Ca2+/calmodulin-stimulated Adenylyl Cyclases are Required for the Potentiating Effect of Glucocorticoid Exposure in the Rat 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. Glucocorticoids have been shown to augment synaptic potentiation processes during stress, however the underlying mechanisms have not been fully described. In this study, we have investigated the role of the Adenylyl cyclases in glucocorticoid enhancement of synaptic potentiation.

We report that stress-induced glucocorticoid secretion augments phosphorylation and postsynaptic localisation of GluA1. These effects were reproduced in adrenalectomised rats treated with a stress-equivalent dose of corticosterone; and blocked by pre-treatment with the glucocorticoid receptor (GR) antagonist RU-486. Selective pharmacological inhibition of adenylyl cyclase activity with SQ22536 ablated the glucocorticoid-induced increase in phosphorylated GluA1, and reversed glucocorticoid-dependent LTP enhancement. Glucocorticoid exposure also induced induce a rapid increase in translation of hippocampal adenylyl cyclases 1 and 8, indicating a further potential role in mediating the longer-term consequences of stress.

These data support a key role for adenylyl cyclases in facilitating the modulatory actions of glucocorticoid hormones on AMPA receptor regulation of learning and memory; initially as an acute mediator of the hormonally induced stress increase in LTP, then potentially in mediating adaptive changes for subsequent stress effects.

Introduction

During learning, 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-AMPARs e.g. homomeric GluA1-containing AMPA receptors) into synapses, is a key mechanism thought to govern neuro-plasticity processes[7,8].

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)[9–11]. Activity-dependent modulation of synaptic transmission by stress and glucocorticoids utilise multiple mechanisms including CP-AMPAR phosphorylation[12–14]. We have previously shown that stress-level glucocorticoid exposure modulates synaptic transmission via PKA-dependent phosphorylation of CP-AMPAR[15]. This is a key requirement for receptor externalization into activated synapses, leading to

further depolarization of the postsynaptic neuron and hence maintenance of LTP[12,16]. Additionally, *in vitro* studies provide evidence for glucocorticoid facilitation of AMPAR lateral diffusion across the postsynaptic membrane[13,16], another action linked to synaptic potentiation.

Despite the important role for glucocorticoids in regulating synaptic potentiation during periods of elevated hormone exposure (reviewed in *Krugers et al, 2010*[17]), 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-induced PKA-dependent insertion of CP-AMPARs into the synaptic plasma membrane, ready for 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)[18,19]. Both are regulated by calmodulin in response to high intracellular calcium levels[20,21], with AC8 exclusively requiring calcium-dependent activation, and AC1 serving as a coincidence detector requiring both calcium influx and activation of associated receptors[22,23]. Further, several lines of evidence report a role for AC1 and AC8 in hippocampal-dependent LTP[22,24] (also reviewed in *Wang et al, 2003*[23]). These hippocampal calcium-sensitive ACs are potential candidates for glucocorticoid responsiveness as they are well placed to couple rises in intracellular calcium during synaptic stimulation with our previously reported cyclic adenosine monophosphate production and PKA-mediated activation of CP-AMPARs, specifically the Type 1 – GluA1, following an acute glucocorticoid signal[25].

Here we provide evidence that hippocampal adenylyl cyclases are required during enhanced synaptic transmission following a stressful event and involved in glucocorticoid-mediated CP-AMPAR postsynaptic localization, a fundamental criterion for maintaining an active state at the neuronal synapse.

Results

Adrenal factors regulate the effects of acute stress on synaptic potentiation

Previously, we and others have demonstrated effects of acute glucocorticoid exposure on synaptic potentiation[12,13,15,16]. Here, we expand on this finding to further examine the molecular pathway between the initial stress induction and the outcome on synaptic plasticity. Adrenal-intact and adrenalectomised (ADX) rats underwent a 30-minute inescapable restraint stress protocol[26] while control animals were unstressed (figure 1A). Plasma samples were collected from each animal at times 0, 30, 60, 90 and 120 minutes, while whole hippocampi were collected at times 0 and 60 minutes from stress induction. 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 ($p<0.0001$). 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; $p<0.001$ Dunnett's post hoc test, and 3.7±1.0 in time-matched ADX rats; $p<0.0001$ Bonferroni post hoc test). Corticosterone levels were still significantly elevated above baseline at 60 minutes (565.6±136.6ng/ml, compared to 46.8±29.0 at baseline; $p<0.01$ Dunnett's post hoc test, and 3.8±0.8 in time-matched ADX rats; $p < 0.001$ Bonferroni post hoc test) before falling to baseline levels at 120 minutes.

GluA1 is known to be a key player in modulating plasticity at the hippocampal synapse, with PKA-dependent phosphorylation at its Ser-845 residue facilitating receptor insertion into the synapse widely accepted as an early step in the synaptic potentiation processes[6,27,28]. Here, we have measured pSer-845 GluA1 (pGluA1) as well as total GluA1 in synaptic plasma membrane (SPM) and postsynaptic density (PSD) cellular fractions prepared from whole hippocampi taken from adrenal-intact or adrenalectomised (ADX) rats at 60-minutes after the onset of a 30-minute restraint stress, compared to unstressed controls (figure 1 B, C). In the SPM, pGluA1 was enhanced in stressed adrenal-intact rats relative to unstressed controls, and the stress effect was ablated in ADX rats. Two-way ANOVA revealed a significant effect of stress ($p=0.0483$) and significant stress*ADX interaction ($p=0.0381$). Bonferroni post hoc multiple comparison tests showed that the stress-induced increase in pGluA1 in the SPM was significant ($p=0.0104$), and that this stress effect was significantly reduced by ADX ($p=0.0274$). In the PSD, no significant effect of stress, ADX, or interaction was found for pGluA1. No significant effect of stress, ADX, or interaction was found for total GluA1 in either the SPM or the PSD. These data show that the small but significant increase in SPM pGluA1 occurs in response to stress and is adrenal-dependent.

Corticosteroid receptor antagonism with RU-486 further modulates the glucocorticoid effect on pGluA1.

