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Essential involvement of the NMDA receptor in ethanol preconditioning–dependent neuroprotection from amyloid- β *in vitro*

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Abstract

In several epidemiological studies, moderate ethanol consumption has been associated with reduced risks of cognitive decline or Alzheimer’s dementia. Of potential relevance is that brain cultures preconditioned with moderate ethanol concentrations are resistant to neurotoxic Alzheimer’s amyloid- β (A β) peptides. Using rat cerebellar mixed cultures we investigated whether certain membrane receptors were early ‘sensors’ in moderate ethanol preconditioning (MEP). In a 6-day MEP protocol (30 mM ethanol), neuroprotection from A β 25–35 was undiminished by antagonism during the first 3 days of either adenosine A₁ or G $\alpha_{i/o}$ protein-coupled receptors. However, similar cotreatment with memantine or DL-2-amino-5-phosphono-pentanoic acid (AP-5), antagonists of NMDA receptors (NMDAR), abolished neuroprotection, indicating key early involvement of this ionotropic glutamate receptor. Also in these cultures, directly activating NMDAR using subexcitotoxic NMDA preconditioning prevented A β neurotoxicity. By day 2 of MEP, we observed increased levels of NMDAR subunits NR1, NR2B, and NR2C that persisted through day 6. Interestingly, memantine co-exposure blocked elevations in the obligatory NR1 subunit. Furthermore, 2 days of MEP significantly increased two indicators of synaptic NMDAR localization, NR2B phospho-Tyr1472, and post-synaptic density 95 scaffolding protein. The results indicate that ethanol preconditioning-dependent neuroprotection is associated with early increases in NR subunits concomitant with enhancement of synaptic localization and activity of NMDAR.

Keywords

alcohol; neurotoxicity; tolerance

Preconditioning refers to stress-dependent adaptive responses that generate a tolerant cellular state, such that preconditioned tissues or organs are resistant to a normally lethal insult. Neuroprotective stress examples include brief ischemia, hypoxia, seizures, and an array of chemicals that induce cellular tolerance, possibly via a ‘sensor-transducer-effector’ mechanism (Dirnagl *et al.* 2003). Our laboratories reported that subchronic preconditioning of rat brain cultures with concentrations of ethanol (20–30 mM) considered moderate to high-moderate in terms of social consumption blood levels (Eckardt *et al.* 1998; Gunzerath *et al.* 2004) – termed moderate ethanol preconditioning (MEP) – afforded neuroprotection from the dementia-associated neurotoxic peptides, HIV-1 gp120, or amyloid- β (A β) (Collins *et al.* 2000; Belmadani *et al.* 2001, 2004).

This study's objective was to elucidate possible 'sensor' roles of certain cell surface receptors that were identified in neuroprotective ischemic preconditioning, in MEP-mediated neuroprotection *in vitro* against A β , the peptide(s) linked to neurodegeneration in Alzheimer's disease (AD) brain. Although MEP neuroprotection was demonstrated earlier against pathophysiological A β 1–42 (Belmadani *et al.* 2004), these experiments used the pathologically active but nonphysiological A β 25–35 peptide, which has a neurotoxic mechanism apparently identical to A β 1–42 (Frozza *et al.* 2009) as well as being the critical sequence within A β 1–42 responsible for impairment of long-term potentiation (Lee *et al.* 2009). Here our findings with MEP implicate activation of glutamatergic NMDA receptors (NMDAR) – but rule against adenosine A1 or other G $\alpha_{i/o}$ protein-coupled receptors – in attaining a neuroprotected state. The ethanol preconditioning appears to modulate NMDAR by increasing subunit expression while enhancing synaptic receptor localization and connectivity, ultimately triggering pathways favoring neuroprotection against toxic peptides that are implicated in synaptic loss, for example, in AD. As such, the findings are consistent with the neuroprotective mechanism of ischemic preconditioning of brain cultures (Lin *et al.* 2008), in which there is an important role for activation of NMDAR.

We posit that this neuroprotective phenomenon of ethanol may yield insight into epidemiological results often indicating that moderate alcohol consumers have a lower risk of dementia, including AD, compared with abstainers (Zuccala *et al.* 2001; Mukamal *et al.* 2003; Peters *et al.* 2008). A prevalent neurodegenerative disorder of aging, AD has been linked to neurotoxic A β peptides (particularly A β 1–42) that accumulate and form soluble oligomers and amyloid plaques in brain. Soluble oligomeric A β levels in AD brain, more so than amyloid plaque burden, correlate with cognitive deficits among AD patients (Naslund *et al.* 2000). Aside from acetylcholinesterase inhibitors, memantine, which antagonizes the extrasynaptic NMDA subclass of glutamate receptors (NMDAR) to suppress excitotoxic receptor activation (Leveille *et al.* 2008), is the only FDA-approved drug that has shown promise in delaying AD-related symptoms. Given the increasing prevalence of AD, investigations into mechanisms that prevent or delay the disease and lead to new therapeutics are of the utmost importance.

