Caveolin-1 expression is essential for \(N\)-methyl-d-aspartate receptor-mediated Src and extracellular signal-regulated kinase 1/2 activation and protection of primary neurons from ischemic cell death

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ABSTRACT  
N-Methyl-D-aspartate (NMDA) receptor (NMDAR) activation and downstream signaling are important for neuronal function. Activation of prosurvival Src family kinases and extracellular signal-regulated kinase (ERK) 1/2 is initiated by NMDAR activation, but the cellular organization of these kinases in relation to NMDARs is not entirely clear. We hypothesized that caveolin-1 scaffolds and coordinates protein complexes involved in NMDAR signaling and that this organization is necessary for neuronal preconditioning, whereby NMDAR activation protects neurons from subsequent ischemic cell death. We found that sublethal ischemia (SLI) or preconditioning via NMDA treatment of primary cortical neurons from neonatal rats or mice increases expression of phosphorylated (P) caveolin-1, P-Src, and P-ERK1/2. The NMDAR antagonist, MK801, or the Src inhibitor, PP2, attenuated SLI-induced preconditioning. NMDAR2B distributed to buoyant fractions and heavy fractions, partially colocalized with caveolin-1 and the membrane raft marker, cholera toxin B. Cultures of primary neurons treated with caveolin-1 small interfering RNA or from caveolin-1−/− mice lacked the NMDA-mediated increase in P-Src and P-ERK, as well as SLI- and NMDA-induced preconditioning. Adenovirally mediated expression of caveolin-1 in neurons from caveolin-1−/− mice restored NMDA-mediated enhancement of P-Src and P-ERK1/2, redistributed NMDAR2B to buoyant fractions, and enhanced NMDAR2B localization to membrane rafts. We conclude that caveolin-1, perhaps via its ability to scaffold key signaling components, is essential for NMDAR localization to neuronal membrane rafts, NMDAR/Src tyrosine kinase family/ERK signaling, and protection of neurons from ischemic injury and cell death.—Head, B. P., Patel, H. H., Tsutsumi Y. M., Hu, Y., Mejia, T., Mora, R. C., Insel, P. A., Roth, D. M. Drummond, J. C., Patel, P. M. Caveolin-1 expression is essential for N-methyl-D-aspartate receptor-mediated Src and ERK 1/2 activation and protection of primary neurons from ischemic cell death. FASEB J. 22, 000–000 (2008)

Key Words: preconditioning • neuroprotection • neuronal apoptosis • membrane rafts • ionotropic glutamate receptors • colocalization

In the central nervous system, the \(N\)-methyl-D-aspartate (NMDA) receptor (NMDAR), a subtype of excitatory glutamate receptors, plays key roles in synaptic plasticity (1), neuronal development (2), and excitotoxicity (3). Src tyrosine kinase family (SFK) members up-regulate NMDAR activity and contribute to the regulation of NMDAR-mediated synaptic long-term potentiation (4–5). NMDAR activation has also been implicated in triggering cerebral preconditioning (7, 8), a phenomenon whereby stimuli (e.g., sublethal ischemia or pharmacological agents) protect the brain from subsequent lethal ischemia (9–12). However, the mechanisms that control NMDAR-induced preconditioning are poorly understood.

Signal transduction molecules are commonly organized by scaffolding proteins into molecular complexes that help provide coordinated, precise, and rapid regulation of cell function. One such protein, caveolin, scaffolds and regulates multiple types of signaling molecules (13–17); this scaffolding and regulation appear to depend on an intact cytoskeleton (18). Caveolin was originally identified and thought to localize exclusively in caveolae, flask-like invaginations of the plasma membrane that exist in numerous cell types but are absent in neurons; recent data, however, implicate caveolins in physiological roles independent of caveolae (19). Although caveolin can be expressed in neurons independently of caveolae, no role has been established for caveolin in NMDAR signaling. Modulation of caveolin, either in terms of total caveolin expression or posttrans-
lational modification (e.g., phosphorylation) or through cytoskeletal disruption (i.e., microtubule and actin depolymerization) (18), can have a profound impact on cell signaling and physiology (20–22). Caveolin serves as a substrate and scaffold for SFKs, key regulators of NMDAR activity (4, 23–25). The ability of caveolin-1 to regulate SFK activity led us to hypothesize that caveolin organizes NMDAR subtypes with SFKs in discrete molecular complexes in neurons to modulate NMDAR-mediated signal transduction and function.

In the present study we tested the hypotheses that 1) caveolin scaffolds NMDAR subtypes and related signaling components in discrete neuronal microdomains and 2) that caveolin expression is essential for NMDA-mediated neuronal signaling (e.g., preconditioning) via SFK phosphorylation and activation of the prosurvival extracellular signal-regulated kinase (ERK) pathway.

MATERIALS AND METHODS

All animal studies were approved by the Veterans Affairs San Diego Institutional Animal Care and Use Committee, and experimental procedures were performed in accordance with the guidelines established in the U.S. Public Health Service Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23).

