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Authors

Goodell, Dayton J
Zaegel, Vincent
Coultrap, Steven J
et al.

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DAPK1 mediates LTD by making CaMKII/GluN2B binding LTP-specific

Dayton J. Goodell[1,2], **Vincent Zaegel**[1], **Steven J. Coultrap**[1], **Johannes W. Hell**[3], and **K. Ulrich Bayer**[1,2,*]

[1]Department of Pharmacology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045

[2]Program in Neuroscience, University of Colorado Anschutz Medical Campus, Aurora, CO 80045

[3]Department of Pharmacology, School of Medicine, University of California, Davis, California 95616

Abstract

The death associated protein kinase 1 (DAPK1) is a potent mediator of neuronal cell death. Here, we find that DAPK1 also functions in synaptic plasticity by regulating the Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII). CaMKII and T286-autophosphorylation are required for both long-term potentiation (LTP) and depression (LTD), two opposing forms of synaptic plasticity underlying learning, memory and cognition. T286-autophosphorylation induces CaMKII binding to the NMDA receptor (NMDAR) subunit GluN2B, which mediates CaMKII synaptic accumulation during LTP. We find that the LTP-specificity of CaMKII synaptic accumulation is due to its LTD-specific suppression by calcineurin (CaN)-dependent DAPK1 activation, which in turn blocks CaMKII binding to GluN2B. This suppression is enabled by competitive DAPK1 versus CaMKII binding to GluN2B. Negative regulation of DAPK1/GluN2B binding by Ca^{2+}/CaM results in synaptic DAPK1 removal during LTP but retention during LTD. A pharmacogenetic approach showed that suppression of CaMKII/GluN2B binding is a DAPK1 function required for LTD.

eTOC Blurb

Correspondence to: K.U.B. (ulli.bayer@ucdenver.edu).
*Lead Contact

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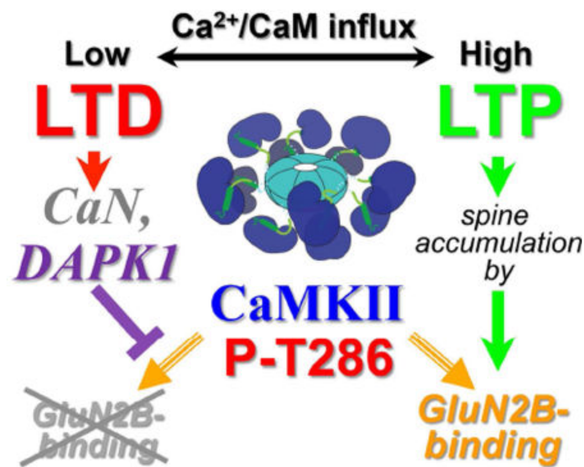

Goodell et al. find that calcineurin (CaN)-dependent activation of the death associated protein kinase 1 (DAPK1) is required for a form of long-term synaptic plasticity, LTD. Specifically, DAPK1 suppresses Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) synaptic accumulation and GluN2B binding during LTD, thus making these CaMKII mechanisms LTP-specific.

Keywords

DAPK1; CaMKII; calcineurin; GluN2B; LTP; LTD; hippocampus; synapse; dendritic spine

INTRODUCTION

Long-term potentiation (LTP) and long-term depression (LTD), are opposing forms of synaptic plasticity thought to underlie learning, memory, and cognition Kandel et al., 2014; Malenka and Bear, 2004; Martin et al., 2000). Both LTP and LTD require the Ca^{2+}/calmodulin (CaM) dependent protein kinase II (CaMKII) α isoform and its autonomous activity that is induced by T286 autophosphorylation (Coultrap et al., 2014; Giese et al., 1998; Silva et al., 1992; Stevens et al., 1994). We have previously demonstrated that autonomous CaMKII may mediate the LTP/LTD decision in part through stimulus-dependent substrate selection (Coultrap et al., 2014). This decision could additionally or alternatively be mediated by the differential subcellular localization of CaMKII during LTP versus LTD. CaMKII is basally enriched in dendritic spines, which form the post-synaptic compartment of excitatory synapses, where it rapidly accumulates further only during LTP but not LTD (Lee et al., 2009; Marsden et al., 2010; Otmakhov, 2004; Shen and Meyer, 1999). The accumulation of CaMKII at excitatory synapses is mediated by its binding to the NMDAR subunit GluN2B, and genetic disruption of this interaction impairs LTP while leaving LTD unaffected (Barcomb et al., 2016; Bayer et al., 2001; 2006; Halt et al., 2012; Leonard et al., 1999; Strack and Colbran, 1998; Zhou et al., 2007). Thus, differential CaMKII/GluN2B binding after LTP- versus LTD-stimuli would provide an attractive mechanism for determining both the sub-cellular localization of CaMKII and the direction of plasticity that is induced. However, both LTP- and LTD-stimuli induce CaMKII T286 autophosphorylation (Barria et al., 1997; Coultrap et al., 2014), which is sufficient to induce CaMKII binding to GluN2B (Bayer et al., 2001; Strack and Colbran, 1998). We here

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hypothesized that the localization of CaMKII during LTP and LTD is mediated by a mechanism that makes CaMKII binding to GluN2B LTP-specific through an LTD-specific suppression of this binding.

In response to ischemic conditions that generate excitotoxic glutamate stimuli, death associated protein kinase 1 (DAPK1), another CaM kinase family member, has been described to bind to extrasynaptic GluN2B at a site overlapping the CaMKII binding site and to phosphorylate GluN2B at the S1303 site that blocks CaMKII binding (O'Leary et al., 2011; Strack et al., 2000; Tu et al., 2010). DAPK1 is a potent mediator of cell death (Bialik and Kimchi, 2013), and it’s activity is accordingly tightly regulated: its kinase activity is suppressed by auto-phosphorylation of S308 within the Ca^{2+}/CaM-binding regulatory region and DAPK1 activation requires both dephosphorylation of this site and Ca^{2+}/CaM binding (Shohat et al., 2001). Increased neuronal DAPK1 activation has been demonstrated directly only after ischemia (Schumacher et al., 2002; Shamloo et al., 2005; Tu et al., 2010), although protein levels are increased also after ceramide induced stress (Pelled et al., 2002) and in Alzheimer’s disease (Kim et al., 2014; 2016). Additionally, while DAPK1/GluN2B binding is required for ischemic neuronal cell death (Tu et al., 2010), nothing is known about the regulation of DAPK1 binding to GluN2B. The DAPK1/GluN2B binding was expected to be governed by similar mechanisms as the well-studied CaMKII/GluN2B binding (Coultrap and Bayer, 2012a; Hell, 2014; Shonesy et al., 2014), because DAPK1 is a member of the CaM kinase family. Surprisingly, we instead found diametrically opposing regulation of DAPK1 versus CaMKII binding to GluN2B, which in turn poised DAPK1 interaction with synaptic GluN2B as an ideal candidate to suppress CaMKII/GluN2B interaction during LTD.

Here, we report that the LTP-specificity of synaptic CaMKII accumulation is due to the suppression of such accumulation during LTD, specifically by DAPK1 activation that leads to DAPK1-mediated blockade of CaMKII binding to GluN2B. DAPK1 activation during LTD was mediated by calcineurin (CaN), a Ca^{2+}-activated protein phosphatase known to be physiologically required for NMDAR-dependent LTD (Mulkey et al., 1994; Zeng et al., 2001). DAPK1 was highly enriched at excitatory synapses, and its binding to GluN2B was negatively regulated by high Ca^{2+}/CaM, resulting in DAPK1 trafficking away from synapses during LTP- but not LTD-stimuli, with retention during LTD-stimuli depending on DAPK1 activation. LTD-stimuli activated DAPK1 in hippocampal slices in a CaN-dependent manner, and inhibition of DAPK1 or CaN allowed the accumulation of CaMKII at excitatory synapses after LTD-stimuli. Conversely, DAPK1 overexpression inhibited LTP-induced synaptic CaMKII accumulation. Finally, DAPK1 inhibition blocked LTD in wild type mice, but not in mutant mice in which CaMKII binding to GluN2B is already genetically disabled. Together, these results demonstrate (i) a role for DAPK1 in physiological synaptic plasticity, (ii) an essential function of CaN activity in LTD, and (iii) a mechanism for the differential translocation of CaMKII during LTP and LTD that explains the dual role of CaMKII in bi-directional plasticity.

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RESULTS

DAPK1/GluN2B binding is negatively regulated by Ca^{2+}/CaM and does not require the kinase domain

The DAPK1/GluN2B binding (Figure 1A) is essential for the DAPK1 function in mediating ischemic neuronal cell death (Tu et al., 2010), yet the regulation of this binding has not yet been investigated. DAPK1 is a CaM kinase with a complex domain sequence, starting with an N-terminal kinase domain and a Ca^{2+}/CaM-binding regulatory domain (Bialik and Kimchi, 2006; 2013) (see Figure 1B). The original description of the DAPK1/GluN2B interaction used blot overlay assays to show that a DAPK1 fragment containing the kinase and regulatory domains binds to the GluN2B region around S1303 (Tu et al., 2010), indicating that DAPK1/GluN2B-binding is homologous to the well-studied CaMKII/GluN2B binding to the same region (Bayer et al., 2001; Strack et al., 2000). Indeed, specific binding of a similar DAPK1 fragment (DAPK1-kr; see Figure 1B) to the GluN2B C-terminal tail (GluN2Bc) was confirmed in our *in vitro* binding assay here (Figure 1C). However, surprisingly and in contrast to the Ca^{2+}/CaM-stimulated CaMKII binding to GluN2B, binding of DAPK1-kr was negatively regulated by addition of increasing Ca^{2+}/CaM concentrations (Figure 1D). Moreover, the DAPK1-kr fragment bound much more weakly compared to the kinase domain-lacking DAPK1 k fragment (Figure 1E), indicating that the main DAPK1/GluN2B interaction does not occur via the kinase domain. Additionally, binding of the kinase domain-lacking DAPK1 k to GluN2B also maintained Ca^{2+}/CaM-sensitivity in the *in vitro* binding assays (Figure 1E).

