Increased Ethanol Resistance and Consumption in Eps8 Knockout Mice Correlates with Altered Actin Dynamics

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SUMMARY

Dynamic modulation of the actin cytoskeleton is critical for synaptic plasticity, abnormalities of which are thought to contribute to mental illness and addiction. Here we report that mice lacking Eps8, a regulator of actin dynamics, are resistant to some acute intoxicating effects of ethanol and show increased ethanol consumption. In the brain, the N-methyl-D-aspartate (NMDA) receptor is a major target of ethanol. We show that Eps8 is localized to postsynaptic structures and is part of the NMDA receptor complex. Moreover, in Eps8 null mice, NMDA receptor currents and their sensitivity to inhibition by ethanol are abnormal. In addition, Eps8 null neurons are resistant to the actin-remodeling activities of NMDA and ethanol. We propose that proper regulation of the actin cytoskeleton is a key determinant of cellular and behavioral responses to ethanol.

INTRODUCTION

Alcoholism affects approximately 300 million people worldwide. Resistance to the acute intoxicating effects of ethanol is a risk factor for the development of alcoholism and is genetically determined, at least in part (Schuckit and Smith, 2000). Thus, the understanding of the molecular mechanisms underlying ethanol resistance might provide important clues about alcohol addiction.
homeostasis (Croce et al., 2004). Conversely, mice carrying a genetic deletion for Eps8 (Eps8-KO mice) are healthy and fertile, with no overt phenotype (Scita et al., 1999). This is likely due to functional redundancy with other members of the family. Indeed, Eps8L1 and Eps8L2 share all known biochemical features of Eps8 and are coexpressed with Eps8 in all tissues, with the notable exception of the central nervous system (CNS) (Offenhäuser et al., 2004).

These observations led us to evaluate the role of Eps8 in behavior. Here we report that Eps8 regulates acute ethanol sensitivity and ethanol consumption in mice, and we provide evidence for mechanisms linking actin dynamics to behavioral responses to ethanol.

**RESULTS**

**Eps8 Knockout Mice Are Less Sensitive to the Hypnotic and Motor-Incoordinating Effects of Ethanol**

We investigated the effects of the loss of Eps8 on the development and function of the CNS. Eps8-KO mice did not show detectable abnormalities in brain architecture (data not shown). Thus, we assessed the basic behavioral and neurological profile of Eps8-KO mice using a modified SHIRPA protocol (Irwin, 1968). No differences with respect to wild-type mice were recorded (see Table S1 in the Supplemental Data available with this article online).

However, in a secondary screen, we discovered that Eps8-KO mice show decreased sensitivity to the hypnotic (sedative) effects of ethanol. Data are expressed as ethanol consumption (g/kg/day) (E) or as ethanol preference ratio, i.e., ethanol consumption per total fluid consumption (F).

We next tested sensitivity to the motor-incoordinating effects of ethanol using the accelerated rotarod (Rustay et al., 2003). Eps8-KO mice (KO) mice showed a normal learning curve (Figure S1A), demonstrating that motor learning was not impaired. However, when challenged with increasing concentrations of ethanol, Eps8-KO mice remained on the rotarod significantly longer than wild-type mice (Figure 1C), demonstrating that motor coordination...
is less affected by ethanol in Eps8-KO compared to wild-type mice. Sensitivity to ethanol is heterogeneous across tasks (Crabbe et al., 2005). Thus, we also tested whether low concentrations of ethanol, known to stimulate locomotor activity in the open field, would differentially affect Eps8-KO mice. Ethanol administration induced a significant increase in locomotor activity in both wild-type and Eps8-KO mice (Figure 1D). Two-way analysis of variance (ANOVA) showed an effect of ethanol treatment versus saline in both wild-type mice ($F_{1,90} = 15.46, p < 0.001$ for treatment; $F_{0,90} = 0.48, p < 0.888$ for time; and $F_{9,90} = 2.99, p < 0.0037$ for the interaction between the factors) and Eps8-KO mice ($F_{1,90} = 24.24, p < 0.0001$ for treatment; $F_{9,90} = 4.09, p < 0.002$ for time; and $F_{9,90} = 1.87, p < 0.0665$ for the interaction between the factors). Interestingly, we did not observe resistance to the locomotor-stimulatory effect of 1.5 g/kg ethanol in Eps8-KO mice. On the contrary, two-way ANOVA showed that locomotor activity was slightly more enhanced by ethanol in Eps8-KO with respect to wild-type mice ($F_{1,186} = 6.56, p = 0.0113$ for genotype; $F_{9,186} = 1.75, p = 0.08$ for time; and $F_{9,186} = 3.40, p = 0.0007$ for the interaction between the factors) (Figure 1D). Thus, Eps8-KO mice are resistant to some, but not all, of the acute effects of ethanol.

