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Functional Analysis of a Potassium-Chloride Co-Transporter 3 (*SLC12A6*) Promoter Polymorphism Leading to an Additional DNA Methylation Site

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The human potassium-chloride co-transporter 3 (*KCC3*, *SLC12A6*) is involved in cell proliferation and in electro-neutral movement of ions across the cell membrane. The gene (*SLC12A6*) is located on chromosome 15q14, a region that has previously shown linkage with bipolar disorder, schizophrenia, rolandic epilepsy, idiopathic generalized epilepsy, autism and attention deficit/hyperactivity disorder. Furthermore, recessively inherited mutations of *SLC12A6* cause Andermann syndrome, characterized by agenesis of the *corpus callosum*, which is associated with peripheral neuropathy and psychoses. Recently, we have demonstrated the association of two G/A promoter polymorphisms of *SLC12A6* with bipolar disorder in a case–control study, and familial segregation of the rare variants as well as a trend toward association with schizophrenia. To investigate functional consequences of these polymorphisms, lymphocyte DNA was extracted, bisulfite modified, and subsequently sequenced. To investigate *SLC12A6* promoter activity, various promoter constructs were generated and analyzed by luciferase reporter gene assays. We provide evidence that the G- allele showed a significant reduction of reporter gene expression. In human lymphocytes, the allele harboring the rare upstream G nucleotide was found to be methylated at the adjacent C position, possibly accountable for tissue-specific reduction in gene expression *in vivo*. Here we demonstrate functionality of an SNP associated with psychiatric disease and our results may represent a functional link between genetic variation and an epigenetic modification. *Neuropsychopharmacology* (2009) **34,** 458–467; doi:10.1038/npp.2008.77; published online 4 June 2008

Keywords: *SLC12A6*; DNA methylation; epigenetics; gene × environment interaction; bipolar disorder; periodic catatonia

INTRODUCTION

The potassium-chloride co-transporter 3 (*SLC12A6*, formerly *KCC3*; OMIM %604878) belongs to a protein family consisting of four transporters so far identified (*SLC12A4-7*; *KCC1-4*). These transporters are involved in cell proliferation (Shen *et al*, 2001) and in the electro-neutral movement of ions across the plasma membrane, thus controlling ionic and osmotic homeostasis of various cell types. The gene *SLC12A6* is located on chromosome 15q13–14 (Hiki *et al*, 1999; Mount *et al*, 1999; Race *et al*, 1999). Several groups have demonstrated that mutations in *SLC12A6* are causative for the recessively inherited Andermann syndrome (ACCPN, OMIM %218000), a severe neurological disorder characterized by agenesis of the *corpus callosum*, peripheral neuropathy (Howard *et al*, 2002b, 2003; Uyanik *et al*, 2006) and psychoses (Filteau *et al*, 1991). The chromosomal region 15q13–14 represents a shared susceptibility locus for a variety of neuropsychiatric disorders including periodic catatonia, bipolar disorder, schizophrenia, rolandic epilepsy, idiopathic generalized epilepsy, autism and attention deficit/hyperactivity disorder (Elmslie, 1997a, b; Elmslie *et al*, 1997; Sander *et al*, 1997).

Interestingly, approximately 1% of the total pool of human genes are coding for solute carrier genes (Gamba, 2005). The genes encoding cation-Cl^- coupled co-transporters are grouped together in the SLC12 family. These co-transporters have recently gained attention in the field of neuroscience, as they facilitate neuronal response to gamma-aminobutyric acid and glycine (Mercado *et al*, 2004). Furthermore, several members of the cation-chloride co-transporter family are involved in hereditary diseases, such as Gitelman's, Bartter's, Gordon's and Andermann's syndromes, and have shown associations with bipolar disorder (Bianchetti *et al*, 1992; Dupre *et al*, 2003; Filteau

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et al, 1991; Gamba, 2005; Gitelman *et al*, 1966; Gordon, 1986; Howard *et al*, 2002b, 2003; Meyer *et al*, 2005; Uyanik *et al*, 2006). The crucial role of cation co-transporters for neuronal development is also supported by animal studies in which knockout mice for cation-chloride co-transporters display a plethora of symptoms such as deafness, locomotor deficits, severe central and peripheral neurodegeneration, and sensorimotor gating defects (Boettger *et al*, 2002, 2003; Hubner *et al*, 2001).

Hence, *SLC12A6* may also be regarded as a valuable candidate gene for complex inherited psychiatric disorders. Initially, two first exons denoted by *SLC12A6-1* and *SLC12A6-2* (formerly *KCC3a* and *KCC3b*), which are under the control of separate promoters (Di Fulvio *et al*, 2001; Mercado *et al*, 2004), have been described where the transcript possessing alternative exon 1a is predominantly expressed in brain. So far, six alternative transcripts have been identified for *SLC12A6* (Mercado *et al*, 2005). All transcripts except for *SLC12A6-2*, are regulated by the same promoter.

Recently, we have reported two G/A polymorphisms in the *SLC12A6* 5′ regulatory region, denoted as 32418760 G/A and 32416574 G/A (numbering of nucleotide positions are relative to chromosome 15 of the UCSC hg18 assembly of the human genome, March 2006; no rs numbers available). These alleles are consecutively referred to as 'upstream G/A' (nt 32418760) and 'downstream G/A' (nt 32416574). Both G variants are rare, with frequencies of about 6%, and in strong linkage disequilibrium with each other. They were found to be significantly associated with bipolar disorder in a case–control study. Furthermore, in a large family with seven affected members, the rare variants were co-segregating with periodic catatonia (OMIM %605419), a subtype of unsystematic schizophrenia according to Leonhard's classification (Meyer *et al*, 2005).

