Chapter VII

General Discussion

Outline

1. Summary

Upon exposure to stressful events, the release of corticosteroids from the adrenal glands is enhanced. These hormones, together with other stress hormones and peptides (Joels and Baram, 2009) enable organisms to react effectively to stressful conditions and help individuals prepare for future events, e.g. by enhancing the consolidation of relevant information (de Kloet et al., 2005).

Corticosteroids regulate consolidation of information via the low affinity glucocorticoid receptors (GRs) (Barrett and Gonzalez-Lima, 2004; Oitzl and de Kloet, 1992; Roozendaal, 2002, 2003; Sandi and Rose, 1994b). The high affinity mineralocorticoid receptors (MRs) appear to be involved in appraisal of novel, stressful information (Oitzl and de Kloet, 1992). Yet, the underlying mechanisms of how these hormones regulate learning and memory processes are far from being resolved. One putative mechanism could involve trafficking of AMPARs, which is important for synaptic plasticity (Beattie et al., 2000; Carroll et al., 1999b; Hu et al., 2007; Malenka, 2003; Malenka and Nicoll, 1999; Plant et al., 2006) as well as learning and memory formation (Hu et al., 2007; Humeau et al., 2007; Whitlock et al., 2006). Recent evidence suggests that corticosteroids modulate the trafficking of AMPARs (Groc et al., 2008; Martin et al., 2009) and AMPAR-mediated synaptic transmission (Karst et al., 2010b; Karst et al., 2005; Karst and Joels, 2005; Pasricha et al., 2011). Therefore, AMPARs might be an important molecular substrate by which stress hormones regulate memory formation (Kessels and Malinow, 2009; Krugers et al., 2010).

In this thesis, we performed behavioral experiments using the classical fear conditioning paradigm to examine the role of GRs and MRs in fear learning. In addition, we examined how fear learning and corticosteroids regulate the function of AMPA receptors. Finally, we examined further how GRs might alter AMPAR function.

The main findings of this thesis are summarized below:

Chapter 2: Blocking GRs prior to training impaired contextual memory one day after training but enhanced tone-cue memory three hours after training. Blocking MRs prior to training impaired contextual memory both three hours and one day after training. In agreement contextual memory was also impaired in forebrain-specific MR knockout mice at three hours after training (24 hours after training was not tested). Blocking MRs prior to training impaired tone-cue memory only at three hours after training. These studies indicate that both MRs and GRs are involved in fear learning.

Chapter 3: Memories become labile upon retrieval of information. Since blocking MRs reduces contextual fear conditioning (chapter 2) we investigated whether blocking MRs prior to retrieval also modulated fear memory. Administration of the MR antagonist spironolactone prior to (but not after) retrieval of contextual information reduced contextual freezing during the retrieval session and 24 hours later, though not one month later. Administration of spironolactone prior to retrieval did not affect extinction of contextual information and tone-cue memories.

Chapter 4: Here we examined whether fear learning is accompanied by altered AMPA receptor mediated synaptic transmission. We report that the mEPSC frequency in the hippocampal CA1 area was largely enhanced when measured shortly (but not one day) after fear conditioning training. In contrast, a significant enhancement in the mEPSC amplitude was found both shortly and one day after training. This effect was accompanied by a significant increase of synaptic GluA2 expression one day after training. These findings suggest that the formation of contextual fear memory - which occurs under stressful conditions - involves slow changes in hippocampal AMPAR-mediated signaling.

Chapter 5: In this chapter we examined the putative pathway between binding of corticosterone to glucocorticoid receptors and the eventual effects

on AMPAR-mediated synaptic transmission. We found that corticosterone slowly enhances the amplitude of mEPSCs in cultured hippocampal neurons. This effect could be fully prevented by co-application of pep2m which inhibits the interaction between N-ethylmaleimide-sensitive factor (NSF) and GluA2. Furthermore, the GR-antagonist RU38486, mTOR inhibitor rapamycin, PI3K kinase inhibitor LY294002 and calcium channel blocker nifedipine could also prevent the corticosterone-induced increase in synaptic transmission and enhanced surface labeling of GluA1 and GluA2 subunits. This supports that corticosterone regulates AMPARs function via NSF/GluA2-dependent trafficking and mTOR signaling.

Chapter 6: Hormones interact to promote the consolidation of fearful memories. We report here that combined administration of corticosterone and the beta-adrenergic receptor agonist isoproterenol (which were ineffective by themselves) rapidly increased phosphorylation of the AMPA receptor GluA1 subunit at S845, increased GluA1 and GluA2 subtype AMPAR surface expression and decreased the inter-event interval (i.e. increased frequency) of AMPAR-mediated mEPSCs. This suggests that cortcosterone in interaction with noradrenaline may also rapidly promote the ability to store information, via an AMPAR-mediated pathway.