Materials

Antibodies to Src and phosphorylated (P)-src kinase were from Cell Signaling Technology Inc. (Beverly, MA, USA), to ERK1/2 and P-ERK1/2 were from Stressgen Biotechnologies Corp. (Victoria, BC, Canada), to P-caveolin-1 were from Chemicon International (Billerica, MA, USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), to total caveolin-1 and caspase-activated deoxyribonuclease (CAD) were from Santa Cruz Biotechnology, Inc., to NMDAR2B were from BD Biosciences (San Jose, CA, USA) and Imgenex (San Diego, CA, USA), and to neuronal nuclear protein (NeuN), β3-tubulin, and double cortin were from Abcam (Cambridge, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase antibody was obtained from Imgenex. Cholera toxin B (CT-B) subunit, fluorescein isothiocyanate (FITC), and Alexa-conjugated secondary antibodies were from Molecular Probes/Invitrogen (Carlsbad, CA, USA). Other secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. All other chemicals and reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless otherwise stated.

In vitro model of neuronal preconditioning

Neonatal rat or mouse neurons were isolated using a papain dissociation kit (Worthington Biochemical Corp., Lakewood, NJ, USA) as described previously (26). Neurons were cultured in Neurobasal A medium supplemented with B27 (2%), 250 mM GLUTMax1, and penicillin/streptomycin (1%). Cells were cultured on poly(D-lysine)/laminin (2 µg/cm²)-coated plates at 37°C in 5% CO₂ for 7 d before experiments. Primary neurons were subjected to preconditioning stimuli [NMDA or sublethal oxygen glucose deprivation (OGD₉₅)] 24 h before exposure to lethal OGD (OGD₉₀) as described (7). Neurobasal medium was replaced with glucose-deprived medium, and cells were placed in an anaerobic chamber (37°C) containing mixed gas (95% N₂/5% CO₂) to remove residual oxygen. Cell death was assayed using trypan blue and phase-contrast light microscopy and/or CAD-positive cells, as determined by immunofluorescence and deconvolution microscopy. Cell death was normalized to the total number of cells imaged.

Small interfering RNA (siRNA) transfection

The expression of caveolin-1 was suppressed in neonatal rat and mouse neurons by using targeted siRNA (Ambion Inc., Austin, TX, USA). The specific caveolin-1 siRNA sequences were as follows: sense, GGGAAUUGAUUCUGGUCAACtt; and antisense, GUUGACGAGUAUUUCCtt. Cells were treated with 0.2 µg of siRNA using RNAiMAX transfection reagent (Invitrogen) for 72 h. Transfection reagent and negative siRNA (scrambled sequence of similar length; Ambion Inc.) served as controls. Functional knockdown of protein expression was assessed by immunoblot and immunofluorescence microscopy.

Sucrose-density fractionation

Membrane rafts were isolated from adult brain and neurons using detergent-free methods. Cells were washed in PBS, scraped in sodium carbonate (150 mM, pH 11.0), and then sonicated with three cycles of 20-s bursts with a 1-min incubation on ice. Homogenate (1 ml) was mixed with 1 ml of 80% sucrose. The mixture was centrifuged at 175,000g using an SW41Ti rotor (Beckman Coulter, Fullerton, CA, USA) for 3 h at 4°C. Samples were removed in 1-ml aliquots.

Immunoprecipitation and immunoblot analysis

Immunoprecipitations were performed using either protein A- or protein G-agarose (Roche, Basel, Switzerland). Antibodies used for immunoprecipitations were the following: caveolin-1 (Santa Cruz Biotechnology, Inc.) and NMDAR2B (Calbiochem, San Diego, CA, USA or Imgenex). Lysates were incubated with primary antibody for 1–3 h at 4°C, immunoprecipitated with protein-agarose overnight at 4°C, and then centrifuged at 15,000g for 5 min. Protein-agarose pellets were washed once in lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1% Igepal CA-630, 1% deoxycholic acid, and 0.2% sodium dodecyl sulfate) followed by subsequent washes in wash buffer 2 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.2% Igepal CA-630) and wash buffer 3 (10 mM Tris-HCl, pH 7.5, and 0.2% Igepal CA-630). Proteins in fractions, immunoprecipitates, and cell lysates were separated by SDS-PAGE using 10% acrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore Co., Bedford, MA, USA) by electroelution. Membranes were blocked in 20 mM PBS Tween (1%) containing 1.5% nonfat dry milk and incubated with primary antibody overnight at 4°C. Primary antibodies were visualized using secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc.) and ECL reagent (Amersham Pharmacia Bio-Tech, Piscataway, NJ, USA). All displayed bands migrated at the appropriate size, as determined by comparison with molecular weight standards (Santa Cruz Biotechnology, Inc.). The amount of protein per fraction was determined using a dye-binding protein assay (Bio-Rad, Hercules, PA).

