N-methyl-D-aspartate Receptor Subtype Mediated Bidirectional Control of p38 Mitogen-activated Protein Kinase

N-methyl-D-aspartate receptor (NMDAR) stimulation activates many downstream mechanisms involved in both cell survival and cell death. The manner in which the NMDAR regulates one of these pathways, the p38 mitogen-activated protein kinase (p38) pathway, is currently unknown. In the present study, we have defined a development-, concentration-, and time-dependent phosphorylation and subsequent dephosphorylation of p38. In cultured hippocampal neurons 7–8 days in vitro (DIV7–8), NMDAR stimulation leads to a concentration-dependent increase in p38 phosphorylation (phospho-p38). However, in more mature neurons (>DIV17) application of NMDA produces concentration-dependent effects, such that low concentrations result in sustained increases in phospho-p38 levels, and high concentrations dephosphorylate p38 within 5 min. Conantokin G, an antagonist of NR1/2A/2B and NR1/2B receptors, inhibits p38 phosphorylation, while NR1/2B-specific antagonists prevent the rapid dephosphorylation of p38 without affecting p38 activation. Furthermore, inhibition of calcein prevents the activation of p38, whereas inhibition of phosphoinositide 3-kinase (PI3K) prevents the rapid dephosphorylation of p38. Our results support the presence of subtype-dependent pathways regulating p38 activation and deactivation: one involves NR1/2A/2B receptors activating calcein and resulting in phospho-p38 phosphorylation, and the other utilizes NR1/2B receptors binding to and activating PI3K and leading to the dephosphorylation of p38 in a manner involving both NR1/2A/2B receptor activation and tyrosine phosphorylation of NR2B. The ability of NMDAR subtype-specific mechanisms to regulate p38 has implications for NMDAR-mediated synaptic plasticity, gene regulation, and excitotoxicity.

The N-methyl-D-aspartate receptor (NMDAR), a member of the ionotropic glutamate receptor family, is required for forms of neuronal synaptic plasticity, for cell death mediated by excitotoxicity and for gene regulation (1, 2). Differential responses mediated through NMDAR stimulation can be caused by the level of activation, the localization of the effect, or by the properties of the receptor. NMDARs likely contain two NR1 and two NR2 subunits (3). Most variations in receptor properties reflect differences in receptor subtype composition involving the four NR2 subunits (NR2A-NR2D) (2).

In the hippocampus, NMDARs are composed mainly of NR1 subunits in combination with NR2A and NR2B subunits. The expression and localization of these NR2 subunits are developmentally regulated. Prenatally and early in postnatal development, hippocampal NMDARs are mostly NR1/2B receptors, localized at developing synapses with the synaptic-associated protein SAP-102 (4–6). During maturation, expression of NR2A subunits increases, and NR2A-containing receptors, bound to post-synaptic density (PSD)-95, predominate in the synapse, whereas NR1/2B receptors become mostly extrasynaptic with SAP102 levels decreasing at the synapse (6–10). NMDAR composition changes similarly in primary culture, in which immature cultures contain mostly NR2B subunits, and NR2A expression increases by 14 days in vitro (DIV) (11). In mature hippocampal cultures, synaptic NMDARs are mainly NR1/2A/2B receptors bound to PSD-95, and NR1/2B receptors are largely located extrasynaptically (11). Studies using pharmacological inhibitors show that differences in receptor subtype composition and localization convey distinct properties to NMDAR function in this in vitro system (2).

Several downstream targets of NMDAR stimulation may be differentially regulated by NMDAR subtype composition and localization (1, 2). Some of these pathways are mediated by calmodulin, which activates calcein, calmodulin-activated protein kinase II (CaMKII), and neuronal nitric-oxide synthase (nNOS). In addition, NMDAR stimulation activates mitogen-activated protein kinases (MAPK). These kinases act on a variety of substrates and play crucial roles in gene regulation (through activation of transcription factors such as the cAMP response element-binding protein, CREB), cell death, and synaptic plasticity (12–16).

Whereas most MAPK investigations have focused on the extracellular signal-regulated kinases 1/2 (ERK1/2), the p38 MAPK (p38) pathway is relatively undefined. p38 is a stress-activated kinase, and its inhibition can attenuate cell death because of neuronal ischemia and glutamate-induced toxicity (14, 17–22). However, p38 also controls the physiological processes of gene regulation through activating transcription factor 2 (ATF-2) and of synaptic plasticity through initiation of long term depression (LTD) and inhibition of long term potentiation (LTP) of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor currents (16, 23–27). Therefore, p38 is important in both excitotoxic mechanisms and physiological synaptic regulation.

Whereas the importance of NMDAR-mediated p38 activation
has been established, the mechanisms by which NMDAR activation controls p38 phosphorylation are undefined. Characterizing these mechanisms could provide important information about both normal physiology and dysregulation during disease states. In the present study, we have identified subtype-dependent control of p38 phosphorylation and dephosphorylation by NR1/2A/2B receptors and NR1/2B receptors, respectively, and defined the pharmacological properties and messenger systems involved in each pathway.

**EXPERIMENTAL PROCEDURES**

**Hippocampal Primary Culture—** Cultures were prepared from the hippocampus of 18–19-day-old Sprague-Dawley rat embryos (E18-E19) as previously described (28). Briefly, the hippocampi from rat embryos were trypan blue-stained (0.027%, 37 °C, 7% CO2 for 20 min) and then washed with Hank's-buffered saline solution (HBSS). The tissue was triturated in warm media (10% defined, heat-inactivated fetal bovine serum, 10% Hams F12 medium, and 80% Dulbecco's modified Eagle's medium with penicillin/streptomycin), and cells were plated at a density of 6 × 10^5 viable cells per 35-mm culture dish or similar relative density onto 6-well or 12-well poly-L-lysine-coated plates. Neurons were maintained at 37 °C, 5% CO2 with neurobasal media containing B27. Cultures, which include a heterogeneous mixture of pyramidal and nonpyramidal hippocampal neurons, were maintained for at least 17 days in vitro (DIV7–17) prior to experimentation, unless otherwise indicated, and are referred to as mature cultures. Immature hippocampal cultures were maintained for 7–8 days in vitro (DIV7–8).

