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A CAG repeat polymorphism of *KCNN3* predicts SK3 channel function and cognitive performance in schizophrenia

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KCNN3, encoding the small conductance calcium-activated potassium channel SK3, harbours a polymorphic CAG repeat in the amino-terminal coding region with yet unproven function. Hypothesizing that *KCNN3* genotypes do not influence susceptibility to schizophrenia but modify its phenotype, we explored their contribution to specific schizophrenic symptoms. Using the Göttingen Research Association for Schizophrenia (GRAS) data collection of schizophrenic patients ($n = 1074$), we performed a phenotype-based genetic association study (PGAS) of *KCNN3*. We show that long CAG repeats in the schizophrenic sample are specifically associated with better performance in higher cognitive tasks, comprising the capacity to discriminate, select and execute ($p < 0.0001$). Long repeats reduce SK3 channel function, as we demonstrate by patch-clamping of transfected HEK293 cells. In contrast, modelling the opposite in mice, i.e. *KCNN3* overexpression/channel hyperfunction, leads to selective deficits in higher brain functions comparable to those influenced by SK3 conductance in humans. To conclude, *KCNN3* genotypes modify cognitive performance, shown here in a large sample of schizophrenic patients. Reduction of SK3 function may constitute a pharmacological target to improve cognition in schizophrenia and other conditions with cognitive impairment.

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INTRODUCTION

SK3, also known as $K_{Ca}2.3$, belongs to the family of tetrameric, small conductance calcium-activated potassium channels (SK) and is encoded by the *KCNN3* gene on human chromosome 1q21.3. This gene is characterized by a polymorphic CAG repeat in the *N*-terminal coding region (Chandy et al, 1998) whose functional significance has not been elucidated yet (Bond et al, 2000; Frei et al, 2006).

Small conductance calcium-activated potassium channels are widely disseminated in the central nervous system and in peripheral tissues (Kohler et al, 1996). In the brain, SK1, SK2 and SK3 are expressed in a partially overlapping but distinct pattern, with comparable regional distributions in rats (Kohler et al, 1996; Stocker & Pedarzani, 2000), mice (Sailer et al, 2004), and humans (Dror et al, 1999; Rimini et al, 2000). SK3 mRNA expression is sparse in the neocortex, but prominent in lateral septal and septohippocampal nuclei, amygdala, thalamus, caudate-putamen, substantia nigra pars compacta, in mono-aminergic neurons in the brain stem, including ventral tegmental area, dorsal raphe nucleus, locus coeruleus, hypothalamus and in Golgi interneurons in the cerebellum (Stocker & Pedarzani, 2000). Expression of SK3 in hippocampus is particularly found in the dentate hilus and the stratum lucidum of CA3 (Sailer et al, 2004). In the ventral midbrain, SK3 mRNA is confined to areas that contain dopaminergic neurons (Sarpal et al, 2004).

Neuronal SK channels are involved in the regulation of excitability and firing patterns, neurotransmitter release, and synaptic plasticity (for reviews see (Faber, 2009; Pedarzani & Stocker, 2008; Stocker et al, 2004)). In dopaminergic neurons of the substantia nigra, SK3 controls frequency and precision of intrinsic pacemaker activity as shown in mouse brain slices (Wolfart et al, 2001). Apamin, a selective SK channel blocker, enhances bursting activity of these neurons in rats *in vivo* (Ji & Shepard, 2006). Due to the lack of SK subtype-specific pharmacological agents, the differential contribution of SK1, SK2 and SK3 to behaviour and cognition is difficult to assess, but mouse models have shed some light on SK3 function. For example, SK3 is upregulated in hippocampus of aged mice, and its downregulation by antisense oligonucleotides reverses age-related deficits in hippocampus-dependent memory tasks and long-term potentiation (Blank et al, 2003). Abnormal respiration after hypoxia and disturbed parturition were reported in SK3 overexpressing mice but no striking deficits were identified in SK3 null mutants (Bond et al, 2000). However, doxycycline-dependent SK3 null mutant mice exhibited increased extra-cellular striatal dopamine, enhanced hippocampal serotonin release, and reduced hippocampal brain-derived neurotrophic factor (BDNF) expression. These mice, exposed to chronic high-dose doxycycline feeding, showed also alterations in tests of depressive behaviour and cognition (Jacobsen et al, 2008, 2009).

Even though the functional significance of a variable glutamine repeat length in the SK3 channel protein has remained unclear, it has been investigated in the context of certain pathologies in humans. An association of the CAG repeat length of *KCNN3* has been discussed for anorexia nervosa (Koronyo-Hamaoui et al, 2002, 2007), migraine (Curtain et al, 2005; Mossner et al, 2005), ataxia (Figueroa et al, 2001), epilepsy (Sander et al, 1999; Vijai et al, 2005) and schizophrenia (Chandy et al, 1998) but most results remain quite equivocal. Regarding schizophrenia, a disease-associated excess of longer CAG repeats was reported (Chandy et al, 1998). In contrast, family-based studies claimed a connection between shorter CAG repeats and schizophrenia (Stober et al, 1998). A meta-analysis concluded that overall, the CAG repeat length of *KCNN3* does not augment the risk of schizophrenia, although a small but significant risk appeared associated with CAG repeats longer than the modal value (Glatt et al, 2003). On the other hand, a longer CAG repeat length has been linked to increased negative symptoms in a British schizophrenic sample (Cardno et al, 1999), and in Jewish schizophrenic patients, where also anergia and paranoid symptoms were found associated (Ritsner et al, 2002).

Based on the above delineated effects of SK3 on cognition in mice (Blank et al, 2003), we hypothesized that SK3 genotypes also influence cognitive performance in humans. In particular, we assumed that SK3 genotypes might modify higher cognition in schizophrenia rather than contributing to the actual risk of developing the disease. The Göttingen Research Association for Schizophrenia (GRAS) data collection enables us to follow this hypothesis (Begemann et al, 2010; Ribbe et al, 2010). With >1000 comprehensively phenotyped schizophrenic patients and >3000 data points *per* subject, the GRAS data collection is an exceptional basis to study genetic causes of or contributions to the schizophrenic phenotype in a 'phenotype-based genetic association study (PGAS)'. This approach is different from and complementary to the genome-wide association studies (GWAS) on schizophrenia as a disease. Rather than searching for 'schizophrenia risk genes', we explore the contribution of genetic variants of candidate genes to schizophrenia-relevant phenotypes.

