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Human dopamine transporter gene: differential regulation of 18-kb haplotypes

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Abstract

Aim—Since previous functional studies of short haplotypes and polymorphic sites of *SLC6A3* have shown variant-dependent and drug-sensitive promoter activity, this study aimed to understand whether a large *SLC6A3* regulatory region, containing these small haplotypes and polymorphic sites, can display haplotype-dependent promoter activity in a drug-sensitive and pathway-related manner.

Materials & methods—By creating and using a single copy number luciferase-reporter vector, we examined regulation of two different *SLC6A3* haplotypes (A and B) of the 5' 18-kb promoter and two known downstream regulatory variable number tandem repeats by 17 drugs in four different cellular models.

Results—The two regulatory haplotypes displayed up to 3.2-fold difference in promoter activity. The regulations were drug selective (37.5% of the drugs showed effects), and both haplotype and cell type dependent. Pathway analysis revealed at least 13 main signaling hubs targeting *SLC6A3*, including histone deacetylation, AKT, PKC and CK2 α -chains.

Conclusion—*SLC6A3* may be regulated via either its promoter or the variable number tandem repeats independently by specific signaling pathways and in a haplotype-dependent manner. Furthermore, we have developed the first pathway map for *SLC6A3* regulation. These findings provide a framework for understanding complex and variant-dependent regulations of *SLC6A3*.

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Keywords

DAT; epigenetics; haplotypic activity; promoter; signaling regulation; single copy number reporter vector

The dopamine (DA) transporter (DAT) is the key regulator of DA transmission [1] for many physiologic processes including reward, voluntary movement, motivation, cognition, attention, working memory and behavioral organization. Consistently, the human DAT gene (*hDAT* or *SLC6A3*) is associated with neuropsychiatric disorders such as substance abuse, depression, bipolar disorder, schizophrenia, attention deficit hyperactivity disorder (ADHD) and Parkinson's disease [2,3]. In knockout or transgenic mice, altered DAT expression levels may cause altered behaviors mimicking various brain disorders [4–8]. These genetic findings all imply the contribution of altered *SLC6A3* activity to related brain disorders.

DAT gene activity is known to be regulated by various internal as well as external factors. For example, substance abuse (such as cocaine abuse) may downregulate *SLC6A3* expression [9–13]. In laboratory animals, DAT mRNA levels can be altered by many small molecules including the DAT inhibitor bupropion, the norepinephrine transporter inhibitor desipramine as well as insulin, estrogen, β -cytotoxics and diabetes-inducing drugs [14–19]. These findings suggest that there could be different regulatory pathways for *DAT* regulations.

Affected by aging [20,21], *SLC6A3* expression levels differ significantly among individuals [22,23], this is attributable to the presence of several functionally polymorphic regulatory regions located throughout the 70-kb *SLC6A3* gene. Known regulatory regions include an 18-kb promoter region [23], two functional variable number tandem repeats (VNTRs) – one located in intron 8 (Int8VNTR) and another located in the 3'-UTR (3'-VNTR) [24–33]. As risk alleles, the longer 6-repeat and 10-repeat alleles of Int8VNTR and 3'-VNTR both conferred lower gene activity than the shorter (5-/9-repeat) alleles [24,25,31–34].

However, these multiple known regulatory regions have not been studied together as a whole. It remains elusive whether and how these polymorphic regulatory regions (entire promoter, Int8VNTR and 3'-VNTR) together affect the regulated promoter activity and which signaling pathways regulate *SLC6A3* via these *cis*-acting elements. This study is designed to address these questions, for the first time, by examining the 18-kb regulatory haplotypes together with the two VNTRs and the haplotype-dependent regulation of the promoter activity by 17 drugs in various model systems.

Materials & methods

Construction of reporter vector pGL3eBelo1

pGL3eBelo1, a single copy number reporter vector that is able to carry large genomic DNA of up to 300 kb and report promoter activity by firefly luciferase (Luc) activity, was generated in our laboratory by shuttling two fragments of Promega's (WI, USA) pGL3-enhancer, one was a 2421 bp fragment containing the synthetic poly(A), *luc+*, Simian vacuolating virus 40 (SV40) late poly(A) and enhancer, the other was a 222 bp fragment containing the SV40 late poly(A) signal, into pBeloBAC11 (NEB, MA, USA). pGL3eBelo1 has seven unique restriction sites including *NotI*, *ApaI*, *BamHI*, *HindIII*, *MluI*, *NheI* and *SrfI* for cloning regulatory regions. *NotI* or *HindIII* digestion inactivates the *LacZ α* for recombination selection. The lambda *cos* site may facilitate cloning of large DNAs by packaging into phage lambda particles. The vector displayed very little background Luc

activity in the cultured cells and was used to construct all the *SLC6A3* expression plasmids in this study.

Haplotype–reporter hybrid (18-kb luc) construction

An 18,909 bp fragment, covering from -16,672 (679 bp upstream of the 3' end of the upstream gene *LPCAT1*, lysophosphatidylcholine acyltransferase 1) to +2236 (5' to the ATG codon), assuming the transcription start site as +1 according to Genbank accession number NM_001044, was cloned into pGL3eBelo1. To generate *SLC6A3* promoter haplotype A and B reporter constructs, we cloned the 18.9-kb fragments from two bacterial artificial chromosome (BAC) clones (AC091933.2 for A and AC026748.7 for B) into the 5' side of *luc+* in pGL3eBelo1, generating 18A and 18B. The construction is described in Supplementary Figure 1 (see www.futuremedicine.com/doi/suppl/10.2217/pgs.13.141).

18-kb luc-VNTRs construction

Cloning of Int8VNTR and 3'-VNTR into the 3' end of *luc+*, at an *FseI* site located between *luc+* and SV40 late poly(A) in 18A and 18B, generating 18A/B-luc-VNTRs. We termed the Int8VNTR 6 repeat as long (L), 5 repeat as short (S), the 3'-VNTR 10 repeat as L and 9 repeat as a S allele as well. We thus prepared four 18-kb luc-VNTRs plasmids termed as 18ALL, 18ASS, 18BLL and 18BSS.

