Corticosteroid effects on glutamatergic transmission and fear memory
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General Discussion
Outline

1. Summary of the thesis
2. Corticosteroid hormones and excitatory synapses
3. Corticosteroid hormones and memory
4. Future perspectives
1. Summary of this thesis

The overall aim of this thesis is to examine the effect of corticosterone on glutamatergic synaptic transmission and memory formation. We focused especially on corticosterone effects on AMPA receptor and NMDA receptor mediated synaptic transmission and contextual fear memory formation.

Chapter 1 provides a brief overview of the literature on how glucocorticoids regulate excitatory synaptic transmission and memory formation. Chapter 2-6 describe experimental studies, which investigate 1) how glucocorticoids regulate synaptic transmission and synaptic plasticity, and 2) whether this is relevant for (fear) learning and memory.

Glucocorticoids are known to enhance AMPA receptor mediated synaptic transmission in various brain areas (Karst et al., 2005; 2010; Liu et al., 2010; Zhou et al., 2010; Chapter 1). An important feature of the brain and its networks is the capacity to undergo activity-dependent changes which allows rapid and persistent adaptation necessary (among other things) for learning and memory formation. In Chapter 2 we investigated whether and how glucocorticoid hormones - within minutes or hours after a brief application - regulate plasticity of AMPA receptor mediated synaptic transmission. Therefore we used an established protocol to enhance synaptic plasticity in cell cultures, by activating NMDA receptors (using glycine and picrotoxin for 3 minutes), and examined how glucocorticoids rapidly or persistently alter the resulting synaptic plasticity. We report that a 20 min incubation of corticosterone can rapidly increase activity dependent changes by enhancing mEPSC frequency. At the same time, a brief 20 min incubation of corticosterone slowly increased the amplitude of mEPSC and prevented (or occluded) the activity-dependent increase in AMPA receptor mediated synaptic transmission. These results indicate that glucocorticoids can i) rapidly amplify an activity dependent effect on AMPARs function, and ii) slowly increase the amplitude of AMPAR-mEPSCs, thus presumably preventing subsequent synaptic plasticity. Preliminary data suggests that the rapid effects on activity-dependent changes in mEPSC frequency are mediated
by MRs, while GRs mediate the slower effects of corticosterone on the amplitude of mEPSCs.

N-Ethylmaleimide-Sensitive Factor (NSF) is critically involved in membrane fusion and its interaction with GluA2 is crucial for insertion and stabilization of AMPARs at the membrane and for maintaining synaptic transmission (Lee et al., 2002, Yao et al., 2008). In Chapter 3, by using different peptides (which specifically disturb the interaction between NSF and GluA2), we examined whether the interaction between NSF and GluA2 is essential for the effects of glucocorticoids on surface expression of AMPARs, AMPA receptor mediated synaptic transmission, AMPA receptor mobility and finally, the effects of corticosterone on fear memory consolidation. Results show that 3 hours application of corticosterone increases surface expression of both GluA1 and GluA2 containing AMPARs; the mobility of synaptic GluA2 containing AMPARs; and the peak amplitude of AMPAR-mEPSCs. These effects can be prevented by application of pep-R845A, which specifically blocks the interaction between GluA2 and NSF. These studies suggest that corticosteroids increase AMPAR mediated synaptic transmission and synaptic insertion of AMPARs via a mechanism that requires NSF/GluA2 interaction. Preliminary data show that pep-R845A -which disturbs the interaction between NSF and GluA2- applied directly after training in a contextual fear-conditioning task enhances freezing behavior 24 hours later, but prevents the memory enhancing effect of corticosterone.