Materials and methods

Chemicals and antibodies

Hoechst 33342, propidium iodide (PI), DL-2-amino-5-phosphonopentanoic acid (AP-5), 8-Cyclopentyl-1,3-dipropylxanthine, pertussis toxin, memantine, NMDA, deoxyribonuclease I, Tweenr 20, Triton-X, protease and phosphatase inhibitor cocktails, and penicillin/streptomycin were obtained from Sigma Co. (St Louis, MO, USA). Neurobasal-A media, B27, trypsin, and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA, USA). American Peptide (Sunnyvale, CA, USA) provided A β 25–35 and A β 35–25. Polyacrylamide gels were obtained from GenScript (Piscataway, NJ, USA). Western blot buffers and enhanced chemiluminescence detection solutions were obtained from Pierce Chemical Co. (Rockford, IL, USA). The antibodies obtained from Upstate Co. (Temecula, CA, USA) were anti NR1-CT (1 : 1000) and anti-NR2B (1 : 1000); anti-NR2C (1 : 1000), post-synaptic density 95 (PSD-95; 1 : 1000), NR2B-pY1472 (1 : 1000), NeuN (1 : 1000), and glial fibrillary acidic protein (GFAP) (1 : 1000) were obtained from Chemicon International (Temecula, CA, USA); and actin (1 : 1000) and horseradish peroxidase-conjugated anti-rabbit IgG (1 : 1000–1 : 20000) were obtained from Cell Signaling (Danvers, MA, USA).

Cerebellar primary cultures

Rats were taken care of according to principles in the *Guidelines for the Use of Animals in Neuroscience Research* by Society for Neuroscience, and procedures were approved by

LUMC IACUC. Rat pups (7-day-old S/D, Harlan, Indianapolis IN, USA) were cold-anesthetized on ice for 2 min and decapitated. Brains were placed in cold Gey's balanced salt solution with 4.5 mg/mL glucose. After removal of meninges, individual cerebella were divided into approximately six pieces and placed in 37°C calcium-/magnesium-free Hank's balanced salt solution containing 0.25% trypsin and 0.5 mg/mL DNase. After 10 min, pieces were transferred to minimal essential medium containing 10% FBS and 5% horse serum. Cells were triturated with two fire-polished pipettes of decreasing size and passing through a 70 µm cell strainer. After determining viable cell density by trypan blue exclusion, cells were diluted to 800 000 cells/mL in Neurobasal-A supplemented with 2% B27, 10% heat-inactivated FBS, 500 µM L-glutamine, and 1% penicillin/streptomycin, and plated on poly-L-lysine-coated 12-well plates at 1 mL/well or 20 mL/10 cm dish. Half of the same growth media less serum was changed every 3 days until 10 days *in vitro* (DIV), when 100% was changed. This allowed glia to proliferate to confluence and produced neurons with robust processes and intricate neural networks (Fig. 1b).

Ethanol preconditioning and A β exposure

Culture media was changed to media containing 30 mM ethanol or media alone at 10 DIV. Culture plates containing ethanol were placed in covered Tupperware containers containing small open dishes of 0.5% ethanol/water to minimize evaporative ethanol loss from experimental wells. The containers were allowed to equilibrate with incubator atmosphere for 15 min and closed. Culture media were changed with fresh ethanol at day 3. At day 6, ethanol media and control media were completely removed and media with/without A β 25–35 or reverse peptide was added. For drug treatments, a parallel set of plates was prepared, differing only by the presence of added drug.

Lactate dehydrogenase assay

Media were collected from cultures and spun at 5000 *g* for 5 min. The manufacturer's microplate protocol was used to determine toxicity. Lactate dehydrogenase (LDH) activity correlated linearly ($R^2 = 0.9992$) with the number of live cerebellar cells lysed over the activity ranges in these experiments. Neurotoxicity was reported as LDH activity/100 µL culture media.

Dual fluorescence staining

Cerebellar cell cultures were incubated 15 min at 37°C with 5 µM Hoechst 33342 which labeled nuclei of all cells and 5 µg/mL PI which stained dead/dying neurons. The extent of cytotoxicity defined as percent of dying neurons was obtained by dividing the number of PI-positive neurons by the total number of Hoechst-stained nuclei 100%. Approximately 500 nuclei were counted per well and six wells per experimental group. Images were subsequently analyzed with NIH ImageJ 1.34s (NIH, Bethesda, MD, USA).