Immunofluorescence and deconvolution microscopy

Neurons were prepared for immunofluorescence microscopy as described previously (27). Antibodies used for immunofluorescence were the following: caveolin-1 (Santa Cruz Biotechnology, Inc. or Cell Signaling Technology, Inc.), NMDAR2B (Calbiochem or BD Biosciences), β3-tubulin and double cortin (Abcam), and P-ERK1/2 (Stressgen Biotechnologies Corp.) as described previously. Secondary antibodies were visualized using secondary antibodies conjugated to Alexa (Molecular Probes/Invitrogen) or IgG (Abcam). Stained neurons, rafts, and membranes were visualized using confocal microscopy (Zeiss, Jena, Germany). Fluorescence images were acquired by deconvolution microscopy (Molecular Dynamics, Sunnyvale, CA).
Tissue and cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, incubated with 100 mM glycine (pH 7.4) for 10 min to quench aldehyde groups, permeabilized in buffered Triton X-100 (0.1%) for 10 min, blocked with 1% bovine serum albumin (BSA)/PBS/Tween (0.05%) for 20 min, and then incubated with primary antibodies (1:100) in 1% BSA/PBS/Tween (0.05%) for 24–48 h at 4°C. Excess antibody was removed by incubation with PBS/Tween (0.1%) for 15 min and incubated with FITC or Alexa-conjugated secondary antibody (1:250) for 1 h. To remove excess secondary antibody, cells were washed six times at 5-min intervals with PBS/Tween (0.1%) and incubated for 20 min with the nuclear stain 4,6-diamidino-2-phenylindole (DAPI) (1:5000) diluted in PBS. Cells were washed for 10 min with PBS and mounted in Gelvatol for microscopic imaging. Deconvolution images were obtained as described (27) and captured with a DeltaVision deconvolution microscope system (Applied Precision, Inc., Issaquah, WA, USA). The system includes a Photometrics charge-coupled device mounted on a Nikon TE-200 inverted epifluorescence microscope. Between 30 and 80 optical sections spaced by ∼0.1–0.3 mm were taken. Exposure times were set such that the camera response was in the linear range for each fluorophore. Lenses included ×100 (numerical aperture (NA) 1.4), ×60 (NA 1.4), and ×40 (NA 1.3). The data sets were deconvolved and analyzed using SoftWorx software (Applied Precision, Inc) on a Silicon Graphics Octane workstation. Image analysis was performed with the Data Inspector program in SoftWorx. Maximal projection volume views or single optical sections are shown as indicated. Colocalization of pixels was assessed quantitatively by CoLocalizer Pro 1.0 software (http://www.homepage.mac.com/colocalizerpro/). An overlap coefficient according to Manders was used to determine the degree of colocalization on whole cells or membrane regions of interest after subtraction of the background through normalized threshold values (28). The values were defined as 0 to 1 with 1 implying that 100% of both components overlap with the other part of the image. Statistical analysis was performed using Prism.

Adenoviral-mediated overexpression of caveolin-1

An adenoviral construct for human caveolin-1 (a gift from Dr. E. Rodriguez-Boulan, Cornell University, Ithaca, NY, USA) was used to overexpress caveolin-1 in primary neurons isolated from caveolin-1 knockout mice. Cells were treated with the caveolin-1-containing adenovirus or LacZ control adenovirus for 48 h (multiplicity of infection = 300).

**Figure 1.** NMDA and OGD$_{SL}$ enhance P-caveolin-1, P-Src, and P-ERK1/2 in cultured cortical neurons. Neurons were subjected to either NMDA (10 μM) or OGD$_{SL}$ for 25 min. A) Immunoblot analysis revealed that NMDA or OGD$_{SL}$ significantly increased P-Cav-1 (21 kDa), P-Src (60 kDa) ($n=4$), and P-ERK1/2 (44/42 kDa) without altering total caveolin-1, Src, or ERK1/2 protein expression in neuronal cell lysates ($n=4$). Values are expressed as mean ± se. *$P < 0.05$ vs. control (Ctrl); $\dagger P < 0.05$ vs. Ctrl. B) Immunofluorescence microscopy of primary neurons revealed enhanced P-Cav-1 (red pixels) after NMDA (ii) or OGD$_{SL}$ (iii) compared with control [neuronal nuclear protein (NeuN), green pixels]. Biv) Pixel analysis reveals that P-Cav-1 (normalized to NeuN) was significantly enhanced in neurons subjected to NMDA or OGD$_{SL}$ ($n=3$). C, D) P-Src (C, red pixels) and P-ERK1/2 (D, red pixels) were enhanced in NMDA-treated (10 μM; 25 min) neurons compared with control ($n=4$). Pixels analysis reveals that P-Src (Ciii) and P-ERK1/2 (Diii) were significantly enhanced when normalized to NeuN (green pixels). Cells were additionally stained with the neuronal marker, NeuN (green pixels), and DAPI (nuclear stain). Values are expressed as mean ± se. *$P < 0.05$ vs. Ctrl; $\dagger P < 0.05$ vs. Ctrl. Scale bar = 20 μm.

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Statistical analysis

Data are expressed as means ± se. Student’s unpaired t test or one-way analysis of variance with a post hoc Bonferroni correction were used to compare treatment groups. Significance was established at a level of $P < 0.05$.