We next tested whether acute corticosterone treatment would be sufficient to reinstate the effect of stress on pGluA1, and conversely whether pre-treatment with the corticosteroid receptor antagonist RU-486 would ablate the corticosterone treatment effect. Hippocampi taken from ADX rats killed exactly 60 minutes after receiving a stress dose of corticosterone (3mg/kg i.p.) with or without pre-treatment with RU-486 (20mg/kg i.p.) were again processed to generate SPM and PSD fractions to assess pGluA1 and total GluA1 (figure 2 A, B). Control ADX rats received subcutaneous injections of vehicle (ethanol diluted in saline). A significant effect of treatment was detected by One-way ANOVA in both SPM ($p=0.0163$) and PSD fractions ($p=0.0005$). Interestingly, Bonferroni post hoc tests revealed that the small but significant corticosterone-induced increase in pGluA1 in the SPM of corticosterone-treated ADX rats ($p=0.0464$), was significantly augmented by pre-treatment with RU-468 ($p=0.0181$). In striking contrast, in the PSD, the significant corticosterone-induced increase in pGluA1 ($p=0.0021$) was blocked by RU-486 ($p=0.0007$).

Significant effects of treatment were also found for total GluA1 in the SPM ($p=0.0054$) and PSD ($p<0.0001$) by One-way ANOVA. Similar to effects seen with pGluA1, Bonferroni post hoc tests revealed that RU486 treatment caused a significant increase in corticosterone-induced total GluA1 levels in the synaptic plasma membrane ($p=0.0055$), and a significant decrease in the postsynaptic density ($p=0.002$). Taken together these results are consistent with a corticosterone-inducible RU-486-sensitive mechanism, potentially involving lateral movement of pGluA1 from the SPM to the PSD.

The glucocorticoid-induced increase in total cellular content of pGluA1 is translation-dependent and transcription-independent.

We further tested whether the corticosterone-dependent effects observed also affected total cellular GluA1 and pGluA1 levels, and whether these effects involved classical transcriptional mechanisms and *de novo* protein synthesis. *Ex vivo* application of the protein translation inhibitor Cycloheximide (CX) and the transcription inhibitor Actinomycin D (Act D) were applied to hippocampal slices, subsequently incubated for 60 minutes with corticosterone then homogenised to release total cellular content (figure 2 C, D). A significant effect of treatment was detected for pGluA1 in these whole cell extracts ($p=0.0068$; One way ANOVA). A significant decrease in corticosterone-induced pGluA1 was detected in the CX pre-treated tissue ($p<0.001$, Bonferroni post hoc test) without a significant reduction in total GluA1. Act D had no effect on the corticosterone-inducible pGluA1 or total GluA1 levels.

Together, these data indicate that a glucocorticoid-induced translation-dependent transcription-independent increase in phosphorylation of GluA1 is seen in total cellular pGluA1 content, and further that glucocorticoid-induced changes in pGluA1 enrichment in defined parts of the plasma membrane (namely the SPM and PSD) is disrupted by antagonism of corticosteroid receptors. While RU-486 is generally thought of as a Type2 corticosteroid receptor (GR) antagonist, we cannot rule out the possibility of off-target effects on the Type1 corticosteroid receptor (MR) which is also highly expressed in the hippocampus, reported to be membrane localised[10,29,30], and involved in synaptic potentiation mechanisms[9]. Importantly, our data suggest that glucocorticoid regulation of GluA1 function may be acting via more than one mechanism. The first mechanism via rapid translation of a protein required for phosphorylation of GluA1, either a kinase or a kinase-regulating accessory protein, and the second potential mechanism via a corticosteroid receptor-dependent lateral movement of pGluA1 from the SPM to the PSD. Considering that pre-treatment with the corticosteroid receptor antagonist RU-486 resulted in far greater accumulation of pGluA1 in the SPM than seen in physiological conditions of stress or corticosterone treatment, a model involving lateral diffusion of pGluA1 (see Park et al, 2018[8] for review) is strongly supported by our data.

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

Activation of GluA1 via phosphorylation by PKA is a well-described mechanism for functional neuroplasticity during stress[31-34]. Yet, the link between signal reception at the synapse, the increase in intracellular calcium and further downstream activation of cAMP[35] to upregulate levels of the active catalytic subunit of protein kinase A, which mediates its phospho-transferase abilities[36] is still unclear. One group of synaptic associated molecules with potential for both processes i.e. utilization of increased calcium following NMDAR activation, with consequent increase in cAMP-mediated activation of PKA, are the calcium stimulated enzymes, the adenylyl cyclases (AC).

We therefore next investigated the requirement of these calcium sensitive enzymes in the glucocorticoid-induced increase in total cellular pGluA1 levels in *ex vivo* hippocampal slices. Pre-treatment with the AC inhibitor (SQ22536) prior to corticosterone treatment resulted in inhibition of the corticosterone-induced increase in pGluA1 without affecting total cellular levels of GluA1 (figure 3A, B). A significant effect of treatment on pGluA1 in the whole cell extracts ($p=0.0083$; One-way ANOVA) was found, with Bonferroni post hoc tests revealing a significant corticosterone-induced increase in pGluA1 ($p=0.021$) and significant inhibition of the corticosterone-induction by SQ22536 ($p=0.020$). Together, these findings support a necessary role for adenylyl cyclases in corticosterone-induced phosphorylation of GluA1.

To assess whether there was a functional sequela to the reduction in corticosterone-dependent pGluA1 after AC inhibition, we next evaluated the effect of SQ22536 on corticosterone-enhanced LTP (figure 3C). A high frequency stimulation (HFS) protocol was applied to acute hippocampal slices. This triggered an increase in field excitatory postsynaptic potentials (150.8 ±2.3% of baseline). As we have shown previously[15], corticosterone treatment markedly augmented the LTP response (184.9 ± 7.9 % of baseline, $p < 0.001$). The augmenting effect of corticosterone was completely abolished by pre-treatment with the AC inhibitor SQ22536 (152.6 ± 10.6 % of baseline, One-way ANOVA $p = 0.011$, with Bonferroni post hoc tests; control vs corticosterone, $p < 0.05$, corticosterone vs corticosterone + SQ, $p < 0.05$; figure 3D). These results demonstrate for the first time that glucocorticoid-mediated amplification of EPSPs following LTP stimulation is dependent on adenylyl cyclases.

NMDAR-dependence for rapid corticosterone-induced increases in both Adenylate cyclase protein expression and phosphorylation of GluA1.

