

Calcium/Calmodulin-Stimulatable Adenylyl cyclases are required for the potentiating effect of acute glucocorticoid exposure in the hippocampal synapse

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Abstract

Experience-dependent synaptic plasticity is important for learning and memory and regulated by the functions of N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors. The stress hormone glucocorticoid, exerts effects on synaptic plasticity through functional modification of these receptors. In this study, we have investigated the mechanisms underlying synaptic potentiation during acute stress and glucocorticoid treatment.

We report that stress-induced glucocorticoid increase augments the postsynaptic localisation and phosphorylation of calcium permeable AMPAR, GluA1 and enhances long-term potentiation (LTP) observed 60 minutes following high frequency stimulation. These effects could be reproduced in adrenalectomized rats by the administration of a stress-equivalent dose of corticosterone. The observed response was blocked by the glucocorticoid receptor (GR) antagonist RU-486, thus potentially mediated by the GR.

Glucocorticoid treatment further increased expression of hippocampal adenylyl cyclases (ACs), AC1 and AC8, implicating this class of enzymes as mediators of hyper-glucocorticoid effect on synaptic plasticity. In support of this, selective pharmacological AC inhibition with SQ22536 ablated the glucocorticoid modulation of AMPA-dependent neuronal plasticity and enhancement of LTP.

These data support a key role for calcium/calmodulin-sensitive adenylyl cyclase enzymes in facilitating the modulatory actions of glucocorticoid hormones on a substrate for cellular mechanism of learning and memory, via AMPA receptor activation.

Introduction

During the learning process, memory formation is encoded by the activation of synapses in brain regions such as the hippocampus. This synaptic process can be modulated by a variety of neuromodulatory factors, including hormones ¹. Experience-dependent synaptic plasticity, a process that is important for learning and memory, is regulated by the actions of NMDA-type receptors and AMPA-type glutamate receptors ²⁻⁴. In synaptic potentiation paradigms, AMPA-type receptors are crucial for the expression of postsynaptic mediated long-term potentiation (LTP) ^{5,6}. Receptor exocytosis, i.e. the activity-dependent exocytosis of calcium permeable AMPA receptors (CP-AMPA; homomeric GluA1-containing AMPA receptors) into synapses, is a key mechanism thought to govern neuro-plasticity processes ⁶.

The glucocorticoid hormones that are released during stress are important modulators of memory function by both genomic and non-genomic actions mediated via the two corticosteroid receptors, the low affinity glucocorticoid receptor (GR) and the high affinity mineralocorticoid receptor (MR) ⁷⁻⁹. Activity-dependent modulation of synaptic transmission by glucocorticoids utilizes multiple mechanisms including CP-AMPA phosphorylation ^{10,11}. We have previously shown that stress-level glucocorticoid exposure modulates synaptic transmission via PKA-dependent phosphorylation of CP-AMPA ¹². This is

a key requirement for receptor externalization into activated synapses, leading to further depolarization of the postsynaptic neuron and hence maintenance of LTP^{10,13}. Additionally, *in vitro* studies provide evidence for glucocorticoid facilitation of AMPAR lateral diffusion across the post-synaptic synapse^{11,13}, another action linked to synaptic potentiation.

Despite the important role for glucocorticoids in regulating synaptic potentiation during periods of elevated hormone exposure, little is known about the signal transduction processes involved in mediating these changes at the synaptic level. For example, it is still unclear which transducer molecule promotes the glucocorticoid-mediated PKA-dependent insertion of CP-AMPARs into the synaptic plasma membrane, ready for its incorporation into the synapse. Such molecule(s) could be specifically targeted for development of therapies aimed at synaptopathologies linked to excessive glucocorticoid exposure.

Membrane bound class III adenylyl cyclases (ACs) include calcium/calmodulin sensitive enzymes expressed in the hippocampal synapse in two forms, type 1 (AC1) and type 8 (AC8)^{14,15}. Both are regulated by calmodulin in response to high intracellular calcium levels^{16,17}, with AC8 exclusively requiring calcium-dependent activation, and AC1 serving as a coincidence detector requiring both calcium influx and activation of associated receptors^{18,19}. These hippocampal calcium-sensitive ACs are potential candidates for glucocorticoid responsiveness as they are well placed to couple the rise in intracellular calcium during synaptic stimulation with our previously reported PKA-mediated activation of AMPARs following an acute glucocorticoid signal²⁰.

Here we provide evidence that these calcium sensitive adenylyl cyclases may not only provide the missing link in the process of enhanced synaptic transmission following a stressful event, but further represent the elusive molecule(s) posited to couple NMDAR-dependent activation to CP-AMPAR post-synaptic expression, a fundamental criterion for maintaining an active state at the neuronal synapse.

Results

Adrenal factors regulate the effects of acute stress on synaptic potentiation

Early phase LTP, which is the molecular correlate of short term/ transient memory formation is thought to be translation-independent, whilst intermediate-phase and late-phase LTP are important for formation of permanent or longer lasting memories and involve synthesis of new proteins^{21,22}. Given the evidence for acute glucocorticoid exposure on synaptic potentiation^{12,23}, it is important to define the temporal expression of synaptic markers indicative of the rapid effects of elevated glucocorticoids on memory.

To address this, adrenal-intact and adrenalectomized (ADX) rats underwent a 30 minute inescapable stress protocol²⁴. Trunk blood and whole hippocampi were collected from these animals at times 30, 60, 120, 240 and 360 minutes after the induction of stress. The control animals in this study did not undergo the restraint stress procedure, thus providing a baseline reference point (time 0) to allow us to assess the temporal dynamics of stress-mediated transcriptional regulation of hippocampal GluA1 and PKA. PKA-

dependent phosphorylation of GluA1 post stress exposure was further examined as a molecular indicator of synaptic potentiation. The plasma corticosterone levels of all rats in this study were assessed by radioimmunoassay (RIA) and analysed by Two-way ANOVA, which revealed a significant effect of time ($p < 0.0001$), a significant effect of ADX ($p < 0.0001$), and a significant interaction of time*ADX ($p < 0.0001$) (figure 1A). ADX rats had extremely low levels of corticosterone at all time points during the restraint protocol, so the RIA minimal detectable concentration (1.7ng/ml) was used for analysis in cases where sample concentration was too low to detect. Adrenal-intact rats exhibited the expected rise in circulating corticosterone by the end of the 30 minute restraint stress protocol (1358.0 ± 260.8 ng/ml, compared to 46.8 ± 29.0 at baseline; $p < 0.001$, and 3.7 ± 1.0 compared to time-matched ADX rats; $p < 0.0001$). Corticosterone levels were still significantly elevated above baseline at 60 minutes (565.6 ± 136.6 ng/ml, compared to 46.8 ± 29.0 at baseline; $p < 0.01$, and 3.8 ± 0.8 compared to time-matched ADX rats; $p < 0.001$) before falling to baseline levels at 120 minutes.

We next assessed the temporal dynamics of Type 1 AMPA receptors (GRIA1) and the catalytic subunit of an isoform of A-type protein kinase (PKACA) transcript expression since in hippocampal neurons these underpin the molecular mechanisms governing synaptic potentiation^{6,25-28}, and activation of the catalytic subunit facilitates the site-specific phosphorylation of Type 1 AMPA receptors²⁹⁻³¹.

The transcriptional dynamics of GRIA1 and PKACA were assessed over the post-stress time course, using RT-qPCR techniques with primers designed to target intronic sites for nascent RNA (hnRNA) and exonic sites for mature RNA (mRNA) on hippocampal cDNA samples prepared from total RNA extracted from the adrenal-intact and ADX rats (figure 1B). In the adrenal-intact rats, GRIA1 hnRNA levels peaked at 120 minutes and mRNA levels peaked at 240 minutes. Both nascent and mature GRIA1 transcript levels remained elevated at 360 minutes. No induction was seen in the ADX rats. Accordingly, two-way ANOVA revealed a significant effect of ADX for both GRIA1 hnRNA ($p < 0.0001$) and mRNA ($p = 0.0127$). A significant effect of time was detected for hnRNA ($p = 0.0457$) but not mRNA ($p = 0.6869$), and no significant interaction was detected between time*ADX for either hnRNA ($p = 0.1134$) or mRNA ($p = 0.0629$). Significant hnRNA induction relative to baseline was seen at 120 minutes ($p = 0.0098$) and 360 minutes ($p < 0.0001$), and relative to ADX at 360mins ($p = 0.009$). Significant mRNA induction relative to baseline was seen at 240 minutes ($p = 0.0259$), and relative to ADX at 240 minutes ($p = 0.0415$) and 360mins ($p = 0.0288$). For PKACA, both nascent and mature transcript induction were similarly delayed until the 360 minute time point (figure 1B). Two-way ANOVA detected a significant effect of time ($p = 0.0074$), a significant effect of ADX ($p < 0.001$), and a significant interaction between time*ADX ($p = 0.0051$) for hnRNA; and a significant effect of ADX ($p = 0.0127$), but no effect of time ($p = 0.6869$) nor interaction between time*ADX ($p = 0.0629$) for mRNA. For both hnRNA and mRNA, significant induction was only detected at the 360-minute time point, relative to baseline (hnRNA $p < 0.0001$, mRNA $p = 0.0409$), and relative to ADX (hnRNA $p < 0.0001$, mRNA $p = 0.0004$).

