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Subunit-Specific Augmentation of AMPA Receptor Ubiquitination by Phorbol Ester

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Abstract

Excitatory neurotransmission relies on the precise targeting of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors to the neuronal plasma membrane. Activity-dependent ubiquitination of AMPA receptor (AMPA) subunits sorts internalised receptors to late endosomes for degradation, which ultimately determines the number of AMPARs on neuronal membrane. Our recent study has demonstrated a functional cross-talk between the phosphorylation and ubiquitination of the GluA1 subunit in mammalian central neurons. However, the existence of such a cross modulation for the GluA2 subunit remains unknown. Here, we have shown that bicuculline induced GluA2 ubiquitination on the same lysine residues (Lys-870 and Lys-882) in the C-terminal as those elicited by the AMPA treatment. Interestingly, bicuculline-induced ubiquitination was markedly enhanced by the phospho-mimetic GluA2 S880E mutant. Pharmacological activation of protein kinase C (PKC) by phorbol ester, which mediates the phosphorylation of GluA2 at Ser-880, augmented bicuculline-induced ubiquitination of GluA2 in cultured neurons. This effect was specific for the GluA2 subunit because phorbol ester did not alter the level of GluA1 ubiquitination. However, phorbol ester-induced enhancement of GluA2 ubiquitination did not require Ser-880 phosphorylation. This suggests that pseudo-phosphorylation of Ser-880 is sufficient but is not necessary for the augmentation of bicuculline-induced GluA2 ubiquitination. Collectively, these data provide the first demonstration of subunit-specific modulation of AMPAR ubiquitination by the PKC-dependent signalling pathway in mammalian central neurons.

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Conflict of interest All authors declare that they have no competing financial interests.

Ethical Approval All animal handling procedures were carried out in accordance to the Australian code for the care and use of animals for scientific purposes by the National Health and Medical Research Council and were approved by the University of Queensland Animal Ethics Committee (AEC approval number QBI/047/18).

Informed Consent This article does not contain any studies with human participants performed by any of the authors. All authors have read and agreed with the submission of the manuscript.

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Keywords

AMPA receptors; Ubiquitination; Phosphorylation; Phorbol ester; Protein kinase C

Introduction

The α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (AMPA receptors) mediate the majority of fast excitatory synaptic transmission in the mammalian central nervous system. They are tetrameric assemblies of two dimers, predominantly composed of GluA1 and GluA2 subunits in principal neurons in the hippocampus and cortex (Lu et al. 2009). Dynamic trafficking of AMPARs into and out of synapses is a major mechanism underlying synaptic plasticity, learning and memory (Anggono and Huganir 2012; Huganir and Nicoll 2013). It is tightly regulated by AMPAR interacting proteins and several post-translational modifications that occur on the carboxyl-terminal tails, including protein phosphorylation, ubiquitination and palmitoylation (Diering and Huganir 2018; Lussier et al. 2015).

Ubiquitination is a reversible modification that covalently attaches one or more (mono- or poly-ubiquitination) highly conserved 76 amino acid ubiquitin moieties to protein substrates (Hershko and Ciechanover 1998). Protein ubiquitination is essential for neuronal physiology and is known to regulate diverse cellular processes such as protein degradation, endocytosis, intracellular trafficking and sorting of transmembrane proteins (Hershko and Ciechanover 1998; Mabb and Ehlers 2010). All four subunits of AMPARs, GluA1–4, undergo activity-dependent ubiquitination which controls their trafficking towards late endosomes for degradation (Widagdo et al. 2015, 2017; Schwarz et al. 2010; Lin et al. 2011; Lussier et al. 2011). In cultured neurons, the ubiquitination of AMPAR subunits is robustly induced by the application of the AMPAR agonist, AMPA, as well as bicuculline, a specific GABA_A receptor antagonist (Widagdo et al. 2015; Lin et al. 2011; Schwarz et al. 2010; Lussier et al. 2011). Although both AMPA- and bicuculline-induced ubiquitination of AMPARs are dependent on Ca²⁺ influx through L-type voltage-gated Ca²⁺ channels (L-VGCCs), each of them engages slightly different signalling pathways (Lussier et al. 2011; Widagdo et al. 2015). AMPA-induced ubiquitination acts by direct binding and activation of both synaptic and extrasynaptic AMPARs. In contrast, bicuculline-mediated ubiquitination relies on presynaptic release of glutamate and should therefore activate only synaptic AMPARs. In addition to AMPARs and L-VGCCs, bicuculline-induced ubiquitination of AMPARs also requires *N*-methyl-D-aspartate- and group I metabotropic glutamate (mGlu) receptor-dependent signalling pathways (Widagdo et al. 2015).

