

# N-Methyl-D-aspartate Receptors Mediate the Phosphorylation and Desensitization of Muscarinic Receptors in Cerebellar Granule Neurons<sup>\*[5]</sup>

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Changes in synaptic strength mediated by ionotropic glutamate N-methyl-D-aspartate (NMDA) receptors is generally considered to be the molecular mechanism underlying memory and learning. NMDA receptors themselves are subject to regulation through signaling pathways that are activated by G-protein-coupled receptors (GPCRs). In this study we investigate the ability of NMDA receptors to regulate the signaling of GPCRs by focusing on the G<sub>q/11</sub>-coupled M<sub>3</sub>-muscarinic receptor expressed endogenously in mouse cerebellar granule neurons. We show that NMDA receptor activation results in the phosphorylation and desensitization of M<sub>3</sub>-muscarinic receptors through a mechanism dependent on NMDA-mediated calcium influx and the activity of calcium-calmodulin-dependent protein kinase II. Our study reveals a complex pattern of regulation where GPCRs (M<sub>3</sub>-muscarinic) and NMDA receptors can feedback on each other in a process that is likely to influence the threshold value of signaling networks involved in synaptic plasticity.

Glutamate neurotransmission mediated through the ionotropic N-methyl-D-aspartate (NMDA),<sup>3</sup> α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors is the primary excitatory stimulus in the central nervous system (1). NMDA and AMPA receptors are found co-expressed at the post-synaptic membrane of glutamatergic synapses. AMPA receptors, permeable to sodium and potassium ions, open in response to glutamate generating rapid excitatory post-synaptic potentials. NMDA receptors allow for the influx

of extracellular calcium into the post-synaptic cell in response to glutamate and a concomitant depolarization that relieves the magnesium block of channel conductance. Through the temporal and spatial summation of excitatory post-synaptic potentials and glutamate release, NMDA receptors can contribute significantly to post-synaptic membrane depolarization.

In addition, NMDA receptors can influence post-synaptic activity in a more subtle manner by allowing for changes in intracellular calcium concentrations that initiate calcium-dependent signaling cascades (2, 3). Prominent among these signaling cascades is the regulation of protein phosphorylation via both calcium/calmodulin-dependent kinase II (CamKII) (3, 4) and the protein phosphatases protein phosphatase 1 and calcineurin (5).

These signaling events are among a number of mechanisms that are proposed to regulate NMDA-mediated changes in synaptic strength (6). Synaptic plasticity of this type is frequently measured as long term potentiation (LTP) and depression (LTD) and is widely regarded as a molecular mechanism that contributes to memory and learning (7, 8). It is now clear that the clustering of signaling molecules around NMDA and AMPA receptors at specialized post-synaptic sites coordinate signaling pathways involved in the adaptation of synaptic activity (3, 9). Among these signaling molecules are members of the G-protein-coupled receptor (GPCR) family (6) where receptors such as metabotropic glutamate (10, 11), adrenergic (12–14), pituitary adenylyl cyclase-activating polypeptide receptor 1 (15), and muscarinic (16) receptors are found closely associated with NMDA receptors. These GPCRs regulate NMDA function through a variety of signaling pathways such as the well established second messenger-regulated pathways of calcium mobilization, protein kinase C (17, 18) and cAMP-dependent protein kinase (19) and additionally via nonreceptor tyrosine kinases Pyk2 (20) and Src (21) through activation of the mitogen-activated protein kinase cascade (22) and control of gene transcription (23). There is, therefore, an extensive range of mechanisms by which GPCRs can influence NMDA receptor function in neurons.

In the current study we investigate further the relationship between NMDA receptors and GPCRs by focusing on the M<sub>3</sub>-muscarinic receptor endogenously expressed in cerebellar granule (CG) neurons. Previous studies have determined that muscarinic receptors regulate both NMDA receptor function

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<sup>3</sup> The abbreviations used are: NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CamKII, calcium/calmodulin-dependent protein kinase II; CG, cerebellar granule; eGFP, enhanced green fluorescent protein; GPCR, G-protein-coupled receptor; GRK, GPCR kinase; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; LTD, long term depression; LTP, long term potentiation; PH, pleckstrin homology; PIP<sub>2</sub>, phosphoinositide 4,5-bisphosphate; PLC, phospholipase C; siRNA, small interfering RNA; GST, glutathione S-transferase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

## NMDA-mediated Regulation of M<sub>3</sub>-muscarinic Receptors

and contribute to LTP and LTD (16, 24–27). Here we demonstrate that the NMDA receptor-mediated activation of CamKII results in the phosphorylation of the M<sub>3</sub>-muscarinic receptor and subsequent uncoupling of the receptor from the G<sub>q/11</sub>/phosphoinositide pathway. Our study demonstrates that there is a complex reciprocal relationship between GPCRs and NMDA receptors where each is able to regulate the activity of the other in a process that is likely to be essential in establishing the threshold activities that contribute to synaptic plasticity.

### EXPERIMENTAL PROCEDURES

**Primary Cell Culture**—Mouse CG neurons were cultured as described previously (28). In brief, cerebella from 7–8-day-old Balb/c pups were mechanically and enzymatically (trypsin) dissociated. The cells were subsequently plated on poly-L-lysine-coated 6-well plates (Nunc) or on round glass coverslips (25-mm diameter) at a cell density of  $0.25 \times 10^6$  cells/cm<sup>2</sup>, in basal medium Eagle (Invitrogen) supplemented with 20 mM KCl, penicillin/streptomycin, 10% fetal calf serum. Cytosine arabinoside (10 μM) was added 48 h after plating to prevent glial cell proliferation. The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> and used after 7–8 days. The experiments were performed in CSS-25 buffer (120 mM NaCl, 1.8 mM CaCl<sub>2</sub>, 15 mM glucose, 25 mM KCl, 25 mM HEPES, pH 7.4) or in a modified CSS-25 buffer as indicated.

For transfection of siRNA duplexes CG neurons cultured for 5 days were transfected with either siRNA duplexes specific for CamKII-β (purchased as verified siRNA from Santa Cruz Biotechnology, Inc.) or scrambled siRNA control. CG neurons were transfected with 80 pmol of scrambled siRNA or 80 pmol of specific siRNA using 5 μl of FuGENE HD transfection reagent (Roche Applied Science) per transfection. The cells were used for phosphorylation experiments 48 h post-transfection.

