Rapid corticosteroid actions in the hippocampus and amygdala

The relevance of timing

Natasha Pasricha

Cover: Proefschrift-AIO.nl
layout: Proefschrift-AIO.nl
Printed by: DPP

ISBN / EAN: 978-90-393-5943-3

Rapid corticosteroid actions in the hippocampus and amygdala

The relevance of timing

Proefschrift
ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 15 mei 2013 des ochtends te 10.30 uur

door

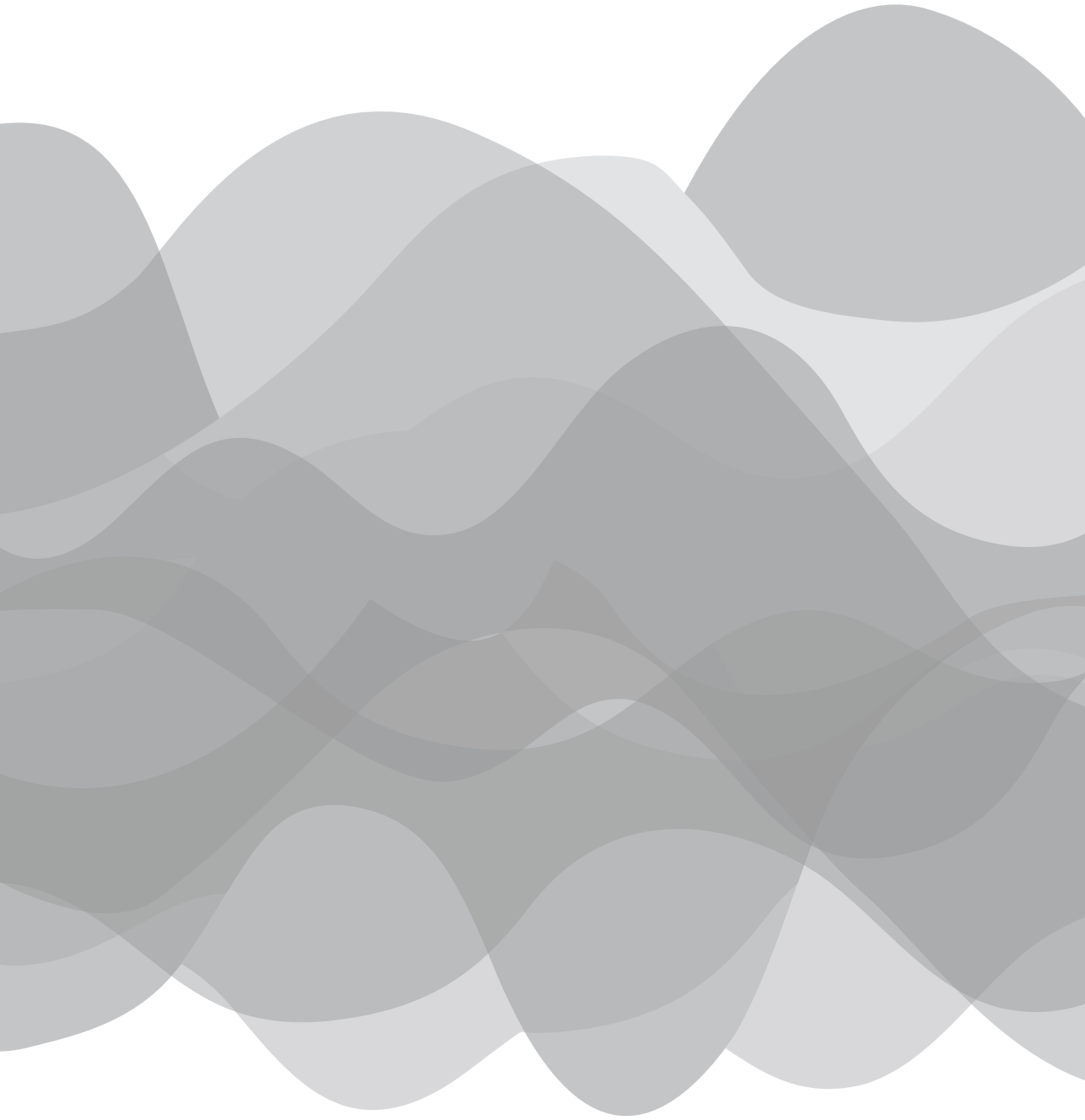
Natasha Pasricha
geboren op 17 augustus 1984
te Yamuna Nagar, India

Promotor: Prof.dr. M. Joëls

Co-promotor: Dr. H. Karst

The research was financially supported by NWO (The Netherlands Organization for Scientific Research) grant 817.02.017.

Table of contents



CHAPTER 1

Introduction

1. Stress

1.1 Stress is 'Good'

Stress is part of our daily lives. It can be induced via environmental factors like loud noise or crowded places, physical situations like intense sports, injury or psychological conditions like social embarrassment or an approaching deadline. The subjective perception of these situations, i.e. stress, activates two main neuroendocrine pathways: the autonomic nervous system (ANS) and the hypothalamo-pituitary-adrenal (HPA) axis which both are aimed at maintaining or reinstating homeostasis (1); (2).

1.2 Brain response to stress

After stress exposure, the locus coeruleus (LC) - where the majority of noradrenergic neurons are located- is activated. These neurons project to the prefrontal cortex (PFC), cerebellum, hippocampus and amygdala (3). In the peripheral nervous system adrenaline is released which indirectly innervates the basal lateral amygdala and LC (4).

HPA axis activation leads to the release of glucocorticoids from the adrenal glands (see Box 1).

The main actors of the HPA axis, glucocorticoids, have many important functions including regulation of glucose, fat and protein metabolism and anti-inflammatory actions, and can affect mood and cognitive functions (6) (7). Under resting conditions, glucocorticoids facilitate the storage of energy supplies as readily available glycogen deposits. Hence the name 'glucocorticoids'. The main glucocorticoid in humans is cortisol; in rodents corticosterone [N.B. In the remainder of this thesis I will use the term 'corticosteroids' rather than 'glucocorticoids']. Corticosteroid hormones are not only released in high amount after stress, but also show circadian and ultradian rhythmicity (see section 2). Due to their lipophylic properties, circulating corticosteroids in the blood can easily cross the blood brain barrier and enter the brain. This affects brain function especially in regions rich in corticosteroid receptors like the hypothalamus, hippocampus, amygdala and PFC. Corticosteroids also cause negative feedback at the level of the hypothalamus and pituitary, to reduce the release of CRH and ACTH (and thus of corticosteroid hormones themselves). The hippocampus, PFC and amygdala are also involved in regulation of the HPA axis (see Box 2).

Box 1: The stress response

Activation of the ANS represents the classic 'fight or flight' response that was first characterized by Walter Cannon and colleagues in the early twentieth century. It leads within seconds to elevated circulating levels of adrenaline primarily from the adrenal medulla, and noradrenaline from sympathetic nerves. This triggers increases in heart rate, force of muscle contraction, peripheral vasoconstriction, and energy mobilization.

The HPA axis is activated a few minutes after the ANS, and eventually leads to elevated levels of circulating glucocorticoids from the adrenal cortex. Stress exposure activates paraventricular nucleus (PVN) neurons in the hypothalamus that secrete releasing hormones, such as corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the portal circulation from the median eminence. These releasing hormones induce the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH in turn acts on the inner adrenal cortex (that is, the zona fasciculata) to initiate the synthesis and release of glucocorticoid hormones (corticosterone in rats and cortisol in humans). The adrenal cortex is also directly innervated by the sympathetic nervous system, which can regulate corticosteroid release. The HPA axis and sympathetic system interact and work together during stressful situations (5).

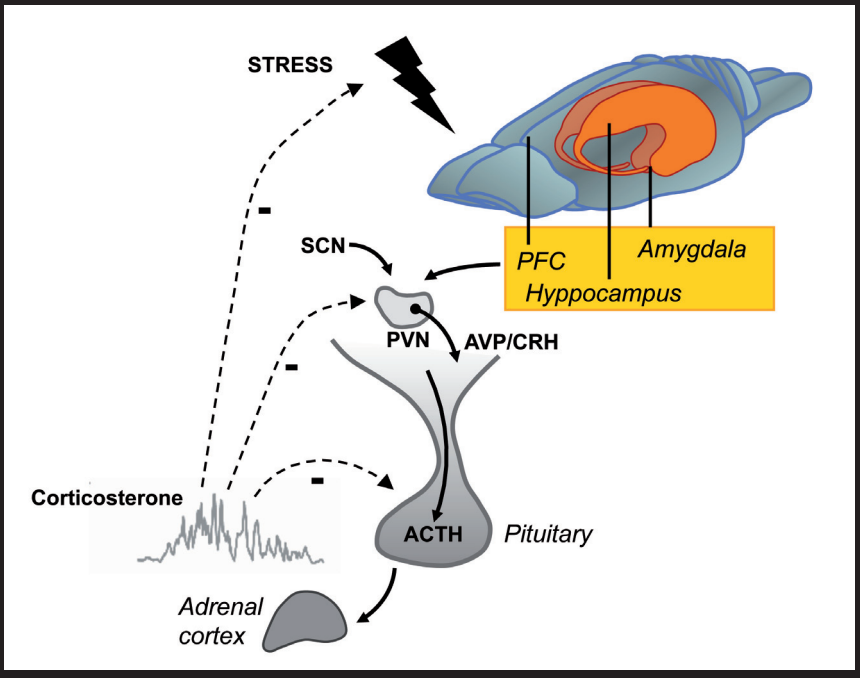


Figure 1: On exposure to stress, the hypothalamus is triggered to release AVP/CRH which induces the anterior pituitary to release ACTH. This in turn acts on the adrenal cortex to release corticosterone. Corticosterone exerts a negative feedback on the hypothalamus and higher brain regions. Due to a delay in the feedforward-feedback loop between the pituitary / brain and the adrenal cortex, corticosterone is released in hourly pulses.

Box 2. Central regulation of the HPA axis

Previous studies link the hippocampus to inhibition of the HPA axis (8)(9). This is for instance evident from the decrease and increase of both corticosterone and CRH upon hippocampal stimulation and lesion respectively (10).

Hippocampal regulation of the HPA axis is region- and stressor-specific. The presence of two types of corticosteroid receptors in the hippocampus (see below) renders this structure responsive to both basal and stressful conditions. Lesions of the hippocampus result in increased corticosterone release following psychogenic but not systemic stressors (11)(12) This implies a context-specific modulation of stress responses by the hippocampus.

The hippocampus also influences autonomic tone. Stimulating the hippocampus decreases heart rate, blood pressure and respiratory rate in awake rats. These effects are blocked by lesions of the medial prefrontal cortex (mPFC). The PFC seems to be less responsive to low corticosterone levels and more responsive during stressful situations.

In contrast to the hippocampus, the amygdala appears to excite the HPA axis. Several studies have established that lesions of the medial and central amygdala result in reduced corticosterone secretion (13) while stimulation of these areas increases the level of stress hormones (14). The medial amygdala was shown to be activated during anticipatory responses and is inactive during reactive episodes of stress (15). A study from Rao et. al suggested that increased levels of corticosterone at the time of acute stress protect against the delayed enhancing effect of stress on BLA synaptic connectivity and anxiety-like behavior (16).

1.3 Corticosteroid receptors in brain

Nuclear receptors

For many years it was assumed that the glucocorticoid receptor (GR) is the only receptor mediating effects of corticosteroid hormones in the brain (17). It has a relatively low affinity, so that it is only partly activated when circulating levels of corticosteroid hormones are low, but becomes substantially occupied after stress or at the circadian peak (18)(19). This receptor is abundantly expressed in nearly all parts of the brain, both in neurons and glial cells. Very high expression levels have been described e.g. for the PVN and the hippocampal CA1 area and dentate gyrus (20) (21).

With the use of selective ligands it became evident in (18) that some parts of the brain express a second receptor-type, which has a high affinity for the mineralocorticoid aldosterone and has the same characteristics as the mineralocorticoid receptor (MR) in the kidney. Unexpectedly this receptor was also found to have a very high affinity for corticosterone and cortisol, in fact a 10-fold higher affinity than GRs (18) (22). In the kidney, corticosterone / cortisol will not bind the MR because these hormones are converted by the enzyme 11β-HSD2, so that the less prevalent hormone aldosterone can bind the receptor (23). In most parts of the brain, however, 11β-HSD2 is hardly expressed, so that corticosterone / cortisol is the main endogenous ligand of the MR. Particularly neurons in all hippocampal subfields, the lateral septum and to a lesser extent some of the amygdala nuclei and cortical layers express MRs.

Both MR and GR belong to the family of nuclear receptors. These receptors act as transcription factors (24) (25) (26) (27). Upon binding of corticosteroids to MR or GR located in the cytoplasm, the receptor-ligand complex moves to the nucleus where it either binds as a homodimer to recognition sites in specific gene promoters or interacts with other transcription factors, thus changing the expression of responsive genes in a slow and persistent manner (28).

Rapid non-genomic actions are not mediated by nuclear receptors

Until a decade ago, corticosteroid hormones were thought to affect brain function mostly through transcriptional regulation, as pointed out above. However, it has increasingly been demonstrated that all steroids, including corticosteroids, can exert their actions also by rapid non-genomic pathways. Different steroids have different non-genomic actions with different underlying mechanisms. It is assumed that non-genomic glucocorticoid effects are mediated by one of three mechanisms: (1) physicochemical interactions with cellular membranes (non-specific non-genomic effects); (2) membrane-bound glucocorticoid receptors (mGR) mediating non-genomic effects; and (3) cytosolic glucocorticoid receptors (cGR) mediating non-genomic effects (29). Although this was formulated for GRs, it most likely is also true for MRs.

Non-genomic effects of corticosterone in the hippocampal CA1 area were found to be mediated by membrane located MRs, causing an increase in glutamate release probability pre-synaptically and a reduction in potassium A-currents post-synaptically. The former was demonstrated by an increase in mEPSC frequency (30)(31). Moreover, corticosterone application on hippocampal slices reduced paired-pulse facilitation, pointing to a presynaptic mechanism of action (30). In addition, corticosterone rapidly changes post-synaptic glutamatergic transmission, by increasing the AMPAR surface diffusion in hippocampal neurons (32). **Whether such rapid corticosteroid actions also take place in other hippocampal subfields was unknown at the start of my project. This will be addressed in Chapter 2.**

However, rapid effects in the hippocampus can also involve GR. For instance, a non-genomic increase in spine density of hippocampal neurons was found to take place via GRs rather than MRs (33). Another study (34) on rapid corticosterone effects did not find evidence for the involvement of either the MR or GR and therefore could point to a novel (so far not identified) membrane-localized receptor. This study showed that corticosterone in the presence of NMDA rapidly enhances NMDA neurotoxicity. This was shown to occur via a non-genomic mechanism, not fitting the profile of MR or GR (34). Other studies reported inhibitory rather than excitatory actions of corticosterone on NMDA signaling ((35) (36). Apparently, hippocampal signaling is rapidly affected by corticosterone in multiple ways, involving membrane-located MRs, GRs or other, still unknown receptors. The existence of an unknown receptor with properties different from MR and GR was previously suggested by biochemical studies (37).

Fast effects of steroids also take place in other brain regions. For instance, (38) showed that in the PVN corticosteroids rapidly cause the release of endocannabinoids from the target cell. These endocannabinoids act locally and pre-synaptically on the excitatory glutamatergic axon. Endocannabinoids released from target neurons reduce the frequency of glutamate-mediated excitatory post-synaptic currents (EPSCs) by 25–40%, acting through cannabinoid CB-1 presynaptic receptors

on incoming glutamate axons. This mechanism is thought to contribute to fast-feedback of the HPA-axis by corticosterone (39)

Membrane receptors

The receptors responsible for non-genomic effects in brain cells are thought to be localized on the plasma membrane. This is partly based on the observation that application of corticosterone-BSA and dexamethasone-BSA, molecules that do not pass the plasma membrane, yielded similar fast effects as corticosterone and dexamethasone (40)(41) (34). The presence of both MR and GR at neuronal membranes was further substantiated by electron microscopy (42)(43). This showed enriched expression of MR and GR at post-synaptic terminals, but also pre-synaptically(43).

Membrane-bound steroid receptors are known for most steroids: Estrogens, androgens, progesterone as well as glucocorticoids (44) (45) (46). The membrane translocation pathways for ERα, GR and MR have been compared and hypothesized to be similar in their involvement of caveolin-1, palmitoylation and/or HSP27 (47). Interestingly, the palmitoylation motif is known to be conserved in many steroid receptors like ERα, ERβ, PR, AR and GR but not in MR (45). This means that MR could be palmitoylated at another sequence or has another pathway for translocation to the membrane

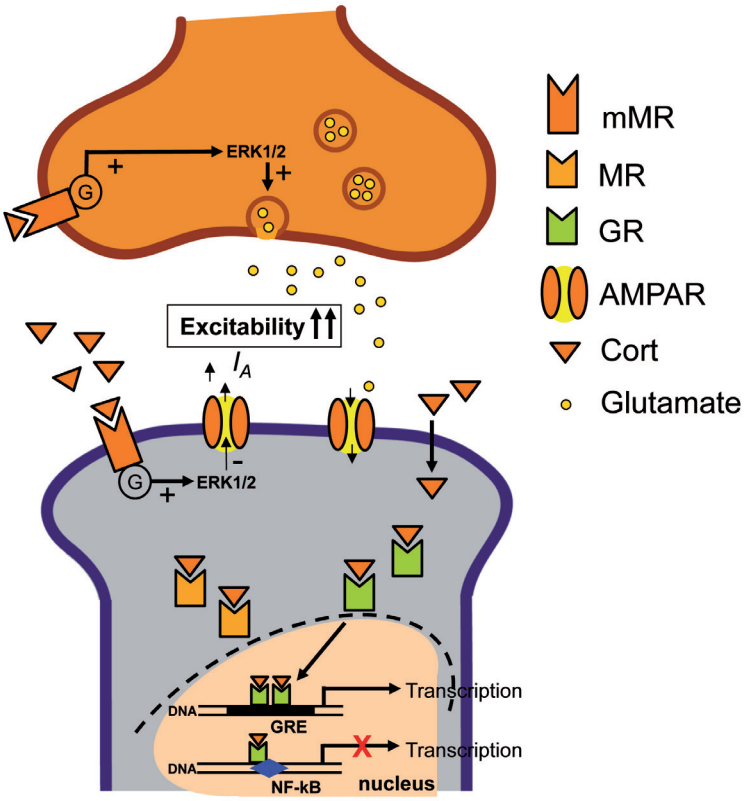


Figure 2: Rapid effects of corticosterone both pre- and postsynaptically. Corticosterone binding to membrane mineralocorticoid receptors 1) presynaptically increases glutamate release probability and 2) postsynaptically decreases potassium A-currents. Both lead to increases in excitability of the cell.

2. Pulsatility

2.1 Circadian and ultradian rhythm in HPA axis - comparison with pulsatile release of other hormones

Biological rhythms are an important component of neuroendocrine systems which play a role in the regulation of reproductive function (48), food intake (49)and adaptation (Lightman S., (50) (19). It has been known for decades that corticosterone is released in a circadian rhythm, which is a cycle of approximately 24 hrs in all bodily processes like biochemical, physiological, endocrine or behavioral function, in all mammals (51)(52). In man, the circadian peak of cortisol is at the end of the resting phase, i.e. in the early morning around awakening. In rodents, the peak of corticosterone is in the late afternoon, at the beginning of the dark phase, to prepare the organism for increased metabolic demand during its active phase. The circadian rhythm in HPA axis is important for physiological functions; disruption in this pattern makes an organism prone to stress-related diseases ((53) (1) (54).

From recent studies mostly by the group of Lightman, it is now known that corticosterone is in fact released in hourly pulses (Box 3), with increasing pulse amplitudes in the light phase and decreasing pulse amplitudes in the dark phase. Joining the peak of these pulses yields the circadian rhythm. This so-called ultradian pattern of corticosteroid release was shown to be present in all the mammals including rodents ((55) (56) rhesus monkeys (57) (58), sheep (59) and humans (60) (61) (62).

Box 3. Ultradian pulses in brain

Using microdialysis, high frequency blood sampling of corticosterone is possible every few minutes. This method allowed the observation of hourly pulses of corticosterone in blood plasma. It was shown in different rodent species. Later on, some gender differences in total corticosterone levels were shown but no differences were illustrated in unbound corticosterone between males and females. To investigate the presence of these hourly fluctuations of glucocorticoid in the brain, microdialysis was done in various brain regions like the hypothalamus, hippocampus, PFC and caudate-putamen. The finding of fluctuations in these areas provided evidence that ultradian pulses are preserved over the blood-brain barrier.

The brain is exposed to ultradian pulses. The physiological response of nuclear or membrane receptors to glucocorticoid pulses are not well investigated. The nuclear MR, due to its high affinity, is always occupied while nuclear GR is occupied only when corticosterone levels are at the peak of the ultradian pulses and during stress (63). Due to the 10 times lower affinity of membrane MRs (mMR) than nuclear MRs, mMRs are occupied when corticosterone levels rise, similar to nuclear GR. This suggests that the main effect of stress may be mediated via nuclear GR and membrane-located MR. It would be interesting to know how these two responses interact and influence the physiological response.

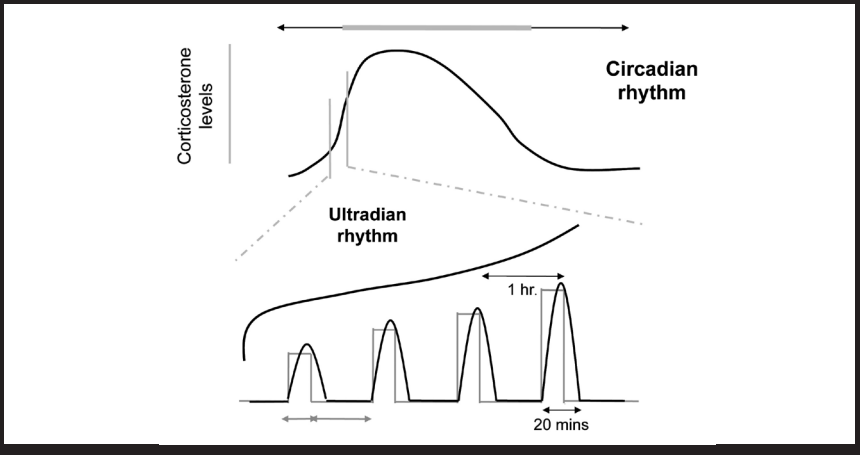


Figure 3: Corticosterone is released in a circadian pattern which is composed of hourly pulses (ultradian rhythm) with a pulse duration of 20 minutes.

Note: One can mimick such physiological pulses (black) experimentally by delivering brief block-pulses (grey). This design was used in Chapters 3 and 4.

The oscillations in hormone levels are important for optimal efficiency. For instance, growth hormone secretary patterns elicit significant sexual dimorphic effects on gene expression(64) while continuous administration attenuates growth (65). Gonadotropin-releasing hormone pulses influence secretary patterns of luteinizing hormone and follicle stimulating hormone, and prevent receptor

desensitization (66) (67) (68). Also insulin is more efficient when given in a phasic pattern rather than tonically (69). Hormones delivered in pulses prevent desensitization or down-regulation of receptors and are thought to be physiologically more efficient (70).

