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Posttranscriptional Regulation of BK Channel Splice Variant Stability by miR-9 Underlies Neuroadaptation to Alcohol

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SUMMARY

Tolerance represents a critical component of addiction. The large-conductance calcium- and voltage-activated potassium channel (BK) is a well-established alcohol target, and an important element in behavioral and molecular alcohol tolerance. We tested whether microRNA, a newly discovered class of gene expression regulators, plays a role in the development of tolerance. We show that in adult mammalian brain, alcohol upregulates microRNA miR-9 and mediates posttranscriptional reorganization in BK mRNA splice variants by miR-9-dependent destabilization of BK mRNAs containing 3′UTRs with a miR-9 Recognition Element (MRE). Different splice variants encode BK isoforms with different alcohol sensitivities. Computational modeling indicates that this miR-9-dependent mechanism contributes to alcohol tolerance. Moreover, this mechanism can be extended to include regulation of additional miR-9 targets relevant to alcohol abuse. Our results describe a mechanism of multiplex regulation of stability of alternatively spliced mRNA by microRNA in drug adaptation and neuronal plasticity.

INTRODUCTION

A challenge to our understanding of addiction is to identify adaptations within individual molecules that underlie tolerance. In this study, we identify dramatic molecular changes attributable to posttransciptional modulation of mRNA stability by microRNA (miRNA) that occur within minutes of alcohol exposure.

Diversity of mRNA species starts with alternative splicing in the cell nucleus, where different exonal combinations of the gene are selected (Black, 2000, 2003; Smith and Valcarcel, 2000) to form various transcripts of the same gene. These transcripts are exported to the cytoplasm, where their stability is regulated by posttranscriptional processes (Guhaniyogi and Brewer, 2001), and protein isoforms with varying properties are produced from the different transcripts (Coetzee et al., 1999; Dredge et al., 2001; Shipston, 2001). These regulatory processes (Blencowe, 2006; Grabowski and Black, 2001) play essential roles in neuronal plasticity, allowing the neuron to quickly fine-tune its protein composition to adapt to different stimuli. These processes are also involved in neurological diseases (Licatalosi and Darnell, 2006; Guhaniyogi and Brewer, 2001). Here, we explore whether a drug of abuse (alcohol) can affect miRNA-based regulatory mechanisms of expression of alternatively spliced mRNA transcripts.

The miRNAs are powerful posttranscriptional regulators of mRNA expression (Jirtle and Skinner, 2007; Filipowicz et al., 2008). They are small (19–25 nt), noncoding RNAs belonging to an important class of endogenous repressors of gene expression (Ambros, 2004; Bartel, 2004). They control expression of target mRNAs by binding to miRNA Recognition Elements (MREs) located in the 3′ untranslated region (3′UTR) of target mRNA.

We investigated whether alcohol, via miRNA, can regulate expression and stability of alternatively spliced mRNAs encoding the main, pore-forming α subunit of the BK channel, a large-conductance calcium- and voltage-activated potassium channel (also called Slowpoke or MaxiK), which is a product of the *KCNMA1* gene. The BK channel (Atkinson et al., 1991) is widely expressed in brain (Knaus et al., 1996; Misonou et al., 2006; Wanner et al., 1999), and influences neuronal excitability, firing frequency, and transmitter release (Storm, 1990). Alternatively spliced variants of the α subunit and their assembly into functional tetramers contribute to functional diversity of the BK channel (Adelman et al., 1992; Atkinson et al., 1991; Butler et al., 1993; Lagrutta et al., 1994; Navaratnam et al., 1997; Rosenblatt et al., 1997; Shipston, 2001; Tseng-Crank et al., 1994) in the brain (Ha et al., 2000; MacDonald et al., 2006). Alternative splicing of BK is dynamically regulated, e.g., the inclusion of the STREX exon is determined by neuronal activity or stress (Xie and McCobb, 1998).

The BK channel is one of the best-described targets of alcohol. Typically, the BK channel is potentiated by alcohol (Butler et al., 1993; Chu et al., 1998; Crowley et al., 2003; Dopico et al., 1998, 1999; Jakab et al., 1997; Knott et al., 2002), although in some tissues, it is inhibited (Dopico, 2003; Walters et al., 2000).

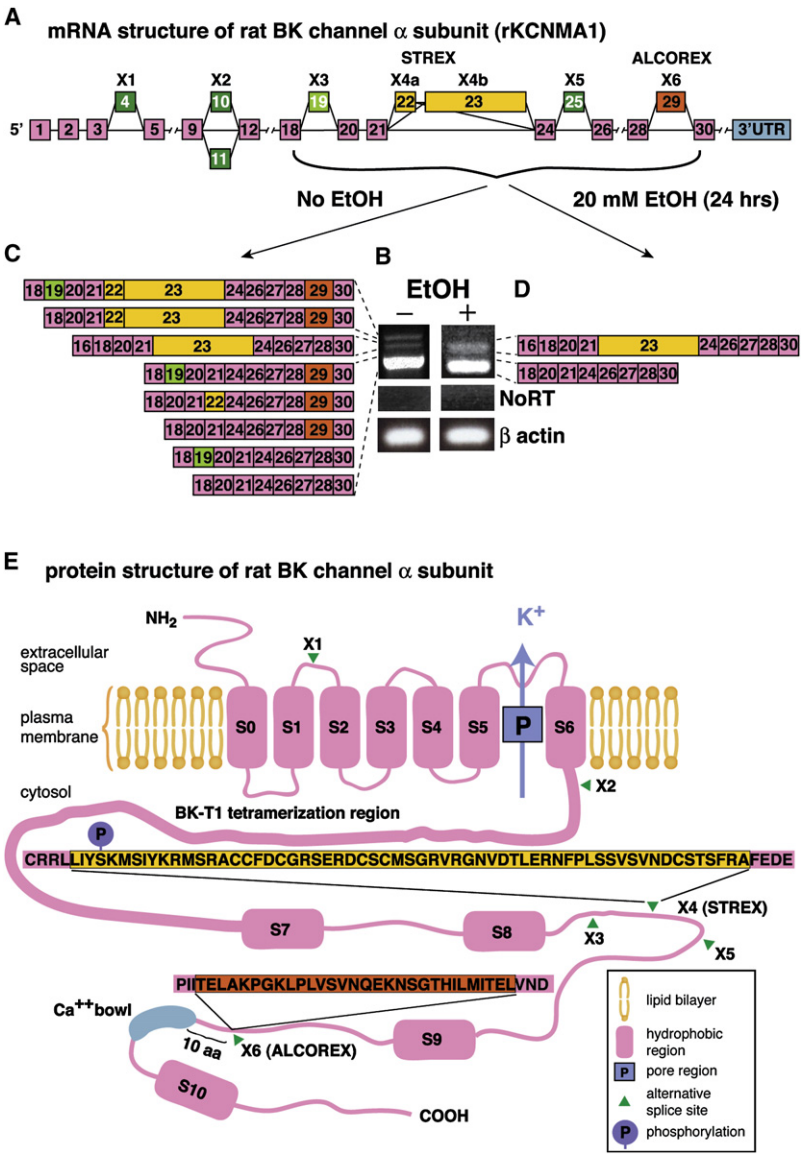

Figure 1. BK mRNA Variants Containing ALCOREX Exon Are Eliminated by Alcohol

(A) A schematic of BK constitutive and alternatively spliced exons within the coding region. Note multiple sites of alternative splicing (marked with X). Exon X4b (referred to here as STREX) corresponds to STREX-1 of Xie and McCobb (1998), while the combination of X4a and X4b corresponds to Xie and McCobb's STREX-2. Exon 29 at the alternatively spliced site X6 has been named ALCOREX, due to its regulation by alcohol (see text). 3′UTR, the 3′ untranslated region of mRNA.

(B) Endpoint PCR was performed using primers bracketing alternative spliced sites X3 through X6 of BK mRNA. Agarose gel electrophoresis revealed different band patterns of BK mRNA isolated from supraoptic nuclei (SON) harvested from naive and alcohol-exposed (20 mM, 24 hr) explants. NoRT, no reverse transcriptase control. β-actin, loading control.

(C) Subcloning and sequencing of cDNA bands from the naive SON indicated that they correspond to eight alternatively spliced variants with different exonal concatenations. Incomplete separation of bands is probably caused by heteroduplexing of BK cDNAs (Mahmoud et al., 2002).

(D) Subcloning and sequencing of two cDNA bands found in the alcohol-exposed SON revealed that they correspond to splice variants carrying only the STREX exon or INSERTLESS, the completely spliced-out variant. A total of 54 clones (28, no alcohol exposure; 26, 24 hr 20 mM alcohol exposure) were isolated and sequenced.

(E) Schematics of BK α protein structure (not drawn to scale). S0–S10, hydrophobic domains; P, pore region; K^+, outward flow of potassium ions; green arrowheads, alternative splicing sites (X1–X6); blown up boxes, polypeptide sequences of exon 22/23 and exon 29 products. Thicker line between S6 and S7 represents BK T1 tetramerization domain (Quirk and Reinhart, 2001). Blue "P" circle indicates position of STREX Ser phosphorylation site described by Tian et al. (2001).

