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Hippocampal NMDA receptor subunits differentially regulate fear memory formation and neuronal signal propagation

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Abstract

Activation of NMDA receptors (NMDAR) in the hippocampus is essential for the formation of contextual and trace memory. However, the role of individual NMDAR subunits in the molecular mechanisms contributing to these memory processes is not known. Here we demonstrate, using intrahippocampal injection of subunit-selective compounds, that the NR2A-preferring antagonist impaired contextual and trace fear conditioning as well as learning-induced increase of the nuclear protein c-Fos. The NR2B-specific antagonist, on the other hand, selectively blocked trace fear conditioning without affecting c-Fos levels. Studies with cultured primary hippocampal neurons, further showed that synaptic and extrasynaptic NR2A and NR2B differentially regulate the extracellular signal-regulated kinase 1 and 2/mitogen-and stress-activated protein kinase 1 (ERK1/2/MSK1)/c-Fos pathway. Activation of the synaptic population of NMDAR induced cytosolic, cytoskeletal and perinuclear phosphorylation of ERK1/2 (pERK1/2). The nuclear propagation of pERK1/2 signals, revealed by up-regulation of the downstream nuclear targets pMSK1 and c-Fos, was blocked by a preferential NR2A but not by a specific NR2B antagonist. Conversely, activation of total (synaptic and extrasynaptic) NMDAR engaged receptors with NR2B subunits, and resulted in membrane retention of pERK1/2 without inducing pMSK1 and c-Fos. Stimulation of extrasynaptic NMDAR alone was consistently ineffective at activating ERK signaling. The discrete contribution of synaptic and total NR2A- and NR2B-containing NMDAR to nuclear transmission versus membrane retention of ERK signaling may underlie their specific roles in the formation of contextual and trace fear memory.

Keywords

contextual and trace fear conditioning; synaptic; extrasynaptic; ERK/MSK; c-Fos

INTRODUCTION

Activation of N-methyl-D-aspartate receptors (NMDAR) in the hippocampus is the critical early molecular event required for the formation of spatial and trace memory (Tonegawa et al., 2003). NMDAR trigger a number of biochemical processes, some serving to maintain attention, working- and short-term memory, others supporting the formation of long-term memory. The latter mechanisms typically depend on the nuclear propagation of membrane-

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generated NMDAR signals by a network of transduction pathways (Dragunow, 1996), such as extracellular signal-regulated kinase 1/2 (ERK1/2) (Sweatt, 2001) and its downstream effectors Rsk1 (Sananbenesi et al., 2002) and MSK1 (Sindreu et al., 2007). These kinases transactivate the expression of *c-Fos* (Ha and Redmond, 2008), an immediate early gene required for long-term memory (Fleischmann et al., 2003).

As in most forebrain areas, NMDAR at hippocampal mature synapses predominantly consist of NR1/NR2A and NR1/NR2B (Cull-Candy et al., 2001; Cull-Candy and Leskiewicz, 2004; Lau and Zukin, 2007). Both NR2A and NR2B subunits have been implicated in memory formation (Tang et al., 1999; Zhao et al., 2005). Whereas general NMDAR antagonists or preferential antagonists of the NR2A subunit impair the formation of virtually any type of memory across various brain areas (Rison and Stanton, 1995; Zhao et al., 2005; Walker and Davis, 2008), behavioral experiments manipulating the level or activity of the NR2B subunit reveal complex effects. In the amygdala and cingulate cortex, NR2B subunits are required for the formation of long-term memory (Zhao et al., 2005; von Engelhardt et al., 2008; Walker and Davis, 2008). Similarly, hippocampal overexpression of NR2B strengthened performance in several learning tasks (Tang et al., 1999). In contrast, blockers of NR2B or deletion of the *GRIN2B* gene coding for NR2B proved ineffective in tasks based on fear conditioning (Zhao et al., 2005; Zhang et al., 2008) and spatial learning (von Engelhardt et al., 2008). It thus remains uncertain which memory processes are regulated by hippocampal NR2B and how the underlying mechanisms differ from those of NR2A.

One possibility is that these subunits distinctively regulate downstream signaling. Effects on the ERK pathway are controversial, however, with evidence for both NR2A or NR2B enhancement and NR2B inhibition of pERK1/2 (Krapivinsky et al., 2003; Kim et al., 2005). In addition to subunit composition, subcellular localization appears to be a key determinant of NMDAR's role in ERK signaling. NMDAR are predominantly found on the postsynaptic membrane of excitatory synapses (O'Brien et al., 1998; Scannevin and Huganir, 2000), but also are present at extrasynaptic sites (Tovar and Westbrook, 2002; Thomas et al., 2006). The distribution of NR2A- and NR2B-containing receptor complexes was originally described as synaptic and extrasynaptic, respectively (Liu et al., 2004; Liu et al., 2007), but recent data demonstrate that both subunits can be located at either compartment (Thomas et al., 2006). Activation of synaptic NMDAR induces ERK1/2 phosphorylation (Krapivinsky et al., 2003; Ivanov et al., 2006), whereas activation of total (synaptic and extrasynaptic) NMDAR either produces weaker activation (Ivanov et al., 2006) or does not activate ERK (Leveille et al., 2008) through an inhibitory action of extrasynaptic NMDAR. The contribution of NR2A and NR2B subunits to the effect of either receptor population is not known.

