Corticosteroid effects on glutamatergic transmission and fear memory
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mTOR is essential for corticosteroid effects on hippocampal AMPA receptor function and fear memory

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Abstract

Glucocorticoid hormones, via activation of their receptors, promote memory consolidation, but the exact underlying mechanisms remain elusive. We examined how corticosterone regulates AMPA receptors (AMPARs) which are crucial for synaptic plasticity and memory formation. Combining a live imaging Fluorescent Recovery After Photobleaching (FRAP) approach with the use of the pH-sensitive GFP-AMPAR tagging revealed that corticosterone enhances the AMPAR mobile fraction and increases synaptic trapping of AMPARs in hippocampal cells. In parallel, corticosterone enhanced AMPAR mediated synaptic transmission. Blocking the mammalian target of rapamycin (mTOR) pathway prevented the effects of corticosterone on both AMPAR trapping – but not on the mobile fraction – and synaptic transmission. Blocking the mTOR pathway also prevented the memory enhancing effects of corticosterone in a contextual fear conditioning paradigm. We conclude that activation of the mTOR pathway is essential for the effects of corticosterone on synaptic trapping of AMPARs and, possibly as a consequence, fearful memory formation.
**Introduction**

Enhanced memory formation of emotionally arousing and stressful situations favors long-term behavioral adaptation to such conditions (De Kloet et al. 1999). Consolidation of emotionally arousing information is facilitated by corticosteroid hormones which are released during and after exposure to stressful situations (Oitzl et al. 2001; Roozendaal et al. 2009). An important question is exactly how these hormones facilitate memory consolidation. Corticosterone, via activation of mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), regulates AMPA receptor (AMPAR) function (Karst and Joels 2005; Groc et al. 2008; Krugers et al. 2010; Martin et al. 2009) a critical endpoint for memory formation (Kessels et al. 2009; Mitsushima et al. 2011).

The intracellular mediators between steroid receptor activation and AMPAR function have not yet been resolved. One potential candidate is mammalian target of rapamycin (mTOR), a serine/threonine-kinase critically involved in synaptic plasticity and memory formation (Glover et al. 2010; Tang et al. 2002) that controls initiation of protein translation through phosphorylation of several signaling targets including the p70-kDa ribosomal S6 kinase (p70S6K) and the eukaryotic initiation factor 4E-binding protein 1 (4EBP1). Activation of the mTOR pathway has also been implicated in the effects of stressful events and corticosteroid hormones on synaptic plasticity since stress exposure and GR activation suppress synaptic plasticity via activation of the mTOR pathway (Yang et al. 2008). These studies suggest that stress and GR activation, via activation of mTOR, enhance synaptic transmission and prevent subsequent synaptic plasticity, a mechanism to preserve stress-related information (Krugers et al. 2010). We tested the hypothesis that corticosterone action requires the mTOR signaling pathway to regulate AMPAR surface mobility, AMPAR function and consequently memory formation.

**Materials and Methods**

*Neuronal cultures*

The experiments were carried out with permission of the local Animal Committee of the
University of Amsterdam and the Centre National de la Recherche Scientifique, Institut de Pharmacologie Moléculaire et Cellulaire University of Nice, Sophia-Antipolis. Primary hippocampal neurons were prepared from E18 pregnant Wistar rats as previously described (Loriol et al. 2013; Loriol et al. 2014). Neurons were plated in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 0.5 mM glutamax and penicillin/streptomycin on 12-mm glass coverslips pre-coated with 0.1 mg/mL poly-L-lysine. Neurons (40,000 cells per coverslip) were fed once a week for 3 weeks in Neurobasal medium supplemented with 2% B27 and penicillin/streptomycin. For live-cell imaging, density of the cultures was 110,000 per 24 mm coverslip.

**Immunocytochemistry**

At DIV13-20 hippocampal neurons were incubated with GluR1 (Calbiochem (1:8) and GluR2 (Zymed (1:80) N-terminal antibodies (10 μg/ml) at 37°C for 15 min (Martin et al. 2008). Cells were preincubated at 37°C in 5% CO₂ for 1 hour in Neurobasal containing the potent mTOR inhibitor Rapamycin (50 nM, Sigma) followed by corticosterone (100 nM, Sigma) or vehicle for 3 hours in the presence of Rapamycin. After washing in DMEM medium, the neurons were fixed for 5 min with 4% formaldehyde/4% sucrose in phosphate-buffered saline (PBS). Neurons were then washed three times in PBS for 30 min at room temperature and incubated with secondary antibody conjugated to Alexa488 (1:400) or Alexa568 (1:400) in staining buffer without TritonX-100 (0.2% BSA, 0.8 M NaCl, 30 mM phosphate buffer, pH 7.4) overnight at 4°C. Neurons were then washed three times in PBS for 30 min at room temperature and mounted. Confocal images were obtained with sequential acquisition settings at the maximal resolution of the microscope (1024 x 1024 pixels). Morphometric analysis and quantification were performed using MetaMorph software (Universal Imaging Corporation).