Pertinent to the mechanisms we have explored in this paper, we next interrogated the upstream role of NMDARs on adenylyl cyclases and PKA activation-dependent phosphorylation of GluA1. While it is already known that AC activity is regulated by calcium influx after NMDAR-dependent membrane depolarisation[35,37], the role that glucocorticoids play in this part of the signalling cascade in relation to

GluA-1 dependent synaptic potentiation is not yet understood. We found that corticosterone treatment of *ex vivo* hippocampal slices was able to rapidly induce an increase in total protein levels for both AC1 and AC8 as early as 60 minutes of treatment time (Figure 4A). However, only the AC8 corticosterone-dependent increase was sensitive to pre-treatment with the selective NMDAR antagonist, AP5. As AP5 competitively binds the receptor's ligand binding site, blocking ligand binding, and consequently preventing calcium influx via the receptor's cation channel[38], our results indicate that rapid glucocorticoid-induced AC8 translation may be NMDAR-dependent in a similar manner to that already described for AC1 activity, albeit in cortical synapses[39]. In contrast, the corticosterone-induced increase in AC1 was not sensitive to AP5 pre-treatment, supporting a lack of NMDAR and calcium-dependence for its rapid translation in the hippocampus. Finally, and as predicted based upon our previous work[15], NMDAR inhibition with AP5 also ablated the corticosterone-induced increase in total pGluA1 levels (figure 4D).

As indicated, significant effects of treatment were found by One-way ANOVA for AC1 ($p=0.0191$), AC8 ($p=0.0007$) and pGluA1 ($p=0.0001$) (figures 4B, C, E). Bonferroni post hoc test results are indicated on each graph, showing that the corticosterone-induced increase in AC1 ($p<0.05$) was not blocked by AP5 (figure 4B), the corticosterone-induced increase in AC8 ($p<0.001$) was ablated by pre-treatment with AP5 ($p<0.01$) (figure 4C) and finally that the corticosterone-induced GluA1 phosphorylation ($p<0.05$) was also completely abolished by AP5 ($p<0.001$; figure 4E).

Taken together, our findings indicate that - in addition to known calcium-dependent activation required for consequent PKA-dependent phosphorylation events[40] - glucocorticoids induce increased phosphorylation of GluA1 via a mechanism involving both NMDAR-dependent and AC-dependent activity.

Discussion

Glucocorticoids exert multiple effects on the body's physiology, through actions that can occur rapidly, as well as over a longer timeframe (reviewed in *Joels et al, 2018*[41]). The rapid transcription-independent effects that take place outside of the classical genomic regulatory actions are of particular interest given that acute glucocorticoid exposure can enhance synaptic potentiation, even within minutes of hormone exposure[9,42]. 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[9,10,30]. For example, glucocorticoid-dependent increases in frequency of miniature excitatory postsynaptic currents (mEPSCs) [4343] have been demonstrated to be mediated by membrane-localised corticosteroid receptors that facilitate synaptic potentiation via CP-AMPARs[9,15,29]. Synapses containing calcium permeable homomeric GluA1 subunit compositions are classically accepted as being fundamental prerequisites for synaptic potentiation processes[34]. 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.

In the current study, we have identified calcium-sensitive adenylyl cyclases (ACs) as key players in the fast-acting glucocorticoid-dependent augmentation of hippocampal synaptic potentiation. Here, we show that both acute stress and glucocorticoid exposure can induce increased GluA1 phosphorylation and synaptic insertion (also see Supplemental figure 1). Phosphorylation of homomeric GluA1 has previously been demonstrated to be PKA-mediated and required for its insertion into the plasma membrane where it is thought to undergo lateral diffusion between the SPM and PSD[44,45]. This mechanism has been shown to involve protein: protein interactions between GluA1 and accessory proteins such as SAP97 that anchor GluA1 receptors at the PSD[46,47]. Indeed, ACs and PKA are linked to GluA1 via these SAP97-containing complexes[48]. Expanding upon this mechanism, we demonstrate a key role of corticosteroid receptors in this process, as blockade with RU-486 induced simultaneous pGluA1 enrichment in the SPM and depletion in the PSD, consistent with inhibition of GluA1 lateral diffusion from the SPM to PSD, and/or a lack of PSD anchorage. Our findings are consistent with recent evidence by Chen et al, 2021. Using 2-photon microscopy, the authors described a role for corticosterone in acute AMPAR mobility between synaptic fractions in-vivo[49]. We further demonstrate that the rapid phosphorylation of GluA1 required for its lateral diffusion across the synaptic plasma membrane may be dependent on *de novo* protein synthesis, since the translation inhibitor cycloheximide blocked the glucocorticoid-induced increase in pGluA1 levels. This could possibly represent the rapid translation of a protein required for phosphorylation of GluA1 i.e. either a kinase or a kinase-regulating accessory protein. For instance, Yuen *et al*. showed an enhancing effect of glucocorticoid on GluA1 synaptic expression [12,50]. Their studies revealed that corticosterone-dependent modulation of synaptic GluA1 may in part be regulated by the immediate early response protein – serum and glucocorticoid-inducible kinase, SGK-1; which is itself rapidly translated *de novo* in response to corticosterone[50].

The fundamental requirement for elevated intracellular calcium in the process of synaptic incorporation of activated GluA1 in excitatory synapses is well recognised[6,27,44,45,51]. The primary source of increased cytosolic calcium during periods of high synaptic activity is influx through NMDARs[37]. While it is known that increased intracellular calcium triggers the activation of second messenger pathways through the calcium/calmodulin stimulation of adenylyl cyclases, thereby inducing activation of PKA[35], and subsequent phosphorylation of GluA1[27], we have now shown that glucocorticoids modulate these processes interdependently during stress to facilitate synaptic potentiation.

Our mechanistic studies into molecular mediators of the glucocorticoid-dependent postsynaptic incorporation of pGluA1 suggest that calcium sensitive adenylyl cyclases located in the hippocampal synapse i.e. AC1 and AC8, are crucial for the glucocorticoid-dependent effects on this mechanism of synaptic potentiation. For instance, our results revealed that GR-dependent phosphorylation of GluA1 requires ACs, as we observed a substantial increase in pGluA1 synaptic expression after glucocorticoid exposure, an effect that was blocked upon treatment by both GR antagonist and inhibition of AC activity.