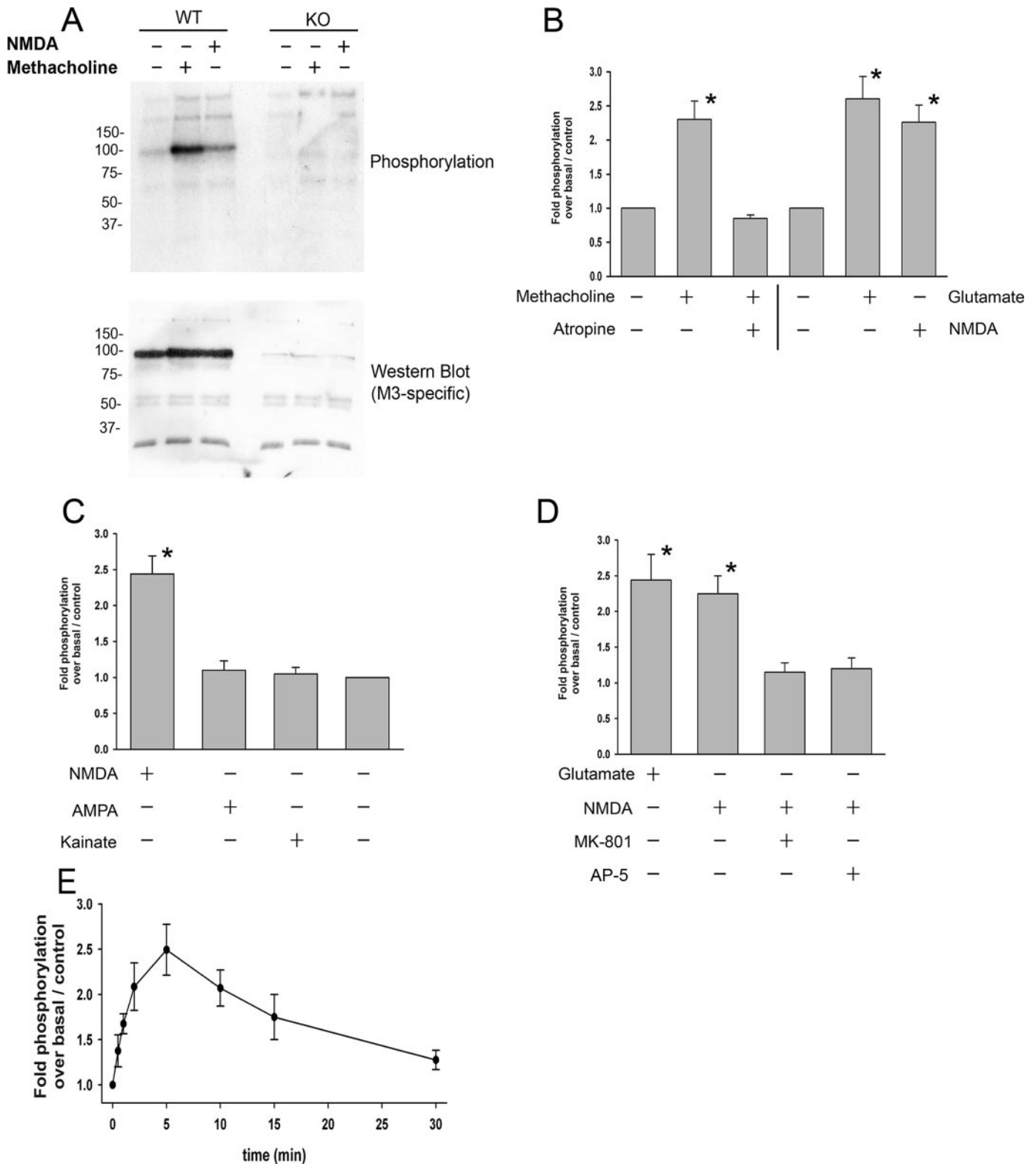
**M<sub>3</sub>-muscarinic Receptor Phosphorylation**—*In vivo* [<sup>32</sup>P]orthophosphate labeling, receptor solubilization, and immunoprecipitation were conducted as described previously (29). In brief, CG neurons in 6-well plates were washed and incubated for 2 h in CSS-25 buffer (1 ml) containing 100 μCi/ml [<sup>32</sup>P]orthophosphate (GE Healthcare). The cells were then stimulated with methacholine, glutamate, NMDA, AMPA, or kainic acid at a final concentration of 100 μM for 5 min unless otherwise stated. Where appropriate, antagonists or other compounds were added before the addition of agonists. The cells were then lysed in lysis buffer (10 mM EDTA, 500 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 10 mM Tris, pH 7.4), and the M<sub>3</sub>-muscarinic receptor was immunoprecipitated using an in house anti-mouse M<sub>3</sub> receptor polyclonal antibody (29). Immunoprecipitated proteins were resolved by 8% SDS-PAGE and visualized by autoradiography or using a STORM phosphor-imager (GE Healthcare). To control for equal receptor loading, immunoprecipitated proteins resolved by SDS-PAGE were transferred to nitrocellulose. The membranes were then exposed before being used in Western blots with M<sub>3</sub>-muscarinic receptor specific monoclonal antibodies (see Figs. 1A and 3A). Quantification of the phosphorylation status of the receptor was determined by analysis using the ImageQuant and AlphaEase softwares.

**Single Cell Imaging of Calcium and Phosphoinositide Responses**—Experiments examining changes in free intracellular Ca<sup>2+</sup> were conducted on CG neurons cultured on coverslips. The cells were loaded with the Ca<sup>2+</sup> indicator fura-2 AM (2 μM, 30 min) (Molecular Probes) in CSS-25 at 37 °C and mounted on to a Zeiss Axiovert 200 inverted epifluorescence microscope fitted with a perfusion chamber (flow rate at 3 ml/min). The cells were excited at 340 and 380 nm at a sample rate of 0.67 Hz by means of an excitation filter wheel. Sequential fluorescent image pairs were collected at wavelengths >510 nm via a cooled CCD camera (ORCA-ER, Hamamatsu) and converted to pseudo color images after background subtraction. Free intracellular Ca<sup>2+</sup> signal was expressed as 340/380 ratio of fluorescence. Approximately 10–15 cells were analyzed in each experiment, and the mean values from different cultures were averaged.

For experiments involving analysis of phosphoinositide signaling, the translocation of eGFP-PH<sub>PLCδ1</sub> from the plasma membrane to the cytosol was conducted as described previously (30, 31). Plasmid DNA carrying the eGFP-PH<sub>PLCδ1</sub> construct was transfected into CG neurons via the calcium-phosphate precipitation method. Briefly, 1 day after preparation of CG neurons, 3 μg of DNA/well in a volume of 10 μl of H<sub>2</sub>O was added to 85 μl of HEPES-buffered saline (137 mM NaCl, 5.6 mM glucose, 50 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 21 mM HEPES, pH 7.1) and to 5 μl 2.5 M CaCl<sub>2</sub>. After 20 min, the solution was added to the cells. The following day, the medium was replaced with fresh medium, and the cells were cultured for a further 6–7 days. The coverslips were then mounted on to the microscope, and images of eGFP were captured at a rate of 1 Hz. Phosphoinositide responses were measured as the relative change in fluorescence intensity in the cytosol. The data are expressed as a self-ratio (*F*/*F*<sub>0</sub>) resulting from subtraction of background fluorescence followed by dividing the fluorescence intensity at a given time by the initial fluorescence within each region of interest. Where NMDA was used to stimulate CG neurons, the cells were perfused with Mg<sup>2+</sup>-free buffer before the addition of NMDA.

**NMDA Desensitization of the M<sub>3</sub>-muscarinic Receptor Phosphoinositide Response**—CG neurons expressing eGFP-PH<sub>PLCδ1</sub> were perfused with CSS-25 containing a maximal concentration of methacholine (100 μM) for 2 min. This generated a phosphoinositide response that was termed S1 and served as an internal control. Methacholine was then removed, and a lag time of 12 min was allowed for the cells to recover. NMDA (100 μM) was then applied for 5 min, followed by a wash-out period of 2 min. The cells were then stimulated with a second application of methacholine, and the resulting phosphoinositide response was termed S2. Desensitization of the M<sub>3</sub>-muscarinic receptor response was determined as the difference in the amplitude between the S1 and S2 responses.