We show here for the first time that a long CAG repeat length in the *KCNN3* gene leads to an electrophysiologically detectable reduction in SK3 conductance. Importantly, long repeat lengths are associated with better cognitive performance of schizophrenic patients in tasks involving the capacity to discriminate, select and execute. Increased SK3 channel activity in turn, as modelled by *KCNN3* overexpression in mice, selectively leads to remarkable deficits in a comparable set of higher brain functions.

RESULTS

Case-control study: the CAG repeat length in the *KCNN3* gene is not associated with schizophrenia

The CAG repeat polymorphism in the *KCNN3* exon 1 coding region has been described in several primate species (Fig 1A and B). We first conducted a case-control study to explore a potential role of the *KCNN3* CAG repeat lengths sum of both alleles as a genetic risk factor for schizophrenia. No significant difference in the distribution of repeat lengths sum between cases ($n = 1060$)
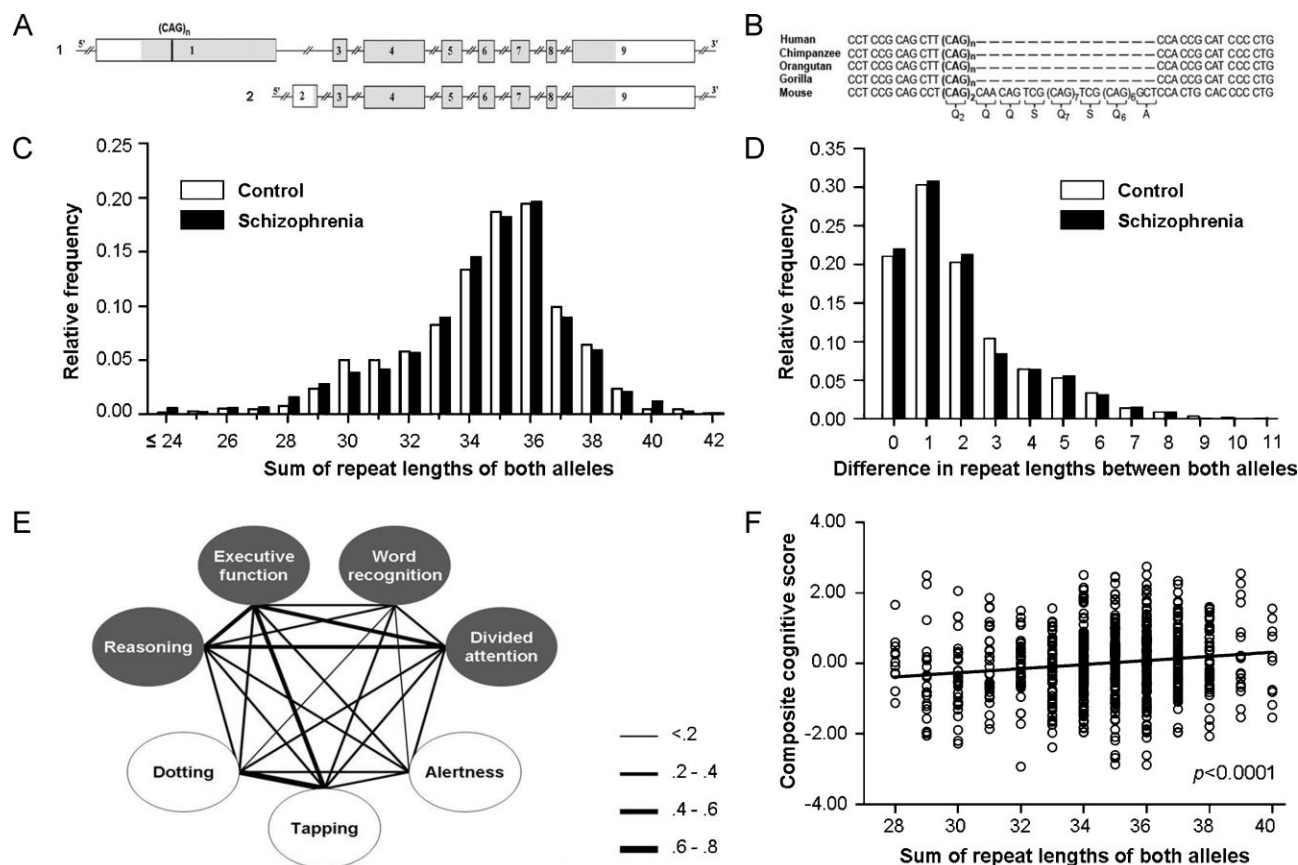
Figure 1. SK3 CAG repeat lengths sum is associated with higher cognitive function in schizophrenia but does not constitute a genetic risk factor for the disease.

A. *KCNN3* is located at 1q21.3 and spans 162.8 kbp. The nine exons (boxes) encode two different splicing variants (1, 2); the coding region is shaded in grey. The black line in exon1 indicates the position of the polymorphic CAG repeat.

B. The region around the CAG repeat is highly conserved among species.

C,D. Neither the distribution of the individual sum of repeat lengths of both alleles (C) nor that of the individual difference between repeat lengths of both alleles (D) is different between schizophrenic patients ($n = 1060$) and healthy controls ($n = 1135$). Hence, these readouts of the SK3 CAG repeat polymorphism do not support a genetic risk for developing schizophrenia.

E,F. In contrast, the PGAS approach allows identification of a role for the SK3 CAG repeat polymorphism in higher cognitive function.

E. Intercorrelation network of cognitive target variables (dark ovals) and cognitive control variables (light ovals) in the GRAS population of schizophrenic patients. Line thickness indicates the degree of correlation between two respective tests after standardization by Blom transformation and adjustment for covariates sex, age, antipsychotic medication and negative symptoms.

F. Scatter plot of the covariate-adjusted composite score calculated as mean of all standardized (Blom transformed) cognitive target variables. Adjusted was for covariates sex, age, antipsychotic medication and negative symptoms. Linear regression analysis reveals a significant effect ($p < 0.0001$) of allelic repeat lengths sum on the composite score.

and healthy controls ($n = 1135$) was found (Fig 1C; $\chi^2 = 5.69$, $p = 0.82$, evaluated with Monte Carlo sampling on 1000 runs; for details see Supporting information). Also, no gender influence was observed. An association analysis of single allele repeat lengths instead of allelic repeat lengths sum between cases and controls did not yield significant distribution differences either (data not shown). Furthermore, the intra individual difference of repeat lengths as a measure of marker heterogeneity did not vary significantly between cases and controls (Fig 1D; $\chi^2 = 4.12$, $p = 0.65$, 1000 Monte Carlo simulations). Thus, as assumed, there is no evidence for a role of the SK3 CAG repeat length in the risk to develop schizophrenia.

