Co-stimulation of mGluR5 and NMDA receptors is required for potentiation of excitatory synaptic transmission in hippocampal neurons

Short Title: Co-incident stimulation of mGluR5 and NMDA receptors

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Summary

In the central nervous system, excitatory synaptic transmission is mediated by the neurotransmitter glutamate and its receptors. Interestingly, stimulation of group I metabotropic glutamate receptors (mGluR) can either enhance or depress synaptic transmission at CA1 hippocampal synapses. Here we report that co-activation of mGluR5, a member of the group I mGluR family, and N-methyl-D-aspartate receptors (NMDARs) potentiates NMDAR currents and induces a long-lasting enhancement of excitatory synaptic transmission in primary cultured hippocampal neurons. Unexpectedly, activation of mGluR5 alone fails to enhance evoked-NMDAR currents and synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR) AMPAR currents. The observed potentiation requires an mGluR5-induced, inositol 1,4,5-trisphosphate receptor (IP₃)-mediated mobilization of intracellular Ca²⁺ which acts in concert with a protein kinase C (PKC), calcium-activated tyrosine kinase cascade to induce a long lasting enhancement of NMDAR and AMPAR currents.
The transmitter glutamate, together with its postsynaptic receptors, mediates much of the excitatory neurotransmission in the central nervous system. Of these postsynaptic receptors, the NMDAR is an ionotropic receptor implicated in neuronal development (1), synaptic plasticity (2) and excitotoxicity (3). In the pyramidal cell synapses of the CA1 region of the hippocampus, the NMDAR is composed of at least one NR1 subunit as well as multiple NR2B or NR2A subunits (4). These postsynaptic NMDARs are associated with a complex of proteins (the "NMDAR complex"), which includes the scaffolding proteins PSD-95, Homer, Shank and GKAP as well as a number of serine/threonine and tyrosine kinases and phosphatases (5). Furthermore, other receptors including mGluR and IP$_3$R are potentially linked to this complex (6). The presence and activity of many of these proteins is integral for LTP and LTD, which are the leading cellular and molecular models for learning and memory (7).

Glutamate also activates postsynaptic mGluRs, which are coupled via G-protein activation to intracellular signaling cascades. Eight mGluRs have been cloned and they are classified into three groups (I, II and III) based upon sequence homology, similarities in signal transduction cascades and pharmacological profiles (8). The group I metabotropic glutamate receptor, mGluR5, is positively coupled to PLC$\beta$ activity (9), PKC and mobilization of intracellular calcium via IP$_3$Rs. Of the group I mGluRs, mGluR5 expression in the CA1 hippocampus has been found to be localized to extrasynaptic and perisynaptic sites (10;11) of CA1 pyramidal neurons while mGluR1 is not highly expressed in CA1 pyramidal neurons and is more predominantly expressed in interneurons (12). Recent evidence demonstrates that mGluR1 and mGluR5 play separate functional roles, via activation of distinct intracellular signaling pathways in CA1 pyramidal neurons (13).
Group I mGluRs can either enhance or depress excitatory synapses (14). The mechanisms by which group I mGluRs act to modulate synaptic performance are not entirely clear but post-translational modifications (15) or increases or decreases in the number of ionotropic glutamate receptors located at excitatory synapses can contribute to either LTP or LTD, respectively (16). At many hippocampal synapses low frequency afferent stimulation induces LTD but brief high frequency stimulation leads to LTP even though both forms of synaptic plasticity require an influx of postsynaptic calcium via NMDARs. In hippocampal slices (17-19) and cultures (14;20-22) bath applications of either NMDA or of a group I mGluR agonist induce LTD, but not LTP, and enhance AMPA receptor endocytosis (14;23-25). However, in cultures selective stimulation of synaptic but not extrasynaptic NMDARs induces LTP and not LTD (22;26-28). The role of mGluRs in the induction of LTP is highly controversial and the exact role of group I mGluRs in modulating NMDARs and LTP is unclear (29;30). In the present study we set out to delineate the mechanism by which group I mGluRs modulate NMDA channel activity.
RESULTS

We first assessed the role of mGluR5 in the regulation of excitatory synaptic transmission in primary hippocampal cultures. Applications of CHPG (100 µM), a selective mGluR5 agonist, but relative insensitive for mGluR1 (31), enhanced miniature excitatory postsynaptic current mediated by NMDARs (mEPSC\textsubscript{NMDA}) but not the AMPA receptor component (mEPSC\textsubscript{AMPA}). The concentration of CHPG employed was chosen to match that used recently to study mGluR5 mediated responses in cultured retinal amacrine neurons (32). The CHPG-induced enhancement of mEPSC\textsubscript{NMDA} was attenuated by co-application of the selective mGluR5 antagonist MPEP. The concentration of MPEP was just maximal (10 µM, Fig. 1a) for antagonism of mGluR5 with little or no effect on mGluR1 (33). In these recordings, and as previously described intracellular Ca\textsuperscript{2+} was sufficiently buffered to prevent the secondary potentiation of mEPSC\textsubscript{AMPA} without preventing the Ca\textsuperscript{2+}-dependent modulation of NMDARs (34;35).

