# Myelin Matters in Schizophrenia : Neurobiological Insights from Rat Model and Human Studies

Dorothea Adriana Maas

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**Proefschrift**

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**Dorothea (Dorien) Adriana Maas**
geboren op 15 november 1991
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# Myelin Matters in Schizophrenia

*Neurobiological Insights from Rat Model and Human Studies*

**Thèse de Doctorat**

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*"his head is just floating in the breeze, man"*

Jim Morrison

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# 1

## General Introduction

**1**

## A  brief history of schizophrenia

Schizophrenia (SZ) is a severe psychiatric disorder that has a similar incidence around the world and was already described in ancient Egyptian texts [1]. Consequently, it is thought that SZ is an ancient disorder that has escorted mankind throughout its existence. Defining SZ as a disorder is complicated because its symptomatology is broad and its etiology remains elusive. SZ was first described as a disorder by Emil Kraeplin who termed it 'dementia praecox' and as such produced a name covering nine different clinical forms of SZ [1,2]. Dementia praecox was diagnosed on the basis of deficits in cognitive and executive functioning that lead to *"Impoverishment and devastation of the whole psychic life"*. It is now recognized that SZ is not a neurodegenerative disorder as suggested by the term dementia, but instead a neurodevelopmental disorder with both genetic and environmental origins. The psychiatrist Eugen Bleuler termed the disorder SZ or rather, the schizophrenias, as he acknowledged that SZ is a spectrum disorder and that no two patients' experiences and symptomatology are the same [1,2]. The word schizophrenia originates from the Greek words σχίζω, meaning split, and φρένα, meaning mind, to highlight fragmented thinking. The schizophrenias covered any mental state that involved what we nowadays term positive symptoms. Since Bleuler's time, positive symptoms, including delusions and hallucinations, have always had a determining role in the diagnosis of SZ. Negative and cognitive symptoms, including flattened affect, anhedonia, executive functioning and memory impairments, are considered less important for diagnosis, even though they have a greater impact on daily life and functional outcome. Recently, it has been suggested that the positive symptoms should not be the main element in the diagnosis of SZ and that it is time to revisit the dogma that started with Bleuler's definition of SZ [3].

## Prevalence, clinical aspects and treatment of SZ

About 0.87% of the individuals in the general population, is diagnosed with SZ during their lifetime [4]. SZ becomes apparent during adolescence and starts at a younger age in males (20-24 years old) than in females (29-32 years old) [5]. The clinical aspects of SZ are complex, but it is generally accepted that they consist of symptoms from different categories: positive, negative, disorganization of thought, motor, mood and cognitive symptoms [6]. The positive symptoms of SZ include all psychosis-related symptoms such as delusions and hallucinations [7]. The most common delusions in SZ are that the patients' thoughts are audible and that their thoughts are controlled by a foreign entity. Hallucinations are sensory perceptions that do not originate from environmental stimuli. The most frequent hallucinations in SZ are auditory although hallucinations in all perceptive domains have been reported. Auditory hallucinations most commonly include hearing voices commenting on the patients' behaviour. The content of both delusions and hallucinations is highly variable and influenced by the patients' life,

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socio-cultural environment and religion [6]. Negative symptoms of SZ include the loss of typical affective functions such as lack of emotional expression, loss of motivation or an inability to feel pleasure. Primary negative symptoms can be attributed to the disorder itself, while secondary negative symptoms are induced by external factors such as a lack of environmental stimulation [6]. Disorganization of thought in SZ highlights the fractionation of the normal thought process and was considered by Bleuler to be the core of the disorder [1]. It refers to an inability to keep a train of thought or dissolution of logical goal-directed thought processes that is reflected in incoherent speech [6]. SZ may also include mood, anxiety or motor symptoms, for example slowing of psychomotor activities or excessive unnecessary movements that go paired with an increase in positive symptoms [8]. In severe form, motor symptoms can also present as catatonia [9]. In contrast to the negative, mood, motor and anxiety symptoms, cognitive symptoms are present in almost all SZ patients [10] and represent a failure to reach the expected general cognitive level of a person's age group or the expected cognitive level based on the individual patient's history. Cognitive deficits in SZ encompass decreases in attention [11], verbal fluency [12], processing speed [13], memory and executive functioning; the latter specifically includes reduced working memory and set-shifting ability [14-16].

Clearly, SZ is a disorder with a highly complex clinical presentation. To be diagnosed with SZ, one should fulfill at least two of the following five DSM-V symptoms for a significant duration of time within one month: delusions, hallucinations, disorganized speech, grossly disorganized or catatonic behaviour and negative symptoms. In addition, daily functioning has to be impaired for at least six months [17].

Currently, no effective treatment for SZ exists and pharmacological therapy focuses on positive symptoms, while neglecting other SZ symptoms [18]. Both first- and second-generation antipsychotics target the dopamine system and effectively reduce positive symptoms in a subset of patients. Second-generation antipsychotics like haloperidol, chlorpromazine and droperidol are the most commonly prescribed antipsychotic medication despite of their metabolic side effects [18]. If second-generation antipsychotic medication is unsuccessful in reducing psychotic symptoms, first-generation antipsychotics and electroconvulsive therapy are prescribed [18]. Psychotherapy and cognitive behavioural therapy are used to improve long-term functional outcome in SZ patients, but no treatments targeting negative or cognitive symptoms specifically are currently available in medical practice [18]. Yet, studies have shown that treatment of SZ patients with antioxidants [19,20] and cognitive remediation therapy [21,22] leads to improvements in cognitive functioning, but further research is necessary to confirm their efficacy and reveal the neurobiological mechanisms underlying their effectiveness.

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## Risk factors for SZ

SZ is a complex disorder caused by a combination of genetic, epigenetic and environmental risk factors [23]. However, the heritability of SZ is 81% as determined by a meta-analysis of twin studies and relatively high compared to the heritability of other complex diseases [24]. The largest genome-wide association study to date showed that 7% of the variability in the risk for SZ in the general population can be attributed to variation in single-nucleotide polymorphisms [25,26]. Some SZ cases carry a mutation in a specific gene, for example a genetic deletion in the chromosomal region 22q11.2 causes SZ in 25% of its carriers [27]. Whole-exome sequencing has revealed that *de novo* mutations in PTPRG, TGM5, SLC39A13, BTK and CDKN3 underlie specific SZ cases [28]. However, in most SZ patients an interaction of several genetic variations with environmental risk factors is hypothesized to underlie the etiology of the disorder.

A number of environmental risk factors convey SZ risk [30] (Figure 1; for an excellent review, see [29]) and include complications around birth, like low birth weight [31,32], and use of forceps [32], preeclampsia [32] and bleeding during pregnancy [31]. In addition, maternal immune activation and maternal stress during pregnancy are well-documented risk factors for SZ [33,34]. Moreover, paternal age of 30-35 years and older at conception conveys risk for SZ [35,36], while the association between maternal age and risk for SZ remains unclear [29]. Likewise, childhood adversity [37], childhood trauma [38] and adult life events [39] bear SZ risk. Also migration and especially being a refugee increases risk for SZ [40,41], although the latter study did not account for trauma or adult-life events as confounding factors. Furthermore, urbanicity increases risk for developing SZ [42,43], while exposure to green nature space during childhood decreases SZ risk [44]. Another widely studied risk factor for SZ is cannabis use, and both the amount of cannabis used and the intake of high-potency cannabis are linked to an earlier psychosis onset [45,46]. Isolated genetic or environmental factors are not sufficient to induce SZ, therefore one must take into account both the accumulative effects of multiple environmental risk factors or multiple genetic risk factors and the interactions between genetic and environmental factors in the etiology of SZ [29,47]. This is a largely unexplored topic, but a recent study found evidence for an additive effect of genetic risk for SZ and regular cannabis use, sexual abuse, emotional abuse, emotional neglect and bullying [48]. Interestingly, the presence of more than one environmental risk factor causes an earlier onset of SZ, providing evidence for a cumulative effect of environmental risk factors [49]. Recently, a tool was developed that may help identifying the environmental risk score of individuals to develop SZ [25]. Altogether, examining SZ risk factors highlights the complexity of SZ etiology and the necessity for research into the neurobiological causes of this disorder that may eventually allow the development of effective treatment strategies.

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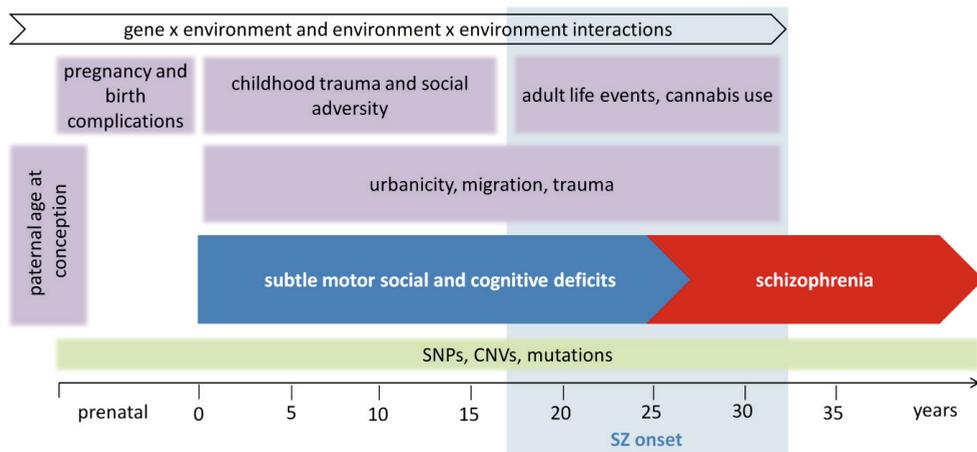


**Figure 1 – Genetic and environmental risk factors in the development of SZ.** Purple: environmental risk factors, green: genetic risk factors, SNP: Single nucleotide polymorphism, CNV: copy number variation. Adapted from Stilo & Murray (2019) (reference 29).

## Cognitive dysfunction in SZ and its link to the prefrontal cortex (PFC)

Cognitive dysfunction in SZ is the topic of this thesis and therefore warrants a more in-depth explanation. Cognitive deficits are considered to be at the core of SZ pathology and have been suggested as a critical diagnostic criterium [50,51]. Cognitive impairment in SZ is independent of psychosis symptoms (but see [52]) and stable after SZ onset throughout disease course [53-56]. There is a developmental aspect to cognitive deficits in SZ as the deficits are already present before disease onset and evident in individuals at high clinical risk to develop SZ [57]. The developmental aspect is underlined by studies showing that premorbid academic achievements during childhood and early adolescence relate to amongst others the severity of working memory deficits, while premorbid academic achievements during late adolescence are associated with a decline in cognitive functioning during SZ [58]. Furthermore, cognitive deficits in SZ get gradually worse during development and stabilize after SZ onset [59].

Cognitive deficits in SZ are thought to arise from the PFC [60] and neurobiological changes in the PFC of SZ patients accompany impairment of executive functioning [56,61]. In functional magnetic resonance imaging studies (fMRI), SZ patients show decreased activation of the dorsolateral (dl)PFC and medial (m)PFC during cognitive tasks [62-64]. Electrophysiological studies have revealed that in SZ patients abnormalities in gamma oscillations in the dlPFC contribute to working memory impairment [65-69]. Longitudinal studies indicate that prior to and during transition to psychosis cognitive deficits increase [57], and changes in the PFC are already present and associated with cognitive

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ability in individuals at high risk to develop SZ [70,71]. Familial SZ risk is associated with decreased fronto-parietal-controlled cognitive ability [72] and reduced mPFC deactivation is more pronounced in individuals at clinical high risk for SZ than in healthy siblings of SZ patients [70]. Moreover, structural brain abnormalities have been observed in high-risk individuals in that clinical high risk to develop SZ is associated with decreased cortical thickness in amongst others the middle frontal gyrus containing the dlPFC, and linked to cognitive ability [73]. In addition, PFC gyrification is higher in such individuals [74].

The degree of cognitive impairment in SZ negatively influences functional outcome and cognitive remediation therapy improves functional outcome [55,75-80]. Therefore, identification of novel treatment strategies that target cognitive symptoms in SZ is important and treatment of cognitive symptoms in high-risk individuals may represent an attractive way to delay or prevent psychosis onset [81].

## Development of the PFC in SZ

To allow the generation of novel treatment strategies that can effectively target PFC-dependent cognitive dysfunction in SZ, a better understanding of the pathophysiology of the PFC in SZ is needed. The first hypothesis concerning the neurobiology of SZ was the dopamine hypothesis that was established in the 1970s and stated that SZ is caused by globally increased dopamine levels in the brain. However, this hypothesis and its later adjustment concerning decreased frontal and increased striatal dopamine levels [82] have failed to satisfactorily explain the emergence of cognitive deficits in SZ [83,84]. Other hypotheses involve glutamatergic abnormalities [85,86] and inflammation [87,88]. The complexity and diversity of SZ illustrate that multiple mechanisms are involved in SZ neurobiology and that these mechanisms might be different or differentially involved in the pathophysiology of individual patients. Nevertheless, from the available literature it seems clear that cognitive symptoms in SZ arise from the PFC and that PFC development before and during SZ onset in adolescence plays a critical role. During adolescence, the PFC undergoes major maturational changes that entail the final increase in developmental myelination and refinement of the intracortical neuronal network. Together these maturational changes allow higher cognitive functions to arise [89]. Figure 2 provides an overview of the major changes in cortical maturation during adolescence and their involvement in SZ.

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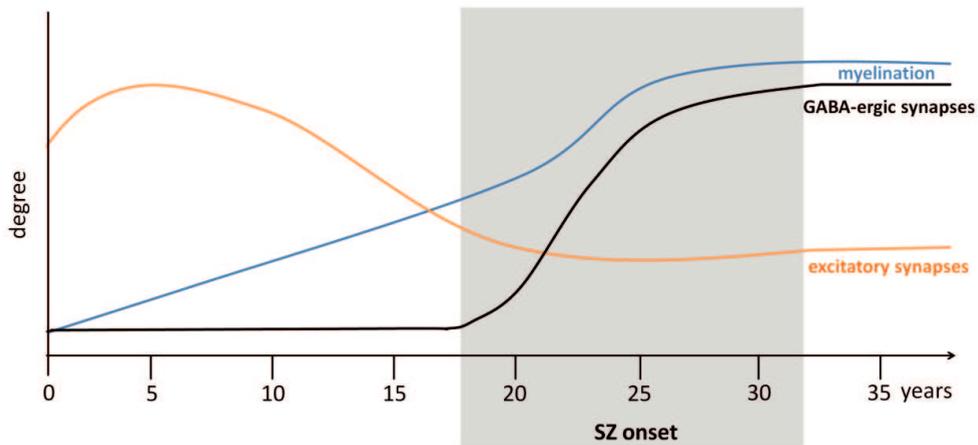
**Figure 2 – Changes during human PFC development.** Schematic representation of changes in the degree of myelination, and the number of GABAergic and excitatory synapses during adolescence (adapted from [89]).

## Maturation of the PFC GABAergic system and its link to SZ

During adolescence, changes in the cannabinoid and glutamatergic systems are evident in the PFC, but the largest maturational changes in the neuronal network concern the GABAergic system. In the adolescent PFC, GABAergic neurotransmission is increased by 30% [90]. There are several subtypes of GABAergic interneurons that include amongst others the fast-spiking parvalbumin [91] and the non-fast-spiking somatostatin, calbindin and calretinin interneurons [92]. During adolescence mRNA expression of parvalbumin is increased, while calretinin mRNA expression is decreased and calbindin mRNA expression is not changed in human dlPFC [92,93]. This might relate to the increase in dopaminergic activation of parvalbumin interneurons that occurs in PFC layer V-VI during adolescence and boosts parvalbumin interneuron activity while not influencing the other interneuron types [92,94,95]. This is in line with the adolescent increase in the mRNA expression of the α1 GABA$_A$ receptor subunit, because this subunit is predominantly present post-synaptically of fast-spiking parvalbumin interneurons [96]. Notably, as opposed to other interneuron types, parvalbumin interneurons get myelinated in an activity-dependent manner and obtain perineuronal nets in the extracellular matrix during adolescence [92,97-99]. Therefore, only parvalbumin and not calbindin or calretinin interneurons undergo changes during adolescence that result in an increased firing rate.

In post-mortem adult SZ PFC, mRNA and protein expression of amongst other GABA-related genes parvalbumin, pan-interneuronal marker glutamate decarboxylase 67 (GAD67) and of the α1 GABA$_A$ receptor subunit is reduced [93,100-103]. One study found not only a lower parvalbumin mRNA expression, but also that parvalbumin-related mRNA

expression patterns in SZ PFC resembled those of immature parvalbumin interneurons [104]. In addition, the perineuronal nets that surround parvalbumin interneurons develop during adolescence and are reduced in SZ dlPFC [105]. However, in the cortical layers no differences in parvalbumin cell density or distribution is found [106,107]. Also, somal size and the density of synapses of parvalbumin interneurons on pyramidal cells are normal, although a decrease in parvalbumin protein expression in these synapses is found [108,109]. Parvalbumin protein expression is dependent on neuronal activity [110]. Therefore, these findings indicate an intrinsic functional abnormality in parvalbumin interneurons that arises during adolescence and renders them less active [111,112]. A reduced activity of parvalbumin interneurons in SZ PFC is supported by the above-described observation that gamma-band oscillations are impaired in SZ PFC, as gamma-band oscillations are produced by a rhythmic inhibition of pyramidal neurons via parvalbumin interneurons [113].

## Maturation of PFC myelination and its link to SZ

Another major maturational process that takes place in the adolescent PFC is a developmental increase in myelination. Myelin is an extension of the oligodendrocyte (OL) cell membrane that envelops long stretches of axons and as such improves conduction velocity of action potentials, and provides metabolic and trophic support to the axon [114]. The myelin sheath is composed of 15-30% protein and 70-85% lipids. Lipids in the myelin membrane include predominantly cholesterol, galactosylceramide and ethanolamine plasmalogen lipids [115]. One OL may produce up to 60 myelin internodes and these internodes can consist of up to 100 membrane folds that contain major dense lipid layers; OLs therefore have high rates of metabolic and lipid biosynthesis (Figure 3a) [114]. Myelin is produced by mature OLs, and myelin production starts in the second trimester of pregnancy and is mostly completed within the first ten years of life. Only myelination of association cortices, including the PFC, continues until the third decade of life [116]. Accordingly, OLs can still differentiate from OL precursor cells (OPCs) in the adult brain to supplement the mature OL pool. During differentiation, OPCs form membrane protrusions that are arranged around the cell body like a spider web and cells that display these protrusions are termed premyelinating OLs (preOLs). PreOLs mature into myelinating OLs and at the myelinating OL stage cell membrane protrusions decrease, and convert into myelin membranes (Figure 3) [117]. The OPC, preOL and mature OL stages can be identified by cellular markers which form an important tool in research investigating OL lineage progression and myelination.
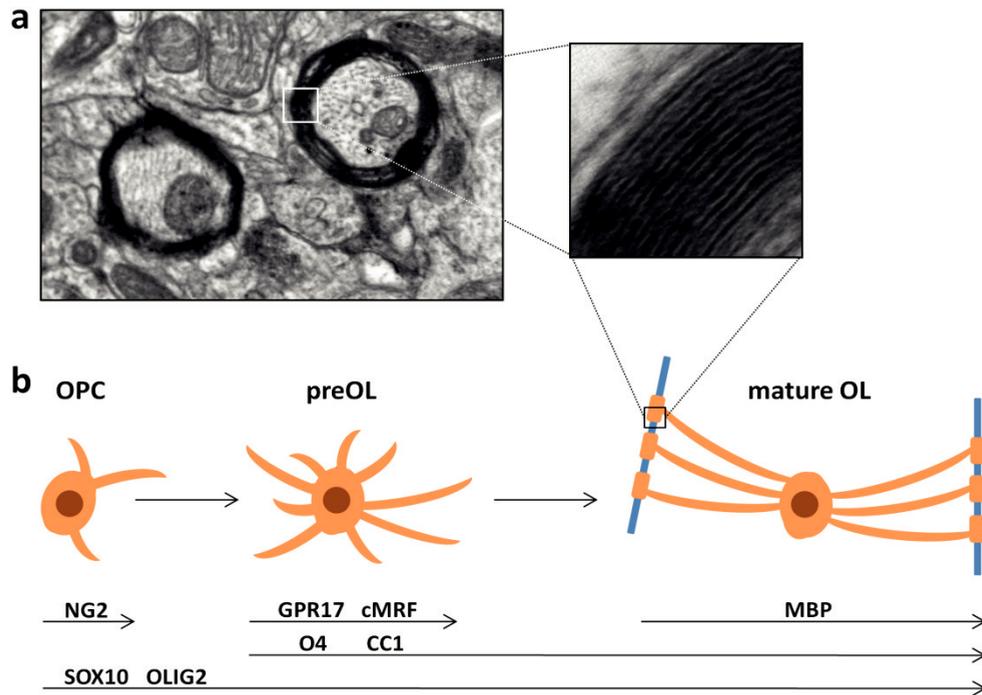
**Figure 3 – Cortical myelination and OL development. (a)** Electron micrograph of myelinated axons and a detail showing the layering of a mature myelin sheath containing major dense lines. **(b)** Schematic representation of the OL differentiation process from OPC to preOL to mature myelinating OL and their corresponding cellular markers. Neuron-glial antigen 2 (NG2), SRY-Box 10 (SOX10), oligodendrocyte transcription factor 2 (OLIG2), glycine rich protein 17 (GPR17), cytoplasmic myelin gene regulatory factor (cMRF), oligodendocyte marker (O4), adenomatous polyposis coli (APC; CC1), myelin basic protein (MBP).

Myelination in the PFC has been reported to occur on both excitatory neurons and parvalbumin interneurons, and its dysregulation is implicated in SZ, as described in detail in Chapter 2. Briefly, declines in white-matter integrity are observed in the PFC in both medicated and unmedicated SZ patients. Findings from genetic, post-mortem as well as animal model studies have revealed that abnormalities in myelination and OLs underlie the widely reported white-matter integrity decrease [118]. White-matter abnormalities in frontal brain regions are associated with cognitive impairment [119-121] and in first-episode SZ patients reduced white-matter integrity predicts a higher degree of cognitive impairment [122,123]. Notably, white-matter integrity deficits are observed in the PFC already in prodromal SZ patients and worsen with the transition to psychosis in adolescence, indicating that an impairment in the maturation of PFC myelination plays an important role in the etiology of cognitive deficits in SZ.

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As myelin consists mainly of lipids, lipid biogenesis may well be affected in SZ. Indeed, SZ is genetically associated with two genes involved in cholesterol homeostasis, namely sterol regulatory element binding protein (SREBP) 1 and 2 [124,125]. Interestingly, in post-mortem brain tissue of SZ patients SREBP1 protein levels are reduced [126] and abnormal levels of fatty acids are found [127]. Moreover, in drug-naïve SZ patients changes in the levels of free fatty acids and ceramide lipids are observed [128]. In addition, in a subgroup of SZ patients reduced levels of amongst others sphingomyelin lipids are correlated with more cognitive impairment [125]. However, it is still unclear whether alterations in lipid homeostasis are contributing to the causal pathophysiology of SZ or arise as a consequence of the disorder [129].

As the maturation process in the adolescent PFC involve both the maturation of parvalbumin interneurons and their myelination, and parvalbumin interneurons display intrinsic functional abnormalities in SZ, it has been hypothesized that defective myelination of specifically parvalbumin interneurons during SZ onset in adolescence is the cause of the cognitive deficits in SZ [111,118].

## Is oxidative stress a key player in the development of cognitive symptoms in SZ?

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the clearance of ROS by antioxidants. Oxidative stress is partly caused by mitochondria and has detrimental effects on cell proliferation and differentiation, and can result in cell death. Chapter 2 provides a detailed literature review concerning oxidative stress in SZ. Briefly, oxidative stress in SZ blood, cerebral spinal fluid and brain has been reported and the presence of oxidative stress in SZ patients is independent of disease state or medication use [130-133]. Mitochondrial dysfunction in SZ is observed in various brain regions and already present in individuals at high risk to develop SZ [134-137]. Notably, decreased levels of antioxidants are correlated with impaired PFC-dependent cognitive ability in first-episode SZ [138]. In addition, low levels of glutathione, the main antioxidant in the brain, are correlated with reduced white-matter integrity in the PFC of SZ patients [139]. Therefore, it seems likely that oxidative stress plays a role in the etiology of myelin-related and PFC-associated cognitive impairment in SZ. I hypothesize that this role is at least partly mediated by the negative effects of oxidative stress on OLs and this hypothesis is described in detail in Chapter 2. In comparison with all other brain cells, OPCs and premyelinating OLs (the cells in transition to become mature myelinating OLs; see above) are extremely vulnerable to oxidative stress due to amongst others their high metabolic demand and free iron use [140-143]. Therefore, I further hypothesize that during adolescence a build-up of oxidative stress in OPCs impairs their differentiation into mature OLs leading to an OL differentiation block and a lack of mature myelinating

OLs in the PFC of SZ patients. As such, PFC neurons do not get adequately myelinated during adolescence, impairing cognitive functioning in SZ.

Parvalbumin interneurons have a higher metabolic demand than other neurons because of their fast-spiking properties and as such have a higher ROS production than their non-fast-spiking counterparts. In addition, the perineuronal nets that surround parvalbumin interneurons have been found to be reduced in SZ dlPFC, while they develop during adolescence and protect against oxidative stress [105]. In mice with genetically engineered oxidative stress, the maturation of parvalbumin interneuron is delayed during adolescence and the formation of the perineuronal net is affected[144]. This indicates that parvalbumin interneurons in SZ during adolescence likely suffer from oxidative stress. Moreover, dysmyelination of specifically parvalbumin interneurons in SZ PFC has been proposed as a neuropathological mechanism involved in cognitive impairment in SZ [112].

Taken together, the negative effects of oxidative stress on the functioning of parvalbumin interneuron in the PFC are caused by two aberrant mechanisms. First, via decreased compensation of ROS produced during fast-spiking parvalbumin interneuron functioning leading to decreased parvalbumin interneuron activity and resulting in decreased cues for the activity-dependent myelination of these interneurons. Second, via impaired OL maturation that leads to a decreased capacity for myelination and reduced parvalbumin interneuron myelination. Conspicuously, both the development of the fast-spiking properties of parvalbumin interneurons and their myelination in the PFC occur during adolescence and are essential for the cognitive functions that are impaired in SZ.

## The APO-SUS/APO-UNSUS rat model for SZ

The use of animal models is pivotal to investigate the etiology and neurobiology of SZ. Animal models for SZ are often genetically engineered on the basis of mutations found in SZ families, but these animal models fail to mimic the complexity of non-familial SZ. Another way to study SZ in animals is with pharmacological models [145] such as the phencyclidine [146-148] or ketamine [149-151] models for SZ. Neurodevelopmental factors that play a role in SZ are also mimicked in for example maternal immune activation animal models [152-156] or maternal stress-induced animal models for SZ [157,158]. These genetic, pharmacological and environmental animal models for SZ are helpful for investigating particular endophenotypes involved in SZ pathology, but do not grasp the gene-environment interactions that are critical in the etiology of SZ. Therefore, applying an animal model that encompasses both genetic and environmental factors appears to be more attractive to explore the mechanisms underlying the complex disorder SZ [159]. One such model is the idiopathic apomorphine-susceptible (APO-SUS) rat model for SZ.

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To create the APO-SUS rat model, Nijmegen Wistar rats were exposed to the dopamine D2-receptor agonist apomorphine and their behavioural stereotypy upon drug injection was measured (Figure 4). A U-curve was found and Wistar rats that displayed an exaggerated behavioural response to apomorphine were selectively bred together to form APO-SUS offspring that was also susceptible to the effects of apomorphine [160]. Their phenotypic counterparts were selected based on the absence of a response to apomorphine and they were therefore termed apomorphine unsusceptible (APO-UNSUS) rats. In this thesis, I have used APO-SUS and APO-UNSUS rats from generations 33-45. APO-SUS rats display SZ-relevant behavioural traits without genetic or pharmacological manipulation and therefore the APO-SUS rat model is an idiopathic rat model that relies on interplay of genetic, epigenetic and environmental factors [161]. APO-SUS rats have a genetic variation in amongst others the gene encoding the γ-secretase component (gene dosage imbalance) and a mutation in the gene encoding the adrenocorticotropic hormone receptor receptor, and epigenetic abnormalities. During post-natal development APO-SUS rats receive less maternal care which influences their behaviour during adulthood [161]. The APO-SUS rats have been extensively studied as a model for SZ and show SZ-relevant behavioural traits in the positive domain, such as reduced prepulse inhibition, increased exploratory behaviour and dopamine-induced stereotypic behaviour [162-164], the negative domain, such as reduced latent inhibition and sucrose preference [161,165], and the cognitive domain, such as memory deficits [166]. In addition, APO-SUS rats display neurobiological similarities with SZ patients that include a hyperactive hypothalamus-pituitary-adrenal axis and elevated dopamine D2-receptor binding in the nucleus accumbens [167,168]. Notably, the PFC of APO-SUS rats shows neurobiological features similar to those described in the PFC of SZ patients, including GABA-signaling-related characteristics. For example, in APO-SUS mPFC the protein level of GAD67 is reduced during adolescence (P20-21). This was not accompanied by alterations in the number of calbindin-, calretinin- and parvalbumin-positive interneurons, suggesting a reduction in GAD67 expression per interneuron rather than a reduction in the number of interneurons. Synaptic input on LII/III pyramidal neurons in the mPFC remains unaltered, while paired-pulse ratio through $GABA_B$ receptor signaling is reduced [167]. This indicates a reduced firing rate of inhibitory synapses onto pyramidal cells in the adolescent APO-SUS mPFC which was associated with an increased protein expression of the perisynaptic $GABA_B$ receptor, suggesting a high sensitivity to GABA spillover-mediated reduction of inhibitory signaling [167]. Taken together, during adolescence the mPFC of APO-SUS rats recapitulates multiple GABA-related features that have been associated with SZ, including a reduced GAD67 expression, no changes in interneuron numbers and reduced inhibitory signaling. These features make the APO-SUS rat an attractive animal model to study the development of interneuron dysfunction and its link to cognitive behaviour with relevance to SZ.
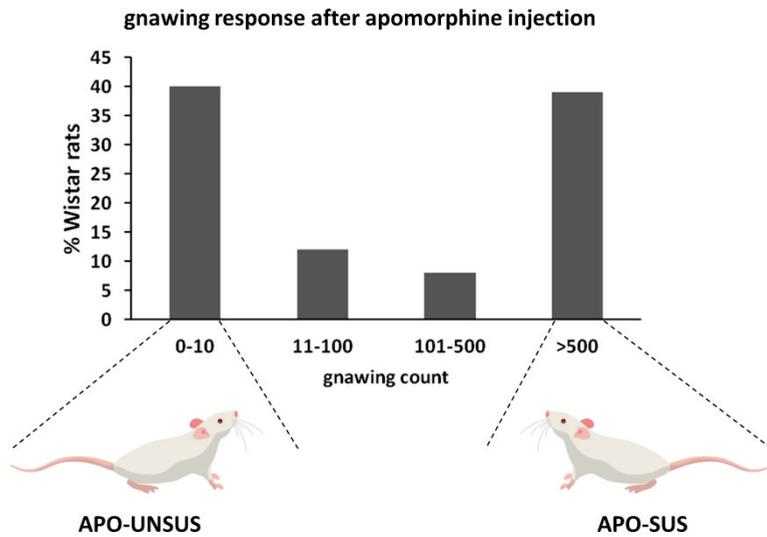
**Figure 4 – Generation of the APO-SUS/APO-UNSUS rat model for SZ.** A population of Wistar rats was injected with apomorphine and their behavioural response to apomorphine was measured in a gnawing test. During this test rats were allowed to gnaw on rubber rings for 45 minutes and the number of gnaws was recorded. Rats that gnawed 0-10 times in 45 minutes were selectively bred together as APO-UNSUS rats and rats that gnawed more than 500 times in 45 minutes were selectively bred together as APO-SUS rats. Graph adapted from Ellenbroek et al., 2002 (reference 159).

## Aims and outline of the thesis

In this thesis, I explore the neurobiological underpinnings of PFC dysfunction in SZ with a focus on the role of oxidative stress and myelination of parvalbumin interneurons, and using the APO-SUS/APO-UNSUS rat model for SZ.

In Chapter 2, I describe the hypothesis that this thesis aims to test. SZ cognitive symptoms are associated with PFC dysconnectivity and oxidative stress may play a key role in this defect. Furthermore, a decrease in white-matter integrity is observed in SZ PFC that is thought to be caused by impaired myelination through OLs. In this literature review, I discuss the available literature on oxidative stress, myelination and OL abnormalities in SZ. Based on this overview, I pose the hypothesis that in SZ a combination of genetic and environmental factors results in elevated oxidative stress in the brain. The oxidative stress affects OPCs more than other brain cell types, because of their high metabolic demand and free iron use. The critical period for the final developmental increase in PFC myelination is during adolescence and as such the PFC is the last of all brain regions to finalize the developmental myelination process. I further hypothesize that the build-up of oxidative stress in OLs during adolescence is such that efficient maturation of OPCs into myelinating OLs is affected, implicating a number of cellular pathways that cause an inactivation of the mTOR-P70S6K pathway

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and hence the PFC does not get myelinated adequately. Hypomyelination of the PFC is then thought to disturb neuronal network functioning in this brain region which leads to impaired cognitive functioning in SZ. This hypothesis will be experimentally explored in Chapters 3-5 of this thesis.

In Chapter 3, mPFC-related cognitive behaviour and the process of myelination in the mPFC of developing APO-SUS and APO-UNSUS rats are studied. I find that adult APO-SUS rats display mPFC-dependent cognitive inflexibility in a task modeled after the Wisconsin card sorting task that has been used to establish cognitive inflexibility in SZ patients. Using a wide range of techniques, including qPCR, western blot, immunohistochemistry, electron microscopy and array-tomography-like experiments, I uncover that specifically parvalbumin interneurons are hypomyelinated in the mPFC of APO-SUS rats. This hypomyelination is accompanied by a block of OL maturation at the premyelinating OL stage that is not caused by an intrinsic impairment of OLs. Intriguingly, brain stimulation by environmental enrichment applied during adolescence restores parvalbumin interneuron hypomyelination and OL maturation as well as cognitive inflexibility.

In Chapter 4, the role of oxidative stress in hypomyelination and cognitive impairment is assessed. Transcriptome analysis of the mPFC of adult APO-SUS rats reveals glutathione antioxidant metabolism as the most-enriched canonical pathway, and indeed I observe lower levels of the naturally occurring tripeptide antioxidant glutathione and mitochondrial changes in the APO-SUS mPFC. Treatment with the glutathione precursor N-acetyl cysteine (NAC) during post-natal development rescues not only glutathione and mitochondrial abnormalities, but strikingly also restores the number of myelinated axons, OL lineage cells and premyelinating OLs to control levels. Thus, glutathione metabolism is essential for developmental interneuron myelination in the mPFC. Moreover, in an mPFC-dependent behavioural test NAC treatment improves cognitive inflexibility of APO-SUS rats, indicating that glutathione metabolism is essential not only for proper myelination of the mPFC, but also for cognitive behaviours that are impaired in SZ.

In Chapter 5, the role of lipids in SZ mPFC is investigated. RNA sequencing analysis of the grey matter of post-mortem dlPFC of SZ patients and controls reveals that genes involved in the liver X receptor (LXR)/retinoid X receptor (RXR) lipid metabolism pathway are enriched among the differentially expressed genes. Moreover, I find that SZ shares genetic etiology with metabolites that are involved in lipid homeostasis including acylcarnitines, lipid content in very-low density lipoprotein (VLDL) particles and fatty acid composition. Intriguingly, LXR/RXR lipid metabolism, acylcarnitines, cholesterol efflux into VLDL particles and fatty acid biosynthesis share common molecular pathways. Moreover, using a publicly available data set we found that reduced cognitive performance is correlated with reduced lipid content (as measured by the MRI MP-RAGE signal) in the dlPFC grey matter in SZ patients. As about 80 percent of brain lipids

are part of myelin sheaths, and both lipid metabolism and myelin abnormalities are associated with cognitive performance, lipid metabolism may be key for the PFC-related cognitive impairment in SZ.

In Chapter 6, I discuss the findings described in this thesis and put the results in a broader perspective.

By studying the relationship between oxidative stress, interneuron myelination and cognitive functioning in the PFC of a rat model with SZ-relevant features, and by testing the effectiveness of two different treatment strategies to improve cognitive functioning, I hope to contribute to the understanding of the cognitive deficits in SZ.

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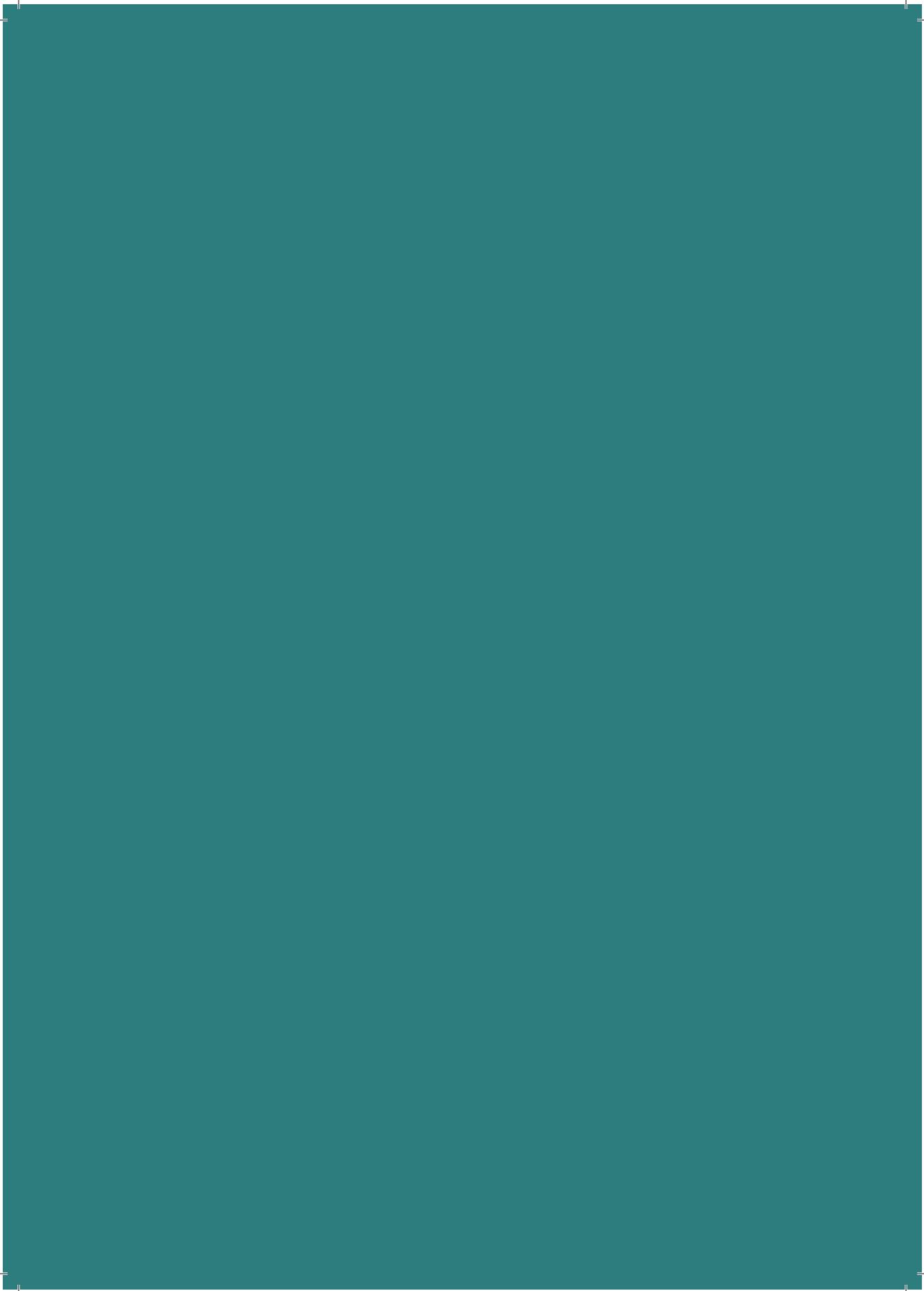
# Oxidative stress, prefrontal cortex hypomyelination and cognitive symptoms in schizophrenia

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## Abstract

Schizophrenia (SZ) is a neurodevelopmental disorder with a broad symptomatology, including cognitive symptoms that are thought to arise from the prefrontal cortex (PFC). The neurobiological aetiology of these symptoms remains elusive, yet both impaired redox control and PFC dysconnectivity have been recently implicated. PFC dysconnectivity has been linked to white matter, oligodendrocyte (OL) and myelin abnormalities in SZ patients. Myelin is produced by mature ols and OL precursor cells (OPCs) are exceptionally susceptible to oxidative stress. Here we propose a hypothesis for the aetiology of cognitive symptomatology in SZ: the redox-induced prefrontal OPC dysfunctioning hypothesis. We pose that the combination of genetic and environmental factors causes oxidative stress marked by a build-up of reactive oxygen species that, during late adolescence, impair OPC signal transduction processes that are necessary for OPC proliferation and differentiation, and involve AMPK, Akt-mtor-P70S6K and pparα signaling. OPC dysfunctioning coincides with the relatively late onset of PFC myelination, causing hypomyelination and disruption of connectivity in this brain area. The resulting cognitive deficits arise in parallel with SZ onset. Hence, our hypothesis provides a novel neurobiological framework for the aetiology of SZ cognitive symptoms. Future research addressing our hypothesis could have important implications for the development of new (combined) antioxidant- and promyelination-based strategies to treat the cognitive symptoms in SZ.

## Introduction

Schizophrenia (SZ) is a neurodevelopmental disorder with positive, negative and cognitive symptoms. Current treatments only target positive symptoms, therefore identifying new treatment strategies that aim at negative and cognitive symptoms is of crucial importance. To achieve this, the elucidation of the neurobiological correlates underlying these symptoms is a necessary first step. Cognitive symptoms of SZ, the focus of this review, include poor executive functioning and are thought to arise from the prefrontal cortex (PFC) [1, 2]. Both redox imbalance and PFC dysconnectivity have been implicated in the aetiology of these symptoms.

## SZ is associated with redox imbalance

Redox imbalance is a state of high oxidative stress caused by an imbalance between the production of reactive oxygen species (ROS) and antioxidants that reduce ROS. A continuous balance between ROS production and reduction is crucial to maintain ROS-dependent cellular processes as well as to prevent ROS-induced cell damage.

### Environmental insults that are associated with SZ cause oxidative stress

One of the most important risk factors for the development of SZ is the activation of the maternal immune system [3,4]. The mechanism by which maternal immune activation affects brain development likely involves oxidative stress [5]. For example, lipopolysaccharide (LPS) exposure during pregnancy induces the release of pro-inflammatory cytokines that induce ROS generation and peroxisomal dysfunction, while antioxidants such as N-acetyl cysteine can reverse the negative effects of LPS exposure on brain development [6]. Other environmental factors associated with redox imbalance and SZ are prenatal malnutrition and maternal stress during pregnancy [7-12]. For example, low protein intake during pregnancy has been show to induce mitochondrial dysfunction and a decrease in endogenous antioxidants resulting in higher ROS production [13]. In addition, obstetric events, such as hypoxia, and environmental insults later in life, such as social stress, are associated with oxidative stress and represent risk factors for SZ [14-20].

### Redox imbalance in SZ patients

Genetic studies have shown associations between oxidative stress gene polymorphisms and SZ [21, 22], including genetic variations in glutathione cysteine ligase (GCL) and several glutathione-S-transferases (GSTs) [23-25], both involved in the synthesis of the endogenous antioxidant glutathione. Fibroblasts of patients carrying genetic variations in GCL display lower glutathione and GCL protein expression, and thus redox imbalance [25]. Unlike genetic association studies, the available genome-wide association studies (GWASs) have not provided convincing evidence for oxidative stress-related genetic

predisposition in SZ and therefore additional GWASs with larger sample sizes may be necessary.

Additionally, both downregulation of components of the antioxidant synthesis pathway and increases in ROS levels have been observed in SZ patients. For instance, total antioxidant and glutathione plasma levels are lower in non-medicated, medicated, first-episode as well as chronic SZ patients [26-29], in line with the reduced glutathione levels found in the PFC and cerebral spinal fluid (CSF) of SZ patients [30, 31] and in *post-mortem* SZ brains [32], in which abnormal redox-related protein expression has also been found [33]. Furthermore, peripheral levels of ROS are increased, and those of glutathione peroxidase (GPX) and superoxide dismutase (SOD) are decreased in SZ patients [34-39], independent of drug use or disease stage. Hence, both lower levels of antioxidants and higher levels of ROS are core features of the disorder and not influenced by disease progression or medication use, indicating that redox imbalance is a primary disease characteristic. Interestingly, in SZ patients, deficits in executive functioning are correlated with higher ROS levels and lower antioxidant-related protein levels [38], directly linking redox imbalance to cognitive dysfunction [35].

### Redox imbalance in SZ rodent models

The MK-801-induced rat model for SZ shows increased oxidative stress specifically in the PFC [40], while higher levels of brain mitochondrial ROS have been found in a ketamine-induced rat model [41]. Inversely, glutathione depletion in rats leads to SZ-like phenotypes [42-44]. Additionally, knock out (KO) mice that lack a crucial subunit of the GCL enzyme show significant reduction of glutathione levels in the anterior cortex [45], especially during the prepuberal period, which are followed by SZ-like behaviour in the time frame of disease onset [46] and SZ-like neural changes in adulthood [47]. Therefore, redox imbalance may represent the main trigger for brain alterations before disease onset, which negatively influence cognition later on.

## Prefrontal dysconnectivity is associated with cognitive symptoms of SZ

Diffusion magnetic resonance imaging (dMRI) reveals alterations in white matter (WM) integrity, i.e. Lower fractional anisotropy (FA) (for a review, see [48]), in both medicated and non-medicated SZ subjects [49, 50]. Importantly, even before SZ disease onset, a reduced WM integrity occurs in frontal areas and advances in further stages of the disorder to more caudal and posterior regions [50-55].

### WM abnormalities in SZ are associated with cognitive symptomatology

Correlations between cognition and frontal WM integrity have been reported in healthy individuals [56, 57]. In chronic SZ, abnormalities in cognitive processing speed are associated with WM disruptions in amongst others frontal areas [58-60] and in first-episode SZ patients a lower frontal WM integrity is correlated with more severe cognitive symptoms [61, 62]. Interestingly, deficit SZ (i.e. SZ with strong cognitive impairment; [63, 64]) is associated with severe WM abnormalities [65-68]. Furthermore, cognitive symptomatology of SZ patients worsens as the disease progresses, in line with the ongoing WM alterations [69, 70].

### Origin of lower FA in SZ PFC

A low FA value in dMRI is indicative of alterations in WM that can be attributed to several cellular factors, including reduced myelination and aberrant axonal properties [71]. Diffusion tensor as well as kurtosis imaging reveal a lower FA and increased radial diffusivity in combination with no changes in axial diffusivity in the frontal lobe of SZ patients [72]. This indicates that myelin rather than axonal abnormalities form the neurobiological basis of the dMRI aberrations in SZ. Other diffusion studies show similar results [73, 74]. Direct evidence for axonal degeneration in SZ is indeed lacking. Furthermore, magnetization transfer ratio (MTR), a more specific imaging measure for myelin, shows lower myelin levels in amongst others the PFC of SZ patients compared to controls [75, 76]. These low myelin levels predict impaired processing speed in SZ and link decreased myelination to cognitive symptoms of the disorder [77,] but see [78].

## SZ is associated with oligodendrocyte abnormalities and decreased myelination

Myelin is produced by oligodendrocytes (OLs) that are derived from OL precursor cells (OPCs) in the developing as well as the adult brain [79-81]. Plasticity in the formation and retraction of myelin sheaths by OLs also occurs from early childhood to adulthood [80, 81]. Neuronal activity can instruct OPCs to divide and mature, and can stimulate myelin sheath production by OLs [82], leading to increased myelination and improved behavioural performance [83]. Conversely, reduced neuronal stimulation by social isolation impairs myelination which correlates with behavioural and cognitive dysfunction [84, 85]. Accordingly, altered myelination dynamics may play a major role in cognition as well as in psychiatric disorders like SZ.

### Evidence from human *post-mortem* studies

In the PFC of SZ patients, lower OL size and regional-specific differences in OL density alongside higher levels of OL apoptosis and necrosis have been observed, accompanied by lower levels of myelin [86-90]. Furthermore, expression of the myelin-associated proteins

2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNP) and myelin associated glycoprotein (MAG) is significantly lower in SZ anterior frontal cortex [90], and differential mRNA expression of these two and other myelin-related genes has been observed in SZ dlPFC [91]. Overall, there is abundant evidence for an OL as well as a myelin deficit in the PFC of SZ *post-mortem* brain.

### Evidence from rodent models

Evidence for a myelin deficit in SZ is also provided by studies on rodent models that range from pharmacological and transgenic to neurodevelopmental models. For example, administration of the NMDA-antagonist MK-801 in adulthood is used as a model for SZ (for a review see [92]) and alters brain expression of amongst others platelet-derived growth factor (PDGF), proteolipid protein (PLP), myelin basic protein (MBP) and CNP [93], decreases WM volume, together with myelin sheath degeneration [94]. Furthermore, mice transgenic for SZ-associated locus G72/G30 show SZ-like behavioural traits and myelin-related protein expression changes [95]. In addition, severe hypomyelination has been observed in mice mutant for the myelination-associated gene *quaking* (a gene downregulated in SZ) alongside structural abnormalities of myelin sheath thickness and composition [96, 97]. Moreover, rodent models for hypomyelination display SZ-like behavioural abnormalities, e.g. Cuprizone demyelination leads to reduced expression of several OL-related transcripts and diminished ability to perform a SZ-relevant cognitive flexibility task [98].