**Incubation in NMDAR Agonists and Selective Antagonists—** Cultures were preincubated with inhibitors for 1 h prior to treatment. All inhibitors were acquired from Sigma-Aldrich, unless otherwise indicated, and included MK801 (1 μM), Ro25-6981 (10 μM), zine (500 nM), conantokin G (50 μM; generous gift from Drs. Francis Castelli and Mary Prorok, Notre Dame), prodinol (1 μM), and prodinol (1 μM; generous gift from Dr. Ted Dawson, Johns Hopkins University), KN62 (10 μM), 7-nitroindazole (7Nz; 100 μM), LY294002 (100 μM; Toceis Cookson, Balwin, MO), ifenprodil (1 μM), dantrolene (10 μM), and wortmannin (10 nM and 100 nM), and SB203580 (1 μM). We also utilized a combination of voltage-dependent calcium channel (VDCC) inhibitors, nifedipine (2 μM), ω-agatoxin IVA (30 nM), and ω-agatoxin IVa (100 nM). FK506, KN62, 7Nz, IF, and LY294002 were dissolved in Me2SO, and experiments involving these inhibitors were compared against a Me2SO control of 1:1000 dilution. Although dantrolene, wortmannin, nifedipine, ω-agatoxin IVA, and ω-agatoxin IVa were also dissolved in Me2SO, the maximal concentration of these inhibitors for these experiments was 15,000, which did not affect the phosphorylation levels of p38. NMDA treatment included 10 μM glycine and NMDA (10, 30, 50, or 100 μM) in neurobasal media with B27 with or without applicable inhibitors. Cultures were maintained at 37 °C and 5% CO2 during preincubation and treatment. After treatment, media were removed from cultures, and cells were immediately snap-frozen in 1.5 × Laemml SDS sample buffer.

**Western Blot Analysis—** Western blot analyses were performed and analyzed by methods previously described (28). Antibodies specific to phospho-p38 (1:1000; Cell Signaling Technology, Beverly, MA) were utilized to detect activation of p38 in these samples. Antibodies specific to phospho-p38 (1:1000; Cell Signaling Technology, Beverly, MA) were utilized to detect activation of p38 in these samples. Antibodies specific to phospho-p38 (1:1000; Cell Signaling Technology, Beverly, MA) were utilized to detect activation of p38 in these samples.

**Fig. 1. NMDAR-dependent p38 phosphorylation and dephosphorylation in mature (÷DIV17) hippocampal cultures.** Lysates from hippocampal neurons were analyzed by Western blotting with antibodies specific to phospho-p38 and total p38. A, representative immunoblot for phospho-p38 and total p38 in NMDA-mediated p38 activation as a function of NMDA applied. Cultures were exposed to NMDA and 10 μM glycine treatment for 5 min with or without 1 μM MK-801 pretreatment. MK-801 significantly inhibited phospho-p38 production in the 10 and 30 μM NMDA conditions, but not significantly with 50 or 100 μM NMDA application (p < 0.0001 by ANOVA; *, p < 0.001 for 10 and 30 μM NMDA conditions by post-hoc Student's t test with Bonferroni correction), p = 0.03 for 50 μM NMDA condition (uncorrected post-hoc Student's t test), and p > 0.8 for 100 μM NMDA condition by post-hoc Student's t test with Bonferroni correction). All values indicate means (from four different experiments), normalized to the total p38 and expressed as a fraction of phospho-p38 immunoreactivity in the 5-min 10 μM NMDA treatment condition. Error bars indicate ± S.E. B, time course of p38 activation, comparing 10 μM NMDA and 100 μM NMDA treatment. Representative immunoblot and quantified data compiled from four different experiments. Differences between the two treatments were first noted at 5 min (*, p < 0.0001 for two-tailed Student's t test; #, p = 0.0016 at 15 min and p = 0.0931 at 30 min by Mann-Whitney test). C, representative immunoblot for phospho-p38 shown, indicating that TTX, dantrolene, and voltage-dependent calcium channel inhibition did not significantly alter p38 activation or deactivation with 5-min treatments. Comparisons completed by two-tailed Student's t tests showed no significant difference between each inhibitor condition and no pretreatment conditions (each inhibitor condition compiled from 3–4 experiments). D, using Fura-2AM, calcium imaging was performed comparing 10 μM NMDA (A) and 100 μM NMDA (B) treatments. Time points from 0 to 5 min were assessed, and indicated values represent mean change in calcium (nM) ± S.E. Data compiled from nine experiments using 2–3 plates per condition in each experiment and 8–10 cells per plate.


**FIG. 2. p38 phosphorylation with immature (DIV7–8) hippocampal cultures.** A, representative immunoblot of phospho-p38 and total p38 and quantification of NMDAR-mediated p38 activation with increasing concentrations of NMDA. Cultures were exposed to NMDA and 10 μM glycine treatment for 5 min with or without 1 μM MK-801 pretreatment. MK-801 significantly inhibited phospho-p38 production in all conditions (*p < 0.0001 by ANOVA, #, p = 0.002 by Mann-Whitney test for the 10 μM NMDA condition, and *, p < 0.001 for 30, 50, and 100 μM NMDA conditions, by post-hoc comparisons with Bonferroni corrections; compiled from four experiments). B, comparison of response to increasing NMDA concentrations between DIV7–8 cultures and cultures maintained for at least DIV17, using data indicated in Fig. 1A and Fig. 2A. C, quantification of phosphorylated ATF-2 using specific antibody to phospho-ATF-2 (represents three experiments for each age of culture). Comparison of phospho-ATF-2 levels for immature and mature cultures. The effects of NMDA concentration on phospho-p38 levels are also noted in control of its downstream transcription factor, indicating that phosphorylation of p38 and activity of p38 both depend on concentration of NMDA and developmental course. D, 1 μM SB203580, a specific inhibitor of p38, was used to show that phospho-p38 is downstream of p38 activation. In both immature and mature hippocampal cultures SB203580 significantly decreased NMDAR-mediated phospho-ATF-2 activation with 10 μM NMDA treatment. *, p < 0.0001 by two-tailed Student's t test compiled from 4–5 experiments.