In acutely isolated CA1 pyramidal neurons, taken from young rat hippocampi, peak NMDAR-mediated whole-cell currents (I\textsubscript{p}) recorded in response to rapid applications of NMDA (Fig. 1b) were similarly enhanced by CHPG. This enhancement was similar whether 100 or 500 µM CHPG was applied. This enhancement of peak currents was also antagonized by MPEP (Fig. 1c\textsubscript{1,c2}). We further demonstrated the absence of a CHPG-induced modulation of NMDAR-mediated currents in CA1 neurons taken from mGluR5 knockout mice even though cells from wild type littermates demonstrated the anticipated enhancement (Figs. 1e\textsubscript{1,e2}). The low yield of mGluR5 knockout mice prevented our evaluation of changes in excitatory synaptic transmission using primary hippocampal cultures that lacked mGluR5 expression.
Note that during the period of simultaneous co-applications of CHPG a rapid but reversible depression of NMDA-evoked currents was observed (e.g. Fig. 1c,e2,f). This transient depression was not blocked by MPEP and it was also seen in cells taken from mGluR5 knockout mice providing evidence that it is due to a direct interaction with NMDARs rather than through mGluR5 signaling. Such a direct effect of mGluR reagents has been previously reported (36-38) and reflects in part an interaction of these agents with the glycine binding site of the NMDA receptor(38). Indeed, at the concentrations of NMDA and glycine used by us, CHPG inhibited recombinant NR1Stop838a/NR2a currents in HEK293 cells by $60.9 \pm 2.0\%$ at $100 \mu\text{M}$ and $98.5 \pm 0.8\%$ at $1 \text{mM}$ ($n = 4$, Fig. 1d1,d2). For this reason the CPHG was not included in the NMDA barrel during perfusion of isolated cells. Thus, the limited depression of NMDA-induced currents simply reflected the rapid recovery of NMDA responses following the washout of CHPG.

One should note that the CHPG-induced potentiation occurs long after (as much as 30 minutes) the application of CHPG whilst the inhibition recovers in less than one second. NMDAR-mediated currents reversed at approximately $0 \text{mV}$ before and after application of all agents demonstrating that there was no change in driving force associated with their use (data not shown).

In order to permit intracellular $\text{Ca}^{2+}$ to vary in the absence of any applied exogenous $\text{Ca}^{2+}$ buffers we also made a series of recordings from isolated CA1 neurons using the perforated patch technique. Under these conditions, and with sub-saturating concentrations of agonists ($10 \mu\text{M} \text{NMDA}, 500 \text{nM glycine}$), peak currents ($I_p$) also demonstrated a time-dependent enhancement following application of $1\text{S},3\text{R}-\text{ACPD}$ ($10 \mu\text{M}; \text{t-ACPD}$) but not following application of it’s inactive analogue, $1\text{R},3\text{S}-\text{ACPD}$ ($10 \mu\text{M}; \text{t-ACPD}$).
µM) or the mGluR5 selective agonist CHPG (not shown). In contrast when near saturating concentrations of agonists (300 µM NMDA, 3 µM glycine) were employed, a long-lasting depression of steady-state currents (I_{ss}) was also revealed following application of CHPG (Fig. 1f). In all subsequent experiments 100 µM CHPG was used to activate mGluR5 responses in isolated CA1 neurons.

These results demonstrate that activation of mGluR5 can selectively enhance mEPSC_{NMDA} and enhance peak currents as well as depress steady-state NMDA-evoked currents accentuating the apparent desensitization of evoked currents. The mGluR5-induced enhancement of NMDAR desensitization (e.g., reduced I_{ss}/I_p) was directly dependent upon the concentration of extracellular glycine (data not shown) and is consistent with enhanced calcium-dependent inactivation of NMDAR-mediated currents (39). However, these results do not exclude the possibility that other forms of receptor desensitization. For instance, it is possible that there might be a change in the number and/or function of NMDAR subtypes.

Many GPCRs activate the mitogen-activated protein kinase (MAPK) cascade through the transactivation of receptor tyrosine kinases (RTKs), such as epidermal growth factor receptors (EGFRs) and platelet-derived growth factor receptors (PDGFRs) (40). GPCR-mediated transactivation of RTKs has been well documented in heterologous cell systems and recently we demonstrated that in CA1 pyramidal neurons, D2/D4 dopamine receptors (GPCRs) transactivate PDGFRs to depress NMDA mediated synaptic transmission (41). Moreover, in glial cells mGluR5-induced activation of MAPK is dependent upon EGFR activity (42). However, inclusion of the PDGFR inhibitor tyrphostin A9 (2 µM) in the recording electrode solution, failed to block the CHPG-induced
potentiation of NMDAR-mediated peak currents in isolated CA1 pyramidal neurons. In addition, applications of either EGF (10 ng/ml) failed to modulate NMDA-evoked currents in isolated CA1 pyramidal neurons (data not shown). Therefore, mGluR5 is unlikely to modulate NMDA responses via transactivation of these growth factor receptors.