Western blot analyses

Following ethanol and drug treatments, cerebellar samples grown on 10 cm dishes were rinsed once with ice-cold Tris-buffered saline, snap-frozen in acetone/dry ice, and stored at –80°C until analysis. Extracts of cells (5–20 µg protein) in the presence of protease and phosphatase inhibitor cocktails were run on 4–12% polyacrylamide gels, transferred to polyvinylidene difluoride membrane blocked with 1–3% bovine serum albumin, and exposed to appropriate primary antibodies, diluted based on manufacturer's recommendations. Horseradish peroxidase-conjugated secondary antibodies were diluted in Tris-buffered saline wash buffer containing 0.1% Tween 20, incubated for 3 h, and developed using enhanced chemiluminescence detection. X-ray films were scanned and analyzed with NIH ImageJ 1.34s. To establish equal loading and normalize intensities of

NMDAR subunits, blots were subsequently stripped and probed for β -actin, which was unaffected by the ethanol preconditioning.

Immunocytochemistry

Following ethanol or control treatment, cultures were washed 2 \times with pre-warmed phosphate-buffered saline (PBS), fixed for 15 min at 37°C with 4% paraformaldehyde, and stored in PBS containing 0.05% NaN₃ for later viewing. Monolayers were permeabilized with PBS containing 0.3% Triton X-100 and blocked with 5% serum of secondary antibody host for 3 h at 27°C. Primary antibodies were incubated overnight at 4°C in blocking buffer, washed, and incubated with corresponding biotinylated secondary antibody. Streptavidin-conjugated Cy2 or Cy3 (1 : 200) was then applied for 2 h at 27°C, washed, and stored in PBS containing 0.05% NaN₃. Primary delete experiments showed specificity of cell-type antibodies. Cultures were digitized on an inverted epifluorescent fluorescent microscope equipped with a Nikon digital camera (Nikon Inc., Melville, NY, USA) for image acquisition. Images were subsequently analyzed with NIH ImageJ 1.34s.

Results

A β causes dose-related neurotoxicity in cerebellar mixed cultures that is inhibited by ethanol preconditioning

Our laboratories previously reported that the MEP protocol in Fig. 1a blocks A β -induced neurotoxicity in hippocampal–cortical slice cultures (Belmadani *et al.* 2004). Although the cerebellum is relatively resistant to amyloid peptides *in vivo* (Kim *et al.* 2003), cerebellar granule neurons are vulnerable to A β *in vitro* (Allen *et al.* 1999). Our mixed cerebellar cultures contained 15–20% NeuN-positive neuronal cell types and a mixture of non-neuronal, primarily GFAP-positive cell types (Fig. 1b). MEP treatment affected neither the neuronal viability (Fig. 1c) nor the total cell number of these cultures (not shown). At 16 DIV, the cultures displayed dose-related A β 25–35 neurotoxicity (Fig. 1d, 25 μ M for 24 h); the toxicity which was because of A β 25–35 was significantly counteracted in ethanol-preconditioned cultures (Fig. 1e) as determined by released LDH. As expected, the reverse peptide A β 35–25 (25 μ M) was not neurotoxic (results not shown). Notably, reduced LDH release in MEP cultures compared with control suggested cytoprotection by MEP from toxicity associated with general medium changes. Similar indications of such protection were observed during other MEP experiments, as well as by other investigators who utilized moderate, subchronic ethanol exposures (Thomas and Morrisett 2000).

Acquisition of ethanol preconditioning-induced neuroprotection is NMDAR-dependent

To determine if adenosine/G $\alpha_{i/o}$ protein-coupled receptors or NMDAR, implicated in other forms of brain preconditioning neuroprotection, were involved in MEP neuroprotection, we examined whether antagonism of these receptors reduced or prevented A β neurotoxicity. Figure 2 shows that when present in culture media for the first 3 days of preconditioning, neither a specific adenosine A₁ receptor antagonist [dipropylxanthine (DPCPX), Fig. 2a] nor the G $\alpha_{i/o}$ protein-coupled receptor inhibitor, pertussis toxin (Ptx, Fig. 2b) significantly counteracted neuroprotection from A β because of 6 days of MEP. However, AP-5, a specific high affinity NMDAR antagonist, completely abolished the neuroprotective effects of MEP (Fig. 2c). Furthermore, memantine, a moderate affinity open channel NMDAR antagonist, blocked most of the MEP neuroprotection (Fig. 2d). These results are entirely consistent with a relatively early ‘sensor’ role for NMDAR in the MEP mechanism.

