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SLC9A9 mutations, gene expression and protein-protein interactions in rat models of attention-deficit/hyperactivity disorder

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Abstract

SLC9A9 (solute carrier family 9, member 9, also known as Na⁺/H⁺ exchanger member (NHE9)) is a membrane protein that regulates the luminal pH of the recycling endosome, an essential organelle for synaptic transmission and plasticity. *SLC9A9* has been implicated in human attention deficit hyperactivity disorder (ADHD) and in rat studies of hyperactivity. We examined the *SLC9A9* gene sequence and expression profile in prefrontal cortex, dorsal striatum and hippocampus in two genetic rat models of ADHD. We report two mutations in a rat model of inattentive ADHD, the WKY/NCrl rat, which affect the interaction of *SLC9A9* with calcineurin homologous protein (CHP). We observed an age-dependent abnormal expression of *SLC9A9* in brains of this inattentive model and in the Spontaneous Hypertensive Rat (SHR) model of ADHD. Our data suggest a novel mechanism whereby *SLC9A9* sequence variants and abnormalities in gene expression could contribute to the ADHD-like symptoms of rat models and possibly the pathophysiology of ADHD in humans.

Keywords

SLC9A9; ADHD; CHP; RACK1

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Conflict of interest

In previous years, Dr. Sagvolden received consulting fees or research support or has been on Advisory Boards or has been a speaker for: Shire, Janssen, and Eli Lilly. In the past year, Faraone has received consulting fees and has been on Advisory Boards for Shire Development and has received research support from Pfizer, Shire and the National Institutes of Health. In previous years, Dr. Faraone has received consulting fees or has been on Advisory Boards or has participated in continuing medical education programs sponsored by: Shire, McNeil, Janssen, Novartis, Pfizer and Eli Lilly. In previous years he has received research support from Eli Lilly, Shire, Pfizer and the National Institutes of Health. Dr. Faraone receives royalties from a book published by Guilford Press: *Straight Talk about Your Child's Mental Health*. Dr. Middleton, Dr Zhang-James, and Tania DasBanerjee reported no biomedical financial interests or potential conflicts of interest.

INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a common, highly-heritable disorder (Faraone and Mick 2010; Gizer and others 2009). Genetic, brain imaging, and animal model studies have implicated numerous candidate genes in its etiology (Faraone and Mick 2010; Gizer and others 2009). One candidate gene is *SLC9A9*, a member of the Na⁺/H⁺ exchanger (NHE) family. *SLC9A9* was first implicated in ADHD by a report of an extended family in which ADHD co-segregated with a pericentric inversion of Chromosome 3 that disrupted both *DOCK3* and *SLC9A9* (de Silva and others 2003). Subsequently, single-nucleotide polymorphisms (SNPs) in *SLC9A9* were identified among the most significant findings in an analysis of 51 candidate genes from the International Multisite ADHD Genetics (IMAGE) project (Brookes and others 2006) and in the IMAGE genome-wide association study (GWAS) of ADHD symptoms, *SLC9A9* achieved one of the lowest p values ($\sim 10^{-5}$; (Lasky-Su and others 2008)). *SLC9A9* was also associated with ADHD in a subsequent association study (Markunas and others) but the association did not achieved genome-wide significance in a meta-analysis of GWAS (Neale and others 2010).

NHEs are large membrane-bound proteins that allow sodium or hydrogen ions to passively diffuse down their concentration gradients across the cell or organelle membrane. There are currently 10 known NHEs. *SLC9A9* is primarily localized on the membranes of late recycling endosomes. Together with *SLC9A6* (which is primarily localized on early/recycling endosomes), *SLC9A9* regulates the pH of endosomal compartments (Nakamura and others 2005; Roxrud and others 2009). Endosomes play important roles in recycling neurotransmitter receptors and transporters, such as glutamate receptors and the dopamine transporter (DAT) (Loder and Melikian 2003; Park and others 2004). This recycling is a critical component of long-term potentiation (LTP) (Park and others 2004; Park and others 2006). Like other NHEs, *SLC9A9* is predicted to have 12 transmembrane domains and a long intracellular C-terminal. The C-termini of the NHEs contain phosphorylation sites and binding domains for regulatory and signaling molecules (Slepko and others 2007). Many NHE binding molecules, such as calcineurin homologous protein (CHP) (Lin and Barber 1996), receptor for activated C-kinase 1 (RACK1) (Ohgaki and others 2008), calmodulin (CaM) (Bertrand and others 1994) and phosphatidylinositol 4,5-bisphosphate (PIP2) (Aharonovitz and others 2000), participate in intracellular Ca²⁺ signaling cascades and protein phosphorylation/dephosphorylation, key cellular mechanisms believed to underlie synaptic transmission and plasticity.

