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RESEARCH PAPER

Involvement of protein kinase C and Src tyrosine kinase in acute tolerance to ethanol inhibition of spinal NMDA-induced pressor responses in rats

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Background and purpose: The present study was carried out to examine the role of protein kinases in the development of acute tolerance to the effects of ethanol on spinal N-methyl-D-aspartate (NMDA) receptor-mediated pressor responses during prolonged ethanol exposure.
Experimental approach: Blood pressure responses induced by intrathecal injection of NMDA were recorded. The levels of several phosphorylated residues on NMDA receptor NR1 (GluN1) (NR1) and NMDA receptor NR2B (GluN2B) (NR2B) subunits were determined by immunohistochemistry and Western blot analysis.
Key results: Ethanol inhibited spinal NMDA-induced pressor responses at 10 min, but the inhibition was significantly reduced at 40 min following continuous infusion. This effect was dose-dependently blocked by chelerythrine [a protein kinase C (PKC) inhibitor, 1–1000 pmol] or PP2 (a Src family tyrosine kinase inhibitor, 1–100 pmol) administered intrathecally 10 min following ethanol infusion. A significant increase in the immunoreactivity of phosphoserine 896 of NR1 subunits (pNR1-Ser896) and phosphotyrosine 1336 of NR2B subunits (pNR2B-Tyr1336) was found in neurons of intermediolateral cell column during the development of tolerance. Levels of pNR1-Ser896 and pNR2B-Tyr1336 were also significantly increased in lateral horn regions of the spinal cord slices incubated with ethanol for 40 min *in vitro*. The increases in pNR1-Ser896 and pNR2B-Tyr1336 levels were inhibited by post-treatment with chelerythrine and PP2, respectively, both in the *in vivo* and *in vitro* studies.
Conclusions and implications: The results suggest that activation of PKC and Src tyrosine kinase during prolonged ethanol exposure leading to increases in the levels of pNR1-Ser896 and pNR2B-Tyr1336 may contribute to acute tolerance to inhibition by ethanol of NMDA receptor function.
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Keywords: alcohol; NMDA receptor; protein kinase C; Src tyrosine kinases; ion channel regulation; tolerance; sympathetic activity

Abbreviations: IML, intermediolateral cell column; NMDA, N-methyl-D-aspartate; NR1, NMDA receptor NR1 (GluN1); NR2B, NMDA receptor NR2B (GluN2B); pNR1-serine 896, phosphoserine 896 on NR1 (GluN1) subunit; pNR1-serine 897, phosphoserine 897 on NR1 (GluN1) subunit; pNR2B-tyrosine 1336, phosphotyrosine 1336 on NR2B (GluN2B) subunit; pNR2B-tyrosine 1472, phosphotyrosine 1472 on NR2B (GluN2B)subunit; pNR2B-serine 1303, phosphoserine 1303 on NR2B (GluN2B) subunit; RVLM, rostral ventrolateral medulla; SPN, sympathetic preganglionic neuron

Introduction

The N-methyl-D-aspartate (NMDA) receptors, a subtype of ionotropic glutamate receptors (nomenclature follows Alexander *et al.*, 2008), are involved in a variety of biological processes (Cull-Candy *et al.*, 2001; Waxman and Lynch, 2005). Sympathetic preganglionic neurons (SPNs), located in the intermediolateral cell column (IML) of the spinal cord, are the final site where neuronal activity related to cardiovascular and visceral function is integrated within the central nervous system (CNS) (Dampney, 1994). Activation of SPNs elicits an increase in peripheral sympathetic activity. Glutamate has been demonstrated to be the fast excitatory neurotransmitter mediating the activity of SPNs by acting on both NMDA and non-NMDA glutamate receptors (Inokuchi *et al.*, 1992; Deuchars *et al.*, 1995). Acute exposure to ethanol (alcohol)

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inhibits NMDA receptor function (Woodward, 2000). Our previous study showed that intravenous injection of ethanol selectively inhibited the pressor responses induced by intrathecal administration of NMDA (Lin *et al.*, 2003b). Inhibition of spinal NMDA-induced pressor responses by continuous infusion of ethanol was reduced over time, indicating the development of acute tolerance. The acute tolerance to ethanol inhibition of NMDA receptors was also found in rat rostral ventrolateral medulla (RVLM) neurons, another key group of neurons involved in the regulation of sympathetic activity and cardiovascular function (Lai *et al.*, 2004). These studies provided *in vivo* evidence of acute tolerance to ethanol inhibition of NMDA receptor activation in central sympathetic-related neurons.

Acute and chronic ethanol exposure affect the function of specific intracellular signalling pathways, including cyclic adenosine 3′, 5′-monophosphate (cAMP)-dependent protein kinase (PKA), protein kinase C (PKC) and tyrosine kinase signalling pathways (Pandey, 1998; Nishio *et al.*, 2001; Newton and Messing, 2006). The effects of ethanol on neuronal signalling may be restricted to certain brain regions (Newton and Messing, 2006). Protein phosphorylation has been recognized as a major mechanism for the regulation of NMDA receptor function (Westphal *et al.*, 1999; Lan *et al.*, 2001; Chen and Roche, 2007). NMDA receptors contain multiple subunits including an NMDA receptor NR1 (GluN1) subunit (NR1), a family of NR2 subunits (A, B, C, D) and two NR3 subunits (A and B) (Cull-Candy *et al.*, 2001). Many serine/threonine and tyrosine phosphorylation sites have been identified in NMDA receptor subunits (Chen and Roche, 2007). For example, the serine residue 897 of the NR1 subunit is phosphorylated by PKA; serine residues 890 and 896 of the NR1 subunit are phosphorylated by PKC. Ca^{2+}-calmodulin dependent kinase II and PKC can phosphorylate the serine residue 1303 of NMDA receptor NR2B (GluN2B) (NR2B) subunits. In addition, Src family tyrosine kinases, such as Src and Fyn, can phosphorylate tyrosine residues 1336 and 1472 of the NR2B subunit. In most neuronal preparations examined, increases in the levels of phosphorylated status of NMDA receptors may result in increased function of the receptor (Chen and Roche, 2007). In addition, protein phosphorylation has been observed to regulate the ethanol sensitivity of NMDA receptors in a few neuronal preparations (Maldve *et al.*, 2002; Yaka *et al.*, 2003).

Because ethanol exposure may activate specific signalling cascades and NMDA receptor activation is regulated by signalling molecules, we hypothesized that changes in the phsophoryated status of NMDA receptor subunits during prolonged ethanol exposure might contribute to the decreased ethanol sensitivity of NMDA receptor function. To test this hypothesis, we examined (i) the effects of PKA, PKC, and Src-family tyrosine kinase inhibitors on acute tolerance of ethanol inhibitory effects, and (ii) changes in the levels of several phosphorylation sites of NMDA receptor NR1 and NR2B subunits *in vitro* and *in vivo* following prolonged ethanol application. Our results suggested that changes in the levels of phosphoserine 896 on NR1 (GluN1) subunit (pNR1-serine 896) and phosphotyrosine 1336 on NR2B (GluN2B) subunit (pNR2B-tyrosine 1336), mediated by activation of PKC and Src tyrosine kinases, during prolonged ethanol application may play an important role in acute tolerance to the inhibitory effects of ethanol on NMDA receptor function in the spinal cord.

Methods

Animals

All animal care and experimental procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tzu Chi University. A breeding colony of Sprague–Dawley rats purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) was established at the Laboratory Animal Center, Tzu Chi University. Adult male rats weighing 250–270 g selected from the colony were used in the present study.

Determination of blood ethanol levels

To avoid perturbing the blood pressure recording, blood ethanol concentrations were measured in another group of male rats under the same conditions as the experimental ones. The rats were anaesthetized with urethane (1.2 $g \cdot kg^{-1}$, i.p.). Additional urethane (0.3 $g \cdot kg^{-1}$, i.p.) was applied if the rats responded to tail pinch. The right femoral vein was cannulated for intravenous injection of ethanol. Ethanol was applied by intravenous bolus injection of 0.16 g followed by continuous infusion at a constant rate of 0.16 $g \cdot h^{-1}$. Blood sample of 0.2 mL was withdrawn from the right femoral artery at 10 and 40 min after intravenous injection. Blood ethanol levels were determined by an alcohol diagnostic kit available commercially (Diagnostic Chemicals Limited, Oxford, CT); the rate of increase in absorbance at 340 nm was recorded with a spectrophotometer (DU650, Beckman, Coulter, Inc., Fullerton, CA, USA).