Preconditioning with NMDA neuroprotects from A β as determined by dual fluorescence staining

If NMDAR activation is essential in MEP neuroprotection, direct stimulation of this receptor via NMDA preconditioning should elicit similar protection from A β insult in our cultures. As shown in Fig. 3a, cultures at 13 DIV were preconditioned for 24 h with 50 μ M NMDA, previously determined to be subneurotoxic in this dose range with cerebellar cultures (R. Mitchell, unpublished data), and then exposed for 24 h to 12.5 μ M A β 25–35 or media only. The 13 DIV time point which corresponds to halfway through the MEP exposure was chosen because of our preliminary findings that both subclasses (NR1 and NR2) of NMDAR subunits were expressed at that time. In Fig. 3b are representative live-cell photomicrographs of dual fluorescence staining with Hoechst 33342 and PI, a combination shown to correlate well in cerebellar cultures with other cytotoxic measures (Bachis *et al.* 2003). Hoechst 33342 labels nuclei of all cells and PI stains dying/dead neurons in the control (C) and NMDA-preconditioned cultures. PI-stained cell densities between control and NMDA preconditioned groups did not differ, verifying that NMDA treatment was not neurotoxic. However, cultures treated with A β demonstrated increased density of PI-labeled degenerating neurons compared with C or NMDA, whereas the NMDA-preconditioned cultures treated with A β (NMDA + A β) appeared similar to C or NMDA alone. Quantification of stained dead cells/total cells, represented as % neurotoxicity in Fig. 3c, showed that A β -induced neurodegeneration was completely prevented by NMDA preconditioning. Thus, NMDAR activation by NMDA ligand binding, or by extension, ethanol preconditioning-augmented NMDAR activity, promoted a neuroprotected state in these cerebellar cell cultures, rendering them resistant to A β insult.

NMDAR subunits are significantly elevated by ethanol preconditioning and memantine abrogates potentiation of obligatory NR1 subunits

As the data implied increased receptor activity because of moderate ethanol exposure, we then examined whether changes in levels of NMDAR subunits could potentially underlie receptor activity. Indeed, the data in Fig. 4 demonstrate that NR1, the obligatory subunit for NMDAR function, and NR2B and NR2C, which contain glutamate binding domain(s), were each significantly elevated by day 2 of 30 mM ethanol exposure and remained elevated after 6 days of preconditioning (Fig. 4a and b). Not shown for simplicity was that the NMDAR subunits were also elevated at day 4 in ethanol preconditioning. It was also apparent that co-treatment with memantine antagonist which counteracted MEP neuroprotection from A β and restored the neurotoxic effects of peptides (Fig. 2d), completely prevented the 2 day elevations in the obligatory NR1 subunit (Fig. 4a and b).

Increases in NR2B phosphotyrosine-1472 and PSD-95 after 2 days of ethanol preconditioning indicate increased synaptically localized NMDAR

Questioning whether MEP-induced NMDAR activation was primarily synaptic, we examined ethanol's effects on levels of tyrosine 1472-phosphorylated NR2B (NR2B-pY1472) and PSD-95 relatively early in the 6-day preconditioning protocol. The activity and synaptic localization of NMDAR are believed to require phosphorylation of subunits, including tyrosine 1472 of NR2B by Src family tyrosine kinases (Yu *et al.* 1997; Goebel-Goody *et al.* 2009). Also, PSD-95 is a cytoskeletal protein involved in the dynamic regulation of synaptic NMDAR complex formation which links the receptors to downstream signaling moieties (Delint-Ramirez *et al.* 2008; Kurisaki *et al.* 2008). As shown in Fig. 4c and d, after 2 days of moderate ethanol exposure, the levels of both NR2B-pY1472 (2B-pY1472) and PSD-95 were significantly elevated. Furthermore, although ethanol preconditioning potentiated NR2B protein levels at this time (Fig. 4a and b), tyrosine kinase activity acting on NR2B – most likely of the src family – was evidently increased as well, as demonstrated by the significantly increased ratio of NR2B-pY1472/total NR2B (pY/2B in

Fig. 4d) in ethanol-treated cultures. The results point to NMDAR subunit up-regulation in association with enhanced synaptic NMDAR localization as relatively early events of the ethanol preconditioning mechanism.

Discussion

One overall conclusion derived from these experiments is that neuroprotection engendered by preconditioning rat cerebellar cultures with moderate ethanol concentrations involves NMDAR activation as a likely neuroprotective sensor. Recapitulating, pharmacological inhibition of NMDAR early during MEP disallowed later neuroprotection. In contrast, antagonism of adenosine A₁ receptors or of Gα_{i/o} protein-coupled receptors generally was without effect. The data agree with evidence for glutamatergic NMDAR involvement in brain preconditioning that originated soon after the discovery of ischemic neuronal tolerance (Kato *et al.* 1992). More recent reports confirm a neuroprotective role for NMDAR in ischemic preconditioning (Grabb and Choi 1999; Raval *et al.* 2003).

The fact that preconditioning with subneurotoxic NMDA ligand itself affords neuroprotection from Aβ in our cerebellar cultures lends additional support to the possibility that NMDAR activation mediates moderate ethanol-initiated prosurvival signaling, and agrees with NMDA preconditioning/neuroprotection results in other primary cultures (Soriano *et al.* 2006; Smith *et al.* 2008). NMDAR excitability appeared critical as well in preconditioning with glutamate or prolonged elevations in electrical activity, as NMDAR antagonists blocked the resultant neuroprotection against subsequent ischemia (Lin *et al.* 2008; Tauskela *et al.* 2008). However, despite this correspondence, we acknowledge that protective pathway of NMDA and mechanism of neuroprotection by ethanol may well differ in other ways.

