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Ethanol withdrawal hyper-responsiveness mediated by NMDA receptors in spinal cord motor neurons

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1 Following ethanol (EtOH) exposure, population excitatory postsynaptic potentials (pEPSPs) in isolated spinal cord increase to a level above control (withdrawal hyper-responsiveness). The present studies were designed to characterize this phenomenon and in particular to test the hypothesis that protein kinases mediate withdrawal.

2 Patch-clamp studies were carried out in motor neurons in rat spinal cord slices. Currents were evoked by brief pulses of glutamate, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) or *N*-methyl-D-aspartic acid (NMDA).

3 Of 15 EtOH-sensitive neurons in which currents were evoked by glutamate, four (27%) displayed withdrawal hyper-responsiveness in the washout period. Mean current area after washout was $129.6 \pm 5\%$ of control.

4 When currents were evoked by AMPA, two of 10 neurons (20%) displayed withdrawal hyper-responsiveness, with a mean current area $122 \pm 8\%$ of control on washout.

5 Of a group of 11 neurons in which currents were evoked by NMDA, nine (82%) displayed withdrawal hyper-responsiveness. Mean increase in current area at the end of the washout period was to $133 \pm 6\%$ of control ($n = 9$, $P < 0.001$). When NMDA applications were stopped during the period of EtOH exposure, mean area of NMDA-evoked responses on washout was only $98.0 \pm 5\%$ of control ($n = 6$, $P > 0.05$).

6 The tyrosine kinase inhibitor genistein ($10 - 20\,\mu$M) blocked withdrawal hyper-responsiveness. Of six EtOH-sensitive neurons, the mean NMDA-evoked current area after washout was $89 \pm 6\%$ of control, $P > 0.05$.

7 The protein kinase A (PKA) inhibitor Rp-cAMP ($20 - 500\,\mu$M) did not block withdrawal hyper-responsiveness. On washout, the mean NMDA-evoked current area was $124 \pm 6\%$ of control ($n = 5$, $P < 0.05$).

8 Two broad-spectrum specific protein kinase C (PKC) inhibitors, GF-109203X ($0.3\,\mu$M) and chelerythrine chloride ($0.5 - 2$ nM), blocked withdrawal hyper-responsiveness. Responses on washout were $108 \pm 7\%$, $n = 5$ and $88 \pm 4\%$, $n = 4$ of control, respectively, $P > 0.05$.

9 NMDA activation during EtOH exposure is necessary for withdrawal hyper-responsiveness. Both tyrosine kinase and PKC, but not PKA, appear to be essential for EtOH withdrawal hyper-responsiveness mediated by postsynaptic NMDA receptors in spinal cord motor neurons.
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Keywords: Ethanol; motor neurons; spinal cord; NMDA; withdrawal; protein kinase C; tyrosine kinase; protein kinase A

Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; APV, D,L-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium; NMDA, *N*-methyl-D-aspartic acid; PKA, protein kinase A; PKC, protein kinase C

Introduction

Alcohol abuse is a serious social and clinical problem. An important component of alcoholism is the syndrome of withdrawal, which includes tremors and hypersensitivity to both noxious and normally innocuous stimuli (hyperalgesia and allodynia) (Landers, 1983; West & Gossop, 1994; Gossop *et al*., 2002). Although little is known about the neurological basis for ethanol (EtOH) withdrawal hyperalgesia or allodynia (Gatch, 1999; 2002; Gatch & Lal, 1999; Gatch & Selvig, 2002), other forms of hyperalgesia are due in part to central sensitization in spinal nociceptive pathways (Yaksh *et al*., 1999; Bridges *et al*., 2001; Rygh *et al*., 2002), including hyperalgesia associated with opioid withdrawal (Mao & Mayer, 2001). In previous studies in intact isolated spinal cord, we have shown that exposure to an anesthetic concentration of EtOH followed by a washout period results in an increase in population excitatory postsynaptic potentials (pEPSPs) to levels significantly above control (Wong *et al*., 1998). We have called this runup of evoked potentials on EtOH washout withdrawal hyper-responsiveness. Withdrawal hyper-responsiveness was specific to the glutamate receptor-mediated pEPSP and was not generalized to slower ventral root-evoked responses. Withdrawal hyperexcitability in this motor pathway may contribute to the behavioral manifestations of EtOH withdrawal. EtOH withdrawal

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hyperalgesia *in vivo* has a time course and pharmacology similar to the withdrawal hyper-responsiveness described in the present study, and is also spinally mediated (Shumilla J., Sweitzer S. and Kendig J., unpublished data).

