The influence of common genetic variations in candidate genes on neuropsychiatric phenotypes

PhD Thesis

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Declaration

I hereby declare that this thesis has been written independently and with no other sources and aids than quoted.

Anne Kästner Göttingen, 01 August 2013

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Table of contents

1. Introduction and aims of the present thesis work

Psychiatric disorders are 'complex diseases' (Meyer-Lindenberg et al. 2006, Craddock et al. 2007). They do not segregate in a Mendelian manner within families and are influenced by both genetic and environmental factors (Visscher et al. 2012). As has been revealed by twin and adoption studies, a large proportion of the phenotypic variation of psychiatric conditions like schizophrenia and autism can be attributed to additive genetic effects (80%-90%) (Cardno et al. 1999, Sullivan et al. 2003). The methodological approaches to unravel the genetic architecture of common psychiatric conditions range from candidate gene studies (Gurling 1986), over linkage studies (Risch et al. 1993) to hypothesis-free designs such as genome-wide association studies (GWAS) (Cichon et al. 2009, Bondy 2011). Although being the most widely applied genetic paradigm in the past decade, GWA studies have lagged behind high expectations (Girard et al. 2012, Visscher et al. 2012).

GWAS rely on the artificial dichotomization of multi-dimensional quantitative traits

GWA studies compare the allele frequency of genetic markers like single nucleotide polymorphisms (SNPs) between unrelated individuals carrying a clinical diagnosis (cases) and healthy subjects (controls) (Cichon et al. 2009, Bondy 2011). These studies draw on the principle of linkage disequilibrium (LD), which refers to the non-random association between alleles at different loci. Although 90% of human genetic variation is ancient, new mutations contributing to an increase or decrease in disease risk constantly emerge in populations (McClellan et al. 2010). By random drift or natural selection some of these mutations can become more frequent in the population (Kimura 1976, Hartl et al. 1997). These causal variants will be associated by LD with SNPs that are contained on SNP arrays produced by commercial companies. The included 'tag SNPs' capture a major part of the common genomic variation in the human population. The scientific community hoped for a majority of causal variants across the entire genome to be accounted for by the 'SNP chips'(Visscher et al. 2012). Thus, GWA studies were expected to explain a major part of the heritability of psychiatric disorders such as schizophrenia (Visscher et al. 2012). This chronic disorder, characterized by bizarre delusions as its unique feature but also by hallucinations, negative symptoms and cognitive decline, affects about one person in 100 and usually strikes in late adolescence or early adulthood (Bleuler 1979). Although not long ago scientists sought to discover the 'schizophrenia gene' (Slater 1958), results in support of the polygenic theory of schizophrenia (Gottesman et al. 1967) emerged in past years. Hundreds of genetic variants

have been found to be differentially distributed between schizophrenic cases and controls. The number of detected variants per study increased with augmenting sample sizes. Disappointingly, only few results could be replicated across studies (Visscher et al. 2012). Among the most replicated GWAS results are markers in the major histocompatibility complex region and in *TCF4*, *ZNF804A* and neuregulin1 genes (Stefansson et al. 2009, Steinberg et al. 2011, Steinberg et al. 2011). The limited reproducibility of GWAS results can be partially attributed to odds ratios (OR) below 1.5 (as they are generally identified by GWAS) which can be explained by cryptic population stratification (McClellan et al. 2010). As a result, the most replicated major 'GWAS hits' together explain less than 1% of the variance in liability to schizophrenia (Visscher et al. 2012).

Unclear biological relevance of common genetic variants associated with schizophrenia

GWAS are by design biased towards detection of association with causal genetic variants that are relatively common in the population because they are based on the principle of LD (Visscher et al. 2012). Besides common genetic variation, highly penetrant rare variants of large effect (such as copy number variations) have been reported to be associated with the risk for schizophrenia in the past years (Bassett et al. 2010, Owen et al. 2010, Rapoport et al. 2011). Presently, whether most genetic variation contributing to complex traits is caused by rare or common variants is controversially discussed (Risch et al. 1996, Visscher et al. 2012). GWA studies were useful in indicating that multiple common SNPs of small effect sizes are implicated in common psychiatric conditions (Purcell et al. 2009). In a recent report it was estimated that 23% of variation in susceptibility to schizophrenia can be explained when all SNPs represented across the current generation of GWAS arrays are considered simultaneously (Lee et al. 2012). Findings from GWA studies imply that we all might carry some risk variants but our systems are robust to their effects due to compensatory mechanisms. In affected individuals, the burden of risk variants might be too high to be efficiently compensated for. Consequently, common genetic variation seems to play an important role in explaining schizophrenia heritability and will be the focus of the present thesis work. Nevertheless, although associated with the endpoint diagnosis of schizophrenia, the GWAS 'top-10' SNPs individually or upon their accumulation do not seem to modulate the severity of lead symptoms of schizophrenia (Papiol et al. 2011) (see attachment, co-authorship III). Putative functional implications of genes emerging from GWAS are often derived from their involvement in particular molecular networks (Luo et al. 2013). It remains unclear, however, how the pathways enriched for genetic variants associated with a

schizophrenia diagnosis; modulate particular phenotypic features of the condition. Consequently, the vast majority of the variants resulting from GWAS and have no established biological relevance or clinical utility for prognosis or treatment (McClellan et al. 2010).

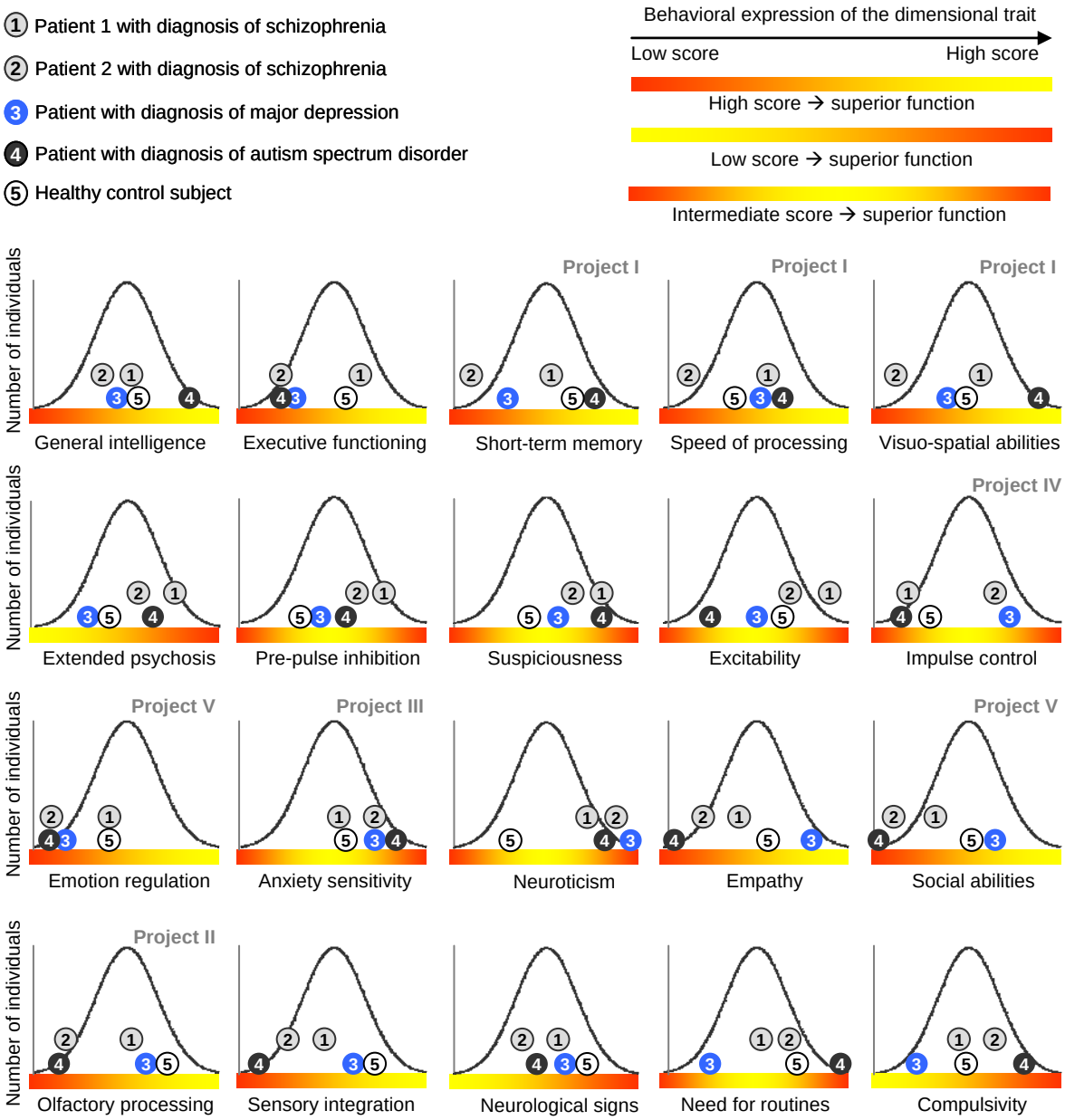

Figure 1 Example of possible subphenotypes resulting from a decomposition of schizophrenia into quantitative traits. When assessed with adequate psychometric measures (i.e. neuropsychological tests, questionnaires, behavioral assays) in a non-selected population of individuals, many dimensional phenotypes are normally distributed. For some assessment tools, high scores may reflect better function (i.e. cognitive variables, first row) whereas as for others low (i.e. neurological signs) or intermediate scores (anxiety sensitivity) may be most functional/adaptive. Individuals belonging to different diagnostic categories and healthy controls can be allocated along the different behavioral dimensions (every person is assigned a score for each 'micro-phenotype'). Schizophrenic patient 1 and 2 (gray circles) differ substantially in terms of the behavioral expression of certain traits exemplifying the tremendous amount of variability within a group of schizophrenic cases. Likewise, an individual who does not have a clinical diagnosis (white circle) might score towards the dysfunctional end of certain behavioral dimensions. The same holds true for individuals carrying an affective or autism spectrum disorder diagnosis suggesting overlapping biological pathways relevant for seemingly distinct diagnostic entities. Some of the depicted phenotypes are relevant for the projects included in this thesis.

Phenotypic approaches to the 'missing heritability' of schizophrenia

How can the 'missing heritability' of schizophrenia be uncovered? Many researchers suppose that genome-wide studies will ultimately lead to a higher amount of explained variance in the liability to schizophrenia when sample sizes are increased such that variants of smaller effects can be detected (Cichon et al. 2009, Sullivan 2012). Others believe that describing the real causal variants using deep-sequencing will substantially contribute to our understanding of the genetic architecture of schizophrenia (Duan et al. 2010, Myers et al. 2011). However, these approaches neglect the very important fact that schizophrenia is a syndrome comprising many different behavioral domains which are differentially pronounced across affected individuals. The present thesis work is based on the assumption that a decomposition of schizophrenia into single quantitative behavioral dimensions will assist in delineating the functional relevance of certain genes by elucidating their influence on particular aspects of the phenotype (phenotype-based genetic association study). Additionally, alternatively to ever increasing sample sizes, it will lead to a substantial increase in statistical power.

Reducing inter-individual variability by decomposing schizophrenia into quantitative traits

The tremendous amount of intra-group variability can be partly ascribed to the fact that schizophrenia is not a one-dimensional phenomenon. It can be conceptualized as composed of various continuous behavioral dimensions which can be assessed quantitatively (Figure 1) (Plomin et al. 2009). Schizophrenia is not homogeneous either. Given the manifold inter-individual phenotypic expressions of schizophrenia symptoms (compare schizophrenia patient 1 and 2 in Figure 1); the integrity of more than one biological pathway is likely to be compromised in this disease (Lee et al. 2013). Thus, certain phenotypically definable subgroups of patients likely differ as to the predominant involvement of certain biological pathways. As a result, a large amount of 'noise' reducing statistical power in GWA studies stems from inter-individual heterogeneity in symptom composition.

Achieving genetic inter-group separation by contrasting the extremes of quantitative traits

GWAS rely on the classification of individuals as cases and controls although this dichotomization is rather artificial (Gottesman et al. 1967). Besides the presence of certain sets of symptoms, a clinical diagnosis requires a person to experience subjective psychological burden or to not function vocationally or socially anymore. This largely depends on the degree of misfit between individual and environment and on how well certain functions are compensated for by the social network. Along the same lines, population

research revealed high rates of psychotic experiences (4-8%) in people who are not diagnosable according to the current classification systems (van Os et al. 2012). Literature suggests a psychometric continuum in the sense of an extended psychosis phenotype sharing etiological factors with the clinical disorder (van Nierop et al. 2011, van Os et al. 2012). Consequently, if schizophrenia is conceptualized as composed of various quantitative behavioral traits, many of the putative 'controls' are nearly cases (Figure 1; e.g. 'excitability' and 'compulsivity') (Plomin et al. 2009). This further diminishes the likelihood of detecting etiologically meaningful differences between the groups. Consequently, statistical power can be significantly enhanced by comparing the low and high extremes of quantitative phenotypes (see projects II and V) or by studying the entire trait distribution with respect to certain candidate genes or functional gene complexes (compare projects I; III and IV).

Aims of the present thesis work

Defining quantitative subphenotypes is indispensable for understanding how *common genetic variants modulate phenotypes of psychiatric disorders* and for how molecular biological processes mediate the relationship between genetic and phenotypic variation. My scientific work has been devoted to the operationalization, quantification and validation of complex phenotypes underlying psychiatric disorder like schizophrenia, using the GRAS (Göttingen Research Association for Schizophrenia) data collection (Ribbe et al. 2010) (for a detailed description see attachment, co-authorship I). Moreover, I was responsible for the statistical analyses of associations of candidate genes with these quantitative phenotypes. Hypotheses for specific genotype-phenotype relationships were derived from the genes' established molecular functions and/or from studies with mouse models of loss-and gain-of function of the respective proteins. Projects I-IV provide proof-of-concept for our phenotype-based genetic association study (PGAS) approach by elucidating the association of certain candidate genes with specific continuous behavioral variables in the schizophrenic GRAS sample (project I: *EPO/EPOR* & higher cognition; project II: *Neuregulin1* & central olfactory processing; project III: *GPM6A* & claustrophobic anxiety; project IV: *MECP2* & impulsive aggression). Ultimately, progress in etiological research leading to the development of new therapeutic targets will require the definition of more homogeneous disease subgroups based on highly intercorrelated quantitative phenotypes. Project V of the present work will give an example of the phenotypic definition of an autistic subgroup of patients with schizophrenia which will provide the ground for a future definition of a clinically relevant biological subgroup of schizophrenic patients.

2. *EPO* and *EPOR* variants modulate cognitive performance in schizophrenia

2.1 Overview of project I

Besides stimulating erythropoiesis (Adamson 1996), erythropoietin (EPO) and its receptor (EPOR) were repeatedly shown to have neurothrophic, angiogenic, anti-oxidative, anti-inflammatory, anti-apoptotic, and stem cell modulating properties *in vitro* and *in vivo* (Brines et al. 2005, Sargin et al. 2010). The observation that EPOR receptor (EPOR) is expressed in neural cells (Morishita et al. 1997) and that EPO is endogenously produced in the brain in a hypoxia-dependent way (Marti 2004), gave rise to the notion that the endogenous EPO/EPOR system serves important neuroprotective functions in non-hematopoietic tissues like the brain. Indeed, evidence supporting a neuroprotective effect for recombinant human erythropoietin (rhEPO) in stroke, schizophrenia, multiple sclerosis and even healthy individuals accumulated over the past years (Ehrenreich et al. 2008, Siren et al. 2009). In these studies beneficial effects of rhEPO regarding stroke outcome measures, cognition and gray matter loss were revealed (Ehrenreich et al. 2002, Ehrenreich et al. 2007, Ehrenreich et al. 2007, Miskowiak et al. 2007, Bartels et al. 2008, Ehrenreich et al. 2011, Wüstenberg et al. 2011) (see co-authorship II). Both in schizophrenic and healthy individuals, the cognitive improvement induced by rhEPO was most pronounced in the domains ‘speed of processing’ and ‘immediate memory’. Importantly, these effects could not be accounted for by an increase in hemoglobin (Miskowiak et al. 2007).

Various studies focus on the phenotypic relevance of the endogenous EPO/EPOR system by searching for associations of *EPO* and *EPOR* genetic markers with myeloprolieferative/-dysplastic syndromes or diabetic retinopathy. Many of them remain inconsistent, especially for the *EPO* gene (de la Chapelle et al. 1993, Sokol et al. 1993, Sokol et al. 1994, Arcasoy et al. 1997, Furukawa et al. 1997, Kralovics et al. 1997, Percy et al. 1997, Watowich et al. 1999, Jedlickova et al. 2003, Petersen et al. 2004, Tong et al. 2008, Abhary et al. 2010, Ma et al. 2010). Although the positive influence of exogenously administered rhEPO on cognition is quite established, the modulatory influence of the *EPO* and *EPOR* genes on cognitive functioning has not been elucidated so far.

As proof-of-concept of our phenotype-based genetic association study approach, we aimed at investigating whether common genetic variants in the *EPO* and *EPOR* genes modulate cognitive functioning in the schizophrenic GRAS sample. To support our assumption that genotype-phenotype relationships are independent of clinical diagnoses, we intended to replicate our findings in a phenotyped control population from Munich (van den Oord et al. 2008).

For the *EPO* gene, two single nucleotide polymorphisms (SNPs) located in functionally interesting regions (5' upstream and the 3' regulatory region) were selected and genotyped: rs167640 (T/G) and SNP rs56444 (T/G). To cover variability in the *EPOR* gene, we chose the short tandem repeat (STR) STR $(GA)_n$ in the *EPOR* 5' upstream area. In order to compare results in patients and healthy controls, relevant cognitive domains (speed of processing and perceptual organization) were operationalized by either using the same test or by using a measure previously shown by factor analysis to share a common source of underlying statistical variance (Berger 1998). Unfortunately, short-term memory function could only be assessed in the patients as no such measure was available for the replicate sample. By employing statistical models correcting for possible confounding factors (such as medication and negative symptoms), we found carriers of the G allele in the *EPO* SNP rs167640 and of *EPOR* STR $(GA)_n$ low repeat sum to be superior in all selected neuropsychological readouts. Interestingly, carriers of one particular combination of *EPO* and *EPOR* genotypes (GG genotype and 21-35 repeat sums, 'GG & 21-35') were found to outperform all other patients. In the healthy control sample, essentially all phenotype-genotype associations could be reproduced, except for the advantage of the 'GG & 21-35' genotype combination. However, when analyzing only those healthy subjects cognitively performing at the level of schizophrenic patients, a similar tendency of genotype superiority could be revealed. These findings suggest that a cognitive advantage of the 'GG & 21-35' constellation may only become obvious when cognitive capacities are reduced. As a next step we wondered, how this phenotype-genotype relationship is biologically mediated. As *EPO* and *EPOR* variants are located in the promoter regions of the respective genes, they are likely to influence transcription factor binding and thus regulability of the system. Mechanistically, we could demonstrate that the cognitively superior genotypes were associated with higher expression/regulability of gene expression. As a conclusion, thesis project I provides evidence for common variants in the *EPO/EPOR* genes to modulate cognitive performance, especially in patients suffering from cognitive decline but also in cognitively inferior healthy subjects.

2.2 Original publication

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*These authors contributed equally to the work.

Personal contribution

I was responsible for the selection and operationalization of the cognitive readouts for the schizophrenic sample and the sample of healthy controls, the statistical analyses combining genetic and phenotypic information, and the interpretation of data for schizophrenic patients and healthy controls and the design of figures and tables. Moreover, I was involved in the conception, design, drafting, revision and publication of the manuscript.

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Erythropoietin Modulates Cognition in Schizophrenia

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Common Variants of the Genes Encoding Erythropoietin and Its Receptor Modulate Cognitive Performance in Schizophrenia

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Erythropoietin (EPO) improves cognitive performance in clinical studies and rodent experiments. We hypothesized that an intrinsic role of EPO for cognition exists, with particular relevance in situations of cognitive decline, which is reflected by associations of *EPO* and *EPO* receptor *(EPOR)* genotypes with cognitive functions. To prove this hypothesis, schizophrenic patients (N > 1000) were genotyped for 5′ upstream–located gene variants, *EPO* SNP rs1617640 (T/G) and *EPOR* STR(GA)$_n$. Associations of these variants were obtained for cognitive processing speed, fine motor skills and short-term memory readouts, with one particular combination of genotypes superior to all others ($p < 0.0001$). In an independent healthy control sample (N > 800), these associations were confirmed. A matching preclinical study with mice demonstrated cognitive processing speed and memory enhanced upon transgenic expression of constitutively active EPOR in pyramidal neurons of cortex and hippocampus. We thus predicted that the human genotypes associated with better cognition would reflect gain-of-function effects. Indeed, reporter gene assays and quantitative transcriptional analysis of peripheral blood mononuclear cells showed genotype-dependent *EPO/EPOR* expression differences. Together, these findings reveal a role of endogenous EPO/EPOR for cognition, at least in schizophrenic patients.

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INTRODUCTION

Erythropoietin (EPO), originally described as a hematopoietic growth factor, has been found to improve cognition almost since its first clinical approval for the treatment of renal anemia, but this effect has long been attributed solely to its indirect action via increased hemoglobin (1). Over the last 15 years, the importance of EPO for nonhematopoietic tissues, particularly the nervous system, has been recognized increasingly. In addition to direct neuroprotective and neuroregenerative functions of the EPO system, its effects on neuroplasticity and cognition have become evident (2–4). EPO treatment has been shown to improve learning and memory functions, not only in disease models, but also in healthy rodents (5–7). In clinical trials on patients suffering from schizophrenia or multiple sclerosis, high-dose rhEPO infusions over several months resulted in better cognitive performance (8,9). Functional magnetic resonance imaging 1 wk after just a single high dose of rhEPO revealed an enhancement of the hippocampal response during memory retrieval in healthy human subjects (10). Importantly, cognitive improvement in these studies was not linked to an increase in hemoglobin. Still-remaining concerns that EPO might exert these cognitive effects via enhanced hemoglobin were further alleviated by the functional separation of hematopoietic and neuro-

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protective actions using EPO variants, devoid of hematopoietic properties (11). In addition, neuroregenerative effects of EPO in insects, lacking hematopoiesis, suggest an intrinsic and phylogenetically ancient significance of EPO for neuroplasticity and cognition (12).

Studies on the role of *EPO* or *EPOR* genotypes have focused on searching for associations of genetic markers in these genes with readouts of the hematopoietic system in healthy individuals and disease states, such as myeloproliferative or myelodysplastic syndromes. The results were mainly negative for *EPO* genotypes (13–18), but several associations for *EPOR* polymorphisms or rare mutations with various forms of familial polycythemia were identified (19,20). Three publications deal with *EPO* genotypes in diabetic retinopathy. One turned out negative (21), whereas the other two found associations, but the results remain contradictory since opposing risk genotypes were described (22,23). Another study reported an association of an *EPO* genotype with age of onset of amyotrophic lateral sclerosis (24). No data are available yet on associations of *EPO* or *EPOR* genotypes with brain functions, including cognition.

We hypothesized that an inherent relevance of EPO for cognitive functioning and neuroplasticity in humans should be reflected by associations of *EPO* and *EPOR* genotypes with selected readouts of cognitive performance and might be uncovered in a disease characterized by cognitive decline. To test these hypotheses, we exploited the GRAS (*Göttingen Research Association for Schizophrenia*) data collection, which provides a unique ground for phenotype-based genetic association studies (PGAS) with information on over 1,000 well-characterized schizophrenic patients (25,26). We show here that in this population, as well as in a healthy replicate sample, *EPO*/*EPOR* genotypes are associated with several domains of higher cognition. Moreover, on the basis of reporter gene assays and mouse studies, we propose that better performance is linked to higher expression/regulability of the endogenous EPO system.

MATERIALS AND METHODS

Subjects

Disease sample. The GRAS data collection was approved by the ethics committee of the Georg-August-University (master committee) and the local internal review boards of the collaborating centers. The project complies with the Helsinki Declaration (27). Patients fulfilling DSM-IV criteria for schizophrenia or schizoaffective disorder were included regardless of the stage of the disease (acute, chronic, residual or remitted). All study participants and, if applicable, their legal representatives gave written informed consent (for detailed information on the GRAS sample, see reference 26). A total of N = 1,050 GRAS patients were successfully genotyped for the *EPO* SNP (single nucleotide polymorphism T/G) rs1617640, N = 1,054 for the *EPO* SNP (T/G) rs564449, and N = 1,054 for *EPOR* short tandem repeat, STR$(GA)_n$, and are included in the present genetic analyses. Most GRAS patients are of European Caucasian ethnicity (Caucasian 95.4%; other ethnicities 1.8%; unknown 2.8%). Peripheral blood mononuclear cells (PBMCs) were taken from a subsample of the GRAS cohort (N = 98) to analyze mRNA expression dependent on genotype.

Case control sample. Healthy voluntary blood donors were recruited by the Department of Transfusion Medicine at the Georg-August-University of Göttingen according to national guidelines for blood donation to serve as control subjects. As such, they widely fulfill health criteria, ensured by a broad predonation screening process including standardized health questionnaires, interviews and assessment of hemoglobin concentration, blood pressure, pulse and body temperature. Of the N = 1,141–1,142 successfully genotyped control subjects, 58.9% were male (N = 672–673) and 41.1% female (N = 469–470). The average age was 34.61 ± 12.30 y (range 18 to 69). The majority of the control subjects were of European Caucasian ethnicity (Caucasian 97.8%; other ethnicities 2%; unknown 0.2%).

Independent healthy control sample (replicate sample). Unrelated volunteers of German descent (that is, both parents German) were selected randomly from the general population of Munich, Germany, and contacted by mail (28). To exclude subjects with central neurological diseases and psychotic disorders or individuals who had first-degree relatives with psychotic disorders, several screenings were conducted before volunteers were enrolled in the study. Firstly, subjects who responded were screened initially by phone for the absence of neuropsychiatric disorders. Secondly, detailed medical and psychiatric histories were obtained for both the patients themselves as well as their first-degree relatives by using a semistructured interview. Thirdly, if inclusion criteria were fulfilled, the subjects were invited for a comprehensive interview including the German version of the structured clinical interview for DSM-IV (SCID I and SCID II) (29) to confirm the absence of any lifetime psychotic disorder. Additionally, the family history assessment module (30) was conducted to exclude psychotic disorders among first-degree relatives. Furthermore, a neurological examination was conducted to exclude subjects with current central nervous system impairment. In case of volunteers being older than 60 years, the Mini-Mental State Examination (MMSE; 31) was performed to exclude subjects with possible cognitive impairment. Written informed consent was obtained from all individuals after providing them with a detailed description of the study, which was approved by the local ethics committee and carried out in accordance with the Helsinki Declaration (27).

Phenotype-Based Genetic Association Study (PGAS)

Disease sample. On the basis of findings of our previous EPO treatment trial with schizophrenic patients (9), neuropsychological measures of processing speed (Digit Symbol-Coding [Zahlen-Symbol-Test], a subtest of German version of Wechsler Adult Intelligence Scale [WAIS; 32]) and perceptual organization (subtests Dotting and Tapping from Mac-

Table 1. Association of *EPO* and *EPOR* genotypes with cognitive target variables in schizophrenic patients (GRAS) and healthy individuals.

	EPO rs1617640				*EPOR* STR $(GA)_n$			*EPO* and *EPOR*		
	GG	GT	TT	Effect (*p*)[a]	Low sum	High sum	Effect (*p*)	GG and 21–35 repeats	All other combinations	Effect (*p*)
	Disease sample (GRAS), mean ± SD (95% CI)[b,c,d]									
	N = 161–172	N = 408–465	N = 345–381		N = 466–509	N = 448–509		N = 60–63	N = 854–955	
Perceptual organization										
Dotting and Tapping	0.08 ± 1.98 (–0.22–0.38)	0.02 ± 1.83 (–0.14–0.19)	–0.11 ± 1.94 (–0.30–0.09)	1.492 (0.222)	0.01 ± 1.85 (–0.16–0.16)	–0.03 ± 1.94 (–0.20–0.14)	4.402 (0.045)	0.65 ± 1.92 (0.18–1.13)	–0.06 ± 1.88 (–0.18–0.06)	10.259 (0.001)
Processing speed										
Digit Symbol-Coding (WAIS)[e]	40.13 ± 14.19 (38.0–42.3)	37.62 ± 12.73 (36.5–38.8)	36.93 ± 13.42 (35.6–38.3)	8.868 (0.003)	37.80 ± 13.20 (36.7–38.9)	37.77 ± 13.38 (36.6–38.9)	1.893 (0.169)	43.57 ± 14.46 (40.0–47.1)	37.40 ± 13.12 (36.6–38.2)	16.166 (<0.001)
Cognition composite[f]	0.08 ± 0.94 (–0.07–0.22)	–0.01 ± 0.88 (–0.09–0.08)	–0.07 ± 0.92 (–0.16–0.03)	3.327 (0.036)	–0.00 ± 0.89 (–0.09–0.07)	–0.02 ± 0.93 (–0.11–0.06)	4.274 (0.039)	0.37 ± 0.90 (0.14–0.59)	–0.04 ± 0.90 (–0.10–0.02)	13.891 (<0.001)
Verbal learning and memory (VLMT)[g]	42.74 ± 13.22 (40.7–44.8)	42.22 ± 12.84 (41.0–43.5)	40.62 ± 12.69 (39.3–42.0)	0.852 (0.356)	42.22 ± 13.00 (41.1–43.4)	41.18 ± 12.82 (40.0–42.4)	5.298 (0.022)	46.72 ± 12.49 (43.6–49.9)	41.36 ± 12.83 (40.5–42.2)	9.063 (0.003)
	Healthy individuals, mean ± SD (95% CI)[h]									
	N = 332	N = 1095	N = 859		N = 447	N = 434		N = 50	N = 831	
Perceptual organization										
Block Design (WAIS)[e]	31.78 ± 9.24 (30.8–32.8)	30.32 ± 9.43 (29.8–30.9)	30.32 ± 9.58 (29.7–31.0)	7.263 (0.007)	32.83 ± 9.18 (32.0–33.7)	30.99 ± 8.76 (30.2–31.8)	9.815 (0.002)	33.46 ± 9.23 (30.9–36.0)	31.83 ± 9.00 (31.2–32.4)	1.068 (0.302)
Processing speed										
Digit Symbol-Coding (WAIS)[e]	53.19 ± 13.22 (51.8–54.6)	51.74 ± 12.96 (51.0–52.5)	51.34 ± 13.33 (50.5–52.2)	4.116 (0.043)	54.29 ± 12.08 (53.2–55.4)	54.13 ± 12.12 (53.0–55.3)	0.004 (0.953)	56.56 ± 10.68 (53.6–59.5)	54.07 ± 12.16 (53.2–54.9)	1.678 (0.196)
Cognition composite[f]	0.12 ± 0.88 (0.02–0.21)	–0.01 ± 0.88 (–0.07–0.04)	–0.03 ± 0.89 (–0.09–0.03)	7.768 (0.005)	0.22 ± 0.84 (0.14–0.29)	0.11 ± 0.79 (0.04–0.19)	4.142 (0.043)	0.34 ± 0.81 (0.11–0.56)	0.16 ± 0.82 (0.10–0.21)	2.025 (0.155)

[a]Effects for GG versus T carriers.
[b]Analysis of covariance (ANCOVA) with age, negative symptoms (PANSS), medication status (chlorpromazine equivalents) and duration of disease as covariates, and Blom-transformed single targets.
[c]Owing to missing data upon phenotyping and the exclusion of nonnative German speakers for language-dependent readouts (VLMT), sample size varies between N = 914–1018 in the total GRAS sample.
[d]CI, confidence interval.
[e]Test from German version of WAIS (32).
[f]Cognition composite represents mean of Dotting and Tapping subtests and Digit Symbol-Coding test (both tests Blom transformed).
[g]Exclusion of nonnative German speakers (N = 92).
[h]Analysis of covariance (ANCOVA) with age as covariate and Blom-transformed single targets.

Quarrie Test for Mechanical Ability; 33) were selected from the GRAS database for phenotype–genotype association analyses (target variables). The "Verbal Learning and Memory Test" (Verbaler Lern- und Merkfähigkeitstest [VLMT]; 34) was included as another target variable to cover aspects of short-term memory. Additionally, to demonstrate the specificity of genotype associations with the selected cognitive readouts, sociodemographic (that is, age, gender, level of education) and clinical variables (age at first episode, duration of disease, medication status, Positive and Negative Syndrome Scale [PANSS; 35] subscales and Global Assessment of Functioning [GAF; 36]) were included in the analysis (see Table 1 for target and Table 2 for sociodemographic and clinical variables).

Healthy individuals (replicate sample). To replicate the phenotype–genotype associations found in GRAS patients in an independent group of healthy individuals, we aimed at covering comparable domains of processing speed and perceptual organization. Digit Symbol-Coding test as measure of processing speed and Block Design (Mosaik-Test) as test of perceptual organization capacities (both from German version of WAIS; 32) were employed. Unfortunately, neuropsychological tests measuring verbal memory were not available in sufficient numbers for replication.

Statistical Analyses

Statistical analyses of phenotype–genotype associations for healthy and schizophrenic individuals were per-

Table 2. Sociodemographic and disease-related variables in schizophrenic patients and healthy controls are not associated with *EPO/EPOR* genotypes.

	EPO rs1617640				*EPOR* STR $(GA)_n$			*EPO* and *EPOR*		
Basic variables	GG	GT	TT	Effect (*p*)[a]	Low sum	High sum	Effect (*p*)	GG and 21–35 repeats	All other combinations	Effect (*p*)
Disease sample (GRAS) (95% CI)[b,c,d]										
	N = 165-171	N = 452-483	N = 366-388		N = 496-519	N = 484-520		N = 60-63	N = 920-976	
Age, mean ± SD, y	40.32 ± 12.48 (38.4–42.2)	39.15 ± 12.43 (38.0–40.3)	39.69 ± 12.74 (38.4–41.0)	(0.316)	39.67 ± 12.39 (38.6–40.7)	39.42 ± 12.71 (38.3–40.5)	(0.540)	37.34 ± 12.50 (34.23–40.5)	39.69 ± 12.55 (38.9–40.5)	(0.166)
Gender, no. (%), male	110 (63.6%) (54.6–72.6)	332 (67.9%) (62.9–72.9)	260 (67%) (61.3–72.7)	1.002 (0.317)	352 (67.2%) (62.4–72.2)	350 (66.4%) (61.5–71.4)	0.094 (0.759)	43 (68.3%) (54.3–82.2)	659 (66.8%) (63.2–70.4)	0.059 (0.808)
Ethnicity, no. (%), Caucasian[e]	171 (98.8%) (97.2–100.4)	461 (94.3%) (92.2–96.4)	368 (94.8%) (92.6–97.1)	1.916 (0.384)	499 (95.4%) (93.6–97.2)	501 (95.1%) (93.2–97.0)	1.691 (0.429)	63 (100.0%) (–)	937 (94.9%) (93.5–96.3)	1.276 (0.528)
Years of education, mean ± SD[f]	12.40 ± 3.15 (11.9–12.9)	12.11 ± 2.93 (11.8–12.4)	11.95 ± 3.23 (11.6–12.3)	(0.208)	12.16 ± 3.06 (10.7–14.5)	12.05 ± 3.10 (12.0–15.7)	(0.528)	12.75 ± 3.25 (11.9–13.6)	12.06 ± 3.07 (11.9–12.3)	(0.143)
Current occupation, no. (%), unemployed	30 (17.3%) (3.8–30.9)	77 (15.7%) (7.6–23.9)	59 (15.2%) (6.0–24.4)	0.275 (0.600)	75 (14.3%) (6.4–22.3)	91 (17.3%) (9.5–25.0)	1.805 (0.179)	14 (22.2%) (0.4–44.0)	152 (15.4%) (9.7–21.1)	1.915 (0.166)
Age at first episode, mean ± SD, y	26.72 ± 9.30 (25.3–28.1)	25.73 ± 8.21 (25.0–26.5)	26.75 ± 8.94 (25.9–27.6)	(0.742)	26.35 ± 8.71 (25.6–27.1)	26.19 ± 8.66 (25.5–26.9)	(0.654)	25.12 ± 9.30 (22.8–27.4)	26.35 ± 8.64 (25.8–26.9)	(0.101)
Duration of disease (first episode), mean ± SD, y	13.25 ± 11.48 (11.5–15.0)	13.28 ± 10.71 (12.3–14.2)	12.83 ± 10.34 (11.8–13.9)	(0.811)	13.27 ± 10.44 (12.4–14.2)	12.94 ± 10.96 (12.0–13.9)	(0.365)	11.80 ± 11.27 (9.0–14.6)	13.19 ± 10.66 (12.5–13.9)	(0.161)
Smoker status, no (%), smoker	57 (33.7) (20.6–40.8)	128 (27.2) (23.2–31.2)	113 (30.5) (25.8–35.2)	2.776 (0.250)	148 (29.1) (25.2–33.0)	154 (30.5) (26.5–34.5)	0.244 (0.621)	22 (35.5) (23.6–47.4)	276 (29.1) (26.2–32.0)	1.135 (0.287)
Chlorpromazine equivalents, mean ± SD	637.1 ± 514.8 (559.5–714.7)	724.29 ± 792.18 (653.6–794.9)	671.7 ± 647.5 (607.0–736.4)	(0.957)	751.5 ± 727.3 (637.9–736.1)	680.6 ± 674.2 (622.5–738.7)	(0.486)	542.6 ± 427.9 (436.9–648.3)	713.1 ± 714.2 (655.3–754.0)	(0.174)
PANSS positive, mean ± SD	13.49 ± 6.17 (12.6–14.4)	14.30 ± 6.75 (13.7–14.9)	13.16 ± 5.75 (12.6–13.7)	(0.669)	13.95 ± 6.45 (13.4–14-5)	13.53 ± 6.16 (13.0–14.1)	(0.355)	13.98 ± 6.59 (12.3–15.6)	13.72 ± 6.30 (13.3–14.1)	(0.805)
PANSS negative, mean ± SD	17.69 ± 7.78 (16.5–18.9)	18.68 ± 8.11 (17.9–19.4)	18.09 ± 7.73 (17.3–18.9)	(0.286)	18.80 ± 8.30 (18.1–19.5)	17.78 ± 7.49 (17.1–18.4)	(0.106)	18.18 ± 7.98 (16.2–20.2)	18.30 ± 7.92 (17.8–18.8)	(0.863)
PANSS general, mean ± SD	33.37 ± 11.17 (31.7–35.1)	34.56 ± 12.51 (33.4–35.7)	32.88 ± 11.28 (31.7–34.0)	(0.809)	34.10 ± 12.43 (33.0–35.2)	33.36 ± 11.25 (32.4–34.4)	(0.610)	33.20 ± 12.29 (30.1–36.3)	33.77 ± 11.83 (33.0–34.5)	(0.570)
PANSS total, mean ± SD	64.34 ± 22.53 (60.9–67.8)	67.43 ± 24.72 (65.2–69.7)	64.03 ± 22.08 (61.8–63.3)	(0.484)	66.89 ± 24.96 (64.7–69.1)	64.38 ± 21.73 (62.4–66.3)	(0.284)	65.30 ± 24.95 (59.0-71.6)	65.67 ± 23.36 (64.2–67.2)	(0.733)
GAF, mean ± SD	45.88 ± 18.48 (43.1–48.7)	45.30 ± 17.26 (43.7–46.9)	46.40 ± 16.67 (44.7–48.1)	(0.934)	46.09 ± 17.78 (44.5–47.6)	45.51 ± 16.72 (44.1–47.0)	(0.836)	47.02 ± 18.60 (42.4–51.7)	45.72 ± 17.16 (44.6–46.8)	(0.749)
Healthy individuals (95% CI)[b]										
	N = 335	N = 1,111	N = 869		N = 449	N = 437		N = 50	N = 836	
Age, mean ± SD, y	51.12 ± 16.37 (49.4–52.9)	51.91 ± 15.45 (51.0–52.8)	52.18 ± 15.42 (51.2–53.2)	(0.686)	47.97 ± 14.16 (46.7–49.3)	48.52 ± 14.42 (47.2–49.9)	(0.427)	46.88 ± 15.14 (42.7–51.1)	48.32 ± 14.24 (47.4–49.3)	(0.605)
Gender, no. (%), male	160 (47.8%) (40.0–55.5)	547 (49.2%) (45.1–53.4)	416 (47.9%) (43.1–52.7)	0.088 (0.767)	221 (44.6%) (38.1–51.2)	198 (40.2%) (33.3–47.0)	1.359 (0.244)	23 (46.0%) (25.6–66.4)	396 (47.4%) (42.5–52.3)	0.035 (0.851)
Education, no. (%),low level[g]	60 (20.6%) (11.0–30.1)	287 (25.8%) (20.8–30.9)	224 (25.8%) (20.1–31.5)	4.316 (0.116)	101 (20.4%) (12.5–28.3)	90 (18.3%) (10.3–26.2)	1.108 (0.575)	7 (14.0%) (–11.7–39.7)	184 (22.0%) (16.0–28.0)	3.386 (0.184)

[a]Effects for GG versus T carriers.
[b]Methods used: Mann-Whitney *U* tests and χ^2 tests.
[c]Owing to missing data upon phenotyping, sample size varies between N = 771–1,049 in the total GRAS sample.
[d]CI, confidence interval.
[e]Exploratory exclusion of non-Caucasian subjects did not appreciably alter any of the main findings of the paper.
[f]Rating according to graduation/certificate; patients currently in school or in educational training are excluded.
[g]Low level education: equal or less than nine years of academic formation.

formed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA; http://www.spss.com). GraphPad Prism, version 5.01 (La Jolla, CA, USA) was used to analyze expression and mouse data.

Case control study (disease sample versus healthy blood donors or replicate sample). For *EPOR* STR $(GA)_n$, the sum of repeat lengths of both alleles was analyzed. To account for intraindividual allelic heterogeneity (that is, the degree of heterogeneity between the two alleles), the difference between allelic repeat lengths was calculated. Distributions of single allele lengths, allelic repeat sum, allelic heterogeneity and of *EPO* SNP genotypes (SNPs rs1617640 and rs564449) between schizophrenic subjects and healthy controls were assessed by χ^2 tests with (*EPOR*) and without (*EPO*) Monte Carlo sampling (1,000 runs).

PGAS. For the phenotype–genotype association analysis using the *EPO* SNP rs1617640 as independent variable, T carriers (GT and TT) were aggregated and contrasted with individuals homozygous for the G allele. Group comparisons for the *EPOR* repeat were based on median splits (21–36 versus 37–54; for the first PGAS approach, Tables 1 and 2) or tercile splits (21–35 versus 36–38 versus 39–54; for more detailed subgroup comparisons, see Figure 1F) of allelic repeat sums. Data on cognitive target variables are presented such that higher values always indicate better performance. They were standardized to mean zero and variance one by Blom transformation (37). In language-dependent tests (VLMT), nonnative German speakers (N = 92) were excluded for analyses. A cognition composite score was calculated for each individual representing the mean of the Blom-transformed data for processing speed and perceptual organization. Genotype differences were assessed by analysis of covariance including covariates age (both healthy individuals and disease controls), duration of disease, chlorpromazine equivalents and severity of negative symptoms (PANSS; the latter three covariates only for the disease sample) as they are known to influence performance on neuropsychological tests. Genotype differences with respect to sociodemographic and clinical readouts were tested using χ^2 (nominal variables) or Mann-Whitney *U* tests (continuous variables). The impact of *EPO* and *EPOR* genotypes on mRNA levels and expression differences dependent on *EPOR* genotype were tested nonparametrically using Kruskal-Wallis and Mann-Whitney *U* tests in the subsample of patients of whom PBMCs were available (N = 98).

DNA Extraction and Normalization

Disease sample and healthy blood donors. Genomic DNA was purified from whole blood using JETQUICK Blood & Cell Culture DNA Spin Kit (Genomed GmbH, Löhne, Germany) according to the manufacturer's protocol. Resulting DNA samples were aliquoted and stored at –80°C. For further analysis, DNA was normalized to 50ng/μL with an automated robotic platform (Microlab Star, Hamilton, Bonaduz, Switzerland). For quality control, each sample was analyzed with a 0.8% agarose gel.

Healthy individuals (replicate sample). DNA extraction was done with the QIAamp Blood Maxi Kit (Qiagen, Hilden, Germany). DNA concentration was adjusted using the PicoGreen quantitation reagent (Invitrogen, Karlsruhe, Germany).

Genotyping—Analysis of SNPs in the *hEPO* Gene

Disease sample and healthy blood donors. The selected SNPs (rs1617640 and rs564449) in the *EPO* gene were analyzed using Simple Probes (TIB Molbiol, Berlin, Germany) and called using the LightCycler 480 Genotyping Software implemented in the LightCycler 480 system (Roche, Mannheim, Germany). The reaction mixture (10 μL) was prepared with 20 ng of DNA in 384 well plates according to standard protocols (Roche). In each run, eight positive controls (hgDNA, Bioline, Luckenwalde, Germany) and negative water blanks were included for quality and internal control purposes. Overall, successfully genotyped markers amounted to 99.7–99.9%.

Healthy individuals (replicate sample). The SNP rs1617640 was genotyped using the iPLEX assay on the MassARRAY MALDI-TOF mass spectrometer (Sequenom, Hamburg, Germany). Genotyping call rates were all >95%.

Genotyping—Analysis of GA Repeats (Both Disease Sample and Healthy Individuals)

The polymorphic GA repeat in the promoter region of *hEPOR* was amplified from genomic DNA by PCR. Primers were chosen from de la Chapelle *et al*., 1993 (38): *hEPOR*_$(GA)_n$ forward: 5′-FAM GGTGA CAGAG CAACA CCCTG-3′; *hEPOR*_$(GA)_n$ reverse: 5′-ATCAG CATCT CTTCC CAGCC-3′ resulting in a PCR fragment of ~186bp. Due to the presence of GGAA repeats in the same region (20), we likely obtained aggregates of all repeats, that is, GGAA as well as GA. Since we assume that the aggregates as a whole are important for modulating function, we did not further analyze the exact composition of the aggregates. In fact, our data validate this assumption. For each sample, the reaction mixture (20 μL) was prepared in 384 well plates, each containing 20 ng of human genomic DNA, 125 μmol/L dNTPs each, 200 nmol/L FAM-labeled forward primer and the reverse primer and 1U Phire polymerase (Finnzymes, Espoo, Finland). The amplicons were separated using size electrophoresis on the ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For this, samples were diluted 1:50 with 0.3 mmol/L EDTA and 4 μL were mixed with 6 μL LIZ-500 Size Standard (Applied Biosystems). Raw data were processed using the Gene Mapper Software 4.0 (Applied Biosystems).

Analysis of Peripheral Blood Mononuclear Cells (PBMCs)

Blood was collected in CPDA (citrate phosphate dextrose adenine) tubes from schizophrenic patients with different genotypes at the selected markers. PBMCs were isolated applying the standard Ficoll-Paque Plus isolation procedure (GE Healthcare, München, Ger-

many). RNA was prepared using Qiagen miRNeasy Mini Kit (Qiagen). 1 μg of RNA per sample was used to synthesize cDNA (SuperScriptIII, Invitrogen). The qRT-PCR was performed with the aid of SYBR Green detection on the LightCycler 480 system (Roche). The starting amount of cDNA was 20 ng; the number of PCR cycles was 30–34 for EPO and 27–31 for EPOR. Primers were added at 0.5 pMol. CT (cycle threshold) values for *EPO and EPOR* were standardized to CT values of *GAPDH*. *hEPO*_qRT-PCR forward: 5′-TCCCA GACAC CAAAG TTAAT TTCTA-3′; *hEPO*_qRT-PCR reverse: 5′-CCCTG CCAGA CTTCT ACGG-3′; *hEPOR*_qRT-PCR forward: 5′-TTGGA GGACT TGGTG TGTTT C-3′; *hEPOR*_qRT-PCR reverse: 5′-AGCTT CCATG GCTCA TCCT-3′; *hGAPDH*_qRT-PCR forward: 5′-CTGAC TTCAA CAGCG ACACC-3′; *hGAPDH*_qRT-PCR reverse: 5′-TGCTG TAGCC AAATT CGTTG T-3′.

Cloning and Transfection Studies

Constructs. The *EPO* promoter constructs were built according to Tong and coworkers (23). Briefly, the promoter site (1.5 kb) including either G or T at rs1617640 was PCR amplified from respective human samples and cloned into the pGL3 basic vector (Promega, Mannheim, Germany). In addition, constructs including the 3′ region of human *EPO*, as this is known to be of major importance for the regulation of EPO expression, were designed. For this, the following primers including *Xba*I sites were used: *hEPO*_3′ forward: 5′-*GCGTC TAGA*C CAGGT GTGTC CACCT-3′; *hEPO*_3′ reverse: 5′-*GCGTC TAGA*A TGACA ATCTC AGCGC-3′.

All constructs were verified by restriction enzyme digestion and complete bidirectional DNA sequencing.

Luciferase Assays. Neuro2a (N2a) cells (LGC Standards GmbH, Wesel, Germany) were plated in 96-well cell culture plates (NUNC, Langenselbold, Germany) at 15,000 cells per well in DMEM supplemented with 5% FCS without antibiotics. At 16–18 h after plating, cells were transfected with Lipofectamine 2000 (Invitrogen) using the manufacturer's protocol. Of the different *EPO* constructs, 30 ng (containing G or T) and 1 ng of pRL-TK-control vector (Promega) were cotransfected. For each treatment, six replicates were performed. At 24 h after transfection, cobalt chloride ($CoCl_2$), a well known mimetic of hypoxic EPO induction (39,40), was added in different concentrations to induce chemical hypoxia to the transfected cells. Concentrations were selected such that after the $CoCl_2$ exposure, no signs of toxicity or increased cell death were observed (Trypan blue counts <1% in all conditions, including normoxic controls). At 48 h after transfection, cells in each well were lysed, using 30 μL passive lysis buffer (Promega). The dual-luciferase reporter assay (Promega) was used according to the manufacturer's protocol. Prior to measurement, lysates were transferred into a black plastic microtiter plate. Measurements were performed with the microplate reader Mitras LB940 (Berthold Technologies, Regensdorf, Switzerland) and associated software MicroWin 2000. Firefly values were divided by the corresponding Renilla readings producing values expressed as relative luciferase units (RLU).

Mouse Studies

The generation and characterization of cEPOR transgenic mice have been reported in detail elsewhere (7). Briefly, $EPOR^{R129C}$ (cEPOR) bears a single point mutation at nucleotide 484, that is, in the exoplasmic domain, causing a substitution of cysteine for arginine at codon 129 of the N terminus (R129C). The cDNA sequence of cEPOR, containing a hemagglutinin (HA; YPYDVPDY) tag inserted five residues downstream of the signal peptidase cleavage site (41,42) was excised with PacI and SalI from the pMX-HA-cEPOR plasmid. The HA-cEPOR cDNA was inserted into pNN265 plasmid, with a modified multiple cloning site, that carries a 5′ hybrid intron and a 3′ intron plus poly-A signal from SV40 through PacI and SalI sites. Finally, the entire DNA fragment of HA-cEPOR, flanked by a hybrid intron at the 5′ end and a polyadenylation signal from SV40 at the 3′ end was cut out from pNN265 vector using *Not*I and placed downstream of the 8.5kb α-CaMKII promoter. The TG founders were produced by pronuclear injection of the linearized DNA into C57BL6/N *(TG1)* or FvB/N *(TG2)* zygotes. The analysis of line TG1 mice was performed after 4–7 backcrosses with C57BL6/N wild type mice (that is, all results reported in this study were obtained from generations 4–7 of the TG1 line). The TG1 line was used (because of its clean C57BL6/N background) for the behavioral experiments presented here. Analysis of line TG2 mice was performed after 8–9 backcrossings to C57BL6/N mice. The genotype of transgenic offspring was analyzed by PCR of tail genomic DNA using primers specific for the 3′ end of the α-CaMKII promoter sequence (5′-GGGAG GTAGG AAGAG CGATG-3′) and the 5′ end of the HA-cEPOR cDNA sequence (5′-CACCC TGAGT TTGTC CATCC-3′) yielding a 769 bp product. PCR amplification of the tail DNA was carried out with the following conditions: 2 min at 94°C (1 cycle); 30 s at 94°C, 30 s at 60°C and 1 min at 72°C (35 cycles), followed by final extension at 72°C for 10 min.

Behavioral Testing

All experiments were approved by the local animal care and use committee in accordance with the German Animal Protection Law. For behavioral testing, mice were housed in groups of 3–5 in standard plastic cages, food and water *ad libitum* (except for the 5-choice [water deprivation] and T-maze [food deprivation] training periods). The temperature in the colony room was maintained at 20°–22°C, with a 12 h light:dark cycle (light on at 7 AM). Behavioral experiments were conducted during the light phase of the day (between 8 AM and 5 PM). Mouse data were analyzed using repeated measures analysis of variance (ANOVA) and Mann-Whitney *U* tests.

Five-choice serial reaction time task (5-CSRTT). The 5-CSRTT measures higher brain functions, ranging from various discrete learning/memory to attentional paradigms (43,44). A detailed description of the procedure and training steps is given

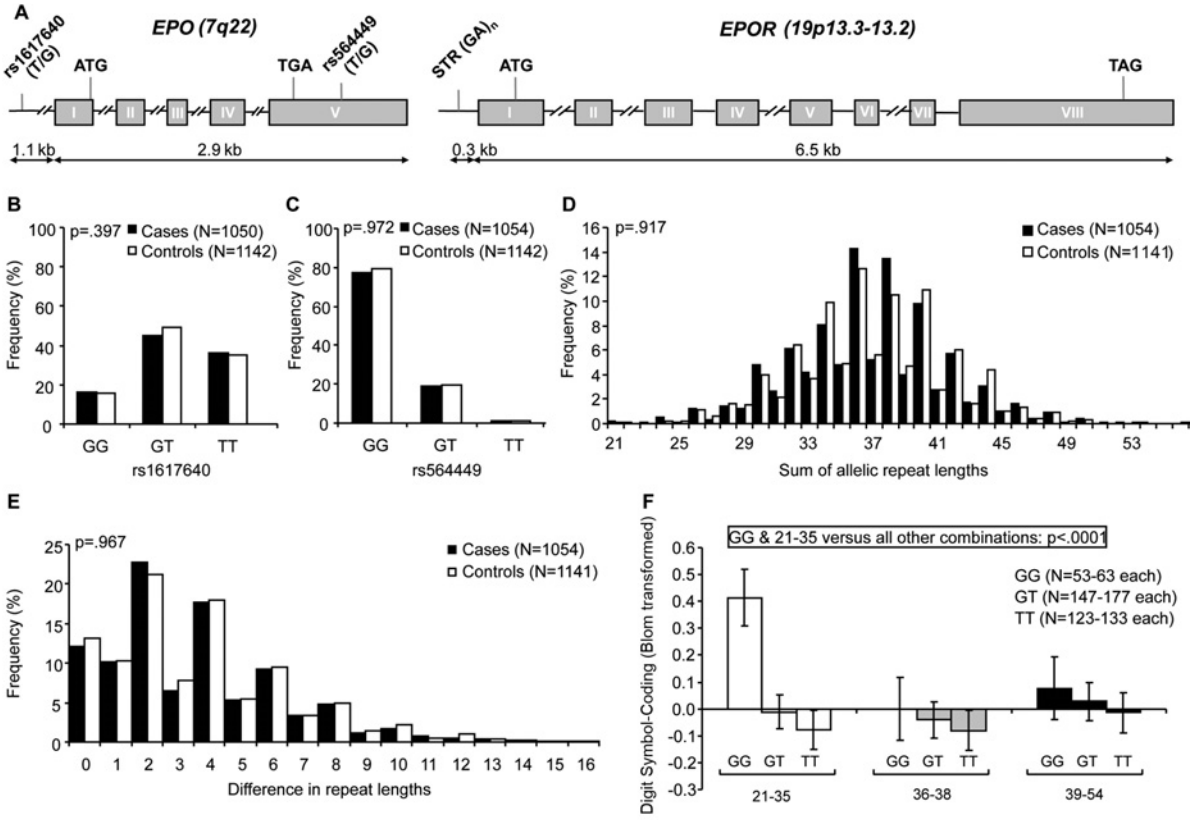

Figure 1. *EPO* and *EPOR* genotype analyses in schizophrenic patients of the GRAS data collection and healthy controls (blood donors). (A) Genetic overview of *EPO/EPOR* including analyzed genetic markers. (B,C) A case control study reveals comparable distribution of *EPO* SNP genotypes in schizophrenic and healthy control subjects, thus excluding *EPO* genotypes as risk factors for schizophrenia. (D,E) Case control analysis of *EPOR* STR $(GA)_n$ repeat lengths shows comparable results for both samples, again excluding a risk constellation of *EPOR* genotypes. (F) Grouping of genotype combinations with respect to Digit Symbol-Coding test performance uncovers one genotype highly superior to all others: GG&21–35 (lowest) repeat sum. Mean ± standard error of the mean (SEM) given; χ^2 tests and analysis of covariance (ANCOVA) applied.

elsewhere (7). In the present study, we report only the results obtained in intervention phase 3 (variable, short stimulus duration, indicative of speed of processing).

Novel object recognition task. Briefly, mice are habituated for 20 min to a gray plastic arena (45 × 45 cm, 35 cm high) with no objects. Next, for the training session, an object is placed in the arena, and the time the mouse spends exploring the object (that is, nose ≤15 mm to the object) is recorded for 10 min (video tracking software Viewer 2; Biobserve, Bonn, Germany). Next, a new, second object is added to the arena and exploration recorded for 10 min (testing session). The whole procedure is repeated several days later with a different set of objects and a 30 min interval between training and testing session. We use plastic objects of similar size (around 3 cm in diameter) but different shape, texture, and color (pilot experiments had confirmed that mice show no spontaneous preference for any of the objects).

T-maze. The T-maze consists of three arms (clear plexiglas, 7 cm wide, 12.5 cm high with the start arm 43.5 cm long, and the goal arms 32.5 cm each). The goal arms contain distinct visual cues on the outer side of the walls; the start arm is plain. A plastic pellet cup (2.2 cm high, 3.5 cm in diameter) is situated in the rear of each goal arm. To have equal olfactory reward cues in both arms, we use pellet cups consisting of two parts separated by a perforated floor: the lower part contains 12 food pellets, which are inaccessible for mice; the accessible upper part holds one food pellet as reward. Before starting the habituation procedure, mice are food deprived (1 h/day access to food in the home cage) for 3 d. Another 3 d of habituation to apparatus and reward (5 min of exposure to the maze each day, with reward placed at both ends of the T-maze) are followed by training for 12 d, six trials/day. On the first (sample) run of each trial, both goal arms are baited, but the mouse is forced to choose one of the goal arms (the other being closed by a removable wooden block: 16 cm × 6.9 cm). After entering the preselected goal arm, the mouse is allowed to consume the reward for 20 s and then placed back in the start box. On the second (choice) run, which during the acquisition phase of training follows 20 s

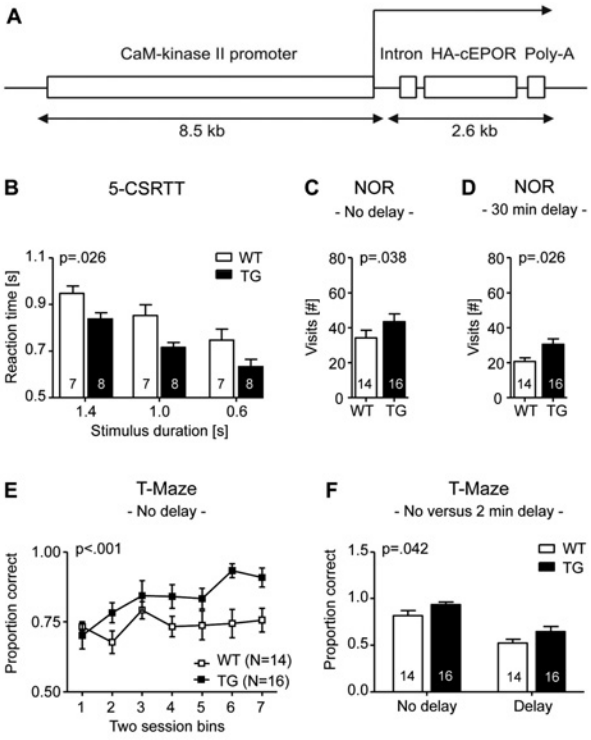

Figure 2. Mice with transgenic expression of constitutively active EPOR (cEPOR) driven by the α-calcium/calmodulin-dependent protein kinase II (α-CaMKII) promoter demonstrate highly superior cognitive performance compared with their wild type littermates. (A) Construct used for transgenic expression. (B) Significant reduction of reaction time in the attentional testing part of the 5-Choice Serial Reaction Time Task (5-CSRTT) reflects superior speed of cognitive processing in transgenic mice. (C–F) Transgenic mice perform better in Novel Object Recognition (NOR) (depicted is the number of visits of the new object) and T-maze tests with or without delay, illustrating their supremacy in memory tasks. Exact N numbers of all experiments are given directly in the bars or the line graph; all male mice, 5–8 month old at the time point of testing; mean ± standard error of the mean (SEM) presented; two-way ANOVA for repeated measures and Mann-Whitney *U* tests applied.

after the sample run, both goal arms are open, and the mouse is rewarded for choosing the previously unvisited arm. The location of the sample arm (left or right) is varied pseudorandomly across trials so that mice received equal numbers of left and right presentations, but no more than two consecutive trials with the same sample location. The mice are run in squads of 6–8 (including both transgenic and littermate control animals) to minimize variation in intertrial intervals (12 min for all mice throughout the 12 d of training). *No-delay trials:* Mice are trained for 14 d in sample and choice runs with 20-s interval (10 trials on d 1; 6 trials/day from d 2–14). *Delay trials:* After finishing the no-delay procedure, mice are tested in the delay procedure using two different time intervals between sample and choice run: 2 min and 6 min (3 d per delay; six trials/day containing three delay and again three no-delay trials each—in an alternating manner—to rule out motivational decrement and to obtain an internal control condition).

Note: All experiments in this manuscript were conducted in a blinded fashion that is, with the respective investigator/rater being unaware of sample assignment.

All supplementary materials are available online at www.*molmed.org*.

RESULTS

Case Control Study

***EPO* SNPs rs1617640 (T/G) and rs564449 (T/G), as well as *EPOR* STR $(GA)_n$, are not associated with schizophrenia.** When conducting phenotype-based genetic association studies (PGAS) to evaluate the contribution of certain genotypes to defined subphenotypes, a potential role of these genotypes as genetic risk factors for schizophrenia should first be explored. Therefore, we performed a case control study on SNP rs1617640 (T/G) and SNP rs564449 (T/G), located in the 5′ upstream region and in the 3′ regulatory region of the *EPO* gene, respectively, as well as the STR $(GA)_n$ in the *EPOR* 5′ upstream area (Figure 1A). To assess the *EPOR* genotype, the repeat lengths sum of both alleles was employed. No significant difference in the distribution of *EPO* genotypes (Figures 1B, C; $\chi^2 = 1.897$ and $p = 0.397$; $\chi^2 = 0.058$ and $p = 0.972$) or of *EPOR* repeat lengths sum (Figure 1D; $\chi^2 = 23.85$, $p = 0.917$, evaluated with Monte Carlo sampling on 1,000 runs) between cases (N = 1,050–1,054) and healthy controls (blood donor sample; N = 1,141–1,142) was found (for details see Figure 1 and Supplementary Table S1). An association analysis of single allele repeat lengths instead of allelic repeat lengths sum between cases and controls also failed to yield significant distribution differences ($\chi^2 = 32.15$, $p = 0.114$, evaluated with Monte Carlo sampling on 1,000 runs). Furthermore, the intraindividual difference of repeat lengths as a measure of marker heterogeneity did not vary between cases and controls (Figure 1E; $\chi^2 = 8.54$, $p = 0.967$, 1000 Monte Carlo simulations). Thus, we could not find any evidence for a role of *EPO*/*EPOR* genotypes regarding the risk to develop schizophrenia. For the following phenotype analyses, the *EPO* SNP rs564449 (T/G) had to be excluded due to its low minor allele frequency (MAF~11%).

Phenotype-Based Genetic Association Study (PGAS)

***EPO* SNP rs1617640 (T/G) and *EPOR* STR $(GA)_n$ are associated with higher**

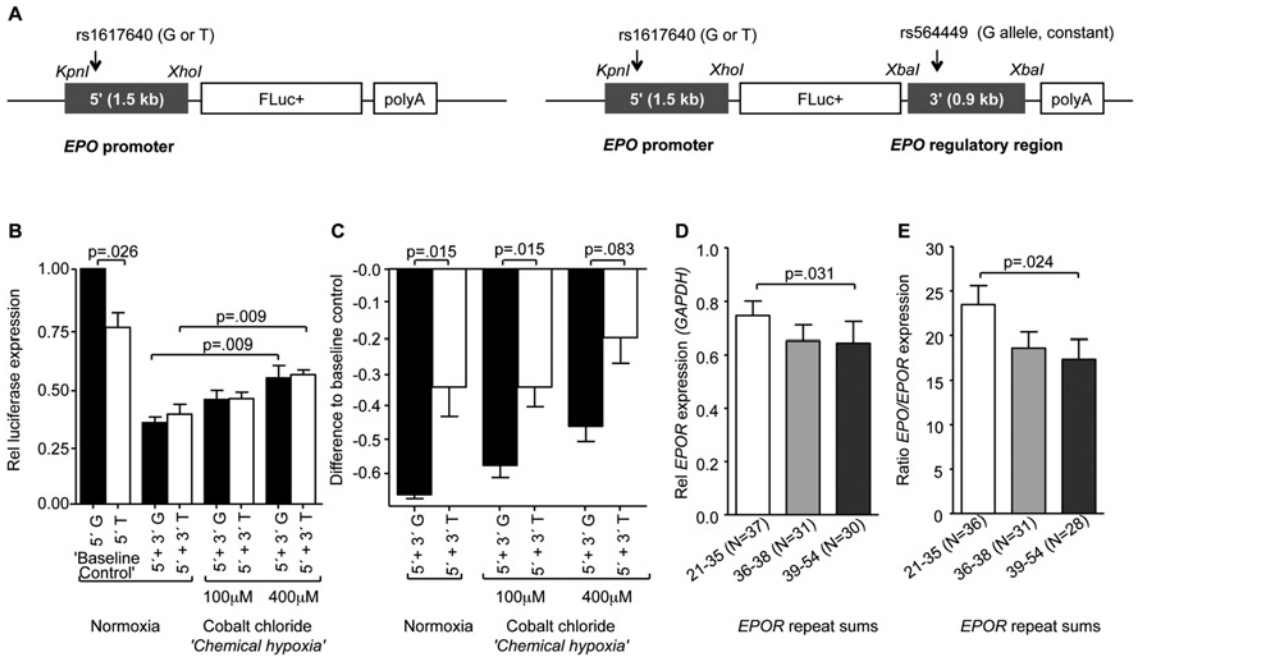

Figure 3. Genotype-dependent *EPO/EPOR* expression differences using reporter gene assays and PBMCs. (A) Reporter gene constructs: 5′ upstream region of the *EPO* gene with either G or T at rs1617640 (left) and in addition with the 3′ regulatory region of *EPO* (right). (B) G at SNP rs1617640 leads to significantly higher basal gene expression than T (baseline control). Addition of the 3′ regulatory region to the construct induces downregulation of gene expression. This suppression is stepwise alleviated by increasing doses of $CoCl_2$ (100 μmol/L and 400 μmol/L). (C) In all conditions shown in (B), the suppressability (regulability) of gene expression compared with the baseline control (that is, normoxic control condition of B) is highest for the G genotype. (D) *EPOR* mRNA levels in peripheral blood mononuclear cells (PBMCs) of patients, determined by qRT-PCR and normalized to GAPDH as the housekeeper, show that the lowest *EPOR* STR $(GA)_n$ repeat length sum is associated with the highest *EPOR* expression. Gender distribution among the three repeat groups is well balanced (males/all: 24/37, 18/31, 20/30; χ^2 test $p = 0.628$). (E) Even when considering the individual *EPO* mRNA levels (which by themselves do not reveal differences; data not shown) in form of an *EPO/EPOR* expression ratio, the significant difference between different STR length carriers remains. N = 28–37 per group; mean ± standard error of the mean (SEM) presented; Mann-Whitney *U* tests applied.

cognition in schizophrenia. In a previous treatment trial, we showed that high-dose EPO, infused weekly over 12 wks, improves cognitive functions and reduces cortical gray matter loss in chronic schizophrenic patients. The domains most prominently influenced by EPO were speed of cognitive processing and short-term memory (Repeatable Battery for the Assessment of Neuropsychological Status (RBANS™): subtests coding, digit span and figure recall) (9). Assuming that an influence of genetic variation within the EPO system on cognitive function would be detectable targeting these domains, we selected of the tests available within the GRAS database those closest to the above (same test or similar test regarding domain or loading on the same factor, that is, tests leading to similar results in an individual due to joint variation in response to the same unobserved latent variable): Digit Symbol-Coding test, VLMT, and Dotting/Tapping (45). Indeed, significant associations were detected for both, *EPO* SNP rs1617640 (T/G) and *EPOR* STR$(GA)_n$ low versus high repeat sum (Table 1): carriers of G at *EPO* SNP rs1617640 and of *EPOR* STR$(GA)_n$ low repeat sum showed superior performance. In contrast, none of the relevant sociodemographic or basic disease variables revealed any significant associations (Table 2). To see whether certain combinations of genotypes of the *EPO* and *EPOR* genes would lead to better performance in the sense of a potential interaction effect, we grouped them accordingly. For *EPOR* genotypes, we assigned all individuals to three equally sized groups of allelic repeat sum carriers from low to high (Figure 1F). Surprisingly, we found one particular genotype combination, GG&21–35 (lowest) repeat sum, to be highly superior compared with all others with respect to performance in Digit Symbol-Coding test ($p < 0.0001$; see Figure 1F) but also in the other tests, where associations had been found for either *EPO* or *EPOR* or both (all $p \leq 0.003$; Table 1).