We also examined the kinetics of the well-characterised glucocorticoid target gene, serum-and-glucocorticoid-inducible kinase (*sgk1*) as a positive control, in view of its status as an immediate early gene^{32,33} and role in glucocorticoid-related actions on synaptic plasticity¹⁰. Indeed, we observed robust

post stress induction of both nascent and mature RNA forms in the adrenal-intact but not ADX animals (Supplemental figure 1) and confirmed by Two-way ANOVA a significant effect of time (hnRNA $p=0.0068$, mRNA $p=0.0127$), of ADX (hnRNA $p<0.0001$, mRNA $p<0.0001$), and a significant interaction between time*ADX (hnRNA $p=0.001$, mRNA $p=0.0019$).

The small but significant changes in GRIA1 and PKACA gene transcription indicate stress responsiveness but do not necessarily indicate functional consequences. Therefore, we also measured GluA1 protein levels and its PKA-dependent post-translational regulation, which is phosphorylation of Ser-845^{27,30}. We did this in adrenal-intact and ADX rats subjected to the 30 minute restraint stress protocol, as well as unstressed controls. Hippocampi were collected at 60 minutes after the onset of stress, as this time-point may be indicative of an intermediate/ translation-dependent plasticity phase³⁴⁻³⁷. Hippocampal synaptic plasma membrane and post-synaptic density extracts were analysed using Western blot with phospho-specific antibodies to examine PKA-dependent phosphorylation of GluA1 at the Serine 845 residue of the receptor (a well-defined marker for GluA1 activation in hippocampal synapses^{29,31}). In the synaptic plasma membrane fraction, we found that PKA-dependent phosphorylation of GluA1 was enhanced in stressed adrenal-intact rats, but not in stressed ADX rats (figure 1 C, D). A significant effect of stress ($p=0.0483$), nonsignificant effect of ADX ($p=0.2285$) and a significant interaction between stress*ADX ($p=0.0381$) were detected by Two-way ANOVA. Post-hoc testing revealed the stress induced increase in pGluA1 in the synaptic plasma membrane fraction was significant ($p=0.0104$), an effect significantly reduced by ADX ($p=0.0274$). There was no significant effect of stress, ADX, or interaction observed for pGluA1 in the PSD, nor for total GluA1 in the SPM or PSD. These data reveal an adrenal stress hormone-dependent phosphorylation of GluA1 receptors localised in synaptic plasma membrane, in response to an acute stress signal at a time point that may arguably be within the non-genomic timeframe.

Modulation of CP-AMPA receptors by glucocorticoids during translation-dependent LTP is non-genomic and facilitated by the type 2 glucocorticoid receptor

Though traditionally thought to act through transcription-dependent mechanisms to regulate synaptic plasticity processes³⁸⁻⁴⁰, it is now known that glucocorticoids can additionally exert rapid, non-genomic effects through actions on membrane-associated, rather than nuclear GRs^{7,41-46}.

To assess whether the stress-dependent effect of pGluA1 could be mediated by adrenal corticosterone, and further differentiate between GR and MR mediated effects, western blot analysis was performed on synaptic fractions generated via differential centrifugation and sequential fractionation of hippocampi from ADX rats treated with a stress dose of corticosterone (3mg/kg) +/- co-treatment with the GR antagonist RU-486 at 20mg/kg. Control animals received subcutaneous injections of vehicle (ethanol diluted in saline). A significant effect of treatment was detected by One-way ANOVA in both synaptic plasma membrane ($p=0.0163$) and post-synaptic density fractions ($p=0.0005$). The small but significant ($p=0.0464$) corticosterone-induced phosphorylation of GluA1 in the synaptic plasma membrane fraction of corticosterone-treated ADX rats, was further enhanced by RU-486 ($p=0.0181$). In striking contrast, the significant corticosterone-induced phosphorylation of GluA1 ($p=0.0021$) was blocked by RU-486

($p=0.0007$) in the post-synaptic density (figure 2 A, B). Significant effects of treatment were also seen for total GluA1 in the synaptic plasma membrane ($p=0.0054$) and post-synaptic density ($p<0.0001$) detected by One-way ANOVA. Similar to results seen with pGluA1, RU486 treatment caused an increase in total GluA1 levels in the synaptic plasma membrane ($p=0.0055$), and a decrease in the post-synaptic density ($p=0.002$). Furthermore, Two-way ANOVA indicated a significant effect of synaptic fraction for both pGluA1 ($p=0.0051$) and total GluA1 ($p<0.0001$), a significant effect of treatment for both pGluA1 ($p=0.0071$) and total GluA1 ($p=0.0035$) and a significant interaction between treatment*fraction for both pGluA1 ($p=0.0009$) and total GluA1 ($p<0.0001$). Taken together these results are consistent with a corticosterone-inducible GR-dependent mechanism, potentially involving lateral diffusion of pGluA1 from the synaptic plasma membrane to the post-synaptic density.

In view of the rapidity of the response to corticosterone, we next assessed whether the corticosterone-induced effects were mediated via membrane-associated GRs rather than cytoplasmic GRs. Abundant levels of activated GR (pSerine211 GR) were detected in the synaptic plasma membrane and post-synaptic density of corticosterone-treated animals (Supplemental figure 2) suggesting the possibility of a non-genomic pathway, so we further tested via *ex vivo* application of modulators of protein translation Cycloheximide (CX) - 50 μ M and gene transcription Actinomycin D (Act D) - 40 μ M to acute hippocampal slices, subsequently treated with 100nM corticosterone for 60 minutes. A significant effect of treatment was detected for pGluA1 in whole cell extracts prepared from the hippocampal slices ($p=0.0068$) (figure 2 C, D). There was a significant decrease in corticosterone-induced GluA1 phosphorylation in the CX pre-treated slices ($p<0.001$) while the reduction of total GluA1 protein levels was not significant. Conversely, Act D had no effect on the corticosterone-inducible pGluA1 or total GluA1 levels (figure 2 C, D).

Taken together, these data suggest that during a potential intermediate-LTP phase, the translation-dependent but transcription-independent augmentation of GluA1 phosphorylation by glucocorticoids is mediated by a non-genomic action of membrane associated GRs.

Calcium/Calmodulin-sensitive Adenylyl cyclases are required for glucocorticoid-mediated synaptic potentiation

Activation of the CP-AMPA GluA1 via PKA is a well-described mechanism for functional neuro-plasticity during stress⁴⁷⁻⁵⁰. Yet, the link between signal reception at the synapse, the resultant increase in intracellular calcium and further downstream activation of cAMP⁵¹ to upregulate levels of the active catalytic subunit of protein kinase A, which mediates its phospho-transferase abilities⁵² is still unclear. One group of synaptic associated molecules with potential for both processes i.e. utilization of increased calcium following NMDAR activation, and resultant increased cAMP-mediated activation of PKA, are the calcium stimulated enzymes, the adenylyl cyclases (AC).

Using the cell membrane permeable selective AC inhibitor SQ22536 - 10 μ M, we investigated the requirement of these calcium sensitive enzymes for the observed mGR-mediated actions on pGluA1 expression. Blocking expression of the transmembrane ACs in whole cell extracts treated with a high dose

of corticosterone (100nM) ablated the glucocorticoid-mediated increase in GluA1 activation (figure 3 A, B). One-way ANOVA detected a significant effect of treatment ($p= 0.0083$) and Bonferroni post-hoc tests revealed that the corticosterone-induced increase in pGluA1 was significant ($p=0.021$) and the SQ22536-dependent inhibition of this induction was also significant ($p=0.020$). There was no significant effect of either corticosterone or SQ22536 treatment for levels of total GluA1 (figure 3A, B).

To confirm the results obtained, we conducted immunocytochemical analysis of cultured neurons to allow the visualization of fluorescent-tagged receptor proteins. We first assessed the temporal kinetics of GluA1 surface expression in DIV 15 hippocampal cultured neurons at 60 and 180 minutes of stress level corticosterone (100nM) or ethanol (control) application. GluA1 dendritic surface expression (as visualized via the neuronal marker-MAP2 and post-synaptic marker – PSD95) was significantly increased by corticosterone treatment at the 180 min time point ($p<0.001$) (Supplemental figure 3 A, B). Next, cultured hippocampal neurons were treated with 10 μ M SQ22536 for 30 minutes prior to application of stress level corticosterone (100nM). Subsets of cover slips containing dissociated hippocampal neurons were co-treated with corticosterone as well as the GR antagonist RU-486 – 500nM, with control cultures treated with ethanol. Cells were fixed at 3 hours following the corticosterone or ethanol application and probed with antibody corresponding to the GluA1 N-terminus. Following membrane permeabilization, the cells were then incubated with post-synaptic marker PSD-95 antibody for highlighting dendritic regions to allow for visualization of co-localized GluA1 and PSD-95. An extremely significant effect of treatment ($p < 0.0001$, One-way ANOVA) was seen for GluA1 dendritic surface localization, as corticosterone exposure induced a significant increase in dendritic GluA1 immunofluorescence ($p<0.0001$), which was blocked by both the AC inhibitor ($p<0.0001$) and the GR antagonist ($p<0.0001$) (figure 3 C, D). Taken together, these findings support a mechanism whereby corticosterone acting via GR induces AC-mediated PKA-dependent phosphorylation of GluA1 presumably via AC-dependent cAMP production that activates PKA.

