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Reduced Nucleus Accumbens SK Channel Activity Enhances Alcohol Seeking during Abstinence

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SUMMARY

The cellular mechanisms underlying pathological alcohol seeking remain poorly understood. Here, we show an enhancement of nucleus accumbens (NAcb) core action potential firing ex vivo after protracted abstinence from alcohol but not sucrose self-administration. Increased firing is associated with reduced small-conductance calcium-activated potassium channel (SK) currents and decreased SK3 but not SK2 subunit protein expression. Furthermore, SK activation ex vivo produces greater firing suppression in NAcb core neurons from alcohol- versus sucrose-abstinent rats. Accordingly, SK activation in the NAcb core significantly reduces alcohol but not sucrose seeking after abstinence. In contrast, NAcb shell and lateral dorsal striatal firing ex vivo are not altered after abstinence from alcohol, and SK activation in these regions has little effect on alcohol seeking. Thus, decreased NAcb core SK currents and increased excitability represents a critical mechanism that facilitates motivation to seek alcohol after abstinence.

INTRODUCTION

Addiction is a chronic disorder characterized by periods of heightened motivation to seek drugs and an increased propensity for relapse (Larimer et al., 1999; Sanchis-Segura and Spanagel, 2006). Thus, there is considerable interest in rodent paradigms that model key aspects of human addiction, since these paradigms help elucidate the molecular and cellular mechanisms that drive drug-seeking behaviors, and as a consequence facilitate the development of novel therapeutic interventions for addiction. There is general agreement that drug-related stimuli can be potent triggers for eliciting relapse in humans and rodents (Epstein et al., 2006; Everitt and Robbins, 2005; Sanchis-Segura and Spanagel, 2006). A number of studies have examined the ability of different stimuli to reinstate drug seeking after extinction (Kalivas and McFarland, 2003; Sanchis-Segura and Spanagel, 2006). Other models examine drug seeking after abstinence from self-administration, since human addicts may not undergo explicit extinction of drug-related stimuli and behaviors (Epstein et al., 2006; Sanchis-Segura and Spanagel, 2006). In particular, drug-related motivation can increase across abstinence, which has been described as an incubation of craving in studies of cocaine and heroin seeking (Epstein et al., 2006), and as an alcohol-deprivation effect, an increase in alcohol intake after abstinence that has been observed in humans (Sanchis-Segura and Spanagel, 2006). Drug-related cues can play an important role in the increased motivation after protracted abstinence for both cocaine and alcohol (Bowers et al., 2008; Epstein et al., 2006).

The nucleus accumbens (NAcb) core is considered critical for allowing a variety of salient stimuli to drive motivated behaviors (Cardinal et al., 2002; Carelli and Wightman, 2004; Epstein et al., 2006; Everitt and Robbins, 2005; Kalivas and McFarland, 2003; Kelley, 2004; Mogenson et al., 1980). NAcb core neurons in rats and nonhuman primates exhibit firing in response to reinforcer-predictive cues (Carelli and Wightman, 2004; Nicola, 2007; Schultz, 2004), and the NAcb is activated by drug-related stimuli in alcoholics (Kareken et al., 2004; Modell and Mountz, 1995; but see Schneider et al., 2001). In addition, NAcb core inactivation can attenuate behavioral responding to cues that predict positive reinforcement (Cardinal et al., 2002; Epstein et al., 2006; Kalivas and McFarland, 2003; Nicola, 2007; Sanchis-Segura and Spanagel, 2006). Since action potential (AP) firing is the predominant mechanism by which neurons transmit information, cue-related NAcb core firing is thought to contribute to the activation of motivated, goal-directed behavior (Carelli and Wightman, 2004; Nicola, 2007). We should note that other regions of the striatum have also been implicated in control of motivated and drug-related behaviors. The lateral dorsal striatum (DStr) is important in well-learned, habitual behavior, while the NAcb shell has been linked to a range of behaviors, including primary reward, reinstatement/relapse induced by drug exposure and some types of drug-related cues, as well as some forms of conditioned phenomena, such as Pavlovian-to-instrumental transfer (Cardinal et al., 2002; Everitt and Robbins, 2005).

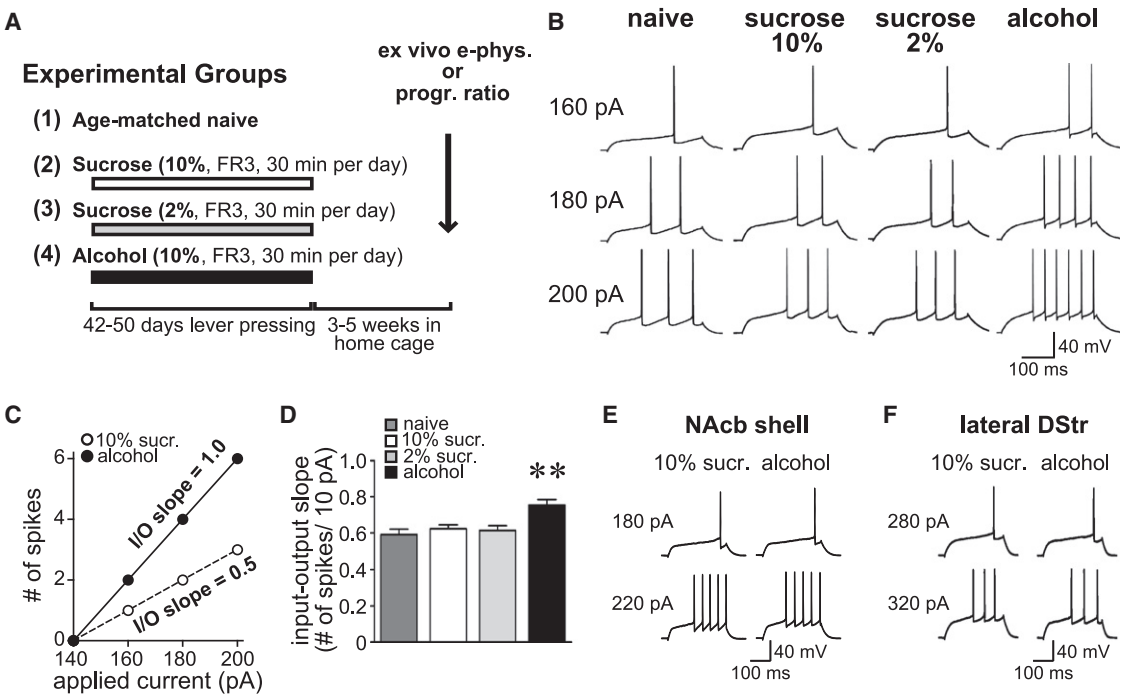

Figure 1. NAcb Core Spike Firing Was Significantly Enhanced after Abstinence from Alcohol Ex Vivo

(A) Animals were allowed to operantly self-administer alcohol (10%) or sucrose (10% or 2%) across 42–50 continuous days on a fixed-ratio 3 schedule (FR-3, 30 min/day) or were age-matched naive controls. For self-administering animals, ex vivo electrophysiology or behavioral responding for alcohol or sucrose was examined after 3–5 weeks abstinence.

(B) Example traces of AP generation evoked in response to depolarizing current steps in NAcb neurons from naive animals or from animals after abstinence from self-administration of sucrose or alcohol.

(C) Example input/output relationships (I/O slope) derived from the alcohol and 10% sucrose traces in (B).

(D) Grouped data showing enhanced spike firing in NAcb core neurons from alcohol- relative to sucrose-abstinent and naive animals.

(E and F) Example traces showing no changes in firing in (E) NAcb shell or (F) lateral DStr neurons after alcohol and abstinence. Error bars indicate SEM. sucr., sucrose; e-phys., electrophysiology; progr., progressive. **p < 0.01 alcohol versus sucrose or naive.

See also Figure S1.

Although molecular neuroadaptations in the NAcb or lateral DStr could potently enhance drug seeking, it is unknown whether alterations in firing in these regions could develop or persist following alcohol intake and thus could facilitate the motivation to seek alcohol that is thought to promote relapse. In this study, we link molecular changes in potassium channel function to enhancement of drug-seeking behavior, identifying a mechanism whereby reduced small-conductance, calcium-activated potassium channel (SK) currents in the rat NAcb core is a critical neuroadaptation that enhances firing ex vivo and facilitates motivation to seek alcohol following protracted abstinence.