It has been recently proposed that various post-translational modifications on AMPARs represent molecular codes that determine the number and/or functional state of synaptic AMPARs in both Hebbian and homeostatic synaptic plasticity (Diering and Huganir 2018). The evidence for dynamic interactions (synergistic or antagonistic) between two different post-translational modifications on AMPAR subunits and their roles in synaptic plasticity has started to emerge. For examples, nitrosylation of Cys-875 enhances GluA1 phosphorylation at Ser-831, whereas GluA1 palmitoylation on Cys-811 inhibits the

phosphorylation of Ser-818 (Lin et al. 2009; Selvakumar et al. 2013). In the context of AMPAR ubiquitination, we have previously demonstrated for the first time a negative cross modulation of GluA1 ubiquitination with the phosphorylation on Ser-845 by protein kinase A (PKA), which mediates amyloid- β -induced removal of surface AMPARs in neurons (Guntupalli et al. 2017). However, the existence of such an interaction in the GluA2 subunit is unknown. In this study, we specifically focussed on the modulation of bicuculline-induced GluA2 ubiquitination by the protein kinase C (PKC) signalling pathway, which is known to directly phosphorylate the GluA2 subunit at Ser-880 and facilitate AMPAR internalisation during long-term depression (LTD) (Chung et al. 2000, 2003).

Materials and Methods

DNA Constructs

Plasmid DNAs encoding full-length pH-sensitive GFP (pHluorin)-tagged GluA2 (pH-GluA2), either wild-type or those C-terminal lysine to arginine mutants have been described previously (Widagdo et al. 2015). Additional pH-GluA2 Ser-880 phospho-mutants (S880A and S880E), as well as the PDZ (postsynaptic density 95/disc large/zona occludens) binding motif mutant (I883E), were generated using the standard polymerase chain reaction (PCR) protocol. Briefly, mutations were introduced in reverse primers containing the HindIII restriction site as shown in Table 1. DNA fragments containing mutated sequences were amplified with those reverse primers and a common forward primer encompassing a unique BstEII restriction site using the Phusion DNA polymerase kit (New England Biolabs) according to manufacturer's protocol. The resulting PCR products were purified using the Qiagen PCR purification kit and subjected to restriction digests before ligated into the unique BstEII and HindIII sites within the pCAG-pH-GluA2 construct (Widagdo et al. 2015).

Antibodies

Specific antibodies against GluA2 (6A, 1:1000) and GFP (JH4030, 1:1000) were produced in-house and have been characterised previously (Widagdo et al. 2015). The following antibodies were purchased from commercial sources: anti-ubiquitin clone P4D1 (Santa Cruz Biotechnology, 1:1000), anti-ubiquitin clone FK2 (Enzo Lifesciences, for IP only), anti-GluA1 clone RH95 (Merck Millipore, 1:1000), anti-GluA1 phospho-Ser-831 (Merck Millipore, AB5847, 1:2500) and anti-GluA2 phospho-Ser-880 clone 022.19.5 (Millipore, 1:1000).

Neuronal Culture and Electroporation

Primary cortical neurons were prepared from E18 rat pups as described previously (Widagdo et al. 2016; Tan et al. 2017). They were plated onto poly-L-lysine-coated dishes in Neurobasal growth medium supplemented with 2% B-27, 2 mM Glutamax, 50 U/ml penicillin, 50 μ g/ml streptomycin and 5% foetal bovine serum (FBS, HyClone, SH30084.03). Neuronal culture media were switched to 1% FBS Neurobasal medium 24 h post-seeding and fed twice a week. Cortical neurons were electroporated at DIV 0 prior to plating using the Amaxa Nucleofector II system (Lonza). Approximately 5 million neurons were put into a cuvette and mixed with 5 μ g of DNA in 100 μ l of Nucleofector solution for

rat neurons (VPG-1003), followed by brief electrical pulses using the pre-set programme G-013 according to manufacturer's protocol. Neurons were plated onto two 6 cm poly-L-lysinecoated dishes, maintained as described above and used at DIV 13 (Widagdo et al. 2015; Guntupalli et al. 2017). Using this protocol, we typically achieve approximately 40% transfection efficiency in primary cortical neurons.