To assess whether there was a functional sequela to the observed responses following AC inhibition, we evaluated the effect on LTP. An established LTP high frequency stimulation (HFS) protocol was applied to acute hippocampal slices (figure 3E). This triggered an increase in field excitatory postsynaptic potentials ($150.8 \pm 2.3\%$ of baseline. Like our previous hormone exposure protocol¹², addition of corticosterone (200nM) markedly augmented the normal LTP response ($184.9 \pm 7.9 \%$ of baseline, $p < 0.001$). This increase was completely abolished by the application of an AC inhibitor ($152.6 \pm 10.6 \%$ of baseline, One-way ANOVA $p = 0.011$, with Bonferroni post-hoc, correction; control vs corticosterone, $p < 0.05$, corticosterone vs corticosterone + SQ, $p < 0.05$; figure 3E, 3F), showing for the first time that glucocorticoid-mediated amplification of EPSPs following LTP stimulation is dependent on hippocampal adenylyl cyclases.

Calcium-sensitive adenylyl cyclases are differentially regulated by glucocorticoids following NMDAR blockade

We next investigated the effect of NMDAR inhibition on the expression of AC1, AC8 and pGluA1 following an exogenous glucocorticoid signal. Acute hippocampal slices were treated with the selective NMDAR

antagonist, AP5 - 50 μ M which competitively binds the receptor's ligand binding site, blocking ligand binding, and consequently prevents resultant calcium influx via the receptor's cation channel⁵³. Slices were then treated with 50nM corticosterone for 60 minutes and prepared for Western blot analysis. Slices treated with ethanol were used as controls (figure 4). One-way ANOVA revealed significant effects of treatment for AC1 ($p=0.0191$), AC8 ($p=0.0007$) and pGluA1 ($p=0.0001$) (figures 4B, C, E). Interestingly, the corticosterone-induced increase in AC1 ($p<0.05$) was not blocked by AP5 (figure 4B). The corticosterone induction of AC8 expression ($p<0.001$) was ablated by pre-treatment with AP5 ($p<0.01$) (figure 4C). Finally, corticosterone-induced GluA1 phosphorylation ($p<0.05$) was also completely abolished by AP5 ($p<0.001$; figure 4E). These findings suggest that glucocorticoid exposure increases pGluA1 in an NMDAR-dependent mechanism that may involve the Type 8 adenylyl cyclase.

Discussion

Glucocorticoids exert multiple effects on the body's physiology, through actions that can occur both rapidly or over a longer term^{34,45,54,55}. The transcription-independent effects that takes place outside of the classical genomic regulatory actions^{56,57} are of interest given findings that acute exposure of the hippocampus to glucocorticoids can enhance synaptic efficacy, even within minutes of hormone exposure^{7,55}. These rapid effects on hippocampal plasticity processes, particularly glutamate-related, are thought to be mediated by membrane-associated versions of the Type 1 and 2 glucocorticoid receptors, mMR and mGR respectively^{7,8,58}. One such example is the ultradian glucocorticoid-dependent increase in frequency of miniature excitatory postsynaptic currents (mEPSCs)⁵⁹ that is mediated by membrane rather than cytosolic glucocorticoid receptors^{7,45} to facilitate synaptic potentiation via CP-AMPA¹². In this study, we have investigated the molecular mechanisms governing both fast and slow-acting glucocorticoid-dependent regulation of hippocampal synaptic potentiation.

Acute glucocorticoid and stress exposure elicited PKA-mediated phosphorylation and increased synaptic GluA1 protein (at the 60 minute time point) prior to the transcription of its nascent transcript (at the 120 minute time point). Interestingly, analysis of the genomic effect of exogenous glucocorticoid on pGluA1 protein expression strongly suggested that the potentiating effect of corticosterone on GluA1 phosphorylation at this early time point is dependent on the synthesis of new protein, since the translation inhibitor, cycloheximide blocked the glucocorticoid-mediated increase in pGluA1 levels. This may potentially be indicative of some form of activity-dependent rapid protein translation, possibly even locally at the synapse, a mechanism that has been described to enable specific control of synaptic strength independently of messenger RNA synthesis in the cell body (reviewed in⁶⁰). While it is possible that the requisite protein could potentially be any in this pathway, the most likely candidates appeared to be AC1 and AC8 as they were both robustly and rapidly translated within a relatively short time of corticosterone exposure (60 minutes).

Consistent with the translation-dependent mechanism, none of the key players in the pathway (AC8, PKA and GLUA1) were transcriptionally upregulated within the first hour after stress exposure, and the

transcriptional inhibitor actinomycin had no effect on the enhancing actions of glucocorticoid on pGluA1. Significant induction of mRNA transcript expression for GluA1 and PKA did not reach significant levels until 4 to 6 hours after the stress, considerably later than the early translation-dependent effects observed in our study. The delayed genomic effects on the expression of PKA and GluA1 is more consistent with a positive feedback mechanism to maintain the rapid membrane-localized action of glucocorticoids on synaptic plasticity. Regulating synaptic strength at glutamatergic synapses is clearly very important and this adds an additional mechanism to the concept of the metaplastic actions of corticosteroid signalling (63, 69).

We next sought to assess whether the Type 2 GRs contribute to these actions, particularly regulation of p-Ser-845-GluA1 during translation-dependent-LTP, for which evidence of the actions of membrane-localised Type 1 receptors is established⁷. Of note, this time point has been linked to GR-dependent effects on the consolidation of newly formed memories, i.e. 60 minutes after the presentation of stress or administration of high levels of glucocorticoid^{35,61}. To do this, we evaluated effects of the Type2 GR antagonist RU-486 on glucocorticoid-dependent activation and expression of GluA1. Following stress, increased GluA1 levels were observed in the SPM. This induction was not seen in the ADX rats. Administration of stress-level glucocorticoids to ADX rats revealed increased GluA1 in the postsynaptic density (PSD). Furthermore, reduced levels of total and phosphorylated GluA1 were found in purified postsynaptic density (PSD) fractions prepared from ADX rats concomitantly administered both high corticosterone dose and RU-486. These findings were confirmed in our immunocytochemistry studies. This occurrence may be mediated by GR, as RU-486 treatment blocked the corticosterone-mediated incorporation of GluA1 into the PSD. Notably, GluA1 levels in the SPM were significantly greater than levels in the PSD, for the RU486 treated animals, suggesting a blockade in lateral diffusion of the receptor to the PSD, or perhaps prevention of interactions between GluA1 and accessory proteins that would aid receptor anchoring at the PSD, such as SAP97, as this would indicate receptor trafficking to the PSD. Indeed, AC and PKA are linked to GluA1 subunit via a SAP97-containing complex, thought to favor synaptic potentiation paradigms {Hell, 2016 #142}. There is no existing data on glucocorticoid regulation of the GluA1/SAP97 complex, and therefore we cannot confirm or conclude a role for this based on our results. Nevertheless, the selective augmented rise in PSD GluA1 levels post glucocorticoid treatment is consistent with previously reported glucocorticoid-mediated enhancement of LTP^{7,12}. Our results are consistent with reported data for RU-486-mediated blockade of the transcription-dependent augmentation of AMPAR expression in hippocampal neurons following stress-level glucocorticoid treatment¹¹ and corroborate reports from Yuen *et al.* and Liu *et al.* that revealed an enhancing effect of GR-activating corticosterone levels on GluA1 synaptic expression in neuronal culture^{10,62}. Their study showed that this action may in part be regulated by the immediate early gene, SGK-1, which is itself transcribed *de novo* in response to the corticosterone signal, a finding we also present in this report.

The results from our glucocorticoid hormone exposure protocols i.e. *ex vivo* administration of high dose of exogenous corticosterone, versus restraint stress, are notable as they showcase selective regulation of synaptic plasticity processes following different types of stressors. Whilst moderate stressors like

immobility/restraint stress can facilitate memory consolidation, predator stress (perhaps more akin to our high exogenous glucocorticoid administration to the ADX rats) has been shown to impair memory consolidation processes^{61,63}. Synapses containing calcium permeable GluA1 subunit compositions are classically accepted as being fundamental prerequisites for synaptic potentiation processes⁶⁴⁻⁶⁸. At the same time, synaptic potentiation will occur independently of solely GluA1 presence at the postsynaptic density, as GluA2 subunits have been found in the synapse during activity-dependent long lasting potentiation of the neuronal synapse⁶⁹. Interestingly, GluA2 levels were also upregulated in the synaptic plasma membrane and the post-synaptic density after stress in adrenal-intact but not ADX rats (Supplemental figure 5) suggesting dynamic regulation of GluA1/2 heteromer. Unlike GluA1, however, GluA2 levels were not upregulated in the synaptic plasma membrane after corticosterone treatment (Supplemental figure 5) suggesting the stress effect on GluA2 is more likely mediated by another adrenal factor, potentially noradrenaline (NA). Yu Liu and colleagues showed that NA can induce GRIA2 transcript expression during stress, as blockade with the NA antagonist propanolol prevented this effect. Furthermore, electrophysiological studies, looking at inward rectifying IV current, an indicator of the presence of GluA2 lacking AMPAR subunits further confirmed the role of NA in inducing a switch in AMPA receptor phenotypes from GluA2-lacking to GluA2-containing receptors during stress, a process requiring calcium influx⁷⁰. Nevertheless, studying GluA2 synaptic trafficking alone does not present a full picture of AMPAR regulation of LTP, and related activity-dependent processes, particularly during stress. GluA2 containing receptors constitutively traffic in and out of synapses. This can occur independently of a neuronal stimulus like stress-mediated activation of NMDARs to increase calcium-mediated signaling cascades, and still take place without influencing synaptic strengthening⁷¹⁻⁷³. Therefore, examining the synaptic insertion of GluA1 containing AMPA receptors is paramount, as may be indicative of synaptic strengthening, and thus potentiation events at the neuronal synapse.

