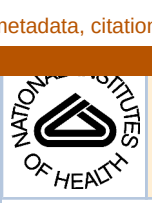

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INVOLVEMENT OF PROTEIN KINASE A IN ETHANOL-INDUCED LOCOMOTOR ACTIVITY AND SENSITIZATION

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Abstract

Rationale—Mutant mice lacking the RIIβ subunit of protein kinase A (regulatory subunit II beta$^{-/-}$) show increased ethanol preference. Recent evidence suggests a relationship between heightened ethanol preference and susceptibility to ethanol-induced locomotor sensitization. It is currently unknown if protein kinase A signaling modulates the stimulant effects and/or behavioral sensitization caused by ethanol administration. To address this question, we examined the effects of repeated ethanol administration on locomotor activity RIIβ$^{-/-}$ and littermate wild-type (RIIβ$^{+/+}$) mice on multiple genetic backgrounds.

Methods—Over three consecutive days, mice were given single i.p. saline injections and immediately placed in a loco-motor activity apparatus to establish a composite baseline for locomotor activity. Next, mice maintained on a hybrid 129/SvEv×C57BL/6J or pure C57BL/6J genetic background were given 10 i.p. ethanol injections before being placed in the activity apparatus. Each ethanol injection was separated by 3–4 days. To determine if changes in behavior were specific to ethanol injection, naïve mice were tested following repeated daily saline injections. The effects of ethanol injection on locomotor behavior were also assessed using an alternate paradigm in which mice were given repeated ethanol injections in their home cage environment.

Results—Relative to RIIβ$^{+/+}$ mice, RIIβ$^{-/-}$ mice, regardless of genetic background, consistently showed significantly greater ethanol-induced locomotor activation. RIIβ$^{-/-}$ mice also showed increased sensitivity to ethanol-induced locomotor sensitization resulting from repeated administration, an effect that was dependent on genetic background and testing paradigm. Increased locomotor activity by RIIβ$^{-/-}$ mice was specific to ethanol injections, and was not related to altered blood ethanol levels.

Conclusions—These data provide novel evidence implicating an influence of protein kinase A signaling on ethanol-induced locomotor activity and behavioral sensitization. The observation that RIIβ$^{-/-}$ mice are more sensitive to the effects of repeated ethanol administration suggests that normal protein kinase A signaling limits, or is protective against, the stimulant effects of ethanol and the plastic alterations that underlie behavioral sensitization.

Keywords

knockout; ethanol; protein kinase A; locomotor activity; sensitization

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Behavioral sensitization has been defined as the long-lasting and progressive enhancement of the locomotor and motivational responses to a drug following repeated administration (Kalivas and Stewart, 1991). Over the past 20 years, many studies have demonstrated the ability of repeated ethanol exposure to elicit locomotor sensitization in mice (Crabbe et al., 1992; Cunningham and Noble, 1992; Lister, 1987; Phillips et al., 1995). Interestingly, a genetic predisposition to alcoholism has been associated with increased sensitivity to this phenomenon. Sons of alcoholics, when compared with sons of non-alcoholics, have been shown to be more sensitive to increases in locomotor activity that emerge over the course of repeated ethanol administrations (Newlin and Thomson, 1991). It has been suggested that increased sensitivity to behavioral sensitization may be an underlying mechanism that increases the risk for developing drug dependence. According to this view, repeated exposure to a drug promotes neural reorganization leading to a hypersensitive state in brain reward circuitry (Robinson and Berridge, 1993, 2000, 2001).

Studies of neural plasticity have shown that intracellular cyclic AMP (cAMP)-dependent protein kinase A (PKA) modulates neurophysiological alterations that are responsible for the sensitization associated with repeated exposure to noxious stimuli (Castellucci et al., 1980). As such, it is possible that PKA signaling also modulates the neural plasticity that is believed to be responsible for the expression of drug-induced behavioral sensitization. In fact, some of the neurochemical systems that have been implicated in drug-induced locomotor stimulation and behavioral sensitization involve guanine nucleotide binding protein-coupled receptors that recruit PKA signaling, including dopamine (Broadbent et al., 1995, 2005; Hamamura et al., 1991; Itzhak and Martin, 1999; Lessov and Phillips, 2003; Mattingly et al., 1994; Palmer et al., 2003), adenosine (Chen et al., 2003), serotonin (Auclair et al., 2004), opioid (Camarini et al., 2000) and GABA (Broadbent and Harless, 1999). Theoretically, since PKA signaling is a basic neuronal mechanism influenced by different neurochemical pathways, neurobiological responses to drugs of abuse with different mechanisms of action on PKA may be a basis for drug cross-sensitization (Itzhak and Martin, 1999; Lessov and Phillips, 2003; McDaid et al., 2005; Muschamp and Siviy, 2002).

It is currently unknown if PKA signaling modulates the stimulant effects and/or behavioral sensitization caused by ethanol administration. To address this question, we examined the effects of repeated ethanol administration on locomotor activity in a PKA-mutant mouse model. In the mouse, PKA includes four regulatory subunits (RIα, RIβ, RIIα and RIIβ) and two catalytic subunits (Cα and Cβ) which are expressed in tissue-specific patterns (McKnight, 1991). Use of mice lacking the RIIβ subunit of PKA ($RII\beta^{-/-}$) allows for the assessment of locomotor sensitization in a model shown previously to have reduced cAMP-stimulated PKA activity in brain regions implicated in behavioral sensitization including the striatum and the nucleus accumbens (Brandon et al., 1998; Thiele et al., 2000b). We have previously shown that $RII\beta^{-/-}$ mice exhibit enhanced ethanol preference and consumption as well as reduced sensitivity to ethanol-induced sedation when compared with wildtype littermate control mice (Fee et al., 2004; Thiele et al., 2000b). Several recent studies have suggested a positive correlation between ethanol preference and ethanol-induced locomotor sensitization in mice (Grahame et al., 2000; Lessov et al., 2001; Palmer et al., 2003). Thus, because $RII\beta^{-/-}$ mice show increased ethanol preference, we hypothesized that $RII\beta^{-/-}$ mice would show increased sensitivity to the stimulant effects of ethanol and enhanced ethanol-induced locomotor sensitization.