The spontaneously hypertensive (SHR) rat obtained from Charles River, Germany (SHR/NCrl) is a well-validated animal model of the ADHD-Combined subtype (ADHD-C), with the Wistar-Kyoto strain obtained from Harlan, UK (WKY/NHsd) serving as its most appropriate control (Sagvolden 2000; Sagvolden and others 2009). A substrain, named the WKHA rat, created from a cross between SHR and WKY, displays high spontaneous activity but low systolic blood pressure. A single genome-wide significant locus (*Act* QTL) on Chromosome 8 that contains *SLC9A9* showed significant linkage to hyperactivity (Moisan and others 2003). The *Act* QTL locus is homologous to the region of human Chromosome 3 where the pericentric inversion disrupting *SLC9A9* segregated with ADHD (de Silva and others 2003). Moreover, the homologous region in mouse Chromosome 9 that contains *SLC9A9* contains an activity-related QTL (Grisel and others 1997; Mathis and others 1995; Miner and Marley 1995).

We reported the behavioral and genetic characterization of a new rat model for the inattentive subtype of ADHD (WKY/NCrl, obtained from Charles River, Germany) (Sagvolden and others 2008). This rat, which shows impaired sustained attention, but normal activity level and impulsiveness, is genetically divergent from the common reference WKY

strain (WKY/NHsd) used as a control for the SHR. The region of genetic divergence includes *SLC9A9* (Sagvolden and others 2008).

The present study tested three hypotheses about *SLC9A9*: 1) that *SLC9A9* gene expression would be dysregulated in WKY/NCrI and SHR/NCrI rats; 2) that *SLC9A9* mutations would be found in these rats and 3) that these mutations would be associated with abnormal interactions of *SLC9A9* protein with other proteins.

METHODS

Animals

We obtained rats from two sources: WKY/NCrI and SHR/NCrI rats from Charles River (Sulzfeld, Germany), and WKY/NHsd control rats from Harlan Europe, (Blackton, Bicester, UK). Animal procedures were approved by the Norwegian Animal Research Authority, and animals were housed and euthanized at the University of Oslo. Experiments were conducted in accordance with the laws and regulations controlling experimental procedures in live animals in Norway and the European Union. Brain parts were dissected and preserved in RNAlater or AllProtect (Qiagen, CA), and shipped at room temperature to SUNY Upstate Medical University. Samples were stored at -80°C upon receipt until use.

Expression analysis of *SLC9A9* and *SYP*

15 adolescent (~28 days old, WKY/NCrI=6, SHR/NCrI=6 and WKY/NHsd=3) and 16 adult (~65 days old, WKY/NCrI=5, SHR/NCrI=3, and WKY/NHsd=8) rats were used for expression analysis of dissected medial prefrontal cortex, dorsal striatum, and hippocampus with real-time quantitative reverse-transcription PCR (qPCR). Two of these brain regions (prefrontal cortex, dorsal striatum) were chosen because they have been implicated in ADHD by a meta-analysis of structural imaging studies in humans (Valera and others 2007). Abnormal hippocampal structure has also been observed in ADHD patients (Plessen and others 2006) and in SHR rats (Amenta and others 1996), and there is an increasing recognition of the relevance of hippocampus in ADHD due to its essential role in learning and memory, attention (Volkow and others 2007), and decision-making (Ernst and others 2003).

Total RNA from all three brain areas were extracted using the RNeasy mini kit (Qiagen, Valencia, CA). Equal amounts of RNA from each brain regions from all three strains of rats were reverse-transcribed using Quantitect Reverse Transcription (RT) kit (Qiagen). Diluted RT reactions were used for qPCR in a Roche LightCycler® 480 Real-Time PCR system using Roche LightCycler 480 SYBR Green I Master reagents. Three stably expressed genes (*CycA*, *Hprt1*, and *Ywhaz*) were used as reference genes for normalization of gene expression (Bonfeld and others 2008). Relative expression levels (in \log_2 scale) were calculated based on differences in the number of cycles required to reach the threshold for target amplicon detection (C_t) versus the geometric mean of the three reference genes (the ΔC_t method) and normalized within each brain region to the ΔC_t value of the adolescent control WKY/NHsd group ($\Delta\Delta C_t$ method).

We also examined the expression of a synaptic marker, the synaptophysin (*SYP*) gene. *SYP* gene was chosen because its expression is correlated with the basic differentiation process of the neurons such as proliferation, fiber outgrowth and the formation of synapses (Bergmann and others 1991; Bergmann and others 1993; Grabs and others 1994). Because developmental differences in neuronal numbers and synaptic connections between different ages and strains of animals could be a confounding factor for gross expression analysis, we calculated the *SLC9A9/SYP* ratio (a \log_2 difference) to estimate the relative amount of gene expression per overall synaptic density in each brain area. First, the level of *SYP* expression

was normalized against the geometric mean of the 3 reference genes (ΔC_t) method. Then, the *SLC9A9/SYP* ratio was calculated as the difference between the specific ΔC_t of each individual gene v.s. the ΔC_t of *SYP* ($\Delta\Delta C_t$). We analyzed *SLC9A9* and *SYP* expression individually, as well as the *SLC9A9/SYP* ratios across all three brain regions.

