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Association between genetic variants of the metabotropic glutamate receptor 3 (*GRM3*) and cognitive set shifting in healthy individuals

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Set-shifting and maintenance are complex cognitive processes, which are often impaired in schizophrenia. The genetic basis of these processes is poorly understood. We aimed to investigate the association between genetic variants of the metabotropic glutamate receptor 3 (*GRM3*) and cognitive set-shifting in healthy individuals. The relationship between 14 selected single nucleotide polymorphisms (SNPs) of the *GRM3* gene and cognitive set-shifting as measured by perseverative errors using the modified card sorting test (MCST) was analysed in a sample of $N = 98$ young healthy individuals (mean age in years: 22.7 ± 0.19). Results show that SNP rs17676277 is related to the performance on the MCST. Subjects with the TT genotype showed significantly less perseverative errors as compared with the AA ($P = 0.025$) and AT ($P = 0.0005$) and combined AA/AT genotypes ($P = 0.0005$). Haplotype analyses suggest the involvement of various SNPs of the *GRM3* gene in perseverative error processing in a dominant model of inheritance. The findings strongly suggest that the genetic variation (rs17676277 and three haplotypes) in the metabotropic GRM3 is related to cognitive set-shifting in healthy individuals independent of working memory. However, because of a relatively small sample size for a genetic association study, the present results are tentative and require replication.

Keywords: Healthy individuals, metabotropic glutamate receptor 3, perseverative errors, schizophrenia, working memory

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L-Glutamate is a major excitatory neurotransmitter in the central nervous system and activates both ionotropic and metabotropic glutamate receptors. Glutamatergic neurotransmission is involved in most aspects of normal brain function (Marenco *et al*. 2006) and can be perturbed in many neuropathological conditions, such as schizophrenia (Egan *et al*. 2004; Marenco *et al*. 2006; Sartorius *et al*. 2008), resulting in cognitive deficits. The metabotropic glutamate receptor 3 (*GRM3*) gene appears to be involved in various domains of cognitive function (Egan *et al*. 2004; Roffman *et al*. 2006). Although the majority of previous research investigated the associations between genetic variants of the *GRM3* gene and cognitive dysfunction (i.e. verbal fluency and verbal list learning) in schizophrenia (Egan *et al*. 2004), only recently a first study in healthy subjects showed an association between a single genetic variant of *GRM3* (SNP rs6465084) and prefrontal function (reduction of *N*-acetylaspartate/creatine levels) but not verbal fluency (Marenco *et al*. 2006). However, a larger range of single nucleotide polymorphisms (SNPs) in the *GRM3* gene has not been investigated simultaneously in relation to neuropsychological function in healthy subjects. Corroborating the role of the metabotropic glutamate receptor (mGluR3) in cognitive function in healthy individuals, a recent functional magnetic resonance imaging study suggested that the integrity of higher executive areas in the dorsolateral prefrontal cortex could be disproportionately compromised and inefficient in the presence of combined deleterious catechol-*O*-methyl transferase and *GRM3* genotypes in normal subjects (Tan *et al*. 2007).

Although various cognitive deficits (working memory, executive function, verbal fluency and episodic memory) have been reported in schizophrenia (Barch 2005), perseverative deficits appear not only to be consistent with a hypothesis of frontal dysfunction in schizophrenia (Everett *et al*. 2001; Szoke *et al*. 2008) but also to play a role as a vulnerability marker to schizophrenia (Erlenmeyer-Kimling & Cornblatt 1992; Kremen *et al*. 1994). In support of this, a recent study among college students reported that those rating high on the Schizotypal Personality Questionnaire (SPQ; Raine 1991) showed more perseverative errors (set-shifting problems) as

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compared with subjects scoring average on the SPQ (Wilson *et al*. 2008).

Overall, the literature points to a potentially important role of the *GRM3* gene in cognitive dysfunction in schizophrenia and possibly also in healthy subjects. Moreover, perseverative error processing appears to be not only a marker of frontal lobe dysfunction in schizophrenia but this psychological measure might also serve as a vulnerability marker of schizophrenia.

