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CaMKII-mediated phosphorylation of GluN2B regulates recombinant NMDA receptor currents in a chloride-dependent manner

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Abstract

Some forms of long-term synaptic plasticity require docking of Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKII α) to residues 1290–1309 within the intracellular C-terminal tail of the GluN2B N-methyl-D-aspartate (NMDA) receptor subunit. The phosphorylation of Ser1303 within this region destabilizes CaMKII binding. Interestingly, Ser1303 is a substrate for CaMKII itself, as well as PKC and DAPK1, but these kinases have been reported to have contradictory effects on the activity of GluN2B-containing NMDA receptors. Here, we have re-assessed the effect of CaMKII on NMDA receptor desensitization in heterologous cells, as measured by the ratio of steady-state to peak currents induced during 3 s agonist applications. CaMKII α co-expression or infusion of constitutively active CaMKII limits the extent of desensitization and preserves current amplitude with repeated stimulation of recombinant GluN1A/GluN2B when examined using low intracellular chloride (Cl^-) levels, characteristic of neurons beyond the first postnatal week. In contrast, CaMKII α enhances the acute rate and extent of desensitization when intracellular Cl^- concentrations are high. The apparent dependence of CaMKII α effects on NMDA receptor desensitization on Cl^- concentrations is consistent with the presence of a Ca^{2+} -activated Cl^- conductance endogenous to HEK 293 cells, which was confirmed by photolysis of caged- Ca^{2+} . However, Ca^{2+} -activated Cl^- conductances are unaffected by CaMKII α expression, indicating that CaMKII affects agonist-induced whole cell currents via modulation of the NMDA receptor. In support of this idea, CaMKII α modulation of GluN2B-NMDA receptors is abrogated by the phospho-null mutation of Ser1303 in GluN2B to alanine and occluded by phospho-mimetic mutation of Ser1303 to aspartate regardless of intracellular Cl^- concentration. Thus, CaMKII-mediated phosphorylation of GluN2B-containing NMDA receptors reduces desensitization at physiological (low) intracellular Cl^- , perhaps serving as a feed-forward mechanism to sustain NMDA-mediated Ca^{2+} entry and continued CaMKII activation during learning and memory.

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Keywords

CaMKII; NMDA receptor; calcium; phosphorylation; synaptic plasticity; electrophysiology; desensitization

Introduction

CaMKII α activation in response to NMDA receptor-mediated Ca²⁺ entry serves as a critical mediator of synaptic plasticity that is thought to contribute to learning and memory (Coultrap and Bayer, 2012; Hell, 2014; Lisman et al., 2012; Merrill et al., 2005; Shonesy et al., 2014). Activated CaMKII α translocates to the postsynaptic density (PSD), in part by binding to residues 1290–1309 within the GluN2B NMDA receptor subunit intracellular C-terminal tail (Bayer et al., 2001; Leonard et al., 1999; Shen and Meyer, 1999; Strack et al., 1997; Strack and Colbran, 1998; Strack et al., 2000). CaMKII targeting to the PSD via GluN2B may be critical for the phosphorylation of other substrates within the PSD, such as GluA1 subunits of the AMPA receptor, perhaps in part due to the ability of GluN2B to stabilize a constitutively active conformation of CaMKII (Bayer et al., 2001; Colbran, 2004; Leonard et al., 2002; Strack et al., 1997; Strack and Colbran, 1998). Indeed, disrupting CaMKII-GluN2B interactions in mice reduces GluA1 phosphorylation (Halt et al., 2012). Several studies also highlight the importance of CaMKII binding to GluN2B in normal synaptic plasticity (Barria and Malinow, 2005; Halt et al., 2012; Sanhueza et al., 2011; Stein et al., 2014; Zhou et al., 2007).

Ser1303 within the CaMKII docking sequence in GluN2B is prominently phosphorylated by CaMKII, which paradoxically promotes CaMKII dissociation (Omkumar et al., 1996; Raveendran et al., 2009; Strack et al., 2000). Physiologically relevant concentrations of ATP may mitigate this effect (O'Leary et al., 2011). While Ser1303 phosphorylation by PKC and DAPK1 was shown to enhance NMDA receptor currents (Liao et al., 2001; Tu et al., 2010), the co-expression of CaMKII was reported to attenuate GluN1A/GluN2B currents by enhancing the extent of desensitization (Sessoms-Sikes et al., 2005). Moreover, the relative importance of CaMKII binding to GluN2B and Ser1303 phosphorylation for the modulation of GluN2B channel kinetics by CaMKII remains unclear and has not been explicitly investigated.

Here we evaluated the mechanisms underlying CaMKII modulation of recombinant GluN1A/GluN2B receptors in HEK293 cells. Surprisingly, we demonstrate a bimodal effect of CaMKII on the apparent desensitization of GluN1A/GluN2B whole-cell currents that is dependent on intracellular Cl[−] concentrations. When intracellular Cl[−] is low, typifying intraneuronal concentrations that predominate beyond first week of postnatal development (Ben-Ari, 2002), CaMKII reduces the extent of desensitization of GluN1A/GluN2B receptors. In contrast, when intracellular Cl[−] is high, CaMKII appears to enhance GluN1A/GluN2B receptor desensitization because whole-cell currents include a significant contribution from an endogenous Ca²⁺-activated Cl[−] conductance that is coupled to NMDA receptor-mediated Ca²⁺ entry. Mutation of Ser1303 to alanine abrogates the effect of CaMKII α , while phospho-mimetic mutation of Ser1303 to aspartate mimics the effect without addition of

CaMKII α . Collectively, these data indicate that Ser1303 phosphorylation alone is responsible for the direct effects of CaMKII on Ca²⁺ entry via GluN1A/GluN2B NMDA receptors. These data help to resolve apparent discrepancies for the role of Ser1303 phosphorylation towards its effects on GluN1A/GluN2B receptor currents. Moreover, these data suggest that Ser1303 phosphorylation may serve as feed-forward mechanism to sustain Ca²⁺ signals that are necessary for synaptic plasticity in mature neurons.

Materials and Methods

Molecular constructs

GluN1A and GluN2B were obtained from Gary Westbrook (Vollum Institute, OHSU, Portland, OR) both in pcDNA1/AMP vector as previously described (Krupp et al., 1996). CaMKII α in pEGFP(C-1) was previously described (Abiria and Colbran, 2010). GluN2B Ser1303 to alanine (S1303A) and Ser1303 to aspartate (S1303D) point mutants had been previously described (Strack et al., 2000). The mutated cDNAs were used as a template for PCR reactions to amplify a 1087 bp region encoding the distal C-terminal domain of GluN2B including position 1303, which was digested with AgeI and EcoRV restriction enzymes (NEB). Mutant fragments were ligated into the complementary AgeI/EcoRV sites within a wild-type GluN2B in pcDNA1/AMP. Appropriate incorporation of these fragments was verified by sequencing.