Transformation with DNAs of large plasmids

A total of 1 μ l of ligation reaction solution was mixed with 10 μ l distilled deionized H₂O and 10 μ l of ice-thawed One Shot[®] TOP10 Electrocomp *Escherichia coli* (Invitrogen, now Life Technologies, NY, USA) in an ice-cold micro-tube, and this mixture was transferred into an ice-cold 2-mm cuvette (Eppendorf, Hamburg, Germany). Electroporation was carried out in an Gibco BRL Cell-Porator[®] Electroporation System (Gibco BRL, now Invitrogen, NY, USA) at 2.5 kV fast charge rate, followed immediately by addition of 0.5 ml of room temperature SOC medium (Invitrogen), and incubation at 225 rpm shaking and 37°C for 1 h before colony selection on LB agar plates.

Cell lines & culture

Four human neuroblastoma cell lines, SK-N-AS, BE(2)-M17, IMR-32 and SHSY5Y, were purchased from ATCC (VA, USA) and maintained at 37°C, 5% CO₂ humidified atmosphere in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 U/ml; Invitrogen). SN4741 cells, an immortalized mouse embryonic substantia nigra-derived cell line (a kind gift from MJ Bannon of Wayne State University, MI, USA), were grown at 33°C in DMEM, supplemented with 10% (v/v) FBS, penicillin (100 U/ml), streptomycin (100 U/ml) and 0.6% D-glucose high glucose (Sigma, MO, USA).

Transfection with *SLC6A3* promoter expression plasmids & Luc assay

Expression of the reporter (Luc) activity varies from cell line to cell line, from well to well, and from cell passage to cell passage, as we have observed during the last 10 years. To control these variations and identify genotype- or drug-specific activity, we used at least three independent preparations of plasmid DNA each with same quality (optical density_{260/280} ratio: ~1.8) for each haplotype to rule out DNA quality-related artefact; we used three wells in each experiment and repeated the experiment four to eight independent times in different plates and cell passages for the same treatment/condition, in order to rule out well/plate-related artefact; we used relative Luc activity within a cell line, for comparison of genotype or drug effects among different cell lines. Since small control plasmids such as pRL-TK cannot be used for large plasmids, transfection efficiency of large plasmid DNAs of different genotypes was controlled by both high quality of multiple

plasmid DNA preparations and multiple independent transfections of cells with different passages. Data are all presented as mean \pm standard error of the mean in this study.

On day 1, cells were split at a ratio of 1:2 into 24-well plates with 30–40% confluence. On day 2, transfection medium was prepared using 0.4 μ g plasmid DNA in 19 μ l of FBS-free medium, mixed briefly with 2.4 μ l of Superfect Transfection Reagent (Qiagen, CA, USA), incubated at room temperature for 20 min, followed by mixing with 0.12 ml of the normal growth medium. The transfection mixture/cells were incubated for 24 h, then added with 0.5 ml of normal growth medium, incubated for another 24 h. Cells in each well were washed with PBS and harvested for Luc activity measurements using the Luciferase Assay System (Promega) in a 96-well format and in Bio-Tek (VT, USA) Synergy™ HT/KC4, according to manufacturer's instructions. Cell number in each well was estimated by protein amount based on Bio-Rad's (CA, USA) Protein Assay Reagent. Arbitrary unit of *SLC6A3* promoter activity was calculated as Luc activity: (readout/protein)_(*SLC6A3*-MOCK) (MOCK, transfection without DNA). 18ALL was used to normalize haplotypic promoter activities.

Drug treatments

On day 1, cells were split at a ratio of 1:2 in 75 cm² flasks with 30–40% confluence. On day 2, transfection mixture was prepared using 20 μ g plasmid DNA of *SLC6A3* promoter expression plasmids in 0.74 ml of FBS-free medium, mixed briefly with 94 μ l of Superfect, incubated at room temperature for 20 min, followed by mixing with 4.7 ml of the normal growth medium. Transfection began by replacing media in each flask with this mixture, gently covering all the cells, and the transfection mixture/cells were incubated for another day. On day 3 (24 h after transfection), cells in each flask were harvested by brief Trypsin/EDTA treatment, resuspended in fresh medium and distributed evenly into 24-well plates. Drugs to be tested were added into the designated wells to a final desired concentration of each of 17 drugs, as listed in Supplementary Table 1 [35]. On day 4 (18 h later), cells in each well were washed with PBS and harvested for Luc activity measurements. We used 10 μ M final concentration for DA, 6-hydroxydopamine (6-OHDA), *d*-amphetamine (*d*-AMPH), cocaine, metham-phetamine, U0126, SB202190 and forskolin; 1 μ M for MK801 and phorbol-12-myristate-13-acetate (PMA); 10 nM for PACAP38, 0.25 μ M for Dynorphin A porcine, 50 ng/ml for IGF-I, 100 ng/ml for TNF- α , 5 mM for valproate, 20 nM for okadaic acid and 3 μ M for LY294002. The treatments were all at 37°C for 18 h unless indicated elsewhere.

Quantitative real-time PCR analysis of endogenous *SLC6A3* mRNA in SK-N-AS

Quantitative real-time PCR analysis of endogenous *SLC6A3* mRNA was carried out as previously described [23]. Quantitative real-time PCR reactions used the SYBR® Green PCR Master Mix (Fermentas, MD, USA). Data were normalized with GAPDH and the primers used in this study were: *SLC6A3* 5'-caaaagct-gcttccatggcacact-3' and 5'-cggtcccaccgagcat-tacct-3'; *hGAPDH* 5'-tgccctcaacgaccactttg-3' and 5'-tctctctctctgtgctcttgc-3'.

^BStop-PCR quantification of relative mRNA abundance of two rs6347 alleles (G/A)

To quantify allelic cDNA levels, ^BStop-PCR was used as previously described [23,36]. First, a 323 bp amplicon was prepared by using forward primer 5'-gggtacatggcagagaagca-3', and reverse primer 5'-tccaggagcgtgaagacgta-3' and a PCR cycle of 95°C for 5 min, 28 cycles of 95°C for 1 s and 57°C for 10 s and 72°C for 30 s, followed by extension at 72°C for 3 min. Biotin labeled primer 5'-biotin-tccaggagcgtgaagacgta-3' was then added to the PCR reaction, followed by incubation at 94°C for 30 s, 62°C for 30 s and 72°C for 1 min. After digestion by *Bbv*I, which was 'CCTCAGC' sensitive, of PCR products with the A allele into biotin-labeled 215 bp and 108 bp (the resistant G allele retained the biotin-labeled 323 bp), the

digests were subjected to 1.5% agarose gel electrophoresis for resolution. After electrophoresis, DNA was capillarially transferred to positively charged nylon membrane (Ambion, now Life Technologies) and biotin on the membrane was detected by using Ambion's BrightStar[®] BioDetect[™] kit. Band intensity was analyzed by FOTO/analyst Investigator (FOTODYNE, CA, USA) and TL100 (Totalab, Newcastle upon Tyne, UK).