In order to increase the understanding of the interaction between both the *GRM3* gene and perseverative errors under non-pathological conditions, in the present study we aimed at investigating the association between a large range of *GRM3* polymorphisms and perseverative error processing in healthy individuals.

Table 1: Sample ($N = 98$) characteristics across gender

	Gender (mean ± SE)		*t*-test, *P* value
	Female ($N = 67$)	Male ($N = 31$)	
Age	22.4 ± 0.24	23.2 ± 0.35	0.032
MWTB IQ	107.3 ± 1.4	109.8 ± 1.8	0.153
Perseverative errors (MCST in %)	4.5 ± 0.41	5.6 ± 0.83	0.10
Verbal working memory (digits backward)	7.6 ± 0.26	8.2 ± 0.35	0.13
BDI	3.7 ± 0.39	3.2 ± 0.54	0.77

MWTB IQ, premorbid intelligence (Mehrfachwahl–Wortschatz–Intelligenztest).

Materials and methods

Sample

The cross-sectional study was performed as part of a larger study effort to investigate genetic influences on cognitive and electrophysiological processes (Beste *et al*. 2009). A sample of 98 (31 males and 67 females) genetically unrelated, healthy subjects of Caucasian descent (mean age of 22.7 ± 0.19 years) was recruited by newspaper announcement. All subjects underwent a detailed screening interview to exclude any current or previous medical and psychiatric disorders. No gender differences were observed for perseverative errors [modified card sorting test (MCST) in %], verbal working memory (digits backward), IQ (MWTB IQ) and depressive symptoms [Beck's Depression Inventory (BDI); see Table 1 for details]. Hardy–Weinberg equilibrium was examined using the program FINETTI provided as an online source (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl; Wienker TF and Strom TM). Gender was equally distributed across genotype groups of the 14 SNPs [Kruskal–Wallis test (*H*-test); data not shown]. The distribution of the genotypes of the 14 SNPs in the *GRM3* gene did not significantly differ from the expected numbers calculated on the basis of observed allele frequencies according to Hardy–Weinberg equilibrium.

The study was approved by the local ethics committee of the University of Muenster, Germany. Participants gave written informed consent after full explanation of all study procedures.

Neuropsychological measures

MCST and premorbid intelligence: MWTB IQ

A modified version of the Wisconsin Card Sorting Test (MCST) (Heaton 1981) was used, which resembles the modification developed by Nelson (1976). This modified version seems to have motivational and interpretative advantages (Nelson 1976). When the standard Wisconsin Card Sorting Test is used, it is not always possible to identify what strategy the subject is using, because 80 of the 128 cards share two or three attributes with a stimulus card. Before the test, subjects were provided with part of the sorting rules and were trained with an automated test version, in which the stimulus card appeared on the screen [part of the neuropsychological tests version 2.2. developed by Ille *et al*. (1992)]. They were told that one sorting category was colour, and that the sorting rule would change during the test. Use of these modified instructions was intended to minimize motivational reasons for performance deficits. The MCST was presented on an IBM-compatible microcomputer. The subject sorted the cards by pressing one of four response-card buttons on a keyboard. Feedback (right or wrong) was provided acoustically and visually on the screen after the sort. The subject had 20 seconds to choose a card. The criterion for shifting category was six correct responses. The test was stopped after six categories had been completed. In addition, there were no test cards sharing two or more attributes with a stimulus card. These modifications were made to obtain greater clarity in categorizing errors. The main measure from the MCST used in this study is called perseverative errors. All participants were tested individually in a quite room free from auditory and visual distractions.

Premorbid intelligence was assessed with a multiple choice verbal intelligence test (Mehrfachwahl–Wortschatz–Intelligenztest MWTB IQ) (Lehr 2005). The MCST and MWTB were given as part of a test battery, which included seven information-processing measures including the 'Digits backward' for assessment of verbal working memory. We selected the MCST to analyse the association with genetic variants of the *GRM3* gene because perseverative errors and the *GRM3* gene are discussed to be involved in impaired executive function, such as in schizophrenia (Drake & Lewis 2003; Harrison *et al*. 2008; Shad *et al*. 2006). The tests were administered in a fixed sequence of presentation.