Plasma ethanol concentrations were not appreciably different between wild-type and Eps8-KO mice after acute administration of ethanol (Figures S1B and S1C), indicating that altered ethanol clearance and/or metabolism are not the underlying causes of the reduced ethanol sensitivity of Eps8-KO mice.

**Eps8 Knockout Mice Show Increased Voluntary Ethanol Consumption**

Studies of inbred or genetically modified mice have shown a positive correlation between resistance to the acute intoxicating effects of ethanol and preference for this drug (Bowers, 2000; Li et al., 1993). Thus, we tested Eps8-KO mice for ethanol consumption using the two-bottle choice paradigm (Belknap et al., 1993). Eps8-KO mice displayed both increased consumption and preference (Figures 1E and 1F). Two-way ANOVA showed an effect of ethanol concentration ($F_{3,18} = 88.4, p < 0.0001$) and genotype ($F_{1,118} = 20.21, p < 0.0001$) and an interaction between these factors ($F_{3,118} = 4.11, p = 0.0082$) for ethanol consumption. For the preference ratio, we found a highly significant effect of ethanol concentration ($F_{3,116} = 37.5, p < 0.0001$) and genotype ($F_{1,116} = 15.24, p = 0.002$), but no interaction between these factors ($F_{3,116} = 1.02, p = 0.3853$).

Differential taste reactivity might influence ethanol consumption (Crabbe et al., 1996). We therefore tested Eps8-KO mice for consumption of sweet, bitter, salty, sour, and umami solutions (Bachmanov et al., 1996). Mutant mice displayed a reduced consumption of sweet solutions containing either high-caloric sucrose (Figure 2A) or noncaloric saccharine (Figure 1G). No significant change was observed in the consumption of other tastants (Figures S2B–S2E), indicating that ablation of Eps8 does not lead to a general impairment in taste perception. An association between sweet preference and ethanol consumption has been reported (Kampov-Polevoy et al., 1999), possibly due to the sweet taste of ethanol-containing solutions. Thus, the negative correlation observed in Eps8-KO mice was unexpected. Nevertheless, to formally exclude the possibility that altered taste accounted for increased ethanol preference, mice were tested for the consumption of a 10% ethanol solution containing saccharine as a sweetener. Eps8-KO mice still displayed a significantly increased preference for ethanol (Figure 1H), demonstrating that altered sweet perception does not account for the increased ethanol consumption. Finally, Eps8-KO mice did not show altered consumption of nicotine-containing solutions (Figure S2F), indicating that lack of Eps8 does not have a general impact on addictive behavior.

**Eps8 Is Expressed in a Subset of Brain Areas Implicated in Ethanol Tolerance**

Several brain areas have been implicated in ethanol-related behavior, including the mesolimbic reward circuit, hypothalamus, cortex, hippocampus, and cerebellum. To gain insight into which areas of the CNS are involved in Eps8 function in ethanol-related behavior, the expression of Eps8 in adult mouse brain was determined. Eps8 was expressed in several brain areas at low/moderate levels (data not shown). High levels of expression were confined to a few scattered neurons in layers II and III of the cerebral cortex (Figure 2A) and in the hippocampus (Figure 2B), two areas classically implicated in higher cognitive functions. In addition, Eps8 was highly expressed in scattered neurons in the prefrontal cortex and amygdala (Figure 2C) but was below detectability in brain areas involved in the mesolimbic reward circuit, such as the nucleus accumbens, ventral tegmental area, and bed nucleus stria terminalis. Thus, Eps8 either is not appreciably expressed or is expressed at low levels in brain areas implicated in the motivational reward leading to increased ethanol consumption. Conversely, Eps8 expression was particularly elevated in the cerebellar granule neuron (CGN) and molecular layers (Figure 2D), suggesting that the resistance of Eps8-KO mice to the motor-incoordinating effects of ethanol might be related to the function of Eps8 in the cerebellum.