Cell culture and transfection

HEK 293 cells (#CRL-1573; ATCC; Manassas, VA) were grown on 15 mm round glass coverslips in standard culture media (Dulbecco's modified Eagle medium, 10% fetal bovine serum + penicillin/streptomycin) (Life Technologies), and transfected with 1 μ g each of GluN1A and GluN2B (or the corresponding phosphomutants) with either 0.3 μ g pEGFP (as transfection marker) or 1 μ g GFP-CaMKII α by the Ca²⁺-phosphate precipitation method. D-AP5 (250 μ M) was added to the culture medium to prevent excitotoxicity.

Expression, purification, and kinase activity assay of constitutively active CaMKII

CaMKII(1–290) in pET100/D-TOPO (Invitrogen, Carlsbad, CA), as previously described (Tavalin 2008), was transformed into Rosetta 2 (DE3) (Novagen) competent cells and grown in Luria-Bertani broth supplemented with ampicillin and chloramphenicol at 37°C to an optical density of 0.5 (600 nm). Protein expression was induced by stimulation with 1 mM isopropyl β -D-1-thiogalactopyranoside at 30 °C for 5.5 hr. Cells were pelleted by centrifugation and frozen overnight at –20 °C. Cells were thawed and lysed in B-PER reagent, and purified by the HisPur Co²⁺ purification kit (Thermo Scientific, Rockford, IL). Contaminating salts were removed by dialysis against kinase storage buffer (100 mM NaCl, 20 mM HEPES, 0.1 mM EDTA, 2 mM dithiothreitol, and 10% glycerol; pH 7.4) and the protein was concentrated by ultrafiltration (Millipore, Bedford, MA). Protein concentration was determined by Bradford assay (Bio-Rad) and the kinase concentration was adjusted to 20 μ M by addition of kinase storage buffer and stored at –80 °C in convenient aliquots.. CaMKII(1–290) (10–100 ng) activity was assayed in 50 μ l reactions using in phosphorylation buffer (10 mM HEPES, 10 mM MgCl₂, and 1 mM DTT, 0.1 mg/ml bovine serum albumin; pH 7.40) and 100 μ M syntide-2 (Genscript) as substrate. Reactions were

initiated by addition of ATP (100 μ M final) and carried out at 30 °C for 60 min in a shaking incubator. Reactions were terminated by the addition of 50 μ l Kinase-Glo Plus reagent (Promega) and luminescence was acquired using a Bio-Rad XRS chemiluminescence documentation system and Quantity One software. Signals from each reaction were compared against a standard curve (0–100 μ M ATP) to determine the concentration of ATP remaining in each sample. The amount of ATP consumed during the reaction was then used to calculate the specific activity which was ~ 634 nmol/min/mg when using 10 ng of the kinase and assessed in two independent assays. CaMKII(1–290) was added to the intracellular pipette solution to the desired concentration.

Electrophysiology and Ca²⁺-uncaging

Twenty-four hours after transfection, cells were visually selected for recording by GFP epifluorescence. Whole-cell recordings of NMDA receptor currents were made with a Multiclamp 700A amplifier (Molecular Devices). Patch pipettes (2 to 4 M Ω) contained (in mM): 140 Cs methanesulfonate (MeSO₄) or CsCl, 10 HEPES, 5 adenosine triphosphate (Na salt), 5 MgCl₂, 0.2 CaCl₂, and 1 EGTA or 10 BAPTA (pH 7.4). The extracellular solution contained (in mM) 150 NaCl, 5 KCl, 1.8 CaCl₂, 10 HEPES, 11.1 glucose, and 0.01 or 0.1 glycine (pH 7.4). To speed solution exchange, cells were lifted from the coverslip after establishment of the whole-cell configuration and placed ~ 20 μ m from the mouth of a series of flow pipes which were controlled by solenoid valves and moved into position by a piezoelectric bimorph. With this configuration, solution exchanges were accomplished within 10–12 ms as assessed by the rise time (20 – 80%) of whole-cell agonist-evoked current responses. Currents were digitized at 2 kHz and filtered at 0.5–1 kHz. Series resistance (90 to 95%) and whole-cell capacitance compensation were employed. Experiments were performed at a holding potential of –60 mV at 20°C. Currents were elicited by a 3 s application of glutamate (1 mM) or NMDA (100 μ M) in the presence of glycine. Desensitization was quantified as the ratio of current remaining at the end of the 3 s agonist application to the peak current; single exponential fits for the desensitizing portion of the current were obtained using Clampfit 10.3 software. Analysis of steady-state to peak current ratios and exponential fits were performed following normalization of each data sweep to its peak and averaging of at least 3 normalized sweeps. Ca²⁺-activated currents were recorded via a Multiclamp 700B amplifier using the same extracellular solution as above and patch pipettes filled with (in mM) 140 CsCl or CsMeSO₄, 10 HEPES, 5 adenosine triphosphate (Mg salt), 0.02 CaCl₂, and 0.1 NP-EGTA (Life Technologies; pH 7.40) in order to maintain a similar free Ca²⁺ as with our standard EGTA-containing intracellular solutions. A fiber optic cable was connected to the output of a mercury arc lamp (Newport) and directed to a shutter (Thorlabs) which was connected to the epifluorescent port of a homemade convertible microscope in the upright configuration. A 495 nm long pass-dichroic beamsplitter (Chroma) was used to direct the light through the back of the objective to the cells. A 470–510 nm excitation filter (Chroma) was transiently moved into place for visualization and selection of cells based on GFP epifluorescence prior to recording and uncaging. To elicit Ca²⁺-activated currents, the shutter was opened for 1.24 s to drive photolysis of NP-EGTA. A voltage ramp protocol was run from –60 to +60 mV during the last 0.24 s of light stimulation to generate the I-V relationship. Currents were corrected for leak currents by subtracting currents obtained with shutter closed just prior to

the photolysis protocol. For each cell, current density was derived by normalizing the current by each cell's capacitance.

For all experiments data are reported as mean $\pm$ S.E. and were subjected to statistical analysis by Student's t-test.

