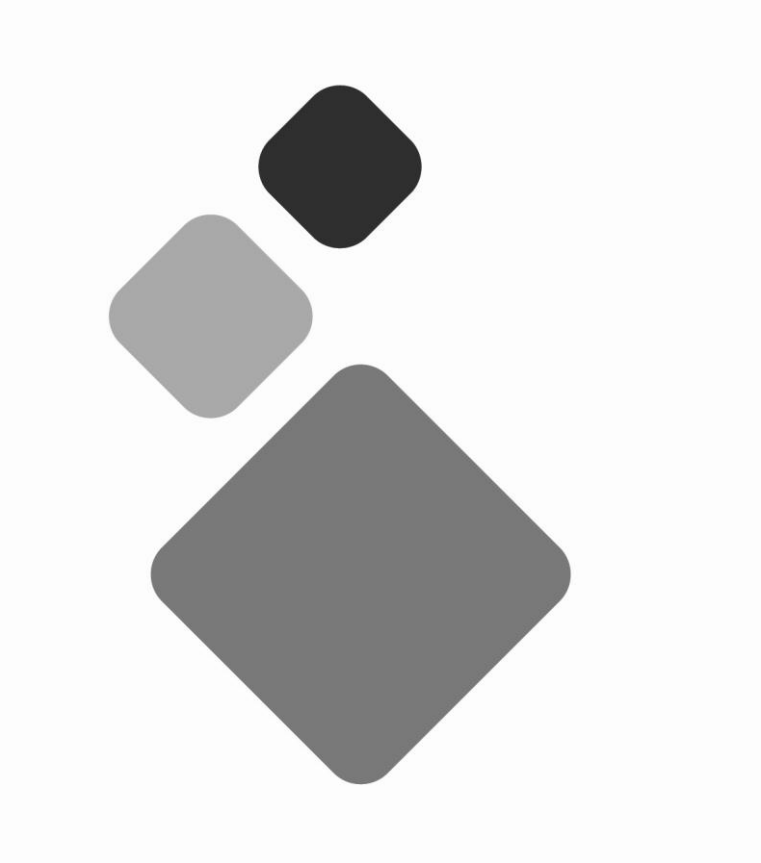

From risk genes to psychiatric phenotypes

Studies of fibroblast growth factor-related and genome-wide genetic variation in humans and mice

Afke F. Terwisscha van Scheltinga

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Van risicogenen naar psychiatrische fenotypes

Studies over aan fibroblast groei factor gerelateerde en genoom-wijde genetische variatie in mensen en muizen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. Dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 18 november 2013 des middags te 2.30 uur

door

Afke Frederike Terwisscha van Scheltinga
geboren op 31 juli 1980 te Delft

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Contents

Chapter 1

Introduction

Schizophrenia

This thesis summarizes research on the relationship between genetic variants and schizophrenia-related traits. Schizophrenia is a severe mental disorder, that not only causes huge personal distress to the affected persons and their family, but is also related to enormous costs for society due to hospitalization and loss of productivity [70]. Symptoms include flattened emotions, social withdrawal, lack of motivation and poverty of speech. These are called negative symptoms, because they are normal abilities that are lost in patients. In addition, patients can experience episodes of psychosis with hallucinations, delusions and disorganized speech, which are called positive symptoms. Onset is typically in early adulthood, with a prevalence of 0.5-0.9% [20]. Both genetic and environmental factors can increase schizophrenia risk. Environmental risk factors include cannabis use at young age [27], advanced paternal age [36], prenatal infections [126], obstetric complications at birth [42], childhood trauma [160] and migration [28]. Genetic factors will be discussed below.

All current antipsychotics are effective in reducing positive symptoms, but no treatments exist that can reduce negative symptoms substantially [166]. This is unfortunate as especially the severity of negative symptoms is related to disease outcome [145]. Thus, improvement of treatment options is urgently needed. The lack of progress so far is due to our limited understanding of the biological processes involved. Fundamental research is therefore crucial to unravel the pathology of schizophrenia and to identify novel drugable targets.

Genetics is currently perceived as one of the most promising research fields in schizophrenia. In the first place, schizophrenia has a large genetic component. Family members of schizophrenia patients are at increased risk of developing the disease themselves, ranging from 9% risk for siblings to 45% risk for identical twins [92]. The heritability (the proportion of variance in disease prevalence in the population that is attributable to genetic factors) is estimated to be around 80% [44]. Second, the techniques to study genetic variations have greatly improved in the last decade. Large-scale genetic studies are now feasible and affordable. These novel genetic tools can greatly help to unravel the pathology of schizophrenia.

Genetics studies of schizophrenia

Arrays have come available with can investigate 0.5-4 million single nucleotide polymorphisms (SNPs, DNA variations at a single A, C, G or T nucleotide) per person in parallel. This allows genome-wide comparison of polymorphisms between a patient group and a healthy control group for association with disease, so-called genome-wide association studies (GWAS). Data from GWAS have such a high density that larger

deletions or duplications in the genome can also be inferred. These copy number variants, or CNVs, range from approximately one thousand base pairs (1kb) to several million base pairs (Mb) in size. After the first three large GWAS on schizophrenia were published in 2009 [190, 207, 214] genetic research on schizophrenia has accelerated.

Common SNPs have minor alleles present in at least 1% of the population. These typically increase schizophrenia risk very little, with odds ratios (ORs) ranging from 1.1-1.5. Because of the small effect sizes, very large samples (>10,000, but rather >100,000 subjects) are required to identify the genetic variants of true positive effect between large numbers of unrelated SNPs. The largest published study to date is performed by the Psychiatric GWAS Consortium on 21,856 subjects and identified 10 genome-wide significant SNPs [200]. An even larger GWAS meta-analysis is on its way, which will very likey provide new convincingly associated variants.

At the other end of the genetic spectrum are the CNVs. CNVs show higher ORs, but have very low frequencies in the population. For instance, a deletion at the long arm of chromosome 22 (the 22q11 deletion syndrome) is the strongest known risk factor for schizophrenia. It is associated with 20-30x increased risk for psychosis and is present in around 1% of the schizophrenia patients [183]. In between the SNPs and CNVs are probably relatively rare variants with moderate effect sizes, which current studies are unable to detect. All together, it is hypothesized that schizophrenia is caused by the interaction of multiple (up to thousands) of common genetic risk variants, each with small effect and rare variants with larger effect sizes.

The additive effects of these recently identified, significantly associated variants explain just a small proportion of the schizophrenia liability (0.01%) [247]. When not only the significantly associated SNPs are included, but also 50,000 weakly associated SNP, the variance explained increased to 3% [190]. This shows that below the stringent threshold of statistical significance many more SNPs with small effects remain hidden. Interestingly, up to 23% of the liability for schizophrenia could be explained when including also non-additive effects (for example gene x gene interactions) [147]. The findings for schizophrenia are comparable to findings in other complex genetic disorders. So, although there is plenty more to be explained, these new techniques for the first time seem to provide a means to tag a substantial portion of the genetic liability.

Future studies will likely increase the variance explained by exploring several other factors contributing to disease liability. These factors may be rare variants with moderate effect sizes for which current GWAS have to little power to detect, gene x gene interactions and epigenetic effects (genetic factors that are not directly related to nucleotide order, but for example to methylation of the nucleotides) [156]. In addition, interaction between genetic factors and environmental risk factors could play an important role. Somebody's genetic

make-up could render him more vulnerable for environmental influences like the psychosis-inducing effects of cannabis [46].

The next step necessary to gain more insight into disease mechanisms is studying whether the variants with diverse functions discovered so far converge onto certain molecular pathways. Do these pathways disturb the development of specific brain cell types or regions? Understanding this is a key challenge for the coming years (or perhaps: decades). The studies in this thesis aim to make a start at answering these questions.

Intermediate phenotypes

In between a genetic variant and the associated disease there are abnormalities at several biological levels. Genetic variants can lead to abnormal protein levels or function. Schizophrenia seems associated with alterations in genes involved in receptor signaling, cell adhesion/myelination, growth factors, transcription factors and immunology [8, 167].

Changes in these proteins can affect the growth and function of cells. Such changes may, for example, result in increased density of white matter neurons, reduced number of oligodendrocytes and reduced dendrite density in schizophrenia patients [52, 180, 224]. The cellular alterations may cause disturbances on the neuronal circuits level, for example in neurotransmitter function. An elevated synthesis and release of dopamine in the striatum is strongly related to the positive symptoms of schizophrenia [110], while glutamatergic dysfunctioning is associated with negative symptoms [158].

Subtle cellular changes can add up to large-scale structural brain abnormalities. An average reduction in brain volume of around 3% is a robust finding in schizophrenia patients [98, 258]. Abnormalities in white matter integrity have been found using diffusion tensor imaging (DTI) [224]. Lastly, on the behavioral and cognitive level, several abnormalities are observed in schizophrenia patients. Patients show lower general intelligence quotient (IQ) scores in childhood [81, 125], with a further decline prior to illness onset [198]. The domains of problem solving, working memory and processing speed are particularly affected [198].

The abnormalities at these different biological levels are called intermediate phenotypes. Endophenotypes are intermediate phenotypes with characteristics that make them useful for genetic studies. Endophenotypes should be highly heritable, cosegregate with illness in families and relatively stable over time [93]. In addition, in order to facilitate genetic studies, they should preferably be objectively assessed at a relatively large scale, and have a normal distribution. Investigating the effects of schizophrenia risk variants on

endophenotypes can inform us about the nature of biological processes involved in schizophrenia. Furthermore, it is argued that because they are 'closer to the relevant actions of the genes' and less heterogeneous, genetic variants would have larger effects on these phenotypes than on the more heterogeneous clinical disease level.

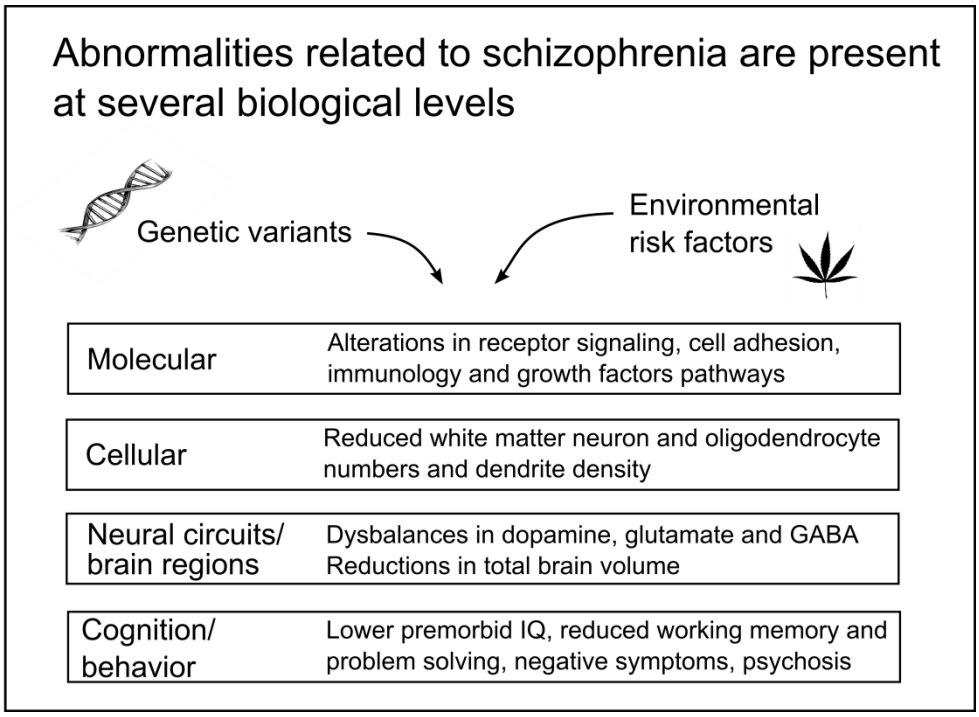

Figure 1.1 Overview of the risk factors and biological levels related to schizophrenia.

Approaches used in this thesis

Brain volume and IQ are examples of schizophrenia endophenotypes. They have a heritability of 90 and 70%, respectively, are strongly related to schizophrenia and are relatively easy and objectively assessed [81, 89, 103]. Therefore, these endophotypes are the main focus in the human genetic studies described in this thesis. Cellular or neurotransmitter abnormalities, other schizophrenia endophenotypes, require either post-mortem brain studies or invasive scanning techniques and are therefore less suitable for human genetic studies. These phenotypes can be assessed in animal studies (see below).

A difficulty in genetic studies is that very many genetic variants can be involved and that their effect sizes are small. Testing up to a million genetic variants in a GWAS dramatically reduces statistical power due to multiple testing. One option to circumvent this problem is to limit the number of tests beforehand, which can be done in several ways.

First, the number of variants tested can be limited to a number of variants in plausible 'candidate genes'. Candidate genes can be identified based on an interesting known function of the gene (functional candidate genes) or the association of genetic variants in or near this gene with the disease in previous studies (positional candidate genes). In this thesis, we have investigated fibroblast growth factors (FGFs). As discussed in **Chapter 2**, FGFs are excellent functional candidate genes for schizophrenia and mood disorders, with positional genetic studies supporting their role. For the study described in **Chapter 3**, we limited the number of SNPs tested to those in the FGF pathway.

Another approach is to combine the effects of multiple SNPs into a single polygenetic score of disease risk. This method was first applied by Purcell et al. to show that thousands of schizophrenia-related common variants combined significantly predict schizophrenia disease status, as well as bipolar disorder, in independent samples [190].

A third approach to reduce the number of tests is to investigate global CNV burden. Since CNVs can alter the function or expression of one or more full genes, their effect sizes might be larger than that of SNPs. While CNVs at specific locations are rare, almost everybody carries CNVs. So, global CNV burden might be a measure with sufficient prevalence and effect size to use in endophenotype studies.

Thus, by carefully choosing the phenotypes and genotypes to study, we maximized power in our studies. There are several other challenges as well. For example, patients differ not only in the amount of schizophrenia risk genes they carry, but also in their entire genetic background. Moreover, factors like the use of medication and illicit drugs or stress exposure can substantially influence brain volume and IQ scores, but are hard to measure reliably.

A way to circumvent these problems is to use animal models. In mice, genes can be genetically deleted or altered and the resulting effects on behavior and brain structure and function can be measured. The controlled environment and accessibility of brain tissue are advantages of animal studies. While it is impossible to create a 'schizophrenic mouse' with human-like symptoms, the effect of genetic variants on intermediate phenotypes like memory deficits or brain volume reductions can very well be studied. Therefore, we have performed a knock out mouse study on one of the candidate genes, FGF2, as described in **Chapter 4**.

Outline by chapter

The first three chapters focus on fibroblast growth factors as candidate genes. **Chapter 2** reviews the functions of FGFs in brain development and in coping with environmental factors. It indicates how FGFs are associated with psychopathology in humans and mice. FGFs affect brain growth and schizophrenia patients are known to have on average smaller brain volumes. Thus, in **Chapter 3** we investigated whether SNPs in the FGF system affect brain volume in schizophrenia patients and healthy controls. In **Chapter 4**, we report a knockout mice study in which the effect of deletion of FGF2 and its interaction with early life stress is investigated. We show abnormalities in behavior, as well as in brain structure.

In the next two chapters, the polygenic score method is used to investigate the combined effects of a genome-wide set of SNPs on schizophrenia endophenotypes. **Chapter 5** describes how this polygenic score relates to brain volume in schizophrenia patients and healthy controls. The association of the polygenic schizophrenia risk score with IQ is reported in **Chapter 6**.

Furthermore, we studied the association of global CNV burden with brain volume, as presented in **Chapter 7**. Lastly, the association of CNVs with paternal age was investigated in schizophrenia patients and healthy controls. This epidemiological risk factor may be especially relevant to genetic studies as an increased mutation rate is a possible explanation for the epidemiological association. The findings are summarized in **Chapter 8**.

Chapter 2

Fibroblast growth factors in neurodevelopment and psychopathology

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Afke F. Terwisscha van Scheltinga, Steven C. Bakker, René S. Kahn, Martien J.H. Kas.

Abstract

In psychiatric disorders, the effect of genetic and environmental factors may converge on molecular pathways and brain circuits related to growth factor functioning. In this review we describe how disturbances in fibroblast growth factors (FGFs) and their receptors influence behavior by affecting brain development. Recently, several studies reported associations of members of the FGF family with psychiatric disorders. FGFs are key candidates to modulate the impact of environmental factors, such as stress. Mutant mice for FGF receptor 1 show schizophrenia-like behaviors that are related to general loss of neurons and postnatal glia dysfunction. Mice lacking FGF2, a FGFR1 ligand, show similar reductions in brain volume and hyperactivity, as well as increased anxiety behaviors. FGFR2 and FGF17 are involved in development of frontal brain regions and impairments in cognitive and social behaviors, respectively. Moreover, treatment with FGF2 was beneficial for depressive and cognitive measures in several animal studies and one human study. These findings indicate the importance of the FGF system with respect to developing novel etiology-directed treatments for psychopathology.

Introduction

Psychiatric disorders, such as schizophrenia, mood disorders and autism, are heterogeneous disorders in which many genetic and environmental factors are involved. Searching for common pathways that are affected by the different etiological factors is a prominent strategy to investigate the pathology of these disorders.

Growth factor functioning may be one of those convergent molecular pathways. Glial growth factors were first hypothesized to be involved in schizophrenia [167], but have been related to autism and mood disorders as well [18, 241]. Glial growth factors are neurotrophic factors produced by glial cells. Well known examples of these are neuregulin 1 (NRG1) and brain derived neurotrophic factor (BDNF) [74, 205], but many more exist, including nerve-, insulin-like-, epidermal- and fibroblast growth factors (FGFs). FGFs are particularly interesting, because they are one of the few growth factors involved in early brain development as well as maintenance and repair throught adult life [219]. While a role for NRG1 and BDNF in psychiatric disorders has been much investigated, the interest for the role of FGFs in psychopathology has been growing relatively recently.

In this review we will explore the wealth of literature on the role of FGFs in psychopathology. There are 22 FGF ligands, which can be divided in several subfamilies based on homology in structure and function, as previously reviewed [112]. Endocrine FGFs (FGF19, FGF21 and FGF23) can act over long distances as endocrine hormones and modulate binding of other FGFs. Intracrine FGFs (FGF11-14) act as receptor-independent intracellular molecules that regulate the function of sodium channels. All other FGFs are paracrine and modulate development by influencing intracellular signaling of neighboring cells. They do so by binding to one of the 4 FGF membrane-bound receptors (FGFRs), which induces dimerization, receptor phosphorylation and activation of four key downstream signaling pathways: RAS/mitogen-actived protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3)/AKT, signal transducer and activator of transcription (STAT) and phospholipase gamma (PLCγ) [112].

The FGF family is crucial for development of the brain, cranium, organs, limbs and inner ear, and for metabolism. The phenotypes of knock-out mice and diversity of human diseases caused by FGF(R) mutations are described in several reviews and include skull malformations, achondroplasia and several cancers [16, 119, 143]. In addition, other reviews have specifically focused on the role of the FGF family in neurodevelopment [14, 113, 159, 199, 219]. Therefore, we will not attempt to give an extensive summary of these functions here. In short, FGFs control growth and patterning of the brain during development, while in adulthood they remain involved in neuro- and gliogenesis, axon outgrowth, myelinogenesis and tissue repair.

In this review, the role of selected FGFs and receptors in psychopathology is described. We focus on four of the FGF family members, namely FGF receptors 1 and 2, FGF2 and FGF17. These were chosen based on available evidence on their association with human psychopathology and on their functions in animal behavior and brain development. These FGFs share a role in neurogenesis, axon outgrowth, brain volume and the excitatory/inhibitatory balance. However, their target regions differ and so do the behaviors that they affect most, ranging from hyperactivity and increased anxiety to disturbed social and cognitive behavior.

Although all FGFRs drive essentially the same signaling pathways, they do act in a highly tissue- and time-specific manner. Specificity is derived by differential expression of binding proteins like heparan sulfate proteoglycans and signaling adapters [143]. Heparan sulfate proteoglycans act as co-receptors in the FGF-FGFR complex, form a storage reservoir for FGF ligands and determine the radius of ligand diffusion [16]. So, although FGFs may be widely released, the distribution of heparan sulfate proteoglycans may help to stimulate activation of targeted cells. Moreover, there may be differences in kinase activity depending on which molecule triggered the signal [240].

The same signaling pathways can be induced by other growth factors, including insulin-like growth factor (IGF), transforming growth factor β (TGFβ), bone morphogenic protein (BMP) and vertebrate homologs of Drosophila wingless (WNT) [223]. In addition, non-canonical ligands such as neuronal cell adhesion molecule (NCAM), L1, N-cadherin and Ephrin A4 can bind to FGFRs [99, 185]. Thus, the final action depends on the combined activity of these ligands and receptors.

Human genetic studies

There are numerous indications that FGFs play a role in schizophrenia and mood disorders [228, 235, 240]. At the DNA level, single nucleotide polymorphisms (SNPs) near *FGFR1* were strongly associated with schizophrenia in a genome-wide association study [208]. Secondly, a SNP located 85 kb from the nearest gene, *FGFR2*, was the only significant finding after several rounds of replication in a linkage fine mapping study on schizophrenia [175, 208]. A neighboring SNP was significantly associated with bipolar disorder [255]. In *FGF2*, a SNP associated with reduced *FGF2* expression was associated with reduced treatment response in depressed patients [120]. Furthermore, the disruption of neuronal PAS domain protein 3 (*NPAS3*) was reported to co-segregate with illness in a small family with schizophrenia [118]. *Npas3* knock-out mice show an 80% reduction in *Fgfr1* mRNA [184] and

express behavioral and physiological abnormalities similar to *Fgfr1* mutant mice, including reduced novel object recognition and prepulse inhibition [34].

At the mRNA expression level, decreased *FGFR2* in the hippocampus and cingulate cortex and slightly raised *FGFR1* in the hippocampus were found in post-mortem brains of schizophrenia patients [86, 121]. In depressed patients, *FGFR1* mRNA was raised and *FGF2* expression decreased independent of medication use [86]. In addition, the level of FGF2 protein in serum was increased in medicated schizophrenia patients and in non-medicated patients with high negative symptoms [104].

Thus, associations have been found across the psychosis-mood disorder spectrum. Genetic variants in FGFs could increase the vulnerability for psychiatric disorders in general, rather than causing a specific symptom.

Environmental risk factors

Two important risk factors for psychiatric disease, that are also related to FGFs, are obstetric complications and stress [42, 127]. The impact of environmental insults may be greater in FGF deficient conditions. For example, it was shown that *Fgf2* knock-out (*Fgf2-/-*) mice suffered much higher rates of cell death than wild type mice after 6-OHDA lesioning, a model for Parkinson's disease [232]. After ischemia, *Fgf2-/-* mice show an increased infarct volume and reduced neurogenesis compared to wild type mice [268]. Unfortunately, the impact of perinatal hypoxia or stress on adult behavior has not been investigated in *Fgf* deficient mice.

What is clear, however, is that acute restrain stress induces an upregulation of hippocampal FGF2 protein and mRNA in adult rats [168]. In contrast, chronic stress reduced *Fgf2* mRNA in the prefrontal cortex [168], while repeated social defeat reduced *Fgf2* and *Fgfr1* mRNA in the hippocampus in adult rats [236]. The increase in *Fgf2* in the prefrontal cortex and hippocampus after stress was particularly high after escapable stress compared to uncontrollable stress [22]. The authors therefore suggest that FGF2 might be involved in emotional regulation during stressful experiences. Prenatal stress affects *Fgf2* expression in both short and long-term. Prenatal exposure to corticosterone produces a significant reduction in *Fgf2* mRNA levels in the hippocampus of male rats [168]. In adult rats who had been exposed to prenatal stress, the baseline *Fgf2* levels were decreased in the prefrontal cortex and increased in the entorhinal cortex and striatum, while the *Fgf2* response to a new stressful event was significantly altered as well [85].

Similarly, perinatal hypoxia showed long-term effects on *Fgf2* expression. Perinatal anoxia leads to lifelong reductions in baseline *Fgf2* mRNA in the ventral tegmental area and an enhancement of the *Fgf2* response to stress [79, 201]. Perinatal hypoxia causes an apparent loss of cortical neurons. In wild type mice, the numbers of excitatory neurons were recovered after 1 month, while the number of inhibitory interneurons remained decreased [73]. In mice lacking *Fgfr1*, however, the deficits in excitatory neurons were persistent and the deficits in interneurons worse compared to wild type mice. So, the FGF system seems to be required for recovery from hypoxia, while hypoxia itself may result in disturbances in FGFs.

The long-term decreases in baseline *Fgf2* levels are thought to increase vulnerability to psychopathology. Baseline hippocampal *Fgf2* mRNA expression correlated with anxiety [69] and *Fgf2* administration reduces anxiety behaviors and depression-like behaviors in rodents, see also the Treatment section [182, 238, 239]. In humans, a *FGF2* SNP is associated with reduced treatment response in depressed patients [120].

The effects of increased *Fgf2* shortly after perinatal stress are less evident. Given the neuroprotective properties of FGF2, it was first hypothesized that this increase in *Fgf2* is a mechanism to compensate for possible subsequent tissue damage [168]. The requirement of *Fgfr1* for recovery of hypoxia has been shown [73]. However, it was recently reported that a single subcutaneous injection of FGF2 on postnatal day 2 resulted in long-term adverse effects [237]. Cocaine sensitization was increased, dopamine D2 receptor expression in the nucleus accumbens increased, and *Fgf2* expression in the ventral tegmental area decreased [50]. These effects may be a result of a disturbed balance between proliferation and differentiation, since FGF2 administration was shown to increase proliferation and delay differentiation of dopamine precursor cells [95, 232].

While adverse events lead to long-term decreases in *Fgf2*, stimulating environments were shown to increase *Fgf2* levels. For example, *Fgf2* mRNA was upregulated in an enriched environment, in the offspring of mothers who showed higher levels of pup licking and grooming (i.e. increased maternal care) and after physical activity [61, 85, 182, 236].

FGFR1

Next, we will subsequently review and discuss the effects of changes in FGFR1, FGFR2, FGF2 and FGF17 on behavior and neurobiology. A summary of the findings can be found in Table 2.1 (behavior) and Table 2.2 (neurobiology).

FGFR1 in behavior

Since full *Fgfr1* knockout mice have severe skull malformations and die embryonically, mutant constructs have been made with loss of functional *Fgfr1* restricted to specific cells or brain regions. For example, *Fgfr1* expression was abolished in radial glial cells by a Cre recombinase-induced deletion of $Fgfr1^{flox}$ alleles (the mice are here abbreviated as rgFGFR1 mice) [176]. Radial glial cells are the primary progenitors of neurons in the dorsal telencephalon and this procedure results in near complete absence of *Fgfr1* in the embryonal telencephalon after embryonic day 13.5, while maintaining expression in other regions. Others have inserted of a dominant negative *Fgfr1* gene under a tyrosine hydroxylase (*TH*) promotor in mice (thFGFR1 mice) [134]. Since TH is the rate-limiting enzyme in dopamine synthesis, this results in absence of *Fgfr1* expression in dopaminergic neurons, but normal expression in other regions, including the cortex and striatum. Furthermore, a line of conditional knock-out mice was generated with loss of *Fgfr1* restricted to the whole brain (bFGFR1 mice) [269]. Lastly, in the dnFGFR1 mice, the expression of *Fgfrs* in the telencephalon was reduced by a dominant negative *Fgfr1* under an Otx1 promoter [209]. This reduces the expression of not only *Fgfr1*, but all *Fgfrs,* during early development.

For these *Fgfr1* models, an increase in locomotor activity is the most replicated finding, with reports in the rdFGFR1, thFGFR1 and dnFGFR1 mice. Besides hyperactivity, dnFGFR1 mice showed occasional head bobbing and turned compulsively in one direction [209]. These abnormalities were absent at birth and developed at around 6 weeks of age (equivalent to adolescence). Since disturbances in most neurotransmitter systems and several brain regions can give rise to hyperactive behavior, it is yet unclear what causes this behavior in the *Fgfr1* mutant mice. Perhaps, the alterations in dopaminergic, serotonergic and histaminergic functioning described hereafter are part of the pathology leading to the observed hyperactivity.

First, alterations were reported in the dopaminergic system. The thFGFR1 mutant mice displayed deficits in prepulse inhibition (PPI) [134]. In PPI, the startle response evoked by a stimulus (usually a sound) is attenuated by a weak stimulus prior to the stimulus (a prepulse). Deficits in PPI are also found in schizophrenia patients, correlate with symptom severity and can arise from a hyperdopaminergic state in the striatum [134]. In these mice, the deficits could be normalized after treatment with flupentixol, an antipsychotic with dopamine D2 antagonistic actions. In addition, a functional hyperactivity of the dopamine system was shown by an increased sensitivity to amphetamine (a dopamine agonist) in dnFGFR1 mice [209]. Reductions in *Fgfrs* other *Fgfr1* might have contributed to this effect. In contrast, rdFGFR1 mice showed unaltered responses to cocaine, amphetamine, dopamine D1 or D2 agonists [171].

Second, a different study on thFGFR1 mice showed that besides deficits in PPI, these mice engage less in social interaction [133]. This could be corrected by treatment with the specific 5-HT2A receptor antagonist M100907 and with quetiapine (an atypical antipsychotic with 5HT affinity) [133]. This effect is specific, as locomotor activity and anxiety measures were unchanged. Similar to the hyperactivity, the behavioral and neurochemical abnormalities were not present at birth, but developed after puberty [133]. This suggests that, while *Fgfr1* deficiency is restricted to catecholaminergic neurons, the disturbed development affects other neurotransmitter systems as well, including the serotonergic system.

Third, behavioral deficits specifically related to histamine were reported in *fgfr1* mutant zebrafish. Zebrafish have two separate *fgfr1* genes and absence of one of these is not lethal due to compensatory mechanisms. *fgfr1a-/-* zebrafish show an increased boldness, as measured by the time needed to reach the most distant point in a novel open field [173]. In addition, these fish showed increased mirror-induced aggression. Both behaviors were specifically related to reduced histamine throughout the brain and not to changes in other neurotransmitters. The concentration of histamine-N-methyltransferase (HMNT), a histamine degrading enzyme, was increased and the phenotype could be rescued by administration of a HNMT inhibitor or the histamine receptor 3 agonist imetit [173]. Fluoxetine (a serotonin reuptake inhibitor- SSRI) or galantamine (acetylcholine antagonist) reduced agression in wild type and *fgfr1a-/-* zebrafish to the same extend.

These observations can be related to the previous findings in several ways. Histamine H3 receptors were found to form heterodimers with dopamine D2 receptors and imetit can inhibit the locomotor activation induced by D1 or D2 receptor agonists [75]. Perhaps imetit was effective in the *fgfr1a-/-* zebrafish because its striatal dopamine levels were relatively increased. Moreover, since the tuberomammillary nucleus, the only region of the brain with histaminergic neurons, receives glutaminergic input from the prefrontal cortex, the changes in histamine could be secondary to disturbed prefrontal cortex development (see FGFR1 in neurobiology) [32].

Other neurotransmitter systems and processes may be affected as well. For example, dnFGFR1 mice showed a stronger and shorter response to guanfacine, an α2 adrenal receptor agonist [209]. The bFGFR1 mice displayed a memory deficit specific to the retention of the learned spatial information in the Morris water maze task [269], which is related to adult neurogenesis (see FGFR1 in neurobiology).

In summary, *Fgfr1* deficiency has been associated with locomotor hyperactivity, deficits in prepulse inhibition and disturbed cognitive and social behavior. Depressive and compulsive behaviors have not been tested in these mice, so a role in other psychopathology is not excluded. In the next section we will describe how these

behavioral abnormalities relate to neurobiological findings in *Fgfr1* mutant mice, for example to reductions in interneurons.

FGFR1 in neurobiology

Similar to schizophrenia patients, dnFGFR1 mice show reductions in total brain size, with most prominent reductions in frontal and temporal regions [98, 209].

Excitatory glutamatergic neurons and inhibitory interneurons seem to be affected differently, which could lead to an imbalance between excitation and inhibition. Such a disturbed excitation/inhibition balance has been suggested to play a role in schizophrenia and autism pathology [158]. In rdFGFR1 mice fewer parvalbumine positive (PV+) interneurons were found in the cerebral cortex of young adults, while glutamatergic neuron numbers were unaltered [171]. The reduction in interneurons correlated with the amount of locomotor hyperactivity, suggesting that the hyperactivity is mediated by reduced inhibitory activity in the cortex. Similarly, the numbers of PV+ and somatostatin+ interneurons were reduced in adult rgFGFR1 mice [176]. This may be the result of postnatal loss, as these numbers were normal at birth. Astrocytes isolated from these mutants were impaired in supporting interneuron development *in vitro*, suggesting that the loss is caused by glia dysfunction [212]. In contrast to previous findings, the dnFGFR1 mouse showed fewer pyramidal neurons and normal numbers of GABAergic and PV+ neurons [209]. The expression of all *Fgfrs* in these mice was reduced during development only, indicating that the survival-promoting role of *Fgfrs* for PV+ neurons increases postnatally.

Furthermore, loss of *Fgfr1* specific to dopaminergic neurons of the midbrain was shown to have secondary effects on the serotonergic system. After blocking of *Fgfr1* signaling in the ventral tegmental area and substantia nigra (by using thFGFR1 mice), cell size and density were decreased in these regions specifically [134]. Despite the loss of dopaminergic neurons, extracellular dopamine levels in the striatum were increased. In addition, serotonergic innervation to these dopaminergic regions was increased [133]. During brain development there is competition between serotonin and dopamine for brain target sites. A complex interaction of dopamine and serotonin in the prefrontal cortex during development may explain the effects of serotonergic drugs on social behavior.

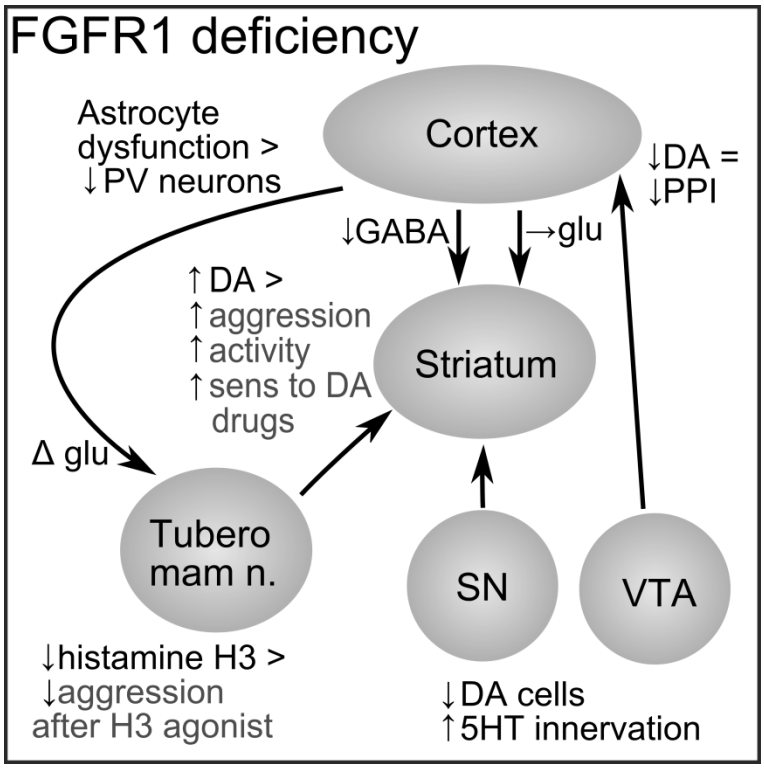

Figure 2.1: Consequences of FGFR1 deficiency. Evidence from several studies is combined to generate hypotheses about brain circuits that are affected by FGFR1. DA=dopamine, 5-HT= serotonin, glu= glutamate, SN=substantia nigra, VTA=ventral tegmental area, Tubero mam n. = tuberomammilary nucleus, PV=parvalbumin, PPI = prepulse inhibition, > = possible causal relationship.

Moreover, the bFGFR1 mice with memory impairments, also displayed deficits in adult neurogenesis and long-term potentiation (LTP) in the hippocampus [269]. In addition, *Fgfr1* deficiency was shown to decrease dendritic outgrowth *in vitro* [134] and *in vivo* [209].

An integrated overview of the findings on FGFR1 can be found in Figure 2.1. In *Fgfr1* mutant mice, locomotor hyperactivity, deficits in prepulse inhibition and disturbed cognitive and social behavior are associated with abnormalities in dopamine, serotonin and histamine neurotransmitter systems. Together this resembles a schizophrenia-like phenotype. FGFR1 has widespread actions and deficiency of this growth factor receptor results in global reductions in neuronal density. Deficiency in *Fgfr1* may lead to a cascade of abnormalities, starting with postnatal increasingly disturbed glia functioning. This

reduces the number of GABAergic PV+ neurons, which is, in turn, associated with locomotor hyperactivity. PV+ interneurons mediate the activity of the glutamatergic cortical projects to dopaminergic areas [45], while reduced development of dopaminergic neurons results in deficits in prepulse inhibition. Due to competition between projecting neurons for target brain sites, this secondarily affects other neurotransmitter systems. Dysfunction in the serotonin system was associated with reduced social interaction in these mice. In addition, memory is disturbed, possibly related to reduced adult neurogenesis.

FGFR2

FGFR2 in behavior

Mice with abolished *Fgfr2* expression in radial glia cells displayed impairments in memory at adulthood [217]. These mice showed significantly slower learning in an 8 day Morris water maze test and performed worse on the probe trial on the 9th day of this test. In an object recognition test on 3 consecutive days, *Fgfr2* mutant mice also performed worse.

When *Fgfr2* expression was specifically abolished in oligodendrocytes, 38% of the mice showed a pronounced hyperactivity after mild environmental stimulation, such as moving the cage or exposure to a novel open field [117]. This behavior developed after 2 weeks of age and could be normalized by dopamine D1 or D2 antagonists, suggesting a postnatal defect in dopaminergic signaling, similar to some findings in *Fgfr1* mutant mice. Other behavioral test were not reported for these mice.

FGFR2 in neurobiology

When *Fgfr2* signaling was abolished in radial glial cells, cortical volume was generally reduced [218]. The reduction was strongest in the medial prefrontal cortex and was due to fewer cortical excitatory pyramidal neurons. This correlated with reduced subcortical inhibitory (PV+) neuron numbers in the cortical projection regions (septum and bed nuclei of the stria terminalis).

In addition, in the hippocampus, volume and number of PV+ neurons were reduced and adult neurogenesis was decreased [217]. Interestingly, these abnormalities were related to specific memory deficits. The number of PV+ neurons correlated with long-term spatial reference memory, while reduced differentiating (doublecortin+) neurons correlated with object recognition. Differentiation and object recognition were reduced even when *Fgfr2* deficiency started in adulthood, while the deficits in interneurons and spatial memory were only seen after *Fgfr2* reductions in early development. This suggests FGFR2 is involved in two distinct processes related to hippocampal function and memory.

Fgfr2 is abundantly expressed in oligodendrocytes and stimulates outgrowth and myelination *in vitro* [117]. Surprisingly, abolishing *Fgfr2* expression in oligodendrocytes did not lead to any observable defects in these cells or myelination *in vivo*. While their hyperactive behaviors were responsive to dopamine receptor antagonists (described above), there were no indications of reduced dopamine and glutamatergic neuron numbers. This suggests a functional rather than structural deficit in the dopamine system. The authors propose that the hyperactive phenotype is caused by abnormal axon-oligodendrocyte interactions in dopaminergic neurons. The findings are summarized in Figure 2.2.

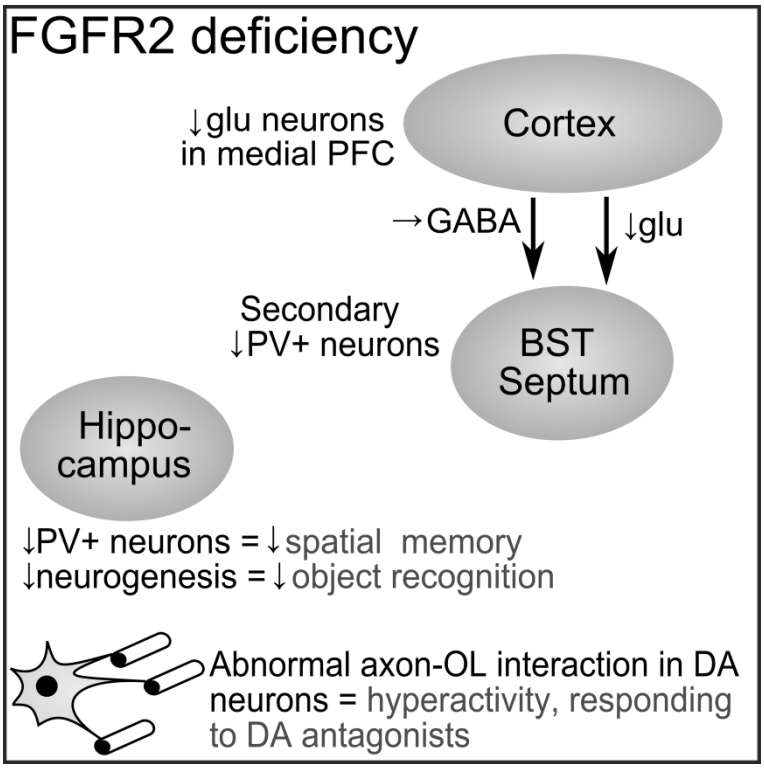

Figure 2.2: Consequences of FGFR2 deficiency. Evidence from several studies is combined to formulate hypotheses about brain circuits that are affected by FGFR2. DA=dopamine, glu= glutamate, PV=parvalbumin, PFC = prefrontal cortex, BST = bed nucleus of stria terminalis, OL = oligodendrocyte.

FGF2

FGF2 in behavior

In mice, *Fgf2* expression was fully abolished by replacing the first exon of the *Fgf2* gene with an *Hprt* minigene [270]. These *Fgf2-/-* mice showed locomotor hyperactivity in the open field, as well as an increased locomotor response to dopaminergic drugs (cocaine, amphetamine and apomorphine) [72]. Sleeping time following administration of GABA receptor agonist sodium pentobarbital was increased [137]. Together, this indicates disturbances in the dopamine and GABA neurotransmitter systems.

In rats, knockdown of *Fgf2* expression resulted in increased anxiety behavior in an elevated plus maze [69]. There was a strong, positive correlation between the *Fgf2* concentration in the hippocampus and the time spent in open arms (i.e. less anxiety). Rats bred for high anxiety behavior showed low expression of *Fgf2* mRNA in the hippocampus [182, 240]. Interestingly, environmental enrichment increased *Fgf2* levels and reduced anxiety in these rats. In contrast, *Fgf2-/-* mice displayed normal anxiety behavior in the open field [72].

Administration of FGF2 can reduce depressive-like behaviors (see Treatment). The effect of loss of FGF2 on depressive behaviors has not been investigated. So, together these studies suggest that FGF2 is related to locomotor activity, anxiety levels and depressive behaviors.

FGF2 in neurobiology

Fgf2-/- mice showed a 10% reduction in cerebral cortex size and decreased neuron density [192]. In the cortex, *Fgf2* seems to affect excitatory pyramidal neurons more than inhibitory neurons, in contrast to *Fgfr1*. Several studies found reductions in pyramidal neurons, most prominent in the deep layers of the frontal cortex [137, 178, 192]. A specific class of neurons arising from the dorsal pseudostratified ventricular epithelium appears to be affected [192]. GABAergic interneurons were relatively spared [137, 192], although one study found reductions in PV+ neurons as well [178]. In addition, increased numbers of ectopic PV+ neurons were reported in *Fgf2* deficient mice [62], indicating a possible migration defect.

Neuron numbers in the hippocampus, striatum and cerebellum were normal [178]. Several studies suggest that hippocampal neurons are sensitive to the effects of FGF2, however. FGF2 administration increased adult neurogenesis in the hippocampus and dentate gyrus volume in wild type rodents in standard conditions and following ischemia, while after *Fgf2* deficiency the opposite was observed: increased infarct volume and reduced neurogenesis after ischemia [268]. Together this suggests that FGF2 is pharmacologically effective, but not physiologically required for neurogenesis in the hippocampus under

normal conditions. Perhaps the lack of FGF2 on its own can be compensated for, but after stress this compensation may be insufficient [95].

It is also possible that FGF2 affects neuron morphology and function, rather than neuron number in the hippocampus under normal conditions. *Fgf2* may be required for correct synapse formation, as spine length was reduced in *Fgf2-/-* mice and LTP disrupted [267]. Conversely, FGF2 administration increases the formation of excitatory synapses *in vitro*, by activating MAPK signaling [149]. In addition, ectopic administration of FGF2 resulted in aberrant targeting of axons in Xenopus [263].

FGF2 also has specific effects on dopaminergic neurons. It increases proliferation and delays differentiation of dopamine precursor cells, protects dopaminergic neurons from neurotoxin-induced cell death and increases survival of transplanted dopaminergic neurons [95, 232]. Remarkably, in *Fgf2-/-* mice the number of dopaminergic neurons in the substantia nigra was 35% *increased* from late embryonic development onwards [196]. Although no compensatory upregulation of other *Fgf* ligands was observed, activation of downstream signaling pathways was normal. Perhaps alternative ligands or co-receptors, such as NCAM or heparan sulfate proteoglycans (see Introduction), are (over)compensating for the lack of FGF2 [195]. An increase in the nuclear isoform of FGFR1 may also explain the finding, as nuclear FGFR1 is known to increase TH expression (the rate-limiting enzyme in dopamine synthesis) [196]. Moreover, it was hypothesized that abnormalities in the dopaminergic system ultimately originate from a dysfunction in glutamatergic inputs from the prefrontal cortex to GABAergic cells in the striatum [72]. Accordingly, fewer cortical glutamatergic fibers were found to innervate the striatum [72].

In addition to the neuronal effects, FGF2 can activate oligodendrocytes, astrocytes and microglia [199]. FGF2 increases proliferation and blocks terminal differentiation of precursor oligodendrocytes, while in mature oligodendrocytes it stimulates outgrowth and inhibits the expression of myelin-related genes [14]. The early effects of FGF2 on proliferation are FGFR3 mediated, while the later effects on outgrowth are FGFR2 mediated, as the oligodendrocytes change in FGFR expression during maturation [80].

The findings on FGF2 are summarized in Figure 2.3. With FGFR1 as its main receptor, some of the abnormalities in *Fgf2-/-* mice overlap with *Fgfr1* mutant mice. Both affect global brain structures, gliogenesis and genetic deletion of either of these genes leads to locomotor hyperactivity. In contrast, the effect on dopaminergic neurons was opposite: a decrease for *Fgfr1*, but an increase for *Fgf2*. Because different behavioral tests were used for the *Fgfr1* and *Fgf2* mice (cognitive and social behaviors for *Fgfr1* and anxiety behaviors for *Fgf2*), overlap in behaviors other than locomotion was not shown.

