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

Supplemental Information

Supplemental Information on Methods

Evaluation of GluA1 localization in rodent brain tissue

Enriched SPM fractions were prepared from homogenized hippocampal tissue samples in accordance with the method previously described by Blackstone *et al*.[1,2]. Cells were homogenised in a hypotonic homogenization buffer [0.32M Sucrose, 4mM HEPES (pH 7.4)], supplemented with 1mm sodium orthovanadate, 0.2 mm sodium fluoride, and Complete Protease Inhibitor (Roche Diagnostics Ltd., Burgess Hill, UK)]. The resulting homogenate was then centrifuged at 4 ^{o}C,1000 g for 5 minutes. The pellet containing chromatin and cellular debris was disposed of, and the remaining supernatant centrifuged at 10000 g for 15 minutes to yield the crude synaptosomal pellet. The resultant supernatant from this spin contained the light membrane and cytosolic fractions, and these were obtained by further spinning in an ultracentrifuge with a fixed angle rotor at 100000 g for 15 minutes to extract the pure cytosol (supernatant) and light membrane pellet (containing endoplasmic reticulum and Golgi membranes) which was re-suspended in the hypotonic extraction buffer. Concomitantly centrifuging at 10000g for 15 minutes yielded a synaptosomal fraction. The resulting pellet was lysed by hypo-osmotic shock using ice-cold distilled water containing protease and phosphatase inhibitors, and then rapidly adjusted back to original pH. The solution was adequately mixed to ensure complete lysis, and then centrifuged at 25000 g for 20 minutes to yield supernatant containing synaptic vesicle proteins (pre-synaptic fraction). The pellet from this spin is the lysed synaptosomal membrane fraction, and this was re-suspended in the extraction buffer, and with the aid of a Pasteur pipette, layered on top of a discontinuous gradient containing 0.8 to 1.0 to 1.2 M sucrose, which equates to 27%/ 34%/ 41. The gradient was spun at 150000g for 2 hours in an ultracentrifuge with a swing bucket rotor. The resultant

pellet was recovered in the layer between the 1.0 M and 1.2 M sucrose by puncturing the ultracentrifuge tubes with a 25G needle and then carefully withdrawing the band of pellet with a 1 ml syringe. The recovered pellet, which had high sucrose content, was diluted back to 0.32 M sucrose, and pelleted out further by spinning at 150000 g for 30 minutes. The pellet containing synaptic plasma membrane fraction was re-suspended in a hypertonic extraction buffer containing 50 mM HEPES pH 7.4 and 2 mM EDTA with protease and phosphatase inhibitors added. The pellet was further cleaned via passage through a 25G needle (Terumo, UK), and a portion aliquoted for use as enriched SPM. In accordance with experimental guidelines described by Cho and others[1,2], proteins localized in the postsynaptic density were further extracted from the remaining re-suspended pellet (synaptic plasma membrane fraction). Addition of 0.5% Triton X-100 (Sigma) breaks down the intact synaptic plasma membrane, and after the protein sample had been subjected to centrifugation at 32000 g for 20 minutes, another pellet (PSD 1T) containing proteins localized in the postsynaptic density was obtained. This pellet was resuspended in the hypertonic buffer and an aliquot stored till further use. To the remaining resuspended pellet, 3% N-lauroyl sarcosine (Sigma) was added to further solubilize the postsynaptic density proteins, and then spun at 200000 g for 1 hour. This allowed specific extraction of a pellet (PSD 1T+S) containing highly enriched postsynaptic density proteins and their associative molecules. Concentrations of all protein extracts were determined using a standard bicinchoninic acid (BCA) protein assay (Pierce, UK). The integrity of the processed samples was verified by western blot analysis using relevant markers for each protein fraction.

Visualization of GluA1 localization in cultured rat primary hippocampal neurons using confocal microscopy (Immunocytochemistry):

Primary hippocampal neurons were prepared from 1-day-old male Wistar rat pups. 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 antibody – GluA1 at 4°C in a dark humidified atmosphere, with gentle shaking. The following day, cells were washed three times with PBS, and permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature, further washed three times before incubation with C-terminus/intracellular epitope antibody for MAP-2. 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-Phenyllindole (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.