Apart from investigating genetic variations as risk factors for psychiatric disease, the study of epigenetic mechanisms is gaining growing importance in the field of neuropsychiatry. Epigenetics comprises all cellular mechanisms that are involved in the 'structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states' (Jaenisch and Bird, 2003). The most prominent mechanisms of epigenetic gene regulation are DNA methylation (Jaenisch and Bird, 2003; Rakyan and Beck, 2006) and histone modifications. It could be demonstrated that differences in epigenetic states such as differential CpG methylation are affected by environmental factors, including maternal care and critical life events (Fraga *et al*, 2005; Weaver *et al*, 2004, 2005). It was also demonstrated that DNA methylation patterns at specific genes are associated with bipolar disorders, schizophrenia, and major depression (Abdolmaleky *et al*, 2006; Grayson *et al*, 2005). Studies of the reelin gene (*RELN*) and the catechol-*O*-methyltransferase gene (*COMT*) promoter identified key bases that function in both positive and negative transcriptional regulation depending on their DNA methylation status. In addition, the methylation pattern of these *cis*-acting elements are significantly different between schizophrenic and non-schizophrenic subjects (Abdolmaleky *et al*, 2004, 2005; Grayson *et al*, 2005, 2006).

The aim of this study is to investigate the functionality of the *SLC12A6* promoter variants. As cytosines, which are directly followed by a guanine, are target for DNA methylation, we hypothesized that the presence of the G nucleotide at the upstream G/A polymorphic site could lead to methylation of the adjacent C *in vivo*. We thus analyzed the DNA methylation state of the upstream CpG site in affected patients and tested *in vitro* methylated *SLC12A6* promoter constructs carrying the G allele or the A allele for their activity in luciferase reporter gene assays.

MATERIALS AND METHODS

Sample Collection

Blood samples of all affected and unaffected members of family 11 were obtained as described before (Meyer *et al*, 2005; Stober *et al*, 2000, 2001), and DNA was extracted according to the salting out procedure (Miller *et al*, 1988).

Bisulfite Modification

Bisulfite modification of DNA was performed as described elsewhere (Frommer *et al*, 1992; Grunau *et al*, 2001) with minor modifications. DNA was obtained from affected and unaffected members of family 11 (Meyer *et al*, 2005) heterozygous for both polymorphisms located in the *SLC12A6* 5′ regulatory region. Two micrograms of genomic DNA were incubated for 20 min at 42°C in 50 µl of 0.3 M NaOH solution. Subsequently, 30 µl of 10 mM hydroquinone (Sigma), and 520 µl of freshly prepared 40.5% sodium bisulfite solution (Sigma) at pH 5 were added to the denatured samples and incubated for 16 h at 55°C in the dark. Modified DNA was desalted using the Wizard DNA purification resin (Promega) according to the manufacturer's protocol and eluted into 50 µl of water. Modification was completed by NaOH treatment (final concentration, 0.3 M) for 10 min at room temperature followed by the addition of 10 M ammonium acetate to a final concentration of 3 M. Additionally, 10 µg of salmon sperm DNA were added as a carrier before ethanol precipitation. Finally, DNA was re-suspended in 25 µl of water and used immediately for PCR or stored at −20°C.

PCR Amplification

The sequence of interest in the bisulfite-treated DNA was amplified by nested PCR with primers specific for the bisulfite-treated sequence (Table 1). To control for efficiency of the bisulfite modification, PCR was also performed with primers selectively targeting untreated genomic DNA.

Bisulfite-specific PCRs were performed in a final volume of 50 µl containing 5 µl of bisulfite-modified DNA (2 µl PCR product for nested PCR; 100 ng DNA for genomic PCR, respectively), 20 pmol of each primer (10 pmol of each primer for nested and genomic PCR), 200 µM of each dNTP, 1.0 or 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C), 0.0025 mg/ml BSA, 0.025% Tween 20, and 2.5 U High Fidelity Taq Polymerase (MBI-Fermentas). To the first bisulfite-specific PCR, 5% dimethylsulfoxide was added.

Amplification was performed in an ABI GeneAmp®9700 cycler: 5 min at 95°C were followed by 35 cycles, (30 s at

Table 1 PCR primer sequences

Region chromosome 15	PCR primers (5′→3′), hybridization temperature $MgCl_2$ concentration and DMSO	Product length (bp)
Accession no.: NM_133647: 32418817–32418400	Forward genomic: TGAATCAAGAAACCCAGACT Reverse genomic: ATTCCATGTTTTCACCACTAC 50°C 1.5 mM	418
Accession no.: NM_133647 32418842–32418564	Forward bisulfite GAGGATGGATGAAAGTTGTGGG Reverse bisulfite CCCAAAAACTAACCCCCAAT 57°C; 1.5 mM 5% DMSO	278
Accession no.: NM_133647 32418839–32418588	Forward bisulfite nested GATGGATGAAAGTTGTGGGTT Reverse bisulfite nested CCTAAAATCTTAACTCCTCACAAATAA 54.2°C; 2 mM	251
Accession no.: NM_133647 32419307–32416464	Forward senseKpn1_for Luciferase ggtaccGTTGGCTGCAGTTCTGCCTTTATCTTA Reverse senseXho_rev Luciferase ctcgagAAGAGCTACCTAGCTAACCCCTCTGGT 64°C 1.5 mM	2844
Accession no.: NM_133647 32419307–32416464	Forward antisenseXho_for Luciferase ctcgagGTTGGCTGCAGTTCTGCCTTTATCTTA Reverse antisenseKpnI_rev Luciferase ggtaccAAGAGCTACCTAGCTAACCCCTCTGGT 64°C 1.5 mM	2844
SLC12A6 cDNA 11060569–11055819	SLC12A6 cDNA specific Primer_for CGGACATAAGAAAGCTCGAAA SLC12A6 cDNA specific Primer_rev CAGTCAACATTGTACAGCAGCA 58°C 2 mM	383

95°C, 45 s at the annealing temperature listed in Table 1, and 30 s at 72°C) ending with a final extension step at 72°C for 7 min. Controls without DNA were performed for each set of PCR. PCR products (10 μl) were separated on a 1.5% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination.

