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9-2015

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Novel human *ABCC9/SUR2* brain-expressed transcripts and an eQTL relevant to hippocampal sclerosis of aging

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Abstract

ABCC9 genetic polymorphisms are associated with increased risk for various human diseases including hippocampal sclerosis of aging. The main goals of this study were 1 > to detect the *ABCC9* variants and define the specific 3′ untranslated region (3′UTR) for each variant in human brain, and 2 > to determine whether a polymorphism (rs704180) associated with risk for hippocampal sclerosis of aging pathology is also associated with variation in *ABCC9* transcript expression and/or splicing. Rapid amplification of *ABCC9* cDNA ends (3′RACE) provided evidence of novel 3′ UTR portions of *ABCC9* in human brain. *In silico* and experimental studies were performed focusing on the single nucleotide polymorphism, rs704180. Analyses from multiple databases, focusing on rs704180 only, indicated that this risk allele is a local expression quantitative trait locus (eQTL). Analyses of RNA from human brains showed increased *ABCC9* transcript levels in individuals with the risk genotype, corresponding with enrichment for a shorter 3′ UTR which may be more stable than variants with the longer 3′ UTR. MicroRNA transfection experiments yielded results compatible with the hypothesis that miR-30c causes down-regulation of SUR2 transcripts with the longer 3′ UTR. Thus we report evidence of complex *ABCC9* genetic regulation in brain, which may be of direct relevance to human disease.

Keywords

KATP; SUR2A; SUR2Ab; SUR2B; TDP-43

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Transcript-specific primers enables PCR to test for the presence of *SUR2A* transcripts with the novel *SUR2A*-specific 3′UTR portion in human tissues.

Figure S2. RT-PCR was performed using additional transcript-specific primers (arrows indicate locations) to test for the presence of *SUR2A, SUR2B, and SUR2Ab* transcripts including the novel *SUR2A*-specific 3′UTR portion in human tissues.

Figure S3. Correlations between qPCR results from the four primer pairs shown in Fig. 9.

Table S1. PCR primers used in this study.

The human ATP-binding cassette, subfamily C, member 9 (*ABCC9*) gene encodes a polypeptide known as sulfonylurea receptor 2 (SUR2); here we employ established terminology referring to the *ABCC9* gene, whereas *SUR2* mRNA and SUR2 protein are products of the same gene (Shi *et al.* 2012 Nichols *et al.* 2013). *ABCC9*/SUR2 is expressed in many human tissues, is relevant to human diseases, and is a targe of therapeutic strategies (Zhang *et al.* 2010; Shi *et al.* 2012 Allebrandt *et al.* 2013; Czeschik *et al.* 2013; Nichols *et al* 2013; Nelson *et al.* 2014). However, much remains to be learned about this intriguing gene, and, in particular, little is known about *ABCC9*/SUR2 neurochemistry in the human brain. Here, we focused on characterizing human brain *SUR2* transcripts and determining whether a previously described gene variant linked to human disease is a local expression quantitative trait locus (eQTL), i.e., whether the gene variant is associated with altered gene expression.

Understanding SUR2 in the human brain is made challenging by multiple levels of biologic complexity. SUR2 is a large (up to 1509 amino acids), evolutionarily conserved protein with multiple membrane-spanning domains. *ABCC9* gene products (*SUR2* transcript and/or SUR2 protein) are expressed robustly in vascular tissue – smooth muscle, pericytes, and endothelium – and also in neurons, astrocytes, oligodendrocytes, microglia, macrophages, and many other cells and tissues (see e.g., Lee *et al.* 1998; Zawar *et al.* 1999; Pelletier *et al.* 2000; Lacza *et al.* 2003; Bondjers *et al.* 2006; Ploug *et al.* 2010; Zhou *et al.* 2012). As SUR2 cellular- and tissue-level functions may also diverge across species, an 'experimental window' to elucidate the gene's role in a human disease is difficult to achieve. Despite the challenges, multiple studies have found that SUR2 plays important roles in regulating vascular tone and in cellular responses to stress including to hypoxia and ischemia (Chutkow *et al.* 2002; Seino and Miki 2003; Elrod *et al.* 2008; Flagg *et al.* 2010; Nichols *et al.* 2013). Two additional key themes have emerged from human studies: first, *ABCC9* gene mutations and polymorphisms are linked to multiple human diseases; and second, *SUR2* mRNA splicing is tissue-specific and functionally important.

ABCC9 genetic polymorphisms are associated with risk for diverse human diseases. Stop codon mutations in *ABCC9* lead to a condition called hypertrichotic osteochondrodysplasia, or 'Cantu syndrome' (van Bon *et al.* 2012; Harakalova *et al.* 2012). Exonic *ABCC9* single nucleotide polymorphisms (SNPs) have been linked to risk for atrial fibrillation, dilated cardiomyopathy, and myocardial infarction (Bienengraeber *et al.* 2004; Nichols *et al.* 2013; Smith *et al.* 2013). In contrast, intronic SNPs that cluster in the 3′ portion of *ABCC9* have been associated with risk for human brain illnesses, including sleep problems, depression, and hippocampal sclerosis of Aging (HS-Aging) (Allebrandt *et al.* 2013; Parsons *et al.* 2013; Nelson *et al.* 2014).

HS-Aging is a prevalent neurodegenerative disease and the present authors identified a particular SNP (rs704180) that is associated with HS-Aging risk (Nelson *et al.* 2014); this finding was replicated subsequently in a separate study sample (Nelson *et al.* 2015). We also found evidence to support the hypothesis that HS-Aging is a 'brain-wide' disease, rather than only affecting the medial temporal lobe structures such as the hippocampal formation (Neltner *et al.* 2014). Unfortunately, detailed analyses of *SUR2* regulation in human brain

have not been published previously. Nor has there been a prior report to describe whether, and how, the pathogenic intronic gene variants are associated with altered *SUR2* genetic regulation.

RNA splicing that produces alternative *SUR2* mRNA transcripts is a conspicuous feature of *ABCC9* regulation, with multiple alternatively spliced exons in the 3′ portion of the gene (Chutkow *et al*. 1999; Shi *et al*. 2005; Ye *et al*. 2009). Two main splice variants have been described: *SUR2A* and *SUR2B*. These transcripts are generated through differential splicing of two *ABCC9* exons (referred to here as Exon38 and Exon39) which encode the polypeptides' carboxy terminal portions (Chutkow *et al*. 1996; Inagaki *et al*. 1996; Isomoto *et al*. 1996; Davis-Taber *et al*. 2000). *SUR2A* has relatively high expression in cardiac and skeletal muscle cells, whereas *SUR2B* has more widespread expression including in the brain (Chutkow *et al*. 1996; Isomoto *et al*. 1996; Davis-Taber *et al*. 2000; Shi *et al*. 2005; Ploug *et al*. 2010). Some other single exons in the gene have been described to be alternatively spliced. For example, a rare non-conventional splicing event skips 25 internal exons to produce a short variant expressed in mitochondria with a new splice junction at exons 4 and 29 of the annotated gene (Ye *et al*. 2009).

Thus guided by both human disease relevance and RNA splicing activity in *ABCC9*, we focused on the 3′ portion of the gene in the current study. The main goals were to improve characterization of brain-expressed *SUR2* transcripts, and to test whether a genetic polymorphism associated with human brain disease is associated with altered gene expression. We found human brain *SUR2* transcript variants that previously were not annotated, indicating novel alternative splicing in the mRNAs' coding region and also 3′ untranslated region (3′UTR) variants. Evidence gathered from multiple sources supports the hypothesis that a gene variant, associated with HS-Aging pathology, is also an eQTL that influences the levels and splice variants of brain mRNA transcripts derived from *ABCC9*.

Methods

In silico studies

To evaluate evidence for association between rs704180 status and *ABCC9* expression, analyses were performed using web servers designed to evaluate eQTLs; thus we used data in the public domain to compare transcript levels between persons with and without the HS-Aging risk genotype. Information about the databases and web servers used in the current study is presented in Table 1. The following web servers were queried: SNPExp (Holm *et al*. 2010), which includes DNA and SNP data from lymphoblastoid cells transformed with human DNA; GTEx (The GTEx Consortium 2013; Moore 2013), and here we used the human brain tissue for which there was the largest sample size (*n* = 34 samples from the basal ganglia); and BRAI-NEAC (Ramasamy *et al*. 2014), which evaluates brain tissue samples from ten different areas isolated from 133 different human subjects. For additional information and acknowledgment regarding public access web servers, please see Supporting Information. The study was performed in accordance with the local (University of Kentucky) IRB and all patients signed informed consent for brain autopsy and research to be performed on their brain tissues.

Total RNA and mRNA isolation

Cases used for isolation of human tissue are described below and in Table 2. Non-brain tissues, and the isolation of total RNA with Trizol LS, were performed as described previously (Wang *et al.* 2014, 2015). Poly-A mRNA isolation used an affinity oligo dT matrix (Oligo (dT)$_{25}$ cellulose beads; New England Biolabs) isolation method following manufacturer's instructions with minor modifications. Briefly, 0.5 –1 mg of total RNA isolated from hippocampus or skeletal muscle was dissolved in 0.5 mL loading buffer (0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) and heated to 65 °C for 5 min. RNA samples were quickly cooled down in ice for 5 min before incubated with 12.5 mg of Oligo (dT)$_{25}$ cellulose beads. Incubation was performed at 23 °C for 5 min with gentle agitation. After incubation, the tubes were centrifuged for 20 s at 5000 *g*, and the RNA supernatant was transferred to a separate tube. The RNA solution was subjected again to heating (65 °C), cooled on ice, and re-incubated with the Oligo (dT)$_{25}$ cellulose beads as above; this procedure was repeated 3 times. The Oligo (dT)$_{25}$ cellulose beads were then suspended in 0.5 mL loading buffer, transferred to a Centrifugal Filter Unit (Millipore) and centrifuged for 20 s. The beads in the filter unit were washed 4 more times with loading buffer, and 1 time in low salt buffer (0.1 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA). Poly-A mRNA was eluted with 2×250 μL elution buffer (20 mM Tris-HCl, pH 7.5), followed by RNA precipitation as in the manufacturer's protocol.