Within each brain region, we assessed the effects of strain, age and the strain by age interaction using linear regression analysis in STATA 11.2. We followed up significant main effects or interactions by computing pairwise comparisons using Wald tests in STATA 11.2.

Genetic sequence analysis of gene *SLC9A9*

We used the whole genome genotyping data acquired from WKY/NCrI and SHR/NCrI rats (from Charles River, Sulzfeld, Germany), and WKY/NHsd control rats (from Harlan Europe, Blacktown, Bicester, UK) to identify genetic divergence in the *SLC9A9* gene region. These data were acquired using Affymetrix Targeted Genotyping Rat Panel (1.0 5K) as described previously (Sagvolden and others 2008). Four adult male rats in each strain that had been behaviorally phenotyped were used for genotyping. SNPs were used for cross-strain comparisons if they met two criteria: (1) complete concordance within each set of strain replicates, and (2) some degree of variation between the three strains.

Because the WKY/NCrI, SHR/NCrI, and WKY/NHsd rats showed genetic divergence in markers spanning the *SLC9A9* locus (Sagvolden and others 2008), we sequenced the entire *SLC9A9* gene in these three strains. PCR primers were designed using ExonPrimer (<http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html>) to amplify each of the exons and adjacent intronic regions with at least 100bp overlap. Template DNAs were selected from those used in the SNP genotyping scan. PCR products were cleaned and sent for sequencing (GeneWiz, Inc). Chromatograms were analyzed using Sequencher software V4.5 (GeneCodes) and compared with the rat reference sequences.

Effects of mutations on protein chemistry and protein-protein interactions

In silico analysis—To examine possible protein alterations produced by the sequence changes, *SLC9A9* and NHE family protein sequences from selected species were downloaded from NCBI. Homology alignments were performed using ClustalX 2.0. Transmembrane domains of human *SLC9A9* protein were predicted by TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Glycosylation and phosphorylation were predicted by the YinOYang server (<http://www.cbs.dtu.dk/services/YinOYang/>). Deleage and Roux amino acid scales (Deleage and Roux 1987) was used to plot the conformational propensity for alpha-helix formation using a linear model with a 21 amino acid window.

Co-Immunoprecipitation—To express c-Myc-tagged *SLC9A9* C-terminal (pMyc-*SLC9A9*^C), we inserted a c-Myc tag sequence between Xho I and Hind III sites of the pdsRed1-N1 plasmid (Clontech). Rat *SLC9A9* coding sequence for the complete C-terminal (residues 494–645 with stop codon) was amplified by PCR and directionally cloned between the EcoR I and Kpn I sites of modified c-Myc plasmid, thus allowing an expression of N-terminally c-Myc-tagged fusion protein. Mutations were introduced using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, TX) and verified by sequencing.

To express C-terminally Flag-tagged CHP and RACK1 proteins (pCHP-Flag and pRACK1-Flag), we replaced the original C-terminal dsRed tag coding sequences in pdsRed1-N1 with a Flag sequence between the AgeI and NotI sites. Rat CHP and RACK1 coding sequence were amplified by PCR with primers containing restriction sites. CHP was directionally cloned into the modified Flag plasmid between the EcoR1 and BamHI sites. RACK1 was

cloned into the same plasmid between the EcoRI and SacII sites. All constructs were sequence-verified.

HEK 293 cells were maintained in DMEM medium with 10% Fetal Bovine Serum, 100U penicillin, 100µg streptomycin, and 1% GlutaMAX™-I supplement (Invitrogen). Cells were co-transfected with a combination of either pMyc-*SLC9A9*^C and pCHP-Flag, or pMyc-*SLC9A9*^C and pRACK1-Flag, using Lipofectamine 2000 reagent. Cells were harvested from 10cm culture dishes and washed twice with PBS buffer. After centrifugation, cell pellets were lysed on ice for 30 min with cold PBS buffer containing 1% Nonidet P-40, 1mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture. Following lysis, cells were centrifuged for 10min at 20,000g at 4°C. The resulting supernatants were mixed with Protein G agarose resins coupled with either Anti-c-Myc antibodies or normal mouse IgG (Pierce, Thermo Scientific). After incubation at 4°C overnight with gentle rotation, beads were washed four times with cold PBS buffer containing 0.1% Nonidet P-40, and immunoprecipitates were separated by SDS-PAGE. *SLC9A9* C-terminal and Flag-tagged binding partners were detected by immunoblotting with horseradish peroxidase-conjugated anti-c-Myc IgG (ab1261, Abcam, MA) and anti-Flag IgG (Sigma, MO) respectively. Immunoreactive bands were quantified in Scion Image for both area and mean optical density (OD), corrected for background OD. The relative binding with the *SLC9A9* C-terminal for CHP and RACK1 was determined by calculating a ratio of the product of area and corrected mean OD for each band detected with anti-Flag antibody compared to that for the anti-c-Myc blots for each co-IP sample. Ratios from three independent experiments were used in a two-tailed t-test.