We aimed to identify the roles of NR2A and NR2B in the formation of contextual and trace fear-provoking memory and elucidate how their localization regulates the compartmentalized activation of the ERK1/2/MSK1/c-Fos pathway. We found that NR2B selectively mediated trace but not contextual fear conditioning whereas NR2A was required for both types of learning. Synaptic activation of NMDAR containing the NR2A subunit resulted in transmission of ERK signaling to the nucleus as revealed by up-regulation of the downstream targets MSK1 and c-Fos signaling. Conversely, activation of the total receptor population resulted in NR2B-mediated phosphorylation and membrane retention of ERK1/2 that did not result in activation of these nuclear targets.

METHODS

Materials

Poly-D-lysine, (–)-Bicuculline Methiodide, CNQX, (+)-MK-801 hydrogen maleate Dizocilpine, nimodipine, N-Methyl-D-aspartic acid, Ro25-6981, strychnine hydrochloride, TTX, mouse pERK1/2 antibody were purchased from Sigma-Aldrich (St. Louis, MO); NeuroBasal medium, B27, were from Invitrogen (Carlsbad, CA); rabbit antiphospho (Thr581)-MSK1 (pMSK1) antibody was from Cell Signaling Technology (Beverly, MA); c-Fos antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); ProteoExtract kit for subcellular proteome extraction was from Calbiochem (San Diego, CA)

Animals

9-10 week-old male C57BL/6N mice (Harlan, Indianapolis, IN), or pregnant female C57BL/6N mice (Harlan) obtained at 16-17 d of gestation, were individually housed in a satellite facility provided with a separate ventilation system (15 air exchanges/hr), a 12/12 dark/light cycle (7 a.m.–7 p.m.), 40%–50% humidity, and 20°C ± 2°C temperature adjacent to the behavioral room. All studies were approved by the Animal Care and Use Committee of Northwestern University in compliance with National Institutes of Health standards. E18-19 mice were used to prepare hippocampal cultures. 9-10 week-old male C57BL/6N mice were used for behavioral testing.

Cannulation and injections

Double cannulae were placed into the dorsal hippocampus (1.5 mm posterior, ±1 mm lateral, 2 mm ventral to bregma) as described earlier (Radulovic et al., 1999). Injections were delivered bilaterally (0.25 µl/side) over a 30 s period at the indicated doses 15 min before training. The cannula position was determined for each mouse by methylene blue injection after the end of experiments, and only data obtained from mice with correctly inserted cannula were analyzed. The antagonists were dissolved in artificial cerebrospinal fluid.

Fear Conditioning

Contextual and tone-dependent fear conditioning was performed in an automated system and consisted of a single exposure to context (3 min) followed by a 30 sec tone (10 kHz, 75 dB SPL) and a footshock (2 sec, 0.7 mA, constant current) as described previously (Radulovic et al., 1998). Context-dependent freezing was measured 24 hrs later every 10th sec over 180 sec by two observers unaware of the experimental conditions and expressed as percentage of total number of observations. Freezing to the tone was scored every 5th sec in a novel context during a 30 sec exposure. Trace fear conditioning was performed as described above except that a 15-sec interval was interposed between tone and shock. All memory tests were performed 24 hrs later. Trace conditioning was additionally assessed during training consisting of 5 context-tone-trace-shock pairings [context (C) 30 sec, tone (T) 30 sec, trace (Tr) 15 sec, and footshock 2 sec]. Freezing during each phase was scored every 5th sec.

Immunohistochemistry

Brains were perfused 1 hr after testing. Mice were anesthetized with an intraperitoneal injection of 240 mg/kg of Avertin and transcardially perfused with ice-cold 4% paraformaldehyde in phosphate buffer (pH 7.4, 150 ml/mouse). Brains were post-fixed for 48 hours in the same fixative and then immersed for 24 hrs each in 10%, 20%, and 30% sucrose in phosphate buffer. After the tissue was frozen by liquid nitrogen, 50 µm-thick coronal sections were used for performing free-floating immunohistochemistry with primary antibodies to c-Fos (1:2000), pERK (1:2000), pMSK1 (1:500), Biotinylated secondary antibody and ABC peroxidase complex were used for signal amplification and DAB,

fluorescein isothiocyanate (FITC) or rhodamine as visualizing substrates. Digital images were captured with a cooled color CCD camera and SPOT software for Macintosh. Image J was used for image processing. Quantification of c-Fos positive cells (c-Fos+) was performed as described previously (Fischer et al., 2007) and the number expressed per 0.1mm² CA1 area. For double labelling, the separate FITC and rhodamine captures were digitally combined to produce composite images. Equal cut-off thresholds were applied to all captures to remove background autofluorescence (Tronson et al., 2009).