In *C. elegans*, deletion of neuronal BK blocks the action of alcohol, while constitutive activation mimics the presence of the drug (Davies et al., 2003). In *D. melanogaster*, deletion of BK results in a loss of a rapid form of behavioral tolerance to alcohol (Cowmeadow et al., 2005).

Previously, we determined that in two mammalian brain regions important in alcohol abuse and addiction, the supraoptic nucleus (SON) and the striatum, the BK channel develops tolerance to alcohol (Knott et al., 2000, 2002; Pietrzykowski et al., 2004). In neurons of both regions, this BK tolerance is manifested as decreased alcohol sensitivity and reduced BK channel density (Pietrzykowski et al., 2004; Martin et al., 2004).

Here, we illustrate that alcohol causes a rapid increase in miR-9 expression in these neurons. Only BK mRNAs containing a miR-9 binding site in their 3′UTRs are degraded, while others are spared. This reorganization of neuronal BK splice variants leads to a change in BK channel isoform profile consistent with the development of tolerance. This mechanism was found to affect ten additional alcohol-relevant miR-9 targets in the brain, providing a potential mechanism for an integrated response to the drug. The data describe a remarkably fast and elegant mechanism of alcohol multiplex regulation of neuronal alcohol tolerance.

RESULTS

Alcohol Rapidly and Specifically Downregulates a Subset of Pre-existing BK mRNA Variants

First, we determined which pre-exposure BK mRNA variants are expressed in the naive SON, using endpoint polymerase chain reaction (PCR). BK has at least six alternative splice sites residing within the coding sequence (CDS) (Beisel et al., 2007; Figure 1A). Using primers unique to each splice site in rat brain, we found alternatively spliced exons at only three (X3, X4, and X6) of these sites (Figures 1 and S1 in the Supplemental Materials, available online). Next, using primers bracketing sites X3 through X6, we observed several distinct bands (Figure 1B), suggesting the expression of several distinct variants of BK, possibly

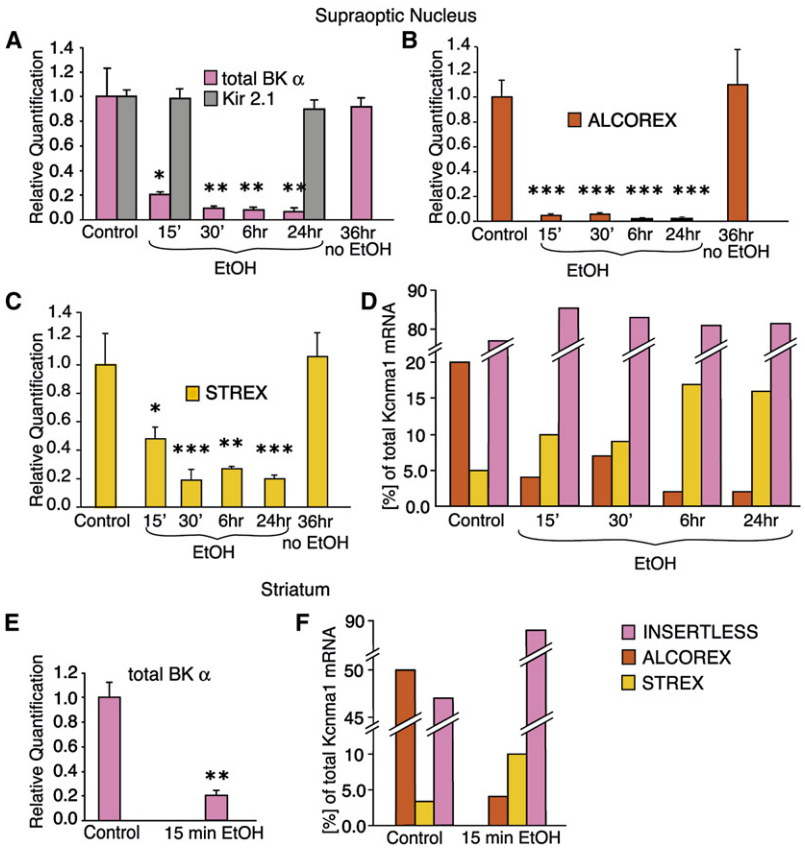

Figure 2. Alcohol Rapidly and Specifically Switches Alternatively Spliced BK mRNA Variants

(A) HNS explants were cultured in regular medium with alcohol for up to 24 hr and without alcohol for up to 36 hr. Twenty millimolar alcohol rapidly downregulates total BK mRNA, but culturing alone does not change its level (n = 9). Moreover, mRNA level of another potassium channel, Kir 2.1, remains unchanged (n = 3).

(B) Within 15 min of 20 mM alcohol exposure, the ALCOREX exon is downregulated to very low levels. Alcohol-free culturing has no effect (n = 9).

(C) STREX exon is also downregulated within 15 min of 20 mM alcohol exposure, but to a lesser extent. A longer exposure times, STREX expression levels off. Alcohol-free culturing has no effect (n = 9).

(D) Initially, ALCOREX constituted 20% of total BK message, while STREX constituted 5%. Short (15–30 min) exposure to 20 mM alcohol decreased ALCOREX level 3- to 5-fold while doubling STREX expression level. Longer exposure to alcohol augmented that effect. The level of the INSERTLESS variant remained almost unchanged. Data calculated from (A), (B), and (C) using BK plasmids as standards (see Experimental Procedures).

(E) In striatal neurons, similarly to the SON neurons, alcohol dramatically decreased total BK message within 15 min of exposure.

(F) In naive striatal neurons BK transcript landscape is different than in SON neurons: ALCOREX expression level also dominates over STREX, but constitutes a larger amount of total BK message (about 50%), while INSERT LESS is less represented. Similarly to SON, alcohol caused a switch in expression of ALCOREX and STREX in striatal neurons. Additionally, the INSERTLESS contribution to the total amount of BK mRNA increased. Data calculated as in (D).

*p < 0.05, **p < 0.01, and ***p < 0.005 compared with control. Error bars represent SEM.

with different exonal concatenations. Indeed, subcloning and sequencing revealed that these bands corresponded to splice variants with differing exonal concatenations (Figure 1C). We observed only 8 of the possible 16 variants, indicating that exonal assembly of BK message within SON neurons is not random. Exon 29 located at site X6 was most frequent.

Exposure of SON neurons to alcohol (20 mM, at or just above legal intoxication levels, up to 24 hr) in hypothalamo-neurohypophysial system (HNS) organotypic explants changed the BK variant landscape dramatically. Endpoint PCR, subcloning, and sequencing revealed the presence of two bands (Figure 1B) encoding two variants (Figure 1D): (1) the STREX variant (STREX-1 in Xie and McCobb [1998]), and (2) a completely spliced-out variant (INSERTLESS).

Thus, alcohol exposure reduced the number of BK mRNA variants by eliminating several variants, five out of six of them containing exon 29. Due to its regulation by alcohol, we refer to exon 29 as ALCOREX (for *alco*hol-*re*gulated *ex*on), by analogy to STREX (for *str*ess axis-*re*gulated *ex*on; Xie and McCobb, 1998). Sequences of products of both exons (ALCOREX and STREX) and their positions in the BK polypeptide are shown in Figure 1E.

Next, we determined the quantitative and temporal changes in total BK mRNA and individual exons using real-time PCR (Experimental Procedures, Figure S2, Bustin, 2002). Alcohol rapidly (after 15 min of alcohol exposure) downregulated total BK mRNA. This effect was BK and alcohol specific and augmented by longer exposures (Figure 2A).

These results, particularly the rapidity of alcohol's downregulation of BK message, suggested that alcohol acts on pre-existing, alternatively spliced BK mRNA variants, rather than regulating alternative splicing during production of BK transcripts. Lack of effect of a transcriptional blocker (10 μg/μl, Actinomycin D) confirmed this hypothesis (Figure S3).

Next, we determined that alcohol exposure resulted in a radical change in the expression of individual BK exons. Alcohol rapidly (after 15 min) and profoundly decreased expression of the ALCOREX exon (Figure 2B). Moreover, longer alcohol exposure (6 hr, 24 hr) augmented that decrease, resulting in almost undetectable levels of this exon, corroborating our endpoint PCR results. The STREX exon was also decreased, but to a lesser degree (Figure 2C).

Thus, we observed that alcohol changed the BK transcript landscape dramatically. Initially, before alcohol exposure of the HNS explant, ALCOREX constituted 20% of total BK message, STREX comprised a smaller fraction (5%) similar to amounts found in other tissues (Zhu et al., 2005), while the INSERTLESS variant (no ALCOREX or STREX exons) constituted about 75% of total BK message (Figure 2D). Short (15 min) alcohol exposure caused a reorganization of alternatively spliced BK transcript

profile, best illustrated as relative amounts: ALCOREX variants decreased 4-fold, STREX doubled, and the INSERTLESS variant was changed the least (Figure 2D). Longer exposure times augmented these effects (Figure 2D; 6 hr, 24 hr).

