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BK channel $\beta 1$ subunit contributes to behavioral adaptations elicited by chronic intermittent ethanol exposure

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Abstract

Background—Large conductance, calcium- and voltage-activated potassium (BK) channels regulate neuronal excitability and neurotransmission. They can be directly activated by ethanol and they may be implicated in ethanol dependence. In the present study, we sought to determine the influence of the auxiliary $\beta 1$ and $\beta 4$ subunits on ethanol metabolism, acute sensitivity to ethanol intoxication, acute functional tolerance, chronic tolerance, and handling-induced convulsions during withdrawal.

Methods—Motor coordination, righting reflex and body temperature were evaluated in BK $\beta 1$ and $\beta 4$ knockout, heterozygous and wildtype mice following acute ethanol administration. Chronic tolerance and physical dependence were induced by chronic intermittent inhalation of ethanol vapor.

Results—Constitutive deficiency in BK $\beta 1$ or $\beta 4$ subunits did not alter the clearance rate of ethanol, acute sensitivity to ethanol-induced ataxia, sedation and hypothermia, nor acute functional tolerance to ataxia. BK $\beta 1$ deletion reduced chronic tolerance to sedation, and abolished chronic tolerance to hypothermia, while BK $\beta 4$ deletion did not affect these adaptations to chronic ethanol exposure. Finally, the absence of BK $\beta 1$ accelerated the appearance, while the absence of BK $\beta 4$ delayed the resolution, of the hyperexcitable state associated with ethanol withdrawal.

Conclusions—Altogether, the present findings reveal the critical role of BK $\beta 1$ in behavioral adaptations to prolonged, repeated ethanol intoxication.

Keywords

alcohol; knockout mice; intoxication; dependence; vapor chambers

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Conflict of interest statement. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Introduction

Large conductance calcium- and voltage-activated potassium (BK) channels regulate firing activity and neurotransmitter release in neurons (N'Gouemo, 2014; Wang et al., 2014; Wang, 2008). Ethanol can potentiate BK-mediated currents in the presence of calcium through direct binding to the C-terminal domain of the pore-forming α subunit and subsequent allosteric facilitation of channel gating (Bukiya et al., 2014). The ability of ethanol to activate BK channels has been shown in a variety of *in vitro* and *ex vivo* systems, but the contribution of this action to the behavioral effects of ethanol *in vivo* remains unclear (see Bettinger and Davies, 2014; Dopico et al., 2014 for reviews).

Studies conducted in invertebrate models provided seemingly contradictory evidence for a role of ethanol-induced potentiation of BK currents in the locomotor effect of ethanol (*Caenorhabditis elegans*, Davies et al., 2003) or in rapid tolerance to the sedative effect of ethanol (*Drosophila melanogaster*, Cowmeadow et al., 2005). Testing in knockout mice has shown that the auxiliary subunit $\beta 4$ prevents rapid tolerance to the effects of ethanol on BK currents, neuronal firing and locomotor activity, and that $\beta 1$ and $\beta 4$ modulate ethanol consumption, particularly in dependent mice (Kreifeldt et al., 2013; Martin et al., 2008). In human populations, the gene encoding BK α (*KCNMA1*) has been repeatedly detected by genome-wide linkage and association studies of the level of response to alcohol and alcohol dependence, although the signals were statistically weak (Edenberg et al., 2010; Kendler et al., 2011; Schuckit et al., 2005). Most convincingly, both *KCNMA1* and the gene encoding $\beta 1$ (*KCNMB1*) were identified as part of a 39-gene functional network selectively associated with a higher risk of developing alcohol dependence (Han et al., 2013). Understanding the molecular and cellular mechanisms underlying the implication of BK channels in alcohol dependence could lead to new opportunities for medication development.

Association of auxiliary β subunits to the pore-forming α subunit can alter the ability of ethanol to potentiate BK currents (Bukiya et al., 2009; Feinberg-Zadek and Treistman, 2007; Martin et al., 2004). One way to probe the role of BK channel activation in the behavioral effects of ethanol is therefore to characterize the phenotype of mice lacking one of these β subunits. In the present study, we investigated the influence of BK $\beta 1$ and $\beta 4$ on acute sensitivity to ethanol intoxication, acute functional tolerance (i.e., the ability to develop tolerance to the behavioral effects of ethanol within a single exposure, which is a risk factor for the development of alcohol-use disorders (Newlin and Thomson, 1990; Schuckit, 1980)), chronic tolerance, and hyperexcitability during ethanol withdrawal. Our findings highlight a critical role of BK $\beta 1$ in behavioral adaptations brought up by chronic intermittent exposure to ethanol.

Materials and Methods

Animals

BK $\beta 1$ and $\beta 4$ knockout (KO) mice were generated by homologous recombination (Brenner et al., 2005; Brenner et al., 2000b) and fully backcrossed on C57BL/6J background. BK $\beta 1$ and $\beta 4$ wildtype (WT), heterozygous (Het) and KO littermates were bred at The Scripps Research Institute in a temperature-controlled (22°C) controlled vivarium. Mice were

maintained on a 12 h/12 h light/dark cycle, and were acclimated for at least one week to changes in circadian cycle. All behavioral testing was performed during the dark phase. Food (standard rodent chow, Harlan Teklad, Frederick, MD, USA) and water were available *ad libitum* at all times. Only males were used in experiments. Mice were at least 10 weeks old when testing was conducted. All procedures were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by The Scripps Research Institute Institutional Animal Care and Use Committee.

