

Can Sodium/Hydrogen Exchange Inhibitors Be Repositioned for Treating Attention Deficit Hyperactivity Disorder? An in silico Approach

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Medications for attention deficit hyperactivity disorder (ADHD) are only partially effective. Ideally, new treatment targets would derive from a known pathophysiology. Such data are not available for ADHD. We combine evidence for new etiologic pathways with bioinformatics data to assess the possibility that existing drugs might be repositioning for treating ADHD. We use this approach to determine if prior data implicating the sodium/hydrogen exchanger 9 gene (*SLC9A9*) in ADHD implicate sodium/hydrogen exchange (NHE) inhibitors as potential treatments. We assessed the potential for repositioning by assessing the similarity of drug–protein binding profiles between NHE inhibitors and drugs known to treat ADHD using the Drug Repositioning and Adverse Reaction via Chemical–Protein Interactome server. NHE9 shows a high degree of amino acid similarity between NHE inhibitor sensitive NHEs in the region of the NHE inhibitor recognition site defined for NHE1. We found high correlations in drug–protein binding profiles among most ADHD drugs. The drug–protein binding profiles of some NHE inhibitors were highly correlated with ADHD drugs whereas the profiles for a control set of nonsteroidal anti-inflammatory drugs (NSAIDs) were not. Further experimental work should evaluate if NHE inhibitors are suitable for treating ADHD. © 2013 Wiley Periodicals, Inc.

Key words: sodium/hydrogen exchange; *SLC9A9*; NHE9; ADHD; drug repositioning; sodium–hydrogen inhibitors; genetics

INTRODUCTION

It has been the hope of genetic studies that discovering new biological pathways would provide new targets for treating attention deficit hyperactivity disorder (ADHD) [Faraone and Mick, 2010]. One new target is sodium/hydrogen exchanger isoform 9 (*SLC9A9*), which was first implicated in ADHD by a report of a family in which ADHD co-segregated with a pericentric inversion that disrupted *SLC9A9* [de Silva et al., 2003]. Significant ADHD-*SLC9A9* associations in humans were reported from candidate gene studies [Brookes et al., 2006; Markunas et al., 2010] and *SLC9A9* achieved one of the lowest *P*-values in an ADHD genome-wide association study (GWAS) ($\sim 10^{-5}$; [Lasky-Su et al., 2008]). In

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another GWAS, *SLC9A9* was nominally associated with nicotine dependence [Uhl et al., 2007], which is also associated with ADHD [Monuteaux et al., 2007; Biederman et al., 2009].

The spontaneously hypertensive (SHR) rat from Charles River, Germany (SHR/NCrl) is an animal model of ADHD [Sagvolden et al., 2009]. We described a new rat model for the inattentive subtype of ADHD (WKY/NCrl, from Charles River, Germany) [Sagvolden et al., 2008], which shows impaired sustained attention, but normal activity level and impulsiveness. Both rat models show increased dopamine transporter (DAT) activity [Roessner et al., 2010]. We subsequently found increased *SLC9A9* expression in hippocampus (WKY/NCrl rats) and prefrontal cortex (SHR/NCrl rats). We found two nonsynonymous mutations in *SLC9A9* for the inattentive WKY/NCrl rats located in a domain where regulatory proteins bind. This region has a similar amino acid structure as other members of the sodium–hydrogen exchange (NHE) protein family (we use the terminology NHE9 to refer to the protein coded by the *SLC9A9* gene and likewise for other family members). We showed that the mutations significantly changed NHE9's interaction with calcineurin homologous protein (CHP),

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but had no effect on its interaction with RACK1 [Zhang-James et al., 2011]. We also examined the expression and relationship of SLC9A9, CHP, and eight additional genes (RACK1, CaM, PPP3R1, PPP1R10, PKC μ , CaMKI, NR2B, and PLC β 1) in the hippocampus of the WKY/NCrl rat and SHR/NCrl models of ADHD. The expression levels of these genes were significantly correlated, suggesting that they may be coregulated [Zhang-James et al., 2012]. Principal component analysis identified two main factors that accounted for 94% of the expression variance of the 10 genes. Significant differences were found for both factors across the three different rat strains, suggesting that SLC9A9-mediated signaling pathways could contribute to the ADHD phenotype of the two rat models [Zhang-James et al., 2012].