The simultaneous enhancement of \(I_p\) and depression of \(I_{ss}\) in pyramidal neurons is reminiscent of what is seen following activation of protein kinase C (PKC) (43). The enhancement results from stimulation of the calcium-activated kinase \(\beta\) (CAK\(\beta\)) or proline rich kinase 2 (Pyk2)/Src cascade (44) while the depression results from a PKC-dependent facilitation of calcium-dependent inactivation (39). The CHPG-induced potentiation was also mediated via activation of PKC as the response was blocked by the selective inhibitor, chelerythrine (45) (10 \(\mu\)M, Fig. 2a). In separate experiments applications of 4\(\beta\)-phorbol 12-myristate 13-acetate (4\(\beta\)-PMA) (100 nM) potentiated \(I_p\) as anticipated but also occluded the mGluR5-induced potentiation (Fig. 2a) while an inactive phorbol ester, 4\(\alpha\)-PMA (100 nM), did neither (\(\alpha\)-PMA: 95 \(\pm\) 5%, \(n=8\); \(\alpha\)PMA + CHPG: 133 \(\pm\) 12%, \(n=8\), \(p<0.001\)). The serine/threonine phosphatases, PP1 and PP2A depress NMDA-evoked currents (46) and appropriate inhibitors enhance and facilitate the PKC-mediated potentiation of \(I_p\) (43). We therefore examined the ability of a phosphatase inhibitor to modulate the mGluR5-induced potentiation. Applications okadaic acid (10 nM) slightly enhanced \(I_p\) but substantially accentuated the potentiation induced by CHPG (OK acid: 112 \(\pm\) 10%, \(n=5\), OK acid + CHPG: 168 \(\pm\) 12%, \(p<0.001\), CHPG: 142 \(\pm\) 8%, data taken at 25 minutes after application of drug, data not illustrated).

There is strong evidence that PKC can activate the non-receptor tyrosine kinase CAK\(\beta\) and this kinase is highly expressed as an unspliced isoform in hippocampal tissue.
CAKβ is also associated with NMDARs (5) (44). We therefore tested the hypothesis that mGluR5 activates PKC and then CAKβ. The CHPG-induced potentiation of I_p was blocked by the functional dominant negative mutant of CAKβ (kinase mutant, CAKβ-K475A, 0.05 μg/ml) (44). Furthermore, recombinant CAKβ (0.05 μg/ml) itself slowly enhanced I_p and subsequently occluded the CHPG-induced potentiation (Fig. 2b). Intracellular applications of CAKβ-K457A also blocked the CHPG enhancement of mEPSC_NMDA (Fig. 2c).

Stimulation of PKC, and/or increases in intracellular calcium, triggers the activation and autophosphorylation of CAKβ on tyrosyl residues, 579/580 and 402. The tyrosine phosphorylation of residue 402 is especially important as this region creates a SH2 ligand by which CAKβ can relieve the autoinhibition of Src tyrosine kinases (48). In CA1 pyramidal neurons Src is downstream of a CAKβ and both the induction of LTP or applications of phorbol esters increase CAKβ phosphorylation (44). Consistent with these observations intracellular applications of recombinant c-Src (50 U/ml) enhanced I_p and occluded the CHPG effect (Fig. 2). In contrast, heat inactivated c-Src (50 U/ml) did not (Fig. 2d). Furthermore, a selective Src kinase inhibitory peptide, Src_{40-58} (25 μg/ml) (49) blocked the CHPG-induced enhancement whereas its control peptide sSrc_{40-58} (a scrambled peptide, 25 μg/ml) failed to do so (Fig. 2e).

To further demonstrate the activation of CAKβ and Src by mGluR5, we performed in vitro phosphorylation assays from isolated CA1 region tissue. CA1 slices were either untreated or was exposed to CHPG or CHPG plus MPEP prior to isolation. Both CAKβ and Src were immunopurified using anti-CAKβ and anti-Src antibodies, respectively. We then probed phosphorylation of CAKβ and Src using tyrosine
phosphorylation-specific antibodies. CHPG, but not CHPG plus MPEP treatment, enhanced tyrosine phosphorylation indicating mGluR5 stimulation activates CAKβ in the CA1 region (44) (Fig. 2f). Similarly CHPG treatment enhanced the phosphorylation of immunoprecipitated Src (Fig. 2f).