In contrast to the clear associations with cognitive parameters found here, and in agreement with previous work by others, for example (14), we did not see any evidence of a potential association of *EPO/EPOR* genotypes with blood indices. In fact, we screened a total of 94 patients (Göttingen participants of the GRAS study) where comprehensive information on blood data was available. Repeated determinations over the year in these pa-

tients (between 2–48 times each) allowed a first step to substantiate each individual's normal values (considering also the reason for hospitalization, for example, exclusion of values obtained after blood loss due to a suicide attempt). These in turn delivered the basis of the mean values given in Supplementary Table S2. As shown there, all parameters are highly similar among genotypes. Interestingly, in this small cohort of individuals (N = 94) with information on blood indices, N = 6 subjects carried the GG&21–35 (low) repeat sum genotype and could be checked against all other genotype combinations. Group comparison by Mann-Whitney *U* test did not reveal any difference between groups for hemoglobin levels ($p = 0.413$), whereas the cognitive composite score already yielded a nearly significant result ($p = 0.093$) (Supplementary Figure S2).

cEPOR Expression in Pyramidal Neurons of Cortex and Hippocampus Increases Speed of Cognitive Processing and Memory Functions in Mice

On the basis of the above findings, we speculated that the best performing genotype combination should be characterized by higher EPO/EPOR expression. This hypothesis is further supported by (i) the cognition-improving effect of high-dose EPO in clinical trials (8,9) and in mouse studies (5,6) and (ii) our previous data on mice with transgenic expression of constitutively active EPOR (cEPOR) driven by the α-calcium/calmodulin-dependent protein kinase II (α-CaMKII) promoter (Figure 2A). These mice show better performance in higher cognitive tasks (7). We reexamined these mice and specifically conducted/analyzed tests measuring speed of cognitive processing and short-term memory, analogous to our neuropsychological findings in humans. In fact, we found a clearly reduced reaction time in the phase addressing selective attention in the 5-CSRTT (2-way ANOVA for repeated measures, $F_{(1,14)} = 6.159$; $p = 0.026$; Figure 2B) as well as superior performance in short-term memory tasks, that is, novel object recognition (no-delay trials $p = 0.038$; 30-min delay trials $p = 0.026$; Mann-Whitney *U* test) and T-maze (2-way ANOVA for repeated measures, no-delay trials $F_{(1,22)} = 19.61$, $p = 0.0002$; 2-min delay trials $F_{(1,22)} = 4.668$, $p = 0.042$; Figures 2C–F). These data further support the hypothesis of higher expression/activity of the EPO system being associated with better cognitive functioning.

Mechanistic Insight: Genotype-Dependent *EPO/EPOR* Expression Differences

To better understand a potential influence of the *EPO* SNP rs1617640 on gene expression, we used reporter gene assays based on the 5′ upstream region of the *EPO* gene with G or T at the respective position. Additionally, we designed constructs including the 3′ regulatory region of human *EPO*, as this is known to be highly homologous between species and thus of major importance for the complex regulation of *EPO* gene expression (39,46) (Figure 3A). As illustrated in Figure 3B, G at SNP rs1617640 leads to significantly higher basal gene (luciferase) expression than T. Upon addition of the 3′ regulatory region to the construct, a remarkable downregulation of gene expression can be observed that is likely due to the interaction with the 5′ region which is essential for hypoxia-related *EPO* regulation (39,40). This suppression is stepwise alleviated by increasing doses of $CoCl_2$ as an inducer of chemical hypoxia. In all conditions, the suppressability (regulability) of gene expression as compared with baseline is highest for the G genotype (Figure 3C).

To explore the role of the *EPOR* STR $(GA)_n$ repeat length sum on gene expression, we determined *EPOR* and *EPO* mRNA in peripheral blood mononuclear cells. The results show that the lowest repeat sum is associated with the highest *EPOR* expression as well as with the highest *EPO*/*EPOR* ratio as readout of the interplay between the two genes (Figures 3D, E). *EPO* mRNA levels per se did not differ significantly between *EPOR* repeat sum groups (data not shown). The exact mechanisms explaining the observed effects on quantitative gene expression are still unclear. Both, *EPO* and *EPOR* gene variants investigated here are located in the promoter areas of the respective genes, where they may, for instance, directly or indirectly influence transcription factor binding. Indeed, regulation of these genes is highly complex and involves many different transcription and cofactors, the roles of which are still widely obscure (46,47).

Replication of the *EPO* and *EPOR* Genotype Associations with Cognitive Performance in a Healthy Control Sample

Having obtained associations of genotypes in the EPO system with cognition in a disease population, that is, individuals suffering from schizophrenia with known disease-typical cognitive deterioration, we wanted to know whether similar findings would be obtained in healthy controls. Fortunately, a phenotyped control population from Munich was available for comparison. Again, a case control study on *EPO* SNP rs1617640 (T/G) as well as *EPOR* STR $(GA)_n$ did not reveal differences between these healthy controls and the GRAS subjects (Supplementary Table S1, Supplementary Figure S1). This healthy control population, however, has several confines with respect to the GRAS sample: (1) Of the relevant cognitive domains, only one has been evaluated with the same test, the other one just with a similar test loading on the same factor (that is, producing similar results in an individual based on a common underlying source of variance); (2) The population is considerably older on average; (3) The sample has a different gender distribution (Table 2). Despite these limitations, the associations are essentially reproduced (Table 1), pointing to very robust effects. Interestingly, having the GG&21–35 repeat sum genotype does not yield an advantage for healthy individuals who generally perform much better on all cognitive tests as compared with schizophrenic individuals (Table 1). This observation suggests that only subjects with an inferior cognitive performance may benefit from this specific genotypic constella-

tion. We wondered whether the superiority of the GG&21–35 group would be revealed when selecting the subgroup of healthy individuals with an average performance equal to the mean performance of the schizophrenic sample on the Digit Symbol-Coding test. Indeed, the low performer group of healthy individuals (Digit Symbol-Coding test performance threshold ≤50) displays a similar tendency of genotype superiority ($p = 0.089$) which is not detectable in the high performer group (Digit Symbol-Coding test performance threshold >50, $p = 0.809$). These results suggest that the GG&21–35 genotype may disclose its benefits particularly in situations of reduced cognitive capacity or (relative) cognitive impairment (Supplementary Figure S3).

DISCUSSION

In the present hypothesis-driven study, we identified novel associations of *EPO* and *EPOR* genotypes with cognition, namely speed of processing, short-term memory and tasks requiring distinct fine motor components, both in schizophrenic patients and in a healthy control population (replicate sample). On a molecular/cellular level, we demonstrate that the cognitively more beneficial genotypes are associated with higher expression/stronger regulability of expression. In supporting preclinical experiments, we show that mice with transgenic expression of constitutively active EPOR in pyramidal neurons of cortex and hippocampus (7) perform better in cognitive domains reminiscent of those influenced by *EPO*/*EPOR* genotypes in humans.

Interestingly, in the schizophrenic population that is defined by overall reduced cognitive performance, one discrete genotype combination (GG&21–35 repeat sum) achieves highly superior cognitive outcome, whereas this same combination in healthy individuals has only the tendency of an advantage. This advantage is restricted to subjects with lower cognitive capacity. Thus, higher EPO/EPOR activity appears to be most beneficial in situations of compromised function. We note that in disease states, such benefit is likely achieved by the upregulation of the endogenous EPO system in the brain, as seen for example, in stroke, schizophrenia or Alzheimer's disease (48–50). In fact, this EPO/EPOR upregulation is further potentiated by rhEPO treatment, resulting in lasting cognitive improvement (8,9,51,52).

The selection of the cognitive domains reported here to show associations with *EPO*/*EPOR* genotypes strictly followed hypotheses derived from the results of earlier rhEPO treatment studies (9). Therefore, multiple testing issues do not apply here. On the other hand, also for a purely hypothesis-driven, exploratory study, it is reassuring to obtain replication of the results in an independent sample, in the present paper, a healthy control population.

Limitations of the present work are particularly (1) the incomplete availability of identical neuropsychological tests in the two populations studied (forcing to use tests loading on the same factor, that is, resulting in highly correlated readouts in a given individual due to shared variance produced by a common underlying variable), and (2) the different age/gender distribution. Despite all these shortcomings, a similar pattern of associations arose, pointing to robust effects and confirming the significance of *EPO*/*EPOR* genotypes for higher cognition.

We are aware that the use of transfection studies/reporter gene assays and of PBMCs cannot answer all questions related to genotype-dependent brain expression of the EPO system. Especially PBMCs are a heterogeneous population of cells. There may be variations in the composition of mononuclear cells such as circulating erythroid progenitor or precursor cells, megakaryocytes, mast cells and macrophages, lymphocytes, and endothelial progenitor cells from sample to sample. Many of these cell types potentially may serve as source of EPO expression in the peripheral blood. We cannot exclude, however, that our data on EPO and EPOR mRNA mainly derive from a small fraction of erythroid progenitors that express these genes. Nevertheless, the pragmatic approach to analyze PBMCs was the only choice presently available for us to study quantitative gene expression in a reasonable number of humans with defined genotype. To obtain further support for increased expression/activation of the EPO system leading to better cognition, we additionally performed mouse studies in a model of targeted upregulation of EPOR in neuronal populations that are known to play a pivotal role in the functions of interest (7). The superiority of these mice included essentially the same cognitive domains as those found to be influenced by *EPO*/*EPOR* genotypes in human populations.

CONCLUSION

To conclude, we identified an intrinsic role of the EPO system for higher cognition, reflected by associations of *EPO*/*EPOR* genotypes with cognitive performance, which may be of particular significance in disease states. These findings further suggest the EPO system as target for treating human brain diseases that are characterized by cognitive decline.

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DISCLOSURE

H Ehrenreich has submitted/holds user patents for EPO in stroke, schizophrenia and MS. Apart from that, the authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Supplemental Data

Common Variants of the Genes Encoding Erythropoietin and Its Receptor Modulate Cognitive Performance in Schizophrenia

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Supplementary Table S1. Case control study: genotype frequencies of *EPO* SNPs in schizophrenic patients (GRAS sample) versus healthy blood donors and versus healthy replicate sample.

(SCZ, GRAS subjects: N=1050 for rs1617640 and N=1054 for rs564449; CON1, healthy blood donors: N=1142; CON2, healthy replicate sample: N=2315).

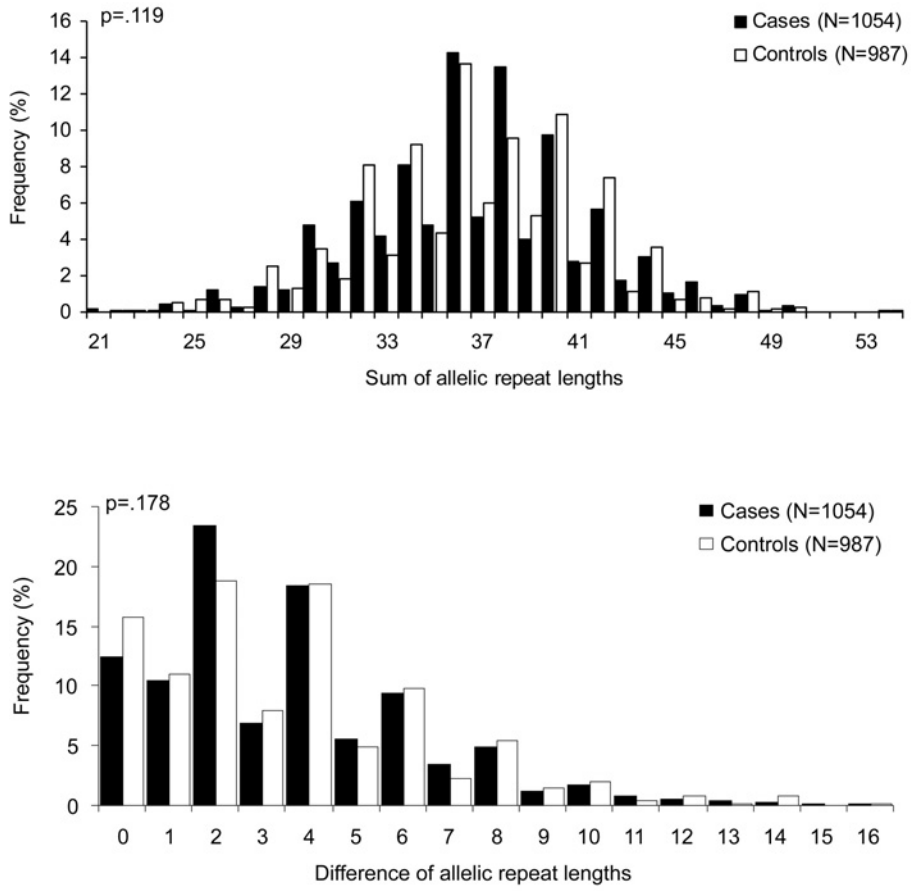
EPO SNPs		Genotypic frequencies GG	GT	TT	χ^2 (p)[a]	HWE (p)
rs1617640	**SCZ** (N=1050)	16.5%	46.5%	37.0%		(.368)
	CON1[b] (N=1142)	15.6%	49.5%	34.9%	1.897 (.397)	(.357)
	CON2[c] (N=2315)	14.5%	48.0%	37.5%	2.297 (.317)	(.508)
		GG	**GT**	**TT**		
rs564449	**SCZ** (N=1054)	79.6%	19.5%	0.9%		(.628)
	CON1[b] (N=1142)	79.5%	19.4%	1.1%	.058 (.972)	(.877)

[a]Comparison of genotype distribution between the respective control group (CON1 or CON2) and the GRAS sample of schizophrenic patients (SCZ); [b]CON1= blood donors, [c]CON2= healthy replicate sample

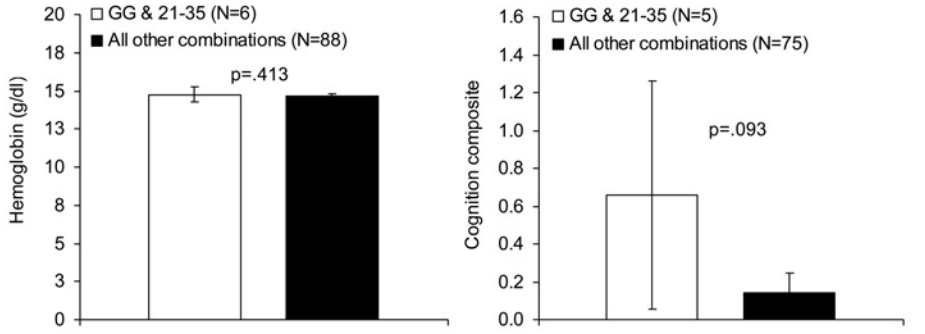
Supplementary Table S2. Blood indices of a total of 94 patients (Göttingen participants of the GRAS study) with comprehensive information on blood data available.

	***EPO* (mean ± SD)**			***EPOR* (mean ± SD)**	
	GG N=13	**GT** N=47	**TT** N=34	**Low sum** N=50	**High sum** N=44
Hemoglobin (g/dl)	14.751 ±1.317	14.703 ±0.923	14.657 ±1.329	14.748 ±1.112	14.631 ±1.157
Hematocrit (%)	43.765 ±3.877	43.484 ±2.611	43.288 ±3.892	43.661 ±3.187	43.166 ±3.384
Erythrocytes (x0^6/µl)	4.860 ±0.439	4.902 ±0.391	4.831 ±0.488	4.894 ±0.442	4.844 ±0.423
Thrombocytes (x0^3/µl)	271.166 ±54.047	254.554 ±44.449	271.043 ±71.662	261.442 ±59.959	264.376 ±54.015

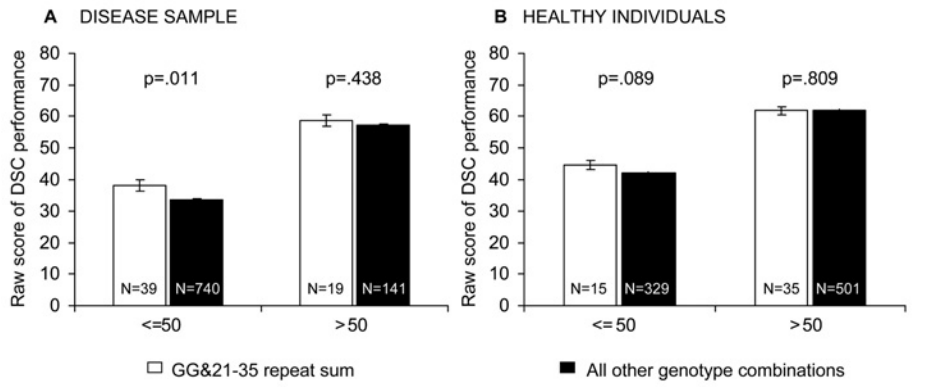
Repeated determinations over the years in these patients (between 2-48 times each) allowed in a first step to substantiate each individual's 'normal' blood values (considering also the reason for hospitalization, e.g. exclusion of values obtained after blood loss due to a suicide attempt). These in turn delivered the basis of the mean values given in the Table.



Supplementary Figure S1. Case control study: frequency distribution of *EPOR* STR $(GA)_n$ repeat lengths sum and repeat lengths difference in the GRAS sample of schizophrenic patients versus subjects of a healthy replicate sample. An association analysis of single allele repeat lengths instead of allelic repeat lengths sum between cases and controls also failed to yield significant distribution differences (χ^2=14.71, p=.903, evaluated with Monte Carlo sampling on 1000 runs).



Supplementary Figure S2. Subgroup analysis: hemoglobin and cognition composite values in GG&21-35 (low) repeat sum genotype carriers versus all other genotype combinations. Of the cohort of patients with blood values available (Supplementary Table 2), N=6 individuals carried the GG&21-35 (low) repeat sum genotype and could be tested against all other genotype combinations. Group comparison by Mann-Whitney *U* test did not reveal any difference between groups for hemoglobin levels (p=.413). In contrast, despite the small number of high performers (GG&21-35 (low) repeat sum carriers), the cognitive composite score (available for most but not all of the patients with blood information - see N numbers) yielded already a nearly significant result (p=.093) (mean±SEM presented).



Supplementary Figure S3. The superiority of the 'GG&21-35 repeat sum' genotype versus all other combinations is most prominent in individuals with low performance. Comparison of the Digit Symbol-Coding (DSC) performance of individuals carrying the genotype combination 'GG&21-35 (lowest) repeat sum' versus subjects with all other combinations. Given are good (raw score > 50) and bad DSC performer groups (raw score <= 50). Mean±SEM of raw scores presented, p-values from ANOVA with Blom-transformed DSC score and age (schizophrenic sample and healthy controls), severity of negative symptoms (PANSS), medication and duration of disease (schizophrenic sample) as covariates.

3. A neuregulin1 risk genotype is associated with performance in central olfactory measures in a schizophrenic sample

3.1 Overview of project II

Central neural circuits underlying olfactory function can be mapped to temporolimbic and frontal brain regions (Benarroch 2010). Dysfunctional connectivity in these olfactory structures has been consistently implicated in the affective and cognitive symptomatology of schizophrenic patients (Turetsky et al. 2009). Consequently, olfactory tasks serve as behavioral probes to assess the functional integrity of neural substrates underlying disturbed sensory-cognitive and emotional processing in schizophrenia (Turetsky et al. 2009). Olfactory input triggers cognitive events, often in form of autobiographical memories (Savic 2001, Larsson et al. 2009, Turetsky et al. 2009). Tests measuring olfactory cognition address the integration of olfactory input into cognitive processing (Atanasova et al. 2008). The spontaneous identification of odorants (odor naming) is a complex task requiring correct smell encoding, semantic memory, and selection of the most appropriate denomination (Schab 1990). First evidence from a very small sample of male schizophrenic patients supports an odor naming deficit in this patient group (Saoud et al. 1998). Likely due to the small sample size, clinical variables did not correlate with odor naming ability (Saoud et al. 1998). The first aim of our study was to build on these findings by evaluating odor naming performance and its relationship with clinical and cognitive variables in a large sample of schizophrenic patients.

Olfactory stimuli do not only induce cognitive activity, they immediately evoke emotional reactions (Soudry et al. 2011). To explore the nature of affective states in response to different scents, available olfactory function tests require the judgement of the hedonic quality of given odors. Schizophrenic subjects are less accurate when deciding whether an odor is edible, pleasant or familiar as compared to healthy controls (Hudry et al. 2002, Moberg et al. 2003, Kamath et al. 2011, Kamath et al. 2011). No study has ever investigated whether schizophrenic patients have difficulties in judging gustatory or semantic properties of odors beyond familiarity, hedonicity and edibility ratings (odor interpretation). Moreover, it has not been evaluated how the inability to accurately interpret olfactory stimuli relates to schizophrenia symptomatology.

As a part of the very comprehensive phenotyping of the GRAS cohort (Ribbe et al. 2010) we modified and extended existing odor interpretation and naming tasks as a basis for a time-economic exploration of the clinical relevance of olfactory processing deficits in our sample. Odor naming and interpretation performance was evaluated in 881 schizophrenic patients and 102 healthy matched controls using 6 semantically diverse odors derived from the University of Pennsylvania Smell Identification Test (Doty et al. 1984) as well as 7 attributes. Statistically, we first analyzed the entire distribution of olfactory performance and then, to clinically characterize the subgroup of patients with a central olfactory impairment, compared the low and high extremes of the odor interpretation phenotype with respect to cognitive and clinical measures and a neuregulin1 marker, a well replicated risk genotype for schizophrenia (Stefansson et al. 2002).

Schizophrenic patients underperformed healthy controls in odor naming and interpretation. Applying multiple linear regression analysis to both olfactory target measures and clinical variables revealed strong relative associations with alertness. Additionally, odor naming was found to depend on the integrity of higher cognitive processing whereas odor interpretation was associated with the severity of positive symptoms. Extreme groups of odor interpretation performance were compared with respect to additional symptomatic parameters. The subgroup of schizophrenic patients defined by a central olfactory dysfunction was found to be characterized by a more severe psychopathology, and extreme cognitive deficits resulting in an overall inferior functional outcome as compared to all other schizophrenic patients. Moreover, the risk genotype (TT) of neuregulin1 SNP8NRG243177, previously shown to be associated with the severity of positive symptoms (Papiol et al. 2011), was found to be overrepresented in the subgroup of patients with extreme odor interpretation impairments. Interestingly, brain regions structurally and functionally altered in individuals carrying the risk allele (T) of SNP8NRG243177 (Hall et al. 2006, Plailly et al. 2006, Barnes et al. 2012) and regions implicated in olfactory processing largely overlap. Thus, the influence of this genotype on odor interpretation may well be (co-)mediated by its effect on brain structure, which was observed to depend on gene expression differences (Law et al. 2006). Together, our findings suggest a central integration deficit in sensory-cognitive networks in a subgroup of schizophrenic patients. Future studies should aim at replicating and refining present findings by using psychometrically improved odor naming and interpretation tasks.

3.2 Submitted manuscript

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*These authors contributed equally to the work.

Personal contribution

I was responsible for the development of the olfactory tasks, the phenotype data collection and entering, the performance of statistical analyses and interpretation of the results and the design of figures and tables. I further did the literature research, designed and wrote the manuscript.

Central olfactory measures, disease severity and a neuregulin1 risk genotype in schizophrenia

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ABSTRACT (187 words)

Background: The clinical relevance of central olfactory measures has not been comprehensively studied in schizophrenia.

Aims: We modified and extended existing odor naming and interpretation tasks and applied them to a large sample of schizophrenic subjects to explore the association with clinical outcome and with a schizophrenia risk genotype, the neuregulin1 marker SNP8NRG243177.

Method: During comprehensive phenotyping of the GRAS *(Göttingen Research Association for Schizophrenia)* cohort, odor naming (active memory retrieval) and odor interpretation (attribute assignment) were evaluated in 881 schizophrenic patients and 102 healthy controls, and associations with emotional processing, disease outcome and SNP8NRG243177 studied.

Results: Patients underperformed controls. Odor naming deficits were correlated with compromised cognition, odor interpretation deficits with positive symptom severity and general alertness. SNP8NRG243177 (neuregulin1) was associated with poor odor interpretation.

Limitations: The psychometric properties of the olfactory measures used here in an exploratory fashion in a large sample have to be improved by e.g. increasing the number of odors for future establishment of a solid clinical test.

Conclusions: The strong association of odor naming and interpretation with disease outcome and a genetic schizophrenia risk factor highlights their clinical and scientific significance.

Introduction

Olfactory information processing in the brain integrates complex neuronal networks with particular relevance of temporolimbic and frontal regions[1]. In schizophrenia, essentially all facets of this information processing are found to be affected, ranging from peripheral receptor insufficiency to disturbances in passive odor identification, odor memory, odor naming and odor interpretation[2-10]. The associations of these deficits with disease severity [9, 11-15], however, have mostly remained incomplete or unclear. The present study has been designed to (I) assess odor naming and interpretation abilities in a large number of schizophrenic patients (N=881) and in healthy matched controls (N=102), (II) evaluate the relationship of olfactory task performance with specific symptom domains, (III) explore extreme groups of odor interpretation regarding determinants of disease severity, and (IV) test for genetic association of odor interpretation with the neuregulin1 marker SNP8NRG243177, a known risk genotype for schizophrenia[16].

METHOD

Subjects

Schizophrenic subjects

The present study (GRAS project) was approved by ethics committees of the Georg-August-University Göttingen (master committee) and collaborating centers. Detailed phenotyping of the GRAS sample[17] contained odor naming and odor interpretation tasks (figure 1A), administered to 999 schizophrenic patients after written informed consent. Present analyses excluded all non-native German speakers (N=89), all patients with known anosmia (N=14, neurological conditions e.g. head injury, or cold) and 6 patients with missing data. Nine patients were excluded as non-admissible based on performance in entry and odor naming tasks (figure 1B). Data analyses were based on the remaining 881 patients.

Healthy controls

As comparison group, 103 healthy subjects matched for age, gender and smoking status (smoker: yes/no) were recruited by public announcements and gave written informed consent. They were free of any physical, neurological and psychiatric disorder, and had no relatives with a history of neuropsychiatric diseases. One individual was excluded due to his performance in entry and odor naming tasks. Data analyses were based on 102 controls.

Measures

Olfaction

Odorants

The University of Pennsylvania Smell Identification Test[18] (UPSIT), the clinically and scientifically most established test, has been widely used as test validation criterion[19]. A total of 10 different scents (scratch and sniff format) were selected from this battery (for detailed description of the administration procedure see[3]). Odorants were chosen by an experienced psychiatrist based on following criteria: (I) For the entry task, odors had to be easily identifiable (low item difficulty) to minimize contribution of higher cognition to task performance. For the naming task, odorants were selected to cover a broad range

of difficulties to differentiate between subjects along the whole spectrum of cognitive abilities. For odor interpretation, items of low difficulty were chosen due to the expected profound interpretation deficit in schizophrenia. (II) The odors had to be of high ecological validity to German subjects. (III) To balance avoidance of redundancy (due to time limitations) and reliability, odors were selected to represent diverse associated contexts (e.g. rose and gasoline) and attribute overlap (both pineapple and licorice are pleasant *and* sweet). Although this study was not designed to introduce a new *ready-to-be-used* olfaction test, preliminary item characteristics and psychometric properties are provided in supplemental table 1.

Entry task

To be able to interpret odor naming and interpretation results as central processing deficits, an entry task (figure 1A; maximum score 4) was introduced. A total of 4 consecutively presented odors are chosen from 4 alternative descriptors each (multiple choice, brief passive odor identification[3]) by pointing to or naming the correct term on the scoring sheet. The entry task should identify potentially anosmic subjects unaware of their condition or failing to report it prior to performing odor naming and interpretation tasks. The 4 odorants *lemon*, *smoke, lilac* and *paint thinner* were chosen to be easily identifiable (supplemental table 1) and representative of all attributes contained in the odor interpretation task. *Lemon* was selected to cover the attributes *sweet* and *edible*, *lilac* is an odor judged as *pleasant* and *natural*, *smoke* is *hot* and *dangerous* and *paint thinner* is often associated with a *technical* context. Figure 1B shows that theoretical binomial probabilities for random guessing (in case of anosmia or pronounced cognitive impairment, assuming equal multiple-choice success probabilities of 0.25 for all 4 odors) markedly differed from relative recognition score frequencies. The vast majority of patients and controls recognized 3-4 odors correctly whereas 95% of guessing subjects would recognize <3 odors correctly in the entry task. Simultaneously, guessing would result in an odor naming score of 0 (free choice test) which is extremely unlikely (supplemental figure 1B). Consequently, subjects were excluded from analysis when they had less than 3 out of 4 correctly recognized odors in the entry task and an odor naming score of 0 (9 schizophrenic subjects, 1 healthy control subject) because of potentially experiencing difficulties at the peripheral processing level. Clearly, this approach risks excluding cognitively extremely impaired individuals. Indeed, excluded individuals cognitively underperformed the 881 schizophrenic subjects (881 subjects: -

0.03±0.78; 9 excluded subjects: -0.92±0.54; mean±SD of higher cognition composite; see below).

Odor naming and odor interpretation

The odor naming task (maximum score 18) measures the correct naming of the 6 odors *pineapple, gasoline, mint, rose, natural gas* and *licorice* (free choice; figure 1A). Three points were scored for the correct name, 1 point if the subject provided an item belonging to the same semantic category (e.g. *pineapple*: 3 points for 'pineapple', 1 point for 'fruit'). Before naming an odor, probands had to decide whether it matched the 7 attributes *pleasant, sweet, hot, technical, natural, edible* and *dangerous* (forced choice: 'yes' or 'no' answer; odor interpretation; same set of odors as for naming) (figure 1A). One point was scored for each attribute assignment consistent with the predefined profile (maximum score 42=6x7). Although task reliability improves with number of items[20], we had to restrict it to 6 items for time-economic reasons (olfactory tasks being only a fraction of the GRAS examination procedure[17]). Nevertheless, the internal consistency of the odor interpretation task, is high for attributes (supplemental table 1).

Disease-relevant possible confounders

As unspecific but disease-relevant (overall severity) possible confounders, duration of disease, number of hospitalizations and medication status (chlorpromazine equivalents) were selected.

Functional outcome, disease severity, psychopathology, cognition and emotional processing

To address schizophrenia relevant target domains, age-at-prodrome, general assessment of functioning (GAF) scale, PANSS (positive and negative syndrome scale) subscales, a higher cognition composite (supplemental table 2), and the alertness subtest (mean of tonic and phasic alertness) of TAP (Testbatterie für Aufmerksamkeitsprüfung) were included. McQuarrie dotting and tapping tests were integrated into a fine motor function composite. Mehrfachwahl-Wortschatz-Test covered premorbid intelligence. Emotional processing was operationalized as sum score of 'blunted affect', 'emotional withdrawal' and 'poor rapport' of PANSS. Higher scores represent worse outcome in PANSS but better performance in GAF and

neuropsychological tests. Healthy controls completed odor naming and interpretation tasks and 4 neuropsychological tests (LPS 3; MWTB; McQuarrie Dotting and McQuarrie Tapping test). All tests are cited in[17].

Schizophrenia severity score

As integrative measure of overall schizophrenia severity, a score of Blom transformed[21] items: *positive*, *negative symptoms* (PANSS), *cognition*[22], *neurological symptoms* (CNI) and *age-at-prodrome* was created with all single variables scaled such that higher values indicate higher symptom severity (figure 2A).

Genetic analysis

The neuregulin1 marker SNP8NRG243177 was genotyped using HypProbes (TIB Molbio, Berlin, Germany) on Light Cycler480 (Roche, Mannheim, Germany).

Statistical analyses

To test for associations of odor naming and interpretation performance with disease-relevant symptom domains, multiple linear regression was applied within the schizophrenic sample (table 1, displaying mutually adjusted *relative* association strengths interpretable in analogy to Cohen's d). Sum scores for higher cognition and fine motor performance are the mean of standardized neuropsychological measures in these domains (larger values represent better performance). Rank-based Blom transformation[21] was applied to standardize all measures by transforming them into standard normally distributed surrogates prior to sum score computation. This maintained the order of the data, but removed skewness from variable distributions. Group comparisons between healthy and schizophrenic subjects (supplemental figure 1A-B), and schizophrenic subgroups (figures 1C, 2B-C, tables 2, 3, supplemental figure 1C, supplemental table 2), or genotype groups (figure 2E-F) were tested by linear regression or analysis of covariance (figure 2F) (estimating difference between group means for standardized normally distributed quantitative targets, with covariate adjustment where indicated), by Fisher's exact test or Chi-square test (for binary targets gender, smoking and genetic risk status), by non-parametric Wilcoxon rank sum test (for quantitative targets which could not be standardized to normal distribution). For linear model analyses, standardized regression coefficients (with 95% confidence limits) were converted to the original variable scale for ease of interpretation by

multiplication with the raw data standard deviation of the target variable within the respective data. Multiple-testing was accounted for by Bonferroni correction and closed testing principle (table 2). All p values are two-sided.

RESULTS

Influence of age, smoking and gender on odor naming and interpretation

Increasing age is associated with a decline in odor naming (average -0.05 points/year, $p=2.2x10^{-11}$, test statistic $t_{978}=-6.770$) and interpretation performance (average -0.06 points/year, $p=1.6x10^{-8}$, $t_{979}=-5.697$) likewise in schizophrenic and healthy individuals (no significant difference). Smoking status had no influence on both central olfactory measures. Women proved to be slightly superior to men in odor naming (average 0.42 points, $p=0.043$, $t_{978}=2.022$) but not interpretation.

Odor naming and interpretation are impaired in schizophrenic patients

The main study results are summarized in figure 1C. Inferior performance of schizophrenic patients (N=881) compared to 102 matched healthy controls became evident for odor interpretation and naming tasks (supplemental figure 1A,B; interpretation: $p=4.0x10^{-10}$; naming: $p=2.9x10^{-9}$).

Odor naming and interpretation are differentially associated with psychopathology and cognition in schizophrenic patients

Odor naming and interpretation correlate substantially (Spearman correlation r=0.51, 95%CI [0.46, 0.56], $p<2.2x10^{-16}$). To investigate relative association strengths of odor naming and interpretation with 3 schizophrenia relevant symptom domains (cognition, negative and positive symptoms) and 2 control variables (fine motor function and premorbid intelligence) (table 1), multiple linear regression was applied in the schizophrenic sample (N=881). Regression coefficients express *relative* association strength (relative to the standard deviation of the target trait). Odor naming and interpretation scores were strongly dependent on alertness. Worse naming results were additionally linked to impaired higher cognition and more severe negative symptoms (PANSS). Deficits in odor interpretation were associated with higher positive symptom

severity (PANSS). No link was detected between odor naming or interpretation and fine motor function or premorbid intelligence.

Extreme group comparisons based on odor interpretation performance reveal strong differences in several symptom domains

Frequency distribution of interpretation and naming scores (supplemental figure 1A,B) show increased probabilities of low scores for schizophrenic patients, especially for odor interpretation. This suggests that olfactory deficits are particularly pronounced in a subgroup of patients. Odor interpretation covers cognitive *and* emotional processing deficits, both representative of a broad spectrum of schizophrenia symptomatology. To explore the informative value of odor interpretation for the domains cognition, psychopathology, emotional processing and functional outcome, schizophrenic high interpretation performers (HIP, scoring >90th percentile) were contrasted with low interpretation performers (LIP, scoring <10th percentile; N=88 each) and compared to healthy controls (Con, N=102). The 3 groups did not differ (pairwise comparisons) with regard to age, gender and smoking status. Expectedly, a highly significant naming deficit became obvious for LIP in comparison to HIP ($p=7.0x10^{-28}$) and healthy controls ($p=9.1x10^{-29}$) (supplemental figure 1C). In terms of reasoning capabilities, healthy controls obtained better results than schizophrenic HIP ($p=1.6x10^{-3}$) with the latter performing superior to LIP (table 2, $p=3.4x10^{-3}$) (figure 1C). For fine motor function, both schizophrenic groups performed comparably and markedly worse than healthy controls (HIP/LIP: p=0.015; HIP/Con: $p=4.4x10^{-6}$; LIP/Con: $p=8.1x10^{-9}$). For premorbid intelligence, a difference was observed between disease groups (HIP/Con: p=0.81; LIP/Con: p=0.017; LIP/HIP: $p=3.6x10^{-3}$) (table 2).

More detailed assessment of group contrast between schizophrenic LIP compared to HIP in disease-related domains, revealed higher severity of negative symptoms ($p=1.7x10^{-4}$) and positive symptoms ($p=1.6x10^{-4}$) for LIP (figure 1C). Additionally, strongly impaired cognitive and emotional processing became evident (higher cognition composite: $p=3.7x10^{-3}$; alertness: $p=1.4x10^{-4}$; emotional processing: $p=3.3x10^{-3}$), resulting in a worse functional outcome ($p=6.5x10^{-6}$; average difference 12 GAF units) for LIP (table 3). No significant differences were observed with regard to possible confounders indicating overall disease severity (age-at-prodrome, duration of disease, number of hospitalizations and chlorpromazine equivalents, all above the Bonferroni

corrected significance threshold p>0.005). Together, the results of the extreme group approach indicate that deficits in odor interpretation are accompanied by more severe psychopathology, worse cognitive and emotional processing and inferior functional outcome.

Schizophrenic carriers of the neuregulin1 SNP8NRG243177 risk allele display high symptom load and poor odor interpretation performance

Employing a composite score of schizophrenia severity (figure 2A) revealed that low odor interpretation ability is closely related to higher symptom load (figure 2B-C). This observation motivated the inclusion of this measure into the schizophrenia severity score for subsequent genetic analyses using the neuregulin1 marker SNP8NRG243177. This SNP was associated with odor interpretation and overall schizophrenia severity based on different statistical approaches: (I) The relative frequency distribution of the schizophrenia severity score including odor interpretation revealed that schizophrenic T allele carriers have a higher probability of severe schizophrenic symptoms (figure 2D). (II) The risk genotype (TT) is overrepresented in the group of extremely poor interpretation performers (figure 2E; Chi-square: 4.380, p=0.036; odds ratio: 2.532 [confidence interval: 1.049, 6.115]). (III) When contrasting Blom transformed means based on SNP8NRG243177 risk status (CC versus T allele), individuals carrying the protective genotype (CC) tended to be less affected on all single measures (figure 2F). The strongest group differences (withstanding Bonferroni correction) were observed for the schizophrenia severity score including odor interpretation (p=0.009) and the odor interpretation performance when homozygous genotypes were contrasted (p=0.004).

DISCUSSION

The present study, building on and extending previous work, demonstrates in a large sample of subjects that the ability to name and qualitatively interpret odors is markedly impaired in schizophrenia. Importantly, it adds new aspects to the available literature by showing distinct associations between central olfactory measures and schizophrenia-relevant clinical variables. Extreme group comparisons as well as an association study using a genetic variant of the schizophrenia risk gene neuregulin1, deliver first hints for a biological subgroup of patients, defined by their severe olfactory dysfunction. This subgroup is characterized by profound abnormalities in higher olfactory processing, together with a more severe psychopathology, extreme cognitive deficits across various neuropsychological measures and pronounced affective flattening, resulting in an overall inferior functional outcome as compared to all other schizophrenic patients.

Odor naming ability relies on higher cognitive processing

Odor naming but not interpretation was found to depend on the integrity of higher cognitive processing. This association was primarily driven by reasoning abilities while all other domains appeared less prominent. In contrast to Saoud and coworkers, we found odor naming, like almost all neuropsychological tests[17], to correlate with PANSS negative scores[9]. This discrepancy is likely explained by the low number of patients included in the Saoud study.

Positive symptoms interfere with odor interpretation ability

Odor interpretation, but not odor naming, was found to be influenced by the severity of positive symptoms. In line with the disconnectivity hypothesis in schizophrenia[23], we hypothesized that the aberrant assignment of hedonic (pleasant), gustatory (sweet, hot) and higher-order semantic attributes (e.g. technical, edible) potentially reflects the dysfunctional integration of sensory input into cognitive-emotional processing. Thus, impaired odor interpretation should reflect positive symptom severity. In fact, while the relationship between basic olfactory processing (e.g. passive odor identification) and severe negative symptoms is quite well established[11, 12, 24, 25], this is the first study to provide evidence for psychotic symptoms to interfere with performance in an olfactory function test. We even show here that odor interpretation impairments may be slightly more marked than deficits in odor naming. Therefore, measuring dysfunctional

integration of olfactory (and likely other sensory) input into cognition should be complemented by assessment of respective dysregulated emotion processing in this patient group.

Alertness modulates performance in central olfactory measures

The large amount of variance shared by odor naming and interpretation can be partially explained by the strong association of both olfactory tasks with the level of alertness. The latter represents the intensity aspect of attention and comprises both a state of general wakefulness (intrinsic alertness) and the ability to increase the readiness to react in response to a cue over a short period of time (phasic alertness)[26]. Importantly, attention is a requirement for assigning the continuous flow of air through the nose to either respiration or olfactory exploration[27]. Non-attended, the olfactory content of inhalations is ignored and not processed further. Effects of attentional modulation on activity in human primary[27] and secondary olfactory cortices[28] and amygdala[29] support this notion. Consequently, the extent to which an individual attends to a presented odor and is able to maintain the level of alertness during the completion of a task, likely influences the amount of resources provided for higher-order mental operations following the perception of an olfactory stimulus. Additionally, it is well established that olfactory (spicy, fruity) and gustatory (salty, sweet) judgements lead to the activation of associated semantic networks. This results in a facilitation of olfactory naming – at least in healthy individuals[7]. However, as the here applied odor interpretation task was the same for all odors, differential depth-of-processing effects, potentially confounding odor naming results, can be excluded.

Schizophrenia susceptibility marker associates with odor interpretation performance

Interestingly, brain regions structurally and functionally altered in individuals carrying the T allele of the neuregulin1 variant SNP8NRG243177[30-32] and regions implicated in higher olfactory processing largely overlap. Therefore, the possibility of a genetic root also for the observed olfactory phenomena arises. Neuregulin1 is among the presently best established schizophrenia risk genes[16]. In a previous study, we had found the protective genotype of the neuregulin1 marker SNP8NRG243177 (CC) underrepresented in schizophrenic patients with most severe positive symptoms[33]. The modulatory effects of this genetic variant on odor interpretation in our schizophrenic sample may well be (co-)mediated through its effect on brain structure, which in turn

may depend on gene expression differences[34]. The strong association of the SNP8NRG243177 risk allele (T) with compromised odor interpretation and overall (positive) symptom severity supports a central olfactory integration deficit in sensory-cognitive or sensory-emotional networks in a defined biological subgroup of schizophrenic patients.

Limitations:

We are aware of the fact that the psychometric properties of the olfactory measures used here in an exploratory fashion have to be improved by e.g. increasing the number of odors for future establishment of a solid clinical test.

Conclusions:

The present work, by simultaneously assessing naming and interpretation deficits in a large schizophrenic sample and combining it with a genetic association, defined a biological subgroup of the disease and prepared the ground for future test development. Importantly, it revealed that both olfactory tasks extract differential information on higher brain functioning in a complementary fashion.

Supplemental information is available at the *Journal of Psychiatry & Neuroscience* website.

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Declaration of interest: None to report.

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Author contributions: HE, MB and AK developed the olfactory tasks. They were advised by DolM. Phenotype data were collected and entered into the database by CH, MB and AK. DM and AK performed all statistical analyses and designed manuscript and figures. HE guided the project and data analysis, together with HB and DolM. AK, together with HE, wrote the paper. All authors discussed the results, read and commented on the manuscript, and have seen and approved the final version.

FIGURE LEGENDS

Figure 1: Design of odor tasks and overview of main results. (A) After completion of the entry task (passive recognition of 4 odors - multiple choice), subjects were asked to assign a set of attributes (odor interpretation) and then to name the respective odorant (odor naming), consecutively for 6 odors. **(B)** The entry task revealed a clear contrast between test results (schizophrenic patients, healthy controls) and theoretical binomial probabilities for guessing subjects. **(C)** The main findings of the study are summarized. Low interpretation performers (LIP) showed worse reasoning abilities, a more severe psychopathology and emotional processing deficits.

Figure 2: Association of odor interpretation performance with a genetic risk variant of neuregulin1. (A) The internal consistency (Cronbach's α) of single variables integrated into a schizophrenia severity score is slightly improved upon inclusion of odor interpretation. **(B, C)** Schizophrenic subjects performing below the 10^{th} percentile (extreme groups) or below the median of odor interpretation performance (total sample) have a profoundly increased schizophrenia severity score (corrected for age and medication). SEM presented. **(D)** The relative frequency distribution of schizophrenia severity score bins (including odor interpretation) shows dissociation between the SNP8NRG243177 CC genotype and T allele carriers. **(E)** Carriers of the protective genotype (CC) are overrepresented in the group of high interpretation performers (Chi-square test). **(F)** Carriers of the CC genotype differ consistently from risk allele carriers (T) in all Blom transformed single variables integrated into the schizophrenia severity scores (all variables except for *age-at-prodrome* corrected for age and medication). Multiple testing adjusted significances (applying Bonferroni: $p \leq 0.006$) are set in boldface. All other p-values ≤ 0.05 are set in italic. SEM presented.

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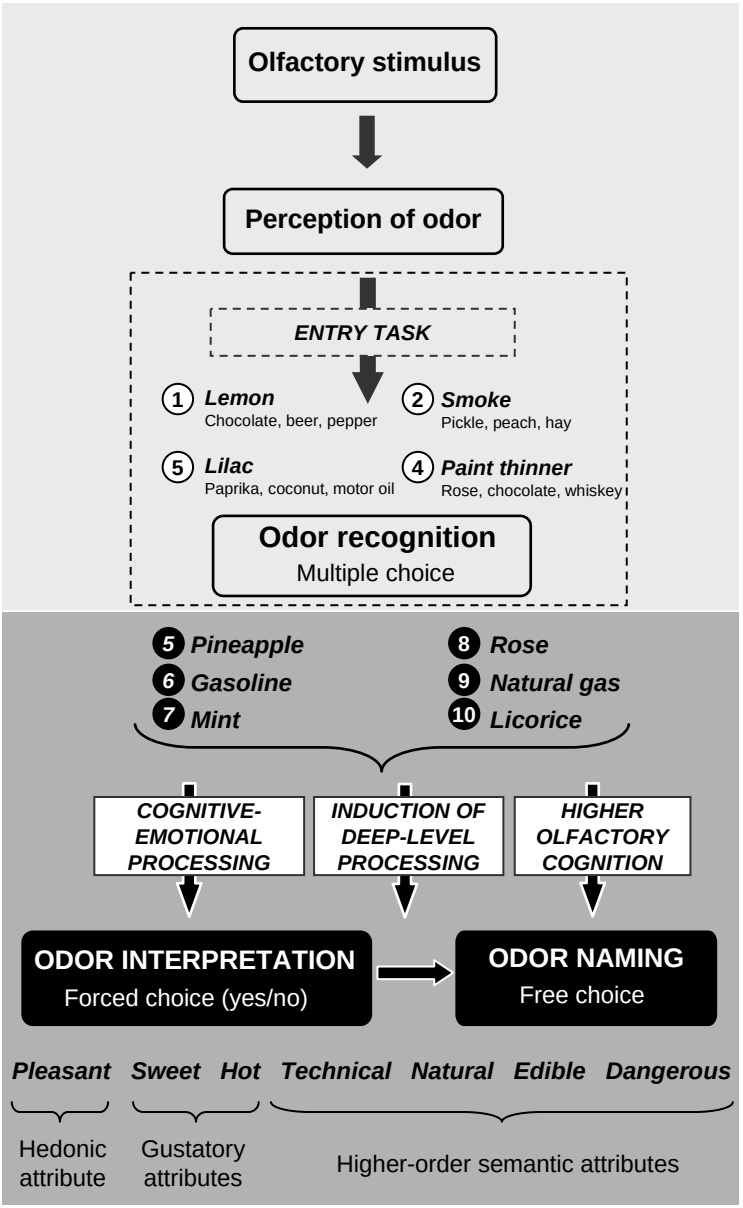
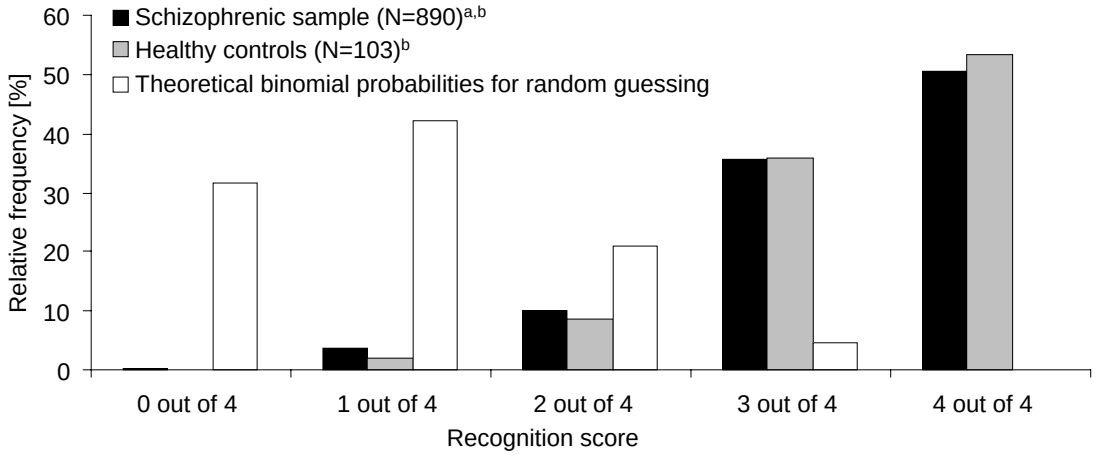
Kästner et al figure 1

A The odor naming and interpretation tasks

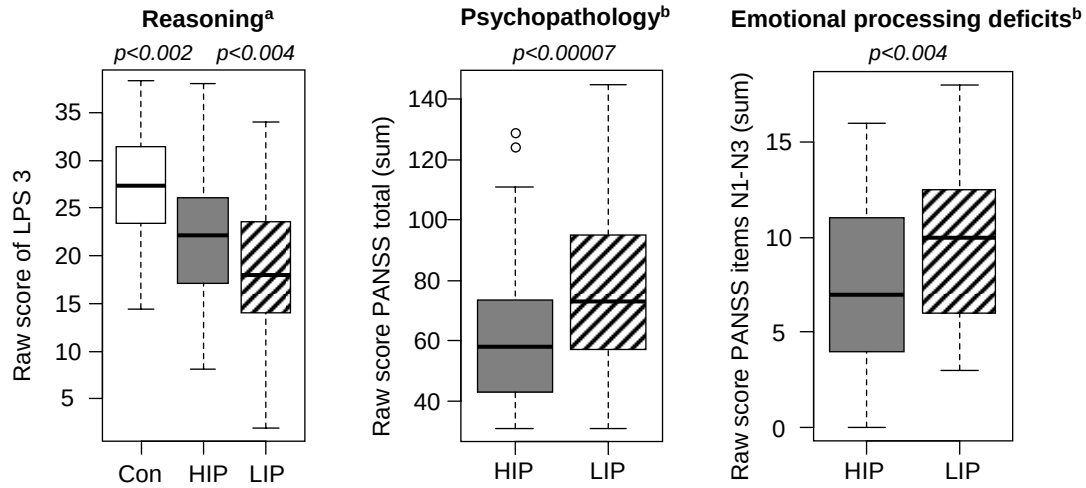


B Entry task



[a]1 or 2 missing values (out of 4) were imputed for N=21 schizophrenic subjects. All other subjects had complete data in the entry task. [b]Still including the N=9 schizophrenic / N=1 healthy subjects that were identified by the entry task and odor naming as non-admissible and excluded from remaining analyses.

C Main findings: Odor interpretation associates with schizophrenia severity



[a]Reasoning: Higher values mean better performance. [b]Psychopathology and emotional processing deficits: Higher values mean more severe symptomatology. Abbreviations: LPS 3=Leistungsprüfsystem (subtest 3); Con=Healthy controls; HIP= High odor interpretation performers; LIP= Low odor interpretation performers; PANSS items N1:Blunted affect, N2:Emotional withdrawal, N3:Poor rapport.

Kästner et al figure 2

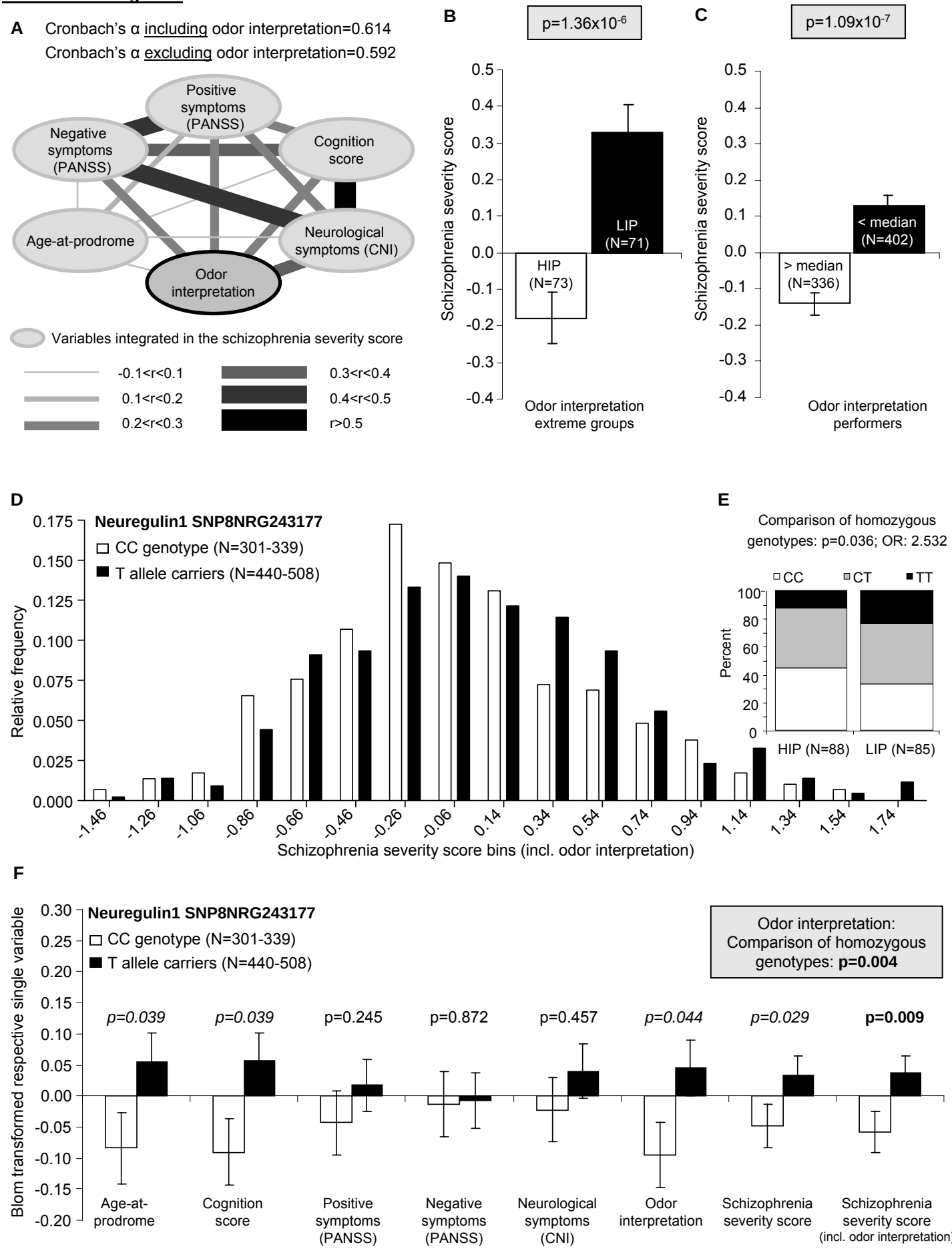


Table 1

Odor naming is associated with cognition and odor interpretation with severity of positive symptoms and alertness in schizophrenic subjects (n=881)

	Odor Naming[a]	Odor Interpretation[a]
Higher cognition composite	**0.165** **(t_{813}= 2.347, p=0.019)**	0.057 (t_{814}= 0.789, p=0.430)
Alertness (TAP)	**0.144** **(t_{813}= 3.401, p=0.001)**	**0.196** **(t_{814}= 4.635, p=0.000)**
PANSS negative	**-0.112** **(t_{813}=-2.717, p=0.007)**	-0.045 (t_{814}=-1.073, p=0.283)
PANSS positive	-0.055 (t_{813}=-1.407, p=0.160)	**-0.107** **(t_{814}=-2.701, p=0.007)**
Fine motor function composite	-0.010 (t_{813}=-0.210, p=0.834)	0.017 (t_{814}= 0.336, p=0.737)
Premorbid intelligence (MWTB)	0.071 (t_{813}= 1.761, p=0.079)	0.059 (t_{814}= 1.420, p=0.156)

[a]Relative association strength of domains with odor naming and odor interpretation (t-statistic t_{df} with df degrees of freedom, p value). Multiple linear regression, adjusted for age (odor naming and odor interpretation) and gender (odor naming), significances (Bonferroni p≤0.025) in boldface.

Table 2

Severely impaired interpretation performers (LIP) show compromised cognition compared to healthy controls (Con) and non impaired interpretation performers (HIP)

Trait		Descriptive		Group Contrast	Effect	[95% CI]	Statistic	p
Basic characteristics								
Age (years)	SZ	39.5 ± 13	(17-78)					
mean ± SD (range)	Con	38.8 ± 14	(18–71)	HIP / Con	2.75	[-0.81, 6.31]	t_{275}= 1.522[a]	0.13
	HIP	41.0 ± 11	(22–64)	LIP / Con	1.25	[-2.31, 4.81]	t_{275}= 0.693[a]	0.49
	LIP	39.7 ± 13	(18–71)	LIP / HIP	-1.40	[-4.58, 1.77]	t_{174}=-0.873[a]	0.38
Gender (% female)	SZ	33						
	Con	32		HIP / Con	OR 1.25	[0.66, 2.38]	Fisher[b]	0.54
	HIP	38		LIP / Con	OR 0.83	[0.42, 1.62]	Fisher[b]	0.64
	LIP	28		LIP / HIP	OR 0.66 for females	[0.33, 1.31]	Fisher[b]	0.26
Smoker status (% yes)	SZ	48						
	Con	63		HIP / Con	*OR 0.45*	[0.21, 0.94]	Fisher[b]	*0.025*
	HIP	43		LIP / Con	OR 0.57	[0.26, 1.23]	Fisher[b]	0.15
	LIP	49		LIP / HIP	OR 1.26 for smokers	[0.52, 3.05]	Fisher[b]	0.68
Disease related higher and basal cognition and premorbid intelligence								
Reasoning[c]	SZ	20.8 ± 6.6	(2-38)					
mean ± SD (range)	Con	27.2 ± 5.2	(14–38)	HIP / Con	-3.00	[-4.86, -1.14]	t_{265}=-3.180[a]	**$1.6x10^{-3}$**
	HIP	21.5 ± 6.5	(8–38)	LIP / Con	-5.49	[-7.63, -3.35]	t_{265}=-5.058[a]	**$7.9 x10^{-7}$**
	LIP	18.2 ± 6.7	(2–34)	LIP / HIP	-2.45	[-4.07, -0.82]	t_{164}=-2.971[a]	**$3.4 x10^{-3}$**
Fine motor function	SZ	-0.12 ± 0.91	(-3.22–3.22)					
composite[c]	Con	0.97 ± 0.68	(-1.09–2.83)	HIP / Con	-0.53	[-0.75, -0.31]	t_{268}=-4.674[a]	**$4.7 x10^{-6}$**
mean ± SD (range)	HIP	-0.002 ± 0.87	(-2.40–1.83)	LIP / Con	-0.78	[-1.03, -0.52]	t_{268}=-5.951[a]	**$8.3 x10^{-9}$**
	LIP	-0.45 ± 0.93	(-2.89–1.90)	LIP / HIP	-0.23	[-0.41, -0.05]	t_{167}=-2.471[a]	*0.014*
Premorbid	SZ	26.2 ± 6.1	(4-37)					
intelligence[d]	Con	30.9 ± 4.0	(18–37)	HIP / Con	-0.22	[-2.06, 1.62]	t_{267}=-0.236[a]	0.81
mean ± SD (range)	HIP	27.8 ± 5.0	(13–37)	LIP / Con	-2.60	[-4.74, -0.46]	t_{267}=-2.393[a]	*0.017*
	LIP	23.8 ± 7.0	(5–35)	LIP / HIP	-2.51	[-4.19, -0.83]	t_{166}=-2.955[a]	**$3.6 x10^{-3}$**

Multiple testing adjusted significances (applying Bonferroni ($p \leq 0.007$) and the closed testing principle) are set in boldface. All other p-values ≤ 0.05 are set in italic; [a]t-Statistic t_{df} with df degrees of freedom for estimation of difference in means (employing a linear model on Blom-transformed trait); [b]Fisher's exact test for count data; [c]Adjusted for age at exam and PANSS negative (value 7 for healthy control). Reasoning ability was measured by LPS 3. Fine motor function composite is the mean from standardized (by Blom-transform) McQuarrie Dotting and McQuarrie Tapping; [d]Adjusted for age (age of onset of psychosis for schizophrenic HIP and LIP, age at exam for healthy control) and PANSS negative (value 7 for healthy control). Premorbid intelligence was assessed by MWTB. Abbreviations: Con= Healthy control (N=102); SZ= Descriptive statistics for whole schizophrenic sample (N=881); HIP= High interpretation performers (N=88); LIP= Low interpretation performers (N=88), OR=Odds ratio.

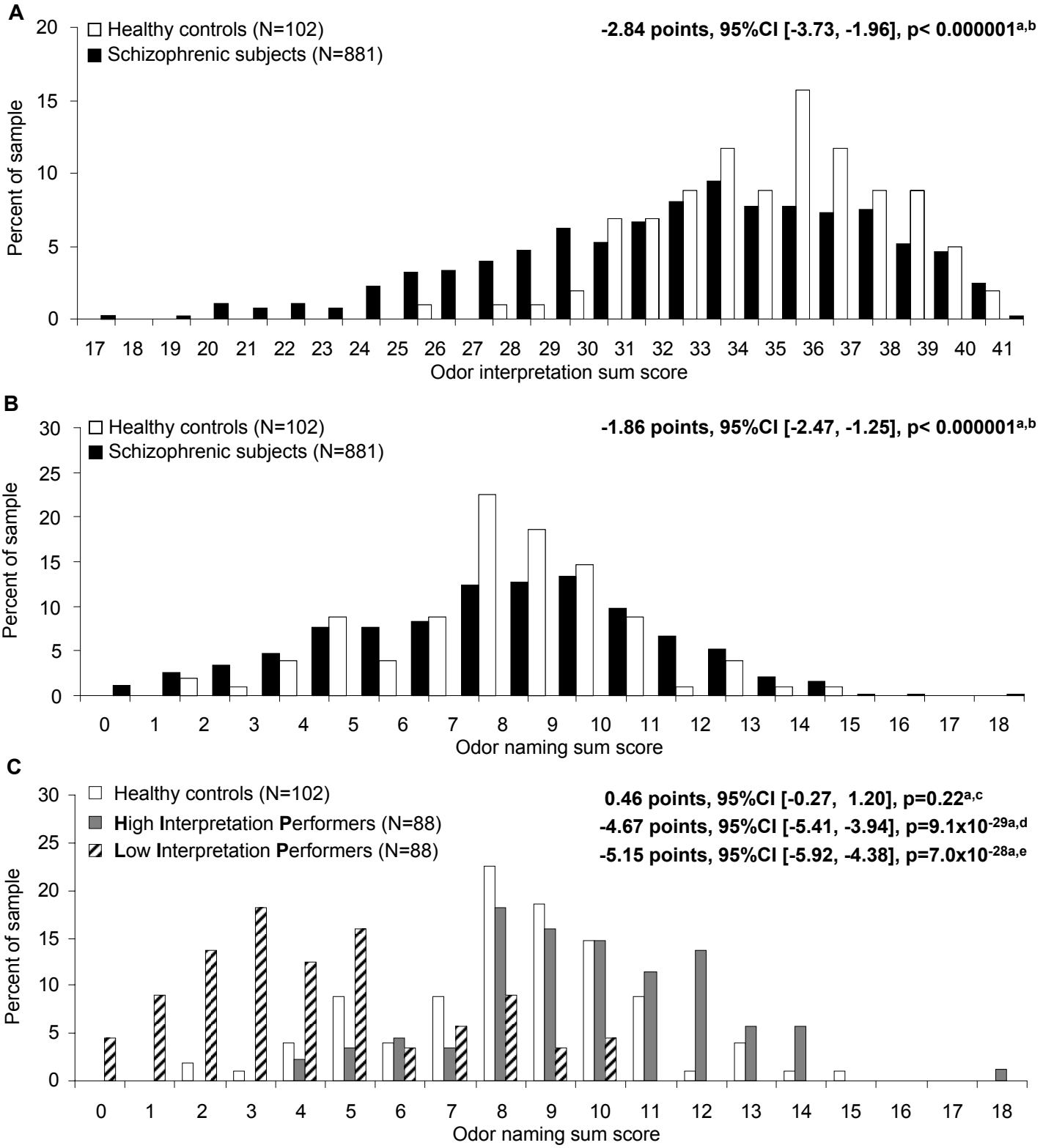
Table 3

Severely impaired interpretation performers (LIP) show a more severe psychopathology, compromised cognition and emotional processing compared to high interpretation performers (HIP)

		Descriptive			LIP compared to HIP	
		SZ (N=881)	**LIP (N=88)**	**HIP (N=88)**	**Effect [95% CI]**	**P (Statistic)**
Disease relevant confounders						
Years of education (years)	median interquartile range	12.0 [10.0, 13.5] (8.0-27.0)	11.0 [9.0,13.0] (8.0-20.5)	13.0 [10.0,15.0] (8.0-24.0)	-1.55 [-2.42, -0.68]	**5.4 x10^{-4}** (t_{174}=-3.525 [a])
Duration of disease (years)	median interquartile range	11.0 [4.7, 20.1] (0.008,58.4)	13.7 [6.4, 21.9] (0.1, 47.4)	11.1 [5.3, 20.9] (0.6, 45.3)	-	0.28 (W=3466[b])
Number of hospitalizations	median interquartile range	3.0 [2.0, 6.0] (0, 55)	3.0 [2.0, 5.0] (1, 44)	4.0 [3.0, 6.0] (1, 50)	-	0.10 (W=2532[b])
Chlorpromazine equivalents	median interquartile range	500 [250, 900] (0, 7375)	600 [300, 1072] (0, 3238)	450 [228, 817] (0, 3064)	-	0.068 (W=3216[b])
Functional outcome, disease severity and psychopathology						
General assessment of functioning (GAF)	mean ± SD range	45.6 ± 17.2 (5, 90)	38.0 ± 17.1 (10, 85)	49.8 ± 17.0 (20, 90)	-11.93 [-16.99, -6.87]	**6.5x10^{-6}** (t_{171}=-4.654[a])
Age-at-prodrome (years)	median interquartile range	20 [17, 26] (2, 66)	19 [16, 24] (12, 66)	23 [17, 30] (2, 47)	-2.70 [-5.44, 0.05]	0.054 (t_{153}=-1.942[a])
PANSS negative	median interquartile range	17.0 [12.0, 23.0] (7.0, 44.0)	22.0 [14.0, 27.0] (7.0, 40.0)	15.0 [11.0, 21.8] (7.0, 38.0)	4.54 [2.21, 6.88]	**1.7 x10^{-4}** (t_{171}= 3.840[a])
PANSS positive	median interquartile range	12.0 [9.0, 17.0] (7.0, 38.0)	15.0 [10.0, 22.0] (7.0, 37.0)	11.0 [8.0, 15.8] (7.0, 38.0)	3.96 [1.94, 5.98]	**1.6 x10^{-4}** (t_{171}= 3.869[a])
Disease-related cognition						
Higher cognition composite[c]	mean ± SD range	-0.03 ± 0.78 (-2.64, 1.93)	-0.41 ± 0.84 (-2.64, 1.40)	0.07 ± 0.83 (-1.92, 1.77)	-0.24 [-0.40, -0.07]	**4.3 x10^{-3}** (t_{169}=-2.896[a])
Alertness (TAP)[c] (msec)	median interquartile range	-282 [-350, -245] (-1288, -163)	-331 [-479, -270] (-1219, -206)	-270 [-311, -237] (-878, -192)	-80.75 [-121.61, -39.89]	**1.4 x10^{-4}** (t_{164}=-3.902[a])
Emotional processing						
PANSS negative N1+N2+N3[d]	median interquartile range	8.0 [5.0, 11.0] (0.0, 21.0)	10.0 [6.0, 12.3] (3.0, 18.0)	7.0 [4.0, 11.0] (0.0, 16.0)	-	**3.3 x10^{-3}** (W=2883[b])

Multiple testing adjusted significances (Bonferroni: p≤0.0045) are set in boldfaces, all other p-values≤0.05 in italic. [a]t-Statistic t_{df} with df degrees of freedom for estimation of difference in means (employing a linear model on Blom-transformed trait; small differences in degrees of freedom are due to low percentages of missing trait values). [b]Wilcoxon rank sum test. [c]Adjusted for age and PANSS negative. [d]N1: Blunted affect, N2: Emotional withdrawal, N3: Poor rapport. Abbreviations: SZ= Descriptive statistics for whole schizophrenic sample (N=881); HIP= High interpretation performers (N=88); LIP= Low interpretation performers (N=88), OR=Odds ratio.

