CaMKII translocation requires local NMDA receptor-mediated Ca^{2+} signaling

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Excitatory synaptic transmission and plasticity are critically modulated by *N*-methyl-D-aspartate receptors (NMDARs). Activation of NMDARs elevates intracellular Ca^{2+} affecting several downstream signaling pathways that involve Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). Importantly, NMDAR activation triggers CaMKII translocation to synaptic sites. NMDAR activation failed to induce Ca^{2+} responses in hippocampal neurons lacking the mandatory NMDAR subunit NR1, and no EGFP-CaMKII α translocation was observed. In cells solely expressing Ca^{2+} -impermeable NMDARs containing NR1^{N598R}-mutant subunits, prolonged NMDA application elevated internal Ca^{2+} to the same degree as in wild-type controls, yet failed to translocate CaMKII α . Brief local NMDA application evoked smaller Ca^{2+} transients in dendritic spines of mutant compared to wild-type cells. CaMKII α mutants that increase binding to synaptic sites, namely CaMKII-T286D and CaMKII-TT305/306VA, rescued the translocation in NR1^{N598R} cells in a glutamate receptor-subtype-specific manner. We conclude that CaMKII translocation requires Ca^{2+} entry directly through NMDARs, rather than other Ca^{2+} sources activated by NMDARs. Together with the requirement for activated, possibly ligand-bound, NMDARs as CaMKII binding partners, this suggests that synaptic CaMKII accumulation is an input-specific signaling event.

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Introduction

Activity-dependent changes in synaptic strength underlie some forms of learning and memory, with the *N*-methyl-D-aspartate receptor (NMDAR) serving as a molecular coincidence detector for pre- and postsynaptic activity (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999).

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The NMDAR-effected postsynaptic Ca^{2+} rise activates calmodulin-dependent protein kinase II (CaMKII) thus enabling synaptic long-term potentiation (LTP) (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999): CaMKII is autophosphorylated, switching to a Ca^{2+} /CaM-independent active state, which can persist after the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) has returned to the resting level. CaMKII thus acts as a molecular memory device translating NMDAR-activity into changes in synaptic strength (Fukunaga *et al*, 1995; Lisman *et al*, 2002), a mechanism corroborated by gene targeting experiments (Elgersma *et al*, 2004).

NMDAR-mediated $[\text{Ca}^{2+}]_i$ elevation is thought to be highly localized at the channel mouth thus encoding information for Ca^{2+} sensors in close proximity of this high- Ca^{2+} nanodomain (Augustine *et al*, 2003). A NMDAR-mediated local Ca^{2+} rise triggers translocation of CaMKII to the post-synaptic density (PSD) where the kinase then directly binds to the NMDAR (Shen and Meyer, 1999; Bayer *et al*, 2001). This binding is facilitated once CaMKII is autophosphorylated (Strack and Colbran, 1998; Leonard *et al*, 1999; Strack *et al*, 2000; Bayer *et al*, 2001). The PSD-localized, activated CaMKII is capable of phosphorylating molecules important for synaptic transmission. Indeed, mass spectrometric analysis showed a multitude of sites in PSD phosphorylated *in vivo* by CaMKII (Trinidad *et al*, 2006).

To investigate if the spatial nature of NMDAR-mediated Ca^{2+} signals determines the specificity of downstream signaling, we used cultured hippocampal neurons from a mouse model expressing Ca^{2+} -impermeable NMDARs (Rudhard *et al*, 2003). In these NR1^{R/-} mice the mandatory NR1 subunit carries the point mutation N598R in the pore domain, abolishing Ca^{2+} permeability of all NMDAR channels (Burnashev *et al*, 1992).

We found that prolonged application of NMDA could still mediate a robust rise of dendritic $[\text{Ca}^{2+}]_i$ in NR1^{R/-} cells which express only Ca^{2+} -impermeable NMDARs. Although a similar protocol routinely induced translocation of CaMKII to the synapse in wild-type neurons, no translocation occurred in NR1^{R/-} cells. Mutations in the EGFP-tagged CaMKII that prolong its synaptic localization (Shen *et al*, 2000) completely recovered NMDAR-dependent accumulation of the kinase at synaptic sites in NR1^{R/-} cells. This suggests that NMDAR-generated local Ca^{2+} signals are primarily responsible for the wild-type CaMKII translocation. Indeed, brief local application of NMDA induced significantly smaller Ca^{2+} transients in dendritic spines of NR1^{R/-} compared with wild-type cells, indicating an important role of Ca^{2+} entry through NMDARs in generating local Ca^{2+} signals.

We conclude that a highly localized rise in Ca^{2+} concentration near the NMDAR pore, rather than a general diffuse increase in $[\text{Ca}^{2+}]_i$ via other Ca^{2+} sources indirectly activated by NMDARs, is required for CaMKII trapping at the PSD. This provides evidence for the concept of functional Ca^{2+} nanodomains at the PSD.

Results

To study the influence of local NMDAR-mediated Ca^{2+} signals on NMDAR-dependent translocation of CaMKII α , we investigated neurons of NR1^{R/-} animals exclusively expressing mutant NMDARs. These receptors contain the NR1(N598R) subunit which leads to Ca^{2+} -impermeable NMDAR channels (Burnashev *et al*, 1992; Rudhard *et al*, 2003).

Hippocampal neurons from wild-type (NR1^{+/+}, NR1^{+/-}) and NR1^{R/-} animals grew in culture and developed comparable morphologies (Supplementary Figure S1A) with no evidence of a significant difference in surface expression levels or distribution of Ca^{2+} -impermeable versus wild-type NMDA receptor complexes (Supplementary Figure S1B and C). Similarly, expression and distribution of other postsynaptic components appeared equivalent in the two genotypes

(Supplementary Figure S2), in line with expression studies in the mutant NR1^{R/-} mice (Rudhard *et al*, 2003).

Ca^{2+} measurements in wild-type and NR1^{R/-} cells

We evaluated NMDA-induced Ca^{2+} responses using fluorescence ratiometric measurements. Intriguingly, although NR1^{R/-} cells express only Ca^{2+} -impermeable NMDARs, selective NMDAR stimulation (50 μM NMDA/10 μM Gly) induced a large Ca^{2+} signal throughout the cell soma and dendrites (Figure 1A).

