Contribution of genetic and environmental factors to symptomatology in psychiatric disorders

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DECLARATION

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

Beata Stepniak Göttingen, 1st December 2014

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ABBREVIATIONS

YLD	*years lived with disability*
DSM	*Diagnostic and Statistical Manual of Mental Disorders*
ICD	*International Classification of Diseases*
SNP	*single nucleotide polymorphism(s)*
MAF	*minor allele frequency(ies)*
GWAS	*genome-wide association study(ies)*
OR	*odds ratio(s)*
CNV	*copy-number variation(s)*
e.g.	*for example (abbr. of Latin 'exempli gratia')*
HPA axis	*hypothalamic-pituitary-adrenal axis*
GRAS	*Göttingen Research Association for Schizophrenia*
PGAS	*phenotype-based genetic association study(ies)*
KO	*knockout*
WT	*wildtype*

1. INTRODUCTION TO THE THESIS TOPIC

Psychiatric disorders are common and of considerable impact. *The Global Burden of Disease, Injury, and Risk Factor Study 2010* estimated mental and substance use disorders to account for the largest portion (22.9%) of the total global years lived with disability (YLD) in 2010. While premature mortality associated with mental and substance use disorders is low, disability attributable to this disorder class is higher than for any other medical condition. Within the group of psychiatric disorders depressive disorders account for 42.5% of YLD followed by substance use disorders (17.3%), anxiety disorders (15.3%), and finally schizophrenia (7.4%) and bipolar disorder (7.4%) (Whiteford et al., 2013). Based on the *National Comorbidity Survey Replication* lifetime prevalence for the most common psychiatric disorders have been estimated to be as high as 28.8% for anxiety disorders, 24.8% for impulse-control disorders, 20.8% for mood disorders, and 14.6% for substance use disorders (Kessler et al., 2005).

The classification of psychiatric disorders and its problems and implications

The diagnosis of mental disorders is often difficult due to the absence of biological disease markers and a considerable overlap of symptoms between different diagnostic categories (Simmons and Quinn, 2014). The classification systems *Diagnostic and Statistical Manual of Mental Disorders* (DSM) (American Psychiatric Association, 2013) and *International Classification of Diseases* (ICD) (World Health Organization, 1993) treat mental disorders as categorical concepts (present/absent) for which a defined combination and numbers of symptoms (often within a defined timeframe) have to be present. Moreover the classification systems are polythetic, meaning that they list multiple symptoms for each disorder, yet not all of them have to be present to consider a specific diagnosis. According to Krueger and Bezdjian (2009) these main characteristics of the DSM and the ICD raise three conceptual problems: comorbidity, within-diagnosis heterogeneity, and subthreshold symptomatology. Comorbidity occurs when patients meet diagnostic criteria for two or more psychiatric disorder (Plomin et al., 2009). The *National Comorbidity Survey Replication* reports a lifetime prevalence of 27.7% for at least two psychiatric disorders and 17.3% for three or more (Kessler et al., 2005). The way we categorize mental disorders poses yet another problem - within-diagnosis heterogeneity. Individuals with very different symptom

constellations and symptom severities will be put in the same diagnostic category because the classification systems do – in most cases – not allow for dimensional severity ratings or symptom based subgroup classification (Krueger and Bezdjian, 2009; Simmons and Quinn, 2014). Finally for individuals with subthreshold symptoms that do not fulfill all diagnostic criteria, the information of proximity to the disease threshold is lost (Krueger and Bezdjian, 2009).

The problems of comorbidity, within-diagnosis heterogeneity, and subthreshold symptomatology do not just remain problems of the diagnostic classification systems. Indeed, they determine how we see and study mental illness. The high prevalence of comorbidity - illustrating the blending of mental disorders into one another – pose the question of whether the disorders really are distinct and we can therefore expect to identify individual, underlying, etiological factors (Krueger and Bezdjian, 2009). Further, when studying one diagnostic group we should be aware of the huge heterogeneity and ask ourselves if we can expect to detect an underlying disease mechanism in individuals displaying very different phenotypes (Krueger and Bezdjian, 2009). Lastly subthreshold symptomatology may become a problem in case-control studies, were the control group may ultimately consist of healthy individuals, some of which fulfill several but not all of the diagnostic criteria.

Many researchers are convinced that advances in therapeutic and prognostic decision-making will only be possible based on the accurate diagnosis of mental illness (Craddock and Owen, 2010) and for this the classification needs to be more directly linked to underlying biological mechanisms (Adam, 2013; Craddock and Owen, 2010; Insel et al., 2010; Krueger and Bezdjian, 2009). So far, clinical neuroscience and genetic studies have failed to confirm the current categorical approach to mental disorders neither have the categories been useful in predicting treatment response (Adam, 2013; Insel et al., 2010; Simmons and Quinn, 2014). In the knowledge of this, researchers have suggested new approaches to the study of psychiatric disorders: (1) the introduction of a dimensional view of mental illness (Craddock and Owen, 2010; Krueger and Bezdjian, 2009), (2) the study of more simple and biologically based intermediate phenotypes across diagnostic borders (Meyer-Lindenberg and

Weinberger, 2006), or even (3) a network approach to psychopathology (Borsboom et al., 2011).

The genetic architecture of psychiatric disorders

Regardless of the diagnostic and classification problems for mental disorders, population, family, and twin studies have provided evidence of overall high heritability, 32-37% for major depressive disorder (Sullivan et al., 2000; Wray and Gottesman, 2012), 62-85% for bipolar disorder (McGuffin et al., 2003; Wray and Gottesman, 2012), 67-85% for schizophrenia (Cardno and Gottesman, 2000; Wray and Gottesman, 2012), and 50-80% for autism spectrum disorder (Lichtenstein et al., 2010; Ronald and Hoekstra, 2011; Sandin et al., 2014). These high estimates encouraged researchers to start searching for the genetic roots of psychiatric disorder. But since the early linkage studies failed to identify so-called mendelian disease genes and after an era of candidate gene studies with mostly inconsistent, non-reproducible results, the conclusion had to be drawn that mental disorders are highly polygenic (Craddock and Sklar, 2013; Flint and Kendler, 2014; Gratten et al., 2014; Hirschhorn et al., 2002; Sullivan et al., 2012). Now, supported by technological advances in high-throughput genotyping and sequencing and by using samples of increasing size, the genetic picture of psychiatric disorders is becoming increasingly complex (Gratten et al., 2014).

One main focus of recent genetic studies has been on common variants – single nucleotide polymorphisms (SNP) – which frequently occur in the population at minor allele frequencies (MAF) >1-5%. These common variants have been estimated to account for one third up to one half of the total heritability of schizophrenia (Lee et al., 2013; Purcell et al., 2009; Ripke et al., 2013), autism spectrum disorder (Gaugler et al., 2014), bipolar disorder (Lee et al., 2013), and major depressive disorder (Flint and Kendler, 2014; Lee et al., 2013; Lubke et al., 2012). So-called genome-wide association studies (GWAS) provide an unbiased approach whereby large numbers of unrelated cases and controls are compared for the frequency distribution of these common SNP. The most recent and largest GWAS identified 108 independent genetic *loci* for schizophrenia (Schizophrenia Working Group of the PGC, 2014), that were genome-wide significantly associated with an increased disease risk ($p<5x10^{-8}$). However, the effects of individual SNP were, as in previous studies, very low with odds ratios

(OR)~1.06-1.3 (Schizophrenia Working Group of the PGC, 2014). The current absence of comparable numbers of genome-wide association findings for other major psychiatric disorders is mainly due to the lack of big enough samples (Gratten et al., 2014; Sullivan et al., 2012) or, as in the case of major depressive disorder, additionally due to greater heterogeneity between affected individuals and lower heritability (Flint and Kendler, 2014; Wray et al., 2012).

Nevertheless, in 2013 two papers were published by the *Cross-Disorder Group of the Psychiatric Genomics Consortium* (Cross-Disorder Group of the PGC, 2013; Lee et al., 2013) which tried to assess the shared genetic risk for five major psychiatric disorders (autism spectrum disorder, attention deficit-hyperactivity disorder, bipolar disorder, major depressive disorder and schizophrenia) using large samples, genome-wide SNP data and two slightly different methodological approaches. Overall both papers provide evidence for shared genetic risk between psychiatric disorders, particularly pronounced between adult-onset disorders and with strongest effects between schizophrenia and bipolar disorder (Cross-Disorder Group of the PGC, 2013; Lee et al., 2013).

Besides those common, also large, rare copy number variations (CNV) with a frequency <1% have been implicated in psychiatric disorders, with OR between ~2 and 20 for individual variants. These structural changes involve deletions or duplications of >100 kilobases of DNA and they often occur *de novo* – meaning that they are present in affected individuals but not their parents (Gratten et al., 2014). CNV seem to be particularly relevant for autism spectrum disorder (Sanders et al., 2011; Sebat et al., 2007) and schizophrenia (The International Schizophrenia Consortium, 2008; Walsh et al., 2008; Xu et al., 2008). The largest meta-analysis of CNV in schizophrenia confirmed previous findings of deletions and duplications as well as specific exon-disrupting deletions in the gene *NRXN1* (all OR≥7.5). However, all of these CNV are not disease specific and have at the same time been associated with mental retardation, autism spectrum disorder, and epilepsy (Levinson et al., 2011). Additionally, in the case of CNV associated with schizophrenia sometimes the reciprocal event (deletion vs. duplication) has been linked to another disorder, e.g. autism (Levinson et al., 2011; Sanders et al., 2011).

Even though a lot of effort has been put in uncovering the genetic architecture of psychiatric disorders, many questions remain. (I) What is the functional importance of the variants identified by genetic studies, considering that >80% of the SNP fall in intronic or intergenic regions (Hindorff et al., 2009)? (II) How do different disease-associated variants combine to cause pathology (Gratten et al., 2014)? (III) Where is the missing heritability to be found, if all markers identified through the largest GWAS on schizophrenia only explain 7% of the variance in case-control status (Schizophrenia Working Group of the PGC, 2014)?

The role of the environment in the development of psychiatric disorders

Concordance rates in monozygotic twins for all psychiatric disorders have been found to be substantially lower than 100%, e.g. 23-50% for major depression (Sullivan et al., 2000), 41-65% for schizophrenia (Cardno and Gottesman, 2000) and, 40-70% for bipolar disorder (Craddock and Sklar, 2013). This indicates that genes are not entirely responsible for disease development. Generally psychiatric disorders are considered *complex traits*, implying that they are caused by genetic and non-genetic factors, as well as their interaction (Gratten et al., 2014; Meyer-Lindenberg and Weinberger, 2006).

The involvement of non-genetic factors in the development of medical conditions [e.g. tobacco smoking and lung cancer (Gandini et al., 2008)] has long been recognized. In the case of mental disorders it took longer for research to appreciate and consider the impact of the environment. There is now a large body of literature available on environmental factors repeatedly associated with increased risk for schizophrenia (Brown, 2011; van Os et al., 2010). However, exposures to early biological hazards (e.g. malnutrition and stress during pregnancy, preterm birth), psychosocial factors and cannabis use have all been associated with an increased risk for major depressive disorder, bipolar disorder, as well as schizophrenia (Uher, 2014). As a psychosocial factor, childhood adversities (e.g. sexual abuse, physical abuse, neglect) have been linked to mental disease outcome across countries (Kessler et al., 2010) and have recently been confirmed by large meta-analyses as important environmental risk factors for schizophrenia (OR=3.6) (Matheson et al., 2013), depression (OR=2.04 for sexual and 1.49 for physical abuse), and anxiety (OR=2.52 for sexual and 1.70 for physical abuse) (Lindert et al., 2014).

One challenge of environmental risk factor research is posed by the fact that it is impossible to establish causative relationships based on epidemiological data. But the information coming from observational studies can help to generate new hypothesis about the etiology of different psychiatric disorders, which can then be tested experimentally. Based on the observation that early psychosocial stress is associated with an increased risk for various mental diseases, research using animal models was able to show that across mammalian species early or chronic stress during development can lead to long lasting alterations of the hypothalamic-pituitary-adrenal (HPA) axis (Sanchez et al., 2001) – dysfunctions, which in turn have been linked to depression (Pariante and Lightman, 2008), post-traumatic stress disorder (de Kloet et al., 2006), and schizophrenia (Walker et al., 2008).

Based on the fact that only few individuals with specific environmental exposures will go on to develop a psychiatric disorder, a new field has emerged, studying gene-environment interactions. Staying with the previous examples of stress affecting HPA axis functioning, Wichers and colleagues (2009) were able to show that genetic predisposition to depression potentiates the effect of developmental stress exposure (Wichers et al., 2009), illustrating an interplay of genes and environment. Overall gene-environment interaction research using human subjects as well as animal models (Caspi and Moffitt, 2006; Hunter, 2005; Kannan et al., 2013) is a growing field baring great potential but at the same time facing difficulties like the need for huge datasets, thorough assessment of environmental exposure, and new statistical methods for the analysis of increasingly complex data comprising various genetic and environmental factors (Thomas, 2010).

2. AIMS OF THE PRESENT WORK

During the introduction I mentioned the purely descriptive, categorical classification of mental disorders, the huge within-diagnosis heterogeneity, and the frequent occurrence of psychiatric comorbidities. I also pointed out that we are confronted with an incredible genetic heterogeneity upon which environmental factors take their effect. In our approach to a better understanding of the etiology of mental disorders we break up the phenotypic heterogeneity of individuals diagnosed with schizophrenia. Our strategy is to define more homogeneous subgroups based on quantitative and qualitative traits. We also try to identify common genetic variants or other biological phenomena associated with the phenotype of the specific subgroup [phenotype-based genetic association study (PGAS)] (Ehrenreich and Nave, 2014). The idea is that a shared phenotype will be linked to shared biological mechanisms within the subgroup.

The aim of the present thesis was to identify psychiatric subphenotypes associated with specific genetic variants (project I), exposure to environmental risk factors (project II) or the presence of serum autoantibodies (projects III and IV) in samples of patients with psychiatric disorders. Most of the analyses were conducted using the Göttingen Research Association for Schizophrenia (GRAS) dataset (Begemann et al., 2010; Ribbe et al., 2010). GRAS is a sample of over 1100 schizophrenic and schizoaffective patients [according to the *Diagnostic and Statistical Manual of Mental disorders Fourth Edition Text Revision* (DSM-IV-TR])] (American Psychiatric Association, 2000), recruited over the last 10 years in participating psychiatric hospitals and other mental health centers throughout Germany. The GRAS cohort comprises a comprehensive assessment of over 3000 phenotypic measures, as well as genetic and serological information for each individual. My work focused on the identification and quantification of symptom-based patient subgroups as well as the operationalization of environmental exposure. My work was complemented by the use of transgenic mouse models and multiple in vitro methods, constituting a translational approach to the study of mental disorders.

3. GENETIC MARKERS OF A MUNC13 PROTEIN FAMILY MEMBER, *BAIAP3*, ARE GENDER SPECIFICALLY ASSOCIATED WITH ANXIETY AND BENZODIAZEPINE ABUSE IN MICE AND HUMANS

3.1 Overview of project I

The brain-specific angiogenesis inhibitor 1-associated protein 3 (Baiap3) is predominantly expressed in the brain and shows sequence homology with Munc13 and synaptotagmin (Shiratsuchi et al., 1998). The cellular function of Baiap3 and its contribution to behavioral phenotypes is unclear, however, the fact that the protein structure contains two C2 domains, implies its involvement in signal-transduction or membrane-trafficking pathways (Koch et al., 2000; Shiratsuchi et al., 1998). Researchers have shown that the mammalian uncoordinated 13 (Munc13) protein family – that Baiap3 shows homology with – is important for synaptic vesicle priming (Brose et al., 1995; Koch et al., 2000; Varoqueaux et al., 2002), a key mechanism for neurotransmitter release. Furthermore synaptotagmin is also crucial for neurotransmitter exocytosis (Chapman, 2002). Based on the Allen Brain Atlas (http://mouse.brain-map.org/) Baiap3 is expressed in the central, medial and basomedial amygdaloid nuclei, the hypothalamus and the periaqueductal gray of the mouse brain – regions involved in regulating autonomic functions.

Inspired by the striking expression pattern of Baiap3 and its possible involvement in synapse function, we wanted to explore the role of this protein. We combined the behavioural analysis of *Baiap3* knockout (KO) mice with the study of SNP-associated phenotypes in a sample of thoroughly phenotyped schizophrenic and schizoaffective patients (DSM-IV-TR) (American Psychiatric Association, 2000) of the GRAS dataset (Begemann et al., 2010; Ribbe et al., 2010).

To assess whether the loss of Baiap3 would lead to any detectable behavioral alterations, we subjected *Baiap3* KO mice and their wildtype (WT) littermates to a battery of standard behavioral tests. We observed anxiety-related behavioral alterations in the open field test for KO animals of both sexes. In all other tests we saw no difference in performance between KO and WT mice. Following up on the open field results, we conducted two

additional tests to assess anxiety, leading to the conclusion that *Baiap3* KO animals of both sexes show an anxiety phenotype (even more pronounced in females) but only in the context of exposure to novel environments.

To explore the association of *BAIAP3* with anxiety in humans we chose to look at three SNP (of which two were finally used for analysis – rs2235632, rs1132358) with minor allele frequencies >30% and determined their association with a human anxiety measure. To operationalize anxiety in our cohort we constructed an anxiety composite score comprised of the trait and state anxiety subscales of the State-Trait Anxiety Inventory (STAI), the anxiousness subscale of the Brief Symptom Inventory (BSI) and the anxiety item from the Positive and Negative Syndrome Scale (PANSS). We found the anxiety composite score to be significantly associated with both SNP in female schizophrenic patients. Due to the fact that anxiety disorders and substance use disorders are highly comorbid (Conway et al., 2006; Grant et al., 2004; Marmorstein, 2012), we decided to check for associations of the chosen SNP genotypes with substances use disorders. We found the same genotypes previously associated with anxiety in females, to be associated with benzodiazepine use disorder in males.

Based on the human results we set up a mouse experiment to compare tolerance development to diazepam (5mg/kg daily intraperitoneal injections for 10 consecutive days) in *Baiap3* KO mice and their WT littermates. In this experiment we observed a faster tolerance development in male *Baiap3* KO mice. When comparing KO and WT mice with regards to self-administration of midazolam in a chronically addicted state, we found no genotype differences. We further injected KO and WT mice with pentylenetetrazole (PTZ), a noncompetitive GABA antagonist with epileptic properties, and saw a higher seizure propensity in *Baiap3* KO mice of both sexes. To study possible differences in the response to benzodiazepines and benzodiazepine withdrawal *in vitro*, we used organotypic hypothalamus slices of *Baiap3* KO and WT mice. Here we found higher basal network activity and no homeostatic adaption to diazepam treatment and withdrawal in *Baiap3* KO slices. Immunostaining analysis did not yield a significant localization of Baiap3 to glutamatergic or GABAergic pre- or postsynapses, leaving the question of the cellular function of Baiap3 open.

In summary, we associated genetic variation in *BAIAP3* with anxiety in females and benzodiazepine use disorder in males. In mice *Baiap3* deficiency was associated with increased novelty-induced anxiety in both sexes (more pronounced in females), and an altered response to benzodiazepines mainly in males. *In vitro* we saw no homeostatic adaption to diazepam in *Baiap3* KO hypothalamus slices. Overall this project implies an etiological connection between anxiety and benzodiazepine abuse in mice and humans. Further research and a better understanding of the underlying biological mechanisms could have the potential to improve the treatment of many affected individuals since anxiety disorders and substance use disorders often co-occur (Conway et al., 2006; Grant et al., 2004; Marmorstein, 2012) and particularly benzodiazepines are often prescribed to individuals suffering from different anxiety disorders (Baldwin et al., 2012; Michael Kaplan and DuPont, 2005).

3.2 Original publication

Stepniak B.*, Wojcik S.M.*, Tantra M.*, Man K.N., Muller-Ribbe K., Begemann M., Ju A., Papiol S., Ronnenberg A., Gurvich A., Shin Y., Augustin I., Brose N., Ehrenreich H. (2013) Genetic markers of a Munc13 protein family member, BAIAP3, are gender specifically associated with anxiety and benzodiazepine abuse in mice and humans. Mol Med **19**:135-48.
**Equally contributing authors*

Personal contribution

I was responsible for the human phenotype-genotype association study using the GRAS data collection. Additionally, I conducted the case-control comparison (see Supplementary Figure S4 D) and I was responsible for the selection and operationalization of the anxiety readouts as well as the development of an anxiety composite score for the schizophrenic sample (Supplementary Figure S2). I conducted all the statistical analyses for the human part (Table 1, Supplementary Table S1, Supplementary Figure S5), contributed to the interpretation of the data, designed the (above mentioned) figures and tables, and wrote the respective figure legends for the publication. Additionally, I wrote the materials and methods section for the human sample of the manuscript. Together with my first author colleagues and under the supervision of Prof Hannelore Ehrenreich I discussed the results and their

interpretation for the discussion section of the paper, responded extensively to queries of reviewers and editors concerning the human data and did the final proof reading of the manuscript together with the other first authors before publication.

PROJECT I
Molecular Medicine

Genetic Markers of a Munc13 Protein Family Member, *BAIAP3*, Are Gender Specifically Associated with Anxiety and Benzodiazepine Abuse in Mice and Humans

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Anxiety disorders and substance abuse, including benzodiazepine use disorder, frequently occur together. Unfortunately, treatment of anxiety disorders still includes benzodiazepines, and patients with an existing comorbid benzodiazepine use disorder or a genetic susceptibility for benzodiazepine use disorder may be at risk of adverse treatment outcomes. The identification of genetic predictors for anxiety disorders, and especially for benzodiazepine use disorder, could aid the selection of the best treatment option and improve clinical outcomes. The brain-specific angiogenesis inhibitor I–associated protein 3 (Baiap3) is a member of the mammalian uncoordinated 13 (Munc13) protein family of synaptic regulators of neurotransmitter exocytosis, with a striking expression pattern in amygdalae, hypothalamus and periaqueductal gray. Deletion of *Baiap3* in mice leads to enhanced seizure propensity and increased anxiety, with the latter being more pronounced in female than in male animals. We hypothesized that genetic variation in human *BAIAP3* may also be associated with anxiety. By using a phenotype-based genetic association study, we identified two human *BAIAP3* single-nucleotide polymorphism risk genotypes (AA for rs2235632, TT for rs1132358) that show a significant association with anxiety in women and, surprisingly, with benzodiazepine abuse in men. Returning to mice, we found that male, but not female, *Baiap3* knockout (KO) mice develop tolerance to diazepam more quickly than control animals. Analysis of cultured *Baiap3* KO hypothalamus slices revealed an increase in basal network activity and an altered response to diazepam withdrawal. Thus, *Baiap3/BAIAP3* is gender specifically associated with anxiety and benzodiazepine use disorder, and the analysis of *Baiap3/BAIAP3*-related functions may help elucidate mechanisms underlying the development of both disorders.
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INTRODUCTION

Anxiety disorders have high lifetime prevalence rates (1) and exhibit a remarkable comorbidity with substance use disorders (2–4). This association worsens treatment outcomes for both conditions (5) and represents a significant burden on individuals and society. Both anxiety disorders and substance use disorders are complex disorders that arise from a combination of genetic influence and environmental factors. To improve upon established treatment options, which include pharmacological as well as cognitive-behavioral therapies (6,7), a more detailed picture of the etiology of these disorders is instrumental. Estimates of heritability from twin and family studies are in the range of 20–40% across the different anxiety disorders (8,9) and in the range of 40–70% for the major substance use disorders (10). Recent studies point to the involvement of a large number of genes with relatively small effect sizes for both anxiety disorder (11,12) and substance use disorder (13–15). Although the interaction between anxiety disorders and substance use disorders is likely bidirec-

*SMW, MT, and BS contributed equally to this study.

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tional and varies by the type of anxiety (16), genetically determined anxiousness personality traits may make the development of an addiction more likely (2,17–19). The recommended first-line pharmacological treatments of anxiety disorders are selective serotonin or serotonin/norepinephrine reuptake inhibitors and the calcium channel modulator pregabaline (6). However, primary care physicians often still prescribe benzodiazepines, which rank among the most frequently abused prescription medications (National Institute on Drug Abuse [http://www.nida.nih.gov]), to patients suffering from anxiety disorders (20). Identifying genetic risk markers would advance our understanding of the biology of anxiety and benzodiazepine abuse and would be a valuable step in improving treatment options for these complex diseases.

In addition to human family, twin and genome-wide association studies, animal models are used to study the genetic basis and neural circuitries of anxiety and addiction. For both animals and humans, anxiety is an adaptive defensive response to threatening stimuli necessary for the survival of the species, whereas anxiety disorders are an extreme and maladaptive manifestation of normal anxiety (21). Somatic anxiety symptoms are mediated by the release of specific neurotransmitters and neuropeptides. The selection of candidate genes that are being investigated in animal studies is still largely driven by hypotheses of the neural circuitries and neurotransmitter systems thought to be involved in mediating fear and anxiety (22). By using a candidate gene approach, we investigate the involvement of the brain-specific angiogenesis inhibitor I–associated protein 3 (Baiap3), which is highly expressed in brain regions involved in processing fear, such as the amygdalae, hypothalamus and periaqueductal gray, in behavioral phenotypes relevant for human psychiatric disorders.

Baiap3 is a member of the mammalian uncoordinated 13 (Munc13) family of synaptic regulators of neurotransmitter exocytosis (23–25). *Baiap3* has a unique and striking expression pattern (Allen Brain Atlas [http://mouse.brain-map.org]) in brain regions such as the central, medial and basomedial amygdaloid nuclei; the hypothalamus; and the periaqueductal gray. These areas are involved in regulating autonomic functions and are also critical in processing fearful stimuli and mediating anxiety-related behaviors (26,27). The cellular function of Baiap3 is currently unknown; however, all other Munc13 members are regulators of vesicle exocytosis in various cell types (28). In the brain, Munc13-1 and Munc13-2 are essential for membrane fusion of synaptic vesicles containing classical neurotransmitters, such as glutamate or γ-aminobutyric acid (GABA) (25). Munc13-4, a non-neuronal Munc13 isoform most closely related to Baiap3 at the sequence level, is involved in exocytosis in cells of the hematopoietic system (29,30).

To explore the function of Baiap3, we combined the behavioral analysis of *Baiap3* knockout (KO) mice with a phenotype-based genetic association study (PGAS) of the human *BAIAP3* gene by using the Göttingen Research Association for Schizophrenia (GRAS) database (31,32). Using this two-pronged approach, we identify *Baiap3/BAIAP3* as the first genetic risk marker for anxiety and benzodiazepine abuse in both mice and humans.

MATERIALS AND METHODS

Animals

Animal maintenance. All experiments were approved by the local Animal Care and Use Committee of Lower Saxony, Oldenburg, Germany. The first three coding exons of the murine *Baiap3* gene were preplaced with a neomycin resistance cassette through homologous recombination in embryonic stem cells (129/Ola) (Supplementary Figure S1A). *Baiap3* mutant mice of mixed 129/Ola;C57BL/6N background were backcrossed for seven more generations to C57BL/6N; all experiments were done with WT and KO littermates of the resulting generation 8. After weaning, mice were group-housed in standard plastic cages (n = 5 per cage) and maintained in a temperature-controlled environment (21 ± 2°C) on a 12-h light–dark cycle with food and water ad libitum, unless stated otherwise.

Drugs used in animal experiments. Two classical benzodiazepines, positive allosteric modulators of GABA type A receptors ($GABA_AR$) were used: (i) the long-acting benzodiazepine diazepam (ratiopharm GmbH, Ulm, Germany) was suspended in saline containing polysorbate 80 for intraperitoneal (IP) injection, and (ii) the short-acting benzodiazepine midazolam (ratiopharm) was added to 2% sucrose solution for oral administration. Antagonists used were as follows: (i) flumazenil (Sigma-Aldrich Chemie, Munich, Germany), routinely applied in the clinic to counteract benzodiazepine overdoses, was dissolved in saline containing polysorbate 80 and HCl; and (ii) pentylenetetrazole (PTZ) (Sigma-Aldrich Chemie), a noncompetitive GABA antagonist with epileptogenic properties, was dissolved in saline for IP injection.

Phenotypical characterization of *Baiap3* KO mice. Behavioral characterization of naive *Baiap3* KO mice and their WT littermates of both sexes began at the age of 8 wks and was performed in the following order: elevated plus-maze, open field, light–dark box, hole board, rotarod and exposure to a fear-conditioning chamber to assess novelty-induced freezing behavior. Mouse numbers of all individual experiments are given in the figure legends.

Elevated plus-maze. The mouse was placed in the central platform, facing an open arm of the plus-maze. Behavior was recorded over 5 min by an overhead video camera. A personal computer equipped with Viewer software (Biobserve, Bonn, Germany) was used to calculate the time each animal spent in open versus closed arms. The proportion of time spent in open arms (natural aversion) was used as a fear equivalent.

Open field. Spontaneous activity in open field was tested in a gray Perspex

arena (120 cm in diameter, 25 cm high), virtually divided into three zones: central, intermediate and peripheral. The mouse was placed in the center, and the test was started when the mouse reached the wall. Over 7 min, the mouse was allowed to freely explore the open field. Behavior was recorded by a personal computer–linked overhead video camera and calculated using Viewer software. Readouts were as follows: velocity, distance traveled, time spent in each zone and initial latency to reach the wall.

Hole board. The hole board apparatus (TSE Systems GmbH, Bad Homburg, Germany) for measuring exploratory activity consisted of a 50 cm × 50 cm × 35 cm transparent Perspex chamber with a nontransparent floor raised above the bottom of the chamber. The floor had 16 equally spaced holes, 2.4 cm in diameter, fitted with a light barrier sensor (8 mm below floor). Mice were allowed to explore the chamber for 5 min, and the number of explored holes (head dips) was recorded.

Rotarod. This test for motor function, balance and coordination consists of a rotating drum (Ugo Basile, Comerio, Varese, Italy), accelerated from 4 to 40 revolutions per minute over 5 min. Each mouse was placed individually on a drum and the latency of falling from the drum was recorded using a stopwatch. To assess motor learning, the test was repeated 24 h later.

Novelty-induced fear response. To assess novelty-induced fear response (indicated by freezing behavior), a chamber designed for training and testing of context fear conditioning was used. Mice were placed inside the chamber and allowed to explore the chamber freely for 2 min, during which time no additional stimulus was presented (equivalent to the assessment of baseline freezing of the fear-conditioning paradigm). Duration of freezing behavior, defined as the absolute lack of movement (excluding respiratory movements), was recorded by a video camera and a personal computer equipped with Video Freeze software (MED Associates, St. Albans, VT, USA).

Pentylenetetrazole-induced seizures. Seizure activity was induced in wakeful mice by using a single IP injection of PTZ (50 mg/kg body weight) (33). After injection of the compound, the mouse was placed in a small, clear home cage and closely observed for 30 min. Latencies to focal (partial clonic), generalized (generalized clonic) and maximal (tonic-clonic) behavioral seizures were recorded. Furthermore, four phases in the continuum of behavioral response to IP PTZ injection were defined as follows: (i) hypoactivity (progressive decrease in motor activity until the animal came to rest in a crouched or prone position with the abdomen in full contact with the cage bottom); (ii) partial clonus (clonus seizure activity affecting face, head and/or forelimb or forelimbs); (iii) generalized clonus (sudden loss of upright posture, whole body clonus involving all four limbs and tail, rearing and autonomic signs); and (iv) tonic-clonic (maximal) seizure (generalized seizure characterized by tonic hindlimb extension—also associated with death). Finally, latencies to partial clonus (PC), generalized clonus (GC) and tonic-clonic (TC) seizures were summed to assign a seizure score to each mouse, used as a quantitative trait measure for mapping according to the following equation: seizure score = $[(0.2) \times (1/\text{PC latency}) + (0.3) \times (1/\text{GC latency}) + (0.5) \times (1/\text{TC latency})] \times 1{,}000$. The weighting factors (0.2, 0.3 and 0.5) in the equation were included as a means of incorporating a measure of the progressive nature of the PTZ-induced seizure phenotype into the severity rating because generalized clonus is regarded as a more significant event than partial clonus and tonic hind limb extension as the most severe component of the phenotype. Therefore, the seizure score reflects the degree of progression of the seizure phenotype in each mouse (33).

Diazepam dependence, tolerance and withdrawal. The mice received injections of diazepam (5 mg/kg body weight IP) for 10 consecutive days. Rotarod test was performed 30 min after each diazepam injection for 7 d, with baseline rotarod training performed for 2 d before starting injections. On d 11, diazepam withdrawal was induced by flumazenil (15 mg/kg body weight IP), followed by injection of PTZ (50 mg/kg body weight IP) to induce withdrawal-related seizures. Seizure induction by PTZ (50 mg/kg body weight IP) was also performed on drug-naive mice.

Midazolam oral self-administration and behavior testing in the addicted state. To induce benzodiazepine dependence as a prerequisite for oral self-administration (document of addiction), group-housed mice received midazolam (ratiopharm) in 2% sucrose (to reduce the bitter taste), instead of drinking water. Midazolam concentration was increased weekly, starting from 0.005 mg/mL until the maximum concentration of 0.05 mg/mL was reached after 10 wks. A respective control group received 2% sucrose only. One set of midazolam mice was then exposed to a midazolam preference test. For this purpose, mice were first switched to single housing with a continued supply of midazolam (0.05 mg/mL) for 2 wks. For the preference test, every mouse had a choice of two bottles containing either midazolam (0.05 mg/mL) in 2% sucrose or 2% sucrose alone for another 2 wks. The relative consumption of midazolam solution was calculated. The other set of mice (midazolam and control mice) stayed group-housed and underwent automated home cage observation using the LABORAS™ system (Metris, Hoofddorp, Netherlands). LABORAS is a fully automated system for continuous behavior recognition and tracking in small rodents. For habituation before testing, mice were temporarily put in single cages similar to the LABORAS cage in the testing room for 2 consecutive nights (1700 to 0900). On the day of testing, Makrolon type 3 cages (840 cm^2), filled with a 2-cm layer of bedding used during the habituation phase, were placed on each triangular sensor platform (95 cm × 75 cm × 75 cm). Food and sucrose solution with midazolam (addicted group) or 2% sucrose (control group) were provided *ad libitum*.

Before each session, LABORAS was calibrated by using the calibration procedure and reference weights supplied by Metris. Movements during nighttime (1800 to 0900) were recorded and distinguished as separate behavioral patterns by the LABORAS software. Locomotion duration and scratching frequency during the dark phase (2000 to 0800) was analyzed.

Statistical analysis. Behavioral data were analyzed separately for males and females by the Mann-Whitney *U* test and two-way analysis of variance (ANOVA), including post hoc Bonferroni testing, where applicable, using Prism4 (GraphPad Software, San Diego, CA, USA). Significance level was set to $p < 0.05$. All data are presented as mean ± standard error of the mean (SEM).

Human Sample

Schizophrenic patient sample. The schizophrenic patient sample (n = 1,086) was recruited across 23 sites throughout Germany in the cross-sectional GRAS study and most comprehensively phenotyped (31,32). The study was approved by the Ethics Committee of the Georg-August-University (Göttingen, Germany) and the review boards of participating centers and complies with the Declaration of Helsinki. Patients fulfilling *Diagnostic and Statistical Manual of Mental Disorders: DSM-IV-TR*, 4th edition, text revision (34) criteria for schizophrenia or schizoaffective disorder were included in the analyses regardless of their disease stage (acute, chronic, residual or remitted). Almost all patients were of European Caucasian descent (Caucasian 94.7%; other ethnicities 1.9%; unknown 3.4%).

Healthy control sample. Voluntary blood donors (n = 1,142) recruited following the national guidelines for blood donation were included for case control analysis (31,32). Also the majority of control subjects are of European Caucasian ethnicity (Caucasian 97.8%; other ethnicities 2%; unknown 0.2%).

Sociodemographic and clinical variables. Sociodemographic data (age, years of education, level of unemployment), information on substance use disorder (summarizing abuse and dependence based on the DSM-IV-TR criteria for alcohol and cannabis) and clinical variables describing disease severity were used to characterize the sample. Clinical variables included Positive and Negative Syndrome Scale (PANSS) positive scale as a measure of positive symptom severity (35) as well as chlorpromazine equivalents to estimate the relative dose of antipsychotic medication. The Global Assessment of Functioning (GAF) scale (DSM-IV-TR) was used as a measure of impairment of psychological, social and occupational functioning.

Target variables. The dichotomous DSM-IV-TR benzodiazepine use disorder diagnosis (summarizing abuse and dependence) and the quantitative anxiety composite score were our target variables. The anxiety composite score is based on the aggregation of four anxiety-related variables: (i) Brief Symptom Inventory (BSI) subscale anxiousness; (ii) State-Trait Anxiety Inventory (STAI) subscale trait anxiety; (iii) STAI subscale state anxiety; and (iv) anxiety item of the PANSS general psychopathology subscale (Supplementary Figure S2).

DNA extraction and normalization. Genomic DNA was purified from whole blood by using JETQUICK Blood and Cell Culture DNA Spin Kit (Genomed, Loehne, Germany) according to the manufacturer's protocol. DNA aliquots were stored at –80°C. For further analyses, DNA was normalized to 50 ng/μL with an automated robotic platform (Microlab Star, Hamilton, Bonaduz, Switzerland). Each sample was analyzed with a 0.8% agarose gel for quality control.

Genotyping. The three selected SNPs (rs11648169, rs2235632, rs1132358) of *BAIAP3* were analyzed by using Simple Probes (TIB Molbiol, Berlin, Germany) and genotyped 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 DNA in 384-well plates following the standard protocol (Roche). In each run, eight positive controls (hgDNA, Bioline, Luckenwalde, Germany) and negative water blanks were included for quality and internal control purposes. Of the GRAS patients, a total of n = 1,082 (99.63%) were successfully genotyped for *BAIAP3* SNP1 (C/G) rs11648169, n = 1,086 (100%) for *BAIAP3* SNP2 (G/A) rs2235632 and n = 1,069 (98.43%) for *BAIAP3* SNP3 (C/T) rs1132358 and included in the analyses. Of the healthy control subjects, all n = 1,142 were successfully genotyped for SNP1, SNP2 and SNP3 of the *BAIAP3* gene.

Statistical analyses. For all analyses, statistical significance was set to 0.05. Statistical analyses of human data were performed by using SPSS for Windows, version 17.0. Group differences in categorical and continuous variables were assessed using χ^2 or Mann-Whitney *U* tests; in cases of normal distribution of the continuous variable, *t* tests were performed. Anxiety score composition was done using *z*-standardized mean subscale scores (BSI anxiousness, STAI trait anxiety, STAI state anxiety) or, in the case of PANSS anxiety, a *z*-standardized single item, organized such that higher values represent higher symptom severity. Intercorrelations and internal consistency of the anxiety composite score was calculated by using Pearson correlation coefficient and Cronbach α (36). In the GRAS sample, the following items or scales were incomplete: BSI anxiousness 7.5% missing, STAI trait anxiety 20.2%, STAI state anxiety 21.6% and PANSS anxiety 3.2%. If all four anxiety variables were available, the mean was calculated for each respective subject as an individual anxiety composite score. In the case of missing data, a linear regression-based multiple imputation model (10 iterations) of missing data was applied, if at least three out of the four variables per subject were available. For the 190 individuals with imputed values, the final anxiety composite score represents the mean of 10 imputed values for the missing item, increasing the availability of the anxiety score from n = 771 to n = 961 schizo-

phrenic subjects (37). Analysis of covariance (adjusted for age, PANSS positive subscale score and chlorpromazine equivalents) was used to analyze the effect of SNP genotypes on the standardized anxiety composite score. For the phenotype-genotype association analyses (including peripheral blood mononuclear cells [PBMCs]; see below) of the *BAIAP3* SNP rs2235632, G carriers (GG and AG) were aggregated and contrasted with individuals homozygous for the A allele, and in the case of SNP rs1132358, C carriers (CC and TC) were aggregated and contrasted with TT individuals. SNP rs11648169 was excluded from further analyses, since it yielded no statistically significant effects.

In Vitro Analyses

Immunofluorescence analysis. Brains were perfusion-fixed, and organotypic hypothalamus slices were immersion fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4). Brains were postfixed for 1 h, cryoprotected with 30% sucrose and frozen. For immunofluorescence analysis, free-floating brain sections of 40 μm thickness or organotypic sections of 300 μm thickness were incubated in primary antibodies for 72 h followed by incubation with IgG-coupled Alexa Fluor 488, Alexa Fluor 555 and Alexa Flour 633 dyes (Invitrogen [Life Technologies, Darmstadt, Germany]) for 24 h. Rabbit and guinea pig antibodies to Baiap3 were raised to a purified fragment (amino acids 617–973) containing the munc homology domain (MHD)-1 and MHD-2 of mouse Baiap3. Commercial primary antibodies used were rabbit and guinea pig anti-vesicular glutamate transporter 1 (VGLUT1), rabbit and guinea pig anti-VGLUT2, rabbit and guinea pig anti-vesicular inhibitory amino acid transporter (Viaat), mouse anti-Gephyrin (mAB7a) (all from Synaptic Systems, Göttingen, Germany), and mouse anti-postsynaptic density protein 95 (PSD-95) (clone K28/48, NeuroMab). False color images of brain sections and organotypic slices were obtained with a fluorescence stereomicroscope (Leica FluoCombi III™) and an ApoTome™ fluorescence microscope (Axio Imager Z1; Zeiss), respectively.

Hypothalamus slice culture. Organotypic hypothalamus slices of 300-μm thickness from postnatal d 5 (P5) and P6 mice were prepared in Hanks balanced salt solution (24020-091; Invitrogen [Life Technologies]) with 20% glucose and 1 mmol/L kynurenic acid (Sigma-Aldrich, Germany) (pH 7.4), by using a McIlwain Tissue Chopper. Slices were cultured in six-well plates on confetti cut from 0.45-μm filters (FHLC04700; EMD Millipore [Millipore Ireland B.V., Tullagreen, Carrigtwohill County Cork, Ireland]) that were placed in 0.4-μm Millicell cell culture inserts (PICM03050; Millipore) for 5 d using a mixture of 41% Earle basal medium Eagle (BME) (F 0225; Biochrom, Berlin, Germany), with 25% Earle balanced salt solution (1.8 mmol/L $CaCl_2$, 1 mmol/L NaH_2PO_4, 0.8 mmol/L $MgSO_4$, 116 mmol/L NaCl, 26.2 mmol/L $NaHCO_3$, 5.4 mmol/L KCl, 5 mmol/L glucose), 20% heat-inactivated horse serum, 10% H_2O, 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Biochrom), 28 mmol/L glucose, 1 mmol/L GlutaMAX™ (35050; Invitrogen [Life Technologies]), 1 μg/mL insulin, 88 μg/mL ascorbic acid, 0.25% MEM Vitamine Solution (K0373; Biochrom) and 0.5% MEM Amino Acids (K0363; Biochrom). On d 5 *in vitro*, the cultures were switched to a medium with identical components but containing 5% horse serum, 55% BME and 2 mmol/L GlutaMAX. At the same time, diazepam was added to the medium from a 60 mmol/L stock solution in dimethylsulfoxide (DMSO) for a final concentration of 10 μmol/L. For control cultures, DMSO was added as a vehicle control at the same dilution of 1:6,000. The CO_2 concentration was 5%, and medium changes were done on the day after culture and every 48 h after that.

Electrophysiological analyses. Organotypic slices containing the ventromedial hypothalamus were transferred to the recording chamber between DIV10 and DIV17. Recordings were started after a 30-min recovery time, the extracellular recording solution contained 120 mmol/L NaCl, 26 mmol/L $NaHCO_3$, 1 mmol/L KH_2PO_4, 2 mmol/L KCl, 20 mmol/L glucose, 2 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$ and 250 nmol/L flumazenil. Cells were whole-cell voltage clamped at –70 or –20 mV or recorded in current clamp mode with an EPC 10 USB Double (HEKA, Lambrecht/Pfalz, Germany) under control of the Patchmaster 2.52 program (HEKA). All analyses were performed by using the Mini Analysis Program (Synaptosoft, Decatur, GA, USA). Recordings of miniature inhibitory postsynaptic currents (mIPSCs) were performed in the presence of 1 μmol/L tetrodotoxin (Tocris [R&D Systems, Wiesbaden-Nordenstadt, Germany]) and 10 μmol/L 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX) (Tocris [R&D Systems]), with an intracellular solution containing 100 mmol/L KCl, 50 mmol/L K-gluconate, 10 mmol/L HEPES, 0.1 mmol/L EGTA, 0.3 mmol/L GTP, 4 mmol/L ATP and 0.2% biocytin. Action potentials and spontaneous inhibitory postsynaptic currents (IPSCs) were recorded with an intracellular solution containing 20 mmol/L KCl, 130 mmol/L K-gluconate, 10 mmol/L HEPES, 0.1 mmol/L EGTA, 0.3 mmol/L GTP, 4 mmol/L ATP and 0.2% biocytin. Action potentials analyzed were from the first minute of a 2-min recording; membrane potentials were measured after setting the current injection to 0 pA at the end of the recording. IPSCs were recorded for 5 min after switching the cell to a holding potential of –20 mV and waiting for 1 min. Statistical analyses were performed using GraphPad Prism5.

Analysis of *BAIAP3* mRNA levels in PBMCs. PBMCs from 121 patients were isolated by using the standard Ficoll-Paque Plus isolation procedure (GE Healthcare, Munich, Germany). For RNA isolation, the miRNeasy Mini Kit (Qiagen, Hilden, Germany) was used. A total of 1 μg RNA, a mixture of oligo dT, hexamer primers, dNTPS (10 mmol/L each) and SuperScriptIII (200 U; Invitrogen [Life Technologies]) were used for transcription into cDNA (20-μL reaction). The mixture

was incubated for 10 min at 25°C and 45 min at 50°C, followed by 45 min at 55°C. For the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), a 1:10 dilution of the cDNA was used and three replicate experiments per sample were performed: 5 μL Power SYBR mix (Applied Biosystems) and 1 pmol of each primer were added. *BAIAP3* qRT-PCR primers used were as follows: 5′-AGCTGGGCCCACCGCATCTCT-3′ with 5′-CTCGGCAGGCACGGAAAAGTAG-3′ and 5′-CTGACTTCAACAGCGACACC-3′ with 5′-TGCTGTAGCCAAATTCGTTGT-3′. The following cycling profile was run on the LightCycler480 system (Roche): preheating at 95°C for 10 min; 45 cycles of 95°C for 15 s, 60°C for 1 min. Cycle threshold values of *BAIAP3* were standardized to cycle threshold values of GAPDH.

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

RESULTS

Generation of *Baiap3* KO Mice

Baiap3 shares the basic domain structure of other Munc13 isoforms, with two munc-homology domains flanked by two C2 domains but lacks the N-termini contained in Munc13-1, -2 and -3 (23). The murine *Baiap3* gene contains 33 coding exons that span 8.7 kb. We generated *Baiap3* KO mice by homologous recombination in embryonic stem cells, replacing the first three coding exons with a neomycin selection cassette (Supplementary Figure S1A). *Baiap3* KO mice are viable, fertile and indistinguishable from their wild-type (WT) littermates in the home cage. In WT brain, the expression pattern of Baiap3 protein analyzed by immunofluorescence staining largely matches the distribution of *Baiap3* mRNA published in the Allen Brain Atlas. Baiap3 protein is prominently expressed throughout the hypothalamus and in the central, medial and basomedial amygdaloid nuclei, as well as in the paraventricular nucleus of the thalamus (Figure 1). Strong expression is further detected in the septum, bed nucleus of the stria terminalis, midbrain including the periaqueductal gray and inferior colliculus, and brain stem including the parabrachial nucleus and nucleus tractus solitarius (Figure 1). Baiap3 immunoreactivity appears punctate, but does not seem to localize to either glutamatergic or GABAergic pre- or postsynapses to a significant degree (Supplementary Figures S1C–G). Adult *Baiap3* KO mice lack any detectable expression of Baiap3 protein by immunofluorescence and Western blot analysis (Figure 1B, Supplementary Figure S1B). Western blot analysis of brains taken from newborn *Baiap3* KO animals revealed the presence of a weak band that most likely corresponds to Baiap3 protein expressed from a start codon present in coding exon 4; however, this putative truncated Baiap3 product is barely detectable by the age of 3 wks and not present in adult animals (Supplementary Figure S1B).

Novelty-Induced Anxiety in *Baiap3* KO Mice

The striking expression pattern of *Baiap3* in the amygdala and other brain regions involved in processing fear piqued our interest, and we chose to assess whether the genetic deletion of *Baiap3* led to any detectable behavioral alterations. We subjected *Baiap3* KO mice and WT littermates of both sexes to a battery of standard behavioral tests (Figures 2A–L; Supplementary Figures S3A–J). In the open field, both male and female *Baiap3* KO mice showed an increased latency to reach the wall upon release in the center zone (Figures 2A, B). Female but not male KO mice also made fewer visits to the center (Figures 2C, D) and spent significantly more time in the periphery (Figures 2E, F). When placed in a novel chamber (fear-conditioning box), both male and female KO mice showed an increased novelty-induced freezing response (Figures 2K, L). Taken together, these findings are indicative of a heightened novelty-induced anxiety level in *Baiap3* KO animals, with a more pronounced effect noted in females. In contrast, classical tests, measuring anxiety in the context of an inherent conflict between a protected and a more anxiogenic area, that is, elevated plus-maze and light–dark box, did not reveal any genotype differences (Figures 2G–J). Furthermore, the distance traveled (motor activity) in open field and elevated plus-maze (Supplementary Figures S3A–D), exploratory behavior (hole board; Supplementary Figures S3E, F), motor learning and coordination (rotarod; Supplementary Figures S3G, H) and body weight (Supplementary Figures S3I, J) were not affected by genotype.

BAIAP3 Is a Risk Marker for Anxiety in Women

To explore the possibility of an association of genetic variability in the human *BAIAP3* gene with specific biological readouts, we made use of the GRAS database of schizophrenic patients (31,32). Our hypotheses regarding Baiap3/BAIAP3 function were based on the anxiety phenotype observed in *Baiap3* KO mice and on the prominent expression of Baiap3 in brain regions involved in processing fearful stimuli as well as in substance use disorders. We selected three single-nucleotide polymorphisms (SNPs) in the *BAIAP3* gene: rs11648169 (C/G, intronic), rs2235632 (G/A, intronic) and rs1132358 (C/T, coding sequence, synonymous Asp1040Asp) (Supplementary Figure S4A) from public databases [http://www.ncbi.nlm.nih.gov/projects/SNP/; http://browser.1000genomes.org; http://hapmap.ncbi.nlm.nih.gov/]. The selection of SNPs was based on (i) a high minor allele frequency (MAF ≥ 0.36) distribution within the European Caucasian population (http://www.ncbi.nlm.nih.gov/SNP/), to increase the power to detect genetic effects, and (ii) the potential for functional consequences. The last criterion could only partially be fulfilled; the exonic SNP rs1132358 (C/T, Asp1040Asp, synonymous) might potentially affect mRNA structure or stability. All SNPs fulfilled Hardy-Weinberg equilibrium criteria, both in cases and in controls ($p > 0.05$). A construction of haplotype blocks of the three SNPs revealed a similarly

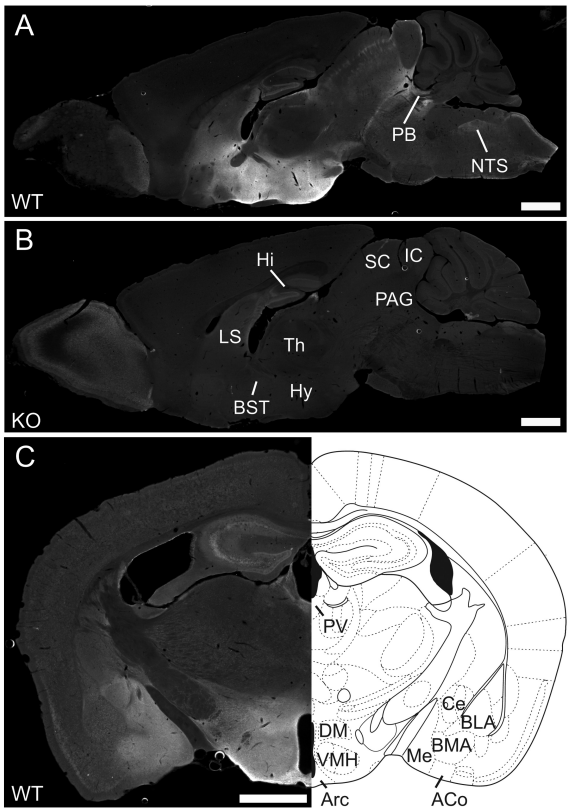

Figure 1. Immunofluorescence analysis of Baiap3 expression in mouse brain. (A) Sagittal brain section of adult *Baiap3* WT mouse stained with rabbit anti-Baiap3 antibody. (B) Sagittal brain section of adult *Baiap3* KO littermate showing the absence of Baiap3 immunoreactivity. Please note that the signal observed in the hippocampus of both WT and KO brain is a nonspecific background staining. (C) Coronal brain section of adult WT mouse stained for Baiap3 with a corresponding coronal diagram, adapted from the mouse Paxinos brain atlas (Bregma –1.46). PB, parabrachial nucleus; NTS, nucleus tractus solitarius; Hi, hippocampus; SC, superior colliculus; IC, inferior colliculus; PAG, periaqueductal gray; LS, lateral septum; Th, thalamus; Hy, hypothalamus; BST, bed nucleus of the stria terminalis; PV, paraventricular thalamic nucleus; DM, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; Arc, arcuate nucleus; Ce, central amygdaloid nucleus; BLA, basolateral amygdaloid nucleus, anterior part; BMA, basomedial amygdaloid nucleus, anterior part; ME, medial amygdaloid nucleus; ACo, anterior cortical amygdaloid nucleus. Scale bars equal 1 mm.

high degree of linkage disequilibrium between them in the GRAS sample (Supplementary Figure S4B) and in healthy controls (Supplementary Figure S4C). Case control analysis of genotype frequencies of the three SNPs did not reveal any significant differences, indicating that the selected genetic variation in *BAIAP3* is not associated with schizophrenia risk (Supplementary Figure S4D). We subsequently used the PGAS approach (32) to analyze the three SNPs for association with specific phenotypic readouts relevant for anxiety disorders and substance use disorders. For this step, an anxiety composite score was constructed using four anxiety-relevant variables (Supplementary Figure S2), which showed a significant association with only two of the three selected SNPs (as expected because of the high linkage disequilibrium between both markers and their similar MAFs) for women but not for men (Table 1). SNP rs11648169 was excluded from further analyses, since it yielded no statistically significant effects.

BAIAP3 Is a Risk Gene for Benzodiazepine Abuse in Men

Because anxiety disorders and substance use disorders often occur together, and *BAIAP3* is expressed in brain regions relevant for emotionality and drug dependence, we also screened for a possible association between genetic variation in *BAIAP3* and substance use disorder. The same risk genotypes (AA for rs2235632, TT for rs1132358) that were associated with anxiety in women showed a statistically significant association with benzodiazepine use disorder in men (Table 1). Even though there was a similar tendency for women (benzodiazepine use disorder associated with 7.0%/7.7% in AA/TT genotypes versus 4.7%/4.6% in G/C carrier status), it did not reach statistical significance, perhaps because of the lower numbers of women than men in the GRAS sample. The genotype frequencies of rs2235632 and rs1132358 did not differ between men and women in the GRAS sample (rs2235632, GG/AG/AA: men 25.1%/49.1%/25.8%; women 28.3%/46.3%/25.5%; rs1132358, CC/TC/TT: men 26.1%/49.4%/24.5%; women 29.8%/46.9%/23.3%), and neither of these two SNPs was associated with disease-related or sociodemographic control variables (Table 1).