Ubiquitination Assay

The ubiquitination of AMPARs was induced by incubating neurons in artificial cerebrospinal fluid (ACSF; in mM, 25 HEPES, 120 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 30 D-glucose, pH 7.4) containing 40 μ M bicuculline (Abcam) or 1 μ M PMA (phorbol 12-myristate 13-acetate, Sigma) for 10 min at 37°C. In some experiments, neurons were pre-incubated with 1 μ M PMA for 10 min prior to bicuculline treatment. For experiments involving the PKC blocker, neurons were pre-treated with 1 μ M Go-6983 (Tocris) or DMSO (vehicle control) for 10 min prior to and present throughout the bicuculline or PMA/bicuculline treatments. Neurons were then lysed in warm 1% SDS (in PBS) and diluted in 10 volumes of ice-cold cell lysis buffer (1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na-pyrophosphate in PBS) supplemented with 10 mM NEM (*N*-ethylmaleimide, Sigma) and Complete EDTA-free protease inhibitor cocktails (Roche). Lysates were centrifuged at 14,000 rpm for 20 min at 4°C and cleared with protein A- or G-Sepharose beads. Pre-cleared lysates were then incubated with antibodies (anti-ubiquitin or anti-GFP) coupled to protein A- or G-Sepharose overnight at 4 °C, followed by four washes with ice-cold lysis buffer and elution in 2× SDS sample buffer. The immunoprecipitated proteins were resolved by SDS-PAGE and probed by western blot analysis with specific antibodies against GluA1, GluA2 and ubiquitin.

Phosphorylation Assay

Phosphorylation of pH-GluA2, either wild-type or mutants, was induced by incubating neurons in neurobasal media containing 1 μ M of PMA for 10 min at 37°C. Neurons were lysed immediately after the PMA treatment with 1× SDS and boiled for 10 min. Proteins were resolved by SDS-PAGE and probed by western blot analysis with specific antibodies against phospho-Ser-880 and GluA2.

Data Analysis

Western blots were detected by enhanced chemiluminescent method and were imaged using X-ray films or on an Odyssey digital imaging system (LI-COR). Quantitative analyses of immunoblots were performed on Image J (NIH) or Image Studio (LI-COR) software. All data sets were subjected to one-way ANOVA statistical test followed by Dunnett's or Tukey's multiple comparison post hoc analyses calculated using Prism7 (GraphPad). Results were presented as mean $\pm$ standard error of the mean (SEM).

Results

Bicuculline-Induced GluA2 Ubiquitination Occurs in the C-Terminal Tail

There are six lysine residues in the GluA2 C-terminal region (Fig. 1a). We have previously mapped the sites of GluA2 ubiquitination induced by AMPA on two distal lysine residues,

Lys-870 and Lys-882, in the C-terminal tails (Widagdo et al. 2015). To determine whether bicuculline-induced ubiquitination targets the same or different lysine residues in GluA2, we electroporated pH-GluA2, either wild-type or GluA2 lysine mutants (single or in combination, Fig. 1a), into primary cortical neurons at DIV 0 and performed ubiquitination assays at DIV 13. Neurons were treated with 40 μ M bicuculline for 10 min, lysed (in denaturing condition to completely dissociate AMPAR subunit complex) and immunoprecipitated with anti-GFP antibodies. We revealed that mutation of all C-terminal lysine residues lysine into arginine completely abolished bicuculline-induced ubiquitination of GluA2 (K838/844/847/850/870/882R, 10.8% $\pm$ 4.7% of wild-type (WT), Fig. 1b, c). This demonstrates that bicuculline-induced ubiquitination of GluA2 occurs solely in the C-terminal domain. Systematic analysis of individual single lysine mutation of GluA2 showed that Lys-870 (55.8% $\pm$ 10.1% of WT) and Lys-882 (49.5% $\pm$ 1.4% of WT) were the major ubiquitination sites (Fig. 1b, c). An additive effect was observed for the K870/882R double mutant in reducing bicuculline-induced ubiquitination of GluA2 (19.7% $\pm$ 5.0% of WT, Fig. 1b, c), indicating that both AMPA and bicuculline treatments lead to ubiquitination of GluA2 on the same sites. These data suggest that they are likely to result in similar functional outcomes in regulating the trafficking of GluA2-containing AMPARs.