Pathway analysis

Drugs or kinase effectors that regulated *SLC6A3* promoter activity *in vitro* or endogenously were used to map out the signaling pathways. The software/database that was used was the frequently updated MetaCore [37].

Expression levels of pathway members in DA (A10) neurons were consulted with a published gene-expression profiling analysis [38]. DRD2 is the auto receptor and is used as a standard active protein of DA neurons [39]. We arbitrarily used 0.25-fold as a cutoff, in order not to miss any informative pathways. At the same time, the Supplementary Material provides the details on all related pathways and relative gene expression in DA neurons. 'Hub genes' highlight the top members that can regulate *SLC6A3* with the most (eight or more) paths.

Results

SLC6A3 18-kb promoter haplotypes A & B

This study included two different 18-kb haplotypes carried by two BAC clones (AC091933.2 and AC026748.7). Sequence alignments showed that these two BAC clones carried 34 informative polymorphisms including 32 SNPs and two VNTRs, one of them was the 5'-VNTR at -11 kb (Figure 1A). Of the 32 SNPs, rs2975226, rs2652511 and rs2975223 are already implicated in ADHD, schizophrenia, treatment efficacy and functional activity [28,40–42]. The 5'-VNTR was associated with mRNA levels in the ventral midbrain tissue of postmortem human brains [23]. We termed AC091933.2 as haplotype A and AC026748.7 as haplotype B (Figure 1A). We cloned these 18-kb haplotypes, as well as VNTRs into pGL3eBelo1 (Figure 1B), as described in Supplementary Figure 1.

In vitro expression systems

We selected cell lines for *SLC6A3* promoter expression by Luc-based screening of the 18-kb haplotypes A and B in five cell lines including four neuroblastoma cell lines (SK-N-AS, BE(2)-M17, IMR-32 and SH-SY5Y) of human origin and one nigral neuron-derived cell line (SN4741) of mouse origin. Luc activity showed that SK-N-AS and SN4741 both supported the expression of the *SLC6A3* promoter while the three other cell lines expressed the promoter at relatively low levels (7.4–8.6% of the SK-N-AS values). In particular, BE(2)-M17 can also express endogenous *SLC6A3*. The high *SLC6A3* promoter activity in SK-N-AS was consistent with the robust endogenous *SLC6A3* expression in this cell line [43]. The endogenous DAT expression in SN4741 has also been con-firmed by different studies [44,45]. Therefore, expression analyses of the four haplotypes in this study were carried out mainly in SK-N-AS and SN4741.

Haplotype-dependent expression

In both SK-N-AS and SN4741, the vector pGL3eBelo1 displayed very low, residual background activity (1.8 and 2.4% of 18ALL on average). Int8VNTR was a *cis*-acting element. Its inhibitory effects were independent of insertion location or orientation, and no matter whether it was or it was not a part of the *luc* RNA (Supplementary Figure 2). Therefore we combined the two VNTRs together before insertion into the end of *luc+*. We

combined the short alleles into SS and the longer alleles into LL because the longer alleles, but not the shorter alleles, were risk factors for ADHD or cocaine addiction. In this sense, LL was the risk factor and SS, the control. Owing to low linkage disequilibrium in between, the four regulatory haplotypes of 18-kb promoter – SS/LL in this study all represent natural regulatory haplotypes in humans. The 18-kb B-shorter alleles of VNTRs (18BSS) haplotype displayed an activity 1.51-fold higher than the 18-kb A-longer alleles (18ALL) in SK-N-AS (Figure 2A). In particular, the 18BSS activity was 3.24-fold higher than the 18ALL's in SN4741 (Figure 2B). The higher activity of 18BSS, was also observed in three other cell lines BE(2)-M17, IMR-32 and SH-SY5Y (data not shown). These haplotypic activities were consistent with the previous findings on higher activity with shorter haplotypes [29,30]. In addition, our data show that even in the presence of the 18-kb promoter region, the long alleles are still associated with lower activity and the short alleles, associated with higher activity.

Drug regulation

To understand whether pathways are involved in haplotype-dependent regulations, 17 different drugs were examined for their effects on *SLC6A3* activity in the four *in vitro* model expression systems (see the drug list in Supplementary Table 1). These four *in vitro* model expression systems were used because of their human origins for this human gene. The listed drugs were common effectors of DA signaling among many other signaling pathways. In particular, these drugs were used to examine the regulation of another human monoamine transporter gene, *SLC18A2* [35]. Results from the regulation studies would not only reveal regulatory pathways for *SLC6A3* but also be comparable between the two DAT genes, helping to identify shared or unique pathways. Among many DAT protein inhibitors, we were focused on only three drugs of abuse, cocaine, d-AMPH and methamphetamine. Therapeutics such as methylphenidate and mazindol were not included. Among the 17 drugs, only valproate has been used to examine *SLC6A3* regulation before [46] but this drug has never been used for haplotype-dependent regulation; forskolin was studied for allele-dependent regulation of Int8VNTR before [34] but has not yet been used to study the *SLC6A3* promoter. Therefore, none of these drugs have been studied for haplotype-dependent regulation of *SLC6A3* before.

Eleven of these drugs (DA, d-AMPH, dynorphin A, IGF-I, (+)MK801, okadaic acid, SB202190, U0126, cocaine, methamphetamine and TNF- α) did not exert any significant regulation and their data are not shown here. We made sure of their activity by using fresh orders of different batches whose qualities were validated by the manufacturers. We present the statistically significant data based upon 18 h treatments of four human cell lines transiently expressing the four regulatory haplotypes with the six other agents: valproate, PACAP38, LY294002, forskolin, PMA and 6-OHDA, as summarized in Figure 3. Overall, the first four drugs upregulated the *SLC6A3* promoter activity on all four haplo types. Uniformly, the neurotoxin 6-OHDA downregulated the activity to 0.2–0.5-fold independently of haplo type or cell type, suggesting that *SLC6A3* be ubiquitously sensitive to oxidative stress. Among the top three upregulators including valproate, PACAP38 and LY294002, dose and time dependences of the endogenous *SLC6A3* upregulation have been reported consistently for valproate before [46] and observed for both PACAP38 and LY294002 as well in SK-N-AS (Supplementary Figure 3).