Our results now demonstrate, that similar to muscarinic receptors (34;44), mGluR5 in CA1 neurons stimulates a PKC/CAKβ/Src cascade to enhance peak NMDA-evoked currents. Activation of this cascade may or may not require a concomitant rise of intracellular Ca^{2+} (47;48). We therefore tested whether the modulation by CHPG depended upon an elevation in Ca^{2+} by employing high concentrations of the chelator BAPTA (20 mM) in the patch pipettes. With this enhanced level of Ca^{2+} chelation CHPG failed to enhance NMDAR-mediated currents (Fig. 3c). In our recordings an increase in Ca^{2+} would result from an influx via NMDARs and perhaps through mGluR5-induced mobilization of internal Ca^{2+} stores by inositol triphosphate receptors (IP_{3}Rs). In support of the latter hypothesis inclusion of the selective IP_{3}R inhibitor, xestospongin-C (Xe-C, 2 µM) (50;51) in the pipette also blocked the response to CHPG (Fig. 3c). Furthermore, acute applications of thapsigargin (50 nM), which acts to promote release of intracellular calcium via IP_{3}Rs (52;53), also enhanced I_{p} (Fig. 3a,b). This led us to hypothesize that activation of IP_{3}Rs results in release of intracellular Ca^{2+}, stimulation of the PKC/CAKβ/Src cascade, and an enhancement of NMDARs. In support of this we found that intracellular applications of chelerythrine (10 µM), Src_{40-58} (25 µg/ml) or CAKβ-K475A (0.05 µg/ml) each blocked the thapsigargin-induced potentiation (Fig. 3b). Furthermore, thapsigargin itself occluded the mGluR5-induced potentiation of peak NMDAR-mediated currents (Fig. 3b).
We next considered whether an influx of Ca\(^{2+}\) through NMDARs was also required to activate the cascade. To this end we initially determined the amplitude of I\(_p\) and then CHPG was applied in the absence of NMDA. NMDA was subsequently applied to determine I\(_p\). This experimental protocol differs from that of Figures 1a,b in that there was no NMDA present during stimulation of mGluR5. As shown in Figure 4a, under this condition CHPG failed to enhance I\(_p\). We further investigated the time dependence of this response. To do so we varied the time between the end of the application of CHPG and the first test application of NMDA (50 \(\mu\)M, glycine 500 nM). We found that the minimal time between mGluR5 and NMDAR stimulation required for enhancement of NMDA channel activity was less than 15 seconds. Surprisingly, a time interval of 30 seconds resulted in a depression of evoked NMDA responses (Fig. 4b). Moreover, we found that NMDA channels must be gated to an open state during stimulation of mGluR5 in order for the enhancement to occur. For example, when CHPG and NMDA were co-applied in the presence of the reversible open-channel blockers, ketamine (50 \(\mu\)M) or magnesium (2 mM), no potentiation of peak NMDA-mediated currents was observed once the blockers were removed (Fig. 4c). In contrast, using cells taken from the same slices CHPG potentiated currents when co-applied with NMDA/glycine in the absence of these channel blockers (data not shown). An influx of Ca\(^{2+}\) was required as stimulation of NMDAR and mGluR5 in the presence of nominal extracellular Ca\(^{2+}\) (0.2 mM + 3 \(\mu\)M neomycin) failed to enhance NMDAR currents (Fig. 4d). Applications of neomycin were made to block the calcium-sensing nonselective cation current (54), and as a control we determined that neomycin failed to block the CHPG stimulated potentiation of NMDAR currents (Fig. 4d, inset).
A long-lasting enhancement of mEPSC_{AMPA} can be induced in primary cultures of hippocampal neurons by employing brief applications of glycine that selectively activate synaptically located NMDARs (22). We anticipated that CHPG should induce LTP in these cultures provided we simultaneously activate synaptic NMDARs Therefore, a concentration of glycine (1 μM) subthreshold for inducing LTP itself was added to all solutions (22). Under this condition of enhanced NMDAR activation applications of CHPG induced a profound and long-lasting potentiation of the amplitude of mEPSC_{AMPA} that was prevented by co-application of the mGluR5 inhibitor, MPEP (Fig. 5). Consistent with our finding that mGluR5 and NMDARs must be co-activated we found that applications of CHPG in the absence of NMDAR stimulation (e.g. APV treated cultures) failed to evoke LTP (Fig. 5). There was also a corresponding increase in the frequency of events in response to CHPG application but not during co-applications of CHPG and MPEP or CHPG plus APV (data not shown).
METHODS

Cell isolation, whole-cell and perforated-patch recordings: CA1 neurons were isolated from hippocampal slices from postnatal rats (Wistar 10-20 days) using previously described procedures (55). To control for variation in response, recordings from control and treated cells were made on the same day, unless otherwise indicated. The extracellular solution consisted of (in mM): 140 NaCl, 1.3 CaCl₂, 5 KCl, 25 HEPES, 33 glucose and 0.0005 TTX, with pH 7.4 and osmolarity between 320 and 325 mosmol. Recordings were done at room temperature (20-22°C). After formation of a whole-cell configuration, the recorded neurons were voltage-clamped at -60 mV and lifted into the stream of solution supplied by a computer-controlled, multi-barreled perfusion system. Continuous perfusion was achieved by using a gravity-fed system. This system allows for a delay in the application of two different drugs from as little as <2 msec. To monitor access resistance, a voltage-step of -10 mV was made before each application of NMDA. Recordings where series resistance varied by more than 10% were rejected. The intracellular solution contained (in mM): 140 CsF or CsCl₂, 11 EGTA, 1 CaCl₂, 2 MgCl₂, 10 HEPES, 2 TEA and 2 K₂ATP with pH 7.2 and osmolarity between 295 and 300 mosmol. Several recordings were also performed from HEK 293 cells expressing recombinant NMDARs under identical recording conditions. For these experiments, HEK cells were transiently transfected using a lipid transfer method ((Invitrogen, Carlsbad, CA, USA) with cDNAs encoding for the NMDA receptor subunits NR2a and a C-terminal truncated form of the NR1 subunit (NR1Stop838) previously demonstrated to abolish Ca²⁺-dependent inactivation of NMDARs (56). In perforated patch recordings, the antibiotic amphotericin B (0.5 µg/ml) was included in the patch electrode.
Once the cell attached configuration was established all negative pressure was relieved and ~10 minutes elapsed before electrical access to the cell was achieved. Some drugs were included in the patch pipette. Data are expressed as mean ± S.E.M. and analyzed using a two-way ANOVA.

**Recordings of miniature postsynaptic excitatory currents:** Procedures for the preparation of primary dissociated cultures of hippocampal neurons have been previously described (57). Whole-cell recordings were made from these cultures 12–17 days after plating. Recordings were performed at room temperature (20–22°C). Recordings from each neuron lasted from at least 45 to 75 min. The series resistance in these recordings varied between 6 to 8 MΩ, and recordings where series resistance varied by more than 10% were rejected. No electronic compensation for series resistance was employed. The patch electrode solution contained the following (mM): 140 CsCl, 2.5 EGTA, or 20 BAPTA, 2 MgCl₂, 10 HEPES, 2 TEA, and 4 K₂ATP (pH 7.3); and osmolarity between 300 to 310 mosmol. The extracellular (perfusion or bathing) solution was of the following composition (mM): 140 NaCl, 1.3 CaCl₂, 5 KCl, 25 HEPES, 33 glucose, 0.0005 TTX, 0.001 strychnine, and 0.02 bicuculline methiodide (pH 7.4), and osmolarity between 325 and 335 mosmol. Each cell was continuously superfused (1 ml/min) with this solution from a single barrel of a computer-controlled multi-barreled perfusion system.