For the purpose of an association analysis of the relevant *BAIAP3* genotypes (GG/AG/AA in rs2235632 and CC/TC/TT in rs1132358) with benzodiazepine use disorder, the GRAS sample delivers an ideal, nearly experimental setting. The distribution of these genotypes among benzodiazepine users versus nonusers is highly comparable, allowing the identification of risk genotypes leading to benzodiazepine use disorder (Supplementary Table S1). Most importantly, the benzodiazepine dose was equal across all genotypes (Supplementary Table S1). Hence, the *BAIAP3* risk genotypes (AA for rs2235632, TT for rs1132358) appear to confer a specific genetic risk of developing benzodiazepine use disorder given equal dose and likelihood of exposure. Interestingly, neither alcohol nor cannabis abuse were found to

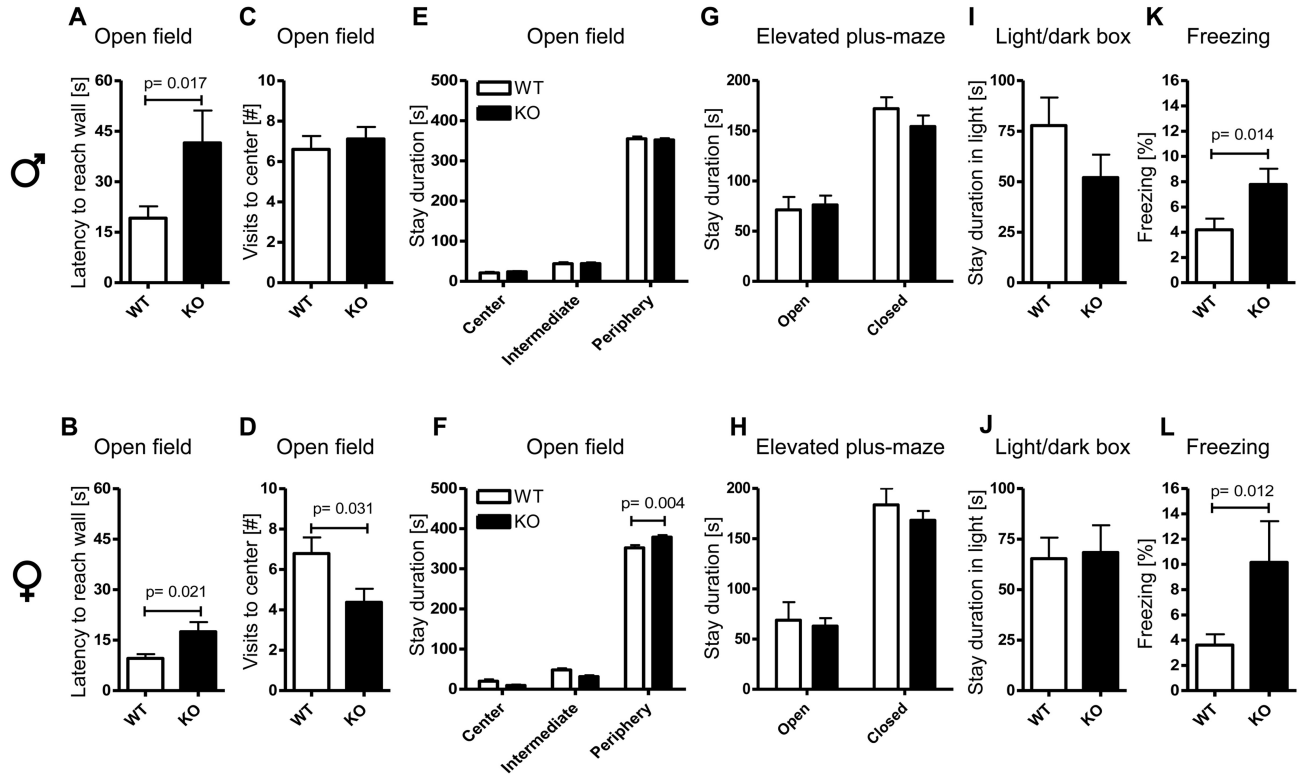

Figure 2. Anxiety phenotype in *Baiap3* KO mice. (A–F) Open field parameters. (A, B) The latency to reach the wall of the open field was significantly increased in *Baiap3* KO mice of both sexes, whereas visits to the center (C, D) and stay in the periphery (E, F) revealed anxiety-like behavior only in females. Elevated plus-maze (G, H) and light–dark box (I, J) revealed no genotype-dependent differences in either sex. (K, L) As readout of unspecific novelty-related anxiety, a higher freezing response was found in male as well as female *Baiap3* KO mice. Numbers tested: males, WT = 16–25, KO = 16–25; females, WT = 18–23, KO = 10–28. Mann-Whitney *U* test (A–D, I–L) and two-way ANOVA (E–H), including Bonferroni testing, were applied. Means ± SEM are presented.

be associated with the two SNPs, pointing to a specific benzodiazepine link with the selected *BAIAP3* genotypes (Table 1).

To determine whether the identified risk genotypes are associated with altered expression of *BAIAP3*, we analyzed the mRNA levels of *BAIAP3* in PBMCs obtained from 121 subjects by qRT-PCR. We found a statistically significant association of the *BAIAP3* risk genotypes (AA for rs2235632, TT for rs1132358) with lower *BAIAP3* mRNA levels in PBMCs of male individuals, which is at least partially comparable to a gene dose reduction or KO situation. This result is not found in women, possibly because of the lower numbers available for analysis (Supplementary Figure S5). However, these findings could also support the interpretation that the effects of *BAIAP3* risk alleles are gender specific.

Male *Baiap3* KO Mice Show Faster Development of Tolerance to Benzodiazepines

On the basis of the identification of human *BAIAP3* risk genotypes for benzodiazepine abuse in male patients, we tested *Baiap3* KO and WT littermates of both sexes in experimental paradigms of chronic benzodiazepine administration to assess the development of tolerance, dependence and withdrawal (Figure 3A). The baseline performance of each mouse in the rotarod test was established on two consecutive days of rotarod training. No significant genotype-dependent differences were detected in baseline performance (Supplementary Figures S3G, H). Benzodiazepine dependence in *Baiap3* KO and WT mice of both sexes was then induced with daily diazepam injections (5 mg/kg IP) for 10 consecutive days. To monitor the development of tolerance to diazepam, motor performance on rotarod at 30 min after each injection was evaluated over the first 7 d of diazepam treatment. Rapid development of tolerance to daily diazepam injections was apparent in both sexes and genotypes by an increase of the latency of falling from rotarod over the course of 7 d (Figures 3B, C). Here, male *Baiap3* KO mice performed significantly better than their WT littermates (Figure 3B), whereas no such difference was detected for females (Figure 3C). Thus, male *Baiap3* KO mice show faster development of tolerance to diazepam.

Baiap3 KO Mice Have an Increased Seizure Propensity

To evaluate whether *Baiap3* genotype would affect the propensity for di-

Table 1. Phenotype comparison of GRAS patients sorted by *BAIAP3* genotypes.

GRAS sample	*BAIAP3* rs2235632			*BAIAP3* rs1132358		
	G carriers (GG/AG)	AA	*p* ($F/T/Z/\chi^2$ value)[a]	C carriers (CC/TC)	TT	*p* ($F/T/Z/\chi^2$ value)[a]
Males	n = 471–538[b]	n = 155–187[b]		n = 474–538[b]	n = 152–175[b]	
Target variables						
Benzo use disorder (n (%))	11 (2.3)	9 (5.4)	**0.047** (χ^2 = 3.93)	11 (2.3)	9 (5.7)	**0.034** (χ^2 = 4.47)
Anxiety composite score (mean ± SD)[c,d]	–0.06 ± 0.74	–0.07 ± 0.70	0.499 (F = 0.46)	–0.07 ± 0.73	–0.05 ± 0.72	0.651 (F = 0.21)
Sociodemographic						
Age (at examination) (years) (mean ± SD (range))	37.33 ± 12.01 (18–78)	36.17 ± 11.91 (17–75)	0.225 (Z = –1.21)	37.28 ± 11.96 (18–78)	36.34 ± 12.06 (17–75)	0.309 (Z = –1.02)
Education (years) (mean ± SD (range))[e]	14.17 ± 3.48 (8–28)	14.42 ± 3.70 (8–27)	0.680 (Z = –0.41)	14.21 ± 3.48 (8–28)	14.32 ± 3.76 (8–27)	0.853 (Z = –0.19)
Unemployment (n (%))	217 (44.7)	76 (44.7)	0.990 (χ^2 = 0.00)	214 (44.2)	75 (46.6)	0.601 (χ^2 = 0.27)
Substance use						
Alcohol use disorder according to DSM-IV-TR (n (%))	221 (42.3)	84 (45.7)	0.435 (χ^2 = 0.61)	217 (41.6)	82 (47.4)	0.180 (χ^2 = 1.80)
Cannabis use disorder according to DSM-IV-TR (n (%))	218 (41.8)	83 (45.1)	0.430 (χ^2 = 0.62)	224 (42.9)	75 (43.4)	0.919 (χ^2 = 0.01)
Clinical						
PANSS positive score (mean ± SD (range))	13.55 ± 6.04 (7–36)	14.02 ± 6.41 (7–38)	0.427 (Z = –0.79)	13.53 ± 6.03 (7–36)	14.23 ± 6.50 (7–38)	0.249 (Z = –1.15)
Chlorpromazine equivalents (mean ± SD (range))	707.90 ± 694.69 (0–6,324.29)	689.45 ± 568.91 (0–3,238.00)	0.678 (Z = –0.42)	701.33 ± 688.16 (0–6,324.29)	712.11 ± 592.75 (0–3,238.00)	0.424 (Z = –0.80)
GAF score (mean ± SD (range))	45.70 ± 16.04 (10–90)	45.35 ± 16.88 (5–90)	0.862 (Z = –0.17)	45.75 ± 16.05 (10–90)	44.90 ± 16.89 (5–90)	0.619 (Z = –0.50)
Females	n = 223–269[b]	n = 75–92 [b]		n = 229–273[b]	n = 71–83[b]	
Target variables						
Benzo use disorder (n (%))	12 (4.7)	6 (7.0)	0.406 (χ^2 = 0.69)	12 (4.6)	6 (7.7)	0.281 (χ^2 = 1.16)
Anxiety composite score (mean ± SD)[c,d]	–0.02 ± 0.77	0.19 ± 0.80	**0.028** (F = 4.91)	–0.02 ± 0.78	0.21 ± 0.77	**0.017** (F = 5.81)
Sociodemographic						
Age (at examination) (years) (mean ± SD (range))	42.36 ± 12.92 (18–79)	44.86 ± 12.52 (21–76)	0.893 (T = 1.61)	42.52 ± 12.90 (18–79)	44.49 ± 12.60 (20–76)	0.221 (T = 1.23)
Education (years) (mean ± SD (range))[e]	14.56 ± 3.95 (7–31)	14.12 ± 3.54 (8–27)	0.447 (Z = –0.76)	14.52 ± 3.94 (7–31)	14.13 ± 3.62 (8–27)	0.454 (Z = –0.75)
Unemployment (n (%))	76 (31.9)	20 (25.0)	0.243 (χ^2 = 1.37)	74 (30.5)	20 (28.2)	0.712 (χ^2 = 0.14)
Substance use						
Alcohol use disorder according to DSM-IV-TR (n (%))	59 (22.4)	14 (15.7)	0.178 (χ^2 = 1.82)	60 (22.4)	13 (16.0)	0.219 (χ^2 = 1.51)
Cannabis use disorder according to DSM-IV-TR (n (%))	30 (11.4)	8 (9.0)	0.525 (χ^2 = 0.40)	29 (10.8)	9 (11.1)	0.941 (χ^2 = 0.01)
Clinical						
PANSS positive score (mean ± SD (range))	13.84 ± 6.66 (7–37)	14.41 ± 6.38 (7–33)	0.288 (Z = –1.06)	13.87 ± 6.69 (7–37)	14.32 ± 6.24 (7–32)	0.366 (Z = –0.91)
Chlorpromazine equivalents (mean ± SD (range))	636.37 ± 776.51 (0–7,375.00)	704.59 ± 762.50 (0–4,370.00)	0.612 (Z = –0.51)	634.40 ± 771.10 (0–7,375.00)	718.83 ± 788.67 (0–4,370.00)	0.616 (Z = –0.50)
GAF score (mean ± SD (range))	46.34 ± 19.42 (8–90)	44.22 ± 17.59 (12–84)	0.435 (Z = –0.78)	46.22 ± 19.25 (8–90)	44.88 ± 18.07 (12–84)	0.645 (Z = –0.46)

SD, standard deviation.
[a]For statistical methods, Mann-Whitney U or χ^2 tests and for normally distributed variables t tests were used. Bolded values: $p < 0.05$.
[b]Because of missing data, sample sizes vary.
[c]Results after multiple imputations (10).
[d]Analysis of covariance with age, positive symptoms (PANSS) and medication status (chlorpromazine equivalent) as covariates.
[e]Total years spent in education system; patients currently in school or educational training were excluded.

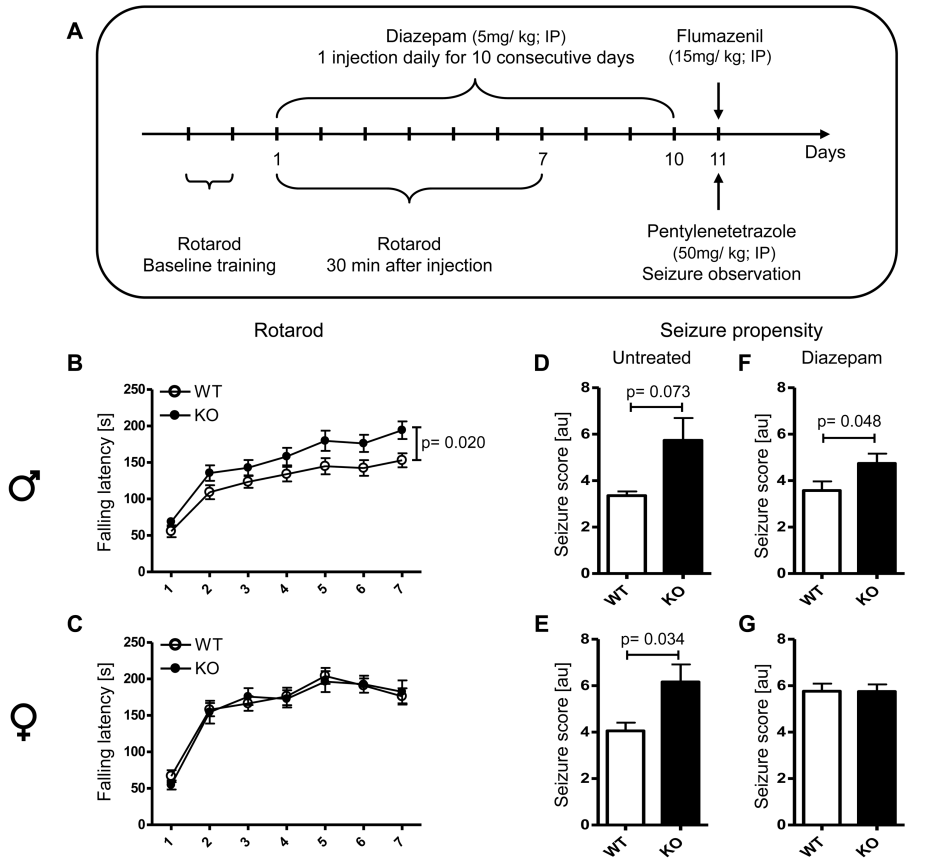

Figure 3. Diazepam tolerance and withdrawal in *Baiap3* KO and WT mice. (A) Experimental design scheme. (B) Male diazepam-treated *Baiap3* KO mice showed significantly faster improvement of performance on the rotarod, consistent with a more rapid development of tolerance to diazepam. (C) Rotarod performance of female mice was comparable between WT and KO. (D, E) Diazepam-naive *Baiap3* KO mice display a higher PTZ-induced seizure propensity compared with WT (significant in females, strong tendency in males). (F, G) Flumazenil-induced diazepam withdrawal does not further increase PTZ-induced seizure propensity in *Baiap3* KO mice. Seizure propensity of female mice became comparable between genotypes, pointing to a ceiling effect. Numbers tested: males, WT = 25, KO = 25; females, WT = 21, KO = 23, except for (D) and (E), where males, WT = 7, KO = 7; females, WT = 8; KO = 10. Mann-Whitney *U* test (D–G) and two-way repeated-measures ANOVA (B, C), including Bonferroni, testing applied. Means ± SEM are presented.

azepam withdrawal-related seizures, the susceptibility to PTZ-induced seizures was first evaluated in diazepam-naive mice. The seizure response of *Baiap3* KO mice of both sexes to PTZ (50 mg/kg IP) was higher than that in WT animals, with the difference just failing to reach significance in males (Figures 3D, E). To assess the effect of genotype on benzodiazepine withdrawal, the diazepam antagonist flumazenil (15 mg/kg IP) was injected on d 11, after 10 d of daily diazepam treatment, immediately followed by PTZ injection (50 mg/kg IP) to trigger withdrawal seizures (Figure 3A). Upon flumazenil-induced diazepam withdrawal, the response to PTZ in male *Baiap3* KO and WT mice did not differ appreciably from the one found in diazepam-naive mice of both genotypes (Figure 3F). In contrast, the genotype-dependent differences in diazepam-naive females regarding seizure scores disappeared under conditions of diazepam withdrawal (Figure 3G), which could be explained by a ceiling effect. Thus, female and male *Baiap3* KO mice are more seizure-prone than their WT littermates, and this propensity is not further increased by benzodiazepine withdrawal.

Drug Self-administration and Basic Behaviors Do Not Differ between *Baiap3* Genotypes upon Chronic Addiction

To assess whether *Baiap3* KO mice, once addicted, would also be more likely to orally self-administer benzodiazepines, we performed an experiment on chronic midazolam addiction, where self-application was assessed after forced long-term exposure to escalating doses of midazolam (Supplementary Figure S6). We detected no genotype or gender differences in the clear preference for midazolam. Moreover, no genotype effects on body weight or basic behavior in the chronically addicted state were noted (Supplementary Figure S6). These data indicate that the *Baiap3* genotype gender specifically affects the development of tolerance, that is, drug abuse at an early stage. In chronic addiction, genotype effects are no longer detectable.

Lack of Homeostatic Adaptation to Diazepam in *Baiap3* KO Hypothalamus Slices

One hypothesis regarding predisposition to the development of addiction at the cellular level is an altered response to the addiction-inducing substance and its withdrawal. Because *Baiap3* KO mice showed an increased seizure propensity and an altered development of tolerance to diazepam, we investigated whether lack of *Baiap3* leads to a measurably altered response to diazepam treatment and withdrawal in neurons *in vitro*. Because Baiap3 expression is highest in the hypothalamus, we cultured organotypic hypothalamus slices prepared from male P5/P6 *Baiap3* KO and WT animals in the presence of either 10 μmol/L diazepam or vehicle (DMSO) and recorded from neurons in the ventromedial hypothalamus in the presence of the diazepam antagonist flumazenil to

mimic diazepam withdrawal conditions *in vitro*. We hypothesized that diazepam treatment would lead to a homeostatic adaptation in the $GABA_A$R-mediated mIPSCs (Figure 4A) that would become apparent under diazepam withdrawal conditions. Although we observed no diazepam treatment–dependent differences that reached statistical significance, there was a significant genotype-dependent effect under diazepam withdrawal conditions. Here, WT mIPSC amplitudes were 27% smaller (Figure 4B) and rise times 13% longer than in KO neurons (Figure 4C), which is suggestive of a homeostatic adaptation to diazepam treatment in WT but not in KO slices. No significant differences in mIPSC decay times and frequencies were observed (Supplementary Table S2). Because the sudden withdrawal of diazepam should lead to an increase in overall network activity, we recorded action potential (AP) frequencies in ventromedial hypothalamus slices in the presence of flumazenil. Surprisingly, KO slices already showed significantly higher AP frequencies than WT slices under control conditions, with no further increase under diazepam withdrawal conditions. By contrast, in WT slices, we observed a significant increase in AP frequency under diazepam withdrawal conditions compared with vehicle-treated WT slices (Figure 4D). There was no significant difference in the resting membrane potentials (Figure 4E), AP rise times, decay times and half-widths (Supplementary Table S2). IPSCs were recorded in the same cells at a holding potential of –20 mV to be able to isolate spontaneous $GABA_A$R-mediated currents without drug application. In WT slices, we observed a significant effect of diazepam withdrawal, with an increase in IPSC amplitude and frequency compared with vehicle-treated WT slices (Figures 4F, G), which is in keeping with the overall higher firing rate and which was not apparent in KO slices. In summary, these data show that neurons in *Baiap3* KO hypothalamus slices have higher AP firing rates, likely consistent with the higher seizure propensity found *in vivo*, and that *Baiap3* KO slices show no obvious homeostatic adaptation to diazepam treatment and withdrawal.

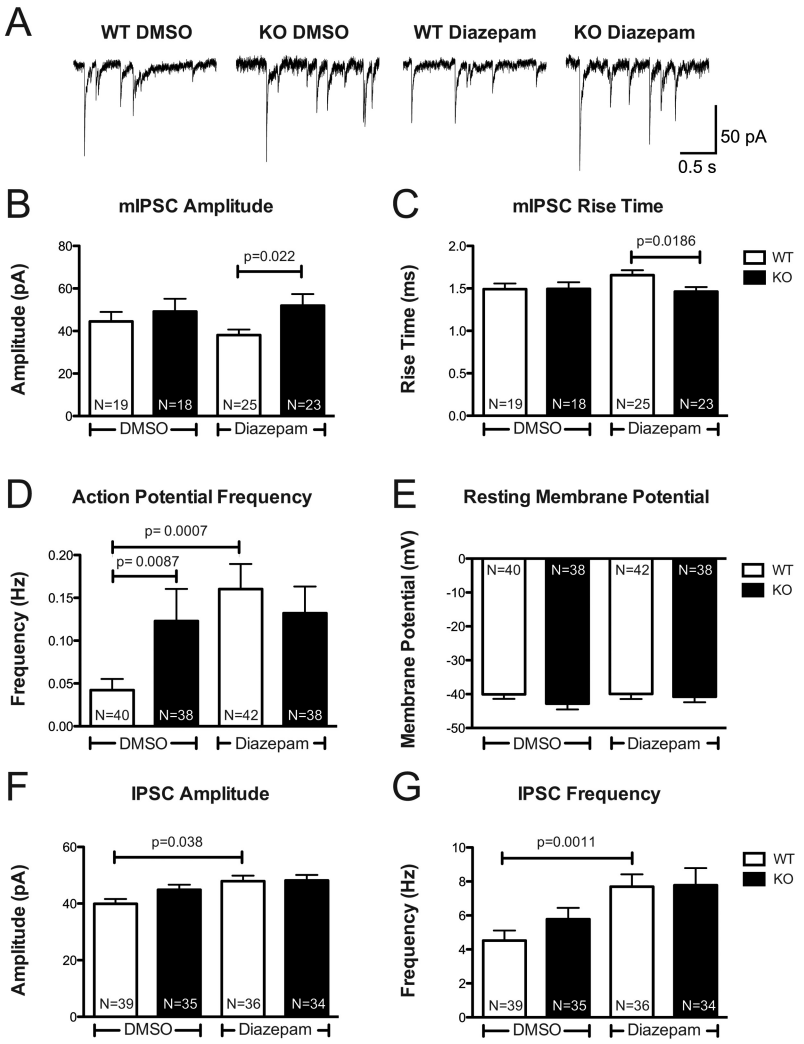

Figure 4. Increased basal network activity and lack of homeostatic adaptation to diazepam treatment in *Baiap3* KO hypothalamus slices. (A) Sample traces of mIPSC recordings from WT and KO hypothalamus slices that were cultured in the presence of diazepam or under vehicle control conditions with DMSO. Under diazepam withdrawal conditions, *Baiap3* WT mIPSC amplitudes were significantly smaller than in KO slices (B), and WT mIPSC rise times were longer than in KO slices (C). (D) *Baiap3* WT slices showed an increase in AP frequency in response to diazepam withdrawal when compared with DMSO-treated WT slices, whereas no such increase was apparent for *Baiap3* KO slices, which already showed an increased AP frequency under DMSO control conditions when compared with WT slices. (E) The resting membrane potential was not affected by experimental condition or *Baiap3* genotype. IPSC amplitudes (F) and IPSC frequencies (G) were increased in *Baiap3* WT slices under diazepam withdrawal compared with DMSO-treated WT slices. Mann-Whitney *U* test was used for AP and IPSC frequencies; Student *t* test was used for all other parameters. Means ± SEM are presented.

DISCUSSION

In this study, we identify two human *BAIAP3* risk genotypes that are associated with anxiety in women and benzodiazepine use disorder in men. We fur-

ther show that *Baiap3* deficiency in mice leads to (i) elevated seizure propensity; (ii) increased anxiety in both genders, with a more pronounced effect in females; and (iii) a faster development of tolerance to benzodiazepines in male mice. *In vitro* analysis of hypothalamic slices revealed an increase in neuronal baseline activity in the absence of *Baiap3*. Withdrawal from chronic benzodiazepine application *in vitro* results in a genotype-specific response pattern.

To the best of our knowledge, no other genetic risk marker that is associated with anxiety and benzodiazepine abuse has been reported to date. We are aware that, pending replication in nonschizophrenic individuals, we cannot be sure that our findings can be applied to the general population. In spite of this limitation, our findings suggest a role for *BAIAP3* and potential interaction partners in the development of anxiety and drug dependence.

Unfortunately, similar data from samples of equally well-phenotyped healthy individuals or even other disease groups are not available. This is particularly true with regard to benzodiazepine abuse, since short-term exposure is a primary goal of controlled and medically surveyed indications. Even looking at other rare situations of long-term exposure (for example, intractable epilepsies), a homogeneous sample comparable to the GRAS collection would be difficult to imagine. In the GRAS sample, there are no differences between *BAIAP3* genotypes regarding benzodiazepine exposure or daily dose (in case of exposure). This constellation allowed us to analyze the specific genetic risk of developing benzodiazepine use disorder in a setting close to an experimental condition.

Importantly, the present study was purely hypothesis-driven. Our hypotheses for performing a human phenotype-based genetic association study of *BAIAP3* were based on the anxiety phenotype we observed during basic behavioral characterization of *Baiap3* KO mice as well as on the distinctive Baiap3 expression pattern in brain, which includes regions relevant for addictive behaviors. We find that in humans, female carriers of the homozygous *BAIAP3* risk genotypes (AA for SNP rs2235632; TT for SNP rs1132358) are more likely to meet criteria for an anxiety disorder, whereas male carriers of the same risk genotypes are more likely to fulfill criteria for benzodiazepine use disorder. Neither SNP was associated with schizophrenia in our case control analysis. Furthermore, no associations with substance use disorder other than benzodiazepine use disorder were observed. In general, both genetic linkage and candidate gene studies suffer from lack of replicability (12). However, in our study, the parallel identification of a gender-specific association of *BAIAP3/Baiap3* with anxiety and an altered response to benzodiazepines in both mice and men, lends strong support to a causal link between *BAIAP3* and the observed phenotypes.

As for other genetic variations associated with anxiety disorders (11,12) or substance use disorders (13–15), the impact of *BAIAP3* genotypes on anxiety disorders or benzodiazepine use disorder is likely to be small. However, the observed effects and their gender specificity (across two species) are intriguing. While we currently have no mechanistic insight into this gender specificity, part of the explanation may lie in the fact that Baiap3 is expressed in sexually dimorphic brain regions such as the hypothalamus, amygdala and the bed nucleus of the stria terminalis (38,39).

Given the higher prevalence of both anxiety disorders and benzodiazepine use disorder in women (9,40) the present findings were surprising at first glance, but the similarity of gender differences in mice and humans underlines their significance, encouraging follow-up work on this gender effect. Admittedly, the gender effects in humans may ultimately turn out to be less prominent, since the total number of individuals with benzodiazepine abuse in the GRAS sample is low, resulting in moderate significance levels only for men. It cannot be excluded that, in a larger sample, an association of benzodiazepine use disorder with the genotypes investigated here might reach significance for women as well. Furthermore, research focusing on gender differences and social desirability in self-reported anxiety suggests an underreporting of fear and distress in men (41–43). Therefore, our use of three self-reported measures in the calculation of the anxiety composite score might partly explain the lack of association of the *BAIAP3* risk genotypes with anxiety in men. Nevertheless, gender differences in *BAIAP3/Baiap3* genotype-phenotype associations most likely exist and are worth pursuing.

Benzodiazepines are positive allosteric modulators of $GABA_AR$ and thus enhancers of inhibitory GABAergic neurotransmission. Their sedative, anti-convulsive and amnesic effects are largely mediated by the $GABA_AR\alpha1$ subunit, the anxiolytic effect by the $\alpha2$ subunit and muscle relaxation by $\alpha2$, $\alpha3$ and $\alpha5$ subunits (44). To date, no specific risk association of these obvious candidate genes has been identified. At present we have no evidence that would suggest that Baiap3 interacts with $GABA_AR$ subunits. However, the increased seizure propensity observed in *Baiap3* KO mice of both sexes, which is already apparent without prior diazepam treatment and withdrawal, is indicative of an altered balance of excitatory and inhibitory systems. Our comparison of neuronal firing rates in hypothalamus slices under baseline and diazepam withdrawal conditions uncovered an increase in basal network activity in the absence of Baiap3. This finding was unexpected, and although presently limited to the hypothalamus, is consistent with the increased seizure propensity observed *in vivo*. Even though we do not know whether the seizures observed in our PTZ-induction model originate in the subcortical regions that express Baiap3, subcortical epileptogenesis with origins in the hypothalamus is a feature seen in hypothalamic hamartomas (45), and the amygdala, which also expresses Baiap3, is known to play a key role in epileptogenesis (46).

Interestingly, the human *BAIAP3* gene is located on chromosome 16p13.3, which has been linked to electroencephalographic traits of idiopathic epilepsy syndromes (47,48). We would thus argue that further investigation of *BAIAP3* as a candidate gene for epilepsy-related phenotypes is warranted. Because we found that Baiap3 did not colocalize with markers of GABAergic or glutamatergic pre- and postsynapses to a significant degree (Supplementary Figures S1C–G), the increased seizure propensity in *Baiap3* KO mice of both sexes and the altered response to benzodiazepines in males is unlikely to be due to a direct effect of Baiap3 at GABAergic or glutamatergic synapses.

The neuronal circuitry underlying the addictive properties of benzodiazepines is less well understood than their molecular mechanism of action. Unlike many other addictive substances, benzodiazepines do not appear to increase dopamine levels in the nucleus accumbens (49–51), although electrophysiological studies suggest that benzodiazepines increase firing of dopaminergic neurons in the VTA through disinhibition of these neurons via inhibition of nearby inhibitory interneurons (52,53). Additional mechanisms, such as neuroendocrine responses to benzodiazepine treatment, may play a critical role in the development of benzodiazepine use disorder (54). Furthermore, because expression of *Baiap3* in both the VTA and in the nucleus accumbens is low (Allen Brain Atlas), a direct effect of Baiap3 on the mesolimbic dopamine pathway does not appear be the most likely explanation for the observed interaction between *Baiap3* genotypes and the response to benzodiazepines. Instead, our findings support the interpretation that the altered response to benzodiazepines could be a consequence of a local or global change in neuronal excitability. Because all other members of the Munc13 protein family have been shown to be regulators of SNARE-mediated exocytosis (25,29), Baiap3 may regulate the release of one or more modulatory neurotransmitters or neuropeptides that influence the balance between GABAergic and glutamatergic neurotransmission. Baiap3 immuoreactivity appears punctate (Supplementary Figures S1C–G) and may localize to peptidergic release sites, some of which may also contain VGLUT2 or Viaat. Although we presently cannot exclude the possibility that Baiap3 might have a postsynaptic function, given what is know about the function of all other members of the Munc13 protein family, we think that a pre-synaptic function is more likely. We can furthermore not exclude the possibility that alterations in the hypothalamic-pituitary-adrenal axis may play a role in the anxiety phenotype or the altered response to benzodiazepines seen in *Baiap3* KO mice. We are currently investigating whether Baiap3 is involved in regulating exocytosis of dense core vesicles and/or intracellular trafficking events that could influence neuropeptide release or extrasynaptic $GABA_A$Rs.

CONCLUSION

To conclude, *BAIAP3* had not previously been considered a candidate gene for either psychiatric disorders or epilepsy. Our study links *BAIAP3/Baiap3* genotypes to anxiety and an altered response to benzodiazepines in both mice and men and thus strongly argues for an involvement of *BAIAP3* in these neuropsychiatrically relevant phenotypes. The identification of human genetic variations that influence the risk for the development of pathological phenotypes as well as the response to pharmacological treatments may pave the way for more efficient treatments with fewer side effects. Rodent models are usually only imperfect representations of human psychiatric conditions; however, the simultaneous identification of *Baiap3* as a biomarker for anxiety and the response to benzodiazepines in mouse and humans suggests that *Baiap3* KO mice will be a valuable tool in further elucidating the genetic, physiological and neuroanatomical underpinnings of anxiety disorders and benzodiazepine use disorder.

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DISCLOSURE

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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PROJECT I
Molecular Medicine

Supplemental Data

Genetic Markers of a Munc13 Protein Family Member, *BAIAP3*, Are Gender Specifically Associated with Anxiety and Benzodiazepine Abuse in Mice and Humans

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Supplementary Table S1. Benzodiazepine use (%) and dose (lorazepam equivalents) in GRAS patients sorted by *BAIAP3* genotypes.

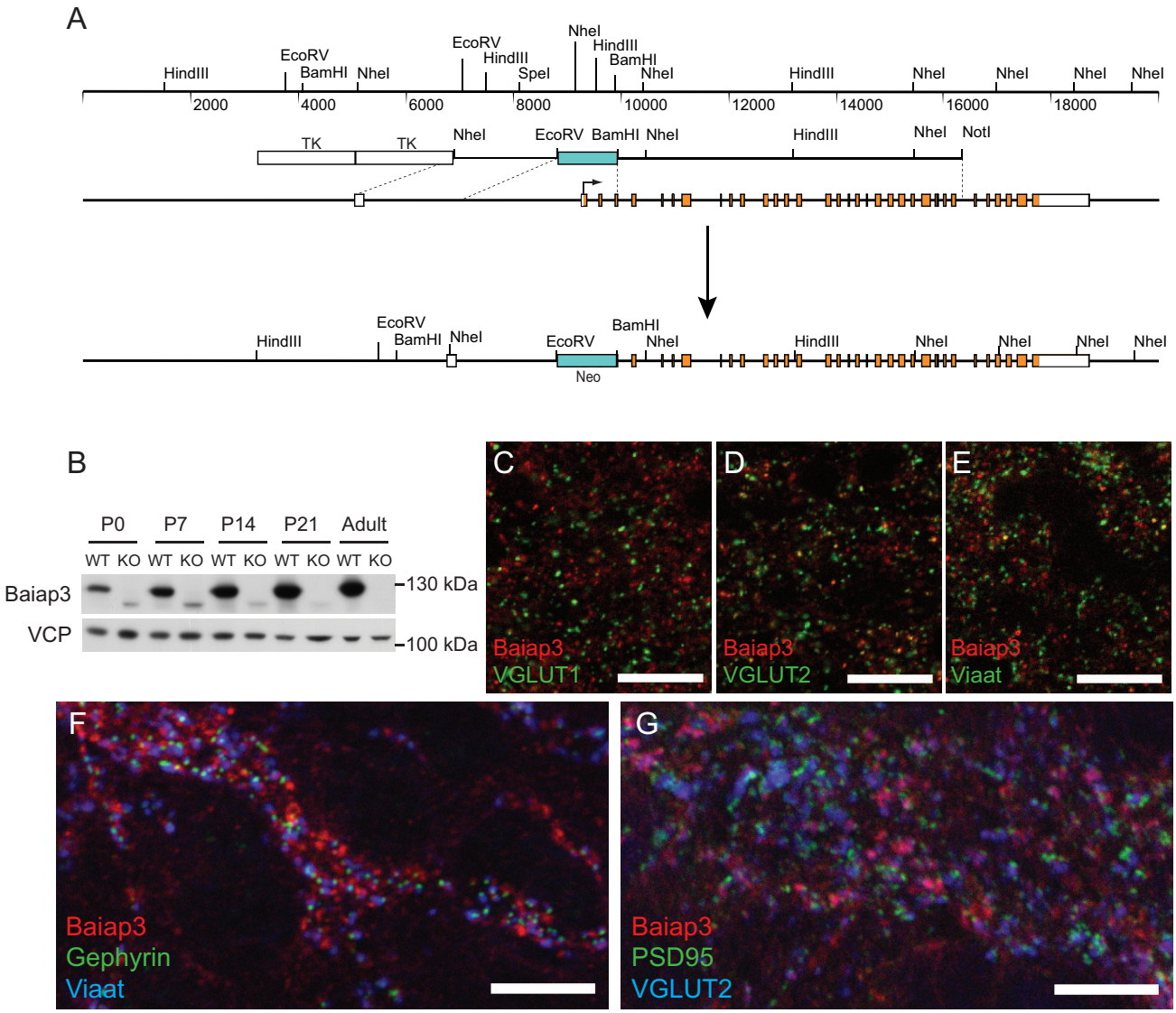
	BAIAP3 rs2235632				*BAIAP3* rs1132358			
Males (GRAS sample)	GC	AG	AA	*p* value (χ^2 value) [a]	CC	TC	TT	*p* value (χ^2 value) [a]
Receiving benzodiazepines, No. (%) [b]	**N=181** 22 (12.2)	**N=351** 57 (16.2)	**N=187** 30 (16.0)	.427 (χ^2 =1.70)	**N=184** 20 (10.9)	**N=347** 57 (16.4)	**N=175** 30 (17.1)	.165 (χ^2 =3.60)
Benzodiazepine dose, mg, Mean±SD [b, c]	**N=21[d]** 3.14±4.14	**N=52[d]** 2.64±3.11	**N=30** 3.04±3.31	.833 (χ^2 =0.37)	**N=19[d]** 2.95±4.31	**N=52[d]** 2.67±3.11	**N=30** 3.00±3.32	.979 (χ^2 =0.04)
Females (GRAS sample)	GC	AG	AA	*p* value (χ^2 value) [a]	CC	TC	TT	*p* value (χ^2 value) [a]
Receiving benzodiazepines, No. (%) [b]	**N=101** 28 (27.7)	**N=167** 34 (20.4)	**N=92** 23 (25.0)	.363 (χ^2 =2.02)	**N=105** 30 (28.6)	**N=167** 34 (20.4)	**N=83** 20 (24.1)	.298 (χ^2 =2.42)
Benzodiazepine dose, mg, Mean±SD [b, c]	**N=27[d]** 2.51±1.51	**N=33[d]** 2.13±2.35	**N=21[d]** 2.33±2.28	.227 (χ^2 =2.97)	**N=29[d]** 2.44±1.49	**N=33[d]** 2.08±2.37	**N=18[d]** 2.52±2.40	.234 (χ^2 =2.90)

[a] For statistical methods *Chi*^{2} or Kruskal-Wallis test was used.
[b] Refers to benzodiazepines as daily medical treatment.
[c] Calculation of lorazepam equivalents according to Bezchlibnyk-Butler, K. Z. & Jeffries, J. J. (Eds) (2003) C*linical Handbook of Psychotropic Drugs (13th ed)*. Cambridge (MA): Hogrefe & Huber.
[D] Discrepancies in N due to missing information on benzodiazepine compound or daily dose.

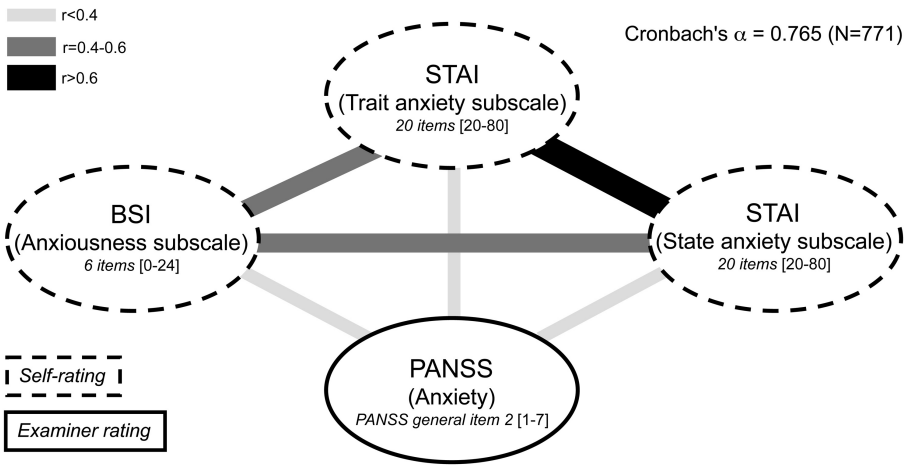
Supplementary Table S2. The mIPSC frequencies and decay times and the AP rise times, decay times and half-width were not affected by experimental condition or *Baiap3* genotype.

	WT DMSO	KO DMSO	WT Diazepam	KO Diazepam
mIPSC Decay Time	20.27±4.35 ms	21.01±4.94 ms	21.40±4.22 ms	20.36±4.21 ms
mIPSC Frequency	3.99±3.26 Hz	5.59±5.78 Hz	4.43±3.74 Hz	3.61±2.80 Hz
AP Rise Time	0.97±0.36 ms	1.02±0.45 ms	1.05±0.37 ms	1.12±0.34 ms
AP Decay Time	1.17±0.17 ms	1.14±0.24 ms	1.14±0.24 ms	1.21±0.36 ms
AP Half-Width	1.22±0.19 ms	1.22±0.29 ms	1.20±0.26 ms	1.27±0.39 ms

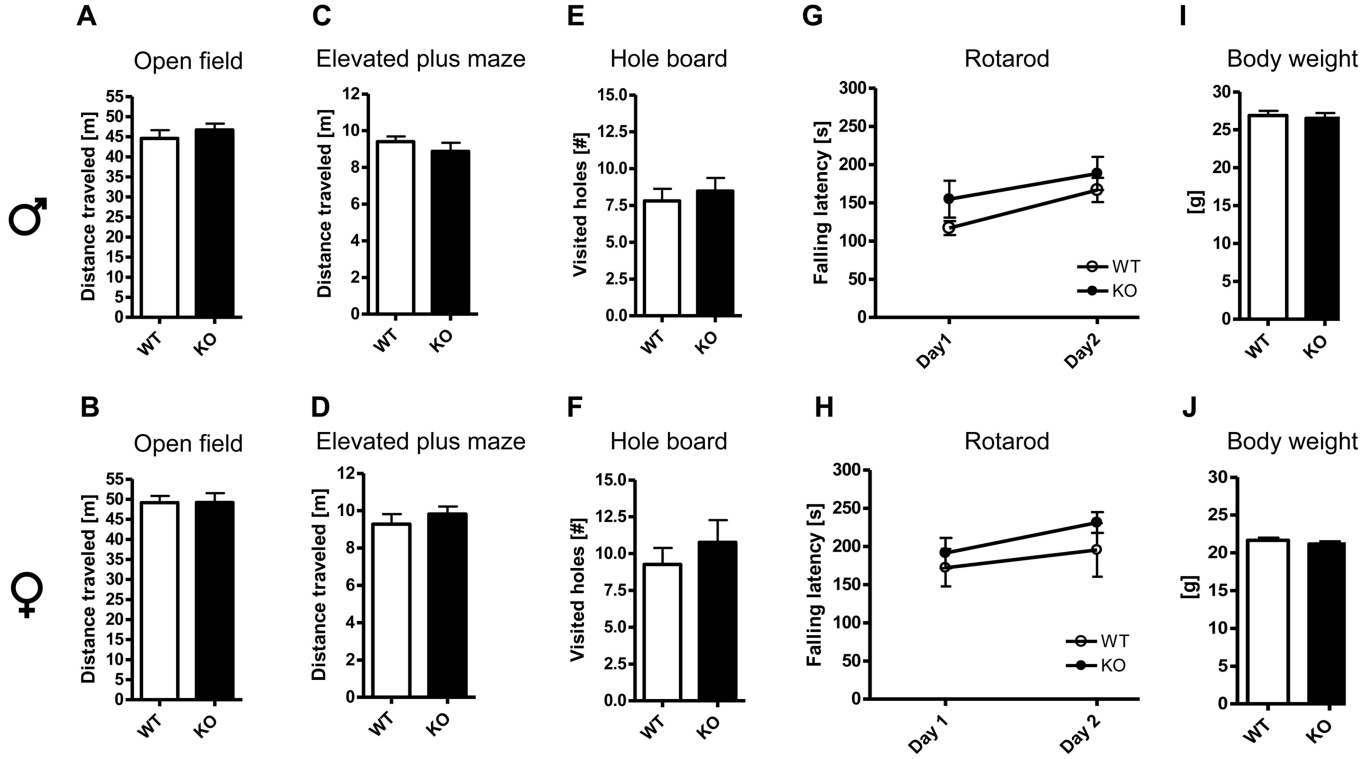
Mann-Whitney *U* test for mIPSC frequencies and Student's *t*-test for all other parameters. Mean±SD presented.



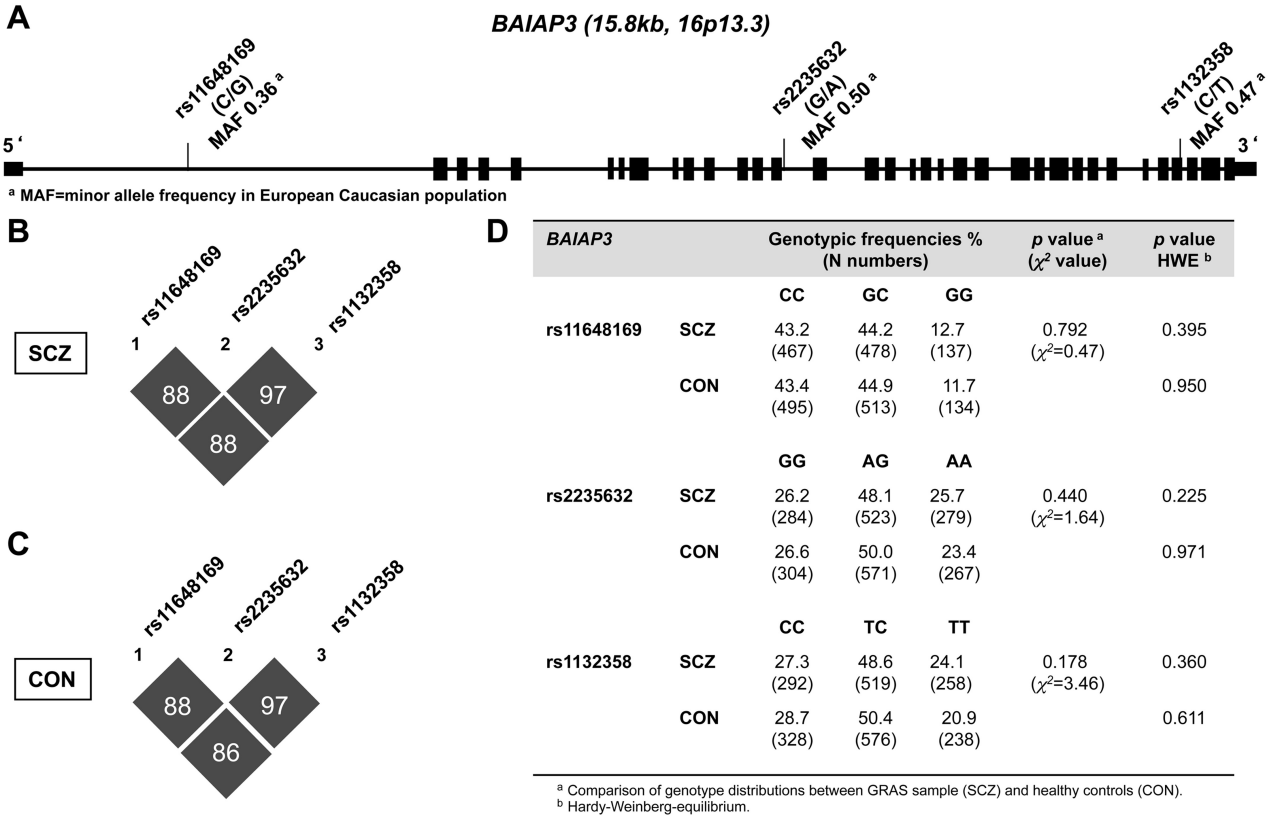
Supplementary Figure S1. Targeting strategy and expression of mouse *Baiap3*. (A) Targeting strategy for the mouse *Baiap3* gene. The first 3 coding exons were replaced with a neomycin selection cassette (Neo). The targeting vector further contained 2 thymidine kinase cassettes (TK) for negative selection. (B) Western blot analysis of the expression of *Baiap3*, in P0, P7, P14, P21 and adult *Baiap3* WT and KO animals. In *Baiap3* KO pups, we detected a truncated Baiap3-immunoreactive product that decreased after P7 and was not detectable in adult mice. An antibody to the valosin-containing-protein (VCP) ATPase was used as a loading control. (C-E) Immunostaining analyses in the hypothalamic medial preoptic area show that Baiap3 largely does not co-localize with markers of glutamatergic and GABAergic synapses. (C) Baiap3 (red) shows no co-localization with the vesicular glutamate tranporter (VGLUT)1 (green). (D) Baiap3 (red) shows only limited co-localization with VGLUT2 (green) (E) Baiap3 (red) shows only limited co-localization with the vesicular inhibitory amino acid transporter (Viaat) (green). (F) In organotypic hypothalamus slices Baiap3 (red) does not show significant co-localization with pre-synaptic (Viaat, blue) or post-synaptic (Gephyrin, green) markers of GABAergic synapses or (G) with pre-synaptic (VGLUT2, blue) or post-synaptic (postsynaptic-density-protein 95, green) markers of glutamatergic synapses. Scale bars equal 10μm.



Supplementary Figure S2. Anxiety composite score. Shown are variables composing the anxiety composite score, their intercorrelations and internal consistency. Pearson's correlation coefficients and Cronbach's α given.



Supplementary Figure S3. *Baiap3* genotype does not affect activity level, exploratory behavior, motor function or body weight. (A,B) The distance traveled during a 7-min session in the open field and (C,D) 5-min session in the elevated plus-maze was comparable among genotypes and genders. (E,F) Exploratory behavior measured in the hole board, (G,H) motor coordination and learning, evaluated by rotarod, as well as (I,J) body weight were comparable for both genders between *Baiap3* KO and WT littermates. Numbers tested: males, WT=16-25, KO=16-25; females, WT=18-23, KO=10-28. Mann-Whitney *U* test (A-F, I,J) and 2-way repeated measures ANOVA (G,H), including Bonferroni testing, were applied. Mean±s.e.m. presented.

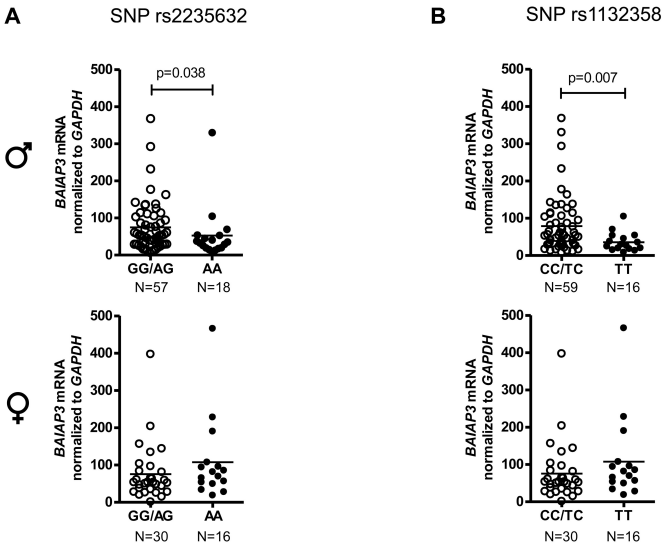


D

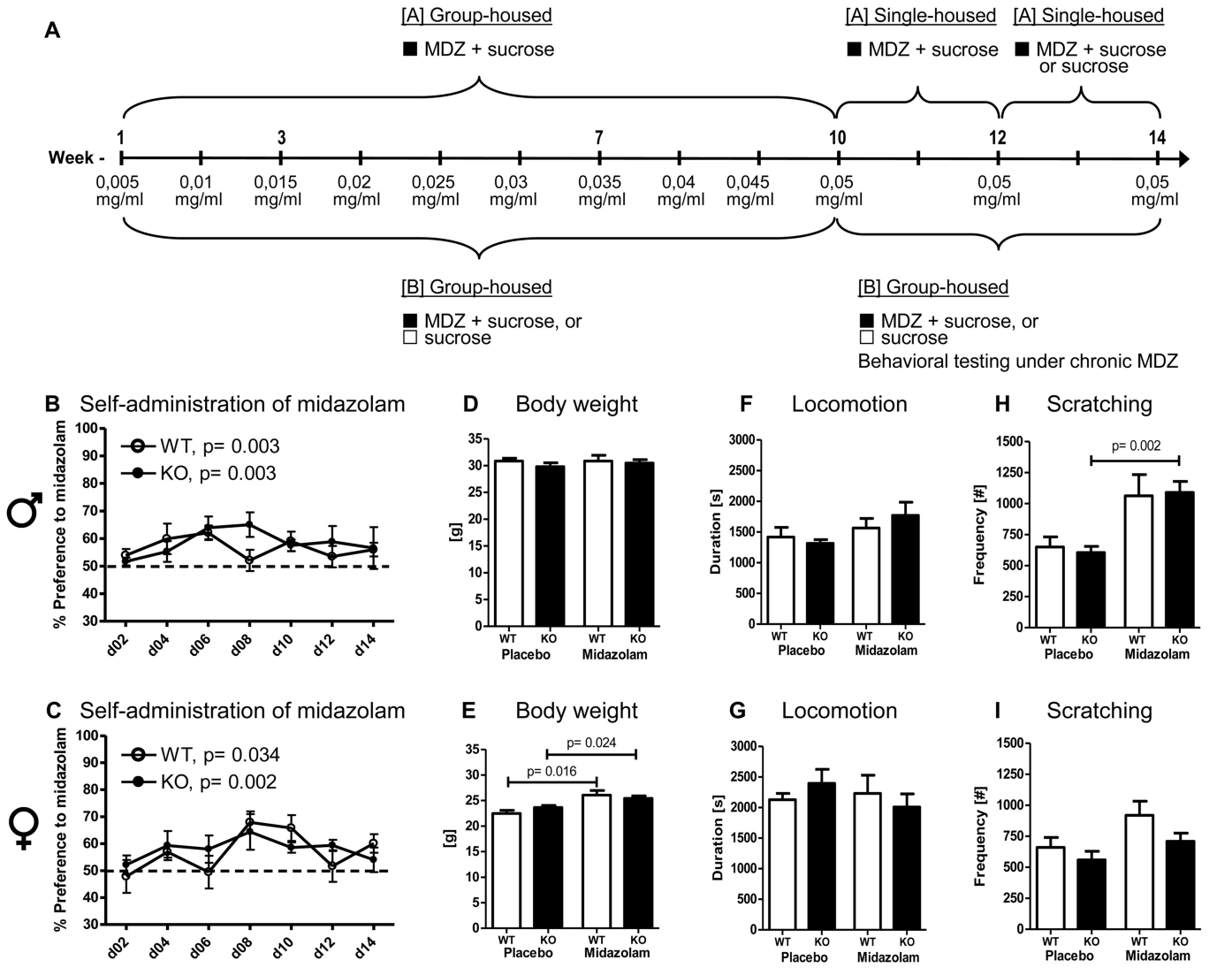
BAIAP3		Genotypic frequencies % (N numbers)			p value [a] (χ^2 value)	p value HWE [b]
		CC	GC	GG		
rs11648169	SCZ	43.2 (467)	44.2 (478)	12.7 (137)	0.792 (χ^2=0.47)	0.395
	CON	43.4 (495)	44.9 (513)	11.7 (134)		0.950
		GG	AG	AA		
rs2235632	SCZ	26.2 (284)	48.1 (523)	25.7 (279)	0.440 (χ^2=1.64)	0.225
	CON	26.6 (304)	50.0 (571)	23.4 (267)		0.971
		CC	TC	TT		
rs1132358	SCZ	27.3 (292)	48.6 (519)	24.1 (258)	0.178 (χ^2=3.46)	0.360
	CON	28.7 (328)	50.4 (576)	20.9 (238)		0.611

[a] Comparison of genotype distributions between GRAS sample (SCZ) and healthy controls (CON).
[b] Hardy-Weinberg-equilibrium.