Only cases with relatively well-preserved RNA integrity (as defined by RNA Integrity Number [RIN] from an Agilent Bioanalyzer 2100 > 4.0, Agilent hails from Santa Clara, CA, USA) were included. Based on this criterion, three cases for which RNA was isolated were not included in this study because of degraded RNA. Final number of samples used was 20 (two brain areas each from 10 different research subjects). The average RIN value was > 8.0 for included cases (Table 2) and there was no difference detected in RIN values between the various groups.

3′RACE: PCR and cloning

To characterize various transcript variants of 3′UTR, Marathon cDNA amplification kit (Clontech, Clontech-Mountain View, CA, USA) was used for 3′RACE. First-strand cDNA was synthesized using poly-A RNA isolated from human hippocampal and skeletal muscle tissues. The synthesis of second-strand cDNA and adapter ligation was performed according to manufacturer's instructions. *ABCC9* gene-specific primers (Table S1) located in Exon38 and Exon39 were designed as forward primers in combination with reverse adapter primer 1 or adaptor primer 2 (supplied with the kit) to amplify 3′ ends of the cDNA. Amplified products were then cloned into PCR cloning vectors Zero-blunt or pCR-XL Topo (Life Technologies, Grand Island, NY, USA). Positive clones were examined by restriction enzyme digestion, and confirmed by sequencing (AGTC, University of Kentucky). Sequencing data were analyzed using NCBI/blastn suite (http://blast.ncbi.nlm.nih.-gov), and UCSC Genome Brower (http://genome.ucsc.edu/). Multiple sequence alignment was carried out by using EMBL-EBI's Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Reverse transcription and PCR (RT-PCR and RT-qPCR)

Total RNA (500 ng) isolated from human tissues was converted into cDNA using Superscript III reverse transcription (RT) kit (Life Technologies) following manufacturer's protocol. The cDNA served as templates for PCR including quantitative real-time PCR (qPCR). Platinum Taq DNA polymerase (Life Technologies) was used in both regular and real-time qPCR. Real-time PCR was carried out in ABI 7000 with SYBR Green as detecting dye. An equivalent quantity of cDNA (10 or 20 ng) was used in the PCR or qPCR. Actin primers were reported previously (Wang *et al.* 2010a) and here were used as reference in normalizing expression levels. Gene-specific primers used in these experiments are listed in Table S1.

Statistical methods

For studies of public access web servers, we relied on the web servers themselves to provide data and statistical tests, and neither further tests nor any other additional analyses were performed on these data (see additional text in Supporting Information). For analyzing qPCR results, the data from three separate qPCR runs (median value) were normalized to three separate qPCR runs (also the median values) for (β-Actin from the same RT reaction. Descriptive statistics were performed using Microsoft Excel (Microsoft-Seattle, WA, USA). Student's *t*-tests were used to compare groups (mean values) and Pearson's correlation was used to test for linear relationships between qPCR amplicons. For the miRNA transfection experiments, simple linear regression was used to test for the replicate averaged Ct (qPCR) values compared between miRNA assays and control. Batch effects were adjusted for via inclusion in the regression model.

MicroRNA (miRNA) transfection in H4 cells to test differential effect on long versus short SUR2 3′UTR

MicroRNA mimics (from Ambion-Life Technologies, Ambion-Grand Island, NY, USA) were used referent to hsa-miR-30c-5p (MIMAT0000244), hsa-miR-200c-3p (MEVIAT0000617), and 'Negative Control #1'. For each, 100 nM was used to transfect H4 cells (Arnstein *et al.* 1974) using RNAiMAX (Life Technologies) according to the manufacturer's instructions. Cells were harvested 48 h post transfection, and RNA was isolated and RT-qPCR performed as described above. Three independent experiments were performed with five replicates in each experiment, for a total of 15 biologic replicates per miRNA transfected.

Results

In silico studies to address whether rs704180 is a local eQTL

For a schematic overview of *ABCC9* genomic arrangement, including 3′ exons and previously identified risk alleles, see Fig. 1; note that the risk alleles cluster in the 3′ portion of the gene. Web servers and datasets used in this study are described in Table 1. Primary data from those web servers are presented in Fig. 2–5; each was queried specifically about whether rs704180 is a cis/local eQTL for *SUR2* transcript levels. Data analyses using the 'SNPExp' website enabled study of correlation between SNP status and transcript levels in

lymphoblastoid cells transformed with human DNA from multiple research volunteers ($n =$ 269). Specifically, the presence of the rs704180 risk allele (A_A) was associated with increased expression of the *ABCC9* gene product, *SUR2* mRNA (for this web server the queried transcript was GI_10947123-A). This is compatible with the hypothesis that rs704180 is a cis (local) eQTL, and the risk genotype is correlated with increased transcript levels.

A second database that was queried was the 'GTEx' web server evaluating the human neuroanatomical area ('caudate and basal ganglia') that included the highest number of samples ($n = 34$ total) among the different brain areas assessed. These analyses indicate that rs704180 is an eQTL in human brain ($p = 0.01$; Fig. 3). The other brain tissues available for eQTL analyses through the GTEx web server all had $n < 30$ and higher p values with the exception of 'nucleus accumbens' ($n = 30$) which returned $p = 0.08$ for rs704180 and $p =$ 0.04 for nearby rs704192 (data not shown). We note that the population frequency of rs704180 varies, depending on study cohort, such that the minor allele may be either A or G, depending on the cohort demographics (Nelson *et al*. 2014). In the GTEx data the identification of the allele (A versus G) is not specified.

The third and final public access database analyzed was the 'BRAINEAC web server that included analyses from multiple human brain areas from 133 research volunteers, focusing on the expression of the *ABCC9* gene product, *SUR2* (Affymetrix transcript ID t3446919, Affymetrix-Santa Clara, CA, USA). These data again supported the hypothesis that the rs704180 risk allele (A_A) is associated with increased expression of *SUR2B* mRNA ($p =$ 0.01; Fig. 4). Additional data analyses were performed using the 'BRAINEAC website because this web tool enabled evaluation of the association between SNP status and individual exon levels (within a transcript) in brain tissue. We queried the association between rs704180 allele status and expression of individual exons from the 3′ portion of *SUR2* mRNA: Affymetrix transcript IDs 3446921 and 3446922/3′UTR, 3446923/Exon 38, 3446925/Exon 37, 3446926/Exon 36, and 3446927/Exon 35. Shown are data expressing p values for each particular exon/SNP association, for all the brain areas combined (Fig. 5). These data provide additional support for the hypothesis that rs704180 status is associated with different expression of Exons 36, 37, and Exon 39 (*SUR2B*), and the distal 3′UTR, but not Exon 38 (*SUR2A*) nor the proximal 3′UTR.

Characterizing SUR2 variants expressed in human brain and directly testing whether rs704180 is an eQTL in brain tissue

Cases were selected from the University of Kentucky Alzheimer's Disease Center (UK-ADC) biobank (Table 2). Information about this research cohort, biobank, neuropathological practices, and SNP genotyping are provided in detail elsewhere (Nelson *et al*. 2009, 2014; Schmitt *et al*. 2012). Cases were selected to represent two groups, those with both HS-Aging pathology and the AA genotype of rs704180, and patients lacking HS-Aging pathology and with the GG genotype of rs704180.

Following RNA isolation and reverse transcription, 3′RACE revealed novel *SUR2* transcript variants and 3′UTR ends (Fig. 6). Notably, a transcript containing Exon38 with a previously unknown 3′UTR was identified in human brain tissue (Fig. 6). We designated this transcript

as *SUR2A*. We also identified a second Exon38-containing transcript that also included the entire length of Exon39; this transcript was evident in human skeletal muscle and other tissues including brain (Figs. 6–8). This is probably the *SUR2* transcript variant reported as *SUR2A* in prior studies (see below). We now designated this transcript as *SUR2Ab*. For *SUR2Ab*, Exon39 may not be translated into a polypeptide since a stop codon is present at the end of Exon38 (Fig. 7). However, the *SUR2Ab* has a completely different 3′UTR in comparison to *SUR2A*. The *SUR2Ab* transcript identified by RACE/sequencing bears a 3′UTR that is identical to 445 bp of the annotated *SUR2B* 3′UTR (NM_020297.3). In addition to that variant, 3′RACE identified several 3′UTR variants that contain Exon39 (Fig. 6, UTR-1.3 kb, UTR-445 bp, UTR-204 bp). These variant 3′UTR s share identical proximal sequence with the previously deposit *SUR2B* transcript (NM_020297.3).

Expression of *SUR2* transcript variants *SUR2A, SUR2B, and SUR2Ab* was examined in human tissues using RT-PCR. Tailored primers (Fig. 8, Table S1) were applied that could detect specifically each of the *SUR2* transcript variants. These studies validated the previously described widespread expression of *SUR2B*, and the novel *SUR2A* 3′UTR portion, while also enabling a comparison of *SUR2* transcripts in human brain cases stratifying by rs704180 genotype. *SUR2B* was readily detected in all tissues tested. *SUR2A* and *SUR2Ab* were also detected in many tissues (Fig. 8, Figure S1 and 2); however, these transcripts were detected at higher levels in skeletal muscle and heart (Fig. 8). Expression levels of *ABCC9* Exon38 and Exon39 in *SUR* transcripts, as well as proximal and distal portion of the *SUR2B* 3′UTR, were evaluated in human brain tissues with or without the rs704180 risk genotype (Fig. 9). RT-qPCR demonstrated that Exon39 (*SUR2B* and *SUR2Ab*) levels were detected at higher levels in brain tissues from patients with the rs704180 risk genotype. Interestingly, detection of the distal portion of the *SUR2B* 3′UTR was negatively correlated with *SUR2B* (Figure S3). We interpret these data, alongside the *in silico* analyses of public domain data (above), to be compatible with the hypothesis that the rs704180 genotype leads to enrichment of a shorter *SUR2B* 3′UTR and a possibly more stable *SUR2B* transcript, in human brain tissue.

