



Intragenic Transcriptional *cis*-Antagonism Across *SLC6A3*

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Abstract

A promoter can be regulated by various *cis*-acting elements so that delineation of the regulatory modes among them may help understand developmental, environmental and genetic mechanisms in gene activity. Here we report that the human dopamine transporter gene *SLC6A3* carries a 5' distal 5-kb super enhancer (5KSE) which upregulated the promoter by 5-fold. Interestingly, 5KSE is able to prevent 3' downstream variable number tandem repeats (3'VNTRs) from silencing the promoter. This new enhancer consists of a 5'VNTR and three repetitive sub-elements that are conserved in primates. Two of 5KSE's sub-elements, E-9.7 and E-8.7, upregulate the promoter, but only the later could continue doing so in the presence of 3'VNTRs. Finally, E-8.7 is activated by novel dopaminergic transcription factors including SRP54 and Nfe2l1. Together, these results reveal a multimodal regulatory mechanism in *SLC6A3*.

Keywords Epistasis · Genetic marker · Haplotype · Primate-specific · Transactivation

Introduction

Besides insulators which play an intervening role, *cis*-acting elements within a gene can be grouped as activating (enhancer) and silencing (repressor) in terms of direct effects on promoter activity [1–3]. It is unknown whether enhancers and silencers interact with each other in transcriptional regulation. Since these *cis*-elements usually carry polymorphisms

[4, 5], dissection of potential *cis*-interplays may provide effective association markers and identify underlying genetic risk factors.

The dopamine transporter (DAT) is a principal regulator of dopamine transmission in the brain [6–8]. Regulated transcription of the human DAT gene (*SLC6A3*: OMIM# 126455, cytogenetic location: 5p15.33, genomic coordinates (GRCh38): 5:1,392,789–1,445,427) determines spatial, temporal, and quantitative aspects of DAT expression in the brain and therefore affects many dopamine-related function including reward, memory, movement, cognition, and behavioral organization. Abnormal regulation may also cause pathological states implicated in a spectrum of neuropsychiatric disorders such as substance use disorders, schizophrenia, bipolar disorder, Parkinson's disease, and ADHD [9]. It is urgent to uncover *cis*-acting elements in *SLC6A3* for two main reasons. The first reason is that identification of these elements may guide us to clone the transcription factors (TFs) for signaling pathway dissection; another reason is that DNA sequence polymorphisms in these functional elements may serve as effective markers for genetic association study.

SLC6A3 spans 70 kb in the human chromosome 5, but only a little is known about its *cis*-acting activity. The best known are two downstream variable number tandem repeats (VNTRs), one in the 3' untranslated region (3'VNTR) [10] and, another, in Intron 8 (In8VNTR) [11]. 3'VNTR has 7–11 repeats of 40 bp and In8VNTR has 5–8 repeats of 30 bp, and both have been shown to display inhibitory activity and used

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as genetic markers in association studies [12, 13]. It has been reported that the TF Hey1 binds to 3'VNTR [14], and there is no information about what proteins recognize Int8VNTR.

The human promoter remains underexploited [15]. This 5' promoter has a 16-kb DNA sequence with hundreds of polymorphisms among various populations (1252 subjects used by the 1000 Genomes Project) [4, 5], but unfortunately, no specific *cis*-acting elements have been identified for the human promoter yet, especially in the 5' distal regions. We and others have found a 121-bp *cis*-acting element located in Intron 1 as an inhibitory activity [16, 17], and like Int8VNTR, no proteins are known to mediate the inhibition except an lncRNA molecule [18]. A few TFs are known to regulate the human core promoter, such as SP1/3, PITX3, and NURR1, but the target sequences are largely unknown [15, 19–21]. Such paucity of transcription information in this critical brain gene prevents us from a better understanding of how *SLC6A3* contributes to the regulation of brain neurochemistry and genetic etiology of related neuropsychiatric disorders.

In this study, we discovered a 5-kb super enhancer (5KSE) located in a distal promoter region that antagonizes the downstream VNTRs and further cloned TFs that regulated 5KSE.

Methods

Plasmid Construction

F plasmid-based luciferase reporters of *SLC6A3* promoters, 18A, 18ALL, 18B, and 18BLL, have been described before [13]. Deletion of a 5-kb (4899 bp) *NheI* fragment from 18A, 18ALL, 18B, and 18BLL resulted in 10A, 10ALL, 10B, and 10BLL.

