

Nova2 Interacts with a *Cis*-Acting Polymorphism to Influence the Proportions of Drug-Responsive Splice Variants of *SCN1A*

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An intronic polymorphism in the *SCN1A* gene, which encodes a neuronal sodium-channel α subunit, has been previously associated with the dosing of two commonly used antiepileptic drugs that elicit their pharmacologic action primarily at this ion-channel subunit. This study sought to characterize the functional effects of this polymorphism on alternative splicing of *SCN1A* and to explore the potential for modulating the drug response in the pharmacologically unfavorable genotype by identification of a splice modifier acting on *SCN1A*. The effects of the genotype at the *SCN1A* IVS5N+5 G→A polymorphism on *SCN1A* splice-variant proportions and the consequences of increased expression of splice modifiers were investigated both in human temporal neocortex tissue and in a cellular minigene expression system. Quantitative real-time polymerase chain reaction was used to quantify the amounts of *SCN1A* transcripts forms. We show that the polymorphism has a dramatic effect on the proportions of neonate and adult alternative transcripts of *SCN1A* in adult brain tissue and that the effect of the polymorphism also appears to be modified by Nova2 expression levels. A minigene expression system confirms both the effect of the polymorphism on transcript proportions and the role of Nova2 in the regulation of splicing, with higher Nova2 expression increasing the proportion of the neonate form. A larger Nova2-mediated effect was detected in the AA genotype that is associated with increased dose requirements. The effects of Nova2 on modulation of the alternative splicing of 17 other neuronally expressed genes were investigated, and no effect was observed. These findings emphasize the emerging role of genetic polymorphisms in modulation of drug effect and illustrate both alternative splicing as a potential therapeutic target and the importance of considering the activity of compounds at alternative splice forms of drug targets in screening programs.

The study of pharmacogenetics has been confined predominantly to the elucidation of genetic variation in processes that control drug exposure, including drug-metabolizing enzymes and transporters. However, there is a growing consensus that genetic variability in drug absorption, distribution, metabolism, and elimination plays only a modest role in variability in treatment response and that variation related to the mode of action of a drug may often be as or more important.

Such genes, however, have not been thoroughly studied, and, to date, there remain relatively few examples of polymorphisms in a pharmacologic target or downstream pathway that contribute to variable drug response in humans. Perhaps the best documented example is the *VKORC1* gene (MIM 608547), which encodes a subunit of the vitamin K epoxide reductase complex and the pharmacological target for the commonly used anticoagulant warfarin. A common polymorphism in this gene has been significantly associated with reduced warfarin dose.¹ Although not yet definitive, it now appears that the molecular mechanism of this variation in warfarin response is regulated at the transcriptional level.²

Another example of a common polymorphism that in-

fluences drug response is one in *SCN1A* (MIM 182389), the gene encoding the target of certain antiepileptic drugs. The *SCN1A* IVS5N+5 G→A polymorphism (*rs3812718*, formerly *SCN1A* IVS4-91G→A) was shown to be significantly associated with maximum dose of both phenytoin and carbamazepine in a cohort of patients with various forms of epilepsy.³ Dosing in epilepsy, as well as in many other therapeutic areas, often involves long periods of trial and error and could benefit from genetic predictors of dosing requirements. In that study, Tate et al.³ found antiepileptic drug dose to be lowest in patients with the GG genotype, intermediate in those with the GA genotype, and highest in those with the AA genotype. In a separate study by the same authors that involved patients of Chinese ancestry, an association was found between *SCN1A* IVS5N+5 G→A and phenytoin serum concentrations at maintenance dose, again in the direction AA→GA→GG for highest→lowest dose.⁴ The phenotype of serum concentration at maintenance dose was chosen, to eliminate the variation between drug doses and serum levels (which are likely to be caused by pharmacokinetic factors) and therefore increase the chance of detecting the effect of pharmacodynamic variation in the drug target.

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Interestingly, *SCN1A* IVSSN+5 G→A is located in the consensus-site sequence located after the neonatal form of exon 5 (exon 5N) of the *SCN1A* gene (fig. 1).³ Collectively, this G→A change, which notably is a frequent mutation associated with mammalian disease,⁶ results in the disruption of the conserved consensus-site sequence, which would theoretically result in a possibly weaker 5' splice site.⁷ Consistent with this theory, preliminary data reported by Tate et al.³ suggest that the *SCN1A* IVSSN+5 G→A polymorphism may control the proportion of the two transcript forms in adults. This exon 5 alternative splicing event is conserved across all neuronally expressed sodium channels.^{8,9} Although not well studied for *SCN1A*, there is extensive evidence of the developmental regulation of this splicing event at other neuronally expressed sodium channels.^{8,9} The sequence conservation across neuronally expressed sodium channels suggests that this also may occur for *SCN1A*, as indicated by semiquantitative assays reported by Tate et al.³ The molecular control of developmentally regulated sodium-channel alternative splicing is unknown.