The impact of corticosteroid ultradian pulses on nuclear GRs has been studied by molecular techniques, again showing the strongest effects on gene expression with pulsatile rather than continuous hormone administration (71). Yet, **the relevance of ultradian pulses for membrane-receptor mediated signaling –via MR or GR- was entirely unknown at the start of my project. I addressed this in Chapters 3 and 4 of my thesis.**

2.2 Mathematical model of pulsatility

The mechanism of ultradian pulse generation has not yet been resolved physiologically. Some secretary patterns are intrinsic, like in the hypothalamus (72) (73). Such a self-generating mechanism of pulses was also shown to be present in the adrenal glands (74) (75). However, it was shown that e.g. the suprachiasmatic nucleus is not the center of corticosteroid pulse generation, as corticosterone pulses are maintained even after lesion of this nucleus (76) (77).

Recently, a mathematical model was proposed explaining the ultradian corticosteroid pulses(78). An important element of this model is the delay between on the one hand the feed forward loop of ACTH to the adrenals and on the other hand the feedback loop of corticosterone to the pituitary / hypothalamus. At the level of the adrenal glands, the dynamic levels of corticosterone, ACTH and GR together with the relatively slow corticosterone synthesis cause the delay of corticosterone release in response to ACTH. This delay was shown to be crucial for the generation of hourly pulses. The computational model assumed that the rapid inhibition of hypothalamic CRH by corticosteroids is not an important factor but that, rather, the major sites for feedback are the anterior pituitary (79) and the slow feedback on CRH gene transcription (80). In this model, the ultradian pulse is an intrinsic property of the pituitary-adrenal system because they still occur with a constant CRH drive, though CRH levels should be within a certain range.

This model also incorporated GR dynamics in the anterior pituitary (81): Application of a GR antagonist increases the amplitude during the peak of circadian CRH drive, with a minor increase in frequency. This has indeed been shown experimentally in a rat study where 5 days of GR antagonist administration was found to elevate levels of corticosterone (82). Therefore, these oscillations are modified by the CRH 'gain' to the pituitary, which can be modified by supra-pituitary feedback mediated via GR (78).

2.3 Plasticity in glucocorticoid release pattern

The glucocorticoid ultradian pattern changes under different physiological conditions. It has not been resolved whether the change in pattern leads to pathology or vice versa, or even that the changes in pulsatility are a mere epiphenomenon. The nature of these changes in pulse characteristics may be genetically determined or imposed by life events, e.g. early life stress. Diseased conditions associated with altered ultradian patterns comprise: Parkinson's disease, inflammatory diseases like arthritis, depression and anxiety disorders (83) (84) (85). More specifically, depression in humans is associated with a flattened circadian rhythm due to increased pulse magnitude particularly

during the circadian trough (86) (87) (53). Not only amplitude but also frequency of the pulses may be changed in disease: Induction of arthritis in rats was associated with an increase in pulse frequency and changes in HPA activation during acute psychological stress(88).

3. Glutamate receptors as target for rapid corticosteroid actions

3.1 Glutamate receptors and their importance in health and disease

Glutamate is the major excitatory neurotransmitter in the brain. Targeting this neurotransmitter system would be a highly effective manner by which rapid corticosteroid actions could profoundly alter brain function. At the start of my project there was already evidence that corticosteroids can indeed quickly change glutamatergic transmission, at least in the hippocampal CA1 area (30).

There are two main ionotropic glutamate receptors, i.e. N-methyl-D-aspartic acid receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPAR). AMPARs are known to play a role in depression and mood disorders (89) (90). For instance, the conventional antidepressant fluoxetine is known to increase AMPAR signaling by altering AMPAR phosphorylation.

Given the key role of AMPARs in health and disease, it is interesting to look into changes in AMPAR function after exposure to corticosterone or stress. As mentioned above, stress and/or corticosterone rapidly change AMPAR properties in hippocampal neurons. This was shown both by electrophysiology methods (30) and in a real-time imaging study, demonstrating an increase in lateral diffusion of AMPARs (32).

NMDAR lateral diffusion was not rapidly influenced by corticosteroids (32) but can be influenced by activation of protein kinase C (91). Rapid action of corticosterone was also shown to suppress NMDA activity which is due to activation of downstream cascade like phospholipase C and protein kinase C (92). Recently, rapid antidepressant-like effects like alleviating anhedonic and anxiolytic behaviour together with synaptic deficits were observed via ketamine-mediated NMDAR blockade or selective NMDA receptor NR2B antagonist, Ro 25-6981 (93).

3.2 AMPA receptor trafficking and synaptic plasticity

AMPARs are heteromeric tetramer complexes (94) formed by different combinations of the subunits GluA1 to 4 (95) (96)(97). Generally, heterodimers of GluA1/GluA2 and GluA2/GluA3 subunits are more abundant than homomeric GluA1 and GluA1/GluA3 AMPAR subunits in adult hippocampal neurons (98). Immature hippocampus and other brain regions are known to form complexes of GluA4 with GluA2 into a receptor (99). The extracellular and transmembrane regions of the various AMPAR subunits are very similar whereas their intracellular tails, which interact with specific cytoplasmic proteins, are distinct (100). These interacting cytoplasmic proteins, involved in the actin cytoskeleton and stabilization of AMPARs at the synapse, may be very important for trafficking (101).

AMPAR subunits are synthesized and assembled in the neuronal cell body (102) and pass through various transport steps to dendrites (103). Once in dendrites, shuttling of AMPAR from and to the synaptic membrane is regulated by two main processes, as shown in the figure 4: 1) recycling between intracellular and membrane

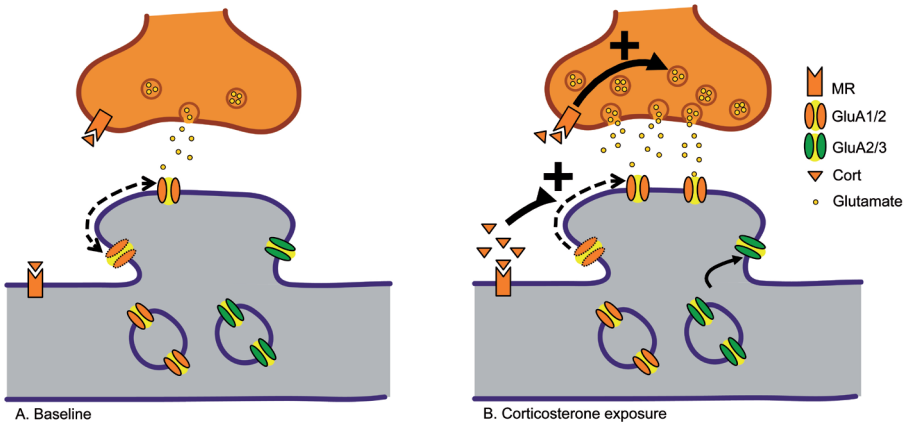


Figure 4: On exposure to corticosterone, glutamate release probability increases pre-synaptically and at the postsynaptic site, AMPAR lateral diffusion increases (B) compared to the baseline condition (A).

receptor pools by exocytosis/endocytosis (104) (105); 2) surface diffusion from extra-/ peri-synaptic regions to the synaptic receptor pool (106) (107) (108) (109). Diffusion time does not change with increase in binding sites at the synapse as it is dependent on number of receptors at extra-/peri-synaptic regions and receptor encountering scaffold proteins; dwell time depends on interaction with binding proteins at the synapse. This phenomenon of stabilization of diffusing receptor due to interaction with binding proteins is known as the 'diffusion trap' (110). The continuous exchange of receptors at the synapse is important for its function, for instance during recovery from LTD where desensitized receptors are exchanged with functional receptors (111).

Corticosterone has time-dependent effects on AMPAR function and mobility (112). The delayed genomic effect of corticosterone leads to an increased amplitude of AMPAR-mediated synaptic currents in hippocampal slices and cultures (41) (113). This could be explained by a recently described GR-dependent increase in the membrane expression of GluA2-containing AMPARs (32)(113). Interestingly, both chemical LTP and corticosterone application seem to target the same process, so that induction of the one occludes the development of the other (32). In a rapid and non-genomic fashion, corticosterone application leads to an increase in mEPSC frequency. Rapid corticosteroid effects on glutamatergic transmission are also expressed as an increased lateral diffusion of GluA2-AMPAR; no increase in receptor number was shown to occur. Both rapid effects on AMPAR-mediated transmission were accomplished via MRs present in the membrane, as was proven by pharmacological means. These studies describing how corticosterone quickly alters AMPAR-mediated mEPSC frequency and mobility so far only involved a single application of corticosterone. However, as argued in section 2, endogenous levels of corticosterone vary in an ultradian manner. **We therefore also examined in this thesis (Chapters 3 and 4) how hourly pulses of corticosterone change AMPAR surface expression and diffusion as well as the properties of mEPSCs mediated by these receptors.**

Glutamate receptor function is highly relevant for behavioral performance.

In view of the non-genomic and genomic actions of corticosteroids on glutamate receptor function, it doesn't come as a surprise that the hormone profoundly affects various types of behavior. For instance, it was shown that MRs are important in the quick appraisal of information and response selection under stressful conditions (114) (115). Conversely, GRs are known to promote long-term consolidation (116)(117)(118). In addition, membrane GRs were shown to promote long-term memory in an object recognition task, via chromatin modification (119).

3.3 NMDA receptor subunit expression and glucocorticoid exposure

NMDA receptors are heteromers composed of two obligatory GluN1 subunits and two regulatory GluN2/3 subunits. The transmembrane domain forms a channel pore with high calcium permeability and voltage dependent magnesium block. Influx of calcium via the NMDA receptor is one of the primary sources of this ion in neurons, additionally to voltage-gated calcium channels. The NMDA receptor is an exclusive ligand gated ion channel. It is activated by glutamate along when the membrane is depolarized to unblock magnesium and increase the probability of opening of the channel (120).

On exposure to stress or corticosterone, NMDA receptor-mediated Ca2+ signals were reported to be rapidly elevated (35). Activation of this receptor is involved in modifying synaptic plasticity after behavioral stress (121). Stress (particularly when chronic) increases NMDAR levels and currents in hippocampal neurons (122) (123), (124) (125). In addition, stress-induced hippocampal atrophy involves NMDARs, because atrophy was shown to be prevented by NMDA blockers (126). Chronic glucocorticoid exposure was found to up-regulate mRNA levels of both NR2A and NR2B subunits in hippocampus (127)(126). This is of relevance, because the ratio between NR2A and NR2B subunits determines the direction of synaptic plasticity (128).

NMDA receptors also have a role in influencing AMPA receptor trafficking (129), providing a possible mechanism by which chronic stress may influence AMPA receptor trafficking indirectly, i.e. by a changed NMDA subunit composition.

4. Interactions between corticosterone and noradrenaline

So far, I have only discussed putative effects of corticosteroid hormones in the brain, particularly in the hippocampus. However, the response to a stressful situation involves actions by corticosterone in concert with various other neurotransmitters like serotonin, neuropeptides, endocannabinoids, dopamine and noradrenaline (130) (131) (132) (133) (134). The overall effect of stress thus not only depends on the response of neurons to these stress-related compounds separately but also on their interactions (135)(112).

In hippocampal neurons, the combined action of the β-adrenergic receptor agonist isoproterenol and corticosteroids affect glutamate transmission and AMPAR surface expression in a complex manner (136). The combined effect of moderately high concentrations of the two hormones leads to optimal synaptic strengthening and further memory enhancement, yet beyond these concentrations the joined effect declines (137). Moreover, the two hormones only synergize when they are present within a certain time-frame. When corticosterone is given prior to isoproterenol it prevents or suppresses the effectiveness of isoproterenol (138).

In the hippocampus, interactions between noradrenaline and corticosterone at the cellular level have only been addressed in a few studies. This is also true for neurons in the basolateral amygdala, an area of great interest because it expresses moderately high amounts of corticosteroid receptors and is a prime terminal region of noradrenergic projections. It is well known that emotional arousal releases noradrenaline in the amygdala and that corticosteroids interact with noradrenaline to enhance memory. Human studies have shown that concurrent noradrenergic activity and stress induce memory enhancement for emotionally arousing experiences which involve the amygdala (139) (140) (141) (142). Noradrenaline seems to be the main actor in these effects, with modulatory actions exerted by corticosteroids. Thus, in vivo microdialysis studies in amygdala have shown that noradrenaline levels rise immediately after stress whereas corticosteroid levels were elevated only 20 minutes after onset of stress exposure (143) (144). A β-adrenoreceptor antagonist infused in the BLA blocked memory enhancement induced by corticosteroids given systemically or directly into BLA (145) (146).The effects on emotional memory may be due to interactions of the two hormones at the postsynaptic level in the BLA, but could also be caused by corticosteroids modulating the bio-availability of noradrenaline. In agreement with the latter, administering a memory enhancing dose of corticosteroids after inhibitory avoidance training rapidly increased noradrenaline levels within amygdala (147) and the levels of noradrenaline positively correlated with retention performance 24 h later. Corticosterone-induced release of noradrenaline is rather rapid (abour 15 mins), suggesting that their interaction is mediated by non-genomic mechanisms.

However, there is also evidence for interactions at the post-synaptic level. Biochemically, corticosterone increases the efficacy of the beta-adrenoceptor-cyclic AMP/protein kinase A system (146). Preliminary electrophysiological evidence at the cellular level also suggests that interaction of corticosteroids with β-adrenoceptor agonists at the postsynaptic level does occur in the BLA, in a time-dependent manner (138). In the BLA, noradrenaline is known to have bidirectional effects on neuronal activity: An inhibitory action is mediated by α2-adrenergic receptors (148)(149)(150), whereas β-adrenergic receptors facilitate excitatory transmission in the BLA (151)(148) (152). It was reported that application of corticosterone together with the β-adrenoreceptor agonist isoproterenol rapidly facilitates AMPA receptor-mediated responses evoked by synaptic stimulation in BLA. However, when corticosterone was applied several hours prior to isoproterenol, the (presumably gene-mediated) GR actions suppressed the isoproterenol effect on AMPA receptor-mediated responses (153). **In this thesis we elaborated the time-sequence of isoproterenol / corticosterone interactions in the BLA, by examining the actions of corticosterone given prior to, concurrent with or after isoproterenol, studying AMPAR mediated mEPSCs properties (Chapter 5).**

5. Stress and mental illness

5.1 Hypercortisolaemia and psychopathology

Chronic stress most likely leads to hypersecretion of corticosteroids, which imposes an increased risk for depression along with abdominal obesity and cardiovascular diseases (154). Emotional arousal, psychotic symptoms and cognitive impairment are also often accompanied by hypercortisolaemia (155). Conversely, patients with Cushing's disease –who have high levels of corticosteroids- can display mood

problems, psychotic symptoms and/or cognitive impairment. This suggests a relationship between hypercortisolaemia and psychopathology.

There is in fact evidence that this relationship is causal. Thus, HPA axis dysfunction appears to be a predictor for depressive symptoms (156) (157). In agreement with a causal relationship, antidepressants were found to enhance limbic mineralocorticoid receptor (MR) and GR expression, presumably normalizing the function of the HPA axis. The degree to which HPA axis function is normalized by antidepressants inversely correlates with the likelihood to develop a relapse (158) (159). Importantly, GR antagonists improve psychotic depressive symptoms whereas MR antagonists worsen antidepressant outcome (1). Therefore, it is evident that sustained hyperactivity of the HPA axis and MR/GR imbalance due to chronic stress or early life stress generate a vulnerable phenotype for mental illness (6)(87)(160).

5.2 Chronic stress in animal models

The mechanism by which hypercortisolaemia alters brain function can be studied under very controlled conditions in rodent models of chronic stress. These conditions of chronic stress differ from 'normal' brief stress exposure. The latter response is known to be protective for the system: it helps in survival and adaptation to the challenge (161). Accordingly, many functions are acutely enhanced after brief stress exposure, including memory and metabolism. However, repeated activation of stress mediators over a period of days, weeks or months causes dysregulation of the HPA-axis and essential brain functions, resulting in deleterious effects on the brain (162).

Chronic stress produced marked reductions in corticosteroid signaling in several brain regions. Numerous chronic-stress regimens cause downregulation of GR and, to a lesser extent, MR mRNA, binding and protein levels. These conditions also result in dendritic atrophy in pyramidal cells of the mPFC and particularly the CA3 region of the hippocampus. In contrast, there is an increase in excitability and dendritic branching in the BLA after chronic stress, supposedly contributing to increased emotional memory (5).

Chronic stress affects the hippocampal formation in many ways. For example, one day after chronic mild stress, both proliferation and apoptosis appear to be reduced in the dentate granular cell layer (163) (164) (165), although this was shown to be reversible (163). Chronic stress was found to reduce synaptic strengthening in all hippocampal subfields (164)(165), which may have been caused by changes in glutamatergic transmission, the GABAergic network and/or calcium influx. In the CA3 area an enhancement of NMDAR-dependent transmission was observed (125). In the dentate gyrus, when chronic mild stress was combined with acute GR activation, AMPAR-dependent synaptic transmission was found to be increased (166). Genetic analysis has provided some insight into the pathways underlying these effects of chronic stress (167). It was found that CREB-signaling plays an important role in mediating effects of chronic stress on LTP, calcium currents and neurogenesis. These effects are partly normalized by treatment with the GR-antagonist RU486(167).

5.3 Hypocortisolaemia or adrenalectomy

Hypocortisolaemia, such as occurs in adrenal insufficiency, has also been linked to psychopathology (168) (169). This can be modeled in animals, in its most extreme

form, by adrenalectomy (ADX). In rats bilateral ADX removes corticosterone entirely from the system. In rats, ADX induces apoptosis 3 days later in some, but not all, dentate granule cells (170). Apoptosis is also associated with functional loss in the dentate network (171). In addition and regardless of the apoptotic process, there could be loss in synaptic strength between the entorhinal cortex and hippocampal cells which is essential for learning and memory processes (172). Currents generated in distal dendrites of dentate cells, like some types of voltage-gated Ca2+ currents, were also influenced after ADX (recorded in soma). Behavioral studies showed impairment in the learning of new information after ADX (114) (173).

In mice, fat cells still produce residual amounts of corticosterone after ADX, sufficient to activate a considerable part of the MRs but not GRs. This prevents acceleration of apoptosis after ADX. Prevention of apoptosis by low doses of corticosterone emphasizes an important role of MRs for DG cell survival (170) (174) as well as for the stability of dendritic trees(175).

While hypo- and hypercortisolaemia thus are associated with psychopathology -in fact may play a causal role- and earlier studies have demonstrated marked changes in structure and function of hippocampal cells, very little was known at the start of my project about how these conditions affect rapid corticosteroid actions. **Therefore I examined whether ADX or prolonged exposure to moderately high levels of corticosterone alter rapid effects of corticosterone in the dentate gyrus (Chapter 6)**

6. Aim of the thesis

At the start of this thesis, rapid corticosteroid actions had been described for neurons in the PVN and CA1 hippocampal neurons, but not for other parts of the hippocampal formation or amygdala nuclei. The overall aim of this thesis was to extend our knowledge about these rapid corticosteroid actions in limbic neurons. More specifically we examined 1) to what extent other hippocampal regions exhibit similar rapid responses; 2) what the relevance is of these rapid responses when neurons are exposed to multiple pulses of corticosterone in succession; 3) how rapid responses to corticosterone interact with rapid responses to noradrenergic agonists; and 4) whether rapid responses are affected by the circulating levels of corticosterone in the days / weeks prior to recording.

Timing was a common theme throughout the thesis. First, we focused on rapid corticosteroid effects, rather than the well-documented slow gene-mediated actions. Second, we examined if rapid corticosteroid actions are the means of the brain to respond to ultradian-like corticosterone pulses. Third, we varied the interval between corticosterone and isoproterenol application. And finally, we investigated the interplay between long-term shifts in corticosteroid levels and acute exposure to the hormone. **The element of timing will be further discussed in the General Discussion (Chapter 7).**

Below I give a brief rationale for each of the studies described in this thesis.

Chapter 2: Rapid non-genomic effects in the dentate gyrus

Regional differences in corticosteroid actions at the genomic level had been shown in several studies. However, region dependency of rapid non-genomic effects was only marginally studied at the start of my project. Thus, fast effects of corticosterone

were shown in hypothalamic PVN neurons and neurons in the hippocampal CA1 region. In chapter 2, I looked into fast non-genomic effects of corticosterone in DG granular neurons which were known to have delayed responses to corticosterone that differ from those seen in CA1 principal neurons.

Chapter 3: Rapid electrical responses to multiple corticosterone pulses

Corticosterone is released every hour in pulses, which is too fast to be translated through genomic pathways. We hypothesized that rapid non-genomic pathways are the only means to translate these pulses to physiological responses in hippocampal neurons.

Chapter 4: Glutamate receptor trafficking in response to multiple pulses of corticosterone

Corticosterone can rapidly increase lateral diffusion of AMPARs. By contrast, delayed effects of corticosterone increase the AMPAR density post-synaptically. We hypothesized that multiple pulses of corticosterone will increase lateral diffusion with every pulse relative to its baseline prior to corticosterone application, but that the baseline might be shifted with later pulses due to genomic effects started by the first pulse of corticosterone.