Nested PCR products were gel purified, fluorescence labeled (Big dye terminator kit; ABI), and directly sequenced in an ABI 310 automated sequencer (Applied Biosystems, Foster City, USA).

Assembly of Luciferase Reporter Gene Plasmids

Reporter gene plasmids were created by cloning four 2844 bp PCR fragments amplified from the 5′-flanking region of *SLC12A6*. Amplicons covered the chromosomal nucleotide positions nt 32419307–32416464 according to UCSC Browser of May 2004. Primer-specific *Kpn*I and *Xho*I recognition sites were added to the PCR products (see Table 1).

The fragments were consistent with the *SLC12A6* G- and A variant promoters, covering both upstream and downstream G/A polymorphic sites. PCR fragments were digested with *Kpn*I and *Xho*I and ligated into the appropriate sites of pGL3-basic luciferase reporter plasmid, which was cut by the same enzymes, with both allelic sequences represented in both sense (pGl3-A-A; pGl3-G-G) and antisense directions (pGl3-A-Ainv; pGl3-G-Ginv).

To distinguish between putative functional consequences of up- and downstream polymorphic promoter sites, a 953 bp fragment (nt 32418869–32417927) including the upstream G/A site was deleted by using *Stu*I (see Figure 1). Subsequently, the plasmids were re-ligated, resulting in constructs differing solely in their downstream G/A polymorphic region (pGL3-del-A; pGl3-del-G). To control for sequence integrity, all constructs were completely sequenced (see Supplementary Table 2).

In Vitro Methylation

SLC12A6 promoter constructs were methylated *in vitro* by using SssI methylase and S-adenosyl methionine under conditions recommended by the manufacturer (New England Biolabs). Complete DNA methylation was ascertained by digesting the methylated DNA with an excess (20 U/mg) of restriction enzymes *Bst*UI or *Tai*I. Only completely methylated DNA preparations were used for transfection under conditions described below.

Transient Transfection and Luciferase Assays

U373MG glioblastoma-astrocytoma (ECACC 89081403), *SLC12A6*-expressing cells, were grown in minimal essential Eagle's medium (Biowest), supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1% penicillin/streptomycin (Pen/Strep), and 1% nonessential amino acids (NEAA) at 37°C in a humified atmosphere at 5% CO_2. Cells were transfected by using Fugene6 transfection reagent (Boehringer) in a ratio of 2 : 1 (μl Fugene6 per μg DNA). Cells were passaged at ~80% confluency and spread in a density of 1×10^5 cells/ml in 24-well plates. After 24 h of incubation, 0.8 μg firefly luciferase constructs with 0.2 μg pGL4.74 (HRLUC/TK, *Renilla* luciferase; Promega), 2 μl Fugene6, and 20 μl serum-free medium were added to the cell culture. The plasmid expressing *Renilla* luciferase was used to control for transfection efficiency. Promoter activity was tested with and without 50 μM forskolin, 2 μM phorbol 12-myristate acetate (PMA), and 50 μM dexamethasone added 24 h after transfection. As internal control, pGL3-control and empty pGL3-basic vectors were transfected separately. Cells were harvested in 100 μl Passive Lysis Buffer (Promega) 48 h after transfection. Additionally, U373MG cells were also transfected after 3 weeks preincubation with 1 mM lithium (Li), 75 μg/ml valproic acid, or 10 ng/ml Haloperidol, respectively. Firefly and *Renilla* luciferase activities were determined sequentially with 20 μl of total cell lysates in 100 μl of luciferase assay reagent followed by addition of 100 μl of Stop & Glo reagent (Promega) per reaction. Chemiluminescence was measured for 10 s in a liquid scintillation spectrophotometer (Berthold).

In vitro methylated constructs were transfected in a ratio 5 : 1 (Fugene: DNA) using 0.4 μg pGL3-*SLC12A6* constructs