The selectivity of the glucocorticoid effect on GluA1 (but not GluA2) identified in our study provides new insight into the nature of corticosteroid receptor actions on modulating functional responses including cognition⁷⁴. We can speculate that post-synaptic increases in pGluA1 and total GluA1 expression following systemic administration of high levels of glucocorticoid may underpin the molecular mechanism by which the stress hormone facilitates neuronal processes, such as LTP saturation, that could impair the consolidation of newly formed memories thereby promoting downstream impairments in memory recall^{75,76}.

GluA1 phosphorylation, though key for synaptic incorporation in excitatory synapses^{6,29,30} still requires the presence of an additional signal, such as a rise in intracellular calcium for functional insertion of activated receptors into the postsynaptic density^{77,78}. The primary source of increased cytosolic calcium during periods of high synaptic activity is influx through NMDARs²⁵. Increased intracellular calcium triggers the activation of second messenger pathways through the calcium/calmodulin stimulation of adenylyl cyclases, thereby inducing the production of PKA⁵¹, and its subsequent phosphorylation activities on GluA1²⁹.

Our mechanistic studies into molecular mediators that facilitate glucocorticoid-dependent postsynaptic incorporation of activated CP-AMPA receptors have identified the calcium sensitive adenylyl cyclases (AC1 and AC8) as the likely candidates for the glucocorticoid-dependent effects on synaptic potentiation. For instance, our results revealed that GR-dependent upregulation of pGluA1 requires ACs, as we observed a substantial increase in pGluA1 synaptic expression after glucocorticoid exposure, an effect that was blocked both by a GR antagonist and an AC inhibitor. Notably, the GR antagonist used in our study has previously been shown to inhibit glucocorticoid effects on distinct cellular parameters, including transcription-dependent augmentation of AMPAR expression in hippocampal neurons following stress-level glucocorticoid treatment ¹¹.

Studies in knock out models suggest the involvement of ACs in mediating normal behavioural responses to stress ^{17,37,79}, with evidence implicating their importance in memory consolidation and retention ^{37,80,81}; behaviours that are clearly linked to the low affinity corticosteroid receptor (GR)-mediated actions during stress ^{35,82-86}. Of the two calcium/calmodulin-stimulatable adenylyl cyclases present in the hippocampal synapse - AC1 and AC8, AC8 is a pure calcium sensor ^{87,88,89}. During periods of increased intracellular calcium, AC8 has been shown to be localized at excitatory synapses in hippocampal neurons, with AC1 more distal ¹⁸, thus AC8 may be maximally stimulated in an activity-dependent manner, such as evident during stress.

Our *in vivo* studies showed that hippocampal synaptic plasma membranes of animals with intact adrenal glands had increased AC8 protein levels following exposure to acute restraint stress without an increase in mRNA levels (Supplemental figure 4) consistent with the transcription-independent translation-dependent mechanism demonstrated by our cycloheximide and actinomycin D inhibitor studies. Interestingly, similar expression dynamics between AC8, GluA1 and GluA2 were observed in subcellular compartments post stress (Figure 1 C, D; Supplemental figures 4 and 5). These findings do not preclude the involvement of AC1 in the observed responses, though corroborate previous report by Kim *et al.* ⁹⁰. Using mathematical simulation models, the authors found that PKA-dependent GluA1 activation is enhanced when the activating kinase is localized near its source molecule, i.e. adenylyl cyclases ⁹⁰. They surmised that localization of PKA near ACs during long-lasting LTP is critical for GluA1-Serine 845 activation by PKA, thus labelling the receptor protein ready for lateral diffusion into post-synaptic terminals ^{29,77,90}. The proximity of calcium sensitive ACs to PKA and GluA1 further prevents inactivation of possibly heteromeric GluA1s (i.e. GluA1/2 subunit compositions) by phosphodiesterases, as well as limited diffusion of AC-derived cAMP ⁹⁰. By preventing calcium-sensitive AC expression with the use of a selective inhibitor, we found an impairment of the potentiating effects of stress-level glucocorticoids on GluA1, as the typical enhancement of PKA-dependent GluA1 phosphorylation was inhibited. These results were mirrored in our electrophysiology experiments. It is possible that the calcium signal for AC mediated actions in the glutamatergic synapse may, at least in part, come from synaptic NMDARs perhaps following membrane depolarisation in the early phases of the stress response, with enhancement of cytosolic calcium from intracellular stores a downstream consequence ²⁰. This may lead to the PKA-dependent phosphorylation of the GluA1 subunit, to enable its exocytosis and subsequent lateral

diffusion into the post-synaptic density to facilitate LTP. PKA also phosphorylates transcriptional regulators such as CREB-binding protein (CBP). By associating with transcriptional factors such as nuclear GR, as well as other transcriptional regulators on response elements of identified promoter regions, transcription of relevant synaptic plasticity modulatory molecules as observed in this study, may be indirectly modulated by the stress response (Figure 5).

Our data showing that AC inhibition prohibits glucocorticoid-dependent GluA1 phosphorylation, synaptic expression and enhancement of LTP suggest that calcium-sensitive hippocampal membrane bound adenylyl cyclase enzymes. may be an essential component for the modulation of plasticity at glutamatergic synapses during stress, and/or glucocorticoid exposure. Additional studies will be needed to define the mechanism by which AC8 modulates glucocorticoid-dependent synaptic potentiation processes. Furthermore, since AC8 is necessary for fear in the presence of stress⁷⁹, future studies could assess the role of glucocorticoids in regulating fear-related learning, as well as memories obtained during high arousal states, as experienced by people who have undergone traumatic situations. Finally, this anxiogenic-linked molecule could potentially be targeted therapeutically for the treatment of anxiety-related conditions.

Conclusion

Though acute exposure to the stress-responsive adrenal glucocorticoid hormones is known to augment synaptic potentiation, which is important for stress-related memory processing, the mechanisms underlying this effect are poorly understood. We show for the first time that calcium/calmodulin-responsive adenylyl cyclases are an essential component of the neuronal machinery necessary for the modulation of plasticity at hippocampal glutamatergic synapses in response to high levels of glucocorticoids, such as are found during emotionally arousing events. The implications of this work are that glucocorticoid-mediated innervation of adenylyl cyclases induce CP-AMPA related synaptic processes to modulate functional activity during stress. These data could inform development of novel strategies targeting stress-related cognitive disorders as well as other conditions involving impairment of synaptic plasticity.

Materials And Methods

Animals: Male Wistar rats (Harlan, Blackthorn Bicester UK) were housed in a 14-hour light/10-hour dark cycle, with *ad libitum* access to food and water. Upon arrival, animals were habituated to handling (minimum - 5 days). Naive (adrenal-intact) animals were used in the studies unless otherwise stated. All rats were culled rapidly by decapitation under terminal isoflurane anaesthesia.

The treatment schedules undertaken for all study experiments is described in Supplemental Table 1.

Adrenalectomy: Bilateral adrenalectomy (ADX) was performed as previously described⁹¹ under general anaesthetic using isoflurane. Peri-operative treatment with dexamethasone (24µg/kg), a non-steroid anti-

inflammatory (Rimadyl, 96µg/kg) and glucose/saline (NaCl 0.45% (w/v), glucose 2.5% (w/v)) was administered via subcutaneous injection to aid recovery. During recovery animals were given 0.9% NaCl (saline) supplemented with corticosterone (25 µg/litre; Sigma-Aldrich, UK) in place of drinking water to maintain electrolyte balance and HPA axis homeostasis. The corticosterone-saline was replaced with saline water 12 hours prior to the start of the treatment protocol.

Induction of acute stress: Rats were physically restrained in Perspex restraint tubes for 30 minutes then returned to their home cage prior to killing at pre-determined time points.