To quantify the rise in $[\text{Ca}^{2+}]_i$, we measured 340/380 ratios from isolated somatic regions (Figure 1A) and dendritic segments (Figure 1D). NMDAR stimulation (60 s) evoked a large increase in the ratio 340/380 at the soma, both in wild-type and NR1^{R/-} cells (Figure 1B). This Ca^{2+} rise was insignificant in the presence of 150 μM 2-amino-5-phospho-

Figure 1 Ca^{2+} imaging in wild-type and NR1^{R/-} mutant cells. (A) Pseudocolor-coded ratio images of emissions evoked by excitation at 340 and 380 nm (ratio 340/380) taken with a CCD camera before ('unstim') and during ('NMDA') stimulation on an NR1^{R/-} cell; scale bar: 10 μm . Application of NMDA led to an increase in Ca^{2+} signal in soma and dendrites. Dashed rectangles indicate the position of the photodiode field during measurements at the soma. (B) Sample traces obtained from somatic measurements on wild-type (+/+; black), NR1^{R/-} (R/-; medium gray), and NR1^{-/-} (-/-; light gray) neurons, shown as 340/380 ratios. Bars indicate application of NMDA ('N', 50 μM , 60 s), APV (150 μM), and Mg^{2+} ions (2 mM). Measurements in Na^{+} -free conditions were made after equilibration with choline chloride-containing Ringer (0 Na^{+} ; indicated by the discontinuity in the time axis). Neurons lacking NR1 subunits (-/-) did not respond to NMDA application at all. However, cells with Ca^{2+} -impermeable NMDARs (R/-) showed a large rise in Ca^{2+} signal which was not blocked by Mg^{2+} ions. Removal of external Na^{+} resulted in reduced Ca^{2+} responses of wild-type cells and abolished responses of NR1^{R/-} cells. (C) Apparent somatic $[\text{Ca}^{2+}]_i$ at baseline ('basal') levels and after maximal response to NMDA ('NMDA', 50 μM) in wild-type and NR1^{R/-} cells ($n_{\text{cells}}/n_{\text{experiments}} = 29/5$ and 21/4, respectively). Paired data from single cells (lines) are shown together with mean $\pm$ s.e.m. (D) DIC image illustrating the typical position of the photodiode field onto a dendrite branch of a pyramidal neuron as used for data in (E + F); scale bar: 20 μm . (E) NMDA (50 μM , 30 s)-evoked changes in dendritic $[\text{Ca}^{2+}]_i$ in cells expressing the wild-type (+/+, black filled symbols) or mutant NR1 subunit (R/-, red open symbols), in the absence (circles) or presence (squares) of 150 μM APV. Dendritic recordings were made as in (D); mean $\pm$ s.e.m. (+/+, $n = 6$; R/-, $n = 8$). Horizontal line indicates agonist application. (F) Apparent dendritic $[\text{Ca}^{2+}]_i$ at baseline ('basal') levels and after maximal response to NMDA ('NMDA', 50 μM) in wild-type and NR1^{R/-} cells ($n_{\text{cells}}/n_{\text{experiments}} = 6/2$ and 8/2, respectively). Paired data from single cells (lines) are shown together with mean $\pm$ s.e.m.

nopentanoic acid (APV), or in neurons lacking the NR1 subunit ($\text{NR1}^{-/-}$). NR1^R-containing receptors are also lacking the Mg^{2+} block (Rudhard *et al*, 2003). The absence of this Mg^{2+} block in R/⁻ cells (Figure 1B) confirms therefore, the presence of mutant NMDARs. As these NMDARs cannot serve as source of Ca^{2+} ions, the rise in $[\text{Ca}^{2+}]_i$ must be caused by Ca^{2+} release or influx secondary to depolarization.

Baseline levels of the somatic apparent free $[\text{Ca}^{2+}]_i$ in NR1^{R/-} cells were similar with those in wild-type neurons (R/⁻, 84 ± 20 nM, $n = 21$; +/+ , 64 ± 8 nM, $n = 29$; mean $\pm$ s.e.m., n_{cells} ; Figure 1C), and in line with (Garaschuk *et al*, 1997; Pozzo-Miller *et al*, 1999; Shuttleworth and Connor, 2001). NMDAR-evoked responses were also comparable between the two genotypes (apparent $[\text{Ca}^{2+}]_i$ after stimulation: +/+ , 631 ± 75 nM, $n = 29$; R/⁻, 629 ± 86 nM, $n = 21$; for derivation of $[\text{Ca}^{2+}]_i$ values, see Supplementary data and Supplementary Methods).

Likewise, the dendritic Ca^{2+} responses evoked by NMDA (30 s) in both wild-type and NR1^{R/-} cells were APV-sensitive and comparable in size (apparent $[\text{Ca}^{2+}]_i$ after stimulation: 116 ± 24 nM, $n = 6$, and 115 ± 20 nM, $n = 8$, respectively; Figure 1E and F).

In summary, these measurements showed a comparable agonist-induced increase of $[\text{Ca}^{2+}]_i$ in wild-type and NR1^{R/-} cells after prolonged (≥ 30 s) NMDA application.

Sources of internal Ca^{2+} rise following NMDAR activation

NMDAR-evoked Ca^{2+} responses were eliminated in the absence of extracellular Na^+ in NR1^{R/-} cells (Figure 1B), whereas in wild-type cells, a residual rise in $[\text{Ca}^{2+}]_i$ could still be observed ($25 \pm 4\%$ of the full response, mean $\pm$ s.e.m., $n = 7$; Supplementary Figure S3). This residual Ca^{2+} rise in wild-type cells is in accordance with earlier observations (Schneeggenburger *et al*, 1993) that about 10% of the ions passing through wild-type NMDAR channels are Ca^{2+} ions and is consistent with a Ca^{2+} influx directly through the activated wild-type, but not NR1(N598R)-mutant, NMDAR channels.