**Live Imaging**

**Neuronal transduction and transfection**

Attenuated Sindbis virus expressing SEP-GluA2 were prepared and used as previously described (Martin et al. 2008; Martin et al. 2009). Neurons were transduced at a MOI of 1 between 18 and 20 DIV and incubated at 37°C under 5% CO₂ for 24h until use.
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For hippocampal neuron transfection, cells were incubated in a mix containing the Lipofectamin 2000 (Invitrogen) with 3 μg of plasmid DNA of palmitoylated membrane-anchored GFP (mGFP) and utilized 48 hours post-transfection.

Imaging

Protocols were performed as previously described (Martin et al. 2008; Martin et al. 2009). Briefly, dendrites from live mGFP or SEP-GluA2 expressing neurons (19-21 DIV) were kept on a heated stage (set at 37°C) on a Nikon Ti inverted microscope and were continuously perfused at 1 ml/min with warm solution. GFP fluorescence was excited through a 100X oil-immersion lens (Numerical Aperture, 1.4) using a 488 nm laser light (50 mW, 1-2%) and time series (1 image every 40 sec) were collected as a single image slice using a Perkin Elmer Ultra-View spinning disk solution. For low pH external solution, equimolar MES (Sigma) was used instead of HEPES and pH adjusted to 6.0 and NH₄Cl (50 mM) was used in place of equimolar NaCl to collapse pH gradient. All SEP-GluA2 experiments included a brief (10 s) low pH wash at the beginning to ensure that the fluorescence from the area of interest comes from surface-expressed AMPARs.

Live SEP-GluA2-expressing neurons were preincubated at 37°C and 5% CO₂ for 1 hour in Neurobasal containing the potent mTOR inhibitor Rapamycin (50 nM, Sigma) followed by corticosterone (100 nM, Sigma) or vehicle for 3 hours in the presence of Rapamycin and finally live-imaged in Earle's buffer (50 mM HEPES-Tris pH 7.4, 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 0.9 g/L glucose) containing the indicated drugs.

Electrophysiology

Coverslips were placed in a recording chamber mounted on an upright microscope (Zeiss Axioskop 2 FS Plus, Germany), kept fully submerged with artificial cerebrospinal fluid (aCSF) containing in (mM): NaCl (145), KCl (2.8), MgCl₂ (1.0), HEPES (10.0), and Glucose (10.0), pH 7.4. Whole cell patch clamp recordings were made using an AXOPATCH 200B amplifier (Axon Instruments, USA), with electrodes from borosilicate glass (1.5 mm outer diameter, Hilgerberg, Malsfeld, Germany). The electrodes were pulled on a Sutter (USA) micropipette puller. The pipette solution contained (in mM):
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120 Cs methane sulfonate; CsCl (17.5); HEPES (10); BAPTA (5); Mg-ATP (2); Na-GTP (0.5); QX-314 (10); pH 7.4, adjusted with CsOH; pipette resistance was between 3–6 MΩ. Under visual control (40X objective and 10X ocular magnification) the electrode was directed towards a neuron with positive pressure. Once sealed on the cell membrane (resistance above 1 GΩ) the membrane patch under the electrode was ruptured by gentle suction and the cell was kept at a holding potential of −70 mV. The liquid junction potential caused a shift of no more than 10 mV, which was not compensated during mEPSCs recording. Recordings with an uncompensated series resistance of <15 MΩ and <2.5 times of the pipette resistance with a shift of <20% during the recording, were accepted for analysis. Data acquisition was performed with Pclamp 8.2 and analyzed off-line with Clampfit 9.0.

Miniature excitatory postsynaptic currents (mEPSCs) were recorded at a holding potential of −70 mV. Tetrodotoxin (0.25 μM, Latoxan, Rosans, France) and bicuculline methobromide (20 μM, Biomol) were added to the buffer to block action potential induced glutamate release and GABA_A receptor mediated miniature inhibitory postsynaptic currents (mIPSCs), respectively. During some recordings the non–NMDA-receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM, Tocris) was perfused to confirm that the mEPSCs were indeed mediated by AMPARs. The events were identified as mEPSCs when the rise time was faster than the decay time. mEPSCs were recorded for 5 min in each cell.

