Cellular/Molecular

Phosphorylation of Ser1166 on GluN2B by PKA Is Critical to Synaptic NMDA Receptor Function and Ca\(^{2+}\) Signaling in Spines

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The NMDA-type glutamate receptor (NMDAR) is essential for synaptogenesis, synaptic plasticity, and higher cognitive function. Emerging evidence indicates that NMDAR Ca\(^{2+}\) permeability is under the control of cAMP/protein kinase A (PKA) signaling. Whereas the functional impact of PKA on NMDAR-dependent Ca\(^{2+}\) signaling is well established, the molecular target remains unknown. Here we identify serine residue 1166 (Ser1166) in the carboxy-terminal tail of the NMDAR subunit GluN2B to be a direct molecular and functional target of PKA phosphorylation critical to NMDAR-dependent Ca\(^{2+}\) permeation and Ca\(^{2+}\) signaling in spines. Activation of β-adrenergic and D\(_1\)/D\(_5\)-dopamine receptors induces Ser1166 phosphorylation. Loss of this single phosphorylation site abolishes PKA-dependent potentiation of NMDAR Ca\(^{2+}\) permeation, synaptic currents, and Ca\(^{2+}\) rises in dendritic spines. We further show that adverse experience in the form of forced swim, but not exposure to fox urine, elicits striking phosphorylation of Ser1166 in vivo, indicating differential impact of different forms of stress. Our data identify a novel molecular and functional target of PKA essential to NMDAR-mediated Ca\(^{2+}\) signaling at synapses and regulated by the emotional response to stress.

Introduction

NMDARs are glutamate-gated ion channels and are enriched at excitatory synapses, where they are strategically positioned to play a crucial role in regulation of synaptic function (Cull-Candy and Leszkiewicz, 2004; Lau and Zukin, 2007; Traynelis et al., 2010). A unique feature of NMDARs is their high permeability to Ca\(^{2+}\). Ca\(^{2+}\) influx through NMDARs is essential for synaptogenesis, plasticity of neural circuitry, and higher cognitive functions, such as learning and memory. NMDARs are tetrameric assemblies of GluN1 and GluN2 subunits, which cotranslationally assemble in the endoplasmic reticulum to form functional channels. In adult hippocampus and neocortex, GluN2A and 2B are the primary GluN2 subunits expressed at synapses (Gray et al., 2011). The precise subunit composition governs the physiological and pharmacological properties of NMDARs. GluN2B-containing receptors exhibit slower decay kinetics (Yashiro and Philpot, 2008), carry more Ca\(^{2+}\) per unit charge (Sobczyk et al., 2005), and preferentially tether to the synaptic plasticity protein CaMKII (Barria and Malinow, 2005; Halt et al., 2012). In addition, GluN2B expression can enhance the magnitude of LTP, promote hippocampal-based learning, plasticity-associated spine growth, and dendritic patterning critical to information processing (Tang et al., 1999; von Engelhardt et al., 2008).

Synaptic NMDARs localize to the postsynaptic density (PSD) where they are structurally organized and spatially restricted in a large macromolecular signaling complex comprised of scaffolding and adaptor proteins, which physically link receptors to kinases, phosphatases, and other downstream signaling proteins (Sheng and Hoogenraad, 2007). Protein kinase A (PKA) is a major kinase known to regulate NMDAR trafficking and gating. At synaptic sites, PKA is kept in close proximity to NMDARs by association of the PKA regulatory subunit with A-kinase anchoring proteins (AKAPs), such as AKAP9 and AKAP5 (Sanderson and Dell’Acqua, 2011).

Emerging evidence reveals that PKA signaling represents a fundamental mechanism by which NMDAR-mediated Ca\(^{2+}\) influx is modulated in neurons. Direct activation of PKA promotes NMDAR Ca\(^{2+}\) permeability, Ca\(^{2+}\) signaling in dendritic spines, and induction of LTP at Schaffer collateral to CA1 pyramidal cell
consistently with this, extracellular signals that modulate cAMP bidirectionally regulate Ca\(^{2+}\) permeation through NMDARs and Ca\(^{2+}\) transients in spines in a PKA-dependent manner. Whereas GABA\(_A\) and dopamine D\(_2\) receptors negatively couple to the cAMP/PKA cascade and inhibit NMDAR-mediated Ca\(^{2+}\) signaling in spines, 2A-type adenosine receptors positively engage PKA signaling, enhance NMDAR Ca\(^{2+}\) permeation, and Ca\(^{2+}\) signaling in spines (Chalifoux and Carter, 2010; Higley and Sabatini, 2010). In addition, activation of \(\beta\)-adrenergic receptors enhances NMDA currents (Raman et al., 1996), and activation of D\(_1/D_5\) dopamine receptors facilitates induction of LTP at CA1 synapses in a PKA-dependent manner (Otmakhova and Lisman, 1996).

Whereas the functional impact of PKA on NMDAR-dependent Ca\(^{2+}\) signaling is well characterized, the molecular target remains unknown. Using a combination of site-directed mutagenesis, electrophysiology, two-photon microscopy, glutamate uncaging, and Ca\(^{2+}\) imaging, we identify Ser1166 in the carboxy-terminal tail of GluN2B as a novel molecular target of PKA phosphorylation critical to Ca\(^{2+}\) signaling in dendritic spines and regulated by the emotional response to stress.

**Materials and Methods**

*Animals.* All animal procedures were in accordance with guidelines approved by the Albert Einstein, Harvard University, and University of California Institutional Animal Care and Use Committee and federal guidelines.

* Constructs. * GluN1–1a and GluN2B(WT) in pRK5 were a kind gift from M.D. Ehlers (Duke University, Durham, North Carolina). The S929/930A and S1166A GluN2B mutants were generated using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions and verified by DNA sequencing. The following primers from 5’ to 3’ were used for GluN2B(S929/930A): forward, GCTCTTACAGCTGAGAGCAGGC GTGCTAGACATCTCTG, and reverse, CAG AGATGCTGTAGACGGTACGACATCTCTG, and S1166A(S929/930A): forward, GACTTCAAGCGAGATCT CGCTTGAAGTC, and reverse, CAGACTTCAAGCGAGATCT CGCTTGAAGTC.