Intrathecal administration and blood pressure measurements

Procedures for intrathecal administration to anaesthetized rats were similar to those described previously (Lin *et al.*, 2003a,b; Malkmus and Yaksh, 2004). The rats were anaesthetized with urethane (1.2 $g \cdot kg^{-1}$, i.p.). Additional urethane (0.3 $g \cdot kg^{-1}$, i.p.) was applied if the rats responded to tail pinch or to intrathecal insertion of polyethylene tubing. The left femoral artery was cannulated with a polyethylene tubing (PE 50) and connected to a pressure transducer with its output to a Gould (Valley View, OH, USA) EasyGraf Recorder (TA420) for recording of blood pressure. The signals from the recorder were sent to a data acquisition system (MP 100, BIOPAC System, Inc., Santa Barbara, CA, USA) for continuous recording of blood pressure, and the built-in function of the acquisition system provides simultaneous measurements of mean arterial pressure (MAP). The right femoral vein was cannulated for intravenous injection of ethanol. Rats were mounted in a stereotaxic header and implanted with a spinal catheter for intrathecal injection. A slit was made in the atlanto-occipital membrane and the catheter (PE-10 tubing) was inserted down into the spinal subarachnoid space so that the tip was placed in the vicinity of T7-T9 segment; the position of the tubing was visually verified at the end of the experiment. The reagents at known concentrations were injected intrathecally at a volume of 10 μL,

which was followed by 10 μL of saline to wash in the agent. As a negative control, intrathecal saline did not elicit any significant changes in blood pressure. NMDA was applied at intervals of 30 min. After NMDA-induced responses were stable over two consecutive tests, experiments were then carried out. Firstly, in order to examine whether kinase inhibitors affected NMDA-induced responses, 10 μL of drugs was injected intrathecally 10 min before the application of NMDA. NMDA-induced pressor effects were recorded at 10 and 40 min after the application of the inhibitors. Secondly, in order to examine the effects of post-treatment with kinase inhibitors on acute tolerance to ethanol inhibition of NMDA-induced responses, a small amount of stock solution of the inhibitors was diluted 100-fold into NMDA solution (0.2 mM) to obtain the desired final concentrations, and 10 μL of the solution was injected intrathecally at 10 and 40 min following the continuous infusion of ethanol. The kinase inhibitors were applied in combination with NMDA for reducing the injection volume.

Immunohistochemistry

The animals were deeply anaesthetized with i.p. urethane. A control rat was killed after the surgery without administration of ethanol; one rat each was killed at 10 and 40 min following continuous ethanol infusion. To evaluate the role of PKAs on ethanol effects, kinase inhibitors were injected intrathecally at 10 min after the initiation of ethanol infusion. Rats were killed by transcardial perfusion with 100 mL of saline followed by 500 mL of paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.4). A laminectomy was performed at vertebrae T7–T9, and the exposed part of the spinal cord was excised. The spinal cord was post-fixed in the same fixative for 4 h and then cryoprotected in 30% sucrose in 0.05 M phosphate buffer (pH 7.4) for 48 h. Frozen serial sections (10 μm) were cut through the segments using a cryostat (CM3050, Leica Microsystems Nussloch GmbH, Nussloch, Germany). Tissue specimens were pretreated with protease (0.05%; Sigma Co., St. Louis, MO) to break the protein cross-links formed by formalin fixation and thereby uncover hidden antigenic sites. The slices were treated with 3% hydrogen peroxide to eliminate endogenous peroxidase activity and incubated in 10% normal goat serum and 0.3% Triton X-100 to block non-specific binding. Before performing immunohistochemical staining, several dilutions of the primary antibody were tested to verify antibody specificity. The sections were incubated in rabbit anti-NR1 polyclonal antibody (1:500; Upstate, Lake Placid, NY), rabbit anti-NR2B polyclonal antibody (1:500; Upstate), rabbit anti-phospho-NR1 antisera (serine 896, 1:200 and serine 897, 1:1000; Upstate), rabbit anti-phospho-NR2B antisera (tyrosine 1336, 1:500; Abcam, Cambridge Science Park, UK; tyrosine 1472, 1:500 and serine 1303, 1:500; Upstate) overnight at 4°C. All sections were then incubated in biotinylated goat–anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA), followed by incubation for an additional 60 min with an avidin-biotin complex solution (1:50, ABC Elite; Vector Laboratories). Following wash, sections were exposed to 3,3′-diaminobenzidine tetrachloride (DAB; 0.2 $mg \cdot mL^{-1}$; Sigma, St Louis, MO, USA) in the presence of hydrogen peroxide (1 $\mu L \cdot mL^{-1}$) to enable visualization of the precipitate. The DAB reaction was intensified with 0.2% nickel chloride. The sections were washed and mounted on gelatin-subbed slides, and the slides were dried, dehydrated in alcohol (70–100% gradually), cleared in xylene, and cover-slipped. The sections were examined under a brightfield microscope at a magnification of 100× to localize neurons in the intermediolateral area. For relative quantification of immunoreactivity, a VideoTest-Master Morphology Image Analysis Package was used. Each image field was captured with gray levels using a CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) mounted on a microscope (Eclipse E800, Nikon Corporation, Tokyo, Japan). A standardized region of interest (200 μm diameter circle) was aligned and consecutively centred on the IML area. Profiles are considered positive only when they were clearly labelled. The average number of immunoreactive (IR) neurons per section counted from 6–10 sections per animal was calculated.

Spinal cord slice preparation and Western blot analysis

Procedures used in obtaining transverse spinal cord slices were similar to those described previously (Lin *et al*., 2003a,b). Under urethane anaesthesia, the thoracic segments of spinal cord were rapidly removed from rats and placed in an ice-cold oxygenated Krebs solution. The Krebs solution consists of (in mM) 117 NaCl, 2.0 KCl, 1.2 KH_2PO_4, 2.3 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$ and 10 glucose, which was saturated with 95% O_2 and 5% CO_2. Coronal 1000 μm-thick sections from T7–T9 segments of spinal cord were prepared with a Vibratome (1000; Ted Pella, Inc., Redding, CA). The slices were incubated in a beaker containing Krebs solution at room temperature for 3–4 h. The slices were then incubated in Kerbs solution containing 100 mM of ethanol for 10 and 40 min. At the end of the incubation period, the slices were removed and quickly frozen by cold spray (FREEZE 75; CRC Industry Europe NV, Zele, Belgium). The lateral horn regions of the slices were punched out by a tissue puncher (0.75 mm in diameter). The isolated tissues were frozen in liquid nitrogen and stored at –85°C until use. To evaluate the effects of kinase inhibitors on ethanol effects, the inhibitors were applied at 10 min after initial incubation with ethanol. Slices without treatment of ethanol or the inhibitors were used as control.

The procedure for Western blot analysis of spinal cord tissue was similar to that described earlier (Lin *et al*., 2006). Before performing Western blot analysis, several dilutions of the primary antibody were tested to verify antibody specificity. The tissue collected from lateral horn regions of six slices was homogenized in 60 μL of lysis buffer (0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 $mTIU \cdot mL^{-1}$ aprotinin) with a homogenizer (Glas-Col, Terre Haute, IN) under ice bath. Sodium dodecyl sulfate (SDS) was added to the sample to a final concentration of 0.1%, and 20 μg of protein was analysed by electrophoresis on 8% denaturing polyacrylamide gels. Separated proteins were transferred to nitrocellulose membrane and then incubated in blocking buffer (20 mM Tris, pH 7.6, 150 mM NaCl) containing 5% skimmed milk powder for 2 h at room temperature on a 2D shaker, followed by three 10-min washes in Tris buffer saline (TBS) supplemented with 0.05% (v/v) Tween 20 (TBS-T). Blots were then probed with primary antibody, rabbit anti-NR1 polyclonal antibody (1:800), rabbit anti-NR2B polyclonal antibody (1:800), rabbit anti-pNR1 antisera (serine 896, 1:1500

and serine 897, 1:1500) and rabbit anti-pNR2B antisera (tyrosine 1336, 1:500; tyrosine 1472, 1:1500; and serine 1303, 1:1500) in TBS-T containing 5% skimmed milk powder overnight at 4°C on a 2D shaker. The blot was then incubated with secondary goat anti-rabbit antibody conjugated to horseradish peroxidase, which was measured with Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA). The chemiluminescent signal was detected by X-ray film (Fuji Photo Film Co., Ltd., Tokyo), and the intensity of the bands was digitalized by scanner and analysed with UN-SCAN-IT gel software version 6.1 for Windows (Silk Scientific Corporation, Orem, UT, USA). Protein concentrations were determined by bicinchoninic acid method (Sigma Co.) using bovine albumin as standard.