Cohorts

BK β 1 mice—A first cohort (WT, n=4; Het, n=4; KO, n=4) was used to determine the rate of ethanol clearance. A second cohort (WT, n=10; Het, n=16; KO, n=11) was tested for ethanol-induced ataxia and acute functional tolerance. A third cohort (WT, n=7; Het, n=8; KO, n=5) was tested for ethanol-induced sedation and hypothermia and was then exposed to CIE (average blood alcohol levels [BALs] during CIE = 179.8 ± 5.5 mg/dl) in order to assess chronic tolerance to these effects, as well as handling-induced convulsions, 26 h into withdrawal.

BK β 4 mice—A first cohort (WT, n=4; Het, n=4; KO, n=4) was used to determine the rate of ethanol clearance. A second cohort (WT, n=12; Het, n=12; KO, n=12) was tested for ethanol-induced ataxia and acute functional tolerance. These mice were also tested for handling-induced convulsions 26 h into withdrawal from CIE (average BALs during CIE = 183.5 ± 7.0 mg/dl; 3 WT, 8 Het and 6 KO mice did not recover from CIE, thereby resulting in smaller group sizes post-CIE). A third cohort (WT, n=9; Het, n=8; KO, n=8) was tested for ethanol-induced sedation and hypothermia and was then exposed to CIE in order to assess chronic tolerance to these effects (average BALs during CIE = 237.6 ± 12.7 mg/dl; all mice recovered from CIE).

Ethanol metabolism

Mice were i.p. injected with 2 g/kg ethanol. Blood samples were collected from the tail vein in heparinized capillaries 45 min, 1.5 h and 3 h post-injection. BALs were measured using a GM7 analyzer (Analox Instruments, Lunenburg, MA).

Ethanol-induced ataxia and acute functional tolerance

Mice were first trained to balance on an accelerating rotarod for 12 trials (10 rpm per min acceleration, Roto-rod Series 8, IITC Life Sciences, Woodland Hills, CA). The effect of ethanol on motor coordination was tested at a fixed speed of 10 rpm and mice were required to stay on the rod for 30 sec, which all mice could achieve by the end of the training phase. All mice met this criterion prior to ethanol exposure. Mice were injected with ethanol (1.5 g/kg, i.p.) and were then tested every 15 minutes until they were able to meet the criterion again. When BK β 4 mice were tested, a 7-min time-point was added in order to better capture the ataxia duration for mice that recovered prior to 15 minutes. Once a mouse had regained motor coordination, retroorbital blood was sampled for BAL determination and the mouse was immediately injected with a second dose of ethanol (1.5 g/kg, i.p.). Rotarod testing was again conducted every 15 min (as well as 7 min post-injection for BK β 4 mice)

until the mouse had recovered. A second retroorbital blood sample was collected upon recovery. Acute functional tolerance was reflected by $BAL_2 > BAL_1$.

Ethanol-induced hypothermia

Body temperature was determined using a digital thermometer (probe inserted 1.5 cm into the rectum). Measures were taken prior to ethanol injection (4 g/kg, i.p.), as well as 60 min and 120 min post-injection.

Ethanol-induced sedation

The injection of ethanol (4 g/kg, i.p.) resulted in the loss of righting reflex (i.e. sleeping). The time at which each mouse regained its righting reflex (i.e. ability to return to a standing position when placed on its back) was recorded. Retroorbital blood was sampled at recovery from sedation for BAL determination.

Handling-induced convulsions

Handling-induced convulsions (HICs) were determined prior to ethanol injection (4 g/kg, i.p.), and 2, 6, 8, 12 and 24 h post-injection. When BK $\beta 4$ mice were tested, a 4-h time-point was added in order to better capture the emergence of HICs following the sedative phase. HIC scoring used a 7-point scale ranging from facial grimace to spontaneous severe tonic-clonic convulsions (Crabbe et al., 1991). It was performed by a single experimenter (AJR) who was blind to the genotype. Briefly, each mouse was picked up by the tail and, if lifting failed to elicit a convulsion, it was gently spun through a 180° arc by rubbing the tail between the thumb and forefinger. The 6–24 h area under the curve (AUC) was calculated to provide a global score of hyperexcitability.

Chronic intermittent ethanol vapor exposure

Mice were subjected to chronic intermittent ethanol (CIE) exposure to induce chronic tolerance to ethanol-induced sedation and hypothermia, as well as to exacerbate withdrawal-induced hyperexcitability and make HICs more readily detectable (Becker et al., 1997) since C57Bl/6J mice show limited HICs (Metten and Crabbe, 1994). CIE was conducted in inhalation chambers (La Jolla Alcohol Research Inc., La Jolla, CA), as previously described (Gorini et al., 2013; Kreifeldt et al., 2013). Before each 16-h vapor exposure period, mice received a loading dose of ethanol (1.5 g/kg, i.p.) and pyrazole (68.1 mg/kg, Sigma, St Louis, MO). Tail vein blood samples were collected periodically at the end of intoxication periods for BAL determination. Ethanol vapor concentration was adjusted so as to yield BALs approximating 200 mg/dl. Mice were exposed to a first series of 4 cycles of intoxication/withdrawal (16 h ethanol vapor/8 h air). The mice were then returned to their home cages for 4–6 weeks before being exposed to a second series of 3 cycles of intoxication/withdrawal. Approximately 26 h into withdrawal from the vapor chambers, mice were i.p. injected with 4 g/kg ethanol and were tested for sedation and hypothermia, and/or HIC.