By preventing calcium-sensitive AC activity with the use of a selective inhibitor, we observed blockade 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. Our finding of AC-dependent phosphorylation of GluA1 by PKA does corroborate a previous report by Kim *et al*. [52]. Using mathematical simulation models, the authors found that PKA-dependent GluA1 activation is enhanced when the activating kinase is localised near its source molecule, i.e. adenylyl cyclases[52]. They surmised that localization of PKA near ACs during long-lasting LTP is crucial for GluA1-Serine 845 activation by PKA, thus labelling the receptor protein ready for lateral diffusion into postsynaptic terminals[27,45,52]. The proximity of calcium sensitive ACs to PKA and GluA1 further prevents GluA1 inactivation by phosphodiesterases, as well as limited diffusion of AC-derived cAMP[52].

Further support for the crucial role of ACs come from knock out mouse studies, suggesting AC involvement in mediating normal behavioural responses to stress[21,53,54], with evidence implicating their importance in memory consolidation and retention[54–56]; behaviours that are clearly linked to the low affinity corticosteroid receptor (GR)-mediated actions during stress[57–62].

Our study does have some methodological limitations that should be considered when interpreting the findings. The AC inhibitor used for the experiments is not selective to any individual AC isoform but instead inhibits known isoforms. To our knowledge, no AC isoform-specific inhibitor has yet been developed[63], thus we can only speculate on the AC isoform(s) contributing to the elucidated pathway. Nevertheless, our experiments have focussed on assessment of the downstream functional consequences of blocking AC activity rather than directly measuring individual AC isoform activity. Given the pathway investigated in this study, the most likely adenylyl cyclases that mediate the cAMP-related activation of PKA for selective GluA1 phosphorylation are AC8 and AC1. Using double-knockout animal models (AC1/AC8 KO), Wong et al showed that these isoforms are critical for providing the cAMP signal to initiate LTP and long-term-memory[24]. Supporting evidence for the role of these two ACs in modulating hippocampal synaptic plasticity have also been provided by others[21], with AC8 shown to be targeted directly to excitatory synapses[22]. Furthermore, of the two calcium/calmodulin-sensitive adenylyl cyclases present in the hippocampal synapse – AC1 and AC8; AC8 is a pure calcium sensor[24,53,64,65]. During periods of increased intracellular calcium, AC8 has been shown to be localised at excitatory synapses in hippocampal neurons, with AC1 more distal[22], suggesting that AC8 may be maximally stimulated in an activity-dependent manner, such as evident during stress, with AC1 mediating previously-described NMDAR-independent early synaptic potentiation during stress[15]; although these hypotheses remain to be tested experimentally.

We therefore propose a mechanistic model whereby stress-induced glucocorticoid exposure acts to potentiate the calcium signal for AC mediated actions at the glutamatergic synapse (see Figure 5). This mechanism may, at least in part, come from synaptic NMDARs following membrane depolarisation in the early phases of the stress response, with enhancement of cytosolic calcium mobilised from intracellular stores an additive phenomenon[25]. We further demonstrated a significant glucocorticoid-dependent augmentation of the PKA-phosphorylation of the GluA1 subunit, to enable its exocytosis and subsequent lateral diffusion into the postsynaptic density which would facilitate AMPAR-mediated LTP[15,66]. In this

way, synaptic potentiation may be directly modulated by glucocorticoids during the stress response. Our key finding that inhibition of AC activity prevents glucocorticoid enhancement of both GluA1 phosphorylation and LTP enhancement, further suggests that calcium-sensitive hippocampal membrane bound adenylate cyclase enzymes may be an essential component for the modulation of plasticity at glutamatergic synapses during stress, and/or glucocorticoid exposure.

Interestingly, our results also revealed an unexpected additional effect of GC exposure on upregulation of both AC1 and AC8 proteins, with AC8 requiring NMDAR activation, potentially indicating a role for AC in adaptive changes in the longer term. As ACs have been found to be necessary for modulating fear-related behaviours during stressful episodes, they may also influence memory processing in people who have undergone traumatic situations[53]. Finally, this anxiogenic-linked molecule could potentially be targeted therapeutically for the treatment of anxiety-related conditions, as well as stress-related memory impairments.

Conclusion

The precise mechanisms underlying the augmentation of synaptic potentiation in response to an acute stress have long remained elusive. 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 the high levels of glucocorticoids that occur during emotionally arousing events. The implications of this work are that glucocorticoids act in concert with calcium activated ACs to induce AMPAR-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 neurological conditions featuring synaptopathologies.

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 are described below.

Adrenalectomy:

Bilateral adrenalectomy (ADX) was performed as previously described[67] 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 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 for a further 30 minutes prior to decapitation and hippocampal dissection, in order to assess the hippocampus at 60 minutes after stress induction in comparison to baseline (0 minutes). To assess the full induction and recovery profile of the stress-induced corticosterone response, trunk blood was collected from all rats used for hippocampal analyses at baseline (0 minutes) and 60 minutes, as well as additional rats at times 30. 90 and 120 minutes.

In vivo drug treatments:

ADX rats were treated with vehicle (ethanol) or GR antagonist (RU-486: 20mg/kg) followed by saline or corticosterone (3mg/kg) 30 mins later via subcutaneous injections. Hippocampi were collected 60 minutes after the Corticosterone or saline injections. Animals in the control group received vehicle and saline injections only.

Evaluation of protein localization and expression in rodent hippocampal tissue:-

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[68,69]. Synaptic plasma membrane (SPM) and postsynaptic density (PSD) fractions were prepared by sequential subcellular fractionation (Detailed description in Supplementary methods).

Preparation of acute hippocampal slices:

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 $NaHCO_3$, 1.25mM $NaH2PO_4$, 2mM $Cacl_2$, 1mM $MgSO_4$ and 10mM glucose). The hippocampi were dissected, and hemispheric slices prepared with a McIlwain tissue chopper (Mickle Laboratory Engineering Company, Guildford, UK). Slices were continuously perfused with aCSF at room temperature for approximately 1-hour, then gently

transferred to nylon meshes in dishes containing aCSF with constant perfusion of the previously outlined gas mixture.

Ex vivo drug treatments in acute hippocampal slices:

Acute hippocampal slices from ADX rats were treated with one of the following drugs; translational inhibitor cycloheximide (CX; 50μM), transcriptional inhibitor Actinomysin-D (ActD; 40μM), AC inhibitor SQ22536 (SQ; 10μM), NMDAR antagonist (AP5; 50μM), or vehicle control (equivalent volume of ethanol used for each drug). Each drug (or matched vehicle control) was added directly to the dish containing slices for 30 minutes, followed by treatment with corticosterone (100nM) for 60 minutes. At the end of the treatment, slices were collected and whole cell extracts processed for Western blot. (Baseline control samples were prepared from hippocampal slices incubated for 90 minutes with the equivalent volume of vehicle (ethanol) for each drug treatment.