Phenotype-based genetic association study: the *KCNN3* CAG repeat length is associated with higher cognition in schizophrenia

Since the essence of the PGAS approach is not to identify potential risk genes for schizophrenia but to understand the contribution of a particular genotype to normal and to disease phenotypes, we moved on to investigate the impact of *KCNN3* on core phenotypes of schizophrenia, i.e. cognition, positive and negative symptoms. Following our hypothesis that SK3 may influence higher cognitive functions, we analysed the CAG repeat polymorphism of the *KCNN3* gene with respect to neuropsychological test performance of the GRAS sample of schizophrenic patients. We first constructed

an intercorrelation network, comprising readouts of higher cognition (encompassing capabilities of discrimination, selection and execution) as target variables, and tests of motor-dependent basic cognitive functions as cognitive control variables (Fig 1E). Target variables included reasoning (LPS3), executive function (TMT-B), word recognition (VLMT) and divided attention (TAP). The target variables were internally consistent (Cronbach's $\alpha = 0.703$), and used for the multivariate models. Cognitive control variables were alertness (TAP), dotting and tapping, all correlating with the cognitive target variables but likely differently regulated. To investigate the influence on general intelligence, a test for premorbid intelligence (MWT-B) was added as additional cognitive control variable. In addition to cognition, PANSS positive and PANSS negative symptoms were analysed as disease-related control variables. Body length served as disease-unrelated control variable (for a detailed description of all variables see Supporting information). Sample characteristics regarding sociodemographic and clinical variables are displayed in Table 1.

The distribution of allelic repeat lengths sum and allelic repeat lengths difference in the schizophrenic population is displayed in Table S1. Only the allelic repeat lengths sum proved to be a genetic readout associated with our phenotypes of interest. The influence of the sum of the repeat lengths of both alleles was studied (i) by a multivariate regression model considering the respective target or control phenotypes as correlated outcomes (Table 2) and (ii) by a classification model (Table S2). Additionally, uncorrected raw data is presented in Table S3. The slope of the regression model estimates the change of covariate-adjusted mean phenotype value *per* additional repeat in the sum of repeat lengths of the two alleles. The classification model estimates the difference in the covariate-adjusted phenotype means between groups with low (below-median; <35) and high (above-median; >35) repeat

lengths sum. The results of the regression and the classification model agree well. Both models show consistently that higher sums of CAG repeats are associated with higher scores (better performance) in the cognitive target but not the cognitive control variables including premorbid intelligence, representing the development-dependent intellectual state at disease onset. Also, PANSS positive, PANSS negative and disease-unrelated control variables are not influenced by repeat sums. Additional individual univariate analyses confirmed that three out of four single target variables included in the multivariate analysis are significantly associated with CAG repeat lengths sum, while this is not the case for any single control variable. For illustration, the influence of the repeat lengths sum on the composite cognitive phenotype is displayed as a simple regression line in Fig 1F. To conclude, the PGAS approach reveals a role of the SK3 CAG repeat lengths for higher cognition, best characterized as the cognitive steps 'discriminate, select and execute', but not for psychopathological symptoms of schizophrenia.

Translational approach: SK3 overexpressing mice show selective impairment in higher cognitive function

Based on above findings and earlier data indicating that SK3 negatively regulates cognition (Blank et al, 2003), we hypothesized that a long CAG repeat length would result in a less functional SK3 channel and better cognition, while a short repeat length would lead to more efficient SK3 function and worse cognitive performance. To test this hypothesis, we analysed basic behaviour and cognition in a transgenic mouse line in which the murine SK3 gene is overexpressed under control of its own regulatory elements, enhanced in *cis* by the tetracyclin-dependent transactivator (tTA) in the absence of any doxyclin (Bond et al, 2000). This SK3 overexpressing allele is referred to as SK3-T in the following. The overexpression of SK3

Table 1. GRAS sample description: total sample and allelic repeat sum groups, contrasted by removal of the median group

	Total GRAS sample (n = 1060)	Low allelic repeat sum[a] (n = 462)	High allelic repeat sum[a] (n = 405)	p[b]
Sociodemographic variables				
Age, mean ± SD (range), y	39.67 ± 12.75 (18–83)	39.37 ± 13.04 (18–78)	39.31 ± 12.18 (18–73)	0.765
Gender, No. (%), male	707 (66.7%)	301 (65.2%)	275 (67.9%)	0.392
Ethnicity, No. (%), caucasian	1008 (95.6%)	437 (94.8%)	387 (96.3%)	0.064
Years of education[c], mean ± SD (range)	12.04 ± 3.05 (8–27)	11.92 ± 2.91 (8–24)	12.15 ± 3.11 (8–24)	0.528
Inpatients at assessment, No. (%)	449 (42.6%)	192 (41.7%)	167 (41.5%)	0.869
Clinical variables				
Age at 1st episode, mean ± SD (range), y	26.46 ± 9.02 (5.40–73.86)	25.95 ± 9.00 (5.40–73.86)	26.41 ± 8.74 (7.95–57.35)	0.423
CPZ, mean ± SD (range)	683.83 ± 700.89 (0–7500.00)	669.70 ± 691.73 (0–6837.43)	710.66 ± 769.41 (0–7500.00)	0.875
Diagnosis, No. (%), schizophrenia[d]	785 (74.5%)	339 (73.5%)	298 (74.1%)	0.972
Numbers of hospitalizations, mean ± SD (range)	8.56 ± 9.81 (0–97)	8.41 ± 9.46 (0–97)	8.74 ± 10.43 (0–82)	0.756
PANSS pos, mean ± SD (range)	1.94 ± 0.89 (1–5.43)	1.91 ± 0.86 (1–5.00)	1.95 ± 0.90 (1–5.43)	0.558
PANSS neg, mean ± SD (range)	2.60 ± 1.13 (1–6.57)	2.56 ± 1.07 (1–5.71)	2.59 ± 1.17 (1–6.29)	0.988
PANSS gen, mean ± SD (range)	2.10 ± 0.74 (1–5.13)	2.09 ± 0.71 (1–4.88)	2.07 ± 0.76 (1–4.69)	0.423
GAF, mean ± SD (range)	45.91 ± 17.31 (5–90)	46.25 ± 17.13 (8–90)	45.41 ± 17.26 (5–90)	0.441
CGI, mean ± SD (range)	5.55 ± 1.09 (2–8)	5.55 ± 1.07 (2–8)	5.56 ± 1.11 (2–8)	0.688

[a]Low (below-median; <35) and high (above median; >35) allelic repeat lengths sum; individuals with an allelic repeat lengths sum of 35 were excluded (n = 193).
[b]Statistical methods used: Mann–Whitney-*U* or χ^2-tests.
[c]Rating according to graduation/certificate; patients currently in school or in educational training are excluded.
[d]*Versus* schizoaffective disorders and other psychotic disorders/yet to be confirmed.