Depressive symptoms

In order to exclude depressive symptoms, BDI (Beck & Beck 1972; Hautzinger *et al*. 1995) was applied at the time of the screening interview (BDI: mean 3.5 ± 0.69; *t*-test for differences between male/female subjects: df = 94, $P = 0.77$). All diagnostic and psychometric evaluations were performed by experienced clinical raters.

SNPs' selection and genotyping

The entire sequence of the *GRM3* gene contains >300 SNPs with minor allele frequency (MAF) >5% (International HapMap Consortium 2007). We used various techniques to limit the number of SNPs assessed to the most relevant. We initially constructed the linkage disequilibrium (LD) pattern of the Utah residents with ancestry from northern and western Europe (CEPH) population of the HapMap Phase II genotype data (Fig. 1) to identify tagging SNPs by an aggressive tagging approach (MAF >5% and $r^2 > 0.8$) using GEVALT version 2 software package (Davidovich *et al*. 2007). The region analysed included about 190 kb of the *GRM3* gene between the positions 86 115 570 and 86 309 849 at chromosome 7 (human genome coordinates hg18). We then selected SNPs with MAF >15% based on power calculations for our sample size ($N = 98$) using QUANTO version 1.2 software (Gauderman 2002). Ultimately, we reduced SNP numbers by assessing the ability of limited numbers of the tagging SNPs to predict the total SNP population using Stampa algorithm (Halperin *et al*. 2005). With this approach, 91.3% of the variation in the gene was captured using 14 tagging SNPs (Table 2; Fig. 2). The mean r^2 of individual tagging SNPs in conjunction with one or more tagged SNPs was 0.963 (Table 2).

Genotyping of the selected 14 *GRM3* tagging SNPs was carried out following published protocols applying the multiplex genotyping assay iPLEX™ for use with the MassARRAY platform (Oeth *et al*.