Because the expression of phenotypes can depend on the genetic background of the knockout mouse model (Fee et al., 2004; Palmer et al., 2003; Thiele et al., 2004), we evaluated ethanol-induced locomotor activity and sensitization in $RII\beta^{-/-}$ and $RII\beta^{+/+}$ mice maintained on differing genetic backgrounds. The sensitization paradigm developed in our laboratory

involved bi-weekly ethanol injections. However, due to the fact that subtle differences in testing paradigm can lead to dramatic differences in observed behavior (Rustay et al., 2003), we also employed an alternate sensitization paradigm that is commonly used in other laboratories (Lessov et al., 2001; Meyer et al., 2005). This paradigm allows for repeated ethanol exposure in the homecage which eliminates many environmental cues associated with the testing chamber that could potentially lead to non-treatment specific increases in locomotor behavior.

EXPERIMENTAL PROCEDURES

Animals

RIIβ$^{-/-}$ mice were created through the disruption of the RIIβ gene by homologous recombination in embryonic stem cells from 129/SvJ mice (Brandon et al., 1998). Chimeras were bred with C57BL/6J mice to obtain heterozygotes (50% 129/SvJ×50% C57BL/6J). These heterozygotes were backcrossed with C57BL/6J mice over eight generations to yield RIIβ$^{\pm}$ mice on a ~100% C57BL/6J genetic background. For some experiments described here, non-littermate RIIβ$^{\pm}$ mice on the 100% C57BL/6J background were bred, to provide RIIβ$^{-/-}$ and RIIβ$^{+/+}$ F2 littermate mice. Additional experiments involved RIIβ$^{-/-}$ and RIIβ$^{+/+}$ F2 littermate mice on a 50% 129/SvEv×50% C57BL/6J background that were created by crossing the RIIβ$^{-/-}$ mice with wild-type 129/SvEv mice. The genetic status of all mice was determined using polymerase chain reaction (PCR) procedures described elsewhere (Thiele et al., 2000b). Animals weighed approximately 20 g, were 3–6 months of age at the beginning of experiments, and were individually housed in polypropylene cages with corncob bedding. Mice had *ad libitum* access to water and standard rodent chow (Tekland, Madison, WI, USA) except where noted. The colony room was maintained at approximately 22 °C with a 12-h light/dark cycle with lights off at 3:00 pm. The number of animals used in the present study was kept to a minimum and all procedures minimized animal suffering. All procedures used in the present study were in compliance with the National Institutes of Health guidelines, and all protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Ethanol-induced locomotor activation: test chamber injections

Drug-naïve RIIβ$^{-/-}$ (male, *n*=14; female, *n*=14) and RIIβ$^{+/+}$ (male, *n*=13; female, *n*=13) mice on a pure C57BL/6J background as well as RIIβ$^{-/-}$ (male, *n*=12; female, *n*=15) and RIIβ$^{+/+}$ (male, *n*=13; female, *n*=15) mice on a mixed 129/SvEv×C57BL/6J background were tested during the light phase of their light/dark cycle. All animals were transported to the testing room in their home cages and allowed to habituate for at least 35-min prior to testing. A fan provided masking noise in the testing room. Mice were removed from their home cages, given an i.p. ethanol or equivolume saline injection according to the dosing schedule outlined in Table 1A, and were placed into the center of an open-field arena that automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA, USA). The open field arena measured 40.64 cm by 40.64 cm by 30.48 cm and was made of clear Plexiglas. Several cm of corncob bedding were placed into the open field chamber to aid in cleaning and to prevent the buildup of odor. Testing sessions were 20-min in duration and soiled bedding was removed from the chamber after each session. In summary, mice received three i.p. injections of isotonic saline (one injection per day) every 2–3 days to establish a locomotor baseline for activity and to allow the mice to habituate to the testing procedure. Following completion of baseline testing, mice received i.p. ethanol injection (one per day) two times a week for up to 10 injections. Mice on the 129/SvEv×C57BL/6J genetic background were given a 1.4 g/kg dose while mice on the C57BL/6J genetic background were given a 2.0 g/kg dose (20% w/v solutions were mixed in isotonic saline). Doses of ethanol for each genetic background were chosen based on pilot observations in our laboratory. Higher doses were shown to have a significant sedative component in mice on the mixed genetic background. After ethanol injections, mice

were tested following a single (129/SvEv×C57BL/6J mice) or two (C57BL/6J mice) i.p. saline injections (one injection per day) to examine specificity of the effects of ethanol on locomotor activity. Mice on the C57BL/6J genetic background received two additional ethanol tests immediately after saline injections. Finally, as an additional test for specificity of the effects of ethanol injection on locomotor activity, naïve RIIβ$^{-/-}$ (male, *n*=10; female, *n*=10) and RIIβ$^{+/+}$ (male, *n*=10; female, *n*=10) mice on the mixed 129/SvEv×C57BL/6J background were run in a study using procedures similar to those described above with the exception that only i.p. saline injections were given over eight trials.