Because very little is known about the structure and biology of NHE9, no studies have yet been conducted to determine if it is targeted by existing drugs. In contrast, a large literature describes molecules that inhibit NHE1–NHE7, with most of this work focused on NHE1–NHE5 [Karmazyn et al., 2003; Masereel et al., 2003]. NHE inhibitors (e.g., amiloride) are used as diuretics, anti-hypertensive agents, and for the prevention of cardiac or neural ischemia [Karmazyn et al., 2003; Masereel et al., 2003]. The potential for these drugs to inhibit NHE9 is unclear because studies of NHE inhibitors have focused on plasma membrane NHEs (NHE1–NHE5) and not organelle specific NHEs (NHE6–NHE9) and the two types of NHEs show some structural divergence in their amino acid sequences [Nakamura et al., 2005]. Although the overall amino acid similarity between the two classes of NHEs is low (23–27%), the membrane regions are more highly conserved, with about 50% amino acid identity between classes [Nakamura et al., 2005]. Of particular relevance to ADHD, clonidine is a modest inhibitor of plasma membrane NHEs [Karmazyn et al., 2003; Masereel et al., 2003], and is also a moderately efficacious drug for ADHD [Palumbo et al., 2008; Jain et al., 2011; Kollins et al., 2011].

In summary, five pieces of evidence suggest that medications which inhibit sodium–hydrogen exchange proteins could treat ADHD symptoms: (1) *SLC9A9* is associated with ADHD in humans; (2) *SLC9A9* mRNA expression is increased in ADHD-relevant brain regions in two rat models of ADHD; (3) one rat model of ADHD has mutations in *SLC9A9*; (4) the rat mutations are associated with greater than normal binding of CHP, which would be expected to increase the activity of NHE9, and (5) clonidine is a modest NHE inhibitor and a moderately effective ADHD medication.

The present work hypothesized substantial similarity of the amino acid sequence in the binding region for NHE inhibitors between NHE9 and NHEs known to be inhibited by NHE inhibitors. Such a finding would suggest that these drugs would inhibit NHE9. We further hypothesized that, if NHE9 inhibition is useful for the treatment of ADHD, it is possible that, in addition to clonidine, other ADHD medications might have a dual mechanism of effect much as clonidine inhibits sodium–hydrogen exchange and also agonizes the adrenergic α -2A receptor. To assess this possibility, we hypothesized that an *in silico* approach from drug repositioning methodology would find a correspondence between the drug–protein binding profile of ADHD medications and those of NHE inhibitors.

METHODS

To compare the amino acid sequences of NHE9 with other NHEs, we multiply aligned human NHE sequences using ClustalW and the BLOSUM62 alignment score matrix as implemented by UniProt, <http://www.uniprot.org/>. A cluster plot of NHE amino acid sequence similarity was created in Jalview 2.6.2. Although Nakamura et al. [2005] had previously presented the amino acid similarities of NHE proteins, we have focused on the NHE inhibitor recognition region which was shown experimentally to involve residues within the fourth and ninth membrane spanning helices (these are bounded by amino acids 150 and 400) Khadilkar et al. [2001].

To assess the correspondence between the drug–protein binding profile of ADHD medications and those of NHE inhibitors, we used the drug repositioning methodology of Luo et al. [2011] and Yang et al. [2009a,b]. This approach uses a collection of 392 structural models of targetable human proteins from UniProt and 582 active forms of drug molecules from DrugBank having known treatment indications. These targets were required to have known, targetable sites with binding pockets that were screened according several criteria to assure the presence of a suitable binding pocket (for details, see Yang et al. [2009a,b]). This database, known as the drug repositioning and adverse reaction via chemical–protein interactome (DRAR-CPI), is accessible at <http://cpi.bio-x.cn/drar/>.

To assess the similarity of our drugs of interest with the drug–protein binding profile of the drugs in DRAR-CPI, we submitted a structural model of the drug molecule to the DRAR-CPI server. The structure of each drug, in SMILES format, was retrieved from PubChem (<http://pubchem.ncbi.nlm.nih.gov/>). These were then input to CORINA (http://www.molecular-networks.com/online_demos/corina_demo) to generate minimal energy conformations. The CORINA files were translated into .ml2 files by VEGA ZZ. These structural models were hybridized *in silico* with each protein target in the DRAR-CPI database using DOCK [Ewing et al., 2001]. The ability of the molecule to dock with the target is quantified as a z-score such that z-scores less than -0.5 indicate that the molecule tends to bind with the protein.