**Eps8 Is Localized in Postsynaptic Structures in Cerebellar Granule Neurons In Vivo and Is Part of the NMDAR Complex**

The above results prompted us to focus on the cerebellum. In sections of the granule cell layer, we detected abundant expression of Eps8 in cerebellar granule cells, with a pattern of expression coinciding with that of F-actin (Figure 3A), which in cerebellar granule cells is known to be concentrated in the postsynaptic granule cell articulations (Capani et al., 2001). By immunoelectron microscopy, Eps8 was localized postsynaptically in the dendritic articulations of CGNs (Figure 3B), while it was absent from presynaptic mossy fibers. Biochemical fractionation of adult...
cerebellum further indicated that Eps8 was present in both the synaptosomal and postsynaptic density fractions (Figure 3C), consistent with the observation that Eps8 is localized both presynaptically in the molecular layer and postsynaptically in the glomeruli of the granule cell layer (Figure 2D).

The presence of Eps8 in the postsynaptic density fraction prompted us to investigate whether Eps8 and NMDAR are physically associated in vivo. In the adult cerebellum, three NMDAR subunits are expressed (Yamada et al., 2001); in Eps8 immunoprecipitates from adult cerebellum of wild-type mice (but not Eps8-KO mice), all three NMDAR subunits could be readily recovered (Figure 3D). This interaction was specific in that no coimmunoprecipitation between Eps8 and GluR1, a subunit of the AMPA receptor also present in the postsynaptic density fraction, was detected (Figure 3D). Similarly, Eps8—and the other two NMDAR subunits, NR2C and NR1—could be recovered in NR2A immunoprecipitates, but not in GluR1 immunoprecipitates (Figure 3E). Thus, Eps8 is part of the NMDAR complex, where it interacts, directly or indirectly, with the NMDAR.

Given the functional redundancy in the Eps8 family, it was important to establish whether Eps8 and NMDAR are physically associated in vivo. In the adult cerebellum, three NMDAR subunits are expressed (Yamada et al., 2001); in Eps8 immunoprecipitates from adult cerebellum of wild-type mice (but not Eps8-KO mice), all three NMDAR subunits could be readily recovered (Figure 3D). This interaction was specific in that no coimmunoprecipitation between Eps8 and GluR1, a subunit of the AMPA receptor also present in the postsynaptic density fraction, was detected (Figure 3D). Similarly, Eps8—and the other two NMDAR subunits, NR2C and NR1—could be recovered in NR2A immunoprecipitates, but not in GluR1 immunoprecipitates (Figure 3E). Thus, Eps8 is part of the NMDAR complex, where it interacts, directly or indirectly, with the NMDAR.

Increased NMDA Currents and Elevated NMDAR Activity after Ethanol Exposure in Eps8-KO Granule Neurons

Based on the above results, and in order to understand the impact of Eps8 ablation on CGN responses to ethanol, we measured excitatory synaptic transmission by whole-cell patch-clamp recordings from CGNs in acute cerebellar slices. Voltage-clamp recordings showed identical passive properties (membrane capacitance and input resistance) in wild-type and Eps8-KO CGNs (Table S2), indicating that basal parameters were not altered. Next, we recorded composite excitatory postsynaptic currents (cEPSCs) after low-frequency mossy-fiber stimulation (0.1 Hz) in the presence of bicuculline, an antagonist of inhibitory GABA receptors. To isolate the non-NMDA (−60 mV) and NMDA (+60 mV) components, two different holding potentials were used. The non-NMDA cEPSC
component showed similar amplitude and rising and decay kinetics in wild-type and Eps8-KO CGNs (Figures 4A–4C; Tables S3 and S4). In contrast, the NMDA cEPSC component was ~2-fold higher in Eps8-KO CGNs due to increased current amplitude and slower decay kinetics (Figures 4D–4F; Table S4).

To exclude the possibility that altered NMDA currents were due to alterations in neurotransmitter release we (1) recorded miniature EPSCs (minis; Figure 4A, inset), which reflect spontaneous release of neurotransmitter quanta, and (2) compared EPSC coefficient of variation (CV = SD/mean), which is altered by changes in release probability. Neither minis nor EPSC parameters were changed in Eps8-KO CGNs (Table S5), indicating that neurotransmitter release is normal in the cerebellum of Eps8-KO mice.