Data analysis

Statview software (SAS Institute Inc.) was used for statistical analysis. Data were subjected to one-way or repeated-measures analysis of variance (ANOVA) as appropriate to determine the effect of genotype, acute and repeated ethanol treatment on intoxication durations, BALs and body temperatures. Interaction between these factors was not significant, unless reported otherwise in the Results section. HICs at each time-point and the AUC were analyzed using Kruskal-Wallis tests. Data are expressed as mean $\pm$ s.e.m. in all graphs.

Results

BK β 1 and β 4 subunits do not influence ethanol metabolism

We first examined whether the rate of ethanol clearance was altered by the constitutive deletion of BK β 1 or β 4 subunits (Fig. 1). There was a linear decrease of BALs over time in all genotypes ($R^2 > 0.89$, $p < 0.001$) and the clearance rate was not sensitive to the absence of BK auxiliary subunits (time $\times$ genotype interaction, BK β 1: $F_{4,16} = 0.02$, n.s.; BK β 4: $F_{4,16} = 1.10$, n.s.).

BK β 1 and β 4 subunits do not influence sensitivity to acute ethanol intoxication

We then investigated whether BK β 1 and β 4 subunits modulate the sensitivity to ethanol intoxication by comparing WT, Het and KO littermates in three different behavioral assays.

The ataxic effect of ethanol (1.5 g/kg, i.p.) was assessed using the rotarod test. The duration of ataxia following a first injection (Fig. 2A and 2C) was similar across genotypes (BK β 1: $F_{2,34} = 0.18$, n.s.; BK β 4: $F_{2,33} = 0.36$, n.s.). BALs were measured upon recovery from ataxia after this first and a subsequent ethanol injection in order to determine the magnitude of acute functional tolerance (i.e., $BAL_2 > BAL_1$, Fig. 2B and 2D). The increase in BALs at recovery was significant in both strains (BK β 1: $F_{1,34} = 143.60$, $p < 0.001$; BK β 4: $F_{1,34} = 167.76$, $p < 0.001$), and there was no main effect of genotype (BK β 1: $F_{2,34} = 1.51$, n.s.; BK β 4: $F_{2,33} = 0.18$, n.s.).

The sedative effect of ethanol (4 g/kg, i.p.) was evaluated using the loss-of-righting-reflex test. Genotype did not influence the duration of sedation in naïve mice (Fig. 3A and 3C, pre-CIE bars, BK β 1: $F_{2,17} = 2.28$, n.s.; BK β 4: $F_{2,22} = 0.69$, n.s.). BALs measured at recovery from sedation were also similar across genotypes (Fig. 3B and 3D, BK β 1: $F_{2,17} = 1.10$, n.s.; BK β 4: $F_{2,22} = 1.05$, n.s.), thereby confirming that the absence of BK β 1 or β 4 does not impact ethanol clearance rate, even at a higher dose of ethanol.

The hypothermic effect of ethanol (4 g/kg, i.p.) was measured over the course of 2 h. Body temperatures prior to the injection (Fig. 4A–B, Pre-CIE 0 min bars) were not affected by genotype (BK β 1: $F_{2,17} = 1.50$, n.s.; BK β 4: $F_{2,22} = 0.07$, n.s.). The drop in body temperature produced by ethanol in naïve mice (Fig. 4A–B, pre-CIE bars) was significant in both strains (BK β 1: $F_{2,34} = 318.24$, $p < 0.001$; BK β 4: $F_{2,44} = 224.72$, $p < 0.001$). There was no main effect of genotype on this hypothermic effect (BK β 1: $F_{2,17} = 2.95$, n.s.; BK β 4: $F_{2,22} = 0.15$, n.s.). Despite a trend for BK β 1 KO mice to be more sensitive than their Het and WT

counterparts, there was no significant interaction between time and genotype ($F_{4,17} = 1.80$, n.s.).

Altogether it appears that BK $\beta 1$ and $\beta 4$ subunits have no detectable influence on the behavioral response to acute ethanol exposure.

BK $\beta 1$ deletion reduces chronic tolerance to ethanol-induced sedation and hypothermia

The loss-of-righting-reflex test was conducted again following CIE exposure in order to determine the magnitude of chronic tolerance to sedation (Fig. 3A–B, post-CIE bars). The decrease in sedation duration produced by CIE was significant in both strains (BK $\beta 1$: $F_{1,17} = 101.35$, $p < 0.001$; BK $\beta 4$: $F_{1,22} = 25.31$, $p < 0.001$). There was a main effect of genotype in BK $\beta 1$ mice ($F_{2,17} = 3.64$, $p < 0.05$) but not in BK $\beta 4$ mice ($F_{2,22} = 0.41$, n.s.).