In the following paragraphs of this chapter I will discuss these findings in detail and focus on the following points:

1) The role of glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) in fear learning and memory formation;

2) The role of MRs and GRs in modulating AMPA receptor function;

3) Combined effects of glucocorticoids and beta-adrenergic receptor activation on fear learning and AMPAR function;

4) Future plans based on results obtained in this thesis.

2. Corticosteroid receptors in Learning and Memory

2.1. The role of Glucocorticoid receptors in learning and memory formation

Two subtypes of corticosteroid receptors are expressed in the brain. Glucocorticoid receptors (GRs) are ubiquitously expressed throughout the brain while mineralocorticoid receptors (MRs) are highly expressed in the limbic system (Arriza et al., 1988; De Kloet et al., 1998; Reul and de Kloet, 1985). GRs have been found to play an important role in promoting memory consolidation but also to suppress memory retrieval. For example, applying selective GR agonists systemically or locally into the basolateral complex of the amygdala (BLA) immediately after inhibitory avoidance training enhanced memory retention (Roozendaal and McGaugh, 1996a, 1997b). Conversely, systemic injection of high dosages of corticosterone impaired the retrieval of already learned information in a water maze task (de Quervain et al., 1998). Similar findings have been reported in humans (de Quervain et al., 2000; Soravia et al., 2006; van Stegeren et al., 2010) (Figure 1).

In line with the view that GRs enhance memory consolidation, we report in chapter 2 that systemic injection of the GR-antagonist RU38486 one hour before training resulted in a reduction in contextual freezing behavior 24 hours after fear conditioning training. Even though the design did not allow to delineate whether this reduction in freezing behavior resulted from the interference with memory acquisition or consolidation, the fact that no effect of GR blockade was found when memory retention was tested shortly after training suggests a slow effect of GRs on contextual memory formation. As reported in chapter 2, contextual memory is relatively unstable 3 hours after training and decreases during retrieval. By contrast, memories are more stable at 24 hours after training. This suggests that time (>3 hrs) is needed for contextual information to become consolidated. Since RU38486 reduced memory at 24 but not 3 hrs after training, the data supports the idea that GRs are indeed involved in the consolidation of information (Oitzl et al., 2001).

Interestingly, application of RU38486 one hour before training resulted in *increased* freezing behavior to the tone cue 3 hours (but not 24 hours) after training. This observation was unexpected since previous studies suggest that corticosteroids enhance memory of aversive stimuli (Roozendaal and McGaugh, 1996a, 1997b). Although the role of MRs in fear learning and memory formation will be discussed further below, we speculate that during this early learning phase, blockade of GRs might uncover the effects of MR activation, which could enhance memory formation. The observation that MR blockade before training impairs tone cue fear memory expression shortly after training further supports this speculation.

Taken together, GRs are generally believed to enhance the consolidation of emotional and stressful memories. It was assumed for a long time that these behavioral effects are exclusively mediated via a genomic action (Oitzl et al., 2001). However, recent studies have shown that corticosteroids can also promote formation of emotional memories via membrane-associated receptors, presumably GRs but not MRs (Barsegyan et al., 2010; Roozendaal et al., 2010). The application of MR / GR antagonists in our studies as well as in other studies using pharmacological approaches do not allow firm conclusions of whether corticosterone effects on memory performance time involve genomic and or-genomic actions. This therefore justifies the question whether corticosteroid effects on fear memories involves genomic as well as non-genomic actions.

Question for future investigation:

-Do GRs regulate learning and memory formation (acquisition, consolidation, retrieval) exclusively via slow genomic actions or also via rapid non-genomic actions?

2.2. The role of Mineralocorticoid Receptors in learning and memory

formation

While GRs play a critical role in consolidation of fearful memories, there is also evidence that mineralocorticoid receptors (MRs) are involved in cognitive function (Figure 1). Recently it has been shown in both rodents and humans that stress exposure immediately before a spatial learning task promotes the use of an egocentric and stimulus-response approach rather than the use of allocentric and spatial map-based approaches (Schwabe et al., 2007; Schwabe et al., 2010). This shift in strategy is mediated by corticosteroids and can be blocked by pretreatment with an MR-antagonist (Schwabe et al., 2010). Accordingly, a critical role for MRs in appraisal of information and response selection has been reported before (Oitzl and de Kloet, 1992; Sandi and Rose, 1994a). MRs are therefore proposed to play an important role in cognitive processes, including learning and memory formation. Indeed, MR overexpression in rat dentate gyrus granular cells enhances consolidation in an object recognition task (Ferguson and Sapolsky, 2007). Conversely, forebrain-specific genetic deletion of MRs leads to impaired acquisition in a Morris water maze task (Berger et al., 2006). In agreement we report in this thesis that the MR-antagonist spironolactone, when injected one hour before fear conditioning training, reduces contextual fear memories shortly and one day after training (Zhou et al., 2010b). Contextual freezing behavior also decreased in forebrain-specific MR knockout mice when tested 3 hours after training (Zhou et al., 2010b). The current view that MRs are involved in the initial phase of a behavioral response to stress, involving strategy, appraisal and response selection, is therefore confirmed by our findings. These early effects may have lasting consequences for learning and memory processes.