Data analysis

Data are presented as mean ± SEM and were plotted and analysed statistically with GraphPad Prism version 4.0 for Windows, GraphPad Software (San Diego, CA). The time–effect relationship of ethanol on NMDA-induced pressor effects and various kinase inhibitors on acute ethanol tolerance were analysed by two-way analysis of variance (ANOVA) with repeated measures (treatment and time as factors) followed by Bonferroni post-test. The statistical evaluation of immunohistochemistry and Western blots were analysed using one-way ANOVA followed by Newman–Keuls post-test. $P < 0.05$ was considered statistically significant.

Materials

Ethanol was purchased from Riedel-de Haen (Deisenhofen, Germany). NMDA was purchased from Sigma Co. The kinase inhibitors, KT5720, chelerythrine chloride and 3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1H-pyrazolo [3,4-d]pyrimidin-4-amine (PP2), were obtained from Tocris (Bristol, UK). Stock solutions of chelerythrine chloride were prepared in distilled water; the others were dissolved in dimethyl sulphoxide. Further dilutions were made in saline. We purchased aprotinin and other reagents used for immunohistochemistry and Western blot analysis from Sigma Co. The reagents for electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA).

Results

Acute tolerance to the inhibition of NMDA-induced pressor effects following prolonged intravenous ethanol infusion

Intrathecal administration of NMDA (2 nmol, 10 μL) produced an increase in MAP of 23 ± 0.5 mmHg ($n = 259$); the increase occurred within 1 min following the injection, reached a maximum by 2–3 min and gradually returned to the baseline levels within 10 min. As in our previous study (Lin *et al.*, 2003b), consecutive intrathecal administration of NMDA (2 nmol) at intervals of 30 min elicited reproducible increases in MAP. Intravenous injection of a bolus of 0.16 g of ethanol followed by continuous infusion at a constant rate of 0.16 g·h^{-1} gradually increased the blood ethanol concentrations (Figure 1C). The blood ethanol concentration after 40 min of continuous ethanol infusion was about 30% higher ($P < 0.01$; $n = 6$) than that after 10 min. Continuous ethanol infusion for 40 min did not cause significant changes in MAP. Under this condition, ethanol significantly reduced NMDA-mediated pressor effect at 10 min, but the inhibition was significantly reduced at 40 min following the infusion. Figure 1A depicts a representative experiment in which NMDA-induced pressor effect at 10 min was inhibited much more than that at 40 min following prolonged application of ethanol, suggesting the development of acute tolerance to ethanol inhibition during continuous ethanol infusion. The time-course of changes in NMDA-induced increase in MAP following continuous infusion of ethanol and saline (as control) is illustrated in Figure 1B. Two-way ANOVA with repeated measures revealed significant effects of treatment ($F = 8.7$, d.f. = 1,21, $P = 0.007$), time ($F = 24.6$, d.f. = 1,21, $P < 0.0001$) and treatment × time interaction ($F = 17.2$, d.f. = 1,21, $P = 0.0005$).

Effects of post-treatment with kinase inhibitors on acute ethanol tolerance

Intrathecal injection of KT 5720 (a PKA inhibitor, at doses up to 200 pmol, $n = 5$), chelerythrine (a PKC inhibitor, 10 nmol, $n = 4$), or PP2 (a Src family tyrosine kinase inhibitor, 10 nmol, $n = 3$) 10 min prior to the application of NMDA did not cause significant changes in NMDA-induced increase in MAP at 10 and 40 min following injection. Post-treatment with chelerythrine (1, 10, 100, 1000 pmol) applied intrathecally in combination with NMDA 10 min following continuous ethanol infusion significantly reduced acute tolerance to ethanol inhibition of pressor responses mediated by NMDA. Representative tracings of the effects of chelerythrine (100 pmol) on NMDA-induced pressor responses before and at 10 and 40 min after continuous administration of ethanol are shown in the left-hand panel of Figure 2A. The dose-response effect of chelerythrine on acute ethanol tolerance is shown in the right-hand panel of Figure 2A. Two-way ANOVA revealed a significant treatment effect ($F = 14.8$, d.f. = 5.41, $P < 0.0001$) and treatment × time interaction ($F = 37.5$, d.f. = 5.41, $P < 0.0001$) with no time effect ($F = 1.2$, d.f. = 1.41, $P = 0.284$). Post-treatment with PP2 (1, 10, 100 pmol) also significantly reduced acute tolerance to ethanol inhibition of pressor responses mediated by NMDA (Figure 2B). Two-way ANOVA showed a significant treatment effect ($F = 11.6$, d.f. = 4.31, $P < 0.0001$) and treatment × time interaction ($F = 27.8$, d.f. = 4.31, $P < 0.0001$) with no time effect ($F = 0.01$, d.f. = 1.31, $P = 0.909$). Post-treatment with KT5720 at doses up to 200 pmol did not affect the development of acute ethanol tolerance (Figure 2C); two-way ANOVA showed a significant effect for treatment ($F = 4.0$, d.f. = 4.34, $P = 0.009$) and time ($F = 84.3$, d.f. = 1.34, $P < 0.0001$) and a treatment × time interaction ($F = 3.9$, d.f. = 4.34, $P = 0.01$).

Changes in the immunoreactivity of NMDA receptor subunit phoshporylation in neurons of the intermediolateral area during continuous ethanol infusion

The effect of continuous infusion of ethanol on the expression of NR1, NR2B, pNR1-serine 896, pNR1-serine 897,

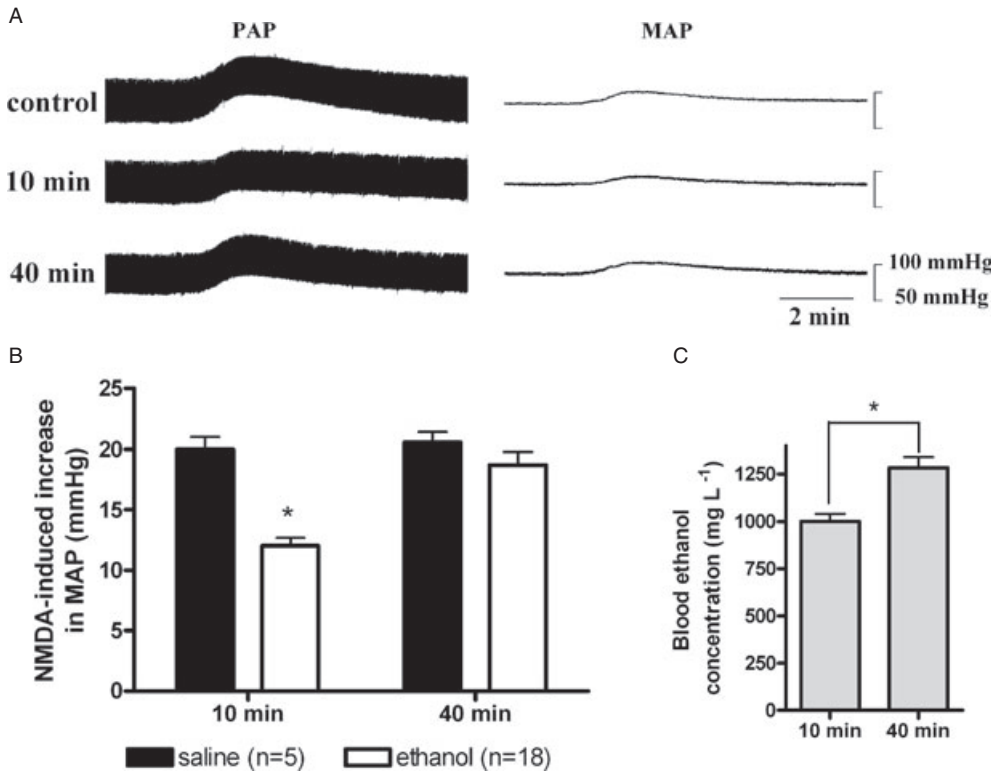

Figure 1 (A) Representative recordings show increases in pulsatile arterial pressure (PAP, left panel) and mean arterial pressure (MAP, right panel) following intrathecal injection of N-methyl-D-aspartate (NMDA) (2 nmol, 10 μL) before the application of ethanol (control) and the subsequent changes in NMDA-induced pressor effects at 10 and 40 min following an intravenous injection of a bolus of 0.16 g of ethanol followed by continuous infusion at a constant rate of 0.16 g·h^{-1}. NMDA was applied every 30 min. (B) Bar graphs show changes in NMDA-induced increases in MAP at 10 and 40 min following continuous infusion of ethanol or saline (as control). Values denote mean + SEM *Significant difference from control analysed using two-way, repeated measures ANOVA followed by Bonferroni post-test. (C) Bar graph showing changes in blood ethanol concentration following continuous infusion of ethanol. *Significantly different from value at 10 min, $P < 0.01$; paired Student's *t*-test.

pNR2B-tyrosine 1336, phosphotyrosine 1472 on NR2B (GluN2b) subunit (pNR2B-tyrosine 1472), and phosphoserine 1303 on NR2B (GluN2B) subunit (pNR2B-serine 1303) in the IML neurons was determined by immunohistochemistry. Ethanol had no significant effects on the numbers of NR1 or NR2B-immunoreactive (IR) neurons at 10 and 40 min following continuous infusion (Figures 3 and 4). The number of neurons in IML area showing immunoreactivity to pNR1-serine 897 was increased at 10 and 40 min and that to pNR1-serine 896 was decreased at 10 min but increased at 40 min (Figure 3). A noticeable increase in the immunoreactivity of pNR2B-tyrosine 1336, but not pNR2B-tyrosine 1472 and pNR2B-serine 1303, was also found at 40 min after continuous ethanol infusion (Figure 4).