Specifically, there was a significant effect of genotype on post-CIE sedation duration in BK $\beta 1$ mice ($F_{2,17} = 4.13$, $p < 0.05$), with BK $\beta 1$ KO mice remaining asleep for a longer time than their WT and Het counterparts ($p < 0.05$ for both comparisons, Student-Newman Keuls *post-hoc* test), thereby indicating weaker chronic tolerance in the absence of BK $\beta 1$. There was no gene dosage effect for this phenotype as BK $\beta 1$ Het mice developed tolerance to the same extent as their WT counterparts.

Body temperatures were also measured again following CIE exposure in order to determine the magnitude of chronic tolerance to hypothermia (Fig. 4A–B, post-CIE bars). CIE did not affect basal body temperatures (BK $\beta 1$: $F_{1,17} = 3.68$, n.s.; BK $\beta 4$: $F_{1,22} = 0.11$, n.s.) but it reduced the hypothermic effect of ethanol 60 min (BK $\beta 1$: $F_{1,17} = 14.27$, $p < 0.01$; BK $\beta 4$: $F_{1,22} = 12.93$, $p < 0.01$) and 120 min post-injection (BK $\beta 1$: $F_{1,17} = 20.30$, $p < 0.001$; BK $\beta 4$: $F_{1,22} = 9.61$, $p < 0.01$) in both strains. In BK $\beta 1$ mice, there was a main effect of genotype on post-CIE body temperatures ($F_{2,17} = 5.40$, $p < 0.05$), and a trend for an interaction between the hypothermic effect of ethanol and genotype ($F_{4,34} = 2.60$, $p = 0.05$). Specifically, genotype differences were significant 60 min ($F_{2,17} = 4.23$, $p < 0.05$) and 120 min ($F_{2,17} = 4.56$, $p < 0.05$) following ethanol injection, with the hypothermic effect of ethanol being stronger in BK $\beta 1$ KO mice than in their WT and Het counterparts, thereby indicating that BK $\beta 1$ deletion reduces the development of chronic tolerance. There was again no gene dosage effect for this phenotype as BK $\beta 1$ Het mice developed tolerance to the same extent as their WT counterparts.

Deletion of BK $\beta 1$ or $\beta 4$ subunits exacerbates withdrawal-induced hyperexcitability

Withdrawal-induced hyperexcitability was assessed using handling-induced convulsions (HICs, Fig. 5). Basal HICs were equivalent across genotypes (BK $\beta 1$: $H = 0.62$, n.s.; BK $\beta 4$: $H = 1.49$, n.s.). HICs were undetectable in all mice 2 h post-injection, as a result of ethanol-induced sedation. HICs peaked 8 h post-injection in WT mice from both strains but were of limited amplitude, in accordance with previous data from C57Bl/6J mice (Metten and Crabbe, 1994). There was a trend for an effect of genotype on the 6–24 h AUC in both strains (BK $\beta 1$: $H = 5.86$, $p = 0.05$; BK $\beta 4$: $H = 5.13$, $p = 0.08$), and genotype differences were significant at selected time-points. Specifically, BK $\beta 1$ deficient mice displayed more HICs 6 h post-injection ($H = 6.70$, $p < 0.05$), and BK $\beta 4$ deficient mice displayed more HICs 12 h post-injection ($H = 6.96$, $p < 0.05$), compared to their respective WT counterparts.

These findings indicate that the absence of BK β 1 accelerates the appearance, while the absence of BK β 4 delays the resolution, of the hyperexcitable state associated with ethanol withdrawal. The abundance of BK β 1 and β 4 appears to be critical for their dampening influence on withdrawal-induced hyperexcitability, as BK β 1 Het mice behaved similarly to their KO counterparts, and BK β 4 Het mice displayed a phenotype that was intermediate between their WT and KO counterparts.

Discussion

Our results show that BK β 1 and β 4 subunits do not influence ethanol metabolism, sensitivity to ethanol intoxication nor acute functional tolerance. By contrast, absence of BK β 1 reduced chronic tolerance to ethanol-induced sedation and hypothermia, suggesting that BK β 1 contributes to the development of behavioral adaptations to chronic ethanol exposure. In addition, lack of BK β 1 or β 4 subunits exacerbated hyperexcitability during ethanol withdrawal. This study complements our previous characterization of BK β 1 and β 4 KO mice in paradigms of voluntary ethanol consumption (Kreifeldt et al., 2013). We had found that the absence of BK β 1 or β 4 does not impact ethanol intake unless the mice are exposed to chronic intermittent ethanol vapors. The present findings support our initial hypothesis that BK auxiliary subunits may be recruited upon prolonged, repeated ethanol intoxication, and highlight the critical role of BK β 1 in behavioral adaptations to ethanol.