### Genetic evidence

OL-related gene variants correlate with reduced WM integrity and cognitive performance [99, 100]. Nevertheless, candidate gene association studies and a large meta-analysis of genetic risk for SZ have shown that myelin- and OL-related genes are not significantly associated with the disorder [101-105]. Therefore, in most cases the myelin pathology observed in SZ likely reflects a secondary phenotype with an indirect, non-genetic cause.

## Redox imbalance can cause an OPC maturation deficit

OPCs and OLs contain exceptionally high amounts of ROS (six times as much), three times lower glutathione concentration and twenty-fold higher free-iron levels [106, 107], probably because their myelin synthesis entails a high metabolic rate [108]. This means that OPCs and OLs are constantly under a high degree of oxidative stress to which the cells are already more susceptible. In fact, redox changes of only fifteen to twenty percent can already influence signal transduction pathways such as PDGFα stimulation of OPC proliferation and maturation [109]. The susceptibility of OPCs and OLs to oxidative stress has serious implications for the process of myelination. For instance, oxidative

stress leads to downregulation of myelin-related gene expression in human OLs *in vitro* [110], and reduced MBP expression and OL number in the rat brain [111-113]. Hence, the myelination abnormalities observed in SZ may well be due to oxidative stress-related OPC dysfunctioning.

## Hypothesis of redox-induced prefrontal OPC dysfunctioning

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Based on the above, we here propose the redox-induced prefrontal OPC dysfunctioning hypothesis of cognitive symptomatology in SZ. This hypothesis states that in SZ the combination of environmental factors and genetic predisposition causes oxidative stress, marked by a build-up of ROS in OPCs (Figure 1). During late adolescence, the high ROS levels impair OPC signal transduction processes that are necessary for their proliferation and differentiation. OPC dysfunctioning coincides with the relatively late onset of PFC myelination, and causes hypomyelination and disruption of connectivity in this brain area. The resulting cognitive symptoms coincide with SZ onset.

In the next sections, evidence for this hypothesis will be presented. First, the relationship between redox imbalance, hypomyelination and cognitive functioning in the PFC will be highlighted. Second, the molecular mechanisms underlying the impairment of OPC functioning by ROS will be discussed. Third, we will consider the critical developmental time period of PFC myelination and in particular of PFC hypomyelination in SZ.
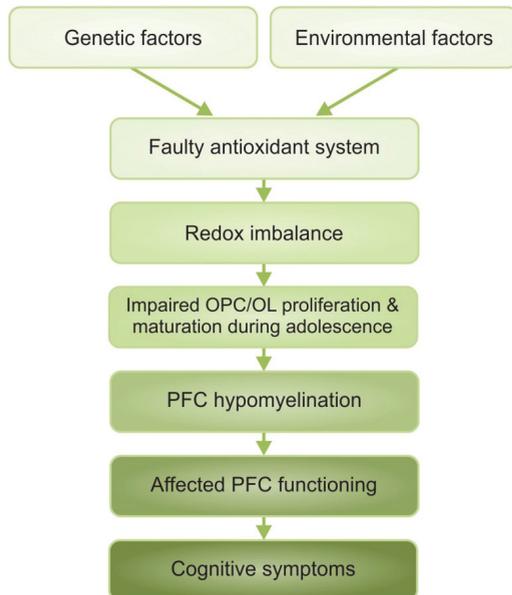


**Figure 1 - Flowchart of the redox-induced prefrontal OPC dysfunctioning hypothesis.** Environmental and genetic factors lead to a faulty antioxidant system, as well as redox imbalance resulting in OPC/OL proliferation and maturation arrest during adolescence, causing hypomyelination of the PFC, insufficient PFC functioning and subsequently the cognitive symptoms observed in SZ.

## ROS can cause OPC dysfunctioning

Baseline levels of oxidative stress in opcs are high. In SZ, oxidative stress levels in opcs are even higher because of extra ROS production by environmental factors as well as intracellular abnormalities that lead to extra ROS production and less ROS clearance (see above). The cause of OPC dysfunction in SZ may be explained by two different, but related, cellular pathways described below. In both pathways, ROS inactivates protein synthesis that is necessary for OPC proliferation and differentiation via the mtor-P70S6K pathway. The inactivation of the latter pathway leads to OPC proliferation arrest, apoptosis and hypomyelination [114].

Figure 2 presents a molecular map that is based on the literature described below and depicts the interactions among various molecules inactivating the mtor-P70S6K pathway in SZ OPCs.

### Inactivation of the mTOR-P70S6 pathway in SZ OPCs

The relatively active metabolism in OPC mitochondria leads to the production of ROS as a by-product of the respiratory chain (Figure 2). The elevated ROS levels cannot be effectively reduced by glutathione, because in opcs glutathione levels are low. Excess ROS leads to an overstimulation of AMP-activated protein kinase (AMPK), which activates the tuberous sclerosis 1/2 (TSC1/2) complex. This complex prevents the activation of the mtor-P70S6K pathway through inhibition of ras homologue enriched in brain (RHEB) [115]. Moreover, RHEB mediation of mTOR activity is necessary for OPC differentiation into myelinating OLs [116]. Also, AMPK stimulation causes enhanced biosynthesis of mitochondria via peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) as well as the upregulation of glutathione and other antioxidants. However, these antioxidant levels are not sufficient to rescue the redox imbalance in SZ OPCs [117]. PGC-1α transactivation of peroxisome proliferator receptor alpha (PPARα) inhibits transcriptional nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [118], preventing efficient transcriptional activation of its target genes, thus contributing to OPC dysfunctioning [118-120]. In addition, environmental factors implicated in SZ (e.g. Prenatal stress and malnutrition) may cause the production of cytokines such as tumor necrosis factor alpha (TNF-α) [121, 122]. This cytokine can activate AMPK, but also directly leads to both the mitochondrial uptake of calcium that might trigger apoptosis and to additional activation of complex I of the respiratory chain, followed by an increase in ROS production [115].

The other cellular pathway that can give rise to reduced activity of the mtor-P70S6K pathway includes signalling via PDGFRα. As stated above, activation of this receptor is necessary for proliferation and maturation of OPCs. The increased levels of ROS in SZ OPCs cause stimulation of Fyn kinase, which in turn activates C-Casitas B-lineage Lymphoma (C-CBL) [109, 123]. This overactivation of C-CBL has been shown to decrease pdgfrα receptor numbers on the OPC cell membrane, reduce mtor-P70S6K pathway

activation, and lower protein synthesis rate for proliferation and differentiation, disrupting OPC cell function [109, 124, 125]. Interestingly, glutathione depletion, both *in vivo* and *in vitro*, inhibits Fyn-dependent maturation of OPCs, accompanied by reduced myelination [126].

Proof of concept for the hypothesis that hypoactivation of the mtor-P70S6K pathway leads to inhibition of OPC proliferation and maturation, and subsequently hypomyelination is provided by the fact that conditional mTOR KO in mouse OPCs leads to various myelination defects [127]. Furthermore, a number of studies have demonstrated that ERK1/2 signalling (which inhibits the TSC1/2 complex and therefore increases mtor-P70S6K signalling) can enhance myelination. For example, ERK1/2 signalling is implicated in the mechanism of action of diosgenin, a drug that enhances OPC differentiation and myelination [128], and of miconazole, which promotes remyelination *in vitro* and in animal models of multiple sclerosis (MS) [129].

In sum, a correct regulation of the AMPK, mtor-P70S6K and ERK1/2 pathways is essential for OPC functioning and myelination. In SZ, these pathways are affected by increased oxidative stress, leading to OPC dysfunctioning and subsequently hypomyelination.

2

**2**
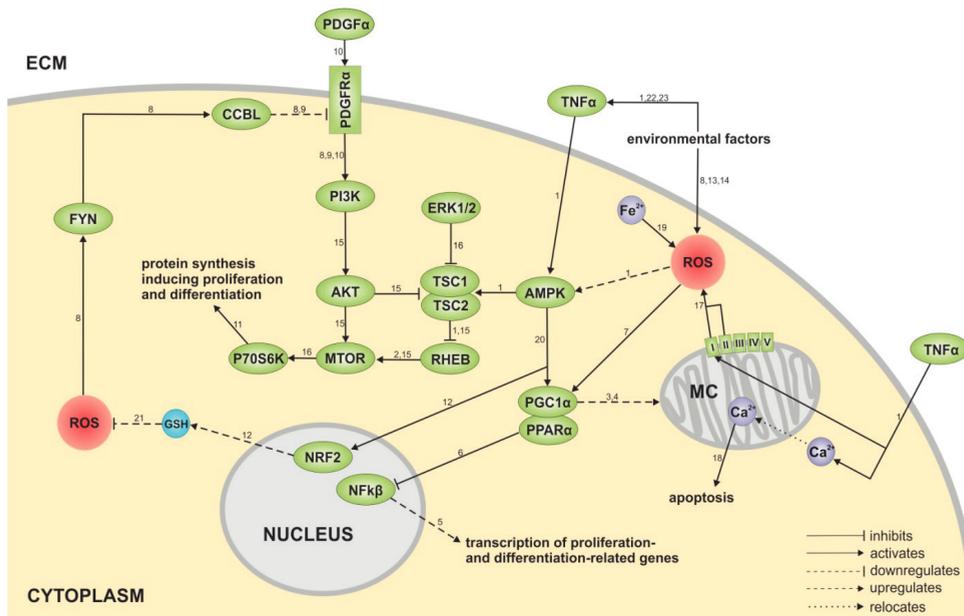


**Figure 2 - Molecular map of pathways that lead ROS to cause OPC dysfunctioning in SZ.** A molecular map is essential to elucidate the neurobiological mechanisms underlying the impairment of OPC functioning by ROS in the SZ PFC. The map shows that two cellular pathways result in a reduced activation of the mtor-P70S6K pathway under conditions of increased ROS production and decreased antioxidant levels. The first pathway involves ROS-induced downregulation of pdgfrα, leading to sub-activation of the mtor-P70S6K pathway. The second AMPK-related pathway leads to inhibition of the mTOR-P70S6K signalling cascade, as well as to downregulation of the transcription of proliferation- and differentiation-related genes. Inactivation of mtor-P70S6K causes decreased protein synthesis for proliferation and differentiation, and consequently leads to OPC dysfunction. See section 'ROS can cause OPC dysfunctioning' for a description, Supplementary Table S1 for the pertinent references, and the list of abbreviations for full names of all components of the molecular map.

## Redox imbalance, aberrant myelination and cognitive functioning are directly related

### Evidence from SZ patients and rodent models

Low glutathione levels are correlated with reduced WM integrity in the mPFC of SZ patients and in GCL KO mice PFC myelin and mature OL numbers are decreased [126]. Environmental risk factors for SZ that are related to oxidative stress are also linked to myelination abnormalities. For example, prenatal stress leads to myelination and WM abnormalities [130, 131], and prenatal infection causes effects on myelination and WM [132, 133]. The effects of prenatal infection on oxidative stress in adulthood are largely unknown, while in young animals the glutathione metabolism is affected [134, 135]. Although a link between prenatal infection and both myelination deficits and redox imbalance has thus

been observed, it is not clear whether the infection-induced effects on myelination are directly mediated by the redox imbalance. An interesting recent investigation studying the relationship between redox imbalance, reduced myelination and cognition has shown that *in vitro* hypoxia leads to oxidative stress that causes OPC maturation defects, which can be rescued by free-radical scavengers [136]. Likewise, under *in vivo* hypoxic circumstances ROS levels are higher, OPC maturation does not take place, myelination is decreased, mice show cognitive impairments, and when free-radical scavengers are provided the cellular as well as behavioural abnormalities are rescued [136]. Hence, redox imbalance causes hypomyelination and cognitive decline.

### Redox-related demyelination leads to cognitive defects in MS

The connection between oxidative stress and myelination defects, as observed in SZ, has also been found in MS, a disease associated with major demyelination. For example, in active demyelinating lesions of *post-mortem* MS brains high levels of oxidized lipids and DNA are present, and apoptotic OLs contain oxidized DNA [137, 138]. It is thought that in MS the elevated oxidative stress is caused by inflammation and leads to the progressive demyelination that characterizes this neurodegenerative disease [139]. The fact that MS patients show cognitive symptoms similar to those observed in SZ (for reviews, see [140, 141]) together with the observation that MS is associated with oxidative stress, decreased myelination and cognitive decline strengthens our hypothesis that an interaction between these factors exists in SZ.

## Is hypomyelination during SZ disease onset PFC-specific?

### Frontal WM development coincides with the prodromal SZ phase/onset of psychosis

WM maturation commences in central and extends to more lateral brain regions over time [142, 143], and WM volume peaks during early adolescence [144]. From this period onwards, the PFC white/grey matter ratio rises with increasing age [145]. In frontal areas, WM and connectivity maturation occurs during late adolescence. Also, the superior longitudinal fasciculus shows increasing connectivity during adolescence [146] and cortico-subcortical WM tracts reach peaks of maturation between the ages of 23 and 39 [147]. These findings indicate that WM maturation in frontal areas is ongoing during SZ disease onset.

High-risk individuals have a lower FA than controls [51], and prodromal patients (at-risk individuals that proceed to psychosis) show a progressive reduction in WM integrity in frontal regions over time [51], in contrast to the increase in integrity leading to the WM maturation peak observed in controls [148-150]. WM tracts of other association areas (e.g. the uncinate and arcuate fasciculi, the anterior and dorsal cingulate, and parts of the corpus callosum) are not different in high-risk *versus* prodromal individuals [151]. Moreover, in prodromal SZ patients WM integrity reductions are observed only

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in frontal areas, while in first-episode patients decreases in WM are found in frontal as well as more caudal regions, including the inferior longitudinal fasciculus and the internal capsule [51-53]. In chronic SZ, lower FA is found in frontal, caudal and more posterior regions, including the corpus callosum, minor and major forceps, inferior fronto-occipital fasciculus, and the splenium [50, 54, 55]. Thus, even before SZ disease onset a reduced WM integrity occurs in frontal areas, that advances in further stages of the disorder, proceeding from frontal towards more caudal and posterior brain regions.

Myelination of most brain regions is completed within the first year of life, whereas the myelination of association areas is ongoing until the thirties, after which myelin levels stabilize and finally decline from the late fifties onwards [152, 153]. The extent of cortical myelination is positively correlated with cognitive performance throughout life [153]. PFC myelination, which occurs during late adolescence, displays a time frame similar to that of PFC WM development [154]. In addition, human PFC myelin-related mrna expression peaks during late adolescence [155]. Thus, the prodromal phase / onset of SZ coincides with the time frame of PFC myelination, and during this stage frontal WM is affected [149]. Furthermore, adult SZ dorsolateral and medial PFC mrna expression patterns of OL-related genes are similar to those in the juvenile healthy developing brain [156]. Therefore, it seems that myelin does not reach the mature state in the SZ PFC during adolescence, as it does in healthy brain development.

## Cognitive symptomatology in SZ is associated with age-related decline in WM integrity

It is important to note that cognitive symptoms of SZ are observed already during the prodromal phase and worsen when psychosis starts. As such, these symptoms follow a developmental pattern that is similar to the decline in WM integrity in SZ. WM maturation and the cognitive functioning of inhibitory control are indeed correlated [157]. Also, the poor working memory of SZ patients correlates with a low WM integrity in the superior longitudinal fasciculus, a frontal structure that matures during adolescence [157].

## The role of OPCs in the PFC and other brain areas during adolescence

In the adult brain, OPCs are necessary for myelin repair following damage [158]. However, as OPCs make up to 4% of the adult brain [159] and appear to be evenly distributed throughout the brain it seems unlikely that they would be involved in only myelin repair. It has been hypothesized that following the major myelination event during the first year of life a subset of OPCs change into a subtype with a morphology and function different from those of precursor cells of OLs [160, 161]. This second type of OPC may play a role in the monitoring of neuronal activity and the immune response [160, 162]. Recently, a brain region-dependent variation in the distribution of various subtypes of OPCs has been shown, which differs between young and adult animals [163]. For example, adult monkey motor cortex OPCs mainly give rise to perivascular cells, not OLs [164]. Likewise,

during adolescence, readily myelinated brain areas may well have a set of OPCs that is functionally different from the set of OPCs in brain areas in which myelination is ongoing, such as the PFC that is likely to have OPCs programmed to become OLs.

### Oxidative stress may cause apoptosis of pre-OLs in the SZ PFC

The cells that are in transition from OPC to OL are called pre-OLs. The detrimental effects of ROS are the largest in this subtype of OLs [165, 166]. The excessive build-up of ROS in pre-OLs during SZ adolescence may lead to apoptosis or a cell cycle arrest followed by an inability to sufficiently produce myelin. In the SZ PFC, a lower number of cells expressing OLIG2 (a marker for all cells of the OL lineage) is observed, with no changes in the number of OPCs, suggesting that indeed PFC OPC maturation impairment in SZ is a likely cause of the lack of myelination in this brain area [167].

In addition to the PFC, demyelination and a decreased WM integrity have also been observed in the hippocampus (HIP) of SZ brains [168-171]. However, HIP WM defects become apparent during first-episode SZ and are fully evident only during the chronic state of SZ [171-173], and as such their development follows a different time course than the PFC WM defects that occur already in the prodromal phase.

Nevertheless, the neurobiological mechanisms causing OL and myelin defects may be similar in the PFC, HIP and other brain areas of SZ patients. Differentiating OPCs are most vulnerable to oxidative stress; therefore, PFC myelination that is dependent on these cells is harmed during early stages of SZ (as discussed above). Oxidative stress levels increase over time and may reach a level at which mature myelinating OLs are also damaged, and thus regions like the HIP and other brain areas, that depend on mature OLs to maintain proper myelination, will be affected during later stages of SZ.

## Neurobiological link between hypomyelination and interneuron abnormalities

A significant body of evidence suggests that interneuron abnormalities in both the PFC and HIP have an important role in SZ pathology [174-179]. Interneurons in the PFC mature during adolescence [179]. Apart from OLs and OPCs, interneurons are also relatively vulnerable to the effects of oxidative stress because of their high mitochondrial demand [180]. Interestingly, oxidative stress-based animal models for SZ display both myelin abnormalities and interneuron defects [181]. Oxidative stress in parvalbumin (PV) interneurons has been proposed as a cause of SZ [182] and interneuron densities are reduced in, amongst other brain regions, SZ PFC [183] and HIP [184]. Thus, the combination of aberrant myelination and reduction in the number of PV interneurons in the PFC and HIP, both caused by oxidative stress, may well lead to an inefficient neuronal network and eventually to SZ-like symptoms (for review, see [185]).

PV interneurons are responsible for the cortical high-frequency gamma-band oscillations that are involved in cognitive functioning and disrupted in SZ [186, 187]. The degree of myelination is dependent on neuronal activity [83], and PV cells are the most active of all interneurons and the only interneuron subtype to be myelinated [188]. Interestingly, a recent review states that the inefficient myelination of specifically PV interneurons, according to our hypothesis caused by high oxidative stress levels, would generate altered gamma band oscillations and cognitive deficits in SZ [188].

## Therapeutic implications

The redox-induced prefrontal OPC dysfunctioning hypothesis of the cognitive symptoms in SZ may have important implications for novel treatment strategies.

### Pharmacological manipulations

Based on the molecular map of the relationship between oxidative stress and OPC functioning (Figure 2) new preventive strategies for individuals at high risk for SZ could include antioxidant treatment. In this regard, antioxidant treatment is effective in rodent models [189] and decreases symptom severity in SZ patients [180, 190]. Therefore, the use of antioxidants, or compounds that generate an increased production of endogenous antioxidants, may be attractive for SZ therapy.

New potential therapeutic targets include components of the mTOR-P70S6K or ERK1/2 pathway (to be activated) and/or AMPK signalling (to be downregulated) in OPCs, and upregulation of the number of PDGFRα receptors in the cell membrane of OPCs. In this respect, increasing mtor signalling by inducing the upregulation of brain-derived neurotrophic factor (BDNF) (e.g. Through 1-amino-1,3-dicarboxycyclopentane) may be considered, and the drugs diosgenin and miconazole could be used to boost ERK1/2 signalling [128, 129, 191]. Moreover, drugs that are known to increase myelination by mature ols and that are tested in the MS field (e.g. benztropine [192]) may prove useful for the treatment of cognitive symptoms in SZ as well.

### Cognitive behavioural therapy

Cognitive behavioural therapy specific for cognitive deficits in SZ reduces symptom severity and improves cognitive performance [193-196]. As learning and neuronal activation upregulate myelin levels in cortical regions, and WM integrity in SZ is directly linked to cognitive functioning, beneficial cognitive and other neuronal activity-dependent therapies may be, at least in part, mediated by an experience-dependent increase of PFC myelination [83, 197-201].

## Conclusions

Here we propose the redox-induced prefrontal OPC dysfunctioning hypothesis for the aetiology of cognitive symptoms in SZ (Figure 1). This hypothesis states that in SZ a combination of increased ROS levels caused by genetic and/or environmental factors and decreased ROS clearance caused by a faulty antioxidant system leads to a build-up of ROS in OPCs. ROS may result in the dysfunctioning of OPCs through a number of cellular pathways, including the ERK1/2 and AMPK signalling cascades that cause an inactivation of the mTOR-P70S6K pathway, and hence negatively influence proliferation and differentiation of this cell type (Figure 2). OPC dysfunctioning occurs in late adolescence, during the critical period of PFC myelination. Therefore, in SZ patients the PFC is hypomyelinated, leading to dysconnectivity and the cognitive symptoms of SZ.

A next step would be the testing of the hypothesis proposed here, in both animal models and SZ patients. For instance, animal models for SZ that show both high oxidative stress levels and PFC hypomyelination (such as the APO-SUS rats, and the rodent prenatal infection and hypoxia models) may be treated with antioxidants from a young age onwards to assess whether lowering oxidative stress can (partially) rescue myelination deficits in the PFC, together with PFC-dependent cognitive functioning, and evaluate the width of the therapeutic window. Furthermore, MTR and dMRI may be used to study PFC myelination over time of individuals at high risk to develop SZ, together with PFC-relevant cognitive assessment. Such studies would establish a relationship between SZ risk, SZ development, PFC WM integrity, myelin levels and cognitive (dys)functioning. In addition, the studies would give insight into whether PFC WM and myelin deficits are indeed caused by a deficiency in prefrontal myelination within the window of SZ disease onset, and whether these shortcomings correlate with cognitive dysfunction in SZ. If confirmed, our hypothesis may significantly contribute to the development of novel antioxidant- and promyelination-based strategies to treat the cognitive symptomatology of this devastating disorder.

### Conflict of interest

The authors declare no conflict of interest.

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## List of abbreviations (in order of appearance):

SZ:             schizophrenia
PFC:            prefrontal cortex
ROS:            reactive oxygen species
LPS:            lipopolysaccharide
GCL:            glutathione cysteine ligase
GST:            glutathione-S-transferase
GWAS:           genome-wide association study
CSF:            cerebral spinal fluid
GPX:            glutathione peroxidase
SOD:            superoxide dismutase
KO:             knock out
dmri:           diffusion magnetic resonance imaging
WM:             white matter
FA:             fractional anisotropy
MRS:            magnetic resonance spectroscopy
OL:             oligodendrocyte
OPC:            oligodendrocyte precursor cell
CNP:            2',3'-Cyclic-nucleotide 3'-phosphodiesterase
MAG:            myelin associated glycoprotein
PDGF:           platelet derived growthfactor
PLP:            proteolipid protein
MBP:            myelin basic protein
AMPK:           AMP-activated protein kinase
TSC1/2: t       uberous sclerosis 1-2 complex
RHEB:           ras homologue enriched in brain
PGC-1α:         peroxisome proliferator-activated receptor gamma coactivator-1 alpha
pparα:          peroxisome proliferator receptor alpha
nfkb:           nuclear factor kappa-light-chain-enhancer of activated B cells
TNF-α:          tumor necrosis factor alpha
C-CBL:          C-Casitas B-lineage Lymphoma
MS:             multipele sclerosis
HIP:            hippocampus
PV:             parvalbumine
BDNF:           brain derived neurotrophic factor
GSH:            glutathione
MT:             mitochondria
ECM:            extracellular matrix

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# Supplementary material

**Supplementary Table S1.** List of references for the interactions/regulations depicted in Figure 2.

| Interaction/ regulation number | Reference for interaction/regulation |
|---|---|
| 1 | Bonora, M., et al., *Tumor necrosis factor-alpha impairs oligodendroglial differentiation through a mitochondria-dependent process.* Cell Death Differ, 2014. **21**(8): p. 1198-208. |
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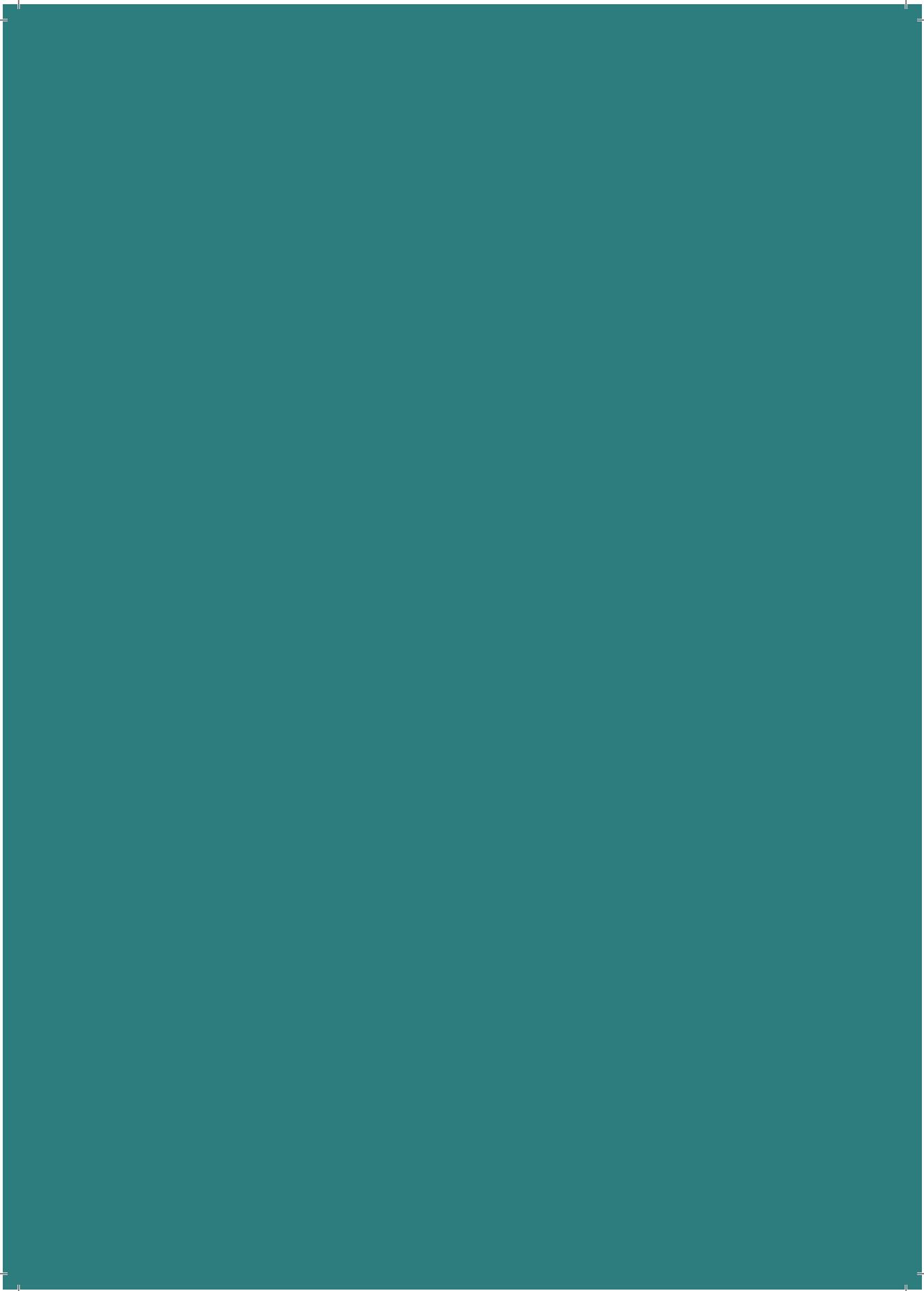
# 3

# Interneuron hypomyelination is associated with cognitive inflexibility in schizophrenia

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## Abstract

Proper cognitive functioning is dependent on a well-operating prefrontal cortex (PFC). Impaired cognitive functioning is a core feature of schizophrenia (SZ), and hypothesized to be due to myelination as well as interneuron defects during adolescent PFC development. Here we report that in a well-characterized rat model with SZ-relevant features these two neuropathological features are linked in that the myelination defect occurred specifically in parvalbumin interneurons. The adult rats displayed medial PFC (mPFC)-dependent cognitive inflexibility, and a reduced number of mature oligodendrocytes and myelinated parvalbumin inhibitory axons in the mPFC. In the developing mPFC, we observed decreased myelin-related gene expression that persisted into adulthood. Notably, environmental enrichment applied during adolescence restored parvalbumin interneuron hypomyelination as well as cognitive inflexibility. Collectively, these findings increase our understanding of the causal pathophysiology of SZ, and support the development of targeted cognitive therapy for individuals at high risk for and in early phases of SZ.

## Introduction

Higher cognitive functions such as working memory and attentional flexibility develop during adolescence, are optimal in early adulthood and are dependent on correct maturation of the prefrontal cortex (PFC) [1]. Interneurons in the PFC microcircuit as well as proper PFC myelination are known to be crucial for proper cognitive functioning [2,3]. Cognitive impairment is a core feature of schizophrenia (SZ), a complex neuropsychiatric disorder affecting 1% of the world population and with a large economic burden [4]. Human genetic and *post-mortem* as well as animal model studies have suggested an intrinsic defect of interneurons in SZ [5-10]. GABAergic interneurons play an important regulatory role in cortical microcircuits and encompass around 20 percent of all myelinated axons in the neocortex [11,12]. The interneuron defect in SZ is characterized by a reduced PFC expression of GABA-related mRNAs, while the number and dendritic arborization of PFC interneurons is not affected [13-15]. In addition, in the PFC of SZ patients lower myelin levels are found [16], *post-mortem* SZ brain tissue shows reduced myelin- and oligodendrocyte (OL)-related gene expression levels [17,18], and rodent models of SZ are characterized by myelination defects [19,20], whereas conversely animal models of demyelination display SZ-related behavioural characteristics [21]. It is further noteworthy that the onset of SZ coincides with both interneuron maturation and PFC myelination during late adolescence. On the basis of these findings, defective myelination of PFC interneurons during adolescence has been hypothesized to underlie cognitive impairment in SZ [14,22]. However, no studies have assessed the development and character of PFC interneuron myelination in this disorder [14].

Understanding the causal pathophysiology of SZ will allow the development of new treatment strategies. However, because of its highly complex nature SZ is mechanistically enigmatic and not easy to mimic in animals. Yet, animal studies are pivotal for mechanistic insight. A number of animal models exist in which a combination of genetic and environmental factors generates a SZ-related phenotype [23]. One such model is the apomorphine-susceptible (APO-SUS) rat which, together with its phenotypic counterpart the apomorphine-unsusceptible (APO-UNSUS) rat, has been extensively studied as an idiopathic model of SZ [24,25]. Without requiring genetic or pharmacological manipulation, APO-SUS rats show SZ-relevant behavioural traits in the positive (e.g. reduced prepulse inhibition, increased exploratory behaviour and dopamine-induced stereotypic behaviour) [26-28], negative (e.g. reduced latent inhibition and sucrose preference) [29,30] and cognitive (e.g. memory deficit) [31] domains as well as neurobiological similarities with SZ patients such as a hyperactive HPA-axis and elevated dopamine D2-receptor binding [15,32]. In particular, APO-SUS rats have a defective PFC microcircuit due to a reduced excitability of GABAergic interneurons, reminiscent of the interneuron abnormalities described in SZ patients [15]. We use the APO-SUS and APO-UNSUS rats as a model to examine the role of (developmental) interneuron myelination in PFC-dependent cognitive impairment. Here, we report that APO-SUS rats display cognitive

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inflexibility, that interneurons are hypomyelinated during PFC development, and that environmental enrichment during adolescence restores interneuron hypomyelination and rescues cognitive impairment.

## Results

### mPFC-specific cognitive inflexibility in APO-SUS rats

To explore cognitive functioning of the APO-SUS rats relative to that of the APO-UNSUS rats, we performed SZ-relevant cognitive behavioural tests in adulthood (postnatal day (P)90). The first test was to examine spatial working memory using the continuous delayed alternation paradigm with 10-second or 60-second delays between trials (each delay was conducted in five separate sessions) (Fig 1a). A trial was counted correct when the first arm entry was into the baited arm and performance was quantified as the percentage correct trials per session. Independent samples T-tests showed no difference in performance per session at the 10-second delay (t=-1.043, p=0.315, df=14), but confirmed a significant decrease in the 60-second delay performance per session (t=-2.947, p=0.015, df=13) in APO-SUS *versus* APO-UNSUS rats. These findings indicate that mPFC-linked spatial working memory is impaired in APO-SUS rats.

To confirm mPFC dysfunction, we next performed an extra-dimensional operant set-shifting test [33]. During this test rats were trained to press the lever above which a cue light was illuminated (visual cue discrimination) until they reached a criterion of ten correct trials in a row. There were no differences in the number of errors made during visual cue discrimination in APO-SUS and APO-UNSUS rats (Supplementary Fig S1). During the next session, the extra-dimensional shift was introduced, during which a reward was provided when the animal pressed the lever on one side of the cage (the non-preferred lever, as revealed by a side-bias test), irrespective of the location of cue light illumination (Fig 1b). The shift was repeated three times with the same group of rats. Relative to APO-UNSUS rats, APO-SUS rats made significantly more errors until the criterion (streak of ten correct trials) was reached during the second and third shifts (independent samples T-test shift 1 t=-0.141, p=0.899, df=36; shift 2 t=3.313, p=0.002, df=36; shift 3 t=2.066, p=0.048, df=36), suggesting reduced mPFC-dependent cognitive flexibility in APO-SUS rats. The increased number of errors made by APO-SUS rats represented perseverative errors, indicating a lack of inhibition of previously learned behaviour (independent samples T-test t=3.638, p=0.001, df=36).

To investigate whether the observed cognitive impairment is also found with behavioural tests dependent on other frontal areas such as the orbitofrontal cortex, we tested reversal learning in both the T-maze and the operant cages [34,35]. In the T-maze, rats were trained to retrieve a reward from one arm until they reached a criterion of 70% correct trials per session (Fig 1c). During the next session the reward could be retrieved from the opposite arm, as such requiring a complete reversal. The number

of trials needed by the APO-SUS and APO-UNSUS rats to reach the criterion of 70% correct performance per session during reversal learning was not significantly different (independent samples T-test t=1.993, p=0.068, df=13). We confirmed this result in the operant setup, where rats were trained to press the lever on one side of the cage until they reached a criterion of a streak of ten correct trials (Fig 1d). A complete reversal was introduced during the next session, when rats were required to press the lever on the opposite side of the operant cage. This reversal learning was performed three times in a row with the same group of rats. For all three reversals, we found no significant difference in the number of errors made until the APO-SUS and APO-UNSUS rats reached a criterion of a streak of ten correct trials (independent samples T-test reversal 1: t=1.579, p=0.123, df=36; reversal 2: t=1.725, p=0.093, df=36; reversal 3: t=0.628, p=0.534, df=36). However, APO-SUS rats made significantly more perseverative errors when the total number of errors during all three reversals was assessed (independent samples T-test perseverative errors: t=4.310, p<0.0001, df=36; regressive errors: t=0.658, p=0.515, df=36). This suggests that orbitofrontal cortex-dependent reversal learning behaviour is only mildly impaired in APO-SUS rats, and not as severely affected as mPFC-dependent working memory and cognitive inflexibility. Taken together, these data indicate that APO-SUS rats show cognitive inflexibility that involves mPFC dysfunction in SZ-relevant tasks.

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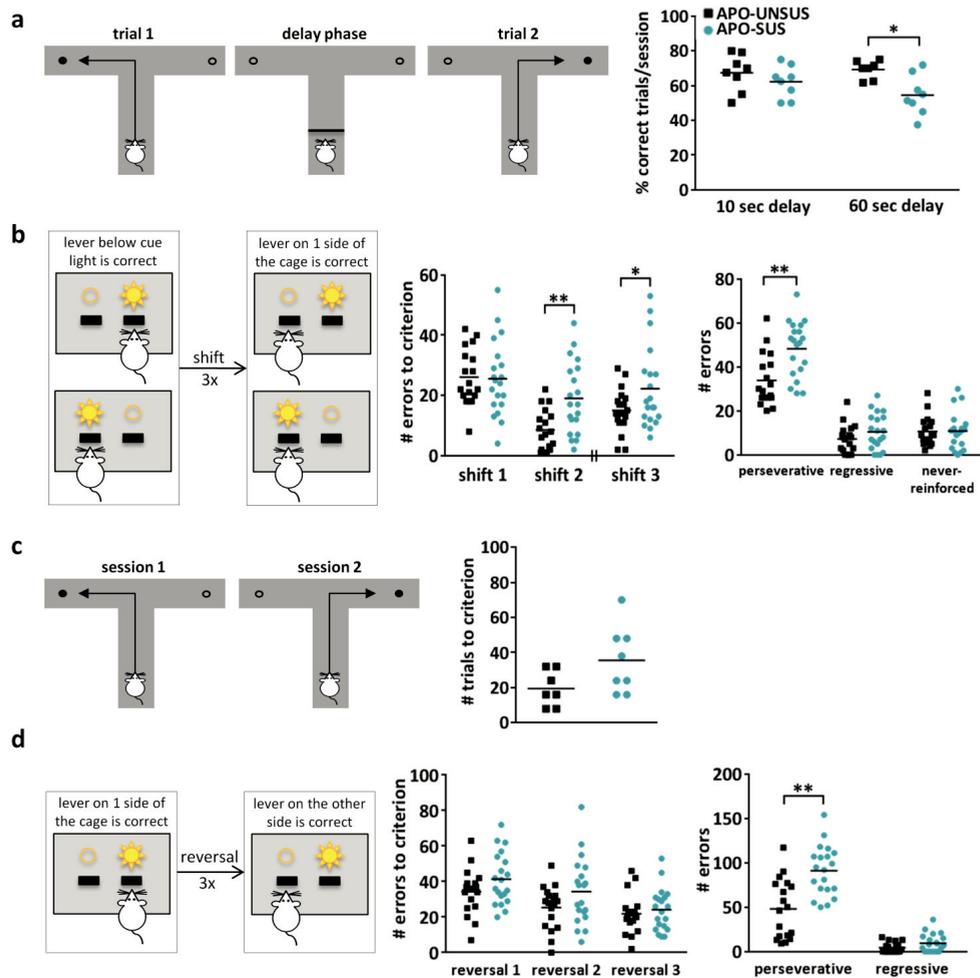
**Figure 1 – mPFC-dependent cognitive flexibility, but not reversal learning, is impaired in APO-SUS versus APO-UNSUS rats. (a)** Schematic representation of the continuous delayed alternation test. Rats were required to retrieve a food reward from alternating arms of a T-maze. Between trials, during the delay period of either 10 seconds or 60 seconds, the rat was placed in the startbox that was closed by a guillotine door. Delay periods were administered in separate sessions. Average percentage (%) of correct trials per session in APO-UNSUS (n=7) and APO-SUS rats (n=8) is depicted. **(b)** Schematic representation of the extra-dimensional set-shifting task. Rats were trained to press the lever above which a cue light was illuminated. From trail 21 of the next session onwards, rats were required to press the lever on one side of the cage irrespective of the cue light. Number of errors to criterion (streak of 10 correct trials) and total number of perseverative (following the 'old rule'), regressive (following the 'old rule' while more than 70% of previous trials were correct) or never-reinforced (pressing a lever that was incorrect during both the 'old rule' and during the current rule) errors in APO-UNSUS (n=18) and APO-SUS rats (n=20) are depicted. Note that between shift 2 and shift 3 the reversal learning trials were conducted (see d) **(c)** Schematic representation of T-maze reversal learning paradigm. Rats were trained to retrieve a reward from one arm of the T-maze until 70% correct performance was reached. During the following session, reward was to be retrieved from the opposite arm. Number of trials until criterion of 70% correct performance in APO-UNSUS (n=7) and APO-SUS rats (n=8) is depicted. **(d)**

Schematic representation of operant reversal learning paradigm. Rats were trained to press the lever on one side of the cage until criterion of a streak of 10 correct trials was reached. In the following session from trial 21 onwards rats were required to press the lever on the other side of the operant cage. Number of errors until criterion of a streak of 10 correct trials, and total number of perseverative and regressive errors in APO-UNSUS (n=18) and APO-SUS rats (n=20) is depicted. *p=<0.05 **p=<0.01 in independent samples T-test. Source data are provided as a Source Data file.

## Reduced myelin-related gene expression in the developing APO-SUS mPFC

Given that cognitive inflexibility in APO-SUS rats was observed in tasks that are dependent on mPFC functioning [33,36], we next investigated neurobiological features of this brain region. It was recently hypothesized that in SZ PFC interneuron dysfunction is associated with dysmyelination of interneurons and that this plays a pivotal role in the pathophysiology of cognitive symptoms of the disorder. Myelination of interneurons occurs during mPFC maturation around P21 in rodents. We have recently shown that in the mPFC of P21 APO-SUS rats there is a reduced inhibitory input onto pyramidal cells, whereas a normal number and morphology of interneurons were observed [15]. These interneuron abnormalities during adolescence resemble interneuron defects described in SZ patients [6,37]. To examine whether the APO-SUS interneuron defect is indeed linked to impaired myelination, we investigated the molecular and cellular correlates of myelin development in the mPFC of APO-SUS and APO-UNSUS rats.

From P21 onwards, qPCR analysis revealed a decrease in the expression of myelin-related mRNAs encoding proteolipid protein (*Plp),* myelin basic protein *(Mbp),* claudin11 *(Cldn11),* myelin oligodendrocyte basic protein *(Mobp),* myelin-associated glycoprotein *(Mag),* and myelin oligodendrocyte glycoprotein *(Mog)* in the mPFC of APO-SUS versus APO-UNSUS rats (Fig 2a; for primer design see Supplementary Table S1; for qPCR statistical values and exact number of samples, see Supplementary Table S2). In the barrel cortex (BC) and anterior corpus callosum (CC), we did not observe differences in the mRNA expression levels of any of these genes (Supplementary Fig S2), indicating unaffected myelin-related gene expression in cortical areas other than the mPFC and in white matter. Additionally, using western blot analysis we found a decrease in the expression of MBP in APO-SUS mPFC, but not BC (Fig 2b; independent samples T-test mPFC: t=-2.564, p=0.033, df=8; BC: t=-0.057, p=0.956, df=10). PLP immunofluorescence staining also revealed a decrease in myelinated areas in both the infralimbic (IL) and prelimbic (PL) subregions of the mPFC, but not in the BC of APO-SUS rats (Fig 2c, independent samples T-test IL: t=-2.757, p=0.017, df=12; PL: t=-2.34, p=0.037, df=12; BC: t=-0.778, p=0.451, df=12). These results suggest that during the development of the mPFC in APO-SUS rats there is a decrease in myelin-related mRNA and protein expression that persists into adulthood.
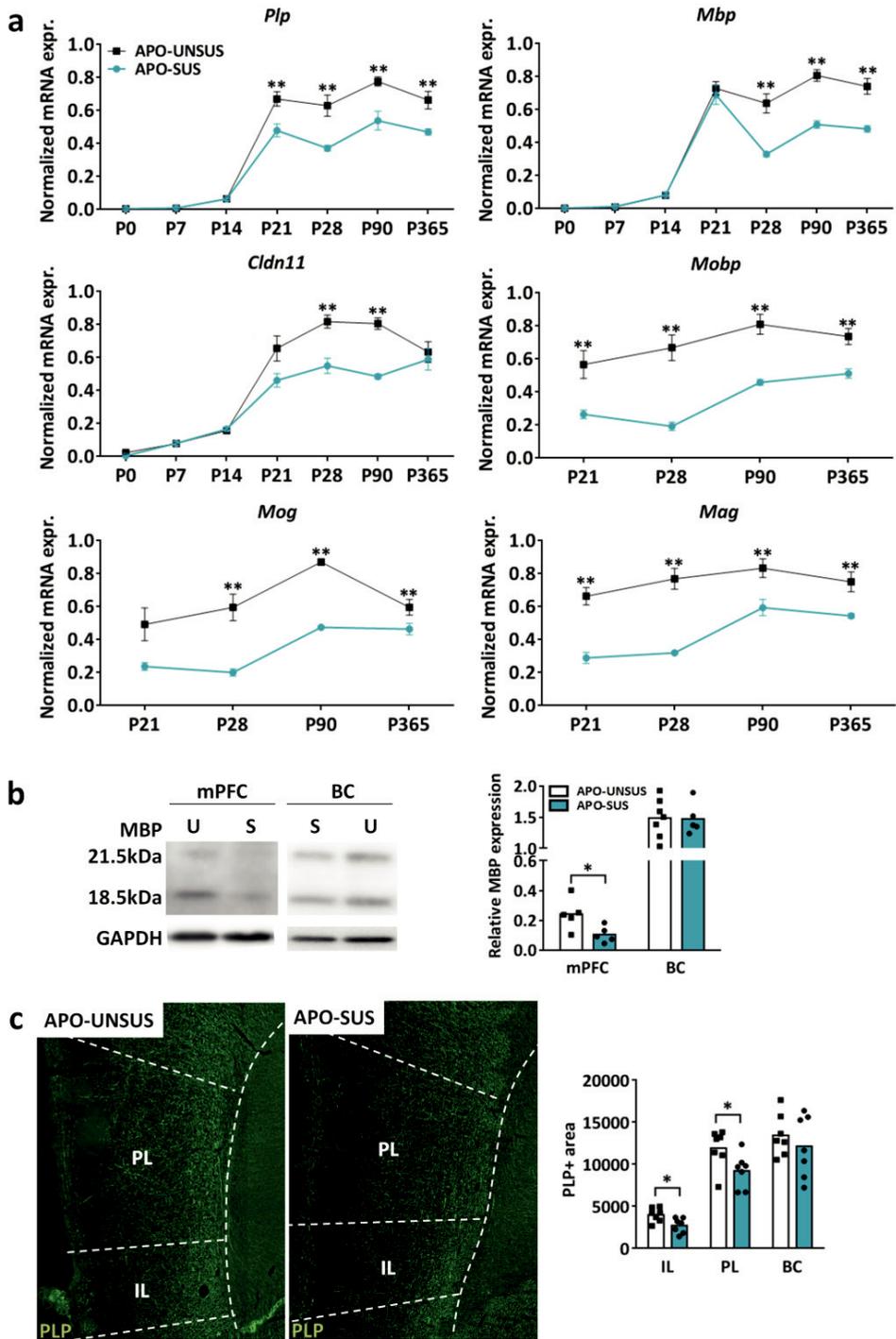
**Figure 2 – Decreased myelin-related gene expression in the development of APO-SUS versus APO-UNSUS mPFC. (a)** Developmental time course of normalized mRNA expression of the myelin-related genes proteolipid protein 1 (*Plp1*), myelin basic protein (*Mbp*), claudin 11 (*Cldn11*), myelin oli-

godendrocyte basic protein (*Mobp*), myelin oligodendrocyte glycoprotein (*Mog*) and myelin associated glycoprotein (*Mag*) in mPFC of P0, P7, P14, P21, P28, P90 and P365 APO-SUS rats versus APO-UNSUS rats. **(b)** Western blot examples for MBP and GAPDH protein expression in APO-SUS (S) and APO-UN-SUS (U) mPFC and BC. Quantification of Western blot analysis of MBP normalized to GAPDH in APO-SUS (n=5) and APO-UNSUS rats (mPFC: n=5 BC: n=7) is depicted. **(c)** Immunohistochemical analysis and quantification of PLP1 protein expression in IL, PL and BC of APO-SUS (n=7) and APO-UNSUS (n=7) rats. *p=<0.05 **p=<0.01 in independent samples T-test with Benjamini Hochberg multiple comparisons correction. Error bars represent standard error of the mean. Source data are provided as a Source Data file.