The present study was designed to explore the mechanisms responsible for withdrawal hyper-responsiveness in spinal cord. Patch-clamp studies were conducted on visually identified motor neurons in spinal cord slices from 7 to 10-day-old rats. Experiments were carried out to test the hypothesis that withdrawal is a postsynaptic phenomenon in motor neurons and is dependent on *N*-methyl-D-aspartic acid (NMDA) receptor activation. Since various kinases have been implicated in the actions of EtOH (Pandey, 1996; McMahon *et al*., 2000; Olive *et al*., 2001; Sanna *et al*., 2002), we probed the role of kinases including tyrosine kinase, protein kinase A (PKA) and protein kinase C (PKC) by employing kinase-specific inhibitors.

Methods

Spinal cord motor neurons were studied using patch-clamp techniques as we have previously described (Cheng & Kendig, 2000, 2002; Wong *et al*., 2001). Experiments were carried out according to protocols approved by the Stanford Institutional Animal Care and Use Committee. Sprague – Dawley rats aged P7 – 10 (P0 = date of birth) were anesthetized with halothane and decapitated, and spinal cords quickly removed and placed in a cold (under 4°C) oxygenated artificial cerebrospinal fluid (ACSF) containing (mM): $NaCl_2$, 123; KCl, 4; NaH_2PO_4, 1.2; $MgSO_4$, 1.3; $NaHCO_3$, 26; dextrose, 10 and $CaCl_2$, 2; pH 7.4. Slices 350 μm thick were sectioned from the lumbar region on a vibratome (Technical Products International, St Louis, MO, U.S.A.), and removed to oxygenated ACSF at room temperature for 1-hour incubation. Individual slices were transferred to a perfusion chamber for recordings. All experiments were carried out at room temperature.

Patch pipettes were pulled on a Flaming-Brown pipette puller (Sutter Instruments, Novato, CA, U.S.A.) and had an impedance of 2 – 5 MΩ when filled with intracellular solution containing (mM) NaCl, 15; K-gluconate, 110; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 10; $MgCl_2$, 2; ethylene glycolbis (*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 11; $CaCl_2 \cdot H_2O$, 1; ATP-Na, 2 and GTP, 0.4; pH 7.3 adjusted with KOH. The osmolarity of the pipette solution was adjusted to 285 – 295 mosm. Whole-cell voltage-clamp recordings were made from visually identified motor neurons using infrared video microscopy and a ×40 water immersion lens (Zeiss Axioskop) and an Axopatch 200B amplifier (Axon Instruments) at a holding potential of – 60 mV in perfusate containing bicuculline methiodide (BMI) 10 μM, strychnine 5 μM, and tetrodotoxin (TTX) 0.5 μM. Postsynaptic currents were evoked by direct pressure application (8 – 10 psi, 100 – 150 ms) of 5 mM glutamate, 2 mM NMDA or 0.02 mM alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) from a pipette positioned near the recorded cell (Picospritzer, General Valve Division of Parker Hannefin, Fairfield, NJ, U.S.A.) at 1 – 2 min intervals. Responses to repeated glutamate, NMDA or AMPA application at these intervals were stable. Either desensitization was minimal or was at steady state. EtOH was obtained from commercial sources (Gold Shield Chemical Company, Hayward, CA, U.S.A.) as the 95% pure compound, diluted to 100 mM (an anesthetic concentration) in ACSF. Concentrations of EtOH in the bath were verified by gas chromatography of the vapor phase in equilibrium with the solution in the chamber. Following a 10 min control period, slices were exposed to 100 mM EtOH for 20 min, followed by an 18 – 20 min wash period in EtOH-free ACSF. Owing to reports that the DHPE plasticizer in polyethylene tubing of the perfusion system would cause a rise in intracellular calcium, particularly when leached out by high EtOH concentrations (Tully *et al*., 2000), the perfusion system employed Teflon throughout. All other drugs used in the experiments were from Sigma (St. Louis, MO, U.S.A.); they are tetrodotoxin (TTX), BMI, strychnine hydrochloride, 6-cy-ano-7-nitroquinoxaline-2,3-dione disodium (CNQX), D,L-2-amino-5-phosphonopentanoic acid (APV), GF-109203X, chelerythrine chloride, genistein and Rp-cAMP.

The area of evoked currents during EtOH application was measured and normalized to the average baseline current area during the 10 min period preceding EtOH application. Data are expressed as mean ± s.e.m. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison test with significance set at $P<0.05$. A single neuron was studied in each slice.