Table 2. Association of SK3 repeat lengths sum with target and control phenotypes

Phenotypes	Change of mean phenotype value *per* additional repeat		Statistical test	
	estimate	95% confidence interval	*t*-value (1df)	*p*-value
Cognitive target variables:				
Combined (multivariate)	0.0269	0.0104, 0.0434	3.1901	**0.0014**[a]
Individual (univariate)				
Reasoning	0.0224	0.0008, 0.0440	2.0317	0.0425[b]
Executive function	0.0281	0.0062, 0.0500	2.5203	**0.0119**[b]
Word recognition	0.0331	0.0096, 0.0565	2.7680	**0.0058**[b]
Divided attention	0.0319	0.0069, 0.0570	2.5004	**0.0126**[b]
Cognitive control variables:				
Combined (multivariate)	0.0132	−0.0039, 0.0304	1.5105	0.1310[a]
Individual (univariate)				
Dotting	0.0163	−0.0059, 0.0385	1.4391	0.1505
Tapping	0.0142	−0.0079, 0.0363	1.2612	0.2076
Alertness	0.0115	−0.0105, 0.0335	1.0281	0.3042
Premorbid intelligence	0.0088	−0.0155, 0.0330	0.7090	0.4785[a]
Disease-related control variables:				
PANSS positive symptoms	0.0051	−0.0186, 0.0288	0.4230	0.6724[a]
PANSS negative symptoms	0.0131	−0.0120, 0.0382	1.0236	0.3063[a]
Disease-unrelated control variable:				
Body length	0.0151	−0.0042, 0.0344	1.5365	0.1248[a]

[a,b] Multiple testing corrected significance thresholds: [a]first level of tests $p \leq 0.0100$ (Bonferroni), [b]second level of tests $p \leq 0.0138$ (permutation test with 50 000 permutations to account for correlations between phenotypes).

Association analyses of allelic repeat lengths sum with mean value of target and control phenotypes. The analysed sample has a range of allelic repeat lengths sums from 28 to 40, $n = 952$. Phenotypes were adjusted for sex and age, additionally for medication (all cognitive phenotypes, PANSS positive and PANSS negative symptoms) and for negative symptoms (all cognitive phenotypes). All phenotypes were standardized to zero mean and variance one: larger values for cognitive phenotypes correspond to better performance. Like Cohen's *d*, the genetically induced effect size (change of mean phenotype value *per* additional repeat) is quantified relative to the standard deviation of the trait. Rare extreme observations of allelic repeat lengths sums (below 28, above 40; $n = 21$) and non-native German speakers with language problems (total of $n = 87$) were excluded from the analyses (to result in *n*: $1060 - 108 = 952$).

in this model is illustrated in Fig 2A and B, comparing hippocampal SK3 immunoreactivity in a wildtype (WT) and a SK3 T/T mouse. Densitometric analysis of the SK3 specific bands obtained by Western blot demonstrates the pronounced increase in SK3 protein in hippocampus ($p = 0.0029$) and heart (used as a control tissue; $p = 0.0005$) of SK3 T/T mice (Fig 2C).

Most basic behavioural tests were unaffected by SK3 over-expression: Time spent in open arms of elevated plus maze (Fig 2D) as well as time spent in the different zones and velocity in the open field (Fig 2E and F) were unchanged, indicating comparable anxiety and activity levels in both experimental groups. However, SK3 T/T mice showed an increase in latency to reach the wall when first put in the open field ($p = 0.029$; Fig 2G). Exploratory activity in the hole board test was reduced in mice overexpressing SK3 ($p = 0.0004$; Fig 2H). Importantly, motor performance and motor learning, as evaluated in the 2-day rota-rod testing, were comparable in both genotypes (Fig 2I). No differences between genotypes were found in sucrose preference, pre-pulse inhibition, and social interaction tests (Fig S1A–D).

In the visible platform training (2 days) of the Morris water maze, there was no overall difference between SK3 T/T and WT mice. Both experimental groups had similar latency to locate the visible platform (2-way repeated measurement ANOVA: effect of genotype $F_{1,26} = 0.23$, $p = 0.637$), similar swim distance to the platform (2-way repeated measurement ANOVA: effect of genotype $F_{1,26} = 0.96$, $p = 0.337$), and similar swim velocity (2-way repeated measurement ANOVA: effect of genotype $F_{1,26} = 1.09$, $p = 0.306$). Notably, however, in the hidden platform testing, assessing the ability to use extra-maze spatial cues to locate a submerged platform, impaired spatial learning in SK3 overexpressing mice became evident, with longer latencies to find the platform over the trials (2-way repeated measurement ANOVA: effect of genotype $F_{1,26} = 5.43$, $p = 0.028$, Fig 2J) as well as longer swim distance to the platform (2-way repeated measurement ANOVA: effect of genotype $F_{1,26} = 11.17$, $p = 0.0025$; not shown). Moreover, in the probe trial, performed to determine whether mice use a spatial strategy to find the platform, SK3 T/T mice spent less time in the target quadrant ($p = 0.033$; Fig 2J, inset and Fig S1E). Along the same lines, SK3 overexpressing mice displayed reduced freezing behaviour in fear conditioning when tested 72 h after training in the same context ($p = 0.036$; Fig 2K), reflecting compromised hippocampal memory. Significant differences between groups were also observed in the cued memory test with SK3 T/T mice showing a decreased freezing response to the conditioned tone ($p = 0.0008$; Fig 2K). Taken together, overexpression of SK3, similar to the short CAG repeat length genotype in humans, leads to reduced performance in higher brain functions.