Supplementary Figure S4. *BAIAP3* genotyping strategy and case-control analyses. (A) Location of the selected single nucleotide polymorphisms (SNPs) in the *BAIAP3* gene. Kb, kilobases. (B) Linkage disequilibrium map for N=1086 schizophrenic and schizoaffective patients and (C) Linkage disequilibrium map for N=1142 healthy blood donors indicating a high degree of linkage between the 3 selected SNPs in both groups. (D) Case-control comparisons reveal a similar distribution of the *BAIAP3* SNP genotypes for patients and healthy individuals, thus excluding the selected *BAIAP3* markers as risk factors for schizophrenia.



Supplementary Figure S5. *BAIAP3* mRNA expression in PBMCs. (A) SNP rs2235632: The risk genotype AA is associated with lower *BAIAP3* mRNA levels in male but not in female patients. (B) SNP rs1132358: The risk genotype TT is associated with lower *BAIAP3* mRNA levels in male but not in female patients. Mann-Whitney *U* test applied, due to non-normal data distribution (A,B).



Supplementary Figure S6. High oral self-administration of midazolam (as readout of addiction), body weight, and basic behaviors in the chronic addicted state are not affected by *Baiap3* genotype. (A) Experimental design scheme. (B,C) *Baiap3* KO and WT mice of both genders displayed significant and comparable preference for midazolam over sucrose. (D,E) Chronic midazolam intake did not affect body weight of male, but increased that of female mice independently of genotype. (F,G) Locomotion duration in LABORAS™ remained unaffected across genotypes and genders. (H,I) Scratching frequency in LABORAS™ tended to be increased upon chronic high-dose midazolam across genotypes and genders. Numbers tested: males, WT= 6-8, KO=10-16; females, WT=6-10, KO=10-13, except for (B) and (C), males, WT=8, KO=4; females, WT=5; KO=4; 2-way repeated measures ANOVA (B,C) as well as 2-way ANOVA (D-I), including Bonferroni testing, where applicable. Mean±s.e.m. presented.

4. ACCUMULATED ENVIRONMENTAL RISK DETERMINING AGE AT SCHIZOPHRENIA ONSET: A DEEP PHENOTYPING-BASED STUDY

4.1. Overview of project II

Research strongly suggests that psychiatric disorders – like schizophrenia – are complex traits, meaning that many genetic and environmental factors are involved in their development (Gratten et al., 2014; Meyer-Lindenberg and Weinberger, 2006). Since common genetic variants have been estimated to account for about one third of the total heritability of schizophrenia (Lee et al., 2013), one major focus of genetic studies are single nucleotide polymorphisms. The most recent GWAS identified 108 genetic *loci* to be genome-wide significantly associated with the disorder (Schizophrenia Working Group of the PGC, 2014). At the same time, research has described environmental factors, which increase the risk to develop schizophrenia. Some of the most important environmental hazards in this context are perinatal brain insults, cannabis use, neurotrauma, childhood adversity (sexual abuse, physical abuse, and neglect), urbanicity, and migration (Brown, 2011; McDonald and Murray, 2000; van Os et al., 2010). For all of these environmental factors OR between 1.65 and 3.6 have been reported (Cantor-Graae and Selten, 2005; Clarke et al., 2006; Henquet et al., 2005; Krabbendam and van Os, 2005; Matheson et al., 2013; Molloy et al., 2011).

As opposed to all this evidence, less is known as to whether genes and environmental exposure during development have the potential to modulate later symptom expression and disease severity in patients with schizophrenia. In fact, research shows that childhood and adolescents are vulnerable phases of brain development and maturation (Andersen, 2003; Sturman and Moghaddam, 2011). Environmental risk exposure during this important time can have long lasting effects (Romeo, 2010; Schneider, 2008), potentially influencing disease presentation and severity. The present study focused on male patients with schizophrenia because men differ from women in incidence, symptom expression and treatment response (Abel et al., 2010; Andersen, 2003), as well as possibly their exposure rates and response to environmental stressors (Kirov et al., 1996; Korosi et al., 2012; Liossi and Wood, 2009; O'Callaghan et al., 1992).

With our study we wanted to answer the question, if the exposure to certain environmental risk factors (perinatal complications, neurotrauma, cannabis use, psychotrauma, urbanicity, and migration) - individually and upon accumulation - up until 18 years of age and before the first psychotic episode, would be associated with measures of disease onset, severity and socioeconomic status later on, in male patients with schizophrenia. Additionally we aimed at comparing the effects of the environmental factors to the combined effects of common genetic variants derived from the latest genome-wide association study on schizophrenia (Schizophrenia Working Group of the PGC, 2014).

In order to answer our research question we studied 750 male patients with the diagnosis of schizophrenia or schizoaffective disorder (DSM-IV-TR) (American Psychiatric Association, 2000) from the GRAS dataset (Begemann et al., 2010; Ribbe et al., 2010). The cohort was retrospectively assessed for exposure to all six environmental factors during childhood and adolescence by the use of semi-structured interviews and questionnaires. Additionally, the whole sample was genotyped using a semi-custom Axiom SNP genotyping array (Affymetrix, Santa Clara, CA, USA), providing a genome-wide coverage for genetic analyses. Since it is thought that a considerable proportion of the genetic markers that do not reach genome-wide significance ($p>5x10^{-8}$) still contribute to disease risk, we calculated polygentic schizophrenia risk scores for increasing *p*-value thresholds ($1x10^{-6}$, 0.0001, 0.001, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0) based on the OR obtained for each risk/protective allele by the latest GWAS (Schizophrenia Working Group of the PGC, 2014).

After strict correction for multiple testing, we found perinatal complications, neurotrauma, and cannabis use to be individually associated with earlier prodrome and disease onset; cannabis use with decreased levels of education; urbanicity with higher unemployment rates; and perinatal complications and cannabis use with more hospital admissions. Since risk burden in our sample was very complex and ranged from no environmental risk exposure in some patients, up to four and more in others, we decided to study the effects of accumulated exposure on the same measures of disease severity and socioeconomic functioning. Here we observed highly significant group differences for age at prodrome and disease onset, years of education, unemployment rates, and number of hospital admissions. Trend tests revealed that with every additional risk factor the outcome is worsened further.

Moreover, the frequency of cannabis consumption up until 18 years of age and prior to the first psychotic episode was also significantly associated with an earlier age at prodrome onset. In contrast, no significant associations between the GWAS-derived schizophrenia risk scores and any of the outcome measure could be observed. Additionally, the analysis of interactions between the environmental load and the schizophrenia risk scores also yielded no significant results. In conclusion, our results show that specific environmental factors (perinatal complications, neurotrauma, cannabis use, psychotrauma, urbanicity, and migration) – especially the amount of them – experienced before disease onset and up until 18 years of age, are a strong predictor of disease onset and socioeconomic functioning in male patients with schizophrenia.

4.2. Original publication

Stepniak B., Papiol S., Hammer C., Ramin A., Everts S., Hennig L., Begemann M., Ehrenreich H. (2014) Accumulated environmental risk determining age at schizophrenia onset: a deep phenotyping-based study. The Lancet Psychiatry **1**:444-453.

Personal contribution

I contributed to the establishment of an extensive database including information on environmental risk factors (perinatal complications, neurotrauma, substance use, psychotrauma, urbanicity, migration) of all GRAS patients (*N*>1200) by conducting data entry as well as by supervising student assistants in their database work. Under the supervision of Prof Hannelore Ehrenreich, I formulated the hypotheses for the project, conducted extensive data analyses (all non-genetic data), interpreted the results and developed the first concept and draft of the paper. Furthermore I designed all the figures and tables and wrote the figure legend for the non-genetic data (see Table 1, 2, 3; Figure B, C, D; Figure legend B, C, D; Appendix page 4, 5, 6) and performed the literature search. I wrote the final manuscript together with my supervisor and was involved in the revisions and publication of the article.

Accumulated environmental risk determining age at schizophrenia onset: a deep phenotyping-based study

Beata Stepniak, Sergi Papiol, Christian Hammer, Anna Ramin, Sarah Everts, Lena Hennig, Martin Begemann, Hannelore Ehrenreich

Summary

Background **Schizophrenia is caused by a combination of genetic and environmental factors, as first evidenced by twin studies. Extensive efforts have been made to identify the genetic roots of schizophrenia, including large genome-wide association studies, but these yielded very small effect sizes for individual markers. In this study, we aimed to assess the relative contribution of genome-wide association study-derived genetic versus environmental risk factors to crucial determinants of schizophrenia severity: disease onset, disease severity, and socioeconomic measures.**

Methods **In this parallel analysis, we studied 750 male patients from the Göttingen Research Association for Schizophrenia (GRAS) dataset (Germany) with schizophrenia for whom both genome-wide coverage of single-nucleotide polymorphisms and deep clinical phenotyping data were available. Specifically, we investigated the potential effect of schizophrenia risk alleles as identified in the most recent large genome-wide association study versus the effects of environmental hazards (ie, perinatal brain insults, cannabis use, neurotrauma, psychotrauma, urbanicity, and migration), alone and upon accumulation, on age at disease onset, age at prodrome, symptom expression, and socioeconomic parameters.**

Findings **In this study, we could show that frequent environmental factors become a major risk for early schizophrenia onset when accumulated (prodrome begins up to 9 years earlier; $p=2\cdot9\times10^{-10}$). In particular, cannabis use—an avoidable environmental risk factor—is highly significantly associated with earlier age at prodrome ($p=3\cdot8\times10^{-20}$). By contrast, polygenic genome-wide association study risk scores did not have any detectable effects on schizophrenia phenotypes.**

Interpretation **These findings should be translated to preventive measures to reduce environmental risk factors, since age at onset of schizophrenia is a crucial determinant of an affected individual's fate and the total socioeconomic cost of the illness.**

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Introduction

Substantial efforts have been made to identify the genetic roots of schizophrenia, in view of heritability estimates of up to 80%.[1] However, awareness is increasing that so-called disease genes of general significance do not exist for the biologically highly heterogeneous, purely clinical construct of schizophrenia. This absence of shared disease genes is supported by the consistently very low odds ratios (ORs) for individual markers derived from genome-wide association studies that are based on ever-increasing numbers of individuals.[1,2]

The most recently published large Psychiatric Genomics Consortium (PGC) genome-wide association study, comprising 36 989 patients with schizophrenia and 113 075 healthy controls, identified 108 genetic loci with genome-wide associations.[2] In addition to these loci, a substantial proportion of schizophrenia risk has been suggested to lie in markers that do not achieve genome-wide significance. Therefore, quantitative polygenic schizophrenia risk scores were calculated on the basis of nominal allele effects. These risk scores now explain up to 7% of variance in the diagnosis of schizophrenia in independent samples.[2] Based on previous genome-wide association studies, the effects of polygenic schizophrenia risk scores on various disease-relevant phenotypes have been explored with variable degrees of success.[3,4]

Importantly, genes alone cannot explain the development of schizophrenia, as indicated by a roughly 50% concordance rate in monozygotic twins.[5] Therefore, intensified research into environmental risk factors is pivotal, also with respect to its inherent preventive potential. Perinatal brain insults, cannabis use, neurotrauma, psychotrauma, urbanicity, and migration are among the most prominently discussed environmental hazards associated with the risk of schizophrenia development.[6–9]

By contrast with most previous work that assessed the effect of different environmental factors on the risk of schizophrenia development, the aim of this study was to assess in patients with schizophrenia the effects of these factors alone and upon accumulation on disease onset, symptom expression, and socioeconomic parameters. Specifically, we retrospectively assessed environmental risk exposure before disease onset. Moreover, we aimed to do back-to-back comparisons of the effects of environmental risk factors and genome-wide association study-derived risk genotypes (grouped into polygenic

schizophrenia risk scores) on the same outcome measures within the same population. Owing to the known differences between male and female patients with schizophrenia in terms of age at onset, psychopathological symptom clusters, vulnerability, and exposure to environmental stressors, we focused on male patients only.[10,11] Similar analyses with female patients remain to be done.

Methods

Study design and participants

The study population for environmental risk assessment consisted of a total of 750 male patients with schizophrenia and schizoaffective disorder (according to *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition Text Revision* [DSM-IV-TR]) from the Göttingen Research Association for Schizophrenia (GRAS) dataset.[12,13] To make a polygenic schizophrenia risk score-based case–control status prediction including both men and women, 1067 patients with schizophrenia (according to DSM-IV-TR) from the GRAS sample (including the 750 male patients in whom we assessed environmental risk) and 1169 healthy controls (anonymous blood donors) were analysed.[12] The ethics committees of the Georg-August-University (Göttingen, Germany) and of the 23 centres participating in GRAS throughout Germany approved the study, which complies with the Declaration of Helsinki. All participants (and/or authorised legal representatives) provided written informed consent.

Phenotyping procedures

A meticulous description of the GRAS data collection standard operating procedures is provided elsewhere.[13] In brief, comprehensive information regarding the prodrome (which precedes schizophrenia onset and is characterised by cognitive decline, social withdrawal, and depression), disease onset (onset of first psychotic episode), symptom expression, and socioeconomic functioning was acquired from a very detailed examination. These assessments included positive and negative syndrome scale (PANSS) rating, assessment based on the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) and other semi-structured interviews (assessing suicidality, education history, present employment status, and history of admissions to hospital), telephone consultations, questionnaires, and an essentially complete collection of hospital discharge letters. For example, for all outcome measures of socioeconomic functioning assessed here, patients' self-reports were always double-checked against hospital letters. Education was measured as total years spent in school, further education, and professional training based on the highest qualification achieved. Present employment status and livelihood were assessed and patients were classified as either unemployed or not unemployed (in full-time or part-time employment, retired, or in education). For number of admissions to hospital, all admissions due to psychiatric diagnoses were counted. To assess cognitive symptoms, a composite score was calculated from different neuropsychological tests, comprising reasoning (Leistungsprüfsystem subtest 3 [LPS3]), executive function (Trail-Making Test, part B [TMT-B]), and verbal learning and memory (Verbal Learning and Memory Test [VLMT]).[12] To estimate family mental illness burden, history of any severe mental illness (schizophrenia, psychosis, depression, or mania) in first-degree relatives was recorded.

Environmental risk exposure

To assess environmental risk exposure, specific information about perinatal complications, neurotrauma, psychotrauma, cannabis use, and migration was derived from the patient's history and extensive semi-structured interviews with patients and relatives or caregivers (GRAS Manual[13]) and from SCID-I. Every patient was dichotomously (yes/no) classified as having or not having been exposed (before disease onset and up to 18 years of age) to perinatal complications, neurotrauma, cannabis, psychotrauma, and migration. To measure urbanicity from birth until 18 years of age, information about place of residence and relocation was collected from hospital discharge letters and social history. If information was missing, patients were contacted by telephone or post with an urbanicity questionnaire. In cases of contradictory information or if the missing data could not be obtained, patients were excluded from the respective analysis. For paternal age at birth and season of birth, information was obtained from files.

Perinatal complications were defined as any deviations from normality during pregnancy (eg, alcohol or substance use, infections, pre-eclampsia, or diabetes), delivery (eg, premature or protracted birth, or hypoxia), and in early postnatal life (eg, pronounced jaundice). Neurotrauma was classified as documented head trauma of any grade of severity, from mild trauma to concussion and contusion. For cannabis use, in addition to the dichotomous classification of all patients into cannabis users and non-users, all people who had used cannabis before 18 years of age and before disease onset were grouped regarding frequency of use into: infrequent users (from five times in life up to six times per year); regular users (from once per month up to every other day); and daily use. In this classification, regular use and daily use referred to the 6-month period in life when use was heaviest. Cases of psychotrauma included loss of a first-degree relative or close attachment figure of high importance (severity of loss ≥5 on a rating scale of 0–10), sexual abuse, severe physical abuse (comprising unpredictability of violence, injury caused by physical reprimand, or use of objects for corporal punishment), or any combination thereof. To measure urbanicity, each city the patients lived in was allocated to one of four categories according to its total population (category 1: ≤10 000, category 2: 10 001–50 000, category 3: 50 001–100 000, or category 4: >100 000 inhabitants). The category was then

multiplied by the number of years that the person had spent living there. In cases of relocation, the same procedure was repeated for each new place of residence and values were added up to obtain one urbanicity score per individual. For further calculations, the urbanicity score was dichotomously grouped into rural (score 18–45) and urban (score 46–72) places of residence. To obtain clean data for the risk of 18 years of urban exposure, patients with a schizophrenia onset before 18 years of age were excluded (n=77). For the risk factor of migration, information from sociodemographic interviews was used to classify any patient who immigrated to Germany up to age 18 years as a migrant.

Statistical analysis of environmental risk

To assess group differences in continuous variables, we used the Mann-Whitney *U* test or, in cases of normal distribution of dependent continuous variables, the *t* test. We used logistic regression analysis to study the effects of more than one variable on dichotomous outcome categories. To compare means of more than two groups, we used the Kruskal-Wallis *H* test if the data followed a non-parametric distribution; otherwise, we used ANOVA if data followed a normal distribution. To assess frequency differences between groups we used the χ^2 test. To assess whether medians of more than two groups ascend or descend, we applied the Jonckheere-Terpstra trend test. Trends in frequency distributions were calculated with the Cochran-Armitage test. Covariate correction was done through calculation of linear regression-based standardised residuals when duration of disease or age, chlorpromazine equivalents of current antipsychotic medication, and PANSS negative subscale score were used as independent variables. We used a linear regression (forced entry) model to calculate the variance explained for age at prodrome and age at onset by either cannabis use alone or all other risk factors (perinatal complications, neurotrauma, psychotrauma, urbanicity, and migration). We generated Kaplan–Meier survival curves for different cannabis use frequencies, with age at prodromal onset as the endpoint. We used the log-rank test to make pairwise comparisons of different curves. For all analyses, statistical significance was set to the 0·05 level. We applied Bonferroni correction as a very conservative method to account for multiple testing. P values that withstand this correction are underlined in the respective tables. Statistical analyses were done using SPSS for Windows version 17.0, except for the Cochran-Armitage test for trend, for which R version 2.15.1 was used.

Genotyping, quality control, population structure, and relatedness

Genotyping of the GRAS patients and control sample was done with a semi-custom Axiom myDesign genotyping array (Affymetrix, Santa Clara, CA, USA), based on a CEU (Caucasian residents of European ancestry from Utah, USA) marker backbone including 518 722 single-nucleotide polymorphisms (SNPs), and a custom marker set including 102 537 SNPs. The array was designed using the Axiom Design center, applying diverse selection criteria.[14] Genotyping was done by Affymetrix on a GeneTitan platform. Several quality control steps were used (SNP call rate >97%, Fisher's linear discriminant >3·6, heterozygous cluster strength offset >–0·1, and homozygote ratio offset >–0·9). These steps were completed with use of either genotyping console software (Affymetrix) or R. In a subsequent step, markers in X, Y, and mitochondrial chromosomes and those with Hardy–Weinberg equilibrium $p<1\times10^{-6}$ (healthy controls) or $p<1\times10^{-10}$ (GRAS patients) were removed, leaving 589 921 SNPs available for analyses. We used this dataset to do a principal components analysis of the whole sample in EIGENSTRAT (SmartPCA module) to identify and exclude ancestral outliers in our participant collection, for which we used a sigma threshold of 5·0.[15] This SNP dataset was pruned further, with minor allele frequency (minor allele frequency ≥0·05) and linkage disequilibrium between markers ($r^2 \leq 0·05$) as inclusion criteria, leaving 33 311 markers ready for relatedness ascertainment. Relatedness was ascertained with PLINK version 1.07 through calculation of a genome-wide pairwise identity-by-descent estimation.[16] In those participant pairs with a PI-HAT relatedness coefficient score greater than 0·2, one of the members of the pair was randomly excluded from analyses, resulting in exclusion of a total of 12 participants (in whom PI-HAT ranged from 0·2706 to 0·9996). This pairwise identity-by-descent estimation was also used to calculate multidimensional scaling components to control for population stratification in polygenic analyses. Similarly, the inbreeding coefficient was calculated from the previously mentioned 33 311 SNPs dataset. PLINK was also used to calculate multidimensional scaling components and inbreeding coefficients.[16]

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Imputation

Genotype imputation was done with the prephasing or imputation approach implemented in IMPUTE2 and SHAPEIT (chunk size 3 Mb).[17,18] A version of the phase 1 integrated variant set release (v3) from the full 1000 Genomes Project dataset (March, 2012)[19] that is limited to variants with more than one minor allele copy ("macGT1"; Aug 26, 2012) was used as imputation reference dataset (INFO value >0·1 and minor allele frequency >0·005).

Derivation of polygenic schizophrenia risk scores

Polygenic schizophrenia risk scores were calculated as described in the most recent international collaborative genome-wide association study of schizophrenia.[2] Briefly, insertions/deletions, low-frequency genetic variants (minor allele frequency <10%), low imputation quality variants (INFO value <0·9), and extended major histocompatibility complex region genetic variants were excluded for these calculations. Variants in r^2 of 0·1 or

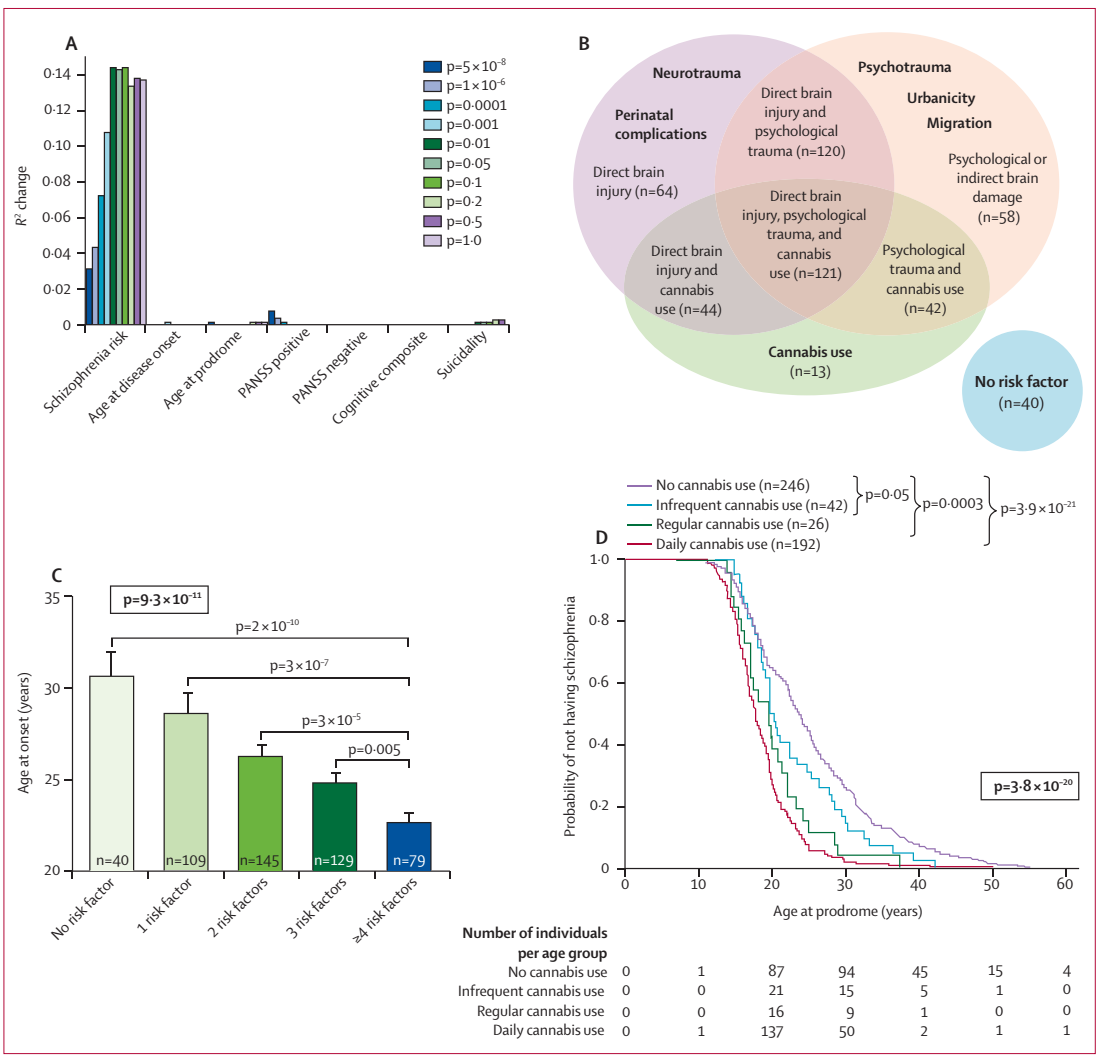

Figure: **Environmental risk contribution to disease onset in schizophrenia versus absence of genetic effects**

(A) Proportion of variance explained (change in adjusted R^2 or Nagelkerke pseudo-R^2) by polygenic genome-wide association study-derived schizophrenia risk scores at increasing p value thresholds. Note that the schizophrenia risk data displayed on the left of the graph are based on 1067 patients (of both sexes) with schizophrenia from the Göttingen Research Association for Schizophrenia sample and 1169 healthy controls (of both sexes), whereas the association with disease phenotypes has been calculated in the male subset of patients with schizophrenia assessed for environmental effects (also see appendix p 3). (B) Overlap of environmental risk exposure in 502 male patients with schizophrenia for whom complete information about all factors was available. (C) Effects of accumulated environmental risk on age at disease onset for the same 502 male patients. The overall p value is shown (in the box) and individual pairwise comparisons of the group with four or more risk factors versus all other groups are presented. The Kruskal-Wallis *H* test and Mann-Whitney *U* test were used for analysis; data are mean (SE) (Bonferroni-corrected significance level: p<0·01). (D) Kaplan–Meier dose–response curves for patients with different frequencies of cannabis use before disease onset and at up to 18 years of age compared with those who have never used cannabis. Regular use ranges from once per month up to every other day, whereas infrequent use includes frequencies from five times in life up to six times per year. The log-rank test was used for group and pairwise intergroup comparisons (Bonferroni-corrected significance level: p<0·02). PANSS=positive and negative syndrome scale.

See Online for appendix

greater and within 500 kb of another marker with stronger effects were discarded, eventually leaving a set of 102 375 SNPs ready for profile scoring. Polygenic schizophrenia risk scores were calculated by application of the *–score function* in PLINK[16] using subsets of SNPs below different p value cutoffs (5×10^{-8}, 1×10^{-6}, 0·0001, 0·001, 0·01, 0·05, 0·1, 0·2, 0·5, 1·0). For each SNP included under these subsets (amounting to a total of 60, 239, 1229, 3359, 10479, 24460, 35486, 51691, 81137, 102375, respectively), the imputation probability for the risk allele was weighted by its respective logarithm of the OR. The individual SNP values were added for each individual participant, leading to the calculation of ten polygenic schizophrenia scores for each person in the target sample.

	Perinatal complications*			Neurotrauma			Cannabis use*		
	No (n=373–424)†	Yes (n=251–284)†	p value (χ^2/Z/t)	No— before first psychotic episode and age ≤18 years (n=292–332)†	Yes— before first psychotic episode and age ≤18 years (n=333–379)†	p value (χ^2/Z/t)	No— before first psychotic episode and age ≤18 years (n=197–217)†	Yes— before first psychotic episode and age ≤18 years (n=188–215)†	p value (χ^2/Z/t)
Disease variables									
Age at disease onset (years)	25·53 (8·01)	23·68 (6·65)	p=0·003 (Z=–2·97)	26·05 (8·25)	24·32 (7·32)	p=0·001 (Z=–3·21)	25·29 (7·50)	23·20 (5·81)	p=0·002 (Z=–3·15)
Age at prodrome (years)	22·71 (7·94)	20·52 (6·57)	p=0·0003 (Z=–3·62)	23·37 (8·20)	21·32 (7·26)	p=0·001 (Z=–3·30)	22·40 (7·19)	20·21 (6·07)	p=0·003 (Z=–2·97)
Positive score on PANSS‡	13·36 (5·94)	13·86 (6·24)	p=0·436 (Z=–0·78)	13·11 (5·89)	13·90 (6·22)	p=0·154 (Z=–1·43)	13·70 (6·56)	13·12 (5·37)	p=0·741 (Z=–0·33)
Negative score on PANSS‡	18·42 (7·70)	17·78 (7·27)	p=0·242 (Z=–1·17)	18·07 (7·75)	18·33 (7·64)	p=0·536 (Z=–0·62)	18·90 (7·88)	17·49 (7·11)	p=0·091 (Z=–1·69)
Cognitive composite§	0·03 (0·81)	0·11 (0·86)	p=0·363 (t=–0·91)	–0·06 (0·87)	0·09 (0·80)	p=0·061 (t=–1·88)	0·06 (0·84)	0·19 (0·74)	p=0·575 (t=–0·56)
Suicidality¶	142 (34·4%)	109 (39·2%)	p=0·196 (χ^2=1·67)	129 (39·7%)	130 (34·7%)	p=0·170 (χ^2=1·89)	74 (35·1%)	88 (41·3%)	p=0·186 (χ^2=1·75)
Socioeconomic variables									
Education (years)‖	11·97 (2·93)	11·68 (2·92)	p=0·095 (Z=–1·67)	12·11 (3·03)	11·86 (2·97)	p=0·357 (Z=–0·92)	12·75 (3·24)	11·18 (2·44)	p<0·00001 (Z=–5·12)
Unemployment**	173 (41·6%)	128 (45·6%)	p=0·300 (χ^2=1·08)	123 (37·4%)	171 (45·6%)	p=0·027 (χ^2=4·86)	79 (37·3%)	104 (48·6%)	p=0·018 (χ^2=5·58)
Number of hospital admissions‡	7·41 (9·45)	8·68 (9·33)	p=0·003 (Z=–2·96)	8·34 (10·93)	8·11 (8·49)	p=0·256 (Z=–1·14)	6·57 (7·98)	10·17 (11·52)	p<0·00001 (Z=–4·69)

Data are uncorrected means (SD) or n (%). For statistical analysis, the Mann-Whitney *U* test or χ^2 test was used, and for normally distributed variables the *t* test was used. Significance values are displayed uncorrected, and p values withstanding Bonferroni correction are underlined. PANSS=positive and negative syndrome scale. *Groups matched for age. †Because of missing data, sample sizes vary. ‡Corrected for duration of disease (standardised residuals after linear regression). §Corrected for age, PANSS negative, and chlorpromazine equivalent (standardised residuals after linear regression). ¶Suicidality=individuals who have attempted suicide in the past. ‖Education refers to the total number of years spent to achieve the highest individual qualification; people presently in education excluded. **Significant results for unemployment were re-examined adding duration of disease as a covariate in a logistic regression model. Neurotrauma (no/yes) did remain a significant predictor of unemployment (no/yes) in the model (Wald test=4·90, p=0·027, odds ratio 1·43). Cannabis use (no/yes) remained a significant predictor of unemployment (no/yes) in the model (Wald test=6·08, p=0·014, odds ratio 1·65).

Table 1: **Effect of environmental risk factors (direct brain injury and cannabis) on measures of disease severity and socioeconomic functioning in male patients with schizophrenia**

Statistical analysis of polygenic schizophrenia risk scores

As dependent variables in a linear regression model, quantitative traits (eg, age at onset and cognitive composite) were used to analyse the effects of polygenic schizophrenia scores. These trait values were corrected when applicable as indicated in the table footnotes. Ten multidimensional scaling components and the inbreeding coefficient were selected as covariates of potential relevance. Adjusted R^2 values derived from a model including all of these covariates were subtracted from adjusted R^2 values from a model including covariates plus the respective polygenic schizophrenia scores. The difference between the adjusted R^2 represents the increase in the variance explained attributable to the score. For dichotomous variables (case–control study and suicidality), in an analogous fashion, Nagelkerke's pseudo-R^2 from a logistic regression containing only covariates (10 multidimensional scaling components and inbreeding coefficient) was compared against the one obtained in a model containing covariates and polygenic schizophrenia scores to estimate the proportion of variance of case–control status explained by the polygenic schizophrenia risk score. The potential effect of an interaction between the risk score and environmental load on phenotypes of interest was also assessed. For this purpose, adjusted R^2 of a model containing only environmental load as predictor was set as the baseline R^2 for comparisons with two models: a model containing environment plus covariates (multidimensional scaling components plus inbreeding coefficient) plus polygenic schizophrenia risk score; and a model containing environment plus covariates (multidimensional scaling components plus inbreeding coefficient) plus polygenic schizophrenia risk score plus the interaction between environment and polygenic schizophrenia risk score (G×E). All calculations were done with SPSS version 17.0.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We studied comprehensively genotyped and phenotyped male patients from our GRAS dataset of patients with schizophrenia.[12–14] We calculated polygenic schizophrenia

	Psychotrauma			Urbanicity			Migration*		
	No—before first psychotic episode and age ≤18 years (n=383–436)†	Yes—before first psychotic episode and age ≤18 years (n=228–257)†	p value (χ^2/Z/t)	Rural upbringing (0–18 years of age) before first psychotic episode (n=299–330)†	Urban upbringing (0–18 years of age) before first psychotic episode (n=213–246)†	p value (χ^2/Z/t)	No—before first psychotic episode and age ≤18 years (n=437–491)†	Yes—before first psychotic episode and age ≤18 years (n=61–71)†	p value (χ^2/Z/t)
Disease variables									
Age at disease onset (years)	25·10 (7·60)	25·02 (8·09)	p=0·567 (Z=–0·57)	26·48 (8·24)	25·90 (6·59)	p=0·640 (Z=–0·47)	23·53 (6·21)	22·56 (4·37)	p=0·595 (Z=–0·53)
Age at prodrome (years)	22·38 (7·44)	21·85 (8·32)	p=0·146 (Z=–1·45)	23·71 (8·50)	22·69 (7·08)	p=0·470 (Z=–0·72)	20·70 (6·20)	19·85 (4·09)	p=0·902 (Z=–0·12)
Positive score on PANSS‡	13·92 (6·44)	13·17 (5·59)	p=0·272 (Z=–1·10)	12·95 (5·72)	13·96 (6·51)	p=0·108 (Z=–1·61)	13·33 (5·85)	12·22 (5·97)	p=0·041 (Z=–2·04)
Negative score on PANSS‡	18·78 (7·64)	17·45 (7·50)	p=0·017 (Z=–2·38)	18·09 (7·55)	18·02 (7·76)	p=0·666 (Z=–0·43)	17·81 (7·32)	17·18 (6·75)	p=0·718 (Z=–0·36)
Cognitive composite§	0·02 (0·86)	0·04 (0·81)	p=0·941 (t=–0·07)	0·03 (0·82)	0·10 (0·82)	p=0·295 (t=–1·05)	0·23 (0·76)	0·01 (0·69)	p=0·0004 (t=3·58)
Suicidality¶‖	145 (33·9%)	103 (40·6%)	p=0·080 (χ^2=3·07)	102 (31·3%)	95 (39·6%)	p=0·041 (χ^2=4·19)	174 (36·1%)	26 (37·1%)	p=0·865 (χ^2=0·03)
Socioeconomic variables									
Education (years)**	12·23 (3·08)	11·57 (2·79)	p=0·006 (Z=–2·75)	12·31 (2·91)	12·23 (3·27)	p=0·547 (Z=–0·60)	11·91 (2·80)	10·18 (2·10)	p<0·00001 (Z=–5·01)
Unemployment‖	185 (42·0%)	104 (40·5%)	p=0·683 (χ^2=0·17)	114 (35·1%)	115 (47·3%)	p=0·003 (χ^2=8·67)	243 (50·0%)	35 (50·7%)	p=0·910 (χ^2=0·01)
Number of hospital admissions‡	8·11 (10·28)	7·85 (7·85)	p=0·927 (Z=–0·09)	7·92 (9·25)	8·42 (10·34)	p=0·877 (Z=–0·15)	7·54 (8·51)	7·17 (12·91)	p=0·147 (Z=–1·45)

Data are uncorrected means (SD) or n (%). For statistical analysis, the Mann-Whitney *U* test or χ^2 test was used, and for normally distributed variables the *t* test was used. Significance values are displayed uncorrected, and p values withstanding Bonferroni correction are underlined. PANSS=positive and negative syndrome scale. *Groups matched for age. †Because of missing data, sample sizes vary. ‡Corrected for duration of disease (standardised residuals after linear regression). §Corrected for age, PANSS negative, and chlorpromazine equivalent (standardised residuals after linear regression). ¶Suicidality=individuals who have attempted suicide in the past. ‖Significant results for suicidality and unemployment were re-examined adding duration of disease as a covariate in a logistic regression model. Urbanicity (rural/urban) remained a significant predictor of suicidality (no/yes) (Wald test=3·95, p=0·047, odds ratio 1·43) and unemployment (no/yes) (Wald test=9·77, p=0·002, odds ratio 1·77). **Education refers to the total number of years spent to achieve the highest individual qualification; people presently in education excluded.

Table 2: **Effect of environmental risk factors (psychological damage) on measures of disease severity and socioeconomic functioning in male patients with schizophrenia**

risk scores for each participant on the basis of p values and ORs available from the latest genome-wide association study of the PGC.[2] Every patient's exposure to selected environmental risk factors up to the age of 18 years and before the onset of psychosis was established on the basis of SCID-I, semi-structured interviews, telephone consultations, questionnaires, and a comprehensive collection of hospitalisation letters.

As figure A and appendix p 3 show, polygenic schizophrenia risk scores based on different levels of significance highly significantly (up to $p=1\cdot15\times10^{-50}$) reproduced the association with schizophrenia diagnosis in the GRAS sample, which supports the validity of the study population. By contrast, the same approach did not show any effect on schizophrenia-relevant phenotypes, thus questioning an appreciable role of polygenic schizophrenia risk score-grouped genome-wide association study-derived genotypes for co-determining lead illness features.

With a focus on the environment, we questioned whether the experience of single risk factors during a vulnerable time of brain development, ie, up to the age of 18 years and before the first psychotic episode, has an effect on the time of illness or prodrome onset. Prodrome is a period of several years, typically preceding schizophrenia onset, which is clinically characterised by cognitive decline, social withdrawal, and depression.[20] A comparison of individuals who had suffered a particular environmental insult of interest, to the remaining patients without this insult (table 1), showed significant results for perinatal complications (defined as any kind of deviation from normality that occurred during pregnancy or around birth), neurotrauma (comprising all levels of severity), and cannabis (ranging from any consumption up to regular use). All these risk factors were associated with younger age at disease onset (first psychotic episode) and at prodrome start (all p values ≤0·003, withstanding Bonferroni correction) (table 1). We note that age at onset co-determines overall prognosis.[20] The earlier individuals develop schizophrenia, the less progressed are their levels of education and socialisation. These factors in turn affect individual social functioning and social role performance and, consequently, societal costs.[20] We did not record any effects on age at disease onset or age at prodrome for psychotrauma, urbanicity, and migration (table 2).

Next, we studied whether risk factor exposure had an effect on schizophrenia severity and socioeconomic

	No risk factor (n=37–40)*	One risk factor (n=93–109)*	Two risk factors (n=132–145)*	Three risk factors (n=115–129)*	Four or more risk factors (n=70–79)*	p value (χ^2, *H*, *F*)†	p value (χ^2, *J*)‡
Disease variables							
Age at disease onset (years)	30·66 (7·68)	28·72 (9·34)	26·20 (7·42)	24·84 (5·64)	22·59 (5·21)	$p=9{\cdot}3\times10^{-11}$ (*H*=52·82)	$p=5{\cdot}2\times10^{-12}$ (*J*=36068·0)
Age at prodrome (years)	28·34 (7·97)	26·07 (9·32)	23·28 (7·82)	21·58 (6·15)	19·40 (5·26)	$p=2{\cdot}9\times10^{-10}$ (*H*=50·46)	$p=5{\cdot}6\times10^{-12}$ (*J*=28005·5)
Positive score on PANSS§	13·05 (6·32)	13·50 (6·50)	13·16 (5·61)	13·84 (6·24)	12·63 (5·94)	p=0·650 (*H*=2·47)	NA
Negative score on PANSS§	18·31 (7·37)	17·71 (7·87)	18·88 (7·58)	17·48 (6·66)	16·37 (7·29)	p=0·189 (*H*=6·13)	NA
Cognitive composite¶	–0·10 (0·85)	–0·02 (0·88)	–0·02 (0·85)	0·19 (0·77)	0·26 (0·74)	p=0·916 (*F*=0·24)	NA
Suicidality‖	8 (20·0%)	36 (33·6%)	50 (35·2%)	48 (37·5%)	32 (41·0%)	p=0·229 (χ^2=5·63)	p=0·039 (χ^2=4·27)
Sociodemographic variables							
Education (years)**	13·71 (3·03)	13·48 (3·38)	12·24 (3·11)	11·93 (2·67)	10·93 (2·56)	$p=2{\cdot}3\times10^{-8}$ (*H*=41·34)	$p=7{\cdot}7\times10^{-10}$ (*J*=34112·5)
Unemployment	7 (17·5%)	33 (30·8%)	60 (42·3%)	55 (43·0%)	45 (57·0%)	p=0·0002 (χ^2=22·34)	$p=6{\cdot}8\times10^{-6}$ (χ^2=20·24)
Number of hospital admissions§	6·95 (9·80)	7·32 (7·77)	7·39 (7·38)	8·75 (12·05)	8·15 (7·94)	p=0·001 (*H*=19·08)	$p=2{\cdot}1\times10^{-5}$ (*J*=56421·0)

Data are uncorrected means (SD) or n (%). NA=not applicable (no trend in data). *Because of missing data, sample sizes vary. †For statistical analysis, the Kruskal-Wallis *H* test or χ^2 test was used, and for normally distributed data ANOVA was used. Significance values are displayed uncorrected, and p values withstanding Bonferroni correction are underlined. ‡To test for statistical trends, the Cochran–Armitage test (qualitative traits) or the Jonckheere-Terpstra test (quantitative traits) was used. Significance values are displayed uncorrected, and p values withstanding Bonferroni correction are underlined. §Corrected for duration of disease (standardised residuals after linear regression). ¶Corrected for age, PANSS negative, and chlorpromazine equivalent (standardised residuals after linear regression). ‖Suicidality=individuals who have attempted suicide in the past. **Education refers to the total number of years spent to achieve the highest individual qualification; people presently in education excluded.

Table 3: **Effect of an accumulation of environmental risk factors on measures of disease severity and socioeconomic functioning in male patients with schizophrenia**

readouts. Indeed, patients with a history of perinatal complications and those who had started to use cannabis before illness onset had more admissions to hospital (all p values ≤0·003, withstanding Bonferroni correction). Neurotrauma, urbanicity, and cannabis use tended to be associated with higher unemployment rates (tables 1, 2). Psychotrauma, including loss of a close attachment figure and physical and sexual abuse, was associated with fewer years of education (p=0·006; table 2), as were cannabis use and migration (both with p values <0·00001, withstanding Bonferroni correction) (tables 1, 2). Season of birth[21,22] and paternal age at birth,[23] also previously discussed as schizophrenia risk factors, did not show associations with any outcome parameters (appendix pp 4–5); except for paternal age, which was significantly associated with years spent in education (p=0·002, withstanding Bonferroni correction; appendix p 5).

The logical question of whether or not an accumulation of up to four or more risk factors would lead to a more severe disease expression has, to our knowledge, never previously been addressed. Figure B exemplifies the complexity of environmental risk exposure in the male sample group, which is categorised for this illustration into three major domains: direct brain injury (perinatal complications and neurotrauma), psychological or indirect brain damage (psychotrauma, urbanicity, or migration), and cannabis use. To systematically study cumulative effects, we compared patients without risk factor exposure and those with one to four or more environmental risks. We recorded highly significant group differences (all withstanding Bonferroni correction) for age at disease onset and prodromal onset (p values around 1×10^{-10}), years of education ($p=2{\cdot}3\times10^{-8}$), unemployment (p=0·0002), and number of admissions to psychiatric hospital (p=0·001) (table 3). Remarkably, every additional risk factor worsens the outcome further, as emphasised by highly significant trend tests (table 3, figure C). Patients with none or one risk factor experience prodromal onset about 8 years later than do those with four or more environmental insults ($p=3{\cdot}7\times10^{-10}$; Cohen's d=0·99; OR for prodrome before age 23 years versus after: OR 10 [95% CI 4·27–21·70, χ^2=36·63]). The strength of these associations could offset potential concerns regarding false-positive results.

Importantly, the effect of cannabis as a preventable environmental risk factor on age at onset ($p=3{\cdot}8\times10^{-20}$) is enormous (figure D). Cannabis use alone can explain 10·2% of variance in age at disease onset, compared with 4·7% explained by all other environmental risks together (linear regression model). This result calls for public education that targets prevention.

As an internal control (accounting for the unavailability worldwide of an adequate replicate sample), we split the male GRAS population into two equally sized samples according to recruitment date. This split-sample approach provided similar results for both halves of the population (appendix p 6).

No appreciable associations between any environmental factor tested here—alone or upon accumulation—with positive or negative symptom load or cognitive performance were detectable. Within some environmental risk constellations, secondary factors predisposing to mental illness might be hidden, such as social status, societal integration, peer group pressure, access to drugs including cannabis, or family history of psychiatric disorders. For family load of mental disease, no differences were noted between risk factor groups (p>0·05; data not shown). Gene–environment interaction analyses based on genome-wide association study-derived polygenic schizophrenia risk scores and individual environmental burden did not show associations withstanding multiple testing

Panel: **Research in context**

Systematic review

Between Jan 1, 2005, and Dec 31, 2012, we did a cross-sectional study—the Göttingen Research Association for Schizophrenia (GRAS) data collection—of patients with schizophrenia, recruited from throughout Germany.[12,13] We aimed to collect a disease population with a level of phenotyping accuracy unprecedented until now, complemented by comprehensive genotype and serological analyses.[14] The deep phenotyping provided the basis for all outcome measures and items used in the present study. Systematic literature searches (on Medline and Google Scholar) in preparation for this work showed an accumulation of four or more environmental risk factors in the same population has never previously been studied, and in reports about more than one risk factor, assessed in the same group of individuals, no comparable numbers of comprehensively phenotyped and genotyped patients were evaluated. However, published studies about single environmental risk factors point to them having an important role not only in disease outbreak (schizophrenia risk),[6–9, 21–23] but also in disease severity or modulation.[30,35,36] So far, no study has provided data about accumulated environmental risk factors back-to-back with genetic data. However, these facts are less surprising when we consider that although funding for, and public awareness of, genetic trials have been substantial during the past decade, appreciable support for research into environmental risk factors has developed only very recently.

Interpretation

Our study is the first to show that the effect of accumulated environmental risk factors on age at schizophrenia onset is huge, as indicated by the fact that the disease develops nearly a decade earlier in individuals with four or more environmental risk factors than in those with no environmental risk. The environmental effect will exert its share of damage in any individual genetically predisposed to schizophrenia. Not all risk factors are avoidable but some, such as cannabis use, certainly are. Here, awareness among clinicians and in the general public needs to grow. Other risk factors, such as migration and urbanicity, could be alleviated by psychosocial and sociopolitical actions. However, some factors (perinatal complications, neurotrauma, and psychotrauma) might not be easily avoidable. Yet even for these, prophylactic measures might apply (eg, better management of at-risk pregnancies, wearing a helmet when cycling, and early post-trauma intervention). Support for controlled studies of the efficiency of these potential prophylactic measures and interventions should be encouraged by our work.

corrections (appendix pp 7–8). However, this finding does not exclude the possibility that interactions might exist between particular environmental risk factors and specific genetic loci that cannot be detected in aggregate.

Discussion

We used a large sample of thoroughly phenotyped male patients with schizophrenia to investigate for the first time back-to-back in the same population the effects of genome-wide association study-derived genetic markers and of environmental risk factors on disease phenotypes (panel). In this population, we show: a qualitatively and quantitatively different effect of defined single environmental hazards on disease onset and socio-economic burden; a substantial effect of accumulated environmental risk factors on age at prodrome and schizophrenia onset; and an absence of detectable effects of genome-wide association study-derived polygenic schizophrenia risk scores on disease-relevant phenotypes. We also show that the relative significance of cannabis as avoidable environmental risk on age at prodrome is substantial.

The absence of detectable effects of case-control genome-wide association study-derived polygenic schizophrenia risk scores on disease-relevant phenotypes might be less surprising when we consider the tremendous heterogeneity of people who fall into the diagnostic category of schizophrenia.[24] Furthermore, detection of any risk score effects on disease variables, if at all relevant, might need huge sample sizes. On the other hand, SNP variants associated with disease risk as aggregated into genome-wide association study-derived polygenic schizophrenia risk scores might not always overlap with risk variants associated with specific syndrome domains (eg, see reference 25). Moreover, schizophrenia risk score-based analyses might not be optimised for study of association with disease-relevant phenotypes.

Environmental hazards differ greatly in time and pattern of occurrence. Whereas perinatal complications happen early during development, neurotrauma and psychotrauma can occur at any point during childhood or adolescence, even repeatedly, and with variable intensity and individual perception. Urbanicity affects people continuously from birth until adulthood. Moreover, environmental hazards differ in their initial mechanisms of action on the brain, even though they might share final common deleterious pathways downstream. As shown in this study, environmental insults that directly—ie, physically or through specific drug effects—affect brain development, maturation, and integrity of cerebral structures have major effects on crucial outcome elements such as age at disease onset. Psychotrauma and migration act indirectly, probably by inducing high amounts of negative psychosocial stress.[26] Urbanicity is often referred to as a proxy for chronic inevitable, and therefore negative, everyday stress.[27]

A substantial amount of published work exists about early cannabis use and the raised risk of developing schizophrenia (eg, see references 28, 29), whereas work analysing the effect of cannabis on age at onset of schizophrenia is less abundant (eg, see a recent meta-analysis[30]). Age at first cannabis use has been falling[31] and the harmful effects, especially of consumption during the teenage years on cognition, development of social competence, and education, have long been known. This specific window of vulnerability could indicate the crucial developmental role of the endogenous cannabinoid system.[32,33] Interference with this system through exogenous cannabinoids has detrimental consequences on consolidation of maturing brain networks as shown in experimental studies.[7,34] We noted that even minor consumption of cannabis has significant effects on age at onset, indicating that the timepoint of exogenous cannabinoid influence is critical, rather than the dose.

Perinatal complications have previously been described to be associated with earlier age at onset of schizophrenia.[35]

Regarding psychotrauma, sexual but also physical abuse has been linked to intensified hallucinations.[36] However, this finding was not replicable in our purely male population.

To obtain a clean and comparable dataset, environmental risk factors were assessed only when they occurred up to the age of 18 years. This timeframe was used to consider the most relevant time of brain development[37] and thus most important susceptibility to environmental effects.[32,33,38] Of course, the effect of environmental factors (eg, adult life events as disease trigger[39]), and certainly disease onset, can also take place much later.

So far, no assessment of environmental risk accumulation regarding schizophrenia onset or severity that was based on more than two risk factors in an appreciable number of patients has been done. Although psychotrauma, urbanicity, or migration per se did not affect age at onset in our study, our accumulation approach suggests that they still contribute to its reduction. This finding would support potential interaction effects, adding to our overall results. Similarly, although the genetic approach in our study using polygenic schizophrenia risk scores did not show any association with age at onset, some published studies indicate that it is determined by a combination of genetic and—to a greater extent—environmental factors.[40]

Patients with up to one environmental risk factor experienced prodromal onset roughly 8 years later than did those with four or more environmental insults. This time difference is highly relevant regarding chances of outcome, since early adulthood is the most crucial time in life during which the groundwork is laid for occupational integration and success, and social inclusion and stability. These processes will all be negatively affected by early onset of prodrome or psychosis.[20]

To summarise, we obtained in the same cohort of male individuals with schizophrenia robust effects of accumulated environmental risk on age-at-onset of schizophrenia or its prodrome, critical determinants of individual prognosis and socioeconomic burden, in contrast to non-detectable effect of accumulated genome-wide association study-derived risk variants (as assessed by the application of polygenic schizophrenia risk scores) on lead phenotypes of schizophrenia. Several important points can be emphasised here: first, increased public awareness about the risks of early schizophrenia onset is needed, especially regarding the effects of cannabis. Second, after the tremendous interest in genome-wide association studies, the present study will hopefully lead to increased support of intensified research into environmental risk factors and their mechanisms of action. Third, the genetic effect is probably highly specific, and definition of biological disease subgroups or syndromes rather than building on the heterogeneous clinical construct "schizophrenia" will be indispensable for successful genome-wide association studies in the future. By contrast, the effect of environmental factors is enormous but rather non-specific, and will exert its share of damage in any genetically predisposed individual.

Contributors

BS, AR, SE, and LH worked on the establishment of the database including information about the environmental risk factors of Göttingen Research Association for Schizophrenia patients. SP and CH did the genetic analyses. BS, SP, and HE analysed and interpreted the data. MB recruited, diagnosed, and assessed patients. HE planned, supervised, and coordinated the project. BS and HE wrote the report. All authors contributed to the final version of the report.

Declaration of interests

We declare no competing interests.

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Supplementary Materials

Accumulated environmental risk determining age at schizophrenia onset

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Table S1: Association of polygenic schizophrenia scores (PSS) derived from GWAS with disease phenotypes and amount of variance explained (R^2 change) in male schizophrenic patients of the GRAS data collection *(note: PSS case-control study is based on males and females of GRAS)*

		PSS according to different *p*-value thresholds									
		PSS≤5x10^{-8}	**PSS≤1x10^{-6}**	**PSS≤0.0001**	**PSS≤0.001**	**PSS≤0.01**	**PSS≤0.05**	**PSS≤0.1**	**PSS≤0.2**	**PSS≤0.5**	**PSS≤1.0**
Case-control study [a]											
Schizophrenia risk [b]	***p*-value**	**3.31E-13**	**1.67E-17**	**1.14E-27**	**1.88E-39**	**2.56E-50**	**2.17E-50**	**1.15E-50**	**1.66E-47**	**1.32E-48**	**2.66E-48**
(N= 1067 SCZ & 1169 HC)	**R^2 change**	**0.031**	**0.043**	**0.072**	**0.108**	**0.144**	**0.143**	**0.144**	**0.134**	**0.138**	**0.137**
Disease variables											
Age at disease onset [c]	***p*-value**	0.517	0.835	0.396	0.224	0.303	0.733	0.557	0.454	0.448	0.441
(N=700 SCZ males)	**R^2 change**	-0.001	-0.001	0.000	0.001	0.000	-0.001	-0.001	-0.001	-0.001	-0.001
Age at prodrome [c]	***p*-value**	0.168	0.741	0.916	0.827	0.521	0.483	0.340	0.253	0.242	0.228
(N=621 SCZ males)	**R^2 change**	0.001	-0.001	-0.002	-0.002	-0.001	-0.001	0.000	0.001	0.001	0.001
PANSS positive [c]	***p*-value**	**0.015**	0.070	0.183	0.563	0.490	0.329	0.417	0.583	0.396	0.351
(N=683 SCZ males)	**R^2 change**	**0.007**	0.003	0.001	-0.001	-0.001	0.000	-0.001	-0.001	0.000	0.000
PANSS negative [c]	***p*-value**	0.478	0.323	0.576	0.823	0.317	0.430	0.306	0.515	0.426	0.443
(N=678 SCZ males)	**R^2 change**	-0.001	0.000	-0.001	-0.001	0.000	-0.001	0.000	-0.001	-0.001	-0.001
Cognitive composite [c]	***p*-value**	0.687	0.763	0.886	0.937	0.659	0.608	0.403	0.393	0.311	0.280
(N=663 SCZ males)	**R^2 change**	-0.001	-0.001	-0.001	-0.002	-0.001	-0.001	0.000	0.000	0.000	0.000
Suicidality [b]	***p*-value**	0.944	0.664	0.989	0.722	0.581	0.546	0.431	0.264	0.268	0.282
N=(684 SCZ males)	**R^2 change**	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.002	0.002	0.002

For all analyses 10 population stratification dimensions and inbreeding coefficient were used as covariates. [a] For case-control study both genders were included in the analyses. [b] Logistic regression was performed for dichotomous phenotypes; Nagelkerke's pseudo R^2 between a model containing only covariates and a model containing covariates+PSS were compared in order the address the effect of PSS. [c] Linear regression was used for continuous variables; adjusted R^2 between a model containing only covariates and a model containing covariates+PSS were compared in order the address the effect of PSS. SCZ, Schizophrenia cases; HC, Healthy controls.