## Parvalbumin interneurons are hypomyelinated in the APO-SUS mPFC

The observed decreased expression of myelin-related genes in APO-SUS mPFC prompted us to assess the number of myelinated axons and myelin integrity using transmission electron microscopy at P90. We found a reduced number of myelinated axons in both IL and PL subregions of the mPFC, but not in BC of APO-SUS rats (Fig 3a; independent samples T-test IL: t=-5.308, p=0.006, df=4; PL: t=-2.832, p=0.047, df=4; BC: t=-1.143, p=0.317, df=4), although axonal density remained unaffected in the APO-SUS versus APO-UNSUS PL (independent samples T-test t=-0.182, p=0.864, df=4). This indicates a reduced number of myelinated axons rather than a reduced total number of axons in APO-SUS mPFC. Furthermore, a significant correlation between the thickness of the myelin sheet (G-ratio) and axon caliber was found in mPFC of both APO-SUS and APO-UNSUS rats (Fig 3b; regression analysis APO-SUS: F=59.830, p<0.001, df=154; APO-UNSUS: F=226.528, p<0.001, df=327). Note that in APO-SUS mPFC myelin thickness, as measured by its G-ratio, as well as myelin structure was normal (Fig 3b-c; independent samples T-test t=-0.431, p=0.684, df=5), suggesting that no demyelination occurred. The lack of demyelination was indicated by the absence of myelin debris in macrophages as revealed by Oil red O staining in the APO-SUS and APO-UNSUS mPFC (Supplementary Fig S3). Cumulative distribution analysis of the axon calibers of myelinated axons confirmed a reduction in the number of myelinated axons in APO-SUS IL and showed that this reduction occurred in axons with a wide range of axon calibers; the 8-10 µm axon caliber category was significantly different in APO-SUS compared to APO-UNSUS IL, the region where the number of myelinated axons was most significantly decreased in the APO-SUS rat (Fig 3d; independent samples T-test p=0.001, no difference in distribution as revealed by a Chi-square test p=0.482, df=8). Together, these findings suggest that a number of axons do not get myelinated in the APO-SUS mPFC.
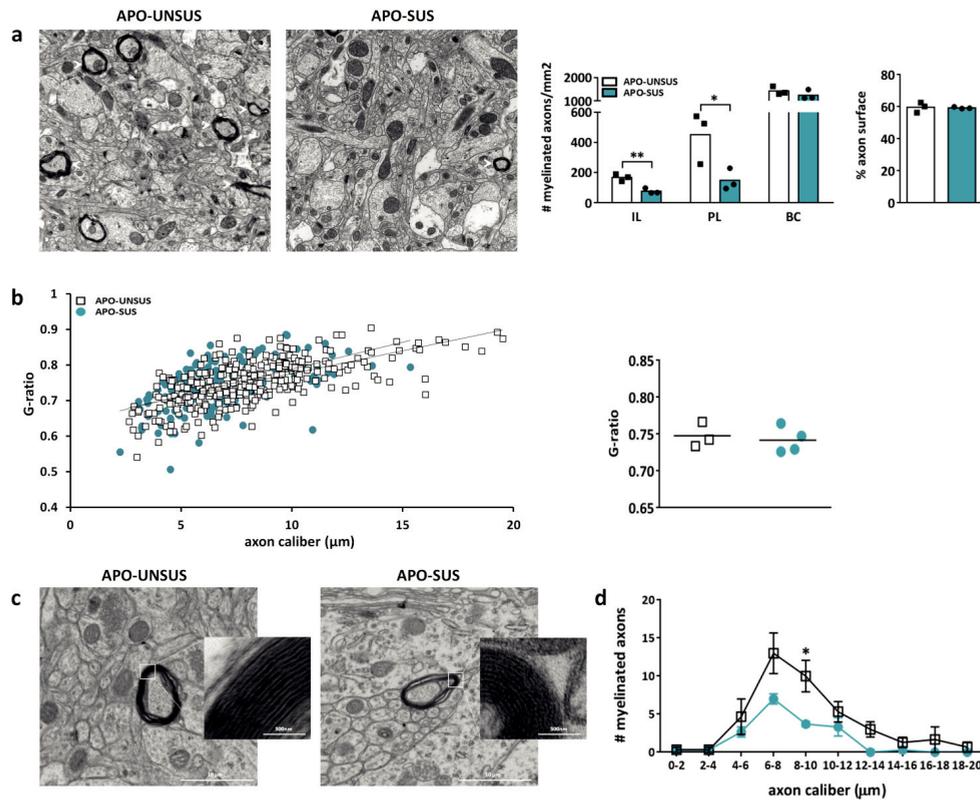
**Figure 3 – Hypomyelination in APO-SUS versus APO-UNSUS mPFC. (a)** Electron microscopy analysis and quantification of the number of myelinated axons in IL, PL and BC, and the percentage (%) of the total surface that represents axon in PL of APO-SUS (n=3) and APO-UNSUS (n=3) rats. **(b)** G-ratio (myelin sheet thickness) *versus* axon caliber and the average G-ratio for all myelinated axons in IL and PL of APO-SUS (n=4) and APO-UNSUS (n=3) rats. **(c)** high magnification electron microscopy images of the myelin ultrastructure in APO-SUS and APO-UNSUS IL **(d)** IL axon caliber frequency distribution in APO-SUS versus APO-USNUS rats. *p=<0.05 **p=<0.01 in independent samples T-test. Error bars represent standard error of the mean. Source data are provided as a Source Data file.

We next investigated whether this group of unmyelinated axons in APO-SUS mPFC corresponds to interneurons by performing immunofluorescent co-staining for MBP and GABA on 70 nm ultra-thin IL sections. This technique permits visualization of MBP+ myelin sheaths surrounding axons of GABAergic inhibitory neurons, allowing the precise quantification of myelinated inhibitory interneurons at the ultrastructural level (Fig 4a). We found that the proportion of MBP+ axons that was also GABA+ amounted to 20% in the mPFC of APO-UNSUS rats, similar to previously published values [11], and this proportion was significantly smaller in APO-SUS mPFC (Fig 4a; independent samples T-test t=-3.685, p=0.021, df=4). Therefore, the percentage of non-GABAergic myelinated axons was higher in APO-SUS mPFC and the reduced number of APO-SUS myelinated axons can be primarily attributed to a reduced number of myelinated GABAergic interneurons in the mPFC of APO-SUS *versus* APO-UNSUS rats. This notion is strengthened by our finding that the number of myelinated axons of different calibers was reduced in APO-SUS IL; myelinated GABAergic interneurons are known to have a range of different axon calibers [11]. To confirm that myelin integrity of GABAergic interneurons was not affected, we measured the length of nodes of Ranvier. We found for the myelinated GABAergic axons a similar average node of Ranvier length of 1.18-1.36 μm in APO-SUS and APO-UNSUS IL (Fig 4b), in line with previously reported values [38]. Myelination of GABAergic interneurons in the PFC has been reported to occur only on parvalbumin interneurons [11,39]. Immunostaining for parvalbumin and MBP on ultrathin tissue sections indeed revealed a reduced number of myelinated parvalbumin axons in APO-SUS relative to APO-UNSUS IL (Fig 4c; Independent samples T-test t=-4.306, p=0.013, df=4), confirming parvalbumin interneuron hypomyelination in the APO-SUS mPFC. This was not caused by a decrease in the number of parvalbumin interneurons in the mPFC of APO-SUS rats (Fig 4d; Independent samples T-test IL: t=-0.467, p=0.650, df=10, PL: t=0.436, p=0.672, df=10).

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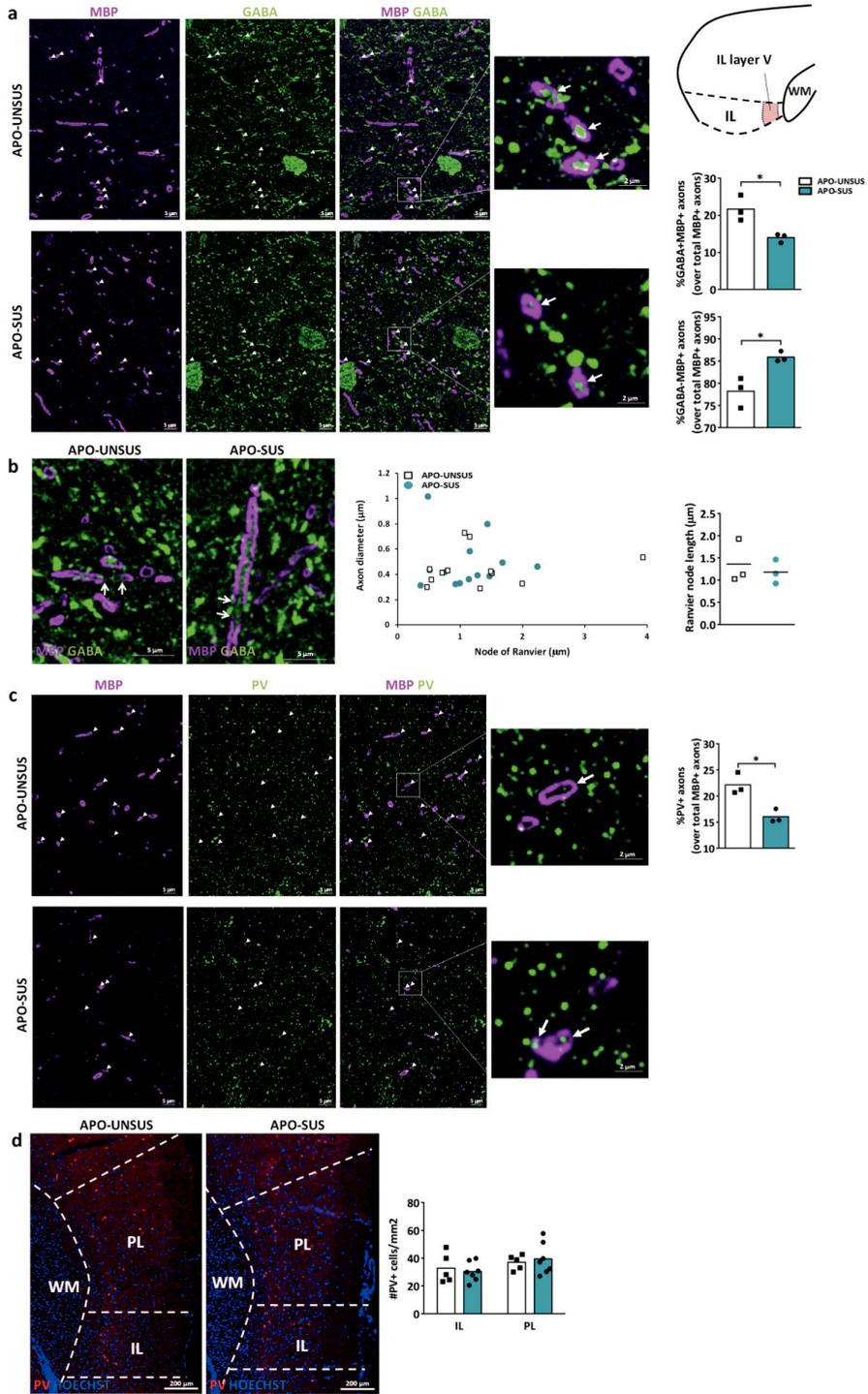


**Figure 4 – Hypomyelination of GABAergic parvalbumin interneurons in APO-SUS versus APO-UNSUS mPFC. (a)** Representative images and quantification of the proportion of myelinated

axons (MBP+) that is GABAergic (% GABA+ of total MBP+ axons) or non-GABAergic (% GABA- of total MBP+ axons) in IL layer V of APO-SUS (n=3) versus APO-UNSUS (n=3) rats. **(b)** Representative images and quantification of the length of the nodes of Ranvier of GABAergic myelinated axons in APO-SUS (n=3) and APO-UNSUS rats (n=3). **(c)** Representative images and quantification of the proportion of myelinated axons (MBP+) that are parvalbumin (PV) interneurons (% PV+ of total MBP+ axons). **(d)** Representative images and quantification of the number of PV interneurons/mm$^2$ in APO-SUS (n=7) and APO-UNSUS (n=5) mPFC. *p=<0.05 in independent samples T-test. Source data are provided as a Source Data file.

## OL maturation is impaired in APO-SUS mPFC

As we identified a lower number of myelinated inhibitory axons, we next examined whether the development of the myelin-producing OLs was impaired in APO-SUS mPFC. Therefore, we assessed the number and maturation state of oligodendroglial cells in APO-SUS and APO-UNSUS mPFC and BC during postnatal development. At P21, we observed no differences in the number of oligodendroglial cells in APO-SUS mPFC and BC (Fig 5a-e). However, in adulthood at P90 we found a decrease in the number of OLIG2+ OL lineage cells in the IL and PL of APO-SUS rats (Fig 5b; independent samples T-test P90 IL: t=-2.662, p=0.030, df=7.502; PL: t=-4.767, p<0.0001, df=12). A trend towards a decreased number of OL precursor cells (OPCs) in the IL and PL of P90 APO-SUS rats was identified (Fig 5c; independent samples T-test P90 IL: t=-2.157, p=0.069, df=6.729; PL: t=-2.099, p=0.058, df=12). Additionally, we observed an increase in the number of MRF+ and GPR17+ premyelinating OLs (Fig 5d; independent samples T-test P90 IL: MRF t=0.151, p=0.882, df=12, GPR17 t=2.013, p=0.069, df=11; PL: MRF t=2.257, p=0.043, df=12, GPR17 t=2.327, p=0.040, df=11; see Supplementary Fig S4 for evidence regarding the specificity of the anti-MRF antibody) and a decrease in the number of OLIG2+CC1+ mature OLs (Fig 5e; independent samples T-test P90 IL: t=-2.817, p=0.015, df=13; PL: -2.797, p=0.015, df=13), suggesting that OLs got arrested at the premyelinating stage and failed to mature in the APO-SUS mPFC. In the APO-SUS BC, we observed only a decrease in the number of mature OLs, indicating that the OPC differentiation impairment is specific to the mPFC.

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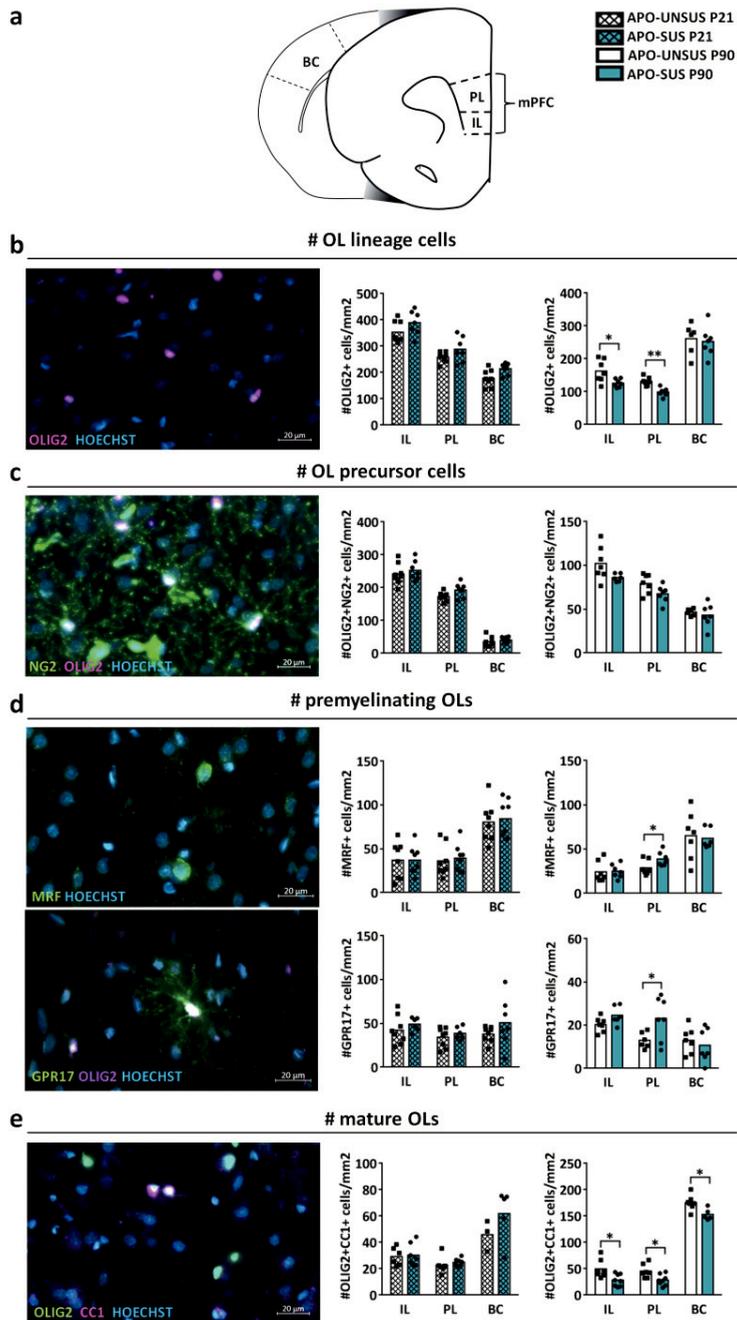
**Figure 5 – OL maturation arrest in APO-SUS versus APO-UNSUS mPFC. (a)** Schematic represen-
tation of IL, PL and BC in rat brain, adapted from the Paxinos and Watson rat brain atlas. **(b)** Number
of OL lineage cells (OLIG2+), **(c)** number of OL precursor cells (OPCs; OLIG2+NG2+), **(d)** number of
premyelinating OLs (MRF+ and GPR17+), and **(e)** number of mature OLs (OLIG2+CC1+) per mm$^2$ in the
IL, PL and BC of APO-UNSUS rats versus APO-SUS rats (P21 n=3-8, P90 n=5-8 specified in the graphs).
*p=<0.05 **p=<0.01 in independent samples T-test. Source data are provided as a Source Data file.

## Hypomyelination in the APO-SUS mPFC is not caused by intrinsic defects of OL lineage progression

To decipher the cause of the impaired OL maturation that leads to hypomyelination in the APO-SUS mPFC, we next examined whether OPCs have a cell-autonomous impairment of proliferation and/or maturation. To test this hypothesis, we used primary oligodendroglial cell cultures from cortex of APO-SUS and APO-UNSUS newborn rats and found no differences in the intrinsic capacity of OPCs to proliferate (Fig 6a; independent samples T-test t=-0.074, p=0.947, df=4) or differentiate (Fig 6b; independent samples T-test OPCs: t=-0.670, p=0.540, df=4; premyelinating OLs: t=0.423, p=0.697, df=4; mature OLs: t=-0.591, p=0.58, df=4). These data indicate that the hypomyelination in APO-SUS mPFC is not due to intrinsic defects of OL proliferation and differentiation. Rather, the results highlight the critical role of cellular dynamics in the APO-SUS mPFC that could negatively affect OPC differentiation and myelination. This notion is strengthened by the fact that during development and in adulthood we did not observe changes in myelin-related gene expression in brain regions other than the mPFC (Supplementary Fig S2).

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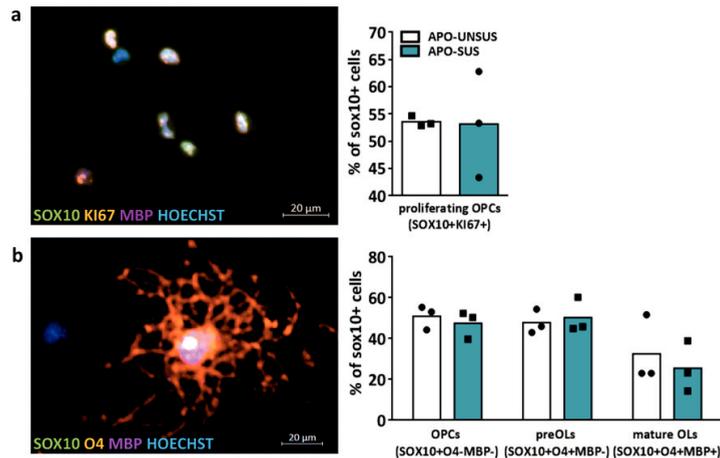


**Figure 6 – No difference in the intrinsic maturation capability of APO-SUS and APO-UNSUS OL precursor cells. (a)** Analysis and quantification of the percentage of all SOX10+ OPCs that are actively proliferating (SOX10+KI67+) in APO-SUS versus APO-UNSUS primary oligodendroglia cell cultures (n=3). **(b)** Analysis and quantification of the percentage of all SOX10+ oligodendroglia cells that is OPC (OPC) (SOX10+O4-MBP-), premyelinating OL (preOL) (SOX10+O4+MBP-) and mature OL (SOX-10+O4+MBP+) in APO-SUS versus APO-UNSUS primary oligodendroglia cultures (n=3). Source data are provided as a Source Data file.

## Environmental enrichment rescues OL maturation defects, hypomyelination and behavioural inflexibility in APO-SUS rats

The development of OLs and the myelination process can be positively influenced by behavioural experiences such as voluntary exercise, socializing and environmental enrichment [40-43]. The effects of behavioural experiences on myelination are thought to be caused by cellular changes such as an increase in neuronal activity, higher levels of neurotransmitters and growth factors, and differential release of metabolites. Importantly, higher rates of sedentary behaviour, less physical activity and decreased sociability in adolescents at high risk to develop SZ correlate with cognitive symptoms in later stages of SZ [44-49]. Based on these findings we applied an environmental enrichment paradigm in order to favorably influence the development of OLs, myelination and cognitive flexibility in the APO-SUS rat model.

We studied OL maturation in APO-SUS and APO-UNSUS rats that were reared under environmental enrichment or standard housing conditions from weaning at P21 onwards, and were sacrificed at P90 for histological analysis (Fig 7a). Immunofluorescence analysis revealed that APO-SUS rats reared in an environmental enrichment had significantly more OL lineage cells in the mPFC than APO-SUS rats reared in standard housing (Fig 7b; one-way ANOVA F=2.497, p=0.097, df=3; APO-SUS standard housing versus environmental enrichment t=-2.960, p=0.018, df=8; APO-SUS standard housing versus APO-UNSUS standard housing t=-2.326, p=0.045, df=9);in the environmental enrichment experiment, we have analyzed IL as there were no substantial differences between IL and PL mPFC subregions in our previous analysis (see Figs 2-5). Environmental enrichment restored the number of OPCs in APO-SUS IL to the IL OPC numbers in APO-UNSUS rats housed under standard conditions (Fig 7c; one-way ANOVA F=1.472, p=0.260, df=3; APO-SUS standard housing versus environmental enrichment t=-3.329, p=0.013, df=7; APO-SUS standard housing versus APO-UNSUS standard housing t=-1.897, p=0.090, df=9). The number of premyelinating OLs was also restored in the IL of APO-SUS rats reared in environmental enrichment as revealed by immunohistochemical experiments with the premyelinating OL markers MRF and GPR17 [50] (Fig 7d; one-way ANOVA MRF: F=4.036, p=0.026, df=3, GPR17: F=2.108, p=0.135, df=3; APO-SUS standard housing versus environmental enrichment MRF: t=2.945, p=0.015, df=10, GPR17: t=0.799, p=0.433, df=10; APO-SUS standard housing versus APO-UNSUS standard housing MRF: t=3.659, p=0.005, df=9, GPR17: t=2.233, p=0.47, df=11). The number of mature OLs, however, was not affected by environmental enrichment in the mPFC of both APO-SUS and APO-UNSUS rats (Fig 7e; one-way ANOVA: F=1.770, p=0.199, df=3; APO-SUS standard housing versus environmental enrichment t=0.326, p=0.754, df=7). These data indicate that environmental enrichment restored the number of OL lineage cells, OPCs and premyelinating OLs, but not mature OLs, in APO-SUS mPFC.

We next investigated whether an environmental enrichment-induced rescue of the numbers of OL-lineage cells, OPCs and premyelinating OLs would be sufficient to restore myelination of interneurons in APO-SUS mPFC. Ultrastructural analysis revealed that in the APO-SUS mPFC environmental enrichment restored the number of myelinated axons to APO-UNSUS standard housing level, while environmental enrichment had no effect on the number of myelinated axons in APO-UNSUS mPFC (Fig 7f; one-way ANOVA F=4.551, p=0.029, df=3; APO-SUS standard housing *versus* environmental enrichment t=-2.291, p=0.062, df=6; APO-SUS standard housing *versus* APO-UNSUS standard housing t=-3.302, p=0.021, df=5). The G-ratio of APO-SUS and APO-UNSUS myelinated axons was not affected by environmental enrichment (Fig 7g; one-way ANOVA F=1.622, p=0.246, df=3), and chi square test on cumulative axon caliber distribution showed that there were no differences in the distribution of myelinated axon caliber between the groups (Fig 7h; Chi square test APO-SUS standard housing *versus* environmental enrichment p=0.338; APO-SUS standard housing *versus* APO-UNSUS standard housing p=0.214). However, environmental enrichment significantly increased the number of myelinated axons of 2-4 µm, 8-10 µm and 10-12 µm axon caliber in APO-SUS mPFC to control levels (Fig 7h; independent samples T-test t=-2.600, p=0.041, df=6; t=-2.926, p=0.047, df=5; t=-2.810, p=0.031, df=6, respectively in APO-SUS standard housing *versus* environmental enrichment). We conclude that environmental enrichment successfully rescued the myelination of interneurons in the APO-SUS mPFC.

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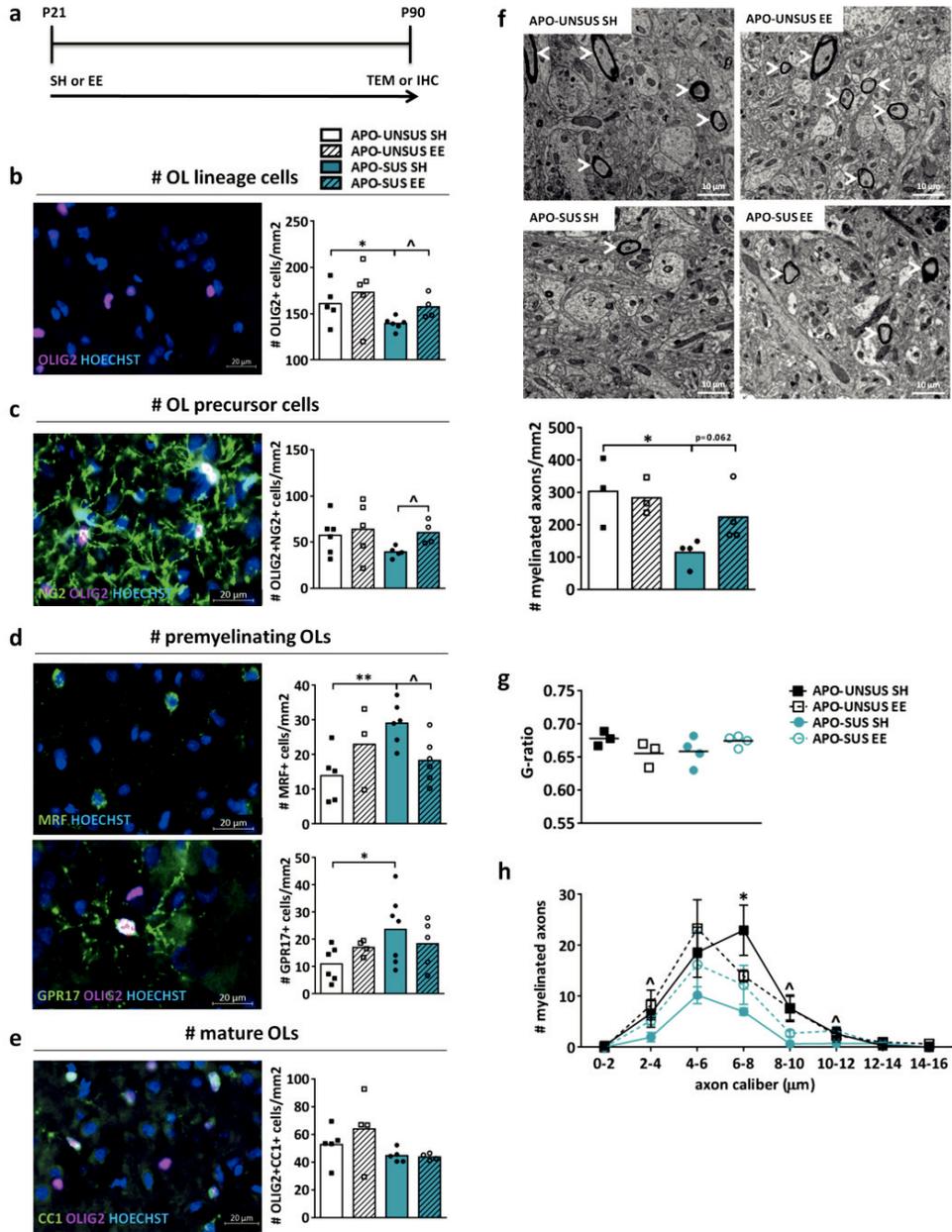
**Figure 7- Environmental enrichment restores number of OLs, OPCs and premyelinating OLs as well as myelination in the mPFC of APO-SUS rats. (a)** Schematic representation of experimental paradigm. APO-SUS and APO-UNSUS rats were housed in standard housing (SH) or in environmental enrichment (EE) conditions starting at P21, and sacrificed at P90 for immunohistochemical (IHC) or transmission electron microscopy (TEM) analyses. **(b)** Number of OL lineage cells (OLIG2+), **(c)** number of OPCs (OLIG2+NG2+), **(d)** number of premyelinating OLs (MRF+ or GPR17+), and **(e)** number of mature OLs (OLIG2+CC1+) per mm2 in the IL subregion of the mPFC of APO-SUS and APO-UNSUS rats in standard housing and environmental enrichment conditions (n=3-6 specified in the graphs). **(f)** TEM

analysis and quantification in the IL of APO-SUS (n=4) and APO-UNSUS (n=3) rats in standard housing and environmental enrichment conditions. **(g)** Average myelinated axon G-ratio in IL of APO-SUS (n=4) and APO-UNSUS (n=3) rats in standard housing and environmental enrichment conditions **(h)** Axon caliber cumulative distributions of myelinated axons in IL of APO-SUS and APO-UNSUS rats in standard housing and environmental enrichment conditions. ^p<0.05 APO-SUS SH versus APO-SUS EE *p<0.05 **p<0.01 APO-SUS SH versus APO-UNSUS SH in independent samples T-test, error bars represent standard error of the mean. Source data are provided as a Source Data file.

Finally, we assessed whether this rescue of interneuron myelination in the mPFC of APO-SUS rats would be sufficient to restore cognitive inflexibility in the extra-dimensional set-shifting test. To answer this question, APO-SUS and APO-UNSUS rats were reared in environmental enrichment or standard housing from weaning at P21 onwards and behavioural testing was performed between P60 and P90 (Fig 8a and Supplementary Fig S5). Multivariate ANOVA revealed a significant effect of the behavioural treatment on the number of errors until criterion (streak of 10 correct trials) during extra-dimensional set-shifting as well as on the number of perseverative errors (Fig 8b; F=2.529, p=0.011, df=9; F=2.367, p=0.017, df=9). Post-hoc testing confirmed a significant improvement in the number of errors until criterion in the third extra-dimensional shift and a reduction of the total number of perseverative errors in APO-SUS rats reared in an environmental enrichment. APO-UNSUS rats reared in an environmental enrichment compared to standard housing-reared APO-UNSUS rats showed a similar number of perseverative errors during extra-dimensional set-shifting, but a decreased error number in the first and an increased error number in the second shift. The number of perseverative errors made during reversal learning was also significantly reduced in APO-SUS rats (Supplementary Fig S5). Taken together, these data demonstrate that environmental enrichment during mPFC development restored the number of OL-lineage cells, OPCs and premyelinating OLs, restored the number of myelinated axons, and reversed behavioural inflexibility in APO-SUS rats.

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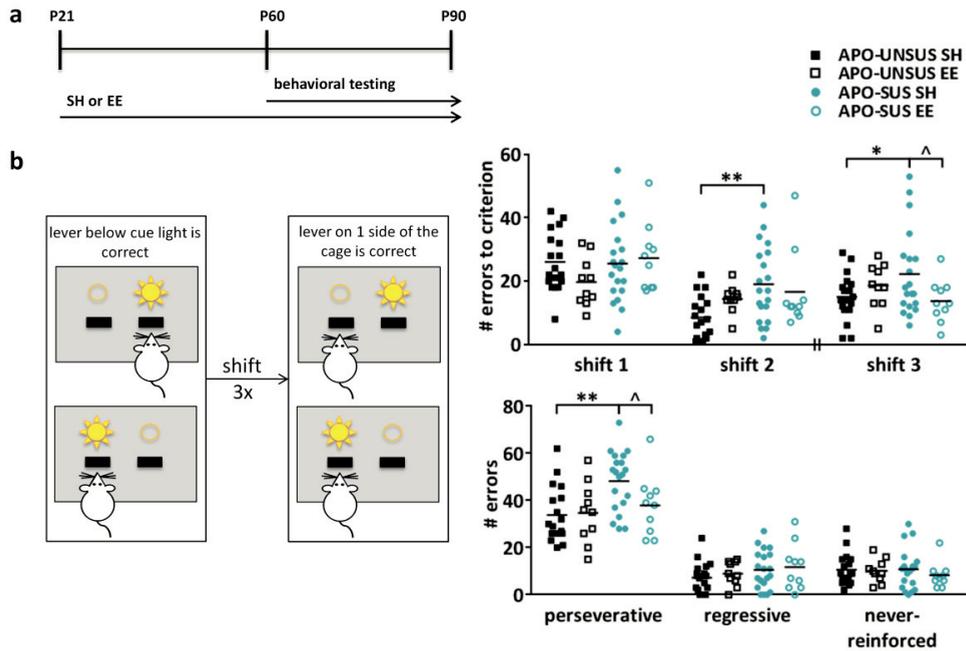
**Figure 8- Environmental enrichment improves cognitive inflexibility APO-SUS rats. (a)** Schematic representation of experimental paradigm. APO-SUS and APO-UNSUS rats were housed in standard housing or in environmental enrichment conditions starting at P21, and subjected to behavioural testing between P60 and P90. **(b)** Schematic representation of the extra-dimensional set-shifting task. Rats were trained to press the lever above which a cue light was illuminated. From trail 21 of the next session onwards, rats were required to press the lever on one side of the cage irrespective of the cue light. Number of errors to criterion (streak of 10 correct trials) and total number of perseverative, regressive and never-reinforced errors in APO-UNSUS (n=18) and APO-SUS (n=20) rats in standard housing (the same data as is presented in fig 1b) and environmental enrichment (n=10) are depicted. Note that between shift 2 and shift 3 the reversal learning trials were conducted (see fig S5). ^p<0.05 APO-SUS SH *versus* APO-SUS EE *p<0.05 **p<0.01 APO-SUS SH *versus* APO-UNSUS SH in multivariate ANOVA posthoc testing. Source data are provided as a Source Data file.

## Discussion

In this study, we have provided direct evidence that in a rat model with SZ-relevant features interneurons are hypomyelinated in the mPFC and that restoring interneuron myelination through environmental enrichment applied during mPFC development improves cognitive flexibility. We found mPFC-dependent cognitive inflexibility in both spatial working memory and extra-dimensional set-shifting. Spatial working memory is also impaired in SZ patients [51] and has been linked to reduced cortical GABAergic signalling [52,53]. APO-SUS rats only display spatial working memory impairment on more difficult trials, indicating cognitive inflexibility rather than working memory failure. The extra-dimensional set-shifting task we performed is a rodent version of the Wisconsin card sorting test that has been used to reveal impaired set-shifting in SZ patients [54]. Interestingly, like in SZ patients the set-shifting impairment in the APO-SUS rats was characterized by a disinhibition of previously learned behaviour [55]. Subtle alterations in SZ PFC inhibitory circuits are thought to underlie these set-shifting impairments [37]. The fact that APO-SUS rats showed cognitive inflexibility only when task demand increased, but not on the first extra-dimensional shift, suggests just subtle alterations in mPFC circuit functioning rather than a complete failure to activate the mPFC. In APO-SUS rats, reversal learning, relying more on orbitofrontal cortex and less on mPFC functioning [34], was only mildly impaired since the rats achieved three reversals, but the total number of perseverative errors during all three reversal phases combined was higher than for APO-UNSUS rats. Similarly, in SZ patients perseverative deficits in (probabilistic) reversal learning are found only in a subset of patients and less consistently than abnormalities in extra-dimensional set-shifting [56,57]. Thus, APO-SUS rats show cognitive inflexibility with characteristics reminiscent of cognitive impairment in SZ patients, including features suggestive of alterations in mPFC inhibitory circuits.

Dysfunctioning of interneurons and defective myelination are both well-replicated neurobiological abnormalities observed in SZ, including decreased myelin-related gene expression in *post-mortem* SZ frontal cortex [17,18]. Our data shows that decreased expression of myelin-related mRNAs and proteins starts during adolescence and persists into adulthood in the APO-SUS mPFC, supporting the hypothesis that in SZ defective myelination occurs during adolescent mPFC development, rather than in adulthood by demyelinating insults. Strengthening this notion is the fact that active demyelination does not occur in the mPFC of APO-SUS rats and that myelinated fibers in the APO-SUS mPFC have a typical G-ratio and myelin structure, indicating hypomyelination rather than demyelination in the mPFC. Furthermore, ultrastructural analysis revealed a reduced number of myelinated axons in the adult APO-SUS mPFC. Importantly, we found that the population of unmyelinated axons corresponded to parvalbumin interneurons, while the number of parvalbumin interneurons remained the same, demonstrating the occurrence of parvalbumin interneuron hypomyelination in the mPFC of the APO-SUS model with SZ-relevant features. In SZ, parvalbumin

interneurons have an intrinsic defect that leads to impaired inhibitory control and disturbed cognitive functioning [37]. Interestingly, it has been recently suggested that a myelination deficiency of parvalbumin interneurons may underlie defective inhibitory regulation in the PFC [14]. We here show that in the mPFC of a rat model for SZ parvalbumin interneurons are indeed hypomyelinated.

A plausible reason for the observed parvalbumin interneuron hypomyelination may be the reduced number of OL lineage cells in the mPFC of APO-SUS versus APO-UNSUS rats. The fact that we found similar numbers of OPCs, increased numbers of premyelinating OLs and decreased numbers of mature OLs suggests a delay or a maturation block at the premyelinating OL stage. Likewise, in *post-mortem* PFC of SZ patients the total number of OL lineage cells is reduced and no changes in the total number of OPCs have been observed [58,59]. The reduced number of mature OLs in APO-SUS BC was not accompanied by changes in OL differentiation or myelination, and suggests an increased myelin production by mature OLs in this brain region. In contrast, the differences in OL differentiation and maturation observed in the mPFC do lead to affected myelination. Primary oligodendroglial cell cultures from APO-SUS and APO-UNSUS cortex revealed that the intrinsic capability of APO-SUS OPCs to proliferate and differentiate was still intact. This suggests that APO-SUS oligodendroglia do have the capacity to terminally differentiate and myelinate axons. We therefore conclude that the cellular dynamics within the APO-SUS mPFC plays a pivotal role in inducing the OL maturation block observed in the APO-SUS rats.

Behavioural experiences are known to influence the development of oligodendroglia and myelination in the PFC [40]. For example, in rodents voluntary exercise increases OL proliferation in the PFC [60], sociability influences myelination and OL development in the PFC during adolescence [41], and environmental enrichment stimulates OLs to produce more myelin [42,43]. The effects of behavioural experiences are strongest within the critical period of PFC myelination, i.e. during adolescence [41]. Importantly, adolescent individuals at high risk for SZ show higher rates of sedentary behaviour, less physical activity and decreased sociability that correlate with cognitive symptoms in later stages of SZ [44,45,61]. Furthermore, levels of academic achievement and social interactions prior to disease onset during adolescence are associated with the degree of impairment in working memory and executive functioning during later phases of SZ [47-49]. This indicates that socializing and cognitive challenges can positively influence cognition during the course of SZ. Indeed, exposing APO-SUS rats to an environmental enrichment paradigm that included voluntary exercise, increased sociability and opportunity for novelty exploration during adolescent mPFC development restored the number of OL lineage cells. The increased density of OPCs together with the reduced number of premyelinating OLs observed following environmental enrichment suggests that under this condition more OLs remain in the OPC pool. Other studies that have applied environmental enrichment to treat glial abnormalities in rat brain have reported

similar increases in the number of OPCs [62-64].Environmental enrichment did not increase the total number of mature OLs, but restored the number of myelinated axons in APO-SUS mPFC. This is in line with previous studies in rodents showing that environmental enrichment increases myelin production [40,42,43]. The exact mechanism underlying the myelin increase is at present unclear, but it has been hypothesized that environmental enrichment causes changes in neuronal activity, axon-OPC synapses, growth factor release, neurotransmitter release, excretion of metabolites and inflammatory factors that can influence oligodendroglial cells and myelination [40]. Environmental enrichment during mPFC development was also sufficient to rescue cognitive flexibility in the extra-dimensional set-shifting test, clearly indicating that developmental interneuron hypomyelination contributes to cognitive impairment observed in the APO-SUS rat model of SZ.

Altogether, our findings help to understand the causal pathophysiology of cognitive impairment in SZ and argue for stimulating interneuron myelination during adolescence as a therapy to treat early stages of SZ. Environmental enrichment, exercise and sociability training in individuals at high risk to develop SZ have the potential to rescue aberrant PFC myelin development. High-risk individuals show a significant decrease in white-matter integrity in frontal circuits after transitioning into psychosis [65], indicating a developmental aspect to psychosis transitions that indeed involves white matter and myelination (for review see ref [22]). Furthermore, behavioural therapy enhances white-matter integrity and improves cognition in SZ patients [66,67]. Together with these earlier findings, our study suggests that stimulating myelination in the PFC by applying environmental enrichment or other targeted behavioural therapies during adolescence is an attractive preventive measure for individuals with a high risk of transitioning to SZ as well as an appealing remedy to improve cognition in SZ patients.

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## Methods

### Animal model

Selection and breeding procedures of the APO-SUS and APO-UNSUS rat lines have been described in detail elsewhere [30,68]. Briefly, we selectively bred rats from an outbred Nijmegen Wistar rat population that displayed stereotyped behaviour upon injection of apomorphine (APO-SUS rats). The same selective breeding was performed with the rats that showed a weak apomorphine-induced stereotypy (APO-UNSUS rats). Apomorphine injection and behavioural selection were only performed with the first 15 generations of APO-SUS and APO-UNSUS rats. In the subsequent breedings, APO-SUS rats displayed SZ-relevant features without this pharmacological treatment. In this study naïve male APO-SUS and APO-UNSUS rats from the 38th-43rd generation were used. Rats were housed in pairs in a temperature and humidity controlled room with a 12-h light-dark cycle (lights on at 7.00 a.m.) and *ad libitum* access to water and standard laboratory chow (V1534-703, SSNIFF, Germany), unless otherwise indicated. Animal experiments were approved by the Animal Ethics Committee of Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, and were conducted in accordance with Dutch legislation (Herziene Wet op Dierproeven, Art 10.a.2, 2014).

### T-maze behavioural tests

During the continuous delayed alternation test, APO-SUS and APO-UNSUS rats were kept on a food restriction schedule. A limited amount of standard laboratory chow was given to maintain the rats at 85-90% of their free-feeding weight. Rats were habituated for 20 minutes to a T-maze with food pellets scattered around the maze (arm length 50 cm, arm height 40 cm and arm width 14 cm with a start box of 15 x 14 x 40 cm that could be closed off by a guillotine door). Food pots (4 x 4 x 4 cm) were present 4 cm from the end of each arm. The animals were trained to perform a minimum of 7 trials within 20 minutes (i.e., retrieving a food pellet from both food pots), after which the continuous delayed alternation paradigm started. Rats were tested during the light phase and performed 9 trials per day (i.e. a session) for 10 days. During the start trial of each session, a food pellet was present both in the left and the right arm of the T-maze. The rat was placed in the start box and upon lifting of the guillotine door the rat was allowed to move freely around the maze until one of the food pellets was retrieved. After that the rat was placed back in the start box for the delay period of either 10 or 60 seconds. In the first test trial only the arm that the animal did not visit during the start trial was baited. During following trials a food pellet was present only in the arm that was not baited during the previous trial. Hence, rats had to alternate between the left and right arms of the T-maze (Fig 1a). Trials were considered correct when the first arm entry (defined as having four paws in one arm) was into the baited arm and the start trial of each session was not taken into account during data analysis. For both

the 10- and 60-seconds delay conditions, five sessions were conducted and the average percentages (%) of correct trials for these five sessions were calculated.

For T-maze reversal learning, the rats were trained until they could retrieve a food pellet from one arm of the maze with a performance of 70% correct trails per session. During the following session, bait could be retrieved from the opposite arm, hence requiring a complete reversal (Fig 1c). We counted the number of trials until a performance of 70% correct trials per session was achieved.

### Operant attentional set-shifting tests

Upon the start of behavioural training, rats were food restricted and received 5-8 grams of food per 100 grams of rat daily. Rats were pre-exposed once to grain reward pellets (Rodent Tablet [5TUM], 45mg, TestDiet, USA) in the home cage. Operant conditioning chambers (29.5 cm L, 24 cmW25 cm H;Med Associates, Georgia, VT) were situated in light and sound-attenuating cubicles equipped with a ventilation fan. Each chamber was equipped with two 4.8-cm-wide retractable levers, placed 11.7 cm apart and 6 cm from the grid floor. A cue light (28 V, 100 mA) was present above each lever. At the same wall, a reward pellet could be delivered in a magazine between the levers and a house light (28 V,100 mA) was located on the same wall. The lever presentation and cue light illumination sides were counterbalanced between rats. Operant extra-dimensional set-shifting and reversal learning procedures are based on previous reports [33].

During pretraining the rats were first habituated to the operant cage and received 50 food pellets with an inter-trial interval varying between 10 and 50 seconds. During the second training phase, one of the levers was extended during the entire session and each lever-press resulted in the delivery of a reward pellet. After the criterion of 50 lever presses was reached, this was repeated with the other lever. During the third training phase, rats were familiarized with the house light and the insertion of the levers into the chamber and were required to press within ten seconds to receive a food pellet. Upon pressing, the lever retracted, a reward pellet was delivered and the house light remained illuminated for another four seconds. If the rat did not press the lever within ten seconds, the lever retracted and the house light went off. In each pair of trials, the left or right lever was presented once, and the order within the pair of trials was random. Five of these retractable lever-press training sessions were performed, each consisting of 90 trials with 20 second inter-trial intervals. Immediately after the last session of retractable lever-press training, without being removed from the operant chamber, the rats performed a side bias test as described previously [33].

Upon completion of pretraining, the rats were trained to perform visual cue discrimination. The cue light was randomly presented above the left or right lever for three seconds, then both levers extended and the house light turned on. The rat was required to press the lever above which the cue light was illuminated within ten seconds in order to receive a reward pellet. When rats reached the criterion of 10 subsequent

correct trials, the session finished. During consecutive phases of the experiment, rats were either required to perform an extra-dimensional set-shift or a reversal learning procedure. During extra-dimensional set-shifting, the first 20 trials of the session consisted of the 'old rule', in which rats had to press the lever above which a cue light was illuminated, and from trial 21 onwards, rats were required to press their non-preferred lever (as indicated by the side bias test) irrespective of the cue light illumination (Fig 1b). During reversal learning, the first 20 trials consisted of pressing the rats' non-preferred lever, and from trial 21 onwards, rats were required to press the opposite lever to receive a reward pellet (Fig 1d). Rats performed two extra-dimensional set-shifts, then three reversal learning shifts and then another extra-dimensional set-shift. We measured the number of errors made until criterion of 10 subsequent correct trials was reached, and classified the errors as perseverative errors (following the 'old rule'), regressive errors (following the 'old rule' while more than 70% of previous trials were correct) or never-reinforced errors (pressing a lever that was incorrect during both the 'old rule' and during the current rule).

### Environmental enrichment

Rats were weaned at P21 and placed in environmental enrichment or standard housing conditions. For environmental enrichment rats were housed in groups of 10 animals in 100 x 54,5 x 48cm cages with cage enrichment in the form of toys, running wheels, tunnels and nesting places. Enrichment was of different colours and textures and was changed three times a week to promote exploration behaviour and rats were handled once a week. Rats in standard housing were housed in pairs in standard cages with standard enrichment in the form of a rat retreat. Rats in standard housing and environmental enrichment conditions were housed in the same room. Rats were either sacrificed at P90 for immunohistochemistry or transmission electron microscopy experiments, or did the operant attentional set-shifting task starting at P60.

### Micropunching and RNA isolation

Naïve P0, P7, P14, P21 (+/- 1 day), P60 (+/- 1 day), P90 (+/- 1 day), P120 (+/- 1 day) and P365 (+/- 14 days) APO-SUS and APO-UNSUS rats were sacrificed by direct decapitation and brains were isolated, frozen on dry ice and stored at -80 ℃. Brains of P0, P7 and P14 were freshly dissected, while brains of P21, P60, P90, P120 and P365 were micropunched. Micropunching was performed in a cryostat (Leica) at -15 ℃ and the Paxinos and Watson rat brain atlas was used to aid dissection. mPFC was collected with a 1.20 or 2.00-mm punch needle (Harris) from 300 µm coronal sections at Bregma 4.00-2.20. Corpus callosum (CC) was microdissected at room temperature (RT) and striatum (STR) punched with a 2.00-mm punch needle from Bregma 1.60- -0.20. Barrel cortex (BC) was punched with a 2.00-mm needle at Bregma 8.08-6.10. Dissected tissues were stored at -80 ℃ until further analysis. Tissue samples were homogenized using Trizol

(Sigma) and RNA was extracted with chloroform, precipitated with isopropanol and glycogen (Fermentas), washed with 75% ethanol, dissolved in MilliQ H2O and stored at -80 ˚C until further analysis.

## Quantitative PCR

For quantitative PCR (qPCR) analysis, RNA samples were treated with DNase I (Fermentas) and cDNA was synthesized using the Revert Aid H-minus first strand cDNA synthesis kit (Thermo Scientific). cDNA was diluted in MilliQ H2O and stored at -20 ˚C. qPCR samples were pipetted using a robot (Corbett Robotics) and contained 2.0 µL diluted cDNA, 0.8 µL 5 µM forward primer, 0.8 µL 5 µM reverse primer, 5 µL SybrGreen mix (Roche) and 1.8 µL MilliQ H2O. qPCR was performed with a Rotor Gene 6000 Series (Corbett Life Sciences) using a 3-step paradigm with a fixed gain of 8. 45-50 cycling steps of 95, 60, and 72 ˚C were applied and fluorescence was acquired after each cycling step. Primers were designed with NCBI Primer-Blast or Primer Express 2.0 and synthesized by Sigma (for primer pair sequences, see Table S1). Take off and amplification values of the housekeeping genes (Ywhaz, B-actin, Ppia and Gapdh) were used to determine the normalization factor with GeNorm 39 [69] after which normalized mRNA expression levels were calculated.

## Western blot analysis

For Western blot analysis, tissue samples were homogenized in RIPA buffer (150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% Na-deoxycholate, 50 mM Tris-HCL pH 8.0, protease inhibitor) using 20 (mPFC and BC) or 25 (CC) strokes in a glass potter and protein levels were determined with a BCA assay (Thermo Scientific). 80 µg of mPFC, 5 µg of CC and 30 µg of BC protein were used for SDS-PAGE and Western blotting onto PVDF membranes. Blots were blocked in 1x PBS, 0.1% Tween and 10% non-fat milk (Elk, Campina) and incubated with MBP antibody (MAB386, Millipore) at 1:4000 or 1:1000, and GAPDH antibody (2118, Cell Signaling) at 1:5000 in 1x PBS, 0.1% Tween and 5% milk at 4 ˚C overnight. Secondary antibodies (anti-mouse or anti-rabbit peroxidase; GAM/IgG(H+L)/PO  and GAR/IgG(H+L)/PO, Nordic Immunology) were incubated for 2hr at RT and images were acquired using Lumi-Light Western blotting substrate (Sigma) in an ImageQuant LAS-4000 digital imaging system (GE Healthcare) and analysed using FIJI.