To examine the generality of the subtractive mechanism of alcohol reorganization of BK transcript landscape in other brain regions, we extended our study to the striatum—a brain region important in the development of various types of addiction (Hyman et al., 2006; Koob, 1999). Using striatal cell culture we observed similar changes (Figures 2E and 2F). Interestingly, alcohol reorganization of BK splice variants in medium spiny neurons was even more profound than that in the SON, since ALCOREX was found in half of the total BK transcripts in unexposed striatum, and alcohol caused greater changes in relative expression: 10-fold downregulation of ALCOREX and a larger increase in INSERTLESS (Figure 2F).

These data suggest that, in alcohol-relevant brain regions, alcohol downregulates BK mRNA by a posttranscriptional mechanism.

miR-9 Mediates Posttranscriptional Alcohol Regulation of BK mRNA

miRNAs are small (21–26 nt), endogenous, single-stranded RNA molecules that act mainly as posttranscriptional repressors of gene expression (He and Hannon, 2004) in many tissues, including CNS (Kosik, 2006). In addition, miRNAs can downregulate their mRNA targets in mammals (Farh et al., 2005; Lewis et al., 2005; Yekta et al., 2004) by binding to target mRNA, resulting in rapid mRNA cleavage (Valencia-Sanchez et al., 2006; Yekta et al., 2004). These features make them ideal candidates for mediators of the observed alcohol regulation of BK transcripts.

miRNA regulation of its target depends upon (1) degree of complementarity between the miRNA and its binding site (the MRE), typically located in the 3′UTR of the target (Kiriakidou et al., 2004; Yekta et al., 2004), and (2) number of MREs (Valencia-Sanchez et al., 2006). In contrast to plants, complementarity is usually partial in mammals (He and Hannon, 2004). The highest likelihood of successful miRNA:MRE binding in mammals is determined by a perfect match between miRNA seed sequence (nt #2 through #7 or #8) and adenosine-flanked MRE (Lewis et al., 2005). It is believed that a single MRE of high complementarity generally results in mRNA cleavage (Valencia-Sanchez et al., 2006; Yekta et al., 2004), while multiple sites result in translational repression (Doench and Sharp, 2004; Kiriakidou et al., 2004).

To determine which miRNAs would form the most complementary and stable duplex with the BK mRNA 3′UTR, we base-paired 109 rodent neuronal miRNAs with the known rat BK mRNA 3′UTR (AF135265), using RNAHybrid (Kruger and Rehmsmeier, 2006), and visually inspected each hybridization. Among promising miRNA species (Figure 3A), miR-9 was the best candidate, with high complementarity (Figure 3B), one of the lowest predicted free energy of hybridization (−22.3 kcal/mol), and a single miR-9 MRE (Figure S6). We confirmed our analysis using TargetScan (Lewis et al., 2003, 2005; Grimson et al., 2007). These characteristics strongly suggested that miR-9 could bind to BK mRNA and cause its cleavage and downregulation.

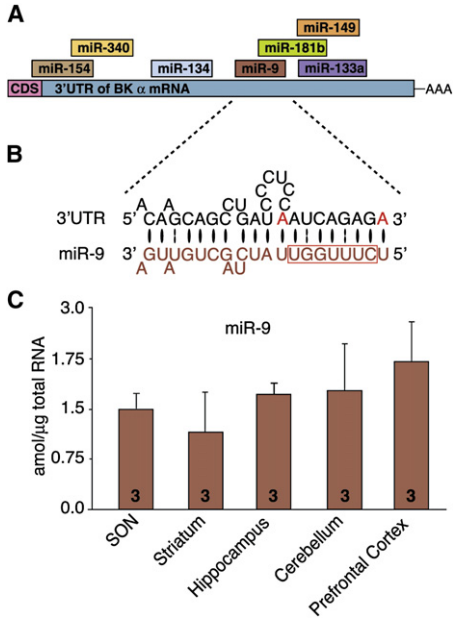

Figure 3. miR-9 Is the Best Candidate for Mediator of Alcohol Regulation of Alternatively Spliced BK mRNA Variants

(A) Schematic showing a subset of miRNAs, which can potentially bind to BK 3′UTR. CDS, a coding sequence; AAA, poly(A) tail.

(B) The detailed alignment between miR-9 and the best-fitting sequence within the BK 3′UTR. The BK 3′UTR and miR-9 5′ nucleotides #1 through #12 have perfect complementarity (including the miR-9 seed sequence: nucleotides #2–8, depicted within box). Additionally, BK 3′UTR anchoring adenosines (red) flank the seed sequence. Bases paired by Watson-Crick bond are depicted by a large black oval, G:U pairs, by two dots.

(C) miR-9 is expressed in alcohol-relevant brain regions.

Error bars represent SEM.

miR-9 is considered a brain-specific miRNA important for neurogenesis (Krichevsky et al., 2003), but there is conflicting evidence for its expression in adult brain (Farh et al., 2005; Wu et al., 2006). We establish, using mature miR-9-specific primers (Figure S4), that miR-9 is expressed in alcohol-relevant brain regions, including SON and striatum (Figure 3C). Using the same primers, serial dilutions of synthetic miR-9 (Figure S4), and individually dispersed SON neurons (Figures 4A and 4B) and striatal neurons (Figure 4C), we determined that a single neuron expressed miR-9 at the level of ~300 mature miR-9 molecules per SON neuron (Figures 4D and 4E) and ~600 per striatal neuron (Figure 4F). Alcohol increased expression of miR-9 in both SON (Figure 4E) and striatal (Figure 4F) neurons on a timescale comparable to the downregulation of BK mRNA (Figure 2). These results suggest that miR-9 can mediate alcohol-mediated posttranscriptional regulation of neuronal BK mRNA.

Alcohol Downregulates only BK 3′UTRs with miR-9 MREs

Since miRNA targets 3′UTRs, one possible explanation for the selective destruction of transcripts is the presence of multiple 3′UTRs (3′UTR heterogeneity) with only some of them containing miR-9 MRE. 3′UTR heterogeneity is a common feature of many genes (Hughes, 2006), and can result from (1) alternative

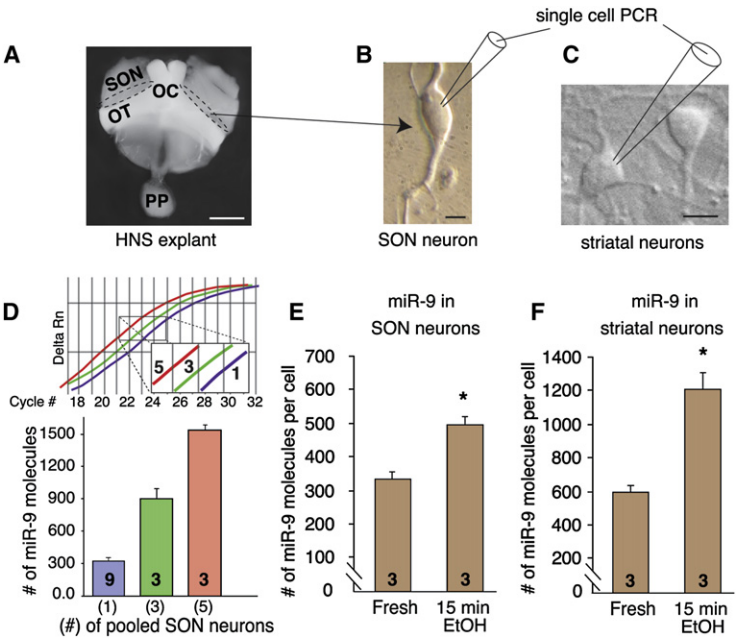

Figure 4. miR-9 Is Regulated by Alcohol on a Single-Neuron Level

(A) Supraoptic nuclei (dotted line) of the HNS explant were the source of the dispersed, single neurons. SON, supraoptic nucleus; OC, optic chiasm; OT, optic tract.

(B) Single-cell PCR was performed using dispersed SON neurons to determine miR-9 levels in individual SON neurons.

(C) Medium spiny neurons were isolated from the striatum, cultured, and used for single-cell real-time PCR. For both neurons, the cell body content was aspirated into the micropipette in RNase-free conditions.

(D) Single-cell real-time PCR for miRNA detection indicates miR-9 levels in pooled and individual SON neurons (inset shows an example of the real-time PCR plot). Both approaches indicate that individual SON neurons contain approximately 300 miR-9 molecules.

(E) Alcohol upregulates miR-9 in SON neurons on a timescale similar to that of BK mRNA downregulation (see Figure 2).

(F) Similarly, alcohol rapidly doubles the expression of miR-9 in striatal neurons.