Ethanol is known to acutely inhibit and chronically potentiate NMDAR currents, although exact mechanisms remain a matter of active investigation (Nagy 2008). We find that persistent increases in subunits NR1, NR2B, and NR2C occur by the first 2 days of moderate ethanol exposure. Such increases in receptor subunits could be a principal reason for increased NMDAR activity leading to neuroprotection. This possibility is borne out by memantine's blockade of ethanol-dependent increases in NR1, the receptor's obligatory subunit, thus contributing to a reduction in NMDAR activity and the annulment of MEP neuroprotection. The above results coincide with hippocampal culture expression data (Maler *et al.* 2005), in which memantine, with negligible effect alone on NMDAR subunits, suppressed the up-regulation of NR1 by 5 days of 50 mM ethanol. Of *in vivo* relevance to our *in vitro* findings is that rats chronically fed moderate ethanol-containing diets demonstrated improved memory performance which was dependent on NR1 (Kalev-Zylinska and During 2007). RNA interference knockdown of hippocampal NR1 abolished ethanol's facilitatory effects in the model, while NR1 over-expression mimicked ethanol's effects. Although NR2 subunits are known ethanol targets (Woodward *et al.* 2006), levels of NR1 subunits invariably exert critical effects on NMDAR function.

A further critical aspect of NMDAR dynamics related to MEP is our evidence for early MEP-dependent receptor increases that reflect synaptic as opposed to extrasynaptic NMDAR – specifically, levels of NR2B phospho-tyrosine 1472, which are associated with decreased endocytosis and synaptic enrichment of NMDAR (Yu *et al.* 1997; Goebel-Goody *et al.* 2009), and of PSD-95, which implicate increased synaptic receptor localization (Delint-Ramirez *et al.* 2008; Kurisaki *et al.* 2008). These findings are in harmony with data showing that increases in synaptic relative to extrasynaptic NMDAR signaling are associated with (or signal) downstream pro-survival events (Soriano *et al.* 2006; Soriano and Hardingham 2007). Furthermore, the significantly augmented ratio of NR2B-pY1772/total

NR2B at an early MEP timepoint of 2 days indicates potentially increased activity of Src-family kinase(s) (Goebel-Goody *et al.* 2009). This additional appealing aspect of MEP-dependent NMDAR modulation that requires further investigation is consistent with our findings that MEP increases cerebellar levels of a critical non-receptor kinase linked to Src activation, focal adhesion kinase (Sivaswamy *et al.* 2008).

A recent report showed that A β 1–42-containing monomeric and oligomeric peptides bind to post-synaptic NMDAR-containing synapses, inhibited NMDAR, and decreased NMDAR immunoreactive spines and surface expression of NR2B-containing NMDAR (Dewachter *et al.* 2009). The authors also found decreased concentrations of NR2B and PSD-95 in a transgenic mouse model of AD. Others have reported that the suppressive effects of A β on NMDAR surface expression correlated with decreased NR2B-pY1472 (Snyder *et al.* 2005). Our results indicate that ethanol preconditioning may combat neurodegenerative processes induced by A β and other neurotoxic proteins (e.g., gp120) by preserving NMDAR-associated synaptic density and connectivity. Speculatively, MEP-mediated neuroprotection could entail the ability of synaptically localized NMDAR to enhance intrinsic antioxidant defenses – specifically, peroxiredoxin and thiol redoxin pathways (Papadia *et al.* 2008).

Although growing evidence suggests a physiological function of amyloid peptide fragments pertaining to regulation of synaptic transmission and homeostasis (Pearson and Peers 2006), aberrant A β peptide overproduction is considered central to AD pathophysiology and possibly age-related mild cognitive decline. The facts that synaptic loss correlates well with cognitive decline *in vivo* (Terry *et al.* 1991), and that A β peptides experimentally induce alterations in synapse composition and density (Lacor *et al.* 2007) underscore such losses. AD is reaching epidemic proportions among aging populations (Wang and Ding 2008); however, effective pharmaceutical approaches to slow AD progression are lacking. Investigations into the molecular mechanisms of ethanol preconditioning may help to clarify roles of NMDAR in AD etiology and offer new strategies for halting synaptic degeneration while preserving synaptic connectivity.