## Immunohistochemistry

For immunohistochemistry, naïve APO-SUS and APO-UNSUS rats of P21 (+/- 1 day) and P90 (+/- 1 day) were perfused with 2% paraformaldehyde (PFA). Perfused brains were removed, postfixed overnight and placed in 30% sucrose in PBS for 3-5 days, frozen on dry ice and stored at -80 ˚C until further processing. Coronal cryosections of 10-14

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µm were collected in a cryostat (Leica) and rehydrated in 1x PBS, 0.1% Triton X-100 or for OLIG2 NG2 immunohistochemistry in 1x PBS, 0.05% Tween-20. For OLIG2-CC1 staining antigen retrieval was performed in a microwave using citric acid-based antigen unmasking solution (Vector). Tissue was blocked in 4% BSA, 0.1% Triton X-100 or 5% NGS/NDS/NHS, 1% BSA, 1% glycine, 0.1% lysine, 0.4% triton X-100 for 1 hour at RT. Primary antibodies were anti-OLIG2 (AB9610, Millipore 1:1000, ab109186, Abcam 1:400 or MABN50, Millipore 1:1000), anti-CC1 (OP80, Calbiochem 1:100), anti-NG2 (MAB5384, Millipore 1:200 or AB5320, Millipore 1:100), anti-MRF (ABN45, Millipore 1:100; directed towards the C-terminal cytoplasmic domain of MRF and used as a marker for premyelinating OLs [70]), anti-GPR17 (10136, Cayman Chemical 1:100) or anti-PLP (MCA839G, Biorad 1:200) and incubated overnight at 4 ˚C. Secondary antibodies were 488-, 555- and 568-conjugated anti-rabbit or anti-mouse (IgG and IgG2b, Alexa) or TRITC-conjugated anti-mouse (IGg1, Southern Biotech) and incubated for 1-2 hours at RT. Hoechst (H6024, Sigma 1:1000) was added as a nuclear counterstain in MilliQ H2O, rinsed with 60% isopropanol and incubated in 60% isopropanol with 0.3% Oil red O (O0625, Sigma) for 15 min at RT and rinsed with 60% isopropanol and MilliQ H2O. As positive control for Oil red O staining, a mouse spinal cord lesion with active demyelination induced by experimental autoimmune encephalomyelitis was used. Sections were mounted in Fluoromount (0100-01, Southern Biotech) and 20x images were obtained using an Axioscan (Leica) and analysed with FIJI. Regions of interest were drawn and cells were counted manually for OLIG2+NG2+, MRF+ and OLIG2+CC1+ cells. OLIG2+ cell counts were obtained in the OLIG2-NG2 or OLIG2-CC1 images. Positive areas for PLP and Oil red O were quantified using a threshold of 25/255 and 0/133, respectively. For PLP images, background was subtracted with a rolling ball radius of 50 pixels.

**Electron microscopy**

For electron microscopy, naïve animals of P90 (+/- 1 day) were perfused with 2% PFA/2% glutaraldehyde and perfused brains were removed, postfixed overnight in 2% PFA/2% glutaraldehyde and stored at 4 ˚C in PBS/0.01% azide until further processing. Sagittal sections of 100 µm were collected using a vibratome (Leica), fixed with 2% osmium tetroxide and contrast was obtained with 5% uranyl acetate. Following ethanol dehydration, sections were embedded in epon resin and ultrathin (70-100 nm) sections were obtained with an ultramicrotome (Leica). Sections were contrasted using lead citrate and 40 non-overlapping 26000x images were obtained in each region of interest. Myelinated axons were counted in all 40 images per brain region and the G-ratio and axon caliber of all myelinated axons perpendicular to the field of view were measured in FIJI. The percentage of axon surface was calculated using 49 equally (200 µm) spaced crosses superimposed over 10 randomly picked images. The percentage of crosses that touched an axon was calculated.

## Immunofluorescent staining of ultrathin sections

Naïve APO-SUS and APO-UNSUS rats of P90 (+/- 1 day) were perfused with 2% PFA/2% glutaraldehyde and perfused brains were removed, postfixed overnight in 2% PFA/2% glutaraldehyde and stored at 4 °C in PBS/0.01% azide until further processing. Sagittal sections of 100 μm were collected using a vibratome (Leica), and IL was dissected according to Figure 81 of the Paxinos and Watson Rat Brain Atlas. IL samples were dehydrated in ethanol (50%, 70%, 80% and twice 90% ethanol for 15 minutes), embedded in LRwhite resin (Electron Microscopy Sciences) at RT for 14 hours and polymerised in LRwhite resin in airtight capsules at 60 °C for 2-3 days. Ultrathin (70-100 nm) sections were obtained with an ultramicrotome (Leica), collected on gelatin-coated glass slides and stained with anti-MBP (MBP, Aves 1:200) and anti-GABA (AB175, Millipore 1:500) or anti-parvalbumin (PV27 Swant 1:300) antibodies using a protocol based on refs [11,38] with adjustments. Briefly, sections were immersed in 100 mM glycine in milliQ water, washed with 1x PBS and primary antibodies diluted in 2% BSA in 1x PBS were applied at 4 °C overnight. Sections were then washed with 1x PBS, secondary antibodies (Alexa anti-chicken 594, anti-Rabbit 488 and anti-guinea pig 488 in 1% BSA in 1x PBS) were added for 30 minutes at RT, and sections were subsequently washed with 1x PBS and coverslipped using Fluoromount (Thermo Fisher). Images were obtained from IL layer V, 300 μm from the CC, using a wide field fluorescent microscope with a 63x oil lens (Leica) and Zen 2 (Leica, blue edition) software. The number of MBP+GABA+ or MBP+parvalbumin+ axons as well as the total number of MBP+ axons was quantified. Because of the reduction in the number myelinated axons in APO-SUS mPFC, we calculated the percentage of MBP+ axons that was also GABA+ or PV+. Nodes of Ranvier were measured in Zen software in MBP+GABA+ axons that were horizontal to the field of view.

## Primary oligodendroglial cell cultures

Mixed glia cultures were obtained from cortex of P1 APO-SUS and APO-UNSUS rats, n=3 replicates per condition. The tissue was homogenized in mixed glia culture medium (GlutaMAX (Invitrogen), 10% fetal bovine serum (Thermo Fisher) 1% Pen-Strep (Thermo Fisher) and 1% non-essential amino acids (Thermo Fisher)) and cells were kept in this medium on 1:10 poly-L ornithine coated T75 flasks at 37 °C 5% CO2. Medium was refreshed after 7 and 13 days in culture and at day 14 OPCs were purified using a shaking protocol. Mixed glia cultures were shook at 250rpm for 1 hour to discard microglia cells, then cultures were shook again at 250rpm for 18 hours to purify OPCs. Supernatant containing OPCs was placed on petridishes (Falcon) for 3x 5 minutes to eliminate astrocyte contamination. OPCs were then plated on PLO-coated coverslips in 24 well plates. After 4 hours medium was changed to either proliferation medium (DMEM-F12 (Invitrogen), 0.5% B27 (Sigma) 1% Pen-Strep (Thermo Fisher), 0.05%FGF (Fibroblast Growth Factor, 25 ng/ml; Sigma) and 0.05% PDGF-BB (Platelet Derived

Growth Factor BB, 10 ng/ml; Sigma)), or differentiation medium for 4 days (DMEM-F12 (Invitrogen), 0.5% B27 (Sigma) 1% Pen-Strep (Thermo Fisher) and 0.05% 40 ng/ml of T3 thyroid hormone (Sigma)). After 4 days cultures were stained with homemade O4 antibody for 1 hour and fixed in 2% PFA for 7 minutes. Subsequently OPCs were incubated with anti-SOX10 (1/100) and anti-MBP (1/200) primary antibodies for 1 hour at RT, washed with 1x PBS and incubated with secondary antibodies (donkey anti-TRITC IgM 1/100 (Southern Biotech), donkey anti-goat Alexa 488 1/1000, donkey anti-rat Alexa 647 1/750 and Hoechst) for 1 hour at RT, washed and mounted with fluoromount. Images were obtained in an Axioscan.

## Statistical analyses

For qPCR, Western blot, immunohistochemistry, electron microscopy, delayed alternation, set-shifting and reversal learning data analysis, statistical significance was calculated using the independent samples T-test and, when appropriate, with Benjamini-Hochberg correction for multiple comparisons in IBM SPSS Statistics 24. Chi-square test was performed on axon caliber frequency data and a linear regression with two-way ANOVA was used to test statistical significance of the regression between G-ratio and axon caliber. For the environmental enrichment experiment, immunohistochemistry and electron microscopy data analysis was done using one-way ANOVAs and effects were assessed with independent samples T-tests, and analysis of the set-shifting task was done using multivariate ANOVAs. For all analyses, outliers were discarded beforehand as indicated by the Grubbs outlier test using Graphpad quickcalcs, and the level of significance was set at $p=0.05$.

## Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request. The source data underlying all Figures and Supplementary Figures are provided as a Source Data file.

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## Author contributions

D.A.M., B.N.O. and G.J.M.M. designed the project. D.A.M. performed data acquisition and analysis and wrote the manuscript. V.D.E. performed data acquisition of P0-P14 qPCRs, J.v.H. performed the animal work for P0-P14 qPCRs. M.S. helped in the design of the operant set-shifting and operant reversal learning task. J.R.H. and P.d.W. contributed to the supervision of the project, A.V. designed and supervised part of the project, and B.N.O. and G.J.M.M. supervised the project. All authors discussed the results and commented on the manuscript.

## Competing interests

We have no conflict of interest to disclose.

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# Supplementary Material



**Supplementary Figure S1 – No difference in the performance of APO-SUS and APO-UNSUS rats during retractable lever-press training and visual cue discrimination. (a)** Schematic representation and performance of APO-SUS and APO-UNSUS rats during retractable lever press training. Rats were required to press the lever they were presented with during 5 consecutive sessions in 1 session per day. The number of rewards in each session in APO-SUS versus APO-UNSUS rats (n=18-20) is depicted. **(b)** Schematic representation of visual cue learning in the operant set-shifting paradigm. Rats were required to press the lever above which a cue light was illuminated in order to receive a reward pellet. The number of errors until criterion of a streak of 10 correct trials was reached in APO-SUS (n=20) and APO-UNSUS (n=18) rats is depicted. Source data are provided as a Source Data file.

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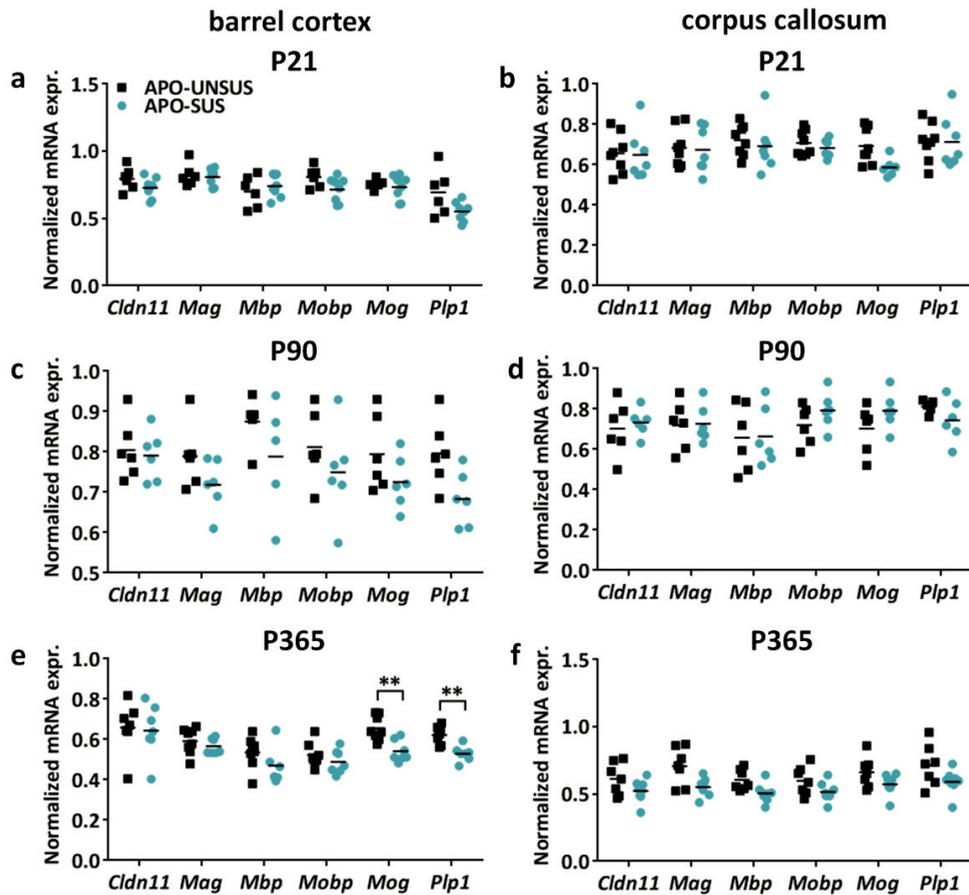
**Supplementary Figure S2 – No difference in myelin-related mRNA expression in APO-SUS versus APO-UNSUS barrel cortex (BC) and corpus callosum (CC).** Normalized mRNA expression of the myelin-related genes proteolipid protein 1 (*Plp1*), myelin basic protein (*Mbp*), claudin 11 (*Cldn11*), myelin oligodendrocytes basic protein (*Mobp*), myelin oligodendrocyte glycoprotein (*Mog*) and myelin associated glycoprotein (*Mag*) in BC and CC of P21 **(a-b),** P90 **(c-d)** and P365 **(e-f)** APO-SUS versus APO-UNSUS rats (P21 n=5-8; P90 n=5-6; P365 n=6-8). **p<0.01. Source data are provided as a Source Data file.
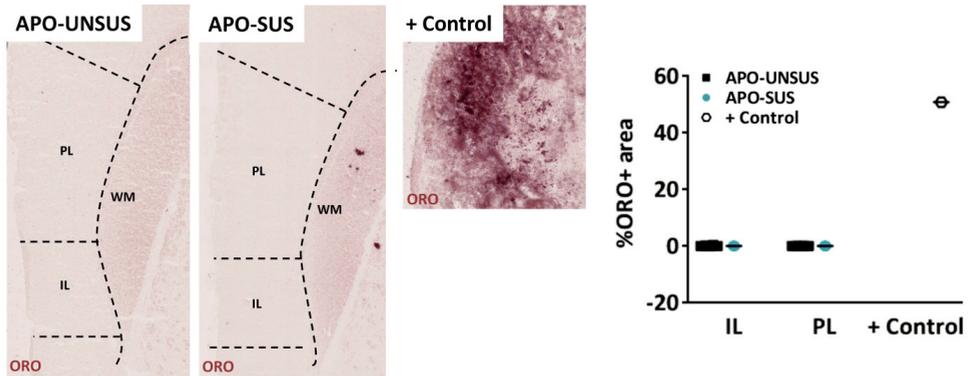
**Supplementary Figure S3 – No active demyelination in APO-SUS and APO-UNSUS mPFC.** Immunohistochemical images and quantification of ORO staining, labeling myelin debris-containing macrophages, in APO-SUS (n=7) and APO-UNSUS (n=6) mPFC compared to a positive control (a demyelinating lesion induced by experimental autoimmune encephalomyelitis). Source data are provided as a Source Data file.
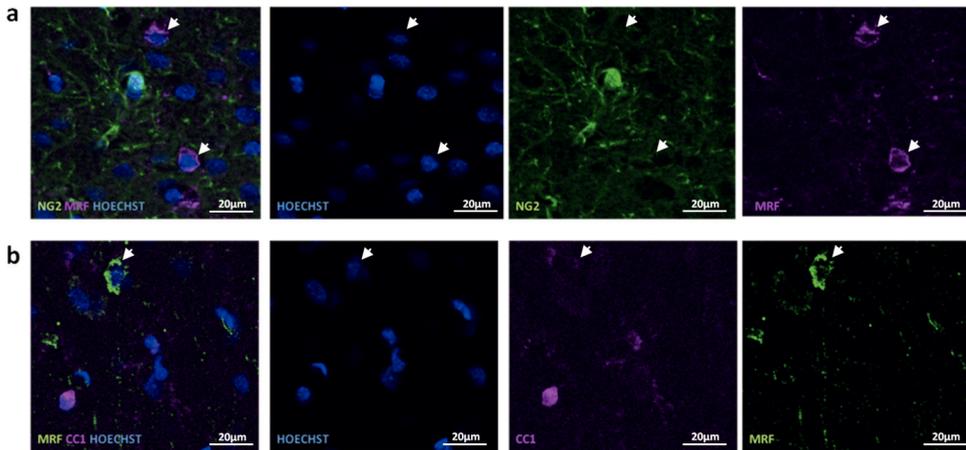


**Supplementary Figure S4 – The cytoplasmic form of MRF is not expressed in NG2+ OPCs, nor in CC1+ mature OLs.** Coimmunostaining for (a) MRF and NG2 and (b) MRF and CC1. Note that MRF+ cells (arrows) are not NG2+ OPCs, and also not CC1+ mature OLs.
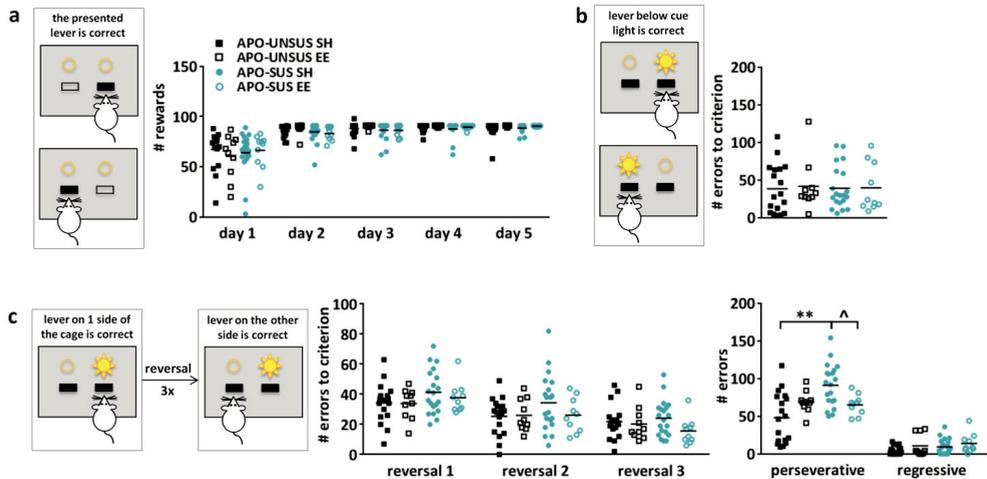
**Supplementary Figure S5 – EE does not affect retractable lever press training or visual cue discrimination, but decreases the number of errors during reversal learning. (a)** Schematic representation and performance of APO-SUS and APO-UNSUS rats during retractable lever press training. Rats were required to press the lever they were presented with during 5 consecutive sessions in 1 session per day. The number of rewards in each session in APO-UNSUS and APO-SUS rats in SH (n=18-20) and EE (n=10) are depicted. **(b)** Schematic representation of visual cue discrimination in the operant set-shifting paradigm. Rats were required to press the lever above which a cue light was illuminated in order to receive a reward. The number of errors until criterion of a streak of 10 correct trials was reached in APO-UNSUS and APO-SUS rats in SH (n=18-20) and EE (n=10) are depicted. Error bars represent standard error of the mean. **(c)** Schematic representation of operant reversal learning paradigm. Rats were trained to press the lever on one side of the cage until criterion of a streak of 10 correct trials was reached. In the following session from trial 21 onwards rats were required to press the lever on the other side of the operant cage. Number of errors until criterion of a streak of 10 correct trials, and total number of perseverative and regressive errors in APO-UNSUS and APO-SUS rats in SH (n=18-20) and EE (n=10) are depicted. **p<0.01 APO-SUS SH versus APO-UNSUS SH ^p<0.05 APO-SUS SH versus EE. Source data are provided as a Source Data file.

**Supplementary Table S1 –** Nucleotide sequences of primers used for qPCRs.

| Gene and abbreviation | | Forward primer 5'-3' | Reverse primer 5'-3' |
| --- | --- | --- | --- |
| Housekeeping genes | | | |
| β-actin | Beta-actin | CCTTCCTGGGTATGGAATCCTGT | TAGAGCCACCAATCCACACA |
| *Ppia* | Peptidyl-prolyl cis-trans isomerase A | AGCACTGGGGAGAAAGGATT | AGCCACTCAGTCTTGGCAGT |
| *Gapdh* | Glyceraldehyde-3-phosphate | GGGTGTGAACCACGAGAAAT | ACTGTGGTCATGAGCCCTTC |
| *Ywhaz* | Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta | TTGAGCAGAAGACGGAAGGT | GAAGCATTGGGGATCAAGAA |
| Myelin-related genes | | | |
| Cldn11 | Claudin 11 | CGCAAAATGGACGAACTGGG | TGCACGTAACCAGGGAGGAT |
| *Mag* | Myelin-associated glycoprotein | AAGCCAGACCATCCAACCTTC | CTCCTGATTCCGCTCCAAGT |
| *Mbp* | Myelin basic protein | CCCTACTCCATCCTCAGACTTTCTT | TGGCGGTGTGCCTGTCTAT |
| *Mobp* | Myelin-associated oligodendrocytebasic protein | AATACCTGCAGGGCAACAAAG | TCTGGTTCTTGGAGAGCCTGG |
| *Mog* | Myelin oligodendrocyte glycoprotein | CGCCGTGGAGTTGAAAGTAG | GCACGGAGTTTTCCTCTAGT |
| Plp1 | Proteolipid protein 1 | GGGCCTGAGCGCAACGGTAA | CAGGCACAGCAGAGCAGGCAA |

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**Supplementary Table S2 –** qPCR timeline statistical values

| Brain region | Gene | Age | t-value | p-value | Sample size APO-SUS | Sample size APO-UNSUS | Significant after Benjamini Hochberg correction |
|---|---|---|---|---|---|---|---|
| PFC | *Plp1* | P0 | -1.248 | 0.230 | 10 | 8 | No |
| | | P7 | 0.804 | 0.432 | 8 | 12 | No |
| | | P14 | -0.432 | 0.670 | 12 | 11 | No |
| | | P21 | -3.256 | 0.006 | 7 | 8 | Yes |
| | | P28 | -3.915 | 0.004 | 8 | 8 | Yes |
| | | P90 | -3.749 | 0.006 | 5 | 5 | Yes |
| | | P365 | -3.419 | 0.008 | 7 | 8 | Yes |
| | *Mbp* | P0 | 0.067 | 0.948 | 10 | 8 | No |
| | | P7 | 0.941 | 0.359 | 8 | 12 | No |
| | | P14 | 0.101 | 0.921 | 12 | 12 | No |
| | | P21 | -0.568 | 0.576 | 10 | 12 | No |
| | | P28 | -5.150 | 0.001 | 8 | 8 | Yes |
| | | P90 | -6.684 | <0.0001 | 4 | 5 | Yes |
| | | P365 | -5.097 | 0.001 | 8 | 8 | Yes |
| | *Cldn11* | P0 | -1.705 | 0.125 | 10 | 8 | No |
| | | P7 | -0.432 | 0.670 | 8 | 12 | No |
| | | P14 | 0.378 | 0.709 | 12 | 11 | No |
| | | P21 | -2.149 | 0.051 | 7 | 8 | No |
| | | P28 | -4.313 | 0.001 | 8 | 7 | Yes |
| | | P90 | -7.245 | <0.0001 | 4 | 6 | Yes |
| | | P365 | -0.495 | 0.628 | 8 | 8 | No |
| | *Mobp* | P21 | -3.195 | 0.009 | 6 | 7 | Yes |
| | | P28 | -5.848 | 0.001 | 8 | 7 | Yes |
| | | P90 | -4.751 | 0.002 | 4 | 6 | Yes |
| | | P365 | -3.954 | 0.002 | 8 | 8 | Yes |
| | *Mog* | P21 | -2.164 | 0.051 | 6 | 8 | No |
| | | P28 | -4.736 | 0.001 | 8 | 8 | Yes |
| | | P90 | -17.44 | <0.0001 | 4 | 5 | Yes |
| | | P365 | -2.263 | 0.040 | 8 | 8 | Yes |
| | *Mag* | P21 | -5.526 | <0.0001 | 6 | 8 | Yes |
| | | P28 | -7.122 | <0.0001 | 6 | 8 | Yes |
| | | P90 | -3.132 | 0.012 | 5 | 6 | Yes |
| | | P365 | -3.393 | 0.010 | 7 | 8 | Yes |

| | | | | | | | |
|----|--------|-------|--------|-------|---|---|-----|
| BC | *Plp1* | **P21** | -1.946 | 0.097 | 8 | 5 | No |
| | | **P90** | -2.581 | 0.027 | 6 | 6 | No |
| | | **P365** | -4.447 | 0.001 | 7 | 8 | Yes |
| | *Mbp* | **P21** | 0.751 | 0.467 | 8 | 6 | No |
| | | **P90** | -1.249 | 0.247 | 5 | 5 | No |
| | | **P365** | -1.431 | 0.178 | 6 | 8 | No |
| | *Cldn11* | **P21** | -1.722 | 0.113 | 7 | 6 | No |
| | | **P90** | -0.360 | 0.726 | 6 | 6 | No |
| | | **P365** | -0.217 | 0.832 | 7 | 7 | No |
| | *Mobp* | **P21** | -2.032 | 0.065 | 8 | 6 | No |
| | | **P90** | -1.068 | 0.311 | 6 | 6 | No |
| | | **P365** | -1.108 | 0.288 | 7 | 8 | No |
| | *Mog* | **P21** | -0.625 | 0.546 | 8 | 5 | No |
| | | **P90** | -1.490 | 0.167 | 6 | 6 | No |
| | | **P365** | -2.844 | 0.002 | 7 | 8 | Yes |
| | *Mag* | **P21** | -0.375 | 0.714 | 8 | 6 | No |
| | | **P90** | -1.718 | 0.117 | 6 | 6 | No |
| | | **P365** | -0.936 | 0.366 | 7 | 8 | No |
| CC | *Plp1* | **P21** | -0.005 | 0.996 | 7 | 8 | No |
| | | **P90** | -1.363 | 0.206 | 6 | 5 | No |
| | | **P365** | -1.900 | 0.080 | 8 | 7 | No |
| | *Mbp* | **P21** | -0.561 | 0.584 | 7 | 8 | No |
| | | **P90** | 0.065 | 0.949 | 6 | 6 | No |
| | | **P365** | -2.669 | 0.019 | 8 | 7 | No |
| | *Cldn11* | **P21** | -0.143 | 0.889 | 7 | 8 | No |
| | | **P90** | 0.496 | 0.630 | 6 | 6 | No |
| | | **P365** | -1.653 | 0.128 | 8 | 7 | No |
| | *Mobp* | **P21** | -0.842 | 0.416 | 6 | 8 | No |
| | | **P90** | 1.337 | 0.211 | 6 | 6 | No |
| | | **P365** | -1.803 | 0.095 | 8 | 7 | No |
| | *Mog* | **P21** | -2.910 | 0.014 | 6 | 8 | No |
| | | **P90** | 1.461 | 0.175 | 6 | 6 | No |
| | | **P365** | -1.819 | 0.092 | 8 | 7 | No |
| | *Mag* | **P21** | -0.182 | 0.858 | 7 | 8 | No |
| | | **P90** | 0.114 | 0.912 | 6 | 6 | No |
| | | *P365* | -2.776 | 0.016 | 8 | 7 | No |

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# Appendix

## Medial prefrontal cortex-dependent behavior is impaired in APO-SUS rats

To further investigate cognitive impairment in APO-SUS rats, we performed a battery of behavioural tests. Medial prefrontal cortex (mPFC)-dependent spatial working memory of the APO-SUS rats was examined using the spatial win-shift task in which rats have to remember which arms of an 8-arm radial maze they have previously visited and which arms they did not visit yet (Figure A1a). The average memory score of APO-SUS rats was significantly lower than that of APO-UNSUS rats, mainly driven by differences found in the first four sessions (Figure A1a). We conclude that in APO-SUS rats mPFC-dependent spatial working memory is impaired. This was confirmed by using a Morris water maze task in which rats are placed in a pool of water laced with milk powder and need to find a platform hidden under the water surface (Figure A1b). APO-SUS rats swam a longer distance than APO-UNSUS rats before they found the platform during trial 3 of block 1, indicating a defect in APO-SUS mPFC-dependent spatial working memory [1]. However, we saw no differences in the performance of APO-SUS and APO-UNSUS rats during blocks 2 and 3 that require hippocampus-dependent spatial memory (Figure A1b). Taken together, we conclude that mPFC-dependent spatial working memory [2-4] is impaired in APO-SUS rats, but that hippocampus-dependent spatial memory is unaffected [5,6].

If the spatial working memory deficit we observed in APO-SUS rats arises from mPFC dysfunction, one would expect other behaviours that depend on this brain region to be affected as well. To test this hypothesis, we investigated the social behaviour of APO-SUS and APO-UNSUS rats, because the mPFC is involved in regulating social behaviours [7-10]. APO-SUS rats spent significantly less time associated with social behaviour and significantly more time showing aggressive behaviour than APO-UNSUS rats (Figure A1c). We also observed a trend towards more time spent with non-social behaviour in APO-SUS *versus* APO-UNSUS rats. These results confirm that mPFC-dependent behaviour is impaired in APO-SUS rats.

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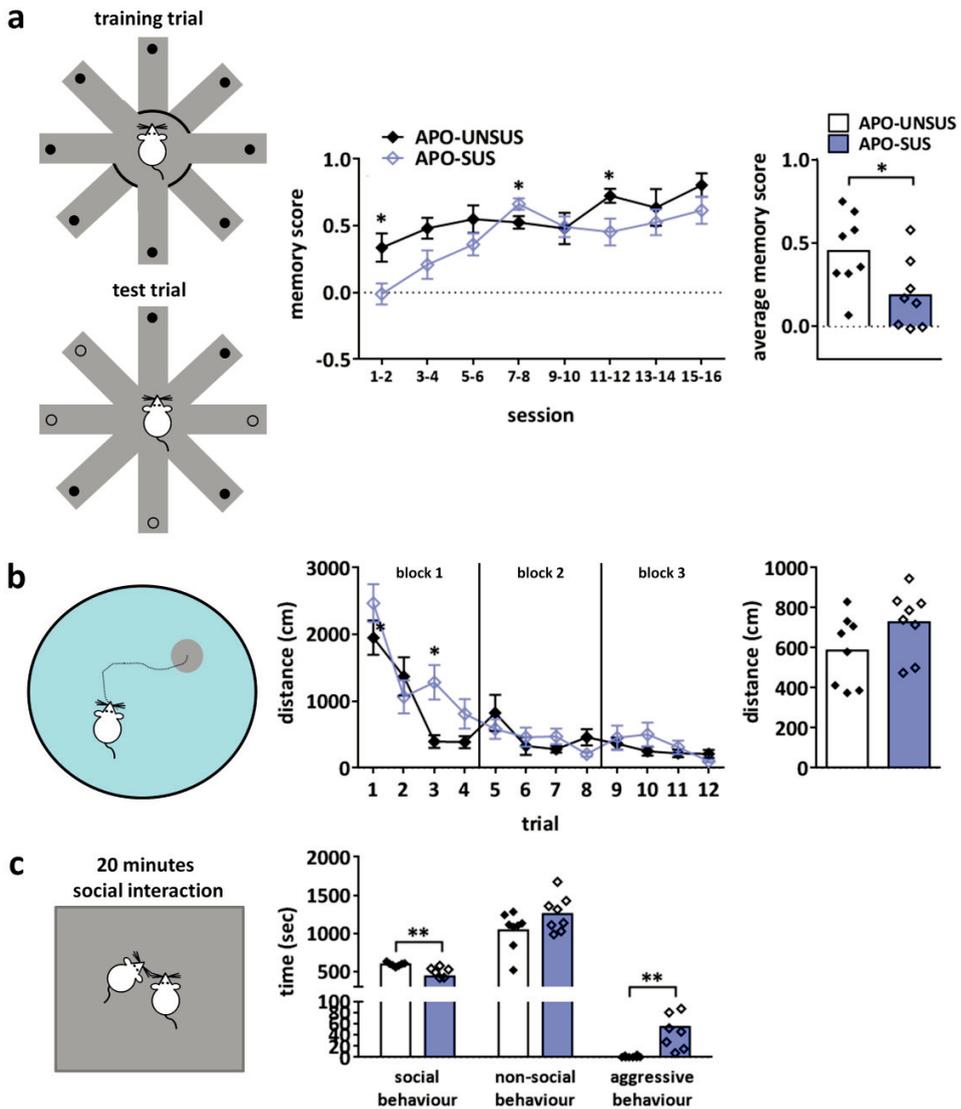
**Figure A1 – mPFC-dependent behaviour is impaired in APO-SUS rats. (a)** Schematic representation and quantification of the spatial win-shift task. During the training trial, all arms of an 8-arm radial maze are baited, four arms are closed and the other four arms are open. The rat explores the maze and eats the four rewards. After a one-minute delay, the rat was placed back in the maze for the test trial during which all arms were accessible and the rat was required to find the four remaining rewards located in the maze's arms that were closed during the test trial. As such, rats were required to remember where they had already been, and where they still had to go. The number of correct and incorrect arm-entries during the test trial was recorded. A memory score is calculated as the number of correct arm-entries minus incorrect arm-entries divided by the total number of arm-entries during the test trial. A memory score of one thus represents optimal performance. Memory score per session and average memory scores are displayed. Rats perform one session of the spatial win-shift task per day for sixteen days in a row. **(b)** Schematic representation and quantification of Morris water maze behavioural test. Rats were placed in a pool and required to find the platform hidden under the water

surface. The ability of rats to remember the location of the platform was tested during 12 two-minute trials spread over three blocks. Block 1 took place on the first testing day, block 2 during the morning and block 3 during the afternoon of the second testing day. The distance swam before reaching the platform is displayed per trial and on average. **(c)** Schematic representation and quantification of the social interaction test. Unfamiliar, weight-matched pairs of APO-SUS and pairs of APO-UNSUS rats were allowed to interact for 20 minutes in an environment they had previously been habituated to. Social interactions were recorded and quantified using JWatcher software. The behaviours were classified into social behaviour (allogrooming, social contact and crawling, sniffing or following the other rat), non-social behaviour (active exploration of the environment, self-grooming, solitary actions) and aggressive behaviour (play-fighting or sniffing the anogenital region). Duration of time spent on social, non-social and aggressive behaviour is displayed. $*p<0.05$ $**p<0.001$ in independent samples T-test, error bars represent standard error of the mean.

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## Myelination is unaffected, but oligodendrocyte numbers are decreased in the APO-SUS cingulate cortex

To investigate whether the hypomyelination we observed in APO-SUS mPFC is specific to this prefrontal subregion, we assessed the myelination status of the cingulate cortex (CG), a prefrontal subregion adjacent to the mPFC. In the CG, we did not find significant differences in the mRNA expression of six myelin-related genes during development at P21, nor during adulthood at P90 or P365 (Figure A2a). There was a reduced expression of Claudin 11 (*Cldn11*) at P21 and P90. However, PLP myelin staining revealed no differences between myelination in the CG of APO-SUS and APO-UNSUS rats (Figure A2b). We did find a decreased number of oligodendrocyte (OL) lineage cells that was caused by a lack of mature OLs, but no changes in the number of OL precursor cells in the APO-SUS CG (Figure A2c). We conclude that the hypomyelination in APO-SUS rats is specific to the mPFC and not present in the CG, while OL defects are observed in both prefrontal regions. Why OL numbers are affected in the CG of APO-SUS rats without affecting myelination in this brain region remains at present unclear.
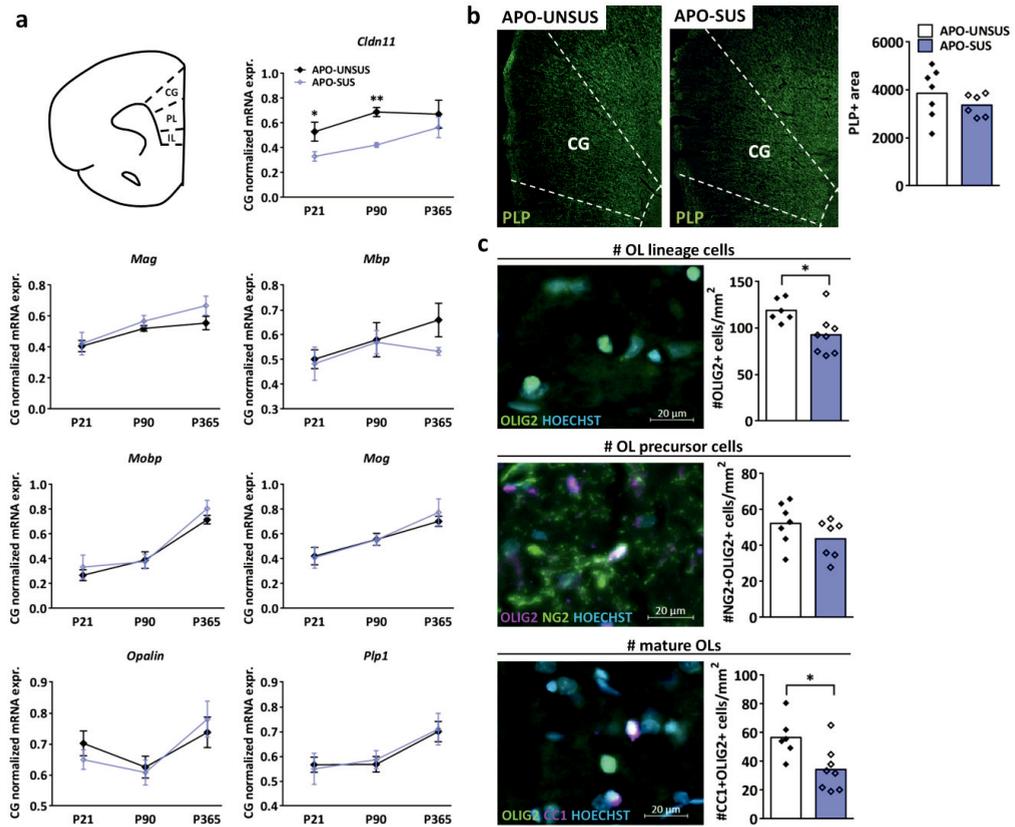
**Figure A2 – No differences in myelination, but decreased number of mature OLs in the cingulate cortex (CG). (a)** Schematic representation of the rat CG, adapted from the Paxinos and Watson rat brain atlas. Normalized mRNA expression of myelin-related genes claudin 11 (Cldn11), myelin associated glycoprotein (*Mag*), myelin basic protein (*Mbp*), myelin oligodendrocyte basic protein (*Mobp*), oligodendrocyte glycoprotein (*Mog*), Oligodendrocytic myelin paranodal and inner loop protein (*Opalin*), proteolipid protein 1 (*Plp1*) in CG of APO-SUS and APO-UNSUS rats of P21, P90 and P365. **(b)** Representative images and quantification of PLP immunofluorescent staining in the CG of APO-SUS and APO-UNSUS rats. **(c)** Number of OLIG2+ OL lineage cells, OLIG2+NG2+ OL precursor cells and OLIG2+CC1+ mature OLs per mm$^2$ in the CG of APO-SUS and APO-UNSUS rats. *p<0.05 **p<0.001 in independent samples T-test, error bars represent standard error of the mean.

## Parvalbumin interneurons are less active in APO-SUS mPFC and this is restored by environmental enrichment

To investigate the activity of parvalbumin interneurons in the infralimbic (IL) region of the mPFC of APO-SUS and APO-UNSUS rats reared under standard housing conditions or in an enriched environment, we performed immunofluorescent staining for parvalbumin and neuronal-activity marker CFOS . We found under SH conditions a reduced percentage of parvalbumin interneurons that was CFOS+ in the IL of APO-SUS rats as compared to that in the IL of APO-UNSUS rats, confirming that parvalbumin interneurons are less active in APO-SUS mPFC (Figure A). The percentage of CFOS+ parvalbumin interneurons was significantly increased in the IL of APO-SUS rats reared in environmental enrichment as compared to that in APO-SUS rats reared in standard housing, indicating that environmental enrichment increases the activity of parvalbumin interneurons in APO-SUS mPFC. environmental enrichment did not affect the percentage of CFOS+ IL parvalbumin interneurons and thus did not increase parvalbumin interneuron activity in APO-UNSUS mPFC (Figure A3).
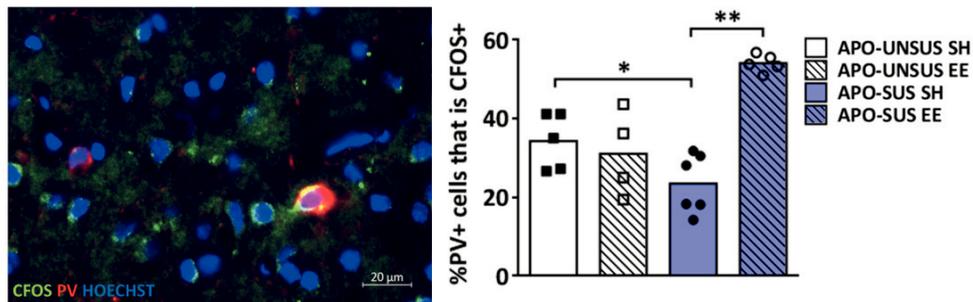


**Figure A3 – Parvalbumin interneurons are less active in APO-SUS mPFC and this is restored by EE.** Representative image and quantification of parvalbumin (PV) and CFOS staining in the IL of APO-SUS and APO-UNSUS rats reared in standard housing (SH) or environmental enrichment (EE). *p<0.05 **p<0.001 in independent samples T-test.

## No apoptosis in the APO-SUS and APO-UNSUS mPFC

To investigate whether OLs in the APO-SUS mPFC were apoptotic we performed a TUNEL assay (S7165 Millipore) that fluorescently labels apoptotic cells. We did not find any signs of cell death in both the APO-SUS and APO-UNSUS IL and PL (Figure A4), and therefore concluded that OLs are not apoptotic in the APO-SUS mPFC.
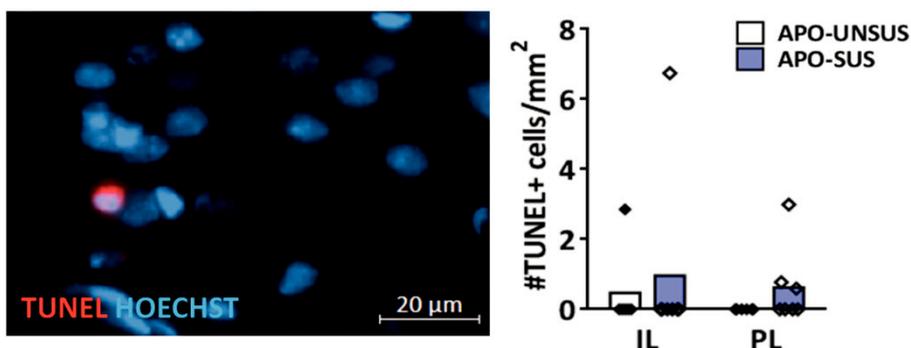
**Figure A4 – No cell apoptosis in the APO-SUS and APO-UNSUS mPFC.** Representative image and quantification of TUNEL staining in the IL and PL of APO-SUS and APO-UNSUS rats.

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## Environmental enrichment does not affect blood glutathione levels in APO-SUS and APO-UNSUS rats

To investigate whether environmental enrichment has any effects on glutathione metabolism, we performed glutathione assays on the blood plasma of APO-SUS and APO-UNSUS rats reared under SH or environmental enrichment conditions. We found that under standard housing conditions glutathione levels were reduced in APO-SUS as compared to APO-UNSUS blood plasma. environmental enrichment did not affect glutathione levels in APO-SUS nor in APO-UNSUS blood plasma (Figure A5).
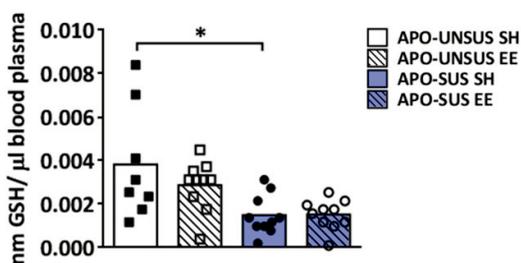


**Figure A5 – No effect of EE on glutathione levels in APO-SUS and APO-UNSUS rats.** nM glutathione (GSH) per µl blood plasma in APO-SUS and APO-UNSUS rats reared in standard housing (SH) or environmental enrichment (EE) conditions. *p<0.05 in independent samples T-test.

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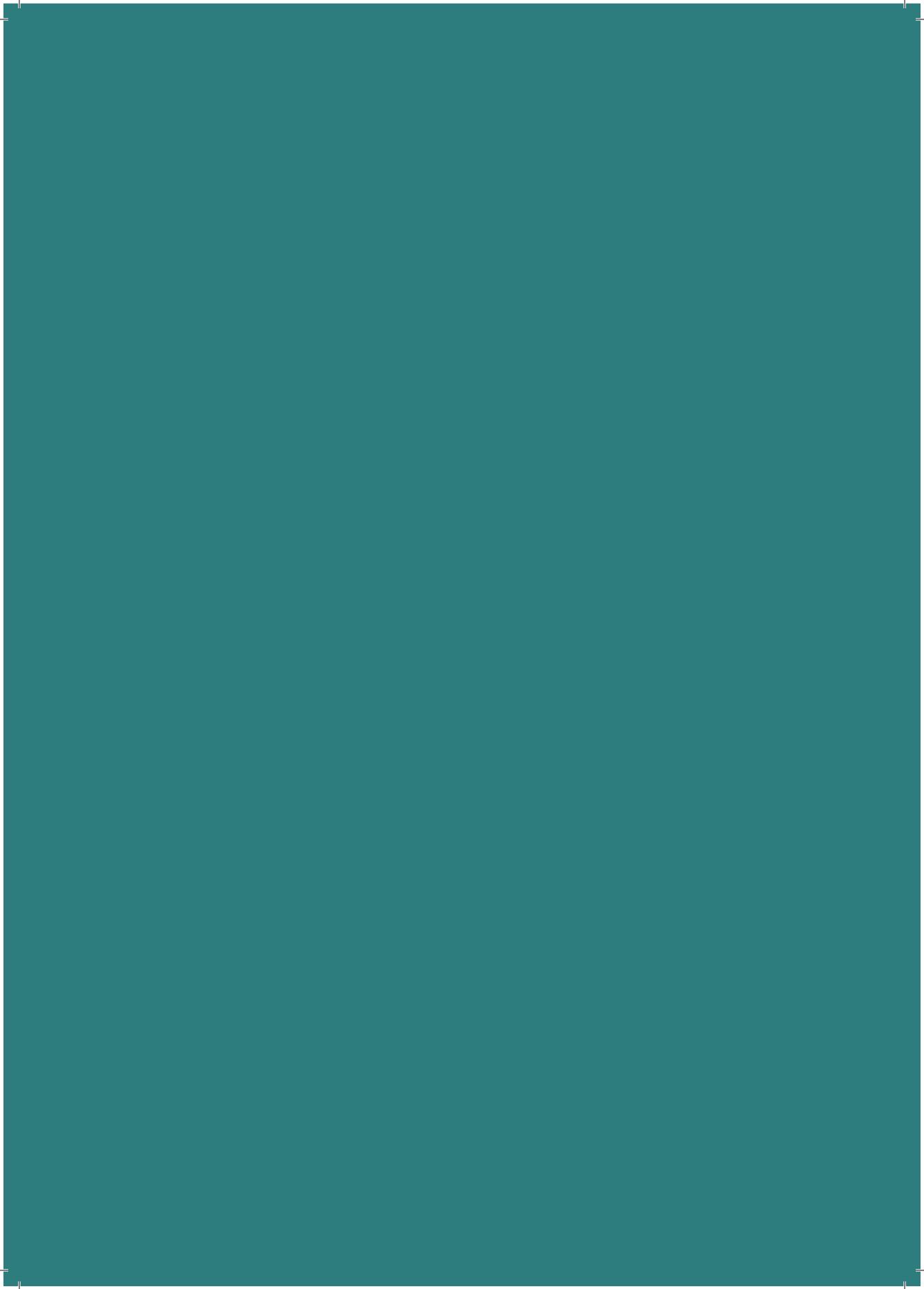
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## Neurobiological basis and repair of interneuron hypomyelination and cognitive inflexibility in rat model for schizophrenia

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## Abstract

Cognitive dysfunction is a core feature of schizophrenia (SZ) and arises from developmental abnormalities in the prefrontal cortex (PFC), the neurobiological underpinnings of which remain elusive. To explore SZ pathophysiology, we use a well-characterized rat model with SZ-relevant features, including the occurrence of medial PFC (mPFC) interneuron hypomyelination. Here we report that transcriptomic analysis of the mPFC of the rat model revealed antioxidant metabolism as the molecular pathway most enriched among the differentially expressed genes. The analysis further showed reduced expression of myelin-related genes. Interestingly, antioxidant-related gene expression was altered from postnatal development onwards and as such preceded interneuron hypomyelination that started during adolescence. Furthermore, a reduced level of the antioxidant glutathione and an increased number of mitochondria were observed in the mPFC of the SZ rat model. Strikingly, chronic treatment of the rats with the glutathione precursor N-acetyl cysteine (NAC) during postnatal development restored not only antioxidant-related mRNA expression, glutathione levels and mitochondria numbers, but also myelin-related mRNA expression, myelinated axon numbers as well as impaired mPFC-dependent cognitive behaviour. The promyelinating effects of NAC were at least partly due to alleviation of an oxidative stress-induced impairment of oligodendrocyte maturation. Together, these findings highlight the neurobiological basis of cognitive symptoms in SZ, and argue for the development of antioxidant therapy for individuals at high risk for and in early phases of SZ.