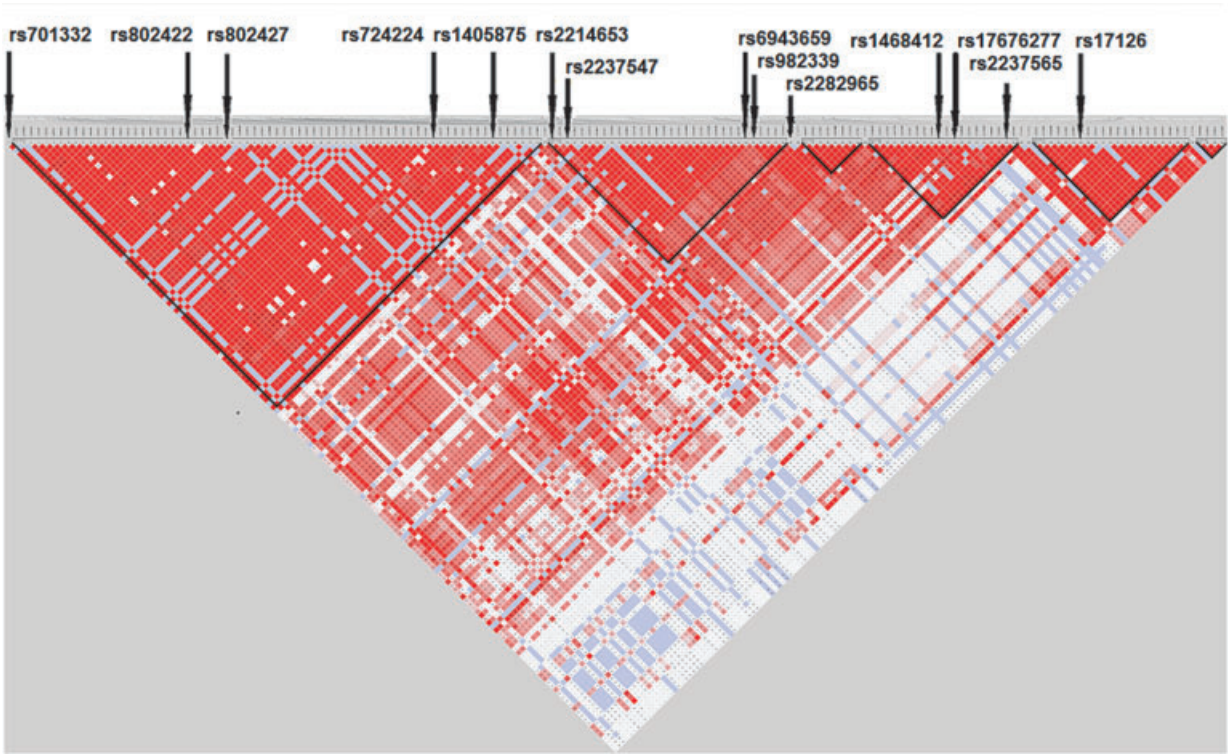

Figure 1: Analysis of LD across 164 *GRM3* SNPs in HapMap CEPH cohort. Deep red, strong LD; red and white, moderate to no LD among the SNPs.

2007), yielding a genotyping completion rate of 98.2%. Genotypes were determined by investigators blinded for the study.

Statistical analyses

Differences of means of continuous variables between groups were tested using two-sample *t*-test (Table 1). Multivariate analysis of covariance (MANCOVA) was performed to investigate the association between *GRM3* SNPs and perseverative errors (MCST in %) considering age, gender, MTWB-IQ and BDI as covariates (Table 3; Fig. 3). In case individual genotypes had small numbers, they were collapsed to a combined genotype (e.g. combined AA/AT genotype of SNP rs17676277). Bonferroni correction for multiple comparisons was carried out *post hoc* for 14 SNPs yielding a corrected *P* value of 0.0036. While no corrections for multiple comparisons were made during parsing of the haplotype analyses, *P* values derived from haplotype analyses were compared with the corrected *P* value of 0.0036.

To reduce computational demands and because of relatively small sample size, haplotypes were analysed using a 'sliding window' approach with a three-marker window size. Haplotypes were inferred using the expectation maximization algorithm from unphased genotype data and a global test of haplotype association was initially performed taking the most common haplotype as baseline and comparing all other haplotypes simultaneously. Statistically significant haplotypes were then explored individually and tested for potential confounders such as gender, age, BDI and log-transformed MWTB IQ. All associations were assessed under additive, dominant and recessive models of inheritance and Akaike's information criterion was used for selecting among models (Akaike 1974). Three SNPs were set to create the haplotypes because of computational limitations and decreasing frequency of longer haplotypes. All computations were performed using SIMHAP version 1.0.2 software (Carter *et al*. 2008).

Results

Neuropsychological performance

Table 3 presents results of MANCOVAs (covariates age, gender, MWTB IQ and BDI) analysing the association between individual *GRM3* SNPs and perseverative errors (in %). Among the 14 *GRM3* SNPs, only rs17676277 showed a significant association with perseverative errors (in %) ($F_{2,40} = 6.4$; $P = 0.0035$). We repeated this analysis with the combined AA/AT genotype because of small numbers of the AA genotype ($N = 4$) and found that the association between rs17676277 and perseverative errors (in %) of the MCST was even stronger ($F_{1,39} = 12.8$; $P = 0.0008$).