The Cross-Talk Between GluA2 Phosphorylation and Ubiquitination

PKC-dependent phosphorylation of GluA2 at Ser-880 is well characterised and essential for AMPAR endocytosis and LTD (Chung et al. 2000, 2003; Seidenman et al. 2003; Steinberg et al. 2006). Our previous work has demonstrated an antagonistic interaction between GluA1 ubiquitination and PKA-dependent phosphorylation at Ser-845 in neurons (Guntupalli et al. 2017). We proposed that an interaction between GluA2 phosphorylation and ubiquitination must also exist. To address this hypothesis, we examined the effect of GluA2 ubiquitin-defective mutants, namely K870R, K882R and K870/882R (dKR), on Ser-880 phosphorylation induced by PMA, a potent activator of PKC, in cultured cortical neurons. We found that neurons that overexpressed pH-GluA2 K870R mutant displayed significantly enhanced level of Ser-880 phosphorylation (GluA2 K870R, 153.1% $\pm$ 8.3% of WT-PMA, Fig. 2a, b). This suggests that GluA2 ubiquitination at Lys-870 negatively regulates Ser-880 phosphorylation. However, GluA2-K882R and dKR mutants had markedly reduced Ser-880 phosphorylation (K882R, 35.0% $\pm$ 3.4% of WT-PMA; dKR, 44.4% $\pm$ 13.3% of WT-PMA, Fig. 2a, b). These data indicate that ubiquitination of GluA2 at Lys-882 might play a dominant role over Lys-870 to synergistically promote GluA2 phosphorylation at Ser-880.

We next tested the effect of manipulating GluA2 Ser-880 phosphorylation site on bicuculline-induced ubiquitination in neurons. We electroporated cortical neurons with pH-GluA2, either WT (SVKI), Ser-880 phospho-deficient (AVKI) or phospho-mimetic (EVKI) mutants at DIV0. At DIV13, primary neurons were treated with bicuculline, lysed in denaturing condition and immunoprecipitated with anti-GFP antibodies. We observed a significant increase in the level of GluA2 ubiquitination in the EVKI phosphomimetic, but not in the AVKI phospho-deficient mutants (GluA2 EVKI, 195% $\pm$ 32.1% of WT; GluA2 AVKI, 129.3% $\pm$ 19.6% of WT, Fig. 3a, b). This suggests that Ser-880 phosphorylation is likely to positively regulate GluA2 ubiquitination, presumably due to its effect in promoting

GluA2 internalisation and/or intracellular retention (Chung et al. 2000; Seidenman et al. 2003; Lin and Huganir 2007).

GluA2 contains a type II PDZ binding motif at the extreme C-terminal tail, which mediates interactions with two PDZ domain-containing proteins, glutamate receptor-interacting protein 1 (GRIP1) and protein interacting with C-kinase 1 (PICK1) (Dong et al. 1997; Xia et al. 1999). Phosphorylation of Ser-880 disrupts the interaction of GluA2 with GRIP1, which stabilises GluA2-containing receptors at synapses (Chung et al. 2000). The same phosphorylation also promotes GluA2 binding to PICK1 (Chung et al. 2000), which facilitates the endocytosis and intracellular retention of AMPARs (Lin and Huganir 2007; Widagdo et al. 2016; Rocca et al. 2008; Fiuza et al. 2017; Madsen et al. 2012; Anggono et al. 2013). To determine whether PDZ-dependent protein–protein interaction influences the level of GluA2 ubiquitination upon bicuculline treatment, we electroporated cortical neurons with pH-GluA2 constructs, either WT or PDZ binding motif mutant (SVKE), and performed the same ubiquitination assay. Interestingly, GluA2-SVKE mutant had no effect on bicuculline-induced ubiquitination (GluA2 SVKE, 95.7% $\pm$ 17.2% of WT, Fig. 3a, b). Collectively, these data indicate that while pseudo-phosphorylation of GluA2 at Ser-880 enhances the ubiquitination of GluA2, it does not require GluA2 binding to either PICK1, GRIP1 or any other PDZ-dependent GluA2-interacting proteins.