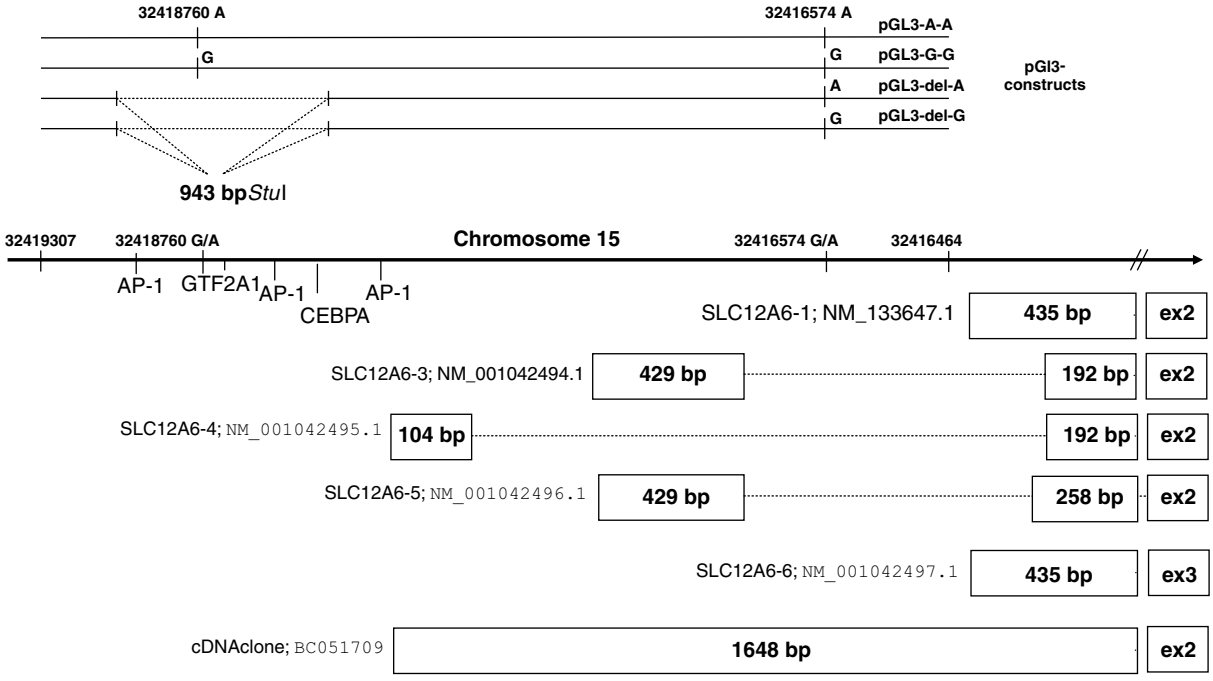

Figure 1 Overview of the *SLC12A6* 5′ regulatory region. Luciferase constructs, polymorphic 32418760 G/A, and 32416574 G/A nucleotides (Meyer *et al*, 2005) are indicated. Transcription factor-binding sites, *Stu*I cutting sites, alternative *SLC12A6* transcripts (Mercado *et al*, 2005), and a putative new *SLC12A6* transcript (BC051709) identified by *in silico* analysis are shown according to their positions on chromosome 15.

and 0.1 μg pGL4.74 control vector. Luciferase expression was assayed under basal conditions, dexamethasone, and PMA-stimulated conditions.

Experiments were done in triplicates and derived from four independent trials. Promoter activities are presented as relative light units calculated as the quotient of the individual firefly luciferase driven value (promoter activity) divided by the correspondent *Renilla* luciferase driven value (internal control). Nonparametric Mann–Whitney *U*-test was used to calculate statistical differences between the groups investigated. Calculations were done both with and without statistical outliers, resulting in almost identical outcome. The results presented here are based on a sample where statistical outliers are included.

Pharmacological Studies

EBV-transformed lymphocytes of heterozygous patient 834 (Meyer *et al*, 2005) were cultured in RPMI 1640, supplemented with 15% FCS, 2% L-glutamine, 1% Pen/Strep, and 0.2% NEAA at 37°C in a humified atmosphere at 5% CO_2. Cells were incubated with and without 1 mM Li, 75 μg/ml valproic acid, or 10 ng/ml haloperidol for 21 days. DNA was isolated and assayed for *SLC12A6* promoter methylation status as described above.

Bioinformatic Analysis

Alternative promoters of *SLC12A6* were computationally analyzed using the BIMAS promoter scan software (BIMAS promoter scan; http://www-bimas.cit.nih.gov/molbio/proscan), and by comparison with recent predictive annotation of default epigenetic states and predicted DNA methylation of all human CpG islands (Bock *et al*, 2006, 2007). Predictions were obtained from http://rd.plos.org/10.1371_journal.pcbi.0030110_01. Transcription factor-binding sites were analyzed using TRANSFAC 10.1.

RESULTS

Polymorphic promoter sites as well as transcription initiation sites of *SLC12A6* are shown in Figure 1. Location of luciferase constructs, restriction sites, and transcription factor-binding sites revealed by using TRANSFAC 10.1 software are indicated.

Methylation

Genomic DNA was extracted from blood of patients carrying the rare promoter allele. As a CpG site is created in the upstream G allele but not the A allele, we examined the methylation status of the adjacent 5′ cytosine. For this purpose, DNA samples were treated with bisulfite and subsequently sequenced as described above (for results, see Figure 2). DNA of healthy and affected individuals heterozygous for the *SLC12A6* promoter variants were assayed for their DNA methylation pattern. The 5′ cytosine of the upstream G variant was found methylated in all healthy controls (853 and 743) and affected family members (834, 744, and 999; Meyer *et al*, 2005).

The aim of this experiment was to investigate in principal whether the cytosine neighboring the upstream SNP can be found in a methylated state. Therefore, quantification of DNA methylation (as described elsewhere; Moser *et al*, 2007) was not performed. For reasons of sample availability, DNA methylation analysis was performed on lymphocyte

DNA. As tissue-specific DNA methylation seems to be confined to no more than 5% of human promoters (Eckhardt *et al*, 2006; Song *et al*, 2005), we believe that it is plausible to extrapolate our findings from blood to brain tissue. To assess the functional effect of both the SNP and its DNA methylation status on gene regulation, we subsequently analyzed the effect of the different alleles on gene expression.