To examine in more detail how corticosteroid hormones regulate AMPA receptor function and fear memory formation we studied the role of the mammalian Target of Rapamycin (mTOR) pathway, which is important for translation, synaptic plasticity and memory formation (Tang et al., 2001; Glover et al., 2010). In Chapter 4, by combining electrophysiology, immunocytochemistry, live cell imaging and contextual fear conditioning, we examined the role of this pathway in corticosterone effects on AMPARs and contextual fear memory formation. Corticosterone enhanced the amplitude of mEPSCs in a time dependent manner through Glucocorticoid Receptors (GRs) and via activation of a protein synthesis dependent pathway. Moreover, corticosterone
increased the mobile fraction of AMPARs as well surface expression and reduced the diffusion coefficient. The effects of corticosterone on AMPA receptor mediated synaptic transmission and the diffusion coefficient were prevented by rapamycin (which blocks the mTOR pathway), indicating that this pathway is involved in highly specific processes of AMPA receptor function. In addition, we report that corticosterone enhanced fear memory and that this effect is prevented by blocking the mTOR pathway. These studies suggest that corticosterone binds to GRs, which increases AMPAR mobility, but also facilitates the synaptic retention of AMPARs via the mTOR pathway, which may contribute to enhanced memory consolidation.

Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine-specific protein kinase that is regulated by the Ca²⁺/calmodulin complex. CaMKII is involved in many signalling cascades and is thought to be an important mediator of synaptic plasticity and learning and memory (Yamauchi, 2005; Lohmann and Kessels, 2014). Interestingly, CaMKII has been implicated in the memory enhancing effects of corticosterone (Hu et al., 2007; Li et al., 2013). In Chapter 5 we therefore studied the role of CaMKII in the effects of glucocorticoids on AMPA receptor mediated synaptic transmission. We used electrophysiological and immunocytochemistry methods to examine whether CaMKII is involved in corticosterone effects on AMPAR-mEPSCs and surface expression. Our results show that corticosterone increases AMPA receptor mediated synaptic transmission and surface expression of GluA1 and GluA2. Pharmacologically interfering with CaMKII function through an inhibitor applied 30 min before and during the entire period of incubation with corticosterone or only during the last 30 min of corticosterone application to the primary hippocampal cultures prevented the corticosteroid-induced enhancement of peak mEPSC amplitude. These data suggest that CaMKII is involved in the corticosteroid regulation of AMPARs function.

The NMDA receptor (NMDAR) is critically involved in activity-dependent changes in synaptic weight as well as memory formation (Tsien et al., 1996; Lu et al., 2001; Kessels and Malinow, 2009). In Chapter 6 we examined the effects of corticosterone on NMDA receptor function. By using electrophysiological techniques we monitored alterations of
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NMDAR-mEPSCs after we applied corticosterone to primary hippocampal cultures. We report that corticosterone – briefly after its application - increased the peak amplitude of NMDAR-mEPSCs, together with an increase of area under the curve (charge). These effects were prevented by application of an NMDA receptor 2B antagonist, suggesting that corticosterone enhances NMDA receptor function via GluN2B receptors. In line with this, Groc et al found that the mobility of NR2B was also increased by corticosterone (unpublished observations). These data suggest that corticosterone is able to enhance NMDAR function.

Conclusion:
In the introduction of this thesis, various questions were asked. The answers to these questions can be summarized as follows:

- Corticosterone regulates activity dependent manner AMPA receptor function
- GluA2-NSF interaction is involved in corticosterone effects on AMPA receptor function and possibly fear memory formation
- mTOR is essential for corticosterone effects on AMPA receptor function and memory formation
- CaMKII is required for corticosterone effects on AMPA receptor function
- NMDA receptor function is regulated by corticosterone

2. Corticosteroid hormones and excitatory synapses.

Exposure to stressful situations increases activity of the autonomic nervous system which induces the release of (nor) adrenaline into the circulation and noradrenaline in the brain (de Kloet et al., 2005; Joëls and Baram, 2009). In addition, stress activates the hypothalamus-pituitary-adrenal axis, which elicits the release of corticosterone from the adrenal glands (de Kloet et al., 2005). Corticosterone can enter the brain and bind to two types of receptors; the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Via activation of their receptors, noradrenaline (Hu et al., 2007) and corticosteroid hormones (Sandi and Rose, 1994; Roozendaal et al., 2009; Krugers et al., 2009).
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2010; Zhou et al., 2010) released in the context of a learning situation enhance memory formation. Moreover, noradrenaline and corticosterone in synergy optimally promote memory consolidation (Roozendaal et al., 2006).