Measurement of hormone levels in plasma: Plasma samples were prepared by centrifuging whole blood collected from the trunks of decapitated animals. Blood samples were spun at 15 000 g for 15 minutes at 4 °C, collected into pre-labelled eppendorf tubes and stored at -20 °C until use in immunoassay. For the assay, plasma samples were thawed and diluted 1:50 in an acidic (pH 3) buffer (B-buffer; consisting of bovine serum albumin, tri sodium citrate and sodium hydrogen orthophosphate). 50 µl of rabbit anti-rat corticosterone primary antibody

(kindly donated by Prof G Makara – Institute of Experimental medicine, Budapest, Hungary), also diluted 1:50 in B buffer, and 50µl of a corticosterone I^{125} tracer (at counts between 3500-4000 cpm in B buffer; Izotop, Budapest, Hungary) were added to 100µl of diluted sample. This mixture was incubated overnight at 4 ^{o}C. The next day, samples were precipitated using a charcoal-dextran (Fluka Analytical, Steinham, Germany) solution (10:1), and spun in a refrigerated centrifuge cooled down to 4°C at 15000 g for 15 minutes, so that the free fraction of corticosterone (i.e. corticosterone unbound to the antibody) would be absorbed onto the activated charcoal. The supernatant was then aspirated, and the resultant charcoal pellet containing the total corticosterone in each sample was counted on a gamma counter (E5010 Cobra II Auto-Gamma, Perkin-Elmer, Groningen, Netherlands).