Figure 3 (AA vs. AT vs. TT genotypes) presents mean perseverative errors (in %) across rs17676277 genotypes showing that individuals with the TT genotype (mean 3.4; SE 0.5) had significantly less perseverative errors (in %) as compared with the AT (mean 6.6; SE 0.6; $P = 0.0005$) and AA (mean 7.3; SE 3.2; df = 91; $P = 0.025$) genotypes (Fig. 3) as well as compared with the combined AA/AT genotypes (mean 6.7; SE 0.6; df = 90; $P = 0.0005$). All results on SNP rs17676277 withstood Bonferroni correction for multiple

Table 2: Selection of SNPs within *GRM3* gene

Gene	Gene position	Total no. of SNPs (MAF ≥ 0.05)	No. of tagging SNP	Mean r^2	Selected SNPs	Position	Function	Alleles	MAF	Alleles captured	Prediction (STAMPA)
GRM3	Chromosome 7	302	164	0.963	rs701332	86 115 570	Intron 1	C/T	0.233 (C)	9	91.3%
					rs802422	86 131 629	Intron 1	A/G	0.304 (A)	7	
	7q21.1–q21.2				rs802427	86 134 271	Intron 1	C/T	0.375 (T)	28	
					rs724224	86 163 556	Intron 1	A/C	0.442 (A)	2	
	86 111 166–86 332 128				rs1405875	86 168 009	Intron 1	C/T	0.271 (C)	15	
					rs2214653	86 183 431	Intron 1	A/G	0.396 (A)	2	
					rs2237547	86 185 973	Intron 1	A/G	0.283 (G)	19	
					rs6943659	86 233 862	Intron 2	C/T	0.350 (C)	2	
					rs982339	86 237 879	Intron 2	A/G	0.267 (A)	12	
					rs2282965	86 239 740	Intron 2	C/T	0.242 (C)	1	
					rs1468412	86 271 387	Intron 3	A/T	0.241 (A)	9	
					rs17676277	86 277 676	Intron 3	A/T	0.217 (A)	2	
					rs2237565	86 293 472	Intron 3	C/T	0.230 (T)	2	
					rs17126	86 309 849	Intron 4	A/G	0.182 (A)	3	

r^2, LD statistic (Carlson *et al*. 2005). MAF data relate CEPH population from HapMap Phase I and II (International HapMap Consortium, 2005).

comparisons (corrected *P* value for individual tests of 14 SNPs as presented in Table 3: $P = 0.0036$), except the result on the single AA genotype ($P = 0.025$) most likely because of small numbers ($N = 4$).

Haplotype analyses of the *GRM3* gene showed significant associations between three haplotypes and perseverative errors in a dominant model of inheritance (adjusted for age, gender, BDI, MWTB IQ; Table 4). While the ACG haplotype (rs17676277 – rs2237565 – rs17126, $P = 0.0373$) contained the significant SNP rs17676277 from previous single SNP analyses, the other two significant haplotypes contained SNPs without previous significant single SNP associations with perseverative errors (TGA haplotype: rs1405875 – rs2214653 – rs2237547, $P = 0.0379$; GAT haplotype: rs2214653 – rs2237547 – rs6943659, $P = 0.0326$).

Overall, haplotype analyses suggest that various SNPs of the *GRM3* gene are related to perseverative error processing in a dominant model of inheritance. Furthermore, haplotype analyses not only support the role of rs17676277 in this association but also suggest that various other SNPs of the *GRM3* gene in conjunction with rs17676277 impact on perseverative error processing in healthy individuals. However, haplotype analyses would not withstand Bonferroni correction for multiple testing (Bonferroni corrected *P* value = 0.0036).

Discussion

In this study, we investigated the association between a large range of genetic variants of *GRM3* (covering 91.6% of the *GRM3* gene) and perseverative errors in healthy individuals. Among 14 SNPs in the *GRM3* gene, the SNP rs17676277 showed significant associations with perseverative error processing. The A-allele was related to higher numbers of perseverative errors as compared with the TT genotype. Haplotype analyses confirmed this association and the effect was best seen under a dominant model of inheritance,