In HEK cells, mCherry-labelled full-length DAPK1 wild type significantly co-localized with co-expressed GFP-labelled GluN2Bc under basal conditions (Figure 1F, G). DAPK1 fragments lacking either the kinase domain (DAPK1 k) or the regulatory domain (DAPK1 r) showed the same extent of co-localization; by contrast, localization of the DAPK1-kr fragment was undistinguishable from mCherry control (Figure 1F, G). Thus, the DAPK1 kinase and regulatory domains are neither necessary nor sufficient to mediate significant GluN2B binding within a cellular environment.

Upon Ca^{2+}-stimulation of the HEK cells by ionomycin treatment, the DAPK1/GluN2B co-localization dispersed (Figure 1F, H). The same regulation was observed for full-length DAPK1-WT and for the DAPK1 k fragment; for the DAPK1-kr fragment that did not show any basal co-localization in the first place, no significant change was seen (Figure 1F, H). These results indicate that the negative regulation of DAPK1/GluN2B binding by Ca^{2+}/CaM (i) is maintained for full-length DAPK1, (ii) occurs within a cellular environment, and (iii) does not require the kinase domain. Furthermore, DAPK1/GluN2B binding *in vitro* was not only reduced by presence of Ca^{2+}/CaM during the binding reaction (Figure 1I), but was also reversed when pre-formed DAPK1/GluN2B complexes were washed with Ca^{2+}/CaM (Figure 1J). Thus, Ca^{2+}/CaM can disrupt pre-established DAPK1/GluN2B binding both within cells and for purified protein *in vitro*.

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DAPK1 S308 phosphorylation negatively regulates GluN2B binding

DAPK1 autophosphorylation at S308 within its regulatory domain occurs basally and prevents stimulation by Ca^{2+}/CaM, making S308 de-phosphorylation a necessary step in DAPK1 activation (de Diego et al., 2010; Shohat et al., 2001). Thus, we decided to test if and how DAPK1/GluN2B binding may be regulated by its S308 phosphorylation state. For this purpose, DAPK1 mutants that mimic either the phosphorylated state (S308D) or the de-phosphorylated state (S308A) were compared. In HEK cells, the S308A mutation increased co-localization with GluN2B mildly but significantly (Figure 2A, B). Localization of DAPK1 WT and S308D was indistinguishable from each other (Figure 2A, B), consistent with DAPK1 S308 phosphorylation in its basal state. Ca^{2+} influx induced by ionomycin treatment reduced the GluN2B co-localization for all DAPK1 constructs (Figure 2A, C). However, the relative reduction of co-localization after Ca^{2+}-stimulation was significantly smaller for the S308A mutant compared to the S308D mutant (Figure 2A, C). Additionally, GluN2B co-localization of the S308A mutant after Ca^{2+}-stimulation remained as strong as seen for DAPK1 wild type or S308D before Ca^{2+}-stimulation (Figure 2A, C). The negative regulation of DAPK1/GluN2B binding by DAPK1 S308 phosphorylation was also observed in our *in vitro* assays, where the DAPK1 S308A mutant bound to GluN2B much better than the S308D mutant (Figure 2D).

Together, these results strongly indicate that DAPK1 S308 phosphorylation weakens the DAPK1/GluN2B binding and makes it more sensitive to disruption by Ca^{2+}/CaM. However, this also posed a conundrum: As S308 phosphorylation inhibits DAPK1 activation by Ca^{2+}/CaM, how can it enhance the negative effect of Ca^{2+}/CaM on GluN2B binding? An answer was provided by additional experiments, which showed that the Ca^{2+}/CaM-binding sites responsible for DAPK1 activation versus DAPK1/GluN2B disruption are different from each other: Ionomycin stimulation dispersed DAPK1/GluN2B co-localization in HEK cells for a DAPK1 mutant that lacks the regulatory domain (DAPK1 r); both basal co-localization and the ionomycin-induced dispersal of DAPK1 r was indistinguishable from DAPK1 wild type (Figure 2E, F). The *in vitro* binding assays also showed strong DAPK1 r binding to GluN2B that remained sensitive to negative regulation by Ca^{2+}/CaM (Figure 2G). Thus, while DAPK1 activation is mediated by Ca^{2+}/CaM-binding to the regulatory domain that contains S308, the Ca^{2+}/CaM-induced disruption of DAPK1/GluN2B binding is mediated by Ca^{2+}/CaM-binding to a different undetermined site.

GluN2B S1303 phosphorylation positively regulates DAPK1 binding

CaMKII binding to GluN2B is inhibited by S1303 phosphorylation (O'Leary et al., 2011; Strack et al., 2000). Thus, we decided to test the effect of S1303 phosphorylation also on DAPK1/GluN2B binding. These experiments were done with the DAPK1 S308A mutant that mimics the LTD-induced de-phosphorylated state of DAPK1 that enhances GluN2B binding. Our results indicated that DAPK1/GluN2B binding is even positively regulated by S1303 phosphorylation: In HEK cells, DAPK1/GluN2B co-localization was significantly higher for GluN2B wild type compared to the phosphorylation-incompetent S1303A mutant (Figure 2H, I). Ca^{2+}-stimulation induced by ionomycin treatment reduced DAPK1 co-localization to both the GluN2B wild type and S1303A mutant; however, co-localization with GluN2B wild type after Ca^{2+}-stimulation remained as strong as seen with the S1303A

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mutant before such stimulation (Figure 2H, J). These results implied that GluN2B must be basally phosphorylated at S1303 in HEK cells, which was indeed observed (Figure 2K). While DAPK1 can phosphorylate GluN2B at S1303 (Figure S2A), the basal S1303 phosphorylation in HEK cells did not depend on co-expression with DAPK1 (Figure 2K). In addition to the GluN2B S1303A mutant, we also tested a S1303D mutant. Notably, S to D mutations introduce significantly less charge and bulk compared to phosphorylation, which thus can be functionally closer to the de-phosphorylated rather than the phosphorylated state. Indeed, with respect to DAPK1 binding, the GluN2B S1303D mutant mimicked the de-phosphorylated state: Its binding to DAPK1 was undistinguishable from the S1303A mutant and significantly lower compared to the S1303-phosphorylated GluN2B wild type (Figure 2H–J). Compared to non-phosphorylated GluN2B wild type, both S1303 mutants showed the same DAPK1 binding (Figure S2B).

DAPK1 is removed from dendritic spines by LTP- but not LTD-stimuli

We next decided to test if DAPK1 localization to dendritic spines is regulated similarly as the DAPK1/GluN2B binding. Consistent with the observed DAPK1/GluN2B binding, immunostaining of DAPK1 in fixed hippocampal cultures shows a significant enrichment in dendritic spines (Tu et al., 2010). Such enrichment was seen here also by live-imaging of mCherry-labelled full-length DAPK1 wild type (Figure 3A). As observed for GluN2B binding *in vitro* and in HEK cells, the DAPK1 kinase domain was neither necessary nor sufficient for dendritic spine localization: The DAPK1 k fragment that lacks the kinase domain showed the same localization as the DAPK1 wild type, whereas the DAPK1-kr fragment that contains only the kinase and regulatory domains showed no enrichment in dendritic spines at all (Figure 3A).

Chemical LTP stimuli, which cause accumulation of CaMKII in dendritic spines, instead caused removal of DAPK1 from spines (Figure 3B), consistent with the opposing regulation of the GluN2B binding of these two CaM kinases by strong Ca^{2+}/CaM-stimuli. The same removal from spines was also seen for the DAPK1 k fragment (Figure 3B), as expected based on the same regulation of GluN2B binding in HEK cells and *in vitro* (see Figure 1F–J). The removal of DAPK1 wild type from spines was LTP-specific and was not observed after chemical LTD stimuli (Figure 3C, D). Notably, spine retention of DAPK1 during LTD stimuli required DAPK1 activity, as inhibition of DAPK1 activity with 10 μM TC-DAPK-6 (TC) or deletion of the kinase domain in the DAPK1 k fragment resulted in spine removal in response to LTD stimuli (Figure 3C, D). This indicates that DAPK1 spine retention is favored by the lower rise in Ca^{2+} caused by LTD- compared to LTP-stimuli (Marsden et al., 2010), but additionally requires a DAPK1-mediated phosphorylation reaction, such as the GluN2B S1303 phosphorylation reaction that enhances DAPK1/GluN2B binding (see Figure 2H–K) and that indeed occurs in a DAPK1-dependent manner after LTD (see Figure 4).

Taken together, these results show that the dynamic DAPK1 localization to dendritic spines during plasticity-inducing stimuli is regulated as expected from the mechanisms shown here to govern the DAPK1/GluN2B binding. Notably, both the regulation of GluN2B binding and

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localization to dendritic spines is completely opposite for DAPK1 compared to CaMKII, even though both are related members of the CaM kinase family.

LTD stimuli cause CaN-dependent DAPK1 activation and DAPK1-dependent GluN2B phosphorylation

The kinase activity-dependence of DAPK1 spine retention during LTD stimuli indicated that DAPK1 is activated during LTD, and then causes a phosphorylation reaction that favors retention, such as at GluN2B S1303. Such a CaN/DAPK1/GluN2B signaling cascade has been observed after ischemic stimuli and has been implicated in the resulting neuronal pathology (Tu et al., 2010). Thus, we tested here if these signaling steps can also be induced in by non-pathological LTD-stimuli in CA1 of the hippocampus, a brain area required for learning and memory (Squire and Wixted, 2011). LTD was induced chemically in hippocampal CA1 mini-slices (Figure 4A) by bath application of 20 μM NMDA for 3 min (Supplemental Figure 3). This mild chemical LTD stimulation paradigm caused robust and persistent de-phosphorylation of GluA1 at S845 (Figure 4B), a hallmark feature of LTD (Lee et al., 1998). Assessment of DAPK1 activation by de-phosphorylation of S308 within its regulatory domain revealed a transient LTD-induced activation that returned to baseline within 15–30 min (Figure 4C). Consistently, phosphorylation of the DAPK1 substrate site S1303 on GluN2B showed a transient increase (Figure 4D).

LTD requires CaN activity (Mulkey et al., 1994; Zeng et al., 2001), and so did LTD-induced DAPK1 activation: After inhibiting CaN with 10 μM cyclosporine A (CycA), the LTD-stimulus no longer caused any significant S308 de-phosphorylation (Figure 4E). Likewise, the LTD-induced GluN2B phosphorylation was DAPK1-dependent: After inhibiting DAPK1 with 10 μM TC, the LTD-stimulus no longer caused a significant increase in S1303 phosphorylation (Figure 4F).

