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The studies described in this thesis were mainly performed at the department of Structural and Functional Plasticity of the Nervous System of the University of Amsterdam, the Netherlands.

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Corticosteroid effects on glutamatergic transmission and fear memory

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CONTENTS

General Introduction

Introduction, aim and outline of this thesis

The stress response

In neurobiology, stress can be defined as the perception and subjective interpretation of any condition that potentially interrupts the physiological or psychological homeostasis of an organism (Kim and Diamond, 2002). When an individual experiences a stressful situation, the previously achieved homeostasis is potentially disturbed, which elicits a complex of physiological and behavioral changes that promote adaptive responses to such disturbances (Krugers et al., 2010).

The core reaction to a stressor is the immediate activation of the autonomic nervous system (ANS), which induces the release of adrenaline and noradrenaline into the circulation. In addition, this provokes the release of noradrenaline in multiple brain areas from presynaptic terminals that arise from the locus coeruleus (Gibbs and Summers, 2002). Another important reaction that occurs after exposure to a stressor is activation of the hypothalamus-pituitary-adrenal (HPA) axis (Lightman and Conway-Campbell, 2010). After stress-exposure, the adrenal cortex secretes high levels of corticosterone (in human cortisol), which easily enters the brain. In the mammalian brain, there are two types of receptors that corticosterone can bind to: glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) (de Kloet et al., 2005; Krugers et al., 2010). MRs have a ten-fold higher affinity for corticosterone than GRs. As a consequence, MRs are largely occupied by corticosterone even when circulating levels of the hormone are low, whereas GRs are mainly activated when corticosterone level rise after stress or during the circadian peak.

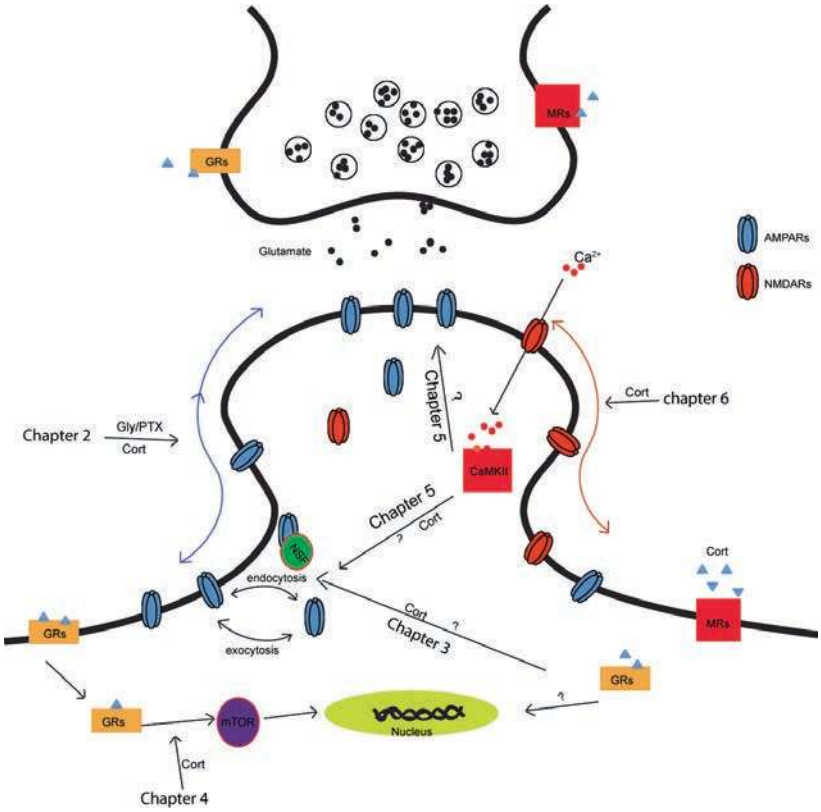
Through these receptors corticosteroids influence the function of numerous brain regions in which they are expressed, such as the hippocampus, amygdala, and prefrontal cortex (Kim and Diamond, 2002; Krugers et al., 2010). Classically, MRs and GRs exert their function through transcriptional regulation of responsive genes (de Kloet et al., 2005). However, corticosterone can also induce rapid, non-genomic effects through MRs and GRs, which are present in the vicinity of neuronal membranes in various brain areas (Di et al., 2003; Karst et al., 2005; Groc et al., 2008).

Via MRs and GRs, corticosteroid hormones promote behavioural adaptation to stressful events (de Kloet et al., 1999). These effects include enhanced consolidation of relevant information (Oitzl and de Kloet, 1992; Sandi and Rose, 1994; Roozendaal et al., 2009); impaired retrieval of already stored information (de Quervain et al., 1998) and shifting behavioural strategies (Schwabe et al., 2010). These effects involve glucocorticoids acting in multiple brain areas (Roozendaal et al., 2009), acting in concert with other neurotransmitters systems and neuromodulators (Joëls and Baram, 2009).

Stress and memory: the aim of the thesis

In this thesis we examined how corticosteroid hormones regulate synaptic function, which is critical for learning and memory (Kessles and Malinow 2009; Rumpel et al., 2006; Nabavi et al., 2014). In particular we studied how corticosteroid hormones modulate excitatory synaptic transmission and whether these effects are relevant for synaptic plasticity and memory formation. We investigated these effects in the hippocampus, an area relevant for learning and memory (Kessels and Malinow, 2009) which contains both MRs and GRs. At the time that the studies described in this thesis were started, there was already an existing literature on how corticosteroid hormones modulate behavioral adaptation and excitatory synaptic transmission and whether these effects are relevant for memory formation. In **Chapter 1** this body of literature is briefly reviewed.

From the description in this review, however, it is clear that there were still many open questions (see scheme 1). Some of these were addressed in novel experimental studies, described in Chapters 2-6. The overarching aim of the thesis was to investigate 1) the mechanisms via which corticosteroid hormones regulate synaptic transmission and synaptic plasticity in the hippocampus, and 2) whether these effects / mechanisms are relevant for (fear) learning and memory.



Scheme 1. Illustration showing the open questions in the thesis. Corticosteroid hormones potentially regulate excitatory synaptic transmission via various mechanisms (for details see text). Cort=Corticosterone; GRs=Glucocorticoid Receptors; MRs=Mineralocorticoid Receptors; CaMKII=Ca^{2+}/calmodulin-dependent protein kinase; NSF=N-ethylmaleimide-sensitive factor; mTOR= mammalian target of rapamycin; Gly/PTX=Glycine/Picrotoxin.

Specific questions addressed in this thesis

Corticosteroid hormones are known to enhance AMPA receptor mediated synaptic transmission in various brain areas (Karst et al., 2005; Zhou et al., 2009; Karst et al., 2010; Liu et al., 2010). Via activation of MRs and GRs, corticosterone rapidly alters the frequency of miniature excitatory postsynaptic currents (mEPSCs), not only in the hippocampus but also in the amygdala (Karst et al., 2005; 2010). Moreover, at a longer time scale, corticosterone enhances the peak amplitude of AMPAR-mediated mEPSCs (Karst et al., 2005; Martin et al., 2009; Liu et al., 2010; Chen et al., 2012). Yet, an important feature of the brain and its networks is the capacity to undergo activity-dependent

changes in synaptic transmission, which allows rapid and persistent adaptation. In **Chapter 2** we therefore investigated whether and how glucocorticoid hormones - within minutes or hours after a brief application - regulate plasticity of AMPA receptor mediated synaptic transmission. To investigate this, we used an established protocol to induce synaptic plasticity at the single cell level by activating NMDA receptors and examined how glucocorticoids rapidly and persistently alter synaptic plasticity.

An important question that needs to be addressed is via which routes and mechanisms corticosteroid hormones modulate AMPA receptor mediated synaptic transmission. A key step in regulating synaptic function is the insertion of AMPA receptors in the cellular membrane. N-Ethylmaleimide-Sensitive Factor (NSF) is critically involved in membrane fusion; its interaction with the AMPA receptor GluA2 subunit is crucial for insertion and stabilization of AMPARs at the membrane and maintaining synaptic transmission (Lee et al., 2002; Yao et al., 2008). By using different peptides (which specifically disturb the interaction between NSF and GluA2), we examined in **Chapter 3** whether the interaction between NSF and GluA2 is essential for the effects of glucocorticoids on surface expression of AMPARs, AMPA receptor mediated synaptic transmission and AMPA receptor mobility. We also examined whether the interaction between NSF and GluA2 is essential for the effects of corticosterone on fear memory consolidation.

To examine more in detail how corticosteroid hormones regulate AMPA receptor function and fear memory formation we next examined -in **Chapter 4**- 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). By combining electrophysiology, immunocytochemistry, live cell imaging and behavioral testing of contextual fear conditioning, we examined the critical role of the mTOR-pathway in the effects of corticosterone on AMPAR function and contextual fear memory formation.

Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II or CaMKII) is a serine/threonine-specific protein kinase that is regulated by the Ca^{2+}/calmodulin complex. CaMKII is involved in many signaling cascades and is thought to be an important

mediator of learning and memory (Elgersma et al., 2002; Silva and Josselyn, 2002; Yamauchi, 2005; Lohmann and Kessels, 2014). Ca^{2+} acts as a second messenger to activate CaMKII, which can promote the insertion of AMPA receptors in synapses and increase the conductance of AMPA receptors by phosphorylation (Sweatt, 1999; Hayashi et al., 2000; Lee et al., 2009). The CaMKII–GluN2B interaction is crucial for the induction of LTP (Barria and Malinow, 2005). Also, after LTP induction CaMKII can reside at the synapse for prolonged periods of time (Otmakhov et al., 2004). Interestingly, CaMKII has earlier been implicated in the memory enhancing effects of corticosterone (Hu et al., 2007; Li et al., 2013). In **Chapter 5** we therefore studied whether CaMKII modulates the effects of corticosteroid hormones on AMPA receptor mediated synaptic transmission. By using electrophysiological and immunocytochemistry methods we examined whether CaMKII regulates the effects of corticosterone on surface expression and synaptic function of AMPARs.

Finally, in **Chapter 6** we addressed the notion that the NMDA receptor (NMDAR) is critically involved in activity-dependent changes in synaptic weight as well as memory formation (Isaac et al., 1995; Tang et al., 1999; Huerta et al., 2000; Lu et al., 2001; Kessels and Malinow, 2009; Lohmann and Kessels, 2014). NMDAR-dependent Ca^{2+} influx in the post-synapse triggers synaptic potentiation and a CaMKII- and Stargazin-dependent decrease in AMPAR diffusional exchange at synapses, which maintains synaptic transmission (Opazo et al., 2010). Moreover, hippocampal NMDARs and NMDAR-dependent synaptic plasticity are considered to be an important substrate of long-term memory processes (Tang et al., 1999; Suzuki et al., 2004; Barria and Malinow, 2005; Kessels and Malinow, 2009). In particular, hippocampal GluN2B subunits determine the calcium permeability of NMDARs, which promotes synaptic plasticity and memory formation (Tang et al., 1999). Corticosterone rapidly enhances synaptic potentiation (Wiegert et al., 2006). We therefore examined in this chapter the effects of corticosterone on NMDA receptor function. By using electrophysiological techniques we monitored alterations of NMDARs-mEPSCs after we applied corticosterone to primary hippocampal cultures. Using pharmacological tools we dissected whether corticosterone – within minutes after its administration – alters the GluN2B component

of NMDA receptor function.

In the final part of this thesis (**Summary and General Discussion**), all results are summarized and critically evaluated in the light of existing literature. Some ideas for future experiments are proposed in this chapter.

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Chapter 1
Tuning hippocampal synapses by stress-hormones: relevance for emotional memory formation

Hui Xiong, Harm J Krugers

Brain Research
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Abstract

While stress is often associated with an increased risk to develop (psycho) pathology, the initial response after exposure to stressors is often highly beneficial and allows individuals to optimally cope with challenging situations. Various neurotransmitters and neuromodulators – such as catecholamines and glucocorticoids - are released upon exposure to stressors and regulate behavioural adaptation to stress and enhance the storage of salient information. Studies over the past years have revealed that catecholamines and glucocorticoids regulate synaptic function and synaptic plasticity - which underlie memory formation - in a highly dynamic manner. In this brief review we will summarize how catecholamines and glucocorticoids regulate synaptic function and discuss how these effects may contribute to acquisition and storage of emotional information.

Introduction

Cognitive processes such as attention, perception and storage of information allow individuals to optimally perform in a complex environment such as a society. In any environment however, events or situations can occur which differ in the degree of salience. This requires fine tuning of cognitive processes to adapt to those salient – often important - conditions, by increasing alertness, changing to adequate behavioural strategies and remembering and using information which is relevant for that particular context.

While rapid and persistent alterations in neuronal function, neuronal communication and network function allow behavioural adaptation, the release of hormones and neurotransmitters such as catecholamines (e.g. (nor)adrenaline), corticotropin releasing hormone (CRH) and glucocorticoids during and after stressful and challenging situations are highly capable to facilitate optimal behavioural adaption to salient events (de Kloet et al., 1999; McGaugh, 2004; Joëls et al., 2006; Joëls and Baram, 2009; Roozendaal et al., 2009). The number of neuromodulators that facilitate coping and behavioural adaptation to stressors is large (Joëls and Baram, 2009) and various studies over the past years have provided evidence that several of these modulators steer cellular processes such as synaptic function, synaptic plasticity and activity in networks which are fundamental for attention, perception and learning and memory (Kim and Diamond, 2002; de Kloet et al., 2005; Joëls and Baram, 2009; Hermans et al., 2011). The time frame at which these modulators modify cellular responses, ranging from effects within seconds and minutes to hours, is highly relevant for behavioural adaptation to stressors (Joëls et al., 2011). Although many hormones and neurotransmitters can modify cellular properties and behaviour we will mainly focus in this review on the role of catecholamines (noradrenaline) and glucocorticoid hormones.

Studies in humans and animals show that activation of the autonomic nervous system is one of the earliest responses after exposure to a stressor (Figure 1A). Noradrenaline,

via projections from the Locus Coeruleus, is released in the brain almost immediately after exposure to stressful experiences (de Kloet et al., 2005; Joëls and Baram, 2009). In addition, noradrenaline and adrenaline are released from the adrenal medulla during and after stressful conditions (Figure 1B). Noradrenaline and adrenaline regulate neuronal function via G-protein coupled α and β-adrenergic receptors. Activation of these receptors results in a cascade of cellular responses which involve activation of various kinases such as cyclic AMP (cAMP), Calcium-calmodulin-dependent kinase II (CaMKII) and protein kinase A (PKA) (Hu et al., 2007). Although adrenaline and noradrenaline levels decline within 30-60 minutes after activation of the autonomic nervous system (autonomic nervous system), they can also exert long-lasting genomic actions via activation of for example cAMP response element-binding protein (CREB) (Chai et al., 2014).

Exposure to stressful situations also activates the Hypothalamus-Pituitary-Adrenal (HPA)-axis (de Kloet et al., 2005) (Figure 1A). This involves the release of corticotropin releasing hormone (CRH) from the hypothalamus which stimulates the release of adrenocorticotropin releasing hormone (ACTH) from the anterior pituitary gland and finally the release of glucocorticoids (corticosterone in rodents and cortisol in humans) from the adrenal glands (Figure 1B). Corticosteroid hormones can bind to the high affinity mineralocorticoid receptors (MRs) and lower affinity glucocorticoid receptors (GRs) (de Kloet et al., 2005). These receptors are present in the brain and have been reported to regulate cellular function in the brain within minutes via membrane receptors but they can also have delayed effects - via regulation of gene transcription and protein synthesis – by activating cytosolic receptors (de Kloet et al., 2005; Karst et al., 2005; Tasker et al., 2006; Karst et al., 2010; Pasricha et al., 2011; Liston et al., 2013) (Figure 1C). (Nor)adrenaline and glucocorticoids can therefore regulate cellular function within minutes, but can also exert long-lasting effects which involve protein synthesis (Figure 1B). There is also emerging evidence that (nor)adrenaline and glucocorticoid hormones can interact at the functional level to modify cellular responses (Joëls et al., 2011). We will discuss how (nor)adrenaline and glucocorticoid hormones rapidly but also persistently regulate synaptic function, synaptic plasticity and neuronal activity

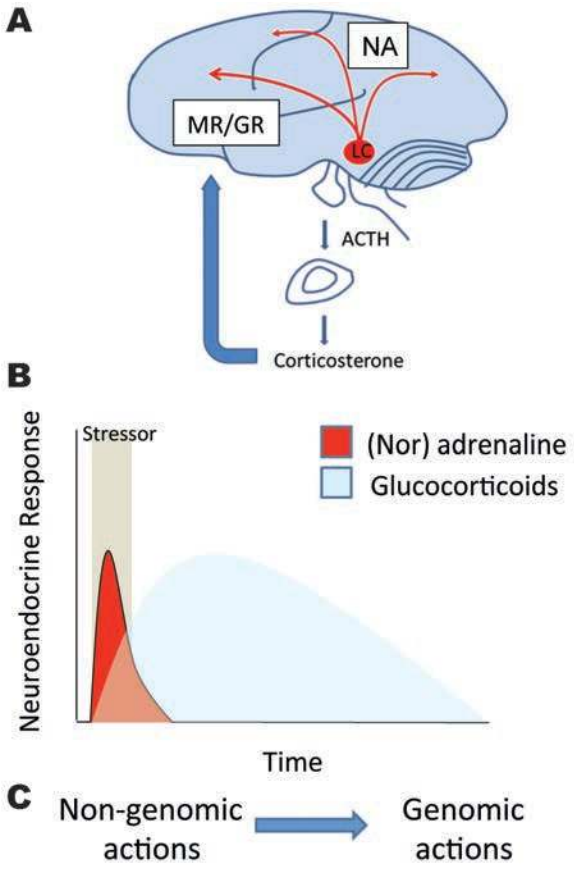

Figure 1. Stress, synapses and behavioural adaptation
A. Exposure to a stressor activates the Autonomic Nervous system. Noradrenergic projection from the Locus Coeruleus (LC) project to brain areas involved in emotional memory formation. In addition, exposure to a stressor activates the Hypothamalus-Pituitary-Adrenal Axis thereby increasing plasma cortisol (humans) and corticosterone (rodents) levels. These hormones regulate neuronal excitability and behavior via mineralocorticoid receptors (MR) and glucocorticoid receptors (GRs). **B.** Stress rapidly increases noradrenaline and glucocorticoid levels. **C.** Via their receptors, noradrenaline and glucocorticoid hormones can exert rapid non-genomic as well as genomic actions.

and discuss whether and how these effects may be relevant for memory formation.