**Radioligand Binding and Internalization Assays**—CG neurons cultured in 6-well plates were incubated with CSS-25 containing NMDA (100 μM) and/or methacholine (100 μM) for the indicated times. The cells were then washed three times with ice-cold CSS-25 and incubated with CSS-25 containing a saturating concentration of the muscarinic receptor antagonist [<sup>3</sup>H]N-methylscopolamine (0.5 nM) for 4 h at 4 °C. The cells



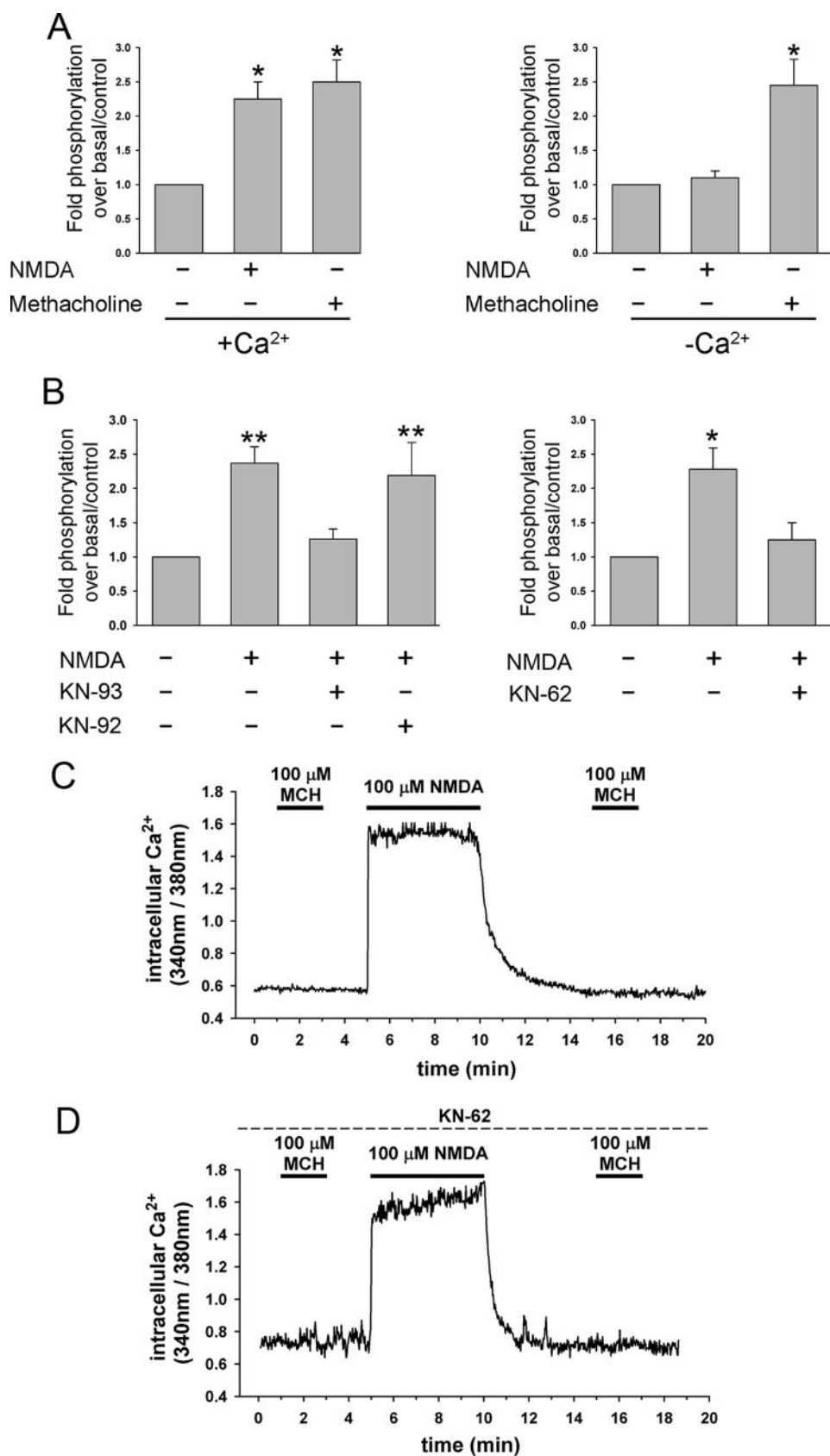
**FIGURE 1. Activation of NMDA receptors induces phosphorylation of M<sub>3</sub>-muscarinic receptor.** *A*, representative autoradiograph of M<sub>3</sub>-muscarinic receptors immunoprecipitated from [<sup>32</sup>P]orthophosphate labeled CG neurons prepared from wild type (*WT*) mice or M<sub>3</sub>-muscarinic receptor knock-out (*KO*) mice. The CG neurons were stimulated for 5 min with either; vehicle, methacholine (100 μM), or NMDA (100 μM). In these experiments immunoprecipitated proteins resolved by SDS-PAGE were transferred to nitrocellulose, and after exposure for phosphorylation the nitrocellulose was probed using a receptor-specific monoclonal antibody. This served as a loading control for the M<sub>3</sub>-muscarinic receptor. *B*, phosphorylation of the M<sub>3</sub>-muscarinic receptor in response to methacholine (100 μM), with or without atropine (10 μM), and to glutamate (100 μM) or NMDA (100 μM). *C*, phosphorylation of the M<sub>3</sub>-muscarinic receptor in CG neurons stimulated with the ionotropic glutamate receptor agonists NMDA, AMPA, and kainic acid (all at 100 μM). *D*, effect of the NMDA receptor antagonists MK-801 (5 μM, 10 min) and AP-5 (20 μM, 10 min). *E*, time course of M<sub>3</sub>-muscarinic receptor phosphorylation following stimulation with NMDA (100 μM). Graphs represent quantification of phosphorylation (means ± S.D.) from at least three replicates, normalized to the basal phosphorylation of the receptor in control cells with no stimulation. \*, significant difference between basal and stimulated samples (*p* < 0.01).



## NMDA-mediated Regulation of M<sub>3</sub>-muscarinic Receptors

were then washed three times with ice-cold CSS-25 and solubilized by the addition of lysis buffer (200  $\mu$ M). Receptor expression was determined by liquid scintillation counting. Nonspecific binding was determined by the inclusion of 10  $\mu$ M atropine.

**In Vitro Phosphorylation of GST-Third Intracellular Loop Fusion Protein**—GST fusion constructs containing the full mouse third intracellular loop R<sup>252</sup> to T<sup>491</sup> (GST-3loop) or GST alone were expressed in *Escherichia coli* BL21 (DE3) IRL bacteria and purified as described previously (32). 5  $\mu$ g of protein was incubated with 200 ng of CaM kinase II (New England Biolabs) in assay buffer (10 mM HEPES, pH 7.4, 2.5 mM  $\beta$ -glycerophosphate, 0.5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.03 mg/ml calmodulin (Calbiochem)) containing 50  $\mu$ M ATP and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. The reactions were incubated for 30 min at 37 °C and stopped by the addition of an equal volume of 2 $\times$  SDS-PAGE sample buffer. The reactions were separated by SDS-PAGE on 12% gels, dried, and exposed to autoradiography film. For mass spectrometric experiments, the reactions were carried out in assay buffer containing 1 mM ATP. After separation by SDS-PAGE, the proteins were transferred to nitrocellulose, and the protein bands were revealed by staining with Ponceau S (Sigma). The protein bands were excised from the membrane and blocked with 0.5% polyvinylpyrrolidone in 0.6% acetic acid for 30 min at 37 °C before digestion with trypsin (1  $\mu$ g) in 50 mM ammonium bicarbonate, overnight at 37 °C. Tryptic peptides were collected, dried in a rotary evaporator, and resuspended in 50% acetonitrile, 0.1% formic acid. Enrichment of phosphopeptides was carried out using titanium dioxide contained in a MonoTip (GL Sciences Inc) according to the manufacturer's instructions, and the phosphopeptides were eluted in a solution of 5% ammonium hydroxide containing 20% acetonitrile. Where indicated, the enriched phosphopeptides were dried and resus-



pending in 10 mM Tris, pH 7.4, 10 mM CaCl<sub>2</sub> and subjected to further proteolytic digestion by the addition of 1  $\mu$ g of chymotrypsin (Roche Applied Science) for 2 h at 25 °C.