mEPSCs were recorded using an Axopatch 1-B amplifier (Axon Instruments, Inc.), and records were filtered at 2 kHz, stored on tape, and subsequently acquired offline with an event detection program (Mini Analysis; Justin Lee). Cells that demonstrated a change in "leak" current of more than 10% (usually less than 10 pA) were rejected from the analysis. The trigger level for detection of events was set approximately
three times higher than the baseline noise. Inspection of the raw data was used to eliminate any false events, and ~300 mEPSCs were averaged for display purposes. The same number of events was used when averaged mEPSCs were compared. The AMPAR component of mEPSC was determined by selecting the area under the event from the start of the event to 8 msec after the start of the event. The NMDAR component was determined by selecting the area under the event from 8 msec onwards. All population data were expressed as mean ± SEM. The Students paired t-test or the ANOVA test (two-way) was employed when appropriate to examine the statistical significance of the differences between groups of data.

**In vitro phosphorylation assays:** CA1 tissue was treated with CHPG (20 min CHPG + 5 min of ECF without CHPG), CHPG + MPEP (25 min MPEP, 20 min CHPG + MPEP), or no treatment (25 min in ECF). Three CA1 regions were pooled together. The tissue was homogenized in ice-cold lysis buffer containing (in mM): 50 Tris-HCl (pH 8.0), 150 NaCl, 2 EDTA, 0.1% SDS, 1% NP-40, 1 Na orthovanadate, protease inhibitors pepstatin A (20 µg/mL), leupeptin (20 µg/mL), and aprotinin (20 µg/mL), and 1 phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation at 14,000 × g for 10 min at 4°C. The protein content of soluble material was determined by BCA protein assay. Soluble protein (500 µg) were incubated overnight with 2 µL of either anti-CAKβ or anti-Src. Immune complexes were isolated by addition of 40 µL of protein G–Sepharose beads, followed by incubation for 1–2 hr at 4°C. Immunoprecipitates were washed several times with SDS lysis buffer. Samples were subjected to 10% SDS-PAGE. Membranes were immunoblotted with a monoclonal antibody to phosphorylated tyrosine (1:1,000 dilution). Signals were detected with
enhanced chemiluminescence (ECL, Amersham) and developed on X-ray film. The membrane was then stripped and reprobed with anti-CAKβ or anti-Src (1:3000 and 1:5000 dilution, respectively). The film images were digitized and imported into Corel Draw for presentation purposes.
DISCUSSION

The objective of the present study was to elucidate the mechanism by which mGluR5 modulates NMDARs and excitatory synaptic transmission. An understanding of the mechanism by which mGluR5 modulates ionotropic glutamate receptors is of particular interest as Group I mGluRs have been reported to both potentiate and, paradoxically depress excitatory synaptic transmission in the CA1 hippocampus (30). We tested the novel hypothesis that co-stimulation of NMDARs and mGluR5, and the timing requirements for the activation of these two receptors, can potentiate or depress excitatory synaptic transmission. We found that co-activation of NMDARs and mGluR5 results in a profound enhancement of peak NMDAR currents and mEPSC_{AMPA}. If, however, mGluR5 was activated in the absence of NMDAR stimulation, the resulting activity of NMDARs and mEPSC_{AMPA} demonstrated a modest depression. Calcium influx via NMDARs was especially important for the mGluR5-induced enhancement of NMDAR currents as blockade of calcium flux (by applications of the NMDA open channel blockers ketamine or magnesium), or by removing extracellular calcium during simultaneous NMDAR and mGluR5 stimulation failed to subsequently enhance NMDAR currents. Finally, recordings from primary cultured hippocampal neurons revealed that mEPSC_{AMPA} were enhanced in response to CHPG application but not when co-applications of CHPG and the NMDAR antagonist APV were made. Collectively these results demonstrate that co-activation of NMDA receptors and mGluR5 is required for the enhancement of NMDAR currents and a long-lasting potentiation of excitatory synaptic transmission in hippocampal cultures.
It is tempting to speculate that co-stimulation of mGluR5 and NMDARs results in potentiation of synaptic transmission in primary hippocampal cultures serves as a model for events that occur during LTP induction \textit{in vivo}. In support of this notion is our finding that co-stimulation of mGluR5 and NMDARs results in an enhancement of miniature excitatory postsynaptic currents mediated by AMPARs that lasts for 45 minutes and in several instances for more than 1 hour. The degree of enhancement is consistent with other culture models of LTP (26;27), however, the interplay of other receptor systems and presynaptic effects, as found \textit{in vivo}, would likely modify the amplitude and kinetics of the response.