MiRNA transfection in H4 cells to test differential effect on long versus short SUR2 3′UTR

Since discovering that the length of the 3′UTR was associated with a change in transcript levels, we wanted to test for a possible mechanism. One mechanism by which 3′UTR sequence confers altered mRNA stability is through providing a target for miRNA binding (Guo *et al.* 2010). We found that there were predicted miRNA targets in the 3′UTR of SUR2. Specifically, TargetScan6.2 (http://www.targetscan.org/) predicted that there are evolutionarily conserved miRNA recognition elements for miR-30 and miR-200 paralogs (Fig. 10). We tested the specific hypothesis that miR-30c, which has putative recognition elements near the 3′ end of the long version of the SUR2 3′UTR, would alter the proportion of *SUR2* transcripts harboring a sequence near the 5′ end of the 3′UTR, versus a sequence near the 3′ end of the 3′UTR (Fig. 11). For these experiments we used H4 cells which have a 'glioneuronal' phenotype (Arnstein *et al.* 1974), and which we have used in prior miRNA transfections (Wang *et al.* 2010a,b). Employing a directional (i.e., 1-sided) hypothesis test, for average miR-30c differences being less than those for the negative control, resulted in a *p*-value of 0.026. When testing for miR-200c differences being greater than control, the

resulting *p*-value is 0.091. If these tests were two-sided, neither would be significant at the 5% nominal alpha level (for the two-sided test, *p* = 0.052 and 0.183, respectively). The data indicate that miR-30c leads to a relative increase in the proportion of 3′UTR amplicons from the 5′ end, in comparison to amplicons from the 3′ end, as predicted. This is compatible with the hypothesis that miR-30c binding is one mechanism underlying the relative instability of *SUR2* transcripts that harbor the longer 3′UTR.

Discussion

We report evidence of complex genetic regulation of the *ABCC9* gene in the human brain. Our study employed experimental and *in silico* analyses, focusing on the 3′ portion of gene. Prior annotation of the *ABCC9* was incomplete. We describe novel human *SUR2* transcripts, including 3′UTR variants. Furthermore, we find evidence that a SNP (rs704180) associated with risk for HS-Aging pathology is a 'cis' (local) eQTL for *ABCC9*. The data indicate that rs704180 is associated with a shorter 3′UTR that is also correlated with increased *SUR2B* transcript level in those samples. One mechanism that may underlie the relative instability of the SUR2 transcript with the longer 3′UTR is down-regulation by miR-30 paralogs such as miR-30c. As far we know this is the first detailed analysis of transcripts and expression patterns of *SUR2* in post-mortem human brain.

Although we report here some *SUR2* splice variants that were not yet annotated, there previously were indications for the existence of some of those transcripts. Prior studies showed that a splice variant exists with both Exon38 and Exon39 included (Isomoto *et al*. 1996; Davis-Taber *et al*. 2000; Ye *et al*. 2009). Importantly, mice have been shown to express full-length transcripts harboring both exons (Isomoto *et al*. 1996). It is theoretically possible that a read-through of that stop codon could occur during some conditions, leading to a true Exon 37–38–39 containing SUR2AB polypeptide, but testing that hypothesis would require substantial additional experiments. We know of no previous description of a *SUR2A*–specific 3′UTR, which is a potentially important novel observation, although it was detected with 3′RACE only. Considering the prior literature and our data together, it is probable that full-length versions of the splice variants described in this study are expressed in brain, in other human tissues, and in many other organisms. We also note that prior studies have reported clones and expressed sequence tags that indicate the presence of differently sized *SUR2B* 3′UTRs (e.g., see expressed sequence tag clones AK_094171 and AK_092535 from Ref, Ota *et al*. 2004), but these also were not full-length clones. There also are shorter, 5′ end annotated *SUR2* transcripts with completely different 3′UTRs (ENST00000538350 and ENST00000326684), which is an additional level of complexity. Future work is required to better understand the RNA processing factors responsible for the many splicing events linked to *ABCC9*/SUR2 function.

Our interest in *ABCC9* stems from discovery of an intronic risk allele (rs704180) for HS-Aging (Nelson *et al*. 2014, 2015). HS-Aging is a neurodegenerative disease that mimics Alzheimer's disease (AD) clinically, and is common in the aged population – the prevalence of HS-Aging in autopsy series is up to 25% among the 'oldest-old' (Nelson *et al*. 2013; Brenowitz *et al*. 2014). The neuropathology of HS-Aging is characterized by cell loss and astrocytosis in the hippocampal formation that is out of proportion to the AD-type plaques

and tangles (Montine *et al*. 2012). Pathogenetic mechanism(s) of HS-Aging are incompletely understood, but may relate to arteriolar vascular disease, and/or a neurodegenerative disease class referred to as frontotemporal lobar degeneration (Nelson *et al*. 2013; Brenowitz *et al*. 2014; Neltner *et al*. 2014). There have not been many prior studies of *ABCC9* in neurodegenerative disease research, but a systematic study of molecular pathways disrupted in *ABCC9* knockout mice identified AD-related molecular variation as the single most impacted pathway associated with that molecular lesion (Gao *et al*. 2014). As SUR2 function has been linked to cellular response to injury in many contexts (Olson and Terzic 2010; Nichols *et al*. 2013), and is targetable by various drugs, the protein represents a credible molecular target for potential disease modification as was recently pointed out (Nelson *et al*. 2014; Macauley *et al*. 2015).

One unexpected outcome from these analyses is that the risk genotype for HS-Aging, rs704180 A_A, is associated with relatively high levels of *SUR2B* transcripts in the post-mortem aged human brain, and also in transformed human-derived lymphoblastoid cells. The reason we did not expect this result is that we found previously that exposure to a drug class (sulfonylureas) that *antagonizes* SUR2 activity is associated with *increased* HS-Aging pathology among individuals over 85 years of age at death in the National Alzheimer's Coordinating Center cohort (Nelson *et al*. 2014). Therefore, we had hypothesized that the *ABCC9* risk genotypes would be associated with decreased *SUR2* expression – the opposite of what was observed. There are at least three possible explanations for this apparent discrepancy. First, the prior published analyses (Nelson *et al*. 2014) may have yielded 'false-positive' results; the association between sulfonylurea drug exposure and HS-Aging pathology was not particularly strong ($p < 0.03$), so may have been the result of random variation. Second, the individuals with greatest risk for HS-Aging pathology may indeed be those with higher, not lower, SUR2 levels in brain, since those persons may be relatively dependent on this molecular pathway and thus more vulnerable to a drug that antagonizes SUR2 function. A third possibility is that the direct impact of sulfonylureas may be exerted on the brain through *ABCC8/SUR1* rather than *ABCC9*/SUR2. Indeed the therapeutic effect of sulfonylureas is exerted through antagonizing pancreatic SUR1 for diabetes control (Proks *et al*. 2002; Nagashima *et al*. 2004). To date, there has been more scholarship published on potential associations between *ABCC8*/SUR1 and brain pathologies (Jiang *et al*. 2007; Mehta *et al*. 2013; Jayakumar *et al*. 2014) than *ABCC9*/SUR2. However, we could find no signal in terms of gene polymorphisms in *ABCC8* being associated with HS-Aging risk (data not shown).

Some factors and potential limitations should be taken into account when interpreting these experiments. The number of samples used in our human brain tissue analyses was low in terms of statistical power, so our results require further replication. Although we use levels of cDNA from processed human brain samples as a signifier of 'gene expression', the relevance to protein 'expression' is not assured, because many factors affect polypeptide levels after mRNA transcription; mRNA translation is another key node of gene expression regulation so mRNA and protein levels are often poorly correlated (Nelson and Keller 2007). The 3′UTR of *SUR2* mRNA, which we here describe to have alternative start-and end-sites in human brain, may regulate translational efficiency, transcript stability, cellular

domain localization, and other factors. It was previously reported that the 3′UTR of *SUR2* may affect mRNA stability (Yang *et al*. 2012) and artifactual variation including post-mortem changes cannot be completely ruled out. Furthermore, although we found evidence that rs704180 is an eQTL for *ABCC9*, that particular SNP could well be a proxy for more complex genomic features including the many SNPs that are in linkage disequilibrium with rs704180. We previously reported that SNPs associated with risk for HS-Aging span at least ten different introns of the *ABCC9* gene (Nelson *et al*. 2014). The study of eQTLs is made challenging because the genotype/transcript change may be labile to anatomical region, cell type(s), agonal state of the patient, and other factors. Our study of eQTL was focused on the single SNP, and therefore both unconventional and also blind to other polymorphisms that may be associated with altered expression of the *ABCC9* gene.

A basic unanswered question is: which cell or tissue compartment(s) is/are important in a clinical-pathological sense? For example, SUR2A and SUR2B proteins are expressed in arteriolar smooth muscle cells, endothelial cells, pericytes, neurons, astrocytes, microglia, oligodendrocytes, and many other CNS cells (see, Lee *et al*. 1998; Zawar *et al*. 1999; Pelletier *et al*. 2000; Bondjers *et al*. 2006; Lacza *et al*. 2003; Zhou *et al*. 2012), and thus the overall impact on human diseases such as HS-Aging may reflect a complicated synergy. Unfortunately, there is no animal (much less cellular) model of HS-Aging, and thus no other known context that could serve as an accurate experimental system to study pathogenetic changes related directly to HS-Aging, other than the aged human brain itself.