Stress-hormones and memory formation

Studies in humans and rodents have revealed that noradrenaline enhances emotional memory formation via activation of β-adrenergic receptors (Cahill et al., 1994; Hu et al.,

2007; Roozendaal et al., 2009). In addition, post-training administration of corticosterone – in various emotionally arousing learning tasks (such as Morris Water maze, Fear conditioning, Inhibitory avoidance) and in various species (including chicks, mice and rats) enhances the consolidation of emotionally arousing information (Oitzl and de Kloet, 1992; Sandi and Rose, 1994; Pugh et al., 1997a; b; Oitzl et al., 2001; Zhou et al., 2010). Various lines of evidence suggest that activation of GRs promotes consolidation via genomic actions (Oitzl et al., 2001), although these effects might also be mediated via glucocorticoids acting via membrane GRs (Roozendaal et al., 2010). In addition to the role of GRs, also MRs have been implicated in memory formation. Human and rodent studies show that stress causes a transition from using hippocampus-dependent spatial strategies to striatum-dependent habitual learning (Schwabe et al., 2010; 2012). These studies suggest that stress hampers (more flexible) spatial learning and enhances stimulus-response learning, which might promote adaptation and survival by relying on learned automated behaviour when exposed to acute stressors. This switch in behavioral strategies is mediated by MRs (Schwabe et al., 2010). Finally, catecholamines and glucocorticoids (via activation of GRs) also interact to optimally promote memory consolidation (Roozendaal et al., 2006; 2009).

Synapses and memory formation

An important question that needs to be addressed is how catecholamines and glucocorticoid hormones (alone and together) regulate learning and memory. Synaptic plasticity - the ability of synapses to change their strength in response to altered activity in synaptic pathways - is a major cellular substrate for learning and memory and behavioural adaptation (Malinow and Malenka, 2002). Long-term potentiation (LTP) and long-term depression (LTD) are two major forms of synaptic plasticity and reflect lasting increased and decreased synaptic transmission respectively (Abraham and Williams, 2008). Both LTP and LTD have been implicated in learning and memory (Neves et al., 2008). This evidence is based on studies demonstrating the targeting molecular mechanisms that underlie synaptic plasticity (often) also affect learning and memory (Rumpel et al., 2005; Neves et al., 2008; Kessels and Malinow, 2009;

Mitsushima et al., 2011); that changes in synaptic plasticity during the learning process e.g. (Rogan et al., 1997; Whitlock et al., 2006) and the notion that occlusion of synapses hampers memory processing (Moser et al., 1998; Whitlock et al., 2006). A recent study further demonstrated the link between LTP, LTD and memory from (Nabavi et al., 2014). By using fear conditioning paradigm and optogenetic tools, they showed that fear conditioning, a type of associative memory, can be inactivated and reactivated by LTD and LTP respectively, which is direct evidence of the link between synaptic processes and memory.

Plasticity at synapses can be regulated in two ways: (1) at the presynaptic site by changing the release of neurotransmitter molecules; (2) at the postsynaptic site by changing the number, types, or properties of neurotransmitter receptors (Kessels and Malinow, 2009; Huganir and Nicoll, 2013). In particular AMPA receptors and NMDA receptors have been implicated in synaptic plasticity. During LTP induction, activation of NMDARs by glutamate, in concurrence with depolarization of the postsynaptic membrane relieves the magnesium channel block which allows the entry of calcium through the NMDARs and results in increased intracellular calcium levels (Nicoll and Malenka, 1998). Calcium activates various kinases that in turn regulate the number and properties of synaptic AMPA receptors. AMPA receptors mediate basal synaptic transmission and consist of four major core subunits (GluA1-4) that form heteromeric tetrameric complexes (Traynelis et al., 2010), although also homomers have been reported in the brain (Plant et al., 2006). The major AMPA receptor isoforms are GluA1/2 and GluA2/3 AMPARs (Lu et al., 2009). Studies from the past years have shown that these subunits (GluA1-4) are phosphorylated at serine, threonine, and tyrosine residues by several protein kinases including CaMKIIα, Protein Kinase A, Protein Kinase C, Protein Kinase G, tyrosine kinase FYN, and Jun amino-terminal kinase (JNK) (Shepherd and Huganir, 2007; Lu and Roche, 2012), which highly is important for synaptic transmission and plasticity (Huganir and Nicoll, 2013).

In recent years, substantial evidence has been gathered that AMPA receptor trafficking to and from synapses is involved in LTP and LTD (but see also (Granger et al., 2013)). This

was directly visualized in 1999 using GFP-tagged receptors expressed in organotypic hippocampal slices by using Sindbis virus (Shi et al., 1999). This study showed that GFP-GluA1 was recruited to synapses after LTP induction together with synaptic transmission enhancement. The current view is that AMPA receptors exocytose to endocytic zones at the membrane and traffick to synapses via lateral diffusion (Makino and Malinow, 2009; Petrini et al., 2009; Kennedy et al., 2010), which is stimulated under LTP-like conditions (Makino and Malinow, 2009).

Several lines of evidence indicate that the dynamic regulation of AMPAR is highly relevant for learning and memory. First, learning increases the expression of synaptic AMPARs and results in LTP-like changes (Whitlock et al., 2006). Second, synaptic insertion of AMPARs in amygdala and hippocampal synapses underlies cue and context conditioning respectively (Rumpel et al., 2005; Mitsushima et al., 2011). Third, studies in transgenic mice demonstrate that GluA1 mutant mice are impaired in short-term working memory (Sanderson et al., 2011a; b).

Stress, synapses and plasticity

Stress has a major impact on synaptic plasticity and synaptic function. These effects range from enhancing synaptic plasticity to reducing synaptic plasticity. The direction of the effects on synaptic plasticity depend a.o. on timing (i.e. when does stress occur with respect to synaptic stimulation); history of the animal and the nature stress-exposure (brief versus chronic exposure to stress) (Joëls and Krugers, 2007). We will discuss how catecholamines and glucocorticoid hormones regulate synaptic function and synaptic plasticity with a focus on the hippocampal formation, unless stated otherwise.

In vitro and ex vivo studies show that noradrenaline - within minutes after activation of β-adrenergic receptors - activates CaMKII and PKA and increases the phosphorylation of AMPA receptors (Hu et al., 2007). Importantly, this reduces the threshold to evoke synaptic potentiation and facilitates the ability to elicit long-term potentiation (Thomas et al., 1996; Winder et al., 1999; Hu et al., 2007; Tully et al., 2007; Gelinas et al., 2008;

Tenorio et al., 2010) (Figure 2).

In vitro studies in the hippocampus show that increased corticosterone levels, within minutes, enhances the frequency of mEPSCs and the release of glutamate from presynaptic terminals (Karst et al., 2005; Olijslagers et al., 2008; Pasricha et al., 2011). In the same time domain, corticosterone increases AMPA receptor lateral diffusion in hippocampal primary neurons (Groc et al., 2008). These effects require activation of MRs and may increase within minutes the ability to enhance synaptic plasticity (Wiegert et al., 2006) (Figure 2). At least one hour after brief application, corticosterone enhances AMPA receptor lateral diffusion, AMPA receptor exocytosis and synaptic retention of AMPA receptors. Consequently, AMPA receptor mediated synaptic transmission is enhanced (Karst and Joëls, 2005; Martin et al., 2009). Stress and corticosterone also enhance excitatory synaptic transmission in the prefrontal cortex via activation of and mechanisms which require activation of serum- and glucocorticoid-inducible kinase (SGK) regulation and Rab GTP-ases GRs (Yuen et al., 2009; Liu et al., 2010; Yuen et al., 2011). At this time, approximately one hour after activation of GRs, the activity-dependent synaptic insertion and AMPA receptor mediated synaptic function is occluded ((Groc et al., 2008); Xiong unpublished observations). This supports earlier studies which have demonstrated that brief stress exposure reduces the ability to elicit hippocampal LTP - both when measured in vivo as well as in ex vivo slice preparations (Foy et al., 1987; Shors et al., 1989; Kim et al., 1996; Pavlides et al., 1996) (Figure 2). In the same time domain, glucocorticoid hormones facilitate the ability to induce long-term depression (Coussens et al., 1997; Xu et al., 1997) indicating that corticosteroid hormones can weaken synapses when they receive low frequency input. These studies reveal that glucocorticoid hormones can rapidly facilitate synaptic plasticity, but also – via a slower mode of action – can reduce the ability to elicit LTP.

Importantly, corticosterone and noradrenaline act in synergy not only to enhance memory formation (Quirarte et al., 1997; Roozendaal et al., 2006; 2009). Also at the synaptic level, noradrenaline and corticosterone interact; briefly after co-application, synaptic plasticity (Pu et al., 2007); AMPA receptor mediated synaptic transmission,

AMPA receptor surface expression and phosphorylation of AMPA receptors are enhanced (Zhou et al., 2011; Krugers et al., 2012).

Stress, synapses and memory

In the previous paragraphs we have discussed that catecholamines and glucocorticoid hormones rapidly increase the ability to elicit long-term potentiation, which reflects an increased ability to acquire information. Approximately one hour later, the ability to evoke LTP is occluded which might reflect a process to prevent overwriting of already stored information, thereby enabling memory consolidation (Figure 2). An important question is whether the effects of stress and stress-hormones on synapses are relevant for memory formation. Recent studies suggest that the effects of catecholamines and glucocorticoid hormones on synaptic function are causally related. First, the memory enhancing effects of noradrenaline on emotional learning as assessed in a fear conditioning task is critically dependent on phosphorylation of GluA1 containing AMPARs (Hu et al., 2007). Moreover, several behavioural studies support the hypothesis that corticosteroid hormones regulate memory via mechanisms that underlie synaptic plasticity such as the MAPK pathway, synapsin-Ia/Ib and CaMKII (Revest et al., 2005; 2010; Chen et al., 2012; Revest et al., 2014) and enhance memory formation via regulation of AMPA receptors at excitatory synapses (Conboy and Sandi, 2010). In the Morris water maze synaptic insertion of AMPARs is required for memory enhancing effects of learning under stress (Conboy and Sandi, 2010). Finally, there is substantial evidence that corticosteroid hormones regulate memory formation that requires prefrontal cortex function via AMPA receptors (Yuen et al., 2009; 2011).

Early life experience and sensitivity of synapses

Early life adversity has profound effects on cognitive function such as learning and memory. In general, early life adversity hampers spatial learning but enhances emotional memory formation (Champagne et al., 2008; Oomen et al., 2010). Early life adversity has also substantial and long lasting effects on synaptic function and

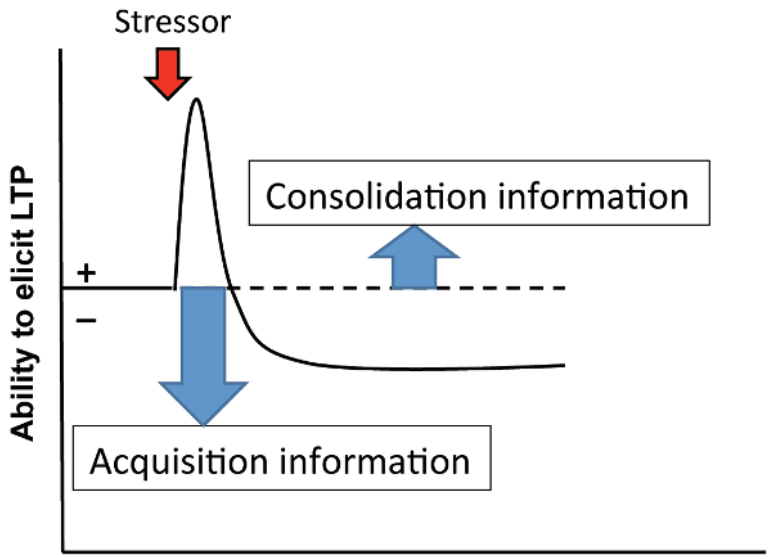

Figure 2. Stress effects on long-term potentiation.
Exposure to a stressor rapidly increases the ability to elicit long-term potentiation (LTP). After exposure to stress, the ability to evoked LTP is hampered. These effects may be related to acquisition and consolidation of information. For more details, see text.

plasticity. Low levels of maternal care and chronic early life stress reduce synaptic potentiation in the hippocampus (Brunson et al., 2005; Champagne et al., 2008; Bagot et al., 2009; 2012) while increasing NMDA receptor mediated synaptic transmission (Bagot et al., 2012; Rodenas-Ruano et al., 2012). Interestingly, early life adversity alters the sensitivity of synapses for stress and stress-hormones. While corticosterone - hours after administration (or release) usually hampers the ability to elicit synaptic plasticity, several studies indicate corticosterone (and activation of beta-adrenergic receptors) enhances synaptic plasticity in animals with low levels of maternal care (Champagne et al., 2008; Bagot et al., 2009) or in animals which have been exposure to maternal deprivation (Oomen et al., 2010).

Summary and perspective

In this review, we summarized recent evidence that stress – via activation of catecholamines and glucocorticoid hormones regulate synaptic plasticity and regulate

learning and memory. These studies suggest that noradrenaline and corticosterone (in interaction) affect emotional memory formation via dynamically regulating AMPARs. A number of critical questions are important to address in the future.

1) Activity-dependent regulation of glutamatergic receptors

While studies showing that corticosterone and noradrenaline dynamically regulate AMPARs, it remains to be determined how corticosterone and noradrenaline regulate activity-dependent changes in AMPA receptor and NMDA receptor function. Moreover, although many proteins are involved in careful regulation of AMPA and NMDA receptors at the membrane and synapse (Anggono and Huganir, 2012; Huganir and Nicoll, 2013) detailed knowledge on how stress and stress-hormones regulate AMPA and NMDA receptor function is lacking.

2) Region specific effects of stress and stress-hormones

Many studies on stress, stress-hormones and synaptic plasticity have focused on the hippocampus. It is important to note that stress hormones have different effects on synaptic plasticity along the rostro-caudal axis (Maggio and Segal, 2007; 2009). It will therefore be important to examine how stress and stress-hormones regulate synaptic function and plasticity in various brain regions which are involved in memory formation (Karst et al., 2010).

3) Behavioural relevance

Ultimately, it will be essential to understand how regulation of excitatory synapses underlie the effects of stress and stress-hormones on (the different phases of) memory formation (such as acquisition and consolidation) as well as on processes such as attention, perception, habit learning, behavioural flexibility and decision making.

4) Early life experience and synapses

Early life experiences lastingly program behavioural programs, synaptic plasticity and the sensitivity of synapses for stress/hormones. Understanding how early life adversity determines the sensitivity of synapses for stress-hormones will contribute to our

fundamental understanding of individual variation in behavioral adaptation to stressful events (Champagne et al., 2008) and is crucial for a better insight in the development of stress-related psychopathology.

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Chapter 2

Corticosterone regulates activity-dependent changes in AMPA receptor mediated synaptic transmission

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In preparation

Abstract

Corticosterone exerts both fast and slow effects on hippocampal glutamatergic synaptic transmission. Here we tested whether this hormone also exerts rapid and slow effects on activity-dependent changes in hippocampal glutamatergic synapses using patch-clamp electrophysiology in hippocampal primary cultures. Our results show that brief administration of corticosterone rapidly increases activity-dependent changes in mEPSC frequency. Via a slower mode of action, corticosterone was found to prevent or occlude activity-dependent changes in the amplitude of mEPSCs. These results verify that corticosterone via different mechanisms alters rapid and slow effects on activity-dependent changes in synaptic transmission. These effects may contribute to enhanced encoding and consolidation of salient information.

Introduction

Exposure to stressful experiences promotes the release of corticosteroid hormones from the adrenal glands (de Kloet et al., 2005). These hormones enter the brain and bind to high-affinity mineralocorticoid receptors (MRs) and lower affinity glucocorticoid receptors (GRs). Classically, these receptors act as transcription factors, but recent studies indicate that corticosteroid hormones via MRs and GRs can also exert rapid non-genomic actions on cellular activity in various brain areas (Karst et al., 2005; Groc et al., 2008; Di et al., 2009; Karst et al., 2010; Joëls et al., 2011)

In the hippocampal formation, corticosteroid hormones, via activation of mineralocorticoid receptors rapidly increase the mobility of AMPA receptors (AMPARs) (Groc et al., 2008), increase neurotransmitter release probability and enhance the frequency of miniature excitatory postsynaptic currents (mEPSCs) (Karst et al., 2005). Via glucocorticoid receptors, these hormones have been reported to slowly increase hippocampal synaptic retention of AMPARs (Groc et al., 2008) and increase the amplitude of mEPSCs (Karst and Joëls, 2005; Martin et al., 2009). Similar effects have been observed in the prefrontal cortex (Liu et al., 2010; Yuen et al., 2011).

These studies indicate that corticosteroid hormones – in different time domains – affect synaptic function by regulating synaptic AMPARs. An important question that remains to be addressed is whether and how these hormones regulate activity-dependent changes in AMPAR function, a critical mechanism for adjustments of synaptic strength contributing to learning and memory (Kessels and Malinow, 2009; Nabavi et al., 2014). This is particularly relevant to get a detailed understanding of the cellular mechanisms that underlie the memory enhancing effects of corticosteroid hormones (de Kloet et al., 1999; Roozendaal et al., 2009). We therefore examined in the present study whether corticosteroid hormones – via MRs and/or GRs - regulate activity-dependent changes in AMPAR mediated synaptic function.

Material and Methods

Primary hippocampal neurons

The experiments were carried out with permission of the local Animal Committee of the University of Amsterdam. Primary hippocampal neurons were prepared from the hippocampus of E18 Wistar rats; after dissection, the hippocampal lobes were digested with 2.5% trypsin (sigma, USA). Neurons were plated in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 0.5 mM glutamax and penicillin/streptomycin (GIBICO USA) and 5% Fetal Bovine Serum (FBS, GIBICO, USA, only for the first day) 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. 5-Fluoro-2′-Deoxyuridine (FUDR) 10 μM was used to inhibit glial growth. All the experiments were carried out during days in vitro (DIV) 14-DIV21.

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), $MgCl_2$ (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): 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 and 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 3 min in each cell.

Corticosterone (100 nM, Sigma) or vehicle (<0.01% ethanol, Merck) was applied to the neuronal cultures for 20 minutes. Chemical LTP (cLTP; picrotoxin1 µM + glycine 200 µM for 3 min; called ptx/gly) or vehicle (<0.01% ethanol, Merck) was applied to the cultures to stimulate network activity (Lu et al., 2001; Groc et al., 2008). The cLTP and recordings were carried out either immediately after 20 min corticosterone administration or after a 160 minutes' period of wash out following a 20 min corticosterone incubation (see Fig.1A and 2A). Spironolactone (100nM, Sigma) and RU486 (100nM, Sigma) were used to identify the role of mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) respectively. These compounds were administered starting 30 min before and during the corticosterone treatment.

Statistics

Statistical analyses were calculated using Prism 5 (GraphPad software, Inc). Data are expressed as mean ± S.E.M. One-way ANOVA was performed and a Bonferroni post-hoc test for multiple comparisons was applied when required. P values < 0.05 were considered significantly different.

Results

Rapid effects of corticosterone

The experimental design is shown in Figure 1A. ANOVA revealed a significant effect on the amplitude of mEPSCs (Figure 1C, $F(3,78)=5.5414$; $p=0.002$). Post-hoc analysis indicated that brief application of corticosterone did not alter the amplitude of mEPSCs (Figure 1B, C). Glycine/picrotoxin (gly/ptx) enhanced the amplitude of mEPSCs, which was not significantly further enhanced by co-administration of corticosterone (Figure 1C). ANOVA revealed a significant effect on the frequency of mEPSCs (Figure 1C, $F(3,78)=7.170$; $p=0.0003$). Post-hoc analysis indicated that brief application of corticosterone or gly/ptx did not alter the frequency of mEPSCS (Figure 1B, D). However, co-administration of corticosterone and gly/ptx significantly increased the frequency of mEPSCs (Figure 1D). Taken together, these observations suggest that the largest increase in amplitude and frequency of mEPSCs is apparent after co-administration of corticosterone and gly/ptx.