RESULTS

NAcb Core AP Firing Was Enhanced after Alcohol Self-Administration and Abstinence

We performed ex vivo whole-cell patch-clamp recording using adult rat brain slices to examine whether NAcb core AP firing was altered after 3–5 weeks abstinence from 42–50 continuous days of operant self-administration of 10% alcohol (Figure 1A and Table 1). Animals at this time of abstinence exhibit enhanced motivation for alcohol relative to animals without abstinence (Bowers et al., 2008). Results were compared with NAcb core neurons from animals after abstinence from self-administration of 10% sucrose, an equivalent concentration of reinforcer as alcohol but with higher operant responding than for alcohol ($t_{(170)}$ = 19.83, p < 0.001, unpaired t test) or after abstinence from self-administration of 2% sucrose, which elicited similar levels of operant responding during self-administration as observed in alcohol-abstinent animals ($t_{(145)}$ = 0.040, p = 0.968, unpaired t test) (Figure 1A and Table 1). Electrophysiological experiments were performed in current-clamp mode, where depolarizing current pulses (300 ms, both sub- and suprathreshold for firing) were applied to elicit AP firing (Figure 1B). The resting membrane potential in each neuron was set to ~–90 mV before analysis of firing. Also, because a different number of neurons was recorded for each animal, basal spike firing and voltage-clamp parameters were averaged for all cells obtained from a given animal, thus obtaining a single value of each parameter for each individual animal.

AP firing was significantly enhanced in NAcb core neurons after abstinence from alcohol but not sucrose self-administration (Figure 1B). To quantify spike firing, the number of APs generated in response to a range of depolarizing current pulses was

Table 1. Lever Pressing and Reinforcer Intake across the Last 10 Days of Self-Administration before the Beginning of Abstinence

	Active Lever Presses	Inactive Lever Presses	g/kg Reinforcer
10% Alcohol	115.1 ± 7.1	10.1 ± 0.9	0.42 ± 0.02
10% Sucrose	426.2 ± 16.6	9.0 ± 0.9	2.12 ± 0.09
2% Sucrose	114.5 ± 12.5	9.0 ± 0.9	0.12 ± 0.01

Active and inactive lever presses and g/kg of reinforcer (in the 30 min operant session) averaged across the last 10 days of self-administration before abstinence. These data include animals from ex vivo electrophysiology experiments and in vivo behavioral experiments.

determined in order to describe the input/output relationship. This ''input/output slope'' (I/O slope) was calculated from the number of spikes generated in the last subthreshold current pulse and the first three suprathreshold current pulses from a given neuron (Figure 1C). NAcb core neurons from alcohol-abstinent animals exhibited a significantly larger basal input/output slope than neurons from naive or sucrose-abstinent animals (Figure 1D, naive: n = 14, 0.59 ± 0.03 AP/10 pA; 10% sucrose: n = 20, 0.62 ± 0.02 AP/10 pA; 2% sucrose: n = 14, 0.61 ± 0.03 AP/10 pA; alcohol: n = 21, 0.76 ± 0.03 AP/10 pA; $F_{(3,65)}$ = 7.946, p < 0.001, one-way ANOVA; p < 0.01 alcohol versus each other group), suggesting that basal excitability in the NAcb core was enhanced after abstinence from alcohol self-administration. In contrast, there was no change in firing after alcohol and abstinence in neurons from the NAcb shell or lateral DStr (Figures 1E, 1F, and S1; NAcb shell: $F_{(3,30)}$ = 0.842, p = 0.481; lateral DStr: $F_{(3,17)}$ = 0.712, p = 0.558; both one-way ANOVA). Thus, alcohol self-administration and protracted abstinence only altered AP firing in the NAcb core, with no change in AP firing in NAcb shell or lateral DStr neurons.

SK Inhibition Differentially Enhanced Firing in NAcb Core Neurons from Alcohol- and Sucrose-Abstinent Animals

Many ion channels can contribute to firing by regulating the afterhyperpolarization (AHP), the refractory period that occurs before a subsequent AP can be generated. SK-type potassium channels are potent regulators of AP firing, since they can enhance the AHP and depress AP firing, while SK inhibition facilitates AP firing (Bennett et al., 2000; Hille, 2001; Pineda et al., 1992). Basal SK function is greater in the lateral DStr (Figure S1; Pineda et al., 1992) and weaker in the NAcb shell (Ishikawa et al., 2009), but the contribution of SK to NAcb core firing remains unknown. Here, we first examined SK function in the NAcb core by determining whether the SK-selective blocker apamin (100 nM) would alter AP firing. If greater basal AP firing in NAcb core neurons from alcohol-abstinent animals reflects reduced SK currents, then apamin should have a smaller effect on firing in neurons from alcohol- versus sucrose-abstinent animals.

SK inhibition with apamin enhanced NAcb core firing ex vivo in all groups. However, basal firing was significantly greater in NAcb core neurons from alcohol-abstinent animals, but there were no differences in firing among groups after exposure to apamin (Figures 2A and 2B and Table S1; 10% sucrose: n = 14 from 10 rats; 2% sucrose: n = 9 from 6 rats; alcohol: n = 11 from 8 rats; apamin: $F_{(1,31)}$ = 261.5, p < 0.001; group: $F_{(2,31)}$ = 3.861, p = 0.032; apamin × group: $F_{(2,31)}$ = 6.336, p = 0.005; two-way repeated-measures ANOVA [RM-ANOVA]; p < 0.05 alcohol versus sucrose before apamin). Thus, SK inhibition produced a significantly greater enhancement in firing in NAcb core neurons from sucrose- relative to alcohol-abstinent animals (Figure 2C and Table S1; $F_{(2,31)}$ = 10.18, p < 0.001, one-way ANOVA; p < 0.05 alcohol versus sucrose). The similar input/output slope after apamin exposure across groups suggests that basal differences in firing reflected differential basal SK function, in particular a reduction of basal SK currents that enhanced excitability of neurons from alcohol-abstinent animals.

To further examine SK regulation of firing, we determined the effect of SK inhibition on the number of APs generated (measured for each cell at the current step with four APs at baseline, or five APs if no current step at baseline had four APs). Apamin inhibition of SK produced a significantly greater increase in AP generation in NAcb core neurons from sucrose- versus alcohol-abstinent animals (Figure 2D and Tables S1 and S2; $F_{(2,31)}$ = 5.242, p = 0.011, one-way ANOVA; p < 0.05 alcohol versus sucrose). Thus, apamin differentially enhanced NAcb core AP generation, with a smaller effect in neurons from alcohol- versus sucrose-abstinent animals. Furthermore, the basal input/output slope for any given neuron was negatively correlated with the change in input/output slope with apamin in all groups (Figure S2; R^2 = 0.363 for 10% sucrose, 0.417 for 2% sucrose, and 0.564 for alcohol; all p < 0.05 Pearson correlation), suggesting that greater basal firing may, in general, reflect reduced basal SK currents. Taken together, these results indicate a critical role for decreased SK currents in enhancing basal excitability of NAcb core neurons after abstinence from alcohol self-administration.

In addition to SK regulation of firing, strong SK currents can also regulate the peak magnitude of the AHP (Bennett et al., 2000). Here, the basal NAcb core peak AHP magnitude, determined 3–4 ms after the AP threshold, was not different across groups (Table S3). Further, apamin did not significantly alter the peak AHP magnitude in any group (Table S1, apamin: $F_{(1,31)}$ = 0.311, p = 0.581; group: $F_{(2,31)}$ = 1.102, p = 0.345; apamin × group: $F_{(2,31)}$ = 0.627, p = 0.541; two-way RM-ANOVA), suggesting that SK currents in NAcb core neurons were relatively moderate compared to some other types of neurons (Bennett et al., 2000). In addition, no changes were observed in many other AP waveform parameters, perhaps indicating that several channels other than SK did not show functional changes after alcohol and abstinence (Table S3). However, the magnitude of the slower component of the AHP, determined relative to the AP threshold at 15 ms after the AP threshold (Figure 2E), was significantly reduced in NAcb core neurons from alcohol- versus sucrose-abstinent animals at baseline (Figures 2E and 2F and Table S1; $F_{(2,31)}$ = 5.415, p < 0.001, one-way ANOVA; p < 0.01 alcohol versus sucrose). Furthermore, the apamin reduction of this delayed AHP component was significantly smaller in NAcb core neurons from alcohol-abstinent animals (Figures 2E and 2G and Tables S1 and S2; $F_{(2,31)}$ = 5.415, p = 0.010, one-way ANOVA, p < 0.05 alcohol versus sucrose). Thus, reduced NAcb core SK regulation of firing contributes significantly to the enhanced spike firing observed after long-term self-administration and protracted abstinence from alcohol but not sucrose.