**ATF-2 is downstream of p38 activation.** In both immature and mature hippocampal cultures SB203580 significantly decreased NMDAR-mediated phospho-ATF-2 activation with 10 μM NMDA treatment. *, p < 0.0001 by two-tailed Student's t test compiled from 4–5 experiments.
p38 in an NMDAR-dependent fashion (17, 31). To identify the optimal concentration of NMDA for phosphorylating p38, we treated hippocampal cultures maintained for at least DIV17 (mature cultures) for 5 min with varying concentrations of NMDA and 10 μM glycine and detected the active state of p38 using an antibody specific to the double phosphorylated form of p38 (Thr180/Tyr182), 5-min treatment with 10 and 30 μM NMDA increased p38 phosphorylation ~10-fold over baseline, but higher concentrations of NMDA reduced p38 phosphorylation, such that 100 μM NMDA treatment did not increase phospho-p38 levels above baseline (Fig. 1A). No differences were observed in total p38 between different conditions, and pretreatment with MK-801, a specific NMDAR antagonist (32), prevented p38 activation, indicating that phosphorylation of p38 with lower concentrations of NMDA required NMDAR activation.

To understand this concentration-dependent phenomenon and to determine if 100 μM NMDA can activate p38 at other time points, we examined the time course of p38 activation, comparing low and high concentrations of NMDA. 100 μM NMDA treatment resulted in peak p38 activation at 1 min that returned to baseline levels by 5 min, whereas 10 μM NMDA treatment resulted in relatively sustained activation with a peak at ~2.5 min (Fig. 1B). As no changes in total p38 and no appearance of breakdown products of either phospho-p38 or total p38 were noted, the decrease with 100 μM NMDA likely reflected a rapid dephosphorylation of p38. In contrast, phospho-p38 levels treated with 10 μM NMDA decreased only slightly over 30 min and did not return to baseline. To rule out endogenous glutamate release or downstream effects of neurotransmission resulting from NMDAR activation and neuronal depolarization as the cause of p38 activation/deactivation, we inhibited neurotransmission with 1 μM tetrodotoxin (TTX). TTX did not significantly alter p38 phosphorylation with either 10 or 100 μM NMDA treatment (Fig. 1C). Furthermore, neither inhibition of calcium release from intracellular calcium stores with dantrolene nor inhibition of voltage-dependent calcium channels (VDCC), using a combination of nifedipine (2 μM), ω-agatoxin IVA (30 nM), and ω-conotoxin GVIA (100 nM), altered p38 phosphorylation with either 10 or 100 μM NMDA (Fig. 1C), supporting the possibility that signals originating specifically from the NMDAR-controlled p38 phosphorylation.

One possible explanation for the acute decrease in phospho-p38 levels with 100 μM NMDA in mature cultures could be a decline in NMDAR-mediated intracellular calcium transients in the presence of 100 μM NMDA. To rule out this possibility, we measured the temporal course of the NMDAR-mediated intracellular calcium response with low and high concentrations of NMDA. 100 μM NMDA treatment resulted in peak intracellular calcium changes by 30 s that were sustained through 5 min of treatment (Fig. 1D). 10 μM NMDA treatment increased intracellular calcium levels rapidly over 30 s to only one-third of the peak observed with 100 μM NMDA, followed by a decrease over 5 min of treatment. Therefore, the decreased activation of p38 after 5 min of 100 μM NMDA treatment was not because of a loss of the NMDAR-mediated intracellular calcium response.

Because NMDAR composition and localization change over the first 2 weeks in culture (11, 33), we explored p38 activation in less mature cultures. Previous reports suggest that 100 μM NMDA treatment results in a more sustained increase in phospho-p38 in cultures matured with the subtype-specific inhibitors 6 μM ConG (†) (compiled from two experiments containing 6 plates, 43 cells) and 10 μM Ro25-6981 (○) (compiled from six experiments containing 13 plates, 111 cells) with 100 μM NMDA treatment when compared with 100 μM NMDA alone (●).
phospho-p38 levels in less mature hippocampal cultures (31). In DIV7–8 hippocampal cultures (immature cultures), NMDAR activation caused a concentration-dependent increase in phospho-p38 production that was completely blocked by 1 μM MK-801 pretreatment (Fig. 2A). Comparing the concentration-dependent data for both ages of cultures showed that the patterns of p38 phosphorylation between the two ages of cultures were distinct, with the decrease with higher concentrations of NMDA observed only in mature cultures (Fig. 2B). To prove that the p38 pathway, and not just p38, was activated in this concentration- and development-dependent manner, we used an antibody specific to phosphorylated ATF-2, which is downstream of p38 in this signal transduction pathway in neurons (34, 35). Phosphorylation of ATF-2 increased in a concentration-dependent fashion in immature cultures, and decreased with higher concentrations of NMDA in mature cultures, matching the results observed with phospho-p38 (Fig. 2C). To verify that ATF-2 is downstream of NMDAR-activated p38, we utilized the p38 inhibitor SB203580. p38 inhibition significantly reduced phospho-ATF-2 immunoreactivity in the presence of 10 μM NMDA in both immature and mature cultures (Fig. 2D). Therefore, NMDAR-mediated p38 activation led to phosphorylation of ATF-2 in this system, and this concentration-dependent, developmental phenomenon affected the entire p38 pathway.