Cloning of a 352-bp fragment (which was located at –8.7 kb and termed as E-8.7 whose sequence is listed below) from the 5 kb of haplotype A into the *NheI* site of 10A or 10ALL resulted in 10A_E-8.7 or 10ALL_E-8.7. Similarly, cloning of E-10.3 and E-9.7 resulted in 10A_E-10.3 or 10A_E-9.7. The PfuUltra High-fidelity DNA Polymerase (Agilent Technologies Inc., Santa Clara, CA, USA)-based PCR used the primers 2.6F aaaaaagctagcgttaagttaa aatacatagct and 2.6R aaaaaagctagcgggggtcttactatgtgt for E-10.3; 3.2F tttttgctagctgggcacagtggctct and 3.2R tttttgctagctttttgttttttatgaga for E-9.7; and 4.2F tttttgctagc-ggctggctaggtgcagatt and 4.2R tttttgctagctttttctttcttttgagat (synthesized by IDT, Skokie, IL, USA; *NheI* site: underlined) for E-8.7 to amplify the fragments, followed by *NheI*-digestion, cloning back to 10A or 10ALL and verification for correct orientation and PCR fidelity by DNA re-sequencing.

ggctggcta ggtgcagatt aactccctta aatgtaagaa aagtgaatgg gggccGgacg cagtggtca tgctgtaat ccaaacctt tgggagctg aggcatttag atcacctaag gttaggagt ccagaccagt gtggccatca cagtgaacc ctgtctctac taagaatatg aaaattagct ggggtgtggtg

gtgcacgct gtaatccag gtactctgga ggctgaggca agagaattgc ctgaaccag gaagcagaag ttgcagagag ctgagatcgc accactgcac tccagctgg gcaacagat gagactccat ctcaaagaaa agaaaagaaa aag (bold, E-8.7; underline, PCR primers; upper case, an rs1478435 allele)

Human SRP54 cDNA clone from Origene Inc. (SC118182 for variant 1, NM_003136) was switched from vector pCMV6-XL5 into pcDNA3.1+ by *NotI* site for pcDNA3.1 + SRP54 where insert orientation was verified by *NheI* digestion. Human Nfe211 (2342 bp), Slitrk2 (2558 bp), or Skp1 (527 bp) cDNA was cloned by PfuUltra high-fidelity DNA polymerase-based PCR and ligated into the indicated sites in pcDNA3.1+, resulting pcDNA3.1 + Nfe211, pcDNA3.1 + Slitrk2, and pcDNA3.1 + Skp1. Nfe211 PCR used the primers tttttgctagctcagcaatgctttctctgaag and tttttgctggcgcctct tccccaggtcactt, Slitrk2 PCR used the primers aaaaag ctagcccgaaggtgcctaaagatgctga and aaaaagcggcgcctt ccaagctgactgattgca, and Skp1 PCR used the primers tttttgctagcgtctcctaacaccgaacacca and tttttgctggcgcaggc acaacattcacttctt all for *NheI/NotI* sites. PCR templates used were cDNA synthesized by using mixed human RNAs isolated from human SK-N-AS, IMR-32, and SH-SH5Y cells combined [13]. PCR fidelity was verified by DNA re-sequencing.

Cell Culture

SK-N-AS and SN4741 were cultured in Dulbecco's modified Eagle's medium (DMEM, ATCC, VA, USA) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, GA, USA) in a 75-cm² Falcon flask (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in humidified air with 5% CO₂. SN4741 cells were cultured at 32 °C for maintenance and at 37 °C for gene expression after transfection.

Cloning of TFs via Y1H System

The 352 bp E-8.7 fragment of haplotype A was used as bait after cloning to pB300/pASZ_Y1H as the vector [22], and a human adult brain cDNA library was constructed into pP6, a pGADGH derivative plasmid, both used in yeast one hybrid (Y1H) in search for proteins that bound to E-8.7 (Hybrigenics Services, Paris, France). The bait construct was checked by sequencing the entire insert and transformed into the yeast strain Y1H300 (MAT a, Gal4Δ, ade2-101, trp1-901, leu2-3,112, his3Δ200) to integrate the DNA bait into the yeast genome. Screening was performed against the random-primed cDNA library. Clones were screened using a mating approach with YHG13 (Y187 ade2-101::loxP-kanMX-loxP, mataα) and the E-8.7-containing yeast (mata) strain as previously described [23]. His⁺ colonies were selected on a medium lacking adenine, leucine, and histidine. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences

were used to identify the corresponding interacting proteins in the GenBank database (NCBI).

Cell Transfection for Luc Activity Assay and RNA Extraction

For luciferase (Luc) activity assay, SN4741 cells in each well of a 24-well plate were transfected with 400 ng cDNA plasmid DNA of pcDNA3.1+ or a cDNA clone, using SuperFect Transfection Reagent (Qiagen, Germantown, MD, USA) following the manufacturers' procedures. After overnight incubation, 400 ng reporter plasmid DNA of the vector pGL3eBleo1, 18A, 10A, 10A_E-10.3, 10A_E-9.7, 10A_E-8.7, 10ALL_E-9.7, or 10ALL_E-8.7 Luc plasmid was further introduced by transfection. Twenty-four hours later, the cells were harvested and lysed with firefly luciferase buffer; Luc activity was analyzed using Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions and read out in a Synergy HT luminometer (BioTek, Winooski, VT, USA). Cell density was estimated by protein quantification by using a BCA (bicinchoninic acid) kit. Luc activity was a result of normalizing the readout by protein amount.