Phorbol Ester Augments Bicuculline-Induced GluA2 Ubiquitination

PMA potently induces Ser-880 phosphorylation and GluA2 internalisation, and thus decreasing the surface expression of GluA2-containing AMPARs (Chung et al. 2000). We have previously demonstrated that ubiquitination of AMPARs occurs post-endocytosis (Widagdo et al. 2015). We therefore hypothesised that PMA treatment might enhance GluA2 ubiquitination due to increased level of receptor internalisation. To test this, we treated primary cortical neurons with PMA prior to bicuculline stimulation. We showed that activation of PKC-mediated phosphorylation by PMA alone did increase the level of GluA2 ubiquitination, albeit significantly weaker compared to bicuculline-induced ubiquitination (59.7% $\pm$ 9.2% of Bic-GluA2, Fig. 4a, b). Interestingly, PMA pre-treatment robustly augmented the level of bicuculline-induced ubiquitination of GluA2 in primary cortical neurons (210.6% $\pm$ 29.4% of Bic-GluA2, Fig. 4a, b). This phenomenon was specific to the GluA2 subunit, because PMA pre-treatment prior to bicuculline stimulation did not enhance the level of GluA1 ubiquitination (116.4% $\pm$ 23.8% of Bic-GluA1, Fig. 4a, c). These data demonstrate for the first time a differential requirement of PKC-dependent phosphorylation in regulating subunit-specific ubiquitination of AMPARs.

Phorbol Ester-Induced Augmentation Does Not Require GluA2 Ser-880 Phosphorylation

Given that PMA robustly induces GluA2 phosphorylation at Ser-880 and that GluA2 phospho-mimetic S880E mutant exhibits enhanced ubiquitination, we proposed that PK induced phosphorylation of Ser-880 underlies the synergistic effect of phorbol ester and bicuculline. To test our hypothesis, we electroporated pH-GluA2 (either WT or AVKI mutant) on primary cortical neurons and performed the same ubiquitination assays following PMA and bicuculline treatments. As expected, PMA pre-treatment robustly enhanced the level of ubiquitination in neurons expressing pH-GluA2 WT (226.2% $\pm$ 31.7% of Bic-WT,

Fig. 5a, b). Surprisingly, pH-GluA2 AVKI mutant also displayed a similar level of augmentation by PMA ($226.4\% \pm 40.3\%$ of Bic-AVKI, Fig. 5a, b). To ascertain that PMA-induced augmentation of GluA2 ubiquitination following bicuculline treatment was indeed mediated by the activation of PKC, we incubated primary cortical neurons with a broad-spectrum PKC inhibitor Go-6983 for 10 min prior to bicuculline or PMA/bicuculline treatments. Our ubiquitination assays revealed that inhibiting PKC activity significantly dampened the PMA-mediated augmentation of GluA2 ubiquitination (PMA/Bic, $360.6\% \pm 51.3\%$ of Bic; PMA/Bic + Go, $190.1 \pm 51.7\%$ of Bic, Fig. 5c, d). Interestingly, Go-6983 pre-treatment had no significant effect on bicuculline-induced ubiquitination of GluA2 (Bic + Go, $125.6 \pm 32.9\%$ of Bic, Fig. 5c, d).

These data clearly demonstrate that although PKC activity itself is essential, PKC-dependent Ser-880 phosphorylation is not required for the enhancement of bicuculline-induced ubiquitination of GluA2 by phorbol ester in primary cortical neurons.

Discussion

Ubiquitination has started to emerge as one of the post-translational modifications critical for controlling the trafficking, intracellular sorting and degradation of AMPARs in mammalian central neurons (Widagdo et al. 2017; Goo et al. 2015). An acute treatment of primary neuronal cultures with bicuculline results in presynaptic release of glutamate, which subsequently induces robust ubiquitination of all AMPAR subunits (Lussier et al. 2011; Widagdo et al. 2015). We found that bicuculline-induced ubiquitination occurs on the same lysine residues (Lys-870/Lys-882) in the GluA2 C-terminal tail as those following AMPA treatment in primary cortical neurons (Widagdo et al. 2015). Interestingly, the contribution of each lysine residue towards GluA2 ubiquitination is similar regardless of the stimuli. Previous work has demonstrated that AMPA- and bicuculline-induced ubiquitination of all AMPAR subunits is completely blocked by inhibiting the GTPase activity of dynamin, indicating that activity-dependent ubiquitination of AMPARs must occur post receptor internalisation (Lussier et al. 2011; Widagdo et al. 2015). Given that bicuculline-induced ubiquitination of GluA2 occurs post-endocytosis, these data indicate that ubiquitination functions to direct the sorting of internalised GluA2 into late endosomes for degradation (Lussier et al. 2011; Widagdo et al. 2015). However, it remains a possibility that bicuculline may induce the attachment of a different type of ubiquitin chain than the K63-linked ubiquitination elicited by the AMPA treatment (Widagdo et al. 2015), which will have different consequences on AMPAR functions.