*In vitro phosphorylation assay.* To assess PKA-dependent phosphorylation of NMDARs, HEK293 cells were transfected with WT and mutant GluN2B with Lipofectamine 2000 (Invitrogen) as described previously (Lin et al., 2004). Forty-eight to 72 h after transfection, lysates were immunoprecipitated with an antibody to GluN2B (Millipore), and immunocomplexes were incubated with PKA-C (Sigma). Samples were run on 3–8% Tris-acetate gels (Invitrogen) for subsequent autoradiography and Western blotting (adapted from Leonard and Hell, 1997).

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Whole-cell patch-clamp recording. Primary cultures of hippocampal neurons were generated from embryonic day (E)18 Sprague Dawley rats and transfected at 5–7 days in vitro (DIV) with WT or mutant GluN2B and GEP by means of calcium phosphate as described previously (Jiang et al., 2004). To minimize NMDAR-induced toxicity, cells were maintained in the presence of the NMDAR antagonist 2R-amino-5-phosphonovaleric acid (2AP) during whole-cell patch-clamp recording. Primary cultures of hippocampal neurons were generated from embryonic day (E)18 Sprague Dawley rats and transfected at 5–7 days in vitro (DIV) with WT or mutant GluN2B and GEP by means of calcium phosphate as described previously (Jiang et al., 2004). To minimize NMDAR-induced toxicity, cells were maintained in the presence of the NMDAR antagonist 2R-amino-5-phosphonovaleric acid (2AP) during whole-cell patch-clamp recording.

*Figures.* Figure 1. Ser1166 in GluN2B is a direct target of PKA phosphorylation critical to PKA potentiation. a, HEK293 cells expressing WT or mutant GluN2B subunits were immunoprecipitated and incubated with PKA-C in the presence of γ\(^{-32}\)P-ATP. Top, Autoradiogram depicting incorporation of γ\(^{-32}\)P into GluN2B. Bottom, Western blot probed with an antibody to GluN2B. Right, Summary data. Values for mutant receptors were normalized to values for WT receptors. b, PKA potentiation of NMDA-evoked currents is abolished in HEK293 cells expressing GluN1 with GluN2B(S1166A); bar above sample records represents duration of NMDA + glycine application (N). Left, Sample traces at 0 (1) and 7 (2) min. Right, Summary time course data of the peak NMDAR current, defined as the initial maximum current. Potentiation is defined as the ratio of the peak current recorded from the same cell at 7 min versus 0 min. Values for WT and mutant receptors in the presence (PKA-C) and absence (Control) of PKA-C were normalized to values for WT receptors in the absence of PKA-C (Control). WT + PKA-C, n = 9 cells; S1166A + PKA-C, n = 11 cells; WT control, n = 7 cells; S1166A control, n = 6 cells from 4 to 9 independent experiments. *c, PKA potentiation of NMDA-evoked currents is abolished in hippocampal neurons expressing GluN2B(S1166A). Left, Representative traces. Right, Summary time course data as in b. WT + PKA-C, n = 5 cells; S1166A + PKA-C, n = 8 cells; WT control, n = 5 cells; S1166A control, n = 5 cells from 3 or 4 independent experiments. d, Far left, Representative traces for individual GluN1/GluN2B(S1166A) (left), WT (center), and S1166D (right) channels. Low gating activity of S1166A relative to that of WT is restored with the phosphomimetic mutation, S1166D. Single-channel activity was recorded with cell-attached pipettes as inward currents through one S1166A, WT, or S1166D channel expressed in a HEK293 cell. Far right, Summary of single-channel parameters for S1166A (gray, n = 14), WT (black, n = 19), and S1166D (white, n = 16). The apparent differences in Po and closed time values for either the S1166A or S1166D mutant versus WT were not significant, consistent with variability in the basal phosphorylation state of WT GluN1/2B receptors. *p < 0.05, **p < 0.01, ****p < 0.0001. Here and in all figures, error bars indicate SEM.
acid (AP5) (Tocris Bioscience) from the time of transfection until recording. Whole-cell patch-clamp recordings were obtained from hippocampal neurons at 11–15 DIV as described previously (Skeberdis et al., 2006). The external solution contained the following (in mM): 145 NaCl, 5.4 KCl, 10 HEPES, 10 glucose, 10 M glycine. PKA-C was infused to a final concentration of 100 nM. Cells were washed for 1 min to allow recovery to basal levels before the next stimulation.

**Single-channel recording and analyses.** Currents were recorded and analyzed with the cell-attached patch-clamp technique as described previously (Kussius et al., 2009). The pipette solution contained the following (in mM): 150 NaCl, 2.5 KCl, 1 EDTA, and 10 HEPES and NMDAR agonists glutamate (1 mM) and glycine (0.1 mM). The pipette voltage was clamped at +100 mV (membrane potential = −90 mV), and inward sodium currents were recorded and amplified (Axopatch 200B, low pass filtered (10 kHz), and sampled (40 kHz, NIDAQ board) into digital files. Only records originating from one-channel patches were retained for analyses. The selected records were idealized with the SKM algorithm in QuB software to calculate average unitary current amplitude, open probability, and durations for open and closed intervals for each record. Statistical significance was evaluated by ANOVA, followed by Tukey’s post hoc analysis.

Ca\(^{2+}\) imaging in HEK293 cells. To image NMDA-evoked Ca\(^{2+}\) signals, cells were loaded with the Ca\(^{2+}\) indicator Fura-2 AM (Invitrogen) in external recording solution or DMEM (1 h at 37°C). NMDA-evoked Ca\(^{2+}\) rises were elicited by application of NMDA (300 μM + 10 μM glycine). PKA-C was infused to a final concentration of 100 nM. Cells were washed for 1 min to allow recovery to basal levels before the next stimulation. The Ca\(^{2+}\) signal at 340 to 380 nm was calculated using MetaFluor software.