To investigate the precise timing requirement of this co-incident activation we varied the interval between the application of CHPG and NMDA/glycine. If these two receptor systems were activated within seconds of one another we noted an enhancement of NMDAR currents, yet longer intervals resulted in a modest depression of peak NMDAR currents. This timing may be consistent with LTP and LTD induction \textit{in vivo}. Given the spatial orientation of NMDARs and mGluR5 (extra-synaptic and peri-synaptic) it is conceivable that there exists a delay in the activation of NMDARs and mGluR5. However, one would expect that NMDARs and mGluR5 would be co-incidently activated during glutamate spillover (i.e., during strong tetanus stimulation), yet a delay in activation of these two receptor systems may occur under circumstances that induce LTD. We anticipate that other receptor or signaling systems in addition to mGluR5 and NMDARs are likely involved in the onset of synaptic plasticity. This would account for our observation that stimulation of mGluR5 alone modestly depresses excitatory synaptic transmission. Indeed, activation of group II and III mGluR receptors on the presynaptic.
membrane are integral for LTD at CA1 synapses (58). It is difficult to evaluate the effects of co-incidence mGluR5 and NMDAR activation in a hippocampal slice preparation given that complete perfusion of drugs into such a preparation would take more than several seconds and there exists direct inhibitory effects of mGluR agonists on NMDA channels.

We have previously shown that Gq-linked GPCRs enhance NMDAR currents and this effect is mediated via a PKC-CAKβ-Src cascade (35;43). Our finding that mGluR5 stimulates the serine/threonine kinase PKC and then non-receptor tyrosine kinase Src to enhance NMDARs and long-term synaptic transmission is entirely consistent with these reports. Given that PKC activates Src kinase via the intermediate tyrosine kinase CAKβ in cell lines (47;59) and in CA1 pyramidal neurons (35), the role of CAKβ was also examined. We determined that pre-applications of CAKβ occluded the mGluR5 response of NMDAR currents and intracellular perfusion of a functional dominant negative, CAKβ-K457A, inhibited the response to CHPG. Moreover, CHPG induced the tyrosine phosphorylation of CAKβ and Src isolated from the CA1 region and these effects were blocked by co-applications with MPEP.

A rise in intracellular Ca²⁺ resulting from either release from intracellular stores or from an influx, as well as via stimulation of PKC, can lead to activation of CAKβ (47). In CA1 neurons inclusion of a strong Ca²⁺ buffer in the patch pipettes prevented the CHPG-induced potentiation of NMDA currents indicating that activation of CAKβ requires both stimulation of PKC and elevated Ca²⁺. It also required mobilization of intracellular calcium, via IP₃Rs as the selective blockade of IP₃Rs blocked the mGluR5 effect. Acute applications of thapsigargin, which promotes calcium release, closely
mimicked activation of mGluR5 and occluded the CHPG-induced potentiation. Moreover, the thapsigargin-induced enhancement of NMDA currents was blocked by inhibitors of PKC, CAKβ and Src. These results collectively suggest that release of calcium from IP3R dependent stores serves as an upstream signal to the activation of the PKC-CAKβ-Src cascade. However, this signal was not sufficient on its own to activate the cascade as an influx of Ca\(^{2+}\) via NMDARs was also required.

The co-incidence of an influx of Ca\(^{2+}\) and its release from intracellular stores under our experimental conditions had to occur on the order of seconds, as longer intervals results in depression of NMDA evoked responses, a finding consistent with observations of a depression of these currents when mGluR5s are stimulated on their own (18;19) or when the influx of Ca\(^{2+}\) via NMDARs is impaired (e.g. cells are depolarized) (14). Our results imply that a threshold concentration of intracellular Ca\(^{2+}\) must be achieved in the vicinity of synaptic NMDARs in order for the activation of the PKC/CAKβ/Src cascade and the resulting potentiation of excitatory synaptic transmission. Alternatively, an influx of Ca\(^{2+}\) through NMDARs may be required to “load” intracellular stores such that upon the subsequent stimulation of IP3Rs (via mGluR5) sufficient Ca\(^{2+}\) is mobilized to activate or facilitate the PKC/CAKβ/Src cascade. Indeed in hippocampal neurons, the Ca\(^{2+}\) influx through NMDARs or voltage-gated Ca\(^{2+}\) channels can act to load intracellular Ca\(^{2+}\) stores (60) and stores are only partially loaded or functionally empty at rest (61;62;62). Another possible explanation is that NMDARs maybe required to ‘sensitize’ mGluR5 sufficiently to permit activation of the PKC/CAKβ/Src cascade. For example, stimulation of NMDARs reverses the desensitization of mGluR5 via a PKC-dependent pathway (63). Also, the scaffolding
protein Homer may retain mGluR5 in an inactive state, as upon dissociation mGluR5 demonstrates constitutive activity (64;65).

However, it is possible that not all conditions may require co-incident activation of mGluR5 and NMDARs to potentiate excitatory synaptic transmission. For instance, strong depolarization of the post-synaptic neuron may allow sufficient calcium entry via NMDARs to trigger the release of calcium via internal stores and initiation of the PKC/CAKβ/Src cascade to enhance NMDAR activity. Under this scheme, the calcium influx via NMDARs may over-ride the necessity of calcium mobilization in response to mGluR5 stimulation (66). One must also consider the possibility that strong stimulation of mGluR5 may initiate the IP3R/PKC/CAKβ/Src cascade independently of NMDAR activity. Such a mechanism seems less likely given that mGluR5 is spatially localized at perisynaptic and extrasynaptic sites and as such their activation would require glutamate spill-over which is likely to activate NMDARs.

The induction of LTP at CA1 synapses requires an elevation of intracellular calcium, most likely through calcium influx via NMDARs, which results in an increase in AMPAR currents (15). This increase in post-synaptic function may be the result of increased AMPAR gating, an increase in AMPARs at the membrane surface or a combination of both (15;67). There is convincing evidence that demonstrates that at least one mechanism for the induction of CA1-LTP requires an upregulation of NMDAR activity (34;35), and NMDAR surface expression (68) and that this amplification is achieved in part through the sequential stimulation of CAKβ and Src (34;35;44).