Recently, several neuronally expressed splice-modifier proteins have been identified, including neuro-oncological ventral antigen 1 and 2 (Nova1 and Nova2, respectively) and sodium channel modifier 1 (Scnm1).^{10–12} It is possible that these neuronally expressed splice-modifier proteins may contribute to the alternative splicing of exon 5 of *SCN1A*. Importantly, the genomic region in and around exon 5 contains several putative Nova-binding sites (fig. 1), suggesting a possible role of Nova proteins in regulation of transcript splicing.⁵

Given the association of this *SCN1A* polymorphism with altered doses of antiepileptic drugs, it is possible that drug doses found clinically may be influenced by the proportion of *SCN1A* transcripts in the neonatal form. Elucidating the genetic and molecular process that regulates this event not only may help in the prediction of how patients may respond to particular antiepileptic drugs but also may reveal a means for driving the splice-variant transcript proportion to the drug-responsive state in patients with the unfavorable (AA) genotype. Therefore, this study sought to quantify the effect of *SCN1A* IVSSN+5 G→A genotype on exon 5 splice-variant proportions and to determine the effects of neuronally expressed splice-modifier proteins on the controlling of this splice event.

Material and Methods

Subjects

This study was approved by the Joint Research Ethics Committee of the Institute of Neurology and the National Hospital for Neurology and Neurosurgery and by the Duke University Institutional Review Board. All patients gave written informed consent for use of resected brain tissue for research. Forty-three temporal neocortical tissue samples were obtained from material resected from patients who underwent therapeutic surgery for drug-resistant mesial temporal lobe epilepsy, in accordance with routine clinical protocols. All tissue used for research was surplus to diagnostic

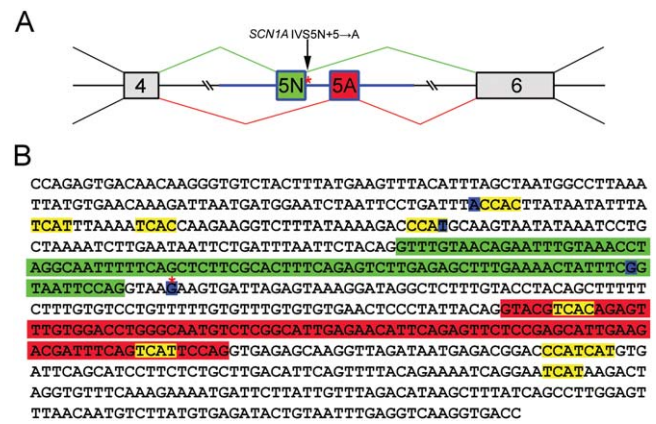


Figure 1. Schematic of exons 4–6 of the *SCN1A* gene. *A*, The adult (5A [red]) and neonatal (5N [green]) mRNA transcript forms of exon 5. The location of the *SCN1A* IVSSN+5 G→A polymorphism (*rs3812718*) is indicated by the red star located in the 5' splice-donor site after exon 5N. The blue line stretching from intron 4 to intron 5 indicates the gene region covered by the minigene construct. *B*, Sequence of the minigene construct with exon 5N (highlighted in green) and exon 5A (highlighted in red). The nine putative Nova-binding sites ([T/C]CA[T/C])⁵ are highlighted in yellow. Highlighted in blue are SNPs that are located in the minigene construct (in order of appearance in the sequence: *rs2195144* [A→G], *rs2217199* [T→C], *rs3812719* [G→T], and *rs3812718* [G→A]).

requirements. Subjects were aged 18–60 years, and the group consisted of 15 men and 28 women. The population of patients with mesial temporal lobe epilepsy was the same as in the 2005 study by Tate et al.,³ although random subjects were selected for both studies (thus it is possible that there may be an overlap of subjects in this study and subjects in the study by Tate et al.). All subjects had received a diagnosis of hippocampal sclerosis that was confirmed histologically. The hippocampal tissue was excluded from analysis because of potential confounding issues of neuronal loss. All tissue was flash frozen in liquid nitrogen and was stored at –80°C until use.

Twenty-nine nonepileptic control temporal cortex tissue samples were obtained through the Kathleen Price Bryan Alzheimer's Disease Brain Bank at Duke University. All patients gave written informed consent for use of the brain tissue for research. The control tissue was collected postmortem (time to collection ranged from 1 to 30 h postmortem, with controlling for agonal state). Subjects were aged 56–90 years, and the group consisted of 14 men and 15 women. Ethical permission for the use of this tissue was provided by Duke University Medical Center Institutional Review Board.

DNA Extraction and Genotyping

DNA was extracted using the Trizol method (Invitrogen), per the manufacturer's instructions. DNA quantity and quality were assessed spectrophotometrically.

Samples were genotyped for the *SCN1A* IVSSN+5 G→A polymorphism (*rs3812718*) by use of a custom designed TaqMan-based allelic discrimination assay (Applied Biosystems). Assay primer and fluorescently labeled probe sequences were as follows

(all are 5'→3'): forward primer, TTTCAGAGTCTTGAGAGCTTTGAAA; reverse primer, TGTGACGTACCTGTAATAGGGAGTTC; G probe, FAM-AATTCCAGGTAAGAAAGTGA-MGB; and A probe, VIC-AATTCCAGGTAAGAAAGTGA-MGB. Assay conditions were in accordance with manufacturers' standard protocols. Fluorescence outputs were quantified in real time by use of a 7900HT Fast Real Time PCR System, and the data were analyzed using SDS software, version 2.2.2 (Applied Biosystems).