Chapter 5: Interaction between rapid actions of corticosterone and noradrenaline

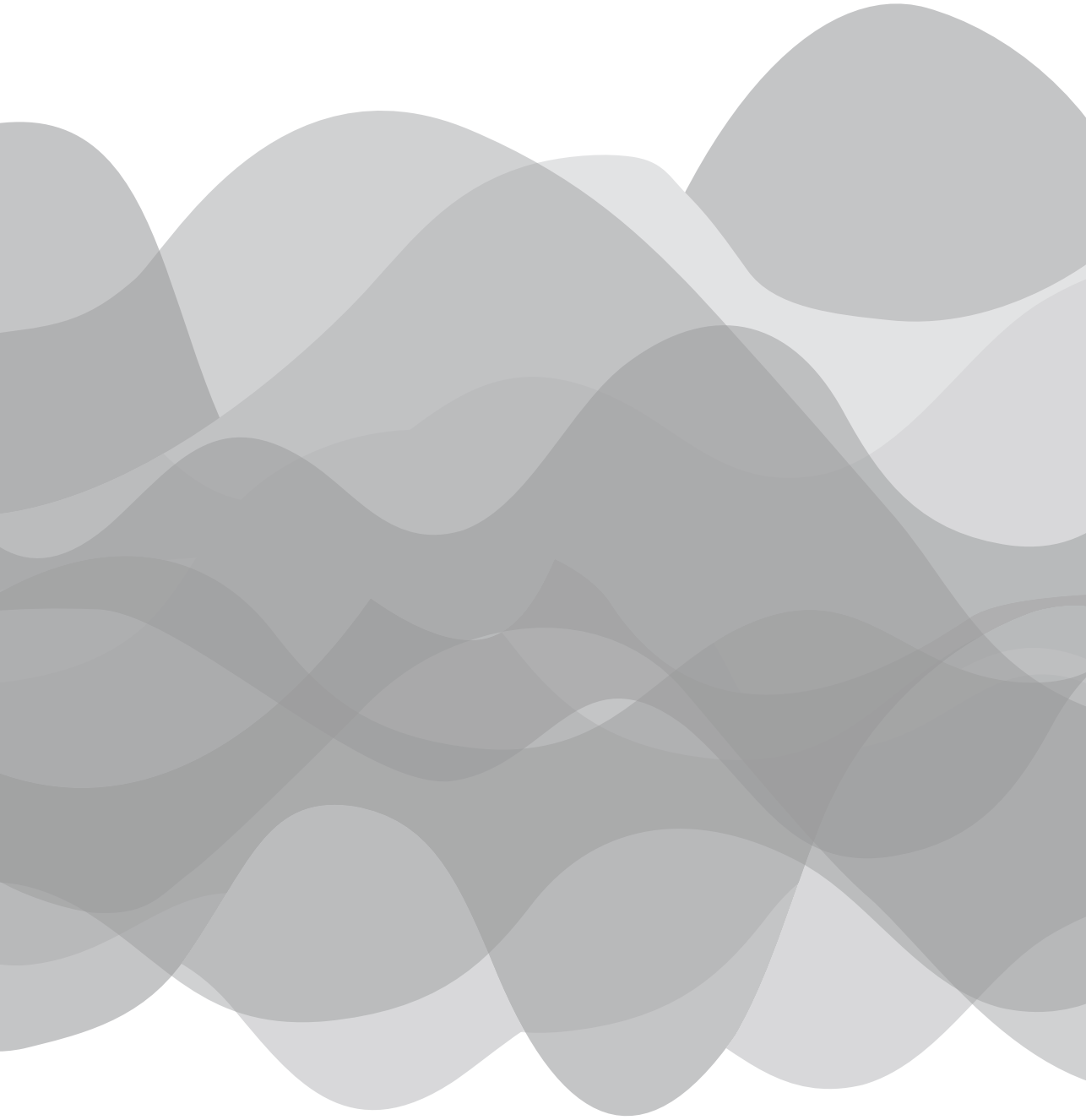
Exposure to stress leads to rapid responses by two systems, i.e. the ANS and the HPA axis. Under physiological conditions, neurons are first exposed to noradrenaline and slightly later to corticosterone. Since noradrenaline (like corticosterone) rapidly changes glutamatergic transmission, we hypothesized that noradrenaline exposure will facilitate the response to subsequently administered corticosterone, while corticosterone exposure *before* noradrenaline will not show this facilitated response and might even be suppressive.

Chapter 6: Changes in non-genomic responses to corticosterone after prolonged hypo- or hypercortisolaemic conditions

Rapid non-genomic effects of corticosterone in the hippocampus are dose-dependent, with a just sub-threshold dose of 3 nM and a near-maximal response at 100 nM. We wondered if extreme variations in circulating corticosterone levels in the days to weeks prior to recording –ADX or moderately high levels of corticosterone in the drinking water for 3 weeks - affect rapid effects induced by these two doses of corticosterone. If so, this might contribute to the pathological conditions related to such extreme variations in corticosteroid hormones.

Chapter 7: General Discussion

In this final chapter, I summarize the main findings of this thesis. Moreover, some considerations which go beyond the discussion of the separate chapters will be highlighted, such as the importance of timing for corticosteroid actions and the functional relevance of rapid corticosteroid actions in health and disease.



CHAPTER 2

Rapid effects of corticosterone in the mouse dentate gyrus via a non-genomic pathway

N Pasricha, M Joels and H Karst

Department of Neuroscience and Pharmacology, University Medical Center, Utrecht, The Netherlands.

J Neuroendocrinol. 2011 Feb;23(2):143-7. doi: 10.1111/j.1365-2826.2010.02091.x.

Abstract

Corticosterone activates two types of intracellular receptors in the rodent brain, i.e. the high affinity mineralocorticoid receptor (MR) and lower affinity glucocorticoid receptor (GR). These receptors act as transcriptional regulators and mediate slow changes in neuronal activity in a region-dependent manner. For instance, in CA1 pyramidal cells corticosterone slowly changes Ca^{2+} currents and glutamate transmission but dentate granule cells seem resistant. Recent studies have shown that corticosteroids also exert rapid MR-dependent, non-genomic effects on hippocampal CA1 cells, e.g. increasing the frequency of miniature excitatory postsynaptic currents (mEPSCs). We here investigated if dentate granule cells are also resistant to rapid effects of corticosterone. We found that, comparable to the CA1 area, corticosterone quickly and reversibly increases mEPSC frequency but not amplitude of dentate cells. This effect did not require protein synthesis and displayed the pharmacological profile of an MR- rather than GR-dependent event. The data supports the hypothesis that, unlike the slow gene-mediated effects of corticosterone, rapid hormonal actions are quite similar for CA1 and dentate cells.

Introduction

On exposure to stress, two systems are activated - i.e. the autonomic nervous system and the hypothalamo-pituitary-adrenal (HPA) axis- which collectively result in elevated levels of catecholamines, neuropeptides and corticosteroid hormones in the rodent's brain (5). After release from the adrenal glands (176), corticosteroids enter the brain and bind to two intracellular receptors, i.e. high affinity mineralocorticoid receptors (MR) and lower affinity glucocorticoid receptors (GR) (1). In the brain, GRs are widely distributed, both in neuronal and glial cells while MR is expressed in neurons only, with high abundance in all subfields of the hippocampus and in the central amygdala (177).

Until recently it was generally thought that noradrenaline and peptides are the main actors in the initial phase of the stress response. Corticosteroids were thought to be important later on, for normalization of brain activity and consolidation of the event, via a genomic pathway (134). The genomic glucocorticoid pathway was shown to contribute importantly to these delayed effects of corticosterone in specific limbic regions like the basolateral amygdala, dentate gyrus (DG) and hippocampal CA1 area (178), (166), (30). In the latter region, high-voltage activated Ca2+ currents and miniature excitatory postsynaptic current (mEPSC) amplitude are increased 1-4 hrs after a brief application of corticosterone (100 nM). Interestingly, though, no effect was observed in dentate granule cells of the same mice (179). These findings pointed to differences between CA1 and DG with respect to the intracellular or extracellular context, causing regional differences in the response to corticosterone.

From recent work, it has become evident that corticosteroids also exert a rapid non-genomic effect on hippocampal CA1 cells. The most conspicuous effect is a rapid and reversible increase in mEPSC frequency after corticosterone administration in vitro. With the use of MR (and GR) knock out mice, it was shown that the rapid effect depends on the gene encoding the MR (41). Very recently, it was reported that these rapid non-genomic effects of corticosterone also display regional differences (180). Thus, in slices containing the basolateral amygdala and prepared from non-stressed mice, corticosterone induced a rapid yet long-lasting (rather than reversible) enhancement in mEPSC frequency. We here questioned if non-genomic responses to corticosterone show regional differentiation between CA1 and DG, as earlier found with respect to the gene-mediated events.

Materials and Methods

Animals

All experiments were carried out with permission of the Animal Ethical Commission from Utrecht University. Male C57BL/6 mice (Harlan, The Netherlands), approx. 5 weeks of age, were group-housed in cages with 12-h light, and 12-h dark schedule (lights on at 8.00 am). Food and water access was ad libitum. One mouse per day was decapitated in basal conditions, before 10.00 am, i.e. when plasma corticosterone levels are low (181).

Slice Preparation and Recording

Animals were decapitated within a few minutes after taking them out of the homecage, which is short enough to not induce any discernable rise in plasma corticosterone concentration (181). The brain was removed from the skull and kept in carbogenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (aCSF; 4°C)

containing (mmol/l): NaCl 120, KCl 3.5, $MgSO_4$ 1.3, NaH_2PO_4 1.25, $CaCl_2$ 2.5, D-glucose 10, and $NaHCO_3$ 25.0. The frontal lobes and cerebellum were removed, and the caudal side of the brain was glued to the platform. Coronal hippocampal slices (350 μm) were made using a vibratome (Leica VT 1000S, Germany) and stored at room temperature, continuously gassed with carbogen.

One slice at a time was submerged in the recording chamber mounted on an upright microscope (Axioskop 2 FS plus; Zeiss, Oberkochen, Germany) with differential interference contrast and a water immersion objective (× 40) to identify the cells. The slices were continuously perfused (flow rate 1.5 ml/min, temperature 30°C, pH 7.4) with aCSF to which was added TTX (0.5 μM; Latoxan, Valence, France), to block sodium channels and bicuculline (50 μM; Enzo), to block $GABA_a$ receptors (182), (183). Parallel perfusion lines were allowed for application of corticosterone and other drugs.

Corticosterone (cort; Sigma, The Netherlands) was dissolved in 90% ethanol (1 mM); this stock was further diluted just before the experiments in aCSF to a final cort concentration of 100 nM (the final concentration of ethanol was 0·009% or lower). As the final ethanol concentration was negligible, no ethanol was added in the control/vehicle aCSF. Also, it has been shown that aCSF with 0.009% ethanol does not effect any of the here tested cell properties (41).

Patch pipettes (borosilicate glass pipettes, inner diameter 0.86 mm, outer diameter 1.5 mm; Hilgenberg, Malsfeld, Germany) were pulled on a Sutter micropipette puller and had a tip resistance of 3-6 MΩ when filled with the pipette (intracellular) solution, containing (in mM): 120 Cs methane sulfonate, 17.5 CsCl, 10 Hepes, 2 MgATP, 0.1 NaGTP, 5 BAPTA; pH was 7.4, adjusted with CsOH. BAPTA was obtained from Molecular Probes (Leiden, The Netherlands) and all other chemicals were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands).

An Axopatch 200B amplifier (Axon Instruments, USA) was used for whole cell recordings, operating in the voltage-clamp mode. The patch-clamp amplifier was interfaced to a computer via a Digidata (type 1200; Axon Instruments, USA) analog-to-digital converter.

Routinely, we cleaned the surface of the slice, in order to have better vision of the cells in the deeper layers of DG. After establishing a gigaseal, the membrane patch was ruptured and the cell was held at a holding potential of -70 mV. The liquid junction potential caused a shift of 8 mV at most. We did not compensate for this potential shift. Recordings with an uncompensated series resistance of <2.5 times the pipette resistance were accepted for analysis. Series resistances were typically between 6-15 MΩ and if the series resistance changed by more than 10% with time or upon application of the drug the recording was not incorporated in the analysis. In view of the small current amplitudes, the recordings were not corrected for series resistance.

Miniature EPSC recording and drugs

Data acquisition and storage was done with PClamp (version 9.2). The currents were recorded at a holding potential of -70 mV with the sampling rate set at 10 kHz and filters at 5 kHz. mEPSCs were accepted if the rise time was faster than the decay time (30). In all cells measured, the following mEPSC characteristics were determined: inter-mEPSC interval, the frequency, rise time, peak amplitude, and tau (τ) of decay (30).

Approximately 10-15 mins after establishing the whole-cell configuration, mEPSCs were recorded under baseline conditions for 5 mins. Next, we applied one of the following drugs for 20 minutes: 100 nM corticosterone (Sigma), to activate both MR and GR; 10 nM of the MR agonist aldosterone (Fluka), with or without the GR antagonist RU38486 (500 nM; Sigma), to selectively activate MR; 10 nM aldosterone and 500 nM RU38486, in the presence of the MR-antagonist spironolatone (100 nM; Sigma-Aldrich), to examine if effects caused by selective activation of the MR could also be blocked with an MR antagonist; or the GR agonist RU 28362 (100 nM; Roussel-UCLAF), to selectively activate GRs. We also tested the effectiveness of the membrane-impermeable conjugate CORT-BSA (100 nM; Sigma) and the ability of the protein synthesis inhibitor cycloheximide (100nM; Fluka) to block corticosteroid actions. Whenever possible, after testing these drugs the perfusion was again switched back to aCSF, to record wash out for 5 mins.

Data analysis and statistics

Data was analyzed offline using ClampFit 9.2. The Kolmogorov-Smirnov test was used (significance of $p<0.05$) to determine if the amplitude and frequency of mEPSCs displayed a normal distribution and whether the distributions before versus during corticosterone application differed significantly. Each distribution (under baseline and corticosterone conditions) was fitted with a Gaussian curve, yielding the value at the x-axis associated with the peak of the curve and the σ value. In addition, we compared for all treatments the averaged amplitude or frequency of mEPSCs during the 5 min baseline period (just prior to drug application) with the last 5 min period during drug application, using a two-tailed paired Student's *t* test, as described earlier (41), (180). The significance was in all cases set at $p < 0.05$.

Results

Miniature excitatory potential postsynaptic currents (mEPSCs) were recorded in 64 cells (from 40 animals), identified under the microscope as DG granular cells; thus, they displayed a round cell-body in the granule cell layer of the supra-pyramidal blade, with generally one or two primary dendrites extending in the direction of the molecular layer. Only one cell was recorded per slice and no more than two recordings were obtained per animal. These mEPSCs are presumably mediated by AMPA receptors because all recordings were carried out at a holding potential of -70 mV, i.e. when NMDA receptors are blocked by Mg ions (120). Once the recording was stable (baseline conditions, 5 min prior to drug application), corticosterone (100nM) was added to the slice.

The mEPSC frequency was found to increase remarkably within a few minutes of corticosterone administration, as shown for a typical example in Fig.1 A and the minute-by-minute average of all cells (n=6) in Fig. 1 B. The effects were quickly reversible upon wash out. As shown in Figure 1C, the frequency distribution of (log-transformed) mEPSC inter-event interval was normally distributed and shifted significantly to the left at the end of the corticosterone application period, compared to the 5 min period prior to hormone application (KS-test, $p<0.05$). Similarly, the distribution of (log-transformed) mEPSC amplitude, both before and during corticosterone application, was normally distributed (Figure 1D). Although there was trend towards a difference between the distribution prior to and during corticosterone application, this did not reach significance (KS-test, $p>0.05$). In

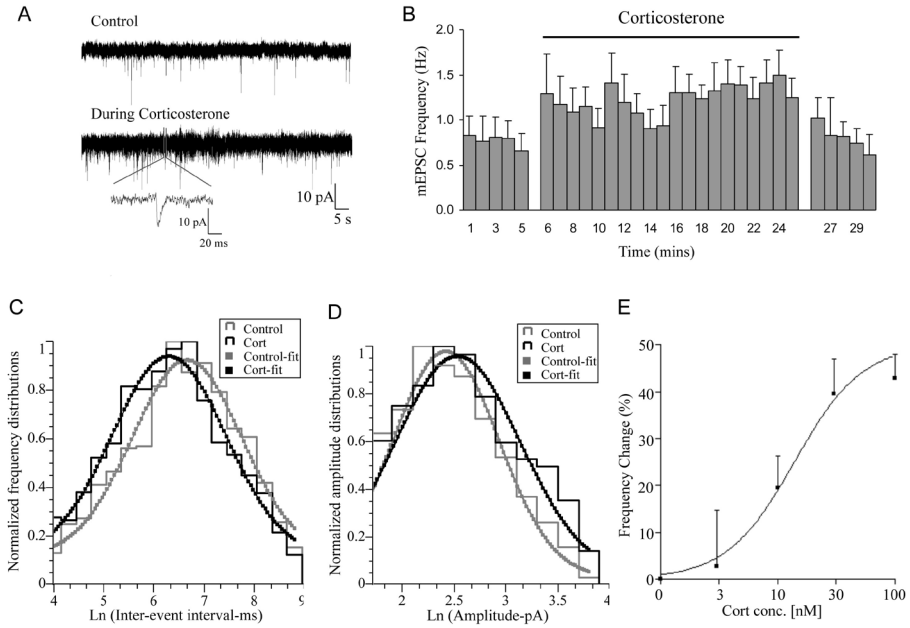


Figure 1. Rapid effects of corticosterone are shown in DG hippocampal cells.
A) Example showing enhanced mEPSC frequency in a DG granule cell during 100 nM corticosterone application (middle) compared to control (only aCSF; top). The inset at the bottom shows a typical example of a mEPSC. B) Averaged mEPSC frequency in time, showing the rapid and reversible increase in mEPSC frequency with application of corticosterone (100 nM, n = 5 cells). Each bin represents the averaged mEPSC frequency (+SEM) over a 1-min period. C) The *log-transformed* frequency distribution of the inter-mEPSC intervals (bars) observed in 5 cells could be fit by a Gaussian curve (drawn squares), both at baseline (i.e. in the 5-min period before corticosterone application; grey) and during the last 5-min period of a 20-min application of corticosterone (black). For the control curve, the $r > 0.90$, with a peak at 6.65 (corresponds to 750 ms) and σ values of 1.09. The Gaussian fit during corticosterone administration had the following characteristics: $r > 0.91$, peak at 6.30 (corresponds to 550 ms) and σ value of 1.13. During corticosterone application the distribution was significantly (KS-test, $p < 0.05$) shifted towards shorter time intervals (n = 5 cells). D) The *log-transformed* amplitude distribution was also nicely fit by a Gaussian curve, both under baseline conditions (grey squares; $r > 0.82$, peak at 2.42 corresponds to 11 pA, σ values, 0.51) and during corticosterone (black squares; $r > 0.81$, peak of the curve at 2.53 corresponds to 12 pA, σ values of 0.60). Although the distribution at baseline and during corticosterone administration had a somewhat different shape, especially in the range of larger amplitudes, this difference did not reach significance (KS-test, $p > 0.05$, n = 5 cells). E) Dose-response curve based on the averaged (+SEM) percentual increase in mEPSC frequency caused by various concentrations (3, 10, 30, 100 nM) of corticosterone (n = 6, 6, 6 and 7 cells respectively). When each of these doses was tested separately against the baseline prior to corticosterone administration, significant ($p < 0.05$) increases in mEPSC frequency were observed with concentrations of 10 nM or higher, but not with lower concentrations of corticosterone.

accordance, when we applied a two-tailed paired Student's t-test to the averaged values recorded in the 5 min period prior to corticosterone and a 5 min period at the end of the drug application, we observed a significant increase in the mEPSC frequency ($p<0.05$) but not amplitude. The average rise-time and τ of decay were determined for mEPSCs recorded under control conditions (mean ± sem over the 5-min baseline period: 1.40 ± 0.99 ms and 2.73 ± 1.14 ms respectively), and at the end of the corticosterone application period (mean ± sem over the last 5 min of corticosterone: 1.41± 1.04 ms and 2.90±1.12 ms respectively; n=6 cells). Apparently, kinetic properties are not liable to rapid changes induced by 100 nM of corticosterone.

We next established the relationship between various concentrations (3, 10, 30, 100 nM) of corticosterone and the increase in mEPSC frequency relative to baseline (n = 6, 6, 6 and 7 cells respectively). The Hill plot of the data shows that the curve fitting was excellent (R^2=0.98), yielding a slope of 1.5 and a half-maximal effect at 14 nM (Fig. 1E). Paired statistical analysis for each dose separately indicated that mEPSC frequency was significantly ($p<0.05$) enhanced by corticosterone (compared to the baseline frequency prior to that particular dose of corticosterone) at concentrations of 10 nM or higher but not at lower concentrations.

This rapid and quickly reversible effect by corticosterone is most likely non-genomic, since a genomic pathway cannot be activated in such a short period of time. In support, corticosterone showed a substantial increase in mEPSC frequency in the presence of cycloheximide (100 nM, $p<0.05$, n=5) (Fig. 2A), added to the vehicle solution at least 30 mins prior to corticosterone application. Cycloheximide is an effective inhibitor of protein biosynthesis in eukaryotes only for genes expressed in the nucleus, not for those expressed in the organelles (184). Also, application of cort-BSA (100nM), a membrane-impermeable conjugate of serum albumin protein with corticosterone, resulted in a significant ($p<0.05$; n=6) enhancement of mEPSC frequency (Fig. 2A). This shows that the rapid non-genomic effects were induced without corticosterone entering the cell, most likely via a receptor residing in the membrane, and activated extracellularly.

We next examined whether these rapid effects of corticosterone are mediated by MR or GR. Application of the selective GR agonist RU 28362 (100 nM, $p>0.05$; n=5) did not cause an increase in mEPSC frequency (Fig. 2B). By contrast, application of the MR agonist aldosterone (10nM, $p<0.05$; n=7) led to an increase in mEPSC frequency which was entirely comparable to the response induced by corticosterone (n=6). The effect of aldosterone was unaffected by concurrent application of the GR antagonist RU 38486 (500 nM, $p<0.05$; n=5). The effect of the MR antagonist spironolactone (100 nM; n=5) was further studied under conditions of a 'clean' activation of MR (10 nM aldosterone + 500 nM RU38486). As is evident from Fig. 2B, spironolactone completely reversed the effect of aldosterone (plus RU38486), underlining the involvement of the MR in the rapid mEPSC frequency observed in the DG.

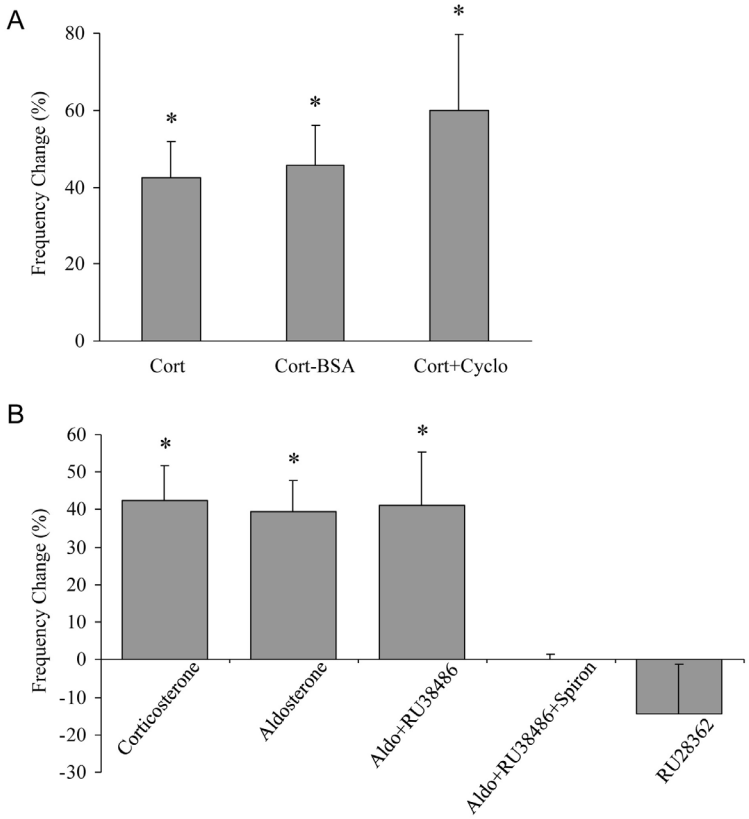


Figure 2. Corticosterone enhances mEPSC frequency in the DG via MRs present in the membrane. A) Corticosterone (100 nM) caused a 40% increase in the mEPSC frequency of DG granule cells compared to baseline conditions (average of n = 6 cells + SEM). A highly comparable increase in mEPSC frequency was observed with the membrane impermeable BSA-corticosterone conjugate (cort-BSA, 100 nM; n = 6 cells), pointing to a membrane location of the receptor mediating the rapid effects. In the presence of the protein synthesis inhibitor cycloheximide (100nM; n = 5 cells), corticosterone was still able to increase mEPSC frequency. This supports the involvement of a non-genomic effect.