Kästner et al supplemental figure 1



Supplemental figure 1: Odor naming and interpretation sum scores in patients and healthy controls and extreme group comparison based on odor interpretation performance. (A) Schizophrenic subjects experience severe problems in correct interpretation of odors compared to matched healthy controls (p<0.000001). **(B)** Schizophrenic subjects are significantly impaired in the odor naming task compared to matched healthy controls (p<0.000001). **(C)** Extremely poor interpretation performers (LIP) perform worse in odor naming compared to high interpretation performers (HIP) and healthy controls.

[a]Linear regression on Blom-transformed trait, adjusted for age and gender (naming). Smoking status did not significantly influence naming, nor interpretation. [b]Schizophrenic compared to healthy subjects (test statistic t_{979}=-6.318 (interpretation), t_{978}=-5.993 (naming)). [c]Schizophrenic HIP compared to healthy subjects (t_{273}=1.234). [d]Schizophrenic LIP compared to healthy subjects (t_{273}=-12.533). [e]Schizophrenic LIP compared to schizophrenic HIP (t_{172}=-13.183). Abbreviations : CI= Confidence interval; HIP= High odor interpretation performers; LIP= Low odor interpretation performers.

Supplemental table 1

Psychometric properties of odor interpretation and odor naming tasks in the schizophrenic sample (N=881)

	Odor naming[a]	Odor interpretation (odors)[b]		Odor interpretation (attributes)[b]
Item difficulty (weighted % correct naming ± 95%CI)				
Pineapple	21.71 ± 0.01	73.0 ± 1.7	Pleasant	81.7 ± 1.2
Gasoline	38.67 ± 0.02	78.2 ± 1.3	Sweet	72.6 ± 1.1
Mint	76.35 ± 0.03	78.0 ± 1.2	Hot	69.5 ± 1.4
Rose	39.99 ± 0.02	80.2 ± 1.3	Technical	81.3 ± 1.1
Natural gas	16.61 ± 0.02	70.9 ± 1.6	Natural	75.1 ± 1.3
Licorice	54.48 ± 0.03	82.3 ± 1.3	Edible	80.4 ± 1.2
			Dangerous	79.5 ± 1.1
Item discrimination, part-whole corrected [95%CI]				
Pineapple	0.24 [0.18, 0.30]	0.21 [0.15,0.27]	Pleasant	0.46 [0.40,0.51]
Gasoline	0.25 [0.19, 0.31]	0.25 [0.19,0.31]	Sweet	0.33 [0.27,0.39]
Mint	0.30 [0.24, 0.36]	0.27 [0.20,0.33]	Hot	0.18 [0.11, 0.24]
Rose	0.28 [0.21, 0.34]	0.18 [0.11,0.24]	Technical	0.58 [0.53, 0.62]
Natural gas	0.13 [0.07, 0.20]	0.16 [0.09,0.22]	Natural	0.45 [0.40,0.50]
Licorice	0.27 [0.20, 0.33]	0.20 [0.14,0.27]	Edible	0.52 [0.47,0.56]
			Dangerous	0.52 [0.47,0.56]
Item discrimination, not part-whole corrected [95%CI]				
Pineapple	0.41 [0.35, 0.46]	0.57 [0.52,0.61]	Pleasant	0.63 [0.59,0.67]
Gasoline	0.50 [0.45, 0.54]	0.53 [0.48,0.58]	Sweet	0.52 [0.47,0.56]
Mint	0.60 [0.55, 0.64]	0.52 [0.47,0.57]	Hot	0.44 [0.38,0.49]
Rose	0.56 [0.51, 0.61]	0.45 [0.40,0.50]	Technical	0.72 [0.69,0.75]
Natural gas	0.33 [0.26, 0.39]	0.50 [0.45, 0.55]	Natural	0.64 [0.60,0.68]
Licorice	0.65 [0.62, 0.69]	0.48 [0.43,0.53]	Edible	0.68 [0.64,0.71]
			Dangerous	0.66 [0.62,0.70]
Internal consistency (standardized Cronbach´s α, equals average split-half reliability)				
	0.50 [0.47, 0.54]	0.44 [0.40,0.48]		0.72 [0.71,0.74]

Abbreviations: CI, confidence interval.
[a]Psychometric measures were calculated based on Spearman correlation (ordinal data).
[b]Psychometic measures were calculated based on Pearson correlation (metric data).

Supplemental table 2

Presentation of high (HIP) and low (LIP) interpretation performer profiles with respect to higher cognition composite components and emotional processing score subscales

		Descriptive			LIP compared to HIP	
		SZ	LIP	HIP	Effect [95% CI]	p (Statistic)
Higher cognition composite components						
Reasoning[c]	mean ± SD range	20.8 ± 6.6 (2-38)	18.2 ± 6.7 (2–34)	21.5 ± 6.5 (8–38)	-2.61 [-4.35, -0.86]	**$3.6x10^{-3}$** (t_{164}=-2.951[a])
Executive functioning[c]	median interquartile range	-56.5 [-103.0, -36.0] (-868– 40)	-79.0 [-139.0, -47.0] (-680– -9)	-47.0 [-122.0, -34.0] (-563– -10)	-24.77 [-52.69, 3.15]	0.082 (t_{155}=-1.753[a])
Working memory[c]	mean ± SD range	13.1 ± 3.9 (1–24)	11.7 ± 4.4 (4–22)	13.6 ± 3.9 (4–24)	-1.25 [-2.50, -0.00]	*0.049* (t_{158}=-1.981[a])
Processing speed[c]	mean ± SD range	38.4 ± 13.2 (4–88)	32.5 ± 13.7 (5–68)	39.3 ± 13.5 (6–71)	-4.52 [-7.99, -1.04]	*0.011* (t_{167}=-2.566[a])
Verbal memory[c]	mean ± SD range	42.0 ± 12.6 (6–72)	37.3 ± 12.5 (11–62)	43.0 ± 14.0 (6–70)	-3.38 [-7.22, 0.45]	0.083 (t_{161}=-1.742[a])
Divided attention[c]	median interquartile range	-725 [-806, -659] (-1663– -355)	-764 [-833, -651] (-1167– -448)	-714 [-797, -647] (-1160– -416)	4.14 [-35.50, 43.78]	0.84 (t_{158}= 0.206[a])
Emotional processing subscales						
Blunted affect (PANSS N1)	median interquartile range	3 [2, 4] (1–7)	4 [3, 5] (1–7)	3 [2, 4] (1–6)	-	**$7.5x10^{-3}$** (W=2947[b])
Emotional withdrawal (PANSS N2)	median interquartile range	3 [1, 4] (1–7)	3 [1, 4] (1–7)	3 [1, 4] (1–6)	-	*0.029* (W=3119[b])
Poor rapport (PANSS N3)	median interquartile range	2 [1, 3] (1–7)	3 [1, 4] (1–6)	2 [1, 3] (1–5)	-	**$3.1 x10^{-3}$** (W=2875[b])

Multiple testing adjusted significances are set in boldface (p≤0.0083 for components of higher cognition composite, p≤0.0167 for subscales of emotional processing score). All other p-values≤0.05 in italic. [a]t-Statistic t_{df} with df degrees of freedom for estimation of difference in means (employing a linear model on Blom-transformed trait). [b]Wilcoxon rank sum test. [c]Adjusted for age and PANSS negative. Reasoning ability was measured by LPS3, executive functioning is represented as difference of execution times between TMTA and TMTB, working memory was assessed by BZT, processing speed by ZST, verbal memory by VLMT and divided attention by negative execution time (-d3mdg) of TAP. In this table, and when building the cognition composite, all measures were presented such that larger values represent better cognitive performance. Abbreviations: SZ= Descriptive statistics for schizophrenic sample (N=881); HIP= High interpretation performers (N=88); LIP= Low interpretation performers (N=88).

4. Loss-of-function genotypes of the neuronal glycoprotein GPM6a are associated with claustrophobia

4.1 Overview of project III

Prolonged exposure to stress leading to dysregulation of the limbic-hypothalamo-pituitary-adrenal axis (LHPA) has been shown to confer susceptibility to depression and anxiety disorders (Shin et al. 2010); two conditions which frequently co-occur (Tronson et al. 2008). Besides evoking subjective distress, elevated levels of glucocorticoids induce physiological and morphological changes in amygdala and hippocampus (Magarinos et al. 1995, McEwen 2008). More specifically, it has been demonstrated across different species that dendritic branching is increased in amygdala while it is found to be decreased in hippocampus after exposure to chronic stress (McEwen 2001, Mitra et al. 2005, Conrad 2006, McEwen 2008). Amygdala and hippocampus have been implicated in fear conditioning and extinction learning, respectively (Shin et al. 2010). Thus, the reported stress-induced morphological alterations might contribute to changes in anxiety-related behaviors, like e.g. facilitation of fear conditioning and impaired extinction learning (Eiland et al. 2012). Both processes have been suggested to be involved in the etiology of anxiety disorders like specific phobias (Stein et al. 2006).

The neuron-specific tetraspan membrane glycoprotein Gpm6a is among the genes identified to be down-regulated in hippocampus in response to chronic stress (Alfonso et al. 2004). It belongs to the myelin proteolipid protein family (Lagenaur et al. 1992) and is abundantly expressed in all subregions of hippocampus (Alfonso et al. 2005). *In vitro* approaches inducing both loss- and gain of function of Gpm6a revealed its putative role in neurite outgrowth, and dendritic spine formation in hippocampal neurons (Alfonso et al. 2005, Michibata et al. 2009). As a result, glycoprotein M6a likely influences stress-induced morphological alterations in hippocampus. Whether the Gpm6a-mediated hippocampal neuropathology contributes to the emergence of anxiety-related phenotypes has not been studied so far.

To investigate the behavioral consequences of prolonged stress in the absence of Gpm6a, *Gpm6a* null mutant mice were generated and behaviorally characterized. Whereas these mice did not display any abnormalities in development and basic behavior, a striking phenotype

emerged in the null mutants independent of the severity of previously applied stress. Mild social isolation stress (i.e. single housing) was sufficient to reveal clear avoidance of the closed arms of the elevated plus maze in the mice lacking Gpm6a; a phenotype reminiscent of human claustrophobia. Further behavioral assays in several cohorts of mice substantiated the claustrophobia-like phenotype in male and female Gpm6a null mutant mice.

Interestingly, the human *GPM6A* gene is located on chromosome 4q32-q34, a region linked to panic disorder (Domschke et al. 2008) but also other anxiety disorders such as agoraphobia or specific phobia (Kaabi et al. 2006). These findings support the notion of susceptibility loci being shared between different diagnostic categories which are highly co-morbid. Along the same lines, depressive symptoms have been shown to be modulated by *GPM6A* in a subgroup of schizophrenic individuals (Boks et al. 2008). To translate our findings in mice to human subjects and thus to explore a potential role of *GPM6A* in human claustrophobia, 115 adult subjects (N=47 claustrophobics and N=68 non-claustrophobic controls; matched for age, gender, marital status and co-morbid disease state) were recruited and interviewed. After validation of claustrophobia status applying DSM-IV criteria (APA 1994), subjects underwent detailed claustrophobia relevant phenotyping using the abbreviated German version of the Claustrophobia Questionnaire (Radomsky et al. 2001) (Short CLQ-G) which was specifically developed and psychometrically evaluated for this project. Claustrophobic cases displayed higher ratings on all 10 items of the Short CLQ-G than controls while the prevalence of DSM-IV anxiety disorders other than claustrophobia did not substantially differ between groups. Genomic sequencing of *GPM6A* covering all exons and flanking noncoding regions identified nine rare single-base substitutions in the noncoding regions of *GPM6A* that were more frequent in the affected individuals as compared to the controls. Moreover, one variant in the 3' untranslated region was found to be genetically linked to claustrophobia in two small pedigrees. Mechanistically, this variant is in an interesting position as it is located in the seed sequence of microRNA (miR) 124. The latter is brain expressed and upregulated in amygdalae of mice in response to restraint stress. Overexpression of miR124 in peripheral blood mononuclear cells of human subjects revealed a down-regulation of *GPM6A* mRNA in carriers of the variant *not* associated with claustrophobia. As a conclusion, we suggest that loss of the dynamic regulation of neuronal *GPM6A* expression poses a genetic risk for claustrophobia.

4.2 Original publication

El-Kordi, A.*, **Kästner, A.***, Grube, S.*, Klugmann, M., Begemann, M., Sperling, S., Hammerschmidt, K., Hammer, C., Stepniak, B., Patzig, J., de Monasterio-Schrader, P., Strenzke, N., Flugge, G., Werner, H. B., Pawlak, R., Nave, K. A. and Ehrenreich, H. (2013). "A single gene defect causing claustrophobia." Transl Psychiatry **3**: e254.

*These authors contributed equally to the work.

Personal contribution

I was responsible for the development and evaluation of the abbreviated German version of the Claustrophobia Questionnaire, for the recruitment and examination of the claustrophobic patients, the control subjects and pedigree members. I conceptualized and carried out the telephone interviews. Moreover, I performed the human data analyses and assisted in the interpretation of the results. I participated in the conception, design, drafting, revision and publication of the manuscript.

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A single gene defect causing claustrophobia

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Claustrophobia, the well-known fear of being trapped in narrow/closed spaces, is often considered a conditioned response to traumatic experience. Surprisingly, we found that mutations affecting a single gene, encoding a stress-regulated neuronal protein, can cause claustrophobia. *Gpm6a*-deficient mice develop normally and lack obvious behavioral abnormalities. However, when mildly stressed by single-housing, these mice develop a striking claustrophobia-like phenotype, which is not inducible in wild-type controls, even by severe stress. The human *GPM6A* gene is located on chromosome 4q32-q34, a region linked to panic disorder. Sequence analysis of 115 claustrophobic and non-claustrophobic subjects identified nine variants in the noncoding region of the gene that are more frequent in affected individuals ($P = 0.028$). One variant in the 3′untranslated region was linked to claustrophobia in two small pedigrees. This mutant mRNA is functional but cannot be silenced by neuronal miR124 derived itself from a stress-regulated transcript. We suggest that loosing dynamic regulation of neuronal *GPM6A* expression poses a genetic risk for claustrophobia.

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Introduction

The neuronal tetraspan membrane glycoprotein Gpm6a has been implicated in neurite outgrowth and dendritic spine formation,[1–3] but the lack of a mouse mutant has prevented any *in vivo* analysis of *Gpm6a* function. Specifically, the observation that *Gpm6a* expression in rodent brain is downregulated by cortisol or following physical restraint stress[4] has been puzzling. As stress is a key factor for triggering mental disorders,[5] we investigated the behavioral consequences of resident-intruder stress in mice lacking the *Gpm6a* gene. We report here the unexpected finding that the neuronal gene *Gpm6a* constitutes a genetic cause of a highly unusual 'claustrophobia-like' phenotype in null mutant mice, which otherwise develop completely normally. In fact, only *Gpm6a* mouse mutants that have experienced a mild 'social stress' exhibit this 'claustrophobia-like' behavior. Moreover, we translate this finding to human individuals, where we find rare sequence variants in the *GPM6A* gene associated with claustrophobia. Mechanistic insight is provided by the demonstration of a human variant-specific loss of *GPM6A* regulability. We conclude that regulability of the *GPM6A* gene under stress is required to avoid claustrophobia, which emerges as an unusual stress response.

Materials and methods

Generation and characterization of *Gpm6a* null mutant mice. All experiments were approved by the local Animal Care and Use Committee in accordance with the German Animal Protection Law. Mice with a targeted inactivation of the *Gpm6a* gene were generated. First a gene-targeting vector (Figure 1a) was constructed. From the cloned mouse (129SV) *Gpm6a* gene, a 6.5-kb fragment of intron 2 became the long homologous arm. A 1.5-kb fragment that included the 3′-part of intron 1 and 6 bp at the 5′-end of exon 2 became the short homologous arm. It was cloned with tailored PCR primers introducing *Hin*d3 (5′) and *Bam*H1 (3′) restriction sites. For negative selection, a neomycin-resistance gene (*neo*) under control of the herpes simplex virus (HSV) thymidine kinase (tk) promoter (kindly provided by R Sprengel, MPI Heidelberg) was utilized. The neomycin cassette was subcloned with tailored PCR primers introducing at both the 5′- and the 3′-end *Bam*H1 restriction sites and translation termination codons in all reading frames. For positive selection, a Cla1 fragment of the HSV-tk under control of the HSV-tk promoter was subcloned into the vector. The construct was verified by molecular sequencing, and the vector backbone (pKS$^+$ bluescript, Stratagene Heidelberg, Germany) was linearized with Not1. Using

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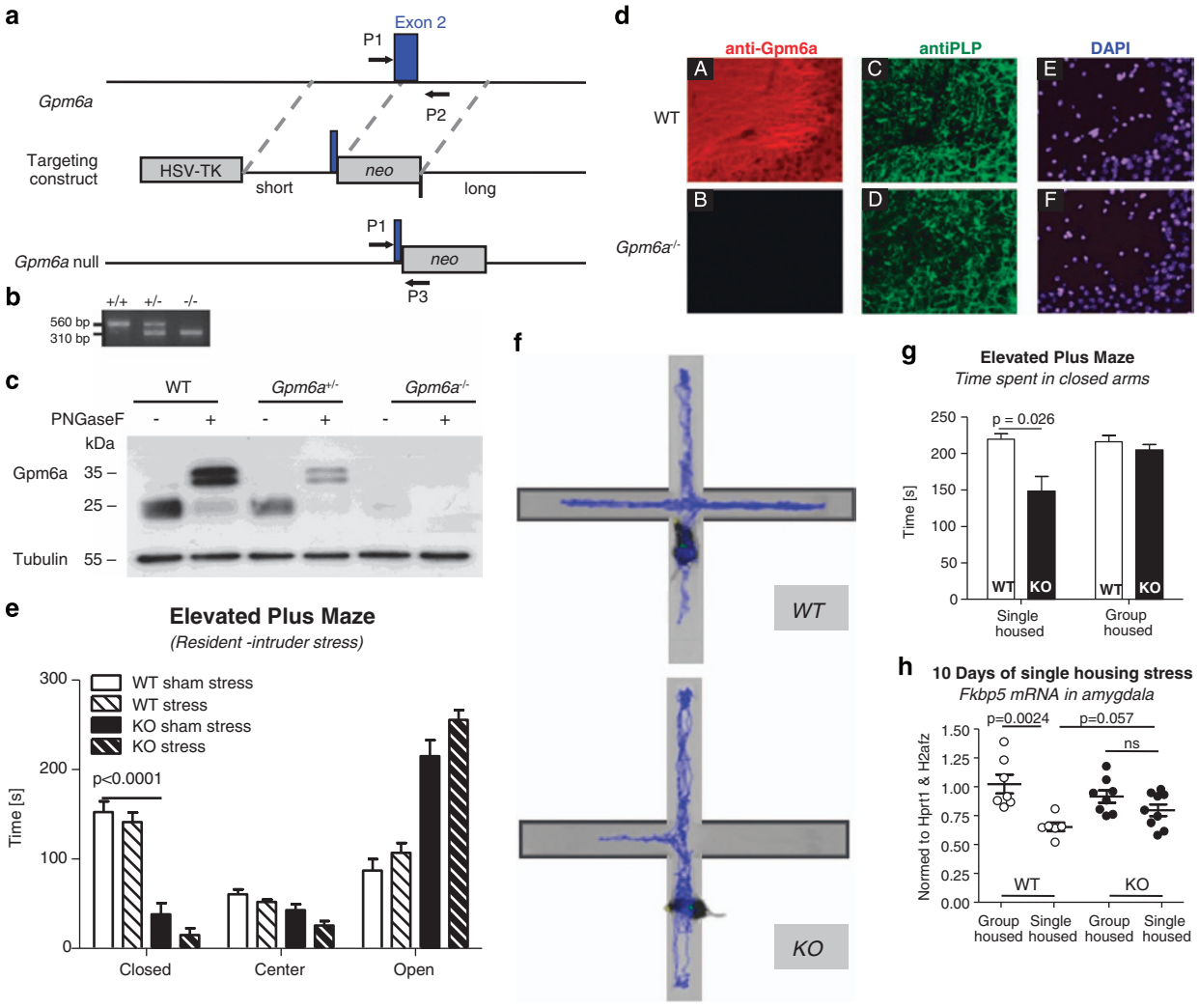

Figure 1 Generation of *Gpm6a* null mutant mice and discovery of behavioral consequences following stress. (**a**) Strategy to inactivate the mouse *Gpm6a* gene. A neomycin resistance cassette flanked by translation stop codons in all reading frames was fused into exon 2, which is the first exon present in all *Gpm6a* transcripts. (**b**) PCR genotyping to identify wild-type (WT) and mutant *Gpm6a* alleles. (**c**) Immunoblot analysis of cortex homogenates using antibodies directed against Gpm6a or tubulin, with or without prior deglycosylation using PNGaseF. Gpm6a was undetectable in *Gpm6a* null mutants. Note that the abundance of Gpm6a was considerably reduced in heterozygous mice. (**d**) Immunohistochemistry of brain sections with antibodies directed against Gpm6a or the related proteolipid protein (PLP). Note that Gpm6a was not detected in *Gpm6a* null mutant mice that showed unchanged PLP expression. (**e**) Elevated plus maze (EPM) behavior of psychosocially stressed (resident-intruder paradigm) as well as of sham-stressed *Gpm6a* null mutants (KO) reveals a prominent claustrophobia-like phenotype ($N = 17$–19 per group). (**f**) Sample track recording of EPM performance, illustrating a *Gpm6a* mutant spending much less time in closed arms compared with its WT littermate. (**g**) Single housing (a prerequisite of performing the resident-intruder stress) is sufficient to induce a claustrophobia-like phenotype in *Gpm6a* mutants ($N = 7$–9 per group), which is absent upon group housing, and (**f**) to reveal a clear genotype difference in the expression of a stress-regulated gene, *Fkbp5,* in mouse amygdala ($N = 7$–9 per group). Mean ± s.e.m. presented. DAPI, 4′-6-diamidino-2-phenylindole; HSK-tk, herpes simplex virus-thymidine kinase.

standard procedures,[6] R1 mouse embryonic stem cells (R1-ES, provided by A Nagy, Toronto, Canada), suspended in phosphate-buffered saline (PBS) with 40 μg linearized targeting vector, were electroporated using a Bio-Rad GenePulser (240 V and 500 μF, Bio-Rad, Munich, Germany). Transfected embryonic stem cells (2×10^7) were cultured on gelatinized 10-cm dishes (Falcon, Heidelberg, Germany) for 1 day and then selected with 300 μg ml^{-1} G418 and 2 μM Gancyclovir. On day 10 after electroporation, 386 resistant clones were picked and one with homologous recombination was identified by semi-nested PCR. Amplification was (1) with forward primer (5′-GGGCTGACTTTTGGATTTTGTGG-3′) and reverse primer (5′-GCCTCTCCACCCAAGCGGCCG GAGAACCTGCGTGC-3′) and (2) on the first PCR product with alternative reverse primer (5′-GCAATCCATCTTGTT CATGGC-3′). Embryonic stem cells were microinjected into C57Bl6/6J blastocysts that were transferred to pseudo-pregnant foster mothers. Highly chimeric males ($N = 4$) were obtained that were bred to C57Bl6/6J females. We interbred heterozygous offspring to obtain homozygous mutant mice, which were born at the expected Mendelian frequency. *Gpm6a* null mutant mice are viable and fertile. For genotyping (Figure 1b), genomic DNA was isolated from tail biopsies using the DNeasy96 kit (Qiagen, Hilden, Germany) according

to manufacturer's directions. In a PCR co-amplification reaction, the presence of the *Gpm6a* wild-type (WT) allele was shown using forward primer #1 (5′-TTGCTCTTCTAC AGGGTGCT-3′) and reverse primer #2 (5′-CCTCCA TCCTCTGTCATTCC-3′), which yielded a 560-bp fragment. We identified the targeted allele with forward primer #1 and reverse primer #3 (5′-GCAATCCATCTTGTTCAATGGC-3′), yielding a 310-bp fragment. For protein analysis (Figure 1c), we prepared total cortex lysates from WT, heterozygous and homozygous mice and determined the protein concentration according to Bradford, and boiled the samples (5 min) before loading. For immunoblot, we separated 40 μg lysate by 12% SDS-polyacrylamide gel electrophoresis and transferred the samples on poly(vinylidene fluoride) membranes (Hybond-P, Amersham Biosciences, Glattbrugg, Switzerland). We blocked the membrane in 5% milk powder in PBS with 0.1% Tween (30 min at 37 °C). Antibodies were directed against the C-terminus of Gpm6a (#24983; 1:500) or tubulin (Sigma, Heidelberg, Germany; 1:5000) and applied in blocking buffer (over night, 4 °C). Following wash, membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany, 1:5000 in blocking buffer). Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce, Bonn, Germany). For immunohistochemistry (Figure 1d), WT and *Gpm6a* null mutant mice were anesthetized with Avertin (250 mg/kg body weight; Sigma), perfused with Hank's balanced salt solution, followed by 4% formaldehyde in PBS and the isolated brains were post-fixed for 1 h. Vibratome sections (thickness 12 μm, Leica VT 1000S, Leica Biosystems, Wetzlar, Germany) were permeabilized with 0.4% Triton X-100 in PBS (30 min, room temperature), blocked in 4% horse serum in PBS (30 min, room temperature) and incubated with antibodies against Gpm6a (M6, rat monoclonal, 1:25; kind gift by Carl Lagenaur,[7] Pittsburgh, USA) or proteolipid protein (A431, rabbit polyclonal, 1:500)[8] at 4 °C for 24 h. After wash, sections were incubated with appropriate fluochrome-coupled secondary antibodies (Dianova, Hamburg, Germany; 2 h, room temperature) and washed three times. Sections were imaged with Leica DMRXA and OpenLab 2.0 software (Improvision, Tübingen, Germany).

Behavioral testing. For behavioral testing, mice were housed in groups of three to five in standard plastic cages, food and water *ad libitum*. The temperature in the colony room was maintained at 20–22 °C, with a 12-h light/dark cycle (light on at 0700 hours). Behavioral experiments were conducted by an investigator, blinded to the genotype, during the light phase of the day (between 0800 hours and 1700 hours). For behavioral experiments, eight different cohorts of mice were used. The order of testing in the first cohort was as follows: elevated plus maze (EPM), open field, hole board, rotarod, pre-pulse inhibition, fear-conditioning, visual cliff. In further cohorts, EPM release in closed arms, EPM in the dark, mouse light/dark box test, mouse wide/narrow box test, EPM retesting ('exposure treatment') and hearing were performed. For electroretinogram, olfaction testing and corticosterone determination upon metabolic cage exposure, separate cohorts were used. Age of mice at the beginning of testing was 19 weeks. Inter-test interval varied depending on the degree of 'test invasiveness' but was at least 1 day. During all tests, the investigator was 'blinded', that is, unaware of mouse genotypes. For comprehensive test description of basic tests, that is, EPM, open field, hole board, rotarod, visual cliff test (vision), buried food finding test (olfaction), sucrose preference test (motivation), pre-pulse inhibition, cued and contextual fear-conditioning, and ultrasound vocalization analysis, please see El-Kordi *et al.*[9] Described in the following are additional, modified or specifically designed tests.

EPM with release in closed arms. In this modified version, mice were placed in the closed arms in the same plus-maze described above. This test was done to address potential motor factors influencing the time spent in arms. The test was otherwise conducted in the same manner as the classical EPM.

EPM in darkness. This test was again performed like the classical EPM, just in full darkness to address potential visual/perceptual factors affecting behavior in open/closed space. The behavior of mice was monitored via infrared camera.

Hot plate test. The hot plate test is used as a measure of pain sensitivity. Mice were placed on a metal plate (Ugo Basile, Comerio, Italy), preheated up to 55 °C. The latency of hind paw licking or jumping was recorded. Mice were removed from the platform immediately after showing the response. A 40-s cutoff time was supposed to prevent wounds, although none of the tested mice reached it.

Assessment of hearing by the acoustic startle response. Individual mice were placed in small metal cages ($90 \times 40 \times 40\,mm^3$) to restrict major movements and exploratory behavior. The cages were equipped with a movable platform floor attached to a sensor that records vertical movements of the floor. The cages were placed in four sound-attenuating isolation cabinets (TSE GmbH, Bad Homburg, Germany). Startle reflexes were evoked by acoustic stimuli delivered from a loudspeaker that was suspended above the cage and connected to an acoustic generator. The startle reaction to an acoustic stimulus (pulse), which evokes a movement of the platform and a transient force resulting from this movement of the platform, was recorded with a computer during a recording window of 100 ms and stored for further evaluation. The recording window was defined from the onset of the acoustic stimulus. An experimental session consisted of a 2-min habituation to 65 dB background white noise (continuous throughout the session), followed by a baseline recording for 1 min at background noise. After baseline recording, stimuli of different intensity and fixed 40 ms duration were presented. Stimulus intensity was varied between 65 and 120 dB, such that 19 intensities from this range were used with 3 dB step. Stimuli of the each intensity were presented 10 times in a pseudorandom order with an interval ranging from 8 to 22 s. The amplitude of the startle response (expressed in arbitrary units) was defined as the difference between the maximum force detected during a recording window and the force measured immediately before the stimulus onset. Amplitudes of responses for each

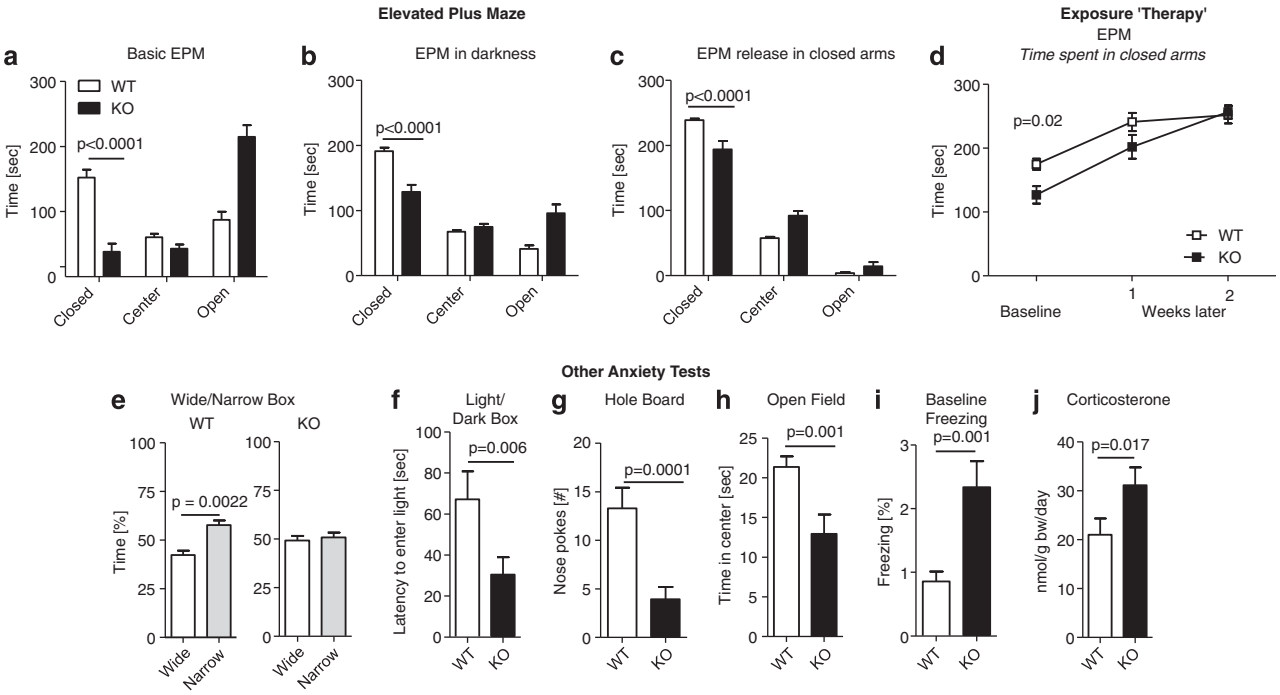

Figure 2 Male *Gpm6a* mutants show a strong claustrophobia-like phenotype on top of mild anxiety features. (**a**) Behavior of *Gpm6a* knockout (KO) and wild-type (WT) littermates in classical elevated plus maze (EPM); (**b**) in EPM performed in the darkness; and (**c**) in EPM upon release in closed arms. (**d**) Weekly exposure to EPM (over 3 weeks) led to reduction of closed arm aversion in *Gpm6a* KO. This adjustment also explains the weaker closed-arm avoidance seen in mice of **b** and **c**, which had had one previous exposure to EPM. (**e**) In a newly developed wide/narrow box test, WT mice spent more time in the narrow area (left graph), whereas *Gpm6a* KO did not show narrow space preference. (**f**) In the light/dark box, *Gpm6a* KO mice entered the light area faster; (**g**) explored less holes (requiring nose pokes in narrow holes); (**h**) spent less time in the center of the open field, and (**i**) exerted higher baseline freezing in the fear-conditioning chamber. (**j**) Exposed to narrow metabolic cages for 3 h, *Gpm6a* KO excreted higher levels of corticosterone via urine. (**a–c**): $N = 17–18$; (**d**): $N = 8$; (**e**): $N = 32–35$; (**g–h**): $N = 17–18$; (**i**, **j**): $N = 12$. Mean ± s.e.m. presented.

stimulus intensity were averaged for individual animals. Mean values for each experimental group were plotted on the graph to provide the stimulus–response curves.

Mouse light/dark box test. The apparatus ($36 \times 20.5 \times 19$ cm^3) consisted of two equal acrylic compartments, one roofed, dark and one white, with a 300 lx light intensity in the white compartment and separated by a divider with an opening (size: $5.7 \times 5\,cm^2$) connecting both compartments. Each mouse was tested by placing it in the black/dark area, facing the white one, and was allowed to explore the novel environment for 5 min. The roof of the dark compartment was closed after releasing the mouse. The number of transfers from one compartment to the other and the time spent in the illuminated side were measured. This test exploited the natural conflict between the animal's drive to explore a new environment and its tendency to rather stay in a closed, dark and protected environment and to avoid bright light.

Mouse wide/narrow box test. This inhouse-made box (test arena: length 60 cm, width 60 cm and height 30 cm) consisted of two equal (each 30 cm length) gray plastic compartments. One compartment was wide and open, the other one narrow (consisting of $30 \times 5 \times 30\,cm^3$ corridor). Mice were placed in the wide compartment, facing the narrow corridor. Light intensity in the wide compartment was 300 lx, in the corridor 150 l×. Time to enter the corridor was recorded by a stopwatch. The behavior was recorded throughout the 10 min testing period by a PC-linked overhead video camera. 'Viewer 2' software was used to calculate velocity, distance travelled, number of visits of and time spent in both compartments.

Electroretinogram. Before the experiments, animals were dark adapted for at least 12 h and all preparations were carried out under dim red light.[10] Mice were anaesthetized by intraperitoneal injection of ketamine ($0.125\,mg\,g^{-1}$) and xylazine ($2.5\,\mu g\,g^{-1}$). Supplemental doses of 1/4 the initial dose were administered when changes in the constantly monitored electrocardiogram or movements indicated that the animals were waking up. Mice were placed on a heated mat (Hugo Sachs Elektronik–Harvard Apparatus, March, Germany) that kept the body temperature constant at 37 °C under the control of a rectal thermometer. The head of the mouse was placed inside a custom-designed Ganzfeld bowl illuminated by a ring of 20 white light-emitting diode. The pupil of the left eye was dilated with 1% atropine sulfate and a silver wire ring electrode was coupled to the corneal surface using electrode gel. The eye and electrode were kept moist by a drop of 0.9% saline applied every 30 min. Subcutaneous needle electrodes were inserted between the eyes (reference) and near the tail (ground). Electrical potentials were amplified 1000 times, filtered between 0.1 and 8 kHz and notch-filtered at 50 Hz using custom-designed hardware. The Tucker Davis System III hardware and BioSig software (Tucker-Davis Technology, Alachua, FL, USA) were used for

stimulus control and recordings. Scotopic responses to 10 white light flashes were averaged for each stimulus condition. Interstimulus intervals were 5 s for light intensities below 1 cds m^{-2} and 17 s for light intensities above 1 cds m^{-2}. The amplitude growth functions and latencies of the A-waves, B-waves and oscillatory potentials in response to 0.1, 1 and 5 ms long-light flashes ranging between 0.0003 and 10 cds m^{-2} was analyzed using custom-written matlab (Mathworks, Natick, MA, USA) software.

Corticosterone excretion. Urine samples were collected using inhouse-made metabolic cages. Mice were placed in small, narrow metal cages ($90 \times 40 \times 40\,mm^3$) to restrict major movements and exploratory behavior, thus resulting in stress-induced corticosterone release. These cages had a wire-mesh floor enabling urine collection via a funnel. The funnel was fixated on top of a collecting flask. Mice (12 per genotype) were placed in the metabolic cages at 2200 hours for 3 h each. Urine was collected at 0100 hours. Concentrations of corticosterone were measured using a commercially available EIA kit (BIOTREND, Cologne, Germany) according to the manufacturer's protocol. Urine creatinine was determined photometrically (Jaffe method). Sample analysis of WT and knockout (KO) animals was performed blinded and in random order. Values were expressed as nmol per day per g body weight.[11]

Resident-intruder (psychosocial stress) test. The procedure is described in detail elsewhere.[12,13] Briefly, male mice of both genotypes (28 days old) were randomly assigned to either the 'stress' or 'sham stress' group. As intruders, they were subjected for 21 days (1 h daily, from 0900–1000 hours) to resident male mice (male FVB, 2–3 months old, habituated to resident cages for $\geqslant$10 days). To prevent injuries, direct interaction was immediately terminated at the first attack (usually occurring after a few seconds) by putting a grid cage ($140 \times 75 \times 60\,mm^3$) over the intruder. Afterwards, intruder mice were placed back in their home cage. Mice were confronted with a different resident every day. Sham stress consisted of placing the intruder mouse in an empty novel cage for 1 h.

Restraint stress paradigm. Mice were kept undisturbed for at least 1 week until a single 6-h restraint stress was performed in a separate room (with mice left in their home cages and put in wire mesh restrainers, secured at the head and tail ends with clips) during the light period of the circadian cycle as described.[14] Control animals were left undisturbed.

Amygdala dissection. Mice were anaesthetized (intraperitoneal sodium pentobarbital 50 mg kg^{-1}) and perfused transcardially (ice-cold PBS). Amygdalae were dissected from a coronal slice -0.58 to -2.3 mm relative to Bregma and stored in *RNA later* (Qiagen) at 4 °C until processed.[14]

Quantitative reverse transcription-PCR from amygdala. Amygdala tissue was homogenized in Quiazol (Qiagen, Hilden, Germany). Total RNA was isolated by using the miRNeasy Mini Kit (Qiagen). First strand cDNA was generated from total RNA using N9 random and Oligo(dT) 18 primers. The relative concentrations of mRNAs of interest in different cDNA samples were measured out of three replicates using the threshold cycle method (deltaCt) for each dilution and were normalized to the normalization factor of *Hprt1* and *H2afz* genes calculated by the geNorm analysis software. Reactions were performed using SYBR green PCR master mix (ABgene, Foster City, CA, USA) according to the protocol of the manufacturer. Cycling was done for 2 min at 50 °C, followed by denaturation at 95 °C for 10 min. The amplification was carried out by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. The specificity of each primer pair was controlled with a melting curve analysis. For quantitative PCR, we used the following primers:

mFkbp5_forward: 5′-ATTTGATTGCCGAGATGTG-3′
mFkbp5_reverse: 5′-TCTTCACCAGGGCTTTGTC-3′
mNpy5r_forward: 5′-TCCCGAGGACTCTAGTATGGA-3′
mNpy5r_reverse: 5′-TCT GTAGTCCTCCCAGGCA-3′
mHPRT1_forward: 5′-GCTTGCTGGTGAAAAGGACCTC TCGAAG-3′
mHPRT1_reverse: 5′-CCCTGAAGTACTCATTATAGTCA AGGGCAT-3′
mH2afz_forward: 5′-ACAGCGCAGCCATCCTGGAGTA-3′
mH2afz_reverse: 5′-TTCCCGATCAGCGATTTGTGGA-3′

miR124. First strand cDNA synthesis and reactions were generated from total RNA using the TaqMan MicroRNA RT Kit, TaqMan MicroRNA Assay for hsa-miR124, TaqMan MicroRNA Assay for sno-RNA142 as a housekeeper and TaqMan 2 × Universal PCR Master Mix (ABgene) according to the manufacturer's protocol. Cycling was done with 10 min denaturation at 95 °C and amplification for 40 cycles at 95 °C for 15 s and 60 °C for 60 s.

Human sample

Claustrophobic subjects. The present study was approved by the Ethics Committee of the Georg-August-University. A total of 47 subjects with clinical diagnosis of claustrophobia according to Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV)[15] were included (Table 1). Healthy subjects ($N = 13$) were recruited by e-mail announcements in the Max Planck Institutes of Experimental Medicine (MPIEM) and Biophysical Chemistry (MPBPC). Patients suffering from psychiatric conditions other than psychotic disorders ($N = 16$; that is, $N = 7$ affective disorder, $N = 5$ anxiety disorder, $N = 2$ substance use disorder, $N = 2$ others) were recruited from the psychiatric hospital of the Georg-August-University Göttingen. In addition, $N = 18$ schizophrenic individuals with a claustrophobic phenotype were selected from the GRAS data collection.[16] Claustrophobic subjects were invited to the outpatient unit of the MPIEM for examination. In the case of GRAS patients, extensive telephone interviews were performed instead. Subjects underwent detailed claustrophobia relevant phenotyping, after validation of diagnoses using DSM-IV criteria[15] by a trained psychologist/psychiatrist. The subsequent examination procedure comprised a short questionnaire regarding sociodemographic information, history of physical and psychiatric diseases, specifically for this project

Table 1. Comparison of sociodemographic, general clinical and anxiety/claustrophobia relevant parameters in claustrophobic and non-claustrophobic subjects independent of mutation status

	Total sample (N = 115) Mean ± s.d.		*Claustrophobic subjects (N = 47) Mean ± s.d.*		*Non-claustrophobic subjects (N = 68) Mean ± s.d.*		*Statistics P*[a]	
Sociodemographics								
Age in years	43.56 ± 13.22		43.87 ± 12.11		43.35 ± 14.02		*0.733*	
Education in years	14.43 ± 3.55		14.31 ± 3.85		14.52 ± 3.35		*0.830*	
	N	*%*	N	*%*	N	*%*	*Effect*	*P*[b]
Gender								
Female	81	*70.4*	13	*27.7*	21	*30.9*	0.139	*0.710*
Male	34	*29.6*	34	*72.3*	47	*69.1*		
Ethnicity								
Caucasian	112	*97.4*	47	*100.0*	65	*95.6*	2.129	*0.546*
African	1	*0.87*	—	—	1	*1.47*		
Other	2	*1.73*	—	—	2	*9.94*		
Marital status								
Single	57	*49.6*	24	*51.1*	33	*48.5*	3.545	*0.471*
Married	33	*28.7*	12	*25.5*	21	*30.9*		
Divorced	19	*16.5*	11	*23.4*	11	*16.2*		
Widowed	3	*2.6*	—	—	3	*4.4*		
Main diagnoses according to DSM-IV								
No clinical diagnosis	27	*23.5*	13	*27.7*	14	*20.6*	1.135	*0.567*
Schizophrenia	43	*37.4*	18	*38.3*	25	*36.8*		
Other clinical diagnoses	45	*39.0*	16	*34.0*	29	*43*		
Prevalence of anxiety disorders								
Comorbid anxiety disorder[c]	68	*59.1*	29	*61.7*	39	*57.4*	*0.702*	
Panic disorder	29	*25.2*	15	*31.9*	14	*20.6*	*0.194*	
Agoraphobia[d]	56	*48.7*	47	*100.0*	9	*13.2*	*< 0.0001*	
Ssocial phobia	18	*15.7*	9	*19.1*	9	*13.2*	*0.440*	
Specific phobia	38	*33.0*	20	*42.6*	18	*26.5*	*0.106*	
Generalized anxiety disorder	13	*11.3*	7	*14.9*	6	*8.8*	*0.375*	
Obsessive compulsive disorder	18	*15.7*	9	*19.1*	9	*13.2*	*0.440*	
	Mean ± s.d.		*Mean ± s.d.*		*Mean ± s.d.*		*P*[a]	
Claustrophobia Relevant Items (Short CLQ-G)								
Subscale 'restriction'								
Dark room	2.18 ± 1.66		3.25 ± 1.22		1.44 ± 1.51		*< 0.000001*	
Well-lit room	1.51 ± 1.46		2.53 ± 1.27		0.78 ± 1.12		*< 0.000001*	
Sleeping bag	1.04 ± 1.45		1.87 ± 1.58		0.47 ± 1.01		*< 0.000001*	
Trunk	2.18 ± 1.67		3.53 ± 1.04		1.25 ± 1.36		*< 0.000001*	
MRI scanner	1.65 ± 1.67		3.17 ± 1.05		0.60 ± 1.11		*< 0.000001*	
Mean of subscale	1.71 ± 1.36		2.87 ± 0.87		0.91 ± 1.00		*< 0.000001*	
Subscale 'suffocation'								
Elevator	1.07 ± 1.39		2.13 ± 1.36		0.34 ± 0.84		*< 0.000001*	
Breathe	0.83 ± 1.09		1.26 ± 1.24		0.54 ± 0.87		*< 0.001*	
Crowded room	1.82 ± 1.57		3.04 ± 1.12		0.97 ± 1.25		*< 0.000001*	
Under a car	1.23 ± 1.44		2.17 ± 1.51		0.59 ± 0.97		*< 0.000001*	
Sauna	1.00 ± 1.44		2.04 ± 1.56		0.28 ± 0.75		*< 0.000001*	
Mean of subscale	1.19 ± 1.08		2.13 ± 0.83		0.54 ± 0.70		*< 0.000001*	
Mean of questionnaire	1.45 ± 1.17		2.50 ± 0.74		0.73 ± 0.82		*< 0.000001*	

Abbreviation: MRI, magnetic resonance imaging.
[a]Mann–Whitney *U*-test.
[b]Fisher's exact test/χ^2-square test.
[c]Anxiety disorders other than agoraphobia.
[d]Agoraphobia includes claustrophobia.

developed abbreviated German version of the Claustrophobia Questionnaire (CLQ)[17] (Short CLQ-G) and the screening questions of the Structured Clinical Interview of Diseases[18] for anxiety disorders.

Non-claustrophobic subjects. A total of 68 subjects, who did not suffer from claustrophobia, were matched to the claustrophobic subjects regarding age, gender and clinical diagnosis where applicable (Table 1). Again, healthy subjects ($N = 14$) were recruited by e-mail announcements in the MPIEM and MPBPC. Patients suffering from psychiatric conditions other than psychotic disorders ($N = 29$; that is, $N = 18$ affective disorder, $N = 4$ general anxiety disorder, $N = 4$ substance use disorder, $N = 3$ others) were recruited

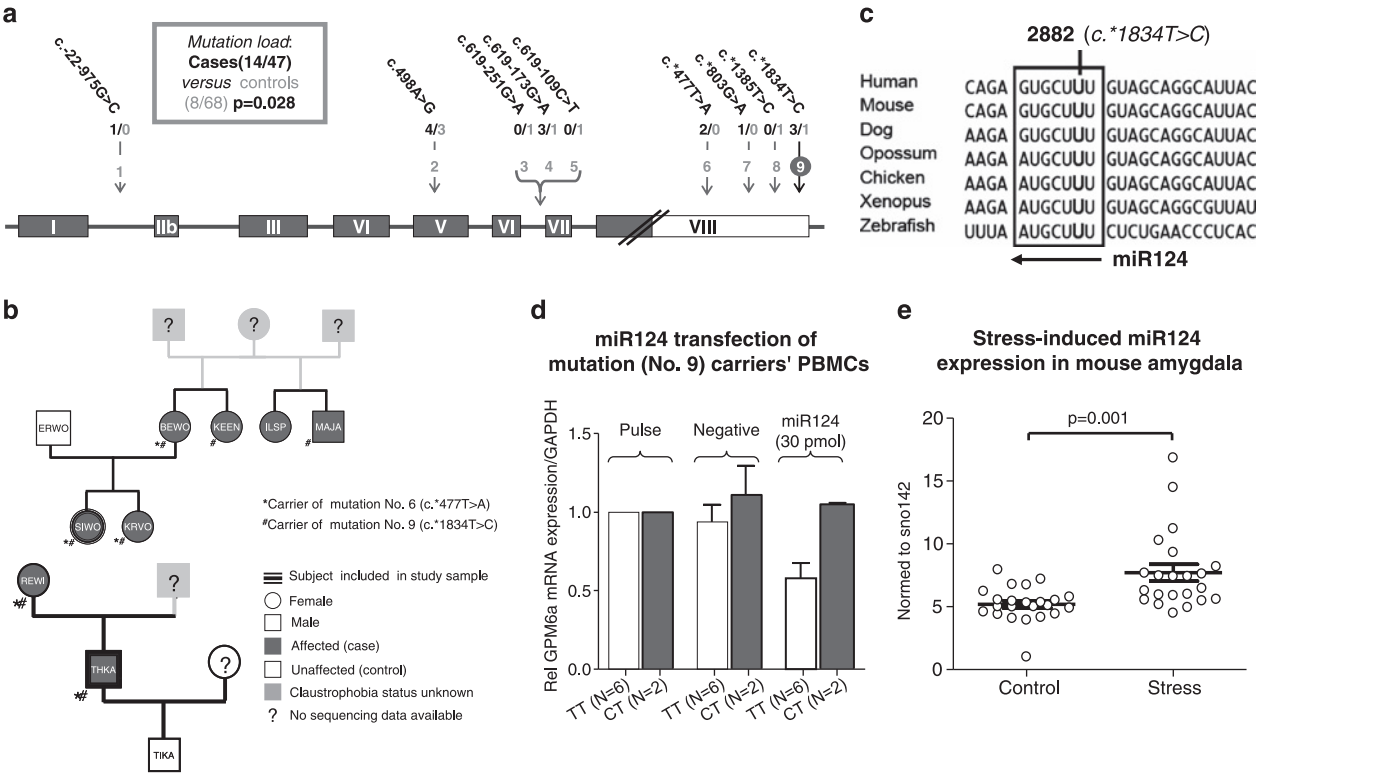

Figure 3 Genetic analysis of *GPM6A*. (**a**) Sequencing strategy and overview of the detected variants. Displayed are the coding exons (filled boxes) and the noncoding region of *GPM6A* (empty box). Arrows indicate rare variants found. Frequencies of rare variants in cases (black) versus controls (gray) are given. (**b**) Pedigrees of two claustrophobic individuals (SIWO and THKA), carrying the mutation at locus c.*1834 (position 2882 in human GPM6A transcript variant 1, mRNA; NM_005277.3), suggesting an association between this mutation and the claustrophobic phenotype. (**c**) Highly phylogenetically conserved genomic structure surrounding c.*1834T>C within the seed sequence of miR124 in the 3′untranslated region of *GPM6A*. (**d**) Expression analysis after miR124 nucleofection. Shown are the results of *GPM6A* RNA expression in peripheral blood mononuclear cells (PBMCs) after nucleofection with miR124 from two patients and six controls (that is, not carrying the variant; age, gender and disease matched; three controls per patient). Results were standardized to the results after just a pulse. (**e**) Restraint stress induces upregulation of miR124 in the amygdala of male mice, identifying this miR as a stress-regulated transcript ($N=22$ per group).

from the psychiatric clinic of the Georg-August-University Göttingen. Furthermore, 25 schizophrenic non-claustrophobic individuals were selected from the GRAS data collection.[16] The examination procedure comprised the same battery of questionnaires as for the claustrophobic subjects (above).

Pedigrees. To explore whether particular variations in *GPM6A* are transmitted in families together with claustrophobia, we tried to contact all available family members of the three claustrophobic individuals carrying the genetic variation at locus c.*1834T>C. Only for two of the subjects, SIWO and THKA (Figure 3b), it was possible to contact a sufficient number of relatives. Claustrophobia diagnosis according to DSM-IV criteria was confirmed by a telephone interview carried out by a trained psychologist. Swabs for genetic analysis and a short sociodemographic questionnaire, also containing items regarding the history of physical and psychiatric diseases, the Short CLQ-G and the screening questions of the Structured Clinical Interview of Diseases for anxiety disorders,[18] were communicated via mail.

Abbreviated German version of the CLQ (Short CLQ-G). To quantitatively assess the severity of claustrophobic anxiety, nine items of the CLQ[17,19] were selected and translated into German language (Supplementary Table 1). One item measuring fear experienced during magnetic resonance imaging was added to the restriction subscale because this situation may induce claustrophobia.[20,21] The CLQ is the most commonly used questionnaire for the psychological assessment of claustrophobia and has excellent psychometric properties (Cronbach's α: 0.95; test–retest reliability: 0.89).[17] It is composed of two subscales measuring two distinct but related fears: fear of restriction and fear of suffocation. Anxiety severity is measured on a 5-point Likert scale. To cover both subscales, five items from the suffocation and four items from the restriction subscale with high ecological validity were selected for construction of the Short CLQ-G. Given the substantial reduction in item number ($\sim$60%), the Short CLQ-G still achieves high internal consistency (total scale: 0.932, restriction: 0.909, suffocation: 0.835) and split-half reliability (0.952, splits matched for mean item difficulty) for the whole subject sample ($N=115$; $N=47$ claustrophobic subjects; $N=68$ non-claustrophobic subjects; Supplementary Table 1).

GPM6A sequencing. DNA from all subjects participating in this study ($N=115$) was isolated from blood with the

JET Quick Kit (Genomed, Loehe, Germany). For analysis of pedigree members (swabs), DNA was isolated with the Isohelix DNA Swab Kit (Biolab Products, Goedenstorf, Germany). *PCR reaction*: All exons, the putative promoter region of Ex2B and the 3′ untranslated region (3′UTR) of *GPM6A* were PCR-amplified from respective samples. Primers are listed below. *Sequencing*: The PCR amplicons were purified from unincorporated primers and deoxyribonucleotide triphosphates by digesting with 1 U Shrimp Alkaline Phosphatase und 5 U Exonuclease I (Exo) according to the manufacturer's instructions (USB Europe GmbH, Staufen, Germany). Sequencing was carried out using the dideoxy chain termination method with the BigDye Terminator v3.1 Cycle Sequencing Kit on a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Raw data were processed with Sequencing Analysis 5.2 (Applied Biosystems) and with different modules of the software package Lasergene 7.0 (DNASTAR, Madison, WI, USA).

Primers for GPM6A sequencing approach

Amplified region		Primer sequence (5′→3′)	Size (bp)
Exon 1	fw	GAAGAAAGAGGAGATGACAAAGG	653
	rv	GTCTGAGGCCGAGGAACATT	
Promoter region Exon 2b	fw	GTGCTGGCTGATTTGGAGATG	810
	rv	CTAACATGAAGCCGACCACCAAC	
Exon 2b	fw	GAGGAGAGAAAAGGAAAACACAG	755
	rv	GAAACATTCATTAGCCTTACTGG	
Exon 3	fw	GAAAGTCTGGGTTGGGAAGGA	788
	rv	GATTTGTACCTGGCACTATTCTA	
Exon 4	fw	GAACCAGGGAAGAGGAGAAG	694
	rv	CCATACATCAATCAACAGTG	
Exon 5	fw	GCCAAGATATGATTTTCCAGCAG	709
	rv	GGGAGGATAAAAGTAGAATGC	
Exon 6_7	fw	GGAACTTGCTTAGATTTGATTAG	955
	rv	GACTTACTTACCCATTGTTTTCC	
Exon 8*	fw	CGAGATAGCAAGGTGTAATGAAG	904
	rv	CATAAACATGAGTAATCTGAGG	
3′UTR*	fw	GAAGATCAGTGGCCATATTAC	1543
	rv	ATTGTACTTGAAAAGAATTCACAC	

*For sequencing exon 8 and the associated 3′UTR additional primers were designed to cover the full sequence.

Exon8rv2: 5′- GGTCCCTTTGAAGGTTACCT-3′
3′UTRfw2: 5′- GAGCAATCAGTATTATTGGACC-3′
3′UTRrv2: 5′- CACTTTACAGCATTCTTGTAGC-3′

Computational micro RNA (miRNA) search. To explore putative miRNA-binding sites in the *GPM6A* 3′UTR, several analyses were performed. TargetScan, version 6.2 (http://www.targetscan.org/) was used to identify miRNA-binding sites. Screening and $\Delta\Delta$G prediction analysis for both alleles of *GPM6A* were carried out using established algorithms (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html).

Expression analysis after nucleofection. Peripheral blood mononuclear cells (PBMCs) of claustrophobic patients with the mutation in the 3′UTR ($N=2$) and three matches per subject were freshly isolated using the standard Ficoll-Paque Plus isolation procedure (GE Healthcare, Munich, Germany). Using the Amaxa Nucleofector II Device (T-020), 6×10^6 cells were transfected with neg miRNA #2 or hsa-miR124 (Applied Biosystems) and cultured in RPMI supplemented with 10% fetal calf serum. After 24 h, cells were harvested and RNA extracted with the miRNeasy Mini Kit (Qiagen). cDNA was synthesized using 200 U SuperScriptIII (Invitrogen, Karlsruhe, Germany). For quantification with quantitative reverse transcription-PCR, the cDNA was used 1:10 diluted and four replicates per sample were performed; to 4 μl diluted cDNA, 5 μl Power SYBR mix (Applied Biosystems) and 1 pmol of each primer (see below) were added. Cycle threshold (CT) values for *GPM6A* were standardized to CT values of *GAPDH*.

hGPM6A_forward: 5′-TGAGATGGCAAGAACTGCTG-3′
hGPM6A_reverse: 5′-CCTTCCACCATCAGCAAAAT-3′
*hGAPDH_*forward: 5′-CTGACTTCAACAGCGACACC-3′
*hGAPDH_* reverse: 5′-TGCTGTAGCCAAATTCGTTGT-3′

Statistical analyses. Data were analyzed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA; http://www.spss.com) (human data analyses) and Prism 4 for Windows version 4.03 (GraphPad Software, Inc., La Jolla, CA, USA) (mouse data analyses). Unless otherwise stated, the data given in figures and text are expressed as mean ± s.e.m., and were compared by two- or three-way analysis of variance with *post-hoc* planned comparisons or by analysis of variance for repeated measurements, Mann–Whitney *U*-test and χ^2 test, where appropriate.

Results

***Gpm6a* null mutant mice appear essentially normal in development and basic behavior.** We have generated *Gpm6a* null mutant mice (KO) to explore the role of *Gpm6a* in the behavioral response to stress (Figures 1a–d). Homozygous KO mice were born at the expected Mendelian frequency and are long-lived. By western blot analysis, heterozygous mice expressed about 50% of the protein (Figure 1c), demonstrating that Gpm6a abundance can be regulated at the transcriptional level *in vivo* (see below). *Gpm6a* KO mice reproduce well and exhibit no obvious developmental defects (data not shown). Also, in a basic behavioral test battery, which included the analysis of motor and sensory functions, motivation and sensorimotor gating, we found no difference from WT littermate controls (Supplementary Figure 1).

Mild stress induces a claustrophobia-like phenotype in Gpm6a null mutant mice. Unexpectedly, when applying the resident-intruder paradigm[13] in order to assess the response to experimental stress, we noticed that sham-stressed *Gpm6a* null mutant mice exhibit a prominent phenotype in the EPM, consisting of a specific avoidance of closed arms. To our knowledge, such a behavioral response, which we like to term 'claustrophobia' in mice, has not been reported before. This phenotype is specifically striking, because normal rodents rapidly seek closed and narrow spaces to hide, which is a protective trait. Interestingly, the claustrophobia-like phenotype was only marginally amplified in those mutant mice that had experienced the resident-intruder stress (Figures 1e,f). As a prerequisite for applying this stress paradigm is prior single housing (of all

mice), we asked whether the relatively mild stress of social withdrawal might have been sufficient to trigger the claustrophobia-like phenotype in *Gpm6a* mutants. Indeed, single-housed, but not group-housed, *Gpm6a* mutants showed claustrophobia (Figure 1g). In these experiments, 10 days of single housing were sufficient to cause downregulation of the stress-responsive gene *Fkbp5*[22,23] in the amygdala of WT mice. Importantly, this downregulation was absent in *Gpm6a* mutant mice, demonstrating a perturbation of the normal stress response even at the molecular level (Figure 1h). A comparable result was obtained for *Npy5r* as another marker of stress (data not shown).[24]

Extra behavioral tests underline the claustrophobia-like phenotype in Gpm6a$^{-/-}$ mice. As claustrophobia-like behavior in mice has to our knowledge never been reported before, we performed a large number of extra behavioral tests in eight independent cohorts of male mice in order to substantiate this unusual phenotype. In fact, claustrophobia upon single housing was found in all cohorts of *Gpm6a* mutants and maintained when EPM was performed in darkness, using infrared cameras or when mice were released in closed arms (Figures 2a–c). This behavioral response did not rely on whisker functions or vocalizations, as confirmed by whisker cutting and ultrasound recording, respectively (data not shown). Similar to an 'exposure therapy' in humans, repeated EPM testings of mutants reduced and ultimately eliminated the claustrophobia-like behavior (Figure 2d; note also the weaker closed arm avoidance of mutants in Figures 1g and 2b,c; Supplementary Figure 2B, showing cohorts that already had one previous EPM test session). Also, other tests confirmed our diagnosis of 'claustrophobia', such as a specifically designed wide/narrow box, a light/dark box and the hole board test, in all of which mutant mice lacked preference for narrow and dark spaces (Figures 2e–g), that is, displayed a highly abnormal behavior, considering that rodents naturally prefer these spaces to hide and thereby protect themselves from predators.

Further tests demonstrated slightly increased general anxiety, again reminiscent of the known human claustrophobic phenotype. Mutants spent less time in the center of the open field and showed increased 'baseline freezing' in the fear-conditioning box (Figures 2h,i). The collection of urine from mutant mice that were kept for 3 h in narrow metabolic cages, revealed a significantly higher corticosterone excretion compared with their WT littermates (at similar urine creatinine values: WT 0.35 ± 0.08 versus KO 0.39 ± 0.06 mg per g body weight and day; $N = 12$/group; $P > 0.1$), indicative of an increased stress level (Figure 2j). As phobias/panic disorders in humans are more prevalent in females than in males,[25] we additionally examined female mutant mice and confirmed a very similar behavioral pattern as in male mice, that is, an unaltered basic behavior and the avoidance of closed arms in EPM (Supplementary Figure 2).

First considerations on a functional compensation for loss of *Gpm6a* in null mutant mice. Interpreting stress at the level of gene expression changes is difficult, because the encoded proteins can be 'upstream' or 'downstream' of stress perception, and either contribute to or protect from abnormal stress response. This complicates the prediction of cause and effect in a pathological situation. *Gpm6a* mRNA is downregulated by chronic social stress and also following prolonged cortisol treatment.[26] As stimulation of the hypothalamus–pituitary–adrenal (stress) axis leads to cortisol release, it is likely that downregulated *Gpm6a* expression mediates adaptation of the brain to stress and is therefore a healthy response that serves a feedback function in neuronal circuits exposed to stressful signals. The loss of *Gpm6a* in null mutant mice is clearly tolerated, presumably by the functional compensation of structurally related membrane proteins that are co-expressed in development (but are likely not stress regulated). One candidate for functional compensation is the neuronal *Gpm6b* gene, which encodes a highly related protein[27] with a similar (but not identical) spatio-temporal expression in brain[28,29] and which is, unlike *Gpm6a*, not among the identified stress-regulated genes.[26,30] In fact, this gene is upregulated under basal conditions in the amygdalae of *Gpm6a* mutant mice (KO: 1.04 ± 0.06; WT: 0.86 ± 0.05, normed to Hprt1 and H2afz; $P < 0.05$). To further investigate compensatory functions between the two genes, we cross-bred *Gpm6a* mutant mice with a newly generated line of *Gpm6b* null mutant mice.[31] The resulting double-mutant mice develop normally and reproduce well, but show 20% unexplained mortality at age 1 month. Further evidence that Gpm6a and Gpm6b have overlapping functions was found in cultured cortical neurons, in which the loss of both proteins reduced the collapse response of growth cones to soluble ephrin-B5, a repulsive signal.[31] This significant but clearly limited evidence of compensation strongly suggests that several (but not all) Gpm6a functions are redundantly served by Gpm6b and presumably other neuronal proteins. If stress-induced downregulation of *Gpm6a* expression *in vivo* were part of a neuroprotective stress response, it would be plausible that *Gpm6a* null mutant mice can develop normally but are selectively affected at the behavioral level, simply because *Gpm6a* compensating genes (such as *Gpm6b*) lack the necessary downregulation following stress exposure.

Selected genomic sequencing of *GPM6A* reveals associations with claustrophobia. As polymorphisms of human *GPM6A,* specifically in the noncoding region, could likewise interfere with dynamic gene regulation, we explored the association of this gene with a predisposition to human claustrophobia. A sample of 115 adult subjects ($N = 47$ self-reported claustrophobics and $N = 68$ non-claustrophobic controls) were recruited and interviewed with special emphasis on general anxiety and claustrophobia (Table 1). The sociodemographic description of the human sample revealed similar distributions between claustrophobic and non-claustrophobic individuals with regard to age, educational background, gender, ethnicity and marital status. Moreover, cases and controls were well matched for comorbid disease state. The prevalence of DSM-IV anxiety disorders other than claustrophobia (Table 1, included under agoraphobia) did not substantially diverge between claustrophobic cases and controls. More than half of the total sample (59%) reported to suffer from at least one (additional)

anxiety disorder. Expectedly, most individuals suffered from any kind of specific phobia (33%), followed by panic disorder (25%), social phobia and obsessive-compulsive disorder (both 16%). Generalized anxiety disorder was least frequent in our sample (11%). Claustrophobic subjects displayed higher severity ratings on all 10 items of an abbreviated German version of the CLQ[17] (Short CLQ-G; essentially all $P<000001$). Despite a 60% reduction in item number, the Short CLQ-G showed still very good psychometric properties comparable to the original instrument (Supplementary Table 1).

On all 115 subjects, we performed genomic sequencing of *GPM6A* covering all exons and flanking noncoding regions. This identified nine single-base substitutions in *GPM6A,* all of which were rare (most of them previously unreported) variants in the noncoding regions. Interestingly, in claustrophobic individuals, the sequenced regions were significantly more polymorphic than in non-claustrophobic controls ($P=0.028$; Figure 3a). To investigate whether particular variants of *GPM6A* are also genetically linked to claustrophobia, we examined two families that shared sequence abnormalities in the 3′UTR. This allowed us to include information on more than one family member ($N=10$) within two small pedigrees (Supplementary Table 2). Indeed, the sequence variants in the 3′UTR/noncoding region exon8 were consistently found in claustrophobic (but not in non-claustrophobic) individuals (Figure 3b). Unfortunately, the pedigrees were too small to assess significance. Interestingly, however, when comparing all mutation carriers in our sample of 115 individuals with all non-mutation carriers (independent of the claustrophobia diagnosis) significantly higher scores for most claustrophobia-relevant items were found associated with the mutation status (Supplementary Table 3).

A single-base substitution in the 3′UTR of *GPM6A* delivers first mechanistic insight. To gain mechanistic insight into the possible role of *GPM6A* sequence variants in the noncoding region, we focused on the newly identified substitution T to C at position c.*1834 in the 3′UTR of exon8, consistently associated with claustrophobia in the two pedigrees. In vertebrates, the c.*1834-T allele is conserved from human to zebrafish (Figure 3c). Mechanistically, this position is of particular interest because it is located within the seed sequence of miR124. This miRNA is expressed in brain and highly conserved.[32] Indeed, *in silico* analysis of the T-to-C substitution predicts the complete loss of miR124 binding ($\Delta\Delta G = -8.11\,\mathrm{kJ\,mol^{-1}}$).