**MALDI-TOF Mass Spectrometry**—Samples resulting from trypsin or chymotrypsin digestion were acidified with formic acid and mixed 1:1 with a solution containing 10 mg/ml of 2,5-dihydroxybenzoic acid (Sigma) in 50% acetonitrile, 1% phosphoric acid. An aliquot of the resulting sample (0.5  $\mu$ l) was spotted onto a stainless steel target plate.

Analysis of peptide digests was carried out using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Warrington, UK) in positive ion reflectron mode over the  $m/z$  range 800–7000.

**LC-MS/MS**—LC-MS/MS was carried out upon each sample using a 4000 Q-Trap mass spectrometer (Applied Biosystems, Warrington, UK). Peptides resulting from proteolytic digestion were loaded at high flow rate onto a reverse phase trapping column (0.3 mm inner diameter  $\times$  1 mm), containing 5  $\mu$ M C18 300 Å Acclaim PepMap media (Dionex) and eluted through a reverse phase capillary column (75  $\mu$ m inner diameter  $\times$  150 mm) containing Jupiter Proteo 4  $\mu$ M 90 Å media (Phenomenex, UK) that was self-packed using a high pressure packing device (Proxeon Biosystems, Odense, Denmark). The output from the column was sprayed directly into the nanospray ion source of the 4000 Q-Trap mass spectrometer. The analysis was carried out in positive ion mode using data-dependent switching.

Fragment ion spectra generated by LC-MS/MS were searched using the MASCOT search tool (Matrix Science Ltd., London, UK) against an updated copy of the SwissProt protein data base using appropriate parameters. The criteria for protein identification were based on the manufacturer's definitions (Matrix Science Ltd.) (33) Candidate peptides with probability-based Mowse scores exceeding threshold ( $p < 0.05$ ), thus indicating a significant or extensive homology, were referred to as "hits." Protein scores were derived from peptide ion scores as a non-probability basis for ranking proteins.

For the phosphopeptides elucidated by MASCOT, individual MS/MS spectra were interrogated manually to validate both the peptide identity and position of assignment. In each case it was clear as to the identity of the peptide (and that the peptide was indeed phosphorylated), but for some MS/MS spectra it was not possible to validate the MASCOT assignment of the residue position for phosphorylation, because of low abundance of the required fragment ions. In these cases (because the sample amount was not limiting), the samples were repeated using multiple reaction monitoring inclusion lists comprising ion pairs consisting of the precursor ion  $[M+2H]^{2+}$  and that of the neutral ion loss  $[M+2H-H_3PO_4]^{2+}$ , such that the MS/MS data were obtained over a longer time period (5.86 s), which resulted in significantly improved data quality. This allowed unambiguous assignment of the position of phosphorylation.

**Data Analysis**—The statistic used was S.D. of at least three determinations, and significance was determined using Stu-

dent's  $t$  test or one-way analysis of variance for multiple comparisons. Significance was accepted when  $p < 0.05$ .

## RESULTS

**NMDA Receptors Mediate M<sub>3</sub>-muscarinic Receptor Phosphorylation in CG Neurons**—The phosphorylation status of the M<sub>3</sub>-muscarinic receptor can be monitored by prelabeling cells with [<sup>32</sup>P]orthophosphate followed by solubilization of the receptor and immunoprecipitation with receptor specific antibodies (34, 35). Experiments of this type have previously demonstrated that the M<sub>3</sub>-muscarinic receptor endogenously expressed in mouse CG neurons is phosphorylated upon stimulation with the muscarinic receptor agonist methacholine (29). In the current study we demonstrate that stimulation of glutamate receptors using NMDA resulted in an increase in the phosphorylation state of the M<sub>3</sub> muscarinic receptor (Fig. 1A). In these experiments the phosphorylated M<sub>3</sub>-muscarinic receptor appears as a band running at just less than 100 kDa. Western blots of the nitrocellulose membrane containing the radiolabeled phosphorylated receptor demonstrated that equal amounts of receptor are immunoprecipitated under various stimulation conditions (Fig. 1A). Importantly, no M<sub>3</sub>-muscarinic receptor was detected in immunoprecipitates from CG neurons prepared from M<sub>3</sub>-muscarinic receptor knock-out mice (Fig. 1A).

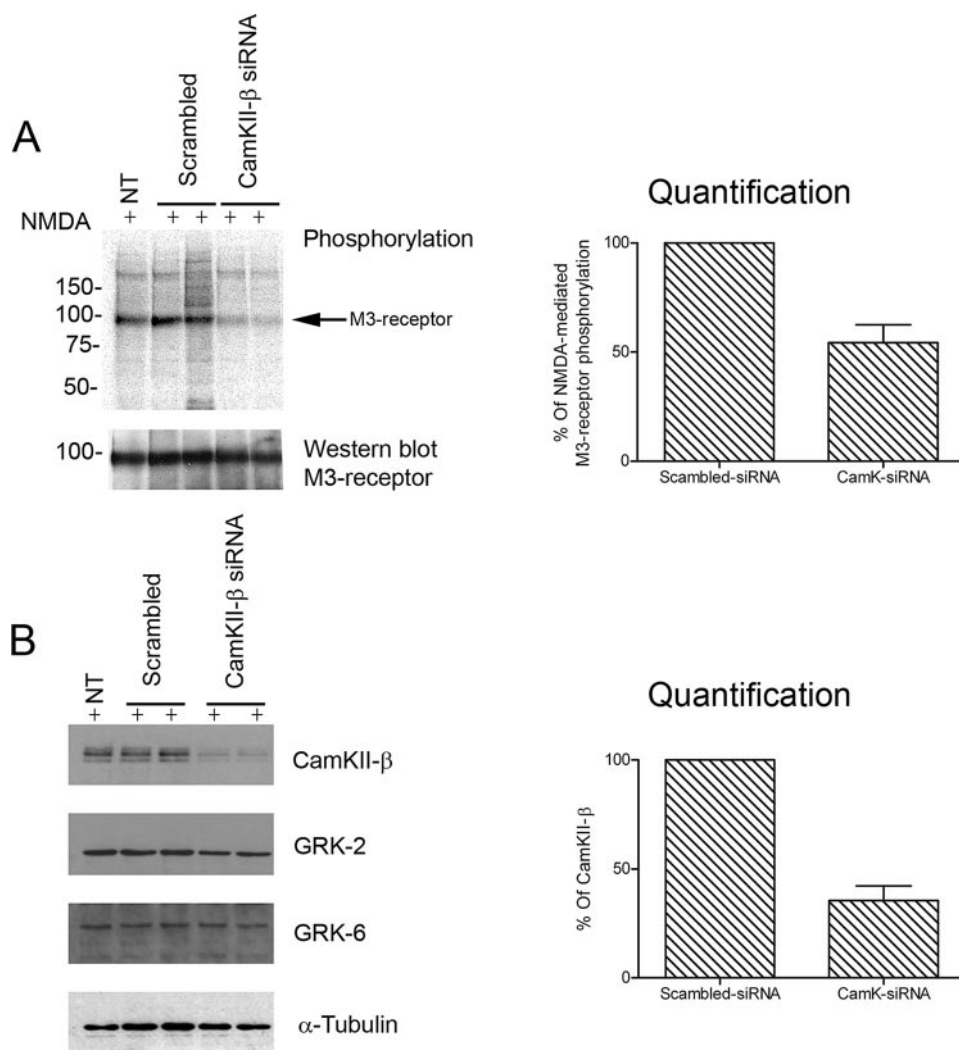
Glutamate increased muscarinic receptor phosphorylation by  $2.21 \pm 0.25$ -fold ( $n = 5$ ) over basal and NMDA by  $2.55 \pm 0.33$ -fold [ $n = 5$ ] (Fig. 1B). This response appears to be mediated via NMDA receptors because neither AMPA nor kainate had any significant effect on M<sub>3</sub>-muscarinic receptor phosphorylation (Fig. 1C).