Hippocampal cultures and treatments

The hippocampus from E18-19 mouse was isolated and dissociated with trypsin as described before (Gao et al., 2006). Cells were plated on coverslips coated with poly-D-lysine in 24-well culture plates at a density of 60 000-80 000 cells/well or 6-well culture plates coated with poly-D-lysine at a density of 300 000-350 000 cells/well and grown in NeuroBasal medium containing 2 mM GlutaMax, 0.5% gentamicin and 2% B27. One-half of the medium was replaced with identical medium every 4 d. Under these conditions, more than 85% cells were viable, more than 90% cells were neurons, and cultures could be maintained for 3 weeks. Neurons were cultured for 12-14 d *in vitro* (DIV) before stimulation and fixation.

One hour before stimulation, the extracellular (perfusion or bathing) solution [in mM: 140 NaCl, 1.3 CaCl₂, 5 KCl, 25 HEPES (pH 7.4), 33 glucose, 0.001 TTX, 0.001 strychnine] with 40 μM CNQX and 5 μM nimodipine was changed. During the stimulation, the TTX, CNQX, and nimodipine were removed from the bath solution. To activate the synaptic NMDAR, neurons were treated with 20 μM bicuculline and 100 μM glycine for 3 min; To activate the extrasynaptic NMDAR, neurons were first treated with 10 μM MK-801, 20 μM bicuculline and 100 μM glycine for 3min and then were stimulated with 20 μM NMDA and 20 μM glycine for another 3 min; To activate the total population of NMDAR (both synaptic and extrasynaptic NMDAR), neurons were treated with 20 μM NMDA and 20 μM glycine for 3 min. After stimulation, the cultures were rinsed and recovered in bath solution with TTX for about 20-30 min at room temperature (RT).

Electrophysiology

Whole-cell patch-clamp recordings were performed on DIV12-14 mouse primary hippocampal neurons. The extracellular solution was the same as above and lacked Mg²⁺. The intracellular solution contained (in mM) 95 CsF, 25 CsCl, 10 HEPES (pH 7.3), 10 EGTA, 2 NaCl, 2 Mg-ATP, 10 QX-314, 5 TEA-Cl, and 5 4-AP. Patch electrodes were pulled from thick-walled borosilicate glass and fire polished to a resistance of 3–4 mΩ. Series resistance was maintained between 5-15 MΩ. Recordings were carried out using a Multiclamp 700A amplifier at a holding potential of −70 mV. To measure channel block of synaptic NMDAR, bicuculline (20 μM) and glycine (20 μM) were added to the bath for 3mins in the absence or presence of MK-801 (10 μM). Whole-cell currents also were evoked by bath application of NMDA (20 μM) before or after application of MK-801, in the presence of bicuculline and glycine, in order to assess the contribution of synaptic NMDAR to the total receptor-mediated whole-cell current. Individual EPSCs arising from both AMPA and NMDA receptors were analyzed for alterations in decay kinetics using Clampfit 10 software and Mini-Anal.

Immunocytochemistry and quantitative immunofluorescence

After stimulation, the neurons were fixed with 4% formaldehyde in PBS containing 4% sucrose for 15 min at RT. Cells were permeabilized and blocked simultaneously in PBS containing 0.1% Triton X-100, and 5% goat serum for 1 hr at RT. Primary antibodies in blocking solution were added at 4°C overnight: rabbit antiphospho-p44/42 ERK (pERK1/2)

antibody (1:500) or mouse pERK1/2 antibody (1:500), rabbit antiphospho (Thr581)-MSK1 (pMSK1) antibody (1:500). After rinsing with PBS, Rhodamin-conjugated (1:500) or FITC-conjugated (1:300) secondary antibodies were added in blocking solution for 1 hr at RT. After four rinses, coverslips were mounted using antifading reagent. Images were acquired and analyzed as described previously (Gao and Wolf, 2007) with an imaging system consisting of a Nikon inverted microscope, CoolSNAP-EZ digital camera and MetaMorph software. All images were taken using identical acquisition parameters. For each experimental group, we selected approximately six cells each from at least four different wells. Processes located about one soma diameter from the soma were chosen for analysis under phase-contrast imaging, to avoid the possibility of experimenter bias based on the intensity of fluorescence staining. For fluorescence intensity measurements, changes in pERK1/2 were measured along 40 μ m of dendrite and average gray value (average immunofluorescence intensity) was determined for each condition.

Protein Extraction and Immunoblot

Cultures were harvested by scraping in ice-cold buffer containing protease and phosphatase inhibitors [in mM: 50 Tris-HCl (pH 7.4), 154 NaCl, 1 EDTA, 1 phenylmethyl sulfonyl fluoride, 1 sodium orthovanadate, 1X protease inhibitor cocktail set I and 1% Nonidet P-40 (v/v)]. Then cells were centrifuged at 10 000 g for 5 min. The supernatant was aliquoted and stored at -80°C . Protein concentration was determined by the Bio-Rad assay. Cytoplasmic (F1), membrane (F2), nuclear (F3), and cytoskeletal (F4) fractions were prepared by using the ProteoExtract kit for subcellular proteome extraction according to the instructions. After determining the protein concentration, the lysates (5–20 μ g/well) were subjected to SDS polyacrylamide gel electrophoresis and subsequently blotted to PVDF membranes and developed as described previously (Sananbenesi et al., 2002). Western blots were replicated with 3–6 samples, each obtained from a different culture (each culture prepared from 6–9 E18–E19 pups).