We included five drugs approved by the US Food and Drug Administration for the treatment of ADHD (methylphenidate, amphetamine, guanfacine, atomoxetine, and clonidine), each having substantial data indicating efficacy for ADHD [Faraone and Buitelaar, 2010; Faraone and Glatt, 2010]. We examined six NHE inhibitors chosen due to prior knowledge about their pattern of selectivity for NHE subtypes [Masereel et al., 2003]: Amiloride and benzamil have limited NHE subtype selectivity; Cariporide selectively inhibits NHE1; S3226 selectively inhibits NHE3; Quinine selectively inhibits NHE7; Harmaline selectively inhibits NHE5. To control for the possibility that DRAR-CPI might produce spurious correlations, we also tested the association of ADHD drugs with five nonsteroidal anti-inflammatory drugs (NSAIDs), for which we had no prior reason to believe would be associated with ADHD drugs: naproxen, aspirin, ibuprofen, celecoxib, and rofecoxib. We refer to all of these drugs as our study drugs.

We used DRAR-CPI to compute z-scores indicating the degree to which each study drug is predicted to bind to the 389 protein targets in the DRAR-CPI database. Thus, for each of our study

drugs, we computed 389 z-scores. These z-scores are informative because two study drugs that bind proteins similarly (and would thus be hypothesized to have similar biological effects) should have similar z-scores. To assess the similarity of the profile of 389 z-scores among study drugs, we correlated the z-scores between each ADHD drug and (a) other ADHD drugs; (b) the NHE inhibitors and (c) the NSAIDs drugs used as controls. A positive correlation means that two study drugs share similar patterns of drug–protein binding across all of the targets in the DRAR-CPI database.

To visualize the similarity of drug–protein binding predictions among the study drugs, we used classical multidimensional scaling using Euclidean distance of their z-scores as the similarity metric, the stress criterion as the loss function and principal normalization as the normalization method. These analyses were accomplished with STATA [StataCorp, 2007].

We used E-Dragon, <http://146.107.217.178/lab/edragon/>, to compute molecular descriptors. A molecular descriptor refers to a quantitative feature of a molecule that can be used to compare it with other molecules. Because two drugs with a similar profile of molecular descriptors are likely to have similar structures, they are also likely to have similar biological effects. We used molecular descriptors from four categories: 48 constitutional descriptors, 261 three-dimensional descriptors, 121 topological descriptors, and 22 molecular property descriptors. To assess the similarity of drugs as regards these molecular descriptors, we used multidimensional scaling as described above. We used seemingly unrelated regression analyses to assess the association of drug subsets with drug–protein binding and with molecular descriptors. This regression method provides joint estimates from several regression models when the error terms of the models are correlated.

RESULTS

As Figure 1 shows, in the predicted transmembrane region, NHE9 is most similar to NHE6 and NHE7. There is one large cluster

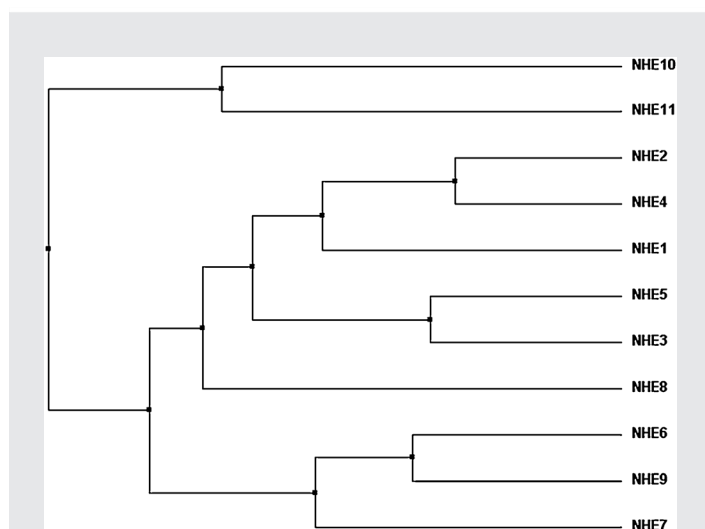


FIG. 1. Amino acid sequence similarities among sodium hydrogen exchanger proteins.

comprising NHE1–5 and NHE8 and a third cluster with NHE10 and NHE11. The percent of identical or similar positions between NHE9 and NHEs shown experimentally to be sensitive to NHE inhibitors are NHE1: 68%, NHE2: 72.4%; NHE3: 65.6%; NHE4: 72.8%, and NHE5: 64.4%.