Whereas addition of the muscarinic receptor antagonist atropine was able to completely block the phosphorylation mediated by direct activation of the M<sub>3</sub>-muscarinic receptor using the muscarinic agonist methacholine (Fig. 1B), atropine had no effect on the NMDA- or glutamate-mediated phosphorylation (data not shown). Furthermore, combined stimulation with NMDA and methacholine did not enhance the M<sub>3</sub>-muscarinic receptor phosphorylation over that induced by the agonists individually (data not shown).

A role of NMDA receptors was confirmed by use of the NMDA receptor-specific antagonists MK-801 and AP-5, which were able to block NMDA-mediated phosphorylation of the M<sub>3</sub>-muscarinic receptor (Fig. 1D). The NMDA showed a transient time course that reached a maximum at 5 min followed by a steady decrease to basal levels by 30 min (Fig. 1E).

**CamKII Activity Is Required for the Phosphorylation of M<sub>3</sub>-muscarinic Receptors by NMDA**—NMDA receptor activation will result in an influx of extracellular calcium and the subsequent activation of a number of calcium-dependent sig-

**FIGURE 2. Extracellular calcium and CamKII activity is required for NMDA-mediated phosphorylation of M<sub>3</sub>-muscarinic receptors.** CG neurons were [<sup>32</sup>P]orthophosphate labeled in calcium containing medium. *A*, cells were subsequently washed with calcium-containing (*left panel*) or nominally calcium-free (*right panel*) medium, and stimulated for 5 min with NMDA (100  $\mu$ M) or methacholine (100  $\mu$ M). *B*, phosphorylation of the M<sub>3</sub>-muscarinic receptor in response to NMDA (100  $\mu$ M, 5 min) in the presence or absence of inhibitors of CamKII, KN-93 and KN-62, and the inactive analogue, KN-92 (10  $\mu$ M). *C* and *D*, time course of the free intracellular calcium concentration in response to transient stimulation by methacholine or NMDA in the absence (*C*) or presence (*D*) of KN-62. The phosphorylation of M<sub>3</sub>-muscarinic receptors was quantified and normalized to the basal phosphorylation and presented as the mean  $\pm$  S.D. \*, significant difference between basal and stimulated samples ( $p < 0.01$ ). \*\*, significant difference between basal and stimulated samples ( $p < 0.05$ ). Calcium traces correspond to the changes in free intracellular calcium in single CG neurons and represent the response of at least 20 cells in three different assays.



**FIGURE 3. siRNA knock-down of CamKII reduces NMDA-mediated M<sub>3</sub>-muscarinic receptor phosphorylation.** CG neurons on day 5 of culture were sham-transfected (NT) or transfected with scrambled siRNA duplexes or siRNA directed against CamKII-β (80 pmol). *A*, after a further 2 days the cells were metabolically labeled with [<sup>32</sup>P]orthophosphate and stimulated with NMDA (100 μM for 5 min). The cells were then solubilized, and M<sub>3</sub>-muscarinic receptor was immunoprecipitated. The gel was transferred to nitrocellulose and exposed to reveal the phosphorylated receptor, after which the membrane was processed by Western blot to determine equal receptor loading. *B*, a small sample of the lysate from each condition was retained for Western blotting for CamKII-β, GRK-2, and GRK-6. Shown are the data from a typical experiment carried out at least three times. The graphs represent the cumulative data (mean ± S.E.) of at least three experiments carried out in duplicate.

naling molecules in particular CamKII (3, 4). We show here that the ability of NMDA receptors to stimulate the phosphorylation of M<sub>3</sub>-muscarinic receptors is dependent on the presence of extracellular calcium in the medium (Fig. 2). Following [<sup>32</sup>P]orthophosphate labeling, the cells were washed with medium (CSS-25) containing calcium or with nominally calcium-free medium. A subsequent stimulation with NMDA resulted in a robust phosphorylation of the M<sub>3</sub>-muscarinic receptor only in cells that were incubated in calcium containing medium (Fig. 2A). In contrast, phosphorylation of M<sub>3</sub>-muscarinic receptors in response to the muscarinic receptor agonist methacholine was not affected by the absence of calcium in the medium (Fig. 2A).

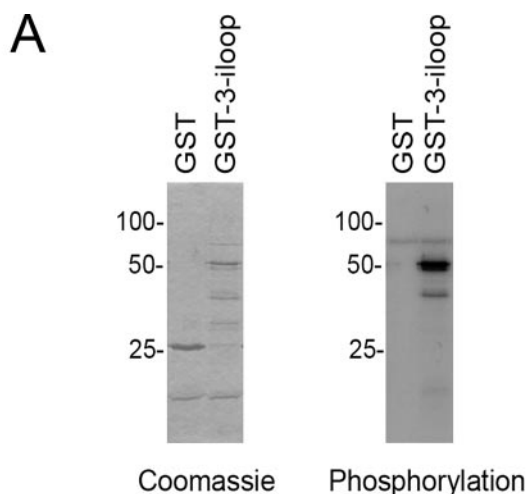
To investigate the role of CamKII in the NMDA-mediated response CG neurons were pretreated with the selective CamKII inhibitors KN-93 or KN-62. Both of these pharmaco-

logical inhibitors prevented NMDA-mediated muscarinic receptor phosphorylation, whereas the inactive analogue KN-92 had no effect (Fig. 2B). To control for the possibility that the CamKII inhibitors were working in an indirect manner by affecting free intracellular calcium concentrations, we measured the rise in intracellular calcium following NMDA treatment in the absence or presence of KN-62. As illustrated in Fig. 2 (C and D), the CamKII inhibitor did not alter the ability of NMDA to increase intracellular free calcium.

These pharmacological studies indicated that CamKII was involved in phosphorylation of M<sub>3</sub>-muscarinic receptors in response to NMDA-stimulation. To further test this, we used siRNA duplexes against the β-isoform of CamKII, which we found to be highly expressed in mouse CG neurons (supplemental Fig. S1). These siRNA duplexes significantly decreased CamKII-β by more than 70% but had no off target effects on kinases that have previous been shown to phosphorylate the GPCRs, *i.e.* GRK2, GRK6 (Fig. 3). Under conditions where CamKII-β was knocked down, NMDA-mediated phosphorylation of the M<sub>3</sub>-muscarinic receptor was decreased by 45.6 ± 8.1% (*n* = 3 + S.D.). These results indicated that CamKII-β, acting downstream of the NMDA receptor, either directly phosphorylates the M<sub>3</sub>-muscarinic receptor or is on a kinase cascade that results in the phosphorylation of the receptor.