Finally, we tested the effect of ethanol perfusion on cEPSCs. One hundred micromolar ethanol inhibited both non-NMDA and NMDA currents in wild-type and Eps8-KO CGNs to a similar extent (Figures 4G and 4H). Ethanol perfusion at 400 mM did not result in further significant inhibition of non-NMDA currents. Instead, 400 mM ethanol increased the reduction of the NMDA current (~40% reduction in either 100 mM or 400 mM ethanol, Figures 4G and 4H). Inhibition of NMDA currents by ethanol was immediate and without apparent recovery during the time frame measured (Figure 4I). Finally, analysis of EPSC CV after ethanol perfusion showed no difference with respect to basal conditions in either genotype, indicating that altered presynaptic release is not responsible for the ethanol effect (Table S6).

In Eps8-KO CGNs, after the initial reduction of NMDA current elicited by 100 mM ethanol, there was no further reduction at higher concentrations. This suggests the existence of two components in the ethanol-induced reduction of NMDA current: an immediate one, apparently largely Eps8 insensitive, and a gradual one, which relies on the presence of Eps8. While further studies will be needed to verify this possibility, it is worth noting that the immediate component might be connected, in principle, to the reported ability of ethanol to bind and inhibit the NMDAR (Honse et al., 2004; Peoples and Stewart, 2000). The gradual Eps8-dependent component is most likely linked, as will subsequently become clear, to actin stability. (We note that this does not imply that the hypothesized immediate component is not linked to actin dynamics.) Whatever the case, given the higher charge transfer through the NMDAR in Eps8-KO neurons under basal conditions, application of either 100 or 400 mM ethanol...
Figure 4. NMDA Currents in Eps8-KO Mice

(A) Superimposed average non-NMDA components in cEPSCs of WT (black) and KO (red) neurons (from acute cerebellar slices, in this and all other electrophysiological experiments) normalized for WT peak amplitude. Currents were recorded at −60 mV of holding potential. The inset shows mini recordings. Minis showed a similar size and frequency in WT (13.8 ± 0.16 ± 0.07 Hz; n = 8) and Eps8-KO mice (13 ± 1.1 pA, 0.15 ± 0.06 Hz; n = 10).

(B) Peak amplitude of non-NMDA currents in WT (white bar) and KO (black bar) neurons.

(C) Decay time constants of non-NMDA currents in WT (white bars) and KO (black bars) neurons; the decay time course was fitted with a double exponential function.

(D) Superimposed average NMDA components in cEPSCs of WT (black) and KO (red) neurons normalized for WT amplitude, recorded at +60 mV from the same cells as in (A).

(E) Amplitude of NMDA currents in WT (white bar) and KO neurons (black bar). NMDA amplitude was calculated after 25 ms from EPSC onset (see vertical broken line in [D]).

(F) Weighted decay constant of NMDA current in WT (white bar) and KO (black bar) neurons; the decay time course was fitted with a double exponential function.
reduced the total current in Eps8-KO neurons to levels present in wild-type neurons before ethanol exposure (Figure 4J). These findings provide a plausible explanation for the resistance of Eps8-KO mice to the acute intoxicating effects of ethanol.

**NMDAR-Mediated Signaling Leading to Actin Remodeling Requires Eps8**

Converging evidence suggests that the effect of the ablation of Eps8 on ethanol-dependent phenotypes is due at least in part to altered actin dynamics upon NMDAR activation: (1) Eps8 is physically associated with NMDAR (this paper), (2) the major electrophysiological alteration in Eps8-KO CGNs consists of an increase in NMDA currents (this paper), (3) Eps8 regulates actin-cytoskeleton dynamics (Disanza et al., 2004; Scita et al., 1999), (4) NMDAR causes rapid actin remodeling in dendritic spines of hippocampal or cerebellar granule neurons (Fischer et al., 2000; Furuyashiki et al., 2002; Halpain et al., 1998; Shiraiishi et al., 2003; Star et al., 2002), and (5) modulation of NMDAR and of NMDAR-originated signals has been widely implicated in neuronal responses to ethanol (Hoffman, 2003). Thus, we directed our attention to the role of Eps8 in NMDAR-induced actin remodeling.