RESULTS

Expression profiles of *SYP* and *SLC9A9* in ADHD rat models

We first examined the expression of the *SYP* gene in each brain region and the correlation between *SLC9A9* and *SYP* expression to determine if gross anatomical differences in neurons and synapses across different strains might confound our analyses. The expression of these two genes was significantly correlated ($r=.40$, $p=.0001$). We found a significant effect of strain on *SYP* expression in both dorsal striatum ($F_{(2,26)} = 9.56$, $p= 0.0008$) and prefrontal cortex ($F_{(2,26)} = 3.56$; $p=0.04$). There was also a significant strain by age interaction in these two regions (dorsal striatum $F_{(2, 26)} = 4.82$, $p=0.02$; prefrontal cortex $F_{(2,26)}=3.81$, $p=0.04$). The effects of strain or strain by age interactions for the hippocampus were not significant (strain $F_{(2, 26)} = 2.10$, $p=0.1433$; strain by age $F_{(2, 26)}=1.46$, $p=0.2515$). Figure 1A shows that there was a similar magnitude of age-dependent decrease of *SYP* expression for the control WKY/NHsd rats in hippocampus (Fold Change (FC)= $-.526$, $p=.047$) and in dorsal striatum (FC= $-.547$, $p=.1$). SHR/NCrI rats lacked this pattern of decrease in these two regions, however they showed an abnormally large age-dependent decrease in *SYP* in the prefrontal cortex ($t_7=-2.9$, $p=.02$), which the other strains did not (p 's $>.05$). The WKY/NCrI rats also lacked the normal age-dependent decrease of *SYP* expression in hippocampus, but showed a larger age-dependent decrease in *SYP* ($t_7=-5.0$, $p=.001$) in dorsal striatum.

Because *SYP* potentially could have confounded our analyses of *SLC9A9* expression for prefrontal cortex and dorsal striatum, these analyses were statistically adjusted using *SYP* as a covariate. In dorsal striatum, we found a significant effect of strain ($F_{(2,25)}=3.51$, $p=0.045$), and a significant effect of *SYP* expression ($F_{(1, 25)}=4.65$, $p=0.04$) on *SLC9A9* expression. The strain by age interaction was not significant ($F_{(2,25)}=2.6$, $p=0.09$). For prefrontal cortex, the effect of *SYP* expression on the *SLC9A9* expression was also significant ($F_{(1, 25)} = 6.27$, $p=0.02$), as well as the effect of strain by age interactions ($F_{(2, 25)} = 4.53$, $p=0.02$). In the hippocampus, age ($F_{(1,25)} = 19.42$, $p=0.0002$), strain by age interaction ($F_{(2, 25)} = 3.85$,

$p=0.035$) and *SYP* expression ($F_{(1,25)}=14.50$, $p=0.0008$) all showed a significant influence on the *SLC9A9* expression.

SLC9A9 expression is plotted in two ways. Figure 1B plots the expression pattern of *SLC9A9*, unadjusted for *SYP*. Figure 1C plots the *SYP* adjusted expression of *SLC9A9* using the *SLC9A9/SYP* ratios. The *SLC9A9/SYP* ratios show that the strain effect for dorsal striatum was primarily accounted for by decreased *SLC9A9/SYP* values for the WKY/NCrI compared to other strains. The interaction effects observed for hippocampus and prefrontal cortex were due to different effects. For both regions, there were no strain differences in adolescence. In adulthood, the hippocampal results showed an increase in *SLC9A9/SYP* for WKY/NCrI rats compared to WKY/NHsd controls ($F_{(1,14)}=5.3$, $p=0.04$) and the SHR/NCrI rats ($F_{(1,14)}=5.6$, $p=0.03$) but the two latter strains did not differ from one another ($F_{(1,14)}=0.38$, $p=0.55$). In contrast, in adulthood, the prefrontal cortex results showed that *SLC9A9/SYP* was increased for SHR/NCrI ($F_{(1,14)}=4.76$, $p=0.047$) and reduced in WKY/NCrI rats ($F_{(1,14)}=7.49$, $p=0.02$) compared with WKY/NHsd control rats. The difference between the two ADHD rat models was also highly significant ($F_{(1,14)}=17.46$, $p=0.0009$).