For effects of SRP54 or Nfe211 overexpression on endogenous *SLC6A3* expression, SK-N-AS or BE(2)-M17 cells were cultured in a 24-well plate for a confluency level of about 80% next day. For each well, 400 ng plasmid DNA was mixed with 20 μ L DMEM; then, 2.4 μ L SuperFect Transfection Reagent was added into DNA solution. The tube was vortexed for 10 s and then was incubated at room temperature (RT) for 10 min. After that, 190 μ L fresh complete medium was added into DNA-SuperFect complex. The liquid in each well was aspirated before adding 200 μ L of the complex to each well. DNA-SuperFect complex solution was changed with 400 μ L fresh complete medium in 2–3 h. Cells were incubated overnight before RNA purification.

qRT-PCR

Cells of each well in a 24-well plate were extracted for total RNA in TRIZOL (Invitrogen, CA, USA) following the TRIZOL User Guide, and RNA was quantified by NanoDrop Lite (Thermo Fisher Scientific, Waltham, MA, USA). RNA (0.6 μ g) was subjected to cDNA synthesis in a volume of 10 μ L, by using the Verso cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). All PCR reactions were performed using SsoAdvanced Universal SYBR green supermix (BIO-RAD, Hercules, CA, USA). Primers were selected based on an amplification coefficient (AC) of near "2" and single peak in melting curve. Fold differences in mRNA expression were calculated as per $AC^{(\Delta CT_{\text{test}} - \Delta CT_{\text{control}})}$, where *GAPDH* was used as an internal control.

Immunofluorescent Staining of Cells, Human Postmortem Midbrain Tissue, and Mouse Brain

For cell staining, SN-K-AS or SN4741 cells were cultured on coverslip until 80% confluence, fixed in 4% paraformaldehyde (PFA) in PBS for 15 min, and washed three times for 5 min each time with 0.01 M PBS. The cells were incubated with 3% bovine serum albumin (BSA) at 37 °C for 30 min. After incubated at 4 °C with rabbit anti-NFE2L1 (sc721) 1:50 dilution or rabbit anti-SRP54 (AB154796) at 1:100 dilution antibodies in 0.03% Triton X-100 PBS for 24 h, the cells were washed three times for 5 min each with 0.01 M PBS and then incubated with Alexa Fluor 568 goat anti-rabbit IgG (1:500, Life technologies, NY, USA) at RT for 2 h in darkness. Slides were mounted with VECTASHIELD anti-fade mounting medium containing DAPI stain (Vector Laboratories, Burlingame, CA, USA) for labeling cell nuclei. Images were captured in confocal laser scanning microscopy (Leica TCS SP8, IL, USA).

For frozen human nigral midbrain tissue which was from healthy controls as described before [24], 10 μ m sections were collected on Superfrost Plus Gold slides (Fisher Scientific USA), fixed in 4% PFA for 30 min, and rinsed three times with PBS and then incubated with 3% BSA at 37 °C for 30 min. After incubated at 4 °C with the same primary rabbit antibodies as for the cells, together with chicken anti-tyrosine hydroxylase (TH; 1:1000; Abcam, Cambridge, MA, USA) antibodies, the sections were washed three times for 5 min with 0.01 M PBS and then incubated with Alexa Fluor 568 goat anti-rabbit IgG (1:500, Life technologies) and Alexa Fluor 488 goat anti-chicken IgY (1:500, Abcam) at RT for 2 h in darkness. Slides were mounted with VECTASHIELD anti-fade mounting medium containing DAPI stain. For the control experiments, the same protocols were applied, but the SRP54 or Nfe211 primary antibodies were substituted by normal rabbit IgG. No positive immunolabeling was observed in any sections in which the primary antibody was substituted. Use of de-identified human postmortem tissue was approved by McLean Hospital IRB Committee.

For mouse midbrain, C57BL/6 adult male brains were fixed in 4% PFA in PBS for 20 h and cryoprotected in 30% (g/v) sucrose overnight prior to embedding in OCT. Frozen sections were coronally collected at 40 μ m thickness. Midbrain sections containing the substantia nigra and the ventral tegmental area were kept at 4 °C in 0.01 M PBS and processed for floating immunofluorescence staining and image capture through the same procedures as for the on-slide human postmortem sections.