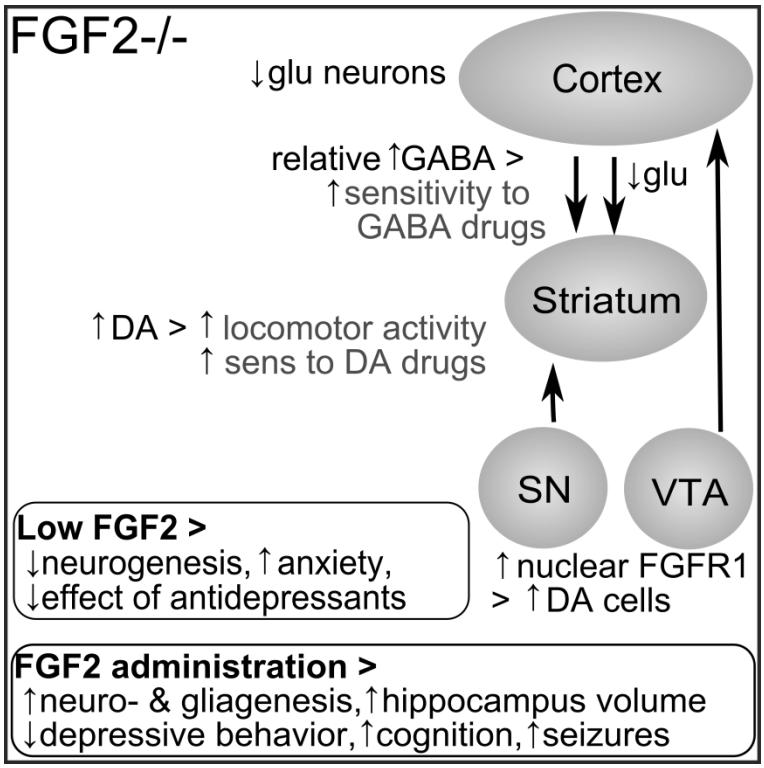

Figure 2.3: Schematic model of FGF2 functioning. Evidence from several studies is combined to generate hypotheses about brain circuits that are affected by FGF2. DA=dopamine, glu= glutamate, SN=substantia nigra, VTA=ventral tegmental area, DG = dentate gyrus, Alz. = Alzheimer's disease, > = possible causal relationship.

FGF17

FGF17 in behavior

Fgf17-/- mice display a specific deficit in social behavior: less interest in a novel female after habituation to the first female. The authors suggest that these mice have an inability to compare and respond to novel social information [203]. In addition, pups showed reduced ultrasonic vocalisations when separated from the dam at postnatal day 8. Male to female aggression, novelty suppressed feeding, anxiety behavior, locomotion and PPI were normal.

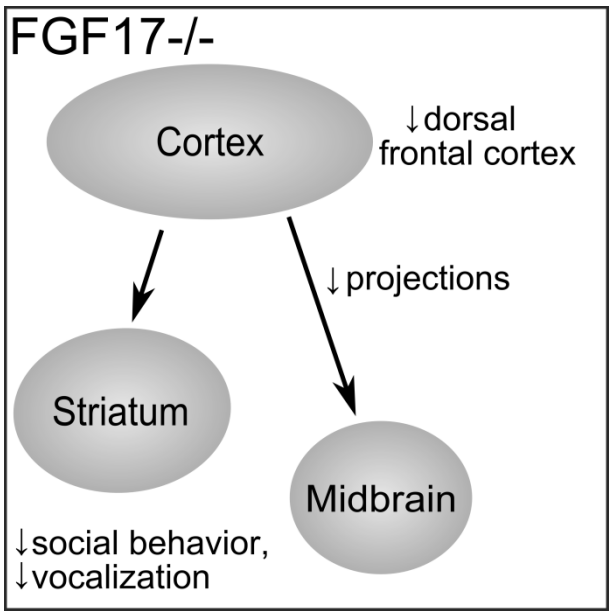

Figure 2.4: Schematic model of FGF17 functioning. Evidence from several studies is combined to formulate hypotheses about brain circuits that are affected by FGF17.

FGF17 in neurobiology

In *Fgf17-/-* mice volume reductions were specific to the dorsal frontal cortex and cerebellum [48]. In addition, the projections from the cortex to the striatum and ventral midbrain were reduced, which may have contributed to the observed social deficits in these mice. The dorsal frontal cortex is involved in working memory, attention and social valuation and is important in schizophrenia and autism [55, 249]. A schematic representation of the findings is displayed in Figure 2.4.

Treatment

The neuroprotective properties of FGF2 instigated investigations on possible therapeutic effects. While increases in FGF2 in early life may disturb developmental processes, as described in Environmental factors, administration of FGF2 at adult age has been proven beneficial. Perhaps FGF2 functions in more specific and subtle processes at this age. The examples of beneficial effects of FGF2 administration in animal models include reduced anxiety behavior in rats that are prone to anxiety [182], reduced depression-like behavior in the forced swim test [239] and a reduction in depression-like behavior induced by chronic unpredictable stress in the sucrose consumption test [65]. In a mouse model of Alzheimer's

disease, increasing gene expression of FGF2 through viral infection significantly restored spatial learning, even after onset of the symptoms [132]. FGF2 facilitated recovery of perinatal cortical injury, whether it was given before or after injury [51].

Interestingly, the positive effects of FGF2 on behavior coincide with effects on neuro- and gliogenesis. The reduction in anxiety was accompanied by an increase in survival of adult born neurons and glia in the hippocampus [182]. In the Alzheimer's model too, neurogenesis was increased after inducing *Fgf2* levels [132]. FGF2 infusion into the prefrontal cortex, but not into the striatum, restored the reduced gliogenesis induced by chronic unpredictable stress [65]. This produced antidepressant actions in the novelty suppressed feeding and forced swim tests [65]. Furthermore, this study showed that the antidepressants fluoxetine and imipramine partially require FGF2/FGFR-signaling for their behavioral as well as gliogenic actions. An independent study showed that FGF2 was able to reverse the depressive behaviors induced by olfactory bulbectomy, as well as ameliorate its effects on hippocampal adult neurogenesis [115]. Moreover, endogenous FGF2 was necessary for the behavioral effects of amitryptiline.

Studies like these suggest that some of the conventional psychiatric treatments may exert their effect by influencing FGF functioning. Antidepressant and antipsychotic medication can increase neurogenesis and several of these drugs were reported to increase FGF expression [63, 228]. Although they increase neurotransmitter levels within minutes to hours, their therapeutic effects may take weeks to develop, with a lag time similar to that necessary for neuronal maturation [63].

In humans, treatments targeting the FGF family are being explored for a wide range of disorders, as reviewed by Beenken et al. [16]. FGFR inhibition is trialed for treatment of malignancy, and paracrine FGFs have a potential in angiogenesis, cytoprotection and tissue repair. Regarding psychiatric disease, there is one study in children with mental retardation caused by perinatal hypoxia [1]. A large developmental and cognitive improvement (+10 IQ points) was reported after treatment with intramuscular injections of FGF2, compared to placebo treated children. FGF2 was generally well tolerated, did not cause toxic or allergic reactions and is easily administrated as it crosses the blood-brain barrier. Unfortunately, administration of FGF2 led to higher mortality rates in two clinical trials with acute stroke patients, due to a dose-dependent hypotension [259]. The dosage used was substantially higher than in the treated children (125 µg/kg versus 0.4 µg/kg).

Thus, although treatment with FGF2 or other drugs influencing the FGF system is promising for psychiatric disorders, more research is needed to investigate its efficacy in specific disorders, as well as the dosages and age groups in which it can safely be applied. Peripheral side effects might be reduced by applying FGF2 intranasally. This was proven a successful way to deliver FGF2 to the brain without affecting blood pressure [152]. Another

option is to design agents that specifically bind to a dimer of two receptors, in order to increase specificity of the drug action. FGFR1 forms a heterodimer with adenosine A_{2A} receptors and together these receptors oppose the actions of dopamine D_2 receptors, located on the same cells [78]. In addition, FGFR1 has recently been shown to form heterodimers with the 5-HT1A (serotonin) receptor in the hippocampus and raphe nucleus [26]. Activation of this dimer may be important for the antidepressant and neurotrophic effects of serotonin. Developing agents that specifically target the FGFR1 heterodimers could be a completely novel strategy to influence the dopamine system in schizophrenia patients.

Summary of the findings

To unravel the pathophysiology of psychiatric disorders, studies are focusing on molecular pathways on which genetic and environmental risk factors converge. Growth factor functioning could be such a convergent pathway. Genetic variants in *FGFR1, FGFR2* and *FGF2* are associated with schizophrenia and depressive disorder. Moreover, early life disturbances, such as hypoxia or social stress, may lead to lifelong reductions in *Fgf2* expression. Several FGFs and FGFRs are involved in regulation of neuronal growth and patterning during development and in neuro- and gliogenesis, axon outgrowth, myelinogenesis and tissue repair into adulthood. Disturbances of the FGF system can therefore have a major impact, especially during critical phases of brain development.

All described mutant *Fgf* mice show a reduction in brain volume. This is likely due to decreased proliferation and earlier differentiation, rather than increased apoptosis or cell death [199]. Other common features include effects on axon growth, synapse formation and long-term potentiation.

In *Fgfr1* and *Fgf2* mutant mice, neurons of the prefrontal cortex are particularly affected, which results in less innervation of the striatum. Together with 'unsupportive astrocytes' this may impair the number and functionality of GABAergic interneurons and dopaminergic neurons. In concert, these subtle but widespread abnormalities may explain the schizophrenia-like symptoms, including locomotor hyperactivity, impaired prepulse inhibition, reduced working memory, altered responsiveness to drugs, and depressive-like behaviors. FGF2 is involved in gliogenesis, which may be part of its antidepressive properties.

More localized brain regions are affected by FGFR2 (hippocampus and medial prefrontal cortex) and FGF17 (dorsal frontal cortex). However, absence of these proteins leads to disturbances not restricted to these regions and may also initiate a cascade of events. For

example, reductions in cortical neuron numbers resulted in dysfunctional subcortical projections in *Fgfr2* and *Fgf17* mutant mice models. This affected the development of other brain regions, such as the bed nucleus of stria terminalis and septum in *Fgfr2* mutant mice. These models show how the development of the different brain regions is highly interrelated. What starts as subtle disturbances in a specific cell type or region results secondarily in widespread abnormalities. The *Fgfr2* and *Fgf17* mutant mouse models display aberrant cognitive and complex social behaviors, respectively.

Future research and concluding remarks

Interestingly, the observed deficits after disruption of FGF2 / FGFR1 signaling resemble those seen in schizophrenia patients. Phenotypic overlap at the behavioral as well as biological level give this model reasonable face validity. For example, disturbances in working memory, prepulse inhibition, locomotor activity and responsiveness to drugs were reported for schizophrenia patients as well as *Fgfr1* mutant mice [153, 163]. On a biological level, overlapping features include reduced brain volumes, especially in frontal and temporal regions [98] and predominant alterations in PV+ interneurons [158]. In *Fgfr1* mutant mice, the interneuron deficit was related to astrocyte dysfunction. Abnormalities in glia cells and white matter play an important role in schizophrenia [19]. However, relatively little is known about the role of astrocytes in schizophrenia and this would be interesting to follow up.

In addition, several other questions may be addressed in future research. Abnormal glia cell functions seem to be central in the abnormalities in *Fgfr1*, *Fgfr2* and *Fgf2* deficient mice. It is unknown which glial cell types are affected most, how these cells respond to loss or gain of FGF(R)s, and how this in turn influences glia-neuron interactions. These findings can be compared to the abnormalities in these cell types in schizophrenia patients. Second, depressive behaviors have not been investigated in *Fgfr1* deficient mice, social behaviors not in *Fgfr2* deficient mice and cognitive and social behaviors not in *Fgf2-/-* mice. It is likely that these genes are involved in those behaviors, based on similarities between the genes and the brain circuits that they are involved in. Third, based on its effects on social behavior, it would be very interesting to investigate the association of genetic variations in *FGF17* with schizophrenia or autism in humans.

The effects of FGFs and their receptors can be compared with those of other neurotrophic factors or candidate genes for psychiatric disorders. The behavioral and neuro-anatomical dysfunctions observed in *Fgfr1* and *Fgf2* deficient mice resemble those that were observed in models using disrupted in schizophrenia 1 (*Disc1*), *Nrg1* and *Bdnf* [30, 74, 205]. These genes have been associated with schizophrenia and mood disorders and have

functions in neurogenesis, migration and synapse formation during development and in adulthood. Perhaps the functions of these growth factors converge to influence similar brain circuitry.

To conclude, several members of the FGF gene family are associated with psychopathology in human and animal studies. They are not only important in brain development, but also modulate the impact of environmental stressors on the brain. Studies in animal models with genetic variations in FGFs or FGFRs will provide an important basis for understanding the neurobiological mechanisms underlying aberrant behavior. Moreover, administration of FGF2 or other agents targeting the FGF system is a promising new treatment option for neuropsychiatric disorders.

Table 2.1

	Genetic association with disease	Environment
FGFR1	Schizophrenia (Shi, 2011; Gaughran, 2006; Katsel, 2005)	NA
FGFR2	Schizophrenia, bipolar disorder (O'Donovan, 2008; Wang, 2012; Gaughran, 2006; Katsel, 2005)	NA
FGF2	Schizophrenia, SSRI response (Gaughran, 2006; Kato, 2009)	Lifelong changes in *Fgf2* expression after perinatal hypoxia or stress. Injury, stress and seizures increase *Fgf2* (Ganat, 2002; Riva, 2005; Fumagalli, 2005; Frank, 2007)
FGF17	Not specifically assessed	Normal (Scearce-Levie, 2007)

Table 2.1: Summary of findings on disease associations and behavior. For precise description and references, see the text. Depressive behaviors were tested with the sucrose consumption test and forced swim test. OL = oligodendrocytes, NA= not assessed. *Fgfr1* mutant mice are indicated by abbreviations with *Fgfr1* deficiency induced in: radial glial cells (rgFGFR1), tyrosine hydroxylase (TH) expressing (dopaminergic) neurons (thFGFR1 mice), whole brain (bFGFR1) or in the telencephalon during development (dnFGFR1). **Table continues on next pages.**

Table 2.1 (continued)

	Sensitivity to drugs	Other
FGFR1	Increased (dnFGFR1) and normal (rgFGFR1) response to amphetamine. Phenotype rescued by histamine H3 receptor agonist (*Fgfr1a-/-* fish) (Shin, 2004; Muller Smith, 2008; Norton, 2011)	Increased extracellular dopamine; serotonergic hyperinnervation in midbrain. Levels of GABA, serotonin and dopamine transporter were normal (Klejbor, 2006; Klejbor, 2009; Shin, 2004; Muller Smith, 2008)
FGFR2	NA	NA
FGF2	Increased response to dopaminergic and GABAergic drugs (Fadda, 2007; Korada, 2002)	Increased dopaminergic neurons. Dopamine increases FGF2 release. FGF2 affects dopamine sensitivity (Ratzka, 2012; Reuss, 2003; Grothe, 2007)
FGF17	NA	NA

Table 2.1 (continued)

	Locomotor activity	Cognition	PPI
FGFR1	Increased activity (rgFGFR1, thFGFR1, dnFGFR1), head bobbing after 6 weeks of age (dnFGFR1) and exploration of novel open field (*Fgfr1a-/-* fish) (Muller Smith, 2008; Klejbor, 2006; Shin, 2004; Norton, 2011)	Reduced memory retention (bFGFR1) (Zhao, 2007)	Reduced PPI, normalized after antipsychotic (thFGFR1) (Klejbor, 2006)
FGFR2	Increased activity in novelty (*Fgfr2* disrupted in OL) (Kaga, 2006)	Reduced spatial and object memory (Stevens, 2012)	NA
FGF2	Increased activity (Fadda, 2007)	NA	NA
FGF17	NA	Normal (Scearce-Levie, 2007)	Normal (Scearce-Levie, 2007)

Table 2.1 (continued)

	Social behaviors	Anxiety	Depressive behaviors
FGFR1	Less social interaction (thFGFR1), increased aggression (FGFR1a-/- fish) (Klejbor, 2009; Norton, 2011)	Normal (thFGFR1) (Klejbor, 2009)	NA
FGFR2	NA	NA	NA
FGF2	NA	Increased anxiety after *Fgf2* knockdown, normal in *Fgf2-/-* (Eren-Kocak, 2011; Fadda, 2007)	Less depressive behavior after FGF2 administration (Elsayed, 2012; Turner 2008)
FGF17	Inability to compare and respond to novel social information. Less vocalisation of pups (Scearce-Levie, 2007)	Normal (Scearce-Levie, 2007)	NA

Table 2.2

	Brain size	Neurites	Excitatory / inhibitory neurons
FGFR1	Reduced brain volume, frontal and temporal most (dnFGFR1, *Fgfr1a-/-* zebrafish), less dopamine neurons (thFGFR1) (Shin, 2004; Norton, 2011; Klejbor, 2006)	Decreased neurite outgrowth (Klejbor, 2006; Shin, 2004)	Normal glut+, reduced PV+ neurons, correlated with hyperactivity (rgFGFR1). Reduced glut+ and normal PV+ neurons (dnFGFR1) (Muller Smith, 2008; Smith, 2010; Shin, 2004)
FGFR2	Reduced brain volume, medial prefrontal most (Stevens, 2010)	NA	Reduction of PV+ neurons in hippocampus, correlated with spatial memory. Secondary reduced PV+ neurons in BST and septum (Stevens, 2012)
FGF2	Reduced cortex, normal hippocampus and striatum (Raballo, 2000; Ortega, 1998)	Reduced spine length; affects excitatory synapses (Zechel, 2006; Li, 2002)	Reduced glut+, normal GABA+ neurons in cortex, reduced glut+ fibers to striatum (Korada, 2002; Ortega, 1998; Fadda, 2007)
FGF17	Reduced dorsal frontal cortex and cerebellum, reduced projections to striatum and midbrain (Cholfin, 2007)	NA	NA

Table 2.2: Summary of neurobiological findings. For precise description and references, see text. Finding concern FGF mutant mice, unless otherwise stated. glut = glutamate, PV= parvalbumin, BST= bed nucleus of stria terminalis, OL= oligodendrocytes, DCX = doublecortin, LTP = long term potentiation, DG = dentate gyrus, NA = not assessed. *Fgfr1* mutant mice are indicated by abbreviations with *Fgfr1* deficiency induced in: radial glial cells (rgFGFR1), tyrosine hydroxylase (TH) expressing (dopaminergic) neurons (thFGFR1 mice), whole brain (bFGFR1) or in the telencephalon during development (dnFGFR1). **Table continues on next page.**

Table 2.2 (continued)

	White matter	Adult neuro-genesis	LTP
FGFR1	Astrocytes impaired of supporting PV+ neurons (rgFGFR1) (Muler Smith, 2008)	NA	Disrupted (Zhao, 2007)
FGFR2	Normal OL and myelin (FGFR2 disrupted in OL) (Kaga, 2006)	Reduced DCX+ cells, correlated with object memory (Stevens, 2012)	NA
FGF2	FGF2 can activate glia cells. Increased gliogenesis after FGF2 administration in prefrontal cortex, is possible mechanism of antidepressive effects (Reuss, 2003; Elsayed, 2012)	Increased neurogenesis and DG volume after FGF2 injection (Zechel, 2010)	Disrupted (Zechel, 2006)
FGF17	NA	NA	NA

2

Chapter 3

Association study of FGF genes and brain volume in schizophrenia patients and healthy controls

Submitted

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Abstract

Background Fibroblast growth factors (FGFs) play crucial roles in brain development and neuroprotection and have been implicated in the susceptibility to schizophrenia. We tested whether genetic variants in FGFs also affect potential schizophrenia endophenotypes.

Methods Intracranial volume and total brain volume were measured on a 1.5T MRI scanner in 162 schizophrenia patients and 151 healthy controls. Subsequently, 915 single nucleotide polymorphisms (SNPs) in 83 genes in the FGF system were analyzed for effects on these volumes.

Results After Bonferroni correction, no single SNP reached statistical significance for effect on brain volume nor intracranial volume. For intracranial volume, there was a significant overrepresentation of SNPs in CACNA1D among the highest-ranking SNPs (top 5 p-values CACNA1D SNPs ranging 0.001-0.009 (ANOVA), overrepresentation p-value = 1.0×10^{-5} (Fisher exact)). This suggests that multiple SNPs with small effects in CACNA1D influence intracranial volume. These primary results were not seen in an independent sample of 892 healthy controls.

Conclusions Altogether, SNPs in the FGF system do not seem to have major effects on brain volume in schizophrenia patients and healthy controls. More general, our data suggest that the effects of individual SNPs on this endophenotype may be not as large as previously expected.

Introduction

One of the leading hypotheses on the pathogenesis of schizophrenia concerns abnormal neuronal connectivity [242], which may be caused by deficiencies in glial growth factors such as fibroblast growth factors FGFs [167]. FGFs are signaling proteins with important roles in brain development by regulating brain patterning, axon outgrowth, neuro- and gliogenesis, differentiation and migration [199]. Later in life, FGFs modulate survival of neurons and glia, neuroprotection, learning and memory [199]. Multiple lines of evidence suggest an involvement of the FGF pathway in schizophrenia etiology, as we previously reviewed [228]. SNPs near FGFR1 were identified as the most significant associations with schizophrenia in a genome-wide association study (rs16887244, $p=1.3x10^{-10}$) [208]. Moreover, SNP rs17101921, located 85 kb from the FGFR2 gene, was the only significant finding after several rounds of replication in a fine mapping study of a linkage study [175]. Another interesting finding is the disruption of the neuronal PAS domain protein 3 (NPAS3) gene that was reported to cosegregate with illness in a small family with schizophrenia [118]. NPAS3 knock-out mice show an 80% reduction in FGFR1 mRNA [184], suggesting that NPAS3 encodes an important regulator of this FGF receptor. Furthermore, our research group found suggestive evidence for an association of FGF1 and FGFR1 SNPs with schizophrenia [116].

Several studies suggest that variations in FGFs can affect brain volume *in vivo*. First, in FGF2 knock-out mice, a 10% reduced volume and decreased neuron number and density was found in the cerebral cortex [192]. Second, mice with artificially reduced cerebral FGFR1 displayed a decreased thickness of the cerebral cortex, as well as other 'schizophrenia-like' characteristics, such as increased locomotor behavior and decreased prepulse inhibition [134, 209]. Interestingly, mutant FGFR1 and FGF2 mice show reduced volumes the whole brain, but most prominent in the frontotemporal cortex, a pattern similar to schizophrenia patients [97]. In addition, SNPs in FGF1 and FGFR1 were associated with grey and white matter volume in a sample of schizophrenia patients and healthy controls [109]. It is noteworthy, however, that FGFs do not only influence brain development, but also contribute significantly to skull growth and bone patterning. Mutations in FGF receptors are associated with craniosynostosis [119], a skull malformation that may also affect brain volume.

Integrating genotypic information with quantitative brain imaging data can help identify the function of candidate genes at the level of brain structure or function [2, 186]. Brain imaging data is well suited to be linked to disease-associated SNPs, since alteration of brain structure is robustly associated with schizophrenia and is highly heritable in schizophrenia patients as well as in healthy subjects [11, 43, 89, 103]. Average reductions in total brain volume of about 3% are observed in schizophrenia patients compared to healthy

individuals [111, 258]. The largest twin study (n=684) to date reported that 77% of the phenotypic overlap between schizophrenia and total brain volume was of genetic origin [103]. Moreover, our research group recently showed that schizophrenia-associated SNPs jointly predict total brain volume [227]. Total brain volume and intracranial volume are highly correlated and grow at a similar rate (25-27%) between early childhood and adolescence [54]. Thereafter, intracranial volume remains stable, while total brain volume slowly declines, at least after the age of 35 [105]. Therefore, intracranial volume (corrected for age and sex) can be considered an indirect measure of early brain development, while total brain volume corrected for age, sex and intracranial volume represents the brain volume changes in late adolescent and adult life. Recent meta-analyses of genome-wide association studies of brain volumes and intracranial volume identified several SNPs with significant effects, although effect sizes were not as large as previously expected [21, 216, 216]. Possibly, the power to identify small effects can be increased using a relatively small set of hypothesis-based SNPs in a genetically homogenous sample.

To summarize, abnormalities in FGFs might increase schizophrenia risk and disturb brain development. Therefore, the aim of the current study was to systematically screen genetic variants in the FGF system for association with intracranial volume, a developmental phenotype affected by FGFs and with total brain volume, a schizophrenia endophenotype in schizophrenia patients and healthy controls.

Methods

Subjects and brain imaging (Stage I)

The study was approved by the Humans Ethics Committee of the University Medical Center Utrecht and conducted in accordance with the Declaration of Helsinki. Participating subjects (162 cases and 151 controls) were derived from a study previously described by Van Haren et al. (n=164) [101] and the Genetic Risk and Outcome of Psychosis (GROUP) study (n=149) [87]. Psychopathology was assessed using the Comprehensive Assessment of Symptoms and History [4]. There were 146 schizophrenia patients and 16 with schizo-affective disorder. Six control subjects had a history of a single depressive episode of mild severity and three had a history of anxiety disorder, obsessive compulsive disorder and adjustment disorder. None of the control subjects had a first- or second-degree family member with psychotic illness. None of the subjects had major cranial bone malformations, such as craniosynostosis.

Magnetic resonance images were acquired on a 1.5 Tesla Philips NT scanner (Release 5) and 1.5 Tesla Philips Achieva scanner (Philips, Best, the Netherlands). On the NT scanner a three-dimensional-Fast Field Echo (3D-FFE: TE=4.6 ms, TR=30 ms, flip angle=30 degrees,

FOV=256x256 mm^2) with 160-180 contiguous coronal 1.2 mm slices, voxel size 1x1x1.2 mm^3, and a T2-weighted Dual Echo-Turbo Spin Echo (DE-TSE:TE1=14 ms, TE2=80 ms, TR=6350 ms, flip angle=90 degrees, FOV=256x256 mm^2) with 120 contiguous coronal 1.6 mm slices of the whole head were used for the quantitative measurements. On the Achieva scanner a three-dimensional T1-weighted coronal spoiled-gradient echo scan of the whole head (256x256 matrix, TE=4.6 ms, TR=30 ms, flip angle=30˚, 160-180 contiguous slices; 1x1x1.2 mm^3 voxels, Field-Of-View (FOV) = 256 mm/70%) was acquired. Furthermore, a single-shot EPI (echo planar imaging) scan was made as part of a diffusion tensor imaging (DTI)-series (SENSE factor 2.5; flip angle=90˚;60 transverse slices of 2.5 mm; no gap; 128 x 96 acquisition matrix; FOV=240 mm; TE=78 ms) together with a magnetization transfer imaging (MTI) scan (60 transverse slices of 2.5 mm; no gap; 128 x 96 acquisition matrix; FOV=240 mm; flip angle=8˚; TE=4.5 ms; TR=37.5 ms). Post-processing was done on the neuro-imaging computer network of the Department of Psychiatry at the University Medical Centre Utrecht. All images were coded to ensure blindness for subject identification. Scans were automatically put into Talairach frame (no scaling), and corrected for inhomogeneities in the magnetic field [211]. Quantitative assessment of the intracranial volume was performed with use of a full-automated computer program based on histogram analyses followed by mathematical morphology operators from the DE-TSE image (NT scanner) or the combined EPI and MTI scans (Achieva scanner) [101, 181]. For the latter, these scans were registered to the T1-weighted image by minimizing a mutual information joint entropy function [154]; the co-registered scans were used for automatic segmentation of the intracranial volume, based on histogram analysis and mathematical morphology operations. All intracranial segmentations were visually checked and corrected where necessary. Next, quantitative assessment of the total brain was performed based on histogram analyses followed by mathematical morphology operations in the T1-weighted image, using the intracranial volume as mask [204].

Genetic analyses (Stage I)

Samples were genotyped at the University of California in Los Angeles (UCLA) using the Illumina HumanHap550 beadchip. In genes of interest all SNPs on the array, including a 10 kb flanking region were selected, or a 50 kb flanking region when less than 3 SNPs were available. Within the 22 FGF ligands and 5 FGF receptors, 530 SNPs were selected. Dixon's mRNA browser [60] was then searched for SNPs with effect on the expression of FGF genes, leading to the inclusion of five cis-acting SNPs that altered the expression of FGF2 or FGF3. Evidence for genes in true positive protein interactions with FGF genes was obtained by software that was developed in house [84], which provides a systematic search of gene interaction databases. This resulted in the inclusion of another 1039 SNPs in 51 genes. In a manual literature search evidence for protein interactions with 5 additional genes was found, adding another 97 SNPs. The PLINK package [189] was then used for quality control. Form the initial set of 1231 SNPs, all SNPs were removed that showed minor allele

frequencies < 0.1 (359 SNPs), 5% or more genotyping errors (5 SNPs) or deviation from the Hardy-Weinberg equilibrium with p<0.05 (76 SNPs). Lastly, 316 SNPs in high linkage disequilibrium with other SNPs in the set ($r^2 > 0.8$) were removed. The final set therefore consisted of 915 SNPs. Identity-by-state pair wise distances between individuals were calculated in PLINK using the multidimensional scaling plot option to check for the genetic homogeneity of the sample and population stratification.

Statistical analyses (Stage I)

Volume measures of the intracranium were determined and corrected for age and sex by taking the unstandardized residuals of the intracranial volumes using linear regression. For each subject, the unstandardized residual was added to the group mean of the intracranial volume, to obtain a corrected intracranial volume measure. Total brain volumes were corrected for age, sex and intracranial volume in a similar way. In ANOVA analyses corrected brain volumes were used as dependent variables. First, the 915 SNPs (additive genotype coding) were analyzed separately as independent variables to investigate the effects of the SNPs on intracranial volume irrespective of disease status. Secondly, disease status and SNP*disease status interactions were also included in the analyses. For total brain volume the main effects of the SNPs were calculated in an analysis including disease status and SNP*disease status interactions, because there were significant differences in brain volume between patients and controls (see results). P-values were corrected using a Bonferroni correction for analyzing 915 SNPs, resulting in an α of 0.00005. Using Genetic Power Calculator [188], we estimated that we had 81% power to detect a SNP with minor allele frequency 0.3 explaining 7.5% of the variance in brain volume or 48% power if the SNP explained 5% of the variance in brain volume in stage I. In stage II there was 83% power to detect 1 of the 57 SNPs explaining 2% of the variance in intracranial volume.

Overrepresentation of high-ranking SNPs per gene was tested with a Fisher exact test, only for the gene that strongly suggested overrepresentation by visual inspection of p-values (CACNA1D). For this gene, the results obtained in our dataset were compared to the results obtained when randomly permuting intracranial volume 10,000 times, while leaving genotype data (and LD structures) unchanged. Each permutation, all 57 CACNA1D SNPs were tested individually for association to the “random” intracranial volume and the number of SNPs with a p-value below cut-off (p<0.1) were counted. The overrepresentation was regarded significant when the observed result was present in less than 1% of the permutations (100 out of 10,000 times). The association of CACNA1D SNPs with body height was tested in 232 subjects of our sample.

Stage II analysis

We attempted validation of the overrepresentation of SNPs with low p-values for effect on intracranial volume in the CACNA1D gene in an independent sample of 892 healthy controls, with 1.5 or 3 Tesla MRI scans, from Radboud University Nijmegen (41% males,

mean age 22.4 years). For a full description of this sample, see [29]. In short, subject specific tissue probability maps were first calculated using the unified segmentation procedure as implemented in SPM5 [6]. Then total gray matter (GM), total white matter (WM) and total cerebrospinal fluid (CSF) volumes were calculated by volume-wise summation of these probability maps and adjusted for effects of MR scanning sequence settings and hardware configurations using linear regression modeling. Finally, total brain volume was calculated as the sum of GM and WM, and total intracranial volume as the sum of GM, WM and CSF. Genetic data was genotyped on Affymetrix GeneChip 6.0 SNP array. All 57 SNPs in CACNA1D were tested, 18 of which were overlapping with the Illumina SNPs used in the initial sample. All 57 SNPs were analyzed separately as independent variables for effect on intracranial volume, including scanner type as factor. There was no difference in mean corrected intracranial volume between the 1.5 and 3T scans.

Results

For demographic measures of the stage I analysis see Table 3.1. There were significantly more males in the patient group than in the control group (80 versus 54% male, $p<0.001$). There were no significant differences in intracranial volume between patients (1519.2 (sd 118.6) ml) and controls (1535.5 (sd 115.6) ml) after correction for age and sex. Corrected brain volume was significantly lower in the patient group (mean 1308.1 (sd 47.8) ml) relative to controls (mean 1330.8 (sd 39.5) ml; $p<0.0001$ after correction for age, sex and intracranial volume), as was expected. The groups did not differ in age and parental education (which is an estimate for socioeconomic status), or in body height (as a measure of general growth rather than brain-specific growth). The multidimensional scaling plot showed a genetically homogeneous sample (data not shown).

SNPs with effects on intracranial volume in stage I

The SNPs with the largest effect on intracranial volume are shown in Table 3.2. SNPs were analyzed for their main effects on intracranial volume as well as for difference in effect in the patient and control groups (interaction with disease status). After Bonferroni correction for all SNPs tested, no single SNP was associated with statistical significance (uncorrected p-value for FGF2 SNP rs308428: $p=0.0009$, corrected $p= 0.82$). Interestingly, however, five out of the eleven SNPs with $p<0.01$ for effect on intracranial volume were located in CACNA1D. Even though this is one of the larger genes in the set, this was far more than to be expected. Indeed, among all SNPs with p-value <0.1 (n=105), CACNA1D SNPs are again significantly overrepresented (observed number of SNPs =20, expected=7, Fisher exact $p=1.0 \times 10^{-5}$).

More stringent cut-off p-values results in larger overrepresentations (observed / expected number of significant SNPs), but higher p-values due to the small number of SNPs left in the analysis. We determined the significance of this result with an additional permutation analysis. By randomly permuting intracranial volume 10,000 times, while keeping genotypes (and thereby linkage disequilibrium patterns) identical we observed a number of CACNA1D SNPs with $p<0.1$ equal to or greater than 20 only forty times, resulting in an empirical p-value of 0.004. When taking into account disease status, CACNA1D SNPs were not significantly overrepresented, because some SNPs had main effects predominantly in patients, others in controls. However, there were no main effects of the CACNA1D SNPs on disease status (lowest p=0.01), nor a gene-based overrepresentation. We observed no overrepresentation of CACNA1D SNPs for association with height. Since there were significantly more males in the patient group, we repeated the analysis using male subjects only. Results, including the CACNA1D overrepresentation, were very similar to those found in the total sample (data not shown). Excluding patients with schizo-affective disorder and control subjects with any disorder did not significantly alter the results.

No single SNP was significantly associated with differential effects on intracranial volume in patients and controls (SNP*disease status interaction), see Table 3.2. SNP rs1336714 in FGF14 was the most significant SNP in this study, showing an interaction with disease status on intracranial volume (uncorrected p=0.0006, corrected p=0.55). Post hoc analysis showed that the minor allele was associated with decrease intracranial volume in schizophrenia patients (uncorrected p=0.01) and increased intracranial volume in controls (uncorrected p=0.02).

SNPs with effects on total brain volume in stage I

Table 3.3 shows the SNPs with effects on total brain volume. The strongest effect was found for rs757331 in FGF22 for an effect on total brain volume irrespective of disease status (uncorrected p=0.009, corrected p=0.82).

Stage II analysis

Because we observed an overrepresentation of low p-values for SNPs in CACNA1D in the initial analysis, we tested the CACNA1D SNPs also in a second, independent sample. Since the overrepresentation was found independent of disease status, a relatively large sample of healthy controls that we had access to was used for the stage II analysis. In this sample, none of the CACNA1D SNPs had a significant effect on intracranial volume and no overrepresentation was observed (expected number of SNPs with $p<0.1$ =7, observed=6), see Table 3.4.

Discussion

We systematically screened genes from the fibroblast growth factor (FGF) system for association with intracranial volume and total brain volume in schizophrenia patients and healthy controls. Total brain volume, relative to intracranial volume, was regarded as a measure of volume-loss at adolescent and adult age, whereas intracranial volume was regarded as a measure of brain growth during early development. After Bonferroni correction, none of the associations for single SNPs remained significant. An interesting observation was a significant overrepresentation of SNPs in the CACNA1D gene among the high-ranking SNPs with an effect on intracranial volume. We observed 2.6 times more CACNA1D SNPs with $p<0.1$ than expected by chance. An overrepresentation of this magnitude was only observed in 0.4% of the intracranial volume permutations, which is suggestive of a true overrepresentation. It is unlikely that this overrepresentation is explained by correlation between the high-ranking CACNA1D SNPs, since r^2 between the SNPs tested never exceeded 0.3. One could argue that CACNA1D SNPs have an effect on growth in general rather than just skull or early brain development. However, these SNPs were not associated with body height in our sample, nor in a GWAS on body height involving 183,727 subjects [146]. Thus, the effects seem to be brain-/ skull-specific.

In an independent second sample of 892 healthy controls, we did not find an overrepresentation of CACNA1D SNPs. This could mean that our initial finding was based on stochastic variation. However, differences between the samples could also have affected the results. In the stage II sample only healthy subjects were included, while in the original sample some SNPs showed predominant effects in patients and other SNPs showing larger effects in controls. In the stage II sample MRIs were acquired on different scanners with two field strengths, 1.5T and 3T, while in stage I only 1.5 T MRI scans were used. Refining the stage II analysis to subjects with 1.5 T scans only (n=416) did not change the results, however.

It was previously thought that SNPs may have substantially larger effects on endophenotypes compared to complex (psychiatric) disorders, since they are assumed to involve the same biological pathways as diseases but are less removed from the relevant gene action and are less heterogeneous [2]. However, recent genome-wide studies of brain volumes suggest that these effects may be more subtle, albeit still larger than on clinical disease phenotypes [21, 216]. While this study had sufficient power to detect large effects, the power for smaller effects was limited. The results of this study should therefore be considered explorative rather than as prove that the FGF system has no role in brain and intracranial volume in human.

CACNA1D (calcium channel, voltage-dependent, L-type, alpha 1D subunit, also called Cav1.3) codes for a calcium channel present in all excitable cells in mammals. In neurons,

L-type calcium channels couple membrane depolarization to numerous processes including gene expression, synaptic efficacy and cell survival [150]. Activation of FGF receptors cause L-type calcium channels to flicker open and shut [5]. This activates axon outgrowth without raising bulk calcium concentrations and may provide a crucial mechanism for axon guidance during development. Interestingly, variation in another gene coding for a similar subtype of a L-type calcium channel, CACNA1C, is reportedly associated with bipolar disorder, schizophrenia, recurrent depressive disorder and autistic features of Timothy syndrome [76, 94, 170, 200, 213]. In addition, variants in this gene have been associated with gray matter and brain stem volume [83, 124, 251]. CACNA1C was not included in our study, because no *a priori* evidence for interaction with FGF genes could be established. So, the effect of CACNA1D on intracranial volume may be exerted through a role in early brain development.

SNP rs1336714 in FGF14 is the most significant single SNP in this study, with a genotype-by-disease interaction effect on intracranial volume. The association with a reduced intracranial volume in schizophrenia patients, but not in controls, however was not significant after Bonferroni correction for multiple testing (uncorrected p=0.0006, corrected p=0.55). Interestingly, disruptions of the FGF14 gene have previously been described to cause microcephaly, cerebellar ataxia and cognitive impairment in several European families [165]. In mice it was shown that FGF14 acts independent of FGF receptors to modulate synaptic transmission in striatal neurons [254]. Unfortunately, effects on brain or skull volume were not specifically tested in mice lacking FGF14.

Although intracranial volume and brain volume are highly correlated (r^2 = 0.84, both corrected for age and sex only), the latter has yielded less convincing findings in genetic studies (e.g. Stein et al., 2012 and the current study). It could be that this measure is confounded by the use of medication or illicit drugs like cannabis [169, 194, 233]. Furthermore, in this study we only analyzed global volumes, and SNPs might have regional brain effects that could have gone undetected. For example, lack of FGF2 or FGFR1 disrupts frontal and temporal regions more strongly than other brain regions [192, 209]. In addition, since deficits in FGF functioning become especially important after stressors, like perinatal hypoxia or social defeat [232, 236], the effect of genetic variants in these genes could depend on this history of environmental stressors the subjects were exposed to. We did not include this in our analyses, however.

To conclude, CACNA1D SNPs were significantly overrepresented among the high-ranking SNPs with an effect on intracranial volume in a sample of schizophrenia patients and healthy controls. This finding was not observed in an independent second sample of healthy controls.

	Schizophrenia patients	Healthy controls	Significance
n	162	151	
Gender (m/f)	131/31	82/69	p<0.0001
Age in years (sd)	31.8 (10.6)	32.2 (12.2)	ns
Handedness (r/l/ambidexter/u)	125/14/4/19	123/15/4/9	ns
Level of education in years	12.3	13.9	p<0.0001
Level of parental education in years	13.2	13.0	ns
Age of first psychotic symptoms in years	22.5	na	
Duration of illness in years	8.6	na	
Intracranial volume in ml (sd)*	1519.2 (118.6)	1535.5 (115.6)	ns
Total brain volume in ml (sd)**	1308.1 (47.8)	1330.8 (39.5)	p<0.0001
Height in cm (sd)*	180.0 (7.5)	178.5 (7.3)	ns

Table 3.1: Demographic information of stage I analysis. sd = standard deviation, u=unknown handedness, na = not applicable, ns = not significant. * Intracranial volume and height were corrected for age and sex. Height was based on data of 232 subjects. ** Total brain volume was corrected for age, sex and intracranial volume.

Main effects of SNP on intracranial volume				
chr	**snp**	**gene**	**F**	**P**
4	rs308428	FGF2	7.2	0.0009
20	rs753381	PLCG1	7.2	0.0009
13	rs4772445	FGF14	7.1	0.001
3	rs2612018	CACNA1D	6.9	0.0011
12	rs10437827	FGF23	6.1	0.0026
3	rs11707976	CACNA1D	5.7	0.0038
9	rs735740	SHB	5.6	0.0042
3	rs3796345	CACNA1D	4.8	0.0086
3	rs3774421	CACNA1D	4.8	0.0089
3	rs3821856	CACNA1D	4.8	0.009
11	rs4756195	CD44	4.8	0.0092

Different effects in patients and controls				
chr	**snp**	**gene**	**F**	**P**
13	rs1336714	FGF14	7.7	0.0006
3	rs4605535	CACNA1D	7.3	0.0008
13	rs4772456	FGF14	6	0.0029
12	rs10506806	SYT1	5.9	0.003
3	rs893363	CACNA1D	5.8	0.0033
3	rs3774605	CACNA1D	5.3	0.0052
3	rs13078747	CACNA1D	5.2	0.0061
17	rs2075555	COL1A1	5.2	0.0062
22	rs3747152	PIB5PA	5	0.0073
19	rs12975781	FGF21	4.8	0.0088
11	rs4937993	NCAM1	4.8	0.009
19	rs838133	FGF21	4.7	0.0099

Table 3.2: SNPs with largest effect on intracranial volume (p<0.01). In 'Main effects of SNP on intracranial volume' results are shown for the effect of the SNPs on intracranial volume irrespective of disease status. In 'Different effects in patients and controls' the interaction effects between SNP and disease on intracranial volume (analyzed in the total Stage I sample) are shown. chr = chromosome, snp = single nucleotide polymorphism, F = ANOVA test statistic, P = p-value uncorrected for multiple testing. Bonferroni corrected p-values are not shown, because almost all are greater than 0.99.

Main effects of SNP on total brain volume				
chr	**snp**	**gene**	**F**	**P**
19	rs757331	FGF22	7.1	0.0009
13	rs9518598	FGF14	6.6	0.0016
3	rs1460916	FGF12	6.5	0.0018
22	rs9624447	ADORA2A	5.6	0.004
13	rs7334198	FGF14	5.5	0.0046
3	rs6763768	CACNA1D	5.1	0.0066
13	rs1407785	FGF14	5	0.0072
13	rs2476227	FGF14	4.9	0.0083
2	rs3770181	EphA4	4.8	0.0091

Different effects in patients and controls				
chr	**snp**	**gene**	**F**	**P**
4	rs7682912	PDGFRA	6.6	0.0016
7	rs6593206	EGFR	6.3	0.002
9	rs943936	SHB	5.6	0.0042
19	rs7249518	FGF22	5.5	0.0045
13	rs9518579	FGF14	5.2	0.0059
5	rs2302273	PDGFRB	5.2	0.0059
10	rs1649200	FGFR2	5.2	0.0062
12	rs11613495	FGF6	5.1	0.0069
10	rs3135831	FGFR2	4.8	0.0085
5	rs3806929	FGF18	4.8	0.009
11	rs12279261	NCAM1	4.8	0.0092

Table 3.3: SNPs with largest effect on total brain volume (p<0.01). In 'Main effects of SNP on total brain volume' results are shown for the effect of the SNPs on total brain volume irrespective of disease status. In 'Different effects in patients and controls' the interaction effects between SNP and disease on total brain volume (analyzed in the total Stage I sample) are shown. chr = chromosome, snp = single nucleotide polymorphism, F = ANOVA test statistic, P = p-value uncorrected for multiple testing. Bonferroni corrected p-values are not shown, because almost all are greater than 0.99.

Effect on intracranial volume in Stage II		
snp	**F**	**P**
rs10490772	2.96	0.052
rs2359453	2.95	0.053
rs6763245	2.68	0.069
rs6797014	2.66	0.070
rs4605535	2.45	0.086
rs7638857	2.43	0.089

Table 3.4: CACNA 1D SNPs with largest effect (p<0.1) on intracranial volume in stage II. Since there were only healthy controls in this sample, only main effects were calculated. chr = chromosome, snp = single nucleotide polymorphism, F = ANOVA test statistic, P = p-value uncorrected for multiple testing

Chapter 4

Lack of FGF2 results in increased locomotor activity and aggression and exacerbates effects of early life stress on anxiety and structural plasticity

In preparation

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Abstract

Background Early life stress is an environmental risk factor for psychopathology. In humans and animals, sensitivity to stress may be altered by their genetic background. Fibroblast growth factors (FGFs) have been implicated in the etiology of psychiatric disorders. Since *Fgf2* is an important modulator of neurogenesis and repair after stress, we hypothesized that disruption of the *Fgf2* gene in mice increases their vulnerability to early life stress regarding brain development and behavior.

Methods *Fgf2*-/- mice and wild type littermates were either subjected to early life stress applied by 24 hours of maternal deprivation on postnatal day 3, or left undisturbed. The brains of adult male offspring were analyzed for neurogenesis, dendritic arborization and the balance between excitatory and inhibitory neurons. In addition, the mice were tested for their behavioral performance on locomotor activity, anxiety, memory and social interaction.

Results Early life stress resulted in a much larger increase in anxiety-like sheltering behavior in *Fgf2-/-* mice than in wild type mice. This increase coincided normalisation of a robust locomotor hyperactivity phenotype in stressed compared to unstressed knockout mice. A gene by early life environmental interaction was also observed in hippocampal neuronal differentiation. Independent of early life stress, *Fgf2*-/- mice were more aggressive in a direct social interaction task, which correlated with lower *Gad1+* inhibitory neuron densities in the hippocampus.

Conclusion Our results provide clear examples of gene (*Fgf2*) by environment (early life stress) interactions that impact on behavior as well as neuronal substrates. Such interactions may contribute to disordered adult behavior in carriers of *FGF* genetic variations.

Introduction

Early life stress in humans is a well-documented risk factor for psychiatric disorders [127]. Humans and animals vary greatly in their sensitivity to early life stress, which may be explained by their genetic background [47]. Identifying the genetic variations that contribute to the consequences of early life adversity, as well as the neurodevelopmental pathways through which this is accomplished, might help to unravel the pathophysiology of psychiatric disorders. We hypothesized that alterations in fibroblast growth factors (*Fgfs*) are among the genetic variants that can contribute to increased sensitivity to stress.

FGFs are signaling proteins involved in brain development. They regulate differentiation, migration and survival of neurons and glia from early embryonic development into adulthood [199]. FGF2, a prototypical ligand of the FGF family, is widely expressed in the brain. Aberrations in FGF2 and its receptors have been implicated in the etiology of psychiatric disorders [86, 120, 208, 229].

The relevance of FGF2 for the consequences of stress exposure is supported by rodent studies. Hippocampal *Fgf2* expression in mice is increased after acute stress in adulthood [82]. Interestingly, this *Fgf2* response to stress in adulthood was disturbed when animals had been exposed to prenatal or early postpartum stress [85, 201]. Conversely, *Fgf2* levels were increased in stimulating environments or with increased maternal care [61, 229]. Given the neuroprotective properties of FGF2 [199], it was hypothesized that increased FGF2 expression or function may compensate for damage caused by stress [168]. *Fgf2* expression seems particularly important during the vulnerable perinatal period. To test the critical importance of FGF2 levels for vulnerability to early life adversity, we here examined the effects of 24 hours maternal deprivation in the absence of FGF2, i.e. in *Fgf2* knockout (*Fgf2*-/-) mice on young adult age. Generally, *Fgf2*-/- mice are viable and fertile and show no gross physical abnormalities [270].