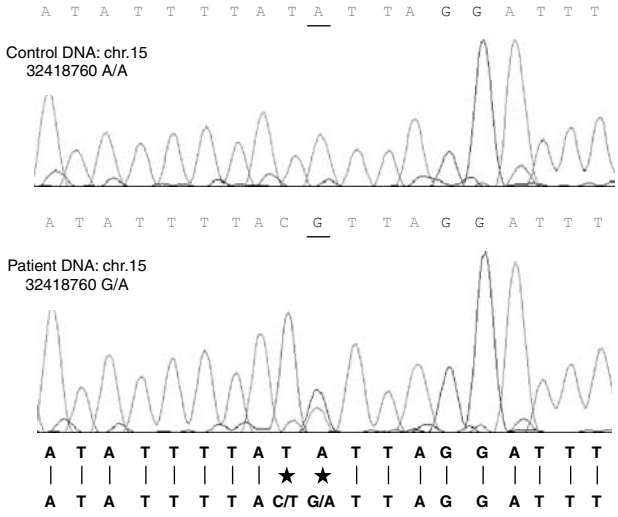

Figure 2 Electropherogram indicating directly sequenced PCR products of *SLC12A6* promoter fragments, of bisulfite-modified DNA deriving from individuals homozygous (A/A) and heterozygous (G/A) for the nt 32418760 SNP. The position of the upstream SNP is underlined; methylation of the adjacent C nucleotide is indicated by an asterisk.

Luciferase Reporter Gene Assays

To investigate the impact of the polymorphic sites on gene regulation, the entire portion of the promoter as well as subfragments were fused to a luciferase reporter gene as described above. As shown in Figure 3, the presence of G alleles (pGl3-G-G) reduced basal luciferase expression in comparison to the abundant A alleles (pGl3-A-A; $p<0.001$). Dexamethasone significantly stimulated luciferase expression in the presence of A (pGl3-A-A) but not G alleles (pGl3-G-G; $p<0.001$), thus pointing to selective glucocorticoid-mediated regulation of the abundant promoter variants even as there is no GRE motif present in the promoter sequence. PMA stimulates expression of both G and A alleles but the promoter activity of the A allele remained significantly higher ($p<0.001$). Therefore, protein kinase C-mediated regulation is likely, but probably does not act directly at the polymorphic sites. As there are several activator protein 1-binding elements present in the *SLC12A6* promoter, increased promoter activity may be induced by the PMA-induced PKC activation of these transcription factors.

Forskolin did not affect promoter activity, and the results were almost identical to unstimulated conditions for both *SLC12A6* promoter constructs. This provides evidence that PKA-mediated signal-transduction pathways are not involved in the regulation of *SLC12A6* expression. Under forskolin stimulation, activity of the A allele is, as before, significantly higher compared to the G alleles ($p<0.001$).

To distinguish the functionality of the polymorphic sites, the upstream polymorphic site was deleted as described. As shown in Figure 4, deletion of the respective upstream site by *Stu*I digestion equalizes expression rates of both A- and G-specific promoter constructs (pGl3-del-A; pGl3-del-G;

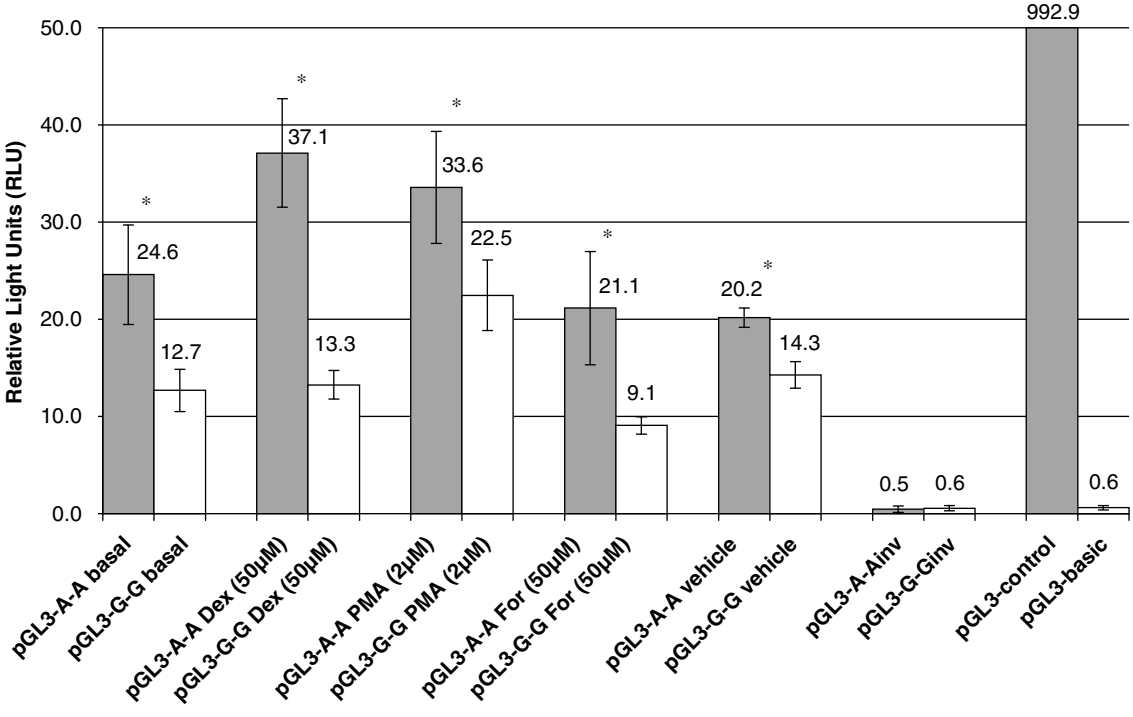

Figure 3 Basal and induced activity of the *SLC12A6* 5′ regulatory region: pGL3-A-A *vs* pGL3-G-G-specific *SLC12A6* promoter constructs. (*$p<001$).

$p = 0.16$) to a medium level, pointing to functional relevance exclusively of the 5′ polymorphic site. Interestingly, the promoter activity was found to be decreased for the pGl3-del-A construct, whereas it was slightly increased in the vector carrying the G variant *SLC12A6* promoter fragment (pGl3-del-G).