**Slice culture preparation.** Organotypic hippocampal slice cultures were prepared from 6- to 7-d-old mice as described previously (Carter and Sabatini, 2004). Two days later, DNA constructs were biolistically transfected with a Helios Gene Gun. Bullets were loaded with 50 μg of WT

### Table 1. Summary of statistics for Figures 1–3

<table>
<thead>
<tr>
<th>Figure</th>
<th>N</th>
<th>Mean ± SEM</th>
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<th>Statistical test</th>
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<tr>
<td>1a</td>
<td>4</td>
<td>WT: 1.000 ± 0.0</td>
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<td>One-way ANOVA with Dunn’s post-test</td>
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<td>1b</td>
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<td>WT: 1.584 ± 0.0763</td>
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<td>One-way ANOVA with Tukey’s post-test (WT vs S1166A, WT vs WT (control), and WT vs S1166A (control))</td>
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<td>WT: 1.04 ± 0.03; P&lt;sub&gt;e&lt;/sub&gt; 0.22 ± 0.04; MOT 5.0 ± 0.5; MCT 27.6 ± 4.5</td>
<td>P&lt;sub&gt;e&lt;/sub&gt;, 0.0317</td>
<td>One-way ANOVA with Tukey’s post-test (P&lt;sub&gt;e&lt;/sub&gt;, S1166A vs S1166D; MCT, S1166A vs S1166D)</td>
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<td>WT: ΔE&lt;sub&gt;rev&lt;/sub&gt; = −6.79 mV</td>
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<td>Two-tailed paired t test for WT vs S1166A</td>
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<tr>
<td>2f</td>
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<td>WT % inhibition: 59.14 ± 7.20%</td>
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<td>Two-tailed unpaired t test for % inhibition by H-89 of WT vs S1166A; one-way ANOVA with Dunn’s post-test (WT control vs H-89)</td>
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<td>WT (control): −17.03 ± 1.58</td>
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<td>3f</td>
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<td>WT (control): −9.677 ± 1.048</td>
<td>* &lt; 0.001</td>
<td>Two-tailed unpaired t test for WT (control) vs WT (ISO)</td>
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\(<p<0.05; **p<0.01; ****p<0.0001.\)
GluN2B or GluN2B(S1166A) and tdTomato (30 μg) to assess efficiency of transfection and identify transfected cells.

**Two-photon laser glutamate uncaging.** For two-photon laser uncaging experiments, synaptic Ca2+ transients were measured and uncaging-evoked EPSCs (uEPSCs) were simultaneously recorded at individual spines from tdTomato+ CA1 neurons expressing wild-type (WT) or mutant GluN2B(S1166A) before and after glutamate photolysis in organotypic hippocampal slice cultures at 10–14 d after transfection. Photolysis of MNI glutamate was performed by focal illumination with a 0.5 ms pulse of light at 720 nm at ~0.5 μm from the target spine head. In all conditions, Mg2+-free ACSF (in mM) 127 NaCl, 2.5 KCl, 25 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, and 25 glucose included 20 μM NBQX to block AMPAR-mediated currents, 1 μM TTX to block action potentials, and 2.5 mM MNI glutamate to elicit laser-induced glutamate release. Glass electrodes (2–3.5 mΩ) were filled with internal solution containing the following (in mM): 130 CsMeSO4, 10 HEPES, 1.8 MgCl2, 10 Na2ATP, 0.3 NaN3CTP, and 8 sodium creatine phosphate. 10 CsCl, 300 μM Fluo-5F, 20 μM AlexaFluor-594, pH 7.3. Voltage-clamp recordings were performed at a holding potential of −70 mV using an Axopatch 200A amplifier; data were filtered at 2 kHz and digitized at 10 kHz. Intracellular Ca2+ imaging and glutamate uncaging were performed on a custom microscope, as described previously (Carter and Sabatini, 2004). Neurons were filled via the patch electrode for 15 min before imaging.

For isoproterenol-treated cells, 10 μM isoproterenol was added 5 min after break-in. Fluo-5F (green) and AlexaFluor-594 (red) were excited using 840 nm light to monitor Ca2+ signals and spine morphology, respectively. To measure Ca2+ transients in spines, green and red fluorescence was acquired during 500 Hz line scans across a spine and a neighboring (control) spine. Reference frame scans were taken between acquisitions to correct for slight drift. Ca2+ signals were quantified as increases in green fluorescence from baseline normalized to the average red fluorescence (ΔG/R) and represented as percentage of the G/R ratio measured in saturating Ca2+ (G/Rmax).

Imaging and physiology data were acquired using a National Instruments data acquisition boards and custom software written in MATLAB as described previously (Pologruto et al., 2003). Off-line analysis was performed using custom routines written in MATLAB and Igor Pro. For each analyzed dendritic spine, the amplitude of NMDAR-mediated transients data acquisition boards and custom software written in MATLAB was acquired during 500 Hz line scans across a spine and a neighboring target spine head. In all conditions, Mg2+-free ACSF (in mM) 127 NaCl, 2.5 KCl, 25 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, and 25 glucose was added 5 min after break-in.

**Slice treatments.** Acute hippocampal slices were prepared as described previously (Lu et al., 2007) and treated with vehicle (H2O or EtOH), 1 μM isoproterenol, 1 μM isoproterenol + propranolol for 10 min, 10 μM SKF81297, or 10 μM SKF81297 + 5 μM SCH23390 (0.5–15 min). After treatment, spines were flash-frozen and extracted with 1% deoxycholate (DOC) in buffer containing the following (in mM): 50 Tris pH 8.5, 10 EDTA, 10 EGTA, and 135 NaCl, and cleared by ultracentrifugation (Leonard and Hell, 1997). All buffers included the following protease and phosphatase inhibitors: 1.5 μM peptatin A, 2.1 μM leupeptin, 0.3 μM aprotinin, 0.2 mM PMSF, 1 mM n-p-tolualdehyde, 25 mM NaPPi, 25 mM NaF, and 2 μM microcin-LR. Immunoprecipitation for GluN2B and GluN1 was as described previously (Lu et al., 2007). Samples were run on 7.5% polyacrylamide gels followed by quantitative immunoblotting with antibodies against pSer1166-GluN2B (PhosphoSolutions), GluN2B, pSer897-GluN1, GluN1, pSer845-GluA1, and GluA1 as described previously (Lu et al., 2007). Chemiluminescent immunosignals were digitized by scanning films and quantified with Adobe Photoshop.

**Fox urine assay.** Postnatal day (P30) rats were placed in a cage containing a 50 ml tube with kimwipes soaked in fox urine (Wildlife Research Supplies) or 0.9% saline for 5 min, and immediately anesthetized with isoﬂurane and decapitated and decapitated as described previously (Liu et al., 2010). Hippocampi were isolated and processed for immunoprecipitation with GluN2B and GluA1 antibodies and Western blot analysis as above.