The profound complexity and uniqueness of human brain pathologies therefore indicates the appropriateness of caution in interpreting our results, while at the same time confirming the need for more human brain analyses. We were gratified to be able to utilize some of the expanding repertoire of research tools – including human biobanks, improved genomic annotation, and publicly accessible genome databases and web servers – to study human brain biology. Using these resources enabled discovery of evidence for novel gene expression phenomena in the *ABCC9* gene that are perhaps relevant to human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used

AD	Alzheimer's disease
HS-Aging	hippocampal sclerosis of aging

RIN	RNA integrity number
SNPs	single nucleotide polymorphisms
SUR2	sulfonylurea receptor 2

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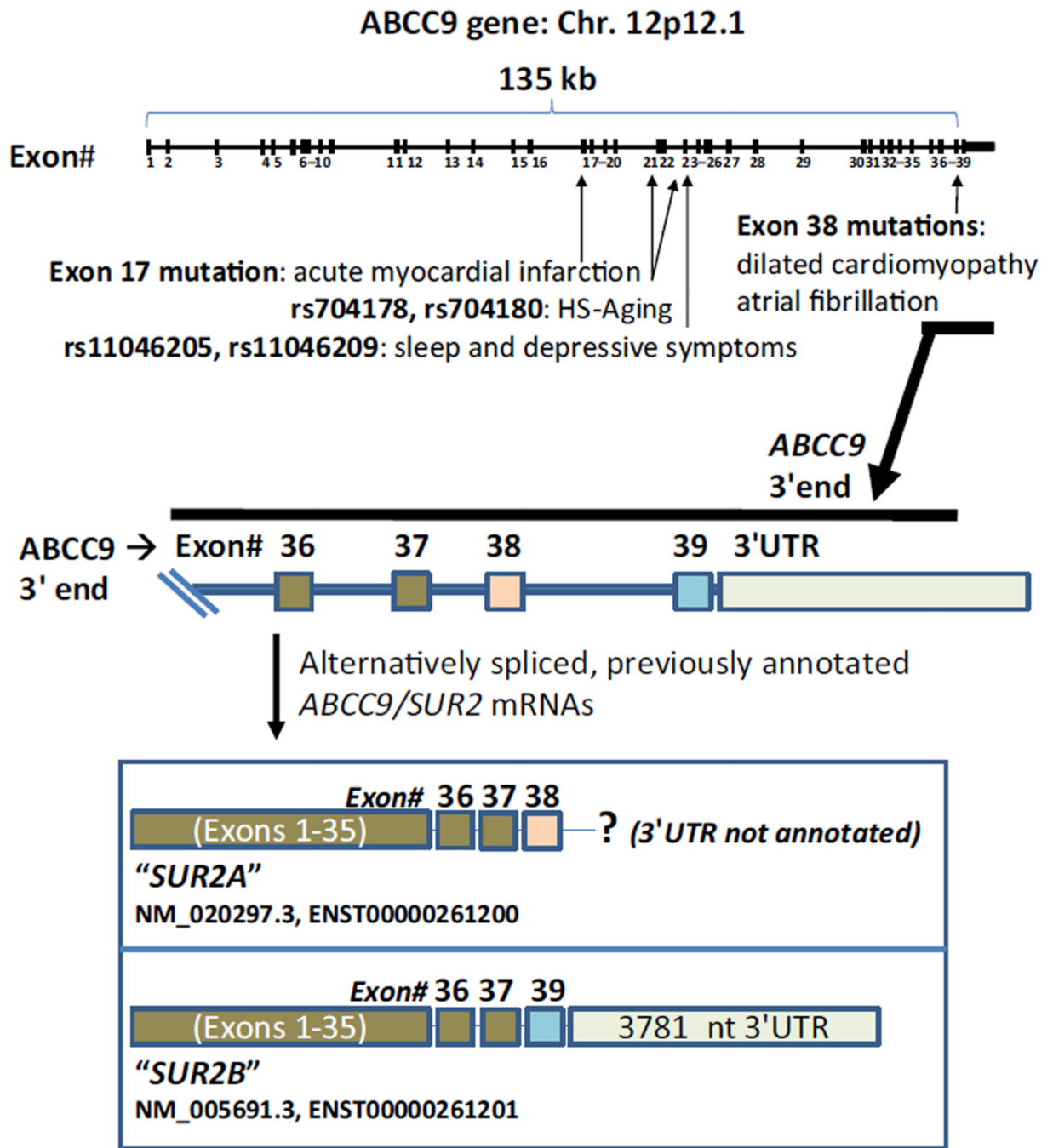
Fig. 1.
Schematic depiction of the *ABCC9* gene including known polymorphisms that are associated with differential risk for human diseases. Citations for these risk alleles are Refs (Bienengraeber *et al.* 2004; Minoretti *et al.* 2006; Olson *et al.* 2007; Allebrandt *et al.* 2013; Parsons *et al.* 2013; Smith *et al.* 2013; Nelson *et al.* 2014). Note that the polymorphisms cluster in the 3′ portion of the gene. Also shown are some of the currently known and annotated *ABCC9* gene products – splice variants that encode SUR2 protein. The two most frequently studied *SUR2* transcripts are termed *SUR2A* (Refseq NM_005691.3, for which a