Chromatin Immunoprecipitation

One million SN4741 or SK-N-AS cells were used for each ChIP reaction. For mouse tissue, 4 mg of VTA tissue was used for each ChIP. Cells were cross-linked using 1%

formaldehyde at RT for 10 min; tissue was homogenized with a motor pellet-pestle before the cross-linking. Cross-linked sample was lysed in lysis buffers (1% SDS, 10 mM EDTA—pH 8, 50 mM Tris-HCl—pH 8). The DNA was then sheared with Diagenode Bioruptor Sonicator (Denville, NJ, USA) for 12 min. The lysate was precleared with Protein G magnetic beads (GenScript, Piscataway, NJ, USA) at 4 °C for 1 h. A total of 4 µg of rabbit anti-NFE2L1 (sc721) or anti-SRP54 (AB154796) was added and incubated at 4 °C overnight with gentle rotation. Next day, 80 µL of protein G magnetic beads was added to each sample and incubated at 4 °C for 2 h. The beads were eluted, and elute was incubated with 200 mM NaCl at 65 °C for 5 h to reverse the cross-linking, followed by treatment with Proteinase K (Cat no: 19131, Qiagen) at 45 °C for 2 h to digest proteins. The treated DNA was purified by using QIAquick PCR Purification Kit (Cat: 28104, Qiagen). Protein-DNA binding activity was examined by PCR using primers 5'-tgtggccatcacagtgaaa-3' and 5'-cttcctgggtcaggcaa-3'; PCR products were resolved by 1.5%-agarose electrophoresis and visualized via ethidium bromide staining and UV illumination.

Experimental Design and Statistical Analysis

All numeric data were analyzed statistically using algorithms implemented in GraphPad Prism 5 software. Student's *t* tests were used for comparing two conditions, and ANOVA was used with Bonferroni post hoc tests for multiple comparisons. All data are expressed as means ± SEM. The statistical significance level was defined as $p < 0.05$: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

Results

Identification of 5KSE in *SLC6A3*

During a fresh search for *cis*-acting elements in the human *SLC6A3*, we found that deletion of a 5-kb (4899 bp, from –8329 to –13,227 bp, assuming “+ 1” at transcription start site or TSS) *NheI* fragment from the 18 kb promoter of haplotype A decreased the promoter activity by almost 5-fold in two cultured models of dopamine neurons (Fig. 1). When we increased the deletion size from 5 to 12 kb (–3.5 to –15.6 kb), no additional effects were found (data not shown). These data suggest that this 5 kb is a super enhancer (5KSE) of *SLC6A3*. DNA sequence analysis of 5KSE suggested that 5KSE carried four repetitive elements: the 5'VNTR (rs70957367, from –11.28 to –10.86 kb), and three sub-elements named E-10.3 (348 bp), E-9.7 (301 bp), and E-8.7 (299 bp) localized from –10.62 to –10.33 kb, from –10.05 to –9.75 kb, and from –9.13 to –8.78 kb. 5'VNTR had no extensive homology to the three other sub-elements except 53.3% identity to E-8.7. By

contrast, there were extensive sequence identities (75.6–80.7%) among the three sub-elements (75.6% between E-10.3 and E-8.7; 80.7% between E-9.7 and E-8.7).

Intragenic Antagonism Between 5KSE and Downstream VNTRs

Previous studies have shown that the two downstream variable number tandem repeats (VNTRs), one located in Intron 8 (Int8VNTR) and another in the last exon (3'VNTR), displayed silencing activity on promoter activity. Int8VNTR has two common alleles, 5 repeats (S) and 6 repeats (L); 3'VNTR has two common alleles, 9 repeats (S) and 10 repeats (L). When the two L alleles were combined into LL, the most common haplotype of the two 3'VNTRs, and LL was inserted into the 3' end of the luciferase gene in the 18A or 10A plasmids, LL decreased the *SLC6A3* promoter activity significantly with similar extents in both constructs (Fig. 2a, b), suggesting that LL inhibited the promoter independently of 5KSE. In the presence of LL, addition of 5KSE into 10ALL increased the promoter by 5.2-fold in 18ALL. Together, an intragenic transcriptional antagonism was revealed between 5KSE and 3' VNTRs, in terms of regulating the *SLC6A3* promoter (Fig. 2c).