Results

Glutamate-evoked currents display withdrawal hyper-responsiveness in some but not all motor neurons

In previous studies in intact spinal cord isolated from neonatal rats, we have shown that the population excitatory postsynaptic potential generated by motor neurons is depressed by EtOH and recovers to levels above control on washout (Wong *et al*., 1998). This phenomenon was also observed in glutamate-evoked currents in motor neurons, under conditions in which synaptic transmission was blocked. As we have reported elsewhere, motor neurons in spinal cord are heterogeneous with respect to EtOH sensitivity (Li *et al*., 2002). We arbitrarily set a criterion of more than 10% depression of glutamate-evoked currents by EtOH as indicating an EtOH-sensitive neuron. Currents were evoked by pressure application of 5 mM glutamate in the presence of 0.5 μM TTX, 10 μM BMI and 5 μM strychnine to block Na^+ channels, $GABA_A$ and glycine receptors, respectively. Of 15 EtOH-sensitive neurons, four (27%) displayed withdrawal hyper-responsiveness (Figure 1a). Mean evoked current area was 129.6 ± 5% of control ($P<0.05$, $n=4$, Figure 1b) after 18 min washout. No corresponding changes of input resistance or holding currents were observed in these neurons during EtOH exposure and washout.

AMPA receptors make only a limited contribution to postsynaptic withdrawal hyper-responsiveness

In spinal cord motor neurons, glutamate-evoked currents are mediated by both AMPA and NMDA receptors at a holding potential of – 60 mV (Wang *et al*., 1999), as are motor neuron responses to dorsal root stimulation (Evans *et al*., 1982; Long *et al*., 1990). To determine the contribution of AMPA receptors to withdrawal hyper-responsiveness in spinal cord

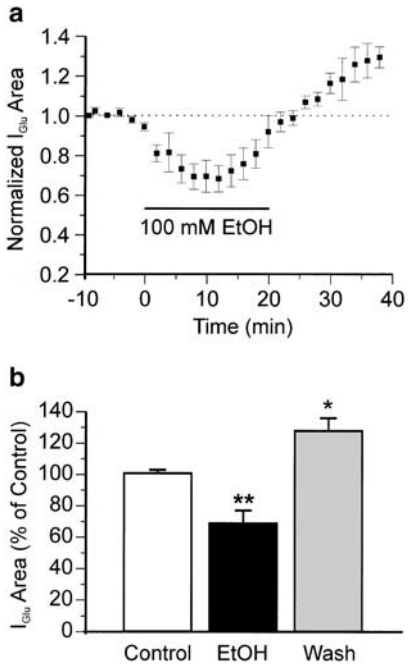

Figure 1 Glutamate-evoked postsynaptic currents display EtOH withdrawal hyper-responsiveness. (a) Time course of EtOH effects on 5 mM glutamate-evoked currents ($n=4$). (b) Histogram showing significant withdrawal hyper-responsiveness. One-way ANOVA test: $^{*}P<0.05$, $^{**}P<0.01$ compared to control.

motor neurons, we carried out experiments in which currents were evoked by pressure application of AMPA in the presence of TTX, BMI and strychnine. We first tested whether AMPA-evoked postsynaptic currents were exclusively mediated by CNQX-sensitive receptors. Currents evoked by 0.02 mM AMPA were nearly completely blocked by 10 – 20 μM CNQX (Figure 2a). Although there are reports of CNQX interaction with NMDA receptors at low agonist concentrations (Lester *et al*., 1989), this antagonist is predominantly selective for non-NMDA glutamate receptors (Andreasen *et al*., 1989). The mean residual current area measured after 10 min CNQX treatment was less than 5% of control ($4.8\pm1\%$, $n=5$, Figure 2b, c), suggesting AMPA-evoked responses are predominantly CNQX-sensitive currents.

The effects of EtOH on AMPA currents were examined in a group of 10 EtOH-sensitive neurons. Of these, two (20%) displayed withdrawal hyper-responsiveness after 20 min exposure to 100 mM EtOH. The mean increase in current area after 18 min washout was 22 ± 8 ($n=2$, data not shown).

NMDA-receptor-mediated currents have a high incidence of withdrawal hyper-responsiveness

We then tested the effect of EtOH on NMDA-receptor-mediated currents. Currents evoked by NMDA (2 mM) were completely blocked by 50 – 100 μM APV in all six motor neurons tested. In spinal cord, APV is considered to be highly