First, we established a suitable model system of CGNs cultured in vitro, which, similar to in vivo CGNs, displayed no significant redundancy of expression of Eps8 family members (Figure 5A). In mature cultured CGNs (>10 days in vitro), Eps8 displayed a distribution similar to that observed in vivo, being localized to the soma and, more importantly, to prominent, F-actin-rich clusters along neurites (Figure 5B). These structures probably represent postsynaptic-like structures, as also witnessed by enrichment of the NMDAR in these structures (Figure 5C). Treatment of cultured CGNs with glutamate or NMDA, but not with AMPA, resulted in almost complete loss of F-actin from the postsynaptic-like structures along the neurites (Figure 5D). Moreover, the specific NMDAR inhibitor MK-801, but not the AMPAR inhibitor CNOX, was able to prevent the effect of glutamate or NMDA (Figure 5D). Finally, the depolymerizing effect of glutamate and NMDA was severely reduced in Eps8-KO neurons (Figure 5E), indicating that Eps8 is required for NMDAR-induced actin remodeling.

One of the best characterized NMDAR-dependent pathways, which leads to actin remodeling in neurons, relies on an increase in intracellular calcium, which in turn causes the sequential activation of the phosphatases calcineurin and Slingshot, ultimately controlling cofilin phosphorylation status and activity (Bamburg et al., 1999; Fukazawa et al., 2003; Niwa et al., 2002; Sarmiere and Bamburg, 2004; Wang et al., 2005). Dephosphorylation of cofilin is known to induce its actin depolymerizing and severing activity (Bamburg et al., 1999). In keeping with these observations, treatment of cultured CGNs with glutamate or NMDA led to a significant reduction in the levels of phosphocofilin; however, this effect was severely reduced in Eps8-KO CGNs (Figure 5F). Of note, treatment with ethanol did not have any effect on the status of phosphocofilin in either wild-type or Eps8-KO CGNs (Figure 5F), a finding that will be subsequently discussed. As a control, we tested the ability of NMDAR to activate ERK in wild-type versus Eps8-KO CGNs and found no significant differences (data not shown). This result, together with the shown sustained NMDA currents in Eps8-KO CGNs (Figure 4), indicates that the effect of the ablation of Eps8 on NMDA-induced dephosphorylation of phosphocofilin is not due to a general impairment of the signaling ability of the NMDAR in Eps8-KO CGNs. We concluded that Eps8 is required in the NMDAR signaling pathway leading to activation of cofilin and actin-filament turnover.

**Functional links among Eps8, Actin, and NMDAR**

The above results led to the testable hypothesis that a reduction in actin dynamics might be directly responsible for the increase in NMDA currents in Eps8-KO neurons. The electrophysiological alterations detected in Eps8-KO neurons could be due directly to the lack of Eps8 function on actin dynamics or to more indirect adaptive changes. Thus, it was important initially to establish whether Eps8 could acutely decrease NMDA currents in Eps8-KO neurons. In order to do this, we perfused recombinant Eps8 intracellulary through the patch pipette. Buffer alone had no effect on either the non-NMDA or the NMDA component of cEPSCs in Eps8-KO neurons (data not shown). Eps8 instead reduced NMDA currents by ~60% in 4 out of 7 Eps8-KO neurons tested and increased NMDA currents (~20%) in wild-type neurons (Figures 6A–6C). Interestingly, non-NMDA currents were also slightly reduced in Eps8-KO neurons upon Eps8 infusion (Figure 6A).

If increased NMDA currents in Eps8-KO neurons are caused by increased actin stability, then they should be sensitive to latrunculin A (LTA)-induced actin depolymerization. We therefore perfused LTA through the patch pipette and recorded cEPSCs. Buffer alone had no effect on cEPSCs (data not shown), and non-NMDA currents were not affected by LTA in either genotype (Figures 6D and 6E). Conversely, the NMDA component of cEPSCs...
was significantly reduced in Eps8-KO but not in wild-type CGNs (Figures 6D–6F). This observation likely reflects an effect of LTA on NMDAR activity that is evidenced only under conditions of altered actin dynamics, such as those induced by Eps8 removal. Whatever the case, increased NMDA currents in Eps8-KO neurons can be reversed by...
either Eps8 or the actin-depolymerizing drug LTA, in keeping with the hypothesis that these currents are due to increased actin stability.