RNA Extraction and cDNA Generation

Total RNA was extracted from brain tissue by use of the RNeasy Lipid Tissue Purification Kit (Qiagen), in accordance with the manufacturer's instructions. RNA from cells was extracted using the RNeasy Purification Kit (Qiagen). The resulting RNA was quantified spectrophotometrically, and 1 µg of total RNA was reverse transcribed into cDNA by use of a High Capacity cDNA Synthesis Kit (Applied Biosystems), in accordance with product instructions.

Plasmid and Minigene Constructs

An Exon Trapping system (Gibco) was used to assess the genetic and molecular control of the alternative splicing of exon 5N and 5A in *SCN1A*. A genomic DNA fragment of 696 bp containing exon 5N and 5A of *SCN1A* was PCR amplified using a primer set—forward primer, GATCGAATTCCCAGAGTGACAACAAGGGTGT, and reverse primer, GATCGGATCCGGTCACCTTGACCTCAAAT-TACA—and was subcloned into a pSPL3 vector. The A and G forms of the minigene were obtained by separate amplification of homozygous individuals. *SCNm1* (MIM 608095), *NOVA1* (MIM 602157), and *NOVA2* (MIM 601991) cDNA with a full-length ORF was PCR amplified with cDNAs from neuroblastoma cells, SK-N-BE(2), by use of primer sets (all are 5'→3'): *SCNm1* forward primer, GATCAGATCTATGTCTTTCAAGAGGGAAGGA; *SCNm1* reverse primer, GATCGAATTCTCAGTCCAAGGGGAGATCAGG; *NOVA1* forward primer, ATTACTCGAGAACATGATGGCGGCAGCTCC; *NOVA1* reverse primer, ATTAGGTACCTCAACCCACTTCTGAG-GATT; *NOVA2* forward primer, TATTACTCGAGATGGAGCCCGA-GGCCCGGAT; and *NOVA2* reverse primer, ATTAGGTACCCAG-GCCTCATCCCACCTTCT. Total RNA was extracted from the SK-N-BE(2) cells and was converted to cDNA by use of a High-Capacity cDNA Archive Kit (Applied Biosystems) for *NOVA2* cloning. PCR was performed with a high-fidelity DNA polymerase, Platinum Pfx (Invitrogen). The PCR product was subcloned into pcDNA3.1 (Invitrogen), with an N-terminal hemagglutinin (HA) tag. All the sequences were verified by DNA sequencing.

To confirm the causal polymorphism of the observed effects in the minigene system, the *SCN1A* IVSSN+5 G→A polymorphism (*rs3812718*) was introduced in the G construct by use of a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene).

Cell Transfection

HEK293 cells were grown to 80% confluence in 6-well plates. The cells were transfected with 0.1 µg of *SCN1A* minigene (A, G, or equal quantities of A and G) and varying amounts of *NOVA2* plasmids by use of Lipofectamine 2000 (Invitrogen), in accordance with the manufacturer's guide. After 48 h, cells were harvested for RNA extraction and western-blot analyses. The same experimental design was employed for transfection of *SCNm1* and *NOVA1* plasmids; however, this was limited to a single transfection of 1 µg.

Real-Time PCR

TaqMan-based real-time PCR was used to determine the percentage of *SCN1A* transcripts containing exon 5N and 5A. Assays were custom designed through Applied Biosystems, and primers or probe sequences were used in these assays as follows (all are 5'→3'). For *SCN1A* 5A transcript quantification in human brain tissue, forward primer AACTGGCTCGATTTCAGTGCATTA, reverse primer ATTGCCAGGTCCACAACT, and probe FAM-CTGTGACGTACGCAAATG-NFQ were used. For *SCN1A* 5N transcript quantification, forward primer TGGCTCGATTTCAGTGC-ATTACAT, reverse primer TCTGAAAGTGCGAAGAGCTGAAAA, and probe FAM-CCTAGGTTTACAAATTCTG-NFQ were used. For *SCN1A* 5A transcript quantification in HEK293 cells, forward primer GTGAACTGCACTGTGACAAGCTGC, reverse primer CACCTGAGGAGTGAATTGGTTCG, and probe FAM-CCGAGCAT-TGAAGACGA-NFQ were used. For *SCN1A* 5N transcript quantification, forward primer GTGAACTGCACTGTGACAAGCTGC, reverse primer TCTGAAAGTGCGAAGAGCTGAAAA, and probe FAM-CCTAGGTTTACAAATTCTG-NFQ were used. *SCNm1*, *NOVA1*, *NOVA2*, and β-actin mRNA expression levels were quantified using commercially available TaqMan assays (Applied Biosystems). Fluorescence outputs were quantified in real time by use of a 7900HT Fast Real Time PCR System, and the data were analyzed using SDS software, version 2.2.2 (Applied Biosystems). In subjects with the AA genotype, the output data often were below the limit of detection for the 5N assay. In these cases, the percentage of *SCN1A* transcripts containing exon 5N was considered to be zero.