MRs appear not only to be involved in the appraisal of novel fearful information, but also play a role during retrieval of already stored information (chapter 3). Current evidence shows that the retrieval of information renders the memory labile which enables updating of relevant information

(Eisenberg et al., 2003; Lee et al., 2006; Monfils et al., 2009; Nader et al., 2000b). We report that MRs are involved in the re-appraisal, updating and/or reconsolidation of contextual information. This was particularly evident when the retrieval process was relatively brief: when exposure to already stored information was relatively long – thereby inducing extinction learning – blocking MRs did not further enhance extinction of contextual and tone cue memory formation. Moreover, MRs are only involved in re-appraisal / updating / reconsolidation of information when the stored memory trace(s) was actively recruited, as no effects on freezing behavior was observed if MRs were blocked after or without retrieval. This suggests that MRs act in concert with synaptic activation to promote the storage of relevant information. Our design does not allow to determine whether MRs are specifically involved in re-appraisal, updating or reconsolidation of information, but the current ideas are summarized in Figure 1. Involvement in reconsolidation seems unlikely, given that spironolactone was ineffective when given after the re-exposure. Dedicated experiments, though, are necessary to resolve this issue. It also remains to be established whether the MR antagonist-spironolactone can be effectively used to reduce fear e.g. in humans, since the effects that we observed lasted for only 24 hours after retrieval. Finally, the behavioral experiments do not allow insight into the mechanism by which MR affects fear learning.

Question for future investigation:

-How do MRs affect learning and memory processes? Are slow genomic and /or non-genomic effects involved?

3. Hormonal Regulation of AMPA receptors

3.1. Glucocorticoid Receptors and AMPA receptor function

As discussed before, corticosteroids, via activation of MRs and GRs, play a critical role in learning and memory processes. Yet, the underlying

mechanisms of how these hormones regulate learning and memory processes are far from being conclusive. AMPARs mediate the majority of rapid excitatory synaptic currents in the mammalian central nervous system. The trafficking of AMPARs to and away from excitatory synaptic membrane is critically required for long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission (Malenka, 2003; Plant et al., 2006) and underlies fearful learning (McKernan and Shinnick-Gallagher, 1997; Rumpel et al., 2005; Tsvetkov et al., 2002).

Activation of GRs has been reported to slowly enhance hippocampal AMPAR-mediated mEPSCs, AMPAR surface expression, lateral diffusion and synaptic insertion (Groc et al., 2008; Karst and Joels, 2005; Martin et al., 2009). Such effects are protein synthesis-dependent. In chapter 5 we further identified that these slow effects of corticosterone on AMPARs-mediated synaptic transmission involve NSF-GluA2 dependent trafficking. Even though synaptic expression of AMPARs should be further investigated in this regard, the electrophysiological results support the current view that NSF-GluA2 interaction is necessary for maintaining synaptic transmission (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Yao et al., 2008) (Figure 2).

In addition to NSF, GluA2 also interacts with other proteins, such as PDZ domain-containing protein interacting with C kinase (PICK1) (Dev et al., 1999; Xia et al., 1999). Contrary to the role of NSF-GluA2 interaction, PICK1 is found to promote the internalization of GluA2 content of AMPARs or stabilizes its intracellular pools (Perez et al., 2001; Rocca et al., 2008; Terashima et al., 2004). Recently it is shown that PICK1 regulates spine size in hippocampal neurons via inhibition of the Arp2/3 complex and thus plays a role in hippocampal LTD (Nakamura et al., 2011). Furthermore, NSF ATPase activity has been reported to disrupt PICK1-GluA2 interaction (Hanley et al., 2002). Therefore, it would be interesting to investigate in detail how glucocorticoids modulate PICK1 synaptic expression and function

as well as its interaction with NSF in the context of AMPAR modulation by glucocorticoids.

Behaviorally, these effects of GRs on AMPA receptor function may be relevant for learning and memory. For example, intracranial injection of the AMPAR antagonist-CNQX impaired the memory-potentiating effect of corticosterone (Venero and Sandi, 1997). Intracerebroventricular infusion of pep2m, the peptide that blocks GluA2 synaptic trafficking by interfering with the interaction between NSF and GluA2, impaired long-term memory retrieval (Conboy and Sandi, 2010). Thus, trafficking of AMPARs might be one of the possible targets for glucocorticoids to facilitate learning and memory processes under stressful conditions.