Statistical analysis

Values are presented as mean $\pm$ SEM. Independent group *t*-tests were used for comparisons between two experimental groups. For multiple groups, one or two-way ANOVA was used followed by a Dunnett's test for *post hoc* analyses. Data were statistically significant if $P < 0.05$ or lower.

RESULTS

Different roles of NR2A and NR2B subtype receptor in hippocampus-dependent contextual and trace fear conditioning

To determine the roles of NR2A and NR2B subunits in memory processes, we infused a preferential antagonist of NR2A-containing NMDAR (NVP-AAM077) or a specific antagonist of NR2B-containing NMDAR (Ro25-6981) intrahippocampally before training in the contextual, delay tone-dependent, or trace fear conditioning paradigms. Two doses of NVP-AAM077 (1 or 2 μ g/ μ l, 0.25 μ l/side) or Ro25-6981 (2 or 10 μ g/ μ l, 0.25 μ l/side) were injected into the dorsal hippocampus 15 min before training. Long-term memory tests were performed 24 hrs later (Fig. 1). The preferential NR2A antagonist NVP-AAM077 impaired contextual fear conditioning, as indicated by a significant reduction of freezing ($F_{(4,40)} = 9.34$, $P < 0.05$), whereas the specific NR2B antagonist Ro25-6981 was ineffective (Fig. 1A; 2 μ g/ μ l: $P = 0.075$, 10 μ g/ μ l: $P = 0.209$ vs vehicle). Neither antagonist affected delay tone-dependent fear conditioning (Fig. 1B; Ro25-6981: 2 μ g/ μ l: $P = 0.067$, 10 μ g/ μ l: $P = 0.135$ vs vehicle; NVP-AAM077: 1 μ g/ μ l: $P = 0.403$, 2 μ g/ μ l: $P = 0.627$ vs vehicle), whereas both impaired trace fear conditioning (Fig. 1C; $F_{(2,15)} = 7.14$, $P < 0.05$). Consistent with observations with contextual fear conditioning (Fig. 1A), the NR2A antagonist NVP-

AAM077 impaired contextual fear induced by the trace conditioning task (Fig. 1C; $F_{(2,15)} = 11.35$, $P < 0.05$).

To further elucidate the role of NR2A and NR2B subunits in different phases of memory, we examined the effects of subunit acting antagonists on acquisition of trace-dependent fear. The number of trials was increased from one to five because one trial is insufficient to trigger within-session freezing. Half of the mice were perfused 1 hr after training to determine c-Fos production and the other half were used to determine long-term memory 24 hrs later. Both antagonists impaired acquisition of trace fear conditioning (Fig. 2A; two way ANOVA, Group x Test interaction: $F_{(26,238)} = 1.992$; $P < 0.01$). Both antagonists impaired long-term contextual and trace fear memory consistent with the results presented in Fig. 1B,C (data not shown). Analyses of c-Fos levels revealed that only the NR2A antagonist NVP-AAM077 decreased c-Fos ($P < 0.05$) expression in CA1 area (Fig. 2B).

These results indicated that hippocampal NR2A- and NR2B-containing receptors play distinctive roles in memory processes, with a general involvement of NR2A in hippocampus-dependent associative learning and selective role of NR2B in the encoding of temporally spaced events.

Activation of synaptic and extrasynaptic NMDAR

To elucidate the molecular mechanisms activated by individual NMDAR subunits, we established a protocol for chemically-induced activation of synaptic, extrasynaptic, and total NMDAR in hippocampal cultures. We verified that MK-801 treatment occluded synaptic NMDA receptors using whole cell patch-clamp recording from cultured hippocampal neurons. Application of bicuculline, a blocker of GABAA receptors (Hardingham et al., 2001) and glycine, the NMDAR co-agonist (20 μ M each for 3 min) resulted in robust enhancement of excitatory synaptic input. Neurons were initially bathed in CNQX, nimodipine and tetrodotoxin to establish a baseline in which activation of synaptic NMDAR was prevented without blocking extrasynaptic NMDAR (Ivanov et al., 2006). In concordance, we observed that MK801 application significantly reduced the EPSC charge transfer ($52.6 \pm 8.6\%$, $P < 0.05$) induced by bicuculline and glycine treatment when compared to control (Fig. 3A; $110 \pm 9.8\%$). Whole-cell currents evoked by bath application of 20 μ M NMDA and glycine also were significantly reduced by MK-801 treatment during elevated excitatory synaptic input. Thus, NMDA current amplitudes were reduced by $67.1 \pm 6.6\%$ relative to control currents elicited before application of MK-801 (Fig. 3B). We conclude from these experiments that synaptic NMDAR comprise a substantial proportion of the total NMDAR population in our cultured neurons, and that the synaptic receptors are effectively occluded by MK-801 in the presence of bicuculline and glycine. These observations are consistent with previous studies that used similar stimulation protocols (Lu et al., 2001; Ivanov et al., 2006). We therefore are able to selectively activate synaptic or extrasynaptic NMDAR.