In this thesis we addressed how specifically corticosteroid hormones regulate memory formation and focused on excitatory synapses as an important mechanism for memory formation. At these synapses, AMPA-type glutamate receptors mediate fast excitatory synaptic transmission. The number of functional AMPA receptors at synapses is critically regulated and an important mechanism for learning and memory (Rumpel et al., 2005; Whitlock et al., 2006; Kessels and Malinow, 2009). Activation of NMDA receptors at these synapses is critical for activity-dependent changes in synaptic plasticity such as long-term potentiation (LTP) as well as learning and memory (Tang et al., 1999; Shimizu et al., 2000; Lisman et al., 2012).

Slow effects of corticosterone on AMPAR mediated synaptic transmission

Earlier studies reported that corticosterone can evoke enhanced amplitudes of mEPSCs hours after application (Karst and Joëls, 2005; Martin et al., 2009). Our current studies confirm that corticosterone slowly enhances AMPAR mediated synaptic transmission. This effect requires time, activation of GRs and protein synthesis. This process is accompanied by enhanced surface expression levels of AMPARs, an increase in the mobile fraction of AMPARs and a decrease in the diffusion coefficient. Our studies suggest that membrane insertion of AMPARs via an NSF/GluA2 mediated mechanism is crucial for the effect of corticosterone on AMPAR surface expression and AMPAR mediated synaptic transmission (Xiong et al., 2016). An earlier excellent series of experiments in the prefrontal cortex (Yuen et al., 2009; Liu et al., 2010; Yuen et al., 2011) has shown that the effects of corticosterone on exocytosis might be mediated by Rab-GTPases. Altogether the data indicates that exocytosis (Yuen et al., 2009; Liu et al., 2010; Yuen et al., 2011), as well as lateral diffusion (Groc et al., 2008), are important steps in the effects of corticosterone on AMPAR mediated synaptic transmission. Possibly, this is region-dependent.
Importantly, interfering with NSF/GluA2 only during the last 30 minutes of corticosterone administration was able to prevent the hormone effects, indicating that even a brief interference with NSF/GluA2 can disturb hormonal effects on synaptic transmission. This implies that a continuous supply of AMPARs is required for corticosteroid hormones to enhance synaptic transmission. This is confirmed by our observation that a CaMKII inhibitor elicits comparable effects, i.e. inhibiting CaMKII hours after administration of corticosterone prevents the effects of corticosterone on synaptic transmission. This indicates that interference with mechanisms which are key for AMPAR function can relatively rapidly prevent the effects of corticosterone on synaptic transmission.

Imaging experiments that we performed also indicate that corticosteroid hormones can increase retention of AMPARs in the synaptic membrane (Groc et al., 2008; Sarabdjitsingh et al., 2014). More specifically, corticosterone reduced the diffusion coefficient of GluA2 containing AMPARs (Xiong et al., 2015). Interestingly, this effect was blocked by rapamycin, which blocks the mTOR pathway. Since mTOR regulates protein translation, these effects might provide a mechanism how corticosterone – in a protein synthesis / translation-dependent manner – regulates AMPAR mediated synaptic transmission. At this point it remains to be determined exactly how corticosterone activates the mTOR pathway. Preliminary evidence suggests that pS6k levels may be enhanced by corticosterone, but this needs to be further investigated. Also, it needs to be determined exactly how corticosteroid hormones regulate synaptic retention of AMPARs. Possibly CaMKII is involved since -via phosphorylation of Stargazin- it has been reported to immobilize AMPARs at synapses (Opazo et al., 2010).

*Activity-dependent effects on synaptic transmission*

Corticosterone has been reported to rapidly increase the frequency of mEPSCs (Karst et al., 2005). In our studies we found no rapid effects of corticosterone on the frequency of mEPSCs which may have been the result of the fact that we used primary cultures (with relatively young neurons in a more dispersed network than in adult slices) or that we did not record in the presence of the hormone. Yet, we did find that corticosterone
enhanced the activity-dependent increase in mEPSC frequency. This indicates that the hormone, possibly by enhancing neurotransmitter release (Karst et al., 2005), promotes synaptic plasticity, which confirms observations that the hormone is able to rapidly promote synaptic plasticity in hippocampal slices (Wiegert et al., 2006).