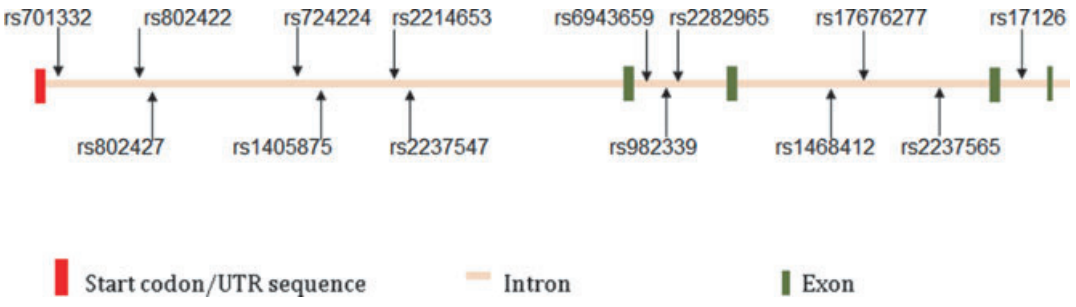

Figure 2: Human *GRM3* gene scheme describing the selected SNP positions.

Table 3: Association between 14 metabotropic *GRM3* SNPs and percentage perseverative errors of the MCST among healthy subjects ($N = 98$) using MANCOVA

14 *GRM3* SNPs	MANCOVA*, *F* value	*P* value*
rs1405875	0.74	0.48
rs1468412	1.41	0.25
rs17126	0.01	0.98
rs17676277	6.4	0.0035†
rs2214653	0.21	0.81
rs2237547	1.25	0.29
rs2237565	0.13	0.88
rs2282965	1.96	0.15
rs6943659	2.65	0.08
rs701332	0.59	0.56
rs724224	1.76	0.18
rs802422	0.52	0.59
rs802427	1.95	0.15
rs982339	0.23	0.79

*P value from MANCOVA with covariates: age, gender, MWTB IQ and BDI. †P value withstands adjusted P value (Bonferroni correction for 14 tests): $P = 0.0036$.

suggesting that only a single copy of each haplotype was required to show a significant association with perseverative error processing. Given the relative high frequency of the haplotypes (between 22.8% and 28.5%), it can be concluded that they have impact on perseverative error processing in a significant number of healthy individuals. However, the haplotype analyses need to be interpreted with caution in this relatively small sample and require replication in larger samples, because the individual haplotype P values would not withstand Bonferroni correction for multiple comparisons.

The SNP rs17676277 is located in a non-translating region (intron 3, position 86 277 676), thus does not result in an amino acid change in the GRM3 protein. Figure 1 shows that rs17676277 is located within the 39kb 'block 4' and all SNPs present in this block are located in intron 3. Recent studies show that nucleotide changes in intronic regions may result in clinical manifestation (Lin *et al*. 2006; Meerson *et al*. 2010). Sequences in these non-protein-coding regions can encode micro ribonucleic acids (miRNAs) that are responsible for RNA-mediated gene silencing through RNA interference (Lin *et al*. 2006). We can hypothesize that SNP rs17676277 can be a part or close to miRNA participating in mGluR3 protein expression regulation. A recent literature research did not show any data confirming or contradicting to presence of miRNAs within the *GRM3* gene. Therefore, more genetic and epidemiological research has to be undertaken to clarify the possible role of the SNP rs17676277 in mGluR3 expression control. Although a mechanistic rationale to explain this association remains to be investigated, our data strongly support