Corticosterone (100 nM, Sigma) or vehicle (<0.01% ethanol) was applied for 1 or 3 hours. In one set of experiments, corticosterone was applied for 3 hours, washed out and cultures were recorded 21 hours after treatment. Cycloheximide (100 μM, Sigma), the GR antagonist RU486 (500 nM, Sigma) and rapamycin (50 nM, Sigma) were applied for 1 hour before co-application with corticosterone or vehicle.

Fear Conditioning
Animals were housed individually one week before the start of the experiment. Rats were trained in a fear conditioning chamber (Context A; W x L x H: 30 cm x 24 cm x
26 cm) that contained a grid floor with 37 stainless steel rods and was connected to a shock generator and sound generator (Med-Farm LION-ELD) developed in-house. During training, one animal at a time was placed in the cage. After three minutes of free exploration, one foot shock (2 seconds, 0.2 mA; Cordero and Sandi, 1998) was delivered and the animal was allowed to stay in the cage for 30 seconds after the end of the foot shock. Immediately after training, corticosterone (2 mg/kg, Sigma) or vehicle (Ethanol, < 0.01 %) was injected intraperitoneally (i.p). At the same time rapamycin (6 mg/kg, Sigma) or DMSO (0.01%) was administered i.p. We have used a single dose of corticosterone in a range which mimics plasma corticosterone levels induced by a substantial stressor (Cordero and Sandi, 1998) and facilitates memory formation (Cordero and Sandi, 1998; Miranda et al., 2008; Atsak et al., 2015). Then, the animals were transferred back into their home cage. Freezing behaviour, defined as no body movements except those related to respiration, was determined every 2 seconds throughout training (Zhou et al. 2010). Twenty four hours later, one animal at a time was placed in context A for 3 minutes without receiving foot shock and freezing behavior was scored.

Statistical analysis
Statistical analyses were calculated using Prism 4 (GraphPad software, Inc). Data are expressed as mean ± S.E.M. Unpaired Student’s t-tests and one-way ANOVA were performed with a Bonferroni post-test for multiple comparison data sets when required.

Results

Imaging AMPA receptors
We first tested the involvement of the mTOR pathway on the surface expression of GluA1 and GluA2 AMPAR subunits in hippocampal cells. Corticosterone increased surface expression of both subunits, which was not affected by co-application of rapamycin (Figure 1A-C). However, by combining a Fluorescent Recovery After Photobleaching (FRAP) approach with the use of the pH-sensitive GFP-AMPAR tagging, we found that corticosterone alters the surface mobility of GluA2 containing AMPARs (Figure 1D-I). More specifically, corticosterone increased the mobile fraction (Figure 1E, G), the
half time of fluorescence recovery $T^{\frac{1}{2}}$ (Figure 1E, H) and consequently, the diffusion coefficient of GluA2-containing AMPARs in dendritic spines is decreased (Figure 1E, I). These effects could not be explained by altered surface diffusion since membrane-GFP diffusion remained unaffected by the corticosterone treatment (Figure 2) indicating

Figure 1. mTOR signaling is involved in the regulation of plasma membrane AMPAR lateral diffusion of corticosterone-treated rat hippocampal neurons. A. Representative images of rat hippocampal neurons with labeling of GluA1 (in green) and GluA2 (in red) AMPAR subunits after treatment with vehicle (veh), corticosterone (cort, 100 nM), rapamycin (rapa, 50 nM) and rapamycin + corticosterone (rapa + cort). B-C. Histograms showing the mean (± S.E.M.) quantification of surface GluA1 (B) or GluA2 (C) AMPAR subunits. Data are expressed as ratio of control (vehicle condition). *p<0.05, ** p<0.01, *** p<0.001, One-way ANOVA, n > 10 cells in each group. D. Sequential images from representative FRAP experiments performed on surface SEP-GluA2 from individual spine head (arrowheads) in control vehicle (veh) or in corticosterone (Cort, 100 nM)
conditions, in absence or in the presence of the potent mTOR inhibitor Rapamycin (Rapa, 50 nM). E. Normalized pooled and averaged FRAP curves from vehicle (n = 13 cells) and corticosterone (100 nM; n = 15 cells) treated hippocampal neurons. F. Normalized pooled and averaged FRAP curves from rapamycin (50 nM; n = 13 cells) and rapamycin + corticosterone (Rapa, 50 nM; Cort, 100 nM; n = 16 cells) treated hippocampal neurons. G-I. Histograms showing the means (± S.E.M.) of synaptic SEP-GluA2 mobile fractions (G), half time of fluorescence recovery (H) and diffusion coefficients (I). One-way ANOVA were performed with a Bonferroni post-test for multiple comparison data sets. *p<0.05, **p<0.01.