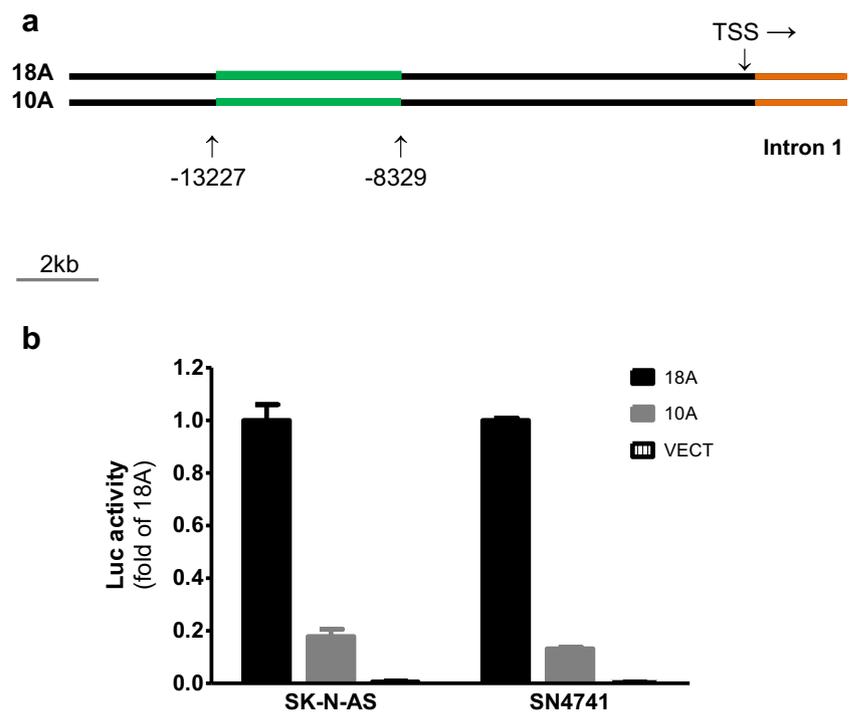
E-8.7 Is a Key Sub-enhancer of 5KSE

In order to better understand 5KSE, we conducted a database search (via NCBI Blast), aiming to identify in other species homology to 5KSE. As a result, three sub-elements were found to share homologies with primate genomes only (Fig. 3a). Among the three, E-10.3 had bit lower conservation (83–86% identity among the top 200 hits) than the other two sub-elements (86–91%). No extensive homologies were found in the rest of the human genome or in non-primate species, suggesting that 5KSE be a primate-specific enhancer.

These primate homologies implied potential functionality. To verify the possibility, we cloned these three sub-elements, one at a time, back to the *NheI* site in the 10A plasmid, substituting 5KSE. Adding back of E-10.3 had no effect on the promoter activity. However, adding back of E-9.7 and E-8.7 each restored at least a half of 5KSE's enhancing activity (2.1-fold and 2.4-fold; Fig. 3b). That meant that E-9.7 and E-8.7 together restored 5-fold, the full enhancing activity of 5KSE.

Next, we examined whether E-9.7 and/or E-8.7 could antagonize the silencing activity of 3'VNTRs. In the presence of LL, E-9.7 was unable to restore any of 5KSE's enhancing activity but E-8.7 was able to increase the promoter activity by 2.9-fold (Fig. 3c). That is, E-8.7 contributed to 5KSE's antagonizing activity.

Fig. 1 Identification of a 5-kb fragment as a super enhancer (green bar, 5KSE) in the 5' promoter regions of *SLC6A3*. **a** Eighteen-kilobyte (haplotypes A) promoter regions and a deletion construct (10A) were generated and studied in this study. **b** Luc reporting of promoter activities on 18A and 10A in two cultured models for dopamine neurons ($***P < 0.0001$ by ANOVA Tukey tests for significance; $n = 3$). Vect, pGL3eBelo1



TF Activation of E-8.7

In order to better understand the *cis*-activating activity of E-8.7, we searched for proteins that mediated the transactivation. E-8.7 was used as bait and a human adult cDNA library used as the prey in the search. One hundred twenty-nine million interactions (12-fold the complexity of the library) were analyzed and 214 clones processed. Potential clones were selected based on in-frame infusion, repeated show-ups, and known expression in dopamine neurons. Four candidate cDNA clones were identified, including SRP54, Nfe211, Slitrk2, and Skp1.

Figure 4a shows that SRP54 and Nfe211 displayed the largest E-8.7-dependent upregulations of the *SLC6A3* promoter; Slitrk2 and Skp1 showed smaller extents of the upregulation. None of the screened cDNA clones showed any downregulations. When SRP54 and Nfe211 combined, a 2.26-fold of upregulation was observed (Fig. 4b), explaining E-8.7's activating capacity (Fig. 3b). Endogenously, overexpression of both proteins each upregulated *SLC6A3* in two cell lines (Fig. 4c).

In cultured cells, SK-N-AS of human origin and SN4741 of murine origin, expression of both SRP54 and