**CamKII Is Able to Directly Phosphorylate the Third Intracellular Loop of the M<sub>3</sub>-muscarinic Receptor**—The mouse M<sub>3</sub>-muscarinic receptor contains a large third intracellular loop with 33 serine and 20 threonine residues. This compares with only three threonine and one serine in the short C-terminal tail. For this reason, and for that fact that the sites for agonist-regulated phosphorylation have previously been shown to be in the third intracellular loop (29, 34, 35), it was decided to test whether CamKII could phosphorylate the third intracellular loop *in vitro*. These experiments demonstrated that a bacterially expressed glutathione *S*-transferase fusion protein containing the third intracellular loop of the mouse M<sub>3</sub>-muscarinic receptor (Arg<sup>252</sup>–Thr<sup>491</sup>) was phosphorylated specifically in the receptor portion of the fusion protein by CamKII (Fig. 4A).





**B** Mouse M<sub>3</sub>-muscarinic receptor (3-i-loop underlined)

1	MTLHSNSTTS	PLFPNISSSW	VHS PSEAGLP	LGTVSQLDYS	NI SQTSGNFS
51	SNDTSSDPLG	GHTIWQVVF	AFLTGFALV	TIIIGNILVIV	AFKVNKQLKT
101	VNNYFLLSLA	CADLIIGVIS	MNLFTTYIIM	NRWALGNLAC	DLWLSIDYVA
151	SNASVMNLLV	ISFDRYFSIT	RPLTYRAKRT	TKRAGVMIGL	AWVISFVLWA
201	PAILFWQYFV	GKRTVPPGEC	FIQFLSEPTI	TFGTAIAAFY	MPVTIMTILY
251	<u>WRIYKETEKR</u>	<u>TKELAGLQAS</u>	<u>GTEAEAENFV</u>	<u>HPTGSSRSRCS</u>	<u>SYELQQQGTK</u>
301	RSSRRKYGGC	HFWFTTKSWK	P <sup>S</sup> AEQMDQDH	SSSDSWNNND	AAASLENSA <sup>S</sup>
351	<u>SDEEDIGSET</u>	<u>RAIYSIVLKL</u>	<u>PGHSTILNST</u>	<u>KLPSSDNLQV</u>	<u>PDKDLGTM<sup>D</sup>V</u>
401	<u>ERNAHKLQ<sup>A</sup>Q</u>	<u>KSMDDRNCQ</u>	<u>KDFSKLPIQL</u>	<u>ESAVDTAKTS</u>	<u>DTNSSVDKTT</u>
451	<u>AALPLSFKEA</u>	<u>TLAKRFALKT</u>	<u>RSQITKRKRM</u>	<u>SLIKEKAAQ</u>	<u>TL<sup>S</sup>AILLAFI</u>
501	ITWT <sup>S</sup> PYNIMV	LVNT <sup>S</sup> FCDSCI	PKT <sup>S</sup> YWNLGW	LCY <sup>S</sup> INSTVNP	VC <sup>S</sup> YALCNKTF
551	RTTFKMLLLC	QCDKRKRKQ	QYQQRQSVIF	HKRVP <sup>S</sup> EQAL	

FIGURE 4. *In vitro* phosphorylation of the M<sub>3</sub>-muscarinic receptor within the third intracellular loop by CamKII. A, bacterially expressed GST or GST-3i-loop (containing the third intracellular loop of the mouse M<sub>3</sub>-muscarinic receptor) were used in an *in vitro* phosphorylation using purified CamKII (200 ng). Shown is a representative experiment where the gel is first stained with Coomassie Blue and then an autoradiograph obtained. B, diagram representation of the CamKII phospho-acceptor sites Ser<sup>322</sup> and Ser<sup>350</sup> as determined by LC-MS/MS (in red) and a peptide that is singularly phosphorylated at one of three sites; serines 384/385 and threonine 397 (indicated in blue). (See supplemental Figs. S2–S4 for detailed traces.)

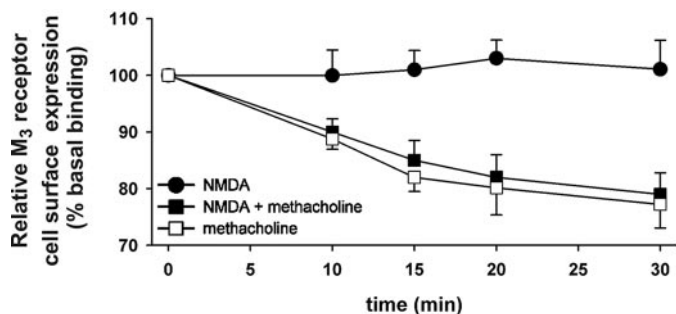


FIGURE 5. NMDA-mediated phosphorylation of the M<sub>3</sub>-muscarinic receptor does not mediate receptor internalization. CG neurons were stimulated with agonists (100 μM) for the indicated times. Surface M<sub>3</sub>-muscarinic receptor expression was then determined using the hydrophilic muscarinic antagonist [<sup>3</sup>H] *n*-methyl scopolamine. The data represent the means ± S.D. of three experiments carried out in duplicate.

Mass spectrometry analysis of tryptic and chymotryptic peptides established that Ser<sup>322</sup> (supplemental Fig. S2) and Ser<sup>350</sup> (supplemental Fig. S3) were phosphorylated *in vitro* by CamKII. Furthermore, one of the residues, serine 384/385 or threonine 397, are also phosphorylated (supplemental Fig. S4). These

studies demonstrate that CamKII can directly phosphorylate specific sites on the M<sub>3</sub>-muscarinic receptor *in vitro*. The data from these experiments are summarized in Fig. 4B.

**NMDA Receptor Stimulation Desensitizes M<sub>3</sub>-muscarinic Receptor Phosphoinositide Signaling but Does Not Affect Muscarinic Receptor Internalization**—Our laboratory and others have shown a clear link between the phosphorylation of M<sub>3</sub>-muscarinic receptors and receptor internalization (29, 36). Here we tested whether stimulation of M<sub>3</sub>-muscarinic receptor phosphorylation via NMDA receptors was able to mediate receptor internalization in CG neurons. Despite there being significant levels of receptor phosphorylation at early time points following NMDA stimulation, there was no evidence of a change in the cell surface expression of M<sub>3</sub>-muscarinic receptors as determined by radioligand binding (Fig. 5). In contrast, stimulation of the muscarinic receptors with the muscarinic agonist methacholine resulted in robust receptor internalization (Fig. 5).

M<sub>3</sub>-muscarinic receptors are coupled via G<sub>q/11</sub>-proteins to the activation of PLC, which catalyzes the hydrolysis of the phospholipid phosphoinositide 4,5-bisphosphate (PIP<sub>2</sub>) to generate two second messengers; diacylglycerol and inositol 1,4,5-trisphosphate (IP<sub>3</sub>) (37). It is possible to track the activation of this second messenger cascade in individual neurons in real time by using a biosensor consisting of the pleckstrin homology (PH) domain of PLCδ<sub>1</sub> fused to eGFP (38). This fusion protein (eGFP-PH<sub>PLCδ1</sub>) has high affinity for PIP<sub>2</sub>, and therefore when expressed in CG, neurons will locate to the inner leaflet of the plasma membrane (39). On hydrolysis of PIP<sub>2</sub> the eGFP-PH<sub>PLCδ1</sub> fusion protein translocates from the plasma membrane to the cytoplasm. This is in response to a depletion of membrane PIP<sub>2</sub> but also because the PH domain for PLCδ<sub>1</sub> has an ~20-fold higher affinity for IP<sub>3</sub> than for PIP<sub>2</sub> (40). By monitoring the translocation of eGFP-PH<sub>PLCδ1</sub> from the plasma membrane to the cytoplasm, it is possible therefore to measure the coupling of the muscarinic receptor to the phosphoinositide signal transduction cascade (39).