Preparation of acute hippocampal slices from adrenalectomized (ADX) rats: ADX rats were decapitated and the brains rapidly removed from the skull and placed in ice-cold perfused artificial cerebrospinal fluid (aCSF) (124mM NaCl, 3mM KCl, 26mM NaHCO₃, 1.25mM NaH₂PO₄, 2mM CaCl₂, 1mM MgSO₄ and 10mM glucose). The hippocampi were dissected, and the two hemispheres separated. Right hippocampi were collected as existing data suggests that this area is important for the hippocampus prepared with a McIlwain tissue chopper (Mickle Laboratory Engineering Company, Guildford, UK) were continuously perfused with aCSF at room temperature for approximately 1-hour. Slices were gently transferred to nylon meshes in small dishes also containing aCSF with constant perfusion of the previously outlined gas mixture for use in experiments. Drugs were added directly to the dish containing slice(s) according to pre-determined treatment schedules (Supplemental Table 1).

For hippocampal protein analysis, whole cellular extracts were prepared from lysed tissue using a high sucrose lysis buffer (10mM Tris pH7.6, 0.32M sucrose, 1mM EDTA, 1% SDS, 2x protease inhibitor cocktail, 1mM NaF and NaVan phosphatase inhibitor). At relevant time points following treatment, samples were transferred to 1.5ml eppendorf tubes and snap frozen in liquid nitrogen. Lysis buffer (100µl) was added to each tube and samples were homogenized thoroughly in the buffer, before being spun (4°C, 10000g, 15 minutes). The resultant pellet containing cell debris and nuclei was discarded, and the supernatant (~ 90µl) retained for analysis of protein expression by standard immunoblot analysis (western blot).

Evaluation of protein localization and expression in rodent brain tissue: To assess the intracellular translocation of steroid receptor proteins, as well as their relative expression in the hippocampus, nuclear and cytoplasmic proteins were extracted from tissue as described by Vallone and others⁹²⁻⁹⁴. To assess the intracellular translocation of synaptic proteins of interest, as well as their relative expression in the hippocampus, brain cellular compartments were extracted using the differential centrifugation procedure described by Blackstone and others^{95,96}. Study parameters: nuclear translocation, synaptic protein lateralized diffusion, subcellular localization and protein expression, were investigated via western blot. Synaptic plasma membrane (SPM) and post-synaptic density (PSD) fractions were prepared by sequential subcellular fractionation (Detailed description in Supplementary methods).

Visualization of protein localization in cultured rat primary hippocampal neurons using confocal microscopy(Immunocytochemistry):

Primary hippocampal neurons were prepared from 1-day-old male Wistar rat pups (detailed in Supplementary methods). On DIV 12-15, coverslips containing cultured hippocampal neurons were treated per pre-determined schedule (Supplemental Table 1). Following treatment protocols, neuronal cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 30 minutes at room temperature, then washed three times with PBS, and blocked with 1% bovine serum albumin (BSA) PBS, for 15 minutes at room temperature. Cells were incubated overnight with relevant N-terminal/extracellular-epitope primary antibodies at 4°C in a dark humidified atmosphere, with gentle shaking. The following day, cells were washed three times with PBS, and permeabilised with 0.1% Triton X-100 for 10 minutes at room temperature, further washed three times before incubation with relevant C-terminus/intracellular epitope antibodies. The cells were further washed in PBS, and in a dark room, secondary antibodies conjugated to appropriate fluorophores were incubated with the cells for two hours. Cultures were washed in PBS and mounted on pre-labeled glass slides using vectashield mounting medium containing 6-Diamidino-2-Phenylindole (DAPI) to identify nuclear morphology, then stored in the dark at -20 °C until viewing on the confocal microscope.

Image acquisition and data quantification: The confocal micrographs were captured with Leica application suite advanced fluorescence (LASIF) software. Using sequential acquisition settings at the maximal resolution of the microscope (1024 X 1024), confocal images were obtained with the use of a 63 X OIL immersion laser objective. The confocal settings were kept the same for all the scans; and brightness, contrast and color balance were adjusted identically across treatments to allow for even and unbiased quantification.

Morphometric analysis and quantitation were performed using the Volocity 3D Image Analysis Software (Perkin-Elmer, Groningen, Netherlands) and Image J (version 1.45, NIH, Bethesda, USA). Here, the baseline/ untreated group values were also set at 100% and treatment groups were calculated as fold changes in relation to the control group.

Electrophysiology: Brains were removed into ice-cold ACSF (same concentration as above) with 95%O₂/5%CO₂. Transverse hippocampal slices (400µM) were cut using a McIlwain tissue chopper and allowed to recover in ACSF at least 1hr at room temperature. A stimulating electrode was placed on the Schaffer collateral pathway. Recording electrodes (5-6 MΩ) containing 3M NaCl filing solution were used for long-term potentiation (LTP) experiments. After 30 minutes of baseline, high frequency tetanic stimulation (2 x 100 pulses; 100 Hz) was used as the LTP induction protocol. Data are analysed using LTP114j software. Statistical significance of observed effects between groups was analysed using unpaired *t*-test.

Western blot analysis: Protein samples were run on SDS polyacrylamide gel (SDS-PAGE) electrophoresis as described by Laemmli ⁹⁷ then transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, UK). Membranes were blocked (1x TBS/0.1 %Tween-20/5 % non-fat skimmed milk powder) to prevent non-specific binding and then incubated with primary antibodies corresponding to the proteins of interest or loading controls. Specific bands were detected via incubation with the appropriate HRP-

conjugated secondary antibodies, then chemiluminescent reagent (ECL, Amersham) and visualised with a G:Box (Syngene). Densitometric analysis was performed using Quantity-one software (Bio-Rad Laboratories, Hercules, CA) and Image J software (version 1.45, NIH, Bethesda, USA) where adjusted volumes of the optical density values for all the immunoblot protein bands, were normalized to levels of endogenous protein markers. Baseline/untreated group value was set at 100% and treatment groups were calculated as relative fold change from this. Except otherwise stated, all Western Blot analysis graphs presented are densitometry quantification of protein bands in the immunoblot, and show fold induction values of the % adjusted OD, relative to the relevant control from each experiment. Given that Western blot analysis was performed in an investigator-blinded manner, the immunoblots presented are representative figures corresponding to the results shown. Unprocessed blots depicting annotated protein bands for each individual experiment are provided as Supplemental material. Expression of either β -actin or relevant fraction-specific marker (as indicated in each figure) was used as loading control to normalize values of the investigated proteins.

Analysis of gene transcription in rodent brain tissue: A two-step real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) protocol was used to determine relative expression of the genes of interest (detailed in Supplementary methods).

qPCR amplification reactions were performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, UK) with Power SYBR Green Master Mix (Applied Biosystems) and custom SYBR green forward and reverse primers corresponding to the gene of interest (Supplemental Table 2). The comparative $\Delta\Delta C_t$ method was used to calculate relative expression of genes of interest for the different experimental conditions⁹⁸, with β -actin expression used as an endogenous control for normalization. *Gene expression levels were represented as fold induction values calibrated against the baseline (control) group, and normalized to endogenous b-actin levels for each sample.*

Measurement of hormone levels in plasma: To determine the levels of glucocorticoid hormone in rodent plasma, a radioimmunoassay was conducted, using an anti-rat corticosterone primary antibody (kindly donated by Prof G Makara – Institute of Experimental medicine, Budapest, Hungary) and corticosterone I¹²⁵ tracer (Izotop, Budapest, Hungary) as previously described^{99,100} and according to the detailed protocol in supplementary methods. Results were obtained as concentration of corticosterone in the plasma samples (ng/ml) and this was ascertained from comparisons to standard curve samples containing known concentrations of corticosterone. The limit of detection for this assay was 11ng/ml. The intra and inter-assay coefficients of variation were 11 and 14.7% respectively.

Statistical analysis of results: All data in this study are presented as mean value \pm SEM. GraphPad Prism 8 software (Graph Pad, UK) and IBM SPSS (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.) statistical software were used. A One-way analysis of variance (ANOVA) followed by a multiple comparison correction procedure to correct for Type 1 error (Bonferroni *post hoc* test) was applied to determine the statistical differences among study groups in all the experiments. Where groups showed statistical significance, further analysis was performed using the Dunnett's *post hoc* test to

compare each data set with the control group, particularly for the time course studies. For experiments requiring comparisons between only two groups, the students unpaired t test was used. A Two-way ANOVA, with Bonferroni correction was applied where analysis of two independent factors influenced an experimental condition. All statistical tests used are denoted in the results. All images included in this manuscript are representative and error bars represent mean \pm SEM. Statistical significance was set at $p < 0.05$, and significant differences between groups are indicated by: * or # $P < 0.05$, ** or ## $P < 0.01$, *** or ### $P < 0.001$, **** or #### $P < 0.0001$.

Declarations

Data Availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

Ethics statement

All animal experimental procedures described in this manuscript were carried out in accordance with UK Home Office and University of Bristol animal welfare regulations.

Ethical approval for all animal experimental procedures was received from The University of Bristol Animal Welfare and Ethical Review Body (UOB-AWERB), part of the University of Bristol Animal Services Unit (UOB-ASU), Bristol, UK.

All experiments were performed in accordance with relevant guidelines and regulations, and in compliance with the ARRIVE guidelines, for reporting of *in vivo* experiments {Percie du Sert, 2020 #101}.