Effects of post-treatment with kinase inhibitors on ethanol-induced increases in neurons IR for pNR-serine 897, pNR1-serine 896 and pNR2B-tyrosine 1336

Intrathecal injection of KT5720 (200 pmol, n = 3) had no significant effects on the basal number of neurons staining positively for pNR1-serine 897 and pNR2B-tyrosine 1336 at 40 min after the injection; injection of chelerythrine (100 pmol, n = 3) had no effects on the basal number of pNR1-serine 896 and pNR2B-tyrosine 1336-IR neurons at 40 min post-injection; injection of PP2 (100 pmol, n = 3) had no effects on the basal number of the pNR2B-tyrosine 1336, pNR1-serine 896 and pNR1-serine 897-IR neurons at 40 min post-injection. Post-treatment with KT5720 (200 pmol) applied at 10 min after ethanol infusion, which significantly reduced the increase in the number of pNR1-serine 897-IR neurons observed at 40 min after continuous ethanol infusion (Figure 5A), was without significant effects on ethanol-induced changes in pNR2B-tyrosine 1336 (Figure 5C). Intrathecal post-treatment with chelerythrine (100 pmol) blocked the increases in the number of pNR1-serine 896-, and pNR2B-tyrosine 1336-IR neurons at 40 min post-injection (Figure 5B,C). Post-treatment with PP2 (100 pmol) blocked ethanol-induced increases in the number of pNR2B-tyrosine 1336-IR neurons (Figure 5C) but was without significant effects on ethanol-induced increases in the number of pNR1-serine 897- and pNR1-serine 896-IR neurons (Figure 5A,B).

Changes in the levels of phosphorylated NMDA receptor subunits in the lateral horn regions of spinal slices incubated with ethanol in vitro

In the *in vitro* study, slices of thoracic segments of the spinal cord were incubated in 100 mM ethanol for 10 or 40 min. The

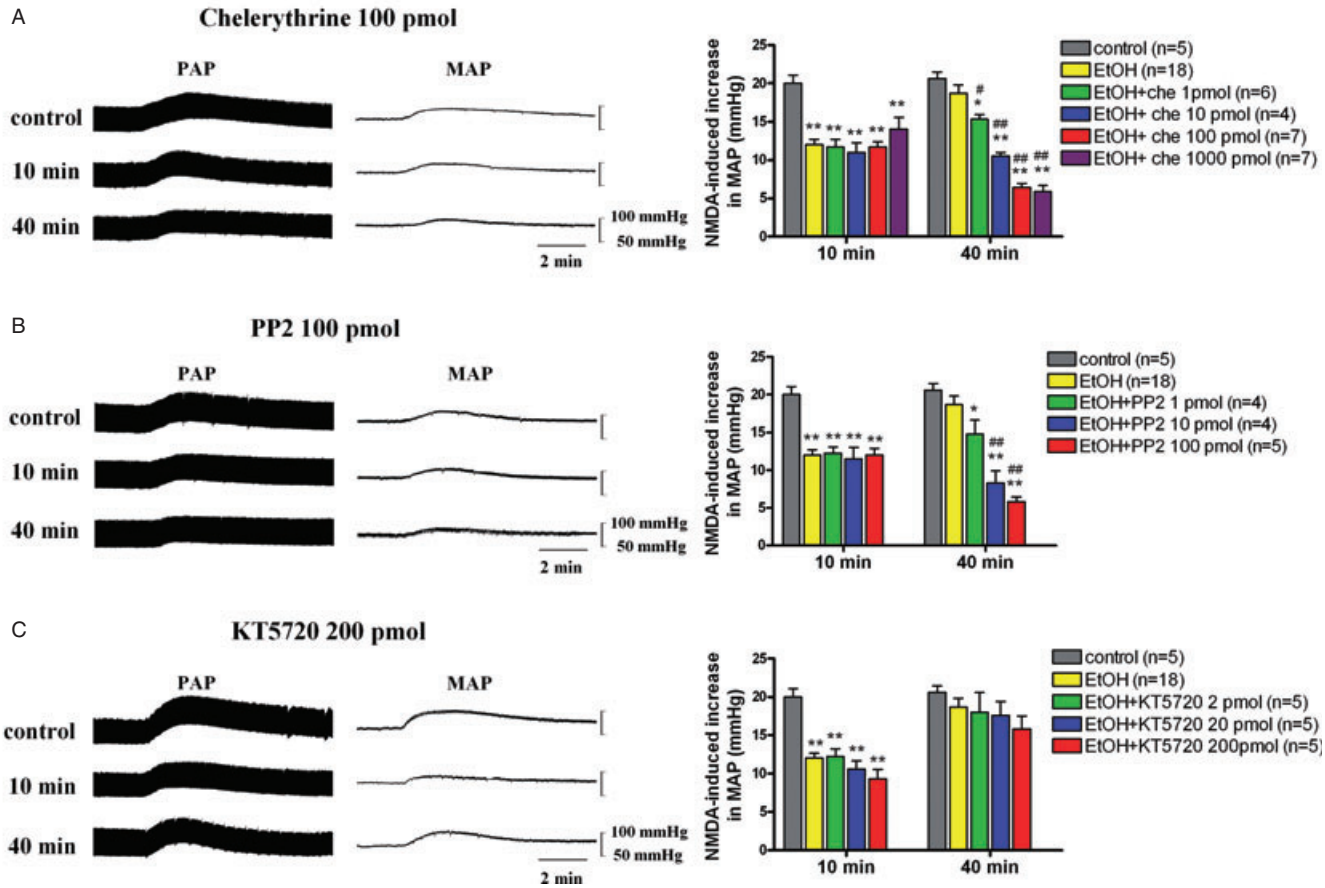

Figure 2 (A) Left panel: representative recordings of N-methyl-D-aspartate (NMDA)-induced increases in pulsatile arterial pressure (PAP, left) and mean arterial pressure (MAP, right) before application of ethanol (control) and the pressor effects induced by NMDA in combination with chelerythrine (a PKC inhibitor) at 10 and 40 min after application of ethanol. Ethanol was applied by intravenous injection of a bolus of 0.16 g followed by a continuous infusion at a constant rate of 0.16 g·h^{-1}. Right panel: bar graphs show changes in NMDA-induced increases in MAP at 10 and 40 min in rats following infusion of saline (as control) and ethanol without and with post-treatment with various doses of chelerythrine (che). Values denote mean + SEM $*P < 0.05$, $**P < 0.01$ versus control group; $\#P < 0.05$, $\#\#P < 0.01$ versus ethanol alone group analysed using two-way repeated measure ANOVA followed by Bonferroni post-test. (B) and (C) are similar to (A) except that chelerythrine was replaced by PP2 (a Src-family tyrosine kinase inhibitor) and KT5720 (a protein kinase inhibitor), respectively.

protein contents of NR1 and NR2B subunits and the levels of several phosphorylated residues on NR1 and NR2B subunits in the lateral horn regions of the slices before and after incubation of ethanol were estimated by immunological staining of Western blots. The level of pNR1-serine 897 was increased after incubation with ethanol for 10 and 40 min and that of pNR1-serine 896 was decreased at 10 min but increased at 40 min following incubation with ethanol (Figure 6A). A significant increase in the level of pNR2B-tyrosine 1336, but not pNR2B-tyrosine 1472 and pNR2B-serine 1303, was found following incubation of ethanol for 40 min (Figure 6B). The increase in the level of pNR1-serine 897 at 40 min was reduced by KT5720 (20 nM) applied 10 min after incubation with ethanol (Figure 7A). Post-treatment with chelerythrine (10 μM) blocked the increases in the levels pNR1-serine 896 at 40 min following ethanol incubation (Figure 7B); Post-treatment with PP2 (25 nM) or chelerythrine (10 μM) blocked the increases in the level of pNR2B-tyrosine 1336 (Figure 7C).