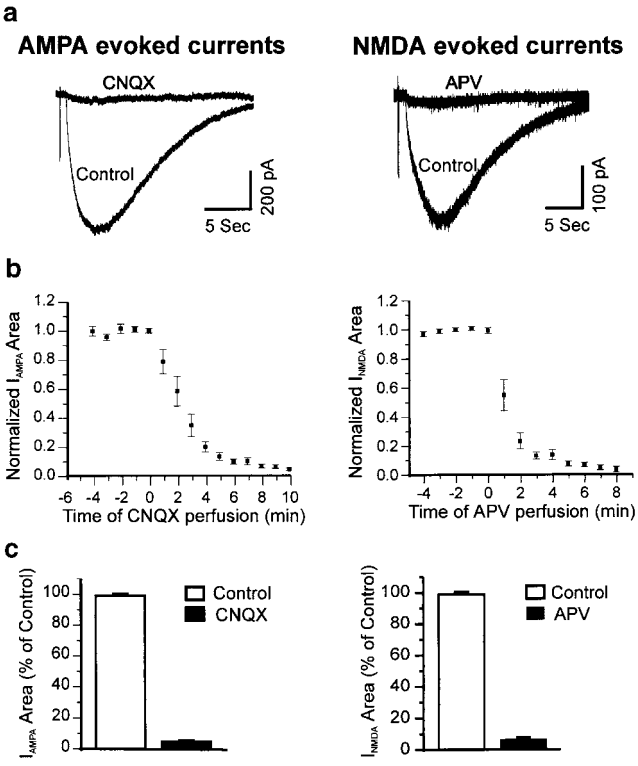

Figure 2 CNQX completely blocked AMPA-evoked currents, and APV completely blocked NMDA-evoked currents. (a) Individual traces elicited by 0.02 mM AMPA in the same motor neuron before (control) and after (CNQX) application of 10 μM CNQX, or by 2 mM NMDA before and after application of 50 μM APV. (b) Time course of mean effects of CNQX on AMPA-evoked currents ($n=5$) and of APV on NMDA-evoked currents ($n=6$). (c) Histogram showing that the AMPA- and NMDA-evoked currents were completely blocked by CNQX and APV, respectively.

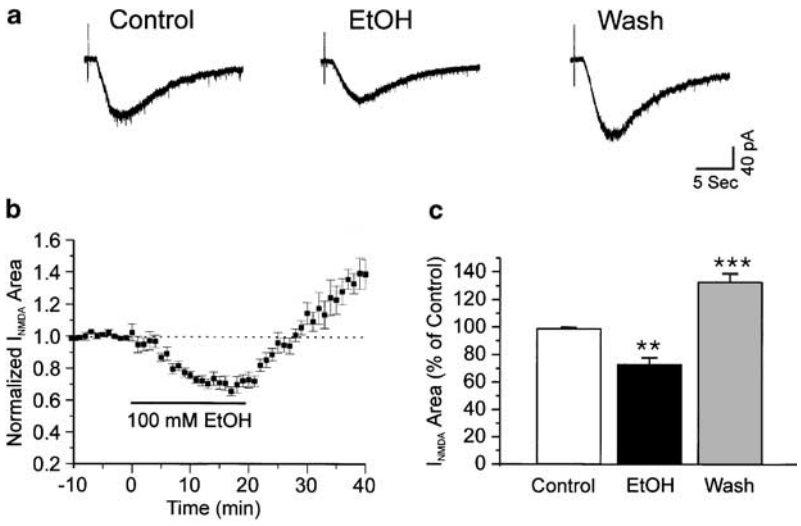

Figure 3 NMDA-evoked currents in individual spinal cord motor neurons display withdrawal hyper-responsiveness. (a) Individual traces from a motor neuron showing withdrawal hyper-responsiveness. (b) Time course of the mean effects of EtOH ($n=9$) on the area of NMDA-evoked currents. (c) Histogram showing the withdrawal hyper-responsiveness. EtOH and wash were measured at 18 min after 100 mM EtOH application and washout, respectively. One-way ANOVA test: $^{**}P<0.01$, $^{***}P<0.001$ compared to control.

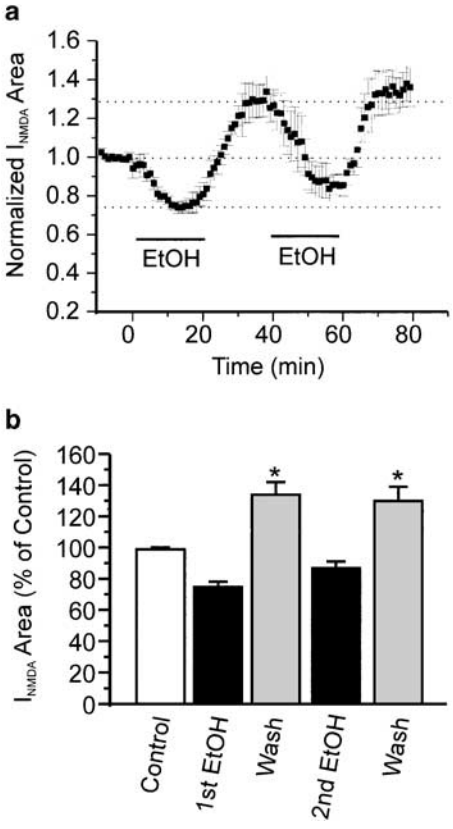

Figure 4 A second exposure to EtOH does not induce further hyper-responsiveness. (a) Time course of the mean effects of EtOH showing the withdrawal effect after the first exposure to EtOH but not after the second exposure. (b) Histogram showing the statistically significantly larger area of currents after first exposure to EtOH. The second exposure to EtOH produces greater depression than the first. $n=6$, one-way ANOVA test: $^{*}P<0.05$ compared to control.

