoding)
Patient	chr3	51741512	SNP	T> <u>G</u>	0.41	245	novel		GRM2	Intron 1

Patient	chr3	51743789	SNP	G> <u>A</u>	0.22	88	<i>novel</i>		GRM2	Intron 2
Patient	chr3	51743972	SNP	G> <u>A</u>	0.27	15	<i>novel</i>		GRM2	Intron 2
Patient	chr3	51744568	SNP	G> <u>T</u>	0.48	182	<i>novel</i>		GRM2	Intron 2
Patient	chr3	51745321	SNP	C> <u>T</u>	0.47	249	rs149905463	0.02/0.0006	GRM2	Intron 2
Patient	chr3	51746173	SNP	C> <u>T</u>	0.23	26	<i>novel</i>		GRM2	Intron 2
Patient	chr3	51746239	SNP	G> <u>A</u>	0.41	157	rs113426529	0.05/0.011	GRM2	Intron 2
Patient	chr3	51744609	Indel	AG> <u>--</u>	0.36	159	rs140701867		GRM2	Intron 2
Patient	chr3	51748891	Indel	TT> <u>--</u>	0.26	249	<i>novel</i>		GRM2	Intron 3
Control	chr19	49563243	SNP	C> <u>A</u>	0.33	21	<i>novel</i>		NTF4	3' UTR
Control	chr19	49563812	SNP	C> <u>G</u>	0.26	35	<i>novel</i>		NTF4	3' UTR
Control	chr19	49564944	SNP	A> <u>C</u>	0.32	247	<i>novel</i>		NTF4	Exon2
Control	chr19	49565936	SNP	G> <u>T</u>	0.24	21	<i>novel</i>		NTF4	Intron 1
Control	chr19	49566491	SNP	C> <u>T</u>	0.21	33	<i>novel</i>		NTF4	Exon 1
Control	chr19	49566492	SNP	A> <u>G</u>	0.21	33	<i>novel</i>		NTF4	Exon 1
Control	chr19	49566727	SNP	A> <u>C</u>	0.23	248	<i>novel</i>		NTF4	Exon 1
Control	chr19	49567376	SNP	G> <u>A</u>	0.65	250	rs34619524		NTF4	3' UTR
Control	chr3	51741138	SNP	C> <u>T</u>	0.42	166	rs149387441	0.06/0.019	GRM2	Exon 1 (NonCoding)
Control	chr3	51745540	SNP	C> <u>T</u>	0.59	250	<i>novel</i>		GRM2	Intron 2
Control	chr3	51747245	SNP	A> <u>C</u>	0.26	248	<i>novel</i>		GRM2	Exon 3
Control	chr3	51747288	SNP	G> <u>A</u>	0.45	227	rs116567227	0.02/0.01	GRM2	Exon 3
Control	chr3	51749957	SNP	A> <u>C</u>	0.29	245	<i>novel</i>		GRM2	Exon 4
Control	chr3	51750091	SNP	A> <u>C</u>	0.26	247	<i>novel</i>		GRM2	Exon 4
Control	chr3	51751642	SNP	A> <u>C</u>	0.23	247	<i>novel</i>		GRM2	Intron 4
Control	chr3	51751994	SNP	A> <u>C</u>	0.29	245	<i>novel</i>		GRM2	Intron 5
Control	chr10	64575010	SNP	T> <u>C</u>	0.39	247	<i>novel</i>		EGR2	Intron 1
Control	chr10	64575034	SNP	A> <u>C</u>	0.29	248	<i>novel</i>		EGR2	Intron 1
Control	chr10	64575357	SNP	A> <u>C</u>	0.24	247	<i>novel</i>		EGR2	Intron 1
Control	chr10	64575417	SNP	T> <u>C</u>	0.64	250	rs7893472	0.04/0.14	EGR2	Intron 1

Control	chr10	64576915	SNP	A> <u>C</u>	0.3	244	<i>novel</i>	EGR2	5' UTR
Control	chr10	64577757	SNP	A> <u>G</u>	0.48	247	<i>novel</i>	EGR2	5' UTR
Control	chr10	64577870	SNP	G> <u>A</u>	0.45	62	<i>novel</i>	EGR2	5' UTR
Control	chr10	64577871	SNP	C> <u>A</u>	0.41	63	<i>novel</i>	EGR2	5' UTR
Control	chr10	64577986	SNP	A> <u>G</u>	0.21	19	<i>novel</i>	EGR2	5' UTR
Control	chr2	73519479	SNP	A> <u>G</u>	0.46	11	<i>novel</i>	EGR4	Exon 2
Control	chr19	49566045	Indel	--> <u>GT</u>	0.29	17	<i>novel</i>	NTF4	Intron 1
Control	chr19	49568047	Indel	-> <u>C</u>	0.47	209	<i>novel</i>	NTF4	5' UTR

*chr3/19/10/2 = chromosome 3/ 19 /10/2; Indel = insertion or deletion; SNP = single nucleotide polymorphism; UTR = un-translated region;

MAF = minor alleles frequency (dbSNP)

*'Frequency' refers to that of the variant allele and 'Depth' refers to the read depth coverage for that particular variant

* Variant is underlined in 'Variation' column

3.5.2. Genotyping

Genotyping was performed for the 92 patients and 194 controls for which CTQ score data was available as well as in an expanded sample of 626 patients and 638 controls (without CTQ data). As noted previously, there were significantly higher numbers of females in both the patient and control cohorts; gender was thus entered as covariate into the analyses.

In the following sections, I will firstly report on the bi-allelic loci association testing, and then GxE correlations and GxE interactions.

3.5.2.1. Bi-allelic loci association testing

Table 3.9 depicts the results of case-control association analyses for unadjusted, adjusted and interaction with CTQ total score for TaqSNPs and NGS SNPs considered, and genotyped in the extended genotyping cohort. Of the 42 polymorphisms identified using NGS and the 19 SNPs using a TagSNP approach, 26 were found to be informative and were considered for further analyses (Table 3.9). Statistically significant associations were observed for 5 SNPs (*MMP9*: rs3918242, rs1805088; *BDNF*: rs6265 (Val66Met), rs10835210; and *GRM2*: rs116567227). Deviation from Hardy Weinberg Equilibrium (HWE) was observed for SNPs rs1805088 and could not be calculated for rs116567227 due to its low frequency within the screened cohort. It is generally accepted that HWE deviation in cases (where HWE is met within controls), points to possible association of that polymorphism with the specific disorder under investigation. This was observed for SNP rs2049045 in *BDNF* only. For *MMP9*'s rs17576 and *BDNF*'s rs925948 HWE was found to be met within the cases cohort, but deviated in controls. *MMP9*'s rs1805088 presented with HWE deviation in both the case and control groups. The increased frequency for the CC genotype (ancestral) within both cases and controls appears responsible for the skew.

Table 3.9: Genotype, allele frequencies and significant associations for polymorphisms in anxiety disorder cases vs. healthy controls, as well as CTQ total score interaction with designated polymorphisms on anxiety disorders diagnosis

	Controls		HWE	Anxiety Cases		HWE	Association P-Val		CTQtot Interaction ^e
	Count ^a	Freq ^b	P-Val	Count ^a	Freq ^b	P-Val	Unadjusted ^c	Adjusted ^d	
<i>MMP9</i>									
rs3918242	210		1	279		0.24			
C/C	201	0.96		231	0.83		0	0	0.179
C/T	9	0.04		48	0.17				
C	411	0.98		510	0.91		0	0	0.179
T	9	0.02		48	0.09				
rs17577	183		0.170	331		0.352			
G/G	142	0.78		249	0.75		0.766	0.775	0.127
G/A	36	0.2		74	0.22				
A/A	5	0.03		8	0.02				
G	320	0.87		572	0.86		0.651	0.651	0.894
A	46	0.13		90	0.14				
rs2274755	170		0.312	313		0.439			
G/G	131	0.77		242	0.77		0.95	0.96	0.134
G/T	35	0.21		65	0.21				
T/T	4	0.02		6	0.02				
G	297	0.87		549	0.88		0.879	0.906	0.73
T	43	0.13		77	0.12				
rs2236416	186		0.314	332		0.619			
A/A	145	0.78		255	0.77		0.896	0.902	0.104
A/G	37	0.2		71	0.21				
G/G	4	0.02		6	0.02				
A	327	0.88		581	0.88		0.852	0.835	0.541
G	45	0.12		83	0.12				
rs17576	187		0.017	325		0.713			
A/A	84	0.45		137	0.42		0.081	0.072	0.235
A/G	71	0.38		151	0.46				
G/G	32	0.17		37	0.11				
A	239	0.64		425	0.65		0.641	0.671	0.453
G	135	0.36		225	0.35				
rs3918253	183		0.066	326		0.427			

T/T	70	0.38		107	0.33		0.121	0.145	0.695
T/C	76	0.42		166	0.51				
C/C	37	0.2		53	0.16				
T	216	0.59		380	0.58		0.821	0.725	0.914
C	150	0.41		272	0.42				
rs1805088	201		0	362		0			
C/C	166	0.83		325	0.9		0.002	0.002	0.309
C/T	23	0.11		13	0.04				
T/T	12	0.06		24	0.07				
C	355	0.88		663	0.92		0.168	0.164	0.158
T	47	0.12		61	0.08				
rs3787268	172		0.097	308		0.316			
G/G	103	0.6		191	0.62		0.628	0.634	0.309
G/A	55	0.32		99	0.32				
A/A	14	0.08		18	0.06				
G	261	0.76		481	0.78		0.453	0.476	0.925
A	83	0.24		135	0.22				
rs13925	177		0.180	331		0.33			
G/G	136	0.77		252	0.76		0.928	0.934	0.102
G/A	36	0.2		71	0.21				
A/A	5	0.03		8	0.02				
G	308	0.87		575	0.87		0.949	0.959	0.644
A	46	0.13		87	0.13				
BDNF									
rs6265	210		0.236	370		0.109			
G/G	145	0.69		173	0.47		0	0	0.007
G/A	56	0.27		170	0.46				
A/A	9	0.04		27	0.07				
G	346	0.82		516	0.7		0	0	0.034
A	74	0.18		224	0.3				
rs925948	125		0.012	346		1			
G/G	123	0.98		344	0.99		0.255	0.258	NA
G/A	1	0.01		2	0.01				
A/A	1	0.01		0	0				
G	247	0.99		690	1		0.182	0.175	NA
A	3	0.01		2	0				
rs7103411	180		0.826	330		1			
T/T	111	0.62		227	0.69		0.181	0.162	0.924

T/C	60	0.33	94	0.28			
C/C	9	0.05	9	0.03			
T	282	0.78	548	0.83	0.068	0.06	0.589
C	78	0.22	112	0.17			
rs11030104	150		0.795	252		0.831	
A/A	98	0.65	170	0.67	0.774	0.758	0.402
A/G	46	0.31	75	0.3			
G/G	6	0.04	7	0.03			
A	242	0.81	415	0.82	0.552	0.524	0.67
G	58	0.19	89	0.18			
rs2049045	162		0.051	306		0.02	
G/G	117	0.72	236	0.77	0.244	0.239	0.023
G/C	45	0.28	70	0.23			
G	279	0.86	542	0.89	0.244	0.239	0.023
C	45	0.14	70	0.11			
rs1401635	164		0.144	306		0.509	
G/G	82	0.5	146	0.48	0.716	0.633	0.149
G/C	62	0.38	127	0.42			
C/C	20	0.12	33	0.11			
G	226	0.69	419	0.68	0.894	0.834	0.099
C	102	0.31	193	0.32			
rs10835210	164		0.52	292		0.641	
C/C	52	0.32	73	0.25	0.036	0.043	0.009
C/A	85	0.52	142	0.49			
A/A	27	0.16	77	0.26			
C	189	0.58	288	0.49	0.016	0.02	0.067
A	139	0.42	296	0.51			
rs11030107	114		0.157	220		0.075	
A/A	63	0.55	128	0.58	0.811	0.783	0.036
A/G	39	0.34	73	0.33			
G/G	12	0.11	19	0.09			
A	165	0.72	329	0.75	0.529	0.492	0.19
G	63	0.28	111	0.25			
rs11030109	146		1	313		1	
G/G	137	0.94	295	0.94	0.861	0.852	0.58
G/A	9	0.06	18	0.06			
G	283	0.97	608	0.97	0.861	0.852	0.58
A	9	0.03	18	0.03			

rs28383487	172		1	287		1			
G/G	163	0.95		271	0.94		0.875	0.879	0.873
G/T	9	0.05		16	0.06				
G	335	0.97		558	0.97		0.875	0.879	0.873
T	9	0.03		16	0.03				
NTF4									
rs77113200	104		1	81		1			
G/G	100	0.96		77	0.95		0.718	0.749	1
G/A	4	0.04		4	0.05				
G	204	0.98		158	0.98		0.718	0.749	1
A	4	0.02		4	0.02				
GRM2									
SNP_06_GRM2	103		1	79		1			
T/T	100	0.97		77	0.97		0.876	0.887	0.101
T/G	3	0.03		2	0.03				
T	203	0.99		156	0.99		0.876	0.887	0.101
G	3	0.01		2	0.01				
rs149905463	104		1	80		1			
C/C	101	0.97		78	0.98		0.873	0.882	0.097
C/T	3	0.03		2	0.02				
C	205	0.99		158	0.99		0.873	0.882	0.097
T	3	0.01		2	0.01				
rs113426529	101		1	77		1			
G/G	95	0.94		74	0.96		0.532	0.495	0.042
G/A	6	0.06		3	0.04				
G	196	0.97		151	0.98		0.532	0.495	0.042
A	6	0.03		3	0.02				
rs149387441	102		1	79		1			
C/C	95	0.93		75	0.95		0.613	0.646	0.302
C/T	7	0.07		4	0.05				
C	197	0.97		154	0.97		0.613	0.646	0.302
T	7	0.03		4	0.03				
rs116567227	105		1	81		-999			
G/G	100	0.95		81	1		0.016	0.016	1
G/A	5	0.05		0	0				
G	205	0.98		162	1		0.016	0.016	1
A	5	0.02		0	0				
EGR2									

rs7893472	103	1	81	1			
T/T	98	0.95	78	0.96	0.702	0.736	1
T/C	5	0.05	3	0.04			
T	201	0.98	159	0.98	0.702	0.736	1
C	5	0.02	3	0.02			

^aGenotype and allelic counts; ^bGenotype and allelic frequency; ^cP-value for association between polymorphism and anxiety disorder status, unadjusted. An additive model was used for the allelic effect; ^dP-value for association between polymorphism and anxiety disorder status, adjusted for gender and age. An additive model was used for the allelic effect. ^eP-value for interaction between CTQ_{tot} with relevant SNP, adjusted for gender and age. *-.999: HWE could not be calculated due to exceedingly small sample numbers for that particular scenario. **NA: implies insufficient genotyping information available to perform analysis. ***Blue: indicates trends towards significance or borderline significant values. ****Red: significant values.

3.5.2.2. Gene-environment correlation

Genetic risk, as identified by polymorphisms within a gene, may be inferred by the observation of the increased likelihood that an individual be exposed to a particular environment, and that that individual may be at increased likelihood to develop a disorder. In the case of anxiety disorders, genetic risk may be identified by investigating variation within an anxiety disorders cohort for which higher severity of childhood trauma has been reported. Genetic risk can therefore be expressed by investigating variation in individuals with greater exposure towards environmental stressors such as childhood trauma (gene-environment correlation) and then subsequently the increased susceptibility to develop anxiety disorders (gene-environment interaction), by looking at the interaction these variables may have with environmental influence (Lau and Eley, 2008).

Significant case-control associations for the presence of anxiety disorders diagnoses were observed for *MMP9* (rs3918242 and rs1805088), *BDNF* (rs6265 and rs10835210) and *GRM2* (rs116567227). Upon further statistical evaluation the rs3918242 SNP identified with an OR value much higher than 1 (4.674) (Table 3.10) for the *CT* genotype, suggesting that this

genotype is associated with > 4X increased susceptibility risk to develop anxiety disorders. Considering allelic contribution, a significant p -value was identified for the T allele as well; however, no homozygous TT genotypes were identified within the current study. This scenario has been previously reported in the evaluation of this SNP (Holliday et al., 2007). SNPs rs1805088 (*MMP9*) and rs6265 (*BDNF*) presented with OR values less than 1 for the significantly identified homozygous CC (OR = 0.287) and AG (OR = 0.001) genotypes, respectively, suggesting the presence of these genotypes to be associated with a protective effect for the presence of anxiety disorders. Similarly, the rs10835210 (*BDNF*) polymorphism presented with ORs less than 1 for both AC (OR = 0.586) and CC (OR = 0.504) genotypes. This polymorphism was therefore also associated with the decreased susceptibility risk for anxiety disorders, and this decreased susceptibility was associated with the presence of the C allele. In this case, however, although the presence of the homozygous C allele indicated a decreased susceptibility risk, the heterozygous genotype presented with a similar OR, indicated that the C allele was not associated with a logarithmic decreased susceptibility advantage. Considering *GRM2*, although significant association was found for rs116567227, due to the absence of the A allele from the case group (only present in controls), subsequent analyses could not be performed and further investigation in a larger cohort is warranted.

These polymorphisms were assessed in a cases cohort with a significantly higher severity of childhood trauma ($p < 0.001$), than the controls. This is suggestive of anxiety diagnosed patients being more likely to find themselves in traumatic situations during early developmental years, and that the polymorphisms identified and statistically described here could be correlated with the development and/ or progression of anxiety disorders.