Corticosterone prevented activity-dependent synaptic plasticity when the hormone was applied hours before inducing plasticity. It has been hypothesized that this may be the result of corticosterone-induced occlusion of synapses which – via a metaplastic mechanism – prevents subsequent synaptic potentiation (Wiegert et al., 2005; Krugers et al., 2010). Likewise, corticosteroid hormones generally suppress synaptic plasticity in slices when the hormones are administered hours before high frequency stimulation (Wiegert et al., 2005; 2006). Occlusion could be induced e.g. by increasing the number and retention of AMPARs at synapses (Groc et al., 2008; Xiong et al., 2015; 2016) and might provide a mechanism that potentially prevents overwriting of information and will maintain information in a network.

Corticosterone and NMDA receptors
Since NMDA receptors are critical for activity dependent changes in synaptic transmission (Shimizu et al., 2000; Lu et al., 2001) we examined whether their activity can be modified by corticosterone. We report that corticosterone increases NMDAR mediated synaptic transmission, an effect which was prevented by blockade of GluN2B receptors. These effects confirm other observations that corticosteroid hormones and stress can activate NMDA receptors (Yuen et al., 2009; 2011), although the latter studies were conducted in the prefrontal cortex and at another time interval after application of acute stressors to the animals. This rapid effect of corticosterone on NMDARs might potentially increase the ability of synapses to undergo synaptic potentiation (Wiegert et al., 2006).

Taken together, a picture emerges that corticosteroid hormones rapidly increase the frequency of hippocampal mEPSCs (Karst et al., 2005), enhance activity-dependent changes in AMPA mediated synaptic transmission (chapter 2), increase AMPA mobility
Various studies have suggested that glucocorticoids activate BDNF-TrkB through the Erk1/2 MAPK pathway to promote fear memory formation (Revest et al., 2014).

Scheme 1: Illustration showing how corticosterone affects AMPARs and NMDARs mediated synaptic transmission. MRs=Mineralocorticoid Receptors; GRs=Glucocorticoid Receptors, LTP=Long term potentiation; NSF=N-ethylmaleimide-sensitive factor; mTOR=mammalian target of rapamycin; CaMKII=Ca\(^{2+}\)/calmodulin-dependent protein kinase; GDI-Rab4=GTP dissociation inhibitor-Rab4 complex.

3. Corticosteroid hormones, excitatory synapses and memory

Various studies have suggested that glucocorticoids activate BDNF-TrkB through the Erk1/2 MAPK pathway to promote fear memory formation (Revest et al., 2014).
Also, MAPK and Egr-1 are involved in stress and corticosterone-induced memory enhancement (Revest et al., 2005). Chen et al. (2012) reported that GRs affect long-term memory formation by recruiting the CaMKIIα-BDNF-CREB-pathway. In addition, Liu et al. (2010) reported that corticosterone increases synaptic AMPA receptors via Serum- and Glucocorticoid-inducible Kinase (SGK) regulation of the GDI-Rab4 complex in vitro, which contributes to memory formation.

In this thesis we investigated whether two (other) potential pathways are involved in corticosterone effects on AMPARs, i.e. NSF/GluA2 interaction and the mTOR pathway. We found that the mTOR pathway is critically involved in the effects of corticosterone on memory consolidation (Xiong et al., 2015). This may suggest that corticosterone, via the mTOR pathway and by promoting retention of AMPARs enhances memory consolidation. Future studies need to further determine whether the interaction between corticosterone, mTOR and AMPARs is causally related to the effects of corticosterone on memory formation.