Since rapid effects of corticosterone on synaptic transmission are mediated by MRs (Karst et al., 2005), we examined here whether these effects of corticosterone on activity-dependent changes in AMPAR mEPSCs were also mediated by MRs. We therefore administered spironolactone (100 nM) to neurons 30 minutes before and during corticosterone administration (Figure 1A). In this experiment no overall effects of corticosterone and/or gly/ptx were found on the amplitude of mEPSCs (Figure 1F), although on average the highest amplitude of mEPSCs was again found in the cort + gly/ptx treated cells. ANOVA revealed a trend towards significancy on mEPSC frequency (Figure 1G, $F(3,50)=2.739$; $p=0.053$) with the highest frequency in cells with co-application of corticosterone and gly/ptx. In the presence of spironolactone, this effect was not present (Figure 1G). Spironolactone itself did not affect the amplitude or frequency of mEPSCs (data not shown).

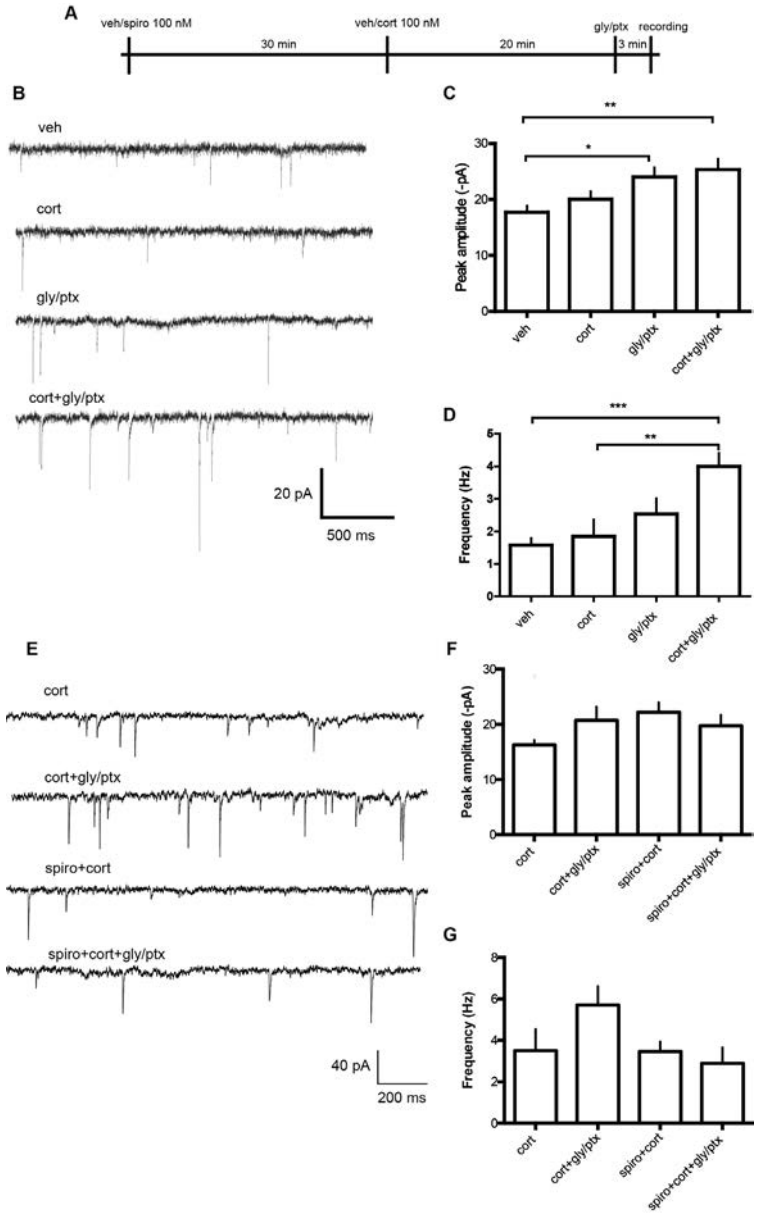

Figure 1. Rapid effects of corticosterone on AMPA receptor function. A. Experimental design: vehicle (<0.01% ethanol) or corticosterone (100 nM) was applied for 20 minutes (Figure 1B-D, n_{veh}=23 cells; n_{cort}=19 cells; $n_{gly/ptx}$=20 cells; $n_{cort+gly/ptx}$=20 cells). Vehicle (<0.01% ethanol) or spironolactone (100nM) was applied 30 min before and during corticosterone treatment (Figure 1E-G, n_{cort}=12 cells; $n_{cort+gly/ptx}$=14 cells; $n_{sprio+cort}$=16 cells; $n_{sprio+cort+gly/ptx}$=12 cells). **B.** Representative mEPSCs traces after 20 minutes vehicle or corticosterone and 3 minutes glycine (200 µM)/ picrotoxin (1 µM) (gly/ptx) treatment. **C.** Histograms showing the amplitude of mEPSCs after treatment with corticosterone (100 nM) and gly/ptx. *p<0.05, **p<0.01. **D.** Histograms showing the frequency of mEPSCs after treatment with corticosterone (100 nM) and gly/ptx. **p<0.01, *** p<0.001. **E.** Representative traces of mEPSCs after treatment with corticosterone or vehicle and

co-application with vehicle or spironolactone (100 nM). **F.** Histograms showing the amplitude of mEPSCs after pre-application of 30 min vehicle or spironolactone and during 20 minutes treatment with corticosterone (100 nM) and 3 minutes glyc/ptx. **G.** Histograms showing the frequency of mEPSCs after pre-application of 30 min vehicle or spironolactone and 20 minutes treatment with corticosterone (100 nM) and 3 minutes gly/ptx.

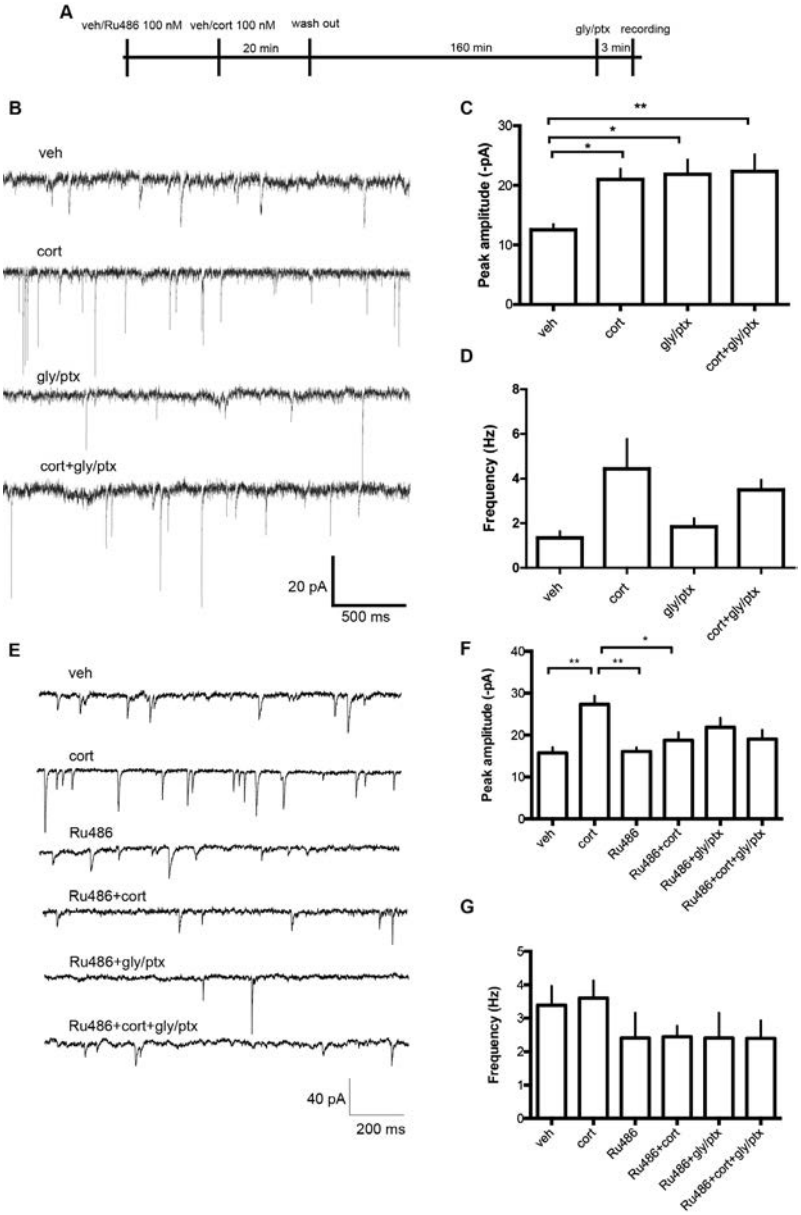

Figure 2. Slow effects of corticosterone on AMPA receptor function. A. Experimental design: vehicle (<0.01% ethanol) or corticosterone (100 nM) was applied for 20 minutes; mEPSCs were recorded after 3 hours (Figure 1B-D, n_{veh}=17 cells; n_{cort}=17 cells; $n_{gly/ptx}$=16 cells; $n_{cort+gly/ptx}$=15 cells). Vehicle (<0.01% ethanol) or RU486 (100nM) was applied 30 min before and during corticosterone treatment (Figure 1E-G, n_{veh}=7 cells; n_{cort}=14 cells; n_{Ru486}=13 cells; $n_{Ru486+cort}$=16 cells; $n_{Ru486+gly/}$

$_{ptx}$=15 cells; $n_{Ru486+cort+gly/ptx}$=8 cells). **B.** Representative mEPSCs traces of 20 minutes vehicle or corticosterone together with 3 minutes glycine (200 µM)/picrotoxin (1 µM) (gly/ptx) treatment and washed out for 160 minutes. **C.** Histograms showing the amplitude of mEPSCs 3 hours after treatment with corticosterone (100 nM) and gly/ptx. *p<0.05, **p<0.01. **D.** Histograms showing the frequency of mEPSCs 3 hours after treatment with corticosterone (100 nM) and gly/ptx. **E.** Representative traces of mEPSCs after treatment with corticosterone or vehicle and co-application with vehicle or RU486 (100 nM). **F.** Histograms showing the amplitude of mEPSCs 3 hours after pre-application of 30 min vehicle or RU486 and during 20 minutes treatment with corticosterone (100 nM) and 3 minutes gly/ptx. *p<0.05, **p<0.01. **G.** Histograms showing the frequency of mEPSCs 3 hours after pre-application of 30 min vehicle or RU486 (100 nM) and during 20 minutes treatment with corticosterone (100 nM) and 3 minutes gly/ptx.

Slow effects of corticosterone

The experimental design is shown in Figure 2A. ANOVA revealed a significant effect on the amplitude of mEPSCs (Figure 2C, $F(3, 61)=5.204$; $p=0.002$). Post-hoc analysis indicated that brief (20 minutes) application of corticosterone enhanced the amplitude of mEPSCs 3 hours later. Also glycine/picrotoxin (gly/ptx) increased the amplitude of mEPSCs (Figure 2A-C). The effect of corticosterone was not further enhanced by gly/ptx (Figure 2C). Application of corticosterone or gly/ptx did not significantly alter the frequency of mEPSCs (Figure 2D). Moreover, gly/ptx did not alter the frequency of mEPSCs in cells which were pretreated with corticosterone (Figure 2D). Taken together, these observations suggest that the amplitude, but not the frequency of mEPSCs is enhanced by corticosterone, and that gly/ptx is not able to alter the amplitude of mEPSCs in cells which were pretreated with corticosterone.

Since slowly-developing effects of corticosterone on synaptic transmission are mediated by GRs (Karst and Joels, 2005; Xiong et al., 2015) we examined the effects of corticosterone and synaptic activation in the absence and presence of the GR antagonist RU38486. ANOVA revealed a significant effect on the amplitude of mEPSCs (Figure 2F, $F(5, 67)=4.843$; $p=0.0008$). Post-hoc analysis indicated that RU38486 itself did not alter synaptic transmission (Figure 2F) but prevented the effect of corticosterone on synaptic transmission (Figure 2F). Interestingly we found no effect of gly/ptx on the amplitude of mEPSCs was present in cells that were pretreated with RU486 (Figure 2F). No effects on the frequency of mEPSCs were present which might be due to the variation in the results (Figure 2G).

Discussion

Corticosteroid hormones, which are released during and after stressful experiences promote memory consolidation (Sandi and Rose, 1994; Roozendaal et al., 2009; Xiong et al., 2015). Since activity-dependent changes in synaptic transmission are generally believed to underlie learning and memory (Nabavi et al., 2014) we examined whether corticosterone regulates activity-dependent changes in synaptic transmission. We report that corticosterone, hours after its application, enhances the amplitude of mEPSCs, and prevents or occludes the activity-dependent increase in synaptic transmission. Briefly after its administration, corticosterone did not alter synaptic transmission, but increased the frequency of mEPSCs after gly/ptx.

Corticosterone has been shown to rapidly increase the frequency of mEPSCs in hippocampal slices (Karst et al., 2005). These effects are believed to be mediated by an increase in glutamate release probability. Here we report that corticosterone did not alter the frequency of mEPSCs rapidly after its application. One possible explanation is that we currently used hippocampal primary cultures, which exhibit more sparse synaptic connectivity when compared to acute slices and also yield more variable effects, possibly due to differences in cell density between batches of cultures. All of these factors may potentially reduce the ability to detect changes in synaptic transmission elicited by corticosterone. Also, although we recorded cells rapidly after corticosterone administration, the hormone was not present during recordings and the fast effects that were reported earlier (Karst et al., 2005) may have been missed. Yet, we did find that corticosterone increased the frequency of mEPSCs after gross synaptic activation using picrotoxin/glycine. This is in line with earlier observations that corticosterone rapidly facilitates synaptic potentiation (Wiegert et al., 2006) and increases synaptic retention of AMPARs (Groc et al., 2008). Our observations therefore suggest that the increase in synaptic plasticity that is elicited by corticosterone is accompanied by an increase in mEPSC frequency. Preliminary observations from our study suggest that these effects were mediated by MRs since spironolactone tended to prevent these effects, though this did just not reach statistical significance, maybe due to the relatively large degree of

variation. However, these preliminary findings are in line with earlier observations that rapid effects of corticosterone on synaptic transmission are mediated via MRs (Karst et al., 2005).

The currently observed increase in mEPSC frequency briefly after corticosterone administration in concert with synaptic activation suggests that corticosterone promotes synaptic plasticity thereby providing a mechanism how corticosteroid hormones promote the encoding of salient information (McGaugh, 2000).

Via a slower mode of action, corticosterone has been reported to increase the amplitude of mEPSCs (Karst and Joëls, 2005; Xiong et al., 2015) which was confirmed in our present study. These effects may be explained by increased lateral diffusion (Groc et al., 2008), exocytosis (Liu et al., 2010) and synaptic retention of AMPARs (Groc et al., 2008; Xiong et al., 2015). We also found that corticosterone seemingly prevents the activity-dependent effects on the amplitude of mEPSCs. Likewise, corticosterone has been reported to prevent the activity-dependent increase in retention of synaptic AMPARs (Groc et al., 2008). This may indicate that corticosterone and activity-dependent processes share common pathways in hippocampal synapses, so that prior exposure to corticosterone reduced the ability to increase synaptic transmission through occlusion (Alfarez et al., 2002; Wiegert et al., 2005; Krugers et al., 2010). The corticosterone induced increase in mEPSC amplitude was prevented by the GR antagonist RU38486. This confirms earlier findings that corticosterone increases AMPA receptor mediated synaptic transmission via activation of GRs. An unexpected finding was that no cLTP could be induced in cells which were pretreated with RU38486. This indeed supports that activation of GRs and glycine/picrotoxin use (at least in part) similar pathways which are required to elicit changes in synaptic plasticity. This is in line with the notion that corticosterone treatment can occlude cLTP-induced enhancement of mEPSC amplitudes. Such a mechanism of occlusion of synaptic transmission may represent a mechanism to prevent the overwriting of information stored at synapses, thereby promoting memory consolidation (Roozendaal et al., 2009).

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Chapter 3

Interactions between N-Ethylmaleimide-Sensitive Factor and GluA2 contribute to effects of glucocorticoid hormones on AMPA receptor function in the rodent hippocampus

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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 receptor (AMPAR) availability in the synapse, which is important for synaptic plasticity and memory formation. Peptides which specifically block the interaction between N-Ethylmaleimide-Sensitive Factor (NSF) and the AMPAR-subunit GluA2 prevented the increase in synaptic transmission and surface expression of AMPARs known to occur after corticosterone application to hippocampal neurons. Combining a live imaging Fluorescence Recovery After Photobleaching (FRAP) approach with the use of the pH-sensitive GFP-AMPAR tagging revealed that this NSF/GluA2 interaction was also essential for the increase of the mobile fraction and reduction of the diffusion of AMPARs after treating hippocampal neurons with corticosterone. Blocking the interaction between NSF and GluA2 furthermore prevented the memory enhancing effects of corticosterone in a contextual fear conditioning paradigm. We conclude that the interaction between NSF and GluA2 contributes to the effects of corticosterone on AMPAR-mediated synaptic transmission and fearful memory formation.

Introduction

Exposure to stressful events increases the synthesis and release of glucocorticoids (GCs) from the adrenal glands (de Kloet et al., 1999). These hormones enter the brain and act via intracellular receptors to enhance behavioural adaptation to stressful events (de Kloet et al., 1999; Xiong et al., 2015). In the brain, two types of receptors for GCs are present; Mineralocorticoid Receptors (MRs) and Glucocorticoid Receptors (GRs). Via activation of MRs, glucocorticoid hormones regulate behavioural flexibility and selection of behavioural strategies (Schwabe et al., 2010). Via activation of GRs, these hormones promote memory consolidation of emotionally arousing events (Sandi and Rose, 1994; Oitzl et al., 2001; Roozendaal et al., 2009; Zhou et al., 2010).

The molecular pathways that underlie the memory enhancing effects of GCs remain elusive. Understanding how GCs determine AMPA receptor (AMPAR) function is important to delineate how GCs facilitate memory formation, since AMPARs are crucial for synaptic transmission and synaptic plasticity (Malinow and Malenka, 2002) and their dynamic regulation at synapses has been implicated in (fear) memory formation (Rumpel et al., 2005; Mitsushima et al., 2011). Various lines of evidence have demonstrated that GCs regulate AMPAR function (reviewed in Krugers et al., 2010). Corticosterone quickly increases hippocampal mEPSC frequency, glutamate release probability and AMPAR-subunit lateral diffusion (Karst et al., 2005; Groc et al., 2008). Hours after corticosterone application, the number of synaptic AMPARs are increased, lateral diffusion and the mobile fraction of AMPARs are enhanced and AMPAR mediated synaptic transmission is increased (Karst et al., 2005; Groc et al., 2008; Martin et al., 2009; Liu et al., 2010).