selective for NMDA receptors (Ault & Hildebrand, 1993). The mean residual current area measured after 8 min APV treatment was less than 5% of control ($4\pm2\%$, $P<0.05$, $n=6$, Figure 2b, c), suggesting the currents evoked by NMDA were mainly mediated by APV-sensitive receptors. The incidence of withdrawal hyper-responsiveness was much higher in NMDA-evoked currents compared to those evoked by AMPA. In a group of 11 EtOH-sensitive neurons, nine (82%) displayed withdrawal hyper-reponsiveness after 20 min exposure to 100 mM EtOH. The average current area after 18 min washout was $133\pm6\%$ of control ($n=9$, $P<0.001$). The mean area of currents on washout across all neurons tested was also significantly larger than control ($124\pm8\%$, $n=11$, $P<0.01$). Figure 3a shows an example of the increase in NMDA-evoked currents on washout of EtOH. Figures 3b and c show the mean effects of EtOH on nine neurons tested.

In a subgroup of EtOH-sensitive motor neurons that displayed withdrawal hyper-responsiveness, we were able to apply EtOH for a second time following washout of the first application. New controls were the current areas measured at the end of the first washout. The occurrence of withdrawal after the second exposure to the same concentration of EtOH was much lower than the first exposure. Among six neurons tested, one (17%) showed withdrawal hyper-responsiveness as an increase in current area compared to the second control. Figure 4a shows the mean effects of the first and second exposure to EtOH on NMDA-evoked currents. The average current area on the first washout was $139\pm9\%$ of control ($n=6$, $P<0.01$). The average current area after the second exposure to EtOH and washout was not different from the second set of controls (139 ± 9 *vs* $137\pm10\%$ of initial control, first and second washout EtOH, respectively ($n=6$, $P>0.05$)). However, since in neurons given only one ethanol treatment, we did not observe hyper-responsiveness for a prolonged time equivalent to the second EtOH exposure and washout, failure to observe withdrawal on second exposure may be because of some neuron deterioration. In addition to the difference in

occurrence of withdrawal effects, the magnitude of EtOH depression following a second exposure of EtOH was significantly larger than the first one (25 ± 4 *vs* $44 \pm 6\%$, first and second exposure to EtOH, respectively, each relative to its own control ($P < 0.01$, Figure 4b)), although the absolute current magnitudes were greater in the second exposure.

Withdrawal hyper-responsiveness is dependent on NMDA receptor activity during EtOH exposure

To find out whether withdrawal is dependent on NMDA receptor channel openings during EtOH exposure, we carried out another set of experiments. In a group of six EtOH-sensitive neurons, we stopped pressure application of NMDA during EtOH exposure, and resumed it in the washout period. Under these conditions, withdrawal hyper-responsiveness was markedly reduced (Figure 5a). The average area of NMDA-evoked currents measured after 18 min washout was $98 \pm 5\%$ of control ($n = 6$, Figure 5b), suggesting that the occurrence of withdrawal hyper-responsiveness is dependent on the activation of NMDA receptors during EtOH exposure.

Protein kinase cascades participate in NMDA-receptor-mediated EtOH withdrawal hyper-responsiveness

Tyrosine kinase has been suggested to play a role in EtOH actions, particularly those involving NMDA receptors (Miyakawa *et al*., 1997; Anders *et al*., 1999; Yagi, 1999). To test the role of tyrosine kinase in this withdrawal hyper-responsiveness