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## Introduction

Schizophrenia (SZ) is a severe neurodevelopmental disorder caused by a combination of genetic and environmental factors [1]. SZ includes cognitive symptoms which are thought to arise from the prefrontal cortex (PFC). The neurobiological basis of the cognitive symptoms of SZ remains unclear, but oxidative stress may play a role [2]. Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the clearance of ROS by antioxidants. In SZ patients, oxidative stress has been reported in the blood and cerebrospinal fluid as well as the medial PFC (mPFC), and is assumed to be caused by decreased glutathione antioxidant levels [3]. Notably, decreased levels of antioxidants are correlated with impaired PFC-dependent cognitive ability in first-episode SZ [4]. Current hypotheses of PFC dysfunction in SZ state that during PFC development in adolescence oxidative stress has a negative impact on the maturation of interneurons, contributing to the cognitive symptoms of the disease [5].

Glutathione antioxidant levels are associated with white-matter integrity in the PFC [6]. In SZ, the observed decrease in white-matter integrity is thought to be caused by defective myelination and correlates with cognitive symptoms [7]. Myelination of the PFC occurs during adolescence and decreased white-matter integrity is already observed before SZ onset and deteriorates further following transition to psychosis [8,9]. In animal models for SZ, white-matter abnormalities likely reflect hypomyelination [10,11], and we have recently shown that hypomyelination occurs specifically in interneurons and can be rescued by environmental enrichment behavioural therapy during adolescence (Chapter 3). As decreased levels of glutathione are associated with both white-matter abnormalities and interneuron maturation defects in the PFC of SZ patients, the question remains whether oxidative stress is indeed a key player and possibly a causative factor in the development of mPFC interneuron hypomyelination in SZ.

To explore the neurobiological underpinnings of mPFC dysfunction in SZ, we use a well-characterized, idiopathic rat model with SZ-relevant features, namely the apomorphine-susceptible (APO-SUS) rat along with its phenotypic counterpart, the apomorphine-unsusceptible (APO-UNSUS) rat [12,13]. APO-SUS rats do not require genetic or pharmacological manipulation to display SZ-relevant behavioural characteristics. These characteristics are related to the positive, negative and cognitive symptom domains, and include reduced prepulse inhibition, increased exploratory behaviour, dopamine-induced stereotypic behaviour [14-16], reduced latent inhibition, reduced sucrose preference [17,18] and memory deficits [19]. Furthermore, the APO-SUS rats display neurobiological features similar to those found in SZ, e.g., a hyperactive hypothalamus-pituitary-adrenal (HPA)-axis and elevated dopamine D2-receptor binding in the nucleus accumbens [20,21].

Here, we report that RNA-sequencing (RNA-seq) analysis of the mPFC of APO-SUS and APO-UNSUS rats revealed that among the differentially expressed genes there is an enrichment of genes involved in glutathione antioxidant metabolism. Intriguingly,

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treatment of the rats with the glutathione precursor N-acetyl-cysteine (NAC) restored not only glutathione metabolism, but also interneuron hypomyelination and improved cognitive functioning in our rat model for SZ.

## Results

### Glutathione metabolism-related mRNAs were enriched among the differentially expressed genes in APO-SUS *versus* APO-UNSUS mPFC

We used RNA-seq to investigate the neurobiological basis of mPFC dysfunction in the APO-SUS rat model with SZ-relevant features. Genome-wide mRNA expression profiling of the mPFC of post-natal day (P) 365 APO-SUS and APO-UNSUS rats showed that 858 transcripts were differentially expressed (fold change ≥|1.2|, Likelihood Ratio test corrected p-value p<0.05) with 371 genes upregulated and 487 genes downregulated in APO-SUS compared to APO-UNSUS mPFC. Ingenuity pathway analysis of the differentially expressed genes revealed that the two top-enriched canonical pathways were 'glutathione-mediated detoxification' and 'glutathione redox reactions I' (p=9.24E-10 and ratio=0.258, and p=1.52E-04 and ratio=0.25, respectively, in Benjamini-Hochberg corrected T-test; Supplementary Table S1; Supplementary Table S2 and S3 for the genes involved in these top two pathways). Quantitative real-time PCR (qPCR) analysis confirmed a dysregulation of glutathione-related genes from these two pathways in the mPFC of APO-SUS rats that started early in development (P0) and persisted into late adulthood (P365) (Figure 1a; for exact sample sizes and statistical values in Independent samples T-test with Benjamini-Hochberg multiple comparisons correction, see Supplementary Table S4). Furthermore, a kinetic assay showed significantly lower levels of the naturally occurring antioxidant tripeptide glutathione in P90 APO-SUS *versus* APO–UNSUS mPFC (Figure 1b; Independent samples T-test t=-2.817, p=0.015, df=13). Therefore, we conclude that glutathione antioxidant metabolism was reduced in APO-SUS mPFC throughout postnatal development and adulthood. In addition, the top 35 most significantly differentially expressed genes in APO-SUS mPFC encompassed five downregulated myelin-related mRNAs (Supplementary Table S5), in line with our previous observation of mPFC hypomyelination in APO-SUS rats (Chapter 3); note that the Ingenuity pathway analysis results did not include the myelin-related genes because these genes do not constitute a canonical pathway.
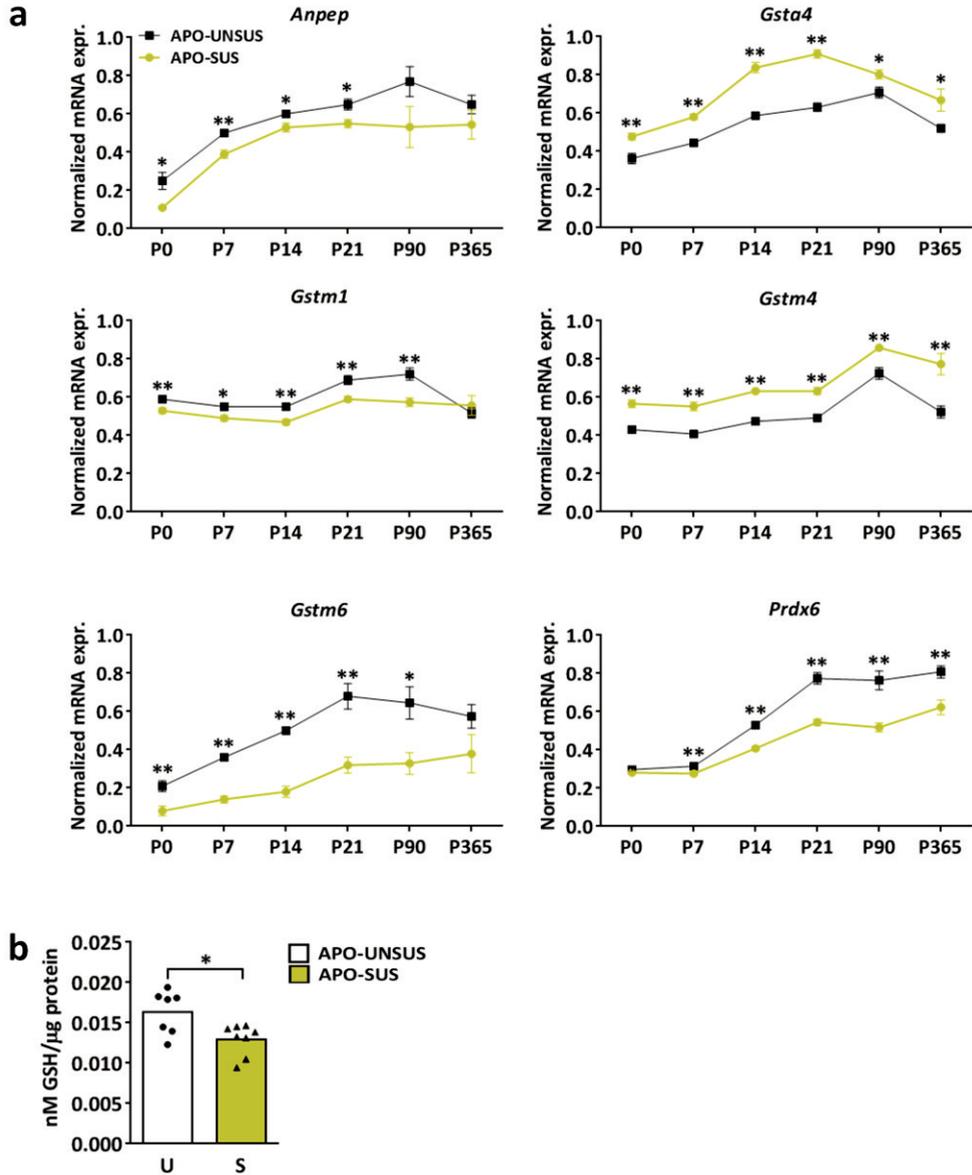
**Figure 1** – **Decreased glutathione-related mRNA expression and glutathione levels in APO-SUS *versus* APO-UNSUS mPFC. (a)** Developmental timeline of mRNA expression of the glutathione-related genes  alanyl aminopeptidase-N (*Anpep*), glutathione-s-transferase-α4 (*Gsta4*), glutathione-s-transferase-μ1 (*Gstm1*), glutathione-s-transferase-μ4 (*Gstm4*), glutathione-s-transferase-μ6 (*Gstm6*) and peroxiredoxin 6 (*Prdx6*) in APO-SUS and APO-UNSUS mPFC. **(b)** nanoMol GSH per microgram protein in P90 APO-SUS *versus* APO-UNSUS mPFC. *p<0.05 and **p<0.01 in independent samples T-test with Benjamini Hochberg multiple comparisons correction. Error bars represent standard error of the mean.

## Increased number of mitochondria in the mPFC of APO-SUS *versus* APO-UNSUS rats

Mitochondria play a key role in the formation of oxidative stress. In SZ, oxidative stress is associated with alterations in mitochondria [22]. To investigate whether the decreased glutathione levels observed in APO-SUS mPFC were accompanied by ultrastructural changes in the mitochondria or by altered mitochondria numbers, we performed transmission electron microscopy analysis. We found an increased surface area containing mitochondria in APO-SUS as compared to APO-UNSUS mPFC (Figure 2a-b; Independent samples T-test t=4.809, p=0.009, df=4). This was true for both axonal and non-axonal mitochondria indicating that the increase in mitochondrial surface in APO-SUS mPFC was not specific to neuronal or non-neuronal cells (Figure 2b; Independent samples T-test axonal mitochondrial surface: t=4.235, p=0.013, df=4, non-axonal mitochondrial surface: t=3.543, p=0.024, df=4). Mitochondrial sizes were not different in APO-SUS *versus* APO-UNSUS mPFC (Figure 2b; Independent samples T-test t=-0.687, p=0.530, df=4). We therefore conclude that the APO-SUS mPFC contains an increased number of mitochondria compared to APO-UNSUS mPFC.
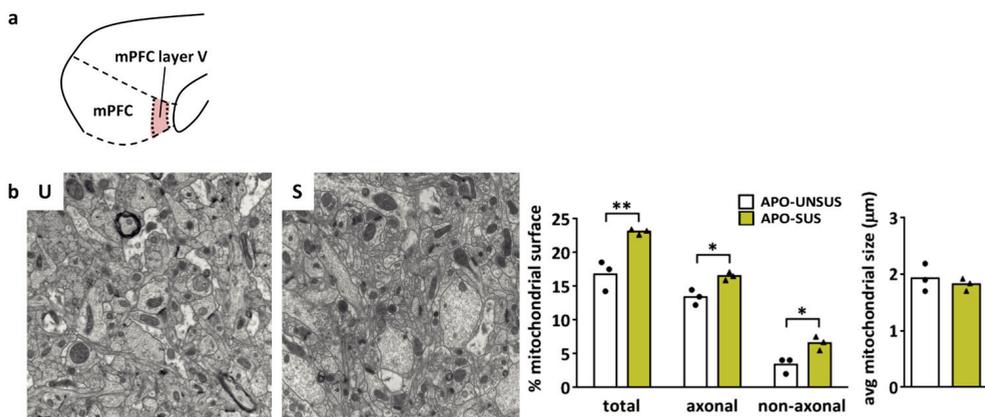


Figure 2 – Increased mitochondrial surface, but no change in mitochondrial size in APO-SUS mPFC. **(a)** Schematic representation of mPFC adapted from the Paxinos and Watson rat brain atlas. **(b)** Electron microscopic representative images and analysis of APO-SUS (n=3) and APO-UNSUS (n=3) average total, axonal and non-axonal mitochondrial surface areas and mitochondrial size in 20 randomly-picked images per animal.

## NAC treatment restored glutathione metabolism and mitochondria numbers in APO-SUS mPFC

Having established impairment in glutathione metabolism and an increased number of mitochondria in the APO-SUS mPFC, we next wondered whether treatment of the rats with the glutathione precursor NAC during post-natal development could restore these aberrations in adulthood. APO-SUS and APO-UNSUS rats were treated with NAC from P5 onwards and sacrificed at P90 for molecular and ultrastructural analyses (Figure 3a). A glutathione assay revealed that in APO-SUS mPFC NAC treatment restored glutathione levels to untreated APO-UNSUS levels, while the treatment had no effect on glutathione levels in APO-UNSUS mPFC (Figure 3b; One-way ANOVA F=3.635, p=0.022, df=3, Independent samples T-test APO-SUS *versus* APO-UNSUS t=-3.777, p=0.002, df=16; APO-SUS *versus* APO-SUS+NAC t=-1.406, p=0.178, df=17). Moreover, NAC treatment significantly restored mRNA expression of a number of glutathione-related genes in the mPFC of APO-SUS rats to APO-UNSUS levels, while glutathione-related gene expression remained unaffected in the mPFC of NAC-treated APO-UNSUS rats (Figure 3c; Multivariate ANOVA F=4.997, p<0.001, df=24, for exact statistical values and sample sizes in Independent samples T-test with Benjamini-Hochberg multiple comparisons correction, see Supplementary Table S6). Interestingly, NAC treatment increased the mRNA expression of genes that are involved in the production and breakdown of glutathione, as well as the binding of glutathione to target oxidative molecules (Figure 3d). *Gstm4* mRNA expression levels were not influenced by NAC treatment. It is at present unclear why the expression of Gstm4, with a function similar to that of Gstm1, Gstm6, Gstm7 and Gsta4, is not affected by NAC-treatment. The fact that NAC-treatment, and thus a change in glutathione levels, did not influence mRNA expression levels of Prdx6 may be linked to its glutathione-independent antioxidant actions, namely through lysophosphatidylcholine acyl transferase and acidic calcium-independent phospholipase A$_2$-dependent activities [23], in contrast to the solely glutathione-dependent actions of the Gstm and Gsta proteins. Furthermore, ultrastructural analysis showed that the mitochondrial surface in the mPFC was significantly restored to control levels in NAC-treated APO-SUS rats (Figure 3e; One-way ANOVA F=11.360, p<0.001, df=3. Independent samples T-test APO-SUS *versus* APO-UNSUS t=3.288, p=0.009, df=9; APO-SUS *versus* APO-SUS+NAC t=5.972, p<0.001, df=9). We conclude that NAC treatment during post-natal development alleviates glutathione metabolism and mitochondrial abnormalities in the APO-SUS mPFC.

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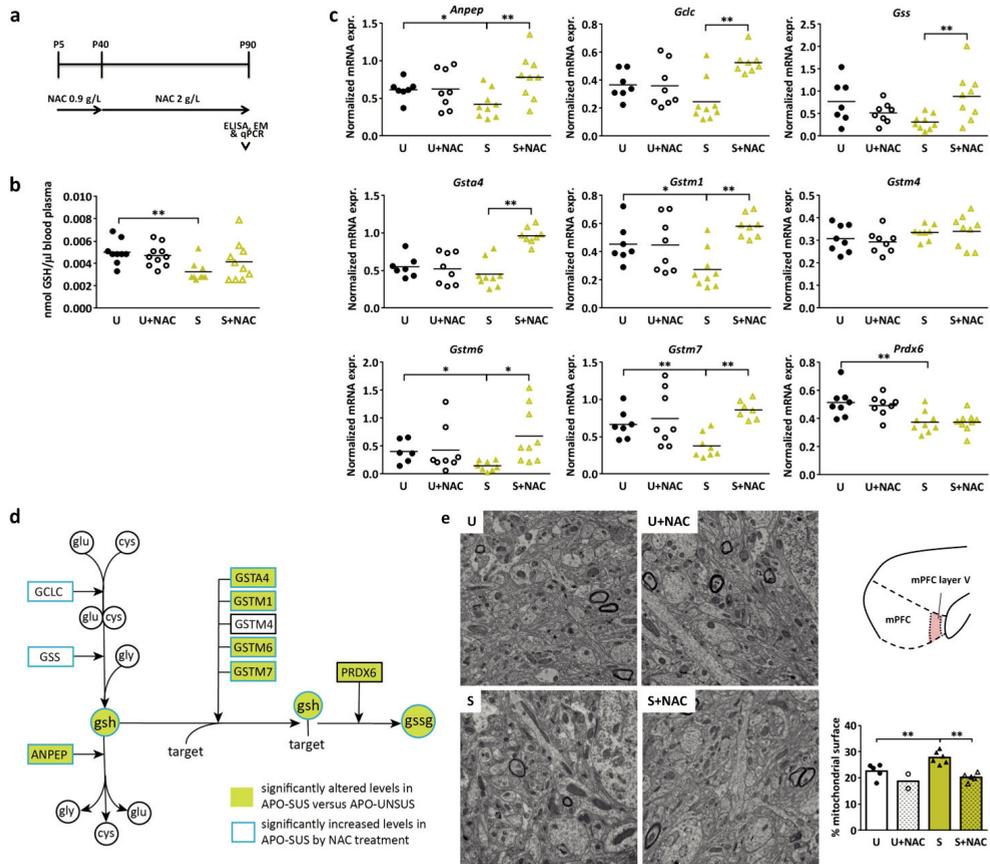
**Figure 3 – NAC treatment restored glutathione levels, glutathione-related mRNA expression, and mitochondrial surface in APO-SUS mPFC. (a)** Schematic representation of experimental paradigm. APO-SUS and APO-UNSUS rats were treated with NAC 0.9 g/L from P5 to P40 and with NAC 2 g/L from P40-P90 and rats were sacrificed for ELISA, EM or qPCR at P90. **(b)** Glutathione (GSH) levels per microliter blood plasma in APO-SUS (S n=9, S+NAC n=10) and APO-UNSUS (U n=9, U+NAC n=10) rats with and without NAC treatment. **(c)** qPCR analysis of mRNA expression of the glutathione-related genes alanyl aminopeptidase-N (*Anpep*), glutathione cysteine ligase catalytic subunit (*Gclc*), glutathione synthethase (*Gss*), glutathione-s-transferase-α4 (*Gsta4*), glutathione-s-transferase-μ1 (*Gstm1*), glutathione-s-transferase-μ4 (*Gstm4*), glutathione-s-transferase-μ6 (*Gstm6*), glutathione-s-transferase-μ7 (*Gstm7*) and peroxiredoxin 6 (*Prdx6*) in mPFC of APO-SUS and APO-UNSUS rats with and without NAC treatment (exact sample sizes displayed in graphs). **(d)** Schematic representation of glutathione (GSH) metabolic pathway. Circles represent amino acids, squares represent enzymes, green symbols refer to components with significantly altered levels in APO-SUS *versus* APO-UNSUS mPFC, blue outlines refer to components with significantly increased levels in APO-SUS after NAC treatment **(e)** Electron microscopic representative images and analysis of the average mitochondrial surface in mPFC of APO-SUS (S n=6, S+NAC n=5) and APO-UNSUS (U n=5, U+NAC n=2) rats with and without NAC treatment from 20 randomly-picked images per animal. *p<0.05, **p<0.01 in Independent samples T-test with Benjamini-Hochberg multiple comparisons correction.

## NAC treatment restored interneuron hypomyelination in APO-SUS mPFC

Decreased levels of glutathione are associated with decreased white-matter integrity in the PFC of SZ patients that is thought to result from deficient myelination [6,24]. In the APO-SUS rats, we have recently reported that interneurons are hypomyelinated during adolescent mPFC development (Chapter 3). The maturation of interneurons occurs during adolescent mPFC development and is thought to be affected by oxidative stress in SZ [5]. However, it remains unclear whether redox imbalance is a causative factor that induces interneuron hypomyelination in the SZ PFC. To investigate whether decreased levels of glutathione antioxidant in APO-SUS mPFC were indeed the cause of the interneuron hypomyelination, we tested whether normalizing glutathione metabolism during post-natal development with NAC treatment could rescue interneuron hypomyelination. Strikingly, qPCR analysis revealed a rescue of the mPFC mRNA expression levels of the myelin-related genes examined in APO-SUS rats treated with NAC, while mPFC myelin-related mRNA expression was unaltered in NAC-treated APO-UNSUS rats (Figure 4a; Multivariate ANOVA F=1.864, p=0.037, df=21, for exact statistical values in Independent samples T-test with Benjamini-Hochberg multiple comparisons correction, see Supplementary Table S7). Electron microscopy confirmed that NAC treatment restored the number of myelinated axons in APO-SUS IL to APO-UNSUS levels (Figure 4b; One-way ANOVA F=2.419, p=0.121, df=3, Independent samples T-test APO-SUS *versus* APO-UNSUS t=-2.867, p=0.024, df=7; APO-SUS *versus* APO-SUS+NAC t=-2.226, p=0.061, df=7). Furthermore, myelin thickness as measured by the G-ratio remained unaffected, and a significant correlation between G-ratio and axon caliber was found in mPFC of APO-SUS and APO-UNSUS rats with and without NAC treatment (Figure 4c; linear regression APO-UNSUS F=5.495, p=0.029, APO-UNSUS+NAC F=18.637, p<0.001 APO-SUS F=26.548, p<0.001, APO-SUS+NAC F=14.398, p<0.001). Therefore, NAC treatment rescued the hypomyelination phenotype in mPFC of APO-SUS rats without affecting myelin structure.

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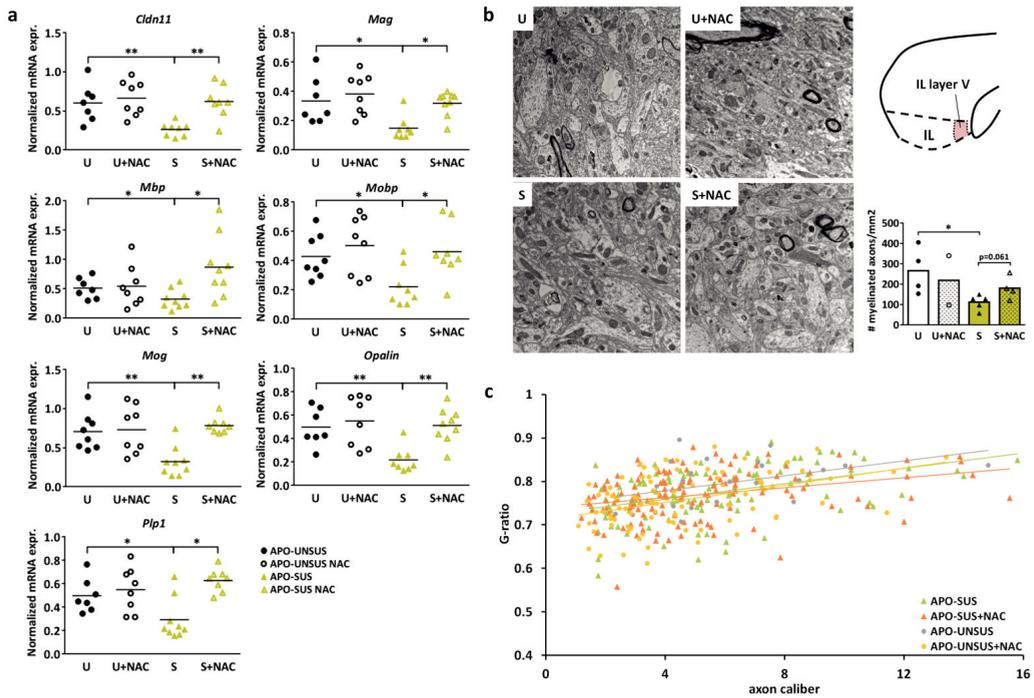
**Figure 4 – NAC treatment rescues hypomyelination in APO-SUS mPFC. (a)** Normalized mRNA expression of myelin-related genes claudin 11 (Cldn11), myelin associated glycoprotein (*Mag*), myelin basic protein (*Mbp*), myelin oligodendrocyte basic protein (*Mobp*), oligodendrocyte glycoprotein (*Mog*), Oligodendrocytic Myelin Paranodal And Inner Loop Protein (*Opalin*), proteolipid protein 1 (*Plp1*) in mPFC of APO-SUS and APO-UNSUS rats with and without NAC treatment (exact sample sizes displayed in graphs). **(b)** Electron microscopic representative images and quantification of the number of myelinated axons in APO-SUS and APO-UNSUS rats with and without NAC treatment. **(c)** G-ratio versus axon caliber for all myelinated axons in IL of APO-SUS and APO-UNSUS rats with and without NAC treatment. *p=<0.05 **p=<0.01 in Independent samples T-test. Error bars represent standard error of the mean.

## NAC treatment restored the number of OL lineage cells and premyelinating OLs in APO-SUS rats

Interneuron hypomyelination in APO-SUS mPFC is caused by impaired maturation of OLs (Chapter 3). OLs and in particular premyelinating OLs are highly sensitive to oxidative stress [25-27]. Importantly, the expression of glutathione-related mRNAs is dysregulated in APO-SUS mPFC from P0 onwards, and as such precedes the decreased expression of myelin-related mRNAs that becomes apparent only during adolescence in APO-SUS mPFC. As myelination of mPFC interneurons takes place during adolescence, we hypothesized that prolonged oxidative insult to OLs prior to and during adolescence, hinders proper myelination of interneurons (Chapter 2). To test this hypothesis, we investigated whether NAC treatment would alleviate the OL maturation impairment in APO-SUS mPFC. Immunofluorescent staining revealed that in NAC-treated APO-SUS rats

the number of OL lineage cells was significantly increased (Figure 5b; One-way ANOVA F=7.530, p=0.001, df=3, Independent samples T-test APO-SUS *versus* APO-UNSUS t=-4.725, p=0.001, df=8.021, APO-SUS *versus* APO-SUS+NAC t=-3.316, p=0.011, df=8). The number of OL precursor cells (OPCs) (Figure 5c) and mature OLs remained unaffected (Figure 5e), while the number of premyelinating OLs was significantly restored in NAC-treated APO-SUS rats (Figure 5d; One-way ANOVA F=7.275, p=0.001, df=3, Independent samples T-test APO-SUS *versus* APO-UNSUS t=3.420, p=0.006, df=11, APO-SUS *versus* APO-SUS+NAC t=3.850, p=0.003, df=10). These findings suggest that the promyelinating effects of NAC are at least partially caused by a positive effect on OL maturation.
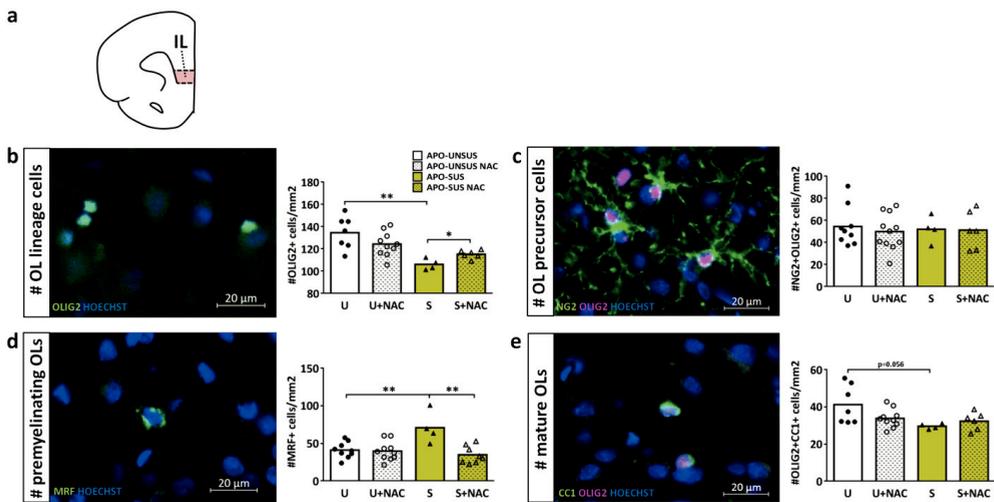


**Figure 5 - NAC treatment restored the number of OL lineage cells and the number of premyelinating OLs in APO-SUS IL. (a)** Schematic representation of IL in rat brain adapted from the Paxinos and Watson rat brain atlas. Numbers of **(b)** OL lineage cells (OLIG2+), **(c)** OL precursor cells (OLIG2+NG2+), **(d)** premyelinating OLs (MRF+) and **(e)** mature OLs (OLIG2+CC1+) per mm$^2$ in the IL of APO-SUS *versus* APO-UNSUS rats with and without NAC treatment (n=4-11 specified in the graphs). *p=<0.05 **p=<0.01 in independent samples T-test.

## Oxidative insult blocked APO-SUS and APO-UNSUS OL maturation *in vitro*

To verify whether an oxidative insult could indeed block the maturation of APO-SUS and APO-UNSUS OLs, we performed *in vitro* experiments with primary OPCs isolated from cortex of newborn APO-SUS and APO-UNSUS rats. OPCs were differentiated in the presence or absence of 1μM cobalt chloride hexahydrate (CoCl$_2$), a chemical that causes oxidative stress, and we analyzed the percentages of all SOX10+ oligodendroglial cells that were OPCs (SOX10+O4-MBP-), preOLs (SOX10+O4+MBP-) or mature OLs (SOX10+O4+MBP+). We confirmed that no differences occur in the intrinsic capacity

of APO-SUS and APO-UNSUS OPCs to mature *in vitro* (Figure 6a and 6b; Chapter 3). However, $CoCl_2$ significantly reduced the percentage of mature OLs in differentiated APO-SUS and APO-UNSUS OPC cultures (Figure 6b; One way ANOVA F=10.436, p=0.004, df=3, Independent samples T-test APO-SUS *versus* APO-SUS+$CoCl_2$ t=2.972, p=0.041, df=4, APO-UNSUS *versus* APO-UNSUS+$CoCl_2$ t=4.646, p=0.010, df=4). In addition, there was an increase in the percentage of OPCs and a decrease in the percentage of preOLs in APO-UNSUS OPC cultures exposed to $CoCl_2$ (Figure 6b; Independent samples T-test APO-UNSUS *versus* APO-UNSUS+$CoCl_2$ OPCs t=-4.897, p=0.008, df=4, preOLs t=-4.897, p=0.008, df=4). We therefore conclude that oxidative stress indeed hinders OL maturation in OPCs of both APO-SUS and APO-UNSUS rats.
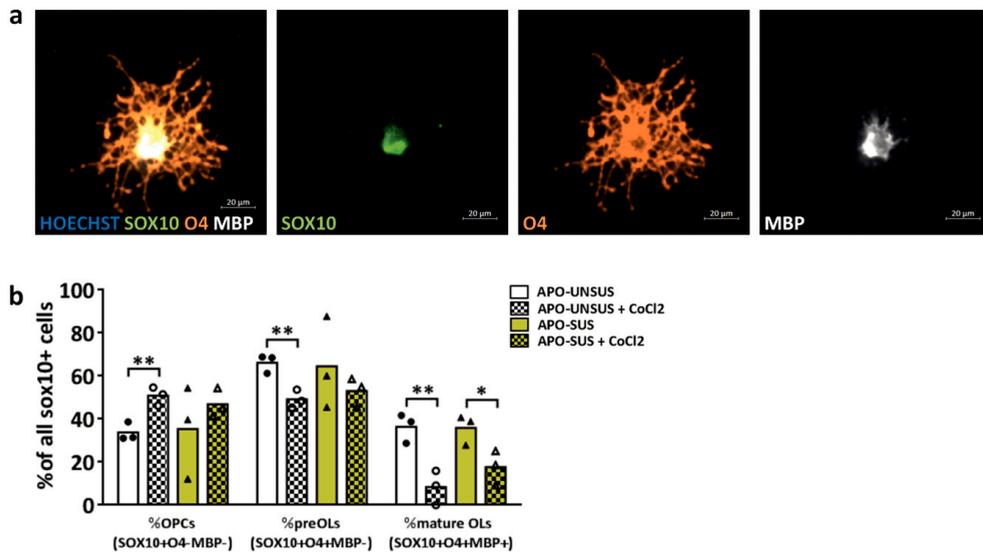


**Figure 6** – **Oxidative stress induced OL maturation impairment in APO-SUS and APO-UNSUS primary OPCs. (a)** Representative images and **(b)** quantification of the percentages of all SOX10+ oligodendroglia cells that are OPC (SOX10+O4-MBP-), premyelinating OL (preOL) (SOX10+O4+MBP-) and mature OL (SOX10+O4+MBP+) in APO-SUS *versus* APO-UNSUS primary oligodendroglia cultures treated with and without $CoCl_2$ during 4 days differentiation (n=3). *p=<0.05 **p=<0.01 in independent samples T-test.

## NAC treatment improved mPFC-dependent cognitive inflexibility in APO-SUS rats

Lastly, we assessed whether restoring glutathione metabolism, mitochondria numbers, myelin-related gene expression, the number of myelinated axons, OL lineage cells and premyelinating OLs in the APO-SUS mPFC would lead to an improvement of cognitive behaviour in NAC-treated APO-SUS rats. Using the extra-dimensional set-shifting task, we have previously observed mPFC-dependent cognitive inflexibility in APO-SUS rats (Chapter 3). The set-shifting task is modeled after the Wisconsin card sorting test used to examine cognitive symptoms in SZ patients [28,29]. We performed the extra-dimensional set-shifting task with APO-SUS and APO-UNSUS rats with and without NAC treatment. We found no differences in initial learning phases of the task (Supplementary Figure S1). In extra-dimensional shift 2 and shift 3, APO-SUS rats showed an increased number of errors until a criterion of a streak of ten correct trials was reached (Multivariate ANOVA F=3.654, p<0.001, df=9 posthoc testing with Benjamini-Hochberg multiple comparisons correction APO-SUS *versus* APO-UNSUS: shift 1 p=0.548, shift 2 p<0.001, shift 3 p=0.008; APO-SUS *versus* APO-SUS+NAC: shift 1 p=0.470, shift 2 p=0.702, shift 3 p=0.272). The number of perseverative errors was significantly increased in untreated APO-SUS rats *versus* untreated APO-UNSUS rats and significantly normalized in APO-SUS rats by NAC treatment (Figure 7b; Multivariate ANOVA F=3.124, p=0.002, df=9 posthoc testing with Benjamini-Hochberg multiple comparisons correction APO-SUS *versus* APO-UNSUS: perseverative errors p<0.001, regressive errors p=0.425, never-reinforced errors p=0.648; APO-SUS *versus* APO-SUS+NAC: perseverative errors p=0.009, regressive errors p=0.012, never-reinforced errors p=0.338). Taken together, our data show that NAC treatment strengthens glutathione antioxidant defenses, normalizes the number of mitochondria, improves OL maturation and as such rescues interneuron hypomyelination and improves cognitive inflexibility in the APO-SUS rat model SZ-relevant features.

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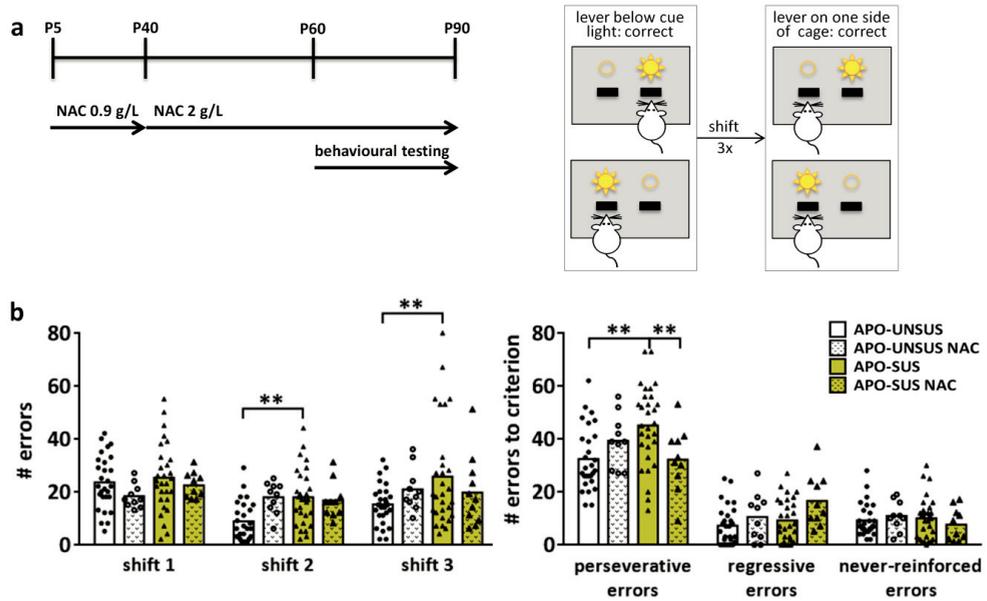
**Figure 7 - NAC treatment improved cognitive inflexibility in APO-SUS rats. (a)** Schematic representation of the experimental design and extra-dimensional set-shifting task. APO-SUS and APO-UNSUS rats were treated with NAC 0.9 g/L from P5 to P40 and with NAC 2 g/L from P40-P90. Behavioural testing was performed between P60 and P90. Rats were trained to press the lever above which a cue light was illuminated. From trial 21 of the next session onwards, rats were required to press the lever on one side of the cage irrespective of the cue light. **(b)** Number of errors to criterion (streak of 10 correct trials) and total number of perseverative, regressive and never-reinforced errors in APO-UNSUS (U n=28, U+NAC n=10) and APO-SUS (S n=30, S+NAC n=10) rats with and without NAC treatment. **p<0.01 in multivariate ANOVA posthoc testing with Benjamini Hochberg multiple comparisons correction.

# Discussion

Animal models recapitulating (some of) the positive, negative and cognitive symptoms of SZ are instrumental to understand the molecular and cellular mechanisms underlying these symptoms. Here we used the well-characterized APO-SUS rat model with SZ-relevant features and an unbiased transcriptomics approach to explore the elusive neurobiological basis of PFC-related cognitive symptoms in SZ. Among the genes differentially expressed in mPFC of APO-SUS *versus* APO-UNSUS rats, genes related to glutathione metabolism were enriched. Glutathione is an endogenous antioxidant that decreases oxidative stress. In SZ patients, glutathione levels are reduced in blood plasma, cerebrospinal fluid, PFC and *post-mortem* brain regions, and are independent of disease stage or medication use [3,30-32]. The fact that in APO-SUS rats impaired glutathione metabolism was observed already at birth and persisted into late adulthood suggests that oxidative stress in SZ may occur before the clinical symptoms of the disorder become apparent.

The reduced levels of glutathione in APO-SUS mPFC were accompanied by an increase in the number of mitochondria. Mitochondrial dysfunction has been reported in various SZ brain regions, and the interplay between mitochondrial dysfunction and oxidative stress has been hypothesized to play a role in SZ etiology [33,34]. In individuals at high risk to develop SZ, mitochondrial dysfunction has been linked to symptoms of the disorder [35], indicating that mitochondrial abnormalities are present already before disease onset. As genetic risk for SZ includes genetic variations in both mitochondria- and oxidative stress-related genes [22], it is unclear whether mitochondrial dysfunction arises as a consequence of oxidative stress or induces oxidative stress.

Reduced PFC glutathione levels have been associated with decreased PFC white-matter integrity that is thought to arise from defective myelination [6,24]. Expression of myelin-related mRNAs and proteins is decreased in *post-mortem* SZ frontal cortex [36,37] and myelin content is reduced in frontal areas of SZ patients [38]. We have recently found that during mPFC development in adolescent APO-SUS rats hypomyelination occurs specifically in interneurons (Chapter 3). Notably, during adolescent mPFC development interneurons undergo maturational changes that include myelination and the formation of perineuronal nets, both of which are thought to be affected in SZ by oxidative stress [5]. To investigate whether oxidative stress causes interneuron hypomyelination in the APO-SUS mPFC, we treated APO-SUS rats with the antioxidant glutathione precursor NAC. Chronic post-natal treatment of APO-SUS rats with NAC normalized blood levels of glutathione as well as the levels of mPFC mRNA expression of glutathione metabolism-related genes in adulthood. In addition, NAC treatment significantly reduced the number of mitochondria in APO-SUS mPFC to the number in APO-UNSUS mPFC, indicating that oxidative stress boosts mitochondria numbers. In the mPFC of APO-UNSUS rats, NAC treatment did not affect glutathione levels, glutathione-related mRNA expression nor mitochondria numbers, suggesting that the antioxidant does not influence the redox

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state when it is already in balance. Remarkably, besides its antioxidative properties, NAC also had promyelinating effects in the APO-SUS mPFC. Restoring the redox balance led to a rescue of the number of myelinated axons and myelin-related mRNA expression in the APO-SUS mPFC. Thus, glutathione metabolism appears to be essential for developmental interneuron myelination in SZ. The notion that oxidative stress underlies PFC hypomyelination in SZ is corroborated by the results of our analysis of the developmental time course of APO-SUS mPFC mRNA expression which show that the dysregulation of glutathione-related genes is evident already at birth and as such preceded reduced expression of myelin-related genes that starts during adolescence (Chapter 3).

Oxidative stress has detrimental effects on cellular proliferation and differentiation, and relative to other cell types OLs, and in particular premyelinating OLs, are extremely vulnerable to oxidative stress [25,26,39,40]. We therefore hypothesized that the causative role of oxidative stress on interneuron hypomyelination in SZ may be mediated by adverse effects on OL maturation. We have previously shown that OL maturation is impaired in APO-SUS mPFC (Chapter 3). Strikingly, we now find that treatment with NAC restores the number of OL lineage cells and premyelinating OLs in APO-SUS mPFC to APO-UNSUS levels. Moreover, our *in vitro* experiments with APO-SUS and APO-UNSUS OPCs confirmed that an oxidative insult is capable of impairing OPC maturation. The fact that NAC treatment positively influences OL maturation supports the notion that oxidative damage to OLs contributes to interneuron hypomyelination in the APO-SUS mPFC and may well underlie hypomyelination in SZ.

Intriguingly, the antioxidative and promyelinating effects of NAC in APO-SUS mPFC led to a significant improvement in cognitive inflexibility in the extra-dimensional set-shifting task. This task is equivalent to the Wisconsin card sorting task that is used to reveal cognitive inflexibility in SZ patients. As an add-on treatment to antipsychotic medication, NAC has moderate effects on the positive, negative and cognitive symptoms of chronic SZ patients [41-43]. The effects on cognition were replicated in early-psychosis patients and accompanied by increases in PFC glutathione levels [44]. However, treatment with N-acetylcysteine amide (NACA), a more hydrophobic and lipophilic derivative of NAC allowing higher ability to cross the blood brain barrier [45], might boost the effects seen in SZ patients treated with NAC. The myelination process can also be accelerated by behavioural experiences, such as physical exercise, social interactions and environmental enrichment [46-49]. Behavioural therapy is known to enhance white-matter integrity and improve cognition in SZ patients [50,51]. We have recently shown that chronic environmental enrichment during adolescence can indeed rescue interneuron hypomyelination as well as cognitive inflexibility in the APO-SUS rats (Chapter 3).

Collectively, the findings increase our understanding of the neurobiological sequence of events leading to cognitive symptoms in SZ and provide new avenues for the development of treatment strategies for this devastating neurodevelopmental

psychiatric disorder. Our studies support the use of chronic NAC (or NACA) treatment in combination with PFC-directed behavioural therapy as preventative measures for individuals at high risk for developing SZ and early-phase SZ patients.

## Methods

### Animal model

Generation of the APO-SUS and APO-UNSUS rat lines has been described in detail elsewhere [18,52]. Briefly, we selectively bred rats from an outbred Nijmegen Wistar rat population that displayed stereotyped behaviour upon injection of apomorphine (APO-SUS rats). The same selective breeding was performed with the rats that showed a weak apomorphine-induced stereotypy (APO-UNSUS rats). Apomorphine injection and behavioural selection were only performed with the first 15 generations of APO-SUS and APO-UNSUS rats. In the subsequent breedings, APO-SUS rats displayed SZ-relevant features without this pharmacological treatment. In this study, naïve male APO-SUS and APO-UNSUS rats from the 32th-44rd generation were used. Rats were housed in pairs in a temperature- and humidity-controlled room with a 12-h light-dark cycle (lights on at 7.00 a.m.) and *ad libitum* access to water and standard laboratory chow (V1534-703, SSNIFF, Germany), unless otherwise indicated. Animal experiments were approved by the Animal Ethics Committee of Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, and were conducted in accordance with Dutch legislation (Herziene Wet op Dierproeven, Art 10.a.2, 2014).

### Micropunching

For micropunching, naïve P21 (+/- 1 day), P60 (+/- 1 day), P90 (+/- 1 day), P120 (+/- 1 day) and P365 (+/- 14 days) APO-SUS and APO-UNSUS rats were sacrificed by direct decapitation and brains were isolated, frozen on dry ice and stored at -80 ˚C. Micropunching was performed in a cryostat (Leica) at -15 ˚C and the Paxinos and Watson rat brain atlas was taken as a reference to aid the dissection. mPFC was collected with a 1.20 or 2.00-mm punch needle (Harris) from 300 µm coronal sections at Bregma 4.00-2.20. Dissected tissues were immediately frozen on dry ice and stored at -80 ˚C until further analysis.

### RNA isolation

RNA for RNA-seq analysis was extracted from P365 APO-SUS and APO-UNSUS mPFC with a Nucleospin RNA II kit following the instructions of the manufacturer (Macherey-Nagel, Dueren, Germany). For all other RNA isolations, tissues were homogenized using Trizol reagent (Sigma) and stainless-steel beads in a Tissue Lyser (Qiagen), followed by chloroform extraction and RNA precipitation with isopropanol in the presence of 20

µg of glycogen (Fermentas). Pellets were washed twice with 75% ice-cold ethanol and dissolved in MilliQ $H_2O$. RNA concentration and purity were measured using a DS-11 spectrophotometer (Denovix). RNA was kept at -80 °C until further analysis.

### RNA-seq and data analysis

For RNA-seq analysis, total RNA quality of the samples was assayed using Agilent 2100 Bioanalyser (Applied Biosystems). Total RNA concentration was estimated by Qubit Fluorometer (Invitrogen). RNA samples (all with an RNA integrity number RIN ≥ 8.60) prepared from P365 APO-SUS (n=4, pooled) and APO-UNSUS (n=4, pooled) mPFC were analyzed by RNA-seq at the Hudson Alpha Institute for Biotechnology (Huntsville, AL, USA). RNA-seq libraries were formed from approximately 850 ng total RNA of each sample. RNA-seq was performed using paired end sequencing on Illumina HiSeq (Illumina), at 100 base pairs, generating over 30 million paired reads. RNAseq FASTQ files were analyzed using GeneSifter software (VizX Labs). Transcript abundance was calculated by estimating the reads per kilobase of exon per million mapped reads (RPKM) and normalization to the number of mapped reads was used for comparison. The mRNAs differentially expressed in the APO-SUS *versus* APO-UNSUS mPFC and meeting our two predefined criteria (fold change ≥ 1.2; Likelihood Ratio test corrected p-value < 0.05) were used as input data for analysis with the Ingenuity Pathway Analysis (IPA) software package (Qiagen) to identify overrepresented biological pathways with a focus on 'Canonical Pathways'.

### Quantitative real-time PCR.

For quantitative real-time PCR (qPCR) analysis, RNA samples were treated with DNase I (Fermentas) and cDNA was synthesized using the Revert Aid H-minus first strand cDNA synthesis kit (Thermo Scientific). cDNA was subsequently diluted 1:20 in MilliQ $H_2O$ and stored at -20 °C until qPCR analysis. qPCR samples were pipetted using a robot (Corbett Robotics) and contained 2.0 µL diluted cDNA, 0.8 µL 5 µM forward primer, 0.8 µL 5 µM reverse primer, 5 µL SybrGreen mix (Roche) and 1.8 µL MilliQ $H_2O$. qPCR was performed with a Rotor Gene 6000 Series (Corbett Life Sciences) using a 3-step paradigm with a fixed gain of 8. Fifty cycling steps of 95, 60, and 72 °C were applied, and fluorescence was acquired after each cycling step. Primers were designed with NCBI Primer-Blast or Primer Express 2.0 and synthesized by Sigma (for primer pair sequences, see Supplementary Table S8). Melting temperature was used to check whether a single PCR product was generated and the take off and amplification values of the housekeeping genes (*Ywhaz, B-actin, Ppia* and *Gapdh*) were used to determine the normalization factor with GeNorm [43] after which normalized mRNA expression levels were calculated. For analysis of the developmental time course of mRNA expression, P0, P7, P14 and P21 (+/- 1 day) APO-SUS and APO-UNSUS rats were sacrificed by direct decapitation, brains were immediately removed, and mPFCs were freshly dissected,

frozen on dry ice and stored at -80 °C for subsequent RNA extraction and qPCR analysis. Furthermore, for the developmental time course analysis, P21 (+/- 1 day), P28 (+/- 1 day), P90 (+/- 1 day) and P365 (+/- 14 days) APO-SUS and APO-UNSUS brains were removed immediately following decapitation, frozen on dry ice and stored at -80 °C for subsequent micropunching, RNA extraction and qPCR analysis.

## Glutathione assay

Levels of the natural antioxidant glutathione were determined by a kinetic glutathione assay (CS0260 Sigma) that measured the level of total glutathione (oxidized and reduced glutathione). The assay was performed according to the instructions of the manufacturer and glutathione levels were normalized by protein content determination through a bicinchoninic acid (BCA) assay (23225; Thermo Fisher).