Association of BK β 1 to the pore-forming α subunit blocks the ability of ethanol to potentiate BK currents (Feinberg-Zadek and Treistman, 2007; Martin et al., 2004). Accordingly, in the dendrites and/or soma of hypothalamic and accumbal neurons, presence of β 1 in the absence of β 4 results in ethanol-insensitive BK currents (Martin et al., 2004; Wynne et al., 2009). Association of BK β 1 was also shown to convert ethanol into a BK channel inhibitor in myocytes by shifting the activation-to-inhibition crossover of ethanol action to lower calcium concentrations (Bukiya et al., 2009). In addition, BK β 1 promotes cell surface trafficking of the VEDEC isoform, a splice variant of the pore-forming α subunit that produces ethanol-insensitive BK currents (Kim et al., 2007; Pietrzykowski et al., 2008). CIE-induced recruitment of BK β 1 in specific neurons and/or neuronal compartments may therefore serve as a molecular tolerance mechanism to prevent excessive activation of BK currents by ethanol, thereby maintaining proper neuronal functioning despite continued exposure to ethanol through allostatic adaptation. Studies in *C. elegans* have highlighted the critical role of the *slo-1* gene, which encodes the worm BK α subunit, and ethanol binding to this subunit in mediating the depressive effect of ethanol on locomotion (Davies et al., 2003; Davis et al., 2014). Assuming ethanol-induced activation of BK channels also contributes to the sedation and hypothermia induced by acutely administered ethanol in mice, putative recruitment of BK β 1 during CIE could generate tolerance to these effects, which would accordingly be reduced in BK β 1 deficient mice. Potential mechanisms of BK β 1 recruitment include expression induction, in accordance with the low levels of basal expression of this subunit in the brain (Behrens et al., 2000; Brenner et al., 2000a; Chang et al., 1997; Jiang et al., 1999), and subcellular translocation, as was recently demonstrated in myocytes upon activation of nitric oxide signaling (Leo et al., 2014).

The absence of phenotypic differences between WT and BK $\beta 4$ KO mice in the tolerance experiments was puzzling. The $\beta 4$ subunit has been shown to prevent the development of tolerance to ethanol's effects at the molecular (BK single channel activity in HEK293 cells and striatal medium spiny neurons), cellular (firing rate in striatal medium spiny neurons) and behavioral (locomotor activity) levels (Martin et al., 2008). We were therefore expecting to detect a larger degree of acute functional tolerance and/or chronic tolerance in BK $\beta 4$ KO mice; however, BK $\beta 4$ deletion had no impact on these behavioral adaptations to repeated ethanol exposure. One possibility is that the absence of BK $\beta 4$ accelerates the development of tolerance without affecting its magnitude. It is possible that our chronic intermittent ethanol exposure paradigm (3–4 cycles of intoxication separated by 4–6 weeks) induced the maximal extent of tolerance and that we failed to capture enhanced tolerance in BK $\beta 4$ KO mice at intermediate time-points. Another possibility is that BK $\beta 4$ plays a distinct role in neurons mediating ethanol-induced sedation and hypothermia than in striatal medium spiny neurons. Despite its broad expression pattern throughout the central nervous system, BK $\beta 4$ distribution varies between brain regions, cell populations, and even subcellular compartments, which could underlie the differential effect of BK $\beta 4$ deletion on the development of tolerance to distinct behavioral effects of ethanol (Behrens et al., 2000; Brenner et al., 2000a; Martin et al., 2004; Sugino et al., 2006; Weiger et al., 2000; Wynne et al., 2009).

Alcohol withdrawal seizures are generated by neuronal hyperexcitability, in particular in the inferior collicular cortex (Rogawski, 2005). Reduced desensitization of BK channels to ethanol-induced potentiation in BK $\beta 1$ deficient mice would likely lead to the development of counteradaptive molecular changes meant to maintain neuronal homeostasis despite continued activation of BK channels by ethanol. The earlier appearance of HICs in ethanol-withdrawn BK $\beta 1$ KO mice may therefore reflect a rebound increase in neuronal excitability resulting from such compensatory adaptations. Notably, BK $\beta 1$ Het mice also displayed premature emergence of HICs, while they developed chronic tolerance to sedation and hypothermia to the same extent as their WT counterparts. This discrepancy indicates there are different BK $\beta 1$ reserve pool requirements for the development of chronic tolerance and physical dependence in CIE-exposed mice.

On the other hand, the phenotype of BK $\beta 4$ mice may result from a more general (i.e., not specific to ethanol withdrawal) increase in excitability/seizure susceptibility. The BK $\beta 4$ subunit exerts an inhibitory influence on several aspects of BK channel activity (see Wang et al., 2014 for review). In particular, association of $\beta 4$ with the pore-forming α subunit slows down the gating kinetics of BK channels, conferring both slow activation and slow deactivation rates (Behrens et al., 2000; Brenner et al., 2000a; Ha et al., 2004; Wang et al., 2006; Weiger et al., 2000). Under certain circumstances, $\beta 4$ can also shift their voltage dependence to more depolarized potentials (Brenner et al., 2000a; Weiger et al., 2000), oppose their activation by phosphorylation (Petrik et al., 2011; Velazquez-Marrero et al., 2014), and downregulate their cell surface expression (Bai et al., 2011; Shruti et al., 2012). In hippocampal granule cells, $\beta 4$ precludes BK channels from participating in membrane repolarization during an action potential (Brenner et al., 2005). As a result, deletion of BK $\beta 4$ results in sharper action potentials, higher firing rate and temporal lobe seizures, similar to the epileptic phenotype generated by a gain-of-function mutation in BK α (Du et al.,

2005). It is therefore possible that increased excitability of inferior colliculus neurons mediates the exacerbated HICs of BK $\beta 4$ deficient mice during ethanol withdrawal.