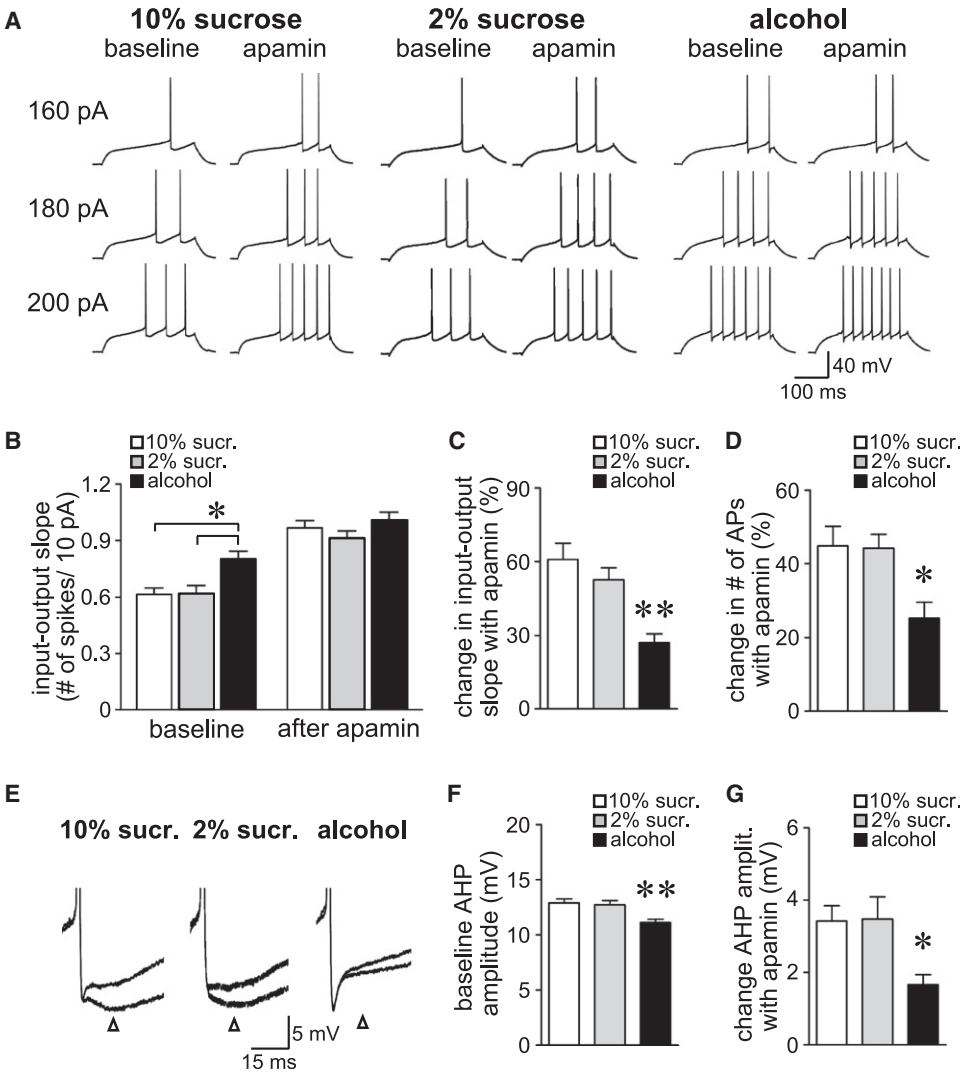

Figure 2. Reduced NAcb Core SK Regulation of Firing Enhanced Firing Ex Vivo after Alcohol and Abstinence

Examples (A) and grouped data (B–D) illustrating that SK inhibition produced a greater enhancement of firing in neurons from sucrose- versus alcohol-abstinent animals. Baseline example traces in (A) are the same as those in Figure 1B.

(C and D) The apamin-induced increases in (C) the input/output slope and (D) AP generation were significantly greater in neurons from sucrose- versus alcohol-abstinent animals.

(E and F) Examples ([E], magnification of the AHP for examples in [A]) and grouped data (F) showing that the amplitude of the AHP, determined relative to the AP threshold at 15 ms after the AP threshold (open arrow), was significantly reduced in neurons from alcohol- versus sucrose-abstinent animals.

(G) The apamin reduction in the AHP amplitude was significantly smaller in neurons from alcohol-abstinent animals.

In (B)–(D), the average input/output slope or APs generated was determined by averaging three sweeps just before addition of apamin and three sweeps 8–10 min after addition of apamin. Error bars indicate SEM. sucr., sucrose. *$p < 0.05$ or **$p < 0.01$ alcohol versus sucrose. See also Figure S2 and Tables S1 and S2.

We should note that increased NAcb core firing ex vivo was observed in nearly all alcohol-abstinent rats independent from the amount of alcohol previously consumed during the last 10 days of self-administration before abstinence (Figure S2; $R^2 = 0.006$, $p = 0.762$, Pearson correlation). This suggests that, although each rat may have its own set-point for the preferred amount of alcohol consumed during self-administration, decreased SK regulation of firing occurred independently of alcohol intake levels.

NAcb Core SK Currents Were Reduced after Alcohol and Abstinence

To directly examine NAcb core SK function after abstinence from either alcohol or sucrose self-administration, we used voltage-clamp methods to isolate SK currents (Hille, 2001; Hopf et al., 2007; Paul et al., 2003). Neurons were held at –70 mV, then depolarized for 400 ms to steps ranging from –40 to –10 mV (with 10 mV between steps) prior to being brought back to –70 mV. A tail current was evident upon returning to –70 mV

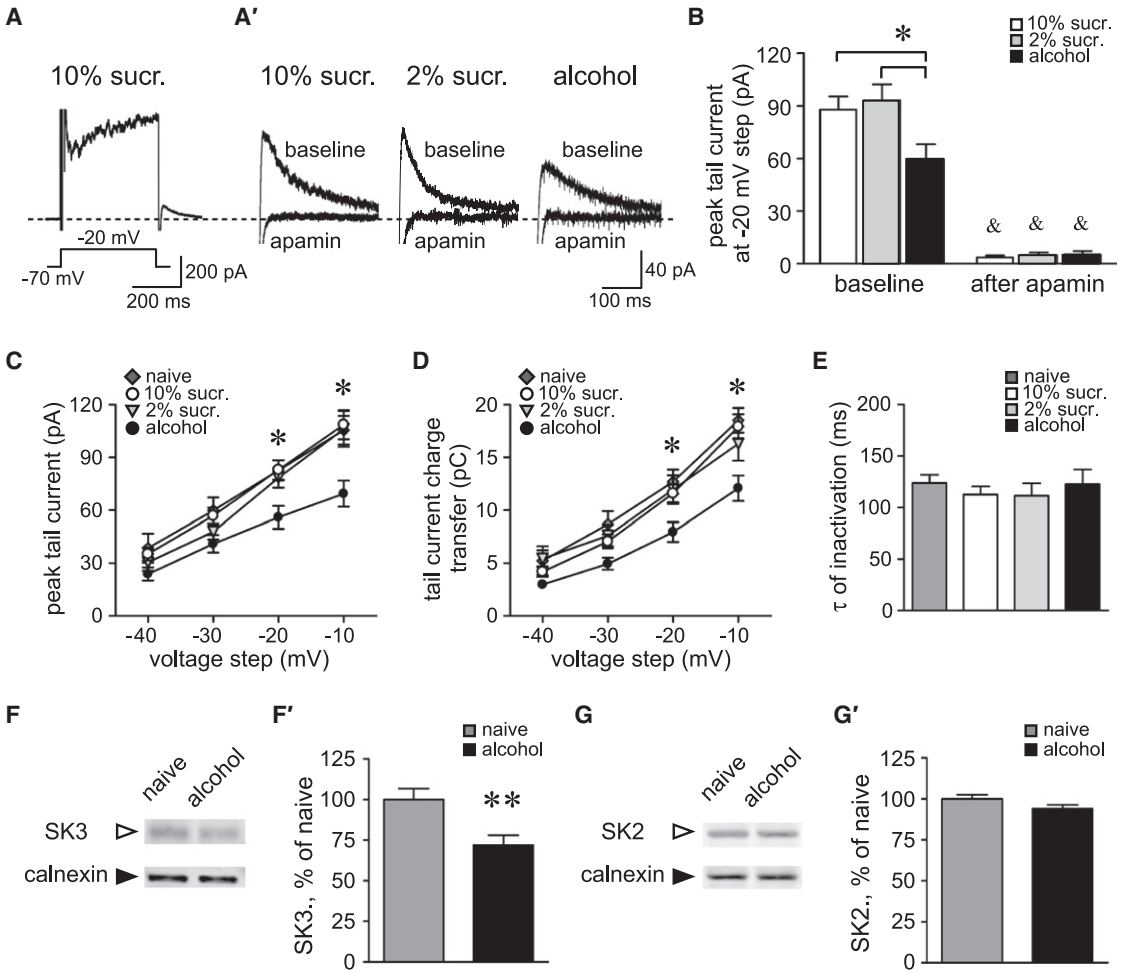

Figure 3. Alcohol and Abstinence Significantly Reduced SK Currents in NAcb Core Neurons Measured under Voltage Clamp

(A) Example of an entire current response upon depolarization to –20 mV from a –70 mV holding potential, with a tail current apparent after returning to –70 mV following the depolarization; (A') example tail currents magnified.