## Electron microscopy

For electron microscopy, naïve animals of P90 (+/- 1 day) were perfused with 2% PFA/2% glutaraldehyde and perfused brains were removed, postfixed overnight in 2% PFA/2% glutaraldehyde and stored at 4 °C in PBS/0.01% azide until further processing. Sagittal sections of 100 μm were collected using a vibratome (Leica), fixed with 2% osmium tetroxide and contrast was obtained with 5% uranyl acetate. Following ethanol dehydration, sections were embedded in epon resin and ultrathin (70-100 nm) sections were obtained with an ultramicrotome (Leica). Sections were contrasted using lead citrate and 40 non-overlapping 26000x images were obtained in IL or PL subregions of the mPFC. Myelinated axons were counted in all 40 images of IL region and the G-ratio and axon caliber of all myelinated axons perpendicular to the field of view were measured in FIJI. The percentage of mitochondrial surface was calculated using 49 equally (200 μm) spaced crosses superimposed over 20 randomly picked PL images. The percentage of crosses that touched a mitochondrion over the total number of crosses was calculated and the size of each mitochondrion that touched a cross was measured.

## NAC treatment

APO-SUS and APO-UNSUS rats were treated between P5-P40 (+/- 1 day) with 0.9 grams per liter and from P40-P90 (+/- 1 day) with 2 grams per liter NAC (A7250 Sigma) in the drinking water. pH was adjusted to 8.6 using 5M NaOH, equivalent to the pH of control drinking water, and NAC as well as control drinking water was offered in dark drinking bottles to prevent NAC oxidation by light. Both NAC-laced and control drinking water were refreshed three times per week. Animals treated with NAC-laced and control drinking water were either sacrificed at P90 (+/- 1 day) for electron microscopy or

immunofluorescent analysis, or were subjected to behavioural testing between P60 and P90 (+/- 6 days) and sacrificed at P90 for glutathione assays and qPCR analyses.

**Immunohistochemistry**

For immunohistochemistry, APO-SUS and APO-UNSUS rats of P90 (+/- 1 day) were perfused with 2% paraformaldehyde (PFA). Perfused brains were removed, postfixed overnight and placed in 30% sucrose in PBS for 3-5 days, frozen on dry ice and stored at -80 °C until further processing. Coronal cryosections of 10 μm were collected in a cryostat (Leica) and rehydrated in 1x PBS, 0.1% Triton X-100 or for OLIG2 NG2 immunohistochemistry in 1x PBS, 0.05% Tween-20. For OLIG2-CC1 staining antigen retrieval was performed in a microwave using citric acid-based antigen unmasking solution (Vector). Tissue was blocked in 4% BSA, 0.1% Triton X-100 or 5% NGS/ NDS/NHS, 1% BSA, 1% glycine, 0.1% lysine, 0.4% triton X-100 for 1 hour at room temperature (RT). Primary antibodies were anti-OLIG2 (AB9610, Millipore 1:1000, ab109186, Abcam 1:400 or MABN50, Millipore 1:500), anti-CC1 (OP80, Calbiochem 1:100), anti-NG2 (MAB5384, Millipore 1:200 or AB5320, Millipore 1:100), anti-MRF ABN45, Millipore 1:100) and incubated overnight at 4 °C. Secondary antibodies were 488-, 555- and 568-conjugated anti-rabbit or anti-mouse (IgG and IgG2b, Alexa) or TRITC-conjugated anti-mouse (IGg1, Southern Biotech) and incubated for 1-2 hours at RT. Hoechst (H6024, Sigma 1:1000) was added as a nuclear counterstain. Sections were mounted in Fluoromount (0100-01, Southern Biotech) and 20x images were obtained using an Axioscan (Leica) and analysed with Zen (Blue edition). Regions of interest were drawn and cells were counted manually. OLIG2+ cell counts were obtained in the OLIG2-CC1 images.

**Primary oligodendroglial cell cultures**

Mixed glia cultures were obtained from cortex of P1 APO-SUS and APO-UNSUS rats, n=3 replicates per condition. The tissue was homogenized in mixed glia culture medium (GlutaMAX (Invitrogen), 10% fetal bovine serum (Thermo Fisher) 1% Pen-Strep (Thermo Fisher) and 1% non-essential amino acids (Thermo Fisher)) and cells were kept in this medium on 1:10 poly-L ornithine coated T75 flasks at 37 °C 5% $CO_2$. Medium was refreshed after 7 and 13 days in culture and at day 14 OPCs were purified using a shaking protocol. Mixed glia cultures were shook at 250 rpm for 1 hour to discard microglia cells, then cultures were shook again at 250rpm for 18 hours to purify OPCs. Supernatant containing OPCs was placed on petridishes (Falcon) three times for 5 minutes to eliminate astrocyte contamination. OPCs were then plated onto PLO-coated coverslips in 24-well plates. After 4 hours, medium was changed to differentiation medium for 4 days (DMEM-F12 (Invitrogen), 0.5% B27 (Sigma) 1% Pen-Strep (Thermo Fisher) and 0.05% 40 ng/ml of T3 thyroid hormone (Sigma)) with or without 1μM $CoCl_2$. After 4 days, cultures were stained with homemade O4 antibody for 1 hour and fixed

in 2% PFA for 7 minutes. Subsequently OPCs were incubated with anti-SOX10 (1/100, R&D Systems AF2864) and anti-MBP (1/200, Abcam ab7349) primary antibodies for 1 hour at RT, washed with 1x PBS and incubated with secondary antibodies (donkey anti-TRITC IgM 1/100 (Southern Biotech), donkey anti-goat Alexa 488 1/1000, donkey anti-rat Alexa 647 1/750 and Hoechst) for 1 hour at RT, washed and mounted with fluoromount. Images were obtained in an Axioscan and analysed in Zen (Bleu edition) manually.

### Operant attentional set-shifting tests

Upon the start of behavioural training, rats were food restricted and received 5-7 grams of food per 100 grams of rat daily. Rats were pre-exposed once to grain reward pellets (Rodent Tablet [5TUM], 45mg, TestDiet, USA) in the home cage. Operant conditioning chambers (29.5 cm L, 24 cmW25 cm H;Med Associates, Georgia, VT) were situated in light and sound-attenuating cubicles equipped with a ventilation fan. Each chamber was equipped with two 4.8-cm-wide retractable levers, placed 11.7 cm apart and 6 cm from the grid floor. A cue light (28 V, 100 mA) was present above each lever. At the same wall, a reward pellet could be delivered in a magazine between the levers and a house light (28 V,100 mA) was located on the same wall. The lever presentation and cue light illumination sides were counterbalanced between rats. Pretraining, operant extra-dimensional set-shifting were performed as described in Chapter 3. Rats performed three extra-dimensional set-shifts (note that between extra-dimensional shifts 2 and 3 reversal learning was performed). We measured the number of errors made until a criterion of 10 subsequent correct trials was reached, and classified the errors as perseverative errors (following the 'old rule'), regressive errors (following the 'old rule' while more than 70% of previous trials were correct) or never-reinforced errors (pressing a lever that was incorrect during both the 'old rule' and during the current rule).

### Statistical analyses

For qPCR, glutathione assay and electron microscopy analysis, statistical significance was calculated using the independent samples T-test and, when appropriate, with Benjamini-Hochberg correction for multiple comparisons in IBM SPSS Statistics 24. Linear regression with two-way ANOVA was used to test statistical significance of the regression between G-ratio and axon caliber. For the NAC-treatment experiment, immunohistochemistry and electron microscopy data analysis was performed using one-way ANOVAs and effects were assessed with independent samples T-tests, and analysis of the set-shifting task was performed using multivariate ANOVAs. For all analyses, outliers were discarded beforehand as indicated by the Grubbs outlier test using Graphpad quickcalcs, and the level of significance was set at p=0.05.

**Data availability**

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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## Author contributions

D.A.M., B.N.O. and G.J.M.M. designed the project. D.A.M. performed data acquisition and analysis and wrote the manuscript. V.D.E. performed data acquisition of P0-P14 qPCRs and RNA sequencing, J.v.H. performed the animal work for P0-P14 qPCRs. J.R.H. and P.d.W. contributed to the supervision of the project, A.V. designed and supervised part of the project, and B.N.O. and G.J.M.M. supervised the project. All authors discussed the results and commented on the manuscript.

## Competing interests

We have no conflict of interest to disclose.

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# Supplementary Material

**Supplementary Table S1** – Ingenuity pathway analysis results of RNA sequencing data from mPFC of APO-SUS versus APO-UNSUS rats.

| Ingenuity Canonical Pathways | -log(Benjamini Hochberg-corrected -H p-value) | Ratio of genes present in our data over the total number of genes in the canonical pathway |
|---|---|---|
| Glutathione-mediated Detoxification | 2.69 | 0.258 |
| Glutathione Redox Reactions I | 2.02 | 0.25 |
| Complement System | 1.87 | 0.189 |
| Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells | 1.6 | 0.188 |
| Calcium-induced T Lymphocyte Apoptosis | 2.56 | 0.167 |

**Supplementary Table S2 -** Genes in IPA canonical pathway 'Glutathione-mediated detoxification'. Transcriptome-wide analysis of APO-SUS and APO-UNSUS medial prefrontal cortex (mPFC) combined with IPA revealed that this pathway represents the most significantly enriched pathway.

| Symbol | Entrez Gene Name | Corrected p-value |
|---|---|---|
| Gsta1 | glutathione S-transferase alpha 1 | 0.0264 |
| GSTA1 | glutathione S-transferase alpha 1 | 0.00111 |
| Gsta4 | glutathione S-transferase, alpha 4 | 0.00025 |
| GSTM4 | glutathione S-transferase mu 4 | 0.0354 |
| GSTM5 | glutathione S-transferase mu 5 | 0.000000969 |
| Gstt1 | glutathione S-transferase, theta 1 | 0.00713 |
| HPGDS | hematopoietic prostaglandin D synthase | 0.000794 |
| MGST1 | microsomal glutathione S-transferase 1 | 0.00282 |

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**Supplementary Table S3 -** Genes in IPA canonical pathway 'Glutathione redox reactions I'. Transcriptome-wide analysis of APO-SUS and APO-UNSUS medial prefrontal cortex (mPFC) combined with IPA revealed that this pathway represents the second-most significantly enriched pathway.

| Symbol | Entrez Gene Name | Corrected p-value |
|---|---|---|
| GPX3 | glutathione peroxidase 3 | 0.0183 |
| Gsta1 | glutathione S-transferase alpha 1 | 0.0264 |
| GSTA1 | glutathione S-transferase alpha 1 | 0.00111 |
| Gstt1 | glutathione S-transferase, theta 1 | 0.00713 |
| MGST1 | microsomal glutathione S-transferase 1 | 0.00282 |
| PRDX6 | peroxiredoxin 6 | 0.00E+00 |

**Supplementary Table S4 –** Statistical values of Independent samples T-test with Benjamini Hochberg multiple comparisons correction in every age group on qPCR expression data presented in Figure 1.

| Age | Gene | t-value | df | Corrected p-value |
|---|---|---|---|---|
| P0 | gstm4 | 4.841 | 19 | 0.000* |
| | gsta4 | 3.652 | 18 | 0.002* |
| | prdx6 | -1.402 | 19 | 0.177 |
| | gstm6 | -3.468 | 19 | 0.003* |
| | anpep | -3.070 | 8.378 | 0.015* |
| | gstm1 | -3.184 | 19 | 0.005* |
| P7 | gstm4 | 5.494 | 15.065 | 0.000* |
| | gsta4 | 7.191 | 20 | 0.000* |
| | prdx6 | -3.100 | 20 | 0.006* |
| | gstm6 | -8.665 | 21 | 0.000* |
| | anpep | -3.979 | 20 | 0.001* |
| | gstm1 | -2.296 | 20 | 0.033* |
| P14 | gstm4 | 10.977 | 22 | 0.000* |
| | gsta4 | 8.099 | 20 | 0.000* |
| | prdx6 | -8.349 | 22 | 0.000* |
| | gstm6 | -8.912 | 22 | 0.000* |
| | anpep | -2.374 | 22 | 0.027* |
| | gstm1 | -4.385 | 22 | 0.000* |
| P21 | gstm4 | 6.433 | 20 | 0.000* |
| | gsta4 | 8.913 | 18 | 0.000* |
| | prdx6 | -6.683 | 16.914 | 0.000* |

|  | gstm6 | -4.280 | 20 | 0.000* |
|---|---|---|---|---|
|  | anpep | -2.784 | 19 | 0.012* |
|  | gstm1 | -3.829 | 16.795 | 0.001* |
| P90 | gstm4 | 4.446 | 5.128 | 0.006* |
|  | gsta4 | 2.526 | 10 | 0.030* |
|  | prdx6 | -3.513 | 10 | 0.006* |
|  | gstm6 | -3.111 | 10 | 0.011* |
|  | anpep | -1.783 | 10 | 0.105 |
|  | gstm1 | -3.760 | 10 | 0.004* |
| P365 | gstm4 | 3.921 | 14 | 0.002* |
|  | gsta4 | 2.399 | 14 | 0.031* |
|  | prdx6 | -3.719 | 14 | 0.002* |
|  | gstm6 | -1.673 | 14 | 0.117 |
|  | anpep | -1.170 | 14 | 0.261 |
|  | gstm1 | 0.684 | 14 | 0.505 |

* Significant after Benjamini Hochberg multiple comparisons correction

**Supplementary Table S5 –** Differentially expressed genes (fold change ≥ |1.2|, Likelihood Ratio test corrected p-value p < 0.05) in APO-SUS *versus* APO-UNSUS medial prefrontal cortex (mPFC) as revealed by RNA sequencing analysis**.**

Table can be accessed via this link:
https://drive.google.com/open?id=1QbdMSOyIDgYJmLgkR3tdQOtad9Zn-jKP

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**Supplementary Table S6 –** Statistical values of Independent samples T-test with Benjamini Hochberg multiple comparisons correction on qPCR expression data presented in Figure 3.

| | APO-SUS versus APO-UNSUS | | | APO-SUS versus APO-SUS+NAC | | |
|---|---|---|---|---|---|---|
| Gene | t-value | df | p-value | t-value | df | corrected p-value |
| Prdx6 | -3.157 | 15 | 0.007* | 0.021 | 16 | 0.983 |
| Gstm4 | 1.115 | 10.736 | 0.289 | -0.197 | 11.573 | 0.847 |
| Anpep | -2.362 | 14 | 0.033* | -3.070 | 16 | 0.007* |
| Gstm6 | -2.837 | 6.303 | 0.028* | -3.139 | 8.533 | 0.013* |
| Gsta4 | -1.190 | 14 | 0.254 | -6.897 | 15 | 0.000* |
| Gstm1 | -2.568 | 14 | 0.022* | -5.476 | 15 | 0.000* |
| Gclc | -1.782 | 14 | 0.096 | -4.570 | 15 | 0.000* |
| Gss | -2.415 | 7.096 | 0.046* | -3.013 | 9.440 | 0.014* |
| Gstm7 | -3.175 | 13 | 0.007* | -6.484 | 13 | 0.000* |

* Significant after Benjamini Hochberg multiple comparisons correction

**Supplementary Table S7 –** Statistical values of Independent samples T-test with Benjamini Hochberg multiple comparisons correction on qPCR expression data presented in Figure 4.

| | APO-SUS versus APO-UNSUS | | | APO-SUS versus APO-SUS+NAC | | |
|---|---|---|---|---|---|---|
| Gene | t-value | df | p-value | t-value | df | corrected p-value |
| Mog | -3.719 | 15 | 0.002* | -6.022 | 15 | 0.002* |
| Plp | -2.495 | 14 | 0.026* | -4.769 | 15 | 0.026* |
| Mag | -2.901 | 13 | 0.012* | -4.253 | 15 | 0.012* |
| Mobp | -2.929 | 14 | 0.011* | -2.942 | 14 | 0.011* |
| Cldn11 | -3.740 | 13 | 0.002* | -4.424 | 14 | 0.002* |
| Mbp | -2.162 | 14 | 0.048* | -3.010 | 16 | 0.048* |
| Opalin | -4.025 | 13 | 0.001* | -4.667 | 15 | 0.001* |

* Significant after Benjamini Hochberg multiple comparisons correction

**Supplementary Table S8 –** Primer sequences.

| Gene and abbreviation | | Forward primer 5'-3' | Reverse primer 5'-3' |
|---|---|---|---|
| Housekeeping genes | | | |
| β-actin | Beta-actin | CCTTCCTGGGTATGGAATCCTGT | TAGAGCCACCAATCCACACA |
| *Ppia* | Peptidyl-prolyl cis-trans isomerase A | AGCACTGGGGAGAAAGGATT | AGCCACTCAGTCTTGGCAGT |
| *Gapdh* | Glyceralde-hyde-3-phosphate | GGGTGTGAACCACGAGAAAT | ACTGTGGTCATGAGCCCTTC |
| *Ywhaz* | Tyrosine 3-monoox-ygenase/tryptophan 5-monooxygenase activation protein, zeta | TTGAGCAGAAGACGGAAGGT | GAAGCATTGGGGATCAAGAA |
| Myelin-related genes | | | |
| *Cldn11* | Claudin 11 | CGCAAAATGGACGAACTGGG | TGCACGTAACCAGGGAGGAT |
| *Mag* | Myelin-associated glycoprotein | AAGCCAGACCATCCAACCTTC | CTCCTGATTCCGCTCCAAGT |
| *Mbp* | Myelin basic protein | CCCTACTCCATCCTCAGACTTTCTT | TGGCGGTGTGCCTGTCTAT |
| *Mobp* | Myelin-associated oligodendrocytebasic protein | AATACCTGCAGGGCAACAAAG | TCTGGTTCTTGGAGAGCCTGG |
| *Mog* | Myelin oligodendro-cyte glycoprotein | CGCCGTGGAGTTGAAAGTAG | GCACGGAGTTTTCCTCTAGT |
| *Opalin* | Oligodendrocytic Myelin Paranodal And Inner Loop Protein | ACCCTGATCCAGCGAAGAAG | TGACTGCCTAGGATTCTCGGATA |
| *Plp1* | Proteolipid protein 1 | GGGCCTGAGCGCAACGGTAA | CAGGCACAGCAGAGCAGGCAA |
| Glutathione-related genes | | | |
| *Gsta4* | Glutathione-S-trans-ferase alpha4 | GCCGCCAAGTACAACTTGTA | CACTGCTAAAGCTAGGCTCTCTTCTT |
| *Gstm4* | Glutathione-S-trans-ferase mu 4 | GCCTAGGCCCCTGGTTTTC | TCTTCACAGCAGCACAGCAACT |
| *Prdx6* | Peroxiredoxin 6 | TGACTGGAAGAAGGGAGA-GAGTGT | ATGGGAGCTCTTTGGTGAAGAC |
| *Gclc* | Glutamate—cysteine ligase catalytic subunit | AGAGGACAAACCCCAACCAC | TCGTGCAAAGAGCCTGATGT |
| *Gss* | Glutathione synthetase | AGCGTGCCATAGAGAACGAG | GCTTCCCAGTTCTGTGCGTT |
| *Gstm1* | Glutathione-S-trans-ferase mu 1 | GTCATGCCCACATAGTCTTCATTC | AGTTCAGGGCAGACCTCAAATC |
| *Gstm6* | Glutathione-S-trans-ferase mu 6 | CTGAGCGTTGCTATCTCGGAG | TTCTGTGTATTCCAGGAGCAGC |
| *Anpep* | Alanyl Aminopepti-dase | CCCATCAGTGGTTTGGCAAC | CATAGTCAGCACCCAGAAATTCC |

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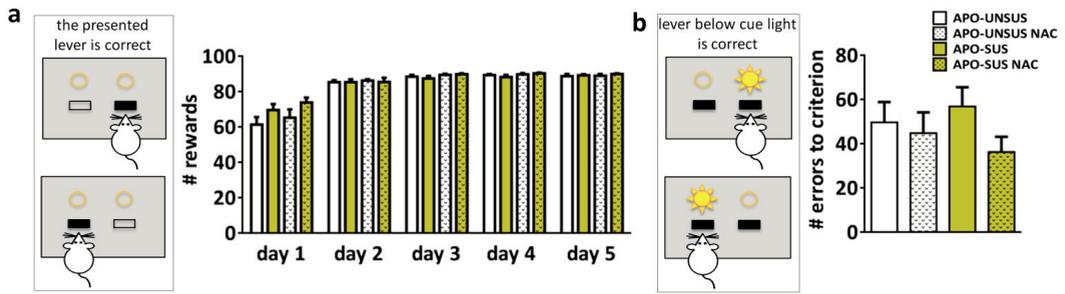
**Supplementary Figure S1 – No differences in the performance of APO-SUS and APO-UNSUS rats during retractable lever-press training and visual cue discrimination with and without NAC treatment. (a)** Schematic representation and performance during retractable lever press training. Rats were required to press the lever they were presented with during 5 consecutive sessions in 1 session per day. The number of rewards in each session in APO-SUS versus APO-UNSUS rats with and without NAC treatment is depicted. **(b)** Schematic representation of visual cue learning in the operant set-shifting paradigm. Rats were required to press the lever above which a cue light was illuminated in order to receive a reward pellet. The number of errors until criterion of a streak of 10 correct trials was reached in APO-SUS and APO-UNSUS rats with and without NAC treatment is depicted. One-way ANOVA F=0.228, p=0.877, df=3. Error bars represent standard error of the mean.

4

# Appendix

## Is the deficit in glutathione metabolism specific to the medial prefrontal cortex of APO-SUS rats?

To investigate whether the glutathione metabolism deficit in APO-SUS rats is specific to the medial prefrontal cortex (mPFC), we performed qPCR analysis of glutathione-related mRNA expression and glutathione assays in three brain regions of APO-SUS and APO-UNSUS rats. We found altered mRNA expression of glutathione-related genes in the barrel cortex, corpus callosum and striatum (Figure A1a). This was accompanied by a lower level of glutathione in APO-SUS relative to APO-UNSUS rats in the corpus callosum and striatum (Figure A1b). In the barrel cortex we found no difference in the level of glutathione, possibly due to a low sample size. We conclude that glutathione metabolism is decreased not only in the mPFC, but also in other APO-SUS brain regions.
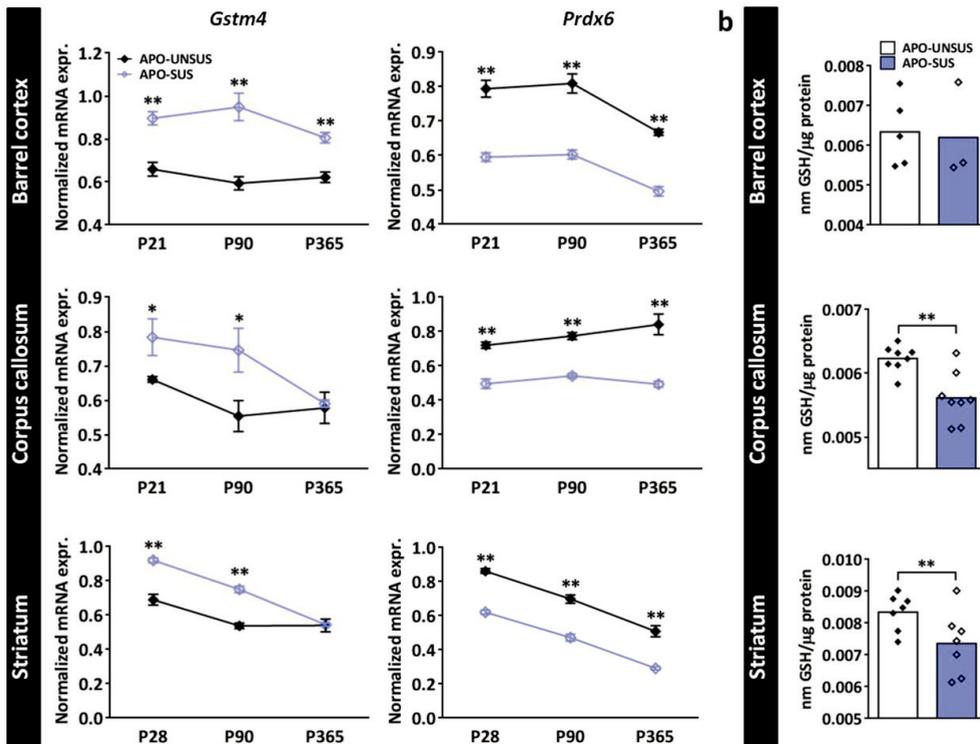


**Figure A1 – Glutathione metabolism is impaired in barrel cortex, corpus callosum and striatum of APO-SUS rats. (a)** Normalized mRNA expression of glutathione-S-transferase mu 4 (*Gstm4*) and peroxiredoxin 6 (*Prdx6*) in APO-SUS and APO-UNSUS barrel cortex, corpus callosum and striatum and **(b)** nM glutathione (GSH) per µg protein. *p<0.05 **p<0.001 in independent samples T-test, error bars represent standard error of the mean.
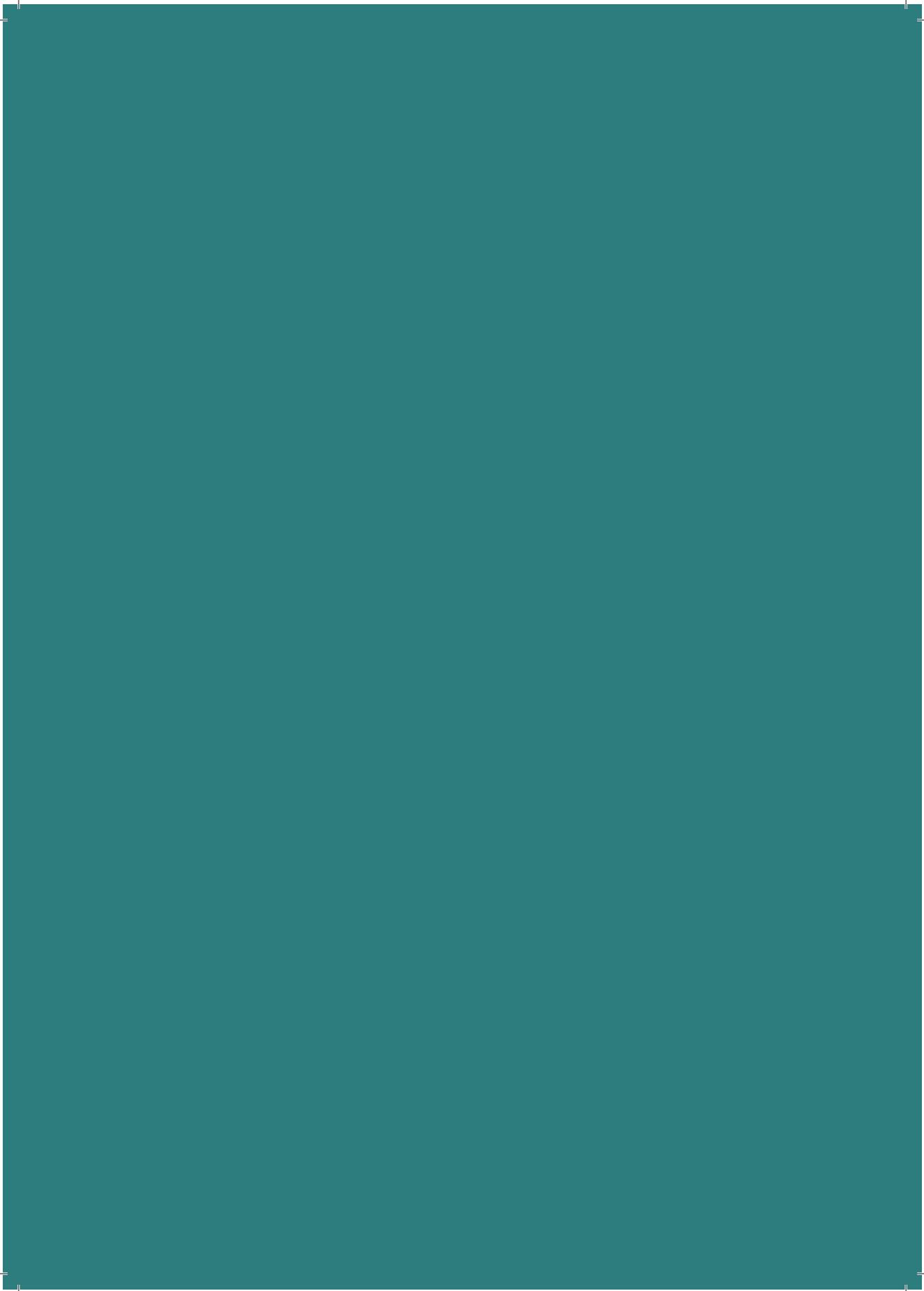
# 5

# Key role for lipids in cognitive symptoms of schizophrenia

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**Submitted**

## Abstract

Schizophrenia (SZ) is a psychiatric disorder with a convoluted etiology that includes cognitive symptoms which arise from amongst others a dysfunctional dorsolateral prefrontal cortex (dlPFC). In our search for the molecular underpinnings of cognitive deficits in SZ, we here performed RNA-sequencing of grey matter from the dlPFC of SZ patients and controls. We found that the differentially expressed RNAs were enriched for mRNAs involved in the Liver X Receptor/Retinoid X Receptor (LXR/RXR) lipid metabolism pathway. Components of the LXR/RXR pathway were upregulated in grey matter, but not in white matter of SZ dlPFC. Intriguingly, an analysis for shared genetic etiology, using two SZ genome-wide association studies (GWASs) and GWAS data for 514 metabolites, revealed genetic overlap between SZ and acylcarnitines, VLDL lipids and fatty acid metabolites that are all linked to LXR/RXR signaling pathways. Furthermore, analysis of structural $T_1$-weighted magnetic resonance imaging and cognitive behavioural data showed that the lipid content of dlPFC grey matter is lower in SZ patients than in controls and correlates with reduced accuracy in the dlPFC-dependent task-switching test. We conclude that aberrations in lipid metabolism, in particular regarding LXR/RXR-signaling, lead to a diminished lipid content in SZ dlPFC that correlates with reduced cognitive performance.

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## Introduction

Schizophrenia (SZ) is a psychiatric disorder with a convoluted etiology and it is thought that an interplay between genetic, epigenetic and environmental risk factors are involved [1]. Symptoms of SZ include positive, negative and cognitive symptoms [2]. The positive symptoms comprise delusions and hallucinations [3], the negative symptoms are a loss of typical affective functions [2] and the most prominent cognitive symptoms of SZ are deficits in attention [4] and executive functioning [5-7]. There are currently no effective pharmacological treatment strategies that target the negative and cognitive symptoms of SZ [8]. The lifetime prevalence of SZ is 0.84%, emphasizing the importance of research into the molecular underpinnings of SZ as a fundament for the development of novel treatment strategies [9].

Cognitive symptoms and related changes in the prefrontal cortex (PFC) of SZ patients are already present before disease onset [10] and contribute negatively to functional outcome [13-16]. Cognitive deficits can be found, albeit to a lesser degree, in individuals at high risk to develop SZ [11] and family members of SZ patients [12]. The various subregions of the PFC are involved in deficits in specific cognitive domains [17]. For example, ventro-lateral PFC functioning remains largely unaffected, while impaired dorso-lateral (dl)PFC-dependent processes are thought to underlie a range of cognitive deficits in SZ [18-20]. In addition dlPFC activation during the performance of cognitive tasks is decreased in SZ patients [19,21,22]. Transcriptomic studies on the PFC of SZ patients have increased our understanding of the molecular mechanisms contributing to the PFC-dependent cognitive impairment in SZ. The majority of transcriptomic studies (RNA sequencing [23-31] or microarray analyses [32,33]) performed on SZ dlPFC were conducted on a mix of grey and white matter. However, grey and white matter display discrete gene expression patterns [34] and therefore investigating the transcriptome of a grey and white matter mix likely complicates the detection of gene expression differences between SZ patients and controls that arise from and are specific to either grey or white matter. Two studies have performed transcriptomic analysis of SZ PFC grey matter, but these did not specify the prefrontal cortical subregion that was used. The first study reported the results for only one downregulated gene, sodium voltage-gated channel alpha subunit 2, which is involved in the propagation of action potentials [35] and the second revealed enrichment of an axon-guidance pathway involving semaphornis and plexins, implicating a role for action potential propagation as well as axon guidance signaling in SZ PFC grey matter [36]. However, spatial differences in gene expression patterns exist throughout the cortex [37] and PFC subregions have distinct contributions to the cognitive deficits in SZ [17]. The only study published to date in which solely the grey matter of the SZ dlPFC was subjected to RNA sequencing has focused on the altered expression of just one gene, namely delta 4-desaturase sphingolipid 2 (DEGS2) [38]. DEGS2 is involved in the synthesis of lipids and a single nucleotide polymorphism in this gene is associated with cognitive performance in SZ [38]. Interestingly, a polygenic risk score analysis has

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revealed that the severity of cognitive deficits is linked to genetic variations in genes involved in retinoid signaling, a pathway that is allied to lipid metabolism [39].

In the current study, we sequenced the transcriptome of the grey matter of dlPFC in SZ and control post-mortem brain tissue. Since we found that the differentially expressed genes were enriched in Liver X Receptor/Retinoid X Receptor (LXR/RXR)-mediated lipid metabolism genes, we next investigated whether SZ has a genetic link with lipid metabolism. We indeed identified shared genetic etiology between SZ and amongst others acylcarnitines, very-low-density lipoprotein (VLDL) lipids and fatty acid metabolites. Finally, exploratory analyses of structural magnetic resonance imaging (MRI) data were in accordance with a lower lipid content of the dlPFC grey matter in SZ patients as compared to controls and correlated with reduced cognitive performance. Thus, distortions in lipid homeostasis play a key role in cognitive symptoms of SZ.

## Results

### RNA-sequencing reveals LXR/RXR activation as the top-enriched canonical pathway in SZ dlPFC grey matter

RNA-sequencing was performed on grey matter dlPFC tissue of four SZ patients and four controls (see Supplementary Table S1 for subject and tissue characteristics). Gene expression density was similar for all samples (Figure 1a) and differential expression analysis revealed 132 significantly upregulated genes and 5 significantly downregulated genes in SZ dlPFC (Figure 1b).

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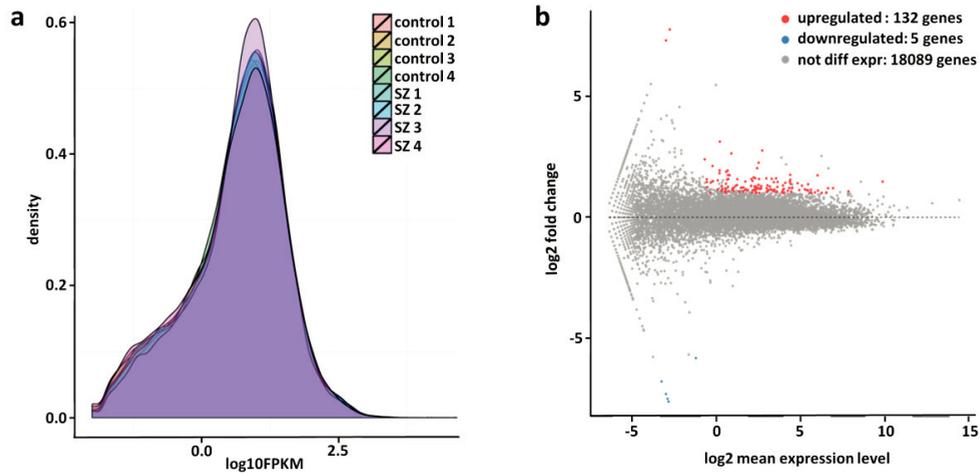
**Figure 1 – mRNA expression profiles of SZ *versus* control dlPFC.** **(a)** Gene expression density profiles over FPKM for all samples. **(b)** Scatter plot for the log2 fold change versus the log2 mean FPKM expression level for all transcripts. Red and blue dots represent significantly (|fold change| > 1 and probability > 0.8) up- and downregulated transcripts, respectively. Grey dots represent transcripts that were not significantly differentially expressed.

Ingenuity pathway analysis of the significantly differentially expressed genes revealed that 'LXR/RXR activation' was the most significantly enriched canonical pathway in the dlPFC of SZ patients (p=3.89E-07 in Benjamini Hochberg corrected T-test; See Table 1 for the top five canonical pathways with statistical values and molecules involved); the other canonical pathways were at least 30 times less enriched. The LXR/RXR pathway regulates cholesterol homeostasis in the brain. We find that in SZ dlPFC grey matter there is an increased abundance of transcripts that are associated with activation of the LXR/RXR pathway, indicating a change in cholesterol metabolism.

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**Table 1 - Ingenuity pathway analysis of genes differentially expressed in SZ versus control dlPFC grey matter.**

| Canonical pathway | P-value (BH corrected) | Genes |
|---|---|---|
| LXR/RXR Activation | 3.89E-07 | AGT, APOC2, C4A/C4B, IL1RL1, S100A8, SERPINA1, TNFRSF11B |
| Antigen Presentation Pathway | 1.43E-05 | HLA-DMA, HLA-DQB1, HLA-DRB3, HLA-DRB5 |
| Complement System | 1.15E-05 | C1QA, C1QB, C1QC, C4A/C4B |
| PD1-PD-L cancer immunotherapy pathway | 5.45E-05 | HLA-DMA, HLA-DQB1, HLA-DRB3, HLA-DRB5, TNFRSF11B |
| T Helper Cell Differentiation | 1.71E-04 | HLA-DMA, HLA-DQB1, HLA-DRB5, TNFRSF11B |

Quantitative polymerase chain reaction (qPCR) confirmed upregulation of angiotensinogen (*Agt*), apolipoprotein C2 (*Apoc2*) and complement 4b (*C4b*) components of the 'LXR/RXR activation' canonical ingenuity pathway in SZ *versus* control dlPFC grey matter (Figure 2a; Independent samples T-test t=2.407, p=0.053, df=6, t=2.673, p=0.056, df=3.986, t=2.155, p=0.083, df=3.059, respectively; see Supplementary Table S2 for primer sequences). We next investigated whether other mRNAs related to LXR/RXR activation were also differentially expressed in SZ dlPFC grey matter. LXRβ is the isoform of LXR that is expressed most abundantly in the brain and LXRβ forms heterodimers with RXRβ [40]. We found an upregulation of *Rxr*β, but no changes in the mRNA expression of *Lxr*β in the SZ dlPFC grey matter as compared to controls (Figure 2b; Independent samples T-test *Rxr*β t=2.202, p=0.070, df=6, *Lxr*β t=0.156, p=0.885, df=3.378). The LXRβ/RXRβ pathway activates the transcription factor sterol regulatory element-binding proteins (e.g. SREBP1) and as such stimulates cholesterol and oxysterol efflux from the cell via ATP-binding cassette transporter A1 (*Abca1*), which is regulated by peripheral myelin protein 22 (*Pmp22*) [40-42]. Upon efflux from the cell, cholesterol is packed in the brain in high-density lipoprotein (HDL)-like particles containing apolipoproteins, predominantly apolipoprotein E (ApoE) [40]. qPCR analysis shows that there is no difference in the mRNA expression of *Srebp1* and *Apoe*, although there was an upregulation of *Abca1* and *Pmp22* in SZ versus control dlPFC grey matter (Figure 2b; Independent samples T-test *Srebp1* t=1.047, p=0.335, df=6, *Apoe* t=1.606, p=0.206, df=3.032, *Abca1* t=3.836, p=0.023, df=3.538, *Pmp22* t=2.219, p=0.068, df=6), indicating increased cholesterol efflux in SZ dlPFC grey matter. Notably, in the dlPFC white matter no changes in LXR/RXR-related mRNA expression were found (Figure 2c and 2d), highlighting the importance of studying mRNA expression patterns in the grey and white matter separately.

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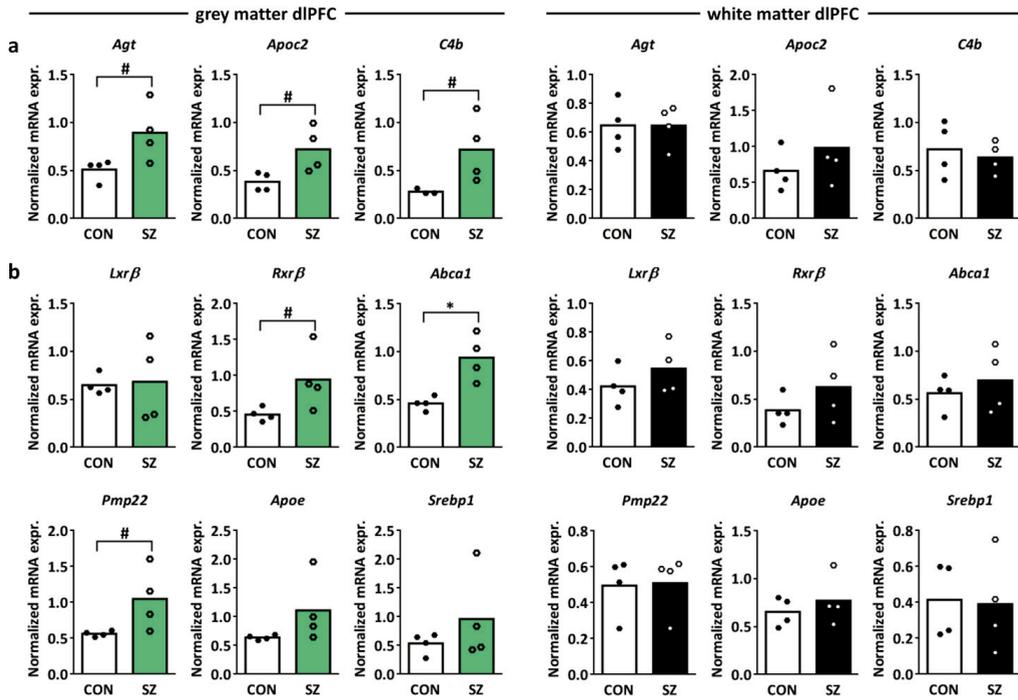
**Figure 2 – mRNA expression of LXR/RXR-related mRNAs in SZ versus control dlPFC grey and white matter. (a)** Normalized mRNA expression of angiotensinogen (*Agt*), apolipoprotein C2 (*Apoc2*) and complement 4b (*C4b*) in SZ *versus* control dlPFC grey matter. These mRNAs were components of the canonical pathway 'LXR/RXR activation' in the Ingenuity pathway analysis. **(b)** Normalized mRNA expression of components of the LXR/RXR signaling cascade Liver X receptor β (*Lxr*β), retinoid X receptor β (*Rxr*β), ATP-binding cassette transporter A1 (*Abca1*), peripheral myelin protein 22 (*Pmp22*), apolipoprotein E (*ApoE*), and sterol regulatory element-binding protein 1 (*SREBP1*) in SZ *versus* control dlPFC grey matter. **(c)(d)** Normalized mRNA expression of the same genes in the dlPFC white matter. n=4 samples per group, #p<0.1 *p<0.05 in Independent samples T-test.

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In addition to the LXR/RXR activation pathway, the canonical pathway analysis also revealed significant enrichment of the "Antigen Presentation Pathway" and the "Complement System" pathways in SZ dlPFC grey matter (Supplementary Table S1; p=1.43E-05, p=1.15E-05 respectively). These findings are in line with previously published results showing that mRNA expression of genes related to inflammation and the immune system are dysregulated in SZ PFC [26,43]. In addition, the top upstream regulator in the Ingenuity pathway analysis was IFNγ (Supplementary Table S3; p=2.22E-16), and this and other proinflammatory cytokines are associated with SZ [44-46].

## Shared genetic etiology between SZ and lipid metabolism

To further investigate the role of lipid metabolism in SZ, we analysed the shared genetic etiology between SZ and 514 circulating metabolites, including amino acids, nutrients, organic compounds,  lipids, cytokines and growth factors. Following Bonferroni correction (Table 2; Supplementary Table S4), we found significant overlap between genetic risk for SZ and 35 metabolites (Table 2; Supplementary Table S4; p<0.05) using the results of the SZ GWAS study published by the Bipolar and Schizophrenia working group of the Psychiatric Genomics Consortium in 2018 [47] . The genetic association between SZ risk and 25 of the 35 metabolites was replicated using a second SZ GWAS dataset provided by the Psychiatric Genomics Consortium in 2014 [48] and we identified 21 additional metabolites that share genetic etiology with SZ (Table 2; Supplementary Table S5). Strikingly, the 56 metabolites that share significant genetic etiology with SZ are all related to lipids (except for IP10 and IL16) and fall within three themes: acylcarnitines, VLDL lipids and fatty acid metabolites. We conclude that disruptions in lipid homeostasis are genetically associated with SZ. In addition, we found shared genetic etiology of SZ with two immune-related metabolites, IP10 and IL16 (Table 2), in line with the involvement of the immune system in SZ.

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Table 2 – Metabolites that share significant genetic etiology with SZ.

| Metabolite | Lowest significant p-value treshold | Bonferroni-corrected p-value | Lowest significant p-value treshold | Bonferroni-corrected p-value |
|---|---|---|---|---|
| | SZ GWAS 2018 reference 48 | | SZ GWAS 2014 reference 49 | |
| C5.1.DC[1] | 0.1 | 0.000246 | 0.05 | 0.015176 |
| IP10[5] | 0.3 | 0.000997 | 0.05 | 0.048767 |
| CH2.DB.ratio[2] | 0.1 | 0.00153 | 0.1 | 0.014121 |
| LPE16_0_LIPID[4] | 0.001 | 0.001988 | 0.001 | 0.000768 |
| XS.VLDL.TG[3] | 0.2 | 0.002406 | 0.3 | 0.039835 |
| C14.1.OH[1] | 0.05 | 0.002534 | 0.05 | 0.008502 |
| DB.in.FA[2] | 0.1 | 0.003962 | 0.1 | 0.010729 |
| XS.VLDL.P[3] | 0.3 | 0.004685 | 0.2 | 0.008044 |
| IDL.C[3] | 0.05 | 0.006454 | n/a | n/a |
| PC38_2_LIPID[4] | 0.3 | 0.007062 | 0.2 | 0.000573 |
| CH2.in.FA[2] | 0.2 | 0.008944 | 0.1 | 0.006678 |
| Bis.DB.ratio[2] | 0.2 | 0.009453 | 0.05 | 0.018628 |
| DHA[2] | 0.4 | 0.010114 | 0.05 | 0.00935 |
| SM.C26.0[4] | 0.1 | 0.010245 | 0.05 | 0.002634 |
| XS.VLDL.L[3] | 0.05 | 0.010881 | 0.2 | 7.49E-05 |
| TAG54_6_LIPID[4] | 0.001 | 0.016009 | 0.001 | 0.009793 |
| SM.OH.C24.1[4] | 0.1 | 0.017865 | 0.05 | 0.006078 |
| FAw3[4] | 0.05 | 0.01807 | 0.05 | 0.002175 |
| fumarate_maleate_valerat_CMH | 0.05 | 0.019523 | 0.05 | 0.001032 |
| Ratio_PC3806_LPC2206_LIPID[4] | 0.05 | 0.023256 | n/a | n/a |
| IDL.FC[3] | 0.4 | 0.024663 | n/a | n/a |
| LPC20_3_LIPID[4] | 0.3 | 0.026304 | n/a | n/a |
| XS.VLDL.PL[3] | 0.05 | 0.027834 | 0.2 | 0.00178 |
| XL.VLDL.TG[3] | 0.3 | 0.032077 | 0.3 | 0.001178 |
| PC32_0_LIPID[4] | 0.1 | 0.032928 | 0.1 | 0.000627 |
| PC.ae.C44.3[4] | 0.1 | 0.034516 | n/a | n/a |
| IDL.L[3] | 0.1 | 0.034726 | n/a | n/a |
| S.VLDL.C[3] | 0.4 | 0.035649 | 0.4 | 0.047852 |
| IDL.P[3] | 0.1 | 0.036891 | n/a | n/a |
| lysoPC.a.C20.4[4] | 0.3 | 0.039526 | 0.2 | 0.019734 |
| Bis.FA.ratio[2] | 0.1 | 0.040777 | 0.05 | 0.005426 |

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| | | | | |
|---|---|---|---|---|
| S.VLDL.L[3] | 0.5 | 0.0408 | n/a | n/a |
| GROa | 0.001 | 0.042828 | n/a | n/a |
| MCP1 | 0.001 | 0.045366 | 0.001 | 0.001432 |
| LPC22_6_LIPID[4] | 0.1 | 0.048797 | n/a | n/a |
| Cit | n/a | n/a | 0.05 | 0.00031 |
| PCB36_4_LIPID[4] | n/a | n/a | 0.05 | 0.000894 |
| PC38_6_LIPID[4] | n/a | n/a | 0.1 | 0.002651 |
| CE20_5_LIPID[3] | n/a | n/a | 0.2 | 0.002948 |
| TAG56_6_LIPID[4] | n/a | n/a | 0.2 | 0.005974 |
| PC40_6_LIPID[4] | n/a | n/a | 0.3 | 0.008719 |
| PC.aa.C24.0[4] | n/a | n/a | 0.1 | 0.010428 |
| TAG56_8_LIPID[4] | n/a | n/a | 0.2 | 0.012203 |
| IL16[5] | n/a | n/a | 0.05 | 0.01252 |
| TAG58_10_LIPID[4] | n/a | n/a | 0.3 | 0.014247 |
| TAG56_6_LIPID[4] | n/a | n/a | 0.3 | 0.020381 |
| XXL.VLDL.PL[3] | n/a | n/a | 0.3 | 0.027232 |
| L.VLDL.P[3] | n/a | n/a | 0.05 | 0.030895 |
| XL.HDL.L[3] | n/a | n/a | 0.5 | 0.032042 |
| aconitate_CMH | n/a | n/a | 0.1 | 0.032102 |
| XL.VLDL.L[3] | n/a | n/a | 0.4 | 0.03703 |
| TAG58_11_LIPID[4] | n/a | n/a | 0.3 | 0.037887 |
| PC38_4_LIPID[4] | n/a | n/a | 0.4 | 0.038142 |
| LDL.D[3] | n/a | n/a | 0.001 | 0.042824 |
| FAw6[4] | n/a | n/a | 0.05 | 0.044601 |
| ADP_CMH | n/a | n/a | 0.4 | 0.049512 |

[1]acylcarnitines [2]fatty acids [3]cholesterols [4]other lipids [5]immune-related cytokines

### Lipid content in SZ dlPFC grey matter is lower than in controls and correlates with reduced accuracy in the task-switching test

We further investigated the effect of disrupted lipid homeostasis in SZ dlPFC using a publicly available dataset from the Consortium for Neuropsychiatric Phenomics. This dataset contains amongst others structural MRI scans and performance in the task-switching cognitive test of 50 SZ patients and 125 control individuals [49]. From this dataset, we analyzed the $T_1$-weighted Magnetization Prepared Rapid Gradient Echo (MP-RAGE) signal. The macromolecular pool in the brain consists mainly of lipids, as illustrated by the typical grey-white matter contrast obtained in $T_1$-weighted MRI scans. The $T_1$ inversion pulse saturates the free-water pool and the macromolecule pool. Following

the saturation, the macromolecular pool quickly relaxes and subsequently accelerates the relaxation of the free-water pool in a process termed magnetization transfer. We hypothesized that a difference in lipid content and thus macromolecular pool, would contribute to a change in magnetization transfer. We tested this by comparing the dlPFC grey matter MP-RAGE signal between SZ patients and controls. However, we were not able to distinguish between the contributions of magnetization transfer, spin density and inherent free-water relaxation rate to the MP-RAGE signal. We found that the MP-RAGE signal was significantly decreased in the dlPFC grey matter of SZ patients as compared to controls, both in the left and right hemispheres, and accounting for age, sex, motion and scanning site (Figure 3a; Supplementary Table S6; linear model left dlPFC estimate=-26.025, t=-4.433, p<0.001, right dlPFC estimate=-25.249, t=-4.319, p<0.001; Supplementary Figure 1). These results are in accordance with a lower macromolecular content and thus a lower lipid content of the SZ dlPFC grey matter. Notably, we found a correlation between the accuracy on the dlPFC dependent task-switching test and the MP-RAGE signal in both the left and right dlPFC accounting for age and motion (Figure 3b; linear model left dlPFC estimate=4.286 , t=1.946, p=0.0579, right dlPFC estimate=4.330, t=1.969, p=0.0551). These data are in accordance with a lower lipid content of the SZ dlPFC grey matter that correlates with a reduced accuracy in the dlPFC-dependent task-switching test and as such confirms the importance a distorted lipid metabolism in cognitive deficits in SZ.