Taken together, these findings confirm that the NMDA-induced Ca^{2+} responses in NR1^{R/-} cells result from a Ca^{2+} influx that is triggered by NMDAR-driven depolarization, but carried through sources other than the activated NR1(N598R) NMDAR channels. The potential sources of Ca^{2+} entry include VGCCs, $\text{Na}^+/\text{Ca}^{2+}$ exchanger, and intracellular stores (see Supplementary data and Supplementary Figure S3).

NMDAR-dependent local Ca^{2+} entry in dendritic spines

Because a long-term, space-equilibrated elevation of Ca^{2+} could in principle originate anywhere in the cell, it may not necessarily reflect large Ca^{2+} rises inside dendritic spines: endogenous Ca^{2+} buffers and pumps restrict Ca^{2+} rises away from the source. Therefore, we assessed the profile of NMDAR-dependent Ca^{2+} entry in the immediate spine proximity on a faster time scale (Figure 2).

A brief (100–150 ms) pulse of NMDA evoked a rapid, prominent Ca^{2+} response in both the spine head and the dendrite (Figure 2A–D). In wild-type cells, the average Ca^{2+} transient was larger in the spine than the dendrite (619 ± 127 and 366 ± 82 nM, respectively, $n = 7$, $p < 0.04$; Figure 2A–D) reflecting the predominant occurrence of NMDARs at the spine head (Racca *et al*, 2000). In the NR1^{R/-} cells, local

application of NMDA also triggered Ca^{2+} responses, which, however, displayed smaller amplitudes and a slower time course compared with the wild-type (Figure 2E–H). Importantly, these data represent the contribution of local Ca^{2+} sources to intracellular Ca^{2+} rises, whereas results obtained with bath application protocols are likely to reflect long-term equilibration of Ca^{2+} across large distances (Figure 1, and dendritic spines at high resolution in Supplementary Figure S5). In addition, NMDA-induced Ca^{2+} transients in the NR1^{R/-} cells were of similar size in spines and shafts (to 63.7 ± 5.6 and 65.4 ± 5.7 nM, respectively, $n = 5$), with the shaft transient generally showing faster rises (Supplementary Figure 2H). Again, this is likely to reflect the lacking NMDAR Ca^{2+} channel sources at spine heads.

In summary, these Ca^{2+} imaging data indicate that (a) wild-type NMDAR Ca^{2+} channels provide a disproportionately large source of rapid Ca^{2+} entry in dendritic spines compared with shafts (Figure 2), (b) there still exist rapid local sources of Ca^{2+} entry in the dendritic spine vicinity in the NR1^{R/-} cells, and (c) long-term activation of NMDAR in wild-type and mutant cells results in similar elevation of global internal Ca^{2+} .

Translocation of CaMKII α

Our Ca^{2+} measurements indicate that wild-type and NR1-mutant cells differ in their sources of free Ca^{2+} in response to NMDAR activation. Thus, the question arises whether signaling pathway molecules downstream of the NMDAR can discriminate between Ca^{2+} entry directly through NMDAR channels and Ca^{2+} originating from other sources.

The translocation of CaMKII α from the cytosol to the PSD is known to depend on NMDAR activation and rise of $[\text{Ca}^{2+}]_i$ (Strack *et al*, 1997; Shen and Meyer, 1999). To test if this process depends on the direct influx of Ca^{2+} through NMDARs, we transfected NR1^{+/+}, NR1^{R/-} and NR1^{-/-} knockout cells with EGFP-CaMKII α and analyzed the intracellular fluorescence distribution in Glu-treated and untreated cells (Figure 3A).

The treatment of wild-type cells (50 μM Glu/10 μM Gly, 2–3 min) changed the uniform intracellular distribution of CaMKII α to a pattern with clusters of high fluorescence intensity within the dendritic arbor in line with previous observations (Shen and Meyer, 1999). The treatment was comparable to that used for Ca^{2+} imaging experiments with prolonged agonist application (1 min). The translocation effect was specific for the tagged kinase; the same experimental conditions did not influence the distribution of plain green fluorescent protein (GFP) (Supplementary Figure S6).

CaMKII clustering was observed mainly at synaptic sites, but also occurred at extrasynaptic sites as reported for similar stimulation protocols (Hudmon *et al*, 2005). To confirm that the increase in the number of CaMKII puncta indeed reflected a translocation to the PSD, we determined the number of CaMKII puncta localized in the proximity of structures that were immunoreactive for the synaptic marker synapsin1 (Figure 4A, bar graph).

Quantification revealed a three-fold increase in synaptically localized CaMKII puncta (Figure 4A) in Glu-stimulated wild-type cells. This increase was statistically significant compared with unstimulated controls or agonist-treated NR1^{-/-} and NR1^{R/-} cells, where Glu-induced CaMKII α translocation was absent (Figure 3A). Moreover, no increase in

Figure 2 Rapid local application of NMDA evokes a local transient rise of $[\text{Ca}^{2+}]_i$ in dendrites and dendritic spines. (A) A dendritic fragment of a wild-type cell (wt) filled, in whole-cell mode, with cell-impermeable $20\text{ }\mu\text{M}$ Alexa Fluor 594 and $200\text{ }\mu\text{M}$ Fluo-4; upper panel Alexa (red) channel; lower panel, Fluo-4 (green) channel. The fine morphology of dendritic spines is clearly seen. Dotted lines indicate the line scan position. (B–D) Line-scan recordings (at 500 Hz for 3 s , dotted lines in A show scan positioning) of Alexa and Fluo-4 fluorescence (B, upper and lower panels, respectively) following pressure application of NMDA ($100\text{ ms} \times 15\text{ psi}$ pressure pulse; Alexa added to the pipette medium for pulse monitoring) through a patch pipette located in a $\sim 15\text{ }\mu\text{m}$ vicinity (shown by a circle in C). (B) Upper panel (red channel) depicts the spatio-temporal profile of NMDA applied through the pipette together with Alexa and diffusing in the bath medium; the corresponding time course is shown in (D, red trace). (B) Lower panel (green channel) depicts Ca^{2+} transients evoked in spine head and in dendritic stem; the corresponding time course traces are shown in (D; green, spine head; blue, dendrite; red, Alexa). Scale bars; (C), $30\text{ }\mu\text{m}$; (D), 1 s and 100 nM . (E–H) The same experiment as in (B–D) but in NR1 mutant (R/–) cells. The corresponding notations are the same as in (E–H). Scale bars; (E), $1\text{ }\mu\text{m}$; (F) 500 ms ; (G), $30\text{ }\mu\text{m}$; (H) 1 s and 100 nM .

puncta could be observed after selective stimulation of AMPARs (Figure 3B). When cells were cotransfected with plain mRFP, we analyzed the translocation in relation to the red fluorescence acting as cell filler and observed the same genotype dependency for the translocation (Supplementary Figure S8), confirming that the change in fluorescence distribution, and therefore CaMKII translocation, is independent of spine volume effects potentially induced by the agonist.