(B) Grouped data showing that peak tail currents, determined for the step to –20 mV, were significantly reduced in NAcb core neurons from alcohol- versus sucrose-abstinent animals and were almost abolished by apamin.

(C and D) (C) Peak tail currents and (D) area under the curve (the tail current charge transfer) were significantly reduced in neurons from alcohol- versus sucrose-abstinent and naive animals.

(E) Tail current τ of inactivation, fit from 55 to 550 ms of the tail current (Abel et al., 2004), was not different across groups.

(F and F′) Reduced NAcb core SK3 subunit protein expression in alcohol relative to naive animals (naive: 100% ± 6.7% optical density, O.D., of naive; alcohol: 72.0% ± 6.0% O.D. of naive).

(G and G′) No change in NAcb core SK2 subunit protein expression with alcohol (naive: 100% ± 2.6% O.D. of naive; alcohol: 94.1% ± 2.3% O.D. of naive). The protein calnexin was used as a procedural control (Bowers et al., 2004, 2008). Apparent molecular weights: open triangle, ~70 kD; closed triangle: ~90 kD. Error bars indicate SEM. sucr., sucrose; pC, picocoulombs. *$p < 0.05$ or **$p < 0.01$ alcohol versus naive and sucrose, $^{\&}p < 0.001$ inhibition by apamin.

See also Figure S3 and Table S1.

(Figures 3A and 3A′), which may reflect slow ion channel deactivation (Hille, 2001). Analyses of apamin-sensitive peak currents and the τ of inactivation was determined for the step to –20 mV because of occasional apamin-insensitive, rapidly activating and inactivating currents at steps to –10 mV, which likely represent currents through the large-conductance calcium-activated potassium channel (BK; Sah and Faber, 2002).

Peak tail currents were significantly smaller in NAcb core neurons from alcohol- versus sucrose-abstinent animals, and SK inhibition with apamin nearly eliminated the tail current in all three groups (Figures 3A and 3B and Table S1; 10% sucrose: n = 12 from 9 rats; 2% sucrose: n = 6 from 5 rats; alcohol: n = 10 from 7 rats; apamin: $F_{(1,25)} = 246.4$, $p < 0.001$; group: $F_{(2,25)} = 3.615$, $p = 0.042$; apamin × group: $F_{(2,25)} = 5.286$, $p = 0.012$; two-way RM-ANOVA; $p < 0.05$ alcohol versus sucrose before apamin), suggesting that the peak tail current predominantly reflected SK-mediated currents (Hopf et al., 2007; Paul et al., 2003). Reduced basal tail currents in neurons from alcohol- versus sucrose-abstinent and naive animals were also evident in a larger group of cells, including those not tested with apamin

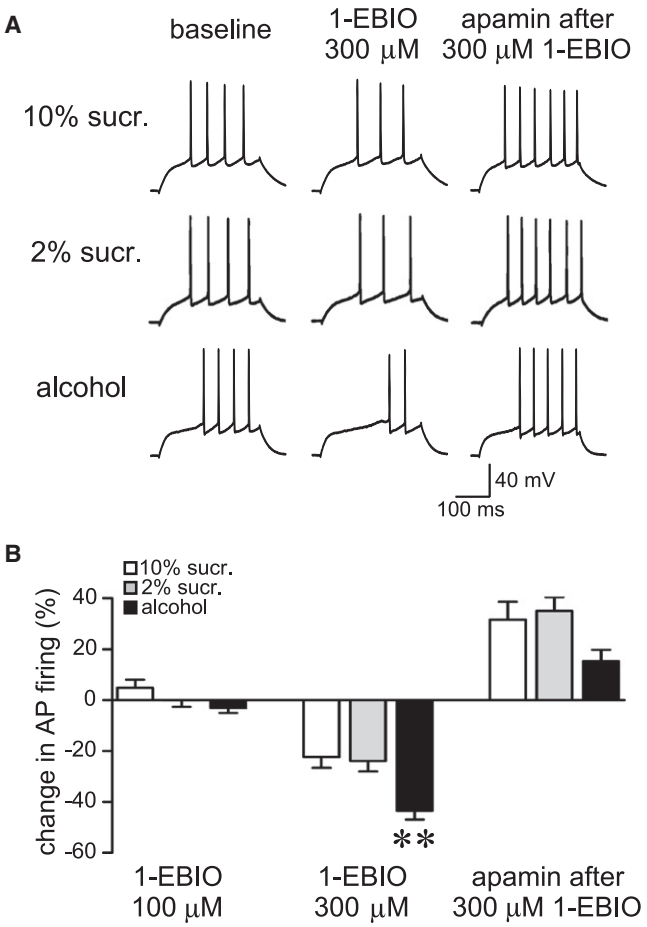

Figure 4. SK Activators Differentially Reduced NAcb Core Firing Ex Vivo in Neurons from Alcohol- and Sucrose-Abstinent Animals

Example traces (A) and grouped data (B) illustrating that the SK positive modulator 1-EBIO (300 μM) reduced NAcb core firing ex vivo, with a greater effect in NAcb core neurons from alcohol- versus sucrose-abstinent animals. Moreover, apamin fully reversed the effect of 300 μM 1-EBIO, indicating that 1-EBIO depressed firing through SK activation. The current steps in (A) were 160 pA, 160 pA, and 200 pA for the 10% sucrose, 2% sucrose, and alcohol examples, respectively. Error bars indicate SEM. sucr., sucrose. **p < 0.01 alcohol versus sucrose. See also Tables S1 and S2.

(Figure 3C; n = 9 for naive, 17 for 10% sucrose, 14 for 2% sucrose, and 17 for alcohol; voltage step: $F_{(3,206)} = 246.8$, $p < 0.001$; group: $F_{(3,206)} = 4.289$, $p = 0.009$; voltage step × group: $F_{(9,206)} = 3.240$, $p = 0.001$; two-way RM-ANOVA; $p < 0.05$ alcohol versus other groups at –20 and –10 mV steps). The tail current charge transfer, calculated by integrating the tail current evoked following the depolarizing pulse (from ~55 to 550 ms into the tail current; Abel et al., 2004), was also significantly reduced in neurons from alcohol- versus sucrose-abstinent and naive animals (Figure 3D; voltage step: $F_{(3,206)} = 336.8$, $p < 0.001$; group: $F_{(3,206)} = 4.806$, $p = 0.005$; voltage step × group: $F_{(9,206)} = 3.117$, $p = 0.002$; two-way RM-ANOVA; $p < 0.05$ alcohol versus other groups at –20 and –10 mV steps). However, the SK tail current τ of inactivation at the –20 mV step was not different between groups (Figure 3E, naive: 123.8 ± 8.0 ms; 10% sucrose: 112.5 ± 8.0 ms; 2% sucrose: 111.4 ± 12.1 ms; alcohol: 122.7 ± 14.2 ms; $F_{(3,53)} = 0.297$, $p = 0.828$, one-way ANOVA), nor were the tail current decay time, rise time, or the relative proportion of the SK current activated across the different voltage steps (Figure S3). Moreover, reduced SK currents did not reflect changes in basic membrane properties such as series or input resistance, which were not different across groups (Table S3). These results demonstrate that NAcb core SK currents were reduced after protracted abstinence from alcohol self-administration. Also, since SK inactivation kinetics were not altered after alcohol and abstinence, these results suggest that decreased SK currents could reflect a decrease in SK channel number rather than a change in SK channel kinetics.