Mechanistic insight: SK3 channel conductance depends on CAG repeat length

To test if the CAG repeat length indeed has an impact on channel function, we expressed three different human SK3 isoforms,
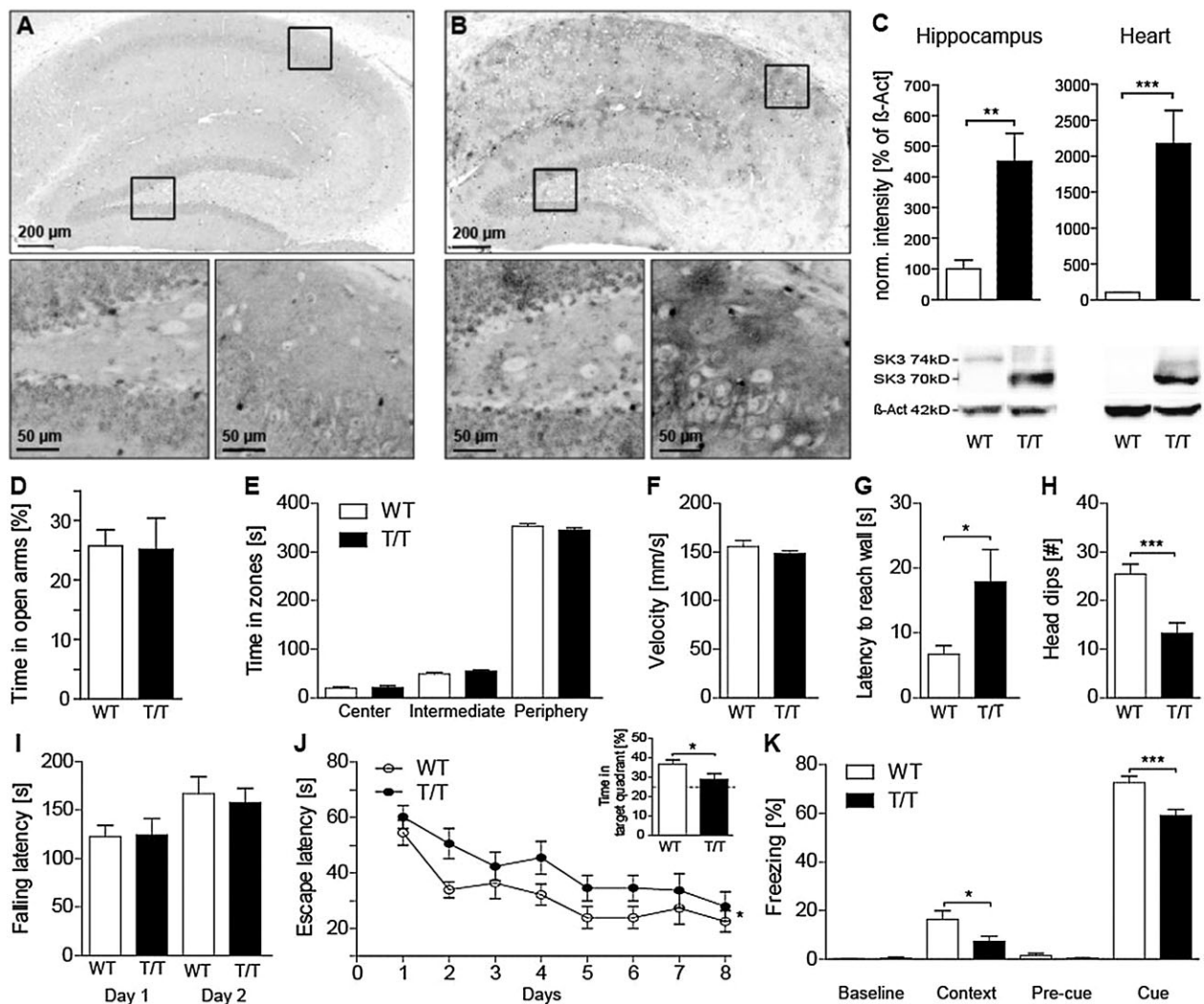
Figure 2. SK3 overexpressing (T/T) mice show selective deficits in higher cognition.

A,B. Comparative distribution of SK3 immunoreactivity in hippocampus (sagittal section) is presented for wildtype (**A**) *versus* SK3 T/T mice (**B**). Insets show magnifications of stained granule neurons in dentate gyrus and pyramidal neurons in Ammon's horn (CA2).

C. Densitometrical quantification of SK3 protein expression in hippocampus and heart of WT and SK3 T/T mice ($n = 6–7$; **Student's t-test, $t_{1,12} = 3.724$, $p = 0.0029$; ***Student's t-test, $t_{1,11} = 4.902$, $p = 0.0005$). SK3 expression is presented as % of ß-actin (ß-Act) and normalized to the WT. Sample Western blots are shown below the bar chart. For quantification, the 74 kDa band was used for WT (endogeneous SK3) and the 70 kDa band for T/T (transgenic SK3 expression).

D. WT and T/T behaviour is unaltered in elevated plus maze. Presented is the percentage of time spent in open arms against the total time spent in both open and closed arms.

E-G. No differences in open field are observed between genotypes regarding time spent in different zones (**E**) or velocity (**F**). The latency to reach the wall after initial start from the centre of the open field (**G**) is significantly higher in SK3 T/T mice compared to WT (*Student's t-test, $t_{1,26} = 2.318$, $p = 0.029$).

H. In hole board, SK3 T/T mice show significantly less exploratory activity than WT (***Student's t-test, $t_{1,26} = 4.110$, $p = 0.0004$).

I. The two genotypes do not differ in motor performance/motor learning on rota-rod.

J. SK3 T/T mice demonstrate longer escape latency in the hidden platform task (*two-way repeated measures ANOVA, $F_{1,26} = 5.43$, $p = 0.028$); in the probe trial (inset), SK3 T/T mice display absence of preference for the target quadrant (*Student's t-test, $t_{1,26} = 2.250$, $p = 0.033$).