Table 3.10: Association analysis of polymorphisms investigating genotype distributions between cases and controls indicating genes, SNPs, p -values and OR ratios

Gene	SNP	Genotype	p -Val	OR	CI	
					2.50%	97.50%
<i>MMP9</i>	rs3918242	CT	< 0.001	4.674	2.340	10.40
	rs1805088	CC	< 0.001	0.287	0.138	0.575
<i>BDNF</i>	rs6265	AG	< 0.001	0.001	<0.001	1.317
	rs10835210	AC	0.042	0.586	0.346	0.971
		CC	0.018	0.504	0.284	0.883

OR: Odds ratio where $OR < 1$ implies a decreased risk for anxiety disorders and $OR > 1$ implies an increased risk for anxiety disorders; CI: confidence interval

3.5.2.3. Gene-environment interaction

Gene-environment interaction in the context of this study refers to the phenotypic effect of interactions between genes and the environment, whereby a particular genotype (or allele) can be associated with altered susceptibility risk to developing anxiety disorders within a particular environment. This was investigated by assessing a genotype-by-CTQ interaction term in the R Genetics package of RStudio. Whilst assessing for gene-environment correlation above, it was seen that individuals with a genetic risk for anxiety disorders present with increased exposure to childhood trauma, and the following results indicate that this exposure influences susceptibility risk to anxiety disorders, demonstrated here by changes in odds ratios associated with a particular genotype.

The interaction of the severity of childhood trauma (as represented by the CTQ total score) with the presence of a particular polymorphism was assessed in a case-control fashion. This was to ascertain whether the association between the severity of childhood trauma and the presence of anxiety disorders was modified by the presence of a genetic polymorphism (Table 3.11). The *p*-values for polymorphism-by-CTQ total score interaction are depicted in the last column of Table 3.9 above. Significant interaction statistics were identified for four SNPs (rs6265, rs2049045, rs10835210, and rs11030107) all found to be located within the *BDNF* gene. A significant interaction was also identified for the rs113426529 polymorphism in the *GRM2* gene, however, due to the low frequency of the minor allele this was not considered for further statistical analyses.

Focusing on *BDNF*, the rs2049045 and rs11030107 polymorphisms did not show significant gene-environment correlation (Table 3.11), however presented with *p*-values of 0.047 and 0.037, respectively for polymorphisms-CTQ total score interaction assessment (Table 3.11). Evaluation of the ORs associated with these polymorphisms within the current dataset indicated the rs2049045 variant to be associated with a slight decreased susceptibility risk for anxiety disorders (OR = 0.916), whereas the rs11030107 presented with a possible increased

susceptibility risk (OR = 1.081) (Table 3.11). The remaining two variants (rs6265 and rs10835210) also presented as possible increased susceptibility risk for anxiety disorders upon interaction with CTQ total score; however, these polymorphisms were also previously identified to be significant in the gene-environment correlation assessments above. When including the CTQ total score interaction statistic the rs6265 polymorphism previously predicted protective effect (OR = 0.001) is completely abolished, OR = 1.132) (Table 3.11). The rs10835210 polymorphism falls into the same category as rs6265, where the consideration of childhood trauma interaction with this polymorphism negates the predicted protective advantage associated with it previously (rs10835210 gene-environment correlation: AC, OR = 0.586; CC OR = 0.504 and rs10835210 gene-environment interaction: AC, OR = 1.084) (Table 3.11).

Table 3.11: Gene, SNP, *p*-values and OR ratios for significant interaction of CTQ total score (CTQtot) and polymorphisms in anxiety cases vs. controls

Gene	SNP	Genotype	<i>p</i> -Val	OR	CI	
					2.50%	97.50%
<i>BDNF</i>	rs2049045	GG	0.047	0.916	0.83	0.99
	rs10835210	AC	0.004	1.084	1.02	1.15
	rs11030107	AG	0.037	1.081	1.01	1.17
	rs6265	AG	< 0.001	1.132	0.93	1.33

OR: Odds ratio where an OR < 1 implies a decreased susceptibility risk for anxiety disorders and OR > 1 implies an increased susceptibility risk for anxiety disorders; CI: confidence interval

3.5.3. Haplotype analyses

Here, again, I report on GxE correlations and subsequently on GxE interactions.

3.5.3.1. Gene-environment correlation

As previously mentioned, gene-environment correlation refers to the ability of genetic risk to be expressed through anxiety cases presenting with a greater exposure to environmental stressors, specifically childhood trauma. Cases have been shown to be associated with a significantly higher severity of childhood trauma, relative to controls ($p < 0.001$) and they are therefore assessed here for haplotype analyses.

Haplotype analyses was performed for the identified polymorphisms using Haploview and confirmed using the LD genetics package in RStudio V 0.97. Linkage disequilibrium (LD) plots with D' values according to Gabriel et al., 2002's recommended CI criteria (default setting in Haploview) can be seen in Figure 3.11. Haplotype blocks pertain to polymorphisms identified in *MMP9* and *BDNF* genes.

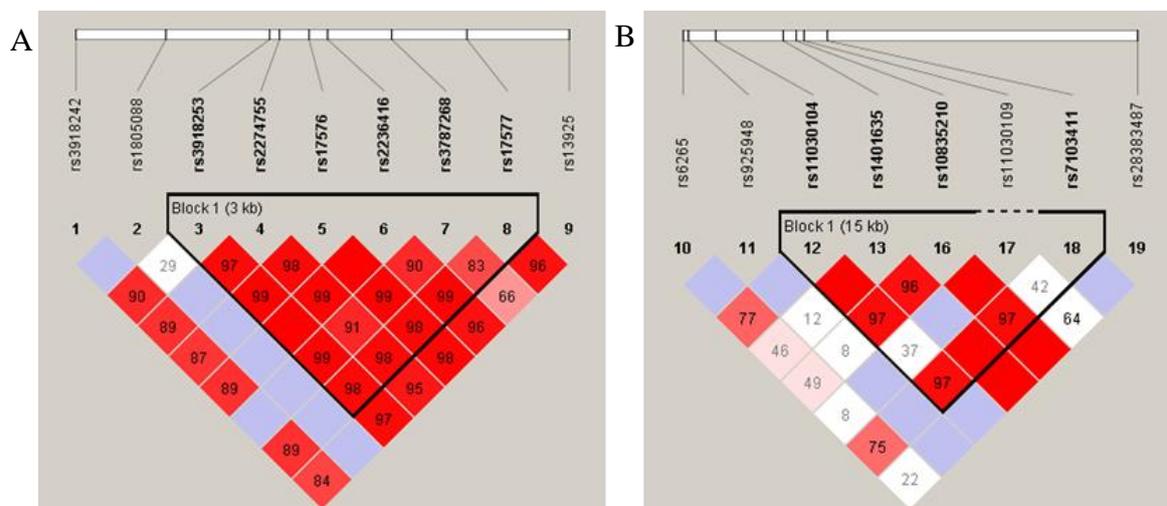


Figure 3.11: LD plots (indicating D' values) generated using polymorphisms genotyped for (A) *MMP9* and (B) *BDNF*. Haplotype blocks were identified based on criteria outlined in Gabriel et al., 2002 (if 95% of informative comparisons are strong LD).

No significant haplotype associations were found for the *MMP9* gene, however one (*BDNF* *rs11030104-rs1401635-rs10835210-rs7103411: AGAT) in the *BDNF* gene was found to trend towards significance, with increased frequency of the haplotype amongst cases (Table 3.12).

Table 3.12: Haplotype frequencies, χ^2 and *P*-value associations (Gabriel et al., 2002)

*rs3918253-rs2274755-rs17576-rs2236416-rs3787268-rs17577 (<i>MMP9</i>)						
*rs11030104-rs1401635-rs10835210-rs7103411 (<i>BDNF</i>)						
Block	Haplotype	Freq.	Case, Control Ratios	Case, Control Frequencies	χ^2	<i>p</i> -value
<i>MMP9</i>	TGAAGG	0.582	362.8:265.2, 201.0:141.0	0.578, 0.588	0.081	0.776
	CGGAAG	0.215	129.8:498.2, 80.0:262.0	0.205, 0.234	1.105	0.293
	CTGGGA	0.123	78.8:549.2, 40.0:302.0	0.126, 0.117	0.179	0.672
	CGAAGG	0.061	43.1:584.9, 16.0:326.0	0.069, 0.047	1.919	0.166
<i>BDNF</i>	AGAT	0.461	303.1:320.9, 142.3:197.7	0.484, 0.419	3.814	0.050
	ACCT	0.291	174.0:450.0, 105.2:234.8	0.281, 0.310	0.893	0.345
	GGCC	0.168	96.0:528.0, 66.3:273.7	0.153, 0.195	2.76	0.097
	AGCT	0.057	35.1:588.9, 20.0:320.0	0.056, 0.059	0.02	0.886

Freq.: frequency of cases and control haplotypes respectively; χ^2 : chi-squared

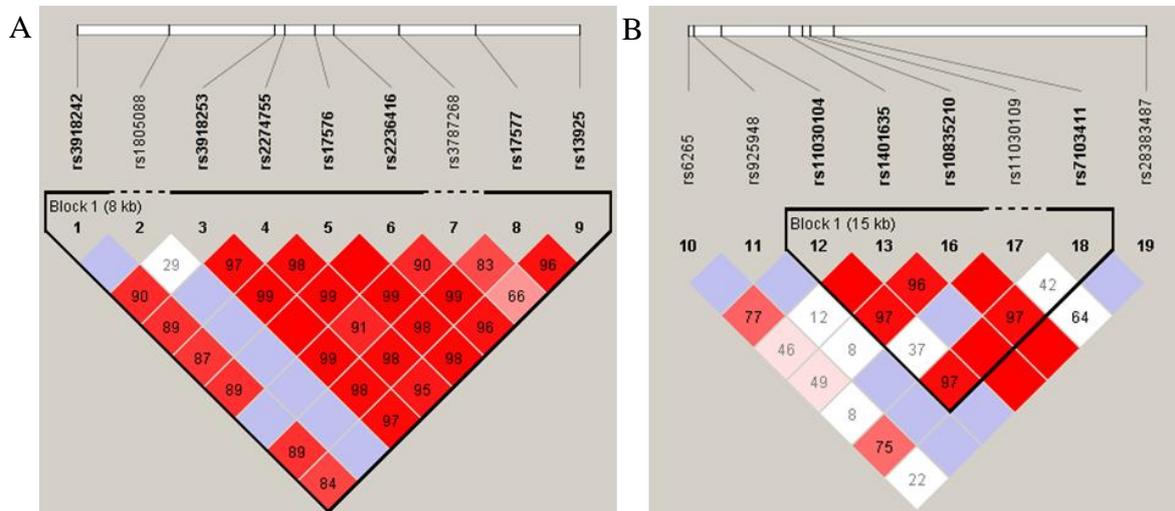


Figure 3.12: LD plots (indicating *D'* values) generated using polymorphisms genotyped for (A) *MMP9* and (B) *BDNF*. Haplotype blocks were identified based on criteria outlined in Gabriel et al., 2002 (if 95% of informative comparisons are strong LD) for *BDNF*, but modified to include rs3918242 and exclude rs3787268 for *MMP9*.

Expansion of the haplotype block for *MMP9* to include rs3918242 for which significant D' values were obtained and exclude rs3787268 which resulted in a break in LD revealed significant associations of two haplotypes in this gene with possible anxiety disorder susceptibility (Figure 3.12 and Table 3.13).

Table 3.13: Haplotype frequencies, χ^2 and *P*-value associations, modified Block

*rs3918242-rs3918253-rs2274755-rs17576-rs2236416-rs17577-rs13925(<i>MMP9</i>)						
*rs11030104-rs1401635-rs10835210-rs7103411 (<i>BDNF</i>)						
Block	Haplotype	Freq.	Case, Control Ratios	Case, Control Frequencies	χ^2	<i>p</i> -value
<i>MMP9</i>	CTGAAGG	0.581	363.1:264.9, 199.9:142.1	0.579, 0.585	0.031	0.860
	CCGGAGG	0.218	130.9:497.1, 82.0:260.0	0.207, 0.240	1.413	0.235
	CCTGGAA	0.063	30.0:598.0, 31.0:311.0	0.048, 0.091	6.791	0.009
	CCGAAGG	0.059	40.7:587.3, 16.0:326.0	0.065, 0.047	1.357	0.244
	TCTGGAA	0.054	42.9:585.1, 8.9:333.1	0.069, 0.026	7.892	0.005
	AGAT	0.461	303.1:320.9, 142.3:197.7	0.484, 0.419	3.814	0.050
<i>BDNF</i>	ACCT	0.291	174.0 : 450.0, 105.2:234.8	0.281, 0.310	0.893	0.345
	GGCC	0.168	96.0:528.0, 66.3:273.7	0.153, 0.195	2.76	0.097
	AGCT	0.057	35.1:588.9, 20.0:320.0	0.056, 0.059	0.02	0.887

Freq.: frequency of cases and control haplotypes respectively; χ^2 : chi-squared

Two haplotypes within the *MMP9* gene (rs-3918242-rs3918253-rs2274755-rs17576-rs2236416-rs3787268-rs17577-rs13925: CCTGGAA and TCTGGAA) were found to be statistically significant for susceptibility to anxiety disorders (Table 3.13). CCTGGAA was found to be present at lower frequency within cases compared to controls whereas TCTGGAA was higher in cases compared to controls.

Considering the observation of significant haplotype frequency between cases and controls identified (Table 3.13), the *MMP9* CCTGGAA haplotype demonstrated a predicted reduced susceptibility risk for the development of anxiety disorders with an OR of 0.469 (Table 3.14). The other significant haplotype frequency observation in *MMP9*, TCTGGAA, presented with

a predicted 4.6-fold increased susceptibility risk to develop anxiety disorders (OR = 4.608). This coincides with the inclusion of the T allele for rs3918242 which presented with a > 4-fold increased risk in gene-environment correlation bi-allelic association testing in section 3.4.1.2 (Table 3.10). One haplotype was identified for the *BDNF* gene, AGAT. This haplotype was associated with a 2.5-fold increased susceptibility genes to develop anxiety disorders (OR = 2.519), and can appropriately be found at higher frequency amongst the cases (Table 3.14).

Table 3.14: Significant *p*-values and OR ratios for significantly identified haplotype effects associated with anxiety disorder susceptibility in bottom 33rd percentile (lower CTQ total scores) cases vs. control

*rs3918242-rs3918253-rs2274755-rs17576-rs2236416-rs17577-rs13925(MMP9)					
*rs11030104-rs1401635-rs10835210-rs7103411 (BDNF)					
Gene	Haplotype	<i>p</i> -Val	OR	CI	
				2.50%	97.50%
<i>MMP9</i>	CCTGGAA	0.009	0.469	0.429	0.514
	TCTGGAA	0.005	4.608	2.641	8.039
<i>BDNF</i>	AGAT	0.050	2.519	1.990	3.190

OR: Odds ratio where an OR < 1 implies a decreased susceptibility risk for anxiety disorders and OR > 1 implies an increased susceptibility risk for anxiety disorders; CI: confidence interval

3.5.3.2. Possible gene-environment interaction?

The statistical assessment of haplotype association in a case-control fashion for a polymorphism-CTQ total score interaction component was not possible using the employed statistical programming. To evaluate a possible interaction of the influence that the severity of childhood trauma may have on the presentation of haplotype associations between cases and controls, analyses were performed for the top 33rd percentile (30 cases, highest CTQ total score; mean = 64.36, median = 60.50) and bottom 33rd percentile (30 cases, lowest CTQ total scores, mean = 28.9, median = 29) for cases, relative to controls (194 controls representative of the general population, mean CTQ total score = 34.60, median CTQ total score = 30.50).

Analyses were performed using Haploview. Linkage disequilibrium (LD) plots with D' values according to Gabriel et al., 2002's recommended CI criteria (default setting in Haploview) can be seen in Figures 3.13 and 3.14. Haplotype blocks pertain to polymorphisms identified in the *MMP9* and *BDNF* genes.

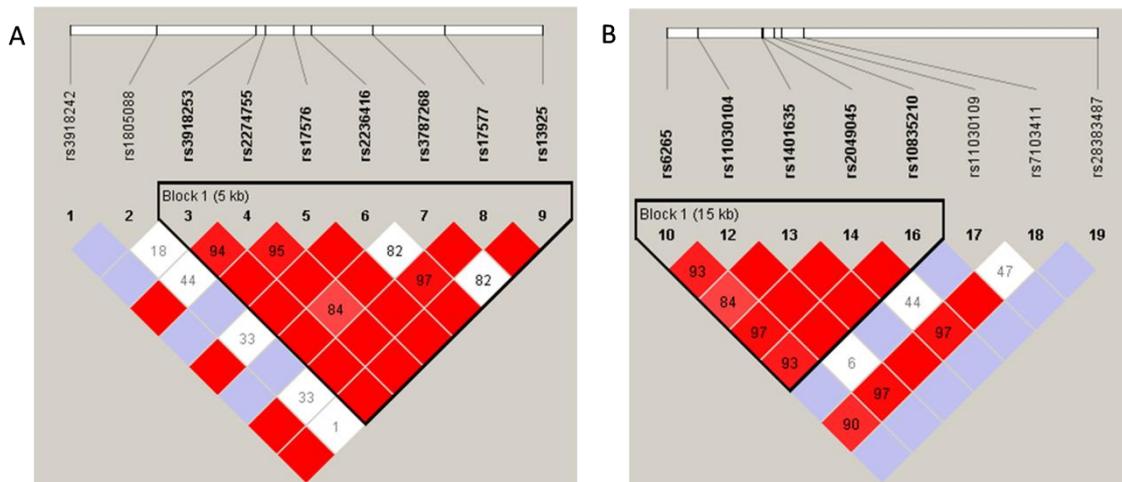


Figure 3.13: LD plots (indicating D' values) generated using polymorphisms genotyped for (A) *MMP9* and (B) *BDNF* comparing the bottom 33rd percentile (low CTQ total score) cases to controls. Haplotype blocks were identified based on criteria outlined in Gabriel et al., 2002.