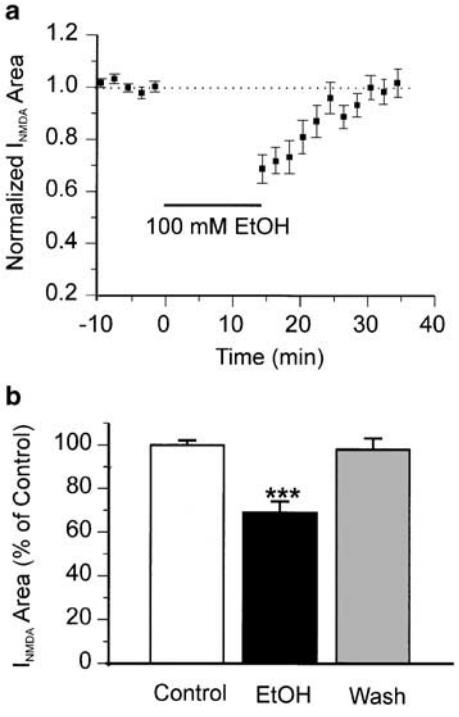

Figure 5 Activation of NMDA receptors during EtOH exposure plays a critical role in EtOH withdrawal hyper-responsiveness. In these experiments, NMDA puffing was stopped for the period of EtOH exposure. (a) Time course of the mean effects of EtOH ($n = 6$). (b) Histogram showing no EtOH withdrawal hyper-responsiveness after wash when NMDA receptors are not stimulated during ethanol exposure. One-way ANOVA test: $***P < 0.001$ compared to control.

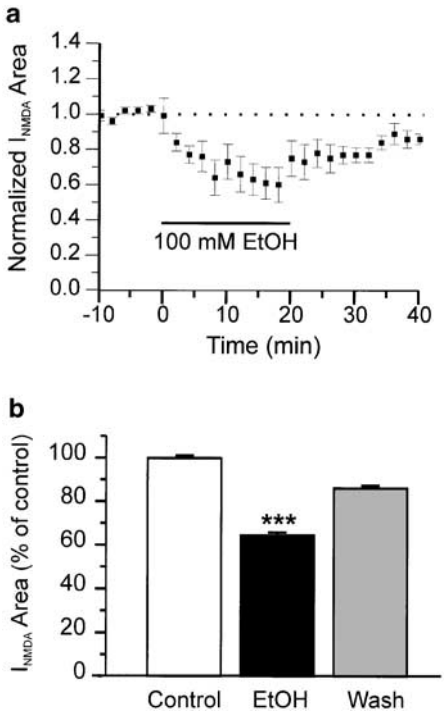

Figure 6 Inhibition of tyrosine kinase eliminates EtOH withdrawal hyper-responsiveness. (a) Time course of the mean effects of EtOH ($n = 6$) in the presence of $10 - 20\,\mu$M tyrosine kinase inhibitor genistein. (b) Histogram showing no EtOH withdrawal hyper-responsiveness. One-way ANOVA test: $***P < 0.001$ compared to control.

model, we inhibited tyrosine kinase by applying the membrane permeable tyrosine kinase inhibitor genistein (Constantinou & Huberman, 1995) ($10 - 20\,\mu$M) in the bath solution. Concentrations of kinase inhibitors were exploratory; if the effect was identical over the range, the results of all tested concentrations were used. EtOH was applied 10 min after the NMDA-evoked currents were stable in the presence of genistein. In a group of six EtOH-sensitive neurons tested, genistein blocked withdrawal hyper-responsiveness (Figure 6a). The average NMDA-evoked current area after wash was $89 \pm 6\%$ of control ($P > 0.05$) (Figure 6b), suggesting that tyrosine kinase participates in withdrawal hyper-responsiveness.

We then examined the role of PKA in withdrawal effects. The PKA inhibitor Rp-cAMP (Rothermel *et al*., 1984; Botelho *et al*., 1988) ($20 - 500\,\mu$M) was loaded into the patch pipette. Five EtOH-sensitive motor neurons were examined. Withdrawal hyper-responsiveness was not eliminated by Rp-cAMP. The average area of NMDA-evoked currents measured at 18 min wash was $124 \pm 6\%$ of control ($n = 5$, $P < 0.05$, Figure 7). However, the time course of EtOH wash was changed. Inhibition of PKA accelerated the development of withdrawal hyper-responsiveness compared to its rate of development in control conditions. Alteration of time course suggests that PKA, although not essential for withdrawal, may modify it by slowing its rate of development.

Two inhibitors specific for PKC were tested. GF-109203X is a membrane-permeable broad-spectrum specific PKC inhibitor (Toullec *et al*., 1991). GF-109203X ($0.3\,\mu$M) was applied in the bath, followed by EtOH applied 10 min after NMDA-evoked currents were stable. GF-109203X