Analysis of whole cell protein expression in acute hippocampal slices:

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, protease inhibitor cocktail, 1mM NaF and NaVan phosphatase inhibitor). Following treatment, hippocampal slices were transferred to 1.5ml eppendorf tubes and snap frozen in liquid nitrogen. Lysis buffer (100μl) was added to each tube and samples homogenised, and then centrifuged (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 western blot.

Western blot:

Protein samples were run on SDS polyacrylamide gel (SDS-PAGE) electrophoresis as described by Laemmli[70] 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. The following monoclonal antibodies used include: anti-β-actin (1/10 000), anti-PSD-95 (1/1,000) and anti-synaptophysin (1/1,000) from Abcam; anti-AC8 (1/1,000) from Novus biologicals; anti-phospho GluR1 S845 (1/2,000) from Millipore; anti-GluR1 (1/250 dilution) and anti-AC1 (1/500) from Santa Cruz biotechnology. Membranes were then incubated in either rabbit or mouse IgG antibodies (1/5000 dilution, Millipore) conjugated to horseradish peroxidise and immunoblotted using the ECL detection system (Thermo Scientific Inc.), and visualised with a G:Box (Syngene).

Western blot analysis:

Densitometric analysis was performed using Quantity-one software (Bio-Rad Laboratories, Hercules, CA). Adjusted volumes of optical density values for all immunoblot protein bands were normalised to levels of endogenous protein markers, either β-actin or relevant fraction-specific marker as indicated in each figure. To account for interblot variability, a control sample (baseline/untreated) was included on each blot so that each treated sample could be calculated as fold change relative to control.

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.

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 I^{125} tracer (Izotop, Budapest, Hungary) as previously described[71,72] and according to the detailed protocol in provides as supplemental 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 ± 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. Two-way ANOVA, with Bonferroni correction was applied for the *in vivo* experiments, where analysis of two independent factors influenced an experimental condition. Where statistical significance was found, further analysis was performed using multiple comparison post hoc tests. For the *in vivo* stress induction experiment, Dunnett's post hoc tests were used to compare each time point relative to baseline (time 0), and Bonferroni post hoc tests were used to compare between ADX and intact rats at matched time points.

For all remaining drug treatment studies, both *in vivo* and in *ex vivo* hippocampal slices, one-way analysis of variance (ANOVA) followed by multiple comparison correction procedure to correct for Type 1 error (Bonferroni *post hoc* test) was applied. Statistical significance was set at $p < 0.05$, and significant differences determined by multiple comparison post hoc tests are indicated on graphs: *or # P <0.05, ** or ## P <0.01, *** or ### P<0.001, **** or #### P<0.0001.

Declarations

Data Availability and Accessibility Statement

All data generated or analysed during this study are included in this published article (and the Supplemental Information files). Any further information on raw data will be made available upon request to the corresponding author.

Ethics statement:

The 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 [73].

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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 with BC. 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