Results

Previous studies indicated that CaMKII decreased NMDA receptor currents by enhancing the extent of desensitization of GluN1A/GluN2B NMDA receptors in HEK293 cells (Sessoms-Sikes et al., 2005). Other studies indicated that PKC and DAPK can phosphorylate the major CaMKII phosphorylation site on GluN2B and enhance GluN2B NMDAR currents (Liao et al., 2001; Tu et al., 2010), although several technical differences might contribute to the disparate results. Replicating conditions used for the prior study of CaMKII modulation of NMDARs (100 μ M NMDA/10 μ M glycine; 1 mM ATP; Cl^- as the major anion), we first confirmed that CaMKII α co-expression enhances the apparent extent of GluN1A/GluN2B desensitization as evaluated by steady-state to peak current ratios (GluN1A/GluN2B: 0.42 ± 0.03 , $n = 4$ vs. + CaMKII α : 0.23 ± 0.04 , $n = 4$; $p < 0.01$; Fig. 1A, E). In contrast, desensitization was significantly decreased by CaMKII α co-expression when whole-cell current responses to 1 mM glutamate/100 μ M glycine were recorded using 5 mM ATP and MeSO_4^- in the pipet solutions (GluN1A/GluN2B: 0.35 ± 0.05 , $n = 5$. +CaMKII α : 0.59 ± 0.05 , $n = 6$; $p < 0.01$; Fig. 1B, E), similar to the effects of other kinases that target Ser1303 for phosphorylation. Therefore, we systematically examined the impact of the technical differences (i.e. ATP concentration, major anion, and agonist) towards generating these disparate responses to CaMKII α co-expression. Because ATP is critical for kinase activity, we first examined the effect of reducing ATP concentrations from 5 mM to 1 mM in the CsMeSO₄-based intracellular recording solution, continuing to use 1 mM glutamate/100 μ M glycine as agonist/co-agonist. However, under these conditions, CaMKII α still reduced NMDAR desensitization, albeit to a slightly lesser extent (GluN1A/GluN2B: 0.34 ± 0.04 , $n = 4$ vs. + CaMKII α : 0.47 ± 0.04 , $n = 6$; $p < 0.05$; Fig. 1C, E). In contrast, exchanging the predominant intracellular anion from MeSO_4^- to Cl^- (while continuing to use 1 mM glutamate/100 μ M glycine as agonist/co-agonist and 5 mM intracellular ATP) resulted in a CaMKII-dependent enhancement of GluN1A/GluN2B desensitization (GluN1A/GluN2B: 0.50 ± 0.04 , $n = 6$ vs. + CaMKII α : 0.33 ± 0.05 , $n = 7$; $p < 0.05$; Fig. 1D, E). Moreover, the extent of GluN1A/GluN2B desensitization in the absence of CaMKII also was sensitive to the identity of the major intracellular anion (GluN1A/GluN2B (MeSO_4^-): 0.35 ± 0.05 , $n = 5$ vs. GluN1A/GluN2B (Cl^-): 0.50 ± 0.04 , $n = 6$; $p < 0.05$; Fig. 1E). These results indicate that the effects of CaMKII are independent of the agonist used or the ATP concentration (at least in this range), but that intracellular concentrations of anions (i.e., Cl^-) dictate the apparent direction by which CaMKII regulates GluN1A/GluN2B receptor desensitization

High concentrations of the fast calcium chelator BAPTA blocked the apparent effect of CaMKII to enhance GluN1A/GluN2B receptor desensitization with high intracellular Cl^- concentrations (Sessoms-Sikes et al., 2005). Similarly, we found that BAPTA occludes the ability of CaMKII α to reduce desensitization in the presence of low intracellular Cl^- (GluN1A/GluN2B: 0.66 ± 0.05 , $n = 5$ vs. + CaMKII α : 0.68 ± 0.04 , $n = 5$; Fig 2). Although

effects of BAPTA on CaMKII-dependent regulation are somewhat difficult to interpret because BAPTA reduces the baseline GluN1A/GluN2B desensitization in the absence of co-expressed CaMKII, it seems likely that Ca^{2+} influx via GluN1A/GluN2B receptors drives CaMKII-dependent modulation of receptor desensitization.

Since intracellular Cl^- concentrations dictate the direction by which CaMKII modulates the apparent extent of GluN1A/GluN2B receptor desensitization and strong Ca^{2+} buffering abrogates the ability of CaMKII to modulate the receptor, we hypothesized that a Ca^{2+} - and/or CaMKII-dependent Cl^- conductance endogenous to HEK cells contributed to these phenomena. Indeed, there is evidence that neuronal NMDA receptor responses can be shaped by similar Cl^- conductances (Huang et al., 2012; Wang et al., 2006) and secondary coupling of recombinant NMDA receptors to endogenous Ca^{2+} -activated Cl^- currents within a heterologous expression system can contribute to the appearance of strongly desensitizing agonist-evoked responses (Leonard and Kelso, 1990). To address this issue we used wide-field UV-induced photolysis of the caged- Ca^{2+} compound NP-EGTA (Ellis-Davies and Kaplan, 1994) in HEK 293 cells transfected with GluN1A/GluN2B in the absence or presence of CaMKII α . Ca^{2+} -uncaging activated an outwardly rectifying current that reversed near 0 mV when using high Cl^- recording electrodes but negligible currents when using MeSO_4^- -filled electrodes, consistent with activation of a Ca^{2+} -activated Cl^- current (Fig 3A, B). However, CaMKII α co-expression did not modify the Ca^{2+} -activated Cl^- conductance, as measured by the current density of (GluN1A/GluN2B: 2.2 ± 1.0 pA/pF at -60 mV, $n = 4$ vs. + CaMKII α : 2.4 ± 0.7 , $n = 4$; Fig 3A, B), or the activation time course as assessed by the 20 – 80 % rise time of the current (GluN1A/GluN2B: 320 ± 87 ms, $n = 4$ vs. + CaMKII α : 290 ± 63 ms, $n = 4$). This suggests that the mechanism by which CaMKII affects GluN1A/GluN2B receptor desensitization primarily involves its ability to directly modulate Ca^{2+} entry via the NMDA receptor, as opposed to an indirect effect on the coupled Ca^{2+} -activated Cl^- conductance.

Activation of a Ca^{2+} -activated Cl^- conductance secondary to NMDA receptor-mediated Ca^{2+} entry may be expected to alter the apparent desensitization rate. Commensurate with this idea, single exponential fits revealed that CaMKII α co-expression selectively accelerated the decay time constant of the agonist-evoked current only when intracellular Cl^- was elevated (GluN1A/2B: 665 ± 53 ms, $n = 5$ vs. + CaMKII α : 795 ± 78 ms, $n = 6$ for MeSO_4^- filled pipettes; Fig 1B, 4C; GluN1A/2B: 709 ± 80 ms, $n = 6$ vs. + CaMKII α : 481 ± 54 ms, $n = 7$ for Cl^- filled pipettes; $p < 0.05$; Fig 1D, 4C).