K. In contextual and cue memory test of fear conditioning, SK3 T/T mice freeze less (*Student's t-test, $t_{1,26} = 2.212$, $p = 0.036$, ***Student's t-test, $t_{1,26} = 3.779$, $p = 0.0008$). For all behavioural experiments: $n = 13–15$; data presented as mean ± s.e.m.; two-sided Student's t-tests used; * indicates $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

characterized by different repeat lengths and fused with eGFP, in HEK293 cells and performed whole-cell patch clamping (Fig 3). Cells transfected with the constructs (eGFPhSK3(CAG)$_{11}$, eGFPhSK3(CAG)$_{18}$, eGFPhSK3(CAG)$_{24}$) showed intense fluorescence signal compatible with cell surface expression (Fig 3A and B). No obvious localization or quantitative differences among clones were observed under epifluorescence microscopy, and the size of the expressed
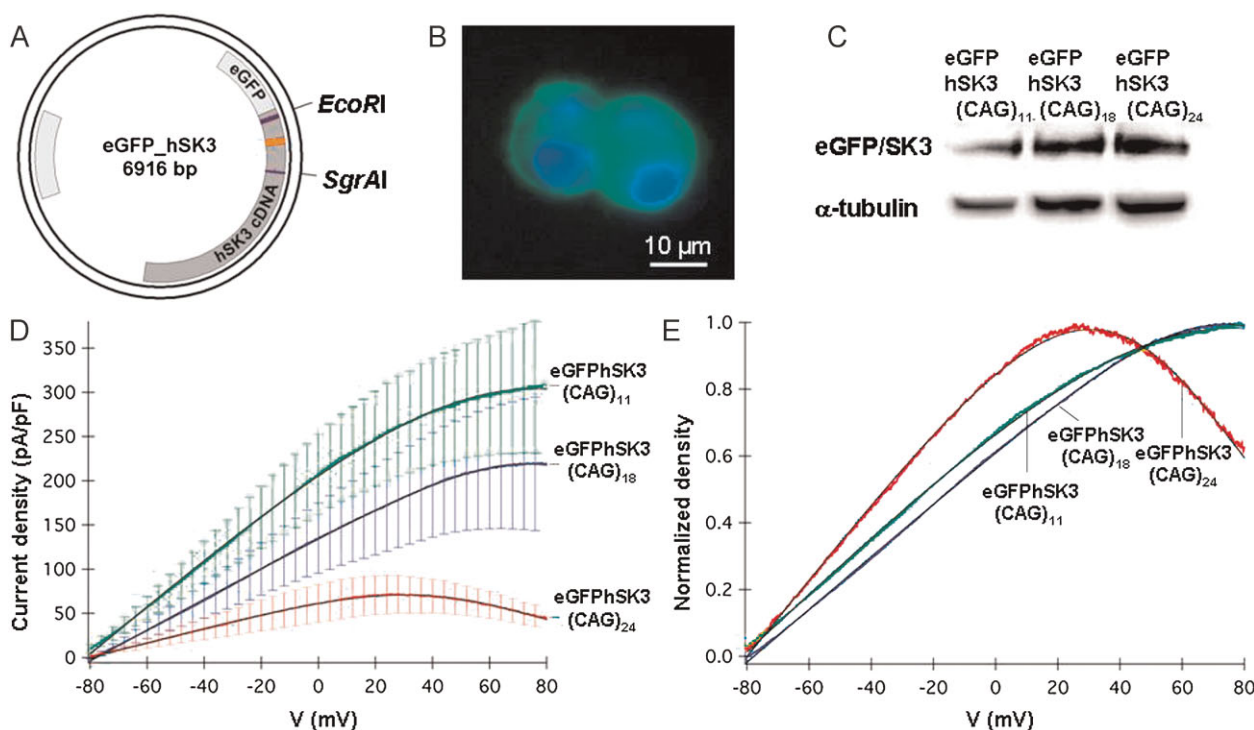
Figure 3. *KCNN3* CAG repeat length affects electrophysiological properties of SK3.

A. For electrophysiological measurements, the eGFPhSK3(CAG)$_n$ construct has been employed to generate clones with different repeat lengths. Indicated are locations of the CAG repeat (labelled in orange), of the primers (labelled in purple) and of the restriction enzyme sites used for cloning.

B. Live transfected HEK293 cells show intense green fluorescent signal on the plasma membrane; cell nuclei are stained with Hoechst33342 (blue).

C. Western blot analysis of eGFP and SK3 demonstrates comparable SK3 fusion protein size and expression level of the different constructs in transfected cells.

D. The average SK3 current response (expressed as apamin-sensitive current density) for eGFPhSK3(CAG)$_{11}$ ($n = 8$; green), eGFPhSK3(CAG)$_{18}$ ($n = 7$; blue) and eGFPhSK3(CAG)$_{24}$ ($n = 9$; red) is displayed. The result of fitting is superimposed as a solid line. Error bars represent s.e.m.

E. Normalized apamin-sensitive current density (*versus* the corresponding individual current density maximum) for clones eGFPhSK3(CAG)$_{11}$, eGFPhSK3(CAG)$_{18}$ and eGFPhSK3(CAG)$_{24}$. Inward rectification starting as early as at $+20$ mV is evident for eGFPhSK3(CAG)$_{24}$. Colours as in panel D.

Note: Colour version is available online.

proteins was comparable as predicted (Fig 3C), excluding truncation artifacts. Measurement of apamin-sensitive current density showed a significant reduction in eGFPhSK3(CAG)$_{24}$ as compared to eGFPhSK3(CAG)$_{18}$ and eGFPhSK3(CAG)$_{11}$. Overall conductance ($\pm 95\%$ confidence interval) calculated was 0.83 ± 0.002 ($n = 9$), 1.75 ± 0.002 ($n = 7$) and 2.72 ± 0.011 ($n = 8$) pA/pF, respectively (Fig 3D). The shape of the current/voltage relationship for SK3 channels is strongly inwardly rectifying (Grunnet et al, 2001). We observed a clear rectification in cells expressing eGFPhSK3(CAG)$_{24}$ already at less depolarized potentials than in eGFPhSK3(CAG)$_{11}$ and eGFPhSK3(CAG)$_{18}$. Plotting the average normalized current against voltage, a gradual increase in the degree of rectification between the different isoforms was observed. Voltage for half-maximal block was 117.22 ± 0.395, 113.89 ± 0.402 and 61.251 ± 0.136 mV for eGFPhSK3 (CAG)$_{11}$, eGFPhSK3(CAG)$_{18}$ and eGFPhSK3(CAG)$_{24}$, respectively (Fig 3E). Qualitatively identical results were obtained in control experiments with an untagged expression of hSK3 clones (Fig S2). Overall, these data support our hypothesis that a long CAG repeat length reduces SK3 channel conductance.

DISCUSSION

We report here the surprising finding that a normal variant of the gene encoding the small conductance calcium-activated potassium channel SK3 predicts cognitive performance of patients with schizophrenia. The discovery of *KCNN3* CAG repeat lengths influencing higher cognition in man was facilitated by a phenotype-based genetic association study (PGAS) on the grounds of our new schizophrenia patient database, the GRAS data collection.