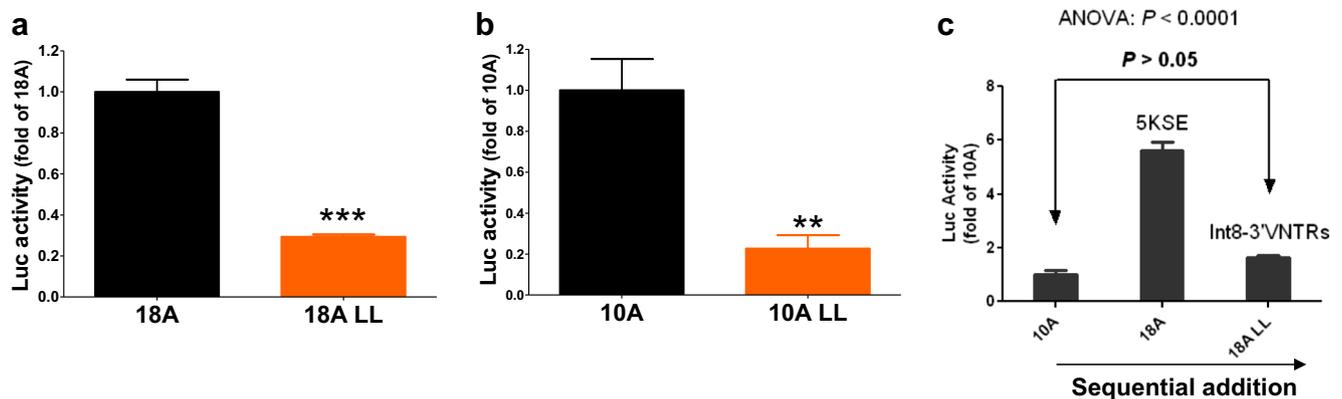


Fig. 2 *SLC6A3* promoter activity of haplotype A silenced by Int8-3' VNTRs in the presence (a) or absence (b) of 5KSE in SK-N-AS. LL, combination of 6-repeat allele of Int8VNTR and 10-repeat allele of 3' VNTR; VNTRs were inserted at an *FseI* site immediately after *luc+* in

pGL3eBelo1. **c** *Cis*-acting mode: upstream 5KSE and downstream Int8-3'VNTRs antagonized each other in regulating 10 kb *SLC6A3* promoter activity; $**p < 0.01$; $***p < 0.0001$ by *t* tests ($n = 4-5$)

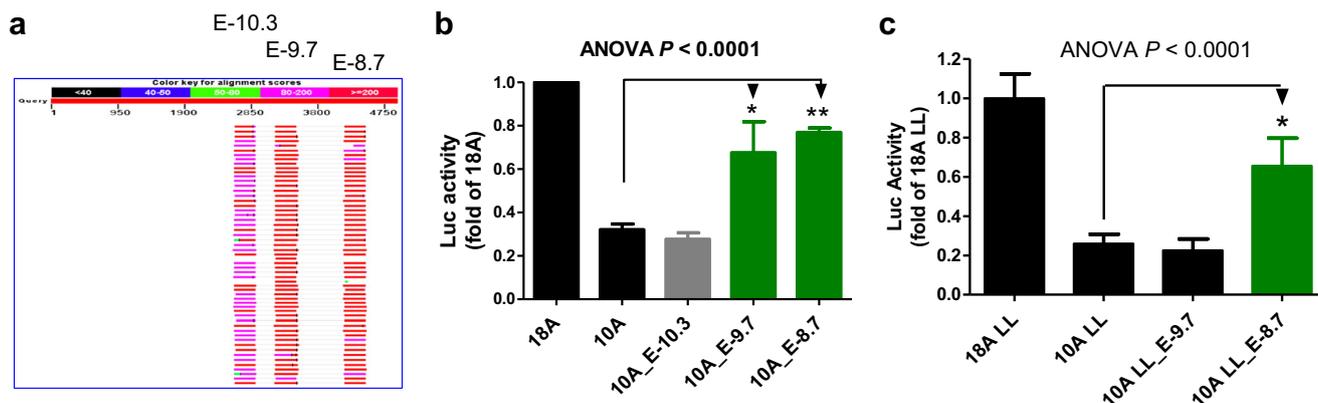


Fig. 3 Identification of two repetitive enhancers within 5KSE in SK-N-AS. **a** Conservation of E-10.3, E-9.7 & E-8.7 of 5KSE in primates only. **b** Enhancing activity of E-9.7 and E-8.7 PCR-cloned into *NheI* site of 10A ($n = 3$). **c** E-8.7 (not E-9.7) antagonized Int8-3'VNTRs ($n = 4$)

Nfe211 was localized to the nuclei but unlike Nfe211, SRP54 was mainly expressed in the cytoplasm, especially of SN4741 (Fig. S1). In human postmortem midbrain tissue, both SRP54 and Nfe211 were found expressed in the nuclei of dopamine neurons (Fig. 4d, e). More of SRP54 was expressed in the cytoplasm and more of Nfe211, in the nuclei. These human data were replicated in another healthy subject (Fig. S2). Consistently with the nuclear expression, ChIP-PCR analysis of naïve human SK-N-AS cells showed that both SRP54 and Nfe211 bound to E-8.7 in the *SLC6A3* promoter (Fig. 4f).

Discussion

This study identified a main enhancer, 5KSE, in the human *SLC6A3* and cloned two TFs that functionally recognized the enhancer. SKSE carries two sets of tandem repeats, which could play more critical role in health and disease than simpler polymorphisms [25]. Another finding is the discovery of transcriptional antagonism between 5KSE and downstream VNTRs. The distribution of the main alleles at the three VNTRs considered here and their level of linkage disequilibrium has been described before [26].

