Stress, emotional learning and AMPA receptors: from behavior to molecule
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Chapter V

Glucocorticoids increase AMPA receptor mediated synaptic transmission via N-Ethylmaleimide-Sensitive Factor/GluA2 dependent trafficking and PI3K-mTOR signaling

(In preparation)

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Abstract

Emotionally arousing events promote the release of corticosteroid hormones from the adrenal glands. This facilitates the consolidation of these events which is considered to be behaviorally relevant. AMPA receptors (AMPARs) are critically involved in stressful learning and memory processes. The trafficking of AMPARs to and from the postsynaptic membrane can be modulated by glucocorticoid receptors (GRs). However, the mechanism underlying this process is still unknown. Here we explored the involvement of i) N-ethylmaleimide-sensitive factor (NSF)/GluA2 dependent trafficking of AMPARs and ii) the PI3K-mTOR pathway which regulates translational control of synaptic scaffolding proteins. In hippocampal primary cultures we found that corticosterone (100 nM) slowly enhanced the peak amplitude of AMPAR-mediated synaptic transmission. This effect could be fully prevented by co-application of pep2m which inhibits the interaction between NSF and GluA2. The GR-antagonist RU38486, mTOR inhibitor rapamycin and PI3K kinase inhibitor LY294002 prevented the increase in synaptic transmission and (although preliminary) also the surface labeling of GluA1 and GluA2 subunits. We conclude that corticosterone-induced increases in synaptic transmission involve NSF/GluA2 dependent trafficking of AMPARs as well as the PI3K-mTOR pathway.
**Introduction**

Memories for emotionally arousing and stressful events are consolidated better than memories for neutral events (McGaugh, 2000). The formation of these memories is promoted by glucocorticoid hormones which are released in high levels during and after exposure to stress. These hormones are lipophilic, can easily cross the blood-brain barrier and bind to two types of receptors in the brain: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). While MRs are involved in appraisal of stressful events and response selection (Oitzl and de Kloet, 1992; Sandi and Rose, 1994a), for several learning paradigms it has been reported that post-training application of glucocorticoids or GR agonists promotes the consolidation of emotionally loaded information (Quirarte et al., 2009; Quirarte et al., 1997; Roozendaal and McGaugh, 1996a, b; Roozendaal et al., 1996). Conversely, application of GR antagonists hampers the storage of information (Oitzl and de Kloet, 1992; Pugh et al., 1997a; Roozendaal and McGaugh, 1997b; Zhou et al., 2010a). An important question that remains to be addressed is the exact mechanism by which these glucocorticoids promote memory formation.

AMPA receptors (AMPAR) mediate the majority of rapid excitatory synaptic signals in the mammalian central nervous system. The trafficking of AMPARs to and from the synaptic membrane is critically involved in long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission (Malenka, 2003; Plant et al., 2006), the two major cellular models that underlie memory formation. Accordingly, AMPAR function is enhanced in aversive learning paradigms (McKernan and Shinnick-Gallagher, 1997; Tsvetkov et al., 2002; Zhou et al., 2009) and pharmacological enhancement of AMPARs accelerates fear conditioning (Rogan et al., 1997a). The trafficking of AMPARs to and from the synapse was shown to be critical for fear learning (Clem and Huganir, 2010; Rumpel et al., 2005).
Interestingly, glucocorticoids enhance AMPAR function. These hormones slowly enhance AMPAR mobility, promote synaptic insertion of AMPARs, and enhance AMPAR-mediated synaptic transmission (Groc et al., 2008; Martin et al., 2009). This effect requires activation of the GR (Groc et al., 2008; Martin et al., 2009) as well as protein synthesis (Martin et al., 2009). Exactly how glucocorticoids persistently increase synaptic transmission is unknown. Here we explored the effect of glucocorticoids on the interaction between N-ethylmaleimide-sensitive factor (NSF) and the intracellular C terminus of GluA2 AMPAR subunit, which regulates basal AMPAR trafficking (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Yao et al., 2008). In addition, we explored glucocorticoid actions on the PI3 Kinase-mTOR pathway. This pathway is involved in protein translational control and long-lasting synaptic plasticity (Hoeffer and Klann, 2009). It can be activated by hormones (Akama and McEwen, 2003; Lee et al., 2005) and stimulates translation of the dendritic scaffolding protein PSD-95 which is critically involved in basal synaptic transmission (Stein et al., 2003).