Thus, our observations suggest that Ca^{2+} influx directly through the NMDARs was required for Glu-evoked CaMKII translocation, hence that CaMKII can discriminate among different Ca^{2+} sources.

Translocation of CaMKII α to synapses is dependent on Ca^{2+} influx through the NMDA receptor channel pore

To test the contribution of secondary, depolarization-induced, Ca^{2+} sources to the translocation, we minimized NMDA-induced depolarization by reducing extracellular Na^+ to nominally zero mM (0 Na^+ , Figure 3C). Under these conditions, NMDAR activation still led to the formation of GFP–CaMKII puncta in dendrites of wild-type cells, revealing successful kinase translocation (Figure 3B).

Our 0 Na^+ data further suggest that a possible buildup of internal Ca^{2+} owing to inactivity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger has no influence on the accumulation of CaMKII.

To test for the influence of VGCCs activated secondary to NMDAR depolarization, we studied CaMKII translocation in the presence of Cd^{2+} ($100\text{ }\mu\text{M}$; Figure 3C). VGCCs blocked by Cd^{2+} did not eliminate the increase in puncta, suggesting that the mere influx through NMDAR channels in wild-type cells is sufficient for translocation of CaMKII. Even depletion of intracellular stores by CPA (10 min , $15\text{ }\mu\text{M}$) in addition to Cd^{2+} block still resulted in puncta formation (see Supplementary Data).

These results clearly show that direct Ca^{2+} influx through the NMDAR is necessary and sufficient for the agonist-evoked translocation of CaMKII α to the synapse.

Translocation of CaMKII mutants; retention at the PSD

Activated CaMKII is translocated to the PSD, where it can bind to NR2B subunits (Bayer *et al*, 2001). Autophosphorylation facilitates and strengthens the binding of CaMKII to PSD components (Leonard *et al*, 1999; Bayer and Schulman, 2001; Bayer *et al*, 2001). Indeed, the introduction of an autophosphorylation mimicking mutation into CaMKII (T286D) results in prolonged retention times of the kinase at the PSD (Shen *et al*, 2000).

The clustering of the constitutively active form T286D CaMKII in unstimulated cells was not different from that of

Figure 3 Translocation of CaMKII α to synapses is dependent on Ca^{2+} influx through the NMDA receptor channel pore. (A) Stimulation of neurons ($50 \mu\text{M}$ Glu/ $10 \mu\text{M}$ Gly; 3 min) expressing the wild-type (+/+), but not the Ca^{2+} -impermeable NMDARs (R/-), increased the synaptic translocation of CaMKII α . Axial projection of confocal overviews (EGFP-CaMKII fluorescence, green, overlaid with anti-synapsin1, red; scale bar: $10 \mu\text{m}$); details enclosed in the white rectangles are expanded underneath (EGFP-CaMKII α , CaMKII α , and anti-synapsin1 fluorescence, 'syn', shown individually and merged; scale bar: $5 \mu\text{m}$). Note the juxtaposition of EGFP-CaMKII puncta with anti-synapsin1 structures in wild-type, but not NR1^{R/-} cells, after Glu treatment. (B) Agonist-induced CaMKII translocation: Glu- or NMDA-induced formation of CaMKII puncta occurred in wild-type cells (+/+), but not in cells lacking NR1 subunits (-/-), or in NR1^{R/-} neurons (R/-). $n = 4-15$. Agonists used at $50 \mu\text{M}$ with $10 \mu\text{M}$ Gly. No formation of puncta was observed with kainate or AMPA (both $100 \mu\text{M}$). (C) NMDAR activation is sufficient for formation of CaMKII puncta. NMDA, $50 \mu\text{M}$; Cd^{2+} , $100 \mu\text{M}$. $n = 4-9$; mean $\pm$ s.e.m. Brackets indicate statistically significant changes by treatment: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. For a detailed listing of n for each condition, see Supplementary data.

wild-type CaMKII (Figure 4B), an observation previously reported for wild-type neurons (Shen and Meyer, 1999). In addition, Glu-induced translocation of T286D CaMKII in NR1^{+/+} cells was comparable to that of wild-type CaMKII. Interestingly, application of Glu or NMDA led to a striking recovery of the translocation effect in cells of the Ca^{2+} -impermeable NR1 genotype (Figure 4B). This result suggests that the constitutively active T286D CaMKII is able to overcome the absence of direct Ca^{2+} influx through NMDARs, thereby recovering the NMDAR-dependent CaMKII translocation to the PSD in NR1^{R/-} neurons to levels observed in wild-type cells. Furthermore, this recovery could be blocked by addition of Cd^{2+} (Figure 4D), suggesting that NMDAR-mediated depolarization-dependent Ca^{2+} sources are necessary and sufficient for the NMDAR-evoked accumulation of constitutively active CaMKII (T286D) at the PSD.

Additionally, we made use of the EGFP-tagged CaMKII double mutant TT305/306VA, where two major inhibitory autophosphorylation sites are removed (Patton *et al*, 1990; Colbran, 1993). The translocated TT305/306VA CaMKII has been shown to have increased net synaptic localization (Shen *et al*, 2000). In line with these findings, we observed increased basal synaptic kinase puncta in wild-type and NR1^{R/-} cells in the absence of Mg^{2+} (Figure 4C), as reported *in vivo* by Elgersma *et al* (2002).

Glutamate stimulation increased the number of synaptic CaMKII-TT305/306VA in wild-type cells. Crucially, CaMKII-TT305/306VA was also able to recover the NR1 wild-type phenotype in NR1^{R/-} cells after stimulation with Glu or NMDA.