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Author contributions: CU, TP, SL and KC designed the experiments; TP, EW, GW, DW, SL, KC and BC supervised the research; CU, JY and BC performed the experiments and analysed the data; CU wrote the manuscript; All authors critically reviewed the manuscript for important intellectual content.

Conflict of interest statement: The authors declare no competing financial interests

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Figures

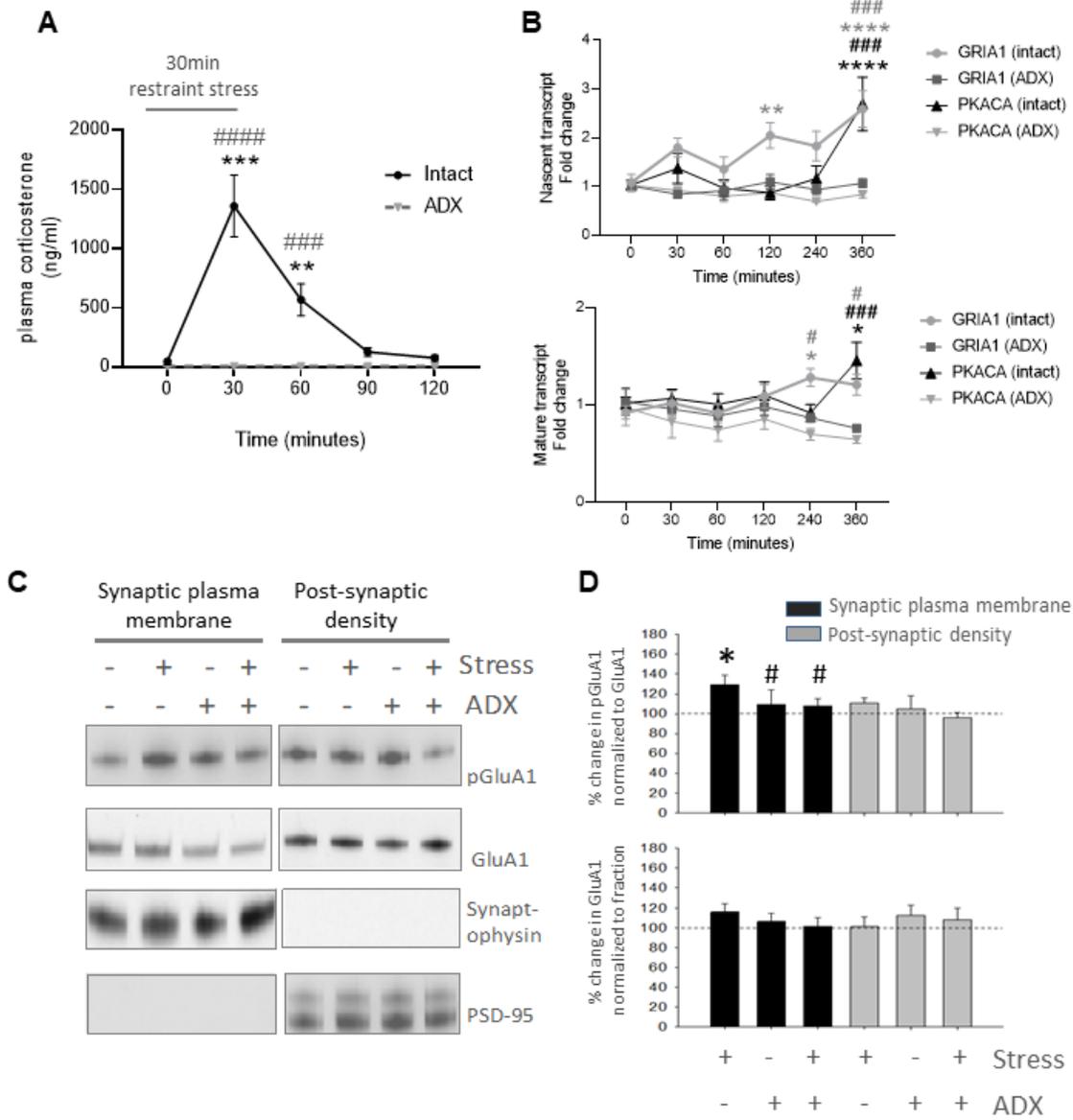


Figure 1

GluA1 regulation after acute stress is adrenal-dependent. A. Temporal dynamics of corticosterone secretion following 30 mins restraint stress exposure. Blood and hippocampi were collected from adrenal intact or ADX rats at 30, 60, 120, 240 and 360 mins after the onset of a 30 minute restraint stress. Control rats for baseline comparisons were unstressed (and shown as time 0 in graph). In adrenal-intact rats, a significant rise in corticosterone levels was seen immediately after termination of stress protocol, with steady decline back to baseline levels by 120 minutes. In ADX rats, corticosterone levels remained low throughout the time course. Symbols: *Comparison to time 0 in intact rats (Dunnett's test). #Comparison between intact rats and ADX rats at matched time points (Bonferroni). Data is plotted as mean \pm SEM for two independent experiments; n=6 per time point group. B. Adrenal-mediated regulation of synaptic plasticity transcripts over time. Graph depicts stress effects on expression levels of GRIA-1 and PKACA nascent and mRNA in hippocampus from intact and ADX rats. Stress-induced rise in GRIA1 and PKACA (late timepoints) transcripts observed in intact rats was ablated with ADX. n=4 animals were used for each time point. Symbols: *represents a significant difference (Dunnett's test) in the comparison to time 0 and #represents a significant difference (Bonferroni) in comparison to ADX group at the matched timepoint. Grey symbols represent GRIA1 and black symbols PKACA. C, D. Stress-induced adrenal hormones modulate GluA1 proteins in the rat hippocampus at 60 minutes after the onset of stress. (C) Western blot illustrating phosphorylated and total GluA1, as well as region-specific markers to determine integrity of the different cellular extracts i.e. synaptophysin for synaptic plasma membrane (SPM) fractions and PSD-95 for proteins from postsynaptic density compartment. (D) Increased stress-induced pGluA1 was observed in the synaptic plasma membrane (SPM) of intact rats, and ablated by ADX. No differences were seen in the post-synaptic density. Total GluA1 expression was unchanged in both intact and ADX animals. n=4 animals were utilized for each group. Symbols: *represents significant difference in comparison between baseline control and stress, and #represents significant difference in comparison between intact rats' stress and ADX rats' stress (Bonferroni tests).