B) This graph shows the percentual increase in mEPSC frequency during selective activation of MR and GR. The percentual increase observed with the mixed endogeneous ligand corticosterone (same data as shown in panel A, here included for reasons of comparison), was very comparable to that seen with a 10-fold lower concentration of the endogenous mineralocorticoid aldosterone (10 nM; n = 7 cells). Additional inclusion of the GR antagonist RU 38486 in the perfusion medium (500nM; n = 5 cells; application started 30 min. before aldosterone), did not change the efficacy of aldosterone. However, if we also included the MR antagonist spironolactone into the perfusion medium (100 nM; n = 5 cells), the response to aldosterone was completely abolished. This supports the idea that rapid changes in mEPSC frequency are effectively evoked by activation of the MR but not GR. In agreement, application of the selective GR agonist RU28362 (100 nM; n = 5 cells) had no significant effect on mEPSC frequency.

Statistical significance was tested with a paired t-test ($p < 0.05$, *), comparing the averaged mEPSC frequency during the final 5 min of treatment with the averaged frequency over the 5-min period prior to drug application.

Discussion

Shortly after stress the brain is exposed to a wave of corticosteroids which lasts for approximately two hours (5), (134). In view of the lipophilic nature of corticosteroids, the hormone will in principle reach all cells. Its effectiveness is (among other things) determined by the distribution of the receptors. In this respect, CA1 and dentate granule cells are quite comparable, since both express very high levels of MRs as well as GRs (1).

Recently, though, we found that despite the abundant presence of MRs and GRs, dentate granule cells as opposed to CA1 neurons do not respond to corticosterone with a change in Ca^{2+} current amplitude (179). It was concluded that the regional difference is due to differences in the cellular context (be it intracellularly or extracellularly) which alter the functional outcome of corticosteroid receptor activation. This finding underlined that corticosteroid exposure of the brain induces a complex regional pattern of responses which is not exclusively determined by the receptor distribution (22).

Over the past decade it has become increasingly evident that corticosteroids also affect limbic cell function via non-genomic effects (41), (180), (32). These effects are accomplished via pathways that differ from the classic gene-mediated signaling, but instead use e.g. G-proteins and ERK1/2 as an intermediate (31). In view of these different signaling pathways - not involving genomic effects, it is very well possible that rapid non-genomic responses to corticosterone are actually quite comparable for CA1 and DG cells. We here demonstrated that this is indeed the case. Thus, DG neurons showed a rapid enhancement in mEPSC frequency, with the exact same kinetic properties (i.e. a rapid onset and quick reversibility) and the same pharmacological profile as earlier reported for CA1 pyramidal cells (41).

The similarity between CA1 and DG cells with respect to the rapid non-genomic effect of corticosterone suggests that shortly after stress, when CORT levels rise, hippocampal neurons carrying MRs in the membrane may respond uniformly with enhanced excitability. Interestingly, neurons in the basolateral amygdala show a similar MR-dependent enhancement in mEPSC frequency; however, this transgresses into a long-lasting enhancement, via a mechanism that requires both MRs and GRs (180). This suggests a dichotomy in rapid corticosteroid responses between hippocampal (CA1 and DG) neurons on the one hand and basolateral amygdalar neurons on the other hand. Moreover, the subsequent GR-dependent normalization also shows region-dependency and appears to be less efficient in the DG but particularly the basolateral amygdala (178), (179), (185). If so, stressful conditions which heavily involve the basolateral amygdala (and to a lesser extent DG) may show an extended window for encoding.

Acknowledgements

This work was supported by NWO grant 817.02.017. Pilot experiments were performed in the Swammerdam Institute of Life sciences, Center for Neuroscience, UvA, Amsterdam.

CHAPTER 3

Amygdala but not hippocampal cells become gradually resistant to repetitive corticosterone exposure

N Pasricha, M Joels and H Karst

Dept. Neuroscience & Pharmacology, Division of Neuroscience, UMC Utrecht, Rudolf Magnus Institute, Utrecht, The Netherlands

In preparation

Abstract

Corticosteroid hormones are released in a circadian pattern which overarches ultradian pulses with an approximate inter-pulse interval of one hour. Pulse amplitudes are high prior to onset of the active period of an organism and low at the circadian trough. This pattern is retained in the brain. Stress-induced surges of corticosterone are superimposed on the ultradian pulses. Limbic cells, more specifically in the CA1 area and dentate gyrus of the hippocampal formation and the basolateral amygdala, express both mineralocorticoid and glucocorticoid receptors to which costicosterone bind with differential affinity. These receptors mediate rapid nongenomic and slow gene-mediated actions. We hypothesized that the nongenomic pathway provides limbic cells with the means to quickly alter neuronal activity in response to repetitive fluctuations in corticosteroid level. To test this we exposed brain slices to four pulses of corticosterone (100 nM) with an interpulse interval of one hour and measured spontaneous miniature excitatory postsynaptic currents (mEPSC) as an index for glutamatergic activity. Both CA1 and dentate cells showed a transient increase in the frequency (but not other properties) of mEPSCs upon repeated exposure, although a temporary attenuation was seen in response to the third pulse. The latter was prevented when protein synthesis was inhibited. Basolateral amygdala cells responded to the first pulse with a sustained enhancement of mEPSP frequency, after which renewed exposure caused a significant reduction in mEPSC frequency. Importantly, basolateral amygdala cells became fully resistant to subsequent pulses of corticosterone, even in the presence of a protein synthesis inhibitor. This suggests a gradual dissociation between hippocampal and amygdalar activity during repetitive corticosterone exposure, with potential implications for ultradian shifts in corticosterone level.

Introduction

Corticosteroid hormones are released from the adrenal gland in a circadian pattern, yielding high levels just before the onset of the active period (1). Several studies over the past decades have shown that this circadian rhythm in fact overarches ultradian pulses with an interpulse interval of approximately one hour (50); (55); (78); (53). While hormone levels during the troughs of these ultradian pulses do not substantially vary over the day, the peak amplitudes follow a clear circadian pattern (19); (186). Ultradian pulses have been observed in many species, including rodents and men (see for review (187)). With in vivo microdialysis it was demonstrated that ultradian corticosteroid pulses measured in rat plasma are quite accurately reflected in the brain, more specifically in the hippocampus (144). Stress-induced surges of corticosterone are superimposed on the ultradian pulses (186).

Recent studies investigated the relevance of ultradian corticosterone exposure for the functionality of neurons. In the brain, corticosterone acts through two nuclear receptors, i.e. the high-affinity mineralocorticoid receptor (MR) and the lower affinity glucocorticoid receptor (GR) (1). These receptors are enriched in limbic areas, such as the CA1 hippocampal region, the dentate gyrus (DG) and to a lesser extent the basolateral amygdala (BLA) which expresses high levels of GR but less MR; all of these three areas play an important role in the cognitive effects of corticosteroids (188); (189); (118). Due to the difference in affinity, the nuclear MR is already substantially activated with trough levels of corticosterone, but activation of GR depends on rises in corticosteroid level such as occur during ultradian peaks or after stress (50). In cell lines, nuclear translocation of GRs as well as heteronuclear RNA levels of GR-responsive genes were found to closely follow the pulsatile pattern of corticosteroid application (190). This pulsatility was lost at the level of the mRNA, but changes in mRNA level of GR-responsive genes turned out to be much more efficient with pulsatile compared to constant administration of corticosteroid hormones (190); (71). It was concluded that the functional outcome of GR activation with respect to genomic regulation critically depends on pulsatile release of the hormone.

It has become increasingly evident, though, that MR and GR also mediate nongenomic actions in limbic cells. For instance, in hippocampal CA1 and DG neurons corticosterone causes a rapid and reversible increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs; (41); (191), each of which represents the postsynaptic response to the spontaneous release of one glutamate-containing synaptic vesicle. These effects are thought to be mediated by MRs inserted into the presynaptic terminal (31). In the BLA, corticosterone also causes an MR-dependent increase in mEPSC frequency, but this effect is more sustained in nature (180). Interestingly, subsequent exposure to corticosterone rapidly activates a GR-dependent pathway, resulting in a decrease of the mEPSC frequency.

These nongenomic pathways are ideally suited to quickly translate repetitive exposure to corticosteroids into changes in activity, which could render these cells more susceptible to input e.g. during the peak of ultradian pulses or after stress (192). If so, this might reveal a second mode by which ultradian pulses are important for brain function, i.e. by synchronizing the activity of neurons in limbic regions. We therefore examined to what extent electrical activity of neurons in the CA1 hippocampal area, the DG and BLA is indeed changed by a sequence of four high-amplitude corticosterone pulses.

Materials and Methods

Animals

All experiments were approved by the Animal Ethical Commission from Utrecht University. Male C57BL/6 mice (Harlan, The Netherlands, approx. 5-6 weeks of age at arrival; n=91 in total) were group-housed with food and water provided ad libitum. Lights were on from 07:00 until 19:00 h. After approximately 1-2 weeks mice (one at a time) entered the experiment; at that time the mice were on average 8 weeks old, which is just beyond the period of adolescence. On the morning of the experiment, the mouse was decapitated within 2 minutes after being taken from the home cage and always before 10.00 am, so that plasma corticosterone levels are expected to be low (181). The latter was confirmed in a sample of the current set of animals, which displayed on average corticosterone plasma levels of 13.5 ± 3.3 ng/ml (n=48) as determined with a radioimmuno assay in trunk blood collected at the moment of decapitation. At this time of the circadian corticosterone release pattern, endogenous pulses are of such low amplitude(19) that the 'corticosterone-history' of individual animals at this point is negligible.

Slice Preparation and Recording

Directly after decapitation, the brain was removed from the skull and placed in chilled (approximately 4°C) artificial cerebrospinal fluid (aCSF) containing (mmol/l): NaCl 120, KCl 3.5, $MgSO_4$ 5.0, NaH_2PO_4 1.25, $CaCl_2$ 0.2, D-glucose 10, and $NaHCO_3$ 25.0. Coronal slices (350 µm) containing the dorsal hippocampus or BLA were made using a vibratome (Leica VT 1000S, Germany), stored in aCSF at room temperature and continuously gassed with carbogen. In the hippocampus, investigations were confined to the dorsal half in view of the functional differences and differential properties of neurons in the ventral-most part of hippocampus (193); (194).

Recording method

One slice at a time was transferred to the recording chamber mounted on an upright microscope (Axioskop 2 FS plus; Zeiss, Oberkochen, Germany) with differential interference contrast and a water immersion objective (× 40) to identify the cells. The slices were continuously perfused (flow rate 1.5 ml/min) with warm aCSF (temperature 30°C, pH 7.4) containing TTX (0.5 µM; Latoxan, Valence, France) to block sodium channels and bicuculline (50 µM; Enzo) to block $GABA_a$ receptors (Zhou et al., 2006). A second perfusion line was installed for application of corticosterone.

Patch pipettes (borosilicate glass pipettes, inner diameter 0.86 mm, outer diameter 1.5 mm; Hilgenberg, Malsfeld, Germany) were pulled on a Sutter micropipette puller (Novato, California, USA) and had a tip resistance of 3-6 MΩ when filled with the pipette (intracellular) solution, containing (in mM): 120 Cs methane sulfonate, 17.5 CsCl, 10 Hepes, 2 MgATP, 0.1 NaGTP, 5 BAPTA; pH was 7.4, adjusted with CsOH. BAPTA was obtained from Molecular Probes (Leiden, The Netherlands), all other chemicals were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands).

An Axopatch 200B amplifier (Axon Instruments, USA) was used for whole cell recordings, operating in the voltage-clamp mode. The patch-clamp amplifier was interfaced to a computer via a Digidata (type 1200; Axon Instruments, USA) analog-to-digital converter.

Visually identified granular neurons in DG and pyramidal neurons in CA1 and BLA were selected for recording. After establishing a gigaseal, the membrane patch was ruptured and the cell was held at a holding potential of -70 mV. The liquid junction potential caused a shift of 8 mV at most. We did not compensate for this potential shift. Recordings with an uncompensated series resistance of <2.5 times the pipette resistance were accepted for analysis. In view of the small current amplitudes, the recordings were not corrected for series resistance.

Corticosterone pulses and miniature EPSC recording

In all cells measured, the following mEPSC characteristics were determined: the frequency, peak amplitude, tau of rise time and tau of decay. The digitized data (stored on PC by Digidata interface) were analyzed off-line using Pclamp vesion 9.2 software, which uses a threshold-based event detection algorithm, with detection threshold levels set at 5 pA. The currents were identified as mEPSCs when the rise time was faster than the decay time(30). Earlier, the decay of each mEPSC was fitted with a mono- and biexponential curve in (Strathclyde software, WCP). This program uses the Levenberg–Marquardt algorithm to iteratively minimize the sum of the squared differences between the theoretical curve and data curve. As a criterion for the goodness of the fit the residual SD should be <0.3. As described (30) fitting with a biexponential instead of a monoexponential curve did not increase the goodness of the fit. Therefore we currently used a monoexponential fit.

Four pulses of corticosterone (100 nM) were given, with an inter-pulse interval of one hour, to the slice submerged in the recording chamber (Figure 1A1). Ten minutes of corticosterone perfusion is expected to gradually increase corticosterone levels in the chamber and upon 10 min washout levels should gradually decrease to baseline, thus mimicking the shape of corticosterone pulses reported in vivo (19). To check this, we perfused a solution containing a dye and fluid samples were collected from the recording chamber for each consecutive minute during a 20 min period. Absorbance of these samples was measured with a spectrophotometer. As shown in Figure 1A2, the selected paradigm indeed resulted in a pulsatile pattern, with a 20 min pulse-width. Peak amplitudes were only reached during part of this 20 min period. We cannot exclude the possibility that the intended concentration (100 nM) was not fully reached, although earlier experiments with a 20 min period of application confirmed that the corticosterone concentration in the recording chamber was comparable to that in the perfusion fluid (unpublished observation). If the intended concentration was not fully reached, this would affect all pulses and all experiments equally and therefore not change the interpretation of our observations. If anything, it would bring the concentrations closer to the physiological range.

In our experience, slices from young-adult mice are quite stable with respect to their electrophysiological properties for approximately 5 hrs (12.00-17.00 hrs), before some slices become liable to run-down. For this reason we restricted the number of pulses to four, to ensure stable recordings and reduce variability caused by the condition of the tissue. In some experiments the issue of possible run-down was directly tested (see Results section).

Approximately 10-15 mins after establishing the whole-cell configuration, mEPSCs were recorded under baseline conditions, followed by recording during 10 minutes application of corticosterone (100 nM, Sigma) and 10 minutes after wash-out. In this study we were primarily interested in the relative changes in mEPSC

properties after each of four pulses of corticosterone, comparing the mEPSCs during the peak of corticosterone application to the baseline before hormone administration. Since earlier experiments indicated that vehicle (0.09% ethanol in aCSF) application is entirely ineffective(41) in changing mEPSC properties we did not run separate experiments with pulsatile vehicle application, except when we had reasons to believe that manipulations of the slices (including repetitive shifts in perfusion medium) might have influenced the results; in those cases we performed dedicated experiments, also involving exposure to the vehicle.

In our hands and in tissue from these young-adult animals (as opposed to the very young animals generally used in mEPSC studies) it was not possible to record mEPSCs under stable conditions for more than one hour. This precluded investigation of multiple pulses in the same cell. Therefore, another cell was patched in the same slice which had already been exposed to the first corticosterone pulse. Baseline mEPSC properties were recorded before exposure of this second cell to another pulse of corticosterone; these baseline properties were compared to the mEPSC characteristics during and after the subsequent corticosterone pulse. We tried to record (different) cells for all 4 pulses in each slice, but this was usually not possible. In that case, we transferred another slice, which had been exposed to multiple pulses in a separate chamber, to the recording set-up. Even so, we were only able to obtain on average 2 cells per animal, given the difficulty of the method and the very narrow time-window (dictated by the pulse rhythm) in which a cell had to be patched. Therefore, the population of slices and mice on which the averaged data for each pulse is based only partly overlaps.

To estimate the contribution of genomic pathways, we also exposed hippocampal or BLA-containing slices to repetitive corticosterone pulses in the presence of cycloheximide, a protein synthesis inhibitor (100 µM; Sigma-Aldrich, Germany). All slices were perfused with aCSF containing cycloheximide at least 30 minutes before the first pulse of corticosterone was given. Cycloheximide was present throughout the experiment while slices were exposed to four pulses of corticosterone or vehicle.

Data analysis

Data was analyzed offline using ClampFit 9.2. Responses to pulses of corticosterone, i.e. the final 5 min of corticosterone application, were compared to the 5 min of baseline just preceding the respective pulse (1^{st}, 2^{nd}, 3^{rd}, 4^{th} pulse). For each cell the frequency, amplitude, tau of risetime and tau of decay of mEPSCs were determined. Throughout the manuscript, data are expressed as mean (determined for all cells in the group) ± standard error of the mean.

Statistics

Cumulative frequency distribution of mEPSC frequency was analyzed with the Kolmogorov-Smirnov-test. The relative changes in mEPSC properties were analyzed for each corticosterone pulse (compared to the baseline just before hormone application), with a two-tailed paired Student's *t* test. For both the statistical tests, significance was set at $p < 0.05$.

To also investigate putative genomic effects that developed over time, we compared baseline mEPSC properties prior to each of the four pulses of corticosterone as well as gradual shifts in the responsiveness to corticosterone; we also examined to what extent the response to corticosterone was affected by

concurrent perfusion of cycloheximide. Since we generally obtained multiple but not all pulse responses in slices from one animal, we analyzed the results with a mixed model, in which there are both within group (time as variable) and between group (with two treatments: cort and cort + cyclo) comparisons (shown in Table 1 and 2). Here, time was defined as a dependent factor and we did not correct for missing values (they were treated as missing). Significance was set at $p<0.05$. Since one can also argue that all data points were independent, we also applied a one way ANOVA, followed by a post hoc (Tukey) analysis, with significance set at $p < 0.05$; this analysis gave highly comparable results as obtained with the mixed model analysis and are therefore not explicitly mentioned in the text.

Results

Overall we recorded mEPSC properties from 132 limbic cells, i.e. 24 CA1 neurons in the dorsal hippocampus, identified under the microscope as pyramidal neurons; 48 identified DG granule cells located in the middle of the granule cell layer; and 60 principal pyramidal-like neurons in the BLA.

In the CA1 area, pyramidal neurons responded to a brief (10 min) pulse of corticosterone in a comparable manner as earlier observed with a 20 min pulse: the mEPSC frequency was quickly elevated and returned to baseline after washout of the hormone (see example in Figure 1B). The shift towards shorter inter-event intervals was apparent from the cumulative frequency histogram (Figure 2A). On average, the frequency in the final 5 minutes of corticosterone application was significantly enhanced compared to the baseline in the 5-min period just prior to hormone application (Figure 3A). CA1 neurons responded similarly to a second pulse applied 1 hr later (Figures 2 and 3; Table 1). Unexpectedly, a clear attenuation was apparent upon application of the 3rd pulse. In fact, the mEPSC frequency was no longer significantly enhanced during the 3rd pulse of corticosterone (Figure 2A, KS: p=0.25; Figure 3A, paired t-test: p=0.39). During the 4th application of the hormone, responsiveness was somewhat restored. This was reflected in a significant enhancement in the mean mEPSC frequency during the final 5 min period of 4th corticosterone application compared to the baseline just before application (Figure 3A; paired t-test: p=0.04). When analyzing the cumulative frequency distribution of the inter-event intervals for the 4th pulse, a significant shift towards shorter intervals was observed (Figure 2; KS: p=0.05). Other mEPSC properties in the CA1 were not affected by corticosteroid treatment at any moment in time (Table 1). We also did not observe time-dependent effects on baseline properties (Table 1), with the exception of the tau of risetime prior to the 2nd and 3rd pulse, which was slightly but significantly enhanced compared to that seen prior to the 1st pulse (Table 1).

Results in the DG were highly comparable to those obtained in the CA1 region. Here too, neurons responded to the 1st pulse with a transiently enhanced mEPSC frequency. The cumulative frequency distribution of the mEPSC intervals was significantly shifted towards shorter intervals (Figure 2B; KS: p=0.02). A significant effect of corticosterone was also observed when analyzing the mean mEPSC frequency during corticosterone application compared to baseline (Figure 3B; paired t-test: Pulse1, p=0.01 and Pulse2, p=0.03). The response was attenuated and no longer significant during the 3rd pulse (paired t-test: p=0.24; KS: p=0.42). Upon the 4th and final application of corticosterone, the response somewhat restored (t-test: p=0.002; KS: p=0.04). The mEPSC amplitude and kinetic properties were

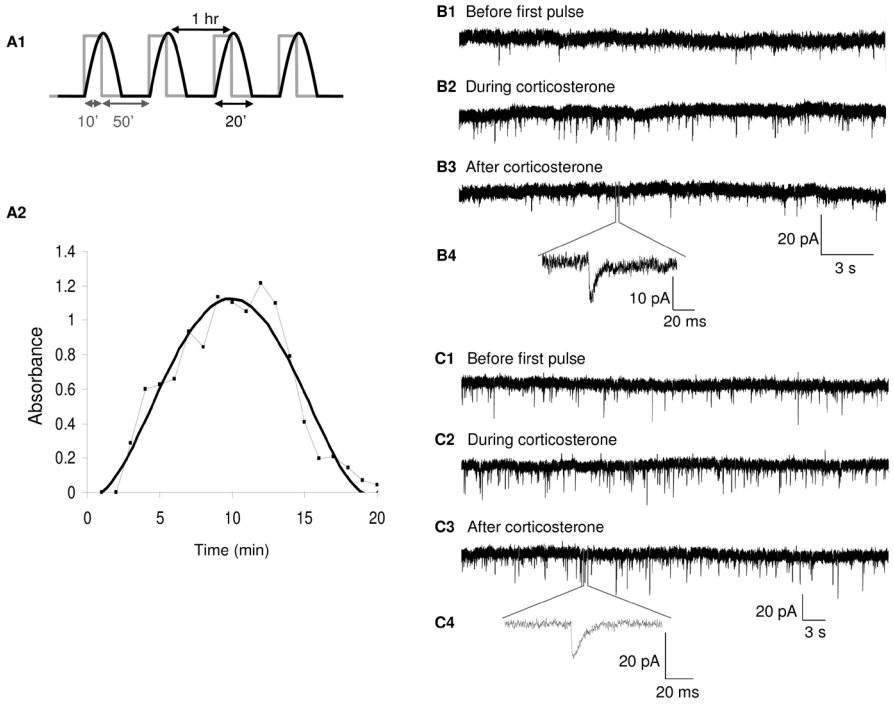


Figure 1.