To determine blood ethanol levels, 6 μl tail nick blood samples were taken from 129/SvEv×C57BL/6J mice immediately after the 1st, 5th, and 10th 20-min test sessions that were run on ethanol injection days. For C57BL/6J mice, blood samples were collected following the 20-min session on the last (twelfth) ethanol injection day. Procedures for assessing blood ethanol levels are described below.

Ethanol-induced locomotor activation: home-cage injections

Drug-naïve RIIβ$^{-/-}$ (male, *n*=10; female, *n*=10) and RIIβ$^{+/+}$ (male, *n*=10; female, *n*=10) mice on a pure C57BL/6J background were tested during the light phase of their light/dark cycle. An overview of the sensitization paradigm, adapted from (Lessov et al. (2001), is described in Table 1B. This paradigm was used for its ability to induce locomotor sensitization in wildtype C57BL/6 mice. On test days 1–3, all mice received i.p. injections of isotonic saline (one injection per day) based on the equivalent volume for a 2.0 g/kg ethanol injection prior to being placed into the center of the open-field arena for 20-min sessions. On day 4, all mice received an i.p. injection of 2.0 g/kg ethanol prior to placement in the locomotor chamber in order to determine basal ethanol responsiveness during the 20-min session. Mice were then assigned to treatment groups equated for locomotor activity during the initial ethanol response (day 4). Over the next 10 days, mice received daily i.p. injections of 2.5 g/kg ethanol and were immediately returned to their home cage. On day 15, half of the mice received an i.p. injection of 2.0 g/kg ethanol and were immediately placed into the center of the activity apparatus to assess ethanol-induced locomotor sensitization. The remaining mice received an i.p. injection of saline to test for general activity not attributable to ethanol.

Blood ethanol concentrations

Six microliter tail nick blood samples were collected into capillary tubes and dispensed into 12×75 mm borosilicate glass tubes containing 375 μl of water and 0.5 g of NaCl. These liquid samples were capped and refrigerated until processing by gas chromatography. Liquid ethanol standards (also 6 μl, 0–200 mg%) and samples were similarly prepared and heated in a water bath at 55 °C for 10 min. Subsequently, a 1.5 ml sample of headspace gas was removed from the glass tubes with a plastic 3.0 ml syringe and injected directly into a SRI 8610C gas chromatograph (Torrance, CA, USA) equipped with an external syringe adapter and 1.0 ml external loading loop. Samples were run at 140 °C through a Hayesep D column and detected with a flame ionization detector at approximately 2 min post-inject. Hydrogen gas, carrier gas (also hydrogen), and internal air generator flow rates were 13.3, 25, and 250 ml/min, respectively. Areas under the curve for blood samples were analyzed with SRI PeakSimple software for Windows running on a Dell Inspiron 3500® laptop computer (Dell, Austin, TX, USA) and converted to mg% in blood based on the curve generated for the standards.

Data analyses

As a measure of the stimulant effects of ethanol on locomotor activity, the data for each ethanol test day data were expressed as change from average saline baseline (activity following ethanol injection–daily average of activity following initial saline injections). As a measure of sensitization to the effects of ethanol on locomotor activity, locomotor activity on the first

ethanol test day was subtracted from each of the subsequent ethanol test day data points (activity following the second and subsequent ethanol injection–activity following the first ethanol injection). Locomotor activity data (raw and converted) were analyzed using analyses of variance (ANOVA). In all analyses, sex was included as a variable of interest but failed to reveal significant main effects or interactions. For this reason, the sex of the mice was excluded as a variable from discussion in the proceeding results section. All data are presented as mean ±standard error of the mean (S.E.M.) and *t*-tests were used for planned comparisons (Winer et al., 1991). Significance was accepted at $P<0.05$ (two-tailed).

RESULTS

Ethanol-induced locomotor sensitization: test chamber injections

Locomotor activity data from mice maintained on the 129/SvEv×C57BL/6J are presented in Fig. 1. There were no differences between genotypes in locomotor activity following baseline saline injections or the saline injection given after the ethanol test days as determined by non-significant ANOVAs (Fig. 1A). Interestingly, the RIIβ$^{-/-}$ mice showed significantly greater locomotor activity over the 10 days of ethanol injections. A repeated measures ANOVA performed on ethanol-induced locomotor activity data revealed a significant main effect of genotype [$F(1, 51)=7.18$, $P=0.01$]. Post hoc tests indicated that the RIIβ$^{-/-}$ mice were significantly more active on ethanol injection days 1, 2, 4, 5, 6, 9, and 10 relative to RIIβ$^{+/+}$ mice. Similarly, when locomotor activity data were expressed as change from baseline activity (Fig. 1B), a repeated measures ANOVA run on ethanol test days showed a significant genotype main effect [$F(1, 51)=6.88$, $P<0.05$] and post hoc tests indicated greater locomotor activity by RIIβ$^{-/-}$ mice on days 2, 4, 6, 9, and 10. There were no genotype differences during the final saline injection. Surprisingly, when locomotor activity data were expressed as change relative to activity following the first ethanol injection–the assessment of locomotor sensitization (Fig. 1C), ANOVAs revealed no significant genotype differences during ethanol test days or during the final saline injection. Taken together, these data suggest that while RIIβ$^{-/-}$ mice on the mixed 129/SvEv×C57BL/6J genetic background were more sensitive to the stimulant effects of ethanol relative to RIIβ$^{+/+}$ mice (Fig. 1A and B), there were no genotype differences in the development of behavioral sensitization (Fig. 1C). Fig. 2 shows blood ethanol levels following the 1st, 5th, and 10th days of ethanol testing. Repeated measures ANOVA revealed no significant genotype or day effects. Thus, differences in ethanol-induced locomotor activity between RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice are not associated with altered blood ethanol levels. Importantly, RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice on the mixed 129/SvEv×C57BL/6J genetic background did not differ in locomotor activity following 8 days (3 baseline days, averaged; 5 test days) of saline injection (Fig. 3).