Administration of catalytically active CAKβ or a peptide activator of Src has been shown to enhance AMPAR activity. However, this enhancement is indirect as it is
abrogated by blocking NMDARs or by buffering intracellular Ca\(^{2+}\) using a relatively slow buffer such as EGTA, (34;35). In contrast, buffering with EGTA fails to block the Src-induced potentiation of NMDA channel activity (34;35). Similarly, the enhancement of NMDA currents in response to activation of both mGluR5 and muscarinic receptors can be observed when EGTA is employed (43;44) but is blocked with the rapid buffer BAPTA. These results suggest that the Ca\(^{2+}\) signal responsible for activation of the PKC-CAK\(\beta\)-Src cascade is more spatially restricted to the vicinity of NMDARs than that required for upregulation of AMPARs.

High frequency stimulation depolarizes CA1 neurons and relieves the voltage-dependent blockade of NMDA channels by Mg\(^{2+}\) which acts to promote Ca\(^{2+}\) entry and induces LTP. This strong stimulation may also enhance glutamate "spill-over" to the perisynaptic sites of metabotropic glutamate receptors. Activation of NMDARs and mGluR5 will then give rise to a Ca\(^{2+}\) and PKC-dependent CAK\(\beta\)/Src cascade which feeds back to further enhance NMDAR responses. This conclusion fits with observations that mGluR5 is highly expressed in CA1 pyramidal neurons at perisynaptic as well as extrasynaptic sites (11;69) (69)and with the demonstration that mGluR5 deficient mice display impaired LTP of NMDAR-mediated transmission (70;71;71).
**Fig. 1.** mGluR5 enhances synaptic NMDAR currents in cultured hippocampal neurons and peak currents in isolated CA1 pyramidal neurons. **a.** CHPG (100 µM) enhanced the mEPSC\textsubscript{NMDA} component but not mEPSC\textsubscript{AMPA}, and this effect was blocked by co-applications of MPEP (10 µM). **Inset.** Current traces illustrating that CHPG enhances mEPSC\textsubscript{NMDA}, but not mEPSC\textsubscript{AMPA}, and this effect is blocked by co-applications of MPEP. **b.** Stability of NMDA-evoked currents in both whole-cell and perforated patch configurations in acutely isolated hippocampal neurons. **c1.** Application of CHPG (100 µM; included in the control barrel for the period indicated by the bar but not in the NMDA-containing barrel) potentiates peak NMDA-evoked currents (I\textsubscript{p}) but not steady-state currents (I\textsubscript{ss}) when subsaturating concentrations of NMDA (30 µM) and glycine (500 nM) were applied and this effect was blocked by co-applications of the mGluR5 antagonist MPEP. **c2.** The CHPG effect was attenuated by co-application of MPEP (CHPG: 135 ± 12%, n=7; CHPG + MPEP: 95 ± 8%, n=8, p<0.001, data obtained at 35 minutes) **d1-2.** CHPG directly blocks NMDA-evoked currents in HEK293 cell expressing recombinant NMDA receptors. The onset and offset of the block was rapid occurring in less than 1 second. **e1-2.** Applications of CHPG failed to enhance I\textsubscript{p} in mGluR5 knockout mice (-/-), but not in control (+/+: 153 ± 11%, n=5; +/-: 97 ± 5%, n=4, p<0.001, data obtained at 30 minutes). **f.** In perforated-patch recordings, CHPG depressed I\textsubscript{ss} with near saturating concentrations of agonist (3 µM glycine, 300 µM NMDA, I\textsubscript{ss}: 69 ± 4%, n=8, p<0.001, I\textsubscript{p}: 112 ± 8%, n=8, data obtained at 40 minutes). The black bar in b, c, d2 and e represent the time and duration of drug application.
Fig. 2. mGluR5-induced enhancement of NMDAR currents is dependent upon PKC, CAKβ and Src. a. The PKC inhibitor chelerythrine (10 µM) was included in the recording electrode solution and was found to block the CHPG induced enhancement, while the active phorobol ester (β-PMA, 100 nM) occluded the CHPG effect (β-PMA: 133 ± 6%, n=7, data obtained at 15 minutes; β-PMA + CHPG: 129 ± 9%, n=7, data obtained at 35 minutes; chelerythrine + CHPG: 94 ± 5%, n=8, p<0.001, data obtained at 35 minutes). b. The functional dominant negative CAKβ-K457A blocked the CHPG-induced enhancement. Inclusion of recombinant CAKβ in the recording electrode solution enhanced NMDAR currents and occluded the CHPG response (CAKβ: 146 ± 13%, n=6, data obtained at 12 minutes; CAKβ + CHPG: 155 ± 18%, n=6, data obtained at 30 minutes; CAKβ-K457A + CHPG: 94 ± 8%, n=7, data obtained at 30 minutes). c. Recordings from primary cultured hippocampal neurons indicate that CHPG enhanced mEPSC_{NMDA} and this effect was attenuated with CAKβ-K457A in the patch electrode solution (CHPG: 21.1 ± 5.4%, n=8; CHPG + CAKβ-K457A: 7.52 ± 4.1%, n=7, p<0.001, data obtained after 25 minutes). In contrast, mEPSC_{AMPA} were not affected in response to CHPG (CHPG: 3.8 ± 1.9%, n=8; CHPG + CAKβ-K457A: 2.4 ± 1.5%, n=7, data obtained after 25 minutes). Inset. Current traces illustrate that CHPG enhances mEPSC_{NMDA}, but not mEPSC_{AMPA}, and this effect is abrogated in the presence of CAKβ-K457A. d. Inclusion of Src in the intracellular solution occluded the CHPG response, whereas inactive Src failed to do so (active Src: 123 ± 4.2%, n=6, data obtained at 10 minutes; active Src + CHPG: 126 ± 3.6%, n=6, data obtained at 35 minutes; inactive Src: 110 ± 6%, n=7; inactive Src + CHPG: 123 ± 6%, n=7, p<0.001). e. The selective Src kinase peptide inhibitor Src40-58 blocked the CHPG response but the scrambled peptide (inactive Src40-58) did not (CHPG + Src40-58: 104 ± 8%, n=6; CHPG + sSrc40-58: 138 ± 8%, n=6, p<0.001, data obtained at 30 minutes). f. In vitro phosphorylation assays reveal that immunopurified CAKβ and Src become tyrosine phosphorylated (immunoblotted with a phosphotyrosine antibody) in response to CHPG but not CHPG plus MPEP. The black bar in a, b, d and e represents the time and duration of CHPG application.
Fig. 3. IP₃Rs stimulate a PKC/CAKβ/Src cascade and are needed for mGluR5-effect. a. Intracellular administration of BAPTA (25 mM) blocked the CHPG effect as did Xe-C (CHPG: 137 ± 8%, n=6; CHPG + BAPTA: 106 ± 6%, n=5, p<0.001; CHPG + Xe-C: 96 ± 5%, n=6, p<0.001, data obtained at 30 minutes). The black bar indicates the time and duration of CHPG application. b. Thapsigargin enhanced Iₚ with sub-saturating concentrations of agonist (500 nM glycine, 10 μM NMDA; Iₚ: 153 ± 12%, n=6, p<0.001; Iₜₘ: 102 ± 4%, n=6, data obtained at 25 minutes of recording) yet depressed Iₜₛ with near saturating concentrations of agonist (3 μM glycine, 300 μM NMDA; peak: 100 ± 5%, n=5; steady-state: 53 ± 6%, n=5, p<0.001, data obtained at 25 minutes of recording). c. The thapsigargin effect was blocked by inhibitors to IP₃Rs, PKC, Src and CAKβ (thapsigargin: 142 ± 8.1%, n=7, thapsigargin + chelerythrine: 109 ± 9%, n=5, p<0.001; thapsigargin + Src₄₀-₅₈: 105 ± 6%, n=8, p<0.001; thapsigargin + CAKβ-K457A: 108 ± 9%, n=6, p<0.001, data obtained at 25 minutes of recording) but not to the inactive Src kinase peptide inhibitor (thapsigargin + sSrc₄₀-₅₈: 139 ± 12%, n=5, data obtained at 25 minutes of recording). Thapsigargin occluded the CHPG effect (thapsigargin: 139 ± 12%, n=6, p<0.001, data obtained at 15 minutes of recording; thapsigargin + CHPG: 144 ± 5%, n=6, data obtained at 25 minutes of recording).
Fig. 4. Co-activation of NMDAR and mGluR5 is required for the mGluR5-induced enhancement of NMDAR currents.  