We focused on three structural endpoints that might be targeted by FGF2 and/or early life stress, namely neurogenesis, dendritic arborization and the balance between excitatory and inhibitory neurons. First, we studied gene-by-environment interactions on adult hippocampal neurogenesis. Postnatally, neurogenesis is restricted to the subgranular zone of the hippocampal dentate gyrus and the subventricular zone. Animal models show that neurogenesis is disrupted subsequent to stress [164], including prenatal or early life stress [139, 177]. FGF2 has an important role in neurogenesis both in early brain development, as well as in the adult hippocampus [192]. Administration of FGF2 in the lateral ventricles induced neuron proliferation and differentiation [193, 266]. Furthermore, the formation of young neurons by differentiation was disrupted in *Fgf2*-/- mice, while proliferation was unaffected [257].

Second, FGF2 may affect the outcome of early life stress by changing dendritic arborization. In support, prenatal stress reduced dendritic arborization [39]. Conversely, administration of FGF2 to cultured neurons greatly enhanced axonal branching [179, 191]. Both neurogenesis and neurite outgrowth have been implicated in the etiology of psychiatric illnesses [180].

A third mechanism by which FGFs may interact with early life stress is through influencing the balance between excitatory and inhibitory neurons, another process which is implicated in psychiatric disorders [158]. Early life stress and hypoxia disturb the balance between excitatory and inhibitory neurons, specifically by affecting Parvalbumin-positive (*Pv+*) interneurons [41, 88]. In *Fgf*2-/- mice, reductions in the number of glutamate+ [137] and *Pv+* GABAergic [178] neurons were reported.

As a final step, we probed several behavioral domains, including anxiety and memory formation that are targeted by FGF2 and/or early life stress. For instance, hippocampal *Fgf2* mRNA expression negatively correlates with anxiety behaviors [69], and FGF2 administration reduces anxiety and depression-like behaviors in rodents [182, 238, 239]. There is also extensive literature supporting early life effects on anxiety behaviors and hippocampal learning [15, 140, 177, 202]. Overall, we expect that the vulnerability of mice to early life stress regarding brain development and behavior is exacerbated by disrupted *Fgf2* expression.

Methods

Animals

Fgf2 knock-out mice with a 129Sv:Black Swiss background (Fgf2tm1Doe/J), originally generated by Zhou et al. [270], were ordered from The Jackson Laboratory (www.jax.org). Wild type Black Swiss mice (BLKSW-M) were ordered from Taconic (www.taconic.com) [3]. All tested mice were obtained from heterozygous crossings. After weaning at age 4 weeks, mice were housed in groups of 2-4 same sex littermates, until they were individually housed from age 8 weeks onwards. Animals were kept in a controlled 12-hr light–dark cycle with a room temperature of 22 ± 1°C and a humidity of 60% and food and water available *ad libitum*. All experimental procedures were approved by the ethical committee for animal experimentation of the University Medical Center Utrecht, The Netherlands.

Early life stress

Maternal deprivation was performed as previously described [68]. Mothers nursing litters were removed from their cage and placed in a clean cage on post-natal day 3. The home cage containing the pups was placed in an adjacent room on a heating pad (30-33°C). Pups

were not fed during the deprivation period. After 24 hours, the mother was reunited with her pups and thereafter left undisturbed. Control litters were left undisturbed.

Immunohistochemistry

Stainings for Ki-67, doublecortin (DCX) and 5-bromo-2-deoxyuridine (BrdU) were performed as previously described [177] on mice not used for behavioral testing, because of possible confounding effects of the BrdU injections on behavior. Mice were intraperitoneally injected with 50mg/kg BrdU (Sigma) twice a day for three consecutive days at 10 weeks of age. At 21 days after the last injection, mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. The brains were 24 hours postfixed in 4% PFA and kept overnight in 30% sucrose prior to slicing the brains on a sliding microtome (6 series of 40 µm sections). Free-floating sections were stored at -20ºC in a cryoprotectant until further processing.

For detection of ki-67 labeled cells, brain sections were first mounted to slides (Superfrost plus slides, Menzel). For antigen retrieval, slides were heated in 0.01M pH 6.0 citrate buffer in a standard microwave (Samsung M6235) to a temperature of approximately 95°C for 15 minutes (5 minutes at 800 Watt, 5 minutes at 400 Watt and 5 minutes at 200 Watt) and subsequently exposed overnight to polyclonal rabbit anti-Ki-67 antibody (1:10000, Novocastra) and incubated for 2 hours with the goat-anti rabbit biotynilated secondary antibody (Vector). For the DCX staining, sections were exposed overnight to polyclonal goat anti-DCX antibody (1:800 Santa Cruz) and subsequently incubated for 2 hours with donkey-anti goat biotinylated secondary antibody (Jackson). Amplification of the DCX and Ki-67 stainings was performed with the avidin-biotin complex and tyramide in 0.01% H_2O_2. The BrdU antigen was retrieved with 30 minutes incubation in 2M HCL at 37°C. Sections were exposed overnight to the rat anti-BrdU primary antibody (1:200 Bio-connect), incubated with the goat anti-rat biotynilated secondary antibody (Vector) and underwent a 2 hour amplification step with avidin-biotin complex. All stainings were visualized through a reaction with diaminobenzidine (0.5 mg/ml) and 0.01% H_2O_2. Stained and mounted sections were counterstained with hematoxylin. Per animal, seven sections along the rostrocaudal axis of the dentate gyrus, at determined distances from the bregma, were selected for quantification. Dentate gyrus surface was calculated by adding the surface area of the seven sections. Cells were counted manually at a 40x magnification on a Zeiss microscope and cell densities were calculated. See Figure 4.1 for representative examples.

Golgi-Cox stainings

Mice were decapitated 1 week after finishing the behavioral tests (at age 16 weeks). Per experimental group six mice were used, of which 1 or 2 were not subjected to behavioral tests. Behavioral testing did not significantly affect the outcome measures. Brains were placed in Golgi-Cox impregnation solutions to incubate for 9 days according to the

protocol of the FD Rapid GolgiStain Kit (FD NeuroTechnologies) [141]. This stained a random subset (estimates are around 1% [135]) of the neurons in the brain. After cutting the brains in 200 µm coronal sections using a vibratome, sections were mounted on gelatin-coated slides. Stainings were visualized with the solutions D and E provided by the kit. Hereafter, slides were dehydrated in ethanol and stored in the dark at 4°C. Per animal, three fully stained neurons of which the dendrites were clearly traceable were used for analyses. Only neurons with cell bodies in the middle two-third of the granular cell layer in the dentate gyrus were selected, to avoid the inclusion of relatively young granular cells. Image stacks (about 350 per neuron, 0.5 µm thick) of the neurons were recorded with Zen pro (Zeiss) using an Imager.M2 microscope (Zeiss), equipped with a AxioCam MRm camera, at a magnification of 40x. Neurons were traced using the Neurolucida drawing tool (MicroBright Field), see Figure 4.1. For every neuron, dendrite length and number of nodes were determined and used as main outcome parameters.

In situ hybridizations

RNA expression of vesicular glutamate transporter 1 (*Vglut1*), a measure of glutamatergic neurons, glutamate decarboxylase 1 (*Gad1*), the rate limiting enzyme of GABA synthesis and parvalbumine (*Pv*), expressed in a subset of the GABAergic neurons, was visualized with in situ hybridisations on coronal brain sections.

Probe synthesis

The following primers were used in a PCR on a cDNA library of wild type adult mouse brains with annealing temperature 60°C (*Vglut1* and *Gad1*) and 58°C (*Pv*): *Vglut1*, forward: 5'-CACATAATGTCCACTACCAA-3', reverse: 5'-CACTGCCAGCCAGCTGGTCG-3', resulting in a 454 bp fragment [13]; *Gad1*, forward: 5'-ATGGCATCTTCCACGCCTTCG-3', reverse: 5'-CCAAATTAAAACCTTCCATGCC-3' encoding a 466 bp fragment [157] and *Pv*, forward: 5'-GGGCCTGAAGAAAAAGAACC-3', reverse: 5'- AGTACCAAGCAGGCAGGAGA-3', encoding a 563 bp fragment [225]. The PCR products were ligated in pGemT Easy (Promega) and transformed to Escherichia coli DH5α. Resulting white colonies were grown overnight on LB medium with 1 µg/ml ampicillin. After purification, the PCR-fragments from the vectors were sequenced. A DIG RNA labeling kit (Roche) was used to synthesize RNA probes.

In situ hybridizations

In situ hybridisations on 14 mouse brains per study group were performed as previously described [114]. Of each group, nine mice were behaviorally tested and five were not. Behavioral testing did not significantly affect the outcome measures. In short, brains were snap frozen and kept at -80°C until cutting in 10 series of 16 µm sections using a cryostat. Sections were thaw-mounted onto slides and fixed in 4% paraformaldehyde. After acetylation and a blocking step, sections were hybridized overnight at 68°C with 400 ng/ml DIG-labeled probe. DIG was detected with an alkaline phosphatase-conjungated mouse

anti-DIG Fab fragment (Roche), using NBT/BCIP (Roche) as substrate. Slides were dehydrated with ethanol and mounted using entellan (Merck).

Quantification
Brain regions were observed under light microscopy (Zeiss) at a magnification of 5x (hippocampus), 10x (motor and somatosensory cortex) or 20x (prelimbic cortex). Digital images of the regions were captured in software (AxioVision, Zeiss). Region of interest, encompassing the whole thickness of the cortex or the entire hippocampus, were initially selected on images of *Vglut1* stained tissue (or *Gad1* for the hippocampus) and were subsequently projected over the remaining images within each animal, see Figure 4.1. Then, positive cell numbers and area size in pixels were automatically assessed by specialized software (Leica Application Suite). Cell densities (number of positive cells / 10^6 pixels) were used for the analyses.

Behavioral tests
Mice were behaviorally tested starting at age 9 weeks. Measurements were performed during the second half of the dark (active) phase (13.00–17.00 hour) in the same order as the tests are described below. Tests lasted until age 16 weeks, after which brains were harvested for Golgi and in situ hybridization analyses.

Elevated Plus maze (EPM)
The maze was made of four arms (50 × 10 cm) in a cross and a central platform (10 × 10 cm) and was elevated 75 cm above the floor. Two opposite arms were enclosed by 30-cm-high non-transparent walls. The mice were placed on the center platform and behavior was recorded for 5 minutes with Ethovision 7.0 (Noldus Information Technology, Wageningen, the Netherlands). The following parameters were used for scoring: percentage time spent on open arms (as a measure of anxiety) and percentage closed arm entries (as a measure of activity).

Home cage (HC)
The home cage test was used for analysis of baseline behavior of mice in a home cage-like environment for 5 consecutive days. Specifications of the home cage were described previously [248], except that in this experiment no running wheel was used. In short, each cage contained 2 feeding stations (on an open and a closed platform), water bottle and a shelter (10 x 10 x 5 cm) and the floor was covered with sawdust (default) or dark paper shreds (EnviroDri®, TecniLab; to improve contrast for white mice). Home cage activity was automatically recorded with video tracking (PhenoTyper and Ethovision 3.0, Noldus Information Technology). The following parameters were used for analysis: distance moved and duration of shelter visits. All parameters were calculated in 1 hour bins and subsequently lumped into 12 hour fragments to distinguish dark/light periods.

Open field test (OF)
Movement of the mice in an circular open field (diameter 80 cm, height 30 cm) was recorded during 5 minutes with Ethovision. For the analyses, the arena was divided into 2 zones: the outer 15 cm and the remaining inner zone. The distance moved in the total arena was used as a measure of activity and the percentage time spent in the inner zone as a measure of anxiety.

Object discrimination test (OD)
The object discrimination test for object memory makes use of the preference of mice to explore novel objects over familiar objects. The objects are made of glass, metal or plastic and are of sufficient height to prevent mice climbing on the objects. In the habituation trial on day 1, the mice were exposed for 5 minutes to 2 identical objects. After a short-term interval of 1 hour, each mouse was re-exposed to 1 familiar object (from the habituation trial) and 1 novel object for 5 minutes. On day 2, 24 hours after the first trial, each mouse was again re-exposed to a familiar and another novel object for 5 minutes. Behavior was recorded and manually scored using Observer (Noldus Information Technology). Any active exploration (approaching, sniffing, touching) of the object, but not passive contact (sitting next to the object) was scored as object exploration. The percentage time exploring the novel object of the total object exploration time was used for analysis.

Object location test (OL)
Testing took place in empty transparent plastic cages (25 x 25 x 30 cm) with 2 transparent walls and 2 white walls with a black circle on 1 wall. Two glass 100ml Erlenmeyers or 2 glass 100ml bottles were used as objects. In the habituation trial on day 1, the mice were exposed for 16 minutes to 2 identical objects placed in adjacent corners. On day 2, 24 hour after the first trial, each mouse was re-exposed for 6 minutes to the 2 objects, one of which was moved to the opposite corner. Behavior was scored using Observer and the percentage time exploring the moved object was used for analysis.

Social discrimination test (SD)
This test, modified from [67], was used to test social memory and aggressive interaction. Mice were tested on 2 days in standard cages with sawdust bedding and a lid with air holes on top. Adult male A/J mice, ordered from Charles River, were used as intruders, because this strain shows little aggressive behavior. On day 1, each testing mouse was allowed 5 minutes exploration of its novel cage. Directly after this, it was exposed to 1 intruder for 2 minutes for habituation. After a short term interval of 5 minutes (in the same cage), each mice was re-exposed for 2 minutes to the familiar intruder and a novel intruder. On the second day (24 hours after the first trial), each mice was again re-exposed to the familiar intruder and another novel intruder for 2 minutes. Behavior was recorded and scored with Observer. Social sniffing, anogenital sniffing and allogrooming were

scored as social interaction, while tail rattle, biting or chasing was scored as aggressive behavior. The time exploring the novel intruder as percentage of the total intruder exploration time and the total time the animals were engaged in aggressive interaction were used for analyses.

Fear conditioning (FC)

In this classical conditioning test, two memory components were analyzed: the associations of an unconditioned stimulus (foot shock) with a conditioned stimulus (a tone, called cued memory) and with a particular context (a cage, called contextual memory). Testing took place in 30 x 30 x 40 cm cages with stainless steel walls with Phenotyper top units to apply the sound cue and record the behavior. During training on day 1, each mouse explored the conditioning context in the fear conditioning box for 180 seconds. Thereafter, a single presentation of a 30 second tone (2.3 kHz, 70 dB) was followed at tone offset by a 2 second foot shock (0.7 mA, constant current), provided through the stainless steel floor grid (ø 1 mm, distance 5 mm). Mice were returned to their home cages 30 seconds after the shock. On the second day (after 24 hours) context-dependent memory was tested by re-exposure to the conditioning box for 180 seconds without any phasic stimulation such as tone and shock (contextual trial). The tone-dependent memory test was performed immediately hereafter in a novel context. The novel context was a similarly sized cage with a plain floor instead of the shock grid, black and white dotted plastic cards at the walls and a honey flavored tea bag attached to the ceiling. First, behavior was monitored for 180 seconds without tone presentation (habituation to novel context). Then, the tone was replayed for 180 seconds (cued trial). The behaviors were scored in Observer. The percentage time spent freezing (the lack of any movement besides respiration) and scanning (no walking, but small head movements are allowed) were used as outcome measures.

Statistical analysis

For all outcome measures, two-way ANOVAs were used as primary analyses, with genotype, stress paradigm and genotype*stress interaction as fixed factors, batch as random factor and age at testing (or decapitation) as covariate. We *post hoc* tested genotype and stress effects with one-way ANOVAs comparing two groups at a time when suggestive effects were found in the two-way ANOVAs. While the two-way ANOVAs have more power, the results may be biased in some situations, for example when stress has opposite effects in the wild type and *Fgf2-/-* groups. Where applicable, repeated measure analyses were performed using a linear mixed-effects model. For the Golgi-analyses, 3-level analyses were performed using neurons, mice and the experimental groups as levels. All together, we tested 8 measures of behavior and 7 measures of brain structure (correlated outcome measures were counted as one). Therefore, an α of 0.05/8 = 0.006 was set as a cut off of significance for the behavioral tests and an α of 0.05/7 = 0.007 for the brain tests.

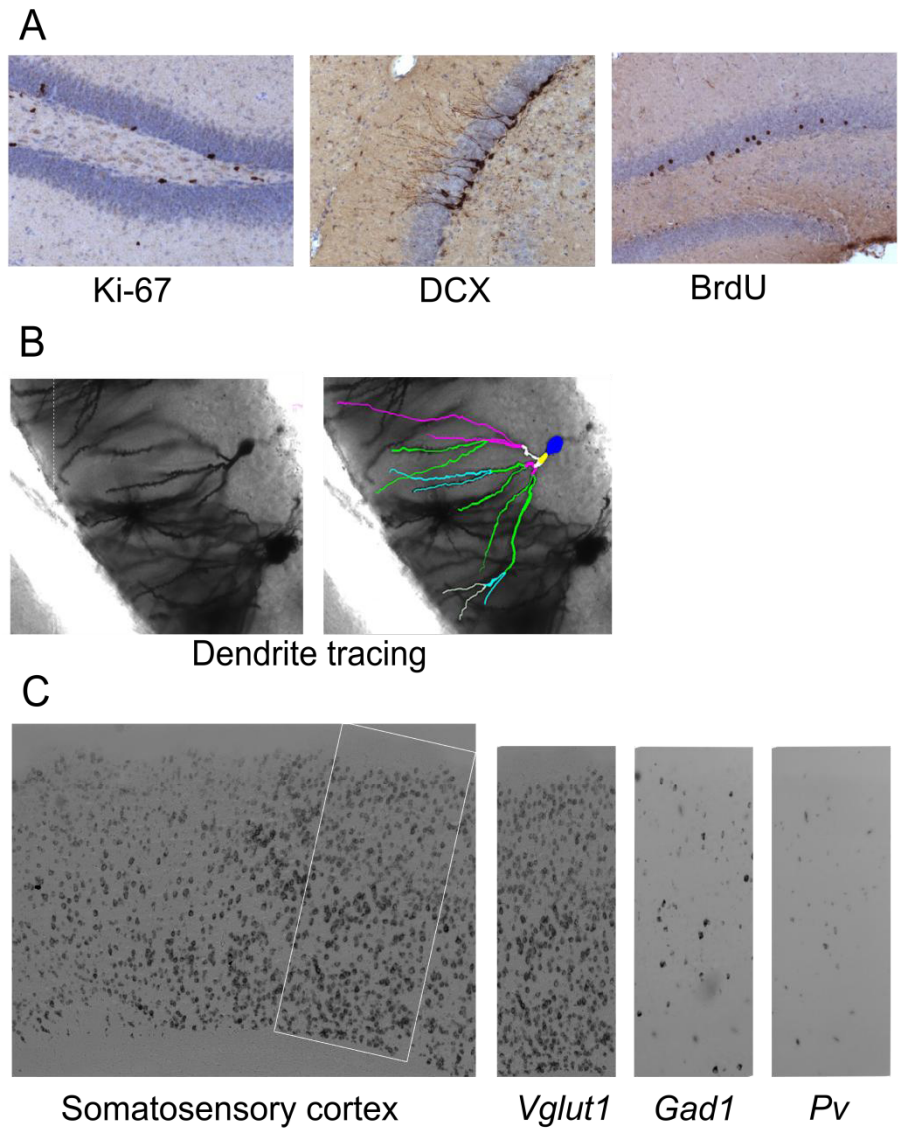

Figure 4.1 Representative examples of the neuromorphological stainings.
A. Immunohistochemistry stainings for Ki-67, doublecortin (DCX) and 5-bromo-2-deoxyuridine (BrdU). **B.** Golgi staining of dendrites in the dentate gyrus (left) and an example of traced dendrites (right). **C.** In situ hybridizations for vescicular glutamate transporter 1 (*Vglut1*), a measure of glutamatergic neurons, glutamate decarboxylase 1 (*Gad1*), the rate limiting enzyme of GABA synthesis and parvalbumine (*Pv*), present in a subset of GABAergic neurons, in the somatosensory cortex.

Results

Generally, *Fgf2-/-* mice bred well with large litter sizes with relatively more males than females (57.3% males, male:female ratio = 1.34, p=0.001; n=548 pups). Genotype frequencies were normal distributed (wild type:heterozygous:homozygous = 1:2:1). For an overview of the F and p-values of all tests, see Table 4.1 and Table 4.2.

Gene-by-environment interaction on differentiation

Stainings for Ki-67, doublecortin (DCX) and 5-bromo-2-deoxyuridine (BrdU) in the dentate gyrus were used as measures for proliferation, differentiation and cell survival respectively [32, 122]. We observed a main effect of early life stress on the amount of DCX-positive neurons (F=10.0, p=0.004, Figure 4.2b), while there was no main effect of *Fgf2* genotype. There was a trend towards a genotype*stress interaction (F=4.7, p=0.038). Post-hoc comparisons revealed that DCX staining was lowest in the *Fgf2*-/- group exposed to early life stress, see Table 4.2. For BrdU+ staining a similar trend was observed for stress, but this did not reach significance (Figure 4.2c, Table 4.1). Ki-67+ cell density and dentate gyrus surface were not significantly affected by either stress, *Fgf2* background or their interaction (Figure 4.2a and 4.2d).

No differences in dendrite length between the groups

After staining neurons using to the Golgi-Cox protocol, dendrite length and the number of branching points of the dendrites of granular neurons in the dentate gyrus were calculated. No differences in these measures were observed between the groups, see Figure 4.2e and 4.2f.

No difference in indices of excitatory and inhibitory transmission

RNA expression of *Vglut1*, *Gad1* and *Pv*, as measures of glutamatergic, GABAergic and a subset of GABAergic neurons, respectively, was visualized with in situ hybridisations on coronal brain sections. We analyzed the hippocampus and prelimbic cortex given their important roles in cognitive and social behavior. We also included the motor and somatosensory cortex, because previously the effects of *Fgf2* disruption were found throughout the cortex and analysis of these regions was relatively straightforward. The number of *Vglut1*+ cells could not be analyzed in the hippocampus and the number of *Pv*+ cells could not be analyzed in the prelimbic region, respectively due to an abundance or paucity of positive cells. A trend was observed for the number of *Gad1*+ neurons in the somatosensory cortex (stress*genotype interaction F=5.9, p=0.019, Figure 4.3d). Early life stress resulted in an increased *Gad1*+ cell density in the wild type mice, while in *Fgf2*-/- mice early life stress had a reversed effect, see Table 4.2. In other regions and for the other markers, there were no significant differences between the groups (see Figure 4.3 and 4.4).

Figure 4.2 (legend see page 73)

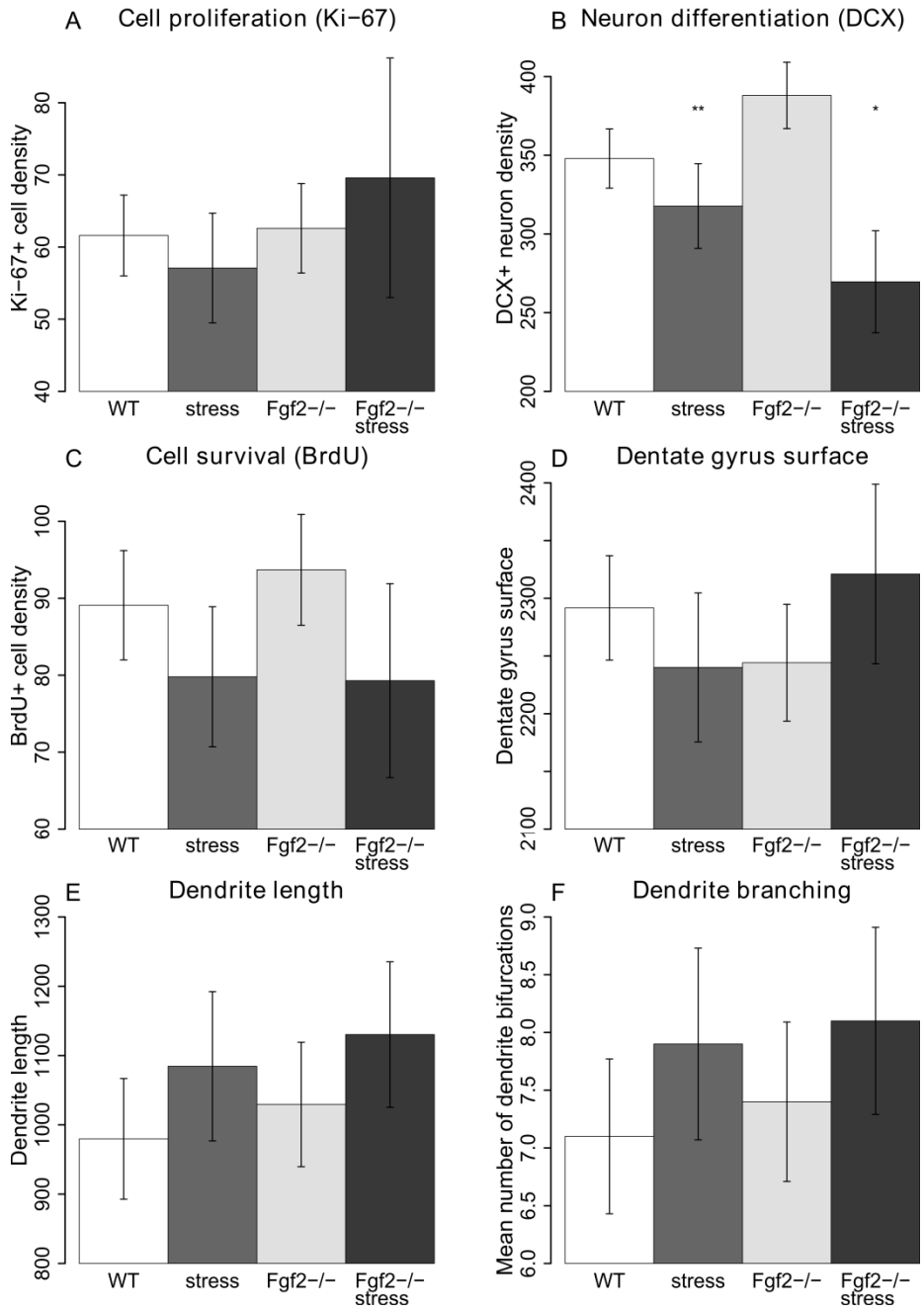

Figure 4.3 (legend see page 73)

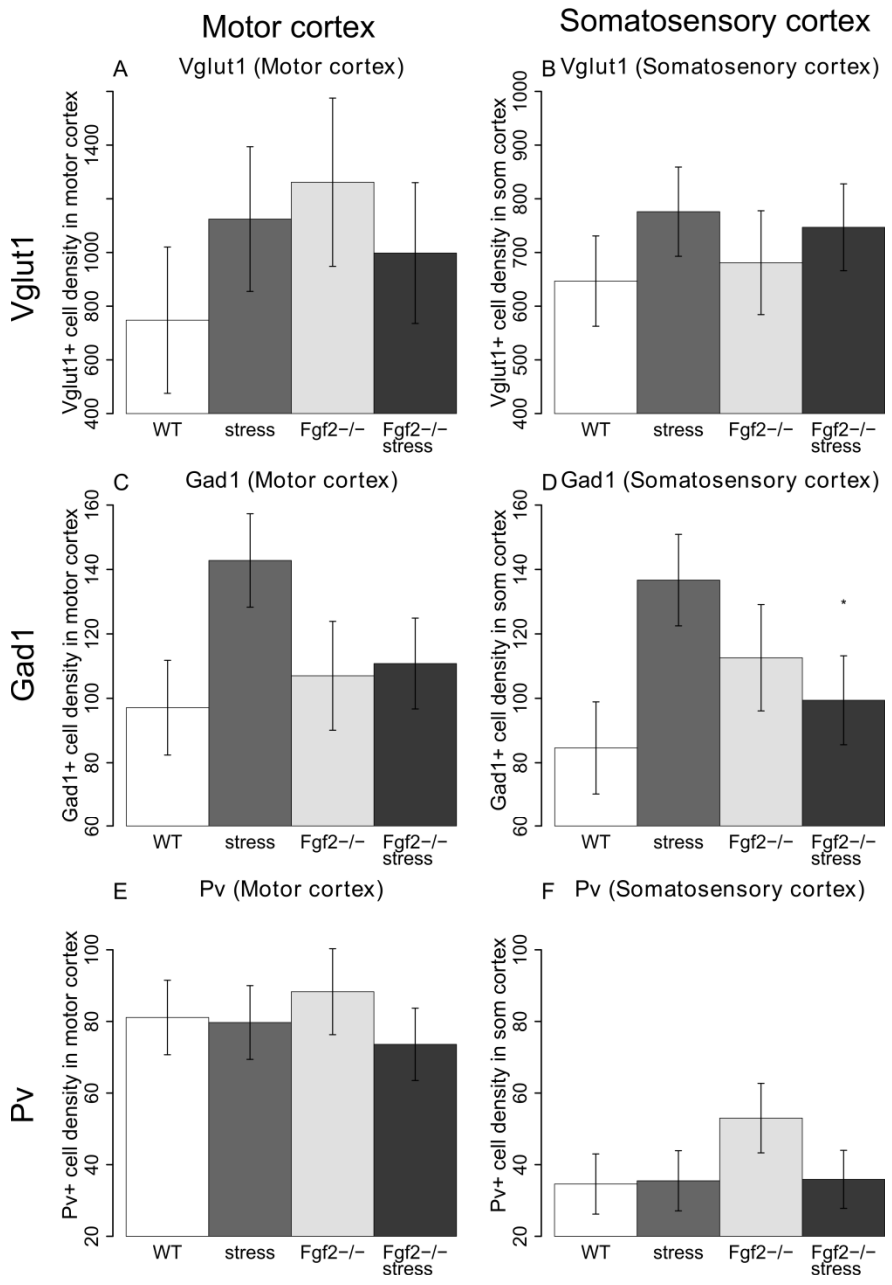

Figure 4.4 (legend see page 73)

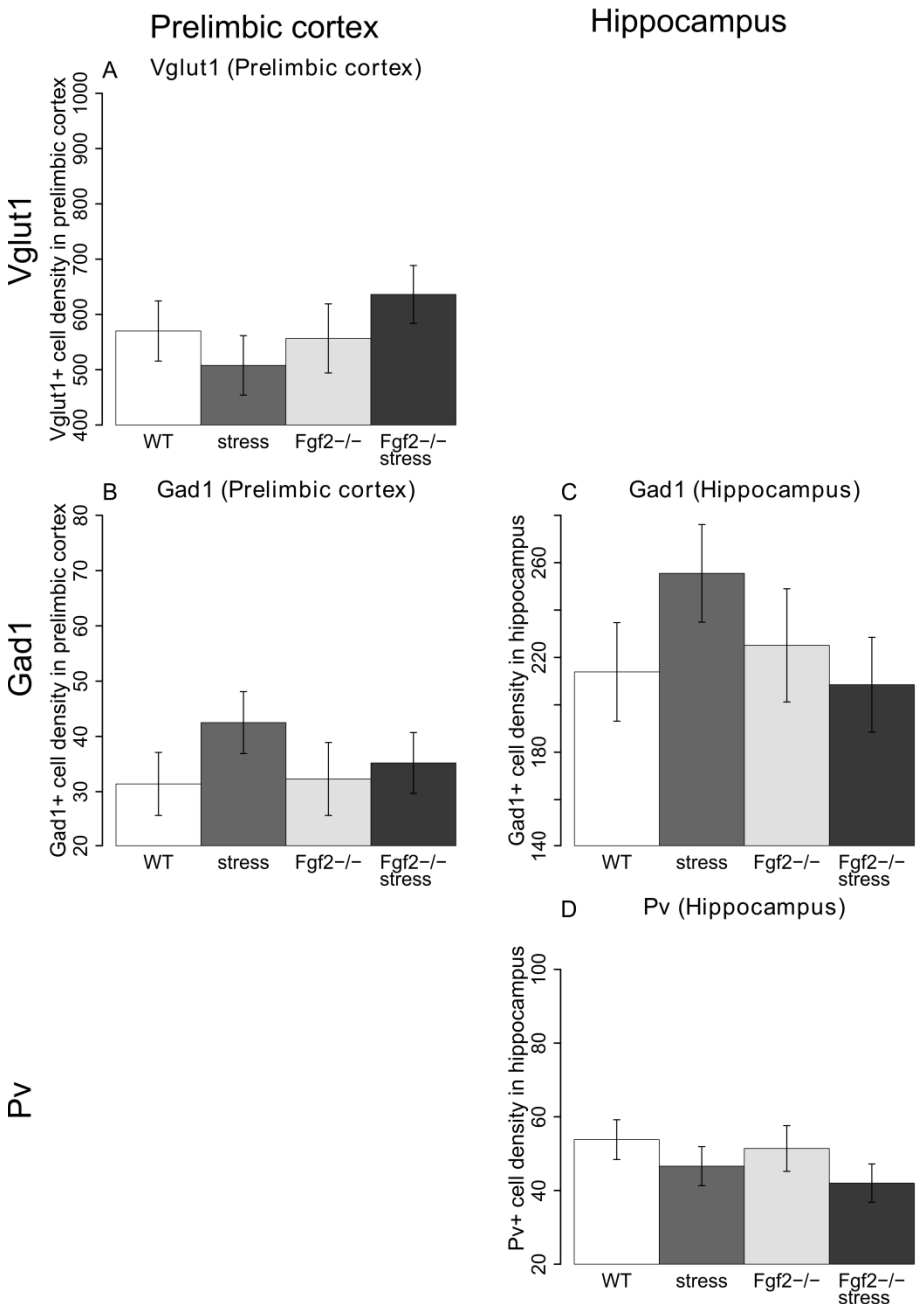

Figure 4.2 (Page 70) Results from tests for neurogenesis and dendritic arborization. All tests were performed in the dentate gyrus (DG) of the hippocampus. Bars represent the means and whiskers the standard error of the means. Ten to fourteen animals per group were analyzed (WT (wild type): 14, stress: 10, *Fgf2-/-*: 11, *Fgf2-/-*stress: 11). ** indicates a p-value <0.007 (significant), * indicates a p-value of <0.05 and >0.007 (suggestive). Early life stress reduced the number of DCX+ neurons (B), a measure of neuronal differentiation. This effect was more pronounced in *Fgf2-/-* mice (stress overall F=10.0, p=0.004, genotype*stress interaction F=4.7, p=0.038, see Table 4.1 and 4.2). There were no significant differences in Ki-67 (A, a measure of proliferation) and BrdU (C, a measure of cell survival) cell densities, nor dentate gyrus surface (D) and in dendrite length (E) or dendrite branching (F) in granule neurons of the dentate gyrus.

Figure 4.3 (Page 71) *Vglut1, Gad1* and *Pv* positive cell densities in the motor cortex (A,C,E) and somatosensory cortex (B,D,F). Bars represent the means and whiskers the standard error of the means. Per experimental group, 14 mice were analyzed. WT = wild type. * indicates a p-value of <0.05 and >0.007 (suggestive). None of these measures showed a significant difference between the groups. The largest effect was found for the interaction between early life stress and *Fgf2-/-* genotype on the number of *Gad1+* neurons in the somatosensory cortex (interaction F=5.9, p=0.019, see Table 4.1 and 4.2), shown in D.

Figure 4.4 (Page 72) No significant differences between the groups in *Vglut1, Gad1* and *Pv* positive neuron densities in the prelimbic cortex (A,B) and the hippocampus (C,D). Bars represent the means and whiskers the standard error of the means. Per experimental group 14 mice were analyzed. WT = wild type. The number of *Pv+* cells in the prelimbic cortex and *Vglut1+* cells in the hippocampus could not be analyzed, respectively due to a paucity and abundance of positive cells.

No differences in memory or fear conditioning between the groups

In view of the effects on neurogenesis, we tested mice in several tasks that involve the hippocampus. No significant effects of genotype or stress on short term and long term memory tests, including social discrimination, object discrimination and object location, were observed, see Figure 5b-d. All groups explored the novel or moved objects more than the familiar objects, indicating sufficient memory function. Post hoc, a trend was observed for a stronger social discrimination in stressed animals, specifically on the long term trial (F=6.309, p=0.015). Furthermore, we tested fear conditioning in a contextual and a cued paradigm (same cage and same sound as during the original fear provoking stimulus, respectively). In the contextual conditioning, a gene*stress interaction was observed at trend level (F=4.77, p=0.032, see Figure 4.5e and Table 4.1). *Fgf2-/-* mice that had received early life stress tended to freeze less, while stress had little effect in wild type mice. In the cued conditioning, early life stress tended to reduce freezing behavior in both wild type and *Fgf2-/-* mice (F=4.58, p=0.036).

Gene by environment interactions on locomotor activity and sheltering behavior

We observed strong effects on locomotor activity during a 5 days continuous recording of home cage behavior (stress F=44.1, $p=2.2x10^{-9}$, genotype F=37.5, $p=8.4x10^{-11}$, stress*genotype interaction F=25.3, $p=6.9x10^{-7}$, see Figure 4.6a and 4.6b and Table 4.1). *Fgf2-/-* mice without early life stress showed a robust increase in baseline locomotor activity; mean distance moved per day was 1.28 times larger on all days. Adaptation (i.e. the sharp drop in activity seen during the first 24 hours) and day-night rhythms were normal. Interestingly, the hyperactive phenotype in *Fgf2-/-* mice normalized when they had been subjected to early life stress, while early life stress did not affect locomotor activity in wild type mice. This normalization appears to be the result of an increase in time spent in the shelter, as *Fgf2-/-* mice subjected to early life stress displayed a significantly increased sheltering time compared to unstressed *Fgf2-/-* mice (see Figure 4.6c and 4.6d). So here too, there is stress*genotype interaction (F=10.7, p=0.001). In *Fgf2-/-* mice, locomotor activity correlated negatively with time spent in the shelter (r^2=-0.54, p=0.025).

In contrast, short term novelty-induced measures of locomotor activity and anxiety were not significantly different between the groups. These include locomotor activity and anxiety (time spent in the outer zone) in the 5 minute open field test (see Figure 4.6e and 4.6f) and anxiety in the 5 minute elevated plus maze test as measured by the time spent in the closed arms, see Figure 4.5f.

***Fgf2-/-* mice are more aggressive independent of stress**

Fgf2-/- mice were more aggressive towards the intruder mice during the social discrimination test (genotype F=14.2, $p=3.9x10^{-4}$, Figure 4.5a). This was observed for *Fgf2-/-* mice with and without early life stress and was present in both the short term and long term tests. Aggression was directed towards the novel and familiar intruders.

Next, we tested the correlation between the aberrant behaviors (aggression and locomotor activity) and the density of excitatory and inhibitory neurons. The correlation with neurogenesis could not be investigated, as these tests were performed in different groups of mice. The time spent in aggressive interaction with the intruder mice during the social discrimination test showed a suggestive correlation with the density in *Gad1*+ cells in the hippocampus (r^2=-0.34, p=0.04), see Figure 4.7a. Post-hoc we observed that the correlation was fully due to a strong correlation between *Gad1*+ cell density and aggression in the *Fgf2-/-* groups (r^2= -0.85, p=0.003), while the effect was insignificant in the other groups. A lower *Gad1*+ cell density was associated with more aggressive behavior. For *Gad1*+ cell density in other regions similar trends were observed, but with smaller effect sizes. Locomotor activity correlated with the density of *Vglut1*+ cells in the motor cortex (r^2=0.47, p=0.006), but this turned out to be due to one outlier in the *Fgf2-/-* group (data not shown).

Figure 4.5 (legend see page 77)

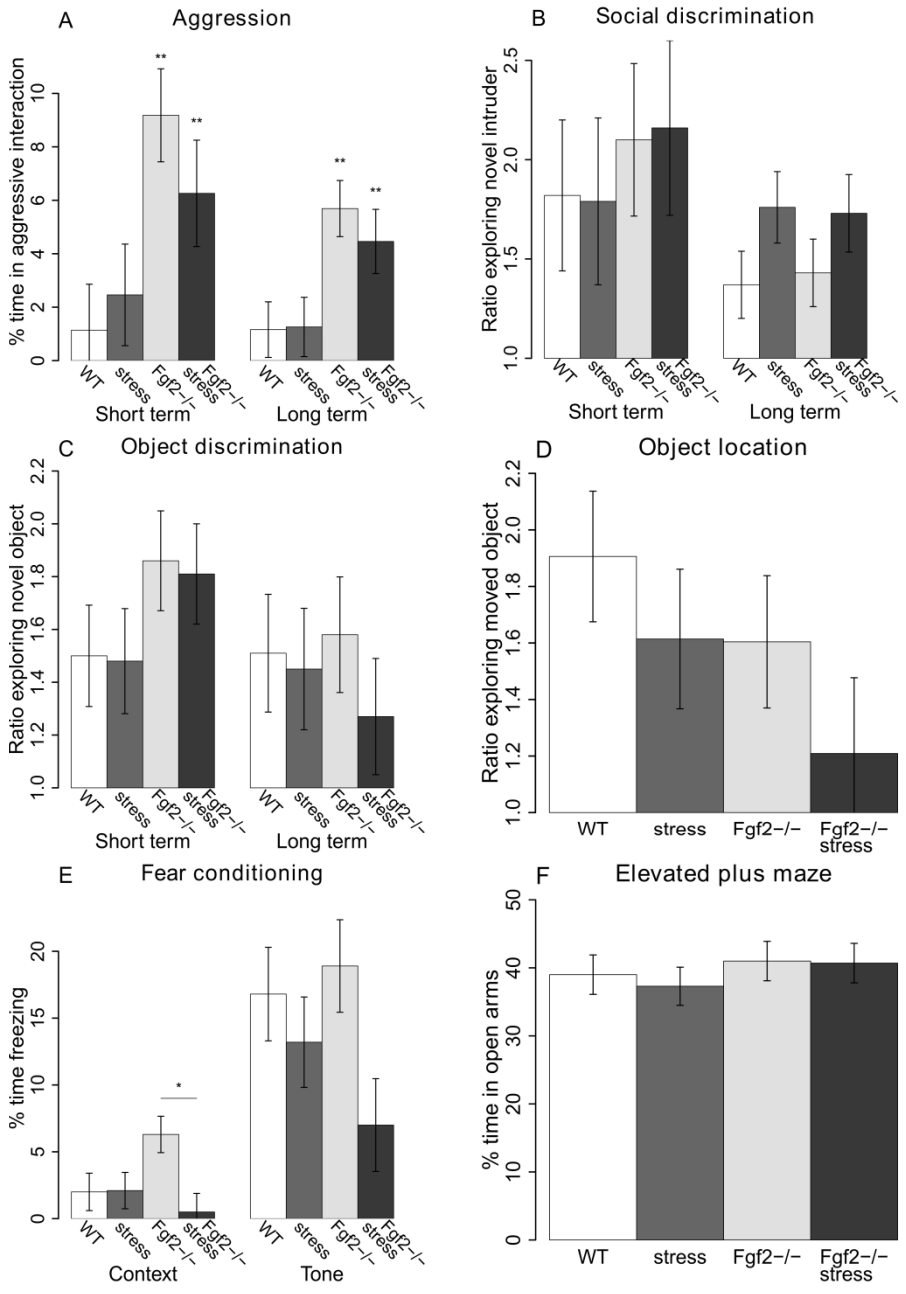

Figure 4.6 (legend see page 77)

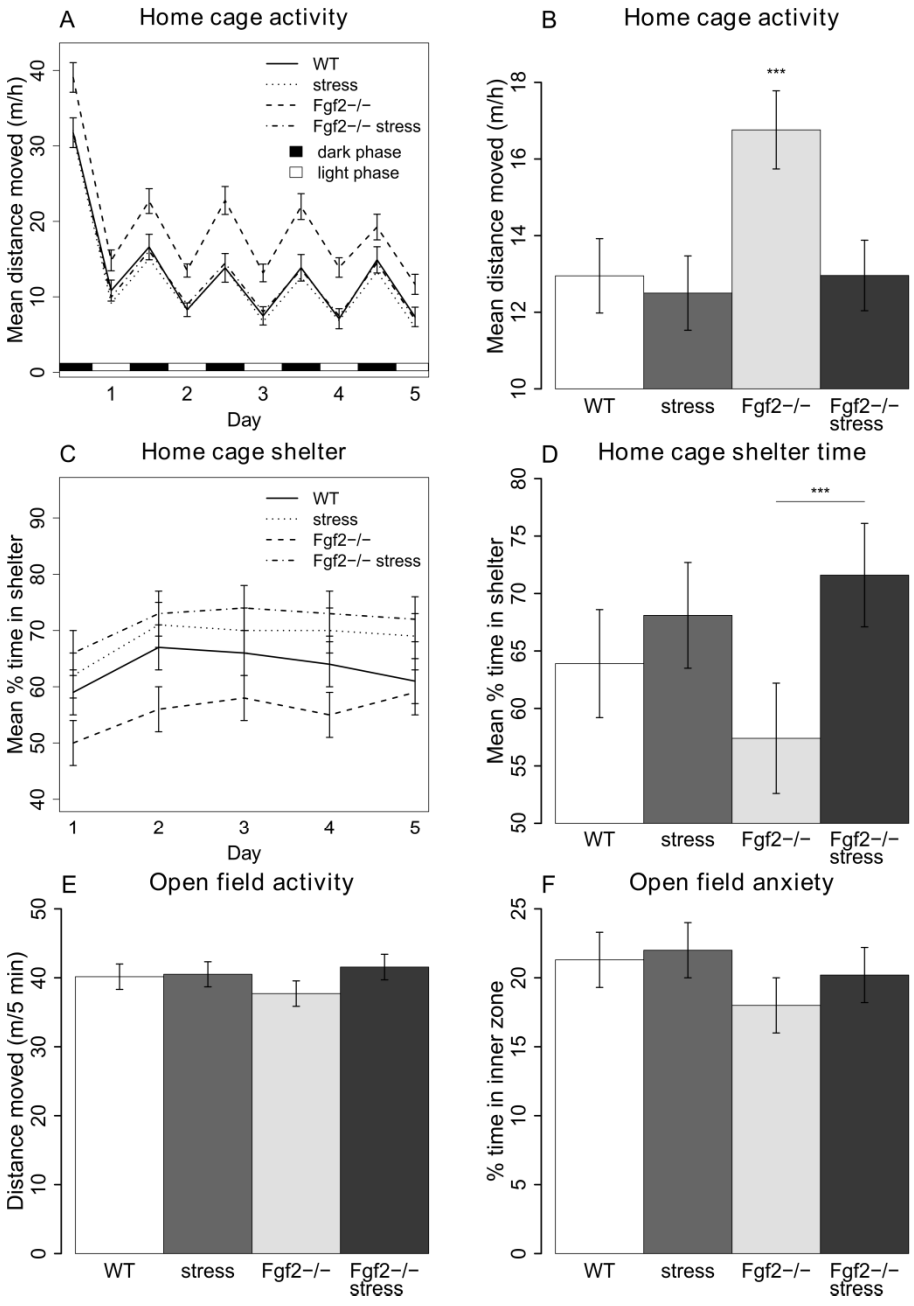

Figure 4.5 (Page 75) Results of SD, OD, OL, FC and EPM behavioral tests. Bars represent the means and whiskers the standard error of the means. Per experimental group, 22 mice were analyzed. WT = wild type. 'Long term' means tested 24 hours after the habituation trial, while 'short term' means tested 5 minutes (**A,B**) or 1 hour (**C**) after the habituation trial. **D** and **E** were only tested after a 24 hour interval. 'Ratio exploring novel intruder' (**B**) is the time exploring the novel intruder divided by the time exploring the familiar intruder. Similar ratios were calculated for novel object (**C**) and moved object (**D**). ** indicates a p-value <0.006 (significant) and * indicates a p-value of <0.05 and >0.006. **A.** *Fgf2-/-* mice with and without early life stress show significantly more aggressive behavior in both the short term and long term trial, genotype F=14.2, $p=3.9x10^{-4}$, see Table 4.1 and 4.2. No significant differences were observed in short term and long term memory tests (**B,C,D**), in fear conditioning (**E)**, nor in the time spent in the open arms of the EPM, a measure of anxiety **(F)**.