Proteins that bind to the cytoplasmic tails of AMPAR-subunits regulate their surface expression and synaptic function. In particular the interaction between the clathrin adaptor complex AP2, the N-Ethylmaleimide-Sensitive Factor (NSF, which is involved in membrane fusion) and GluA2 is crucial for insertion and stabilization of AMPARs at the plasma membrane and to maintain synaptic transmission (Nishimune et al., 1998; Hanley et al., 2002; Lee et al., 2002; Beretta et al., 2005). Although AP2 and NSF

have overlapping sites at GluA2, they exert different roles in AMPAR trafficking. While NSF is required to maintain AMPAR-mediated synaptic transmission, AP2 recruited to surface AMPARs promotes endocytosis (Lee et al., 2002). Importantly, the interaction between NSF and GluA2 is also critical for maintaining fear memory formation (Joels and Lamprecht, 2010; Migues et al., 2014). We here examined whether the interaction between NSF/AP2 and GluA2 is critical for the effects of GCs on AMPAR function, AMPAR mobility and fear 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; 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.

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), $MgCl_2$ (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): 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 Mini-Analysis 6.0.

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 3 min in each cell.

Corticosterone (100 nM, Sigma) or vehicle (<0.01% ethanol, Merck) was applied to the neuronal cultures for 3 hours. To examine whether the interaction between NSF, AP2 and GluA2 is essential for the effects of corticosterone on AMPAR function we treated cells with Pep2m (myr-KRMKVAKNAQ), which blocks the interaction between GluA2 and both AP2/NSF (Lee et al., 2002); R845A (myr-KAMKVAKNPQ), which specifically blocks the interaction between GluA2 and NSF (Lee et al., 2002); or Δ849-Q853 (myr-KRMKLNINPS), which specifically blocks the interaction between GluA2 and AP2 (Lee et al., 2002). Scr-peptide (myr-VRKKNMAKQA) was used as control peptide (Lee et al., 2002). Peptides were obtained from Genscript (USA) and applied at a dosage of 50 µM either i) 30 minutes before and during corticosterone treatment; or ii) only during the

last 30 min of corticosterone incubation (Figure 1).

Immunocytochemistry

At DIV14-20, 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% CO_2 for 1 hour in Neurobasal before treatment. Corticosterone (100 nM, Sigma) or vehicle (<0.01% ethanol) was applied to the neuronal cultures for 3 hours. To examine whether the interaction between NSF, AP2 and GluA2 is essential for the effects of corticosterone on AMPA receptor function we treated cells with Pep2m or R845A peptide; Scr-peptide was used as control peptide. Peptides were applied at a dosage of 50 µM for 30 minutes before and during the corticosterone treatment, or at a dosage of 25 µM only during the last 30 min of corticosterone incubation.

After washing in Dulbecco's Modified Eagle's medium (DMEM), 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 as described elsewhere (Martin et al., 2009; Xiong et al., 2015) by using MetaMorph software (Universal Imaging Corporation).

Live Imaging

Neuronal transduction and transfection

Attenuated Sindbis virus expressing SEP-GluA2 was prepared and used as previously described (Martin et al., 2008; 2009). Neurons were transduced at a MOI of 1 between 18 and 20 DIV and incubated at 37°C under 5% CO_2 for 24h until use.

Imaging

Protocols were performed as previously described (Martin et al., 2008; 2009). Briefly, dendrites from SEP-GluA2 expressing neurons (19-21 DIV) were kept on a heated stage (set at 37°C) on a Nikon 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 NH_4Cl (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% CO_2 for 1 hour in Neurobasal before treatment. Corticosterone (100 nM, Sigma) or vehicle (<0.01% ethanol) was applied to the neuronal cultures for 3 hours. To examine whether the interaction between NSF, AP2 and GluA2 is essential for the effects of corticosterone on AMPA receptor function we treated cells with Pep2m, R845A peptide or Scr-peptide as a control. Peptides were derived from Genscript (USA) and applied during the last 30 minutes of corticosterone treatment at a dosage of 25 µM and finally live-imaged in Earle's buffer (50 mM HEPES-Tris pH 7.4, 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.9 g/L glucose) containing the indicated drugs.

Fear Conditioning

Surgery

3 month old C57BL6 mice were anesthetized with avertin (1.2% (wt/vol), 0.02 ml/g, intraperitoneal) and chronically implanted with double guide cannulas (Plastics One) in the CA1 region of the dorsal hippocampus using a high-precision stereotaxic system. Cannulas were fixed to the skull using dental cement. Coordinates were based on the stereotaxic mouse brain atlas (Rao et al., 2011). Anterior-posterior coordinates relative to bregma were 1.6 mm, and lateral coordinates relative to the midsagittal suture line were

± 1.03 mm. Buprenorphine was injected (0.1 mg/kg, subcutaneous) as an analgesic. Mice were allowed to recover for a period of at least 5 days before experimentation (Rao et al., 2011).

Animals were housed individually one week before the start of the experiment. Mice 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 mild foot shock (2 seconds, 0.2 mA) was delivered and freezing behaviour, defined as no body movements except those related to respiration, was determined every 2 seconds throughout training (Zhou et al. 2010). The animal stayed 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 i.p. To examine whether the interaction between NSF and GluA2 is essential for the effects of corticosterone on fear memory formation either the R845A peptide or the scrambled control peptide Scr-peptide was applied at a dosage of 2.5 pmol/side immediately after the training session via the cannulas under brief isoflurane anesthesia. Then, the animals were transferred back into their home cage. Twenty-four hours later, one animal at a time was placed in the same context as in which the shock was delivered for 3 minutes, now without receiving a foot shock. Freezing behaviour was scored during a 3 min period.

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

Blocking the NSF-AMPAR interaction prevents the increase in AMPAR surface expression induced by corticosterone

Application of corticosterone (100 nM for 3 hrs) to cultured hippocampal cells increased the surface expression of both GluA1 (Figure 1A,B) and GluA2 (Figure 1A,C) AMPAR

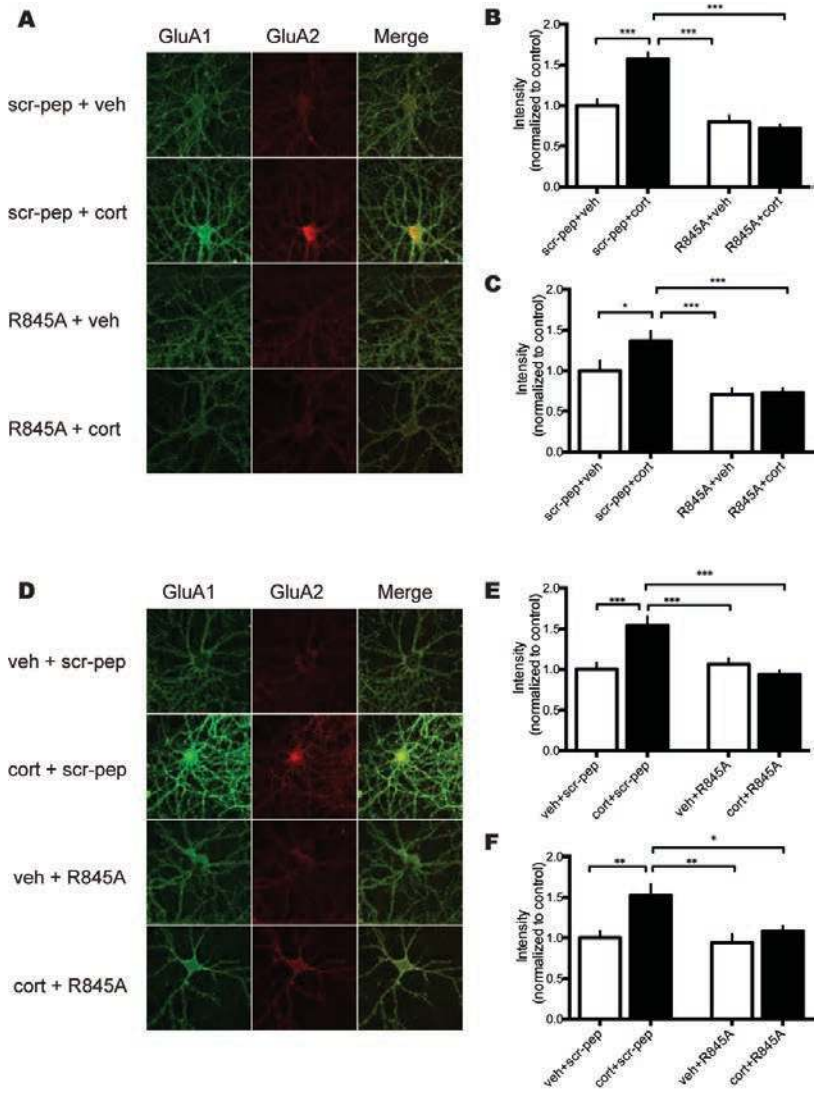

Figure 1. NSF interfering peptide prevents corticosterone effects on surface expression of AMPARs. A. Representative images of rat hippocampal neurons with surface labeling of GluA1 (in green) and GluA2 (in red) AMPAR subunits after treatment with corticosterone (cort, 100 nM) and peptides. Corticosterone (100 nM) was applied for 3 hours. R845A and scrambled-peptide

were applied at a dosage of 50 μM 30 minutes before and during corticosterone treatment. **B-C.** Histograms showing the mean (± S.E.M.) quantification of surface GluA1 (B) or GluA2 (C) AMPAR subunits. Corticosterone (100 nM) was applied for 3 hours. R845A and scrambled-peptide were applied at a dosage of 50 μM 30 minutes before and during corticosterone treatment. Data are expressed as ratio of control (vehicle condition). n>15 cells, one-way ANOVA was performed with a Bonferroni post-test for multiple comparison data sets, *p<0.05, **p<0.01, *** p<0.001. **D.** Representative images of rat hippocampal neurons with labeling of GluA1 (in green) and GluA2 (in red) AMPAR subunits after treatment with corticosterone (cort, 100 nM) and peptides. Corticosterone (100 nM) was applied for 3 hours. R845A and scrambled peptide were applied at a dosage of 50 μM during the last 30 minutes of corticosterone treatment. **E-F.** Histograms showing the mean (± S.E.M.) quantification of surface GluA1 (B) or GluA2 (C) AMPAR subunits. Corticosterone (100 nM) was applied for 3 hours. R845A and scrambled peptide were applied at a dosage of 50 μM during the last 30 minutes of corticosterone treatment. Data are expressed as ratio of control (vehicle condition). n>15 cells, one-way ANOVA was performed with a Bonferroni post-test for multiple comparison data sets, *p<0.05, **p<0.01, *** p<0.001.

subunits. We tested whether the effects of corticosterone on surface expression could be prevented by inhibiting the interaction between NSF and GluA2 subunits. Indeed, treatment of cells with R845A before and during treatment with corticosterone prevented the effect of corticosterone on surface expression of GluA1 and GluA2 (Figure 1A-C).

To examine whether the interaction between NSF and GluA2 is also critical for the effects of corticosterone on surface expression of AMPARs once hormone treatment has started we also applied R845A to cells during the last 30 minutes of hormone treatment (Figure 1D-F). Brief treatment with R845A during the last 30 minutes of hormone administration also blocked the effect of corticosterone on surface expression of both GluA1 and GluA2 (Figure 1D-F).

Blocking the NSF-AMPAR interaction prevents the effect of corticosterone on membrane receptor mobility

We next tested the involvement of NSF in the effects of corticosterone on the surface mobility of GluA2-containing AMPARs (Figure 2A). By combining a Fluorescence Recovery After Photobleaching (FRAP) approach with the use of the pH-sensitive GFP-AMPAR tagging (SEP-GluA2), we found that corticosterone alters the surface mobility of GluA2-containing AMPARs (Figure 2A). More specifically, corticosterone increased the mobile fraction (Figure 2B, D), increased the half time of fluorescence recovery T1/2 (Figure 2E) and decreased the diffusion coefficient of GluA2-containing AMPARs

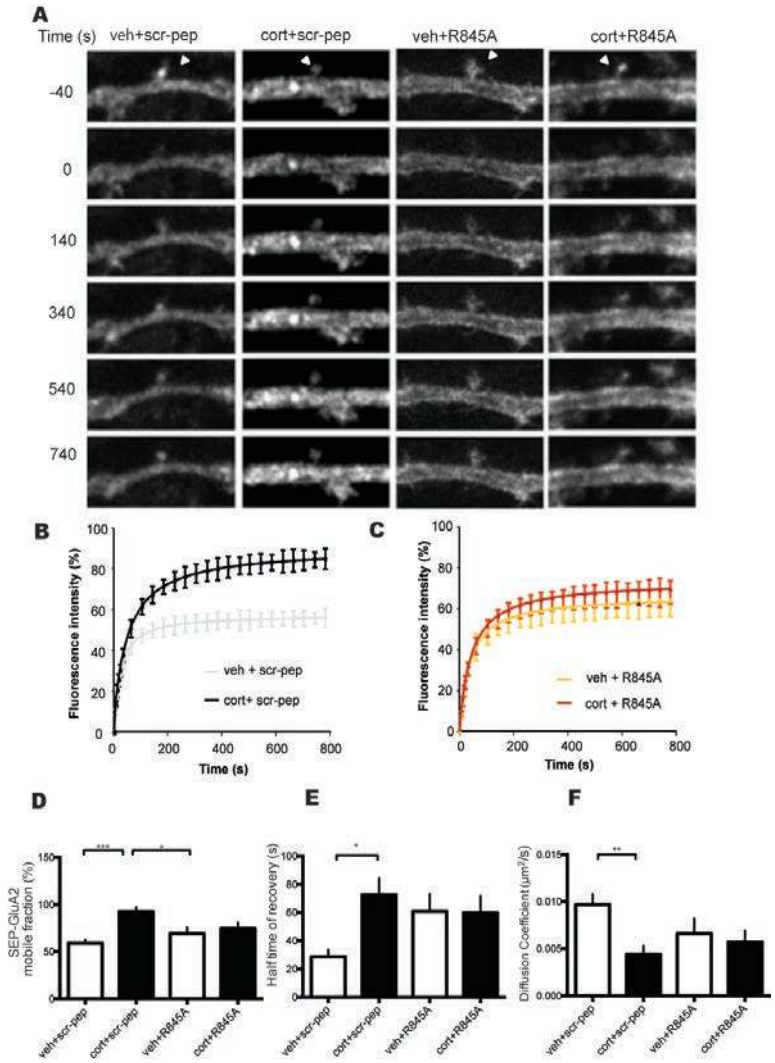

Figure 2. NSF interfering peptide prevents corticosterone effects on AMPAR mobility.
A. Sequential images from representative FRAP experiments performed on surface SEP-GluA2 from individual spine head (arrowheads) in control vehicle (veh) or in corticosterone (Cort, 100 nM) conditions, in absence or in the presence of R845A. Corticosterone (100 nM) was applied for 3 hours; R845A and scrambled peptide were applied at a dosage of 25 µM only during the last 30 minutes of corticosterone treatment. **B.** Normalized pooled and averaged FRAP curves from vehicle (n = 20 cells) and corticosterone (n = 18 cells) treated hippocampal neurons in the presence of scrambled peptide. Corticosterone (100 nM) was applied for 3 hours, scrambled peptide was applied at a dosage of 25 µM during the last 30 minutes of corticosterone treatment. **C.** Normalized pooled and averaged FRAP curves from R845A (n = 13 cells) and R845A + corticosterone (n = 17 cells) treated hippocampal neurons. Corticosterone (100 nM) was applied for 3 hours, R845A peptide was applied at a dosage of 25 µM during the last 30 minutes of corticosterone treatment. **D-F.** Histograms showing the means (± S.E.M.) of synaptic SEP-GluA2 mobile fractions (D), half time of fluorescence recovery (E) and diffusion coefficients (F). Corticosterone (100 nM) was applied for 3 hours, R845A and scrambled peptide were applied at a dosage of 25 µM during the last 30 minutes of corticosterone treatment. One-way ANOVA was performed with a Bonferroni post-test for multiple comparison data sets. *p<0.05, **p<0.01.

in dendritic spines (Figure 2F). These effects could not be explained by altered surface diffusion since membrane-GFP diffusion remained unaffected by the corticosterone treatment (Xiong et al., 2015) indicating that the stress hormone selectively facilitates the mobility of GluA2, and promotes the synaptic trapping of AMPARs. The effects of corticosterone on the mobile fraction, the half time of fluorescence recovery $T^{1/2}$ and the diffusion coefficient of GluA2-containing AMPARs in dendritic spines were not observed upon pretreatment of cells with R845A (Figure 2C-F) whereas treatment with R845A by itself did not significantly impact the surface mobility of SEP-GluA2 (Figure 2C-F).

Blocking the NSF-AMPAR interaction prevents the effect of corticosterone on AMPAR-mediated synaptic transmission

Application of corticosterone (100 nM) for 3 hrs to cultured hippocampal cells increased the amplitude of mEPSCs (Figure 3A, B). In separate series of experiments, application of pep2m, which blocks the interaction between GluA2 and both AP2/NSF (Lee et al., 2002) as well as R845A, which specifically blocks the interaction between GluA2 and NSF (Lee et al., 2002), before and during corticosterone treatment completely prevented this effect (Figure 3A, B). By contrast corticosterone still increased the amplitude of mEPSCs in the presence of Δ849-Q853, which specifically blocks the interaction between GluA2 and AP2 (Lee et al., 2002) (Figure 3A, B). Altogether, this indicates that the effect of corticosterone on the amplitude of mEPSCs is specifically regulated by an effect of the hormone on the interaction between GluA2 and NSF. Corticosterone did not consistently affect the frequency of mEPSCs in any of the experiments (Figure 3C).

To examine whether the interaction between NSF and GluA2 is also critical for the effects of corticosterone on synaptic transmission once hormone treatment has started we applied Pep2m and R845A to cells only during the last 30 minutes of hormone treatment (Figure 3D-F). Brief treatment with either Pep2m or R845A blocked the effect of corticosterone on mEPSC amplitude (Figure 3D, E). No effects of corticosterone or peptide treatment on mEPSC frequency were measured (Figure 3F).