However, as for wild-type CaMKII, stimulation protocols did not have any effect on the distribution of mutant

Figure 4 CaMKII mutants rescue PSD accumulation of CaMKII in NR1^{R/-} neurons. Fluorescent puncta for EGFP-tagged wild-type (A, 'CaMKIIα'), constitutively active form (B, 'T286D'), and inhibitory autophosphorylation-deficient form (C, 'TT305/306VA') of CaMKIIα in stimulated (Glu or NMDA; 50 μM with 10 μM Gly) and unstimulated neurons from specific NR1 genotypes (wild type: +/+; NR1^{R/-}: -/-; NR1^{R/-}: R/-). Left: Examples taken from the groups depicted in the graphs on the right (axial-projected confocal stacks showing fluorescent EGFP-CaMKII puncta, scale bar: 10 μm); insets: details of dendritic segments (scale bar: 2 μm). Right: Densities (counts/100 μm dendrite length) of synaptically localized EGFP-CaMKII puncta. Data are shown as mean ± s.e.m.; n = 7–14, number of independent experiments: 2–4. For clarity, statistical significance is shown only for NR1^{R/-} genotype; (**) P < 0.01, (***) p < 0.001. (D + E) Influence of Cd²⁺ (100 μM) (D) and of AMPA (100 μM) (E) on synaptic puncta levels of EGFP-tagged CaMKII clones expressed in wild-type and NR1 N589R-mutant cells.

CaMKII in NR1^{R/-} cells, suggesting that the presence of NMDARs is required for CaMKII translocation to the PSD.

Specific stimulation of AMPARs with 100 μM AMPA failed to induce translocation of CaMKII-T286D and CaMKII-TT305/306VA to synaptic sites (Figure 4E). We obtained equivalent

results with the nondesensitizing agonist kainate (100 μM) (Supplementary Figure S7). These findings suggest a principal functional difference between NMDA- and AMPA-mediated depolarization, and perhaps a requirement of ligand binding to the NMDAR leading to a conformational change, in addition to opening of the channel (Vissel *et al*, 2001).

In summary, two different mutated forms of CaMKII that affect the duration time of the kinase at the PSD, namely T286D and TT305/306VA, were found to accumulate at synaptic sites upon stimulation of Ca^{2+} -impermeable NMDA receptors. Thus, conditions that facilitate the binding of CaMKII to PSD components and reduce its dissociation from the synaptic compartment can overcome the lack of a Ca^{2+} signal beneath the NMDA receptor pore. This effect, however, requires an agonist-induced activation of NMDARs.

Discussion

CaMKII translocation requires direct Ca^{2+} influx through the NMDAR channel

The activation of NMDARs and the subsequent rise in Ca^{2+} are necessary and sufficient for CaMKII translocation (Strack and Colbran, 1998; Shen and Meyer, 1999 and this manuscript).

The lack of NMDA-induced translocation of CaMKII in neurons derived from $\text{NR1}^{-/-}$ animals was not surprising, as these cells do not express functional NMDARs and consequently do not exhibit NMDA-evoked Ca^{2+} rises (Figure 1B). Although Ca^{2+} rises were evoked by AMPAR-dependent depolarization in these cells (Supplementary Figure S4), no CaMKII translocation was observed with Glu (Figure 3B), indicating the requirement for NMDAR activation.

Deletion of NMDARs in $\text{NR1}^{-/-}$ cells, however, removes not only NMDAR-dependent Ca^{2+} signals but also the corresponding CaMKII-binding sites at PSDs. To test whether CaMKII translocation requires Ca^{2+} entry through NMDARs, in addition to NMDAR binding, we used the $\text{NR1}^{\text{R}/-}$ mouse model.

In $\text{NR1}^{\text{R}/-}$ neurons, distribution of NR1 subunits and the integrity of PSDs appeared preserved (Supplementary Figures S1 and S2). However, prolonged activation of NMDARs in these cells failed to translocate CaMKII (Supplementary Figures S3 and S4A) even though it produced long-term Ca^{2+} elevation, throughout the dendritic compartments, similar to that in the wild-type. The most likely explanation is that in $\text{NR1}^{\text{R}/-}$ neurons, ligand-bound Ca^{2+} -impermeable NMDARs depolarized the cell via Na^{+} influx and thus activated VGCCs and/or the reverse Na^{+} - Ca^{2+} exchange. Prolonged activation of these Ca^{2+} sources led to a rise and equilibration of Ca^{2+} throughout the cell. However, this global Ca^{2+} rise was insufficient to trigger CaMKII translocation, which suggests that the latter may require high-concentration Ca^{2+} domains inside the spines.

Indeed, the concept of local Ca^{2+} nanodomains has been well established (Augustine *et al*, 2003). In line with this, activated wild-type NMDAR Ca^{2+} channels, which remain open for tens of millisecond, could generate an exceptionally sharp local Ca^{2+} 'hot-spot' (Supplementary Figure S9). It would then be reasonable to assume that each individual Ca^{2+} source produces its own Ca^{2+} domain determined by the source opening kinetics.

We found that a brief local pulse of NMDA evoked clear Ca^{2+} responses inside the dendritic spines, both in wild-type and mutant cells. In $\text{NR1}^{\text{R}/-}$ cells, however, Ca^{2+} entry into spines was reduced (Figure 2G and H). This indicated that, although local Ca^{2+} sources (along with their respective Ca^{2+} nanodomains) still existed, their numbers were greatly reduced because of the lack of Ca^{2+} permeability by NMDARs. The non-NMDAR Ca^{2+} sources and their associated Ca^{2+} domains thus appeared unable to induce CaMKII translocation.

In view of these findings, we propose that Ca^{2+} nanodomains generated by Ca^{2+} entry directly through wild-type NMDAR channels are critical for the process of CaMKII translocation.

A model for the lack of CaMKII translocation in cells with Ca^{2+} -impermeable NMDARs

What are the possible reasons for the lack of CaMKII translocation in cells with Ca^{2+} -impermeable NMDARs?