To assess the effect of miR124 on expression of the endogenous human *GPM6A* gene, we obtained PBMCs, in which the *GPM6A* transcript can be detected and quantified by reverse transcription-PCR. When miR124 was over-expressed by nucleofection of freshly isolated PBMCs, steady-state levels of *GPM6A* mRNA were significantly decreased in cells that were homozygous for the c.*1834-T (WT) allele, but not in PBMCs from the heterozygous carriers of the mutant c.*1834-C allele (Figure 3d). miR124 is expressed in the adult brain, but has only been studied in neuronal development[32,33] and for its role in neuroplasticity.[34,35] We asked whether miR124 is also found in the amygdalae of mice and stress regulated. To this end, WT mice were exposed to restraint stress for 6 h, followed immediately by amygdala dissection. Indeed, we detected a significant upregulation of miR124 (Figure 3e) under stress.

Discussion

The behavioral analysis of *Gpm6a* mutant mice has led to the unexpected finding that a single neuronal gene can cause an isolated behavioral defect, best described as claustrophobia. Belonging to the category of agoraphobia/panic disorder, claustrophobia is often assumed to be a conditioned response, following a related traumatic experience.[25,36] In our model, claustrophobia-like behavior was observed in mice with a strong genetic predisposition (that is, *Gpm6a* deficiency) when combined with rather mild chronic stress. Interestingly, there was no obvious relationship between the quality of stress (that is, single-housing) and the very specific avoidance behavior. This not only suggests that loss of *Gpm6a* expression is a key genetic determinant of claustrophobia, but also sufficient to turn an unrelated stressor into a trigger of a unique behavioral response. We note that *Gpm6a* itself is widely expressed in the CNS, including hippocampus and amygdala as known sites of fear conditioning. Thus, there are no reasons to believe that the encoded membrane protein has evolved in the context of specific behavioral functions. It is much more likely that membrane protein Gpm6a, similar to other proteolipids,[37,38] is a cholesterol-associated tetraspan,[39] that binds other neuronal membrane proteins, which provide functional specificity. It is thus intriguing that Gpm6a has been found to stimulate endocytosis of μ-opioid receptors from the surface of neuronal cells.[40,41] We note that opioids are well known to be involved in regulation of fear/anxiety and their extinction in mouse and man.[24]

Virtually nothing was known about the cause of claustrophobia. Typically, anectodal evidence suggested traumatic experiences, such as in individuals that became trapped alive, but these incidents cannot explain the high frequency of claustrophobia in otherwise normal people. The cause or trigger of some cases of claustrophobia may still be related to exposure to narrow spaces,[36] traumatic brain injury[42] and other traumatic experiences, such as surviving of mining accidents, but these are mostly poorly documented. Our report of a mutant mouse model for claustrophobia suggests that also human claustrophobia can have a familial predisposition. We could identify a genetic component of claustrophobia, involving *GPM6A* expression and its post-transcriptional regulation by the (stress-regulated) neuronal miR124. These data suggest that *GPM6A* may contribute to the normal stress response in mouse and human. Larger studies in human samples would be required to assess exactly to what extent variants of *GPM6A* act as a claustrophobia-susceptibility gene.

At first glance, the two findings in mouse and human appear contradictory, because the claustrophobic phenotype was associated with the murine *Gpm6a* null mutation and the human *GPM6A* c.*1834-C allele. The latter is predicted to encode a more stable mRNA, due to the loss of its miR124-binding site. However, both findings can be reconciled with the compensation of Gpm6a (in the null mutant) by related proteins, such as Gpm6b. These proteins substitute for

Gpm6a in neurons and allow mutant animals to develop and behave normally. However, when exposed to stress the expression of these genes is not downregulated (unlike *Gpm6a*), as evidenced by the gene expression profiling that had identified and later confirmed *Gpm6a* as the only stress-responsive proteolipid in the adult brain.[1,30] Along these lines, we note that miR124, which acts as a stress-regulated mediator of *GPM6A* downregulation, as shown here, does not have comparable functional binding sites in *GPM6B*. Thus, loss of dynamic proteolipid expression in neurons (and the inability to downregulate these proteins) may predispose to abnormal stress response, rather than the loss of *Gpm6a per se*.

The detailed downstream mechanisms will have to be explored in other conditional mouse mutants in the future. Gpm6a drives the rate of endocytosis that downregulates the steady-state level of μ-opioid receptors at the surface of neuronal cells.[40,41] Thus, our data are compatible with a hypothetical model, in which a stress-induced phobia/panic disorder might be caused (in part) by a reduced feedback regulation of endogenous opioid receptor signalling. Obviously, interactions with other proteins that also influence behavior may be functionally relevant, and we note that the human serotonin transporter has been reported to interact *in cis* with GPM6A and GPM6B[43] (and Jana Haase, Dublin, Ireland, personal communication), whereas another study has implicated this serotonin transporter in human panic disorders.[44] In turn, *GPM6A* may also be relevant as a modifier of other diseases, and it is intriguing that an association has been found between *GPM6A* and the severity of depression in patients with schizophrenia.[45] The ramification of *GPM6A* downstream mechanisms are therefore likely complex and beyond the scope of this study. However, by placing the dynamic expression of *GPM6A/Gpm6a* both upstream and downstream of stress perception in the brain, we suggest a working model of GPM6A/Gpm6a as a neuronal 'brake' for maintaining a healthy stress response.

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

MK and HW in the lab of K-AN generated the Gpm6a KO mice. PdeM-S and JP carried out the molecular biological characterization of the mice under supervision of HW and K-AN. All behavioral experiments of mice were designed, performed and analyzed by AEl-K. Electroretinogram measurements were done by NS, ultrasound vocalizations by KH. Mouse amygdala dissections and quantitative reverse transcription-PCR from amygdala tissue were performed by SS under supervision of RP. The corticosterone assay was conducted by SS. AK developed and evaluated the Short CLQ-G, was responsible for telephone interviews, recruiting and examination of claustrophobic patients, control subjects, family members as well as data analyses. She was assisted by MB and BS. SG designed the genetic study, performed *GPM6A* sequencing/data analysis, cell culture, nucleofection and corresponding expression analysis. She was supported by CH. MB did the computational miRNA search. GF and RP gave input to data analysis, interpretation and literature citation. K-AN and HE initiated the project, designed the whole translational study and wrote the manuscript. HE and K-AN had full access to all data of the study and take responsibility for data integrity and accuracy of data analysis.

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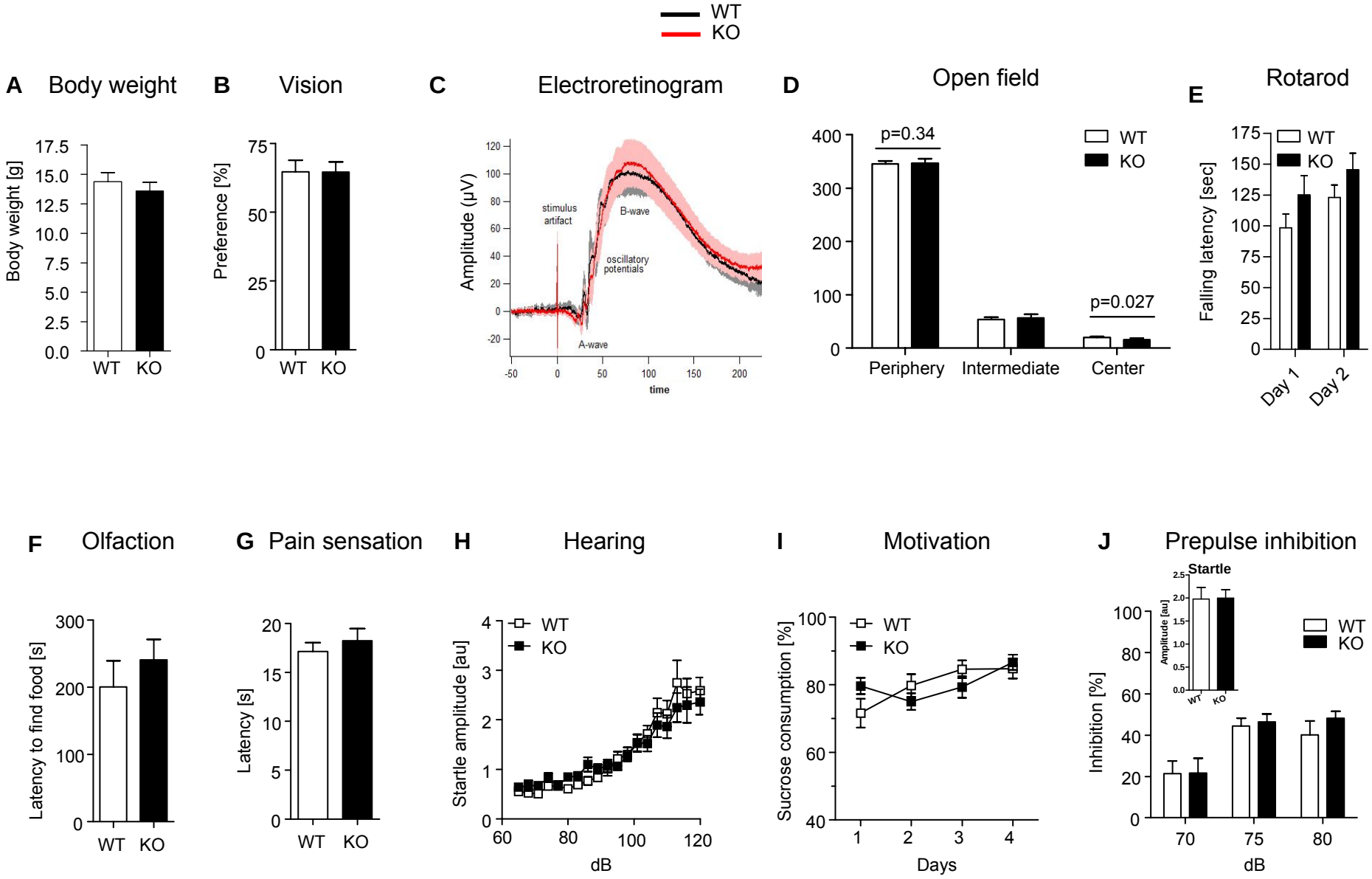
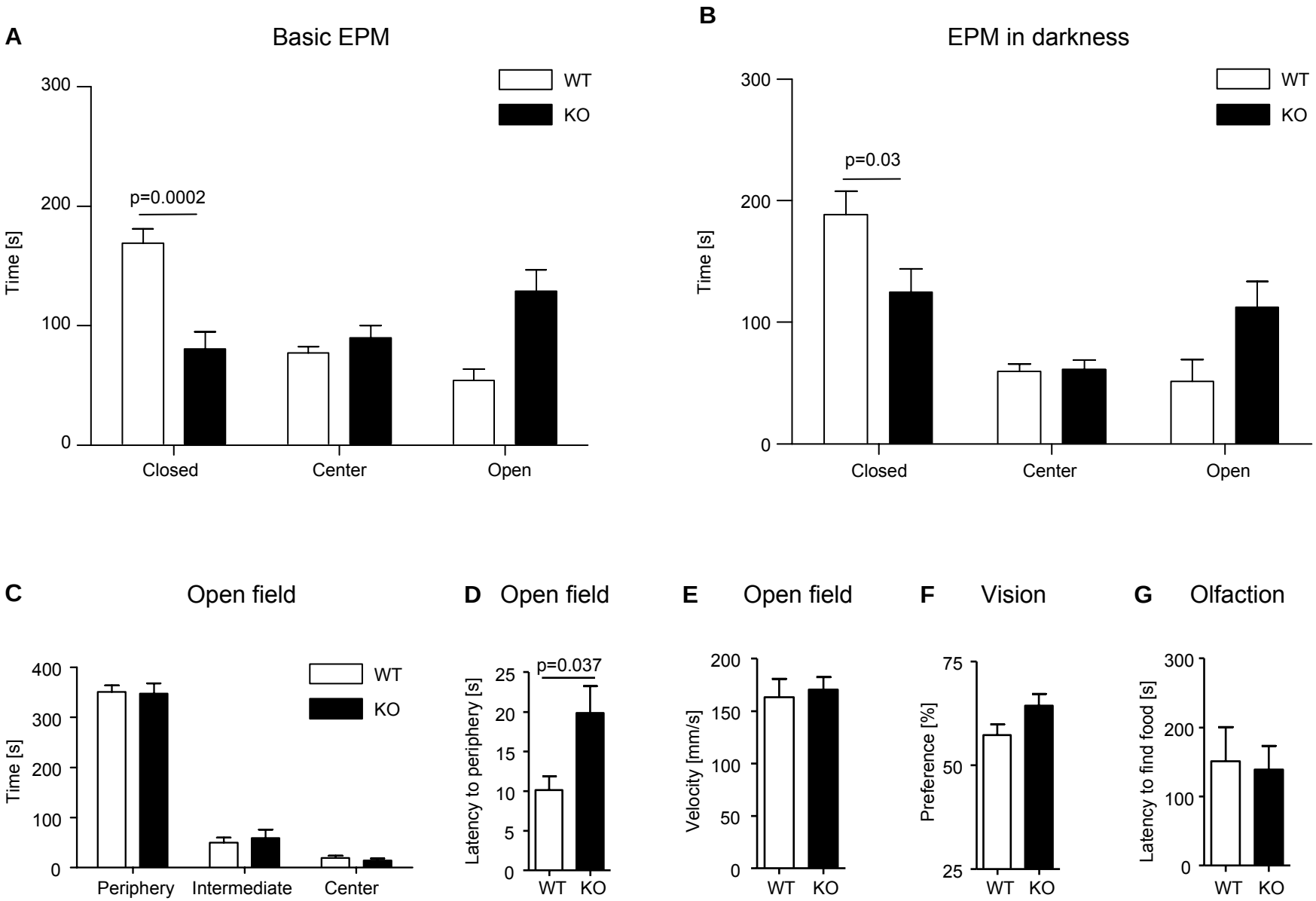
Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)

Supplementary Figure 1



Supplementary Figure 2



Supplementary Table 1

Psychometric properties of the Short CLQ-G

	TOTAL SAMPLE (N=115)	CLAUSTROPHOBIC SUBJECTS (N=47)	NON-CLAUSTROPHOBIC SUBJECTS (N=68)
INTERNAL CONSISTENCY (CRONBACH´S ALPHA)			
CLQ total	0.932	0.764	0.909
'RESTRICTION' (SR)	0.909	0.739	0.870
'SUFFOCATION' (SS)	0.835	0.764	0.790
SPLIT-HALF RELIABILITY[A]			
	0.952	0.803	0.947
ITEM DISCRIMINATION[B]			
SR			
to be locked in a small, **dark room**	0.821	0.688	0.787
to be locked in a small, **well-lit room**	0.822	0.587	0.800
to lie in a **sleeping bag**	0.651	0.413	0.628
to lie in the **trunk** of a car	0.842	0.444	0.826
to lie in an **MRI scanner**	0.773	0.371	0.578
SS			
to be in an **elevator**	0.739	0.533	0.558
having difficulties to **breathe** through nose	0.518	0.327	0.586
to be in the middle of a **crowded concert hall**	0.789	0.409	0.715
to work **under a car**	0.684	0.315	0.716
to be in a **sauna**	0.655	0.296	0.592
ITEM DIFFICULTY (MEAN ±SD)			
SR			
to be locked in a small, **dark room**	2.18 ± 1.66	3.25 ± 1.22	1.44 ± 1.51
to be locked in a small, **well-lit room**	1.51 ± 1.46	2.53 ± 1.27	0.78 ± 1.12
to lie in a **sleeping bag**	1.04 ± 1.45	1.87 ± 1.58	0.47 ± 1.01
to lie in the **trunk** of a car	2.18 ± 1.67	3.53 ± 1.04	1.25 ± 1.36
to lie in an **MRI scanner**	1.65 ± 1.67	3.17 ± 1.05	0.60± 1.11
SS			
to be in an **elevator**	1.07 ± 1.39	2.13 ± 1.36	0.34 ± 0.84
having difficulties to **breathe** through nose	0.83 ± 1.09	1.26 ± 1.24	0.54 ± 0.87
to be in the middle of a **crowded concert hall**	1.82 ± 1.57	3.04 ± 1.12	0.97 ± 1.25
to work **under a car**	1.23 ± 1.44	2.17 ± 1.51	0.59 ± 0.97
to be in a **sauna**	1.00 ± 1.44	2.04 ± 1.56	0.28 ± 0.75

[A]Correlation between split a (elevator, breathe, sauna, dark room, trunk) and split b (crowded room, under a car, well-lit room, sleeping bag, MRI scanner). Both splits matched for mean item difficulty; [B]Correlation between each item and the mean of the respective subscale. Indicates how well a certain item represents the whole subscale.

Cronbach's alpha coefficients are given as measures of the internal consistency of the items included in the claustrophobia questionnaire. To calculate the split-half reliability (Spearman-Brown correlation between 2 test halves) reflecting the questionnaires' homogeneity, the 10 single items were divided into 2 clusters such that both splits did not differ in mean item difficulty (split a: elevator, breathe, sauna, dark room, trunk and split b: crowded room, under a car, well-lit room, sleeping bag, MRI scanner). Item discrimination represents the correlation of a single item and the mean of the total scale or the subscale the item belongs to. It indicates how much a single test item influences the overall result, i.e. how representative the item is for the entire measure. Item difficulties are given as means with lower values indicating higher item difficulty.

Supplementary Table 2 **Description of the pedigree sample with respect to sociodemographic and anxiety/claustrophobia relevant readouts**

	SOCIODEMOGRAPHICS					ANXIETY & CLAUSTROPHOBIA RELEVANT READOUTS				
	Degree of relationship[a]	Age at examination (years)	Education (years)	Marital status	Main diagnosis (DSM-IV)	Comorbid anxiety disorder	Claustrophobia	Claustrophobia Questionnaire (total mean)	Claustrophobia Questionnaire (mean of subscale ‚restriction')	Claustrophobia Questionnaire (mean of subscale ‚suffocation')
Pedigree 1										
SIWO	Included in study sample	28	17	Single	No clinical diagnosis	Specific phobia; acrophobia	**YES**	**2.40**	**3.40**	**1.40**
BEWO	Mother	54	13.5	Married	No clinical diagnosis	Specific phobia; acrophobia	**YES**	**2.10**	**2.40**	**1.80**
ERWO	Father	57	12.5	Married	No clinical diagnosis	None	**NO**	**0.40**	**0.20**	**0.60**
KRVO	Sister	32	13.5	Married	No clinical diagnosis	Specific phobia; acrophobia	**YES**	**0.80**	**1.00**	**0.60**
KEEN	Aunt	48	12	Married	No clinical diagnosis	Specific phobia; acrophobia	**YES**	**0.80**	**1.20**	**0.40**
ILSP	Aunt	58	12	Married	No clinical diagnosis	Specific phobia	**YES**	**5.70**	**8.80**	**2.60**
MAJA	Uncle	43	12	Single	No clinical diagnosis	None	**YES**	**1.50**	**2.40**	**0.60**
Pedigree 2										
THKA	Included in study sample	46	13	Divorced	Affective disorder	None	**YES**	**3.40**	**3.40**	**3.40**
REWI	Mother	66	12	Married	No clinical diagnosis	None	**YES**	**0.50**	**0.80**	**0.20**
TIKA	Son	8	-	-	No clinical diagnosis	None	**NO**	**0.0**	**0.0**	**0.0**

[a]Degree of familial relationship to subject included in the study sample (SIWO & THKA)

Supplementary Table 3

Comparison of sociodemographic and anxiety/claustrophobia relevant parameters in mutation carriers independent of claustrophobia diagnosis

	MUTATION CARRIERS (N=22)		NON- MUTATION CARRIERS (N=93)		STATISTICS	
SOCIODEMOGRAPHICS						
	Mean ± SD		**Mean ± SD**		***p*[a]**	
Age in years	46.26±13.64		42.92±13.64		*0.221*	
Education in years	15.11±3.50		14.27±3.56		*0.367*	
	N	***%***	**N**	***%***	**Effect**	***p*[b]**
Gender						
female	19	*86.4*	62	*66.7*	3.315	*0.069*
male	3	*13.6*	31	*33.3*		
Ethnicity						
caucasian	22	*100.0*	90	*96.8*	0.729	*0.866*
african	-	-	1	*1.1*		
other	-	-	2	*2.1*		
Marital status						
single	11	*50.0*	46	*49.5*		
married	4	*18.2*	29	*31.2*	4.035	*0.399*
divorced	7	*31.8*	15	*16.1*		
widowed	-	-	3	*3.2*		
MAIN DIAGNOSES ACCORDING TO DSM-IV						
	N	***%***	**N**	***%***	**Effect**	***p*[b]**
no clinical diagnosis	4	*18.2*	23	*24.7*		
schizophrenia	9	*40.9*	34	*36.6*	0.437	*0.804*
other clinical diagnoses	9	*40.9*	36	*38.7*		
PREVALENCE OF ANXIETY DISORDERS						
	N	***%***	**N**	***%***	***p*[b]**	
comorbid anxiety disorder[c]	14	*63.6*	54	*58.1*	0.810	
panic disorder	9	*40.9*	20	*21.5*	0.098	
agoraphobia	14	*63.6*	42	*45.2*	0.156	
claustrophobia	14	*63.6*	33	*35.5*	*0.028*	
social phobia	3	*13.6*	15	*16.1*	1.00	
specific phobia	9	*40.9*	29	*31.2*	0.452	
generalized anxiety disorder	3	*13.6*	10	*10.8*	0.712	
obsessive-compulsive disorder	6	*27.3*	12	*12.9*	0.109	
CLAUSTROPHOBIA RELEVANT ITEMS (SHORT CLQ-G)						
	Mean ± SD		**Mean ± SD**		***p*[a]**	
Subscale 'restriction'						
dark room	3.00±1.45		1.99±1.65		*0.005*	
well-lit room	2.00±1.57		1.38±1.41		0.085	
sleeping bag	1.73±1.55		0.88±1.38		*0.012*	
trunk	2.77±1.57		2.04±1.67		0.057	
magnetic resonance imaging scanner	2.32±1.46		1.49±1.68		*0.023*	
mean of subscale	2.36±1.27		1.56±1.33		*0.010*	
Subscale 'suffocation'						
elevator	1.73±1.55		0.91±1.32		*0.018*	
breathe	1.31±.132		0.72±1.00		*0.035*	
crowded room	2.50±1.54		1.66±1.54		*0.030*	
under a car	1.77±1.54		1.11±1.39		0.059	
sauna	1.45±1.60		0.89±1.39		0.089	
mean of subscale	1.75±1.07		1.08±1.05		*0.006*	
Mean of questionnaire	2.06±1.08		1.31±1.16		*0.006*	

[a]Mann-Whitney U-Test; [b] Fisher's exact test/ Chi-square test; [c]Anxiety disorders other than agoraphobia;

Supplementary Figure 1: Basic behavior of male *Gpm6a* KO mice as well as visual function determined by electroretinography is normal. (A) Body weight; **(B)** Vision (visual cliff test); **(C)** Electroretinography; this was performed to exclude potential developmental alterations in the retina[1] of mutant mice affecting visual perception (N=6 per group); grand averages±SEM of electroretinograms evoked by 0.075cds/m^2 light flashes recorded from M6A mutant mice and wildtype littermates (n=6 each); over a range of 7 light intensities between 0.0003cds/m^2 and 10cds/m^2, amplitudes and latencies of A- and B-waves (reflecting synchronous activity of photoreceptors and bipolar/retinal ganglion cells, respectively), as well as the amplitude and frequency of oscillatory potentials overlying the ascending B-wave (reflecting electric activity of amacrine cells), were normal in M6A mutants. No significant differences were obtained for any of the intensities tested. **(D)** Open field pattern (except for time spent in the center); **(E)** Rotarod; **(F)** Olfaction (buried food finding); **(G)** Pain sensation (hot plate); **(H)** Acoustic startle response (hearing); **(I)** Motivation (sucrose preference test); **(J)** Sensorimotor gating, measured by prepulse inhibition (PPI), and startle response are all indistinguishable between KO and WT. N=13-28 per group except for **(C).** Mean±SEM presented.

Supplementary Figure 2: Brief behavioral characterization of female *Gpm6a* KO mice also reveals a claustrophobia-like phenotype. (A) Comparable to male KO mice, female KO have an aversion towards closed arms in the classical EPM as well as **(B)** in EPM conducted in darkness. **(C)** Open field behavior pattern, vision and olfaction were unaltered in KO. N=7-18; Mean±SEM presented.

Supplementary Table 1: Psychometric properties of the 'short German version' of the CLQ

Supplementary Table 2: Description of the pedigree sample with respect to sociodemographic and anxiety/claustrophobia relevant readouts

Supplementary Table 3: Comparison of sociodemographic and anxiety/claustrophobia relevant parameters in mutation carriers independent of claustrophobia diagnosis

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[1] Zhao J, Iida A, Ouchi Y, Satoh S, Watanabe S. M6a is expressed in the murine neural retina and regulates neurite extension. *Mol Vis* 2008; **14:** 1623-1630.

5. Slightly modified expression of the gene encoding methyl-CpG binding protein-2 influences impulsivity in mouse and man

5.1 Overview of project IV

The gene encoding methyl-CpG binding protein-2 (MECP2) maps to chromosome Xq28 and is one of the most dosage-sensitive genes transcriptionally regulating genes associated with neuronal functional integrity (Chahrour et al. 2008). Loss-of-function mutations of MECP2 lead to Rett syndrome, characterized by a gender- and mutation-type-dependent array of symptoms ranging from loss of speech and acquired motor skills to autistic symptoms, severe mental retardation and neonatal encephalopathy (Bienvenu et al. 2006). Interestingly, gene duplication can cause very similar symptoms (Ramocki et al. 2010), which points to the necessity of a tight regulation of gene expression. Accumulating evidence from studies with transgenic mouse models of complete loss-of-function, reduced expression or overexpression of Mecp2, further substantiates that certain levels of the protein are required for the neuronal system to maintain its inhibitory and excitatory balance (Ramocki et al. 2008). Very mild overexpression of Mecp2 (~1.5-fold) already disrupts neuronal homeostasis resulting in higher seizure propensity together with alterations in neuronal branching sites and increased spine density (Bodda et al. 2013). Behaviorally, disturbed neuronal homeostasis across different Mecp2 mouse models expresses as abnormal social behavior and increased aggression (Shahbazian et al. 2002, Moretti et al. 2005, Fyffe et al. 2008, Kerr et al. 2008, Samaco et al. 2009, Chao et al. 2010, Pearson et al. 2012, Samaco et al. 2012, Bodda C 2013). Interestingly, behavioral effects of altered Mecp2 levels vary dependent of the genetic background (C57Bl6 versus FVB) of the mice.

Our first aim of the present study was to explore the impact of the genetic background on basic behaviors, seizure propensity and spontaneous home cage behavior in transgenic mice with mild overexpression of Mecp2. Indeed, increased aggression in transgenic mice as compared to wildtype littermates could only be detected for the FVB/N background but not for C57Bl6/N mice. We further observed that while mild Mecp2 overexpression in FVB and C57Bl/6 mice left most basic behaviors unaltered, it modulated seizure propensity (gender-dependent) and home cage behavior independent of the genetic background.

In humans, data on aggression/impulsivity in Rett or *MECP2* gene duplication syndrome are scarce. A family study characterizing the neuropsychiatric phenotypes of 9 males and 9 females carrying *MECP2* duplications revealed a high prevalence of hostility (63%) in the carrier females. Strikingly, females exhibited mild psychiatric symptoms despite 100% skewing of inactivation of the mutation carrying allele and normal *MECP2* mRNA levels (Ramocki et al. 2009). Moreover, one of three very mild cases of Rett syndrome who carried a mutation located in the deletion hotspot of the 3' end of the *MECP2* gene, has been reported to experience episodes of uncontrolled aggression (Huppke et al. 2006). Consequently, also in humans, very mild loss and gain of function of MECP2 might result in a range of neuropsychiatric symptoms including aggression.

Surprisingly, despite the obvious importance of MECP2 for nervous system development and function, the effect of common genetic variations of this gene on human behavior remains widely unknown. It was previously shown that common genetic variants such as 3' UTR SNP rs2734647 of *MECP2* alter the risk for autism (Loat et al. 2008). Moreover, carriers of the minor allele of SNP rs2239464 have decreased cortical surface area in brain regions such as the cuneus (Joyner et al. 2009). Interestingly, this brain region has been shown to be associated with inhibitory control in patients suffering from bipolar disorder (Haldane et al. 2008). In the framework of the present project, we wondered whether slight MECP2 expression differences mediated by common genetic variations would also be associated with aggression relevant behavioral phenotypes in humans and how possible genotype-phenotype relationships would be mechanistically mediated.

Two *MECP2* SNPs, rs2239464 and rs2734647, were considered for the phenotype-based association study (inclusion criterion $r^2<0.8$). Translating our findings from the Mecp2 overexpressing mouse model to humans, we selected impulse control, excitement and uncooperativeness (Positive and Negative Syndrome Scale, PANSS) as aggression equivalents from the GRAS data collection (Ribbe et al. 2010). Our PGAS approach was applied to the three target measures and possible sociodemographic and clinical confounders such as age and educational level. Significant genotype-dependent group differences for both selected SNPs were shown for male patients with respect to poor impulse control while only nominal significances/tendencies could be observed for excitement and uncooperativeness. In women, no statistically significant results were obtained. Importantly, no genotype differences withstanding correction for multiple testing were observed for the

sociodemographic and clinical measures. In terms of functional implications of the 3'UTR SNP rs2734647, we identified two microRNAs (miR) with seed binding sites including the 3'UTR SNP position. In vitro luciferase assays demonstrated a loss of down-regulation upon co-transfection with these miRs in case of the allele associated with higher impulsivity. A co-evolutionary effect on one specific miR sequence, adapting for binding to the respective 3'UTR sequence in mouse and man, may emphasize the importance of this regulatory interaction. As a conclusion, well in line with the aggression phenotype observed in transgenic FVB mice, already mildly elevated MECP2 levels seem to be sufficient to modulate impulsive/aggressive behavior in human subjects.

5.2 Manuscript in preparation

Tantra, M.*, Hammer, C.*, **Kästner, A.***, Begemann, M., Bodda, C., Stepniak, B., Castillo Venzor, A., Erbaba, B., Tarami, A., Hammerschmidt, K., Schulz-Schaeffer, W., Mannan, A. and Ehrenreich, H. (In preparation). "Slightly modified expression of the gene encoding methyl-CpG binding protein-2 influences behavior of mouse and man."

*These authors contributed equally to the work.

Personal contribution

I was responsible for the operationalization of impulsivity and aggression in the human sample. I developed the severity scoring of aggression based on the patients' medical reports and a clinical interview contained in the examination booklet of the GRAS study. I supervised the student performing the aggression scoring and database entering for all schizophrenic patients. Moreover, I performed the human PGAS data analyses and assisted in the interpretation of the results. I designed the tables and participated in the conception, design, drafting, revision and publication of the manuscript.

6. Operationalization and validation of an autistic phenotype in the GRAS sample

Introduction

Our current psychiatric classification largely relies on the operationalization of categories introduced by experienced diagnosticians from the 19th century such as Emil Kraepelin, Eugen Bleuler or Kurt Schneider. They highlighted few salient clinical features which they determined to signify separate nosologic entities (Kendler 2009). However, many clinical features such as psychotic symptoms, mood dysregulation or anxiety transcend diagnostic categories (Craddock et al. 2005) (also compare schizophrenic, depressive and autistic patients Figure 1). DSM-IV (APA 1994) lists 522 criteria for diagnosing 201 distinct psychiatric conditions. Hence, several symptoms function as criteria for more than one disorder which challenges the view of psychiatric conditions as entirely distinct entities (Borsboom et al. 2011, Rosen et al. 2012). Also from a genetic point of view, the boundaries between different psychiatric diagnostic categories begin to blur. In the last years, support for genetic risk factors simultaneously associating with several psychiatric diseases accumulated (Burbach et al. 2009, Carroll et al. 2009, Purcell et al. 2009, Smoller et al. 2013).

Evidence challenging the symptomatic demarcation between autism spectrum disorders (ASD) and schizophrenia exemplify phenomenological similarities described for various psychiatric conditions (Kendler et al. 1993, Craddock et al. 2007). Interestingly, the following findings seem to mark the beginning of a reinstatement of previous conceptualizations of autism as a schizophrenia subtype (Kanner 1943, Crespi 2010). Since it was shown that autistic individuals often report psychotic experiences in adolescence or early adulthood, a diagnosis of autism does not exclude symptoms typical for schizophrenia (Stahlberg et al. 2004, Bevan Jones et al. 2012). Recent evidence illustrates that 10 out of 18 cases with early-onset schizophrenia also fulfilled symptom criteria for Asperger's syndrome (Waris et al. 2013). Some argue that symptomatic similarities of ASD and schizophrenia are only superficial and do not reflect overlapping underlying pathogenic factors, due to supposedly diverse developmental trajectories and ages of onset (Sasson et al. 2011). In contrast to autism spectrum disorder (ASD), schizophrenia is still widely considered as adult onset mental illness (Häfner et al. 1997). To strengthen the case for altered neurodevelopment in schizophrenia, a plethora of neuropathological findings implicating developmentally reduced synaptic connectivity can be put forward (Murray 1987, de Haan et al. 2004, Owen et al.

2011). Longitudinal studies have revealed that neurodevelopmental abnormalities such as a delay in motor development or impairments in receptive language are found to prevail in those later diagnosed with schizophrenia (Jones et al. 1994, Cannon et al. 2002, Rapoport et al. 2009). Relationship and adjustment difficulties in childhood, core features of autism, have also been reported to be prevalent in this condition (Cannon et al. 2001, Rutter et al. 2006). Along the same lines, recent studies convincingly demonstrate that childhood-onset schizophrenia is preceded by a diagnosis of an autism spectrum disorder in 30%-50% of the cases (Rapoport et al. 2009). Most importantly, however, among those patients suffering from schizophrenia, some exhibit a prominent autistic phenotype while psychotic symptoms are less prominent (King et al. 2010, Bastiaansen et al. 2011). This autistic subgroup of schizophrenic patients can be characterized by behavioral abnormalities such as difficulties in social interaction, communication, emotion processing, and motor abnormalities (Cheung et al. 2010, King et al. 2010). Additionally, schizophrenic individuals predominantly suffering from negative symptoms obtain high scores on the Autism Diagnostic Observation Schedule (ADOS); an instrument developed to support the diagnosis of ASD (Bastiaansen et al. 2011). Taken together, the strong phenotypic relationship supports overlapping genetic susceptibility and pathogenesis of ASD and a subgroup of schizophrenic individuals.

Aims of thesis project V

To provide the ground for investigating biological pathways common to ASD and a subgroup of schizophrenic patients, project V is aimed at defining an autistic subgroup of schizophrenic patients using specific highly intercorrelated readouts from the GRAS data collection (Ribbe et al. 2010). These autism relevant items were merged into a composite score reflecting the overall severity of autistic behaviors. As the selected items have not originally been designed to assess autistic behaviors, a validation study in an ASD sample has been initiated to evaluate construct (convergent and discriminant validity) and criterion-related validity. Although the recruitment of ASD patients will be ongoing, preliminary results from the validation study will be reported in the following.

Methods

Operationalization of autistic symptoms using the GRAS data collection

From the detailed phenotyping of the GRAS sample (Ribbe et al. 2010), items indicative of autistic behavior (covering all three symptom domains according to DSM-IV) (APA 1994) were selected from the Positive and Negative Syndrome Scale (PANSS) (Kay et al. 1987) and

the Cambridge Neurological Inventory (CNI) (Chen et al. 1995). Both are standardized third-party clinical observation tools, well-evaluated and widely applied to assess positive, negative, general psychopathology and neurological symptom severity in schizophrenia (Kay et al. 1988, Chen et al. 2000, Bottlender et al. 2013, Salavera et al. 2013). The severity scoring of PANSS items ranges from 1 (definition does not apply) to 7 (severe dysfunction) whereas CNI item scores can vary between 0 (normal function), 2 and 3 (function grossly abnormal). To cover the diagnostic domain of *difficulties in social interaction*, items 1 ('blunted affect'); 3 ('poor rapport') and 4 ('social withdrawal') of the PANSS negative subscale were used. *Difficulties in communication* were measured employing items 5 ('difficulties in abstract thinking') and 6 ('lack of spontaneity and flow of conversation') of the PANSS negative subscale as well as the CNI items 'aprosodic speech', 'echophenomena' and 'perseveration'. The third diagnostic symptom cluster assessing *limited, repetitive and stereotypic patterns of behavior* was accounted for by using item 5 ('mannerism') and 15 ('preoccupation') of the PANSS general subscale and item 7 of the negative subscale ('stereotyped thinking') as well as the item 'mannerism' assessed by the CNI.

Creation of an autism severity score for every schizophrenic GRAS patient

To generate an integrative measure reflecting the overall severity of autistic behaviors (autism severity score), a composite score was created (mean of all items) for every schizophrenic GRAS patient. Prior to merging single items, phenotypes were standardized to zero mean and variance one by rank based Blom transformation (Blom 1958).

Participants of the validation study

So far, 53 probands (36 males, 17 females) have been included in the validation study. They were partly recruited to the outpatient unit of the Max Planck Institute of Experimental Medicine, Göttingen by public announcements or to the Autism Diagnosis Center of the University Hospital of Rostock and the Psychiatric Clinic in Taufkirchen. Some probands presented with an assured ASD diagnosis, while others had not been diagnosed before. For 44 individuals (29 males, 15 females; age [mean±SD]: 34.27±10.35) an ASD diagnosis based on DSM-IV criteria (infantile autism [N=12], Asperger's syndrome [N=24]; atypical autism [N=8]) (APA 1994) could be confirmed by an invariant team of specially trained and experienced psychiatrists and psychologists. Nine individuals (7 males, 2 females; age [mean±SD]: 35.9±12.60) did not receive an ASD diagnosis and were included in the control group. The control subjects presented with isolated symptoms resembling an ASD condition

but were classified as suffering from personality disorder (N=6), attention deficit hyperactivity disorder (N=1) or as healthy control (N=2). Besides an established ASD diagnosis, inclusion criteria consisted in average intelligence (Hamburger-Wechsler Intelligence scale (Tewes 1994)), legal age (≥ 18 years) and language abilities permitting the conductance of anamnestic interviews.

Measures and procedure of the validation study

As both PANSS and CNI have not been developed and evaluated for the assessment of autism relevant behaviors, construct validity and criterion-related validity of the autism severity score were evaluated. Construct validity is assured, if it can be demonstrated that the autism severity score specifically measures autism relevant behavior. One possibility is to show that the newly developed score correlates substantively with a clinical rating of autistic behaviors with already established construct validity (convergent validity). A high correlation results if a person who scores high on the established instrument also obtains high values on the new measure and vice versa. At the same time, the new tool should not correlate with an established measure assessing a construct different from autism (discriminant validity). The items contained in the autism severity score were rated on the basis of a semi-structured interview which was also designed to explore developmental delays and abnormalities as well as current symptoms relevant for the confirmation or exclusion of an ASD diagnosis. To provide convergent validity, module 4 of the Autism Diagnostic Observation Schedule (ADOS) (Lord et al. 2000) was scored based on the 'original algorithm' relying on the social interaction and communication domain only (Lord et al. 2000). The ADOS is a standardized clinical rating instrument assessing social interaction, communication and imagination during a semi-structured interaction with an examiner. Modul 4 has been developed for adolescents and adults with fluent speech and has good criterion-related validity (Lord et al. 2000, Bastiaansen et al. 2011). All ADOS raters of the present study received a special training to guarantee standardized administration and scoring. To assure discriminant validity, the positive symptoms subscale score of the PANSS was used because it comprises items very different from core features of autism. Additionally, criterion-related validity referring to the quality of differentiation between diagnosed and non-diagnosed individuals should be assessed for the autism severity score. Importantly, the ADOS and the autism severity items were always scored by two different examiners both of which were blind to the clinical diagnosis in 66% of the cases.

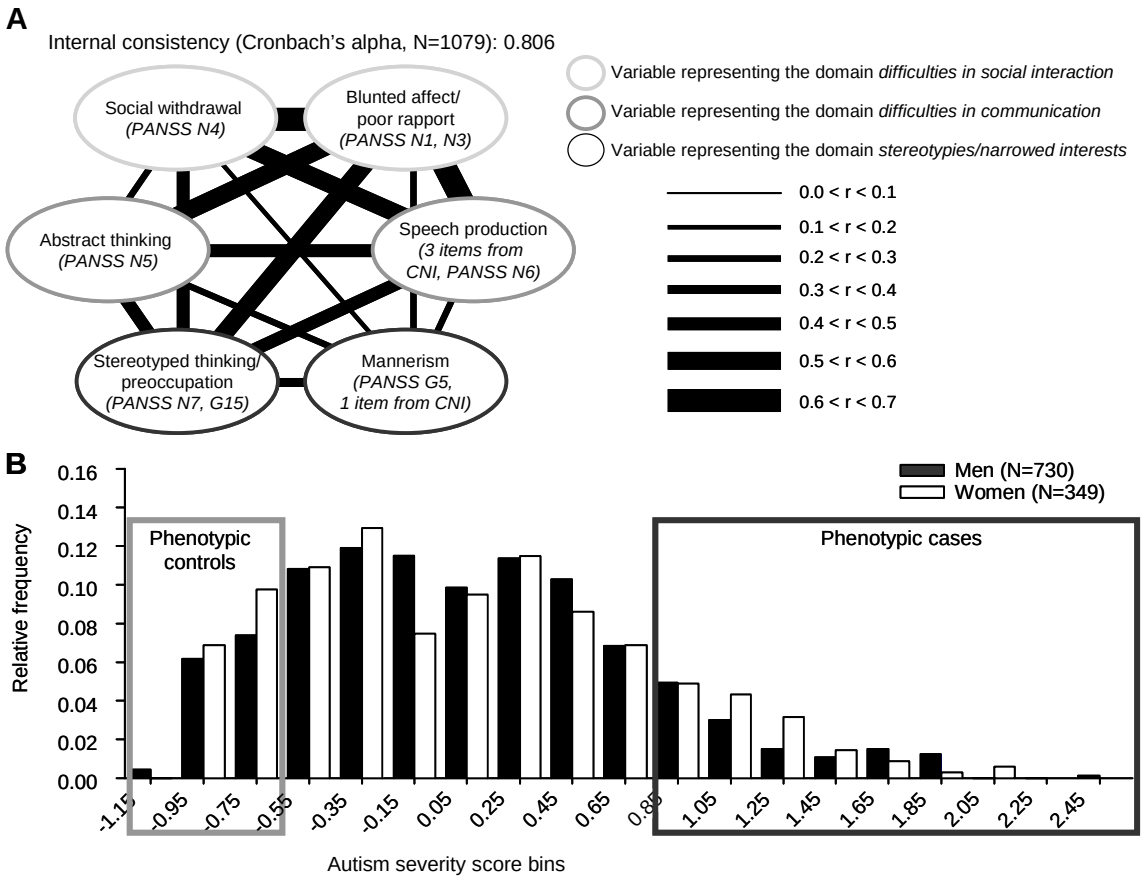

Figure 2 Operationalization of autistic symptoms in the schizophrenic GRAS sample. (A) Intercorrelation pattern of 6 autism relevant variables created from 12 individual items extracted from the Positive and Negative Syndrome Scale (PANSS) and the Cambridge Neurological Inventory (CNI) of the GRAS data collection. As revealed by the substantial internal consistency all variables are very likely to be indicative of the same underlying psychological construct. **(B)** Relative frequency distribution of the autism severity score in the schizophrenic GRAS sample. The autism severity score is composed of the 6 variables presented in panel A and provides the ground for future genetic approaches. The latter will involve a comparison of the extremes of the distribution (phenotypic cases versus phenotypic controls) with respect to constellations of common variants of genes implicated in certain biological pathways such as synaptic transmission.

Statistical analyses

Spearman rank correlation coefficients are reported for the individual PANSS and CNI items and the ADOS (Figure 3A). Pearson correlation coefficients were calculated for the z-standardized autism severity score and the ADOS (Figure 3B; convergent validity) and the PANSS positive subscale (Figure 3C; discriminant validity). In the present study, criterion-related validity refers to the degree to which ADOS and autism severity score ratings are in agreement with the clinical diagnosis of having ASD or not. Logistic regression was used to predict dichotomous ASD status based on both scores. The continuous scores instead of cut-off based status were used as for the autism severity score no such cut-off has been defined yet. Receiver Operating Characteristic (ROC) curves were calculated to provide information on the sensitivity and specificity of all possible threshold settings for the ADOS and the

autism severity score (Figure 3D). In addition, Area under the Curve (AuC) statistics representing the overall level of agreement between criterion (i.e. clinical diagnosis of ASD) and instrument (i.e. ADOS and autism severity score) were determined. The higher the AuC (1 = perfect agreement), the higher the probability for a randomly chosen ASD patient to score higher on the respective instrument than a randomly chosen proband without ASD. Group differences were assessed by Mann-Whitney U tests (Figure 3E).

Results

Autism severity score in the GRAS sample allows extreme group definition

The high internal consistency (Cronbach's alpha, N=1079: 0.806) of all individual autism variables (Figure 2A) indicated that they are very likely to be indicative of one underlying construct. Hence, they were integrated into an autism severity score supposed to reflect the overall severity of the dimensional trait. The distribution of the autism severity score in the schizophrenic GRAS cohort (range: -1.15 to 2.5) allows the definition of extreme groups contrasting maximally with respect to the severity of autistic symptoms (Figure 2B).

First evidence for construct and criterion-based validity of the autism severity score

As the items used to operationalize autistic features have not originally been developed for this purpose, the autism severity score was construct validated in a sample of ASD patients and controls. Construct validity can be assessed by evaluating the convergence between a newly developed rating scale and a measure with established construct validity for the trait of interest. For this purpose the ADOS was chosen, which has high diagnostic validity for the diagnosis of ASD. Almost all single items included in the autism severity score correlated with the ADOS (Figure 3A) although both measures had been rated by independent examiners. Strongest correlations ($0.4<r<0.7$) were observed for the items belonging to the diagnostic domains *difficulties in social interaction and communication*. Its highly significant correlation with the ADOS ($r=0.654$; Figure 3B) and the lack thereof with the positive symptom subscale of the PANSS ($r=0.044$; Figure 3C), points towards high construct validity of the autism severity score. As revealed by logistic regression analysis based on ASD diagnosis, the autism severity score was able to correctly classify 83% of the probands whereas the ADOS classified 86.8% of the probands correctly. ROC curves for the ADOS and autism severity scores resulted in AuC values of 0.832 and 0.745, respectively (Figure 3D). Lastly, significant score differences between the control group and the ASD group were observed for both the ADOS ($p=0.001$) and the autism severity score ($p=0.02$). Taken

together, the high convergence between the autism severity scores and the ADOS supports its high construct validity and criterion-related validity.

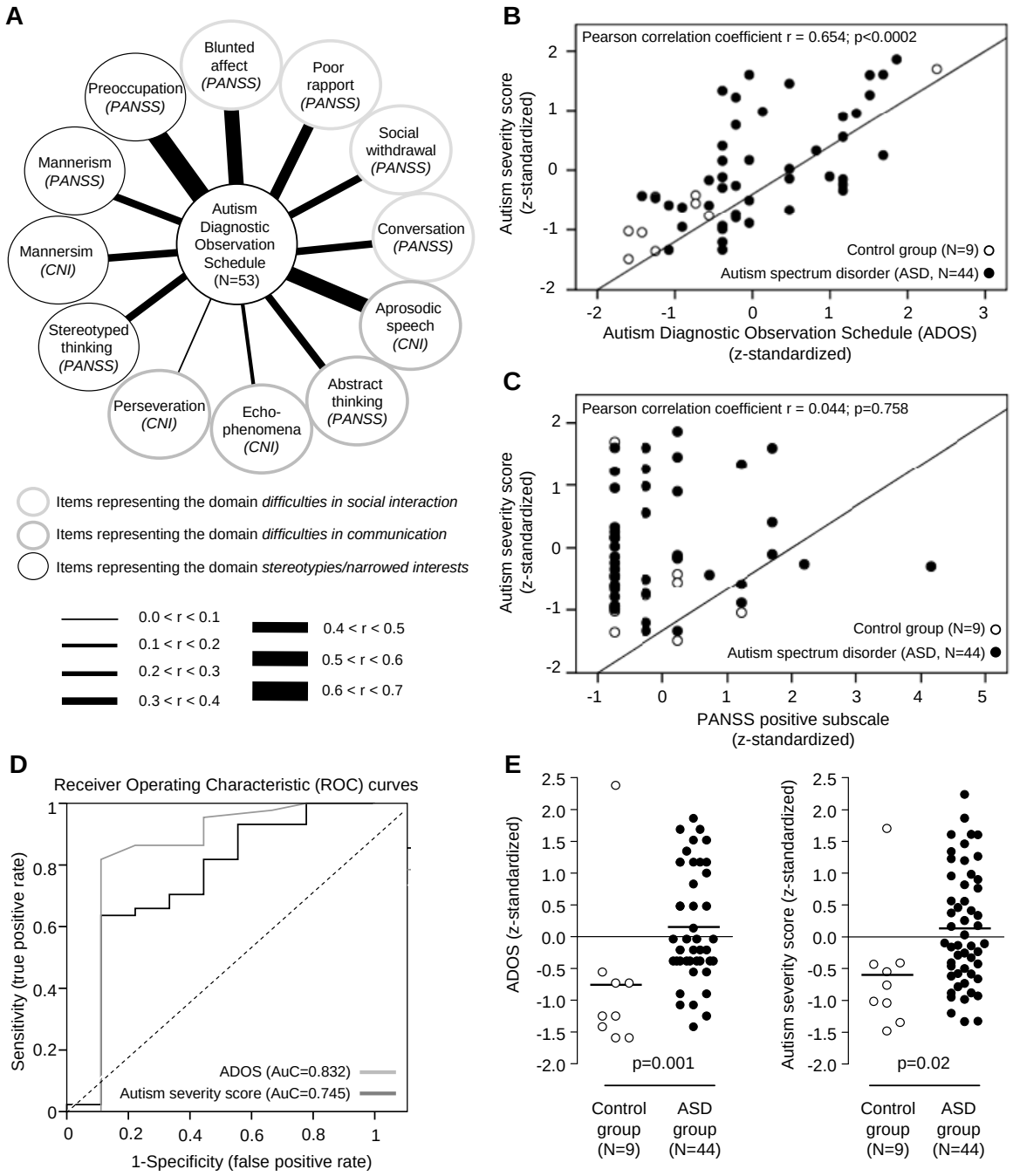

Figure 3 Results of the construct and criterion-related validation of the autism severity score in a sample of ASD patients and controls. (A) Intercorrelations of individual autism severity score items and the Autism Diagnostic Observation Schedule (ADOS). The strength of correlation (Spearman rank correlation) is symbolized by the thickness of the horizontal bars. **(B)** Convergent validity of the autism severity score is underlined by a highly significant correlation of z-standardized ADOS and autism severity scores (Pearson correlation). **(C)** Discriminant validity of the autism severity score is supported by a lack of correlation between the z-standardized Positive and Negative Syndrome Scale (PANSS) positive subscale and autism severity score. (**D)** Receiver Operating Characteristic curves for the ADOS and the autism severity score illustrate high Area under the Curve (AuC) statistics which indicates that both scores adequately predict whether someone has an ASD diagnosis or not. **(E)** Scatterplots reveal significant group differences (autism spectrum disorder [ASD] group versus control group) for the ADOS and the autism severity score. P-values were obtained from Mann-Whitney U test.

Discussion

A multitude of genetic and clinical studies converge on the notion that similar biological pathways may be involved in the etiology of ASD and a subgroup of schizophrenic individuals. Selected items from clinical rating instruments originally developed to assess the severity of schizophrenia psychopathology (PANSS) and neurological symptoms (CNI) were used to characterize autistic symptoms in the schizophrenic GRAS sample. Probands with a clinical ASD diagnosis as well as control subjects were recruited to evaluate construct and criterion-related validity of the autism severity score. As could be demonstrated by the correlation with the ADOS and the lack of co-variation with the PANSS positive symptoms score, first evidence for the autism severity score to be a construct valid instrument could be obtained. In addition, the autism severity score was shown to have good general criterion-related validity. It is able to correctly classify the majority of individuals and higher scores on the autism severity score predict a higher probability of having a clinical ASD diagnosis. Moreover, group comparisons between ASD and a control group demonstrate that the autism severity score is able to differentiate between ASD cases and individuals who themselves suspected to deserve a clinical diagnosis of ASD and therefore participated in the study but for whom a clinical ASD diagnosis according to DSM-IV criteria could not be confirmed. As a consequence, the items developed to assess the severity of negative symptomatology in schizophrenia might also be useful to evaluate autistic symptoms in individuals diagnosed with ASD.

Interestingly, correlations strengths of ADOS and individual autism score items largely varied. Single measures representing the stereotypies and restricted interest domain (i.e. ‘mannerism’, ‘perseveration’) were least comparable to the ADOS score. This might be due to the fact that the original ADOS algorithm used to generate the score in the present study does not contain items covering this symptom domain (Lord et al. 2000). In future studies, a revised algorithm will be used to decide whether the lack of co-variation is due to the respective items not being valid or to the ADOS not covering this behavioral aspect. Not surprisingly, as correspondent ratings are included in the ADOS, the items ‘blunted affect’, ‘poor rapport’ and ‘aprosodic speech’ correlated substantively with the ADOS. Although there is no direct equivalent contained in the ADOS, highest correlations were obtained for the PANSS item ‘preoccupation’. This measure assesses the state of being absorbed with “internally generated thoughts and feelings and with autistic experiences to the detriment of

reality orientation and adaptive behavior" Thus, in follow-up evaluation studies, this item might turn out to complement the revised ADOS algorithm.

Across all measures, better criterion-related validity was obtained for the ADOS as compared to the autism severity score. However, this could be based on the fact that the clinical diagnosis of ASD is often assisted by the ADOS due to the lack of an established diagnostic tradition for ASD in adults (Bastiaansen et al. 2011). Thus, both parameters might not be completely independent from each other.

Several limitations should be taken into account when interpreting the results of the study. First of all, data on psychometric properties of ADOS, module 4 are scarce. Existing studies demonstrating good psychometric properties rely on very small samples (Lord et al. 2000, Bastiaansen et al. 2011). Thus, it can be questioned whether the ADOS is an adequate comparison standard. Nevertheless, to date, the ADOS is the only standardized third-party clinical rating instrument available. Second, samples sizes in the present study are quite small. To substantiate the preliminary findings reported here, recruitment of the ASD patients and controls is still ongoing. Moreover, because our semi-structured interviews relied on full speech capacity, the ASD group studied is likely biased towards the high-functioning end of the autistic spectrum. Besides the small sample size, this limits generalizability of the results. Also, raters were blind to ASD diagnosis for only 66% of recruited individuals. Hence, for the remaining part, their severity ratings could have been influenced by their awareness about the diagnosis.

Our findings are well in line with a previous study illustrating that some patients with treatment-resistant schizophrenia have autistic symptoms and that these co-vary with negative but not with positive symptoms (Sheitman et al. 2004). Importantly, this subgroup of patients did not respond to neuroleptic treatment which strongly points to an involvement of biological pathways not targeted by conventional dopaminergic agents. The definition of extreme groups based on the here presented autism severity score, permits a future investigation of genetic constellations reflecting the integrity of biological pathways shared by individuals suffering from ASD and a subgroup of schizophrenic individuals. This might stimulate the development of novel pharmacological targets resulting in therapeutic benefits for phenotypically defined subgroups of schizophrenic patients.

7. Summary and conclusions

Applying a phenotype-based genetic association approach, the present thesis work provides evidence for several genes modulating rather specific behavioral dimensions in a schizophrenic sample. This complements existing genetic paradigms by elucidating possible relationships between common genetic variants, mildly altered biological networks and behavioral phenotypes relevant for psychiatric disorders. We could show that the endogenous EPO/EPOR system has a role in aspects of cognitive functioning in schizophrenic patients and cognitively inferior healthy controls (project I). Moreover, an association of the well-replicated schizophrenia risk gene neuregulin1 with central olfactory processing could be detected (project II). The gene encoding glycoprotein M6a was identified to be involved in the risk to develop claustrophobia (project III). Lastly, common genetic variants in the transcriptional regulator MECP2 were identified to influence the severity of impulsivity and aggression (project IV). The functional relevance of certain candidate genes for specific aspects of behavior could be further substantiated by mouse models of loss and gain of function of the respective proteins. Mechanistic insight was obtained by studying the influence of SNPs located in the regulatory regions (5' and 3' regions) of the genes on the regulability of expression. Behavior has to be highly flexible in order to be adaptive in a constantly changing social environment. Thus, loss of dynamic regulation of the expression of certain genes might give rise to dysfunctional behaviors. Projects I, III and IV converge on the finding that those genotypes associated with superior expression of the phenotype (superior cognition, no claustrophobia, less impulsivity) showed elevated flexibility in gene expression. For project I (*EPO/EPOR* variants), this higher regulability might potentially be mediated by genotype-dependent transcription factor binding whereas for projects III and IV flexible regulation of gene expression was shown to be influenced by certain brain-expressed microRNAs.

Prior to analyzing specific subphenotypes of schizophrenia, schizophrenic GRAS patients and healthy blood donors were compared with respect to differential distributions of certain alleles of the respective markers of interests (data not reported in the overview sections). Importantly, none of the presented candidate genes (*EPO*, *EPOR*, neuregulin1, *MECP2*) was found to be associated with an overall disease risk for schizophrenia. Researchers in favor of this case-control approach might ascribe this result to the GRAS sample lacking the statistical power to detect the presumably small effects. We believe that a concept such as 'overall disease risk' does not exist. We rather expect different genes to differentially associate with

particular phenotypes/inter-correlated phenotypes more or less pronounced in individuals carrying a psychiatric diagnosis which can also be assessed in 'healthy' subjects ('healthy' meaning non-diagnosed). The considerable variability in symptom composition and severity likely obscures potential effects of variants playing a role in a subsample of schizophrenic individuals only. Thus, ultimately, an understanding of the genetic architecture of schizophrenia bearing relevant therapeutic implications requires the definition of more homogeneous disease subgroups based on highly intercorrelated quantitative phenotypes that share common etiologies. These biologically relevant subgroups might represent the extremes of continuously distributed phenotype severity scores. Project V of the present thesis gave a description of the operationalization of a prevailing autistic phenotype in a subset of schizophrenic patients. Future studies will have to reveal whether the severity of autistic traits in schizophrenic individuals is modulated by genes implicated in the etiology of autism spectrum disorders. Loci or pathways mainly involving synaptic proteins also implicated in familial forms of autism might be interesting candidates (Garber 2007, Carroll et al. 2009).

As a conclusion, the present thesis work comprises several examples highlighting the importance of defining subphenotypes of schizophrenia to reduce intra-group variability and to be able to genetically compare phenotypic cases and controls. The introduced phenotype-genotype association approach may eventually assist the description of biological pathways contributing to the emergence of dysfunctional behaviors. This is crucial for the development of novel therapeutic targets addressing predominantly affected symptom clusters.

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9. Appendix

9.1 Accepted co-author publications

Co-author publication I

Ribbe, K.*, Friedrichs, H.*, Begemann, M., Grube, S., Papiol, S., **Kästner, A.**, Gerchen, M. F., Ackermann, V., Tarami, A., Treitz, A., Flogel, M., Adler, L., Aldenhoff, J. B., Becker-Emner, M., Becker, T., Czernik, A., Dose, M., Folkerts, H., Freese, R., Gunther, R., Herpertz, S., Hesse, D., Kruse, G., Kunze, H., Franz, M., Lohrer, F., Maier, W., Mielke, A., Muller-Isberner, R., Oestereich, C., Pajonk, F. G., Pollmacher, T., Schneider, U., Schwarz, H. J., Kroner-Herwig, B., Havemann-Reinecke, U., Frahm, J., Stuhmer, W., Falkai, P., Brose, N., Nave, K. A. and Ehrenreich, H. (2010). "The cross-sectional GRAS sample: a comprehensive phenotypical data collection of schizophrenic patients." BMC Psychiatry **10**: 91.

Personal contribution

I performed a substantial part of the data entry and participated in the continuous improvement of data validation and quality control.

Co-author publication II

Ehrenreich, H., **Kästner, A.**, Weissenborn, K., Streeter, J., Sperling, S., Wang, K. K., Worthmann, H., Hayes, R. L., von Ahsen, N., Kastrup, A., Jeromin, A. and Herrmann, M. (2011). "Circulating damage marker profiles support a neuroprotective effect of erythropoietin in ischemic stroke patients." Mol Med **17**(11-12): 1306-1310.

Personal contribution

I carried out the statistical analyses and designed the figures and tables.

Co-author publication III

Papiol, S.*, Malzahn, D.*, **Kästner, A.**, Sperling, S., Begemann, M., Stefansson, H., Bickeboller, H., Nave, K. A. and Ehrenreich, H. (2011). "Dissociation of accumulated genetic risk and disease severity in patients with schizophrenia." Transl Psychiatry **1**: e45.

Personal contribution

I contributed to the data acquisition, participated in the analysis and interpretation of data and assisted in the design of the figures.

Co-author publication IV

Hagemeyer, N.*, Goebbels, S.*, Papiol, S.*, **Kästner, A.**, Hofer, S., Begemann, M., Gerwig, U. C., Boretius, S., Wieser, G. L., Ronnenberg, A., Gurvich, A., Heckers, S. H., Frahm, J., Nave, K. A. and Ehrenreich, H. (2012). "A myelin gene causative of a catatonia-depression syndrome upon aging." EMBO Mol Med **4**(6): 528-539.

Personal contribution

I coordinated and supervised the recruitment of subjects for the diffusion tensor imaging study and performed the human genetic association analyses. I participated in the writing of the manuscript.

Co-author publication V

El-Kordi, A.*, Winkler, D.*, Hammerschmidt, K., **Kästner, A.**, Krueger, D., Ronnenberg, A., Ritter, C., Jatho, J., Radyushkin, K., Bourgeron, T., Fischer, J., Brose, N. and Ehrenreich, H. (2013). "Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism." Behav Brain Res.

Personal contribution

I created the gender-specific autism composite scores, carried out the linear regression based multiple imputation and performed the autism composite score related statistical analyses. Moreover, I was involved in the conception, design, drafting, revision and publication of the manuscript.

*These authors contributed equally to the work.

BMC Psychiatry

RESEARCH ARTICLE Open Access

The cross-sectional GRAS sample: *A comprehensive phenotypical data collection of schizophrenic patients*

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Abstract

Background: Schizophrenia is the collective term for an exclusively clinically diagnosed, heterogeneous group of mental disorders with still obscure biological roots. Based on the assumption that valuable information about relevant genetic and environmental disease mechanisms can be obtained by association studies on patient cohorts of ≥1000 patients, if performed on detailed clinical datasets and quantifiable biological readouts, we generated a new schizophrenia data base, the GRAS (Göttingen Research Association for Schizophrenia) data collection. GRAS is the necessary ground to study genetic causes of the schizophrenic phenotype in a 'phenotype-based genetic association study' (PGAS). This approach is different from and complementary to the genome-wide association studies (GWAS) on schizophrenia.

Methods: For this purpose, 1085 patients were recruited between 2005 and 2010 by an invariable team of traveling investigators in a cross-sectional field study that comprised 23 German psychiatric hospitals. Additionally, chart records and discharge letters of all patients were collected.

Results: The corresponding dataset extracted and presented in form of an overview here, comprises biographic information, disease history, medication including side effects, and results of comprehensive cross-sectional psychopathological, neuropsychological, and neurological examinations. With >3000 data points per schizophrenic subject, this data base of living patients, who are also accessible for follow-up studies, provides a wide-ranging and standardized phenotype characterization of as yet unprecedented detail.

Conclusions: The GRAS data base will serve as prerequisite for PGAS, a novel approach to better understanding 'the schizophrenias' through exploring the contribution of genetic variation to the schizophrenic phenotypes.

Background

Schizophrenia is a devastating brain disease that affects approximately 1% of the population across cultures [1]. The diagnosis of schizophrenia or - perhaps more correctly - of 'the schizophrenias' is still purely clinical, requiring the coincident presence of symptoms as listed in the leading classification systems, DSM-IV and ICD-10 [2,3].

Notably, one of the core symptoms of schizophrenia, namely cognitive deficits, from mild impairments to full-blown dementia, has not yet been considered in these classifications. Biologically, schizophrenia is a 'mixed bag' of diseases that undoubtedly have a strong genetic root. Family studies exploring relative risk of schizophrenia have led to estimates of heritability of about 64-88% [4,5]. Monozygotic twin studies showing

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concordance rates of 41-65% [6,7] indicate a considerable amount of non-genetic causes, in the following referred to as 'environmental factors'. Already in the middle of the twentieth century, schizophrenia was seen as a 'polygenetic' disease [8] and, indeed, in numerous genetic studies since, ranging from segregation or linkage analyses, genome scans and large association studies, no major 'schizophrenia gene' has been identified [9]. Even recent genome-wide association studies (GWAS) on schizophrenia confirm that several distinct loci are associated with the disease. These studies concentrated on endpoint diagnosis and found odds ratios for single markers in different genomic regions ranging from 0.68 to 6.01 [10], essentially underlining the fact that - across ethnicities - in most cases these genotypes do not contribute more to the disease than a slightly increased probability.

We hypothesize that an interplay of multiple causative factors, perhaps thousands of potential combinations of genes/genetic markers and an array of different environmental risks, leads to the development of 'the schizophrenias', as schematically illustrated in Figure 1. There may be cases with a critical genetic load already present without need of additional external co-factors, however, in most individuals, an interaction of a certain genetic predisposition with environmental co-factors is apparently required for disease onset. In fact, not too much of an overlap may exist between genetic risk factors from one schizophrenic patient to an unrelated other schizophrenic individual, explaining why it is basically impossible to find common risk genes of schizophrenia with appreciable odds ratios. One GRAS working hypothesis is that in the overwhelming majority of cases, schizophrenia is the result of a 'combination of unfortunate genotypes'.

If along the lines of traditional human genetics all attempts to define schizophrenia as a 'classical' genetic disease have largely failed, how can we learn more about the contribution of genes/genotypes to the disease phenotype? Rather than searching by GWAS for yet other schizophrenia risk genes, we designed an alternative and widely complementary approach, termed PGAS (phenotype-based genetic association study), in order to

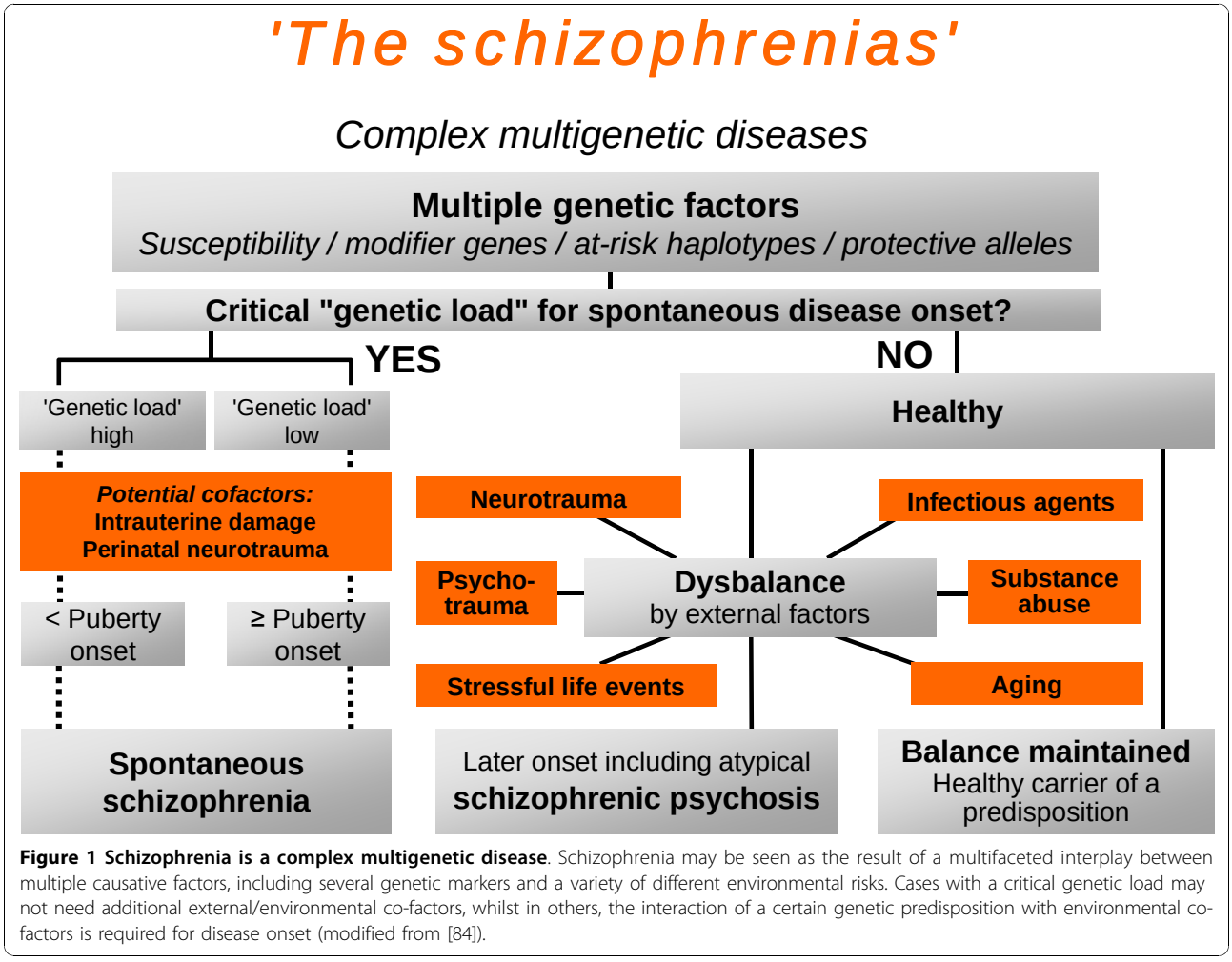


Figure 1 Schizophrenia is a complex multigenetic disease. Schizophrenia may be seen as the result of a multifaceted interplay between multiple causative factors, including several genetic markers and a variety of different environmental risks. Cases with a critical genetic load may not need additional external/environmental co-factors, whilst in others, the interaction of a certain genetic predisposition with environmental co-factors is required for disease onset (modified from [84]).

explore the contribution of certain genes/genetic markers to the schizophrenic phenotype. To launch PGAS, we had to establish a comprehensive phenotypical data base of schizophrenic patients, the GRAS (Göttingen Research Association for Schizophrenia) data collection. Very recently, we have been able to demonstrate proof-of-concept for the PGAS approach [[11], and Grube et al: Calcium-activated potassium channels as regulators of cognitive performance in schizophrenia, submitted].