In this study, stimulation of the CG neurons with methacholine evoked a rapid and substantial translocation of eGFP-PH<sub>PLCδ1</sub> to the cytosol. The level of the phosphoinositide response (named S1) remained elevated for the length of the stimulation ( $F/F_0 = 1.81 \pm 0.12$ ;  $n = 20$ ; Fig. 6A). The value of S1 served as an internal standard. Following withdrawal of

## NMDA-mediated Regulation of $M_3$ -muscarinic Receptors

methacholine, the eGFP-PH<sub>PLC</sub> $\delta_1$  probe returned to the plasma membrane within 2 min (Fig. 6A). The cells subjected to a second stimulation with 100  $\mu$ M methacholine gave rise to a phosphoinositide response (named S2) of very similar shape and amplitude ( $F/F_0$   $1.73 \pm 0.10$ ;  $n = 12$ ; Student's  $t$  test,  $p > 0.05$ ) to the first (S1). However, when cells were treated with 100  $\mu$ M NMDA for 5 min before the second stimulation with methacholine, the amplitude of S2 was significantly reduced ( $F/F_0$   $1.43 \pm 0.08$  [ $n = 18$ ]; Fig. 6B). These data demonstrate that NMDA receptor stimulation was able to desensitize the  $M_3$ -muscarinic receptor phosphoinositide response.

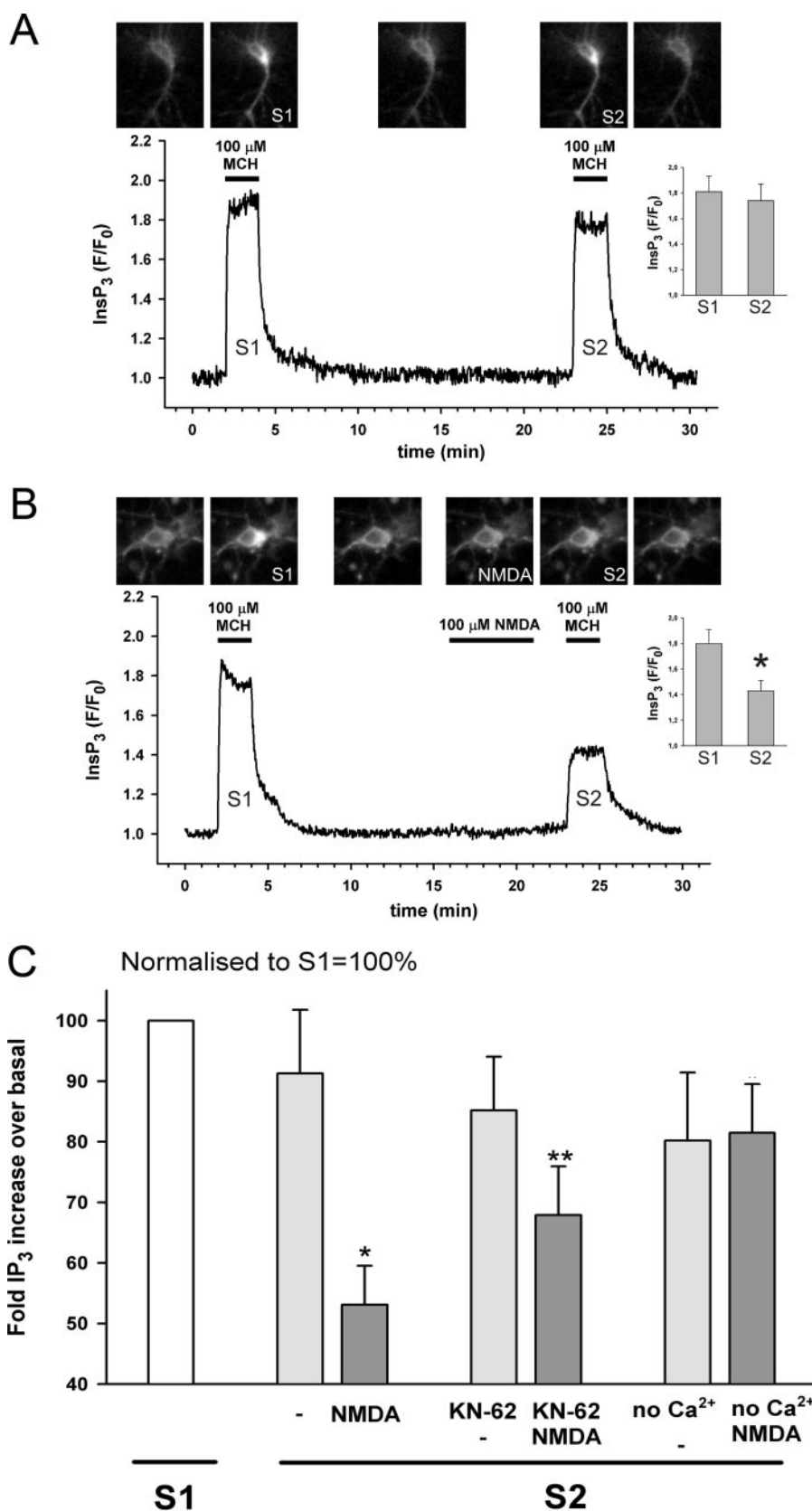
**NMDA-mediated Desensitization of the  $M_3$ -muscarinic Receptor Is Dependent on Extracellular Calcium and Calcium/Calmodulin-dependent Kinase II Activity**—The ability of NMDA to mediate desensitization of the  $M_3$ -muscarinic receptor phosphoinositide response was completely dependent on the presence of extracellular calcium in the medium (Fig. 6C). Withdrawing extracellular calcium for the period of NMDA stimulation resulted in an S2 response to methacholine, which was not significantly different from controls (Fig. 6C).

Inhibition of CamKII using KN-62 significantly inhibited the ability of NMDA treatment to desensitize the methacholine response (Fig. 6C). Control experiments established that KN-62 did not interfere nonspecifically with the muscarinic receptor phosphoinositide response (data not shown). Importantly, KN-62 had no indirect effects on the ability of NMDA receptors to mediate an influx of extracellular calcium (Fig. 2, C and D). These data indicate that the ability of NMDA to desensitize the  $M_3$ -muscarinic receptor phosphoinositide response is dependent on extracellular calcium influx and CamKII activity.

## DISCUSSION

It is clear from a large number of studies that GPCRs can influence the activity of NMDA receptors and in so doing regulate physiological responses such as LTP and LTD (6). The

current study extends these findings by demonstrating that NMDA receptors can also feedback onto GPCR signaling. Hence, there appears to be a reciprocal regulatory process





where both receptor types are able to mediate cross-talk allowing for fine tuning of the signaling mechanisms that ultimately lead to physiological processes such as synaptic plasticity.