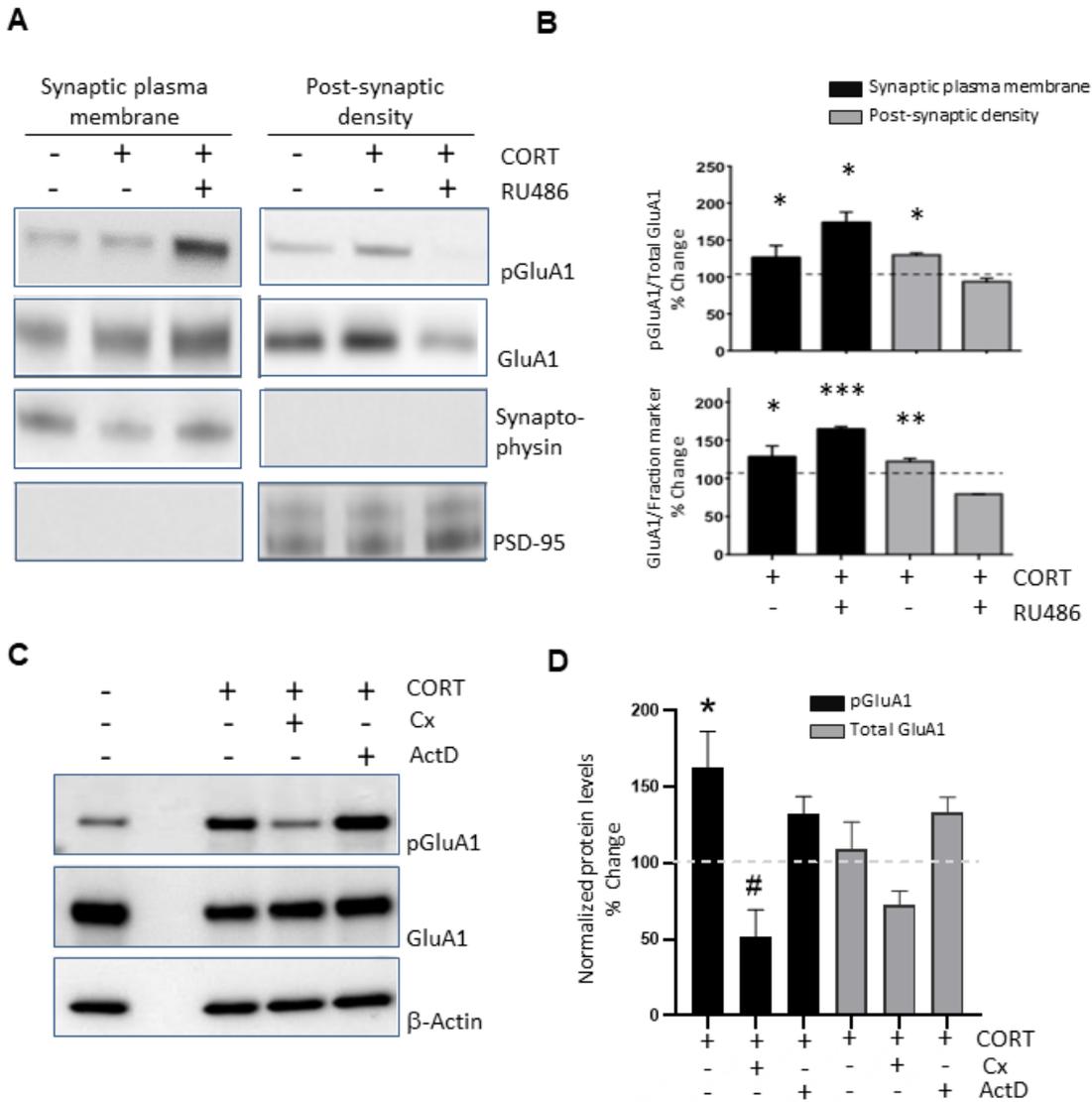


Figure 2

Glucocorticoids act via the Corticosteroid Type 2 receptor to upregulate hippocampal GluA1 synaptic expression in a transcription-independent manner. For (A/B), to assess effect of GR blockade on synaptic plasticity parameters, ADX rats were injected with GR antagonist (RU-486: 20mg/kg) 30 minutes prior to corticosterone injection (3mg/kg). Hippocampi were collected 60 minutes after corticosterone injection for subcellular fractionation. Control animals received ethanol and saline injections only. (A) Immunoblot

of investigated proteins pGluA1 and total GluA1, plus fraction-specific markers to determine integrity of the subcellular extracts i.e. synaptophysin for synaptic plasma membrane (SPM) fractions and PSD-95 for postsynaptic density (PSD) compartment proteins. (B) Significantly higher levels of both phosphorylated GluA1 and total GluA1 were evident in the SPM following glucocorticoid exposure. Administration of GR antagonist RU-486 augmented the increased protein levels seen in this fraction. In the PSD, the glucocorticoid increase in both pGluA1 and total GluA1 by glucocorticoid exposure was completely blocked by RU-486. For (C/D), to assess genomic versus non-genomic responses, acute hippocampal slices from ADX rats were treated with cycloheximide (50 μ M) or Actinomycin-D (40 μ M) for 30 minutes followed by 100nM corticosterone. Whole cell extracts were prepared from the hippocampal slices after 60 minutes of corticosterone treatment. (C) Immunoblot of pGluA1, total GluA1 and endogenous loading control β -actin. (D) Graph shows decreased expression of corticosterone-induced pGluA1 in cycloheximide + corticosterone treated slices. Application of Act-D had no effect on corticosterone-dependent regulation of GluA1 phosphorylation or expression. No significant difference was found for total GluA1. Error bars on all graphs represent mean \pm SEM from one experiment; $n \geq 4$ per group. Symbols: *indicates significant difference (Bonferroni test) in the comparison between vehicle treated control and corticosterone treated rats (2B) or hippocampal slices (2D) and # indicates significant difference (Bonferroni tests) in comparison between corticosterone and corticosterone + modulator RU486 (2B) or corticosterone + modulator CX (2D).

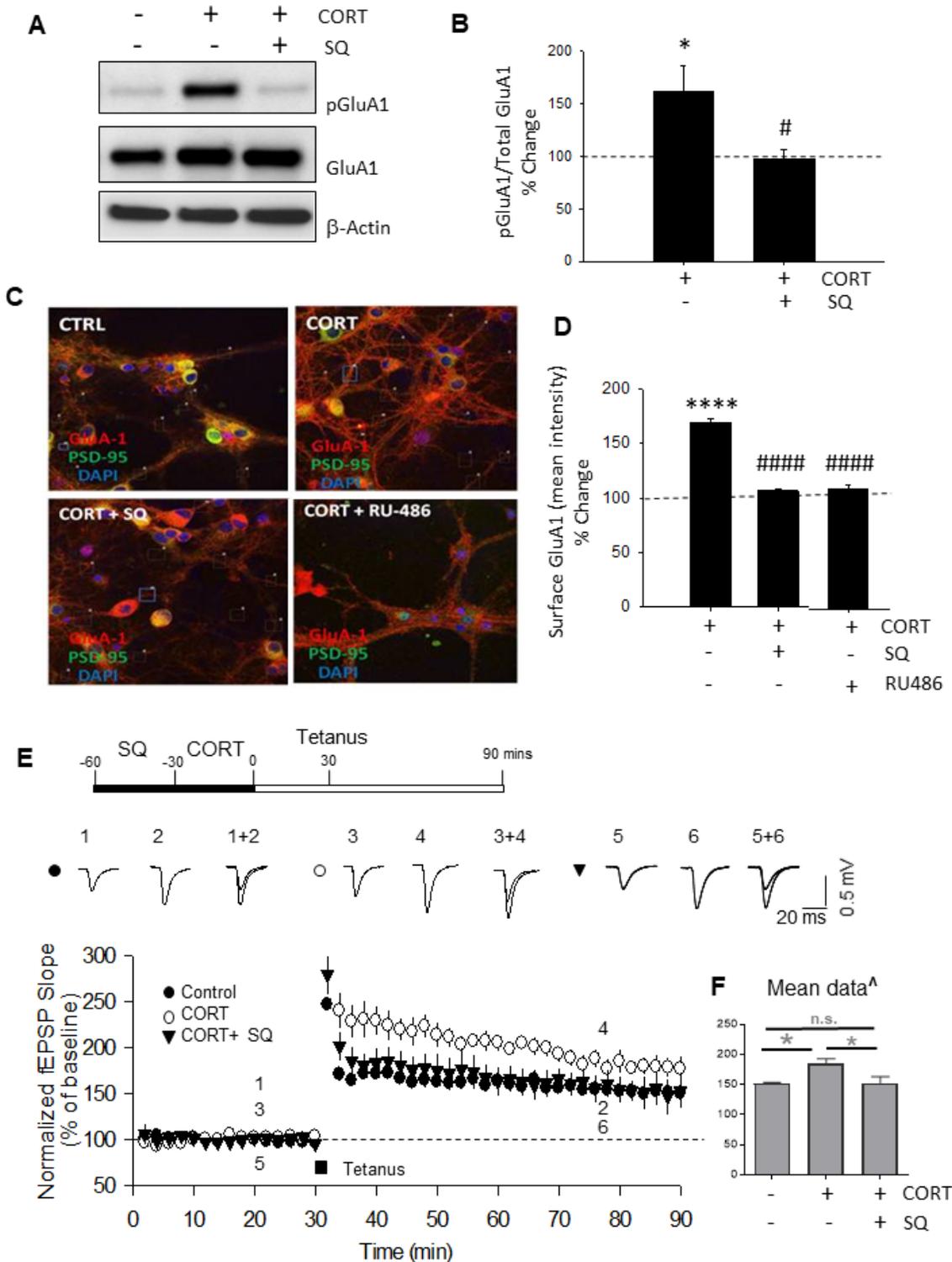


Figure 3

Calcium-sensitive Adenylyl cyclases drive the potentiating action of Type-2 glucocorticoid receptor on hippocampal synaptic plasticity For (A/B) administration of AC inhibitor: SQ22536 - SQ (10 μ M) was applied to hippocampal slices or 30 minutes, followed by 60 minutes of 100nM corticosterone treatment. (A) Immunoblot for pGluA1, total GluA1 and β -actin. (B) Graph showing SQ ablation of corticosterone-induced pGluA1. Error bars represent mean \pm SEM from one experiment; $n \geq 4$ per group. Symbols:

*significant difference between vehicle control and corticosterone treated slices, and #significant difference between corticosterone and corticosterone+SQ (Bonferroni tests). (C) Modulation of GluA1 surface expression in dendritic regions of cultured hippocampal neurons (D1V15) following 100nM corticosterone treatment, showing effects of pre-treatment with vehicle, 10 μ M SQ or 500nM RU486 for 30 minutes. Overlay image shows GluA1 (red), PSD-95 (green) and DAPI (blue) captured with confocal microscopy [Scale = 10 μ m]. (D) Graph showing the significant increase in corticosterone-induced GluA1 co-localization with PSD-95 at 3 hours of corticosterone treatment, which is blocked with both adenylyl cyclase inhibition (SQ) and GR antagonist (RU486). Graph shows quantified mean intensity values from immunofluorescent images. Dendrites were highlighted with a PSD-95 marker (green fluorescence). Values are fold induction from mean of the control (ethanol) group values (assigned 100%). Error bars represent mean \pm SEM from two experiments; $n \geq 30$ dendrites per group. Symbols: *indicates significant difference between vehicle treated control and corticosterone-treated cells, and #indicates significant difference between corticosterone and corticosterone+modulator (SQ or RU486 as indicated) (Bonferroni post-tests). (E) Corticosterone (200nM) enhanced field excitatory postsynaptic potentials (184.9 ± 7.9 % of baseline, $n = 6$, open circle, $p < 0.001$) above normal LTP-derived induction (150.8 ± 2.3 % of baseline, $n = 6$, closed circle) is abolished with pre-treatment of AC inhibitor: SQ22536 (152.6 ± 10.6 % of baseline, $n = 6$, inverted triangle, $p < 0.001$). (F) Averaged data ^from the final 10 minutes of recording. Normalized fEPSP slope (% of baseline). Error bars represent mean \pm SEM from one experiment; $n = 6$ per group.