Number on each bar represents n value. Numbers in parentheses in (D) represent number of neurons pooled. Scale bars in (A) = 1 mm, (B) = 10 μm, (C) = 10 μm. Error bars represent SEM.

polyadenylation within the same 3′UTR, and/or (2) the presence of multiple 3′UTRs. Alternative polyadenylation signal (PAS) sites can produce mRNAs with 3′UTRs of different lengths (Legendre et al., 2006). We observed that the rat BK 3′UTR (AF135265) has two potential PAS sites bracketing miR-9 MRE, which potentially could produce a long 3′UTR with a miR-9 MRE, and a shorter 3′UTR lacking the miR-9 MRE (Figure S6).

3′ rapid amplification of cDNA ends (3′RACE) indicated that this is unlikely. We detected only the full-length version of this 3′UTR in fresh tissue (Figure 5A, lower band, Figure S6). Surprisingly, we observed two additional larger bands (Figure 5A). Indeed, two novel, longer BK 3′UTRs have recently been cloned (Beisel et al., 2007) in rat inner ear hair cells. We determined that they are expressed in neurons and constructed a schematic summarizing BK α 3′UTR heterogeneity (Figures 5B and S5) using a rat genome database to determine chromosomal position of 3′UTR sequences, and 3′UTR sequence described by us and others (Beisel et al., 2007). The three different 3′UTRs associated with BK α are a result of alternative splicing in the 5′ end of the CDS and the 3′UTR region. Despite partial homology of one of the new 3′UTRs (2.2) with the previously known 3′UTR (2.1), only 3′UTR-2.1 has the miR-9 MRE (Figures 5B and 5C).

We next established, using specific primers to each 3′UTR (Figures S7 and S8), that although all three 3′UTRs are present in the SON, their relative amounts vary substantially (Figure 5D). 3′UTR-2.1 was the most abundant (~90% of total 3′UTR), 3′UTR-2.2 was less abundant (~10% of total 3′UTR), while 3′UTR-1.0 was hardly detectable (Figure 5D). Therefore, we determined alcohol regulation of expression of the two most abundant 3′UTRs: 3′UTR-2.1 and -2.2. Alcohol did not affect the expression of 3′UTR-2.2; however, it caused a dramatic downregulation of 3′UTR-2.1 (Figure 5E), corresponding temporally to the upregulation of miR-9 expression (Figures 4E and 4F) and to the downregulation of specific BK mRNA variants (Figure 2).

Thus, we observed that alcohol differentially affects transcripts with specific 3′UTRs, similar to its differential regulation of transcripts with distinct exonal concatenations (ALCOREX versus STREX) located in the protein CDSs. These data led us to hypothesize that association of specific CDS exons with specific 3′UTRs is not coincidental. Indeed, we observed that CDS exons associate with 3′UTRs in a deliberate manner: STREX exon was associated with both 3′UTRs in very similar amounts, while ALCOREX exon was exclusively associated with 3′UTR-2.1 (Figure 5F).

To further test the hypothesis that miR-9 downregulates BK mRNA by binding directly to the BK mRNA 3′UTR-2.1 region, we constructed BK plasmids containing the rat BK 3′UTR region with or without miR-9 MRE (miR-9 MRE$^+$, miR-9 MRE$^-$, respectively; based on the AF135265 sequence) and transfected HEK293 cells (which lack endogenous miR-9 and BK) with each of these constructs in the presence or absence of miR-9 and miR-135b (a miRNA with poor complementarity to BK mRNA; Figure 5G). This approach revealed that miR-9 MRE in the BK 3′UTR is required for miR-9-specific downregulation of BK mRNA, as determined by real-time PCR (Figure 5H).

BK mRNA Splice Variants Encode Channels with Varied Alcohol Responsiveness

To determine whether reorganization of BK transcript profile can contribute to the development of alcohol tolerance, we used heterologous expression to measure alcohol's effect on activity of individual BK splice variants: INSERTLESS, ALCOREX, and STREX.

Ion flux through a channel is determined by (1) conductance of a single channel, (2) its open probability (the fraction of the time the channel remains open), and (3) its density (the number of channels in the plasma membrane). In neurons, alcohol changes the open probability (NP_o) of BK channels (sensitivity) and their density, but not their conductance (Pietrzykowski et al., 2004).

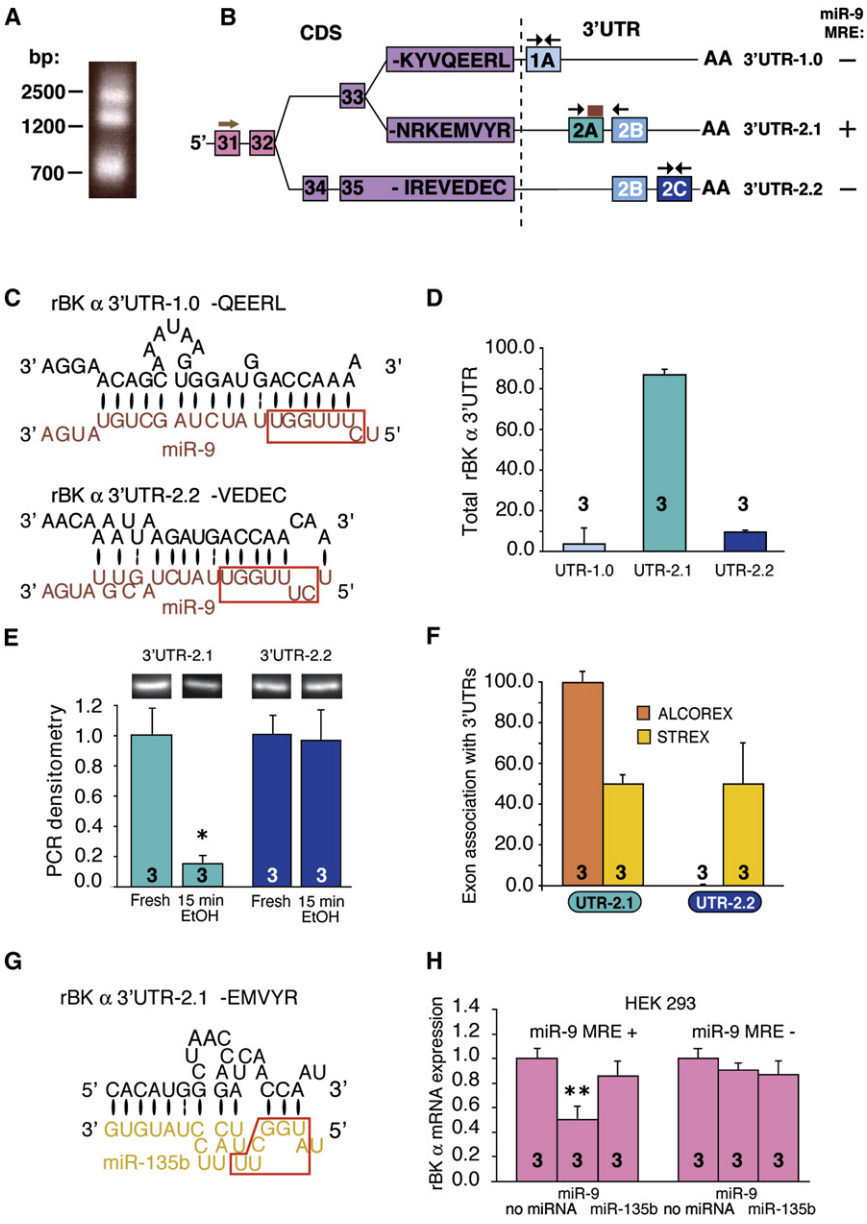

Figure 5. miR-9 Controls Expression of Alternatively Spliced BK mRNA Variants by Binding to Specific BK 3′UTR (-2.1)

(A) Agarose gel electrophoresis of BK 3′RACE products reveals the presence of three possible 3′UTR regions of BK mRNA. (B) Schematic constructed based on our results. Rat BK gene information (rat Ensembl; Hubbard et al., 2007) and data from reference (Beisel et al., 2007) show options for exonal assembly of the 3′ end of the coding sequence (CDS) and 3′UTR. Numbers in boxes represent exons. Letters represent the eight last amino acid residues characteristic for each BK isoform. The modular structure of 3′UTR is depicted. Note that the latter part of 3′UTR-2.1 and the former part of 3′UTR-2.2 are homologous (see also Figure S5). Note also that among three BK 3′UTR regions, a miR-9 MRE is present on 3′UTR-2.1 (brown box), but not on the two others. An arrow above exon 31 indicates position of 3′-RACE forward primer; black arrows in each 3′UTR region show position of a pair of real-time PCR primers used to detect and quantify individual 3′UTRs. (C) A detailed juxtaposition of 3′UTR-1.0 and 3′UTR-2.2 with miR-9 shows poor binding of miR-9 to 3′UTR-1.0 and 3′UTR-2.2. Bases paired by Watson-Crick bond are depicted by a large black oval; G:U pairs, by two dots. (D) Quantification of BK 3′UTRs in naive neurons. (E) Endpoint PCR and gel densitometry were used to quantify the alcohol effect on expression of 3′UTR-2.1 and -2.2 variants. Alcohol rapidly and profoundly downregulated BK 3′UTR-2.1, but not 3′UTR-2.2. Similar real-time PCR data not shown. (F) Real-time PCR data revealed that ALCOREX is associated with 3′UTR-2.1, while STREX is associated with both 3′UTR-2.1 and 3′UTR-2.2. (G) A detailed juxtaposition of BK 3′UTR-2.1 with miR-135b (control miRNA with poor complementarity to BK mRNA) used in (H). (H) miR-9 specifically downregulates only BK mRNA with the miR-9 MRE as determined using real-time PCR. HEK293 cells were transiently transfected with BK constructs with or without the 3′UTR region carrying miR-9 MRE (miR-9 MRE +, miR-9 MRE −, respectively) alone or together with one of two miRNAs (miR-9 or miR-135b). BK message is downregulated by miR-9 only when this message contains the 3′UTR sequence. miR-135b was unaffected in both cases.

p was determined by independent samples t test. Number on each bar represents n value. *$p < 0.05$, **$p < 0.01$. See also Figures S5–S8 for primer positions and Table S1 for their sequences. Error bars represent SEM.