Discussion

Our previous study found that ethanol selectively inhibited NMDA-induced responses and the development of acute tolerance during prolonged ethanol exposure in SPNs *in vivo* and *in vitro* (Lin *et al.*, 2003b). In that study, the magnitude of increases in blood pressure induced by intrathecal injection of NMDA and the amplitude of membrane depolarizations induced by superfusion of NMDA were used as indices of NMDA receptor activation *in vivo* and *in vitro*, respectively. In the present study, we evaluated the role of PKA, PKC and tyrosine kinase signalling pathways in the development of acute ethanol tolerance using pharmacological approach. The tolerance to NMDA-induced pressor responses was attenuated by intrathecal post-treatment with a selective PKC or Src tyrosine kinase inhibitor, but not with a PKA inhibitor. Immunohistochemistry and Western blot analysis showed significant increases in the IR neurons for and the levels of pNR1-serine 897, pNR1-serine 896, and pNR2B-tyrosine 1336

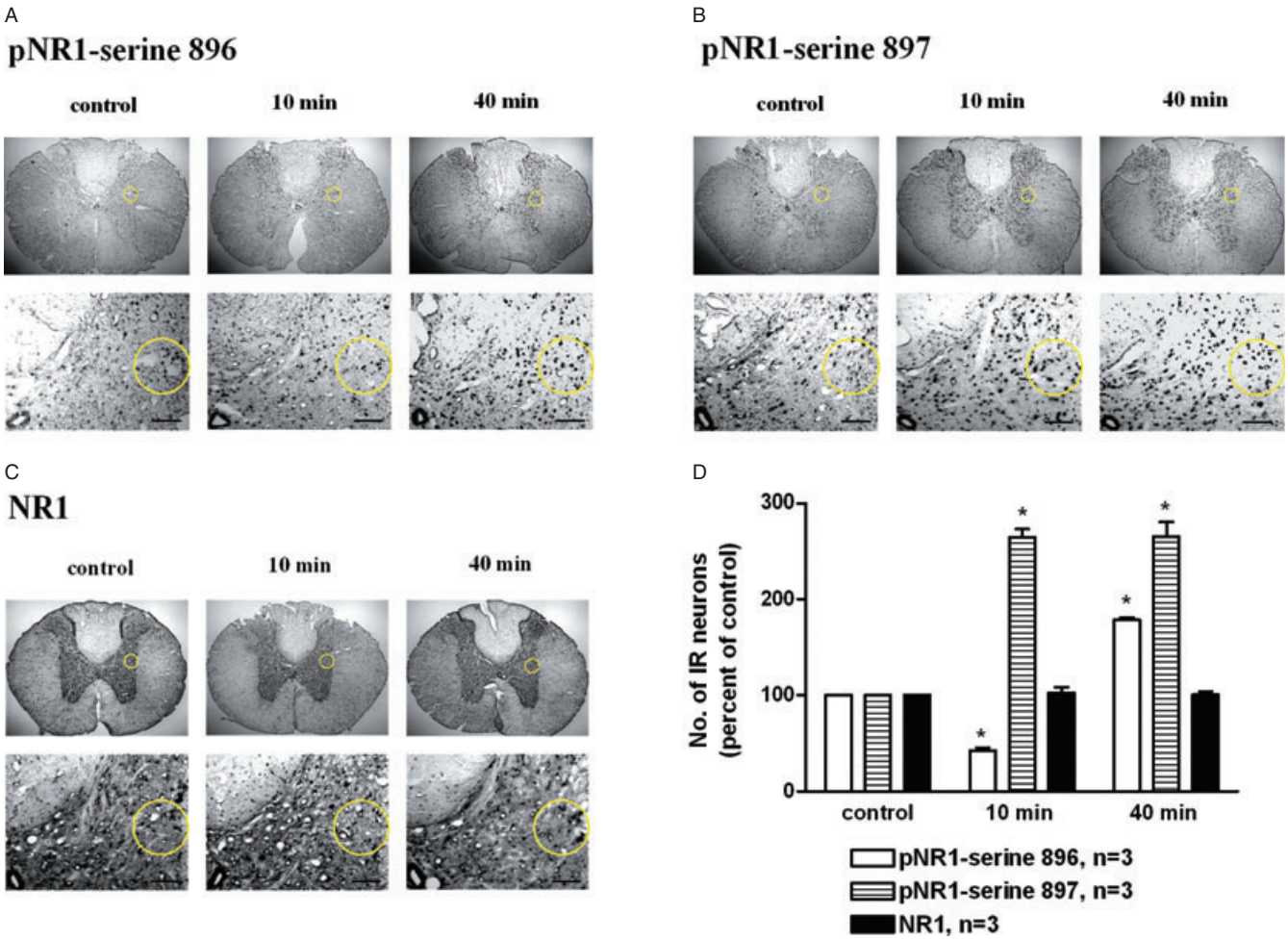

Figure 3 (A–C) Representative immunohistochemical analysis of the expression of phosphoserine 896 on NR1 (GluN1) subunit (pNR1-serine 896, A), phosphoserine 897 on NR1 (GluN1) subunit (pNR1-serine 897, B), and N-methyl-D-aspartate (NMDA) receptor NR1 (GluN1) (NR1) subunit (C) in IML area (labelled with circle) of T7–T9 segments of rat spinal cord before (control) and at 10 and 40 min following intravenous continuous infusion of ethanol. Scale bar = 100 μm. (D) Graph shows the time course of changes (as % control) in the expression of pNR1 (serine 896 and serine 897) and NR1 following continuous application of ethanol. A group of three rats was used to determine the expression of immunoreactive (IR) neurons in the IML area. A control rat was killed after the surgery without administration of ethanol; one rat each was killed at 10 and 40 min following ethanol infusion. The average number of IR neurons per section in rats without administration of ethanol is taken as the control value and set to 100%. Values denote mean + SEM from three separate experiments (n = 3). *Statistically significant difference from control analysed by one-way ANOVA followed by Newman–Keuls post-test.

in the *in vivo* and *in vitro* experiments, respectively, during prolonged application of ethanol. The increases in pNR1-serine 897, pNR1-serine 896 and pNR2B-tyrosine 1336 were blocked by specific kinase inhibitors, supporting the specific regulation of these amino acid residues by PKA, PKC, and Src tyrosine kinase signalling pathways, respectively. Our results provide the first *in vivo* evidence that changes in the levels of pNR1-serine 896 and pNR2B-tyrosine 1336 induced by the activation of PKC and Src tyrosine kinases during prolonged ethanol application may play an important role in acute tolerance to ethanol inhibition of NMDA receptor function in the spinal cord.

As mentioned in the Introduction, the function of NMDA receptors is regulated by kinases and phosphatases (Westphal *et al*., 1999; Lan *et al*., 2001). The present study examined the changes in the phosphorylation of several serine and tyrosine residues of NR1 and NR2B subunits in the lateral horn regions of the spinal cord during prolonged ethanol application both *in vivo* and *in vitro*. We found that continuous infusion of ethanol for 40 min caused increases in the immunoreactivity for pNR1-serine 896, pNR1-serine 897, and pNR2B-tyrosine 1336 but leaving that of pNR2B-serine 1303 and pNR2B-tyrosine 1472 unaltered. Post-treatment with a PKC or tyrosine kinase inhibitor blocked acute tolerance to ethanol inhibition of spinal NMDA-induced pressor responses, suggesting that activation of PKC and tyrosine kinase signalling pathways leading to increases in the levels of pNR1-serine 896 and pNR2B-tyrosine 1336 may be involved in the development of the tolerance. In the *in vitro* study, significant increases in the levels of pNR1-serine 896, pNR1-serine 897 and pNR2B-tyrosine 1336 were also observed in the slices incubated with ethanol for 40 min. The results from Western blot analysis in the lateral horn region of spinal cord slices were similar to those from immunohistochemical staining *in vivo*. Our previous study (Lin *et al*., 2003b) found reduced inhibitory effects of ethanol on NMDA-induced