**Ethanol Induces Actin Reorganization in Neurons in an Eps8-Dependent Manner**

Ethanol affects actin remodeling in nonneuronal cells (Allansson et al., 2001; Qian et al., 2003), suggesting that it might exert similar effects in neurons. We investigated this possibility in CGNs in vitro. Acute ethanol treatment (200 mM, 30 min) delocalized Eps8, particularly in the postsynaptic-like structures along the neurites, where discrete Eps8 clusters were greatly reduced as a consequence (Figure 7A). This correlated with pronounced loss of F-actin from the same postsynaptic-like structures (Figures 7A and 7B), whereas a synaptic marker, synapsin, did not change upon ethanol treatment (Figures 7A and 7B), arguing against both nonspecific effects of ethanol and major changes in synaptic morphology. Dose- and time-dependence analysis revealed that the effects were already visible after 5 min of ethanol treatment (200 mM) or at doses as low as 50 mM (30 min) (Figures S3A and S3B). The effects of ethanol on F-actin redistribution were reversible within 30 min of ethanol washout (Figure S3C). Application of the NMDAR antagonist MK-801 in conjunction with ethanol did not prevent actin depolymerization, demonstrating that the effects of ethanol were not due to activation of the NMDAR (Figures S3D and S3E).

Eps8-KO neurons displayed resistance to the ethanol-induced remodeling of F-actin (Figures 7C and 7D), and doses as high as 400 mM were needed to observe some F-actin redistribution (Figure 7E). Importantly, the same effects were observed on acute cerebellar slices treated with ethanol ex vivo (Figure 7F). In particular, at 400 mM ethanol—the concentration previously shown to severely inhibit wild-type NMDA currents—almost complete actin depolymerization was observed in wild-type but not Eps8-KO slices (Figure 7F).

The lack of Eps8 therefore renders the actin cytoskeleton less sensitive to the remodeling action of ethanol, in line with the demonstrated role of Eps8 in actin dynamics. Part of the action of Eps8 is mediated through its direct interaction with F-actin (Innocenti et al., 2002, 2003; Scita et al., 1999; Croce et al., 2004; Disanza et al., 2004). However, both the in vitro binding activity of Eps8 to F-actin and the cellular consequences of Eps8 expression were disrupted in Eps8-KO neurons.
Figure 7. Ethanol Induces Eps8-Dependent Actin Depolymerization in Cultured CGNs

(A) Cultured CGNs from WT mice were treated with ethanol (200 mM, 30 min) or were mock treated (Control), followed by detection by immunofluorescence of Eps8, F-actin, and synapsin. Typical images of neurites are shown. Bar = 2 μm.

(B) Quantitative assessment of the experiment in (A). Data in (B), (D), and (H) are expressed as relative pixel intensity ± SD (n = 10–16 random neurites per condition).

(C) CGNs from WT or KO mice were treated with ethanol (as in [A]) or were mock treated, followed by detection with phalloidin. Bar = 2 μm.

(D) Quantitative assessment of the experiment in (C).

(E and F) Cultured CGNs (E) or cerebellar slices (F) from WT or KO mice were treated with ethanol as indicated (30 min) or were mock treated (Ctr/Control), followed by detection with phalloidin. Bar in (E) = 2 μm; bar in (F) = 25 μm.

(G) Cultured CGNs from WT or KO mice were treated with LTA as indicated (30 min) or were mock treated, followed by detection with phalloidin. Bar = 2 μm.

(H) Quantitative assessment of the experiment in (G).
and the in vitro actin barbed-end-capping activity of Eps8 were unaffected by treatment with ethanol (data not shown). These results argue against a direct effect of ethanol on the Eps8:F-actin interaction and rather suggest, also in light of the results in Figure 5 and Figure 6, that the absence of Eps8 renders the actin cytoskeleton less dynamic and therefore less sensitive to actin-depolymerizing stimuli. To test this possibility, we investigated the effect of LTA on wild-type and Eps8-KO neurons. Consistent with the hypothesis, Eps8-KO CGNs displayed reduced sensitivity to the actin-depolymerizing effect of LTA (Figures 7G and 7H).