Role of NMDAR populations in ERK1/2 signaling

Brief (3min) application of glycine (100 μ M, Lu et al., 2001) and bicuculline (20 μ M) drove an increase in glutamatergic activity of the neuronal network and significantly increased ($P < 0.05$) pERK1/2 (pERK1/2; Fig. 4A). Similarly, activation of all NMDAR by bath application of NMDA (20 μ M) and glycine (20 μ M) resulted in a significant increase of pERK1/2 ($P < 0.05$). In contrast, activation of extrasynaptic NMDAR (following occlusion of synaptic receptors with MK-801) did not exert a significant effect on ERK 1/2 phosphorylation ($P > 0.05$). The lower panel in Fig. 4A showed the ratio of pERK1/2 and total ERK1/2 for each condition.

Immunocytochemical analyses further demonstrated that synaptic NMDAR activation not only increased intensity of pERK in the processes of cultured hippocampal neurons, but also maximally increased the intensity of pERK1/2 signal in the neuropil and perinuclear space, whereas activation of total NMDAR preferentially enhanced pERK1/2 in the processes of cultured hippocampal neurons (Fig. 4B). The lower panel in Fig. 4B showed the intensity of pERK1/2 in the processes for each condition.

NMDAR populations differentially affect the subcellular activation of ERK1/2

We next examined the role of NMDAR in the activation of ERK1/2 in distinct subcellular compartments. Fractions of cytosol (F1), membrane (F2), nucleus (F3) and cytoskeleton (F4) were analyzed by immunoblot (Fig. S1A,B). Anti-Na/K-ATPase, LDH, β -catenin and CREB antibodies were used to test the purity of membrane, cytosolic, cytoskeletal and nuclear fractions, respectively (Fig. S1B). As expected, β -catenin was abundant in cytoskeleton and was also found in the membrane and nucleus (Kim et al., 2008). All other markers showed exclusive enrichment in the membrane (Na/K-ATPase), cytosol (LDH) and nucleus (CREB). Activation of synaptic NMDAR caused a ~2-3-fold increase of ERK1/2 phosphorylation in the cytosol, and moderate (~1.5-fold) but significant ($P < 0.05$) increase in the cytoskeleton and nucleus. Activation of total NMDAR primarily triggered membrane (~3-fold) and cytosolic (~2.5 fold) phosphorylation of ERK1/2 ($P < 0.05$; Fig. 5). Extrasynaptic NMDAR did not increase pERK1/2 levels in any of the examined subcellular compartments ($P > 0.05$).

NR2A-containing subunits mediate nuclear signaling induced by synaptic NMDAR

To delineate the roles of NR2A and NR2B subunits in ERK1/2 signaling triggered by the synaptic and extrasynaptic NMDAR populations, we tested nuclear propagation of ERK1/2 signaling in the presence of a specific antagonist of NR2B-containing NMDAR, Ro25-6981 (Ro, 1 μ M) and a preferential antagonist of NR2A-containing NMDAR, NVP-AAM077 (NVP, 0.4 μ M) (Fig. 6). NVP-AAM077 completely blocked ERK1/2 phosphorylation induced by synaptic NMDAR activation (Fig. 6A; $P < 0.05$), whereas Ro25-6981 had no effect ($P > 0.05$). Both antagonists prevented ERK1/2 phosphorylation induced by activation of total NMDAR ($P < 0.05$). In addition to pERK1/2, activation of synaptic NMDAR also increased MSK1 phosphorylation and c-Fos levels (Fig. 6B; $P < 0.05$), indicative of nuclear transmission of synaptically generated signals. NVP-AAM077 abolished MSK1 phosphorylation and c-Fos production induced by synaptic NMDAR, while Ro25-6981 had no effect. Double staining of pMSK1 and pERK1/2 further demonstrated that synaptic NMDAR activation increased the intensity of pMSK1, pERK1/2 and their perinuclear colocalization (Fig. 6C; Fig. S2). We analyzed approximately 100 perinuclear pERK1/2 positive (pERK1/2+) and a similar number of nuclear pMSK1 positive (pMSK1+) cells in each group. In the control group (no activation of NMDAR), 23.8% pyramidal neurons were pERK+, 7.9% were pMSK1+, and 50% of pMSK1+ cells were colocalized with pERK+ cells. In the synaptic activation group, the number of pERK+ cells was increased for 52.4%, pMSK1+ cells for 36.9%, and pMSK1+ cells colocalized with pERK+ cells for 81.6%. Similar results were found in CA1 area of hippocampus (Fig. 6D), where pMSK1+ cells, pERK+ cells, and colocalization were increased in fear conditioning mice compared to naïve mice. Thus, synaptic NR2A-containing NMDAR have the potential to trigger the dendrite-to-nucleus ERK1/2/MSK1/c-Fos signaling pathway in cultured hippocampal neurons as well as in the hippocampal CA1 area in vivo.