3′ untranslated region (3′UTR) has not been annotated) and *SUR2B* (Refseq NM_020297.3). These two splice variants are produced by differential inclusion and exclusion of Exon38 and Exon39 in the annotated *ABCC9* gene.
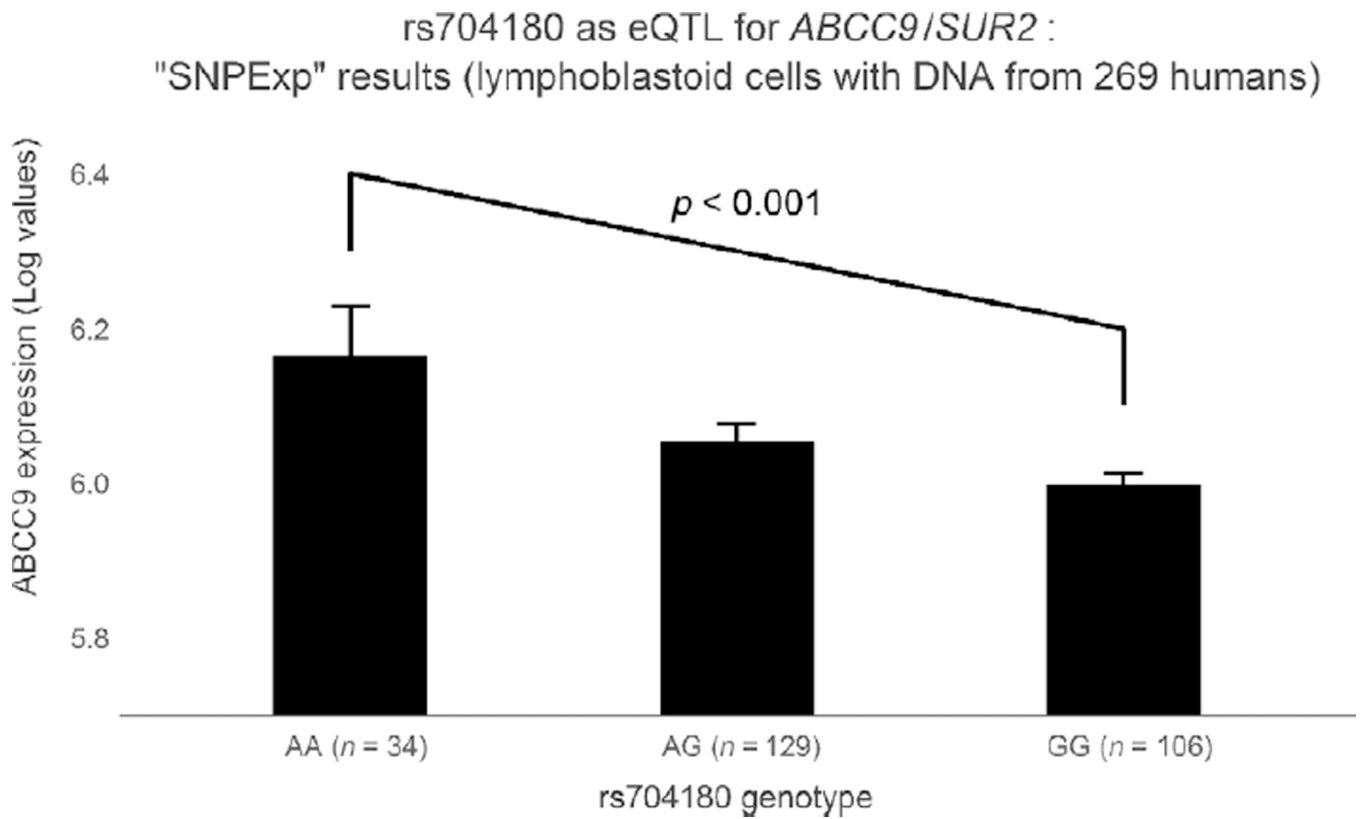
Fig. 2.
Data analyses using the 'SNPExp' website (see Table 1) showing that in transformed lymphoblastoid cells ($n = 269$), the rs704180 risk allele (A_A) correlates with increased expression of the *ABCC9* gene product, *SUR2* mRNA, indicating that rs704180, which is a risk allele for hippocampal sclerosis of aging, is a cis (local) expression quantitative trail locus (eQTL).
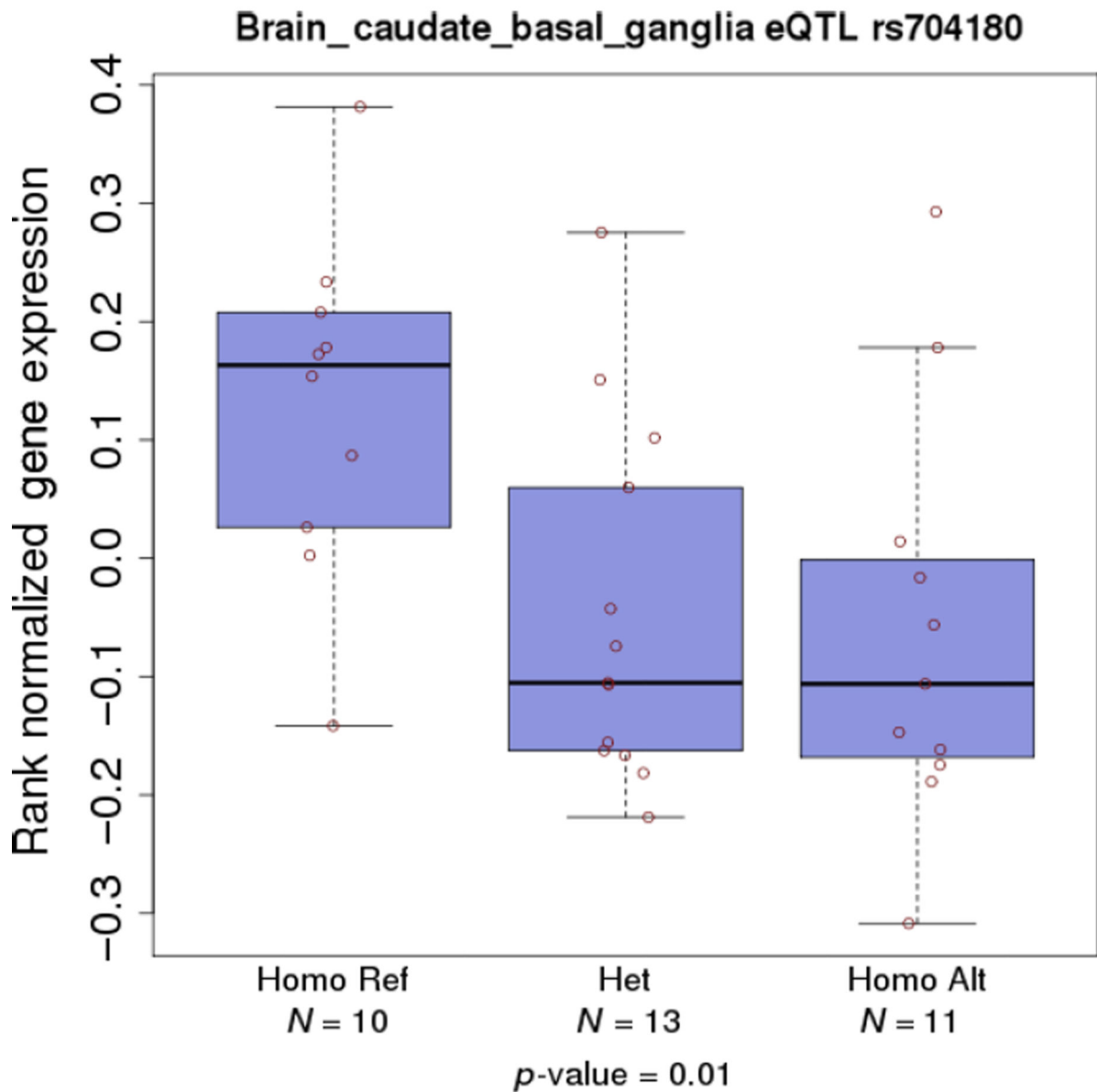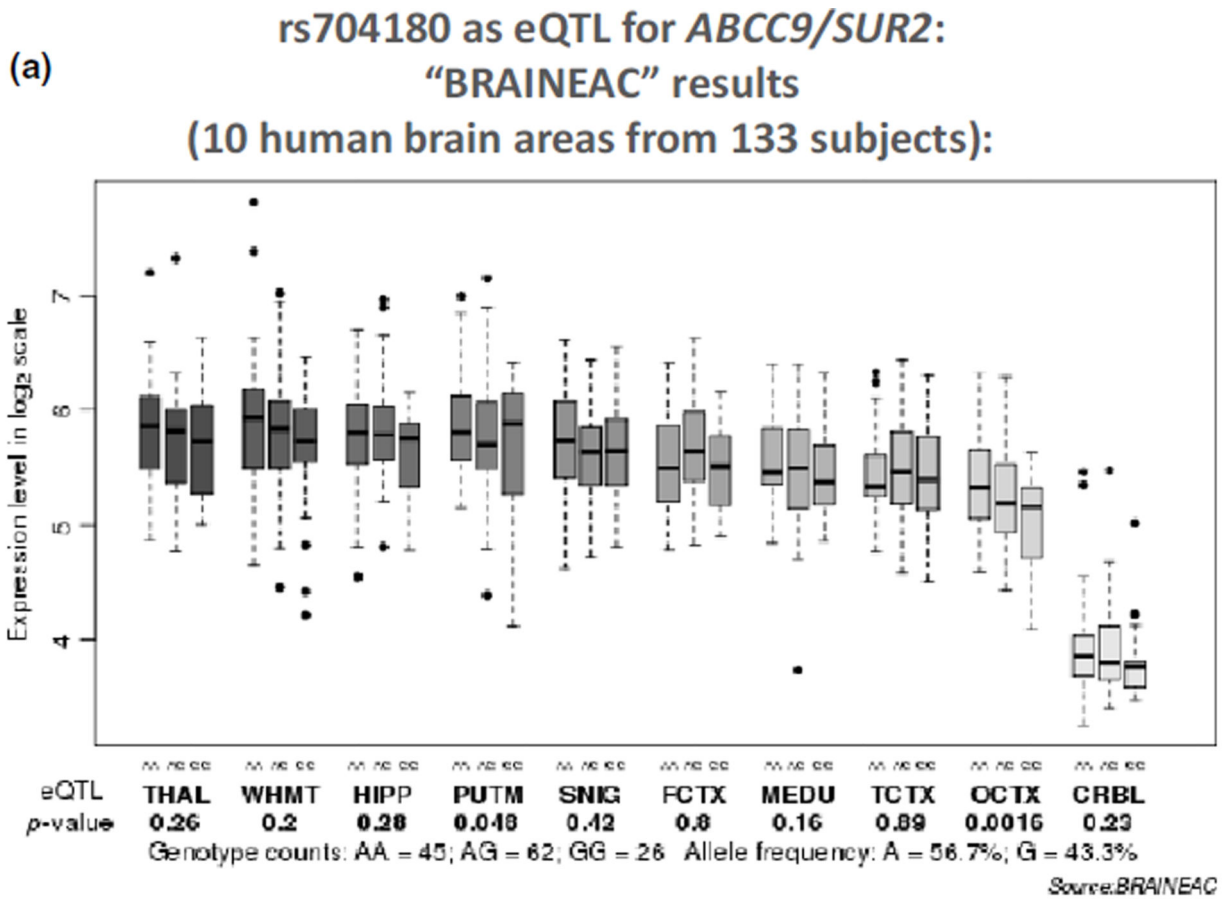
Fig. 3.
Data analyses using the 'GTEx' website (see Table 1) evaluating the human brain area from that database ('caudate and basal ganglia') with the most samples ($n = 34$ total). These analyses again indicate that rs704180 is an eQTL. Note that in these data the Ref allele correlates with increased expression for *ABCC9* ($p = 0.01$) but the identification of the allele (A vs. G) is not specified.

(a)

rs704180 as eQTL for *ABCC9/SUR2*: "BRAINEAC" results (10 human brain areas from 133 subjects):

eQTL	THAL	WHMT	HIPP	PUTM	SNIG	FCTX	MEDU	TCTX	OCTX	CRBL
p-value	0.26	0.2	0.28	0.048	0.42	0.8	0.16	0.89	0.0016	0.23

Genotype counts: AA = 45; AG = 62; GG = 26   Allele frequency: A = 56.7%; G = 43.3%

Source:BRAINEAC

(b)	Results from "BRAINEAC" data: p value for association between SNP status and ABCC9 transcript for cerebellum (CRBL), frontal cortex (FCTX), hippocampus (HIPP), medulla (MEDU), occipital cortex (OCTX), putamen (PUTM), substantia nigra (SNIG), temporal cortex (TCTX), thalamus (THAL), and white matter (WHMT)										Overall average p value factoring in "directionality"
	THAL	WHMT	HIPP	PUTM	SNIG	FCTX	MEDU	TCTX	OCTX	CRBL	aveALL
t3446919 (*ABCC9/SUR2*)	0.26	0.2	0.28	0.048	0.42	0.8	0.16	0.89	0.0016	0.23	**0.011**
rs704180 GG allele trend for decreased expression ?	Yes	Yes	Yes	No	Yes	No	Yes	No	Yes	Yes	

Fig. 4.
Data analyses using the'BRAINEAC website (see Table 1) evaluating multiple human brain areas (*n*=133 cases), focusing on the expression of the *ABCC9* gene product, *SUR2* mRNA (Affymetrix transcript ID t3446919). These data provide support for the hypothesis that the rs704180 risk allele (A_A) is correlated with increased expression of *SUR2* mRNA in multiple brain areas. The data for each brain area are shown in (a), and digested numbers in (b). The overall *p* value for the association between the rs704180 genotype and t3446919 expression is 0.011.