In $\text{NR1}^{\text{R}/-}$ cells, the number and distribution of NMDARs may differ from wild-type cells. However, wild-type and mutant NMDARs showed equivalent membrane expression, synaptic distribution (Supplementary Figure S1B/C), and similar amplitudes of whole-cell currents (Rudhard *et al*, 2003). Moreover, we failed to detect differences in number and synaptic localization of two key components of NMDAR complexes, namely PSD-95 and NR2B (Supplementary Figure S2). Therefore, the lack of NMDA-induced CaMKII translocation in $\text{NR1}^{\text{R}/-}$ cells is unlikely to arise from changes in number, distribution, and structural integrity of the Ca^{2+} -impermeable NMDAR complexes.

A restricted or otherwise changed Ca^{2+} availability for activation of CaMKII in the mutant neurons in comparison to wild-type cells might affect the translocation of CaMKII. Most likely, CaMKII is recruited from the dendritic area to the synaptic site upon NMDAR stimulation (Meyer and Shen, 2000). However, with the experimental paradigm used in the translocation assays (i.e., prolonged agonist application, time scale of minutes), we found no difference between genotypes in the Ca^{2+} signal in dendritic segments. On the other hand, at a much faster time resolution (hundreds of ms), line scanning measurements in spines revealed strikingly smaller and slower Ca^{2+} responses to brief NMDA puffs. This suggests that the direct NMDAR Ca^{2+} signal is indeed required for trapping CaMKII at the PSD.

The results with CaMKII mutants suggest that a dynamic model for CaMKII binding to and unbinding from the NMDAR can provide a way to compensate for the lack of direct Ca^{2+} signals in $\text{NR1}^{\text{R}/-}$ cells (Figure 5).

The translocation process itself (Figure 5A, pathway (a)) has been shown to depend only on the binding of Ca^{2+} /CaM to CaMKII. Nevertheless, this process is reversible within seconds, hence the ability to observe CaMKII translocated to the synapse critically depends on the time scale in which the kinase is present at the PSD (Shen and Meyer, 1999). Autophosphorylation of CaMKII at T286 prolongs this time scale (Shen *et al*, 2000), thus enabling CaMKII to form, or strengthen, its interactions with multiple binding partners, for example, NR2B, NR1, NR2A, densin-180 (Strack and Colbran, 1998; Gardoni *et al*, 1999; Bayer *et al*, 2001, 2006; Walikonis *et al*, 2001; Leonard *et al*, 2002; Robison *et al*, 2005).

Figure 5 Local NMDA receptor-mediated Ca^{2+} signaling in CaMKII translocation. **(A)** In wild-type neurons, stimulation of NMDA receptors (NMDAR) leads to an increase in $[\text{Ca}^{2+}]_i$ directly (i), as Ca^{2+} ions enter via the receptor to form a locally restricted Ca^{2+} hot-spot beneath the NMDAR channel mouth and indirectly (ii) as NMDAR activation triggers other Ca^{2+} sources. Consequently, CaMKII is translocated to the PSD (a) where it can directly interact with and bind to the NMDAR (b) in a process favored by phosphorylation at residue T286. The translocation is reversible and CaMKII dissociation from the PSD (c) is furthered by inhibitory autophosphorylation. **(B)** In cells with Ca^{2+} -impermeable NMDARs the path (i) is defective and CaMKII translocation (a) is possibly counterbalanced by pathways (b) and (c). The wild-type phenotype can, therefore, be rescued either by the autophosphorylation-mimicking CaMKII mutant T286D, which strengthens interaction (b), or by the inhibitory autophosphorylation-deficient mutant TT305/306VA, which weakens pathway (c).

Indeed, a CaMKII mutant that mimicks autophosphorylation (T286D) overcame the apparent lack of CaMKII translocation in cells with Ca^{2+} -impermeable NMDARs (Figure 4B). The recovery of wild-type translocation behavior in $\text{NR1}^{\text{R}/-}$ cells with the constitutively active form of CaMKII suggests that in the absence of a high local $[\text{Ca}^{2+}]$ at the NMDAR, wild-type CaMKII might not reach sufficient levels of activity necessary for efficient autophosphorylation to interact with its binding partners (Figure 5, pathway (b)) and thus may not acquire a Ca^{2+} -independent active state.

Phosphorylation at residues 305/306 favors the dispersion of CaMKII subunits from the PSD (Strack *et al*, 1997) (Figure 5, pathway (c)), but is inhibited after the enzyme is trapped at the NMDAR (Bayer *et al*, 2001). Introducing TT305/306VA mutations into CaMKII rescued the wild-type translocation phenotype in $\text{NR1}^{\text{R}/-}$ cells (Figure 4C). Most likely, net inhibitory TT305/306 phosphorylation is favored in these cells, as presumably less autophosphorylation at T286 and hence, less binding to NMDARs occurs.

Thus, we propose that in $\text{NR1}^{\text{R}/-}$ cells the local Ca^{2+} signal at the NMDAR pore is at a subthreshold level and is only compensated by mutations in CaMKII that strengthen retention of the kinase in the PSD. Consequently, the local Ca^{2+} signal created by direct influx through the NMDA receptor is necessary to maintain sufficient Ca^{2+} /CaM-dependent CaMKII activity for autophosphorylation to out-compete the main protein phosphatase at the PSD, PP1. This would then result in the ability of T286-phosphorylated CaMKII to bind several PSD components (Figure 5, pathway (b)), locking it at the PSD.

Alternatively, the rescue by the CaMKII mutants could be explained in terms of CaM sensitivity. The T286D mutation mimicks autophosphorylation that increases affinity to CaM (Meyer *et al*, 1992). In CaMKII-TT305/306VA, the inhibitory autophosphorylation sites that prevent CaM binding (Colbran, 1993) are abolished. This net increase in CaM

affinity in both mutants should allow translocation also in response to a less-pronounced elevation of internal Ca^{2+} , possibly recruiting the kinase from longer distances or compensating for an eventual restricted availability of CaM in $\text{NR1}^{\text{R}/-}$ cells, as the shuttle of CaM between membrane and cytosolic compartments is itself a process regulated by Ca^{2+} (Persechini and Stemmer, 2002).