Large data bases of schizophrenic patients have been instigated for decades to perform linkage/family studies, treatment trials, genetic or epidemiological studies applying either a cross-sectional or a longitudinal design (e.g. [12-20]). However, for the above introduced PGAS approach, another type of data base is required, and only few of the existing data banks are suited for phenotypical analyses. An example is the 'Clinical Antipsychotic Trial of Intervention Effectiveness (CATIE)', originally set up as a treatment study comparing a first generation antipsychotic drug with several second generation antipsychotics in a multisite randomized double-blind trial [17,21]. The huge amount of data accumulated in the frame of this trial serves now also for GWAS and genotype-phenotype association studies [22-25]. Disadvantages may be that the CATIE data were collected by different examiners in 57 US sites and that comprehensive data for phenotypical analyses are only available for subsamples of the originally included 1493 patients. Another example of a large data base with considerable phenotypical power is the 'Australian Schizophrenia Research Bank (ASRB)' [26]. ASRB operates to collect, store and distribute linked clinical, cognitive, neuroimaging and genetic data from a large sample of patients with schizophrenia (at present nearly 500) and healthy controls (almost 300) [27,28]).

The present paper has been designed (1) to introduce the GRAS data collection, set up as prerequisite and platform for PGAS; (2) to exemplify on some selected areas of interest the potential of phenotypical readouts derived from the GRAS data collection and their internal consistency; (3) to provide a first panel of epidemiological data as a 'side harvest' of this data base; and (4) to enable interested researchers worldwide to initiate scientific collaborations based on this data base.

Methods

Ethics

The GRAS data collection has been approved by the ethical committee of the Georg-August-University of Göttingen (master committee) as well as by the respective local regulatories/ethical committees of all collaborating centers (Table 1). The distribution of the centers over Germany together with information on the numbers of recruited patients per center is presented in Figure 2.

GRAS patients

From September 2005 to July 2008, a total of 1071 patients were examined by the GRAS team of traveling investigators after giving written informed consent, own and/or authorized legal representatives. Since then, low-rate steady state recruitment has been ongoing, among others to build up a new cohort for replicate analyses of genotype-phenotype associations. As of July 2010, 1085 patients have been entered into the data base. They were examined in different settings: 348 (32.1%) as outpatients, 474 (43.7%) as inpatients in psychiatric hospitals, 189 (17.4%) as residents in sheltered homes, 54 (5%) as patients in specific forensic units, and 20 (1.8%) as day clinic patients. Inclusion criteria were (1) confirmed or suspected diagnosis of schizophrenia or schizoaffective disorder according to DSM-IV and (2) at least some ability to cooperate. Recruitment efficiency over the core travel/field study time from 2005 to 2008 and patient flow are shown in Figures 3a and 3b. Of the 1085 patients entered into the data base, a total of 1037 fulfilled the diagnosis of schizophrenia or schizoaffective disorder. For 48 patients the diagnosis of schizophrenia could not be ultimately confirmed upon careful re-check and follow-up. Of the schizophrenic patients, 96% completed the GRAS assessment whereas about 4% dropped out during the examination. Almost all patients agreed to be re-contacted for potential follow-up studies, only 1.5% were either lost to follow-up (present address unknown or deceased) or did not give consent to be contacted again.

Healthy control subjects

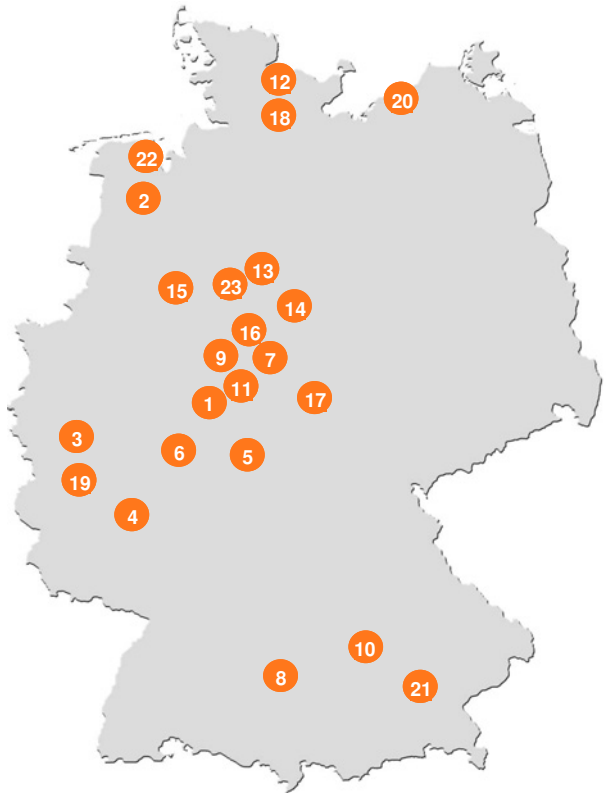
(1) For genetic analyses, control subjects, who gave written informed consent, were voluntary blood donors, recruited by the Department of Transfusion Medicine at the Georg-August-University of Göttingen according to national guidelines for blood donation. As such, they widely fulfill health criteria, ensured by a broad pre-donation screening process containing standardized questionnaires, interviews, hemoglobin, blood pressure, pulse, and body temperature determinations. Of the total of 2265 subjects, 57.5% are male (n = 1303) and 42.5% female (n = 962). The average age is 33.8 ± 12.2 years, with a range from 18 to 69 years. Participation as healthy controls for the GRAS sample was anonymous, with information restricted to age, gender, blood donor health state and ethnicity. Comparable to the patient population (Table 2), almost all control subjects were of European Caucasian descent (Caucasian 97.8%; other ethnicities 2%; unknown 0.2%). **(2)** For selected cognitive measures and olfactory testing, 103 additional healthy volunteers were recruited as control subjects (matched with respect to age, gender, and smoking habits). These healthy controls include 67.0% male (n = 69) and 33.0%

Table 1 GRAS data collection manual: Table of contents

category	content	reference in the paper
legal documents/ethical requirements	patient information, informed consent form, confidentiality form, and others...	
patient history	general information (age, sex, ethnicity,...)	→ table 2
	education/employment	→ table 2
	living situation	→ table 2
	legal history	
	medication including side effects	→ table 4
	medical history	
	family history	
	global quality of life[a]	→ table 2 and figure 6
	birth history/traumatic brain injury	
	stressful life events	
	suicidal thoughts/suicide attempts	
	hospitalization history	→ table 2 and figure 6
clinical interviews/ratings	parts of SCID-I: addiction, anxiety, affective disorders, psychotic disorders*[b]	
	Positive and Negative Syndrome Scale* (PANSS)[c]	→ table 2 and figure 6
	Clinical Global Impression* (CGI)[d]	→ table 2 and figure 6
	Global Assessment of Functioning* (GAF)[e]	→ table 2 and figure 6
questionnaires	State-Trait-Anxiety-Inventory* (STAI)[f]	→ table 2 and figure 6
	Brief Symptom Inventory* (BSI)[g]	→ table 2 and figure 6
	Toronto Alexithymia Scale* (TAS)[h]	→ table 2
cognitive tests	premorbid IQ (MWT-B)[i, j]	→ table 3 and figure 7
	reasoning (LPS-3)[k]	→ table 3 and figure 7
	letter-number-span (BZT)[l]	→ table 3 and figure 7
	finger dotting and tapping[m]	→ table 3 and figure 7
	trail making tests (TMT-A and TMT-B)[n]	→ table 3 and figure 7
	verbal fluency (DT/RWT)[o, p]	
	digit-symbol test (ZST)[q]	→ table 3 and figure 7
	verbal memory* (VLMT)[r]	→ table 3 and figure 7
physical examination	Testbatterie zur Aufmerksamkeitsprüfung (TAP)[s]	→ table 3 and figure 7
	general physical examination	
	Cambridge Neurological Inventory (CNI)[t]	→ table 5 and figure 8
	Contralateral Co-Movement Test (COMO)[u]	
	Barnes Akathisia Rating Scale (BARS)[v]	→ figure 8
	Simpson-Angus Scale (SAS)[w]	→ figure 8
	Tardive Dyskinesia Rating Scale (TDRS)[x]	→ figure 8
	Abnormal Involuntary Movement Scale (AIMS)[y]	→ figure 8
	odor testing (ORNI Test)[z]	
	blood sampling (DNA, serum)	

*questionnaires and cognitive tests in respective German versions

[a] Based on a visual analogue scale (Krampe H, Bartels C, Victorson D, Enders CK, Beaumont J, Cella D, Ehrenreich H: The influence of personality factors on disease progression and health-related quality of life in people with ALS. Amyotroph Lateral Scler 2008, 9:99-107). [b]Wittchen H-U, Zaudig, M. and Fydrich, T.: SKID-I (Strukturiertes Klinisches Interview für DSM-IV; Achse I: Psychische Störungen). Göttingen: Hogrefe; 1997. [c]Kay SR, Fiszbein A, Opler LA: The positive and negative syndrome scale (PANSS) for schizophrenia. Schizophr Bull 1987, 13(2):261-276. [d]Guy W: Clinical Global Impression (CGI). In ECDEU Assessment manual for psychopharmacology, revised National Institue of Mental Health. Rockville, MD; 1976. [e]AmericanPsychiatricAssociation: Diagnostic and statistical manual of mental disorders, 4th edition (DSM-IV). Washington, DC: American Psychiatric Press; 1994. [f]Laux L, Glanzmann P, Schaffner P, Spielberger CD: Das State-Trait-Angstinventar (STAI). Weinheim: Beltz; 1981. [g]Franke GH: Brief Symptom Inventory (BSI). Goettingen: Beltz; 2000. [h]Kupfer J, Brosig B, Braehler E: Toronto Alexithymie-Skala-26 (TAS-26). Goettingen: Hogrefe; 2001. [i]Lehrl S, Triebig G, Fischer B: Multiple choice vocabulary test MWT as a valid and short test to estimate premorbid intelligence. Acta Neurol Scand 1995, 91(5):335-345. [j]Lehrl S: Mehrfach-Wortschatz-Intelligenztest MWT-B. Balingen: Spitta Verlag; 1999. [k]Horn W: Leistungsprüfsystem (LPS). 2 edition. Goettingen: Hogrefe; 1983. [l]Gold JM, Carpenter C, Randolph C, Goldberg TE, Weinberger DR: Auditory working memory and Wisconsin Card Sorting Test performance in schizophrenia. Arch Gen Psychiatry 1997, 54(2):159-165. [m]Chapman RL: The MacQuarrie test for mechanical ability. Psychometrika 1948, 13(3):175-179. [n]War-Department: Army Individual Test Battery. Manual of directions and scoring. Washington, D.C.: War Department, Adjutant General's Office; 1944. [o]Kessler J, Denzler P, Markowitsch HJ: Demenz-Test (DT). Göttingen: Hogrefe; 1999. [p]Aschenbrenner S, Tucha O, Lange KW: Der Regensburger Wortflüssigkeits-Test (RWT). Göttingen: Hogrefe; 2000. [q]Tewes U: Hamburg-Wechsler Intelligenztest fuer Erwachsene (HAWIE-R). Bern: Huber; 1991. [r]Helmstaedter C, Lendt M, Lux S: Verbaler Lern- und Merkfähigkeitstest (VLMT). Goettingen: Beltz; 2001. [s]Zimmermann P, Fimm B: Testbatterie zur Aufmerksamkeitsprüfung (TAP). Version 1.02c. Herzogenrath: PSYTEST; 1993. [t]Chen EY, Shapleske J, Luque R, McKenna PJ, Hodges JR, Calloway SP, Hymas NF, Dening TR, Berrios GE: The Cambridge Neurological Inventory: a clinical instrument for assessment of soft neurological signs in psychiatric patients. Psychiatry Res 1995, 56(2):183-204. [u]Bartels C, Mertens N, Hofer S, Merboldt KD, Dietrich J, Frahm J, Ehrenreich H: Callosal dysfunction in amyotrophic lateral sclerosis correlates with diffusion tensor imaging of the central motor system. Neuromuscul Disord 2008, 18 (5):398-407. [v]Barnes TR: The Barnes Akathisia Rating Scale - revisited. J Psychopharmacol 2003, 17(4):365-370. [w]Simpson GM, Angus JW: A rating scale for extrapyramidal side effects. Acta Psychiatr Scand Suppl 1970, 212:11-19. [x]Simpson GM, Lee JH, Zoubok B, Gardos G: A rating scale for tardive dyskinesia. Psychopharmacology (Berl) 1979, 64 (2):171-179. [y]Guy W: Abnormal involuntary movement scale (AIMS). In ECDEU Assessment manual for psychopharmacology, revised National Institute of Mental Health. Rockville, MD; 1976. [z]ORNI Test (Odor Recognition, Naming and Interpretation Test; developed for the purpose of odor testing in schizophrenics; manuscript in preparation)



	center (city)	numbers of recruited patients
1.	Bad Emstal-Merxhausen	241 *(22.2%)*
2.	Bad Zwischenahn	40 *(3.7%)*
3.	Bonn	19 *(1.8%)*
4.	Eltville-Eichberg	20 *(1.8%)*
5.	Fulda	30 *(2.8%)*
6.	Giessen-Haina	36 *(3.3%)*
7.	Göttingen	114 *(10.5%)*
8.	Günzburg	31 *(2.9%)*
9.	Hofgeismar	10 *(0.9%)*
10.	Ingolstadt	27 *(2.5%)*
11.	Kassel	19 *(1.8%)*
12.	Kiel	26 *(2.4%)*
13.	Langenhagen	24 *(2.2%)*
14.	Liebenburg	27 *(2.5%)*
15.	Lübbecke	30 *(2.8%)*
16.	Moringen	4 *(0.4%)*
17.	Mühlhausen	53 *(4.9%)*
18.	Rickling	56 *(5.2%)*
19.	Rieden	91 *(8.4%)*
20.	Rostock	80 *(7.4%)*
21.	Taufkirchen	32 *(2.9%)*
22.	Wilhelmshaven	27 *(2.5%)*
23.	Wunstorf	48 *(4.4%)*
total number of patients		**1085**

Figure 2 Collaborating centers and patient numbers. Map of Germany displaying the locations of all 23 collaborating centers that were visited by an invariable team of traveling investigators. The table next to the map provides numbers of patients examined in each center. Some centers were visited more than once.

(n = 34) female subjects with an average age of 39.02 ± 13.87 years, ranging from 18 to 71 years.

Traveling team

The GRAS team of traveling investigators consisted of 1 trained psychiatrist and neurologist, 3 psychologists and 4 medical doctors/last year medical students. All investigators had continuous training and calibration sessions to ensure the highest possible agreement on diagnoses and other judgments as well as a low interrater variability regarding the instruments applied. Patient contacts were usually prepared by colleagues/personnel in the respective collaborating psychiatric centers (Figure 2) to make the work of the travel team as efficient as possible.

The GRAS manual

A standardized procedure for examination of the patients has been arranged with the GRAS manual, composed for the purpose of the GRAS data collection. Table 1 presents its contents, including established instruments, such as clinical interviews/ratings, questionnaires, cognitive and neurological tests [2,29-53].

GRAS operating procedure

The GRAS data base operating procedure leading from the large set of raw data provided by the travel team to the data bank with its several-fold controlled and verified data points is illustrated in Figure 4. Already during the time when the travel team examined patients all over Germany, a team of psychologists started to work on the development of the GRAS data base, integrating the raw data to ultimately result in over 3000 phenotypic data points per patient (total of over 3.000 000 data points at present in the data collection) (Figure 5). Most importantly, the chart records/medical reports of all patients were carefully screened, missing records identified and, in numerous, sometimes extensive and repeated, telephone and written conversations, missing psychiatric discharge letters of every single patient organized. After careful study and pre-processing of raw data and chart records, the confirmation of the diagnoses, determination of age of onset of the disease and prodrome as well as other essential readouts were achieved by meticulous consensus decisions.

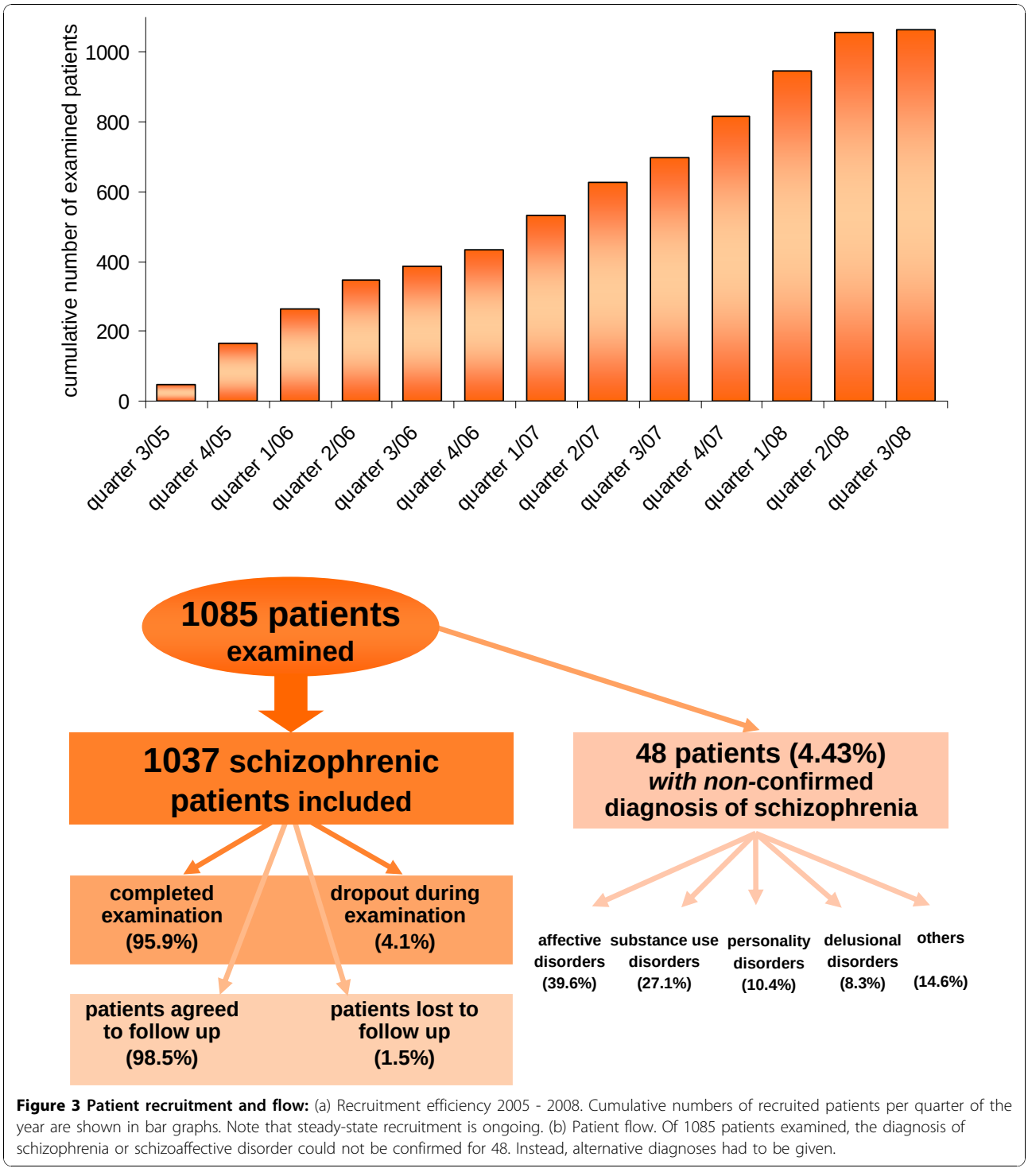


Figure 3 Patient recruitment and flow: (a) Recruitment efficiency 2005 - 2008. Cumulative numbers of recruited patients per quarter of the year are shown in bar graphs. Note that steady-state recruitment is ongoing. (b) Patient flow. Of 1085 patients examined, the diagnosis of schizophrenia or schizoaffective disorder could not be confirmed for 48. Instead, alternative diagnoses had to be given.

Statistical analyses

For the establishment of the data base and for basic statistical analyses of the data, SPSS for Windows version 17.0 [54] was used. Comparisons of men and women in terms of sociodemographic and clinical picture as well as neurological examination were assessed using either Mann-Whitney-U or Chi-square test. Prior to correlation and regression analyses, selected metric phenotypic variables were standardized by Blom transformation [55]. The Blom transformation is a probate transformation into ranks and the resulting standardized values are normally distributed with zero mean and variance one.

Table 2 GRAS sample description

		total				men				women				statistics	
		N	%	mean (sd)	median	N	%	mean (sd)	median	N	%	mean (sd)	median	χ^2/Z	P
sociodemographics															
total n		1037	*100*			693	*100*			344	*100*				
age (in years)				39.52 (12.56)	39.05			37.57 (11.97)	36.67			43.46 (12.80)	42.85	Z = -6.980	< 0.001*
education (in years)				11.94 (3.37)	12.00			11.71 (3.34)	12.00			12.42 (3.39)	12.00	Z = -2.714	0.007*
ethnicity:	caucasian	992	*95.66*			661	*95.38*			331	*96.20*				
	african	7	*0.68*			6	*0.87*			1	*0.30*				
	mixed	10	*0.96*			7	*1.01*			3	*0.90*			χ^2 = 1.202	0.753
	unknown	28	*2.70*			19	*2.74*			9	*2.60*				
native tongue:	German	902	*86.98*			591	*85.71*			311	*90.67*				
	bi-lingual German	46	*4.44*			38	*4.33*			8	*1.46*			χ^2 = 6.899	0.032*
	other	89	*8.58*			64	*9.96*			25	*7.87*				
marital status:	single	748	*72.13*			575	*82.97*			173	*50.44*				
	married	129	*12.44*			48	*6.93*			81	*23.32*				
	divorced	124	*11.96*			57	*8.23*			67	*19.53*			χ^2 = 121.516	< 0.001*
	widowed	13	*1.25*			3	*0.43*			10	*2.92*				
	unknown	23	*2.22*			10	*1.44*			13	*3.79*				
living situation:	alone	292	*28.16*			201	*29.00*			91	*26.45*				
	alone with children	17	*1.64*			0	*0*			17	*4.94*				
	with partner (± children)	137	*13.20*			50	*7.22*			87	*25.29*				
	With parents	157	*15.14*			121	*17.46*			36	*10.47*				
	with others (family members, friends)	71	*6.85*			53	*7.65*			18	*5.23*			χ^2 = 116.823	< 0.001*
	sheltered home	282	*27.19*			212	*30.59*			70	*20.35*				
	forensic hospital	54	*5.21*			43	*6.20*			11	*3.20*				
	homeless	4	*0.39*			4	*0.58*			0	*0*				
	unknown	23	*2.22*			9	*1.30*			14	*4.07*				
clinical picture															
diagnosis:	classical schizophrenias	852	*82.16*			615	*88.74*			237	*68.90*			χ^2= 61.794	< 0.001*
	schizoaffective disorders	185	*17.84*			78	*11.26*			107	*31.10*				
age of onset of first psychotic episode				25.75 (8.81)	23.00			24.49 (7.71)	22.00			28.28 (10.23)	26.00	Z = -5.705	< 0.001*
duration of disease (in years)				13.23 (10.71)	10.87			12.57 (10.38)	10.16			14.54 (11.24)	13.02	Z = -2.600	0.009*
hospitalization (number of inpatient stays)				8.60 (9.76)	6.00			8.49 (9.95)	5.00			8.83 (9.38)	6.00	Z = -0.727	0.467

Table 2: GRAS sample description *(Continued)*

chlorpromazine equivalents		687.36 (696.85)	499.98	706.67 (668.43)	520.00	648.35 (750.50)	450.00	Z = -2.428	0. 015*
PANSS[a]:	positive symptoms	13.76 (6.32)	12.00	13.94 (6.16)	12.00	13.92 (6.64)	12.00	Z = -0.130	0.990
	negative symptoms	18.23 (7.85)	17.00	18.14 (7.57)	17.00	18.11 (8.44)	17.00	0.886	0.376
	general psychiatric symptoms	33.73 (11.83)	32.00	33.37 (11.31)	32.00	34.50 (12.81)	33.00	-0.886	0.376
	total score	65.64 (23.40)	63.00	65.32 (22.41)	63.00	66.31 (25.37)	62.00	-0.025	0.980
Clinical Global Impression scale[b]		5.57	6.00	5.57 (1.03)	6.00	5.57 (1.18)	6.00	Z = -0.121	0.894
Global Assessment of Functioning[c]		45.76 (0.68)	45.00	45.60 (16.30)	45.00	46.09 (19.11)	45.00	Z = -0.323	0.747
global quality of life [d]		5.41 (2.37)	5.00	5.43 (2.31)	5.00	5.38 (2.49)	5.00	Z = -0.378	0.705
Brief Symptom Inventory [e]:	general severity index	0.88 (0.68)	0.71	0.87 (0.66)	0.71	0.92 (0.72)	0.71	Z = -0.687	0.492
State-Trait-Anxiety Inventory [f] :	state anxiety	43.54 (10.89)	43.00	43.48 (10.45)	43.00	43.65 (11.79)	43.00	Z = -0.121	0.904
	trait anxiety	44.96 (11.34)	45.00	44.67 (11.09)	45.00	45.56 (11.82)	46.00	-0.983	0.326
Toronto Alexithymia Scale [g]		2.59 (0.56)	2.61	2.58 (0.54)	2.55	2.60 (0.60)	2.66	Z = -0.607	0.544

[a]Kay SR, Fiszbein A, Opler LA: The positive and negative syndrome scale (PANSS) for schizophrenia. Schizophr Bull1987,13(2):261-276. [b]Guy W: Clinical Global Impressions (CGI). In ECDEU Assessment manual for psychopharmacology, revised NationalInstitue of Mental Health. Rockville, MD; 1976. [c]AmericanPsychiatricAssociation: Diagnostic and statistical manual of mental disorders, 4th edition (DSM-IV). Washington, DC: American Psychiatric Press; 1994. [d]Based on a visual analogue scale (Krampe H, Bartels C, Victorson D, Enders CK, Beaumont J, Cella D, Ehrenreich H: The influence of personality factors on disease progression and health-related quality of life in people with ALS. Amyotroph Lateral Scler 2008, 9:99-107). [e]Franke GH: Brief Symptom Inventory (BSI). Goettingen: Beltz; 2000. [f]Laux L, Glanzmann P, Schaffner P, Spielberger CD: Das State-Trait-Angstinventar (STAI). Weinheim: Beltz; 1981. [g]Kupfer J, Brosig B, Braehler E: Toronto Alexithymie-Skala-26 (TAS-26). Goettingen: Hogrefe; 2001.

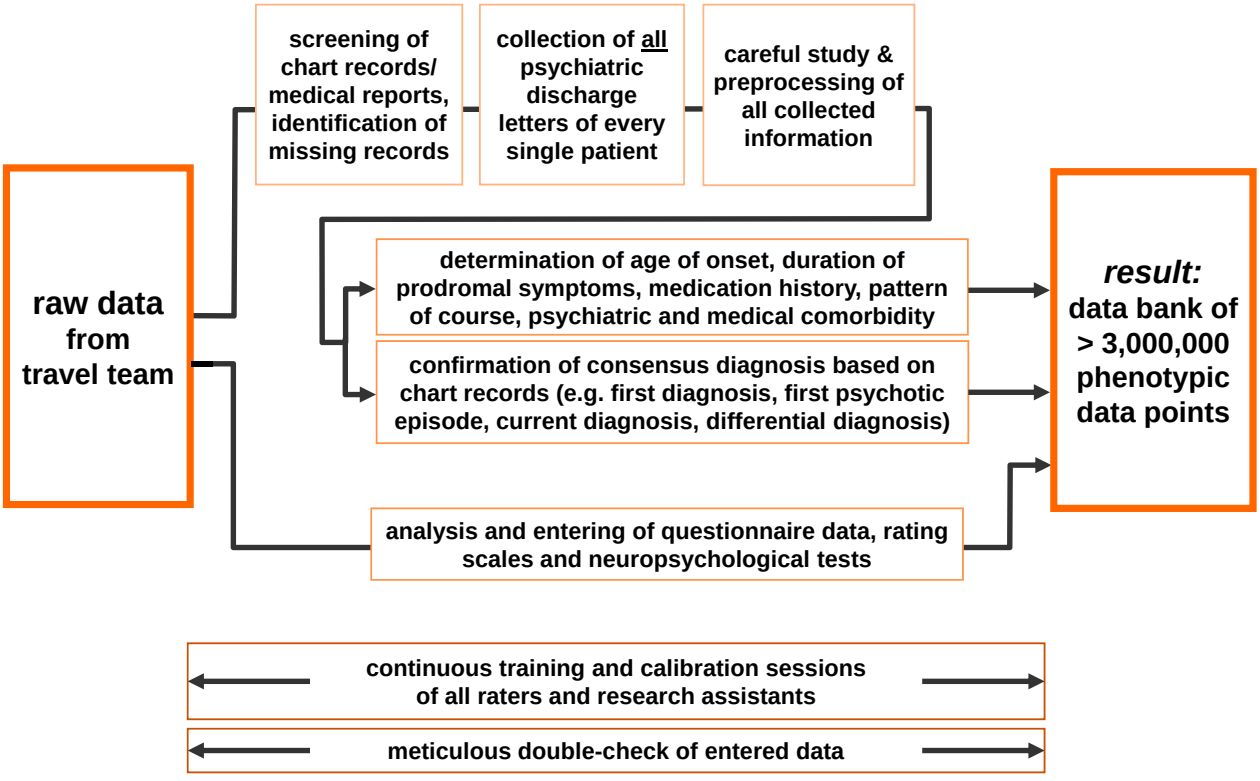


Figure 4 Development of the GRAS data bank. Raw data, brought to Göttingen by the traveling team of examiners, were only entered into the data base after careful and comprehensive data re-checking, also based on patient charts and discharge letters. During the whole process, continuous calibration sessions and repeated re-checking of the entered data took place.

Comparisons of men and women in terms of cognitive performance were assessed by analyses of covariance, using age, duration of disease, years of education and chlorpromazine equivalents as covariates. For all intercorrelation patterns, correlations of the particular target variables were assessed using Pearson product-moment correlation. Cronbach's alpha coefficient was determined for estimation of internal consistency of the target variables within a defined intercorrelation pattern. Multiple regression analyses using the enter method were conducted to evaluate the contribution of selected disease related variables (duration of disease, positive symptoms, negative symptoms, catatonic signs and chlorpromazine equivalents) to 3 dependent variables: basic cognition/ fine motor functions, cognitive functions and global functioning (GAF) [2]. The dependent variables basic cognition/fine motor functions and cognitive functions are both composite score variables. The basic cognition/ fine motor function score comprises alertness (TAP), dotting and tapping (Cronbach's alpha = .801) [39,46] and the cognition score consists of reasoning (LPS3), 2 processing speed measures (TMT-A and digit-symbol test, ZST), executive functions (TMT-B), working memory (BZT), verbal learning & memory (VLMT) and divided attention (TAP) [37,38,41,44-46] (Cronbach's alpha = .869). For both scores, a Cronbach's alpha >.80 indicates a high internal consistency as prerequisite for integrating several distinct items into one score. Multiple regression analyses were conducted for the total sample and separated for men and women.

Results

Biographic and clinical data

The GRAS data collection comprises presently (as of August 2010) 1037 patients with confirmed diagnosis of schizophrenia (82.2%) or schizoaffective disorder (17.8%). A total of 693 men and 344 women fulfilled the respective diagnostic requirements of DSM-IV. Table 2 provides a sample description, both total and separated for male and female patients, with respect to sociodemographic data and clinical picture. There are some differences between genders in the GRAS sample: Women are older, less single, have more years of education, more diagnoses of schizoaffective disorders, longer duration of disease, later age of onset of first psychotic episode and lower doses of antipsychotics. However, regarding determinants of the clinical picture, e.g. PANSS scores [30], genders do not differ significantly.

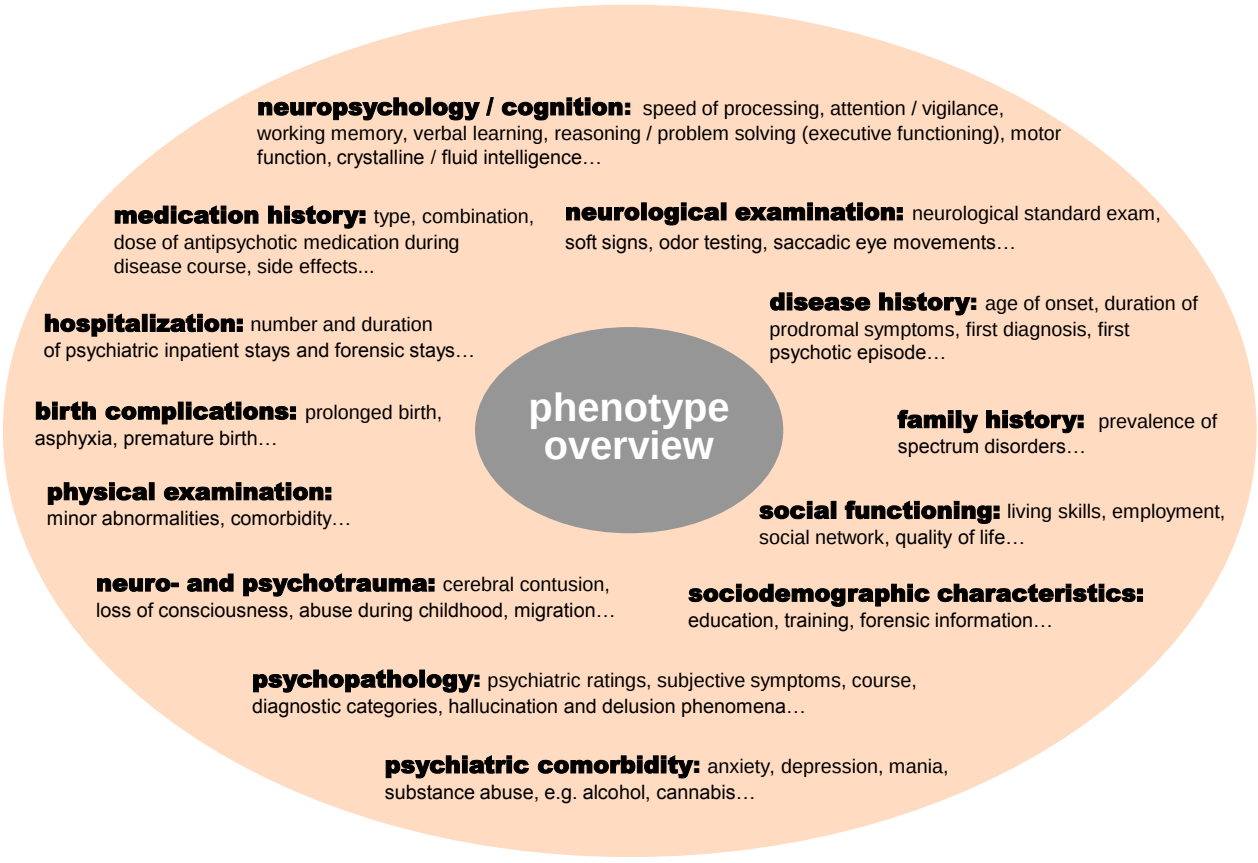


Figure 5 Phenotype overview. Various different domains covered by the GRAS data collection are displayed. These domains will also deliver the basis for further sophistication of phenotypical readouts.

An intercorrelation pattern of selected clinical readouts, obtained by (1) clinical ratings and (2) self-ratings of the patients and complemented by (3) 'objective data', in this case medication and hospitalization, is presented in Figure 6. The Cronbach's alpha of .753 suggests that items derived from the 3 different perspectives harmonize well. Whereas patient ratings of quality of life and state anxiety (STAI) [32] are only weakly correlated with professional clinical ratings and objective data, the patients' self-estimated symptom burden as measured with the BSI [33] shows moderate to good correlation.

Cognition

For the ongoing/planned genetic analyses, not only the clinical picture with its schizophrenia-typical positive and negative symptoms, but particularly cognition plays an important role. The cognitive tests applied in the GRAS data collection show an intercorrelation pattern that further underlines quality and internal consistency of the data obtained by the invariable team of investigators (Figure 7). Table 3 represents the cognitive performance data of the complete GRAS sample in the respective domains. In addition, the performance level of men and women is given as well as - for comparison - available normative data of healthy individuals. Since for dotting and tapping [39], no normative data were available in the literature, the values shown in Table 3 were obtained from the healthy GRAS control population for cognitive measures (n = 103; see patients and methods).

Comparing cognitive performance of schizophrenic men and women, analyses of covariance have been conducted, with age, duration of disease, years of education and chlorpromazine equivalents as covariates, which revealed significant gender differences in discrete cognitive domains. Men performed better in reasoning ($F = 17.62$, $p < .001$), alertness ($F = 28.30$, $p < .001$ for reaction time and $F = 10.39$, $p = .001$ for lapses), and divided attention ($F = 14.07$ $p < .001$ for reaction time and $F = 22.12$, $p < .001$ for lapses). In contrast, female schizophrenic patients were superior in verbal memory tasks ($F = 12.38$, $p < .001$) and digit symbol test ($F = 19.24$, $p < .001$). With respect to normative data obtained from healthy controls, cognitive data of all schizophrenic patients are in the lower normal range (percentile rank = 16 for digit symbol test) or even below (percentile

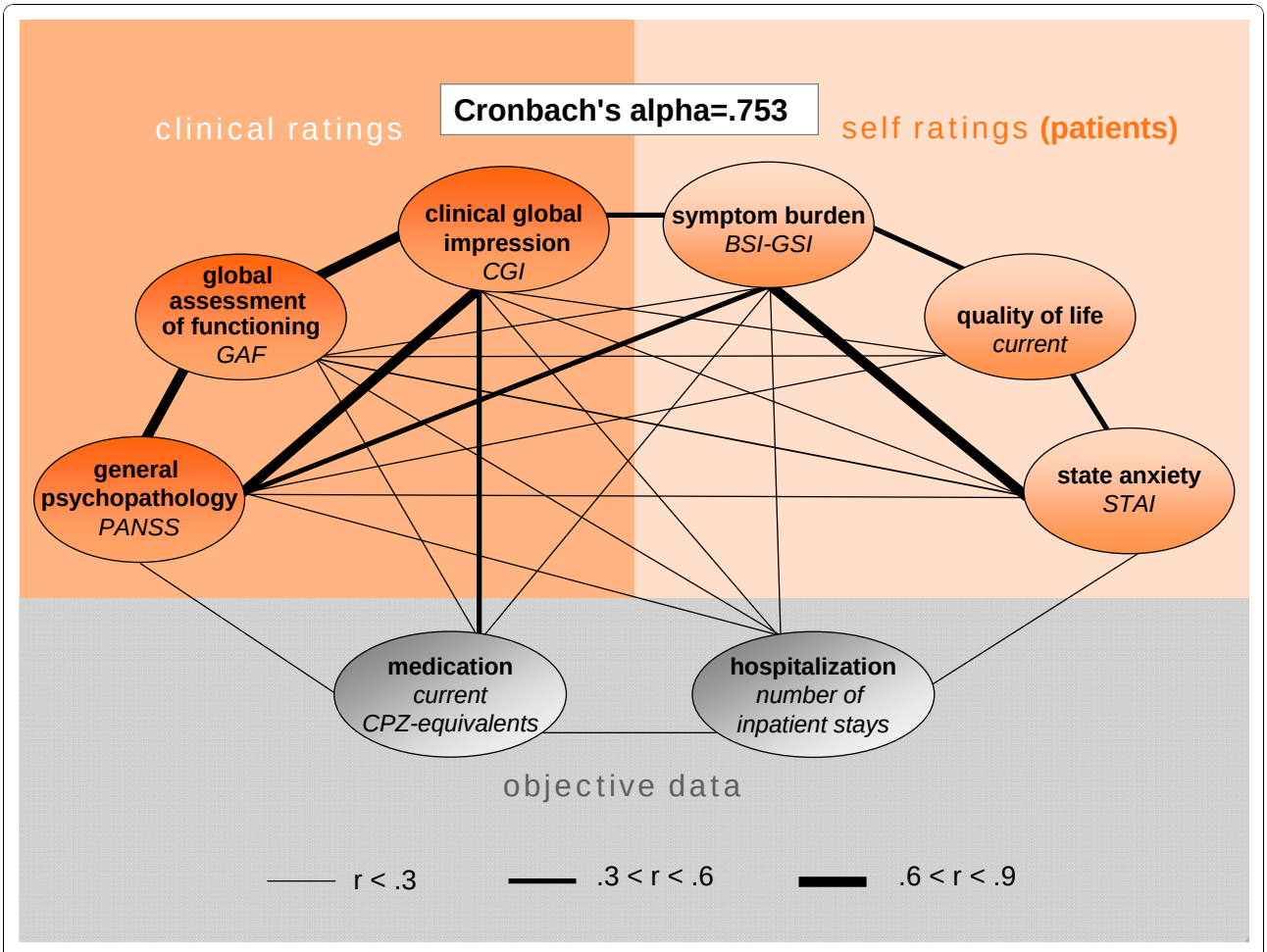


Figure 6 Clinical intercorrelation pattern. Correlations between measures of the clinical picture derived from different approaches: Patient self-ratings, clinical rater judgement and 'objective data'. Thickness of the lines represents the strength of correlation between two measures; only significant correlations are displayed. Note the strong internal consistency expressed by a Cronbach's alpha of .753.

ranks 10 for verbal memory, TMT-A, TMT-B, alertness and divided attention). Only for reasoning (LPS) [37] and premorbid intelligence (MWT-B) [36], schizophrenic subjects lie in the average range (percentile ranks of 31 and 43.5 respectively).

Antipsychotic medication and side effects

Another important feature of schizophrenic patients that may influence their every-day functioning and performance, and result in a considerable number of side effects, is their antipsychotic medication. The GRAS data collection contains information on type, dose, duration of medication and drugs prescribed over the years. The mean dose of present antipsychotic medication of the whole GRAS population, expressed as chlorpromazine equivalents [56] amounts to 687.36 (± 696.85). Chlorpromazine equivalents in male are significantly higher as compared to female patients (Table 2). An overview of self-reported side effects of current antipsychotic medication in the GRAS sample, again sorted by gender, is given in Table 4. Of the 1037 patients with confirmed diagnosis of schizophrenia/schizoaffective disorder, 24 were presently not on antipsychotic drugs, whilst for 1 patient the current medication was unknown. Of the remaining 1012 patients who currently receive antipsychotic medication (16.5% first generation antipsychotics, 54.1% second generation antipsychotics and 29.4% mixed) and were all explicitly interviewed regarding medication side effects, only 423 reported any. The discrepancy between side effects measured versus side effects based on patients' reports becomes obvious when considering for instance the number of patients with clear extrapyramidal symptoms: A total of 335 subjects measured by Simpson-Angus Scale (mean score >.3) [50] contrasts only 117 patients self-reporting extrapyramidal complaints. External rating of extrapyramidal side effects in the GRAS population was comprehensively performed, utilizing a number of respective instruments which all showed significant

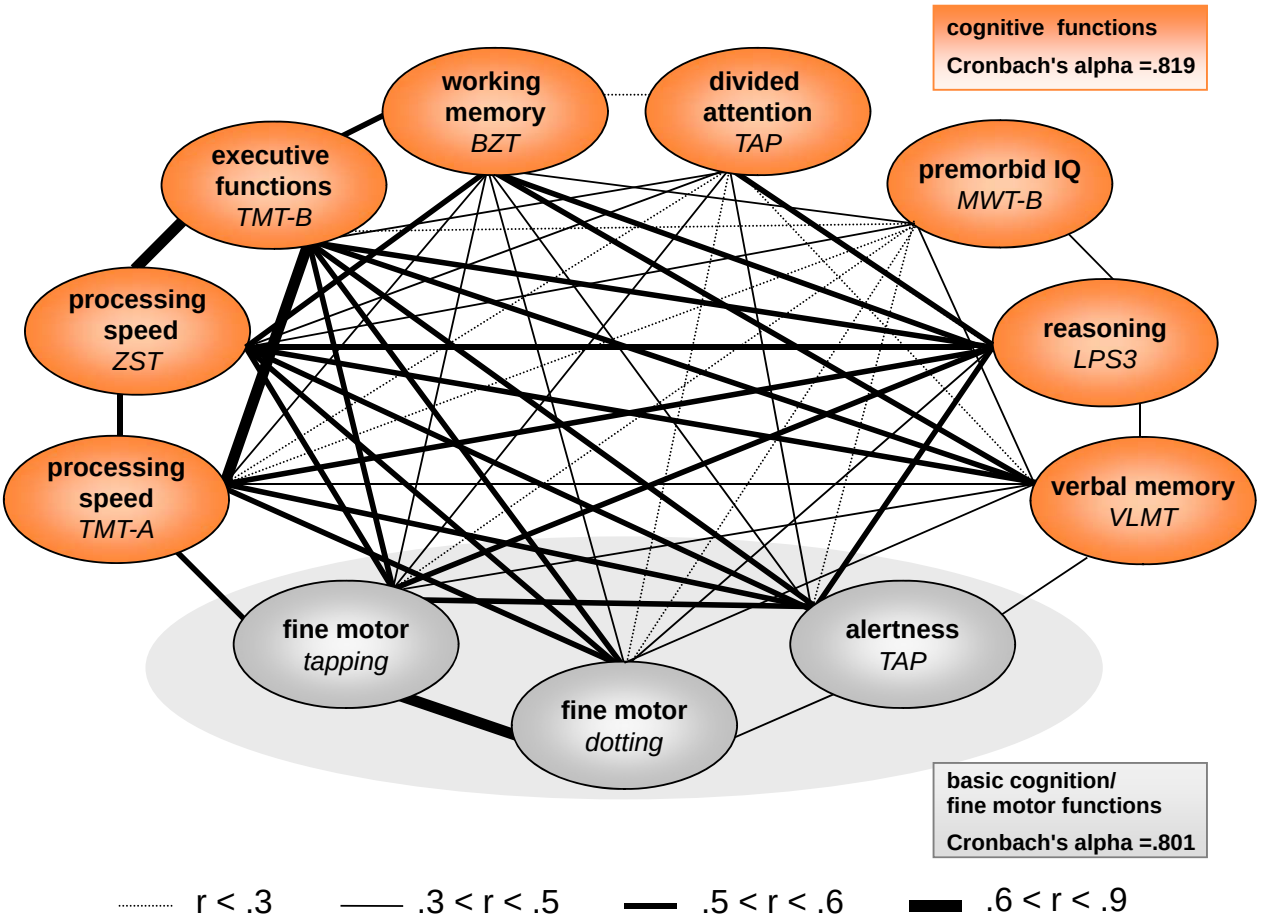


Figure 7 Cognitive intercorrelation pattern. Shown are all neuropsychological tests performed, together with their respective cognitive domain. Thickness of the lines represents the strength of correlation between two tests; only significant correlations are displayed. Tests for higher cognitive functions are labelled in orange; tests for basic (mainly basic cognition/fine motor dependent) functions in grey. Measures of higher cognitive functions as well as measures of basic cognition/fine motor functions show powerful internal consistency (Cronbach's alpha of .819 and .801 respectively).

intercorrelation (Figure 8) [47,49-52,57]. A composite score of the 6 Blom transformed scales, used for testing potential gender effects, yielded no significant differences in extrapyramidal symptoms in men versus women (Z = -0.022, p = 0.982).

Neurological symptoms

Similar to cognitive readouts, evaluation of inherent neurological symptoms in the schizophrenic patient population are of tremendous interest, not only for understanding the contribution of particular genes/genetic markers and/or environmental factors to the schizophrenic phenotype but also for estimating the impact of potential neurological comorbidities. Table 5 provides an overview of neurological symptoms based on the Cambridge Neurological Inventory (CNI) [47]. Only in the subscale 'Failure to suppress inappropriate response', significant differences between men and women (Z = -3.175, p = 0.001) became evident. Women were less able to hold respective responses back, e.g. to blink with one eye, leaving the other eye open, or to perform saccadic eye movements without moving the head.

Prediction of functioning

In order to delineate the influence of disease on functioning in the GRAS sample, multiple regression analyses have been employed. These procedures assessed the contribution of 5 disease-related variables, i.e. duration of disease, PANSS positive and negative scores [30], catatonic signs [47], and dose of antipsychotic medication, to 3 dependent performance variables: (a) basic cognition/fine motor functions, (b) cognitive performance and (c) global functioning (Table 6). Regarding basic cognition/fine motor function, multiple regression analysis revealed a significant model accounting for

Table 3 Cognitive performance of GRAS patients. For comparison, normative data are presented wherever available2.

	men			women			ANCOVA		total			normative data (PR) or mean sample values of healthy controls		
	N	mean (sd)	median	N	mean (sd)	median	F	p	N	mean (sd)	median	N	PR (Percentile Rank)	mean (sd)
reasoning (LPS)	663	21.26 (6.70)	22.00	324	18.79 (6.31)	18.00	17.62	< .001*	987	20.45 (6.67)	21.00	1556[a]	31	-
working memory (BZT)	627	13.24 (3.79)	14.00	312	12.62 (3.91)	13.00	1.20	.274	939	13.03 (3.84)	13.00	30[b]	-	15.70 (2.6)
executive functions (TMT-B)°	631	131.42 (104.21)	99.00	307	147.65 (121.09)	108.00	0.00	.956	938	136.73 (110.22)	100.00	24[c]	10	71.5 (31.07)
verbal memory[1)] (VLMT)	602	41.15 (12.63)	41.00	302	42.68 (13.02)	42.00	12.38	< .001*	904	41.66 (12.78)	42.00	89[d]	10	52.39 (7.87)
premorbid IQ[1)](MWT-B)	613	25.96 (6.22)	27.00	311	26.21 (6.13)	27.00	0.69	.405	924	26.04 (6.19)	27.00	1952[e]	43.5	-
divided attention (TAP)°														
reaction time	651	759.67 (114.25)	743.43	308	805.16 (150.99)	780.04	14.07	< .001*	959	774.28 (128.89)	755.05	200[f]	8	-
lapses		3.35 (7.15)	1.00		6.41 (13.18)	2.00	22.12	< .001*		4.33 (9.62)	1.00			
processing speed														
trail making test A (TMT-A)°	676	49.18 (35.22)	40.00	332	55.32 (42.22)	43.00	0.17	.683	1008	51.20 (37.76)	41.00	24[c]	< 5	33.04 (7.89)
digit-symbol test (ZST)	674	37.46 (12.58)	37.00	329	38.58 (14.14)	39.00	19.24	< .001*	1003	37.83 (13.12)	38.00	200[g]	16	-
basic cognition/fine motor function														
alertness (TAP)°														
reaction time	665	319.62 (116.13)	284.08	326	379.11 (161.80)	328.04	28.30	< .001*	991	339.19 (135.73)	298.41	200[f]	10	-
lapses		0.52 (2.04)	0.00		1.18 (3.57)	0.00	10.39	.001*		0.73 (2.66)	0.00			
dotting	673	46.10 (13.08)	46.00	320	45.36 (14.96)	46.00	1.62	.203	993	45.86 (13.71)	46.00	103[h]	-	63.24 (11.03)
tapping	671	29.01 (8.57)	29.00	319	27.58 (9.00)	27.00	0.76	.783	990	28.55 (8.73)	28.00	103[h]	-	37.63 (7.04)

° Higher scores reflect better performance, except for TMT-A, TMT-B, Alertness and Divided Attention (TAP)

* For statistical comparison (ANCOVA) between men and women values are corrected for age, duration of disease, chlorpromazine equivalents and years of education (except MWT-B).

[1)] Non-native and non-bilingual German speaking patients are excluded (n = 89).

[2)] Percentile ranks (PR) < 15 indicate that the mean or the median of the total sample is below average in comparison to a normative sample.

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32.4% of variance in the total sample. In fact, duration of disease, negative symptoms, catatonic signs, and medication (chlorpromazine equivalents) contributed significantly to basic cognition/fine motor function, whereas positive symptoms did not (β = -.006, p = .856). According to the standardized regression coefficients, duration of disease and negative symptoms are the best predictors of basic cognition/fine motor function (β = -.346, p < .001 and β = -.334, p < .001). For higher cognitive functions, the set of disease-related variables explained 33% of variance in the total sample. Again, duration of disease and negative symptoms are the best predictors of higher cognitive functions (β = -.335, p < .001 and β = -.351, p < .001). Positive symptoms did not

Table 4 Self-reported medication side effects of patients (N = 423)* according to treatment type

	FGA[1]		SGA[2]	
	men	women	men	women
Parkinson symptoms	17%	15.6%	3.8%	11.6%
dyskinetic/dystonic symptoms	35.8%	31.3%	9.4%	9.7%
akathisia	22.6%	12.5%	6%	6.8%
hyperprolactinaemia	-	-	-	1.9%
hormonal dysfunctions (gynecomastia, absence/changes of menorrhea)	-	9.4%	-	5.8%
sexual dysfunction	7.5%	-	10.3%	-
vertigo (incl. hypotonia)	5.7%	12.5%	5.1%	8.7%
weight gain	9.4%	18.7%	38.3%	39.8%
diabetes mellitus	-	-	0.4%	-
sialorrhea ('drooling')	-	-	20.4%	6.8%
skin abnormalities, loss of hair	1.9%	-	1.7%	5.8%
gastrointestinal symptoms	1.9%	6.3%	5.9%	7.8%
hyperhidrosis	-	-	2.6%	-
psychological symptoms (loss of concentration, no drive, tiredness)	33.9%	28.1%	44.2%	31.1%
cardiovascular symptoms (tachycardia, hypertension)	-	-	1.3%	1.9%
impaired vision	-	-	1.7%	3.9%
dry mouth	5.7%	9.4%	5.1%	4.9%
urinary retention	-	3.1%	1.3%	-
number of patients who reported side effects	53	32	235	103

[1]FGA - first generation antipsychotics, typical antipsychotics

[2]SGA - second generation antipsychotics, atypical antipsychotics

*Only N = 423 patients (out of 1012 patients who were on antipsychotic medication) reported side effects (see text for details).

reach significance (β = - .015, p = .658). With respect to global functioning, all chosen disease-related factors accounted for 59.6% of variance in the total sample. Only duration of disease *per se* did not reach significance (β = -.028, p = .198). Positive and negative symptoms were the strongest predictors of global functioning (β = - .441, p < .001 and β = -.380, p < .001).

Discussion

The present paper provides an overview of the GRAS data collection, including (1) study logistics and procedures, (2) sample description regarding sociodemographic data, disease-related variables, cognitive performance and neurological symptoms, paying particular attention to gender differences, and (3) a first presentation of intercorrelation patterns for selected areas of interest to phenotype studies. (4) In addition, disease-related factors influencing important criteria of daily functioning are evaluated in the >1000 GRAS patients. Overall, the GRAS sample represents a typical schizophrenic population in contact with the health system and is - last not least due to its homogeneous data acquisition - ideally suited for the ongoing and planned phenotype-based genetic association studies (PGAS) (e.g. [[11], and Grube et al: Calcium-activated potassium channels as regulators of cognitive performance in schizophrenia, submitted]).

The GRAS data collection has several remarkable advantages, two of which are of major importance for its ultimate goal, PGAS: (i) Different from other studies dealing with the establishment of a schizophrenia data base, *all* data for GRAS were collected by one and the same traveling team of examiners, who frequently performed calibrating sessions and rater trainings. This effort has clearly paid off in terms of reliability and quality of the data, considering the internal consistencies of the GRAS phenotypes, as exemplified in the displayed correlation patterns. (ii) Even though the GRAS study has been implemented as a cross-sectional investigation, the GRAS data collection also includes solid longitudinal information derived from the almost complete psychiatric chart records/discharge letters of all schizophrenic patients. This longitudinal set of data has been essential to e.g. reliably estimate prodrome versus disease onset, i. e. occurrence of the first psychotic episode.

Comparable to other schizophrenia samples, the GRAS sample comprises around two thirds of male and one third of female patients [17,58]. Assuming that the gender ratio in schizophrenia were 1:1 as claimed in text books, but recently also questioned [59,60], then two principal reasons may account for the gender distribution observed here: (1) Schizophrenic women generally seem to have less contact with the health system due to being better socially settled (later age of onset of

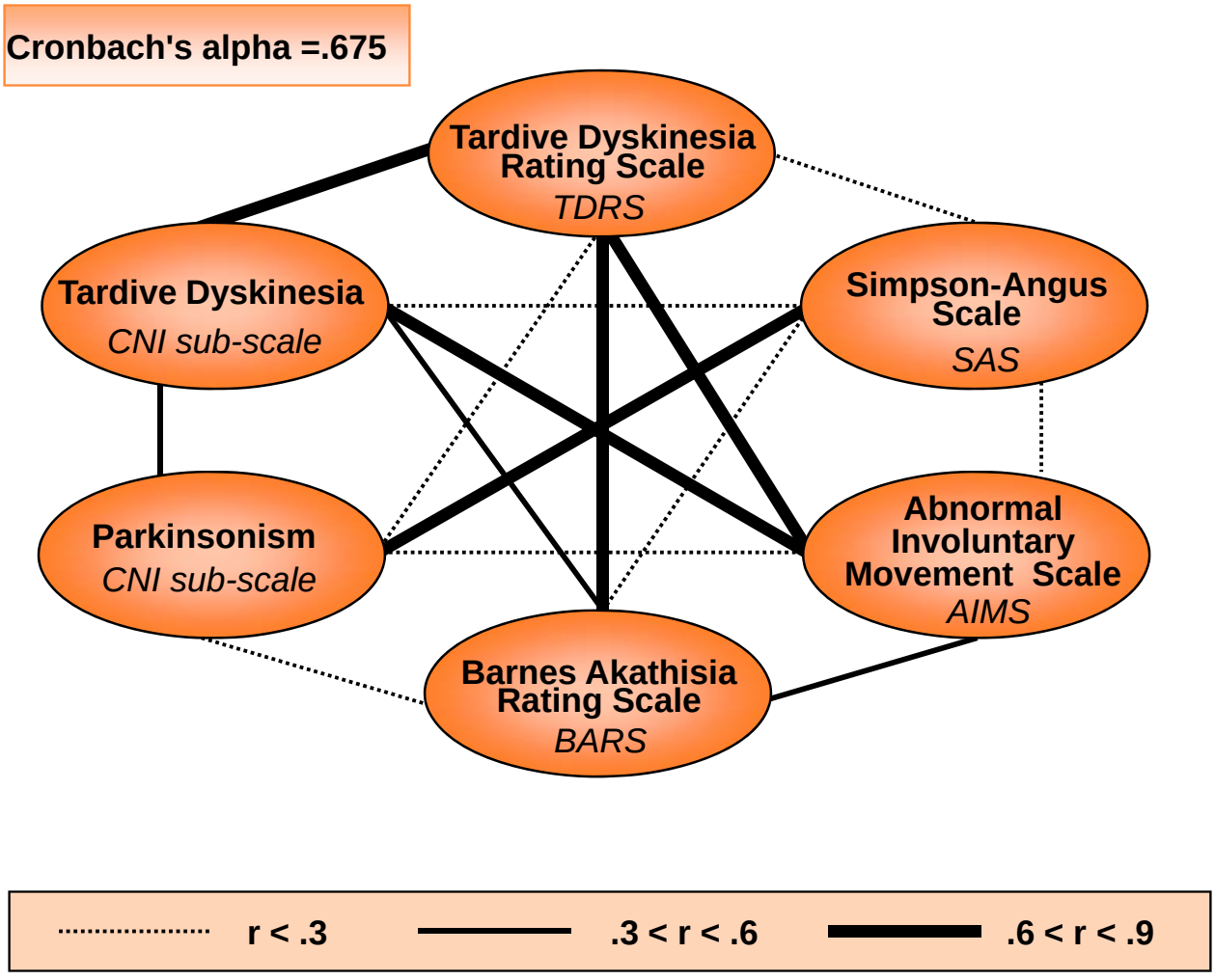

Figure 8 Extrapyramidal intercorrelation pattern. Shown are correlations between different neurological tests for measuring extrapyramidal symptoms. Thickness of the lines represents the strength of correlation between two tests; only significant correlations are displayed. Cronbach's alpha of .675 shows that these measures have a decent internal consistency.

disease) and protected within their families [61]; (2) A certain (smaller) recruitment bias may be explained by the fact that the traveling team of examiners visited some institutions with an overrepresentation of males, e. g. specialized forensic units or a hospital for psychotic patients with co-morbid substance use disorders.

With the purposeful strategy to visit several different facilities of psychiatric health care covering inpatients, outpatients, residents of sheltered homes and forensic patients, the GRAS approach tried to avoid biases inherent to pure inpatient samples [58]. Nevertheless, patients who are not in contact with the health care system are unlikely to be integrated in any comparable data bases. For instance, only 4 of the 1085 examined patients are currently homeless, whereas among homeless people a considerable proportion suffers from schizophrenia [62]. To reach them as well, different and more cost intensive recruitment strategies would be required [13]. On the other hand, the schizophrenic phenotype required for the GRAS-PGAS studies pursued here, might be veiled in this severely affected subsample of patients that is additionally characterized by other specific problems, e. g. a highly elevated incidence of multiple substance use disorders and severe downstream medical comorbidities [63,64].

Gender differences in schizophrenia as obvious from the present data collection have been known for a long time [65]. In agreement with the literature, men and women in the GRAS sample differ by diagnosis, with women having a higher rate of schizoaffective disorders [66,67]. With respect to age of onset, education, indicators of social integration (e.g. marital status, living situation) and medication, the present results are also in perfect agreement with previous findings: Male patients

Table 5 Cambridge Neurological Inventory (CNI)a subscale sum scores (N = 893-942)

sub scales	total		men		women		statistics	
	Mean (sd)	Median (range)	Mean (sd)	Median (range)	Mean (sd)	Median (range)	Z	p
Hard neurological signs								
plantar reflexes (le/ri*), power in upper and lower limb (le/ri), and reflexes (hyper- and hyporeflexia) in upper and lower limb (le/ri)	1.12 (1.70)	0.0 (0 - 10)	1.07 (1.66)	0.0 (0-8)	1.22 (1.78)	0.0 (0-10)	-1.467	n.s
Motor coordination								
finger-nose test (le/ri), finger-thumb tapping (le/ri), finger-thumb opposition (le/ri), pronation-supination (le/ri); fist-edge-palm test (le/ri), Oseretsky test	4.11 (4.27)	3.0 (0-20)	3.95 (4.17)	2.0 (0-20)	4.44 (4.45)	3.0 (0-20)	-1.629	n.s
Sensory integration								
extinction, finger agnosia (le/ri), stereoagnosia (le/ri), agraphesthesia (le/ri), left-right disorientation	3.66 (3.32)	3.0 (0-15)	3.63 (3.32)	3.0 (0-15)	3.73 (3.31)	3.0 (0-14)	-0.521	n.s
Primitive reflexes								
snout reflex, grasp reflex, palmo-mental reflex (le/ri)	0.84 (1.14)	0.0 (0-5)	0.80 (1.11)	0.0 (0-5)	0.91 (1.19)	0.0 (0-5)	-1.363	n.s
Tardive dyskinesia								
dyskinetic, sustained or manneristic face and head movement, simple or complex abnormal posture, dyskinetic, dystonic or manneristic trunk/limb movement	0.55 (1.17)	0.0 (0-9)	0.58 (1.25)	0.0 (0-9)	0.49 (0.98)	0.0 (0-7)	-0.132	n.s
Catatonic signs								
gait mannerism, gegenhalten, mitgehen, imposed posture, exaggerated or iterative movement, automatic obedience, echopraxia	0.43 (0.96)	0.0 (0-8)	0.45 (0.98)	0.0 (0-8)	0.38 (0.91)	0.0 (0-7)	-1.717	n.s
Parkinsonism								
increased tone in upper and lower limb (le/ri), decreased associated movements in walking, shuffling gait, arm dropping, tremor postural or resting, rigidity in neck	1.76 (2.90)	0.0 (0-15)	1.70 (2.85)	0.0 (0-15)	1.89 (3.02)	0.5 (0-15)	-1.172	n.s
Failure to suppress inappropriate response								
blinking or head movement in saccadic eye movement, winking with one eye	1.23 (1.49)	1.0 (0-6)	1.12 (1.42)	1.0 (0-6)	1.48 (1.62)	1.0 (0-6)	-3.175	.001*

*le/ri - left and right

[a]Chen EY, Shapleske J, Luque R, McKenna PJ, Hodges JR, Calloway SP, Hymas NF, Dening TR, Berrios GE: The Cambridge Neurological Inventory: a clinical instrument for assessment of soft neurological signs in psychiatric patients. Psychiatry Res 1995, 56(2):183-204.

are younger when the first psychotic episode occurs, are more frequently single, more often dependent on supported living conditions (e.g. residential homes) and show lower educational status [61,67,68]. Among the explanations for the observed gender differences in schizophrenia are the protective role of female hormones [69] and social aspects like earlier marriage of young women leading to a more protected environment at disease onset [13]. In line with these considerations is the work of Häfner and colleagues [12]. In a prospective design he could show that 'the social course (of schizophrenia) is determined by individual stage at illness onset and by early illness course' [70].

With respect to psychopathology and premorbid functioning, the GRAS sample may be slightly different from other schizophrenia samples reported in the literature [67]. Several studies published in this area show that men exhibit more negative symptoms, even in a geriatric sample [71,72], and that females have poorer premorbid cognitive functioning than males [73]. In the GRAS patients, there are no gender differences regarding psychopathology and premorbid cognition. Importantly, clear support for a comparable severity of psychopathology in men and women of the GRAS sample is provided by the lack of gender differences in numbers of hospitalizations, clinical severity ratings, including global functioning (CGI, GAF [2,31]), and self-ratings of symptom severity and anxiety. One potential explanation for the discrepancies between the GRAS sample and other studies regarding psychopathology may be that patient numbers in some of the other studies have been too low to give conclusive results. In the assessment of premorbid cognitive functioning of the GRAS sample, a methodological limitation could be the retrospective determination of premorbid intelligence using a so-called 'hold' measure, i.e. a multiple choice vocabulary test [35]. Even though this is an accepted and valid instrument to retrospectively estimate premorbid intelligence [74], a prospective procedure might be more accurate. In fact, Weiser and colleagues had the opportunity to base their assessments on cognitive testing performed on adolescents before starting their military service [73], potentially explaining the deviating results.

Gender differences regarding current cognitive performance are similar within the GRAS sample (even though

Table 6 Multiple regression analyses predicting a) basic cognition/fine motor functions, b) cognitive performance, c) global functioning

	total			male			female		
	β	t	p	β	t	p	β	t	p
a) basic cognition/fine motor functions[1]									
duration of disease (*years*)	-.346	-11.92	< .001	-.353	-9.68	< .001	-.318	-6.59	< .001
positive symptoms (*PANSS*)	-.006	-0.18	.856	-.028	-0.69	.489	.065	1.08	.283
negative symptoms (*PANSS*)	-.334	-10.05	< .001	-.293	-7.32	< .001	-.415	-7.01	< .001
catatonic signs (*CNI*)	-.126	-4.26	< .001	-.128	-3.45	.001	-.161	-3.27	.001
medication (*CPZ-equivalents*)	-.080	-2.70	.007	-.066	-1.83	.068	-.147	-2.84	.005
regression model		*r^2 = .324 p < .001*			*r^2 = .306 p < .001*			*r^2 = .383 p < .001*	
b) cognitive performance[2]									
duration of disease (*years*)	-.335	-11.54	< .001	-.356	-9.72	< .001	-.294	-6.12	< .001
positive symptoms (*PANSS*)	-.015	-0.44	.658	-.033	-0.80	.427	.023	0.38	.704
negative symptoms (*PANSS*)	-.351	-10.47	< .001	-.320	-7.92	< .001	-.396	-6.56	< .001
catatonic signs (*CNI*)	-.132	-4.46	< .001	-.103	-2.76	.006	-.204	-4.16	< .001
medication (*CPZ-equivalents*)	-.082	-2.74	.006	-.060	-1.62	.105	-.140	-2.70	.007
regression model		*r^2 = .330 p < .001*			*r^2 = .305 p < .001*			*r^2 = .394 p < .001*	
c) global functioning[3]									
duration of disease (*years*)	-.028	-1.29	.198	-.008	-0.28	.780	-.062	-1.78	.076
positive symptoms (*PANSS*)	-.441	-17.33	< .001	-.458	-14.45	< .001	-.415	-9.60	< .001
negative symptoms (*PANSS*)	-.380	-15.02	< .001	-.345	-10.97	< .001	-.430	-10.0	< .001
catatonic signs (*CNI*)	-.060	-2.67	.008	-.050	-1.71	.088	-.093	-2.58	.011
medication (*CPZ-equivalents*)	-.106	-4.71	< .001	-.122	-4.29	< .001	-.078	-2.07	.040
regression model		*r^2 = .596 p < .001*			*r^2 = .559 p < .001*			*r^2 = .662 p < .001*	

[1]A basic cognition/fine motor composite score was used as a dependent variable comprising alertness (TAP), tapping, and dotting (Chronbachs alpha = .801).