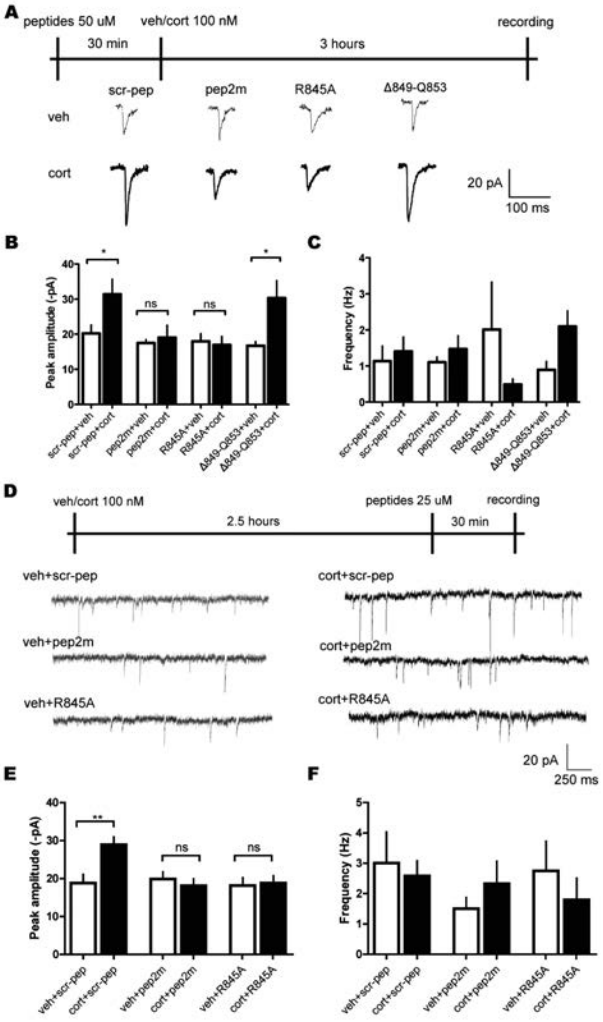

Figure 3. NSF interfering peptide prevents corticosterone effects on AMPAR function.
A. Experimental design: corticosterone (100 nM) was applied for 3 hours. Pep2m (n=15 cells); R845A (n=10 cells); Δ849-Q853 (n=6 cells) or scrambled-peptide were applied at a dosage of 50 μM 30 minutes before and during corticosterone treatment. Traces reflect individual mEPSCs recorded at 3 hours after corticosterone/peptide treatment. **B.** Histograms showing the amplitude of mEPSCs after treatment with corticosterone (100 nM) and peptides (50 μM). n>10 cells; One-way ANOVA was performed with a Bonferroni post-test for multiple comparison data sets, *p<0.05, ns: not significant. **C.** Histograms showing the frequency of mEPSCs after treatment with corticosterone (100 nM) and peptides (50 μM). **D.** Experimental design: corticosterone (100 nM) was applied for 3 hours. Pep2m; R845A or scrambled-peptide were applied at a dosage of 25 μM during the last 30 minutes of corticosterone treatment. Traces reflect individual mEPSCs recorded after corticosterone/peptide treatment. **E.** Histograms showing the amplitude of mEPSCs after treatment with corticosterone (100 nM) and peptides (25 μM). n>10 cells in each group, **p<0.01, ns: not significant. One-way ANOVA was performed with a Bonferroni post-test for multiple comparison data sets. *p<0.05, **p<0.01, ns: not significant. **F.** Histograms showing the frequency of mEPSCs after treatment with corticosterone (100 nM) and peptides (25 μM).

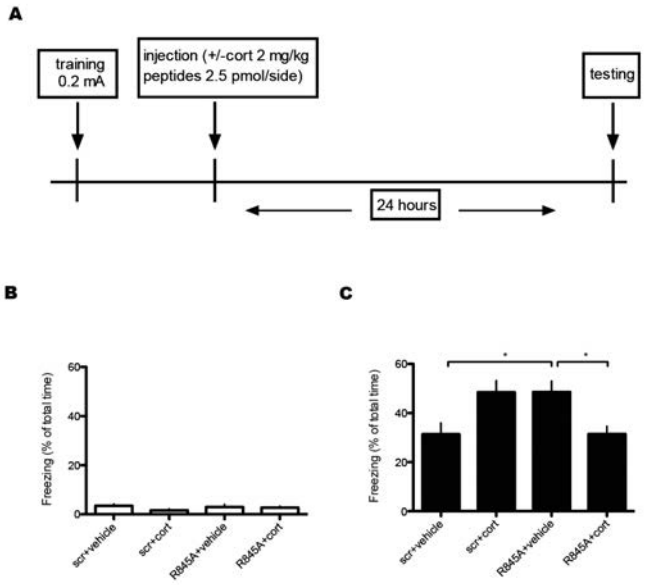

Figure 4. Corticosterone enhances memory consolidation via NSF/GluA2 interaction. A. Timeline of the behavioral experiment. Animals received a foot shock of 0.2 mA and were immediately injected with vehicle (veh) or corticosterone (cort, 2 mg/kg). At the same time, R845A or scrambled peptide were injected intra-hippocampally. Contextual fear was tested 24 hours after training. **B.** Histograms showing freezing behavior (% of total time; Mean ± S.E.M) on day 1 of animals during free exploration before foot shock exposure and before being treated with vehicle, corticosterone and peptides. **C.** Histograms showing freezing behavior (% of total time; Mean ± S.E.M) of animals 24 hours after being injected intraperitoneally immediately after training with vehicle (veh, n=5) or corticosterone (cort, 2 mg/kg) in the presence (n=7)/absence (n=7) of pep-R845A or scrambled peptide. One-way ANOVA was performed with a Bonferroni post-test for multiple comparison data sets. *p<0.05.

Fear Conditioning

Finally we tested whether the memory-enhancing effects of corticosterone can be prevented by interfering with the AMPAR-NSF interaction (Figure 4). 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 4C). Post-training application of R845A also increased freezing behavior at 24 hours after training. Animals treated with both R845A and corticosterone showed freezing levels which were comparable with vehicle injected animals (Figure 4).

Discussion

Glucocorticoids (GCs) affect the synaptic regulation of AMPAR function, which is crucial for memory formation (Rumpel et al., 2005; Krugers et al., 2010; Mitsushima et al., 2011; Nabavi et al., 2014). The pathways that determine how corticosteroid hormones regulate synaptic AMPARs remain elusive. We report that interfering with N-Ethylmaleimide-Sensitive Factor (NSF, which is involved in membrane fusion of GluA2) prevents the effects of corticosterone on AMPAR mediated hippocampal synaptic transmission, surface expression of AMPARs, AMPAR mobility and contextual fear memory formation.

Various lines of evidence indicate that GCs regulate AMPARs. Within minutes after application of corticosterone, the surface mobility of AMPA receptors (Groc et al., 2008) and frequency of mEPSCs (Karst and Joëls, 2005) is reversibly enhanced. At a longer time-scale, and in agreement with earlier observations (Karst and Joëls, 2005; Groc et al., 2008; Martin et al., 2009), we report here that corticosterone lastingly increases the surface expression of GluA1/GluA2 AMPARs, the mobile fraction of GluA2, synaptic trapping of GluA2 and the amplitude of mEPSCs. Thus, corticosterone, via a slow mode of action enhances synaptic delivery and retention of hippocampal GluA2-containing AMPARs (Xiong et al., 2015).

Synaptic insertion and removal of GluA2 containing AMPARs is largely dependent on the interaction between intracellular proteins and the carboxyl terminus of the GluA2 subunit (Nishimune et al., 1998; Kim et al., 2001; Lee et al., 2002). In particular, the interaction between NSF and GluA2 is critical for maintaining AMPARs in the synapse. This may involve interference by NSF of the interaction between PICK1 and GluA2, thereby preventing internalization and intracellular retention of AMPARs. In this view, NSF maintains AMPAR function (Nishimune et al., 1998; Song et al., 1998; Lüscher et al., 1999; Noel et al., 1999; Evers et al., 2010). The internalization of AMPARs is mediated by dynamin-dependent clathrin mediated processes (Carroll et al., 1999; Lee et al., 2002), via clathrin adaptors such as the AP2 complex which bind to cytoplasmic domains of AMPARs. Since the AP2 complex associates with a region of GluA2 that overlaps

with the NSF binding site, differentially interfering with these peptides is required to determine whether NSF (or AP2) is required for the effects of GCs on AMPARs.

Here we observed that peptides that specifically interfere with NSF (R845A) or AP2 (Δ849-Q853) or both with NSF and AP2 (Pep2m, Lee et al., 2002) did not alter synaptic transmission by themselves, which is in contrast to earlier observations (Lee et al., 2002). This discrepancy may be due to different experimental approaches such as dosages of peptides that are applied. Other studies used 2-4 times higher dosages (Lee et al., 2002; Noel et al., 1999) as currently applied. Alternatively, other studies have used different experimental procedures to interfere with the peptides, such as using transfection (Lee et al., 2002), which do not precisely allow control of targeting the protein of interest. Regardless of this discrepancy, our results do demonstrate that NSF is required for a GC-evoked increase in AMPAR-mediated synaptic transmission since R845A but not Δ849-Q853 (which interferes with AP2) was effective in preventing the effects of corticosterone.

To examine whether the effects of corticosterone and NSF on AMPAR-mediated synaptic transmission are accomplished through by the surface expression of AMPARs we examined the expression of membrane GluA1 and GluA2. Corticosterone - at a dosage of 100 nM - increased both GluA1 and GluA2 expression as reported before (Martin et al., 2009; Groc et al., 2008). These effects are prevented by interfering with NSF supporting that regulation of GluA2 is important for the functional effects of corticosterone. Since interfering with NSF also prevented the corticosterone-induced increase in surface expression of GluA1, our results suggest that preventing the interaction between NSF and GluA2 is able to regulate GluA1 as well and that corticosterone enhances the expression of GluA1/GluA2 containing AMPARs, with functional consequences. Interestingly, the effects of corticosterone on AMPA receptor-mediated synaptic transmission and surface expression of AMPARs could still be prevented by interfering with NSF once hormone treatment was started. This indicates that corticosterone drives AMPARs to the synaptic membrane and that preventing membrane stabilization of AMPARs prevents the effects of corticosterone. Thus, corticosterone activates a

mechanism that is required for trafficking to the neuronal membrane and the functional effects can be overruled by preventing insertion of AMPARs.

We furthermore showed that the effect of corticosterone on the dynamics of surface-expressed AMPARs could be prevented by interfering with NSF. This confirms that NSF is required for membrane insertion of AMPARs. The notion that NSF is required for synaptic trapping might suggest that AMPARs need to be transported from endocytic zones (Petrini et al., 2009) by lateral diffusion (Groc et al., 2008) to the synapse where they can be retained, a process that is facilitated by corticosterone. Thus, NSF is critical for GC-induced membrane retention of AMPARs, synaptic trapping of AMPARs and the increase in AMPAR-mediated synaptic function.

Corticosteroid hormones enhance fear memory formation, and there is evidence that this involves regulation of AMPARs (Conboy and Sandi, 2010; Yuen et al., 2011; Xiong et al., 2015). Since earlier studies have reported that NSF is required for consolidation of contextual fear memory formation (Joels and Lamprecht, 2010; Migues et al., 2014), we examined whether the specific interaction between NSF and GluA2 was crucial for corticosterone increased contextual fear memory. We found that corticosterone enhances contextual fear memory formation in a weak fear conditioning paradigm as reported earlier (Cordero and Sandi, 1998). Interestingly, R845A applied directly after training also enhanced contextual fear, which was somewhat unexpected. Earlier studies have reported that R845A reduces fear memory formation when applied 1 day after training, which may indicate that NSF is involved in maintenance of fear memories (Migues et al., 2014). Our current results show that interfering with NSF at training enhances the expression of fear 24 hours later. Although it is difficult to explain this effect at this point, it supports that interfering with AMPAR insertion at learning determines memory formation. Interestingly, disrupting the interaction between NSF and GluA2, did prevent corticosterone to enhance memory formation. This suggests that enhanced contextual fear memory formation that is mediated by corticosterone requires stabilization of GluA2 containing AMPARs. Whether this effect involves enhanced memory consolidation is not clear at the moment. Therefore, peptides

that interfere with NSF should be applied at later time-points after training and GC administration.

In summary, our results indicate that GCs increase AMPAR mediated synaptic transmission and synaptic insertion of AMPARs via a mechanism that requires NSF/GluA2 interaction. This may contribute to the memory enhancing effects of GCs.

Acknowledgements

F.C. and S.M. designed and analyzed the live imaging experiments. F.C. performed the live imaging experiments. HX performed electrophysiological recordings and surface labeling. HX, LS and SS performed behavioural experiments. HX, FC, SM, ZQ, MJ, and HK wrote the manuscript. We thank Jeremy Henley (University of Bristol, UK) for the generous gift of pSinRep5 SEP-GluA2 and membrane-GFP plasmids. We gratefully acknowledge the 'Agence Nationale de la Recherche' (ANR-2011-JSV4-003 1) and the French Government for the "Investments for the Future" LABEX 'SIGNALIFE' (ANR-11-LABX-0028-01) to SM for financial support. We also thank the Royal Netherlands Academy for Arts and Sciences for support to HX, MJ and HK (Grants 05CDP013 and 11CDP017).

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Chapter 4

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% CO_2 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% CO_2 for 24h until use.

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 NH_4Cl (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% CO_2 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 $CaCl_2$, 0.8 mM $MgCl_2$, 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), $MgCl_2$ (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):

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 $T^{1/2}$ (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

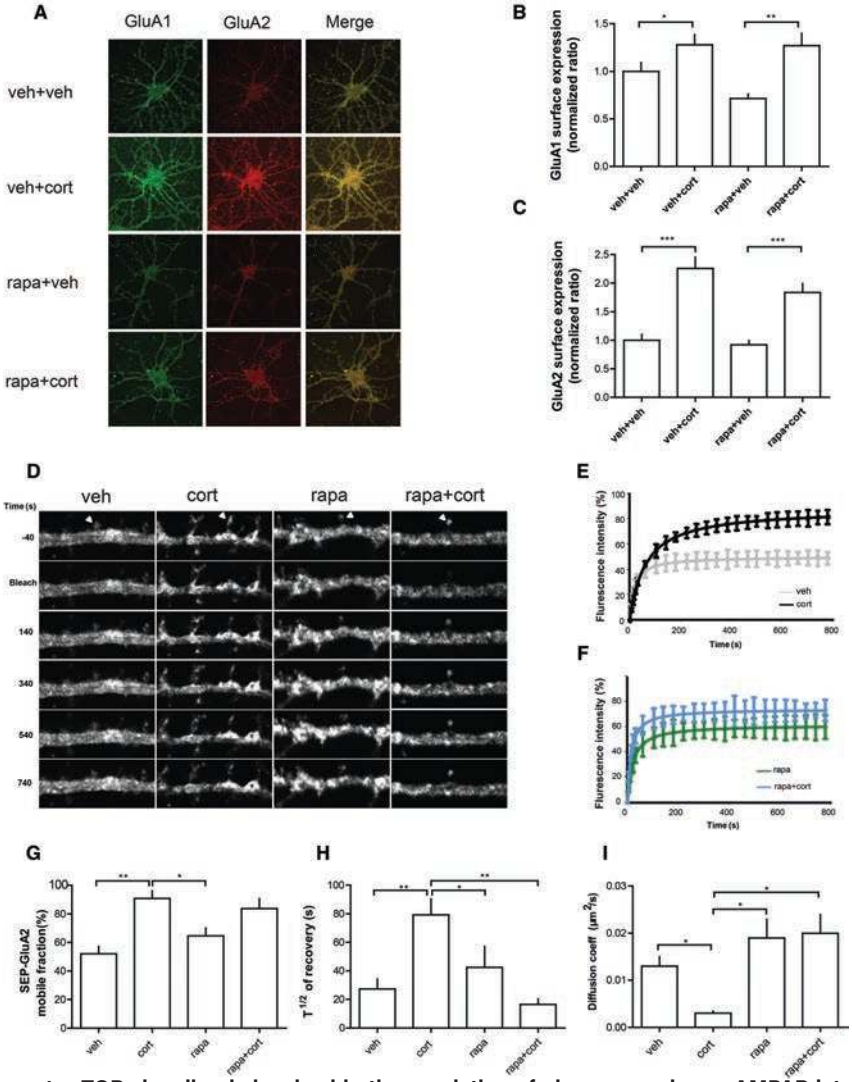

Figure 1. mTOR signaling is involved in the regulation of plasma membrane AMPAR lateral diffusion of corticosterone-treated rat hippocampal neurons. A. Representative images of rat hippocampal neurons with labeling of GluA1 (in green) and GluA2 (in red) AMPAR subunits after treatment with vehicle (veh), 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 rapamycine + 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.

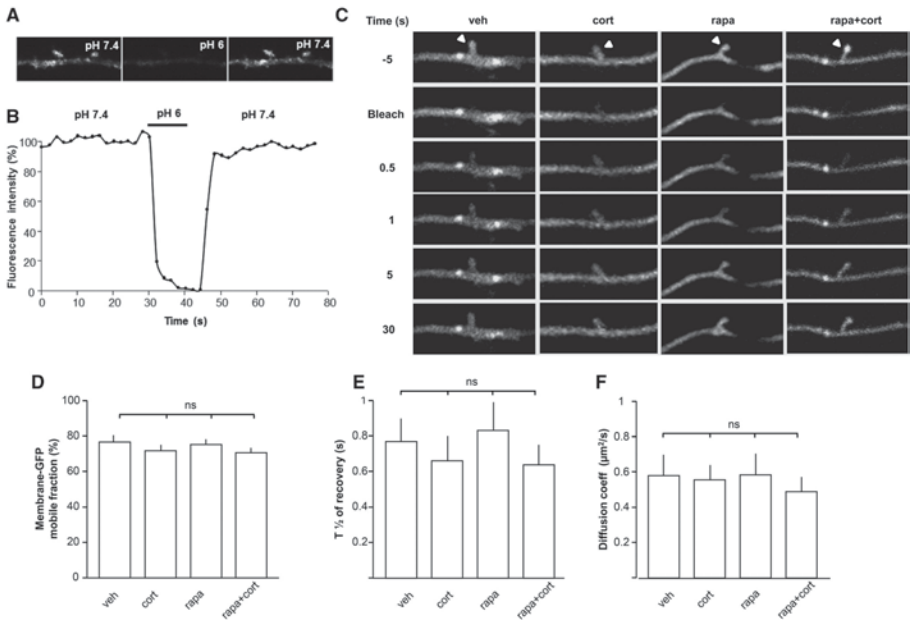

Figure 2. Blocking the mTOR signaling pathway does not impact the synaptic diffusion of membrane GFP. A. Sequential confocal images of SEP-GluA2 in living rat hippocampal neurons. Bright SEP-GluA2 fluorescence is mainly due to surface expressed receptors and fluorescence is rapidly lost in pH 6.0 external 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 $T^{1/2}$ 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