RESULTS

Pharmacological (NMDA) or ischemic (OGD$_{SL}$) preconditioning stimuli increase the expression of P-caveolin-1, P-Src, P-ERK1/2, and P-p38 in primary rat cortical neurons

Immunoblot analysis of NMDA-stimulated (10 μM; 25 min) or OGD$_{SL}$-treated (25 min, 5 min reoxygenation) neurons revealed a significant enhancement in expression of P-caveolin-1, P-Src, and P-ERK1/2 (Fig. 1A). These findings were confirmed by immunofluorescence microscopy, which revealed that expression of P-caveolin-1 was enhanced after exposure to NMDA (Fig. 1Bii) or OGD$_{SL}$ (Fig. 1Biii); total caveolin-1 expression was unchanged (data not shown). NMDA treatment also increased neuronal expression of P-Src (Fig. 1Cii) and P-ERK1/2 (Fig. 1Dii).

Caveolin-1 and NMDAR2B colocalize and coimmunoprecipitate in primary rat cortical neurons

NMDAR2B is tyrosine-phosphorylated (via SFK), found in postsynaptic densities (29), and contains two consen-
Figure 3. NMDAR2B colocalizes with the membrane raft marker, CT-B, and is present in BFs from primary neurons. Primary neurons were stained for caveolin-1 and CT-B or NMDAR2B and CT-B. A–C) Approximately 90% of the membrane raft marker, CT-B (A), colocalized with caveolin-1 (B), whereas ~50% of caveolin-1 colocalized with CT-B. D–F) Approximately 40% of CT-B (D) and NMDAR2B (E) colocalized. G) Quantitation of colocalization data. H) Sucrose density fractionation revealed that under basal conditions NMDAR2B was present in both BFs and HFs. After a pharmacological preconditioning stimulus (10 μM NMDA; 25 min), NMDAR2B redistributed to only HFs. Scale bar = 10 μm.

Figure 4. NMDA or OGD_{SL} treatment protects neurons against OGD_{L}-mediated cell death. Primary neurons were exposed to NMDA (10 μM; 25 min) or OGD_{SL} (25 min) 24 h before OGD_{L} (60 min), and 24 h later neuronal cell death was assessed with trypan blue staining. A) OGD_{L} (ii, n=3) significantly increased neuronal cell death (trypan blue-positive cells) compared with control cells (i). NMDA (iii) or OGD_{SL} (iv) treatment of neurons 24 h before OGD_{L} reduced neuronal cell death compared with neurons subjected to OGD_{L} alone. B) The NMDAR antagonist, MK801, and the Src kinase inhibitor, PP2, blocked both NMDA and OGD_{SL}-mediated neuronal cell preconditioning in vitro (n=3). Values are expressed as mean ± se. *P < 0.05 vs. Ctrl; #P < 0.05 vs. OGD_{L} vehicle; &P < 0.05 vs. NMDA-OGD_{L} vehicle; ϕP < 0.05 vs. OGD_{SL}-OGD_{L} vehicle.
sus sequences (amino acids 635–642, WAffAVIF and 1042–1049, FSKSDRY) that are necessary for binding to caveolin (to what is termed the caveolin binding motif) (15, 16). Immunofluorescence microscopy showed that caveolin-1 (Fig. 2A, D) colocalizes with NMDAR2B (Fig. 2B, E) on both the cell body and along neuronal extensions resembling dendritic shafts and spines, both of which are highly enriched in
NMDAR2B-containing NMDARs (30, 31) (Fig. 2C, F). Coimmunoprecipitation experiments using neuronal lysates and antibodies to caveolin-1 and NMDAR2B provided further evidence for the interaction of these proteins (Fig. 2H). These findings thus document the fact that caveolin-1 and NMDAR2B colocalize in the soma and along dendritic shafts of primary rat cortical neurons in culture.

NMDAR2B partially colocalizes with CT-B subunit and is present in buoyant fractions but can be redistributed by NMDA

Because >50% of NMDAR subtypes localize to neuronal membrane rafts (32), we performed immunofluorescence microscopy on primary rat neurons to assess whether NMDAR2B is present in neuronal membrane rafts. We found that ~90% of the membrane raft marker, CT-B (Fig. 3A), colocalized with caveolin-1 (Fig. 3B), whereas ~50% of caveolin-1 colocalized with CT-B (Fig. 3C, G, left graph), with ~40% of CT-B (Fig. 3D) and NMDAR2B (Fig. 3E) colocalizing with each other (Fig. 3F, G, right graph). We assessed whether NMDAR2B distributes to buoyant, low-density fractions (BFs) after sucrose density fractionation and found that under basal conditions NMDAR2B was present in both BFs and heavy fractions (HFs). After agonist stimulation (10 μM NMDA; 25 min), NMDAR2B redistributed to only HFs (Fig. 3H). Thus, NMDAR2B localizes to neuronal membrane rafts, and this localization is altered by agonist.

NMDA or OGDSL stimulation protects rat cortical neurons from subsequent OGD

Cortical neurons exposed to NMDA (10 μM; 25 min) or OGD (25 min) 24 h before OGD (1 h) have reduced cell death compared with neurons exposed only to OGD (Fig. 4). NMDA- and OGD-induced preconditioning were significantly attenuated by treatment of neurons with the NMDAR antagonist, MK801 (10 μM, 15 min), or the Src kinase inhibitor, PP2 (10 μM, 1 h) (Fig. 4B), results that are consistent with the idea that NMDAR agonism and Src activation contribute to neuronal preconditioning. Brief episodes of sublethal OGD or brief exposure to NMDA thus activate NMDAR and Src kinase to protect neurons from a subsequent lethal event.