Haplotype-dependent drug regulation

Among the drug regulations in Figure 3, two drugs displayed statistically significant haplotype dependence, which is detailed in Figure 4. The PI3K inhibitor LY294002 upregulated the shorter alleles more significantly (with a greater magnitude) than the longer ones, without affecting the 18-kb promoter. LY294002 upregulated the SS more than the LL

activities, with the largest extents (1.51-fold on haplotype A and 1.56-fold on haplotype B) in the background of 18-kb promoter region (Figure 4A). The PKC activator PMA upregulated only the promoter region of haplotype B in IMR-32, without affecting the VNTRs (Figure 4B).

Cell type-dependent drug regulation

Of the six drugs, valproate upregulated the promoter activity in all constructs and in all expression systems. The extent of regulation was smaller in SH-SY5Y compared to other cell lines including SK-N-AS, BE(2)-M17 and IMR-32. The PACAP38 regulation depended on cell type, without any effects in SK-N-AS. Forskolin's upregulations had larger extents in BE(2)-M17 and IMR-32 than in SK-N-AS and SH-SY5Y. 6-OHDA downregulated the promoter activity in all constructs and in all cell lines (Figure 3).

Allele-dependent drug regulation of endogenous *SLC6A3* expression

To support the overall haplotype-dependent regulation of *SLC6A3* observed in the transient expression systems, we further investigated whether these drugs regulated endogenous *SLC6A3* expression from chromosome 5 in an allele-dependent manner by using a modification of hot-stop PCR, ^BStop-PCR. We used the exonic SNP rs6347 to monitor allele-dependence in BE(2)-M17 because this cell line carried informative (both alleles) rs6347. SK-N-AS was included as a homo-zygote A/A carrier for allele and digestion controls (lanes 1 and 2 in Figure 5A where 323 bp represented allele G and the digestion product 215 bp, allele A). Imbalanced intensities between the two bands indicated allele-dependent regulations (Figure 5A). Quantification of the regulations by the six drugs in Figure 5A is summarized in Figure 5B. Valproate, PACAP38, LY294002 and forskolin upregulated G- more than A-associated *SLC6A3* expression (1.52-, 1.46-, 1.36- and 1.39-fold), all reaching statistical significance (Figure 5B). Cocaine did not regulate *SLC6A3* in this cell line, as opposed to previously observed reduced expression in postmortem brain tissue [10–12], probably because BE(2)-M17 lacked the cocaine-related pathways. 6-OHDA did not display any allele dependence but appeared to downregulate *SLC6A3* on both chromosomes, consistent with the findings from the transient expression systems (Figure 3).

Haplotype-dependent signaling pathways

The purpose of this pathway analysis was to develop an initial regulatory map for DA neurons based on findings from *in vitro* studies and on active genes in DA neurons. Based on the significant regulation findings mentioned above, we used the 'build network' function with the building algorithm of 'shortest paths' in Meta-Core to generate a preliminary pathway map that collected information from all five types of cells (see Supplementary Figure 4). Manually, we screened all the proteins in this preliminary map for expression levels in DA neurons, based on DA neuron-specific gene-expression profiling [38]. Any expression levels below a quarter of the DRD2 receptor level were considered nonexpression and the related pathways were removed from the map, resulting in a DA neuron-related pathway map as in Figure 6. Among the six drugs that endogenously regulated *SLC6A3* haplotypes, 6-OHDA was not yet included in the MetaCore database. The pathways mediating the valproate, LY294002, PMA, forskolin and PACAP38 regulation were mapped out but only approximately two-thirds of the members were expressed at levels of at least 25% of the DRD2 receptor (the DA neuron autoreceptor) expression levels (Supplementary Figure 4). After removing those with expression <25% of the DRD2 levels, a map that likely works in DA neurons was described (Figure 6). In the valproate pathway, histone deacetylation was a major hub because HDAC1, for example, had more than ten interactions with other targets. LY294002 regulated *SLC6A3* mainly through the CK2 α chains–SP1/SP3 complex as well as the DNA–PK–PKC–SP1/SP3 complex pathways. PMA

regulated *SLC6A3* through the PKC–SP1/SP3 complex pathways. Forskolin regulated *SLC6A3* through FXR (also known as NR1H4, a bile acid-binding transcription factor), a pathway that might work only in our *in vitro* assay systems because of very low FXR expression levels in DA neurons. PACAP38 regulated *SLC6A3* through cAMP but how cAMP regulated *SLC6A3* remained unclear. cAMP was supposed to regulate ERK1/2 via MEK1 but in the different assay systems the ERK1/2 inhibitor did not affect *SLC6A3* in our assays. Instead, PACAP38, perhaps through the cAMP, regulated *SLC6A3* indirectly through unknown transcription factors. These known pathways all targeted *SLC6A3* through the three known transcription factors HEY1, NURR1, GCR- α and the SP1/SP3 complex. All of these five drugs differentially regulated *SLC6A3* between the two haplotypes or the two rs6347 alleles under this study.

Discussion

For the first time, we have examined drug regulation of the large regulatory haplotypes, especially the promoter regions, of *SLC6A3*. To analyze these large regulatory haplotypes, we have developed the first single copy number plasmid Luc-reporter constructs facilitating the mapping of the first *SLC6A3* signaling pathways. These comprehensive *SLC6A3* haplotypes covering from the 18-kb promoter region to the 3'-VNTR display promoter activity of more than threefold difference, the largest haplotypic difference reported to date for this gene (Figure 2). Our data, especially the drug regulation data, suggest that genetic evaluation of *SLC6A3* activity needs to consider the contribution from both VNTRs and the 5' promoter region (Figure 4).

It has been known for the last two decades that F-plasmid-based single copy number vectors may allow for cloning and maintenance of chromosome DNA fragments of up to 300 kb in length stably in *E. coli* [47]. In this study, we have adopted this single copy number plasmid approach and developed pGL3eBelo1 for investigation of regulatory activity of large chromosomal fragments (Figure 1). As more and more information is revealed by genome-wide association studies and gene–gene interaction studies, it is critical to have the ability to assess functional relevance of the statistical findings in human genetics across different fields [48–51]. It is therefore likely that the combination of single copy number vectors and gene activity reporters will find wide applications in the near future.