The effect of *Nova2* on alternative splicing events in 17 ion-channel genes (splicing events are defined in table 1) was investigated in cells transfected with 0.1 µg of *SCN1A* minigene with and without 1 µg *NOVA2* cDNA transfection (the same preparation was used for investigation of the effects of *Nova2* on *SCN1A* exon 5 splicing). TaqMan-based real-time PCR assays were used to quantify splice-variant ratios (variant:common transcript

Table 1. Alternative Splicing Events in Ion-Channel Genes That Were Screened for *Nova2*-Mediated Regulation

Gene	GenBank Accession Number for Splice Form	
	Common	Variant
<i>CACNA1A</i>	NM_023035	BE972738
<i>CACNA1B</i>	NM_000718	M94173
<i>CACNA1G</i>	NM_018896	NM_198376
<i>CACNB1</i>	NM_000723	NM_199247
<i>CACNB4</i>	NM_000726	AY054985
<i>CLCN7</i>	NM_001287	AK096963
<i>FXND6</i>	NM_022003	BP372334
<i>GRIA1</i>	NM_000827	A46050
<i>GRIA3</i>	NM_007325	NM_000828
<i>HTR3A</i>	NM_000869	BG341613
<i>KCNJ15</i>	NM_170736	CQ732921
<i>KCNK1</i>	NM_002245	AV733795
<i>KCNN1</i>	NM_002248	BM718136
<i>KCNN2</i>	NM_021614	BG769522
<i>KCNQ2</i>	NM_004518	AY358189
<i>MCOLN1</i>	NM_020533	CA489568
<i>SCN1A</i>	NM_006920	CQ727958

forms, as defined in table 1) by targeting unique exon-exon boundaries.

Western-Blot Analysis

Protein was extracted with 2% SDS from cell pellet. Protein was separated on an NuPAGE 4%–12% Bis-Tris gel (Invitrogen). The gel was blotted to nitrocellulose membrane (BioRad). The membrane was blocked with 5% skim milk. Then, the membrane was incubated with rabbit anti-HA antibody (1:500 [Abcam]) overnight at 4°C and subsequently was incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:5,000 [Amersham]) for 1 h at room temperature. The signal was detected by Amersham ECL kit. Rabbit anti-β-actin antibody (1:2,000 [Abcam]) was used for a loading control. ImageJ software was used to perform densitometric analysis.

Data Analysis

One-way analysis of variance (ANOVA) was used to determine the statistical significance of the *SCN1A* exon 5N splice variant compared with *SCN1A* IVS5N+5 G→A genotype correlation in human brain tissue. Statistical analyses were performed separately in control and mesial temporal lobe epilepsy samples, in addition to a combined subject analysis. Tukey post hoc tests were used to determine the statistically significant differences between splicing proportions in the three genotype groups.

The effects of *NOVA2* mRNA expression (*NOVA2*) on the percentage of *SCN1A* mRNA transcripts containing exon 5N (*SCN1A* 5N%) were fit to a sigmoidal E_{max} model defined as follows:

$$SCN1A\ 5N\% = 5N\%_0 + \frac{E_{max} \times E(NOVA2^{\gamma}_{50})}{E(NOVA2^{\gamma}_{50}) + NOVA2^{\gamma}}, \quad (1)$$

where E_{max} is the maximum *NOVA2*-mediated change in *SCN1A* 5N%, $E(NOVA2^{\gamma}_{50})$ is amount of *NOVA2* needed to elicit one-half the maximum effect on *SCN1A* 5N%, γ is the shape factor governing the relationship between *SCN1A* 5N% and *NOVA2* mRNA expression, and $5N\%_0$ is the percentage of transcripts containing exon 5N in the presence of no or very low quantities of *NOVA2*.

Linear regression was used to assess the effects of splice-modifier protein mRNA expression on the proportion of *SCN1A* transcripts for all subjects and for the individual genotypes. Student's *t* test was used to compare splice-variant ratios of ion-channel genes in HEK293 cells transfected with *SCN1A* minigene and in the presence and absence of 1-μg *NOVA2* cDNA transfection ($n = 3$ independent replications per group).

Results

In human brain tissue, the *SCN1A* IVS5N+5 G→A polymorphism had a substantial effect on the percentage of transcripts containing exon 5N (neonatal form) of *SCN1A* (fig. 2A). Individuals with the AA genotype had a mean (\pm SEM) of $0.7\% \pm 1\%$ of *SCN1A* transcripts in the neonatal form, whereas subjects with the GG genotype had $41\% \pm 9\%$ of transcripts containing exon 5N. The G allele elicited a dominant effect, with those with the AG genotype having $28\% \pm 4\%$ of transcripts in the neonatal form. The presence or absence of a history of temporal lobe epilepsy did not affect the correlation between ge-