Sequence variants of *SLC9A9* in WKY/NCrI Rats

The gene for *SLC9A9* lies within a region of Chromosome 8 that we previously identified in a genome-wide SNP scan as genetically divergent between the WKY/NCrI and WKY/NHsd strains (Supplementary Table 1; (Sagvolden and others 2008)). We resequenced all 18 exons and their flanking intronic regions, as well as 1Kb upstream of the start codon of *SLC9A9*. Consistent with our SNP panel results, The SHR/NCrI rats showed no sequence variations in the *SLC9A9* coding region. We found five novel SNPs in the inattentive WKY/NCrI rats, including two nonsynonymous mutations in highly-conserved regions (V512G and K534R; Supplementary Figure 1A). These mutations were always found together. Retrospective examination of behavioral data for the one K534R/V512G heterozygote did not reveal any difference compared with K534R/V512G homozygotes, suggesting a likely dominant effect of the mutations (data available upon request). Although the SNP for the second mutation (K534R) lies at the exon and intron boundary, it did not change gene splicing. Only normally spliced transcripts were amplified from cDNAs of the WKY/NCrI rat brains and verified by sequencing using primers flanking exon 16 (data not shown).

Effects of *SLC9A9* mutations on protein binding

The two non-synonymous mutations are located in the intracellular C-terminal juxtamembrane region of *SLC9A9*, where regulatory proteins normally bind. The two residues mutated in the inattentive WKY/NCrI rats are highly conserved across mammalian species (Supplementary Figure 1B). Specific binding partners of *SLC9A9* in the region harboring the mutations have not been fully identified. This region shares some degree of homology with other members of the NHE family, whose binding partners are well-characterized. One binding partner that is essential for Na^+/H^+ exchanger function is CHP. The CHP-binding domain in SLC9A1 (510–540aa) forms an alpha-helix, inserting into a hydrophobic cleft formed by CHP (Ammar and others 2006; Pang and others 2001). To predict whether CHP also binds to *SLC9A9*, we first examined whether the *SLC9A9* juxtamembrane region is able to form an alpha-helix secondary structure using an algorithm developed by Deleage and Roux (Deleage and Roux 1987). We found a high propensity for alpha-helix formation in the juxtamembrane region (Supplementary Figure 1C). The mutations slightly reduced the alpha helix conformational score. Alignment of this putative alpha-helix forming region with the CHP-binding domains in SLC9A1-5s reinforced the existence of conserved hydrophobic residues that may mediate an interaction of CHP with *SLC9A9* (Supplementary Figure 1D).

To test this possible interaction and examine the effects of the mutations on the interaction, we transiently co-expressed Myc-tagged *SLC9A9* C-terminals (with or without the double mutations) and Flag-tagged CHP in HEK293 cells, and performed co-immunoprecipitation using an anti-Myc antibody. Since the two *SLC9A9* mutations were always found together in WKY/NCrl rats, we tested the joint effect of both mutations on interactions between the C-terminal of *SLC9A9* and CHP. We confirmed the interaction of the C-terminal of *SLC9A9* with CHP. Furthermore, the mutations significantly increased the amount of CHP precipitated with *SLC9A9* by almost two-fold ($p=0.027$ by two tailed t-test, Figure 2, last two lanes).

Co-immunoprecipitation assays evaluated the effects of the joint mutations on the interaction with RACK1, the only previously described binding partner for *SLC9A9*. The K534R mutation is located within the binding region for RACK1, although multiple sites for RACK1 may exist in this region, which require further characterization (Ohgaki and others 2008). We confirmed the interaction of *SLC9A9* with RACK1. However, the mutations had no effect on this interaction (Figure 2, first two lanes).

Although the main bands for the Myc-tagged *SLC9A9* C-terminal appeared to be ~25kDa in SDS-PAGE in both cell lysates and immunoprecipitates, the predicted molecular weight of this peptide is 17.8kDa. We verified the specificity of the anti-Myc bands using another commercially available Myc antibody as well as an anti-*SLC9A9* antibody (Abcam, data not shown). Predictions using the YinOYang web server indicated that the C-terminal of the *SLC9A9* is heavily glycosylated and also contains several phosphorylation sites (data not shown). We suspect that the higher than expected molecular weight was due to post-translational modifications.

DISCUSSION

We found substantial strain differences in *SYP* expression for dorsal striatum and prefrontal cortex, but not hippocampus. We also found evidence for strain differences in *SLC9A9* expression in each brain region and that these differences among strains were age dependent. After adjusting for potentially confounding differences between strains in neuronal numbers and synaptic connections as measured by the synaptic marker *SYP*, we found that the expression of *SLC9A9* in ADHD rats, particularly the WKY/NCrl rats, significantly deviated from the control rats in the dorsal striatum at both ages, and in the hippocampus and the prefrontal cortex of the adult rats. For SHR/NCrl rats, the expression of *SLC9A9* was elevated only in the prefrontal cortex at the adult age. We also found two co-transmitted mutations of *SLC9A9* in the WKY/NCrl rats. The effects of mutations on the interaction of *SLC9A9* with other proteins were predicted and confirmed with co-immunoprecipitation. It is not clear whether the *SLC9A9* expression abnormalities in the WKY/NCrl rats were a compensatory response of the effect of mutations on the protein functions. Nevertheless, our results suggest that the gene expression and sequence abnormalities in *SLC9A9* may play a role in the ADHD phenotype of the ADHD rat models.