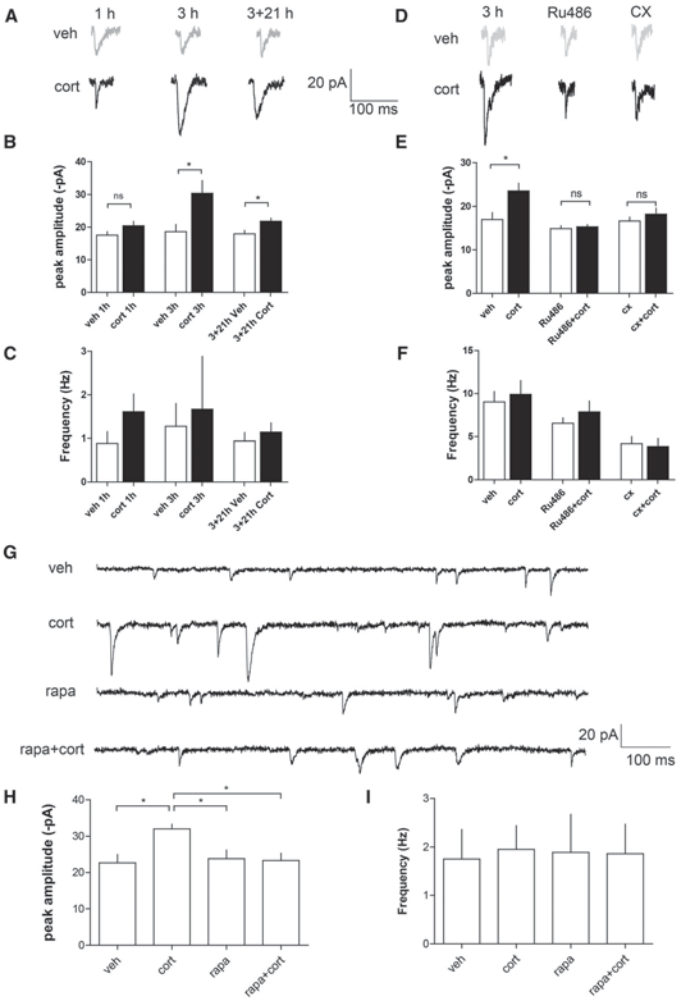

Figure 3. Corticosterone regulates AMPAR function via the mTOR pathway. A. Representative traces of mEPSCs at 1, 3 or 24 hours after vehicle (veh) or corticosterone (Cort, 100 nM) treatment on rat hippocampal neurons. **B.** Amplitude of mEPSCs at 1, 3 or 24 hours after vehicle (veh) or corticosterone (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

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

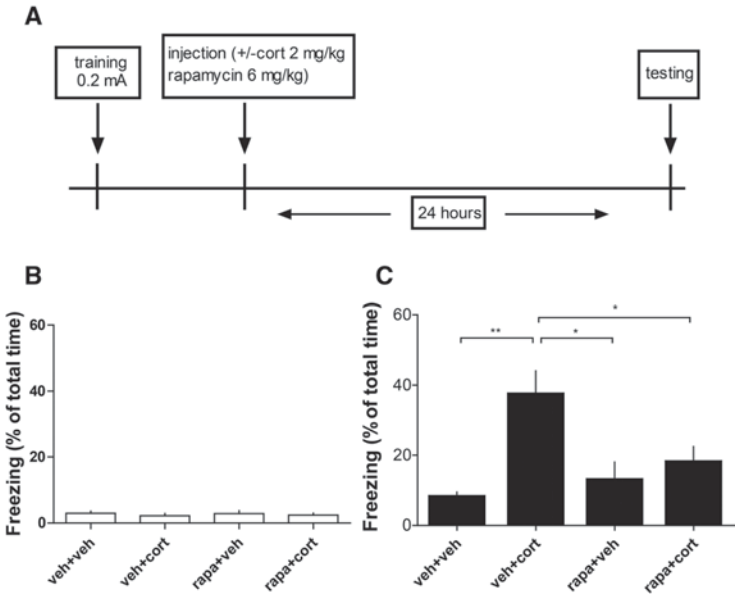

Figure 4. Corticosterone enhances memory consolidation via mTOR pathway. A. Timeline of behavioral experiment. Animals received a foot shock of 0.2 mA and were immediately injected with vehicle (veh), corticosterone (cort, 2 mg/kg), rapamycin (rapa, 6 mg/kg) or corticosterone (cort, 2 mg/kg) + rapamycin (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 intraperitoneal 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.

Acknowledgements

F.C. and S.M. designed and analyzed the live imaging experiments. F.C. performed the live imaging experiments. HX, MZ YZ, performed electrophysiological recordings. HX performed surface labeling and behavioural experiments. FC, SM, MJ, and HK wrote the manuscript. We thank Jeremy Henley (University of Bristol, UK) for the generous gift of pSinRep5 SEP-GluA2 and membrane-GFP plasmids. We gratefully acknowledge the 'Agence Nationale de la Recherche' (ANR-2011-JSV4-003 1) and the French Government for the "Investments for the Future" LABEX 'SIGNALIFE' (ANR-11-LABX-0028-01) to SM for financial support. We also thank the Royal Netherlands Academy for Arts and Sciences for support to HX, MZ, MJ and HK (Grants 05CDP013 and 11CDP017).

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Chapter 5
Glucocorticoids regulate hippocampal AMPA receptor function via activation of Calcium-calmodulin dependent Kinase II

Hui Xiong, Marian Joëls, Harm J Krugers

In preparation

Abstract

Emotionally important events are well remembered. Although memories of emotional experiences are known to be mediated and modulated by glucocorticoids, the underlying molecular mechanisms remain elusive. Here we examined whether Calcium-calmodulin dependent Kinase II (CaMKII) is involved in the effects of glucocorticoids on AMPA receptor (AMPAR) function, a critical endpoint for memory formation. We report that incubation of primary hippocampal cultures for 3 h with corticosterone increases AMPAR mediated synaptic transmission and increases surface expression of GluA1 and GluA2. These effects were prevented by the CaMKII blocker KN-93, administered prior to and during corticosterone application or during the final 30 minutes of corticosterone application. These results suggest that CaMKII is required to initiate and maintain the effects of corticosterone on AMPAR function.

Introduction

Emotionally arousing events, whether positive or negative, are well remembered in general (de Kloet et al., 1999), which reflects an adaptive mechanism that has evolved to remember salient and relevant information (de Kloet et al., 1999). Exposure to emotionally arousing events activates among others the hypothalamus pituitary adrenal axis which provokes the release of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal glands (de Kloet et al., 2005). Various lines of evidence indicate that glucocorticoids promote memory consolidation, thereby stabilizing newly formed memories (Oitzl and de Kloet, 1992; Roozendaal et al., 2009; Yuen et al., 2011; Xiong and Krugers, 2015). These effects are mediated via glucocorticoid receptors, which are enriched in brain areas such as the hippocampus, amygdala and prefrontal cortex, which play crucial roles in memory formation (Reul and de Kloet, 1985; de Kloet et al., 2005; Roozendaal et al., 2009).

Recent studies have demonstrated that the memory enhancing effects of glucocorticoids may require activation of the MAPK–Erk pathway (Revest et al., 2014), regulation of Synapsin–1a/1b (Revest et al., 2010) and CaMKII (Chen et al., 2012). Yet, how these pathways contribute to the effects of glucocorticoids on learning and memory remains elusive.

Corticosterone, via activation of glucocorticoid receptors, regulates AMPA receptor (AMPAR) function (Karst et al., 2005; Groc et al., 2008; Martin et al., 2009; Conboy and Sandi, 2010; Krugers et al., 2010; Yuen et al., 2011; Xiong and Krugers, 2015), a critical endpoint for memory formation (Kessels and Malinow, 2009; Mitsushima et al., 2011). Via a genomic mode of action, glucocorticoids lastingly increase AMPAR function (Karst et al., 2005; Martin et al., 2009), AMPAR mobility (Groc et al., 2008) and synaptic retention of AMPARs (Xiong et al., 2015).

Here we examined whether corticosteroid hormones regulate AMPAR function via activation of Calcium calmodulin dependent Kinase II (CaMKII), which is critical for

regulation of AMPAR function (Chen et al., 2012), memory formation (Lisman et al., 2012) and the memory enhancing effects of glucocorticoids (Chen et al., 2012).

Materials and Methods

Hippocampal primary cultures

Primary hippocampal cultures were prepared from embryonic day 18 (E18) rat brains. Cells were plated on coverslips coated with poly-D-lysine (0.5 mg/ml) at a density of 40-50K/well/coverslip for electrophysiology and immunosurface labeling experiments. Hippocampal cultures were grown in Neuronbasal medium supplemented with (per 100ml): B27 2 ml, GlutaMaxl 1 ml, Pen/Streptomycin 1 ml, Fetal bovine serum (FBS) 5-10 ml (plating medium) for the first day (DIV0-DIV1). Plating medium was changed by culture medium (without FBS) from the 2nd day (DIV2) onwards. 5-Fluoro-2′-Deoxyuridine (FUDR, 10 μM, Sigma) was added into the culture medium to inhibit glial growth. All reagents except FUDR (Sigma) were from GIBCO Invitrogene, USA. The experiments were carried out in accordance with and approved by the local Animal Committees of University of Amsterdam.

Electrophysiology

Coverslips were placed in a recording chamber mounted on an upright microscope (Zeiss Germany), and kept fully submerged with artificial extracellular solution [containing (in mM): NaCl (145), KCl (2.8), $MgCl_2$ (1), HEPES (10), $CaCl_2$ (2), and Glucose (10), 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): 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 6.0.

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 3 min in each cell.

Corticosterone (100 nM, Sigma) or vehicle (<0.01% ethanol) was applied to the neuronal cultures for 3 hours. To examine whether CaMKII is involved in corticosterone effects on AMPARs mediated transmission, KN93 (inhibitor of CaMKII, 5 µM, Calbiochem, USA) was applied to the neuronal cultures either 30 minutes before and during corticosterone treatment or during the last 30 min of corticosterone incubation. KN92 (Calbiochem, USA) was used as a control reagent of KN93 (Opazo et al., 2010).

Immunocytochemistry

At DIV14-20 hippocampal neurons were incubated with GluR1 (Calbiochem (1:8) and GluR2 (Zymed (1:80) N-terminal antibodies (10 mg/ml) at 37°C for 15 min (Martin et al. 2009). Cells were preincubated at 37°C in 5% CO_2 for 1 hour in Neurobasal before treatment. Corticosterone (100 nM, Sigma) or vehicle (<0.01% ethanol) was applied to the neuronal cultures for 3 hours. To examine whether CaMKII is involved in corticosterone effects on AMPARs surface expression, KN93 (inhibitor of CaMKII, 5 µM, Calbiochem) was applied to the neuronal cultures either 30 minutes before and during

corticosterone treatment or during the last 30 min of corticosterone incubation. KN92 (Calbiochem) were used as control reagent of KN93.

After washing in Dulbecco's Modified Eagle's Medium(DMEM), 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) (both from Invitrogen, USA) in staining buffer without TritonX-100 (0.2% BSA, 0.8 M NaCl, 30 mM phosphate buffer, pH 7.4) overnight at 4 oC. 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).

Statistical analysis

Statistical analyses were calculated using Prism 5 (GraphPad software, Inc). Data are expressed as mean ± S.E.M. One-way ANOVA were performed with a Bonferroni post-test for multiple comparison data sets when required.

Results

Blocking CaMKII during the entire 3 h corticosterone administration.

Application of corticosterone (100nM) for 3 hours to cultured hippocampal cells increased the amplitude of mEPSCs. To examine whether CaMKII is critical for the effects of corticosterone on AMPARs-mEPSCs (Figure 1B,C), we applied KN93 (5 μM), an inhibitor of CaMKII activity, to cultured hippocampal cells 30 min before and during the corticosterone application. The increased amplitude of mEPSCs induced by corticosterone was prevented by KN93 application, but not by KN92 (an inactive analog of KN93) (Figure 1A-E).

We next tested the involvement of CaMKII in corticosterone effects on surface

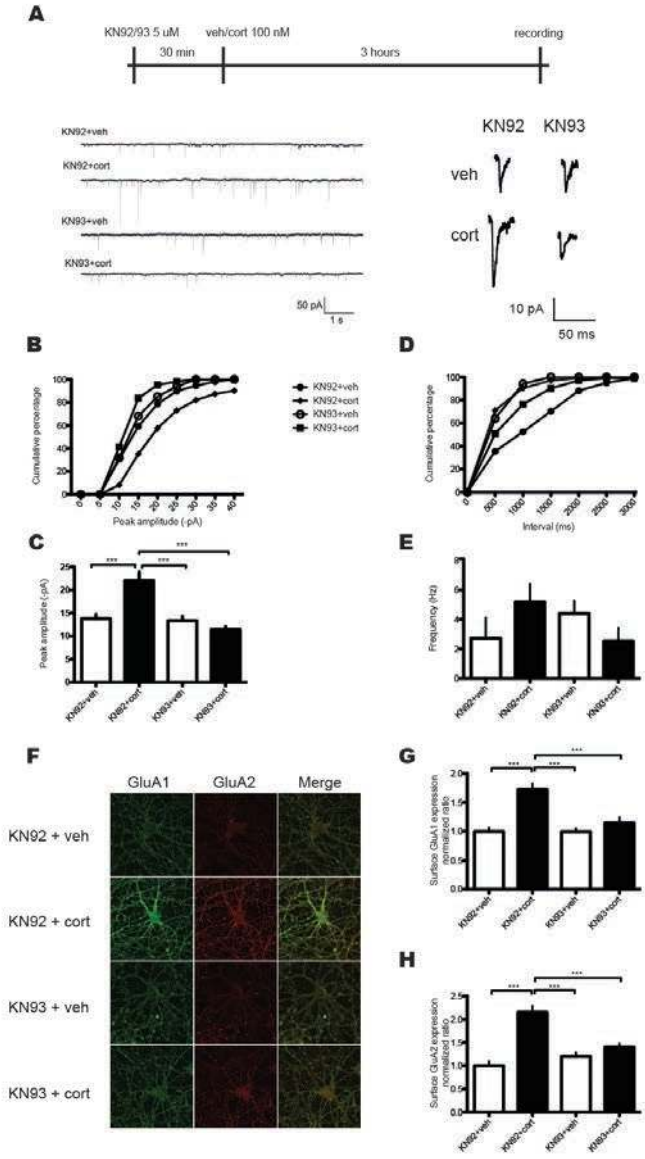

Figure 1: Blocking CaMKII during corticosterone administration prevents corticosterone effects on AMPARs-mEPSCs and surface expression. A. Top: experimental design: corticosterone (100nM) was applied for 3 hours. KN92 or KN93 were applied at a dosage of 5 μM 30 minutes before and during corticosterone administration; bottom: representative traces and individual mEPSCs after KN92 or KN93 +/- corticosterone treatment. **B-C:** Cumulative percentage distribution and histograms showing the amplitude of mEPSCs after treatment with KN92 or 93 (5 μM) +/- corticosterone (100 nM). n>10 cells in each group, *p<0.05, ns: not significant (One way-ANOVA). **D-E:** Cumulative percentage distribution and histograms showing the frequency of mEPSCs after treatment with KN92/93 and corticosterone treatment. **F.** Representative images

of rat hippocampal neurons with surface labeling of GluA1 (in green) and GluA2 (in red) AMPAR subunits after treatment with corticosterone (cort, 100 nM) and KN92 or 93. Corticosterone (100 nM) was applied for 3 hours. KN92 and KN93 were applied at a dosage of 5 μM 30 minutes before and during corticosterone treatment. **G-H.** Histograms showing the mean (± S.E.M.) quantification of surface GluA1 (G) or GluA2 (H) AMPAR subunits. Corticosterone (100 nM) was applied for 3 hours. KN92 and KN93 were applied at a dosage of 5 μM 30 minutes before and during corticosterone treatment. Data are expressed as ratio of control (vehicle + KN92 condition). n>15 cells; One-way ANOVA followed by a Bonferroni post-test for multiple comparison data sets, *p<0.05, **p<0.01, *** p<0.001.

expression of AMPARs. Both GluA1 and GluA2 surface expression was enhanced by 3 hours of corticosterone (100 nM) application. This was blocked by administration of KN93 (5 μM), not by KN92 (Figure 1F-H). These results indicate that corticosterone effects on AMPARs-mEPSCs and surface expression require CaMKII.

Blocking CaMKII during the final 30 min of corticosterone administration.

To examine whether CaMKII is also critical for the effects of corticosterone on synaptic transmission once hormone treatment has started, we applied KN92/KN93 to cultured hippocampal cells only during the last 30 minutes of hormone treatment (3 hours in total) (Figure 2A). Brief (30 min) treatment of KN93 (5 μM) blocked the effect of increasing peak amplitude of AMPARs-mEPSCs, which was induced by corticosterone (100 nM) (Figure 2A-E).

We next investigated the role of CaMKII in corticosterone effects on surface expression of AMPARs in the last 30 minutes of corticosterone treatment. Again, both GluA1 and GluA2 subunit of AMPARs surface expression was enhanced by corticosterone treatment. These effects were prevented by 30 minutes treatment with KN93 (5 μM) (Figure 2F-H), but not by KN92. These results indicate that either the development of corticosterone effects on AMPAR mEPSCs amplitude enhancement critically depends on activation of CaMKII during the final 30 min of corticosterone application or that blockade of CaMKII in this period might be able to reverse earlier established effects of corticosterone on glutamate transmission.

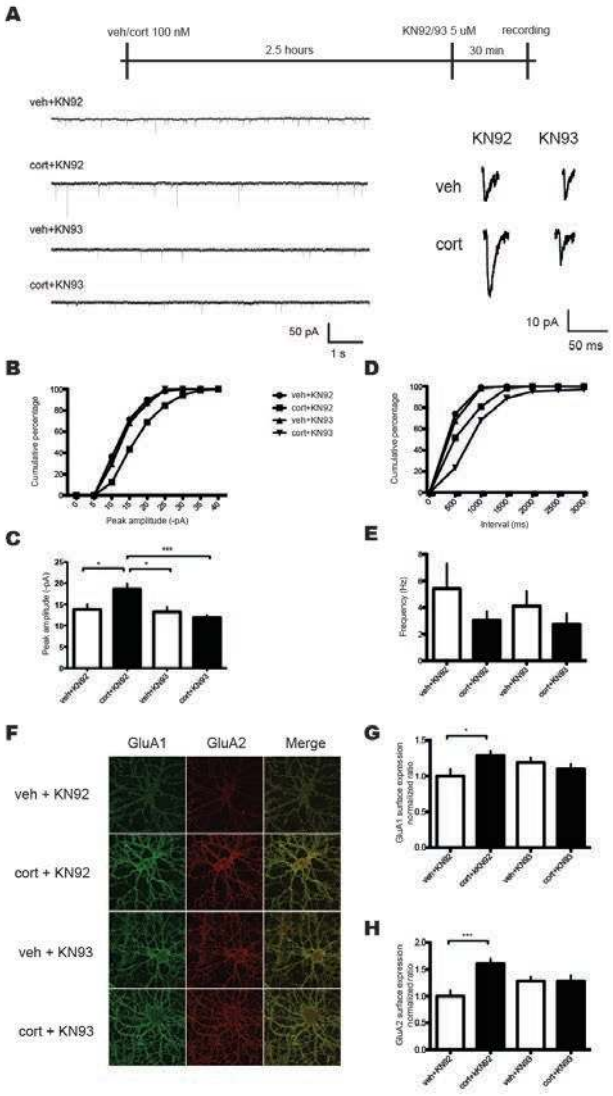

Figure 2: Blocking CaMKII after corticosterone administration prevents corticosterone effects on AMPARs-mEPSCs but not the surface expression. A. Top: experimental design: corticosterone (100nM) was applied for 3 hours. KN92 or KN93 were applied at a dosage of 5 μM during the last 30 minutes of a 3 h corticosterone treatment; bottom: representative traces and individual mEPSCs after KN92 or KN93 +/- corticosterone treatment. **B-C:** Cumulative percentage distribution and histograms showing the amplitude of mEPSCs after treatment with KN92 or 93 (5 μM) +/- corticosterone (100 nM). n>10 cells in each group, *p<0.05, ns: not significant (One way-ANOVA). **D-E:** Cumulative percentage distribution and histograms showing the frequency of mEPSCs after treatment with KN92/93 and corticosterone treatment. **F.** Representative images of rat hippocampal neurons with surface labeling of GluA1 (in green) and GluA2 (in red) AMPAR subunits after treatment with corticosterone (cort, 100 nM) and KN92 or 93. Corticosterone (100

nM) was applied for 3 hours. KN92 and KN93 were applied at a dosage of 5 μM 30 minutes during the last 30 minutes of a 3 h corticosterone treatment. **G-H.** Histograms showing the mean (± S.E.M.) quantification of surface GluA1 (G) or GluA2 (H) AMPAR subunits. Corticosterone (100 nM) was applied for 3 hours. KN92 and KN93 were applied at a dosage of 5 μM during the last 30 minutes of a 3 h corticosterone treatment. Data are expressed as ratio of control (vehicle + KN92 condition). n>15 cells; One-way ANOVA followed by a Bonferroni post-test for multiple comparison data sets, *p<0.05, **p<0.01, *** p<0.001.