A. Method of pulsatile application of corticosterone. A1 illustrates the application schedule in which corticosterone (100 nM) is applied four times for 10 min, with an inter-pulse interval of 1 hr (grey line). In view of the gradual distribution of the perfusion solution in the recording chamber we hypothesized that slices were exposed to drugs as depicted by the black line. A2: typical example of optical measurements showing the absorbance in samples of aCSF to which we added a dye and which were collected every minute from the recording chamber. The points connected by a drawn line depict the actual values, while the smooth line is the best fit.

B. Typical mEPSC before (B1), during (B2) and 10 min after (B3) the first corticosterone application, in an identified CA1 pyramidal cell. An example of an mEPSC with higher time resolution is shown in B4.

C. Typical mEPSC before (C1), during (C2) and 10 min after (C3) the first application of corticosterone to a principal cell of the BLA. Example with higher time resolution is shown in C4.

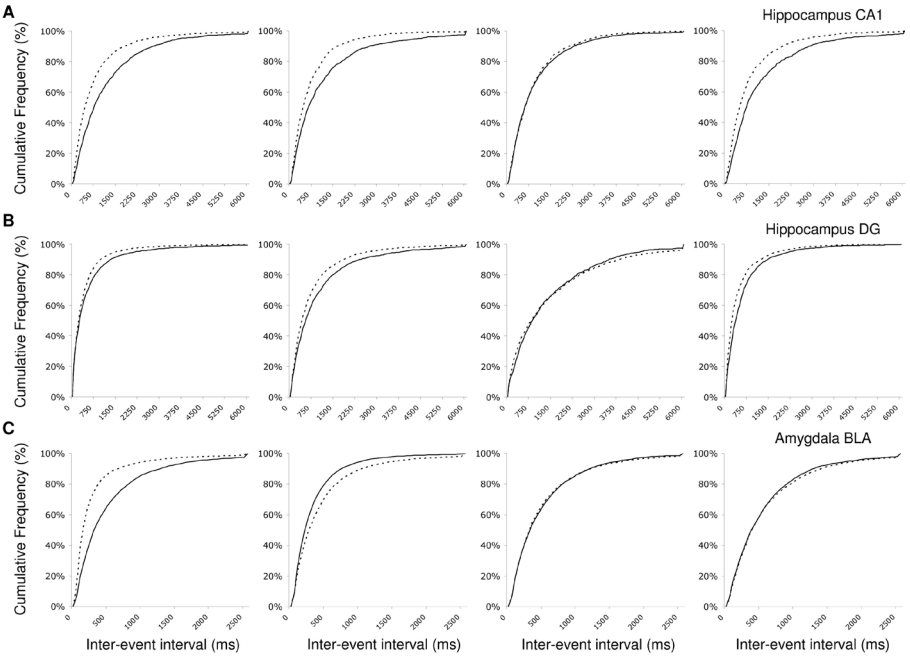


Figure 2. Cumulative frequency distributions of the inter-event intervals for mEPSC recorded in the CA1, DG and BLA during 4 corticosterone pulses. Frequency distributions before (drawn line) and during corticosterone administration (striped line) were compared for each area and each pulse with a Kolmogorov-Smirnov test. Both in the CA1 area (panel A) and in the dentate gyrus (DG; panel B) corticosterone application resulted in a shift to the left, causing smaller inter-event intervals. This was not observed, though, during the 3rd pulse (p=0.25 and p=0.42 for CA1 and DG respectively). During the 4th pulse, corticosterone again induced a shift towards shorter inter-event intervals, which was significant for the DG (p=0.04) and just reached significance in the CA1 area (p=0.05). In the BLA (panel C), the 1st pulse caused a significant shift towards smaller inter-event intervals (p=0.02). This was reversed during a 2nd exposure to corticosterone (p=0.04). No significant changes were observed during the 3rd (p=0.40) or 4th pulse (p=0.14). Number of cells in the CA1 area: n=6, 6, 6 and 6, for 1st, 2nd, 3rd and 4th pulse respectively; in the DG: n=7, 6, 6 and 6 granule cells; and for the BLA: n=9, 8, 7 and 6 cells.

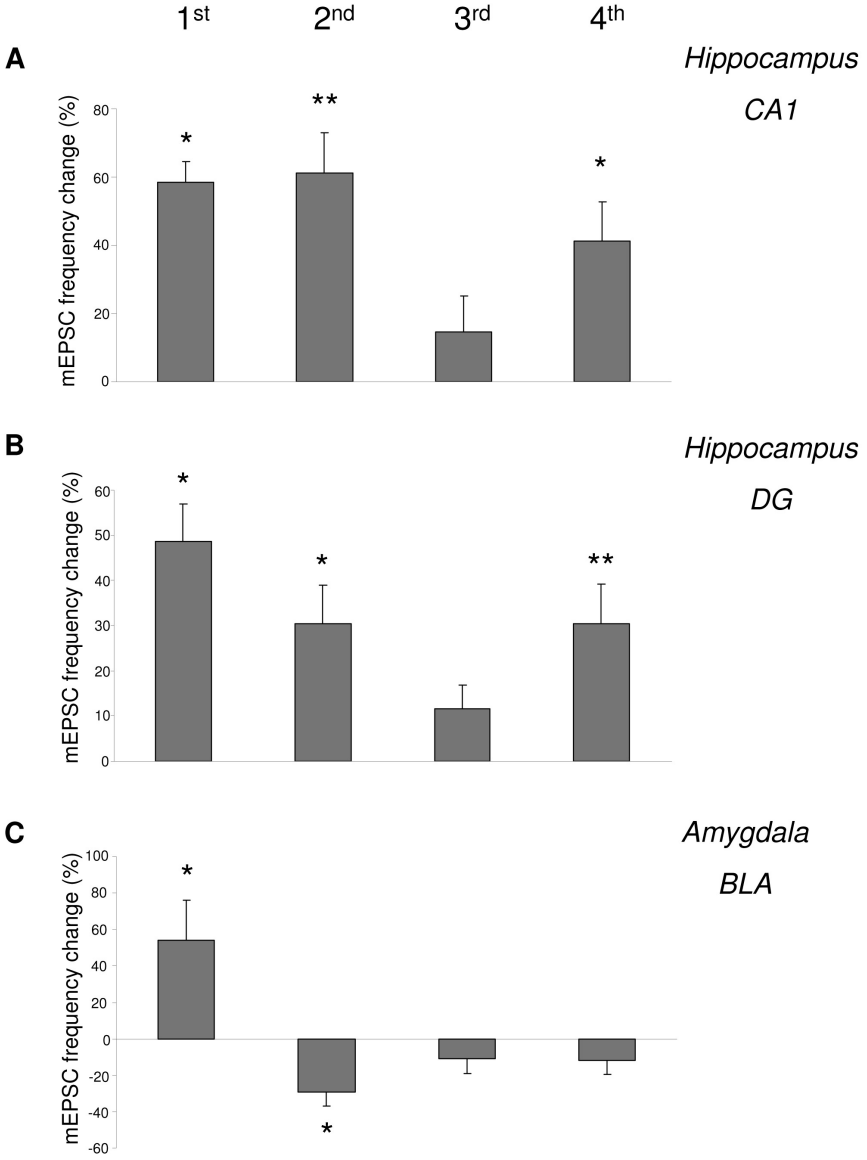


Figure 3. Percentual change in mEPSC frequency compared to baseline (=0%) after application of corticosterone. Panel A shows the change in mEPSC frequency in response to the 4 pulses for CA1 pyramidal neurons, using the experimental protocol shown in Figure 1A. Data show the mean (±SEM) change, based on the following number of cells for the 1st, 2nd, 3rd and 4th pulse respectively: n=6, 6, 6 and 6. In panel B, data for the dentate gyrus (DG) are summarized. Data are based on n=7, 6, 6 and 6 granule cells, for 1st, 2nd, 3rd and 4th pulse respectively. The lower panel (C) depicts the averaged data for principal cells in the basolateral amygdala (BLA). Data are based on n=9, 8, 7 and 6 cells, for 1st, 2nd, 3rd and 4th pulse respectively. Statistically significant changes in mEPSC frequency due to application of corticosterone (compared to baseline) are indicated by * ($p<0.05$) or ** ($p<0.01$).

	pulse 1 absolute value during baseline	% change during cort	pulse 2 absolute value during baseline	% change during cort	pulse 3 absolute value during baseline	% change during cort	pulse 4 absolute value during baseline	% change during cort
CA1								
Frequency (events/sec)	0.70 ± 0.09	58.6 ± 6.0*	0.56 ± 0.11	61.1 ± 12.0**	0.56 ± 0.11	19.3 ± 11.1&	0.52 ± 0.04	41.3 ± 11.6*
Amplitude (pA)	-15.2 ± 1.9	5.4± 4.9	-9.4 ± 1.0	-6.4 ± 8.1	-7.8 ± 1.0	10.0 ± 4.7	-6.7 ± 1.2	4.4 ± 7.3
Tau rise (ms)	1.7 ± 0.4	16.3 ± 9.4	2.9 ± 0.2	-2.0 ± 8.7	3.3 ± 0.2	-5.0 ± 5.7	3.32 ± 0.3	10.4 ± 4.02
tau of decay (ms)	14.6± 1.7	-1.5 ± 12.4	13.0 ± 1.2	9.2 ± 12.1	13.3 ± 0.8	-4.6 ± 6.1	11.8 ± 1.3	-2.2 ± 4.8
DG								
Frequency (events/sec)	0.79 ± 0.21	48.6 ± 8.3*	0.60 ± 0.14	30.4 ± 8.7*	0.73 ± 0.21	11.7 ± 5.2&	0.93 ± 0.20	30.5 ± 10.0**
Amplitude (pA)	-11.9 ± 3.0	-5.6 ± 5.2	-13.3 ± 24	10.3 ± 7.4	-11.4 ± 2.7	11.3 ± 8.3	-12.0 ± 2.1	-0.93 ± 2.23
Tau rise (ms)	2.5 ± 0.2	11.9 ± 5.9	3.5 ± 0.1	-2.4 ± 7.3	2.9 ± 1.0	4.6 ± 8.3	2.9 ± 0.1	7.7 ± 3.5
tau of decay (ms)	10.0 ± 1.1	9.2 ± 7.9	12.4 ± 1.0	0.39 ± 4.97	14.0 ± 0.2	-1.1 ± 4.4	10.0 ± 0.9	0.8 ± 6.6
BLA								
Frequency (events/sec)	2.2 ± 0.4	54.2 ± 21.6*	3.7 ± 0.6	-29.1 ± 7.8*&	2.5 ± 0.4	-11.0 ± 8.3&	2.1 ± 0.3	-12.0 ± 7.7&
Amplitude (pA)	-14.7 ± 1.1	-7.2 ± 5.4	-13.1 ± 1.5	-6.4 ± 4.5	-12.7 ± 1.1	-3.1 ± 7.0	-11.5 ± 1.2	-3.7 ± 5.1
Tau rise (ms)	1.7 ± 0.1	-0.5 ± 8.8	1.5 ± 0.1	0.5 ± 11.6	1.6 ± 0.1	5.5 ± 6.4	1.5 ± 0.1	-12.4 ± 5.3
tau of decay (ms)	8.9 ± 1.0	4.4 ± 9.9	7.6 ± 0.9	-3.3 ± 14.8	8.6 ± 0.9	-6.5 ± 10.5	8.2 ± 0.8	7.5 ± 11.8

Table 1. Percentual changes in mEPSC frequency are relatively stable for a sequence of 4 pulses of corticosterone (cort) in hippocampal CA1 and DG cells, but not in neurons of the basolateral amygdala (BLA) nucleus. In nearly all cases the absolute (mean ± SEM) baseline mEPSC frequency, amplitude, the timeconstant (tau) for the risetime and time constant of decay prior to the 4 cort pulses , nor the relative change in the latter three properties changed over time. *, **: relative to baseline prior to corticosterone application, $p<0.05$ or 0.01 respectively; #: $p<0.05$ relative to baseline before 1st pulse. &: $p<0.05$ relative to effect of 1st cort pulse. See for number of cells for each area / pulse the legend of Figure 2.

not changed by any of the pulses (Table 1). We also did not observe significant effects over time with regard to the baseline mEPSC frequency prior to the pulses nor in the amplitude, tau of rise time or tau of decay (Table 1).

Pulsatile corticosterone application affected pyramidal-like cells in the BLA very differently. As in the CA1 and DG, BLA neurons responded to the 1st pulse with enhanced mEPSC frequency, which remained high after wash-out of the

hormone (example in Figure 1C). Statistical analysis revealed that the inter-event interval was significantly shifted towards lower intervals during corticosterone administration compared to baseline conditions prior to corticosterone (Figure 2C; KS: p=0.02). A highly significant shift towards higher frequency was also observed when comparing the mean mEPSC frequency during corticosterone with the baseline (Figure 3B; paired t-test: p=0.04). The second pulse caused the mEPSC frequency to decrease (Figures 2; KS: p=0.04 and 3; paired t-test: p=0.03). Thereafter, BLA cells appeared to become entirely unresponsive to pulses of corticosterone. We considered the possibility that BLA cells may be more sensitive to manipulations and/or run-down of the slices, so that the 3rd and 4th pulse of corticosterone would hit the cells by the time that the slice condition had deteriorated. To test this, we exposed slices first to two pulses of vehicle and then administered corticosterone. Under those conditions, however, corticosterone did significantly increase the mEPSC frequency (n=6, 35.2 ± 8.3 % increase compared to baseline, p=0.02), supporting that BLA cells are still viable and responsive to corticosterone 3-4 hrs after preparation of the slices. Other mEPSC properties were not affected by the hormonal pulses (Table 1). Also, baseline values of the parameters measured did not change significantly over time (Table 1).

We next wondered to what extent the slight attenuation in corticosterone responsiveness observed in CA1 and DG cells particularly upon the 3rd application and the complex pattern of responses observed in the BLA depend on genomic actions of the corticosterone, which might gradually change background conditions as a result of the early applications. To test this, all experiments were run in the presence of the protein synthesis inhibitor cycloheximide. For these experiments we focused on DG and BLA cells, which are illustrative for the two different response patterns. Treatment with cycloheximide did not affect most of the baseline characteristics of DG and BLA neurons, although we did observe significant changes in the baseline mEPSC rise-time for some pulses, both in the DG and BLA (Table 2), compared to the corresponding pulses in the absence of cycloheximide; the baseline tau of decay was only different between cycloheximide and control conditions prior to pulse 3 in the BLA (Table 2).

As shown in Figure 4, the attenuation in responsiveness to the 3rd pulse of corticosterone with respect to mEPSC frequency, as described above for DG granular cells, disappeared in the presence of cycloheximide. The pattern in the BLA was also affected (Figure 4B). Thus, BLA neurons still responded to the 1st pulse of corticosterone with enhanced mEPSC frequency, but frequency values returned to baseline upon wash-out (4.9 ± 10.3 % change compared to the baseline before 1st pulse, p=0.7; n=8 cells). In the continued presence of cycloheximide, the second application of corticosterone to BLA cells caused a significant *enhancement* in frequency, similar to what is seen in hippocampal cells. However, even in the presence of cycloheximide, BLA cells still did not respond to a 3rd and 4th application of corticosterone. This did not depend on the condition of the slices, since exposure of slices to two pulses of vehicle followed by a pulse of corticosterone caused a transient increase in mEPSC frequency (60.0 ± 18.3% increase compared to baseline, n=6, $p<0.05$), similar to what was seen when slices were immediately exposed to a pulse of corticosterone.

As in control conditions, other mEPSC properties remained unaffected by pulses of corticosterone in the presence of cycloheximide, both in the DG and BLA (Table 2).

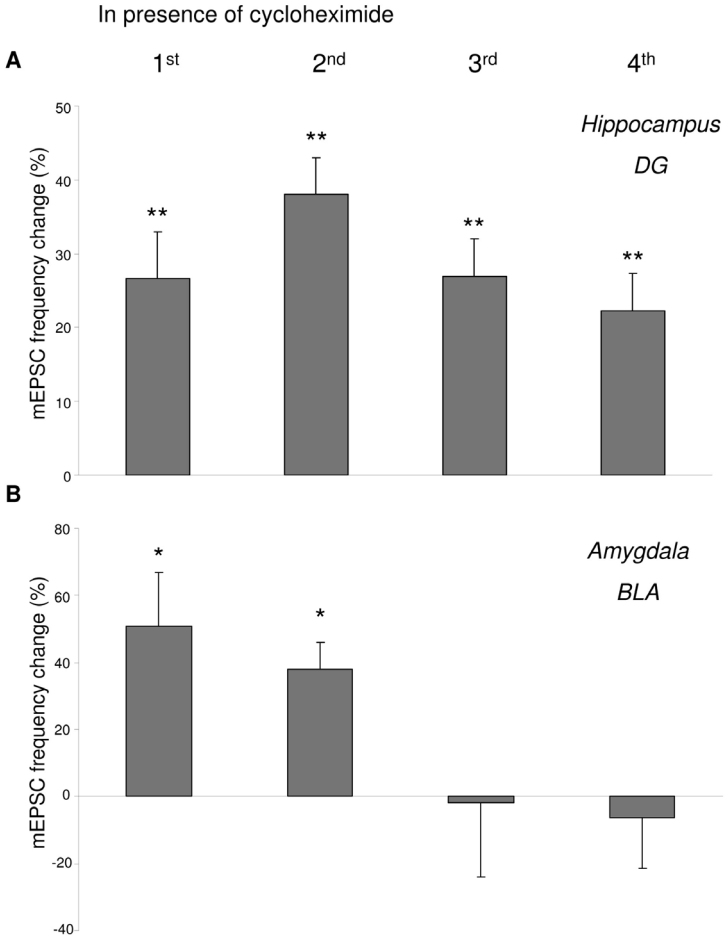


Figure 4. To examine the dependence of corticosteroid effects on protein synthesis, application of the four high-amplitude (100 nM) corticosterone pulses was carried out in the presence of cycloheximide. Panel A shows, for dentate gyrus neurons, the percentual change in mEPSC frequency in response to the 4 pulses of corticosterone compared to the corresponding baseline period (=0%). Data show the mean (±SEM), based on the following number of cells for the 1st, 2nd, 3rd and 4th pulse respectively: n = 7, 5, 6 and 5. The lower panel (B) depicts the averaged data for principal cells in the basolateral amygdala (BLA). Data are based on n=8, 8, 4 and 4 cells, for the 1st, 2nd, 3rd and 4th pulse respectively. Statistically significant changes in mEPSC frequency due to application of corticosterone (compared to baseline) in the presence of cycloheximide are indicated by * ($p<0.05$) or ** ($p<0.01$).

	pulse 1 absolute value during baseline	% change during cort	pulse 2 absolute value during baseline	% change during cort	pulse 3 absolute value during baseline	% change during cort	pulse 4 absolute value during baseline	% change during cort
DG								
Frequency (events per sec)	0.68 ± 0.12	26.7 ± 6.2**	0.75 ± 0.10	38.0 ± 5.0**	1.09 ± 0.23	27.0 ± 6.2**	1.09 ± 0.24	22.3 ± 5.0**
Amplitude (pA)	-8.8 ± 1.2	-0.34 ± 0.87	-9.5 ± 0.7	7.0 ± 8.5	-6.8 ± 0.3	15.1 ± 10.0	-6.4 ± 0.6	9.2 ± 6.6
Tau rise (ms)	3.8 ± 0.1	-5.6 ± 5.3	3.3 ± 0.2§	17.3 ± 1.5&	4.4 ± 0.3#	-1.0 ± 7.5	3.7 ± 0.1§	0.85 ± 4.73
Tau of decay (ms)	8.8 ± 1.1	11.7 ± 3.4	10.7 ± 0.5	16.1 ± 6.1	11.0 ± 1.6	4.4 ± 9.0	12.0 ± 1.2	8.7 ± 5.2
BLA								
Frequency (events per sec)	3.0 ± 0.7	50.8 ± 15.9*	2.2 ± 0.5	37.9 ± 8.0*	2.7 ± 0.3	-1.8 ± 22.2&	2.7 ± 0.8	-6.4 ± 15.1&
Amplitude (pA)	-13.0 ± 1.0	2.0 ± 3	-14.2 ± 1.1	-2.6 ± 3.8	-15.9 ± 1.7	1.6 ± 2.5	-14.6 ± 0.4	9.8 ± 10.2
Tau rise (ms)	1.7 ± 0.1§	11.7 ± 6.5	1.6 ± 0.2	-1.8 ± 17.4	1.5 ± 0.1§	-8.0 ± 8.8	1.6 ± 0.1	-6.8 ± 7.5
Tau of decay (ms)	8.1 ± 1.3	24.4 ± 16.5	6.8 ± 0.6	-1.6 ± 19.5	6.9 ± 0.8#§	-1.6 ± 19.5	5.7 ± 1.0	21.8 ± 27.8

Table 2. The attenuated response to a 3rd pulse of cort in DG cells and the reversed response to a 2nd cort pulse in the BLA are prevented by cycloheximide. Absolute values for baseline mEPSC frequency prior to the 4 pulses of cort are mostly not affected in the presence of cycloheximide compared to vehicle; cort in the presence of cycloheximide also did not significantly change baseline mEPSC amplitude, tau rise and time constant of decay, nor the relative change in any of these properties during the 4 pulses of corticosterone. *: $p<0.05$ relative to baseline prior to cort application; #: $p<0.05$ relative to baseline before 1st pulse; §: $p<0.05$ compared to corresponding baseline values in the absence of cycloheximide; &: $p<0.05$ relative to cort effect by 1st pulse. Number of cells for each area / pulse in legend of Figure 3.

Discussion

We earlier hypothesized that the nongenomic mechanisms by which corticosteroids affect limbic cells are ideally suited to quickly translate multiple shifts in corticosteroid levels into changes in neuronal activity (192); this could render cells more (or less) susceptible to input at various stages of e.g. ultradian pulses. We now tested the experimental evidence for this assumption. The main finding is that CA1 and DG cells - despite some attenuation - indeed quite reliably translate repetitive shifts in corticosteroid level into altered glutamate transmission. However, principal cells in the BLA show a very complex pattern of responses to repeated corticosteroid exposure, in which BLA cells over time become fully resistant to the effects of hormone treatment on mEPSC activity. The pattern of BLA responses becomes more comparable to hippocampal cells when protein synthesis is inhibited, but still gradually turns refractory to hormone treatment. This underlines that, as observed earlier for gene-mediated corticosteroid actions (195); (179), nongenomic effects display a distinct regional specificity, so that exposure of the brain to a wave of corticosteroids does not induce a uniform response in all areas expressing receptors.