Table I shows the results of our analyses correlating protein binding affinity z-scores between drugs. With the exception of clonidine, all ADHD drugs were highly and significantly correlated with other ADHD drugs. This suggests that the ADHD drugs have similar drug–protein binding features to one another, which would be consistent with them having similar biological activities. Thus, the DRAR-CPI drug–protein database recovers most of the expected associations among ADHD drugs.

For the NHE inhibitors amiloride, cariporide, S3226, and benzamil, drug–binding profiles were significantly positively correlated with methylphenidate, amphetamine, atomoxetine, and guanfacine. Benzamil and amiloride were also positively correlated with clonidine. Quinine showed modest negative association with two ADHD drugs. Harmaline was negatively correlated with amphetamine and positively correlated with clonidine. No NSAID drug was positively correlated with any ADHD drug. We did, however, observe several significant, albeit, modest negative correlations.

We used multidimensional scaling to visualize the similarity of drug–protein binding among the drugs in Tables I and II. Two dimensions accounted for 95.8% of the variance. The mean correlation between observed drug differences in drug–protein binding and the Euclidean distances of the two-dimensional solution was 0.97, indicating an excellent fit of the model. Figure 2 plots the two-dimensional solution. These dimensions summarize the similarity of drugs across the many drug–protein interactions recorded in DRAR-CPI. They do not correspond to specific protein or drug features. The lower right hand corner of this figure shows a cluster of drugs comprising all ADHD drugs (except clonidine) and four NHE inhibitors: amiloride, benzamil S2226, and cariporide. The small cluster on the lower left contains three NSAIDs. The upper-middle cluster contains two NSAIDs and two NHE inhibitors (harmaline and quinine). Clonidine did not cluster with other drugs.

Because the analyses of drug–protein interaction indicated that two of the NHE inhibitors (quinine and harmaline) and one ADHD drug (clonidine) did not associate with other ADHD drugs, we sought to determine if that could be accounted for by the properties of these molecules. A multidimensional scaling of the molecular descriptors of the ADHD and NHE inhibitor drugs, extracted three dimensions accounting for 86% of the variance. The mean correlation between observed drug differences in molecular descriptors and the Euclidean distances of the two-dimensional solution was 0.96, indicating an excellent fit of the model. The three-dimensional solution is given in Figure 3. These dimensions summarize the similarity of drugs across the many molecular descriptors derived from E-Dragon. They do not correspond to specific drug features.

To further understand clarify these differences among drugs, we used regression analysis to compare drug types on the molecular feature dimensions. These analyses used drug type as the predictor variable and the molecular feature dimensions as dependent

TABLE I. Significant Drug–Protein Binding Correlations Between ADHD Drugs and Other Drugs

	MPH	AMPH	ATX	GUAN	CLON
Attention deficit hyperactivity disorder drugs					
Methylphenidate	1.00	0.73	0.78	0.74	
Dextroamphetamine	0.73	1.00	0.75	0.84	
Atomoxetine	0.78	0.75	1.00	0.81	
Guanfacine	0.74	0.84	0.81	1.00	
Clonidine					
Sodium/hydrogen exchange inhibitors					
Cariporide	0.65	0.65	0.70	0.71	
Amiloride	0.70	0.86	0.79	0.88	0.21
S3226	0.61	0.60	0.72	0.72	
Benzamil	0.68	0.71	0.75	0.72	0.23
Quinine		−0.31		−0.21	
Harmaline		−0.21			0.55
Nonsteroidal anti-inflammatory drugs					
Naproxen	−0.59	−0.49	−0.56	−0.50	
Aspirin	−0.53	−0.37	−0.53	−0.45	
Ibuprofen	−0.56	−0.44	−0.56	−0.53	
Celecoxib					
Rofecoxib		−0.27		−0.20	

MPH, methylphenidate; AMPH, amphetamine; ATX, atomoxetine; GUAN, guanfacine; CLON, clonidine; only correlations significant at the Bonferroni corrected level of $P < 0.0004$ are presented.

variables. They showed that clonidine, quinine, and harmaline significantly differed from the other drugs on the third molecular features dimension ($z = 3.4$, $P = 0.001$) but not the first two molecular feature dimensions (P 's > 0.5). We also used regression analyses to assess the association between the drug–protein binding dimensions of Figure 2 and the molecular descriptor dimensions of Figure 3. Regression analyses also found that drug–protein binding dimension one was significantly associated with molecular descriptor dimension three ($z = 2.98$, $P = 0.003$). Drug–protein binding dimension two was associated with molecular descriptor dimensions one ($z = 3.46$, $P = 0.001$) and three ($z = 2.68$, $P = 0.007$). From these results, separation of harmaline, quinine, and clonidine from the other drugs are best highlighted by plotting drug–protein dimension 1 against molecular descriptor dimension 3 (Fig. 4). As can be seen in the lower left quadrant of Figure 4, harmaline, quinine and clonidine cluster away from the other drugs.