Our previous study has demonstrated a functional cross-talk between GluA1 ubiquitination with PKA-dependent phosphorylation at Ser-845 (Guntupalli et al. 2017). In the present study, we sought to determine whether the ubiquitination of GluA2 could also be cross-modulated by the phosphorylation at Ser-880 by PKC, which is known facilitate GluA2 internalisation during LTD (Chung et al. 2000, 2003; Seidenman et al. 2003). We found that PMA, which potently activates PKC and Ser-880 phosphorylation, also induced the ubiquitination of GluA2, albeit slightly weaker compared to those elicited by bicuculline or AMPA stimulations. Individual mutations of Lys-870 and Lys-880 to arginine residues, both of which significantly reduce GluA2 ubiquitination, give rise to an opposite effect on PMA-

induced phosphorylation at Ser-880. While GluA2 K870R promotes the phosphorylation of Ser-880, the GluA2 K882R mutant completely inhibits it. Previous studies have shown that mutation of Lys-882 to alanine abolishes PKC-mediated phosphorylation of Ser-880 (Steinberg et al. 2006; Seidenman et al. 2003) due to a disruption in the PKC consensus recognition motif (S/T-X-K/R) (Kemp and Pearson 1990). Although mutation of Lys-882 into arginine is not expected to affect PKC recognition in GluA2, it is plausible that Lys-882 is part of the epitope recognised by the GluA2 phospho-Ser-880 antibodies used in this study.

To address our original question, we took an alternative approach by investigating the effects of Ser-880 phospho-mutants have on the levels of GluA2 ubiquitination. Our observation that the GluA2 S880E phospho-mimetic mutant significantly enhanced bicuculline-induced ubiquitination led us to hypothesise that phosphorylation of Ser-880 synergistically promotes the ubiquitination of GluA2 in neurons. Indeed, a brief pre-treatment with PMA prior to bicuculline stimulation resulted in a robust augmentation of GluA2 ubiquitination. Importantly, the effect of PMA is specific to the GluA2 as it does not modulate the level of GluA1 ubiquitination following bicuculline treatment. These data are largely consistent with our previous finding that demonstrates the lack of effect of GluA1 Ser-831 phospho-deficient or -mimetic mutants on AMPA-induced ubiquitination of GluA1 in primary cortical neurons (Guntupalli et al. 2017). Given that PMA can also induce the phosphorylation of Ser-818 within the GluA1 membrane proximal region (Boehm et al. 2006), it is also likely that Ser-818 phosphorylation may not modulate the ubiquitination of GluA1 in neurons. The differential effects of PMA in modulating the levels of ubiquitination on GluA1 and GluA2 subunits may have implications on the subunit-specific trafficking and the regulation of AMPAR subunit composition in neurons.

In contrast to our initial hypothesis, the effect of PMA was independent of Ser-880 phosphorylation as PMA induced a similar level of augmentation in GluA2 S880A phospho-deficient mutant as in wild-type GluA2. Given the lack of effects of GluA2 S880A phospho-deficient mutant or inhibiting PKC on bicuculline-induced GluA2 ubiquitination, we concluded that pseudo-phosphorylation of GluA2 is sufficient, but is not necessary for bicuculline-induced ubiquitination of the GluA2 subunit of AMPARs.

It is well established that a PDZ-dependent interaction between GluA2 with PDZ domain-containing proteins such as PICK1 and GRIP1/2 is essential for the function of GluA2-containing AMPARs in synaptic plasticity [reviewed in (Anggono and Huganir 2012; Bissen et al. 2019)]. However, a mutation in the GluA2 PDZ binding motif is completely dispensable for bicuculline-induced ubiquitination. One interpretation from this finding is that the binding of GluA2 to its E3 ligase does not involve the C-terminal PDZ binding motif. Even in the case where the interaction between GluA2 and its E3 ligase is indirect, it does not require an intermediate that contains a PDZ domain. Although the lack of binding to PICK1 or GRIP1 does not affect the levels of GluA2 ubiquitination induced by bicuculline, it remains possible that the binding of GluA2 to either PICK1 or GRIP1 may differentially modulate the ubiquitination of GluA2 in neurons.