**Injections and forced swim assay.** P24–P32 rats were subjected to forced swim in a 4 L beaker chamber filled to 3 L at a temperature of 16–22°C for 5 min. The chamber was cleaned after every one or two rats. One hour prior to forced swim, rats were anesthetized with isoﬂurane, stereotaxically injected into the hippocampal CA1 with the PKA inhibitor Rp-8-Br-CAMPS (25 nmol, 4 µl) (Enzo Life Sciences) or with saline. The coordinates for injection into the hippocampus were ~3 anterior/posterior, ~2 medial/lateral, and ~4 dorsal/ventral from bregma. Immediately after forced swim, rats were anesthetized with isoﬂurane, decapitated, and the hippocampi transferred to ice-cold PBS containing protease and phosphatase inhibitor cocktails as above. Hippocampi were microdissected around the injection site, flash-frozen in liquid N2, and stored at −80°C. For Western blot analysis, hippocampi were processed as described above.

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**Figure 2.** Ser1166 is critical to modulation of NMDAR Ca2+ permeability and NMDA-evoked Ca2+ rises by PKA. Representative traces (a) and current–voltage relationship (b) for NMDA-elicited currents recorded from HEK293 cells expressing GluN1 with WT and mutant GluN2B(S1166A) in the absence and presence of H-89. H-89 induced a negative shift in reversal potential in cells expressing WT (ΔErev = −6.79 mV, but not GluN2B(S1166A) (ΔErev = 0.46 mV). **c,** Summary data depicting reversal potential (Erev); WT, n = 6 cells; S1166A, n = 6 cells from 3 independent experiments. *p = 0.0207; n.s., Not significant. d–f, PKA potentiation of NMDA-evoked rises in Ca2+ is diminished in HEK293 cells expressing GluN1 with GluN2B(S1166A). d, Representative images of basal and NMDA-evoked Ca2+ rises before (control), during (H-89), and after washout of H-89 (recovery) for cells expressing GluN1 with WT or S1166A receptors. e, Time course data of cells in d. f, Left, Summary data for NMDA-evoked Ca2+ rises. Right, Summary data expressed as percentage inhibition by H-89; WT, n = 7 cells; S1166A, n = 5 cells from 5 independent experiments. *p = 0.0165. **p < 0.01. Scale bar, 50 μm.
Table 1

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<th>Reaction</th>
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<tr>
<td>ΔGluN2B</td>
<td>60</td>
<td>60</td>
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<td>10% ΔGluN2B</td>
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Results

PKA phosphorylates GluN2B at Ser1166

To identify the molecular target of PKA relevant to regulation of NMDAR Ca$^{2+}$ permeability and Ca$^{2+}$ signaling in spines, we used NetPhosK and scansite.mit.edu software set at medium confidence algorithms to identify putative PKA consensus sequences in the GluN2B carboxy-tail. The GluN2B carboxy-terminal tail sequence harbors three sites that conform to a PKA consensus phosphorylation motif (K/R-K/R-X-(X)-S/T): Ser929, Ser930, and Ser1166. We used site-directed mutagenesis to mutate these residues singly or together to the nonphosphorylatable residue alanine. To examine the ability of PKA to phosphorylate GluN2B at these sites, we expressed GluN2B(WT), GluN2B(S929/930A), or GluN2B(S1166A) in HEK293 cells and immunoprecipitated cell lysates with an antibody directed to GluN2B. Immunocomplexes were incubated with the recombinant, constitutively active catalytic subunit of PKA (PKA-C) or vehicle in the presence of γ-32P-labeled ATP, and samples were subjected to autoradiography and Western blotting (Fig. 1a). In HEK293 cells, GluN2B subunits migrate as doublets, corresponding to the glycosylated and nonglycosylated forms of the receptor (Petralia et al., 1994). Background phosphorylation (defined as phosphorylation in the absence of PKA-C) of WT and mutant receptor subunits was negligible (Fig. 1a). PKA-C induced striking phosphorylation of WT and mutant GluN2B(S929/930A) receptors (Fig. 1a; Table 1). In contrast, PKA-induced phosphorylation of mutant GluN2B (S1166A) receptors was greatly diminished but not abolished (Fig. 1a; Table 1). The small residual signal may reflect additional sites of PKA phosphorylation on GluN2B, at least under the conditions of this assay. WT and mutant receptors exhibited comparable levels of total GluN2B abundance, assessed by Western blot analysis (Fig. 1a), and surface expression, assessed by surface biotinylation assays (data not illustrated). These results indicate that Ser1166 of GluN2B is a main site of PKA phosphorylation.

PKA potentiation of NMDA-evoked currents is abolished in cells expressing GluN2B(S1166A)

To address whether Ser1166 is a functional target of PKA, we undertook several experimental approaches. First, we examined the impact of Ser1166 on PKA-dependent potentiation of NMDA-elicited currents (300 μM with 10 μM glycine) recorded from HEK293 cells expressing GluN1 together with WT or mutant GluN2B(S1166A) receptors and GFP to enable visualization of transfected cells (Fig. 1b). HEK293 cells are relatively small, geometrically simple, and more nearly isopotential than are neurons and enable examination of NMDAR function in a system expressing a homogeneous population of NMDARs (see Materials and Methods). In these experiments, the same cell was stimulated repeatedly with agonist (once per minute; 5 s per application) for a total of 10 applications under computer control before and after action of PKA-C. PKA-C was delivered directly into the cell via the patch pipette at the time of break-in (t = 0 min). Potentiation was defined as the ratio of peak currents recorded from the same cell at two different time points (7 vs 0 min). In cells expressing WT NMDARs, PKA-C potentiated the peak phase of NMDA-evoked currents (300 μM with 10 μM glycine) recorded from HEK293 cells expressing GluN1 together with WT or mutant GluN2B(S1166A) receptors, the peak to steady-state ratio of the basal current was markedly reduced, and PKA potentiation was abolished (Fig. 1b; Table 1). The peak phase of NMDA-evoked currents recorded from cells expressing WT or mutant receptors and treated with vehicle was not detectably altered within the time course of the experiment.