We next tested for an effect of mutating the major CaMKII phosphorylation site on GluN2B (Omkumar et al., 1996; Strack et al., 2000), which is embedded within the CaMKII binding site, on receptor desensitization while using MeSO_4^- as the major intracellular anion. Mutation of Ser1303 to Ala to abrogate phosphorylation of GluN2B at this site had no effect on desensitization in the absence of CaMKII, but the co-expression of CaMKII had no effect on desensitization of GluN1A/GluN2B-S1303A receptors (GluN1A/S1303A: 0.38 ± 0.02 , $n = 4$ vs. + CaMKII α : 0.37 ± 0.05 , $n = 5$; Fig. 4A, C). Importantly, a phospho-mimetic Ser1303 to aspartate mutation (S1303D) reduced GluN1A/GluN2B receptor desensitization in the absence of CaMKII α , and CaMKII α co-expression had no further effect (GluN1A/S1303D: 0.56 ± 0.05 , $n = 5$ vs. + CaMKII α : 0.55 ± 0.04 , $n = 5$; Fig 4A, C). Of note, neither

phospho-mutant nor CaMKII co-expression affected the current decay kinetics (GluN1A/S1303A: 707 ± 98 ms, $n=4$ vs. + CaMKII α : 708 ± 122 ms, $n=5$; GluN1A/S1303D: 609 ± 86 ms, $n=5$ vs. + CaMKII α : 782 ± 36 ; $n=5$; Figure 4C). Collectively, these data suggest that CaMKII phosphorylation of Ser1303 is responsible for enhancing GluN1A/GluN2B currents via a reduction in the extent of desensitization with low intracellular Cl⁻ concentrations. Similarly, while using Cl⁻ as the major intracellular anion, the phospho-deficient S1303A mutant had no effect on the extent or rate of desensitization in the absence of CaMKII, and CaMKII had no effect on these parameters (GluN1A/S1303A: 0.48 ± 0.03 and 788 ± 89 ms, $n=5$ vs. + CaMKII α : 0.49 ± 0.02 and 715 ± 25 ms, $n=5$; Fig. 4B and D). However, phospho-mimetic S1303D mutation enhanced the extent of GluN1A/GluN2B receptor desensitization in the absence of CaMKII α , and CaMKII α co-expression had no further effect (GluN1A/S1303D: 0.28 ± 0.05 , $n=5$ vs. + CaMKII α : 0.34 ± 0.04 , $n=6$; Fig 4B). Importantly, this increase in the extent of desensitization due to phospho-mimetic substitution at Ser1303 using Cl⁻ as the major intracellular anion was accompanied by acceleration of the decay time constant of the agonist-evoked current similar to that observed due to CaMKII co-expression with wild-type GluN1A/GluN2B receptors (GluN1A/S1303D: 444 ± 29 , $n=5$ vs. + CaMKII α : 492 ± 66 , $n=6$; Fig 4B). Together, these data further support the idea that phosphorylation of GluN2B at Ser1303 represents the principal target by which CaMKII modulates recombinant GluN1A/GluN2B currents regardless of transmembrane Cl⁻ gradients.

CaMKII α co-expression with GluN2B-containing receptors, carried out with high intracellular Cl⁻ concentrations, does not appear to increase the amplitude of NMDA receptor currents when compared on a population basis. Similarly, we could not detect any systematic increase in initial peak current due to CaMKII α co-expression or phosphorylation state of the receptor, regardless of intracellular Cl⁻ concentration. This likely stems from a wide variation of current density across all transfections conditions ($13 \pm 2 - 86 \pm 26$ pA/pF). Therefore, in order to explore whether CaMKII regulates GluN1A/GluN2B current amplitude, we investigated changes in whole cell currents with repeated agonist applications. In the absence of co-expressed CaMKII, peak GluN1/GluN2B current amplitude declined ($-24.6 \pm 5.6\%$ relative to initial current, $n=5$; Fig. 5A) over the course of 8 agonist applications, when using MeSO₄⁻ as the intracellular anion to avoid confounds associated with the endogenous Ca²⁺-activated Cl⁻ conductance. This decline was independent of intracellular Ca²⁺ as it occurred even when 10 mM BAPTA was included in the whole-cell recording solution ($-20.6 \pm 6.4\%$ relative to initial current, $n=4$; Fig. 5A), reminiscent of a use-dependent downregulation described for GluN1A/GluN2A receptors (Vissel et al., 2001). However, the extent of desensitization during individual agonist applications was extremely stable even in the face of the decline in whole cell current amplitudes (Fig. 5B). In contrast, peak GluN1A/GluN2B current amplitudes were unaffected during repeated agonist applications when constitutively active CaMKII was infused into the cell or when CaMKII α was co-expressed (CaMKII(1–290) infusion: $-3.6 \pm 6.4\%$ relative to initial current, $n=6$; $p < 0.05$ compared to GluN1A/GluN2B alone and CaMKII α co-expression: $-1.8 \pm 7.8\%$, $n=3$; $p < 0.05$). Thus, in the presence of CaMKII, whole cell current amplitudes after 8 agonist applications were increased by ~30% compared to control. Significantly, the extent of reduction of GluN1A/GluN2B current desensitization of was

very similar following the loading constitutively active CaMKII or the co-expression of CaMKII α (GluN1A/2B alone: 0.34 ± 0.05 ; $n = 5$; CaMKII infusion: 0.56 ± 0.05 , $n = 6$; $p < 0.05$ compared to GluN1A/2B; CaMKII co-expression: 0.55 ± 0.06 , $n = 3$; $p < 0.05$ compared to GluN1A/2B; all measured at application # 3; Fig 5B). Thus, CaMKII not only reduces desensitization but also augments GluN1A/GluN2B current amplitude as has been observed for other kinases that can phosphorylate Ser1303.

Discussion

The present studies resolve a longstanding controversy concerning the role of CaMKII in regulating GluN2B-NMDARs. Our data show that CaMKII decreases the extent of desensitization of these receptors and preserves current amplitude, resulting in a net increase in Ca^{2+} influx. This is evident when whole-cell recordings are conducted using low intracellular Cl^- concentrations that match levels that prevail in neurons beyond the 1st week of postnatal development. In the presence of high intracellular Cl^- concentrations, Ca^{2+} -activated Cl^- currents are inwardly directed at -60 mV, and summate with agonist-evoked GluN1A/GluN2B currents to shape the overall agonist-induced whole-cell response. This coupling of NMDA receptors to Ca^{2+} -activated Cl^- channels camouflages the apparent effect of CaMKII α as an apparent increase in desensitization.