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Abbreviations used

AD	Alzheimer's disease
A β	amyloid- β
AP-5	DL-2-amino-5-phosphono-pentanoic acid
DIV	days <i>in vitro</i>
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
LDH	lactose dehydrogenase
MEP	moderate ethanol preconditioning
NMDAR	NMDA receptors
PBS	phosphate-buffered saline
PI	propidium iodide

PSD-95 post-synaptic density 95

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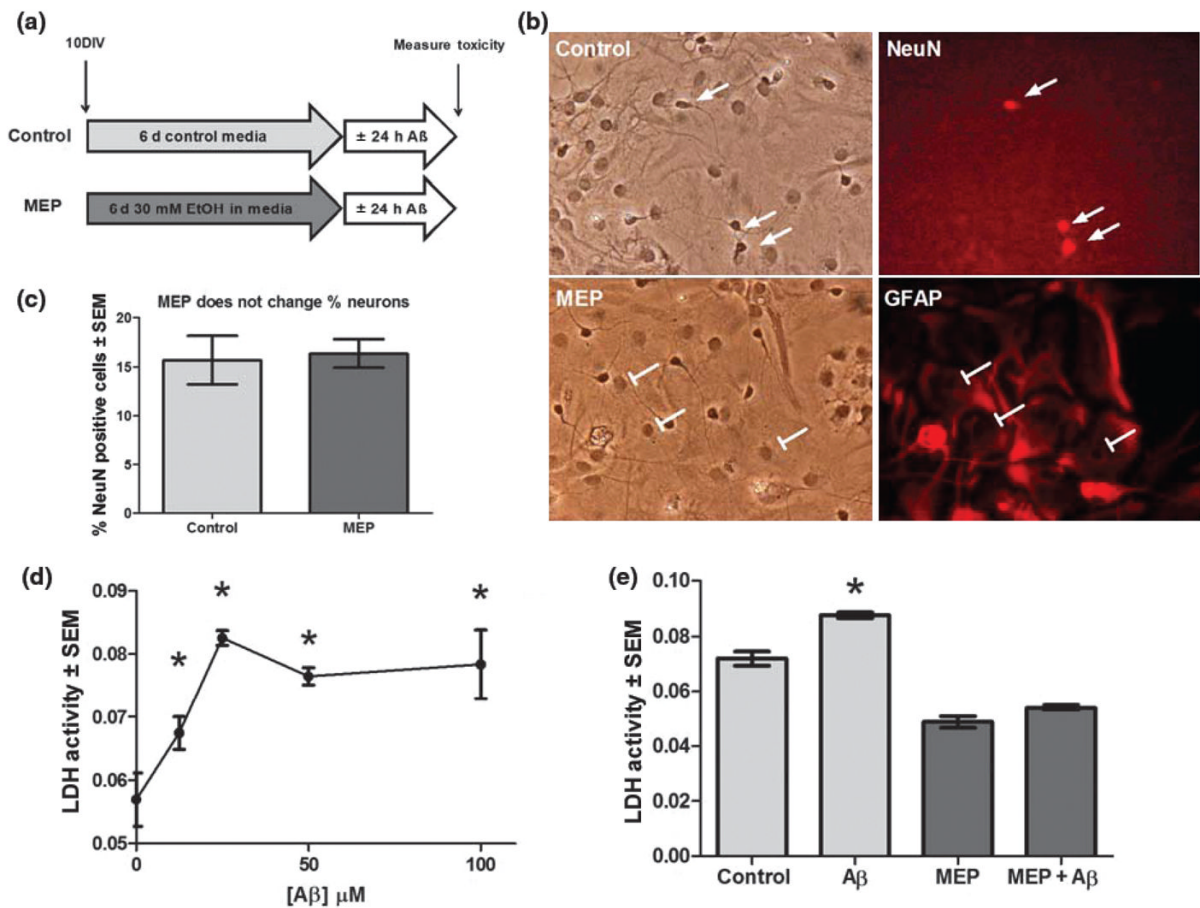


Fig. 1. Dose-related Aβ neurotoxicity and neuroprotection by moderate ethanol preconditioning (MEP). (a) MEP protocol: cerebellar cultures (10 DIV) were treated with 30 mM EtOH or control media for 6 days, rinsed, and exposed for 24 h to 25 μM Aβ25–35 or vehicle. (b) Control (top row) and MEP-treated (bottom row) cerebellar cultures (16 DIV) were fixed and analyzed by immunocytochemistry for neuronal (NeuN+) and glial (GFAP+) cell types and co-stained with Hoechst 33342, identifying all nuclei. (c) MEP did not alter neuronal composition of cerebellar cultures. Cerebellar cultures were treated as in b and the percent of total (Hoechst 33342-positive) cells that were positive for NeuN were quantified in MEP and control-treated cultures ($n = 3$ at least 100 cells counted per well). (d) Dose-related neurotoxicity of cerebellar cultures (16 DIV) to Aβ25–35 as measured by LDH release; * $p < 0.05$ versus no Aβ25–35 ($n = 6$). (e) Aβ neurotoxicity in cerebellar cultures was prevented by MEP. * $p < 0.05$ versus control; 1-way ANOVA with Scheffe *post hoc* test ($n = 6$).