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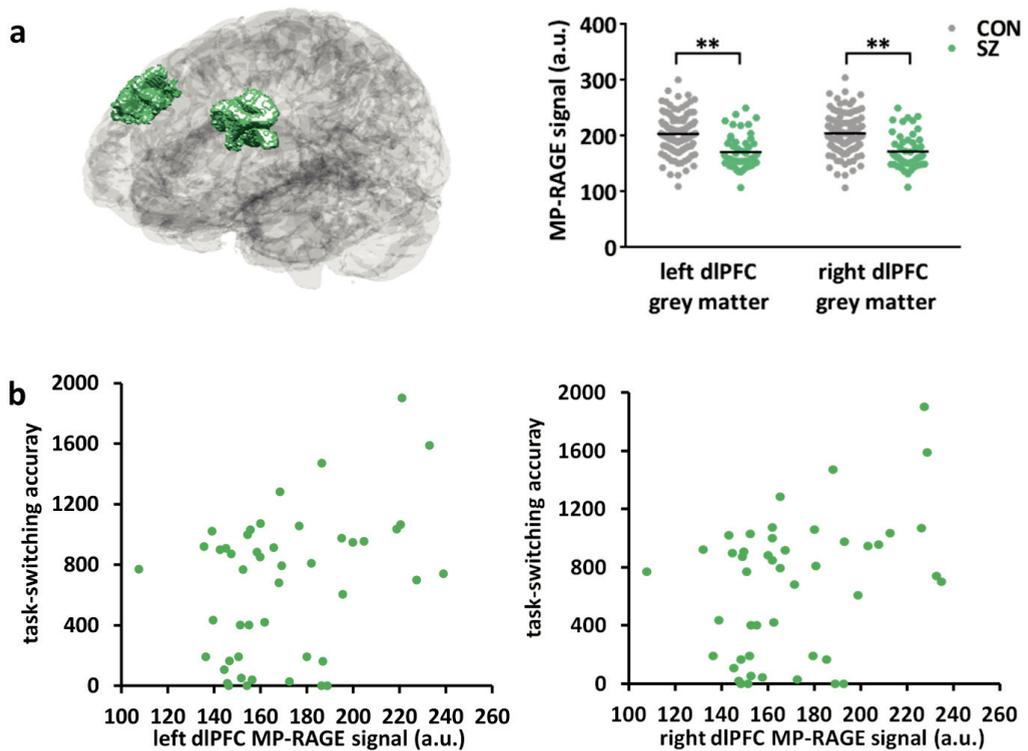
**Figure 3 – MP-RAGE signal in SZ and control dlPFC grey matter and correlation with task-switching accuracy.** **(a)** Schematic representation of the dlPFC grey matter in the left and right brain hemispheres. Average MP-RAGE signal from the left and right dlPFC grey matter in SZ *versus* control (CON). **p<0.001 in a linear model. **(b)** Scatterplot of the accuracy on the task-switching test and the MP-RAGE signal from the left and right hemisphere dlPFC grey matter in SZ patients.

## Discussion

SZ is a psychiatric disorder with an unknown etiology. Cognitive deficits in SZ patients are associated with the dlPFC and here we performed RNA sequencing of post-mortem dlPFC grey matter of SZ patients and controls to gain insight into the molecular mechanisms contributing to cognitive dysfunction in SZ. We found an enrichment of differentially expressed genes in the LXR/RXR activation pathway and validated upregulation of components of the LXR/RXR lipid metabolism pathway in SZ dlPFC grey, but not white, matter. We further revealed shared genetic etiology between SZ and a number of lipid-related metabolites, confirming a genetic link between SZ and lipid metabolism, and using the MP-RAGE signal from structural MRI data we found results that are in accordance with a reduced lipid content in the dlPFC grey matter of SZ patients correlating with reduced performance in the task-switching cognitive test.

Grey and white matter have a different cellular composition and function, and distinct transcriptomes [34]. Grey matter of the cortex consists mainly of neurons and glial cells, while the white matter consists primarily of myelinated axons. Therefore, RNA sequencing results from a mix of grey and white matter inevitably represent combined findings. RNA sequencing studies on such mixes of grey and white matter from the dlPFC of SZ patients have shown amongst others altered abundance of transcripts involved in glucocorticoid signaling [30], presynaptic function [33], inflammation [26], nuclear receptor signaling [24], synaptic vesicle recycling, transmitter release and cytoskeletal dynamics [32]. Our RNA sequencing results from the SZ dlPFC grey matter confirm the dysregulation of inflammation-related genes. Among the genes differentially expressed in grey matter of SZ dlPFC *versus* controls, we further found an enrichment of genes involved in LXR/RXR-mediated cholesterol lipid homeostasis. LXR/RXR-related genes were upregulated in the dlPFC grey matter of SZ patients, but remained unaltered in the dlPFC white matter. The previous transcriptomic studies on a mix of dlPFC grey and white matter have likely missed this enrichment because of discord introduced by the high lipid content of the white matter.

The LXR/RXR pathway is activated by binding of oxysterols to LXR. Oxysterols are metabolites that are produced during the breakdown of cholesterol and cross the blood brain barrier. In the brain, LXRβ forms hetrodimers with RXRβ and their activation leads to increased efflux of cholesterol via ABCA1- and PMP22-regulated mechanisms into HDL-like particles containing apolipoprotein, inhibited uptake of cholesterol into the cell and stimulated fatty acid synthesis[40-42]. We indeed found upregulation of *Rxrβ, Apoc2*, *Abca1* and *Pmp22* in SZ dlPFC grey matter as compared to controls. The role of LXR activation in SZ has not been extensively studied. However, LXR signaling is involved in the development of ventral midbrain dopaminergic neurons [40,50] and there is a genetic association between PMP22 and SZ [51]. *In vitro* studies have shown contradictory effects of antipsychotics on LXR signaling in that one study reported an increased mRNA expression of *Abca1* and *Apoe* [52], while a second study showed that antipsychotics reduce

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cholesterol synthesis and export from the endoplasmatic reticulum and do not induce LXR activation [53]. Nevertheless, a disturbance of LXR-mediated cholesterol homeostasis appears to have a role in SZ etiology, but further studies are necessary.

A number of links exist between SZ and distorted lipid homeostasis. For example, a meta-analysis has revealed that metabolic syndrome in SZ patients, a condition in which cholesterol and triglyceride levels are abnormal, is associated with a high degree of cognitive impairment [54]. Metabolic syndrome also impairs cognition in otherwise healthy individuals [55]. Blood triglyceride levels are correlated with positive symptom severity and blood HDL levels to global functioning of SZ patients [56]. Unmedicated SZ patients have lower total cholesterol, HDL and apolipoprotein levels [56,57], and lower short chain acylcarnitine levels in the blood [58]. Moreover, in SZ patients using antipsychotic medication the occurrence of metabolic syndrome is increased and cholesterol levels are correlated with cognitive impairment [55,59], implicating a role for peripheral lipid homeostasis in brain functioning and cognitive deficits in SZ. In the present study, we find that SZ shares genetic etiology with a number of metabolites, most of which we could replicate using a second SZ GWAS study. Among the metabolites that share genetic etiology with SZ, we found an enrichment of acylcarnitines, VLDL lipids and fatty acid metabolites. This finding confirms a genetic contribution to alterations in lipid homeostasis in SZ and indicates that such alterations are at least partially genetically determined and thus intrinsic to the disorder and not solely caused by antipsychotic treatment.

Notably, acylcarnitines, fatty acid production, cholesterol efflux into HDL-like particles and LXR/RXR activation share a common molecular pathway (Figure 4). During fatty acid oxidation unsaturated fatty acids esterify with acyl-CoA to form acylcarnitine that is subsequently transported into the mitochondrial inner membrane. Once inside the inner mitochondrial membrane, acylcarnitines are subjected to beta-oxidation which produces acetyl-coA which can either enter the citric acid cycle, or is transported to the cytosol where it participates in lipid biosynthesis (fatty acid as well as cholesterol synthesis). Cholesterol can be transported out of the cell via HDL-like particles. Based on our transcriptomic study as well as the shared genetic etiology between SZ and several lipid-related metabolites, we conclude that lipid homeostasis involving fatty acid oxidation and cholesterol efflux, production and transport may well play a role in SZ.
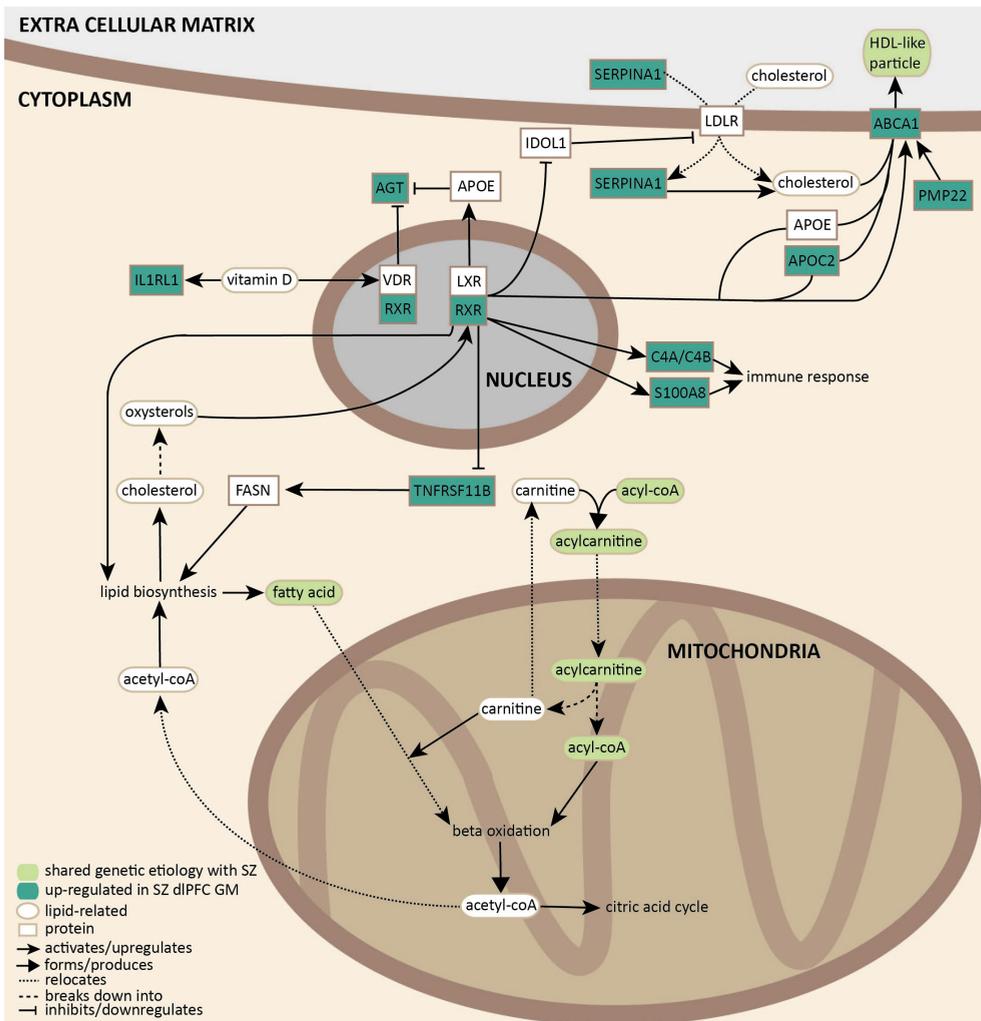
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**Figure 4 –Molecular pathways involving LXR/RXR signaling, acylcarnitines, VLDL lipids and fatty acid metabolites.** Dark green rectangles represent genes upregulated in SZ dlPFC. Light green ovals represent lipid-related metabolites that share significant genetic etiology with SZ. Arrows indicate the nature of molecular interactions (see legend in Figure 4 for details). References [39,42,60-70] have been used to construct the molecular pathways.

Using a publicly available dataset [49], we show that the $T_1$-weighted MP-RAGE signal is significantly decreased in the SZ dlPFC grey matter. The $T_1$-weighted MP-RAGE signal creates contrast between grey and white matter, which is thought to be due to magnetization transfer effects, where increased lipid content results in increased signal. Our results therefore are in accordance with decreased lipid content in the dlPFC of SZ patients. This interpretation discards added effects from differences in spin density or

in the relaxation rate of the free water pool itself to the MP-RAGE signal. However, the relaxation rate of the free water pool itself is largely homogeneous across the brain [71,72]. Nevertheless, our interpretation of decreased lipid content in the SZ dlPFC grey matter warrants future validation with quantitative magnetization transfer methods. Notably, decreased MP-RAGE signal in SZ dlPFC grey matter correlates with decreased accuracy in the task-switching test in SZ patients. The task-switching test examines executive functioning and relies on the dlPFC [73-75]. In SZ patients, reduced accuracy [76] as well as reaction time differences [77] in this test have been reported. We here show that in SZ altered performance in the task-switching test in SZ might arise from a decreased lipid content in the dlPFC.

About half of the dry weight of the brain is attributed to lipids and 80% of brain lipids are part of myelin sheaths. In SZ PFC, abnormalities in myelination are evident and decreased PFC myelin content contributes to disease symptomatology [78]. Furthermore, polyunsaturated fatty acid levels in the blood are correlated with white-matter integrity in frontal regions of the SZ brain [79], increased LDL levels are associated with white-matter alterations [80] and white matter as well as myelin abnormalities in the PFC contribute to cognitive deficits in SZ [78]. Moreover, myelin lipids are produced by oligodendrocytes (OLs) and LXRβ knockout mice show a hypomyelination phenotype because cholesterol deficiency inhibits OL differentiation and myelination [81,82]. During brain development LXRβ is also involved in the formation of OL precursor cells [83] and LXRβ exerts transcriptional control over myelin-related genes [84].

Taken all findings together, the SZ-associated disturbances in lipid homeostasis may mediate the myelination deficits and as such contribute to the etiology of the cognitive symptoms of SZ.

## Methods

### Samples and RNA-sequencing

Human post-mortem brain tissue was obtained from the Dutch Brain Bank. Four samples of chronic SZ patients and four samples of controls from the dlPFC in the middle frontal gyrus were obtained. From each tissue sample a 300 μm section was obtained in a cryostat (Leica) at -15 °C and 2-3 punches were collected using a 2.00-mm punch needle (Harris) from different places in the grey matter. Punches were frozen on dry ice and stored at -80 °C until RNA isolation. RNA isolation was performed using RNeasy lipid tissue mini kit (74804 Qiagen) and isolated RNA was sent for quality control, RNA sequencing and bioinformatics data analysis to BGI Genomics. Agilent 2100 Bio Analyzer was used to determine RNA quality and RIN values of all RNA samples were 6.7 or higher. RNA sequencing was performed using BGISEQ-500 platform generating 6.71 Gb bases per sample. Using HISAT clean reads were mapped to the reference genome UCSC HG38  with an average of 92.06% mapped reads. Gene expression levels (FPKM) were

calculated using RSEM and NOIseq algorithms were then used to determine differentially expressed genes between SZ patients and controls. Significantly differentially expressed genes (probability>0.8) were used for analysis with the Ingenuity Pathway Analysis software package (Qiagen) to identify overrepresented canonical pathways.

## Quantitative real-time PCR

For quantitative real-time PCR (qPCR) analysis, 350 µg RNA per sample was treated with DNase I (Fermentas) and cDNA was synthesized using the Revert Aid H-minus first strand cDNA synthesis kit (Thermo Scientific). cDNA was subsequently diluted 1:20 in MilliQ $H_2O$ and stored at -20 °C until qPCR analysis. qPCR samples contained 2.0 µL diluted cDNA, 0.8 µL 5 µM forward primer, 0.8 µL 5 µM reverse primer, 5 µL SybrGreen mix (Roche) and 1.8 µL MilliQ $H_2O$. qPCR was performed with a Rotor Gene 6000 Series (Corbett Life Sciences) using a 3-step paradigm with a fixed gain of 8. Fifty cycling steps of 95, 60, and 72 °C were applied, and fluorescence was acquired after each cycling step. Primers were designed with NCBI Primer-Blast and synthesized by Sigma (for primer pair sequences, see Supplementary Table 4). Melting temperature was used to check whether a single PCR product was generated and the take off and amplification values of the housekeeping genes (*Ppia* and *Gapdh*) were used to determine the normalization factor with GeNorm [43] after which normalized mRNA expression levels were calculated.

## Shared genetic etiology

Two SZ GWAS and four metabolite GWAS datasets were used to calculate shared genetic etiology between SZ and metabolite levels. We first calculated shared genetic etiology between 561 metabolites and SZ using previously published SZ GWAS data that was obtained from 33,426 SZ patients from European ancestry [47]. We then replicated the calculation using a second SZ GWAS data set, namely the GWAS data from 36,989 SZ patients as provided by the Psychiatric Genomics Consortium [48] that includes the same patients from European descent, but also includes individuals with East-Asian ancestry. The metabolite GWAS data was obtained from Rhee et al. (2013) [91] including 268 metabolite GWASs, Draisma et al. (2016) [92] including 129 metabolite GWASs, Kettunen et al. (2016) [93] including 123 metabolite GWASs and Ahola-Olli et al. (2017) [94] including 41 cytokine GWASs and included 2076, 7,478 and 24,925 participants of European decent, and 2019 Finnish participants respectively. Shared genetic etiology was calculated using PRSice [85] based on the method of Johnson et al. 2013 [86]. Metabolite GWAS data were taken as base samples, and SZ GWAS data as the target sample, and correlation results were weighted by the SZ group size. Using PRSice, SNPs were clumped to remove linkage disequilibrium (LD) with a LD threshold of 0.1, a distance threshold of 250Kb and the 1000 Genomes Project data as genotype reference [87]. A range of SNP significance thresholds was used ($p_T$ < 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5) to calculate shared genetic etiology and the p-values obtained using those thresholds

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were corrected with Bonferroni multiple comparisons correction for the number of metabolites tested.

## Analysis of the dlPFC grey-matter MP-RAGE signal and correlation with task-switching accuracy

The Consortium for Neuropsychiatric Phenomics made available an MRI dataset including 125 healthy individuals (median age = 28 years old, 53% female) and 50 individuals (median age = 37.5 years old, 76% female) diagnosed with SZ or schizoaffective disorder. This dataset includes amongst others a $T_1$-weighted MP-RAGE sequence (TR=1.9 s, TE=2.26 ms, FOV=250 mm, matrix=256×256, slice thickness=1 mm, 176 slices) as well as cognitive behavioural data from the task-switching test. For details on the dataset, see [49]. The MP-RAGEs were corrected for $B_0/B_1$ inhomogeneities using the N4 algorithm. A study-specific template of the MP-RAGE scans was created in the common space between the scans with an iterative diffeomorphic warp estimate using the ANTS package [88]. The template was diffeomorphically registered to the MarsAtlas [89]. A segmentation of the dlPFC was extracted from the atlas and projected to each individual scan. The dlPFC regions-of-interest were corrected at the individual level with a gray-matter mask made with FSL-FAst and the output was visually verified. The average MP-RAGE signal in the dlPFC grey matter of SZ patients and controls was examined. Two linear models were fitted including the average left and right grey-matter dlPFC MP-RAGE signal as the dependent variable and age, sex and group as the independent variables. Given that a SZ patient may move more in an MRI environment which affects the signal intensity, a retrospective motion-estimate (Average Edge Strength; AES) was also calculated with the homonymous Matlab toolbox [90] and entered as an independent variable. Because the MRI-data were acquired with 2 separate 3T scanners (Trio, Siemens Healthineers), the analyses were repeated for data acquired at each scanner. We then utilized a linear model to analyse the correlation between dlPFC grey matter MP-RAGE signal and accuracy in the task-switching test in SZ patients accounting for age and motion. For details on the task-switching test, see [49].

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5

# Supplementary Material

**Supplementary Table S1–** Patient and tissue characteristics

| Group | Sex | Age | Post-mortem delay | pH |
|---|---|---|---|---|
| Control | Male | 71 | 05:45 | unknown |
| Control | Female | 78 | 07:10 | 6.32 |
| Control | Male | 75 | 07:10 | 6.45 |
| Control | Female | 83 | 06:40 | 6.55 |
| SZ | Male | 67 | 05:45 | 6.29 |
| SZ | Female | 63 | 05:00 | 6.50 |
| SZ | Female | 79 | 04:45 | 6.34 |
| SZ | Female | 55 | 09:50 | 6.82 |

**Supplementary Table S2** – Primer sequences of primers used for qPCR.

| Gene | Primer sequence 5' to 3' |
|---|---|
| APOC2 forward | ACACTATGGGCACACGACTC |
| APOC2 reverse | GGTCCCCTGGACCTCAAATC |
| C4B forward | CATAGGAGCATGCAGGGGG |
| C4B reverse | TGATGAAGGGCGATGGTCAC |
| AGT forward | GATGGAAGACTGGCTGCTCC |
| AGT reverse | AAGCCCTTCATCTTCCCTTGG |
| LXRb forward | CTGTAAAGGAGGAGGGTCCG |
| LXRb reverse | ATCTGGGATGACCCAGTCTGT |
| RXRB forward | CCTCCTTCTCACACCGATCC |
| RXRB reverse | CCTGGAGAGGGACCGATCAA |
| APOE forward | GGGCCTCTAGAAAGAGCTGG |
| APOE reverse | CTTGGCCTGGCATCCTGC |
| ABCA1 forward | ACTTGGTGGGACGAAACCTC |
| ABCA1 reverse | TACAGGTCTGGGCCTGATGA |
| SREBP1 forward | CTGACCGACATCGAAGGTGA |
| SREBP1 reverse | AAGTGCAATCCATGGCTCCG |
| PMP22 forward | TCACCAAACGAATGGCTGC |
| PMP22 reverse | GATGTAAAACCTGCCCCCCTT |

5

**Supplementary Table S3 -** Top upstream regulators in Ingenuity pathway analysis of SZ versus control dlPFC.

| Upstream regulator | P-value |
| --- | --- |
| IFNG | 2.22E-16 |
| Dexamethasone | 1.05E-12 |
| TNF | 1.68E-11 |
| FOS | 2.10E-08 |
| IL1B | 8.21E-08 |

**Supplementary Table S4** - Metabolites that share significant genetic etiology with SZ using SZ GWAS from 2018 [47] as target.

Table can be accessed here:
https://drive.google.com/open?id=1QbdMSOyIDgYJmLgkR3tdQOtad9Zn-jKP

**Supplementary Table S5** - Metabolites that share significant genetic etiology with SZ using SZ GWAS from 2014 [48] as target.

Table can be accessed here:
https://drive.google.com/open?id=1QbdMSOyIDgYJmLgkR3tdQOtad9Zn-jKP

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**Supplementary Table S6 -** : Linear model explaining variance in left and right dlPFC grey matter MP-RAGE signal for both scanning sites.

| Coefficients | Estimate | t-value | p-value | 95% CI |
|---|---|---|---|---|
| Left dlPFC grey matter | | | | |
| SZ > Healthy controls | -26.025 | -4.433 | < 0.001 | [-37.616. -14.434] |
| AES | 7226.472 | 2.057 | 0.041 | [291.293, 14161.651] |
| Age | 0.569 | -1.983 | 0.049 | [-1.135, 0.003] |
| Sex | 0.727 | 0.118 | 0.906 | [-12.858, 11.404] |
| Right dlPFC grey matter | | | | |
| SZ > Healthy controls | -25.249 | -4.319 | < 0.001 | [-36.79. -13.707] |
| AES | 6.877.356 | 1.966 | 0.051 | [-28.15, 13782.861] |
| Age | 0.637 | -2.230 | 0.027 | [-1.2, 0.073] |
| Sex | 0.592 | 0.097 | 0.923 | [-12.671, 11.487] |
| Left dlPFC grey matter – scanning site 1 | | | | |
| Schizophrenic > Normal | -34.179 | -2.869 | 0.008 | [-58.542, -9.817] |
| AES | 6342.165 | 0.586 | 0.563 | [-15806.928, 28491.258] |
| Age | 0.104 | 0.137 | 0.892 | [-1.453, 1.662] |
| Sex | -17.290 | -1.130 | 0.268 | [-48.579, 13.999] |
| Right dlPFC grey matter – scanning site 1 | | | | |
| Schizophrenic > Normal | -35.157 | -2.981 | 0.006 | [-59.275. -11.038] |
| AES | 5.164.139 | 0.482 | 0.634 | [-16763.375. 27091.653] |
| Age | 0.001 | 0.002 | 0.998 | [-1.543, 1.54] |
| Sex | -16.044 | -1.059 | 0.298 | [-47.02, 14.932] |
| Left dlPFC grey matter – scanning site 2 | | | | |
| Schizophrenic > Normal | -30.376 | -2.959 | 0.004 | [-50.789. -9.964] |
| AES | 10487.644 | 2.328 | 0.022 | [1530.604. 19444.683] |
| Age | 0.699 | -1.693 | 0.094 | [-1.521, 0.122] |
| Sex | 0.164 | 0.019 | 0.985 | [-17.327, 16.998] |
| Right dlPFC grey matter – scanning site 2 | | | | |
| Schizophrenic > Normal | -29.820 | -2.896 | 0.005 | [-50.297, -9.344] |
| AES | 10553.526 | 2.336 | 0.022 | [1568.473, 19538.579] |
| Age | 0.684 | -1.651 | 0.103 | [-1.508. 0.14] |
| Sex | 0.843 | 0.097 | 0.923 | [-18.059, 16.373] |

5
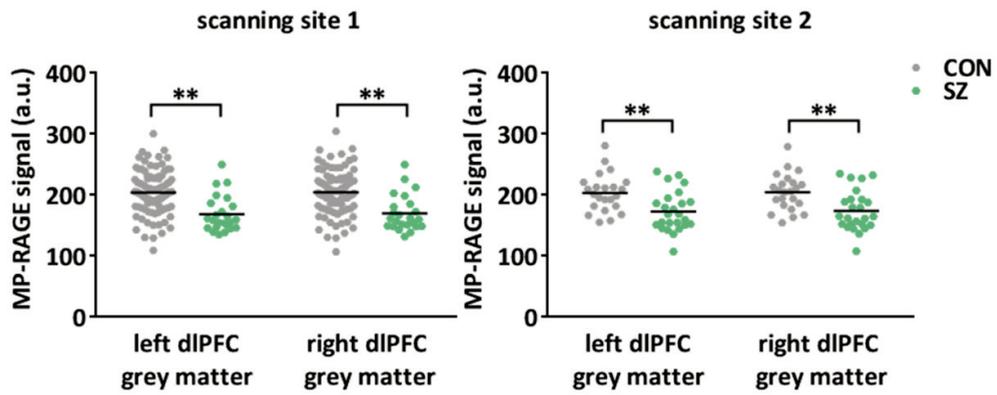
**Supplementary Figure S1 – Average MP-RAGE signal in SZ versus control dlPFC grey matter in the left and right hemispheres and at both scanning sites**. **p<0.001 in a linear model.

5
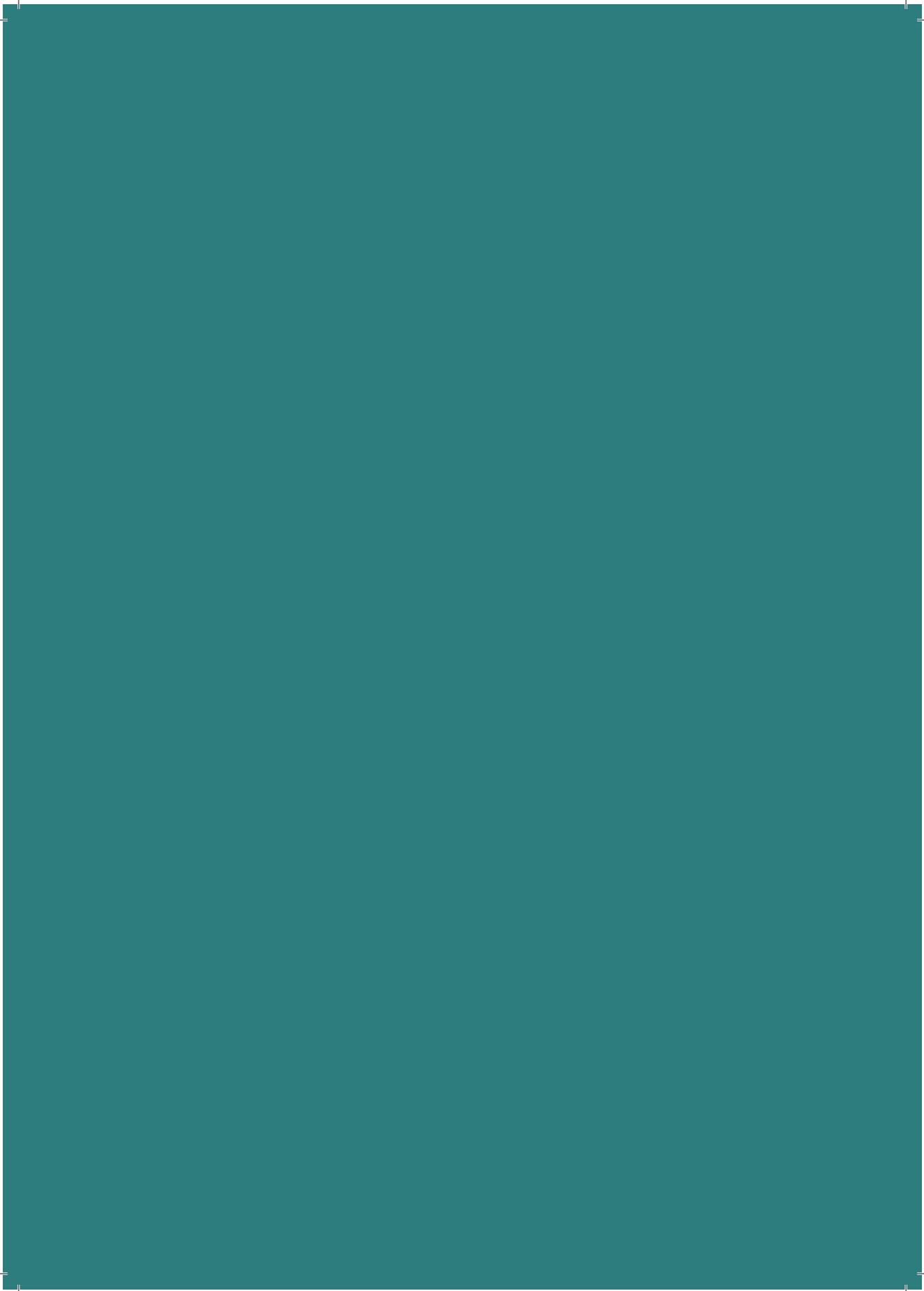
# 6

## General Discussion

Schizophrenia (SZ) is a severe psychiatric disorder with a convoluted etiology that includes amongst others positive, negative and cognitive symptoms [1]. The positive symptoms comprise delusions and hallucinations [2] and are reasonably well-treated with antipsychotic medication. The negative symptoms of SZ represent loss of typical affective functions [1] and cognitive symptoms of SZ include deficits in attention [3] and executive functioning [4-6]. Cognitive symptoms of SZ remain currently untreated and their etiology remains unknown [7]. Nevertheless, the degree of cognitive impairment in SZ is associated with functional outcome [8-14]. Therefore, neurobiological insights into the underpinnings of cognitive symptoms of SZ are necessary to fuel the development of novel treatment strategies that target these symptoms. In Chapters 1 and 2 of this thesis I have outlined my hypothesis concerning the neurobiology of cognitive deficits in SZ. During adolescence, the critical period for prefrontal cortex (PFC) myelination, the build-up of oxidative stress in oligodendrocytes (OLs) is such that efficient maturation of OL precursor cells (OPCs) into myelinating OLs is affected, leading to hypomyelination of the PFC that ultimately hinders proper cognitive functioning. Effects of PFC hypomyelination are hypothesized to be largest in parvalbumin interneurons, because in SZ their activity is decreased which renders them unattractive for OLs to myelinate. In the research presented in Chapters 3 and 4 of this thesis, I have explored these hypotheses using the apomorphine-susceptible (APO-SUS) rat model with SZ-relevant features. In Chapter 5, I have used an unbiased transcriptomic approach to analyse the post-mortem PFC of SZ patients, and investigated the role of lipids, the main components of myelin sheaths, in cognitive deficits in SZ using genetic as well as functional magnetic resonance imaging (fMRI) and cognitive behavioural data from SZ patients. Below I will discuss my main findings and put the results into perspective regarding existing knowledge.

## APO-SUS rat as a model for mPFC-dependent cognitive dysfunction and its relevance to SZ

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To gain insight into the neurodevelopmental processes that underlie SZ PFC pathophysiology, the use of an animal model that shares cognitive features with SZ patients is attractive. Cognitive symptoms of SZ encompass decreases in attention [3], verbal fluency [15], processing speed [16], and memory and executive functioning [4-6]. Impairments in executive functioning include working memory and attentional set-shifting problems [4-6]. Cognitive deficits in SZ arise from the PFC and different PFC subregions are involved in deficits in specific cognitive domains [17]. For example, ventro-lateral PFC functioning remains largely unaffected, while impaired dorso-lateral (dl)PFC-activation underlies a range of cognitive deficits in SZ, including executive functioning, working memory, insight and cognitive control [18-21]. During cognitive tasks the dlPFC is overactivated in SZ patients, which is thought to arise from cortical disinhibition of the dlPFC [19,22,23]. This notion is corroborated by the observation that gamma-band oscillations in the

PFC of SZ patients are abnormal [24], and these oscillations are produced through a rhythmic inhibition of pyramidal neuron firing by parvalbumin interneurons leading to an overactivated PFC and cognitive impairment [25]. Another neurobiological correlate of dlPFC-dependent cognitive deficits in SZ is decreased white-matter integrity [26,27]. However, to which degree these two phenomena, namely parvalbumin interneuron dysfunction and decreased white-matter integrity, are contributing to the etiology of SZ cognitive deficits remains unknown. Its elucidation requires the application of an appropriate animal model. The extensively-studied, idiopathic APO-SUS rat model with SZ-relevant features and its phenotypic counterpart apomorphine unsusceptible (APO-UNSUS) rat is an attractive animal model for investigating mPFC dysfunction in SZ [28,29]. Without requiring genetic or pharmacological manipulation, APO-SUS rats show SZ-relevant behaviours relevant to the positive [30-32], negative [33,34] and cognitive [35] symptoms of SZ and neurobiological similarities with SZ patients [36,37]. Importantly, APO-SUS rats have a defective PFC microcircuit due to a reduced excitability of GABAergic interneurons [36], reminiscent of the interneuron abnormalities described in SZ patients.

In Chapter 3, we subjected the APO-SUS rats to a number of cognitive tests that are relevant to SZ. We examined attentional set-shifting and spatial working memory behaviour that are dlPFC-dependent and impaired in SZ patients [18-21], and in the rat require mPFC functioning [39,138]. We found that APO-SUS rats show a deficit in spatial working memory and attentional set-shifting tasks, while in orbitofrontal cortex-dependent reversal leaning tasks [38] APO-SUS rats are only mildly impaired. We confirmed that mPFC-related spatial working memory in the spatial win-shifting task [39] and the initial acquisition phase of the Morris water maze task are impaired in APO-SUS rats (Figure A1a and A1b, Appendix to Chapter 3). Yet, hippocampus-dependent long-term spatial memory in APO-SUS rats is not affected (Figure A1b, Appendix to Chapter 3). Thus, APO-SUS rats show mPFC-dependent cognitive deficits with characteristics reminiscent of cognitive impairment in SZ patients. Notably, social behaviour [40-42] is also impaired in APO-SUS rats, who spend less time with social, and more time with non-social and aggressive behaviours than APO-UNSUS rats (Figure A1c, Appendix to Chapter 3). Social behaviour is associated with both mPFC GABAergic transmission[43], and OL differentiation and myelination [44,45] and is as such dependent on maturational changes that occur in the mPFC during adolescence and are implicated in SZ. Indeed, social behaviours are impaired in SZ and linked to cognitive deficits [46].

Taken together, in SZ dlPFC-dependent cognitive functioning is impaired and associated with the malfunctioning of GABA-transmission and white-matter structure. In the APO-SUS rat model, we observe an impairment of cognitive functions similar to that in SZ. In the rat, these cognitive functions are dependent on the mPFC, in which both GABA-neurotransmission and white matter undergo their final maturation during adolescence, the timeframe in which SZ first manifests itself. As such, these

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maturational processes are of interest in a study on the neurobiological underpinnings PFC dysfunction in SZ cognitive deficits.

## mPFC hypomyelination in APO-SUS rats and its relevance to SZ

In the PFC of both medicated and unmedicated SZ patients, decreases in white-matter integrity are observed. Notably, white-matter abnormalities in the frontal cortex correlate with cognitive impairment in SZ patients [47-49]. Findings from genetic, post-mortem as well as animal model studies have revealed that abnormalities in myelination likely underlie the decrease in white-matter integrity in SZ [27]. White-matter abnormalities in SZ PFC are already evident before disease onset and worsen with transition to psychosis in adolescence and as such have a developmental aspect. It is therefore thought that the final maturational increase in myelination of the PFC during adolescence is affected in SZ patients [50]. This is supported by studies showing hypomyelination in animal models with SZ-like behaviours and the fact that genetic risk factors for SZ influence a molecular pathway that stimulates myelination [51]. However, because SZ shares symptoms with demyelinating diseases such as multiple sclerosis [52] and in post-mortem SZ brain tissue deformation of myelin sheaths has been observed [53] it has also been suggested that breakdown of myelin occurs in SZ PFC. Although, ultrastructural studies on SZ PFC are scarce. To shed light on the role of (de)myelination in SZ-related cognitive functioning, we investigated mPFC myelination in the APO-SUS and APO-UNSUS rats. During adolescence, mRNA expression levels of myelin-related genes increase in APO-UNSUS mPFC, while this increase is less pronounced in APO-SUS mPFC (Chapter 3). As a result, mRNA and protein expression of myelin-related genes are reduced in adult APO-SUS mPFC. Using ultrastructural analyses of the APO-SUS mPFC, we confirmed a lack of myelinated axons in adulthood, whereas no signs of demyelination were observed. Conspicuously, hypomyelination was not observed in the APO-SUS cingulate cortex, a prefrontal subregion adjacent to the mPFC (Figure A2a and A2b, Appendix of Chapter 3), nor in the barrel cortex, striatum or corpus callosum, suggesting that hypomyelination is specific to the mPFC.

## Hypomyelination is specific to parvalbumin interneurons in APO-SUS mPFC

Apart from myelination, maturation of the PFC during adolescence also involves refinement of GABAergic neurotransmission. During adolescence, GABAergic signaling in the PFC is increased by 30% [54], which is caused by an increase in the activity of fast-spiking parvalbumin interneurons [55-57]. This is accompanied by increased mRNA and protein expression of parvalbumin, and the formation of perineuronal nets around parvalbumin interneurons [55,58]. Interestingly and contrary to popular belief, parvalbumin interneurons become myelinated in the cortex [59-61], a process that likely contributes to the maturation of these interneurons during adolescence. Studies on SZ post-mortem brain tissues indicate that during adolescence parvalbumin interneuron

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maturation is severely reduced. Expression of parvalbumin-related mRNAs is decreased [58,62-65] and mirrors the expression patterns in immature parvalbumin interneurons [66]. Also decreases in parvalbumin perineuronal nets are observed in SZ PFC [67]. Notably, parvalbumin interneuron numbers, somal size and synapse density remain unaffected [68,69]. Reduced levels of parvalbumin protein have been found in synapses in SZ PFC [70,71]. Parvalbumin protein expression is dependent on neuronal activity [72] suggesting that these parvalbumin interneurons are less active in SZ PFC than in healthy individuals. In sum, SZ PFC parvalbumin interneurons have an intrinsic functional abnormality that arises during adolescence and renders them less active [73,74].

In APO-SUS rats, we have observed mPFC interneuron abnormalities during adolescence that resemble those found in SZ PFC, namely reduced GAD67 protein levels that were not accompanied by alterations in the number of amongst others parvalbumin-positive interneurons, suggesting a reduction in GAD67 expression per interneuron [36]. Paired-pulse ratio through GABA-B receptor signaling is reduced in the APO-SUS mPFC [36], indicating a reduced firing rate of inhibitory synapses onto pyramidal cells in the adolescent APO-SUS mPFC. It remains unclear why mPFC interneurons are less active in APO-SUS, although a heightened sensitivity to GABA spillover-mediated reduction of inhibitory signaling was indicated by the increased protein expression of the perisynaptic GABA-B receptor in APO-SUS mPFC [36]. The fact that both myelination and interneuron abnormalities in the APO-SUS mPFC arise in adolescence suggests that interneuron myelination might represent the hub on which various neurobiological alterations converge in the APO-SUS mPFC. In fact, it has been suggested that specifically myelination of parvalbumin interneurons could be affected in SZ PFC [73]. Using an array-tomography-like technique, we discovered that percentages of non-GABA myelinated axons were similar in APO-SUS and APO-UNSUS mPFC, however in APO-SUS mPFC only around 10% of the myelinated axons indeed belong to parvalbumin interneurons, while in APO-UNSUS mPFC this was around 20% (Chapter 3) and consistent with previously published percentages [61]. Yet, like in adolescence, also in adulthood there are no differences in parvalbumin interneuron numbers in the APO-SUS mPFC, indicating parvalbumin interneurons are present, but get myelinated less frequently than in the APO-UNSUS mPFC. This is the first time that interneuron myelination was assessed in an animal model for SZ and our results support a role for developmental aberrations that lead to interneuron hypomyelination in SZ PFC. Future studies are needed to establish whether interneuron hypomyelination is indeed a core feature of SZ PFC neuropathology.

## Why is APO-SUS mPFC hypomyelination specific to parvalbumin interneurons?

APO-SUS mPFC hypomyelination is specific to parvalbumin interneurons, leading to the question why this neuronal subtype is preferentially affected. Whether or not

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an axon gets myelinated is dependent on a range of factors, including axon diameter, neuronal activity, neurotransmitter signaling and growth factor excretion, and can also be influenced by metabolic changes and cellular stress [76]. From the findings discussed above, it becomes clear that parvalbumin interneurons in SZ PFC are less active and less neuronal activity has the potential to negatively affect myelination [86]. It has recently been confirmed that parvalbumin interneurons undergo activity-dependent myelination [59] and it is therefore an attractive hypothesis that a lower activity of parvalbumin interneurons during adolescence hinders their myelination. Relative to APO-UNSUS, the APO-SUS mPFC contains a lower percentage of parvalbumin interneurons positive for the neuronal activity marker c-FOS (Figure A3, Appendix to Chapter 3), in line with our previously published APO-SUS results [36] and a lower parvalbumin interneuron activity in SZ PFC [73,74].

### Environmental enrichment (EE) restores parvalbumin interneuron hypomyelination and cognitive deficits in APO-SUS rats.

The question arises whether interneuron hypomyelination in APO-SUS mPFC is a causative factor leading to the mPFC-dependent cognitive impairment we observed in APO-SUS rats. To investigate this, we applied a behavioural paradigm during adolescence that is known to increase PFC myelination, namely EE including social enrichment, voluntary exercise opportunities and cage enrichment [44,75-77]. EE during adolescence restored cognitive functioning in APO-SUS rats and rescued the number of myelinated axons in APO-SUS mPFC (Chapter 3). These data confirm that interneuron hypomyelination contributes to mPFC-dependent cognitive impairment in APO-SUS rats and that in SZ a developmental hypomyelination of the mPFC is likely hindering proper cognitive functioning. As such, EE, exercise and sociability training in individuals at high risk to develop SZ have the potential to rescue aberrant PFC myelin development. On the one hand, Behavioural therapy in SZ patients indeed reduces cognitive symptoms and is associated with an increase in white-matter integrity [78,79]. On the other hand, higher rates of sedentary behaviour, less physical activity and decreased sociability in adolescents at high risk to develop SZ correlate with more severe cognitive symptoms in later stages of SZ [80-85].

Interestingly, the increase in myelinated interneurons observed in the APO-SUS mPFC following EE was accompanied by an increased percentage of active parvalbumin interneurons (Figure A3, Appendix to Chapter 3). These preliminary results indicate that a low parvalbumin interneuronal activity could imply hypomyelination and increased neuronal activity, e.g. through EE during adolescence, entails promyelinating effects. However, further studies are needed corroborate this assumption because, other than neuronal activity, EE mediates changes in axon-OL precursor cell (OPC) synapses, growth factor production, neurotransmitter release, excretion of metabolites and inflammatory factors [76], all of which may contribute to the promyelinating effects of

EE. Thus, the exact neurobiological mechanism underlying the promyelinating effects of EE remain to be established.

### A key role for oxidative stress in parvalbumin interneuron hypomyelination

Oxidative stress in blood, cerebral spinal fluid and brain is an intrinsic and well-replicated feature of SZ and is independent of disease state or medication use [87-90]. Specifically, glutathione antioxidant levels are consistently found to be reduced in SZ blood plasma and several brain regions of post-mortem brain tissue, and accompanied by increases in the levels of reactive oxygen species (ROS) [89-92]. Also, changes in mitochondria have been identified in SZ post-mortem brain tissue and are related to oxidative stress [93,94]. Likewise, we find oxidative stress in the APO-SUS mPFC, illustrated by a decreased glutathione antioxidant metabolism and increased numbers of mitochondria (Chapter 4). Reduced glutathione metabolism was observed throughout post-natal life in APO-SUS mPFC and also found in striatum, corpus callosum and barrel cortex of APO-SUS rats (Figure A1a and A1b, Appendix to Chapter 4). These data suggest a global increase in oxidative stress in APO-SUS brain, reminiscent of findings in SZ [89-92]. The fact that in APO-SUS rats we found an impaired glutathione metabolism that started at birth and persisted into late adulthood suggests that oxidative stress in SZ may already occur before the clinical symptoms of the disorder become apparent.

Oxidative stress has detrimental effects on cellular proliferation, differentiation and survival, and could as such have a major impact on circuit establishment during adolescent mPFC development in SZ. Oxidative stress has been hypothesized to affect interneuron functioning in the SZ PFC in several ways. First, parvalbumin interneuron dysfunction in SZ may arise from oxidative damage to these interneurons [74]. The fast-spiking property of parvalbumin interneurons is accompanied by a high metabolic demand, which causes increased ROS production that in combination with the decreased antioxidant defenses observed in SZ PFC could damage the interneurons. In addition, perineuronal nets protect parvalbumin interneurons against oxidative stress, and these perineuronal nets are decreased in SZ PFC [67,95]. Second, oxidative stress in SZ PFC may hinder myelination via OL damage. Relative to other brain cells, OLs are extremely vulnerable to oxidative insults, because of their high metabolic demand and free iron use necessary for myelin membrane production (OLs produce myelin membranes that can be more than 100 times their soma weight). High levels of oxidative stress in SZ PFC during adolescence could therefore impair myelination.

### Treatment with N-acetylcysteine (NAC) rescues interneuron hypomyelination and improves cognitive deficits in APO-SUS rats

To investigate the role of oxidative stress in parvalbumin interneuron hypomyelination and cognitive dysfunction in APO-SUS rats, we treated APO-SUS and APO-UNSUS rats during post-natal development with the antioxidant NAC, a direct precursor of

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glutathione that passes the blood brain barrier and enhances glutathione production. NAC treatment increased the blood plasma levels of glutathione and the mPFC mRNA expression of glutathione-related genes in APO-SUS rats (Chapter 4). In addition, the number of mitochondria was significantly reduced in APO-SUS mPFC following NAC treatment. This implies that the NAC-treated APO-SUS rats were not exposed to oxidative stress during adolescent mPFC development. Remarkably, the mPFC of the NAC-treated APO-SUS rats had a similar number of myelinated axons and similar levels of myelin-related mRNA expression as APO-UNSUS mPFC. As such, antioxidant treatment during post-natal development can rescue interneuron hypomyelination in the APO-SUS mPFC. Treatment of SZ patients with NAC increases white-matter integrity in the fornix and this correlates with increased glutathione levels in the mPFC [96,97]. NAC also improves working memory, attention and executive functioning in SZ patients [98,99], and we observed indeed improved cognitive functioning of APO-SUS rats following NAC treatment. We conclude that NAC treatment alleviates oxidative stress and allows normal development of parvalbumin interneuron myelination during adolescence leading to improved cognitive functioning in the APO-SUS rats. This indicates that NAC treatment could be an attractive way to prevent cognitive deficits in individuals at high-risk to develop SZ as well as an appealing treatment strategy for cognitive symptoms in SZ patients.