The PI3K-mTOR signaling pathway also plays an important role in AMPAR insertion (Man et al., 2003; Wang et al., 2006) and is critically involved in synaptic plasticity (Gobert et al., 2008; Man et al., 2003; Tang et al., 2002; Tsokas et al., 2007) and memory formation (Parsons et al., 2006; Tischmeyer et al., 2003). In chapter 5 we report that the PI3K inhibitor LY294002 and mTOR inhibitor rapamycin were able to block the effects of corticosterone on AMPAR-mediated mEPSC peak amplitude as well as AMPAR surface expression in cultured hippocampal neurons. This indicates that the PI3K-mTOR pathway might be an important mechanism via which corticosteroid hormones promote the synaptic retention of AMPARs (Figure 2). The fact that application of the mTOR pathway blocker rapamycin impairs late (but not early)-phase LTP (Gobert et al., 2008; Tang et al., 2002) as well as later phase memory consolidation (Tischmeyer et al., 2003) suggests that translational control of relevant protein synthesis is required for maintenance of LTP and formation of long term memory. This might be the process targeted by corticosteroids.

Components of the mTOR pathway, such as mTOR, eukaryotic initiation factor 4E and its binding proteins, have a completely overlapping distribution in cultured hippocampal neurons with PSD-95 (Tang et al., 2002),

a postsynaptic protein that is critically involved in retention of AMPA receptors (Stein et al., 2003). Overexpression of PSD-95 selectively enhances AMPAR synaptic clustering and AMPAR-mediated synaptic transmission (Beique and Andrade, 2003; El-Husseini et al., 2000; Stein et al., 2003). Interestingly, enhanced PSD-95 expression suppresses the ability to induce LTP and enhances the ability to elicit LTD as shown with the transfection or viral infection (Beique and Andrade, 2003; Stein et al., 2003), indicating a synaptic stabilization role of PSD-95. This is very reminiscent of the effects of corticosteroids: GR activation slowly reduces the ability to elicit LTP, while LTD under these conditions is facilitated (Alfarez et al., 2002; Wiegert et al., 2005). These slow/genomic GR-mediated effects therefore facilitate normalization of previously increased excitability and might protect earlier encoded information (de Kloet et al., 2008; Joels et al., 2008).

Taken together, our data suggest that activation of GRs slowly promotes AMPAR-mediated synaptic transmission via NSF-GluA2 dependent trafficking, involving PI3K-mTOR signaling. This does not exclude the possibility that other signaling pathways also play a role in the facilitatory effect of corticosteroids on glutamate transmission, for instance, corticosterone treatment was found to enhance AMPAR-mediated synaptic transmission and AMPAR surface expression via the induction of serum-and-glucocorticoid-inducible kinase (SGK) and the activation of Rab4, which mediates receptor recycling between early endosomes and the plasma membrane (Liu et al., 2010; Yuen et al., 2011). Therefore, it is relevant to further examine the role of corticosteroids on AMPAR exocytosis/endocytosis by targeting proteins involved in those two processes, such as the members of the Rab family of membrane sorting proteins (Gerges et al., 2004; Glodowski et al., 2007; Liu et al., 2010; Ward et al., 2005).

Questions for future investigation:

-What are the molecular mechanisms via which GRs modulate exocytosis/endocytosis and lateral diffusion of AMPA receptors ?

-(How) do GRs promote retention of AMPARs? Are these effects mediated via genomic and/or non-genomic effects?

-Is hormonal regulation of AMPAR function critical for the memory enhancing effects of GRs?

3.2. Mineralocorticoid Receptors and AMPA receptor function

The fact that MR-mediated actions of corticosteroids modulate learning and memory processes justifies the question how these receptors affect synaptic function. Apart from the classic slow / genomic effects of corticosteroids mediated via GRs on synaptic transmission, rapid, presumably non/genomic effects on synaptic transmission via MRs have been reported recently (Karst et al., 2010b; Karst et al., 2005; Pasricha et al., 2011).

Recent data show that MRs are not only localized in the cytosol, but also believed to reside at the plasma membrane with 10-fold lower affinity than classic cytosolic MRs (Joels et al., 2008; Karst et al., 2005). Evidence shows that this type of MR mediates rapid and non-genomic effects and is probably involved in many rapid functions in the central nervous system (de Kloet et al., 2008). It has been shown that corticosterone application rapidly (within minutes) increases the frequency of AMPAR-mediated miniature excitatory synaptic currents (mEPSCs) presumably by increasing the pre-synaptic glutamate release probability (Karst et al., 2005). In addition, Groc et al. (2008) - using single quantum-dot imaging in live hippocampal neurons - demonstrated that corticosterone and also a membrane-impermeable BSA-corticosterone conjugate rapidly increase GluA2-AMPAR surface diffusion (Groc et al., 2008). These effects can be mimicked by a selective MR agonist (aldosterone) and blocked by a selective MR antagonist. At the same time, corticosterone amplified the glycine/picrotoxin- induced increase in synaptic surface GluA2-AMPAR

density (Groc et al., 2008).