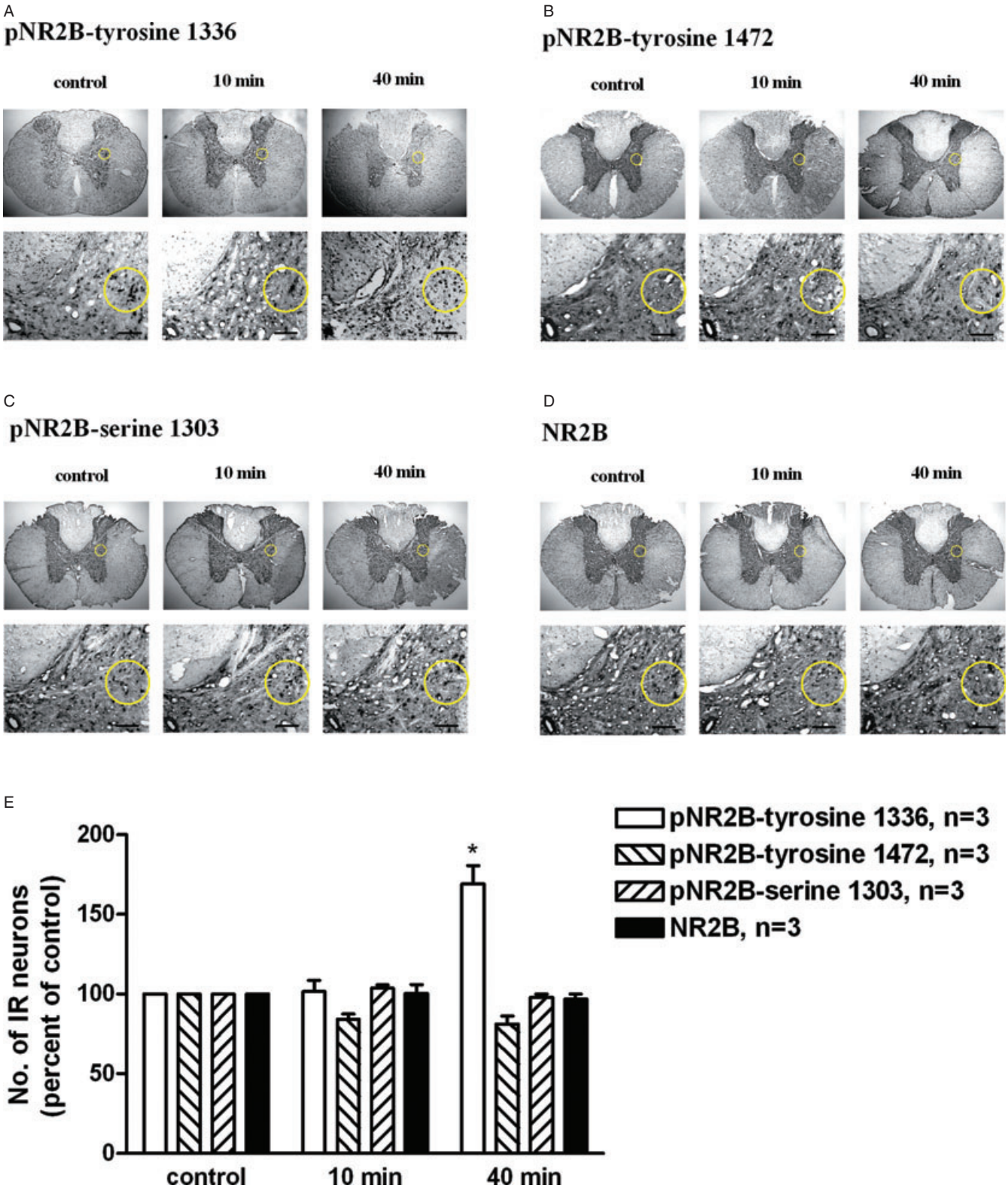

Figure 4 (A–D) Representative immunohistochemical analysis of the expression of phosphotyrosine 1336 on NR2B (GluN2B) (pNR2B-tyrosine 1336, A), phosphotyrosine 1472 on NR2B (GluN2B) (pNR2B-tyrosine 1472, B), and phosphoserine 1303 on NR2B (GluN2B) (pNR2B-serine 1303, C), and NR2B subunit (D) in IML area (labeled with circle) of T7-T9 segments of rat spinal cord before (control) and at 10 min and 40 min following intravenous continuous infusion of ethanol. Scale bar = 100 μm. (E) Graph shows the time course of changes (as % control) in the expression of pNR2B (tyrosine 1336, tyrosine 1472, and serine 1303) and NR2B following continuous application of ethanol. The average number of immunoreactive (IR) neurons per section in control rats is taken as the control value and set to 100%. Values denote mean + SEM. *Statistically significant difference from control analysed by one-way ANOVA followed by Newman–Keuls post-test.