The role of simple sequence repeats as genetic modulators of brain function and behaviour is quite well established, however, in most cases the biological mechanisms involved are far from clear (Fondon et al, 2008). Notably, the present study allows mechanistic insight, elucidating the functional role of the polymorphic glutamine repeats in the *N*-terminal coding region of SK3. Although the *N*-terminus of the channel does not directly contribute to the core structure of the pore, it may, as in many other channels, form part of the internal vestibule and thereby influence permeation properties. As documented here by whole-cell patch-clamping, long CAG repeats reduce current amplitude

at depolarized potentials in the presence of internal Ca^{2+}. This reduced channel conductance is associated in schizophrenic patients with better performance in higher cognitive tasks, comprising the capabilities to 'discriminate, select and execute'. On the contrary, SK3 overexpression in mice, as a model of increased channel function, leads to reduced cognitive abilities in a similar set of tasks. Thus, SK3 conductance appears to be inversely correlated with higher cognition.

The electrophysiological results obtained here with transfected cells help to explain this conclusion which is somewhat unexpected at first glance. Our data indicate an enhanced Ca^{2+} block in the SK3 isoform with the longest repeats as compared to the shorter repeat variants, leading to an overall decreased conductance at a given Ca^{2+} concentration in a physiologically relevant range. This would generate different afterhyperpolarization depending on the length of the repeat, and ultimately result in increased excitability, as shown for dopaminergic neurons, where decreasing the activity of SK channels by decreasing the apparent Ca^{2+} affinity changes the firing pattern from a pacemaker to an irregular or bursting one (Ji et al, 2009). The phenotypic effect of different CAG repeat or polyglutamine lengths can also be independent of the electrophysiological properties of the resulting channel, since it might alter the affinity of RNA binding proteins and/or protein–protein interactions (Jasinska et al, 2003; Orr & Zoghbi, 2007).

A 'less is more' feature does not seem to be entirely unusual for potassium channels influencing cognitive readouts. Along these lines, apamin treated mice show superior cognitive behaviour, while overexpression of SK2 specifically leads to cognitive impairment in similar tasks (Hammond et al, 2006; Stackman et al, 2002). Also, disruption of the gene encoding another type of potassium channel, the voltage-gated channel BEC1/KCNH3, changes hippocampal neuronal activity as well as synaptic plasticity, and enhances cognitive function in mice (Miyake et al, 2009). A primate-specific isoform of yet another member of the voltage-gated ether-à-go-go family, KCNH2 (ERG1), is highly expressed in post mortem brains of schizophrenics. This shorter isoform appears to be inversely correlated with cognition and to impair channel function (Huffaker et al, 2009).

In our study, focusing on the CAG repeat polymorphism in KCNN3, the gene encoding SK3, a small conductance calcium-activated potassium channel, the distribution of repeat lengths was comparable in both schizophrenic and healthy subjects. This finding confirms the meta-analysis by Glatt et al (2003) and points against a major role of this particular genetic marker for the risk to develop schizophrenia. Also, the present study did not substantiate an effect of glutamine repeat length on positive or negative symptoms as reported for Jewish (Ritsner et al, 2002) or British schizophrenic patients (Cardno et al, 1999). This discrepancy may be explained by ethnical differences or by a potential bias due to the small sample sizes used in these earlier studies (Cardno et al, 1999; Ritsner et al, 2002). In agreement with the present findings on cognitive readouts in schizophrenic patients, our results obtained with the KCNN3 overexpressing mice further support a role of this gene in higher cognition, whereas readouts of positive symptoms (e.g. hyperactivity) or negative symptoms (e.g. social interaction, sucrose preference) were not seen in the SK3 overexpressing as compared to WT mice (Fig S1).

The observation that long glutamine repeats, and thus reduced SK3 channel function, are equally disseminated in healthy and schizophrenic subjects makes it very likely that the 'long repeat SK3 effect' on higher cognition is not restricted to schizophrenia, although functional proof in healthy individuals and in other disease cohorts is still required. Nevertheless, the SK3 genotype clearly contributes to the cognitive phenotype of schizophrenic patients. Even if traits of interest in schizophrenia (here: cognition) are never explained by a single modifier gene only, candidate alleles like the SK3 variants shown here co-determine, together with other trait-relevant genes and environmental factors, the outcome of an individual suffering from schizophrenia.

In fact, we hypothesize that an interplay of multiple causative factors, perhaps thousands of potential combinations of genes/genetic markers and an array of different environmental risks, leads to the development of a schizophrenic phenotype. Not too much of an overlap may exist between genetic risk factors from one schizophrenic patient to an unrelated other schizophrenic individual, explaining why it is basically impossible to find common risk genes of schizophrenia with appreciable odds ratios. In the overwhelming majority of cases, schizophrenia seems to be the result of a 'combination of many unfortunate genotypes', and a short SK3 repeat variant may be one of them.

Given the obvious influence of SK3 conductance on higher cognition, this channel may be an interesting pharmacological target for addressing cognitive function in disease conditions associated with cognitive deficits.

MATERIALS AND METHODS

For a more comprehensive version of Materials and methods see Supporting information.

Human study

Subjects

The present study has been based on the GRAS data collection of $n = 1074$ patients (as of October 2009) diagnosed according to DSM-IV-TR with schizophrenia (73.2%), schizoaffective disorder (14.8%) or other psychotic disorders/yet to be confirmed (12.0%). Control subjects were healthy blood donors ($n = 1143$). The GRAS data collection as well as the healthy control population is described in greater detail elsewhere (Begemann et al, 2010; Ribbe et al, 2010).

Genotyping

Standard methods were used for DNA extraction from whole blood (Genomed GmbH, Löhne, Germany). The polymorphic CAG repeat in exon1 of KCNN3 was amplified from genomic DNA by PCR. Primers were chosen according to Austin et al (1999), resulting in a PCR fragment of ~121 bp. The amplicons were separated using size electrophoresis on the ABI 3730 XL DNA Analyser (Applied Biosystems, Foster City, USA). Raw data were processed using the Gene Mapper

The paper explained

PROBLEM:

Schizophrenia has a strong genetic component but it is unclear how genetic variants contribute to the disease phenotype. A polyglutamine repeat in a calcium-activated potassium channel, SK3, which is important for synaptic plasticity, has been discussed for some time as potential risk factor for schizophrenia. However, up to now it has remained unclear which domains of the phenotype might be affected and how a 'risk role' of SK3 might translate to the biological level.

RESULTS:

We report here that SK3 does not increase the general risk for schizophrenia, but that it contributes in a significant way to the cognitive abilities of schizophrenic patients. Specifically, longer polyglutamine stretches are associated with better cognitive performance. On the molecular level, longer stretches result in a reduced conductance of the channel. On the other hand, mice overexpressing this gene (as a model of humans with shorter polyglutamine sequence) perform cognitively worse than their wildtype littermates. The chain of interactions thus reads: Shorter repeats or SK3 overexpression—enhanced channel function—reduced cognitive performance.