Figure 2. Blocking the mTOR signaling pathway does not impact the synaptic diffusion of membrane GFP. A. Sequential confocal images of SEP-GluA2 in living rat hippocampal neurons. Bright SEP-GluA2 fluorescence is mainly due to surface expressed receptors and fluorescence is rapidly lost in pH 6.0 external solution. The fluorescence associated to SEP-GluA2 is almost totally abolished at low pH. B. Representative trace showing the dynamic SEP-GluA2 fluorescence changes upon pH treatment described in A. C. Sequential images from representative FRAP experiments performed on palmitoylated mGFP from individual spine head (arrowheads) in control vehicle (Ctrl; n = 28 cells) or in corticosterone (Cort, 100 nM; n = 28 cells) conditions, in absence (Rapa, n = 22 cells) or in the presence of the potent mTOR inhibitor Rapamycin (Rapa, 50 nM; n = 25 cells). D-F. Histograms showing the means (± S.E.M.) of synaptic mGFP mobile fractions (D), half time of fluorescence recovery (E) and diffusion coefficients (F) under the various conditions tested in C. One-way ANOVA were performed with a Bonferroni post-test for multiple comparison data sets. ns: not significantly different.

that the stress hormone selectively facilitates the mobility of GluA2, and promotes the synaptic trapping of AMPARs. Corticosterone effects on the mobile fraction were not affected by the mTOR antagonist rapamycin (Figure 1F, G, H), but rapamycin incubation completely prevented the effect of corticosterone on the T½ and AMPAR diffusion coefficient (1F, I).
Electrophysiology

We next examined the role of the mTOR pathway in hippocampal AMPAR function. Corticosterone increased the amplitude of mEPSCs three hours but not one hour after treatment (Figure 3A, B). These effects were long lasting, since the increase in

Figure 3. Corticosterone regulates AMPAR function via the mTOR pathway. **A.** Representative traces of mEPSCs at 1, 3 or 24 hours after vehicle (veh) or corticosterone (Cort, 100 nM) treatment on rat hippocampal neurons. **B.** Amplitude of mEPSCs at 1, 3 or 24 hours after vehicle (veh) or corticosterone (Cort, 100 nM) treatment. *p<0.05, unpaired t test, n > 10 in each group. **C.** Frequency of mEPSCs at 1, 3 or 24 hours after vehicle (veh) or corticosterone (Cort, 100 nM) treatment. **D.** Representative traces of mEPSCs after treatment with corticosterone (Cort) and co-application with vehicle (veh), the GR-antagonist RU486 (500 nM), and translation inhibitor cycloheximide (CX, 100 μM) for 3h. **E.** Amplitude of mEPSCs after treatment with corticosterone (Cort) and co-application with vehicle (veh), the GR-antagonist RU486 (500 nM), and translation inhibitor cycloheximide (CX, 100 μM). Each condition (+ control) was tested in a separate experimental series. *p<0.05, unpaired t test, n=12 cells in each group. **F.** Frequency of mEPSCs after treatment

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with corticosterone (Cort) and co-application with vehicle (veh), the GR-antagonist RU486 (500 nM), and translation inhibitor cycloheximide (CX, 100 μM). G. Traces of mEPSCs after treatment with vehicle (veh), corticosterone (Cort, 100 nM), rapamycin (rapa, 50 nM) or co-application of rapamycin and corticosterone (rapa+cort). H. Amplitude of mEPSCs after corticosterone treatment (Cort, 100 nM) and co-application of vehicle (veh) or rapamycin (rapa, 50 nM). *p<0.05, One-way ANOVA, veh (n=8), cort (n=7), rapa (n=8), rapa + cort (n=8).