Because *SYP* expression correlates with neuronal differentiation and synaptic formation (Bergmann and others 1991; Bergmann and others 1993; Grabs and others 1994), it has been used as a marker for quantitative measurements of synapses (Calhoun and others 1996; Mouton and others 1997). Overall the number of synapses that we measured by *SYP* may represent a net effect of dynamic postnatal synaptogenesis and pruning. We found a similar age-dependent decrease of *SYP* expression for the control WKY/NHsd rats in hippocampus and in dorsal striatum, indicating that active pruning is predominant in these areas. In contrast, for controls *SYP* expression was not changed between the two ages in prefrontal cortex, suggesting they may still be forming new synapses to counter balance the effect of

pruning or that pruning was not robust during the two age points that we examined, which is consistent with the fact that prefrontal cortex matures later than other brain regions.

Examining the *SYP* expression profiles in two ADHD animal models suggests different developmental abnormalities in these two strains. SHR/NCrl rats lack the normal age-dependent decrease in *SYP* in both hippocampus and dorsal striatum, suggesting a defective pruning. In prefrontal cortex, SHR/NCrl rats showed a significantly more robust decrease in *SYP* with age. One possibility is that early postnatal pruning was delayed and followed by excessive or inappropriate pruning from adolescent to adulthood, resulting in a higher than normal number of synapses in adolescence and a lower than normal number of synapses in adulthood. Our *SYP* observations in SHR/NCrl rats are consistent with the anatomical alterations reported by others (Amenta and others 1996; Nelson and others 1993; Nelson and Boulant 1981; Nelson and Boulant 1983). WKY/NCrl rats were statistically no different from the controls in both the prefrontal cortex and the hippocampus, although there was some indication of a lack of hippocampal pruning. In dorsal striatum, however, WKY/NCrl rats significantly deviated from the two other strains in *SYP* expression, suggesting a delay in early postnatal pruning followed by excessive pruning. Overall, the *SYP* results suggest that anatomical defects during brain development and maturation may be associated with the ADHD phenotypes in animal models. These defects are more wide-spread in SHR brains, consistent with the presence of all three ADHD symptom clusters: hyperactivity, impulsivity and inattentiveness. The limited effects seen for the WKY/NCrl rats may explain why that strain only displays inattentive symptoms.

The significant correlation of *SLC9A9* and *SYP* expression and the relative stability of the *SLC9A9/SYP* ratios over age in control rats suggest that *SLC9A9* expression is proportional to the number of synapses. This is consistent with Morrow et al.'s (2008) study of hippocampal neurons, which suggested that *SLC9A9* was one of the "activity-regulated" genes. Consistent with this idea, deleterious sequence variants of *SLC9A9* have been found in patients with autism and epilepsy (Morrow and others 2008). Mutations of *SLC9A6*, a close family member that also resides in endosome membranes, were found in patients with Angelman Syndrome, involving autistic symptoms and epilepsy (Gilfillan and others 2008). *SLC9A6* knockout mice showed increased seizure susceptibility. Mutations or targeted disruption of *SLC9A1*, an ubiquitous plasma membrane protein, produced seizures in mice (Bell and others 1999) (Cox and others 1997). Epilepsy often co-exists with ADHD and autism (Canitano 2007; Deonna and Roulet 2006; Gurrieri and others 1999; Hamoda and others 2009; Hesdorffer and others 2004). SHR rats also have increased susceptibility to seizures (Goldberg and others 1975), but we do not know whether WKY/NCrl rats are susceptible to seizures.

SLC9A9 may serve as a local signaling scaffold protein. Mammalian Na⁺/H⁺ exchangers have a long intracellular C-terminal, which recruits various signaling molecules, and may promote the assembly of local signaling complexes and coordinate divergent signaling pathways (Baumgartner and others 2004). In our study we showed that the *SLC9A9* C-terminal interacts with the signaling molecules CHP and RACK1. These molecules and other binding partners of Na⁺/H⁺ exchangers (such as CAM and PIP2) are not only regulators of Na⁺/H⁺ exchanger activity, but also important signaling molecules in calcium signaling and protein phosphorylation. These signaling pathways are essential for synaptic transmission and plasticity. Mutations that affect the protein-protein interactions or expression of *SLC9A9* may affect synaptic signaling and alter neuronal activities. In turn, the activity-dependent synaptogenesis and pruning can lead to changes in *SYP*.