NAcb Core SK3 Subunit Protein Expression Was Reduced during Abstinence from Alcohol

SK channels can be formed from one or more of three subunits, with SK3 subunit expression particularly high in the NAcb core and striatum relative to the SK2 and SK1 subunits (Sailer et al., 2004; Stocker and Pedarzani, 2000). Here, immunoblotting revealed that SK3 subunit expression was significantly reduced in the NAcb core of alcohol-abstinent rats relative to age-matched naive rats (Figure 3F; naive: n = 17; alcohol: n = 14; $t_{(29)} = 3.050$, $p = 0.005$, unpaired t test). In addition, no changes were observed in NAcb core protein expression of SK2 subunits (Figure 3G; naive: n = 12; alcohol: n = 12; $t_{(22)} = 1.720$, $p = 0.099$, unpaired t test). Thus, abstinence from long-term alcohol self-administration was associated with enhanced NAcb core firing (Figure 1), reduced SK regulation of firing (Figure 2), reduced SK currents under voltage clamp (Figures 3A–3E), and decreased NAcb core SK3 but not SK2 subunit protein expression (Figures 3F and 3G), suggesting that decreased SK3 subunit protein expression likely contributed to decreased SK currents after alcohol self-administration and abstinence.

SK Activation Differentially Suppressed Firing in NAcb Core Neurons from Alcohol- versus Sucrose-Abstinent Animals

Our firing and voltage-clamp results indicate that SK is a potent and differential regulator of neuronal activity in NAcb core neurons from alcohol- and sucrose-abstinent animals, since SK inhibition with apamin produced a smaller increase in firing in neurons from alcohol-abstinent animals that correlated with a greater basal input/output slope (Figures 2 and S2). Thus, we examined whether SK activation with 1-EBIO, which can enhance SK function by increasing the apparent calcium sensitivity (Pedersen et al., 1999; Walter et al., 2006), and the subsequent inhibition of firing might also be altered after alcohol and abstinence. 100 μM 1-EBIO had no effect on firing (Figure 4 and Tables S1 and S2; 10% sucrose: n = 6 from 5 rats; 2% sucrose: n = 5 from 4 rats; alcohol: n = 6 from 5 rats; $F_{(2,14)} = 2.510$, $p = 0.117$, one-way ANOVA). In contrast, 300 μM 1-EBIO significantly decreased AP generation in NAcb core neurons, with a significantly greater depression of firing in neurons from alcohol- versus sucrose-abstinent animals (Figure 4 and Tables S1 and S2; 10% sucrose: n = 13 from 9 rats; 2% sucrose: n = 7 from 6 rats; alcohol: n = 8 from 6 rats; $F_{(2,25)} = 7.671$, $p = 0.003$,

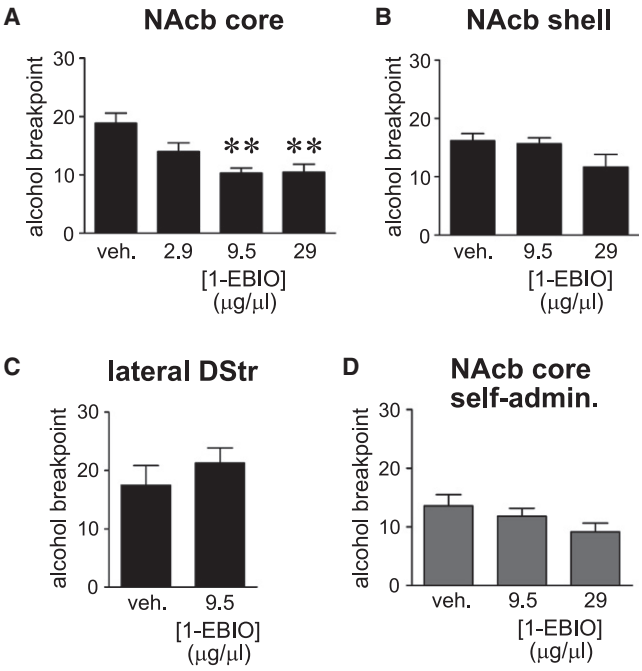

Figure 5. NAcb Core SK Activation Reduced the Motivation to Seek Alcohol

(A) Intra-NAcb core 1-EBIO microinjection dose-dependently reduced the motivation expressed to obtain alcohol after abstinence, measured as breakpoint under a progressive ratio schedule of reinforcement.

(B and C) The breakpoint obtained for alcohol was not reduced by SK activation in the (B) NAcb shell or (C) lateral DStr. Intra-NAcb core 1-EBIO also reduced the g/kg alcohol consumed during the progressive ratio test session (vehicle: 0.23 ± 0.01 g/kg; low 1-EBIO: 0.20 ± 0.01 g/kg; medium 1-EBIO: 0.17 ± 0.01 g/kg; high 1-EBIO: 0.18 ± 0.1 g/kg; $F_{(3,76)} = 5.025$, $p = 0.003$, one-way ANOVA; $p < 0.05$ vehicle versus medium and high 1-EBIO).

(D) Breakpoint for alcohol during self-administration without abstinence was not significantly reduced by 1-EBIO in the NAcb core. Animals were tested under PR with one dose of 1-EBIO, or two different doses of 1-EBIO, in a counterbalanced manner, with 1 week of home-cage abstinence between sessions, except in (D), where animals self-administered alcohol each day between PR sessions. Also, the high 1-EBIO dose (29 μg/μl) was chosen because it was the highest solubility we could obtain in the 10% DMSO/90% saline vehicle. Error bars indicate SEM. veh., vehicle; self-admin., self-administration. **$p < 0.01$ vehicle versus medium and high dose 1-EBIO.

See also Figures S4 and S5 and Table S4.

one-way ANOVA, $p < 0.05$ alcohol versus sucrose). In addition, the 1-EBIO-mediated reduction in firing was strongly reversed by subsequent application of apamin (Figure 4 and Table S1; 10% sucrose: n = 9 from 7 rats; 2% sucrose: n = 6 from 6 rats; alcohol: n = 9 from 6 rats; two-way RM-ANOVA across baseline, 1-EBIO, and apamin: drug: $F_{(2,36)} = 101.2$, $p < 0.001$; group: $F_{(2,36)} = 4.041$, $p = 0.036$; drug × group: $F_{(4,36)} = 2.125$, $p = 0.098$; $p < 0.001$ apamin versus 1-EBIO), strongly suggesting that 1-EBIO suppressed NAcb core firing through SK activation. Also, there was a trend toward a significantly smaller apamin enhancement of firing after alcohol and abstinence ($F_{(2,21)} = 3.101$, $p = 0.066$, one-way ANOVA), in agreement with reduced SK regulation of NAcb core firing in alcohol-abstinent animals. Taken together, these results suggest that SK activation produced a significantly greater inhibition of firing in neurons from alcohol- versus sucrose-abstinent animals.

SK Activation in the NAcb Core Reduced the Motivation to Seek Alcohol but Not Sucrose

We hypothesized that the decreased NAcb core SK currents and increased excitability observed ex vivo would increase NAcb core responsiveness to alcohol-related stimuli and thereby facilitate motivation to obtain alcohol. Thus, we examined operant responding for alcohol or sucrose after abstinence on a progressive ratio schedule of reinforcement, where the response requirement for reinforcement increased with each subsequent reinforcer obtained. The point at which the rat stops responding, the breakpoint, is considered a quantitative indicator of motivation (Roberts et al., 2007; Sanchis-Segura and Spanagel, 2006). We utilized 1-EBIO to examine SK modulation of motivation for reinforcers under progressive ratio since 1-EBIO has previously been used for intracranial infusion to activate SK channels during behavior (Walter et al., 2006; Zavala-Tecuapetla et al., 2008).

The breakpoint for alcohol after abstinence was significantly and dose-dependently reduced by 1-EBIO infusion into the NAcb core (Figure 5A and Table S4; vehicle: n = 23; low dose [2.9 μg/μl] 1-EBIO: n = 17; medium dose [9.5 μg/μl] 1-EBIO: n = 23; high dose [29 μg/μl] 1-EBIO: n = 17; Kruskal-Wallis statistic = 17.35, $p < 0.001$; $p < 0.01$ vehicle versus medium and high 1-EBIO). In strong contrast, the breakpoint for alcohol after abstinence was not significantly reduced by 1-EBIO in the NAcb shell (Figure 5B and Table S4; vehicle: n = 17; medium 1-EBIO: n = 11; high 1-EBIO: n = 6; Kruskal-Wallis statistic = 2.544, $p = 0.280$) or in the lateral DStr (Figure 5C and Table S4; vehicle: n = 8; medium 1-EBIO: n = 8; Mann-Whitney U = 16.50, $p = 0.105$). Lever-pressing for alcohol during the progressive ratio session showed a similar effect of intracranial 1-EBIO as breakpoint (Figure S4). Thus, only SK activation within the NAcb core was able to reduce motivation to obtain alcohol after abstinence.