CaN-mediated DAPK1 activation prevents synaptic CaMKII accumulation after LTD stimuli

LTD-stimuli cause the CaMKII autophosphorylation at T286 that is sufficient to induce the CaMKII/GluN2B binding that mediates CaMKII accumulation at excitatory synapses during LTP (Bayer et al., 2001; Coultrap et al., 2014; Strack and Colbran, 1998); yet no such accumulation of endogenous CaMKII occurs during LTD (Marsden et al., 2010). We hypothesized that this synaptic accumulation is suppressed during LTD by CaN-dependent DAPK1 activation. Then, inhibition of either CaN or DAPK1 should allow synaptic CaMKII accumulation also during LTD stimuli. Here, we expressed a GFP-labelled intrabody against CaMKII (Mora et al., 2013) in hippocampal neurons in order to enable live-imaging of the movement of endogenous CaMKII during LTD stimuli (Figure 5A; Movies 1–3). The monitoring of endogenous rather than overexpressed CaMKII is important in LTD: While endogenous CaMKII accumulates in dendritic spines only during LTP, overexpressed GFP-CaMKII shows such accumulation also after LTD (Marsden et al., 2010). Under basal conditions, CaMKII was present throughout the dendritic shaft and slightly enriched in dendritic spines (Figure 5A). While chemical LTP-stimuli with glutamate/glycine induce robust further CaMKII accumulation in dendritic spines (Barcomb et al., 2013; Bayer et al., 2001; 2006; O'Leary et al., 2011), no such accumulation was seen after chemical LTD-stimuli (Figure 5A; Supplemental Movie 1). However, inhibiting either DAPK1 (with 10 μM

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TC) or CaN (with 10 μM CycA) allowed significant translocation of endogenous CaMKII into spines even after LTD-stimuli (Figure 5A; Supplemental Movies 2–3), as expected based on our hypothesis. Importantly, basal localization of CaMKII was unaffected by either inhibitor (Figure S4A).

DAPK1 overexpression inhibits synaptic CaMKII accumulation after LTP stimuli

After assessing the effect of DAPK1 loss-of-function during LTD-stimuli, we next tested the effect of DAPK1 gain-of-function on synaptic CaMKII accumulation during LTP-stimuli. For this purpose, we overexpressed mCherry-labelled DAPK1 and mCerulean-labelled CaMKIIα in hippocampal cultures along with an intrabody to PSD-95 (Gross et al., 2013) to mark excitatory synapses. DAPK1 was highly enriched in dendritic spines under basal conditions. However, after one day of expression, not all spines contained DAPK1 (Figure 5B), thereby providing an internal control for the effect on CaMKII movement to different spines within the same neuron. Basal CaMKII localization was not affected by DAPK1 overexpression (Figure S4B). Chemical LTP-stimuli caused robust and rapid movement of endogenous CaMKII to excitatory synapses that lacked over-expressed DAPK1 (Figure 5B). By contrast, movement to synapses that contained over-expressed DAPK1 was significantly reduced (Figure 5B). This block was dependent on the DAPK1 kinase domain, as no effect was detected after overexpression of the kinase domain-lacking DAPK1 k fragment (Figure 5B).

DAPK1-mediated suppression of CaMKII/GluN2B binding is required for LTD

With DAPK1 control of stimulation-induced CaMKII movement to synapses established, we set out to determine the function of this mechanism in the expression of LTD. First, we examined the effect of DAPK1 inhibition on LTD that is induced in acute hippocampal slice by low-frequency stimulation. DAPK1 inhibition with 1 μM TC completely blocked this LTD (Figure 5C; Figure S4C).

These results provide evidence for DAPK1 requirement in LTD. In order to elucidate the contribution of DAPK1-mediated suppression of CaMKII synaptic accumulation and GluN2B binding during LTD, we employed a pharmacogenetic combination approach using mice with mutant GluN2B that is incapable of CaMKII binding in the first place (GluN2B^{CaMKII}) (Halt et al., 2012). The principle behind such pharmacogenetic combination approaches is that genetic removal of the relevant drug target should eliminate the pharmacological effect of the drug; this provides powerful evidence for the specific mechanism by which a drug exerts its effect. DAPK1 inhibition in either wild type or GluN2B^{CaMKII} mice had no effect on basal transmission, as assessed by input/output slope and paired pulse facilitation (Figure S4E–H). Without DAPK1 inhibition, the GluN2B^{CaMKII} mice showed normal LTD (Figure 5D; Figure S4D), consistent with a previous report (Halt et al., 2012) and with the specific requirement for CaMKII/GluN2B binding in LTP but not LTD. However, the LTD in the GluN2B^{CaMKII} mice was no longer sensitive to DAPK1 inhibition (Figure 5D; Figure S4D). This demonstrates that the GluN2B^{CaMKII} mutant eliminates the drug target that makes LTD sensitive to DAPK1 inhibition in wild type mice. Thus, DAPK1 is required in LTD by suppressing CaMKII/

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GluN2B binding (a function that is no longer necessary when this binding is already suppressed by the GluN2B ^{CaMKII} mutation).

Contribution of competition, S1303 phosphorylation, and Ca^{2+}/CaM-regulation in the DAPK1-mediated control of CaMKII/GluN2B binding

Based on the DAPK1-mediated regulation of CaMKII localization to dendritic spines, we decided to test how DAPK1 affects CaMKII binding to GluN2B *in vitro*. T286-phosphorylated CaMKII effectively bound to GluN2Bc even in absence of Ca^{2+}/CaM (Figure 6A–C), as expected (Bayer et al., 2001; Strack and Colbran, 1998). In order to mimic the situation found within cells, DAPK1 was pre-bound to GluN2Bc, followed by the addition of T286-phosphorylated CaMKII. Interestingly, CaMKII binding was completely blocked by both DAPK1-S308A and DAPK1 k, consistent with competition for an overlapping binding site on GluN2B, and indicating that binding of DAPK1 alone without the LTD-induced phosphorylation of S1303 on GluN2B is sufficient to inhibit CaMKII/GluN2B binding (Figure 6C). Upon stimulation with increasing Ca^{2+}/CaM concentrations *in vitro*, an increasing amount of DAPK1 was displaced and an increasing amount of CaMKII bound to GluN2B (Figure 6D). Thus, DAPK1 and CaMKII compete for GluN2B binding, and T286-phosphorylated CaMKII requires additional Ca^{2+}/CaM-stimulation to displace DAPK1. Notably, T286-phosphorylated CaMKII also requires such additional Ca^{2+}/CaM-stimulation to potentiate synaptic transmission (Barcomb et al., 2014). Importantly, while CaMKII won the competition at high Ca^{2+}/CaM concentrations that are related to LTP stimuli, DAPK1 still won the competition when Ca^{2+}/CaM was added at lower concentrations that are related to LTD stimuli (Figure 6D). While GluN2B S1303 phosphorylation inhibits CaMKII/GluN2B binding (O'Leary et al., 2011; Strack et al., 2000), these results indicate that competition alone is sufficient to block the binding under low Ca^{2+}/CaM concentration conditions (as CaMKII binding was done in presence of ADP instead of ATP, meaning that the Ca^{2+}/CaM concentration did not affect S1303 phosphorylation). In order to test sufficiency of DAPK1 competition (without S1303 phosphorylation) to block CaMKII/GluN2B binding more formally and within cells, co-localization of the CaMKII T286D mutant with the GluN2B S1303A mutant in HEK cells was assessed with or without co-expression of DAPK1 wild type (Figure 6E). As expected, the CaMKII T286 mutant basally colocalized with the GluN2B S1303A mutant (Figure 6E, F). Co-expression of DAPK1 significantly reduced co-localization of the CaMKII T286D mutant with GluN2B (Figure 6E, F); by contrast, co-expression with CaMKII did not reduce co-localization of DAPK1 with GluN2B (Figure 6E, G). Thus, even without DAPK1 activation or phosphorylation of GluN2B at S1303, DAPK1 wins the competition with autonomous CaMKII for GluN2B binding.

Overall, our results implicate the following model for DAPK1-mediated regulation of CaMKII localization and function (Figure 7): Both LTP- and LTD-stimuli induce the CaMKII T286 autophosphorylation that can induce CaMKII/GluN2B binding in principle, but only LTP-stimuli (associated with high Ca^{2+}/CaM concentrations) cause the displacement of DAPK1 from GluN2B (and dendritic spines) that allows CaMKII binding to GluN2B (and accumulation in dendritic spines). DAPK1 activation by CaN during LTD-stimuli (associated with lower Ca^{2+}/CaM concentrations) allows retention of DAPK1

binding to GluN2B (and localization in dendritic spines); this DAPK1 retention blocks CaMKII binding to GluN2B (and accumulation in dendritic spines) during LTD-stimuli, in a manner that is further aided by (but not dependent on) GluN2B S1303 phosphorylation.

DISCUSSION

This study demonstrates a physiological function of DAPK1 in neurons: DAPK1-mediated suppression of CaMKII/GluN2B binding during LTD is essential for this form of synaptic plasticity. This mechanism also provides direct explanation for the roles of two other Ca^{2+}-activated enzymes prominently involved in synaptic plasticity: CaN and CaMKII. CaN activity has long been known to be required for LTD (Collingridge et al., 2010; Malenka and Bear, 2004; Mulkey et al., 1994; Xia and Storm, 2005; Zeng et al., 2001), and CaMKII and its T286 autophosphorylation mediates both LTP and LTD (Coultrap et al., 2014; Giese et al., 1998; Silva et al., 1992). Our results demonstrate that the direction of CaMKII-mediated plasticity is regulated by CaN through the activation of DAPK1 during LTD. Briefly, during LTP-stimuli, DAPK1 is displaced from synaptic GluN2B, which allows CaMKII binding to GluN2B and synaptic accumulation, which in turn has been shown previously to be required for normal LTP (Halt et al., 2012). By contrast, during LTD-stimuli, DAPK1 remains localized to synaptic GluN2B, thereby preventing synaptic accumulation of T286-phosphorylated CaMKII. Inhibition of DAPK1 (or CaN) activity prevented synaptic DAPK1 retention during LTD and allowed synaptic CaMKII accumulation in response to LTD-stimuli. Importantly, DAPK1 inhibiton also blocked expression of LTD in hippocampal slices of wild type mice. This DAPK1 function in LTD was indeed mediated by suppression of CaMKII/GluN2B binding, as LTD was no longer sensitive to DAPK1 inhibition in mutant mice in which CaMKII/GluN2B binding is already suppressed genetically.