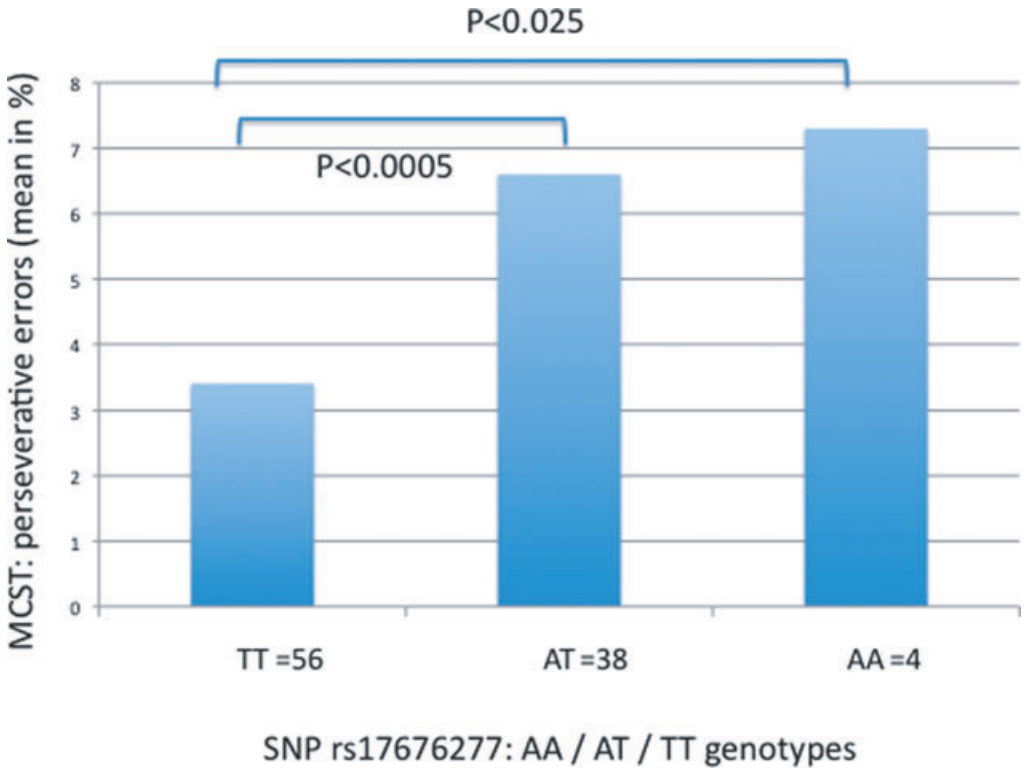

Figure 3: Percentage perseverative errors in $N = 98$ healthy individuals grouped by single *GRM3* SNP rs17676277 genotypes (AA/AT/TT). MANCOVA with covariates: age, gender, MWTB IQ and BDI.

Table 4: Association of *GRM3* gene haplotypes with perseverative errors

				Additive model					Dominant model					Recessive model				
SNP	Haplotype	Frequency	Copy	Coefficient	SE	*P*	Mean	AIC	Coefficient	SE	*P*	Mean	AIC	Coefficient	SE	*P*	Mean	AIC
rs1405875	TGA	0.2853	0	–	–	–	4.1223	504.9 NS	–	–	–	4.1220	502.9 0.0356	–	–	–	4.7394	507.1 NS
rs2214653			1	1.688	0.850	0.0506	5.8099		1.711	0.809	0.0379	5.8332						
rs2237547			2	1.792	1.323	0.1791	5.9143							1.175	1.308	0.3710	5.9143	
rs2214653	GAT	0.2384	0	–	–	–	4.1861	504.8 NS	–	–	–	4.1870	502.8 0.0305	–	–	–	4.7753	507.2 NS
rs2237547			1	1.715	0.850	0.0471	5.9010		1.765	0.812	0.0326	5.9516						
rs6943659			2	2.043	1.709	0.2351	6.2293							1.454	1.715	0.3990	6.2293	
rs17676277	ACG	0.2283	0	–	–	–	4.1644	504.1 NS	–	–	–	4.1644	503.5 0.0348	–	–	–	4.8890	507.2 NS
rs2237565			1	1.740	0.777	0.0276	5.9047		1.632	0.772	0.0373	5.7965						
rs17126			2	−2.469	3.681	0.5041	1.6949							−3.194	3.749	0.3960	1.6949	

Copy, copy number of haplotypes; coefficient, coefficient of linear regression analysis; AIC, Akaike's information criterion; NS, non-significant model.