Considering the bottom 33rd percentile of cases, representative of lower CTQ total scores, two haplotype blocks were identified (Figure 3.13), one for *MMP9* and one for *BDNF*. When looking at the haplotype analyses performed for the entire cohort in section 3.4.2.1 above, the haplotype block for *MMP9* was similar (Figure 3.12), except here the rs13925 polymorphism is included. The *BDNF* haplotype block presented was quite different to that seen in Figure 3.11 and includes rs6265 and rs11030104, whilst excluding rs7103411 (Figure 3.14). Although different haplotype blocks were obtained using this strategy, there were no haplotype associations found to be significantly associated with anxiety disorders susceptibility for the *MMP9* or *BDNF* genes (Table 3.15), when considering a group of low incidence severity for childhood trauma (CTQ total score below 30).

Table 3.15: Haplotype frequencies, χ^2 and P -value associations for bottom 33rd percentile (low CTQ total score) cases vs. controls

*rs3918253-rs2274755-rs17576-rs2236416-rs3787268-rs17577-13925 (MMP9)							
*rs6265-rs11030104-1401635-rs2049045-rs10835210 (BDNF)							
Block	Haplotype	Freq.	Case, Control Ratios	Case, Control Frequencies	χ^2	p -value	
MMP9	TGAAGGG	0.589	18.0:12.0, 201.0:141.0	0.600, 0.588	0.017	0.896	
	CGGAAGG	0.239	9.0:21.0, 80.0:262.0	0.300, 0.234	0.664	0.415	
	CTGGGAA	0.113	2.0:28.0, 40.0:302.0	0.067, 0.117	0.697	0.404	
	CGAAGGG	0.043	0.0:30.0, 16.0:326.0	0.000, 0.047	1.467	0.226	
BDNF	GAGGA	0.42	12.6:17.4, 143.0:197.0	0.419, 0.421	0.000	0.991	
	GACGC	0.301	6.2:23.8, 105.3:234.7	0.208, 0.310	1.352	0.245	
	AGGCC	0.168	3.9:26.1, 58.4:281.6	0.131, 0.172	0.332	0.565	
	GAGGC	0.071	1.9:28.1, 24.3:315.7	0.065, 0.071	0.020	0.888	
	GGGGC	0.018	1.2:28.8, 5.5:334.5	0.041, 0.016	0.989	0.320	

Freq.: frequency of cases and control haplotypes respectively; χ^2 : chi-squared

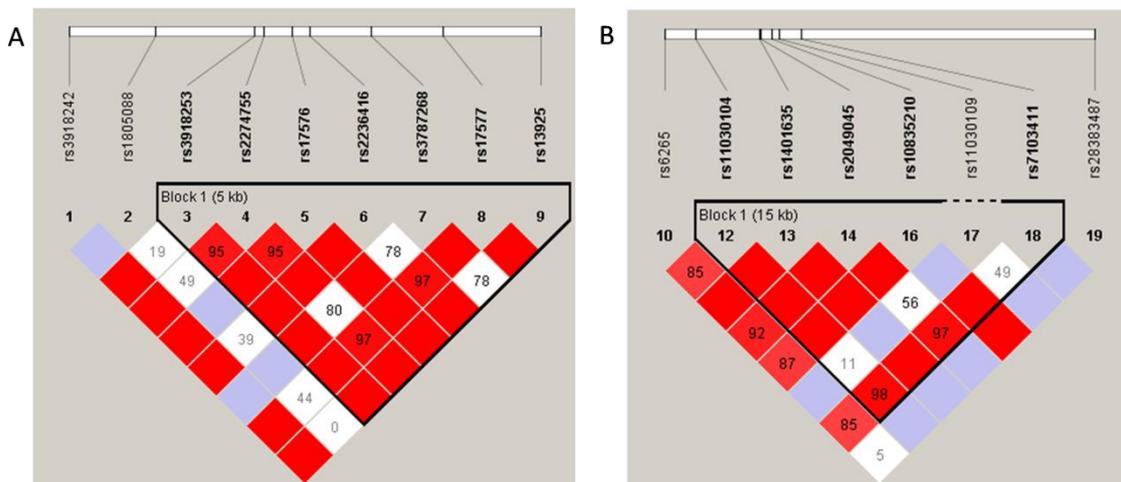


Figure 3.14: LD plots (indicating D' values) generated using polymorphisms genotyped for (A) *MMP9* and (B) *BDNF* comparing the top 33rd percentile (high CTQ total score) cases to controls. Haplotype blocks were identified based on criteria outlined in Gabriel et al., 2002.

The top 33rd percentile of cases, representative of higher CTQ total scores, also maintained a single haplotype block per gene presentation pattern (Figure 3.14). Considering the haplotype analyses performed for the bottom 33rd percentile CTQ total scores individuals above, the haplotype block for *MMP9* was identical to that seen in Figure 3.13. The *BDNF* haplotype block for the top 33rd percentile presented with a block identical to that reported in section 3.4.2.1 above for haplotype analyses of the entire cohort (Figure 3.12). Although an identical haplotype block was obtained for *MMP9* here: where there were no significant associations identified in the bottom 33rd percentile – here two significant haplotype associations were found (*MMP9*: *rs3918253-rs2274755-rs17576-rs2236416-rs3787268-rs17577-13925: CGGAAGG and CGAAGGG) (Table 3.16). The CGGAAGG haplotype presented with a higher frequency in cases (suggestive of increased susceptibility risk for anxiety disorders), whereas CGAAGGG presented with a higher frequency in controls (suggestive on decreased susceptibility risk for anxiety disorders). No significant haplotype associations for anxiety disorders were identified for the *BDNF* gene in the context of the top 33rd percentile CTQ total score cases relative to controls (Table 3.16), but this may be attributed to sample cohort sizes considering the trend towards significance identified for the gene-environment correlation analyses above (section 3.4.2.1).

Table 3.16: Haplotype frequencies, χ^2 and *P*-value associations for top 33rd percentile (high CTQ total score) cases vs. controls

*rs3918253-rs2274755-rs17576-rs2236416-rs3787268-rs17577-13925 (<i>MMP9</i>)						
*rs11030104-1401635-rs2049045-rs10835210-rs7103411 (<i>BDNF</i>)						
Block	Haplotype	Freq.	Case, Control Ratios	Case, Control Frequencies	χ^2	<i>p</i> -value
<i>MMP9</i>	TGAAGGG	0.595	24.0:12.0, 201.0:141.0	0.667, 0.588	0.843	0.359
	CGGAAGG	0.217	2.0:34.0, 80.0:262.0	0.056, 0.234	6.096	0.014
	CTGGGAA	0.116	4.0:32.0, 40.0:302.0	0.111, 0.117	0.011	0.917
	CGAAGGG	0.056	5.0:31.0, 16.0:326.0	0.139, 0.047	5.266	0.022
<i>BDNF</i>	AGGAT	0.432	19.8:16.2, 142.5:197.5	0.549, 0.419	2.251	0.134
	ACGCT	0.304	8.9:27.1, 105.6:234.4	0.246, 0.311	0.645	0.422
	GGCCC	0.16	4.0:32.0, 56.2:283.8	0.111, 0.165	0.713	0.398
	AGGCT	0.053	0.4:35.6, 19.7:320.3	0.010, 0.058	1.462	0.227

GGGCC	0.033	2.0:34.0, 10.2:329.8	0.056, 0.030	0.672	0.412
AGGCC	0.012	0.0:36.0, 4.7:335.3	0.000, 0.014	0.499	0.480

Freq.: frequency of cases and control haplotypes respectively; χ^2 : chi-squared

Two *MMP9* haplotype associations were identified when considering the top 33rd percentile CTQ total score cases (representative of the higher CTQ total scores) (Table 3.16). The *MMP9* CGGAAGG demonstrated a predicted reduced susceptibility risk for the development of anxiety disorders with an OR of 0.890 (Table 3.17). A second haplotype, CGAAGGG, was associated with a 1.7-fold increased susceptibility risk (OR = 1.721). In this case the haplotype did not contain the rs3918242 polymorphism associated with the > 4-fold risk for developing anxiety disorders, inferring that alternate polymorphisms may exist within the *MMP9* gene that could negatively influence susceptibility risk.

Table 3.17: Significant *p*-values and OR ratios for significantly identified haplotype effects associated with anxiety disorder susceptibility in top 33rd percentile (higher CTQ total scores) cases vs. control

*rs3918253-rs2274755-rs17576-rs2236416-rs3787268-rs17577-13925 (<i>MMP9</i>)					
Gene	Haplotype	<i>p</i> -Val	OR	CI	
				2.50%	97.50%
<i>MMP9</i>	CGGAAGG	0.014	0.890	0.865	0.916
	CGAAGGG	0.022	1.721	1.548	1.913

OR: Odds ratio where and $OR < 1$ implies a decreased susceptibility risk for anxiety disorders and $OR > 1$ implies an increased susceptibility risk for anxiety disorders; *CI:* confidence interval

Chapter 4

Discussion

4. Discussion

Chapter 4 presents a detailed discussion of the findings and summarized descriptions of the results presented in Chapter 3. The relevance and relatedness of the novel susceptibility genes that were identified are addressed and an account of the genotyping findings after extrapolation onto a human cohort is provided.

4.1. Animal Work

4.1.1. Environmental intervention animal model

The Sprague Dawley rats used in this study comprised an outbred model. This allowed for a better representation of the molecular genetics involved than an inbred model considering the genetic heterogeneity that would be observed in an unrelated human study cohort. The environmental stressors implemented in the present study (i.e. maternal separation and restraint stress) are well established models for eliciting anxiety-like behaviours in rodents (Section 2.4), and although alternative models for eliciting anxiety-like behaviours (such as strain differences, bi-directional breeding or various combinations thereof) might prove to be better models upon implementation (Schmidt and Müller, 2006; Egan et al., 2010) – the candidate genes identified here allude to environmental intervention alone as also being effective. The use of alternative models such as strain differences and bi-directional breeding, in conjunction with environmental interventions, may be helpful in validating the data produced here.

The results of the rat model and interventions used in this study are discussed in some detail below.

4.1.2. Behavioural data

Maternal separation in mice and rats is believed to represent early-life trauma and is expected to result in increased anxiety amongst exposed subjects (George et al., 2010). It may be argued that our MS rats experienced significantly increased anxiety, as indicated by the increased immobility times (during the FST task) observed relative to control rats ($p < 0.0001$) (Figure 3.1 and 3.2). Maternal separation has been previously reported to result in increased anxiety-like behaviour in rats (Kuhn and Schanberg, 1998; Romeo et al., 2003; George et al., 2010; Uchida et al., 2010) and increased immobility time as an indirect measure of this (Mineur et al., 2013). We further hypothesized that rats exposed to an early-life stressor (*i.e.* maternal separation), and subsequently exposed to a mild adulthood stressor (*i.e.* restraint stress), would show increased anxiety-like behaviours compared to healthy controls due to a possible additive mechanism of perpetuation (Uchida et al., 2010). However, this was not confirmed in the present study. Although significantly higher immobility times (suggesting higher anxiety) were observed in the MS+RS and MS groups compared to the control group, no significant differences were observed between MS+RS and MS groups in this regard. This is not consistent with a recent study (Uchida et al, 2010), which reported that early developmental trauma with subsequent stressors resulted in increased anxiety in adulthood. An alternative explanation to our findings may be that synaptic preconditioning (referring to the ability of neurons to regulate their overall action potential firing rate - (Turrigiano, 2007, 2008) may have occurred, *i.e.* when exposed to a major-life trauma, changes in gene expression occurs, causing synaptic remodeling, subsequently altering the phenotypic response to further stressors (Figure 3.1 and 3.2). In the present study, rats exposed to restraint stress only showed lower immobility times (FST) than the control group, reflecting greater activity and indicative of hypervigilance, *i.e.* more anxious behaviour compared to controls. So, in summary, MS and MS+RS rats showed behaviours indicative of higher anxiety levels compared to controls. The RS group, in contrast, had lower anxiety than MS and MS+RS groups, contrary to expectation. The RS group showed “inconsistent” behaviour, *i.e.* lower anxiety than the other experimental groups in one task (FST), but higher anxiety in the 2 other tasks (OFT and EPM). It may be hypothesized that this unexpected finding resulted from the rats exhibiting panic due to the lack of preconditioning to trauma during early life. Considering the inconsistent RS findings,

it is important to note that the core focus here was early developmental trauma perpetuating observable increased anxiety-like behaviours in adulthood, which was seen in the MS and MS+RS groups. This is in line with current literature regarding childhood trauma and anxiety disorders in humans (Heim and Binder, 2012; Chu et al., 2012).

In the OFT, increased total distance moved is indicative of increased time spent within the outer-zone of the maze (as with time kept constant, the rat would need to travel to the outer-zone to yield higher total distance travelled times), and this is subsequently associated with increased anxiety levels amongst subjects (Hulshof et al., 2011). The distance travelled by the control group was lowest, suggesting lowest levels of anxiety in this group. The MS group travelled furthest i.e. showed the most anxiety-related behaviour, suggesting that this group experienced the most anxiety. Again, there was no statistically significant difference between the MS and MS+RS groups in terms of the level of anxiety-like behaviour observed. Given these findings, it is plausible to assume that early trauma exposure allows for gene expression facilitating synaptic modeling that is less responsive to subsequent traumatic events. As mentioned earlier, this contradicts previous work (Uchida et al., 2010) stipulating that early-life trauma predisposes to increased anxiety response in adulthood upon subsequent stress application. The methodology employed by Uchida et al. differed slightly to that used in this study, which could possibly account for deviation in observed results. Uchida and co-workers, for example, used a female rat cohort, whereas only male rats were used in the present study. A precedent for synaptic scaling has been established in literature before (Citri and Malenka, 2008; Schmidt et al., 2013) and therefore replication of this study in female rat populations, or rats of a different breed, to establish whether the trend observed here is maintained, may be warranted. Nonetheless, to reiterate, the focus of the present study was the observation of anxiety-like behaviours after the application of an early-life stressor, and this was observed in the MS and MS+RS groups, relative to controls.

In summary, our findings suggest that rats exposed to maternal separation during early-development exhibit significantly increased anxiety-like behaviours in adulthood. It was hypothesized that rats exposed to MS and subsequent adulthood stress (*i.e.* MS + RS) would have even higher anxiety than those exposed to MS only, but this was not confirmed. A preconditioning effect arguably allowed for less anxiety-like behaviour in the combination RS + MS rat group, relative to the MS rats. The behavioural differences observed between

rats exposed to an early-life stressor (maternal separation) and controls, could be (at least partially) explained by differential expression in genes responsible for synaptic plasticity.

The synaptic plasticity pathway was subsequently investigated for differential expression in the rat striatum. The striatum is an area of high synaptic plasticity, and the primary input nucleus receiving excitatory impulses from the cortex and thalamus – previously implicated in anxiety disorders literature (Citri and Malenka, 2008; Greer and Capecchi, 2002; Welch et al., 2007).

4.1.3. RT² Profiler Array, Synaptic Plasticity

Several genes were found to be differentially expressed between the four experimental groups of rats, namely *Mmp9*, *Bdnf*, *Ntf4*, *Arc*, *Egr2*, *Egr4* and *Grm2*. The human homologues (*MMP9*, *BDNF*, *NTF4*, *ARC*, *EGR2*, *EGR4* and *GRM2*) for these genes and their plausible candidacy as susceptibility genes for increased risk for anxiety disorders are discussed below:

4.1.3.1. *Matrix metalloproteinase 9 (MMP9)*

Matrix metalloproteinases (MMPs) comprise of a family of over 25 distinct zinc-dependent members with a primary role comprising the degradation and alteration of the intrasynaptic extracellular matrix (ECM) structure and function (Matrisian, 1992; Bauvois, 2012). The elucidation of the role of MMPs in nervous system function remains relatively recent; however, initial studies have implicated MMP2, MMP3 and MMP9 in cognitive impairment related to multiple nervous system disorders, such as autoimmune demyelinating disease and nervous system lymphoma (Gold et al., 2009; Yong, 2010; Hottinger et al., 2011). MMP's brain involvement has, however, not been limited to neurodegenerative diseases but also to the areas of neural development and response to injury. MMP9 specifically, is required for oligodendrocyte process extension and myelination (Oh et al., 1999; Kim et al., 2012) and has increased expression during postnatal cerebellar development (Vaillant et al., 1999). In the hippocampus, increased levels of MMP9 can be observed in response to kainic acid and

bicuculline-induced seizures, correlating with subsequent synapse formation (Matrisian, 1992; Huntley, 2012). Literature indicates the role of MMPs to be vital in synaptic remodeling and therefore possibly vital for synaptic plasticity (Phillips and Reeves, 2001; Reeves et al., 2003; Huntley, 2012).