Locomotor activity data from mice maintained on the C57BL/6J are presented in Fig. 4. ANOVA of average baseline locomotor activity following the initial saline injections revealed that relative to the RIIβ$^{-/-}$ mice, RIIβ$^{+/+}$ mice showed greater locomotor activity (Fig. 4A) [$F(1, 51)=5.627$, $P<0.05$]. However, there were no significant differences in locomotor activity following saline injections given between the 10th and 11th ethanol injection (as noted as S4 and S5 in Fig. 4A). These injections were conducted to demonstrate the specificity of the locomotor response. Despite showing lower basal locomotor activity, RIIβ$^{-/-}$ mice on the C57BL/6J genetic background showed significantly greater ethanol-induced locomotor activity as revealed by a significant geno-type main effect following a repeated measures ANOVA run on the first 10 days of ethanol testing [$F(1, 47)=23.86$, $P<0.001$]. Post hoc tests indicated that RIIβ$^{-/-}$ mice were significantly more active at each of the 10 days. Similarly, a repeated measures ANOVA run on ethanol tests days 11 and 12 showed a significant main effect of genotype [$F(1, 48)=24.088$, $P<0.001$] that reflected the increased activity of RIIβ$^{-/-}$ mice relative to wild-type animals. When expressed as change from baseline activity (Fig. 4B), repeated measures ANOVAs revealed genotype differences during the initial 10

ethanol injections [$F(1, 47)=30.368$, $P<0.001$] and during the two ethanol injections given at the end of the study [$F(1, 48)=33.281$, $P<0.001$]. Post hoc tests indicated that RIIβ$^{-/-}$ mice showed greater locomotor activity relative to RIIβ$^{+/+}$ mice at every ethanol test day. There were no genotype differences in change from baseline data following saline injections (S4 and S5). In general, the overall pattern of data in Fig. 4B indicates that RIIβ$^{-/-}$ mice are more sensitive to the stimulant effects of ethanol. However, it is important to note that following the first ethanol injection, both RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice showed reduced activity relative to baseline activity. Significant genotype differences at this point are consistent with previous observations indicating that RIIβ$^{-/-}$ mice are less sensitive to the sedative properties of ethanol (Fee et al., 2004;Thiele et al., 2000b).

C57BL/6J locomotor data expressed as change relative to the first ethanol injection are shown in Fig. 4C. In contrast to the mixed background mice, C57BL/6J RIIβ$^{-/-}$ mice exhibited significantly greater expression of ethanol-induced locomotor sensitization over the course of ethanol injections. This conclusion was supported by a repeated measure ANOVA that revealed a significant main effect of genotype [$F(1, 47)=13.428$, $P=0.001$]. Post hoc analyses showed significant genotype differences on ethanol test days 4–10, 11, and 12. Additionally, a repeated measures ANOVA run on the final two ethanol test days was significant ([$F(1, 49)=11.674$, $P=0.001$]), again indicating that RIIβ$^{-/-}$ mice showed greater expression of locomotor sensitization relative to RIIβ$^{+/+}$ mice. On the other hand, a genotype main effect following a repeated measures ANOVA revealed that RIIβ$^{+/+}$ mice showed significantly greater locomotor activity relative to RIIβ$^{-/-}$ mice following saline injections (S4 and S5) [$F(1, 49)=18.415$, $P<0.001$]. Finally, there were no significant differences between RIIβ$^{-/-}$ (219.20±6.59 mg%) or RIIβ$^{+/+}$ (217.27±6.18 mg%) mice in blood ethanol levels immediately following the last ethanol test session on day 12. Thus, similar to mice on the mixed genetic background, RIIβ$^{-/-}$ mice in the pure C57BL/6J genetic background were more sensitive to the stimulant effect of ethanol relative to RIIβ$^{+/+}$ mice (Fig. 4A and B). Additionally, the greater increases in ethanol-induced locomotor activity by RIIβ$^{-/-}$ mice following repeated ethanol injections (Fig. 4C) indicated that RIIβ$^{-/-}$ mice on the C57BL/6J genetic background were also more sensitive to the development of locomotor sensitization.

Ethanol-induced locomotor activation: home cage injections

Locomotor activity data following home cage ethanol injections in mice maintained on a C57BL/6J genetic background are presented in Fig. 5. Similar to the previous experiment, RIIβ$^{-/-}$ mice on the C57BL/6J genetic background showed less average locomotor activity following baseline saline injections (Fig. 5A), as evidenced by a significant ANOVA [$F(1, 38)=5.685$, $P<0.05$]. Furthermore, an ANOVA run on loco-motor data collected following the first ethanol injection (day 4) revealed that RIIβ$^{-/-}$ mice showed significantly greater activity relative to RIIβ$^{+/+}$ mice [$F(1, 38)=9.311$, $P<0.005$]. A two-way mixed factor ANOVA run on locomotor activity data from test day 15 showed significant main effects of drug [$F(1, 36)=97.204$, $P<0.001$] and genotype [$F(1, 36)=7.681$, $P<0.01$], and a significant interaction between these variables [$F(1, 36)=5.245$, $P<0.05$]. Post hoc tests indicated that while locomotor activity was enhanced by ethanol injection (relative to saline injection) in both genotypes, such increases were greater in RIIβ$^{-/-}$ mice.