Altogether our data point to a critical role of BK auxiliary subunits in the behavioral adaptations to chronic intermittent ethanol exposure, and suggest that modulation of BK channel activity may be a viable therapeutic approach to reverse neurophysiological adaptations developing in heavy alcohol drinkers.

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References

- Bai JP, Surguchev A, Navaratnam D. $\beta 4$ -subunit increases Slo responsiveness to physiological Ca^{2+} concentrations and together with $\beta 1$ reduces surface expression of Slo in hair cells. *Am J Physiol Cell Physiol*. 2011; 300:C435–446. [PubMed: 21178105]
- Becker HC, Diaz-Granados JL, Weathersby RT. Repeated ethanol withdrawal experience increases the severity and duration of subsequent withdrawal seizures in mice. *Alcohol*. 1997; 14:319–326. [PubMed: 9209546]
- Behrens R, Nolting A, Reimann F, Schwarz M, Waldschutz R, Pongs O. hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel β subunit family. *FEBS Lett*. 2000; 474:99–106. [PubMed: 10828459]
- Bettinger JC, Davies AG. The role of the BK channel in ethanol response behaviors: evidence from model organism and human studies. *Front Physiol*. 2014; 5:346. [PubMed: 25249984]
- Brenner R, Chen QH, Vilaythong A, Toney GM, Noebels JL, Aldrich RW. BK channel $\beta 4$ subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat Neurosci*. 2005; 8:1752–1759. [PubMed: 16261134]
- Brenner R, Jegla TJ, Wickenden A, Liu Y, Aldrich RW. Cloning and functional characterization of novel large conductance calcium-activated potassium channel β subunits, hKCNMB3 and hKCNMB4. *J Biol Chem*. 2000a; 275:6453–6461. [PubMed: 10692449]
- Brenner R, Perez GJ, Bonev AD, Eckman DM, Kosek JC, Wiler SW, Patterson AJ, Nelson MT, Aldrich RW. Vasoregulation by the $\beta 1$ subunit of the calcium-activated potassium channel. *Nature*. 2000b; 407:870–876. [PubMed: 11057658]
- Bukiya AN, Kuntamallappanavar G, Edwards J, Singh AK, Shivakumar B, Dopico AM. An alcohol-sensing site in the calcium- and voltage-gated, large conductance potassium (BK) channel. *Proc Natl Acad Sci U S A*. 2014; 111:9313–9318. [PubMed: 24927535]
- Bukiya AN, Liu J, Dopico AM. The BK channel accessory $\beta 1$ subunit determines alcohol-induced cerebrovascular constriction. *FEBS Lett*. 2009; 583:2779–2784. [PubMed: 19616547]
- Chang CP, Dworetzky SI, Wang J, Goldstein ME. Differential expression of the α and β subunits of the large-conductance calcium-activated potassium channel: implication for channel diversity. *Brain Res Mol Brain Res*. 1997; 45:33–40. [PubMed: 9105668]
- Cowmeadow RB, Krishnan HR, Atkinson NS. The slowpoke gene is necessary for rapid ethanol tolerance in *Drosophila*. *Alcohol Clin Exp Res*. 2005; 29:1777–1786. [PubMed: 16269907]
- Crabbe JC, Merrill C, Belknap JK. Acute dependence on depressant drugs is determined by common genes in mice. *J Pharmacol Exp Ther*. 1991; 257:663–667. [PubMed: 2033513]
- Davies AG, Pierce-Shimomura JT, Kim H, VanHoven MK, Thiele TR, Bonci A, Bargmann CI, McIntire SL. A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. *Cell*. 2003; 115:655–666. [PubMed: 14675531]