The response of BK activity to acute alcohol challenge varied among isoforms (Figure 6A). The presence of the ALCOREX exon produced BK channels with the greatest alcohol sensitivity of those tested: the magnitude of potentiation was largest, and persisted the longest (Figure 6B). INSERTLESS BK channels were also potentiated by alcohol, but to a much smaller degree than ALCOREX, and returned to baseline faster (Figures 6A and 6B). In contrast, the mRNA variant encoding a BK channel with STREX produced channels which were resistant to alcohol (Figure 6B). Measurements made in cell-attached mode at lower Ca^{2+} levels, designed to provide optimal opportunity for alcohol to potentiate gating by lowering NP_o, confirmed the absence of alcohol potentiation in STREX channels (data not shown). Integrated measurement of the magnitude and the duration of alcohol potentiation (area under the curve in Figure 6B, see Experimental Procedures), which corresponds to ion flow through the channel, shows the differences in acute alcohol sensitivity of BK variants (Figure 6C).

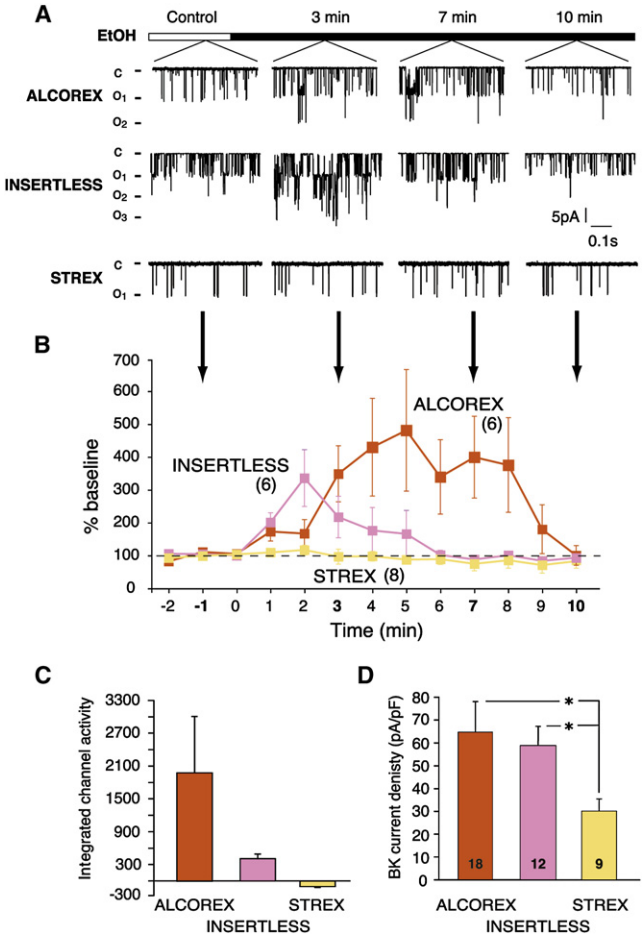

Figure 6. BK Channel Isoforms Encoded by Alternatively Spliced Messages Adapt Differently to Acute Alcohol Exposure

(A) Representative single-channel currents from BK-ALCOREX, BK-INSERTLESS, and BK-STREX recorded from transiently transfected HEK293 cells at different time points of a continuous alcohol exposure, using inside-out patches, in 135 mM symmetrical K^+ and 5 μM-free calcium.

(B) The presence of ALCOREX produced channels of greatest alcohol sensitivity in terms of both magnitude of potentiation by alcohol and persistence of that potentiation. INSERTLESS channels were also potentiated by alcohol, but to a lesser extent, and more transiently. STREX-containing channels were completely resistant to alcohol. Baseline activity is defined here as open probability (NP_o) before alcohol application. Number in parenthesis represents n value.

(C) Quantitation of (B) performed by measurement of the integrated area under the curve using the trapezoid formula (see Supplementary Materials).

(D) Innate current density of ALCOREX and INSERTLESS isoforms is higher than STREX current density. Current density was calculated as previously described (Pietrzykowski et al., 2004) as current/membrane surface area (determined from capacitance measurements).

Number on each bar represents n value. *$p < 0.05$. Error bars represent SEM.

Native current density differed among isoforms similarly to acute alcohol sensitivity, which contrasted ALCOREX and INSERTLESS with STREX (Figure 6D, Controls). ALCOREX and INSERTLESS BK channels had similar current density (65 pA/pF ± 13 pA/pF and 59 pA/pF ± 9 pA/pF, respectively), while STREX current density was lower (30 pA/pF ± 8 pA/pF) (Figure 6D).

Modeling the Consequences of miR-9 Regulation of BK Transcripts

Although we observed that alcohol, via miR-9, regulated alternatively spliced BK transcripts encoding channel isoforms exhibiting differing alcohol responsiveness, it was still unclear how these changes contribute to tolerance. The most abundant isoform (INSERTLESS) had only a mild response to alcohol, and its relative amount changed the least. In contrast, the relative amounts of isoforms with the least (STREX) and the greatest (ALCOREX) alcohol responses changed the most (Figure 2D), but their relative contribution to overall BK channel amount was rather small.

To determine how these changes in various transcripts might contribute to alcohol tolerance, we developed a computational model integrating our molecular biological and electrophysiological outcomes. Importantly, BK channels can function only as tetrameric assemblies of four monomers, where each monomer is encoded by an individual BK transcript. To assess the functional consequences of alcohol regulation of BK transcripts, we incorporated into the model published data describing differential effects of PKA on different BK tetramers (Tian et al., 2004), and our new data describing the relative functional weighting of monomers in determining alcohol sensitivity of assembled BK channel tetramers.

First, based on the quantities of individual BK transcripts encoding individual splice variant monomers (Figure 2), we calculated the probabilities of all possible combinations of monomers assembled into tetramers in both SON and striatal neurons (Equation 1, Supplementary Materials). We determined that in naive SON neurons four types of tetramer were most prevalent: ALCOREX/INSERTLESS heteromers (almost half of the total amount of the channel), INSERTLESS homomers (about one-third), INSERTLESS/STREX heteromers (about 10% of total amount), and ALCOREX/INSERTLESS/STREX heteromers (also about 10% of total amount) (Figure 7A).

Alcohol significantly changed the contribution of certain tetrameric assemblies to the total amount of BK channel. The most striking change was a several-fold drop in the ALCOREX/INSERTLESS assembly contribution, and a collateral increase in the INSERTLESS/STREX assembly. Modeling also predicted a concurrent moderate increase in INSERTLESS homomers. Interestingly, in naive striatal neurons the calculated distribution of BK channel assemblies differed from that in SON neurons, with a higher percentage of ALCOREX/INSERTLESS heteromers and ALCOREX homomers, and smaller amounts of STREX-containing assemblies and INSERTLESS homomers (Figure 7B). Nevertheless, similar to that in SON neurons, the alcohol-dependent shift away from ALCOREX-containing assemblies and toward STREX-containing assemblies was observed. Additionally, in striatal neurons, alcohol caused a larger increase in the INSERTLESS homomer.