amplitude of mEPSCs was still present when recorded 21 hours after washing out of corticosterone (Figure 3A, B). Both the GR-antagonist RU486 and the protein synthesis inhibitor cycloheximide (Figure 3D, E) prevented the effects of corticosterone on the amplitude of mEPSCs, indicating that corticosterone-induced changes in AMPAR function start through genomic GR actions. The increase in the amplitude of mEPSCs in corticosterone was also fully blocked when rapamycin was co-incubated with corticosterone (Figure 3G, H), pointing to mTOR as a necessary intracellular mediator of GR-dependent signaling. Frequencies of mEPSCs were not significantly altered by

Figure 4. Corticosterone enhances memory consolidation via mTOR pathway. A. Timeline of behavioral experiment. Animals received a foot shock of 0.2 mA and were immediately injected with vehicle (veh), corticosterone (cort, 2 mg/kg), rapamycin (rapa, 6 mg/kg) or corticosterone (cort, 2 mg/kg) + rapamycin (rapa, 6 mg/kg intraperitoneally). Contextual fear was tested 24 hours after training. B. Freezing behavior (% of total time; Mean ± S.E.M) of animals during free exploration, before foot shock exposure and before being treated with vehicle (veh), corticosterone (cort, 2 mg/kg) or rapamycin (6 mg/kg) intraperitoneally immediately after training. C. Freezing behavior (% of total time; Mean ± S.E.M) of animals 24 hours after being injected intraperitoneally immediately after training with vehicle (veh), corticosterone (cort, 2 mg/kg), rapamycin (6 mg/kg) intraperitoneally immediately after training. *p<0.05, **p<0.01. One-way ANOVA test, n= 7 animals (vehicle), 7 animals (corticosterone), 8 animals (rapamycin), 8 animals (rapamycin + corticosterone).
the pharmacological treatments (Figure 3C, F, I).

**Fear Conditioning**

Finally we tested whether hippocampus-dependent memory enhancing effects of corticosterone are mediated via the mTOR pathway. Application of corticosterone (2 mg/kg) immediately after training in a weak contextual fear conditioning paradigm (0.2 mA) enhanced the expression of contextual fear 24 hours after training (Figure 4). Post-training application of rapamycin by itself did not affect freezing behavior at 24 hours after training. However, post-training administration of rapamycin prevented the corticosterone induced increase in contextual fear memory at 24 hours after training (Figure 4).

**Discussion**

Various studies have shown that activation of GRs enhance hippocampus-dependent memory formation (Oitzl et al. 2001; Roozendaal et al. 2009; Zhou et al. 2010). Enhanced memory formation may involve BDNF-TrkB-MAPK-synapsin Ia/Ib signaling (Revest et al. 2005; Revest et al. 2010; Revest et al. 2014) and CaMKII-BDNF-CREB signaling (Chen et al. 2012). Yet, it remains to be determined how corticosteroid hormones regulate synaptic function, which is fundamental for memory formation (Rumpel et al. 2005; Kessels et al. 2009). We report that corticosterone via the mTOR pathway enhances synaptic retention of AMPARs, AMPAR function - which is a critical mechanism for memory formation (Kessels et al. 2009) - and improves contextual memory formation.

Corticosteroid hormones, via activation of GRs, have been reported to increase exocytosis of AMPARs (Yuen et al. 2011) and lateral diffusion of AMPARs (Groc et al. 2008). We report here that corticosterone not only enhances the mobile pool of AMPARs, but also enhances the synaptic retention of AMPARs and AMPAR mediated synaptic function. This yields a picture that corticosterone acts on various pathways (exocytosis, lateral diffusion and retention) to increase AMPAR function.
Previous studies have shown that serum- and glucocorticoid-inducible kinase and the activation of Rab4 – which are involved in exocytotic processing – are involved in enhanced synaptic function by corticosteroid hormones (Yuen et al. 2011). Our results reveal that the corticosterone-induced increase in retention of AMPARs is regulated via the mTOR pathway. This effect is highly specific, since blocking the mTOR pathway did not prevent corticosterone effects on the mobile fraction of AMPARs. Exactly how mTOR regulates synaptic retention and synaptic function of these receptors is not clear. Most likely, this effect involves translation of proteins, which regulate synaptic trapping of AMPARs. Importantly, these effects are highly relevant for behaviour since preventing activation of the mTOR pathway prevented the effect of corticosterone on contextual memory consolidation. Taken together, a picture now emerges that corticosterone binds to GRs and increases AMPAR mobility via exocytosis (Yuen et al. 2011), lateral diffusion (Groc et al. 2008), but also by facilitating the synaptic retention of AMPARs via the mTOR pathway, contributing to enhanced memory consolidation.

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References


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