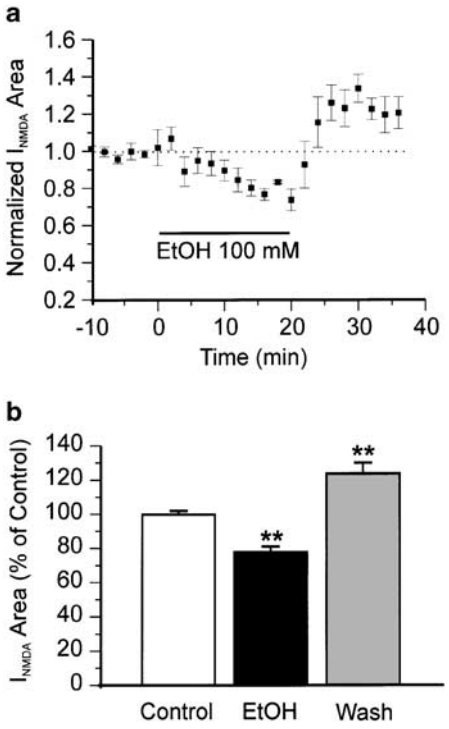

Figure 7 The PKA inhibitor Rp-cAMP did not block EtOH withdrawal hyper-responsiveness. (a) Time course of mean effect of EtOH on NMDA-evoked currents ($n=5$) while PKA was inhibited by intracellular dialysis of $20-500\,\mu$M Rp-cAMP. (b) Histogram showing EtOH withdrawal effects on NMDA-evoked currents. One-way ANOVA test: $^{**}P<0.01$ compared to control.

dramatically depressed withdrawal hyper-responsiveness. Figure 8 summarizes the effects of GF-109203X on withdrawal hyper-responsiveness. GF-109203X accelerated the recovery of EtOH-depressed currents during wash (Figure 8a). However, the average area of NMDA-evoked excitatory postsynaptic currents (EPSCs) did not recover significantly above control following wash ($108\pm7\%$ of control at 18 min of wash, $n=5$, $P>0.05$, Figure 8c).

We then examined another broad-spectrum specific PKC inhibitor, chelerythrine chloride (Herbert *et al.*, 1990). The effects of this agent were concentration dependent. At 33–500 nM, chelerythrine chloride appeared to enhance withdrawal hyper-responsiveness (data not shown). All three motor neurons tested showed withdrawal hyper-responsiveness. The average area of NMDA-evoked currents at 18 min of wash was $149\pm5\%$ of control ($n=3$, $P<0.01$). In contrast, at concentrations of 0.5–2 nM, chelerythrine chloride eliminated withdrawal (Figure 8b). The average area of NMDA-evoked currents at 18 min of wash was $88\pm4\%$ of control ($n=4$) (Figure 8d). The dual concentration-dependent effects of this PKC inhibitor suggest that different PKC isoforms may have diverse roles in withdrawal.

Discussion

NMDA receptors and withdrawal

Although withdrawal hyper-responsiveness was observed in a few cases in which currents were evoked by glutamate or

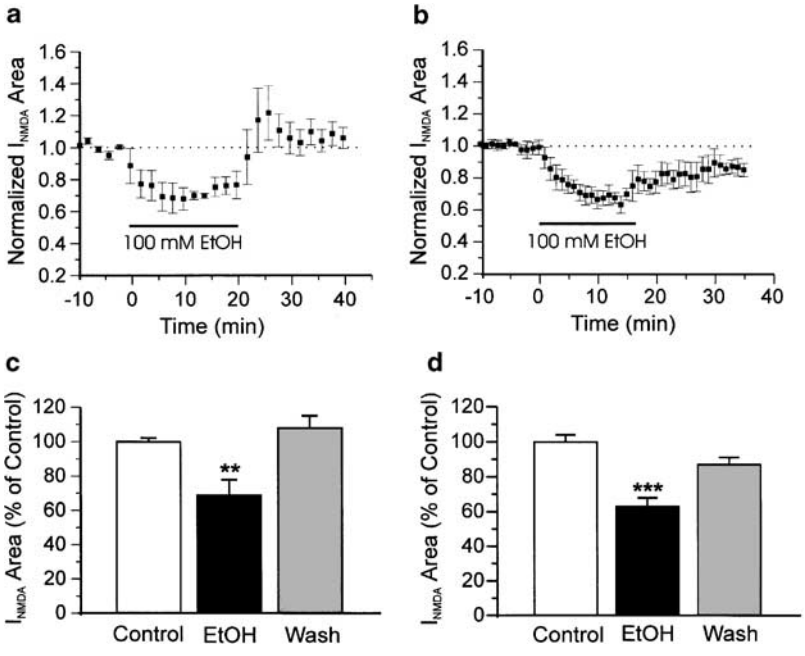

Figure 8 The PKC inhibitors GF-109203X and chelerythrine chloride depressed EtOH withdrawal hyper-responsiveness. (a) Time course of mean effect of EtOH on NMDA-evoked currents ($n=5$) in the presence of $0.3\,\mu$M GF-109203X. (b) Time course of mean effect of EtOH on NMDA evoked currents in the presence of 0.5–2 nM chelerythrine chloride ($n=4$). (c) Histogram showing no withdrawal hyper-responsiveness after 18 min washout of EtOH in the presence of GF10923X. (d) Histogram showing no withdrawal hyper-responsiveness after 18 min washout of EtOH in the presence of chelerythrine chloride. One-way ANOVA test: $^{**}P<0.01$, $^{***}P<0.001$ compared to control.