(a) Application of CHPG in the absence of NMDAR activity failed to subsequently enhance NMDA evoked responses (control: 100%, $t=25$ min: $88 \pm 9\%$, $n=6$, data obtained at 25 minutes of recording). (b) Time intervals less than 15 seconds between stimulation of mGluR5 and NMDARs were required for the mGluR5-induced enhancement of NMDAR currents. All data obtained at 20 minutes of recording. (c) Ketamine (50 µM) and magnesium (2 mM), reversible, open channel NMDA blockers were applied during CHPG in order to block ion flux. During subsequent wash NMDAR currents were not enhanced (for magnesium, $t=25$ min: $89 \pm 7\%$, $n=6$; for Ketamine, $t=35$ min: $85 \pm 12\%$, $n=7$). (d) The influx of extracellular calcium via NMDARs was required as stimulation of NMDARs and mGluR5s with 0.2 mM extracellular calcium (in the presence of neomycin) failed to result in a long-lasting potentiation of NMDA responses (no CHPG: $103 \pm 8\%$, $n=6$; CHPG: $108 \pm 13\%$, $n=6$, data obtained at 20 minutes of recording). **Inset.** Current traces illustrate that neomycin failed to block the CHPG-induced enhancement of NMDAR currents in recordings.
Fig. 5. Co-activation of mGluR5 and NMDARs induces LTP in primary cultured hippocampal neurons. a-b. Administration of CHPG under low intracellular calcium buffering conditions (0.1 mM EGTA/0 mM CaCl₂) potentiates mEPSC<sub>AMPA</sub> (CHPG: 1.38 ± 0.09, n = 14, data obtained at 45 minutes of recording). This effect was blocked by co-applications of MPEP (CHPG + MPEP: 1.09 ± 0.11, n=15, data obtained at 45 minutes of recording) and a modest depression of mEPSC<sub>AMPA</sub> was observed when CHPG was applied in the presence of APV (CHPG + APV: 09.1 ± 0.17, n=11, data obtained at 45 minutes of recording) or magnesium (not shown).

Reference List


Figure 1
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Figure 3
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Figure 5
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Co-stimulation of mGluR5 and NMDA receptors is required for potentiation of excitatory synaptic transmission in hippocampal neurons

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