A second mechanism underlying the role of *SLC9A9* in neurotransmission is through the Na⁺/H⁺ exchanger activity of the *SLC9A9*. CHP interaction with Na⁺/H⁺ exchanger C-

terminals plays an important role in regulating the activity of the exchanger (Pang and others 2001). The mutations that enhanced the interaction of CHP with *SLC9A9* may influence the antiporter activity of the *SLC9A9*. In addition, expression abnormalities in *SLC9A9* can also produce changes in the antiporter activity. By regulating endosomal pH, *SLC9A9* modulates the functions of the endosome that are important in synaptic transmission. For example, recycling endosomes function as reservoirs for AMPA receptors (AMPA receptors), enabling their rapid insertion in the postsynaptic membrane and promoting dendritic spine formation during long term potentiation (LTP) (Park and others 2004),(Park and others 2006). Blocking the endosomal trafficking pathway prevents LTP. The dopamine transporter (DAT), a target for ADHD medications, is constitutively recycled through the endosome (Melikian and Buckley 1999). Impaired DAT internalization and recycling as a result of altered endosomal function could thus result in an excess of membrane-associated DAT. In turn, this could lead to hypodopaminergic synapses, one of the hypothesized neurochemical changes in ADHD (Brown and others 2010; Spencer and others 2005). In fact, we recently obtained evidence for increased DAT expression and activity in the nigrostriatal system of the WKY/NCrl rat as well as SHR/NCrl rat (Roessner and others), supporting the possibility that DAT regulation is abnormal in these models.

The implications of *SLC9A9* variants for neuropsychiatric conditions extend beyond ADHD. Variants of *SLC9A9* have been reported in patients with autism and epilepsy (Morrow and others 2008). These findings are notable for ADHD, given the known comorbidity and shared genetic and neuropathophysiological features between ADHD and autism (Corbett and Constantine 2006; Grady and others 2005; Kent and others 2008; Mulligan and others 2008; Nijmeijer and others; Reiersen and others 2007; Sinzig and Lehmkuhl 2007). Interestingly, epilepsy is also more frequent among ADHD patients compared with the general population (Hamoda and others 2009).

There are several limitations of the current study. First, the co-immunoprecipitation studies could not conclusively prove a direct binding between *SLC9A9* and CHP. However, based on previous data regarding the interactions between CHP and other Na⁺/H⁺ exchanger members, and the sequence homology and structure prediction (supplementary figure 1), it is highly likely that *SLC9A9* binds directly with CHP. Secondly, the sample sizes used in our expression studies are relatively small, particularly for the young WKY/NHsd and adult SHR/NCrl groups (represented by only 3 animals in each group). We only examined two different ages, and expression of *SLC9A9* and *SYP* clearly varies with age. It is worth noting that the expression in adult SHR rats may be influenced by hypertension developed later in life. Thus, hypertension could account for the SHR findings for PFC in adulthood. An increased sample size and more developmental time points, particularly the prenatal and early postnatal time points may reveal more significant findings. Finally, we could not draw conclusion that the abnormal expression or mutations of *SLC9A9* is the causal variant for the ADHD phenotype in the WKY/NCrl rats, because that there may be other genetic causes, and that the expression abnormalities are merely a compensatory response to other genetic defects. Future studies utilizing transgenic or knockout animals would be useful to clarify these issues.

In summary, we found deleterious mutations of *SLC9A9* in the inattentive WKY/NCrl rat and significantly dysregulated *SLC9A9* expression in their brains and in the brains of SHR rats (who do not carry the mutations). Considering the complex network of signaling molecules that *SLC9A9* may interact with and the importance of endosomal recycling in synaptic transmission, we hypothesize that this gene is critical for normal synaptic development and transmission.