High intracellular Cl^- concentrations also reduced the apparent extent of GluN1A/GluN2B receptor desensitization in the absence of CaMKII α . This may be expected if GluN1A/GluN2B-mediated Ca^{2+} influx is rather weakly or remotely coupled to the endogenous Ca^{2+} -activated Cl^- channels. Ca^{2+} would be expected to stimulate a relatively slow rise and decay of remote inward Cl^- currents, summing with the GluN1A/GluN2B current to increase agonist-induced steady-state currents to a greater extent than the peak currents. CaMKII α co-expression significantly reduces the extent of GluN1A/GluN2B desensitization when examined using low intracellular Cl^- concentrations, presumably reflecting the direct effect of CaMKII on these NMDA receptors. However, in the presence of high intracellular Cl^- , the enhanced Ca^{2+} influx caused by CaMKII α co-expression results in more efficient coupling to the Ca^{2+} -activated Cl^- current. This would preferentially augment the peak agonist-induced current to a greater extent than the steady-state current, resulting in an apparent enhancement of the extent of desensitization as previously described (Sessoms-Sikes et al., 2005) and confirmed here. Notably, we also found that CaMKII accelerated GluN1A/GluN2B desensitization in high Cl^- . In previous studies, CaMKII accelerated the desensitization of GluN1F/GluN2B splice variant receptors, but not GluN1A/GluN2B receptors. The reason for this discrepancy is unclear, but may relate to the faster agonists application conditions used in the present studies which may have allowed us to better resolve an impact of the Ca^{2+} -activated Cl^- current on the apparent desensitization kinetics with the generally smaller GluN1A/GluN2B-mediated currents. It must be noted that current density for recombinant GluN1A/2B currents is highly variable within and across transfection conditions ($13 \pm 2 - 86 \pm 26$ pA/pF), such that we could not reliably detect potential changes in the absolute peak or steady-state currents. Nevertheless, we observed a consistent pattern of effects on the rate and extent of desensitization when using elevated intracellular Cl^- , suggesting that these parameters are influenced by local, rather than bulk, Ca^{2+} entry.

Our analysis of whole-cell Ca^{2+} -activated Cl^- currents induced by photolysis of caged- Ca^{2+} suggests that these currents were typically on the order of 2 pA/pF. This may appear insufficient to significantly impact steady-state to peak NMDA receptor current ratios. However, wide-field photolysis of caged- Ca^{2+} under the conditions of our experiments likely generated submaximal intracellular Ca^{2+} concentrations for activation of the Ca^{2+} -activated Cl^- current. Indeed, our photolysis conditions likely achieved “global” free Ca^{2+} concentrations on the order of 3–5 μM based on the concentration of caged- Ca^{2+} included in the pipette (20 μM) and the quantum yield of NP-EGTA (Ellis-Davies and Kaplan, 1994). Endogenous Ca^{2+} -activated Cl^- channels can be an order of magnitude larger when maximally activated by Ca^{2+} (Sala-Rabanal et al., 2015). It seems likely that NMDA receptor activation generates larger sub-membrane Ca^{2+} elevations than the global increases we achieved by uncaging Ca^{2+} (Sabatini et al., 2002). Thus, Ca^{2+} -activated Cl^- currents should be sufficiently large to significantly modify the relatively small agonist-evoked peak GluN1A/GluN2B glutamate-gated currents, especially after CaMKII-dependent enhancement of Ca^{2+} influx via the NMDA receptor.

The identity of the Ca^{2+} -activated Cl^- current in HEK 293 cells remains unknown. TMEM16A and CLC-3 are potential candidates as both have been reported to be endogenous to HEK 293 cells (Matsuda et al., 2008; Sala-Rabanal et al., 2015), and TMEM16A is thought to be the endogenous Ca^{2+} -activated Cl^- current in *Xenopus* oocytes that promotes apparent desensitization of recombinant NMDA receptor currents (Leonard and Kelso, 1990; Schroeder et al., 2008; Yang et al., 2008). However, CaMKII-mediated phosphorylation was reported to directly enhance CLC-3 currents (Cuddapah and Sontheimer, 2010; Huang et al., 2001; Robinson et al., 2004), whereas CaMKII has been implicated in the downregulation of TMEM16A activity (Wang et al., 2012). In contrast, we found that CaMKII α expression had no effect on the endogenous Cl^- conductance. It is possible that CaMKII was not activated by our uncaging protocol CaMKII, although some degree of kinase activation would be expected as CaMKII has an overlapping Ca^{2+} sensitivity with Ca^{2+} -activated Cl^- channels (Shifman et al., 2006; Yang et al., 2008). Nevertheless, our demonstration that even in the absence of CaMKII, phospho-mimetic substitution of GluN2B at Ser1303 recapitulates the modulation of GluN1A/GluN2B currents by CaMKII further supports the idea that direct CaMKII-mediated modulation of the Cl^- conductance is not responsible for the Cl^- -dependent effects on NMDA receptor desensitization. Rather, the effects of CaMKII on the NMDA receptor-mediated responses reflect primarily a direct modulation of GluN1A/GluN2B receptors, which secondarily drives the Ca^{2+} -activated Cl^- conductance when intracellular Cl^- is high. Notably, CaMKII reduces the desensitization of GluN1/GluN2A currents even in the presence of elevated intracellular Cl^- (Sessoms-Sikes et al., 2005), suggesting that GluN2B-containing NMDA receptors are preferentially coupled to the Ca^{2+} -activated Cl^- conductance. This may relate to the preferential binding of CaMKII to GluN2B rather than GluN2A, as well as to importance of CaMKII-GluN2B interactions for synaptic plasticity (Barria and Malinow, 2005). Given these observations, it is interesting to speculate that the effects of CaMKII on neuronal NMDA receptors and synaptic plasticity may be modified during development, when Cl^- concentrations are dramatically altered in concert with expression of the K^+ -dependent Cl^- transporter KCC2 (Chevy et al., 2015; Ferando et al., 2016). Clearly, the

impact of Cl^- channel coupling on neuronal NMDA receptors and synaptic plasticity during development requires further study.