Discussion

Glucocorticoids (GCs), via activation of GRs, have been reported to increase exocytosis of AMPARs (Yuen et al., 2011), lateral diffusion of AMPARs (Groc et al., 2008) and synaptic retention of AMPARs ((Sarabdjitsingh et al., 2014; Xiong et al., 2015) which is accompanied by an increase in hippocampal AMPAR function (Karst et al., 2005; Martin et al., 2009; Xiong et al., 2015). In line with these studies we report here that a brief administration of corticosterone increases AMPAR mediated synaptic function at three hours after GC administration and increases surface expression of GluA1 and GluA2 in cultured hippocampal neurons. These GC-induced changes were prevented by inhibiting the Calcium/calmodulin-dependent kinase II (CaMKII) inhibitor KN93, but not by the inactive control KN92.

The function, trafficking and synaptic signalling of AMPA receptors are tightly regulated by phosphorylation (Lisman et al., 2012). CaMKII phosphorylates the GluA1 AMPA subunit at Ser831 to increase single channel conductance (Kristensen et al., 2011). In addition, increased activity of CaMKII induces the delivery of AMPARs into synapses (Hayashi et al., 2000). At present we report that inhibiting CaMKII by using the inhibitor KN93 did by itself not modify AMPAR mediated synaptic transmission. This is in line with the hypothesis that activity-dependent changes are required to activate CaMKII which then increases AMPAR function and AMPAR delivery to synapses (Lisman et al., 2012). Yet, KN93 did inhibit the effects of GCs on AMPAR mediated synaptic transmission, both when applied prior to and during GC administration as well as 2.5 hrs after GC administration had started. This indicates that corticosterone has a CaMKII-dependent slow-onset but long lasting effect on AMPAR function (Xiong et al., 2015) and that interfering with CaMKII is able to rapidly prevent GC effects on AMPAR

function. The rapid effect of KN93 may imply that corticosterone effects via CaMKII are only established during the final 30 min of a 3 hrs corticosterone administration period or perhaps that earlier established effects can be reversed by later blockade of CaMKII.

An important question is exactly how corticosterone effects on AMPAR mediated are mediated by CaMKII. One possible intermediate step could involve an increase in intracellular calcium levels (and subsequent activation of CaMKII), e.g. via activation of L-type calcium channels (Chameau et al., 2007; Karst and Joëls, 2007). Indeed inhibiting L-type calcium channels with nifedipine is also able to prevent the effects of GCs on AMPAR mediated synaptic transmission (M.Zhou, personal communication). It is important to mention that KN93 may also inhibit other ion channels. While KN93 inhibits the activation of CaMKII (Pellicena and Schulman, 2014) is has also been reported to modulate activation of the L-type Ca^{2+} channel by CaMKII (Li et al., 1992; Anderson et al., 1998). Moreover, KN-93 blocks voltage-dependent K^{+} current in smooth muscle cells at concentrations used to inhibit CaMKII (Ledoux et al., 1999). Whether the effects of GCs on AMPAR currents are exclusively regulated by CaMKII therefore remains to be determined.

Our data suggest that glucocorticoids via CaMKII regulate AMPARs which are crucial for learning and memory (Roozendaal et al., 2009; Silva et al., 1996; Rumpel et al., 2005). In agreement, GCs can modify memory consolidation via a glucocorticoid receptor-dependent phosphorylation of CaMKII (Chen et al., 2012). Via this activation, CaMKII has been reported to result in a CREB-BDNF-dependent increase in fear memory consolidation (Chen et al., 2012). To examine whether this model holds true at the cellular level, it would be important to examine if the effects of GCs on AMPAR function are mediated via this pathway.

In conclusion, we report that activation of CaMKII contributes to the effects of GCs on AMPAR function. It is tempting to speculate that these effects may contribute to the memory enhancing effects of GCs.

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Chapter 6
Corticosterone regulates NMDA receptor mediated synaptic function via GluN2B subunits

Hui Xiong, Laurent Groc, Marian Joëls, Harm J Krugers

In preparation

Abstract

Corticosteroid hormones, within minutes, increase AMPA receptor mediated synaptic transmission and facilitate long-term potentiation (LTP). Here we examined in hippocampal primary cultures whether corticosterone also alters NMDA receptor function which is critical for activity-dependent changes in synaptic transmission. We report that brief application of corticosterone (50-100 nM) rapidly increases the surface expression and synaptic content of GluN2B but not GluN2A subunits in primary hippocampal cell cultures, an effect that remained stable for at least 30 minutes. Furthermore, corticosterone enhanced the amplitude as well the area under the curve of spontaneous NMDA receptor currents, but did not affect the frequency or decay time. These physiological effects were absent in cells which were treated with the GluN2B antagonist Ro 25-6981. Overall, the results suggest that corticosteroid hormones rapidly increase NMDA receptor function via regulation of the GluNR2B subunit. Since this subunit determines calcium permeability of NMDA receptors and facilitates LTP, this may provide a cellular mechanism via which corticosteroid hormones facilitate synaptic plasticity.

Introduction

Exposure to stressful experiences increases the release of corticosteroid hormones from the adrenal glands (de Kloet et al., 2005). Corticosteroids (cortisol in humans, corticosterone in rodents) activate the high-affinity mineralocorticoid receptor (MR) and when plasma corticosteroid levels rise they also activate the lower affinity glucocorticoid receptor (GR) (de Kloet et al., 2005). Via activation of MRs and GRs, corticosteroids facilitate behavioural adaptation to stressful experiences which involves a.o. selection of adaptive behavioural strategies (Schwabe et al., 2010) and enhanced memory consolidation of emotionally arousing experiences (Roozendaal et al., 2009).

Glutamatergic synaptic transmission is critical for activity-dependent changes in synaptic function in networks which underlie behavioural adaptation such as memory formation (Malinow and Malenka, 2002; Kessels and Malinow, 2009). Corticosterone dynamically regulates hippocampal glutamatergic synaptic transmission; within minutes after administration. The hormone increases neurotransmitter release probability, AMPA receptor (AMPAR) mobility and facilitates synaptic long-term potentiation (LTP) in hippocampal neurons (Karst et al., 2005; Wiegert et al., 2006; Groc et al., 2008; Martin et al., 2009). Hours after a brief administration, corticosterone increases AMPAR postsynaptic transmission via a process that requires activation of GRs and protein synthesis, promotes retention of AMPARs and suppresses LTP, both in hippocampal and prefrontal neurons (Alfarez et al., 2002; Karst and Joëls, 2005; Wiegert et al., 2005; Groc et al., 2008; Yuen et al., 2011).

Postsynaptic NMDA receptors (NMDAR) are required for activity-dependent synaptic plasticity such as LTP and various forms of learning and memory (Tsien et al., 1996; Kessels and Malinow, 2009; Bliss and Collingridge, 2013). NMDARs are hetero-tetramers comprising various combinations of GluN1, GluN2A-D, and GluN3 subunits (Paoletti et al., 2013). In the hippocampal formation, the most abundant NMDAR subtypes are composed of GluN1 subunits associated with GluN2A and/or GluN2B subunits (Paoletti et al., 2013). The ratio between GluN2A and GluN2B-NMDARs is not uniform and can be

rapidly altered; it may play a role in metaplasticity (Tovar and Westbrook, 2002; Groc et al., 2006; Bellone and Nicoll, 2007; Zhao et al., 2008; Matta et al., 2011).

Recent studies have suggested that corticosterone alters NMDA receptor mediated synaptic transmission and plasticity in hippocampus (Tse et al., 2011) and prefrontal cortex (Yuen et al., 2009; 2011), possibly through altering the synaptic GluN2A/GluN2B ratio. We investigated here whether corticosterone, within minutes after application, alters NMDA receptor function in hippocampal primary cultures. We focused on changes in GluN2B subunits localization and functionality following corticosterone exposure, using live cell imaging and electrophysiological approaches.

Materials and Methods

Primary hippocampal culture

The experiments were carried out with permission of the local Animal Committee of the University of Amsterdam. Primary hippocampal neurons were prepared from E18 pregnant Wistar rats. The hippocampus was dissected from E18 embryos and digested with 2.5% trypsin (Sigma, USA). Neurons were plated in Neurobasal medium (Invitrogen USA) supplemented with 2% B27 (Invitrogen USA), 0.5 mM glutamax and penicillin/streptomycin (GIBICO USA) and 5% Fetal Bovine Serum (FBS) (GIBICO, USA, only for the first day) 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. 5-Fluoro-2′-Deoxyuridine (FUDR) 10 μM was used to inhibit glial growth. All experiments were carried out in cultures DIV14-DIV21. For live imaging, neurons were transfected at 7 to 14 DIV using the effecten transfection kit (QIAGEN), using the provider's protocol.

Time-lapse imaging

Neurons co-transfected with Homer1c-DsRed and either GluN2A-SEP, GluN2B-SEP or GluN1-SEP were placed on the heated stage (37°C) of an inverted confocal spinning-disk microscope (Leica, Germany). To test the population of surface GluN subunits-SEP,

we used a low pH-solution adjusted to pH 5.4 which quenched all the fluorescence confirming that SEP allows the specific visualization of surface receptors. Fluorescence was excited using a monochromator, and cluster fluorescence intensity was followed over time to assess synaptic receptor content. Corticosterone (50 nM) was applied after a 5 min baseline; next, the medium was carefully replaced by new equilibrated and heated medium after the protocol application). Clusters were imaged over a total period of 35 minutes. Fluorescence intensity was measured using Metamorph software (Universal imaging, USA) and corrected for photobleaching and background noise.

Electrophysiology (mEPSCs)

Coverslips were placed in a recording chamber mounted on an upright microscope (Zeiss Axioskop 2 FS Plus, Germany). We used Mg2+ free extracellular solution which contained the following component (in mM); 140 NaCl, 5 KCl, 3 $CaCl_2$, 10 Glucose, 10 HEPES, 0.0025 TTX, 0.02 bicuculline, 0.005 NBQX, pH 7.4 (310 mOsm), and kept the coverslip fully submerged. Corticosterone (100 nM) or vehicle solution (<0.1% ethanol) was added directly into the extracellular solution while recording. In a separate series of experiments, testing the involvement of GluN2B subunits, the selective GluN2b-blocker Ro 25-6981 (3 µM, Tocris) or vehicle (DMSO) was also directly added into the extracellular solution while recording. The two series unfortunately could not be combined, because recordings became too unstable when the solution was altered several times in succession (data not shown).

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 and 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 6.0.

Statistics

Statistical analyses were calculated using Prism 5 (GraphPad software, Inc). Data are expressed as mean ± S.E.M. Unpaired and paired Student's t-tests were performed when required.

Results

Corticosterone acutely alters GluN2B but not GluN2A-NMDAR surface distribution and dynamics.

In order to explore the dynamic interplay between corticosterone exposure and the trafficking of GluN2-NMDAR subtypes in live hippocampal neurons, we first expressed in cultured hippocampal neurons the GluN2A or GluN2B subunit fused to a Super Ecliptic pHluorin (SEP) at its extracellular N-terminus (GluN2B-SEP). SEP is a pH sensitive variant of GFP that only emits fluorescence at neutral pH; fluorescence is quenched at acidic pH, such as in intracellular vesicles. This tool allowed us to study surface GluN2A/B-NMDAR separately from intracellularly located subunits.

We imaged the surface clusters of GluN2A- and GluN2B-NMDAR before and during exposure to 50 nM corticosterone (Figure 1A). For GluN2A-NMDAR surface clusters (colocalizing with synaptic markers), there was no significant change in the cluster fluorescence intensity over time (Figure 1A-C), indicating that the synaptic content of GluN2A-NMDAR remains stable after an acute exposure to corticosterone. For GluN2B-NMDAR, however, there was a rapid increase in the cluster fluorescence intensity, already observed 5 min after corticosterone incubation (Figure 1A-C). In addition,

the corticosterone-induced GluN2B-NMDAR synaptic content increase remained stable over a period of more than 30 min (Figure 1B). Together, these data show that

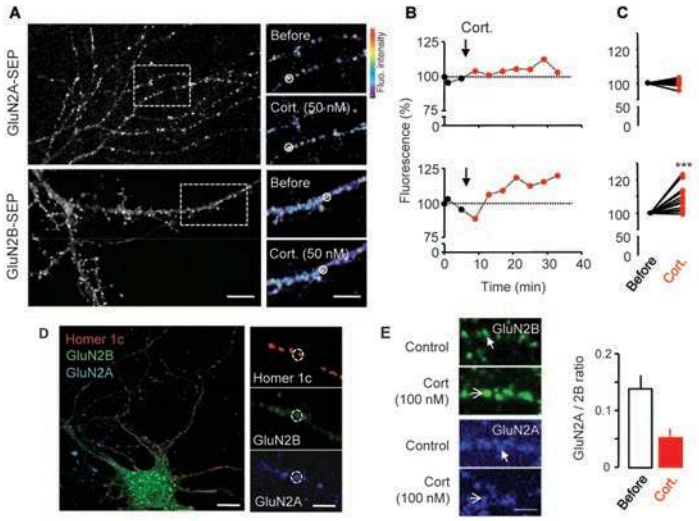

Figure 1: Corticosterone acutely alters GluN2B but not GluN2A-NMDAR surface distribution and dynamics. A. Representative images of the surface clusters of GluN2A- and GluN2B-NMDAR before and after exposure to 50 nM corticosterone. **B-C.** Quantification of Fluo. Intensity of surface GluN2A- and GluN2B-NMDAR before and after exposure to 50 nM corticosterone. **D.** Representative image of Homer 1C (red)/GluN2A (blue)/GluN2B (green) staining. **E.** Representative image of synaptic GluN2A-, GluN2B-NMDAR and quantification of synaptic GluN2A/GluN2B ratio before and after 100 nM corticosterone treatment. (*Data from L. Groc, Bordeaux*)

corticosterone acutely favors the clustering of surface GluN2B-NMDAR, likely changing the GluN2A/GluN2B-NMDAR synaptic ratio.

To directly address this point, we then transfected hippocampal neurons with GluN2A and GluN2B subunits containing different extracellular tags in order to measure their relative content in synapses (labeled by the expression of Homer 1c-DsRed) (Figure 1D). The application of corticosterone rapidly increased the synaptic content of GluN2B and significantly reduced the 2A/2B synaptic ratio (Figure 1E).

Corticosterone increases amplitude and charge area of NMDAR mEPSCs

To examine the effect of corticosterone on NMDA receptor function, we applied corticosterone (100 nM) to hippocampal cultures neurons. Traces of NMDA mEPSCs

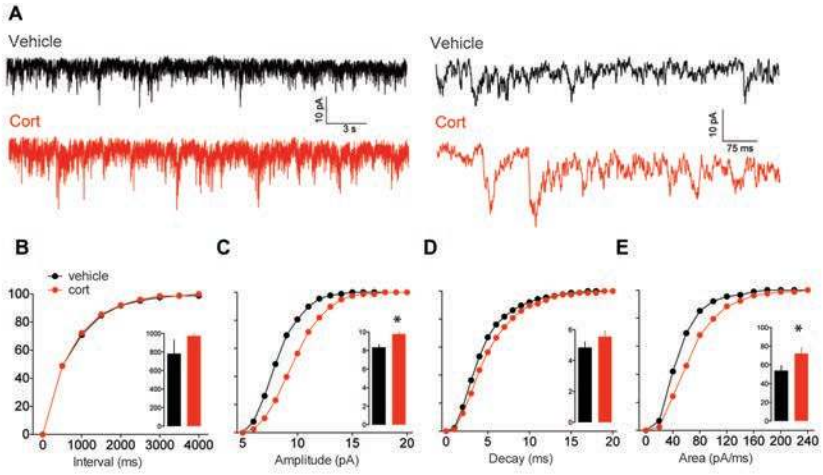

Figure 2: Corticosterone increases amplitude and charge of NMDARs-mEPSCs.
A. Representative traces (at two time resolutions, see calibration bars) of NMDARs-mEPSCs after vehicle (<0.1% ethanol) and corticosterone (100 nM). B. Cumulative percentage distribution and histograms showing the interval of NMDARs-mEPSCs after vehicle (n=14 cells) and corticosterone (n=19 cells) treatment. C. Cumulative percentage distribution and histograms showing the peak amplitude of NMDARs-mEPSCs after vehicle (n=14 cells) and corticosterone (n=19 cells) treatment. D. Cumulative percentage distribution and histograms showing the decay of NMDARs-mEPSCs after vehicle (n=14 cells) and corticosterone (n=19 cells) treatment. E. Cumulative percentage distribution and histograms showing the charge of NMDARs-mEPSCs (area under the curve) after vehicle (n=14 cells) and corticosterone (n=19 cells) treatment.

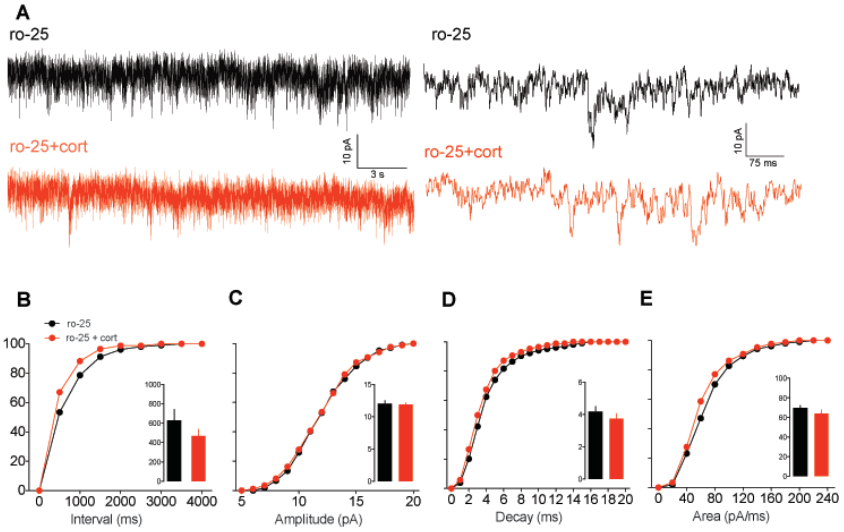

Figure 3: Corticosterone effects on NMDARs function via modulating GluN2B subunit. A. Representative traces (at two time resolutions, see calibration bars) of NMDARs-mEPSCs after Ro 25-6981 (3 μM) and Ro 25-6981 (3 μM) plus corticosterone (100 nM). **B.** Cumulative percentage distribution and histograms showing the interval of NMDARs-mEPSCs after Ro 25-6981 (n=9

cells) and Ro 25-6981 plus corticosterone (n=7 cells). **C.** Cumulative percentage distribution and histograms showing the peak amplitude of NMDARs-mEPSCs after Ro 25-6981 (n=9 cells) and Ro 25-6981 plus corticosterone (n=7 cells). **D.** Cumulative percentage distribution and histograms showing the decay of NMDARs-mEPSCs after Ro 25-6981 (n=9 cells) and Ro 25-6981 plus corticosterone (n=7 cells). **E.** Cumulative percentage distribution and histograms showing the charge of NMDARs-mEPSCs after Ro 25-6981 (n=9 cells) and Ro 25-6981 plus corticosterone (n=7 cells).

are shown in Figure 2A. Analysis of the data indicates that corticosterone increases the peak amplitude and the area under the curve (charge) of NMDAR mEPSCs when compared to vehicle treated cells (Figure 2C, E). Corticosterone did not affect the frequency (Figure 2B) or decay time (Figure 2D) of NMDAR mEPSCs.