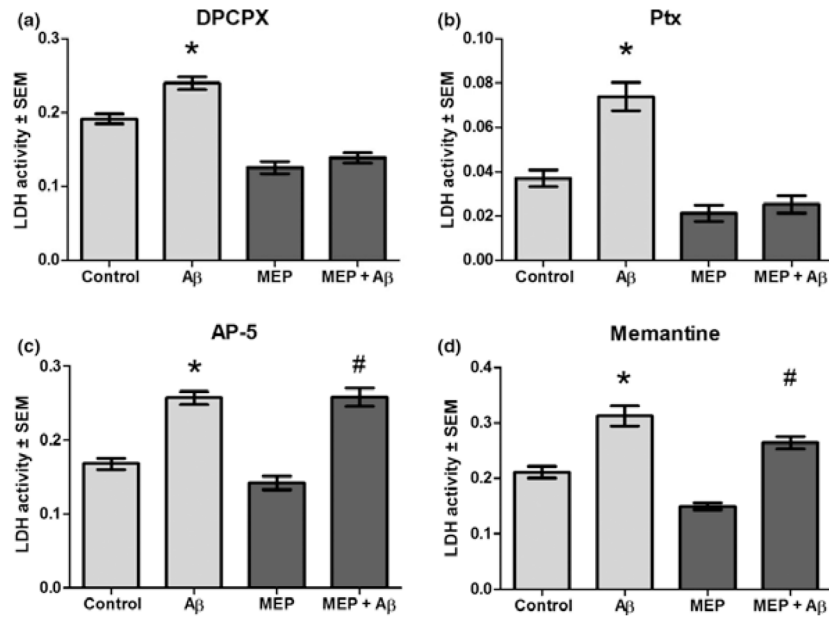


Fig. 2. MEP-induced neuroprotection against A β : NMDAR antagonists abolished protection but antagonism of adenosine A1 receptors or Gi/o protein-coupled receptors had no effect. MEP of cerebellar cultures (as in Fig. 1) was performed with the respective receptor antagonists present for the first 3 days in a 6-day protocol, followed by 25 μ M A β 25–35 or vehicle for 24 h and media LDH assays. (a) dipropylxanthine (DPCPX) (100 nM), adenosine A1 receptor antagonist, did not prevent MEP neuroprotection. (b) Pertussis toxin (Ptx, 50 ng/mL), Gi/o protein-coupled receptor antagonist, also did not alter MEP neuroprotection. (c) AP-5 (45 μ M), NMDAR antagonist, completely abolished MEP neuroprotection. (d) Memantine (50 μ M), NMDAR antagonist, significantly inhibited MEP neuroprotection. * p < 0.001 versus control; # p < 0.001 versus MEP; 1-way ANOVA with Scheffe *post hoc* test (n = 6).

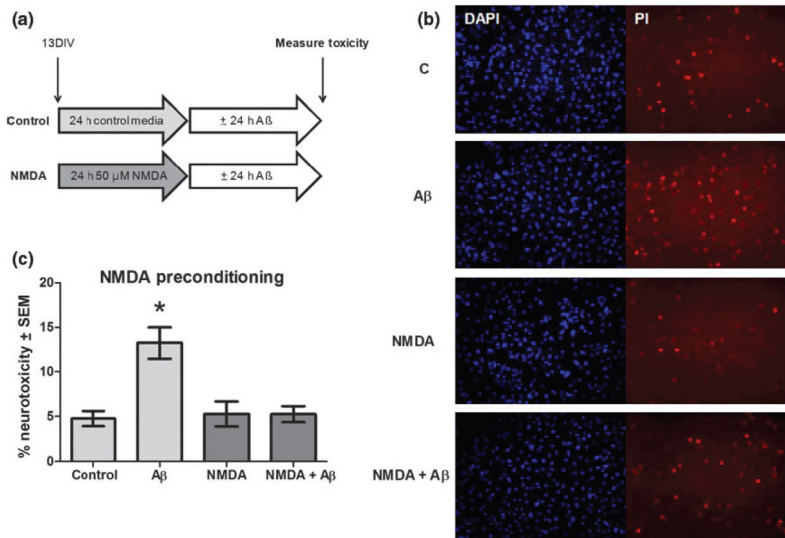


Fig. 3. NMDA preconditioning protects against Aβ neurotoxicity as determined by dual fluorescence staining with Hoescht 33342 and PI. (a) Cerebellar cultures (13 DIV) were treated for 24 h with NMDA (50 μM) or control media followed by 24 h in 25 μM Aβ_{25–35}. (b) Representative images of neurotoxicity assayed by live cell dual fluorescence staining. (c) Quantification of Aβ_{25–35} neurotoxicity (% Hoescht stained nuclei that are PI-positive) in NMDA-preconditioned or control cultures. **p* < 0.001 versus control; 1-way ANOVA with Scheffe *post hoc* test (*n* = 6).