Our data significantly enhance our mechanistic understanding of CaMKII regulation of the NMDA receptor. As noted above, CaMKII binds tightly to GluN2B and phosphorylates Ser1303 within the CaMKII binding domain. In principle, both of these events could contribute to the reduced desensitization observed in low Cl^- conditions. However, these events are coupled, potentially clouding the picture. Pre-phosphorylation at Ser1303 blocks subsequent *de novo* binding of CaMKII to a GluN2B fragment *in vitro* (Strack et al., 2000), but Ser1303 phosphorylation subsequent to CaMKII binding induces a rather slow dissociation of CaMKII rather than immediate dissociation (Strack et al., 2000). Physiological concentrations of adenine nucleotides also appear to stabilize CaMKII binding to Ser1303-phosphorylated GluN2B (O'Leary et al., 2011). Although the rates of CaMKII binding and dissociation from NMDA receptors in intact cells are poorly understood, there is qualitative support for the view that Ser1303 phosphorylation controls complex formation in intact cells based on analyses of CaMKII co-localization with GluN1A/GluN2B containing S1303A or S1303D mutations to abrogate or mimic phosphorylation at this site (Raveendran et al., 2009; Strack et al., 2000). Moreover, a recent study indicated that a brief application of NMDA induces slow dissociation of CaMKII from synaptic NMDA receptors (Aow et al., 2015). However, since CaMKII binding to GluN2B is stabilized by S1303A mutation, our observation that the activity of S1303A mutated receptors is not affected by co-expression of CaMKII indicates that CaMKII binding alone has no direct effect on NMDA receptor activity, at least under these conditions. Moreover, the efficacy of S1303D mutation, which disrupts CaMKII binding, to maximally reduce desensitization without CaMKII α co-expression indicates that Ser1303 phosphorylation alone is sufficient to reduce desensitization when intracellular Cl^- is low. Thus, Ser1303 phosphorylation might be expected to enhance Ca^{2+} entry into spines, which could sustain or further stimulate CaMKII activity (Barcomb et al., 2014; Coultrap et al., 2010), enabling feed-forward CaMKII-mediated control of other synaptic substrates, such as GluA1 subunits of the AMPA receptor (see Introduction).

In summary, the present findings have clarified the role of CaMKII in regulating NMDA receptors. Unexpectedly, the findings also revealed the dramatic impact of intracellular Cl^- concentrations on the coupling to NMDA receptors. It will be important to determine the roles of these mechanisms in regulating synaptic plasticity, particularly when intra-neuronal Cl^- concentrations are dramatically altered across development.

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References

- Abiria SA, Colbran RJ. CaMKII associates with CaV1.2 L-type calcium channels via selected beta subunits to enhance regulatory phosphorylation. *J Neurochem.* 2010; 112:150–161. [PubMed: 19840220]

- Aow J, Dore K, Malinow R. Conformational signaling required for synaptic plasticity by the NMDA receptor complex. *Proc Natl Acad Sci U S A*. 2015; 112:14711–14716. [PubMed: 26553983]
- Barcomb K, Buard I, Coultrap SJ, Kulbe JR, O’Leary H, Benke TA, Bayer KU. Autonomous CaMKII requires further stimulation by Ca²⁺/calmodulin for enhancing synaptic strength. *FASEB J*. 2014; 28:3810–3819. [PubMed: 24843070]
- Barria A, Malinow R. NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. *Neuron*. 2005; 48:289–301. [PubMed: 16242409]
- Bayer KU, De Koninck P, Leonard AS, Hell JW, Schulman H. Interaction with the NMDA receptor locks CaMKII in an active conformation. *Nature*. 2001; 411:801–805. [PubMed: 11459059]
- Ben-Ari Y. Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci*. 2002; 3:728–739. [PubMed: 12209121]
- Chevy Q, Heubl M, Goutierre M, Backer S, Moutkine I, Eugene E, Bloch-Gallego E, Levi S, Poncer JC. KCC2 Gates Activity-Driven AMPA Receptor Traffic through Cofilin Phosphorylation. *J Neurosci*. 2015; 35:15772–15786. [PubMed: 26631461]
- Colbran RJ. Targeting of calcium/calmodulin-dependent protein kinase II. *Biochem J*. 2004; 378:1–16. [PubMed: 14653781]
- Coultrap SJ, Bayer KU. CaMKII regulation in information processing and storage. *Trends Neurosci*. 2012; 35:607–618. [PubMed: 22717267]
- Coultrap SJ, Buard I, Kulbe JR, Dell’Acqua ML, Bayer KU. CaMKII autonomy is substrate-dependent and further stimulated by Ca²⁺/calmodulin. *J Biol Chem*. 2010; 285:17930–17937. [PubMed: 20353941]
- Cuddapah VA, Sontheimer H. Molecular interaction and functional regulation of CLC-3 by Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) in human malignant glioma. *J Biol Chem*. 2010; 285:11188–11196. [PubMed: 20139089]
- Ellis-Davies GC, Kaplan JH. Nitrophenyl-EGTA, a photolabile chelator that selectively binds Ca²⁺ with high affinity and releases it rapidly upon photolysis. *Proc Natl Acad Sci U S A*. 1994; 91:187–191. [PubMed: 8278362]
- Ferando I, Faas GC, Mody I. Diminished KCC2 confounds synapse specificity of LTP during senescence. *Nat Neurosci*. 2016
- Halt AR, Dallapiazza RF, Zhou Y, Stein IS, Qian H, Juntti S, Wojcik S, Brose N, Silva AJ, Hell JW. CaMKII binding to GluN2B is critical during memory consolidation. *EMBO J*. 2012; 31:1203–1216. [PubMed: 22234183]
- Hell JW. CaMKII: claiming center stage in postsynaptic function and organization. *Neuron*. 2014; 81:249–265. [PubMed: 24462093]
- Huang P, Liu J, Di A, Robinson NC, Musch MW, Kaetzel MA, Nelson DJ. Regulation of human CLC-3 channels by multifunctional Ca²⁺/calmodulin-dependent protein kinase. *J Biol Chem*. 2001; 276:20093–20100. [PubMed: 11274166]
- Huang WC, Xiao S, Huang F, Harfe BD, Jan YN, Jan LY. Calcium-activated chloride channels (CaCCs) regulate action potential and synaptic response in hippocampal neurons. *Neuron*. 2012; 74:179–192. [PubMed: 22500639]
- Krupp JJ, Vissel B, Heinemann SF, Westbrook GL. Calcium-dependent inactivation of recombinant N-methyl-D-aspartate receptors is NR2 subunit specific. *Mol Pharmacol*. 1996; 50:1680–1688. [PubMed: 8967993]
- Leonard AS, Bayer KU, Merrill MA, Lim IA, Shea MA, Schulman H, Hell JW. Regulation of calcium/calmodulin-dependent protein kinase II docking to N-methyl-D-aspartate receptors by calcium/calmodulin and alpha-actinin. *J Biol Chem*. 2002; 277:48441–48448. [PubMed: 12379661]
- Leonard AS, Lim IA, Hemsworth DE, Horne MC, Hell JW. Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. *Proc Natl Acad Sci U S A*. 1999; 96:3239–3244. [PubMed: 10077668]
- Leonard JP, Kelso SR. Apparent desensitization of NMDA responses in *Xenopus* oocytes involves calcium-dependent chloride current. *Neuron*. 1990; 4:53–60. [PubMed: 1690016]
- Liao GY, Wagner DA, Hsu MH, Leonard JP. Evidence for direct protein kinase-C mediated modulation of N-methyl-D-aspartate receptor current. *Mol Pharmacol*. 2001; 59:960–964. [PubMed: 11306676]