Activity-dependent changes in synaptic adhesion, reflected in structural molecule modifications within synaptic elements, could underlie the functional and morphological plasticity of synapses during learning (Agnihotri et al., 1998; Lynch, 1998; Lynch et al., 2007; Caroni et al., 2012). The ECM contains components which regulate the activity of multiple cell adhesion molecules (CAMs), and it is believed that changes in the ECM could alter synaptic architecture in ways that could change the efficiency of synaptic transmission. In addition to CAM regulation, the ECM also houses a number of neurotransmitters and growth factors which can be utilized accordingly in response to ECM changes (Meighan et al., 2006). ECM modulation is therefore paramount in the modification of synaptic plasticity and learning.

Molecules that play a role in the process of synaptic plasticity need to be able to conform to a system that is under constant, necessary revision, and the molecules of the ECM appear to meet this criterion. Matrisian et al. (1992) have shown that MMPs (specifically MMP 3 and 9) are required for spatial learning and that MMP modulation is governed by NMDA and/ or glutamate receptor function (Matrisian, 1992; Bauvois, 2012). This is interesting within the context of the results presented here, as the only significantly down-regulated gene was *Grm2* (a glutamate receptor), in the RS MS rats, relative to controls. MMP9 has previously been reported to work indirectly on glutamate receptors through the cleavage of β -dystroglycan (Michaluk et al., 2007). The role of MMP9 in glutamate receptor trafficking, in particular GluR2, is observed by its over-expression allowing for a neurotoxic role. MMP9 may be responsible (if only partially) for the *Grm2* down-regulation seen in this study (Michaluk et al., 2009). Over- and constant stimulation of glutamate receptors resulting in their damage allows for *MMP9* over-expression to play a role in mediating excitotoxic neuronal loss. MMP9 is found at higher levels in response to higher levels of plasticity response post neuronal damage, aiding in the formation of new synapses in response to damage (Michaluk et al., 2009; Szklarczyk et al., 2002). It is therefore noteworthy that *Mmp9*, was not flagged as significantly altered in this MS+RS group, but was significant in the MS group. The

double-edged nature of the Mmp9 protein characterized within the literature just described could allude to different Mmp9 function (plasticity-promoting or excitotoxic) depending on the application of adulthood stress in MS+RS group vs. MS only. Further studies, such as protein quantification and interaction experiments, are warranted to determine when MMP9 is acting in a plasticity-promoting, excitotoxic or dual manner relating to a specific stressor group. The exact interaction that may be at play in this particular instance therefore requires further investigation. It is important to note, however, that different neurotransmitters or receptors and their intracellular signaling mechanisms appear to be responsible for regulating specific subsets of MMPs (Schroen and Brinckerhoff, 1996; Mott and Werb, 2004; Michaluk et al., 2007, 2009).

An alternative mechanism of MMP function includes the modification of the ECM and synaptic plasticity through cadherins, neural CAMs and integrins. These are highly expressed within synaptic structures and are vital for synaptic plasticity. Recent studies have shown that the neuronal cadherin, *Cdh2*, was associated with OCD-like behaviour in a dog model (Dodman et al., 2010), strengthening hypotheses for *MMP9* involvement in the pathogenesis of anxiety disorders. Previous work shows direct MMP interaction with cadherin regarding synaptic remodeling and the formation of new synaptic pathways post traumatic brain injury (Warren et al., 2012). This, the aforementioned work implicating *Cdh2* in OCD within a dog model, and the identification of *Mmp9* as aberrantly expressed in rats exposed to environmental stressors in this study makes strong supporting evidence for its consideration as a target susceptibility gene for anxiety disorders in humans.

MMP9 has therefore been associated with synaptic plasticity within literature before, but to the best of our knowledge this is the first study to implicate the gene specifically within an anxiety disorders setting. The identification of *Mmp9* as a susceptibility candidate using a rat model and subsequent identification of polymorphisms within *MMP9* in a gene-environment correlation and interaction approach makes for the first molecular evidence for the involvement of *MMP9* in the underlying molecular etiology of anxiety disorders, as represented by OCD, SAD and PD in this study.

4.1.3.2. *Brain-derived neurotrophic factor (BDNF)*

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin super-family, is synthesized as pro-forms, which are cleaved intracellularly to release mature, secreted ligands. Mature neurotrophins are then able to selectively bind to tyrosine kinase receptors (Trk) which promote Trk-mediated differentiation and survival of neural circuitry (Hall et al., 2003). With *BDNF* belonging to the neurotrophin family, the neurotrophin hypothesis stipulates that neuronal activity is able to enhance the synthesis, secretion and signaling of neurotrophins (Fig 4.1), enabling the modification of synaptic transmission. This ultimately provides a connection between neuronal activity and synaptic plasticity (Schinder and Poo, 2000). Neurotransmitters are able to portray an instructive role, whereby they act at the synapse to directly modify presynaptic transmitter release, synaptic morphology and postsynaptic sensitivity to allow for a persistent synaptic modification (Kotaleski and Blackwell, 2010; Mayford et al., 2012).

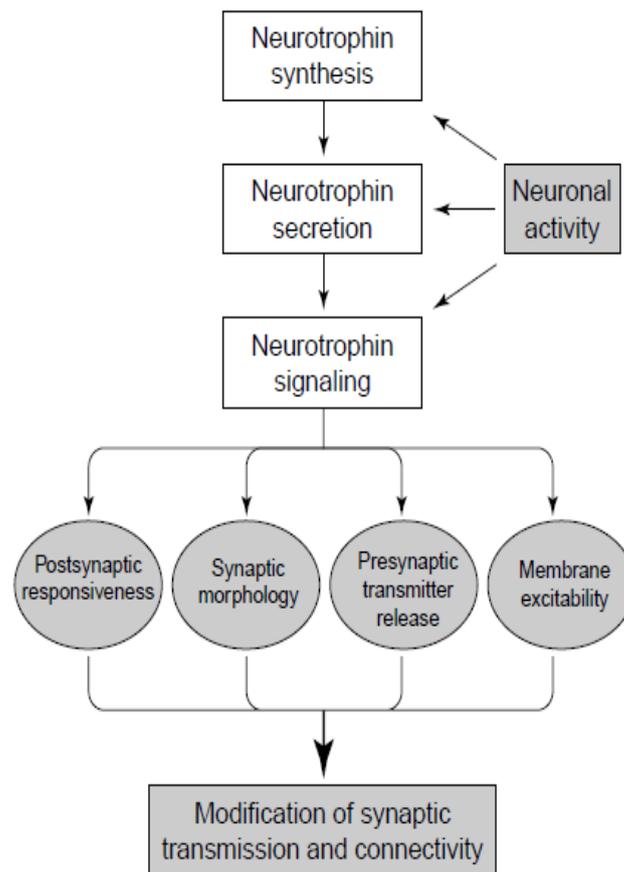


Figure 4.1: The neurotrophin hypothesis for plasticity of synaptic transmission. Neuronal activity regulates neurotrophins at three different levels: synthesis, secretion and signaling. Synaptic transmission and connectivity are modified as a consequence of specific changes in the pre- and postsynaptic neurons. There is experimental evidence to support the existence of all three levels of regulation (figure adapted from Schinder and Poo, 2000).

Neurotrophins permit rapid morphological change allowing for axonal and dendritic sprouting, and ultimately persistent synaptic modifications (McAllister et al., 1995; Boulanger and Poo, 1999; van Ooyen, 2011; Maya-Vetencourt and Caleo, 2013). In the present study we observed over-expression of *Bdnf* and another neurotrophin gene, *Ntf4*, in rats exhibiting increased anxiety-like behaviours relative to controls, and considering the aforementioned literature, this is most likely for effective synaptic modification. The persistent presence of neurotrophins throughout the adult nervous system suggests multi-functional roles throughout the lifespan of the organism (Rothman and Mattson, 2012). The over-expression of *Bdnf* and *Ntf4* observed in adult rats exposed to restraint stress, an adulthood stressor, supports this

finding, assuming its literature specified involvement in synaptic plasticity and the assumption that synaptic plasticity changes are involved in stress response adaptations. Whether neurotrophins play a permissive or instructive role most likely depends on a number of factors including, but not limited to, the type of synapse, the stage of neuronal development and ultimately the pattern of neuronal activity controlling its expression (Schinder and Poo, 2000).

BDNF has been linked to serotonin, with studies indicating that neurochemical and behavioural analysis of heterozygous *Bdnf*^{+/-} mice with partial impairment of *Bdnf* expression show physiological disturbances in central serotonergic neurons in early adulthood and that this leads to structural deterioration with advancing age (Hall et al., 2003). *BDNF* has further been specified to promote neuronal proliferation, regeneration and connectivity during development and maintenance of plasticity during adulthood (Bramham and Messaoudi, 2005). This is strengthened by the findings in this study with *Bdnf* being highlighted as a candidate in all stress-application rat groups, relative to controls (Alonso et al., 2008). *BDNF* may therefore be involved in the pathogenicity of anxiety conditions via serotonergic neurons specifically, but also via alternative pathways by altering or modifying synapses.

Multiple lines of evidence exists associating *BDNF* polymorphism with anxiety disorders. Hall et al. (2003) identified two haplotypes [*rs988748-rs2049046-rs6265-a20: CTGC, P = 0.007; GAAG, P = 0.002 (Sklar et al., 2002)] associated with OCD in a probands cohort of 164 American Caucasian individuals for both over- and under-transmission, respectively. *BDNF* and OCD association was further concreted by Alonso and co-workers (2008) who employed a tagSNP approach to generate haplotype data in a Spanish Caucasian cohort of OCD patients and controls. The research allowed for haplotype generation similar (although not identical) to that of Hall et al. (2003), but containing the rs6265 polymorphism defining the same protective haplotype against OCD. The protective nature associated with the rs6265 polymorphism has been previously investigated, and confirmed, within our group as well (Hemmings et al., 2008; Katerberg et al., 2009). The most recent study by our group has identified the rs6265 (*BDNF* Val66Met) polymorphism to specifically interact with the presence of childhood trauma, and that the A allele (met-allele) specifically results in increased risk to develop OCD in the presence of emotional abuse (Hemmings et al. 2013). This coincides with the modifying interactive effects observed in this study discussed in the

human work later on (Section 4.2) *BDNF* has also been investigated in SAD and PD (Gorman, 2000; Morreale et al., 2009; Schumacher et al., 2011; Goes, 2012), with findings suggesting varied contribution of *BDNF* to anxiety disorders etiology. There are however, still relatively few studies that focused on PD and SAD specifically and therefore it may be entirely plausible that different genetic contributions within the *BDNF* gene may contribute to these disorders differently. The current study, which encompassed an anxiety disorders cohort, i.e. a cohort that expanded beyond individual phenotypic classifications, alludes to an underlying mechanism of involvement for *BDNF* in the etiology of anxiety disorders.

Focusing specifically on plasticity, *BDNF* signaling at synapses has been shown to enhance long-term potentiation (LTP) (a process of synaptic strengthening associated with learning and memory) and this is mediated by cAMP (Mattson et al., 2004). The mechanisms underlying LTP were evaluated in the hippocampus, and therefore striatal application warrants further investigation. This strengthens the plausibility of the identification of *Bdnf* (in rats) as a susceptibility candidate in our synaptic plasticity assays and as a subsequent plausible susceptibility candidate for anxiety pathogenesis involving the striatum and plasticity in humans.

4.1.3.3. Neurotrophin 4 (NTF4)

Neurotrophin 4 (NTF4) is also known as *neurotrophin 5 (NTF5)*. As mentioned (Section 4.1.2.2), neurotrophins exert their effect through two main receptor subunits, namely Trk and p75 (McAllister et al., 1995; Schinder and Poo, 2000; Huang and Krimm, 2010). Tyrosine kinase receptor B (trkB) is the main receptor of NTF4 and is shared by *BDNF* (Mattson et al., 2004; Huang and Krimm, 2010). The trkA receptors are expressed mainly on large cholinergic interneurons, whereas trkB (the receptor for NTF4) is expressed mainly on striatal projection neurons and some interneurons (Ardelt et al., 1994), tying into a possible explanation for *Ntf4* differential expression in this study.

There is evidence to suggest that *NTF4* is involved in the modulation of synaptic efficacy (Machado Dias, 2011; Runge et al., 2012). Furthermore, hippocampal cell induction in cell culture with *BDNF*, *NTF3* and *NTF4* has been shown to result in rapid potentiation of

glutamate-mediated synaptic transmission (Lessmann et al., 1994; Schinder and Poo, 2000). *Ntf4* and *Bdnf* may therefore be involved in anxiety-like behaviour manifestation by facilitating for *Grm2*-mediated synaptic transmission (glutamate receptor excitation). Experimental data has shown that NTF4 is a requirement for neuronal survival and was observed to be essential for the survival of striatal neurons in an organotypic slice culture (Ardelt et al., 1994; Huang and Krimm, 2010; Harrington and Ginty, 2013). This was shown again in subsequent investigations (Huang and Krimm, 2010; Harrington and Ginty, 2013). NTF4-treated cultures indicated an increase in the number of neurons, which may have been as a result of stimulated proliferations and/ or differentiation of neural precursor cells, or the rescuing of cells that were undergoing apoptotic death. The role of this gene in reshaping synaptic circuitry and synaptic plasticity processes is therefore important, and its involvement most likely paramount for inclusion in anxiety pathogenicity considering synaptic reshaping in response to early-life stress. The literature described above indicates *Bdnf* and *Grm2* interact or influence *Ntf4* function and this supports the candidacy of these genes as susceptibility targets for investigation in human anxiety pathogenicity.

The application of neurotrophins at developing and mature synapses acutely stimulates neurotransmitter release (Kang and Schuman, 1996; Wang and Poo, 1997). Neuromuscular cell culture work shows both pre- and postsynaptic effects of neurotrophins, increasing the spontaneous and evoked release of synaptic vesicles and changing the kinetics of acetylcholine receptors (Wang and Poo, 1997). Low levels of neurotrophins act synergistically with synaptic terminal depolarization, and to be effective, depolarization must take place during the time of neurotrophin exposure. cAMP synergizes with BDNF to potentiate both spontaneous and action potential based evoked neurotransmitter release (Boulanger and Poo, 1999). Literature therefore clearly outlines an important and multifaceted role for BDNF in neuronal circuitry shaping and plasticity, again in conjunction with NTF4.

Literature reports that adverse life events during early developmental stages can produce long lasting changes in the hypothalamic-pituitary-adrenal (HPA) axis, increasing susceptibility to stress and placing one at an increased risk to develop mental disorders (Roceri et al., 2004). Neurotrophins may be an important factor or downstream target of stress considering their regulation integrates changes in response to hormones and neurotransmitters (Runge et al.,

2012; Harrington and Ginty, 2013). Literature indicates that traumatic life experiences (such as those replicated by maternal separation rat model employed in this study) can affect the levels of neurotrophins, namely BDNF. This is exactly what has been found in the present study, where we observed increased levels of BDNF and NTF4 in stress application rat groups, relative to controls. Roceri and co-workers (2004) showed that in a maternal separation rat model, a significant reduction in *BDNF* expression was observed within the prefrontal cortex and striatal brain regions, and speculated that this may render these brain regions less plastic and more vulnerable under challenging conditions (increase susceptibility to psychiatric disorders associated with these brain regions). In this study we find that both *Ntf4* and *Bdnf* were up-regulated relative to controls (*Bdnf* in all stress-application groups) contradicting Roceri (2004). We hypothesize that this may be necessary for neuronal survival and for allowing for a higher level of plasticity in response to environmental stressors. This may contribute to the synaptic scaling observed in the RS MS group, which was not observed in Roceri and co-workers (2004). BDNF is a regulator of ARC (the protein product of another gene identified to be differentially expressed relative to controls), and this may support the contradictory increase in *Bdnf* expression identified in this study compared to Roceri and co-workers (2004). Regardless of the contradiction stated here, the identification of neurotrophins, specifically *BDNF* and *NTF4* as susceptibility candidates in the development of anxiety disorders, warrants further investigation within a human anxiety disorders cohort.

Although neurotrophins have been shown to be involved in striatal neuronal survival, synaptic plasticity, neuronal differentiation and neuronal cell rescue (as described above), there is no data regarding the specific involvement of *NTF4* via the aforementioned mechanism in OCD, SAD, PD or any of the other anxiety disorders. This is therefore, to the best of our knowledge, the first study implicating *NTF4* in the molecular etiology of anxiety disorders, and considering the more present (although still yet not fully understood) *BDNF* involvement stated above, *NTF4* is another novel susceptibility gene for investigation in anxiety disorders.