In vitro methylation of pGL3-A-A and pGL3-G-G constructs led to almost silenced promoter activity. After *in vitro* methylation, pGL3-G-G activity was significantly lower (all $p < 0.02$) compared to that of the pGL3-A-A (Figure 5)

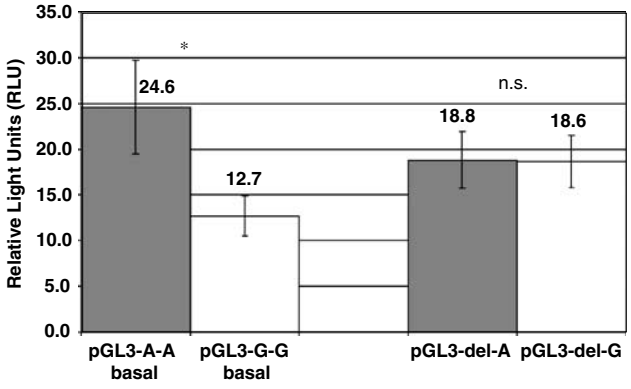

Figure 4 Basal *SLC12A6* promoter activity before and after deletion of the upstream G/A polymorphic site (*$p < 001$).

under all conditions assessed, may be due to the additional methylation site in the G-allele.

Effects of Mood Stabilizers and Antipsychotic Drugs on CpG Methylation and *SLC12A6* Promoter Activity

As it has been proposed that *SLC12A6* resembles a positional candidate gene for bipolar disorder and subtypes of schizophrenia (Delpire and Mount, 2002; Meyer *et al*, 2005), we investigated whether the *SLC12A6* polymorphic promoter sites are prone to stimulation by common mood stabilizers, such as Li and valproic acid, which are both commonly used in the treatment of bipolar disorder, and the antipsychotic drug haloperidol, frequently used in the treatment of psychoses. However, presence of haloperidol, valproic acid, or Li had no effect on reporter gene expression in our test system, as they also had no influence on promoter methylation (data not shown).

Computional Epigenetics

Genes with strong CpG island promoters are often ubiquituously expressed, showing robust transcription across a wide range of tissue types (Vinogradov, 2005). In constrast, genes with less CpG-rich promoters are more susceptible to regulation by variable methylation levels (Eckhardt *et al*, 2006). By comparison with a recently published prediction of the strength and expected epigenetic state of all CpG-rich

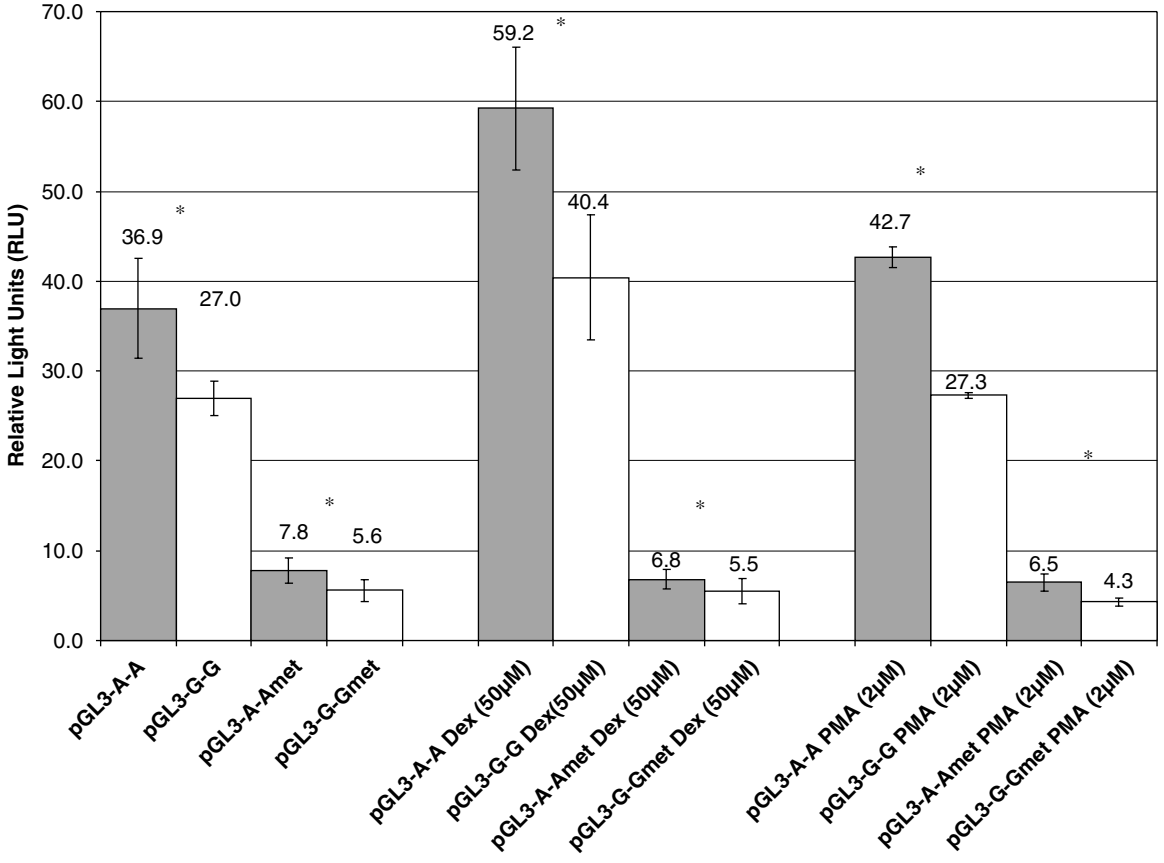

Figure 5 Basal and induced activity of the *SLC12A6* 5′ regulatory region before (pGL3-A-A *vs* pGL3-G-G) and after *in vitro* methylation (pGL3-A-Amet *vs* pGL3-G-Gmet; all *$p < 002$).