The M<sub>3</sub>-muscarinic receptor subtype is a “classical” G<sub>q/11</sub>-coupled receptor that has been shown to couple to PLC- $\beta$  isoforms in CG neurons (41). Furthermore, muscarinic receptor subtypes are among a group of GPCRs that are known to regulate NMDA function through a number of mechanisms (16, 24–27). Hence, CG neurons are an ideal native cell type to test the possibility that NMDA receptors might cross-talk with GPCRs. We have done this here by focusing on the post-translational modification and signaling properties of the M<sub>3</sub>-muscarinic receptor.

Our previous studies have determined that the M<sub>3</sub>-muscarinic receptor is phosphorylated in response to agonist occupation by a range of receptor-specific kinases that include members of the well characterized G-protein-coupled receptor kinase (GRK) family (29, 42) and also by protein kinases CK2 (29) and CK1 $\alpha$  (34). In these studies we have found no evidence for a role of calcium-regulated protein kinases in agonist-mediated phosphorylation of M<sub>3</sub>-muscarinic receptors (32, 43). Confirmation of this fact is reported in the current study where withdrawal of extracellular calcium from the medium had no significant effect on the agonist-regulated phosphorylation of the M<sub>3</sub>-muscarinic receptor in CG neurons. In contrast, we show here that stimulation of NMDA receptors results in phosphorylation of M<sub>3</sub>-muscarinic receptors in CG neurons via a mechanism that is completely dependent on extracellular calcium. This immediately suggested that the elevation of intracellular calcium mediated via calcium entry through the NMDA receptors was activating calcium-regulated protein kinases responsible for M<sub>3</sub>-muscarinic receptor phosphorylation. CamKII is a well established downstream effector of calcium entry through NMDA receptors (4). By the use of pharmacological inhibitors and siRNA, we established here that CamKII activity was necessary for NMDA-mediated phosphorylation of the M<sub>3</sub>-muscarinic receptor.

The data presented here does not, however, show conclusively that CamKII directly phosphorylates the M<sub>3</sub>-muscarinic receptor. The possibility that CamKII is on a protein kinase cascade that results in receptor phosphorylation cannot be eliminated.

Our previous work had determined that the M<sub>3</sub>-muscarinic receptor is phosphorylated on different sites by different protein kinases and that this results in differential regulation of receptor signaling (29). This is illustrated by the fact that M<sub>3</sub>-muscarinic receptor internalization is mediated by GRK phosphorylation (36), whereas phosphorylation by protein kinase CK2 has no effect on receptor internalization but regu-

lates the ability of the receptor to signal to the Jun-kinase pathway (29). In this regard it is interesting to observe in the current study that CamKII-dependent phosphorylation of the M<sub>3</sub>-muscarinic receptor in CG neurons does not mediate receptor internalization. It is likely, therefore, that CamKII directs phosphorylation of the receptor on different sites from that of the GRKs and that the signaling outcome of phosphorylation is therefore different (44). Our mass spectrometry analysis demonstrated that the third intracellular loop of the M<sub>3</sub>-muscarinic receptor can act as a substrate for CamKII. Ongoing work in the laboratory is aimed at determining whether the sites of CamKII are indeed distinct from that of established receptor kinases (*i.e.* GRK2/6, CK1 $\alpha$ , and CK2).

To determine the functional significance of NMDA-mediated phosphorylation of the M<sub>3</sub>-muscarinic receptor, we examined the coupling of the receptor to the G<sub>q/11</sub>/phosphoinositide signaling pathway. Although it is possible to measure bulk IP<sub>3</sub> production (45), this approach is not sufficiently sensitive for reliable use in CG neurons. We therefore turned to measuring the activation of the phosphoinositide signaling cascade via the translocation of the PIP<sub>2</sub>/IP<sub>3</sub> biosensor eGFP-PH<sub>PLC $\delta$ 1</sub> (39). A short pulse of NMDA was able to significantly desensitize the M<sub>3</sub>-muscarinic receptor-mediated phosphoinositide response. This was dependent both on extracellular calcium and CamKII activity and as such mimicked characteristics of NMDA-mediated receptor phosphorylation. It appears, therefore, that the process of NMDA-mediated desensitization of the M<sub>3</sub>-muscarinic receptor response is a consequence of CamKII-dependent phosphorylation of the receptor.

This study, coupled with our previously published work, suggests a complex process of by which NMDA receptors can regulate the M<sub>3</sub>-muscarinic receptor phosphoinositide response in CG neurons. Here we demonstrate that pre-exposure to NMDA results in desensitization of the phosphoinositide response, whereas our previous work showed that concomitant NMDA/muscarinic receptor activation results in an enhancement of phosphoinositide signaling (41). Both of these effects are via calcium entry-mediated by the NMDA receptor. In the pre-exposure experiments, calcium entry results in CamKII activation and receptor phosphorylation, which uncouples the receptor from G<sub>q/11</sub>. If, however, the NMDA receptor is simultaneously stimulated with the M<sub>3</sub>-muscarinic receptor, then the elevated intracellular calcium levels will feed forward to sensitize PLC- $\beta$  and enhance the receptor-mediated response in a process that allows for coincidence detection (41, 46). Thus, the signaling outcome of the interaction between NMDA and M<sub>3</sub>-muscarinic receptors is dependent on the temporal pattern of NMDA and muscarinic receptor stimulation.

**FIGURE 6. Effect of NMDA on the M<sub>3</sub>-muscarinic receptor phosphoinositide response is dependent on calcium entry and CamKII activity.** CG neurons were transfected with eGFP-PH<sub>PLC $\delta$ 1</sub> and grown for 7–9 days before being imaged on an inverted epifluorescence microscope. *A*, continuously perfused cells were subjected to two stimulations with methacholine (100  $\mu$ M). Shown is the time course of eGFP-PH<sub>PLC $\delta$ 1</sub> translocation from the plasma membrane to the cytoplasm in a representative single cell expressed as changes in the cytoplasmic F/F<sub>0</sub> self-ratio (see “Experimental Procedures”). Shown also are representative fluorescent images of CG neurons stimulated with methacholine (100  $\mu$ M) for 0, 3, 15, 24, and 29 min. *B*, same as *A* but including a 5-min stimulation with NMDA (100  $\mu$ M) prior to the second methacholine stimulation. Shown are images of a cell at times 0, 3, 15, 18, 24, and 29 min. *C*, the effects of the CamKII inhibitor KN-62 (10  $\mu$ M) added 10 min before NMDA or the effects of removal of extracellular calcium on the NMDA-mediated desensitization of the M<sub>3</sub>-muscarinic receptor phosphoinositide response. The data are representative traces of 12–18 cells taken from 8–10 independent experiments. The insets represent the means  $\pm$  S.D. of the S1 and S2 phosphoinositide responses. \*, significant difference between S1 and S2 ( $p < 0.01$ ). \*\*, significant difference between S1 and S2 ( $p < 0.05$ ).

This complex relationship between the NMDA and muscarinic receptors in CG neurons is consistent with an emerging understanding of the multilayered regulatory mechanisms that control NMDA-mediated synaptic plasticity. The fact that neuronal plasticity is itself a dynamically regulated process is now acknowledged and described by some as metaplasticity or “plasticity of plasticity” (6, 47). Signaling pathways regulated by GPCRs have been identified as major players in regulating NMDA receptor function and defining the subtle changes in NMDA receptor activity that determines whether a threshold is obtained that drives synaptic strengthening (LTP) or alternatively the threshold associated with synaptic weakening (LTD) is the one that is attained. Our identification, in this study, of a further layer of interaction between GPCRs and NMDA receptors reveals an additional regulatory mechanism that can be recruited by neurons in fine tuning the signaling networks that converge on synaptic plasticity.

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