After determining the tetrameric makeup of BK channels in neurons, we considered alcohol sensitivity of various assemblies. In Figure 6C we described different alcohol effects on different BK homomers (potentiation of INSERTLESS and ALCOREX by alcohol versus STREX alcohol resistance). Interestingly, recent reports (Tian et al., 2001, 2004) described a similar differential effect, with respect to the action of PKA phosphorylation on BK

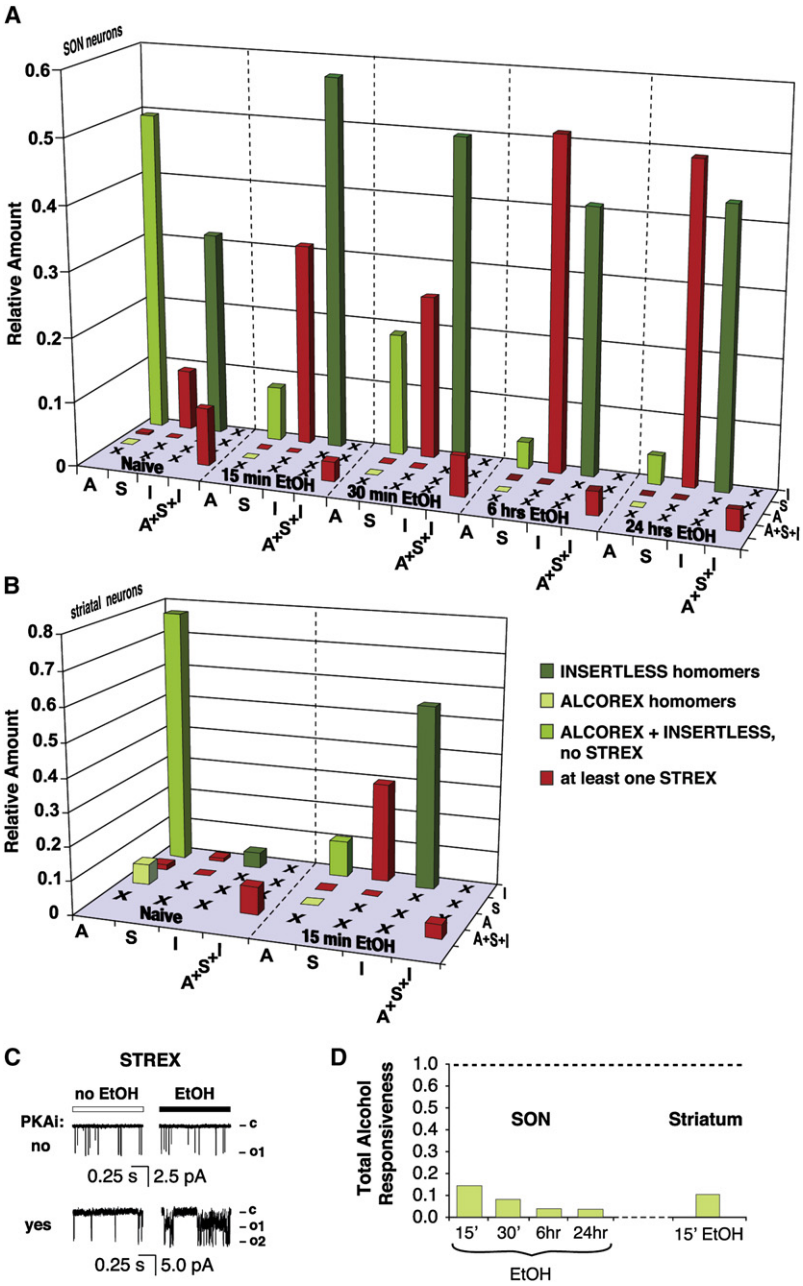

Figure 7. Mathematical Modeling Indicates that Shift in BK Transcript Landscape Changes Tetrameric Makeup of BK Channels in Neurons, Decreases Overall BK Channel Responsiveness to Alcohol, and Causes the Development of Tolerance

(A) A matrix of BK splice variant monomer assembly created to estimate BK tetrameric assembly and to calculate their alcohol sensitivity in SON neurons. Height of columns corresponds to the amount of a tetramer as a fraction of the total BK channel amount. Color of a column represents level of BK potentiation by alcohol: light green, high potentiation; dark green, low potentiation; medium green, intermediate level of potentiation. In naive SON neurons the most abundant BK tetrameric assemblies were ALCOREX/INSERTLESS heteromers, INSERTLESS/STREX heteromers, ALCOREX/INSERTLESS/STREX heteromers, and INSERTLESS homomers. Alcohol decreased the amount of ALCOREX-containing assemblies (ALCOREX/INSERTLESS and ALCOREX/INSERTLESS/STREX heteromers) and increased the amount of INSERTLESS/STREX heteromers. The amount of INSERTLESS homomers also increased, but to a lesser extent. A, ALCOREX; S, STREX; I, INSERTLESS.

(B) A similar matrix was created for striatal neurons. In naive striatal neurons the vast majority of BK channels consisted of ALCOREX/INSERTLESS heteromers, with very low amounts of ALCOREX/INSERTLESS/STREX heteromers, ALCOREX homomers, and STREX homomers. Alcohol decreased the level of ALCOREX/INSERTLESS heteromers and increased the level of STREX/INSERTLESS heteromers and INSERTLESS homomers. Color code as in (A). A, ALCOREX; S, STREX; I, INSERTLESS.

(C) Alcohol inhibition of STREX is PKA dependent. In the absence of PKA inhibitor (14-22 Amide), alcohol exposure decreased activity of BK channels containing STREX (upper traces). Block of PKA BK-STREX phosphorylation allowed potentiation of the channel by alcohol (lower traces).

(D) The development of alcohol tolerance of the entire BK channel population in SON and striatal neurons is expressed as a ratio of BK alcohol responsiveness after alcohol exposure to BK alcohol responsiveness before alcohol exposure (Equation 2, Supplementary Materials). Only the outcomes of the most conservative calculation (alcohol sensitivity of ALCOREX/INSERTLESS heteromers equals alcohol sensitivity of the INSERTLESS homomers) are shown. Fifteen to thirty minutes of alcohol exposure profoundly decreased total BK alcohol responsiveness in both SON and striatal neurons. Longer alcohol exposures augmented this effect. Assuming equal alcohol sensitivity of ALCOREX/INSERTLESS heteromers and ALCOREX homomers, the decrease in responsiveness is even more profound (data not shown). These changes are fully compatible with the development of alcohol tolerance.

homomer activity: i.e., INSERTLESS potentiation and STREX inhibition. Moreover, the STREX channel was inhibited even when only a single STREX monomer was present within a heteromer. We reasoned that if STREX alcohol resistance was mediated by PKA, then similar dominance of the STREX insert (i.e., alcohol resistance) within heteromers might occur. Using a specific PKA inhibitor (14-22 Amide), we found that the alcohol resistance of STREX is indeed PKA dependent (Figure 7C). Thus, we assumed that any BK channel containing a STREX monomer is alcohol resistant to the same degree that a STREX homomer is.

Therefore, BK tetrameric groups can be placed into four categories of alcohol sensitivity (color-coded in Figures 7A and 7B): (1) assemblies with at least one STREX, yielding alcohol-resistant channels (red), (2) ALCOREX homomers, highly activated by alcohol (light green), (3) INSERTLESS homomers, with low potentiation by alcohol (dark green), and (4) ALCOREX/INSERTLESS heteromers, for which the relative alcohol sensitivity is unknown (medium green).

In the final step, we calculated the overall responsiveness of the composite BK channel population to alcohol (Equation 2,

Table 1. Additional, CNS-Specific miR-9 Targets Relevant to Alcohol Actions

	TargetScan miR-9 Target Genes				
#	TargetScan #	Symbol	Score	Name	Effect of 15 min., 20 mM EtOH Exposure
1	4	*KCNJ2*	−0.80	potassium inwardly-rectifying channel, subfamily J, member 2	no change
2	35	*GABRB2*	−0.50	gamma-aminobutyric acid (GABA) A receptor, beta 2	− −
3	93	*KCNMB2*	−0.39	BK channel, subfamily M, beta member 2	− −
4	95	*CLOCK*	−0.39	clock homolog (mouse)	− −
5	101	*PPARA*	−0.38	peroxisome proliferator-activated receptor alpha, 2 isoforms	− −
6	117	*NPY2R*	−0.36	neuropeptide Y receptor Y2	no change
7	132	*SYNJ1*	−0.35	synaptojanin 1	− −
8	144	*TGFBR2*	−0.34	transforming growth factor, beta receptor II (70/80 kDa)	+ + +
9	157	*HDAC5*	−0.33	histone deacetylase 5	−
10	243	*CACNB1*	−0.26	calcium channel, voltage-dependent, beta 1 subunit	− −
11	273	*NOX4*	−0.24	NADPH oxidase 4	+
12	346	*DRD2*	−0.20	dopamine receptor D2	− − −
13	749	*PRKCA*	−0.04	protein kinase C, alpha	no change
14	not a target	*KCNMB1*	X	BK channel, subfamily M, beta member 1	no change

A total of 13 targets were determined using TargetScan. For each target, its TargetScan-specific number, symbol, total context score, full name, and alcohol effect is shown. A more negative score is associated with a more favorable miR-9 binding. Classification of alcohol effect: ±25% (no change), 26%–50% upregulation (+), 51%–75% upregulation (+ +), 76%–100% upregulation (+ + +), 26%–50% downregulation (−), 51%–75% downregulation (− −), 76%–100% downregulation (− − −).