These findings at the single cell level indicate that MRs may rapidly promote the capacity to store information at the cellular level. Accordingly, rapid corticosteroid effects on synaptic plasticity have been reported. For example, corticosterone rapidly facilitates synaptic potentiation in the mouse hippocampal CA1 area, but only when high levels of the hormone and high frequency of stimulation coincide in time (Wiegert et al., 2006). While the role of MRs remained unclear in this study, others have reported that MR activation can promote LTP in the dentate gyrus (Korz and Frey, 2003). Moreover, Pu et al., (2007) found in a slightly different experimental setting that corticosterone application can increase the early phase LTP in the hippocampal dentate gyrus, when applied around the time of high frequency stimulation.

Earlier studies have demonstrated that MR activation enhances LTP in the dentate gyrus (Pavlides et al., 1995) and CA3 area (Pavlides and McEwen, 1999) in anesthetized rats, and in the hippocampal CA1 area in vitro (Pavlides et al., 1996). In these experiments the delay between the time brain tissue was first exposed to MR agonist or antagonist till synaptic potentiation was usually > 1 hour, making a distinction between rapid (non-genomic) and slower (genomic) effects impossible.

Recently Karst *et al* (2010) showed that corticosterone rapidly enhances glutamatergic synaptic transmission via MRs in BLA neurons. In contrast to previous findings in the hippocampus (Karst et al., 2005), this enhancement was long-lasting, potentially in favor of encoding information related to the stress experience (Karst et al., 2010b). Subsequent application of corticosterone resulted in a reduction of synaptic transmission (which was absent in hippocampal CA1 neurons), via a process involving GRs and cannabinoid receptor-1. Importantly, these findings demonstrate that corticosteroids can rapidly affect synaptic transmission in the hippocampus and amygdala - which are critically engaged in fear learning -

although critical differences between the two areas are present. Overall these studies demonstrate that corticosteroids can rapidly enhance hippocampal AMPA receptor mediated synaptic transmission, presumably via activation of MRs. Although it remains speculative, these effects may contribute to fearful learning and memory formation. The current ideas on this topic are summarized in Figure 2.

3.3. Relevance of Mineralocorticoid Receptors

Due to their 10-fold higher affinity than GRs (De Kloet et al., 1975), cytosolic MRs are normally occupied under basal conditions or during the trough of corticosteroid secretion (de Kloet et al., 2005).This raises the question to what extent MRs can be further activated and via which mechanisms corticosteroids can modulate brain function via MRs in vivo, in response to an exogenous or endogenous hormone surge?

Previous studies have demonstrated that the affinity of membrane-located MRs in mammalian brain neurons (Groc et al., 2008; Karst et al., 2005) is 10-fold lower than that of the classic cytosolic MRs, i.e. membrane-located MRs have more or less comparable affinity as the classic cytosolic GRs. The finding of membrane-located low-affinity MRs provides a possible explanation to the previous question. Despite the nearly full occupancy of classic MRs under basal conditions, it is possible that membrane-located MRs compete with cytosolic GRs during stress. Thus, a new model has been proposed (Joels et al., 2008), which highlights the existence of membrane located MRs and their role in various brain functions such as synaptic plasticity and learning and memory formation during stress (in addition to the role of classic MRs and GRs). Plasma membrane-located MRs are also ideally suited to follow the pulsatile pattern of circulating hormones levels (de Kloet and Sarabdjitsingh, 2008). Via membrane MRs, corticosteroids could also amplify the effect of other stress hormones (Groc et al., 2008). Thereby, these hormones may contribute to the fast behavioral

effects and encoding of stress-related information, although the underlying signaling pathways still need to be addressed. Such initial fast effects may be counteracted by subsequent slow/genomic effects via GR, which facilitate suppression of previously increased excitability, thus favoring storage of information for the future (de Kloet et al., 2008; Joels et al., 2008).

Questions for future investigation:

-How does MR activation enhance synaptic insertion of AMPARs? Are these effects mediated via genomic and/or non-genomic effects?

-Is hormonal regulation of AMPAR function critical for the memory enhancing effects of MRs?

-How are MRs inserted into the plasma membrane?