Table S2: Impact of season of birth on readouts of disease severity and socioeconomic functioning in male schizophrenic individuals

	Season of birth			
	February & March**	June & July**	other months	p [a] (χ^2/Z/T) [a]
	n=104-117[b]	n=113-126[b]	n=439-503[b]	
Disease variables				
Age at disease onset, mean±SD	25.89±7.73	25.61±8.42	24.75±7.61	p=0.179 (H=3.44)
Age at prodrome, mean±SD	22.80±7.98	22.74±8.25	21.91±7.63	p=0.301 (H=2.40)
PANSS positive, mean±SD [c]	14.22±5.78	13.01±6.08	13.64±6.18	p=0.194 (H=3.28)
PANSS negative, mean±SD [c]	19.04±8.13	18.14±7.83	18.13±7.50	p=0.557 (H=1.17)
Cognitive composite, mean±SD [d]	0.03±0.76	-0.04±0.82	0.05±0.86	p=0.370 (F=1.00)
Suicidality, n (%) [e]	36 (32.1)	48 (39.3)	182 (36.6)	p=0.511 (χ^2=1.34)
Socioeconomic variables				
Education, years, mean±SD [f]	12.30±3.31	11.91±3.09	11.84±2.89	p=0.593 (H=1.04)
Unemployment, n (%)	44 (38.6)	51 (41.1)	213 (42.8)	p=0.706 (χ^2=0.70)
Number of hospitalizations [c]	8.67±11.63	8.23±10.47	8.14±9.08	p=0.678 (H=0.78)

**Previous work reported highest risk for schizophrenia in individuals born in February and March[22] or associations with deficit schizophrenia upon summer birth (June, July)[21].
Uncorrected means±standard deviations (SD) displayed. [a] For statistical methods, Kruskal-Wallis-H or χ^2-test and for normally distributed data ANOVA was used. Bolded values, p<0.05. Significance values are displayed uncorrected with p-values withstanding Bonferroni correction underlined. [b] Due to missing data, sample sizes vary. [c] Corrected for duration of disease (standardized residuals after linear regression). [d] Corrected for age, PANSS negative, chlorpromazine equivalents (standardized residuals after linear regression). [e] Suicidality = individuals with history of suicide attempts. [f] Education refers to total of years spent to achieve the highest individual qualification; individuals currently in education excluded.

Table S3: Impact of paternal age on readouts of disease severity and socioeconomic functioning in male schizophrenic subjects

	Paternal age (at birth)*			
	age < 25**	age 25-35	age > 35**	p [a] (χ^2/Z/T) [a]
	n=122-137[b]	n=340-381[b]	n=106-118[b]	
Disease variables				
Age at disease onset, mean±SD	25.53±8.96	25.08±7.58	25.19±7.74	p=0.896 (H=0.22)
Age at prodrome, mean±SD	22.25±9.21	22.33±7.40	22.46±8.28	p=0.387 (H=1.90)
PANSS positive, mean±SD [c]	13.09±5.77	13.36±6.12	14.36±6.31	p=0.357 (H=2.06)
PANSS negative, mean±SD [c]	18.70±7.36	17.53±7.71	18.49±7.32	p=0.132 (H=4.05)
Cognitive composite, mean±SD [d]	0.05±0.84	0.11±0.81	0.00±0.79	p=0.802 (F=0.22)
Suicidality, n (%) [e]	51 (38.6)	131 (34.8)	41 (35.0)	p=0.727 (χ^2=0.64)
Socioeconomic variables				
Education, years, mean±SD [f]	11.56±2.62	12.53±3.12	11.70±3.00	**p=0.002** (H=12.69)
Unemployment, n (%)	57 (42.2)	148 (39.2)	50 (42.4)	p=0.733 (χ^2=0.62)
Number of hospitalizations [c]	9.20±12.40	7.75±8.77	8.19±7.37	p=0.249 (H=2.78)

**High and low paternal age has previously been associated with increased risk for schizophrenia[23].
Uncorrected means±standard deviations (SD) displayed. [a] For statistical methods, Kruskal-Wallis-H or χ^2-test and for normally distributed data ANOVA was used. Bolded values, p<0.05. Significance values are displayed uncorrected with p-values withstanding Bonferroni correction underlined. [b] Due to missing data, sample sizes vary. [c] Corrected for duration of disease (standardized residuals after linear regression). [d] Corrected for age, PANSS negative, chlorpromazine equivalents (standardized residuals after linear regression). [e] Suicidality = individuals with history of suicide attempts. [f] Education refers to total of years spent to achieve the highest individual qualification; individuals currently in education excluded.

Table S4: Impact of accumulation of environmental factors on disease and prodrome onset in male schizophrenic individuals: Internal control via group split by recruitment date

GROUP 1	No risk factor	One risk factor	Two risk factors	Three risk factors	Four or more risk factors	*p* [a] (*H*) [a]	*p* [b] (*J*) [b]
	n=18-20[c]	n=47-54[c]	n=62-72[c]	n=51-64[c]	n=32-39[c]		
Disease variables							
Age at disease onset, mean±SD	29.20±6.53	28.92±9.21	26.50±8.01	25.05±5.38	22.18±5.44	**$p=4.5\times10^{-6}$** (*H*=30.19)	**$p=3.8\times10^{-7}$** (*J*=8730.5)
Age at prodrome, mean±SD	26.90±7.31	26.37±9.24	24.47±8.81	21.27±6.14	18.73±5.90	**$p=9.8\times10^{-6}$** (*H*=28.52)	**$p=1.5\times10^{-7}$** (*J*=5927.5)

Uncorrected means and standard deviations (SD) displayed, bolded values, $p<0.05$ (two-tailed).
[a] For statistical methods, Kruskal-Wallis-*H* test was used. [b] To test for statistical trend Jonckheere-Terpstra test (quantitative traits) was used. [c] Due to missing data, sample sizes vary.

GROUP 2	No risk factor	One risk factor	Two risk factors	Three risk factors	Four or more risk factors	*p* [a] (*H*) [a]	*p* [b] (*J*) [b]
	n=19-20[c]	n=46-55[c]	n=70-73[c]	n=64-65[c]	n=38-40[c]		
Disease variables							
Age at disease onset, mean±SD	32.13±8.60	28.52±9.56	25.91±6.84	24.64±5.96	22.99±5.01	**$p=6.1\times10^{-5}$** (*H*=24.58)	**$p=3.1\times10^{-6}$** (*J*=9312.0)
Age at prodrome, mean±SD	29.71±8.52	25.78±9.50	22.23±6.72	21.83±6.19	19.96±4.66	**$p=7.8\times10^{-5}$** (*H*=24.04)	**$p=1.7\times10^{-5}$** (*J*=8269.0)

Uncorrected means and standard deviations (SD) displayed, bolded values, $p<0.05$ (two-tailed). Significance values are displayed uncorrected with *p*-values withstanding Bonferroni correction underlined.
[a] For statistical methods, Kruskal-Wallis-*H* test was used. [b] To test for statistical trend Jonckheere-Terpstra test (quantitative traits) was used. [c] Due to missing data, sample sizes vary.

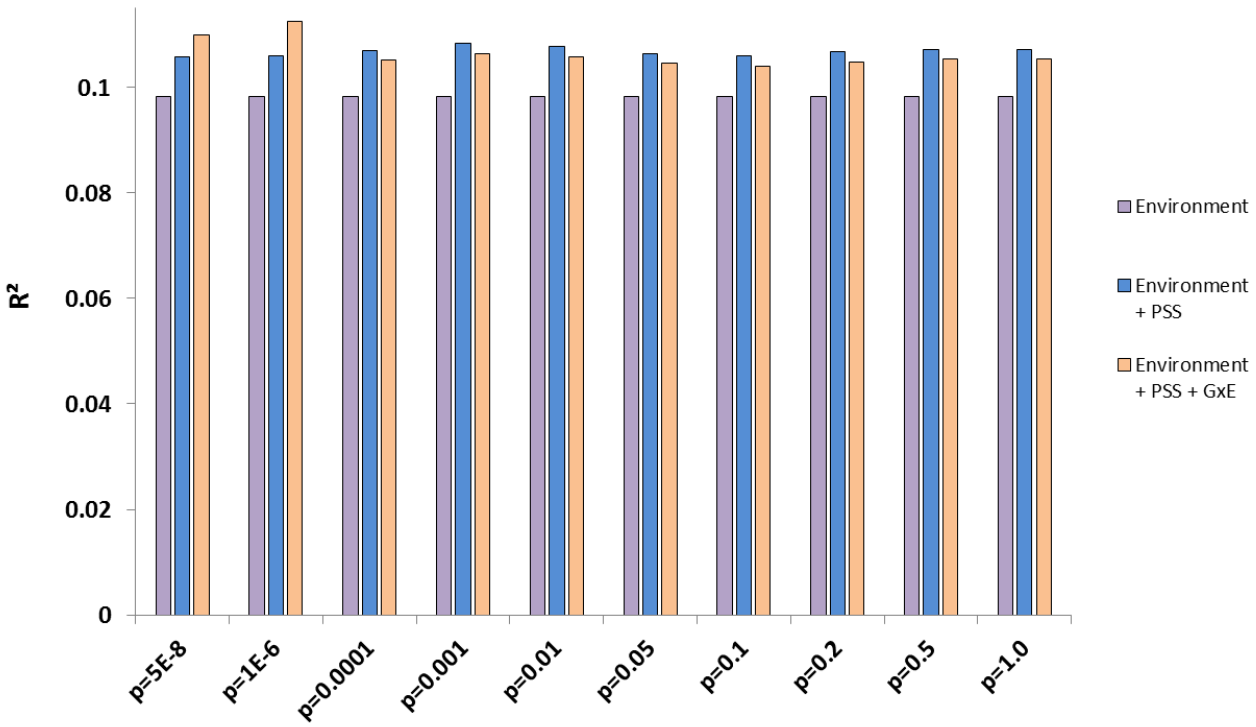
Table S5: Association of polygenic schizophrenia scores (PSS) or their interaction with environmental factors (GxE) with disease phenotypes and amount of variance explained (R^2 change) in male schizophrenic patients. *Models containing only environmental load as predictor were used to set the baseline R^2 in order to estimate the potential additional effect of PSS or GxE.*

Disease variables			PSS according to different *p*-value thresholds									
			PSS≤5x10^{-8}	**PSS≤1x10^{-6}**	**PSS≤0.0001**	**PSS≤0.001**	**PSS≤0.01**	**PSS≤0.05**	**PSS≤0.1**	**PSS≤0.2**	**PSS≤0.5**	**PSS≤1.0**
Age at disease onset [a]	PSS	***p*-value**	0.524	0.459	0.308	0.189	0.243	0.415	0.497	0.346	0.288	0.289
		R^2 change	0.008	0.008	0.009	0.010	0.009	0.008	0.008	0.008	0.009	0.009
	GxE	***p*-value**	0.080	**0.039**	0.860	0.950	0.834	0.683	0.897	0.844	0.768	0.768
		R^2 change	0.012	0.014	0.007	0.008	0.008	0.006	0.006	0.007	0.007	0.007
Age at prodrome [a]	PSS	***p*-value**	0.145	0.943	0.802	0.731	0.427	0.444	0.451	0.286	0.262	0.257
		R^2 change	0.010	0.005	0.006	0.006	0.007	0.007	0.007	0.008	0.008	0.008
	GxE	***p*-value**	0.126	**0.014**	0.178	0.399	0.531	0.454	0.683	0.736	0.638	0.669
		R^2 change	0.013	0.017	0.007	0.005	0.006	0.006	0.005	0.006	0.007	0.007
PANSS positive [a]	PSS	***p*-value**	0.104	0.388	0.192	0.472	0.921	0.891	0.997	0.844	0.979	0.997
		R^2 change	0.001	-0.003	-0.001	-0.004	-0.005	-0.005	-0.005	-0.005	-0.005	-0.005
	GxE	***p*-value**	0.427	0.643	0.989	0.865	0.637	0.777	0.925	0.839	0.927	0.995
		R^2 change	0.000	-0.005	-0.004	-0.006	-0.007	-0.007	-0.007	-0.007	-0.007	-0.007
PANSS negative [a]	PSS	***p*-value**	0.687	0.715	0.582	0.521	0.991	0.997	0.874	0.863	0.896	0.916
		R^2 change	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001
	GxE	***p*-value**	0.147	0.904	0.261	0.654	0.983	0.810	0.930	0.856	0.906	0.855
		R^2 change	0.004	-0.001	0.002	0.000	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001
Cognitive composite [a]	PSS	***p*-value**	0.853	0.595	0.613	0.336	0.694	0.928	0.891	0.885	0.890	0.888
		R^2 change	0.004	0.005	0.005	0.006	0.005	0.004	0.004	0.004	0.004	0.004
	GxE	***p*-value**	**0.015**	0.096	0.145	0.310	0.332	0.380	0.805	0.616	0.736	0.745
		R^2 change	0.016	0.009	0.007	0.006	0.004	0.004	0.002	0.002	0.002	0.002
Suicidality [b]	PSS	***p*-value**	0.191	0.308	0.900	0.460	0.588	0.794	0.745	0.629	0.618	0.691
		R^2 change	0.042	0.041	0.038	0.039	0.038	0.038	0.038	0.038	0.038	0.038
	GxE	***p*-value**	0.457	0.910	0.424	0.260	0.256	0.875	0.837	0.980	0.936	0.829
		R^2 change	0.044	0.041	0.039	0.043	0.042	0.038	0.038	0.038	0.038	0.038

These analyses included only those male schizophrenic patients with full genetic and environmental data (N=472). For all analyses involving genetic data 10 population stratification dimensions and inbreeding coefficient were used as covariates. Bolded values, $p<0.05$. [a] Linear regression was used for continuous variables; adjusted R^2 of a model containing only environmental load as predictor was set as the baseline R^2 for comparisons with the additional effect of PSS or GxE. [b] Logistic regression was performed for dichotomous phenotypes; Nagelkerke's pseudo R^2 of a model containing only environmental load as predictor was set as the baseline for comparisons with the additional effect of PSS or GxE.

Figure S1: Evaluation of environmental and genetic contributions to age at disease onset in male schizophrenic individuals

The amount of variance (adjusted R^2) explained by different models regarding age at disease onset is shown. Purple model contains only environmental load as predictor. Blue model contains environmental load and polygenic schizophrenia score (PSS) as predictors. Finally, orange model contains environmental load, PSS and the interaction between them (GxE) as predictors. Overall, in the sample under analysis, genetic effects (either as main factor or in interaction with environment) are not statistically significant and do not significantly improve the original (purple) environmental model. A very similar picture emerges if other schizophrenia-related phenotypes are considered (see Table S5).



X axis shows the different *p*-value thresholds used to define the polygenic scores. *Y* axis shows the adjusted R^2 value. These analyses included only those male schizophrenic patients with full genetic and environmental data (N=472). For all analyses involving genetic data (Environment + PSS or Environment + PSS + GxE) 10 population stratification dimensions and inbreeding coefficient were used as covariates.

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5. AUTOANTIBODIES AGAINST BRAIN ANTIGENS AND PSYCHIATRIC DISORDERS

5.1. Overview of project III

In 2007/2008 the *anti-NMDA-receptor encephalitis* was described as a disorder associated with high levels of *N*-methyl-D-aspartate (NMDA) receptor IgG antibodies, directed against the NR1 subunit of the receptor, in patients' cerebrospinal fluid and blood (Dalmau et al., 2008; Dalmau et al., 2007; Sansing et al., 2007). Individuals that received the diagnosis showed, besides severe general and neurological symptoms, personality changes, irritability, anxiety, memory loss, agitation, bizarre behavior, delusions, paranoid thoughts, and hallucinations – psychiatric symptoms reminiscent of schizophrenia (Dalmau et al., 2011; Dalmau et al., 2008; Florance et al., 2009; Gable et al., 2009; Irani et al., 2010b). As the underlying mechanism Hughes and colleagues (2010) suggested a titer-dependent reduction of NMDA receptor surface and total cluster density due to receptor internalization.

There are different lines of evidence that support an involvement of the NMDA receptors in schizophrenia pathophysiology. NMDA glutamate receptor antagonists (phencyclidine, ketamine) can induce schizophrenia-like symptoms in healthy human subjects (Cohen et al., 1962; Davies and Beech, 1960; Luby et al., 1959; Rosenbaum et al., 1959), and postmortem as well as imaging studies report NMDA receptor abnormalities in schizophrenic patients (Kristiansen et al., 2007; Pilowsky et al., 2006). The most recent GWAS, found markers in genes involved in glutamatergic neurotransmission to be among the most significantly associated with schizophrenia (Schizophrenia Working Group of the PGC, 2014) and the largest exome sequencing study of *de novo* mutations in schizophrenia to date, describes a significant enrichment of these mutations in the postsynaptic NMDA receptor complex (Fromer et al., 2014).

Based on all previous evidence and intrigued by the description of the anti-NMDA receptor encephalitis we wanted to elucidate the role of serum NMDA receptor antibodies in brain diseases. Therefore the present study had five aims: (I) Describe the overall prevalence of antibodies directed against the NR1 subunit of the NMDA receptor in patients with schizophrenia, affective disorders, Parkinson as well as healthy control subjects; (II) Examine

the frequency of different immunoglobulin class (IgM, IgA, IgG) antibodies and their titers in healthy and ill subjects, and compare the functionality of IgM, IgA, and IgG; (III) Evaluate if patients with schizophrenia, tested positive for NMDA receptor antibodies, differ from schizophrenic patients tested negative, in symptomatology or disease onset; (IV) Study the importance of the blood-brain barrier for the development of pathology in mice and humans; (V) Search for genetic and environmental factors that are associated with antibody formation.

Across all groups (schizophrenia, affective disorders, Parkinson, healthy) about 10% of individuals were tested positive for anti-NR1 NMDA receptor antibodies in serum. Immunoglobulin classes as well as titer were also comparable between all tested groups. Primary cortical neurons from mouse embryos (E16) were incubated with either IgM, IgA or IgG antibodies or seronegative Ig extract. The samples were obtained from patients (schizophrenia, affective disorders) and healthy controls. Antibodies of all Ig classes led to increased endocytosis compared with seronegative extracts regardless of disease state. When comparing Ig-positive and Ig-negative patients with schizophrenia we found no significant differences in measures of disease onset, disease severity, as well as cognitive and neurological functioning. We further studied *ApoE* homozygous KO mice (Piedrahita et al., 1992), which have an impaired blood-brain barrier (Fullerton et al., 2001). *ApoE* KO and WT mice received intravenous tail injections of purified human IgM, IgA, and IgG antibodies on three consecutive days, upon which spontaneous activity was measured. Only *ApoE* KO mice showed a further reduction of spontaneous activity after the antibody application. Similarly only KO mice that had received the antibody treatment responded to MK-801 (non-competitive NMDA receptor antagonist) challenge with increased activity – a psychosis related response. In an attempt to translate these findings back to humans, we compared schizophrenic patients that had or had not suffered birth complications or a neurotrauma with and without NMDA receptor antibodies. We assumed that birth complications as well as a head trauma are factors that can lead to an – at least temporarily – impaired blood-brain barrier. In fact, NMDA receptor antibody positive patients with schizophrenia, which had experienced birth complications or a neurotrauma and were tested positive for antibodies experienced more neurological symptoms than any of the other groups. Our final goal was the identification of factors that would trigger antibody formation. Whilst we did

not find human leukocyte antigen (HLA) alleles to be associated with anti-NR1 seropositivity, a genome-wide association approach led the discovery of one significantly associated SNP (intergenic region on chromosome 1). Additionally we detected an association of past influenza A and B infection (IgG antibodies) with an increased prevalence of NMDA receptor antibodies in men. In summary we were able to show that NMDA receptor antibodies, comparable in isotypes, titers, and functionality, are rather common and not specific to brain disease. Only when blood-brain barrier integrity is compromised, we saw antibody related effects on neurological functions. Our study uncovered one genetic (SNP rs524991) and one environmental (influenza AB status) risk factor for antibody formation.

5.2. Original publication

Hammer C., **Stepniak B.**, Schneider A., Papiol S., Tantra M., Begemann M., Siren A.L., Pardo L.A., Sperling S., Mohd Jofrry S., Gurvich A., Jensen N., Ostmeier K., Luhder F., Probst C., Martens H., Gillis M., Saher G., Assogna F., Spalletta G., Stocker W., Schulz T.F., Nave K.A., Ehrenreich H. (2014) Neuropsychiatric disease relevance of circulating anti-NMDA receptor autoantibodies depends on blood-brain barrier integrity. Mol Psychiatry **19**:1143-9.

Personal contribution

As part of the anti-NMDA receptor autoantibody project I was responsible for the extensive phenotypical analyses of the human patient as well as the control samples in the study (Table 2, Figure 1d). I prepared the table and figure and wrote the figure legend as well as the respective part of the materials and methods section (‘Participants’, ‘Phenotypical analyses’, ‘Statistical analysis – Human data’). I was involved in the literature search before the writing of the paper. Together with the first author and under the supervision of Prof Hannelore Ehrenreich I discussed the results and their interpretation for the discussion section of the manuscript. During the review process I responded to queries concerning the human data and did the final proof reading of the manuscript together with the first author before publication.

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ORIGINAL ARTICLE

Neuropsychiatric disease relevance of circulating anti-NMDA receptor autoantibodies depends on blood–brain barrier integrity

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In 2007, a multifaceted syndrome, associated with anti-NMDA receptor autoantibodies (NMDAR-AB) of immunoglobulin-G isotype, has been described, which variably consists of psychosis, epilepsy, cognitive decline and extrapyramidal symptoms. Prevalence and significance of NMDAR-AB in complex neuropsychiatric disease versus health, however, have remained unclear. We tested sera of 2817 subjects (1325 healthy, 1081 schizophrenic, 263 Parkinson and 148 affective-disorder subjects) for presence of NMDAR-AB, conducted a genome-wide genetic association study, comparing AB carriers versus non-carriers, and assessed their influenza AB status. For mechanistic insight and documentation of AB functionality, *in vivo* experiments involving mice with deficient blood–brain barrier (ApoE$^{-/-}$) and *in vitro* endocytosis assays in primary cortical neurons were performed. In 10.5% of subjects, NMDAR-AB (NR1 subunit) of any immunoglobulin isotype were detected, with no difference in seroprevalence, titer or *in vitro* functionality between patients and healthy controls. Administration of extracted human serum to mice influenced basal and MK-801-induced activity in the open field only in ApoE$^{-/-}$ mice injected with NMDAR-AB-positive serum but not in respective controls. Seropositive schizophrenic patients with a history of neurotrauma or birth complications, indicating an at least temporarily compromised blood–brain barrier, had more neurological abnormalities than seronegative patients with comparable history. A common genetic variant (rs524991, $P = 6.15E-08$) as well as past influenza A ($P = 0.024$) or B ($P = 0.006$) infection were identified as predisposing factors for NMDAR-AB seropositivity. The >10% overall seroprevalence of NMDAR-AB of both healthy individuals and patients is unexpectedly high. Clinical significance, however, apparently depends on association with past or present perturbations of blood–brain barrier function.

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Keywords: *APOE*; ApoE null mutant mice; autoimmunity; GWAS; influenza; *NFIA*

INTRODUCTION

N-methyl-D-aspartate receptors (NMDAR) are glutamate-gated ion channels, abundantly expressed in mammalian brain.[1] They form heteromers of NR1, NR2 and NR3 subunits, and are pivotal in regulating synapse function.[2] In schizophrenia, NMDAR hypofunction has been hypothesized due to induction of psychotic symptoms by antagonists.[3] In 2007, Dalmau *et al.*[4,5] described a paraneoplastic syndrome, based on 12 women with ovarian teratoma, carrying IgG autoantibodies (AB) against the NMDAR NR1/2 subunits. The syndrome, termed 'anti-NMDAR encephalitis', variably consisted of psychosis, memory deficits, seizures, dyskinesia, decreased consciousness and autonomic instability. Since its initial description, a flood of publications appeared. The search for anti-NR1 IgG AB in small samples ($N = 46$–80) of schizophrenic patients yielded discordant results.[6–8] Recently, >400 previously collected cases of anti-NMDAR encephalitis have been reviewed, most without associated tumor.[9] Similarly, immunomodulatory treatment outcomes of these and around 100 more cases have been summarized.[10] As a syndrome-pertinent pathophysiological mechanism, an AB-induced decrease of NMDAR-mediated currents, due to enhanced receptor internalization and thus reduced surface expression, has been suggested.[11]

Few studies explored a role of other classes of immunoglobulins (Ig) in an NMDAR-AB syndrome. In individuals with slow cognitive impairment, anti-NR1 IgA AB were found, which affected synaptic protein expression and decreased NMDAR-mediated currents.[12] Anti-NR1 IgM AB were described in a patient with bipolar disorder[13] and in patients with herpes simplex encephalitis.[14] In the largest study so far, investigating IgG, IgA and IgM, Steiner *et al.*[15] reported a higher prevalence of AB of all isotypes in 121 schizophrenic patients, compared with healthy controls or patients suffering from affective disorders. Apart from tumors, no sound information is available yet on putative susceptibility factors for the development of anti-NR1 AB.

The present study was designed to (1) systematically screen in an unbiased fashion a large number ($N = 2817$) of healthy

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individuals and subjects suffering from schizophrenia or other brain diseases for presence of NMDAR-AB of IgG, IgA or IgM isotype; (2) specifically address the question of why healthy AB carriers might remain healthy, by extending this work to experiments *in vivo* and *in vitro*; (3) search for genetic and environmental factors predisposing to NMDAR-AB formation.

MATERIALS AND METHODS

Participants

Subject data were collected in accordance with ethical guidelines and the Declaration of Helsinki. Sample selection was unbiased, that is sera collection was concluded before analysis of NMDAR-AB was planned. Schizophrenic patients ($N = 1081$) were recruited from 2005–2011 at 23 German sites in the GRAS (Göttingen Research Association for Schizophrenia) study. Patients fulfilling DSM-IV criteria for schizophrenia (81.5%) or schizoaffective disorder (18.5%) were included regardless of disease stage.[16,17] Healthy GRAS controls were anonymized blood donors ($N = 1272$; Transfusion Medicine, Göttingen). Health was ensured by pre-donation screening (questionnaires, interviews, hemoglobin, blood pressure, pulse, temperature). Patients with affective disorders ($N = 148$) were also included (ongoing GRAS extension). Parkinson patients ($N = 263$) and respective controls ($N = 53$) were recruited from 2010–2011 in Italy (Rome area). Of the GRAS patients, three volunteers carrying high titers of anti-NR1 IgG, IgA, or IgM, and three seronegative controls agreed to blood donation for mouse experiments (Supplementary Table S1, Supplementary Appendix).

Phenotypical analyses

On all schizophrenic (GRAS) patients, extensive phenotypical characterization was performed as referenced previously.[16,17] Frequency and duration of prodrome, age at first psychotic episode, positive and negative syndrome scale (PANSS) scores, chlorpromazine equivalents, neurological symptoms (CNI; Cambridge Neurological Inventory) including fine motor skills (MacQuarrie dotting/tapping), current cognitive functioning (composite score comprising reasoning, executive function, verbal learning and memory), premorbid intelligence and global assessment of functioning (GAF) were employed as disease characteristics. As factors affecting blood–brain barrier (BBB) integrity, past neurotrauma (all severity levels) and birth complications (all pre- and perinatal complications) were carefully and comprehensively assessed. The final judgment regarding experience of birth complications or neurotrauma in the schizophrenic (GRAS) patient cohort was derived from a number of different sources. First, the information from semi-structured interviews about birth and neurotrauma history of each patient was used. To verify the data or increase the amount of detailed information, all discharge letters of each single patient were screened. In the case of neurotrauma, other semi-structured interviews on critical life events, suicidality and aggressive behavior toward others were used to explore whether patients had experienced serious accidents (including brain trauma) or committed suicide attempts that included, for example, falls or jumps from great heights or had been involved in serious fights leading to head injuries. Finally, information from the physical exam of each patient was included to check whether any scars on the head or neck were found indicative of an injury to the head. After collecting all the data, each patient was dichotomously (yes/no) classified as having or not having experienced a neurotrauma or birth complication. In case of contradictory information, the treating physician and even the obstetric hospital were contacted, and in case of still missing information or a high level of uncertainty, patients were excluded from the analysis.

Serological analyses

Commercially available recombinant immunofluorescence tests (Euroimmun, Lübeck, Germany), standard procedures for clinical diagnosis (100% sensitivity and 100% specificity), were used to detect NMDAR-AB, based on HEK293 cells transfected with NR1 or NR1/NR2b NMDAR-subunits.[5,18] Seropositivity was assessed by two researchers independently. Titers were double-determined in two laboratories (MPI, Euroimmun) by identifying the maximum dilution at which specific fluorescence was still visible. Few samples with discrepant results were re-analyzed, leading to full concordance. The presence of IgG AB against influenza A and B virus was determined by ELISA (Novagnost-InfluenzaA-IgG, Novagnost-InfluenzaB-IgG, Siemens Healthcare Diagnostics GmbH, Eschborn, Germany), automatically processed on BEPIII (Siemens Healthcare Diagnostics GmbH), and interpreted (manufacturer's instructions) as positive, negative or borderline (the latter negative for statistics).

Immunoglobulin purification

Ammonium sulfate precipitation of a serum fraction containing immunoglobulins (Ig) and dialysis was carried out as described.[19] IgG, IgA, or IgM were quantified by immunodiffusion using NOR partigen immunoplates (Siemens Medical Solutions, Marburg, Germany).

Mouse experiments

Experiments were approved by the local Animal Protection Committee. Male C57Bl/6N ApoE$^{-/-}$[20] and wild-type (WT) mice, aged 12–16 weeks, were used (housed at 4–5 per cage, 12 h light/dark cycle, food/water *ad libitum*). Groups (4–6 each) received either extracted Ig fractions from NMDAR-AB seropositive (IgG, IgA, or IgM) or seronegative individuals (information on titer/concentration in Supplementary Table S1 and Supplementary Appendix). Daily intravenous (tail) injections (150 μl each) were performed on 3 consecutive days. Examiners were not aware of group assignment ('blinded'). Spontaneous activity in open field (8 min) was tested in all mice 3–4 days before the first injection (initial group matching). One day after the last injection, spontaneous activity (8 min) was again measured, followed by intraperitoneal injection (0.3 μg per 10 μl per gram of body weight) of the non-competitive NMDAR antagonist MK-801 (Dizocilpine; [5*R*,10*S*]-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine; Sigma-Aldrich GmbH, Munich, Germany) and extended (120 min) open-field observation.[21] MK-801 acts as use-dependent ion-channel blocker.

Endocytosis assays and quantification

Primary cortical neurons prepared from mouse embryos (E16) were cultured on poly-L-lysine-coated glass-coverslips in MEM + B27 (Invitrogen, Karlsruhe, Germany) for 14 days. Glass coverslips were washed with cold Hank's balanced salt solution (HBSS), and incubated (20 min, 4 °C) with Ig extracts containing either NMDAR-AB of IgG, IgA or IgM isoforms or seronegative Ig extracts (1:100) in HBSS. The examiner was unaware of the nature of the extract ('blinded'). Unbound AB was removed (3 HBSS washes) before placing cells in pre-warmed (37 °C) growth medium for 15 min to allow internalization. After medium wash-off (cold HBSS), remaining surface NMDAR were labeled with anti-mouse NR1-AB (Abcam, Cambridge, UK) for 15 min on ice. After cold HBSS wash, surface-bound NR1-AB was labeled (4 °C, 15 min) with Alexa-488-coupled 2nd AB (anti-mouse IgG; Invitrogen, Karlsruhe, Germany). After wash-off (HBSS; 4 °C) of unbound AB, cells were fixed in 4% paraformaldehyde. For quantification, confocal images of cell surface staining were taken with identical acquisition parameter on SP2 LSM (Leica, Wetzlar, Germany). Signal intensity was quantified with ImageJ, and ratio of intensity per cell surface area calculated.

Genetic analyses

A semi-custom Axiom myDesign genotyping array (Affymetrix, Santa Clara, CA, USA) was used. For description of array-specifications, quality controls and genome-wide genetic association study (GWAS), see Supplementary Appendix.

Statistical analysis

P-values < 0.05 were considered significant. Data in figures are expressed as mean ± s.e.m., in tables as mean ± s.d.

Mouse experiments. Data were compared by analysis of variance, followed by *post-hoc* tests where appropriate using Prism5 (GraphPad-Software Inc., La Jolla, CA, USA) or SPSS (SPSS-Statistics 17.0, IBM-Deutschland GmbH, Munich, Germany). Greenhouse–Geisser correction was applied on violation of sphericity.

Human data. Group differences in categorical and continuous variables were assessed using Chi-square or Mann–Whitney U tests. A generalized linear model was employed upon covariate inclusion. In case of normal distribution of continuous variables, T-tests were performed. To assess main effects and interactions between neurotrauma/birth complications and Ig-positivity regarding CNI scores, two-way independent ANOVA was

Table 1. Prevalence of anti-NR1 AB in patients and controls

	Anti-NR1 seropositivity—N (%)							
Study group	*Any*	*IgG*	*IgA*	*IgM*	*IgG + IgA*	*IgG + IgM*	*IgA + IgM*	*IgG + IgA + IgM*
GRAS[a] patients ($N = 1081$)	93 (8.6)	7 (0.7)	56 (5.2)	46 (4.3)	0 (0)	0 (0)	16 (1.5)	0 (0)
Affective-disorder patients ($N = 148$)	24 (16.2)	5 (3.4)	15 (10.1)	7 (4.7)	1 (0.7)	0 (0)	2 (1.4)	0 (0)
GRAS[a] controls ($N = 1272$)	137 (10.8)	5 (0.4)	75 (5.9)	80 (6.3)	2 (0.2)	3 (0.2)	19 (1.5)	1 (0.1)
Parkinson patients ($N = 263$)	35 (13.3)	1 (0.4)	17 (6.5)	25 (9.5)	1 (0.4)	1 (0.4)	7 (2.7)	1 (0.4)
Parkinson controls ($N = 53$)	6 (11.3)	0 (0)	3 (5.7)	3 (5.7)	0 (0)	0 (0)	0 (0)	0 (0)
Total ($N = 2817$)	295 (10.5)	18 (0.6)	166 (5.9)	161 (5.7)	4 (0.1)	4 (0.1)	44 (1.6)	2 (0.1)

[a]GRAS patients are schizophrenic individuals of the GRAS data collection (Göttingen Research Association for Schizophrenia). GRAS controls are the respective healthy control collective (see also Materials and methods).

conducted. Corrected values reflect linear regression-based residuals when age, chlorpromazine and PANSS negative scores were independent variables. PLINK (v1.07) was used to test association between single-nucleotide polymorphisms (SNPs) and anti-NR1 serological status (allelic test) and deviations from Hardy–Weinberg equilibrium.[22] Principal components were generated using EIGENSTRAT (http://genetics.med.harvard.edu/reich/Reich_Lab/Software.html). Human leukocyte antigen (HLA) types were imputed for seven HLA genes using HiBag0.9.1 at four-digit resolution, based on a pre-fit European ancestry model (http://cran.r-project.org/web/packages/HIBAG/index.html). *P*-values were multiple-testing corrected (Bonferroni) where indicated, but are displayed uncorrected.

RESULTS

NMDAR-AB seroprevalence in 2817 individuals

AB of all here analyzed isotypes (IgG, IgA and IgM), directed against the NMDAR-NR1 subunit, were identified in 10.5% of subjects (Table 1). Importantly, seroprevalence did not differ between schizophrenic (GRAS) patients (8.6%) and GRAS controls (10.8%, $P = 0.078$). An apparently higher incidence in affective-disorder patients (16.2%) is explained by a higher mean age. In fact, seroprevalence increases with age (Supplementary Figure S1, Supplementary Table S2, Supplementary Appendix) and is higher in male than female subjects (Supplementary Table S3, Supplementary Appendix, 11.53% versus 8.68%, $P = 0.017$). Seropositivity between Parkinson patients (13.3%) and respective controls did not differ (11.3%, $P = 0.694$).

Seroprevalence and titer distribution of NMDAR-AB Ig isotypes

Considering each Ig class separately, again no differences in seroprevalence among groups arose (Table 1). Occurrence of IgG anti-NR1 was infrequent (0.6% in total) compared with IgA (5.9%) or IgM (5.7%). A combination of IgA/IgM AB was present in 1.6%, combinations including IgG in only 0.1% each. AB exclusively against the NR1/NR2b heterodimer, that is without reactivity against NR1 alone, were not identified. Titer distributions in patient and control groups as possible explanation of NMDAR-AB pathology did not differ (Supplementary Table S4, Supplementary Figure S2, Supplementary Appendix).

NMDAR-AB functionality in a neuronal endocytosis assay

We next wondered whether AB from controls and patients differ in functionality. Extracts from seropositive subjects, independently of isotype or disease state, resulted in increased endocytosis, compared with seronegative extracts (Figure 1a, Supplementary Table S1, Supplementary Appendix).

Relevance of BBB integrity for NMDAR-AB effects in mice

Having comparable serological (% seropositivity, Ig-isotype, titer distribution) and functional results in controls and patients, we asked why healthy AB carriers remain healthy. We hypothesized that a compromised BBB might decide on the pathophysiological significance of NMDAR-AB. To approach this hypothesis experimentally, we employed ApoE$^{-/-}$ mice[20] (with known BBB leakage)[23–25] versus WT. Intravenous injection of purified Ig fractions from NMDAR-AB seropositive (IgM, IgG, IgA) subjects led to alterations in spontaneous open-field activity and the response to MK-801 exclusively in ApoE$^{-/-}$ mice. Trends were comparable in groups receiving IgM, IgG or IgA extracts, resulting in significant differences on pooling (Figures 1b and c, Supplementary Figure S3, Supplementary Appendix).

Translating experimental BBB findings to schizophrenic (GRAS) patients

Overall, schizophrenic anti-NR1 carriers and non-carriers do not differ with respect to disease phenotypes, covering the symptom clusters of anti-NMDAR encephalitis (Table 2). Also, occurrence and duration of prodromal phase and age of disease onset are similar between the two groups, arguing against a sudden/atypical syndrome start in AB carriers (Table 2). Following our BBB hypothesis, we compared individuals with birth complications or past brain trauma—conditions known to provoke temporary or persistent (albeit often minor) BBB abnormalities.[26,27] Indeed, also in humans, a clear impact, that is a more severe neurological phenotype, arises from the combination of compromised BBB function and circulating NMDAR-AB (Figure 1d).

Identification of first genetic susceptibility factors

GWAS have been successful in identifying associations between genomic variants and autoimmune disorders, such as rheumatoid arthritis or systemic lupus erythematosus.[28] We performed GWAS to spot SNPs potentially predisposing to formation of NMDAR-AB (Supplementary Appendix). We identified a genome-wide significant SNP, rs524991 (A/G, $P = 6.15E{-}08$; Bonferroni threshold $P = 8.62E{-}08$), with an odds ratio (OR) of 2.22 (95% confidence interval (CI) = 1.654–2.991; Supplementary Figure S6, Supplementary Appendix). This variant with a minor allele frequency of 12.45% in seropositive versus 6.01% in seronegative individuals is located in an intergenic region on chromosome1 (Supplementary Figure S7, Supplementary Appendix). Its closest neighboring gene is nuclear factor I/A (*NFIA*, 218.59 kb downstream), a transcription factor reported to mediate neuroprotective effects of NMDAR activation.[29] Separate analysis of SNP rs524991 association with NMDAR-AB seropositivity (Table 3) showed a similar tendency for all study groups (except Parkinson) and no gender difference (Supplementary Table S8, Supplementary Appendix). Search for a predisposing role of HLA alleles for NMDAR-AB formation did not deliver hits, apart from a nominally significant association of HLA-A03 with seropositivity ($P = 0.01$; Supplementary Table S9, Supplementary Appendix).

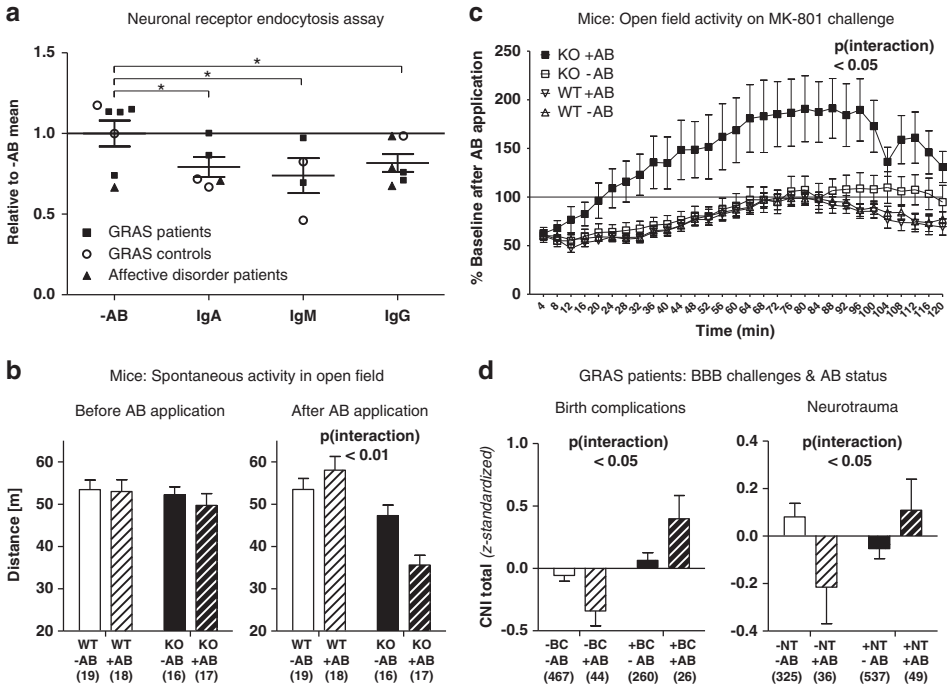

Figure 1. NMDAR-AB functionality and relevance of the blood–brain barrier. (**a**) Reduced AB binding to primary cortical neurons indicates increased endocytosis of NMDAR after incubation with Ig extracts containing either NMDAR-AB of IgG, IgA or IgM isoforms (all $P<0.05$), or seronegative Ig extracts (one-tailed *T*-tests). Mean values upon AB extracts (each tested in 1–3 independent experiments, dependent on serum availability) were normalized to the mean of the respective seronegative control extracts. (**b**) ApoE$^{-/-}$ (KO) and WT mice do not differ in spontaneous activity in the open field before AB injection. However, 1 day after the last of three daily injections with seropositive (+AB) or seronegative (−AB) Ig extracts, a decrease in spontaneous activity was evident exclusively in seropositive ApoE$^{-/-}$ (KO+AB) mice: two-way ANOVA revealed a significant interaction effect of genotype and serotype ($F=8.96$, $P<0.01$), as well as a significant main effect of genotype ($F=27.81$, $P<0.0001$), but not of serotype. (**c**) Only ApoE$^{-/-}$ (KO) mice with their known compromised BBB respond to intravenous NMDAR-AB extracts with a hypersensitive (psychosis-related) response in the open field to the NMDAR antagonist MK-801. Using a generalized linear model for repeated measures to evaluate results after MK-801 treatment, we obtained significant main effects of time ($F=36.25$, $P<0.001$), genotype ($F=9.54$, $P<0.01$) and serotype ($F=4.85$, $P<0.05$) as well as a significant genotype × serotype interaction effect ($F=5.75$, $P<0.05$). (**d**) In the GRAS sample of schizophrenic patients, birth complications and history of neurotrauma as readouts for temporarily/persistently compromised BBB were examined in NMDAR-AB-positive versus negative subjects with respect to their impact on neurological symptom severity (CNI total score, z-standardized). Birth complications showed an interaction with serotype ($F=5.80$, $P<0.05$) regarding CNI and a main effect ($F=11.24$, $P=0.001$). Likewise, past neurotrauma showed an interaction with serotype ($F=4.02$, $P<0.05$). Group sizes are given as numbers in brackets. *$P<0.05$; WT, wild type; KO, ApoE$^{-/-}$ knockout; −AB, NMDAR-AB seronegative; +AB, NMDAR-AB seropositive; BC, birth complications; NT, neurotrauma.

Identification of environmental susceptibility factors

As first risk factor for NMDAR-AB formation, the presence of a tumor, preferentially an ovarian teratoma was identified.[4] Other predisposing factors have remained speculative. Infections have been suggested as triggers of AB formation in autoimmune diseases,[30] for example, Epstein-Barr virus in multiple sclerosis.[31] We hypothesized that a similar role might be attributed to influenza for NMDAR-AB. Anti-NMDAR encephalitis was reported in a patient with influenza H1N1 infection and two subjects after respective vaccination.[9] NMDAR-AB were described in pediatric cases of encephalitis lethargica, a condition—not unequivocally—associated with influenza.[32,33] Strikingly, we found an increased anti-NR1 AB prevalence in individuals carrying anti-influenza A ($P=0.024$, OR = 1.366, CI 95% = 1.042–1.790) and B ($P=0.006$, OR = 1.453, CI 95% = 1.109–1.904) IgG (Table 3). This association was present in males only (Supplementary Table S10, Supplementary Appendix).

DISCUSSION

The present study provides (1) the first large-scale systematic screen for presence of NMDAR-AB in serum of healthy and neuropsychiatrically diseased subjects. In >2800 unbiasedly selected individuals, >10% seroprevalence of anti-NR1 AB, independent of group affiliation, was found. (2) From this unexpected observation, the fundamental question arose of why healthy AB carriers have remained healthy, despite comparable distribution of AB isotypes, titers and *in vitro* functionality. An experimental mouse model supports our central hypothesis, that is, the essential role of BBB integrity. Only in ApoE mutant mice, but not in respective controls, we find that human NMDAR-AB cause psychosis-related behavioral perturbation.[21] The BBB role is further underlined by a hypothesis-driven outcome analysis of schizophrenic (GRAS) patients with history of birth complications or neurotrauma indicating past/present BBB insufficiency. (3) Ultimately, with a genome-wide significant marker, SNP rs524991, and an association of seropositivity with influenza AB status, we provide genetic and environmental risk factors of NMDAR-AB formation.

The most remarkable finding of the present work is the high seroprevalence of NMDAR-AB in healthy individuals. Only one other study included a considerable number of healthy subjects in a screen of psychiatric patients but reported seropositivity for only 1 in 240 controls (0.4%) and 2 in 108 (<2%) affective-disorder patients.[15] Seroprevalence in schizophrenic patients (9.9% of 121)

Table 2. Baseline characteristics, according to study group (means ± s.d. (range) are displayed, unless indicated otherwise)

Patients and control groups	*Total sample*	*Ig-positive individuals*	*Ig-negative individuals*	P-*value* (χ^2, *Z, T value*)[a]
Schizophrenic (GRAS) patients	*N = 774–1081*[b]	*N = 63–93*[b]	*N = 711–988*[b]	
Age, years	39.37 ± 12.59 (17–79)	41.15 ± 11.98 (18–75)	39.20 ± 12.63 (17–79)	0.115 ($Z = -1.58$)
Gender, *N* male (%)	723 (66.9)	68 (73.1)	655 (66.3)	0.181 ($\chi^2 = 1.79$)
Prodrome, *N* cases (%)	754 (80.6)	64 (79.0)	690 (80.8)	0.698 ($\chi^2 = 0.15$)
Duration of prodrome, years	2.81 ± 3.57 (0–28.2)	2.64 ± 3.01 (0–13.0)	2.83 ± 3.62 (0–28.2)	0.853 ($Z = -0.19$)
Age at first episode, years	25.88 ± 8.90 (5–68)	25.85 ± 8.98 (12–51)	25.89 ± 8.90 (5–68)	0.890 ($Z = -0.11$)
PANSS positive score	13.74 ± 6.25 (7–38)	13.28 ± 5.71 (7–31)	13.78 ± 6.31 (7–38)	0.597 ($Z = 0.60$)
PANSS negative score	18.25 ± 7.90 (7–46)	17.00 ± 7.42 (7–37)	18.37 ± 7.93 (7–46)	0.122 ($Z = -1.55$)
PANSS general score	33.74 ± 11.76 (16–82)	32.48 ± 10.80 (16–75)	33.86 ± 11.85 (16–82)	0.373 ($Z = -0.89$)
CPZ	686.53 ± 697.43 (0–7375)	628.04 ± 537.82 (0–2620)	691.97 ± 710.43 (0–7375)	0.580 ($Z = -0.55$)
CNI[c,d]	0.00 ± 1.00 (−2.71–3.07)	−0.03 ± 0.92 (−2.07–2.11)	0.00 ± 1.01 (−2.71–3.07)	0.742 ($T = 0.33$)
MacQuarrie dotting[e]	0.00 ± 1.00 (−3.61–3.22)	0.14 ± 1.14 (−2.67–3.07)	−0.01 ± 0.98 (−3.61–3.22)	0.172 ($T = -1.37$)
MacQuarrie tapping[e]	0.00 ± 1.00 (−4.83–3.14)	0.11 ± 0.99 (−2.00–3.10)	−0.01 ± 1.00 (−4.83–3.14)	0.261 ($T = -1.13$)
Cognitive composite score	−0.02 ± 0.84 (−2.57–2.98)	−0.01 ± 0.89 (−2.12–2.03)	−0.02 ± 0.84 (−2.57–2.98)	0.946 ($T = -0.07$)
Premorbid IQ (MWT-B[f])	25.67 ± 6.36 (4–42)	26.64 ± 6.28 (6–36)	25.57 ± 6.36 (4–42)	0.093 ($Z = -1.68$)
GAF	45.70 ± 17.18 (5–90)	46.26 ± 16.54 (10–80)	45.65 ± 17.25 (5–90)	0.642 ($Z = -0.47$)
Neurotrauma, *N* cases (%)	648 (62.4)	55 (62.6)	593 (59.8)	0.592 ($\chi^2 = 0.29$)
Birth complications, *N* cases (%)	307 (39.7)	27 (42.9)	280 (39.4)	0.589 ($\chi^2 = 0.29$)
Healthy (GRAS) controls	*N = 1272*	*N = 137*	*N = 1135*	
Age, years	37.43 ± 13.24 (18–69)	40.90 ± 12.17 (19–68)	37.01 ± 13.31 (18–69)	**< 0.001** ($Z = -3.56$)
Gender, *N* male (%)	780 (61.3)	95 (69.3)	685 (60.4)	**0.041** ($\chi^2 = 4.17$)
Affective-disorder patients	*N = 148*	*N = 24*	*N = 124*	
Age, years	49.70 ± 15.49 (20–92)	47.38 ± 11.87 (25–76)	50.15 ± 16.09 (20–92)	0.314 ($Z = -1.01$)
Gender, *N* male (%)	70 (47.3)	11 (45.8)	59 (47.6)	0.875 ($\chi^2 = 0.03$)
Parkinson patients	*N = 253–263*[b]	*N = 33–35*[b]	*N = 220–228*[b]	
Age, years	66.04 ± 10.08 (36–86)	69.06 ± 8.33 (45–81)	65.59 ± 10.26 (36–86)	0.055 ($Z = -1.92$)
Gender, *N* male (%)	175 (66.5)	28 (80.0)	147 (64.5)	0.070 ($\chi^2 = 3.29$)
Parkinson controls	*N = 51–53*[b]	*N = 6*[b]	*N = 45–47*[b]	
Age, years	63.31 ± 11.68 (22–80)	67.50 ± 12.58 (44–78)	62.76 ± 11.59 (22–80)	0.188 ($Z = -1.33$)
Gender, *N* male (%)	21 (39.6)	2 (33.3)	19 (40.4)	0.738 ($\chi^2 = 0.11$)

Abbreviations: CPZ, chlorpromazine equivalents; GAF, global assessment of functioning; GRAS, Göttingen Research Association for Schizophrenia; PANSS, positive and negative syndrome scale. Bolded values, $P < 0.05$. [a]For statistical methods, Mann–Whitney *U* or χ^2 tests and for normally distributed variables, *T*-tests were used. [b]Due to missing data, sample sizes vary. [c]Cambridge Neurological Inventory mean value if more than 95 items were available. [d]Corrected for age and CPZ. [e]Corrected for age, PANSS negative and CPZ. [f]Mehrfach-Wortschatz-Intelligenz test (multiple choice vocabulary test).

was comparable to our study. Reasons for this discrepancy are unclear but perhaps related to the smaller number of controls and their younger age.[15] Importantly, analytical materials/methods of both studies were identical, schizophrenia patients show comparable seroprevalence, and the here randomly selected positive specimens for *in vitro* analyses all confirmed AB functionality.

For exerting pathological effects, NMDAR-AB have to reach NMDAR in the brain. This brain presence may occur via (1) AB transfer over a compromised BBB, which normally restricts large molecules from directly entering the brain in appreciable amounts (expected transfer over an intact BBB, for example, of IgG is only 1/500, of IgA 1/600, and of IgM 1/3000 of the serum concentration); (2) slow accumulation of these large molecules due to reduced cerebrospinal fluid (CSF) flow,[34] in which case, however, a retrograde CSF circulation would have to deliver the AB back to brain tissue, or (3) intrathecal synthesis by B lymphocytes.[34,35] Our seroprevalence data do not allow any conclusion on AB production in brain. Therefore, healthy AB carriers may differ at least in part from seropositive disease groups by lack of intrathecal AB synthesis. Nevertheless, with *in vivo* experiments in mice and a hypothesis-driven human database screening, we underscore the likely critical role of an intact BBB as protective mechanism against circulating AB-mediated pathology in mouse and man.

Wild-type mice were not behaviorally affected after injection of human serum extract containing IgG, IgA or IgM NMDAR-AB. In contrast, ApoE$^{-/-}$ mice showed differences in behavior on AB injection, that is reduced spontaneous activity in the open field and hyperlocomotion following MK-801. These behavioral phenomena may be explained by the reported receptor internalization and hypofunction after hippocampal infusion of NMDAR-AB,[11] as exactly opposite effects were described after NMDA application in rats.[36] Stimulation of locomotion by the NMDAR antagonist MK-801 may be caused by hyperexcitability of limbic circuits through NMDAR blockade on inhibitory GABAergic neurons.[37,38] This consequence of NMDA receptor inhibition would be amplified by NMDAR-AB. Similarly, increased motor cortex excitability in mice was provoked by NMDAR-AB injection into the prefrontal area.[39]

In the GRAS sample of schizophrenic individuals, well-documented history of birth complications and brain trauma were evaluated as proxy variables of past or present BBB impairment.[26,27] Indeed, affected individuals show more severe neurological abnormalities when carrying NMDAR-AB. These findings strengthen the hypothesis of BBB involvement in NMDAR-AB pathology, and—replication provided—may even justify recommendations of anti-NR1 serum screening in case of neurotrauma or other conditions with anticipated BBB dysfunction.

Importantly, we did not find any clinically relevant differences when comparing all schizophrenic NMDAR-AB carriers with all non-carriers. Perhaps with information on CSF (which we do not have in our large cohort), an expected 30% of individuals with

Table 3. Susceptibility factors for NMDAR-AB seropositivity, according to study group

Study group	*GRAS patients*		*Affective-disorder patients*		*GRAS controls*		*Parkinson patients*		*Parkinson controls*		*Total*	
Anti-NR1 seropositivity	+	−	+	−	+	−	+	−	+	−	+	−
rs524991												
N, GG (%)	65 (70.7)	867 (88.8)	18 (75.0)	104 (87.4)	111 (82.2)	977 (87.6)	31 (88.6)	187 (85.0)	4 (66.7)	41 (91.1)	229 (78.4)	2176 (87.9)
N, GA (%)	27 (29.3)	105 (10.8)	6 (25.0)	15 (12.6)	19 (14.1)	135 (12.1)	4 (11.4)	32 (14.5)	2 (33.3)	4 (8.9)	58 (19.9)	291 (11.8)
N, AA (%)	0 (0.0)	4 (0.4)	0 (0.0)	0 (0.0)	5 (3.7)	3 (0.3)	0 (0.0)	1 (4.6)	0 (0.0)	0 (0.0)	5 (1.7)	8 (0.3)
P (allelic, d.f. = 1)	**3.23E–06**		0.133		**0.006**		0.551		0.091		**7.48E–07**	
OR (CI 95%)	2.80 (1.78–4.39)		2.12 (0.78–5.79)		1.78 (1.17–2.72)		0.72 (0.25–2.11)		4.30 (0.70–26.52)		1.99 (1.51–2.63)	
Influenza A												
N, seropositive (%)	71 (77.2)	648 (66.2)	16 (66.7)	91 (74.0)	97 (70.8)	764 (67.4)	25 (71.4)	141 (61.8)	6 (100.0)	28 (59.6)	215 (73.1)	1672 (66.6)
P (Pearson's chi^2)	**0.032**		0.461		0.417		0.274		0.052		**0.024**	
OR (CI 95%)	1.727 (1.043–2.860)		0.703 (0.275–1.799)		1.174 (0.796–1.732)		1.543 (0.707–3.367)		NA		1.366 (1.042–1.790)	
Influenza B												
N, seropositive (%)	30 (32.6)	202 (20.6)	5 (20.8)	32 (26.0)	37 (27.0)	221 (19.5)	12 (34.3)	79 (34.6)	0 (0.0)	8 (17.0)	84 (28.6)	542 (21.6)
P (Pearson's chi^2)	**0.008**		0.593		**0.039**		0.966		0.273		**0.006**	
OR (CI 95%)	1.861 (1.172–2.956)		0.748 (0.258–2.169)		1.529 (1.020–2.291)		0.994 (0.769–1.286)		NA		1.453 (1.109–1.904)	

Abbreviations: +, anti-NR1 seropositive; −, anti-NR1 seronegative; CI, confidence intervals; d.f., degree of freedom; GRAS, Göttingen Research Association for Schizophrenia; NA, not available; OR, odds ratio.
Bolded values, $P<0.05$.

permanent barrier dysfunction[40] could have been extracted and would have allowed us to uncover a clinically relevant difference between AB carriers and non-carriers among them. Instead, we found a clinical difference between AB carriers and non-carriers with past birth complication or neurotrauma as a proxy for at least temporary BBB disturbance.[26] It is interesting to speculate that the reported 30% of schizophrenic patients with compromised barrier function[40] and the post-trauma individuals recognized here might represent an (partly) overlapping subpopulation of schizophrenic subjects. Along these lines of thought, future studies may be initiated, analyzing CSF samples of a large number of schizophrenic patients for NMDAR-AB titers and determining the CSF-serum albumin quotient[34] as marker of blood–CSF barrier (dys)function.