**NMDAR Subtype Dependence of p38 Phosphorylation**—Developmental changes in hippocampal cultures include synaptic NMDAR composition changes from NR1/2B to NR1/2A/2B, and an increase in the number of extrasynaptic NR1/2B receptors (11). We utilized subtype-specific inhibitors to define the pharmacological requirements for both p38 phosphorylation and dephosphorylation. Mature cultures were treated with either 10 μM NMDA or 100 μM NMDA for 1 or 5 min to identify the effects of NMDAR subtypes on both p38 activation and deactivation. Pretreatment with 6 μM ConG, a competitive inhibitor of the glutamate binding site in NR1/2B and NR1/2A/2B receptors (36, 37), inhibited NMDAR-induced p38 phosphorylation (Fig. 3A). Zinc (500 nM), which selectively inhibits NR1/2A receptors, has minimal effects on NR1/2A/2B receptors, and has no effect on NR1/2B receptors (38–40), did not significantly alter p38 activation or deactivation (Fig. 3B). In contrast, 1 μM ifenprodil, a noncompetitive inhibitor with higher efficacy at NR1/2B than NR1/NR2A/NR2B receptors (11, 40, 41), and 10 μM Ro25-6981, a NR1/2B-specific inhibitor that is mechanistically and structurally related but more potent and selective than ifenprodil (30, 42), did not alter the activation of p38, but prevented the return of phospho-p38 levels to baseline after 5 min of 100 μM NMDA treatment (Fig. 3C). Therefore, p38 activation in mature hippocampal cultures was mediated by NR1/2A/2B receptors with the time-dependent decrease at high NMDA concentrations mediated by NR1/2B receptors. Because inhibition of NR1/2B receptors did not alter p38 phosphorylation with 10 μM NMDA and only prevented the deactivation of p38 with 100 μM NMDA treatment in mature cultures, NR1/2B receptors did not appear to contribute to the activation of p38. In immature cultures, NMDAR-dependent activation of p38 was almost entirely inhibited by Ro25-6981, ConG, and MK-801, suggesting that p38 activation occurs mostly through NR1/2B receptors in immature neuronal cultures (Fig. 3D). Because each subtype-selective inhibitor produced distinct effects on phospho-p38 levels, and particularly since NR1/2B
receptor inhibition showed no effect with low concentrations of NMDA in mature cultures, we examined the effects of each subtype-specific inhibitor on the overall intracellular calcium changes produced by NMDAR stimulation in mature hippocampal cultures. Calcium transients with 10 μM NMDA and 10 μM glycine treatment were only moderately inhibited by pretreatment with 10 μM Ro25-6981, with a significant decrease only at 30 s after agonist application (Fig. 3E). In fact, Ro25-6981 prevented the decline of calcium transients noted at later time points with 10 μM NMDA treatment. Therefore, with low concentrations of NMDA, NR1/2B receptors did not contribute to the overall increase in cellular calcium, but did contribute to the recovery of intracellular calcium to baseline levels. Upon application of 100 μM NMDA and 10 μM glycine, ConG prevented NMDAR-mediated calcium transients almost entirely, and Ro25-6981 decreased the intracellular calcium response significantly but to a much lesser degree (Fig. 3F).

Therefore, our mature hippocampal cultures contained mostly NR1/2A/2B receptors and a large number of NR1/2B receptors, with a relative lack of NR1/2A receptors, matching the subtype composition noted by others in cultured hippocampal neurons, mature hippocampus, and most of the forebrain (11, 43–46).

Because Ro25-6981 significantly decreased intracellular calcium transients in mature hippocampal cultures, the lack of p38 dephosphorylation during NR1/2B receptor inhibition could have resulted from either an overall decrease in intracellular calcium response or from specific NR1/2B receptor inhibition. To distinguish between global changes in intracellular calcium transients and specific inhibition of NR1/2B receptors, we increased the extracellular calcium concentration during NMDA treatments to augment the intracellular calcium response during our NMDAR activation paradigm (47). If nonspecifically attenuating the intracellular calcium response prevented p38 dephosphorylation, then increasing intracellular calcium transients using additional extracellular calcium chloride (CaCl₂) would increase the dephosphorylation of p38. Addition of 5 mM CaCl₂ (above the 1.36 mM CaCl₂ contained in the media) to 10 μM NMDA treatment increased the intracellular calcium response significantly above that of 10 μM NMDA alone (Fig. 4A), but it did not decrease the activation of p38 after 5 min of treatment (Fig. 4B).

To directly test the effect of NR1/2B receptor inhibition without the observed inhibition of the intracellular calcium response, we treated cultures with 100 μM NMDA and Ro25-6981 in the presence or absence of 5 mM CaCl₂. Addition of 5 mM CaCl₂ to 100 μM NMDA and Ro25-6981 increased intracellular calcium transients to approximately those of 100 μM NMDA alone (Fig. 4C) with differences between the two treatments only within the first 30 s of agonist application. However, a 5-min 100 μM NMDA, Ro25-6981, and 5 mM CaCl₂ treatment significantly increased phospho-p38 levels above that of 100 μM NMDA treatment alone, even though both treatments showed similar calcium responses (Fig. 4D). Furthermore, there was no significant difference in phospho-p38 levels with a 5-min 100 μM NMDA and Ro25-6981 treatment with and without the addition of 5 mM CaCl₂. Therefore, our data suggest NR1/2B receptor activation, rather than overall NMDAR-induced calcium elevations, as the primary mediator of acute p38 dephosphorylation.