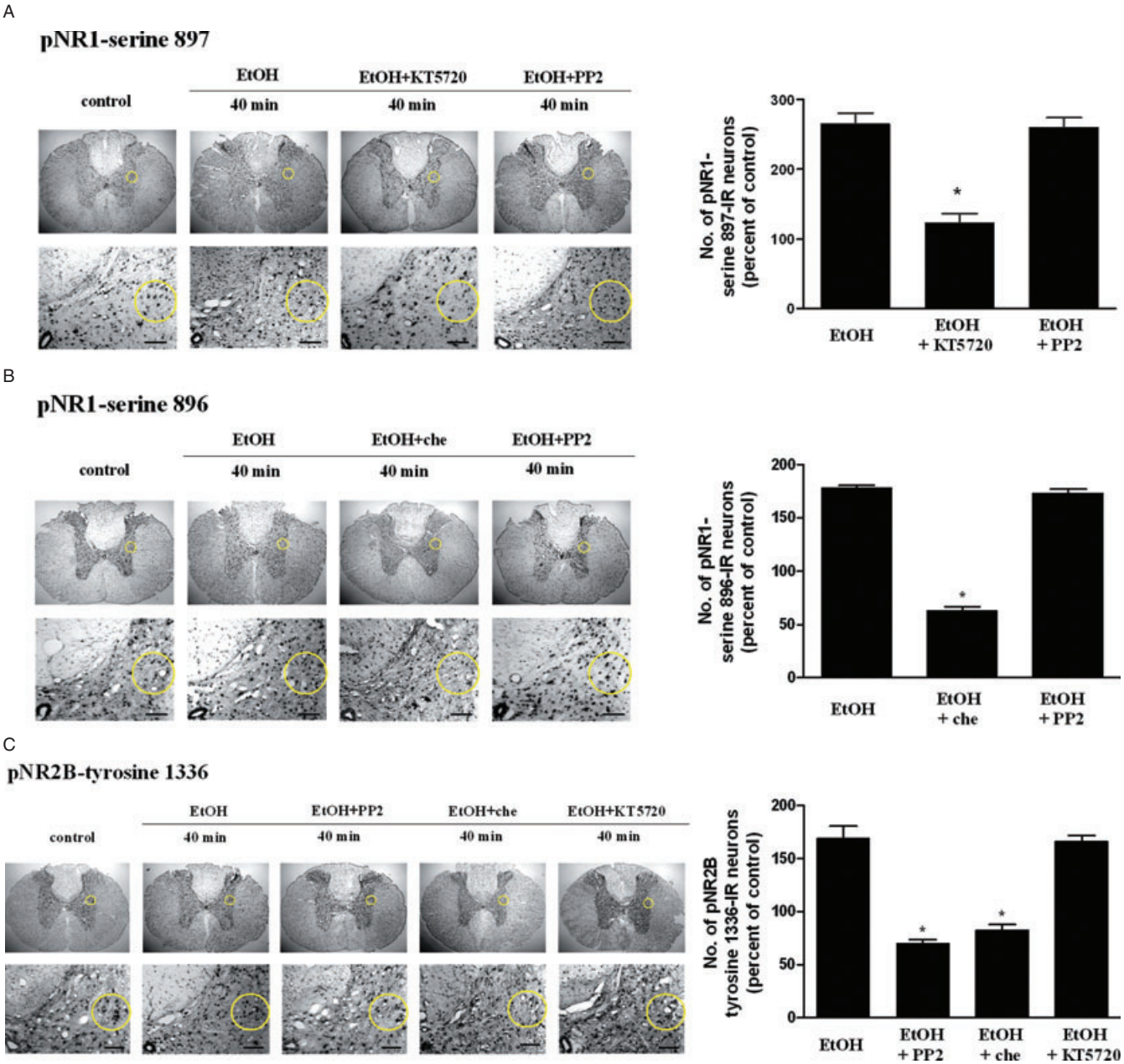

Figure 5 (A) Left panel shows representative immunohistochemical analysis of the expression of phosphoserine 897 on NR1 (GluN1) subunit (pNR1-serine 897) in the intermediolateral cell column (IML) area (labelled by circle) of T7–T9 segments of rat spinal cord at 40 min following without or with intrathecal KT5720 (a protein kinase inhibitor, 200 pmol) or PP2 (a Src-family tyrosine kinase, 100 pmol) applied at 10 min following continuous ethanol (EtOH) infusion. The control group was not treated with ethanol. The changes (as % control) in the number of pNR1-serine 897-IR neurons are shown in the right graph. The average number of the IR neurons (9.36 ± 0.45, $n = 3$) per section in control rats is taken as the control value and set to 100%. (B) similar to (A) except that that levels of phosphoserine 896 on NR1 (GluN1) subunit (pNR1-serine 896) was determined in rats post-treated with chelerythrine (che, 100pmol), a protein kinase C inhibitor, or PP2 (100 pmol) following ethanol infusion. The average number of the pNR1-serine 896-immunoreactive (IR) neurons (16.23 ± 0.58, $n = 3$) per section in control rats is taken as the control value and set to 100%. (C) similar to (A) except that levels of phosphotyrosine 1336 on NR2B (GluN2B) subunit (pNR2B-tyrosine 1336) was determined in rats post-treated with PP2 (100 pmol), che (100 pmol), or KT5720 (200 pmol) following ethanol infusion. The average number of the pNR2B-tyrosine 1336-IR neurons (11.67 ± 0.58, $n = 3$) per section in control rats is taken as the control value and set to 100%. Values denote mean + SEM from three separate experiments. Scale bar = 100 μm. *Statistically significant difference from 40 min following ethanol infusion without post-treatment with inhibitors analysed by one-way ANOVA followed by Newman–Keuls post-test.

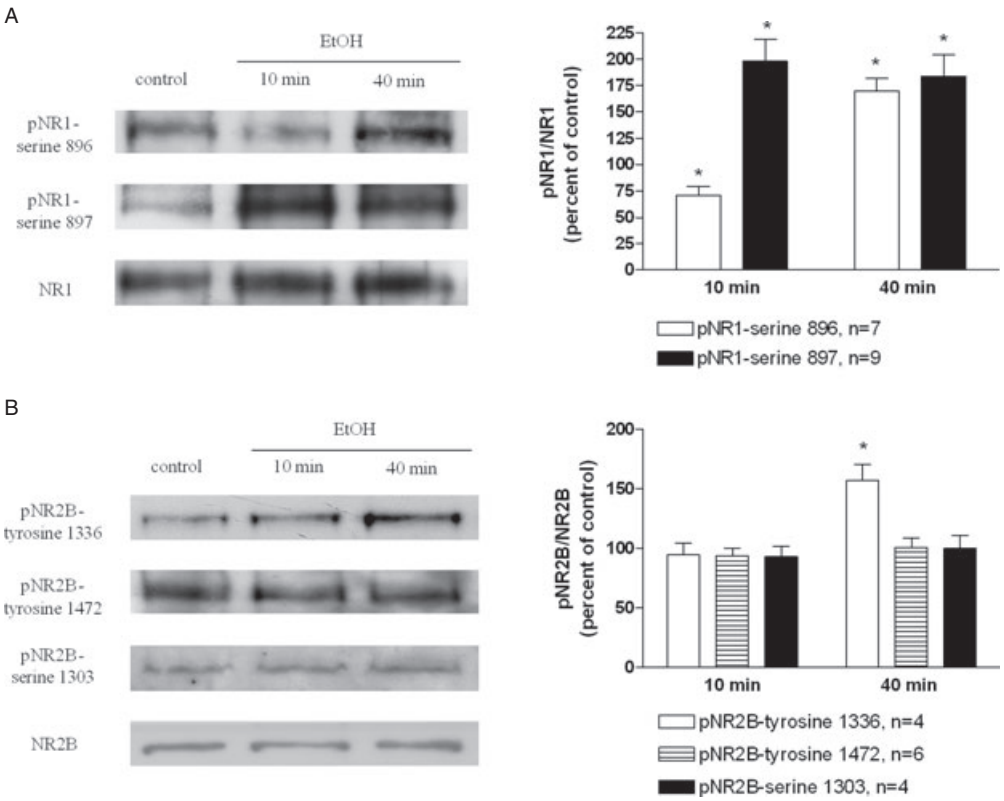

Figure 6 (A) Left panel: Western blot analysis of the levels of phosphoserine 896 on NR1 (GluN1) subunit (pNR1-serine 896), phosphoserine 897 on NR1 (GluN1) subunit (pNR1-serine 897), and N-methyl-D-aspartate (NMDA) receptor NR1 (GluN1) (NR1) subunit in lateral horn regions of spinal cord slices. Spinal cord slices (1000 μm thick) were incubated in 100 mM of ethanol (EtOH) for 10 and 40 min. In control group, the slices were not treated with ethanol. Right panel: bar graph shows changes (as % control) in the expression of pNR1 (serine 896 and serine 897) and NR1 following incubation of ethanol for 10 and 40 min. The ratio of pNR1 to NR1 in control group is taken as the control value and set to 100%. (B) similar to (A) except that the level of phosphotyrosine 1336 on NR2B (GluN2B) (pNR2B-tyrosine 1336), phosphotyrosine 1472 on NR2B (GluN2B) (pNR2B-tyrosine 1472), phosphoserine 1303 on NR2B (GluN2B) (pNR2B-serine 1303) and NR2B subunit in lateral horn regions of spinal cord slices was determined. Values denote mean + SEM. *Statistically significant difference from control analysed by one-way ANOVA followed by Newman–Keuls post-test.

depolarizations in some of SPNs at 40–50 min after superfusion of ethanol (100 mM) onto the spinal cord slices for 50 min, indicating the tolerance development *in vitro*. The results of Western blot analysis not only confirmed the findings observed in the immunohistochemical experiments but also provided evidence that changes in the phosphorylated status of NMDA receptors may be the mechanisms underlying the development of acute tolerance in the SPNs *in vitro*. Post-treatment with a PKA inhibitor, at a dose significantly inhibiting the increases in neurons staining for pNR1-serine 897, failed to block acute tolerance development. In addition, a significant increase in the immunoreactivity and the level of pNR1-serine 897 was observed not only at prolonged exposure (40 min), but also at short exposure (10 min) following continuous ethanol infusion. These results suggest that pNR1-serine 897 may not play a major role in mediating the acute tolerance development in the present study.

Various kinase-mediated phosphorylation of NMDA receptor subunits have been shown to enhance NMDA receptor function (Salter, 1998; Lu *et al*., 1999; Westphal *et al*., 1999) and to reduce NMDA receptor sensitivity to ethanol (Snell *et al*., 1994; Maldve *et al*., 2002; Yaka *et al*., 2003). The attenuation of ethanol inhibition of NMDA receptors by activation of dopamine receptors in the nucleus accumbens has been suggested to be mediated by the activation of PKA (Maldve *et al*., 2002). Our previous study also demonstrated that cAMP-PKA signalling pathway is involved in acute tolerance to ethanol inhibition of the pressor responses induced by microinjection of NMDA into rat RVLM (Lin *et al*., 2006). Phosphorylation of NR2B subunits of the NMDA receptor by Fyn tyrosine kinases would counteract the inhibitory actions of ethanol in the hippocampus (Miyakawa *et al*., 1997; Yaka *et al*., 2003). The present study demonstrated that increases in the levels of pNR1-serine 896 and pNR2B-tyrosine 1336 via PKC and Src tyrosine kinase signalling pathways may be the mechanism underlying acute tolerance to ethanol inhibition of spinal NMDA-induced pressor responses. The results strengthen the notion that the development of acute ethanol tolerance is a kinase-dependent mechanism and imply that the tolerance is derived from the multiple effects of ethanol rather than from self-regulation of NMDA receptors. The results also suggest that ethanol-activated kinases participating in the tolerance development may vary in different brain areas.