The NSF-GluA2 interaction is also involved in memory formation (Lee et al., 2002; Yao et al., 2008; Joels and Lamprecht, 2010; Migues et al., 2014). Pep-R845A is a specific NSF-GluA2 interaction inhibitory peptide, and infusion of pep-R845A into the lateral amygdala 30 min before fear conditioning led to an impairment of long-term fear memory formation, but did not affect short-term memory formation (Joels and Lamprecht, 2010). Another study reported that object location and contextual fear memory is impaired 5 and 28 days respectively after pep-R845A administration into the dorsal hippocampus (Migues et al., 2014). Our preliminary data show that pep-R845A applied into the dorsal hippocampus immediately after training in a contextual fear-conditioning task enhanced freezing behavior 24 hours later, and prevented the memory enhancing effect of corticosterone. At this point it is difficult to explain how interfering with the interaction between NSF-GluA2 by itself enhances fear memory. However, it has been reported that NSF also directly interacts with β2 adrenergic receptor (Cong et al., 2001). The β2AR-NSF interaction is required for efficient internalization of β2 adrenergic receptors and for their recycling to the cell surface (Cong et al., 2001). We
hypothesize that the dysfunction of NSF induced by Pep-R845A application may also interrupt the interaction of β2AR-NSF, inducing failure of β2AR internalization. It has also been reported that noradrenaline is released after stress, and via activation of β2 adrenergic receptor, may phosphorylate and enhance synaptic delivery of GluA1, thereby lowering the threshold for LTP and memory (Hu et al., 2007). Thus, the fear memory enhancing effect induced by pep-R845A infusion after training in the weak fear conditioning paradigm may be due to preventing the interaction of NSF with the β2 adrenergic receptor. Corticosterone effects on memory enhancement were prevented by pep-R845A in the same experiment. We propose that corticosterone promotes the mobility of AMPAR subunits and increases the AMPAR retention at synapses (Martin et al., 2009; Xiong et al., 2015), an effect that can be blocked by pep-R845A in vitro, so that the interaction of GluA2 with NSF may contribute to the memory enhancing effect of corticosterone.

Taken together, our data suggests that interruption of GluA2-containing AMPARs trafficking contributes to memory formation, and corticosterone effects on fear memory enhancing requires the interaction between NSF and GluA2. To further explore the role of the NSF-GluA2 interaction in corticosterone effects on contextual fear memory enhancement, it is necessary to investigate the timing of corticosterone driving the AMPARs into synapses in this specific weak fear conditioning paradigm.

4. Future perspectives

In this thesis, we used several approaches, e.g. electrophysiology (whole cell patch clamp), immunochemistry (surface expression of AMPARs), live cell imaging (FRAP), and behavioral testing (contextual fear conditioning paradigm) to determine how corticosteroid hormones regulate synaptic function - a critical endpoint for learning and memory - and memory formation. While these studies reveal novel insights how corticosteroid hormones regulate synaptic function and memory formation, there remain multiple challenges for the future.
1) In chapter 4 and chapter 5, we performed studies to examine the role of corticosterone on synaptic transmission and memory consolidation. It will be important to determine via which pathways MRs and GRs regulate AMPAR mediated synaptic transmission and whether these pathways are critically involved in the memory enhancing effects of corticosterone. For example, how exactly does corticosterone regulate the mTOR pathway and which proteins are targeted by mTOR to enhance synaptic retention of AMPARs, AMPAR mediated synaptic transmission and ultimately, memory consolidation?

2) Memory formation involves different processes such as attention/perception, encoding, consolidation, retrieval, behavioural flexibility, and response selection. Some of these processes are known to be regulated by corticosteroid hormones, e.g. response selection and consolidation (Oitzl et al., 2001; Roozendaal et al., 2009; Schwabe et al., 2010; Zhou et al., 2010; Xiong et al., 2015). Yet, it will be important to determine whether and how corticosteroid hormones and MR/GR activation affect processes such as attention/perception. What is the role is of different brain areas and what are the cellular and molecular mechanisms? For this, a combination of techniques such as cellular recordings, pharmacology and optogenetics, in combination with detailed behavioural studies, will be required.

3) AMPARs are composed of different subunits with different different kinetics and different roles in synaptic function (Shi et al., 1999; 2001; Kessels and Malinow, 2009; Huganir and Nicoll, 2013). Corticosterone affects AMPARs, but whether / how corticosteroid hormones regulate different types of AMPARs and whether this is relevant for different phases of memory formation remains elusive.