Despite its identified role in neuronal pathology (Tu et al., 2010), regulation of DAPK1/GluN2B binding has not been investigated previously. By contrast, CaMKII/GluN2B binding has been studied intensively (for review see Coultrap and Bayer, 2012a; Hell, 2014; Shonesy et al., 2014). DAPK1 and CaMKII are related CaM kinases, and both have been reported to bind to the same region on GluN2B, near S1303 (Bayer et al., 2001; 2006; Strack et al., 2000; Tu et al., 2010), suggesting similar regulation of their binding. We indeed found here that DAPK1 and CaMKII compete for the same binding site on GluN2B, but surprisingly, we found that the mechanisms and regulation of DAPK1 versus CaMKII binding to GluN2B are essentially opposite. DAPK1 binds to GluN2B without stimulation, and while Ca^{2+}/CaM activation is required to induce CaMKII/GluN2B binding, it instead disrupts DAPK1/GluN2B binding. Additionally, the CaMKII kinase domain mediates GluN2B binding, but although the kinase domain of DAPK1 is sufficient for DAPK1/GluN2B binding *in vitro*, this interaction is much weaker than for full length DAPK1 or for DAPK1 lacking the kinase domain and is insufficient to localize DAPK1 to GluN2B in cells. Further, phosphorylation in their regulatory domains (at T286 and S308, respectively) enables CaMKII binding even after Ca^{2+}/CaM has unbound, but instead reduces DAPK1 binding. Finally, phosphorylation of GluN2B at S1303 blocks CaMKII binding, but instead enhances DAPK1 binding.

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The regulation of DAPK1/GluN2B binding is also consistent with the neuronal DAPK1 trafficking observed here. Under basal conditions, DAPK1 was found to be highly enriched in dendritic spines, and after LTP-stimuli that induce strong Ca^{2+}/CaM signals, DAPK1 was removed from spines. By contrast, DAPK1 was retained at spines during LTD-stimuli that induce weaker Ca^{2+}/CaM signals and additionally promote the effects that positively regulate DAPK1/GluN2B binding: the CaN-dependent DAPK1 dephosphorylation at S308 and the resulting DAPK1-dependent GluN2B phosphorylation at S1303. By contrast, neuronal CaMKII trafficking could not be solely explained by its intrinsic regulation of GluN2B binding, and instead additionally requires the DAPK1-mediated regulation described here: Without suppression by DAPK1, the CaMKII T286 phosphorylation that is induced by both LTP and LTD would result in GluN2B binding and synaptic CaMKII accumulation also during LTD.

By determining the CaMKII localization after plasticity stimuli, DAPK1 should affect the substrates that CaMKII can phosphorylate. Notably, CaMKII has at least two phosphorylation sites on the AMPA-type glutamate receptor (AMPAR) subunit GluA1: S831, which increases AMPAR single-channel conductance after LTP (Barria et al., 1997; Benke et al., 1998; Derkach et al., 1999; Kristensen et al., 2011; Poncer et al., 2002), and S567, which decreases synaptic localization of AMPARs and occurs during LTD (Coultrap et al., 2014; Lu et al., 2010). LTP-induced GluA1 S831 phosphorylation is dependent on CaMKII/GluN2B binding, as the GluN2B $^{\Delta CaMKII}$ mutant completely abolishes this phosphorylation while reducing LTP by half (Halt et al., 2012). By contrast, LTD is normal in the GluN2B $^{\Delta CaMKII}$ mutant mice, suggesting that GluA1 S567 phosphorylation does not require the CaMKII/GluN2B interaction. Indeed, in C. elegans, which lacks a CaMKII-binding GluN2B subunit, synaptic removal of AMPARs remains dependent on CaMKII (Hoerndli et al., 2015).

This study provides direct evidence of DAPK1 activation in neurons outside of injury or disease. Notably, the DAPK1 activation after LTD was transient, and this temporal limitation is likely essential for enabling its physiological function in LTD without initiation of pathological pathways and cell death. It also suggests that while DAPK1 activity is required for LTD induction it may not be necessary for the maintenance of LTD. This activation timeline is much shorter than that seen after ischemic injury (the only other stimulus directly shown to activate DAPK1 in neurons), where DAPK1 activation also occurs at immediate timepoints, but remains elevated for at least 24 hours (measured by dephosphorylation of S308) (Shamloo et al., 2005) and up to 8 days post injury (measured by increased protein levels and activity toward a specific substrate) (Schumacher et al., 2002). DAPK1 protein levels (and thus likely also activity) are also chronically upregulated in human Alzheimer’s disease patients, and DAPK1 plays a role in two hallmark features of this disorder: aberrant tau processing and increasing neural release of soluble Amyloid-β (Kim et al., 2014; 2016). Here we show that DAPK1 overexpression inhibits LTP-induced CaMKII translocation, which should reduce LTP (Halt et al., 2012). Thus, upregulation of DAPK1 protein levels in injury or disease could result in LTP deficits and cognitive dysfunction through this mechanism, although this remains to be tested.

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Similarly to the transient activation of DAPK1 seen here, another pro-cell death protein, caspase-3, is transiently activated during, and required for, the induction of LTD (Li et al., 2010), where it is necessary for spine shrinkage and pruning (Erturk et al., 2014) as well as cleavage of the glycogen synthase kinase-3β (GSK3β) inhibitor protein kinase B (Akt) (Li et al., 2010). DAPK1 is a major contributor to several apoptosis pathways where it potentially acts upstream of caspase-3 activation (Bialik and Kimchi, 2013; Gozuacik et al., 2008) and could thus play a role in caspase-3 activation also during LTD. However, an upstream requirement for DAPK1 in this LTD signaling pathway is unlikely, as the pharmacogenetic approach used here indicates that DAPK1 blockade of CaMKII/GluN2B binding is the essential function of DAPK1 in LTD. DAPK1 binding to GluN2B has also been prominently implicated in the pathological functions of DAPK1 (Tu et al., 2010). However, this binding occurs several hours after ischemic injury, and is thus likely also separate from non-pathological binding of DAPK1 at synaptic sites during LTD. Here, we show that DAPK1 is removed from synaptic sites during high levels of Ca^{2+} influx with LTP stimuli, and the pathological and longer lasting Ca^{2+} rise in post synaptic cells during ischemic insult should similarly displace DAPK1 from synaptic sites. Therefore, the subsequent pathological binding to extrasynaptic GluN2B could be a bi-product of this long-lasting displacement during the initial injury. Thus, when examining common pathways in LTD and cell death-induction, mild, rather than strong (and possibly also death inducing), LTD stimuli should be used to separate the distinct functions of these proteins in LTD from their roles in cell death.

Traditional thought holds that LTP is mediated by protein kinases while LTD is mediated by protein phosphatases. This view is clearly too simplistic, as LTD requires the protein kinases CaMKII (Coultrap et al., 2014; Stevens et al., 1994), PKA (Kameyama et al., 1998; Lu et al., 2008; Sanderson et al., 2016) and GSK3β (Peineau et al., 2007). Our results not only add another protein kinase that is required for LTD, DAPK1, but also demonstrate that one essential phosphatase function in LTD is actually kinase activation: The DAPK1 mechanism described here required upstream activation by CaN. Similarly, GSK3β is activated during LTD through the dephosphorylation of S9 by protein phosphatase 1 (PP1). Activation of GSK3β is also further increased by PP1 mediated dephosphorylation of T308 of Akt, which acts to phosphorylate S9 of GSK3β to inhibit its activity (Peineau et al., 2007). Thus, phosphatase inhibition of one kinase (Akt) during LTD results in the activation of another kinase (GSK3β), further highlighting the complexity of phosphatase/kinase interactions during LTD. Importantly, in the current study, inhibition of CaN not only prevented the activation of DAPK1, but it also prevented the essential function of DAPK1 in LTD shown here: LTD-specific suppression of CaMKII/GluN2B binding. Together the results provide evidence for a non-pathological function of the DAPK1/GluN2B interaction and thus help to bridge a gap in the interplay between normal physiological signaling and pathological disruptions that lead to cell death. Further, they establish the regulatory mechanisms underlying the DAPK1/GluN2B interaction, and thus have direct implications outside of the physiological role demonstrated here.

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EXPERIMENTAL PROCEDURES

A complete list of materials and detailed methods can be found in the online Supplemental Experimental Procedures. All procedures involving animals were carried out in accordance with the National Institutes of Health best practices for animal use and were approved by the University of Colorado Institutional Animal Care and Use Committee. The University of Colorado Anschutz Medical Campus is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). Mice of both genders were used for electrophysiology and biochemical experiments. The GluN2B ^{CaMKII} mice are described previously (Halt et al., 2012).

Cell culture, transfections, protein preparations, and Western analysis were done as previously described (Barcomb et al., 2013; 2014; 2015; Bayer et al., 2001; 2006; Coultrap and Bayer, 2012b; 2014; Goodell et al., 2016; 2014; O'Leary et al., 2011; Singla et al., 2001; Vest et al., 2007) and are detailed in the Supplemental Experimental Procedures. Antibodies and dilutions are shown in Supplemental Table 1.

Electrophysiology and chemical (c)LTD in slices from mixed sex p13–17 mice was done as described (Coultrap et al., 2014; Goodell et al., 2016). Electrophysiological responses were recorded at 30.5 +/– 0.5°C in the CA 1 in response to Schaffer collateral stimulation. LTD was induced with low frequency stimulation (LFS, 900 pulses at 1 Hz) for electrophysiology and chemically (cLTD, 20 μM NMDA for 3 min) for Western analysis of phosphorylation changes in CA1 mini slices.