Next, we addressed whether Ser1166 is a molecular target of PKA relevant to potentiation of NMDA currents by PKA in primary cultures of hippocampal neurons, a more physiologically relevant system to assess NMDAR function. NMDARs and the machinery required for proper NMDAR targeting and function, such as NMDAR-interacting proteins and AKAPs, are highly expressed in neurons and enriched in dendritic spines (Lau and Zukin, 2007; Sanderson and Dell’Acqua, 2011). Toward this end, we transfected hippocampal neurons at 5–7 DIV with WT or mutant GluN2B(S1166A) together with GFP and performed recording from GFP+ neurons at 11–15 DIV. Thirty minutes before recording, we applied the adenyl cyclase inhibitor SQ22536 (100 μM) to deplete basal levels of cAMP and reduce endogenous PKA.

Surface biotinylation assay. To assess surface expression of GluN2B WT and mutant constructs, HEK293 cells were transfected with GluN1 and WT or mutant GluN2B constructs with Lipofectamine 2000 as above. Forty-eight to 72 h after transfection, total and surface extracts were isolated and processed for Western blots as described previously (Lin et al., 2004). Membranes were probed with antibodies directed to GluN1, GluN2B, and β-actin (loading control) and bands quantified as above.

Statistical analyses. Data are represented as mean ± SEM. All statistical analyses were performed using Prism software (GraphPad). Significance was defined as p < 0.05.

Figure 3. Ser1166 of GluN2B is essential to β-adrenergic-induced Ca$^{2+}$ rises in spines. a, Representative 2PLSM image of a dendritic spine from a CA1 hippocampal neuron filled with the red fluorophore AlexaFluor-594 and the green Ca$^{2+}$-sensitive indicator Fluo-5F. The yellow arrowhead indicates the location of a 500 μs pulse of 720 nm laser light used to focially uncage glutamate near the spine head. White dashed line indicates the region used for line scan analysis of fluorescence. Scale bar, 1 μm. b, Red and green fluorescence in the spine head (sp) and neighboring dendrite (dend) measured in a line scan over the region indicated by the dashed line in a. Inset scale bars, 100 ms and 5% ΔG/ΔG0. Yellow arrowhead indicates time of uncaging. The increase in green fluorescence indicates increased intracellular Ca$^{2+}$ and the corresponding spine Ca$^{2+}$ transient (white trace) is below. c, Average trace of uncaging-evoked ΔCa$^{2+}$signals produced by single uncaging stimuli in neurons expressing GluN2B(WT) (left) or GluN2B(S1166A) (right) under control conditions (black) or after isoproterenol incubation (red). Mean (solid lines) and mean ± SEM (shaded regions) are shown. d, Average trace of uEPSCs produced by single uncaging stimuli in neurons expressing GluN2B(WT) (left) or GluN2B(S1166A) (right) under control conditions (black) or after isoproterenol incubation (red). e, Population data for peak ΔCa$^{2+}$spine and (f) uEPSC elicited by single uncaging stimuli in neurons expressing GluN2B(WT) or GluN2B(S1166A) under control conditions (Ctrl) or after isoproterenol incubation. Red bars represent the mean of distribution ± SEM. *p < 0.001.
activity. In neurons expressing WT or mutant NMDARs and treated with vehicle, NMDA-evoked responses exhibited rundown (Fig. 1c), consistent with previous findings (Skeberdis et al., 2006). Direct delivery of PKA-C via the patch pipette poteniated the peak phase of NMDA-elicted currents by ~1.3-fold (Fig. 1c; Table 1). In neurons expressing WT GluN1 with mutant GluN2B(S1166A), PKA potentiation was essentially abolished (Fig. 1c; Table 1), indicating the ability of overexpression of mutant GluN2B to outcompete endogenous GluN2B and GluN2A for assembly with GluN1 and to dominate NMDA-elicted currents.

The results thus far indicate a role for Ser1166 in potentiation of NMDA currents by PKA, but they do not address the mechanism by which this occurs at the single-channel level. Toward this end, we recorded activity in patches containing only a single channel from brain lysates in the presence or absence of phosphatase (PP) inhibitors as indicated (+/− 1. Top, GluN2B immunocomplexes were incubated with no enzyme (control), PKA-C, or alkaline phosphatase (AP). Membranes were probed with antibodies to pSer1166-GluN2B in the absence or presence of phosphopeptide and total GluN2B to detect IP levels. Middle, Input samples (lysates). Membranes were probed with antibodies to pSer1166-GluN2B in the absence or presence of phosphopeptide and total GluN2B to detect IP levels. Middle, Input samples (lysates). Membranes were probed with antibodies to pSer1166-GluN2B, pSer897-GluN1, and pSer845-GluA1 to assess phosphorylation or to GluN2B, GluN1, or GluA1 to determine total subunit levels.

Ser1166 is critical to the PKA-dependent shift in NMDAR reversal potential

The results thus far indicate that Ser1166 of GluN2B is critical to PKA potentiation of NMDA-elicted currents, but they do not address a direct role for Ser1166 in regulation of NMDAR Ca2+ permeability by PKA. Toward this end, we undertook two experimental approaches. First, we examined current–voltage relations for HEK293 cells expressing GluN1 together with WT or mutant GluN2B receptors. The nonphosphorylatable mutant, S1166A, exhibited an open probability that was markedly reduced (Po, 0.13 ± 0.02, n = 14) relative to that of the phosphomimetic mutation S1166D (0.26 ± 0.03, n = 16, p = 0.0317; Fig. 1d; Table 1). The WT receptor exhibited an open probability (0.22 ± 0.04, n = 20) that was intermediate in value but did not differ statistically from that of S1166A or S1166D (p > 0.05, ANOVA) (Fig. 1d; Table 1), consistent with the substantial variability in GluN2B channel activity noted previously (Amico-Ruvio and Popescu, 2010; Borschel et al., 2012; Maki et al., 2012) and with the concept that WT GluN2B-containing NMDARs are a combination of both phosphorylated and nonphosphorylated receptor species. The large difference in activity between the S1166A and the S1166D mutants is largely attributable to a change in mean closed durations, as neither mean open duration nor unitary current amplitude was altered (Fig. 1d; Table 1). As with Po values, mean closed durations of WT NMDARs (28 ± 5 ms) were intermediate in value but did not differ significantly between that of S1166A (61 ± 19 ms) and S1166D (16 ± 2) mutant receptors (p > 0.05, ANOVA).