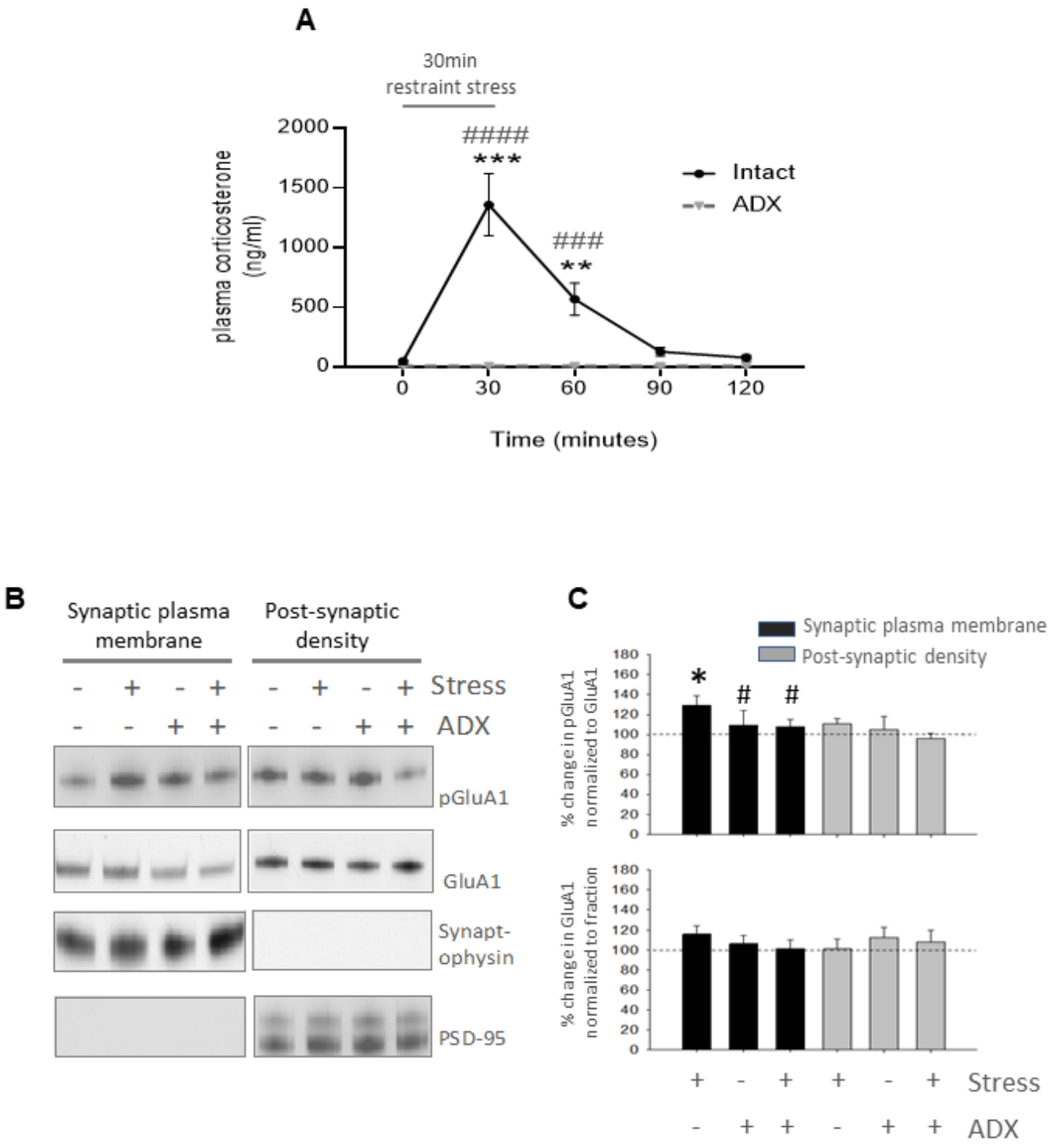

Figure 1

GluA1 regulation after acute stress is adrenal-dependent. A. Temporal dynamics of corticosterone secretion following 30 mins restraint stress exposure. Blood was collected from adrenal intact or ADX rats at times 0, 30 and 60 mins after the onset of a 30-minute restraint stress. Hippocampi were collected at times 0 and 60 mins. 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 ± SEM for two independent experiments; n ≥6 per time point group. B, C. Stress-induced adrenal hormones modulate GluA1 proteins in the rat hippocampus at 60 minutes after the onset of stress. (B) 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. (C) 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 postsynaptic density. Total GluA1 expression was unchanged in both intact and ADX animals. n ≥4 animals were utilised 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).

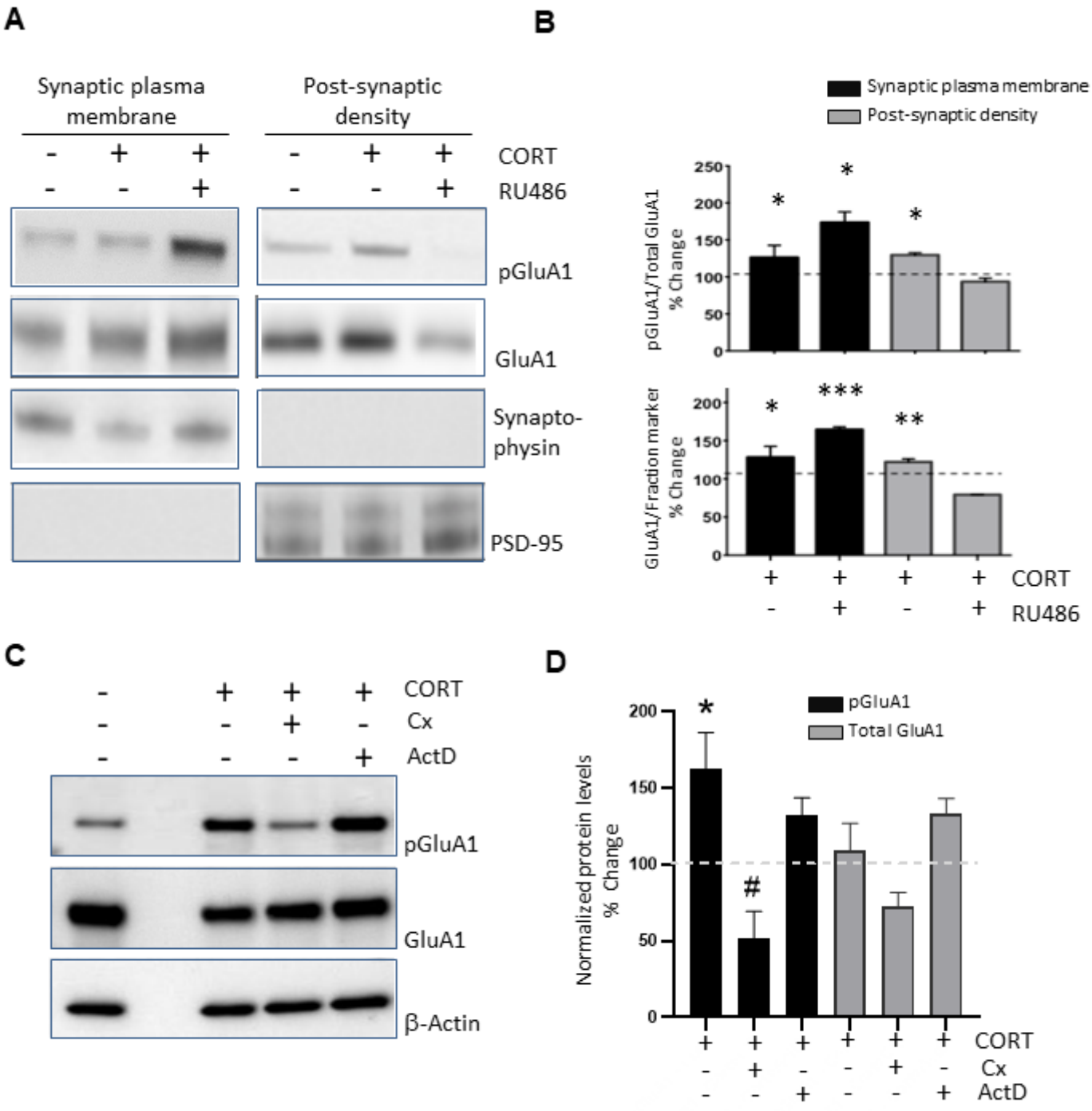

Figure 2

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 (50μM) or Actinomycin-D (40μM) 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 ± SEM from one experiment; n≥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).