Our study is the first to investigate putative genetic susceptibility factors for the formation of anti-NR1 AB. A GWAS approach led to the identification of the genome-wide significant risk SNP rs524991. Further experiments providing mechanistic insight as well as replication analyses are warranted. By a hypothesis-driven approach,[9,32,33] we uncovered an association of influenza A or B AB with anti-NR1 seropositivity, suggesting molecular mimicry. This phenomenon induces generation of AB reacting both against pathogenic elements and autoantigens,[30] and has a role in autoimmune diseases.[41,42] As the influenza A M2 channel and NMDAR share a common ligand, the antiviral compound amantadine,[43] a putative structural homology might act as inducer of NMDAR-AB. The observed association was found in males only. Interestingly, males have a higher incidence of influenza,[44] and male mice exert a more vigorous immune response on influenza infection.[45] This gender disposition might also explain the higher anti-NR1 AB prevalence in males.

In conclusion, our study draws an increasingly complex picture of NMDAR-AB pathology, with anti-NMDAR encephalitis possibly constituting the extreme end of a broad spectrum of mild to severe phenotypes associated with NMDAR autoimmunity. Beyond the NMDAR-AB studied here, loss of blood–brain barrier integrity may generally constitute a major risk factor for detrimental effects of peripheral AB against central nervous system epitopes.

CONFLICT OF INTEREST

Dr Stöcker is a full-time employee of and holds stocks in Euroimmun AG. Dr Martens is a full-time employee of Synaptic Systems GmbH. All other authors declare no conflict of interest.

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

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information on their work.

Neuropsychiatric disease relevance of circulating anti-NMDA receptor antibodies depends on blood-brain barrier integrity

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Table S1. Characteristics of sera used for in vivo and in vitro experiments

List of sera of patients and healthy controls used for determination of AB functionality *in vitro* (receptor endocytosis assay) and *in vivo* (mouse intravenous injection experiments). Total concentrations of the respective Ig subclass in the ammonium sulfate precipitated serum fractions are consistent with published serum levels of immunoglobulins in the general adult population *(Gonzalez-Quintela A, et al. Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. Clin Exp Immunol 2008;151:42-50).*

Ig isotype	ID (code)	Age (years)	Gender	Diagnosis	Serum titer	Titer in Ig extract	Total concentration in fraction (g/l)			Usage of purified serum	
							IgG	IgA	IgM	Endocytosis	Injection
IgG	706594	49	m	healthy control	32	10	16.84	4.35	1.44	√	
	BPD00045	45	f	bipolar affective disorder	100	32	4.25	NA	NA	√	
	SCZ00979	27	m	schizoaffective disorder	320	100	11.50	NA	NA	√	
	SCZ01025	40	f	schizoaffective disorder	100	100	14.37	NA	NA	√	
	UPD00066	32	f	major depressive disorder	320	100	10.41	NA	NA	√	
	UPD00071	43	m	major depressive disorder	1000	1000	20.13	1.92	1.26	√	√
IgA	706026	39	m	healthy control	3200	3200	13.78	1.57	2.09	√	
	706472	37	m	healthy control	1000	1000	18.13	3.42	2.44	√	
	SCZ00547	31	m	schizophrenia	1000	1000	14.98	2.98	2.02	√	
	SCZ00857	43	f	schizophrenia	1000	1000	20.81	4.86	1.15	√	√
	UPD00035	46	m	major depressive disorder	320	320	13.78	4.86	1.50	√	
IgM	707334	47	m	healthy control	3200	1000	14.98	3.09	1.44	√	
	708436	49	m	healthy control	3200	320	21.51	2.88	1.32	√	
	SCZ00996	46	m	schizoaffective disorder	1000	1000	32.20	4.86	1.56	√	
	SCZ00926	35	m	schizoaffective disorder	1000	100	25.12	2.38	0.66	√	√
Negative	707340	37	f	healthy control	NA	NA	9.89	0.81	1.26	√	
	708438	48	f	healthy control	NA	NA	11.50	1.41	0.89	√	
	BPD00028	41	m	bipolar affective disorder	NA	NA	16.21	5.52	1.21	√	√ (control for IgA)
	SCZ00071	41	m	schizophrenia	NA	NA	25.12	1.74	1.62	√	√ (control for IgG)
	SCZ00097	46	f	schizophrenia	NA	NA	13.20	1.17	1.69	√	
	SCZ00397	37	m	schizophrenia	NA	NA	16.84	3.20	1.56	√	
	SCZ00437	43	m	schizophrenia	NA	NA	17.48	1.10	1.38	√	√ (control for IgM)

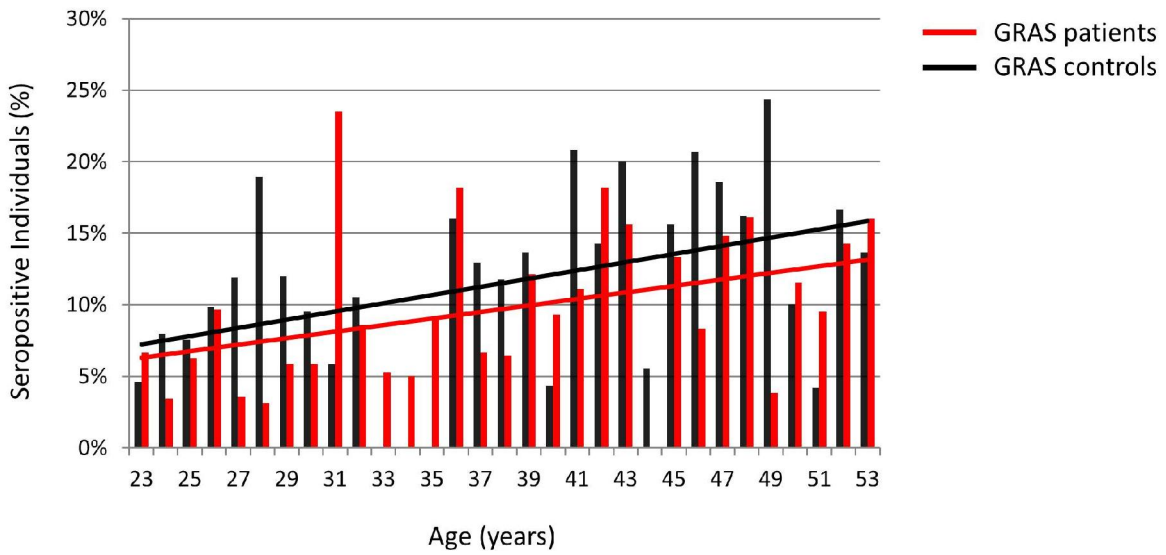
m, male; f, female; NA, not available.

Table S2. Mean age of study cohorts by Ig isotype

Study group	Mean age in years (SD)				
	Seropositive				Seronegative
	all	IgG	IgA	IgM	
GRAS patients	41.1 (12.0)	34.0 (11.7)	42.0 (9.9)	43.4 (13.7)	39.2 (12.6)*
Affective disorder patients	47.4 (11.9)*	45.6 (9.8)	50.6 (9.8)*	41.9 (11.0)	50.2 (16.1)*
GRAS controls	40.9 (12.2)	43.8 (12.6)	39.6 (13.0)	42.2 (13.9)	37.0 (13.3)
Parkinson patients	69.1 (8.3)	52.00 (NA)	68.8 (8.4)	68.1 (8.4)	65.6 (10.3)
Parkinson controls	67.5 (12.6)	NA	70.3 (6.5)	64.7 (18.1)	62.8 (11.6)

* denotes significant difference ($p \leq 0.05$) of patient group versus respective controls. *p* values were calculated using 2-tailed independent samples t-tests, equal variances not assumed in case of significant Levene's test. SD, standard deviation; NA, not applicable.

Figure S1. Age dependence of seropositivity



Dotted lines connect single data points (seroprevalence per year of age), solid lines represent linear trends. Only GRAS patients and GRAS controls were included due to the significantly smaller size of the other cohorts. Patients younger than 23 and older than 53 were not included due to low numbers per year of age.

Table S3. Gender distribution of seropositive subjects by Ig isotype

Ig Isotype	% Seropositive		p (Pearson Chi²)
	Male (N=1769)	Female (N=1048)	
all	11.53%	8.68%	**0.017**
IgG	0.51%	0.86%	0.260
IgA	6.56%	4.77%	0.052
IgM	6.33%	4.68%	0.067

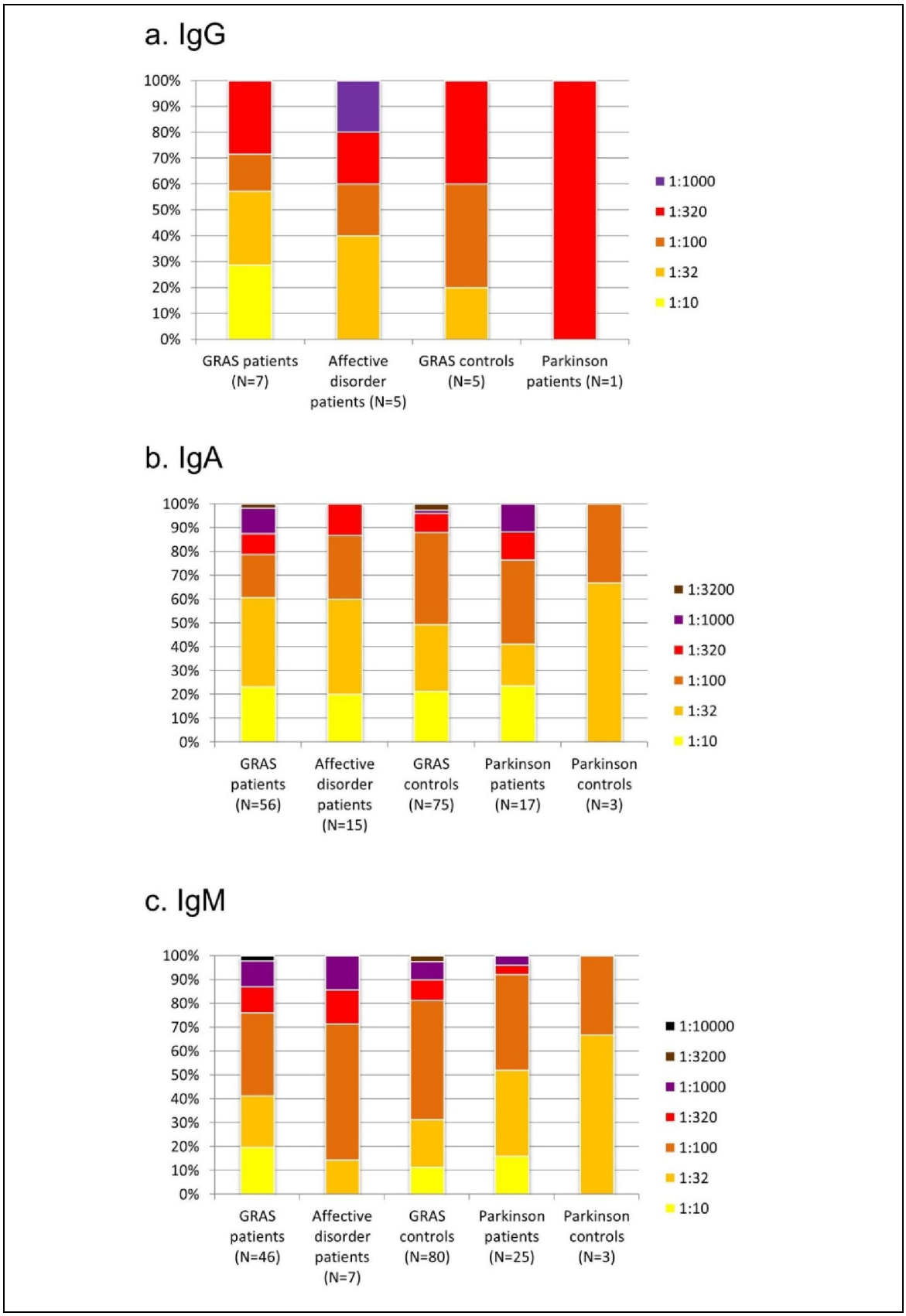
Bolded values, $p<0.05$.

Table S4. Distribution of anti-NR1 AB titers

		Anti-NR1 AB titer (% of seropositive subjects)							
Study cohort	**Isotype**	**1:10**	**1:32**	**1:100**	**1:320**	**1:1000**	**1:3200**	**1:10000**	**all**
GRAS patients	IgG	2 (28.6)	2 (28.6)	1 (14.3)	2 (28.6)	0 (0.0)	0 (0.0)	0 (0.0)	7 (100.0)
	IgA	13 (23.3)	21 (37.5)	10 (17.9)	5 (8.9)	6 (10.7)	1 (1.8)	0 (0.0)	56 (100.0)
	IgM	9 (19.6)	10 (21.7)	16 (34.8)	5 (10.9)	5 (10.9)	0 (0.0)	1 (2.2)	46 (100.0)
Affective disorder patients	IgG	0 (0.0)	2 (40.0)	1 (20.0)	1 (20.0)	1 (20.0)	0 (0.0)	0 (0.0)	5 (100.0)
	IgA	3 (20.0)	6 (40.0)	4 (26.7)	2 (13.3)	0 (0.0)	0 (0.0)	0 (0.0)	15 (100.0)
	IgM	0 (0.0)	1 (14.3)	4 (57.1)	1 (14.3)	1 (14.3)	0 (0.0)	0 (0.0)	7 (100.0)
GRAS controls	IgG	0 (0.0)	1 (20.0)	2 (40.0)	2 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (100.0)
	IgA	16 (21.3)	21 (28.0)	29 (38.7)	6 (8.0)	1 (1.3)	2 (2.7)	0 (0.0)	75 (100.0)
	IgM	9 (11.3)	16 (20.0)	40 (50.0)	7 (8.8)	6 (7.5)	2 (2.5)	0 (0.0)	80 (100.0)
Parkinson patients	IgG	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)
	IgA	4 (23.5)	3 (17.6)	6 (35.3)	2 (11.8)	2 (11.8)	0 (0.0)	0 (0.0)	17 (100.0)
	IgM	4 (16.0)	9 (36.0)	10 (40.0)	1 (4.0)	1 (4.0)	0 (0.0)	0 (0.0)	25 (100.0)
Parkinson controls	IgG	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	IgA	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (100.0)
	IgM	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (100.0)

Mann-Whitney U-rank test revealed no difference between groups regarding titers.

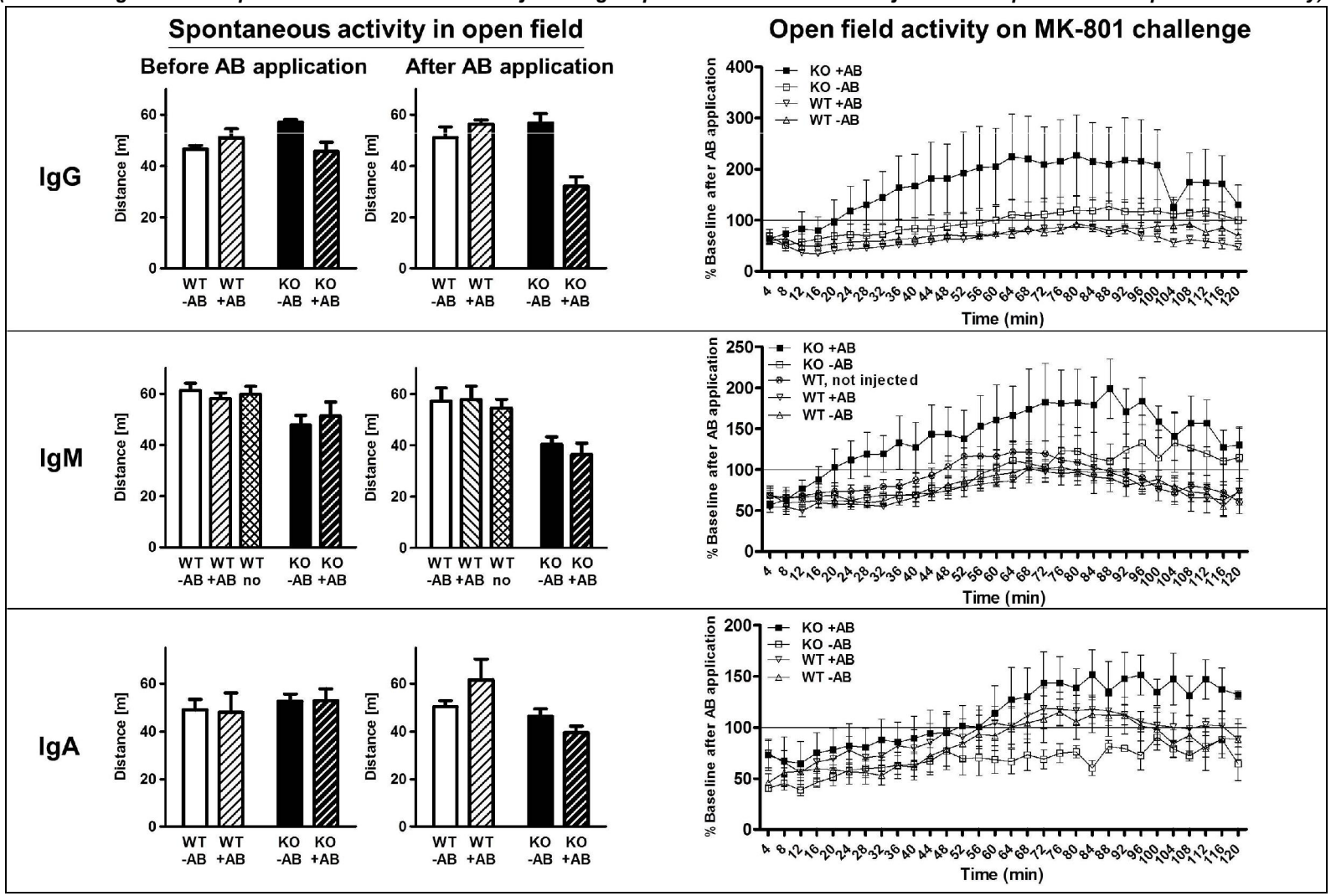
Figure S2. Anti-NR1 AB titers: 100% stacked chart



100% stacked column chart, depicting cumulative distribution of titers, separated by Ig isotype

Figure S3. Mouse AB injection experiments presented separately for isotypes

(Note: The IgM set of experiments includes a non-injection group to exclude effects of injection on spontaneous open field activity)



Left panels: Spontaneous activity in the open field - Total distance traveled before and after Ig injection. Right panels: Open field activity upon MK-801 challenge. m, meter; WT, wildtype; KO, ApoE$^{-/-}$ knockout; -AB, NMDAR-AB seronegative; +AB, NMDAR-AB seropositive; no, non-injection control; N=4-6 per group.

SNP array specifications and genotyping quality control

Genotyping was performed using a semi-custom Axiom® myDesign™ genotyping array (Affymetrix, Santa Clara, CA, USA), based on a CEU (Caucasian residents of European ancestry from Utah, USA) marker backbone including 518.722 SNPs, and a custom marker set including 102.537 SNPs. The array was designed using the Axiom® Design center (www.affymetrix.com), applying diverse selection criteria (Table S5). Genotyping was performed by Affymetrix on a GeneTitan® platform.

Table S5. Marker selection criteria

Marker group	Threshold	# of markers
Axiom CEU backbone	MAF > 0.025, direct selection	518,722
Functional Variants (CDS, UTR, exonic)	MAF > 0.2, direct selection	27,369
Markers contained in RefSeq / UCSC genes (+/- 2kbp)	MAF > 0.2, r^2 > 0.8	31,190
Markers in the MHC genomic region	best tag	15,395
Markers in CpG islands	MAF > 0.2, direct selection	5,511
Markers in known splice sites	MAF > 0.1, direct selection	95
Markers in microRNA regions	MAF > 0.2, direct selection	56
Manual selection of candidate SNPs	MAF > 0.2, best tag	22,921
Affymetrix SNPs for quality control		3,334
TOTAL		**624,593**

MAF, minor allele frequency; CDS, coding sequence; UTR, untranslated region; RefSeq, NCBI reference sequence; UCSC, University of California Santa Cruz Genome Browser; kbp, kilo base pairs; MHC, major histocompatibility complex.

In total, 3128 individuals were subjected to genotyping (Table S6).

Table S6. Patient and healthy control groups selected for genotyping

Study group	N (%)
GRAS patients	1177 (37.6)
Affective disorder patients	173 (5.5)
GRAS controls	1274 (40.7)
Parkinson patients	276 (8.8)
Parkinson controls	182 (5.8)
Autism patients	29 (0.9)
Other conditions	17 (0.5)
TOTAL	**3128 (100)**

Quality control (QC) was performed on sample and SNP level, according to the manufacturer's recommendations (Table S7). A total of 3.086 individuals and 612.121 SNPs passed all quality controls to be included in genetic analyses.

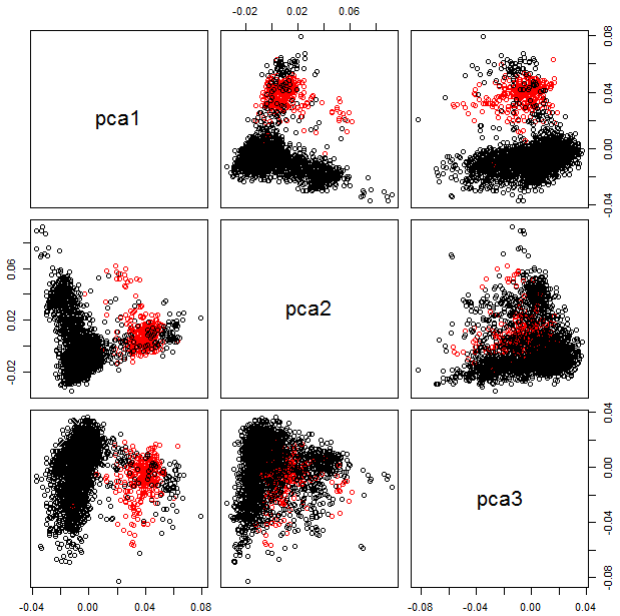
Table S7. Axiom® array quality control

	Filter criteria	Threshold for inclusion	N
Sample level			Samples passing QC step (%)
	Pre QC		3,128 (100)
	Dish QC	≥ 0.82	3,124 (99.8)
	Cluster call rate	≥ 0.97	3,086 (98.7)
SNP level			SNPs passing QC step (%)
	Pre QC		624,593 (100)
	SNP call rate	≥ 0.97	
	Fisher's Linear Discriminant (FLD)	≥ 3.6	
	Heterozygous Cluster Strength Offset (HetSO)	≥ -0.1	
	Homozygote Ratio Offset (HomRO)	≥ 0.3	612,121 (98.0)

Genome-wide association study (GWAS)

GRAS and affective disorder patients, as well as the healthy GRAS controls, were collected in Germany, whereas the Parkinson samples and controls were collected in Italy. An analysis of principal components (PCA) was performed, showing a clear clustering of samples by descent (Figure S4). Therefore, to reduce population stratification bias, GWAS was performed including only GRAS and affective disorder patients, as well as GRAS controls.

Figure S4. Principal component analysis



Graphical presentation including all individuals showing the first 3 principal components. Samples collected in Germany are depicted in black, samples collected in Italy in red.

For the GWAS, PLINK v1.07 was used to calculate association statistics, applying a basic allelic model, and using the following filter criteria for SNP and sample exclusion:

- Markers located on sex chromosomes
- Hardy-Weinberg $p < 5x10^{-7}$ in controls
- Minor allele frequency < 0.01
- missingness per marker > 0.05
- missingness per individual > 0.02

Overall, a total of **580.297** SNPs remained for analysis. The final dataset consisted of **245 cases** (anti-NR1 positive) and **2152 controls** (anti-NR1 negative).

A Q-Q plot was generated to visualize possible confounders (Figure S5). The genomic inflation factor was **λ=1.01** (Devlin B, Roeder K. Genomic control for association studies. Biometrics 1999;55:997-1004). This low genomic inflation value justifies not including principal components as covariates after removal of the Italian Parkinson patient and control samples.

Figure S5. Q-Q plot

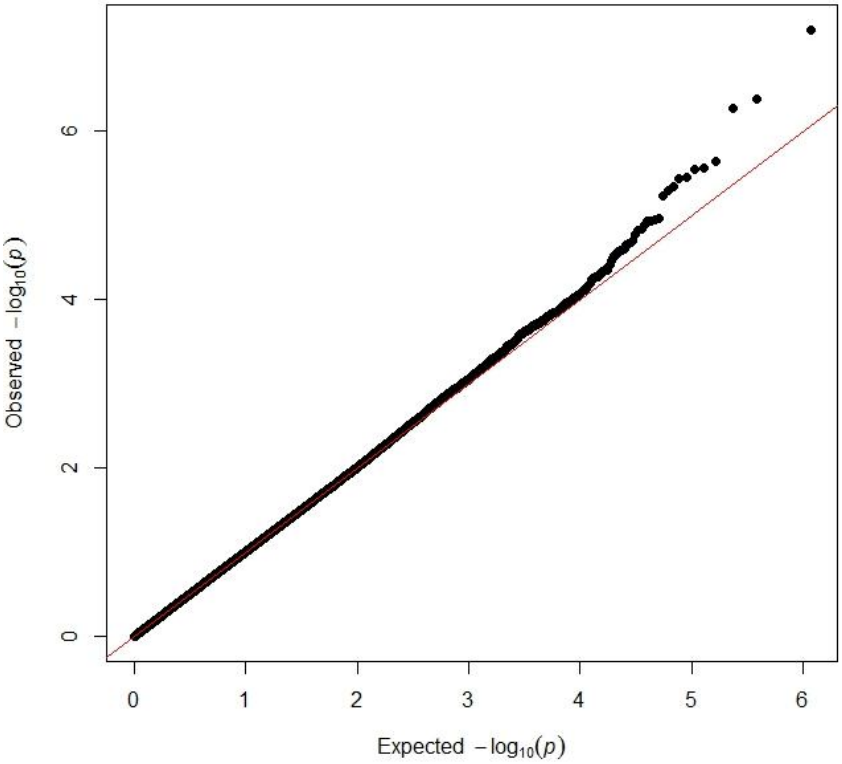
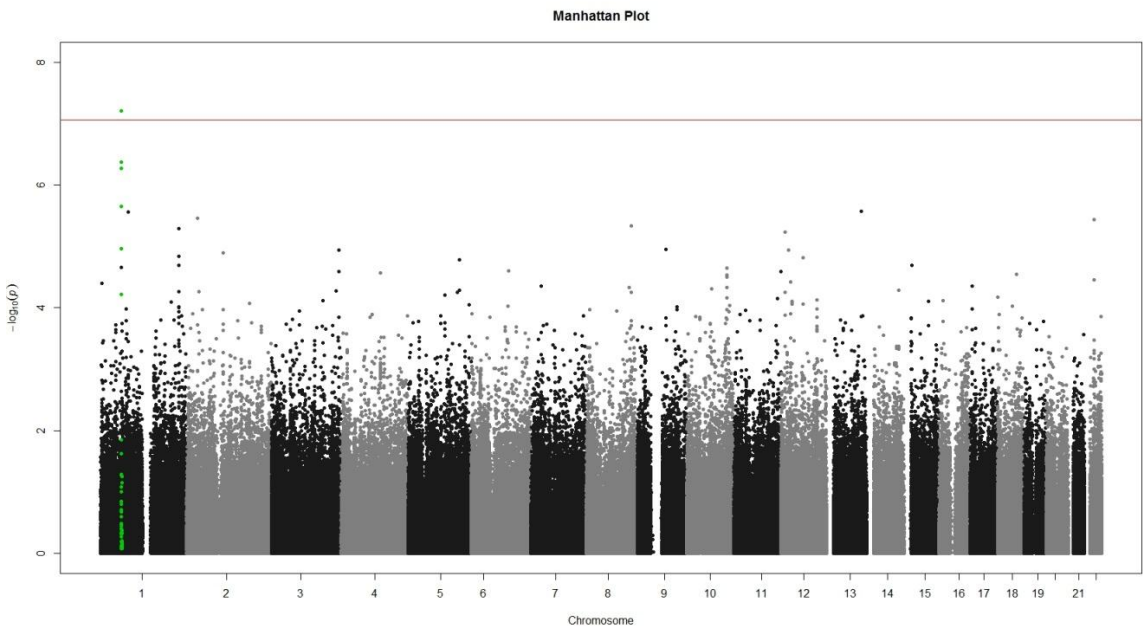
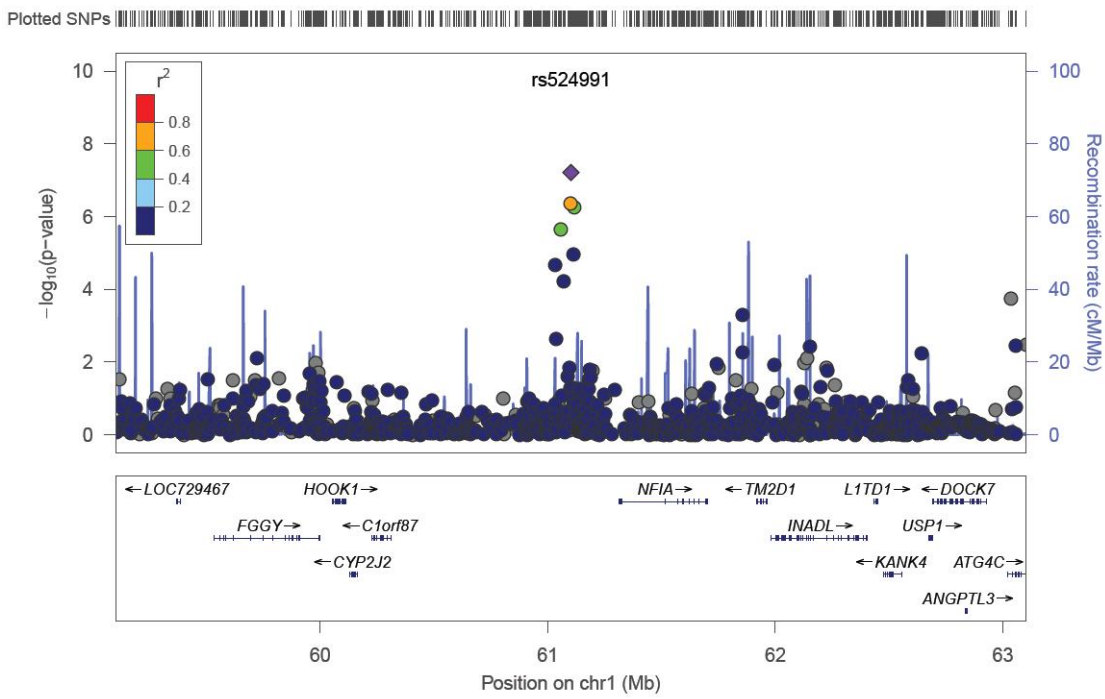


Figure S6. Manhattan plot



Manhattan plot of single SNP test statistics. The genomic position is shown on the X axis, and the -$\log_{10}$(p) on the Y axis. The horizontal line denotes the threshold for significance after Bonferroni correction for 580.297 tests (*p*=8.62E-08). A marker on chromosome 1 (rs524991) showing genome-wide significance, as well as surrounding SNPs (+/- 50kbp) are depicted in green.

Figure S7. SNP rs524991 regional association plot



Regional association plot showing 2000kbp 5' and 3' of rs524991 (*p*=8.62E-08). The positions of neighboring genes are shown on the X axis, and the -$\log_{10}$(p) on the Y axis. Color coding denotes LD information (see legend within the plot).

Table S8. SNP rs524991 association statistics by study group

Study group	Anti-NR1	N	Genotypes (frequencies)			*p* (trend test) d.f.=1	*p* (OR, [95% CI]) allelic, d.f.=1
Both genders			GG (%)	GA (%)	AA (%)		
GRAS patients	+	92	65 (70.7)	27 (29.3)	0 (0.0)	**2.92E-06**	**3.23E-06 (2.80, [1.78 - 4.39])**
	-	976	867 (88.8)	105 (10.8)	4 (0.4)		
Affective disorder patients	+	24	18 (75.0)	6 (25.0)	0 (0.0)	0.118	0.133 (2.12, [0.78 - 5.79])
	-	119	104 (87.4)	15 (12.6)	0 (0.0)		
GRAS controls	+	135	111 (82.2)	19 (14.1)	5 (3.7)	**0.007**	**0.006 (1.78, [1.17 - 2.72]**
	-	1115	977 (87.6)	135 (12.1)	3 (0.3)		
Parkinson patients	+	35	31 (88.6)	4 (11.4)	0 (0.0)	0.547	0.551 (0.72, [0.25 - 2.11])
	-	220	187 (85.0)	32 (14.5)	1 (4.6)		
Parkinson controls	+	6	4 (66.7)	2 (33.3)	0 (0.0)	0.081	0.091 (4.30, [0.70 - 26.52])
	-	45	41 (91.1)	4 (8.9)	0 (0.0)		
TOTAL	+	292	229 (78.4)	58 (19.9)	5 (1.7)	**7.64E-07**	**7.48E-07 (1.99, [1.51 - 2.63])**
	-	2475	2176 (87.9)	291 (11.8)	8 (0.3)		
Males only			GG (%)	GA (%)	AA (%)		
GRAS patients	+	67	51 (76.1)	16 (23.9)	0 (0.0)	**0.004**	**0.004 (2.28, [1.28 - 4.04])**
	-	649	579 (89.2)	67 (10.3)	3 (0.5)		
Affective disorder patients	+	11	9 (81.8)	2 (18.2)	0 (0.0)	0.251	0.262 (2.65, [0.45 - 15.45])
	-	55	51 (92.7)	4 (7.2)	0 (0.0)		
GRAS controls	+	92	76 (82.6)	13 (14.1)	3 (3.3)	0.064	0.063 (1.64, [0.97 - 2.76])
	-	646	562 (87.0)	83 (12.8)	1 (0.2)		
Parkinson patients	+	28	25 (89.3)	3 (10.7)	0 (0.0)	0.465	0.465 (0.63, [0.18 - 2.18])
	-	140	118 (84.3)	21 (15.0)	1 (0.7)		
Parkinson controls	+	2	1 (50.0)	1 (50.0)	0 (0.0)	**0.04**	**0.046 (12.3, [0.61 - 250.5])**
	-	19	18 (94.7)	1 (5.3)	0 (0.0)		
TOTAL	+	200	162 (81.0)	35 (17.5)	3 (1.5)	**0.002**	**2.04E-03 (1.74, [1.22 - 2.48])**
	-	1509	1328 (88.0)	176 (11.7)	5 (3.3)		
Females only			GG (%)	GA (%)	AA (%)		
GRAS patients	+	25	14 (56.0)	11 (44.0)	0 (0.0)	**2.11E-05**	**2.97E-05 (4.33, [2.06 - 9.09])**
	-	327	288 (88.1)	38 (11.6)	1 (0.3)		
Affective disorder patients	+	13	9 (69.2)	4 (30.8)	0 (0.0)	0.272	0.300 (1.90, [0.55 - 6.52])
	-	63	52 (82.5)	11 (17.5)	0 (0.0)		
GRAS controls	+	39	31 (79.5)	6 (15.4)	2 (5.1)	**0.024**	**0.022 (2.28, [1.11 - 4.69])**
	-	421	371 (88.1)	49 (11.6)	1 (0.2)		
Parkinson patients	+	7	6 (85.7)	1 (14.3)	0 (0.0)	0.969	0.970 (1.04, [0.12 - 8.82])
	-	80	69 (86.3)	11 (13.7)	0 (0.0)		
Parkinson controls	+	4	3 (75.0)	1 (25.0)	0 (0.0)	0.461	0.477 (2.33, [0.21 - 25.66])
	-	26	23 (88.5)	3 (11.5)	0 (0.0)		
TOTAL	+	88	63 (71.6)	23 (26.1)	2 (2.3)	**7.42E-06**	**8.81E-06 (2.68, [1.71 - 4.21])**
	-	917	803 (87.6)	112 (12.2)	2 (0.2)		

Bolded values, $p<0.05$; d.f., degrees of freedom; OR, odds ratio; CI, confidence intervals; -, seronegative; +, seropositive.

Table S9. HLA type association with seropositivity

HLA allele	Anti-NR1 seropositivity (%)		*p* (Pearson's chi²)	OR	CI 95%		Imputation
	- (N=2483)	+ (N=294)			low	high	quality (mean)
HLA-A							
1	14.0%	12.6%	0.343	0.88	0.68	1.14	0.954
2	29.7%	28.7%	0.615	0.95	0.79	1.15	0.937
3	15.6%	19.7%	**0.010**	*1.33*	*1.07*	*1.65*	0.944
11	5.7%	4.8%	0.370	0.83	0.56	1.24	0.943
24	9.6%	10.0%	0.727	1.05	0.79	1.40	0.907
HLA-B							
7	13.3%	13.1%	0.874	0.98	0.76	1.26	0.833
8	9.8%	8.5%	0.305	0.85	0.63	1.16	0.940
15	8.4%	8.5%	0.917	1.02	0.75	1.38	0.616
18	5.5%	4.9%	0.554	0.89	0.60	1.32	0.570
35	10.6%	12.4%	0.168	1.20	0.93	1.56	0.476
40	6.4%	7.1%	0.467	1.13	0.81	1.58	0.554
44	11.9%	9.9%	0.150	0.81	0.61	1.08	0.869
51	6.2%	5.4%	0.490	0.88	0.60	1.27	0.752
HLA-C							
2	5.2%	5.3%	0.937	1.02	0.69	1.49	0.973
3	13.2%	13.1%	0.927	0.99	0.77	1.27	0.945
4	12.8%	15.3%	0.095	1.23	0.96	1.56	0.967
5	7.1%	7.0%	0.932	0.99	0.70	1.38	0.967
6	10.2%	11.2%	0.463	1.11	0.84	1.45	0.963
7	31.0%	30.4%	0.793	0.98	0.81	1.17	0.957
12	7.1%	6.8%	0.798	0.96	0.68	1.34	0.947
HLA-DPB1							
1	5.2%	4.8%	0.624	0.91	0.61	1.35	0.885
2	13.9%	14.3%	0.785	1.03	0.81	1.32	0.826
3	9.7%	9.2%	0.696	0.94	0.70	1.27	0.713
4	56.5%	58.0%	0.497	1.06	0.89	1.26	0.859
HLA-DQA1							
1	41.8%	43.9%	0.331	1.09	0.92	1.29	0.892
2	12.5%	11.6%	0.531	0.92	0.70	1.20	0.941
3	14.3%	14.6%	0.819	1.03	0.81	1.31	0.823
5	28.3%	27.7%	0.771	0.97	0.80	1.18	0.926
HLA-DQB1							
2	19.8%	17.9%	0.273	0.88	0.71	1.10	0.947
3	35.5%	36.4%	0.669	1.04	0.87	1.24	0.927
5	17.4%	17.3%	0.985	1.00	0.80	1.25	0.937
6	24.4%	26.5%	0.254	1.12	0.92	1.36	0.882
HLA-DRB1							
1	10.5%	11.4%	0.511	1.09	0.84	1.43	0.838
3	11.0%	9.5%	0.266	0.85	0.64	1.13	0.886
4	13.7%	13.6%	0.932	0.99	0.77	1.27	0.727
7	12.4%	11.6%	0.540	0.92	0.70	1.20	0.889
11	14.1%	14.1%	0.968	1.00	0.79	1.28	0.603
13	12.0%	14.5%	0.092	1.23	0.97	1.58	0.822
15	14.0%	13.9%	0.995	1.00	0.78	1.28	0.892

HLA types were imputed at 4-digit resolution for all genotyped subjects. However, for statistical power issues, statistics were performed at 2-digit resolution. A minimal allele frequency of 5% in seronegative or seropositive subjects was set as inclusion criterion for statistical analysis. Bolded values, $p<0.05$; -, seronegative; +, seropositive; OR, odds ratio; CI, confidence intervals.

Table S10. Influenza association with anti-NR1 seropositivity, gender-specific

Study cohort		GRAS patients		Affective disorder patients		GRAS controls		Parkinson patients		Parkinson controls		TOTAL	
anti-NR1 seropositivity		+	-	+	-	+	-	+	-	+	-	+	-
Influenza A (males)	N seropositive (%)	55 (80.9)	427 (65.7)	8 (72.7)	45 (76.3)	68 (71.6)	454 (66.4)	19 (67.9)	85 (57.8)	2 (100.0)	11 (57.9)	152 (74.5)	1022 (65.6)
	p (Pearson's chi²)	**0.011**		0.461		0.312		0.322		0.243		**0.011**	
	OR [CI 95%]	2.210 [1.182 - 4.131]		0.830 [0.193 - 3.559]		1.276 [0.795 - 2.048]		1.540 [0.653 - 3.632]		NA		1.536 [1.102 - 2.140]	
Influenza B (males)	N seropositive (%)	26 (38.2)	135 (20.8)	3 (27.3)	19 (32.2)	28 (29.5)	146 (21.3)	11 (39.3)	54 (36.7)	0 (0)	4 (21.1)	68 (33.3)	358 (23.0)
	p (Pearson's chi²)	**0.001**		0.746		0.075		0.798		0.471		**0.001**	
	OR [CI 95%]	2.362 [1.398 - 3.990]		0.789 [0.188 - 3.315]		1.540 [0.955 - 2.482]		1.114 [0.486 - 2.554]		NA		1.677 [1.225 - 2.296]	
Influenza A (females)	N seropositive (%)	16 (66.7)	221 (67.2)	8 (61.5)	45 (71.4)	29 (69.0)	310 (68.9)	6 (85.7)	56 (69.1)	4 (100.0)	17 (60.7)	63 (70.0)	649 (68.2)
	p (Pearson's chi²)	0.959		0.48		0.983		0.356		0.122		0.732	
	OR [CI 95%]	0.977 [0.406 - 2.355]		0.640 [0.184 - 2.220]		1.007 [0.508 - 1.996]		2.679 [0.306 - 23.433]		NA		1.086 [0.678 - 1.739]	
Influenza B (females)	N seropositive (%)	4 (16.7)	67 (20.4)	2 (15.4)	13 (20.6)	9 (21.4)	75 (16.7)	1 (14.3)	25 (30.9)	0 (0)	4 (14.3)	16 (17.8)	184 (19.3)
	p (Pearson's chi²)	0.663		0.665		0.433		0.356		0.419		0.718	
	OR [CI 95%]	0.782 [0.259 - 2.365]		0.699 [0.138 - 3.553]		1.364 [0.627 - 2.968]		0.373 [0.043 - 3.266]		NA		0.901 [0.513 - 1.584]	

Bolded values, $p<0.05$; +, anti-NR1 seropositive; -, anti-NR1 seronegative; OR, odds ratio; CI, confidence intervals

5.3. Overview of project IV

We previously reported ~10% of *N*-methyl-D-aspartate (NMDA) receptor serum antibodies in patients with brain diseases (schizophrenia, affective disorders, Parkinson) as well as healthy controls with no differences in Ig class distribution, titers or functionality (Hammer et al., 2014). The aim of project IV was to screen an even larger number of individuals with brain diseases (schizophrenia, affective disorders, ischemic stroke, Parkinson disease, amyotrophic lateral sclerosis, borderline personality disorder) and healthy controls for presence of NMDA receptor as well as 24 other antibodies directed against brain antigens. Most of the additionally screened antigens had previously been associated with inflammatory brain disease (Dalmau et al., 1999; Dalmau et al., 2007; De Camilli et al., 1993; Irani et al., 2010a; Jarius et al., 2010; Kaufman et al., 1992; Peterson et al., 1992; Voltz et al., 1999; Xiao et al., 1991).

The final sample consisted of 1703 healthy controls and 1152 patients with the diagnosis of schizophrenia or schizoaffective disorder from the GRAS dataset (Begemann et al., 2010; Ribbe et al., 2010) as well as 211 patients with affective disorders recruited as an extension of GRAS to other disease groups and 74 GRAS patients with 'mental disease not yet classified'. 367 patients (schizophrenia, affective disorders, borderline personality disorder) were recruited at the University of Magdeburg (Department of Psychiatry) and 258 Parkinson patients in Rome (Italy). Finally 442 ischemic stroke patients and 29 patients with amyotrophic lateral sclerosis, enrolled in Göttingen and Hannover, participated in the study. The serum samples of all 4236 participants were tested for antibodies against the NMDA receptor NR1 subunit, amphiphysin, ARHGAP26, CASPR2, MOG, GAD65, Ma2, Yo, Ma1, AMPA receptor GluR1 subunit, AMPA receptor GluR2 subunit, AQP4, CV2, Tr/DNER, DPPX-IF1, GABA type B receptor subunit 1, GABA type B receptor subunit 2, GAD67, GLRA1b, GRM1, GRM5, Hu, LGI1, recoverin, Ri, and ZIC4.

Antibodies against the NMDA receptor were the most frequent type across all studied groups (disease and healthy) with a prevalence of ~10%, followed by amphiphysin (2.0%), ARHGAP26 (1.3%), CASPR2 (0.9%), MOG (0.8%), GAD65 (0.5%), Ma2 (0.5%), Yo (0.4%), and Ma1 (0.4%). All other antigens were even less frequent making final conclusions about their

association with brain disease impossible. For the 9 most common antigens we did not observe any differences regarding prevalence, Ig class or titers between disease groups and healthy controls. One striking observation was that the antibodies directed against extracellular antigens (NMDA receptor, CASPR2, MOG) were rarely of the IgG type, while the antibodies directed against intracellular antigens (amphiphysin, ARHGAP26, GAD65, Ma2, Yo, Ma1) were mostly IgG (p=2.8x10^{-48}). This finding indicates that antigen location might be a determinator of immunoglobulin class. To conclude, we were able to replicate our previous findings of ~10% prevalence of NMDA receptor antibodies in an even larger sample and additionally report the prevalence, Ig class, and titers for 24 other brain antigens. Our results do not suggest a per se pathological role of serum antibodies directed against brain antigens.

5.4. Original publication

Dahm L.*, Ott C.*, Steiner J.*, **Stepniak B.**, Teegen B., Saschenbrecker S., Hammer C., Borowski K., Begemann M., Lemke S., Rentzsch K., Probst C., Martens H., Wienands J., Spalletta G., Weissenborn K., Stocker W., Ehrenreich H. (2014) Seroprevalence of autoantibodies against brain antigens in health and disease. Ann Neurol **76**:82-94.
**Equally contributing authors*

Personal contribution

I was responsible for the establishment of an extensive database with information on all human study groups (disease and healthy) and their autoantibody seroprevalence. Besides the data management I conducted the statistical analysis and provided the data presented in Table 2 and 3. Furthermore I conducted the analysis and prepared Figure 1A and B and wrote the respective figure legend. I wrote the 'Participants' and 'Statistical Analysis' section for the methods part of the manuscript. Finally I contributed to the interpretation of the results for the discussion section of the paper and was involved in the proof reading of the manuscript before publication.

RESEARCH ARTICLE

Seroprevalence of Autoantibodies against Brain Antigens in Health and Disease

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Objective: We previously reported an unexpectedly high seroprevalence (~10%) of N-methyl-D-aspartate-receptor subunit-NR1 (NMDAR1) autoantibodies (AB) in healthy and neuropsychiatrically ill subjects (N = 2,817). This finding challenges an unambiguous causal relationship of serum AB with brain disease. To test whether similar results would be obtained for other brain antigen-directed AB previously connected with pathological conditions, we systematically screened serum samples of 4,236 individuals.
Methods: Serum samples of healthy (n = 1,703) versus neuropsychiatrically ill subjects (schizophrenia, affective disorders, stroke, Parkinson disease, amyotrophic lateral sclerosis, personality disorder; total n = 2,533) were tested. For analysis based on indirect immunofluorescence, we used biochip mosaics of frozen brain sections (rat, monkey) and transfected HEK293 cells expressing respective recombinant target antigens.
Results: Seroprevalence of all screened AB was comparable in healthy and ill individuals. None of them, however, reached the abundance of NMDAR1 AB (again ~10%; immunoglobulin [Ig] G ~1%). Appreciable frequency was noted for AB against amphiphysin (2.0%), ARHGAP26 (1.3%), CASPR2 (0.9%), MOG (0.8%), GAD65 (0.5%), Ma2 (0.5%), Yo (0.4%), and Ma1 (0.4%), with titers and Ig class distribution similar among groups. All other AB were found in ≤0.1% of individuals (anti–AMPAR-1/2, AQP4, CV2, Tr/DNER, DPPX-IF1, GABAR-B1/B2, GAD67, GLRA1b, GRM1, GRM5, Hu, LGI1, recoverin, Ri, ZIC4). The predominant Ig class depended on antigen location, with intracellular epitopes predisposing to IgG (chi-square = 218.91, $p = 2.8 \times 10^{-48}$).
Interpretation: To conclude, the brain antigen-directed AB tested here are comparably detectable in healthy subjects and the disease groups studied here, thus questioning an upfront pathological role of these serum AB.

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The occurrence in serum of autoantibodies (AB) directed against brain antigens has long been recognized in classical autoimmune diseases and in paraneoplastic syndromes (for review, see eg Diamond et al,[1] Sutton and Winer[2]). Recent work reports the presence of AB directed against brain epitopes in serum of ~90% of individuals, independent of any illness.[3] In this pivotal work, however, no antigen specificity of the brain-targeting AB has been assessed. Some authors even propose the term *immunculus* for a normal network of constitutively expressed natural AB interacting with different extracellular, membrane, cytoplasmic, and nuclear self-

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antigens.[4,5] Serum diversity of these antibodies is strongly influenced by age, individually remarkably stable over time, and interestingly also conserved among mammals.[6]

Originally stimulated by literature describing an association of N-methyl-D-aspartate-receptor subunit-NR1 (NMDAR1) AB with a multifaceted neuropsychiatric syndrome,[7–9] we determined the seroprevalence of NMDAR1 AB in 1,325 healthy and 1,492 neuropsychiatrically ill subjects, among them 1,081 schizophrenic, 263 Parkinson disease, and 148 affective disorder patients. We wondered whether, in some of the ill individuals, NMDAR1 AB might have accounted for or worsened their symptoms. Surprisingly, however, we found a comparable seroprevalence of ~10% NMDAR1 AB in all investigated groups, with similar distribution of titers and immunoglobulin (Ig) isotypes (IgM and IgA being most frequent and IgG amounting overall to only ~1%) and indistinguishable AB functionality in healthy and ill subjects.[10] Another study on mentally ill versus healthy individuals ultimately came to equal conclusions.[11,12]

At first assuming a potential pathological role of circulating NMDAR1 AB, the most obvious question that arose for us from this unexpected discovery was to ask why healthy individuals have remained healthy. In a series of mouse experiments, we showed that the integrity of the blood–brain barrier (BBB) determines whether brain epitope–specific serum AB can exert any measurable symptoms.[10] This result is perfectly in line with the hypothesis of Diamond and coworkers[1] that under conditions of BBB compromise, AB can alter brain function in otherwise healthy individuals. Closing the circle to the above-cited work of Levin and colleagues,[3] these authors propose that the normal immunocompetent B-cell repertoire is full of B-cells making AB that recognize brain antigens.[1] The relevance of these AB is not understood yet but likely goes far beyond clear-cut pathology.

The present study has been designed to systematically screen for the first time serum samples of a large number of healthy individuals (n = 1,703) versus subjects with neuropsychiatric diseases (schizophrenia, affective disorders, ischemic stroke, Parkinson disease, amyotrophic lateral sclerosis [ALS], borderline personality disorder; total n = 2,533) for the presence of a panel of 25 distinct AB directed against defined brain antigens.[13–22] Most of these AB have been associated in the past with some kind of inflammatory brain disease (for more detailed description, see Table 1). Specifically, we aimed (1) to replicate and extend our recent work on NMDAR1 AB to >1,400 more individuals, and using this extended data set as a comparator matrix, (2) to evaluate frequency, disease group distribution, Ig classes, and titers of 24 other distinct brain-specific AB.

Subjects and Methods

Participants

Subject data were collected in accordance with ethical guidelines and the Helsinki Declaration. Sample selection was unbiased, that is, sera collection in >90% of individuals was concluded before analysis of AB was planned. Schizophrenic and schizoaffective patients (fulfilling Diagnostic and Statistical Manual of Mental Disorders, 4th edition criteria) were recruited during 2005–2011 at 23 German sites (n = 1,152) in the Göttingen Research Association for Schizophrenia (GRAS) study[23,24] as well as independently at the Department of Psychiatry, University of Magdeburg (n = 226). In a total of n = 74 patients initially suspected to have schizophrenia, the diagnosis had to be revised to "mental disease not yet classified" (referred to as "others"). Healthy GRAS controls were anonymized blood donors (n = 1,265; Transfusion Medicine, Göttingen).[24] As such, they widely fulfill health criteria, ensured by a broad predonation screening process containing standardized questionnaires, interviews, hemoglobin and other blood parameters, blood pressure, pulse, and body temperature determinations. Similarly, Magdeburg controls (n = 357) included blood donors, students, and hospital personnel, undergoing regular health screening. Patients with affective disorders were recruited as part of an ongoing GRAS extension to other disease groups (n = 211) or at Magdeburg University (n = 99). Parkinson disease patients (n = 258) and respective controls (n = 81) were recruited during 2010–2011 in Italy (Rome area).[10] Patients with ischemic stroke (n = 442) and patients with ALS (n = 29) were enrolled in Göttingen and Hannover. Patients with borderline personality disorder (n = 42) were from Magdeburg University.

Serological Analyses

Serum samples of all 4,236 participants were tested for the presence of NMDAR1 AB (independent of whether they had had previous determinations of NMDAR1 AB[10]) and a large panel of further defined antineural AB (see Table 1 for description) as follows: Biochip mosaics (Euroimmun, Lübeck, Germany) contained nonfixed nitrogen-frozen tissue cryosections (4 μm; rat hippocampus, monkey cerebellum) and recombinant cell substrates (formalin- or acetone-fixed recombinant HEK293 cells), each expressing a different neural antigen (NMDAR1, amphiphysin, ARHGAP26, CASPR2, MOG, GAD65, Ma2, Yo, Ma1, AMPAR-1/2, AQP4, CV2, Tr/DNER, DPPX-IF1, GABAR-B1/B2, GAD67, GLRA1b, GRM1, GRM5, Hu, LGl1, recoverin, Ri, ZIC4). Expression of the individual recombinant protein (autoantigen) was validated by immunological methods employing human or commercially available monospecific animal antibodies.