Figure 4.6 (Page 76) Locomotor activity and anxiety measures from the 5 x 24 hour home cage (A-D) and 5 minute open field test (E,F). Bars represent the means and whiskers the standard error of the means. Per experimental group, 22 mice were analyzed. WT = wild type, m/h= meter per hour. *** indicates a p-value < 0.0001 (strongly significant, see Table 4.1 and 4.2). In **A.** mean activity per hour measures are lumped together in 12-hour bins representing the dark and light phases. *Fgf2-/-* mice are significantly more active than the other groups on all days (stress F=44.1, $p=2.2x10^{-9}$, genotype F=37.5, $p=8.4x10^{-11}$, stress*genotype interaction F=25.3, $p=6.9x10^{-7}$). **B.** mean activity per hour lumped together over all 5 days. In **C.** percentage time in the shelter per hour, possibly an anxiety measure, is lumped together in 24-hour bins. Early life stress results in a significantly larger increase in the time spent in the shelter in *Fgf2-/-* mice than in wild type mice (F=10.7, p=0.001). **D.** mean time spent in the shelter lumped together over all 5 days. No hyperactivity (**E**) or anxiety (**F**) of *Fgf2-/-* mice was observed in the open field test.

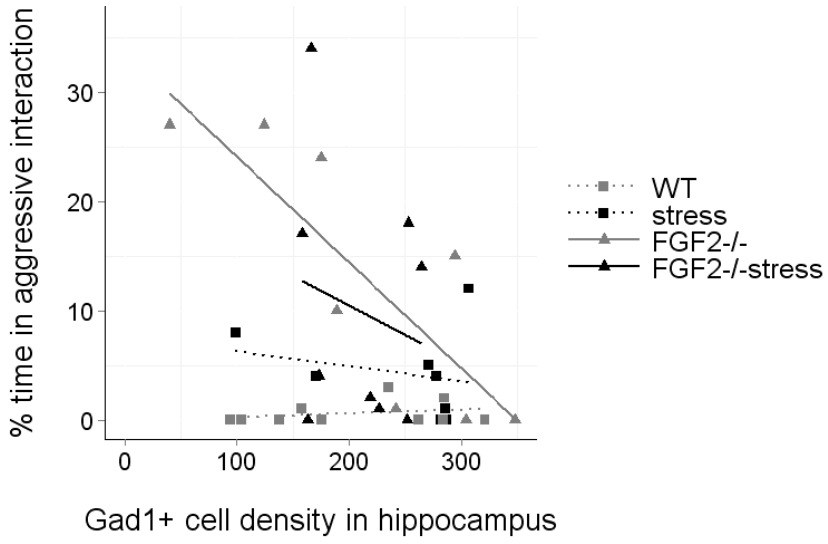

Figure 4.7 Correlations between aggressive behavior and *Gad1+* neuron density in the hippocampus. The correlation is only significant in the Fgf2-/- mice group (r^2= -0.85, p=0.003). Per group, nine mice were included. WT= wild type.

Discussion

We tested the hypothesis that lack of *Fgf2* may provide a vulnerable genetic background to the effects of early life stress, possibly by targeting neurogenesis, neurite outgrowth or the balance between excitatory and inhibitory neurons; and that these processes might exacerbate the long term behavioral outcome of early life stress.

With respect to structural plasticity, we report a significant reduction in DCX+ neuron density (a marker for differentiating young neurons) after early life stress. This stress effect was most pronounced in *Fgf2-/-* mice. *Fgf2* expression appeared to target differentiation rather than other aspects of structural plasticity, as markers for proliferation and cell survival (Ki-67 and BrdU) and dendritic arborization were unaffected by the *Fgf2* background and/or early life environment. Possibly, early differences on those measures might have been compensated by the time the mice are adults.

Previously, reductions in neurogenesis in *Fgf2-/-* mice were observed only after environmental challenges in adulthood, such as ischemia, or administration of kainic acid or 6-hydroxydopamine [232, 266]; only one study found reduced differentiation in (unstressed) *Fgf2* knockout mice [257]. Conversely, administration of FGF2 in the lateral ventricles induced neuron proliferation and differentiation [193, 266]. Together this suggests that FGF2 is pharmacologically effective, but may not be physiologically required for neurogenesis in the hippocampus under unchallenged conditions. Perhaps the lack of FGF2 can be compensated for under 'normal' conditions, as the activation of signaling pathways downstream of the FGF receptors was normal in *Fgf2-/-* mice [195]. Other FGF ligands were not up-regulated in *Fgf2-/-* mice, but perhaps availability of FGF receptor 1 (FGFR1) or alternative ligands of FGF receptors, such as NCAM, is increased [196, 229, 257]. After environmental challenges, including early life stress, this compensation may be insufficient. Nevertheless, the reduced neuronal differentiation in *Fgf2* deficient mice exposed to early life stress indicates a functional relationship of FGF2 signaling with neuronal adaptation in the hippocampus during stressful early life events.

Because changes in hippocampal function and structure are often reflected in altered memory performance [57, 128, 246], one could expect altered memory performance in stressed mice and especially in stressed *Fgf2-/-* knockouts. However, we found no effects of genotype, stress nor interactions on memory tests. The mice used in this study had an outbred Black Swiss background. Black Swiss mice have been reported to show poor memory, high locomotor activity and high aggressiveness compared to other mice strains (A/J and Bl6) [49, 77]. We cannot exclude that this behavioral phenotype of the background strain, in particular the poor memory performance, might have influenced the outcome, possibly due to floor effects.

No major differences in *Vglut1*, *Gad1* or *Pv* expression, as indices for glutamatergic, GABAergic and a subset of GABAergic neuron densities, were observed in *Fgf2* null mutants with or without early life stress. This contrasts with previous studies reporting reduced numbers of glutamate+ and Pv+ neurons in *Fgf2* knockouts [137, 178]. Our results could have potentially been influenced by the behavioral testing that was done on two thirds of the mice used for in situ hybridizations. Importantly though, there was no significant difference between the behaviorally tested and naive mice and there were no significant differences in cell densities between the four groups of naïve mice. We cannot exclude that possible differences appear at levels other than transcripts, such as at the protein level, or in other ways than the mere density of cells. For example, fewer cortical glutamatergic fibers were found to innervate the GABAergic cells of the striatum in *Fgf2* knockouts [72].

Strong genotype by environment interactions were observed for locomotor activity and sheltering behavior. Unstressed *Fgf2* knockouts showed a robust increase in home cage locomotor activity levels, but, interestingly, *Fgf2* knockouts subjected to early life stress displayed normal activity levels. This might be explained by a strong increase in shelter time in *Fgf2-/-* mice subjected to early life stress, as home cage locomotor activity and shelter time are mutually exclusive. Time spent in the shelter might be a measure of stress-induced anxiety, as it was reported to increase acutely after stress and correlated with anxiety measures [58, 248]. This suggests that *Fgf2-/-* mice are particularly sensitive to the long term anxiety inducing effects of early life stress. In contrast to a previous study [72], we did not observe increased activity or anxiety in short lasting tests for anxiety-like behavior (OF and EPM). These findings indicate that the increased home cage sheltering behavior in early life stressed *Fgf2-/-* mice is not induced by novelty, but only becomes apparent in longitudinal and stable home cage environmental conditions.

Earlier, hyperactivity in *Fgf2-/-* mice was reported to correlate with reduced corticostriatal glutamatergic function and increased responsiveness to dopaminergic drugs [72]. Increased locomotor activity was also observed in mice mutant for *Fgfr1* and *Fgfr2* [117, 229]. In *Fgfr1* mutant mice, hyperactivity correlated with a decrease in Pv+ neurons in the cerebral cortex and not with the number of glutamatergic neurons [171]. Thus, locomotor hyperactivity seems to be a general characteristic of disturbed FGF functioning and may be related to glutamatergic, dopaminergic or GABAergic dysfunctioning. Alterations in these neurotransmitters may be related to the number of neurons expressing these neurotransmitters, but also to functional cell properties. Our current data support the latter possibility, because we did not observe significant correlations between the *Vglut1*, *Gad1* and *Pv*+ cell densities and locomotor activity or shelter time.

Regardless of the early life environment, *Fgf2* knockouts displayed increased aggression, indicating that this phenotype is related to the *Fgf2* deficiency rather than to the influence

of early life stress. Aggressive behavior was negatively correlated with *Gad1+* neuron density in the hippocampus of *Fgf2-/-* mice, so perhaps this behavior was induced by reduced GABAergic interneuron signaling. Aggressiveness has not been investigated in *Fgf* mutant mice before, but was reported in *fgfr1a-/-* zebrafish [173]. In these zebrafish, aggressiveness was specifically related to reduced histamine levels throughout the brain and was rescued by administration of histamine H3 receptor agonist imetit [173].

Altogether, disruption of the abundant growth factor FGF2 results in a specific phenotype with increased locomotor activity and aggressiveness and no gross morphological abnormalities or cognitive dysfunction. *Fgf2-/-* mice are more sensitive to the effects of early life stress compared to wild type mice, resulting in increased sheltering behavior and a stronger reduction in hippocampal neuronal differentiation, but surprisingly without apparent signs of impaired contextual memory. Locomotor hyperactivity and aggressiveness can both be considered as impulsive behaviors or signs of impaired behavioral inhibition. Interestingly, impairments in inhibitory control, dysorganized psychomotor behavior and abnormalities in GABAergic and dopaminergic transmission are observed in schizophrenia patients, as well as in other psychiatric disorders such as attention deficit hyperactivity disorder (ADHD). It would therefore be of great interest to specifically target in future studies the possible existence of *Fgf2**stress interactions in frontal areas important for impulsive behaviors and behavioral control.

	Stress		Genotype		Gen*stress	
	F	**P**	**F**	**P**	**F**	**P**
Neurogenesis						
BrdU	2.63	0.12	0.09	0.77	0.15	0.70
Ki-67	0.02	0.89	0.63	0.44	0.68	0.42
DCX	10.02	**4.00×10^{-3}**	0.03	0.86	4.73	**0.038**
Dentate gyrus surface	1.24	0.27	0.53	0.59	2.48	0.10
Golgi						
Dendrite length	0.31	0.59	1.46	0.25	0.64	0.44
Number of nodes	0.00	1.00	1.66	0.20	0.00	0.98
In situ hybridisations						
HIP_gad	0.28	0.60	0.75	0.39	2.21	0.14
HIP_pv	1.80	0.19	0.42	0.52	0.05	0.83
PLC_vglut	0.02	0.89	1.12	0.29	1.92	0.17
PLC_gad	1.17	0.29	0.32	0.58	0.58	0.45
MOT_vglut	0.03	0.86	0.51	0.48	1.56	0.22
MOT_gad	2.19	0.15	0.57	0.45	2.29	0.14
MOT_pv	0.46	0.50	0.00	0.96	0.45	0.51
SOM_vglut	1.04	0.31	0.00	0.98	0.16	0.69
SOM_gad	1.43	0.24	0.11	0.75	5.89	**0.019**
SOM_pv	0.71	0.40	1.25	0.27	1.27	0.27
Behavioral tests						
EPM_durOpen	0.12	0.74	0.90	0.35	0.08	0.79
EPM_closedEntry	0.03	0.86	0.01	0.92	0.07	0.80
HC_distance	44.14	**2.22×10^{-9}**	37.50	**8.41×10^{-11}**	25.33	**6.86×10^{-7}**
HC_shelter	37.44	**1.54×10^{-9}**	0.85	0.36	10.67	**0.001**
OF_distance	1.19	0.28	0.15	0.71	0.95	0.33
OF_durInner	0.51	0.48	1.57	0.21	0.14	0.71
OD_novelratio	0.06	0.81	1.11	0.29	0.82	0.37
SD_novelratio	3.64	0.06	0.01	0.92	0.18	0.67
SD_aggression	0.01	0.95	18.26	**4.26×10^{-5}**	1.17	0.28
OL_moveratio	1.51	0.22	1.22	0.27	0.54	0.46
FC_tone	4.58	**0.036**	0.37	0.54	1.51	0.22
FC_context	4.07	**0.047**	1.03	0.31	4.77	**0.032**

Table 4.1: Result of two-way ANOVAs. The effect of genotype, stress and genotype*stress interaction were all evaluated in one test. For example, for the stress effect this means that unstressed wild type and *Fgf2-/-* animals are compared to stressed wild type and *Fgf2-/-* animals. Testing batch was included as random factor and age at testing (or decapitation) as covariate. An α of 0.05/8 = 0.006 was set as a cut off of significance for the behavioral tests and an α of 0.05/7 = 0.007 for the brain tests. Values in bold and black are significant and values in bold and gray

between 0.05 and 0.006. HIP = hippocampus, PLC = prelimbic cortex, MOT = motor cortex, SOM = somatosensory cortex, for abbreviations of the behavioral tests, see Methods.

	Genotype in unstressed		Genotype in stressed		Stress in WT		Stress in Fgf2-/-	
	F	**P**	**F**	**P**	**F**	**P**	**F**	**P**
DCX	3.50	0.08	1.24	0.30	1.31	0.27	9.05	**0.013**
SOM_gad	3.73	0.07	2.68	0.11	3.82	0.06	0.01	0.93
HC_distance	40.96	**9.06×10^{-10}**	0.58	0.45	3.83	0.05	39.53	**1.09×10^{-8}**
HC_shelter	6.37	**0.012**	6.89	**0.009**	1.55	0.21	57.50	**3.71×10^{-13}**
SD_aggression	14.21	**3.92×10^{-4}**	4.66	**0.035**	3.16	0.08	0.49	0.49
FC_tone	0.08	0.78	2.31	0.14	0.67	0.42	3.92	0.06
FC_context	2.70	0.11	1.52	0.23	0.42	0.52	7.12	**0.011**

Table 4.2: Results of the one-way ANOVAs. While the two-way ANOVAs (Table 4.1) have more power, the results may be biased in some situations, for example when stress has opposite effects in the wild type (WT) and *Fgf2-/-* groups. Therefore, we post hoc tested the genotype and stress effects with one-way ANOVAs comparing two groups at a time when significant results were found in the two-way ANOVAs. Testing batch was included as random factor and age at testing (or decapitation) as covariate. An α of 0.05/8 = 0.006 was set as a cut off of significance for the behavioral tests and an α of 0.05/7 = 0.007 for the brain tests. Values in bold and black are significant and values in bold and gray between 0.05 and 0.006. SOM = somatosensory cortex, HC = home cage, SD = social discrimination, FC= fear conditioning.

Chapter 5

Genetic schizophrenia risk variants jointly modulate total brain and white matter volume

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Abstract

Background Thousands of common single nucleotide polymorphisms (SNPs) are weakly associated with schizophrenia. It is likely that subsets of disease-associated SNPs are associated with distinct heritable disease-associated phenotypes. Therefore, we examined the shared genetic susceptibility modulating schizophrenia and brain volume.

Methods Odds ratios for genome-wide SNP data were calculated in the sample collected by the Psychiatric GWAS Consortium (8,690 schizophrenia patients and 11,831 controls, excluding subjects from the present study). These were used to calculate individual polygenic schizophrenia ("risk") scores (PSSs) in an independent sample of 152 schizophrenia patients and 142 healthy controls with available structural MRI scans.

Results In the entire group, the PSS was significantly associated with total brain volume (R^2=0.048, p=1.6x10^{-4}) and white matter volume (R^2=0.051, p=8.6x10^{-5}) equally in patients and controls. The number of (independent) SNPs that substantially influenced both disease risk and white matter (n=2,020) was much smaller than the entire set of SNPs that modulated disease status (n=14,751). From the set of 2,020 SNPs, a group of 186 SNPs showed most evidence for association with white matter volume and an explorative functional analysis showed that these SNPs were located in genes with neuronal functions.

Conclusions These results indicate that a relatively small subset of schizophrenia genetic risk variants is related to the (normal) development of white matter. This in turn suggests that disruptions in white matter growth increase the susceptibility to develop schizophrenia.

Introduction

Schizophrenia is a disabling mental disorder with a heritability of around 80% [44]. The Psychiatric Genomics Consortium (PGC) recently published a large genome-wide association study (GWAS) on schizophrenia [200]; this analysis of 17,836 cases and 33,859 controls yielded seven loci with common alleles that subtly increase schizophrenia risk. However, there are most likely many more single nucleotide polymorphisms (SNPs) involved in schizophrenia susceptibility: Purcell and colleagues described the additive effects of thousands of disease-associated SNPs combined into a single polygenic schizophrenia score (PSS) [190]. This PSS based on >30,000 (mostly independent) SNPs explained around 3% of the variance in schizophrenia in an independent sample. Another recent study estimated that 23% of the variation in liability to schizophrenia is captured by the combined effect of >900,000 SNPs [147]. These data support a complex mode of inheritance, with thousands of genetic variants of small effect contributing to disease. This large genetic heterogeneity is further complicated by substantial variation in clinical presentation, disease course and associated phenotypes. It is likely that subsets of disease-associated SNPs are associated with distinct heritable disease-associated phenotypes (also called endophenotypes [93]).

One such phenotype is brain volume and it is well suited to be linked to disease associated SNPs. Brain volume is robustly associated with schizophrenia, with average reductions in total brain volume of about 3% in schizophrenia patients compared to healthy individuals [111, 258]. It is highly heritable in healthy subjects as well as in schizophrenia patients [9, 43, 89, 103] and reduced brain volumes are inherited together with illness in families [24]. In fact, the largest twin study (n=684) to date recently reported that 77% of the phenotypic overlap between schizophrenia and total brain volume was of genetic origin [103], with white matter loss in schizophrenia patients largely (94%) attributable to genetic factors (although gray matter volume was determined by unique and common environmental factors [103]). Thus, white matter volume is an excellent candidate endophenotype to be linked to schizophrenia-associated SNPs.

The aim of the current study was to investigate the combined effect of schizophrenia-associated loci on brain volume in order to answer several questions. First, is brain volume in schizophrenia patients indeed determined by disease-associated SNPs, and if so, by which proportion of these SNPs? Second, do disease-associated SNPs affect brain volume in patients only, or do they modulate brain volume in general? Finally, is the involvement of genetic factors on white matter volume in particular, as previously suggested by heritability calculations from twin studies, supported by genotype data?

Methods and materials

Discovery sample

Data from the Schizophrenia Psychiatric GWAS Consortium (PGC) was used as a discovery sample to identify the schizophrenia risk variants, their p-values and odds ratios. Analysis and quality control was performed according to the consortium's standards [200]. All subjects from the PGC sample were included, except for the 1,342 cases and controls from our own schizophrenia genome-wide association study (UCLA/UMC Utrecht).

Target sample

The target sample consisted of 152 schizophrenia patients and 142 controls with available magnetic resonance imaging (MRI) data. Subjects were included for the Genetic Risk and Outcome of Psychosis (GROUP) study (n=162) [87] and a study described previously [101] (n=132), performed in the University Medical Centre Utrecht. For both patients and controls psychopathology was assessed using the Comprehensive Assessment of Symptoms and History [4]. Of the target sample 138 subjects were diagnosed with schizophrenia and 14 with schizo-affective disorder. Unaffected subjects had no history of psychiatric illness except for four subjects who had a history of depressive disorder, anxiety disorder, obsessive-compulsive disorder and adjustment disorder respectively. None of the control subjects had first-degree family members with psychotic illness.

MRI analysis

Brain images were acquired on either a Philips NT or a Philips Achieva scanner at 1.5 Tesla. Scanner type showed no main effect or interaction effect with disease status on total brain and white matter volume (after correction for age, gender and intracranial volume). Gray matter volumes were slightly lower with the Philips Achieva scanner (mean 615.0 ml (sd 25.0) versus 624.3 ml (sd 26.7), p=0.002). Correcting for scanner type did not influence the results. MRI acquisition and processing methods have been previously described [25, 111]. Post-processing was done on the neuro-imaging computer network of the Department of Psychiatry at the University Medical Centre Utrecht. All images were coded to ensure blindness for subject identification. Scans were put into Talairach frame (no scaling), and corrected for inhomogeneities in the magnetic field [211]. Volume measures of the intracranium, total brain, cerebral gray and white matter were determined. Quantitative assessment of the intracranial volume was performed with use of a full-automated computer program based on histogram analyses followed by mathematical morphological operators in either the DE-TSE image (NT) or a single-shot echo planar imaging scan (as part of a diffusion tensor imaging series) together with a magnetization transfer imaging scan (Achieva). All intracranial segmentations were visually checked and corrected where necessary. Quantitative assessment of the total brain, gray and white matter volumes

were performed based on histogram analyses followed by mathematical morphological operators in the 3D-FFE image, using the intracranial volume as mask [31].

Genetic analysis

Subjects in the target sample were genotyped at UCLA Neurosciences Genomics Core (UNGC) using the Illumina HumanHap550 beadchip. Initial quality control was performed by the PGC, removing individuals with more than 5% missing SNPs or with evidence of more than random genetic similarity (c.q. distant relatedness) and SNPs on chromosomes X, Y and mitochondrial DNA. Only SNPs genotyped in the target sample were included (in the discovery sample part of the SNPs were imputed due to the use of different genotyping platforms). These SNPs were filtered based on minor allele frequencies of less than 0.02 (removing 4,528 SNPs) and >1% missing genotypes per SNP (7,552 SNPs). There was no evidence of deviation from Hardy-Weinberg equilibrium with $p< 1x10^{-6}$, non-random genotyping errors with $p< 1x10^{-6}$, such as systematic batch effects. There was a marginally increased call rate in patients compared to controls (e.g. for SNPs with a p-value for association with schizophrenia <0.01 : mean genotyping rate in patients: 4,038/ 4,040 versus 4,036/4,040 in controls, p=0.02). To remove all SNPs in linkage disequilibrium, SNPs were pruned based on a pair wise r^2 threshold of 0.25 and a sliding window of 50 SNPs wide, shifting 5 SNPs at each step, using PLINK [189]. In this way another 341,261 SNPs (74.3%) were removed, leaving 117,924 SNPs for analysis.

Statistical analysis

Measures of total brain, cerebral gray and white matter volume were corrected for the covariates age, sex and intracranial volume by taking the unstandardized residuals of the volumes using linear regression in the total group. For each subject the unstandardized residual was added to the intercept + $beta_i$*$mean_i$, where i represents the different covariates. Intracranial volume explained a large part of the variation in brain volume, resulting in a correlation between the uncorrected and corrected brain volumes of 0.36. Intracranial volumes were corrected for age and sex in a similar way. All corrected brain volume measures were normally distributed in the total group and in the patient and control groups separately.

For each individual in the target sample a polygenic schizophrenia score (PSS) was calculated using PLINK. For each SNP, the number of 'risk variants' an individual carried (0, 1 or 2) was multiplied by the logarithm of the odds ratio for that particular variant. 'Risk variants' are the alleles (nominally) associated with disease, including both true risk alleles and stochastic variation. Sets of SNPs with p-values below different cutoffs for effect on schizophrenia (p-value cutoffs for effect on schizophrenia or Pcutoff-SZ) were defined. First, the following Pcutoff-SZ were used: 0.01; 0.06; 0.1; 0.2; 0.3; 0.4 and 0.5. When the largest R^2 values were found at relatively low cutoffs, we added smaller Pcutoff-SZ (0.002; 0.004; 0.006; 0.008; 0.02; 0.04 and 0.08). The score was summed over all SNPs in the

Pcutoff-SZ-SNP sets for each individual in the target sample to obtain the individual polygenic scores.

We first performed a logistic regression with disease status as dependent (outcome) variable and subsequently performed linear regressions using total brain, gray matter and white matter volumes as dependent (outcome) variables. Sex and intracranial volume were analyzed as negative controls. Total brain volume and intracranial volume develop at a similar rate until the age of 14, after which intracranial volume is practically stable [54]. Total brain volume corrected for intracranial volume thus represents the brain volume changes in late adolescent and adult life. Because intracranial volume is not substantially different between schizophrenia patients and healthy controls, it is not likely to be influenced by the PSS. A full model with the PSS and the covariates was compared with a model only containing the covariates, to investigate whether PSS significantly improved phenotype prediction. We compared the difference in R^2 adjusted for the number of predictors in the model, a measure of the variance explained. For disease status Nagelkerke's pseudo R^2 was used. Ten population stratification dimensions were used as covariates in the analyses as measures of hidden genetic population substructures. These dimensions were calculated in PLINK based on identity-by-state pair wise distances between individuals using the multidimensional scaling plot option.

Other covariates were the number of non-missing SNPs used for scoring, the inbreeding coefficient (the ratio of the observed versus expected number of homozygous alleles, calculated in PLINK) and the number of copy number variants (CNVs). For a description of CNV calling see [38]. For the brain volume analyses we subsequently included disease status and the interaction between disease status and PSS in the analyses. P-values < 0.008 were considered significant (α 0.05/6 phenotypes (disease status, total brain, white matter, gray matter and intracranial volume and sex), not corrected for the different Pcutoff-SZ, since these are highly correlated).

Functional analyses

Refseq genes within 100kb from the SNPs associated with both schizophrenia and white matter (n=186) were identified. These genes were functionally clustered using the database for annotation, visualization and integrated discovery (DAVID) [256]. This database uses functional annotations, including gene ontology (GO) terms, KEGG pathways and Biocarta pathways to cluster genes into biological meaningful groups. These settings were used: high classification stringency and minimal 4 genes per group.

Results

For demographic information see Table 5.1. While there were significantly more males in the patient group than in the control group, brain volumes were corrected for sex and sex chromosomal SNPs were removed in all analyses. As expected, patients showed on average smaller brain volumes. They also received fewer years of education, most likely due to their illness. The level of parental education, an estimate of socioeconomic status, was not different between the patient and control groups. There was a good prediction of disease status in the target sample by the PSS based on the PGC sample (with a maximum R^2=0.046 (increase in R^2 after adding PSS to the model), p=0.001, at Pcutoff-SZ 0.1 (14,751 mostly independent SNPs, see Figure 5.1), indicating that the polygenic score is a reliable measure of schizophrenia risk. Testing this association in the full GWAS sample from Utrecht (n= 1,342 subjects, adding all subjects for which no MRI data was available) resulted in a similar variance explained (R^2=0.035), as expected with much smaller p-values ($4.0x10^{-7}$, data not shown).

The polygenic schizophrenia score was significantly associated with total brain volume at different Pcutoff-SZ, irrespective of disease status (with a maximum at Pcutoff-SZ 0.006: R^2=0.048, p=1.6x10-4, see Figure 5.1 and Supplementary Table I). The association was in the expected direction: higher genetic risk scores were associated with smaller total brain volumes. When including disease status in the analysis, the effect of PSS on brain volume remained significant (R^2=0.038, p=0.001) and there was no significant interaction between PSS and disease status, indicating that the association was similar in the patient and control groups. PSS was specifically associated with reduced white matter volume (R^2=0.051, p=8.6x10-5 at Pcutoff-SZ 0.006) and did not explain variance in gray matter volume (R^2=0.002, p=0.232 at Pcutoff-SZ 0.006). As expected, no association was found between PSS and either sex or intracranial volume, which can be regarded as negative controls. From graphical inspection of the data we concluded that there were no outliers in brain volume or in PSS that could significantly influence the results.

	Schizophrenia patients	Healthy controls	Significance
n	152	142	
Gender (m/f)	121/31	79/63	$p=1x10^{-5}$
Age in years (sd)	32.0 (10.9)	32.3 (12.2)	ns
Handedness (r/l/ambidexter /unknown)	120/13/5/14	115/14/4/9	ns
Level of education in years	12.2 (2.5)	13.9 (2.9)	$p=1.1x10^{-7}$
Level of parental education in years	13.2 (3.1)	13.2 (3.2)	ns
Age of first psychotic symptoms in years (sd)	22.5 (5.4)	na	
Duration of illness in years (sd)	8.5 (9.6)	na	
Duration of untreated illness in years (sd)	1.4 (2.7)	na	
Duration of treatment with medication (sd)	7.1 (10.1)	na	
Intracranial volume in ml (sd)**	1671.3 (116.1)	1683.6 (118.0)	ns
Total brain volume in ml (sd)*	1492.0 (55.0)	1513.0 (43.4)	$p=3.5x10^{-4}$
Gray matter volume in ml (sd) *	617.0 (27.6)	623.5 (24.6)	p=0.035
White matter volume in ml (sd) *	502.6 (34.3)	515.72 (32.0)	p=0.001

Table 5.1: Demographic information. sd=standard deviation, ns=not significant, na = not applicable. Level of parental education is a measure of socioeconomic level. * = corrected for age, sex and intracranial volume. ** = corrected for age and sex

While the 14,751 SNPs with p-values up to 0.1 (for effect on schizophrenia) substantially contributed in explaining variation in disease status, a much smaller subset of 2,020 schizophrenia risk variants explained most variance in white matter volume (with Pcutoff-SZ 0.01). Within this set of 2,020 mostly independent SNPs, which were selected based on their association to schizophrenia, we explored which SNPs contributed most to the effect on white matter volume. SNPs were tested for their individual effect on white matter volume (in the same target sample). With a 'white matter polygenic score' analysis we selected an optimal p-value cutoff. White matter p-values were used to construct sets of SNPs with p-values below different cutoffs (p-value cutoff for white matter, or Pcutoff-WM): 0.06; 0.08; 0.1; 0.2; and 0.3. Of these sets of SNPs, the set with Pcutoff-WM 0.1 jointly explained most variance in white matter volume compared to the other Pcutoff-WM. Therefore we included SNPs with white matter p-value < 0.1 for further analyses. This small subset of 186 SNPs explaining variance in both schizophrenia and white matter volume thus represents the most important SNPs in determining the genetic overlap between the two phenotypes. Together these 186 SNPs explain 0.7% of the variance in disease status, which is a relatively large part (i.e. 9.2% of 2,020 SNPs explain 35% (0.007/ 0.02) of the variance explained by these 2,020 SNPs. This set included one of the ten SNPs with genome-wide significance in the PGC sample (rs17662626 near PCGEM1).

Next, we investigated the type and function of the 375 genes located within 100kb on either side of these 186 SNPs. An overview of these genes can be found in supplementary table II. Genes can be grouped in 7 functional clusters (Table 5.2): immunoglobulin-like cell-adhesion molecules, signal peptides, zinc fingers, ion channels, (neurotransmitter) receptors, WD repeat proteins and transcription factors. The cluster of immunoglobulin-like cell-adhesion molecules is enriched compared to the reference database, having enrichment scores of 1.42 (an enrichment score of >1.3 is regarded as significant enrichment [256]). The most prevalent gene ontology (GO) categorie is cell-cell adhesion (GO:0016337, p=0.0007). However, after correction for testing multiple GO categories, this is not significantly enriched compared to the reference database. Similar results were found with flanking regions of 10kb (data not shown).

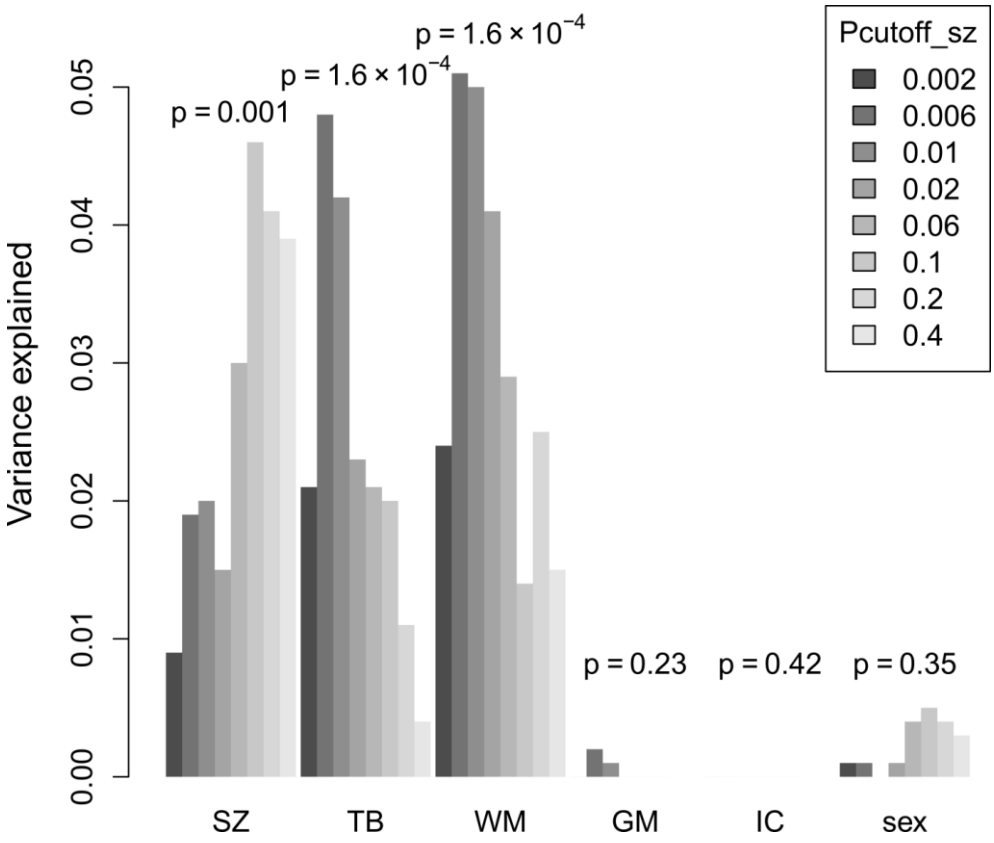

Figure 5.1: The variance explained of different phenotypes by PSS for different Pcutoff-SZ SNP sets. SZ= schizophrenia, TB= total brain volume, WM= white matter volume, GM= gray matter volume, IC= intracranial volume. y-axis = explained variance by the PSS of this phenotype. For dichotomous traits Nagelkerke's pseudo R^2 was compared between a model with only covariates and a model including covariates and the PSS. For continuous traits the difference in R^2 was used. Intracranial volume and sex were included as negative controls. For more information see supplementary table I.

Clustername	Genes	Enrichment score
Immunoglobulin-like cell-adhesion molecules	LSAMP, SIGLEC1, BOC, CNTN4, IGSF8, IGSF9, SLAMF9, CEACAM16	1.42
Signal peptides	TMEM18, TMEM59L, TMEM74, TMEM139, TMC2, TMC5, GPR157, CEACAM16, SLAMF9, SLC35F1, TMCC3, SLC37A2, CHODL, KIAA0319, LRTM1, TAS2R40, C16orf62, C7orf44, GPM6A, LRP3, NKAIN2, CEACAM19	1.23
Zinc fingers	ZDHHC7, MTF2, SMYD2, TRIM10, TRIM15, TRIM26, TRIM31, TRIM40, RNF39, DEF8, ZC3H14	1.02
Ion channels	CACNA2D3, KCNJ9, KCNJ10, KCNAB2, TRPM3, ACCN2	0.93
(Neurotransmitter) receptors	GRM3, GABRB2, HTR1F, ADCYAP1R1, TAS2R40, PTGER1, GPR157	0.91
WD repeat proteins	WDR17, WDR52, WDR88, EML5, PPP2R2C, KIAA1239	0.82
Transcription factors	ASXL3, TCF4, TCF20, HIVEP2, FOXI1, CRTC1, MTF2, EBF4, ZNF23, ZNF100, ZNF707, ESRRG, GLIS3	0.55

Table 5.2: Clusters of genes selected for variance explained in both schizophrenia and white matter volume. An enrichment score of >1.3 is considered a significant enrichment compared to a reference database.

Discussion

We investigated the combined effect of schizophrenia-associated SNPs on brain volume, a highly heritable phenotype associated with this disease. Schizophrenia-associated SNPs explained around 5% of the variance in total brain and white matter volume, in patients as well as in healthy controls. This effect was largely exerted by only a fraction (n=2,020) of all SNPs with effect on disease status (n=14,751). Our data not only suggest that a relatively small subset of all schizophrenia-associated variants is related to white matter development, but also that disruptions in white matter development contribute to schizophrenia in susceptible individuals. Disease status was successfully predicted by the PSS, indicating that the genetic risk variants do indeed reflect schizophrenia risk in this smaller sample. Our finding that genes regulating white matter development are relevant to schizophrenia is consistent with our earlier report that the phenotypic overlap between schizophrenia and white matter volume is almost completely explained by genetic factors [103]. Indeed, diffusion tensor imaging (DTI) studies show that abnormalities in white matter integrity are present before the onset of schizophrenia [224], while increased density and altered distribution of white matter neurons [52] and a reduction in oligodendrocyte numbers have repeatedly been found in post mortem brains of schizophrenia patients [224]. Taken together, these data are consistent with a model in which genetic risk variants increase schizophrenia vulnerability through aberrant development of brain connectivity [108]. Transition to the disease proper most likely occurs in interaction with other genetic or environmental risk factors.

Another important observation is that a much smaller number of common variants appears to determine the overlap between schizophrenia and brain volume than the entire set that modulates schizophrenia. While almost 15,000 SNPs with Pcutoff-SZ up to 0.1 substantially contributed to the variance explained in disease status, only a little over 2,000 SNPs with Pcutoff-SZ up to 0.01 contributed to the variance in white matter volume. In fact, the variance explained in white matter volume *decreased* when adding more SNPs (with higher p-values for association to schizophrenia). This is in agreement with a model in which only a subset of the schizophrenia-associated SNPs influences brain volume. In this case, adding extra SNPs *with* effect on schizophrenia, but *without* a substantial effect on brain volume (OR>1, logOR>1) changes the PSS so that the variance explained in brain volume is diminished at higher cutoffs. These results suggest a specific subset of the disease-associated SNPs that is related to brain volume. A schematic representation of these subsets is shown in Figure 5.2.

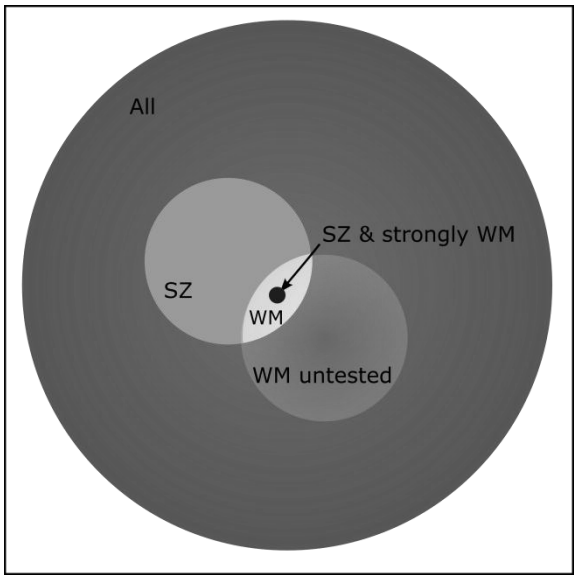

Figure 5.2: Schematic representation of the SNPs involved in different phenotypes. The large green circle represents all 117,924 SNPs included after quality control and removing SNPs in LD. The yellow circle represents the 14,751 SNPs having the largest effect on schizophrenia in the target sample. The small white area stands for the subset of these SNPs (n=2,020) that do also explain variance in white matter volume. There could be more SNPs influencing white matter volume (represented by the translucent white circle), but these were not investigated. The small blue circle represents the 186 SNPs who are likely to contribute most to both schizophrenia and white matter volume. The size of the circles represents the number of SNPs in that group.

Perhaps there are other SNPs with more subtle effects on white matter volume, also outside the 2,020 SNPs set, but their effect might easily be overshadowed by the relatively large amount of SNPs without effect on white matter volume. Since only 2% of the 2,020 SNPs were located in the HLA region, these are unlikely to have confounded the analyses. Applying the same strategy to other phenotypes related to schizophrenia, such as cognitive dysfunction, dopamine receptor binding, or other suitable endophenotypes, could similarly result in subsets of candidate variants that help elucidate the biology of the disorder. The same method can obviously be applied to other diseases and their endophenotypes.

Several other points are important to address. First, only a small amount of the total variance in brain volume and disease status is captured by the PSS. This is comparable with data reported previously [190] and can be explained by incomplete capture of rare variants and gene-gene interactions, among others [147]. Using a polygenic score method based on common variants, we cannot aim to explain the majority of the variance in brain

volume. Second, since reductions in gray matter volume are commonly reported in schizophrenia patients [111, 258], it might seem remarkable that the PSS is not associated with gray matter volume. However, a recent study indicates that in schizophrenia patients gray matter volume is mostly determined by unique environmental factors (explaining 43% of the variance) [103]. This is in contrast to white matter volume, which has a much stronger genetic component (63%). Our data provide genetic support for the observation in this twin study. Furthermore, brain volume can be influenced by environmental factors such as the use of psychotropic medication [169]. The association between the PSS and brain volume was found not only in the total group, but also within the control group. Since psychotropic medication is used by patients only, it is unlikely that the observed effect could be explained for a substantial part by the use of medication. Because medication use is unreliably assessed in retrospect, we did not include it in the analyses. Lastly, the results do not imply that brain volume is influenced by a smaller number of common variants than schizophrenia. The effects of SNPs that do influence brain volume, but not schizophrenia, are not captured by the PSS.

Among the 2,020 independent schizophrenia risk variants explaining most variance in white matter volume, 186 SNPs were shown to affect white matter volume most strongly. We functionally clustered the 375 genes located near these 186 SNPs in order to generate hypotheses about the biological processes affected by these variants. Functional clustering of the genes should be regarded as explorative, and should be interpreted with caution, since these analyses are biased towards the effect of larger genes and well-investigated pathways [64]. Besides, functional relationships between SNPs and nearby genes are often assumed, but not necessarily present. Still, the identified gene clusters have highly relevant functions. The significantly overrepresented cluster of immunoglobulin-like cell adhesion molecules contains proteins that are involved in axon guidance or cell-cell interactions [71]. LSAMP is expressed at dendrites of neurons in cortical and subcortical regions of the limbic system and mediates neurite outgrowth [17]. A significant increase in LSAMP expression was found in the dorsolateral prefrontal cortex of patients with schizophrenia and bipolar disorder [17]. Contactin 4 (CNTN4) regulates neural network formation and has previously been implicated in autism and anorexia nervosa [53, 253]. Also other clusters contain many genes with relevant, neuronal functions, such as the receptors for glutamate, GABA and serotonin, which have been previously implicated in schizophrenia [123]. Each of these genes may make relevant targets for further research.

To conclude, these results indicate that a relatively small subset of schizophrenia genetic risk variants is related to the (normal) development of white matter. This in turn suggests that disruptions in white matter growth increase the susceptibility to develop schizophrenia.

Chapter 6

Schizophrenia genetic variants are not associated with intelligence

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Abstract

Background Schizophrenia is associated with lower premorbid intelligence (IQ) as well as (premorbid) cognitive decline. Both schizophrenia and IQ are highly heritable traits. Therefore, we hypothesized that genetic variants associated with schizophrenia, including copy number variants (CNVs) and a polygenic schizophrenia risk score (PSS), influence intelligence.

Methods IQ was estimated with the Wechsler Adult Intelligence Scale. CNVs were called with QuantiSNP and PennCNV. For the PSS, odds ratios for genome-wide SNP data were calculated in a sample collected by the Psychiatric GWAS Consortium (8,690 schizophrenia patients and 11,831 controls). These were used to calculate individual PSSs in our independent sample of 350 schizophrenia patients and 322 healthy controls.

Results While significantly more genes were disrupted by deletions in schizophrenia patients compared to controls (p=0.009), there was no effect of CNV measures on IQ. The PSS was associated with disease status (R^2 = 0.055, p = 2.1 x 10^{-7}) and with IQ in the entire sample (R^2 = 0.018, p = 0.0008), but the effect on IQ disappeared after correction for disease status.

Conclusions Our data suggest that rare and common schizophrenia-associated variants do not explain the variation in IQ in healthy subjects nor in schizophrenia patients. Thus, reductions in IQ in schizophrenia patients may be secondary to other processes related to schizophrenia risk.

Introduction

Schizophrenia is a severe mental disorder with cognitive deficits as a core feature [66, 142]. It has been associated with lower premorbid general intelligence (IQ) [81, 125], with cognitive decline prior to illness onset [198] and (although this is controversial) cognitive decline during the course of the illness [106]. Both schizophrenia and IQ are largely determined by genetic factors, with estimated heritabilities of 80% [44] and 70% [81], respectively. Several studies have identified genetic variants that affect these respective phenotypes. For schizophrenia, the findings include an excess of *de novo* copy number variants (CNVs, genomic microdeletions or microduplications from 1kb to multiple Mb in size) [215, 250, 262] and gene-containing CNV deletions [38]. Moreover, ten individual single nucleotide polymorphisms (SNPs) have been associated with schizophrenia in genome-wide association studies (GWAS) [200], while the combination of many (>900,000) SNPs captures 23% of the variation in liability to develop schizophrenia [147]. Similarly, rare CNVs are associated with general intelligence [265] and mental retardation [96]. While no large GWAS on intelligence in healthy subjects has been reported, the combined effects of multiple SNPs captures 48% of the variation in intelligence in childhood [59]. These data suggest that both common and rare alleles contribute to schizophrenia disease susceptibility as well as to general intelligence.

It is likely that subsets of schizophrenia-related SNPs are associated with distinct heritable disease-associated phenotypes. These are called endophenotypes when they are 1. associated with the illness, 2. heritable, 3. primarily state independent, 4. cosegregate with illness in families [93]. Brain volume is an example of an endophenotype of schizophrenia, since it is robustly associated with disease [258], is highly heritable [103] and cosegregates with illness in families [24]. It is an objectively assessed quantitative measure. We previously showed that a combined score of common variants associated with schizophrenia (polygenic schizophrenia score or PSS) significantly predicts total brain and white matter volume in schizophrenia patients and in healthy subjects [227], suggesting that schizophrenia-associated SNPs may be involved in (normal) white matter development. Thus, novel candidate genes can be identified based on the overlap in high-ranking SNPs associated with schizophrenia and its endophenotypes. IQ is another candidate endophenotype for this approach, because of its strong association with disease [161], high heritability [81], normally distributed quantitative measures and relatively straightforward assessment.

While schizophrenia and IQ are phenotypically correlated and are both largely determined by genetic factors, these factors do not necessarily overlap. To what extent IQ-related genetic variants explain variation in schizophrenia is still unclear. A twin study in a sample of schizophrenia and healthy twins (n=263 subjects) found that IQ measured after disease onset explained 37% of the variation in disease status [234]. Shared genetic influences

accounted for 92% of this phenotypic correlation. However, a large Swedish population cohort study of twins and sib pairs (n=374,199 subjects) found that the broader phenotype of psychosis explained a much smaller amount (1%) of variation in premorbid intelligence at age 18, although this phenotypic correlation was also largely determined by shared genetic influences (for 91%). However, since the correlation was small, the genetic variance for premorbid IQ shared with psychosis was only 7% [81]. This suggests that of all genetic variants associated with premorbid IQ, few will influence psychosis, and vice versa. However, so far studies have not investigated the predictive value of measured genetic variants.

The premorbid cognitive deficits in schizophrenia patients were largest in the domains of problem solving, working memory and processing speed [198]. It is possible that schizophrenia-associated genetic factors influence these specific cognitive functions rather than general intelligence. However, previous studies indicate that IQ and working memory have a similar high heritability and a comparable genetic overlap with schizophrenia [7, 234]. None of the cognitive subtests was a significantly better schizophrenia endophenotype than total IQ. Therefore, we used total IQ as our main outcome measure.