Our results above suggest that NAcb core SK regulation of firing ex vivo was reduced after abstinence from alcohol but not sucrose self-administration, and that activating SK within the NAcb core significantly reduced the motivation expressed to obtain alcohol. Thus, we next examined whether intra-NAcb core 1-EBIO would reduce motivation for an equivalent concentration of a natural reinforcer, a highly sweet 10% sucrose solution (Avena et al., 2008). The breakpoint for 10% sucrose after abstinence from operant sucrose self-administration was not reduced by 1-EBIO in the NAcb core (Figure 6A and Table S4; vehicle: n = 20; medium 1-EBIO: n = 21; high 1-EBIO: n = 15; Kruskal-Wallis statistic = 12.14, $p = 0.002$; $p > 0.05$ vehicle versus medium or high 1-EBIO). Lever pressing for 10% sucrose during the progressive ratio session also showed little effect of intracranial 1-EBIO (Figure S4). Thus, intra-NAcb core 1-EBIO did not reduce motivation for 10% sucrose at doses that significantly reduced the motivation expressed to obtain alcohol.

Since the breakpoint achieved for 10% sucrose or 5% sucrose is higher than that observed for 10% alcohol (Figures 5A and 6A; Bowers et al., 2008), we trained additional animals

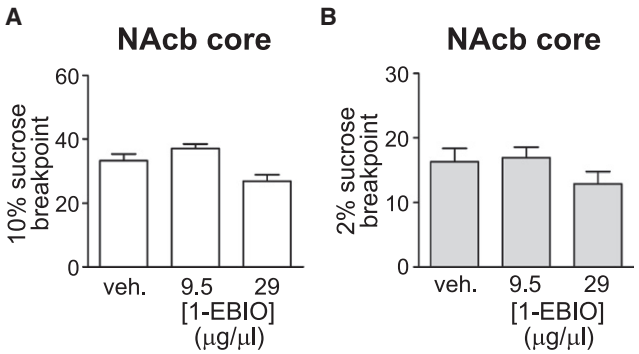

Figure 6. Moderate NAcb Core SK Activation Did Not Reduce the Motivation for Sucrose

(A) Intra-NAcb core 1-EBIO microinjection did not reduce breakpoint for 10% sucrose, although a trend for reduced breakpoint was observed at the high 1-EBIO dose that reduced motoric activity (Figure S4).

(B) Intra-NAcb core 1-EBIO microinjection did not reduce breakpoint for 2% sucrose. Error bars indicate SEM. veh., vehicle; sucr., sucrose.

See also Figures S4 and S5 and Table S4.

to self-administer 2% sucrose. Lever pressing across the last 10 days of self-administration (Table 1) and breakpoint after abstinence in vehicle-injected animals (Figures 5A and 6B and Table S4; alcohol: 17.4 ± 1.1 breakpoint, n = 47; 2% sucrose: n = 14) were not different between alcohol and 2% sucrose rats (presses: $t_{(145)} = 0.040$, p = 0.968; breakpoint: $t_{(60)} = 0.608$, p = 0.546; both unpaired t test). However, although the levels of responding for 10% alcohol and 2% sucrose were comparable, the breakpoint for 2% sucrose after abstinence was not reduced by 1-EBIO in the NAcb core (Figure 6B and Table S4; medium 1-EBIO: n = 13; high 1-EBIO: n = 11; Kruskal-Wallis statistic = 3.460, p = 0.177). Lever pressing for 2% sucrose during the progressive ratio session also showed little effect of intracranial 1-EBIO (Figure S4). Thus, intra-NAcb core SK activation significantly reduced motivation for alcohol but had no effect on motivation for sucrose at sucrose concentrations that elicited greater (10%) or similar (2%) levels of responding as alcohol.

We should note that there was a trend toward reduced breakpoint and responding after abstinence with the high 1-EBIO dose in NAcb core 2% sucrose-abstinent animals (Figures 6B and S4), NAcb core 10% sucrose-abstinent animals (Figures 6A and S4), and NAcb shell alcohol-abstinent animals (Figures 5B and S4). Accordingly, intra-Nacb core infusion of the high but not medium 1-EBIO dose reduced the acute locomotor-activating effects of the psychostimulant cocaine (Figure S4). Thus, the highest 1-EBIO dose may have reduced the apparent motivation through more nonspecific effects on motivational or motoric function, but the medium dose of 1-EBIO only reduced alcohol breakpoint when infused into the NAcb core. A lack of general motor impairment by the medium dose of 1-EBIO is also supported by no differences in inactive lever responding under progressive ratio (Table S5). Together, these data support the hypothesis that neuroadaptations in NAcb core SK after alcohol and abstinence allowed the SK activator 1-EBIO to reduce the motivation expressed to obtain alcohol but not sucrose.

To determine whether 1-EBIO inhibition of motivated responding was particular to alcohol in general versus sucrose, we examined the effect of NAcb core 1-EBIO on the motivation expressed to obtain alcohol after only 24 hr abstinence, a time point equivalent to that experienced during the daily self-administration training. However, the breakpoint for alcohol during self-administration was not significantly reduced by 1-EBIO microinjection into the NAcb core (Figure 5D and Table S4; n = 16 for all groups; Kruskal-Wallis statistic = 3.796, p = 0.150), suggesting that 1-EBIO reduction of alcohol breakpoint after abstinence was not simply an effect of alcohol intake per se.

DISCUSSION

Our results suggest that reduced SK regulation of AP firing in the NAcb core after longer-term alcohol self-administration and protracted abstinence may represent a critical regulator of motivation for alcohol after abstinence. AP firing ex vivo was enhanced in neurons from the NAcb core but not the NAcb shell or lateral DStr after alcohol self-administration and abstinence, with no change in NAcb core firing after sucrose self-administration and abstinence. Firing and voltage-clamp analyses revealed that enhanced NAcb core excitability after alcohol and abstinence was due to reduced SK channel currents, with a concurrent reduction of NAcb core SK3 but not SK2 subunit protein expression. Further, SK activation with the positive modulator 1-EBIO suppressed firing ex vivo to a greater extent in NAcb core neurons from alcohol- versus sucrose-abstinent animals. SK activation with 1-EBIO in the NAcb core in vivo significantly reduced the motivation expressed to obtain alcohol after abstinence. In contrast, NAcb core 1-EBIO did not reduce motivation for sucrose, and 1-EBIO in the NAcb shell and lateral DStr did not alter alcohol seeking, at doses that significantly reduced alcohol seeking in the NAcb core. This suggests that the 1-EBIO-mediated reduction in the motivation expressed to obtain alcohol did not occur simply through nonspecific motor or motivational impairments. Thus, altered SK channel function in the NAcb core represents a crucial mechanism that facilitates motivation for alcohol after abstinence.

Understanding the molecular mechanisms that develop in relation to repeated drug self-administration and abstinence is a critical step in identifying potential therapies for relapse, since these neuroadaptations can potently facilitate drug seeking. Here, we identified a relationship between SK function and motivation for reinforcers, whereby intracranial infusion of SK activators only altered seeking behavior under conditions where SK function measured ex vivo was reduced, namely in the NAcb core of alcohol-abstinent animals. AP firing is the predominant mechanism for neuronal transmission of information, and phasic NAcb core firing in relation to behaviorally relevant stimuli (Carelli and Wightman, 2004; Nicola, 2007; Schultz, 2004), including increased firing after abstinence from cocaine (Hollander and Carelli, 2007), is particularly interesting given that drug-related stimuli can potently drive relapse in rodent models of addiction (Epstein et al., 2006; Kalivas and McFarland, 2003; Katner and Weiss, 1999) and in human addicts (Larimer et al., 1999; Sanchis-Segura and Spanagel, 2006). Although the impact of drug-related stimuli can be studied using operant models where

responding for the drug is extinguished before examining whether drug-related stimuli can reinstate responding (Kalivas and McFarland, 2003; Sanchis-Segura and Spanagel, 2006), we utilized a model where animals underwent protracted abstinence before relapse testing, since human addicts may not undergo explicit extinction of drug-related stimuli and behaviors (Epstein et al., 2006; Sanchis-Segura and Spanagel, 2006). Increasing motivation to obtain alcohol across abstinence, termed the alcohol deprivation effect, has been considered a model for pathological or aberrant alcohol seeking and relapse (Heyser et al., 1997; Sanchis-Segura and Spanagel, 2006; Sinclair and Senter, 1968). Although speculative, motivation for alcohol after abstinence in the paradigm used here persists when alcohol-related cues are present but access to alcohol is not allowed; however, responding is greatly reduced when both alcohol-related cues and alcohol are removed (Bowers et al., 2008), suggesting the importance of alcohol-related cues in maintaining motivation for alcohol (Bowers et al., 2008), and previous studies have highlighted the importance of the NAcb core in many cue-related behaviors (Cardinal et al., 2002; Carelli and Wightman, 2004; Epstein et al., 2006; Everitt and Robbins, 2005; Kalivas and McFarland, 2003; Kelley, 2004; Mogenson et al., 1980). Also, although the levels of alcohol and sucrose intake in the present study were relatively moderate compared to those in other studies demonstrating the development of physical dependence (Table 1; Avena et al., 2008; Bell et al., 2006), we observed enhanced motivation for alcohol during abstinence compared to animals without abstinence (Mann-Whitney U = 113.0, p = 0.044, see also Bowers et al., 2008). Since activation of NAcb core SK channels reversed the motivation expressed to obtain alcohol during abstinence, with little effect on motivation for sucrose, neuroadaptations in NAcb core SK channels represent critical modulators of maladaptive motivation for alcohol after abstinence.