[2]A cognitive composite score was used as a dependent variable consisting of reasoning (LPS3), 2 processing speed measures (TMT -A and digit-symbol test, ZST), executive functions (TMT-B), working memory (BZT), verbal memory (VLMT) and divided attention (TAP) (Chronbachs alpha = .869).

[3]Global assessment of functioning (GAF) was used as a dependent variable.

at a lower functioning level [75]) compared to healthy controls [76] after considering age of onset, duration of disease, education and medication as covariates. Men perform better in reasoning, alertness and divided attention but worse in verbal memory, confirming reports on first-episode as well as chronically ill schizophrenic patients [77].

Women in the GRAS study receive significantly lower doses of chlorpromazine equivalents, confirming that they require less medication to achieve a reasonable treatment effect [78]. Importantly, regarding medication side effects, there were no gender differences in extrapyramidal symptoms. There were also no differences in the overall proportion of men and women who self-reported side effects, but the pattern of complaints was slightly different. For instance, women mentioned more often hormonal dysfunction and vertigo (or related symptoms like hypotonia), whilst men complained mainly about sexual dysfunction. Altogether, it is worth pointing out that the percentage of patients self-reporting side effects is low when compared to that with objectively measured side effects, e.g. extrapyramidal symptoms (11.3% versus 32.3%).

Explicit studies on gender differences in antipsychotic medication side effects found a somewhat different distribution of complaints, e.g. more weight gain, diabetes and specific cardiovascular diseases in females [78,79]. Here, one reason is certainly the still preliminary data set of the GRAS collection evaluated, based at this point exclusively on cross-sectional patient reports. For a more appropriate coverage of medication side effects, all charts/discharge letters of every GRAS patient (also of those patients who did/could not report them), will have to be screened and entered into the data base. Comprehensive information on antipsychotic (and other) drugs and their side effects in the GRAS sample has been collected and is waiting for analyses to support e.g. future pharmacogenomic approaches, perhaps also in collaboration with industry partners.

In line with the findings of a recent meta-analysis [80], positive symptoms of the GRAS patients do not influence higher cognitive function or basic cognition/fine

motor performance, whilst negative symptoms, catatonic signs, duration of disease and antipsychotic medication have a significant effect on both. The clinical ratings of global functioning, however, strongly rely on positive as well as negative symptoms, medication and catatonic signs [81-83].

Conclusion

GRAS enables a novel phenotype-based approach to understand the molecular-genetic architecture of schizophrenia. The GRAS data collection encompasses a large sample of comprehensively phenotyped, moderately to severely affected schizophrenic patients. Proof-of-principle for the suitability of the GRAS data collection for PGAS has already been demonstrated [[11], and Grube et al: Calcium-activated potassium channels as regulators of cognitive performance in schizophrenia, submitted]. Further extensive analyses of the accumulated information on every single patient are ongoing.

Abbreviations

GRAS: Göttingen Research Association for Schizophrenia; GWAS: genome-wide association study; PGAS: phenotype-based genetic association study; CATIE: clinical antipsychotic trials of intervention effectiveness; CNI: Cambridge Neurological Inventory; ASRB: Australian Schizophrenia Research Bank; FGA: first generation antipsychotics; SGA: second generation antipsychotics; CPZ: chlorpromazine.

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Authors' contributions

MB coordinated and supervised the traveling team of investigators and had a considerable impact on design and establishment of the data collection. KR and HFr were part of the traveling team of investigators, conducted statistical analyses of the clinical data, assisted in manuscript writing, and supervised data entry, substantially performed by SG, SP, AK, MFG, VA, ATa, ATr, and MF. Of the collaborating centers, LA, JBA, MBE, TB, AC, MD, HFo, RF, RG, SH, DH, GK, HK, MFr, FL, WM, AM, RMI, CO, FGP, TP, US, HJS and UHR enabled the work of the traveling team of examiners, by pre-selecting and preparing patients and organizing respective facilities and working conditions. HE, KAN, NB, PF, WS, and JF developed the concept of GRAS (Göttingen Research Association for Schizophrenia, founded in 2004), and guided the project, data analysis, and paper writing, hereby supported by BKH. All authors discussed the results, commented on the paper draft and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Circulating Damage Marker Profiles Support a Neuroprotective Effect of Erythropoietin in Ischemic Stroke Patients

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The German Multicenter EPO Stroke Trial, which investigated safety and efficacy of erythropoietin (EPO) treatment in ischemic stroke, was formally declared a negative study. Exploratory subgroup analysis, however, revealed that patients not receiving thrombolysis most likely benefited from EPO during clinical recovery, a result demonstrated in the findings of the Göttingen EPO Stroke Study. The present work investigated whether the positive signal on clinical outcome in this patient subgroup was mirrored by respective poststroke biomarker profiles. All patients of the German Multicenter EPO Stroke Trial nonqualifying for thrombolysis were included if they (a) were treated per protocol and (b) had at least two of the five follow-up blood samples for circulating damage markers drawn (n = 163). The glial markers S100B and glial fibrillary acid protein (GFAP) and the neuronal marker ubiquitin C-terminal hydrolase (UCH-L1) were measured by enzyme-linked immunosorbent assay in serum on d 1, 2, 3, 4 and 7 poststroke. All biomarkers increased poststroke. Overall, EPO-treated patients had significantly lower concentrations (area under the curve) over 7 d of observation, as reflected by the composite score of all three markers (Cronbach $\alpha = 0.811$) and by UCH-L1. S100B and GFAP showed a similar tendency. To conclude, serum biomarker profiles, as an outcome measure of brain damage, corroborate an advantageous effect of EPO in ischemic stroke. In particular, reduction in the neuronal damage marker UCH-L1 may reflect neuroprotection by EPO.

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INTRODUCTION

Since 1998, nearly 200 preclinical studies have proven erythropoietin (EPO) to have neuroprotective and neuroregenerative potential, ranging from ischemia and neurotrauma to inflammation and neurodegeneration. Essentially all of the few clinical studies performed in the neuroscience field have yielded positive results with respect to EPO treatment effects (reviewed in [1,2]). The first encouraging clinical study was the Göttingen EPO Stroke Study, which showed beneficial outcome of ischemic stroke patients upon EPO (3).

Unfortunately, the respective follow-up study, the German Multicenter EPO Stroke Trial (ClinicalTrials.gov identifier NCT00604630), building on these positive results, turned out to be a formally negative trial, because of the unexpectedly large percentage of recombinant tissue plasminogen activator (rtPA) treatments with a high violation rate of thrombolysis contraindications (4). Patients with rtPA application despite prior anticoagulation had inferior outcome under additional EPO, whereas patients treated with rtPA "*lege artis*" ("correctly"; "by law of art") did not have any disadvantage of EPO treatment (www.epo-study.de) (4). Potential mechanisms explaining a negative interplay between EPO and rtPA were recently reported in preclinical work (5,6). In contrast, patients who did not receive rtPA likely benefitted from EPO with a clinical course/outcome (National Institutes of Health Stroke Scale [NIHSS]) comparable to that obtained in the first EPO stroke study (3,4). In the absence of any other neuroprotective or neurore-

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Table 1. Patient characteristics on inclusion.

Variable	Patients included in subgroup analysis (n = 163)		
	EPO (n = 76)	Placebo (n = 87)	*P*
Age (years) (mean ± SD)	71.14 ± 11.45	71.37 ± 10.78	1.00[a]
Sex (M/F ratio) (%)	40/36 (52.6/47.4)	44/43 (50.6/49.4)	0.875
Number of deaths (%)	6 (7.9)	8 (9.2)	1.00
Hemisphere (n (%))			
Left	35 (46.1)	40 (46.0)	0.879
Right	41 (53.9)	46 (52.9)	
Both	0 (0.0)	1 (1.1)	
Stroke subtype (n (%))			
Cardiogenic embolism	34 (44.7)	42 (48.3)	0.985
Arterial embolism	18 (23.7)	17 (19.5)	
Large artery occlusion	13 (17.1)	14 (16.1)	
Paradox embolism	2 (2.6)	2 (2.3)	
Lacunar infarction	5 (6.6)	6 (6.9)	
Unknown	4 (5.3)	6 (6.9)	
Prior anticoagulation (n (%))			
No	42 (55.3)	45 (51.7)	0.601
Yes	34 (44.7)	41 (47.1)	
Unknown	0 (0.0)	1 (1.1)	
Hypertension (n (%))			
No	18 (23.7)	22 (25.3)	0.246
Yes	58 (76.3)	62 (71.3)	
Subclinical/borderline	0 (0.0)	3 (3.4)	
Unknown	0 (0.0)	0 (0.0)	
Diabetes (n (%))			
No	58 (76.3)	60 (69.0)	0.503
Yes	15 (19.8)	24 (27.6)	
Subclinical/borderline	3 (3.9)	3 (3.4)	
Unknown	0 (0.0)	0 (0.0)	
NIHSS			
Mean ± SD (range)	12.08 ± 5.90 (4–27)	11.47 ± 5.52 (4–27)	0.539[a]
MRI diffusion-weighted imaging (cm^3)			
Mean ± SD (range)	34.84 ± 44.11 (0–186)	42.39 ± 65.52 (0.2–298)	0.688[a]
MRI FLAIR (cm^3)			
Mean ± SD (range)	4.06 ± 12.10 (0–77)	2.26 ± 4.9 (0–23)	0.895[a]
Time to treatment (min)			
Mean ± SD (range)	275.32 ± 79.10 (42–442)	278.98 ± 65.85 (78–485)	0.977*

[a]*P* values from group comparison by Mann-Whitney *U* test. All other *P* values obtained from the two-sided χ^2 test or Fisher exact test.

generative strategy available for stroke patients, this promising signal encourages further work along these lines.

Circulating biomarkers of brain damage are increasingly considered as additional outcome measures for stroke complementing clinical and imaging data. Among the markers selected for the present analysis, the glial damage markers S100B and glial fibrillary acidic protein (GFAP) have been in clinical use for many years, whereas the neuronal marker ubiquitin C-terminal hydrolase (UCH-L1) has been integrated recently in the repertoire of stroke biomarkers. All these damage markers correlate well with clinical severity, course and outcome of brain injury (7,8).

UCH-L1 is a highly abundant protein that resides in almost all neurons and averages between 1% and 5% of total soluble brain proteins. It has been suggested that UCH-L1 plays a critical role in the removal of excessively oxidized or misfolded proteins both during normal and neuropathological conditions (9–12). On the basis of this important neuronal function and its high specificity and abundance in the central nervous system, we selected UCH-L1 here as a candidate biomarker for poststroke brain injury and readout of neuroprotection.

S100B is a low-molecular-weight glial protein of a multigenic family of calcium-binding proteins, highly specific to the nervous system and found in abundance in the astroglia compartment in the cerebral cortex, in peripheral Schwann cells, but also extra-neuronally in melanocytes, adipocytes and chondrocytes (13). In previous studies, we could show that S100B release was associated with stroke severity and clinical outcome (14). S100B was also postulated to be a marker of generalized blood-brain barrier dysfunction, rather than of specific glia damage only (15).

GFAP is a monomeric filament protein localized to astrocytes in the brain. GFAP is involved in various neuronal processes, including maintenance of the blood-brain barrier (reviewed in [16]). Increased serum concentrations of GFAP were described after ischemic stroke and traumatic brain injury and to correlate with clinical severity and outcome (17,18).

About 10 years ago, we argued that molecular markers of brain damage might be a useful tool in translational stroke research and that the analysis of the release patterns of biomarkers might be a promising strategy to evaluate neuroprotective approaches in stroke treatment (19). Here we report poststroke biomarker profiles of an exploratory subgroup comprising per-protocol treated ischemic stroke patients of the German Multicenter EPO Stroke Trial who did not receive rtPA.

MATERIALS AND METHODS

Patients

The present predefined exploratory subgroup analysis is based on all pa-

tients of the randomized, double-blind, placebo-controlled German Multicenter EPO Stroke Trial (4) who fulfilled the following requirements: (a) they were treated per-protocol, (b) they had not received rtPA, and (c) they had at least two out of five follow-up blood samples for circulating damage markers drawn, resulting in a total of n = 163 patients (exclusion of n = 3 due to missing serum samples). Main inclusion criteria were acute ischemic stroke in the middle cerebral artery territory leading to a score ≥4 in the NIHSS.

Study Intervention

Intravenous infusion of recombinant human EPO (Epoetin-α, 40,000 IU) or placebo was given within 6 h after symptom onset (day 1) and repeated 24 and 48 h later (4). The dose was chosen according to the previous EPO study (3).

Biomarker Assays

Blood for biomarker analysis was drawn on d 1, 2, 3, 4 and 7. Serum was stored at –80°C. Enzyme-linked immunosorbent assays of S100B, GFAP and UCH-L1 were performed blindly (that is, without clinical information) using antibodies from Banyan (Alachua, FL, USA), Sigma (St. Louis, MO, USA) and Dako (Carpentaria, CA, USA).

Statistical Analysis

For each marker, a linear regression–based multiple imputation (10 iterations) model of missing data (UCH-L1 5.8%; S100B 6.5%; GFAP 20.6% missing) was applied, if at least two out of five values per subject were present (resulting in n = 163 subjects for UCH-L1 and S100B and n = 154 for GFAP). All per-protocol treated non-rtPA individuals not meeting this criterion were excluded from further analysis (UCH-L1 and S100B n = 3; GFAP n = 12). Areas under the curve (AUCs) for every marker were determined for each imputation matrix by the composite trapezoidal rule for numerical integration. The pooled AUC represents the mean of the 10 AUC matrices per marker. Two composite scores were calculated reflecting the mean of the *z*-standardized pooled AUC values for UCH-L1, S100B and GFAP (Cronbach α = 0.811) and for S100B and GFAP (Cronbach α = 0.755). For a total of n = 9 individuals, the composite scores had to be based on the *z*-standardized pooled AUC values for UCH-L1 and S100B only. Mann-Whitney *U* tests (two-tailed) and $\chi 2$ tests or Fisher exact test were used for intergroup comparisons. Analysis of variance for repeated measures was applied to compare EPO versus placebo with respect to Δ NIHSS

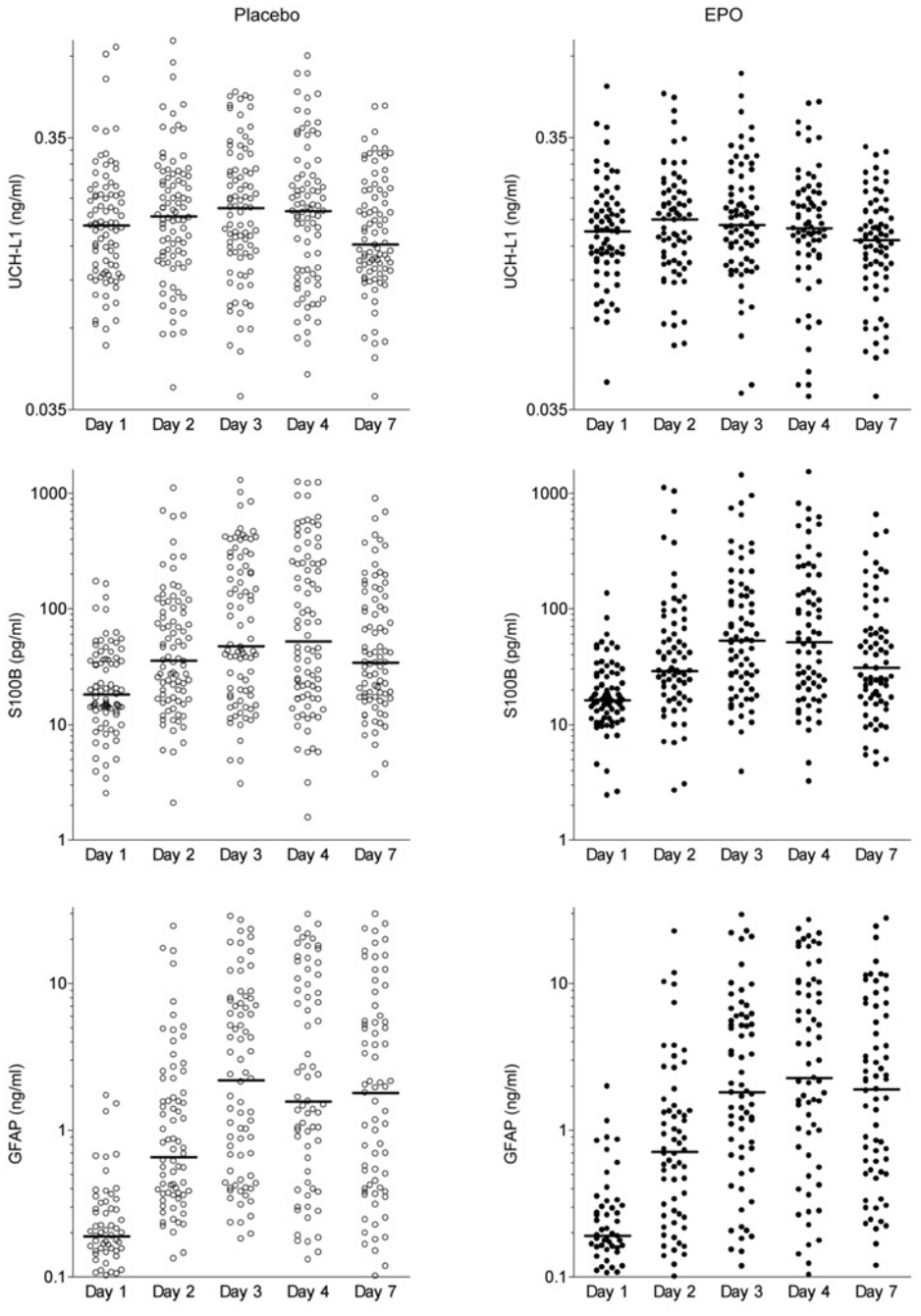

Figure 1. Course of circulating brain damage markers after ischemic stroke. All three biomarkers measured in serum over time increase after stroke (placebo white, left panels; EPO black, right panels). Note the logarithmic scale of presentations. Medians are given.

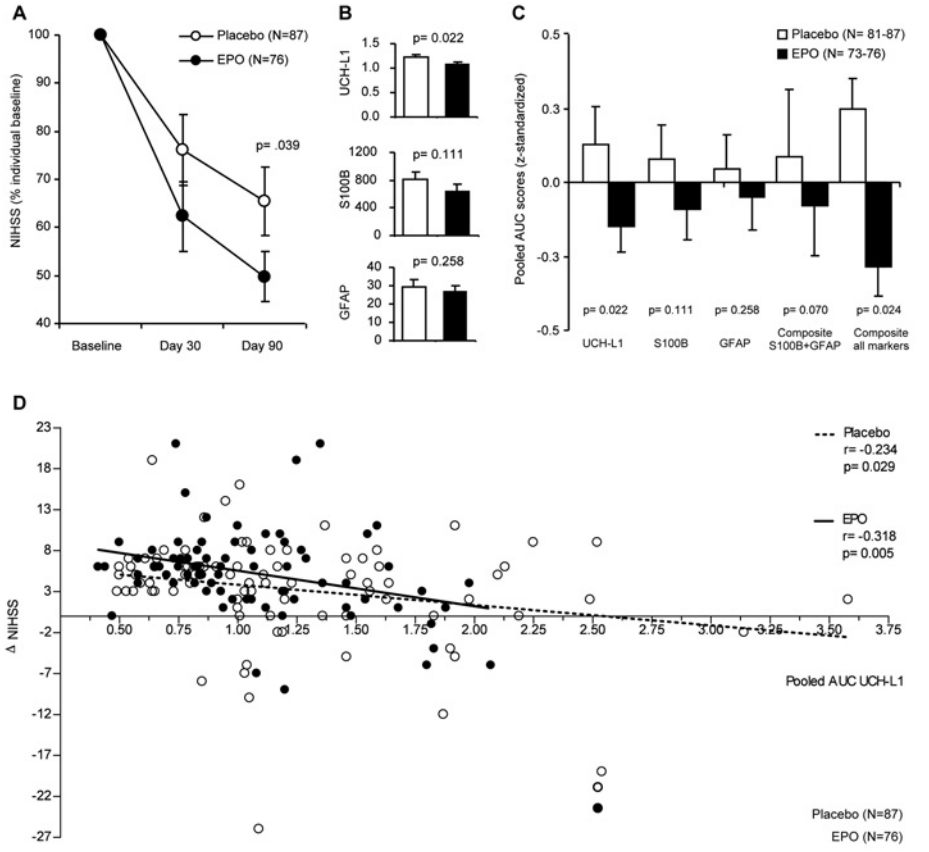

Figure 2. Biomarkers substantiate the positive signal on clinical outcome of EPO compared with placebo patients. (A) EPO patients show improved clinical outcome (NIHSS) after stroke compared with the placebo group. AUC mean ± SEM values (B) and AUC *z*-standardized values (C) demonstrate differences in biomarkers poststroke between EPO and placebo patients. (D) Δ NIHSS and UCH-L1 AUC correlate in both treatment groups with a numerically higher correlation coefficient in EPO patients.

(NIHSS at baseline – NIHSS day 90). Analysis of covariance with NIHSS score at baseline as the covariate compared both groups with respect to pooled single-marker AUC values and AUC composite scores. Further, a correlation analysis (Pearson) of Δ NIHSS and UCH-L1 AUC was performed. Data are presented as mean ± SD in text/tables and median or mean ± SEM in figures.

RESULTS

Patient characteristics at inclusion were well balanced between EPO and placebo groups in all important baseline variables, representative of a typical stroke population (Table 1). Biomarker profiles in serum displayed the expected increases between d 2 and 4 poststroke, with peak time points varying considerably among different markers and individual patients (Figure 1).

The clinical course of included per-protocol treated non-rtPA patients (n = 163) demonstrates a slightly better outcome of the EPO compared with the placebo group (mean Δ NIHSS of 5.3 ± 5.3 in EPO versus 3.3 ± 6.5 in placebo; P = 0.039) (Figure 2A). As a best estimate of the total increase in circulating damage marker concentrations, AUCs were calculated for each marker in all patients. AUCs, corrected for NIHSS d 1 (severity of stroke symptoms upon inclusion, that is, before any study drug treatment), turned out to be significantly lower in EPO versus placebo patients for UCH-L1 and showed a similar tendency for S100B and GFAP (Figure 2B).

To make use of the complete biomarker information, a *z*-standardization of the AUC scores for each marker was performed. AUC composite scores of the two glial markers and of all three biomarkers were calculated. The internal consistency of these composite scores turned out to be sufficiently high (n = 154; Cronbach $\alpha = 0.755$; and n = 154; Cronbach $\alpha = 0.811$, respectively) to justify their use as composites. Figure 2C illustrates *z*-standardized biomarker AUC levels for all single markers and the two composites showing that all three biomarkers discriminate between EPO and placebo groups, with UCH-L1 as a single marker and the three-marker composite score reaching statistical significance. Correlation coefficients of Δ NIHSS and UCH-L1 AUC were found to be significant for both treatment groups, with a numerically higher value in EPO patients (Figure 2D). This result again emphasizes the neuroprotective property of EPO in stroke.

DISCUSSION

The present exploratory subgroup study builds on the observation that stroke patients of the German Multicenter EPO Stroke Trial, nonqualifying for rtPA treatment, seemed to have a better clinical course/outcome under EPO than placebo (4). This observation is further supported here by respective stroke biomarker profiles. The blunted increase in the neuronal damage marker UCH-L1 under EPO especially points to neuroprotection. The results obtained with this marker may actually suggest its extended use as a surrogate marker of stroke severity in future neuroprotection trials.

Similar to the first EPO stroke study (3), the S100B increase tended to be lower in EPO patients but failed to reach statistical significance here. The third evaluated marker, GFAP, turned out to be the least responsive one in the present analysis, perhaps due to the necessity (insufficient sample volume left) to im-

pute circa 20% of missing data (as compared with only around 6% for S100B and UCH-L1). The composite score of both glial markers, S100B and GFAP, produced a "near-significant" result. The composite of all three markers, although different between treatment groups, does at first look not add to the information obtainable with the neuronal marker UCH-L1 alone. However, both composite scores reveal that all contributing markers, be it of glial or neuronal origin, essentially behave synergistically.

In conclusion, stroke is a very common, devastating and frequently severely disabling condition with only thrombolysis and supportive measures presently available for treatment. The former still reaches just a small percentage of patients, and the increasing violation of rtPA contraindications (as also experienced in the German Multicenter EPO Stroke Trial) reflects desperation and fatalism of treating personnel in the absence of alternative therapeutic options. Importantly, stroke patients are extremely heterogeneous with respect to genetic and environmental predisposing factors, including comorbidities, explaining why huge effects of novel treatment strategies can never be expected over all patients. Therefore, even the slightest signal of benefit of neuroprotective treatment strategies has to be vigorously pursued. In this regard, the course of circulating brain damage markers upon EPO (in association with the documented clinical improvement) should encourage further work on EPO or EPO variants/analogs in ischemic stroke patients who are not eligible for thrombolysis.

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DISCLOSURE

H Ehrenreich holds/has submitted patents on EPO for stroke, schizophrenia and multiple sclerosis. J Streeter, KK Wang, RL Hayes and A Jeromin are employees of Banyan Biomarkers, Inc., a company developing biomarkers for brain diseases. All other authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Dissociation of accumulated genetic risk and disease severity in patients with schizophrenia

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Genotype–phenotype correlations of common monogenic diseases revealed that the degree of deviation of mutant genes from wild-type structure and function often predicts disease onset and severity. In complex disorders such as schizophrenia, the overall genetic risk is still often >50% but genotype–phenotype relationships are unclear. Recent genome-wide association studies (GWAS) replicated a risk for several single-nucleotide polymorphisms (SNPs) regarding the endpoint diagnosis of schizophrenia. The biological relevance of these SNPs, however, for phenotypes or severity of schizophrenia has remained obscure. We hypothesized that the GWAS 'top-10' should as single markers, but even more so upon their accumulation, display associations with lead features of schizophrenia, namely positive and negative symptoms, cognitive deficits and neurological signs (including catatonia), and/or with age of onset of the disease prodrome as developmental readout and predictor of disease severity. For testing this hypothesis, we took an approach complementary to GWAS, and performed a phenotype-based genetic association study (PGAS). We utilized the to our knowledge worldwide largest phenotypical database of schizophrenic patients ($n>1000$), the GRAS (Göttingen Research Association for Schizophrenia) Data Collection. We found that the 'top-10' GWAS-identified risk SNPs neither as single markers nor when explored in the sense of a cumulative genetic risk, have any predictive value for disease onset or severity in the schizophrenic patients, as demonstrated across all core symptoms. We conclude that GWAS does not extract disease genes of general significance in schizophrenia, but may yield, on a hypothesis-free basis, candidate genes relevant for defining disease subgroups.
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Introduction

In complex disorders lacking clearly identifiable disease-causing factors, such as schizophrenia, the overall genetic risk is often >50%[1] but genotype–phenotype relationships are obscure. Recent genome-wide association studies (GWAS) on schizophrenia, building on very large cohorts of cases and controls, have uncovered and replicated a risk for several single-nucleotide polymorphisms (SNPs). Among the 10 'top hits', reaching genome-wide significance in different populations after multiple-testing correction (even though with low odds ratios), are markers in the major histocompatibility complex region and in *TCF4*, *ZNF804A* and *NRGN* genes.[2–6] The biological relevance of these SNPs, however, for phenotypes or severity of schizophrenia has remained unclear.

All current GWAS data on schizophrenia rely on endpoint diagnosis only and do not allow for genotype–phenotype correlations. In an approach complementary to GWAS, we employed the 'top-10' schizophrenia-associated SNPs that have emerged as genome-wide significant from several GWAS, and explored their potential contribution to the disease phenotype, including positive and negative symptoms, cognitive deficits and neurological signs (including catatonia), and age of onset of the prodrome as developmental readout and predictor of disease severity. For this phenotype-based genetic association study (PGAS), we utilized the GRAS (Göttingen Research Association for Schizophrenia) Data Collection.[7,8]

Materials and methods

Schizophrenic patients. The GRAS study was approved by the Ethics Committees of the Georg-August-University of Göttingen and of participating centers, and comprises at present 1041 patients with confirmed Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV)[9] diagnosis of schizophrenia (82.2%) or schizoaffective disorder (17.8%), examined between 2005 and 2010 in 23 collaborating centers all over Germany (Supplementary Table 1).[7,8]

Healthy subjects. Healthy subjects for the case–control study were blood donors ($n=1144$), recruited according to national guidelines for blood donation.[7] Comparable to the

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patient population (Caucasian 95.5%; other ethnicities 1.8%; unknown 2.7%), almost all controls were of European–Caucasian descent (Caucasian 97.8%; other ethnicities 2%; unknown 0.2%).

Phenotyping. Comprehensive interviews, testing and clinical ratings were conducted by an invariable team of trained examiners (psychiatrists and psychologists) using the 'GRAS Manual'.[7,8] Diagnoses of schizophrenia/schizoaffective disorders were based on the structured clinical interview for DSM-IV (SCID),[10] substantiated by information from medical records/discharge letters of all the patients. Psychopathological state, symptom severity and functional outcome were evaluated by clinical ratings (positive and negative syndrome scale (PANSS)[11] and global assessment of functioning (GAF).[9]) Neuropsychological testing (subtest 3 of Leistungsprüfsystem,[12] Trail-Making Test,[13,14] Verbal Learning and Memory test[15]) including pre-morbid intelligence (Mehrfachwahl-Wortschatz-Intelligenz test-B[16]) and neurological examination (Cambridge Neurological Inventory[17]) yielding respective composite scores, are described in detail elsewhere.[7,8]

Genotyping. SNP genotyping was performed with SimpleProbes (TIB Molbiol, Berlin, Germany) on LightCycler480 (Roche, Mannheim, Germany). All markers fulfilled the Hardy–Weinberg equilibrium.

Statistical methods. *PGAS*: Phenotype data were standardized to be normally distributed with expectation zero and variance one, and presented such that higher values always indicate better performance. Expected values of univariate and multivariate phenotypes were analyzed with linear models (Rv2.12.0), covariate adjusted and tested for additive effects of GWAS risk alleles with single-locus models (Table 1) and with a 10-loci model (Table 2). The latter simultaneously estimates for all the 10 loci regression coefficients with number of respective GWAS risk alleles. To assess statistical significance, the commonly used Bonferroni correction was employed; however, raw *P*-values are given.

Results

First, we proved in a case–control study (cases $n=1041$; healthy controls $n=1144$) that the GRAS population (for patient characteristics see Supplementary Table 1) provides a 'genetic data matrix' that essentially replicates the GWAS results (Table 1, upper part). In fact, screening of the 10 genome-wide hits resulted in a significant association of markers rs6913660, rs13211507 and rs3131296 in the major histocompatibility complex region (chromosome 6), and marker rs2312147 in chromosome 2 (*VRK2* gene) with an increased risk for the disease. Due to the smaller sample size compared with the GWAS studies, leading to lower power (power 70–90% for the SNPs found to reach allelic *P*-values $\leqslant 0.05$, average power of 43% over all markers), not all SNPs turned out to be significantly associated with the schizophrenia risk. Nevertheless, all of them exhibit the same direction of association as reported in GWAS.[2–6] In fact, the GRAS sample has been included in a recent large GWAS follow-up study.[6]

We next conducted PGAS single-locus and PGAS multilocus quantitative association analyses. In both the procedures we searched for the hypothesized association of markers with the lead symptoms of schizophrenia including developmental readouts/disease severity, both in the form of a composite construct (multivariate phenotype) and of its components separately (individual phenotypes), that is, positive and negative symptoms (PANSS), cognitive deficits (composite of executive function, reasoning, verbal learning and memory) and neurological signs (including catatonia), and/or with the age of onset of the disease prodrome. We further hypothesized that, if the markers were disease specific, they should not be associated with the schizophrenia-unrelated, general disease control variables. Hence, such variables referring to symptoms that are not in any way specific for schizophrenia were also included PANSS general psychopathology (depression, anxiety and others), global assessment of functioning and pre-morbid intelligence (basic cognitive capabilities of an individual before disease onset) (Tables 1 and 2; Supplementary Table 2).

With >1000 patients, the single-locus analysis found none of the schizophrenia phenotypes, neither individual nor multivariate, significantly influenced by any of the markers after multiple-testing correction by Bonferroni (Table 1). We thus wondered whether—instead of single genotypes—the 'genetic load' of a patient, that is, the accumulation of risk genotypes, would give us a clearer signal regarding the contribution of genetic risk to disease phenotype and severity. As illustrated in Figure 1, the composite severity score, built on the basis of the five core symptom variables (see inset), shows an essentially equal distribution of schizophrenia severity from the lowest to the highest genetic load group. As more symptoms do not necessarily reflect more severe disease and certain symptom groups may better associate with genetic load, all five variables were considered separately, too. But again, for the 10 top hits of GWAS, none of the schizophrenia symptom groups was dependent regarding its severity on the increase in the 'genetic load' (Supplementary Table 2, and Supplementary Figure 1). This becomes evident when the composite score data are presented alternatively as percentage severity over all the genetic load groups; there is no tendency of an increase in severe cases with an increase in the number of risk markers (Supplementary Figure 2). Interestingly, grouping the distribution of accumulated risk genotypes in the Icelandic GWAS sample ($n=582$ schizophrenic individuals) yields a pattern of overall risk distribution that is comparable to that of the GRAS database (Supplementary Figure 3), further supporting the validity of our sample for the PGAS approach to the GWAS hits.

We now pursued the question whether better insight into the phenotypical contribution of the GWAS hits could be obtained by performing a multilocus additive joint model analysis. This analysis weighs each marker according to its estimated relative importance as a risk genotype. Also this approach failed to uncover statistically significant associations after multiple-testing correction. Close to significance are two

Table 1 GWAS replication and PGAS single-locus quantitative association analysis

	Region/*neighboring gene* (Chromosome)		MHC *HIST1H2BJ* (Chr.6)		MHC *PGBD1* (Chr.6)		MHC *NOTCH4* (Chr.6)		*NRGN* (Chr.11)		*ZNF804A* (Chr.2)		MHC *PRSS16* (Chr.6)		*VRK2* (Chr.2)		*TAOK2* (Chr.16)		*TCF4* (Chr.18)		*TCF4* (Chr.18)	
	SNP ID		rs6913660		rs13211507		rs3131296		rs12807809		rs1344706		rs6932590		rs2312147		rs4583255		rs9960767		rs4309482	
GWAS Replication	Genotype Case(%) / Control(%)		C C	73.4/65.8	T T	86.2/81.9	G G	79.3/74.4	T T	69.7/66.7	T T	38.2/35.2	T T	61.0/57.1	C C	40.8/35.3	T T	33.8/31.5	A A	88.3/89.0	A A	37.7/35.6
			C A	24.3/31.3	T C	13.2/17.5	G A	19.6/24.1	T C	27.8/30.1	T G	49.0/49.3	T C	33.7/37.4	C T	43.3/49.4	T C	47.7/47.5	A C	11.4/11.0	A G	46.3/47.2
			A A	2.3/2.9	C C	0.6/0.6	A A	1.1/1.5	C C	2.6/3.2	G G	12.9/15.4	C C	5.3/5.5	T T	15.9/15.3	C C	18.5/20.9	C C	0.3/0.1	G G	16.0/17.3
	P value		**0.0007**		**0.0195**		**0.0255**		0.3036		0.1562		0.1707		**0.0130**		0.3054		0.5058		0.5301	
	Allelic Case(%) / Control(%)		C	85.5/81.4	T	92.8/90.6	G	89.1/86.5	T	83.5/81.8	T	62.6/59.9	T	77.8/75.8	C	62.5/60.0	T	57.6/55.3	A	94.0/94.4	A	60.9/59.2
			A	14.5/18.6	C	7.2/9.4	A	10.9/13.5	C	16.5/18.2	G	37.4/40.1	C	22.2/24.2	T	37.5/40.0	C	42.4/44.7	C	6.0/5.6	G	39.1/40.8
	P value		**0.0004**		**0.0094**		**0.0081**		0.1264		0.0695		0.1122		0.1042		0.1321		0.5568		0.2628	
	OR (Risk Allele) (95% CI)		1.34 (C) (1.14, 1.59)		1.34 (T) (1.07, 1.68)		1.28 (G) (1.06, 1.55)		1.13 (T) (0.96, 1.33)		1.12 (T) (0.99, 1.27)		1.12 (T) (0.97, 1.30)		1.11 (C) (0.98, 1.25)		1.10 (T) (0.97, 1.24)		1.08 (C) (0.83, 1.41)		1.07 (A) (0.95, 1.21)	
	OR (Risk Allele) (95% CI) in previous GWAS		1.15 (C) (1.10, 1.21)		1.24 (T) (1.16, 1.32)		1.19 (G) (1.13, 1.25)		1.15 (T) (1.10, 1.20)		1.10 (T) (1.07, 1.14)		1.16 (T) (1.11, 1.21)		1.10 (C) (1.06, 1.13)		1.08 (T) (1.05, 1.11)		1.23 (C) (1.15, 1.32)		1.09 (A) (1.06, 1.12)	
PGAS	Multivariate phenotype[a]	Allele Effect	0.0054		0.0286		-0.0697		**0.0979**		-0.0174		0.0027		0.0263		0.0064		**0.1158**		-0.0066	
		P value	0.8837		0.5731		0.0987		**0.0057**		0.5280		0.9322		0.3107		0.8072		**0.0346**		0.8014	
	PANSS positive	Allele Effect	-0.0014		0.0736		-0.0245		0.0317		-0.0043		0.0157		0.0278		0.0522		0.1054		-0.0380	
		P value	0.9811		0.3767		0.7237		0.5838		0.9233		0.7597		0.5121		0.2228		0.2396		0.3779	
	PANSS negative[a]	Allele Effect	0.0004		0.1260		0.0542		**0.1572**		0.0066		0.0025		-0.0031		0.0813		0.1673		0.0587	
		P value	0.9951		0.1398		0.4471		**0.0081**		0.8862		0.9624		0.9441		0.0653		0.0677		0.1870	
	Cognitive score[a]	Allele Effect	-0.0521		-0.1136		0.0441		**0.1301**		0.0003		-0.0133		-0.0342		0.0433		0.0851		-0.0298	
		P value	0.2802		0.0857		0.4225		**0.0048**		0.9932		0.7459		0.3169		0.2063		0.2382		0.3870	
	Total CNI[a]	Allele Effect	0.0458		-0.0135		-0.1239		**0.1626**		-0.0213		0.0229		-0.0137		0.0410		0.0436		-0.0030	
		P value	0.4307		0.8655		0.0639		**0.0033**		0.6197		0.6401		0.7373		0.3190		0.6070		0.9425	
	Prodromal Onset	Allele Effect	0.0148		0.0462		**-0.1974**		0.0530		-0.0453		-0.0204		**0.0941**		**-0.0976**		0.1480		0.0097	
		P value	0.8256		0.6259		**0.0103**		0.4174		0.3683		0.7179		**0.0428**		**0.0387**		0.1472		0.8378	
	PANSS general	Allele Effect	-0.0025		0.0656		-0.0040		**0.1392**		-0.0350		-0.0026		0.0340		0.0678		0.1334		0.0244	
		P value	0.9679		0.4506		0.9562		**0.0213**		0.4586		0.9604		0.4433		0.1290		0.1524		0.5885	
	GAF[a]	Allele Effect	0.0035		-0.0382		-0.1233		0.1147		-0.0139		0.0378		0.0157		0.0307		0.1199		0.0196	
		P value	0.9552		0.6569		0.0858		0.0558		0.7658		0.4743		0.7204		0.4891		0.1960		0.6616	
	Premorbid Intelligence[b]	Allele Effect	-0.0435		-0.0434		0.0309		0.0542		0.0242		-0.0020		0.0612		-0.0824		0.0100		-0.0067	
		P value	0.4770		0.6083		0.6622		0.3589		0.6008		0.9700		0.1617		0.0589		0.9134		0.8786	

Abbreviations: CNI, Cambridge neurological inventory; CI, confidence interval; Chr, chromosome; GAF, global assessment of functioning; GWAS, genome-wide association studies; MHC, major histocompatibility complex; OR, odds ratio; PGAS, phenotype-based genetic association study; PANSS, positive and negative syndrome scale; SNP, single-nucleotide polymorphism; SNP ID, SNP identifier.
GRAS sample of schizophrenic patients, $n=1041$; healthy control sample, $n=1144$. Upper part shows the case–control genetic association study essentially replicating previous GWAS results. SNPs are presented from left to right in the order of OR (odds ratio). Pearson χ^2-test and Fisher's exact test (both two sided) were used for genotypic and allelic comparisons, respectively. Lower part (PGAS) gives additive effect per copy of GWAS risk allele on expected value of schizophrenia-relevant quantitative phenotypes. The multivariate phenotype combines five schizophrenia core features (positive PANSS, negative PANSS, cognitive score, total CNI and prodromal onset). All phenotypes were standardized to zero mean and variance one and presented such that larger values correspond to better performance (for this purpose, PANSS scores and total CNI were multiplied by -1). General PANSS, GAF and premorbid intelligence were included in the analyses as disease control variables. The estimate of allele effect is negative if carriers of a GWAS risk variant perform worse. It is positive, if carriers of a GWAS risk variant perform better with respect to the expected trait value for the schizophrenic sample. Allele effect size on mean trait is quantified relative to trait variability (standard deviation). *P*-values below 0.05 were highlighted for optical guidance but are not significant after multiple-testing adjustment.
[a]Corrected for age: PANSS negative, cognitive score and total CNI (for separate analyses and within multivariate phenotype), GAF.
[b]Corrected for language problems: pre-morbid intelligence (898 with no language problems and 108 with correction for language problems).
Exploratory exclusion of non-Caucasian subjects from the GRAS sample ($n=48$; 4.5%) did not qualitatively alter any of the main findings in this Table.

associations, one between the marker rs3131296 in *NOTCH4* (chromosome 6) and prodromal onset (developmental readout), the other between rs12807809 in chromosome 11 (near *NRGN* gene) and severity of the neurological signs (Table 2). In fact, when ignoring the multiple-testing issue, the latter marker which has a relatively low odds ratio in our case–control study (OR 1.13 here and 1.15 in the GWAS study of Stefansson *et al*. [5]), shows several association 'signals' that make it attractive for follow-up in future subgroup analyses. However, this marker did not associate with the cognition of schizophrenic subjects in a recent study.[18]

Discussion

How can the overall negative result regarding the phenotypical significance of basically all GWAS 'top-10' genotypes be explained? First, in clear contrast to monogenic diseases,[19] the genetic risk for schizophrenia may not simply be reflected by phenotypical disease severity or by core symptoms of the disease. Here, an array of environmental risk factors that cannot easily be controlled for might also have a modulating role.[20] Second, the genotype –to phenotype translation may only be visible and valid for a relatively small subgroup of individuals, but still leads to significant (even though low) genetic risk odds ratios in very large GWAS samples. Third, one risk genotype may partly 'neutralize' another one, resulting in risk reduction upon combination rather than accumulation of the genetic load. This latter, seemingly paradox interaction is supported by the observation that the few effects on phenotypes found here in marker rs12807809 (chromosome 11; near *NRGN*) unexpectedly go into the opposite direction (risk genotype shows less severity). Fourth and finally, we cannot rule out that for some analyses the GRAS sample may not have enough power to detect the (certainly weak if any) phenotypical consequences of the 'top 10' GWAS hits. In this context, however, the general question

Table 2 PGAS multilocus quantitative association analysis.

Region/neighboring gene (chromosome)		MHC *HIST1H2BJ* (Chr.6)	MHC *PGBD1* (Chr.6)	MHC *NOTCH4* (Chr.6)	*NRGN* (Chr.11)	*ZNF804A* (Chr.2)	MHC *PRSS16* (Chr.6)	*VRK2* (Chr.2)	*TAOK2* (Chr.16)	*TCF4* (Chr.18)	*TCF4* (Chr.18)
SNP ID		***rs6913660***	***rs13211507***	***rs3131296***	***rs12807809***	***rs1344706***	***rs6932590***	***rs2312147***	***rs4583255***	***rs9960767***	***rs4309482***
PGAS											
Multivariate phenotype[a]	Allele effect	−0.0024	0.0996	**−0.1081**	**0.0966**	−0.0209	−0.0024	0.0241	−0.0038	**0.1233**	−0.0147
	P-value	0.9669	0.1277	**0.0204**	**0.0062**	0.4466	0.9582	0.3498	0.8846	**0.0254**	0.5749
PANSS positive	Allele effect	−0.0475	0.1224	−0.0710	0.0280	0.0026	0.0144	0.0295	0.0464	0.1124	−0.0428
	P-value	0.6211	0.2615	0.3593	0.6302	0.9542	0.8508	0.4902	0.2844	0.2160	0.3268
PANSS negative[a]	Allele effect	−0.0424	0.1669	0.0004	**0.1455**	−0.0016	−0.0253	0.0023	0.0671	0.1462	0.0462
	P-value	0.6627	0.1285	0.9955	**0.0145**	0.9717	0.7449	0.9573	0.1288	0.1130	0.3024
Cognitive score[a]	Allele effect	−0.0468	−0.1442	0.0972	**0.1262**	−0.0012	0.0449	−0.0274	0.0434	0.0834	−0.0398
	P-value	0.5390	0.0927	0.1108	**0.0066**	0.9738	0.4590	0.4246	0.2077	0.2524	0.2530
Total CNI[a]	Allele effect	0.0617	0.0484	**−0.1535**	**0.1663**	−0.0306	0.0041	−0.0141	0.0247	0.0498	−0.0091
	P-value	0.5025	0.6402	**0.0392**	**0.0027**	0.4770	0.9552	0.7292	0.5490	0.5595	0.8263
Prodromal onset	Allele effect	0.0587	0.1900	**−0.2631**	0.0545	−0.0527	−0.0536	0.0757	**−0.1099**	0.1715	−0.0006
	P-value	0.5733	0.1125	**0.0020**	0.4038	0.2967	0.5183	0.1057	**0.0212**	0.0966	0.9903
PANSS general	Allele effect	−0.0049	0.0916	−0.0425	**0.1312**	−0.0391	−0.0239	0.0368	0.0583	0.1184	0.0150
	P-value	0.9609	0.4169	0.5992	**0.0311**	0.4110	0.7644	0.4089	0.1962	0.2089	0.7418
GAF[a]	Allele effect	−0.0260	0.0129	−0.1548	0.1096	−0.0157	0.0685	0.0192	0.0229	0.1166	0.0141
	P-value	0.7905	0.9073	0.0531	0.0686	0.7394	0.3825	0.6637	0.6096	0.2136	0.7547
Pre-morbid intelligence[b]	Allele effect	−0.0744	−0.0148	0.0496	0.0533	0.0335	0.0449	0.0576	−0.0739	0.0097	−0.0086
	P-value	0.4439	0.8928	0.5299	0.3714	0.4741	0.5648	0.1920	0.0946	0.9171	0.8469

Abbreviations: CNI, Cambridge neurological inventory; Chr, chromosome; GAF, global assessment of functioning; MHC, major histocompatibility complex; PGAS, phenotype-based genetic association study; PANSS, positive and negative syndrome scale; SNP, single-nucleotide polymorphism; SNP ID, SNP identifier.
GRAS sample of schizophrenic patients, $n = 1010$ individuals with complete genotype information for 10 GWAS loci. SNPs are presented from left to right in the order of OR (odds ratio; compare Table 1). Instead of considering the SNPs separately as in Table 1, their joint additive effect is estimated simultaneously, allowing for different relative weights of the loci. Allele effect coefficients (and their associated *P*-values) for each additive variant in a multiple regression model are displayed. The multivariate phenotype combines five schizophrenia core features (positive PANSS, negative PANSS, cognitive score, total CNI and prodromal onset). All phenotypes were standardized to zero mean and variance one and presented such that larger values correspond to better performance (for this purpose, PANSS scores and total CNI were multiplied by −1). General PANSS, GAF and pre-morbid intelligence were included in the analyses as disease control variables. The estimate of allele effect is negative if carriers of a GWAS risk variant perform worse. It is positive, if carriers of a GWAS risk variant perform better with respect to the expected trait value for the schizophrenic sample. Allele effect size on mean trait is quantified relative to trait variability (standard deviation). Weight estimates with *P*-values below 0.05 were highlighted for optical guidance but are not significant after multiple-testing adjustment.
[a]Corrected for age: PANSS negative, cognitive score and total CNI (for separate analyses and within multivariate phenotype), GAF.
[b]Corrected for language problems: pre-morbid intelligence (886 with no language problems and 107 with correction for language problems).
Exploratory exclusion of non-Caucasian subjects from the GRAS sample ($n = 48$; 4.5%) did not qualitatively alter any of the main findings in this Table.

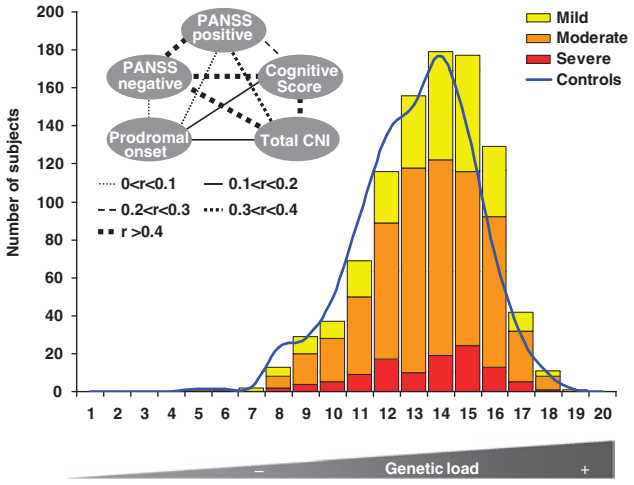

Figure 1 Distribution of phenotype severity and cumulative genetic load with respect to the number of GWAS-identified 'top-10' risk SNP alleles in the GRAS population (bar graph). Phenotype severity is based on a composite score of the five core features of schizophrenia, displayed as inset (including intercorrelations between items). Score range in the GRAS sample is divided into three equal parts and ranked as mild, medium and severe disease phenotype. The blue line denotes the comparison of the risk SNP allele distribution in the healthy control sample.

arises of how much clinical significance a genetic association may have if several thousand patients are needed to reveal a tiny effect on disease severity or phenotype. Thus, building on >1000 patients, one would at least have expected some more signals to pop up (that is, more nominal *P*-values around 0.05 in the PGAS part of Table 1). In fact, the estimated power assessed according to Lettre *et al.,*[21] who performed simulations in a similar context (sample size, normally distributed phenotype), amounts to overall around 80% at the $\alpha = 0.05$ level for the PGAS approach. The presented additive model, chosen to match our hypothesis of cumulated risk, performed similarly to a co-dominant one but appeared to be slightly more powerful.

After all, we note that entirely different genotypes that were never found to be significantly associated with any schizophrenia risk in GWAS, still may profoundly modulate the schizophrenic phenotype, for example, of genes encoding neuregulin-1, complexin 2 or COMT.[7,22,23] On the other hand, GWAS finds may be of general rather than disease-specific significance. Several studies have for instance suggested that schizophrenia and affective disorders are on a continuum of liability. Genetic linkage and association studies have proposed common disease loci for both the disorders.[2,24] Likewise, family studies show that first-degree relatives of bipolar patients have a higher risk for schizophrenia compared with first-degree relatives of healthy controls.[25,26] Also other psychiatric diseases like alcoholism or major depression have been found to be associated with certain schizophrenia risk genes, for example, *DISC1*.[27] Thus, exploration of many other phenotypes available in the GRAS database, including candidate intermediate phenotypes[28,29] or those reflecting a more dimensional approach to the disease,[30] might potentially be interesting. Purpose of the present study, however, was to cover the lead symptoms of schizophrenia in the first place.

To conclude, GWAS approaches in diseases as complex as schizophrenia do not lead to the reconstruction of a 'common disease mechanism' or to the discovery of 'classical disease genes', as such genes obviously do not exist. What makes our study important for the clinician is that we can show, for the first time, that the combination of a whole battery of genetic pre-disposing factors (the 'top 10' GWAS finds in schizophrenia) in individual patients will not make their schizophrenic phenotype any different or worse than that of those patients who do not carry these genetic factors. Importantly, however, GWAS results may guide, on a hypothesis-free basis, to the identification of totally unexpected candidate genes involved in certain disease aspects in subgroups of patients, as they can be defined by PGAS. In order to get closer to understanding the disorders as complex and heterogeneous as schizophrenia, GWAS and PGAS will have to go hand in hand.

Conflict of interest

The authors declare no conflict of interest.

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Author Contributions: H.E. and S.P. had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. The study concept and design was given by S.P. and H.E. Data were acquired by M.B., A.K., S.P., S.Sp., H.S. and H.E. Analysis and interpretation of data was done by S.P., D.M., A.K., H.B., H.S. K-A.N. and H.E. Manuscript was drafted by H.E., S.P. and K-A.N. Critical revision of the manuscript for important intellectual content was done by S.P., D.M., A.K., S.Sp., M.B., H.S., H.B., K-A.N. and H.E. Statistical analyses were carried out by D.M. and H.B. Administrative, technical and material support was given by S.Sp. and M.B. The study was supervised by H.E.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)

Supplementary Information

Dissociation of accumulated genetic risk and disease severity in patients with schizophrenia

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Outline:

Supplementary Table 1: GRAS patient sample description.

	Variable	Mean ± s.d.	Median
GRAS SAMPLE	Age in GRAS patients (years)	39.54 ± 12.55	39.05
	PANSS positive	13.76 ± 6.32	12.00
	PANSS negative	18.23 ± 7.86	17.00
	Cognitive score	-0.01 ± 0.85	0.02
	Total CNI	19.76 ± 18.63	15.00
	Prodromal Age of Onset (years)	22.81 ± 8.70	20.00
	PANSS general	33.73 ± 11.83	32.00
	GAF	45.73 ± 17.26	45.00
	Premorbid Intelligence	26.13 ± 6.15	27.00
	Gender proportion	66.7% men / 33.3% women	-

Gender and age distribution in the sample of patients (GRAS, n=1041). Mean ± s.d. and Median of the phenotypical variables analyzed in the GRAS sample.

Supplementary Table 2: PGAS: Association between cumulative genetic load and expected value of quantitative schizophrenia-relevant phenotypes

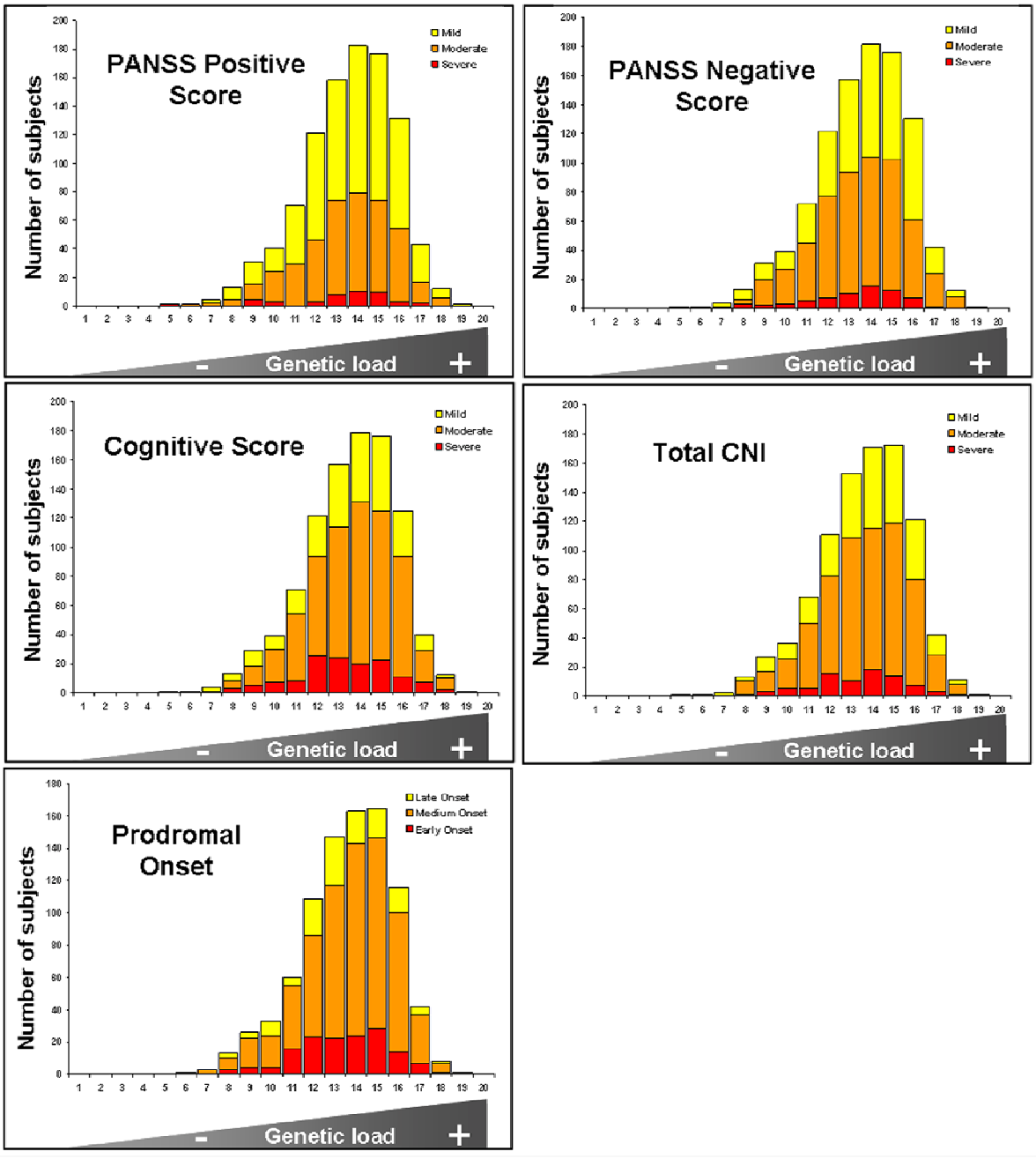
PGAS	Multivariate phenotype[#]	Effect per unit increase of genetic load	0.0083
		P value	0.3391
	PANSS positive	Effect per unit increase of genetic load	0.0106
		P value	0.4557
	PANSS negative[#]	Effect per unit increase of genetic load	**0.0333**
		P value	**0.0220**
	Cognitive score[#]	Effect per unit increase of genetic load	0.0033
		P value	0.7745
	Total CNI[#]	Effect per unit increase of genetic load	0.0111
		P value	0.4163
	Prodromal Onset	Effect per unit increase of genetic load	-0.0051
		P value	0.7502
	PANSS general	Effect per unit increase of genetic load	0.0216
		P value	0.1445
	GAF[#]	Effect per unit increase of genetic load	0.0122
		P value	0.4073
	Premorbid Intelligence[‡]	Effect per unit increase of genetic load	0.0014
		P value	0.9248

[#]Corrected for age: PANSS negative, cognitive score, total CNI (for separate analyses and within multivariate phenotype); GAF.
[‡]Corrected for language problems: premorbid intelligence (886 with no language problems, 107 with correction for language problems).

Analysis based on the GRAS sample of schizophrenic patients, i.e. n=1010 individuals with complete genotype information for 10 GWAS loci. The cumulative genetic load is defined as sum of the number of all GWAS risk variants at the 10 loci of Table 1. Estimated was the mean effect of the genetic load per unit increase (additive model with equal weighting of risk variants at each locus). Phenotypes were standardized to zero mean and variance one and presented such that larger values correspond to better performance (i.e. PANSS scores and total CNI were multiplied by -1). General PANSS, GAF and premorbid intelligence were included in the analyses as disease control variables. A positive estimate of the effect of increased genetic load suggests a milder phenotype, a negative estimate a more severe phenotype. Effect size is quantified relative to trait variability (standard deviation). *P* values below 0.05 were highlighted for optical guidance but are not significant due to multiple-testing adjustment. Exploratory exclusion of non-Caucasian subjects from the GRAS sample (n=48; 4.5%) did not qualitatively alter any of the main findings in this Table.

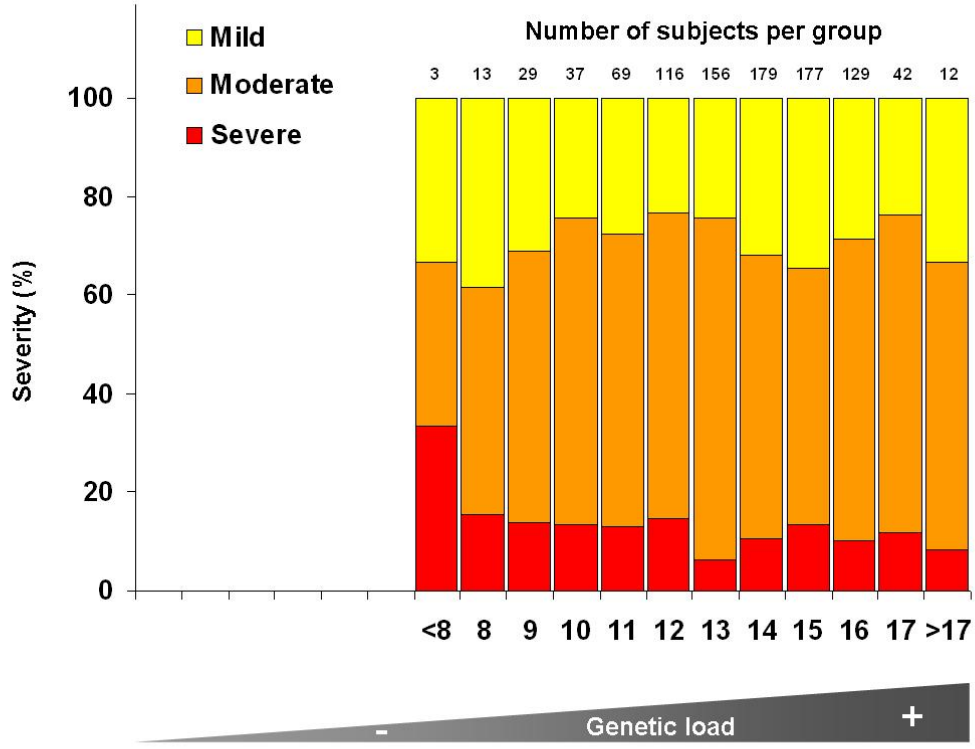
Supplementary Figure 1

Distribution of phenotype severity and cumulative genetic load with respect to the number of GWAS-identified 'top-10' risk SNP alleles in the GRAS population (bar graph). Phenotype severity in each of the figures is based on one of the 5 core items of schizophrenia. Score range for each item in the GRAS sample is divided into 3 equal parts and ranked as mild, medium and severe phenotype, respectively.



Supplementary Figure 2

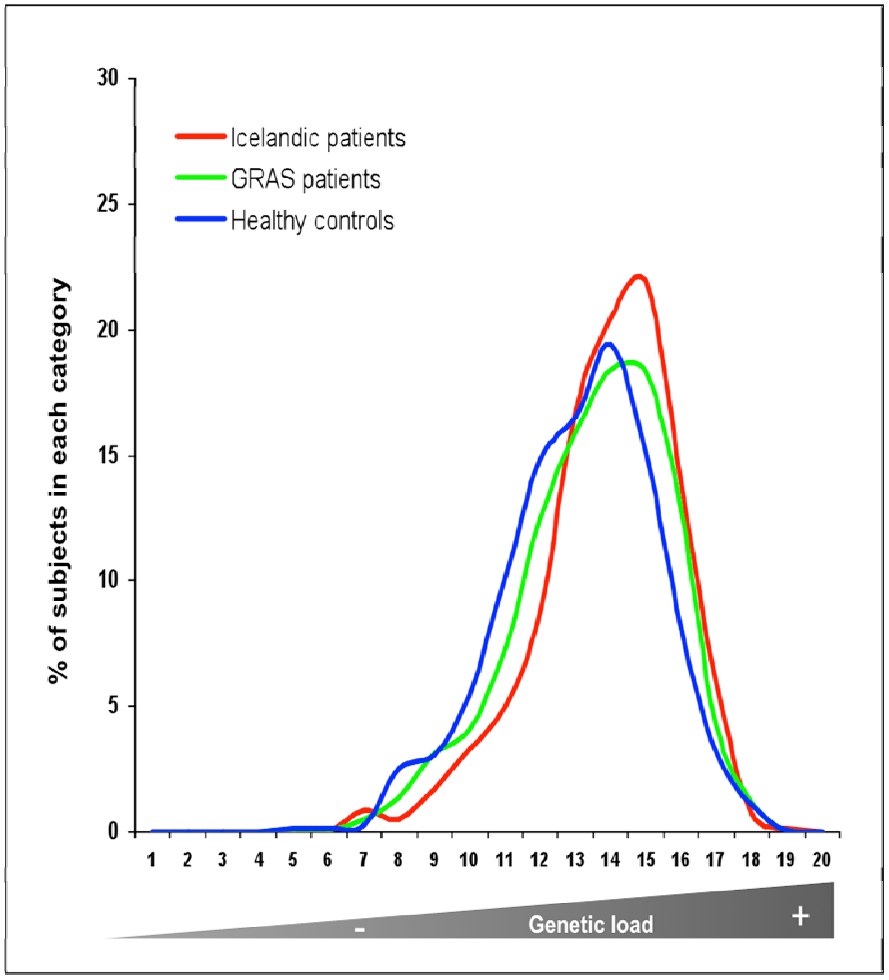
Percentage distribution of phenotype severity in genetic load groups with respect to the number of GWAS-identified 'top-10' risk SNP alleles in the GRAS population. Phenotype severity is based on a composite score of 5 core features of schizophrenia (compare inset of Figure 1). Score range in the GRAS sample is divided into 3 equal parts and ranked as mild, medium and severe disease phenotype.



Supplementary Figure 3

Distribution of cumulative genetic load in Icelandic and German populations

Grouping the distribution of accumulated risk genotypes in the Icelandic GWAS sample (n=582 schizophrenic individuals) yields a pattern similar to the GRAS population (n=1041), further supporting the validity of the GRAS sample for the PGAS approach to the GWAS hits. German healthy controls are from the GRAS case-control study (n=1144).



EMBO Molecular Medicine

A myelin gene causative of a catatonia-depression syndrome upon aging

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Keywords: anxiety; axonal degeneration; diffusion tensor imaging; low-grade inflammation; social withdrawal

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Severe mental illnesses have been linked to white matter abnormalities, documented by postmortem studies. However, cause and effect have remained difficult to distinguish. *CNP* (2′,3′-cyclic nucleotide 3′-phosphodiesterase) is among the oligodendrocyte/myelin-associated genes most robustly reduced on mRNA and protein level in brains of schizophrenic, bipolar or major depressive patients. This suggests that CNP reduction might be critical for a more general disease process and not restricted to a single diagnostic category. We show here that reduced expression of *CNP* is the primary cause of a distinct behavioural phenotype, seen only upon aging as an additional 'pro-inflammatory hit'. This phenotype is strikingly similar in *Cnp* heterozygous mice and patients with mental disease carrying the AA genotype at *CNP* SNP rs2070106. The characteristic features in both species with their partial *CNP* 'loss-of-function' genotype are best described as 'catatonia-depression' syndrome. As a consequence of perturbed CNP expression, mice show secondary low-grade inflammation/neurodegeneration. Analogously, in man, diffusion tensor imaging points to axonal loss in the frontal corpus callosum. To conclude, subtle white matter abnormalities inducing neurodegenerative changes can cause/amplify psychiatric diseases.

INTRODUCTION

The *CNP* gene encodes the enzyme 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) which is present in non-compacted myelin areas such as the inner mesaxon, paranodal loops and Schmidt-Lantermann incisures (Braun et al, 2004; Yu et al, 1994), and accounts for about 4% of total central nervous system myelin proteins (Braun et al, 2004). *CNP* is expressed early in development of oligodendrocytes (Yu et al, 1994), increases with onset of myelination and remains detectable in these cells throughout life (Scherer et al, 1994). *In vitro* and *in vivo* studies demonstrated a regulatory function of CNP for process outgrowth in oligodendrocytes (Gravel et al, 1996; Lee et al, 2005; Yin et al, 1997), as well as an interaction with microtubules, cytoskeleton and RNA (Bifulco et al, 2002; De Angelis & Braun, 1996; Gravel et al, 2009; Lee et al, 2005).