Biochip mosaics were incubated each with 70 μl of phosphate-buffered saline (PBS)-diluted serum, starting at 1:10, for 30 minutes at room temperature, washed with PBS-Tween, and immersed in PBS-Tween for 5 minutes. Bound antibodies were stained with fluorescein isothiocyanate–labeled goat anti-human IgA, IgG, and IgM (Euroimmun) for 30 minutes at

TABLE 1. Brief Sketch of the 9 Defined Brain Antigens Screened in the Present Study with the Most Abundant Autoantibody Seroprevalence

Antigen	Full Name	Location	Function	Association	First Description of Autoimmune Association
NMDAR NR1	N-methyl-D-aspartate receptor subunit NR1	Extracellular/synaptic	Transmembrane protein, ionotropic neurotransmitter receptor subunit, ligand-gated ion channel, mediates excitatory glutamatergic synaptic transmission, involved in synaptic plasticity	Anti-NMDAR encephalitis	Dalmau 2007,[13] *Annals of Neurology*
Amphiphysin	Amphiphysin	Intracellular/synaptic	Intracellular presynaptic vesicular protein, dynamin-binding partner, involved in clathrin-mediated synaptic vesicle endocytosis	Stiff-person syndrome, encephalomyelitis, limbic encephalomyelitis, sensory/sensorimotor neuropathy	De Camilli 1993,[14] *Journal of Experimental Medicine*
ARHGAP26, GRAF	Rho GTPase activating protein 26, GTPase regulator associated with focal adhesion kinase, oligophrenin-1–like protein	Intracellular/somata and neuropil	Intracellular protein, binds to focal adhesion kinase and mediates the activity of the GTP binding proteins RhoA and Cdc42, involved in clathrin-independent endocytosis	Subacute inflammatory cerebellar ataxia	Jarius 2010,[15] *Journal of Neuroinflammation*
CASPR2	Contactin-associated protein 2	Extracellular/neuropil	Transmembrane protein, involved in cell adhesion, mediates juxtaparanodal Kv1 channel clustering on myelinated axons	Morvan syndrome, neuromyotonia, limbic encephalitis	Irani 2010,[16] *Brain*
MOG	Myelin oligodendrocyte glycoprotein	Extracellular/outer myelin sheet	Transmembrane protein, cell adhesion molecule, involved in maintenance of the structural integrity of myelin, only expressed by myelinating oligodendrocytes	Acute disseminated encephalomyelitis, multiple sclerosis, clinically isolated syndrome, neuromyelitis optica and spectrum disorders	Xiao 1991,[17] *Journal of Neuroimmunology*

TABLE 1. Continued

Antigen	Full Name	Location	Function	Association	First Description of Autoimmune Association
GAD65	Glutamate decarboxylase 65	Intracellular/synaptic	Intracellular and presynaptic protein, catalyzes the decarboxylation of glutamate to the inhibitory neurotransmitter GABA	Stiff-person syndrome, cerebellar syndrome, limbic encephalitis, epilepsy	Kaufman 1992,[18] *Journal of Clinical Investigation*
Ma2	Ma2/PNMA2	Intracellular/nuclei	Function still unknown, expressed in neuronal nuclei	Limbic encephalitis, brainstem encephalitis, cerebellar syndrome, polyneuropathy	Voltz 1999,[19] *New England Journal of Medicine*
Yo	CDR62/CDR2, CDR34/CDR1	Intracellular/cytoplasm	Nuclear DNA binding protein, acts possibly as transcription factor	Brainstem encephalitis, cerebellar syndrome	Peterson 1992,[20] *Neurology*
Ma1	Ma1/PNMA1	Intracellular/nuclei	Acts possibly as proapoptotic protein in neurons	Limbic encephalitis, brainstem encephalitis, cerebellar syndrome, polyneuropathy	Dalmau 1999,[21] *Brain*

Information partly derived from Probst et al.[22]
GABA = γ-aminobutyric acid; GTP = guanosine triphosphate.

TABLE 2. Overview of 9 Defined Brain Antigens Leading to the Most Abundant Autoantibody Seroprevalence in Healthy and Neuropsychiatrically Ill Individuals

	Study Group									
	Schizophrenia	**Affective disorders**	**Parkinson**	**Stroke**	**ALS**	**Borderline**	**Others[b]**	**Disease Group Total**	**Healthy Controls**	**Total**
Male, %	**65.1**	**48.1**	**66.3**	**54.8**	**58.6**	**33.3**	**60.8**	**60.6**	**57.9**	**59.5**
Age, yr ± SD	**39.0 ± 12.6**	**45.9 ± 15.6**	**65.9 ± 10.1**	**68.3 ± 12.5**	**60.3 ± 10.2**	**27.0 ± 8.5**	**39.2 ± 15.5**	**47.7 ± 18.0**	**37.7 ± 13.5**	**43.7 ± 17.1**
No.	**1,378**	**310**	**228–258[c]**	**442**	**29**	**42**	**74**	**2,503–2,533[c]**	**1,693–1,703[c]**	**4,196–4,236[c]**
Antigen[a]										
NMDAR NR1										
Total No.	1,378	310	258	442	29	42	74	2,533	1,703	4,236
Seropositives, No. (%)	129 (9.4)	33 (10.6)	21 (8.1)	85 (19.2)	5 (17.2)	1 (2.4)	4 (5.4)	278 (11.0)	145 (8.5)	423 (10.0)
Seropositive, No. male	81	15	15	47	4	1	3	166	90	256
IgM/IgA/IgG, No.	69/73/8	12/19/6	20/16/12	56/39/6	5/2/0	0/1/0	0/2/2	162/152/34	74/76/20	236/228/54
Titer range	1:10–1:320	1:10–1:320	1:10–1:1,000	1:10–1:1,000	1:10–1:320	1:32	1:10–1:32	1:10–1:1,000	1:10–1:320	1:10–1:1,000
Amphiphysin										
Total No.	1,378	310	228	442	29	42	74	2,503	1,694	4,197
Seropositives, No. (%)	28 (2.0)	8 (2.6)	1 (0.4)	10 (2.3)	0 (0.0)	0 (0.0)	1 (1.4)	48 (1.9)	38 (2.2)	86 (2.0)
Seropositive, No. male	21	4	1	6	0	0	1	33	22	55
IgM/IgA/IgG, No.	1/6/24	3/4/7	0/1/0	0/6/6	0/0/0	0/0/0	0/1/0	4/18/37	4/13/28	8/31/65
Titer range	1:10–1:320	1:10–1:100	1:32	1:10–1:100	—	—	1:32	1:10–1:320	1:10–1:320	1:10–1:320
ARHGAP26										
Total No.	1,378	310	228	442	29	42	74	2,503	1,694	4,197
Seropositives, No. (%)	14 (1.0)	7 (2.3)	0 (0.0)	10 (2.3)	0 (0.0)	1 (2.4)	0 (0.0)	32 (1.3)	23 (1.4)	55 (1.3)
Seropositive, No. male	12	5	0	8	0	1	0	26	17	43
IgM/IgA/IgG, No.	0/4/14	0/1/6	0/0/0	0/2/9	0/0/0	0/1/0	0/0/0	0/8/29	0/9/18	0/17/47
Titer range	1:10–1:320	1:10–1:320	—	1:10–1:100	—	1:320	—	1:10–1:320	1:10–1:100	1:10–1:320

TABLE 2. Continued

	Study Group									
	Schizophrenia	Affective disorders	Parkinson	Stroke	ALS	Borderline	Others[b]	Disease Group Total	Healthy Controls	Total
Male, %	**65.1**	**48.1**	**66.3**	**54.8**	**58.6**	**33.3**	**60.8**	**60.6**	**57.9**	**59.5**
Age, yr ± SD	**39.0 ± 12.6**	**45.9 ± 15.6**	**65.9 ± 10.1**	**68.3 ± 12.5**	**60.3 ± 10.2**	**27.0 ± 8.5**	**39.2 ± 15.5**	**47.7 ± 18.0**	**37.7 ± 13.5**	**43.7 ± 17.1**
No.	**1,378**	**310**	**228–258[c]**	**442**	**29**	**42**	**74**	**2,503–2,533[c]**	**1,693–1,703[c]**	**4,196–4,236[c]**
Antigen[a]										
CASPR2										
Total No.	1,378	310	258	442	29	42	74	2,533	1,703	4,236
Seropositives, No. (%)	21 (1.5)	2 (0.6)	0 (0.0)	2 (0.5)	0 (0.0)	0 (0.0)	1 (1.4)	26 (1.0)	12 (0.7)	38 (0.9)
Seropositive, No. male	16	1	0	0	0	0	0	17	4	21
IgM/IgA/IgG, No.	12/3/6	1/1/1	0/0/0	1/0/1	0/0/0	0/0/0	1/0/0	15/4/8	7/0/5	22/4/13
Titer range	1:10–1:32	1:10–1:32	—	1:10	—	—	1:32	1:10–1:32	1:10–1:100	1:10–1:100
MOG										
Total No.	1,378	310	228	442	29	42	74	2,503	1,694	4,197
Seropositives, No. (%)	15 (1.1)	1 (0.3)	1 (0.4)	8 (1.8)	1 (3.4)	0 (0.0)	0 (0.0)	26 (1.0)	9 (0.5)	35 (0.8)
Seropositive, No. male	8	1	0	3	1	0	0	13	7	20
IgM/IgA/IgG, No.	10/4/1	0/1/0	1/0/0	6/2/1	1/0/0	0/0/0	0/0/0	18/7/2	6/2/1	24/9/3
Titer range	1:10–1:320	1:10	1:32	1:10–1:100	1:10	—	—	1:10–1:320	1:10–1:320	1:10–1:320
GAD65										
Total No.	1,378	310	258	442	29	42	74	2,533	1,703	4,236
Seropositives, No. (%)	9 (0.7)	1 (0.3)	0 (0.0)	3 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	13 (0.5)	9 (0.5)	22 (0.5)
Seropositive, No. male	8	1	0	2	0	0	0	11	7	18
IgM/IgA/IgG, No.	0/1/8	0/0/1	0/0/0	0/0/3	0/0/0	0/0/0	0/0/0	0/1/12	1/4/5	1/5/17
Titer range	1:10–1:100	1:320	—	1:32–1:100	—	—	—	1:10–1:320	1:10–1:320	1:10–1:320
Ma2										
Total No.	1,378	310	228	442	29	42	74	2,503	1,694	4,197

TABLE 2. Continued

	Study Group									
	Schizophrenia	Affective disorders	Parkinson	Stroke	ALS	Borderline	Others[b]	Disease Group Total	Healthy Controls	Total
Male, %	**65.1**	**48.1**	**66.3**	**54.8**	**58.6**	**33.3**	**60.8**	**60.6**	**57.9**	**59.5**
Age, yr ± SD	**39.0 ± 12.6**	**45.9 ± 15.6**	**65.9 ± 10.1**	**68.3 ± 12.5**	**60.3 ± 10.2**	**27.0 ± 8.5**	**39.2 ± 15.5**	**47.7 ± 18.0**	**37.7 ± 13.5**	**43.7 ± 17.1**
No.	**1,378**	**310**	**228–258[c]**	**442**	**29**	**42**	**74**	**2,503–2,533[c]**	**1,693–1,703[c]**	**4,196–4,236[c]**
Antigen[a]										
Seropositives, No. (%)	8 (0.6)	3 (1.0)	0 (0.0)	2 (0.5)	0 (0.0)	0 (0.0)	1 (1.4)	14 (0.6)	8 (0.5)	22 (0.5)
Seropositive, No. male	5	2	0	1	0	0	1	9	2	11
IgM/IgA/IgG, No.	3/3/2	0/3/0	0/0/0	1/0/1	0/0/0	0/0/0	0/1/0	4/7/3	3/2/4	7/9/7
Titer range	1:10–1:320	1:32	—	1:10–1:32	—	—	1:10	1:10–1:320	1:10–1:320	1:10–1:320
Yo										
Total No.	1,378	310	228	442	29	42	74	2,503	1,693	4,196
Seropositives, No. (%)	5 (0.4)	1 (0.3)	1 (0.4)	1 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)	8 (0.3)	10 (0.6)	18 (0.4)
Seropositive, No. male	4	1	1	1	0	0	0	7	6	13
IgM/IgA/IgG, No.	0/2/3	0/1/0	0/1/0	0/0/1	0/0/0	0/0/0	0/0/0	0/4/4	1/4/7	1/8/11
Titer range	1:10–1:100	1:10	1:32	1:32	—	—	—	1:10–1:100	1:10–1:100	1:10–1:100
Ma1										
Total No.	1,378	310	228	442	29	42	74	2,503	1,694	4,197
Seropositives, No. (%)	3 (0.2)	1 (0.3)	0 (0.0)	2 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	6 (0.2)	9 (0.5)	15 (0.4)
Seropositive, No. male	2	1	0	2	0	0	0	5	5	10
IgM/IgA/IgG, No.	0/0/3	0/0/1	0/0/0	0/0/2	0/0/0	0/0/0	0/0/0	0/0/6	2/0/7	2/0/13
Titer range	1:32–1:320	1:32	—	1:32–1:100	—	—	—	1:32–1:320	1:10–1:100	1:10–1:320

[a]Ig class numbers do not always add up to the total number of seropositives due to double and triple positives.
[b]Patients for whom initial diagnosis of schizophrenia was not confirmed (ie, other mental disease, not yet classified).
[c]Range accounts for missing determinations.
ALS = amyotrophic lateral sclerosis; yr = years; Ig = immunoglobulin; SD = standard deviation.

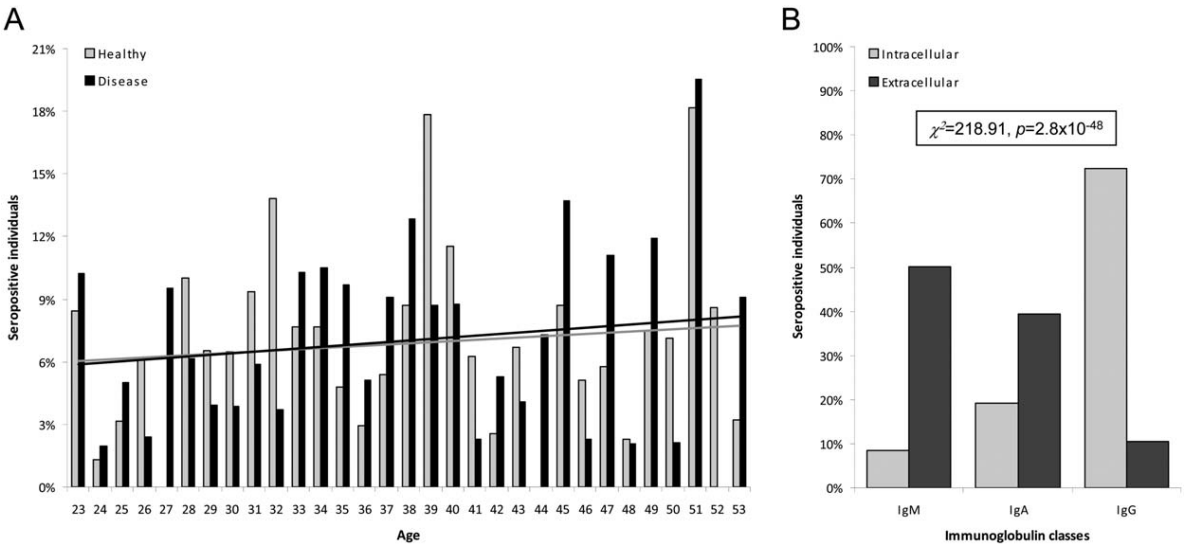

FIGURE 1: (A) Seropositivity for autoantibodies (AB) directed against 8 defined brain antigens (amphiphysin, ARHGAP26, CASPR2, MOG, GAD65, Ma2, Yo, and Ma1) by age. Displayed is the relative frequency (%) of seropositive individuals in the respective age group of healthy (gray) versus ill (black) individuals. N-methyl-D-aspartate-receptor subunit-NR1 (NMDAR1) AB are not included; for information on these AB see Hammer et al.[10] (B) Immunoglobulin (Ig) class depends on antigen location. Note the inverted pattern of distribution of the predominant Ig isotype in individuals carrying AB against intracellular versus extracellular epitopes. NMDAR1 AB are included. For clarity, individuals who tested positive for AB of >1 Ig class/antigen (n = 96) or for >1 antigen (n = 29) were excluded.

room temperature. Slides were washed again with a flush of PBS-Tween and then immersed in PBS-Tween for 5 minutes. Drops of PBS-buffered, 1,4-diazabicyclo[2.2.2]octane–containing glycerol (approximately 20 μl per field) were placed onto a cover glass, and the biochip slides were embedded in this mounting medium simultaneously and examined by fluorescence microscopy. Positive and negative controls were included with every test procedure. Samples were classified as positive or negative based on fluorescence intensity of the transfected cells in direct comparison with nontransfected cells and control samples. Endpoint titers refer to the last dilution showing a measurable degree of fluorescence, with 1:10 being the cutoff for positivity. The biochip mosaics have been validated and established in previous studies.[22,25]

Statistical Analysis

Group differences in categorical variables were assessed using chi-square test. Logistic regression analysis was employed to study predictive effects of age and disease status on seropositivity. For all analyses, statistical significance was set to the 0.05 level. Statistical analyses were performed using SPSS for Windows (v17.0; IBM-Deutschland, Munich, Germany).

Results

In full agreement with our recent work,[10–12] NMDAR1 AB showed an overall frequency of ~10%, increasing with age, with comparable distribution across essentially all disease groups as well as healthy individuals (Table 2). Only age ($b = 0.02$, Wald = 42.09, $p = 8.7 \times 10^{-11}$), but not disease versus health status ($b = 0.06$, Wald = 0.29, $p = 0.594$) predicted seropositivity in a logistic regression model ($R^2_{\text{Nagelkerkes}} = 0.02$, model chi-square = 49.21, $p = 2.1 \times 10^{-11}$). Again, distribution of titer range (IgM: chi-square = 5.75, $p = 0.218$; IgA: chi-square = 3.65, $p = 0.302$; IgG: chi-square = 2.31, $p = 0.511$) and Ig class (IgM, IgA, and IgG: chi-square = 0.55, $p = 0.759$) were similar in healthy and ill subjects, with IgG once more constituting the rarest isotype. Remarkably, in the Parkinson disease sample analyzed here, despite an average age close to the stroke group, NMDAR1 AB seropositivity amounted to only 8.1%. The reasons for this low percentage are presently unclear and call for replication.

Only 8 of the 24 other AB showed appreciable seropositivity (range = 0.4–2.0%), but each was well below the NMDAR1 AB frequency (Table 2): amphiphysin (2.0%), ARHGAP26 (1.3%), CASPR2 (0.9%), MOG (0.8%), GAD65 (0.5%), Ma2 (0.5%), Yo (0.4%), and Ma1 (0.4%). As seen with NMDAR1 AB, frequencies of AB against the other 8 antigens were similar over all subject groups (healthy and disease: chi-square = 1.25, $p = 0.535$), and titer ranges were comparable. In contrast to NMDAR1 AB, however, seroprevalence of these 8 AB tended to slightly (but not significantly) ascend with age (Fig 1A). A total of 96 individuals carried AB of >1 Ig class against the same antigen (Table 2).

Interestingly, comparable to NMDAR1 AB, the 2 other AB directed against extracellular antigens with still substantial seropositivity (Table 2), CASPR2 and MOG,

TABLE 3. Defined Brain Antigens Leading to Less Abundant Autoantibody Seroprevalence in Healthy Subjects and Neuropsychiatric Disease Groups

	Study Group									
	Schizophrenia	Affective Disorders	Parkinson	Stroke	ALS	Borderline	Others[b]	Disease Group Total	Healthy Controls	Total
Male, %	**65.1**	**48.1**	**66.3**	**54.8**	**58.6**	**33.3**	**60.8**	**60.6**	**57.9**	**59.5**
Age, yr ± SD	**39.0 ± 12.6**	**45.9 ± 15.6**	**65.9 ± 10.1**	**68.3 ± 12.5**	**60.3 ± 10.2**	**27.0 ± 8.5**	**39.2 ± 15.5**	**47.7 ± 18.0**	**37.7 ± 13.5**	**43.7 ± 17.1**
No.	**1,378**	**310**	**228–258[c]**	**442**	**29**	**42**	**74**	**2,503–2,533[c]**	**1,693–1,703[c]**	**4,196–4,236[c]**
Antigen[a]										
AMPAR-1 (AMPA receptor subunit GluR1)	1 [0.1]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	1 [0.0]	2 [0.1]	3 [0.1]
AMPAR-2 (AMPA receptor subunit GluR2)	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	2 [0.1]	2 [0.0]
AQP4 (aquaporin 4)	1 [0.1]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	1 [0.0]	0 [0.0]	1 [0.0]
CV2 (collapsin response mediator protein 5)	1 [0.1]	0 [0.0]	0 [0.0]	1 [0.2]	0 [0.0]	0 [0.0]	0 [0.0]	2 [0.1]	0 [0.0]	2 [0.0]
Tr/DNER (Delta/Notch-like epidermal growth factor-related receptor)	1 [0.1]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	1 [0.0]	0 [0.0]	1 [0.0]
DPPX-IF1 (dipeptidyl-peptidase–like protein 6)	1 [0.1]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	1 [0.0]	1 [0.1]	2 [0.0]
GABAR-B1/B2 (γ-amino butyric acid type B receptor subunit 1 and 2)	0 [0.0]	1 [0.3]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	1 [0.0]	0 [0.0]	1 [0.0]
GAD67 (glutamate decarboxylase 67)	1 [0.1]	1 [0.3]	0 [0.0]	1 [0.2]	0 [0.0]	0 [0.0]	0 [0.0]	3 [0.1]	1 [0.1]	4 [0.1]

TABLE 3. Continued

	Study Group									
	Schizophrenia	Affective Disorders	Parkinson	Stroke	ALS	Borderline	Others[b]	Disease Group Total	Healthy Controls	Total
Male, %	**65.1**	**48.1**	**66.3**	**54.8**	**58.6**	**33.3**	**60.8**	**60.6**	**57.9**	**59.5**
Age, yr ± SD	**39.0 ± 12.6**	**45.9 ± 15.6**	**65.9 ± 10.1**	**68.3 ± 12.5**	**60.3 ± 10.2**	**27.0 ± 8.5**	**39.2 ± 15.5**	**47.7 ± 18.0**	**37.7 ± 13.5**	**43.7 ± 17.1**
No.	**1,378**	**310**	**228–258[c]**	**442**	**29**	**42**	**74**	**2,503–2,533[c]**	**1,693–1,703[c]**	**4,196–4,236[c]**
Antigen[a]										
GLRA1b (glycin receptor alpha 1)	2 [0.1]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	1 [1.4]	3 [0.1]	1 [0.1]	4 [0.1]
GRM1 (metabotropic glutamate receptor 1)	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]
GRM5 (metabotropic glutamate receptor 5)	2 [0.1]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	2 [0.1]	0 [0.0]	2 [0.0]
Hu (HuB, HuC, HuD)	1 [0.1]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	1 [0.0]	0 [0.0]	1 [0.0]
LGI1 (leucine-rich glioma-inactivated protein 1)	1 [0.1]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	1 [0.0]	1 [0.1]	2 [0.0]
Recoverin	3 [0.2]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	3 [0.1]	1 [0.1]	4 [0.1]
Ri (Nova-1 and Nova-2)	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]
ZIC4 (zinc finger protein 4)	1 [0.1]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	1 [0.0]	0 [0.0]	1 [0.0]

Data are shown as No. [%] seropositive.
[a]For more information on antigens see Probst et al.[22]
[b]Patients for whom initial diagnosis of schizophrenia was not confirmed (ie, other mental disease, not yet classified).
[c]Range accounts for missing determinations.
ALS = amyotrophic lateral sclerosis; yr = years; SD = standard deviation.

were rarely of the IgG class. In contrast, the remaining relatively frequent 6 AB (Table 2), recognizing intracellular antigens (amphiphysin, ARHGAP26, GAD65, Ma2, Yo, Ma1), were predominantly IgG. These highly significant differences in immunoglobulin class frequencies (chi-square = 218.91, $p = 2.8 \times 10^{-48}$) may indicate that the antigen location codetermines Ig class selection (Fig 1B).

All other AB screened were found in ≤0.1% of individuals (AMPAR-1/2, AQP4, CV2, Tr/DNER, DPPX-IF1, GABAR-B1/B2, GAD67, GLRA1b, GRM1, GRM5, Hu, LGl1, recoverin, Ri, ZIC4), making solid conclusions difficult (Table 3). Overall, distribution in healthy and ill subjects was again similar (seropositives/tested, ill subjects: 19/2,503 = 0.8% vs healthy subjects: 9/1,693 = 0.5%; chi-square = 0.79, $p = 0.375$).

Of the n = 4,196 individuals who could ultimately be successfully screened for all 25 antigens, 83.2% (n = 3,493) were seronegative, 16% (n = 670) were seropositive for 1 (of 25), 0.7% (n = 29) for 2, and 0.1% (n = 4) for 3 specific antigens. No significant difference in overall seroprevalence was found between healthy and ill individuals.

Discussion

A large number of individuals (N = 4,236), healthy or suffering from different neuropsychiatric diseases, were for the first time systematically screened for seroprevalence of 25 AB directed against defined brain antigens. For NMDAR1 AB, the previously reported high overall seroprevalence of ~10%[10] could be confirmed in the present sample, extended in total number (2,817 plus 1,419 new subjects) and disease groups (schizophrenia, affective disorders, and Parkinson disease, plus additionally ischemic stroke, ALS, and borderline personality disorder).

In contrast to NMDAR1 AB, all other 24 newly tested brain-targeting AB revealed a much lower seroprevalence (≤2.0%). As seen before[10] and now replicated with NMDAR1 AB, these newly explored 24 AB also showed comparable frequency, titer, and Ig class distribution across all investigated groups. As predisposing factors for NMDAR1 AB seropositivity, we previously identified a common genetic variant (rs524991) as well as anti-influenza A or B seropositivity.[10] The low frequency of the 24 newly screened AB would require >20,000 deeply phenotyped and genotyped subjects for an investigation of this kind, which clearly limits the possibilities even of the present large study. Also, potential conclusions regarding symptom aggravation by these circulating AB in disease states would have to be built on much larger numbers of comprehensively characterized subjects. An exploratory analysis of the 8 most prevalent AB taken together showed that seropositivity was not predicted by positive influenza A or B titers (data not shown), thus rendering a major role of influenza as a general, nonspecific autoimmunity trigger unlikely.

It is important to mention that the autoimmune diseases that have been previously associated with the 25 autoantibodies under study (see Table 1) have not been the subject of the present investigation. This investigation focused solely on seroprevalence of these AB in healthy and neuropsychiatrically ill subjects. Moreover, the potential presence of a nascent, not yet detected tumor in one or the other individual under study, healthy or ill, cannot be entirely excluded.

The data of the present study support earlier hypotheses of a physiological presence in serum of AB directed against brain antigens.[1,3] In contrast to previous work investigating only overall serum IgG immunoreactivity with brain tissue, the AB reported here are not only well defined regarding the distinct brain antigens but were also systematically tested for all 3 major Ig classes, IgG, IgM, and IgA. IgG and IgA AB are formed by class switch (from IgM), spontaneously or for instance in conditions of inflammation.[26] An interesting observation in the present study is the rarity of the IgG class in AB directed against extracellular epitopes, NMDAR1, MOG, and CASPR2, as compared to the highly significant predominance of this Ig isotype in the 6 other rather frequently detected AB that all recognize intracellular antigens (amphiphysin, ARHGAP26, GAD65, Ma2, Yo, Ma1). Hence, antigen location (extracellular vs intracellular) apparently plays a major role in isotype determination in "physiological autoimmunity." We note in this context that "pathological or harmful autoimmunity" such as that associated with anti-NMDAR1 encephalitis has been linked to AB of the IgG class directed against an extracellular epitope, namely NMDAR1.[27]

The present work aimed exclusively at analyzing serum AB. For obvious reasons, cerebrospinal fluid (CSF) was not available for the same large set of individuals. The obtained data may serve as a reference for clinicians advising caution with respect to any conclusions on a causal association of serum AB with brain disease. The presence of these AB in the circulation obviously does not allow any firm assumption as to whether they play a pathophysiological role in any brain-related syndrome, and certainly does not on its own justify immunosuppressive treatment, unless they are also proven to exist in an appreciable amount in the CSF. Importantly, a certain rate of basic transfer is expected to take place also in healthy individuals with

intact BBB, for example, for IgG, 1/500 of the serum concentration.[28] Therefore, detection of substantial CSF AB levels appears mandatory for allowing conclusions on a causal or symptom-aggravating association with any central nervous system disorder, such as any kind of encephalitis, epilepsy, psychosis, extrapyramidal symptoms, or cognitive decline. Similar conclusions were drawn in a recent study on cases of anti-NMDAR1 encephalitis where CSF and serum levels were retrospectively evaluated.[27]

In the absence of a temporary or permanent disturbance of the BBB, serum AB would not be expected to enter the brain in noticeable amounts. However, in case of AB passage through a "leaky" BBB, facilitated by genetic predisposition (eg, *APOE4* carrier status), during fetal life or upon brain trauma or inflammation, symptom aggravation may well evolve.[1,10,29,30] On the other hand, considering earlier attempts to treat stroke or epilepsy with AB against NMDAR1, there may even be situations where AB entering the brain are protective.[31] Importantly, AB may also penetrate into the brain in other (noninflammatory) situations, for example, stress.[1] Thus, the individual repertoire of physiological AB may modulate brain function and/or codetermine outcome from brain disease. Undeniably, exploring the role of brain antigen-directed serum AB is just in its infancy, and much more research is needed to understand their pathophysiological significance.

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Authorship

M.B., G.S., and K.W. recruited, diagnosed and assessed patients. B.S., J.S., C.H., K.W., H.M., and G.S. worked on the establishment of the respective databases. B.T., S.S., S.L., K.R., and C.P. prepared and conducted all AB analyses, supervised and guided by W.S. Furthermore, L.D., C.O., B.S., J.W., and H.E. analyzed and interpreted all final data. H.E. and W.S. planned, supervised, and coordinated the project. H.E., together with L.D., C.O., and J.S., wrote the manuscript. All authors contributed to the current version of the paper regarding either conception, design, data analysis, or editing. L.D., C.O., and J.S. contributed equally.

Potential Conflicts of Interest

C.P.: stock, Euroimmun. G.S.: board membership, Avanir Pharmaceuticals; consultancy, Novartis, Lundbeck, FB Health; speaking fees, paid manuscript preparation, Novartis. W.S.: board membership, stock, Euroimmun.

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6. SUMMARY AND CONCLUSIONS

It can be considered common knowledge that psychiatric disorders have a multifactorial etiology. Accounting for this multifactorial etiology, this thesis comprises projects that focused on the association of genetic markers, environmental exposure, and the presence of serum autoantibodies with psychiatric phenotypes. With project I, on the brain-specific angiogenesis inhibitor 1-associated protein 3 (Baiap3), we were able to provide first evidence for a direct biological connection between anxiety and benzodiazepine use disorder. Using a translational approach with mice and humans, we identified a very similar phenotype associated with *Baiap3*/*BAIAP3* across species. This finding could, if replicated in independent human samples, and more specifically in individuals with anxiety disorders, provide important implications for a more personalized treatment. Our finding suggests that in the therapy of (male) patients diagnosed with anxiety disorders, which carry the *BAIAP3* risk SNP genotypes – possibly predisposing to the development of a benzodiazepine use disorder – the usage of benzodiazepines should be avoided.

In project II I was able to show that specific environmental factors (perinatal complications, neurotrauma, cannabis use, psychotrauma, urbanicity, and migration) experienced before disease onset and up until 18 years of age were associated with disease onset and socioeconomic functioning in male patients with schizophrenia, particularly when accumulated. This finding bares implications regarding the prevention of multiple risk factor exposure in at-risk populations. Furthermore, this study is an attempt to predict important outcome variables in patients with schizophrenia. The development of multivariate outcome prediction models, including environmental and genetic factors, could be very useful for making more objective treatment decisions. To get some insight on the biological mechanisms that might mediate the environmental effects, a follow-up study of epigenetic changes in individuals with none versus many risk factors could be of interest, since DNA methylation changes have been associated with the experience of e.g. childhood trauma (Klengel et al., 2013; Labonte et al., 2012).

In project III and IV we studied the association of 25 serum autoantibodies with schizophrenia, affective disorders, borderline personality disorder, Parkinson's disease, ischemic stroke, and amyotrophic lateral sclerosis, concluding that these antibodies directed

against brain antigens are not associated with the studied diseases. However, in the schizophrenic patients of the GRAS sample we identified a subtle neurological phenotype in individuals with NMDA receptor autoantibodies, which had experienced events (birth complication, neurotrauma), at least temporarily compromising blood-brain barrier function. There is increasing interest in studying the crosstalk between the immune system and the brain, which go far beyond the classical examples of systemic lupus erythematosus or multiple sclerosis (Diamond et al., 2009; Hornig, 2013). Human and animal studies provide hints for effects of cytokines, the microbiome, or microbes on behavior and suggest their involvement in the development of psychiatric disorders, too (Ezenwa et al., 2012; McCusker and Kelley, 2013; Miller et al., 2013). A better understanding of these mechanisms bears the potential for completely new treatment approaches in psychiatry.

The future of genetic research on psychiatric disorders will certainly also involve more whole exome or even whole genome sequencing studies, which will lead to the association of an increasing number of genetic variants with mental disorders. One important question will therefore remain, how all the genetic variants combine to cause pathology. In an attempt to study the combined effects of genetic variants, another project of my PhD (not featured in this thesis) focused on the fragile-X family of genes (*FMR1*, *FMR2*, *FXR1*, *FXR2*) and how SNPs across these genes – in accumulation – mediate autistic features in male schizophrenic patients.

Many researchers agree that the classification of mental disorders needs to be more directly linked to the biological mechanisms involved (Adam, 2013; Craddock and Owen, 2010; Insel et al., 2010; Krueger and Bezdjian, 2009). Studying subgroups of individuals that share similar phenotypic features could therefore be one way to discover common underlying biological mechanisms.

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8. APPENDIX

8.1 Accepted co-author publications

Co-author publication I

Kästner A*, Grube S*, El-Kordi A*, **Stepniak B**, Friedrichs H, Sargin D, Schwitulla J, Begemann M, Giegling I, Miskowiak KW, Sperling S, Hannke K, Ramin A, Heinrich R, Gefeller O, Nave KA, 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.
**Equally contributing authors*

Personal contribution

I assisted in the interpretation of the data for the schizophrenic as well as the healthy control sample and the design of figures and tables. Furthermore I was involved in the revision and publication of the manuscript.

Co-author publication II

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 HB, Pawlak R, Nave KA and Ehrenreich H (2013). A single gene defect causing claustrophobia. Transl Psychiatry **3**: e254.
**Equally contributing authors*

Personal contribution

I assisted in the human data analyses as well as the revision and publication of the manuscript.

Co-author publication III

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 U, Ehrenreich H (2014). Mild expression differences of *MECP2* influence aggressive social behavior. EMBO Mol Med: 1-22.

**Equally contributing authors*

Personal contribution

I assisted in the operationalization of impulsivity and aggression in the human sample. I contributed to the development of a severity scoring of aggression, based on the patients' medical reports and a clinical interview contained in the examination booklet of the GRAS study. I contributed to the revision and publication of the manuscript.

Molecular Medicine

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

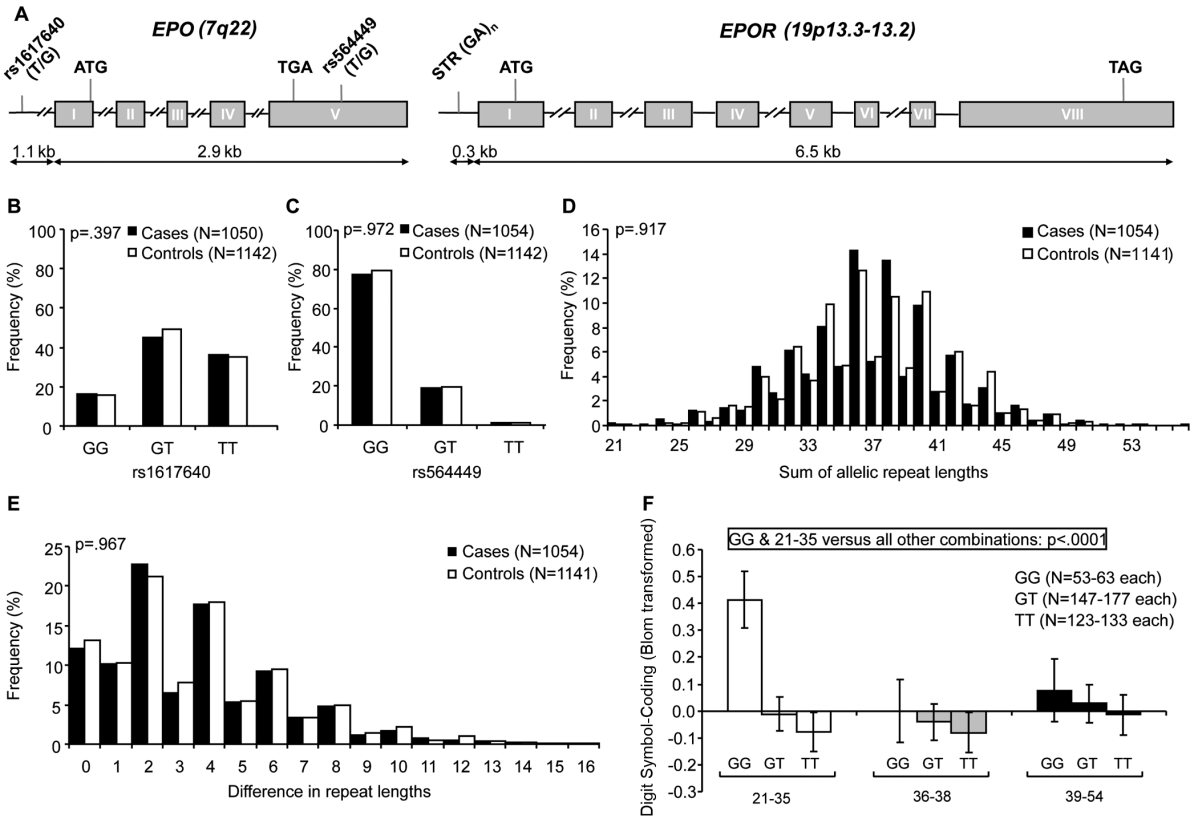

Figure 1. *EPO* and *EPOR* genotype analyses in schizophrenic patients of the GRAS data collection and healthy controls (blood donors). (A) Genetic overview of *EPO/EPOR* including analyzed genetic markers. (B,C) A case control study reveals comparable distribution of *EPO* SNP genotypes in schizophrenic 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

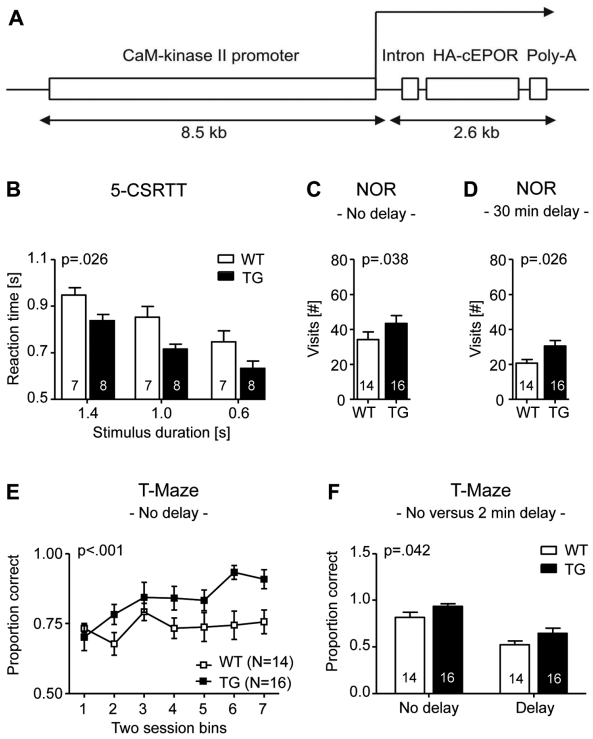

Figure 2. Mice with transgenic expression of constitutively active EPOR (cEPOR) driven by the α-calcium/calmodulin-dependent protein kinase II (α-CaMKII) promoter demonstrate highly superior cognitive performance compared with their wild type littermates. (A) Construct used for transgenic expression. (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**

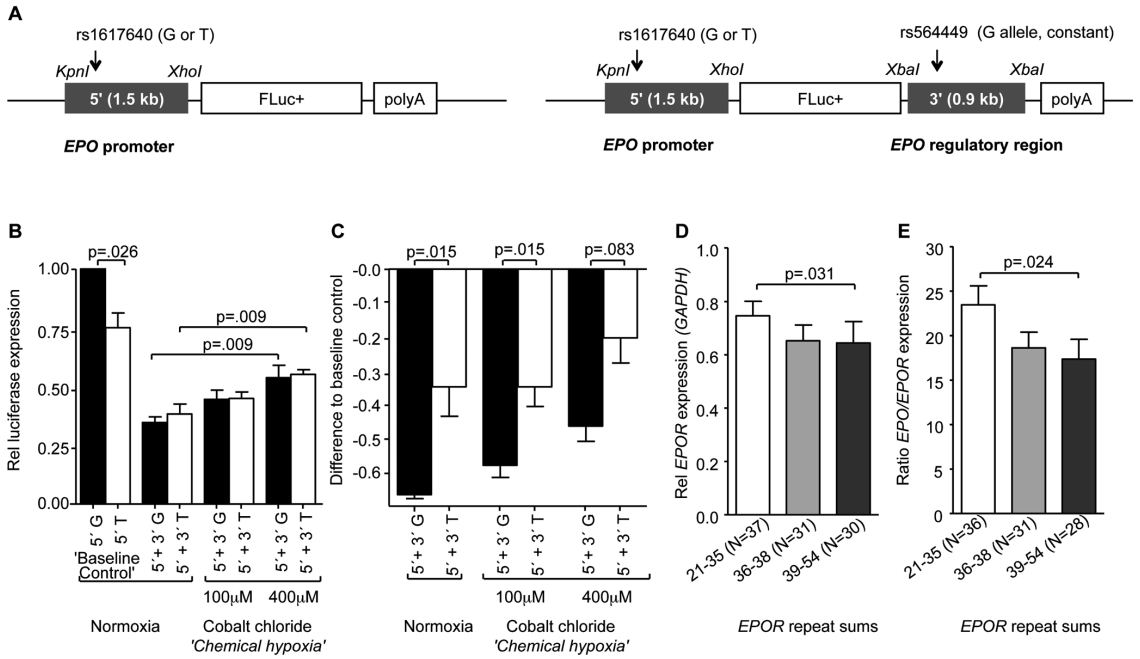

Figure 3. Genotype-dependent *EPO/EPOR* expression differences using reporter gene assays and PBMCs. (A) Reporter gene constructs: 5′ upstream region of the *EPO* gene with either G or T at rs1617640 (left) and in addition with the 3′ regulatory region of *EPO* (right). (B) G at SNP rs1617640 leads to 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.

ACKNOWLEDGMENTS

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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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Molecular Medicine

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).

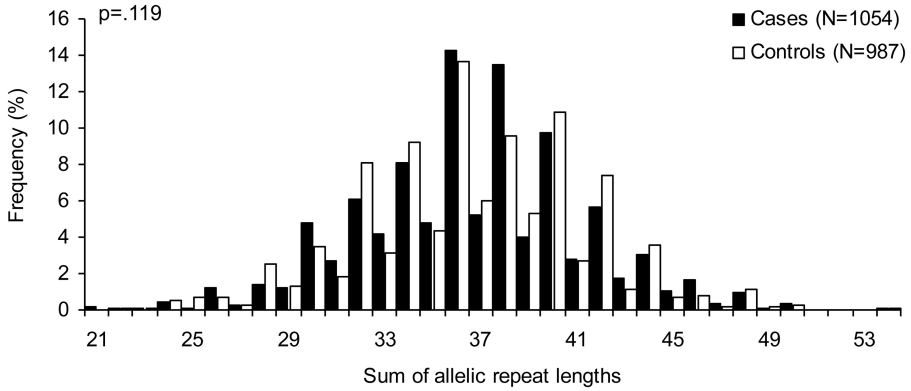
EPO SNPs		Genotypic frequencies			χ^2 (p)[a]	HWE (p)
		GG	GT	TT		
rs1617640	**SCZ** (N=1050)	16.5%	46.5%	37.0%		(.368)
	CON1[b] (N=1142)	15.6%	49.5%	34.9%	1.897 (.397)	(.357)
	CON2[c] (N=2315)	14.5%	48.0%	37.5%	2.297 (.317)	(.508)
		GG	GT	TT		
rs564449	**SCZ** (N=1054)	79.6%	19.5%	0.9%		(.628)
	CON1[b]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

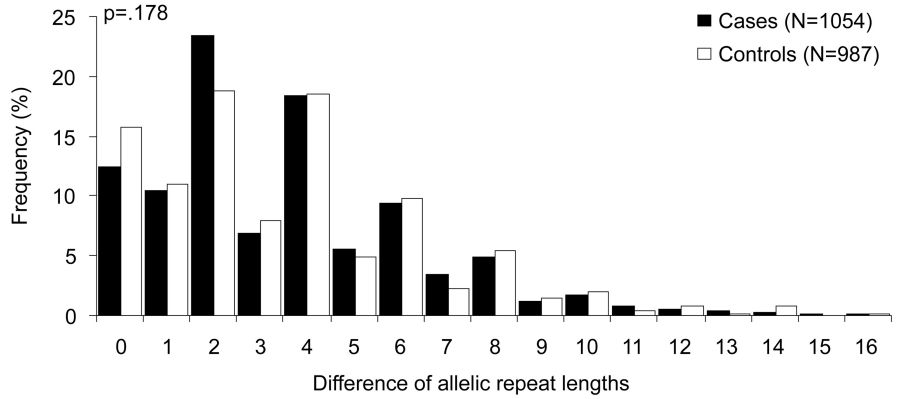
[a]Comparison of genotype distribution between the respective control group (CON1 or CON2) and the GRAS sample of schizophrenic patients (SCZ); [b]CON1= blood donors, [c]CON2= healthy replicate sample

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

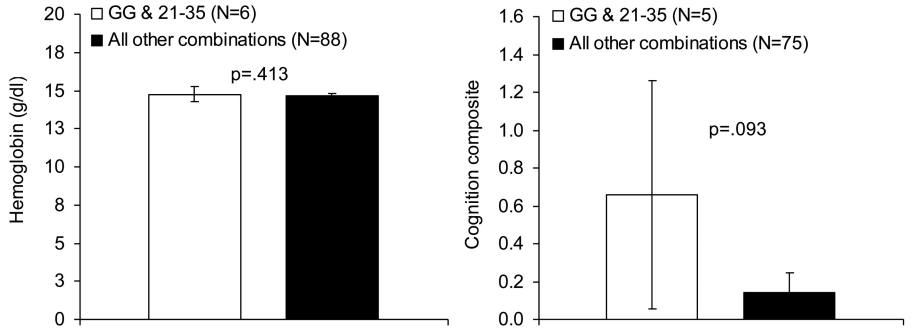
	EPO (mean ± SD)			*EPOR* (mean ± SD)	
	GG N=13	GT N=47	TT N=34	Low sum N=50	High sum N=44
Hemoglobin (g/dl)	14.751 ±1.317	14.703 ±0.923	14.657 ±1.329	14.748 ±1.112	14.631 ±1.157
Hematocrit (%)	43.765 ±3.877	43.484 ±2.611	43.288 ±3.892	43.661 ±3.187	43.166 ±3.384
Erythrocytes (x0^6/µl)	4.860 ±0.439	4.902 ±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.

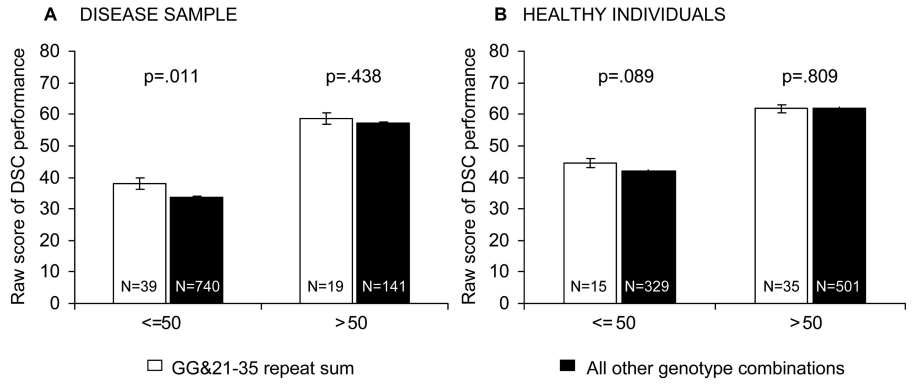




Supplementary Figure S1. Case control study: frequency distribution of *EPOR* STR $(GA)_n$ repeat lengths sum and repeat lengths difference in the GRAS sample of schizophrenic patients versus subjects of a healthy replicate sample. An association analysis of single allele repeat lengths instead of allelic 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.