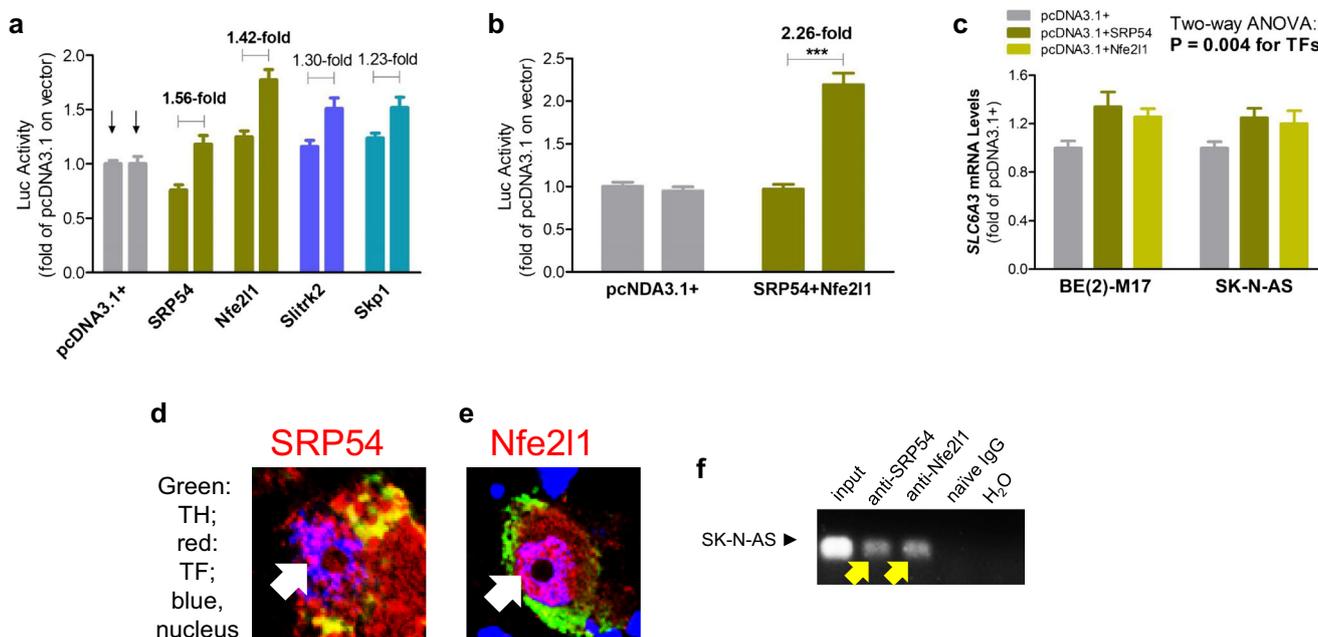


Fig. 4 Identification of TFs for E-8.7. **a** Overexpression of individual cDNA clones regulated the *SLC6A3* promoter. **b** More significant regulation by combined cDNA clones in human SK-N-AS cells: 1st bar, plasmid 10A (18A without 5KSE); 2nd bar, 10A_E-8.7; bracket, clones for combinations; two-way ANOVA: $*P < 0.05$ or $***0.0001$

($n = 4$ for both **a** and **b**). **c** Overexpression of TFs upregulated endogenous *SLC6A3* mRNA levels in two cell lines ($n = 6$). Nfe211 and SRP54 showed nuclear expression (white arrow) in human postmortem midbrain (**d**, **e**) and binding (yellow arrow) to E-8.7 in SK-N-AS by ChIP-PCR (**f**)

Increased *SLC6A3* Activity Can Be a Risk for Psychiatric Disorders

Previous studies have shown that environmental risks such as nicotine [27, 28], various stressors [29, 30], and high-fat diet [31] all upregulate the DAT gene activity [24]. Particularly, cigarette smoking is considered as a gateway drug for substance use disorders [32]. Therefore, delineation of the molecular mechanisms for *SLC6A3* upregulation is an urgent matter in order to understand genetic contribution of *SLC6A3* to related neuropsychiatric disorders. Cloning of *trans*-activators, such as SRP54 and Nfe2l1, may provide an opportunity to elucidate signaling pathways to endogenous and external risks. In this regard, investigation of 5KSE may enhance our understanding of the role of *SLC6A3* in brain function or pathophysiology.

Regulation of *SLC6A3* Does Not Follow a Simple Mode

For the last decades, the widely used genetic markers 3'VNTR and Int8VNTR both downregulated heterogeneous promoters in vitro. It remains unknown whether the silencing activity interacts with activating events. Findings from this study suggest for the first time that different *cis*-acting elements may interact with each other selectively in controlling the gene activity (Fig. 5). An example of the selectivity is that E-8.7, not E-9.7, may attenuate the silencing activity from the downstream VNTRs, excluding a simple additive mode. However, E-8.7 and E-9.7 together appear to follow an additive mode in enhancing the promoter activity (Fig. 2c vs Fig. 3b). Although E-9.7 and E-8.7 share 80.7% identity, it is unknown why E-9.7 fails to attenuate the downstream VNTRs. An assumption is that different TFs bind differentially to these two sub-elements. Previously, we have reported that 5'VNTR in 5KSE is correlated with the mRNA levels in healthy midbrain tissues [26] but the functionality of 5'VNTR is not revealed by this study. We failed to observe that 5'VNTR can regulate the promoter on its own, suggesting that additional mechanisms remain to be elucidated. Interestingly, the transcriptional

antagonism between 5KSE and downstream VNTRs is different from a traditional competition model where different TFs compete for binding to the same *cis*-acting site [33–38]. That is, in *SLC6A3*, different TFs on different sites might compete for the same core promoter complex.