Figure 5. siRNA-mediated knockdown of caveolin-1 decreases NMDA-mediated phosphorylation of Src and ERK1/2

We used siRNA to knock down caveolin-1 (Cav1-siRNA) to assess the potential role of caveolin-1 in NMDA receptor signaling and preconditioning of rat neurons in culture. After a 72-h treatment with Cav1-siRNA, immunoblot analysis (Fig. 5A, B) and immunofluorescence microscopy (Fig. 5C, D) showed that caveolin-1 protein expression was decreased by ~67% compared with the control or siRNA-scrambled control. Cav1-siRNA-treated neurons showed a complete inhibition of NMDA (10 μM; 25 min)-mediated activation of ERK1/2 and attenuated NMDA-mediated activation of P-Src (Fig. 5A, B) compared with NMDA-treated controls and NMDA-treated scrambled siRNA controls. In addition, siRNA-mediated knockdown of caveolin-1 attenuated both NMDA- and OGDSL-induced neuronal preconditioning (Fig. 5E). Thus, siRNA-mediated knockdown of caveolin-1 disrupts NMDA-mediated phosphorylation of Src and ERK1/2 and neuronal preconditioning.

NMDA and OGDSL fail to modulate Src and ERK1/2 and do not precondition caveolin-1 knockout neurons

As a further test of the role of caveolin-1, we exposed primary neurons cultured from caveolin-1 knockout and wild-type (WT) mice to NMDA (10 μM; 25 min) or OGD (25 min; 5 min reoxygenation). Under basal conditions, expression of both P-Src and P-ERK1/2 was enhanced in neurons from caveolin-1 knockout mice (Fig. 6A, right panel vs. left panel), results akin to findings in non-neuronal cells (21). Relative to responses of WT neurons, neurons from caveolin-1 knockout mice that were treated with either NMDA or OGD showed a prominent blunting in the increase over basal of P-Src or P-ERK1/2 expression. Moreover, treatment of neurons from WT but not caveolin-1 knockout mice with NMDA or OGD reduced cell death, as assessed by trypan blue staining, compared with lethal levels of OGD (OGDL, 60 min) (Fig. 6B). We confirmed the latter results by using an alternative assay: immunofluorescence microscopy and assessment of an apoptotic marker, CAD (red pixels: i, iv, vii, and x), expression of which was elevated in WT neurons (Fig. 6C) exposed to OGD (Fig. 6Cii, vi), but not if pretreated with NMDA (Fig. 6Cvi, ix) or OGD (Fig. 6Cx, xii). In contrast (Fig. 6D), CAD immunofluorescent staining is minimal.
Figure 6. NMDA or OGD_{sl} fails to enhance P-Src or P-ERK1/2 or to protect caveolin-1 knockout neurons from exposure to lethal levels of OGD. A) Immunoblot analysis revealed that exposure of caveolin knockout (Cav-1^-/-) neurons to NMDA (10 \mu M; 25 min) or OGD_{sl} (25 min) failed to enhance P-Src or P-ERK1/2 compared with nontreated Cav-1^-/- neurons (n=4). B) In WT neurons trypan blue staining revealed that NMDA (10 \mu M; 25 min) or OGD_{sl} (25 min) reduced neuronal cell death compared with OGD_{sl} (60 min) 24 h later (WT: OGD_{sl} vs. CTRL, n=4). In contrast, in Cav-1^-/- neurons, pretreatment with NMDA or OGD_{sl} failed to protect neurons against OGD_{sl} (B, Cav-1^-/-: NMDA or OGD_{sl} vs. CTRL, n=4). Values are expressed as mean ± se. *P < 0.001 vs. CTRL-WT; †P < 0.05 vs. Cav-1 KO CTRL; ‡P < 0.05 vs. WT NMDA/OGD_{sl}; §P < 0.05 vs. WT OGD_{sl}/OGD_{sl}. C) Immunofluorescence microscopy demonstrated that CAD (red pixels: i, iv, vii, x), an apoptotic marker, was enhanced in WT neurons exposed to OGD_{sl} (iv, vi) but not in neurons pretreated with NMDA (vi, ix) or OGD_{sl} (x, xii). In contrast to WT neurons, CAD immunofluorescence was significantly enhanced in Cav-1^-/- neurons exposed to OGD_{sl} (iv, vi). In Cav-1^-/- neurons, CAD immunofluorescence was significantly enhanced in Cav-1^-/- neurons pretreated with NMDA (xii, ix) or OGD_{sl} (x, xii) before OGD_{sl} 24 h (n=3). Neurons were also stained for the neuronal microtubular marker, β3-tubulin (green pixels in Ci, vi, viii, xi, Dii, vi, viii, x) and the nuclear marker, DAPI (blue pixels). E) Values from C and D are expressed as mean ± se of CAD pixels normalized to β3-tubulin. †P < 0.001 vs. Ctrl-WT; ‡P < 0.05 vs. Ctrl-Cav1 KO; §P < 0.05 vs. NMDA/OGD_{sl}/WT; γP < 0.05 vs. OGD_{sl}/OGD_{sl}-WT. Scale bar = 15 \mu m.
cence was significantly enhanced in caveolin-1 knock-
out neurons pretreated with NMDA (Fig. 6Dvii, ix) or
OGD_{SL} (Fig. 6Dv, xii) before OGD_{L} (Fig. 6Div, vii). We
quantitated (Fig. 6E) the results by normalizing CAD
staining (red pixels, left column) to the neuronal
marker, β3-tubulin (green pixels, middle column; Fig.
6Ci, vi, viii, xi, Di, vi, viii, xi). The inability of NMDA or
OGD_{SL} to precondition neurons from the knockout
mice indicates that caveolin-1 protein expression is
essential for NMDA-mediated phosphorylation of Src
and ERK1/2 and neuronal preconditioning in vitro.