DISCUSSION

Our results provide two sources of information that support the hypothesis that NHE inhibitors may act on NHE9 and may have anti-ADHD efficacy. First, NHE9 shows a high degree of amino acid similarity between NHE inhibitor sensitive NHEs in the region of the NHE inhibitor recognition site that has been defined for NHE1. Second, we found highly significant, positive associations among drugs approved by the US Food and Drug Administration for the treatment of ADHD and drugs known to inhibit sodium hydrogen exchange. We did not find positive associations between ADHD drugs and the control NSAID drugs.

The high correlations among ADHD drugs validate the idea that the DRAR-CPI database can recover meaningful associations among drugs based on their predicted binding affinities with many different proteins. This result is particularly impressive given that the database did not include the primary targets of these

TABLE II. Pearson Correlations Between Drug–Protein Binding and Molecular Description Dimensions

	Drug–protein binding dimensions	
	1	2
Molecular descriptor dimension 1	0.196 [0.56]	−0.625 [0.04]
Molecular descriptor dimension 2	0.048 [0.89]	−0.126 [0.71]
Molecular descriptor dimension 3	0.654 [0.03]	−0.484 [0.13]

Drug–protein binding and molecular description dimensions derived from multidimensional scaling. P -values are in parentheses. See text for details.

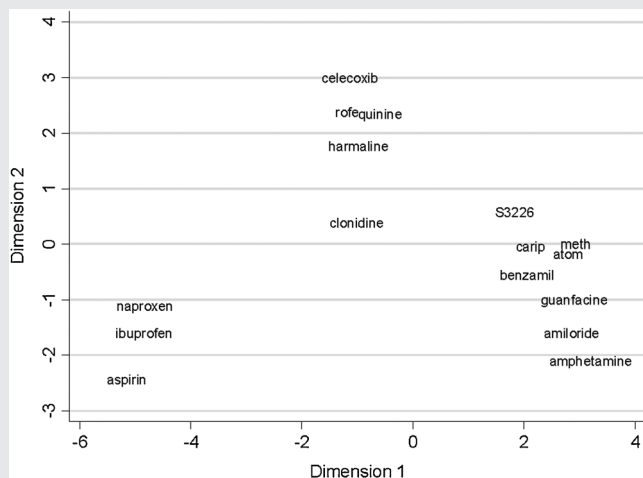


FIG. 2. Multidimensional scaling of drug-protein binding similarity among drugs.

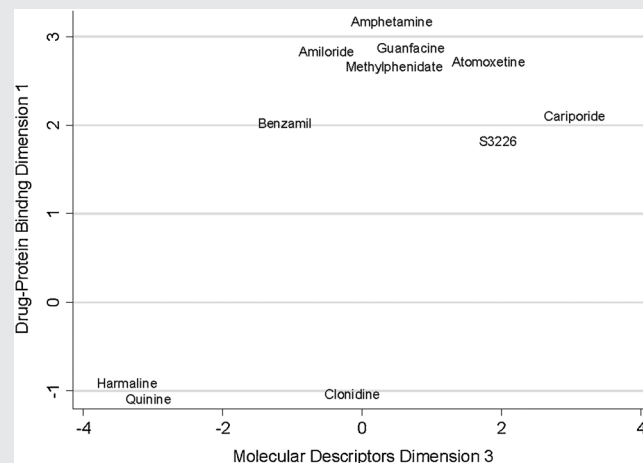


FIG. 4. Association between drug-protein binding and molecular descriptors.

medications, which are believed to mediate their therapeutic effects on ADHD (the dopamine transporter for methylphenidate and amphetamine; the norepinephrine transporter for atomoxetine and the adrenergic, alpha-2A, receptor for guanfacine and clonidine). The correlational data for guanfacine are particularly intriguing. Its putative mechanism of action is via the alpha-2A receptor, yet it did not correlate with clonidine, which is believed to have a similar mechanism of action. In contrast, it correlated highly with the stimulants and atomoxetine, the primary targets of which are the dopamine and norepinephrine transporters, respectively. This could indicate that there are additional mechanisms of action shared among this latter set of drugs that have been captured by

DRAR-CPI. Determining if such mechanisms exist and if they are relevant to therapeutic efficacy will require additional research.