Live imaging of DIV-13–14 neurons or HEK-293 cells was done at 32°C in HEPES buffered imaging solution containing (in mM) 130 NaCl, 5 KCl, 10 HEPES pH 7.4, 20 Glucose, 2 $CaCl_2$, 1 $MgCl_2$, adjusted to proper osmolarity with sucrose on a Zeiss Axiovert 200M inverted microscope (Carl Zeiss GmbH, Oberkochen, Germany) fitted with a 63× objective (1.4NA, plan-Apo). Focal plane z-stacks (0.3 μm steps, over 1.8–2.4 μm) were acquired and deconvolved to eliminate out of focus light. 2D maximum intensity projection images were then generated and analyzed by an experimenter blinded to condition using Slidebook 6.0 software (Intelligent Imaging Innovations (3i)).

In neurons, cLTD was induced with 30 μM NMDA, 10 μM glycine, 10 μM CNQX for 1 min and cLTP was induced with 100 μM glutamate, 10 μM glycine for 1.5 min. The cLTD stimuli results in very little CaMKII accumulation at dendritic spines (shown here and in Marsden et al., 2010) while our cLTP stimuli induces robust CaMKII accumulation (Barcomb et al., 2013; Bayer et al., 2001; 2006; O'Leary et al., 2011). These stimuli were previously demonstrated to decrease or increase AMPAR surface expression, respectively (shown in the supplement of Marsden et al., 2010), and are similar to other documented plasticity stimuli (Malgaroli and Tsien, 1992; Molnár, 2011). Starting and changes in spine- or PSD-to-shaft ratios were compared between groups. Ca^{2+} influx in HEK-293 cells was generated with 10 μM ionomycin for 5 min and Pearson’s correlation of fluorescence overlap was compared between groups.

In vitro protein binding experiments were done as previously described (Goodell et al., 2014) using immobilized GST-GluN2Bc on sepharose or magnetic beads. DAPK1

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constructs (varying concentrations or 1 μM for comparison between constructs) were bound in either EGTA or varying Ca^{2+}/CaM as indicated. For CaMKII/DAPK1 competition experiments, DAPK1 was prebound to GST-GluN2Bc at saturating concentrations prior to adding autonomous (pT286) CaMKII.

All data are shown as mean +/– standard error of the mean (SEM). Statistical significance is indicated in the figure legends. Data was analyzed with analysis of varience (ANOVA) or Kruskal-Wallice test for 3 or more groups followed by Dunnet's-T, Student's-Newman-Keuls, Dunn's test or Dunn-Bonferoni as appropriate. Two groups were compared with non-paired two-tailed Student's t-test or two-tailed Mann-Whitney test. A Repeated-Measures-ANOVA using Time × Group as independent variables was used for comparisons over multiple timepoints.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- DAPK1 binding to GluN2B competes with CaMKII binding and is disrupted by Ca^{2+}/CaM
- DAPK1 is removed from synapses during LTP but retained during LTD
- DAPK1 activation and GluN2B binding prevents synaptic CaMKII accumulation during LTD
- DAPK1-mediated suppression of CaMKII/GluN2B binding is required for LTD.

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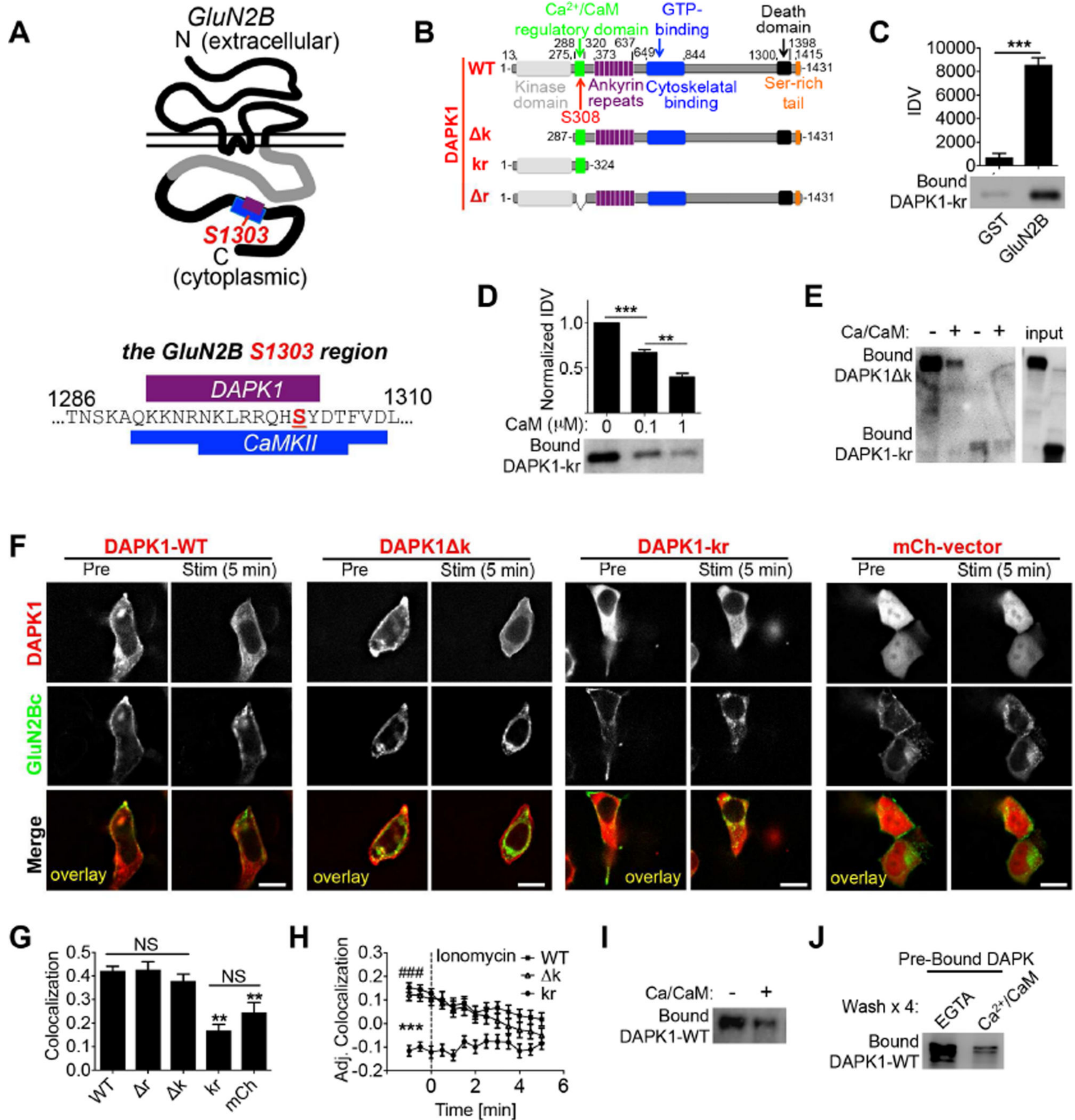

Figure 1. DAPK1/GluN2B binding is negatively regulated by Ca^{2+}/CaM and does not require the kinase domain

DAPK1 interaction with the GluN2B C-terminus was investigated *in vitro* and in HEK-293 cells. Data are shown as mean and SEM. * p<0.05, ** p<0.01, *** p<0.001, NS, not significant, difference from control or from indicated group unless otherwise indicated. Scale bars represent 10 μm. IDV, immuno-detection value.

(A) Schematic of GluN2B and the sequence of the S1303 region containing overlapping DAPK1 and CaMKII binding sites.

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(B) DAPK1 construct map showing the domain sequence and the truncations used in the current study.

(C) DAPK1-kr was sufficient for specific binding to GluN2B *in vitro*. n=4 each group.

(D) DAPK1-kr to GluN2B binding was negatively regulated by Ca^{2+}/CaM. n=3 each group.

(E) DAPK1 k bound to GluN2B far more than DAPK1-kr and retained sensitivity to Ca^{2+}/CaM. DAPK1 input example is from a lighter exposure of the same blot.

(F) Example images of basal and stimulated colocalization of DAPK1 constructs with GluN2Bc. Cells were transfected with mCh-DAPK1 constructs and pDisplay-eGFP-GluN2Bc (GluN2Bc). Cells were stimulated with ionomycin (10 μM) at timepoint 0 and imaged for 5 min post stimulation.

(G) Quantification of DAPK1/GluN2Bc colocalization by Pearson's correlation. n=18 WT, n=24 r, n=29 k, n=17 kr, n=25 mCh, ** p<0.01, *** p<0.001 difference from WT, k and r.

(H) DAPK1-WT and k GluN2Bc binding was negatively regulated by Ca^{2+}/CaM stimulation. ###, p<0.001, Group × Time interaction, *** p<0.001, DAPK1-kr differed from DAPK1 WT and DAPK1 k in starting value and Ca^{2+} induced dissociation.

(I) Ca^{2+}/CaM reduced binding of DAPK1 WT *in vitro*.

(J) Ca^{2+}/CaM (2 mM, 1 μM) was sufficient to compete off pre-bound DAPK1 *in vitro*.

See also Figure S1.