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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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Keywords: chromosome 4; *GPM6A*; human pedigree; miR124; mouse mutant; panic disorder
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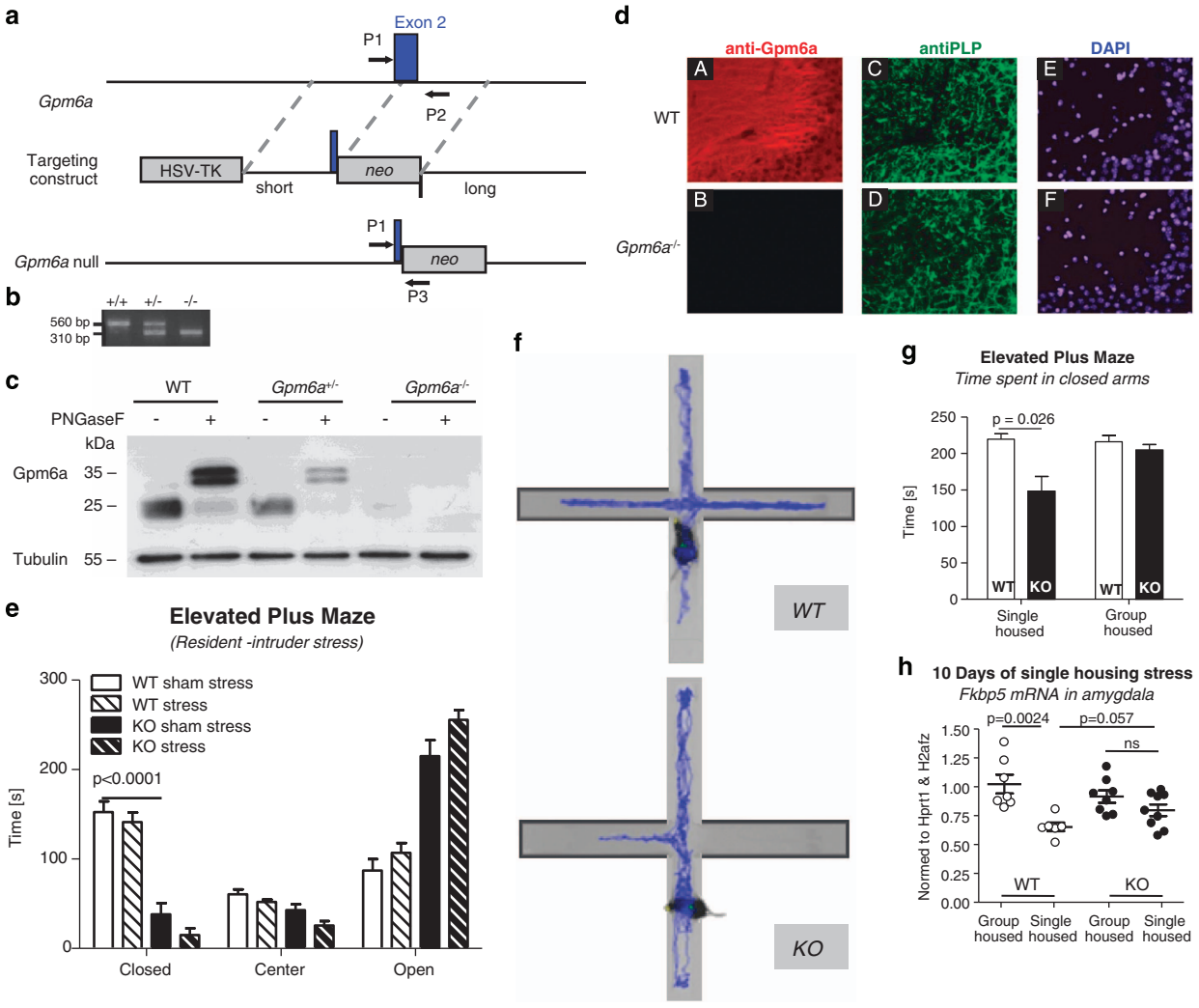

Figure 1 Generation of *Gpm6a* null mutant mice and discovery of behavioral consequences following stress. (**a**) Strategy to inactivate the mouse *Gpm6a* gene. A neomycin resistance cassette flanked by translation stop codons in all reading frames was fused into exon 2, which is the first exon present 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

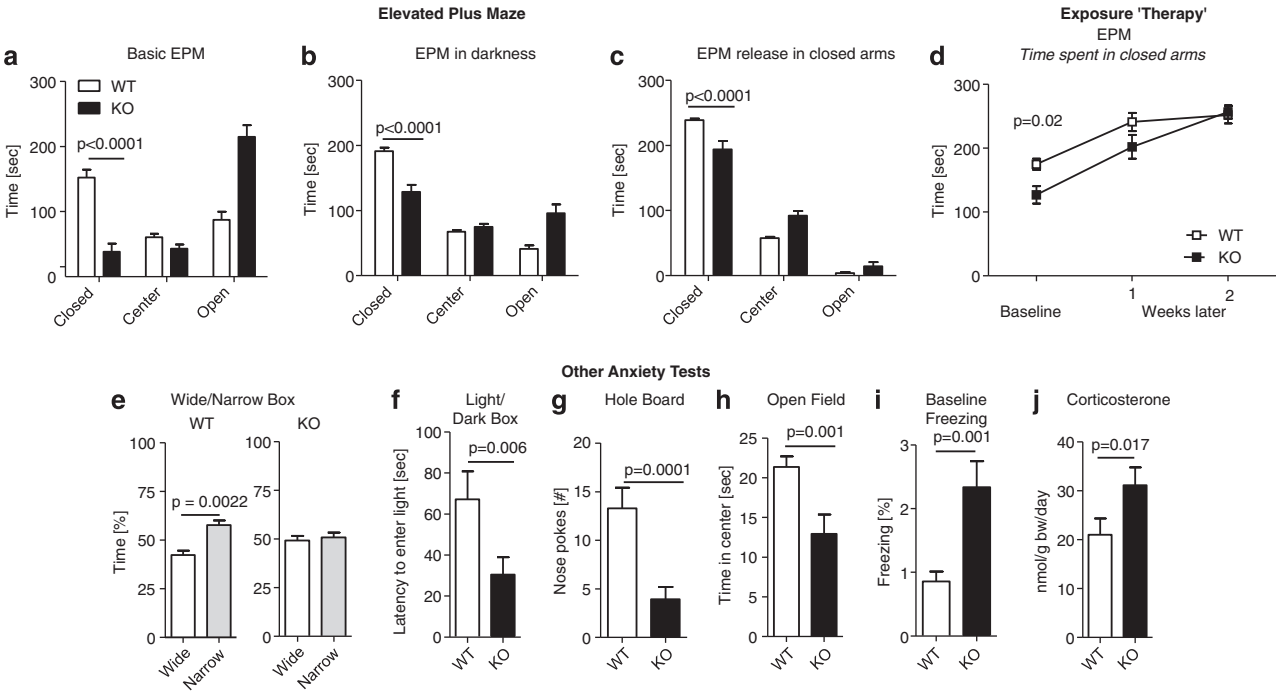

Figure 2 Male *Gpm6a* mutants show a strong claustrophobia-like phenotype on top of mild anxiety features. (**a**) Behavior of *Gpm6a* knockout (KO) and wild-type (WT) littermates in classical elevated plus maze (EPM); (**b**) in EPM performed in the darkness; and (**c**) in EPM upon release in closed 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

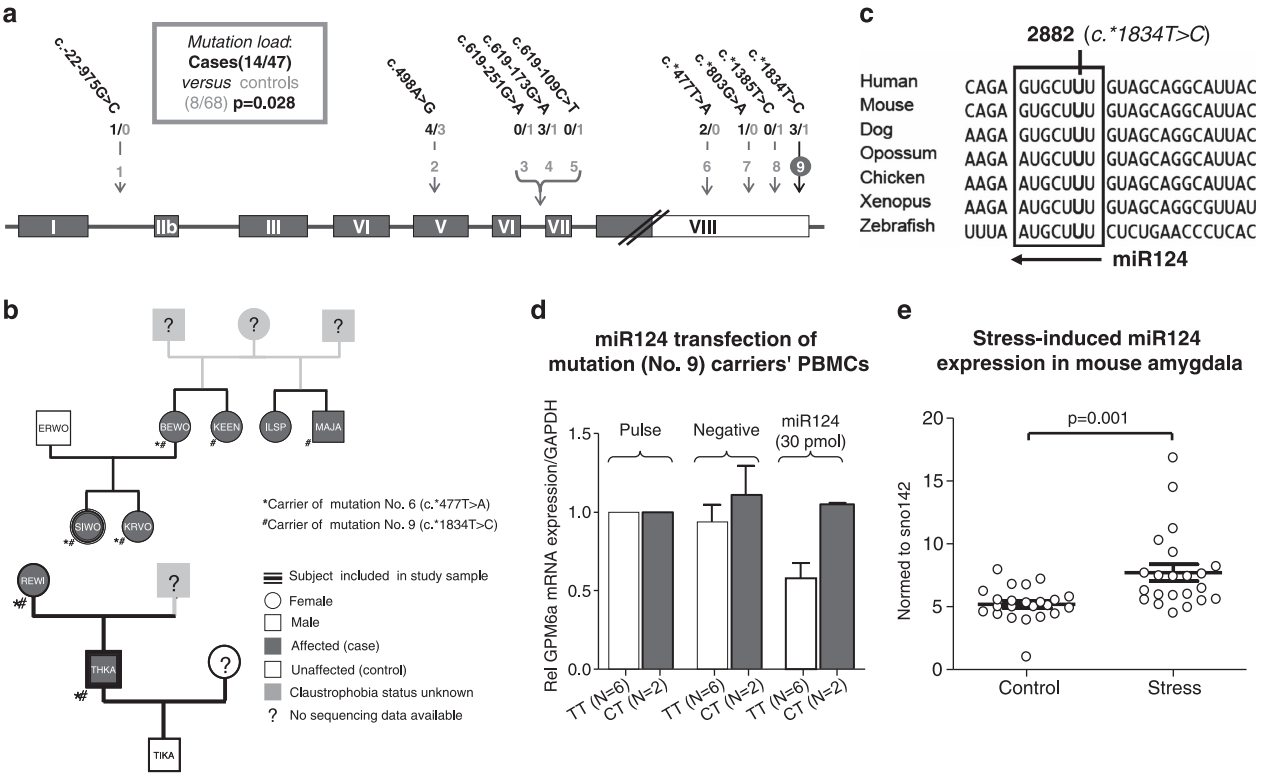

Figure 3 Genetic analysis of *GPM6A*. (**a**) Sequencing strategy and overview of the detected variants. Displayed are the coding exons (filled boxes) and the noncoding region of *GPM6A* (empty box). Arrows indicate rare variants found. Frequencies of rare variants in cases (black) versus controls (gray) 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 ΔΔG 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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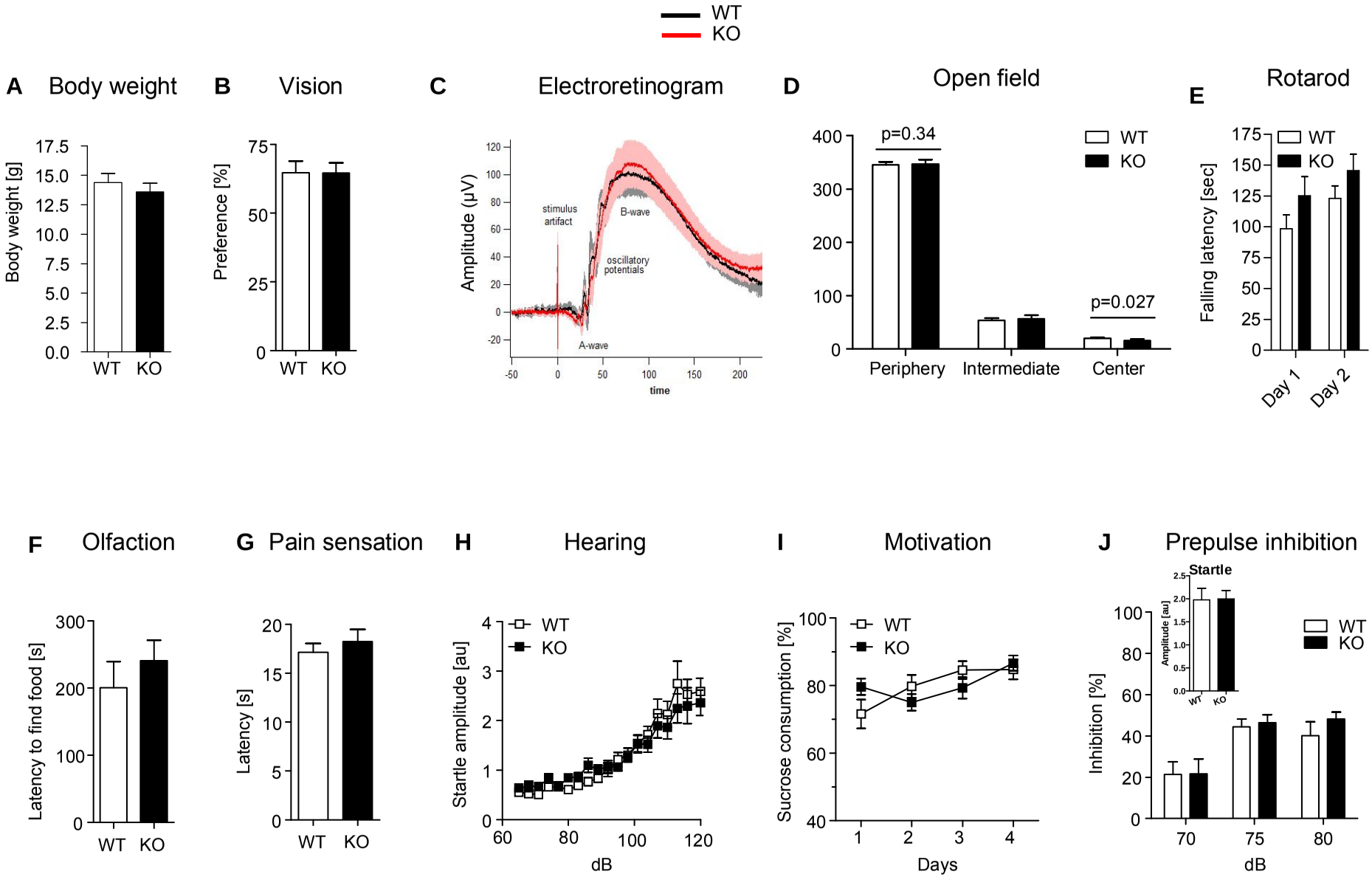
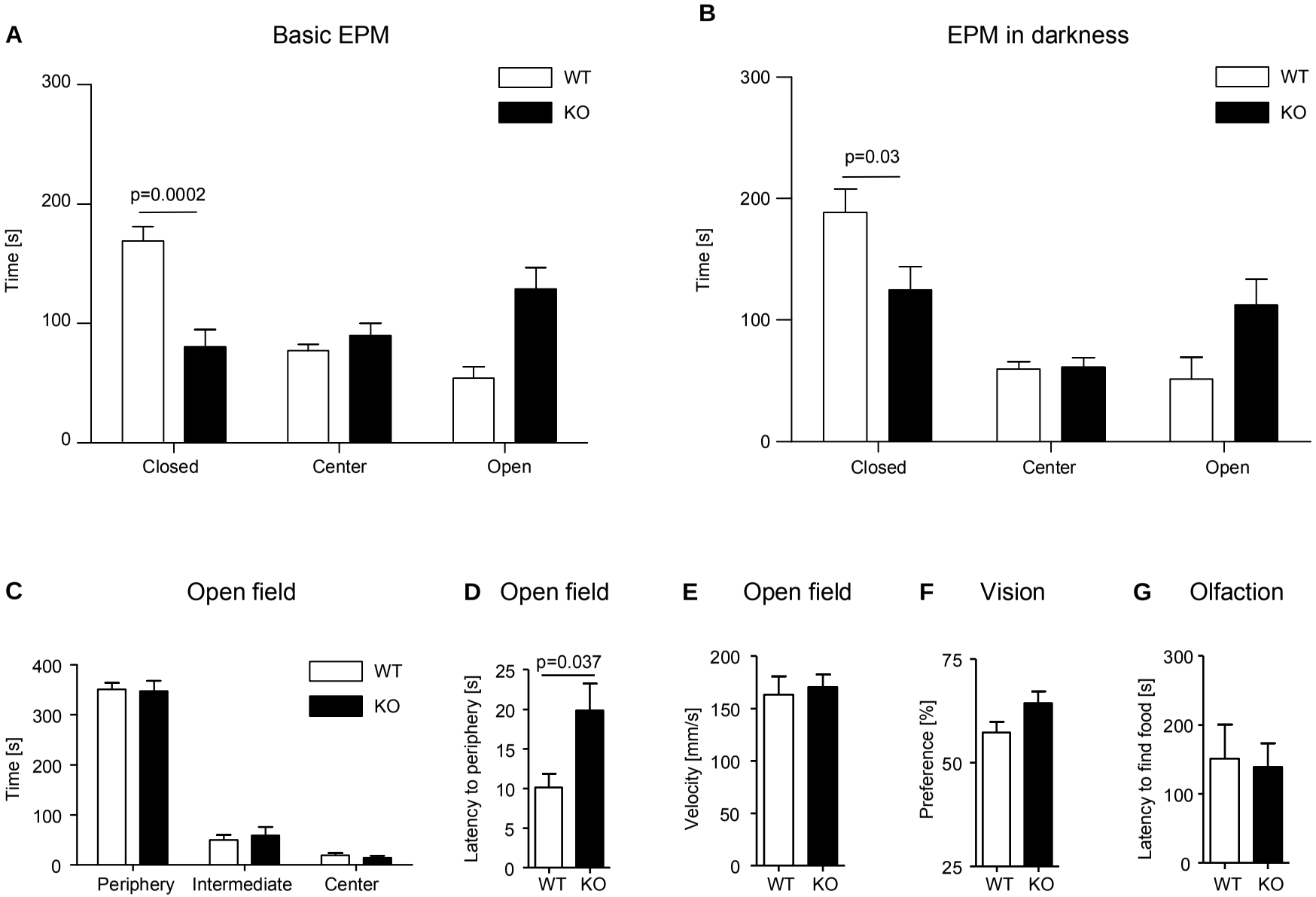
Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)

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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Research Article

TRANSPARENT PROCESS OPEN ACCESS

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Mild expression differences of *MECP2* influencing aggressive social behavior

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Abstract

The X-chromosomal *MECP2/Mecp2* gene encodes methyl-CpG-binding protein 2, a transcriptional activator and repressor regulating many other genes. We discovered in male FVB/N mice that mild (~50%) transgenic overexpression of *Mecp2* enhances aggression. Surprisingly, when the same transgene was expressed in C57BL/6N mice, transgenics showed reduced aggression and social interaction. This suggests that *Mecp2* modulates aggressive social behavior. To test this hypothesis in humans, we performed a phenotype-based genetic association study (PGAS) in >1000 schizophrenic individuals. We found *MECP2* SNPs rs2239464 (G/A) and rs2734647 (C/T; 3′UTR) associated with aggression, with the G and C carriers, respectively, being *more* aggressive. This finding was replicated in an independent schizophrenia cohort. Allele-specific *MECP2* mRNA expression differs in peripheral blood mononuclear cells by ~50% (rs2734647: C > T). Notably, the brain-expressed, species-conserved miR-511 binds to *MECP2* 3′UTR only in T carriers, thereby suppressing gene expression. To conclude, subtle *MECP2/Mecp2* expression alterations impact aggression. While the mouse data provides evidence of an interaction between genetic background and mild *Mecp2 over*expression, the human data convey means by which genetic variation affects *MECP2* expression and behavior.

Keywords genetic background; human; microRNA; mouse; phenotype-based genetic association study

Subject Categories Genetics, Gene Therapy & Genetic Disease; Neuroscience

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Introduction

The X-chromosomal *MECP2/Mecp2* gene encodes for methyl-CpG-binding protein 2, which can act both as a transcriptional activator and repressor (Chao & Zoghbi, 2012). Indeed, hundreds of genes have been estimated to be regulated, directly or indirectly, by this protein (Chahrour *et al*, 2008; Cohen *et al*, 2008). Complete or partial *loss-of-function* mutations of *MECP2* lead to Rett syndrome, characterized by a gender-dependent array of symptoms, ranging from early loss of acquired speech and motor skills to severe mental retardation and neonatal encephalopathy, among many others (Amir *et al*, 1999; Bienvenu & Chelly, 2006). Interestingly, gene duplication can cause very similar symptoms (Ramocki *et al*, 2010), and both down- and upregulation of *MECP2* are associated with behavioral core features of autism spectrum disorders (ASD) (Peters *et al*, 2013).

Diverse genetic mouse models, ranging from complete loss-of-function to reduced or enhanced expression, have been generated to study consequences of *Mecp2* mutations (Chen *et al*, 2001; Guy *et al*, 2001; Shahbazian *et al*, 2002; Collins *et al*, 2004; Moretti *et al*, 2005; Samaco *et al*, 2008; Bodda *et al*, 2013). In fact, there is a correlation between *Mecp2* "dosage" and phenotype severity, indicating a narrow normal expression range, with deviance in both directions being disadvantageous (Chao & Zoghbi, 2012). Along these lines, we have previously shown that mild overexpression of *Mecp2* of ~1.4–1.5 times the wildtype (WT) level induces increased seizure propensity and aggression in male FVB/N mice, together with alterations in neuronal branching sites and augmented spine density (Bodda *et al*, 2013). Application of an epileptogenic compound, pentylenetetrazole, to transgenic (TG) neurons *in vitro* leads to a marked increase in amplitude and frequency of calcium spikes (Bodda *et al*, 2013).

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While epileptic seizure susceptibility is a well-established result of alterations in *MECP2* expression (Shahbazian *et al*, 2002; Collins *et al*, 2004; Chahrour & Zoghbi, 2007; Chao *et al*, 2010; Chao & Zoghbi, 2012; Bodda *et al*, 2013), information on the behavioral consequences of mild overexpression is still rather limited. Doubling the *Mecp2* expression level in mice resulted in impaired social interaction (Samaco *et al*, 2012). *Mecp2* deficiency, however, also led to abnormal social behavior such as deficits in nest building, altered social interaction and enhanced aggression (Shahbazian *et al*, 2002; Moretti *et al*, 2005; Fyffe *et al*, 2008; Kerr *et al*, 2008; Samaco *et al*, 2008, 2009; Chao *et al*, 2010; Pearson *et al*, 2012). Taken together, tightly regulated *Mecp2* expression is obviously critical for normal function of genes involved in social behavior (Moretti *et al*, 2005).

Importantly, the reported phenotypes arose from diverse genetic backgrounds of mice carrying *Mecp2* mutations, not considering basic phenotypical differences among mouse strains (Wolfer & Lipp, 2000; Moy *et al*, 2009; Pietropaolo *et al*, 2011; O'Leary *et al*, 2013; Samaco *et al*, 2013). For example, behavioral comparisons between FVB/N and C57BL/6N strains revealed that FVB/N mice showed a higher frequency of bouts during behavioral paradigms of aggression (Mineur & Crusio, 2002; Pugh *et al*, 2004). Thus, the genetic background might well mask or modulate phenotypical changes induced by alterations in *Mecp2* expression.

In humans, data on aggression/impulsivity in Rett or *MECP2* gene duplication syndrome are scarce. A family study characterizing neuropsychiatric phenotypes of 9 males and 9 females with *MECP2* duplications revealed a high prevalence of hostility (63%) in female carriers. Moreover, an asymptomatic Rett mutation carrier with the mutation located in the deletion hotspot of the *MECP2* 3′ region has been reported to experience episodes of uncontrolled aggression (Huppke *et al*, 2006).

This study has been designed (1) to explore the role of gender and genetic background (FVB/N versus C57BL/6N) for behavioral phenotypes associated with mild *Mecp2* overexpression in mice, that is, altered aggression, spontaneous home cage behavior and predisposition to epileptic seizures, and (2) in parallel to search in humans for social behavioral consequences of common single-nucleotide polymorphisms (SNPs) within *MECP2* and (3) to strive for mechanistic insight into SNP-related *MECP2* expression differences in man.

Results

Mild *Mecp2* overexpression in FVB/N and C57BL/6N mice of both genders leaves most basic behaviors unaltered but modulates spontaneous home cage activity

To estimate the expression of the TG protein $Mecp2^{WT\_EGFP}$ (~100 kDa) compared to endogenous Mecp2 (~70 kDa) in TG mice, we performed Western blot analysis with total protein extracted from hippocampus and cerebellum. As described previously for FVB/N mice (Bodda *et al*, 2013), the relative expression of $Mecp2^{WT\_EGFP}$ in brain amounts to 40–50% of endogenous Mecp2 in both strains, leading to a subtle overexpression of ~1.4- to 1.5-fold compared to WT. $Mecp2^{WT\_EGFP}$ TG mice of both genders and strains (FVB/N and C57BL/6N) develop and reproduce normally, and are devoid of any immediately obvious phenotype. Also, upon comprehensive testing of several independent cohorts of both genotypes and genders at different ages, all major domains of basic behavior, that is, general activity, anxiety, motor functions, exploratory behavior, sensory and cognitive functions were unaltered when compared to the respective WT littermate controls (Table 1a, b), thus confirming and extending our earlier report on male FVB/N TG mice (Bodda *et al*, 2013).

We wondered whether subtle behavioral differences upon mild overexpression of *Mecp2* are perhaps not captured by the usual behavioral test battery. Therefore, spontaneous home cage behavior was monitored continuously overnight using LABORAS™. Indeed, we found significant differences between TG and WT mice. There was an overall tendency of reduced locomotion, including climbing, and increased immobility in TG as compared to WT, noticeable across strains and genders, except for male FVB/N mice that failed to show significant changes in locomotion (Fig 1).

Male *Mecp2* transgenic mice of both genetic backgrounds exhibit altered territorial social behavior and aggression as compared to their WT littermates

Based on the unexpected observation that mild overexpression of *Mecp2* leads to increased territorial aggressive behavior of male TG versus WT FVB/N mice when exposed to FVB/N intruders (Bodda *et al*, 2013), we started here a series of new experiments on the resident-intruder paradigm: (1) We aimed at testing reproducibility of this phenomenon in FVB/N mice exposed to younger intruder males of C57BL/6N background, that is, *per se* less aggressive intruders (Mineur & Crusio, 2002; Pugh *et al*, 2004). This experiment yielded similar results (Fig 2A), that is, increased territorial aggression in TG FVB/N. (2) We were interested to see whether another genetic background, C57BL/6N, would modify the effect of *Mecp2* overexpression. However, using the same resident–intruder protocol as in FVB/N males, no attack by C57BL/6N males was observed (Table 1b). As male C57BL/6N mice are generally less aggressive compared to male FVB/N (Mineur & Crusio, 2002; Pugh *et al*, 2004), the resident–intruder protocol had to be slightly modified by extending the cut-off time to 10 min and increasing the basal level of aggression using warming (Greenberg, 1972; Gaskill *et al*, 2012). Even though the necessary increase in surrounding temperature (<38°C) is far below the temperature used to test pain sensitivity (hot plate test; temperature set to 55°C), we note here that the pain threshold in TG and WT mice was identical (Table 1b). Prior to testing, the home cage of the resident mouse was placed under a heat-emitting red lamp for 20 min to obtain mild prewarming. Further, during testing, the home cage of the resident was positioned on a warming pad (set to 38°C). Surprisingly and opposite to FVB/N mice, the attack latency of TG C57BL/6N was longer, pointing to reduced territorial aggression as compared to WT littermates. In fact, within the 10 min observation, 30% of WT and 55% of TG residents of the C57BL/6N background did not attack the intruder at all (Fig 2A). In contrast to the clear alteration of territorial aggression in TG versus WT male mice of both strains, the sociability test (mouse preferred over empty cage) revealed entirely normal behavior, indistinguishable between TG and WT (Fig 2B).

Table 1. (A) FVB/N mice.

Behavioral paradigms (order according to domains)	Cohort 1							Cohort 2							Cohort 3						
		Male			Female				Male			Female				Male			Female		
	Age[a]	WT (*N* = 14)	TG (*N* = 18)	*P*	WT (*N* = 9)	TG (*N* = 4)	*P*	Age[a]	WT (*N* = 10–27)	TG (*N* = 6–22)	*P*	WT (*N* = 15–27)	TG (*N* = 14–37)	*P*	Age[a]	WT (*N* = 10–28)	TG (*N* = 10–12)	*P*	WT (*N* = 10–14)	TG (*N* = 10–21)	*P*
Anxiety-like behavior																					
Elevated plus maze (time in open versus closed arms [s])	4				Genotype: *P* = 0.2, *F* = 1.825			12	Genotype: *P* = 0.3, *F* = 1.120			Genotype: *P* = 0.8, *F* = 0.082			5	Genotype: *P* = 0.4, *F* = 0.567			Genotype: *P* = 0.6, *F* = 0.342		
Activity																					
Open field (total distance [m])	4	73 ± 6	80 ± 5.6	n.s.	74 ± 10	85 ± 3	n.s.	12	78 ± 6.3	69 ± 4.3	n.s.	72 ± 5.6	71 ± 3	n.s.	5	87 ± 2.8	84 ± 3.9	n.s.	94 ± 3.7	89 ± 3.4	n.s.
Exploratory behavior																					
Hole board (holes visited [#])								12	12 ± 1.4	10 ± 1.4	n.s.	23 ± 2.6	21 ± 1.7	n.s.	6	15 ± 1.2	15 ± 1.7	n.s.	19 ± 2.8	21 ± 2.2	n.s.
Impulsivity																					
Marble burying and digging (marbles buried [#])								14	3.9 ± 0.5	4.6 ± 0.6	n.s.				7	4.5 ± 0.5	4.6 ± 0.7	n.s.	3.8 ± 0.9	4.3 ± 0.7	n.s.
															16	4.5 ± 1	3.6 ± 1	n.s.	1.5 ± 0.7	3.5 ± 1.0	n.s.
Motor leaning and coordination																					
Rotarod (latency to fall [s])								13	Genotype: *P* = 0.2, *F* = 1.383			Genotype: *P* = 0.4, *F* = 0.834									
Motor strength																					
Grip strength (grip strength [P])								16	95 ± 6.3	94 ± 7.8	n.s.	97 ± 4.3	97 ± 3	n.s.							
Hearing																					
Hearing curve (startle amplitude [AU])								13	Genotype: *P* = **0.003**, *F* = 15.53 (TG: increased startle)			Genotype: *P* = **0.01**, *F* = 12 (TG: increased startle)									
Vision																					
Visual cliff (preference for "ground" [%])								15	52 ± 0.7	55 ± 1.3	n.s.	54 ± 0.9	52 ± 1	n.s.							
Olfaction																					
Food finding test (latency to find hidden cookie [s])								18	83 ± 29	114 ± 33	n.s.	234 ± 32	270 ± 15	n.s.							
Food finding test (latency to find visible cookie [s])								18	12 ± 3.2	11 ± 2	n.s.	14 ± 2.2	16 ± 2.4	n.s.							
Pain sensation																					
Hot plate (latency to lick [s])								24				18.6 ± 1.3	16.5 ± 1.2	n.s.							
Anhedonia-like behavior																					
Sucrose preference test (preference for sucrose [%])								20	69 ± 3.9	65 ± 3.9	n.s.	70 ± 2.1	64 ± 2.5	n.s.							
Sociability																					
Social interaction: 3-partite chamber (sociability index [AU])								16	5.4 ± 2.3	5.8 ± 4.3	n.s.	7.9 ± 3.4	3.2 ± 3.0	n.s.	8	17 ± 4.4	13 ± 5.2	n.s.	22 ± 3.1	13 ± 2.9	0.05
Communication																					
Ultrasound vocalization (calls [#])															13				456 ± 100	434 ± 103	n.s.
Social competence																					
Nest building (Nest score [AU])																					

Table 1. (A) (continued)

Behavioral paradigms (order according to domains)	Cohort 1							Cohort 2							Cohort 3						
		Male			Female				Male			Female				Male			Female		
	Age[a]	WT (*N* = 14)	TG (*N* = 18)	*P*	WT (*N* = 9)	TG (*N* = 4)	*P*	Age[a]	WT (*N* = 10–27)	TG (*N* = 6–22)	*P*	WT (*N* = 15–27)	TG (*N* = 14–37)	*P*	Age[a]	WT (*N* = 10–28)	TG (*N* = 10–12)	*P*	WT (*N* = 10–14)	TG (*N* = 10–21)	*P*
Social dominance																					
Tube test (dominance score [AU])																					
Aggression																					
Resident-intruder paradigm (latency to attack [s]); 5 min session; FVB/N as intruder	8	200 ± 33	109 ± 19	**0.049**				25	55 ± 12	49 ± 8.1	0.7	no attack			9	36 ± 11	19 ± 3.6	0.4			
Resident-intruder paradigm (latency to attack [s]); 5 min session; C57BL/6N as intruder															16	209 ± 22	84 ± 33	**0.002**			
Resident-intruder paradigm (latency to attack [s]); 10 min session with pre-warming; C57BL/6N as intruder																					
Sensorimotor gating																					
Startle reactivity - PPI set up (startle amplitude [AU])															7	8.5 ± 0.8	10.6 ± 1.1	0.06	6 ± 0.6	10 ± 0.7	**0.0003**
															16	9.6 ± 1.2	15 ± 2	0.05	5.4 ± 1.3	12 ± 1.2	**0.005**
Prepulse inhibition (percentage inhibition [%])								12	Genotype: *P* = 0.3, *F* = 1.314			Genotype: *P* = 0.2, *F* = 2.005			7	Genotype: *P* = 0.9, *F* = 0.036			Genotype: *P* = 0.4, *F* = 0.899		
															16	Genotype: *P* = 0.5, *F* = 0.499			Genotype: *P* = 0.6, *F* = 0.323		
Novel object recognition																					
No delay paradigm (preference for new object [%])																					
30´ delay paradigm (preference for new object [%])																					
Working memory																					
Y-maze (percentage alternation [%])															7	55 ± 3.0	61 ± 2.8	n.s.	67 ± 5.4	61 ± 3.4	n.s.
Hole board (errors [#])																					
Reference memory																					
Hole board (errors [#])																					
Home cage activity (LABORAS)																					
Locomotion frequency [#]															35	6246 ± 724	7822 ± 1155	n.s.	20466 ± 2598	6393 ± 919	**0.0002**
Immobility frequency [#]															35	26679 ± 2510	23322 ± 1678	n.s.	5623 ± 2198	23903 ± 3181	**0.0002**
Climbing frequency [#]															35	331 ± 100	196 ± 45	n.s.	451 ± 78	277 ± 115	n.s.
Seizure propensity																					
PTZ- seizure induction (seizure score [AU])								30	4.8 ± 0.7	9.1 ± 2	**0.042**	6 ± 1	8 ± 1	**0.006**							
Body weight [g]	6	25 ± 0.5	25 ± 0.3	n.s.	21 ± 0.3	22 ± 0.6	n.s.	16	30 ± 0.5	30 ± 0.7	n.s.	25 ± 0.5	25 ± 0.3	n.s.	6	21 ± 0.5	21 ± 0.7	n.s.	17 ± 0.5	17 ± 0.4	n.s.

Gray-shaded cells denote experiments that are presented in the figures. All P-values ≤ 0.05 are set in bold face.
[a]Age in weeks.

Table 1. (B) C57BL/6N mice

Behavioral paradigms (*order according to domains*)	Cohort 1 and 2							Cohort 3			
		Male			Female				Male		
	Age[a]	WT (N = 10–26)	TG (N = 12–13)	P	WT (N = 9–28)	TG (N = 11–13)	P	Age[a]	WT (N = 16)	TG (N = 10)	P
Anxiety-like behavior											
Elevated plus maze (time in open versus closed arms [s])	5	Genotype: P = 0.07, F = 3.398			Genotype: P = 0.6, F = 0.244						
Activity											
Open field (total distance [m])	5	55.3 ± 2	58.1 ± 3	n.s.	60 ± 1.9	59 ± 2.7	n.s.				
Exploratory behavior											
Hole board (holes visited [#])	7	11 ± 1	12 ± 1.7	n.s.	18 ± 2.5	20 ± 2.9	n.s.				
Impulsivity											
Marble burying and digging (marbles buried [#])	8	9.5 ± 1.4	8.2 ± 2.2	n.s.	5.9 ± 0.8	7.7 ± 1.2	n.s.				
Motor leaning and coordination											
Rotarod (latency to fall [s])	6	Genotype: P = 0.4, F = 0.552			Genotype: P = 0.8, F = 0.054						
Motor strength											
Grip strength (grip strength [P])	30	134 ± 3.9	138 ± 5.8	n.s.	123 ± 3.2	117 ± 5.3	n.s.				
Hearing											
Hearing curve (startle amplitude [AU])	7	Genotype: P = 0.2, F = 1.434			Genotype: P = 0.1, F = 2.773						
Vision											
Visual cliff (preference for "ground" [%])	10	53 ± 1.3	53 ± 1.3	n.s.	52 ± 1.3	56 ± 3.4	n.s.				
Olfaction											
Food finding test (latency to find hidden cookie [s])	16	107 ± 15	89 ± 17	n.s.	96 ± 14	113 ± 30	n.s.				
Food finding test (latency to find visible cookie [s])	16	8.6 ± 1.5	12 ± 3.5	n.s.	6.5 ± 1.8	7.5 ± 2.0	n.s.				
Pain sensation											
Hot plate (latency to lick [s])	32	13 ± 0.6	15 ± 1.2	n.s.	16 ± 0.7	15 ± 1.2	n.s.				
Anhedonia-like behavior											
Sucrose preference test (preference for sucrose [%])	14	91 ± 1.3	88 ± 1.4	n.s.	75 ± 2.6	71 ± 4.9	n.s.				
Sociability											
Social interaction: 3-partite chamber (sociability index [AU])	12	6.4 ± 1.8	6.2 ± 3.2	n.s.	9.0 ± 1.3	7.2 ± 3.4	n.s.				
Communication											
Ultrasound vocalization (calls [#])	13	126 ± 33	26 ± 12	**0.03**	345 ± 57	150 ± 62	0.08				
Social competence											
Nest building (Nest score [AU])								7	3.8 ± 0.2	2.4 ± 0.5	**0.02**

Table 1. (B) (continued)

Behavioral paradigms (*order according to domains*)	Cohort 1 and 2							Cohort 3			
		Male			Female				Male		
	Age[a]	WT (*N* = 10–26)	TG (*N* = 12–13)	*P*	WT (*N* = 9–28)	TG (*N* = 11–13)	*P*	Age[a]	WT (*N* = 16)	TG (*N* = 10)	*P*
Social dominance											
Tube test (dominance score [AU])								14	1.1 ± 0.3	0.4 ± 0.2	0.1
Aggression											
Resident-intruder paradigm (latency to attack [s]); 5 min session; FVB/N as intruder											
Resident-intruder paradigm (latency to attack [s]); 5 min session; C57BL/6N as intruder	32	No attack						13	No attack		
Resident-intruder paradigm (latency to attack [s]); 10 min session with pre-warming; C57BL/6N as intruder								14	349 ± 52	460 ± 53	0.06
Sensorimotor gating											
Startle reactivity - PPI set up (startle amplitude [AU])	7	7.7 ± 0.5	7.7 ± 0.6	n.s.	6.8 ± 0.4	8.9 ± 0.6	**0.01**				
Prepulse inhibition (percentage inhibition [%])	7	Genotype: $P = 0.7$, $F = 0.135$			Genotype: $P = 0.7$, $F = 0.115$						
Novel object recognition											
No delay paradigm (preference for new object [%])	8	56 ± 3.9	66 ± 3.7	n.s.	53 ± 2.4	56 ± 3.5	n.s.				
30′ delay paradigm (preference for new object [%])	8	57 ± 2.9	51 ± 4.4	n.s.	54 ± 2.5	62 ± 3.7	n.s.				
Working memory											
Y-maze (percentage alternation [%])	10	64 ± 2.0	63 ± 4.2	n.s.	63 ± 2.1	66 ± 2.6	n.s.				
Hole board (errors [#])	20	Genotype: $P = 0.07$, $F = 3.511$ (TG: more errors)			Genotype: $P = 0.8$, $F = 0.0228$						
Reference memory											
Hole board (errors [#])	20	Genotype: $P = 0.09$, $F = 3.174$ (TG: more errors)			Genotype: $P = 0.6$, $F = 0.262$						
Home cage activity (LABORAS)											
Locomotion frequency [#]	37	5110 ± 279	2584 ± 220	**<0.0001**	5423 ± 376	3579 ± 442	**0.009**				
Immobility frequency [#]	37	13921 ± 1258	27359 ± 1250	**<0.0001**	12266 ± 1121	22697 ± 1118	**<0.0001**				
Climbing frequency [#]	37	105 ± 10	49 ± 12	**0.004**	349 ± 73	75 ± 21	**0.0003**				
Seizure propensity											
PTZ- seizure induction (seizure score [AU])	40	6.7 ± 1.1	10 ± 0.8	**0.03**	7.7 ± 0.8	8.1 ± 1	**0.02**				
Body weight [g]	12	27 ± 0.4	27 ± 0.4	n.s.	23 ± 0.3	23 ± 0.4	n.s.	14	29 ± 0.6	30 ± 1.3	n.s.

Gray-shaded cells denote experiments that are presented in the figures. All P- values ≤ 0.05 are set in boldface.
[a]Age in weeks.

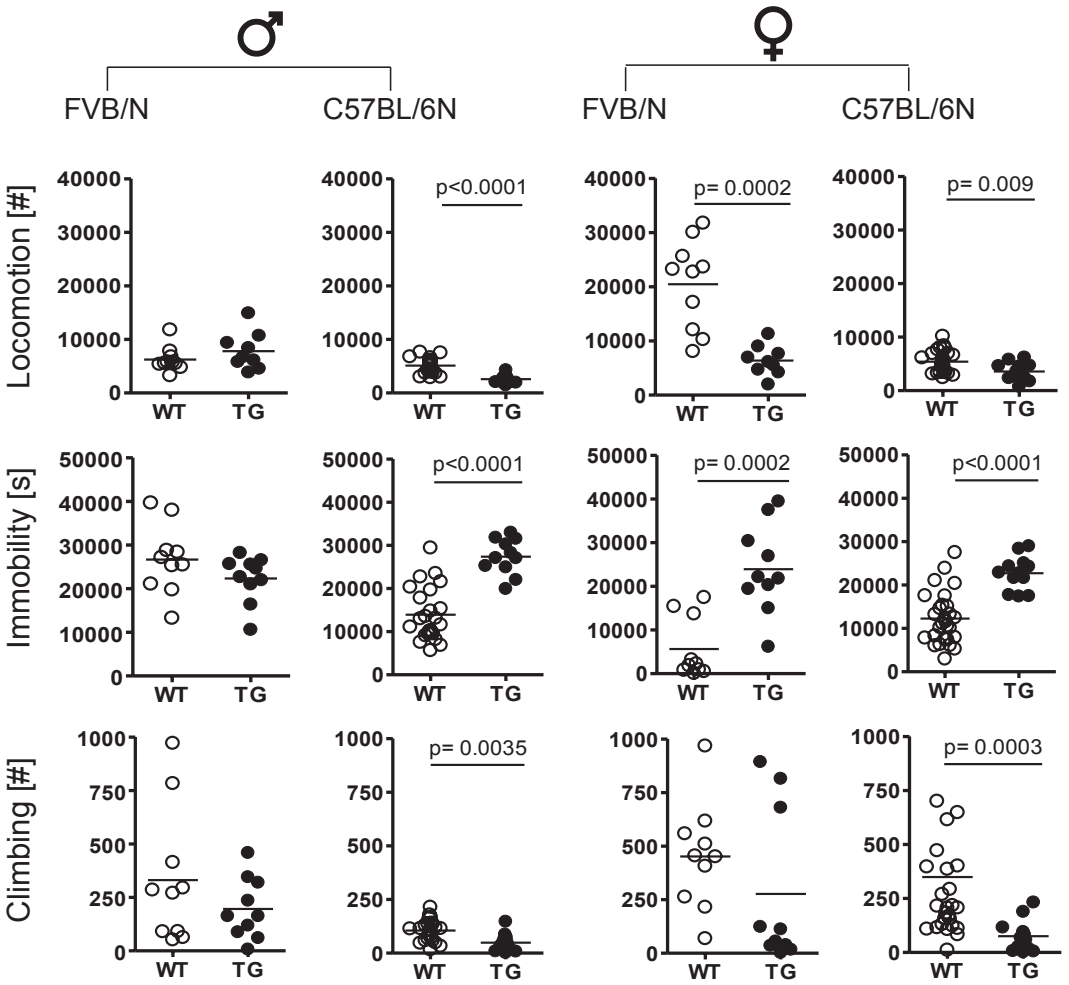


Figure 1. Spontaneous home cage activity of FVB/N and C57BL/6N mice of both genders is modulated by mild Mecp2 overexpression.
Results for male and female mice of both strains are presented. With the exception of male FVB/N mice, locomotion, immobility and climbing reveal similar TG effects across genders and genetic backgrounds. $N = 10$–28; mean ± s.e.m. given.

The unforeseen result of reduced aggression in male TG C57BL/6N prompted us to investigate the pattern of their territorial behavior during the resident–intruder test. We measured frequency and duration of agonistic encounters of the resident mouse for the first 3 min upon introducing the intruder. Whereas the latency to initiate the first contact was comparable between male WT and TG C57BL/6N mice (8.5 ± 1.3 s versus 10.7 ± 1.9 s; $P = 0.35$), TG mice exhibited lower frequency and duration of follow/chase behavior, as well as sniffing of facial and anogenital areas of the intruder mice than their WT littermates (Fig 3).

Since this territorial behavioral pattern of male C57BL/6N TG mice indicated somewhat reduced social interest, we hypothesized that these mice might also display other signs of changed social interest/competence. Indeed, nest building capacity/quality, social dominance measured by the tube test, and ultrasound vocalization in response to an anesthetized female intruder, revealed inferiority or at least a strong tendency thereof in TG animals (Fig 3). Together, these data indicate that also in *Mecp2* overexpressing mice of C57BL/6N background, territorial aggressive behavior is a central target phenotype. In these intrinsically non-aggressive mice, however, the direction of change is exactly opposite to the FVB/N strain and accompanied by reduced social interest and competence.

Seizure propensity is increased upon mild *Mecp2* overexpression, independent of genetic background and gender

A common characteristic neurological phenotype found in different mouse models of mutant *Mecp2,* ranging from complete *loss-of-function* to overexpression, is epileptic seizures (Guy *et al*, 2001; Shahbazian *et al*, 2002; Collins *et al*, 2004; Chahrour & Zoghbi, 2007). We previously reported in male FVB/N TG mice a higher susceptibility to seizure induction by the $GABA_A$ receptor antagonist, pentylenetetrazole (PTZ) (Bodda *et al*, 2013). This work could now be expanded to female FVB/N TG as well as to male and female C57BL/6N TG mice. Indeed, PTZ-induced seizure propensity is increased across genetic backgrounds and genders (Fig 4), pointing to a strong overall phenotypical consequence of mild *Mecp2* overexpression.

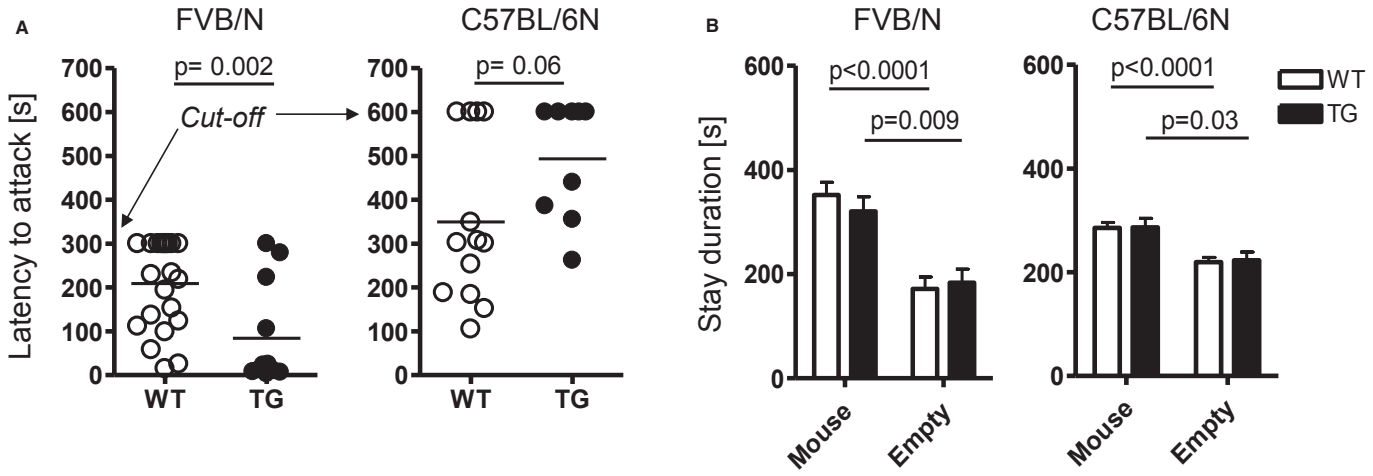


Figure 2. Territorial aggressive behavior in male mice is influenced by Mecp2 overexpression and genetic background.

A Latency to attack in the resident-intruder test is significantly reduced in male FVB/N TG mice but increased in C57BL/6N. Note the different cut-off for the 2 strains.

B Sociability testing in the tripartite chamber reveals a highly significant preference of male mice, independent of the genetic background, for a stranger mouse as compared to an object (empty cage).

Data information: N = 10–24; mean ± s.e.m. given.

The startle response to acoustic stimulation is augmented upon mild *Mecp2* overexpression in female mice of both genetic backgrounds

Deletion of *Mecp2* in GABAergic neurons resulted in reduction in the startle response to acoustic stimuli of 120db (Chao *et al*, 2010), but nothing has been known regarding a potential influence of mild *Mecp2* overexpression on startling. Since *Mecp2* TG mice of both genetic backgrounds exerted an exaggerated seizure reaction to a $GABA_A$ receptor antagonist, PTZ, we wondered whether mild overexpression of *Mecp2* might also alter the startle reflex and/or the prepulse inhibition of the startle response (PPI). Indeed, we found enhanced startling in female but not male *Mecp2* TG mice of both FVB/N and C57BL/6N background (Fig 4). No changes in the percentage of PPI were observed in any strain or gender (Table 1a,b). Similar findings that the startle response at 120db does not necessarily affect overall PPI were reported in the context of a study on mouse inbred strain differences (Paylor & Crawley, 1997).

MECP2 SNP distribution is comparable in healthy and schizophrenic individuals

Since our mouse studies revealed aggressive social behavior as a central phenotype modulated by subtle *Mecp2* overexpression, we started a hypothesis-driven analysis on subjects of the GRAS (Göttingen Research Association for Schizophrenia) data collection (Ribbe *et al*, 2010). Genotyping for association analyses was performed for 4 SNPs located in the X-chromosomal *MECP2* gene with reasonably high minor allele frequency (Fig 5A). At first, a potential genetic risk condition of the selected SNPs for schizophrenia was assessed: A case–control study comparing allele frequencies of the 4 genotyped SNPs rs2239464, rs3027933, rs2075596, and rs2734647 in 1052 GRAS patients versus 1248 healthy controls was performed separately for men and women. All markers fulfilled Hardy–Weinberg criteria, and no significant associations with diagnosis were detected (Fig 5B,C). Due to strong linkage disequilibrium (LD) between markers, only rs2239464 and rs2734647 were considered for the phenotype-based genetic association study (PGAS, inclusion criterion $r^2 < 0.8$) (Fig 5D).

Normal genetic variation of *MECP2* influences aggression and impulsivity in man

We next selected aggression-related variables from the GRAS phenotypical data collection and tested their association with the 2 selected *MECP2* SNPs separately in men and women. *Poor impulse control* and a further trait reflecting behavioral expression of impulsive aggression (*excitement*) were chosen. For both, SNP rs2734647 in the 3′UTR (Table 2) and for SNP rs2239464 (Table 3), significant associations with these traits were found in men, with C carriers and G carriers (the major alleles), respectively, being more aggressive. Sociodemographic and clinical measures potentially confounding these results did not differ between genotype groups. The nominally significant result for years of education in males (SNP rs2734647) was accounted for by including it as a covariate in all models with target measures as dependent variables. In contrast, for women, no statistically significant results were obtained. The phenotype–genotype relationships could be reproduced in a small independent sample of schizophrenic men (Table 4). Taken together, an association of *MECP2* genotypes with readouts of aggression was found in two independent cohorts of men.

SNP rs2734647 in the 3′UTR affects miR-511 binding and gene expression

To examine possible functional implications of SNP rs2734647 in the 3′UTR on microRNA (miR)-dependent regulation of gene expression, *in silico* analyses were performed using TargetScan (Release 6.2) (Lewis *et al*, 2005; Grimson *et al*, 2007) and PITA (Kertesz *et al*, 2007), resulting in the prediction of 4 miRs with seed binding

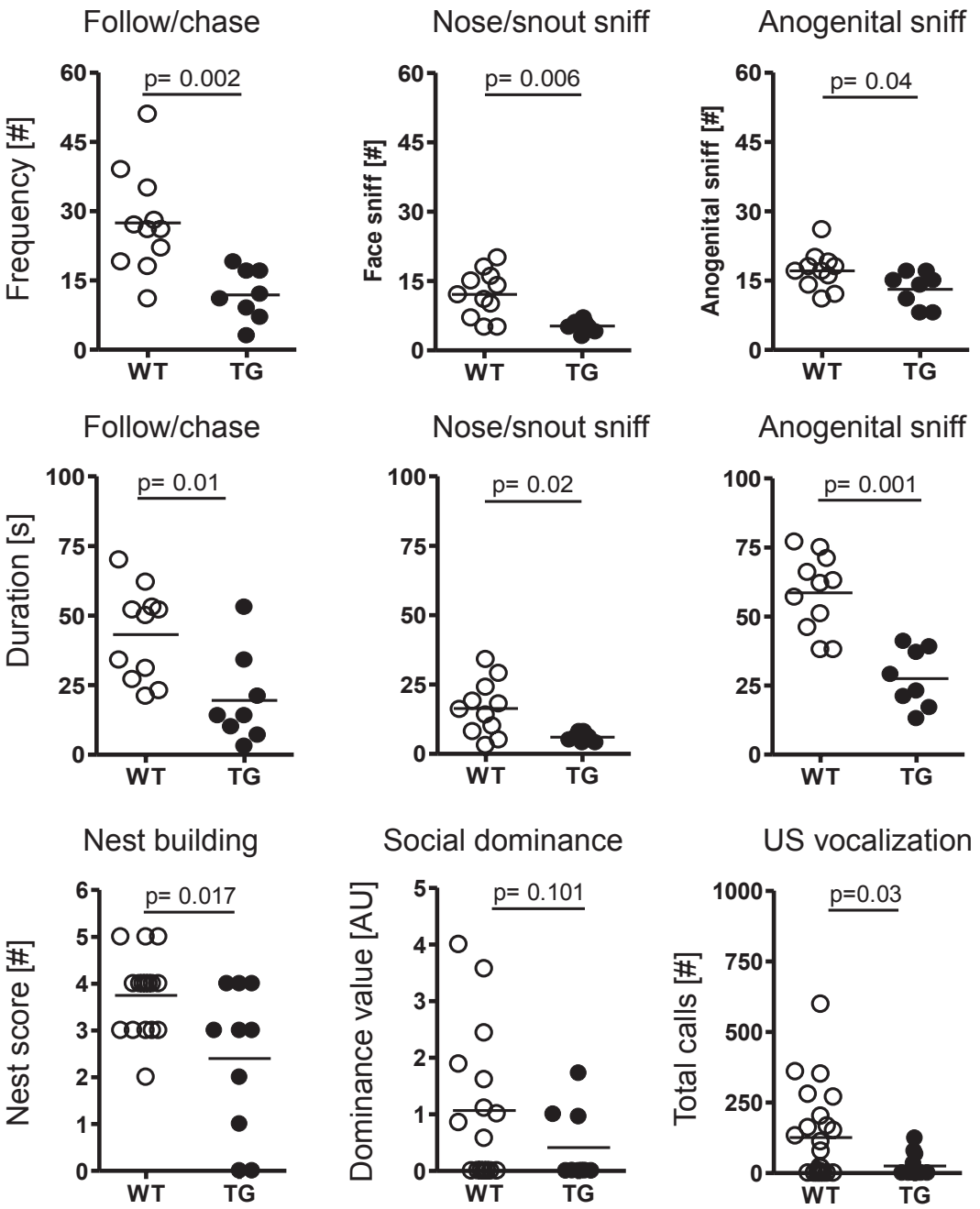


Figure 3. Male TG C57BL/6N mice show reduced territorial social interaction as well as inferior social competence.
Upper 2 rows: Frequency and duration of determinants of territorial social interaction, that is, follow/chase behavior, nose/snout sniff and anogenital sniff, are consistently reduced in TG carriers. Lower row: TG mice are inferior in nest building, social dominance behavior and ultrasound vocalization. $N = 10–23$; mean ± s.e.m. given.

sites comprising the SNP rs2734647 position (i.e., c.*3638A>G). Allele-specific differences of the predicted ΔΔG values, indicating strength of miR binding, are summarized in Fig 6A. While miR-4711-3p and miR-511 are predicted to show preferential binding in case of the presence of the T-allele, miR-515-3p has a strong negative ΔΔG only in case of the C-allele, and miR-519e lacks a strong allele preference. *In vitro* luciferase assays using HEK293 cells revealed significantly reduced luciferase activity in case of co-transfection of the plasmid carrying the T-allele with both miR-4711-3p and miR-511 (Fig 6B). Co-transfection with miR-515-3p or miR-519e did not lead to luciferase activity reduction for either rs2734647 T or C (Fig 6B). In luciferase assays using N2a cells, the positive result was only replicated for miR-511 (Fig 6C). In summary, these data strongly suggest miR-511 as rs2734647 genotype-dependent candidate for *MECP2* regulation in humans. For the sake of completeness, we determined endogenous expression of miR-511

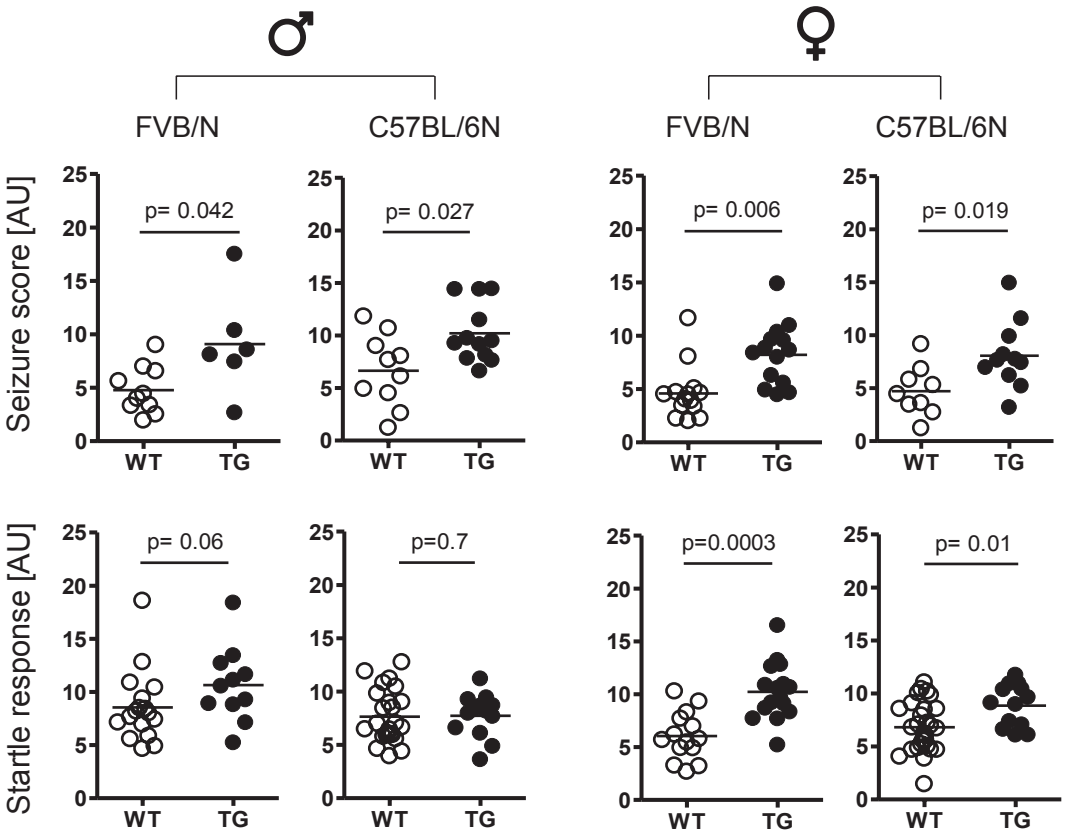


Figure 4. Pentylenetetrazole-induced seizure propensity is increased upon mild Mecp2 overexpression independent of strain and gender, whereas the startle response is augmented in females only.
Upper row: Higher seizure scores are found in TG carriers across gender and genetic backgrounds. $N = 6$–14; Lower row: Significant increase in the startle response is observed only in female mice of both genetic backgrounds. $N = 11$–28; mean ± s.e.m. given.

in the cell lines used for transfection. Whereas mmu-miR-511 levels were under the detection limit in N2a cells, hsa-miR-511 was clearly expressed in HEK293 cells (3.59×10^{-3}). However, since we always used negative, that is, non-miR-transfected controls, Luciferase assay results are unlikely to have been affected.

miR-511 is expressed in aggression-relevant human brain regions

Since miR-511 seems to be an important modulator of rs2734647 genotype-dependent *MECP2* expression in humans, we asked whether this miR would be detectable in brain regions relevant for impulsivity and aggression (frontal and prefrontal cortex, temporal cortex, occipital cortex, hippocampus, and amygdala). Placenta was used as developmental control tissue. In all regions examined, miR-511 expression was found (Fig 6D). Importantly, the same holds true for *MECP2* using primers amplifying either *MECP2*_e2 only or both isoforms (Fig 6E). In contrast, miR-4711-3p expression was not detected (data not shown), at least questioning a role of this miR for MECP2 regulation in the adult human brain. In the adult C57BL/6N versus FVB/N mouse brain, the expression of miR-511 was low and comparable between strains in cortex (2.39×10^{-5} versus 3.71×10^{-5}), hippocampus (6.45×10^{-5} versus 3.78×10^{-5}), and cerebellum (2.22×10^{-5} versus 2.36×10^{-5}). Strong expression was found in embryonic tissue (C57BL/6N embryo head E17: 1.83×10^{-3}; embryo body: 2.26×10^{-3}), and highest in cultured microglia (13.02×10^{-3}).