Our study in these large regulatory haplotypes confirms the previous findings on allelic activity of the individual VNTRs. As mentioned above, the 5-repeat or 9-repeat alleles were found to be associated with higher promoter activity whereas the longer alleles, were found to be associated with lower activity, especially in healthy subjects. These reported results were obtained when each VNTR was studied independently. In the 18-kb promoter region background, these results remain valid. Conceivably, these VNTRs responded to different signaling pathways for *SLC6A3* regulation. This explains why the 9-repeat allele is also associated with the higher DAT protein density in human brains carrying this allele, based upon imaging analysis [33].

The signaling pathways turned out to be complicated already, based on only a limited number of transcription factors for *SLC6A3*, making three implications. Foremost, the new finding on the involvement of histone deacetylation, together with recently reported promoter DNA methylation, suggests that the DAT gene is also subject directly or indirectly to extensive epigenetic regulation [21,52,53]. Therefore, when a polymorphism in the promoter is associated with a disease but does not confer any functional variation in transient expression systems, it is worth considering examining the epigenetic status including DNA methylation and histone modifications around the polymorphic site in endogenous systems. Second, signaling regulation can be achieved through different

regulatory regions. AP-1, GCR- α and the SP1/SP3 complex act through the promoter region while HEY1 regulates *SLC6A3* through 3'-VNTR [54,55]. Specifically, forskolin may upregulate *SLC6A3* through NURR1-SP1/SP3 complex, but downregulate *SLC6A3* through HEY1. Since HEY1 did not differentiate between the 9-repeat and 10-repeat alleles of 3'-VNTR in transcriptional regulation *in vitro*, it could be various expression levels of HEY1 that underlie the reported 3'-VNTR associations with ADHD [56–58]. Third, *SLC6A3* could be regulated by complicated signaling cascades in various DA neurons. Only five *SLC6A3*-selective drugs already imply relatively complex signaling cascades here. In fact, the DAT gene is regulated by many external and internal factors, suggesting that additional related signaling cascades wait for future delineation. Furthermore, balance or imbalance between different pathways may partly explain the cell-type dependence in *SLC6A3* promoter regulation (Figure 3). Importantly, the regulation by these signaling cascades can be affected by genetic variation in their genomic target (Figures 4 & 5), providing mechanistic insight into individual variation in *SLC6A3* activity and in response to environment. This initial pathway map merits experimental validation in future studies.

SLC6A3 variant-dependent regulation by valproate could imply individual dependent efficacy of this medicine for mood stabilization. Valproate has been used as a mood stabilizer for patients with bipolar disorder and schizophrenia [59–63]. Mice with reduced expression of DAT display hyperactive activity and perseverative motor behavior, mimicking certain symptoms observed in these patients. Treatment of these transgenic mice with valproate was able to attenuate these symptoms significantly [6]. Indeed, it was shown that valproate could upregulate *SLC6A3* activity [46]. We have shown that valproate upregulates the G more than the A allele of rs6347 endogenously in BE(2)-M17. This data is consistent with the clinical observations that valproate treatment can be effective in some patients but not in others [64–68]. Future study will need to verify these pathways in DA neurons as well as the underlying polymorphisms in order to provide evidence-based guidelines for individualized treatment [69].

Finally, we have shown that 6-OHDA down-regulated *SLC6A3* in all haplotypes and all expression systems used (Figure 3). By contrast, 6-OHDA was able to upregulate *SLC18A2* that encodes VMAT2 [35]. As a result, exposure to this neurotoxin reduces accumulation of cytosolic DA molecules by decreasing the DAT-mediated entry of extracellular DA and increasing the VMAT2-mediated sequestering of cytosolic DA into intra-cellular vesicles. Although we do not yet understand the pathways, there might be a feedback mechanism to respond to oxidative environment for self-defense.

Conclusion

To date, most studies of functional variation of *SLC6A3* have focused on pharmacologic regulation of single polymorphisms or promoter activity of short haplotypes (<7.4 kb). No reported study has looked at activity of large haplotypes containing multiple functional or potentially functional alleles, mainly owing to the lack of appropriate methodologies for large haplotypes. In this study, we have developed a single copy number reporter vector, pGL3eBelo1, which can potentially harbor up to 300 kb of haplotypes. By using pGL3eBelo1, we are able to analyze the 18-kb promoter region haplotypes together with the two well-studied downstream VNTRs in *SLC6A3*. Findings from these analyses show that 18-kb haplotypes may display large differences in *SLC6A3* promoter activity. Specific signaling pathways that regulate *SLC6A3* may target various regulatory regions throughout the 70-kb gene and be affected by both haplotype and cell type. In conclusion, our data help build the first pathway map for *SLC6A3* regulation, suggesting that both transcription factors and epigenetic events, and perhaps miRNAs and other ncRNAs, may contribute to varying

SLC6A3 activity. It is anticipated that a comprehensive map may play an instructive role in the development of individualized medicine for *SLC6A3* -related brain disorders.

Future perspective

With a single copy number reporter vector available, we are for the first time able to measure overall promoter activity of 18-kb haplotypes in *SLC6A3*. Additionally, by investigating 17 drugs' effects, this study provides the most comprehensive regulatory information to date on this important brain gene. The pharmacological data have suggested that there are more transcription factors for this gene but there is no clue about what transcription factors are mediating the regulatory pathways (e.g., PACAP38) and where these transcription factors bind in the regulatory regions. By progressive deletions of the 18-kb reporter constructs, we are currently mapping novel *cis*-acting elements in the promoter regions and will further clone transcription factors that bind to polymorphic sites in these elements, delineating individual *cis*-acting elements. We will, therefore, better understand how *SLC6A3* variants are differentially regulated through its promoter regions. This large-construct-guided approach can also be applied to the transcribed and downstream regions, generating a comprehensive regulatory map for individualized treatment of *SLC6A3*-related diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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▪ of interest

▪▪ of considerable interest

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Executive summary

Background

- *SLC6A3* encodes the plasma membrane dopamine transporter that regulates dopamine transmission in the brain and is associated with more than ten different brain disorders.
- Previous analyses have focused on regulation of downstream variable number tandem repeats (VNTRs) by a few chemicals or on haplotypic activity of the core promoter regions of *SLC6A3*.

pGL3eBelo1-based measurement of comprehensive haplotypic activity in *SLC6A3*

- In order to analyze transcriptional activity of 18-kb chromosomal fragments in *SLC6A3*, a single copy number luciferase reporter vector, pGL3eBelo1, was created.
- By using pGL3eBelo1, we demonstrated that two 18-kb regulatory haplotypes containing the entire 18-kb promoter regions and two known downstream VNTRs display up to 3.2-fold difference in *SLC6A3* promoter activity.
- Haplotypic difference in promoter activity varies in different expression systems.