AMPA, NMDA currents were much more likely to display increases in current area above control on washout. It thus appears that withdrawal hyper-responsiveness is largely caused by actions on NMDA receptors. There is a large literature implicating NMDA receptors in alcohol dependence and withdrawal both *in vivo* (Kumari & Ticku, 2000; Narita *et al*., 2000; Bienkowski *et al*., 2001; Davis & Wu, 2001) and *in vitro* (Thomas *et al*., 1998; Kumari & Ticku, 2000; al Qatari *et al*., 2001; Nagy & Laszlo, 2002). In most studies, however, withdrawal is examined following chronic EtOH administration over a period of days, a very different protocol from the short-term exposure for 20 min employed in the present study, and one that may be expected to include more and different mechanisms including changes in gene expression. However, the results of the present study show that withdrawal following a single brief EtOH exposure is also dependent on NMDA receptors, and furthermore that it requires activation of the receptors during EtOH exposure to develop.

Withdrawal hyper-responsiveness is an apparent increase in excitability dependent on EtOH exposure and appearing during EtOH removal. It is not clear, however, whether the increase in excitability develops during the presence of EtOH and is only revealed when the depressant effects are removed, or whether it develops as a consequence of the disappearance of EtOH. In the former case, one would predict that withdrawal would contribute to the apparent potency of ethanol by decreasing it. Indeed, the biphasic actions of EtOH during exposure, initial depression followed by partial recovery, which we have described as acute tolerance (Li *et al*., 2002), might be thought actually to represent withdrawal excitation developing during EtOH exposure. However, tolerance is a different phenomenon, dependent on activation of metabotropic glutamate receptors rather than direct actions on the ligand-gated glutamate receptors (Li *et al*., 2002). Tolerance was not observed in the NMDA-evoked currents in the present study. It thus appears that withdrawal is a function of the removal of EtOH, however, one triggered by NMDA receptor activation during EtOH exposure.

In spinal cord, motor neuron responses to dorsal root stimulation include both AMPA and NMDA components. In our previous study in intact spinal cord, both AMPA and NMDA receptors were required for expression of withdrawal (Wong *et al*., 1998). The results suggest that NMDA receptors mediate withdrawal and feedback to enhance currents at both NMDA and AMPA receptors. However, in the previous study, unlike the present one, NMDA receptor activity did not appear to be essential during EtOH exposure to induce withdrawal. There are two alternative possible explanations for the apparent discrepancy. The difference may be because of the entirely postsynaptic response examined in motor neurons, as opposed to the complete circuit with both pre- and postsynaptic elements in the whole cord. In addition, the slower kinetics of EtOH washout in the whole cord may have left high concentrations of EtOH at the site of action after NMDA receptors were unblocked, thus leaving some NMDA receptors unblocked during part of the exposure to EtOH.

Roles of kinases in withdrawal

The present study suggests that withdrawal is dependent on transduction by kinases including tyrosine kinase and PKC, but not PKA. Kinases, particularly PKC, exist in a number of isoforms with different cellular locations and presumably different functions. The inhibitors used are selective for each kinase but are broad of spectrum for isoforms. In particular, GF-109203X and chelerythrine are inhibitors to all PKC isoforms, those dependent on both calcium and diacylglycerol, on diacylglycerol alone, or on neither. Thus, the results of the present study have no information on the particular isoforms responsible, although the concentration dependence of the direction of chelerythrine actions suggests opposing effects on different PKC isoforms on withdrawal hyper-responsiveness. There are a number of studies describing the roles of various kinases in EtOH behavioral actions, especially tyrosine kinase in relation to NMDA receptors (Miyakawa *et al*., 1997; Anders *et al*., 1999; Yagi, 1999). However, few of these deal with withdrawal. An exception is a study showing that mice deficient in PKC epsilon display attenuated withdrawal symptoms, suggesting a role for this isoform of PKC in withdrawal (Olive *et al*., 2001). The mechanism for kinase-dependent enhancement of currents at glutamate ligand-gated receptors has not been defined. In cultured hippocampal neurons, intracellular application of the catalytic fragment of PKC enhances currents through both NMDA and AMPA/kainate receptors, presumably by a mechanism involving phosphorylation of the receptors (Wang *et al*., 1994; Xiong *et al*., 1998).

Summary

The results of the present study characterize withdrawal hyper-responsiveness as a property of NMDA receptors. Withdrawal requires activation of PKC and tyrosine kinase but not PKA, although PKA modifies the time course of withdrawal. The results shed light on mechanisms of EtOH dependence and may provide a direction for the development of therapeutic targets to treat the problem.

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