Test day activity data expressed as change from average baseline are presented in Fig. 5B. A two-way mixed factor ANOVA revealed significant genotype [$F(1, 36)=18.135$, $P<0.001$] and drug treatment [$F(1, 36)=106.113$, $P<0.001$] main effects. Post hoc tests confirmed that RIIβ$^{-/-}$ mice show greater ethanol-induced increases, and less saline-induced decreases, in locomotor activity relative to RIIβ$^{+/+}$ mice. Test day activity data expressed as change from first ethanol injection are presented in Fig. 5C. A two-way mixed factor ANOVA revealed a significant treatment main effect [$F(1, 36)=90.7$, $P<0.001$], but the genotype main effect was

not significant. Additionally, the interaction effect was significant [$F(1, 36)=5.012$, $P<0.05$]. Post hoc analyses revealed that this interaction could not be attributed to significant differences in locomotor activity in saline or ethanol treated RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice. Taken together, these data indicate that with the home cage injection paradigm, RIIβ$^{-/-}$ mice on the C57BL/6J genetic background are more sensitive to the locomotor stimulant effects of ethanol (Fig. 5B) but did not show enhanced sensitivity to behavioral sensitization (Fig. 5C) when compared with RIIβ$^{+/+}$ mice in this paradigm.

DISCUSSION

In the present report, RIIβ$^{-/-}$ mice consistently showed significantly greater ethanol-induced locomotor activity relative to RIIβ$^{+/+}$ mice. RIIβ$^{-/-}$ mice also showed increased sensitivity to ethanol-induced locomotor sensitization, an effect that may be dependent on genetic background and/or testing paradigm. Importantly, increased locomotor activity by RIIβ$^{-/-}$ mice was specific to ethanol injections (and not seen following saline injections), and was not associated with altered blood ethanol levels. Normal blood ethanol levels and ethanol metabolism by RIIβ$^{-/-}$ mice have previously been documented (Fee et al., 2004; Thiele et al., 2000b). Since RIIβ$^{-/-}$ mice show blunted PKA activity in critical brain regions, such as the striatum and nucleus accumbens (Brandon et al., 1998; Thiele et al., 2000b), the present results suggest that normal PKA signaling is part of a mechanism that protects against ethanol-induced locomotor activity and behavioral sensitization.

A growing body of literature is emerging indicating that phenotypes, including neurobiological responses to ethanol, can depend on the genetic background of the knockout model (Bowers et al., 1999; Howe et al., 2002; Kelly et al., 1998; Palmer et al., 2003; Phillips et al., 1999; Simpson et al., 1997; Thiele et al., 2000a). It is possible that the protective role of the RIIβ subunit of PKA against ethanol-induced behavioral sensitization depends on epistatic interactions with other genes, interactions that may depend on the genetic background of the mouse. This argument is consistent with the observation in the present report that RIIβ$^{-/-}$ mice on the pure C57BL/6J, but not the mixed 129/SvEv×C57BL/6J, genetic background showed enhanced behavioral sensitization when repeated ethanol injections where given immediately before locomotor activity testing. Alternatively, the expression of enhanced behavioral sensitization by RIIβ$^{-/-}$ mice may have depended on procedural differences between experiments, rather than mouse genetic background. In fact, different sensitizing doses of ethanol were used in each of the three experiments reported here, ranging from a 1.4 to a 2.5 g/kg dose. In addition, C57BL/6J RIIβ$^{-/-}$ mice were given repeated ethanol injections either just before locomotor activity testing or in their homecage environment. Recent work has documented that the expression of phenotypes in mouse research can be sensitive to subtle differences in the testing procedures as well as the testing environment (Boehm et al., 2000; Crabbe et al., 1999; Rustay et al., 2003). Along these lines, sensitization has been observed in wildtype mice on a variety of genetic backgrounds including C57BL/6 mice (Broadbent et al., 1995; Lessov et al., 2001; Pastor and Aragon, 2005), however, differences in variables such as ethanol dose and inter-dose interval may have dramatic effects on the expression of locomotor sensitization. We believe that it is for this reason we failed to observe sensitized locomotor activity in RIIβ$^{+/+}$ mice on either genetic background following bi-weekly ethanol administration (Figs. 1A and 4A). Taken together, while the role of the RIIβ subunit of PKA in ethanol-induced behavioral sensitization may depend on mouse genetic background (and thus epistatic interactions between genes), it is also possible that different outcomes between experiments resulted from procedural differences.