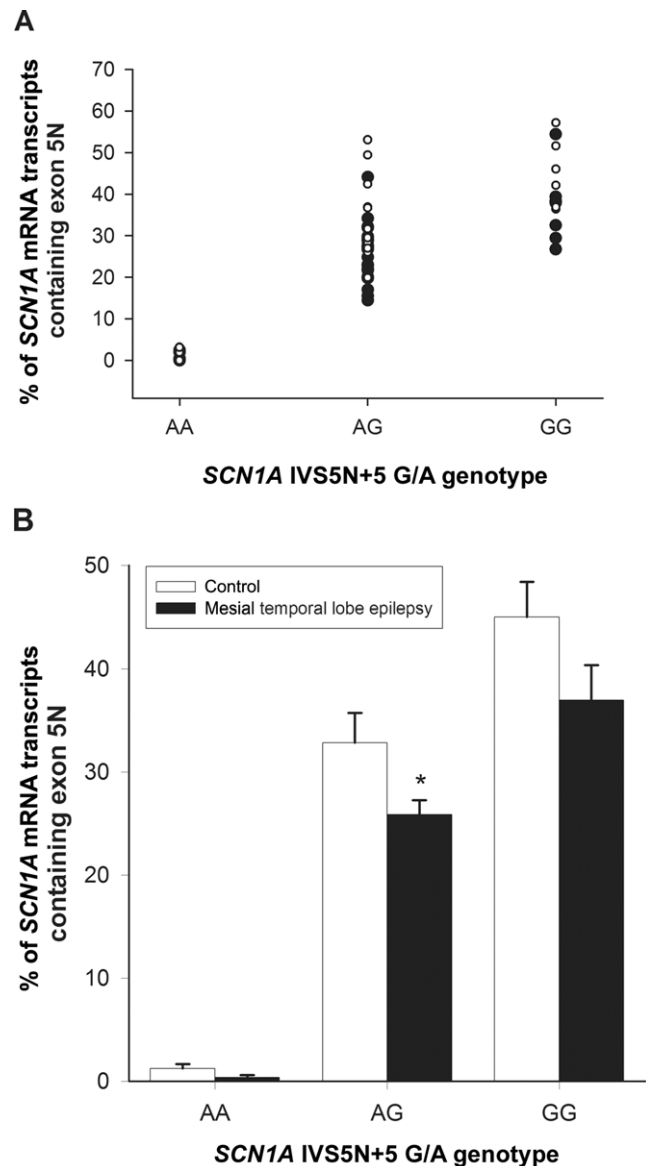


Figure 2. Evidence of the genetic control of *SCN1A* exon 5 alternative splicing in human brain tissue. *A*, Correlation of the *SCN1A* IVS5N+5 G→A genotype with the percentage of transcripts containing neonatal exon 5N in the temporal neocortex of control tissue (unblackened circles) and mesial temporal lobe epileptic brain tissue (blackened circles). $P < .001$ by ANOVA for mesial temporal lobe epilepsy and control samples combined, and $P < .001$ by ANOVA for separate analyses of mesial temporal lobe epilepsy and control samples. By Tukey post hoc statistical analysis, $P < .001$ for AA versus AG, $P < .001$ for AG versus GG, and $P < .001$ for AA versus GG. *B*, Difference in the percentage of transcripts containing neonatal exon 5N in the temporal neocortex of control samples and mesial temporal lobe epileptic brain tissue. Data are presented as mean \pm SEM. $P < .05$ by Student's *t* test for comparison with control.

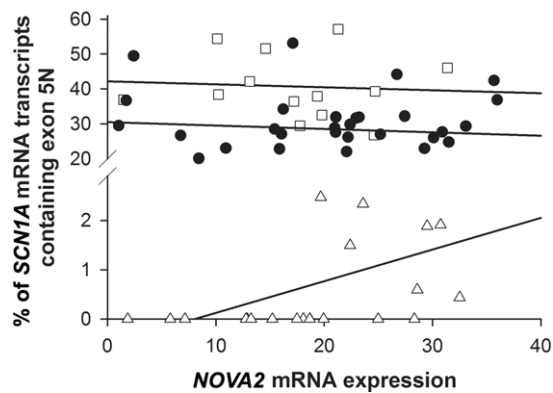


Figure 3. Percentage of *SCN1A* mRNA transcripts containing exon 5N graphed versus *NOVA2* mRNA expression (normalized to β -actin expression) for controls and subjects with mesial temporal lobe epilepsy with the AA (triangles), AG (circles), and GG (squares) genotypes at the *SCN1A* IVS5N+5 G→A polymorphism. *NOVA2* mRNA expression correlates with the extent of *SCN1A* exon 5 splicing for the AA genotype in human brain tissue. Lines indicate the fit of a linear regression model to the data for each genotype subgroup.

genotype and the alternative splicing event. However, the magnitude of the proportion of *SCN1A* neonatal transcripts was slightly altered in the disease state, with a trend toward a lower percentage of transcripts containing exon 5N in patients with mesial temporal lobe epilepsy (fig. 2B).