Input specificity of NMDAR-mediated CaMKII translocation

The requirement of a locally restricted Ca^{2+} signal for CaMKII translocation, as indicated by our data, strongly suggest input specificity of the translocation process. While CaMKII mutants seem to accumulate at synapses with slow and indirect Ca^{2+} signals (as in $\text{NR1}^{\text{R}/-}$ cells), the wild-type CaMKII seems to translocate only to an NMDAR-activated synapse. No accumulation at a nearby synapse would occur. The biochemical properties of wild-type CaMKII make it, therefore, an ideal candidate for input-specific activation of NMDAR-dependent signaling events such as synaptic plasticity. Indeed, activated CaMKII is translocated to the PSD where it can bind to NR2B subunits (Bayer *et al*, 2001), an interaction modulated by the strength of the synaptic input (Bayer *et al*, 2006) and required for expression of LTP (Barria and Malinow, 2005).

Implications of NMDAR Ca^{2+} impermeability and lack of CaMKII translocation for the $\text{NR1}^{\text{R}/-}$ genotype

At the PSD, Ca^{2+} signals arise by influx via VGCCs and NMDARs or by release from intracellular stores and have been studied at single spine level (Emptage *et al*, 1999; Kovalchuk *et al*, 2000; Sabatini *et al*, 2002; Sobczyk *et al*, 2005). The PSD provides a high-density network of signaling molecules where a variety of Ca^{2+} sensors (e.g., CaM, caldendrin) are positioned in close proximity of Ca^{2+} sources. This creates the conditions for a locally restricted Ca^{2+} signal, called 'nanodomain', as Ca^{2+} ions need to diffuse only molecular distances from its source to the target molecule (Augustine *et al*, 2003).

In $\text{NR1}^{\text{R}/-}$ cells, the lack of a local Ca^{2+} concentration right at the NMDAR channel pore will mainly affect proteins in close vicinity, especially those PSD components capable of direct interaction with the NMDAR itself, such as CaMKII.

The failure of NMDAR in $\text{NR1}^{\text{R}/-}$ cells to accumulate CaMKII at the synapse may result in a reduced presence of CaMKII at the PSD with consequences on the PSD composition. However, the overall levels of major neurotransmitter receptor subunits in membranes are normal (Rudhard *et al*, 2003) and expression of crucial synaptic components is not affected by the mutation in the NR1 subunit (Supplementary Figures S1 and S2).

Changes of CaMKII at the PSD can influence learning and memory (Elgersma *et al*, 2004). $\text{NR1}^{\text{R}/-}$ mice die at birth, hence no LTP or learning and memory experiments can be performed. However, the altered recruitment and distribution of CaMKII probably contribute to the observed lack of synaptic refinement of emerging somato-sensory maps in $\text{NR1}^{\text{R}/-}$ mice (Rudhard *et al*, 2003). Effects of the NMDAR mutation on synaptic plasticity are therefore likely.

NMDAR-dependent activation of CaMKII, along with its localized accumulation, will particularly alter the phosphorylation state of its PSD-associated substrates (Trinidad *et al*,

2006). Therefore, a failure of NMDARs to activate and/or translocate CaMKII can affect further downstream signaling in NR1^{R/-} synapses. Apart from CaMKII, other targets for NMDAR-dependent Ca^{2+} signals exist, which in turn are linked to specific downstream signaling cascades (Hardingham *et al*, 2002), which might be affected by a lack of NMDAR-dependent Ca^{2+} rise in the spine. For example, NMDA-induced GluR2 endocytosis was found to require Ca^{2+} influx through a specific NMDAR subtype (Tigaret *et al*, 2006), which can be best explained by the functional Ca^{2+} nanodomain model.

In summary, Ca^{2+} influx through activated NMDAR channels generates a local Ca^{2+} signal at the channel pore that is necessary and sufficient for CaMKII translocation to and its retention at the PSD. This is functional evidence of an exquisite signal processing machinery associated very tightly with NMDARs and thus supports the concept of Ca^{2+} nano/microdomains in spines or at the PSD.

Materials and methods

Chemicals

Unless stated otherwise, all chemicals were from Sigma (St Louis, MO), Tocris (Tocris Cookson Ltd, Avonmouth, UK) or Invitrogen (Carlsbad, CA).

Hippocampal neuron cultures

Hippocampal primary neuronal cultures were prepared in accordance to a protocol by Banker and Goslin (1991), and described in detail by Specht *et al* (2005). Cells from individual pups were cultured separately.

Expression constructs

EGFP-CaMKII α . The expression construct pBS.EGFP-MT-CK2A codes for an N-terminal fusion of CaMKII α with EGFP similar to one described (Shen and Meyer, 1999). For details, see Supplementary methods.

For analysis of overall cell morphology, neurons were infected with Sindbis viral EGFP-expression construct for 5 h (Specht *et al*, 2005).

Transfection

Hippocampal neurons were typically transfected at day 14 *in vitro* (DIV) (range 13–15), with GFP-CaMKII α expression constructs with lipofectamine 2000 used as recommended by the manufacturer (Invitrogen).

Translocation

For CaMKII α translocation experiments, cells were washed 14–15 h after transfection with HEPES-Ringer (in mM: HEPES pH 7.35, 5; KCl, 2.5; NaCl, 126; CaCl_2 , 2; MgCl_2 , 2; glucose, 11.1; tetrodotoxin (TTX), 0.0005; strychnine, 0.001; bicuculline methiodide, 0.02), and subsequently treated for 2–3 min with 50 μM Glu/10 μM Gly, or alternatively with AMPA or kainate at 100 μM in presence of 150 μM APV. For application of 50 μM NMDA/10 μM Gly, the same buffer was used without Mg^{2+} . Controls were treated in parallel with the respective buffers containing no agonists. For solutions lacking Na^+ , NaCl was substituted with choline chloride.

Typically, data were obtained from three independent experiments. One experiment was performed per litter, and only litters were used which included at least one wild-type pup. We analyzed the distribution of EGFP-tagged CaMKII on fixed tissue, in analogy to (Rao and Craig, 1997; Ju *et al*, 2004; Hudmon *et al*, 2005), see below. Whereas this approach does not allow kinetic analysis of the translocation process, it permits reduced experimental variability by parallel processing of multiple samples under exactly the same conditions. In addition, data from more than one cell per sample can be obtained, which is more suited to the limited availability of cultures from mutant mice (see breeding schemes in Supplementary methods).