With the use of RIIβ$^{-/-}$ mice, the present results are the first direct demonstration that normal PKA signaling is protective against the stimulant effects of ethanol and ethanol-induced behavioral sensitization. This being said, caution is necessary when drawing conclusions

because it is becoming increasingly clear that interpretations of phenotypic data from studies with knockout mice are subject to several caveats (Gerlai, 2001). One concern is that constitutive deletion of a gene could lead to compensatory processes (up- or down-regulation of other genes) during development. In fact, the relative distribution of other regulatory subunits up-regulates in an apparent attempt to compensate for the loss of RIIβ in the present model (Amieux et al., 1997; Brandon et al., 1998). However, it stands to reason that this compensation is not complete as evidenced by reduced cAMP-stimulated PKA activity in brain regions such as the striatum, nucleus accumbens, amygdala, hippocampus and hypothalamus (Brandon et al., 1998; Thiele et al., 2000b). A second concern pertaining to knockout models, as noted above, is the possibility that there are epistatic interactions between genes such that observed phenotypes may be dependent on the genetic background of the mouse. One way to address this concern is to test the knockout model on at least two genetic backgrounds as we have done in this report. While increased sensitivity to ethanol-induced behavioral sensitization may depend on the genetic background of RIIβ$^{-/-}$ mice, increased sensitivity to ethanol-induced locomotor activity was observed in RIIβ$^{-/-}$ mice on both genetic backgrounds tested. A third concern is that differences between knockout and wild-type mice may be related to genes other than the mutated gene, a problem that is exacerbated when mice are maintained on a hybrid genetic background (i.e. 129/SvEv×C57BL6J). One solution that has been proposed to address this issue is to test mice that have been backcrossed to one genetic background. Here, we studied RIIβ$^{-/-}$ mice that were backcrossed to a C57BL/6J background over eight generations.

We have previously shown that deletion of the RIIβ subunit results in a mouse that will more readily consume ethanol solutions compared with wildtype littermate controls in the absence of altered taste preference, caloric intake, or ethanol metabolism (Fee et al., 2004; Thiele et al., 2000b). Recently, we found that increased ethanol consumption by RIIβ$^{-/-}$ mice is not reliably predicted by basal levels of anxiety (Fee et al., 2004) despite the observation that blunted PKA activity and phosphorylated CREB (pCREB) in the amygdala are associated with increased anxiety-like behavior (Pandey et al., 2003). In addition to drinking more ethanol and showing increased sensitivity to the stimulant effects of ethanol, the RIIβ$^{-/-}$ mice consistently show reduced sensitivity to the sedative properties of ethanol (Fee et al., 2004; Thiele et al., 2000b) and in the present report RIIβ$^{-/-}$ mice were resistant to the sedative effects of an initial ethanol injection on locomotor activity that was evident in RIIβ$^{+/+}$ mice (Figs. 1A, 4A and B, and 5A). This latter observation raises the possibility that increased sensitivity to ethanol-induced locomotor stimulation and sensitization by RIIβ$^{-/-}$ mice are perhaps secondary to reduced sensitivity to the sedative effects of ethanol. While this issue cannot be completely ruled out, Phillips and colleagues (Meyer and Phillips, 2003; Phillips et al., 1996) have consistently demonstrated a dissociation between tolerance to the sedative/ataxic effects of ethanol and ethanol-induced behavioral sensitization. Furthermore, RIIβ$^{-/-}$ mice show enhanced behavioral sensitization to amphetamine (Brandon et al., 1998), a drug that does not induce sedation.

It has been suggested that increased sensitivity to the stimulant effects of drugs and behavioral sensitization that develops following repeated drug exposure may be an underlying common mechanism that increases the risk for developing drug dependence (Robinson and Berridge, 1993, 2000, 2001). Consistent with this relationship, ethanol-preferring C57BL/6J mice showed increased ethanol preference after the acquisition of behavioral sensitization (Lessov et al., 2001) and a study with selectively bred high alcohol preferring (HAP) and low alcohol preferring (LAP) mice found that high ethanol preference was predictive of the acquisition of ethanol-induced locomotor sensitization (Grahame et al., 2000). Other manipulations of protein expression in mice have suggested a connection between ethanol preference and the development of locomotor sensitization following repeated ethanol injections (Szumlinski et al., 2005). Here we extend these findings by showing a positive correlation between ethanol

preference and ethanol-induced behavioral sensitization in the RIIβ$^{-/-}$ mouse model. At odds with this theoretical perspective is the observation that the DBA/2J strain of mice readily acquires ethanol-induced behavioral sensitization but strongly avoids consuming ethanol (Lessov et al., 2001; Phillips et al., 1994). However, a recent study by Camarini and Hodge (2004) found that repeated ethanol injections in DBA/2J mice significantly increased ethanol intake to levels similar to that observed in C57BL/6 mice.

While the present results are the first direct demonstration that PKA signaling modulates the stimulant effects of ethanol and ethanol-induced behavioral sensitization, previous pharmacological and genetic studies have established that PKA signaling is involved with amphetamine-(Crawford et al., 2004; Crawford et al., 2000; Tolliver et al., 1999) and cocaine-(Miserendino and Nestler, 1995; Park et al., 2000; Schroeder et al., 2004) induced locomotor sensitization. Of direct relevance to the present report is the observation that RIIβ$^{-/-}$ mice are more susceptible to the acquisition of locomotor sensitization following repeated amphetamine exposure (Brandon et al., 1998). Interestingly, repeated amphetamine exposure reduces PKA activity in the nucleus accumbens and striatum (Crawford et al., 2000, 2004). Thus, we speculate that reduced PKA signaling in these regions causes RIIβ$^{-/-}$ mice to be more sensitive to the stimulant effects of ethanol as well as ethanol- and amphetamine-induced behavioral sensitization. Because dynorphin, a neuropeptide that is reduced in the dorsal medial striatum of RIIβ$^{-/-}$ mice (Brandon et al., 1998), plays an inhibitory role in sensitization (Heidbreder et al., 1995), it can be speculated that increased ethanol- and amphetamine-induced behavioral sensitization in RIIβ$^{-/-}$ mice may be the result of low striatal dynorphin activity. However, we suggest that a degree of caution is necessary in that it is still unclear to what extent, if any, the neuronal mechanisms involved in ethanol- and amphetamine-induced locomotor sensitization overlap.