Corticosterone effects on NMDAR function occur via GluN2B

We next investigated how corticosterone regulates NMDA receptor function. By using Ro 25-6981, which is a potent and selective activity-dependent blocker of NR2B containing NMDA receptors, we tested whether corticosterone effects on NMDA receptor function are mediated via GluN2B. Traces of NMDA mEPSCs are shown in Figure 3A. In the presence of Ro 25-6981 corticosterone did not alter the frequency, amplitude, decay time or area of NMDA mEPSCs (Figure 3C-E).

Discussion

NMDA receptor activation is critical for activity-dependent changes in synaptic plasticity such as long-term potentiation (LTP) (Kessels and Malinow, 2009) as well as learning and memory (Tang et al., 1999; Shimizu, 2000; Lisman et al., 2012). Corticosteroid hormones strongly regulate AMPA receptor-mediated synaptic transmission and LTP (Karst et al., 2005; Wiegert et al., 2006). Corticosterone, within minutes, increases synaptic transmission and facilitates synaptic plasticity (LTP) ((Karst et al., 2005; Wiegert et al., 2006; Groc et al., 2008). Here we report that corticosterone, also within minutes, increases the amplitude of NMDA receptor-mediated currents as well the area under the curve of NMDA currents. This agrees with a recent report indicating that exposure to corticosterone increases hippocampal NMDA receptor-mediated synaptic transmission within 0-30 minutes after administration of the hormone (Tse

et al., 2011). Our effects were absent in cells treated with the GluN2B antagonist Ro 25-6981 suggesting that corticosteroid hormones increase NMDA receptor function via regulation of the GluNR2B subunit. Accordingly, the relatively fast effects by corticosterone on receptor function were accompanied by a fast redistribution and increase in synaptically localized GluN2B-NMDAR, as observed with high-resolution at the single molecule level. This supports the view that corticosterone rapidly reorganizes synaptic NMDA receptors in hippocampal networks to promote synaptic alterations.

It remains to be determined how corticosterone rapidly regulates NMDA receptor function. Recent studies have shown that corticosterone within minutes affects AMPAR mediated synaptic function (Karst et al., 2005; Groc et al., 2008) via activation of mineralocorticoid receptors (MRs). In line with this, preliminary evidence suggests that the effects of corticosterone on NMDARs are also mediated by MRs (Laurent Groc, personal communication). The signalling pathway between (putative) MR activation and NMDAR mobility and/or function, however, still needs to be entirely resolved.

Taken together, recent data suggests that corticosterone via MRs and within minutes is able to regulate glutamatergic synaptic transmission via multiple pathways, at least in the hippocampus: via neurotransmitter release probability, AMPAR (particularly GluA2) mobility, NMDAR enrichment in the synaptic fraction and NMDA receptor function, the latter two mainly involving GluN2B. Importantly, the GluN2B subunit largely determines the calcium permeability of NMDA receptors (Lisman et al., 2012) and enhanced GluN2B expression facilitates LTP and memory formation (Tang et al., 1999). The presently observed increase in GluN2B function may therefore provide a molecular mechanism to explain how corticosterone, within minutes after administration, is able to facilitate long-term potentiation (Wiegert et al., 2005), possibly in concert with increased neurotransmitter release (Karst et al., 2005) and enhanced AMPAR mobility (Groc et al., 2008). It is tempting to speculate that these rapid changes in synaptic function and synaptic plasticity reflect adaptations in neuronal network to promote storage of relevant information and facilitate behavioural adaptation to stressful experiences.

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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.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine-specific protein kinase that is regulated by the Ca^{2+}/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

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.,

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).

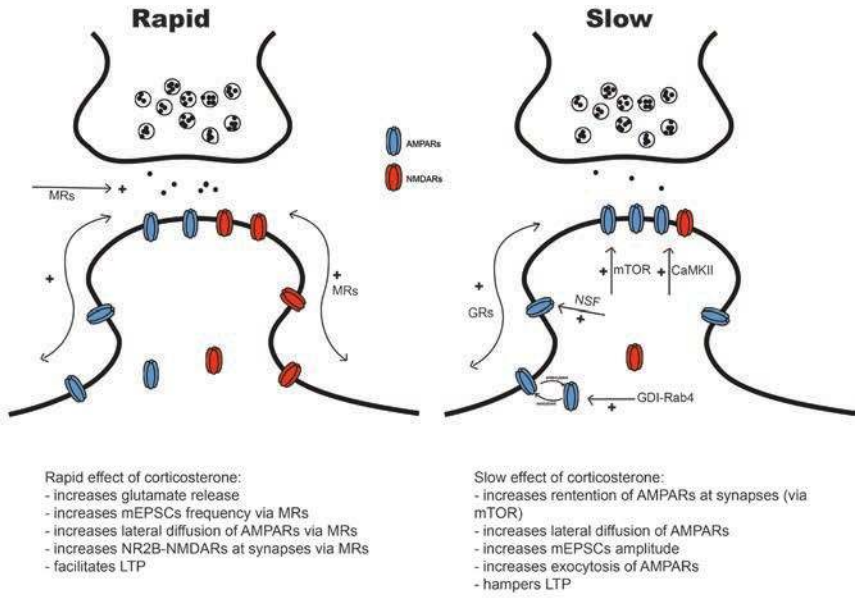
Corticosterone prevented activity-dependent synaptic plasticity when the hormone was applied hours before inducing plasticity. It has been hypothesized that this may be the result of corticosterone-induced occlusion of synapses which – via a metaplastic mechanism – prevents subsequent synaptic potentiation (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

(Groc et al., 2008), and increase GluN2B function (chapter 6). These effects are likely to be mediated by MRs and may all contribute to an enhanced ability to induce synaptic potentiation. Hours after administration of corticosteroid hormones, the amplitude of mEPSCs (Karst and Joëls, 2005; Martin et al., 2009; Xiong et al., 2015; 2016), AMPAR surface expression (Martin et al., 2009; Xiong et al., 2015; 2016), AMPAR lateral diffusion (Groc et al., 2008) and exocytosis and retention of AMPARs (Xiong et al., 2015; 2016) are enhanced. At this time point, the ability of synapses to express 'new' synaptic potentiation is hampered, favouring consolidation of earlier stress-related information. These effects are likely to be mediated by activation of GRs.



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

(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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Nederlandse Samenvatting

Samenvatting

Stressvolle gebeurtenissen worden over het algemeen goed onthouden. Dit komt in belangrijke mate tot stand door glucocorticoiden die worden afgegeven tijdens en na afloop van een stressvolle gebeurtenis. In dit proefschrift hebben we onderzocht hoe de effecten van het stress hormoon corticosteron op de communicatie tussen excitatoire cellen in hersenen en het geheugen tot stand komen. We hebben ons hierbij vooral gericht op de effecten van corticosteron op AMPA receptoren en NMDA receptoren en de vorming van contextuele aspecten van het angstgeheugen.

Hoofdstuk 1 geeft een kort overzicht van de literatuur over wat er bekend is over de effecten van glucocorticoiden op de communicatie tussen excitatoire hersencellen en het geheugen. In hoofdstukken 2-6 worden nieuwe experimentele studies beschreven waarin wordt bestudeerd; 1) hoe glucocorticoiden de communicatie tussen hersencellen en synaptische plasticiteit beïnvloeden, en 2) of dit van belang is voor het (angst) geheugen.

Van glucocorticoiden weten we dat ze synaptische transmissie en de functie van AMPA receptoren in verschillende hersengebieden stimuleren. Een belangrijke eigenschap van de hersenen (en netwerken in de hersenen) is het vermogen om activiteits-afhankelijke veranderingen te ondergaan die het mogelijk maken dat cellen en netwerken zich snel en langdurig kunnen aanpassen en waardoor informatie kan worden opgeslagen.

In hoofdstuk 2 werd onderzocht of (en hoe) glucocorticoiden, binnen minuten na hun toediening of langdurig nadat ze zijn toegediend, de plasticiteit van synaptische transmissie beïnvloeden. Daarvoor werd een protocol gebruikt om synaptische plasticiteit op te wekken (activering van NMDA receptoren door toediening van glycine en picrotoxine gedurende 3 minuten) en werd onderzocht hoe glucocorticoiden snel en langdurig de plasticiteit beïnvloeden. We vonden dat kort na 20 minuten toediening van corticosteron de activiteits-afhankelijke plasticiteit was vergroot doordat de frequentie van de mEPSCs was toegenomen. Indien langer werd gewacht was de amplitude van

de mEPSCs toegenomen en was de activiteits-afhankelijke toename in de amplitude van de mEPSCs afwezig (occlusie). Deze resultaten tonen aan dat 1) glucocorticoiden op korte termijn de activiteits-afhankelijke effecten op synaptische transmissie kunnen versterken, en 2) glucocorticoiden op langere termijn zorgen voor een toename in synaptische transmissie waardoor synaptische plasticiteit mogelijk wordt voorkomen. Voorlopige gegevens tonen aan dat de snelle effecten op activiteits-afhankelijke veranderingen in de frequentie van mEPSCs verlopen via de mineralocorticoid receptor (MR) terwijl de langzamere effecten op de amplitude van de mEPSCs verlopen via de glucocorticoid receptor (GR).

N-Ethylmaleimide-Sensitive Factor (NSF) is betrokken bij fusie processen met de (neuronale) membraan. De interactie tussen GluA2 en NSF is van belang voor insertie en stabilisering van AMPA receptoren in de membraan en voor het in stand houden van synaptische transmissie. Door gebruik te maken van peptiden die de interactie tussen NSF en GluA2 verstoren hebben we in hoofdstuk 3 onderzocht of de interactie tussen GluA2 en NSF essentieel is voor de effecten van glucocorticoiden op de expressie van AMPA receptoren in de membraan, AMPA receptor gemedieerde synaptische transmissie, de mobiliteit van AMPA receptoren en, uiteindelijk, de effecten van glucocorticoiden op het angstgeheugen. We vonden dat toediening van glucocorticoiden (3 uur) zorgt voor een toename van AMPA receptoren (GluA1 en GluA2 subunits) in de membraan, voor een toename in de mobiliteit van GluA2 bevattende AMPA receptoren, en voor een toename in de amplitude van AMPA-receptor gemedieerde mEPSCs. Deze effecten werden voorkomen door toediening van pep-R845A dat specifiek de interactie tussen GluA2 en NSF blokkeert. Deze studies suggereren dat glucocorticoiden voor een toename in AMPA-receptor gemedieerde synaptische transmissie zorgen via een mechanisme waarbij de interactie tussen GluA2 en NSF van belang is. In gedragsexperimenten werd gevonden dat toediening van pep-R845A direct na training in een contextuele angstconditioneringstaak zorgt voor een toename in freezing gedrag 24 uur na die training. Glucocorticoiden zorgen ook voor een toename van het angstgeheugen. Het peptide R845A voorkwam het geheugen bevorderende effect van glucocorticoiden.

Om verder te bestuderen hoe glucocorticoiden de functie van AMPA receptoren en het angstgeheugen beïnvloeden hebben we de rol van mammalian Target of Rapamycin (mTOR) – wat betrokken is bij translatie, synaptische plasticiteit en geheugen - bestudeerd. Met behulp van elektrofysiologie, immunocytochemie, live cel imaging en contextuele angstconditionering hebben we de rol van dit eiwit bestudeerd bij de effecten van glucocorticoiden op AMPA receptoren en het angstgeheugen. Corticosteron zorgde voor een toename in de amplitude van AMPA receptor gemedieerde synaptische transmissie. Dit effect had tijd nodig om zich te ontwikkelen en verliep via glucocorticoid receptoren (GR) en eiwitsynthese. Corticosteron zorgde verder voor een toename in de mobiele fractie van AMPA receptoren, voor een toename van AMPA receptoren in de celmembraan, en voor een afname in de diffusie coëfficiënt. Het effect van corticosteron op AMPA receptor gemedieerde synaptische transmissie en de diffusie van AMPA receptoren werd voorkomen door rapamycine (dat de mTOR cascade remt). Deze studie toont aan dat de mTOR cascade betrokken is bij specifieke regulatie van AMPA receptoren door glucocorticoiden. Daarnaast vonden we dat glucocorticoiden het angstgeheugen bevorderen. Ook dit effect werd voorkomen door remming van de mTOR cascade. Daarmee laten deze experimenten zien dat glucocorticoiden, via binding aan GR, zorgen voor een toename in AMPA-receptor gemedieerde synaptische transmissie, AMPA receptor mobiliteit, en voor retentie van AMPA receptoren via de mTOR cascade. Deze mechanismen verklaren mogelijk waarom glucocorticoiden het angstgeheugen versterken.

Ca2+/calmodulin-dependent protein kinase II (CaMKII) is een serine/threonine-specifiek kinase dat via het Ca2+/calmodulin complex wordt gereguleerd. CaMKII is betrokken bij vele signaal cascades in neuronen en is betrokken bij synaptische plasticiteit en geheugen. CaMKII is ook betrokken bij de geheugen bevorderende effecten van glucocorticoiden. In hoofdstuk 5 hebben we daarom bestudeerd of CaMKII ook betrokken is bij de effecten van glucocorticoiden op AMPA receptoren. Door middel van elektrofysiologische technieken en immunocytochemie hebben we bestudeerd of CaMKII betrokken is bij de effecten van glucocorticoiden op AMPA receptor mEPSCs en de membraan expressie van AMPA receptoren. Onze resultaten lieten

zien dat glucocorticoiden zorgen voor een toename in AMPA receptor gemedieerde synaptische transmissie en een toename in de expressie van AMPA receptor subunits in de membraan. Toediening van een remmer van CaMKII 30 minuten voor en tijdens de toediening van corticosteron voorkwam het effect van corticosteron op AMPA receptor gemedieerde synaptische transmissie en AMPA receptor expressie. Dit effect werd ook gevonden indien de remmer werd toegediend gedurende de laatste 30 minuten waarin corticosteron werd toegediend. Deze resultaten laten zien dat CaMKII betrokken is bij de effecten van glucocorticoiden op de functie van AMPA receptoren.

De NMDA receptor is essentieel voor activiteits-afhankelijke veranderingen in synaptische transmissie en voor geheugenvorming. In hoofdstuk 6 hebben we daarom bestudeerd of glucocorticoiden de functie van NMDA receptoren beïnvloeden. Door middel van elektrofysiologische technieken hebben we veranderingen in NMDA receptor gemedieerde mEPSCs geregistreerd na toediening van corticosteron aan hippocampale primaire cultures. We vonden dat corticosteron, binnen minuten na toediening, zorgde voor een toename in de amplitude van NMDA receptor gemedieerde mEPSCs, en voor een toename in de oppervlakte van de stromen. Deze effecten werden voorkomen door een GluN2B antagonist toe te dienen. Deze resultaten suggereren dan glucocorticoiden, via GluN2B, zorgen voor een toename in de functie van NMDA receptoren. In overeenstemming hiermee heeft Laurent Groc (Bordeaux) gevonden dat corticosteron zorgt voor een toename in mobiliteit van de GluN2B subunit (niet gepubliceerde waarnemingen). Deze studies suggereren dan glucocorticoiden zorgen voor een toename in de functie van NMDA receptoren.

Conclusie:

In de introductie van dit proefschrift werden enkele vragen gesteld. De antwoorden op deze vragen kunnen als volgt worden samengevat:

- Corticosteron reguleert de activiteits-afhankelijke veranderingen in AMPA receptor functie,
- De interactie tussen GluA2 en NSF is van belang voor de effecten van corticosteron op AMPA receptor functie en mogelijk ook voor de effecten van corticosteron op de

vorming van het angstgeheugen,

- mTOR is belangrijk bij de effecten van corticosteron op de functie van AMPA receptoren, de mobiliteit van AMPA receptoren en de vorming van het angstgeheugen,
- CaMKII is nodig voor de effecten van corticosteron op de functie van AMPA receptoren,
- De functie van NMDA receptoren wordt gereguleerd door corticosteron.

Appendices

Dankwoord
List of publications
About the author

Dankwoord/Acknowledgement

I knew it would be a great adventure when I decided to come to Amsterdam in 2010, especially for a boy grew up in a little village never been seeing the world outside of China before. A lot happened during these past years, and as I imagined, it was an adventure indeed. And I' m so happy that finally I' m done with my PhD study since 2010.

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Hui Xiong 熊晖
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List of Publications

Xiong H, Cassé F, Zhou M, Xiong Z-Q, Joëls M, Martin S, Krugers HJ. 2016. Interactions between N-Ethylmaleimide-Sensitive Factor and GluA2 contribute to effects of glucocorticoid hormones on AMPA receptor function in the rodent hippocampus. Hippocampus:n/a–n/a.

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Hui Xiong, Marian Joëls, Harm J Krugers. Glucocorticoids regulate hippocampal AMPA receptor function via activation of Calcium-calmodulin dependent Kinase II. *In preparation*

Lenka Mikasova, **Hui Xiong**, Amber Kerkhof, Delphine Bouchet, Harm J Krugers3and Laurent Groc. Stress hormone acutely controls GluN2B-NMDAR surface dynamics through a membrane mineralocorticoid receptor. *In preparation*

About the author

Hui Xiong (熊晖) was born on the 12nd of January 1986 in a small village of Taojiang (桃江县) Hunan (湖南省) China. He finished his bachelor study in Hainan University in 2009, and in the summer of 2008 he went to the Lab of Neurobiology of Disease, Institute of Neuroscience, Chinese Academy of Science, Shanghai working as an intership student and researcher untill to May of 2010, supervised by Dr. Xiong Zhiqi. Since June of 2010, Hui Xiong started working in the Swammerdam Institute for Life Sciences, University of Amsterdam. In May of 2011, he got an exemption of his master and started to work as a PhD student in University of Amsterdam, supervised by Dr. Harm Krugers and Professor Dr. Marian Joels. Since June of 2015, he got an extension till to the end of November 2016.