Ser1166 is critical to the PKA-dependent shift in NMDAR reversal potential

The results thus far indicate that Ser1166 of GluN2B is critical to PKA potentiation of NMDA-elicted currents, but they do not address a direct role for Ser1166 in regulation of NMDAR Ca2+ permeability by PKA. Toward this end, we undertook two experimental approaches. First, we examined current–voltage relations for HEK293 cells expressing GluN1 together with WT or mutant GluN2B(S1166A) receptors in the absence and presence of H-89. If, indeed, PKA facilitates Ca2+ permeation through NMDARs, inhibitors of PKA should cause a negative shift in the reversal potential (Erev) of the NMDAR current–voltage relationship (I/V curve) (Skeberdis et al., 2006). HEK293 cells are well suited for these experiments because of the relatively small contribution of voltage-gated conductance. NMDA-elicted currents were recorded at holding potentials ranging from ~60 to 60 mV, in 20 mV steps. In cells expressing WT NMDARs, the I/V curve was slightly outwardly rectifying and the NMDA reversal potential (Erev) was ~10 mV (Fig. 2a,b), the known reversal potential for NMDARs. The noise in the responses reflects the stochastic gat-
Table 2. Summary of statistics for Figures 4–6

<table>
<thead>
<tr>
<th>Figure</th>
<th>N</th>
<th>Mean ± SEM</th>
<th>p</th>
<th>Statistical test</th>
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<tr>
<td>4a</td>
<td>2–5 slices</td>
<td>control: 0.2732 ± 0.0585</td>
<td>*, 0.0138</td>
<td>One-way ANOVA with Tukey’s post-test (control vs PKA)</td>
</tr>
<tr>
<td>4b</td>
<td>6–10 slices</td>
<td>S1166: vehicle, 0.2978 ± 0.04566; ISO, 2.019 ± 0.244; ISO + PROP, 0.3674 ± 0.03994</td>
<td>****, &lt; 0.0001</td>
<td>One-way ANOVA with Tukey’s post-test (vehicle vs ISO and ISO vs vehicle + PROP)</td>
</tr>
<tr>
<td>4c</td>
<td>2–5 slices</td>
<td>S1166: vehicle, 0.4482 ± 0.04804; SKF, 0.8647 ± 0.05879; SKF + SCH, 0.4041 ± 0.1217</td>
<td>*&lt; 0.05</td>
<td>One-way ANOVA with Tukey’s post-test (vehicle vs SKF81297)</td>
</tr>
<tr>
<td>5a</td>
<td>4 animals</td>
<td>S1166: control, 0.7940 ± 0.1259; Fox U, 0.7689 ± 0.0638</td>
<td>**, 0.0030</td>
<td>Two-tailed paired t test for GluA1 S845 control vs Fox U</td>
</tr>
<tr>
<td>5b</td>
<td>5 animals</td>
<td>S1166: control, 0.1226 ± 0.0198; FS, 1.073 ± 0.175</td>
<td>***, &lt; 0.0006</td>
<td>Two-tailed unpaired t test for control vs FS</td>
</tr>
<tr>
<td>6b,c</td>
<td>5 animals</td>
<td>S1166: Sal control, 0.818 ± 0.1251; Sal FS, 1.543 ± 0.238; Rp-cAMPS control, 0.4344 ± 0.1228; Rp-cAMPS FS, 0.8291 ± 0.1975</td>
<td>*&lt; 0.05</td>
<td>One-way ANOVA with Tukey’s post-test (S1166 and S845 Sal control vs FS; Sal FS vs Rp-cAMPS FS)</td>
</tr>
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</table>

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Ser1166 of GluN2B is critical to PKA-dependent potentiation of NMDA-evoked Ca\(^{2+}\) rises

Second, we examined the impact of Ser1166 on PKA-dependent NMDAR-mediated Ca\(^{2+}\) rises. Toward this end, we imaged NMDA-evoked Ca\(^{2+}\) rises in HEK293 cells expressing GluN1 together with WT or mutant GluN2B(S1166A) receptors. Cells were loaded with the Ca\(^{2+}\) indicator Fura-2 AM; and after a brief (15–20 s) stable baseline, Ca\(^{2+}\) transients were elicited by a single application (60 s) of NMDA (100 μM) with 10 μM glycine, and images were taken before (control), during (H-89), and after washout (recovery) of H-89. Application of NMDA and glycine elicited a rise in Ca\(^{2+}\) (Fig. 2d,e). In cells expressing WT NMDARs, H-89 markedly inhibited NMDA-evoked Ca\(^{2+}\) rises (Fig. 2d–f; Table 1). After washout of the inhibitor, NMDA-evoked Ca\(^{2+}\) rises exhibited substantial recovery. In cells expressing GluN2B(S1166A), inhibition by H-89 was greatly diminished (Fig. 2d–f; Table 1). Collectively, these findings strongly suggest that Ser1166 is important to regulation of NMDAR Ca\(^{2+}\) signaling by PKA.

Ser1166 is required for regulation of NMDAR-mediated Ca\(^{2+}\) transients in spines

The results thus far indicate that Ser1166 is critical to regulation of NMDA-evoked Ca\(^{2+}\) signaling but do not distinguish between synaptic and extrasynaptic Ca\(^{2+}\) signaling. Ca\(^{2+}\) transients in the dendrites of CA1 pyramidal neurons induced by uncaging of glutamate can be restricted to a single spine and are mainly NMDAR-mediated (Yuste et al., 1999; Bloodgood and Sabatini, 2009). Moreover, spine Ca\(^{2+}\) is under the control of cAMP/PKA signaling (Skeberdis et al., 2006; Chalfoux and Carter, 2010; Higley and Sabatini, 2010). Whereas the functional impact of PKA on NMDARs is well established, the molecular target remains unknown. To investigate whether Ser1166 is critical to regulation of synaptic and extrasynaptic Ca\(^{2+}\) signaling, GluN2B(S1166A) receptors were imaged in the dendrites of CA1 layer II–III pyramidal neurons expressing a single spine and are mainly NMDAR-mediated.
of Ca$^{2+}$ rises in dendritic spines by PKA, we bioisotically overexpressed WT or mutant GluN2B(S1166A) in organotypic hippocampal slices. At 10–14 d after transfection, we activated glutamatergic synapses by focal glutamate uncaging and simultaneously measured Ca$^{2+}$ transients by two-photon microscopy and recorded uEPSCs at individual spines before and after application of isoproterenol, a well-known activator of β-adrenergic receptors and upstream activator of PKA (Fig. 3a,b).