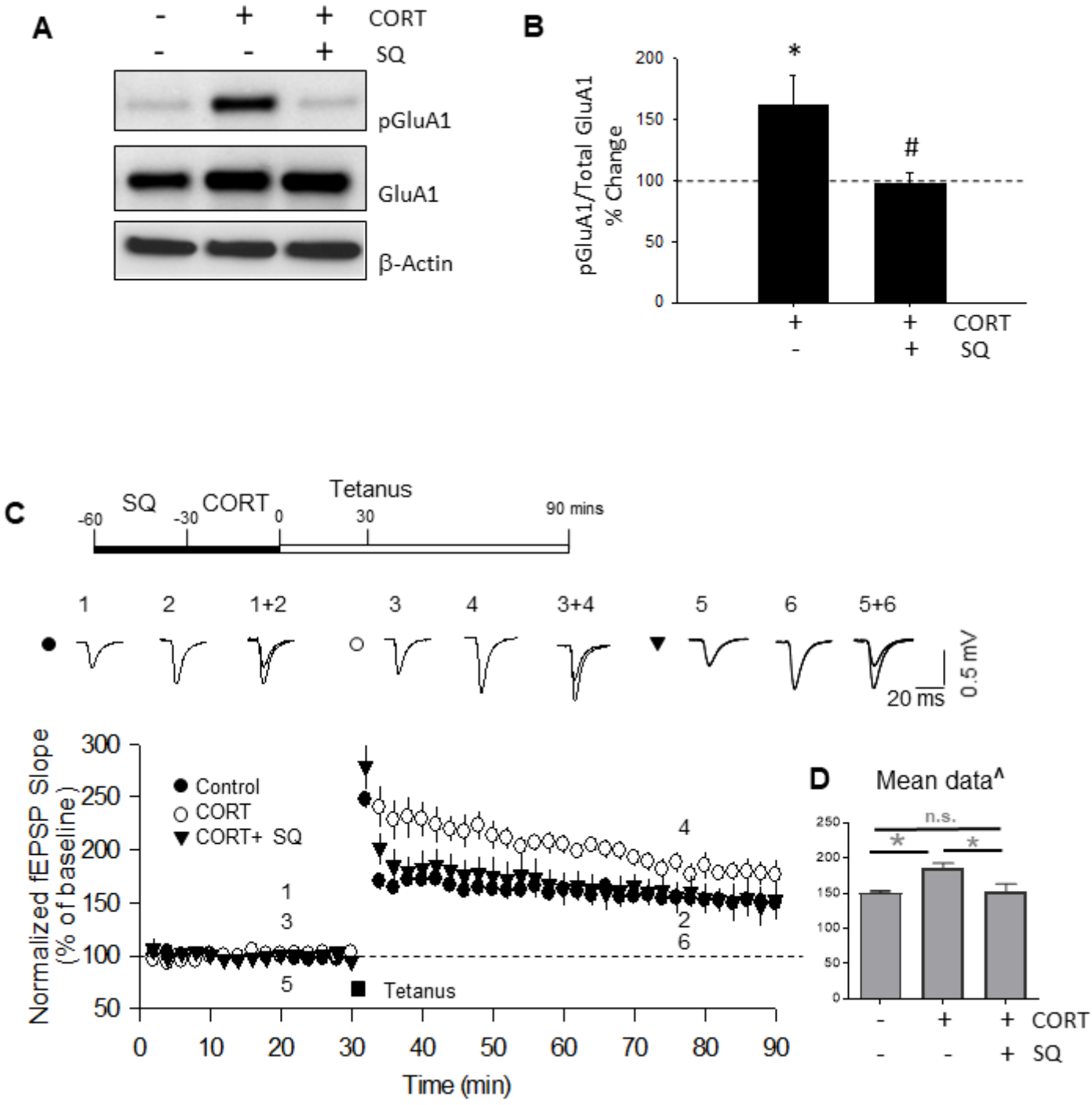

Figure 3

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 (10μM) 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 SEM from one experiment; n≥4 per group. Symbols: *significant difference between vehicle control and corticosterone treated slices, and #significant

difference between corticosterone and corticosterone+SQ (Bonferroni tests). (C) 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). (D) Averaged data ^from the final 10 minutes of recording. Normalised fEPSP slope (% of baseline). Error bars represent mean ± SEM from one experiment; n=6 per group.

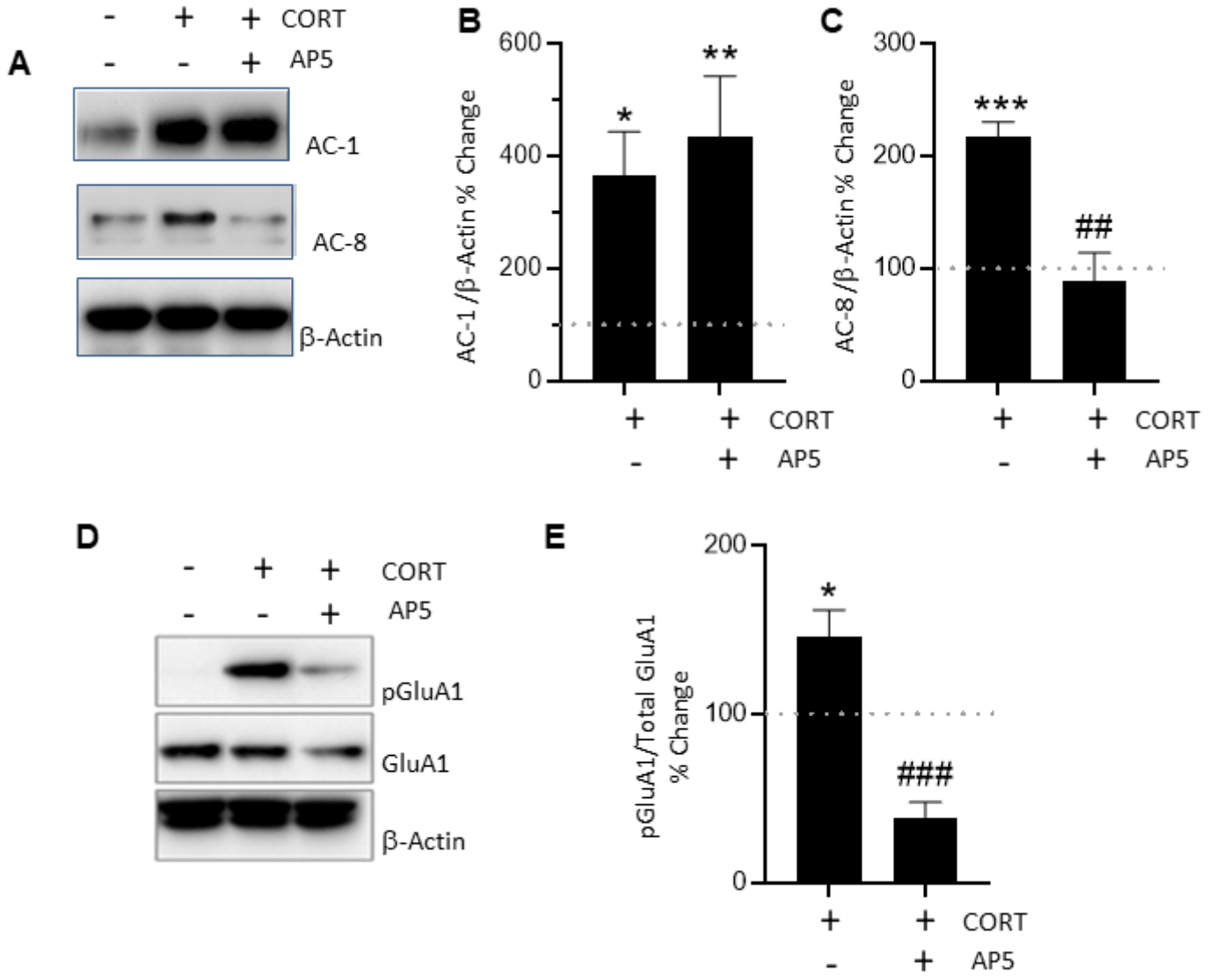

Figure 4

The corticosteroid induced increase in expression of pGluA1 and Type 1 and Type 8 adenylyl cyclases is NMDAR-dependent. Hippocampal slices were incubated with 50μM 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 ± 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 hoc tests).