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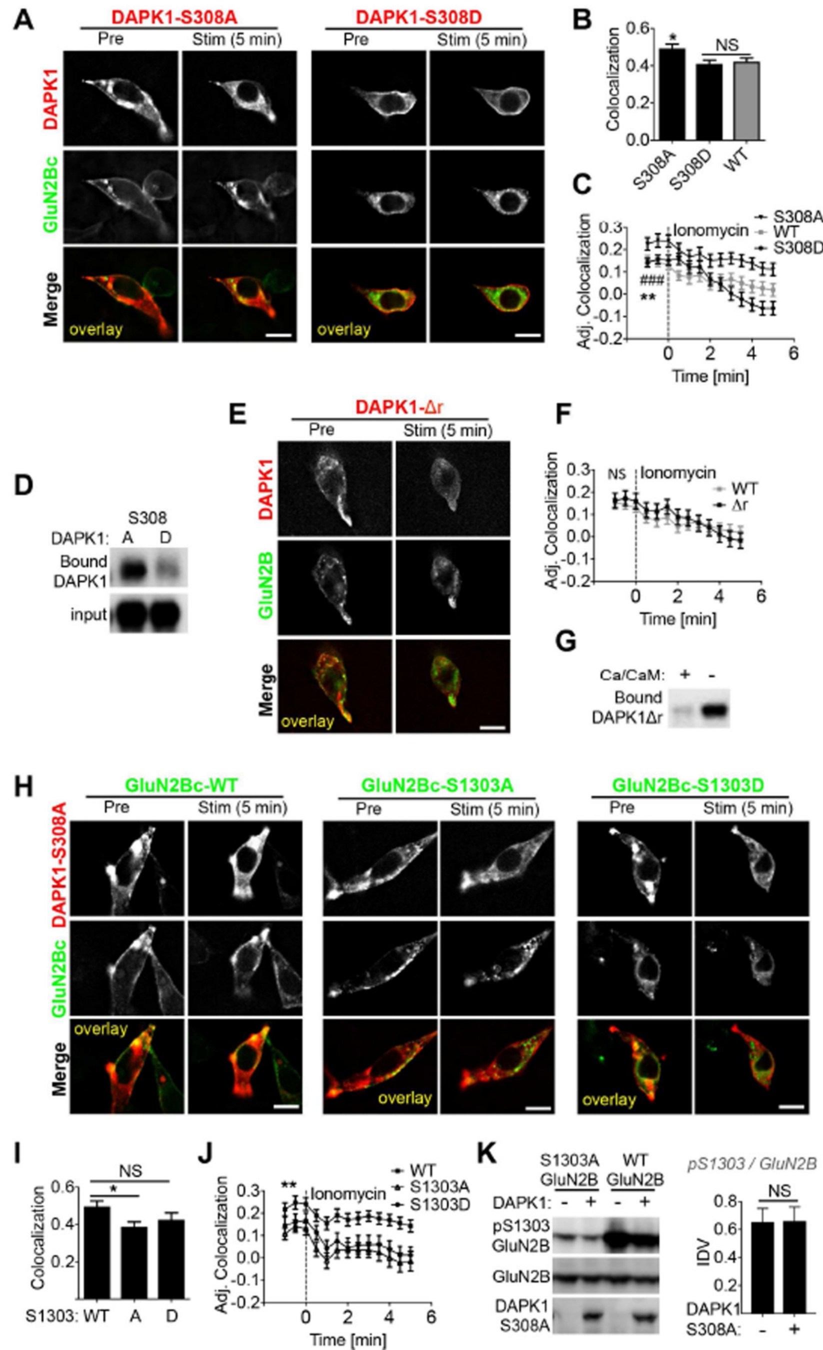

Figure 2. DAPK1/GluN2B binding is positively regulated by DAPK1 S308 dephosphorylation and GluN2B S1303 phosphorylation

DAPK1 interaction with the GluN2B C-terminus was investigated *in vitro* and in HEK-293 cells. Data are shown as mean and SEM. * p<0.05, ** p<0.01, *** p<0.001, NS, not significant, difference from control or from indicated group unless otherwise indicated. Scale bars represent 10 µm. IDV, immuno-detection value.

(A) Example images of basal and stimulated colocalization of DAPK1 S308 mutants with GluN2Bc. Cells were transfected with mCh-DAPK1 constructs and pDisplay-eGFP-

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GluN2Bc (GluN2Bc). Cells were stimulated with ionomycin (10 μM) at time point 0 and imaged for 5 min post stimulation.

(B) DAPK1 S308A showed higher basal colocalization with GluN2Bc in HEK-293 cells. n=31 S308A, n=31 S308D, n=18 WT, * $p<0.05$, difference from S308D and WT. WT quantification (in grey) is the same as in Figure 4G.

(C) DAPK1 S308A was less sensitive to Ca^{2+} induced disociation from GluN2Bc than DAPK1 WT or S308D. WT quantification (in grey) is the same as Figure 4H. ###, $p<0.0001$ Group × Time interaction, ** $p<0.001$, DAPK1 S308A differs from DAPK1 WT and DAPK1 S308D.

(D) DAPK1 S308A bound to GluN2Bc more than DAPK1 S308D *in vitro*. DAPK1 input example is from a lighter exposure of the same blot.

(E) Example images of basal and stimulated colocalization of CaM binding deficient DAPK1 r and GluN2Bc. Cells were transfected with mCh-DAPK1 r and pDisplay-eGFP-GluN2Bc (GluN2Bc). Cells were stimulated with ionomycin (10 μM) at timepoint 0 and imaged for 5 min post stimulation.

(F) DAPK1 r showed the same Ca^{2+} induced dissociation from GluN2B as DAPK1 WT. WT quantification (in grey) is the same as Figure 4H.

(G) DAPK1 r maintained Ca^{2+}/CaM sensitivity *in vitro.*

(H) Example images of basal and stimulated colocalization of DAPK1 S308A with GluN2Bc. Cells were transfected with mCh-DAPK1 S308A and pDisplay-eGFP-GluN2Bc (GluN2Bc) WT or S1303A or D mutants. Cells were stimulated with ionomycin (10 μM) at time point 0 and imaged for 5 min post stimulation.

(I) DAPK1 S308A basal colocalization with GluN2B was reduced by S1303A mutation. n= 28 GluN2Bc WT, n=20 S1303A, n=20 S1303D.

(J) Ca^{2+} induced unbinding of DAPK1 S308A occurred for all GluN2B constructs, but S1303A and S1303D mutation resulted in significantly lower localization after ionomycin stimulation. ** $p<0.01$, GluN2Bc WT differs from S1303A and D mutants.

(K) GluN2B is phosphorylated at S1303 in HEK-293 cells independent of DAPK1 expression. n=5 each condition.

See also Figures S1 and S2.

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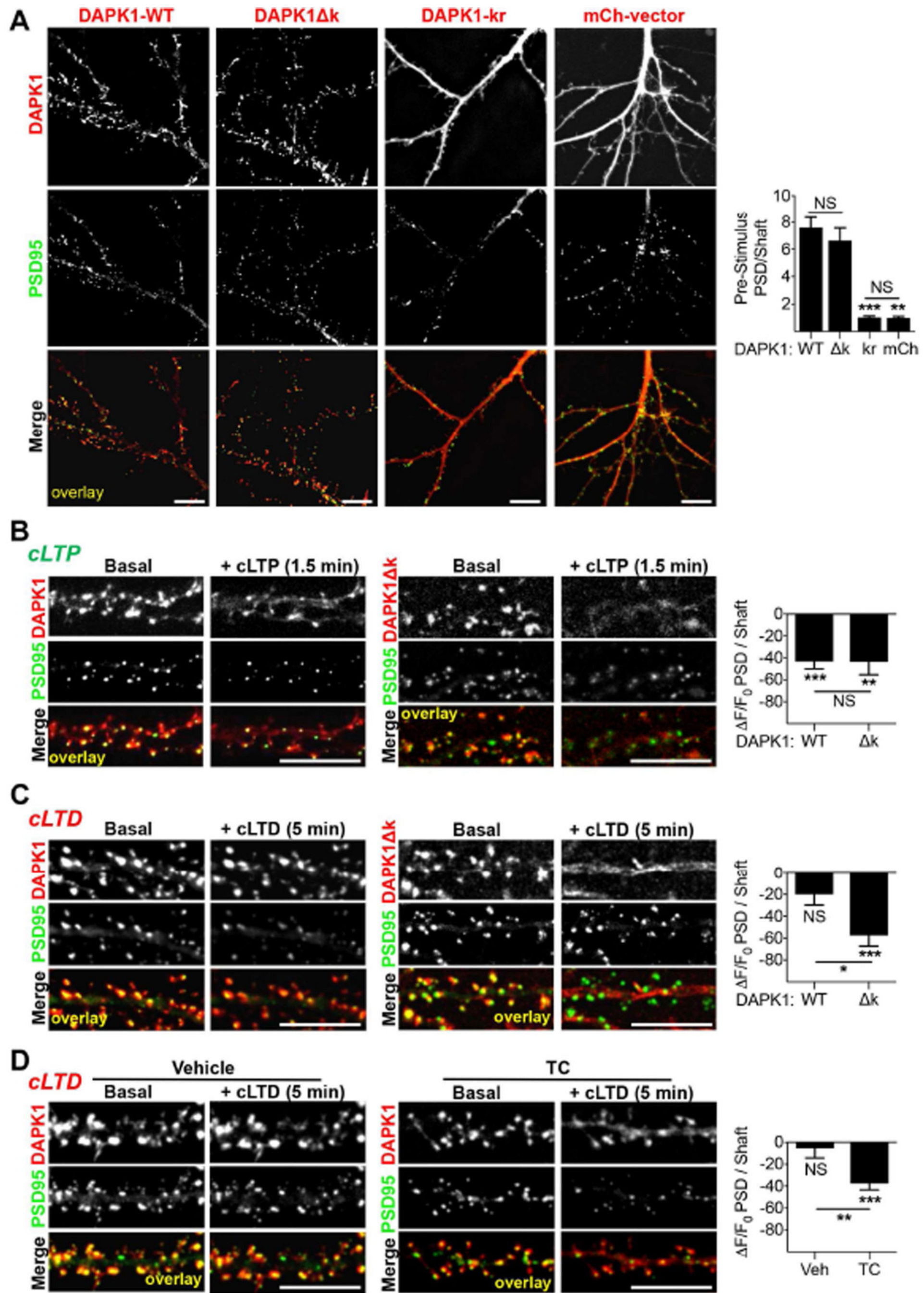

Figure 3. DAPK1 is removed from dendritic spines by LTP- but not LTD-stimuli

Neurons were transfected with mCh-DAPK1 and an intrabody to PSD-95 (GFP). Chemical LTD (cLTD) was induced with 30 μM NMDA, 10 μM glycine, 10 μM CNQX for 1 min. Chemical LTP (cLTP) was induced with 100 μM glutamate, 10 μM glycine for 1.5 min. Data are shown as mean and SEM. * p<0.05, ** p<0.01, *** p<0.001, NS, not significant, difference from 0 or from indicated group unless otherwise indicated. Scale bars represent 10 μm.

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(A) DAPK1-WT and DAPK1 k, but not DAPK1-kr, were highly enriched in dendritic spines. n=33 WT, n=25 k, n=11 -kr, n=7 mCh. ** p<0.01, *** p<0.001, difference from DAPK1 WT and k.

(B) DAPK1 WT and DAPK1 k were both removed from dendritic spines by cLTP. n=12 WT, n=10 k.

(C) DAPK1 WT but not DAPK1 k remained in dendritic spines during cLTD. n=10 WT, n=10 k.

(D) DAPK1 retention in dendritic spines during cLTD depends on kinase activity, as indicated by removal from spines when DAPK1 activity was inhibited by 10 μM DAPK-TC6 (TC). n=21 Vehicle (Veh), n=17 TC.