In this study we exposed cells to four identical pulses, using a high corticosterone concentration. While this can serve as a model to demonstrate how repetitive shifts in corticosteroid level are translated into neuronal activity in general, it only partly mimics the natural situation. We selected a concentration of 100 nM corticosterone to ensure a reliable change in mEPSC frequency (41), but this is a high concentration compared to the naturally circulating hormone levels measured with in vivo microdialysis (196); (197), even when the latter are corrected for the recovery-rate of the microdialysis probe. Moreover, in the natural rhythm, ultradian pulse amplitudes will gradually rise (187). Mimicking the latter situation in this first survey would make it very hard to determine the stability of responses to repetitive corticosteroid exposure. To avoid this added level of complexity we here used a pharmacological approach in which pulses of equal amplitude were mimicked, similar to the approach used in an earlier in vivo study (71). Follow-up studies, though, with lower concentrations of the hormone will be necessary to confirm that the currently observed patterns are not only relevant when an organism is exposed to multiple stressors in rapid succession but also apply to the effects of ultradian corticosteroid pulses on the brain.

Hippocampal cells -be it located in the CA1 area or DG- consistently responded less clearly to the 3rd pulse; remarkably, hippocampal cell responses restored upon the 4th exposure to corticosterone, a consistent finding for which we currently have no explanation. The time-frame of the attenuated response to the 3rd pulse, i.e. approximately 2 hrs after the first exposure to corticosterone, is sufficient to allow gene-mediated corticosteroid effects to develop. In accordance, the attenuation in DG cells was not observed when protein synthesis was prevented. Slow gene-mediated effects of corticosterone on hippocampal cell physiology have indeed been described before (see for review (189)). In this respect, the slow GR-dependent increase in surface expression of the AMPA receptor subunit-2 in cultured hippocampal cells (32); (113) and the enhanced amplitude of AMPA receptor-mediated mEPSCs in CA1 cells (30) seem most relevant. Using a protocol of multiple pulse exposure (as opposed to a single pulse protocol used earlier(30) we presently did not observe a significant slow enhancement in mEPSC amplitude, neither in CA1 pyramidal cells nor in DG granule cells. We did, however, see

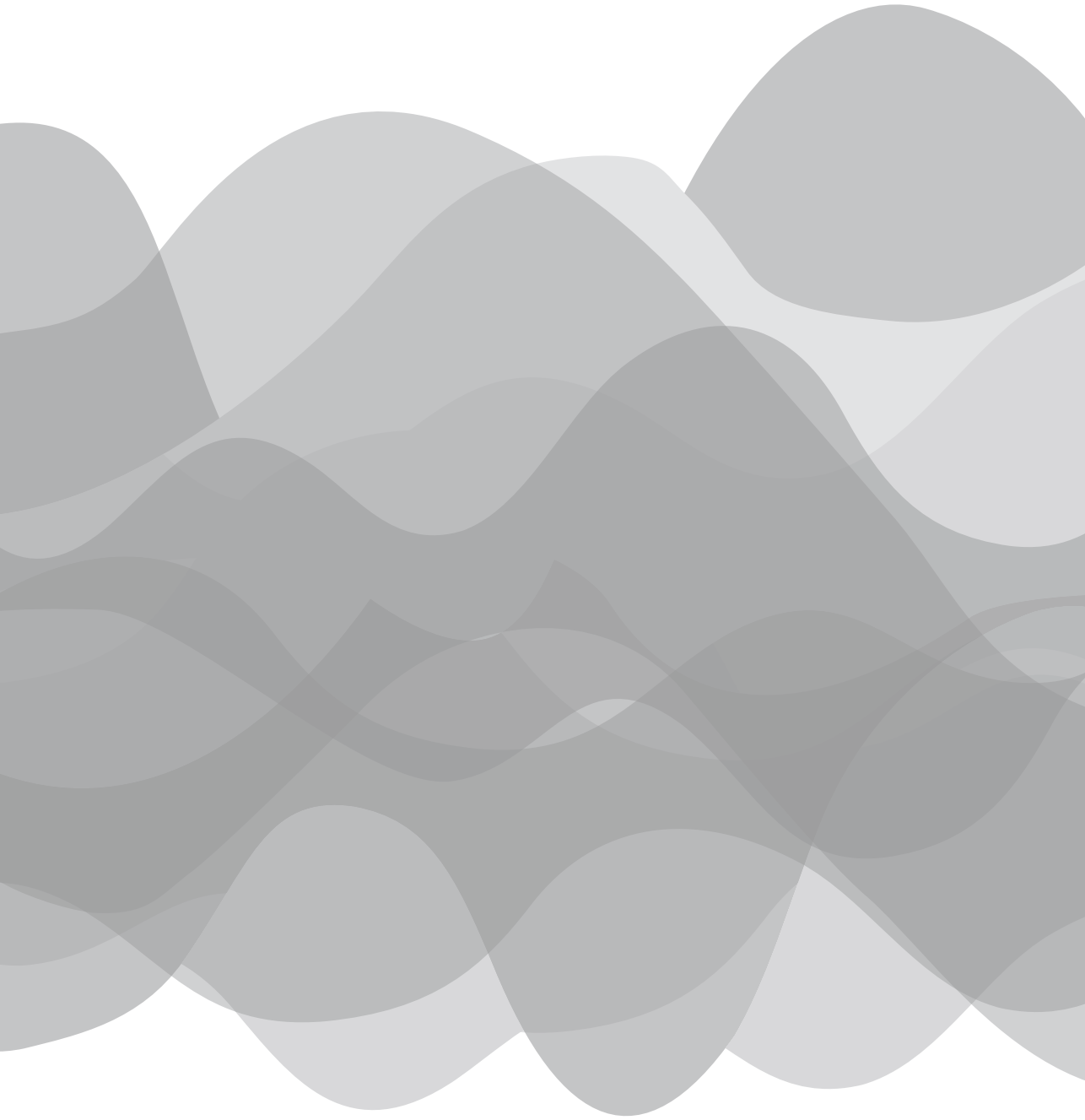
some slow (and in some cases cycloheximide-dependent) changes in the kinetic properties of mEPSCs upon repeated exposure to pulses of corticosterone, which were not seen several hours after exposing hippocampal neurons to a single pulse of corticosterone (30). This underlines that slow (putatively gene-mediated) effects of corticosterone on mEPSC properties in the hippocampus are not entirely the same when cells are exposed to a single pulse as opposed to multiple pulses such as may occur with multiple stressors in rapid succession or, potentially, as a result of ultradian shifts during the circadian peak. Gradual adjustments in presynaptic glutamate release may thus develop secondary to multiple rapid actions on glutamate transmission. Obviously, the mechanism underlying this discrepancy between physiological properties after a single versus multiple hormone pulses needs further investigation.

Previously we showed that the sustained nature of the response in the BLA after initial exposure to corticosterone depends on both MR and GR activation as well as protein synthesis (180). The current study not only confirms the latter but furthermore demonstrates that the flip from enhanced to decreased mEPSC frequency during the first and second pulse respectively requires protein synthesis: without protein synthesis BLA cells responded to two successive corticosteroid pulses as if they were hippocampal cells. This suggests that the sustained nature of the initial enhancement in mEPSC frequency seen in BLA cells is critical for the development of the later nongenomic GR-dependent decrease in mEPSC frequency. The most important finding of the present study, however, is that BLA cells apparently become refractory to corticosterone application (at least with respect to rapid changes in mEPSC frequency) after the first 2 pulses, even in the presence of a protein synthesis inhibitor. This is suggestive of a receptor internalization process, possibly via phosphorylation and arrestin binding of the receptor, such as has been described for G protein-coupled receptors (198). Interestingly, rapid internalization has indeed been reported for nuclear receptors, specifically the estrogen receptor alpha (199); (200), though not (yet) for corticosteroid receptors.

In summary, CA1 and DG cells quite reliably translate multiple exposures to corticosteroids in rapid succession into matching changes in neuronal activity, which could render these cells more sensitive to synaptic inputs e.g. during the peaks of the ultradian pulses. The comparable profile of CA1 and DG cells in this respect may promote synchronization among hippocampal cells during ultradian variations in corticosteroid level, a second mode by which ultradian pulses could be important for brain function. This sharply contrasts with principal cells in the BLA which over time become unresponsive to pulses of corticosterone. This would lead to a gradual dissociation between hippocampal and amygdalar responsiveness to repetitive shifts in corticosterone. Interestingly, regional dissimilar sensitivity to stress during the various phases of ultradian pulses was earlier observed with regard to c-fos activation, as reported by (195); they showed steady c-fos activation in hippocampal cells regardless of the moment of stress exposure relative to the ultradian pulse, whereas the activity of amygdalar cells strongly depended on timing of the stressor. It is tempting to speculate that this dissociation between BLA and hippocampal cells responsiveness to corticosteroids may have consequences for the degree to which emotional and neutral aspects of stress-related information can be encoded (201); (202); (203).

Acknowledgments

This work was supported by grant 817.02.017 from the Dutch Organization for Scientific Research NWO (awarded to HK). Our sincere thanks to Marek Brandys for his help in statistical analysis.



CHAPTER 4

GluA2 surface diffusion in response to pulses of corticosterone

N Pasricha[1], A Kerkhof[1], L Mikasova[2], M Joëls[1], H Karst[1] and L Groc[2]

[1] Dept. Neuroscience & Pharmacology, Division of Neuroscience, UMC Utrecht, Rudolf Magnus Institute, Utrecht, The Netherlands
[2] CNRS, Interdisciplinary Institute for Neurosciences, Universite de Bordeaux, Bordeaux, France

In preparation

Abstract

Corticosterone is released in hourly pulses, with peak amplitudes following a circadian pattern. Over the past decade, corticosterone was shown to have rapid non-genomic effects on various aspects of glutamatergic transmission in the hippocampus, via mineralocorticoid receptors (MRs) most likely residing in the plasma membrane. We hypothesized that these membrane-located receptors provide the means to translate hourly variations in corticosterone level into a functional outcome. In this exploratory study we selected surface diffusion of the AMPA receptor subunit GluA2 -which was previously found to be quickly altered via MRs- as the parameter of interest. Using quantum dot technology, we observed that lateral diffusion of GluA2 subunits was significantly enhanced after the first pulse of corticosterone (10 min, 100 nM), but the effectiveness of corticosterone attenuated in subsequent pulses. Synaptic dwell-time was not affected by any of the pulses. Interestingly, GluA2 subunit diffusion was not significantly enhanced by a pulse of corticosterone conjugated to bovine serum albumin (cort-BSA, which in contrast to corticosterone cannot pass the membrane), but decreased significantly when cort-BSA was applied for the third time. The latter was also seen when the mineralocorticoid aldosterone was administered in the presence of the glucocorticoid antagonist mifepristone, supporting the involvement of a membrane-located MR. In agreement, the decreased GluA2 surface diffusion seen during the third pulse of cort-BSA was blocked by the MR-antagonist spironolactone. Overall, these preliminary results suggest that hourly pulses of corticosterone are only mildly translated into changes in GluA2 surface diffusion. Possibly, effects mediated via membrane-located MRs are masked by other corticosterone actions.

Introduction

When an individual encounters a stressful situation, two systems are activated: the sympatho-adrenomedullar system and the hypothalamic-pituitary-adrenal (HPA) axis, leading to increased release of (nor)adrenaline and corticosteroid hormones respectively. Stress-induced rises in corticosteroid level are superimposed on a circadian release pattern, with high corticosteroid levels at the start of the wake phase (204). The circadian rhythm in fact overarches the peaks of brief hourly (ultradian) bursts, which can be measured in both plasma and brain ((144).

Corticosterone -the prevailing rodent corticosteroid hormone- is highly lipophilic and easily crosses the blood-brain barrier. In the brain, it binds to two corticosteroid receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These receptors are located in the cytoplasm and upon binding of the hormone translocate to the nucleus where they act as transcription factors (24) (25) (26)(27). Both receptors are abundantly present in principal cells of the CA1 hippocampal area and the dentate gyrus; in CA3 neurons, MR but not GR levels are high (205). Due to a high affinity of MRs for corticosteroids, these receptors are under most circumstances occupied and remain occupied in-between ultradian pulses. By contrast, GRs are mostly unbound when corticosteroid levels are low, but gradually become occupied when hormone levels rise (206). GR is occupied in a phasic manner, i.e. it binds high concentrations of corticosterone and unbinds at lower concentration within 30 mins, which roughly coincides with the inter-pulse interval of ultradian pulses (63). This means that nuclear MRs are still largely occupied at the onset of a new pulse, while the GR is then already cleared from the nucleus and ready to respond to a new pulse. Recent experiments showed that GR-dependent transcriptional activity is most optimal when corticosterone is administered in hourly pulses rather than continuously (187).

Corticosteroid receptors have also been shown to mediate effects within minutes; this is not easily explained by genomic pathways. In the hippocampus, these fast non-genomic effects are thought to involve MRs inserted in the plasma membrane (32); (41), although to date such membrane MRs have only been visualized in the amygdala, not (yet) in the hippocampus (43). Because of their rapid mode of action, membrane located MRs are well-suited to translate ultradian corticosterone pulses into altered neuronal function.

Glutamatergic transmission in the hippocampus is sensitive to both rapid (via MR) and slow (via GR) effects by corticosteroids. Thus, GR activation causes a slow (2-4 hours) increase in transmission mediated by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (41). Also, corticosterone via GR has a delayed (after 1-4 hours) effect on NMDA-dependent synaptic plasticity (207). Conversely, corticosterone via presynaptic MRs rapidly increases the release probability of glutamate-containing vesicles (41). Rapid MR-dependent changes were also reported for GluA2-AMPA receptor subunit mobility (32). Similar fast pre- and postsynaptic effects on glutamate transmission were also observed with corticosterone conjugated to BSA, which cannot pass the membrane. This supports involvement of a membrane-located receptor (32); (41). Corticosterone binding to hippocampal MRs also influences LTP and memory performance (208); (209).

Recent findings support that hourly corticosterone pulses are quite well translated into changes in glutamatergic transmission (Chapter 3 of this thesis). Repetitive pulsatory application of corticosterone (100nM for 10 minutes at hourly intervals) to hippocampal slices increased mEPSC frequency at the peak of the pulse through

a presynaptic mechanism, returning back to baseline at the nadir. Interestingly, the increase in mEPSC frequency was attenuated during the third pulse. In the current explorative study, we investigated whether changes in AMPA receptor surface diffusion, caused by rapid *postsynaptic* actions of corticosterone, also 'follow' this pattern of hourly administration. To test this, corticosterone was applied to hippocampal cell cultures in hourly pulses and GluA2-AMPAR surface diffusion was measured using a single-molecule tracking approach. We used MR or GR analogues to get more insight in the receptor involved in these postsynaptic actions, and examined if the effects could be mimicked by the membrane-impermeable conjugate of corticosterone to BSA.

Materials and Methods

Hippocampal cell cultures

Cultures of hippocampal neurons were prepared from 18 day old Sprague-Dawley rat embryos which were decapitated to dissect the hippocampus from the brain. Hippocampal cells were plated at a density of 300,000 cells per ml and grown on poly-L-lysine-coated coverslips in MEM supplemented with Serum Supreme (*BioWhittaker*). This medium was replaced after 4 days *in vitro* (DIV) by a serum-free neurobasal medium. A HEPES buffer was used as medium for the experiments: 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$*$2H_2O$, 1.6 mM $MgCl_2$*$6H_2O$, 10 mM Hepes and 24 mM D-glucose. Cultures were maintained at 37 °C in 5% CO2. Quantum dot experiments were performed in cells 14-21 DIV.

Drug administration

To investigate the effect of a pulsatile rhythm of corticosterone exposure, corticosterone (100 nM) was applied in four consecutive pulses. The pulses were given every hour for 10 minutes, which resulted in ten minutes of build-up, followed by ten minutes of wash-out, and a forty minutes inter-pulse interval (Figure 1A). Pulses were given to the neuronal cell culture dish with four coverslips (the dish was stirred at a constant speed to ensure an equal distribution of the drugs). One coverslip was used per pulse. The first coverslip was exposed to a pulse under the microscope. Simultaneously, 3 other coverslips were exposed to a first pulse. After exposure to this first pulse in the culture dish, a second coverslip was taken to expose to a 2nd pulse under the microscope for recording and so on for the 3rd and 4th pulse. Perfusion under the microscope was realized by mounting the coverslip onto a ludin chamber that contained two tube-holders, one for perfusion and the other for suction. Cells were kept at ~37° C.

The following drugs were used in the experiments to determine the role of MR and GR: Corticosterone (water soluble 2-hydroxypropyl-β-cyclodextrin complex, Sigma-Aldrich; 100 nM; dissolved in PBS); corticosterone-BSA (bovine serum albumin) (Steraloids Inc, UK; 100 nM; dissolved in PBS); aldosterone, a selective MR agonist (Sigma-Aldrich; 10 nM; dissolved in 0.0003% ethanol (EtOH)); spironolactone, a selective MR antagonist (Sigma-Aldrich; 50 nM; dissolved in 0.005% EtOH); mifepristone, a selective GR antagonist (Sigma- Aldrich; 500 nM; dissolved in 0.014% EtOH).

Quantum dot staining and microscopy

Coverslips were incubated at 37°C for ten minutes with a GluR2- mouse antibody (1:800, Millipore). After washing with warm medium, cells were incubated at 37°C

for ten minutes with a mix of quantum dot (QD) anti-mouse 655 (1:1000, Invitrogen) and casein, to prevent nonspecific binding (1:1000, Vector Laboratories, Paris, France). Incubation for one minute with mitotracker (1:10000, Green Mitotracker, Molecular Probes) was applied for later detection of synapses. A custom wide-field single-molecule fluorescence inverted microscope equipped with x100 oil-immersion objective was used for QD visualization. Recording was performed by an EMCCD camera; image acquisition was performed using MetaMorph (Universal imaging Corp.). Twenty frames (50 ms per frame) were recorded with the transmission light and averaged to have a clear overall picture of the neuronal structure. We visualised synapses by using Green Mitotracker and appropriate emission/excitation filters; here again, twenty frames (50 ms per frame) were recorded and averaged. QDs were detected by using a xenon lamp (560RDF55, Omega) and appropriate emission filters (655WB20; Omega Filters). QD-labeled GluA2 subunits were followed in randomly selected dendritic regions. To track the receptor dynamics, a thousand frames were recorded (50 ms per frame) and saved as a stack file. Two movies were recorded prior to giving the pulse (for baseline recording) and two movies were recorded during the last 5 minutes of the rising phase of a corticosterone pulse.

Tracking trajectories

Quantum dots are photostable and bright. Analysis of trajectories by QD analysis is complicated by the fact that they have an on- and off state, and therefore appear to blink. This can be turned into an advantage with the appropriate analysis methods: because of the blinking, individual QDs can easily be distinguished. The methods used for tracking QDs have been described elsewhere (32).

Recorded trajectories of single molecules were reconstructed by correlation analysis between consecutive images, using a Vogel algorithm, and performed by multiple imaging analysis software (MIA), a plug-in for Metamorph. Consecutive tracking of single QDs was performed by custom-made software written in MATLAB. Subtrajectories of single QD-receptor particles were continuously tracked between QD blinks and reconnected across dark blink periods to produce a complete trajectory based on a maximal allowable displacement of 3 pixels between two frames and a maximal allowable dark period of 25 frames. Non-specifically bound QDs and endogenous QD-receptor trajectories were removed from the analysis. The instantaneous diffusion coefficient, D, was calculated for each trajectory, from linear fits of the first 4 points of the mean-square-displacement versus time function using $MSD(t) = \langle r^2 \rangle (t) = 4Dt$.

Data and Statistical analysis

For each experiment, we used 3 to 6 sets of 14-21 DIV hippocampal cultures, obtained from different rat embryos. In this chapter, the instantaneous diffusion coefficient (membrane diffusion) is expressed in $\mu m^2/s$ and group values are represented with the median ± 25-75% (interquartile range, IQR). All of the other group values are expressed as mean ± s.e.m. Comparisons between groups for instantaneous diffusion coefficients were performed using non-parametric statistical tests, Mann Whitney test (pair comparison) or Kruskal-Wallis followed by Dunn's Multiple. A mixed model with two factors (treatment, time) was used for between-group comparisons of time-dependent effects of corticosterone and other drugs. Comparisons between before-after conditions in different pulses for

synaptic dwell time and synaptic content were performed using a non-parametric Mann Whitney test or Kruskal-Wallis statistical test. The significance was set at *p < 0.05, **p< 0.01, ***p < 0.001 compared to the appropriate controls.

Results

The effect of corticosterone pulses on GluA2 receptor subunit surface diffusion

Pulses of corticosterone (Figure 1A) were applied to primary hippocampal cultures to investigate their effect on GluA2 receptor trafficking using single quantum-dot tracing (Figure 1B). Corticosterone treatment differentially affected GluA2 receptor trafficking during the four consecutive pulses (mixed models, Interaction Pulses x Corticosterone $F(1,3) = 5.6$; $p = 0.001$). Follow-up analysis revealed that a gradual attenuation occurred. Thus, GluA2 receptor trafficking was significantly increased at the peak of the first pulse (Figure 1C and 1D; Table 1). This result agrees with previous findings on rapid effects of corticosterone (32). The effect of the second, third and fourth pulse of corticosterone differed from that of the first pulse. While GluA2 surface diffusion was on average increased during the 2nd and 3rd pulse, this did not reach significance (Figure 1E and F; Table 1). No effect at all of corticosterone on GluA2 surface diffusion was found during the 4th pulse (Figure 1E and F; Table 1). The total number of molecules tracked did not change during the whole experiment (Kruskal-Wallis comparing medians, p=0.1548), indicating that the differences observed between diffusion coefficients in different pulses were not due to overall GluA2 receptor number changes.

GluA2 receptor trafficking did not change after application of any of four pulses of HEPES buffer without corticosterone (Table 2). To study the specificity of the corticosteroid action on GluA2 trafficking, we also examined the diffusion coefficients of the GluN1 receptor. These did not change during pulsatile exposure to corticosterone (Figure 2B; Table 3). The baseline before pulse 3 and pulse 4 appeared to be increased compared to the baseline prior to pulse 1, but given the rather low number of observations especially for the third pulse, no conclusions can be drawn at this time.

Corticosterone and synaptic dwelling

The GluA2 receptor was found to stay significantly shorter in the synaptic region after the first and second corticosterone pulse (synaptic dwell time. Baseline before pulse 1: mean = 0.4749 ± 0.02536, N=2798; and during pulse 1: 0.3180 ± 0.01797, N=2145, p=0.0016; baseline before pulse 2: 0.4812 ± 0.03615, N=2638 baseline; and during pulse 2: 0.3728 ± 0.02075, N=2612, p=0.0093; data not shown). No significant differences were seen after the third or fourth pulse (baseline before pulse 3: 0.3583 ± 0.02454, N=1216 baseline; during pulse 3: 0.4208 ± 0.03168, N=957, p=0.1188; baseline before pulse 4: 0.4365 ± 0.04204, N=1376; and during pulse 4: 0.4414 ± 0.03850, N=780, p=0.9309). To measure whether the diffusion of molecules had a certain direction towards either the synaptic or extra-synaptic regions, we also analyzed the number of molecules in the synaptic region versus the total number of molecules. There appeared to be no significant difference between pulses with respect to the change of the percentage of synaptic molecules versus the total (Figure 3A and 3B; 2-way ANOVA, Factor Pulse: $F(1,3) = 1.97$, $p = 0.12$; Corticosterone $F(1,3) = 0.37$, $p = 0.54$). We also found no support that the synaptic content of the GluA2 receptor changed for any of the pulses separately (i.e. compared to its own baseline; pulse 1: $p = 0.4688$, pulse 2: $p = 0.4465$, pulse

Table 1: GluA2 total diffusion coefficient ($\mu m^2\ s^{-1}$) during 4 consecutive corticosterone (cort) pulses. The first pulse caused a significant increase in GluA2 trafficking, but subsequent responses did not reach significance.