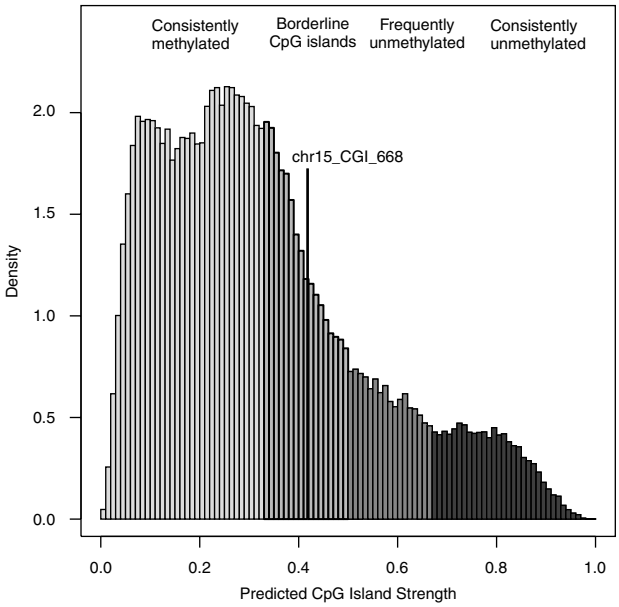

Figure 6 Histogram of the predicted CpG island strengths for all CpG islands in the human genome. The *x*-axis gives the CpG island strength as predicted by the combined epigenetic score (Bock *et al*, 2007), and the *y*-axis gives a density estimate, normalized to a total area of one. From left to right, CpG islands are predicted to exhibit a more open and transcriptional permissive chromatin structure. The orthogonal line marks the promoter CpG island of *SLC12A6*.

regions in the human genome (Bock *et al*, 2007), we found that *SLC12A6* is regulated by a weak CpG island promoter (Figure 6). With a CpG island strength prediction of 0.418 on a scale between zero and one (highlighted by a vertical line in Figure 6), it may be sensitive to a single additional CpG upstream of the core promoter. A recent study of the genomic determinants of DNA methylation variation (Bock *et al*, 2008) lends further support to a potential epigenetic function of the G/A polymorphism in the upstream region of *SLC12A6*. Using the methodology of this study, the genomic region between the SNP and the transcription start site was predicted to exhibit relatively large interindividual variation but also consistent conservation of the DNA methylation status of specific CpGs (the region's predicted h_i score is 0.03). Furthermore, we note that a genomic polymorphism that becomes functional by its *cis* effect on epigenetic regulation has been reported previously by a study on Beckwith–Wiedemann syndrome (Murrell *et al*, 2004), in which loss of imprinting at the *IGF2* gene could be associated with four neighboring SNPs. In conclusion, while a rigorous proof that the upstream G/A polymorphism is functional due to epigenetic rather than genetic regulatory effects is still outstanding, we have accumulated several lines of experimental and computational evidence that DNA methylation may contribute to its effect on gene expression.

DISCUSSION

The potassium-chloride co-transporter 3 gene represents a strong positional and functional candidate for psychoses, including bipolar disorder and schizophrenia (Delpire and Mount, 2002; Meyer *et al*, 2005; Uyanik *et al*, 2006). Here, we provide a functional mechanism for a rare promoter variant of *SLC12A6*, associated with bipolar disorder (Meyer *et al*, 2005). It could be demonstrated that the less frequent allele of the investigated SNP can lead to DNA methylation at a CpG site, and was associated with differences in the regulation of gene expression in our test system.

As shown by Escher *et al* (2005), plasmids can be methylated after transfection. Therefore, we tested *SLC12A6* promoter activity comparing G *vs* A alleles. To ensure complete plasmid methylation, constructs were also assayed after *in vitro* methylation. Under all conditions tested the *SLC12A6* G promoter allele showed significantly lower promoter activity compared to the A-allele. Deletion of the upstream polymorphic region led to equal promoter activities of both constructs, indicating that the *SLC12A6* upstream promoter SNP is functional and responsible for altered promoter activity. The SNP investigated here was found to be associated with bipolar disorder in a case–control study (Meyer *et al*, 2005). Results of this study now demonstrate functionality of the upstream SCL12A6 promoter SNP. We propose that its functionality might be modulated by DNA methylation, known to be influenced by developmental and environmental factors in a time- and tissue-dependent manner and therefore may contribute to the pathogenesis of (psychiatric) disorders.

SLC12A6 is involved in cell proliferation processes (Shen *et al*, 2004) and mutations of this gene cause a severe neurological disorder frequently associated with psychosis (Filteau *et al*, 1991; Howard *et al*, 2002a, b, 2003). The involvement of this gene in psychopathology supports the developmental hypothesis of psychoses (Isohanni *et al*, 2001; Jones *et al*, 1994; Weinberger, 1995) ,which suggests that subtle malfunction of axon guidance processes in the developing brain may lead to cognitive and behavioral impairment. It is noteworthy that we are not studying novel mutations here but polymorphic variants, of which the G alleles are rare but nonetheless present in the gene pool in Hardy–Weinberg equilibrium with their respective pendants. According to the theory of complex disorder inheritance (Cannon, 2005), functional variants of this kind may act together with others in the pathogenesis of certain complex diseases and personality traits (Kohn, 2005).

In addition, it has been proposed (Liu *et al*, 2005) that rare variants may be of importance to the understanding of pathogenesis of complex inherited disorders.