4.1.3.4. *Activity-regulated cytoskeleton-associated protein (ARC)*

ARC, also known as the *activity-regulated gene 3.1 (ARG3.1)*, produces mRNA which is immediately and rapidly transported to dendrites and specifically accumulates at sites of synaptic activity (Link et al., 1995; Steward et al., 1998). *ARC* accumulates within dendritic cells at the site of local synaptic activity, suggesting a local synthesis (Steward et al., 1998; Rodriguez-Murillo and Greenberg, 2008; Whalley, 2012). The *ARC* gene produces a single protein, whose sequence is highly conserved amongst vertebrate species (Plath et al., 2006; Whalley, 2012). It was initially predicted to be a cytosolic protein (Lyford et al., 1995). Subsequent studies using yeast two-hybrid systems revealed interaction with two proteins, namely dynamin and endophilin, known to play an endocytotic role (Chowdhury et al., 2006). When co-expressed in neurons, *ARC* and endophilin associate with vesicles that selectively traffic AMPARs (AMPA-type glutamate receptors). The rate of surface AMPAR endocytosis is increased and the level of surface AMPAR therefore reduced. The protein similarly reduces the level of synaptic AMPAR responses, as evidenced in experiments using spontaneous miniature excitatory synaptic currents. During these processes the *ARC* protein also appears to be the rate limiting molecule, as endophilin over-expression did not alter the AMPAR trafficking (Whalley, 2012). Models using *ARC* knockouts show synaptic AMPAR levels to be at steady-state, with reduced endocytosis, and also shows increased amplitude of spontaneously evoked miniature excitatory synaptic currents (Bramham et al., 2008). The identification of *Arc* as a susceptibility candidate in our synaptic plasticity assays is therefore well routed within the literature, considering its active role in synaptic activity and glutamatergic regulation. It can also be hypothesized that the reduction in *Grm2* (an AMPAR) may be as a result of *Arc* up-regulation resulting in receptor endocytosis and degradation.

Excitatory synaptic strength and *ARC* expression suggest the role of *ARC* in homeostatic plasticity (Rial Verde et al., 2006). This could explain the differential expression of *Arc* seen in nRS MS and RS nMS groups, relative to controls where *Grm2* expression was not significantly different to controls. *ARC* expression blocks homeostatic increases in AMPAR function as a result of chronic neuronal activity (Hayashi et al., 2012; Whalley, 2012). *ARC*

therefore evokes continuous and precise control over synaptic strength and cellular excitability (Bramham et al., 2008). It was initially thought that ARC action would be limited to late-phase plasticity events, however considering the rapid local translation of *ARC* mRNA, studies revealed it is able to act in an NMDA receptor-independent form of Hebbian plasticity (referring to basic synaptic plasticity in which an increase in synaptic efficacy arises from the presynaptic cell's persistent stimulation of the postsynaptic cell (Park et al., 2008; Waung et al., 2008; Ljaschenko et al., 2013). This form of plasticity requires rapid, *de novo* protein synthesis in dendrites, and it was observed that ARC is translationally induced within 5 min of group 1 metabotropic glutamate receptors (mGluR). This is essential for mGluR-dependent long-term depression (LTD) and once again ties into the susceptibility candidates identified in the RS MS rat group as seen by the down-regulation of *Grm2*. This is characteristic of LTD (Kanhema et al., 2006; Park et al., 2008).

ARC has been shown to be involved in early-phase LTP enhancement and the blockage of late-phase LTP (Plath et al., 2006). Early ARC synthesis is necessary for LTP expression, whilst late expression is involved in LTP consolidation. LTP consolidation is modulated by signaling events initiated post LTP induction, and one of the major regulators of this process is BDNF (Bramham and Messaoudi, 2005; Lynch et al., 2007), further strengthening our plausible susceptibility targets identified. The highlighting of BDNF as a regulator of ARC, acts as support for the contradictory increase in *Bdnf* expression identified in this study compared to Roceri and co-workers (2004). This may support a theory of pathway-based approach for complex trait disorders, such as the anxiety disorders. This means that a single gene may have varied observable effects due to the involvement of entire pathways in disorder pathogenicity.

ARC is therefore capable of neural activity pattern translation at many different levels of protein synthesis-dependent synaptic plasticity. It serves a critical role in AMPAR endocytosis underlying LTD and homeostatic plasticity. Its regulation by BDNF ties it in with *Bdnf* and *Grm2* in the hypothesis of synaptic plasticity and gene expression changes therein being associated with the manifestation of anxiety-like behaviours in rats and supports our the hypothesis for it as a plausible susceptibility gene for anxiety disorders pathogenicity in humans. Again, although *ARC* has been studied with regards to plasticity; it has not been investigated specifically in relation to OCD, PD or SAD. This makes for the first molecular

evidence for the involvement of *ARC* in anxiety disorders pathogenicity and makes for the identification of a novel gene for susceptibility thereto.

4.1.3.5. *Early-Growth Response 2, 4 (EGR2, EGR4)*

EGRs are members of a family of transcription-regulatory factors with a defining characteristic, namely the highly conserved DNA-binding domain composed of three zinc-finger motifs (Gashler and Sukhatme, 1995; O'Donovan et al., 1999). Due to the highly conserved DNA-binding domains and zinc-finger motifs amongst the individual EGR molecules, it is plausible to assume that they might have similar or related functions in the brain (Gashler and Sukhatme, 1995; O'Donovan et al., 1999; Gashler and Sukhatme, 1995). The EGR family has been extensively implicated in the gene expression changes required for synaptic or neuronal plasticity, learning and memory (Milbrandt, 1988; O'Donovan et al., 1999; Li et al., 2005; Poirier et al., 2007). Little is known about the function of the EGR2 protein in the adult brain but it has been associated with peripheral nerve myelination (Topilko et al., 1994), hindbrain segmentation (Giudicelli et al., 2003) and endochondral bone formation (Levi et al., 1996). Due to the incompatibility of *Egr2* knock-out mice with life, EGR2 studies have been limited to those examining regulated expression after a pharmacological input or, as in this study, environmental stressor (maternal separation and restraint stress). Studies by Poirier et al. (2007) using conditional mutant mice showed that, contradictory to the current model of similar function based on EGR1 and EGR3, *Egr2*-deficiency may facilitate learning and memory function (Poirier et al., 2007). This implies that *Egr2* may be involved in antagonistic mechanisms toward brain function, whereby deficient regulation could hamper memory and learning. In the current study, the up-regulation of *Egr2* expression might therefore be advantageous to mechanisms of synaptic remodeling (supporting memory and learning) required in response to early-developmental trauma.

To date, no functional work regarding EGR4 functionality in cognitive and behavioural phenotypic manifestation has been performed and very little is known about the role of the protein in synaptic plasticity (Poirier et al., 2007). This specific member of the EGR family

has been previously associated with male infertility and autonomous germ-cell defects (O'Donovan et al., 1999; Cooke and Saunders, 2002), however no work has been performed in OCD, SAD, PD or any of the other anxiety disorders. The up-regulation of this gene in all stress application groups is therefore an interesting finding and warrants further investigation with regards to functionality in regulation concerning plastic remodeling and homeostasis. Its marked up-regulation in all three stress groups alludes to a possible functional role in synapse modification in response to a stressor in both early-developmental and adulthood stress (although the exact nature of this role has not been elucidated in literature before). This data therefore suggest a role for this gene in manifestations of anxiety in rats. This warrants its consideration in the etiology of anxiety disorder in humans.

4.1.3.6. *Glutamate receptor 2 (GRM2)*

Glutamate is a major excitatory neurotransmitter in the central nervous system (Iihara et al., 2001). The metabotropic glutamate receptors (mGluRs) are members of a class of GTP-binding protein coupled receptors comprising eight subtypes. GRM2 belongs to glutamate receptors subtype 2 and has been previously associated with mental health disorders such as schizophrenia (Joo et al., 2001; Tamandani et al., 2012), which may have phenotypic overlap with anxiety and stress disorders (Watson and Naragon-Gainey, 2010; Achim et al., 2011; Wakabayashi et al., 2012). The significant identification of this gene in the synaptic plasticity pathway of rats exhibiting anxiety-like behaviours suggests that it could prove pivotal to synaptic modeling that may be involved in anxiety disorders (Achim et al., 2011). GRM2 is involved in adenylyl cyclase inhibition thereby suppressing the production of cAMP, and has been involved in pathways such as excitatory neuronal transmission, synaptic plasticity, neural cell death and learning and memory (Joo et al., 2001). The *Grm2* gene was the only gene to show down-regulation relative to the control rats, and only in the combination stressor group (MS+RS). We hypothesized that the down-regulation of this gene in rats exposed to adulthood stress, post early-life trauma, is involved in synaptic preconditioning, allowing neural pathways involved in stress-response to better adapt to a stressful environment. The down-regulation observed for *Grm2* could therefore be the reason for the plateau seen in the behavioural results between MS rats and MS+RS, when we

initially expected to find an increase in anxiety-like behaviour with combination stressors. The possible involvement of *GRM2* in synaptic scaling in response to early-life trauma seems paramount to the understanding of gene-environment interaction and warrants the investigation of this gene in the pathogenicity of anxiety disorders. The glutamate receptors have become quite popular with regards to the involvement in anxiety disorder pathogenicity considering their potential as drug targets for anxiety disorders and schizophrenia treatment strategies (Swanson et al., 2005; Conn and Jones, 2009). The glutamate receptors and glutamatergic-associated plasticity is a hot topic regarding anxiety etiology to date and it has been associated with OCD, SAD and PD pathogenicity (Schoepp et al., 2003; Swanson et al., 2005; Ting and Feng, 2008; Conn and Jones, 2009; Nasca et al., 2013). The elucidation of *GRM2* as a susceptibility candidate in this study is therefore in accordance with the literature and in its identification, adds confidence to the completely novel genes identified alongside it in this study with regards to anxiety disorders.

4.2. Work in Humans

4.2.1. Childhood trauma as a susceptibility risk

Traumatic events occurring during early developmental years have been associated with increased risk for anxiety disorders (Gunnar and Quevedo, 2007; Danese et al., 2009; Irish et al., 2010; Nugent et al., 2011). Results obtained in this study provide further evidence for this hypothesis; patients had significantly increased childhood trauma compared to controls.

Patients reported increased exposure to childhood trauma with regard to all the abuse and neglect subscales of the CTQ, except for physical neglect. Compared to controls, emotional neglect amongst patients demonstrated the highest level of significance ($p < 0.0001$) followed closely by emotional abuse ($p < 0.001$) and sexual abuse ($p < 0.001$). Physical abuse yielded a significant p -value of $p = 0.014$, representing significantly higher levels of physical abuse amongst cases, relative to controls. Although various emotional and physical forms of trauma during childhood appear to be associated with increased susceptibility to develop one or more anxiety disorders, our data suggest that the emotional forms (*i.e.* emotional abuse and

emotional neglect) bear the most weight for this increased risk. Of the physical forms of trauma, sexual abuse carried the most weight for increased susceptibility risk. Interestingly, physical neglect did not yield significant findings in this study; however, this was also previously demonstrated in specifically SAD and anxiety in general (Wessel et al., 2001; Kuo et al., 2011). It is possible that physical neglect is not as weighted as the other components of the CTQ scale, or that based on the presence of a possible support system, physical neglect may be of the variables to nullify more easily.

In summary, our data is consistent with literature that supports a role for childhood trauma as a susceptibility risk factor for the development of anxiety disorders. The role that the selected polymorphisms and haplotypes may have in the susceptibility risk for anxiety disorders will be discussed in the following sections of this chapter.

4.2.2. tNGS

Amplicon-based targeted next generation sequencing (tNGS) was performed on a subset (12 patients and 19 controls) of the research cohort, as described in the methodology. 103 SNPs and 9 indels were identified for both patients and controls. Of these 112 variants, 30 (27 SNPs and 3 indels) were found to be previously identified. This means that 73% of the variation identified was novel to this study (Table 4.1). Considering the 112 variants again, 38 SNPs and 4 indels were unique to either patient or control individuals. Many of these variants are found within the 5' or 3' UTR region and could account for transcriptional regulation changes. These could be especially relevant in considering the role of early life trauma in the pathogenesis of anxiety disorders – a core gene-environment interactive component of this study as well. When focusing on the exome-related data generated: a number of novel exonic variants were found, as well as intronic variants that may disrupt splicing (Table 4.1).

Table 4.1: Summary of the variation identified with NGS

	SNPs	Indels
Total	103	9

Previously Identified (%)	26.21	33.33
This study (%)	73.79	66.66
Patients (%)	49.51	77.78
Controls (%)	50.49	22.22
Unique to Patients (%)	11.65	50.00
Unique to Controls (%)	88.35	50.00
UTR (%)	26.21	11.11
Intron (%)	43.70	77.77
Exon (%)	30.09	11.11

**SNP = single nucleotide polymorphism; Indel = insertion/ deletion; UTR = untranslated region*

The large number of novel polymorphisms identified (in these already novel anxiety-related susceptibility genes) attests to the alluded unique nature of the South African population (specifically Caucasian individuals in this study) (Wright et al., 2011). Genotyping of the aforementioned polymorphisms in the extended cohort provided valuable insight into polymorphism frequencies specifically in the South African Caucasian population.

Determining the genetic architecture of susceptibility for complex neuropsychiatric disorders, like the anxiety disorders, continues to be a challenge. Initial strategies suggested that the advent of genome-wide association studies (GWAS) would result in the elucidation of multiple new targets for further research under the assumption that a number of common genetic variants are most likely the culprits in complex disorders, and not rare mutations (Kricka and Di Resta, 2012; Ripke et al., 2013). What has been found however, is that common variants represent a rather small effect size and that the genetic architecture of some of the more complex disorders are most likely the result of a combination of common and rare variation (Ezewudo and Zwick, 2013). A number of research articles have indicated that with the advent of NGS, *de novo*, rare and novel population-specific variations in the genetic code could be identified and could present with a better association with disorder etiology in complex disorders like schizophrenia and autism (O’Roak et al., 2011; Xu et al., 2011). The same is most likely true for the anxiety disorders and the large quantity of novel polymorphisms identified here (in only five susceptibility genes) supports this assumption.

Although no significant differences were found in terms of case-control association analyses when considering the SNPs identified using tNGS, substantially larger or smaller MAFs were observed compared to previously described data (Table 3.8). The non-significance finding

may be due to small sample sizes despite large discrepancies reported relative to database entries (Evans and Purcell, 2012). In complex disorders, such as the anxiety disorders, the genetic components may be spread across several loci and therefore large sample sizes are necessary to assess a (most likely) small contribution of multiple genes and/ or polymorphisms to disorder pathogenesis. To achieve an 80% power assuming a significance level of 0.05 ($\alpha = 0.05$; $\beta = 0.20$) with an OR of 1.2 or greater for small effects for rare and/ or common alleles, population cohorts much larger than that used here would be necessary (Evans and Purcell, 2012; Pfeiffer and Gail, 2003). Considering the cohort size in this study and assuming a 30.1% life-time prevalence rate for anxiety disorders in the South African population (Kessler et al., 2007), a 83% power can be reached assuming a false-positive maximum of 3 for the polymorphisms investigated for a relative genotype risk of 1.5. This refers to common polymorphisms of a MAF of at least 0.2 (Center for Statistical Genetics, University of Michigan, <http://www.sph.umich.edu/csg/abecasis/CaTS/tour1.html>) (Skol et al., 2007). For polymorphisms with MAF less than 0.2, the power is greatly reduced and findings need to be replicated and validated in a much larger cohort (such as the polymorphisms identified using tNGS).

In summary, the application of tNGS sequencing of five potential susceptibility genes in anxiety disorders yielded 112 polymorphisms (109 SNPs and 3 Indels). Of these the 83% found to be novel (considering the 42 polymorphisms unique to either cases or controls only) suggests that a combination of *de novo*, rare and common population-specific genetic mutations may contribute to disorder susceptibility, and that these may be a result of recent, sudden and immense population expansion rates (Ezewudo and Zwick, 2013; Tennessen et al., 2012). The large discrepancies in MAFs reported for the 7 previously reported tNGS SNPs identified (Table 3.8) in this study further merit the aforementioned statement, and although no significant *p*-values were obtained, due to small sample and effect sizes these non-significant results can best be described as inconclusive until replicated with greater power. As previously mentioned, 112 passed all filter criteria, however only the 42 unique to either cases or controls only were considered for further analyses to minimize SNPs for investigation to those that were most likely to be informative for increased or decreased susceptibility risk, respectively. The remaining 70 polymorphisms were not included for further genotyping in an expanded cohort due to budget constraints, this does not negate their possible association with disorder pathogenesis, and they too should be considered for further

analysis in studies with greater power. The results represented here warrant, at the very least, further consideration for NGS application in the anxiety disorders, in a population specific fashion.

4.2.3. Hardy-Weinberg Equilibrium (HWE)

The Hardy-Weinberg Equilibrium principle states that in the absence of migration, mutation, natural selection and assortative mating, genotype frequencies at any locus are a simple function of allele frequencies (Wigginton et al., 2005; Wittke-Thompson et al., 2005). Deviation in the aforementioned principles could therefore result in an absence of agreement for a HWE model and it is generally deemed that the absence could be associated with the model of disease or disorder being investigated. It is generally accepted that the presence of a variant increasing risk for the development of a particular disease/ disorder could result in deviation from HWE and subsequently that this variant may therefore be a disease-causing or affiliated mutation. A HWE deviation is therefore generally accepted to be found within the cases for variant associated with disease, whilst HWE is met within the controls; however this is not always the case. The reverse may also be possible where deviation from HWE is found within the controls but met within the patients, as is the case with rs17576, and rs925948 in this study. The assumption that can be made in these cases is that deviation from HWE in the control group is possibly associated with disorder pathogenicity and if so in a protective capacity i.e. the controls are adapting whereas the cases are not and this is making cases more susceptible to disease (as discussed by Wigginton et al., 2005; Wittke-Thompson et al., 2005). This may be the case in this study considering the rs925948 polymorphism in *BDNF* and rs17576 polymorphism in *MMP9*; however no statistically significant associations were found for either of these variants. This can therefore best be described as inconclusive and requires validation in a larger cohort.