In slices overexpressing WT GluN2B, application of isoproterenol (10 μM, 5 min) induced a marked increase in the pharmacologically isolated, NMDA component of uEPSC amplitude and Ca$^{2+}$ transients in individual dendritic spines (Fig. 3c–f; Table 1). In contrast, in slices overexpressing GluN2B(S1166A), isoproterenol did not detectably alter either the uEPSC amplitude or Ca$^{2+}$ transients (Fig. 3c–f; Table 1). Thus, the loss of Ser1166 is sufficient to abolish the impact of β-adrenergic receptor (and hence PKA) activation on NMDAR-mediated Ca$^{2+}$ transients and currents in individual spines. The finding that isoproterenol increases not only NMDAR-mediated Ca$^{2+}$ transients, but also NMDA uEPSCs, is consistent with our earlier observation that, in the case of GluN2B-containing NMDARs, PKA modulates not only NMDAR Ca$^{2+}$ signaling but also a substantial fraction of NMDA-evoked currents (Skeberdis et al., 2006). Collectively, these data indicate that Ser1166 is critical to β-adrenergic receptor-dependent potentiation of NMDAR-mediated Ca$^{2+}$ signaling and uEPSCs in individual spines. Moreover, these findings, together with our findings in Figure 1c, demonstrate that GluN2B(S1166A) serves as a dominant-negative mutant in hippocampal neurons.

**Activation of β-adrenergic and D$_1$/D$_5$ dopamine receptors promote phosphorylation of Ser1166**

Our findings thus far indicate that Ser1166 of GluN2B is a molecular and functional target of PKA, but they do not address whether physiological stimuli that engage and activate PKA signaling promote phosphorylation of Ser1166. Toward this end, we generated a phospho-specific antibody directed to Ser1166 of GluN2B. To validate the antibody, we performed several control experiments. First, we documented the ability of PKA-C to phosphorylate whether physiological stimuli that engage and activate PKA signaling promote phosphorylation of Ser1166. Toward this end, we generated a phospho-specific antibody directed to Ser1166 of GluN2B. To validate the antibody, we performed several control experiments. First, we documented the ability of PKA-C to phosphorylate Ser1166 of GluN2B (Fig. 6a). Second, we showed that incubation with the phosphopeptide (p-peptide) blocked the phospho-Ser1166 signal, indicating specificity of the antibody (Fig. 6a). Third, we showed that omission of phosphatase inhibitors to permit endogenous phosphatase activity or addition of alkaline phosphatase abolishes the phospho-Ser1166 signal (Fig. 6a).

The β-adrenergic (Hall, 2004; Dai et al., 2009) and D$_1$/D$_5$ dopamine receptors signal through the cAMP/PKA pathway (Beaulieu and Gainetdinov, 2011) to upregulate GluN2B phosphorylation at Ser1166. To test the hypothesis that physiological stimuli that engage and activate PKA signaling promote phosphorylation of Ser1166, we examined the effects of forced swim, fox urine, and D$_1$/D$_5$ dopamine receptor activation on phosphorylation levels of GluN2B at Ser1166 and GluN1 at Ser897 in a quantifiable in vivo assay. For this purpose, we used Western blot analysis to determine the phosphorylated forms of GluN2B and GluN1 in hippocampal lysates from rats exposed to fox urine or forced swim. Western blots were probed with antibodies to pSer1166-GluN2B, pSer897-GluN1, and pSer845-GluA1 and then with antibodies to total GluN2B, GluN1, and GluA1. Exposure to fox urine increased phosphorylation of GluN1 at Ser897 and GluA1 at Ser845, with little or no effect on phosphorylation levels of GluN2B at Ser1166 or GluN1 at Ser897 (Fig. 5a). Similar results were obtained for forced swim. Western blots (top) and summary data (bottom) for hippocampal lysates from rats exposed to a single episode of forced swim for 5 min. Western blots were probed with antibodies as above for exposure to fox urine. Forced swim induced striking phosphorylation of GluN2B at Ser1166, GluN1 at Ser897, and GluA1 at Ser845. Values for phosphorylated protein were normalized to the corresponding value for total protein and then to the corresponding value for control animals. n = 5 animals per treatment group. ***p = 0.0006 (S1166 control vs FS). +++p = 0.0002 (S845 control vs FS). ****p < 0.0001.

Phosphorylation of Ser1166 on GluN2B by PKA

**Figure 5.** Phosphorylation of GluN2B at Ser1166 is induced by forced swim, but not exposure to fox urine, *in vivo*. **a**, Representative Western blots (top) and summary data (bottom) for hippocampal lysates from rats exposed to fox urine for 5 min. Western blots were probed with antibodies to pSer1166-GluN2B, pSer897-GluN1, and pSer845-GluA1 and then with antibodies to total GluN2B, GluN1, and GluA1. Exposure to fox urine increased phosphorylation of GluN1 at Ser897 and GluA1 at Ser845, with little or no effect on phosphorylation levels of GluN2B at Ser1166 or GluN1 at Ser897. n = 4 animals per treatment group. **p = 0.0030. **b**, Representative Western blots (top) and summary data (bottom) for hippocampal lysates from rats exposed to a single episode of forced swim for 5 min. Western blots were probed with antibodies as above for exposure to fox urine. Forced swim induced striking phosphorylation of GluN2B at Ser1166, GluN1 at Ser897, and GluA1 at Ser845. Values for phosphorylated protein were normalized to the corresponding value for total protein and then to the corresponding value for control animals. n = 5 animals per treatment group. ***p = 0.0006 (S1166 control vs FS). +++p = 0.0002 (S845 control vs FS). ****p < 0.0001.
Phosphorylation of Ser1166 is induced by the emotional response to stress in vivo

The results thus far indicate that phosphorylation of GluN2B at Ser1166 is critical to modulation of NMDAR function by PKA, but do not address experience-driven regulation of NMDAR phosphorylation at this site. Adverse experience in the form of stress promotes the release of norepinephrine and β-adrenergic signaling (McGaugh and Roozendaal, 2002; Padgett and Glaser, 2003). We examined whether adverse experience promotes phosphorylation of GluN2B at Ser1166 in the hippocampus. A single, brief exposure to fox urine stimulates release of norepinephrine and induces freezing behavior, indicative of fear (Hu et al., 2007; Liu et al., 2010). Exposure of animals to fox urine (5 min) increased phosphorylation of GluA1 at Ser845 in the hippocampal CA1, in corroboration of others (Hu et al., 2007) (Fig. 5a; Table 2), with little or no effect on phosphorylation of the NMDAR subunits GluN2B at Ser1166 and GluN1 at Ser897 (Fig. 5a; Table 2). These findings indicate that phosphorylation of NMDARs and AMPARs in response to emotional stress is under the control of different intracellular signaling pathways in vivo.