	Number of trajectories (N)	Median ($\mu m^2\ s^{-1}$)	Inter-quartile range ($\mu m^2\ s^{-1}$)	p-value (paired-t test, two tailed)
Before Pulse 1	2198	0.00154	0.000368 – 0.2024	
Cort Pulse 1	1911	0.01045	0.000461 – 0.2569	0.0024
Before Pulse 2	2445	0.00175	0.00038 – 0.17065	
Cort Pulse 2	2185	0.00173	0.0003525 – 0.20996	0.0546
Before Pulse 3	1837	0.00194	0.000443 – 0.1022	
Cort Pulse 3	1769	0.001325	0.000386 – 0.1307	0.0871
Before Pulse 4	1531	0.00088	0.000284 – 0.0301	
Cort Pulse 4	1441	0.00079	0.000261 – 0.047966	0.4319

Table 2: Control experiment with HEPES buffer. GluA2 total diffusion coefficient ($\mu m^2\ s^{-1}$) did not change during the 4 vehicle pulses.

	Number of trajectories (N)	Median ($\mu m^2\ s^{-1}$)	Inter-quartile range ($\mu m^2\ s^{-1}$)	p-value (paired-t test, two-tailed)
Before Pulse 1	371	0.0837	0.00165 – 0.266857	
Vehicle Pulse 1	356	0.09125	0.0013775 – 0.26907	0.4847
Before Pulse 2	452	0.0264	0.000406 – 0.20792	
Vehicle Pulse 2	548	0.02005	0.00079 – 0.148039	0.5489
Before Pulse 3	190	0.02755	0.00027 – 0.148703	
Vehicle Pulse 3	308	0.00163	0.00020 – 0.145152	0.0839
Before Pulse 4	173	0.0015	0.0002735 – 0.216	
Vehicle Pulse 4	153	0.00116	0.000175 – 0.12822	0.079

Table 3: NR1 subunit total diffusion coefficient ($\mu m^2\ s^{-1}$) did not change during 4 corticosterone pulses.

	Number of trajectories (N)	Median ($\mu m^2\ s^{-1}$)	Inter-quartile range ($\mu m^2\ s^{-1}$)	p-value (paired-t test, two tailed)
Before Pulse 1	387	0.0216	0.000395 - 0.3189	
Cort Pulse 1	309	0.00318	0.000371 - 0.3725	0.6854
Before Pulse 2	639	0.3531	0.000376 - 0.3531	
Cort Pulse 2	507	0.00348	0.000308 - 0.3634	0.2301
Before Pulse 3	205	0.2486	0.000714 - 0.4514	
Cort Pulse 3	38	0.1524	0.001673 - 0.3853	0.9559
Before Pulse 4	451	0.1664	0.000537 - 0.3740	
Cort Pulse 4	249	0.0348	0.000559 - 0.3577	0.6870

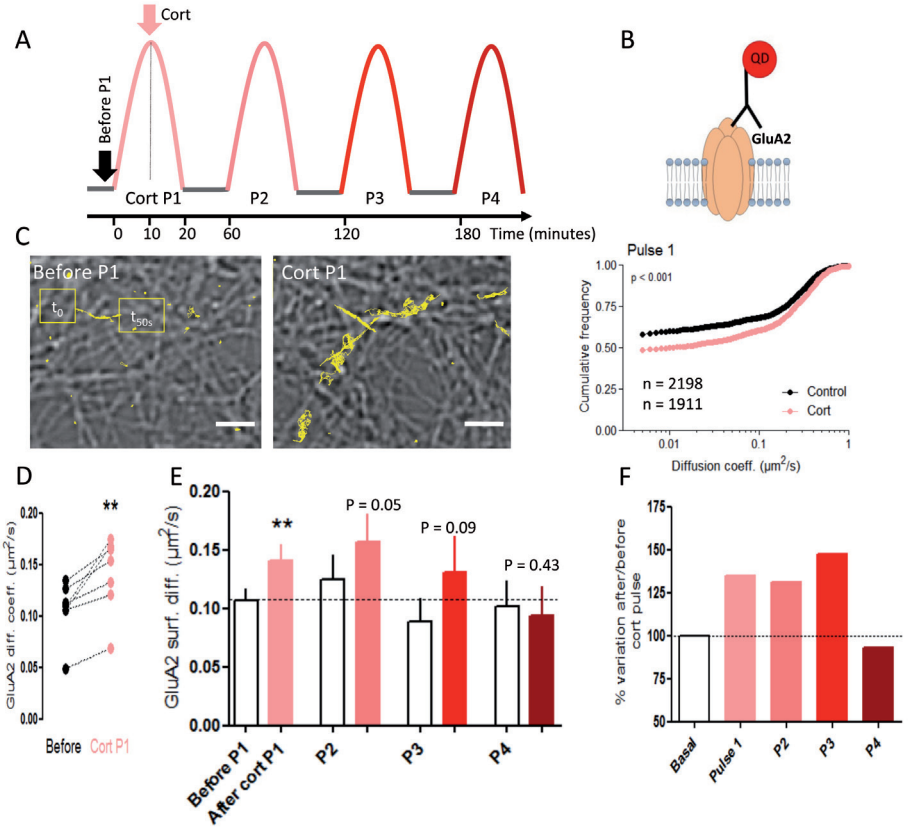


Figure 1. Consecutive corticosterone pulses differentially influence GluA2-AMPAR trafficking

A. Corticosterone pulses were applied once every hour. Ten minutes of build-up of the corticosterone concentration was followed by ten minutes of washout. During the following 40 minutes of rest, no corticosterone was present in the medium. Arrows represent measurement points; two recordings were made before the pulse (black arrow), two recordings were made 5-10 minutes after the start of the pulse (colored arrow).

B. Quantum-dots (QD) were bound to the GluA2 receptor via a specific antibody for GluA2.

C. Left: representative trajectory (10-50 s duration) of surface GluA2 before and after the first pulse of corticosterone (peak at 100 nM). Scale bars represent 5 μm Right: The total diffusion coefficient was significantly increased after the first pulse of corticosterone (cumulative frequency, n=2198 for control and n=1911 for corticosterone)

D. Left: The first corticosterone pulse (peak at 100 nM) significantly increased the mean surface diffusion of the GluA2 receptor (mean, n=7).

E. The corticosterone-induced increase in GluA2 surface diffusion was only observed after the first pulse (bars represent mean ± s.e.m., n=1441-2445 trajectories per group, n = 7 experiments). No significant changes were found during pulse 2, 3 and 4.

F. Percentual change of the mean surface diffusion during corticosterone compared to the baseline, for 4 consecutive pulses. Although the percentual increase was comparable for the 1st, 2nd and 3rd pulse, only the 1st pulse of corticosterone resulted in a significant enhancement. * P<0.05 ** P<0.01 ***P<0.001

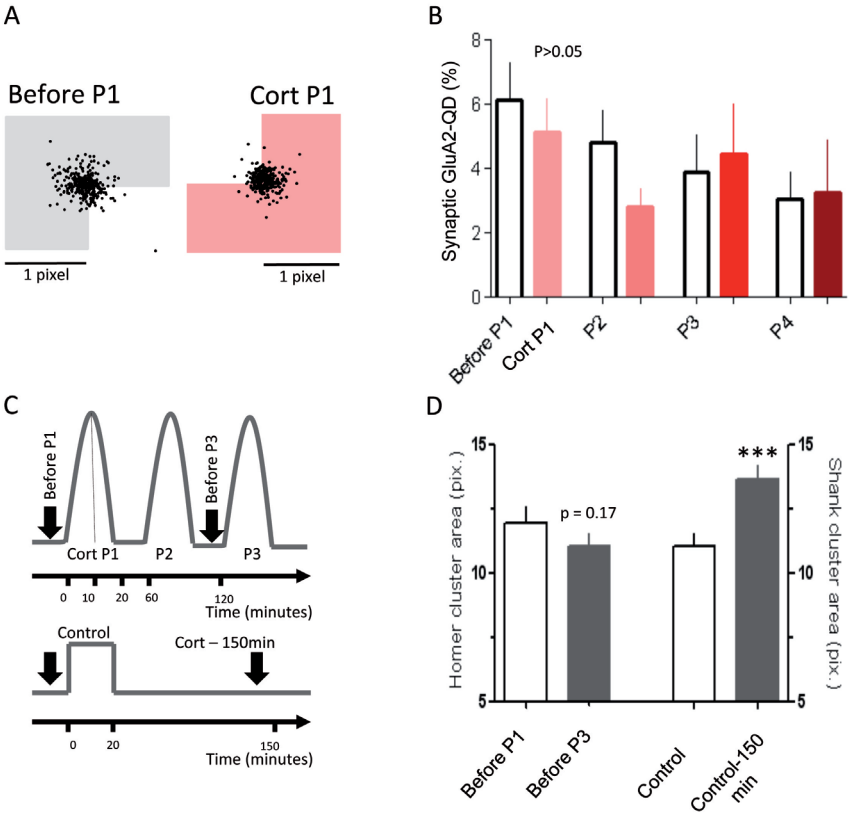


Figure 2. GluA2 synaptic content and postsynaptic density (PSD) size do not change after 150 mins during hourly pulses of corticosterone.

A. Synaptic content was measured by calculating the number of molecules present in synaptic and extrasynaptic domains over 1000 frames (50 ms per frame).

B. Hourly corticosterone pulses rendered no significant differences in synaptic content.

C. PSD size was measured before the experiment and before pulse 3 (120 minutes after onset of the 1st pulse; size measured as area of Homer; top). In an earlier experiment, PSD size was measured 150 minutes after onset of a 20-min application of corticosterone (size measured as area of Shank; bottom).

D. Results show that while a single 20-minutes application of corticosterone increases PSD size (average area size in pixels) after 150 minutes, this is not observed after a comparable interval with pulsatile corticosterone application.

* P<0.05 ** P<0.01 ***P<0.001

3: p = 0.9134, pulse 4: p = 0.6163, Mann-Whitney test). Thus, even though the diffusion coefficient changed in response to the first corticosterone pulses, the content of GluA2 receptors stayed constant.

Interestingly, pulsatile administration of corticosterone did affect the size of the postsynaptic density (PSD). Thus, when PSD size (measured as area of Shank) was determined 150 minutes after onset of a single 20-min application of corticosterone, it was found to be significantly increased (Figure 3C and D). When PSD size (measured as area of Homer) was compared between the baseline prior to the 1st pulse and before the 3rd pulse, i.e. 120 minutes after onset of the 1st pulse with the 2nd pulse in-between, the two values were highly comparable (Figure 3C and D).

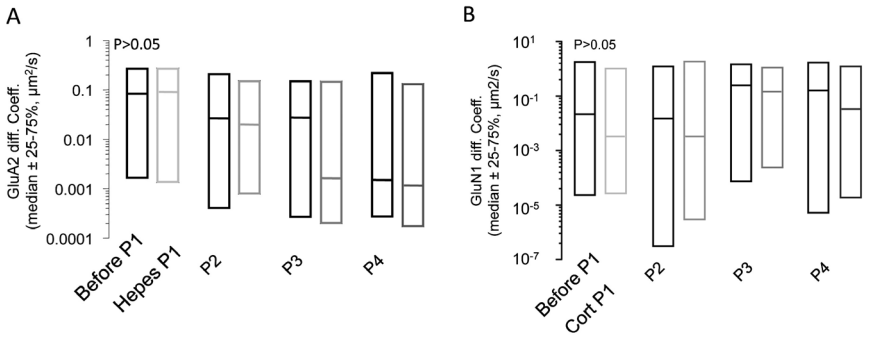


Figure 3. No effect of vehicle pulses on GluA2 surface diffusion or corticosterone pulses on GluN1 surface diffusion.

A. Hepes buffer without corticosterone was applied in pulses. GluA2 receptor surface diffusion was measured before the pulse and 5-10 minutes after onset. No statistically significant effects were observed for any of the pulses.

B. To underline the specificity of the effect of corticosterone on GlA2 surface diffusion, pulses of corticosterone were applied for their effect on GluN1 surface diffusion. Corticosterone did not affect GluN1 surface diffusion.

The effect of corticosterone-BSA on GluA2 receptor dynamics

Consecutive pulses of corticosterone conjugated to bovine serum albumin (cort-BSA) significantly changed GluA2 receptor trafficking (Figure 4; Kruskal-Wallis, $p < 0.0001$; Interaction Pulses x cort-BSA $F(1,3) = 22.8$; $p<0.0001$). GluA2 receptor trafficking was significantly increased after the first pulse of cort-BSA, similar to corticosterone (Figure 4C and 4D; Table 4). GluA2 receptor trafficking did not change at all after application of a second or fourth pulse of cort-BSA (Figure 4D). Mean GluA2 receptor trafficking was significantly reduced after a third cort-BSA pulse (Figure 4B and 4C; Table 4). Synaptic dwell-time of the GluA2 receptor was only influenced after a third pulse of cort-BSA ($p< 0.0001$, based on all trajectories), showing that the GluA2 receptor stayed shorter in the synaptic region.

Involvement of MR or GR

When we applied aldosterone in the presence of the GR antagonist mifepristone, thus selectively activating the MR, the total diffusion coefficient increased after the first pulse ($p< 0.0001$, based on all trajectories), whereas in the second and fourth pulse no effect was observed (see Table 5). During the third pulse, GluA2 receptor

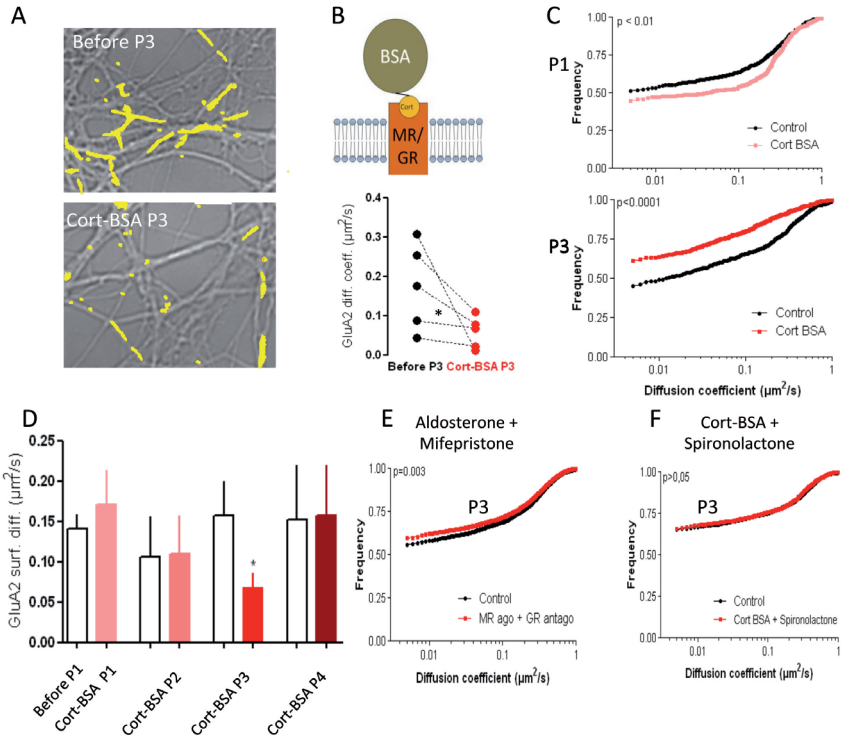


Figure 4. The third pulse responds differently to cort-BSA than to corticosterone.

A. Representative trajectories (10-50 s duration) of surface GluA2 diffusion before and after the first pulse of cort-BSA (peak at 100 nM, scale bars represent 5 µm).

B. Top: The involvement of membrane receptors in translating the effects of a corticosterone pulse was assessed by applying cort-BSA, which cannot pass the plasma membrane. Bottom: The third pulse of cort-BSA, unlike corticosterone itself, lowered surface diffusion of the GluA2 receptor in each experiment performed (n=5).

C. Total diffusion coefficient of all experiments with cort-BSA combined. Top: The first pulse of cort-BSA gives a similar increase in GluA2 receptor surface diffusion as corticosterone itself (cumulative frequency, n = 638 – 791). Bottom: The third pulse of cort-BSA significantly lowers surface diffusion (cumulative frequency, n = 776 – 823).

D. Cort-BSA significantly increased GluA2 surface diffusion during the first pulse and lowered surface diffusion after the third pulse (mean ± s.e.m., n = 638 - 1181 trajectories per group) while no significant changes were observed during pulse 2 and 4.

E. An experiment was performed with 4 pulses of the MR agonist aldosterone in the presence of the GR-antagonist mifepristone. The results indicate that GluA2 surface diffusion was decreased in response to the third pulse of aldosterone / mifepristone, similar to what was seen with cort-BSA (cumulative frequency, n = 3657 – 3715).

F. The decreased GluA2 receptor trafficking during the third pulse of cort-BSA was blocked by the MR-antagonist spironolactone (cumulative frequency, n = 2280 - 2609).

* P<0.05 ** P<0.01 ***P<0.001

Table 4: GluA2 total diffusion coefficient ($\mu m^2\ s^{-1}$) during 4 consecutive cort-BSA pulses. The first cort-BSA pulse yielded a significant increase in GluA2 trafficking. A significant decrease in GluA2 surface diffusion was seen with the third pulse.

trafficking was significantly reduced (p=0.003, Figure 4E). During the first pulse

	Number of trajectories (N)	Median (µm2 s-1)	Inter-quartile range (µm2 s-1)	p-value (paired-t test, two-tailed)
Before Pulse 1	791	0.00405072	0.0006088 – 0.2337	
Cort-BSA Pulse 1	638	0.043371	0.000841 – 0.29195	p=0.0066
Before Pulse 2	1018	0.00293381	0.000618 – 0.09669	
Cort-BSA Pulse 2	666	0.00181685	0.000549 – 0.08370	0.0545
Before Pulse 3	776	0.012139	0.0009086 – 0.2241	
Cort-BSA Pulse 3	823	0.00185265	0.00050 – 0.053861	p< 0.0001
Before Pulse 4	1181	0.00137098	0.00054 – 0.04047	
Cort-BSA Pulse 4	1005	0.00133911	0.000427 – 0.07516	p=0.7663

Table 5: GluA2 total diffusion coefficient ($\mu m^2\ s^{-1}$) during 4 consecutive pulses of aldosterone in the presence of mifepristone. The first pulse showed a highly significant increase in GluA2 trafficking. A significant decrease in GluA2 surface diffusion was seen during the third pulse.

	Number of trajectories (N)	Median (µm2 s-1)	Inter-quartile range (µm2 s-1)	p-value (paired-t test, two-tailed)
Before Pulse 1	2763	0.00863001	0.000587 – 0.22053	
Aldo+Mife Pulse 1	2599	0.0387911	0.000699 – 0.28248	< 0,0001
Before Pulse 2	3600	0.003375	0.00049 – 0.181589	
Aldo+Mife Pulse 2	2094	0.00563	0.0004467 – 0.2310	0.0506
Before Pulse 3	3715	0.00209255	0.000427 – 0.18065	
Aldo+Mife Pulse 3	3657	0.00159912	0.000395– 0.14647	0.003
Before Pulse 4	2207	0.00207	0.000489 – 0.22797	
Aldo+Mife Pulse 4	2034	0.00151441	0.000457 – 0.16687	0.0931

of aldosterone / mifepristone, the synaptic dwell-time was significantly reduced (p= 0.03), whereas the time spent by the GluA2 receptor in the synaptic region increased after a third pulse of aldosterone in presence of mifepristone (p=0.04).

The response to four pulses of aldosterone in the presence of mifepristone resembled the response to cort-BSA rather than corticosterone itself. To further examine the involvement of the mMR, we therefore next applied cort-BSA in the presence of the specific MR blocker spironolactone and focused on the third pulse. The effect of the third pulse of cort-BSA on the GluA2 receptor diffusion coefficient was effectively blocked by the antagonist of the MR (p=0.1371; Figure 4F). Application of cort-BSA / spironolactone did not influence the synaptic dwell-time after the third pulse (p = 0.0754).

Discussion

It has been hypothesized that membrane-located MRs mediating rapid effects of corticosterone are well-suited to translate ultradian corticosterone pulses into altered neuronal function (192). Recent findings support that glutamate release

probability is indeed changed by each of four consecutive pulses of corticosterone, although the response was attenuated during the 3rd exposure (Chapter 3 of this thesis). In the current explorative study, we examined whether changes in GluA2 surface diffusion, caused by rapid *postsynaptic* actions of corticosterone, also 'follow' this pattern of hourly administration.

The results show that surface dynamics of the GluA2 receptor is particularly sensitive to the 1st application of corticosterone and is less affected by subsequent pulses. Furthermore, we found evidence for reduced GluA2 surface diffusion in response to activation of membrane-located receptors that pharmacologically resemble MRs. Since this was not seen with corticosterone itself -which in addition to activating membrane-located MRs can exert many other actions-, we tentatively conclude that pulses of corticosterone through multiple pathways affect GluA2 diffusion such that the response to consecutive pulses attenuates.

A consistent finding in the present study was that GluA2 surface diffusion markedly increased in response to the 1st pulse of corticosterone, which agrees with earlier findings (32). Importantly, increased GluA2 surface diffusion was not found with the HEPES medium, nor did corticosterone change diffusion of molecules along the membrane indiscriminatively: Repetitive corticosterone pulses did not change NR1 subunit trafficking. The latter is in line with an earlier study, although the NR2A/NR2B subunit composition was found to increase 1 to 2 hours after a single 20-minute corticosterone application (210). Interestingly, regulatory mechanisms of glutamate receptor mobility differ between distinct subunits of AMPAR. Among the AMPAR subunits, which range from GluA1 to 4, the role of GluR1 in synaptic plasticity paradigms, such as long-term potentiation (LTP) and long-term depression (LTD), has been extensively studied. GluR1 knockout mice lack LTP in the CA1 region of adult hippocampus (211) (212) suggesting a critical role. It will be interesting to look into GluA1 trafficking during pulses of corticosterone and to examine the functional consequences of pulsatile corticosterone application for LTP induction in hippocampal neurons.

Previously it was demonstrated that the rapidly increased GluA2 receptor trafficking in response to a single application of corticosterone is maintained even after two hours (32). In our experiment with hourly pulses, we observed that the level of GluA2 receptor trafficking was rapidly elevated after a first pulse of corticosterone, but returned to baseline after the first pulse but also subsequent pulses. The fact that a static rise in receptor trafficking only occurs when no pulse is intervening implies an important role of the pulsatile rhythm of corticosterone in keeping the diffusion of the GluA2 receptor at basal levels. Moreover, not only the diffusion coefficient went back to baseline, but also the GluA2 receptor synaptic content did not change over time when cells were exposed to 4 consecutive pulses of corticosterone. This is different from the earlier experiment with a single corticosterone exposure (32), where GluA2 receptor content was elevated two hours after a 20-minute corticosterone application. Both observations indicate that intervening pulses can keep the synaptic- and extrasynaptic GluA2 content stable. This is in concert with the view that the pulsatile rhythm is involved in maintaining homeostasis (187). It should be noted, though, that the longer duration of corticosterone application in the previous compared to the current study (20 versus 10 min) may have contributed to the difference. A control experiment in which corticosterone is administered only once for 10 minutes (or in which 4 pulses of 20 min duration are given) is necessary to exclude this possibility.