Focusing on the SNP rs1805088, HWE deviation was observed in both the case and control populations. When evaluating the allelic distribution, HWE was met. The establishment of common trends of HWE and deviation from there in complex disorders (such as the anxiety disorders) is not as well characterized as that of common models of disease (Wittke-Thompson et al., 2005). Additional confounders to HWE deviation in complex disorders

include (but are not limited to) gene-gene interactions, gene-gene-environment, as well as age, gender and population stratification. It is true that deviation from HWE can be a result of genotyping error (Wigginton et al., 2005), however in this study SNPs genotyped using TaqMan probe assays were repeated for randomized selected samples to confirm results. Genotyping performed using assays by LGC Genomics/ KBiosciences using KASP[®] technology were wet-benched tested prior to mass genotyping and quality controlled to ensure accurate typing. Thus it may be argued that genotyping error was not the reason for HWE deviation here. Considering the novelty of the South African Caucasian population (i.e. that fact that it has never been utilized for the screening of the variant before), as well as this polymorphism not having been characterized in a cohort of collective anxiety disorders patients (OCD, SAD and PD), deviation from HWE could be expected due to a number of variables (e.g. population stratification, the combination of different anxiety disorders grouped into a single cohort, and/ or environmental interaction), some of which were not considered here or are unknown. The reason for deviation, however, is not known and before results can be considered sound for this variant, screening in larger cohorts to ascertain whether the genotype and allelic frequencies observed here are maintained, is necessary.

4.2.4. Genotyping, significant associations and interactions

As noted previously, severity of childhood trauma rates were significantly higher in anxiety disorder patients than controls. The following genes and polymorphisms were found to be significantly associated with anxiety disorder pathology, considering childhood trauma as a correlating variable: *BDNF*: rs6265, rs10835210, *MMP9*: rs3918242, rs1805088 and *GRM2*: rs116567227.

4.2.4.1. BDNF

The rs6265 (also known as Val66Met) polymorphism has been well studied in anxiety, as well as other psychiatric disorders (depression, attention deficit hyperactivity disorder and Alzheimer's) (Huang et al., 2007; Krishnan et al., 2007; Sánchez-Mora et al., 2010). The *G*

allele (Val-allele) has previously been reported to be protective against the development of anxiety disorders in American and Spanish Caucasian cohorts, whereas the A allele (Met-allele) affects the intracellular processing of the pro-BDNF polypeptide preventing or hampering its release from active neuronal cells. This protective allelic attribute described was investigated in OCD (Hall et al., 2003) and the intracellular polypeptide mechanism in schizophrenia (Egan et al., 2003) cases. The significant association of rs6265 with anxiety disorders pathogenesis in this study is therefore in line with that reported previously (Chen et al., 2006; Sánchez-Mora et al., 2010, 66), and the OR of < 0.001 in favor of the AG genotype validates the G allele predicting a protective role for this variant. Whereas the significance of this polymorphism in OCD and other psychiatric disorders has been studied (Egan et al., 2003; Hall et al., 2003), this is the first study highlighting its involvement in the underlying etiology of anxiety disorders in an anxiety disorders cohort extended beyond disorder based classifications, as the cases cohort comprised a combination of three anxiety disorders main diagnoses (OCD, SAD and PD).

The rs10835210 has previously been associated with phobic disorders (Xie et al., 2011), as well as associated with a 1.761 X increased likelihood to develop internalizing disorders (Meng et al., 2011). In the present study rs10835210 AC and CC genotypes were found to be significantly correlated with anxiety disorders in protective capacity with ORs of 0.586 and 0.504, respectively suggesting a ~50% reduced susceptibility risk to anxiety disorders. Contradictory to recent reports regarding phobic and internalizing disorders implicating the C allele to result in increased occurrence for internalizing disorders, the presence of the C allele here coincides with an apparent protective effect. This is further validated by the presence of the A allele in the identified *BDNF* AGGAAT haplotype found with increased frequency amongst anxiety disorders cases in this study.

Focusing on a gene-environment interaction, four genetic variants (*BDNF*: rs6265, rs2049045, rs10835210, and rs11030107) were found to significantly interact with the severity of childhood trauma (as measured by CTQ total score) to modify the predicted susceptibility risk to anxiety disorders (as indicated by OR). A single variant was also found to approach significance (*BDNF*: rs1401635). The only variants also found to be statistically significant under gene-environment correlation assessment were rs6265 and rs10835210. Looking at the CTQ total score, although highly significant (at least two tier) *p*-values were

obtained [rs6265: $p = 0.007$ (genotype), 0.034 (allele) and rs10835210: $p = 0.009$ (genotype) and 0.067 (allele)], ORs deviating only slightly from 1 were identified when looking at gene-environment interaction. This would generally be inconclusive at best, however, considering the rs10835210 polymorphism, a decreased risk for anxiety disorders was predicted when assessing gene-environment correlation (OR = 0.287) for this variant. When considering an interaction with childhood trauma (as indicated by CTQ total score), this predicted risk is not observed and the presence of this polymorphism interacting with childhood trauma trends towards increased susceptibility to anxiety disorders (OR = 1.084, CI = 1.02 – 1.15). The rs6265 variant demonstrates this environmental interaction with even more clarity. Gene-environment correlation with childhood trauma indicated the A allele of the heterozygous AG genotype for the rs6265 polymorphism to present with the reduced risk to develop anxiety disorders within this study with an OR of < 0.001 . Considering a gene-environment interaction with the severity of childhood trauma this reduced risk is no longer observed and the presence of the A allele is associated with an up to 1.33-fold increased susceptibility to develop anxiety disorders (OR = 1.132, CI = 0.93 – 1.33). Previous work performed by our group has demonstrated an interaction of the severity of childhood trauma and A allele of the rs6265 polymorphism with increased susceptibility to OCD (Hemmings et al.2013). Validating these findings in larger cohorts for anxiety disorders in general is necessary to confirm results, as well as investigating whether these findings hold true for the individual anxiety disorders.

The rs2049045 (*BDNF*) has been previously reported to be associated with the progression of bipolar disorder with psychiatric comorbidities (Neves et al., 2011). It was found that the presence of this variant in heterozygous or homozygous mutant states was not associated with bipolar disorder itself, but with increased risk for smoking, drinking and alcoholism in these patients. That is a representation of genetic risk by gene-environment correlation. A number of other studies also support the association of rs2049045 with smoking and substance abuse tendencies in psychiatric conditions (Li et al., 2008; Novak et al., 2010), and therefore makes the interaction of this variant with increased severity of childhood trauma to influence anxiety disorder phenotype an interesting finding. The interaction of rs2049045 with CTQ total score in this study yielded an OR of 0.916 (CI: 0.83 – 0.99) suggesting reduced susceptibility risk to having anxiety disorders with the presence of this polymorphism in an individual with early developmental trauma. The final polymorphism within the *BDNF* gene associated with

childhood trauma severity interaction, rs11030107, had a heterozygous genotype presenting with an OR of 1.081 (CI: 1.01 1.17), suggesting that presence of this genotype is associated with increased risk for having an anxiety disorder, when there is a history of childhood trauma to interact with. This same risk, however, was not seen for the homozygous mutant genotype and this could be explained by heterozygous advantage. Heterozygous advantage is where there is a selective preference for the heterozygous genotype of higher fitness as the phenotype of the heterozygote lies outside the phenotype range of both homozygotes (Penn et al., 2002; Charlesworth and Willis, 2009).

The aforementioned gene-environment interactions attest to a definite effect of the severity of childhood trauma in the development and/ or progression of anxiety disorders. These results support the consideration of early life trauma as an interacting co-variable in the underlying neurobiology of anxiety disorders.

4.2.4.2. MMP9

Polymorphisms found within the *MMP9* gene have been well documented to date, but never within an anxiety disorders context. The rs3918242 polymorphism has previously been associated with cardiovascular disease, cancer, and obesity to name a few (Wu et al., 2010; Zhou et al., 2011; Andrade et al., 2012). The MMP proteins are able to degrade nearly all of the endogenous ECM ligands of adhesion molecules, and it is therefore plausible that variation found within the gene has been associated with a large number of disorders/diseases. Anxiety prognosis has been extensively characterized to be a comorbidity in cardiovascular disease and cancer afflicted patients (Martens EJ et al., 2010; Vahdaninia et al., 2010) where the MMP (and specifically MMP9) proteins have been found to be involved in disease progression. The identification of *MMP9* in this study and significant association of polymorphisms with increased susceptibility to anxiety disorders presents molecular evidence for the increased frequency of anxiety phenotypes in these diseases. In this study rs3918242 was found to be significantly associated with anxiety disorders with a high prevalence for the heterozygous genotype containing the mutant T allele amongst patients. Further analyses revealed an OR of 4.674 for the *CT* genotype indicating a > 4-fold increased

likelihood to develop anxiety disorders. Furthermore, no homozygous *TT* cases were found in this study, and this has been the case in previous studies (Holliday et al., 2007). Although this is the first study reporting on this variant and gene in strictly an anxiety disorders setting, previous literature supports the increased susceptibility risk nature of this polymorphism and *T* allele with disease progression with a 9.1 OR in a breast cancer study (Hughes et al., 2007).

The rs1805088 SNP indicated statistically significant association for gene-environment correlation analyses. Further analyses indicated a predicted reduced susceptibility for anxiety disorders associated with *CC* genotype (OR = 0.287). When focusing on the *CT* and *TT* genotypes for this variant, there appeared to be an increased prevalence for the *CT* heterozygous genotype amongst the cases, and increased frequency for the *TT* genotype amongst the controls. This is rather an unusual phenomenon and is most likely responsible for the deviation in HWE identified for this polymorphism. As previously mentioned, deviation in HWE is mostly likely an indicator of errors in genotyping; however this particular variant was typed using a TaqMan assay, with repeat samples typed at a different experimental time-point for quality control purposes. This polymorphisms has not been previously been screened in an anxiety disorders cohort, or the South African Caucasian population. Considering the deviation from HWE associated with this polymorphism, validation of its frequency within a larger cohort as well as alternative South African population is necessary to expand on the uncertainty regarding this polymorphism, and subsequently validate results presented in this study.

4.2.4.3. GRM2

The *GRM2* gene is of particular interest considering its presentation as the only down-regulated gene found in the synaptic plasticity arrays performed on rats exhibiting anxiety-like behaviour. Polymorphisms identified in *GRM2* were achieved by tNGS in a subset of the controls and anxiety cases and then extended to the remainder of the cohort via probe-based genotyping techniques (KASP[®] genotyping). The results revealed very low genotypic and allele frequencies for polymorphisms identified within the study cohort, however one previously identified polymorphism, rs116567227, was found to be significant. Literature

regarding the specifics of this polymorphism with regards to function and/ or effect is lacking except for its identification in the 1000 Genomes project, making its elucidation here a novel one within the context of anxiety disorders and synaptic plasticity. SIFT and PolyPhen are online programmes designed to detect whether the presence of a variant will result in an amino acid change that could affect normal protein function. SIFT and PolyPhen predictions (Ensembl database) indicated low toleration scores and possibly damaging predications, respectively. Due to the low frequency of this polymorphism, ORs predicting decreased or increased susceptibility risk effects were not calculated. Further analyses in larger sample cohorts are necessary to investigate and validate the significance identified here.

Considering gene-environment interaction, the rs113426529 in *GRM2* showed statistical significance for CTQ total score interaction; however, larger cohorts are needed to effectively discern how the presence of childhood trauma affects the phenotypic outcome in the presence of this SNP. It is also noteworthy to mention that *BDNF*'s rs1401635 approached significance for interaction with increased severity of childhood trauma. This variant should be considered for possible significance in larger cohort studies or meta-analysis.

4.2.5. Haplotype Analyses

4.2.5.1. Gene-environment correlation

The ability of genetic risk to be expressed through anxiety cases presenting with a greater exposure to environmental stressors, specifically childhood trauma for haplotype associations was assessed for the polymorphisms identified. Cases have been shown to be associated with a significantly higher severity of childhood trauma, relative to controls ($p < 0.001$) and they are therefore assessed considering this, relative to controls for haplotype associations.

LD analyses were performed on candidate genes for the polymorphisms identified. The majority of the analyses were conducted using the default algorithm designed by Gabriel et al., 2002 in which 95% confidence bounds on D' were generated for each comparison. That

is, if 95% of informative comparisons are in strong LD, a haplotype block is created. Under the confidence intervals analyses, haplotype blocks were identified for polymorphisms in *MMP9* *BDNF* genes. Under the standard confidence interval settings no significant haplotype associations between cases and controls were identified in the *MMP9* gene. *BDNF*, however, yielded one significant haplotype, * rs11030104-rs1401635-rs10835210-rs7103411 *AGAT* which was associated with a higher frequency within cases, compared to controls, and indicated a 2.5-fold increased susceptibility risk to developing anxiety disorders (OR = 2.519). The rs10835210 polymorphism was previously implicated in the aetiology of phobic disorders (including SAD and PD) (Xie et al., 2011), providing further evidence for increased susceptibility risk with this haplotype.

The rs3918242 polymorphism demonstrated high LD, although its inclusion within a haploblock was impeded by the rs3787268 polymorphism which broke LD. Modification of the *MMP9* haplotype block to include rs3918242 for which considerable D' was seen, and to exclude the rs3787268 polymorphism which broke LD from rs3918242, yielded two significant haplotypes for the *MMP9* gene (*MMP9* *rs3918242-rs3918253-rs2274755-rs17576-rs2236416-rs17577-rs13925 *CCTGGAA* and *TCTGGAA*). The *CCTGGAA* haplotype was found with higher frequency in cases compared to controls, yet found to be associated with a decreased susceptibility risk to develop anxiety disorders (OR = 0.469). This is an unusual finding but, and it is possibly attributed to the presence of the C allele of the rs3918242 polymorphism in this haplotype, protective over its T allele counterpart. *TCTGGAA*, however, was found to be present predominantly amongst cases and a > 4.6-fold increased susceptibility risk (OR = 4.608). This haplotype contains the rs3918242 polymorphism previously associated with cardiovascular disease, cancer, and obesity to name a few (Wu et al., 2010; Zhou et al., 2011; Andrade et al., 2012) and 9-fold increased risk for breast cancer (Hughes et al., 2007); conditions known to be associated with high rates of comorbid anxiety prognosis (section 4.2.4.2) (Martens EJ et al., 2010; Vahdaninia et al., 2010). This data, however, represents the first haplotype data that supports association of variation within *MMP9* gene with anxiety aetiology in specifically an anxiety disorders cohort. This therefore poses the questions: should cancer and cardiac compromised patients who carry the associated alleles be monitored (and possibly pre-treated) for anxiety disorders? And also should patients being treated for anxiety disorders who test positive for these polymorphisms be considered at increased risk for heart disease and cancer? These

questions have large clinically, economic and administrative complications when it comes to treatment strategies. The results presented here need to be validated in larger, and alternative cohorts to evaluate whether the considerations the proposed questions imply outweigh the complications mentioned.

4.2.5.2. Possible gene-environment interaction?

To assess gene-environment interaction with regards to haplotype associations, the top (30 cases, highest CTQ total score; mean = 64.36, median = 60.50) and bottom (30 cases, lowest CTQ total scores, mean = 28.9, median = 29) 33rd percentile of patients were tested for association. A direct haplotype-CTQ total score interaction was not able to be assessed within Haploview. Separation of the anxiety disorders cases into high and low CTQ total score sub-groups was therefore performed to assess for differences in the haplotype associations identified. LD analyses were conducted using the same default algorithm designed by Gabriel et al., 2002 in which 95% confidence bounds on D' were generated for each comparison. Under these confidence intervals analyses, two haplotypes were identified within the *MMP9* gene, however only within the top 33rd percentile comparisons (*MMP9* *rs3918253-rs2274755-rs17576-rs2236416-rs3787268-rs17577-13925, *CGGAAGG* and *CGAAGGG*) (Table 3.18). The *CGGAAGG* haplotype presented with an OR of 0.890 and was associated with decreased susceptibility risk towards developing anxiety disorders. The remaining significantly identified haplotype association, *CGAAGGG*, was found to be present in higher frequency within the cases and was appropriately found to be associated with a 1.7-fold increased susceptibility risk (OR = 1.721).

From the gene-environment correlation and gene-environment interaction analyses it is clear that haplotypes within the *MMP9* gene are significantly associated with the pathogenicity of anxiety disorders, and predominantly with substantial increased risk. This association also appears to be influenced by the severity of childhood trauma, observed by the absence of haplotype associations in the bottom 33rd percentile cases (associated with low severity of childhood trauma in patients – relatable to that seen in controls) but with the identification of two within the top 33rd cases sub-group (associated with high severity of childhood trauma).

The validation of these results using statistical consultation to allow for direct haplotype-childhood trauma interaction analyses is necessary to more concretely ascertain the involvement of haplotype interaction with the severity of childhood trauma. The identification of a ‘severity of childhood trauma-single polymorphism’ interaction earlier provides some foundation for this assumption being perpetuated on a haplotype level as well. The identification of haplotype associations considering high severity of childhood trauma, but not low severity childhood trauma in a case-control association fashion further merits the further consideration of haplotype associations in a gene-environment interactive setting.