Materials and methods

Hippocampal primary cultures

Primary hippocampal cultures were prepared from embryonic day 18 (E18±1) rat brains. Cells were plated on coverslips coated with poly-D-lysine (0.5 mg/ml) at a density of 75,000/well for electrophysiology and immunosurface labeling experiments. Hippocampal cultures were grown in Neurobasal medium supplemented with (per 100ml): B27 2ml, GlutaMax1 1ml, Penicillin/Streptomycin 1ml, FBS 5-10ml (plating medium) for the first day. Half of the total volume was changed once a week by culturing medium (plating medium without FBS) from the 2nd day onwards, with 5-Fluoro-2'-Deoxyuridine (FUDR) 10µM to inhibit glial growth. All the reagents except FUDR (Sigma) were from Gibco Invitrogen. The
experiments were carried out in accordance with and approved by the local Animal Committees of the University of Amsterdam.

**Treatment**

DIV 14-21 hippocampal neurons were treated with corticosterone (CORT, 100nM, Sigma) or its vehicle (<0.01%EtOH) for 3 hours, prior to and during recording. Pep2m (100 µM,Tocris) or vehicle (distilled water) was applied 30 minutes prior to corticosterone incubation: GR antagonist-RU38486 (500 µM, Sigma), mTOR antagonist-rapamycin (50 nM), specific PI3K inhibitor-LY294002 (50 µM) or L-type calcium channel blocker-nifedipine (5 µM, Sigma) was added into medium one hour before corticosterone incubation; EtOH (<0.01%) was used as the vehicle for all the groups except DMSO in the case of rapamycin (final concentration of DMSO <0.1%).

**Electrophysiology**

Coverslips (DIV 14-21 neurons) were placed in a recording chamber mounted on an upright microscope (Zeiss Axioskop 2 FS Plus, Germany), filled with HEPES buffer at room temperature (20°C ± 2) containing (in mM): NaCl (145), KCl (2.8), CaCl₂ (2.0), MgCl₂ (1.0), H-HEPES (10.0), pH 7.4, osmolarity 300±10 mOsm/L. 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): 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 MINIANALYSIS (version 6.0.9).

As described before (Karst et al., 2005; Karst and Joels, 2005), 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. mEPSCs were recorded for 2 min in each cell. The events were analyzed manually and identified as mEPSCs when the rise time was faster than the decay time, with an event detection threshold of -5 pA. The average frequency (inter-even intervals) and peak amplitude of the mEPSCs were determined for each cell.

**Immunosurface labeling and confocal microscopy**

DIV14-21 hippocampal neurons were incubated with GluA1 (Calbiochem, 1:8) and GluA2 (Zymed, 1:80) N-terminal antibodies (10 mg/ml) at 37°C for 15 min. 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 on a Zeiss laser scanning microscope 510 (USA)
were obtained with sequential acquisition settings at the maximal resolution of the microscope (1024x1024 pixels). Morphometric analysis and quantification were performed using MetaMorph software (Universal Imaging Corporation, USA). The averaged whole cell surface immunodensity from 8-10 neurons per group was measured.

**Statistics and data analysis**

Data are expressed as mean ± SEM. Analyses were performed in SPSS 11.0 for windows, using Kolmogorov-Smirnov (KS) tests, one-way ANOVA followed by post hoc Tukey multiple comparisons or independent two-tailed t-test. Data was normalized to control groups for figure presentation. A P-value of 0.05 was set as the level of significance. Data in each experiment was collected from 2-3 batches of cultured neurons, with all the treatments randomized to avoid possible batch/time differences.

**Results**

*Corticosterone enhances AMPAR-mediated mEPSCs via NSF/GluA2 dependent trafficking*

We first examined whether glucocorticoid regulation of synaptic transmission involves NSF/GluA2 dependent trafficking. An example of mEPSCs traces in response to either vehicle or corticosterone treatment is shown in Figure 1A. Application of 100 nM corticosterone significantly enhanced the amplitude of mEPSCs (Figure 1B, $F_{3, 50}=5.6$, $P<0.01$). Post-hoc Tukey multiple comparisons revealed that corticosterone significantly enhanced peak amplitude compared to vehicle group ($P<0.01$), which was prevented by co-application of pep2m (vehicle_Cort versus pep2m_Cort: $P>0.05$). Pep2m itself had no effect (vehicle versus pep2m: $P>0.05$). Although an overall significant effects was found on the mEPSC frequency (Figure 1C. $F_{3, 50}= 3.6$, $P<0.02$), Post-hoc Tukey analysis revealed only significant differences between corticosterone and pep2m...
treated cells (P<0.05). Overall, these data indicate that NSF/GluA2-dependent trafficking underlies the corticosterone-induced increase in AMPA receptor mediated synaptic transmission.