We also investigated a possible role for neuronally expressed splice-modifier proteins—Nova1, Nova2, and Scnm1—in controlling the *SCN1A* exon 5 splicing event, by assessing the correlation between the percentage of transcripts containing exon 5N and the mRNA expression within individual genotypes. We found no effect of Nova1 and Scnm1 on modulation of the *SCN1A* exon 5 splicing event, collectively or for individual genotypes. Although no *NOVA2*-mediated modulation of *SCN1A* exon 5 splicing was detected when all subjects were evaluated, a statistically significant correlation (uncorrected for multiple testing) was observed in human brain tissue between *NOVA2* mRNA expression and the percentage of *SCN1A* transcripts containing exon 5N for the AA genotype (fig. 3). *NOVA2* mRNA expression was equivalent in both mesial temporal lobe epilepsy and control brain tissue, and a similar correlation was observed in both experimental groups (data not shown).

We used a minigene expression system to confirm a causal relationship between these correlations observed in human adult brain tissue. First, we tested the role of the *SCN1A* IVS5N+5 G→A polymorphism. In this cellular system, the experimentally simulated GG genotype resulted in 51% of transcripts containing exon 5N, whereas the percentage was significantly reduced for the AA genotype, to 7% (fig. 4). The AG genotype resulted in 40% of transcripts being in the neonatal form (fig. 4); however, we

note that we did not quantify the true copy numbers of the A and G alleles in this study.

Because of the presence of other SNPs in the minigene construct (fig. 1), we also performed mutagenesis studies to confirm that the *SCN1A* IVS5N+5 G→A polymorphism was eliciting the observed splicing changes and that the alternations were not due, in whole or in part, to other polymorphisms in the minigene construct. Direct sequencing of the G minigene construct revealed the reference sequence indicated in figure 1. The A construct had the alternate sequence at all the SNP locations (*rs2195144* [A→G], *rs2217199* [T→C], *rs3812719* [G→T], and *rs3812718* [G→A]). To confirm that the *SCN1A* IVS5N+5 G→A polymorphism is the causal SNP in the minigene system, we mutated the *SCN1A* IVS5N+5 G→A polymorphism in the G construct to the A form (at the IVS5N+5 site) and tested the effects of this change on the percentage of transcripts containing exon 5N. This experiment revealed no statistical difference in transcripts containing exon 5N between the original AA minigene system and the GG-mutated system (data not shown). These findings confirm that the *SCN1A* IVS5N+5 G→A polymorphism is, in fact, the casual variant in the modulation of the alterations in *SCN1A* exon 5 splicing.

Next, we tested how overexpression of the common neuronally expressed splice-modifier proteins Scnm1, Nova1, and Nova2 influences *SCN1A* splicing. Overexpression of neither Scnm1 nor Nova1 showed any effects on *SCN1A* exon 5 alternative splicing in the minigene cellular system investigated here (data not shown). However, increasing Nova2 decreased the quantity of *SCN1A* transcripts containing the adult form of exon 5 while it simultaneously increased the number of transcripts con-

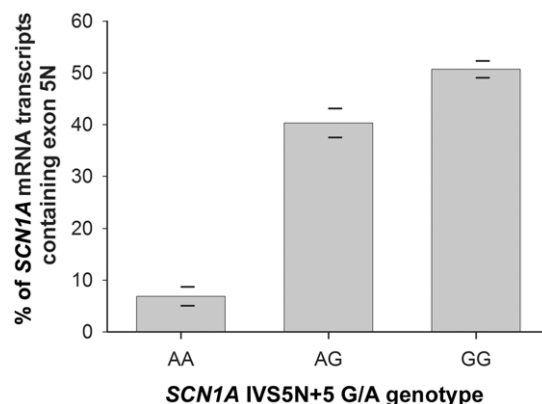


Figure 4. Evidence of the genetic control of *SCN1A* exon 5 alternative splicing in a minigene construct. Shown is the change in the percentage of exon 5N expression with *SCN1A* IVS5N+5 G→A genotype in HEK293 cells transfected with the genomic DNA fragment containing the G, A, or A and G alleles (1 μ g plasmid in total). Data are presented as the mean of two independent replications (values for independent observations are superimposed by use of dashes [-]).

taining the neonatal form. Overall, this translated into Nova2 increasing the percentage of *SCN1A* transcripts containing exon 5N (fig. 5). However, the effects of Nova2 varied for the different *SCN1A* IVS5N+5 G→A genotypes. For the GG genotype, *NOVA2* mRNA expression at the observed maximum elicited an ~2.5-fold increase in the proportion of *SCN1A* transcripts containing exon 5N, whereas, for the AA genotype, the expression change produced an ~20-fold change in the splicing event. Similar to the observations in human brain tissue, these findings support a greater effect of Nova2 in the AA genotype.

We note that, in these experiments, the maximum changes in splicing elicited by Nova2 in the AA genotype were predicted only and were not directly observed (i.e., an experimentally derived maximum plateau of effect was not obtained in these experiments) (fig. 5). Despite the failure to reach the plateau, a much larger change from baseline in *SCN1A* alternative splicing was observed, despite a lower maximum-observed increase in *NOVA2* mRNA expression in the AA minigene cellular system. Increases in *NOVA2* mRNA expression were associated with increases in protein expression for both genotypes (fig. 5), which is consistent with the hypothesis that expression of the splice-modifier protein is modulating the observed

effects on *SCN1A* alternative splicing. Importantly, we emphasize that, with modulation of Nova2 expression, the *SCN1A* exon 5 splice-variant percentage in the AA system approached that observed for the GG minigene expressing endogenous levels of *NOVA2* mRNA (fig. 5).