4.3. Limitations

4.3.1. Animal models of anxiety

For an animal model to be optimal in mimicking a particular condition or disorder, it needs to mimic said condition with regards to etiology, symptomatology and treatment (Bloom and Kupfer, 1995). In complex psychiatric conditions, however, many of the cardinal features are based on subjective verbal report – making the ‘ideal’ animal model somewhat infeasible. This means that when looking at an animal model for human anxiety disorders it is important to redefine the usual requirements and to focus on approaches relying on the mimicking of specific signs, symptoms, behaviours or responses as opposed to trying to replicate the entire condition (Müller and Holsboer, 2006; Schmidt and Müller, 2006). Rodents are the most widely used animals of choice in animal research. In particular, mice and rats are the most commonly used animals in models used for mimicking anxiety disorder traits.

Subjecting rats to maternal separation and restraint stress yielded anxiety-like behaviours as well as changes in gene expression. Significance was maintained for some of the identified genes when extrapolated onto a human cohort suggesting the utilized animal model was effective for this study.

In this study, there were 13 stressor (referring per stressor group) and 13 control rats. This number was found to be acceptable based on literature guidelines (Guidelines for the Care

and Use of Mammals in Neuroscience and Behavioral Research, 2003) and consistent with recent literature pertaining to anxiety disorders (Slattery and Neumann, 2010; Molander et al., 2011). These studies, as well as the present one, comprised of only male rats (to control for any possible sex-linked and hormonal effects in candidate identification) and made use of only environmental interventions.

4.3.2. Cohort size and power

Interrelated variables such as effect size, the type of genetic model used, agreement between susceptibility and marker allele frequency and LD all contribute to the power of an association study. In etiologically complex disorders, such as the anxiety disorders, often very large sample sizes are required to obtain significance since a number of common and rare variants spread across several loci may all make a contribution to the phenotype.

To achieve an 80% power at $\alpha = 0.05$ with an odds ratio of 1.2 or greater in a case-control association study assuming variants with small effects, cohorts in the tens of thousands are needed. Therefore in this study, findings should be considered as preliminary, requiring validation in a much larger sample. Specifically, in situations where statistical significance was not obtained, it can best be described as inconclusive, until validated in a larger study or subjected to meta-analyses. And where statistical significance was obtained, replication is required for validation.

To maximize statistical power within this study, association testing was not performed on the population stratifying for the individual main diagnoses (OCD, SAD and PD). This warrants further investigation in a larger study cohort to ascertain whether significance is maintained, enhanced or lost when stratified.

4.3.3. OCD as an anxiety disorder

OCD has been classified as an anxiety disorder in DSM-II through to DSM-IV; however, recent developments and new knowledge have brought this categorization under scrutiny. Notably, in ICD-10, OCD is classified separately to the other anxiety disorders under the category of “Neurotic, stress-related, and somatoform disorders” (Stein et al., 2010; Bienvenu et al., 2012). Changing its classification would not change which individuals would receive a particular diagnosis, however, it would bare influence on disorder conceptualization and ultimately treatment regime (Stein et al., 2010). There are many overlapping features between OCD and the other anxiety disorders (e.g. frequent comorbidity with depression, avoidance behaviours, response to SSRIs, etc.). However, over time, research and clinical evidence suggested that OCD may be more closely related to other conditions characterized by driven, non-functional, repetitive nature (e.g. body-focused repetitive behaviour disorder, hair-pulling disorder (trichotillomania) and skin-picking disorder). Nevertheless, there is considerable overlap between OCD and the anxiety disorders and this project rests on these. Considering a neuronal circuitry perspective, for example, there is evidence to suggest similarities in neuronal circuitry between OCD and the anxiety disorders (e.g. OCD, PD, SAD and PTSD share dopaminergic and serotonergic neuronal pathway involvement considering SSRI effectivity in treatment) (Stein et al., 2010). Family studies have also linked OCD to anxiety disorders, particularly general anxiety disorder (GAD), (Stein et al., 2010). Reviews of the current literature have led to recommendations that DSM-5 change the section of anxiety disorders to include OC-spectrum disorder (Phillips et al., 2010; Stein et al., 2010) and be called “Anxiety and Obsessive Compulsive-Spectrum Disorders”.

Drawing on evidence from the above literature, the inclusion of OCD in this study of some of the anxiety disorders thus seems warranted. Although the reclassification of OCD in DSM-5 may be clinically relevant, it may be argued that the significant overlap with other anxiety disorders warrants its inclusion in a study to elucidate the molecular etiology of anxiety disorders.

4.3.4. The absence of other DSM-IV anxiety disorders: PTSD, GAD and specific phobia

PTSD is an anxiety disorder in the DSM-IV and also believed to be associated with early-developmental trauma, although characteristically manifesting after an immediate life threatening event (Perry, 1994; Yehuda et al., 2001; Shavitt et al., 2010). A GAD diagnosis requires an uncontrollable, unrealistic worry about multiple topics as well as an accompanying physiological symptom, over a long period of time (Stein, 2004). Development of GAD has also been associated with childhood trauma (Afifi et al., 2010; Nugent et al., 2011; Weems and Varela, 2011). Specific phobia is characterized by severe anxiety in response to, and subsequent avoidance of, a specific stimulus, and often develops after a particular traumatic event (First and Gibbon, 2004). Specific phobia has also previously been associated with childhood trauma resulting in increased susceptibility for the disorder (Briggs-Gowan et al., 2010). Arguably, it would have been useful to include individuals with a primary diagnosis of PTSD, GAD or specific phobia, in addition to the current population. Unfortunately we did not have access to large enough numbers of samples with these diagnoses. Repeating this work in such cohorts is warranted.

4.3.5. Use of the dorsal striatum

Another limitation is the use of the rat striatum only. There are several other brain regions associated with anxiety disorder pathology (e.g. the hippocampus and amygdala). Using the methods presented here on these other brain structures may provide further insight into the pathogenicity of anxiety disorders.

4.4. Future research directions

The present study forms part of a larger collaborative research effort to investigate and identify the molecular etiology of anxiety disorders. The aim here was to identify novel genes that may be involved in the pathogenesis of anxiety and also to ascertain gene-environment interaction effects in disorder pathogenicity.

The use of rats exhibiting anxiety-like behaviours to identify novel susceptibility genes for anxiety disorders in humans was successfully demonstrated in this study, with the identification of several genes that have previously been reported to be involved in synaptic plasticity and other neuronal pathways, but not directly in an anxiety disorders disorder cohort.

The identification of more than 100 polymorphisms within five genes of which 80% were novel, attests to the unique nature of the South African population from a genetic perspective (Wright et al., 2011). With NGS becoming more affordable and easily accessible, the application of targeted deep sequencing for known anxiety-associated genes within a South African context (or other geographical delineations) could yield many new polymorphisms. Novel polymorphisms may be working in an epistatic fashion with already characterized polymorphisms (such as the rs6265 polymorphism in *BDNF*), with each other or with environmental influences (such as childhood trauma), and identifying these may allow for a more complete picture of the genetic architecture of anxiety disorders.

A further consideration is the statistically significant association between childhood trauma and the presence of an anxiety disorder. Gene-environment interactions were identified between certain polymorphisms and the severity of childhood trauma. Childhood trauma is but one of the environmental variables that can play a role in the pathology of anxiety disorders and further gene-environment interplay (and combinations thereof) (*e.g.* pharmacotherapy and nutrition) should be investigated in future studies.

4.5. Conclusion

In this study a Sprague Dawley rat model subjected to environmental stressors was implemented to determine the effect of developmental stress on gene expression. The objectives of this study were to determine if there are differentially expressed genes in rats with a trauma history vs. those without, to determine whether the identified differentially expressed genes are potential susceptibility genes for anxiety disorders in humans, using case-control association studies, and to determine whether genes that are differentially expressed in rats with a trauma history and with consequent anxiety-like behaviours point to

susceptibility genes for anxiety disorders in humans for which childhood trauma severity scores are known.

Firstly, study findings confirmed that rats exposed to environmental stressors were observed to exhibit significantly increased anxiety-like behaviours compared to unexposed controls. When differences in gene expression were assessed comparing rat groups exhibiting anxiety-like behaviours to controls, several genes (*i.e. Bdnf, Mmp9, Arc, Ntf4, Grm2, Egr2, and Egr4*) were found to be differentially expressed in the synaptic plasticity pathway of striatal tissue. This provides evidence for the usefulness of an environmental intervention rat model displaying subsequent anxiety-like behaviours in evaluating the molecular underpinnings regarding a gene-environment interaction in anxiety disorders. Secondly, considering the human homologues for the susceptibility candidates identified, *BDNF* was the only gene to be well characterized within the anxiety disorder literature. The remaining candidates, although prominent in synaptic plasticity, have not been specifically investigated within anxiety disorder pathology. This study, therefore, successfully produced novel candidate genes to act as susceptibility targets in anxiety disorders. Moreover, in addition to identifying novel susceptibility candidates using a pathway specific approach, differentially expressed genes were associated with anxiety-like behaviours, making a strong argument for susceptibility candidacy in anxiety disorder pathology. For example, the under-regulation of the *GRM2* gene (involved in excitatory neuronal responsiveness) in the combination stressor groups points to an attenuating or scaling response to further stressors.

And thirdly, the work presented here provides further validation for childhood trauma as a risk factor for susceptibility to anxiety disorders. Candidate genes, assessed in a case-control association fashion, yielded significant variant associations with anxiety disorders in either a protective or increased susceptibility risk fashion. Four significant SNP interactions with childhood trauma were also identified suggesting a gene – environment interaction with the presence of early life trauma in the manifestation of anxiety disorders. The rs10835210 polymorphism presented with protective attributes associated with the *AC* genotype; however, this seemed to skew to an increased susceptibility in the presence of childhood trauma. The same was true for Val66Met (rs6265) in *BDNF*, whose highly protective effect was eliminated when considering increased severity of childhood trauma. This is the kind of gene – environment interaction observed that needs to be evaluated when considering the

etiology of the anxiety disorders, to allow for a better understanding, and ultimately better treatment of these conditions. The rs2049045 polymorphism and its identification as a genetic risk factor by gene-environment correlation in substance abuse is interesting, considering substance abuse being a commonly associated comorbidity amongst anxiety disorders and its identification as a gene-environment interaction variable in this study. Substance abuse as an environmental contributor to the phenotypic manifestation of a disorder or disease as a result of gene – environment interaction has been documented previously (Douglas et al., 2010; Pani et al., 2010). Validating its role as a gene – environment component in anxiety disorders in a cohort for which substance abuse information is available is warranted. To summarize, there is a definite gene – environment interaction to be considered when there is an observable increased incidence in the severity of childhood trauma in cases, and in the presentation of anxiety disorders. There are also grounds for the consideration of other environmental contributants (smoking, alcohol abuse etc.) in addition to the effect of childhood trauma in investigating and understanding the molecular etiology of anxiety disorders.

This study represents one of the first to employ a tNGS approach to identify novel and previously characterized genetic variants associated with anxiety disorders. In excess of 100 polymorphisms (>100 SNPs and 4 Indels) were identified of which over 80% were novel, contributing valuable knowledge to current database capacities regarding anxiety disorder etiology.

In conclusion, the work presented here yields important findings pertaining to the neurobiological underpinnings of anxiety disorders and has contributed novel susceptibility genes, SNP and haplotype associations for consideration in the molecular etiology of anxiety disorders. These variants have been investigated in the context of gene-environment interaction and gene-environment correlation in the context of childhood trauma, and significant associations have been found. The sample size remains to be one of the most important limitations warranting follow up in a larger sample for validation, as well as assessment in individual anxiety disorders to determine whether the significance identified here holds true. The genetic heterogeneity of the anxiety disorders is challenging; however, the findings presented here contribute substantially to a better understanding of the etiology of anxiety disorders.

Appendix I

Buffers and Solutions

1. DNA extraction solutions

Cell Lysis Buffer

Sucrose	0.32 M
Triton-X-100	1%
MgCl ₂	5 mM
Tris-HCl	10 mM
H ₂ O	1 L

3 M Sodium Acetate

Sodium Acetate

H₂O

pH adjusted to 5.2 with glacial acetic acid (Merck)

ddH₂O – brought to volume of 100 ml

Na-EDTA solution

NaCl (Sigma)	18.75 ml of 4 M Stock
EDTA (Sigma)	250 ml of 100 mM stock solution

Phenol/Chloroform

Phenol	50 ml
Chloroform	48 ml

8-hydroxyquinone	2 ml
------------------	------

Mix and store at 4°C

Chloroform/ octanol (24:1)

Chloroform	96 ml
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Octanol	4 ml
---------	------

Store at 4°C

TE-Buffer (10X stock)

Tris OH	108 g
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EDTA (pH 8.0)	0.01 M
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H ₂ O	150 ml
------------------	--------

2. Electrophoresis Solutions**TBE-Buffer (10X Stock)**

Tris-HCl (Sigma)	108 g
------------------	-------

Boric Acid (Sigma)	58 g
--------------------	------

Na ₂ EDTA (Sigma)	9.3 g
------------------------------	-------

ddH₂O to final volume of 1 L

Ethidium Bromide

Ethidium Bromide	500 mg
------------------	--------

ddH ₂ O	50 ml
--------------------	-------

Cresol loading dye

Cresol Red (Sigma) Solution	10 mg in 1 ml ddH ₂ O
-----------------------------	----------------------------------

Sucrose Solution 3.4 g sucrose in 9.8 ml ddH₂O

Add 200 µl to 10 ml of sucrose solution

2% Agarose Gel

Agarose (Sigma) 2 g

ddH₂O bring to volume of 100 ml

Appendix II

Experion output data for RNA quality and quantity assessment

Sample Name	RNA Area	[RNA] (ng/ μ l)	Ratio [28S:18S]	RQI	RQI Classification
S1	328.28	96.05	1.47	9.2	Green
S2	304.74	89.16	0.88	7.7	Green
S3	370.52	133.66	1.78	9.7	Green
S3	222.39	65.07	1.53	9.3	Green
S4	250.13	73.18	1.18	8.9	Green
S5	338.46	99.03	1.45	9.1	Green
S6	321.65	94.11	1.57	9.4	Green
S7	267.17	78.17	1.18	9.2	Green
S8	254.61	74.5	1.02	8.9	Green
S9	355.64	104.05	1.37	9.2	Green
S10	272.13	79.62	1.6	9.5	Green
S11	340.46	99.61	1.3	9.2	Green
S12	411.69	148.51	1.71	9.5	Green
S14	269.42	78.83	1.06	9	Green
S15	377.24	136.09	1.46	9.4	Green
S16	240.07	86.6	1.74	9.5	Green
S17	19.08	6.88	1.35	N/A	Red
S18	181.15	98.14	1.06	8.9	Green
S19	299.97	108.21	1.74	9.6	Green
S20	277.02	99.93	1.61	9.5	Green
S21	361.5	130.41	1.72	9.5	Green
S22	221.96	80.07	1.35	9.2	Green
S23	258.99	93.43	1.28	9.1	Green
S24	244.64	88.25	1.32	9	Green

S25	235.11	84.81	1.35	9.1	Green
S26	180.18	64.17	1.09	9	Green
S27	64.55	22.99	1.59	9.2	Green
S28	161.33	57.45	1.36	8.8	Green
S30	84.83	45.95	1.27	8.8	Green
S31	45.22	24.5	1.37	8.7	Green
S32	18.95	10.26	1.25	7.2	Green
S33	64.2	34.78	1.35	9	Green
S34	75	40.63	1.65	8.9	Green
S35	56.94	30.85	1.31	8.6	Green
S36	89.17	48.31	1.71	9.1	Green
S37	101.49	54.98	1.61	9	Green
S38	55.19	19.65	1.85	9.2	Green
S39	29.45	10.49	1.7	9.1	Green
S40	35.63	12.69	1.83	9.6	Green
S41	71.19	38.57	1.31	8.7	Green
S42	46.03	24.94	1.24	8.8	Green
S43	66.21	23.58	1.46	9.6	Green
S44	37.74	13.44	1.23	9.2	Green
S45	142.07	50.59	1.7	9.4	Green
S46	118.32	42.14	1.43	9	Green
S47	103.64	36.91	1.51	8.9	Green
S48	112.19	39.95	1.61	9.2	Green
S49	13.84	7.5	1.91	N/A	Red

**RQI = RNA quality indicator; RQI Classification: Red = failed, Green = passed; S refers to Striatal tissue*

***S50 and S51 were failed samples and are not represented here*

Appendix III

RT² Profiler™ Array gene list

Type	Unigene	GeneBank	Symbo l	Description	Gene Name
5, 6, 9	Rn.42924	XM_217197	Adam1 0	ADAM metallopeptidase domain 10	MADM
3	Rn.21414 5	XM_223616	Adcy1	Adenylate cyclase 1 (brain)	Ac1
3	Rn.10382	NM_017142	Adcy8	Adenylate cyclase 8 (brain)	Ac8
7	Rn.11422	NM_033230	Akt1	V-akt murine thymoma viral oncogene homolog 1	Akt
1, 9	Rn.10086	NM_019361	Arc	Activity-regulated cytoskeleton-associated protein	rg3.1
1, 3	Rn.11266	NM_012513	Bdnf	Brain derived neurotrophic factor	MGC10525 4
3	Rn.10749 9	NM_012920	Camk2 a	Calcium/calmodulin-dependent protein kinase II alpha	PK2CDD, PKCCD
3, 7	Rn.10961	NM_133605	Camk2 g	Calcium/calmodulin-dependent protein kinase II gamma	-
3, 5	Rn.23200	NM_031333	Cdh2	Cadherin 2	N-cadherin
1	Rn.6479	NM_024125	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	Il6dbp, LAP, NF- IL6, TCF5
1	Rn.20262 0	NM_013154	Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	C, EBPd, CELF
3	Rn.89774	NM_012784	Cnr1	Cannabinoid receptor 1 (brain)	SKR6R
1	Rn.90061	NM_031017	Creb1	CAMP responsive element binding protein 1	Creb
1	Rn.10251	NM_013086	Crem	CAMP responsive element modulator	Icer