Figure 1. Corticosterone enhances synaptic transmission via NSF/GluA2 dependent trafficking. Bar diagram represents the normalized value of peak amplitude and frequency of mEPSCs for all treatment groups relative to respective vehicles. Data are collected from 13-17 neurons per group. A) An example of mEPSCs traces treated with either corticosterone or vehicle. B) A significant between-group effect was found in peak amplitude of mEPSCs (one-way ANOVA,
P<0.01). Corticosterone significantly enhanced the mEPSC peak amplitude (post hoc Tukey comparison, **P<0.01). This effect was completely blocked by pep2m (**P<0.01). C) A significant between-group effect was found in frequency of mEPSCs (one-way ANOVA, P<0.01), while corticosterone did not affect the frequency of mEPSCs (P>0.05).

**Corticosterone enhances AMPAR-mediated synaptic transmission via the PI3K-mTOR pathway.**

In a second experiment we examined whether corticosterone regulates AMPA receptor mediated synaptic transmission via the PI3K-mTOR pathway. One-way ANOVA revealed a between-group effect in the peak amplitude of mEPSCs (Figure 2 A, F₉,₁₂₅=4.4, P<0.01,). Application of corticosterone (100nM) to hippocampal cultures significantly enhanced the peak amplitude of mEPSCs (post hoc Tukey multiple comparison, P<0.01). Application of corticosterone in the presence of the GR-antagonist RU38486 (500 nM) did not affect the peak amplitude of the mEPSCs (P>0.05). Likewise, corticosterone did not change the mEPSC amplitude in cells pretreated with either the PI3K inhibitor-LY294002 (50 µM), rapamycin (50 nM) or nifedipine (5 µM) compared to respective controls (Figure 2A, P>0.05). No effects on the frequency were observed between the treatments (Figure 2 B, F₉,₁₂₅=1.2, P>0.05).
Figure 2. Corticosterone enhances AMPA receptor mediated synaptic transmission via PI3K-mTOR pathway. A) Corticosterone (100 nM) significantly enhances the peak amplitude of mEPSCs compared to vehicle (post hoc Tukey comparison, **P<0.05). Co-application of LY294002, rapamycin, RU38486 and nifedipine before and during corticosterone treatment prevented the effect of corticosterone on the amplitude of mEPSCs. In contrast, B) No between-group effect was found in frequency of mEPSCs (one-way ANOVA, P>0.05).

**Corticosterone enhances surface AMPARs labeling via the PI3K-mTOR pathway.**

Finally we examined (in an ongoing experiment) whether corticosterone regulates AMPA receptor surface expression via the PI3K-mTOR pathway. Only drug treatments in the presence of either corticosterone or its vehicle
has been so far analyzed and thus reported here. Immunosurface labeling showed that incubation with 100 nM corticosterone for 3 hours enhanced the surface expression of both GluA1 (t_{14}=2.36, P<0.05) and GluA2 (t_{14}=2.38, P<0.05) AMPA receptor subunits (Figure 3A & B). This increase in surface labeling by corticosterone appears to be regulated by GRs since in the presence of the GR antagonist RU38486 (500 nM) GluA1 and GluA2 surface labeling was not affected by corticosterone (t_{14}=1.99, P>0.05 and t_{14}=0.72, P>0.05, respectively) when compared to RU38486 treatment alone. Moreover, corticosterone application of cells pretreated with either the PI3K inhibitor LY294002 (50 µM) or the mTOR inhibitor rapamycin (50 nM) was ineffective in changing GluA1 and GluA2 surface labeling (LY294002: t_{14}=1.99, P>0.05 and t_{14}=0.72, P>0.05; rapamycin: t_{14}=1.99, P>0.05 and t_{14}=0.72, P>0.05) when compared to treatment with the respective drugs alone. Finally, we found that the increase in AMPAR requires calcium influx, since corticosterone was also ineffective in enhancing GluA1 (t_{6}=1.06, P>0.05) and GluA2 (t_{6}=1.31, P>0.05) surface expression expression when applied in the presence of the L-type calcium channel blocker nifedipine.
Figure 3. Corticosterone enhances surface expression of AMPA receptors via PI3K-mTOR pathway. Corticosterone (100nM) enhanced the surface expression of GluA1 A) and GluA2 B) subunits compared to its vehicle (t-test, *P<0.05). Application of LY294002, rapamycin, RU38486 and nifedipine prior to and during corticosterone incubation prevented this effect of corticosterone when compared to respective drug treatment in the presence of corticosterone vehicle (t-test, P>0.05).