NAcb shell AP firing ex vivo was not altered after alcohol and abstinence, and NAcb shell 1-EBIO infusion did not alter the motivation expressed to obtain alcohol at doses that reduced alcohol seeking when injected in the NAcb core. This is consistent with the possibility that the NAcb shell generally plays little role in well-learned appetitive behaviors and instead is implicated in novelty and primary reward and in some forms of learning, including in relation to Pavlovian stimuli (Cardinal et al., 2002; Everitt and Robbins, 2005). However, the NAcb shell is also implicated in drug-primed reinstatement of cocaine seeking (Schmidt et al., 2005), and the lack of role of the NAcb shell observed here suggests that the primary reinforcing effects of alcohol may not be required to maintain motivation expressed to obtain alcohol after abstinence. Perhaps more surprising is that, despite more than 40 continuous days of alcohol self-administration, which could recruit habit circuitry (Everitt and Robbins, 2005), SK activation with 1-EBIO in the lateral DStr did not reduce the motivation to seek alcohol after abstinence, and lateral DStr AP firing was not altered after alcohol and abstinence. A recent study found that the NAcb core and DStr can interact to maintain habitual behavior (Belin and Everitt, 2008), and it is possible that more potent inhibition of the lateral DStr could reduce alcohol seeking. In addition, brain areas other than the striatum also likely regulate motivation for alcohol (Spanagel, 2009). For example, decreased SK function in VTA dopamine neurons after repeated passive alcohol exposure enhances burst firing (Hopf et al., 2007), which could enhance motivation for alcohol by increasing NAcb dopamine levels (Spanagel, 2009). Interestingly, SK3-deficient mice show elevated striatal dopamine levels and altered dopamine-dependent emotional behaviors (Jacobsen et al., 2008); this could occur through reduced midbrain SK3 levels leading to increased dopamine release or through enhanced excitability in other brain areas. However, the observation that a more moderate reduction of firing by a NAcb core SK activator was able to significantly decrease motivation for alcohol supports the importance of NAcb core SK neuroadaptations in regulating the motivation to obtain alcohol after abstinence.

Interestingly, NAcb core 1-EBIO did not alter motivation for sucrose, even though the NAcb core can regulate sucrose seeking under some conditions (Bari and Pierce, 2005). We hypothesize that an inability of 1-EBIO to alter reward-seeking behavior, even though SK activation with 1-EBIO reduced firing somewhat ex vivo, could in part reflect a floor effect where the 1-EBIO influence on firing is more moderate under conditions of strong basal SK function. These results also suggest that NAcb core 1-EBIO inhibition of alcohol seeking arose from a reduction in the motivation for alcohol rather than a global, nonspecific reduction in motor or motivational capacity. Further, although SK channels have been localized to presynaptic glutamatergic terminals in brain regions other than the striatum (Brosh et al., 2007), the SK antagonist apamin did not alter the amplitude of evoked excitatory postsynaptic potentials (EPSPs) in the NAcb core of naive rats (–0.7% ± 5.1% change in EPSP amplitude with apamin; $t_{(6)} = 0.385$, p = 0.714, paired t test; n = 7), suggesting that NAcb core SK did not regulate glutamate release and, thus, that the in vivo 1-EBIO effects reported here are more likely mediated by postsynaptic SK channels. In addition, although our preliminary waveform analyses suggested that SK neuroadaptations contribute prominently to changes in NAcb core firing, we cannot unequivocally rule out the possibility that neuroadaptation in channels other than SK also contributed, especially in vivo. Although NAcb core firing has been studied in vivo during ongoing alcohol self-administration (Janak et al., 1999), and SK changes ex vivo lasting several days after learning have been observed (Brosh et al., 2007), our study links the molecular changes in potassium channel function in a particular brain region, the NAcb core, to an enhancement of drug-seeking behavior.

Decreased NAcb core SK currents after alcohol and abstinence could reflect reduced SK subunit expression or, given SK activation by calcium, decreased calcium flux into NAcb core neurons (Bargas et al., 1999). SK3 subunits are expressed at high levels in the naive NAcb core (Sailer et al., 2002; Stocker and Pedarzani, 2000), and SK3 but not SK2 subunit protein expression was reduced during abstinence from alcohol, which likely contributed to the decreased SK currents and increased excitability in the NAcb core that is postulated to facilitate the motivation to obtain alcohol. Thus, we consider it more likely that NAcb core neurons from alcohol-abstinent animals contained fewer SK channels, which reduced overall SK function and enhanced excitability, but that the remaining SK channels

in alcohol neurons exhibited normal properties. Also, if reduced SK function were due to decreased calcium sensitivity of SK channels, the effect of 1-EBIO on firing would likely be reduced rather than enhanced in alcohol- versus sucrose-abstinent animals. Instead, we hypothesize that a reduced 1-EBIO effect on firing in sucrose-abstinent animals reflected a floor effect where strong basal SK function decreased the ability of 1-EBIO to further reduce firing. Thus, our results suggest that decreased NAcb core SK function after alcohol and abstinence reflected reduced SK3 subunit levels. However, the mechanism underlying the reduced SK3 protein expression after alcohol and abstinence remains unclear. Genetic regulation of SK subunits is generally poorly understood. SK3 subunit gene expression can be increased through estrogen receptor activation in vitro (Bosch et al., 2002; Jacobson et al., 2003), and SK2 subunit gene expression can be decreased by glucocorticoids acting through NF$\kappa\beta$ (Kye et al., 2007). Although acute estrogen can positively influence alcohol seeking (Ford et al., 2004), long-term alcohol exposure can reduce estrogen function (Dees et al., 2000). A similar pattern is apparent for NFkB (Okvist et al., 2007) and perhaps other regulators of gene expression, such as CREB (Spanagel, 2009). Since the acute reinforcing effects of alcohol that sustain self-administration can occur through a number of molecular targets, and self-administration could in part be sustained by conditioned responses mediated by receptors for several neurochemicals (Spanagel, 2009), it is challenging to identify a priori how acute alcohol exposure induced the NAcb core SK neuroadaptation after alcohol and abstinence. However, since the degree of SK neuroadaptation did not correlate with the level of alcohol self-administration prior to abstinence, we speculate that changes in NAcb firing after alcohol and abstinence may reflect associative learning. For example, given the important role for the NAcb core in appetitive learning (Kelley, 2004; Spanagel, 2009), signaling systems in the NAcb that maintain alcohol self-administration through rewarding or conditioned effects could secondarily cause genetic changes. Future experiments are required to address the molecular, genetic, and behavioral interactions that may lie upstream of reduced SK3 protein expression.

In conclusion, we propose that reduced NAcb core SK regulation of firing represents a crucial mechanism underlying the heightened motivation to seek alcohol. Our evidence that the behavioral efficacy of SK activators is dependent upon an alcohol-induced decrease in SK activity, coupled with the behavior- and brain-region-specific reduction in alcohol seeking by positive SK modulators, suggests that positive SK modulators represent a promising therapeutic intervention against excessive alcohol drinking. Our data are particularly exciting because the FDA-approved drug chlorzoxazone, which has been used for more than 30 years as a centrally acting myorelaxant (Chou et al., 2004), can activate SK channels in a similar manner as 1-EBIO (Cao et al., 2001). It is important to note that SK is not the only target of chlorzoxazone (Dong et al., 2006) and that this molecule can present a variety of clinical side effects (Chou et al., 2004). Still, this FDA-approved compound provides an unexpected and very exciting opportunity to design human clinical trials to examine whether chlorzoxazone reduces excessive or pathological alcohol drinking, and our study highlights the potential of SK channel activators as therapeutic agents against pathological alcohol consumption.