Supplemental Data

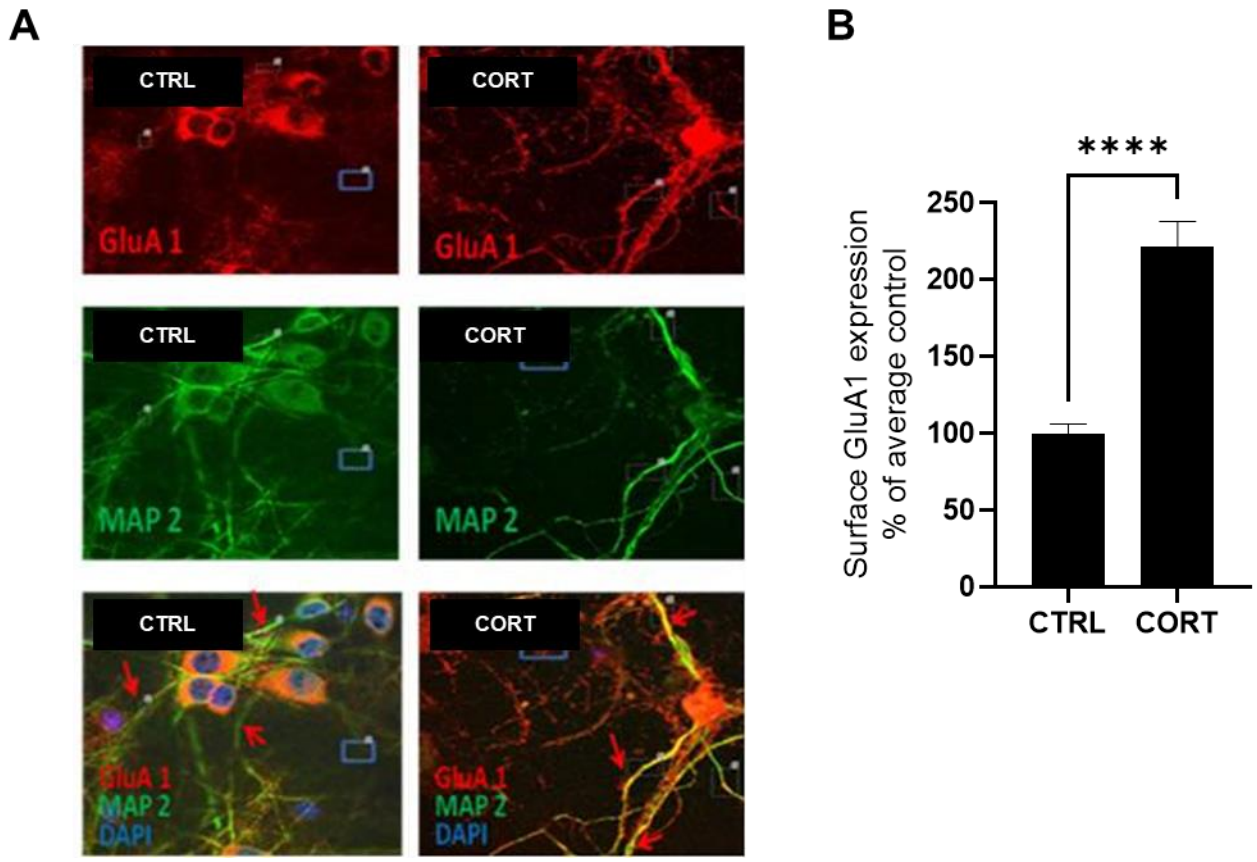

Figure S1: Temporal kinetics of AMPAR surface expression in dendritic regions of hippocampal neurons in response to glucocorticoid treatment

Immunocytochemistry time course analysis depicting surface expression of AMPAR proteins in DIV15 cultured hippocampal neurons. Neuronal cultures were treated with stress level corticosterone (100nM) or ethanol as control. The dissociated neurons were fixed at 3 hours for analysis of GluA1. (A) Representative image of GluA1 (stained red), MAP2 (neuronal marker stained green) and merged images (depicted in yellow) including nuclear marker-DAPI (stained blue), as obtained via confocal microscopy [Scale = 10μm]. (B) Graphical representation showing quantification of surface GluA1 immunofluorescence from dendritic projections over time is shown. In line with established protocol from previous studies, corticosterone significantly increased GluA1 proteins (stained red) at 3 hours. *Error bars represent mean ± SEM from two experiments; n≥30 dendrites per group. Graphs presented*

here are quantified mean intensity values from immunofluorescent images obtained with velocity program. Dendrites were highlighted with the MAP2 neuronal marker and AMPAR expression was measured with Image J. Values are fold induction from mean of the control (ethanol) group values (assigned 100%). Symbols: *represents a significant difference (Dunnet's post-test) in the comparison to *time 0 (untreated control cells).*

References

1 Cho, K. O., Hunt, C. A. & Kennedy, M. B. The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. *Neuron* **9**, 929-942 (1992).

2 Grab, D. J., Carlin, R. K. & Siekevitz, P. The presence and functions of calmodulin in the postsynaptic density. *Ann N Y Acad Sci* **356**, 55-72 (1980).

Fractionation Experiment: Effect of Stress on PSer845-GluA1 (Figure 1B/C)								
SYNAPTIC PLASMA MEMBRANE					POST SYNAPTIC DENSITY			
CTRL	**STRESS**	**ADX**	**ADX+STRESS**		**CTRL**	**STRESS**	**ADX**	**ADX+STRESS**
1.000	1.444	1.343	1.208		1.000	1.004	1.256	1.054
1.000	1.330	1.095	1.071		1.000	1.196	1.088	0.946
1.000	1.107	0.844	0.966		1.000	1.122	0.810	0.882

Fractionation Experiment: Effect of Stress on Total GluA1 (Figure 1B/C)								
SYNAPTIC PLASMA MEMBRANE					POST SYNAPTIC DENSITY			
CTRL	**STRESS**	**ADX**	**ADX+STRESS**		**CTRL**	**STRESS**	**ADX**	**ADX+STRESS**
1.000	1.194	1.163	1.170		1.000	1.059	1.310	1.284
1.000	1.282	1.123	1.002		1.000	1.154	1.119	0.888
1.000	1.004	0.897	0.871		1.000	0.819	0.948	1.074

Fractionation Experiment: Effect of CORT+/- RU-486 on PSer845-GluA1 (Figure 2A/B)						
SYNAPTIC PLASMA MEMBRANE				POST SYNAPTIC DENSITY		
CTRL	**CORT**	**CORT+RU-486**		**CTRL**	**CORT**	**CORT+RU-486**
1.000	1.572	1.905		1.000	1.348	1.024
1.000	1.020	1.450		1.000	1.246	0.927
1.000	1.199	1.856		1.000	1.294	0.856