The aim of the current study was therefore to investigate the relationship between IQ and two distinct types of schizophrenia-related genetic variants, i.e. CNVs and common SNPs. There are several possible scenarios. The first option is that these genetic variants are associated with IQ in both patients and healthy controls. If so, this would imply that novel schizophrenia risk genes could be identified using population-based samples with IQ data. A second option is that these variants affect IQ in schizophrenia patients only, which would suggest that schizophrenia genes affect processes involved in cognition. Finally, if disease-associated variants do not predict IQ in schizophrenia patients nor in healthy subjects, this could indicate that the cognitive deficits in patients are secondary to other processes related to the risk for schizophrenia, for example environmental factors like the use of cannabis or stress. Or the cognitive deficits can be related to indirect genetic effects, for example by genes influencing early brain development, which in turn affects cognition.

Methods

Subjects and IQ measurements

The study was approved by the Medical Ethical Committee of the University Medical Center Utrecht. The sample consisted of 350 patients (90% with schizophrenia, and 10% with schizoaffective disorder) and 322 controls. Clinical diagnosis was determined using the Comprehensive Assessment of Symptoms and History [4]. Unaffected controls had no

history of psychiatric illness except for three subjects who had a history of anxiety disorder, obsessive-compulsive disorder and adjustment disorder, respectively. None of the control subjects had first-degree family members with psychotic illness. Total IQ was estimated with the Wechsler Adult Intelligence Scale (WAIS) [220]. Four out of 11 subtests of WAIS versions III (n=97) and IIIR (n=575) were used (WAIS III: block design, comprehension, vocabulary and picture arrangement; WAIS IIIR: block design, information, symbol search and arithmetic). Raw scores were multiplied by 11/4 to obtain an estimate of total IQ. Due to adjustment of the norm scores, mean IQ measures were significantly lower in the newer version compared to the older version. Therefore, IQ scores were corrected for version, by taking the undstandardized residual in a linear regression, with the newer version (IIIR) as reference. Estimated IQ scores were normally distributed both before and after correction for version.

Genotyping, CNV and PSS measurements

Subjects were genotyped at the UCLA Neurosciences Genomics Core (UNGC) using the Illumina HumanHap550 beadchip. CNVs were determined from SNP data using the QuantiSNP and PennCNV algorithms as described previously [38, 226]. Briefly, deviations in log R ratios (a measure of the magnitude of the combined fluorescent signals from both sets of probes) and B allele frequencies (relative ratio of the fluorescent signals from one allelic probe to the other) for 10 consecutive SNPs were regarded as CNVs. Refseq genes within 50kb of the CNV borders were counted using the March 2006 assembly on the UCSC browser (http://genome.ucsc.edu). The total numbers of CNVs and the total number of genes affected by CNVs were calculated for each individual.

Secondly, a polygenic schizophrenia score (PSS) was calculated as described before [227]. In short, data from the Schizophrenia Psychiatric GWAS Consortium (PGC) on 8,690 schizophrenia patients and 11,831 controls was used as a discovery sample to identify the schizophrenia risk variants, their p-values and odds ratios. For each individual in our independent sample of 350 patients and 322 controls the PSS was calculated using PLINK [189]. For each SNP, the number of 'risk variants' an individual carried (0, 1 or 2) was multiplied by the logarithm of the odds ratio for that particular variant. 'Risk variants' are the alleles (nominally) associated with disease, including both true risk alleles and stochastic variation. Sets of SNPs with p-values below different cutoffs for effect on schizophrenia (p-value cutoffs for effect on schizophrenia or Pcutoff) were defined. The following Pcutoff were used: 0.01; 0.05; 0.1; 0.2; 0.3; 0.4 and 0.5. The score was summed over all SNPs in the Pcutoff-SNP sets for each individual in the current sample to obtain the individual polygenic scores. PSSs were normally distributed.

Statistical analyses

First, the difference in the 2 CNV measures (the total number of CNVs and the total number of genes affected by CNVs) between patients and controls were calculated using a

T-test. Second, the 2 CNV measures were used as factors in ANOVAs together with disease status and a CNV*disease status interaction as factors and total IQ as dependent variable in the total sample. All analyses were repeated for deletions and duplications separately. For the PSS, we performed linear regressions using the PSS as predictor and the total IQ estimates as outcome measure. Ten population stratification dimensions, an inbreeding coefficient, the number of SNPs used for scoring and the number of CNVs were used as covariates (for details see [227]). Similar to the previous analysis, we performed secondary analyses while including effects for disease status and PSS*disease status interactions. The association of the PSSs with disease status was analyzed using a logistic regression analysis. The variance explained adjusted for the number of predictors in the model (R^2) was used as the main outcome parameter. P-values were Bonferroni corrected for multiple comparisons with $\alpha = 0.008$ (0.05/6 (since there are 3 'independent' genetic measures (2 CNV and 1 PSS) x 2 tests: main effect and interaction effect)). Post-hoc we analyzed the association between the PSS and the 4 subtests of the WAIS IIIR separately.

Results

There were significantly more males in the patient group than in the control group (78% v. 49% male, p<0.0001), see Table 6.1. The groups did not differ in age and parental education. The years of education and estimated IQ were significantly lower in schizophrenia patients.

There was a trend towards more deletions in schizophrenia patients compared to healthy controls (p=0.09), while significantly more genes were affected by deletions in patients (p=0.009), see Table 6.2. Neither of the CNV measures showed a significant association with IQ.

The PSS significantly predicted disease status in the current sample ($R^2 = 0.055$, $p = 2.1x\ 10^{-7}$, for Pcutoff 0.3). A significant association was found between the PSS and IQ ($R^2 = 0.018$, $p = 0.0008$, for Pcutoff 0.3) as shown in Figure 6.1. Results for other Pcutoff are also shown in Figure 6.1. However, after correction for disease status the association was no longer significant (PSS: p = 0.97; PSS*disease status: p = 0.17). Furthermore, no association of the PSS with IQ was found within the patient and control groups (patients: $R^2 = 0.009$, p = 0.067 and controls: $R^2 = -0.003$ (negative value due to adjustment for the number of predictors in the model), p = 0.82). Excluding subjects tested with the older version of the WAIS did not change the results.

Discussion

We have investigated the association of schizophrenia-related genetic variants with IQ -a highly heritable phenotype associated with schizophrenia- in 350 schizophrenia patients and 322 healthy subjects. While deletions disrupted significantly more genes in schizophrenia patients, these deletions and the other CNV measures did not exert major effects on IQ in schizophrenia patients nor in healthy controls.

The combined score of schizophrenia-associated SNPs was associated with disease status, indicating that a polygenic schizophrenia score (PSS) can predict disease in an independent sample. The PSS was also significantly associated with IQ in the entire sample of patients and controls. However, after correcting for disease status this association disappeared. The PSS does not predict IQ within the patient and control groups. This result may seem puzzling at first sight, but can be explained by assuming that both IQ and the PSS are independently associated with schizophrenia.

Another possibility is that schizophrenia-associated genetic factors influence specific cognitive functions rather than general intelligence. Therefore we post-hoc repeated the analyses for each of the 4 subtests of the WAIS IIIR separately. Similar results were found as for the total IQ scores: the PSS explained some of the variation in the subtest in the whole sample, but the association disappeared after correction for disease status. We estimated that we had 80% power to detect a significant effect with α 0.008 when the PSS explained 1.8% or more of the variance in IQ. Thus, if there is any effect of schizophrenia-related SNPs on total IQ or one of the cognitive subdomains, this effect is likely to be small.

The findings are in agreement with the small (7%) genetic variance for psychosis shared with premorbid IQ [81]. Thus, although there is a robust association between schizophrenia and intelligence, this may not be mainly due to shared genetic factors. Instead, the cognitive deficits in schizophrenia patients may be secondary to other processes. We previously showed, in a partly overlapping sample, that the variance in total brain and white matter volume explained by the PSS is much larger (4.8 and 5.1%) [227], This effect was found in schizophrenia patients, as well as in healthy controls. Hence, the cognitive deficits in schizophrenia patients could be a result of a generalized effect of schizophrenia genes on early brain growth and maturation, perhaps in interaction with the effects of disease-related environmental factors.

There are several other factors that may have influenced the results and which should be acknowledged as potential limitations of this study. First, IQ measurements in patients could be influenced by the presence of psychotic symptoms, the use of medication or illicit

drugs, which could have partly masked the genetic effects. Secondly, total IQ was estimated by 4 out of 11 WAIS subtests, which is less precise than an estimate based on all 11 subtests, although the shortened version is more widely used [81]. Furthermore, the lack of effect of global CNV measures on IQ does not exclude an effect of individual CNVs at specific chromosomal locations on schizophrenia susceptibility and IQ. For example, CNVs at 15q11.2 are specifically found in patients with schizophrenia and intellectual disability (Derks et al, unpublished observations). However, since CNVs at specific locations are usually rare, these are likely to go unnoticed in the current sample.

To conclude, rare and common schizophrenia-related genetic variants do not predict variation in IQ in healthy subjects, nor within the schizophrenia patient group. This suggests that cognitive deficits and the risk for schizophrenia cannot be attributed to shared genes. The cognitive dysfunction in schizophrenia patients is therefore more likely to be secondary to other processes related to schizophrenia risk. So, although we did not investigate this directly, our study suggests that population-based studies aiming to identify schizophrenia candidate genes by investigating the association of genetic variants with IQ may not be fruitful.

	Schizophrenia patients	Healthy controls	Significance
n	350	322	na
Gender (m/f)	272/78	158/164	p<0.0001
Age in years	30.2 (9.0)	31.6 (11.9)	ns
Age of first psychotic symptoms in years	22.5 (5.4)		na
Duration of illness in years	7.5 (7.9)		na
Level of education in years	12.6 (2.4)	13.9 (2.3)	p<0.0001
Level of parental education in years	13.4 (3.1)	13.3 (3.1)	ns
Estimated IQ	95.8 (16.6)	111.2 (14.1)	p<0.0001

Table 6.1: Demographic information. Means are displayed with the standard deviation in brackets (sd). n=number of subjects, m=male, f=female, estimated IQ= a measure of intelligence quotient as calculated from the scores on 4 WAIS subtests, na= not applicable, ns= not significant.

	P sz	P IQ	P interaction
CNVs	0.67	0.99	0.53
Genes in CNVs	0.22	0.97	0.24
Deletions	0.09	0.82	0.95
Genes in deletions	0.009	0.43	0.21
Duplications	0.38	0.60	0.73
Genes in duplications	0.63	0.74	0.97

Table 6.2: The association between CNV measures and IQ. P sz is the p-value of the main effect of the CNVs on schizophrenia disease status tested with a T-test. P IQ is the p-value of the main effect of the CNV measures on IQ, investigated with ANOVAs in which the CNV measures, disease status and the CNV*disease status interactions were included as factors in the analyses. P interaction is the p value of the interaction effect between the CNV measure and disease status on IQ.

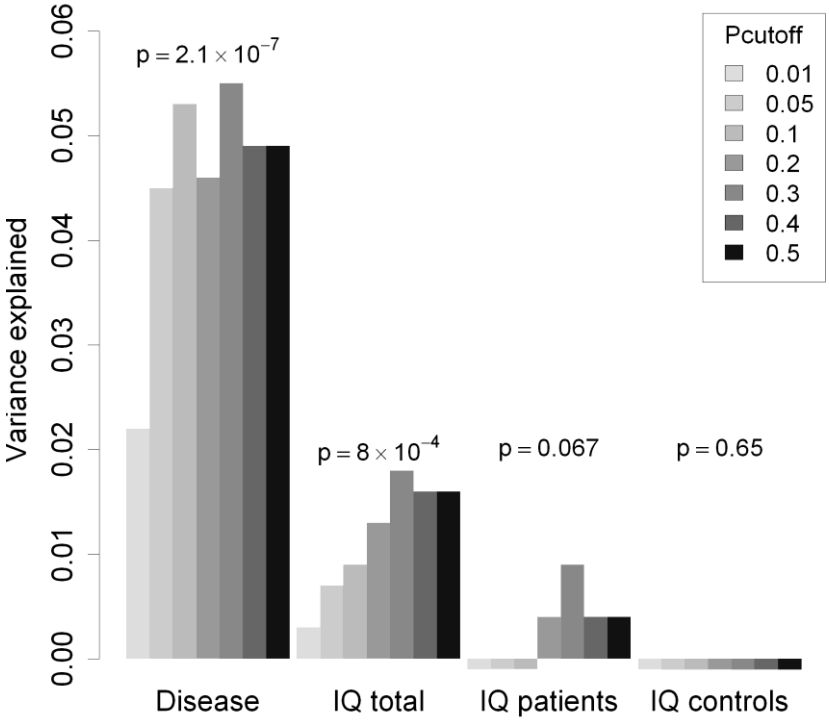

Figure 6.1: The variance explained by PSS in schizophrenia disease status and IQ for different Pcutoff SNP sets. For 'schizophrenia' the association between the PSS and schizophrenia disease status is tested. In 'IQ total' the association between PSS and IQ is tested in the total sample, while in 'IQ patients' and 'IQ controls' this association is tested in respectively the groups of patients and controls only. Variance explained (R^2) is adjusted for the number of predictors in the model. The p-values shown are the p-values for the Pcutoff set with the lowest p-value within that phenotype.

Chapter 7

Association study of CNVs with brain volume in schizophrenia patients and healthy controls

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Abstract

Schizophrenia patients have more copy number variations (CNVs) than healthy controls, and reduced brain volumes. Although this could suggest a causal relationship, we found no association between global CNV burden and three brain volume measures (on a MRI scan) in a sample of 173 schizophrenia patients and 176 healthy controls.

Introduction

The heritability of schizophrenia is around 80% [44], but the underlying genetic determinants are largely unknown. Copy number variants (CNVs) are genomic microdeletions or microduplications from 1kb to multiple Mb in size. These can disrupt genes and thereby change mRNA and protein levels, possibly resulting in disease [222]. An excess of rare CNVs was found in schizophrenia (15%) compared to healthy controls (5%) [250]. Our group previously reported a significantly higher burden of deletions in schizophrenia patients compared to healthy controls [38]. Several specific deletions related to schizophrenia, e.g. on chromosome location 1q21.1 and 22q11, were previously shown to be associated with neuroanatomical abnormalities such as reduced total brain volumes [33, 271]. Total brain volume is highly heritable (80-90%) [10] and patients with schizophrenia have on average 3% smaller brain volumes compared to healthy controls [258]. Investigating the genetic factors underlying brain volume reductions can increase insight into the pathophysiology of schizophrenia. Our group previously reported that white matter loss in schizophrenia patients was largely (94%) attributable to genetic factors, while gray matter volume was determined by unique and common environmental factors [103]. We therefore investigated the association between global CNV burden and total brain, white matter and gray matter volume in schizophrenia patients and healthy controls.

Methods and Materials

Subjects

The sample consisted of 173 patients (158 with schizophrenia, and 15 with schizoaffective disorder) and 176 controls. Psychopathology was assessed using the Comprehensive Assessment of Symptoms and History [4]. Unaffected subjects had no history of psychiatric illness except for three subjects who had a history of anxiety disorder, obsessive-compulsive disorder and adjustment disorder. None of the control subjects had first-degree family members with psychotic illness.

Magnetic resonance imaging

Brain images were acquired on Philips NT and Philips Achieva scanners at 1.5 Tesla. MRI acquisition and processing methods have been previously described [111]. Post-processing was done on the neuro-imaging computer network at our department. Images were coded to ensure blind measurements. Total brain, white matter and gray matter volume were corrected for intracranial volume, age and sex by taking the unstandardized residuals using linear regression.

Genotyping

Subjects were genotyped at the University of California in Los Angeles on the Illumina HumanHap550 beadchip. CNVs were determined from SNP data using the QuantiSNP and PennCNV algorithms as described previously [38]. Deviations for 10 consecutive SNPs were regarded as CNVs. Refseq genes within 50kb of the CNV borders were counted using the March 2006 assembly on the UCSC browser (http://genome.ucsc.edu). Brain expression was determined using expressed sequence tag data of brain tissue in the UCSC browser. Total numbers of CNVs affecting genes, genes affected by CNVs and brain expressed genes affected by CNVs were calculated.

Statistical analyses

Above a certain cut-off point (3+, 4+ or 5+, depending on the frequency distribution) numbers of CNVs were combined to obtain categories of at least 20 subjects (see Table 7.1). First, the 3 CNV measures were used as factors (one-by-one) in ANOVAs with the unstandardized residuals of the brain volume measures as dependent variables in the total sample. Second, to study if the impact of CNVs on brain volume differs between patients and controls, disease status and CNV*disease status interaction were included in the analyses. All analyses were repeated for deletions and duplications separately.

Results

There were significantly more males in the patient group than in the control group (80 v. 54% male, $p<0.0001$). The groups did not differ in age and parental education (an estimate for socioeconomic status). Total brain volume was significantly smaller in patients (mean 1482.1ml, sd 50.8) than in controls (mean 1505.4ml, sd 43.7; $p=5.3x10^{-6}$), as were gray matter and white matter volume. After correction for multiple comparisons ($\alpha=0.003$ (=0.05/18 (3 brain volumes x 3 'independent' CNV measures x 2 tests))), none of the CNV measures showed a significant association with total brain volume, as shown (Table 7.2).

Discussion

The results suggest that global CNV burden does not exert major effects on brain volume in schizophrenia patients nor in healthy controls. We had 80% power to detect a CNV measure explaining 4.3% of the variance in total brain volume with α 0.003. Although the effect of CNVs might be larger than the effect of SNPs and quantitative phenotypes have more power than dichotomous disease traits [187], the combined effects of CNVs may still have been too small to detect in our sample. If only a limited number of specific CNVs have an impact on brain development, these could be missed by analyzing global CNV

burden. Alternatively, CNVs might have regional effects on brain volume that could go undetected by using global brain volume measures. Replication of this negative finding is essential, and for this purpose our raw data are available on request. Should replication studies also fail to demonstrate an association between CNV burden and brain volume, another possible explanation is that CNVs and brain volume are independently associated with schizophrenia, or that at least one of them is associated to schizophrenia through a confounder.

Frequency of CNV measure	0	1	2	3	3+	4	4+	5+
CNVs containing genes	178	92	92	42			35	
Genes in CNVs	178	41	35	30		24		131
Brain genes in CNVs	193	71	55	27		29		62
Deletions containing genes	263	114	41		21			
Genes in deletions	263	60	40		76			
Brain genes in deletions	301	69	34		33			
Duplications containing genes	247	118	51		23			
Genes in duplications	247	38	30	22		27		75
Brain genes in duplications	257	67	37		76			

Table 7.1: Frequency distribution of CNVs. For each CNV measure it is shown how many subjects were observed to have a certain amount of CNVs. 3+ means all subjects with 3 or more deletions were grouped in this category, in order to obtain categories containing at least 20 subjects.

P main	Total brain	White matter	Gray matter
CNVs containing genes	0.15	0.56	0.07
Genes in CNVs	0.88	0.9	0.5
Brain genes in CNVs	0.98	0.66	0.66
Deletions containing genes	0.02	0.14	0.64
Genes in deletions	0.09	0.44	0.13
Brain genes in deletions	0.56	0.98	0.39
Duplications containing genes	0.24	0.53	0.34
Genes in duplications	0.69	0.89	0.66
Brain genes in duplications	0.48	0.8	0.62

P interaction	Total brain	White matter	Gray matter
CNVs containing genes	0.28	0.79	0.07
Genes in CNVs	0.29	0.51	0.66
Brain genes in CNVs	0.19	0.21	0.61
Deletions containing genes	0.58	0.28	0.13
Genes in deletions	0.04	0.07	0.07
Brain genes in deletions	0.26	0.02	0.04
Duplications containing genes	0.19	0.31	0.09
Genes in duplications	0.54	0.52	0.3
Brain genes in duplications	0.47	0.73	0.03

Table 7.2 Association results of CNVs with brain volumes. P main is the effect of the CNV in the total sample. P interaction is the interaction with disease status, which is equivalent to the difference in effect in the patient and control groups. P-values are not corrected for multiple testing.

Chapter 8

Advanced paternal age increases the rate of rare, large deletions in schizophrenia patients and healthy controls

In preparation

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Abstract

Background Advanced paternal age is a risk factor for schizophrenia. This effect might be related to the accumulation of mutations in the sperm of older males. We tested the hypothesis that schizophrenia patients with older fathers carry more copy number variants (CNVs) and focused on large CNVs not flanked by segmental duplications (SDs), since these have a higher chance of being *de novo* and of paternal origin.

Methods A sample of 2,177 schizophrenia patients was genotyped on Affymetrix 6.0 arrays. CNVs were called with Birdsuite. The group was split based on paternal age (<35 and ≥35 years) and odds ratios (ORs) were calculated. The strongest effect observed in the first hypothesis-generating stage was tested in two additional samples consisting of 694 schizophrenia patients and 6,615 healthy controls in total.

Results We observed an increased rate of singleton deletions >500kb not flanked by SDs in patients with older fathers in our initial sample (OR=3.26, uncorrected p=0.02). A similar effect was observed in meta-analysis of odds ratios in the two additional samples of schizophrenia patients and healthy controls combined (OR=1.84, one-sided p=0.028). This effect was independent of disease status. Deletions in DEC1 and duplications in APBA2, GLB1L2, GLB1L3 and PLN were more prevalent in patients with old fathers, although these findings were not significant after correction for multiple testing. We observed no significant enrichment of spermatogenesis-related genes in the deletions of patients with older fathers.

Conclusion The offspring of older fathers have an increased risk of carrying rare, large deletions, independent of schizophrenia disease status. The effect is suggestive of an increased rate of *de novo* mutations, rather than a higher overall CNV burden in the older fathers. The result must be considered preliminary due to limited sample size, modest statistical significance and the lack of full family data to identify *de novo* CNVs.

Introduction

From epidemiological studies, it has long been known that the children of older fathers (typically above 35 years of age) are at increased risk of schizophrenia [36, 100, 162]. It was long unclear whether this effect was related to socioeconomic or genetic factors. Recently, a sequencing study showed that there was a strong correlation between paternal age and *de novo* SNP mutation rates in schizophrenia and autism patients [136]. This suggests that genetic factors may play an important role in the association between advanced paternal age and schizophrenia.

Sex differences in germ cell production can explain why a disease might be related to paternal age more strongly than to maternal age [56]. In the female, germ cells undergo 22 mitotic divisions before birth and then lay dormant until the last meiotic division just before ovulation. In the male, however, germ cells keep renewing every 16 days by mitotic divisions. So, while age does not affect the number of germ cell cycles in females, it has a strong effect in males. Every mitosis carries a (small) risk of introducing mutations. Therefore, mitosis-based mutations might be related to paternal age effects, while meiosis-based mutations can be derived from both paternal and maternal origin.

It was previously thought that novel mutations happen at random positions across the genome. Every now and then these will hit a schizophrenia-related gene, resulting in this disorder in the offspring. This hypothesis is called the 'copy error hypothesis' [91]. There are important limitations to this hypothesis. First, the paternal age risk does not seem to increase linearly. There is a steady or slowly increasing risk until the age of 35, after which there is a stronger (perhaps exponential) increase in schizophrenia risk for the progeny [40, 162]. Second, if the mutations were random, one would expect the risk of all genetic disorders to increase with paternal age. This has been reported for some autosomal-dominant disorders, congenital abnormalities, (childhood) cancers and psychiatric disorders [91]. However, we have not found any report of increased paternal age for other complex disorders, including diabetes, coronary artery disease, Crohn's disease or rheumatoid arthritis.

Based on the study of the paternal age effects in autosomal-dominant full-penetrance disorders, a new hypothesis was proposed [91]. In this theory of 'selfish sperm selection', some mutations cause selective advantage to sperm cells and may therefore accumulate more rapidly in the testis of older males. For example, Apert's syndrome is caused by a specific mutation in fibroblast growth factor receptor 2 (FGFR2, 755C>G). This mutation leads to a gain-of-function of the protein, with stronger binding of natural ligands (like FGF2) and binding of new ligands (like FGF10). Once a sperm stem cell has acquired such a mutation (by a random process) it is more likely to survive and proliferate than its neighbours, resulting in islands of mutated sperm cells in the testes. When transmitted to a child, this mutation causes serious defects in growth of the limbs and skull (syndactily and craniosynostosis). In the sperm of 18-80-year-old healthy males, this mutation

increases at an almost exponential rate, while neighbouring nucleotides showed no increase. Since affected subjects rarely have children, almost all disease burden is caused by *de novo* mutations. The selfish sperm selection hypothesis explains why the frequency of this disease is much higher than would be expected based on randomly distributed mutations alone (incidence 1:80,000 compared to $1.1x10^{-8}$ background mutation rate) [90]. It also explains the paternal bias for Aperts's syndrome: >90% of the *de novo* mutations are of paternal origin. Another example of such a process is seen in achondroplasia, which is caused by FGFR3 mutations.

Similar to these syndromes, *de novo* mutations may play a role in schizophrenia. Schizophrenia has been associated with an increased rate of *de novo* copy number variants (CNVs, deletions or duplications of 1 kb to several Mb in size) [131, 155, 262]. In addition, it was reported that schizophrenia is associated with an increased rate of *de novo* single nucleotide polymorphism (SNP) mutations [260, 261] and that the number of *de novo* SNP mutations showed a strong association with paternal age in a sample of schizophrenia and autism trios [136].

CNVs can be calculated from genome-wide SNP data, while for *de novo* SNP determination sequencing of trios would be required. We hypothesized that schizophrenia patients with older fathers carry more *de novo,* mitosis-based mutations than patients with younger fathers. *De novo* CNVs tend to be larger and less frequent than inherited CNVs [131] and these characteristics can be used as proxy measures when no parental genotype information is available, as was the case in the samples available for the present analysis. For CNVs with frequency <1%, 33% of deletions >500kb are *de novo*, which is 4.1 times more often compared to deletions >100kb [131].

We separately assessed CNVs not flanked by segmental duplications (SDs), because these are more often mitosis-related [210]. Mitosis-based mutations include *de novo* SNPs or non-recurrent CNVs, which are thought to derive from a process to repair double strand breaks, called non-homologous end joining (NHEJ) [231]. Recurrent CNVs are likely caused by non-allelic homologous recombination (NAHR) during meiosis and occur more often in the presence of SDs. An example of a recurrent CNV is the 22q11 deletion, which is equally of paternal and maternal origin [230]. Interestingly, only CNVs not flanked by segmental duplications showed an association with advanced paternal age and increased paternal origin in subjects with intellectual disability [107].

Furthermore, Goriely et al. observed that genes affected in the syndromes with paternal age effects were all found in tyrosine kinase growth factors and their downstream RAS signalling pathway. We hypothesized that these pathways might also play a role in mental disorders. RASopathies and fibroblast growth factor dysfunctioning have been suggested to play a role in schizophrenia and autism spectrum disorders [221, 228, 241, 272]. RAS upregulation suppresses the maturation of cortical neurons and inhibits the formation of dendritic spines and synapses [264]. Therefore, the paternal age effect observed for

schizophrenia patients may partly arise from mutations that have growth promoting effects on sperm and lead to aberrant brain growth at the same time. This might also occur due to the effects of genes that are not involved in RAS signaling but in which mutations can similarly increase sperm cell cycle while having deleterious effects on the brain. Thus, we investigated which genes were affected by CNVs of patients with old fathers and whether these genes were predominantly found in spermatogenesis-, RAS- or cell cycle related pathways.

Methods

Analyses were performed in two stages. In the first (hypothesis-generating) stage, we carried out multiple analyses of the relationship between paternal age and CNV prevalence in the Molecular Genetics of Schizophrenia (MGS) sample. These analyses considered genome-wide counts of deletions and of duplications in 32 tests of different subsets of rare CNVs impacting on exons, as well as counts of exonic CNVs in each RefSeq gene and in 4 defined gene sets relevant to our hypotheses. In the second stage, we tested the one nominally-significant finding in two additional samples of schizophrenia patients and healthy controls.

Subjects of the MGS sample

Clinical methods and sample characteristics were previously described [148, 207]. Briefly, 2,177 unrelated schizophrenia patients were recruited by 10 university-based sites in the United States and Australia under a common protocol. They received consensus diagnoses of DSM-IV schizophrenia (90%) or schizoaffective disorder (with schizophrenia criterion A for at least 6 months) based on available information from interviews, informants, and medical records. Using the same sources, paternal and maternal age at the time of birth of the subjects was estimated by the interviewer. We excluded cases where estimates of parental age were below 14 or above 70 years for fathers or below 10 or above 55 years for mothers. Adopted subjects were excluded from the analyses. European-American (72%) and African-American subjects (28%) were analyzed together. Genotypes were also available for 3,611 control subjects (no lifetime schizophrenia or bipolar disorder), but no parental age information. In the main analyses, these controls were used only for filtering of CNVs with frequency greater than 1% and to contrast case and control frequencies in the analyses of individual genes.

CNV calling in the MGS sample

DNA specimens of all controls and 72% of the patients were extracted from Epstein-Barr virus transformed lymphoblastic cell lines, while 28% of patient specimens were extracted from blood. The specimens were assayed at the Broad Institute (Cambridge, USA) by using

Affymetrix 6.0 genotyping arrays (Affymetrix, Santa Clara, USA). CNVs were detected with the Birdseye module of the Birdsuite software package, version 2 (internal version 1.3) [138]. The data were normalized within plates of up to 92 DNA samples. Human genome build 18 (HG18) locations are reported. We used narrow call criteria as described previously [148]. Only CNVs intersecting exons were included in analyses. Furthermore, we excluded CNVs larger than 4000 kb (observed primarily in cell line DNA and thus probably representing artifacts), those in telomeric and centromeric regions, and in regions where there was evidence of plate effects or other suspected artifacts. CNVs with more than 70% overlap with a segmental duplication were excluded, because large identity with another region in the genome is likely to increase artifacts and annotation errors. A list of segmental duplications (genomic regions of >1kb size and with >90% identity with another region) was downloaded from UCSC, version Built 36, Hg18. Next, CNVs were filtered on frequency to be either rare (frequency <1%) or singletons using PLINK [189]. Filtering on frequency <1% was based on length overlap of >50% between CNVs, in the sample of cases and controls together, for deletions and duplications separately. Singleton CNVs were determined by excluding all CNVs that showed >50% overlap with another CNV in our sample. For both deletions and duplications, four size ranges were analyzed: all size segments, segments <100kb, >100kb and >500kb. Lastly, the CNVs flanked and not flanked by segmental duplications (SDs) were analyzed separately. Proximity to SDs was defined as CNVs that started or ended in or within 15kb from a SD region.

Global CNV burden and paternal age in MGS sample

The schizophrenia patients were divided into two groups based on paternal age of 35 years or older at the time of subject's birth (n=571) vs. below 35 years (n=1606), since especially after this age schizophrenia risk increases. We tested the effect of paternal age on 32 genome-wide CNV measures including all combinations of two CNV types (deletions, duplications), two frequency measures (rare [<1%], singleton), 4 size ranges, and 2 SD-based subsets (flanked or not flanked by SDs). A global burden analysis was performed in PLINK for all 32 CNV measures, considering schizophrenia cases with older vs. younger fathers. Ancestry, DNA source (blood v. cell lines) and mean number of CNVs per plate were used as covariates. Sex, inclusion site and age at inclusion were not related to paternal age and/or the number of CNVs and were therefore not included as covariates. The outcome variable for each individual was the observation of at least one CNV meeting the criteria for that analysis (few individuals had more than one in most of the analyses). To make sure that the use of categorical age groups was not missing any effects, we repeated the same 32 tests with paternal age as a quantitative predictor in Poisson regressions.

Because of the sample size and the expected effect sizes, we considered it highly unlikely that any results in the MGS sample would withstand correction for multiple testing, and thus these analyses were hypothesis-generating. Also, it is difficult to determine a

corrected threshold of significance because many of the tests are strongly inter-correlated. As a rough approximation, we considered p<0.006 as a somewhat liberal threshold based on 8 highly independent tests (4 each in the categorical and continuous analyses, see Table 8.1).

Additional samples for hypothesis testing

After selecting the strongest hypothesis in the MGS sample, we tested it in 2 additional samples from the Netherlands comprising three groups of subjects: a sample of schizophrenia patients and controls [38] and a large sample of control subjects [35]. An overview of sample characteristics and CNV calling can be found in Table 8.2. In short, the case/control sample consisted of 694 schizophrenia patients and 407 healthy controls genotyped on an Illumina 550K array. Quality control was performed using the same criteria as for the MGS sample. The Dutch control sample consisted of 6208 healthy controls genotyped on different Illumina arrays, of which only overlapping SNPs (basically those on the Illumina 300K array) were used for CNV calling. Data acquired with quality control steps described previously [23, 35] was used for this sample, however because we only had access to CNV counts rather than raw data, we included CNVs that intersected genes (rather than specifically identifying intersection with exons); and we were unable to exclude CNVs with >70% overlap with segmental duplications. We only tested the number of subjects with deletions >500 kb. A meta-analysis of odds ratios for the three groups was performed using a one-sided Cochran-Mantel-Haenszel chi-square test in R.

Gene-by-gene analysis in MGS sample

We tested whether more cases with older vs. younger fathers had an exonic duplication or (separately) deletion for each HG18 RefSeq gene, using PLINK (one-sided tests). These tests were only performed for rare deletions and duplications of all sizes (without subdividing for CNVs of different sizes, singleton CNVs or CNVs not flanked by SDs. No covariates were used in the primary analyses, but the effect of ancestry, plate and DNA source was checked afterwards for candidate genes. Empirical p-values were calculated by performing 50,000 permutations. For genes with genome-wide corrected empirical p-values <1, CNVs were visually inspected by plotting Log R ratio and B allele frequency with PennCNV [252]. CNVs with unconvincing plots were excluded from the analysis. Secondly, the presence of CNVs in the candidate genes was checked in the controls of the MGS sample (for whom paternal age was unknown) and in the additional samples.

Gene-set enrichment in MGS sample

Four lists of genes were created based on the annotation of genes to Gene Ontology categories of 'spermatogenesis (GO:0007283, 397 genes)', 'regulation of RAS GTPase activity' (GO:0032318, 162 genes), 'negative regulation of cell cycle' (GO:0045786, 456 genes) and 'positive regulation of cell cycle' (GO:0045787, 110 genes). We used the gene-set-enrichment test for CNVs in PLINK [197] to investigated whether genes in these gene sets were more commonly affected by deletions or duplications than all Refseq genes in

general. Since we were primarily interested in mutations that increase the sperm stem cell cycle, the gene set 'negative regulation of cell cycle' was only tested in deletions, while the gene set 'positive regulation of cell cycle' was only tested in duplications.

CNV	Size	SD - nonSD (rare)		SD - nonSD (singleton)		rare - singleton (nonSD)		rare - singleton (SD)	
		r2	P	r2	P	r2	P	r2	P
deletion	all	0.04	0.08	0.02	0.39	0.53	<0.001	0.33	<0.001
deletion	<100 kb	0.05	0.01	0.01	0.7	0.49	<0.001	0.37	<0.001
deletion	>100 kb	0.03	0.18	0.07	0	0.6	<0.001	0.25	<0.001
deletion	>500 kb	0.01	0.73	0.21	<0.001	0.69	<0.001	0.22	0.001
duplication	all	0.06	0.01	0.01	0.57	0.5	<0.001	0.31	<0.001
duplication	<100 kb	0.02	0.31	0.06	0.01	0.46	<0.001	0.33	<0.001
duplication	>100 kb	0.03	0.12	-0.02	0.28	0.5	<0.001	0.24	<0.001
duplication	>500 kb	0.01	0.55	0	0.85	0.48	<0.001	0.18	<0.001

CNV	Size	with all size CNVs (rare nonSD)		del - dup (rare nonSD)		del - dup (rare SD)	
		r2	P	r2	P	r2	P
deletion	all			0.02	0.43	-0.02	0.31
deletion	<100 kb	0.88	<0.001	0.03	0.12	0.02	0.48
deletion	>100 kb	0.49	<0.001	-0.02	0.47	0	0.99
deletion	>500 kb	0.15	<0.001	-0.02	0.26	-0.03	0.11
duplication	all						
duplication	<100 kb	0.38	<0.001				
duplication	>100 kb	0.33	<0.001				
duplication	>500 kb	0.16	<0.001				

Table 8.1: Correlation between CNV measures It is difficult to determine a corrected threshold of significance because many of the tests are strongly inter-correlated. For example, the correlation between the number of rare and singleton CNVs and between CNVs of different CNV sizes is highly significant. In contrast, the numbers of CNVs flanked by SDs and not flanked by SDs and the numbers of deletions and duplications are more or less independent. Therefore, as a rough approximation, we considered $p<0.006$ as a somewhat liberal threshold based on 8 highly independent tests (4 each in the categorical and continuous analyses).

	MGS	Dutch	UMC
Reference	Levinson 2011	Buizer 2013	Buizer 2013
N schizophrenia patients	2177	0	694
N healthy controls	0	6208	407
Sex (% males)	67.9	44.2	66.6
Ancestry (% EA)	72.7	100	100
DNAsource (% blood)	28.7	100	100
Paternal age (sd)	30.1(7.8)	32.6 (6.9)	31.4 (5.6)
Maternal age (sd)	26.6 (6.7)	30.1 (6.2)	28.8 (5.0)
Parental age difference (sd)	3.5 (5.3)	2.5 (4.5)	2.6 (3.7)
Old fathers (% ≥ 35 years)	26.2	35.5	25.2
SNP array	Affymetrix 6.0	Illumina 300	Illumina 550
CNV calling	Birdsuite	PennCNV	PennCNV
Frequency of rare, exonic CNVs:			
all size deletions	0.97	0.48	0.56
all size duplications	1.00	0.52	0.45
deletions >500kb	0.06	0.01	0.03
duplications >500kb	0.08	0.04	0.05

Table 8.2: An overview of demographic data, CNV calling methods and the number of CNVs in the different samples MGS = Molecular Genetics of Schizophrenia Consortium, UMC = University Medical Centre of Utrecht, EA = European-American, sd = standard deviation, SD = segmental duplication, kb = kilobase. The frequency numbers are the number of exonic CNVs with frequency <1% divided by the number of subjects. Only for the Dutch controls sample genic CNVs were used.

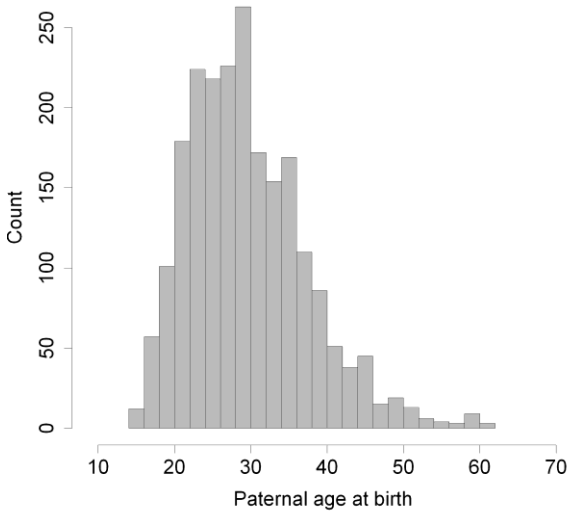


Figure 8.1: Frequency distribution of paternal age in the MGS sample Mean paternal age =30.08, sd = 7.8 for 2,177 subjects.

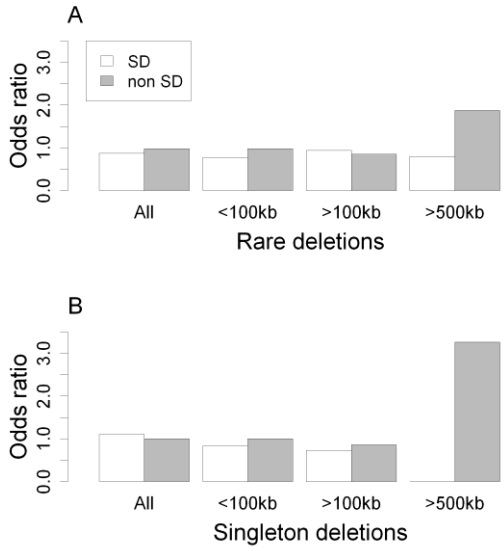


Figure 8.2: Global deletion burden in the MGS sample. Odds ratios for total numbers of CNVs of different sizes in cases with older fathers vs. younger fathers: A, for rare deletions (<1% frequency); B, for singleton deletions. SD = flanked by segmental duplications. P-values for rare deletions >500kb, non SD flanked = 0.05, for singleton deletions >500kb p = 0.09 and for singleton deletions >500kb, non SD flanked = 0.02. All duplications showed odds ratios around 1, see Table 8.5.

Cases with old fathers			Number of subjects carrying the following numbers of CNVs:						
CNV	**Size**	**Flanking**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
deletion	all	SD	432	118	19	2			
deletion	<100 kb	SD	508	60	3				
deletion	>100 kb	SD	485	77	8	1			
deletion	>500 kb	SD	551	18	2				
deletion	all	non SD	305	179	67	19	1		
deletion	<100 kb	non SD	347	156	54	14			
deletion	>100 kb	non SD	508	58	5				
deletion	>500 kb	non SD	557	14					
duplication	all	SD	415	130	23	3			
duplication	<100 kb	SD	500	65	6				
duplication	>100 kb	SD	474	88	7	2			
duplication	>500 kb	SD	544	26	1				
duplication	all	non SD	309	179	66	10	6	1	
duplication	<100 kb	non SD	400	138	28	5			
duplication	>100 kb	non SD	434	117	17	2	1		
duplication	>500 kb	non SD	551	20					
Cases with young fathers									
CNV	**Size**	**Flanking**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
deletion	all	SD	1158	371	69	8			
deletion	<100 kb	SD	1380	209	16	1			
deletion	>100 kb	SD	1338	248	19	1			
deletion	>500 kb	SD	1535	70	1				
deletion	all	non SD	839	558	156	43	10		
deletion	<100 kb	non SD	958	519	106	21	2		
deletion	>100 kb	non SD	1393	191	20	2			
deletion	>500 kb	non SD	1585	20	1				
duplication	all	SD	1149	387	60	9	1		
duplication	<100 kb	SD	1421	173	10	1	1		
duplication	>100 kb	SD	1303	269	33	1			
duplication	>500 kb	SD	1533	69	4				
duplication	all	non SD	823	556	169	49	5	2	2
duplication	<100 kb	non SD	1100	412	83	7	2	2	
duplication	>100 kb	non SD	1198	357	45	5	1		
duplication	>500 kb	non SD	1548	57	1				

Table 8.3: Rare CNV counts for each analysis SD= segmental duplications. These counts are for exonic cnvs with frequency <1%, after quality control in EA and AA subjects together.

Cases with old fathers			Number of subjects carrying the following numbers of CNVs:			
CNV	**Size**	**Flanking**	**0**	**1**	**2**	**3**
deletion	all	SD	554	17		
deletion	<100 kb	SD	560	11		
deletion	>100 kb	SD	565	6		
deletion	>500 kb	SD	571			
deletion	all	non SD	478	87	6	
deletion	<100 kb	non SD	502	64	5	
deletion	>100 kb	non SD	547	23	1	
deletion	>500 kb	non SD	563	8		
duplication	all	SD	553	17	1	
duplication	<100 kb	SD	560	10	1	
duplication	>100 kb	SD	564	7		
duplication	>500 kb	SD	569	2		
duplication	all	non SD	508	57	5	1
duplication	<100 kb	non SD	533	36	2	
duplication	>100 kb	non SD	544	25	1	1
duplication	>500 kb	non SD	566	5		
Cases with young fathers						
CNV	**Size**	**Flanking**	**0**	**1**	**2**	**3**
deletion	all	SD	1547	57	2	
deletion	<100 kb	SD	1569	37		
deletion	>100 kb	SD	1583	22	1	
deletion	>500 kb	SD	1601	5		
deletion	all	non SD	1350	228	27	1
deletion	<100 kb	non SD	1418	175	13	
deletion	>100 kb	non SD	1528	73	4	1
deletion	>500 kb	non SD	1599	6	1	
duplication	all	SD	1574	32		
duplication	<100 kb	SD	1590	16		
duplication	>100 kb	SD	1590	16		
duplication	>500 kb	SD	1604	2		
duplication	all	non SD	1414	177	14	1
duplication	<100 kb	non SD	1489	113	4	
duplication	>100 kb	non SD	1521	83	2	
duplication	>500 kb	non SD	1592	14		

Table 8.4: Singleton CNV counts for each analysis SD= segmental duplications. These counts are for singleton exonic cnvs, after quality control in EA and AA subjects together.

Results

Demographic variables and CNV counts (MGS sample)

Demographic data for the MGS subjects are shown in Table 8.2. Paternal age was highly correlated with maternal age (r^2=0.74) and difference in parental age (r^2=0.55). For the frequency distribution of paternal age see Figure 8.1. For the CNV counts per group per CNV type, see Table 8.3 and 8.4. African-American ancestry was associated with younger paternal age (mean 29.3 years (sd 8.8) versus 30.4 years (sd 7.4) in European Americans (EA), p=0.009) and more small deletions (mean number of deletions <100kb in AA was 0.87 (sd 0.95) versus 0.57 (sd 0.76) in EA, $p<10^{-5}$) and more small duplications (mean number of duplications <100kb in AA was 0.64 (sd 0.83) versus 0.46 (sd 0.70) in EA, $p<10^{-5}$). Counts of CNVs >500kb were not different in AA vs. EA subjects. Ancestry was included as a covariate in the analyses.

Hypothesis-generating analyses (MGS sample)

First, the proportion of CNV carriers was compared between patients with old fathers and patients with younger fathers. As shown in Figure 8.2 and Table 8.5, there was nominal evidence for association for an excess of rare, large deletions not flanked by SDs in patients with old fathers (frequency 14/571 in patients with older fathers v. 21/1606 in patients with younger fathers, OR=1.87, p=0.05). For singleton deletions too, deletions >500 kb not flanked by SDs showed the largest effect (frequency 8/571 in old-father-cases v. 7/1606 in young-father-cases, OR=3.26, p=0.02). The number of duplications showed no differences between the patients with old and young fathers, see Table 8.5. To make sure we were not missing any effects not related to SD flanking, the analyses were post hoc repeated for flanked and non-flanked CNVs combined, with no nominally significant p-values. When using paternal age as continuous predictor, the differences were not significant, see Table 8.6.

Hypothesis-testing analysis of genome-wide CNV counts (stage 2)

The excess of rare deletions >500 kb not flanked by SDs was selected as the primary hypothesis to be tested in the additional datasets. The other of the two nominally significant effects observed in the MGS sample, singleton deletions >500 kb not flanked by SDs, was not tested in the additional samples, since there was little difference between rare and singleton deletion numbers in these samples. Results can be found in Figure 8.3. The results were comparable to the initial analyses (OR=1.84, p=0.028, excluding the MGS sample in the analysis or OR=1.86, p=0.0045, including all samples in the analysis). The Woolf test statistic for heterogeneity of the samples was not significant. This indicates that the effect in the different samples was not significantly different. Thus, the observed effect applies for both schizophrenia patients and healthy controls.