Discussion
Although glucocorticoids have been reported to enhance AMPAR-mediated synaptic transmission (Karst and Joels, 2005; Martin et al., 2009), the underlying mechanism was unknown. NSF/GluA2 dependent trafficking serves to maintain basal synaptic transmission (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Yao et al., 2008). Here we report that
corticosterone enhances the peak amplitude of AMPAR-mediated mEPSCs via a mechanism involving NSF/GluA2 dependent AMPAR trafficking. In addition, corticosterone might enhance AMPA receptor mediated synaptic transmission via the ability of NSF to disrupt GluA2-PICK1 interaction (Terashima et al., 2008). GluA2-containing AMPARs appear to be excluded from synapses by binding to PICK1 (Perez et al., 2001): disrupting GluA2/PICK1 interactions using pep-EVKI increased postsynaptic AMPA receptor mediated synaptic responses (Yao et al., 2008). Moreover, PICK1 is critically involved in endocytosis of AMPA receptors (Hanley et al., 2002). Corticosterone may therefore reduce endocytosis and enhance surface and synaptic expression of AMPA receptors. Moreover, the present study and earlier reports (Groc et al., 2008; Martin et al., 2009) show that corticosterone slowly enhances surface expression of GluA2-containing AMPARs via GR activation. Overall these data supports the notion that slow GR-dependent effects on AMPAR trafficking are directly translated into functional consequences, i.e. a selective increase in mEPSC amplitude.

We next explored the signaling pathway upstream of AMPAR trafficking and the associated changes in mEPSC amplitude, focusing on the PI3K-mTOR pathway. The rationale behind this was two-fold. First, this pathway is critically involved in translational control of synaptic plasticity (Hoeffer and Klann, 2010; Wang et al., 2006), and translational processes are involved in glucocorticoid effects on AMPAR trafficking (Martin et al., 2009). Second, other hormones – such as estrogen and insulin – have been shown to activate this pathway (Akama and McEwen, 2003; Lee et al., 2005) and ultimately regulate translation of PSD-95 which is important for maintaining AMPA receptors at synapses (Stein et al., 2003) and maturation of excitatory synapses (El-Husseini et al., 2000). Our studies suggest that pharmacological blockade of the PI3K-mTOR pathway by either LY294002 or rapamycin prevent the corticosterone-induced increase in mEPSC amplitude and AMPA receptor surface expression. How glucocorticoids
exactly regulate this pathway, and whether these hormones increase PSD95 protein expression remains to be investigated. However, enhanced synaptic PSD-95 expression has been reported to suppress synaptic potentiation and facilitate synaptic depression (Stein et al., 2003) reminiscent of the slow effect of corticosterone on synaptic transmission and synaptic plasticity (Alfarez et al., 2002; Groc et al., 2008; Wiegert et al., 2005). Interestingly, nifedipine blocked the glucocorticoid effects on AMPAR-mediated synaptic transmission. Glucocorticoids have been reported to increase calcium-influx via L-type calcium channels (Chameau et al., 2007). This may indirectly affect the functionality of the PI3K-mTOR signaling pathway. Dedicated biochemical investigations will be required to resolve this in the future.

In conclusion, our data suggests that glucocorticoids increase synaptic transmission via NSF/GluA2-dependent trafficking of AMPARs. Although it requires further evidence (i.e. more extensive surface labeling and biochemical studies), these hormones may promote synaptic retention of AMPARs via the PI3K-mTOR pathway and increase translational regulation of synaptic scaffolding proteins such as PSD-95.