Finally, we tested the specificity of Nova2-mediated alternative splicing by evaluating the effects of increases in expression of this protein on modulation of splicing events in 17 neuronally expressed ion-channels genes. We found that Nova2 exhibited no effect on splice-variant ratios for any of the 17 alternative splicing events defined in table 1 (data not shown).

Discussion

The *SCN1A* IVS5N+5 G→A polymorphism, located in the splice-donor site of exon 5N, had a striking effect on the percentage of transcripts in the neonatal form of the *SCN1A* gene in adults with and without epilepsy (fig. 2A). In those individuals with the GG genotype, up to 50% of the transcripts include the neonatal version of exon 5, compared with an often undetectable level of the neonatal version in some subjects with the AA genotype. This correlation reveals a statistically significant increase in exon

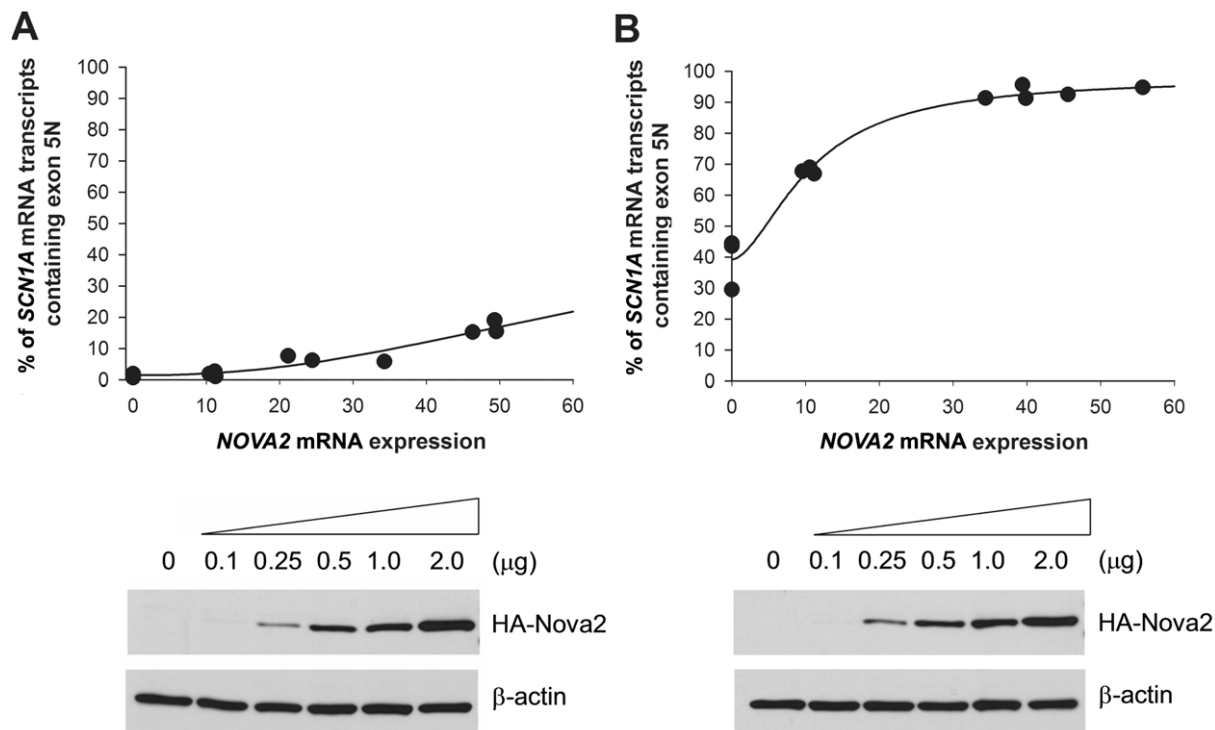


Figure 5. Top panels, Change in the percentage of *SCN1A* transcripts containing exon 5N in the presence of increasing *NOVA2* mRNA expression in the HEK293 minigene construct with genotype AA (A) or GG (B) at the *SCN1A* IVS5N+5 G→A polymorphism. Lines indicate the fit of the E_{max} model in equation (1) to the data. Bottom panels, Western blot of the change in HA-tagged Nova2 protein expression with increasing amounts of *NOVA2* plasmid in the HEK293 cells. Nova2 modulates *SCN1A* splicing in the AA genotype to a greater extent than in the GG genotype.

5 splicing between the AG and GG genotypes; however, the magnitude of the change is less between the AG and GG genotypes compared with that between the AA and AG genotypes both in vivo and in vitro (figs. 2A and 4, respectively). It is unclear why the G allele appears to elicit a dominant effect on exon 5 splicing (figs. 2A and 4).