Subcellular Localization of NMDAR-mediated p38 Activation—Because NR1/2A/2B receptors are generally located synaptically, and NR1/2B receptors are found extrasynaptically and on the soma (9, 11, 48–52), the pharmacological data predict that p38 phosphorylation and dephosphorylation might occur at different cellular locations. At baseline, immunofluorescence with antibodies specific to phospho-p38 (red) was minimal (Fig. 5A, left). NR2B-containing receptors (NR1/2A/2B and NR1/2B receptors) (red) were localized in puncti in the dendrites and on the surface of the cell body (Fig. 5A, right), indicating that such receptors may be found both within the dendrites and on the neuronal soma. With a 1-min 10 μM NMDA treatment, phospho-p38 was visualized mostly in dendrites when compared with no treatment (Fig. 5, A and B), consistent with the localization of NR2B-containing receptors. An antibody specific to MAP2 (green) was utilized to visualize the localization of phospho-p38 in the

![Fig. 5](image-url)
dendrites, as seen by the merged picture of phospho-p38 and MAP2 immunoreactivity after a 1-min 10 μM NMDA treatment (Fig. 5C). By 5 min, phospho-p38 was localized to the neuronal soma and nucleus (blue-DAPI). 100 μM NMDA treatment, however, produced minimal increases in phospho-p38 in dendrites at 1 min with most phospho-p38 localized to the neuronal soma and nucleus. Consistent with our Western blot results, a 5-min treatment with 100 μM NMDA showed phospho-p38 activation levels similar to baseline. These data indicated that phospho-p38 is present in dendrites, soma, and nucleus, though appearing at different time points depending on the treatment utilized, with the dephosphorylation of p38 likely occurring within the soma and/or nucleus of neurons. To see if NR1/2B receptor inhibition would alter phospho-p38 levels in any particular cellular region, we treated neurons with both 100 μM NMDA and Ro25-6981. At 1 min, phospho-p38 levels increased in both the dendrites and in the neuronal soma and nucleus, and by 5 min, phospho-p38 was visualized only in the soma and nucleus. These data suggested that p38 dephosphorylation through NR1/2B receptor activation may occur at two locations, first with a loss of phospho-p38 signal at the synapse around 1 min after stimulation, and second with a loss of phospho-p38 signal at the soma and nucleus, consistent with the localization of NR2B-containing receptors.

Control of NMDAR-mediates p38 Phosphorylation by Intracellular Messenger Systems—To explore the mechanism by which NR1/2B receptors decrease phospho-p38 levels, we originally hypothesized that the phosphatase calcineurin, which is activated by NMDAR stimulation (53), could be responsible. We treated cultures with 100 μM NMDA and a specific inhibitor of calcineurin, 1 μM FK506 (54). Surprisingly, FK506 did not change phospho-p38 levels after 5 min of 100 μM NMDA treatment (Fig. 6A); however, it significantly inhibited p38 activation with 1 min of 100 μM NMDA and at both time points of 10 μM NMDA treatment (Fig. 6, A–C). Although phospho-p38 levels were slightly increased at baseline (0 min) with FK506 pretreatment, these levels were not significantly different from
nM wortmannin were utilized to assess PI3K activation. By ANOVA, compiled from four experiments, 100 nM wortmannin significantly inhibited calcium transients produced by 100 μM NMDA, no significant differences were noted between pretreatments of LY294002, Ro25-6981, or both inhibitors concurrently (p = 0.1918 by ANOVA, compiled from four experiments). B, concentrations of 10 and 100 nM wortmannin were utilized to assess the specificity of PI3K inhibition to prevent p38 dephosphorylation. Neither concentration affected p38 phosphorylation with 10 μM NMDA treatment; however, 100 nM wortmannin significantly increased p38 phosphorylation with 100 μM NMDA treatment (*, p = 0.0024, by Mann-Whitney test, compiled from four experiments).

We assessed the effect of inhibition of other calmodulin-activated enzymes on p38 phosphorylation to establish the specificity of calcineurin inhibition. NMDA-mediated p38 activation and deactivation were not affected by either nNOS inhibition or CaMKII inhibition, as assessed by 7-nitroindazole (7NI) and KN62, respectively (Fig. 6, E and F). Although the nNOS inhibitor increased activation of p38 at baseline (0 min) and with a 5-min 10 μM NMDA treatment, these increases were independent of NMDA activation, because the activation and deactivation profiles of p38 were not altered. Conversely, ERK1/2 activation was significantly attenuated by CaMKII inhibition, as previously reported (Fig. 6F, inset) (55).

As PI3K has been implicated in many of the physiological processes opposing p38, we sought to assess whether it might be involved in control of p38 dephosphorylation. PI3K selectively binds to NR1/2B receptors in the hippocampus in vivo, because antibodies specific to the p85 subunit of PI3K immunoprecipitate NR2B and NR1, but not NR2A, in a manner requiring phosphorylation of Tyr1336 (56, 57). Because of these subtype-specific effects, we hypothesized that PI3K may mediate the NR1/2B receptor-mediated effects of p38 dephosphorylation. As hypothesized, LY294002, a specific PI3K inhibitor, prevented the dephosphorylation of p38 with 5 min 100 μM NMDA treatment without significantly affecting the activation of p38 with 10 μM NMDA (Fig. 7A). As previously reported, inhibition of PI3K decreased ERK1/2 activation (Fig. 7A, inset) (55). Furthermore, LY294002 did not significantly alter calcium transients with 100 μM NMDA at any time point (Fig. 7B), indicating that PI3K is not affecting the activity of the NMDAR. When cells were pretreated with LY294002 and Ro25-6981, no significant increase in p38 phosphorylation was observed with 100 μM NMDA treatment beyond that of 100 μM NMDA and Ro25-6981 alone, suggesting that NR2B and PI3K activation, resulting in p38 dephosphorylation, act through a single pathway (Fig. 7C).

To assess further the specific effects of PI3K inhibition on p38 phosphorylation, we used an independently acting, although less stable, PI3K inhibitor, wortmannin, at both 10 and 100 nM concentrations. Whereas neither concentration of wortmannin affected p38 phosphorylation with 10 μM NMDA treatment without significantly affecting the activation of p38 with 10 μM NMDA (Fig. 7A). As previously reported, inhibition of PI3K decreased ERK1/2 activation (Fig. 7A, inset) (55). Furthermore, LY294002 did not significantly alter calcium transients with 100 μM NMDA at any time point (Fig. 7B), indicating that PI3K is not affecting the activity of the NMDAR. When cells were pretreated with LY294002 and Ro25-6981, no significant increase in p38 phosphorylation was observed with 100 μM NMDA treatment beyond that of 100 μM NMDA and Ro25-6981 alone, suggesting that NR2B and PI3K activation, resulting in p38 dephosphorylation, act through a single pathway (Fig. 7C).