Immunocytochemistry

Fixation and staining. Cells (14–16 DIV for CaMKII translocation studies) were fixed with 4% paraformaldehyde/4% sucrose in PBS for 15–20 min at 35°C, permeabilized with 0.2% Triton X-100 in PBS for 10 min, washed and blocked at 35°C in 5% horse serum (HS) in PBS. They were counterstained with rabbit anti-synapsin1 (1:500; ab8, Abcam, Cambridge, UK) or mouse anti-synapsin1 (1:500; clone 46.1, Synaptic Systems, Göttingen, Germany). Secondary antibodies conjugated with Cy3 and Cy5 were used 1:300 (at 2.5 $\mu\text{g}/\text{ml}$, Jackson ImmunoResearch, West Grove, PA). Coverslips were washed with PBS and mounted in GelMount.

Imaging. Cells were randomly selected on basis of healthy morphology. Confocal images were taken with a LSM 510 Meta laser scanning inverted microscope (Carl Zeiss Ltd, Rugby, UK), using a $\times 63$ magnification oil immersion Plan-Apochromat objective (NA 1.40), sequential line-scan mode, $\times 2$ scan averaging and 0.2 μm distance between optical sections. Images were analyzed using ImageJ software (<http://rsb.info.nih.gov/ij/>): Confocal stacks were Z-projected for each channel individually. Puncta were recognized as spherical structures of 0.2–0.5 μm , $\geq \times 2$ brighter than the surrounding dendritic background and counted blindly with respect to the experimental conditions. This measurement was performed on dendritic segments (50–100 μm length) that typically belonged to second and third order branches. The selected segments fulfilled the criteria of minimal crossings with neighboring processes, and a detectable axial fluorescence. Synaptic location of NR1 or CaMKII puncta was determined by their proximity to synapsin1-containing structures, within a mask of $0.28 \times 0.28 \mu\text{m}$, centered on the CaMKII (or NR1) spot. If immunopositive structures for synapsin1 were found within the field of the mask, the original structure was deemed as postsynaptic.

Ca^{2+} measurements

Solutions. Recording solutions contained in mM: HEPES, 5 (pH 7.35); KCl, 2.5; CaCl_2 , 2; glucose, 11.1; NaCl, 126; TTX, 0.5; TROLOX, 1; glycine, 0.01. In Ca^{2+} -free solutions, Ca^{2+} was exchanged for 1 mM of EGTA. For solutions lacking Na^+ , HEPES was pHed with KOH, KCl was added to 2.5 mM of K^+ , and NaCl was substituted with choline chloride. Agonist solutions contained in mM: NMDA, 0.05; APV, 0.15; nickel, 0.1; nimodipine, 0.01; nifedipine, 0.01; cyclopiazonic acid, 0.015; KB-R7943, 0.03. Experiments testing nifedipine and KB-R7943 effects were performed in carbonate-buffered recording solution as described (Rudhard *et al*, 2003).

Recording. Cells (DIV12–20) were loaded for 20 min with 2 μM fura-2-AM (Molecular Probes Inc., Eugene, OR) in MM at 37°C. Coverslips were transferred into a recording chamber (Model RC-26GLP, Warner Instruments Corp.) on a Nikon Eclipse E600FN microscope equipped with a $\times 40$ and a $\times 60$ oil immersion objective. They were perfused for 20 min with oxygenated recording solution to allow for hydrolysis of the fura-ester. Recording and agonist solutions were applied at a flow rate of 2.2 ml/min. Fura excitation (340 and 380 nm) was achieved via a monochromator (Polychrom II, TILL Photonics, Martinsried, Germany) controlled by CellWorks (npi, Tamm, Germany). Emission was monitored via a photodiode (TILL Photonics), data were recorded with CellWorks and analyzed in Igor Pro (Wavemetrics Inc., Lake Oswego, OR). Alternatively, pictures were acquired with a CCD camera (Retiga 1300, Q Imaging, Burnaby, BC, Canada), and for illustration purposes thresholded to background values, ratio images calculated and false colored in Image J V1.29 (Rasband WS, NIH, Bethesda, MD).

High-resolution Ca^{2+} imaging: fast confocal microscopy

Experiments were carried out in a dedicated BioRad Radiance 2100 imaging system integrated with patch-clamp electrophysiology (Scott and Rusakov, 2006). Cultures were perfused with the bath medium at room temperature. Cells were filled with the cell-impermeable fluorescent tracer Alexa Fluor 594 (20 μM) and Ca^{2+} -sensitive, cell-impermeable indicator Fluo-4 (200 μM , $K_d = 350 \text{ nM}$), in whole-cell mode at $V_h = -70 \text{ mV}$. The dyes were excited, respectively, at 568 and 488 nm, and the emission signals were chromatically separated at 560 nm and recorded using two photomultiplier tubes. Line-scans were quantified as fluorescence

values integrated over the imaged width of the cell compartment, with the background fluorescence subtracted. Recorded image stacks were analyzed offline (Scott and Rusakov, 2006).

To activate local NMDARs, we applied the agonist through a pressurized patch pipette (tip diameter 2–3 μm) placed in the 10–20 μm vicinity of the spine and filled with 100 μM NMDA in bath solution (plus 10 μM Alexa Fluor 594 to monitor pressure puffs).

Because the kinetics of Ca^{2+} sensitive responses in the spine vicinity was slower than the characteristic time of inter-spine Ca^{2+} - and-indicator equilibration due to diffusion reaction, we assessed Ca^{2+} concentration from Fluo-4 fluorescence using the common steady-state formula (Maravall *et al*, 2000).

$$[\text{Ca}^{2+}] = K_d (F_{\text{max}}/F - 1)^{-1}$$

where $K_d = 350 \text{ nM}$, and F and F_{max} stand for, respectively, the recorded fluorescence value and the maximum fluorescence recorded in conditions of dye saturation (achieved through bath application of ionomycin) in each cell.

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Statistical analysis

Statistical analysis of data was performed using a two-tailed Mann–Whitney *U*-test (Altman, 1999).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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