We found many significant, positive correlations between ADHD drugs and NHE inhibitors. In contrast, we found no significant positive correlations between the NSAID drugs and the ADHD drugs. This latter finding provides some reassurance that our methodology was not biased to find positive correlations with ADHD drugs.

We could not determine why some NHE inhibitors cluster with ADHD drugs and others do not. The pattern we observed cannot be explained by the NHE inhibition selectivity of these compounds. The pattern of amino acid similarity among NHE proteins (Fig. 1) shows that NHE7 is more similar to NHE9 than are the other NHE proteins. Despite this similarity, the drug-protein binding profile of quinine (which selectively inhibits NHE7) was not positively correlated with any of the ADHD drugs. Comparing our findings with experimental NHE inhibition results reported by Masereel et al. [2003], the NHE inhibitors that belong to the ADHD drug cluster in Figure 2 do not show a specific pattern of NHE inhibition. Amiloride and benzamil are nonspecific inhibitors, cariporide is a selective NHE1 inhibitor and S3226 selectively inhibits NHE3. We also found no positive correlations for harmaline, which selectively inhibits NHE5.

Our analysis of molecular descriptors is limited by the small sample size, which makes it difficult to show statistical significance. Nonetheless, these results offer additional validation for the idea that the use of drug-protein binding profiles to cluster drugs is sensitive to variability in molecular features. It also provides some insights into why some NHE inhibitors are associated with ADHD drugs and others are not.

Although promising, our results are not entirely supportive of our hypothesis. Notably, clonidine, is approved for the treatment of ADHD and is a modest NHE inhibitor. However, it did not cluster with the other ADHD drugs in our analysis of drug-protein binding. This finding tempers our conclusion that NHE inhibitors

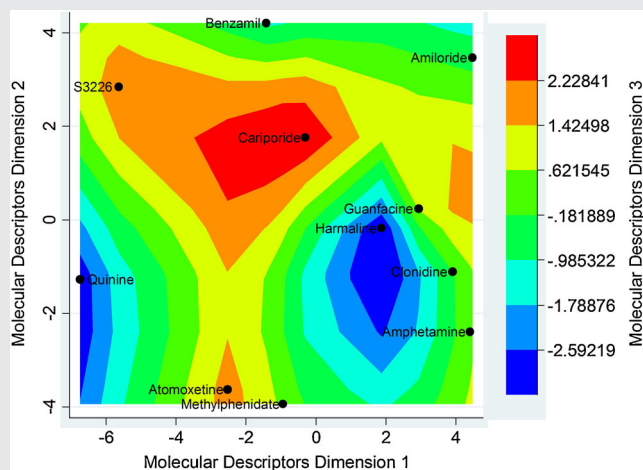


FIG. 3. Multidimensional scaling of molecular descriptor similarity among drugs.

might inhibit NHE9. Another obstacle to NHE9 inhibition is that, unlike NHEs that are currently targeted by these drugs, NHE9 is an intracellular protein, which could complicate drug delivery.

Our results suggest that further experimental work should be done to determine if the NHE inhibitors that cluster with ADHD drugs might be suitable for treating ADHD. If we are successful, the potential impact of this project is broad because (a) ADHD is a common disorder affecting 6% of children [Faraone et al., 2003]; (b) in the majority of cases, the disorder persists into adulthood [Biederman and Faraone, 2005; Faraone et al., 2006]; and (c) the disorder is associated with serious impairments including traffic accidents [Biederman and Faraone, 2005], increased health care utilization [Biederman and Faraone, 2005], substance abuse [Biederman and Faraone, 2005], reading difficulties [Cheung et al., 2012], neuropsychological dysfunction [Bloemsma et al., 2012], brain abnormalities [Paloyelis et al., 2012], unemployment [Biederman and Faraone, 2005], emotional dysregulation [Banaschewski et al., 2012], and divorce [Biederman and Faraone, 2005]. The economic impact of ADHD is between \$77.5 and \$115.9 billion each year [Biederman and Faraone, 2006]. Although medications for ADHD are effective in controlling symptoms for many patients, they do not “cure” the disorder. As we have shown in our longitudinal study of ADHD children [Biederman et al., 1996], even those receiving treatment are at risk for adverse outcomes.

Future work includes screening NHE inhibitors that perform well in silico with in vitro and in vivo assays of NHE9 inhibition. That could be followed by testing in the well-established SHR rat model of ADHD [Sagvolden et al., 2009] as well as human trials for drugs that are currently approved for such use by pertinent regulatory agencies.

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