SLC6A3 promoter regulation by six (35.3%) of the 17 drugs examined

- Valproate, PACAP38, the PI3K inhibitor LY294002, the adenylyl cyclase activator forskolin and the protein kinase activator PMA all upregulated the promoter activity of the 18-kb regulatory haplotypes; the neurotoxin 6-OHDA downregulated the promoter activity.
- The upregulations were both haplotype and cell type dependent. LY294002 and PMA could differentially regulate the two haplotypes, in a cell type-dependent manner.

First pathway map for *SLC6A3* regulation

- *SLC6A3* was regulated by selected pathways, which involved 13 hubs.
- Histone-deacetylase-related epigenetic regulation is a major pathway.

Conclusion & future perspective

- Our pGL3eBelo1-based study with 17 drugs provided the most comprehensive data to date on regulation of *SLC6A3* promoter activity and haplotype or cell-type dependence.
- The 18-kb haplotypes showed a large difference in promoter activity, attributable to independent effect of promoter versus VNTRs; most pathways upregulated the promoters.
- The pGL3eBelo1-based approach may help map novel *cis*-acting elements and build a signaling map of *SLC6A3* regulation, guiding the development of individualized treatment of *SLC6A3*-related brain disorders.

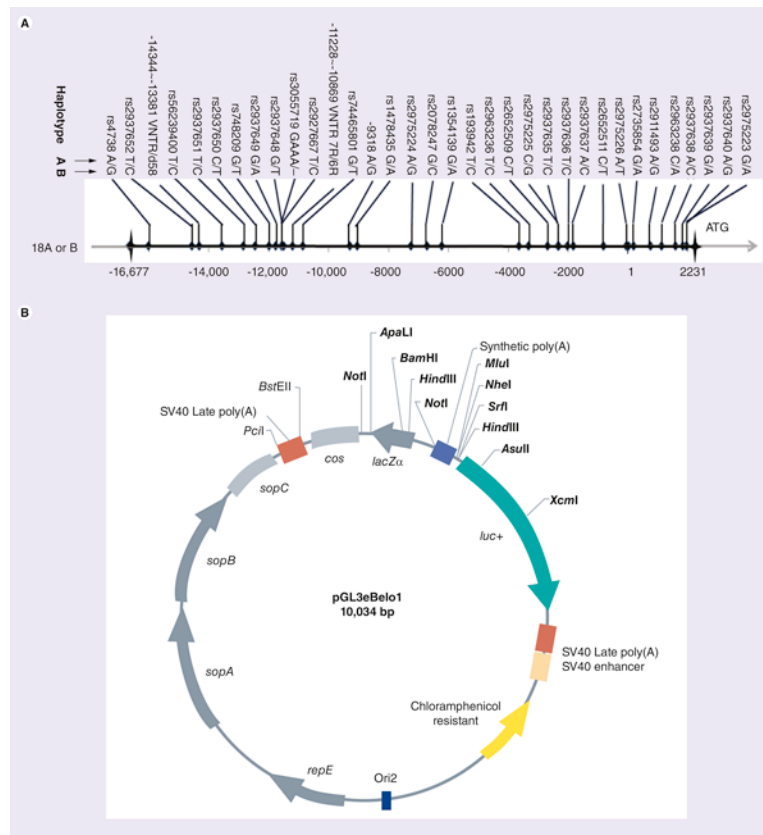


Figure 1. Two 18-kb *SLC6A3* promoter region haplotypes and the expression vector used in this study

(A) Haplotypes A and B of *SLC6A3* 18-kb promoter region: 34 polymorphisms (32 SNPs and two variable number tandem repeats [VNTRs]) revealed by BAC alignment (AC091933 vs AC026748), each polymorphism indicated by two alleles (separated by '/') of two haplotypes; horizontal gray arrow: chromosome DNA, with coordinates for 18 kb upstream of ATG codon (black horizontal line) using the transcription start site as +1 (-14344~-13381 VNTR missed 58 bp in haplotype B; rs3055719 missed 'GAAA' in haplotype B). (B) A single copy number reporter vector pGL3eBelo1 developed to harbor *SLC6A3* regulatory regions. Aquamarine, firefly luciferase gene (*luc+*); orange box, SV40 enhancer for *luc+* expression; *cos*, the lambda *cos* site for packaging into phage lambda particles and facilitating cloning of large fragments; yellow arrow, the chloramphenicol-resistant gene. Cloning sites are labeled in bold; *lacZα* for recombination selection (see 'Materials & methods' section for description of construction). A unique *FseI* site located between *luc+* and SV40 late poly(A) used for VNTR insertion is not shown here. Please see colour figure at <http://www.futuremedicine.com/doi/pdf/10.2217/pgs.13.141>.