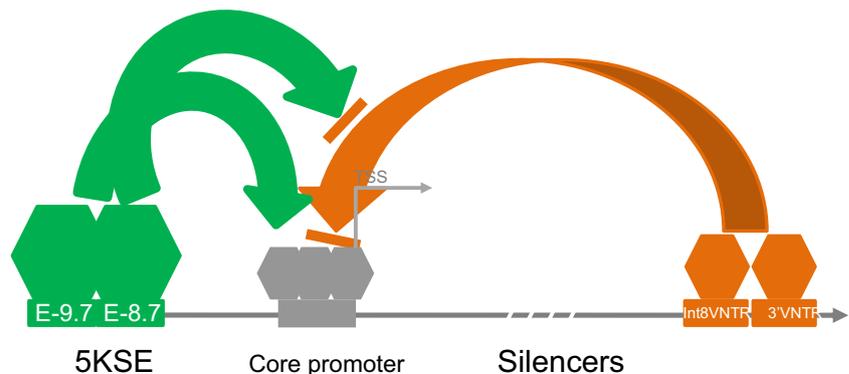
Intragenic Antagonism Implies Haplotype-Dependent Gene Activity

In an additive regulatory mode, different *cis*-acting elements may function independently from each other so that the overall gene activity in a cell at a time is the additive result of these independent regulations. In case of polymorphic *cis*-acting elements such as VNTRs, each allele can be correlated with a gene expression level and serve as an effective genetic marker in a case-control association study. However, in the presence of intragenic antagonism, an activator-bound allele may not necessarily correlate with a higher gene activity due to attenuation by another site so that this functional marker may not serve as an effective genetic marker for “main effect.” In other words, haplotypes of the interacting sites would serve as effective markers in association studies [39–41]. Therefore, elucidating transcriptional antagonism is critical for an understanding of genetic etiology. In this case, E-8.7 carries one common polymorphism, rs1478435, and 12 other low-frequency polymorphisms according to the 1000 Genomes Project [4]. Identifying two TFs for E-8.7, we still do not know whether they target polymorphic sequences. Future study is warranted to clarify the TF binding sequences in E-8.7 and whether these TFs recognize any TFs on the downstream VNTRs.

E-9.7 Is the Most Conserved Sub-element Here

Among the three sub-elements, E-9.7 is the most conserved in two ways. It finds significant homologies (88–89%) in 16 other chromosomes (except 2, 5, 14, 16, 18, 20, 21, and Y) in the human genome. Among different species, it also shows the highest homologies. It is unclear why it has less function

Fig. 5 Transcriptional *cis*-antagonism in *SLC6A3* (dashed arrow, 5' to 3'). Green, enhancing; brown, silencing; gray, core promoter; rectangles, *cis*-acting elements; hexagons, proteins



than E-8.7. As well, the function of E-10.3 remains cryptic. It is possible that these conserved sequences contribute to developmental regulation of *SLC6A3* activity as the *DAT* gene is not active till E14 in rats [42]. This hypothesis is based on recent literature that conserved enhancers are developmentally important [43, 44]. Consistently, Nfe2l1 is already implicated in dopamine neuron development [45]. It remains unknown specifically how *SLC6A3* is developmentally activated; investigation of these enhancing elements would help dissect the developmental mechanism.

SRP54 and Nfe2l1 Are Novel *trans*-Activators for *SLC6A3*

SRP54, the Signal Recognition Particle 54, has a known function in which it helps to insert hydrophobic signaling peptide of a membrane protein into a membrane [46]. It has never been studied for dopamine neurons or as a TF; Nfe2l1 is a known TF, albeit it has not been studied in dopamine neurons. It was reported of an Nfe2l1 binding consensus sequence [47], but this sequence is not matched with E-8.7, possibly because of cell type-specific binding [48, 49]. Similar to the human postmortem observations, we have also confirmed that both proteins are expressed in the nuclei of dopamine neurons of mouse VTA and SNc (data not shown here). The dopaminergic significances of these proteins are unknown and warrant future investigation.

Limitations

SLC6A3 has a human-specific array of *cis*-acting elements, which limits valuable utilization of experimental systems for in vivo validations of intragenic transcriptional antagonism. To better understand the intragenic antagonism, we would need to identify all related TFs including their binding sequences in *SLC6A3* but currently we have an incomplete understanding.

Conclusions

5KSE is a large effect enhancer and exerts its function via previously unknown *trans*-activators including SRP54 and Nfe2l1. *SLC6A3* harbors an intragenic transcriptional antagonism between upstream 5KSE and downstream VNTRs, emphasizing a haplotypic activity of *SLC6A3*.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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