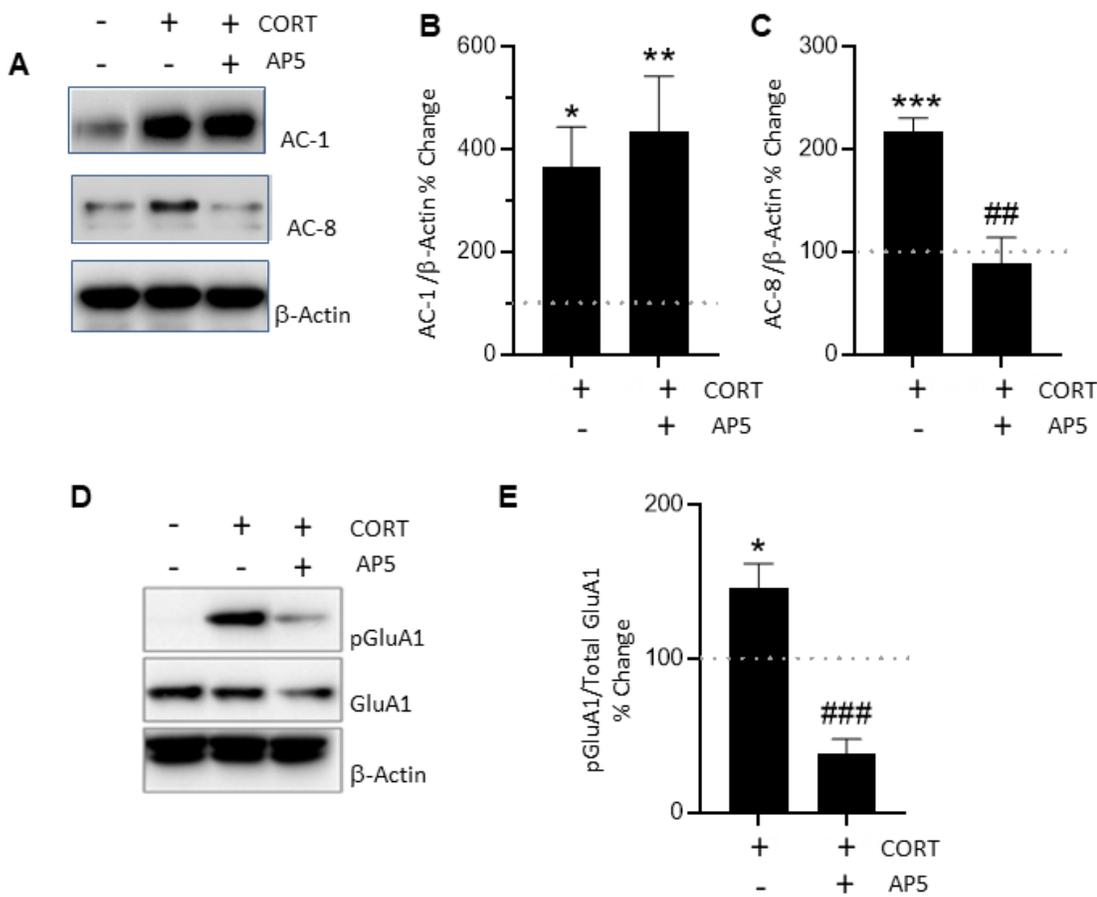


Figure 4

The corticosteroid induced increase in expression of pGluA1 and Type 1 and Type 8 adenylyl cyclases is NMDAR-dependent. Hippocampal slices were incubated with 50 μ M NMDAR antagonist (AP5) for 30 minutes followed by addition of 50nM corticosterone (or ethanol vehicle for control comparison). All slices were collected after 60 minutes of corticosterone/vehicle application. (A) Representative blots showing AC1, AC8 and β actin (loading control for normalization) protein levels. (B-C) Graphs depicting

averaged averaged quantification data show effect of NMDAR antagonist (AP5) on corticosterone-induced rapid increase (at 60 minutes) in protein expression of AC1 (B) and AC8 (C). AP5 had no effect on the corticosterone induction of AC1, but completely blocked the corticosterone-inducible expression of AC8. (D) Representative blots show phosphorylated and total GluA1, as well as β actin (loading control) protein levels. In (E), the averaged quantification data for pGluA1/ totalGluA1 ratio is presented. Corticosterone treatment induced a significant increase in GluA1 phosphorylation, which was abolished upon pre-treatment with the NMDAR antagonist. Error bars represent mean \pm SEM from one experiment; n=4 per group. Symbols: *indicates significant difference in the comparison between vehicle treatment (control) and corticosterone treatment and #indicates significant difference in comparison between corticosterone treatment and corticosterone+AP5 treatment (Bonferroni post-tests).

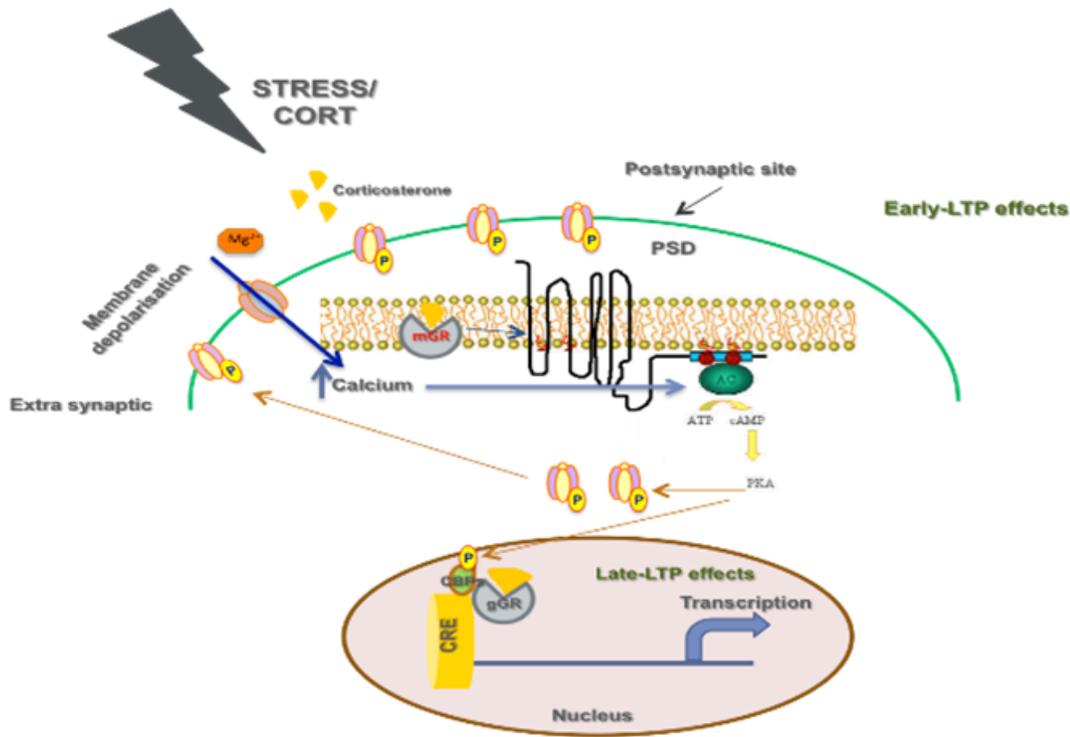


Figure 5

Theoretical model of adenylylation (AC) involvement in the regulation of hippocampal GluA1 activity during stress. Acute exposure to the glucocorticoid stress hormone can trigger postsynaptic responses, including activity-dependent regulation of synaptic transmission by the CP-AMPA receptor, GluA1, with depolarisation of the postsynaptic membrane an ensuing consequence. Membrane depolarisation allows the removal of magnesium ions blocking NMDARs enabling the influx of calcium through the ion pore

channels of the NMDA receptors. Concomitantly, increased glucocorticoid levels following stress exposure can act on membrane localised corticosterone receptors to activate GPCRs such as mGluRs, thereby triggering PLC-mediated enhancement of cytosolic calcium from intracellular stores. Increased calcium activates membrane bound adenylate cyclase enzymes. This leads to the conversion of ATP to cAMP dispelling the regulatory subunit of PKA, thus enabling activation of the catalytic part, that exerts the phospho-transferase properties of the kinase. During translation-dependent LTP, PKA phosphorylates the GluA1 subunit, and activation of these subunits enable its exocytosis into the extra-synaptic membrane. These receptors subsequently diffuse laterally into the post-synaptic density to exert their regulatory effects potentiating the neuronal synapse. PKA also phosphorylates transcriptional regulators such as CREB-binding protein (CBP). By associating with transcriptional factors such as nuclear GR, as well as other transcriptional regulators on response elements of identified promoter regions, transcription of relevant synaptic plasticity modulatory molecules are subsequently regulated.

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