We sought to more directly link PI3K activation to NR1/2B receptor-mediated p38 deactivation. As PI3K interacts with many modulators in the cell, it is difficult to directly identify NMDAR-mediated phosphorylation of substrates by PI3K. However, PI3K activation is a defined process, in which bind-
NR2A and NR2B, but only NR2B antibodies immunoprecipitated the indicated on the left side. Both NR2A and NR2B immunoprecipitated NR2A and NR2B, but only NR2B antibodies immunoprecipitated the phosphorylation of the p85 regulatory subunit to YXXM motifs (in which the tyrosine is phosphorylated) leads to activation (59–62). The NR2B subunit contains one of these motifs, in which the tyrosine amino acid 1336 (Tyr1336) (56, 63). Utilizing antibodies specific to phospho-Tyr1336-NR2B, we examined Tyr1336-NR2B phosphorylation as a potential indicator of binding and activation of PI3K. In mature neurons, 5 min of 10 μM NMDA treatment did not increase phospho-Tyr1336 immunoreactivity (Fig. 8A). However, 5 min of 100 μM NMDA increased phospho-Tyr1336 65% above baseline levels. In addition, treatment of immature cultures (DIV7–8) did not increase phospho-Tyr1336 of NR2B (Fig. 8B). Thus, increases in phospho-Tyr1336-NR2B were only seen under conditions leading to p38 dephosphorylation. Interestingly, pretreatment with the NR1/2B-specific inhibitor Ro25-6981 did not prevent the increase in phospho-Tyr1336 (Fig. 8A), but preincubation with either ConG or MK-801 prevented the increase in phospho-Tyr1336 (Fig. 8C).

Based on these results, while NR2B subunits would bind and activate PI3K, the signal for increasing phosphorylation of Tyr1336-NR2B was mediated by NR1/2A/2B receptors or both NR1/2A/2B and NR1/2B receptors.

Because NR1/2A/2B receptors lead to increases in phospho-Tyr1336-NR2B, we wanted to assess PI3K binding to the different receptor subtypes (NR1/2A/2B versus NR1/2B) to support our pharmacological data. To provide direct evidence of NR2B binding to PI3K, we performed coimmunoprecipitation using specific antibodies to NR2A, NR2B, and the p85 subunit of PI3K. NR2A- and NR2B-specific antibodies immunoprecipitated each other, but only NR2B could immunoprecipitate p85 (Fig. 8D). Furthermore, p85 could immunoprecipitate NR1 and NR2B, but not NR2A (Fig. 8E), consistent with in vivo studies of the hippocampus (56, 57). Whereas p85 appeared to immunoprecipitate NR2B in the absence of NMDAR stimulation, an increased amount of NR2B was immunoprecipitated after a 5-min exposure to 100 μM NMDA, and anti-p85 only immunoprecipitated phospho-Tyr1336-NR2B after 5-min exposure to 100 μM NMDA. These data thus supported that p85 selectively bound to NR2B in a manner that is facilitated by phosphorylation of Tyr1336-NR2B. These results complement pharmacological data showing blockade of p38 dephosphorylation with PI3K inhibitors, a process requiring PI3K binding and activation by NR1/2B receptors, and provide biochemical support for the crucial role of PI3K in control of p38 activity.

**DISCUSSION**

In the present study, we defined mechanisms of p38 activation controlled by stimulation of NMDAR subtypes in conjunction with neuronal maturation and agonist concentration. Phosphorylation of p38 in mature cultures required NR1/2A/2B receptor activation. Conversely, inhibitors of NR1/2B receptors blocked the rapid decrease in phospho-p38 levels with high concentrations of NMDA. In immature cultures, NR2B-containing receptors activated p38 in a concentration-dependent manner lacking acute dephosphorylation. At this developmental stage, NMDARs are primarily composed of NR1/2B receptors that are bound at developing synapses to SAP102 (5). However, by DIV14, expression of NR2A increases, NR1/2A/2B receptors (bound to PSD-95) become the major NMDAR at the synapse, and NR1/2B receptor levels increase at extrasynaptic sites (11). These observations in conjunction with our data suggest that the activation of p38 is a synaptic event in both NR2B phospho-Tyr1336 (Y1336), using a specific antibody. A significant increase in phospho-Tyr1336 was noted after 5 min of 100 μM NMDA both with and without Ro25-6981 pretreatment but not after 10 μM NMDA treatment (p < 0.0001 by ANOVA, *, p < 0.001 comparing both 0 and 10 μM NMDA to 100 μM and 100 μM NMDA + Ro25-6981 by post-hoc Student’s t test with Bonferroni correction; compiled from four experiments). Data represent amount of phospho-Tyr1336 divided by total NR2B (AB1557) and standardized to no treatment condition. B, immature hippocampal cultures (DIV7–8) treated with 10 and 100 μM NMDA showed no increases in phospho-Tyr1336 above that of no treatment (p = 0.5692 by ANOVA, compiled from four experiments). C, mature hippocampal cultures were pretreated with 6 μM ConG or 1 μM MK-801, treated with 100 μM NMDA for 5 min, and were assessed for phospho-Tyr1336 levels. No increase in phospho-Tyr1336 was observed when cultures were pretreated with ConG or MK-801 (p = 0.9646 by ANOVA, compiled from three experiments). D, representative immunoblots from three independent experiments assessing coimmunoprecipitation by indicated antibodies. Immunoprecipitating antibodies are indicated on the top, and antibodies used to probe Western blots are indicated on the left side. Both NR2A and NR2B immunoprecipitated NR2A and NR2B, but only NR2B antibodies immunoprecipitated the p85 subunit of PI3K. E, anti-p85-PI3K immunoprecipitated NR1 and NR2B, but not NR2A, with increased immunoreactivity of NR1 and NR2B observed after 5 min of 100 μM NMDA treatment. Furthermore, phospho-Tyr1336 was immunoprecipitated by anti-p85 only in the presence of 100 μM NMDA treatment.
stages of neuronal cultures, while p38 dephosphorylation is caused through extrasynaptic NR1/2B receptors. Whereas the localization of NMDARs likely plays a role in p38 phosphorylation and dephosphorylation, the phosphorylation of p38 occurred in the dendrites and NR1/2B receptor-mediated p38 dephosphorylation occurred both at the dendrites and in the soma and nucleus. This may be explained by the presence of extrasynaptic NR1/2B receptors both in the dendrites and on the cell body (9, 11, 48–52). Although our data suggest that phospho-p38 migrates from the dendrites to the soma/nucleus, we have not ruled out the presence of separate pools of somatic/nuclear p38, activated by signal transduction mechanisms initiated by NMDAR activation at the synapse.