9	Rn.9765	NM_019621	Dlg4	Discs, large homolog 4 (Drosophila)	Dlgh4, PSD95, Sap90
1	Rn.9096	NM_012551	Egr1	Early growth response 1	Krox-24, NGFI-A, Ngf1, Ngfi, zif-268
1	Rn.89235	NM_053633	Egr2	Early growth response 2	Krox20
1	Rn.44371	NM_017086	Egr3	Early growth response 3	-
1	Rn.31998	NM_019137	Egr4	Early growth response 4	Egr4I1, NGFI-C
8	Rn.27233	XM_233574	Ephb2	Eph receptor B2	RGD15642 32
1	Rn.10375 0	NM_022197	Fos	FBJ osteosarcoma oncogene	c-fos
3	Rn.10368	NM_017295	Gabra5	Gamma-aminobutyric acid (GABA) A receptor, alpha 5	-
3, 4, 8	Rn.11391	NM_013145	Gnai1	Guanine nucleotide binding protein (G protein), alpha inhibiting 1	BPGTPB
3, 4, 8, 9	Rn.29971	NM_031608	Gria1	Glutamate receptor, ionotropic, AMPA 1	gluR-A
3, 4, 8	Rn.91361	NM_017261	Gria2	Glutamate receptor, ionotropic, AMPA 2	GluR-K2, GluR2, gluR-B
4, 8, 9	Rn.74049	NM_032990	Gria3	Glutamate receptor, ionotropic, AMPA 3	GLUR3, GluR-3, GluR-C, GluR-K3
4, 8, 9	Rn.10938	NM_017263	Gria4	Glutamate receptor, ionotropic, AMPA4	GluR-D, GluR4
3, 7, 8, 9	Rn.9840	NM_017010	Grin1	Glutamate receptor, ionotropic, N-methyl D- aspartate 1	NMDAR1, NR1

3, 5, 7, 8, 9	Rn.9710	NM_012573	Grin2a	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	NMDAR2A, NR2A
3, 5, 7, 8, 9	Rn.9711	NM_012574	Grin2b	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	-
3, 7, 8, 9	Rn.9709	NM_012575	Grin2c	Glutamate receptor, ionotropic, N-methyl D-aspartate 2C	NR2C
3, 7, 8	Rn.91209	NM_022797	Grin2d	Glutamate receptor, ionotropic, N-methyl D-aspartate 2D	-
4	Rn.74240	NM_032069	Grip1	Glutamate receptor interacting protein 1	-
4, 8, 9	Rn.87787	NM_017011	Grm1	Glutamate receptor, metabotropic 1	Gprc1a
4, 8	Rn.9681	XM_343470	Grm2	Glutamate receptor, metabotropic 2	-
8, 9	Rn.41715	XM_001062001	Grm3	Glutamate receptor, metabotropic 3	mGluR3
8	Rn.89046	NM_022666	Grm4	Glutamate receptor, metabotropic 4	-
8	Rn.29972	NM_017012	Grm5	Glutamate receptor, metabotropic 5	mGluR5, mGluR5
8	Rn.10409	NM_031040	Grm7	Glutamate receptor, metabotropic 7	-
8	Rn.44420	NM_022202	Grm8	Glutamate receptor, metabotropic 8	Glur8, Gprc1h, Mglur8, mGluR8b, mGluR
1, 9	Rn.37500	NM_031707	Homer 1	Homer homolog 1 (Drosophila)	HOMER1F, Vesl-1
4	Rn.6282	NM_178866	Igf1	Insulin-like growth factor 1	-
2	Rn.9874	NM_017128	Inhba	Inhibin beta-A	-
1	Rn.93714	NM_021835	Jun	Jun oncogene	-

1	Rn.15806	NM_021836	Junb	Jun B proto-oncogene	-
1	Rn.2398	NM_031135	Klf10	Kruppel-like factor 10	Tieg
3, 4, 7	Rn.34914	NM_053842	Mapk1	Mitogen activated protein kinase 1	Erk2
1, 3, 6	Rn.10209	NM_031055	Mmp9	Matrix metalloproteinase 9	-
5	Rn.11283	NM_031521	Ncam1	Neural cell adhesion molecule 1	Cd56, MGC12460 1, N-CAM, NCAM-C, Ncam
1	Rn.2411	XM_342346	Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NF-kB
1	Rn.8395	NM_030867	Nfkbib	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	MGC93398
1	Rn.22168	XM_227525	Ngf	Nerve growth factor (beta polypeptide)	Ngfb
4	Rn.10980	NM_012610	Ngfr	Nerve growth factor receptor (TNFR superfamily, member 16)	LNGFR, RNGFR, p75, p75NTR
4	Rn.10573	NM_052799	Nos1	Nitric oxide synthase 1, neuronal	bNOS
1	Rn.16210	NM_001034	Nptx2	Neuronal pentraxin 2	Narp
1	Rn.10000	NM_024388	Nr4a1	Nuclear receptor subfamily 4, group A, member 1	HMR, Ngf-b, Nur77
1	Rn.9715	NM_031073	Ntf3	Neurotrophin 3	-
3	Rn.44225	NM_013184	Ntf4	Neurotrophin 4	NT4P, Ntf5
3, 8	Rn.11246	NM_012731	Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	RATTKB1, TRKB1, Tkrb
1, 5	Rn.23337	NM_022868	Pcdh8	Protocadherin 8	-
4, 9	Rn.24750	NM_053460	Pick1	Protein interacting with PRKCA 1	Prkcbp

1	Rn.34888	NM_017034	Pim1	Pim-1 oncogene	-
1, 4, 6	Rn.10710 2	NM_013151	Plat	Plasminogen activator, tissue	PATISS, tPA
3	Rn.11243	NM_013187	Plcg1	Phospholipase C, gamma 1	PPLCA
3, 4, 7	Rn.2024	NM_031527	Ppp1ca	Protein phosphatase 1, catalytic subunit, alpha isoform	-
3, 4, 7	Rn.1495	NM_022498	Ppp1cc	Protein phosphatase 1, catalytic subunit, gamma isoform	Ppp1cc1
4	Rn.73852	NM_130403	Ppp1r1 4a	Protein phosphatase 1, regulatory (inhibitor) subunit 14A	Cpi17
4, 5	Rn.1271	NM_017039	Ppp2ca	Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	Pp2a1
3, 4	Rn.6866	NM_017041	Ppp3ca	Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	Calna1
3, 4	Rn.20790 8	XM_343975	Prkca	Protein kinase C, alpha	Pkca
3	Rn.9747	NM_012628	Prkcg	Protein kinase C, gamma	MGC10548 7, PKC, PKCI, Prkc, Prkcc, RATPKCI
4	Rn.20472 4	XM_219805	Prkg1	Protein kinase, cGMP-dependent, type 1	Pkgi, cGk1
3	Rn.44409	NM_013018	Rab3a	RAB3A, member RAS oncogene family	RAB3
1	Rn.19480	NM_199267	Rela	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	NFkB
5, 6	Rn.98353	NM_080394	Reln	Reelin	Reelen, RI, reeler
10	Rn.12325 1	XR_008709	RGD15 62511	Similar to MmKIF17	-

1	Rn.1892	NM_053453	Rgs2	Regulator of G-protein signaling 2	-
1	Rn.859	NM_013216	Rheb	Ras homolog enriched in brain	-
10	Rn.21997 6	XM_228146	Sirt1	Sirtuin (silent mating type information regulation 2 homolog) 1 (<i>S. cerevisiae</i>)	Sir2
1	Rn.1501	NM_001109 302	Srf	Serum response factor (c-fos serum response element-binding transcription factor)	RGD15597 87
2, 9	Rn.42910	NM_021695	Synpo	Synaptopodin	-
6	Rn.25754	NM_053819	Timp1	TIMP metalloproteinase inhibitor 1	TIMP-1, Timp
1, 5	Rn.2275	NM_012675	Tnf	Tumor necrosis factor (TNF superfamily, member 2)	MGC12463 0, RATTNF, TNF-alpha, Tnfa
3	Rn.2502	NM_013053	Ywhaq	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	14-3-3t
11	Rn.973	NM_001007 604	Rplp1	Ribosomal protein, large, P1	MGC72935
11	Rn.47	NM_012583	Hprt1	Hypoxanthine phosphoribosyltransferase 1	Hgprtase, Hprt, MGC11255 4
11	Rn.92211	NM_173340	Rpl13a	Ribosomal protein L13A	-
11	Rn.10789 6	NM_017025	Ldha	Lactate dehydrogenase A	Ldh1
11	Rn.94978	NM_031144	Actb	Actin, beta	Actx

*1: immediate-early response (IEGs), 2: late response, 3: long term potentiation (LTP), 4: long term depression (LTP), 5: cell adhesion, 6: extracellular matrix and proteolytic processing, 7: CREB factors, 8: neuronal receptors, 9: postsynaptic density, 10: other, 11: housekeeping genes

Appendix IV

Patient Information and Informed Consent

UCT/ US MRC UNIT ON ANXIETY AND STRESS DISORDERS

PATIENT INFORMATION AND INFORMED CONSENT

Genetics of Anxiety Disorders

PURPOSE:

This study is part of a research project we are conducting to learn more about the genetic causes and symptoms of anxiety disorders (including obsessive-compulsive and spectrum disorders such as trichotillomania (TTM), panic or social anxiety disorder). We would like to discuss your life experiences and those of your other family members with you. Doctors and scientists at the MRC Unit on Anxiety and Stress Disorders and the University of Stellenbosch, in collaboration with qualified researchers from other research institutions worldwide, hope to identify the genes that may increase susceptibility to these disorders.

This is not a treatment study. Information is being collected for research purposes only.

STUDY PROCEDURE:

If you decide to participate, we shall ask you to attend an interview (which may be videotaped) with a researcher. This interview will include neuropsychological tasks and a number of questions related to your current illness, comorbid conditions like depression,

family functioning, quality of life, your prior history of treatment for psychiatric conditions, and particular symptoms you may have experienced as part of your illness. In addition, we may ask to take photographs of your face and hands. This whole procedure will last about 4-5 hours (two 2-hour sessions with a break in-between).

You will also be asked to have your blood drawn. Approximately 48 ml (3 tablespoons) of blood will be drawn from your arm. We may need to contact you again to get another blood sample should we fail to get a DNA sample from your blood. The blood sample you give may be used to create a cell line. This is done by changing some of your blood cells so that they can grow forever. The cell line is living tissue and it can be used to make more of your DNA at any time in the future. This process will take place at the MRC Centre for Molecular and Cellular Biology and the Division of Medical Biochemistry, Faculty of Health Sciences, at the University of Stellenbosch. The DNA will then be taken from the cell line and saved for scientific analyses which will be performed now, and possibly in the future.

We may contact you later for further information, or request you to complete another interview at a later date, in order to obtain follow-up information that may be of use in our genetic analyses. This may involve an assessment similar to the current assessment, including a series of interviews and/or another blood sample. Your current participation is in no way binding to your future participation.

We would like your permission to contact your relatives in order to get more information about any family history of mental illness, if need be.

If you have been diagnosed with TTM, we hope to also interview one of your close (first-degree) relatives. You can still participate in the study even if your relatives do not. If you are a relative (e.g. a parent) of a person diagnosed with TTM who have participated in this project, we will ask you to complete a number of self-report scales. These scales will ask questions about your current psychiatric symptoms if any, depression, anxiety, family functioning and quality of life. These scales will also ask you whether your child has ties,

OCD and other problems. They will also ask about your child's psychiatric condition and how you respond to it. It can take up to 4 hours to complete these self-report scales – either at home or at our Unit.

Personal information that could be used to identify you (such as your name, contact information, etc.) will not be given out. Your data and DNA is likely to be made available to qualified scientists around the world to study your particular disorder. Your cell line and DNA will be maintained permanently, unless you request to have it removed. If at any time in the future you wish to have your DNA, cell lines or clinical data removed from the storage site, you may do so by contacting the researchers conducting this study (Christine Lochner at 021 - 938 9179).

The researchers who will have access to your DNA include those who work with private and/or for profit companies. These researchers may be interested in eventually developing commercial medical products using the DNA from you and other participants. They may sell or patent discoveries based on this research and thus benefit financially. Please note that you or your heirs will not receive any compensation if this occurs.

We do not expect to discover any information of direct benefit to your condition, or your treatment, during the next few years. If later on, diagnostic tests or new ways to treat your condition are discovered, this information will have to be obtained from properly licensed clinical labs, clinics, or your physician, and will not be available from the research team.

If you are hospitalized at a psychiatry facility or have received any treatment from a mental health professional, we would like your permission to review your treatment records, which will be obtained from your doctor.

RISKS:

There are no more than minimal medical or psychological risks associated with this study. If you feel fatigued, tired, uncomfortable, or in any way upset during any part of the session(s), you may ask to stop for a rest break or have the interview discontinued. The research interview does not take the place of a full psychiatric evaluation. You may experience some emotional discomfort when answering some questions. If any particular question makes you feel uncomfortable, you may discuss its importance with the specially trained interviewer. You may choose not to answer any question which you are still uncomfortable with.

You may feel some pain associated with having blood withdrawn from a vein. You may experience discomfort, bruising and/or other bleeding at the site where the needle is inserted. Occasionally, some people experience fleeting dizziness or feel faint when their blood is drawn.

Some insurance companies may mistakenly assume that your participation in this study is an indication that you are at higher risk of a genetic disease, and this could hurt your access to health or other insurance. We will not share any information about you, or your family, with an insurance company. However, if you discuss your participation in this study with your doctor, and he or she records it in your medical record, it is possible that an insurance company may access the information as part of a medical record review. It is the opinion of the investigators that participation in this study does not constitute genetic testing. Although one long-term goal of this research is the development of a genetic test for the anxiety disorders, at the current time, no information from your DNA sample that would be useful in the treatment of your disorder will be obtained. Therefore, participation in this study should not be reported as genetic testing.

Your unidentified DNA and cell line will be available to qualified researchers permanently.

BENEFITS:

There are no direct benefits to you. However, individuals who might develop one or more of these anxiety disorders in the future, their family members, and future generations may benefit if we can locate the genes that lead to such disorders. That knowledge could then lead to the development of methods for prevention and new treatments for curing these diseases.

CONFIDENTIALITY:

If you consent to participate in this study, your identity will be kept confidential. Your answers will not be shared with other family members or anyone else except for staff members involved in this study. All data will be kept in locked file cabinets accessible only to the research staff. All research information obtained will not be associated with your name; research staff will use only a coded number and/or your initials. Blood samples will be safely stored and identified by code number and access will be limited to authorized scientific investigators. Copies of treatment records from hospitals or mental health professionals are kept in locked files and are reviewed by members of the research team only. Any publications resulting from this study will not identify you by name.

VOLUNTARY PARTICIPATION:

Your participation in this study is voluntary and you may refuse to participate or withdraw from the study at any time without any loss of benefits to which you are otherwise entitled. Some members of the team of investigators conducting this study may be responsible for your clinical care. Refusal to participate in this study will not change your clinical care.

RESEARCH QUESTIONS AND CONTACTS:

If you are interested in genetic counseling, you will be given information about where you can receive such counseling and a new blood sample may be required at that time. DNA information about a relative will be released only if the genetic counsellor confirms that the relative in question is deceased or cannot be found and that the information is essential for clinical counseling.

The researchers will answer any questions you might have about the procedures described above, or about the results of the study. If you have any questions, you may call Dr Christine Lochner at (021) 938 9179.

The University of Stellenbosch Committee for Human Research has approved recruitment and participation of individuals for this study.

You have been given a copy of this consent form to keep.

INFORMED CONSENT/ASSENT TO TAKE PART AND AUTHORIZATION TO USE OR SHARE HEALTH INFORMATION FOR RESEARCH:

I have read the above patient information, the research study has been explained to me, including risks and benefits (if any), all my questions have been answered, and I consent voluntarily to participate in this study.

Print name: _____ Signature: _____

(Adults or Minors younger than 18 years)

Date: _____

OR

I understand the information that was given to me, and would like to give permission for my child/the person I am authorized to represent, to take part in this research study, and also agree to allow his/her health information to be used and shared as described above.

Print name: _____ Signature: _____

(Parent / Guardian of Minor)

Date: _____

OR

Print name: _____ Signature: _____

(Family member / next of kin)

Date: _____

Relationship to patient: _____

I have discussed the proposed research with this subject and, in my opinion, this patient understands the benefits, risks, and alternatives (including non-participation) and is capable of consenting to voluntary participation.

Print name: _____ Signature: _____

(Study Investigator or Designee)

Date: _____

Print name: _____ Signature: _____

(Witness (if applicable))

Date: _____

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