Collectively, these data provide the first direct evidence that normal PKA signaling, and specifically the RIIβ subunit of PKA, plays a protective role against the stimulant effects of ethanol and ethanol-induced behavioral sensitization. Future studies are required to define the specific brain regions in which PKA signaling influences behavioral sensitization and whether PKA signaling influences this phenomenon by affecting pre- and/or post-synaptic neuronal function.

Acknowledgments

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Abbreviations

ANOVA, analysis of variance; cAMP, cyclic AMP; PKA, protein kinase A; RIIβ, regulatory subunit II beta; S.E.M., standard error of the mean; −/−, knockout; +/+, wild-type.

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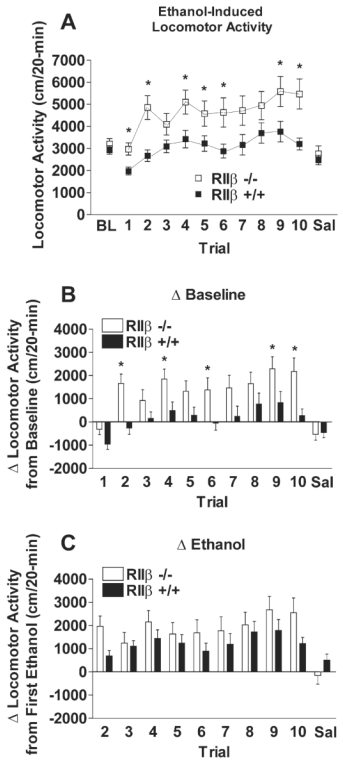

Fig. 1.

Acquisition test for ethanol-induced locomotor activation and sensitization in129/SvEv×C57BL/6J RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice. (A) Represents raw data for locomotor activity during a 20-minute activity session. Average baseline constitutes the numerical average for activity during the three habituation sessions. (B) Represents locomotor activity corrected for basal activity (Ethanol trial activity 1–10 minus average baseline activity). (C) Represents the acquisition of locomotor sensitization by presenting each ethanol exposure corrected for baseline ethanol exposure (Ethanol trials 2–10 minus Ethanol trial 1 activity). All values reported are mean±S.E.M. There were significant genotype differences in ethanol-induced locomotor activity but not in ethanol-induced sensitization.

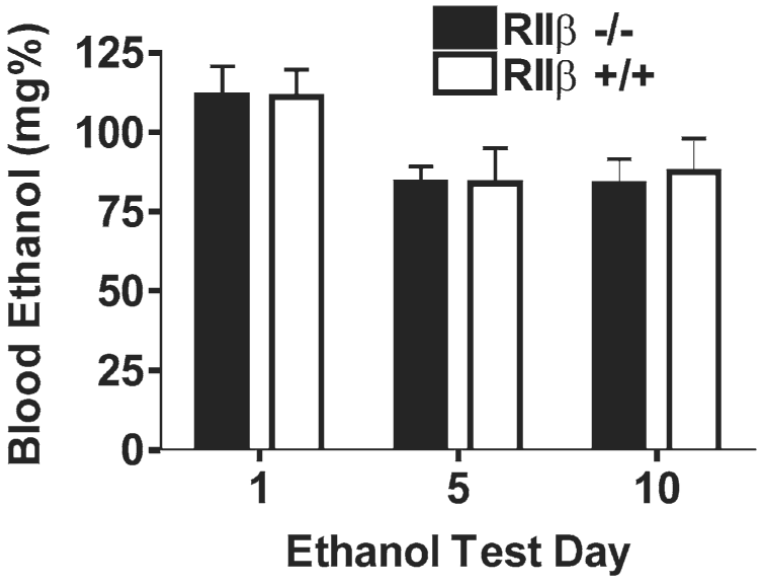

Fig. 2.

Blood ethanol concentrations (mg%) in 129/SvEv×C57BL/6J RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice. Immediately following ethanol test sessions 1, 5, and 10, 129/SvEv×C57BL/6J RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice had tail blood collected for analysis using gas chromatography to assess the possibility of alterations in ethanol pharmacokinetics over the course of the study. All values reported are mean±S.E.M. There were no significant genotype differences.

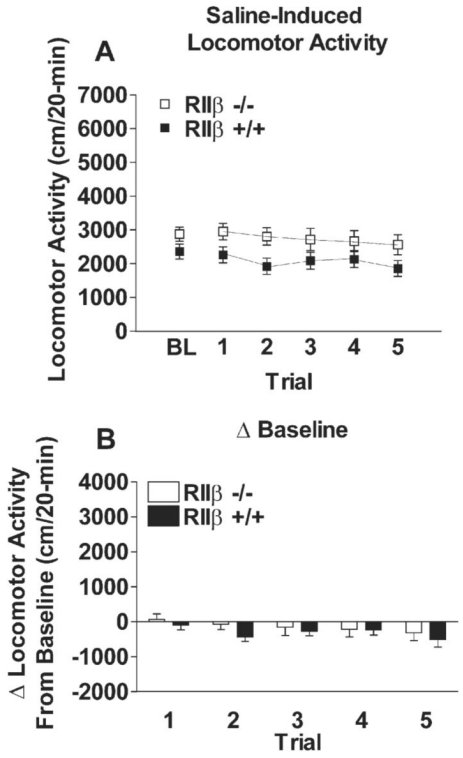

Fig. 3.