Supplementary Materials) as a measure of alcohol tolerance. Since we did not know the level of ALCOREX/INSERTLESS heteromer potentiation by alcohol (category #4), we ran the model through two extreme variations: alcohol potentiation of the ALCOREX/INSERTLESS heteromer is (1) as low as the INSERTLESS homomer, or (2) as high as the ALCOREX homomer. These two extremes provided upper and lower limits of the possible level of ALCOREX/INSERTLESS potentiation by alcohol, and allowed us to determine the potential range of the decrease in total alcohol responsiveness of the BK channel in neurons. Clearly, even in the most conservative calculation (variation #1), a 15–30 min exposure to alcohol resulted in BK channels with only 15% of naive neuron responsiveness, exhibiting almost complete tolerance to alcohol (an even more profound effect was evident in variation #2, not shown), in both SON and striatal neurons (Figure 7D). Moreover, longer alcohol exposures caused an augmentation of alcohol tolerance (Figure 7D; 6 hr, 24 hr).

Additional miR-9 Targets Important for Alcohol Actions in the CNS

Since miRNAs are known to affect multiple targets (Ambros, 2004; Bartel, 2004), we wondered whether alcohol regulated other miR-9 targets. A search by miRNA target prediction software, TargetScan (Release 4.1; Grimson et al., 2007), retrieved 826 predicted miR-9 targets (as of January 18, 2008). From this catalog we selected a list of targets (Table 1) with known roles in alcohol's actions and documented expression in the CNS (12 total; see Supplementary Materials).

Expression of 8 out of the 12 targets was downregulated by alcohol, two targets were upregulated, and the expression of two other targets was unchanged (Table 1). All ten regulated targets play important roles in different aspects of neuronal physiology (Supplementary Materials), including excitability (GABRB2, KCNMB2, CACNB1, and DRD2), gene expression (CLOCK, PPARA, TGFBR2, HDAC5, and CACNB1), lipid metabolism (PPARA), and function of presynaptic terminals (GABRB2,

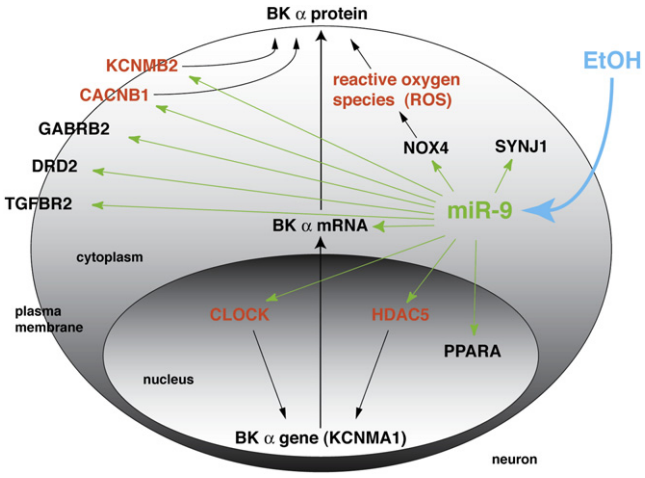

Figure 8. Additional Targets Involved in Alcohol Actions in the CNS Are Modulated by miR-9

A schematic depicts a simplified network of 11 miR-9 targets important in alcohol actions in the CNS, centered on BK expression pathway. miR-9 targets mRNA molecules in the cytoplasm as shown for BK. For clarity, mRNAs of other targets were omitted. Shown are functional proteins of these targets to indicate their main cellular localization. miR-9 targets plus reactive oxygen species (ROS) known to affect BK gene expression or protein function are in bold. Dotted lines show additional pathways of other miR-9 targets, which could potentially regulate BK. For description of symbols see Table 1.

SYNJ1, CACNB1, and KCNMB2). Moreover, some of these targets are known to directly regulate BK channel expression and/or function, while others could potentially contribute to that regulation (Figure 8, Supplementary Materials).

DISCUSSION

Here, we describe a mechanism of alcohol tolerance involving miRNA. Clinically relevant alcohol concentrations rapidly increase miR-9 levels in central neurons. Only one out of three BK channel 3′UTRs contains an MRE with complementarity to miR-9. Thus, there is a selective degradation of message, resulting in reorganization of BK splice variant profile. Modeling, based upon experimental results indicating the baseline levels of the various transcripts, combined with the differential downregulation of BK transcripts by alcohol, allowed a determination of the post-exposure distribution of tetrameric channels. Further, when the model included data (obtained from expression studies) describing differential alcohol responsiveness within this channel population, the resulting prediction was consistent with the development of tolerance. We show also that alcohol, via miR-9 upregulation, regulates additional targets, suggesting a central role for miR-9 in alcohol's actions in the CNS.

miRNA Posttranscriptional Regulation of Transcript Stability via 3′UTR Heterogeneity as a Mechanism of Alcohol Tolerance

In this paper we demonstrate that, in neurons, several different splice variants of the BK channel α subunit are constructed by combining various concatenations of protein coding exons (e.g., STREX alone, STREX and ALCOREX, ALCOREX alone, INSERTLESS) with different 3′UTRs. Since the 3′UTR serves as a major regulatory element of mRNA expression, stability, turnover, and translation efficiency (Garneau et al., 2007; Hughes, 2006), this mechanism allows BK channel plasticity. We showed that, regardless of partial homology of two BK 3′UTRs, only one of them was regulated by alcohol, due to the selective presence of the miR-9 MRE. These results suggest that even subtle differences in miRNA binding profiles can have profound consequences on gene expression regulation. Because many mammalian genes express alternative UTRs (Hughes, 2006), this mechanism of neuronal plasticity is likely to be of great importance in adaptation to many exogenous or endogenous factors. This mechanism might also contribute to alcohol's effects in humans, since the miR-9 MRE in BK mRNA 3′UTR is conserved between rodents and humans (Figure S9).

We observed that the ALCOREX exon is always associated with 3′UTR-2.1, but not 3′UTR-2.2. This is in contrast to the STREX exon, which can associate with either 3′UTR. It will be of interest to understand the mechanisms controlling the association of particular exonal concatenations in the CDS of mRNA with particular 3′UTRs.

Both the rise in miR-9 and the concomitant BK mRNA downregulation were well underway within 15 min of alcohol exposure. Our data indicate that the fast regulation of BK mRNA by alcohol is likely occurring through the posttranscriptional downregulation of pre-existing mRNA variants, via a miRNA-regulated mechanism, which directs them to a degradation pathway. Once destined for destruction, mRNA is degraded quickly (Garneau et al., 2007), which could explain the rapidity of the process observed here.

The rapid upregulation of mature miR-9 by alcohol may result from an increase in miR-9 gene expression, via production of new primary miR-9 gene transcript (pri-miR-9) in the nucleus, and/or accelerated conversion of immediate pre-miR-9 precursors (pre-miR-9) into mature miR-9, possibly by increased activity of enzymes such as Dicer (Bernstein et al., 2001) and/or Armitage (Cook et al., 2004; Tomari et al., 2004).

Interestingly, the cell-to-cell consistency of our single-cell measurements suggests precise regulation of miR-9 levels in neurons. One siRNA molecule with miRNA-like features can degrade several target molecules in a very short time (Hutvagner and Zamore, 2002). Thus, the cell needs to tightly control levels of miRNA, because even subtle changes in miRNA expression could have profound effects on the expression of miRNA targets. This could also explain why we observe such a massive downregulation of BK mRNA in a very short period of time, with relatively slight (2-fold) upregulation of miR-9.

Functional Consequences of miR-9 Regulation of BK mRNA

Blocking the actions of miR-9 with miR-9 inhibitor, or more specifically, blocking an interaction between miR-9 and 3′UTR-2.1 with miRNA target protector (Choi et al., 2007), could help to establish the consequences of miR-9 regulation of BK mRNA. However, these RNAi approaches would be unlikely to produce interpretable data. There are multiple molecular targets for miR-9 (Figure 8), some directly influencing BK gene expression or protein function, raising the possibility of a complicated and

dynamic response pattern. Additionally, we have evidence for multiple forms of BK tolerance, based on several mechanisms (described below), some miR-9 independent. Therefore, in place of the RNAi approach, we used computational modeling. Although in both SON and striatal neurons INSERTLESS is the most abundant transcript, while STREX and ALCOREX are less abundant, a linear extrapolation from the amounts of monomeric transcripts to the profile of the resulting channel population would be misleading. It is necessary to consider the unequal contribution of individual monomers within the tetrameric BK structure to both BK channel activity and its alcohol sensitivity. For example, although INSERTLESS predominates in amount, its influence on alcohol sensitivity can be diminished because of the dominant nature of STREX in a tetramer. Our modeling approach considered this feature, and posits a mechanism by which the reorganization of BK transcripts results in the development of alcohol-tolerant BK current. Of course, as with all such modeling, the results are suggestive rather than definitive. Nevertheless, they are consistent with the development of alcohol tolerance previously reported in neuronal cells (Pietrzykowski et al., 2004).