Few previous studies have systematically assessed NMDAR-mediated control of p38. In 12-day-old hippocampal cultures, treatment with 100 µM NMDA for 15 min activates p38 (31). 5 min of 100 µM glutamate treatment results in p38 activation that is NMDAR-dependent in cerebellar granule cells (17), a neuronal system with different NMDAR subtype composition than hippocampal cultures.

Other studies have concentrated on NMDAR-mediated ERK1/2 and CREB activation. ERK1/2 is activated through CaMKII inhibition of the synaptic Ras GTPase-activating protein SynGAP (16, 64–66) and is dephosphorylated over the course of 30 min in a manner that is not developmentally regulated (67). Alternatively, ERK1/2 may instead be activated through NR2B binding to the guanine nucleotide exchange factor RasGRF1 (68). CREB, although downstream of ERK1/2 activation, is activated in an NMDAR-mediated developmental, concentration-dependent, and subtype-specific manner matching that of p38 (15, 67).

Our data indicate that p38 is modulated independently of ERK1/2 and CREB, because p38 is activated through calcineurin, dephosphorylated by PI3K, and does not involve other calmodulin-dependent signals (Fig. 9). The localizations of these messengers are consistent with the activation and deactivation of p38; calcineurin is localized synaptically (69), and the p85 subunit of PI3K selectively binds to the NR2B subunit of NR1/2B receptors localized extrasynaptically (56, 57). In contrast to p38, ERK1/2 activation is not altered by calcineurin inhibition, and PI3K activates ERK1/2 (55, 70). Because CaMKII inhibition can also decrease ERK1/2 activation (55, 66), the effect of PI3K on p38 dephosphorylation is independent from its effect on ERK1/2. Whereas others (71) hypothesize that p38 is downstream of CaMKII inhibited SynGAP, our data have not connected CaMKII and p38. Furthermore, our data support independent NMDAR-mediated pathways for the activation of CREB and p38, since inhibition of calcineurin does not prevent CREB phosphorylation (67, 72).

Our data suggest a complex pathway in hippocampal neurons in which NR1/2A/2B receptors, although responsible for p38 activation, also independently lead to phosphorylation of NR1/2B receptors at Tyr1336-NR2B. Alternatively, NR1/2A/2B receptors could cause the dissociation of NR2B from RACK1, an inhibitory scaffolding protein that specifically prevents fyn phosphorylation of NR2B (not NR2A) subunits (73). Through this phosphorylation event, PI3K binds to NR1/2B receptors and is activated (56, 57). Because Tyr1336 is not the preferential site of tyrosine phosphorylation on NR2B (63), a higher level of NMDAR activation might be required for this phosphorylation event and PI3K activation to occur. Because there is no detectable increase in phosphorylation of Tyr1336-NR2B in immature cultures, the presence of NR1/2A/2B receptors or their associated synaptic elements may be required for Tyr1336-NR2B phosphorylation. Therefore, this phosphorylation of Tyr1336-NR2B and subsequent PI3K activation provides a mechanism by which the developmentally regulated and concentration-dependent p38 dephosphorylation occurs.

Many studies on p38 concentrate on its role in inducing LTD or inhibiting LTP. LTP and LTD are NMDAR-dependent electrophysiological processes controlled through Ras activation to ERK1/2 and Rap activation to p38, respectively (16, 23). Furthermore, a recent study shows that NMDAR-mediated activation of p38 can lead to LTD through the small GTPase Rab5 (74), the next step downstream from p38 activation. p38 inhibition can both prevent LTD and/or remove impairments on LTP (16, 23–27); therefore the concentration-dependent shut-off of p38 could act as a molecular switch between LTD and LTP. Whereas Krapivinsky et al. (71) have studied p38 in a different paradigm, they also concluded that the inactivation of p38 allows the induction of LTP. Our data provide a mechanism by which physiological facilitation of LTP may occur.

Several related findings support the significance of our data in paradigms of synaptic plasticity. Induction of LTD occurs through a mechanism involving both p38 and calcineurin (16), and calcineurin may be involved in forms of LTP inhibition (75). Furthermore, PI3K is necessary for LTD of AMPA receptor currents (76), and its inhibition attenuates LTP through a mechanism independent of ERK1/2 activity (70). In addition, LTD requires tyrosine phosphorylation of NR2B, which is nec-
NMDA-mediated Control of p38 MAPK

Acknowledgments—We thank Margie Maronski for hippocampal neuron preparation, and Dr. Peter Bannerman, Ashleigh Hahn, and the Confocal Microscopy Core at Children’s Hospital of Philadelphia for aid in confocal microscopy. Calcium imaging experiments and confocal microscopy experiments were supported by the Mental Retardation Research Center of the Children’s Hospital of Philadelphia (P30-26979).