- Lisman J, Yasuda R, Raghavachari S. Mechanisms of CaMKII action in long-term potentiation. *Nat Rev Neurosci.* 2012; 13:169–182. [PubMed: 22334212]
- Matsuda JJ, Filali MS, Volk KA, Collins MM, Moreland JG, Lamb FS. Overexpression of CLC-3 in HEK293T cells yields novel currents that are pH dependent. *Am J Physiol Cell Physiol.* 2008; 294:C251–262. [PubMed: 17977943]
- Merrill MA, Chen Y, Strack S, Hell JW. Activity-driven postsynaptic translocation of CaMKII. *Trends Pharmacol Sci.* 2005; 26:645–653. [PubMed: 16253351]
- O’Leary H, Liu WH, Rorabaugh JM, Coultrap SJ, Bayer KU. Nucleotides and phosphorylation bi-directionally modulate Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) binding to the N-methyl-D-aspartate (NMDA) receptor subunit GluN2B. *J Biol Chem.* 2011; 286:31272–31281. [PubMed: 21768120]
- Omkumar RV, Kiely MJ, Rosenstein AJ, Min KT, Kennedy MB. Identification of a phosphorylation site for calcium/calmodulin-dependent protein kinase II in the NR2B subunit of the N-methyl-D-aspartate receptor. *J Biol Chem.* 1996; 271:31670–31678. [PubMed: 8940188]
- Raveendran R, Devi Suma Priya S, Mayadevi M, Steephani M, Santhoshkumar TR, Cheriyan J, Sanalkumar R, Pradeep KK, James J, Omkumar RV. Phosphorylation status of the NR2B subunit of NMDA receptor regulates its interaction with calcium/calmodulin-dependent protein kinase II. *J Neurochem.* 2009; 110:92–105. [PubMed: 19453375]
- Robinson NC, Huang P, Kaetzel MA, Lamb FS, Nelson DJ. Identification of an N-terminal amino acid of the CLC-3 chloride channel critical in phosphorylation-dependent activation of a CaMKII-activated chloride current. *J Physiol.* 2004; 556:353–368. [PubMed: 14754994]
- Sabatini BL, Oertner TG, Svoboda K. The life cycle of Ca(2+) ions in dendritic spines. *Neuron.* 2002; 33:439–452. [PubMed: 11832230]
- Sala-Rabanal M, Yurtsever Z, Nichols CG, Brett TJ. Secreted CLCA1 modulates TMEM16A to activate Ca(2+)-dependent chloride currents in human cells. *Elife.* 2015:4.
- Sanhueza M, Fernandez-Villalobos G, Stein IS, Kasumova G, Zhang P, Bayer KU, Otmakhov N, Hell JW, Lisman J. Role of the CaMKII/NMDA receptor complex in the maintenance of synaptic strength. *J Neurosci.* 2011; 31:9170–9178. [PubMed: 21697368]
- Schroeder BC, Cheng T, Jan YN, Jan LY. Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell.* 2008; 134:1019–1029. [PubMed: 18805094]
- Sessoms-Sikes S, Honse Y, Lovinger DM, Colbran RJ. CaMKII α enhances the desensitization of NR2B-containing NMDA receptors by an autophosphorylation-dependent mechanism. *Mol Cell Neurosci.* 2005; 29:139–147. [PubMed: 15866054]
- Shen K, Meyer T. Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. *Science.* 1999; 284:162–166. [PubMed: 10102820]
- Shifman JM, Choi MH, Mihalas S, Mayo SL, Kennedy MB. Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is activated by calmodulin with two bound calciums. *Proc Natl Acad Sci U S A.* 2006; 103:13968–13973. [PubMed: 16966599]
- Shonesy BC, Jalan-Sakrikar N, Cavener VS, Colbran RJ. CaMKII: a molecular substrate for synaptic plasticity and memory. *Prog Mol Biol Transl Sci.* 2014; 122:61–87. [PubMed: 24484698]
- Stein IS, Donaldson MS, Hell JW. CaMKII binding to GluN2B is important for massed spatial learning in the Morris water maze. *F1000Res.* 2014; 3:193. [PubMed: 25187880]
- Strack S, Choi S, Lovinger DM, Colbran RJ. Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. *J Biol Chem.* 1997; 272:13467–13470. [PubMed: 9153188]
- Strack S, Colbran RJ. Autophosphorylation-dependent targeting of calcium/calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl-D-aspartate receptor. *J Biol Chem.* 1998; 273:20689–20692. [PubMed: 9694809]
- Strack S, McNeill RB, Colbran RJ. Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. *J Biol Chem.* 2000; 275:23798–23806. [PubMed: 10764765]
- Tu W, Xu X, Peng L, Zhong X, Zhang W, Soundarapandian MM, Balel C, Wang M, Jia N, Zhang W, Lew F, Chan SL, Chen Y, Lu Y. DAPK1 interaction with NMDA receptor NR2B subunits mediates brain damage in stroke. *Cell.* 2010; 140:222–234. [PubMed: 20141836]

- Vissel B, Krupp JJ, Heinemann SF, Westbrook GL. A use-dependent tyrosine dephosphorylation of NMDA receptors is independent of ion flux. *Nat Neurosci.* 2001; 4:587–596. [PubMed: 11369939]
- Wang M, Yang H, Zheng LY, Zhang Z, Tang YB, Wang GL, Du YH, Lv XF, Liu J, Zhou JG, Guan YY. Downregulation of TMEM16A calcium-activated chloride channel contributes to cerebrovascular remodeling during hypertension by promoting basilar smooth muscle cell proliferation. *Circulation.* 2012; 125:697–707. [PubMed: 22215857]
- Wang XQ, Deriy LV, Foss S, Huang P, Lamb FS, Kaetzel MA, Bindokas V, Marks JD, Nelson DJ. CLC-3 channels modulate excitatory synaptic transmission in hippocampal neurons. *Neuron.* 2006; 52:321–333. [PubMed: 17046694]
- Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim WS, Park SP, Lee J, Lee B, Kim BM, Raouf R, Shin YK, Oh U. TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature.* 2008; 455:1210–1215. [PubMed: 18724360]
- Zhou Y, Takahashi E, Li W, Halt A, Wiltgen B, Ehninger D, Li GD, Hell JW, Kennedy MB, Silva AJ. Interactions between the NR2B receptor and CaMKII modulate synaptic plasticity and spatial learning. *J Neurosci.* 2007; 27:13843–13853. [PubMed: 18077696]