Reexpression of caveolin-1 protein in primary
neurons isolated from caveolin-1 knockout mice
reestablishes NMDAR-mediated phosphorylation of
Src and ERK1/2

Because neurons deficient in caveolin-1 (e.g., Cav1-
siRNA or from caveolin-1 knockout mice) did not
respond to NMDA treatment, we tested whether rein-
truction of caveolin-1 into the deficient neurons
would restore NMDA-mediated phosphorylation of Src
and ERK1/2. We incubated neurons isolated from
caveolin-1 null mice with an adenovirus containing
full-length human caveolin-1 (Adv-Cav1) cDNA or con-
trol virus (Adv-LacZ) for 48 h. Under basal conditions
the caveolin-1 knockout neurons incubated with Adv-
Cav1 exhibited reduced expression of P-Src and
P-ERK1/2 compared with Adv-LacZ-incubated neurons
(Fig. 7A). NMDA treatment (10 μM; 25 min) of the
Adv-Cav1-incubated neurons increased P-Src and
P-ERK1/2 compared with basal levels without changing total Src or ERK1/2
expression, results akin to those of WT neurons (Fig.
1). Caveolin-1-deficient neurons incubated with Adv-
LacZ and then with NMDA exhibited no enhancement
of P-Src or P-ERK1/2 compared with basal conditions.

![Figure 7](image-url)

**Figure 7.** Adenoviral expression of caveolin-1 in caveolin-1 knockout neurons reestablishes NMDAR-mediated signaling and enhances NMDAR2B distribution to buoyant fractions and localization to membrane rafts. Primary neurons isolated from caveolin-1 null (Cav-1^{-/-}) mice were incubated with Adv-Cav1 or Adv-LacZ. A) Immunoblot analysis revealed that under basal conditions Cav-1^{-/-} neurons incubated with Adv-Cav1 exhibited reduced P-Src and P-ERK1/2 compared with Adv-LacZ-incubated neurons (n=4). NMDA treatment (10 μM; 25 min) resulted in enhanced P-Src and P-ERK1/2 with no change in total Src or ERK1/2 protein expression (n=4). Adv-LacZ-incubated Cav-1^{-/-} neurons exposed to NMDA exhibited no enhancement of P-Src or P-ERK1/2 compared with basal conditions. B) Values are expressed as mean ± se. *p < 0.05 vs. Adv-Cav1 control (CTRL). C) Sucrose density fractionation of Cav-1^{-/-} neurons treated with Adv-LacZ revealed that NMDAR2B was only present in HFs. Incubation of Cav-1^{-/-} neurons with Adv-Cav1 resulted in the detection of NMDAR2B in both BFs and HFs. NMDA treatment of Adv-Cav1-exposed Cav-1^{-/-} neurons redistributed NMDAR2B from BFs to HFs. D) Incubation of Cav-1^{-/-} neurons with Adv-Cav1 revealed a significant increase in colocalization between CT-B (Div) and NMDAR2B (Dvi) compared with Adv-LacZ (Diii). Data represent mean ± se. Scale bar = 10 μm.

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Adenovirally mediated expression of caveolin-1 in neurons isolated from caveolin-1 knockout mice thus restores NMDAR-mediated Src and ERK1/2 phosphorylation.

**Adenoviral-mediated expression of caveolin-1 in caveolin-1 knockout neurons redistributes NMDAR2B to BFs**

Caveolin-1 knockout neurons were treated with Adv-LacZ or Adv-Cav1 for 48 h, exposed to NMDA, and then subjected to sucrose density fractionation. In caveolin-1/−/− neurons incubated with Adv-LacZ, NMDAR2B was only present in HFs under both basal and NMDA-treated conditions (Fig. 7C, top panels). Incubation of caveolin-1/−/− neurons with Adv-Cav1 resulted in the detection of NMDAR2B in both BFs and HFs (Fig. 7C, bottom panels). Incubation of Adv-Cav1-treated caveolin-1/−/− neurons with NMDA redistributed NMDAR2B from BFs to HFs and increased the colocalization of NMDAR2B and CT-B (Fig. 7Dvii, viii) compared with that of Adv-LacZ-treated neurons (Fig. 7Diii). These findings indicate that adenoviral-mediated expression of caveolin-1 in neurons isolated from caveolin-1 knockout mice redistributes NMDAR2B to BFs and enhances the localization of NMDAR2B to neuronal membrane rafts.