"BRAINEAC" results (human brain): individual exons

Portion of *ABCC9/SUR2* gene and transcript	Affymetrix exon ID#	rs704180 *p* value*
Exon 36	3446927	0.0062
Exon 37	3446926	0.0072
Exon 38 (*SUR2A*)	3446925	0.35 (NS)
Exon 39 (*SUR2B*)	3446923	0.0038
5' portion of 3'UTR	3446922	0.012
3' portion of 3'UTR	3446921	0.85 (NS)

ABCC9 →

3446922   3446921

Exon# 36   37   38   39   3'UTR
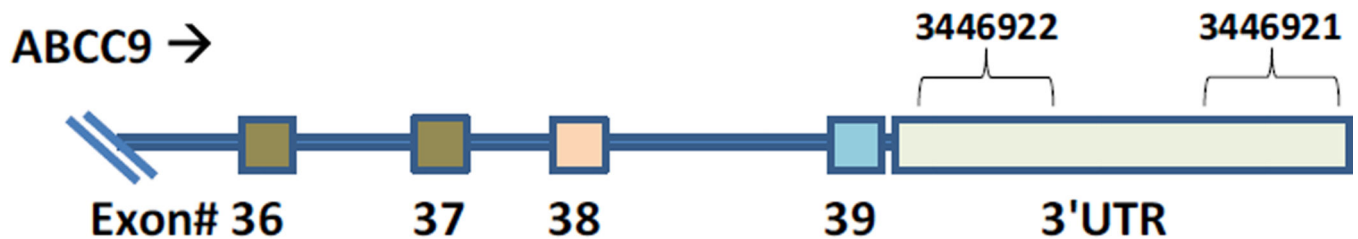
*-p value represents the probability that the rs704180 SNP status is randomly associated with this exon transcript level in all the brain tissue specimens (the "aveALL" parameter, for more details see website http://www.braineac.org)

Fig. 5.
Data analyses using the 'BRAINEAC website (see Table 1) evaluating multiple human brain areas, focusing on the association between rs704180 allele status and expression of individual exons from the *SUR2* mRNA (Affymetrix transcript IDs 3446921/3′UTR, 3446923/Exon 38, 3446925/Exon 37, 3446926/Exon 36, and 3446927/Exon 35). Shown are data expressing *p* values for all the brain areas combined (see Fig. 4). These data provide support for the hypothesis that rs704180 status is associated with different expression of Exons 36, 37, and Exon 39 (*SUR2B*), and the distal 3′ untranslated region (3′UTR), but not Exon 38 (*SUR2A*) or the proximal 3′UTR.
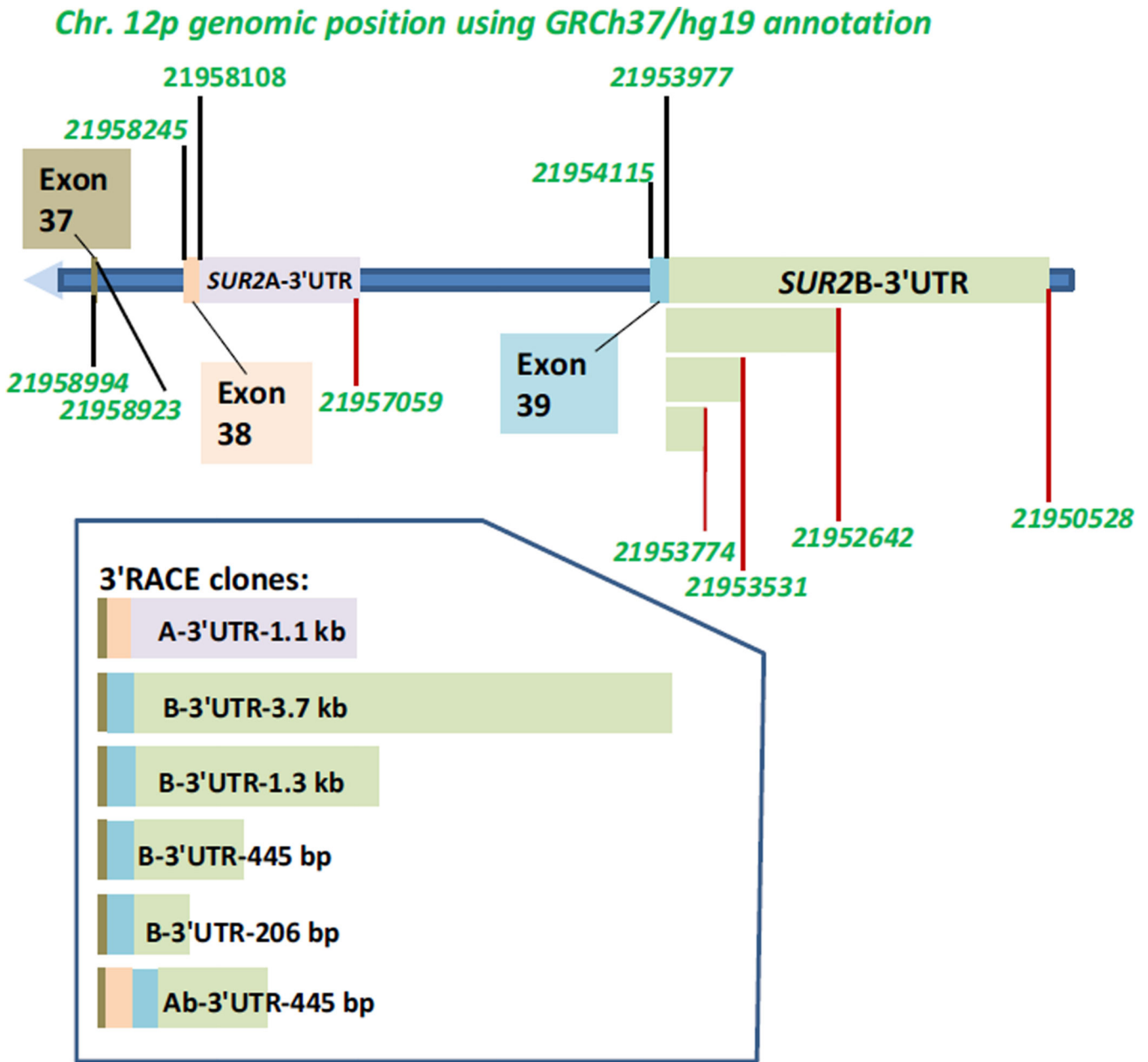
Fig. 6.
3′RACE results with graphic illustration of 3′-end transcripts cloned from human brain. In green are specific genomic locations according to the GRCh37/hg19 annotation. Note that there is a novel 3′ untranslated region (3′UTR) portion that is specific to *SUR2A* transcripts *upstream* of Exon39 (light purple color). Inset depicts clones that were sequenced. Among these are representative sequences corresponding to *SUR2* variants *SUR2A, SUR2B*, and *SUR2Ab*. Notably there are alternative lengths of the cloned 3′UTR of *SUR2B* transcripts identified by 3′RACE.

SUR2Ab: nucleotide sequence from 3′ RACE

```
Exon39      ------------------------------------------------------------
A-B         caccgtgtctcttctattatggatgcaggccttgttttagtcttttctgagggtatttta
Exon38      caccgtgtctcttctattatggatgcaggccttgttttagtcttttctgagggtatttta


Exon39      ------------------------------------------------------------
A-B         gtggagtgtgatactgtcccaaatttgctcgcccacaagaatggcctcttttccactttg
Exon38      gtggagtgtgatactgtcccaaatttgctcgcccacaagaatggcctcttttccactttg


Exon39      --------------------------------------------------------catc
A-B         gtgatgaccaacaagtagACCATCATGATCTATTGCACCAAGTCTGTCATTCTCCGcatc
Exon38      gtgatgaccaacaagtag------------------------------------------


Exon39      gagtacacactattctgacggcagacctggttattgtgatgaagcgaggaaatatttttag
A-B         gagtacacactattctgacggcagacctggttattgtgatgaagcgaggaaatatttttag
Exon38      ------------------------------------------------------------


Exon39      aatatgacactccagaaagcctcttggctcaggaaaatggagtatttgcttcttttgttc
A-B         aatatgacactccagaaagcctcttggctcaggaaaatggagtatttgcttcttttgttc
Exon38      ------------------------------------------------------------


Exon39      gcgcagacatgtga
A-B         gcgcagacatgtga
Exon38      --------------
```

Predicted amino acid sequences: *SUR2A, SUR2B, SUR2Ab*

```
SUR2A       HRVSSIMDAGLVLVFSEGILVECDTVPNLLAHKNGLFSTLVMTNK*
SUR2Ab      HRVSSIMDAGLVLVFSEGILVECDTVPNLLAHKNGLFSTLVMTNK*
SUR2B       HRVHTILTADLVIVMKRGNILEYDTPESLLAQENGVFASFVRADM*
            ***  *   *  **  *     *    * **    ***   ** *      *
```