What could be the mechanism that contributes to the robust augmentation of bicuculline-induced GluA2 ubiquitination by phorbol ester? One potential mechanism could involve the activation of the GluA2 E3 ligase, Ring finger protein 167 (RNF167) by PKC (Lussier et al. 2012) (Fig. 5e). Although it has not been formally tested, PKC is predicted to be a protein kinase that can directly phosphorylate and potentially activate RNF167. Alternatively, PMA may stabilise and target RNF167 to endosomes. A similar mechanism has been observed for RNF13, a close homolog of RNF167, in PMA-treated cells (Bocock et al. 2010). PMA treatment stabilises full-length RNF13 protein by reducing the proteolysis of the N-terminal protease-associated domain, as well as redistributing it to the recycling endosomes. These mechanisms are not mutually exclusive and may occur sequentially or simultaneously. This raises the question whether engaging the PKC signalling pathway leads to subsequent phosphorylation and activation of RNF167, which warrants thorough investigations in the future.

In conclusions, the present study provided the first demonstration of a subunit-specific modulation of GluA2 ubiquitination by the PKC signalling pathway. We envisage that the binding of glutamate on AMPARs in neurons with high level of PKC activity (such as following the activation of mGluRs) will result in subunit-specific regulation of AMPAR ubiquitination and intracellular sorting, which ultimately govern the subunit composition and number of AMPARs, including the Ca²⁺-permeable AMPARs at synapses. Given the potential of “AMPA code” permutations in creating a rich repertoire of synapses with different AMPAR functionality and synaptic weights, future studies should focus on the role of GluA2 ubiquitination in synaptic plasticity, particularly hippocampal and cerebellar LTD.

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Abbreviations

AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA	AMPA receptors
DIV	Days in vitro
DMSO	Dimethyl sulfoxide
GRIP1	Glutamate receptor-interacting protein 1
L-VGCCs	L-type voltage-gated calcium channels
LTD	Long-term depression
mGluRs	Metabotropic glutamate receptors
NEM	<i>N</i> -Ethylmaleimide

NMDA	<i>N</i> -Methyl-D-aspartate
NMDARs	NMDA receptors
PDZ	Postsynaptic density 95/disc large/zona occludens-1
PICK1	Protein interacting with C-kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RNF	Ring finger protein
WT	Wild-type

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Fig. 1.

Bicuculline induces ubiquitination of GluA2 at Lys-870 and Lys-882 in the C-terminal tail. **a** Amino acid sequence of the GluA2 C-terminal tail, with all lysine residues highlighted in red. Ser-880 is highlighted in green. Boxed sequence is the PDZ binding motif. **b** Primary cortical neurons were electroporated with pH-GluA2, either wild-type, individual K/R mutant constructs (K838R, K844R, K847R, K850R, K870R, K882R), the double mutant (dKR, K870/882R) or the combination of all six of them, prior to plating. At DIV13, neurons were treated with bicuculline for 10 min at 37 °C, lysed in denaturing condition and immunoprecipitated with anti-GFP antibodies. Eluted proteins and total lysates were then subjected to western blot and probed with anti-ubiquitin and anti-GluA2 antibodies. **c** Quantification of ubiquitination levels after normalising against the amount of immunoprecipitated pH-GluA2. Data represent mean $\pm$ SEM of band intensities normalised to pH-GluA2 WT (One-way ANOVA; * $P < 0.05$; *** $P < 0.001$; $n = 3$ to 6 per group)

Fig. 2.

Effects of GluA2 ubiquitin-deficient mutants on phorbol ester-induced phosphorylation at Ser-880. **a** Primary cortical neurons were electroporated with pH-GluA2 plasmids, either wild-type (WT), K870R, K882R or dKR (K870/882R) prior to plating. At DIV14, neurons were treated with PMA for 10 min at 37 °C and immediately lysed with 1× SDS sample buffer. Lysates were resolved by SDS-PAGE and subjected to western blot with specific antibodies against GluA2 phospho-Ser-880 and GluA2 antibodies. **b** The effect of lysine/arginine mutants on Ser-880 phosphorylation was quantified after normalising against total GluA2 levels. Data represent mean ± SEM of band intensities normalised to PMA-treated pH-GluA2 WT (One-way ANOVA; * $P < 0.05$; *** $P < 0.001$; $n = 5$ per group)

Fig. 3.