IMPACT:

We conclude that regarding SK3 channel function and cognition, 'less is more'. Therefore, pharmacological reduction of SK3 channel conductance might be an attractive novel strategy to improve cognition in disease states.

Software 4.0 (Applied Biosystems). Successful genotyping was performed for 1060 patients and 1135 controls (99%).

Mouse study

Behaviour

Male WT and SK3 T/T mice (Bond et al, 2000) (littermates) were started at the age of four months on a battery of behavioural tests, performed in the following order: elevated plus maze, open field, hole board, rota-rod, pre-pulse inhibition, social interaction, sucrose preference, Morris water maze and fear conditioning.

Immunohistochemistry

SK3 immunostaining was carried out on paraffin sections using a rabbit polyclonal anti-SK3 antibody (Alomone, Jerusalem, Israel; dilution 1:120 v/v) and peroxidase labelled polymer anti-rabbit antibody (Envision HRP System/DAB for rabbit primary antibodies, DAKO, Glostrup, Denmark). Immunocomplexes were visualized by diaminobenzidine (DAB; Sigma–Aldrich, Taufkirchen, Germany). Bright field images were obtained with an Axiovert 200M microscope (Zeiss, Oberkochen, Germany).

Western blot

Experiments were performed using a rabbit polyclonal anti-SK3 antibody (Alomone, 1:2500), and a mouse monoclonal anti-beta actin antibody (Abcam, Cambridge, UK, 1:10000) for mouse tissues, or a mouse monoclonal anti-α-tubulin antibody (Sigma–Aldrich, 1:20000) for HEK293 cells, and appropriate horseradish-peroxidase-conjugated secondary antibodies (sheep anti-mouse IgG 1:10000 and donkey anti-rabbit IgG 1:10000, GE Healthcare, Munich, Germany, for mouse tissues or anti-rabbit IgG 1:5000 and anti-mouse IgG 1:10000, Sigma–Aldrich, for HEK293 cells). Immunoreactivity was visualized by chemoluminescence (ECL, Millipore, Billerica, USA) and quantified by the Quantity One analysis software (Bio-Rad, Munich, Germany).

In vitro analysis

Cloning

The vector containing eGFP-labelled SK3 was kindly provided by H. Wulff (UC Davis). The different lengths of CAG repeats (11, 18 and 24) were amplified by PCR from respective human samples and cloned into the original vector using *EcoRI* and *SgrAI* restriction sites. The resulting constructs eGFPhSK3(CAG)$_{11}$, eGFPhSK3(CAG)$_{18}$, eGFPhSK3(CAG)$_{24}$ were verified by sequencing. For obtaining constructs without eGFP, vectors were sequentially digested with *AgeI* and *BspEI*.

Transfection

HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) following the manufacturer's guidelines. Stable cell pools were obtained by selection with 300 μg/ml G-418 (Invitrogen). Representative fluorescence images of living cells were taken under an epifluorescence microscope; nuclei were stained with Hoechst33342 (Invitrogen).

Electrophysiology

All measurements were performed by a blinded investigator. Macroscopic currents elicited by a 500 ms voltage ramp from −80 to +80 mV were recorded in the whole-cell configuration of the patch-clamp technique (Hamill et al, 1981). The intracellular solution contained (in mM) 160KCl, 0.5 MgCl$_2$, 10EGTA, 9.5CaCl$_2$ (free Ca^{2+} 1.03 μM), 10Hepes/KOH pH 7.35. The control external recording solution contained (in mM) 160NaCl, 2.5KCl, 2CaCl$_2$, 1MgCl$_2$, 8glucose, 10HEPES/NaOH, pH 7.4. Apamin (100 nM)-sensitive currents were determined by off-line subtraction. To determine overall conductance and voltage of half-maximal block, we used a linear current/voltage function with a block at positive voltages. Goodness of fit was evaluated by Pearson's χ^2, and confidence intervals by Student's *t* distribution.

Statistical analysis

Case-control study

The sum of repeat lengths of both alleles of all individuals was analysed. To account for the degree of heterogeneity between the two alleles (intra individual heterogeneity), the difference between allelic repeat lengths was also calculated. Maximized χ^2 values for distribution of genotypes among schizophrenic and control samples were determined by Monte Carlo tests (using 1000 simulations) with Clump software (Sham & Curtis, 1995) (http://www.mds.qmw.ac.uk/statgen/dcurtis/software.html).

PGAS

Analyses were based on allelic repeat lengths sum. Statistical analyses were carried out with R (v2.10.0). Data on cognitive tests are presented in a way that higher values always indicate better performance. Non-native German speakers with language problems ($n = 87$) were excluded. Metric phenotypes were standardized to zero mean and variance one by Blom transformation (Blom, 1958) and analysed by linear models. Multivariate analysis modelled a target phenotype vector, accounting for individual correlation between vector entries. Variables were adjusted for covariates sex and age, for covariate antipsychotic medication dose (all cognitive phenotypes, PANSS positive symptoms, PANSS negative symptoms) and for covariate negative symptoms (all cognitive phenotypes). Multiple testing adjusted significant thresholds to the 5%-level were determined by Bonferroni-adjustment or by permutation test (50 000 permutations, to account for correlations between cognitive phenotypes).

Animal study

Statistical significance was evaluated using unpaired Student's t-test and two-way repeated measurement ANOVA including Bonferroni testing where applicable. Significance level was set to $p < 0.05$. Data are represented as mean $\pm$ s.e.m. in figures and text. The data were analysed using Prism4 (GraphPad Software, San Diego, CA, USA).

Author contributions

SG carried out genetic analyses and the cloning procedure. She was supported by SP and for sample processing and logistics by FB. The human association study was carried out by MFG. BA performed all behavioural analysis of SK3 T/T mice, and SM the expression analysis. LAP conducted the electrophysiology. DM, MFG and HB performed the genetic statistics. MB coordinated and supervised the travelling team of investigators as well as the database staff. KR and HF were pivotal members of the travelling team and supervised the database. JR provided DNA samples from healthy blood donors. HE, K-AN, NB and WS developed the concept of GRAS, and guided the project, data analysis and paper writing. KAR, PF and MM gave input to data analysis and manuscript preparation. HE, SG, MFG and BA wrote the paper. All authors discussed the results, have read and commented on the manuscript, and have seen and approved the final version.

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