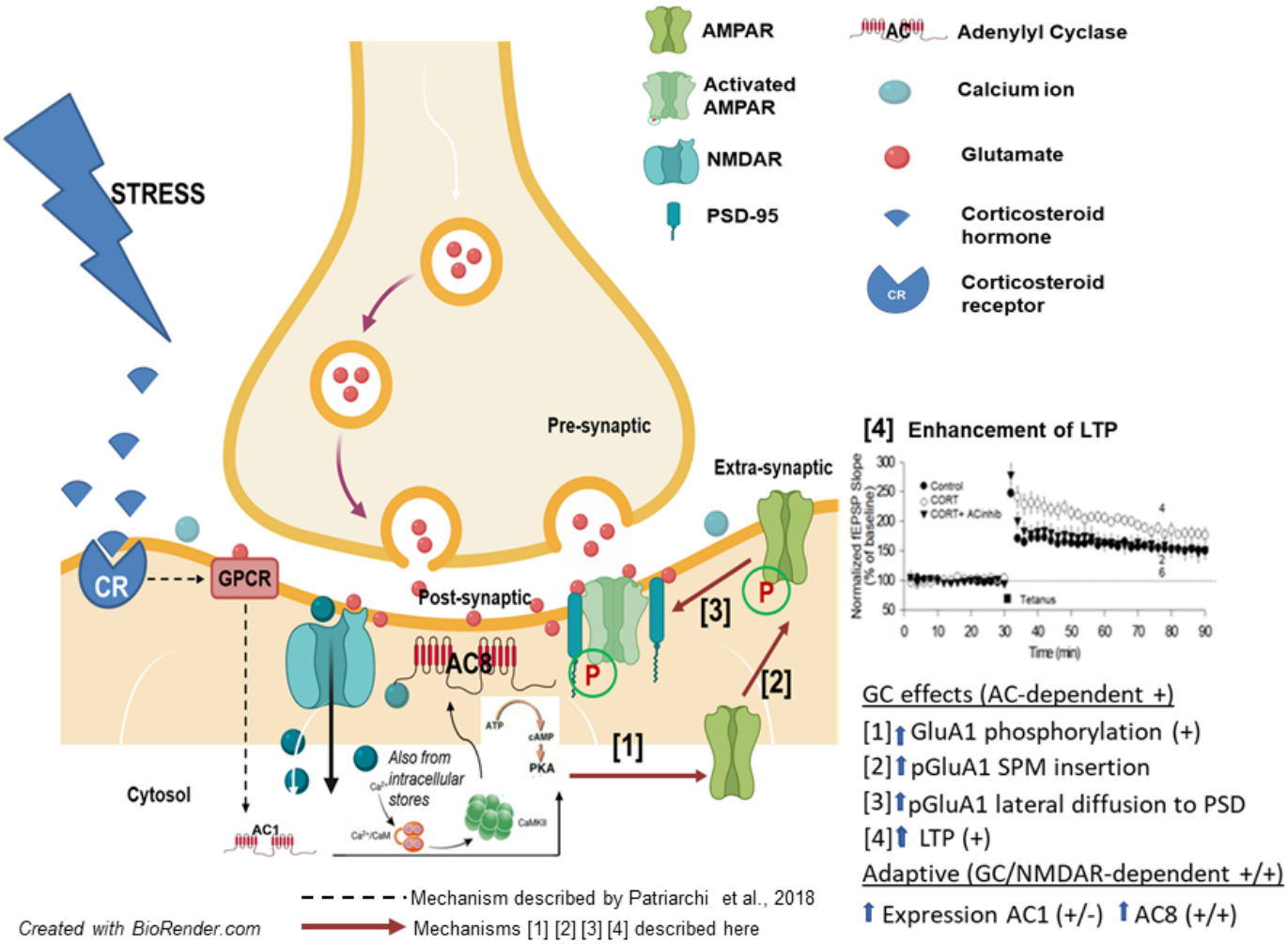

Figure 5

Theoretical model of adenylate cyclase (AC) involvement in the regulation of hippocampal GluA1 activity during stress. In this model we illustrate how rapid glucocorticoid mediated actions facilitated by the Ca2+/calmodulin-stimulated adenylyl cyclases can enable maintenance of LTP in excitatory synapses during stress and/or glucocorticoid exposure. Here we expand upon the findings by Patriarchi et al 2018 and Nunez et al 2020, who showed that membrane-localised corticosteroid receptors can act via a novel

GPCR to induce adenylyl cyclase activity-dependent cAMP production40,66, and our own previous data showing GCs induce increased intracellular calcium and subsequent PKA-dependent phosphorylation of GluA115. We show in the present study that corticosteroid-signalling regulates [1] phosphorylation of GluA1 at the Ser845 residue primed by rises in intracellular Ca2+, [2] incorporation of pGluA1 into the synaptic plasma membrane (SPM) at extra-synaptic sites, [3] lateral diffusion into the postsynaptic density (PSD), resulting in [4] augmentation of long term potentiation at the neuronal synapse. We demonstrate that steps [1] and [4] are ablated by pre-incubation with the adenylyl cyclase inhibitor SQ22536, strongly suggesting that adenylyl cyclases are a key player in the mechanism whereby stress and/or glucocorticoid exposure enhances long term potentiation in excitatory synapses. Glucocorticoid-induced translation of AC1 and AC8, may further act as a mediator of adaptive changes to subsequent effects of stress.

Supplementary Files

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- Supplementalinformationwithrawdata.pdf