**DISCUSSION**

The present article is the first to show a direct relationship between NMDA receptor signaling, preconditioning, and caveolin-1 in primary neurons. The data demonstrate that a NMDA receptor subtype (i.e., NMDAR2B) colocalizes and coimmunoprecipitates with caveolin-1 and that loss of caveolin-1 expression disrupts NMDA receptor signaling and attenuates Src and ERK1/2 phosphorylation in response to NMDA or OGD. The data demonstrate the essential role of caveolin-1 in both NMDAR signaling and ischemic preconditioning, as shown by the inability of caveolin-1/−/− neurons and neurons treated with Cav1-siRNA to undergo preconditioning. Collectively, the data show that caveolin-1 expression is essential for NMDAR-mediated Src and ERK1/2 phosphorylation and for ischemic preconditioning of neurons.

Caveolin-1 is expressed in multiple types of neurons, including hippocampal and dorsal root ganglion neurons, and PC12 cells as well as in excitatory synapses in the CA1 of neonatal and adult hippocampus, all of which express glutamate receptors (33–36). Caveolin-1 knockout mice exhibit neurological abnormalities that include claspings, abnormal spinning, muscle weakness, reduced activity, and gait abnormalities (37), as well as increased cerebral infarct size in response to permanent ischemia (38). The interplay between caveolin-1 and signaling cascades in neurons that underlie these neuropathological conditions has not previously been known. Our immunofluorescence data show that caveolin-1 distributes in a discrete, punctate pattern on neuronal cell bodies and along dendritic shafts. In both cellular regions, caveolin-1 partially colocalizes with NMDAR2Bs, which are highly enriched in dendritic shafts and spines (30, 31), identifying those regions as potential sites for caveolin-1 to regulate neurotransmission and synaptic plasticity. Cerebral ischemia decreases the integrity of membranes and thus may perturb signaling mechanisms through disruption of caveolin-associated signaling complexes by modulating caveolin expression or the stability of caveolin-oligomer complexes.

NMDAR subunits are organized in multiprotein complexes that scaffold proteins and signaling enzymes such as SFKs (23, 39). SFKs are considered hubs for the modulation of NMDAR signaling under both normal and ischemic conditions (4) and in the induction of long-term potentiation (LTP) (40). SFK-mediated tyrosine phosphorylation of NMDAR2B, the predominant tyrosine-phosphorylated NMDAR subtype at postsynaptic densities, increases LTP in the hippocampus (25, 41). Caveolin expression appears to be required for maintaining activation of Src and ERK1/2 after NMDAR stimulation, perhaps via recruitment of C-terminal Src kinase, which deactivates Src (42–44). The NMDAR2B possesses two regions (635–642, WAFFAVIF and 1042–1049, FSFKSDRI) that potentially interact with caveolin-binding motifs (15). In addition, caveolin-1 is a phosphorylated substrate and scaffold for SFKs (20, 45) and, thus, may facilitate NMDAR-mediated neurotransmission. The current data show clearly that caveolin-1 coimmunoprecipitates and colocalizes with NMDAR2B and that expression of caveolin is necessary for neuronal NMDA-mediated signaling (Fig. 8). These findings suggest that caveolin is involved in NMDA receptor-mediated neuronal events in addition to preconditioning, such as postinjury neuronal plasticity and modulation of receptor expression (36, 46). NMDAR2B is the most tyrosine-phosphorylated NMDAR subtype at postsynaptic densities (4, 29); the present findings implicate a role for caveolin in regulating NMDAR-mediated neurotransmission and neuronal plasticity at such densities, perhaps by linking SFK and other NMDAR-mediated signaling events. This latter concept is consistent with our findings indicating that NMDA-stimulated downstream signaling is blunted in neurons treated with caveolin-1 siRNA or isolated from caveolin-1 knockout mice but reestablished if the latter neurons are incubated with a viral vector that increases caveolin-1 expression. Studies that assess the effects of alterations in caveolin expression on glutamate receptor signaling, for example, during development and senescence, merit further investigation.