### The role of OLs in mPFC-specific hypomyelination of the APO-SUS brain

As during development of the APO-SUS mPFC no demyelination, but rather a decrease in myelin production occurs, we decided to study the functioning of OLs and OL lineage progression (Chapter 3). In SZ post-mortem dlPFC, a reduced density of OLs has been found [100-103]. In particular, an increase in the number of OLs over age is observed in control PFC tissue, while in SZ PFC an increase is not evident [104]. Unaltered numbers of OPCs, but a reduction in the total number of OL lineage cells have been found in SZ PFC, indicating a defect in OL differentiation [105]. We found remarkably similar results in the APO-SUS mPFC, including no changes in the number of OPCs, but a decreased number of OL lineage cells. This was accompanied by an increased number of premyelinating (pre)OLs and a reduced number of mature OLs, indicating a maturation block of APO-SUS OLs in the mPFC. Importantly, OLs were not apoptotic in the APO-SUS mPFC (Figure A4 Appendix of Chapter 3). In contrast to the mPFC-specific hypomyelination, abnormalities in OL numbers were also observed in other brain regions. We found a decreased number of OL lineage cells and mature OLs in the CG (Figure A2c, Appendix to Chapter 3) and a reduced density of mature OLs in the BC (Chapter 3).

To investigate whether the OLs of APO-SUS rats have an intrinsic defect that hinders their differentiation, we performed primary culture experiments with APO-SUS and APO-UNSUS oligodendroglial cells (Chapter 3). Under *in vitro* conditions, APO-SUS OPCs proliferate and differentiate into mature OLs to the same degree as APO-

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UNSUS OPCs. However, when we exposed primary OPCs to oxidative stress *in vitro*, a lower percentage of APO-SUS and APO-UNSUS OPCs reached the mature OL stage. This indicates that the oxidative stress in the APO-SUS brain can negatively affect OL differentiation. As oxidative stress was observed in all brain regions tested in APO-SUS rats, it is not surprising that not only OLs in the mPFC, but also in the CG and BC displayed abnormalities.

Since oxidative stress and OL abnormalities were observed in multiple brain regions of APO-SUS rats, the question arises why hypomyelination occurred only in the mPFC? One explanation could be that the critical window for OL maturation and myelination in the mPFC is around adolescence, while myelination in other brain regions takes place in early post-natal life. Furthermore, oxidative stress builds up over time, and the relatively late differentiation and myelination of OPCs in the mPFC may therefore be hindered by high oxidative-stress levels. If so, alleviating oxidative stress with NAC treatment should be sufficient to restore OL abnormalities in the mPFC. However, while indeed the density of OL lineage cells and preOLs in mPFC was restored in NAC-treated APO-SUS rats, the number of mature OLs remained reduced (Chapter 4). It is of interest to note that the negative effects of oxidative stress on OLs are largest in preOLs [106-108]. The fact that NAC treatment did not rescue the number of mature OLs indicates that oxidative damage to OLs only partly explains their differentiation block in APO-SUS mPFC. Notably, in contrast to NAC treatment, EE did not influence glutathione levels (Figure A5, Appendix to Chapter 3) and therefore APO-SUS rats reared in EE were still exposed to oxidative stress. Yet, EE restored the number of OL lineage cells and preOLs, and increased OPC density in APO-SUS mPFC (Chapter 3). Hence, the beneficial effects of EE on OL differentiation in APO-SUS mPFC occur despite of the presence of oxidative stress.

Parvalbumin interneurons undergo activity-dependent myelination, which occurs via communication with OPC and preOLs. OPCs and preOLs are able to sense neuronal activity of the to-be-myelinated axons via activation of amongst others glutatmatergic and purinergic receptors. This receptor activation instructs OPCs and preOLs to mature and myelinate the axon [109-111]. Therefore, because parvalbumin interneurons are less active in the mPFC, OPCs and preOLs may not be properly instructed to mature and myelinate these interneurons. This could also explain why alterations in OL numbers only lead to hypomyelination in the mPFC, and may clarify the beneficial effects of EE on OL differentiation, as EE increased the percentage of parvalbumin interneurons that was active (Figure A3 Appendix to Chapter 3). However, additional experiments are needed to confirm this notion.

Taken together, a negative effect of oxidative stress on OL maturation only partly explains the OL maturation block in the APO-SUS mPFC and it is feasible that the low parvalbumin interneuron activity contributes to the block in OL maturation in the APO-SUS mPFC.

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### Lipid metabolism in SZ PFC

Myelin sheaths consist mainly of cholesterol, galactosylceramide and ethanolamine plasmalogen lipids [112]. Myelin membrane cholesterol is produced by OLs and offers stability to myelin sheaths [113]. Importantly, the rate at which cholesterol is produced is directly linked to the rate of myelin membrane creation and high levels of cholesterol are necessary for myelin growth and layering [113]. As in SZ myelination is decreased, lipids could play a role in disease etiology. Indeed, SZ is genetically associated with two transcription factors involved in cholesterol metabolism, sterol regulatory element binding protein (SREBP) 1 and 2 [114]. In addition, SREBP1 protein levels are reduced and fatty acid levels are altered in SZ post-mortem brain tissue [115,116]. Unmedicated SZ patients have lower total cholesterol, high-density lipoprotein (HDL), apolipoprotein and acylcarnitine blood levels, and altered blood levels of free fatty acids and ceramide lipids [117-120]. Moreover, a role for apolipoproteins, representing an essential part of HDL particles that allow cholesterol efflux from cells, has been proposed in SZ [121]. The question remains whether the lipid metabolism changes observed in SZ are linked to the PFC myelin abnormalities and related cognitive deficits. We therefore performed RNA sequencing analysis of post-mortem SZ and control dlPFC, and identified an enrichment of upregulated genes in the Liver X Receptor (LXR)/Retinoid X Receptor (RXR) activation pathway (Chapter 5). We further identified extensive shared genetic etiology between SZ and lipids, including lipid content in HDL-like particles, confirming a link between SZ and cholesterol efflux. Further shared genetic etiology concerned acylcarnitines, implicated in the breakdown of unsaturated fatty acids, and the ratio of saturated over non-saturated fatty acids. Fatty acids form a substrate for the production of most lipid species, supporting a role in SZ not just for cholesterol efflux, but also for fatty acid biosynthesis.

As about 80% of the lipids present in brain are part of myelin sheaths, a defect in lipid metabolism is likely to influence myelination. Fatty acid and LDL levels in the blood are indeed correlated with white-matter integrity in SZ frontal cortex [122,123]. Moreover, LXRβ knockout mice show a hypomyelination phenotype because cholesterol deficiency inhibits OL differentiation and myelination [124,125], and during brain development LXRβ is involved in the formation of OPCs [126]. Therefore, it is likely that altered lipid metabolism in SZ contributes to myelination defects. This is supported by studies that show a link between lipids and cognition in SZ. For example, the severity of cognitive deficits is associated with genetic variation in genes involved in lipid metabolism [127] and genetic as well as transcriptomic variation in the lipid-related gene delta 4-desaturase sphingolipid 2 (DEGS2) is associated with cognitive deficits in SZ [128]. Moreover, in a subgroup of SZ patients deceased levels of sphingomyelin lipids are correlated with more severe cognitive impairment [129]. Further evidence comes from the fact that metabolic syndrome in SZ patients as well as in non-psychiatrically ill individuals is associated with a higher degree of cognitive impairment [130] and that in

SZ patients who take antipsychotic medication cholesterol levels are correlated with cognitive impairment [131,132]. We confirmed that indeed reduced cognitive performance in SZ patients is correlated with decreased dlPFC lipid content from $T_1$-weighted structural MRI scans (Chapter 5). Taken together, distortions in lipid metabolism play a key role in cognitive dysfunction in SZ, most likely via negative effects on the process of myelination.

## Conclusions and future perspectives

The objective of this thesis was to explore the neurobiological underpinnings of PFC dysfunction in SZ with a focus on the role of oxidative stress and myelination, employing the APO-SUS rat model as well as SZ post-mortem brain tissue, and analyzing genetic, cognitive behavioural and structural MRI data from SZ patients. In the APO-SUS rat model, we have identified cognitive impairment that is similar to that observed in SZ patients and associated with mPFC dysfunctioning. We have revealed developmental hypomyelination in the APO-SUS mPFC that becomes apparent during adolescence and occurs specifically in parvalbumin interneurons. mPFC-specific hypomyelination was accompanied by oxidative stress that also occurred in several other APO-SUS brain regions. We further found that parvalbumin interneuron hypomyelination arises from a maturation block of OLs that was partly caused by detrimental effects of oxidative stress on OLs. We also found indications that a low parvalbumin interneuronal activity may provoke hypomyelination, but further studies are necessary. PFC parvalbumin interneurons undergo their final maturation steps, including myelination, during adolescence, the timeframe in which SZ first manifests itself. Our results indicate that maturation of parvalbumin interneuron myelination is affected. Remarkably, we show that both EE and NAC treatment during adolescent mPFC development can rescue interneuron hypomyelination and improve cognitive functioning in APO-SUS rats, but do not have the same neurobiological mechanism. This highlights the importance of adolescent mPFC development in the etiology of cognitive impairment in SZ. Finally, we show a key role for lipids in the pathophysiology of the SZ dlPFC and that SZ shares genetic etiology with lipid species. A decreased dlPFC lipid content was correlated with a reduced performance in a cognitive task. Therefore, altered lipid metabolism likely contributes to myelination abnormalities and cognitive deficits in SZ that arise from the dlPFC.

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The work presented in this thesis emphasizes the importance of the developmental processes occurring in the PFC during adolescence. Oxidative stress levels are increased in SZ and build up over time. During adolescence in SZ, the developmental increase in PFC myelination does not occur and the degree of GABAergic signaling does not increase (Figure 1a). Our APO-SUS rat studies indicate that oxidative stress, myelination and GABAergic signaling are interrelated and critical for development of cognitive impairment in SZ (Figure 1b). Further developmental studies in animal models for SZ as

well as in individuals at high risk for SZ are needed to increase our understanding of the relationship between oxidative stress, myelination and GABAergic signaling in SZ.
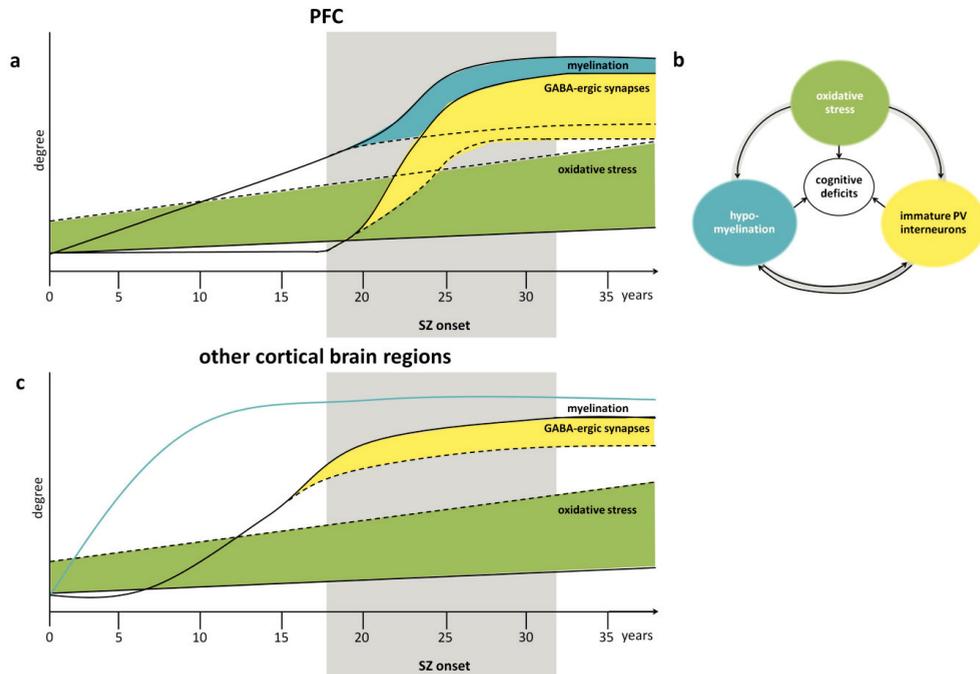


**Figure 1 – Development of oxidative stress, myelination and GABAergic signaling in the SZ PFC.**
**(a)** Oxidative stress levels in SZ are higher than in healthy individuals and increase over time. The developmental increase in the degree of myelination in adolescence does not occur in SZ and during adolescence the degree of GABAergic signaling increases in healthy individuals, this increase is not as pronounced in SZ patients. **(b)** Our results in the APO-SUS rat model reveal that during mPFC development in adolescence there is interplay between oxidative stress, myelination and parvalbumin (PV) interneuron alterations and that all these factors contribute to the development of cognitive impairment. **(c)** Development of myelination, GABAergic synapses and oxidative stress is other brain regions in SZ based on references [133-135].

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In sum, we propose that in APO-UNSUS rats myelinated parvalbumin interneurons provide an inhibitory signal to pyramidal neurons resulting in a low excitatory activity of pyramidal neurons, which contributes to cognitive flexibility (Figure 2). Lipids are produced by OLs and transported to the myelin sheaths. Because of a maturation block of OLs, parvalbumin interneurons are not adequately myelinated in APO-SUS mPFC. In addition, oxidative stress damages both parvalbumin interneurons and myelination in APO-SUS mPFC and these features contribute to cognitive inflexibility, probably via reduced inhibitory signaling of parvalbumin interneurons to pyramidal neurons that in turn are hyperexcited. Moreover, in SZ dlPFC we have identified an increased efflux of lipids, which could also play a role in APO-SUS mPFC dysfunction. We further reveal a positive effect of both EE and NAC treatment on interneuron hypomyelination and cognitive deficits in APO-SUS rats. Yet, following EE OL maturation remains impaired and oxidative stress negatively affects parvalbumin interneurons and OLs. Following NAC treatment, oxidative stress is reduced and does not affect parvalbumin interneurons and OLs anymore, but OLs still show maturation impairment. Taken together, the results indicate that EE and NAC treatment rescue interneuron hypomyelination and cognitive flexibility via distinct neurobiological mechanisms.

Overall, our findings have implications for the development of strategies to treat cognitive symptoms in SZ. NAC treatment has positive effects on cognition in first episode as well as chronic SZ [98,99,136,137]. We reveal the neurobiological correlates of the beneficial effects of NAC treatment on cognitive functioning and emphasize that chronic NAC treatment could be an attractive measure to prevent cognitive decline in individuals at high risk to develop SZ. In addition, EE, exercise and sociability training in individuals at high risk to develop SZ have the potential to rescue aberrant PFC myelin development and cognitive dysfunction. This is an important finding considering that higher rates of sedentary behaviour, lower levels of physical activity and decreased sociability in adolescents at high risk to develop SZ correlate with cognitive symptoms in later stages of SZ [80-85]. Behavioural therapy indeed enhances white-matter integrity and improves cognition in SZ patients [78,79]. Because myelin matters in SZ, we encourage the application of combinatorial pharmacological and PFC-directed behavioural therapy. Combining chronic NAC treatment and sociability training, physical exercise and environmental enrichment could be an attractive preventative strategy as well as an appealing remedy to alleviate cognitive symptoms in SZ.
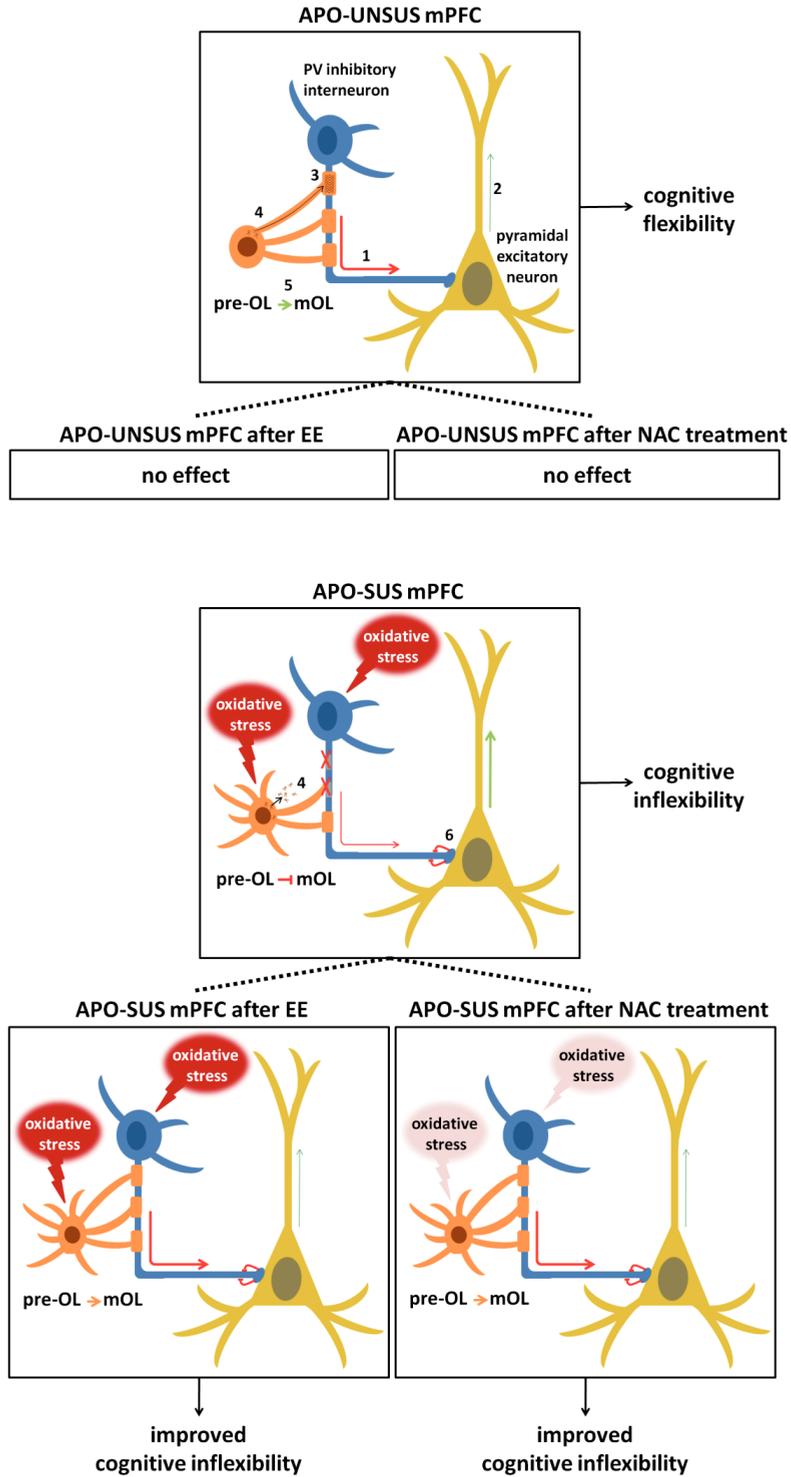
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Figure 2 – The neurobiology of APO-SUS mPFC dysfunction and the effects of NAC and EE treatment. In APO-UNSUS mPFC parvalbumin (PV) interneurons produce a strong inhibitory signal onto pyrami-

dal neurons, which in turn produce a weak excitatory signal contributing to cognitive flexibility. PV interneurons are myelinated by mature (m)OLs that arise from premyelinating (pre)OLs. Myelin membranes consist mainly of lipids that are produced by OLs. In the APO-SUS mPFC, preOLs do not mature into mOLs, PV interneurons do not get myelinated and produce a weak inhibitory signal, and pyramidal neurons are insufficiently inhibited, contributing to cognitive inflexibility. There is a negative feedback loop in the synapse from interneurons onto pyramidal neurons and in SZ dlPFC lipid efflux is increased, which might also play a role in the APO-SUS mPFC. Moreover, oxidative stress negatively affects OLs and PV interneurons. Both EE and NAC treatment rescue PV interneuron hypomyelination and improve cognitive inflexibility. This might be caused by a rescue of the inhibition of pyramidal neurons. However, during both EE and NAC treatment there is still a maturation defect of OLs and after EE treatment there is still exposure of the cells to oxidative stress. 1: PV interneuron produces an inhibitory signal onto a pyramidal neuron; 2: pyramidal neuron produces an excitatory signal; 3: parvalbumin interneuron myelination; 4: lipids are produced by OLs transported into the myelin sheath; 5: preOLs mature to myelinating OLs; 6: in APO-SUS mPFC, there is a negative feedback loop in the interneuron synapse.

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# Summary

Schizophrenia (SZ) is a severe psychiatric disorder with a highly complex clinical presentation and neurobiological correlates. SZ has positive (e.g., hallucinations and delusions), negative (e.g., the loss of typical affective functions) and cognitive (e.g., impairments in memory and executive functioning) symptoms that become apparent during adolescence. Cognitive symptoms of SZ are thought to arise from a dysfunctioning of the prefrontal cortex (PFC), and to involve altered myelination and interneuron abnormalities. Myelin membranes are an extension of the oligodendrocyte (OL) cell membrane and form an insulating layer around axons of (inter)neurons that allows the saltatory conduction of action potentials. In SZ, the severity of cognitive symptoms determines functional outcome and therefore elucidation of the neurobiological mechanisms underlying these symptoms is important for the development of novel treatment strategies.

In Chapters 1 and 2 of this thesis, I have introduced the hypothesis that the maturation of the PFC is affected in SZ. Adolescence is the timeframe during which SZ first manifests itself and during which parvalbumin interneurons in the PFC undergo maturational changes that include their myelination. I further hypothesized that high levels of oxidative stress in the PFC of SZ patients interfere with the maturation of oligodendrocyte precursor cells (OPCs) to OLs and as such hinder proper PFC myelination.

To investigate the neurobiological underpinnings of PFC dysfunction in SZ, I utilized the apomorphine susceptible (APO-SUS) rat model with SZ-relevant features and its phenotypic counterpart the apomorphine unsusceptible (APO-UNSUS) rat (Chapters 3 and 4). Without requiring genetic or pharmacological manipulation, APO-SUS rats show SZ-relevant behavioural traits and neurobiological characteristics. We showed that medial (m) PFC-dependent attentional set-shifting, spatial working memory and social behaviour of APO-SUS rats is impaired as compared to that of APO-UNSUS rats, while cognitive behaviour that is dependent on other brain regions remained unaffected (Chapter 3). During adolescence, expression levels of myelin-related mRNAs increase in APO-UNSUS mPFC, while such an increase is less pronounced in APO-SUS mPFC, resulting in reduced myelin-related mRNA and protein expression in the mPFC of adult APO-SUS rats. Ultrastructural analyses of the APO-SUS mPFC confirmed a reduced number of myelinated axons in adulthood, whereas no signs of demyelination were observed. Moreover, the unmyelinated axons in the APO-SUS mPFC were found to belong to parvalbumin interneurons, while the number of parvalbumin interneurons was unaltered. Therefore, parvalbumin interneurons were present, but the number that got myelinated was lower in APO-SUS than that in APO-UNSUS mPFC. This is the first time that interneuron myelination has been assessed in an animal model for SZ.

Our results support a model in which developmental aberrations during adolescence lead to interneuron hypomyelination in SZ PFC. Remarkably, environmental enrichment (EE) that included voluntary exercise, social enrichment and cage enrichment, restored parvalbumin interneuron hypomyelination and cognitive deficits in APO-SUS rats. The increase in myelinated interneurons observed following EE was accompanied by an increased percentage of active parvalbumin interneurons in the APO-SUS mPFC (Chapter 3). As such, a combination of EE, exercise and sociability training in individuals at high risk to develop SZ has the potential to rescue aberrant myelin development and increase the number of active parvalbumin interneurons in the PFC. However, the exact neurobiological mechanism underlying the promyelinating effects of EE remains to be established.

A key feature of SZ is the occurrence of oxidative stress. Levels of the antioxidant glutathione are consistently found to be reduced and levels of reactive oxygen species to be increased in SZ blood plasma and post-mortem brain tissue, and these changes are accompanied by alterations in mitochondria. We indeed observed oxidative stress in the APO-SUS mPFC, illustrated by a reduced glutathione metabolism and increased numbers of mitochondria (Chapter 4). Reduced glutathione metabolism in APO-SUS mPFC was observed throughout post-natal life and as such preceded hypomyelination that occurred merely during adolescence. Furthermore, oxidative stress has been hypothesized to affect the functioning of parvalbumin interneurons in the SZ PFC by hindering their effective myelination through the extremely oxidative stress-vulnerable OLs. During postnatal development, we treated APO-SUS rats with the antioxidant N-acetylcysteine (NAC) and found restored blood plasma levels of glutathione, mRNA expression of glutathione-related genes and mitochondria numbers in APO-SUS mPFC. The antioxidative effects of NAC rescued interneuron hypomyelination and improved cognitive deficits in the APO-SUS rats. We conclude that NAC treatment alleviates oxidative stress and allows normal development of parvalbumin interneuron myelination during adolescence leading to improved cognitive functioning in the APO-SUS rats. This indicates that NAC treatment could be an attractive way to prevent cognitive deficits in individuals at high-risk to develop SZ as well as an appealing treatment strategy for cognitive symptoms in SZ patients (Chapter 4).

During development of the APO-SUS mPFC, no demyelination, but rather a decrease in myelin production occurred and we therefore investigated the lineage progression of OLs. We found no changes in the number of OPCs, but a decreased number of OL lineage cells and mature OLs, and an increased number of preOLs, indicating a maturation block of OLs in the APO-SUS mPFC. In contrast to the observed hypomyelination that was specific to the mPFC, abnormalities in OL numbers were also observed in other brain regions. Likely, APO-SUS OPCs had no intrinsic defect as under *in vitro* conditions they proliferated and differentiated into mature OLs to the same degree as APO-UNSUS OPCs. However, when we exposed primary OPCs to oxidative

stress *in vitro*, a lower percentage of APO-SUS and APO-UNSUS OPCs reached the mature OL stage. On the one hand, NAC treatment indeed restored the density of OL lineage cells and preOLs in the mPFC of APO-SUS rats, but the number of mature OLs remained reduced. On the other hand, while during EE APO-SUS rats still have lower levels of the antioxidant glutathione, EE did restore the number of OL lineage cells and preOLs, and increased OPC density in APO-SUS mPFC. Nevertheless, NAC administration nor EE led to increased numbers of mature OLs in the treated APO-SUS rats. We concluded that oxidative stress only partly explains the maturation block of OLs in the APO-SUS mPFC. As parvalbumin interneurons are myelinated in an activity-dependent manner, i.e., the activity of these interneurons instructs OLs to mature and myelinate, we suggest that the inactivity of the parvalbumin interneurons in APO-SUS mPFC also contributes to the observed OL maturation block (Chapter 4). However, future experiments are needed to explore this further.

Myelin membranes consist mainly of lipids and lipid homeostasis in OLs is therefore important for proper myelination. RNA sequencing analysis of post-mortem dorsolateral (dl)PFC from SZ patients and control individuals identified an enrichment of upregulated genes in a cholesterol efflux pathway (Chapter 5). Using two separate SZ GWAS datasets and genetic data concerning 514 metabolites, we further identified shared genetic etiology between SZ and lipids, confirming a link between SZ and cholesterol efflux. Additional shared genetic etiology concerned acylcarnitines, lipid particles and the ratio of saturated over non-saturated fatty acids. Furthermore, using a publicly available structural magnetic resonance imaging (MRI) dataset we showed that reduced cognitive performance of SZ patients was correlated with a decreased lipid content in the grey matter of the dlPFC. Thus, distortions in lipid homeostasis play a key role in cognitive dysfunction in SZ, most likely through negative effects on the process of myelination.

The work presented in this thesis emphasizes the importance of the developmental processes that occur in the PFC during adolescence and contributes to our understanding of the neurobiological etiology of cognitive symptoms in SZ. I conclude that myelin matters in SZ and based on the acquired neurobiological insights the application of combinatorial (pharmacological and behavioural) therapy is encouraged, including chronic NAC treatment and sociability training, physical exercise and EE, as a preventative strategy as well as an appealing remedy to alleviate PFC hypomyelination and cognitive symptoms in SZ.

# Samenvatting

Schizofrenie (SZ) is een ernstige psychiatrische aandoening met een zeer complexe klinische presentatie en neurobiologische veranderingen. SZ wordt gekenmerkt door positieve symptomen (bijv. hallucinaties en waanideeën), negatieve symptomen (bijv. het verlies van typische affectieve functies) en cognitieve symptomen (bijv. problemen met geheugen en aandacht) die tijdens de adolescentie aan het licht komen. Men denkt dat de cognitieve symptomen van SZ worden veroorzaakt door veranderingen in myeline (een vettige witte stof die de uitloper, het axon, van de zenuwcel, het neuron, omhult en beschermt) en interneuronen in de prefrontale cortex (PFC). Myelinemembranen zijn een uitbreiding van het celmembraan van de zogenaamde oligodendrociet (OL) en vormen een isolerende laag die effectieve geleiding van actiepotentialen door neuronen mogelijk maakt. De ernst van de cognitieve symptomen is bepalend voor de ernst van SZ. Daarom is opheldering van de neurobiologische mechanismen die ten grondslag liggen aan deze symptomen belangrijk voor de ontwikkeling van nieuwe behandelingsstrategieën voor SZ.

In de Hoofdstukken 1 en 2 van dit proefschrift heb ik de hypothese geïntroduceerd dat er bij SZ iets misgaat bij de ontwikkeling van de PFC. De adolescentie is het tijdsbestek waarin SZ zich voor het eerst manifesteert en waarin tevens zogenaamde parvalbumine interneuronen in de PFC veranderingen ondergaan, waaronder hun myelinisatie. Een belangrijk kenmerk van SZ is de aanwezigheid van een meer dan normale hoeveelheid reactieve zuurstofverbindingen in de cel, zogenaamde oxidatieve stress. Ik veronderstel dat een hoge mate van oxidatieve stress in de PFC van SZ-patiënten de ontwikkeling van zogenaamde OL voorlopercellen (OPC's) tot OL's verstoort en als zodanig een goede myelinisatie van de PFC belemmert.

Om de neurobiologische achtergrond van het niet goed functioneren van de PFC in SZ te onderzoeken, heb ik gebruik gemaakt van het apomorfine-gevoelige (APO-SUS) rattenmodel voor SZ en zijn fenotypische tegenhanger, de apomorfine-ongevoelige (APO-UNSUS) rat (Hoofdstukken 3 en 4). APO-SUS ratten vertonen gedragseigenschappen en neurobiologische kenmerken die relevant zijn bij SZ zonder dat genetische of farmacologische manipulatie van de ratten nodig is. We toonden aan dat mediaal (m)PFC-afhankelijk 'set-shifting' gedrag, ruimtelijk werkgeheugen en sociaal gedrag van de APO-SUS ratten is aangetast in vergelijking met dat van APO-UNSUS ratten. Cognitief gedrag dat afhankelijk is van andere hersengebieden bleef echter onaangetast (Hoofdstuk 3). Tijdens de adolescentie stijgen de expressieniveau's van myeline-gerelateerde mRNAs in de mPFC van APO-UNSUS ratten, terwijl een dergelijke toename minder uitgesproken is in de mPFC van APO-SUS ratten. Dit resulteert in een verminderde hoeveelheid myeline-gerelateerde mRNA- en eiwitexpressie in de mPFC van volwassen APO-SUS ratten. Ultrastructurele analyse van de APO-SUS

mPFC bevestigde een verminderd aantal gemyelineerde axonen op volwassen leeftijd, terwijl er geen tekenen van demyelinisatie werden waargenomen. Bovendien bleken de ongemyelineerde axonen in de APO-SUS mPFC toe te behoren aan parvalbumine interneuronen, terwijl het aantal van deze interneuronen ongewijzigd bleef. Er waren dus wel parvalbumine interneuronen aanwezig, maar het aantal dat gemyelineerd werd, was lager dan in de APO-UNSUS mPFC. Dit is de eerste keer dat myelinisatie van interneuronen werd onderzocht in een diermodel voor SZ. Onze resultaten ondersteunen een model waarin ontwikkelingsafwijkingen tijdens de adolescentie leiden tot verminderde myelinisatie (hypomyelinisatie) van interneuronen in de PFC van SZ-patiënten. Opmerkelijk is dat milieuverrijking (EE), die bestond uit vrijwillige lichaamsbeweging, sociale verrijking en kooiverrijking, leidde tot het herstel van de hypomyelinisatie van parvalbumine interneuronen en cognitieve stoornissen bij APO-SUS ratten. De toename van gemyelineerde interneuronen na EE ging gepaard met een verhoogd percentage actieve parvalbumine internuronen in de APO-SUS mPFC (Hoofdstuk 3). Het toepassen van een combinatie van vrijwillige lichaamsbeweging, sociabiliteittraining en omgevingsverrijking bij personen met een hoog risico om SZ te ontwikkelen kan dus mogelijk een afwijkende myeline-ontwikkeling voorkomen en het aantal actieve parvalbumine interneuronen in de PFC verhogen. Het exacte neurobiologische mechanisme dat ten grondslag ligt aan de myelinisatie-stimulerende effecten van EE moet echter nog worden vastgesteld.

Zoals genoemd, staat oxidatieve stress centraal bij SZ. In bloedplasma en post-mortem hersenweefsel van SZ-patiënten is het niveau van de antioxidant glutathion verlaagd en het gehalte aan reactieve zuurstofverbindingen verhoogd. Deze veranderingen gaan gepaard met veranderingen in de energiefabrieken van de cel, de mitochondriën. We hebben inderdaad oxidatieve stress waargenomen in de APO-SUS mPFC, namelijk een verminderd glutathion metabolisme en verhoogde aantallen mitochondriën (Hoofdstuk 4). Het verlaagde glutathion metabolisme werd geconstateerd na de geboorte van de rattenpups en ging dus vooraf aan de hypomyelinisatie, die pas tijdens de adolescentie plaatsvond. OLs zijn extreem gevoelig voor oxidatieve stress en daarom denkt men dat dergelijke stress de myelinisatie van parvalbumine interneuronen door OLs belemmert, waardoor de werking van deze interneuronen in de PFC van SZ-patiënten negatief wordt beïnvloed. Tijdens hun postnatale ontwikkeling hebben we APO-SUS pups behandeld met de antioxidant N-acetylcysteïne (NAC) en vonden we in de mPFC  van de behandelde APO-SUS ratten een herstel van de bloedplasmaspiegels van glutathion, de mRNA expressie van glutathion-gerelateerde genen en het aantal mitochondriën. De antioxidatieve effecten van NAC leidden ook tot een verbetering van de hypomyelinisatie van interneuronen en van de cognitieve problemen in de volwassen APO-SUS ratten. We concluderen dat NAC-behandeling tijdens adolescentie oxidatieve stress vermindert en een normale myelinisatie van parvalbumine interneuronen mogelijk maakt en leidt tot een beter cognitief functioneren van de APO-SUS ratten.

Dit wijst erop dat NAC-behandeling een aantrekkelijke manier zou kunnen zijn om cognitieve problemen te voorkomen bij individuen met een hoog risico om SZ te ontwikkelen en een aantrekkelijke behandelingsstrategie zou kunnen vormen voor de cognitieve symptomen van SZ-patiënten (Hoofdstuk 4).

Tijdens de ontwikkeling van de mPFC van APO-SUS ratten is er geen sprake van een verlies aan myeline (demyelinisatie), maar wel van een daling van de myelineproductie en daarom hebben we de rijping van OL's onderzocht. We vonden geen veranderingen in het aantal OPC's, maar een verminderd totaal aantal OL's en een verminderd aantal volwassen OL's, in combinatie met een verhoogd aantal voorloper (pre)OL's, wat wijst op een stop in de rijping van OL's in de APO-SUS mPFC. In tegenstelling tot de waargenomen hypomyelinisatie, die specifiek was voor de mPFC, werden ook in andere hersengebieden afwijkingen in het aantal OL's waargenomen. Waarschijnlijk hadden de OPC's in de APO-SUS ratten geen intrinsiek defect, aangezien ze zich onder kweekomstandigheden (*in vitro*) in dezelfde mate als APO-UNSUS OPC's vermeerderden en ontwikkelden. Toen we de primaire OPC's echter *in vitro* aan oxidatieve stress blootstelden, bereikten een lager percentage APO-SUS en APO-UNSUS OPC's het volwassen OL-stadium. Enerzijds herstelde de NAC-behandeling inderdaad het totaal aantal OL's en preOL's in de mPFC van APO-SUS ratten, maar het aantal volwassen OL's bleef lager. Anderzijds, terwijl tijdens EE APO-SUS ratten nog steeds een lager glutathion antioxidantgehalte hadden, herstelde EE wel het totaal aantal OL's en preOL's, en werd de dichtheid aan OPC's verhoogd in APO-SUS mPFC. Niettemin leidde EE, net zoals de toediening van NAC, niet tot een toename van het aantal volwassen OL's in de behandelde APO-SUS ratten. We concludeerden dat oxidatieve stress slechts een gedeeltelijke verklaring is voor de rijpingsstop van OL's in de APO-SUS mPFC. Aangezien myelinisatie van de parvalbumine interneuronen afhankelijk is van hun activiteit, d.w.z. de activiteit van deze interneuronen instrueert OL's om volwassen te worden en te myelineren, stellen we voor dat de inactiviteit van de parvalbumine interneuronen in APO-SUS mPFC ook bijdraagt aan de waargenomen OL rijpingsstop (Hoofdstuk 4). Er zijn echter aanvullende experimenten nodig om dit verder te onderzoeken.

Myelinemembranen bestaan voornamelijk uit lipiden en daarom is in OL's een juist evenwicht in lipiden, zogenaamde lipide homeostase, belangrijk voor een goede myelinisatie. Uit analyses van de hoeveelheden RNA in post-mortem dorsolaterale (dl)PFC van SZ-patiënten en controlepersonen bleek in de groep van differentieel tot expressie komende RNA's een verrijking van RNA's te zijn die bij de afgifte van cholesterol betrokken zijn (Hoofdstuk 5). Met behulp van genetische datasets, identificeerden we tevens een genetische verwantschap tussen SZ en lipiden, inclusief acylcarnitines, lipidepartikels, en de verhouding tussen verzadigde en niet-verzadigde vetzuren. Dit bevestigde het verband tussen SZ en cholesterolafgifte. Met behulp van een vrij-beschikbare set aan structurele magnetische resonantie imaging (MRI) data vonden we bovendien dat verminderde cognitieve prestaties van SZ-patiënten waren

gecorreleerd met een verminderd macromoleculair vetgehalte in de grijze stof van de dlPFC. Veranderingen in lipide homeostase spelen dus een belangrijke rol bij het tot stand komen van de cognitieve symptomen van SZ, waarschijnlijk door een negatief effect op het proces van myelinisatie.

De resultaten van het onderzoek dat gepresenteerd wordt in dit proefschrift benadrukken het belang van de ontwikkelingsprocessen die optreden in de PFC tijdens de adolescentie en dragen bij aan ons begrip van de neurobiologische achtergrond van de oorzaak van de cognitieve symptomen in SZ. Ik concludeer dat myeline van belang is in SZ en raad op basis van de verworven neurobiologische inzichten de toepassing aan van een combinatie van chronische NAC-behandeling en gedragstherapie (zoals sociabiliteitstraining, lichaamsbeweging en omgevingsverrijking) die als preventieve strategie gebruikt kan worden en tevens een aantrekkelijke manier is om hypomyelinisatie in de PFC en cognitieve symptomen van SZ af te zwakken.

# Résumé

La schizophrénie (SZ) est une maladie neuropsychiatrique complexe, dont l'étiologie est encore inconnue et dont les symptômes cognitifs (troubles de la mémoire et des fonctions exécutives) ne sont pas encore complètement traités. La compréhension des mécanismes cellulaires et moléculaires, à l'origine de ces altérations cognitives, est donc importante pour l'élaboration de nouvelles stratégies thérapeutiques. Dans les chapitres 1 et 2 de ma thèse, j'ai émis l'hypothèse que le développement du cortex préfrontal (CPF) est affecté dans cette pathologie neuropsychiatrique. Nous avons émis l'hypothèse que le stress oxydatif, observé dans le CPF de patients SZ, pourrait altérer la maturation des oligodendrocytes, cellules à l'origine de la synthèse de myéline (OLs), et conduire à des altérations de la myélinisation du CPF dans la SZ. Afin de tester cette hypothèse, dans le chapitre 3, nous avons utilisé une souche de rats sensibles à l'apomorphine (APO-SUS), reproduisant certaines caractéristiques de la SZ. Nous avons montré que les rats APO-SUS présentent des altérations de la mémoire spatiale et du comportement social, en comparaison aux rats contrôles (APO-UNSUS). Ces fonctions cognitives sont dépendantes du CPF et ne semblent pas impliquer d'autres aires cérébrales. De l'adolescence à l'âge adulte, les niveaux d'expression des gènes codant pour les protéines de la myéline sont diminués dans le CPF des rats APO-SUS, caractérisés par une hypo-myélinisation sévère. Des analyses ultra-structurales du CPF , nous ont permis de confirmer l'hypo-myélinisation des axones parvalbumines chez le rat APO-SUS, sans signe évident de démyélinisation. Nos données démontrent ainsi, pour la première fois, une hypo-myélinisation sélective des interneurones parvalbumines dans un modèle de la SZ et soulignent l'importance des altérations de la myélinisation des aires corticales dans l'étiologie de la SZ. De façon intéressante, nous avons démontré que l'hypo-myélinisation des axones parvalbumines est corrigée par un environnement enrichi (EE), une activité physique et les interactions sociales. Cette restauration de la myéline du CFP chez les rats APO-SUS est également corrélée à une réduction des troubles cognitifs. Nos résultats suggèrent que l'EE, l'activité physique et les interactions sociales pourraient être proposés pour corriger les symptômes cognitifs chez les personnes atteintes de SZ.

Une des caractéristiques majeures de la SZ est un stress oxydatif élevé. En effet, les niveaux d'antioxydants du glutathion sont fréquemment réduits et ceux des radicaux libres augmentés dans le plasma de patients SZ. De plus, des études ont montré des altérations mitochondriales dans le tissu cérébral post-mortem de cas SZ. Dans le chapitre 4, nous avons retrouvé ce stress oxydatif dans le CFP des rats APO-SUS. Ce stress oxydatif est caractérisé par une diminution du métabolisme antioxydant du glutathion et par une augmentation du nombre de mitochondries. Cette réduction du métabolisme du glutathion chez le rat APO-SUS précède l'hypo-myélinisation du CPF.

RÉSUMÉ

Nous avons émis l'hypothèse que ce stress oxydatif pourrait affecter les focntions des interneurones du CPF, et ce de plusieurs manières: i) le stress oxydatif peut causer des dommages aux interneurones parvalbumines et conduire à leurs dysfonctionnements; ii) le stress oxydatif peut entraver la myélinisation des interneurones parvalbumines, en raison des effets délétères sur les OLs, cellules particulièrement vulnérables au stress oxydatif. Dans le chapitre 4, nous avons montré que le traitement par un antioxydant (N-acétylcystéine) permet de restaurer les taux plasmatiques de glutathion, l'expression des gènes liés au glutathion, ainsi que le nombre de mitochondries chez les rats APO-SUS. Par ailleurs, ce traitement a permis de corriger l'hypo-myélinisation des interneurones parvalbumines et les déficits cognitifs des rats APO-SUS. Nos résultats suggèrent que les antioxydants, comme le NAC, sont d'un grand intérêt thérapeutique pour le traitement des troubles cognitifs de la SZ.

Afin de mieux comprendre les mécanismes à l'origine de l'hypo-myélinisation du CPF chez le rat APO-SUS, nous avons également analysé le développement des cellules oligodendrocytaires dans le CPF des rats APO-SUS et APO-UNSUS. Nos résultats ont démontré une diminution significative du nombre d'oligodendrocytes matures (OLs), due à un blocage de leurs maturations dans le CPF du rat APO-SUS. De plus, nos données *in vitro* ont montré que ce blocage de la maturation des OLs chez le rat APO-SUS est dû à des facteurs extrinsèques. Effet, le stress oxydatif inhibe la maturation des OLs APO-SUS et APO-UNSUS, *in vitro*. A l'inverse, le traitement par le NAC et l'EE restaurent la densité de cellules oligodendrogliales et de pré-OLs, sans toutefois restaurer la densité d'OLs matures dans le CPF du rat APO-SUS. Nos données suggèrent que le stress oxydatif ne permet pas à lui seul d'expliquer le blocage de maturation des OLs chez le rat APO-SUS. D'autres mécanismes dépendants de l'activité des interneurones doivent être envisagés.

Dans la dernière partie de ma thèse, je me suis intéressée au métabolisme des lipides, et plus particulièrement du cholestérol chez le rat APO-SUS. La biosynthèses et l'homéostasie des lipides sont d'une importance majeure pour la production de myéline. Dans le chapitre 5, nous avons effectué une étude RNAseq du CPF dorso-latéral de cerveaux post-mortem SZ et contrôles. Cette étude nous a permis d'identifier un certain nombre de gènes de la voie de signalisation LXR/RXR-cholestérol, dont l'expression est fortement augmentée dans la SZ. L'ensemble de ces résultats suggère que l'homéostasie des lipides pourrait également avoir un rôle prépondérant dans les atteintes cognitives de la SZ, probablement via son action sur le processus de myélinisation.

Les travaux effectués au cours de ma thèse mettent en exergue l'importance du développement du CPF dans les maladies neuropsychiatriques, comme la SZ. Ils permettent également de mieux comprendre certains des mécanismes à l'origine de la SZ. Nos données démontrent un rôle central de la myélinisation du CPF dans l'étiologie de la SZ et l'importance des thérapies pro-myélinisantes dans cette pathologie, avec un fort impact sociétal.

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Liefs,
Dorien

# Curriculum Vitae

Dorien Maas obtained her gymnasium diploma in 2010 at the Kennemer College (Beverwijk, The Netherlands). Her Bachelor degree in Health and Life sciences was completed at the Free University of Amsterdam in 2013, comprising a major in Biomedical Sciences, a minor in Biomolecular Neurosciences and an internship in Neuroproteomics under the supervision of Dr. K.W. Li, and conducted at the Department of Molecular and Cellular Neurobiology (Center for Neurogenomics and Cognitive Research, CNCR, Amsterdam, The Netherlands). From 2013 to 2015 she obtained her Master degree in Cognitive Neuroscience at Radboud University, which included a research internship under the supervision of Dr. Astrid Vallès and Prof. dr. Gerard Martens at the Department of Molecular Animal Physiology (Donders Institute for Brain, Cognition and Behaviour, Centre for Neuroscience, Faculty of Science, Radboud University, Nijmegen, The Netherlands) that layed the foundation for the work presented in this thesis. In 2014, Dorien obtained a grant from the Radboud Honours programme Beyond the Frontiers to perform a research project at the Department of Pharmacological Sciences under the supervision of Prof. Dr. M. Riva at the University of Milan (Milan, Italy). In 2015, the Royal Netherlands Academy of Arts and Sciences (KNAW) awarded Dorien a prize for the best Master thesis in Life Sciences (Chapter 2), sponsored by Pfizer Pharmaceuticals. In the same year, she obtained a TOPtalent research grant from the Donders Institute to perform PhD-research at the departments of Molecular Animal Physiology and Cognitive Neuroscience (Radboud university medical centre, Nijmegen, The Netherlands) under the supervision of Prof. Dr. G.J.M. Martens and Prof. Dr. J.R. Homberg. During her PhD, Dorien set up a collaboration with Prof. Dr. B. Nait-Oumesmar (Laboratory for Myelin Plasticity and Repair at the Brain and Spine Institute, Sorbonne University, Paris, France). A Van Gogh travel grant obtained in 2017 allowed her to perform nearly two years of her PhD research in Paris. The results of her PhD-project are described in this thesis in order to obtain a PhD-degree at Radboud University as well as at Sorbonne University.

# List of Publications

–   Selten M.M., Meyer F., Ba W., Vallès A., **Maas D.A.**, et al., Increased GABAB receptor signaling in a rat model for schizophrenia. *Sci Rep* **6**, 34240 (2016).

–   **Maas D.A.**, Vallès A., Martens G.J.M. Oxidative stress, prefrontal cortex hypomyelination and cognitive symptoms in schizophrenia. *Transl Psychiatry* **7**, e1171 (2017).

–   **Maas D.A.**\*, Jager A.\*, Fricke K., De Vries R.B., Poelmans G.#, Glennon J.C.# Aggressive behavior in transgenic animal models: A systematic review. *Neuroscience & Biobehavioral Reviews* **91**, 198-217 (2018).

–   **Maas D.A.**, Eijsink V.D., Van Hulten J.A., De Weerd P., Homberg J.R., Vallès A., Nait-Oumesmar B. #, Martens G.J.M. # Interneuron hypomyelination contributes to cognitive inflexibility in schizophrenia. *Nature Communications, revised manuscript submitted.*

–   **Maas D.A.**, Eijsink V.D., Spoelder M., Van Hulten J.A., De Weerd P., Homberg J.R., Vallès A., Nait-Oumesmar B. #, Martens G.J.M. # Neurobiological basis and repair of interneuron hypomyelination and cognitive inflexibility in rat model for schizophrenia. *Submitted.*

–   **Maas D.A.**, Martens M.B., Priovoulos N., Zuure W., Homberg J.R., Nait-Oumesmar B. , Martens G.J.M. Key role for lipids in cognitive symptoms of schizophrenia. *Submitted.*

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