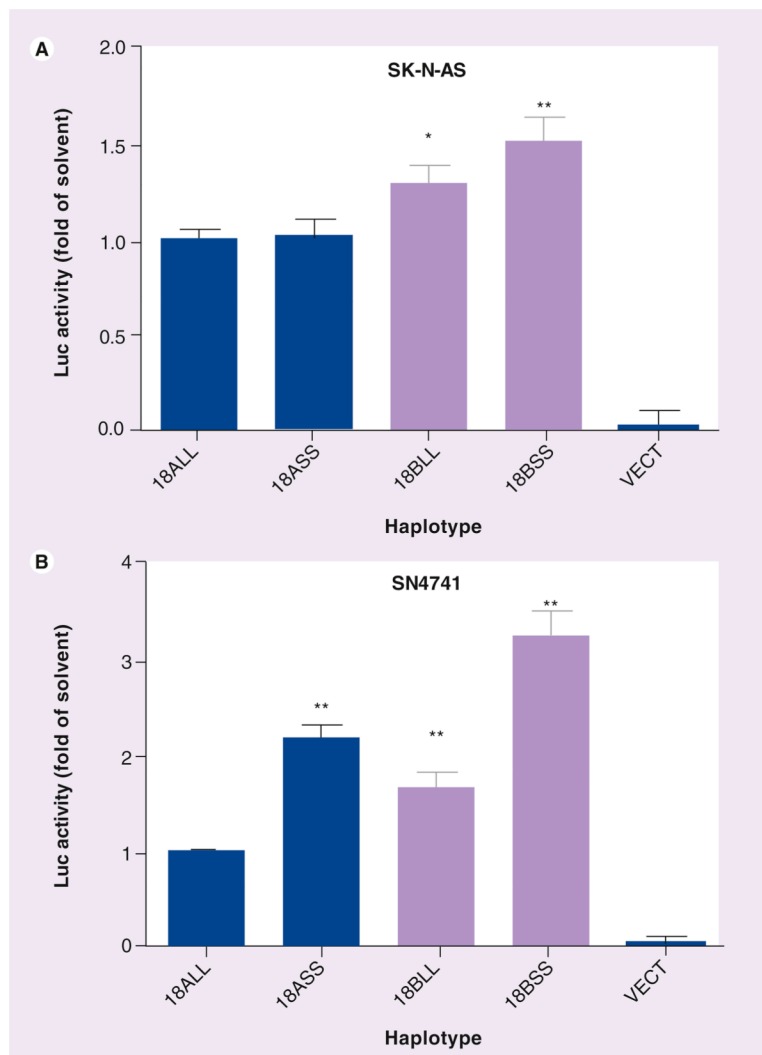


Figure 2. Haplotype-dependent expression of *SLC6A3* in SK-N-AS and SN4741

(A) SK-N-AS and (B) SN4741. Average residual activity of VECT was 1.8% and 2.4% of 18ALL in SK-N-AS and SN4741, respectively. One-way ANOVA Tukey, comparing with 18ALL: * $p < 0.05$, ** $p < 0.01$ ($n = 4-6$). Darker colored bars = 18A, lighter colored bars = 18B.

LL: 6-repeat of Int8 variable number tandem repeat (VNTR) combined with 10-repeat of 3'-VNTR; SS: 5-repeat of Int8VNTR with 9-repeat of 3'-VNTR; VECT: Expression vector pGL3eBelo1.

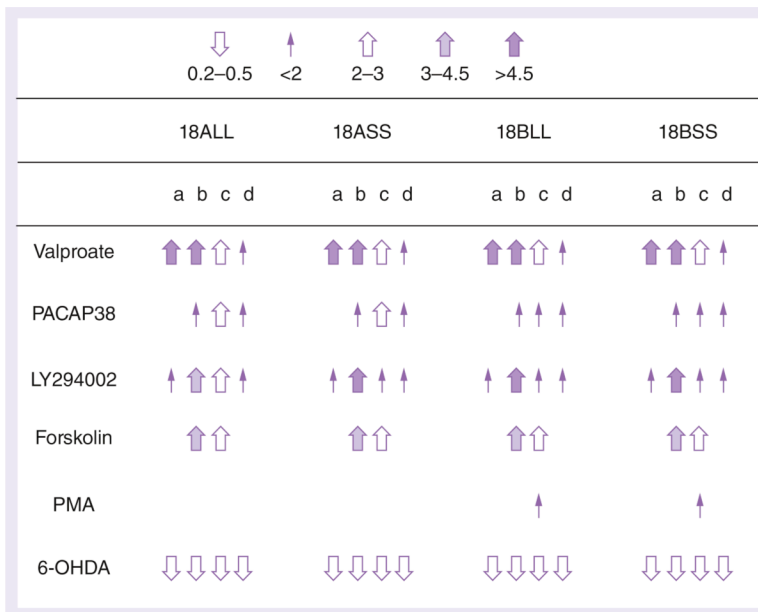


Figure 3. Regulation of *hDAT* promoter activity by 6-OHDA, PACAP38, valproate, LY294002, forskolin and PMA in four cell lines, based on Luc activity analysis

All arrows represent statistical significances by one-way ANOVA Tukey (n = 4–8), compared with solvent controls; no arrow, no statistical significance.

a: SK-N-AS (1.8%)[†]; b: BE(2)-M17 (2.9%); c: IMR-32 (6.2%);

d: SH-SY5Y (0.5%).

[†]Average residual activity of the vector as a percentage of 18ALL activity.

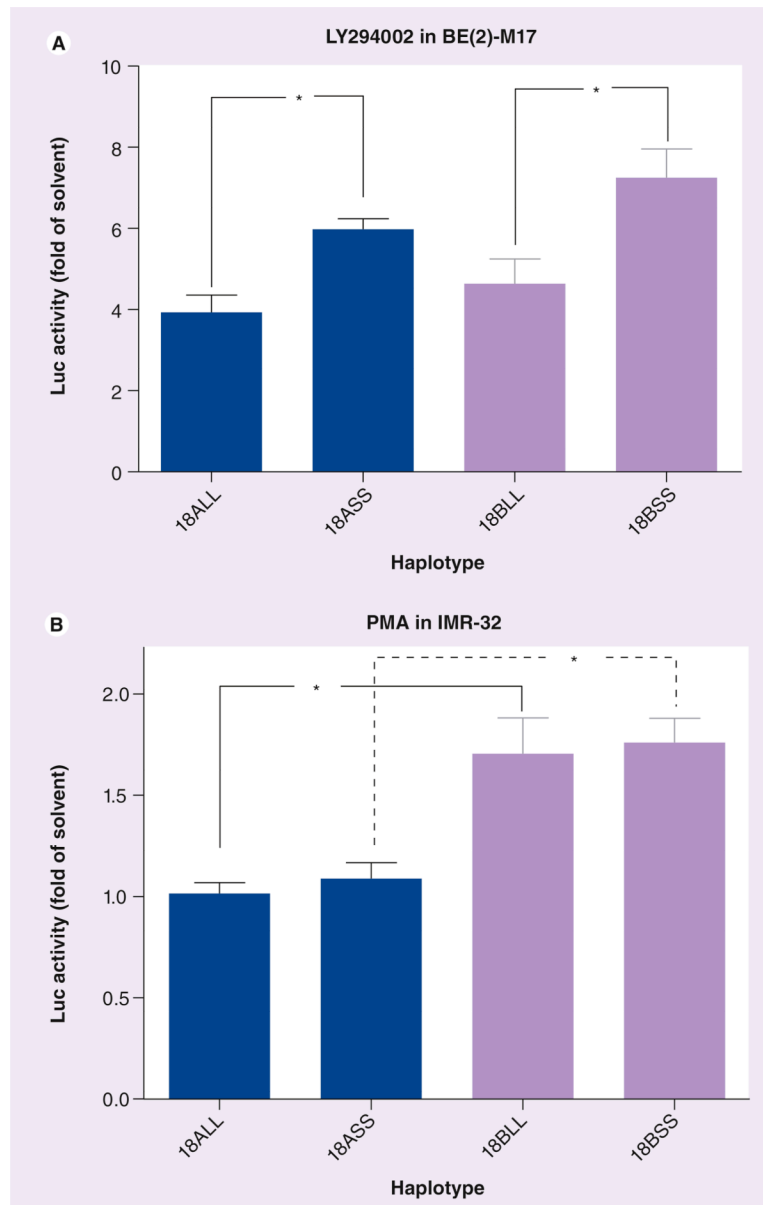


Figure 4. Haplotype-dependent regulation of *SLC6A3* promoter by drugs in BE(2)-M17 and IMR-32, based on Luc activity analysis
(A) BE(2)-M17 and **(B)** IMR-32. One-way ANOVA Tukey: * $p < 0.05$ ($n = 4-8$). Darker colored bars = 18A, lighter colored bars = 18B.