DISCUSSION

Eps8 and Ethanol-Related Behavior

Data in this paper establish that removal of Eps8 in mice causes resistance to the hypnotic and motor-incoordinating effects of ethanol. Eps8-KO mice also show increased ethanol consumption. In Drosophila melanogaster, the two homologs of Eps8, Arouser and CG8907, regulate acute ethanol sensitivity (D.G and U.H., unpublished data). Thus, Eps8 is part of an evolutionarily conserved circuit that modulates sensitivity to ethanol. We further show that alterations in neuronal actin dynamics underlie the observed phenotype in Eps8-KO mice. In an accompanying paper in this issue of Cell, Rothenfluh et al. (2006) demonstrate that mutations in RhoGAP18B affect ethanol sensitivity in the fly. Together, these results argue that regulation of the actin cytoskeleton is a key factor in cellular and behavioral responses to ethanol.

Is the Eps8 expression pattern compatible with the observed phenotypes? The expression of Eps8 in the cerebellum is consistent with the resistance of Eps8-KO mice to the acute intoxicating effects of ethanol since this phenotype involves cerebellar function (Hanchar et al., 2005; Tabakoff et al., 2003). However, the increased ethanol consumption/preference of Eps8-KO mice is less easily conceptualized, as Eps8 is not widely expressed in areas implicated in motivational reward. We cannot exclude that brain areas displaying low/moderate expression of Eps8 are functionally altered in Eps8-KO mice. However, we would like to offer an alternative explanation: It is known that chronic exposure to ethanol leads to increased NMDAR activity in mice by diverse mechanisms, e.g., receptor levels, synaptic localization, or posttranslational modifications (Chandler, 2003; Hoffman, 2003). Intriguingly, increased NMDA currents in naive Eps8-KO mice mimic this condition, which is reached in wild-type mice after chronic ethanol exposure. Thus, Eps8-KO mice might consume more ethanol simply because the rewarding effects of ethanol consumption are not limited (or are less limited) by its intoxicating side effects.

Eps8 in NMDAR Signaling Leading to Actin Remodeling

The major electrophysiological alteration uncovered in Eps8-KO CGNs is the increase in NMDA currents, an event that could be directly linked to reduced actin dynamics. There are two implications of these findings. At the phenotypic level, this might account for the resistance of Eps8-KO mice to the acute intoxicating effects of ethanol since exposure to ethanol reduces NMDA current in Eps8-KO neurons to levels comparable to those of naive (unexposed to ethanol) wild-type neurons. At the molecular level, they implicate Eps8 in the NMDAR pathway. Indeed, the NMDAR signaling pathway leading to activation of cofilin, and hence to actin dynamics, is nonfunctional in Eps8-KO neurons.

The NMDAR is connected to the actin cytoskeleton, which links the receptor to molecules involved in its posttranslational modification and in signaling (Salter and Kalia, 2004; Sheng and Kim, 2002). In addition, the NMDAR is thought to activate a negative feedback loop in which an increase in intracellular calcium leads to activation of the phosphatase Slingshot, which, in turn, dephosphorylates cofilin, liberating the actin-remodeling activity of the latter (Huang et al., 2006; Wang et al., 2005). This in turn is thought to downmodulate NMDAR activity (Morishita et al., 2005; Rosenmund and Westbrook, 1993). In Eps8-KO CGNs, the decreased ability of NMDAR to induce actin remodeling and the increased NMDA currents at steady state could be due to a defect in this negative regulatory mechanism. The exact role of Eps8 in the NMDAR pathway leading to dephosphorylation of cofilin remains to be established and will be further discussed below.

Eps8 in Ethanol-Induced Actin Remodeling

We show that concentrations of ethanol that are easily reached in the CNS in alcohol-related disorders induce actin remodeling in CGNs, while this effect is attenuated in Eps8-KO neurons. Alterations in actin dynamics in dentritic spines are known to accompany and mediate the structural and functional changes associated with synaptic plasticity (Fukazawa et al., 2003; Krucker et al., 2000; Zhou et al., 2004; Zito et al., 2004). Thus, the previously described effect of ethanol on synaptic plasticity (Berry and Matthews, 2004; Blitzer et al., 1990; Krazem et al., 2003) might be mediated in part by alteration of actin-filament turnover.