Fig. 7.
A transcript that we term SUR2Ab is expressed in multiple human tissues and here we present the nucleotide sequence (top) and predicted amino acid sequence (bottom) of this transcript. Note that the transcript includes both *ABCC9/SUR2* Exon38 (like *SUR2A*) and Exon39 (like *SUR2B*) but the translated product would stop before the Exon39 so the mature polypeptide would be identical to SUR2A despite completely different 3′ untranslated region (3′UTR). This transcript has not been annotated in humans but full-length SUR2Ab has been annotated in mice (Isomoto *et al*. 1996).
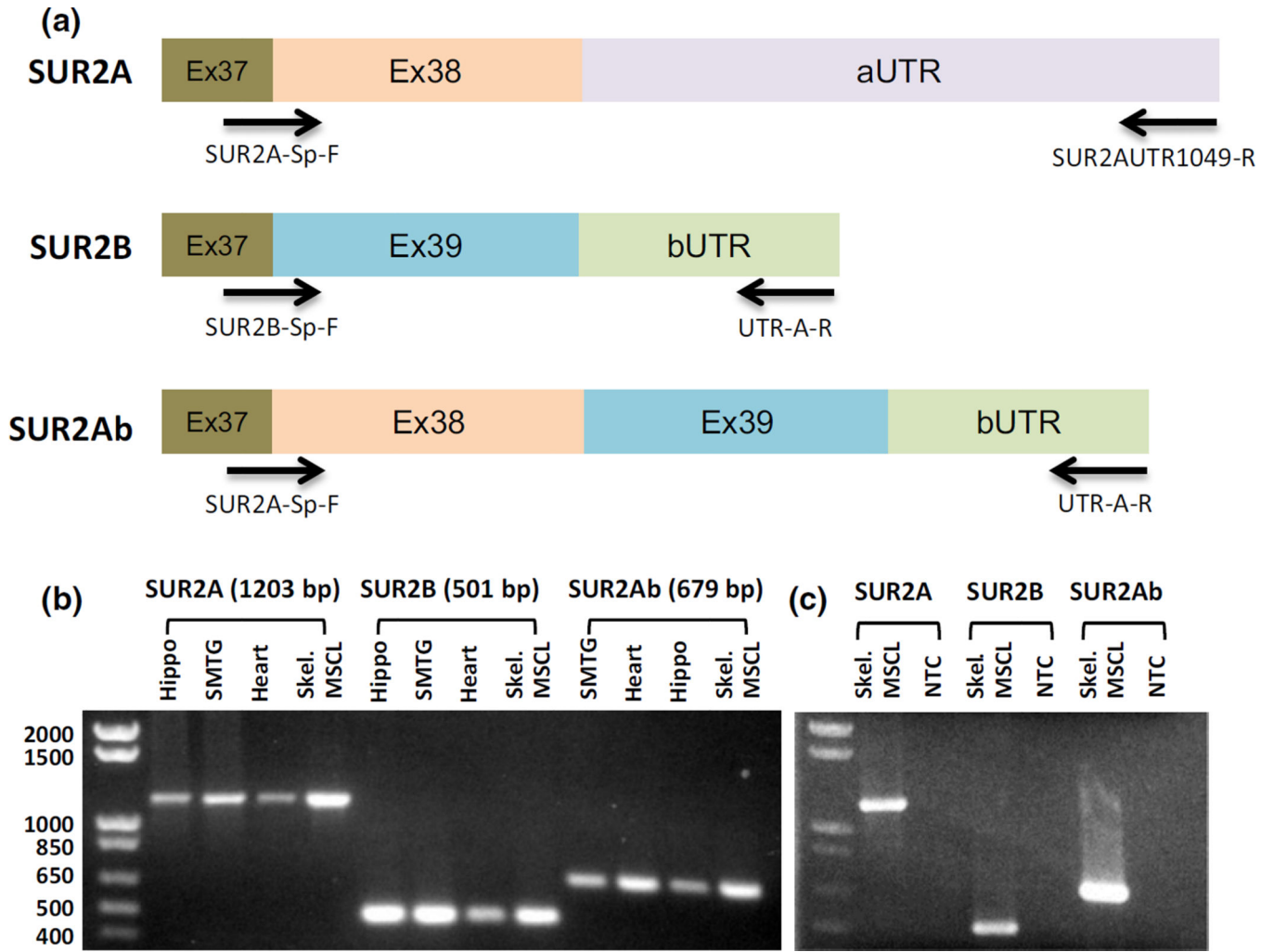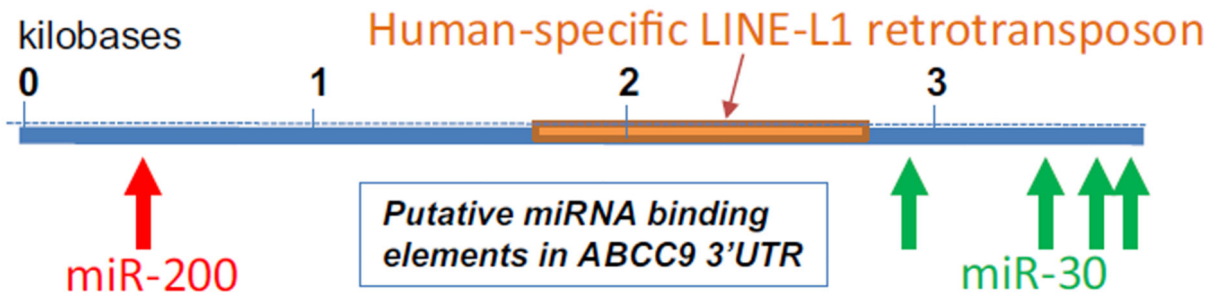
Fig. 8.
PCR to test for the presence of *SUR2A, SUR2B*, and *SUR2Ab* transcripts in human tissues. Primers were developed which span exon junctions to enable transcript subtype-specific PCR (a). Representative PCR results (agarose gel stained with SYBR green stain; b) from human hippocampus (Hippo; in brain), superior and middle temporal gyrus (SMTG; in brain), heart, and skeletal muscle. Panel (c) demonstrates that water only (non-template) controls (NTC) are negative for PCR-amplified DNA.
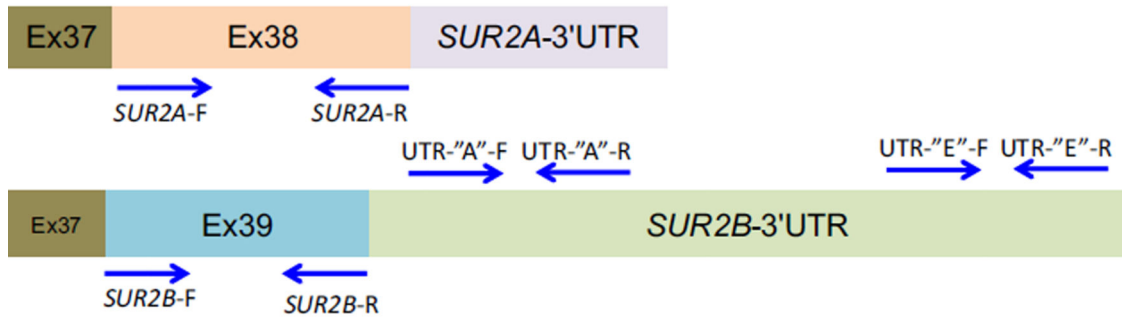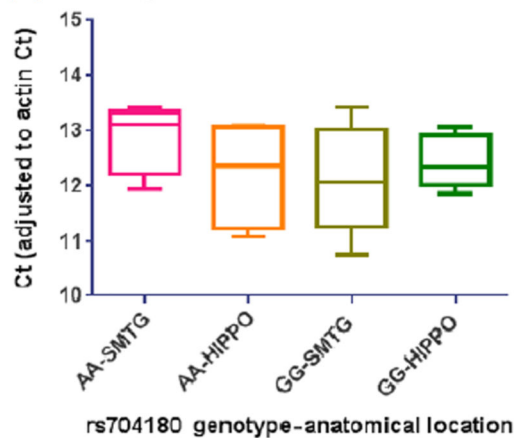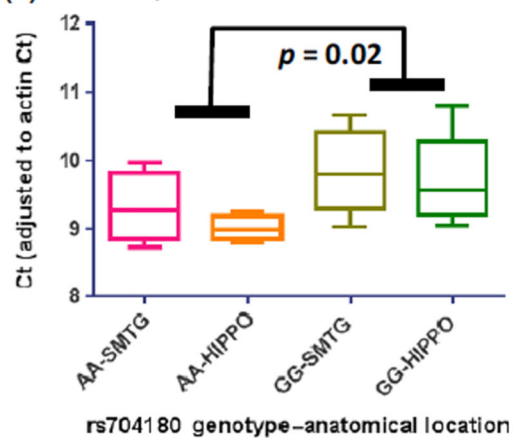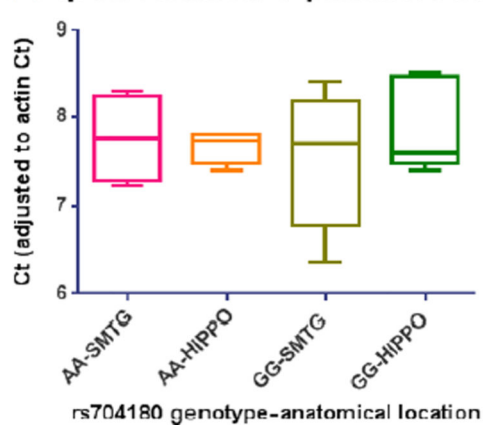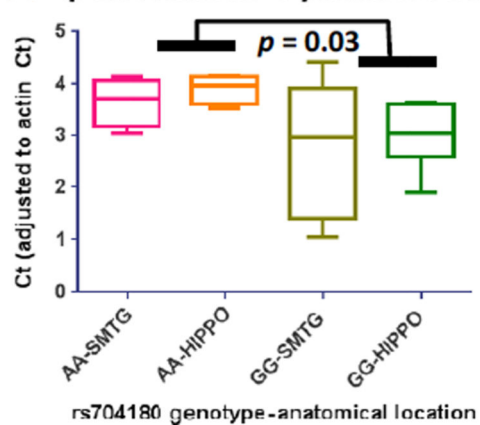
Fig. 9.
Quantitative PCR (qPCR) from human brain tissue samples (see Table S1 for primer sequences), (a) Primers were developed for qPCR to demonstrate amplicons related to Exon38 (*SUR2A*), Exon39 (*SUR2B*), and regions of *SUR2* 3′ untranslated region (3′UTR) as shown. The results of qPCR experiments are shown in (b–e). Note that Ct is lower (expression higher) for *SUR2B* in cases with the rs704180 risk genotype (AA) in comparison to cases with GG rs704180 genotype. There also is a lower level of distal 3′UTR portion detected in the cases with the risk genotype.