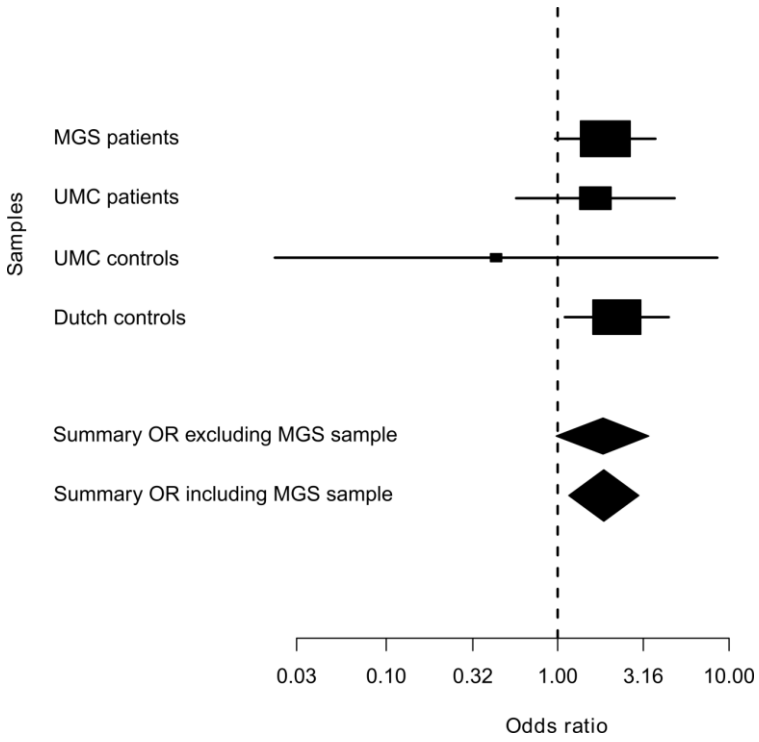


Figure 8.3: Replication analysis for the association between paternal age and rare, large deletions. Shown are the odds ratios for the difference in the proportion of subjects carrying rare, exonic deletions >500kb not flanked by SDs between subjects with old and young fathers. MGS sample is the sample that was initially tested, the other three samples are the replication samples. The combined effect is significant when excluding the MGS sample from the analysis (OR = 1.84, 95% CI = 1.08-infinite, p = 0.028 (uncorrected for multiple testing)). When including the MGS sample in the analysis the p-value is 0.0045 (OR = 1.86, 95% CI = 1.25-infinite).

			Rare CNVs		Singleton CNVs	
CNV	**Size**	**Flanking**	**Proportion OR**	**P**	**Proportion OR**	**P**
deletion	all	SD	0.87	0.96	1.11	0.82
deletion	<100 kb	SD	0.78	0.97	0.84	0.75
deletion	>100 kb	SD	0.94	0.83	0.73	0.81
deletion	>500 kb	SD	0.80	0.86	<0.01	>0.99
deletion	all	non SD	0.98	0.70	1.00	0.44
deletion	<100 kb	non SD	0.98	0.70	1.00	0.43
deletion	>100 kb	non SD	0.85	0.93	0.86	0.77
deletion	>500 kb	non SD	**1.87**	**0.05**	**3.26**	**0.02**
duplication	all	SD	0.96	0.72	1.58	0.08
duplication	<100 kb	SD	1.08	0.30	1.94	0.07
duplication	>100 kb	SD	0.90	0.85	1.23	0.40
duplication	>500 kb	SD	1.04	0.47	2.92	0.28
duplication	all	non SD	0.94	0.89	0.92	0.75
duplication	<100 kb	non SD	0.94	0.77	0.92	0.72
duplication	>100 kb	non SD	0.96	0.77	0.89	0.74
duplication	>500 kb	non SD	0.97	0.59	1.01	0.58

Table 8.5: Odds ratios of proportions of exonic CNV carriers in cases with older vs. younger fathers. OR=odds ratio, SD = segmental duplication. Patients were split in 2 groups based on paternal age >35 or <35 years. P-values are empirical p-values calculated for exonic CNVs by permutation analyses in PLINK, uncorrected for multiple testing.

			Rare (<1%)		Singleton	
CNV	**Size**	**SD flanking**	**Beta**	**P**	**Beta**	**P**
deletion	all	SD + non SD	0.000	0.903	0.001	0.857
deletion	<100 kb	SD + non SD	0.003	0.399	0.007	0.350
deletion	>100 kb	SD + non SD	-0.005	0.311	-0.013	0.272
deletion	>500 kb	SD + non SD	0.013	0.259	0.029	0.309
deletion	all	non SD	0.001	0.696	0.002	0.798
deletion	<100 kb	non SD	0.006	0.127	0.009	0.255
deletion	>100 kb	non SD	-0.017	0.033	-0.017	0.185
deletion	>500 kb	non SD	0.025	0.238	0.029	0.347
duplication	all	SD + non SD	-0.003	0.224	-0.002	0.815
duplication	<100 kb	SD + non SD	-0.002	0.694	0.002	0.843
duplication	>100 kb	SD + non SD	-0.005	0.181	-0.007	0.555
duplication	>500 kb	SD + non SD	-0.005	0.616	0.021	0.403
duplication	all	non SD	-0.004	0.195	-0.007	0.391
duplication	<100 kb	non SD	-0.004	0.424	-0.006	0.586
duplication	>100 kb	non SD	-0.005	0.292	-0.008	0.496
duplication	>500 kb	non SD	-0.011	0.488	0.023	0.410

Table 8.6: Paternal age as continuous predictor of the number of exonic CNVs a subject carries. SD = segmental duplication. Poisson regressions were performed with paternal age as continuous predictor variable, CNV count as outcome variable and ancestry and mean number of CNVs per plate as covariates. Since very few subjects carried more than 1 CNV >500kb, for these variables logistic regressions were performed with all subjects carrying 1 or more CNVs in the same outcome group. P-values are based on the Wald-chi square test statistic.

Gene by gene analysis

Next, we performed gene by gene analyses to investigate whether there were Refseq genes more often affected by CNVs of subjects with advanced paternal age. Nominally significant results were observed in the MGS sample for deletions in DEC1 (disrupted in esophagal cancer 1, p=0.005) and for duplications in PLN (Phospholamban), GLB1L2 and GLB1L3 (Galactosidase beta 1-like 2 and 3) and APBA2 (amyloid precursor protein-binding protein A2) (APBA2) (p=0.018 for each duplication) (see Table 8.7 and Figure 8.4). CNVs in these genes were also more prevalent in patients with old fathers compared to all MGS controls (for whom paternal age was unknown) with p-values ranging from 0.02-0.003). GLB1L2 and GLB1L3 are located head to tail on chromosome 11 and are affected by the same CNVs. DEC1 deletions were only observed in African-American subjects. Apart from DEC1, there were no indications that findings were related to effects of plate, DNA source or ancestry. In the additional samples combined, each of these CNVs was significantly more prevalent in cases with older vs. with younger fathers (Table 8.7), and DEC1 deletions and APBA2 duplications were also significantly more prevalent in schizophrenia cases than in controls (p=0.028 and p=0.0005, respectively).

	Deletion	Duplication	Duplication	Duplication
chr	9	6	11	15
bp start	116943917	118976134	133651484	27001131
bp end	117204744	118988280	133751427	27197808
gene	**DEC1**	**PLN**	**GLB1L3 /2**	**APBA2**
In MGS sample:				
n (old/young/ missing/control)	4/0/4/4	3/0/0/2	3/0/1/2	3/0/1/0
Frequency old	0.0070	0.0053	0.0053	0.0053
Frequency young	0	0	0	0
Frequency controls	0.0011	0.0006	0.0006	0.0000
OR Old v. Young	NA	NA	NA	NA
P Old v. Young	0.005	0.018	0.018	0.018
OR Old v. Control	6.36	9.52	9.52	NA
P Old v. Control	0.015	0.020	0.020	0.003
Size range in kb	11 - 17	24 - 306	60-140	85 - 1510
number of probes in range	7-11	8 - 83	23 - 48	31 - many
Near SD	no	no	no	yes
In DGV	yes	no	yes	yes
Mean Paternal age carriers	39.6	35.5	45.33	38.6
Mean Maternal age carriers	32.4	29.75	40.33	34.6
Including repliction samples:				
OR Old v. Young (patients)	NA	12.70	NA	12.70
P Old v. Young (patients)	0.003	0.013	0.014	0.013
OR Old v. Young (controls)	NA	NA	1.87	NA
P Old v. Young (controls)	1	1	0.460	1
OR Old v. Young (all)	NA	6.65	3.89	8.88
P Old v. Young (all)	0.009	0.092	0.043	0.035
OR Patient v. Control	3.88	3.88	0.77	NA
P Patient v. Control	0.028	0.190	0.784	0.0005

Table 8.7: genes affected more frequently in subjects with advanced paternal age chr= chromosome, bp start = start of the gene, bp end = end of the gene, n = number of subject carrying this cnv, (old/young/missing/control)= groups of old-father-cases, young-father-cases, cases with missing paternal age data and healthy controls (for which no paternal age information was available). OR= odds ratio, P = p-value of chi square test (uncorrected for multiple testing), NA= not applicable, SD = segmental duplication, DGV = database of genomic variants.

Figure 8.4 (DEC1)

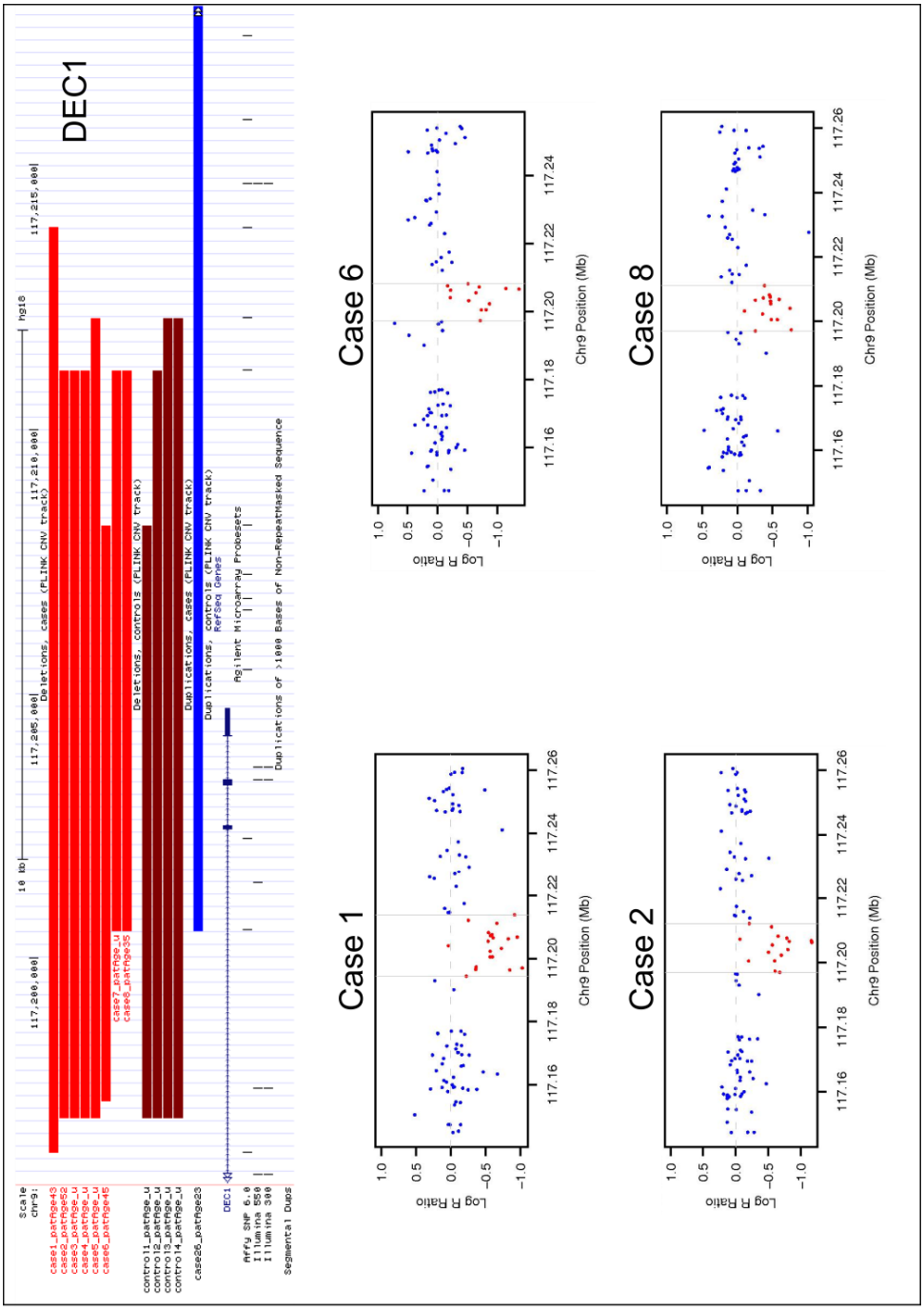


Figure 8.4 (PLN)

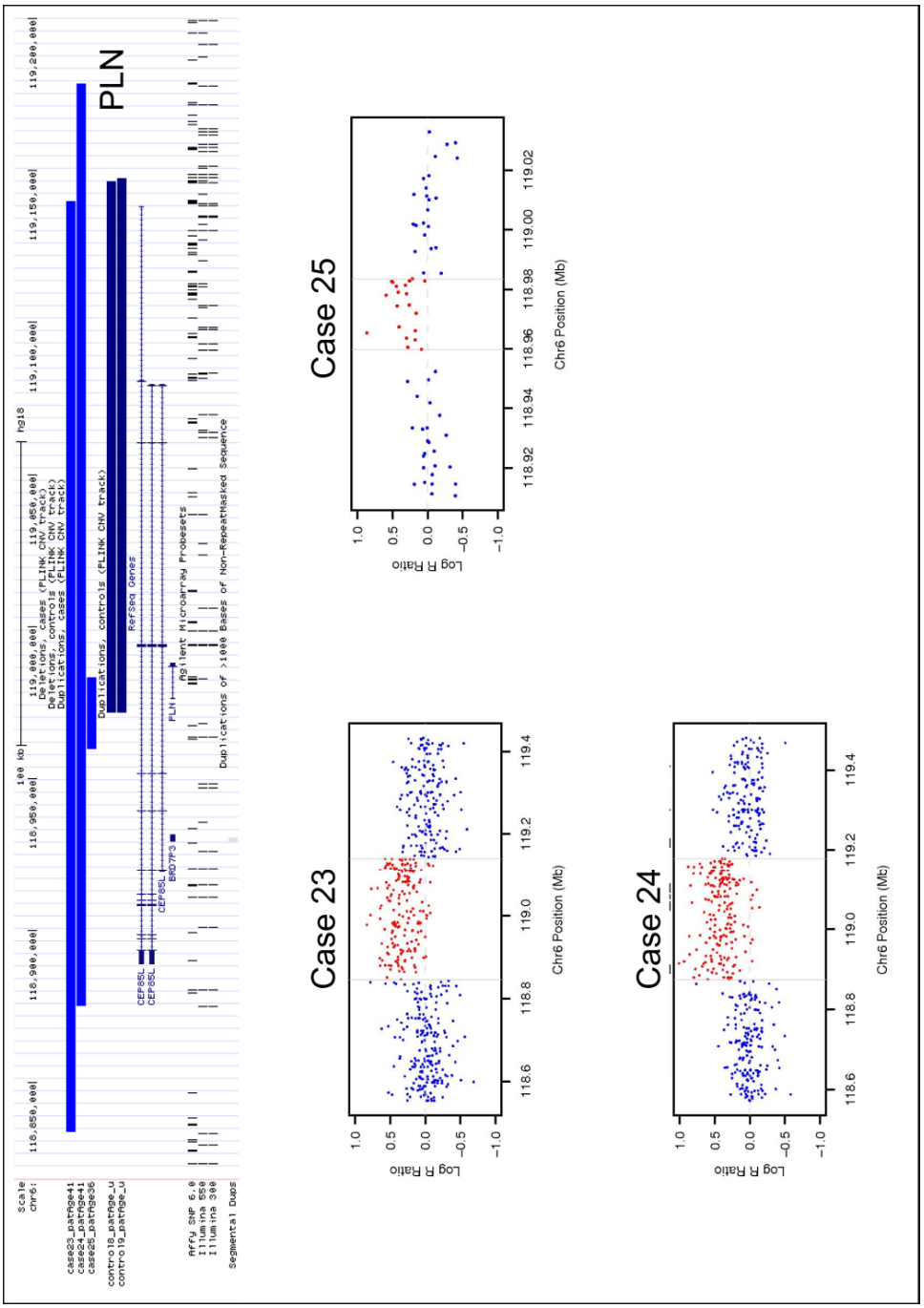


Figure 8.4 (GLB1L2/3)

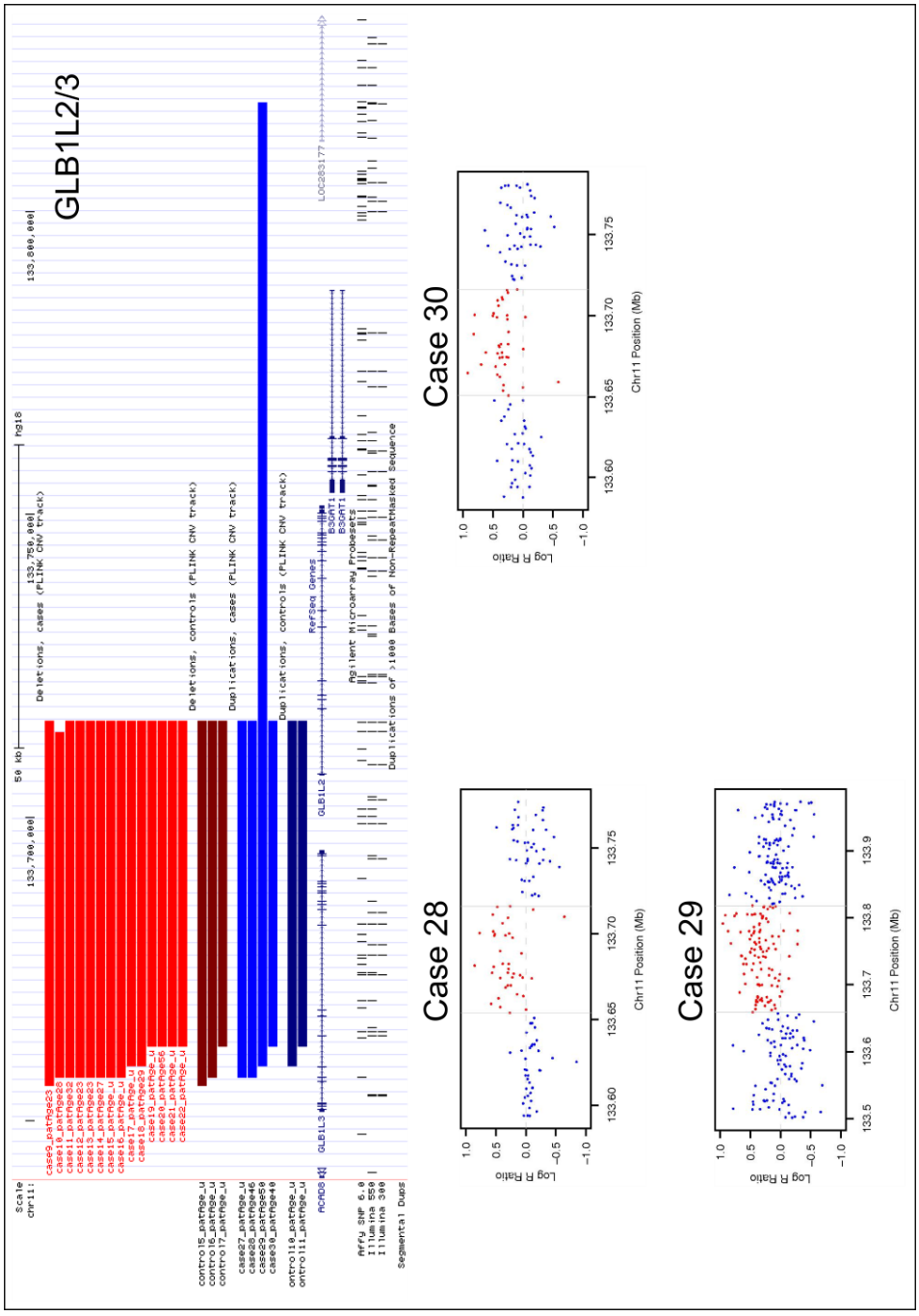
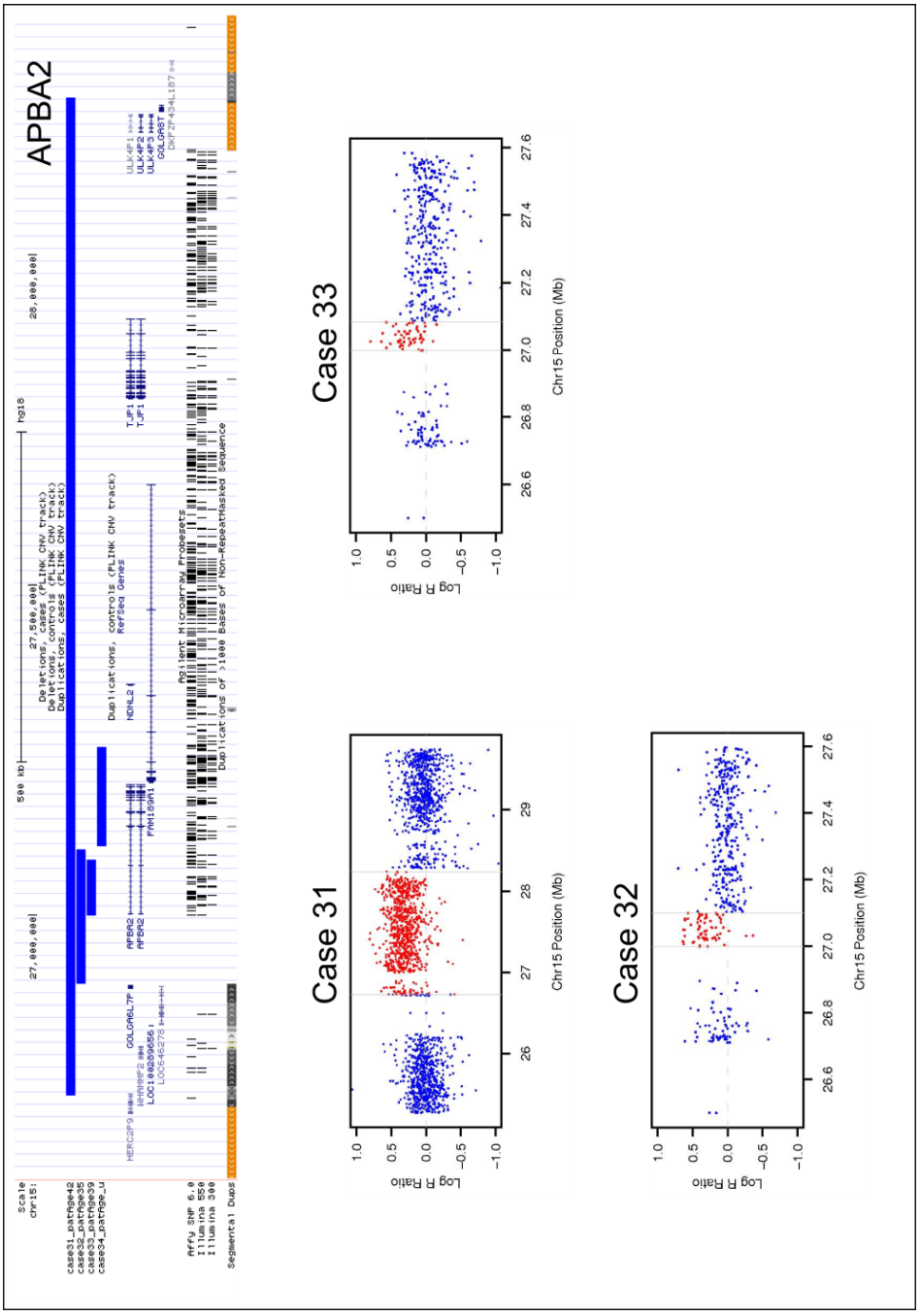


Figure 8.4 (APBA2)



Gene-set enrichment

No significant enrichment of CNVs in any of the four defined (potentially spermatogenesis-related) gene sets in cases with older fathers, see Table 8.8.

Pathway	All deletions		All duplications		Deletions >500kb	
	OR	P	OR	P	OR	P
Sperm	0.201	0.9737	0.378	0.1605	1.278	0.5627
RAS	0.610	0.0845	0.488	0.1203	0.938	0.1308
Negreg	0.317	0.4861			0.938	0.8064
Posreg			0.458	0.2686		

Table 8.8: Pathway enrichment The gene-set-enrichment test for CNVs in PLINK was used to test enrichment of 4 gene sets. The gene sets were based on the annotation of genes to Gene Ontology categories of 'spermatogenesis (sperm, GO:0007283, 397 genes)', 'regulation of RAS GTPase activity' (RAS, GO:0032318, 162 genes), 'negative regulation of cell cycle' (negreg, GO:0045786, 456 genes) and 'positive regulation of cell cycle' (posreg, GO:0045787, 110 genes). Negreg was only tested for deletions and posreg only for duplications.

Discussion

Schizophrenia is associated with advanced paternal age and this effect is possibly related to a larger number of mutations in the sperm of older males [136, 162]. Therefore, we investigated whether schizophrenia patients with older fathers carry more CNVs than patients with younger fathers. In the stage I analysis in the MGS sample, we found suggestive evidence that patients with old fathers carry more deletions >500 kb not flanked by segmental duplications than patients with younger fathers (OR=1.87, p=0.05 for rare deletions, OR=3.26, p=0.02 for singleton deletions). No significant differences in burden of smaller deletions or duplications of any size were observed. The excess of rare, large deletions in subjects with older fathers was then investigated in 2 additional samples (adding 694 patients and 6,615 controls). A similar effect was observed (OR=1.84, p=0.028). The association was of the same magnitude and direction in the patient and control samples and is thus independent of disease status. This suggests that the increase in large deletions may not always increase schizophrenia risk. Instead, disease risk may depend on the genomic location of the deletions.

Interestingly, rare, large deletions not flanked by SDs constitute the CNV measure with the highest chance of being *de novo* and of paternal origin. In a sample of schizophrenia trios, 33% of the rare, large deletions were *de novo* [131]. Many *de novo* mutations are smaller than 500kb, but these cannot be distinguished from inherited CNVs without parental genotype information, which was unavailable for these samples. Our results are in concordance with the hypothesis that the number of *de novo* CNVs is increased, but this might not be the only explanation of the finding. A role for large, inherited deletions, as well as other genetic variants or interaction with socioeconomic factors is also possible.

Furthermore, five genes were more prevalent in CNVs of patients with old fathers compared to patients with younger fathers and compared to controls in the MGS sample. Adding the data of the replication sample, it was observed that for all genes the paternal age effect was stronger in the patients than in the controls. It should be noted that due to different genotyping platforms used, small CNVs could have gone undetected in the Dutch controls sample, influencing the likelihood to find CNVs in the candidate genes. In our samples, DEC1 and APBA2 showed some evidence for association with schizophrenia disease status, while for PLN and GLB1L2/3 the paternal age effect was independent of a disease effect. Duplications in APBA2 have previously been associated with schizophrenia [130, 172] and autism [12] and deletions in APBA2 with mental retardation [206]. Deletions in GLB1L2/3, rather than duplications, were previously reported to relate to schizophrenia (in the MGS sample) [148]. A summary of the functional characteristics of these genes can be found in Table 8.9. All genes show at least some expression in the brain, as well as in germ stem cells. DEC1 and APBA2 have neuronal functions in circadian rhythms and synapse

functioning, respectively. The dual role of these genes in germ cells and the brain fits the selfish sperm selection hypothesis for schizophrenia.

In the pathway analyses, we observed no significant enrichment of spermatogenesis-, RAS or cell cycle related genes in the CNVs of patients with old fathers compared to patients with younger fathers. The GO categories probably do not capture all possible genes involving these processes. For example, fibroblast growth factor 2 (FGF2) and glial derived growth factor (GDNF) are essential for sperm stem cell survival in culture [91] and their expression might influence sperm stem cell proliferation *in vivo* as well. However, FGF2 and GDNF are not included in any of our GO categories. Therefore, our findings do not exclude the possibility that selfish sperm selection plays a role in the paternal age effect observed in schizophrenia patients.

The findings of this study coincide a prior functional hypothesis. However, there are some limitations worth mentioning. We were unable to correct our analyses for possible confounders such as IQ and socioeconomic status. Furthermore, there were considerable differences in genotyping and CNV calling algorhythms between the samples. Birdsuite is especially prone to call more small CNVs [129]. For the CNVs in the candidate genes, we observed a 93% overlap between Birdsuite and PennCNV calls in the MGS sample. Moreover, CNVs >500kb might be less prone to false positive calls. Therefore, we do not believe that our finding of more rare deletions >500kb in subjects with older fathers is primarily attributable to CNV calling errors.

The association between paternal age and CNV burden was previously investigated in the Dutch control sample [35], with no significant findings. In the current study, a suggestive association of paternal age with rare, large deletions not flanked by SDs was observed in the same sample. This difference can be explained by the use of different statistical methods (2 instead of 8 age categories) and a focus on CNVs not flanked by SDs in our study.

To summarize, subjects with older fathers carry on average almost 2 times more rare, large deletions not flanked by SDs compared to subjects with younger fathers. This effect was independent of schizophrenia disease status. Several questions are related to this finding. First, is this excess in large deletions due to *de novo* deletions? Since large deletions have a relatively high chance of being *de novo*, the findings may suggests that the association with advanced paternal age may be driven by effects of *de novo* CNVs, rather than inherited CNVs. We do not currently have access to large datasets of parent-proband trios to answer this question, but we will attempt to pursue this analysis in the future. Secondly, do these deletions survive because they increase the survival of sperm stem cells? No supporting evidence for this notion was found in this study. However, inadequate annotation of genes and pathways involved in sperm stem cell renewal may

have limited the ability to find these effects. And the last question is: do any of the paternal age-related deletions subsequently increase the risk of schizophrenia? No differential effect was observed in schizophrenia patients and healthy controls, but fewer patients were analyzed and there were technical differences between patient and control samples. We hypothesize that only a small proportion of the paternal age-related CNVs may increase schizophrenia risk. Therefore, much larger samples would be necessary to demonstrate such an association for either single CNVs or in aggregate for this class of CNVs. Whether or not a CNV will increase schizophrenia risk, may depend on the genes affected by these deletions. Since autism is associated with advanced paternal age, more *de novo* mutations and with RASopathies [144, 151, 272], it will be interesting to study the association between paternal age and CNVs disrupting RAS-related genes for this disease as well.

	Full name	Alternative names	Association to disease	Expression in testis	Expression in brain	Function in sperm	Function in brain	Function in cell cycle / cancer	Other functions
DEC1	Disrupted in esophagal cancer 1	STRA13 (mouse), SHARP2 (rat)		+	++	?	circadian rhythm	differentiation, apoptosis, associated with various cancers	
PLN	Phospho-lamban			++	+	?	?	?	cardio-myopathy, muscle contractility
GLB1L2/3	Galactosidase beta 1 like 3			++	++	?	?	oligodendro-cytoma, cervicalretina cancer	lactose metabolism, differentiation
APBA2	amyloid precursor protein-binding protein A2	MINT2	associated to SZ, autism, Alzheimer	++	+++	?	synaptic exocytosis, neurite outgrowth	silenced in gastic and colorectal cancer; marker of endometrial carcinoma	

Table 8.9: Functional characteristic of candidate genes Expression levels were based on UCSC data: + = some expression, ++ = high expression, +++ = very high expression. SZ = schizophrenia

Chapter 9

Summary and discussion

Summary

Fundamental research on schizophrenia may lead to new insights in the nature of this disorder and thereby to novel treatment strategies. Since schizophrenia has a high heritability and techniques to study variation in DNA have greatly improved in the last decade, genetics is one of the most promising research fields in schizophrenia. While increasing numbers of genetic risk factors become known, it is still unclear what their functional effects are and which biological processes they disturb. One way to study this is to investigate whether schizophrenia-related genetic variants affect intermediate phenotypes related to schizophrenia. In this thesis, several approaches were used to explore these relationships, including the study of candidate genes, polygenic schizophrenia risk scores, copy number variants and a knockout mouse model. Here I will summarize the findings of these studies and discuss their strengths and limitations. Lastly, I will give some directions for future research.

FGFs

The first three chapters focus on fibroblast growth factors (FGFs) as candidate genes for schizophrenia. In **Chapter 2**, the neuronal functions of FGFs and their associations with psychiatric disorders are reviewed. FGFs control brain patterning, neuro- and gliogenesis, axon outgrowth, myelinogenesis from early development into adulthood. They are key candidates to modulate the impact of environmental factors like stress. Mutant mice for FGFR1, FGFR2, FGF2 and FGF17 showed neuronal and behavioral abnormalities similar to those seen in psychiatric patients, such as reduced brain volume, hyperactivity, impairments in cognition and aberrant social behaviors.

These interesting functions of the FGF family prompted us to further investigate the role of genetic variants in FGF genes in schizophrenia patients. Since schizophrenia patients show smaller brain volumes and FGFs affect brain growth, we studied the effect of SNPs in the FGF system on brain volume. **Chapter 3** describes that we found no evidence for common genetic variants in FGFs influencing brain volume in schizophrenia patients, nor in healthy controls. While this study had sufficient power to detect large effects, the power for smaller effects was limited. The results of this study should therefore be considered explorative rather than as prove that the FGF system has no role in brain and intracranial volume in humans.

In **Chapter 4**, we report a knockout mice study that investigates the effects of deletion of Fgf2 and its interaction with early life stress. Mice lacking Fgf2 showed increased locomotor activity under baseline conditions and increased aggression towards same-sex intruder mice. Interestingly, early life stress normalized the locomotor activity levels of Fgf2 deficient mice, perhaps due to increased sheltering behavior. In addition, early life

stress was associated with reduced neuronal differentiation in the hippocampus and Fgf2-/- mice were shown to be especially sensitive to this. Aggressiveness of Fgf2-/- mice was not altered by early life stress and correlated with lower Gad1+ cell densities in the hippocampus. The relative mild, but specific phenotypic defects of Fgf2 disruption, as well as the increased responsiveness to stress make the mouse model an interesting model for evaluation of gene x environment interactions on psychopathology.

Polygenic score

In the next two chapters, the polygenic score method was used to investigate the combined effects of a genome-wide set of SNPs on schizophrenia endophenotypes. Using a summarized score for genetic risk, rather than to test each SNP separately, reduces multiple testing problems. Odds ratios and p-values for genome-wide SNP data were calculated in the sample collected by the Psychiatric GWAS Consortium. These were used to calculate individual polygenic schizophrenia risk scores (PSSs) in our independent sample. In **Chapter 5**, we describe the association of the PSS with brain volume measured on structural MRI scans. The PSS was significantly associated with total brain volume and white matter volume, equally in patients and controls. A group of 186 SNPs showed most evidence for association with schizophrenia and white matter volume and an explorative functional analysis showed that these SNPs were located in genes with neuronal functions.

We then used the same method to investigate the genetic overlap between schizophrenia and intelligence, as described in **Chapter 6**. Remarkably, the PSS was neither associated with IQ within the patient group, nor within the healthy control group. So, while both brain volume and IQ are strongly related to schizophrenia and are highly heritable, they are not influenced by schizophrenia-related SNPs in the same way. We propose a model in which schizophrenia-related genes primarily affect brain growth and maturation. This could result in higher vulnerability to environmental factors, which may in turn give rise to cognitive defects and schizophrenia symptoms.

CNVs

The last three chapters focus on copy number variations (CNVs). Excess of rare CNVs and particularly of deletions in schizophrenia patients was previously reported. Several specific deletions related to schizophrenia, e.g. on chromosome location 1q21.1 and 22q11, were associated with intellectual disabilities and reduced total brain volumes [33,271]. We hypothesized that not only CNVs at these specific locations, but also global deletion or duplication burden were related to the schizophrenia endophenotypes brain volume and IQ.

In **Chapter 6** we report no association between global deletion and duplication burden and IQ in schizophrenia patients and healthy controls. We also did not observe an association between global deletion and duplication burden and brain volume in these

groups, as described in **Chapter 7**. It is possible that *de novo* CNVs, rather than global CNV burden, are associated with brain volume and IQ. Studying *de novo* CNVs would require genotype data of both parents, which was not available.

In the last chapter, **Chapter 8**, a study on the association between CNVs and paternal age in schizophrenia patients is described. Schizophrenia patients have on average older fathers than healthy controls. We report that subjects with older fathers (35+ years at the time of birth) carry around 2 times more rare, large deletions compared to subjects with younger fathers, while there was no change in the numbers of other types of CNVs. The effect was observed for both schizophrenia patients and healthy controls. These rare, large deletions have a higher chance to be derived *de novo* than the average CNV. Therefore, a possible mechanism is that *de novo* mutations accumulate in the sperm of older males. The exact location and genes involved may determine whether subjects get ill or not.

Discussion

While a wide variety of methods was used in the studies of this thesis, there are some considerations that are general to this type of clinical genetic research. A strength of the reported studies is that they focus on phenotypes of which the variation is likely to be largely determined by genetic factors. The heritability of schizophrenia, brain volume and IQ (around 80, 90 and 70% respectively) are among the highest found in psychiatric phenotypes. Both brain volume and IQ show a robust association with schizophrenia. While this may seem a logical prerequisite for genetic studies, many studies are focused on substantially less heritable phenotypes.

Another advantage of brain volume and IQ as phenotypes is that they are objectively assessed, quantitative phenotypes. This may increase power in statistical analyses compared to, for example, the presence of acoustic hallucinations.

Furthermore, it was thought that genetic variants have larger effects on endophenotypes than on the disease itself. Endophenotypes might be less heterogeneous and are assumed to involve the same biological pathways as the disease, but are less removed from the relevant gene action [2]. However, recent genome-wide studies of brain volumes suggest that these phenotypes are still genetically complex, and that genetic effects may be subtler than expected [103].

A disadvantage of using intermediate phenotypes is that they usually require more labor-intensive data acquisition and that data from different sites (i.e. different MRI scanners)

may be difficult to combine. This results in substantially smaller samples compared to disease-status-only samples. In all our studies, we have therefore tried to find a balance between the number of tests performed and sample size, to retain sufficient power. Generally, we have included few phenotypic measures and a variety of genetic measures. So, we choose total IQ and not all intelligence subtests, such as working memory and processing speed and total brain volume, rather than up to 40 regional brain volumes. This is because the global measures are more reliably assessed, have a higher heritability and were shown to be equally or better endophenotypes for schizophrenia than the sub-measures [7,234]. However, should effects be limited to specific cortical regions, or specific cognitive subtasks, we may have missed them. On the genetic part, we tried to retain power by investigating global CNV burden rather than individual genes affected by CNVs and by testing a polygenic risk score and a selected set of SNPs in candidate genes instead of testing a genome-wide SNP set.

Furthermore, certain confounders can hardly be avoided and have potentially affected the results. For example, schizophrenia patients often use medication, are more likely to smoke, use illicit drugs and have a passive life style. These factors can potentially affect their brain volume and intelligence measurements. This can obscure true genetic effects, but also lead to spurious association. Therefore, we also studied the effects of the genetic variants within the patient and control groups separately. The clinical heterogeneity of the disorder is also important in this aspect. Different genetic factors may predispose to subtypes of schizophrenia. Since all these subtypes are combined in our sample, some real genetic effects may have been obscured. On the other hand, the controls participating in these studies tend to be healthier and more intelligent than the subjects in a true population sample. Therefore, they might carry less CNVs or schizophrenia-related SNPs than the average person, which leads to an overestimation of genetic effect sizes.

Lastly, a word on the use of mice models for schizophrenia genetics. Mice models are well suited to study the effect of genetic variants on several schizophrenia-related neurobiological phenotypes, such as memory deficits, brain volume reductions or alterations in Pv+ neurons. While in patients brain tissue is inaccessible, in mice the alterations in neuron morphology and function can be studied. These alterations can be related to behavioral abnormalities and the modification of these abnormalities by drugs can be tested. Another advantage is the ability to control the genetic background, living environment and food intake of these mice, in order to minimize inter-individual variation other than the one under investigation.

A disadvantage is of course the use of live stock for experiments. According to Dutch law this is considered acceptable if the suffering of the animals is kept to a minimum and the possible benefits of the study are substantial. Since schizophrenia includes many subjective symptoms and affects primate- or human-specific abilities like language and

self-regulation, a full schizophrenia mouse model is impossible to develop. In general, animal studies on candidate genes for schizophrenia like the one described in **Chapter 4**, should be considered an exploration of the basic functions of the gene in the brain and in behavior, rather than as a model for schizophrenia.

Future directions

Genetic and neuroscience studies are beginning to unravel the genetic underpinnings and affected neurobiological processes of schizophrenia. However, more research is needed before insights can be translated to novel treatment strategies for patients. I would like to finish with some suggestions for future research on this topic.

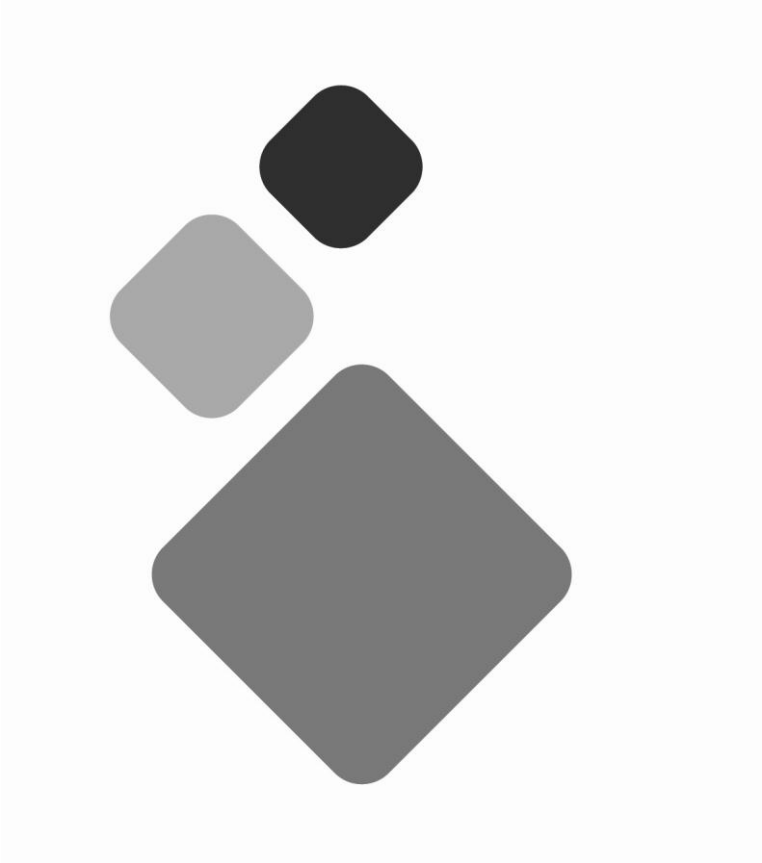
First, genetic studies on complex disorders show that very large samples are needed to detect associated variants with statistical significance. In order to make endophenotype studies work, it is important to collect data on these phenotypes at the same large scale as data on disease status. Technical difficulties hindered the aggregation of MRI data from different sites. When this becomes feasible, this can greatly improve the possibilities of genetic studies on brain volume. An example is the ENIGMA consortium that recently performed genome-wide association meta-analyses on hippocampal, brain and intracranial volume .

Furthermore, both phenotypic and genotypic measurements can be improved. For example, higher resolution structural and functional MRI scans and advanced morphologic studies on post-mortem brains of schizophrenia patients might help to better describe the neurobiological abnormalities of schizophrenia patients. Another interesting development is the use of pluripotent stem cells obtained from a skin biopsy from schizophrenia patients. These stem cells can be converted into neurons and studied for abnormalities and sensitivity to medication.

The genotypic measures can be improved to integrate SNP genotypes with functional data on RNA expression or the biological pathways that they are involved in. Sequencing techniques can help to identify more rare genetic variants. When the DNA of the parents of the patients is sequenced too, it is possible to detect *de novo* SNPs and CNVs. In addition, interactions between genetic variants and environmental risk factors should be included when possible.

To conclude, we studied the effects of schizophrenia-related genetic variants on intermediate phenotypes of schizophrenia. The strongest effect was found for a polygenic schizophrenia risk score on brain volume. In future studies, we will perhaps be able to

specify the brain regions and genes involved in this association. In the long run, it could be investigated whether modification of the processes involved is beneficial to schizophrenia patients.



Nederlandse samenvatting

Schizofrenie

Dit proefschrift beschrijft mijn promotieonderzoek over de genetische achtergronden van schizofrenie. Schizofrenie is een ernstige psychiatrische aandoening, die niet alleen gepaard gaat met grote lijdensdruk voor de patiënten en hun familie, maar ook met hoge kosten voor de maatschappij door zorgkosten en verminderde productiviteit. De symptomen zijn onder te verdelen in twee clusters. Om te beginnen zijn er de negatieve symptomen, zo genoemd omdat het over dingen gaat die normaal aanwezig zijn, maar die patiënten kwijt raken. Dit zijn bijvoorbeeld vervlakte emoties, sociale teruggetrokkenheid, spraakarmoede en verlies van motivatie. Daarnaast kunnen patiënten episodes van psychose doormaken met hallucinaties, wanen en verwarde spraak. Dit zijn de positieve symptomen. Symptomen ontstaan meestal vanaf de jonge volwassenheid en komen bij 0,5-0,9% van de bevolking ooit in het leven voor. Zowel genetische factoren als omgevingsfactoren, waaronder cannabisgebruik, complicaties tijdens de zwangerschap of geboorte en jeugdtrauma, kunnen bijdragen aan het ontstaan van schizofrenie. Deze factoren kunnen elkaar ook versterken, iemand kan bijvoorbeeld een genetische gevoeligheid hebben voor de effecten van cannabis.

Alle huidige antipsychotica zijn effectief in het verminderen van positieve symptomen, maar er bestaat geen behandeling tegen negatieve symptomen. Dit is teleurstellend, omdat vooral de ernst van de negatieve symptomen het functioneren van de patiënt bepaalt. Het is daarom van groot belang betere behandelingen te ontwikkelen. Tot nu toe is dit moeilijk gebleken, omdat we nog maar weinig weten van de achtergronden van schizofrenie en welke hersenprocessen de symptomen veroorzaken. Fundamenteel onderzoek naar het ontstaan van schizofrenie kan hier inzicht in geven en daarmee uiteindelijk bijdragen aan de ontwikkeling van nieuwe behandelingen.

Genetisch onderzoek wordt in dit opzicht als veelbelovend beschouwd. Dit komt allereerst omdat het ontstaan van schizofrenie in behoorlijke mate erfelijk bepaald is. Familieleden van een schizofrenie patiënt hebben een grotere kans deze ziekte ook te krijgen, variërend van 9% voor broers en zussen tot 45% voor eeneiige tweelingbroers en -zussen. De mate waarin een ziekte door genen bepaald wordt, wordt ook wel uitgedrukt in het erfelijkheidsgetal. Dit getal beschrijft in hoeverre of iemand de ziekte krijgt wordt bepaald door erfelijke factoren (in een bepaalde populatie). De erfelijkheid van schizofrenie is 80%, erg hoog voor een ziekte. Een tweede reden voor de populariteit van genetisch onderzoek is dat de technieken om DNA te bestuderen de laatste jaren sterk verbeterd zijn. Grootschalige genetische studies zijn hierdoor haalbaar en betaalbaar geworden.

Genetische studies naar schizofrenie

Elke cel in ons lichaam bevat hetzelfde erfelijke materiaal, wat is verdeeld over 46 chromosomen. De chromosomen bestaan uit lange strengen desoxyribonucleïnezuur

(DNA), waarin vier nucleotiden (bouwstenen) elkaar afwisselen: A, C, G en T. Een gen is een functionele eenheid binnen een chromosoom en is een recept voor een eiwit. De volgorde van de nucleotiden in een gen bepaalt welk eiwit er gemaakt kan worden. Bij elkaar hebben mensen ongeveer 24.000 genen. Alle erfelijke informatie in een cel samen heet het genoom.