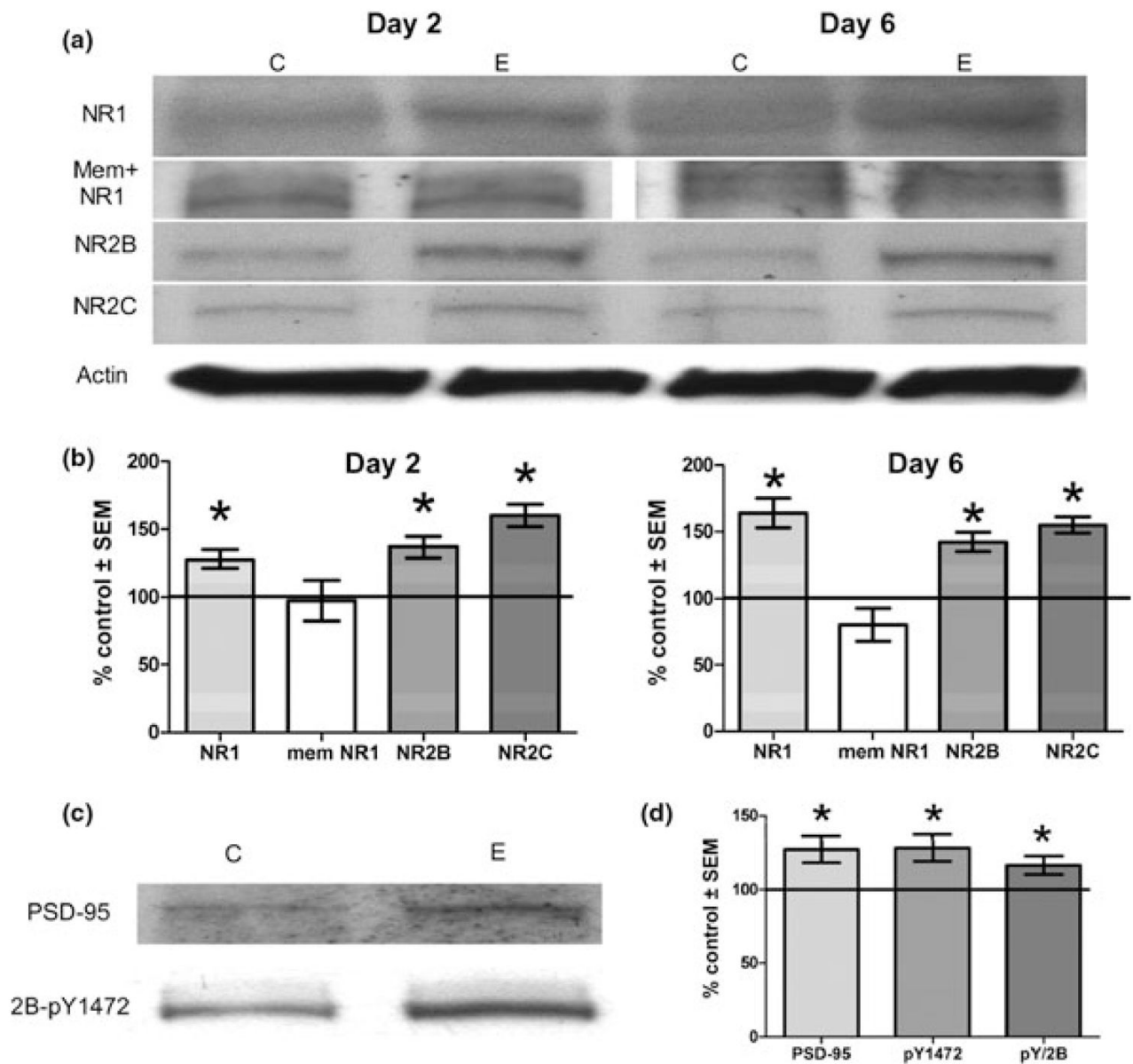


Fig. 4. Increased protein levels of NMDAR subunits by day 2 of MEP persist through day 6 and correlate with indicators of synaptically localized complexes; memantine co-treatment blocks day 2 up-regulation of obligatory NR1 subunit. Cerebellar cultures (10 DIV) were treated with 30 mM ethanol or control media for 2 or 6 days, protein extracted and subjected to immunoblot analyses. (a) Representative immunoblots of NMDAR subunits NR1, NR1 with 45 μ M memantine, NR2B, and NR2C after ethanol (E) or control (C) media. (b) Quantification of immunoblot analyses normalized to actin and the results expressed as % control. (c) Representative immunoblots of NMDAR subunit NR2B phosphorylated tyrosine 1472 (2B-pY1472) and synaptic scaffolding protein PSD-95. (d) Immunoblot quantification of PSD-95, 2B-pY1472 and the ratio of 2B-pY1472 to total NR2B expressed as % control. * $p < 0.05$ versus control by student's t -test ($n = 3-6$).