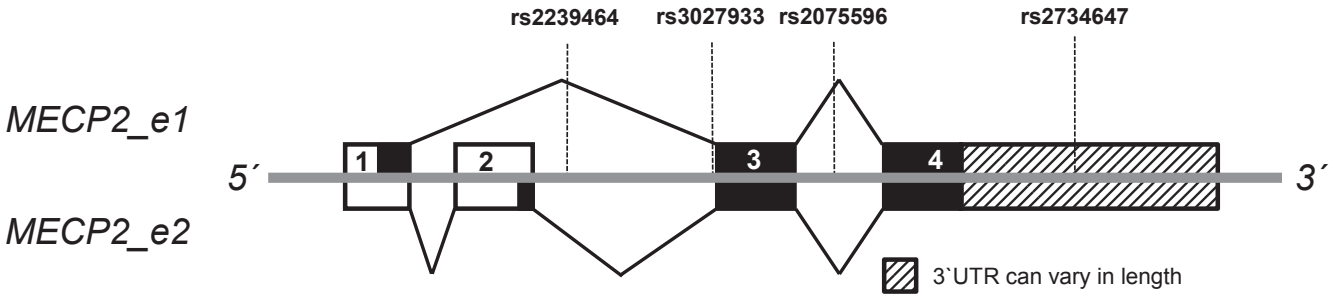
Peripheral blood mononuclear cells (PBMC) of male rs2734647 T-carriers show lower *MECP2* expression

Since miR-511 was shown to be expressed in dendritic cells and macrophages (Tserel *et al*, 2011), we investigated the hypothesized allele-specific downregulation of *MECP2* expression in PBMC of male subjects. Indeed, a significantly lower expression in T carriers versus C carriers (amounting to around 50%) was detected with primers amplifying both *MECP2* isoforms, whereas *MECP2_e2* alone showed no expression difference dependent on the rs2734647 genotype (Fig 6F). This result may indicate that in T carriers mainly the *MECP2_e1* isoform is affected, at least in PBMC, potentially related to isoform-specific different lengths of the *MECP2* 3′UTR (Coy *et al*, 1999).

Sequence variation of miR-511 in mouse and man underlines the importance of an interaction with the *MECP2* 3′UTR

A perfect seed match of human hsa-miR-511 with the *MECP2* 3′UTR in carriers of rs2734647-T is the most likely reason for the difference

A



B

SNP rs#	Test for HWE deviation (females)		
	P (total)	*P* (controls)	*P* (cases)
rs2239464	0.19	0.39	0.41
rs3027933	0.51	0.49	1.00
rs2075596	0.89	0.59	0.68
rs2734647	1.00	0.74	0.70

C

SNP rs#	Genotype	N (%)		*P* (OR, [95% CI])
		GRAS patients	GRAS controls	allelic, d.f.=1
Females				
rs2239464	GG	205 (61.4)	307 (64.0)	0.45 (1.10, [0.86 - 1.40])
	GA	117 (35.0)	158 (32.9)	
	AA	12 (3.6)	15 (3.1)	
rs3027933	CC	236 (70.7)	341 (71.0)	0.80 (1.04, [0.79 - 1.36])
	CG	90 (26.9)	130 (27.1)	
	GG	8 (2.4)	9 (1.9)	
rs2075596	GG	239 (71.3)	344 (71.7)	0.75 (1.05, [0.79 - 1.37])
	GA	87 (26.0)	127 (26.5)	
	AA	9 (2.7)	9 (1.9)	
rs2734647	CC	234 (69.9)	337 (70.2)	0.77 (1.04, [0.80 - 1.36])
	CT	91 (27.2)	132 (27.5)	
	TT	10 (3.0)	11 (2.3)	
Males				
rs2239464	G	582 (81.4)	617 (80.3)	0.60 (0.93, [0.72 - 1.21])
	A	133 (18.6)	151 (19.7)	
rs3027933	C	606 (84.8)	655 (85.3)	0.77 (1.04, [0.78 - 1.39])
	G	109 (15.2)	113 (14.7)	
rs2075596	G	613 (85.2)	660 (85.9)	0.81 (1.04, [0.78 - 1.39])
	A	104 (14.5)	108 (14.1)	
rs2734647	C	608 (84.8)	646 (84.1)	0.71 (0.95, [0.72 - 1.26])
	T	109 (15.2)	122 (15.9)	

D

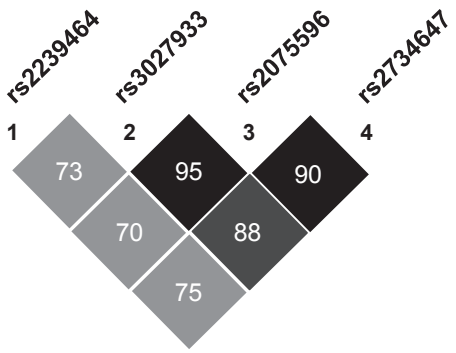


Figure 5. Basic genetics of *MECP2*: Gene structure, Hardy-Weinberg statistics, linkage disequilibrium, and case-control analysis in schizophrenic (GRAS) patients and healthy individuals.

A Schematic overview of MECP2 isoforms e1 and e2, including SNP positions. Digits depict exon numbers, solid black lines exon usage for the respective isoform. Dashed lines denote SNP positions. Black fillings in boxes denote coding sequence, isoform-specific in exons 1 and 2.

B Test for deviation from Hardy-Weinberg-Equilibrium (HWE) in females only due to X-chromosomal location of *MECP2*.

C Case–control association analysis - separate for both genders - reveals similar distribution of SNPs in patients and controls.

D Linkage disequilibrium for all included GRAS patients and controls.

in luciferase activity in contrast to the C-allele, which results in a mismatch in the seed binding region. C is the conserved ancestral allele found in multiple other mammalian species, including mouse. Strikingly, the seed sequence of mouse mmu-miR-511 differs from hsa-miR-511 exactly regarding the nucleotide complementary to the SNP position (Fig 6G). Thus, mmu-miR-511 shows an ideal seed match to the mouse 3′UTR, whereas hsa-miR-511 perfectly fits to the human 3′UTR carrying the T-allele. This observation may emphasize the importance of the miR-511 interaction with *MECP2*. Notably, screening of available data from Jackson Laboratories (http://www.jaxlab.org) revealed that the two mouse lines employed here are not polymorphic for the *Mecp2* 3′UTR allele or the miR-511 sequence. It would, however, be interesting to investigate whether non-inbred mice are polymorphic for the respective alleles.

Discussion

The present study shows that mildly increased *Mecp2/MECP2* expression leads to alterations in male social aggression. Using C57BL/6N versus FVB/N mice (with their known inherent strain differences in aggressive behavior) (Mineur & Crusio, 2002; Pugh *et al*, 2004) as models, we demonstrate that the direction of change upon mild *Mecp2* overexpression in this behavioral target domain is subject to modification by the genetic background. In other words, the resulting lower or higher social aggression depends on the basic genetic make-up of a particular subject. Hints of *MECP2* influencing aggressive behavior could also be shown in two independent cohorts of schizophrenic men, with a polymorphism in the 3′UTR of the gene co-determining both the level of *MECP2* expression as well as of aggression. The genotype-dependent expression difference in

Table 2. Phenotype comparison of GRAS patients by *MECP2* SNP rs2734647 genotypes

Males (GRAS sample)[a]	C	T	*P*-value ($F/Z/\chi^2$ value)[b]
Target variables[c]	*N* = 491 to 608	*N* = 73 to 109	
Poor impulse control, mean ± s.d. [range]	1.72 ± 1.12 [1 to 6]	1.39 ± 0.76 [1 to 4]	**0.0001** (*F* = 14.65)
Excitement, mean ± s.d. [range]	2.05 ± 1.30 [1 to 7]	1.82 ± 1.13 [1 to 6]	**0.034** (*F* = 4.52)
Control variables			
Sociodemographic variables			
Age (at examination), years, mean ± s.d. [range]	37.40 ± 12.09 [17 to 78]	37.02 ± 11.99 [21 to 71]	0.736 (*Z* = −0.34)
Education, years, mean ± s.d. [range][d]	12.11 ± 3.10 [0 to 24]	11.32 ± 2.48 [8 to 18]	**0.020** (*Z* = −2.33)
Unemployment, No. (%)	107 (17.8)	22 (21.0)	0.446 (χ^2 = 0.58)
Clinical variables			
PANSS general score, mean ± s.d. [range][e]	30.57 ± 10.82 [15 to 81]	31.88 ± 9.73 [16 to 55]	0.542 (*Z* = −0.61)
PANSS negative score, mean ± s.d. [range]	18.04 ± 7.67 [7 to 46]	19.23 ± 7.31 [7 to 40]	0.070 (*Z* = −1.81)
PANSS positive score, mean ± s.d. [range][f]	11.61 ± 5.25 [6 to 34]	11.39 ± 5.15 [6 to 30]	0.667 (*Z* = −0.43)
Cognition composite score, mean ± s.d. [range][g]	0.09 ± 0.84 [−2.4 to 2.2]	0.00 ± 0.84 [−2.1 to 1.7]	0.543 (*F* = 0.37)
Chlorpromazine equivalents, mean ± s.d. [range]	688 ± 644 [0 to 4511]	725 ± 734 [0 to 6324]	0.394 (*Z* = −0.85)
GAF score, mean ± s.d. [range]	45.48 ± 16.35 [5 to 90]	46.94 ± 15.45 [15 to 85]	0.375 (*Z* = −0.89)

Females (GRAS sample)[a]	CC	CT	TT	*P*-value ($F/Z/\chi^2$ value)[b]
Target variables[c]	*N* = 194 to 234	*N* = 73 to 91	*N* = 8 to 10	
Poor impulse control, mean ± s.d. [range]	1.73 ± 1.15 [1 to 7]	1.57 ± 0.94 [1 to 5]	1.60 ± 1.07 [1 to 4]	0.729 (*F* = 0.31)
Excitement, mean ± s.d. [range]	2.11 ± 1.30 [1 to 7]	1.93 ± 1.31 [1 to 7]	1.50 ± 0.53 [1 to 2]	0.277 (*F* = 1.29)
Control variables				
Sociodemographic variables				
Age (at examination), years, mean ± s.d. [range]	43.41 ± 12.90 [18 to 76]	41.82 ± 12.47 [19 to 72]	43.52 ± 12.10 [20 to 58]	0.657 (χ^2 = 0.84)
Education, years, mean ± s.d. [range][d]	12.61 ± 3.36 [8 to 27]	12.14 ± 2.83 [8 to 21]	12.15 ± 3.76 [8 to 19]	0.750 (χ^2 = 0.58)
Unemployment, No. (%)	25 (10.9)	12 (13.6)	4 (40.0)	0.176 (χ^2 = 1.83)
Clinical variables				
PANSS general score, mean ± s.d. [range][e]	33.04 ± 12.10 [15 to 69]	32.38 ± 12.25 [15 to 72]	31.30 ± 10.64 [19 to 54]	0.872 (χ^2 = 0.28)
PANSS negative score, mean ± s.d. [range]	18.13 ± 8.50 [7 to 44]	17.82 ± 8.11 [7 to 36]	19.30 ± 9.36 [7 to 39]	0.904 (χ^2 = 0.20)
PANSS positive score, mean ± s.d. [range][f]	12.25 ± 6.03 [6 to 32]	11.41 ± 5.25 [6 to 30]	11.40 ± 4.40 [6 to 18]	0.650 (χ^2 = 0.86)
Cognition composite score, mean ± s.d. [range][g]	−0.02 ± 0.83 [−2.0 to 1.9]	−0.19 ± 0.90 [−2.0 to 2.0]	0.08 ± 0.83 [−1.5 to 1.1]	0.090 (*F* = 2.43)
Chlorpromazine equivalents, Mean ± s.d. [range]	677 ± 839 [0 to 7375]	608 ± 685 [0 to 4375]	640 ± 512 [150 to 1680]	0.614 (χ^2 = 0.98)
GAF score, mean ± s.d. [range]	45.44 ± 19.07 [12 to 90]	47.07 ± 18.60 [8 to 90]	48.80 ± 14.73 [25 to 70]	0.472 (χ^2 = 1.50)

All $P \leq 0.05$ are set in boldface.
GAF, global assessment of functioning
[a]Due to missing data, sample sizes vary.
[b]For statistical methods, Mann–Whitney *U*/Kruskal–Wallis test (women) or Chi-square tests and for models including covariates ANCOVAs (target variables and cognition composite score) were used.
[c]ANCOVA with education, age at examination, negative symptoms (PANSS) and medication status (chlorpromazine equivalent) as covariates.
[d]Years spent in education system; patients currently in school or educational training included (score 0).
[e]Item 14 (target variable) excluded from sum score.
[f]Item 4 (target variable) excluded from sum score.
[g]ANCOVA with age, negative symptoms (PANSS) and medication status (chlorpromazine equivalent) as covariates.

men found in PBMC (around 50%) is in the range of the transgenic overexpression in both mouse strains, emphasizing the physiological significance of these findings as well as of our mouse models for studying behavioral consequences of a normal "*Mecp2* dose range".

Even though in both schizophrenia samples, higher expression of *MECP2* (3′UTR SNP rs2734647 C carriers lack suppressibility by miR-511) was associated with higher aggression, it has to be considered that also humans are not an isogenic population. One might

Table 3. Phenotype comparison of GRAS patients by *MECP2* SNP rs2239464 genotypes

Males (GRAS sample)[a]	G	A	*P*-value (*F*/*Z*/χ^2 value)[b]
Target variables[c]	*N* = 469 to 582	*N* = 94 to 134	
Poor impulse control, mean ± s.d. [range]	1.72 ± 1.12 [1 to 6]	1.44 ± 0.81 [1 to 4]	**0.001** (*F* = 10.84)
Excitement, mean ± s.d. [range]	2.05 ± 1.31 [1 to 7]	1.83 ± 1.11 [1 to 6]	0.056 (*F* = 3.67)
Control variables			
Sociodemographic variables			
Age (at examination), years, mean ± s.d. [range]	37.31 ± 12.07 [17 to 78]	37.27 ± 12.12 [21 to 71]	0.918 (*Z* = −0.10)
Education, years, mean ± s.d. [range][d]	12.10 ± 3.10 [0 to 24]	11.50 ± 2.52 [8 to 20]	0.063 (*Z* = −1.86)
Unemployment, No. (%)	101 (17.6)	28 (21.5)	0.294 (χ^2 = 1.10)
Clinical variables			
PANSS general score, mean ± s.d. [range][e]	31.71 ± 10.84 [15 to 81]	31.36 ± 9.83 [16 to 55]	0.966 (Z = −0.04)
PANSS negative score, mean ± s.d. [range]	18.13 ± 7.66 [7 to 46]	18.61 ± 7.48 [7 to 40]	0.420 (*Z* = −0.81)
PANSS positive score, mean ± s.d. [range][f]	11.67 ± 5.29 [6 to 34]	11.20 ± 4.99 [6 to 30]	0.358 (*Z* = −0.92)
Cognition composite score, mean ± s.d. [range][g]	0.09 ± 0.83 [−2.4 to 2.2]	0.05 ± 0.88 [−2.1 to 1.9]	0.842 (*F* = 0.40)
Chlorpromazine equivalents, mean ± s.d. [range]	691 ± 635 [0 to 4511]	688 ± 700 [0 to 6324]	0.936 (*Z* = −0.08)
GAF score, mean ± s.d. [range]	45.11 ± 16.26 [5 to 90]	45.27 ± 15.7 [15 to 85]	**0.045** (*Z* = −2.00)

Females (GRAS sample)[a]	GG	GA	AA	*P*-value (*F*/*Z*/χ^2 value)[b]
Target variables[c]	*N* = 173 to 206	*N* = 94 to 117	*N* = 10 to 13	
Poor impulse control, mean ± s.d. [range]	1.74 ± 1.18 [1 to 7]	1.62 ± 0.99 [1 to 5]	1.23 ± 0.60 [1 to 3]	0.243 (*F* = 1.42)
Excitement, mean ± s.d. [range]	2.12 ± 1.29 [1 to 7]	1.98 ± 1.31 [1 to 7]	1.62 ± 1.12 [1 to 5]	0.416 (*F* = 0.88)
Control variables				
Sociodemographic variables				
Age (at examination), years, mean ± s.d. [range]	43.65 ± 13.11 [18 to 76]	42.01 ± 12.15 [19 to 72]	42.66 ± 12.37 [20 to 58]	0.665 (χ^2 = 0.82)
Education, years, mean ± s.d. [range][d]	12.66 ± 3.32 [8 to 27]	12.22 ± 3.05 [8 to 21]	11.96 ± 3.28 [8 to 19]	0.538 (χ^2 = 1.24)
Unemployment, No. (%)	20 (10.0)	17 (14.5)	4 (30.8)	0.056 (χ^2 = 5.77)
Clinical variables				
PANSS general score, mean ± s.d. [range][e]	32.92 ± 12.42[15 to 69]	33.11 ± 11.79 [15 to 72]	30.23 ± 11.42 [19 to 54]	0.690 (χ^2 = 0.74)
PANSS negative score, mean ± s.d. [range]	18.07 ± 8.49 [7 to 44]	18.20 ± 8.37 [7 to 39]	17.62 ± 8.48 [7 to 39]	0.961 (χ^2 = 0.08)
PANSS positive score, mean ± s.d. [range][f]	12.28 ± 5.99 [6 to 32]	11.74 ± 5.55 [6 to 32]	10.00 ± 4.26 [6 to 18]	0.440 (χ^2 = 1.64)
Cognition composite score, mean ± s.d. [range][g]	0.01 ± 0.84 [−2.0 to 1.9]	−0.20 ± 0.87 [−2.0 to 2.0]	0.03 ± 0.75 [−1.5 to 1.4]	**0.049** (*F* = 3.06)
Chlorpromazine equivalents, mean ± s.d. [range]	671 ± 852[0 to 7375]	638 ± 702 [0 to 4370]	650 ± 504 [150 to 1680]	0.811 (χ^2 = 0.41)
GAF score, mean ± s.d. [range]	46.07 ± 19.12 [15 to 90]	45.13 ± 18.87 [8 to 90]	51.38 ± 12.35 [25 to 70]	0.323 (χ^2 = 2.26)

All $P \leq 0.05$ are set in boldface.
[a]Due to missing data, sample sizes vary.
[b]For statistical methods, Mann–Whitney *U*/Kruskal–Wallis test (women) or Chi-square tests and for models including covariates ANCOVAs (target variables and cognition composite score) were used.
[c]ANCOVA with education, age at examination, negative symptoms (PANSS) and medication status (chlorpromazine equivalent) as covariates.
[d]Years spent in education system; patients currently in school or educational training included (score 0).
[e]Item 14 (target variable) excluded from sum score.
[f]Item 4 (target variable) excluded from sum score.
[g]ANCOVA with age, negative symptoms (PANSS) and medication status (chlorpromazine equivalent) as covariates.

thus, in analogy to our observation in different mouse strains, predict a human population with reduced aggression as suggested by the C57BL/6N data (i.e., a bimodal aggression distribution when combining different genetic backgrounds).

Although the results for women show a similar tendency for poor impulse control and excitement as aggression readouts, they are far from reaching significance, likely due to the smaller number of individuals, but certainly also to the fact that *MECP2* is X chromosomal. Homozygous T carriers in the female sample are therefore expectedly very rare.

In our translational PGAS approach, we had the chance to explore the aggression association of *MECP2* genotypes in a phenotypically very well-characterized large sample of schizophrenic individuals (Ribbe *et al*, 2010), and we replicated the association findings in a

Table 4. Phenotype comparison of replication sample patients by *MECP2* SNPs rs2734647 and rs2239464

Males (replication sample)	rs2734647 genotypes		*P*-value (Z/χ^2 value)[a]
	C	T	
Target variables[b]	$N = 322$	$N = 63$	
Poor impulse control, mean ± s.d. [range]	3.16 ± 1.91 [1 to 7]	2.86 ± 1.93 [1 to 6]	**0.05** ($Z = -1.64$)
Excitement, mean ± s.d. [range]	3.79 ± 1.60 [1 to 7]	3.52 ± 1.56 [1 to 7]	0.09 ($Z = -1.33$)
Control variables			
Age (at examination), years, mean ± s.d. [range]	35.34 ± 10.91 [19 to 65]	36.63 ± 10.80 [18 to 67]	0.16 ($Z = -1.01$)
Education rating,% low, intermediate, high education level	47.8%, 22.4%, 29.8%	42.9%, 30.2%, 27.0%	0.21 ($\chi^2 = 1.78$)

Males (replication sample)	rs2239464 genotypes		*P*-value (Z/χ^2 value)[a]
	G	A	
Target variables[b]	$N = 308$	$N = 77$	
Poor impulse control, mean ± s.d. [range]	3.19 ± 1.92 [1 to 7]	2.82 ± 1.89 [1 to 6]	**0.03** ($Z = -1.91$)
Excitement, mean ± s.d. [range]	3.83 ± 1.60 [1 to 7]	3.42 ± 1.58 [1 to 7]	**0.02** ($Z = -2.07$)
Control variables			
Age (at examination), years, mean ± s.d. [range]	35.28 ± 11.01 [19 to 65]	36.66 ± 10.40 [18 to 67]	0.12 ($Z = -1.23$)
Education rating,% low, intermediate, high education level	48.7%, 22.1%, 29.2%	40.3%, 29.9%, 29.9%	0.14 ($\chi^2 = 2.53$)

All $P \leq 0.05$ are set in boldface.
[a]For statistical methods, Mann–Whitney *U* or Chi-square tests were used (all *P*-values one-sided).
[b]Mann–Whitney *U*-tests with standardized residuals from linear regression with target variables as dependent variables and age at examination and negative symptoms as independent variables.

second, independent cohort of schizophrenic men. Therefore, we cannot state with certainty at this point that the phenotype association holds true in the same way for healthy individuals. Nevertheless, apart from the supporting data obtained for healthy mice, it has to be emphasized that aggression is not a specific or unique symptom in schizophrenia. Also, the range of aggressive features in the whole GRAS patient sample follows a normal distribution, extending from very low to very high aggression scores.

Along the same lines, the case–control study presented here, including 1052 cases and 1248 controls, fails to attribute to *MECP2* any schizophrenia risk gene role. In some contrast, a recent study reported in a Han Chinese population an association of rs2734647 C with the disease (498 cases versus 2025 controls, replicated in 1027 cases versus 1005 controls) (Wong *et al*, 2013). Although we did not even find a respective trend (Fig 5C), we cannot entirely exclude limited power of our case–control approach. On the other hand, the association might well be population-specific. In any case, a potential risk gene status, even if confirmed by future GWAS including X-chromosomal genotypes, will not be dramatic considering the large number of individuals needed for its demonstration. Instead, *MECP2* is most likely disease-independently involved in the regulation of a basic mammalian behavioral phenotype, that is, aggression. Interestingly, carriers of the minor allele of *MECP2* SNP rs2239464 were previously shown to have decreased cortical surface area in brain regions such as the cuneus (Joyner *et al*, 2009), which is associated with inhibitory control in patients with bipolar disorder (Haldane *et al*, 2008).

In further support of an association between *MECP2* and aggression, impulse control alterations in individuals with *MECP2/Mecp2* gene duplication syndrome have been reported (Ramocki *et al*, 2009), even though the findings of the present study are more relevant for the understanding of physiological gene-dose effects on social behavior. Importantly, Mecp2/MECP2 functions as transcriptional regulator targeting hundreds of other genes (Chen *et al*, 2003; Sun & Wu, 2006; Bird, 2008; Chahrour *et al*, 2008; Ben-Shachar *et al*, 2009; Wu *et al*, 2010). Thus, it is most likely a whole pattern of genes—directly or indirectly influenced by this regulator—that primes nuances of aggressive social behavior. As an example, changes in the expression of a Mecp2 regulated gene, *Prom1,* are associated with domestication and aggressive behavior in animals (Albert *et al*, 2012; Gopisetty *et al*, 2012). Furthermore, Mecp2 is known to control expression of brain-derived neurotrophic factor (Bdnf) (Martinowich *et al*, 2003), which in turn is involved in the regulation of aggression (Ito *et al*, 2011).

Aggression seems to be a strong target phenotype of mild *Mecp2* overexpression independent of the genetic background, since these expression changes did not lead to alterations in basic behavior, including motor, sensory and cognitive functions. Male and female TG mice of both the FVB/N and C57BL/6N genetic backgrounds displayed basic behavior comparable to their WT littermates. Apart from aggression, only home cage activity, seizure propensity and startle response were influenced by mild *Mecp2* overexpression in a fashion widely independent of the genetic background.

There have been reports on sexual dimorphism with respect to *Mecp2* expression and function in the brain. For instance, in amygdalae and ventromedial hypothalamus, male rats express less *Mecp2* as compared to females (Kurian *et al*, 2007, 2008). Furthermore, conditional knockout of *Mecp2* during amygdala development caused subtle modifications of juvenile play behavior in male but not female rats (Kurian *et al*, 2008). These findings may indicate a role of *Mecp2* in gender-specific modulation of behavior. In the present work, however, sexual dimorphism was consistently observed

only with the startle response in a genotype and genetic background independent manner.

Even though the explicit situations are still unknown in which miR-511 regulated *MECP2/Mecp2* expression might be of particular physiological relevance, any kind of inflammation in the brain for instance could play a pivotal role, considering the relatively high expression found here in mouse microglia. The distinct suppression of *MECP2* expression by miR-511 in SNP rs2734647-T carriers reported here may even be considered as a future treatment target in *MECP2* gene duplication syndrome. In any case, the high conservation of the interaction between miR-511 and *MECP2* in both mouse and man makes a specific significance of their interplay very likely. This significance is further supported by the here demonstrated co-expression of *MECP2* and miR-511 in human brain areas pivotal for aggression and impulsivity regulation (Brower & Price, 2001; Horn *et al*, 2003; Berlin *et al*, 2004; Bauman *et al*, 2006; Zetzsche *et al*, 2007; Siever, 2008; Whelan *et al*, 2012). Interestingly, miR-511 expression was found here also in different mouse brain areas, with levels comparable across both genetic backgrounds.

To conclude, MECP2/Mecp2 has been shown here to be a regulator of social aggressive behavior in mouse and man, with the genetic background playing an important modifier role.

Materials and Methods

Mice

All mouse experiments have been approved by the Animal Care and Use Committee of Lower Saxony, Oldenburg, Germany. The generation of $Mecp2^{WT\_EGFP}$ TG mice with a 1.4–1.5-fold *Mecp2* overexpression on FVB/N background has been described in detail previously (Bodda *et al*, 2013). Briefly, a bacterial artificial chromosome (BAC) clone, pBAC_B22804, containing 120 Kb of murine genomic fragment with the intact *Mecp2* gene and the flanking *Opsin1* and *Irak1* genes was used for generating the transgenic construct (Kifayathullah *et al*, 2010). To generate the pBAC_Mecp2WT_EGFP construct, the enhanced green fluorescent protein/kanamycin-resistant gene (*EGFP/kan*) cassette was PCR amplified using pEGFP1 vector as template with primers containing 50-bp flanking sequence from either side of the *Mecp2* stop codon. The endogenous stop codon was replaced by two glycine residues inframe between the Mecp2 protein and the EGFP protein to facilitate the two proteins to fold and function independently. The amplified *EGFP/Kan* cassette was electroporated into E.coli harboring the BAC clone and pGET recombination system, to facilitate the homologous recombination of *EGFP/Kan* cassette at the site of stop codon of *Mecp2*. The correct insertion of *EGFP/Kan* cassette after the recombination event into the BAC clone was confirmed by sequencing. The *Mecp2* flanking genes, *Opsin1* and *Irak1* were deleted from the modified BAC clone using additional BAC recombineering with zeocin selection cassette (containing the BAC homology arms and zeocin antibiotic marker gene driven by EM7 promoter from pSELECT vector) (InvivoGen, Toulouse, France), to avoid any additional phenotype arising from the overexpression of these genes. During the process of *Opsin1* deletion, a MluI restriction site was introduced into BAC clone. The final BAC construct pBAC_Mecp2WT_EGFP was linearized with MluI restriction enzyme and micro-injected into the male pronuclei of the fertilized mouse oocytes derived from the FVB/N strain. Next, the injected oocytes were transplanted into the uteri of the foster mothers. Genomic DNA isolated from tail biopsies was analyzed for the presence of the transgene by PCR. Because the transgene was not confirmed to be localized to the X chromosome, translational relevance with respect to modeling mosaicism resulting from X chromosome loss is limited.

To create a comparator congenic strain for behavioral analyses, *Mecp2* TG FVB/N mice were backcrossed for 10 generations to the C57BL/6N background. For experiments reported here, male and female $Mecp2^{WT\_EGFP}$ TG (hemizygous) and their WT littermates on either FVB/N or C57BL/6N backgrounds were used. Tail biopsies were taken before weaning to obtain genomic DNA for genotyping (Kifayathullah *et al*, 2010; Bodda *et al*, 2013). Western blot and qPCR analyses for TG expression estimation were performed as described previously (Bodda *et al*, 2013).

Behavioral analyses

After weaning and during the whole period of behavioral testing, mice were housed individually in standard plastic cages (26.5 × 20 × 14 cm) and kept under temperature-controlled environment (21 ± 2°C) on 12 h light/dark cycle with food and water *ad libitum*, unless stated otherwise. Single housing was necessary since male FVB/N mice exhibited extremely aggressive behavior in a group-housed setting. In order to avoid housing differences as confounding variables, we decided to single-house all mice, independent of gender and strain. All experiments were conducted by investigators unaware of the genotype ("blinded"), during the light phase of the day (between 8:00 am and 6 pm, except for automated home cage behavioral assessment. Several independent cohorts of mice (genders and strains tested separately, starting at 5 weeks of

Figure 6. SNP rs2734647 in the 3′UTR of MECP2: Search for mechanistic insight.

A Human miRNAs predicted to bind to the *MECP2* 3′UTR in an rs2734647 allele-specific manner. The bases corresponding to the SNP position are black-shadowed. Numbers left and right of the nucleotide sequence refer to its base-pair position within the miRNA sequence.

B, C Luciferase assay results showing relative luciferase activity in HEK293 and N2a cells after co-transfection of candidate miRNAs with phRL-TK rs2734647C, or rs2734647T, respectively; mean ± s.e.m.; $N = 7$ (refers to biological replicates) for all conditions. Statistical significance was calculated relative to the non-transfection control (100%).

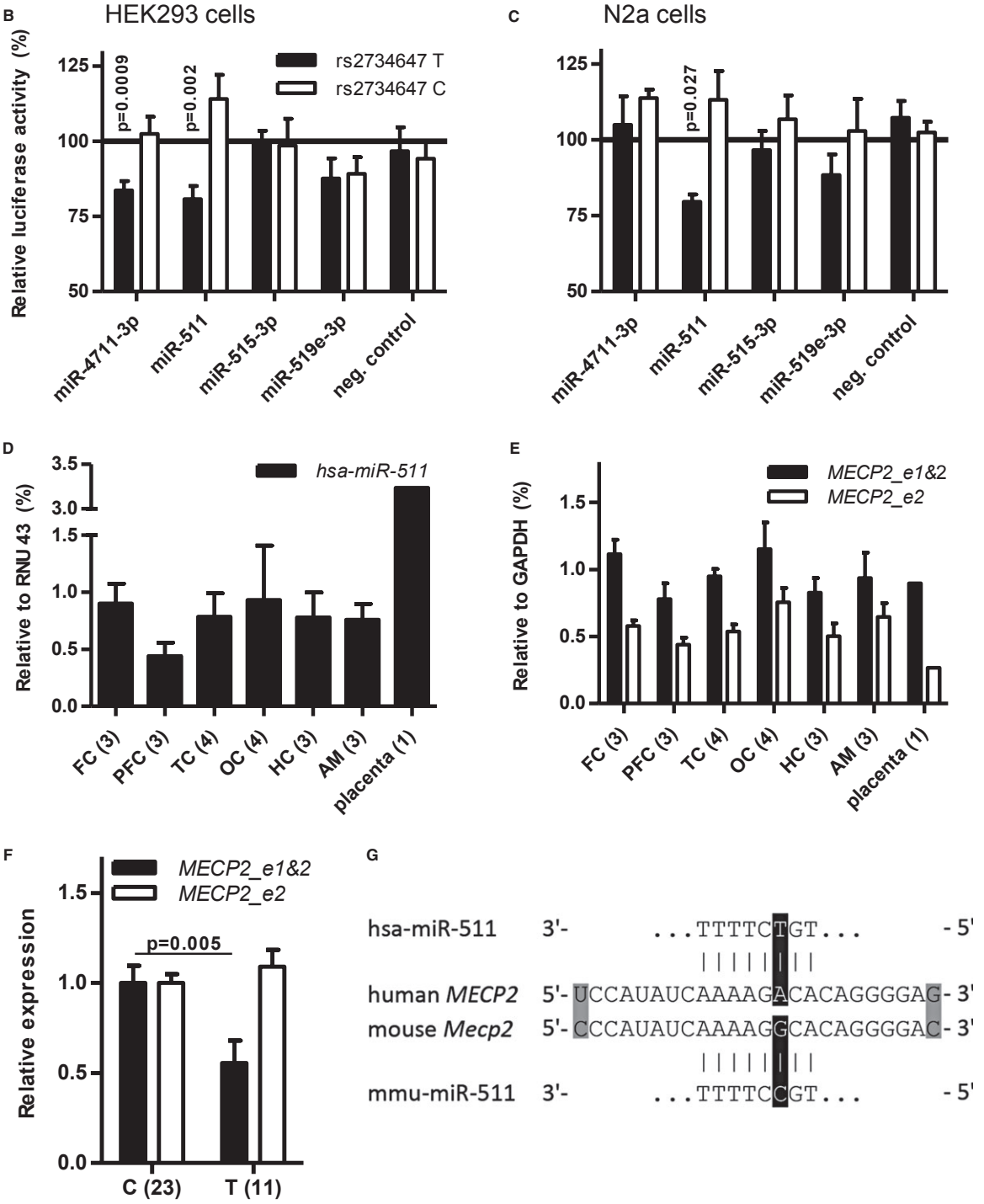
D, E Relative expression of hsa-miR-511 and of MECP2 isoform 2 (*MECP2* e2) or both isoforms (*MECP2* e1&*2*) in aggression/impulsivity – relevant brain areas: FC=frontal cortex, PFC, prefrontal cortex; TC, temporal cortex; OC, occipital cortex; HC, hippocampus; AM, amygdala; as well as in placenta ($N = 1$) as control tissue. Numbers of individual brains included in the analysis are given in brackets; mean ± s.e.m.

F Relative expression of *MECP2* e2 or *MECP2* e1&2 in peripheral blood mononuclear cells (PBMC) of male patients dependent on rs2734647 genotype; N numbers of individuals in brackets; mean ± s.e.m.

G Alignment of human and mouse *MECP2* 3′UTRs around rs2734647 SNP position (black-shadowed) and human and mouse miR-511, illustrating a perfect species-specific seed match; hsa-miR-511 perfectly matches the human *MECP2* 3′UTR in case of rs2734647 T. Additional mismatches are gray-shadowed.

A

miRNA		Mature sequence		ΔΔG value for rs2734647	
				T-allele	C-allele
hsa-miR-4711-3p	45-	CGUGUCUUCUGGCUUGAU	-62	-8.58	-4.85
hsa-miR-511	16-	GUGUCUUUUGCUCUGCAGUCA	-36	-7.82	-4.37
hsa-miR-515-3p	51-	GAGUGCCUUCUUUUGGAGCGUU	-72	NA	-9.48
hsa-miR-519e	52-	AAGUGCCUCCUUUUAGAGUGUU	-73	-4.93	-3.98



age) were run through a battery of tests covering altogether basic behavioral, sensory, motor, cognitive and social functions (for overview see Table 1a,b). The order of tests was always oriented toward increasing invasiveness and performed as published in detail earlier (Jamain *et al*, 2008; El-Kordi *et al*, 2012; Bodda *et al*, 2013): Elevated plus maze (anxiety), open field (spontaneous activity), hole board (exploratory behavior), grip strength and rotarod (motor force, balance and coordination), marble burying (stereotypies and obsessive–compulsive behaviors), prepulse inhibition of the startle response (sensorimotor gating), hearing test (startle curve upon random presentation of stimulus intensities from 65 dB to 120 dB), Y-maze (working memory), novel object recognition, visual cliff (vision), sociability (social preference, i.e., other mouse over object), buried food finding (olfaction), sucrose preference (anhedonia), hot plate test (pain sensation), and hole board (working and reference memory). Moreover, automated home cage behavior analysis (LABORAS™), ultrasound vocalization recording, nest building (social competence), social tube (dominance), resident–intruder paradigm (aggression) and seizure propensity (seizure induction by pentylenetetrazole) were performed as indicated (Table 1a,b). In the following, the here relevant tests (with significant results) will be described in detail, while for the other tests the reader is politely referred to previous publications of ourselves and others (Kuc *et al*, 2006; Jamain *et al*, 2008; Mandillo *et al*, 2008; El-Kordi *et al*, 2012; Bodda *et al*, 2013).

Automated home cage behavior analysis

Automated home cage behavior analysis was performed using LABORAS™ system (Metris b.v., Hoofddorp, the Netherlands), which consists of a triangular shaped sensor platform (Carbon Fiber Plate 1000 mm × 700 mm × 30 mm), 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 × 48 mm). 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 wood-chip bedding covered floors. The cage is placed directly onto the sensing platform, with the upper part of the cage (including top, food hopper and drinking bottle) suspended in a height-adjustable frame separate from the sensing platform. Resultant electrical signals caused by mechanical vibrations as induced by movement of the mouse are transformed by each force transducer, amplified to a fixed signal range, filtered to eliminate noise, digitized and stored on a computer. Stored signals are classified into separate behavioral categories like locomotor activity, immobility and climbing, and quantified by the LABORAS™ software. Prior to each session, LABORAS™ was calibrated. Spontaneous mouse behavior was assessed from 6:00 pm until 9:00am, with 1-h cage habituation prior to initiation of recording. Male (FVB/N: 10TG, 10WT; C57BL/6N: 12TG, 24WT) and female mice (FVB/N: 10TG, 10WT; C57BL/6N: 13TG, 28WT) were tested.

Ultrasound vocalizations (USVs)

Ultrasound vocalizations (USVs) were recorded using a microphone (UltraSoundGateCM16) connected to a preamplifier (UltraSoundGate116), which was linked to a computer. At the day of recording, mice in their home cage (single-housed) were placed in the recording room for 60s. Subsequently, the intruder mouse was put into the resident's cage, and vocalization behavior recorded for 3 min. The intruder mouse was an anesthetized unfamiliar female (anesthetic: intraperitoneal injection of 0.25% tribromoethanol, 0.1 ml/10 g body weight). Number of calls per recording session was counted, and USVs were separated from other sounds using the whistles detection algorithm of Avisoft-SASLab 5.2 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 (El-Kordi *et al*, 2012; Hammerschmidt *et al*, 2012). Avisoft Bioacoustics, Berlin, Germany, delivered all sound recording hardware and software. Male C57BL/6N mice (12TG and 23WT) were tested.

The resident–intruder test

The resident–intruder test was used to study inter-male aggression in various independent cohorts of TG and WT mice of both genetic backgrounds (FVB/N and C57BL/6N) and different age groups, ranging from 8 to 32 weeks (Jamain *et al*, 2008; Bodda *et al*, 2013). As standard opponents (intruders), group-housed males (4 weeks younger than resident test males) of C57BL/6N background were employed (Charles River, Sulzfeld, Germany). An intruder was introduced into the home cage of the test resident. Observation started when the resident first sniffed the opponent and stopped (stop watch) at first attack (defined as bite) to prevent wounding, but lasted for 300s (FVB/N) or 600s (C57BL/6N) if no attack occurred (cut-off) (Mineur & Crusio, 2002; Pugh *et al*, 2004). Male mice (FVB/N: 12TG, 22WT; C57BL/6N: 10TG, 13WT) were tested. Over the first 180s (unless mice attacked before), frequency and duration of following behaviors was additionally quantified in C57BL/6N (10TG, 11WT): nose/snout and anogenital sniff, following/chasing.

The social tube test

The social tube test measures social dominance (Messeri *et al*, 1975; Moretti *et al*, 2005; Garfield *et al*, 2011). The test apparatus comprises a 30-cm-long transparent acrylic tube with an internal diameter of 3 cm. Two mice are placed from opposite ends in the tube and gently pushed to the middle, where they face each other closely. Single-housed TG and WT C57BL/6N mice were challenged with unrelated group-housed mice of C57BL/6N background. A subject was declared "winner" when its opponent completely retreated from the tube ("out") within 300s (cut-off). To account for both, winning/losing and time to win/lose, dominance values are calculated using the following formulas: winner dominance value = 100/time to out and loser dominance value = 1/(300 − time to out). Male C57BL/6N mice (10TG, 16WT) were tested in this paradigm.

Nest building

Nest building is an important behavior in rodents, reflecting social competence in reproduction (Deacon, 2006; Satoh *et al*, 2011; El-Kordi *et al*, 2012). Two hours before dark phase, the nesting towel was removed from home cages of single-housed mice and replaced by a nestlet (pressed 2.7 g cotton square). Nest building quality was

scored in the morning (Deacon, 2006; El-Kordi *et al*, 2012). Male C57BL/6N mice (10TG, 16WT) were tested.

The pentylenetetrazole-induced seizure protocol

The pentylenetetrazole-induced seizure protocol has been described in detail previously (Ferraro *et al*, 1999; Bodda *et al*, 2013; Wojcik *et al*, 2013). Seizure activity was induced in wakeful mice by a single intraperitoneal (i.p.) injection of pentylenetetrazole (PTZ) (50 mg/kg body weight) followed by close observation for 30 min in a small, clear homecage. Latencies to focal (partial clonic), generalized clonic and maximal tonic-clonic behavioral seizures were recorded. Furthermore, 4 phases in the continuum of behavioral response to PTZ injection were defined as follows: (1) Hypoactivity (progressive decrease in motor activity until resting in a crouched or prone position with abdomen in full contact with cage bottom); (2) Partial clonus (clonus seizure activity affecting face, head, and/or forelimb or forelimbs); (3) Generalized clonus (sudden loss of upright posture, whole body clonus involving all 4 limbs and tail, rearing and autonomic signs); and (4) Tonic-clonic (maximal) seizure (generalized seizure characterized by tonic hindlimb extension —also associated with death). Finally, latencies to partial clonus (PC), generalized clonus (GC), and tonic-clonic (TC) seizures were summed to assign each mouse a seizure score that was used as a quantitative trait measure for mapping according to the following equation: Seizure score = (0.2)(1/PC latency) + (0.3)(1/GC latency) + (0.5)(1/TC latency)] × 1000. The weighting factors (0.2, 0.3 and 0.5) in the equation were included as means of incorporating a measure of the progressive nature of the PTZ-induced seizure phenotype into the severity rating because generalized clonus is regarded as a more significant event than partial clonus, and tonic hind limb extension is regarded as the most severe component of the phenotype. Therefore, the seizure score reflects the degree of progression of the seizure phenotype in each mouse.(Ferraro *et al*, 1999) The test was performed on adult *Mecp2* TG of FVB/N and C57BL/6N background (30–40 weeks old). Male (FVB/N: 6 TG, 10 WT; C57BL/6N: 12 TG, 10 WT) and female mice (FVB/N: 14 TG, 14 WT; C57BL/6N: 10 TG, 11 WT) were tested.

Human sample

Schizophrenic patients (discovery sample)

The GRAS (Göttingen Research Association for Schizophrenia) data collection(Ribbe *et al*, 2010) was approved by the ethics committee of the Georg-August-University Göttingen (master committee) and respective review boards of collaborating centers. The project complies with the Helsinki Declaration. Patients fulfilling DSM-IV criteria for schizophrenia or schizoaffective disorder were included regardless of disease stage (acute, chronic, residual, or remitted). All study participants (European Caucasian 95.3%; other 2.0%; unknown 2.7%) and, if applicable, their legal representatives gave written informed consent. Of the included 1052 patients, 68.2% were male ($N = 717$) and 31.8% female ($N = 335$). Average age was 39.14 ± 12.56 years (range 17–78).

Healthy controls

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. 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 $N = 1248$ successfully genotyped control subjects (European Caucasian 97.8%; other 2%; unknown 0.2%), 61.5% were male ($N = 768$) and 38.5% female ($N = 480$). Average age was 37.44 ± 13.23 years (range 18–69).

Independent schizophrenia sample (replicate sample)

To replicate in an independent sample the phenotype–genotype associations found in male schizophrenic GRAS patients, data from male schizophrenic subjects ($N = 385$) of the Munich/Halle collection of Dan Rujescu could be analyzed (Van den Oord *et al*, 2006). Also in this replicate sample, written informed consent had been obtained from all subjects after detailed and extensive description of the study, which was approved by the local ethics committee and carried out in accordance with the ethical standards laid down in the Declarations of Helsinki.

Phenotyping—target variables

All schizophrenic patients of the GRAS data collection were comprehensively phenotyped (Ribbe *et al*, 2010). To prove our hypothesis that *MECP2* genotypes modulate aggressive behavior in human subjects, we selected target variables closely related to (*poor impulse control*) or predicting (*excitement*) aggressive behavior in schizophrenic individuals (Arango *et al*, 1999; Soyka *et al*, 2007; Colasanti *et al*, 2010). To assess the severity of *poor impulse control*, item 14 of the positive and negative syndrome scale (PANSS) (Kay *et al*, 1987) was used ("disordered regulation and control of action on inner urges, resulting in sudden, unmodulated, arbitrary or misdirected discharge of tension and emotions without concern about consequences"). From the subscale covering positive symptomatology, item 4 assessing excitement ("hyperactivity as reflected in accelerated motor behavior, heightened responsivity to stimuli, hypervigilance or excessive mood lability") was employed (Tables 2 and 3). Both PANSS readouts were also available in the independent sample of schizophrenic individuals (replicate sample). The choice of items is supported by the literature: Cheung and colleagues compared aggressive and non-aggressive schizophrenia patients (aggression assessed by the Staff Observation Aggression Scale) with respect to single items of the PANSS (Cheung *et al*, 1997). The largest group difference was found for the PANSS item "poor impulse control". Strikingly, the aggressive group had an average score of 4 (range: 1–7) on this item. Even after controlling for the total level of psychopathology, the associations of "poor impulse control" and aggressive behavior remained significant. Additionally, in a more recent prospective study, "poor impulse control" as measured by PANSS was highly predictive of aggressive behavior (assessed by the Overt Aggression Scale)(Nolan *et al*, 2005).

Phenotyping—control variables

Control variables (and potential confounders) are also presented in Tables 2 and 3. Sociodemographic data (age, years of education, unemployment rate), a cognition composite score and clinical variables describing disease severity were used to characterize the GRAS sample and exclude potential confounding effects explaining

the target phenotype–genotype associations. The cognition composite score(Begemann *et al*, 2011) represents the mean of 3 z-standardized neuropsychological measures of higher cognitive functioning: reasoning ability (Leistungsprüfsystem subtest 3; Horn, 1983), executive functioning (Trail-Making Test B; Reitan, 1958) and verbal learning and memory (Verbal Learning and Memory Test; Helmstaedter *et al*, 2001). As further clinical variables, the general, positive and negative scores of the PANSS (target variables excluded from respective scores), chlorpromazine equivalents (standardized dosage of antipsychotic medication) (Rijcken *et al*, 2003; Woods, 2003) and Global assessment of functioning (GAF; Wittchen *et al*, 1997) as measure of impaired psychological, social and occupational performance were used.

DNA extraction, normalization, and genotyping

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 50 ng/μl with an automated robotic platform (Microlab Star, Hamilton, Bonaduz, Switzerland). For quality control, each sample was analyzed with a 0.8% agarose gel. Genotyping was performed using SimpleProbes (TIB Molbiol, Berlin, Germany) on LightCycler 480 (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions.

Transfection studies

Cell lines

Human embryonic kidney 293 (HEK293) and mouse neuroblastoma (N2a) cells were maintained in DMEM supplemented with 1 g/l glucose, L-glutamine (Glutamax), 5% fetal calf serum, 100U/ml penicillinG sodium and 100 μg/ml streptomycin sulfate. For luciferase assays, cells were seeded into 12-well plates (220,000 cells/well/2 ml medium), cultured for 24 h and co-transfected using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany), following the manufacturer's instructions.

Luciferase reporter constructs and detection assay

The reporter plasmids phRL-TK rs2734647C, or rs2734647T, respectively, were constructed by cloning a 3′UTR fragment of 346 bp and including the SNP downstream of the Renilla luciferase open reading frame, making use of the XbaI restriction site of phRL-TK (forward primer: 5′-ATTATCTAGACCAGGTCTACCCCTCCCGGC-3′, reverse primer: 5′-ATTATCTAGAGGCTGCTCCCTGTCCCAGGT-3′). Sequence integrity was verified using Sanger sequencing. Of the Renilla luciferase reporter construct (phRL-TK rs2734647C, or rs2734647T, respectively), 1 μg (per well), plus 1 μg (per well) of the reference construct pCMV-LacZ (Clontech, Mountain View, CA, USA) were co-transfected in the presence of 10 pg of mirVana miRNA mimic hsa-miR-4711-3p, hsa-miR-511, hsa-miR-515-3p, hsa-miR-519e-3p, negative control #2 (all Life Technologies, Darmstadt, Germany), or no miRNA, respectively. After 24 h, cells were split in 96-well plates, creating four technical replicates for each condition, and separately for luciferase and beta-galactosidase measurement. Enzyme activity was determined using a Mithras LB 940 Plate Reader (Berthold, Bad Wildbad, Germany). Renilla luciferase activity was normalized to beta-galactosidase activity.

RNA isolation and quantitative PCR

Peripheral blood mononuclear cells (PBMC) were isolated from citrate blood of men with rs2734647C or rs2734647T genotype (X-chromosomal gene), applying a standard isolation procedure (Ficoll-Paque Plus, GEHealthcare, München, Germany). Human RNA was extracted from deep-frozen human brain samples of adult male subjects who had been free of neuropsychiatric diseases, from placenta or PBMC, respectively, using a miRNeasy Mini kit (Qiagen, Hilden, Germany). The same kit was employed for isolation of mouse RNA from whole E17 embryo (divided into body and head), hippocampus, amygdala, placenta, and cultured microglia. Synthesis of cDNA was done by the SuperScriptIII system (Invitrogen, Karlsruhe, Germany). Detection of *MECP2* cDNA was performed using SYBR green (Roche, Diagnostics GmbH, Mannheim, Germany) and specific primer pairs amplifying the *MECP2*_e2 isoform (NCBI reference sequence NM_004992), spanning exons 2–3 (forward primer: 5′- CA GCTCCAACAGGATTCCAT-3′, reverse primer: 5′- TGGAGGTCCTGG TCTTCTGA-3′), or both isoforms (NM_004992 and NM_001110792), spanning exons 3–4 (forward primer: 5′- AGCTTAAGCAAAGGAAAT CTGG-3′, reverse primer: 5′-GCTTTTCCCTGGGGATTG-3′). Specific Taqman microRNA assays were used to detect hsa-miR-511, hsa-miR-4711-3p, or mmu-miR-511 (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. *MECP2* expression levels were normalized to *GAPDH*, human miRNA expression levels to RNU43, and mouse miRNA expression levels to sno-142.

Statistics

All experimental data acquisition was done by experimenters unaware of group assignment ("'blinded"). Mouse behavioral data were analyzed by Mann–Whitney *U*-test or 2-way analysis of variance including *post hoc* Bonferroni testing, where applicable, using Prism4 (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± s.e.m., unless stated otherwise. Luciferase assay results from each experiment were normalized to the relative luciferase activity without miRNA co-transfection and analyzed using one-tailed unpaired *t*-tests. qPCR results were normalized to the respective control genes and analyzed using one-tailed unpaired *t*-tests. PLINK (v1.07) (Purcell *et al*, 2007) was used for the analysis of statistical association between single SNPs and case or control status (allelic test), and to test for deviations from Hardy–Weinberg equilibrium. Statistical analyses of phenotype–genotype associations in the human samples (both GRAS and replicate sample) were performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA; http://www.spss.com). As *MECP2* is X-linked, analyses were performed separately for men and women. Genotype differences with respect to the target variables were assessed by analysis of covariance. Covariates age at examination, years of education, chlorpromazine equivalents and severity of negative symptoms (PANSS) were used, as these parameters are likely to influence the extent to which impulsive aggressive behavior becomes obvious in a social situation. For the cognition composite score, analysis of covariance with covariates age, chlorpromazine equivalents and severity of negative symptoms was applied as these variables have been widely reported to confound performance on neuropsychological tests(Bilder *et al*, 1992; Hori *et al*, 2006). Genotype differences

The paper explained

Problem
The transcriptional regulator methyl-CpG-binding protein2, MECP2, is renowned because of the devastating neurodevelopmental disorder Rett syndrome, caused by partial or complete loss of its function. The very same gene, when duplicated, induces a similar disorder, indicating the necessity of tight *MECP2* regulation. Among the vast array of other genes influenced by MECP2, many are involved in modulating behavior. Surprisingly, nothing is known on the physiological (i.e., non-disease-related) effects on behavior of subtle *MECP2* expression differences.

Results
We present here a translational study that explores behavioral consequences of mildly increased *MECP2* expression across species. We find that the behavioral target domain directed by MECP2 is male social aggression. This in turn is subject to modification by the genetic background, as we demonstrate by comparing an inherent aggressive with a less aggressive mouse strain. In two independent cohorts of schizophrenic individuals,that is, a discovery and a replicate sample, we identify a role of *MECP2* in aggressive human behavior and show that a polymorphism in the untranslated region of the gene determines binding efficiency of another brain-expressed regulator, microRNA-511. Notably, the genotype-dependent expression difference of 40–50% (rs2734647-C>T) in man, at least partially mediated by microRNA-511, is comparable to the level of transgenic overexpression in our mice, emphasizing the physiological significance of these findings.

Impact
Genetic regulation of behavior is still poorly understood. We explore for the first time behavioral consequences of mildly (~50%) increased *MECP2* expression in mouse and man. This is achieved by transgenic *Mecp2* overexpression in mice and—in parallel—through genetic variation-induced non-regulability of *MECP2* gene expression (rs 2734647-C: no miR-511-mediated *MECP2* downregulation) in humans. We find MECP2 to be a modulator of social aggression. While the mouse data shows an interaction between genetic background and *Mecp2* expression on behavior, the human data provides means by which genetic variation may affect *MECP2* expression and result in behavioral change.

with respect to sociodemographic and clinical measures were tested non-parametrically using chi-square (nominal variables) or Mann–Whitney U/Kruskal–Wallis tests (continuous variables). All P-values derived from statistical models for the GRAS sample are two-sided (Tables 2 and 3). For the replicate sample one-sided P-values are displayed (Table 4). Nominal significance level for all analyses was set to $P < 0.05$.

Supplementary information for this article is available online: http://embomolmed.embopress.org

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Author contributions

Conceived and designed the study: HE, MT, CH, AK. Prepared the data: MT, CH, AK, MB, LD, CB, KH, BS, ACV, BE, IG, AT, BK, AH, WSS. Analyzed the data: MT, CH, AK, LD, KH, DR, AUM. Wrote the paper: HE, MT, AK, CH. Critical review and approval of the manuscript: all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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8.2 Curriculum vitae

Personal data

Name	Beata Stepniak
Date of birth	18th January 1983
Place of birth	Görlitz
Nationality	German
E-mail	stepniak@em.mpg.de

Education

2011 - present **Max Planck Institute of Experimental Medicine** (Göttingen, Germany)
PhD position in the Clinical Neuroscience group

- PhD thesis: 'Contribution of genetic and environmental factors to symptomatology in psychiatric disorders'
  Supervised by Prof. Dr. Dr. Hannelore Ehrenreich

2011 - present **Georg August University Göttingen** (Göttingen, Germany)
PhD student at the Göttingen Graduate School for Neurosciences, Biophysics and Molecular Biosciences (GGNB)

- PhD program 'Systems Neuroscience'

2002 - 2008 **University of Leipzig** (Leipzig, Germany)
Diploma in Psychology

- Diploma thesis: 'Depression, anxiety and post-traumatic stress disorder in breast cancer patients in general – and on the basis of personality types.'
  Supervised by PD Dr. Marcus Roth
- Focus: Clinical and educational psychology

2000 - 2002 **Friedrich-Ludwig-Jahn Gymnasium** (Grossengottern, Germany)
Abitur

1999 - 2000 **Peers Oxford's Technology College** (Oxford, UK)
GCE Module Mathematics

Work and practical experience

2009 - 2010 **Oxford University** (Oxford, UK)
Graduate Research Assistant at the Department of Experimental Psychology

- Running experiments with babies at the BabyLab using the preferential looking paradigm (IPL) as well as eyetracking. Assisting in running experimental sessions for ERP.

2008 (May - July) **University of Leipzig** (Leipzig, Germany)
Seminar Lecturer: Diagnostic Assessment Practical

- Taught diagnostic assessment practical to undergraduate psychology students, graded projects and gave personal feedback.

9. LIST OF PUBLICATIONS

Stepniak B.*, Papiol S.*, Poggi G.*, Hartmann A., Kästner A., Begemann M., Benseler F., Matuszko G., Brosi C., Fischer U., Dityatev A., Rujescu D., Ehrenreich H. (2015) Common variants in the fragile-X gene family modulate autistic features. In preparation.

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