Human *ABCC9* 3' UTR (full-length, NM_020297.3)

kilobases

Human-specific LINE-L1 retrotransposon

miR-200

Putative miRNA binding elements in ABCC9 3'UTR

miR-30

Annotated ABCC9 mRNA sequences evaluated:

Human *ABCC9*: NM_020297.3   (8325 bp full length)
Mouse *ABCC9*: XM_006506948.2   (7551 bp full length)
Chicken *ABCC9*: XM_003640404.2   (6225 bp full length)

MiR-200c:

                  3' agGUAGUAAUGGGCCGUCAUAAu 5' hsa-miR-200c (3p)

Human *ABCC9*   5001:5' agugucaaugaguaguCAGUAUUa 3'
Mouse *ABCC9*   6590:5' cguuagaagaaaguaaUAGUAUUa 3'
Chicken *ABCC9*   5340:5' cacacuguaguuggcaCAGUAUUg 3'

MiR-30c

                  3' cgacucucacauccuACAAAUGu 5' hsa-miR-30c (5p)

Human *ABCC9*   7618:5' aguaaaugucuaauUGUUUACa 3'
Human *ABCC9*   8052:5' ucgggaccaugucUUGUUUGCc 3'
Human *ABCC9*   8172:5' ccuuuucuauguugUGUUUAUu 3'
Human *ABCC9*   8235:5' cucuacaauaaaugUGUUUAUu 3'
Mouse *ABCC9*   7463:5' uuaugucaccuguaUGUUUACa 3'
Mouse *ABCC9*   7525:5' uaaauuaguccuuuUGUUUACu 3'
Chicken *ABCC9*   6199:5' aaaaauaaagcagaUGUUUACa 3'

Fig. 10.
There are evolutionary conserved putative miRNA target recognition elements in the 3′ untranslated region (3′UTR) of *ABCC9*. The location of these sequences is shown for human (NM_020297.3), mouse (XM_006506948.2), and chicken (XM_003640404.2) *ABCC9* transcripts. For each of these species, the miR-200 paralog miRNAs are predicted to bind the 5′ part of the 3′UTR, whereas the miR-30 paralogs are predicted to bind the 3′UTR near the distal portion of the 'full-length' 3′UTR. Note that the 3′UTR of humans is over 3 kb in

length and contains a human-specific LINE-LI retrotransposon, the functional significance of which remains unknown.
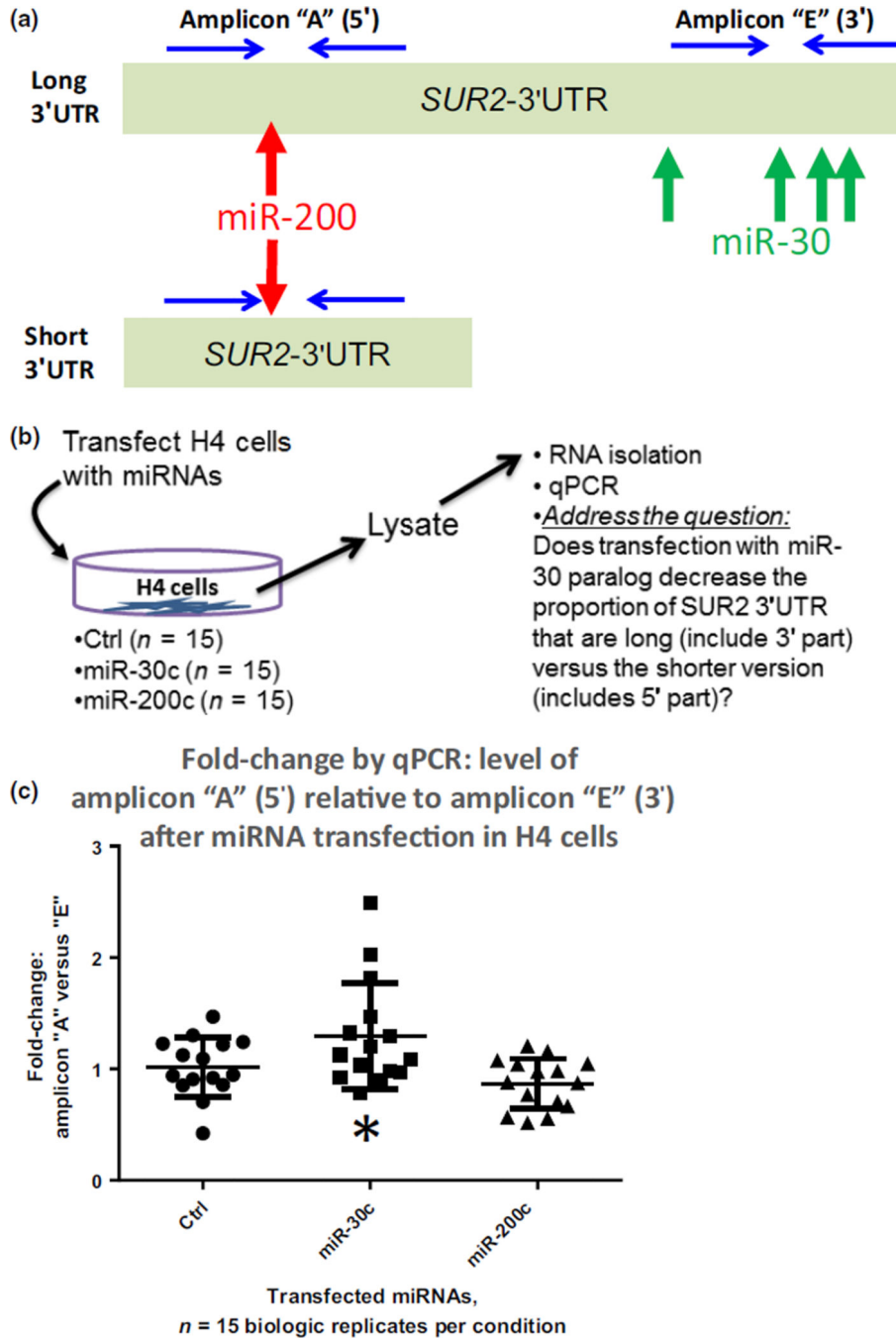
Fig. 11.
An experiment on cultured H4 cells to test the hypothesis that miRNAs may help to explain the differing apparent stability of mRNAs with the longer, versus shorter, 3′ untranslated region (3′UTR) sequence. The experiment involved miRNA transfections followed by qPCR assessment of amplicons related to the 5′ end of the 3′UTR (amplicon 'A') versus an amplicon related to the 3′ end of the 3′ UTR (amplicon 'E'). Primer sequences are provided in Table S1. The dispositions of the amplicons and the putative miRNA recognition sequences on the 3′UTR are shown (a). The overall experiment is described schematically in

(b). Following transfections with miRNAs (100 nm miRNAs, for 48 h, 15 biologic replicates per condition), cells were harvested and the RNA isolated followed by qPCR for Amplicons A and E. (c) Following transfection with miR-30c, there was relative increase in the proportion of *SUR2* transcripts that harbor Amplicon 'A' relative to Amplicon 'E' (*p < 0.03 using 1-tailed Student's *t*-test), normalized to the averaged value of the control miRNA transfections. These data are compatible with the hypothesis that binding by miR-30c is one potential mechanism for *SUR2* transcripts with the longer 3′UTR being less stable.

Table 1

List of datasets and web servers used for this study to evaluate rs704180 as an eQTL

Web server name and (citation)	Types of cells or tissues	Weblink
SNPExp (Holm *et al.* 2010)	Transformed human lymphobastoid cells	http://app3.titan.uio.no/biotools/tool.php?app=snpexp
GTEx (The GTEx Consortium 2013; Moore 2013)	Human tissues including brain – we used brain tissue with largest sample size: 'Caudate basal ganglia'	http://www.gtexportal.org/home/
BRAINEAC (Ramasamy *et al.* 2014)	Human brain tissue from multiple areas	http://www.braineac.org/

Table 2

Brain samples used in current study and characteristics of research volunteers

Sample Name[*]	RIN[*]	rs704180 status	Sex	APOE	Age at death	Final Clinical dx[*]	Final MMSE	HS-Aging	CERAD[*]	Braak stage
Case 1-SMTG	8.4	AA	F	4/4	93	AD	0	Yes	Frequent	V
Case 1-Hippo	8.1									
Case 2-SMTG	8.7	AA	F	2/3	91	MCI	28	Yes	Moderate	V
Case 2-Hippo	8.8									
Case 3-SMTG	8.8	AA	F	2/3	91	AD	29	Yes	Sparse	II
Case 3-Hippo	8.9									
Case 4-SMTG	8.3	AA	F	2/3	94	AD	19	Yes	None	0
Case 4-Hippo	8.7									
Case 5-SMTG	4.6	GG	M	3/4	85	AD	17	No	Moderate	VI
Case 5-Hippo	8.1									
Case 6-SMTG	7.9	GG	F	3/3	87	ND	29	No	None	IV
Case 6-Hippo	7.4									
Case 7-SMTG	9.4	GG	F	2/3	99	MCI	27	No	None	II
Case 7-Hippo	8.7									
Case 8-SMTG	6.9	GG	F	3/3	92	AD	19	No	Frequent	V
Case 8-Hippo	8.5									
Case 9-SMTG	8.3	GG	M	3/4	91	AD	22	No	Moderate	IV
Case 9-Hippo	8.4									
Case 10-SMTG	6.4	GG	M	3/4	93	AD	21	No	Frequent	IV
Case 10-Hippo	8.1									

[*]
For 'Sample Name', 'SMTG' refers to superior and middle temporal gyri (Brodmann areas 21/22), and 'Hippo' refers to hippocampal formation including cornu ammonis; 'RIN' refers to 'RNA Integrity' number from an Agilent BioAnalyzer 2100; 'AD'-Alzheimer's disease; 'MCI'-mild cognitive impairment; 'ND' not clinically demented'; 'CERAD' refers to density of Alzheimer-type neuritic amyloid plaques in neocortical regions.