In CA1 neurons of the hippocampus, PKC-dependent potentiation of NMDA receptor activity is mediated via activation of Src tyrosine kinase signalling cascade leading to

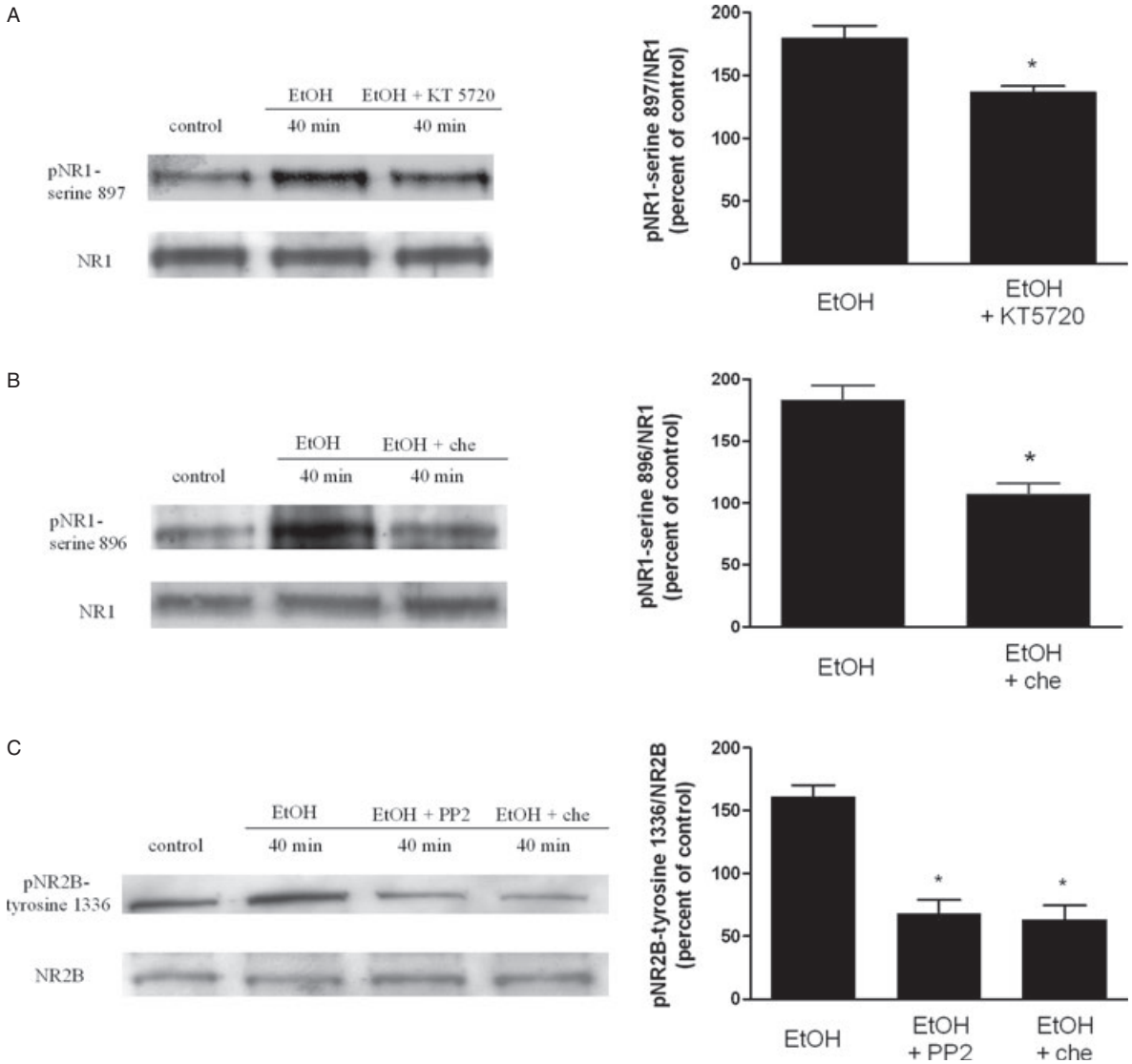

Figure 7 (A) Left panel shows Western blot analysis of the levels of phosphoserine 897 on the NR1 (GluN1) subunit (pNR1-serine 897) and N-methyl-D-aspartate (NMDA) receptor NR1 (GluN1) subunit (NR1) in lateral horn regions of spinal cord slices incubated with ethanol for 40 min without or with addition of KT5720 (20 nM), a protein kinase inhibitor. KT5720 was applied at 10 min after ethanol (EtOH) incubation. In the control group, the slices were not treated with ethanol or/and KT5720. This experiment was repeated three times. The changes (as % control) in the ratio of pNR1-serine 897 to NR1 are shown in the right graph. The ratio of pNR1-serine 897 to NR1 in the control group is taken as the control value and set to 100%. (B) similar to (A) except that that levels of phosphoserine 896 on the NR1 (GluN1) subunit (pNR1-serine 896) was determined in the lateral horn regions of the slices post-treated with chelerythrine (che; 10 μM), a protein kinase C inhibitor, following ethanol incubation. This experiment was repeated five times. (C) similar to (A) except that levels of phosphotyrosine 1336 on NR2B (GluN2B) (pNR2B-tyrosine 1336) was determined in the lateral horn regions of the slices post-treated with 25 nM PP2, an inhibitor of Src-family tyrosine kinase or 10 μM che following ethanol incubation. This experiment was repeated seven times. The ratio of pNR1-serine 896 to NR1 (A), pNR1-serine 897 to NR1 (B), or pNR2B-tyrosine 1336 to NR2B (C) in the control group is taken as the control value and set to 100%. Values denote mean + SEM. *Statistically significant difference from the group incubated with ethanol for 40 min without post-treatment with inhibitors, analysed by one-way ANOVA followed by Newman–Keuls post-test.

tyrosine phosphorylation of NR2A and NR2B subunits (Lu *et al*., 1999; Grosshans and Browning, 2001). We also found that incubation of spinal cord slices with phorbol 12-myristate 13-acetate (5 nM), a PKC activator, for 40 min increased both the levels of pNR1-serine 896 and pNR2B-tyrosine 1336 in the lateral horn regions of the spinal cord slices analysed by Western blot (results not shown). The current study showed that ethanol-induced increase in pNR2B-tyrosine 1336 was blocked by a PKC inhibitor in addition to by a Src tyrosine kinase inhibitor. These results support the notion that PKC regulation of NR2B subunit is via Src tyrosine kinase and suggest the involvement of PKC in ethanol regulation of pNR2B-tyrosine 1336. There is *in vitro* evidence that ethanol can directly affect PKC activity, either activating or inhibiting PKC depending on the experimental conditions (Stubbs and Slater, 1999). On the other hand, PKC is a downstream signalling molecule of many neurotransmitter receptors. Whether ethanol regulates the function of certain neurotransmitter receptors in the neurons of the IML area, leading to the activation of PKC, remains to be clarified.

The number of neurons expressing pNR1-serine 896 was significantly reduced at 10 min after continuous ethanol infusion. Similar results from Western blot analysis were found in spinal cord slices incubated with ethanol for 10 min. In the hippocampus, dephosphorylation of tyrosine residues in NMDA receptor subunits by acute exposure to ethanol has been suggested to play an important role in mediating the inhibitory effects of ethanol on NMDA receptor function (Alvestad *et al*., 2003). It is possible that decreases in the levels of pNR1-serine 896 at 10 min may contribute to the inhibitory effects of ethanol on NMDA receptor function and on spinal NMDA-induced pressor effects. Further work is required to establish this assumption. Phosphorylation of NR2B on serine residues 1303 and 1323 by PKC has been implicated in the enhancement of channel function (Liao *et al*., 2001). However, the current study showed that prolonged ethanol application had no significant effects on pNR2B-serine 1303. It is possible that certain serine and tyrosine residues of NMDA receptor subunits are more sensitive to ethanol-induced kinase signalling cascades.

A positive relationship between ethanol consumption and blood pressure is well established in epidemiologic studies (Puddey and Beilin, 2006). Although the precise mechanisms underlying ethanol regulation of cardiovascular function remain unknown, several studies have suggested an important role of central sympathetic activity in the development of alcohol-induced hypertension (Abdel-Rahman & Wooles, 1987; Randin *et al*., 1995). Ethanol inhibition of spinal NMDA-induced pressor responses and acute tolerance to the inhibition during prolonged exposure in the present study were observed at blood concentrations considered to be mildly intoxicating. Most heavy drinkers reach this range of blood levels (Shpilenya *et al*., 2002). Thus, the current finding showing ethanol regulation of NMDA receptor phsophorylation via PKC and tyrosine kinase signalling cascades is pharmacologically relevant. In view of the importance of NMDA receptor activation in modulation of central control of the cardiovascular system, ethanol may have a profound effect on sympathetic output if it acts to change the action of NMDA receptor function on the central sympathetic circuit.

In conclusion, prolonged ethanol exposure caused an increase in the levels of pNR1-serine 896 and pNR2B-tyrosine 1336 resulting from activation of PKC and Src tyrosine kinases. Such changes may have reduced NMDA receptor sensitivity to ethanol and contributed to the development of acute tolerance to ethanol inhibition of spinal NMDA-mediated pressor effects.

Acknowledgement

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Conflicts of interest

None.

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