DISCUSSION

We demonstrated that in the hippocampus, NR2B significantly contribute to the acquisition and consolidation of trace fear conditioning without affecting contextual fear conditioning.

NR2A, on the other hand, were required for both types of memories. These effects were accompanied by a selective involvement of NR2A, but not NR2B, in the induction of c-Fos, suggesting that individual subunits differentially regulate intracellular signaling. Consistent with this possibility, *in vitro* analyses performed with cultured hippocampal neurons revealed that only NR2A triggered nuclear transmission of ERK1/2 signaling, whereas activation of both synaptic and extrasynaptic NMDAR resulted in NR2B-dependent activation and membrane retention of pERK1/2. The identified subunit- and location-specific NMDAR mechanisms regulating ERK signaling may provide a molecular basis for the differential involvement of individual receptor subunits in memory processes.

Roles of NR2A-containing and NR2B-containing NMDAR in hippocampus-dependent associative learning

Similar to the effects of general NMDAR antagonists (Misane et al., 2005; Quinn et al., 2005) or deletion of the NR1 gene (Tsien et al., 1996; Huerta et al., 2000), the preferential antagonist of the NR2A subunit significantly impaired both types of hippocampus-dependent memory investigated in this study. It should be noted that, based on the relatively low dose range of NVP-AAM077 displaying selectivity for NR2A over NR2B, some actions of this antagonist might involve both subunits (Fox et al., 2006). It is therefore uncertain whether trace fear conditioning solely depends on NR2B or both NR2A and NR2B subunits. It is very likely however, that contextual fear conditioning was mediated by NR2A, given that the highly specific NR2B antagonist Ro25-6981 (Fox et al., 2006) was ineffective in this paradigm. Our results agree with observations that NR2B does not significantly contribute to one-trial conditioning (Zhao et al., 2005; Zhang et al., 2008) and spatial learning (von Engelhardt et al., 2008). Notably, activity of these receptors was required for trace fear conditioning, both during acquisition as well as formation of long-term trace fear memory. Based on similar data found with NR1-deficient mice (Huerta et al., 2000), NR1/NR2B complexes seem to be preferentially required for the processing of temporal over contextual memory. However, it remains unclear whether the NR2B antagonist-induced impairments in long-term trace fear memory were due to a failure of acquisition, consolidation, or both. Hippocampal NR2B may also act to maintain working memory (von Engelhardt et al., 2008), a process that plays an important role in trace fear conditioning (Koch et al., 2003). Because post-training hippocampal injections of NMDAR antagonists are typically ineffective at impairing memory (Young et al., 1994), it is not possible to delineate which of these processes was affected.

Intrahippocampal injection of NVP-AAM077 blocked learning-induced c-Fos production, whereas Ro25-6981 did not. This suggests that up-regulation of c-Fos, as shown here and elsewhere (Weitemier and Ryabinin, 2004), is not sufficient for trace fear conditioning. Thus, other molecular mechanisms, such as localized activation of the c-Fos-inducing kinases ERK1/2/MSK1, are most likely regulated by NMDAR subunits. Accordingly, compartmentalized activity is a key constraint of ERK's interactions with upstream activators, downstream targets, and role in memory processes (Roberson et al., 1999; Shalin et al., 2006; Schrick et al., 2007; Tronson et al., 2009).

NMDAR localization and ERK1/2/MSK1/c-Fos signaling

Because the role of NMDAR subunits critically depends on their synaptic location, we conducted subsequent experiments with cultured hippocampal neurons allowing independent examination of the roles of synaptic, extrasynaptic or total NMDAR population in ERK/MSK1/c-Fos signaling. This pathway was selected based on its marked up-regulation in the hippocampus after fear conditioning (Radulovic et al., 1998; Sananbenesi et al., 2002; Sindreu et al., 2007). In general, synaptic NMDAR are thought to activate ERK and CREB, whereas extrasynaptic NMDAR shut off these pathways (Hardingham et al.,

2002; Leveille et al., 2008). Our results support the view that the synaptic pool of increases ERK phosphorylation. However, selective activation of extrasynaptic NMDAR was consistently ineffective in modulation of ERK1/2. Instead, it specifically influenced ERK signaling induced by co-activation of synaptic NMDAR. This modulatory effect did not involve a reduction of ERK phosphorylation, but instead induced membrane retention of pERK1/2. Furthermore, activation of synaptic NMDAR, but not extrasynaptic or total NMDAR, resulted in nuclear propagation of ERK signals as revealed by induction of its nuclear downstream targets pMSK1 and c-Fos (Brami-Cherrier et al., 2007; Chandramohan et al., 2008). Conversely, combined NMDAR stimulation resulted in membrane retention of pERK1/2 without affecting pMSK1 and c-Fos. These findings support recent data showing markedly higher potency of synaptic vs total NMDAR to stimulate gene expression (Zhang et al., 2007).