In the context of prior human studies (cf. Introduction), our data raise the intriguing possibility that, for some cases of ADHD, upregulation of SLC9A9 could be one of pathophysiology that is potentially treatable with existing medications that inhibit sodium-hydrogen exchangers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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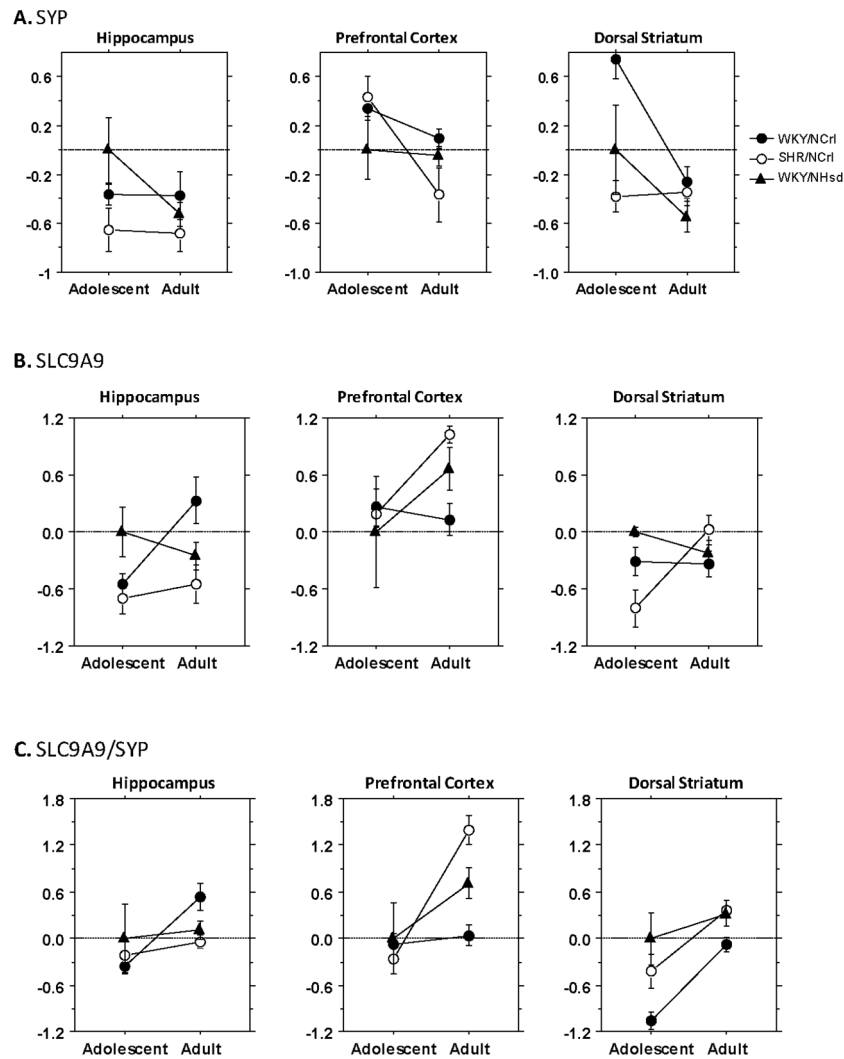


Figure 1. Developmental expression profile of *SYP* and *SLC9A9* in ADHD rat models

A. Relative levels of *SYP* expression (\log_2 difference) in each brain regions were calculated using $\Delta\Delta C_t$ methods with the adolescent WKY/NHsd rats as baseline level in each brain region. **B.** Relative levels of *SLC9A9* expression (\log_2 difference) in each brain region were calculated using $\Delta\Delta C_t$ methods with the adolescent WKY/NHsd rats as baseline level in each brain region. **C.** The \log_2 difference of *SLC9A9* vs synaptaphysin (*SLC9A9/SYP* ratio) in each brain region was calculated using $\Delta\Delta C_t$ methods.

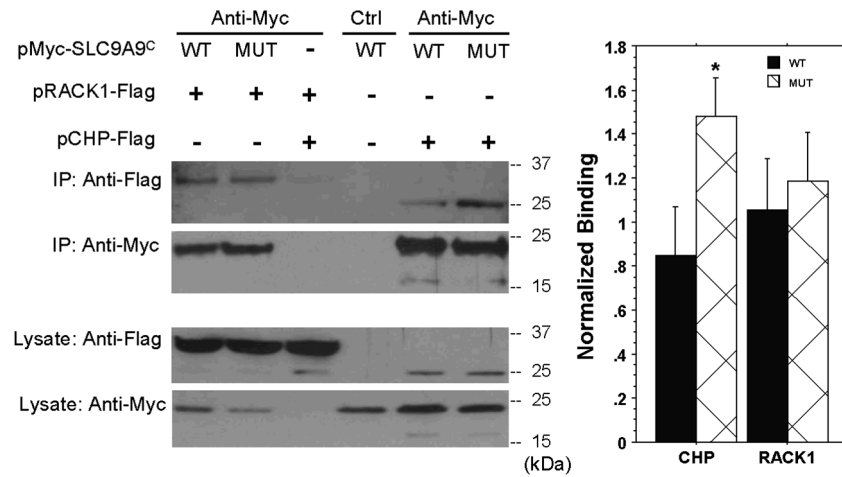


Figure 2.

Co-immunoprecipitation of *SLC9A9* C-terminal with RACK1 or CHP in HEK293 cells. C-Myc-tagged *SLC9A9* C-terminal (WT, wild type; MUT, with both mutations) and Flag-tagged binding partners (RACK1 and CHP) were co-expressed in HEK293 cells. The cell lysates were precipitated with either an anti-Myc antibody or equivalent mouse IgG as negative control (Ctrl). After extensive washes, the immunoprecipitate (IP) was eluted with SDS loading buffer and separated on SDS-PAGE gel. The same membrane was blotted with HRP conjugated anti-Flag and anti-Myc antibodies (the top two blots). Western blots of equal amount cell lysates were also done for both anti-Myc and anti-Flag antibodies to ensure the equal expression of both proteins (bottom two blots). The relative bindings of RACK1 or CHP to *SLC9A9* are normalized and quantified in Scion Image (see Methods for details, * $p=0.027$, two tailed t-test).