4. Combined hormonal effects on memory and AMPA receptors

4.1. Corticosteroids, beta-adrenergic receptors and learning and memory formation

Upon stress, not only corticosteroids but also other stress-related peptides and neurotransmitters -such as CRH, endocannabinoids and noradrenaline- are released in the brain (Joels and Baram, 2009). These hormones, in close harmony with corticosteroids promote the consolidation of relevant information (Joels, 2006). In particular there is evidence that corticosteroids and noradrenaline work together to promote adaptive behavior. For example, infusion of a β-antagonist into the basolateral complex of the amygdala (BLA) inhibits the GR-induced increase in inhibitory avoidance learning (Quirarte et al., 1997; Roozendaal and McGaugh, 1997a; Roozendaal et al., 1999a). Systemic injection of corticosterone was found to increase BLA norepinephrine levels shortly after inhibitory avoidance training (McReynolds et al., 2010). It has been proposed that glucocorticoids enhance memory consolidation, in a permissive fashion, by potentiating β-adrenoceptor-cAMP/PKA signaling in the BLA (Roozendaal et al., 2002b).

4.2. Corticosteroids, beta-adrenergic receptors and AMPA receptor trafficking

Since AMPA receptors are critical for storing information at the cellular level we examined how corticosteroids and noradrenaline – via combined action – modulate AMPAR-mediated synaptic transmission. In *chapter 6*, we report that corticosterone, together with a relatively low (rather than high) concentration of isoproterenol (a β-adrenoceptor agonist), rapidly enhances AMPAR immunosurface labeling, phosphorylation of GluA1 S845 as well as AMPAR-mediated mEPSCs. These studies suggest that the combined action of corticosteroids and noradrenaline rapidly promotes AMPAR signaling and hence presumably the storage of information. Support for this notion comes from earlier studies showing that co-application of isoproterenol and corticosterone leads to very effective synaptic plasticity in the dentate gyrus (Pu et al., 2007). This is very different from the situation where corticosterone is allowed to develop its genomic actions. For instance, noradrenaline has been reported to increase the excitability of CA1 pyramidal neurons from adrenalectomized rats, and this effect is attenuated by application of a GR agonist hours in advance (Joels and de Kloet, 1989). Similar findings have been reported at the network level, where corticosterone can slowly suppress synaptic potentiation (Pu et al., 2007), and even with respect to amygdala-dependent behaviour (Borrell et al., 1984). These findings indicate that corticosteroids on the one hand prime the network functionally to facilitate noradrenergic effect during stress and on the other hand seem to prevent overshooting of network function. The latter effect might favor preservation of previously acquired information from subsequent disturbance, at least for a period of time.

Questions for future investigation:

-Does combined beta-adrenergic and glucocorticoid receptor

activation long-lastingly regulate AMPAR-mediated synaptic transmission?

-Is PKA/cAMP signaling involved in combined actions of noradrenaline and glucocorticoids on AMPAR function?

-Does regulation of AMPAR function by combined beta-adrenergic / glucocorticoid receptor activation underlie hormonal regulation of emotional memories?

5. Closing remarks and future directions

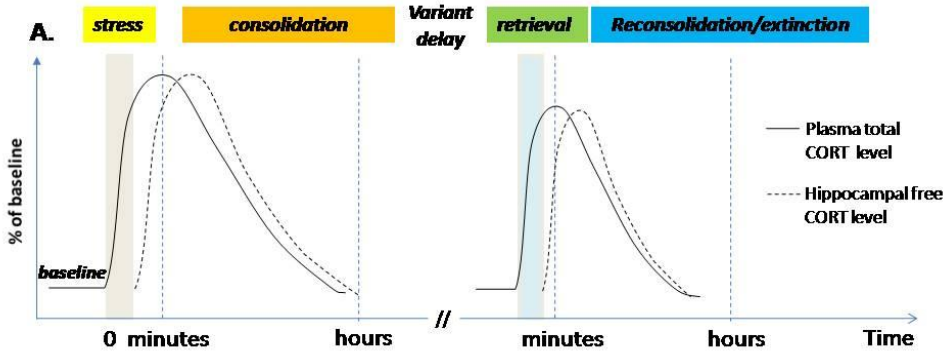
1) In this thesis, we describe that not only GRs, but also MRs are critically involved in fear-related learning and memory processes. To examine in detail the role of MRs versus GRs in the different memory phases (acquisition including appraisal and strategy; consolidation; retrieval; renewed encoding; extinction), it would be interesting to perform behavioral studies and apply specific MR or GR agonists and antagonists systemically or locally in mutant animals with targeted disruption of GRs or MRs. In combination with application of corticosteroids that act on membrane receptors (e.g. corticosterone-BSA conjugate) this will also help to identify the involvement of membrane-located receptor activation in learning and memory processes.

2) GR-antagonists have been reported to ameliorate symptoms of psychotic depression (Belanoff et al., 2001; Belanoff et al., 2002; Flores et al., 2006) and recently human MR gene haplotypes have been associated with perceived chronic stress (van Leeuwen et al., 2010) In this thesis we report that the MR antagonist spironolactone is able to temporary reduce fear memories when applied before retrieval of fearful information. Therefore it is important to further explore the potential of targeting glucocorticoid receptors during memory retrieval in order to (eventually) intervene with maladapted fear / mood disorders in humans.