Studies employing homozygous *Cnp*-null mutant mice revealed that Cnp is essential for axonal survival but not for myelin assembly (Lappe-Siefke et al, 2003). In fact, *Cnp*−/− mice show progressive axonal swellings and brain inflammation with first motor deficits occurring at 4 months that progress to severe hindlimb paralysis and death at 8–15 months (Lappe-Siefke et al, 2003). In contrast, *Cnp*+/− mice with a 50% reduced Cnp expression do not exhibit any signs of inflamma-

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tion nor of abnormalities in neurological scoring or behaviour at least until the age of 12 months (Lappe-Siefke et al, 2003; Wieser et al, in preparation), indicating that lower Cnp levels can be fully compensated for.

Nevertheless, decreased CNP expression could have pathophysiological significance. *CNP* is among the oligodendrocyte/myelin-associated genes identified to be most robustly reduced both on mRNA and protein level in postmortem brains of schizophrenic, bipolar or major depressive patients (Aston et al, 2005; Mitkus et al, 2008; Tkachev et al, 2003). These findings suggest that CNP reduction might be critical in a more general disease process and that the potential role of this molecule is not restricted to a single diagnostic category but of global relevance for severe mental disorders.

Several genetic association studies have explored a potential impact of genetic variability in the *CNP* gene (chr17q21.2, 11Kb) on the overall risk for schizophrenia, with inconclusive results so far (Che et al, 2009; Peirce et al, 2006). Interestingly, however, a synonymous (Gly/Gly) single nucleotide polymorphism (SNP), localized in the fourth exon of the gene (rs2070106), influences *CNP* expression in the human cortex, especially in frontal areas, with the rarer A-allele showing lower expression in comparison to the G-allele (Iwamoto et al, 2008; Mitkus et al, 2008; Peirce et al, 2006).

Recent work indicates that in major psychiatric disorders like schizophrenia and depression, low-grade inflammation constitutes a crucial mechanism in the final common disease pathway (reviewed in Monji et al, 2009). Already the normal aging process is associated with slightly increased brain inflammation characterized by, for example, enhanced levels of pro-inflammatory cytokines, higher microglial numbers and increased reactivity with augmented expression of microglial surface markers (reviewed in, *e.g.* Miller & Streit, 2007; Sparkman & Johnson, 2008; Streit, 2006).

To address the pathophysiological relevance of reduced CNP expression, we chose *CNP* partial 'loss-of-function' genotypes with aging as an additional 'pro-inflammatory hit'. We examined old *Cnp*+/− mice and schizophrenic patients with the AA *versus* GG genotype in the *CNP* SNP rs2070106. We report here the surprising association of *CNP* partial loss-of-function with a catatonia-depression syndrome both in mouse and man upon aging. Importantly, we provide evidence for late-onset low-grade inflammation in mice as a plausible pathophysiological mechanism. In patients carrying the low-expression genotype (AA), a comparable process might be reflected by axonal loss in the frontal corpus callosum as detectable by neuroimaging.

RESULTS

Brains of aging *Cnp*+/− mice are characterized by enhanced inflammation, astrogliosis and axonal degeneration

Immunohistochemical analysis of mouse brains from age 4 to 26 months revealed an age-related increase in the number of ionized calcium-binding adapter molecule 1 (IBA-1) and Mac-3 positive microglia, infiltrating T-lymphocytes (cluster of differentiation 3; CD3) and astrocytes (glial fibrillary acidic protein, GFAP) in corpus callosum, striatum and anterior commissure (month 4 *vs.* month 26: all $p \leq 0.01$; for wild-type (Wt) as well as *Cnp*+/− mice). This increase was significantly more pronounced in old *Cnp*+/− as compared to Wt mice (Fig 1A–H). Axonal swellings (spheroids) as readout of neurodegeneration were determined in corpus callosum, striatum and anterior commissure using amyloid precursor protein (APP) immunoreactivity (Fig 1I/J). At the age of 4 months, no positive APP staining was detected. Thereafter, an age-dependent increase in axonal swellings became evident, again more prominent in *Cnp*+/− mice (Fig 1I/J). Determination of *Cnp* mRNA expression in brains of young *versus* old mice revealed a remarkable decrease upon aging in Wt mice, which, however, still maintained levels above those in *Cnp*+/− mice (Fig 1K). In both Wt and *Cnp*+/− mice, we found a corresponding age-dependent reduction of Cnp protein in purified myelin membranes, with the lowest overall level in aged *Cnp*+/− (Fig 1L). Proteolipid protein (Plp), a control protein for compact myelin, also decreased with age but independent of the *Cnp* genotype (Fig 1L). Taken together, old *Cnp*+/− mice show a more pronounced low-grade inflammatory phenotype with axonal degeneration compared to Wt mice.

Aged *Cnp*+/− mice have a slightly elevated anxiety profile but normal motor activity, coordination and strength

To test whether the pronounced histological changes upon aging are associated with any behavioural consequences, we investigated aged (24 months old) *Cnp*+/− and Wt mice employing a comprehensive test battery. In the open field test, a measure for general locomotor activity and anxiety, *Cnp*+/− mice tended to spend less time in the centre than Wt ($p = 0.096$; Fig 2A). Velocity and total distance travelled in the open field were comparable in both genotypes (Fig 2B and C), indicating normal activity. In the elevated plus maze, a classical anxiety test, open arm visits were reduced in *Cnp*+/− mice ($p = 0.036$; Fig 2D), whereas, the light/dark-box did not yield differences in the time spent in light (Fig 2E). Freezing behaviour is seen as another indicator of anxiety/fear in rodents. *Cnp*+/− mice showed higher percentage of freezing in the fear conditioning chamber already at baseline, that is before measurement of conditioned or cued memory ($p = 0.007$; Fig 2F), precluding the use of fear conditioning for memory assessment in these mice. Like basic motor activity, which proved to be normal, motor performance, coordination and motor learning, as evaluated in a 2-day rota-rod testing, were not different between genotypes (Fig 2G). Also, gait analysis detected no motor abnormalities or ataxia (see, *e.g.* Fig 2H depicts forelimb stride of left and right paw) and muscle strength, measured by the grip strength test, did not differ between genotypes (Fig 2I). To summarize, 24 months old *Cnp*+/− mice show normal overall motor performance and a mildly elevated anxiety profile in different anxiety-relevant tests compared to Wt mice.

Aged *Cnp*+/− mice show impaired social and exploratory behaviour

Social behaviour of aged Wt and *Cnp*+/− mice was tested in a three-partite chamber. This test measures the preference of a

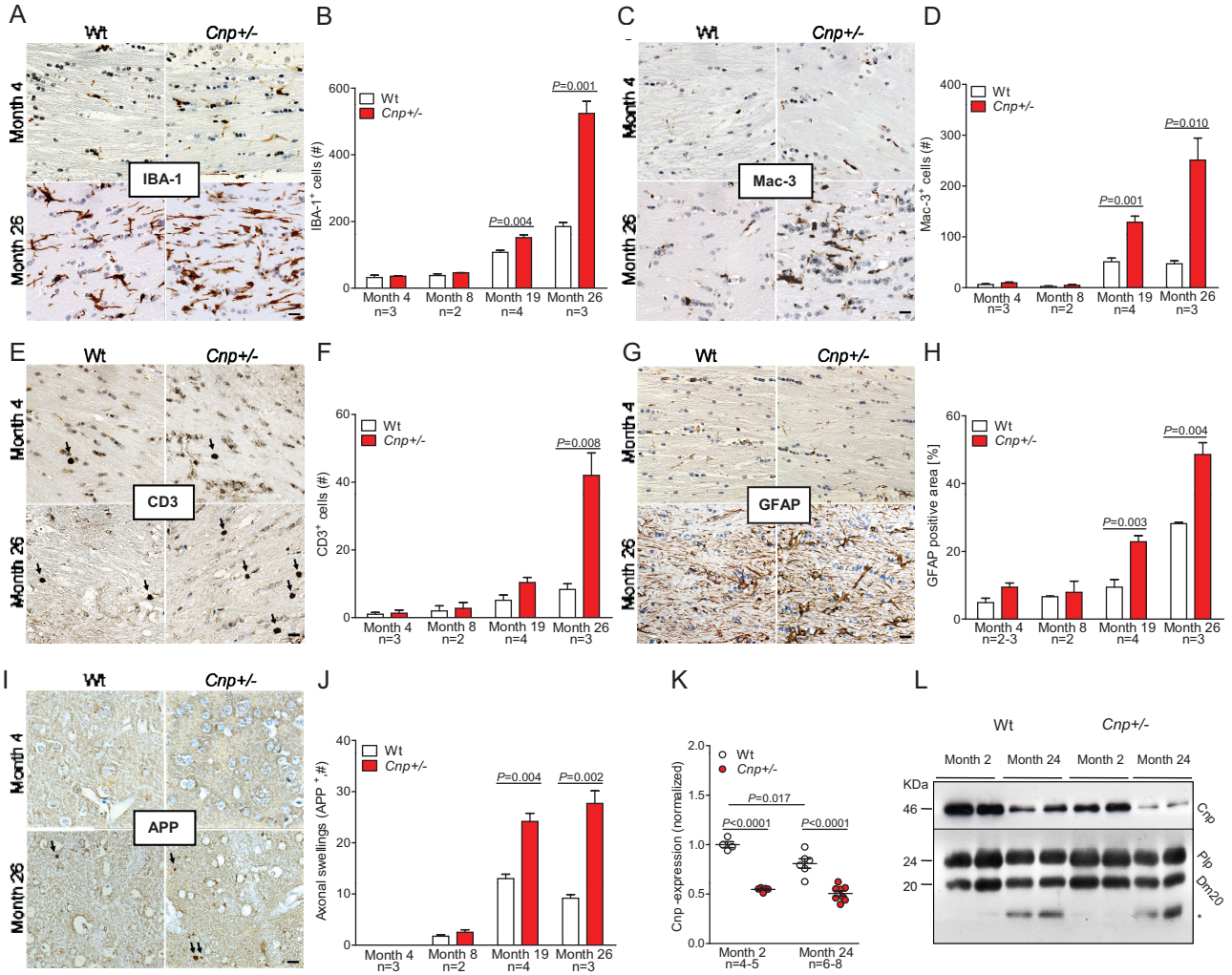

Figure 1. Low-grade brain inflammation and axonal degeneration in aged *Cnp*+/− mice.

A. Representative microscopic images of the corpus callosum from 4 months (upper panels) and 26 months (lower panels) old Wt and *Cnp*+/− mice, immunostained for IBA-1; scale bar 20 μm.

B. Bar graph gives the age-dependent quantification of the total number of IBA-1 positive microglia in the corpus callosum of Wt and *Cnp*+/− mice. For all quantifications (**B**, **D**, **F**, **H**, **J**), *n* numbers indicated; mean ± s.e.m. presented; two-sided Student's *t*-test used.

C. Representative microscopic images of the corpus callosum from 4 months (upper panels) and 26 months (lower panels) old Wt and *Cnp*+/− mice, immunostained for Mac-3; scale bar 20 μm.

D. Bar graph gives the age-dependent quantification of the total number of Mac-3 positive microglia in the corpus callosum of Wt and *Cnp*+/− mice.

E. Representative microscopic images of the corpus callosum from 4 months (upper panels) and 26 months (lower panels) old Wt and *Cnp*+/− mice, immunostained for CD3; black arrows exemplify respective positive cells; scale bar 20 μm.

F. Bar graph gives the age-dependent quantification of the total number of CD3 positive T-lymphocytes in the corpus callosum, striatum and anterior commissure of Wt and *Cnp*+/− mice.

G. Representative microscopic images of the corpus callosum from 4 months (upper panels) and 26 months (lower panels) old Wt and *Cnp*+/− mice, immunostained for GFAP; scale bar 20 μm.

H. Densitometrical quantification of the GFAP positive area in the corpus callosum.

I. Representative microscopic images of the striatum from 4 months (upper panels) and 26 months (lower panels) old Wt and *Cnp*+/− mice, immunostained for APP; black arrows exemplify respective positive cells; scale bar 20 μm.

J. Bar graph gives the age-dependent quantification of the APP positive axonal swellings in the corpus callosum, striatum and anterior commissure of Wt and *Cnp*+/− mice.

K. *Cnp* mRNA expression level of Wt and *Cnp*+/− mice at months 2 and 24, normalized to mean value of ATP synthase subunit beta (*Atp5b*) and acidic ribosomal phosphoprotein P0 (*Rplp0*) as housekeeper genes and to 2 months old Wt (1.0); mean ± s.e.m. presented; two-sided Student's *t*-test used.

L. Cnp protein expression of Wt and *Cnp*+/− mice at months 2 and 24, compared to Plp as control protein of compact myelin; * low-size band detected in aged brain myelin with the Plp antibody directed against the C-terminus of PLP/DM20.

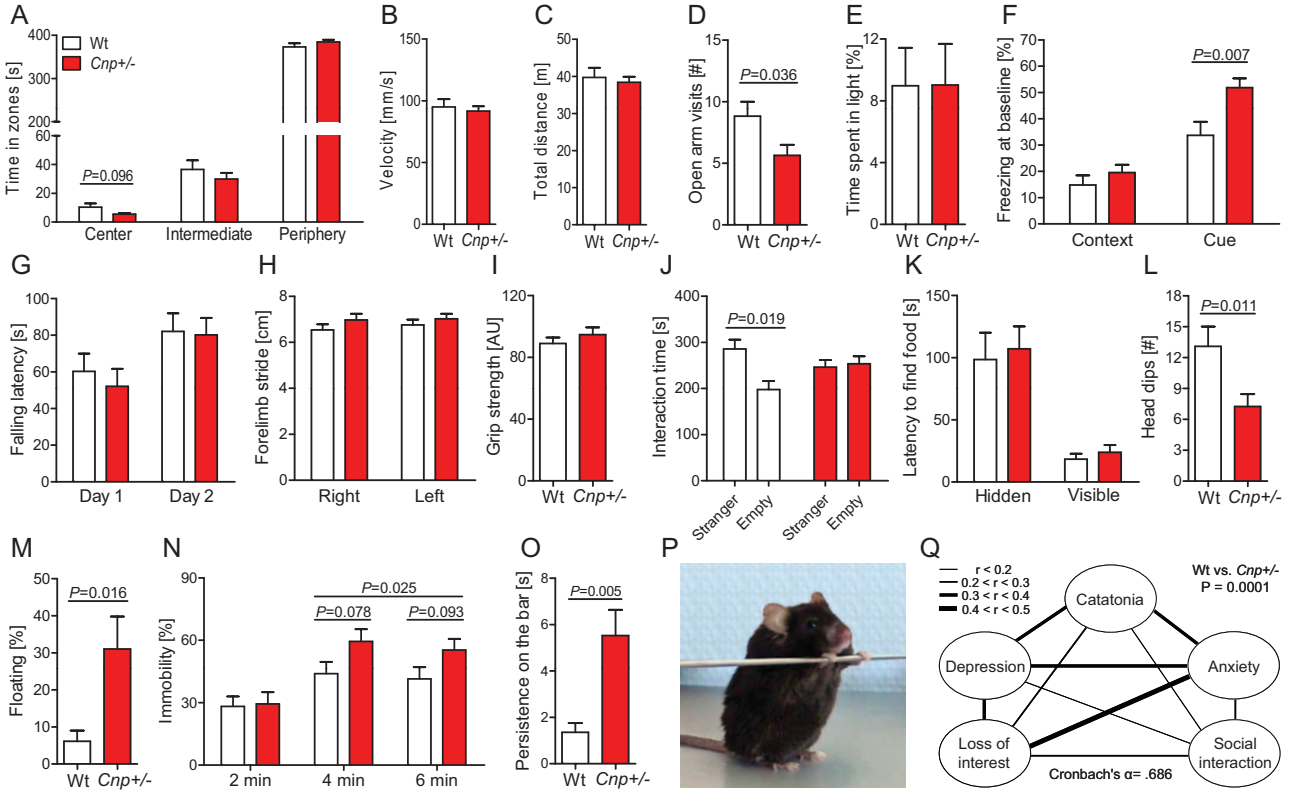

Figure 2. Aged *Cnp*+/− mice show a phenotype composed of catatonia, depression, loss of interest, impaired social interaction and anxiety.

A-C Open arm parameters.

D. Elevated plus maze.

E. Light/dark box paradigm.

F. Baseline freezing in the context and cue memory task of fear conditioning.

G. Rota-rod.

H. Gait analysis.

I. Grip strength.

J. Sociability testing in the three-partite chamber.

K. Buried-food finding test – latency to find hidden *versus* visible food pellets.

L. Hole board.

M. Floating rate in a 90 s swim trial.

N. Tail suspension test.

O. Bar test for catatonia.

P. Typical posture of a catatonic *Cnp*+/− mouse during the bar test; see also videos of Supporting Information.

Q. Behavioural composite score displayed as intercorrelation network of Z-transformed items. Line thickness indicates the degree of correlation between 2 respective items. The composite score differs between genotypes ($p = 0.0001$). For all behavioural experiments, 24 months old mice were used: Wt $n = 9$–11 and *Cnp*+/− $n = 10$–16; mean ± s.e.m. presented; two-sided or paired *t*-tests used where applicable.

mouse for a chamber containing a small wire cage with a stranger mouse in comparison to a chamber with an empty wire cage. Aged Wt mice displayed the expected behaviour, that is spent significantly more time close to the cage with the stranger mouse compared to the empty wire cage ($p = 0.019$), whereas, *Cnp*+/− mice did not show preference. To control for altered olfaction as a potential confounder of social behaviour in mice, the buried-food-finding test was performed, confirming normal olfactory function in both groups (Fig 2K). In the hole board test, measuring exploratory behaviour of mice, old *Cnp*+/− mice had significantly less head dips ($p = 0.011$; Fig 2L), indicating loss of interest (in the absence of any signs of altered basic motor activity). To conclude, old *Cnp*+/− mice demonstrate several facets of a loss of interest in the outside world.

Aged *Cnp*+/− mice exhibit features of depression and catatonia

In the Morris water maze task, *Cnp*+/− mice displayed prominent floating behaviour, precluding analysis of this test for learning and memory. Analysis of the time mice spent floating within a swim trial of 90 s yielded threefold higher floating rates of *Cnp*+/− mice in comparison to Wt, which we interpret as a potential sign of depression ($p = 0.016$; Fig 2M). To further consolidate this hypothesis, we performed an

established test to measure depression in rodents, the tail suspension test, which determines over 6 min the time mice spend immobile. Fractionated analysis revealed that *Cnp*+/− mice had a higher duration of immobility in the second and last third of the test period compared to Wt ($p = 0.025$; Fig 2N), consistent with the typical 'give up' behaviour of depressed individuals. A phenotype, thus far observed in mice only upon induction (*e.g.* body pinch or drug exposure; Amir, 1986; Chaperon & Thiebot, 1999) is catatonia/catalepsy, a state of immobility where mice persist in an externally imposed abnormal posture for a prolonged time period. Mice are put into a position where they have to grab a bar while standing with their hind paws on the floor (as illustrated in Fig 2P; for a striking example see videos of Supporting Information). Wt mice swiftly left this position, whereas, *Cnp*+/− mice persisted in this posture ($p = 0.005$; Fig 2O). Taken together, old *Cnp*+/− mice exhibit a catatonia-depression syndrome.

Creating a mouse behavioural composite, the '*catatonia-depression score*'

For translational purposes and confirmation of the internal consistency of our behavioural readouts in aged mice, we calculated intercorrelations between the observed behavioural sub-phenotypes catatonia, depression, loss of interest, impaired social interaction and anxiety as target variables. These variables, put together in a composite score, were internally consistent (Cronbach's $\alpha = .686$; Fig 2Q). Operationalization of the score items is detailed in the Materials and Methods Section. Expectedly, the score was significantly higher in *Cnp*+/− (0.32 ± 0.44) than in Wt mice (-0.43 ± 0.41; $p = 0.0001$). Based on these findings, we wondered whether reduced expression of the *CNP* gene in aging human patients may have a similar influence on the phenotype.

Exploiting the GRAS data base for a phenotype-based genetic association study on the role of *CNP* genotypes in a 'catatonia-depression syndrome'

To search for potential behavioural consequences of a previously described *CNP* loss-of-function genotype in humans (Iwamoto et al, 2008; Mitkus et al, 2008; Peirce et al, 2006), we conducted a phenotype-based genetic association study (PGAS) targeting the *CNP* SNP rs2070106 (A/G; Fig 3A) in >1000 schizophrenic patients of the Göttingen Research Association for Schizophrenia (GRAS) data collection (Begemann et al, 2010; Ribbe et al, 2010). As a first step, we performed a case–control analysis (schizophrenic patients *vs.* healthy controls) and found that this genetic marker does not contribute to an increased risk of schizophrenia in our population, as proven by the genotypic and the allelic chi-square comparison ($p > 0.05$; Table I of Supporting Information).

Next, a composite score including all variables represented in the mouse behaviour composite was created that also yielded good internal consistency with a Cronbach's $\alpha = .695$ (Fig 3B). The operationalization of the score items is explained in the Materials and Methods Section. As illustrated in Fig 3C, the composite score shows a clear age and genotype (rs2070106) association: AA subjects develop a significantly higher score with increasing age as compared to GG carriers, with the dissociation of the regression lines starting at around the age of 40 years. We therefore set a cut-off of 40 years and focused on the older schizophrenic patients with our further PGAS analysis.

The characteristics of the GRAS patients with an age ≥40 years, separated by AA *versus* GG genotype of rs2070106, are presented in Table 1. These data demonstrate that both genotype groups are comparable with respect to basic socio-demographic and clinical/disease control variables but differ highly significantly in the composite score measuring the catatonia-depression syndrome. Interestingly, heterozygote individuals (GA) are very similar to GG subjects. They do not show an intermediate phenotype in the composite score (Table II of Supporting Information). Importantly, when screening all items of the composite separately, a significant age-associated genotype (GG *vs.* AA) effect, comparable to the mouse findings, becomes evident for all (Fig 1 of Supporting Information).

CNP rs2070106 genotypes influence myelin/axon integrity in the frontal corpus callosum fibres, a candidate region of catatonia-depression

Based on clinical observation of the affected individuals – both mouse and man – and the scarce information in the literature on brain areas potentially involved in the catatonic phenomenon (Arora & Praharaj, 2007; Northoff et al, 2004), we hypothesized that aging AA individuals displaying the catatonia-depression syndrome, in contrast to GG subjects, should show differences in axonal integrity of frontal crossing fibres. To prove this hypothesis, a subset of older patients of both genotypes (GG $n = 11$; AA $n = 10$) from the GRAS sample was selected and matched according to age, gender and duration of disease (Table 1). These patients, living all over Germany, were re-invited to Göttingen for diffusion tensor imaging (DTI). Indeed, DTI identified higher axial diffusivity (AD) and a higher apparent diffusion coefficient (ADC) in the frontal part of the corpus callosum (genu) of AA subjects as compared to GG patients ($p \leq 0.005$ for both values; Fig 3E), consistent with a more progressed axonal loss/degeneration. This effect was specific for the frontal commissural fibres and was not observed in the posterior corpus callosum taken as a control region (Fig 3F). ADC values in the genu were generally correlated with age but, despite the small number of imaged subjects, resulted in a significant difference between genotypes upon linear discriminant analysis (LDA; $p < 0.05$; Fig 3G). Importantly, there were no global brain volume differences detectable between GG and AA subjects that could have accounted for DTI results ($p > 0.05$ for all comparisons; Fig 3H).

DISCUSSION

We report here the unexpected finding that *CNP* loss-of-function genotypes are causative of a mental syndrome, consisting of catatonia, depression, mild anxiety/social withdrawal, impaired social interaction and reduced interest in the outside world, which is remarkably similar in mouse and man. In both species, age becomes an important cofactor, supporting the view that the

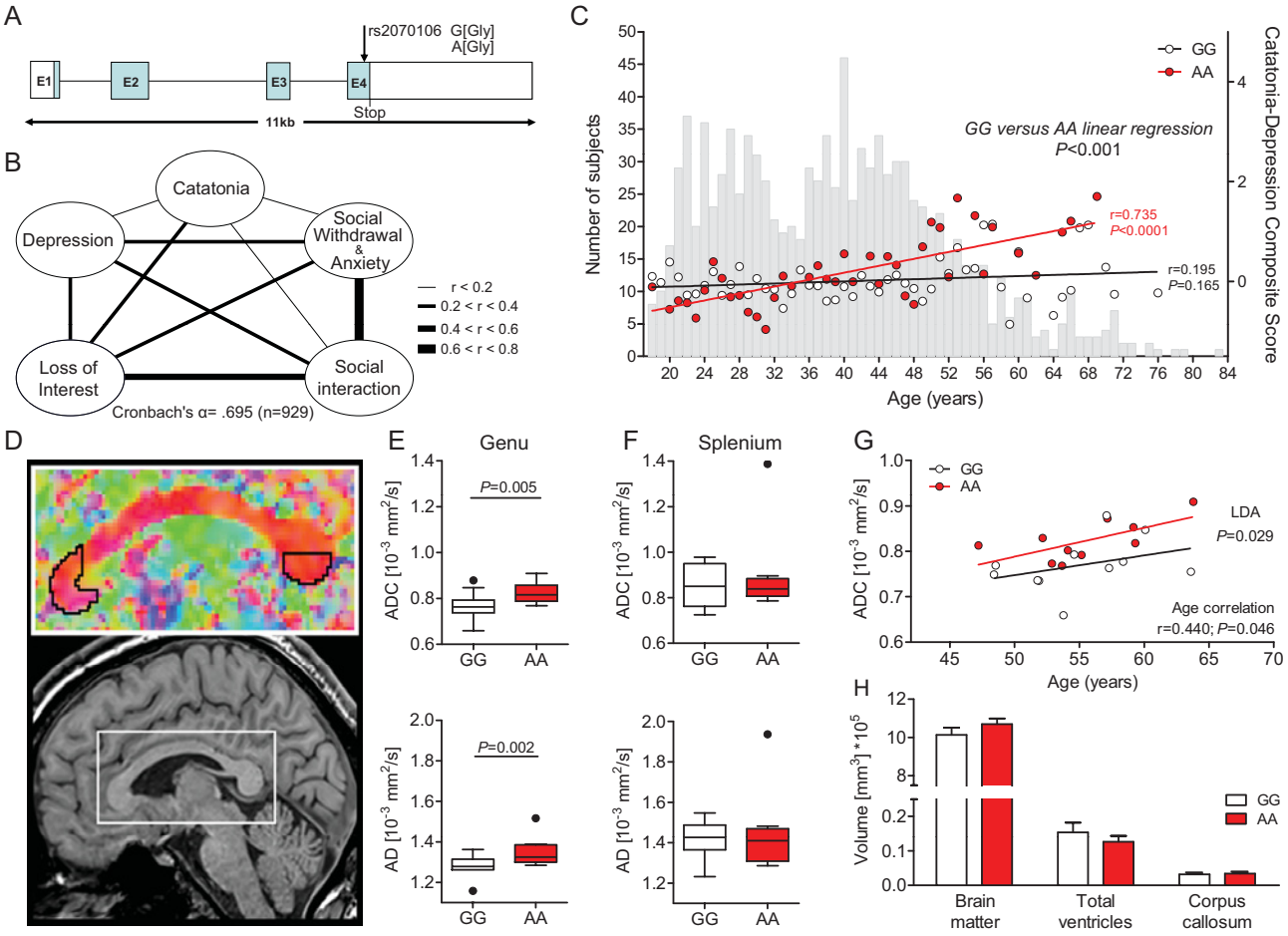

Figure 3. Age- and genotype-dependent association of the CNP rs2070106 SNP with a catatonia-depression syndrome in the GRAS sample of schizophrenic patients.

A. Schematic view of the human *CNP* gene structure and location of the synonymous SNP rs2070106 (A/G).

B. Intercorrelation network of all Z-transformed items of the catatonia-depression composite in the GRAS population. Line thickness indicates the degree of correlation between two respective items.

C. Correlation of genotypes with the catatonia-depression composite score across age groups. Grey bars in the background display the age distribution of the total GRAS sample of schizophrenic patients ($n = 1048$). Red or white circles denote mean values of the composite score for the respective age group and genotype (red, AA; black, GG). Linear regression lines of the genotypes dissociate after the age of 40 years. Pearson product-moment correlation applied.

D. Diffusion tensor imaging (DTI) study selecting the frontal (genu) and caudal (splenium) areas of the corpus callosum as regions of interest.

E,F ADC and AD values plotted according to rs2070106 homozygosity status in genu (**E**, target region) and splenium (**F**, control region) of the corpus callosum in a subgroup of schizophrenic individuals >40 years of age (GG $n = 11$ and AA $n = 10$); results corrected for chlorpromazine equivalents (CPZ). Mean ± s.e.m. presented and ANCOVA applied.

G. Correlation of ADC and age in AA and GG genotypes; linear discriminant analysis (LDA) with genotype as grouping variable and ADC and age as independent variables. Pearson product-moment correlation applied.

H. Magnetic resonance imaging (MRI) volumetric comparison of brain matter, ventricular system and corpus callosum between genotypes. Mean ± s.e.m. presented; two-sided Student's *t*-test applied.

underlying mechanism of this mental syndrome is a slowly progressive neurodegeneration, beginning in subcortical white matter, as described for the more rapid axonal loss in *Cnp* null mutant mice (Edgar et al, 2009; Lappe-Siefke et al, 2003). Importantly, the *CNP* loss-of-function genotype is causative of the here described behavioural syndrome but not of schizophrenia where it may only shape the aging phenotype.

In fact, the human part of this study has been obtained from a phenotypically extremely well characterized schizophrenic population (the 'GRAS data collection'), which was accessible and where all assessed items of the catatonia-depression syndrome are potentially relevant for disease subphenotypes. If a similar database on patients with, for example major depression had been available, the study would have been extended to this population. We expect that in individuals suffering from other mental disorders and even to some (perhaps mild) degree in healthy subjects, the phenotypical consequence of the *CNP* rs2070106 AA genotype will be

Table 1. Sociodemographic variables, composite score (target variable) and clinical/disease control variables of the GRAS sample of schizophrenic patients ≥40 years with homozygosity in *CNP* SNP rs2070106 (A/G) and – for comparison – in the subset of patients selected for DTI

	GRAS sample ≥40 years		P^b (F/χ^2)	DTI subsample	
	AA ($n = 45$)	GG ($n = 235$)		AA ($n = 10$)	GG ($n = 11$)
Sociodemographic variables					
Age, years, mean ± SD (range)	51.04 ± 7.65 (40.44–69.93)	50.16 ± 8.42 (44.03–79.49)	0.515	52.07 ± 4.74 (44.08–60.30)	49.50 ± 5.34 (40.66–58.71)
Gender, No. (%), male	26 (57.8%)	142 (60.4%)	0.740	7 (70%)	8 (72.7%)
Ethnicity, No. (%), Caucasian	43 (95.6%)	225 (95.7%)	0.892	10 (100%)	11 (100%)
Years of education[a], mean ± SD (range)	12.27 ± 3.82 (0–21)	12.32 ± 3.36 (0–27)	0.933	12.45 ± 3.39 (9–19.5)	14.14 ± 3.16 (8–19)
Target variable					
Catatonia-depression composite score, mean ± SD (range)	0.38 ± 0.86 (−1.13–1.91)	0.03 ± 0.74 (−1.20–2.44)	**0.009** **0.006**[c]	0.72 ± 0.94 (−0.99–1.85)	0.02 ± 0.76 (−0.97–0.97)
Clinical/disease control variables					
Age at first episode, years, mean ± SD (range)	29.77 ± 10.18 (15.26–55.61)	30.71 ± 9.58 (14.73–57.35)	0.533	30.80 ± 11.03 (19.35–49.61)	30.80 ± 11.03 (22.10–41.89)
Duration of disease (1st episode), years, mean ± SD (range)	21.02 ± 10.27 (0.16–47.35)	19.39 ± 10.86 (0.04–58.23)	0.359	21.27 ± 11.26 (0.16–39.13)	19.26 ± 9.19 (3.30–34.18)
CPZ, mean ± SD (range)	650.68 ± 515.35 (37.5–2295.0)	805.76 ± 915.14 (0–7375.0)	0.271	525.93 ± 276.29 (175–940)	352.61 ± 338.62 (0–1200)
PANSS pos, mean ± SD (range)	14.00 ± 6.81 (7–36)	14.55 ± 6.63 (7–35)	0.619	12.9 ± 6.01 (7–25)	11.27 ± 3.66 (7–17)
PANSS neg, mean ± SD (range)	19.79 ± 8.48 (7–38)	18.86 ± 8.21 (7–46)	0.503	21.1 ± 8.94 (7–35)	17.55 ± 5.87 (7–27)
PANSS gen, mean ± SD (range)	35.66 ± 14.17 (16–68)	34.65 ± 12.54 (16–82)	0.644	35.1 ± 11.20 (20–55)	29.82 ± 9.97 (17–51)
PANSS total, mean ± SD (range)	69.22 ± 27.27 (30–128)	68.10 ± 24.70 (30–160)	0.795	69.1 ± 23.64 (37–115)	58.64 ± 16.35 (31–90)
GAF, mean ± SD (range)	42.68 ± 20.22 (11–90)	43.82 ± 17.56 (10–90)	0.703	43.80 ± 14.21 (25–63)	56.55 ± 17.41 (35–85)
CGI, mean ± SD (range)	5.75 ± 1.35 (3–8)	5.62 ± 1.13 (2–8)	0.488	6.00 ± 0.94 (5–7)	5.09 ± 1.14 (3–7)

CPZ, chlorpromazine equivalents as measure of antipsychotic drug dose; PANSS, positive and negative syndrome scale (consisting of three parts: pos; positive symptoms; neg, negative symptoms; gen, general psychopathology); GAF, global assessment of functioning; CGI, clinical global impression (see Ribbe et al, 2010 for further details).

Due to missing data upon phenotyping, sample size varies between $n = 242$ and 280 in the sample of individuals with age equal to or above 40 years.

[a]Rating according to graduation/certificate; patients currently in school or in educational training are excluded.

[b]Statistical methods used: ANOVA or χ^2-test.

[c]Result after correction for CPZ.

comparable. Along these lines, we show that many schizophrenic patients (and virtually all patients younger than 40 years) lack this syndrome. We would therefore like to stress again that this syndrome is independent of the diagnosis schizophrenia, which is also supported by the behavioural homology of the *Cnp* mouse model.

Several studies have suggested that schizophrenia and affective disorders are on a continuum of liability. Genetic linkage and association studies have proposed common disease loci for both disorders (Berrettini, 2000; O'Donovan et al, 2008). Family studies document that first-degree relatives of bipolar patients have a threefold higher risk for schizophrenia compared with first-degree relatives of healthy controls (Sham et al, 1994; Valles et al, 2000). Psychopathological syndromes, as the catatonia-depression syndrome shown here, shared by subgroups of both patient populations, would also be compatible with this overlap. Indeed, catatonia has been found to be highly prevalent in elderly patients with major depression (Starkstein et al, 1996). It will be interesting to determine whether depressed individuals that exhibit catatonic signs are also preferentially carriers of the *CNP* rs2070106 AA genotype.

To our knowledge, no spontaneous catalepsy in mice has as yet been reported, in contrast to pinch- or drug-induced catalepsy/catatonia (for review see, *e.g.* Amir, 1986; Chaperon & Thiebot, 1999). The here observed *Cnp*+/− associated catalepsy/catatonia represents, therefore, the first clearly defined genetic catatonia model. Catatonia as a prominent phenotype has been extensively described by Karl Kahlbaum in 1874 (Kahlbaum, 1874) and entered the Diagnostic and Statistic Manual of Mental Disorders (APA, 2000) from its first edition in 1952 on, where it appears until now in connection with mood disorders, schizophrenia, and general medical conditions (Heckers et al, 2010). Nevertheless, reports on potential brain areas involved in this phenomenon in man are still scarce and point to frontal regions, based on, for example pronounced catatonia in a case with butterfly glioma of the frontal corpus callosum (Arora and Praharaj, 2007) or on a functional magnetic resonance imaging (MRI) study in akinetic catatonic patients during negative emotional stimulation (Northoff et al, 2004). We hypothesized that genotype-dependent axonal degeneration should be detectable in the frontal commissural fibres of the corpus callosum. These considerations were supported by the fact that the catatonia presented here in the context of a syndrome is characterized by several features of a primarily executive control (frontal lobe) deficiency in the absence of any 'classical' motor dysfunction. Indeed, we could localize axonal degeneration, determined by an increased axonal diffusivity in DTI, selectively to the genu corporis callosi.

The *CNP* rs2070106 AA genotype leads to reduced expression of CNP (Mitkus et al, 2008; Peirce et al, 2006), constituting 'partial loss-of-function'. Since there is an appreciable degree of linkage disequilibrium across the *CNP* gene (www.hapmap.org), the effects seen with the synonymous SNP rs2070106 might well be due to the influence of another genetic variant in

close vicinity (*e.g.* in the 3′-untranslated region (3′-UTR) of the *CNP* gene). Alternatively, according to previous studies, synonymous SNPs may modify translational timing due to differential codon usage (Kimchi-Sarfaty et al, 2007) or inactivate an exonic splicing silencer that compensates for other genetic variations in exonic splicing enhancers (Nielsen et al, 2007).

We demonstrated increased numbers of inflammatory cells, gliosis and axonal degeneration in old *Cnp*+/− mice suggesting an important role of low-grade inflammation in the described syndrome. Even though brain sections of human patients with the respective *CNP* genotypes were not available in the present study, the axonal degeneration detected by DTI is an intriguing observation that might point to the hypothesis of a comparable disease mechanism in mouse and man. Mechanistic details on the subcellular functions of CNP in myelinating oligodendrocytes have been reported (Gravel et al, 2009) and are under further investigation. The secondary neuroinflammation is a well-known cause of nitric oxide-mediated axonal stress and neurodegeneration (for review see Amor et al, 2010; Smith & Lassmann, 2002). We note that a diverse group of inherited myelinopathies in the nervous system of mice can trigger the recruitment of microglia/macrophages and T-cells (Ip et al, 2006; Kassmann et al, 2007; Martini & Toyka, 2004), demonstrating that low-grade inflammation is a rather unspecific response of myelinating glial cells to cellular stress, possibly related to perturbed lipid metabolism (Dumser et al, 2007). Interestingly, low-grade inflammation has been found to be associated with behavioural consequences in mouse studies (Bercik et al, 2010) and hypothesized to play a role in mental diseases (Gardner & Boles, 2011; Monji et al, 2009; Muller & Schwarz, 2008; Schnieder & Dwork, 2011). Respective first clinical trials employing antiinflammatory strategies in bipolar disease and schizophrenia yielded positive signals (Laan et al, 2010; Muller et al, 2010). Having information available on a predisposing genotype, individualized preventive and therapeutic approaches may be possible in the future.

To conclude, the major finding of the present study is the proof-of-principle that subtle changes of subcortical white matter can be the cause, rather than merely the consequence, of a complex neuropsychiatric syndrome. This distinction is extremely difficult in human patients with a psychiatric disease of unknown etiology, specifically when pharmacologically treated and only diagnosed (by MRI) with minor abnormalities of white matter tracts (Davis et al, 2003). Our analysis was possible by building on genetic variants of the cell type-specific *CNP* gene that lead to a partial loss-of-function genotype in both mouse and man. Importantly, *Cnp* heterozygosity (in mice) and moderately reduced *CNP* expression levels (in humans) are well tolerated until an advanced age. At that point, however, haploinsufficiency causes a striking phenotype in mice and shapes the phenotype of a complex psychiatric disease in humans. Although we have no ultimate proof that moderately reduced *CNP* levels in any individual (diseased or healthy) suffice to trigger a catatonia-depression syndrome upon aging, they clearly add to other genetic factors (here in patients diagnosed with schizophrenia) such that the catatonia-depression syndrome can be well defined and emerges as remarkably similar to the isolated behavioural phenotype of aged *Cnp* heterozygous mice. This amazing similarity of the behavioural phenotype in two different species emphasizes the relevance of glial dysfunction in psychiatric disorders, and supports the exploration of therapeutic strategies to target the associated low-grade neuroinflammation.

MATERIALS AND METHODS

Human studies

Healthy subjects

Blood donors ($n = 1045$; Begemann et al, 2010) were recruited for the case-control study. Ethnicity (Caucasian 97.8%; other ethnicities 2%; unknown 0.2%) was comparable to the patient population (Caucasian 95.5%; other ethnicities 1.8%; unknown 2.7%).

Schizophrenic patients

The GRAS data collection was approved by Ethics Committees of the Georg-August-University of Göttingen and participating centres, and comprises at present 1048 patients with Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV; APA, 2000) diagnosis of schizophrenia (81.7%) or schizoaffective disorder (18.3%), examined between 2005–2010 in 23 centres all over Germany (for details see Ribbe et al, 2010). Interviews, testing and ratings were conducted by an invariable team of trained examiners using the 'GRAS Manual' (Begemann et al, 2010; Ribbe et al, 2010).

Catatonia-depression composite

The score consists of five phenotype domains: *Depression* was operationalized by items 3 (*guilt feelings*) and 6 (*depression*) of general psychopathology subscale of Positive and Negative Syndrome Scale (PANSS) (Kay et al, 1987). *Catatonia* was based on catatonic signs subscale of the Cambridge Neurological Inventory (*gait mannerism*, *gegenhalten*, *mitgehen*, *imposed posture*, *abrupt or exaggerated spontaneous movements*, *iterative movements*, *automatic obedience* and *echopraxia*; Chen et al, 1995). *Deficits in social interaction* were built on items 1 (*blunted affect*) and 3 (*poor rapport*) of PANSS negative subscale, combined with item 44 (*never feeling close to another person*) of Brief Symptom Inventory (Derogatis & Melisaratos, 1983). *Social withdrawal/anxiety* was assessed by item 4 (*social withdrawal*) of PANSS negative subscale and item 12 (*suddenly scared for no reason*) of Brief Symptom Inventory. *Loss of interest in the outside world* was estimated by item 7 (*self-centred attitude*) of PANSS negative and item 15 (*preoccupation*) of general subscale. Phenotype domains were Z-standardized to be normally distributed with expectation zero and variance one. Higher values indicate worse outcome. Composite calculation was based on subjects without missing data ($n = 929$). Correlations of the five target phenotypes were assessed using Pearson product–moment correlation and internal consistency was determined using Cronbach's α.

Genotyping

Genotyping of SNP rs2070106 was performed using SimpleProbes (TIB Molbiol, Berlin, Germany) on LightCycler480 (Roche Diagnostics, Basel, Switzerland).

MRI/DTI

For MRI/DTI analyses, a subset of patients ≥40 years of both genotypes (GG $n = 11$; AA $n = 10$) from the GRAS sample was selected and matched according to age, gender and duration of disease. Studies were conducted at 3T (Tim Trio, Siemens Healthcare, Erlangen, Germany) using a 32-channel head coil. DTI was performed at 2 mm isotropic resolution using diffusion-weighted single-shot stimulated echo acquisition mode (STEAM) sequences (Hofer et al, 2010; Karaus and Frahm, 2009) combining 6/8 partial Fourier encoding and parallel imaging. Protocol comprised 24-independent diffusion gradient directions and *b*-values of 0 and 900 smm^{-2}. A total of 55 transverse sections (2 mm thickness) covered brain parts dorsal and ventral to the corpus callosum. To increase signal-to-noise ratio, acquisition was repeated three times (17 min). Anatomic images were based on T_1-weighted 3D fast low angle shot (FLASH) MRI sequence (repetition time TR = 11 ms, echo time TE = 4.9 ms, flip angle 15°).

DTI regions of interest (ROI)

Before calculation of diffusion tensor, diffusion-weighted MRI data sets were interpolated to 1 mm isotropic resolution and smoothed with a 3D Gaussian filter (half width 1 mm). Individual ROIs were manually defined on colour-coded maps of the main diffusion direction without thresholding. ROIs for the corpus callosum were placed in the midsagittal plane as well as in two directly neighbouring parasagittal sections covering central portions of genu and most posterior part of splenium (Hofer & Frahm, 2006). Mean values of fractional anisotropy (FA), ADC, AD and radial diffusivity (RD) were calculated.

MRI volumetry

Analyses were performed with an automatic brain segmentation tool for surface-based cortical thickness (http://surfer.nmr.mgh.harvard.edu). T_1-weighted images underwent corrections for intensity inhomogeneity, skull strip and registration into Talairach space followed by segmentation into grey matter, white matter and various brain areas. Regional differences of cortical thickness between patient groups were investigated using Qdec (FreeSurfer for multiple comparisons and voxel-based morphometry). Statistics relied on $p \leq 0.05$ (false discovery rate corrected for multiple comparisons). Visualization employed an inflated pial surface model.

Mouse studies

Mouse mutants

Experiments were carried out according to animal policies of the German Federal State of Niedersachsen. *Cnp*+/− mice were genotyped with primers *Cnp*-E3s, 5′-GCCTTCAAACTGTCCATCTC-3′; *Cnp*-E3as, 5′-CCCAGCCCTTTTATTACCAC-3′ and puro3, 5′-CATAGCCTGAAGAACGAGA-3′.

Immunostaining

Mice were anesthetized with Avertin (Sigma–Aldrich, Taufkirchen, Germany) and perfused through the left ventricle with 15 ml of Hank's balanced salt solution (Lonza, Basel, Switzerland), followed by 50 ml of 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were harvested and postfixed in 4% paraformaldehyde overnight at 4°C and then embedded in Paraplast (Surgipath Paraplast; Leica, Wetzlar, Germany). Microtome sections of 5 μm (Microm HM400, Walldorf, Germany) were prepared. For diaminobenzidine (DAB)-based immunostaining of paraffin sections, Dako-$LSAB_2$ system or Vectastain Elite ABC kit (Vector laboratories, Burlingame, CA, USA) were used according to manufacturer's instructions. Primary antibodies were directed against APP (1:750, Chemicon (Millipore) Billerica, MA, USA), CD3 (1:150, Serotec, Oxford, UK), GFAP (1:200, Novocastra (Leica) Newcastle Upon Tyne, UK), IBA-1 (1:1000, Wako, Neuss, Germany) and Mac-3 (1:400, BD Pharmingen, Franklin Lakes, NJ, USA).

Quantitative real time polymerase chain reaction (qRT-PCR)

qRT-PCR was performed using SYBR green master mix (Applied Biosystems, Foster City, CA, USA) and 7500 Fast Real-Time PCR System (Applied Biosystems). Specific qRT-PCR primers were designed by Roche Universal ProbeLibrary Assay Design Center (*Cnp*, forward 5′-TAACCCTCCCTTAGCCCCTG-3′, reverse 5′-GTCCCTAGCATGTGGCAGCT-3′; for normalization: *Atp5b* forward 5′-GGATCTGCTGGCCCCATAC-3′, reverse 5′-CTTTCCAACGCCAGCACCT-3′, *Rplp0* forward 5′-GATGCCCAGGGAAGACAG-3′, reverse 5′-ACAATGAAGCATTTTGGATAATCA-3′). Data were analysed with Microsoft Excel 2010.

Western blot

Myelin purified from protein lysates was performed according to (Norton & Poduslo, 1973). For Western blotting, proteins were size-separated in 12% sodium dodecyl sulphate (SDS)–polyacrylamide gels (0.2 μg/μl), blotted onto polyvinylidene difluoride membranes (Hybond P; GE Healthcare, München, Germany), blocked with 5% milk powder in Tris-buffered saline (TBS) and Tris-buffered saline + Triton X-100 (TBST; 150 mM NaCl, 10 mM Tris/HCl, pH 7.4; 0.1% Tween20), and incubated with primary antibodies (CNPase, 1:5000, Sigma, Saint Louis, MO, USA; Plp (A431), 1:5000; Jung et al, 1996), overnight at 4°C. Blots were washed with TBS/0.05% Tween20, incubated with appropriate secondary horseradish peroxidase-conjugated antibodies (Dianova, Hamburg, Germany), washed with TBS/0.05% Tween20 and developed by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Morphometry

Digitized overlapping light microscopic images (20× if not otherwise stated), fused to a continuous image of a complete corpus callosum (bregma 0.74 mm) by using Photoshop CS5 and ImageJ software were analysed for absolute numbers of IBA-1 and Mac-3 positive cells. To quantify GFAP positive areas, a plug-in for the ImageJ software for semi-automated analysis was implemented (http://www1.em.mpg.de/wieser). APP positive axonal spheroids (analysed at 40× magnification) and CD3 positive T-cells are expressed as total numbers quantified in corpus callosum, anterior commissure and striatum. For all stainings, two sections per mouse were quantified.

Behavioural testing

Tests were performed as described in detail previously, using the following order: Elevated plus maze (Radyushkin et al, 2010), open field (Radyushkin et al, 2010), light/dark box (Finn et al, 2003), rota-rod (Radyushkin et al, 2010), gait analysis (Brooks & Dunnett, 2009), grip strength (Radyushkin et al, 2010), hole board (Radyushkin et al, 2009), sociability (Moy et al, 2004), buried-food-finding test for olfaction (Radyushkin et al, 2009), floating behaviour (analysis of swimming/floating during a 90s trial in the Morris water maze pool; Morris, 1984;

The paper explained

PROBLEM:

Myelin and white matter abnormalities have been documented in neuropsychiatric diseases such as schizophrenia, major depression and bipolar disorder. However, their significance for disease mechanisms, pathogenesis or phenotypes is still obscure. A considerable number of postmortem studies found reduced expression of several myelin genes, including 2′,3′-cyclic nucleotide 3′-phosphodiesterase (*CNP*), in the brains of individuals with severe mental disease. In the present translational approach, we report for the first time phenotypical consequences of moderate *CNP* 'loss-of-function' genotypes, that is genetic variants leading to decreased *CNP* expression, both in man (single nucleotide polymorphism rs2070106) and mouse (heterozygous *Cnp* null mutant mice).

RESULTS:

We show that reduced *CNP* expression causes a distinct behavioural abnormality, seen only upon aging as an additional 'pro-inflammatory hit'. This phenotype is strikingly similar between *Cnp* heterozygous mice and patients with mental disease, carrying the AA genotype at *CNP* SNP rs2070106. The characteristic features in both species are best described as a 'catatonia-depression' syndrome and include bizarre posturing, depression, anxiety, loss of interest in the outside world and social withdrawal. As a consequence of perturbed *CNP* expression, mice show secondary low-grade inflammation and degeneration of nerve fibres. Analogously, in man, diffusion tensor imaging points to axonal loss in the frontal corpus callosum.

IMPACT:

Our genetic data demonstrate that subtle white matter abnormalities can be the cause of a psychiatric syndrome. To our knowledge, *CNP* is the first gene identified to be associated with catatonia, and aged heterozygous null mutant mice are the first animal model of spontaneous catatonia. Moderately reduced *CNP* expression contributes to a distinct phenotype, which is not restricted to a single diagnostic category but could explain features of catatonia-depression in different mental disorders and possibly – to a milder degree – even in aging healthy individuals. This knowledge will help defining subgroups of (aging) subjects who may profit from novel, more specific therapeutic approaches including anti-inflammatory strategies.

Stone & Lin, 2011), tail suspension test (Cryan et al, 2005), bar test (Kuschinsky & Hornykiewicz, 1972; see Fig 2P and videos of Supporting Information) and fear conditioning (Radyushkin et al, 2009).

Mouse score

For the catatonia-depression score, five phenotype domains were created: (I) *Depression* was operationalized by floating time and tail suspension (delta time of immobility in the last 2 min minus first 2 min), (II) *catatonia* by time on bar, (III) deficit in *social interaction* by delta time spent with stranger *versus* empty compartment, (IV) *anxiety* by open field-duration in centre, elevated plus maze – open arm visits, and fear conditioning – freezing at baseline in cue task and (V) *loss of interest* by hole board – number of head dips. Composite score calculation was done in analogy to the human score and based on mice with not more than two variables missing ($n = 27$).

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 17.0 (https://www.spss.com/de) and Prism5 (GraphPad Software, San Diego, CS, USA). Exact procedures are indicated in the respective sections. **Note**: All experiments/analyses in both men and mice were performed by persons unaware of genotypes ('blinded').

Author contributions

NH, SG, SP, KAN and HE developed study concept and design; NH performed all behavioural analysis of *Cnp*+/− mice; SP and AK carried out human genetic analyses and performed the human association study; UCG and GLW performed the histological analyses of *Cnp*+/− mice under supervision of SG; DTI study with human subjects was performed by SH and analysed under the supervision of SB and JF; Administrative, technical and material support was provided by MB, AR and AG in different aspects of the study; MB and AK coordinated and supervised the recruitment of subjects for the DTI study; SHH, SB and JF gave input to data analysis, interpretation, and manuscript preparation; NH, SG, SP, AK, KAN and HE wrote the manuscript; KAN and HE had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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Supporting information

A myelin gene causative of a catatonia-depression syndrome upon aging

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Contents:

Supporting Table I

Case-control analysis for SNP rs2070106 in exon 4 of *2',3'-cyclic nucleotide 3'-phosphodiesterase* (*CNP*) human gene. No statistically significant differences were observed between schizophrenic /schizoaffective cases and controls with respect to genotypic and allelic frequencies.

Case-control				
	Genotypes			***P-value***
	GG	**GA**	**AA**	***Genotypic***
Controls (n=1045)	459 (43.9%)	476 (45.6%)	110 (10.5%)	0.716
Cases (n=1048)	477 (45.5%)	459 (43.8%)	112 (10.7%)	
	Alleles			***P-value***
	G allele		**A allele**	***Allelic***
Controls (2n=2090)	1394 (66.7%)		696 (33.3%)	0.622
Cases (2n=2096)	1413 (67.4%)		683 (32.6%)	

Both cases and controls fulfilled Hardy-Weinberg equilibrium criteria (Chi-square test P=0.99 and P=0.71, respectively)

Supporting Table II

Catatonia-depression composite score: Descriptives and ANOVA results upon inclusion of all 3 genotypes. The data show that heterozygous (GA) subjects have very similar composite score to GG carriers; they do not reveal an intermediate phenotype or A allele dosage-dependent effect.

Descriptives

Composite score

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
AA	40	.3794	.86258	.13639	.1036	.6553	-1.13	1.91
GA	188	.0497	.76584	.05585	-.0605	.1599	-1.20	2.72
GG	202	.0308	.74461	.05239	-.0725	.1341	-1.20	2.44
Total	430	.0715	.77000	.03713	-.0015	.1445	-1.20	2.72

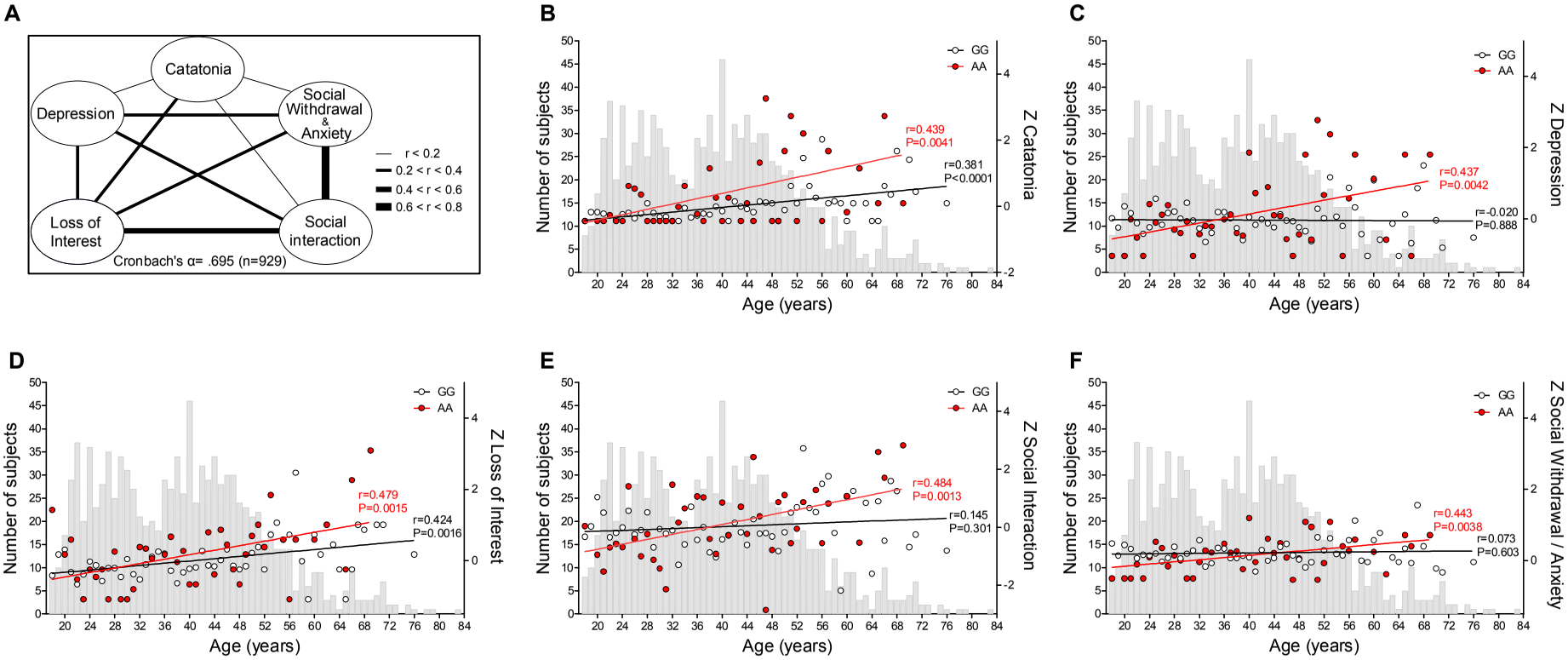
ANOVA

Composite score

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.217	2	2.108	3.599	.028
Within Groups	250.138	427	.586		
Total	254.355	429			

Supporting Figure 1

Correlation of genotypes with the catatonia-depression composite score sub-items across age groups
(A) Composite score presented for overview; **(B)-(F)** Grey bars in the background display the age distribution of the total GRAS sample of schizophrenic patients (n=1048). Red or white circles denote the mean values of the individual sub-item according to the respective age group and genotype (red=AA; black=GG). In most panels, the linear regression lines of the genotypes dissociate clearly after the age of 40 years. **(B)** Catatonia; **(C)** Depression; **(D)** Loss of interest; **(E)** Social interaction; **(F)** Social withdrawal/anxiety. Compare Figure 3C, main text.



Supporting Videos:

1 - Catalepsy Test Cnp+/-

2 - Catalepsy Test Wt

A phenotype, thus far observed in mice only upon pinching or drug exposure (cannabinoids) is catatonia/catalepsy, a state of immobility where mice persist in an externally imposed abnormal posture for a prolonged time period. In the bar test, 24 months old male mice are put into a position where they have to grab a bar while standing with their hind paws on the floor. *Cnp+/-* mice persisted in this posture (Video 1), whereas Wt mice swiftly left this position (Video 2).

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Research report

Development of an autism severity score for mice using *Nlgn4* null mutants as a construct-valid model of heritable monogenic autism

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HIGHLIGHTS

- Loss-of-function mutations of *NLGN4X* are the most frequent monogenic autism cause.
- *Nlgn4*KO mice show reduced social functions/communication and increased stereotypies.
- Females exhibit a slightly milder phenotype.
- For the first time a gender-specific autism severity composite score is presented.
- These data favour *Nlgn4* mutant mice as an ASD model with construct and face validity.

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ABSTRACT

Autism is the short name of a complex and heterogeneous group of disorders (autism spectrum disorders, ASD) with several lead symptoms required for classification, including compromised social interaction, reduced verbal communication and stereotyped repetitive behaviors/restricted interests. The etiology of ASD is still unknown in most cases but monogenic heritable forms exist that have provided insights into ASD pathogenesis and have led to the notion of autism as a 'synapse disorder'. Among the most frequent monogenic causes of autism are *loss-of-function* mutations of the *NLGN4X* gene which encodes the synaptic cell adhesion protein neuroligin-4X (NLGN4X). We previously described autism-like behaviors in male *Nlgn4* null mutant mice, including reduced social interaction and ultrasonic communication. Here, we extend the phenotypical characterization of *Nlgn4* null mutant mice to both genders and add a series of additional autism-relevant behavioral readouts. We now report similar social interaction and ultrasonic communication deficits in females as in males. Furthermore, aggression, nest-building parameters, as well as self-grooming and circling as indicators of repetitive behaviors/stereotypies were explored in both genders. The construction of a gender-specific autism severity composite score for *Nlgn4* mutant mice markedly diminishes population/sample heterogeneity typically obtained for single tests, resulting in p values of <0.00001 and a genotype predictability of 100% for male and of >83% for female mice. Taken together, these data underscore the similarity of phenotypical consequences of *Nlgn4/NLGN4X loss-of-function* in mouse and man, and emphasize the high relevance of *Nlgn4* null mutant mice as an ASD model with both construct and face validity.

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1. Introduction

The term autism spectrum disorders (ASD) describes a group of etiologically heterogeneous conditions, forced under a common label according to presently used clinical classification systems of psychiatric diseases. The purely phenotypical diagnosis – with all its pitfalls and differential diagnoses on top – blinds out the tremendous heterogeneity of ASD and likely also accounts for the low interrater reliability achieved in the clinical diagnosis of adult autism [1,2]. The discovery of monogenic heritable forms of the disorder involving mutations in genes encoding neuroligin-4X (NLGN4X), neurexin-1 (NRXN1), neuroligin-3 (NLGN3), SHANK2, and SHANK3, has not only strengthened the notion that autism might be a disease of the synapse but also led to the development of urgently needed mouse models with proven construct validity [3–9]. In the past, only animal models with some face validity could be used to study ASD and to search for therapeutic strategies [10] but no effective treatment for humans is available to date that would measurably improve core symptoms of ASD. More adequate animal models are expected to improve this situation.

Strikingly, several monogenic mouse models with disruption of only one synaptic gene display an array of entirely different symptoms, reminiscent of autism, i.e. compromised verbal and non-verbal communication, disturbed social interaction and social skills, narrowed interests and stereotypical repetitive behaviors ('routines'), impulsivity and altered aggression (e.g. for review, see [11–13]). Comparable to humans, however, the presentation of symptoms with respect to their severity varies considerably among individual mice even if they carry the same ASD-related mutation. Therefore, an autism severity composite score rather than the selection of single readouts might better assist in the search for new treatments addressing the core features of the disorder.

The present study has been designed (1) to comprehensively characterize the behavior of *Nlgn4* null mutant mice, a construct-valid model of monogenic heritable autism, extending previous work [4] to a greater variety of symptoms, particularly to repetitive stereotyped behaviors, and to the female gender which has never been studied; (2) to develop an autism composite score with highest power of contrasting between autistic and non-autistic individuals as an important basis for future therapeutic strategies.

2. Materials and methods

2.1. Mice

For all experiments reported here, male and female *Nlgn4* null mutant (*Nlgn4*−/−) and wildtype (WT) C57BL/6J mice (littermates) were used. They were derived from revitalized frozen embryos that originated from a C57BL/6J-SV129 mosaic *Nlgn4*−/− line that had been backcrossed into C57BL/6J for 6 generations [4]. After revitalization, *Nlgn4*−/+ mice were bred with C57BL/6J for 2 further generations, and then *Nlgn4*−/+ mice were interbred for colony expansion to generate mice for experiments. The revitalization was initiated since there had been concerns raised by others regarding a potential loss of phenotypical changes in these mice with increasing numbers of generations ('phenotype bleaching'). See also the paper by [14]. These concerns even led us to prematurely unblind an 8-arm preclinical treatment study with *Nlgn4*−/− versus WT mice (littermates) of generation 13, which had been running in our lab. Due to the still small number of untreated (control) mice available for behavioral analysis at the time point of unblinding this study, we cannot make any firm conclusions regarding reduction or stability of the originally reported phenotype [4]. The tendencies obtained, however, clearly do not support a loss of phenotypic changes in *Nlgn4*−/− (see Supplementary Figures 1–3). Nevertheless, such gradual 'bleaching' of phenotypic changes over generations with respect to higher brain functions may well happen, as illustrated by our follow-up studies of another mouse strain, cEPOR transgenic mice, where the transgene expression remained stable over the respective generations, but the initially superior spatial learning and memory, reversal learning/cognitive flexibility and activity gradually returned to control levels ([15]; Supplementary Figure 4).

Mouse genotyping: WT and *Nlgn4*−/− littermates were obtained from *Nlgn4*+/− heterozygous breeding pairs. Genotypes of the offspring were analyzed by PCR of tail genomic DNA using the following primers: Forward primer 5′-CTTCCTATCCTGTTACTCTCAC-3′, WT reverse primer 5′-TAGGGAAAGCGGAATTGAGTGTAAC-3′ (yielding a 475 bp product) and KO reverse primer 5′-ACACTCCAACCTCCGCAAACTCCT-3′ (yielding a 183 bp product). PCR amplification of the DNA was carried out with the following conditions: 5 min, 94 °C (1 cycle); 30 s, 94 °C; 30 s, 64 °C; 1 min, 72 °C (30 cycles), followed by final extension at 72 °C for 7 min.

2.2. Behavioral testing

All experiments were approved by the local Animal Care and Use Committee in accordance with the German Animal Protection Law. For behavioral testing, mice were housed in groups of 3–5 (except where otherwise specified) in standard plastic cages, with food and water ad libitum. The temperature in the colony room was maintained at 20–22 °C, with a 12 h light–dark cycle (light on at 7:00 am). All behavioral experiments were conducted by investigators, unaware of the genotype ('blinded'), during the light phase of the day (between 8:00 am and 5:00 pm). Basic behavioral functions were assessed in 2 large consecutive cohorts of male and female mice (genders tested separately) in the following order: elevated plus maze, open field, hole board, rota-rod, pre-pulse inhibition of the startle response (PPI), social interaction in pairs, ultrasound vocalization, and LABORAS spontaneous home cage behavioral assessment. The second cohort (again genders tested separately) was additionally evaluated in LABORAS, in a modified version of social interaction in the tripartite chamber [16], olfaction, marble burying, nest building, induced self-grooming in LABORAS as well as in enriched environment, and finally aggression, using the resident-intruder paradigm. The age of mice at the beginning of testing was 11–12 weeks. Inter-test interval varied depending on the degree of 'test invasiveness' but was at least 1 day.

Elevated Plus Maze Test: Individual animals were placed on the central platform facing an open arm of the plus-maze (made of gray Perspex with a 5 cm × 5 cm central platform, 2 open arms of 30 cm × 5 cm, and 2 closed arms of 30 cm × 5 cm × 15 cm, with overall illumination at 135 lx). Behavior was recorded for 5 min by an overhead video camera and a computer equipped with Viewer 2 software (BIOBSERVE GmbH, St. Augustin, Germany) to calculate the time each animal spent in open or closed arms. The proportion of time spent in open arms was used for the estimation of open arm aversion, which is an indicator of fear.

Open Field Test: Spontaneous activity in the open field was tested in a gray circular Perspex arena (120 cm in diameter, 25 cm high). Individual animals were placed in the center of the open field and were allowed to explore it for 7 min. The behavior was recorded by a computer-linked overhead video camera. Viewer 2 software (BIOBSERVE GmbH) was used to calculate the distance traveled and the time spent in the central, intermediate, and peripheral zones of the open field.

Hole Board Test: Individual mice were placed in the center of the hole board (transparent Perspex chamber (50 cm × 50 cm × 36 cm), with a non-transparent floor raised 3 cm above the bottom of the chamber with 16 equally spaced holes of 2.2 cm diameter), and allowed to explore the chamber for 5 min. The number of holes explored (head dips) was monitored by 2 layers of infrared photo beams connected to a computer with the AKS software (TSE Systems GmbH, Bad Homburg, Germany).

Rota-Rod Test: The rota-rod (Ugo Basile Srl, Comerio, Italy) comprised a rotating drum which was accelerated from 4 to 40 rpm over the course of 5 min. Individual mice were placed on the drum, and once they were balanced, the drum was accelerated. The time in seconds at which the respective animal fell from the drum was recorded using a trip switch. Each animal went through 3 consecutive trials, one trial per day.

Pre-Pulse Inhibition Test: Individual mice were placed in small metal cages (82 mm × 40 mm × 40 mm) to restrict major movements and exploratory behavior. The cages were equipped with a movable platform floor attached to a sensor that recorded vertical movements of the floor. The cages were placed in 4 sound-attenuating isolation cabinets (TSE Systems GmbH). Startle reflexes were evoked by acoustic stimuli delivered from a loudspeaker that was suspended above the cage and connected to an acoustic generator. The startle reaction to an acoustic stimulus, which evokes a movement of the platform and a transient force resulting from this movement of the platform, was recorded with a computer during a recording window of 260 ms and saved for further evaluation. The recording window was defined from the onset of the acoustic stimulus. An experimental session consisted of a 2 min habituation to 65 dB background white noise (continuous throughout the session), followed by a baseline recording for 1 min at background noise. After baseline recording, 6 pulse-alone trials using startle stimuli of 120 dB intensity and 40 ms duration were applied to decrease influence of within-session habituation. These data were not included in the 120 dB/40 ms analysis of the pre-pulse inhibition. For tests of pre-pulse inhibition, the startle pulse was applied either alone or preceded by a pre-pulse stimulus of 70 dB, 75 dB, or 80 dB intensity and 20 ms duration. An interval of 100 ms with background white noise was used between each pre-pulse and pulse stimulus. The trials were presented in a pseudorandom order with an interval ranging from 8 to 22 s. Amplitude of the startle response (expressed in arbitrary units) was defined as a difference between the maximum force detected during a recording window and the force measured immediately before the stimulus onset. Amplitudes were averaged for each individual animal, separately for both types of trials (i.e. stimulus alone or stimulus preceded by a pre-pulse). Pre-pulse inhibition was calculated as a percentage of the startle response using the following

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formula: % pre-pulse inhibition = 100 − [(startle amplitude after pre-pulse)/(startle amplitude after pulse only) × 100].