Why does the neuron goes to such remarkable lengths to counteract the consequences of the potentiating effects of alcohol on BK, by both (1) minimizing the potentiation of gating by ethanol and (2) dramatically reducing message level and current density of the channel? BK channels play a central role in the regulation of neuronal excitability, controlling neurotransmitter release and the shaping of action potentials in many brain regions. On a cellular level, BK serves as an integrator of regulatory processes, because it is activated by both voltage and intracellular calcium (Salkoff et al., 2006). This channel has a very high conductance, and sustained activation would likely have serious consequences for nervous system function. Because of its large conductance, potentiation by alcohol of even the small population of channels remaining could be undesirable. A related question is how the nervous system retains normal function in the face of such a large reduction of BK. Indeed, BK knockout mice, while able to survive, are seriously impaired (Sausbier et al., 2004). It is possible that the overall, relative shift to the STREX isoform, which has relatively high calcium sensitivity (Xie and McCobb, 1998), helps to maintain adequate levels of BK activity while minimizing the effects of alcohol.

miR-9 Can Serve as a Master Switch for Alcohol Effects in the CNS

The miR-9-dependent downregulation of target mRNA and reorganization of its splice variants may represent a general mechanism of neuronal adaptation to alcohol. We determined that miR-9 affects at least ten additional targets besides BK with documented roles in alcohol actions in the CNS (Table 1, Supplementary Materials), some of which can undergo alternative splicing (SYNJ1 and DRD2). Moreover, we observed that the expression of these targets is regulated by brief alcohol exposure. These additional downstream targets of miR-9 are involved in major aspects of neuronal physiology (for details see Supplementary Materials) including excitability, regulation of gene expression, metabolism of lipids, and function of presynaptic terminals.

miRNA interaction with its mRNA target usually results in downregulation of that target, or translation repression. Surprisingly, in our study two miR-9 targets (TGFBR2 and NOX4) were upregulated. A recent report gives an example of a miRNA species (Let-7) switching its target expression from repression to activation depending upon cell-cycle phase (Vasudevan et al., 2007). Thus, upregulation of genes by miRNA is possible. Activation of miR-9 by alcohol might cause upregulation of some targets and concomitant downregulation of others.

miRNA Involvement in Mechanisms of Tolerance

In addition to the route for adaptation provided by posttranscriptional regulation of ion-channel mRNA stability by miR-9, other cellular mechanisms contribute to the development of BK alcohol tolerance. Alcohol tolerance is a complex phenomenon and has several forms, e.g., acute, rapid, or chronic tolerance, depending upon the length of alcohol exposure and the consequent involvement of cellular mechanisms. Our working hypothesis is that acute tolerance occurs via posttranslational effects of the drug directly on the existing channel, which allow rapid alterations that are relatively short-lived. In contrast, longer forms of tolerance can be attributable to other mechanisms acting upstream of posttranslational changes. They include posttranscriptional regulation of stability of existing transcripts (e.g., via miR-9), followed by protein synthesis and insertion of new functional channels into the plasma membrane. These posttranscriptional mechanisms may result in a slower-developing, but longer-lived adaptation to alcohol exposure. Thus, although the effects of alcohol on upregulation of miR-9 and subsequent subtractive rearrangement of the BK mRNA isoform landscape are surprisingly fast, the functional consequences will be delayed, dependent upon translation of the message and subsequent delivery and insertion of the channel protein into the neuronal plasma membrane.

Moreover, additional upstream pathways, specifically those regulating transcription of BK gene expression (Figure 8), might be involved after longer alcohol exposures. Previous studies of regulation of BK channel alternative splicing by, for example, stress or activity (McCobb et al., 2003; Xie and McCobb, 1998) have focused on splicing decisions during production of pre-mRNA from the gene, with a time frame of weeks. Recently, it has been discovered that BK channel transcription is under epigenetic control (Wang et al., 2007). Could alcohol act via a similar mechanism and could miRNA be involved? For example, alcohol might modulate alternative splicing during transcription and pre-mRNA exonal assembly, skipping the ALCOREX exon and excluding it from the final variants of BK mRNA. Considering the length of the BK gene (~690 kb, *AC 000083*) and the elongation rate of polymerase II (~30 nt/s; Alberts et al., 1994), production of modified BK transcripts would be expected to take at least 6 hr. Indeed, our data indicate the augmentation of tolerance after at least 6 hr of exposure (Figure 7D). It has recently been shown also that another miRNA species (miR-133) can regulate alternative splicing during gene transcription in muscle (Boutz et al., 2007). We also observed that in HEK293 cells, which lack miR-9, several hours of alcohol exposure decreases BK current density in the plasma membrane (data not shown). Therefore, it is possible that for longer alcohol exposures, additional

mechanisms of gene expression, possibly including other miRNA species, could be regulated by alcohol.

We show here also that INSERTLESS and ALCOREX exhibit different patterns of alcohol sensitivity and channel density. The molecular mechanisms underlying these differences are, at this point, unclear. These two BK isoforms do not have apparent differences in consensus phosphorylation sequence (NetPhos 2.0, data not shown), making this an unlikely explanation for the difference in short-term adaptation. Possibly, the insertion of ALCOREX produces a conformational change in the BK polypeptide, resulting in a change in potentiation by alcohol. The ALCOREX insert is located just ten residues upstream of the calcium bowl, and this physical proximity could potentially change the interaction of the calcium bowl with calcium ions and subsequently affect channel activity. Indeed, as reported by Ha et al. (2000), the BK channel with an insert encoded by exon 29 ($rSlo_{27}$ = ALCOREX) activates faster than BK channels without this insert, dependent upon calcium concentration. Previous data (Dopico et al., 1998) indicate that alcohol acts as a partial agonist of the BK channel, with calcium as a full agonist. Another intriguing possibility derives from recent work, which has shown that bilayer thickness can affect alcohol's potentiation of BK activity (Yuan et al., 2007), allowing speculation that if the different isoforms reside in different membrane domains (e.g., lipid rafts), this could lead to different adaptation patterns.

In summary, our data provide an elegant mechanism of alcohol tolerance involving miRNA regulation of mRNA transcript stability. The process described in this study may represent a general mechanism of neuronal adaptation to alcohol, with miR-9 playing a pivotal role in a complex regulatory network. These miR-9-dependent mechanisms may have roles in neuronal plasticity extending beyond adaptation to drugs of abuse, and may have the potential to uncover novel therapeutic targets.

EXPERIMENTAL PROCEDURES

Explant Preparation and Culture

Rat SON explants were prepared as previously described (Pietrzykowski et al., 2004). In transcription block experiments, water-soluble Actinomycin D (10 μg/μl, final concentration, Sigma) was added to the culture medium for the indicated time.

Primary Striatal Cultures

Cultures of dissociated rat striatal neurons were prepared using a modification of a protocol described by Leveque et al. (2003) using postnatal day eight Sprague-Dawley rat pups. All experiments were performed on neurons that were 14–21 days in culture.

Alcohol Treatment

Alcohol concentration in explant and striatal cultures was obtained as described previously (Pietrzykowski et al., 2004). Alcohol levels were measured using a GM7Analyser (Analox Instruments Inc., MA).

Molecular Biology

For a full description of molecular biology methods, including RNA isolation from explants and striatal cultures, endpoint PCR, cloning, sequencing, 3′RACE, real-time PCR detection of BK, miRNA and additional miR-9 targets, single-cell real-time PCR, and plasmid preparation, see the Supplementary Materials.

HEK293 Cell Transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and, for transfection, confluent HEK293 cells were plated on 60 mm Petri dishes and transfected using PolyFect transfection reagent (QIAGEN, Balencia, CA) complexed with BK variants (BK-INSERTLESS, BK-ALCOREX, BK-STREX) in pVAX vector (Invitrogen) cDNAs.

In miRNA experiments, BK 3′UTR+/− plasmids (0.0442 fmol/well) were transfected alone, or with one of two miRNA precursors (miR-9 and miR-135b) (200 pmol/well). BK mRNA was isolated for quantitation 24 hr after transfection.

For electrophysiological analysis cells were transfected with a BK variant (BK-ALCOREX, BK-STREX, or BK-INSERTLESS in pVAX) together with the expression plasmid (πH3-CD-8) for the α subunit of the human CD-8 lymphocyte surface antigen (GeneBank M12824), allowing the identification of transfected cells with CD-8-antibody-coated beads (Dynal/Invitrogen) as previously described (Martin et al., 2004).

Electrophysiology

For a full description of electrophysiological recordings, including whole-cell and single-channel recordings, current density, and alcohol sensitivity, see Supplementary Materials.

Computational Modeling of the miR-9 Regulation of BK Transcripts

For a full description of computational modeling of miR-9 regulation of BK transcripts, including assumptions and equations, see Supplementary Materials.

Statistical Analysis

For a full description of statistical analysis see Supplementary Materials. $p < 0.05$ was defined as statistically significant. n = number of independent biological preparations. All error bars represent standard error of the mean (SEM). Statistical analysis was performed using statistical package for the social sciences.

SUPPLEMENTAL DATA

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/59/2/274/DC1/.

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