Locomotor activation and sensitization in129/SvEv×C57BL/6J RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice following repeated saline injections. (A) Represents raw data for locomotor activity during a 20-minute activity session following saline injection equivalent in volume to a 1.4 g/kg ethanol injection. Average baseline constitutes the average activity for all three habituation sessions. (B) Represents locomotor activity corrected for basal activity (Saline trial activity 1–5 minus average baseline activity). All values reported are mean±S.E.M. There were no significant genotype differences in saline-induced locomotor activity.

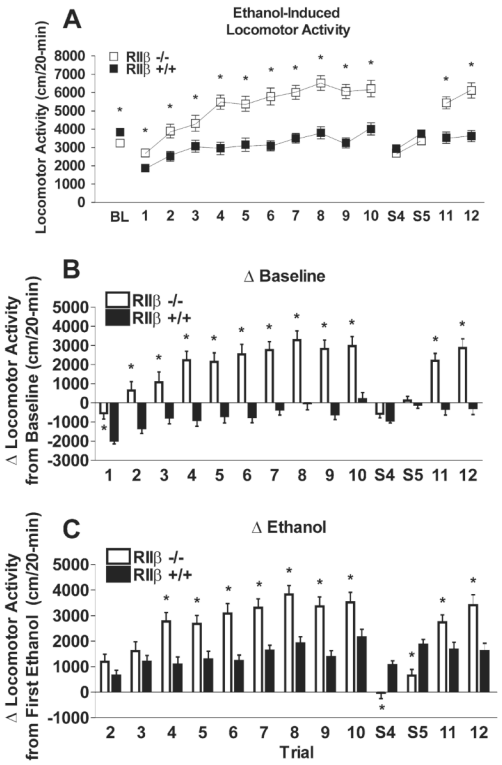

Fig. 4.

Acquisition test for ethanol-induced locomotor activation and sensitization in RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice maintained on a C57BL/6J background. (A) Represents raw data for locomotor activity during a 20-minute activity session. Average baseline constitutes the numerical average for activity during the three habituation sessions. (B) Represents locomotor activity corrected for basal activity (Ethanol trial activity 1–10, Ethanol trials 11+12, and Saline trials 4+5 minus average baseline activity). (C) Represents the acquisition of locomotor sensitization by presenting each ethanol exposure corrected for baseline ethanol exposure (Ethanol trial activity 2–10, Ethanol trials 11+12, and Saline trials 4+5 minus Ethanol trial 1 activity). All values reported are mean±S.E.M. There were significant genotype differences in ethanol-induced locomotor activity and ethanol-induced locomotor sensitization.

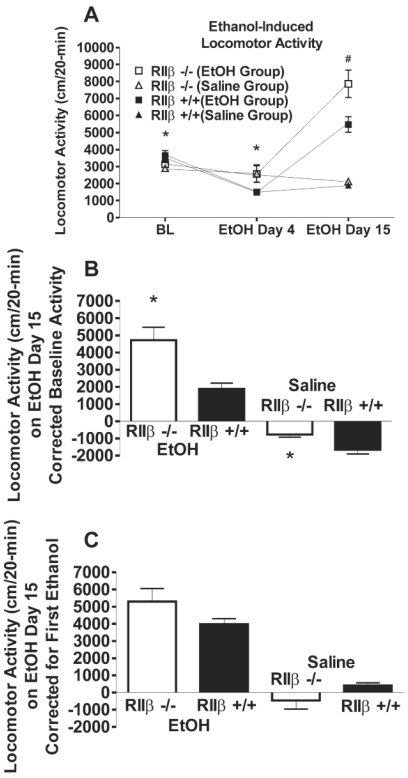

Fig. 5.

Expression test for ethanol-induced locomotor activation and sensitization in RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice maintained on a C57BL/6J background. (A) Represents raw data for locomotor activity during a 20-minute activity session following first ethanol exposure (Ethanol day 4) in locomotor chamber and final ethanol exposure (Ethanol day 15). On days 5–14 ethanol was administered in the home cage and mice were not exposed to the locomotor chamber. Average baseline constitutes the numerical average for activity during the three habituation sessions. (B) Represents locomotor activity on day 15 corrected for basal activity (Ethanol day 15 activity minus average baseline activity). (C) Represents the acquisition of locomotor sensitization by presenting final ethanol exposure corrected for baseline ethanol activity (Ethanol day 15 activity minus Ethanol day 4 activity). All values reported are mean±S.E.M. There were significant genotype differences in ethanol-induced locomotor activity but not ethanol-induced locomotor sensitization.

Table 1A

Dosing schedule for ethanol-induced locomotor activity: test chamber injections

129/SvEv×C57BL/6J			
Test days 1–3	Test days 4–13	Test day 14	
Equivolume i.p. saline C57BL/6J	1.4 g/kg i.p. ethanol	Equivolume i.p. saline	
Test days 1–3	Test days 4–13	Test days 14–15	Test days 16–17
Equivolume i.p. saline	2.0 g/kg i.p. ethanol	Equivolume i.p. saline	2.0 g/kg i.p. ethanol

Table 1B

Dosing schedule for ethanol-induced locomotor activity: home cage injections

Treatment	Days 1–3	Day 4	Days 5–14	Day 15
Group	Habituation	Initial ethanol	Conditioning	Test day
EtOH	Saline, LC	2.0 EtOH, LC	2.5 EtOH, HC	2.0 EtOH, LC
Saline	Saline, LC	2.0 EtOH, LC	2.5 EtOH, HC	Saline, LC

HC, home cage; LC, locomotor chamber.