3) PI3K-AKT-mTOR signaling as well as NSF/GluA2-dependent AMPAR trafficking underlie glucocorticoid-regulation of AMPAR function. Therefore, by pharmacological blockade or genetic up-/down-regulation of the mTOR pathway and/or NSF expression in cultured hippocampal neurons, the combined effects of corticosterone and isoproterenol can be further studied at the mechanistic level. In addition, with quantum-dot and high-resolution imaging approaches AMPAR dynamics such as exocytosis / endocytosis in response to various stimulations (e.g. hormone(s) administration or chemical LTP induction) need to be investigated in detail.

4) Combined application of a beta-adrenergic receptor agonist and corticosteroids regulate AMPAR-mediated synaptic transmission. These findings underline the importance to examine AMPAR function as relevant molecular mechanism by which stress-hormones and neurotransmitters interact and modulate learning and memory processes.

5) Finally, the fact that many stress-related peptides and neurotransmitters (e.g. CRH, endocannabinoids) play important roles in learning and memory formation (Izquierdo et al., 1988; Lee et al., 1992; Radulovic et al., 1999; Roozendaal et al., 1992), justifies a thorough integrated and systemic investigation to understand how all the elements (i.e. each individual stress hormone or peptide) are composed together to make a “symphonic” reaction that helps store relevant information which can be used for future events.



B.

	Acquisition*	Consolidation	Retrieval	Reconsolidation	Extinction
CORT	↑	↑	↓	↑	↑
MR activation	+	?	+	?	?
GR activation	+	+	+	+	+

↑ facilitation; ↓ inhibition; + involved; ? Not known or not determined

* Included variant aspects, e.g. response selection, strategy, appraisal etc.

Figure 1. Corticosterone and Corticosteroid Receptors in Learning and Memory

A) Exposure to stressful experiences increases peripheral circulating plasma corticosterone levels. With a delay of less than 15 minutes hippocampal free corticosterone levels also reach their peak and both peripheral and cerebral levels then decline (Droste et al., 2008). Most likely, similar changes occur during fear conditioning: CS-US training increases plasma corticosterone levels which then slowly decline. Upon re-exposure to the (fearful) CS, a similar increase in plasma corticosterone levels most likely occurs (Conway-Campbell et al., 2007).

B) Elevated corticosterone levels activate MRs and GRs which modulate learning and memory processes.

1) Corticosterone enhances memory acquisition (Akirav et al., 2004; Archer et al., 1981; Beylin and Shors, 2003; Roozendaal and McGaugh, 1996a, b). Both MRs (Berger et al., 2006; Herrero et al., 2006; Zhou et al., 2010b) and GRs play a role in acquisition (Brinks et al., 2007; Oitzl et al., 1997).

2) Corticosterone enhances memory consolidation (Abrari et al., 2009; Pugh et al.,

1997b; Quirarte et al., 2009; Roozendaal et al., 2006a; Roozendaal and McGaugh, 1996a; Roozendaal et al., 2006c; Sandi and Rose, 1994b), via activation of GRs (Jin et al., 2007; Oitzl and de Kloet, 1992; Pugh et al., 1997a; Quirarte et al., 2009; Quirarte et al., 1997; Roozendaal et al., 2001; Roozendaal and McGaugh, 1996a, b, 1997a, b; Roozendaal et al., 2009c; Roozendaal et al., 1999a; Roozendaal et al., 1996; Roozendaal et al., 2002b; Roozendaal et al., 1999b; Sandi and Rose, 1994b). A possible role for MRs in consolidation remains to be verified. In chapter 2 we describe that blocking MRs immediately after training does not affect freezing (Zhou et al., 2010b). However, a possible explanation of the "lack of effect" could be that endogenous MRs were already activated after training, thereby reducing the ability of spironolactone to block MRs.

3) Application of high doses of corticosterone before retrieval impairs memory retrieval (de Quervain et al., 2007; de Quervain et al., 2003; de Quervain et al., 1998; Pakdel and Rashidy-Pour, 2007; Rashidy-Pour et al., 2009; Sajadi et al., 2006, 2007). This effect might involve GRs (Roozendaal et al., 2003; Roozendaal et al., 2004b), Our studies suggest that MRs is required for contextual memory retrieval, while experiments of the role of GRs are being performed.

4) Corticosterone has also been shown to facilitate reconsolidation (Wang et al., 2008a) possibly via GRs (Jin et al., 2007; Wang et al., 2008a). In our experiments we found no clear role of MRs in reconsolidation (chapter 3, but see also #2 of this legend).

5) Corticosterone enhances extinction (Bohus and de Kloet, 1981; Gourley et al., 2009), via GR activation (Gourley et al., 2009; Yang et al., 2006). Our preliminary studies do not provide evidence for a role of MRs in extinction (chapter 3).