Membrane raft formation (47) and signaling may have implications for certain forms of calcium signaling and LTP. Three forms of LTP (LTP1, LTP2, and LTP3) have been described to occur in different subcellular regions of neurons (48). LTP3, which is driven by strong stimuli [8×100 Hz θ-burst stimulation (TBS)] in the soma, requires NMDAR dependent and voltage-
dependent Ca\(^{2+}\) channel sensitive components and leads to gene transcription (49). In contrast, LTP1 (weak stimulus or single TBS) depends on NMDAR and the endoplasmic reticulum (50) (via ryanodine receptors) and posttranslational modification of synaptic proteins and occurs in discrete microdomains in dendritic spines (51, 52). Similar to LTP1, LTP2 (moderate stimuli) requires NMDAR and inositol (1,4,5)-triphosphate receptors and involves new protein synthesis within dendrites, which is independent of de novo gene transcription (53). The compartmentalization of these various forms of Ca\(^{2+}\) signals suggests that calcium sources colocalize with these signaling components in discrete subcellular microdomains to modulate effector systems and second messenger production (54). Previous work has shown that isoforms of caveolin colocalize and coimmunoprecipitate with ER and plasmalemmal calcium channels in various cell types (27). Our data demonstrating that reexpression of caveolin enhances membrane raft formation in the soma and dendrites and colocalization between NMDAR and CT-B (a membrane raft marker) implicates caveolin as a potential modulator of one or more forms of LTP within different subcellular regions in neurons. Our data demonstrating that reexpression of caveolin enhances membrane raft formation in the soma and dendrites and colocalization between NMDAR and CT-B (a membrane raft marker) implicates caveolin as a potential modulator of one or more forms of LTP within different subcellular regions in neurons. Regulation of neuronal function by glutamate depends on assembly of multiprotein complexes in glutamatergic synapses that include neuroligins and neurexins, ionotropic and metabotropic glutamate receptors, and kinases and phosphatases, all of which facilitate synaptogenesis, LTP, learning, and memory (55). High-fidelity signal transduction in neurons involves the organization and communication between membrane receptors, intracellular proteins, and cytoskeletal components that localize in membrane rafts (56, 57).

Recent evidence has linked expression of caveolin to preconditioning in cardiac tissue (44), but the role of caveolin in neuronal preconditioning is unknown. The present data, demonstrating a link between caveolin-1 protein expression and NMDAR-mediated neurotransmission, are consistent with findings that identify a role for caveolin-1 in the formation of signaling complexes involved in synaptic potentiation (i.e., \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) (35, 36) and depression (amyloid precursor protein) (58, 59). Moreover, the current data provide new evidence in support of the importance of caveolin-1 expression for proper NMDAR/SFK/ERK1/2 neuronal signaling.

The key events that modulate expression and phosphorylation of caveolin in the central nervous system are unknown. Our findings that decreased caveolin expression disrupts NMDAR signaling events and that reexpression of caveolin-1 rescues proper NMDAR signaling, strongly suggest that caveolin is necessary for signal transduction activated by glutamate release from presynaptic terminals. Cellular stress events (i.e., superoxide radicals, osmotic stress, and UV exposure) can increase SFK-mediated phosphorylation of caveolin (20), leading us to hypothesize that caveolin is involved in NMDAR signaling and SFK activation. Recent evidence has shown that caveolin-1B, a splice variant of caveolin-1 that lacks the residue (tyrosine 14) required for SFK-mediated phosphorylation, is involved in \(p75\) neurotrophin receptor signaling in neurons (60). Other studies have demonstrated neuroprotective pathways mediated by NMDAR activation of phosphatidylinositol 3-kinase/Akt (61), which is modulated by caveolin-1 (62, 63), thus implying that caveolin-1 may regulate multiple NMDAR-mediated signaling events.

Figure 8. Schematic diagram depicting the role of neuronal caveolin in regulating NMDAR-mediated activation of Src and ERK1/2. A) Under basal conditions, caveolin-1 scaffolds inactive Src tyrosine kinase, ERK1/2 MAPK, and NMDAR2B (NR2B) at the plasma membrane. On NMDAR agonism, inactive Src (Y527) is autophosphorylated at tyrosine 416, leading to caveolin-1 phosphorylation (Y14), NR2B phosphorylation (Y1252, Y1336, and Y1472), and calcium (Ca\(^{2+}\)) entry through the open channel, leading to downstream ERK1/2 activation and cell survival. B) siRNA for caveolin-1 leads to knockdown of caveolin-1 protein expression by \(~67\%\). Reduction of caveolin-1 attenuates NMDAR-mediated activation of Src, blocks activation of the prosurvival ERK1/2 mitogen-activated protein kinase, thus leading to an inability to protect neurons against lethal ischemia. Note: NMDA receptor channels are formed by the assembly of two classes of subunits, NR1A and NR2A–2D. NR2B is the most tyrosine phosphorylated subunit at postsynaptic densities (4).

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involved in neuroprotection. The current studies demonstrate that expression of caveolin-1 is essential for NMDA-mediated Src and ERK1/2 phosphorylation and neuronal preconditioning. In view of the important role of NMDAR-Src signaling in glutamatergic signaling, synaptic plasticity, excitotoxicity, and memory, caveolin-1 may be integral to these processes as well.

This work was supported by an American Heart Association Scientist Development Grant to H.H.P. (0630039N), National Institutes of Health grants to P.M.P. (GM 34107), to E.R.-B. (GM 66232), and a Merit Award from the Department of Veterans Affairs to D.M.R. We are grateful to the University of California, San Diego Cancer Cell Imaging Shared Resource, in particular Jim Feramisco, Keri Peston-Smhap, and Steve McMullen. In some cases, three-dimensional perspective views were made at VisLab in the San Diego Supercomputer Center using National Partnership for Advanced Computational Infrastructure Scalable Visualization Tools.

REFERENCES


Received for publication June 29, 2007. Accepted for publication August 30, 2007.