We next examined the impact of forced swim, another well-established paradigm of emotional as well as mild physical stress (Anisman et al., 2001), on NMDAR phosphorylation in the hippocampus. A single, brief exposure to forced swim stimulates release of norepinephrine (Yoshitake et al., 2004) and increases the magnitude of LTP at synapses onto dentate gyrus granule cells (Korz and Frey, 2003). Exposure of animals to forced swim (5 min) induced striking phosphorylation of GluN2B at Ser1166 and (to a lesser extent) phosphorylation of GluN1 at Ser897 (Fig. 5b; Table 2). Forced swim also induced a relatively modest phosphorylation of GluA1 at Ser845. The finding that adverse experience in the form of forced swim, but not exposure to fox urine, facilitates phosphorylation of GluN2B at Ser1166 suggests that different forms and/or intensities of stress can induce differential phosphorylation of PKA targets in a receptor- and site-specific manner.

To identify the signaling pathway that mediates stress-induced phosphorylation of GluN2B at Ser1166, we injected rats with the PKA inhibitor Rp-8-Br-cAMPS or saline, directly into the hippocampal CA1 at 1 hr prior to forced swim and microdissected the small region of CA1 directly around the injection site (Fig. 6a). Forced swim induced an approximately twofold increase in phosphorylation of GluN2B at Ser1166 and a modest increase in phosphorylation of GluN1 at Ser897. Rp-8-Br-cAMPS markedly reduced phosphorylation of GluN2B at Ser1166 (and, to a lesser extent, GluN1 at Ser897) under both control and forced swim conditions (Fig. 6b; Table 2). These findings are consistent with the concept that both basal and experience-dependent phosphorylation of Ser1166, at least in part, is mediated by cAMP/PKA signaling. Rp-8-Br-cAMPS also reduced basal and forced swim-induced phosphorylation of GluA1 at Ser845 (Fig. 6c; Table 2).

Discussion

The present study identifies Ser1166 in the NMDAR subunit GluN2B as a novel molecular and functional target of PKA phosphorylation critical to potentiation of NMDAR Ca$^{2+}$ signaling at synapses and regulated by the emotional response to stress. We show that physiologically relevant stimuli that engage and activate cAMP/PKA signaling, and are important to the acquisition and consolidation of memory (McGaugh and Roozendaal, 2002; Hu et al., 2007), regulate phosphorylation of NMDARs and Ca$^{2+}$ signaling in spines. Accordingly, activation of β-adrenergic and D$_1$/D$_5$ dopamine receptors stimulates phosphorylation of GluN2B at brain slices. Moreover, activation of β-adrenergic receptors potentiates Ca$^{2+}$ transients and uncaging-evoked synaptic currents in individual spines of neurons expressing WT, but not mutant S1166A, NMDARs. Thus, loss of this single phosphorylation site is sufficient to abolish β-adrenergic-dependent potentiation of NMDA currents and Ca$^{2+}$ transients, linking phosphorylation of NMDARs to Ca$^{2+}$ signaling in spines. This is significant in that central noradrenergic signaling mediates emotional arousal and the emotional response to stress (McGaugh and Roozendaal, 2002; Padgett and Glaser, 2003). We further show that adverse experience in the form of forced swim, but not exposure to fox urine, elicits striking phosphorylation of Ser1166 in a PKA-dependent manner in vivo, revealing experience-dependent phosphorylation of this site in a physiologically relevant context. These findings indicate that different stress
paradigms differentially regulate NMDAR phosphorylation and, by inference, Ca\(^{2+}\) permeation and Ca\(^{2+}\) signaling.

cAMP/PKA signaling represents a pivotal mechanism by which NMDAR-mediated Ca\(^{2+}\) signaling is modulated in neurons under physiological and pathological conditions. PKA promotes NMDAR Ca\(^{2+}\) permeability, Ca\(^{2+}\) rises in spines, and induction of LTP at CA1 synapses (Skeberdis et al., 2006). Moreover, extracellular signals that modulate CAMP bidirectionally regulate Ca\(^{2+}\) permeation through NMDARs and spine Ca\(^{2+}\) in a PKA-dependent manner (Chalifoux and Carter, 2010; Higley and Sabatini, 2010). Although these studies establish a functional role for PKA, they do not address the molecular target. In the present study, we reveal a previously unrecognized mechanism by which activation of major signaling pathways, known to couple positively to CAMP/PKA and play a critical role in learning and memory, can alter Ca\(^{2+}\) signaling in activated dendritic spines. Although not addressed by the present manuscript, phosphorylation of the carboxy-terminal tails of channels is known to affect channel properties, including ion selectivity. Moreover, phosphorylation events that regulate channel activity typically occur in either the N-terminal or C-terminal domains of the channel and not in the pore itself (Levitan and Kaczmarek, 2002). By analogy, phosphorylation of Ser\(^{1166}\) may induce a delocalized conformational change. Alternatively, Ser\(^{1166}\) might form part of a recognition motif for an as yet unidentified protein, which in some capacity regulates the selectivity pore within the NMDA channel.

A novel finding of the present study is that emotional stress in the form of a single exposure to forced swim, but not exposure to fox urine, elicits striking phosphorylation of Ser\(^{1166}\) in a PKA-dependent manner in vivo, revealing experience-driven regulation of phosphorylation at this site. Notably, these effects persist as late as P32, the oldest age examined, consistent with the concept that phosphorylation at this site. Notably, these effects persist as late as P32, the oldest age examined, consistent with the concept that phosphorylation at this site.

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