Het merendeel van ons DNA is identiek tussen twee mensen en zelfs met mensapen hebben we zo'n 95% gemeenschappelijk. Op een aantal plekken bestaat er wel variatie. Hier heeft bijvoorbeeld 30% van de mensen een A en 70% van de mensen een T. Deze plekken heten *single nucleotide polymorphisms* (SNPs, spreek uit: snips). Het aantal SNPs in het menselijke genoom wordt geschat op 10 miljoen. Ook al gaat het om kleine verschillen, soms kan het wel leiden tot een eiwit dat veel korter of minder functioneel is. En soms is niet de geteste SNP zelf, maar een sterk gecorreleerde naburige SNP of andere genetische variant de eigenlijke boosdoener. Door SNPs verspreid over alle chromosomen te testen, kunnen we zo een idee krijgen van alle veel voorkomende genetische variatie. Soms kan een enkele ongunstige SNP op een specifieke plek al leiden tot een ziekte, zoals bijvoorbeeld bij taaislijmziekte het geval is. Andere ziekten, zoals hart- en vaatziekten, diabetes of schizofrenie, ontstaan door een combinatie van vele genetische risico varianten.

Sinds een paar jaar zijn er tests beschikbaar die tot een miljoen SNPs per persoon tegelijk kunnen meten. Genoomwijde associatie studies (GWAS) maken gebruik van deze tests en vergelijken de aanwezigheid van SNPs tussen patiënten en gezonde mensen. De eerste drie grote GWAS over schizofrenie werden gepubliceerd in het prominente tijdschrift Nature in 2009. Sindsdien heeft het genetische onderzoek een grote vlucht genomen.

Van dit soort studies hebben we een aantal belangrijke dingen geleerd. Om te beginnen viel op dat SNPs het risico op ziekte meestal maar een heel kleine beetje verhogen. Als het risico op schizofrenie met de gunstige variant gemiddeld 0,9% is, dan is het voor iemand met de risicovariant 1%. Dit geldt overigens niet alleen voor schizofrenie, maar voor de meeste ziektes. Hoewel de bijdrage van individuele SNPs aan het ontstaan van schizofrenie dus niet groot is, kunnen we uit de clustering van risico SNPs in bepaalde genen of netwerken van genen wel veel leren over het ontstaan van deze ziekte. Omdat de effecten van SNPs zo klein zijn, zijn er hele grote studies nodig (met 10.000, of nog beter: 100.000 proefpersonen) om ze aan te tonen. De grote hoeveelheid SNPs maakt dat we onbevooroordeeld het hele genoom kunnen testen, dat wil zeggen zonder aannames over welk type genen er bij betrokken zullen zijn. Het maakt het echter ook moeilijk om de paar belangrijke SNPs te vinden tussen al die SNPs zonder effect.

Met de SNP-data kunnen we ook nog een ander soort genetische variatie bestuderen, namelijk *copy number variants* (CNVs). Normaal gesproken heeft iemand van elk stuk DNA

twee kopieën, één van zijn vader en één van zijn moeder gekregen. Soms gaat er echter iets mis en heeft iemand maar nul of één kopieën (een deletie) of drie of vier kopieën (een duplicatie), voor duizend tot miljoenen nucleotiden achter elkaar. Omdat deze CNVs meerdere genen in zijn geheel kunnen verstoren, zijn hun effecten vaak groter dan die van SNPs. De meeste CNVs (op specifieke locaties in het genoom) zijn heel zeldzaam, maar −paradoxaal− de meerderheid van de mensen heeft wel ergens in zijn genoom een CNV.

De grootste bekende risicofactor voor schizofrenie is een CNV op de lange arm van chromosoom 22 (het 22q11-deletiesyndroom, ook wel velocardiofaciaal of DiGeorge syndroom geheten). Dragers van deze CNV hebben een 20-30x verhoogd risico op schizofrenie. Ongeveer 1% van alle schizofreniepatiënten bezit deze mutatie. Andere nu bekende CNVs verhogen het risico niet zo sterk als deze, maar wel sterker dan SNPs. Huidige genetische studies zijn nog niet geschikt om varianten te vinden die én zeldzaam zijn én een klein effect hebben, maar deze bestaan vermoedelijk wel. Al met al wordt er gedacht dat schizofrenie veroorzaakt kan worden door een combinatie van honderden tot duizenden veel voorkomende genetische varianten met een klein effect en een verzameling zeldzame unieke varianten met grotere impact.

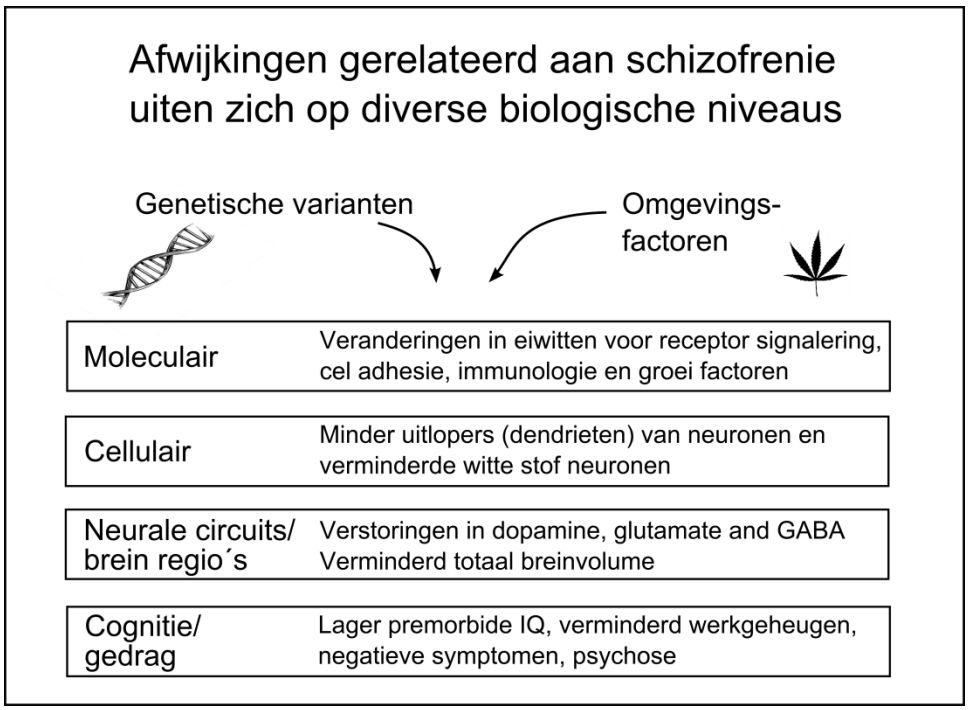
Hoewel we nu dus voor het eerst een rol voor specifieke genetische varianten in schizofrenie hebben kunnen aantonen, blijft er nog veel te onderzoeken. Alle bekende risicovarianten bij elkaar verklaren maar een klein deel van de hoge erfelijkheid van schizofrenie. Of in andere woorden: het risico op schizofrenie voor familieleden van patienten is hoger dan je alleen op grond van overeenkomst in de bekende risico-SNPs zou verwachten. Ook veel gezonde mensen zijn drager van dergelijke risicovarianten. Daarom is genetisch onderzoek nog niet geschikt om vast te stellen of iemand deze ziekte heeft of zal krijgen.

Ook is het vaak nog onduidelijk welke functionele effecten deze genetische varianten hebben. Hebben de veranderde eiwitten een gelijksoortige functie? En op welke celtypes of breinregio's dan? En hoe leidt een verstoorde ontwikkeling van deze breinregio's tot symptomen? Het beantwoorden van deze vragen zal een belangrijke plaats innemen in het schizofrenie onderzoek van de komende jaren (of decennia).

Endofenotypes

Voordat een verandering in het DNA tot ziekte heeft geleid, zijn er vele tussenstappen gemaakt. Allereerst leidt een genetische variant tot een andere vorm of hoeveelheid van een eiwit. Schizofrenie is geassocieerd met veranderingen in genen voor eiwitten die te maken hebben met het contact tussen zenuwcellen (neurotransmitters), groeifactoren en eiwitten die een rol spelen bij de afweer. Deze veranderde eiwitten kunnen de groei en functie van cellen beïnvloeden. De zenuwcellen (neuronen) van schizofrenie patiënten hebben bijvoorbeeld gemiddeld minder uitlopers. De veranderde cellen kunnen op hun

beurt leiden tot verandering in lokale breinprocessen, waardoor er bijvoorbeeld te veel dopamine of te weinig glutamaat (beide neurotransmitters) vrijkomt. Alle kleine veranderingen samen tellen op tot grotere veranderingen in de structuur en functie van de hersenen. Bij schizofreniepatiënten zien we bijvoorbeeld gemiddeld 3% kleinere breinen, een lager IQ (al voorafgaand aan de eerste psychotische symptomen) en problemen in het werkgeheugen. Deze veranderingen in het brein zorgen samen voor de symptomen van schizofrenie.



De kenmerken die niet de kernsymptomen zijn van een ziekte, maar er wel mee geassocieerd zijn noemen we intermediaire fenotypes. Endofenotypes zijn intermediaire fenotypes met een aantal eigenschappen die voordelig zijn voor gebruik in genetische studies. Endofenotypes moeten sterk genetisch bepaald worden, samen overerven met ziekte in aangedane families en redelijk stabiel zijn over de tijd. Het kan een aantal voordelen hebben om endofenotypes te onderzoeken in plaats van de ziekte zelf. Ze kunnen bijvoorbeeld makkelijker of betrouwbaarder te meten zijn of sterker samenhangen met de genetische varianten.

Breinvolume gemeten op een MRI-scan en IQ zijn voorbeelden van endofenotypes van schizofrenie. Wij hebben gekozen om deze te bestuderen, omdat ze sterk met schizofrenie samenhangen, op voldoende grote schaal meetbaar zijn en beide sterk door genetische factoren bepaald worden.

Onderzoek in dit proefschrift

FGFs

Alles bij elkaar zijn er miljoenen erfelijke varianten die theoretisch met schizofrenie samen kunnen hangen. Als we deze allemaal zouden testen, kunnen we de varianten met echt effect niet meer onderscheiden van de ruis veroorzaakt door het uitvoeren van zo veel tests. We moeten dus een selectie maken. Dit hebben we op een aantal manieren gedaan. Allereerst hebben we gekeken naar een aantal 'kandidaatgenen', die door hun belangrijke functie in het brein kandidaten zijn om een rol te spelen bij de ontwikkeling van schizofrenie. In dit proefschrift hebben we gekeken naar de rol van fibroblast groeifactoren (FGFs). **Hoofdstuk 2** geeft een overzicht van de functies van FGFs in het brein en in gedragsafwijkingen. FGFs controleren hoe het brein zich ontwikkeld vanaf de vroegste ontwikkeling tot in de volwassenheid. Ze zijn ook belangrijk in het herstel van stress. Afwijkingen in het FGF-systeem zijn gevonden bij psychiatrische patiënten en kunnen afwijkend gedrag en breinontwikkeling bij muizen veroorzaken, bijvoorbeeld kleinere breinen, hyperactiviteit en geheugenproblemen.

Omdat schizofreniepatiënten gemiddeld kleinere breinen hebben dan gezonde mensen en FGFs de groei van het brein beïnvloeden, wilden we onderzoeken of afwijkingen in FGFs ook een rol speelden bij schizofreniepatiënten. In **hoofdstuk 3** wordt beschreven dat we geen bewijs hebben gevonden dat veel voorkomende variatie in FGF genen het breinvolume van schizofreniepatiënten of gezonde mensen beïnvloedt.

Een lastigheid bij het bestuderen van patiënten (en mensen in het algemeen) is dat ze niet alleen verschillen in het ziek zijn, maar ook verschillen in hun hele genetisch opmaak en in de dingen die ze hebben meegemaakt tijdens hun leven, zoals stress of gebruik van medicijnen en drugs. Deze factoren kunnen ook breinvolume en IQ beïnvloeden en de studies dus verstoren. Om deze lastigheden te omzeilen kan gebruik worden gemaakt van diermodellen.

Muizen worden veel gebruikt voor dit soort studies, omdat het mogelijk is om bij hun een specifieke genetische verandering te induceren. Bij muizen met en zonder een genetische verandering kan worden gekeken of ze verschillen in breinontwikkeling en gedrag, terwijl alle andere omstandigheden gelijk worden houden. Een ander voordeel van het gebruik

van muizen is dat hersenmateriaal beschikbaar is voor analyse, wat bij mensen natuurlijk niet zo is. Aan de andere kant zijn er ook overduidelijke verschillen tussen muizen en mensen in hun breinen. We zullen om die reden nooit een 'muis met schizofrenie' kunnen maken. Wat we wel kunnen doen, is kijken of een kandidaatgen bij muizen invloed heeft op de neuronen en hersenprocessen die verstoord zijn bij mensen met schizofrenie. Wij hebben gekeken of gebrek aan fibroblast groeifactor 2 (FGF2) muizen gevoeliger maakt voor stress op jonge leeftijd.

Hoofdstuk 4 doet verslag van deze muizenstudie. De muizen zonder FGF2 waren hyperactief en sneller agressief naar andere muizen. Als de muizen zonder FGF2 waren blootgesteld aan vroege stress, waren ze opvallend genoeg niet meer hyperactief, maar nog wel agressief. Geheugen en angstmaten (zoals de tijd dat een muis zich aan de rand of in het midden van een open veld begeeft) waren normaal. Stress vroeg in het leven verminderde het aantal jonge neuronen in de hippocampus (een hersengebied) en muizen zonder FGF2 waren hier gevoeliger voor dan normale muizen. Al met al zijn de muizen zonder FGF2 grotendeels normaal, maar hebben ze een aantal specifieke gedragsafwijkingen en een grotere gevoeligheid voor stress. Sommige van deze afwijkingen lijken op wat we zien bij schizofreniepatiënten, bijvoorbeeld de verminderde remming van gedrag en afwijkingen in het glutamaat systeem. Dit maakt het een interessant model om iets te leren over de interactie tussen genen en stress op psychopathologie.

Polygenetisch score

Een andere aanpak om het probleem van de vele mogelijke testen te omzeilen, is om niet alle SNPs één voor één te testen, maar eerst een totaalscore te berekenen die het effect van een genoomwijde set SNPs bij elkaar optelt. Vervolgens kunnen we kijken of deze totaalscore van genetisch risico voor schizofrenie geassocieerd is met breinvolume en IQ. Op deze manier kunnen we in een kleine groep proefpersonen toch een idee krijgen van het effect van vele SNPs. Deze methode is eerder gebruikt om te laten zien dat schizofrenie-risico-SNPs de aanwezigheid van bipolaire stoornis voorspellen.

We konden data uit een internationale database gebruiken om te kijken hoe sterk SNPs met schizofrenie samenhingen. Hiermee konden we voor onze eigen proefpersonen een genetische schizofrenierisicoscore uitrekenen. In **hoofdstuk 5** hebben we gekeken naar de samenhang van deze score met breinvolume gemeten op MRI-scans. De score hing significant samen met totaal breinvolume bij de patiënten én bij de gezonden. Het brein bestaat uit 'grijze stof', gebieden waar neuronen liggen, en 'witte stof', gebieden waar de verbindingen tussen de neuronen lopen. De sterkste samenhang werd gevonden met wittestofvolume, terwijl er met grijzestofvolume geen relatie was. Schizofreniegenen lijken dus de normale (en niet alleen ziekte gebonden) variatie in wittestofvolume te

beïnvloeden. De SNPs die het sterkst effect hadden op beide fenotypes bleken te liggen in genen met een rol in ontwikkeling van de uitlopers van neuronen.

Vervolgens hebben we dezelfde methode gebruikt om te kijken naar de overlap tussen schizofrenierisicogenen en IQ, zoals beschreven in **hoofdstuk 6**. Hier vonden we echter geen associatie. De vermindering van IQ bij schizofreniepatiënten lijkt dus het gevolg van iets anders dan een direct effect van genetische varianten. Mogelijk verstoren de genen de groei van witte stof, wat mensen gevoeliger maakt voor andere invloeden, bijvoorbeeld van stress, drugs of andere genen. Deze factoren tezamen kunnen dan de cognitieve problemen bij patiënten opleveren.

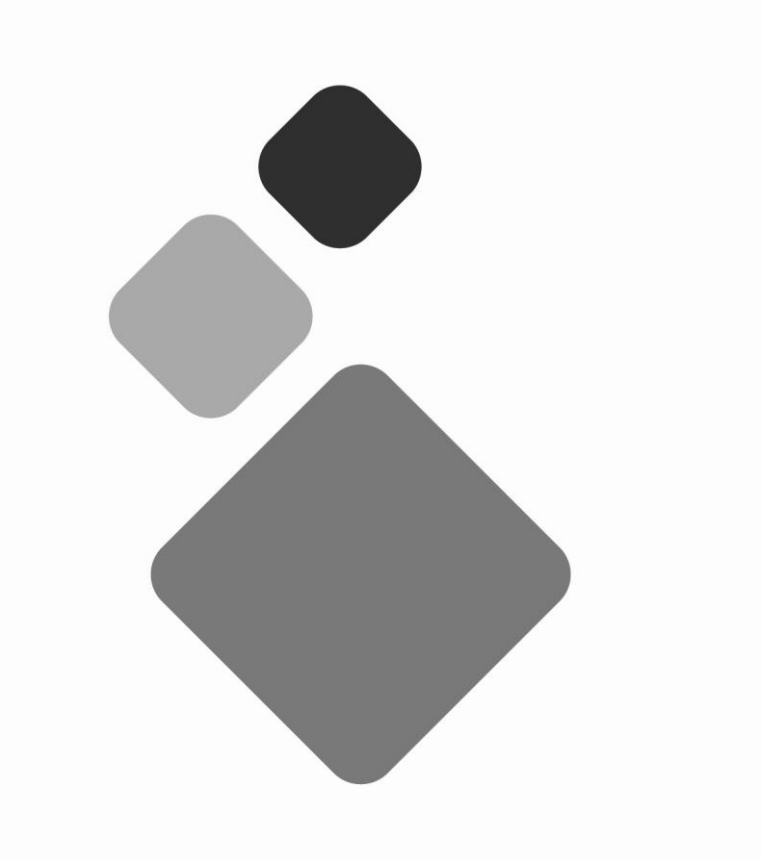
CNVs

Tot slot hebben we gekeken naar CNVs. Aangezien CNVs relatief grote veranderingen in het DNA zijn, hebben ze gemiddeld ook een groter effect. Schizofrenie patiënten hebben gemiddeld meer CNVs dan gezonde mensen. Een aantal specifieke plekken in het DNA staan er om bekend dat ze de kans op schizofrenie verhogen. CNVs op deze plekken leiden ook vaak tot afwijkingen in IQ of breinvolume. Daarom wilden we onderzoeken of ook het totale aantal CNVs per persoon samenhing met IQ of breinvolume bij schizofreniepatiënten en gezonde mensen. Voor zowel IQ als breinvolume hebben we geen relatie gevonden, zoals beschreven in **hoofdstuk 6 en 7**. Mogelijk is niet het totale aantal CNVs van belang, maar de specifieke locatie van de CNVs. Omdat CNVs op specifieke locaties doorgaans zeldzaam zijn, zijn hele grote onderzoeksgroepen vereist om dit te onderzoeken. Dit was nu helaas nog niet haalbaar.

Het laatste hoofdstuk, **hoofdstuk 8**, beschrijft een studie over de relatie tussen het aantal CNVs en de leeftijd van de vader. De vaders van schizofreniepatiënten zijn gemiddeld ouder dan die van gezonde mensen. Of anders gezegd: hoe ouder een man is als hij kinderen krijgt, hoe groter de kans dat zijn kinderen schizofrenie krijgen. Doordat spermastamcellen het hele leven blijven delen, kunnen mutaties geleidelijk ophopen. Een man kan dan een mutatie doorgeven aan zijn kinderen, terwijl hij zelf de mutatie niet in alle cellen heeft. Als iemand een CNV heeft die zijn beide ouders niet hebben, wordt dit een *de novo* CNV genoemd. We hebben gevonden dat zowel schizofrenie patiënten als gezonde mensen met oudere vaders (35 jaar of ouder ten tijde van de geboorte) vaker grote, zeldzame deleties dragen. Deze grote, zeldzame CNVs hebben een relatief grote kans om *de novo* te zijn. Om dit zeker te weten zouden we het DNA van de ouders ook moeten onderzoeken, maar dit was niet beschikbaar. Kennelijk vormt de leeftijd van de vader een risico voor het krijgen van CNVs, maar bepaald iets anders, bijvoorbeeld de locatie van de CNV in het genoom, of die persoon schizofrenie krijgt.

Conclusie

Al met al hebben we uiteenlopende aanpakken gebruikt om de effecten van genetische varianten op endofenotypes van schizofrenie te bestuderen. Het sterkste effect hebben we gevonden voor de relatie tussen een genetische risicoscore voor schizofrenie en breinvolume. Deze, en vele andere genetische en hersenwetenschappelijke studies, dragen bij aan onze kennis over de ontwikkeling van schizofrenie. Het zal echter nog wel even duren voordat deze kennis vertaald kunnen worden in betere behandeling voor patiënten. We zullen in vervolgstudies dan eerst preciezer moeten bepalen welke breingebieden en welke genen er betrokken zijn bij het ontstaan van schizofrenie. Op de lange termijn hopen we te kunnen onderzoeken of schizofreniepatiënten baat hebben bij beïnvloeding van de betrokken processen.



References

References

1. Aguilar LC, Islas A, Rosique P, Hernandez B, Portillo E, Herrera JM *et al.* Psychometric analysis in children with mental retardation due to perinatal hypoxia treated with fibroblast growth factor (FGF) and showing improvement in mental development. *J Intellect Disabil Res* 1993; 37: 507-520.
2. Almasy L, Blangero J. Endophenotypes as quantitative risk factors for psychiatric disease: rationale and study design. *Am J Med Genet* 2001; 105: 42-44.
3. Andoh M, Zhang G, Russell-Lodrigue KE, Shive HR, Weeks BR, Samuel JE. T cells are essential for bacterial clearance, and gamma interferon, tumor necrosis factor alpha, and B cells are crucial for disease development in Coxiella burnetii infection in mice. *Infect Immun* 2007; 75: 3245-3255.
4. Andreasen NC, Flaum M, Arndt S. The Comprehensive Assessment of Symptoms and History (CASH). An instrument for assessing diagnosis and psychopathology. *Archives of General Psychiatry* 1992; 49: 615-623.
5. Archer FR, Doherty P, Collins D, Bolsover SR. CAMs and FGF cause a local submembrane calcium signal promoting axon outgrowth without a rise in bulk calcium concentration. *Eur J Neurosci* 1999; 11: 3565-3573.
6. Ashburner J, Friston KJ. Unified segmentation. *Neuroimage* 2005; 26: 839-851.
7. Aukes MF, Alizadeh BZ, Sitskoorn MM, Kemner C, Ophoff RA, Kahn RS. Genetic overlap among intelligence and other candidate endophenotypes for schizophrenia. *Biological Psychiatry* 2009; 65: 527-534.
8. Ayalew M, Le-Niculescu H, Levey DF, Jain N, Changala B, Patel SD *et al.* Convergent functional genomics of schizophrenia: from comprehensive understanding to genetic risk prediction. *Mol Psychiatry* 2012; 17: 887-905.
9. Baaré WF, Hulshoff Pol HE, Boomsma DI, Posthuma D, De Geus EJ, Schnack HG *et al.* Quantitative genetic modeling of variation in human brain morphology. *Cerebral Cortex* 2001; 11: 816-824.
10. Baaré WF, Hulshoff Pol HE, Boomsma DI, Posthuma D, De Geus EJ, Schnack HG *et al.* Quantitative genetic modeling of variation in human brain morphology. *Cerebral Cortex* 2001; 11: 816-824.
11. Baaré WF, Hulshoff Pol HE, Boomsma DI, Posthuma D, De Geus EJ, Schnack HG *et al.* Quantitative genetic modeling of variation in human brain morphology. *Cerebral Cortex* 2001; 11: 816-824.
12. Babatz TD, Kumar RA, Sudi J, Dobyns WB, Christian SL. Copy number and sequence variants implicate APBA2 as an autism candidate gene. *Autism Res* 2009; 2: 359-364.
13. Bai L, Xu H, Collins JF, Ghishan FK. Molecular and functional analysis of a novel neuronal vesicular glutamate transporter. *J Biol Chem* 2001; 276: 36764-36769.
14. Bansal R. Fibroblast growth factors and their receptors in oligodendrocyte development: implications for demyelination and remyelination. *Dev Neurosci* 2002; 24: 35-46.
15. Barbosa Neto JB, Tiba PA, Faturi CB, de Castro-Neto EF, da GN-M, de Jesus MJ *et al.* Stress during development alters anxiety-like behavior and hippocampal neurotransmission in male and female rats. *Neuropharmacology* 2012; 62: 518-526.

16. Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. *Nat Rev Drug Discov* 2009; 8: 235-253.
17. Behan AT, Byrne C, Dunn MJ, Cagney G, Cotter DR. Proteomic analysis of membrane microdomain-associated proteins in the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder reveals alterations in LAMP, STXBP1 and BASP1 protein expression. *Mol Psychiatry* 2009; 14: 601-613.
18. Bellon A, Krebs MO, Jay TM. Factoring neurotrophins into a neurite-based pathophysiological model of schizophrenia. *Prog Neurobiol* 2011; 94: 77-90.
19. Bernstein HG, Steiner J, Bogerts B. Glial cells in schizophrenia: pathophysiological significance and possible consequences for therapy. *Expert Rev Neurother* 2009; 9: 1059-1071.
20. Bijl RV, Ravelli A, van ZG. Prevalence of psychiatric disorder in the general population: results of The Netherlands Mental Health Survey and Incidence Study (NEMESIS). *Soc Psychiatry Psychiatr Epidemiol* 1998; 33: 587-595.
21. Bis JC, Decarli C, Smith AV, van der LF, Crivello F, Fornage M *et al.* Common variants at 12q14 and 12q24 are associated with hippocampal volume. *Nat Genet* 2012; 44: 545-551.
22. Bland ST, Tamlyn JP, Barrientos RM, Greenwood BN, Watkins LR, Campeau S *et al.* Expression of fibroblast growth factor-2 and brain-derived neurotrophic factor mRNA in the medial prefrontal cortex and hippocampus after uncontrollable or controllable stress. *Neuroscience* 2007; 144: 1219-1228.
23. Blauw HM, Veldink JH, van Es MA, van Vught PW, Saris CG, van der ZB *et al.* Copy-number variation in sporadic amyotrophic lateral sclerosis: a genome-wide screen. *Lancet Neurol* 2008; 7: 319-326.
24. Boos HB, Aleman A, Cahn W, Hulshoff Pol HE, Kahn RS. Brain volumes in relatives of patients with schizophrenia: a meta-analysis. *Arch Gen Psychiatry* 2007; 64: 297-304.
25. Boos HB, Cahn W, van Haren NE, Derks EM, Brouwer RM, Schnack HG *et al.* Focal And Global Brain Measurements in Siblings of Patients With Schizophrenia. *Schizophr Bull* 2011; 38: 814-825.
26. Borroto-Escuela DO, Romero-Fernandez W, Mudo G, Perez-Alea M, Ciruela F, Tarakanov AO *et al.* Fibroblast growth factor receptor 1- 5-hydroxytryptamine 1A heteroreceptor complexes and their enhancement of hippocampal plasticity. *Biol Psychiatry* 2012; 71: 84-91.
27. Bossong MG, Niesink RJ. Adolescent brain maturation, the endogenous cannabinoid system and the neurobiology of cannabis-induced schizophrenia. *Prog Neurobiol* 2010; 92: 370-385.
28. Bourque F, van d, V, Malla A. A meta-analysis of the risk for psychotic disorders among first- and second-generation immigrants. *Psychol Med* 2011; 41: 897-910.
29. Bralten J, rias-Vasquez A, Makkinje R, Veltman JA, Brunner HG, Fernandez G *et al.* Association of the Alzheimer's gene SORL1 with hippocampal volume in young, healthy adults. *Am J Psychiatry* 2011; 168: 1083-1089.
30. Brandon NJ, Sawa A. Linking neurodevelopmental and synaptic theories of mental illness through DISC1. *Nat Rev Neurosci* 2011; 12: 707-722.

31. Brouwer RM, Hulshoff Pol HE, Schnack HG. Segmentation of MRI brain scans using non-uniform partial volume densities. *Neuroimage* 2010; 49: 467-477.
32. Brown RE, Stevens DR, Haas HL. The physiology of brain histamine. *Prog Neurobiol* 2001; 63: 637-672.
33. Brunetti-Pierri N, Berg JS, Scaglia F, Belmont J, Bacino CA, Sahoo T *et al.* Recurrent reciprocal 1q21.1 deletions and duplications associated with microcephaly or macrocephaly and developmental and behavioral abnormalities. *Nature Genetics* 2008; 40: 1466-1471.
34. Brunskill EW, Ehrman LA, Williams MT, Klanke J, Hammer D, Schaefer TL *et al.* Abnormal neurodevelopment, neurosignaling and behaviour in Npas3-deficient mice. *Eur J Neurosci* 2005; 22: 1265-1276.
35. Buizer-Voskamp JE, Blauw HM, Boks MP, van Eijk KR, Veldink JH, Hennekam EA *et al.* Increased paternal age and the influence on burden of genomic copy number variation in the general population. *Hum Genet* 2013; 132: 443-450.
36. Buizer-Voskamp JE, Laan W, Staal WG, Hennekam EA, Aukes MF, Termorshuizen F *et al.* Paternal age and psychiatric disorders: findings from a Dutch population registry. *Schizophr Res* 2011; 129: 128-132.
38. Buizer-Voskamp JE, Muntjewerff JW, Strengman E, Sabatti C, Stefansson H, Vorstman JA *et al.* Genome-wide analysis shows increased frequency of copy number variation deletions in Dutch schizophrenia patients. *Biological Psychiatry* 2011; 70: 655-662.
39. Bustamante C, Bilbao P, Contreras W, Martinez M, Mendoza A, Reyes A *et al.* Effects of prenatal stress and exercise on dentate granule cells maturation and spatial memory in adolescent mice. *Int J Dev Neurosci* 2010; 28: 605-609.
40. Byrne M, Agerbo E, Ewald H, Eaton WW, Mortensen PB. Parental age and risk of schizophrenia: a case-control study. *Arch Gen Psychiatry* 2003; 60: 673-678.
41. Cabungcal JH, Steullet P, Kraftsik R, Cuenod M, Do KQ. Early-life insults impair parvalbumin interneurons via oxidative stress: reversal by N-acetylcysteine. *Biol Psychiatry* 2013; 73: 574-582.
42. Cannon M, Jones PB, Murray RM. Obstetric complications and schizophrenia: historical and meta-analytic review. *Am J Psychiatry* 2002; 159: 1080-1092.
43. Cannon TD, Thompson PM, van Erp TG, Toga AW, Poutanen VP, Huttunen M *et al.* Cortex mapping reveals regionally specific patterns of genetic and disease-specific gray-matter deficits in twins discordant for schizophrenia. *Proc Natl Acad Sci U S A* 2002; 99: 3228-3233.
44. Cardno AG, Marshall EJ, Coid B, Macdonald AM, Ribchester TR, Davies NJ *et al.* Heritability estimates for psychotic disorders: the Maudsley twin psychosis series. *Archives of General Psychiatry* 1999; 56: 162-168.
45. Carlsson A, Waters N, Holm-Waters S, Tedroff J, Nilsson M, Carlsson ML. Interactions between monoamines, glutamate, and GABA in schizophrenia: new evidence. *Annu Rev Pharmacol Toxicol* 2001; 41: 237-260.
46. Caspi A, Moffitt TE. Gene-environment interactions in psychiatry: joining forces with neuroscience. *Nat Rev Neurosci* 2006; 7: 583-590.

47. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H *et al.* Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* 2003; 301: 386-389.
48. Cholfin JA, Rubenstein JL. Patterning of frontal cortex subdivisions by Fgf17. *Proc Natl Acad Sci U S A* 2007; 104: 7652-7657.
49. Clapcote SJ, Lazar NL, Bechard AR, Wood GA, Roder JC. NIH Swiss and Black Swiss mice have retinal degeneration and performance deficits in cognitive tests. *Comp Med* 2005; 55: 310-316.
50. Clinton SM, Turner CA, Flagel SB, Simpson DN, Watson SJ, Akil H. Neonatal fibroblast growth factor treatment enhances cocaine sensitization. *Pharmacol Biochem Behav* 2012; 103: 6-17.
51. Comeau WL, Hastings E, Kolb B. Pre- and postnatal FGF-2 both facilitate recovery and alter cortical morphology following early medial prefrontal cortical injury. *Behav Brain Res* 2007; 180: 18-27.
52. Connor CM, Crawford BC, Akbarian S. White matter neuron alterations in schizophrenia and related disorders. *Int J Dev Neurosci* 2011; 29: 325-334.
53. Cottrell CE, Bir N, Varga E, Alvarez CE, Bouyain S, Zernzach R *et al.* Contactin 4 as an autism susceptibility locus. *Autism Res* 2011; 4: 189-199.
54. Courchesne E, Chisum HJ, Townsend J, Cowles A, Covington J, Egaas B *et al.* Normal brain development and aging: quantitative analysis at in vivo MR imaging in healthy volunteers. *Radiology* 2000; 216: 672-682.
55. Courchesne E, Mouton PR, Calhoun ME, Semendeferi K, Ahrens-Barbeau C, Hallet MJ *et al.* Neuron number and size in prefrontal cortex of children with autism. *JAMA* 2011; 306: 2001-2010.
56. Crow JF. The origins, patterns and implications of human spontaneous mutation. *Nat Rev Genet* 2000; 1: 40-47.
57. Curzon P, Rustay NR, Browman KE. Cued and Contextual Fear Conditioning for Rodents. ed. Buccafusco JJ. Methods of Behavior Analysis in Neuroscience. 2009; 2nd edition. Boca Raton (FL), CRC Press.
58. Dalm S, de VL, Spruijt BM, Oitzl MS. Repeated rat exposure inhibits the circadian activity patterns of C57BL/6J mice in the home cage. *Behav Brain Res* 2009; 196: 84-92.
59. Deary IJ, Yang J, Davies G, Harris SE, Tenesa A, Liewald D *et al.* Genetic contributions to stability and change in intelligence from childhood to old age. *Nature* 2012; 482: 212-215.
60. Dixon AL, Liang L, Moffatt MF, Chen W, Heath S, Wong KC *et al.* A genome-wide association study of global gene expression. *Nat Genet* 2007; 39: 1202-1207.
61. Dono R. Fibroblast growth factors as regulators of central nervous system development and function. *Am J Physiol Regul Integr Comp Physiol* 2003; 284: R867-R881.
62. Dono R, Texido G, Dussel R, Ehmke H, Zeller R. Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *Embo J* 1998; 17: 4213-4225.

63. Eisch AJ, Petrik D. Depression and hippocampal neurogenesis: a road to remission? *Science* 2012; 338: 72-75.
64. Elbers CC, van Eijk KR, Franke L, Mulder F, van der Schouw YT, Wijmenga C *et al.* Using genome-wide pathway analysis to unravel the etiology of complex diseases. *Genet Epidemiol* 2009; 33: 419-431.
65. Elsayed M, Banasr M, Duric V, Fournier NM, Licznerski P, Duman RS. Antidepressant Effects of Fibroblast Growth Factor-2 in Behavioral and Cellular Models of Depression. *Biol Psychiatry* 2012; 72: 258-265.
66. Elvevag B, Goldberg TE. Cognitive impairment in schizophrenia is the core of the disorder. *Critical Reviews in Neurobiology* 2000; 14: 1-21.
67. Engelmann M, Wotjak CT, Landgraf R. Social discrimination procedure: an alternative method to investigate juvenile recognition abilities in rats. *Physiol Behav* 1995; 58: 315-321.
68. Enthoven L, Schmidt MV, Cheung YH, van der Mark MH, de Kloet ER, Oitzl MS. Ontogeny of the HPA axis of the CD1 mouse following 24 h maternal deprivation at pnd 3. *Int J Dev Neurosci* 2010; 28: 217-224.
69. Eren-Kocak E, Turner CA, Watson SJ, Akil H. Short-hairpin RNA silencing of endogenous fibroblast growth factor 2 in rat hippocampus increases anxiety behavior. *Biol Psychiatry* 2011; 69: 534-540.
70. Evers SM, Ament AJ. Costs of schizophrenia in The Netherlands. *Schizophr Bull* 1995; 21: 141-153.
71. Fabre PJ, Shimogori T, Charron F. Segregation of ipsilateral retinal ganglion cell axons at the optic chiasm requires the Shh receptor Boc. *J Neurosci* 2010; 30: 266-275.
72. Fadda P, Bedogni F, Fresu A, Collu M, Racagni G, Riva MA. Reduction of corticostriatal glutamatergic fibers in basic fibroblast growth factor deficient mice is associated with hyperactivity and enhanced dopaminergic transmission. *Biol Psychiatry* 2007; 62: 235-242.
73. Fagel DM, Ganat Y, Cheng E, Silbereis J, Ohkubo Y, Ment LR *et al.* Fgfr1 is required for cortical regeneration and repair after perinatal hypoxia. *J Neurosci* 2009; 29: 1202-1211.
74. Favalli G, Li J, Belmonte-de-Abreu P, Wong AH, Daskalakis ZJ. The role of BDNF in the pathophysiology and treatment of schizophrenia. *J Psychiatr Res* 2012; 46: 1-11.
75. Ferrada C, Ferre S, Casado V, Cortes A, Justinova Z, Barnes C *et al.* Interactions between histamine H3 and dopamine D2 receptors and the implications for striatal function. *Neuropharmacology* 2008; 55: 190-197.
76. Ferreira MA, O'donovan MC, Meng YA, Jones IR, Ruderfer DM, Jones L *et al.* Collaborative genome-wide association analysis supports a role for ANK3 and CACNA1C in bipolar disorder. *Nat Genet* 2008; 40: 1056-1058.
77. Flaisher-Grinberg S, Einat H. Strain-specific battery of tests for domains of mania: effects of valproate, lithium and imipramine. *Front Psychiatry* 2010; 1: 10.

78. Flajolet M, Wang Z, Futter M, Shen W, Nuangchamnong N, Bendor J *et al.* FGF acts as a co-transmitter through adenosine A(2A) receptor to regulate synaptic plasticity. *Nat Neurosci* 2008; 11: 1402-1409.
79. Flores C, Stewart J. Changes in astrocytic basic fibroblast growth factor expression during and after prolonged exposure to escalating doses of amphetamine. *Neuroscience* 2000; 98: 287-293.
80. Fortin D, Rom E, Sun H, Yayon A, Bansal R. Distinct fibroblast growth factor (FGF)/FGF receptor signaling pairs initiate diverse cellular responses in the oligodendrocyte lineage. *J Neurosci* 2005; 25: 7470-7479.
81. Fowler T, Zammit S, Owen MJ, Rasmussen F. A population-based study of shared genetic variation between premorbid IQ and psychosis among male twin pairs and sibling pairs from Sweden. *Archives of General Psychiatry* 2012; 69: 460-466.
82. Frank MG, Der-Avakian A, Bland ST, Watkins LR, Maier SF. Stress-induced glucocorticoids suppress the antisense molecular regulation of FGF-2 expression. *Psychoneuroendocrinology* 2007; 32: 376-384.
83. Franke B, Vasquez AA, Veltman JA, Brunner HG, Rijpkema M, Fernandez G. Genetic variation in CACNA1C, a gene associated with bipolar disorder, influences brainstem rather than gray matter volume in healthy individuals. *Biol Psychiatry* 2010; 68: 586-588.
84. Franke L, Bakel H, Fokkens L, de Jong ED, Egmont-Petersen M, Wijmenga C. Reconstruction of a functional human gene network, with an application for prioritizing positional candidate genes. *Am J Hum Genet* 2006; 78: 1011-1025.
85. Fumagalli F, Bedogni F, Slotkin TA, Racagni G, Riva MA. Prenatal stress elicits regionally selective changes in basal FGF-2 gene expression in adulthood and alters the adult response to acute or chronic stress. *Neurobiol Dis* 2005; 20: 731-737.
86. Gaughran F, Payne J, Sedgwick PM, Cotter D, Berry M. Hippocampal FGF-2 and FGFR1 mRNA expression in major depression, schizophrenia and bipolar disorder. *Brain Res Bull* 2006; 70: 221-227.
87. Genetic Risk and Outcome in Psychosis (GROUP) investigators. Evidence that familial liability for psychosis is expressed as differential sensitivity to cannabis: an analysis of patient-sibling and sibling-control pairs. *Arch Gen Psychiatry* 2011; 68: 138-147.
88. Giachino C, Canalia N, Capone F, Fasolo A, Alleva E, Riva MA *et al.* Maternal deprivation and early handling affect density of calcium binding protein-containing neurons in selected brain regions and emotional behavior in periadolescent rats. *Neuroscience* 2007; 145: 568-578.
89. Goldman AL, Pezawas L, Mattay VS, Fischl B, Verchinski BA, Zoltick B *et al.* Heritability of brain morphology related to schizophrenia: a large-scale automated magnetic resonance imaging segmentation study. *Biol Psychiatry* 2008; 63: 475-483.
90. Goriely A, McVean GA, van Pelt AM, O'Rourke AW, Wall SA, de Rooij DG *et al.* Gain-of-function amino acid substitutions drive positive selection of FGFR2

mutations in human spermatogonia. *Proc Natl Acad Sci U S A* 2005; 102: 6051-6056.

91. Goriely A, Wilkie AO. Paternal age effect mutations and selfish spermatogonial selection: causes and consequences for human disease. *Am J Hum Genet* 2012; 90: 175-200.
92. Gottesman I. Schizophrenia genesis: The origins of madness. ed. Freeman & Co. 1991. New York.
93. Gottesman I, Gould TD. The endophenotype concept in psychiatry: etymology and strategic intentions. *American Journal of Psychiatry* 2003; 160: 636-645.
94. Green EK, Grozeva D, Jones I, Jones L, Kirov G, Caesar S *et al.* The bipolar disorder risk allele at CACNA1C also confers risk of recurrent major depression and of schizophrenia. *Mol Psychiatry* 2009; 15: 1016-1022.
95. Grothe C, Timmer M. The physiological and pharmacological role of basic fibroblast growth factor in the dopaminergic nigrostriatal system. *Brain Res Rev* 2007; 54: 80-91.
96. Guilmatre A, Dubourg C, Mosca AL, Legallic S, Goldenberg A, Drouin-Garraud V *et al.* Recurrent rearrangements in synaptic and neurodevelopmental genes and shared biologic pathways in schizophrenia, autism, and mental retardation. *Archives of General Psychiatry* 2009; 66: 947-956.
97. Gur RE, Keshavan MS, Lawrie SM. Deconstructing psychosis with human brain imaging. *Schizophr Bull* 2007; 33: 921-931.
98. Haijma SV, van Haren NEM, Cahn W, Koolschijn PC, Hulshoff Pol HE, Kahn RS. Brain Volumes in Schizophrenia: A Meta-Analysis in Over 18 000 Subjects. *Schizophr Bull* 2012; Epub ahead of print doi: 10.1093/schbul/sbs118.
99. Hansen SM, Berezin V, Bock E. Signaling mechanisms of neurite outgrowth induced by the cell adhesion molecules NCAM and N-cadherin. *Cell Mol Life Sci* 2008; 65: 3809-3821.
100. Hare EH, Moran PA. Raised parental age in psychiatric patients: evidence for the constitutional hypothesis. *Br J Psychiatry* 1979; 134: 169-177.
101. Haren NEM van, Hulshoff Pol HE, Schnack HG, Cahn W, Brans R, Carati I *et al.* Progressive brain volume loss in schizophrenia over the course of the illness: evidence of maturational abnormalities in early adulthood. *Biological Psychiatry* 2008; 63: 106-113.
103. Haren NEM van, Rijsdijk F, Schnack HG, Picchioni MM, Toulopoulou T, Weisbrod M *et al.* The Genetic and Environmental Determinants of the Association Between Brain Abnormalities and Schizophrenia: The Schizophrenia Twins and Relatives Consortium. *Biological Psychiatry* 2012; 71: 915-921.
104. Hashimoto K, Shimizu E, Komatsu N, Nakazato M, Okamura N, Watanabe H *et al.* Increased levels of serum basic fibroblast growth factor in schizophrenia. *Psychiatry Res* 2003; 120: 211-218.
105. Hedman AM, van Haren NE, Schnack HG, Kahn RS, Hulshoff Pol HE. Human brain changes across the life span: A review of 56 longitudinal magnetic resonance imaging studies. *Hum Brain Mapp* 2011.

106. Hedman AM, van Haren NE, van Baal GC, Brans RG, Hijman R, Kahn RS *et al.* Is there change in intelligence quotient in chronically ill schizophrenia patients? A longitudinal study in twins discordant for schizophrenia. *Psychological Medicine* 2012; epub ahead of print: doi:10.1017/S0033291712000694.
107. Hehir-Kwa JY, Rodriguez-Santiago B, Vissers LE, de LN, Pfundt R, Buitelaar JK *et al.* De novo copy number variants associated with intellectual disability have a paternal origin and age bias. *J Med Genet* 2011; 48: 776-778.
108. Heuvel MPvd, Kahn RS. Abnormal brain wiring as a pathogenetic mechanism in schizophrenia. *Biol Psychiatry* 2011; 70: 1107-1108.
109. Hoogendoorn ML, Haren NEMv, Jungerius BJ, Bakker SC, Sinke RJ, Selten JP *et al.* Myelin- and oligodendrocyte-related genes and brain morphology in schizophrenia. *Published in thesis of Hoogendoorn,M L* 2007.
110. Howes OD, Kapur S. The dopamine hypothesis of schizophrenia: version III--the final common pathway. *Schizophr Bull* 2009; 35: 549-562.
111. Hulshoff Pol HE, Schnack HG, Bertens MG, van Haren NEM, van der Tweel I, Staal WG *et al.* Volume changes in gray matter in patients with schizophrenia. *American Journal of Psychiatry* 2002; 159: 244-250.
112. Itoh N, Ornitz DM. Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. *J Biochem* 2011; 149: 121-130.
113. Iwata T, Hevner RF. Fibroblast growth factor signaling in development of the cerebral cortex. *Dev Growth Differ* 2009; 51: 299-323.
114. Jacobs FM, van der Heide LP, Wijchers PJ, Burbach JP, Hoekman MF, Smidt MP. FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. *J Biol Chem* 2003; 278: 35959-35967.
115. Jarosik J, Legutko B, Werner S, Unsicker K, von Bohlen und HO. Roles of exogenous and endogenous FGF-2 in animal models of depression. *Restor Neurol Neurosci* 2011; 29: 153-165.
116. Jungerius BJ, Hoogendoorn ML, Bakker SC, Van't Slot R, Bardoel AF, Ophoff RA *et al.* An association screen of myelin-related genes implicates the chromosome 22q11 PIK4CA gene in schizophrenia. *Mol Psychiatry* 2007; 13: 1060-1068.
117. Kaga Y, Shoemaker WJ, Furusho M, Bryant M, Rosenbluth J, Pfeiffer SE *et al.* Mice with conditional inactivation of fibroblast growth factor receptor-2 signaling in oligodendrocytes have normal myelin but display dramatic hyperactivity when combined with Cnp1 inactivation. *J Neurosci* 2006; 26: 12339-12350.
118. Kamnasaran D, Muir WJ, Ferguson-Smith MA, Cox DW. Disruption of the neuronal PAS3 gene in a family affected with schizophrenia. *J Med Genet* 2003; 40: 325-332.
119. Kan SH, Elanko N, Johnson D, Cornejo-Roldan L, Cook J, Reich EW *et al.* Genomic screening of fibroblast growth-factor receptor 2 reveals a wide spectrum of mutations in patients with syndromic craniosynostosis. *Am J Hum Genet* 2002; 70: 472-486.
120. Kato M, Okugawa G, Wakeno M, Takekita Y, Nonen S, Tetsuo S *et al.* Effect of basic fibroblast growth factor (FGF2) gene polymorphisms on SSRIs treatment response and side effects. *Eur Neuropsychopharmacol* 2009; 19: 718-725.