REFERENCES

1. Dingledine, R., Borges, K., Bowie, D., and Traynelis, S. F. (1999) Pharmacol. Rev. 51, 71–81
2. Lynch, D. R., and Guttmann, R. P. (2001) Curr. Drug Targets 2, 215–231
3. Lau, L. F., Mammen, A., Ehlers, M. D., Kindler, S., Chung, W. J., Garner, W. W., and Paoletti, P. (2000) Nat. Rev. Neurosci. 1, 212–220
4. Li, H. J., Rojas-Soto, M., Oguni, A., and Kennedy, M. B. (1998) J. Neurochem. 70, 693–699
5. Shiraishi, Y., Mizutani, A., Mikoshiba, K., and Furuchi, T. (2003) Mol. Cell. Neurosci. 22, 188–201
6. Nakazawa, T., Komai, S., Tsuoka, T., Hisatsume, C., Unemori, H., Mishina, M., and Yamamoto, T. (1999) Genes Cells 4, 657–666
7. Walker, E. H., Parold, M. E., Pemisz, O., Stephens, L., Hawkins, P. T., Wymann, M. P., and Williams, R. L. (2000) Nat. Cell. Biol. 2, 352–357
8. Shoelson, S. E., Sirvastava, M. W., Williams, K. P., Hu, P., Schleissinger, J., and Weiss, M. A. (1993) EMBO J. 12, 795–802
9. Holt, K. H., Olson, L., Moyer-Rowley, W. S., and Pesse, J. E. (1994) Mol. Cell. Biol. 14, 42–49
10. Kurosu, H., Maehama, T., Okada, T., Yamamoto, T., Hoshino, S., Fukui, Y., Ui, M., and Goto, K. (1997) J. Biol. Chem. 272, 24252–24256
11. Enomoto, N., Futatsugi, N., Narumi, T., Shirouzu, M., Yokoyama, S., Konagaya, A., and Taji, M. (2005) J. Biochem. 138, 1231–1236
12. Nakazawa, T., Komai, S., Tsuoka, T., Hisatsume, C., Unemori, H., Semb, K., Mishina, M., Manabe, T., and Yamamoto, T. (2001) J. Biol. Chem. 276, 693–699
13. Chan, H. J., Rejas-Soto, M., Oguni, A., and Kennedy, M. B. (1998) Neuron 20, 895–904
14. Kim, J. H., Liao, D., Lau, L. F., and Huganir, R. L. (1998) Neuron 20, 683–691
15. Komiya, N. H., Watabe, A. M., Carlisle, H. J., Porter, K., Charlesworth, F. M., Gendreau, C. M., Krasnow, S. J., Morris, R. G., O’Dell, T. J., and Grant, S. G. (2002) J. Neurosci. 22, 9721–9732
16. Sala, C., Rudolph-Correa, S., and Sheng, M. (2000) J. Neurosci. 20, 3555–3566
17. Krapivinsky, G., Krapivinsky, L., Manasian, Y., Ivanov, A., Tyriz, R., Pellegrino, C., Ben-Ari, Y., Clapham, D. E., and Medina, I. (2003) Neuron 40, 757–774
18. Dell’Acqua, M. L., Dodge, K. L., Tavalin, S. J., and Scott, J. D. (2002) J. Biol. Chem. 277, 4876–48802
19. Opaño, P., Watabe, A. M., Grant, S. G., and O’Dell, T. J. (2003) J. Neurosci. 23, 3679–3688

NMDA-mediated Control of p38 MAPK

essential for NR1/2B receptor-mediated PI3K activation (56, 77). Therefore, the NR2B/P3K-mediated inhibition of p38 may be crucial for the induction of LTP.

Our results suggest that under low levels of NMDAR stimulation, p38 dephosphorylation does not occur because of a relative lack of NR1/2B receptor activation and Tyr1836-NR2B phosphorylation. Whereas our results suggest that NR1/2B receptors do not contribute substantially to the size of intracellular calcium transients at low agonist concentration, inhibition of NR1/2B receptors prolonged the agonist response. The relative lack of NR2B receptors to neuronal currents produced by low levels of agonist stimulation has been noted before (78). Because NR2A and NR2B subunits have a similar affinity for glutamate (79, 80), differences in agonist affinity do not explain this result. Our data thus support pathways for communication between synaptic and extrasynaptic receptors. NR1/2B receptor inhibition may facilitate synaptic responses while decreasing extrasynaptic responses (81).

NMDAR activation controls many downstream pathways, some of which are controlled specifically through subtype composition or localization. Here we have shown that NR2A/2B receptors are opposed by NR1/2B receptors in the activation of p38, but our results imply more complex mechanisms involving cross-talk between synaptic and extrasynaptic receptors. Understanding these molecular mechanisms by which specific subtypes mediate different effects may provide the key to the interplay of synaptic events that occur both physiologically and through aberrant regulation of glutamate in disease states.

...
NMDAR-mediated Control of p38 MAPK

71. Krapivinsky, G., Medina, I., Krapivinsky, L., Gapon, S., and Clapham, D. E. (2004) Neuron 43, 563–574
72. Bito, H., Deisseroth, K., and Tsien, R. W. (1996) Cell 87, 1203–1214
73. Yaka, R., Thornton, C., Vagts, A. J., Phamluong, K., Bonci, A., and Ron, D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5710–5715
74. Brown, T. C., Tran, I. C., Backos, D. S., and Esteban, J. A. (2005) Neuron 45, 81–94
75. Chen, Q. S., Wei, W. Z., Shimahara, T., and Xie, C. W. (2002) Neurobiol. Learn Mem. 77, 354–371
76. Man, H. Y., Wang, Q., Lu, W. Y., Ju, W., Ahmadian, G., Liu, L., D’Souza, S., Wong, T. P., Taghibiglou, C., Lu, J., Becker, L. E., Pei, I., Liu, F., Wymann, M. P., MacDonald, J. P., and Wang, Y. T. (2003) Neuron 38, 611–624
77. Kojima, N., Wang, J., Mansuy, I. M., Grant, S. G., Mayford, M., and Kandel, E. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4761–4765
78. Kew, J. N., Trube, G., and Kemp, J. A. (1996) J. Physiol. 497, 761–772
79. Laurie, D. J., and Seeburg, P. H. (1994) Eur. J. Pharmacol. 268, 335–345
80. Priestley, T., Laughton, P., Myers, J., Le Bourdelles, B., Kerby, J., and Whiting, P. J. (1995) Mol. Pharmacol. 48, 841–848
81. Mallon, A. P., Auberson, Y. P., and Stone, T. W. (2004) Exp. Brain Res. 162, 374–383