EXPERIMENTAL PROCEDURES

All behavioral methods were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and the Ernest Gallo Clinic and Research Center's Institute for Animal Care and Use Committee and were essentially the same as described in Bowers et al. (2008).

Self-Administration Training

Adult male, Wistar rats (250–275 g, Harlan, Livermore, CA) were individually housed and maintained on a 12 hr light/dark cycle (lights on 7 am) with food and water available ad libitum unless stated otherwise. Rats slated for alcohol training were given home cage alcohol (10%, w/v) ad libitum as their sole liquid source for 5 days. Rats slated for sucrose training were water deprived for 2 days. Afterward, rats were placed in standard operant chambers (Coulbourn Instruments, Allentown, PA) fitted with a 100 μl dipper cup, flanked by two levers, each with a cue light. Chambers were outfitted with a house light, cue tone, and sound-attenuation cubicles that contained fans. Shaping of lever pressing occurred over one to three 15 hr overnight sessions for 10% sucrose (w/v) on an FR-1 schedule of reinforcement. Depression of one lever (active) resulted in delivery of the cue light, tone, and liquid reinforcement in a lick-contingent manner (where access to the reinforcer was terminated if licking did not commence within 2 s). Depression of the other lever (inactive) yielded no programmed consequence. Licks at the dipper were recorded with a lickometer to verify that lever presses were followed by consumption of the reinforcer. Rats were randomly assigned to right or left active lever prior to shaping. Rats exhibiting more than 400 active lever presses in the overnight session were considered to have learned the lever-pressing paradigm.

After these overnight sessions, rats were allowed a 45 min session to respond for 10% sucrose (FR-1) and then a 30 min FR-3 session for 10% sucrose the following day. After these sessions, sucrose-drinking rats lever pressed for 10% or 2% sucrose (30 min/day, FR-3) for 42–50 continuous days. Training for alcohol-drinking rats proceeded on a sucrose-fading method, with 1–2 days each of responding for a 10% alcohol solution containing sucrose at 10%, 5%, 2.5%, then 1% (30 min/day, FR-3). After this fading procedure, rats lever pressed for 10% alcohol for 42–50 additional continuous days (30 min/day, FR-3). After 42–50 days of sucrose only or alcohol only self-administration, rats were left in their home cages for 3–5 weeks, at which time brain slices were prepared for patch-clamp electrophysiology or behavioral responding for sucrose or alcohol under progressive ratio was examined.

Surgery

Implantation of microinjection cannulae was performed as previously described (Bowers et al., 2008) except that cannulae were aimed 1 mm above the target region with the following coordinates relative to bregma with a level skull: NAcb core +2.2 mm AP, ±2.41 mm ML, and −6.18 mm DV, θ 8° angle away from midline; NAcb shell +2.2 mm AP, ±2.35 mm ML, and −6.25 mm DV, θ 8° away from midline; lateral DStr +1.0 mm AP, ±3.6 mm ML, −4.0 DV. Cannulae were obturated (33 g, stainless steel) and monitored daily. Surgery occurred 1 week into abstinence, except for animals tested with 1-EBIO during self-administration, where surgery occurred 2.5–3 weeks before progressive ratio testing. See Figure S5 for representative histology.

Progressive Ratio Assessment of Motivation

Microinjection was performed as previously described (Bowers et al., 2008) except that freshly prepared 1-EBIO or vehicle (10% DMSO in 0.9% NaCl) was injected in 0.75 μl per side over 2 min. Microinjectors were left in place for an additional 2 min to prevent reflux and to facilitate diffusion, after which time injectors were removed, obturators replaced, and animals returned to the home cage for 6 min. After this time, the motivation to seek alcohol or sucrose was assessed. Animals were tested under PR with one dose of 1-EBIO, or two different doses of 1-EBIO, in a counterbalanced manner, with 1 week home-cage abstinence between sessions. Animals were assigned

to particular doses based upon prior self-administration levels so that self-administration levels were balanced across dose groups. No ordering effects related to exposure of animals to multiple doses were observed ($p > 0.05$ for all experimental groups, two-way repeated-measures ANOVA).

Breakpoint under PR was determined exactly as described in Bowers et al. (2008). Briefly, the PR session was initiated by presentation of a compound cue (extension of the levers, illumination of the stimulus light over the active lever, tone sounding, and illumination of a raised dipper cup filled with alcohol or sucrose). In addition, rats were presented with an alcohol odor cue (~15 ml of ~87% alcohol sprinkled in the bedding beneath the previously alcohol-paired lever) for 2 min before presentation of the compound cue; the bedding of sucrose rats was sprinkled with water. After the compound cue, responding proceeded under a PR schedule that was the same for alcohol and sucrose rats. Briefly, after the compound cue, rats could lick the dipper cup, press a lever, or do nothing. If rats licked first (in ~50% of rats), a PR schedule of reinforcement of 1, 1, 2, 2, 3, 4, 5, 6, 7, 9, 10, 12, 13, 15, 17, 20, 22, 25, 28, 32, 36, 40, 45, 50, etc., ensued. If rats pressed first (in ~50% of rats), a PR of 1, 2, 2, 3, 4, 5, 6, 7, 9, 10, 12, 13, 15, 17, 20, 22, 25, 28, 32, 36, 40, 45, 50, etc., ensued. If the rat chose to do nothing, a 20 s timeout period occurred, and the rat was re-cued with the compound cue for up to 20 iterations. In the <2% of rats that required re-cue, one or two re-cues were sufficient to elicit a response. Breakpoint was defined as the number of presses contained in the last, successfully completed ratio in either a 1 hr session or after 15 min of nonresponding, whichever came first. >75% of rats ended the session early by omitting response for 15 min. Null responses, where an animal completed the required number of lever presses but did not lick to receive the reinforcer, were not counted toward the breakpoint.

Slice Preparation

Brain slice preparation was performed as described in Martin et al. (2006) and are described in detail in Supplemental Experimental Procedures.

Ex Vivo Electrophysiology

Electrophysiology experiments were performed using previously described methods (Hopf et al., 2003, 2007) and are described in detail in Supplemental Experimental Procedures.

Immunoblotting

SK3 and SK2 subunit protein expression was assayed in the NAcb core during abstinence as previously described, including Ponceau S visualization (Bowers et al., 2008), except that 15 μg protein sample was transferred to 0.22 μm pore nitrocellulose prior to incubation with either a SK3 N terminus antibody (1:800, Alomone, Jerusalem, Israel) or a SK2 antibody C-terminal (1:800, Alomone), overnight at 4°C in 4% milk, then rinsed and probed with an IR800-labeled, anti-rabbit secondary (1:10,000, Rockland, Gilbertsville, PA). To control for loading, transfer, and blotting conditions, membranes were cut after transfer and probed for the integral ER membrane protein calnexin (1:3000, Stressgen, Ann Arbor, MI) followed by the IR800 antisera. Calnexin has been used in this capacity before, since expression was shown not to change during abstinence from cocaine or alcohol (Bowers et al., 2004, 2008). Loading concentration and antisera dilution were determined to be within the linear range of detection using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Blots were quantified by integrated intensity, a measure of pixel density independent of image resolution and the size of the bounding box drawn to define lanes and bands. SK1 subunit expression was not examined due to very low expression in the striatum (Sailer et al., 2004; Stocker and Pedarzani, 2000) and lack of a suitable commercially available antibody.

SK3 and SK2 protein expression was compared between alcohol-abstinent animals and naive animals. SK protein expression was not measured in sucrose-abstinent rats, since SK regulation of firing, measured using electrophysiology ex vivo, was nearly identical in naive and sucrose-abstinent animals. Thus, these more precise electrophysiological data suggest that immunoblotting would not detect differences in SK subunit protein expression between sucrose-abstinent and naive rats, although we cannot completely rule out the possibility that sucrose-abstinent animals may also exhibit altered SK subunit expression.

Statistics

All data were shown as mean plus or minus the standard error of the mean. Unless otherwise indicated, all statistics were performed using a one-way ANOVA followed by a Bonferroni correction or a two-way repeated-measures ANOVA followed by a Tukey post-hoc comparison. Because active lever-pressing requirements under PR were generated from an exponential function and were not normally distributed, data from the PR test sessions were analyzed using the nonparametric Mann-Whitney rank sum test or Kruskal-Wallis test with a Dunn's post-hoc comparison. Statistics were calculated using SigmaStat 3.1 (Systat Software, San Jose, CA).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Discussion, five figures, and five tables and can be found with this article online at doi:10.1016/j.neuron.2010.02.015.

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