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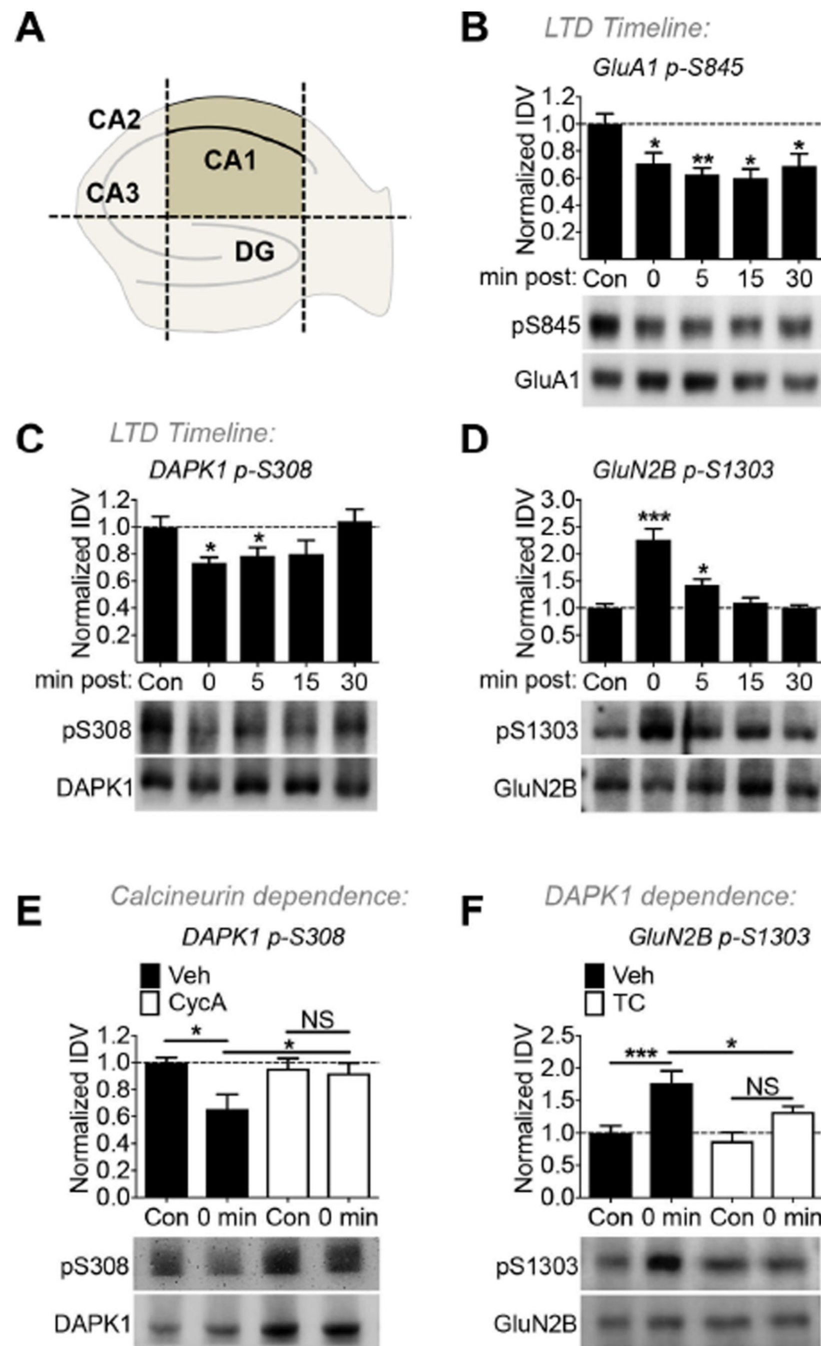

Figure 4. LTD stimuli cause CaN-dependent DAPK1 activation and DAPK1-dependent GluN2B phosphorylation

Hippocampal CA1 mini slices were subjected to 20 μM NMDA for 3 min (cLTD) and recovered for the indicated times. Slices were homogenized and subjected to Western analysis of phosphorylation sites and total protein. IDV, immuno-detection value. n=8 unless indicated otherwise; Data are shown as mean and SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS, not significant, difference from control or from indicated group.

(A) Schematic of a CA1 mini slice.

(B) The hallmark LTD-induced dephosphorylation of S845 of GluA1 occurred stably in response to cLTD.

(C) Autoinhibitory phosphorylation of DAPK1 at S308 was reduced transiently after cLTD.

(D) Phosphorylation of GluN2B at S1303 was increased after cLTD.

(E) Pre-incubation (10 min) with the calcineurin inhibitor cyclosporin A (CycA; 10 μM) blocked cLTD induced dephosphorylation of S308 DAPK1. n=7 for vehicle cLTD, n=9 CycA cLTD,

(F) Pre-incubation (10 min) with the DAPK1 inhibitor TC-DAPK-6 (TC;10 μM) blocked cLTD induced phosphorylation of S1303 GluN2B. n=11 vehicle control, n=10 TC control. See also Figure S3.

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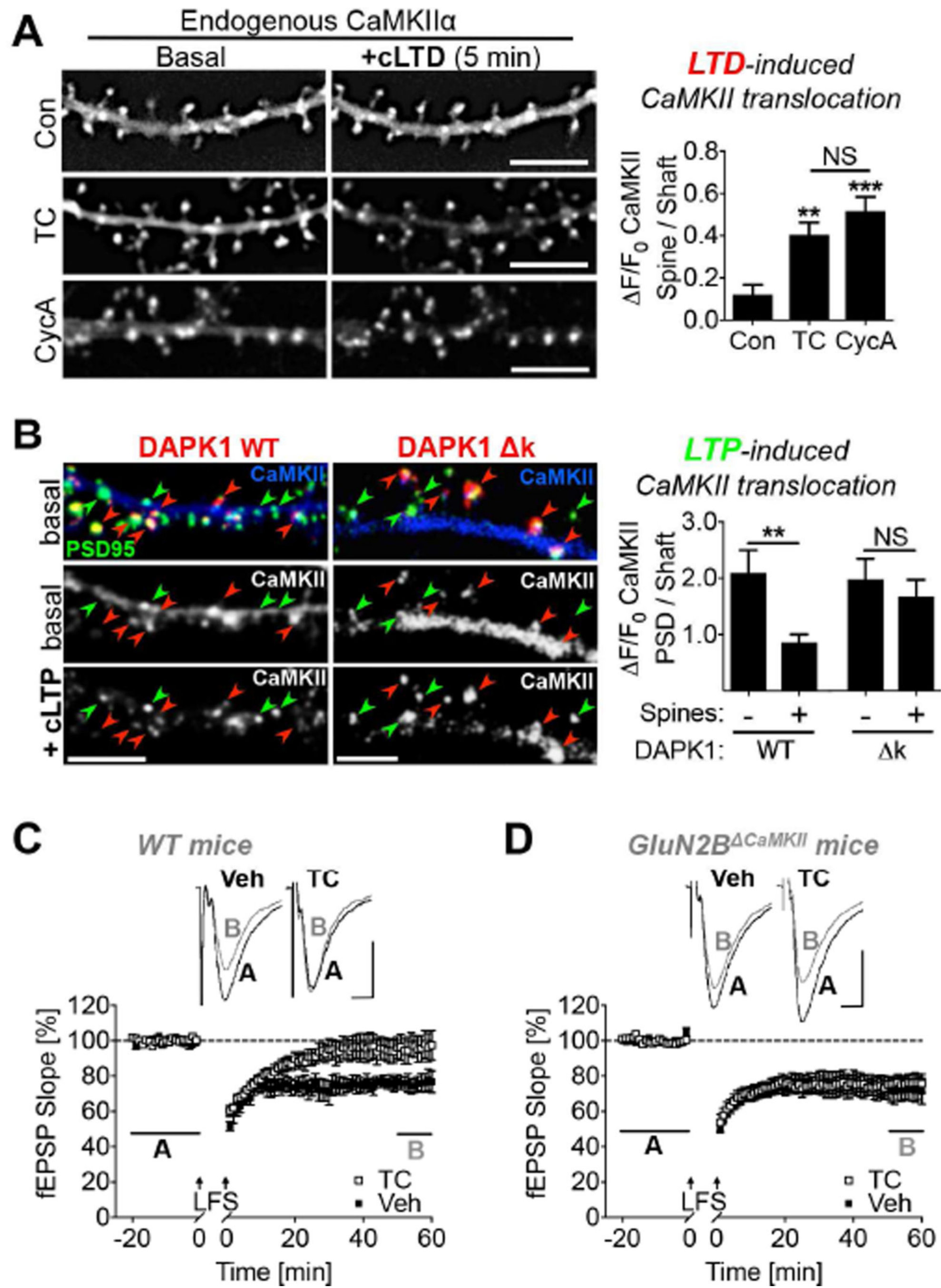

Figure 5. DAPK1-mediated suppression of CaMKII/GluN2B binding is required for LTD

DAPK1 functions in directing synaptic CaMKII localization and in mediating LTD were assessed in hippocampal cultures (panels A, B) and in acute hippocampal slices (panels C, D). Data are shown as mean and SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS, not significant, difference from control or from indicated group. Neuronal scale bars represent 5 μm. Electrophysiology scale bars represent 5 ms (horizontal) and 0.5 mA (vertical).

(A) Inhibition of DAPK1 (10 μM TC-DAPK-6; TC) or CaN (10 μM cyclosporine A; CycA) allowed the accumulation of endogenous CaMKII in dendritic spines after cLTD. Neurons

were transfected with an intrabody to CaMKIIα and chemical LTD was induced with 30 μM NMDA, 10 μM glycine and 10 μM CNQX for 1 min. Quantifications represent the average of at least 15 spines per neuron from n=26 control, n=19 TC, n=24 CycA neurons.

(B) DAPK1 overexpression inhibited LTP induced accumulation of overexpressed CaMKII in overexpressed DAPK1 WT but not DAPK1 k containing dendritic spines. Neurons were transfected with mCh-DAPK1, an intrabody to PSD95 (GFP, imaged in YFP channel), and mCer-CaMKII. Chemical LTP (cLTP) was induced with 100 μM glutamate, 10 μM glycine for 1.5 min. Quantifications represent 7–15 DAPK1 +/− spines per neuron from n=11 DAPK1 WT and n=8 DAPK1 k expressing neurons.