Contrary to our findings, other studies have shown decreased activation of ERK after total NMDAR stimulation (Ivanov et al., 2006; Leveille et al., 2008). Among a number of procedural differences, we believe that the differential use of TTX during stimulation is one of the main factors causing this discrepancy. Our treatments were fully balanced for the presence (pre- and post-incubation) or absence (all stimulations) of TTX. This approach was based on our preliminary experiments showing TTX-induced variability of basal pERK1/2 that significantly affected further ERK phosphorylation upon NMDAR stimulation (data not shown). Thus, the absence of TTX before stimulation (Mulholland et al., 2008; Leveille et al., 2008) might have elevated basal pERK1/2 levels causing reduced phosphorylation in response to stimulation (Chandler et al., 2001). In addition, the selective presence of TTX only during extrasynaptic and total NMDAR stimulation (Ivanov et al., 2006; Mulholland et al., 2008) could be responsible for the decrease of pERK1/2 levels when compared to synaptic NMDAR stimulation without TTX.

NMDAR subunit composition and ERK1/2/MSK1/c-Fos signaling

NR2A subunits were identified as the main component of synaptic NMDAR mediating ERK1/2 phosphorylation and accompanying nuclear activation of MSK1 and c-Fos. Accordingly, stimulation of NR2A-containing NMDAR is known to activate ERK1/2 (Kim et al., 2005), dendritic protein synthesis (Tran et al., 2007) and BDNF gene expression (Chen et al., 2007). Given that both NR2A and NR2B subunits are present at synaptic sites, the predominant activation of NR2A-containing NMDAR may be based on their higher open channel probability when compared to NR2B-containing NMDAR (Erreger et al., 2007).

Data on NR2B-dependent ERK1/2 phosphorylation are less consistent, and show either an increase (Krapivinsky et al., 2003) or an age-dependent decrease (Kim et al., 2005). Under the stimulation conditions employed in our study, NR2B subunits did not contribute to the synaptic effects of NMDAR, but their antagonism fully reversed the increase of membrane pERK1/2 caused by activation of total NMDAR. Interestingly, the NR2A- and NR2B-subunit antagonists did not show additive effects. Instead, either drug completely blocked ERK1/2 phosphorylation. Given the limited selectivity of NVP-AAM077 for NR2A over NR2B, a main role of NR2B cannot be excluded. Alternatively, stimulation of NR2A- and NR2B- NMDAR generated non-redundant signals converging to activate ERK1/2.

Conclusions

We showed that NR2A and NR2B NMDAR differentially contribute to associative learning and signal transduction. Contrary to earlier neuronal models suggesting that extrasynaptic NMDAR attenuate ERK1/2 phosphorylation induced by synaptic NMDAR, we showed that both receptor populations contribute to the phosphorylation of pERK1/2. Whereas synaptic

NR2A-containing NMDAR triggered nuclear transmission of ERK1/2 signals, revealed by up-regulation of the downstream nuclear targets pMSK1 and c-Fos. Co-activation of NR2A- and NR2B-containing NMDAR resulted in activation and membrane retention of this kinase. Thus, instead of producing opposite actions, NR2A-containing and NR2B-containing NMDAR would be expected to regulate different processes underlying memory. Accordingly, NR2A-containing NMDAR were required for the formation of lasting contextual and trace fear memory and nuclear immediate early gene responses. On the other hand, NR2B-containing NMDAR preferentially mediated trace fear conditioning, suggesting that membrane retention of pERK1/2 by NR2A/NR2B may be important for successful association of temporally separated events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Different roles of NR2A- and NR2B-containing NMDAR in hippocampus-dependent long-term fear memory. Mice were injected with the NR2B specific antagonist Ro25-6981 (2 or 10 µg/µl, 0.25 µl/side) or NR2A preferring antagonist NVP-AAM077 (1 or 2 µg/µl, 0.25 µl/side) through microcanula into the dorsal hippocampus 15 min before training. The memory tests were performed 24 hrs later. (A) NVP-AAM077 (NVP) impaired contextual fear conditioning, whereas Ro25-6981 (Ro) was ineffective. $*P < 0.05$ vs vehicle (Veh), $n = 7-8$. (B) Neither antagonist impaired delay tone-dependent fear conditioning. $P > 0.05$, $n = 7-8$. (C) Both antagonists impaired trace fear conditioning, while NVP-AAM077 additionally impaired contextual fear. $*P < 0.05$ vs vehicle, $n = 6-7$. Data are presented as mean $\pm$ S.E.M.

FIGURE 2.