Our findings support observations made in *Slc12a6* knockout studies revealing a gene dosage effect in *Slc12a6* null compared to heterozygous knockout mice (Boettger *et al*, 2003; Howard *et al*, 2002b, 2003; Jentsch, 2005; Rust *et al*, 2006).

In humans, *SLC12A6* deficiency leads to Andermann's syndrome. Interestingly, individuals possessing only one functional *SLC12A6* allele do not show any clinical or even subclinical phenotype. However, here we provide evidence for a gene variant leading to a DNA methylation site with functional consequences on *SLC12A6* promoter activity. Such methylation-sensitive variants may represent targets for epigenetic modification induced by environmental factors (Weaver *et al*, 2004), and become functionally relevant only after the occurrence of disease precipitating factors such as chronic stress, critical life events, or pathogen exposure (Fraga *et al*, 2005; Weaver *et al*, 2004, 2005).

We speculate that the newly identified DNA methylation site may represent a polymorphism with a *cis*-regulatory, epigenetic mode of functioning. As the role of environmental factors in influencing the DNA methylation and other epigenetic modifications is now well-established (Peaston and Whitelaw, 2006), it seems likely that the presence of a *SLC12A6* promoter variant, by introducing an additional DNA methylation site, might mediate a functional connection between environmental factors such as psychosocial stress and the increased susceptibility to psychopathology conferred by a genetic variant (de Kloet *et al*, 2005). The possible impact of environmental factors on the epigenotype has recently been demonstrated. For instance, it could be shown that natural variations in maternal care (Weaver *et al*, 2004), nutrition (Weaver *et al*, 2005; Wolff *et al*, 1998), and drug treatment (Alonso-Aperte *et al*, 1999; Shimabukuro *et al*, 2006; Weaver *et al*, 2004) have an impact on promoter methylation in rats. Interestingly, the epigenotype shows differences even in human monozygotic twins over the lifespan, reflecting the influence of lifestyle and ageing on the epigenotype (Fraga *et al*, 2005).

Promoter activities were found to be decreased after deletion of a 943 bp *Stu*I fragment for the pGl3-del-A construct, whereas it slightly increased in the vector carrying the G variant *SLC12A6* promoter fragment (pGl3-del-G). This is supporting our hypotheses that CpG methylation adjacent to the upstream G/A polymorphic site could lead to a slightly more condensed DNA organization in the G-allele, whereas the DNA organization in the A-allele lies open for transcription. After deletion of the upstream polymorphic region, elements available for epigenetic modulation silencing promoter activity in the G-allele/enhancing promoter activity in the A-allele, respectively, were excised leading to almost identical promoter activities of the constructs investigated.

It has to be mentioned as a limitation that, as we did not have access to brain tissue of the appropriate genotype, DNA methylation pattern in carriers of the G allele was investigated in peripheral tissue. Given the high tissue specificity in gene expression patterns, brain tissue might have a different DNA methylation pattern. However, we could demonstrate in principle that the *SLC12A6* upstream G/A SNP can lead to allele-specific methylation and that presence of the G allele is associated with reduced promoter activity—possibly modulated by DNA methylation.

In silico analysis, using TRANSFAC 10.1® did not reveal altered transcription factor-binding sites comparing A and G-allele. It cannot be ruled out that unknown *cis*- or *trans*-acting factors may affect allele-specific promoter activity. As the upstream palindromic sequence TTAC**A**TT is abolished in the presence of the rare G nucleotide (TTAC**G**TT), putative transcription factor-binding efficiency could be altered and also be fine-tuned by cytosine methylation in a time-, tissue-, and environment-dependent manner.

On the other hand, it was hypothesized that methylated DNA can facilitate histone deacetylation by recruiting chromatin remodeling enzymes such as histone deacetylases by the action of methyl-CpG-binding domain proteins (MBDs) similar to MeCP2 (Tsankova *et al*, 2007). In the G-allele, a palindromic sequence of sense and antisense strand is created (5′-AC**G**T-3′), possibly representing a MBD-binding site. This putative protein-binding site furthermore could be modulated by cytosine methylation leading to enhanced MBD binding and decreased promoter activity. Both possibilities, higher binding efficiency for a positive regulator of gene transcription in the A-allele or higher binding efficiency of a negative regulator in the G-allele, may help explaining the putatively causative effect of this variant in family 11 (Meyer *et al*, 2005).

Future studies are warranted to identify putative transcription factors selectively binding in the *SLC12A6* upstream polymorphic region. It will need to be clarified whether CpG methylation underlies functionality at this site or if transcription factor binding is altered due to the presence of the G allele *per se*. Even if the reduction in gene expression is independent of CpG methylation, this study provides data for the functionality of the investigated SNP.

Furthermore, as DNA methylation and histone deacetylation act in a synergistic manner to regulate gene transcription (Attwood *et al*, 2002), *SLC12A6* SNP-specific chromatin immunoprecipitation assays (ChiP-assays) to identify histone acetylation pattern influencing *SCL12A6* DNA organization are warranted. Additionally, it is conceivable that alternative *SLC12A6* first exon usage, as already described for the neuronal nitric oxide synthase (nNOS, Reif *et al*, 2006; Saur *et al*, 2004), can be selectively modulated by promoter SNPs. Therefore, tissue-specific expressions of the different *SLC12A6* transcripts have to be elucidated in further studies.

In conclusion, this study provides evidence that the upstream *SLC12A6* G/A promoter SNP is functional not only by changing the DNA primary structure but also by influencing the allelic epigenotype and consequently by influencing the chromatin organization. The upstream *SLC12A6* 'G/A' SNP could represent a functional link between genetic variation and epigenetic modification.

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