GluA2 Ser-880 phospho-mimetic mutation enhances bicuculline-induced ubiquitination. **a** Cortical neurons were electroporated with pH-GluA2, either WT (SVKI), S880A (AVKI), S880E (EVKI) and I883E (SVKE) prior to plating. At DIV12, neurons were treated with bicuculline for 10 min at 37 °C, lysed with 1% SDS and immunoprecipitated with anti-GFP antibodies. Eluted proteins and total lysate were then subjected to western blot and probed with anti-ubiquitin and GluA2 antibodies. **b** Quantification of ubiquitination levels after normalising against the amount of immunoprecipitated pH-GluA2. Data represent mean $\pm$ SEM of band intensities normalised to pH-GluA2 WT (One-way ANOVA; ** $P < 0.01$; $n = 5$ to 7 per group)

Fig. 4.

Phorbol ester augments bicuculline-induced ubiquitination of GluA2. **a** DIV 14 primary cortical neurons were treated with either ACSF, bicuculline, PMA or pre-treated with PMA prior to incubation with bicuculline. Neurons were lysed with 1% SDS and immunoprecipitated with anti-ubiquitin antibodies. Eluted proteins and total lysate were then subjected to western blot and probed with anti-GluA2, GluA2 phospho-Ser-880, GluA1 and GluA1 phospho-Ser-831 antibodies. The effects of these pharmacological treatments on GluA1 (**b**) and GluA2 (**c**) ubiquitination were quantified after normalising against total GluA1 or GluA2. Data represent mean $\pm$ SEM of band intensities normalised to WT-Bic groups (One-way ANOVA; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $n = 9$ per group for GluA1; $n = 11$ per group for GluA2)

Fig. 5.

Phorbol ester-induced augmentation of GluA2 ubiquitination does not require Ser-880 phosphorylation. **a** Cortical neurons were electroporated with pH-GluA2, either WT (SVKI) or S880A (AVKI) prior to plating. At DIV12, neurons were treated with either ACSF, bicuculline, PMA or pre-treated with PMA for 10 min prior to incubation with bicuculline for a further 10 min. Neurons were lysed with 1% SDS and immunoprecipitated with anti-GFP antibodies. Eluted proteins and total lysates were then subjected to western blot and probed with anti-ubiquitin, GluA2 phospho-Ser-880 and GluA2 antibodies. **b** Quantification of ubiquitination levels after normalising against the amount of immunoprecipitated pH-GluA2. Data represent mean $\pm$ SEM of band intensities normalised to bicuculline-treated groups (One-way ANOVA; $*P < 0.05$; $n = 7$ per group). **c** DIV 14 primary cortical neurons were incubated with the PKC blocker Go-6983 (Go) or DMSO control (–) for 10 min prior to bicuculline or PMA/bicuculline treatments. Neurons were lysed with 1% SDS and immunoprecipitated with anti-ubiquitin antibodies. Eluted proteins and total lysate were then subjected to western blot and probed with anti-ubiquitin, anti-GluA2 and GluA2 phospho-Ser-880. **d** Quantification of ubiquitination levels after normalising against the amount of immunoprecipitated GluA2. Data represent mean $\pm$ SEM of band intensities normalised to

bicuculline-vehicle-only group (One-way ANOVA; * $P < 0.05$, ** $P < 0.01$; $n = 3$ per group).

e A schematic diagram of the interaction between GluA2 ubiquitination and phosphorylation. Although pseudo-phosphorylation of Ser-880 is sufficient to enhance the level of GluA2 ubiquitination induced by bicuculline (dashed blue arrows), it is not necessary for the PMA-mediated augmentation. We propose that PKC-dependent phosphorylation and activation of a GluA2 E3 ligase (such as RNF167) as a potential mechanism underlying the PMA effect in augmenting the level of GluA2 ubiquitination (magenta arrows)

Table 1

Primers used to generate pH-GluA2 mutants

Primer name	Oligonucleotide sequence (5'–3')
Forward primer	CTGTGGTGGTCACCCACATATTGG
S880A reverse primer	GATAAGCTTCTAAATTTAACACAGCCTCGATGCCATATAC
S880E reverse primer	GATAAGCTTCTAAATTTTAACCTCTCGATGCCATATAC
I883E reverse primer	GATAAGCTTCTACTCTTTAACACTCTCGATGCC

Unique restriction site sequences within primers are underlined. Mutated sequences are indicated in bold and italic