121. Katsel P, Davis KL, Gorman JM, Haroutunian V. Variations in differential gene expression patterns across multiple brain regions in schizophrenia. *Schizophr Res* 2005; 77: 241-252.
122. Kee N, Sivalingam S, Boonstra R, Wojtowicz JM. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. *J Neurosci Methods* 2002; 115: 97-105.
123. Kegeles LS, Mao X, Stanford AD, Girgis R, Ojeil N, Xu X *et al.* Elevated Prefrontal Cortex gamma-Aminobutyric Acid and Glutamate-Glutamine Levels in Schizophrenia Measured In Vivo With Proton Magnetic Resonance Spectroscopy. *Arch Gen Psychiatry* 2012; 69: 449-459.
124. Kempton MJ, Ruberto G, Vassos E, Tatarelli R, Girardi P, Collier D *et al.* Effects of the CACNA1C risk allele for bipolar disorder on cerebral gray matter volume in healthy individuals. *Am J Psychiatry* 2009; 166: 1413-1414.
125. Khandaker GM, Barnett JH, White IR, Jones PB. A quantitative meta-analysis of population-based studies of premorbid intelligence and schizophrenia. *Schizophrenia Research* 2011; 132: 220-227.
126. Khandaker GM, Zimbron J, Lewis G, Jones PB. Prenatal maternal infection, neurodevelopment and adult schizophrenia: a systematic review of population-based studies. *Psychol Med* 2013; 43: 239-257.
127. Khashan AS, Abel KM, McNamee R, Pedersen MG, Webb RT, Baker PN *et al.* Higher risk of offspring schizophrenia following antenatal maternal exposure to severe adverse life events. *Arch Gen Psychiatry* 2008; 65: 146-152.
128. Kim JS, Lee HJ, Kim JC, Kang SS, Bae CS, Shin T *et al.* Transient impairment of hippocampus-dependent learning and memory in relatively low-dose of acute radiation syndrome is associated with inhibition of hippocampal neurogenesis. *J Radiat Res* 2008; 49: 517-526.
129. Kim SY, Kim JH, Chung YJ. Effect of Combining Multiple CNV Defining Algorithms on the Reliability of CNV Calls from SNP Genotyping Data. *Genomics Inform* 2012; 10: 194-199.
130. Kirov G, Gumus D, Chen W, Norton N, Georgieva L, Sari M *et al.* Comparative genome hybridization suggests a role for NRXN1 and APBA2 in schizophrenia. *Hum Mol Genet* 2008; 17: 458-465.
131. Kirov G, Pocklington AJ, Holmans P, Ivanov D, Ikeda M, Ruderfer D *et al.* De novo CNV analysis implicates specific abnormalities of postsynaptic signalling complexes in the pathogenesis of schizophrenia. *Mol Psychiatry* 2012; 17: 142-153.
132. Kiyota T, Ingraham KL, Jacobsen MT, Xiong H, Ikezu T. FGF2 gene transfer restores hippocampal functions in mouse models of Alzheimer's disease and has therapeutic implications for neurocognitive disorders. *Proc Natl Acad Sci U S A* 2011; 108: E1339-E1348.
133. Klejbor I, Kucinski A, Wersinger SR, Corso T, Spodnik JH, Dziewiatkowski J *et al.* Serotonergic hyperinnervation and effective serotonin blockade in an FGF receptor developmental model of psychosis. *Schizophr Res* 2009; 113: 308-321.

134. Klejbor I, Myers JM, Hausknecht K, Corso TD, Gambino AS, Morys J *et al.* Fibroblast growth factor receptor signaling affects development and function of dopamine neurons - inhibition results in a schizophrenia-like syndrome in transgenic mice. *J Neurochem* 2006; 97: 1243-1258.
135. Kolb B, Forgie M, Gibb R, Gorny G, Rowntree S. Age, experience and the changing brain. *Neurosci Biobehav Rev* 1998; 22: 143-159.
136. Kong A, Frigge ML, Masson G, Besenbacher S, Sulem P, Magnusson G *et al.* Rate of de novo mutations and the importance of father's age to disease risk. *Nature* 2012; 488: 471-475.
137. Korada S, Zheng W, Basilico C, Schwartz ML, Vaccarino FM. Fibroblast growth factor 2 is necessary for the growth of glutamate projection neurons in the anterior neocortex. *J Neurosci* 2002; 22: 863-875.
138. Korn JM, Kuruvilla FG, McCarroll SA, Wysoker A, Nemesh J, Cawley S *et al.* Integrated genotype calling and association analysis of SNPs, common copy number polymorphisms and rare CNVs. *Nat Genet* 2008; 40: 1253-1260.
139. Korosi A, Naninck EF, Oomen CA, Schouten M, Krugers H, Fitzsimons C *et al.* Early-life stress mediated modulation of adult neurogenesis and behavior. *Behav Brain Res* 2012; 227: 400-409.
140. Kosten TA, Kim JJ, Lee HJ. Early life manipulations alter learning and memory in rats. *Neurosci Biobehav Rev* 2012; 36: 1985-2006.
141. Koyama Y, Tohyama M. A modified and highly sensitive Golgi-Cox method to enable complete and stable impregnation of embryonic neurons. *J Neurosci Methods* 2012; 209: 58-61.
142. Kraepelin E. Dementia Praecox and Paraphrenia. 1919. Edinburgh, E & S Livingstone.
143. Krejci P, Prochazkova J, Bryja V, Kozubik A, Wilcox WR. Molecular pathology of the fibroblast growth factor family. *Hum Mutat* 2009; 30: 1245-1255.
144. Lampi KM, Hinkka-Yli-Salomaki S, Lehti V, Helenius H, Gissler M, Brown AS *et al.* Parental Age and Risk of Autism Spectrum Disorders in a Finnish National Birth Cohort. *J Autism Dev Disord* 2013; epub ahead of print: doi:10.1007/s10803-013-1801-3.
145. Lang FU, Kosters M, Lang S, Becker T, Jager M. Psychopathological long-term outcome of schizophrenia - a review. *Acta Psychiatr Scand* 2012; 127: 173-182.
146. Lango Allen H, Estrada K, Lettre G, Berndt SI, Weedon MN, Rivadeneira F *et al.* Hundreds of variants clustered in genomic loci and biological pathways affect human height. *Nature* 2010; 467: 832-838.
147. Lee SH, Decandia TR, Ripke S, Yang J, Sullivan PF, Goddard ME *et al.* Estimating the proportion of variation in susceptibility to schizophrenia captured by common SNPs. *Nature Genetics* 2012; 44: 247-250.
148. Levinson DF, Duan J, Oh S, Wang K, Sanders AR, Shi J *et al.* Copy number variants in schizophrenia: confirmation of five previous findings and new evidence for 3q29 microdeletions and VIPR2 duplications. *Am J Psychiatry* 2011; 168: 302-316.

149. Li AJ, Suzuki S, Suzuki M, Mizukoshi E, Imamura T. Fibroblast growth factor-2 increases functional excitatory synapses on hippocampal neurons. *Eur J Neurosci* 2002; 16: 1313-1324.
150. Lipscombe D, Helton TD, Xu W. L-type calcium channels: the low down. *J Neurophysiol* 2004; 92: 2633-2641.
151. Luo R, Sanders SJ, Tian Y, Voineagu I, Huang N, Chu SH *et al.* Genome-wide transcriptome profiling reveals the functional impact of rare de novo and recurrent CNVs in autism spectrum disorders. *Am J Hum Genet* 2012; 91: 38-55.
152. Ma YP, Ma MM, Cheng SM, Ma HH, Yi XM, Xu GL *et al.* Intranasal bFGF-induced progenitor cell proliferation and neuroprotection after transient focal cerebral ischemia. *Neurosci Lett* 2008; 437: 93-97.
153. MacDonald AW, Schulz SC. What we know: findings that every theory of schizophrenia should explain. *Schizophr Bull* 2009; 35: 493-508.
154. Maes F, Collignon A, Vandermeulen D, Marchal G, Suetens P. Multimodality image registration by maximization of mutual information. *IEEE Trans Med Imaging* 1997; 16: 187-198.
155. Malhotra D, McCarthy S, Michaelson JJ, Vacic V, Burdick KE, Yoon S *et al.* High frequencies of de novo CNVs in bipolar disorder and schizophrenia. *Neuron* 2011; 72: 951-963.
156. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ *et al.* Finding the missing heritability of complex diseases. *Nature* 2009; 461: 747-753.
157. Manrique C, Compan V, Rosselet C, Duflo SG. Specific knock-down of GAD67 in the striatum using naked small interfering RNAs. *J Biotechnol* 2009; 142: 185-192.
158. Marin O. Interneuron dysfunction in psychiatric disorders. *Nat Rev Neurosci* 2012; 13: 107-120.
159. Mason I. Initiation to end point: the multiple roles of fibroblast growth factors in neural development. *Nat Rev Neurosci* 2007; 8: 583-596.
160. Matheson SL, Shepherd AM, Pinchbeck RM, Laurens KR, Carr VJ. Childhood adversity in schizophrenia: a systematic meta-analysis. *Psychol Med* 2010; 43: 225-238.
161. Mesholam-Gately RI, Giuliano AJ, Goff KP, Faraone SV, Seidman LJ. Neurocognition in first-episode schizophrenia: a meta-analytic review. *Neuropsychology* 2009; 23: 315-336.
162. Miller B, Messias E, Miettunen J, Alaraisanen A, Jarvelin MR, Koponen H *et al.* Meta-analysis of paternal age and schizophrenia risk in male versus female offspring. *Schizophr Bull* 2011; 37: 1039-1047.
163. Minassian A, Henry BL, Geyer MA, Paulus MP, Young JW, Perry W. The quantitative assessment of motor activity in mania and schizophrenia. *J Affect Disord* 2010; 120: 200-206.
164. Mirescu C, Gould E. Stress and adult neurogenesis. *Hippocampus* 2006; 16: 233-238.
165. Misceo D, Fannemel M, Baroy T, Roberto R, Tvedt B, Jaeger T *et al.* SCA27 caused by a chromosome translocation: further delineation of the phenotype. *Neurogenetics* 2009; 10: 371-374.

166. Miyamoto S, Miyake N, Jarskog LF, Fleischhacker WW, Lieberman JA. Pharmacological treatment of schizophrenia: a critical review of the pharmacology and clinical effects of current and future therapeutic agents. *Mol Psychiatry* 2012; 17: 1206-1227.
167. Moises HW, Zoega T, Gottesman II. The glial growth factors deficiency and synaptic destabilization hypothesis of schizophrenia. *BMC Psychiatry* 2002; 2: 1-14.
168. Molteni R, Fumagalli F, Magnaghi V, Roceri M, Gennarelli M, Racagni G *et al.* Modulation of fibroblast growth factor-2 by stress and corticosteroids: from developmental events to adult brain plasticity. *Brain Res Brain Res Rev* 2001; 37: 249-258.
169. Moncrieff J, Leo J. A systematic review of the effects of antipsychotic drugs on brain volume. *Psychol Med* 2010; 40: 1409-1422.
170. Moskvina V, Craddock N, Holmans P, Nikolov I, Pahwa JS, Green E *et al.* Gene-wide analyses of genome-wide association data sets: evidence for multiple common risk alleles for schizophrenia and bipolar disorder and for overlap in genetic risk. *Mol Psychiatry* 2009; 14: 252-260.
171. Muller Smith K, Fagel DM, Stevens HE, Rabenstein RL, Maragnoli ME, Ohkubo Y *et al.* Deficiency in inhibitory cortical interneurons associates with hyperactivity in fibroblast growth factor receptor 1 mutant mice. *Biol Psychiatry* 2008; 63: 953-962.
172. Need AC, Ge D, Weale ME, Maia J, Feng S, Heinzen EL *et al.* A genome-wide investigation of SNPs and CNVs in schizophrenia. *PLoS Genet* 2009; 5: e1000373.
173. Norton WH, Stumpenhorst K, Faus-Kessler T, Folchert A, Rohner N, Harris MP *et al.* Modulation of Fgfr1a signaling in zebrafish reveals a genetic basis for the aggression-boldness syndrome. *J Neurosci* 2011; 31: 13796-13807.
175. O'donovan MC, Norton N, Williams H, Peirce T, Moskvina V, Nikolov I *et al.* Analysis of 10 independent samples provides evidence for association between schizophrenia and a SNP flanking fibroblast growth factor receptor 2. *Mol Psychiatry* 2009; 14: 30-36.
176. Ohkubo Y, Uchida AO, Shin D, Partanen J, Vaccarino FM. Fibroblast growth factor receptor 1 is required for the proliferation of hippocampal progenitor cells and for hippocampal growth in mouse. *J Neurosci* 2004; 24: 6057-6069.
177. Oomen CA, Soeters H, Audureau N, Vermunt L, van Hasselt FN, Manders EM *et al.* Severe early life stress hampers spatial learning and neurogenesis, but improves hippocampal synaptic plasticity and emotional learning under high-stress conditions in adulthood. *J Neurosci* 2010; 30: 6635-6645.
178. Ortega S, Ittmann M, Tsang SH, Ehrlich M, Basilico C. Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc Natl Acad Sci U S A* 1998; 95: 5672-5677.
179. Patel MN, McNamara JO. Selective enhancement of axonal branching of cultured dentate gyrus neurons by neurotrophic factors. *Neuroscience* 1995; 69: 763-770.
180. Penzes P, Cahill ME, Jones KA, VanLeeuwen JE, Woolfrey KM. Dendritic spine pathology in neuropsychiatric disorders. *Nat Neurosci* 2011; 14: 285-293.

181. Peper JS, Schnack HG, Brouwer RM, van Baal GC, Pjetri E, Szekely E *et al.* Heritability of regional and global brain structure at the onset of puberty: a magnetic resonance imaging study in 9-year-old twin pairs. *Hum Brain Mapp* 2009; 30: 2184-2196.
182. Perez JA, Clinton SM, Turner CA, Watson SJ, Akil H. A new role for FGF2 as an endogenous inhibitor of anxiety. *J Neurosci* 2009; 29: 6379-6387.
183. Philip N, Bassett A. Cognitive, behavioural and psychiatric phenotype in 22q11.2 deletion syndrome. *Behav Genet* 2011; 41: 403-412.
184. Pieper AA, Wu X, Han TW, Estill SJ, Dang Q, Wu LC *et al.* The neuronal PAS domain protein 3 transcription factor controls FGF-mediated adult hippocampal neurogenesis in mice. *Proc Natl Acad Sci U S A* 2005; 102: 14052-14057.
185. Polanska UM, Fernig DG, Kinnunen T. Extracellular interactome of the FGF receptor-ligand system: complexities and the relative simplicity of the worm. *Dev Dyn* 2009; 238: 277-293.
186. Potkin SG, Macciardi F, Guffanti G, Wang Q, Turner JA, Lakatos A *et al.* Identifying gene regulatory networks in schizophrenia. *Neuroimage* 2010; 53: 839-847.
187. Potkin SG, Turner JA, Guffanti G, Lakatos A, Torri F, Keator DB *et al.* Genome-wide strategies for discovering genetic influences on cognition and cognitive disorders: methodological considerations. *Cognitive Neuropsychiatry* 2009; 14: 391-418.
188. Purcell S, Cherny SS, Sham PC. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 2003; 19: 149-150.
189. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *American Journal of Human Genetics* 2007; 81: 559-575.
190. Purcell SM, Wray NR, Stone JL, Visscher PM, O'donovan MC, Sullivan PF *et al.* Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. *Nature* 2009; 460: 748-752.
191. Qiang L, Yu W, Liu M, Solowska JM, Baas PW. Basic fibroblast growth factor elicits formation of interstitial axonal branches via enhanced severing of microtubules. *Mol Biol Cell* 2010; 21: 334-344.
192. Raballo R, Rhee J, Lyn-Cook R, Leckman JF, Schwartz ML, Vaccarino FM. Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. *J Neurosci* 2000; 20: 5012-5023.
193. Rai KS, Hattiangady B, Shetty AK. Enhanced production and dendritic growth of new dentate granule cells in the middle-aged hippocampus following intracerebroventricular FGF-2 infusions. *Eur J Neurosci* 2007; 26: 1765-1779.
194. Rais M, Cahn W, van Haren NEM, Schnack H, Caspers E, Hulshoff Pol HE *et al.* Excessive brain volume loss over time in cannabis-using first-episode schizophrenia patients. *Am J Psychiatry* 2008; 165: 490-496.
195. Ratzka A, Baron O, Grothe C. FGF-2 deficiency does not influence FGF ligand and receptor expression during development of the nigrostriatal system. *PLoS One* 2011; 6: e23564.

196. Ratzka A, Baron O, Stachowiak MK, Grothe C. Fibroblast growth factor 2 regulates dopaminergic neuron development in vivo. *J Neurochem* 2012; 122: 94-105.
197. Raychaudhuri S, Korn JM, McCarroll SA, Altshuler D, Sklar P, Purcell S *et al.* Accurately assessing the risk of schizophrenia conferred by rare copy-number variation affecting genes with brain function. *PLoS Genet* 2010; 6.
198. Reichenberg A, Caspi A, Harrington H, Houts R, Keefe RS, Murray RM *et al.* Static and dynamic cognitive deficits in childhood preceding adult schizophrenia: a 30-year study. *American Journal of Psychiatry* 2010; 167: 160-169.
199. Reuss B, von Bohlen und Halbach O. Fibroblast growth factors and their receptors in the central nervous system. *Cell Tissue Res* 2003; 313: 139-157.
200. Ripke S, Sanders AR, Kendler KS, Levinson DF, Sklar P, Holmans PA *et al.* Genome-wide association study identifies five new schizophrenia loci. *Nature Genetics* 2011; 10: 969-976.
201. Riva MA, Molteni R, Bedogni F, Racagni G, Fumagalli F. Emerging role of the FGF system in psychiatric disorders. *Trends Pharmacol Sci* 2005; 26: 228-231.
202. Sanchez MM, Ladd CO, Plotsky PM. Early adverse experience as a developmental risk factor for later psychopathology: evidence from rodent and primate models. *Dev Psychopathol* 2001; 13: 419-449.
203. Scearce-Levie K, Roberson ED, Gerstein H, Cholfin JA, Mandiyan VS, Shah NM *et al.* Abnormal social behaviors in mice lacking Fgf17. *Genes Brain Behav* 2008; 7: 344-354.
204. Schnack HG, Hulshoff Pol HE, Baare WF, Staal WG, Viergever MA, Kahn RS. Automated separation of gray and white matter from MR images of the human brain. *Neuroimage* 2001; 13: 230-237.
205. Shamir A, Kwon OB, Karavanova I, Vullhorst D, Leiva-Salcedo E, Janssen MJ *et al.* The importance of the NRG-1/ErbB4 pathway for synaptic plasticity and behaviors associated with psychiatric disorders. *J Neurosci* 2012; 32: 2988-2997.
206. Sharp AJ, Hansen S, Selzer RR, Cheng Z, Regan R, Hurst JA *et al.* Discovery of previously unidentified genomic disorders from the duplication architecture of the human genome. *Nat Genet* 2006; 38: 1038-1042.
207. Shi J, Levinson DF, Duan J, Sanders AR, Zheng Y, Pe'er I *et al.* Common variants on chromosome 6p22.1 are associated with schizophrenia. *Nature* 2009; 460: 753-757.
208. Shi Y, Li Z, Xu Q, Wang T, Li T, Shen J *et al.* Common variants on 8p12 and 1q24.2 confer risk of schizophrenia. *Nat Genet* 2011; 43: 1224-1227.
209. Shin DM, Korada S, Raballo R, Shashikant CS, Simeone A, Taylor JR *et al.* Loss of glutamatergic pyramidal neurons in frontal and temporal cortex resulting from attenuation of FGFR1 signaling is associated with spontaneous hyperactivity in mice. *J Neurosci* 2004; 24: 2247-2258.
210. Sibbons C, Morris JK, Crolla JA, Jacobs PA, Thomas NS. De novo deletions and duplications detected by array CGH: a study of parental origin in relation to mechanisms of formation and size of imbalance. *Eur J Hum Genet* 2012; 20: 155-160.

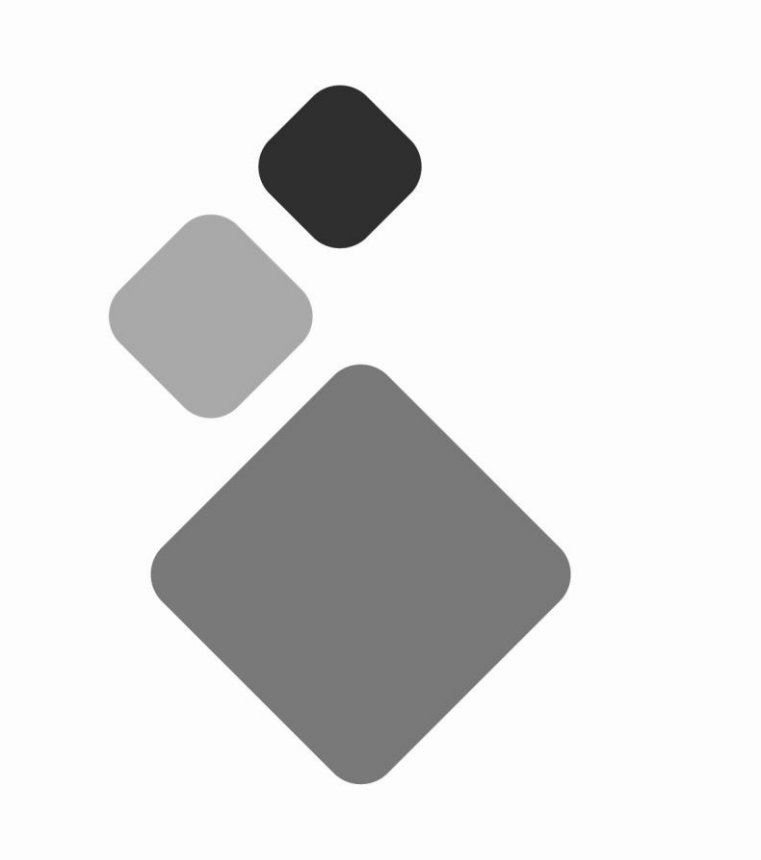
211. Sled JG, Zijdenbos AP, Evans AC. A nonparametric method for automatic correction of intensity nonuniformity in MRI data. *IEEE Transactions on Medical Imaging* 1998; 17: 87-97.
212. Smith KM, Maragnoli ME, Phull PM, Tran KM, Vaccarino FM. Fibroblast growth factor receptor 1 (FGFR1) participates in post-natal interneuron development. *Int J Dev Neurosci* 2010; 28: 698.
213. Splawski I, Timothy KW, Sharpe LM, Decher N, Kumar P, Bloise R *et al.* Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. *Cell* 2004; 119: 19-31.
214. Stefansson H, Ophoff RA, Steinberg S, Andreassen OA, Cichon S, Rujescu D *et al.* Common variants conferring risk of schizophrenia. *Nature* 2009; 460: 744-747.
215. Stefansson H, Rujescu D, Cichon S, Pietilainen OP, Ingason A, Steinberg S *et al.* Large recurrent microdeletions associated with schizophrenia. *Nature* 2008; 455: 232-236.
216. Stein JL, Medland SE, Vasquez AA, Hibar DP, Senstad RE, Winkler AM *et al.* Identification of common variants associated with human hippocampal and intracranial volumes. *Nat Genet* 2012; 44: 552-561.
217. Stevens HE, Jiang GY, Schwartz ML, Vaccarino FM. Learning and memory depend on fibroblast growth factor receptor 2 functioning in hippocampus. *Biol Psychiatry* 2012; 71: 1090-1098.
218. Stevens HE, Smith KM, Maragnoli ME, Fagel D, Borok E, Shanabrough M *et al.* Fgfr2 is required for the development of the medial prefrontal cortex and its connections with limbic circuits. *J Neurosci* 2010; 30: 5590-5602.
219. Stevens HE, Smith KM, Rash BG, Vaccarino FM. Neural stem cell regulation, fibroblast growth factors, and the developmental origins of neuropsychiatric disorders. *Front Neurosci* 2010; 4.
220. Stinissen J, Willems PJ, Coetsier P, Hulsman WWL. Manual of the Dutch Translation of the Wechsler Adult Intelligence Scale. 1970. Amsterdam, the Netherlands, Swets.
221. Stornetta RL, Zhu JJ. Ras and Rap signaling in synaptic plasticity and mental disorders. *Neuroscientist* 2011; 17: 54-78.
222. Sutrala SR, Goossens D, Williams NM, Heyrman L, Adolfsson R, Norton N *et al.* Gene copy number variation in schizophrenia. *Schizophrenia Research* 2007; 96: 93-99.
223. Szebenyi G, Fallon JF. Fibroblast growth factors as multifunctional signaling factors. *Int Rev Cytol* 1999; 185: 45-106.
224. Takahashi N, Sakurai T, Davis KL, Buxbaum JD. Linking oligodendrocyte and myelin dysfunction to neurocircuitry abnormalities in schizophrenia. *Prog Neurobiol* 2011; 93: 13-24.
225. Tanahira C, Higo S, Watanabe K, Tomioka R, Ebihara S, Kaneko T *et al.* Parvalbumin neurons in the forebrain as revealed by parvalbumin-Cre transgenic mice. *Neurosci Res* 2009; 63: 213-223.
226. Terwisscha van Scheltinga AF, Bakker S, Haren NEM van, Buizer-Voskamp J, Boos H, Vorstman J *et al.* Association study of copy number variants with brain volume

in schizophrenia patients and healthy controls. *Psychiatry Research* 2012; 200: 1011-1013.

227. Terwisscha van Scheltinga AF, Bakker SC, Haren NEM van, Derks EM, Buizer-Voskamp JE, Boos HB *et al.* Genetic schizophrenia risk variants jointly modulate total brain and white matter volume. *Biological Psychiatry* 2012; 73: 525-531.
228. Terwisscha van Scheltinga AF, Bakker SC, Kahn RS. Fibroblast Growth Factors in Schizophrenia. *Schizophr Bull* 2009; 36: 1157-1166.
229. Terwisscha van Scheltinga AF, Bakker SC, Kahn RS, Kas MJ. Fibroblast Growth Factors in Neurodevelopment and Psychopathology. *Neuroscientist* 2013; 19: 479-94.
230. Thomas NS, Durkie M, Potts G, Sandford R, Van ZB, Youings S *et al.* Parental and chromosomal origins of microdeletion and duplication syndromes involving 7q11.23, 15q11-q13 and 22q11. *Eur J Hum Genet* 2006; 14: 831-837.
231. Thomas NS, Morris JK, Baptista J, Ng BL, Crolla JA, Jacobs PA. De novo apparently balanced translocations in man are predominantly paternal in origin and associated with a significant increase in paternal age. *J Med Genet* 2010; 47: 112-115.
232. Timmer M, Cesnulevicius K, Winkler C, Kolb J, Lipokatic-Takacs E, Jungnickel J *et al.* Fibroblast growth factor (FGF)-2 and FGF receptor 3 are required for the development of the substantia nigra, and FGF-2 plays a crucial role for the rescue of dopaminergic neurons after 6-hydroxydopamine lesion. *J Neurosci* 2007; 27: 459-471.
233. Tomelleri L, Jogia J, Perlini C, Bellani M, Ferro A, Rambaldelli G *et al.* Brain structural changes associated with chronicity and antipsychotic treatment in schizophrenia. *Eur Neuropsychopharmacol* 2009; 19: 835-840.
234. Toulopoulou T, Picchioni M, Rijsdijk F, Hua-Hall M, Ettinger U, Sham P *et al.* Substantial genetic overlap between neurocognition and schizophrenia: genetic modeling in twin samples. *Archives of General Psychiatry* 2007; 64: 1348-1355.
235. Turner CA, Akil H, Watson SJ, Evans SJ. The fibroblast growth factor system and mood disorders. *Biol Psychiatry* 2006; 59: 1128-1135.
236. Turner CA, Calvo N, Frost DO, Akil H, Watson SJ. The fibroblast growth factor system is downregulated following social defeat. *Neurosci Lett* 2008; 430: 147-150.
237. Turner CA, Capriles N, Flagel SB, Perez JA, Clinton SM, Watson SJ *et al.* Neonatal FGF2 alters cocaine self-administration in the adult rat. *Pharmacol Biochem Behav* 2009; 92: 100-104.
238. Turner CA, Clinton SM, Thompson RC, Watson SJ, Jr., Akil H. Fibroblast growth factor-2 (FGF2) augmentation early in life alters hippocampal development and rescues the anxiety phenotype in vulnerable animals. *Proc Natl Acad Sci U S A* 2011; 108: 8021-8025.
239. Turner CA, Gula EL, Taylor LP, Watson SJ, Akil H. Antidepressant-like effects of intracerebroventricular FGF2 in rats. *Brain Res* 2008; 1224: 63-68.
240. Turner CA, Watson SJ, Akil H. The fibroblast growth factor family: neuromodulation of affective behavior. *Neuron* 2012; 76: 160-174.

241. Vaccarino FM, Grigorenko EL, Smith KM, Stevens HE. Regulation of Cerebral Cortical Size and Neuron Number by Fibroblast Growth Factors: Implications for Autism. *J Autism Dev Disord* 2008; 39: 511-520.
242. van den Heuvel MP, Kahn RS. Abnormal brain wiring as a pathogenetic mechanism in schizophrenia. *Biol Psychiatry* 2011; 70: 1107-1108.
246. Vann SD, Albasser MM. Hippocampus and neocortex: recognition and spatial memory. *Curr Opin Neurobiol* 2011; 21: 440-445.
247. Visscher PM, Brown MA, McCarthy MI, Yang J. Five years of GWAS discovery. *Am J Hum Genet* 2012; 90: 7-24.
248. Visser L de, van den Bos R, Kuurman WW, Kas MJ, Spruijt BM. Novel approach to the behavioural characterization of inbred mice: automated home cage observations. *Genes Brain Behav* 2006; 5: 458-466.
249. Volk DW, Lewis DA. Prefrontal cortical circuits in schizophrenia. *Curr Top Behav Neurosci* 2010; 4: 485-508.
250. Walsh T, McClellan JM, McCarthy SE, Addington AM, Pierce SB, Cooper GM *et al.* Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia. *Science* 2008; 320: 539-543.
251. Wang F, McIntosh AM, He Y, Gelernter J, Blumberg HP. The association of genetic variation in CACNA1C with structure and function of a frontotemporal system. *Bipolar Disord* 2011; 13: 696-700.
252. Wang K, Li M, Hadley D, Liu R, Glessner J, Grant SF *et al.* PennCNV: an integrated hidden Markov model designed for high-resolution copy number variation detection in whole-genome SNP genotyping data. *Genome Res* 2007; 17: 1665-1674.
253. Wang K, Zhang H, Bloss CS, Duvvuri V, Kaye W, Schork NJ *et al.* A genome-wide association study on common SNPs and rare CNVs in anorexia nervosa. *Mol Psychiatry* 2011; 16: 949-959.
254. Wang Q, Bardgett ME, Wong M, Wozniak DF, Lou J, McNeil BD *et al.* Ataxia and paroxysmal dyskinesia in mice lacking axonally transported FGF14. *Neuron* 2002; 35: 25-38.
255. Wang T, Zeng Z, Hu Z, Zheng L, Li T, Li Y *et al.* FGFR2 is associated with bipolar disorder: A large-scale case-control study of three psychiatric disorders in the Chinese Han population. *World J Biol Psychiatry* 2012; 13: 599-604.
256. Wei Huang D, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; 4: 44-57.
257. Werner S, Unsicker K, von Bohlen und HO. Fibroblast growth factor-2 deficiency causes defects in adult hippocampal neurogenesis, which are not rescued by exogenous fibroblast growth factor-2. *J Neurosci Res* 2011; 89: 1605-1617.
258. Wright IC, Rabe-Hesketh S, Woodruff PW, David AS, Murray RM, Bullmore ET. Meta-analysis of regional brain volumes in schizophrenia. *American Journal of Psychiatry* 2000; 157: 16-25.
259. Wu D. Neuroprotection in experimental stroke with targeted neurotrophins. *NeuroRx* 2005; 2: 120-128.

260. Xu B, Ionita-Laza I, Roos JL, Boone B, Woodrick S, Sun Y *et al.* De novo gene mutations highlight patterns of genetic and neural complexity in schizophrenia. *Nat Genet* 2012; 44: 1365-1369.
261. Xu B, Roos JL, Dexheimer P, Boone B, Plummer B, Levy S *et al.* Exome sequencing supports a de novo mutational paradigm for schizophrenia. *Nat Genet* 2011; 43: 864-868.
262. Xu B, Roos JL, Levy S, van Rensburg EJ, Gogos JA, Karayiorgou M. Strong association of de novo copy number mutations with sporadic schizophrenia. *Nature Genetics* 2008; 40: 880-885.
263. Yamaguchi Y. Heparan sulfate proteoglycans in the nervous system: their diverse roles in neurogenesis, axon guidance, and synaptogenesis. *Semin Cell Dev Biol* 2001; 12: 99-106.
264. Yang K, Cao F, Sheikh AM, Malik M, Wen G, Wei H *et al.* Up-regulation of Ras/Raf/ERK1/2 signaling impairs cultured neuronal cell migration, neurogenesis, synapse formation, and dendritic spine development. *Brain Struct Funct* 2012; 218: 669-682.
265. Yeo RA, Gangestad SW, Liu J, Calhoun VD, Hutchison KE. Rare copy number deletions predict individual variation in intelligence. *PLoS One* 2011; 6: e16339.
266. Yoshimura S, Takagi Y, Harada J, Teramoto T, Thomas SS, Waeber C *et al.* FGF-2 regulation of neurogenesis in adult hippocampus after brain injury. *Proc Natl Acad Sci U S A* 2001; 98: 5874-5879.
267. Zechel S, Jarosik J, Kiprianova I, Schober A, Unsicker K, von Bohlen und Halbach O. FGF-2 deficiency does not alter vulnerability of the dopaminergic nigrostriatal system towards MPTP intoxication in mice. *Eur J Neurosci* 2006; 23: 1671-1675.
268. Zechel S, Werner S, Unsicker K, von Bohlen und Halbach O. Expression and functions of fibroblast growth factor 2 (FGF-2) in hippocampal formation. *Neuroscientist* 2010; 16: 357-373.
269. Zhao M, Li D, Shimazu K, Zhou YX, Lu B, Deng CX. Fibroblast growth factor receptor-1 is required for long-term potentiation, memory consolidation, and neurogenesis. *Biol Psychiatry* 2007; 62: 381-390.
270. Zhou M, Sutliff RL, Paul RJ, Lorenz JN, Hoying JB, Haudenschild CC *et al.* Fibroblast growth factor 2 control of vascular tone. *Nat Med* 1998; 4: 201-207.
271. Zinkstok J, Amelsvoort Tv. Neuropsychological profile and neuroimaging in patients with 22Q11.2 Deletion Syndrome: a review. *Child Neuropsychology* 2005; 11: 21-37.
272. Zou H, Yu Y, Sheikh AM, Malik M, Yang K, Wen G *et al.* Association of upregulated Ras/Raf/ERK1/2 signaling with autism. *Genes Brain Behav* 2011; 10: 615-624.



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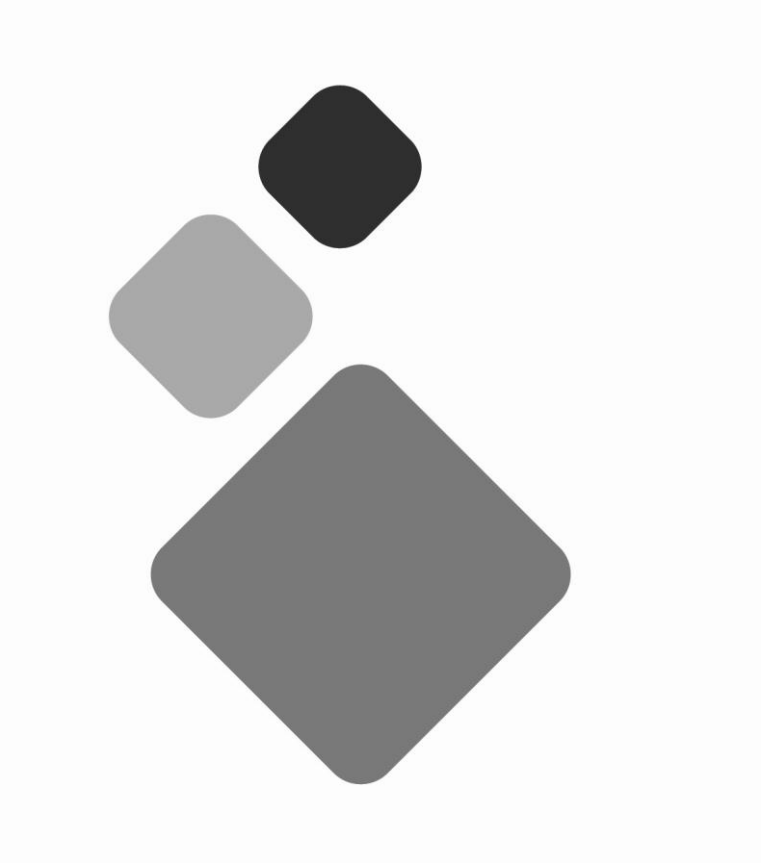
Owen, Christos Pantelis, George Papadimitriou, Michele T Pato, Leena Peltonen, Hannes Petursson, Ben Pickard, Jonathan Pimm, Ann E Pulver, Vinay Puri, Digby Quested, Emma M Quinn, Henrik B Rasmussen, János M Réthelyi, Robert Ribble, Marcella Rietschel, Brien P Riley, Mirella Ruggeri, Ulrich Schall, Thomas G Schulze, Sibylle G Schwab, Rodney J Scott, Jianxin Shi, Engilbert Sigurdsson, Jeremy M Silverman, Chris C A Spencer, Kari Stefansson, Amy Strange, Eric Strengman, T Scott Stroup, Jaana Suvisaari, Lars Terenius, Srinivasa Thirumalai, Johan H Thygesen, Sally Timm, Draga Toncheva, Edwin van den Oord, Jim van Os, Ruud van Winkel, Jan Veldink, Dermot Walsh, August G Wang, Durk Wiersma, Dieter B Wildenauer, Hywel J Williams, Nigel M Williams, Brandon Wormley, Stan Zammit, Patrick F Sullivan, Michael C O'Donovan, Mark J Daly & Pablo V Gejman.



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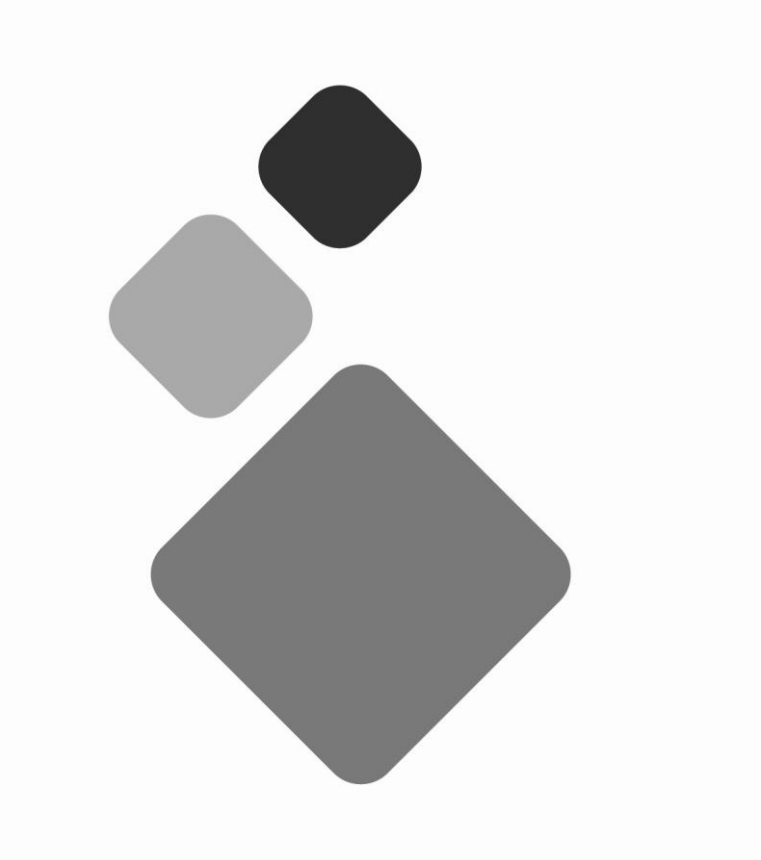


Publications

Publications

1. Smits SM, **Terwisscha van Scheltinga AF**, van der Linden AJ, Burbach JP, Smidt MP. Species differences in brain pre-pro-neurotensin/ neuromedin N mRNA distribution: the expression pattern in mice resembles more closely that of primates than rats. *Brain Research and Molecular Brain Research*, 2004;125:22-8.

2. **Terwisscha van Scheltinga AF**, Bakker SC, Kahn RS. Fibroblast growth factors in schizophrenia. *Schizophrenia Bulletin,* 2010; 36:1157-66.

3. **Terwisscha van Scheltinga AF**, Bakker SC, van Haren NEM, Buizer JE, Boos HBM, Vorstman JAS, Cahn W, Hulshoff Pol HE, Ophoff RA, Kahn RS. Association study of copy number variants with brain volume in schizophrenia patients and healthy controls. *Psychiatry research*, 2012; 200:1011-13.

4. **Terwisscha van Scheltinga AF**, Bakker SC, van Haren NEM, Derks EM, Buizer-Voskamp JE, Boos HBM, Cahn W, Hulshoff Pol HE, Ripke S, Psychiatric Genome-wide association study (GWAS) Consortium, Ophoff RA, Kahn RS. Genetic schizophrenia risk variants jointly modulate total brain and white matter volume in schizophrenia patients and healthy controls. *Biological Psychiatry*, 2013; 73:525:31.

5. **Terwisscha van Scheltinga AF**, Bakker SC, van Haren NEM, Derks EM, Buizer-Voskamp JE, Cahn W, Ripke S, Psychiatric Genome-wide association study (GWAS) Consortium, Ophoff RA, Kahn RS. Schizophrenia genetic variants are not associated with intelligence. *Psychological Medicine*, 2013. epub ahead of print, doi:10.1017/S0033291713000196

6. **Terwisscha van Scheltinga AF**, Bakker SC, Kahn RS, Kas MJH. Fibroblast growth factors in neurodevelopment and psychopathology. *The Neuroscientist*, 2013; 19:479:94.

Submitted articles

7. **Terwisscha van Scheltinga AF**, Bakker SC, van Haren NEM, Boos HBM, Schnack HG, Cahn W, Hulshoff Pol HE, Kahn RS. Association study of fibroblast growth factor genes and brain volumes in schizophrenia patients and controls.

Articles in preparation

8. **Terwisscha van Scheltinga AF**, Prop E, Brouwer A, Kostrzewa E, Vergoossen DLE, Naninck EFG, Korosi A, de Visser L, Bruining H, Bakker SC, Joëls M, Kahn RS, Kas MJH. Lack of FGF2 results in increased locomotor activity and aggression and exacerbates effects of early life stress on anxiety and structural plasticity.

9. **Terwisscha van Scheltinga AF**, Buizer-Voskamp JE, Shi J, Veldink JH, van den Berg LH, Hennekam EAM, Tiemeier H, Uitterlinden AG, Kiemeney LA, Kahn RS, Ophoff RA, Levinson DF. Advanced paternal age increases the rate of rare, large deletions in schizophrenia patients and healthy controls.

Published abstracts

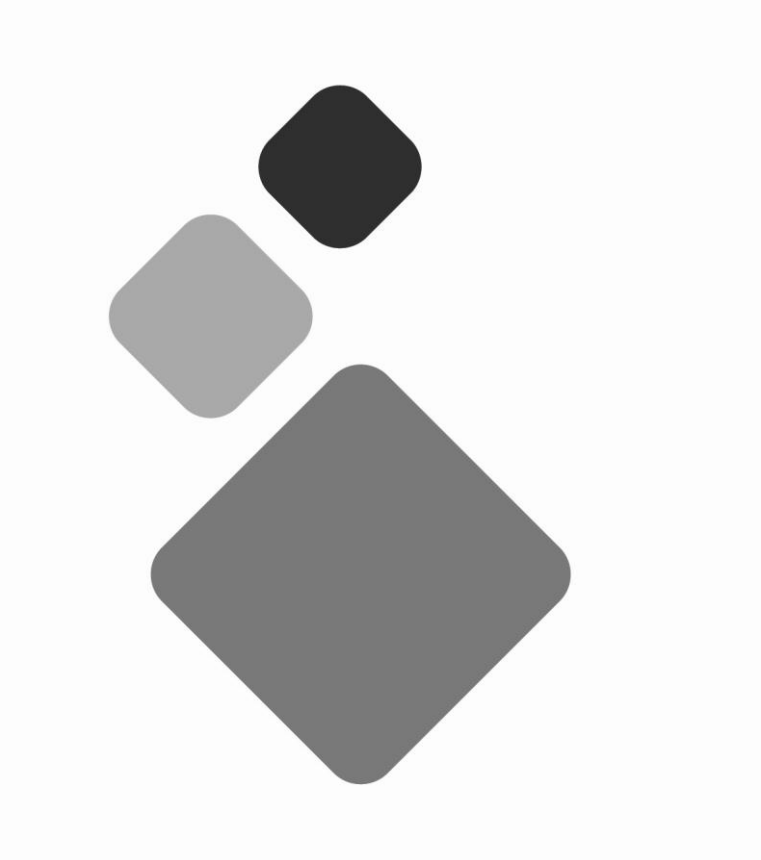
1. **Terwisscha van Scheltinga AF**, Bakker SC, van Haren NEM, Hulshoff Pol HE, Cahn W, Ophoff RA, Kahn RS. Fibroblast growth factors and brain volume in schizophrenia patients and healthy controls. Schizophr Res 2010 April;117(2-3):453.

2. **Terwisscha van Scheltinga AF**, Bakker SC, van Haren NEM, Hulshoff Pol HE, Cahn W, Buizer-Voskamp JE, Gladwin TE, Vorstman JAS, Ophoff RA, Kahn RS. Association study of CNVs with brain volume and brain volume change over 5 years in schizophrenia patients and healthy controls. Schizophr Res 2010 April;117 (2-3):454.

3. **Terwisscha van Scheltinga AF**, Bakker SC, van Haren NEM, Boos HBM, Schnack HG, Cahn W, Hulshoff Pol HE, Kahn RS. Het effect van schizofrenie gerelateerde genetische variaties op structurele breinafwijkingen. Tijdschr v Psychiatrie 2011 Mar; 52 (suppl 1):S59.

Awards

1. De Girard de Mielet van Coehorn foundation travel award in 2009
2. Early Career Investigator award finalist at World Congress of Psychiatric Genetics in 2011



Curriculum vitae

Afke F. Terwisscha van Scheltinga was born in 1980 and grew up in Pijnacker. She graduated gymnasium from Dalton scholengemeenschap in Voorburg in 1998. The same year, she started medical school at the University Medical Centre Utrecht. During her studies she went abroad for a gynaecology internship in Tanzania and an ophthalmology internship in Scotland. She performed an extended research internship on molecular neuroscience under supervision of Marten P. Smidt at the Rudolf Magnus Institute of Neuroscience, Department of Pharmacology and Anatomy, University Medical Centre Utrecht. In addition, from 2002-2005 she studied part-time philosophy at University Utrecht. After her graduation from medical school in 2005, she started to work at a psychiatric ward for acutely ill patients and later at the team for assertive community treatment at Altrecht in Woerden. In May 2007 she started her psychiatric residency combined with the PhD project described in this thesis at the University Medical Centre Utrecht under supervision of René S. Kahn (research and clinical training), Roel A. Ophoff (research) and Jaap Wijkstra (clinical training). As part of her research project she visited Stanford University, California, United States of America in 2012-2013 for 8 months, under supervision of Douglas F. Levinson. She hopes to graduate as a psychiatrist in September 2014.