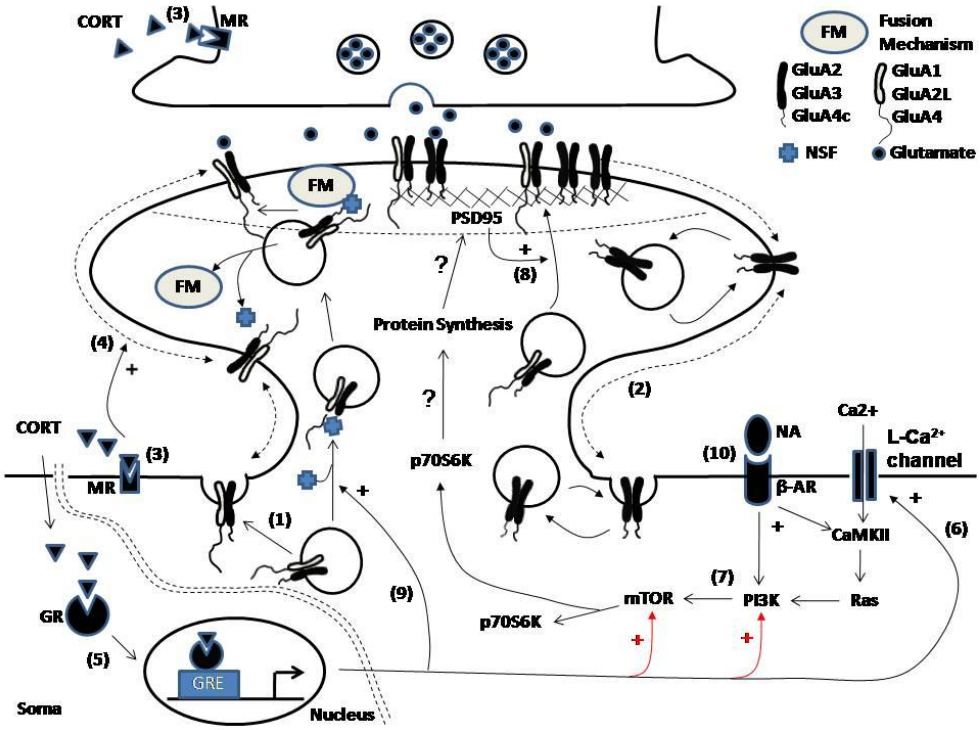


Figure 2. Corticosteroid Regulation of AMPARs.

This figure shows a (preliminary) summary of how glucocorticoids can enhance AMPAR mediated synaptic transmission.

(1-2) Upon delivery to the extrasynaptic membrane, AMPARs diffuse between the extrasynaptic space and synapse via lateral diffusion (Makino and Malinow, 2009; Petrini et al., 2009). GluA1/2 containing AMPA receptors require activity to become synaptically inserted (Hayashi et al., 2000; Kakegawa et al., 2004; Plant et al., 2006; Shi et al., 2001) and these receptors become replaced by GluA2/3 containing AMPARs (Shi et al., 2001).

(3-4) Elevated corticosterone (CORT) levels rapidly increase AMPAR-mediated synaptic transmission via MRs which are located both in the postsynaptic (Karst et al., 2005; Olijslagers et al., 2008) and presynaptic membrane (Olijslagers et al., 2008). These effects most likely involve an increase in release probability (not shown) and lateral diffusion (Groc et al., 2008).

(5-6) Corticosterone activates cytosolic GRs and slowly regulates AMPAR-mediated synaptic transmission and activity. For example, corticosterone enhances L-type calcium current via GR activation (Chameau et al., 2007). This might increase of cytoplasmic calcium levels, which in turn can activate numerous signaling

pathways.

(7-9) In chapter 5, we show that glucocorticoid-induced increases in surface labeling and AMPAR function involves PI3K and mTOR. The main active phosphoform of the downstream substrate of this PI3K-mTOR pathway-Thr-389-phosphorylated-p70 S6 kinase (p70S6K) can be found in the dendritic shaft and a subset of dendritic spines (Cammalleri et al., 2003) and is involved in protein synthesis (such as PSD95). PSD95 overexpression selectively increases the clustering and function of postsynaptic GluA1-containing AMPAR (El-Husseini et al., 2000). Meanwhile noradrenaline (NA) can also potentiate the PI3K pathway (Chenal and Pellerin, 2007) and thus might enhance GR effects (Chenal and Pellerin, 2007).

(10) NSF is found to interact with the C terminal of GluA2 subtype AMPARs in sub-synaptic vesicles, which regulates the “docking” of the vesicles with postsynaptic membrane fusion machinery (FM) and promotes the insertion of AMPARs into the synaptic plasma membrane (Song et al., 1998). Blocking NSF/GluA2 interaction abolishes the effect of corticosterone on AMPAR-mediated synaptic transmission.