Sociability and Social Memory Tests: Sociability and social memory were tested as described [16] with small modifications. The social testing arena was a rectangular, 3-chambered box. Each chamber was 40 cm × 20 cm × 22 cm in size. Dividing walls were made from clear Plexiglas, with rectangular openings (35 mm × 220 mm) allowing access into each chamber. The chambers of the arena were cleaned and fresh wood-chip bedding was added between trials. The test mouse was first placed in the middle chamber and allowed to explore for 5 min. The openings into the 2 side chambers were obstructed by plastic boxes during this habituation phase. After the habituation period, a C57BL/6N male mouse of the same age (stranger 1) without prior contact with the test mouse was placed in one of the side chambers. The location of stranger 1 in the left versus right side chamber was systematically alternated between trials. The stranger mouse was enclosed in a small (140 mm × 75 mm × 60 mm), rectangular wire cage, which allowed nose contact through the bars but prevented fighting. The animals serving as strangers had previously been habituated to placement in the small cage. An identical empty wire cage was placed in the opposite chamber. A weighted cup was placed on the top of the small wire cages to prevent climbing by the test mice. Both openings to the side chambers were then unblocked and the subject mouse was allowed to explore the entire social test arena for a 10 min session. The amount of time spent in each chamber and the number of entries into each chamber were recorded by the video-tracking system Viewer 2 (BIOBSERVE GmbH). An entry was defined as all 4 paws in one chamber. At the end of the first 10 min, each mouse was tested in a second 10 min session where stranger 1 changed the side to avoid bias by side preference. At the end of this session, mice were tested in a third 10 min episode to quantify social preference for a new stranger. A second, unfamiliar mouse of the same age (stranger 2) was placed into the previously empty wire cage. The test mouse had a choice between the already familiar stranger 1, and the novel unfamiliar stranger 2. As described above, time spent in each chamber and the number of transitions between chambers of the apparatus during the first (social preference) and third 10 min session (social memory) were analyzed. Based on the amount of time spent in each chamber, a 'sociability index' and a 'social memory index' were calculated according to the following formulas:

$$\text{sociability index} = \frac{\text{time}_{\text{stranger}}}{\text{time}_{\text{stranger}} + \text{time}_{\text{empty}}} \times 100$$

$$\text{memory index} = \frac{\text{time}_{\text{novel}}}{\text{time}_{\text{novel}} + \text{time}_{\text{familiar}}} \times 100$$

Test of Social Interaction in Pairs, i.e. 'Social Approach': The social interaction test was performed in a neutral cage (gray Plexiglas box, 30 cm × 30 cm × 30 cm). During 2 consecutive days, each individual mouse went through one 10 min session in the neutral cage to habituate to the testing conditions. On day 3 (test day), pairs of unfamiliar mice of the same genotype were placed into the neutral cage for 10 min. Behavior of mice was recorded by a computer and the video-tracking system Viewer 2 (BIOBSERVE GmbH). The time spent in social interaction (defined as staying in close contact) was registered.

Buried Food Finding Test: Starting 6 days before testing, mice were habituated to clear cages (29.5 cm × 18.5 cm × 13 cm) for 20 min, 4× per day. Starting 4 days before testing, mice received a piece of chocolate cookie (1.6 g) with water ad libitum at each habituation. Additionally, mice received 3–5 cookies in their home cage over night which they consumed within 24 h. When mice consumed also the cookies during the habituation phase, testing was performed on the next day. Starting 12 h before testing, mice were deprived of food with water ad libitum. For testing, individual mice were placed into clear cages, in which a piece of chocolate cookie was hidden under 1.5 cm standard bedding at the end of the cage. The mouse was positioned in the right corner at the opposite end of the cage, and the food finding time, i.e. the time from the moment the mouse was placed into the cage to the time it located the cookie and initiated burrowing, was recorded. As soon as the cookie was detected, the mouse was removed from the cage. Fresh bedding was used for each trial; all mice underwent identical testing procedures.

Resident-Intruder Test: Inter-male aggression was studied using the resident–intruder paradigm (e.g. [17,4]). Previously group housed male mice were separated and housed individually for 21 days before testing. As standard opponent males, we used group-caged males of the same age (4 months) from the C57BL/6N strain (Charles River, Sulzfeld, Germany). A standard opponent was introduced into the cage of the tested resident male and observation started when a tested resident male sniffed the opponent for the first time. The observation was stopped immediately after the first attack (an attack being defined as a bite) to prevent wounding, but lasted 10 min if no attack occurred. The latency to attack was recorded by a stop watch.

Ultrasound Vocalization Analysis: We recorded ultrasonic vocalizations (USVs) of male mice using the recording software Avisoft Recorder 4.2 at a sampling frequency of 300 kHz. The microphone (UltraSoundGate CM16) was connected to a preamplifier (UltraSoundGate 116), which was connected to a computer (all sound recording hardware and software was from Avisoft Bioacoustics, Berlin, Germany). For the recording, resident mice (males and females) were housed in single cages. At the day of the recording, mice in their own home cage were placed on the desk in the recording room for 60 s. Subsequently, the intruder mouse was put into the home cage of the resident, and the vocalization behavior was recorded for 3 min. We recorded male mice with unfamiliar female mice in estrous. Female mice were tested with anaesthetized unfamiliar females (anesthetic: intraperitoneal injection of 0.25% tribromoethanol, 0.1 ml/10 g body weight). We counted the number of calls per recording session and separated USVs from other sounds using the whistles detection algorithm of AVISOFT RECORDER 4.2 with following selection criteria: Possible changes per step = 4 (4687 Hz), minimal continuity = 8 ms, possible frequency range = 35–150 kHz. These criteria had been tested in former studies of mouse USVs [4,18].

LABORAS: The LABORAS™ system (Metris b.v., Hoofddorp, The Netherlands) consists of a triangular shaped sensor platform (Carbon Fiber Plate 1000 mm × 700 mm × 700 mm × 30 mm, Metris b.v.), positioned on 2 orthogonally placed force transducers (Single Point Load Cells) and a third fixed point attached to a heavy bottom plate (Corian Plate 980 mm × 695 mm × 695 mm × 48 mm, Metris b.v.). The whole structure stands on 3 spikes, which are adjustable in height and absorb external vibrations. Mice are housed in clear polycarbonate cages (Makrolon type II cage, 22 cm × 16 cm × 14 cm) with a wood-chip bedding covered floor. The cage is placed directly onto the sensing platform, with the upper part of the cage (including the top, food hopper and drinking bottle) suspended in a height-adjustable frame separate from the sensing platform. Resultant electrical signals caused by the mechanical vibrations of the movement of the animal are transformed by each force transducer, amplified to a fixed signal range, filtered to eliminate noise, digitized and then stored on a computer. The computer then processes the stored data using several signal analysis techniques to classify the signals into the behavioral categories of eating, drinking, scratching, circling, climbing, immobility, locomotor activity and grooming (for details see [19]). The behavior which dominates is scored. Spontaneous mouse behavior was assessed from 5:00 pm until 9:00 am, with 1 h habituation to the cages before the initiation of recording. Behaviors during the light as well as the dark cycle were analyzed separately.

Marble Burying Test: This test is used to assess stereotypies and obsessive–compulsive behaviors in mice [20]. Mice were tested in plastic cages (34.5 cm × 56.5 cm × 18 cm) filled with 5 cm deep wood-chip bedding. Evenly spaced (4 cm apart) 24 glass marbles were placed on the surface. Individual mice were put in the cage and left there for 30 min. Illumination was dimmed (6 lx). The number of buried marbles (to 2/3 their depth) during this time was counted. Two groups of mice with different housing conditions were tested. Mice were single housed for at least 10 days. Another group was left in the group housing cages.

Nest Building: Nest construction is an important indicator of social and reproductive behavior [21,22]. The nest building test was conducted according to a previously reported protocol [21]. Approximately 1 h before the dark phase, group housed mice were transferred to single housing cages with wood-chip bedding and nesting towels, but no environmental enrichment items. After 2 nights of habituation, nesting towels were removed and nestlets (pressed cotton squares, weighing ~3 g) placed in the single housing cages. Nest building was assessed on the next morning using a rating scale where high scores indicate an almost perfect nest.

Induced Self-Grooming in LABORAS and Enriched Environment: Grooming is a common and robust behavior in rodents. Excessive grooming has been observed in animal models of obsessive-compulsive disorders as well as ASD [23,8]. The natural tendency to groom might be amplified by various experimental procedures [24]. We used water induced self-grooming according to a previously described protocol [24] with slight modifications. Mice were misted with water and placed on the LABORAS platforms (see above) under red light for a 30 min recording session. Behavior was simultaneously video-recorded. The misting procedure was as follows: Mice were taken by their tail and lifted over a table, then misted with a spray bottle filled with water at room temperature. The misting pattern was 3× on the back (distance 30 cm), 3× on the abdomen and again 3× on the back. The analysis of LABORAS data was done as described above. Video-recorded grooming duration was estimated by 2 independent raters, unaware of the genotype. After an interval of 2 weeks, animals were placed into cages with enriched environment (with paper towel, toilet rolls, running wheel) and kept there for 72 h. Then grooming was induced again as described above and behavior was recorded (under red light in the enriched environment cages) with a video camera. Video ratings were conducted in the same manner as described above.

Statistical Analysis: Unless stated otherwise, the data given in figures and text are expressed as mean ± SEM, and were compared by 2-way analysis of variance (ANOVA) with post hoc planned comparisons, or by ANOVA for repeated measurements, Mann–Whitney U and χ^2 test where appropriate, using SPSS v.17 Software, San Diego, USA. A p value below 0.05 was considered to indicate significance.

Autism Composite Score: For the autism composite score, selected single readouts were z-standardized and presented such that higher values represent higher symptom severity. Relevant items were selected based on significant Mann–Whitney U test results. To build the scores on complete data sets, for each behavioral test, a linear regression based multiple imputations (10 iterations) model of missing data (males: vocal calls 17.6%, nesting score 23.5%, social approach 11.8%, marble burying 23.5%, circling 35.3%, aggression 32.4%; females: vocal calls 2.8%, novelty index 2.8%, social approach 11.1%, marble burying 16.7%, circling 52.8%) was applied to the z-standardized single tests for male and female mice separately. A total of 17 male mice (N = 13 WT and N = 4 KO) had to be excluded prior to imputation because more than

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3 out of 6 readouts were missing. Altogether 4 female mice ($N = 4$ WT) were excluded because more than 3 out of 5 readouts were lacking. Thus, the imputation models were based on 34 ($N = 16$ WT and $N = 18$ KO) male and 36 ($N = 21$ WT and $N = 15$ KO) female individuals. Note that missing values are mainly due to test logistics (limitations of mouse numbers running in a certain test); there was no other systematic reason for exclusion of animals from particular tests, and exclusions were all random. Gender specific composite scores were calculated by integrating the means of the imputation matrices of all behavioral readouts. Genotype dependent group comparisons were conducted by Mann–Whitney U tests. Intercorrelation patterns (pairwise Pearson correlations) were based on available sets of z-standardized raw scores.

3. Results

3.1. Basic behaviors are normal in male and in female Nlgn4 null mutant mice

Basic behavioral test results for activity, anxiety, motor performance, exploratory behavior and sensorimotor gating of both male and female *Nlgn4* null mutant mice are shown separately in Supplementary Figures 5 and 6. Genotypes did not differ in elevated plus maze, open field, rota-rod, hole board, startle response and PPI performance.

3.2. Nlgn4 null mutants of both genders display broadly impaired social behaviors and communication

To comprehensively characterize the social phenotype in adult *Nlgn4* null mutants of both genders, we assessed social approach behavior, vocalization, aggression and nest building (Fig. 1). Consistent with our previously reported findings in male *Nlgn4* null mutants only [4], we again saw significantly reduced interaction time in males (Fig. 1A; $p = 0.0006$). This observation was now also made independently in female mutants (Fig. 1B; $p = 0.015$). Additionally, the number of calls was significantly reduced in both genders of *Nlgn4* null mutants (Fig. 1C, D; $p = 0.006$ and $p = 0.0097$, respectively). Again, as shown earlier [4] male *Nlgn4* null mutants had a higher attack latency in the resident-intruder paradigm (Fig. 1E; $p = 0.025$). In a newly performed test, nest building, only male mutants had a higher proportion of untouched nesting material (Fig. 1F, inset, $p = 0.049$) and built significantly less functional nests (Fig. 1G; $p = 0.006$). Female mutants showed the same tendency as the males. Their potential performance deficits, however, did not reach statistical significance (Fig. 1F, H).

3.3. Deficits in social functions of Nlgn4 null mutants as determined in the tripartite chamber are mild and look different dependent on gender

As reported previously [4], male *Nlgn4* null mutants showed a deficit in the first trial of social interaction in the tripartite chamber (Supplementary Figure 7A; 2-way ANOVA RM, $F_{\text{interaction}(1,39)} = 8.935$, $p = 0.0048$; $F_{\text{compartment}(1,39)} = 94.45$, $p < 0.0001$; $F_{\text{genotype}(1,39)} = 0.6493$, $p = 0.4253$). Post hoc single comparisons revealed that mutant mice spent less time in the compartment with the stranger mouse, and more time in the empty compartment (mouse compartment: $p = 0.018$; empty compartment: $p = 0.02$). Consequently, male mutant mice had a significantly lower sociability index as compared to WT animals (Supplementary Figure 7C; $p = 0.0058$). In contrast, in the present series of tests, social novelty was indistinguishable between WT and mutant males (Supplementary Figure 7E,G). On the other hand, sociability was not affected in female *Nlgn4* null mutants (Supplementary Figure 7B,D) which, however, showed a deficit in social memory (Supplementary Figure 7F; 2-way ANOVA RM, $F_{\text{interaction}(1,37)} = 6.444$, $p = 0.0155$; $F_{\text{compartment}(1,37)} = 3.163$, $p = 0.0835$; $F_{\text{genotype}(1,37)} = 3.826$, $p = 0.0581$). Post hoc single comparisons revealed a significantly reduced time spent with the new mouse in the female mutant group (Supplementary Figure 7F, $p = 0.015$) and thus a reduction in their novelty index (Supplementary Figure 7H; $p = 0.036$).

3.4. Stereotyped repetitive behaviors are detectable in Nlgn4 null mutant mice of both genders

Since stereotypies are an important feature of ASD, we decided to study this trait in *Nlgn4* null mutants and their WT littermates in more detail. To this end, we assessed marble burying both in single and group housed mice. Single housing has previously been used as an environmental stressor leading to subtle alterations in behavior [25]. Interestingly, only single housed null mutants of both genders had an increased number of buried marbles (Fig. 2A, B, left panels; Mann–Whitney U tests, $p = 0.0281$ and 0.0093 respectively). Group housed mice did not demonstrate a genotype effect (Fig. 2A, B, right panels). Spontaneous homecage behavior assessed via LABORAS revealed other compulsive features: *Nlgn4* null mutants of both genders had more circling episodes (Fig. 2C, D, left panels; Mann–Whitney U tests, $p = 0.0244$ and $p = 0.0424$ respectively), with general locomotion being unaffected (Fig. 2C, D, right panels).

Grooming is considered to be an ecologically relevant behavioral trait in mice [26]. Excessive grooming has been reported in various mouse lines [23,27,28] and is regarded to be analogous to human obsessive–compulsive behaviors (e.g. [29]). To analyze grooming under standardized conditions, we misted mice with tap water and recorded self-grooming for 30 min under standard single housing conditions which revealed a significant interaction effect in female mice (Fig. 2F, left panel: $F_{\text{interaction}(2,54)} = 5.888$, $p = 0.0049$; $F_{\text{time}(2,54)} = 303.8$, $p < 0.0001$; $F_{\text{genotype}(1,27)} = 0.5509$, $p = 0.4644$). There were no significant differences between male *Nlgn4* null mutants and WT littermates (Fig. 2E, left panel: $F_{\text{interaction}(2,48)} = 0.3685$, $p = 0.6937$; $F_{\text{time}(2,54)} = 254.9$, $p < 0.0001$; $F_{\text{genotype}(1,24)} = 0.9074$, $p = 0.3503$). Post hoc single comparison detected a significant difference between female mutants and WT in the second 10 min interval (Fig. 2F, left panel; $p = 0.009$), indicating increased grooming time in the mutant group. Since autism is also characterized by reduced interests and higher resistance towards environmental distractors, we tested whether mice generally groomed less in a more attractive and stimulating environment – perhaps paying more attention to the environmental enrichment instead of compulsive self-grooming. Surprisingly, however, induced self-grooming under enriched conditions was even more pronounced and did not differ between genotypes in any of the genders (Fig. 2E, right panel; $F_{\text{interaction}(2,46)} = 1.621$, $p = 0.2088$; $F_{\text{time}(2,46)} = 53.59$, $p < 0.0001$; $F_{\text{genotype}(1,23)} = 1.422$, $p = 0.2453$. Fig. 2F, right panel; $F_{\text{interaction}(2,46)} = 0.123$, $p = 0.8847$; $F_{\text{time}(2,46)} = 96.26$, $p < 0.0001$; $F_{\text{genotype}(1,24)} = 0.1202$, $p = 0.7318$).

3.5. Development of an autism severity composite score for mice

Comparable to a known form of a human monogenic heritable autism, where mutations of the *NLGN4X* gene can lead to a variety of ASD typical symptoms, we found readouts of social interaction, ultrasound communication, and stereotyped behaviors also altered in *Nlgn4* null mutant mice. Thus, disruption of only one single gene results in a diverse array of behavioral abnormalities. To account for individuality of discrete symptom severity in the autistic syndrome as a whole as well as for gender differences, we created an autism severity score, separately for male and female mice. For this autism composite score, selected single symptom readouts were z-standardized and genotype groups were contrasted by Mann–Whitney U tests (Fig. 3A, B). Behavioral tests with a clear genotype dependent dissociation of means

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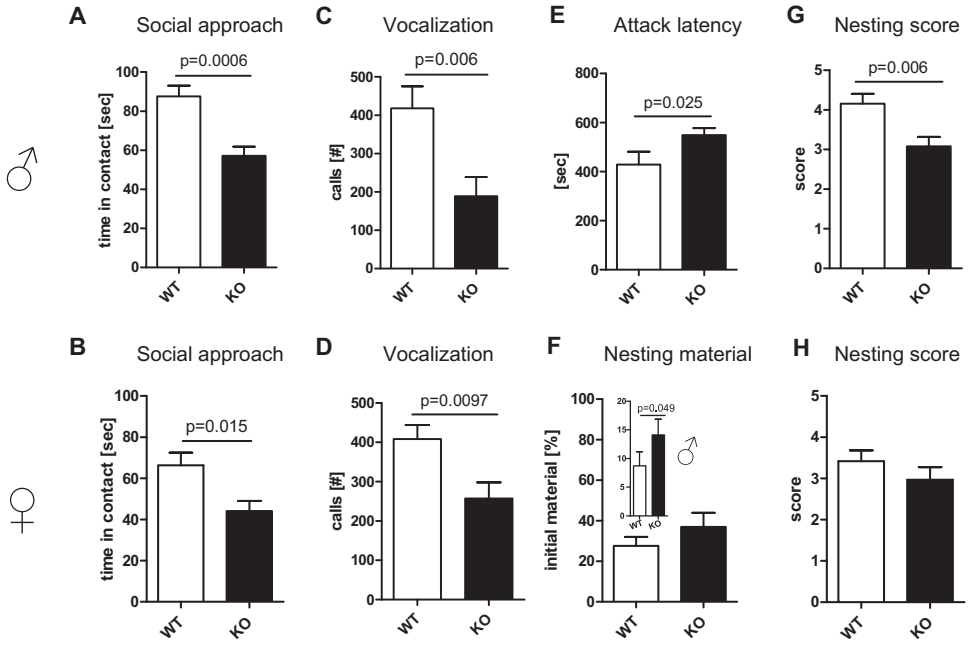

Fig. 1. Diverse features of social behaviors are affected in *Nlgn4* null mutants (KO) of both genders as compared to wildtype (WT) mice. (A, B) Male as well as female *Nlgn4* null mutant mice spent less time in direct social contact with a stranger mouse from the same genotype and gender (♂: WT = 36; KO = 26; ♀: WT = 30; KO = 22). (C, D) Null mutants had significantly fewer calls in the ultrasound vocalization paradigm (♂: WT = 29; KO = 24; ♀: WT = 36; KO = 23). (E) Male *Nlgn4* KO mice had longer attack latency in the resident-intruder paradigm, consistent with reduced social aggression (♂: WT = 12; KO = 11). (F, inset, and G) Only male mutants left a higher proportion of initial nesting material untouched and built significantly less functional nests (♂: WT = 13; KO = 13). (F, H) Nest building readouts in female *Nlgn4* KO mice show only a tendency of a deficit, without reaching significance (♀: WT = 24; KO = 15). Data presented as mean ± SEM.

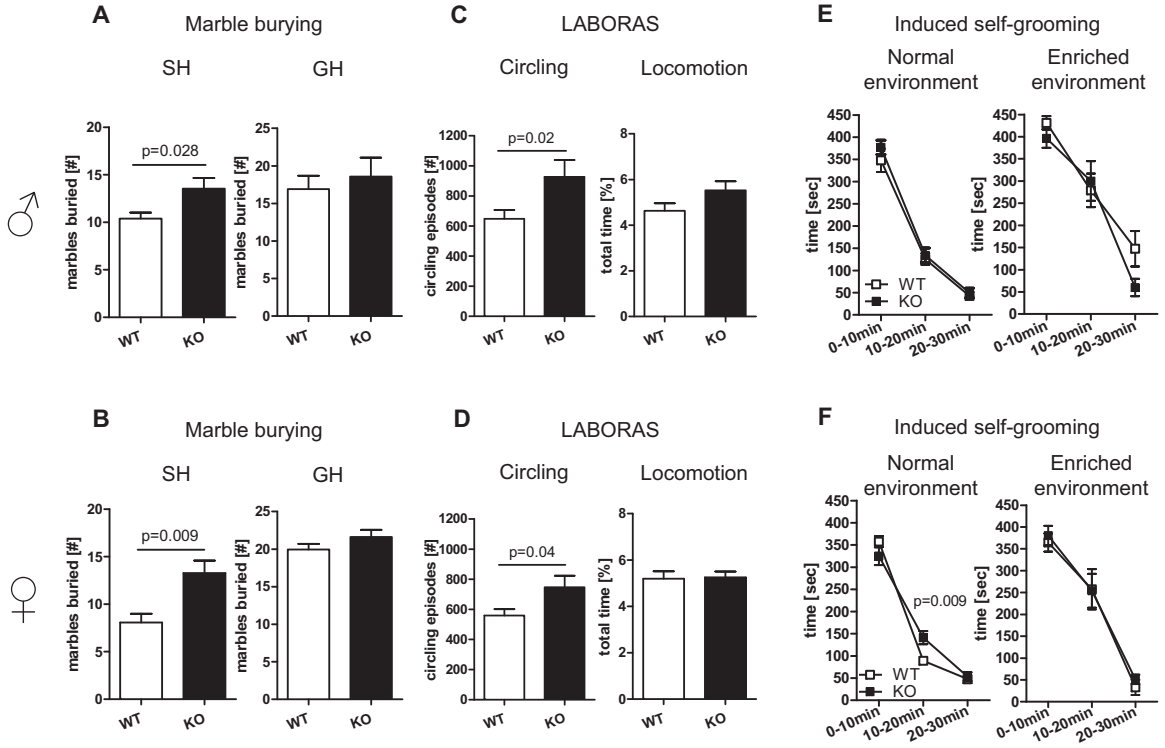

Fig. 2. *Nlgn4* null mutant mice demonstrate increased stereotyped behaviors (A, B) Both male and female *Nlgn4* mutants–when single housed (left panel: ♂: WT = 13; KO = 13; ♀: WT = 15; KO = 15) – buried more marbles in contrast to WT mice. This significant difference disappeared upon group housing (right panel: ♂: WT = 13; KO = 7; ♀: WT = 24; KO = 13). (C, D) Spontaneous circling behavior (left) in LABORAS was increased in *Nlgn4* mutants of both genders without alterations in general locomotor activity (right) (♂: WT = 17; KO = 16; ♀: WT = 14; KO = 15). (E, F) Water misting induced self-grooming, which showed an increase in female, but not in male *Nlgn4* KO mice as compared to WT (left: normal environment: ♂: WT = 13; KO = 13; ♀: WT = 15; KO = 14; right: enriched environment, ♂: WT = 12; KO = 13; ♀: WT = 13; KO = 13). Mean ± SEM presented.

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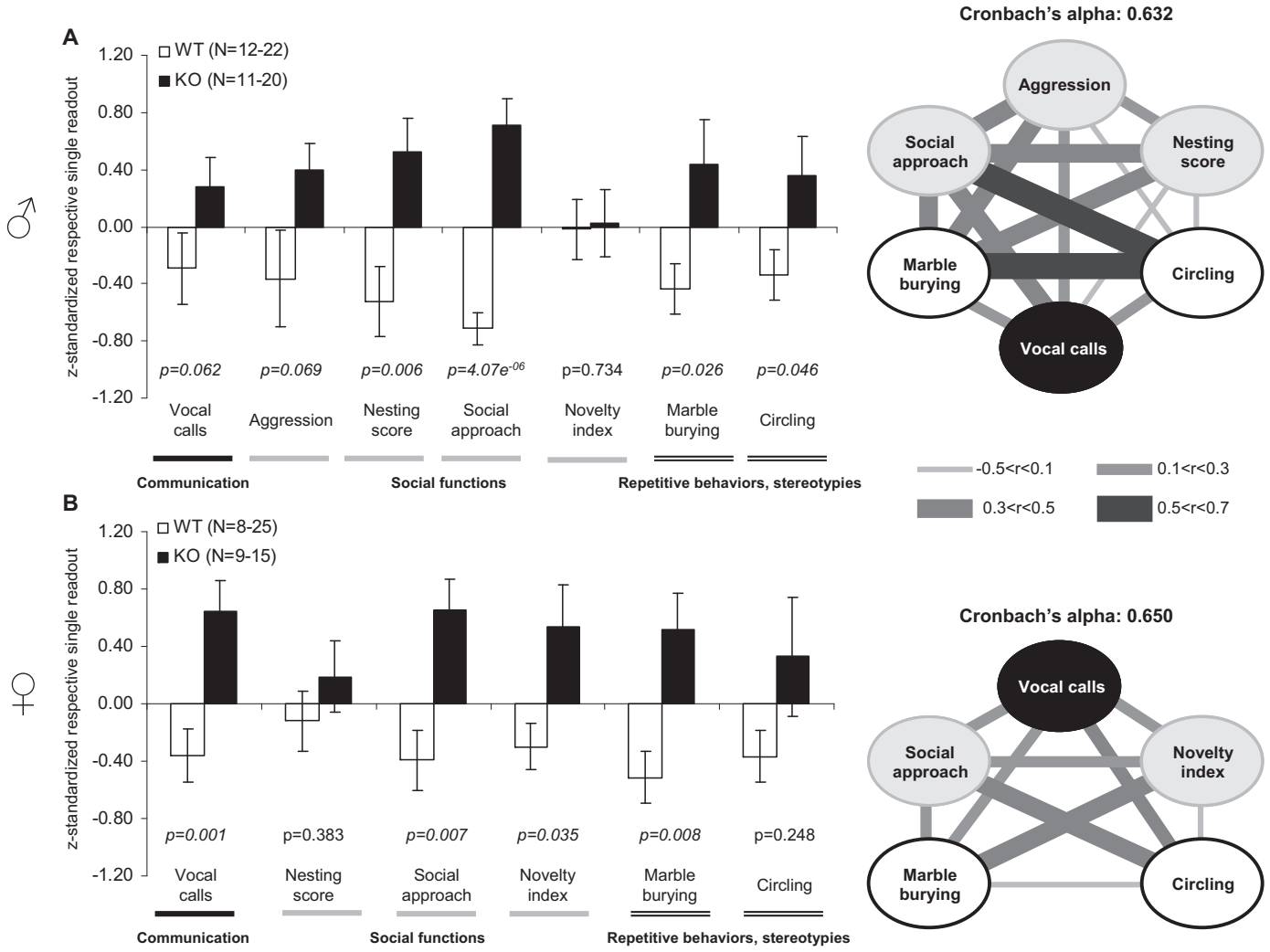

Fig. 3. Transformation and preparation of data for the development of an autism severity composite score (A) Male mice: Contrasting of 7 autism-relevant single behavioral readouts (mean ± SEM) following z-standardization allows all but one (novelty index) to be integrated into the composite score based on a very good Cronbach's alpha of 0.6 This value supports a high internal consistency of the data substantiating the integration of all readouts into one score. Intercorrelations are displayed in the construct, consisting of all 3 lead domains of ASD (B) Female mice: Contrasting of 6 autism-relevant single behavioral readouts (mean ± SEM) following *z*-standardization allows all but one (nesting score) to be integrated into the composite score based on a very good Cronbach's alpha of >0.6. This value supports a high internal consistency of the data substantiating the integration of all readouts into one score. Intercorrelations are displayed in the construct, consisting of all 3 lead domains of ASD.

(group comparison significant or at least showing a strong tendency) were selected with the aim of building gender specific composite scores for each individual mouse, assuring coverage of all 3 diagnostic domains of ASD (i.e. communication, social functions, repetitive behaviors/stereotypies). These composite scores should reflect the overall severity of autistic behaviors in a continuous fashion with higher values indicating higher severity of autistic behaviors. For male mice, the composite score was based on vocal calls, aggression, nesting, social approach, marble burying and circling (resulting in a Cronbach's $\alpha = 0.632$). For female mice, vocal calls, social approach, novelty index, marble burying, and circling (Cronbach's $\alpha = 0.650$) were included in the composite score. Intercorrelations between single score items are displayed in Fig. 3A, B. Gender specific autism severity composite scores, calculated by integrating the means of all behavioral readouts (following imputations as described in materials) yielded highly significant results ($p < 0.0001$) for both genders for contrasting between mutant and WT mice (Fig. 4A, B). The relative frequency distribution of the autism severity composite score discriminates well between WT and null mutant mice, with a slightly higher overlap seen for female as compared to male subjects (Fig. 4C, D). Individual animals can be assigned to the correct genotype with nearly 100% prediction accuracy in males (75% of phenotypic variance explained; calculations based on a binary logistic regression model using the imputed complete data set). In female mice, 54% of phenotypic variance is explained by the respective model and >83% of predictions of genotype status are correct (73% of mutants correctly identified as such and 91% of WT correctly assigned to the WT group by the model) (Fig. 4E, F).

4. Discussion

The present study provides a comprehensive phenotypical characterization of *Nlgn4* null mutant mice, thereby essentially reproducing our own previous work on *Nlgn4*−/− males [4] and extending it – independently – also to females and, notably, to a number of new ASD relevant tests, most of which turned out to be informative. The results show that both genders of *Nlgn4* null mutants display a multifaceted autistic-like syndrome consisting of distinctive symptoms for all 3 lead features of the disorder: Disturbed social interactions, compromised verbal (ultrasound) communication and repetitive behaviors/restricted interests. Most importantly, based on these typical behavioral symptoms (as evaluated by the most informative tests), we have been able to

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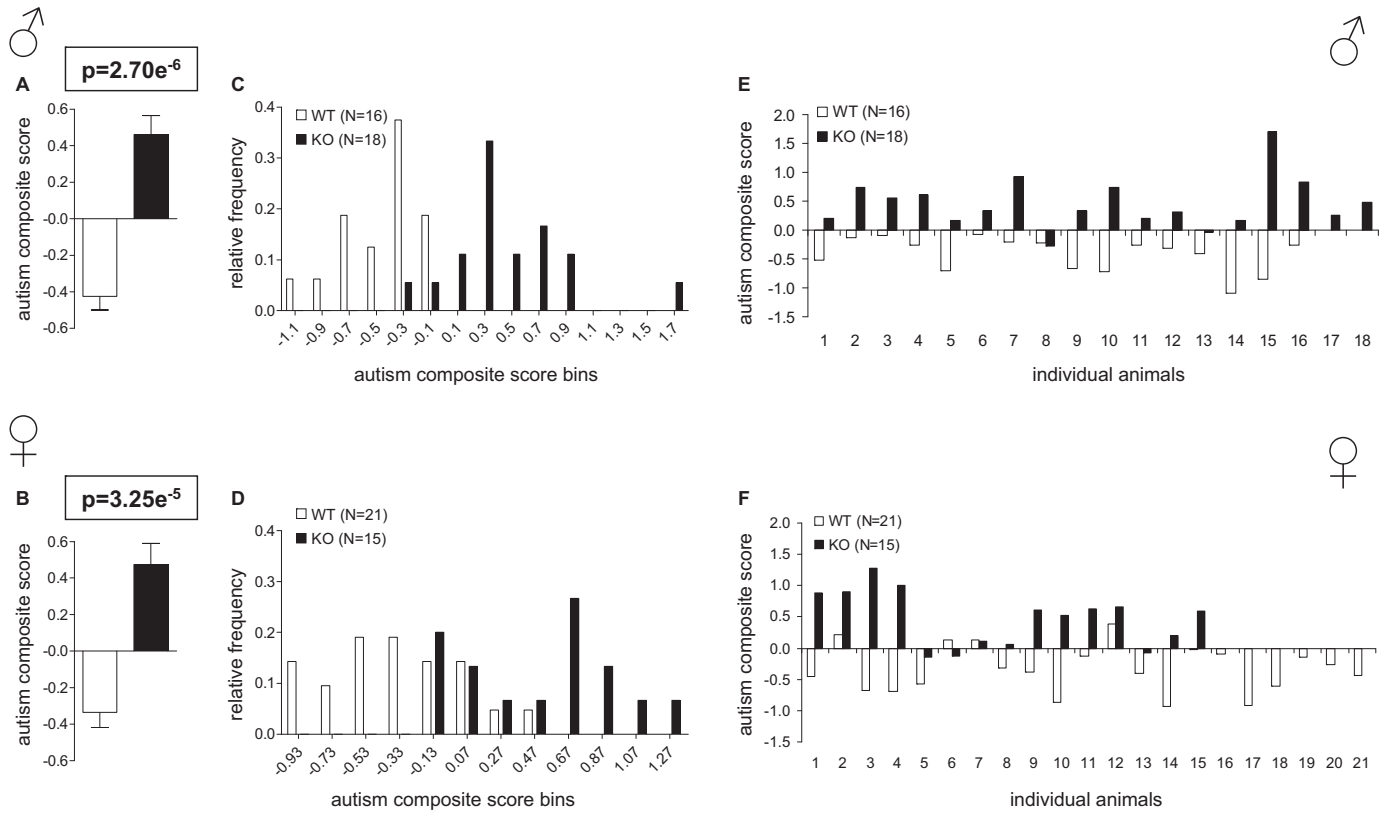

Fig. 4. Presentation of the first autism severity composite score for mice (A, B) The newly developed autism severity composite score (higher values represent higher symptom severity) based on the *z*-transformed raw data of selected tests with significant difference between genotypes (Fig. 3) and imputed values for missing data (as explained in materials), gives highest significance levels for both genders. (C, D) Relative frequency distribution of autism composite score bins separates WT and mutants, with less overlap in males than in females. (E, F) The autism score of individual mice strikingly discriminates between genotypes.

develop for the first time an autism severity composite score for mice. This score allows a highly significant discrimination between autistic and non-autistic *Nlgn4*−/− mice, with an accuracy of 100% for males and of >83% for females to predict the genotype status from the behavioral readouts. In the meanwhile, this same score has already been successfully applied to other genetic mouse models of autism analyzed in our lab (manuscript in preparation).

Compared to the other tests of social behavior with their consistently robust findings (social approach behavior, vocalization, aggression and nest building), demonstrated again in the present work, the discriminatory power of the tripartite chamber tests [16] remains delicate and difficult to reproduce, at least in our hands. In fact, we made similar observations with these tests in various different mouse lines that we have analyzed over the years. Lab-to-lab variations including differences in breeding, upbringing and maintenance or even differential effects of modifier genes may explain at least part of these difficulties. In the present work, these problems of the tripartite chamber tests are reflected by the partly divergent results in male mice (i.e. in social memory) when comparing the previously published [4] and the present cohort. The reason for this discrepancy remains unknown but all other findings of the previous study were entirely reproducible.

The fact that the prediction of genotype from behavior is less accurate for female mice reflects the overall milder and slightly different ASD-like phenotype found here in female as compared to male mice, despite the use of mostly higher *n*-numbers for testing of females. It has to be mentioned, however, that due to space, personnel and logistic limitations, male and female mice were not strictly tested in parallel. Therefore, a direct statistical comparison is not justified. The comparison provided here is descriptive and looks separately at the test results obtained with both genders.

Particularly in nesting but also in induced self-grooming and social interaction readouts, genders differ clearly. Of note, gender-specific findings have been reported in several other mouse models of autism (e.g. [30,9]) and, also in humans, autistic symptom severity and distribution show gender differences [31–36].

Moreover, individuality is an important issue in ASD. Despite having the same mutation, persons affected by the disorder can show different severity and different lead symptom distribution [37–43]. This observation may partly be explained by genetic and environmental modulation of the syndrome. Interestingly, however, the same still holds true for mice where almost identical environmental conditions (cage, group, food, etc.) on top of the highly inbred background tremendously diminish variability as compared to humans.

Nevertheless, a fascinating example of how environment can modify behavior is shown by our results on marble burying behavior in both genders. Here, group housing alleviates the compulsive phenotype. Interestingly, social enrichment and environmental complexity have been reported to improve the autistic phenotype in e.g. fragile X mice [44] as well as in humans [45,46], supporting the important role of the social context and of behavioral psychotherapy in autistic individuals [47–49].

As shown here, the development of an autism severity score has delivered a more robust ground for future treatment studies than single readouts with their higher heterogeneity in the population and their smaller effect sizes. The score provides a powerful and unitary measure for the overall severity of autistic symptoms, covering all ASD relevant behavioral domains. In fact, testing new

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compounds for the treatment of autism should always happen in both genders and across a wide range of autism-relevant tests. Applying integrative gender-specific scores will largely simplify the quantification and validation of treatment outcomes in both male and female experimental groups.

Since autism is usually diagnosed in children before the age of 3 years, assessment of neurobehavioral developmental milestones in neonatal mice up to the age of 3–4 weeks will be interesting for further characterization of the *Nlgn4*−/− mouse model of autism. In the present work, we did not yet address these questions. Moreover, developmental disturbances (e.g. seizures, learning and memory impairments) will have to be assessed. The good construct and face validity of the present mouse model may now encourage in depth studies on the issue of predictive validity e.g. by using pharmacological agents or genetic rescue of the phenotype. In the case studied here, one gene aberration causes the syndrome. Therefore, ideally, a causal treatment should also combat all features of the disorder. In the present study, group in contrast to single housing of *Nlgn4* null mutants obviously attenuated selected compulsive/stereotypic traits, indicating a social component of symptom severity. Further work will have to elucidate the effect of potential modifying environmental factors, e.g. environmental complexity and social enrichment, in the *Nlgn4* null mutant model.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2012.11.016.

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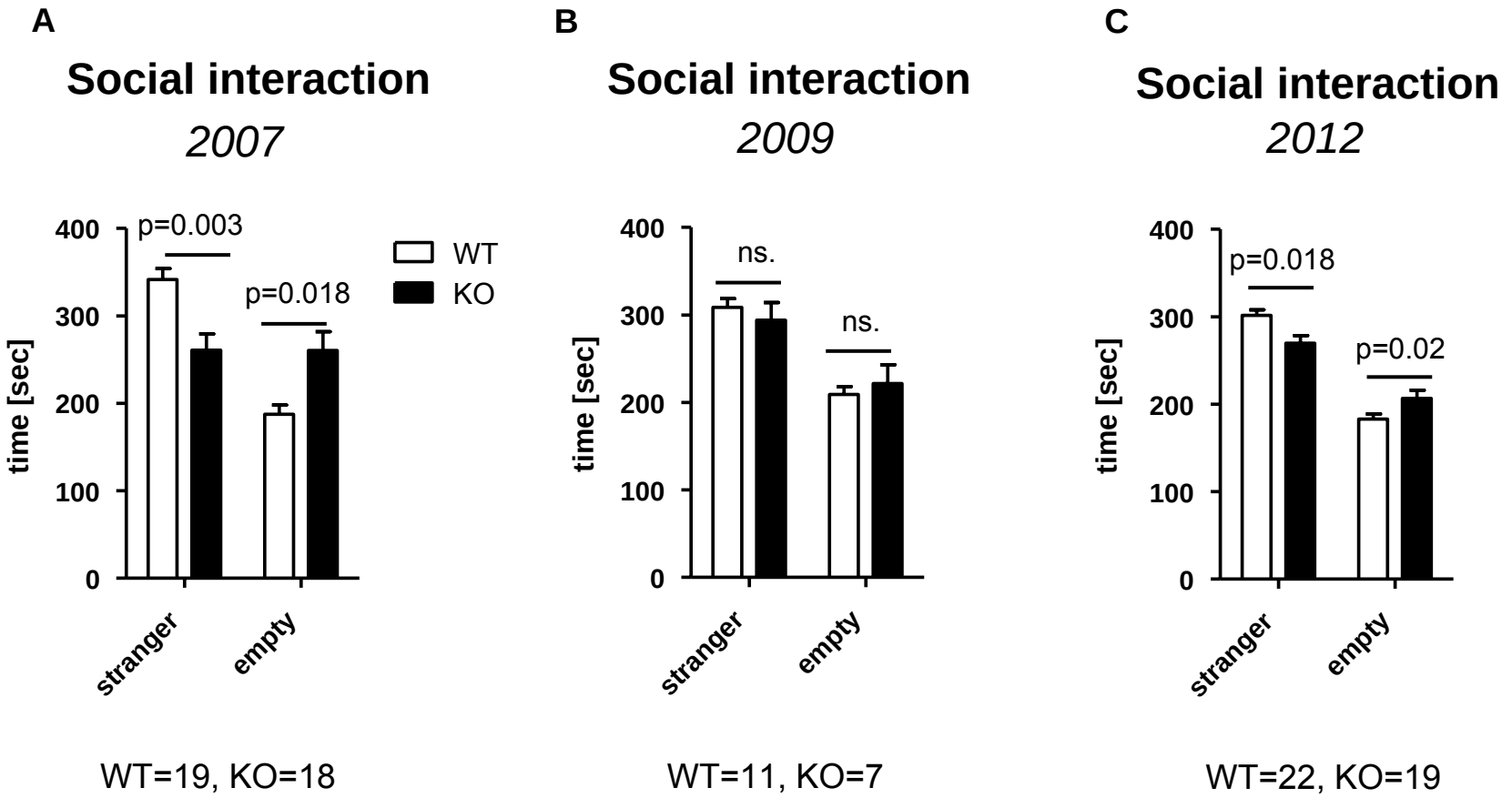
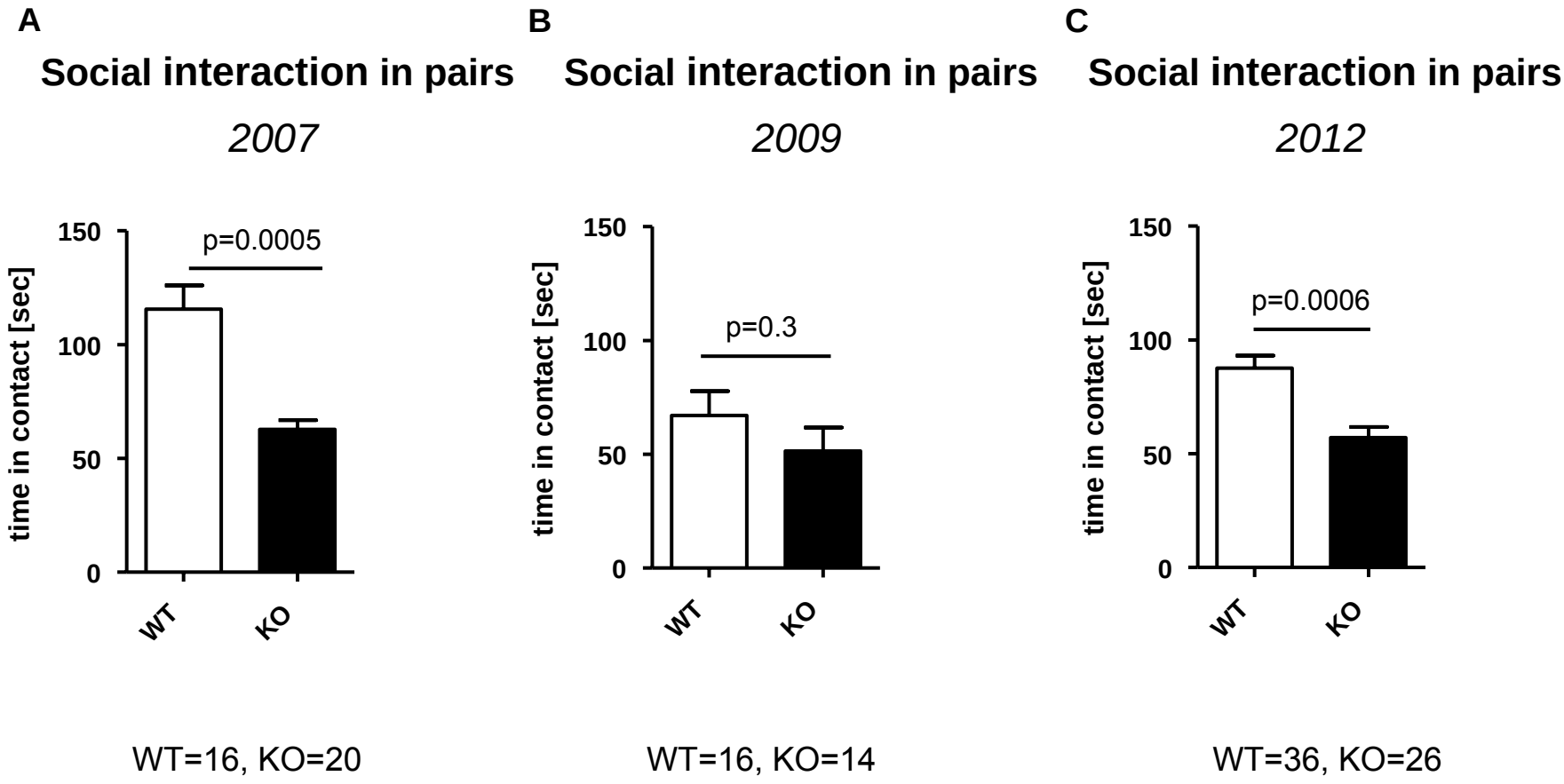
El-Kordi et al Supplementary Figure 1



El-Kordi et al Supplementary Figure 2

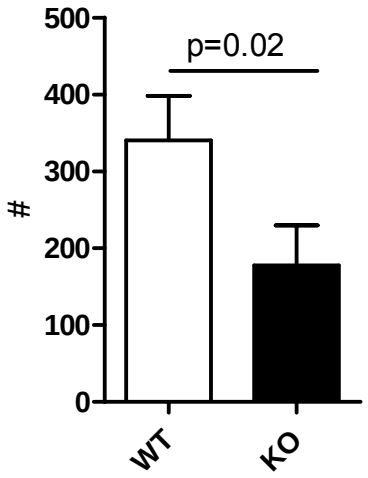


El-Kordi et al Supplementary Figure 3

A

Courtship calls

2007

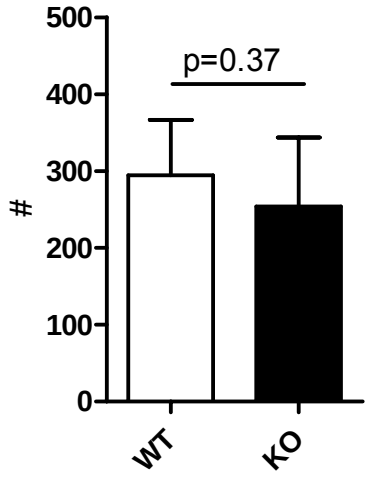


WT=20, KO=16

B

Courtship calls

2009

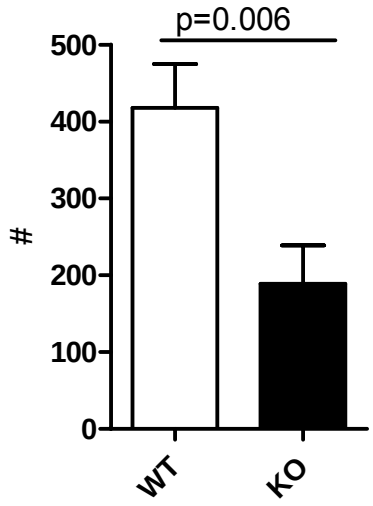


WT=12, KO=7

C

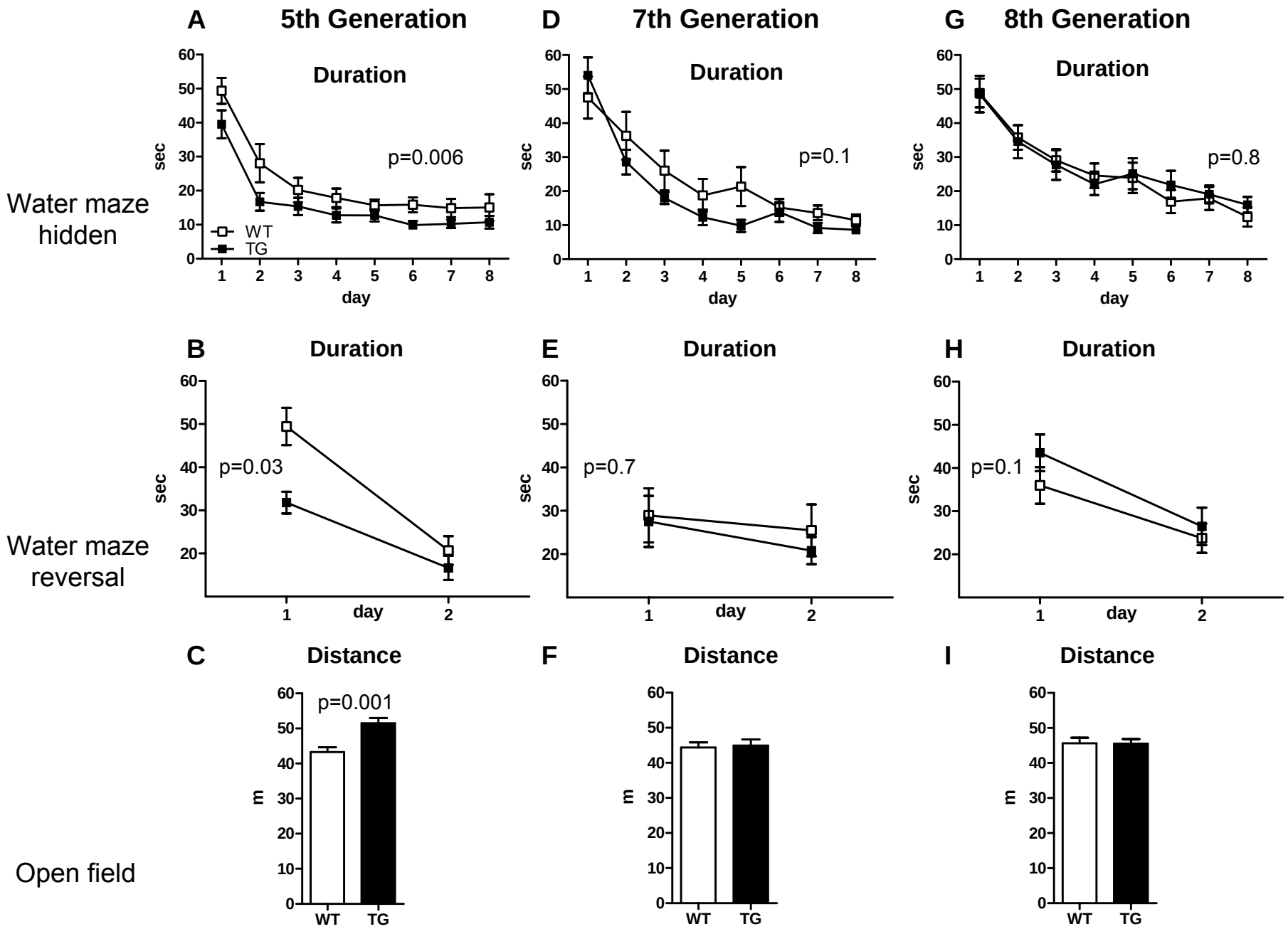
Courtship calls

2012

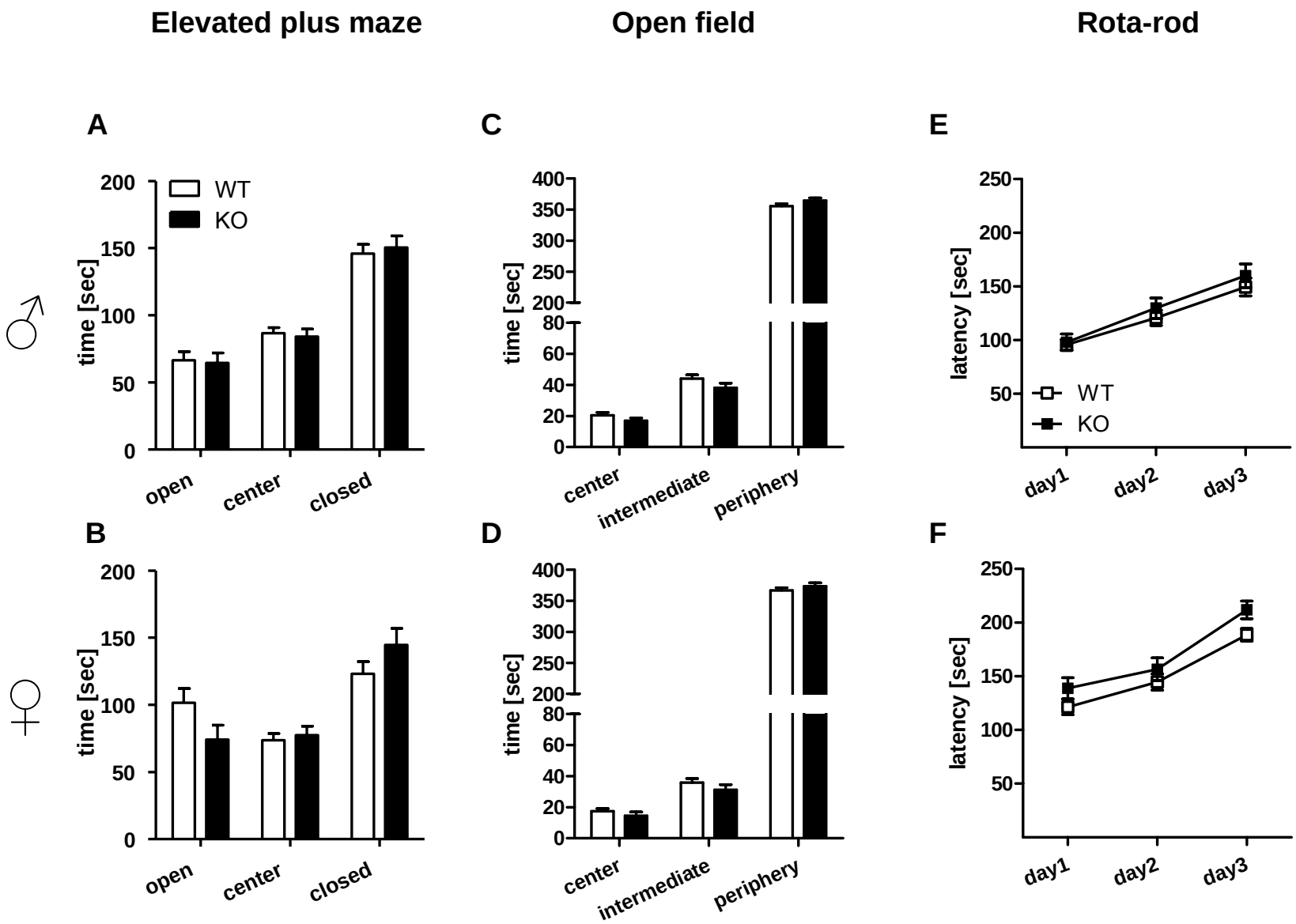


WT=29, KO=24

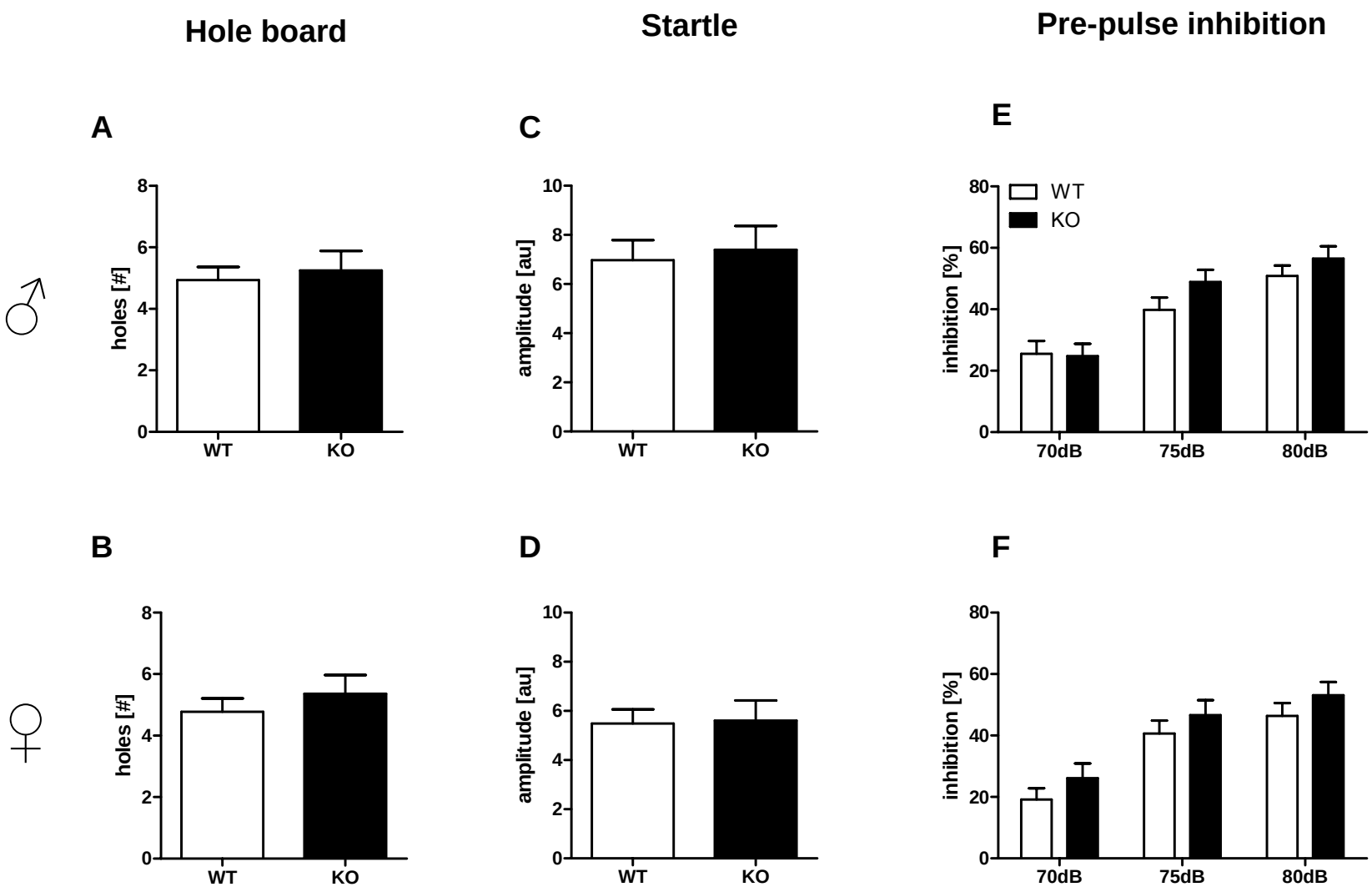
El-Kordi et al Supplementary Figure 4



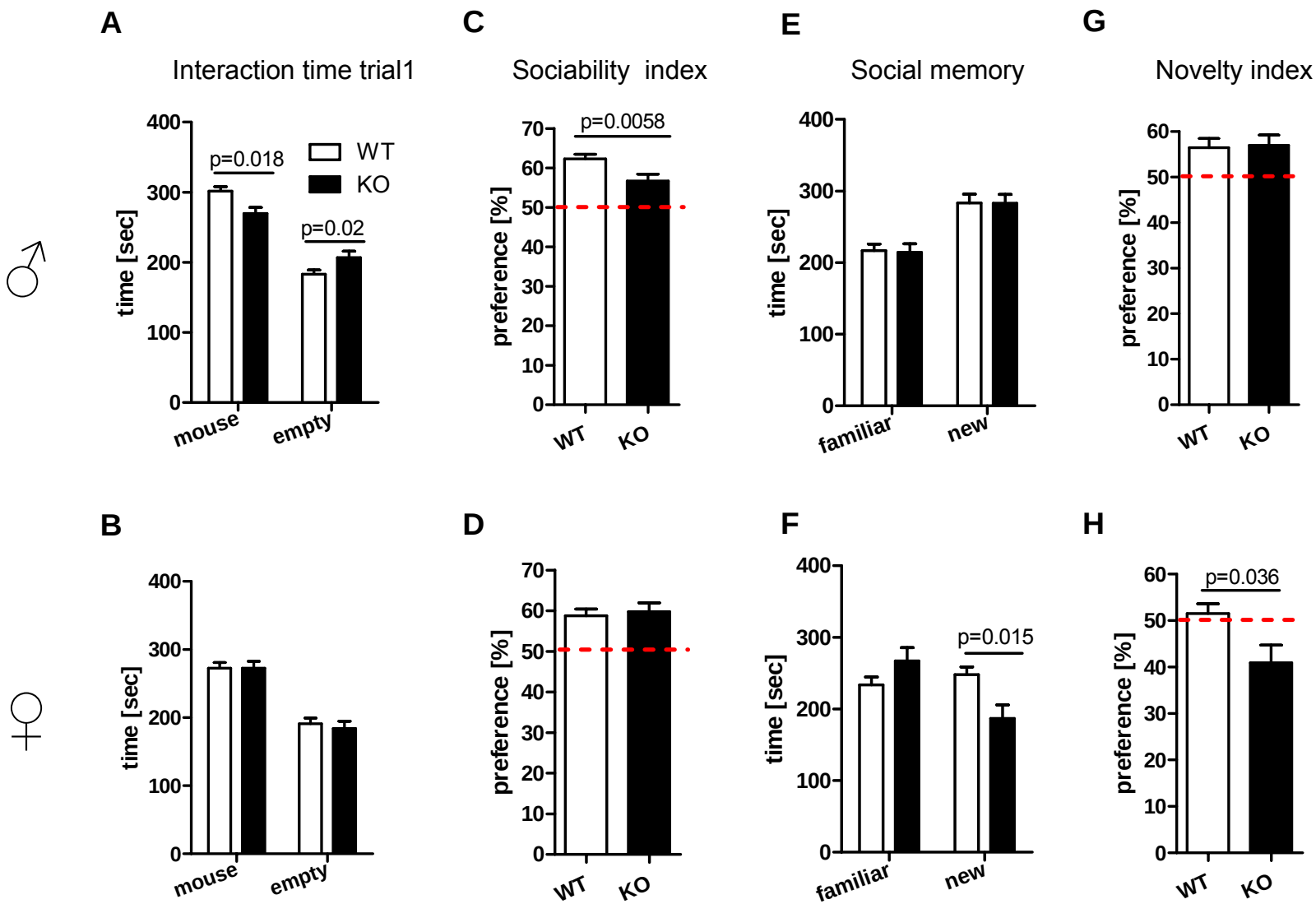
El-Kordi et al Supplementary Figure 5



El-Kordi et al Supplementary Figure 6



El-Kordi et al Supplementary Figure 7



9.2 Curriculum vitae

Personal data

Name	Anne Kästner
Date of birth	16.09.1983
Place of birth	Rheda-Wiedenbrück
Nationality	German
E-mail	kaestner@em.mpg.de

Education

since March 2010	PhD student at Göttingen Graduate School for Neurosciences, Biophysics and Molecular Biosciences (GGNB); PhD program *'Systems Neuroscience'* PhD thesis in the department of Clinical Neuroscience (Prof. Dr. Dr. Hannelore Ehrenreich) of the Max Planck Institute of Experimental Medicine, Göttingen, Germany
2009	Diploma in Psychology, Georg-August-University of Göttingen, Germany Focus: Experimental, clinical and educational psychology Title of diploma thesis: '*Sensing* and *seeing* - two distinguishable processes of change perception.'
2003	Abitur, Greselius Gymnasium, Bramsche, Germany

Work and practical experience

2009-2010	Researcher at the Department of Experimental Psychology (Prof. Dr. Uta Lass), Georg-Elias-Müller Institute of Psychology, University of Göttingen
2006-2009	Student research assistant at the Department of Experimental Psychology (Prof. Dr. Uta Lass), Georg-Elias-Müller Institute of Psychology, University of Göttingen
2007	Internship in an orphanage *(Aldeas de Niños de padre Alfredo Spiessberger)* in Santa Cruz de la Sierra, Bolivia; implementation of an intervention program for the treatment of dyslexia in school children
2007	Internship in a Child and Adolescent Psychiatry, ward for girls of 14-18 years, Germany; diagnostics, neuropsychology, visiting of individual and group therapy sessions, relaxation techniques
2003-2010	Volunteer work in an orphanage *(Aldeas de Niños de padre Alfredo Spiessberger)* in Santa Cruz de la Sierra, Bolivia

9.3 List of publications

Tantra, M.*, Hammer, C.*, **Kästner, A.***, Begemann, M., Bodda, C., Stepniak, B., Castillo Venzor, A., Erbaba, B., Tarami, A., Hammerschmidt, K., Schulz-Schaeffer, W., Mannan, A. and Ehrenreich, H. (In preparation). "Slightly modified expression of the gene encoding methyl-CpG binding protein-2 influences behavior of mouse and man."

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El-Kordi, A.*, Winkler, D.*, Hammerschmidt, K., **Kästner, A.**, Krueger, D., Ronnenberg, A., Ritter, C., Jatho, J., Radyushkin, K., Bourgeron, T., Fischer, J., Brose, N. and Ehrenreich, H. (2013). "Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism." Behav Brain Res.

Kästner, A.*, Grube, S.*, El-Kordi, A.*, Stepniak, B., Friedrichs, H., Sargin, D., Schwitulla, J., Begemann, M., Giegling, I., Miskowiak, K. W., Sperling, S., Hannke, K., Ramin, A., Heinrich, R., Gefeller, O., Nave, K. A., Rujescu, D. and Ehrenreich, H. (2012). "Common variants of the genes encoding erythropoietin and its receptor modulate cognitive performance in schizophrenia." Mol Med **18**: 1029-1040.

Hagemeyer, N.*, Goebbels, S.*, Papiol, S.*, **Kästner, A.**, Hofer, S., Begemann, M., Gerwig, U. C., Boretius, S., Wieser, G. L., Ronnenberg, A., Gurvich, A., Heckers, S. H., Frahm, J., Nave, K. A. and Ehrenreich, H. (2012). "A myelin gene causative of a catatonia-depression syndrome upon aging." EMBO Mol Med **4**(6): 528-539.

Papiol, S.*, Malzahn, D.*, **Kästner, A.**, Sperling, S., Begemann, M., Stefansson, H., Bickeboller, H., Nave, K. A. and Ehrenreich, H. (2011). "Dissociation of accumulated genetic risk and disease severity in patients with schizophrenia." Transl Psychiatry **1**: e45.

Ehrenreich, H., **Kästner, A.**, Weissenborn, K., Streeter, J., Sperling, S., Wang, K. K., Worthmann, H., Hayes, R. L., von Ahsen, N., Kastrup, A., Jeromin, A. and Herrmann, M. (2011). "Circulating damage marker profiles support a neuroprotective effect of erythropoietin in ischemic stroke patients." Mol Med **17**(11-12): 1306-1310.

Ribbe, K.*, Friedrichs, H.*, Begemann, M., Grube, S., Papiol, S., **Kästner, A.**, Gerchen, M. F., Ackermann, V., Tarami, A., Treitz, A., Flogel, M., Adler, L., Aldenhoff, J. B., Becker-Emner, M., Becker, T., Czernik, A., Dose, M., Folkerts, H., Freese, R., Gunther, R., Herpertz, S., Hesse, D., Kruse, G., Kunze, H., Franz, M., Lohrer, F., Maier, W., Mielke, A., Muller-Isberner, R., Oestereich, C., Pajonk, F. G., Pollmacher, T., Schneider, U., Schwarz, H. J., Kroner-Herwig, B., Havemann-Reinecke, U., Frahm, J., Stuhmer, W., Falkai, P., Brose, N., Nave, K. A. and Ehrenreich, H. (2010). "The cross-sectional GRAS sample: a comprehensive phenotypical data collection of schizophrenic patients." BMC Psychiatry **10**: 91.

*These authors contributed equally to the work.