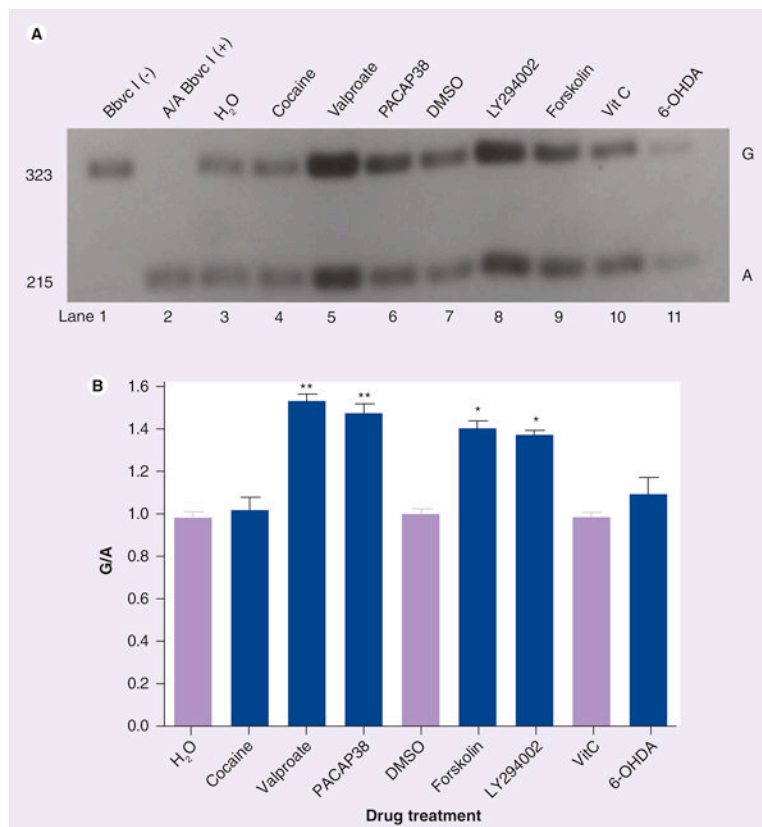


Figure 5. Allele-dependent regulation of endogenous *SLC6A3* activity by drugs in rs6347-carrying BE(2)-M17, based on ^BStop-PCR analysis of mRNA levels

(A) Characteristic data of ^BStop-PCR analysis. Lane 1: PCR production without *BbvcI*-digestion; lane 2: rs6347 homozygote A/A (from SK-N-AS) digested completely by *BbvcI*; lanes 3, 7 and 10: solvents (lighter color) for drugs in following lanes (lanes 4, 5, 6, 8, 9 and 11: drug treatments; darker color). (B) Quantification of regulation data in (A). One-way ANOVA Tukey: * $p < 0.05$; ** $p < 0.01$, compared with solvent control ($n = 3$).

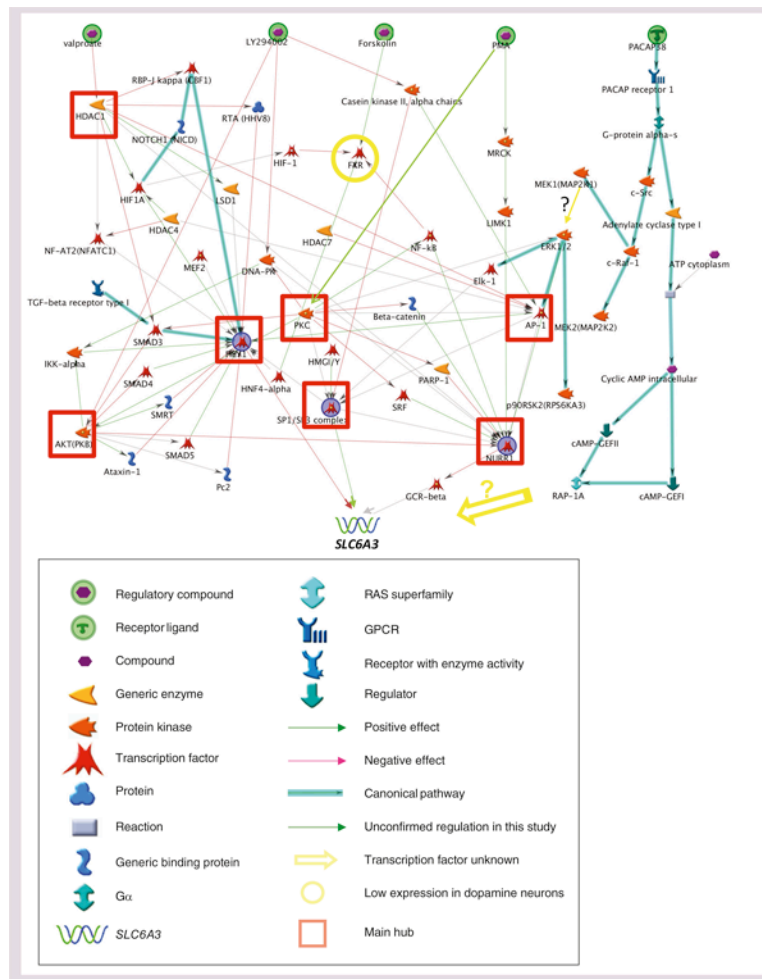


Figure 6. Signaling cascades that regulate *SLC6A3* (located at bottom) in a haplotype-dependent manner

Insert: symbol description. Included are all genes expressed at levels over a quarter of DRD2 expression levels in the dopamine neurons, except the open circle, for over a quarter of DRD2 expression levels (other genes with low expression levels are not shown). Oxidative stress-related (6-hydroxydopamine) pathways are not included.