Highlights

- Cl^- gradients influence CaMKII modulation of GluN2B NMDA receptor desensitization
- Ser1303 phosphorylation of GluN2B reduces desensitization at low intracellular Cl^-
- CaMKII binding to GluN2B does not appear to regulate GluN2B desensitization
- Sustained GluN2B-NMDAR Ca^{2+} entry due to CaMKII may enhance synaptic plasticity

Fig. 1. Anion gradients control the direction of CaMKII-mediated regulation of the extent of GluN1A/GluN2B desensitization

HEK 293 cells were transfected with plasmids encoding GluN1A and GluN2B ± GFP-CaMKIIα. Whole-cell currents were elicited by 3 s applications of agonist/co-agonist combinations in conjunction with varying the composition of the intracellular whole-cell electrode solutions as indicated. (A–D) Representative peak normalized GluN1A/GluN2B current traces (from a holding potential of –60 mV) are shown from cells without (black traces) or with GFP-CaMKIIα (red traces) co-expression for each condition. Conditions for each set of traces (A–D) correspond directly to those shown below in the summary bar graph in panel (E). Individual data points for each condition are superimposed on the summary bar graph. Glycine concentrations are in μM, while ATP concentrations are in mM. Note that CaMKIIα reduces desensitization when MeSO₄⁻ is the predominant intracellular anion, but enhances desensitization when Cl⁻ is the predominant anion. * p < 0.05; ** p < 0.01 compared to their respective controls in the absence of CaMKIIα, or as specified.

Fig. 2. Strong Ca^{2+} buffering appears to occlude the ability of CaMKII to reduce GluN1A/GluN2B desensitization

HEK 293 cells were transfected with plasmids encoding GluN1A and GluN2B $\pm$ GFP-CaMKII α . Whole-cell currents were elicited by 3 s applications of 1 mM glutamate/100 μM glycine. (A) Representative peak normalized GluN1A/GluN2B current traces (from a holding potential of -60 mV) are shown from cells without (black traces) or with GFP-CaMKII α (red traces) co-expression using 10 mM BAPTA in the CsMeSO $_4$ -based whole-cell electrode solution. (B) Bar graph summarizing the results from multiple experiments with the individual data points for each condition superimposed.

Fig. 3. A Ca^{2+} -activated Cl^- current endogenous to HEK 293 cells is resistant to regulation by CaMKII

HEK 293 cells were transfected with plasmids encoding GluN1A and GluN2B $\pm$ GFP-CaMKII α to maintain identical transfection conditions with all previous experiments. Whole-cell recordings were carried out using the CsMeSO $_4$ - or CsCl-based intracellular solutions similar to Fig. 1B and 1D except that 0.1 mM NP-EGTA and 0.02 mM CaCl $_2$ replaced 1 mM EGTA and 0.2 CaCl $_2$. (A) Representative leak subtracted and capacitance normalized current traces are shown for a cells without (black and gray traces) or with GFP-CaMKII α (red and pink traces) co-expression subjected to wide-field UV-induced photolysis of NP-EGTA followed by a voltage ramp from -60 to $+60$ mV. Gray and pink traces recorded with CsMeSO $_4$ while black and red traces recorded with CsCl. (B) Summary graph of the capacitance normalized I-V relationship obtained from multiple cells shows that the photolysis-induced current is outwardly-rectifying and resistant to modification by CaMKII α and virtually undetectable with the impermeant MeSO $_4^-$ anion.

Fig. 4. CaMKII regulates GluN1A/GluN2B desensitization via Ser1303 phosphorylation
 HEK 293 cells were transfected with plasmids encoding GluN1A and either wt GluN2B, phospho-deficient S1303A GluN2B mutant or phospho-mimetic S1303D GluN2B mutant $\pm$ GFP-CaMKII α . Whole-cell currents were elicited by 3 s applications of 1 mM glutamate/100 μ M glycine and recorded using either (A, C) CsMeSO $_4^-$ based or (B, D) CsCl $^-$ based electrode solutions. Representative peak normalized current traces are shown for cells without (black traces) or with GFP-CaMKII α (red traces) co-expression. (A, B) Bar graph summarizing the steady-state to peak current ratios from multiple experiments with the individual data points for each condition superimposed. Data for wild-type GluN1A/GluN2B $\pm$ CaMKII α obtained in Fig. 1 are shown for comparison. (C, D) Bar graph summarizing the desensitization time constant (τ) obtained from exponential fits to the decay of the agonist-evoked currents for the cells shown in A and B. Individual data points for each condition are superimposed. For each panel, statistical comparisons are

between GluN1A/2B alone and +CaMKII α demonstrating the effect of CaMKII, GluN1A/2B + CaMKII α and GluN1A/S1303A +CaMKII α demonstrating block of CaMKII action by the phospho-deficient mutant, and GluN1A/2B and GluN1A/S1303D demonstrating the mimicking of CaMKII action. * $p < 0.05$, ** $p < 0.01$ for these relevant comparisons.

Fig 5. CaMKII preserves GluN1A/GluN2B currents during repetitive agonist exposures
 HEK 293 cells were transfected with plasmids encoding GluN1A and GluN2B $\pm$ GFP-CaMKII α . Whole-cell currents were elicited by repeated 3 s applications of 1 mM glutamate/100 μ M glycine (every min) using CsMeSO₄-based electrode solutions with 1 mM EGTA included except where noted. These sustained recordings reflect a subset from those obtained for GluN1A/GluN2B $\pm$ GFP-CaMKII α (from Fig. 1B), with high intracellular BAPTA (Figure 2), or a separate set in which 50 nM constitutively active CaMKII (CaMKII(1–290)) was included in the whole-cell recording solution. (A) Summary time course of peak current normalized to the peak current obtained from the initial agonist exposure for each of the indicated conditions. Representative first (black) and final (red)

traces are shown for each condition. (B) Corresponding summary time course demonstrating the stability of the steady-state to peak ratio following repeated agonist exposures.

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