4) Recent studies from various labs have shown that there are engram cells in different brain areas (e.g. hippocampus, amygdala, nucleus accumbens) (Suzuki et al., 2004; Liu et al., 2012; Yiu et al., 2014; Tonegawa et al., 2015). For example by using optogenetics, light activation of memory engram cell population can induce memory recall, and it was shown that the hippocampus-amygdala-nucleus accumbens circuit is responsible for
stress induced depression-like behavioral expression (Liu et al., 2012; Redondo et al., 2014; Tonegawa et al., 2015). These findings may raise the question whether engram cells are involved in the memory enhancing effects of corticosterone.

5) We have focused on the effects of corticosteroid hormones on cellular effects in the hippocampus. However, recent studies have shown that corticosteroid hormones can regulate brain function and behavior also at the circuit level. For example, stress, via MRs, regulates the connectivity between amygdala and striatum which may be relevant for altering stimulus response strategies (Vogel et al., 2015). Moreover, corticosterone, in a fear conditioning paradigm, alters neural activity within the hippocampus-amygdala circuitry (Kaouane et al., 2012). Stress induced depression-like behavior is rescued in mice by optogenetically reactivating dentate gyrus cells that were previously active during a positive experience, which involves the hippocampus-amygdala-nucleus-accumbens pathway (Ramirez et al., 2015). To get a complete understanding of how stress regulates brain function and behavior, a major challenge will be to understand how stress and stress-hormones regulate the connectivity between brain areas and whether this is critical for memory formation.

6) We have addressed how corticosteroid hormones regulate neuronal function and behavior. However, after exposure to stress, a series of responses is activated and other neuromodulators such as noradrenaline (Hu et al., 2007) and CRH (Joëls and Baram, 2009; Krugers et al., 2010) may affect synaptic function and memory formation, alone, but also in concert (Roozendaal et al., 2009; Zhou et al., 2012). It will be relevant to understand how the interaction between corticosterone and other hormones (e.g. noradrenaline, CRH) regulates neuronal function and behaviour.

7) Stress is an important risk factor for diseases such as depression and posttraumatic stress disorder in vulnerable individuals. This implies that interactions between genes and environment determine resilience and vulnerability (Caspi and Moffitt, 2006; Klengel and Binder, 2015). One important environmental factor that determines the sensitivity for stress is the mother-infant (or pup) interaction during the early postnatal period.
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(Champagne et al., 2008; Korosi and Baram et al., 2010; Oomen et al., 2010; Krugers and Joels, 2014; Naninck et al., 2015). Interestingly, early postnatal experiences lastingly regulate excitatory synaptic function. Thus, low levels of maternal care and maternal deprivation persistently enhance hippocampal NMDA receptor function (Bagot et al., 2012; Rodenas-Ruano et al., 2012) via enhancing GluN2B-containing NMDA receptors. Interestingly, this is accompanied by suppressed synaptic plasticity in adulthood (Champagne et al., 2008; Bagot et al., 2009; Oomen et al., 2010) which can be prevented by blocking NMDA receptors (Bagot et al., 2012). This may indicate that early life stress, via disruption of NMDA receptor function, may predispose to cognitive impairment and, if occurring in humans, yield an increased risk to develop psychopathology. Plasma corticosterone levels increase after exposure to early life stress, and the sensitivity of synapses to corticosterone is altered after exposure to early life adversity (Champagne et al., 2008). It will be important to understand how early life experience regulates the sensitivity for excitatory synapses and stress hormone later in life. For example, how does early life stress regulate the sensitivity of AMPA receptors NMDA receptors, the CamKII and mTOR pathways or GluA2-NSF interaction for corticosterone? Are epigenetic factors involved and how does this affect fear behavior later in life?

Answers to these exciting questions will enhance our insight in corticosteroid actions on glutamate transmission and fear learning, which in future may open new avenues for the treatment of stress-related psychopathology.
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