Roles of NR2A- and NR2B-containing NMDAR in trace fear conditioning and c-Fos production. Mice were injected with the NR2B specific antagonist Ro25-6981 (Ro, 2 $\mu\text{g}/\mu\text{l}$, 0.25 $\mu\text{l}/\text{side}$) or NR2A preferring antagonist NVP-AAM077 (NVP, 1 $\mu\text{g}/\mu\text{l}$, 0.25 $\mu\text{l}/\text{side}$) through microcanula into the dorsal hippocampus 15 min before training. (A) Acquisition of trace fear conditioning was performed for five trials of context (C)-tone (T)-trace (Tr)-footshock (lightning symbol) pairings. Freezing was measured during each phase. Both antagonists impaired trace fear conditioning ($P < 0.01$) compared to vehicle (Veh), $n = 6-8$. (B) Immunohistochemistry results showed that only NVP-AAM077 decreased the number of c-Fos+ cells in CA1, whereas Ro25-6981 had no effect. $*P < 0.05$ vs Vehicle, $n = 4$. Scale bar, 50 μm . Data are presented as mean $\pm$ S.E.M.

FIGURE 3.

Extrasynaptic NMDAR activation after MK801 block of synaptic NMDAR. (A) 10 μ M MK801 blocked synaptic NMDAR. Black trace illustrates the pre-application spontaneous excitatory post-synaptic current (EPSC) trace and the gray trace illustrates post-application EPSC trace after control (top panel) and MK801 (middle panel) treatment conditions. Bottom panel: Quantification of the charge transfer after control ($n = 4$) and after MK801 ($*P < 0.05$, $n = 3$.) treatment illustrates a reduction in the EPSC charge transfer after MK801 treatment. (B) Extrasynaptic NMDAR activation after MK801 block. Black trace illustrates the first whole cell current elicited by application of 20 μ M NMDA in the presence of 20 μ M glycine and gray trace denotes the second whole cell current elicited by a second application after control (top panel) and 10 μ M MK801 (middle panel) treatment conditions. Bottom panel: Quantification of the second elicited peak whole cell current after control ($n = 3$) and after MK801 ($*P < 0.05$, $n = 4$.) treatment illustrates activation of extrasynaptic NMDAR after MK801 block of synaptic NMDAR. Data are presented as mean $\pm$ S.E.M.

FIGURE 4.

Effects of NMDAR activation on ERK1/2 phosphorylation in cultured hippocampal neurons. (A) Synaptic NMDAR (Syn) activation or total NMDAR (Total) activation resulted in robust ERK1/2 phosphorylation. Extrasynaptic NMDAR (E-syn) activation had no effect. $*P < 0.05$ vs corresponding control (Cont), $n = 4$. (B) Immunocytochemical analyses further demonstrated that synaptic NMDAR increased the intensity of pERK1/2 both in the neuronal processes and perinuclear compartment, while activation of total NMDAR preferentially increased the intensity of pERK1/2 in the processes. Quantification of the intensity of pERK1/2 in neuronal processes: $*P < 0.05$ vs control, $n = 28-30$. Scale bar, 20 μm of top panel, 5 μm of bottom panel. Data are presented as mean $\pm$ S.E.M.

FIGURE 5.

Subcellular distribution of pERK1/2 after activation of different NMDAR populations. Activation of synaptic NMDAR (Syn) increased ERK1/2 phosphorylation in cytosol (F1), cytoskeleton (F4) and nuclear fractions (F3), while activation of total NMDAR (Total) mainly induced pERK1/2 in the membrane (F2). Extrasynaptic NMDAR (E-syn) activation had no effect on any fraction. * $P < 0.05$ vs corresponding control (Cont), $n = 3-4$. Data are presented as mean $\pm$ S.E.M.

FIGURE 6.

NR2A-containing NMDAR mediates nuclear transmission of ERK1/2 signals induced by activation of synaptic NMDAR. (A) Ro25-6981 (Ro, 1 μ M), a specific antagonist of NR2B-containing NMDAR, or NVP-AAM077 (NVP, 0.4 μ M), an antagonist with preferential activity on NR2A-containing receptors was added 20 min before stimulation. NVP-AAM077 completely blocked ERK1/2 phosphorylation induced by synaptic NMDAR (Syn) activation, whereas Ro25-6981 had no effect. Either antagonist blocked ERK1/2 phosphorylation induced by total NMDAR (Total) activation. * $P < 0.05$ compared to vehicle group (Veh), $n = 3-6$. (B) Western Blot showed increased intensity of phospho-MSK1 (pMSK1) and c-Fos after synaptic NMDAR activation. NVP-AAM077 completely blocked these effects, while Ro25-6981 was ineffective. Extrasynaptic (E-syn) or total NMDAR activation did not affect pMSK1 and c-Fos levels. * $P < 0.05$ vs corresponding control (Cont), $n = 3-4$. Data are presented as mean $\pm$ S.E.M. (C) After stimulation, the cells were fixed and double stained with pMSK1 (red) and pERK1/2 (green). Synaptic NMDAR activation increased the intensity of nuclear pMSK1, and its perinuclear colocalization with pERK1/2 (yellow). Scale bar, 10 μ m. (D) Immunofluorescence performed on brain sections further demonstrated that in CA1 area of hippocampus pMSK1+ nuclei (red) and pERK signals in neuronal processes, soma, and perinucleus (green) were up-regulated and colocalized (yellow) in fear conditioned when compared to naïve mice. Arrows indicate pMSK1+ cells. Scale bar, 10 μ m.