The resistance of Eps8-KO neurons to the actin-remodeling effects of both ethanol and NMDA might suggest common mechanisms. In a simple scenario, ethanol might intercept the NMDAR pathway leading to actin remodeling, mimicking, at some levels, the effects of active NMDAR. However, ethanol does not cause dephosphorylation of cofilin (Figure 5F). Our data are therefore more compatible with the possibility that Eps8 is not directly part of a putative ethanol-signaling pathway. Rather, in Eps8-KO mice, the action of ethanol on the actin meshwork might impact on pre-existing reduced actin dynamics, as also supported by the decreased sensitivity of Eps8-KO neurons to the actin-depolymerizing effects of LTA.
A Tentative Molecular Model for the Role of Eps8 and Actin Dynamics in Ethanol Responses

We would like to propose a working model that attempts to rationalize the impact of Eps8, and of its regulation of actin dynamics, on ethanol responses and phenotypes. In this model, a first site of action of Eps8 is downstream of NMDAR and upstream of dephosphorylation/activation of cofilin. In the absence of Eps8, this pathway is interrupted, with predicted reduction in actin-filament turnover. A second site of action is unveiled by ethanol and LTA studies in CGNs. In this case, altered dephosphorylation of cofilin is not involved, and the lack of Eps8 seems to cause an increased stability of the actin cytoskeleton. Interestingly, in both cases, the effects of the ablation of Eps8 seem to converge toward a reduction of actin dynamics.

How does this translate in molecular terms? Eps8 is known to have a dual signaling/structural role in actin dynamics in that it participates in the formation of a multimolecular Rac-GEF complex (Innocenti et al., 2002, 2003; Scita et al., 1999) and is also endowed with actin barbed-end-capping properties (Croce et al., 2004; D’Anza et al., 2004). There is reason to believe that the two functions of Eps8 might be differentially involved in the two points of action outlined above. There is emerging evidence that Rac is involved in dephosphorylation/activation of cofilin, possibly through regulation of the phosphatase Slingshot (Nagata-Ohashi et al., 2003). There is reason to believe that the two points of action outlined above. There is emerging evidence that Rac is involved in dephosphorylation/activation of cofilin, possibly through regulation of the phosphatase Slingshot (Nagata-Ohashi et al., 2003). This suggests a simple mechanism through which Eps8 might participate in the NMDAR→cofilin pathway by regulating Rac activity. On the other hand, the actin barbed-end-capping activity of Eps8 might impact actin-cytoskeleton stability, as this function is required for optimal actin dynamics in that it participates in the formation of a multimolecular Rac-GEF complex (Innocenti et al., 2002, 2003; Scita et al., 1999) and is also endowed with actin barbed-end-capping properties (Croce et al., 2004; D’Anza et al., 2004). There is reason to believe that the two functions of Eps8 might be differentially involved in the two points of action outlined above. There is emerging evidence that Rac is involved in dephosphorylation/activation of cofilin, possibly through regulation of the phosphatase Slingshot (Nagata-Ohashi et al., 2003). This suggests a simple mechanism through which Eps8 might participate in the NMDAR→cofilin pathway by regulating Rac activity. On the other hand, the actin barbed-end-capping activity of Eps8 might impact actin-cytoskeleton stability, as this function is required for optimal actin dynamics in that it participates in the formation of a multimolecular Rac-GEF complex (Innocenti et al., 2002, 2003; Scita et al., 1999) and is also endowed with actin barbed-end-capping properties (Croce et al., 2004; D’Anza et al., 2004). There is reason to believe that the two functions of Eps8 might be differentially involved in the two points of action outlined above. There is emerging evidence that Rac is involved in dephosphorylation/activation of cofilin, possibly through regulation of the phosphatase Slingshot (Nagata-Ohashi et al., 2003).

In conclusion, our data open new perspectives in ethanol-related disorders in connection with the regulation of stability of the actin cytoskeleton. In this framework, it will be of interest to perform analysis of polymorphisms that might affect Eps8 in human alcoholics.
Supplemental Data
Supplemental Data include Supplemental Experimental Procedures, Supplemental References, three figures, and six tables and can be found with this article online at http://www.cell.com/cgi/content/full/127/1/213/DC1/.

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