the role of the *GRM3* gene in cognitive set-shifting processes and the role of SNP rs17676277 as either a causative genetic variant or a biomarker associated with another, possibly functionally relevant polymorphism in LD with this SNP. Studies show that mGluR3 is expressed by glia and neurons in many brain regions and it has a predominantly presynaptic distribution, consistent with its role as an inhibitory autoreceptor and heteroreceptor. The available data suggest particular roles for mGluR3 in long-term depression, in glial function (Luyt *et al*. 2004) and in neuroprotection (Harrison *et al*. 2008) all of which is relevant for complex cognitive processing. Some studies report genetic association of *GRM3* polymorphisms with schizophrenia-related endophenotypes such as impaired cognition, cortical activation and glutamate markers (Harrison *et al*. 2008).

Our study for the first time shows the involvement of the *GRM3* gene in set-shifting in healthy individuals. This result is corroborated by previous findings in various ways. The *GRM3* gene is related first to a reduction of *N*-acetylaspartate/creatine levels in the right dorsolateral prefrontal cortex in humans (Marenco *et al*. 2006) and second to glutamatergic neurotransmission within the medial prefrontal cortex which is required for normal set-shifting performance in rats (Stefani & Moghaddam 2003). Moreover, the latter research showed in a subsequent publication that glutamatergic receptor hypofunction impairs the capacity to modify existing knowledge or to inhibit responses that are no longer appropriate (Stefani *et al*. 2003), both of which are relevant cognitive functions for set-shifting tasks as used in our study.

Alternative to those models indicating the importance of the *GRM3* gene for glutamatergic neurotransmission in set-shifting tasks, glutamatergic neurotransmission has recently been related to working memory function, which might represent an alternate pathway of set-shifting function (Tan *et al*. 2007). Pantelis *et al*. (2009) suggested recently that working memory might contribute to set-shifting impairment in schizophrenic patients as shown for schizophrenia at onset and during the chronic phase of the illness. The authors report that deficits in set-shifting at illness onset are explained by deficits in working memory functions, which are comparable to those observed in chronic illness (Pantelis *et al*. 2009). This observation may imply for our study that the association of the *GRM3* gene with perseverative errors might have been mediated through working memory. However, when we analysed in an analysis of covariance model (dependent variable = perseverative errors) a potential interaction between verbal working memory and the *GRM3* gene (interaction term: F value 1.42, $P = 0.22$), the reported association between SNP rs17676277 and perseverative errors was not altered; in fact, the association appeared stronger than without interaction term (F value 9.47; $P = 0.0006$). Thus, our data suggest that perseverative errors are associated with the *GRM3* gene independent of (verbal) working memory performance.

Our study has strength and limitations. The relatively large coverage of the *GRM3* gene extends previous research, which investigated only a few SNPs. Furthermore, because our study was performed in healthy individuals, the findings extend our knowledge on the role of *GRM3* in physiological

function. However, because the association between the specific SNP and perseverative errors has not been reported before, it is unclear as of yet if this particular SNP plays a role in set-shifting or other neuropsychological functions in a clinical sample such as in schizophrenia. Thus, future clinical research, i.e. in schizophrenia, is required to clarify the role of the *GRM3* gene in general and rs17676277 in particular in executive function. Future studies among healthy individuals would benefit from the assessment of schizotypal personality features which have been shown a relationship with perseverative errors (Wilson *et al*. 2008). Albeit various other genetic association studies in the field study healthy individuals with similar sample sizes (Hashimoto *et al*. 2009a,b), the relatively small sample size of our genetic association study in a relatively homogenous group of healthy subjects requires replication in independent and larger samples. Furthermore, because our finding points to an involvement of the *GRM3* gene in complex cognitive tasks such as set-shifting under 'physiological' conditions, replication in clinical samples of schizophrenic patients is of greater interest.

In conclusion, set-shifting abilities such as perseverative error processing appear to be related to the *GRM3* gene (SNP rs17676277, three haplotypes) in healthy individuals, which does not appear to be mediated through working memory performance. However, because of a relatively small sample size for a genetic association study, the present results are tentative and require replication.

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