The association was observed clearly both in autopsy samples from individuals who do not have known neurological conditions and in resection tissue samples from patients with a history of refractory temporal lobe epilepsy (fig. 2A), providing evidence that this splice variation is likely to be largely genetically controlled, rather than a seizure-induced event. Tate et al.³ reported a very modest effect of genotype on *SCN1A* exon 5 alternative splicing in patient temporal neocortical tissue only, not in control brain tissue.³ The discrepancy could be attributed to the lack of quantitative real-time PCR in the original study, coupled with the comparatively smaller sample sizes evaluated. It is also possible that the difference may simply be due to a different population of control subjects used in this study. We note that animal models of epilepsy also indicate an association between increased exon 5N levels and seizures,^{13,14} which is not apparent in our samples by the quantitative assays used here. In fact, in our samples, there was a slightly reduced percentage of neonatal transcripts in the brain tissue collected from mesial temporal lobe epilepsy for each genotype group (fig. 2B). This opposing finding may simply be due to differences between the animal models and humans. Although additional work is needed to establish the role of seizures in mediating *SCN1A* exon 5 alternative splicing, our data clearly show that the genotype predominantly drives this alternative splicing event, regardless of disease status.

Consistent with the correlation between *SCN1A* IVSSN+5 G→A genotype and alternative transcript splicing in human brain tissue, the exon 5N and 5A minigene system also demonstrated that the expression level of exon 5N was directly affected by the genotype (fig. 4). The proportion found is strikingly similar to the data from human samples. These data reveal virtually no change in the level of exon 5A expression, indicating that the effect of the *SCN1A* IVSSN+5 G→A polymorphism appears to be mainly the result of increased exon 5N expression, a finding that also is consistent with the observations in humans. The *SCN1A* IVSSN+5 G→A polymorphism is located 6 bases into the intron in the 5' splice site following exon 5N (fig. 1). The guanine base at this nucleotide position is highly conserved, with an estimated presence of 75%–86% in human introns, whereas the adenine nucleotide at this base position is far less common, with a presence of ~5%–11%.¹⁵ Given that the *SCN1A* IVSSN+5 G→A polymorphism causes a change from a highly conserved sequence to a lesser conserved sequence, it was not unexpected that a functionally weaker 5' splice site at exon 5N was seen in the presence of this change.

This unambiguous evidence of genetic control of a splicing event in the *SCN1A* gene, in combination with the

new data further supporting genotype dependency of phenytoin dose in patients with epilepsy,⁴ provides an example of a functionally significant genetic polymorphism that appears to directly influence the therapeutic dose of antiepileptic medications and could have other currently unknown phenotypic consequences.

The genetically controlled splicing variation and, consequently, the altered dose requirements of carbamazepine and phenytoin in humans could be the result of the product of transcript variant of the *SCN1A* gene producing altered electrical signaling in patients with epilepsy (i.e., epilepsy is worse in patients with the AA genotype, therefore requiring higher doses of antiepileptic medication to effectively control the seizures). However, our observations also could be the result of altered pharmacology of the antiepileptic drugs in the presence of the different genotypes (i.e., altered potency and/or efficacy of the drugs at the *SCN1A* gene product). Consistent with the latter possibility, an analogous functional splicing alteration in the sodium-channel gene of the German cockroach has been reported to cause altered sensitivity to deltamethrin.^{16,17} Furthermore, we cannot rule out the possibility that the electrical properties of *SCN1A* at baseline and in the presence of drugs differ in the presence of altered transcript-variant quantities. To address this important question, future work will focus on identifying the functional differences between the products of the *SCN1A* exon 5N and 5A transcripts in the absence and presence of drugs.

In addition to the effects of the *SCN1A* polymorphism, we also have documented an important role of Nova2 in modulating the proportion of *SCN1A* alternative transcripts. Because the two transcripts appear to result in different pharmacological properties, this work clearly highlights the potential therapeutic importance of control of splice variation. For example, because the neonate form appears to be more responsive to some antiepileptic drugs, it would seem possible, in principle, to make some forms of epilepsy more easily treatable by increasing the proportion of the neonate transcript through manipulation of *NOVA2* expression or other modifier genes.

Given these findings, Nova2 may, in the future, prove to be a drug target for improvement of pharmacologic responsiveness in a population of patients with refractory epilepsy. However, we note that Nova2 has been shown to modulate numerous alternative splicing events in vivo,¹⁸ and it could be argued that such a strategy may suffer from extensive nonspecific effects. As a preliminary step toward evaluating the generality of *NOVA2* effects on neuronal splicing, we looked at 17 alternative splicing events in ion-channel genes in the presence and absence of increases in Nova2 expression. Of these selected alternative splicing events (table 1), none exhibited Nova2-mediated alternative splicing, although additional work is needed to evaluate comprehensively the specificity of Nova2 in modulating neuronal splicing in humans. These data highlight a specific regulatory role of Nova2 in con-

trolling a clinically significant alternative splicing of *SCN1A* and provide novel avenues to pursue in the attempt to understand and regulate genetic response to medications.

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Web Resources

The URLs for data presented herein are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/>
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for *VKORC1*, *SCN1A*, *SCN1M*, *NOVA1*, and *NOVA2*)

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