- Davis SJ, Scott LL, Hu K, Pierce-Shimomura JT. Conserved single residue in the BK potassium channel required for activation by alcohol and intoxication in *C. elegans*. *J Neurosci*. 2014; 34:9562–9573. [PubMed: 25031399]
- Dopico AM, Bukiya AN, Martin GE. Ethanol modulation of mammalian BK channels in excitable tissues: molecular targets and their possible contribution to alcohol-induced altered behavior. *Front Physiol*. 2014; 5:466. [PubMed: 25538625]
- Du W, Bautista JF, Yang H, Diez-Sampedro A, You SA, Wang L, Kotagal P, Luders HO, Shi J, Cui J, Richerson GB, Wang QK. Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat Genet*. 2005; 37:733–738. [PubMed: 15937479]
- Edenberg HJ, Koller DL, Xuei X, Wetherill L, McClintick JN, Almasy L, Bierut LJ, Bucholz KK, Goate A, Aliev F, Dick D, Hesselbrock V, Hinrichs A, Kramer J, Kuperman S, Nurnberger II Jr, Rice JP, Schuckit MA, Taylor R, Todd Webb B, Tischfield JA, Porjesz B, Foroud T. Genome-wide association study of alcohol dependence implicates a region on chromosome 11. *Alcohol Clin Exp Res*. 2010; 34:840–852. [PubMed: 20201924]
- Feinberg-Zadek PL, Treistman SN. Beta-subunits are important modulators of the acute response to alcohol in human BK channels. *Alcohol Clin Exp Res*. 2007; 31:737–744. [PubMed: 17391339]
- Gorini G, Roberts AJ, Mayfield RD. Neurobiological signatures of alcohol dependence revealed by protein profiling. *PLoS One*. 2013; 8:e82656. [PubMed: 24358215]
- Ha TS, Heo MS, Park CS. Functional effects of auxiliary beta4-subunit on rat large-conductance $\text{Ca}(2+)$ -activated $\text{K}(+)$ channel. *Biophys J*. 2004; 86:2871–2882. [PubMed: 15111404]
- Han S, Yang BZ, Kranzler HR, Liu X, Zhao H, Farrer LA, Boerwinkle E, Potash JB, Gelernter J. Integrating GWASs and human protein interaction networks identifies a gene subnetwork underlying alcohol dependence. *Am J Hum Genet*. 2013; 93:1027–1034. [PubMed: 24268660]
- Jiang Z, Wallner M, Meera P, Toro L. Human and rodent MaxiK channel beta-subunit genes: cloning and characterization. *Genomics*. 1999; 55:57–67. [PubMed: 9888999]
- Kendler KS, Kalsi G, Holmans PA, Sanders AR, Aggen SH, Dick DM, Aliev F, Shi J, Levinson DF, Gejman PV. Genomewide association analysis of symptoms of alcohol dependence in the molecular genetics of schizophrenia (MGS2) control sample. *Alcohol Clin Exp Res*. 2011; 35:963–975. [PubMed: 21314694]
- Kim EY, Zou S, Ridgway LD, Dryer SE. Beta1-subunits increase surface expression of a large-conductance $\text{Ca}2+$ -activated $\text{K}+$ channel isoform. *J Neurophysiol*. 2007; 97:3508–3516. [PubMed: 17329633]
- Kreifeldt M, Le D, Treistman SN, Koob GF, Contet C. BK channel beta1 and beta4 auxiliary subunits exert opposite influences on escalated ethanol drinking in dependent mice. *Front Integr Neurosci*. 2013; 7:105. [PubMed: 24416005]
- Leo MD, Bannister JP, Narayanan D, Nair A, Grubbs JE, Gabrick KS, Boop FA, Jaggar JH. Dynamic regulation of beta1 subunit trafficking controls vascular contractility. *Proc Natl Acad Sci U S A*. 2014; 111:2361–2366. [PubMed: 24464482]
- Martin G, Puig S, Pietrzykowski A, Zadek P, Emery P, Treistman S. Somatic localization of a specific large-conductance calcium-activated potassium channel subtype controls compartmentalized ethanol sensitivity in the nucleus accumbens. *J Neurosci*. 2004; 24:6563–6572. [PubMed: 15269268]
- Martin GE, Hendrickson LM, Penta KL, Friesen RM, Pietrzykowski AZ, Tapper AR, Treistman SN. Identification of a BK channel auxiliary protein controlling molecular and behavioral tolerance to alcohol. *Proc Natl Acad Sci U S A*. 2008; 105:17543–17548. [PubMed: 18981408]
- Metten P, Crabbe JC. Common genetic determinants of severity of acute withdrawal from ethanol, pentobarbital and diazepam in inbred mice. *Behav Pharmacol*. 1994; 5:533–547. [PubMed: 11224305]
- N’Gouemo P. BKCa channel dysfunction in neurological diseases. *Front Physiol*. 2014; 5:373. [PubMed: 25324781]
- Newlin DB, Thomson JB. Alcohol challenge with sons of alcoholics: a critical review and analysis. *Psychol Bull*. 1990; 108:383–402. [PubMed: 2270234]