Fractionation Experiment: Effect of CORT+/- RU-486 on Total GluA1 (Figure 2A/B)						
SYNAPTIC PLASMA MEMBRANE				POST SYNAPTIC DENSITY		
CTRL	**CORT**	**CORT+RU-486**		**CTRL**	**CORT**	**CORT+RU-486**
1.000	1.515	1.583		1.000	1.143	0.786
1.000	1.016	1.705		1.000	1.264	0.792
1.000	1.313	1.648		1.000	1.258	0.798

Effect of CORT+/- CX/ActD (Fig. 2C/D)/SQ (Fig. 3A/B) on P-GluA1					
Whole Cell Extract					
CTRL	**CORT**	**CORT+CX**	**CORT+ACT-D**	**CORT+SQ**	**CORT+RU**
0.575	1.797	0.462	1.027	0.456	0.568
0.605	1.098	0.292	0.961	0.410	1.391
0.739	1.538	0.491	1.294	0.679	0.613
0.805	1.803		0.004	1.353	0.729
0.560	0.982			1.113	0.732
0.735	1.181			0.941	0.004
0.790	1.056			0.917	0.006
0.003	0.003			0.006	0.006
0.007	0.008				

Effect of CORT+/- CX/ActD (Fig. 2C/D)/SQ (Fig. 3A/B) on T-GluA1					
Whole Cell Extract					
CTRL	**CORT**	**CORT+CX**	**CORT+ACT-D**	**CORT+SQ**	**CORT+RU**
0.649	0.684	0.583	1.222	0.437	0.474
1.660	0.961	0.957	1.224	1.292	1.188
0.503	1.017	0.174	1.623	1.357	1.173
1.081	1.277		0.187	1.186	0.122
1.010	1.171				0.140
1.161	1.278				0.236
0.118	0.039				
0.152	0.198				
0.251	0.272				

Effect of CORT+/- AP5 (Fig. 4A/B)		
Whole Cell Extract_AC1		
CTRL	**CORT**	**CORT+CX**
1.000	4.479	5.269
1.000	4.380	5.571
1.000	2.118	2.179
1.000	10.871	14.690

Effect of CORT+/- AP5 (Fig. 4A/B)		
Whole Cell Extract_AC8		
CTRL	**CORT**	**CORT+CX**
1.000	2.487	1.509
1.000	1.936	1.081
1.000	1.934	0.537
1.000	2.305	0.425

Effect of CORT+/- AP5 (Fig. 4C/D)

Effect of CORT+/- AP5 (Fig. 4C/D)

Whole Cell Extract_P-GluA1		
CTRL	CORT	CORT+CX
1.000	7.212	3.548
1.000	0.575	0.175
1.000	1.329	0.198
1.000	4.772	0.660

Whole Cell Extract_T-GluA1		
CTRL	CORT	CORT+CX
1.000	4.393	5.452
1.000	1.918	0.508
1.000	0.836	0.609
1.000	4.159	2.991

Supplemental Table 1: Raw data of densitometric quantification from all Western blot analysis presented. All Western blot experiments and analysis were conducted in an investigator-blinded manner; therefore, samples were run in the assays without reference to expected results. Densitometric quantification was performed via normalisation to β-actin (39KDa), except otherwise stated in text (e.g. for fractionation studies, blots were normalized to corresponding fraction marker). Raw data of the densitometric analysis showing fold induction from averaged control are provided here.

- Fractionation experiments: Effect of stress on phosphorylated and total GluA1 (Figure 1C). SPM fractions were normalized to synaptophysin, while PSD fractions were normalized to PSD-95 protein levels
- Fractionation experiments: Effect of CORT +/- GR-inhibitor (RU486) on phosphorylated and total GluA1 (Figure 2A)
- Whole cell extract experiments: Effect of CORT +/- Cyclohexamide (CX) or Actinomycin-D (Act-D) (Figure 2C) or AC inhibitor (SQ) (Figure 3A) on phosphorylated and total GluA1
- Whole cell extract experiments: Effect of CORT +/- NMDAR inhibitor (AP5) on AC8 and AC1 (Figure 4A); and on phosphorylated and total GluA1 (Figure 4D)