(C) Field excitatory post synaptic potentials (fEPSPs) were recorded in the CA1 dendritic layer in response to CA1 Schaffer collateral stimulation. LTD was induced with 900 pulses at 1 Hz (LFS) in slices incubated in TC (1 μM) or vehicle (Veh) (0.08% DMSO). TC (1 μM) blocked LFS induced LTD in WT mice. n=9 TC, n=7 Veh.

(D) TC (1 μM) failed to block LFS induced LTD in GluN2B ^{CaMKII} mice. n=9 TC, n=8 Veh.

See also Figure S4 and Supplemental Movies 1–3.

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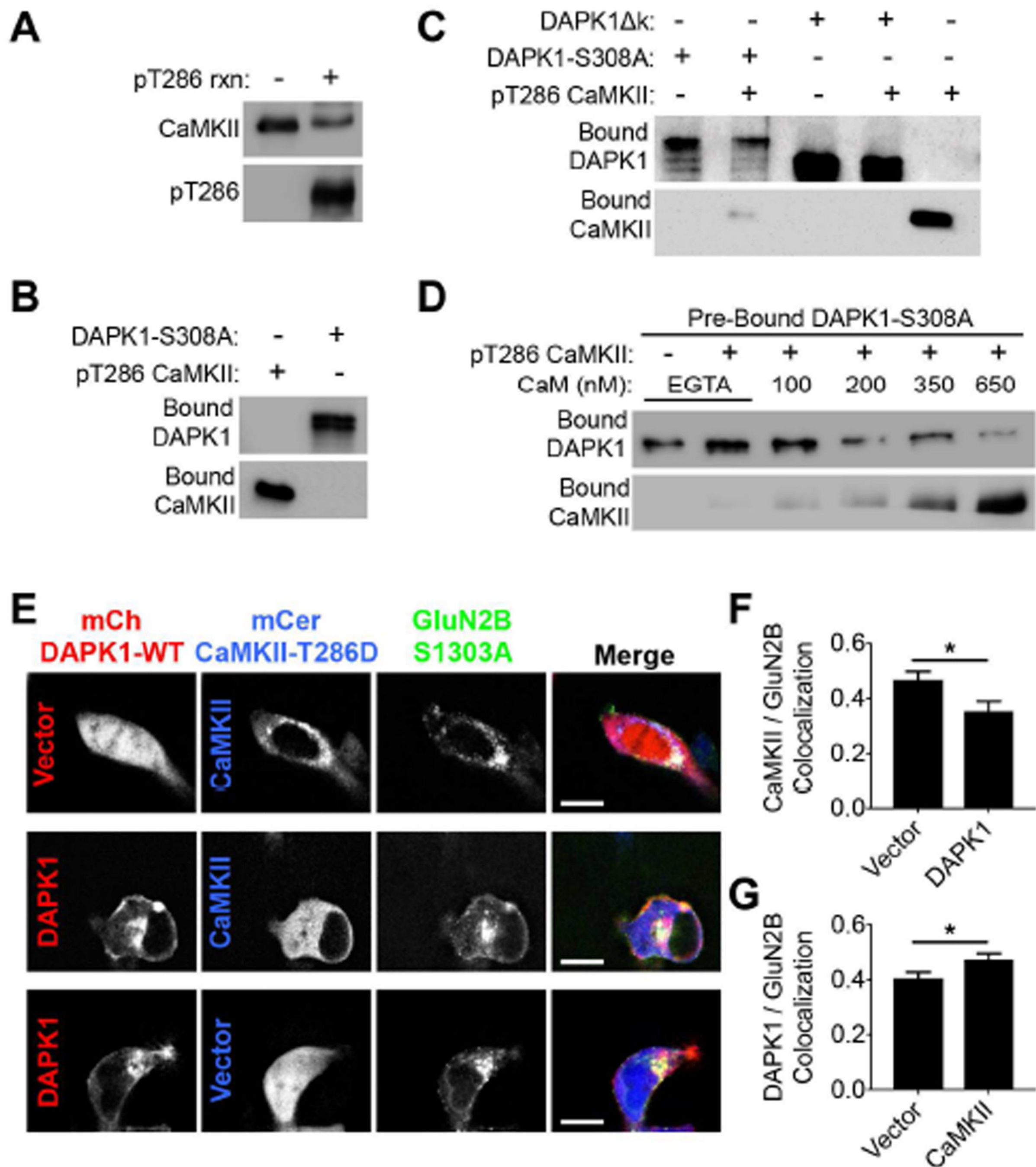

Figure 6. pT286 CaMKII requires additional Ca^{2+}/CaM stimulation to displace DAPK1 from GluN2B

The effects of pre-bound DAPK1 on autonomous (pT286) CaMKII/GluN2B binding were assessed *in vitro* and in HEK-293 cells. *p<0.05, difference from indicated group.

(A) CaMKII autophosphorylation at T286 was induced *in vitro*.

(B) pT286 CaMKII bound to GluN2B without further Ca^{2+}/CaM stimulation.

(C) Pre-binding of DAPK1 S308A or DAPK1 k blocked pT286 induced binding.

(D) pT286 CaMKII displaced DAPK1 from GluN2B with additional Ca^{2+}/CaM stimulation in a concentration dependent manner.

(E) Example images of basal colocalization of DAPK1-WT and CaMKIIα T286D with GluN2Bc. Cells were transfected with mCh-DAPK1-WT, mCer-CaMKIIα T286D (or respective empty vectors) and pDisplay-eGFP-GluN2Bc S1303A (imaged in YFP channel).
(F) CaMKII T286D colocalization with GluN2B S1303A was reduced by DAPK1 WT coexpression.
(G) DAPK1 WT colocalization with GluN2B S1303A was not reduced by CaMKII T286D coexpression. n= 31 DAPK1 WT + CaMKIIα T286D, n=26 mCh-Vector + CaMKIIα T286D, n=23 DAPK1 WT + mCer-Vector.

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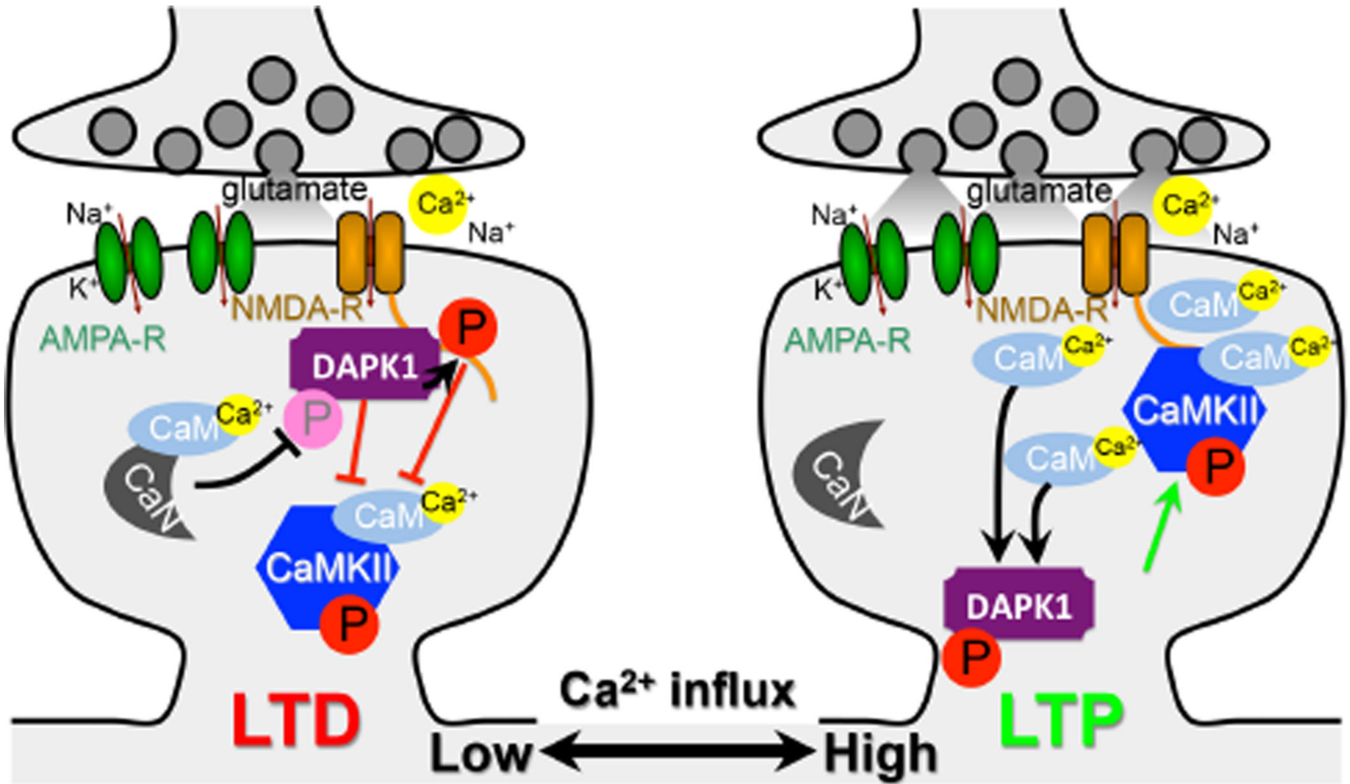

Figure 7. Model of DAPK1 regulation of the CaMKII binding site on GluN2B

DAPK1 and GluN2B are basally colocalized in cells. During low Ca^{2+} influx such as that which occurs with LTD-stimuli, CaN activates DAPK1 by de-phosphorylation of S308, which in turn triggers DAPK1-mediated phosphorylation of GluN2B at S1303 GluN2B. Both DAPK1 S308 de-phosphorylation and GluN2B S1303 phosphorylation enhance the DAPK1/GluN2B interaction, and DAPK1 activation promotes retention of the binding to GluN2B during LTD, which in turn prevents CaMKII/GluN2B binding during LTD. During high Ca^{2+} influx such as that which occurs with LTP-stimuli, high levels of Ca^{2+}/CaM negatively regulate DAPK1/GluN2B binding, which eliminates DAPK1 competition for the binding site on GluN2B and in turn allows CaMKII/GluN2B binding during LTP.