- Petrik D, Wang B, Brenner R. Modulation by the BK accessory beta4 subunit of phosphorylation-dependent changes in excitability of dentate gyrus granule neurons. *Eur J Neurosci.* 2011; 34:695–704. [PubMed: 21848922]
- Pietrzykowski AZ, Friesen RM, Martin GE, Puig SI, Nowak CL, Wynne PM, Siegelmann HT, Treistman SN. Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron.* 2008; 59:274–287. [PubMed: 18667155]
- Rogawski MA. Update on the neurobiology of alcohol withdrawal seizures. *Epilepsy Curr.* 2005; 5:225–230. [PubMed: 16372057]
- Schuckit MA. Self-rating of alcohol intoxication by young men with and without family histories of alcoholism. *J Stud Alcohol.* 1980; 41:242–249. [PubMed: 7374142]
- Schuckit MA, Wilhelmsen K, Smith TL, Feiler HS, Lind P, Lange LA, Kalmijn J. Autosomal linkage analysis for the level of response to alcohol. *Alcohol Clin Exp Res.* 2005; 29:1976–1982. [PubMed: 16340454]
- Shruti S, Urban-Ciecko J, Fitzpatrick JA, Brenner R, Bruchez MP, Barth AL. The brain-specific Beta4 subunit downregulates BK channel cell surface expression. *PLoS One.* 2012; 7:e33429. [PubMed: 22438928]
- Sugino K, Hempel CM, Miller MN, Hattox AM, Shapiro P, Wu C, Huang ZJ, Nelson SB. Molecular taxonomy of major neuronal classes in the adult mouse forebrain. *Nat Neurosci.* 2006; 9:99–107. [PubMed: 16369481]
- Velazquez-Marrero C, Seale GE, Treistman SN, Martin GE. Large conductance voltage- and Ca²⁺-gated potassium (BK) channel beta4 subunit influences sensitivity and tolerance to alcohol by altering its response to kinases. *J Biol Chem.* 2014; 289:29261–29272. [PubMed: 25190810]
- Wang B, Jaffe DB, Brenner R. Current understanding of iberiotoxin-resistant BK channels in the nervous system. *Front Physiol.* 2014; 5:382. [PubMed: 25346692]
- Wang B, Rothberg BS, Brenner R. Mechanism of beta4 subunit modulation of BK channels. *J Gen Physiol.* 2006; 127:449–465. [PubMed: 16567466]
- Wang ZW. Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Mol Neurobiol.* 2008; 38:153–166. [PubMed: 18759010]
- Weiger TM, Holmqvist MH, Levitan IB, Clark FT, Sprague S, Huang WJ, Ge P, Wang C, Lawson D, Jurman ME, Glucksmann MA, Silos-Santiago I, DiStefano PS, Curtis R. A novel nervous system beta subunit that downregulates human large conductance calcium-dependent potassium channels. *J Neurosci.* 2000; 20:3563–3570. [PubMed: 10804197]
- Wynne PM, Puig SI, Martin GE, Treistman SN. Compartmentalized beta subunit distribution determines characteristics and ethanol sensitivity of somatic, dendritic, and terminal large-conductance calcium-activated potassium channels in the rat central nervous system. *J Pharmacol Exp Ther.* 2009; 329:978–986. [PubMed: 19321803]

Figure 1. Ethanol metabolism in BK β 1 and β 4 deficient mice

BALs were measured 45 min, 1.5 h and 3 h following the injection of ethanol (2 g/kg, i.p.) in BK β 1 (**A**, WT, n=4; Het, n=3; KO, n=4) and BK β 4 (**B**, WT, n=4; Het, n=3; KO, n=4) mice. Linear regression was significant in all genotypes ($R^2 > 0.89$, $p < 0.001$).

Figure 2. Ethanol-induced ataxia and acute tolerance in BK β 1 and β 4 deficient mice

The effect of ethanol (1.5 g/kg, i.p.) on motor coordination was assessed using the rotarod test in BK β 1 (A–B, WT, n=10; Het, n=16; KO, n=11) and BK β 4 (C–D, WT, n=12; Het, n=12; KO, n=12) mice. Sensitivity to ethanol intoxication was reflected by the duration of ataxia (A, C). Acute functional tolerance was reflected by higher BALs at recovery after a second injection administered as soon as the mice recovered from the first injection (B, D, black stars, $p < 0.001$).

Figure 3. Ethanol-induced sedation and chronic tolerance in BK β 1 and β 4 deficient mice

The sedative effect of ethanol (4 g/kg, i.p.) was assessed using the loss-of-righting reflex test in BK β 1 (A–B, WT, n=7; Het, n=8; KO, n=5) and BK β 4 (C–D, WT, n=9; Het, n=8; KO, n=8) mice. There was no effect of genotype on sedation duration (A and C) and BALs measured at recovery (B and D) pre-CIE. CIE exposure produced tolerance to sedation in both strains, as reflected by a shorter sleeping duration post-CIE (black stars, $p < 0.001$). There was a significant effect of genotype in BK β 1 mice (white star, $p < 0.05$).

Figure 4. Ethanol-induced hypothermia and chronic tolerance in BK β 1 and β 4 deficient mice
 The hypothermic effect of ethanol (4 g/kg, i.p.) was measured in BK β 1 (A, WT, n=7; Het, n=8; KO, n=5) and BK β 4 (B, WT, n=9; Het, n=8; KO, n=8) mice. CIE exposure produced tolerance to hypothermia in both strains, as reflected by a smaller drop in body temperature post-CIE (black stars: two, $p < 0.01$; three stars, $p < 0.001$). There was a significant effect of genotype in BK β 1 mice post-CIE 60 and 120 min post-injection, as well as in BK β 4 mice prior to the injection (white stars, $p < 0.05$).

Figure 5. Ethanol withdrawal-induced hyperexcitability in BK β 1 and β 4 deficient mice
A time course of handling-induced convulsions (HICs) was determined after the injection of ethanol (4 g/kg, i.p.) in CIE-exposed BK β 1 (A, WT, n=7; Het, n=8; KO, n=5) and BK β 4 (B, WT, n=9; Het, n=4; KO, n=6) mice. There was a significant effect of genotype in BK β 1 mice 6 h post-injection and in BK β 4 mice 12 h post-injection (white stars, $p < 0.05$).