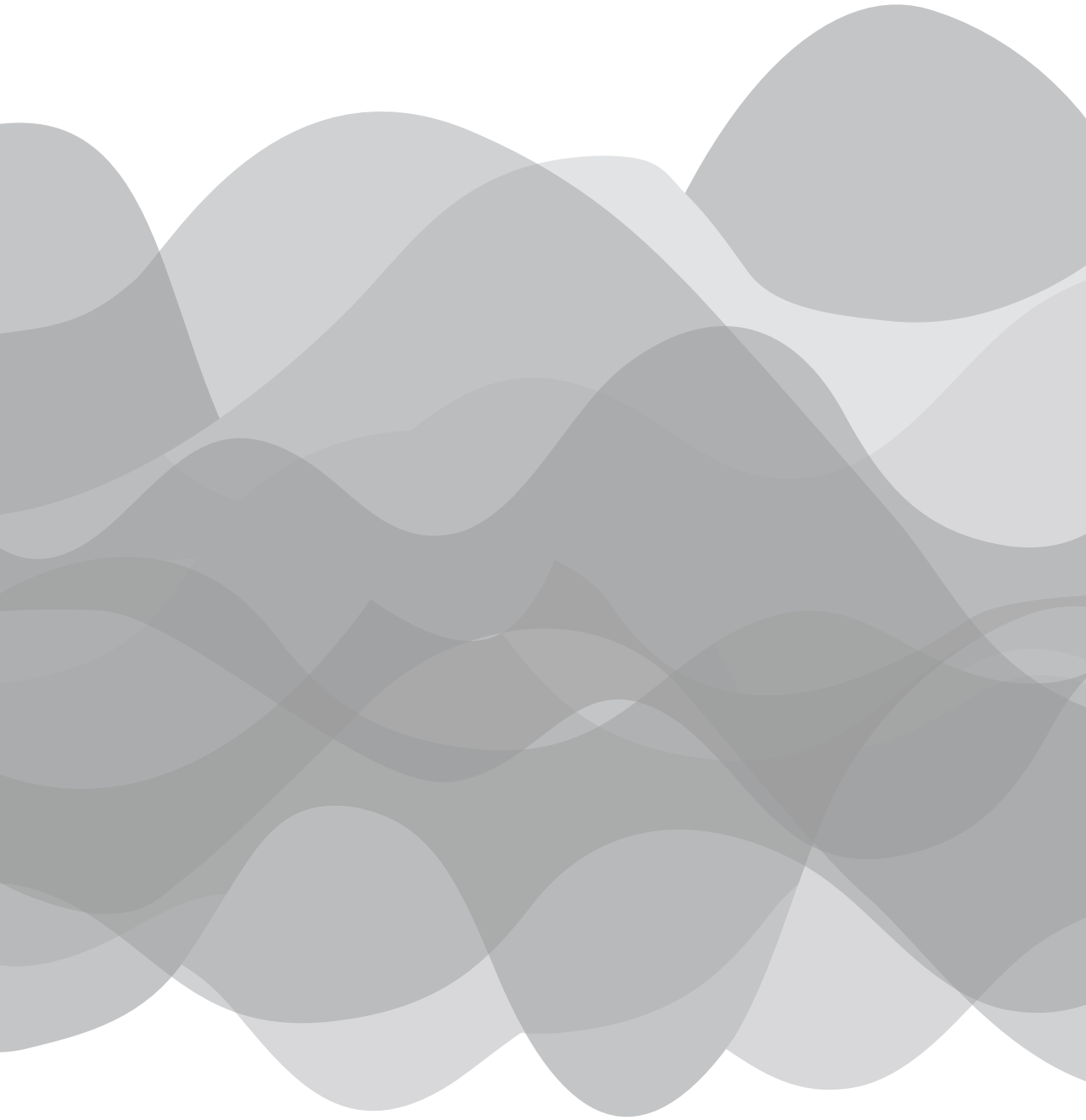
AMPAR synaptic content has been reported to correlate positively with PSD size (213); (214). When corticosterone causes AMPAR subunits to concentrate in the postsynaptic density and attract other proteins, one would expect the PSD size to increase simultaneously. Correspondingly, we found that simultaneously with an increase in GluA2 synaptic content, PSD size was increased 150 minutes after a 20 minutes 100 nM corticosterone application. Yet, we did not observe a change in size at 120 minutes after the onset of the first pulse when in-between another pulse of corticosterone was delivered. The experimental conditions for the two sets of data were not exactly the same. Thus, in the single pulse experiment corticosterone was applied for 20 minutes and PSD size was measured 150 minutes later by the area illuminated via a Shank construct. In the experiment with 4 pulses, corticosterone was administered for 10 minutes and PSD size was determined 120 minutes later by calculating the average size of the Homer areas. While the two data-sets suggest that intervening pulses ensure the synapse to stay at a basal level so that excitation is still possible, proper control experiments are still warranted.

In Chapter 3, we showed with electrophysiological techniques in acutely prepared mouse brain slices of hippocampus that mEPSC frequency increases -suggesting an increased glutamate release probability (180)- during the 1st and 2nd pulse of corticosterone, but that the response is attenuated during a 3rd pulse. On application of corticosterone pulses in the presence of cycloheximide (a protein synthesis inhibitor that blocks the genomic pathway), there was no attenuation at the 3rd pulse, supporting the involvement of genomic pathways in the modulation of these later pulses. Most likely, during early pulses, both membrane and intracellular receptors are activated. The latter could over time change the sensitivity to the former. This also seems to be true for corticosteroid actions on GluA2 surface diffusion, because responses to corticosterone gradually attenuated. Attempts to study the effects of corticosterone in the presence of cycloheximide unfortunately failed; hippocampal cultures consisting of relatively immature rat hippocampal cells seem to be less tolerant to the use of this protein synthesis inhibitor than slices from adult mice.

However, even when we ruled out the involvement of nuclear receptors, i.e. by applying cort-BSA (which presumably does not pass the plasma membrane and hence cannot reach intracellular receptors), responses were not comparable for the consecutive pulses. While we observed a significant increase in GluA2 surface diffusion upon the 1st pulse of cort-BSA, the 3rd pulse caused a significantly reduced diffusion, with a pharmacological profile that fits MR involvement. The differential response between the 1st and 3rd pulse suggests that even non-genomic effects of corticosterone are liable to changes upon repeated exposure. These complex interactions between genomic and non-genomic pathways with repeated hormone exposure are not unprecedented. For instance, also for estradiol it has been shown that membrane-initiated actions change the effect of a subsequent pulse, for example for gene transcription and sexual behavior (215); (216).

While repeated exposure to the endogenous MR and GR ligand corticosterone quite consistently alters *presynaptic* elements of the glutamatergic system in CA1 hippocampal and dentate principal cells (Chapter 3), *postsynaptic* aspects of glutamatergic transmission seem unable to keep up their response (Chapter 4). It should be realized, however, that the methodological conditions of the two studies were very different. Thus, while in Chapter 3 we visually identified hippocampal cells in slices acutely prepared from young-adult mice, results in Chapter 4 were

obtained from cultured rat hippocampal neurons of unknown identity kept 14-21 DIV. Although we tried to keep the design during the actual experiments comparable (e.g. using the same application protocol for corticosterone in both studies), the conditions prior to the experiments nevertheless were considerably different. For future experiments, it is advisable to also carry out electrophysiological recordings in the cultured cells and attempt QD tracking in slices, to further align the experimental conditions. Until then, it remains complex to predict how overall glutamatergic transmission in hippocampal cells responds to multiple exposures to corticosterone. Resolving this issue is important, because hippocampal cells are not only exposed to ultradian variations in corticosterone level but also to variable levels caused by multiple stressors in rapid succession.



CHAPTER 4

Interactive effects of corticosterone and isoproterenol on spontaneous glutamatergic transmission in the mouse basolateral amygdala

H Karst, N Pasricha and M Joels

In preparation

Abstract

Shortly after stress, neurons in the brain -- including in the basolateral amygdala (BLA)--are exposed to noradrenaline and slightly later to corticosterone. We here examined the effects of these two hormones alone and in interaction on spontaneous glutamatergic transmission in the BLA. To this end we recorded the frequency, amplitude and decay timeconstant of miniature excitatory postsynaptic currents (mEPSC) in identified BLA principal neurons. The β-adrenoceptor agonist isoproterenol, like 100 nM corticosterone, increased the mEPSC frequency but did not affect other properties. The dose-dependency was bell-shaped, with the highest response at 3 μM. Co-administration of corticosterone markedly increased the response to 0.1-3 μM isoproterenol; the enhancement was only transient, though, with the lower concentrations of isoproterenol. When corticosterone was not administered simultaneously with but at variable delays after isoproterenol, the efficacy of the steroid to increase mEPSC frequency was greatly reduced, particularly with delays of >40 min. When the order was reversed, i.e. corticosterone was applied >1 hr prior to isoproterenol, the effectiveness of the latter was not altered. Overall, this suggests that isoproterenol initiates a pathway that alters the downstream effects of corticosterone. This may be of relevance for the effectiveness of endogenous corticosterone released after stress, since this hormone reaches BLA neurons *after* they have been exposed to noradrenaline.

Introduction

When an organism is exposed to a potential threat, two systems are being activated, i.e. the sympatho-adrenomedullar system and the hypothalamo-pituitary-adrenal axis(1)(217)(218). As a consequence, the brain is first exposed to a short-lasting wave of noradrenaline(219)(220) and slightly later(144)to a more prolonged wave of glucocorticoids (corticosterone in rodents). The delay between the peak of exposure to noradrenaline and corticosterone was reported to be approximately 20 minutes(144)(221), but corticosterone is most likely already present at earlier time-points after stress, though at a lower concentration.

The co-exposure to these two stress mediators sets the stage for interactive modulation of neuronal activity, at least in neurons carrying receptors for noradrenaline --particularly β-adrenoceptors, which were found to be important in mediating stress effects on behavior(137)(222)(118)-- and corticosterone(138). Interactions are highly relevant for cellular properties that are affected by noradrenaline as well as corticosterone, such as glutamatergic transmission(151)(223)(153)(180). Earlier, we have indeed shown that such interactions do occur, e.g. with respect to evoked glutamatergic transmission and synaptic plasticity. For instance, synaptic plasticity is facilitated by the β-adrenoceptor isoproterenol in the mouse dentate gyrus; this was found to be accelerated by co-applied corticosterone(224). If corticosterone was applied several hours in advance of isoproterenol, the β-adrenoceptor mediated facilitation of synaptic plasticity was suppressed. In the BLA, corticosterone was ineffective in accelerating isoproterenol-mediated facilitation of synaptic plasticity (225)or of AMPA-receptor mediated transmission evoked by synaptic stimulation . However, a pulse of corticosterone administered 1-4 hrs prior to isoproterenol significantly suppressed the β-adrenoceptor mediated facilitation of both synaptic plasticity and synaptically evoked AMPA currents (225)(153). To what extent spontaneous glutamatergic transmission is also affected by isoproterenol and corticosterone alone and/or in interaction was not studied so far.

To address this we focused on the properties of miniature excitatory postsynaptic currents (mEPSCs) -- each of which represents the postsynaptic response to the spontaneous release of one glutamate-containing vesicle-- in identified principal BLA neurons. Previously it was shown that mEPSC frequency, but not amplitude, is rapidly changed by corticosterone via mineralocorticoid receptors (MR) or glucocorticoid receptors (GR), depending on the recent history of the animal(180). We here *i*) examined whether mEPSC properties are also affected by the β-adrenoceptor agonist isoproterenol, administered at various concentrations. We next investigated *ii*) whether co-administration of 100 nM corticosterone altered the response to various concentrations of isoproterenol. To mimic the natural pattern of exposure, *iii*) we first exposed BLA neurons to the dose of isoproterenol yielding the largest response and subsequently, with a variable delay, to 100 nM corticosterone. In a final series, *iv*) we reversed the order of hormone administration (a pharmacological condition), i.e. BLA neurons first received a brief (20 min) pulse of 100 nM corticosterone and 1-4 hrs later were exposed to the dose of isoproterenol yielding the largest response.

Materials and Methods

Male young-adult C57/Bl6 mice (6–9 wk old; Harlan CPB) were used in all experiments. The mice were decapitated under rest in the morning (i.e., when

corticosteroid levels are very low). Immediately after decapitation, the brain was removed from the skull and chilled (at □4°C) in artificial cerebrospinal fluid (aCSF) containing (in mmol/L): NaCl 120, KCl 3.5, MgSO4 1.3, NaH2PO4 1.25, CaCl2 2.5, D-glucose 10, and NaHCO3 25.0, gassed with 95% O2-5% CO2. The frontal lobes and cerebellum were removed and then the caudal side of the brain was glued on the slicing plateau of a vibroslicer (Leica VT 1000S; Leica Instruments, Nussloch, Germany); this allowed to prepare coronal slices (350 μm thick) containing the BLA. Slices were stored at room temperature in aCSF.

One slice at a time was placed in a recording chamber mounted on an upright microscope (Axioskop 2 FS plus; Zeiss) with differential interference contrast, water-immersion objective (40×), and 10× ocular. The slices were continuously perfused with aCSF (flow rate 2–3 mL/min, temperature 32 °C, pH 7.4) to which bicuculline methobromide (20 μm; Santa Cruz Biotechnology) and tetrodotoxin (0.5 μm; Latoxan) were added to block GABAA receptor-mediated signals and action potentials, respectively. Whole-cell voltage-clamp recordings were made with an Axopatch 200B amplifier (Axon Instruments) using borosilicate glass electrodes (impedance 4–6 MΩ, 1.5-mm outer diameter, 0.86 mm inner diameter ; Harvard) pulled with a micropipette puller (Brown/Flaming P-87; Sutter Instruments). For mEPSC recordings, the intracellular pipette solution contained 120 mM Cs methane sulphonate, 17.5 mM CsCl, 10 mM Hepes, 5 mM BAPTA, 2 mM MgATP, and 0.1 mM Na GTP (295 mOsm, pH 7.4 adjusted with CsOH). BAPTA was obtained from Molecular Probes; all other chemicals were purchased from Sigma.

Neurons in the BLA were selected for recording if they displayed a pyramidal-shaped cell body. All mEPSCs were recorded with a holding potential of −70 mV. From each cell, we determined the following mEPSC properties: frequency, amplitude and time constant for decay. Series resistance and capacitance were monitored during the whole recording. Responses were filtered at 5 kHz and digitized at 10 kHz (Digidata 1322A; Axon Instruments). All data were acquired, stored, and analyzed on a PC using pClamp 9.2 and Clampfit 9.2 (Axon Instruments).

To test the influence of the β-adrenergic agonist isoproterenol-bitartrate (Sigma-Aldrich; dissolved to its final concentration in ACSF) on mEPSC properties, one specific concentration (0.3, 1, 3, 10 or 30 μM) was applied for 15 min. Corticosterone (Sigma Aldrich; 100 nM in 0.01% ethanol) was either *i*) co-applied with isoproterenol; *ii*) perfused to a cell recorded with a delay of 15-90 minutes after termination of the previous cell, which was exposed to isoproterenol only; or *iii*) administered 1-4 hrs prior to isoproterenol. Previous studies have shown that the latter treatment is sufficient to cause gene-mediated changes in cellular properties(181).

Unless stated otherwise, we applied paired *t* test comparisons between mEPSC properties determined during the final 5 min of baseline recording and the final 5 min of recording in the presence of isoproterenol or corticosterone. If mEPSC properties determined 1–4 h after corticosterone treatment were compared with those after vehicle treatment, we used unpaired statistics.

Results

Dose-dependent effects of isoproterenol on mEPSC frequency

Isoproterenol was found to increase the mEPSC frequency of BLA neurons in a dose-dependent manner (see Figure 1). The lowest dose tested (0.3 μM) on average

increased the frequency with approximately 15%, but this did not attain statistical significance. However, 1 □M isoproterenol yielded a significant enhancement in mEPSC frequency, as did the slightly higher dose of 3 µM. Interestingly, a further increase in the dosage of isoproterenol led to a reduction in the change in frequency, so that the effects observed with 10 or 30 µM isoproterenol were no longer significant. Overall this resulted in an inverted U-shaped dose-dependency. None of the doses of isoproterenol caused significant changes in either the mEPSC amplitude or the decay time constant (Table I).

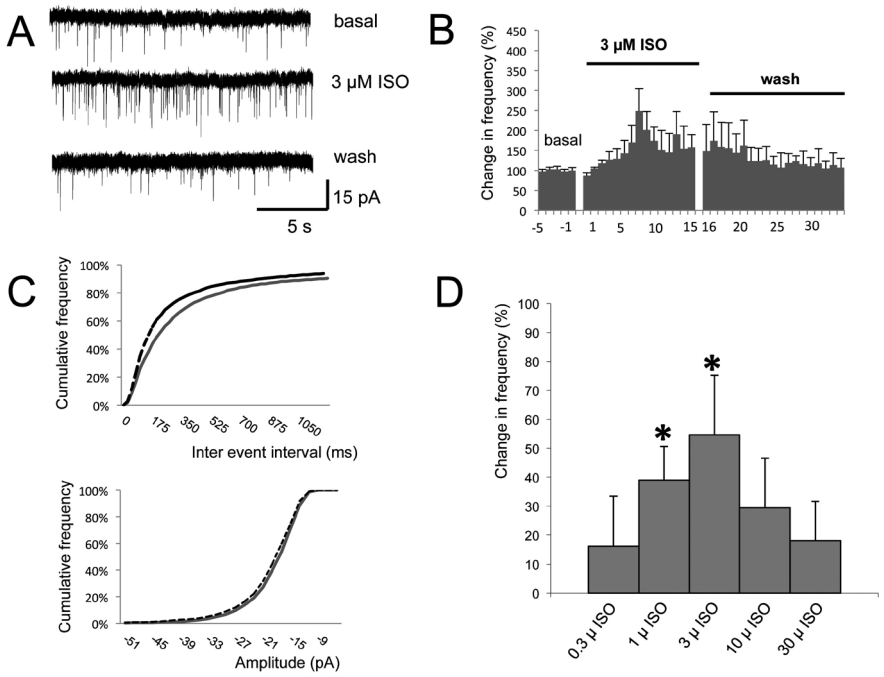


Figure 1. Isoproterenol enhances mEPSC frequency of BLA principal neurons (A). The mEPSC frequency over time is shown for BLA neurons (average of n = 6 cells) before, during and after application of 3 µM isoproterenol in B. During the incubation of isoproterenol the increase in the frequency peaked between 5 and 10 minutes and stabilized during the following 5 minutes. Comparison of the averaged frequency during baseline the final 5 min during isoproterenol administration (t = 10–15 min) revealed a statistically significant difference (paired t test, $P < 0.05$). Note that the increased mEPSC frequency returned to baseline levels during washout. The cumulative distribution of mEPSC frequencies in BLA neurons (C) showed a significant enhancement during isoproterenol administration (grey line; Kolmogorov–Smirnov analysis, $P < 0.05$) compared with baseline (black line). No change was observed in the frequency distribution of mEPSC amplitudes during isoproterenol application (baseline in black bars, isoproterenol application in grey). Isoproterenol was found to increase the mEPSC frequency of BLA neurons in a dose-dependent manner (D). A maximal effect was reached with a dosage of 3 µM isoproterenol. Higher dosages were less effective. Overall this resulted in an inverted U-shaped dose-dependency.

Table 1. None of the dose of isoproterenol causes changes in the decay-tau and amplitude of the mEPSCs in the BLA.

	Tau-decay (ms)	Amplitude (nA)	Capacitance (pF)	Rinput
basal	8.42 ± 0.95	16.84 ± 1.45	32.3 ± 7.0	16.0 ± 2.2
0.3 µM ISO	8.74 ± 1.24	16.33 ± 2.02		
basal	6.79 ± 0.75	17.47 ± 1.32	26.3 ± 5.4	17.0 ± 3.0
1.0 µM ISO	6.78 ± 0.59	16.88 ± 1.54		
basal	8.66 ± 0.60	19.32 ± 1.90	36.5 ± 3.6	14.3 ± 2.3
3.0 µM ISO	7.20 ± 0.36	19.53 ± 2.14		
basal	9.86 ± 1.39	16.52 ± 1.51	24.4 ± 3.4	18.4 ± 1.3
10 µM ISO	8.20 ± 0.52	15.67 ± 0.58		
basal	7.09 ± 0.95	17.55 ± 1.58	30.8 ± 4.6	16.2 ± 1.8
30 µM ISO	6.06 ± 0.35	16.08 ± 1.30		

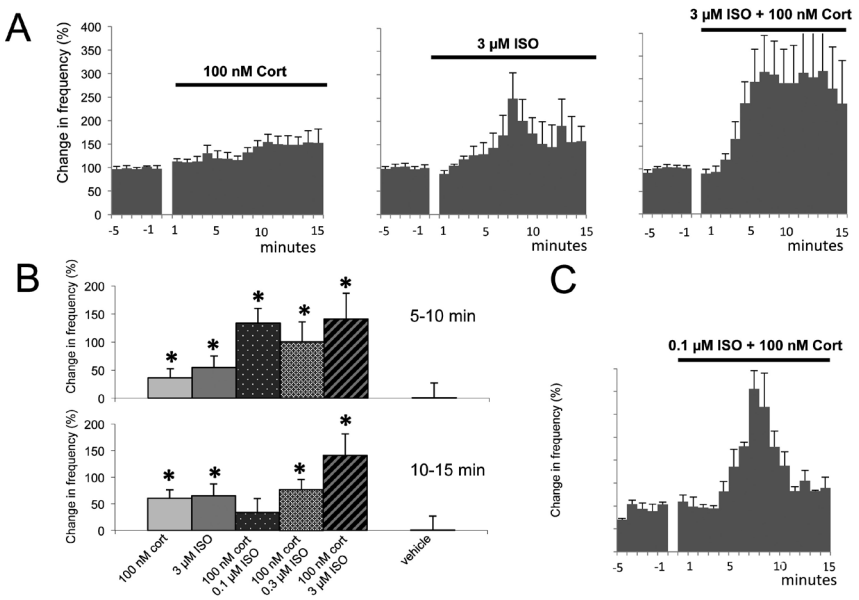


Figure 2. Co-application of 100 nM corticosterone and 3 µM Isoproterenol approximately cumulated the effect of each of the compounds (A). Accumulation of the effects was obtained for other dosages of isoproterenol (0.1 and 0.3 µM) at the time interval of 10-15 minutes after the start of application (B). However, low dosages of isoproterenol, which were not affective when applied solely (see fig 1), cause a significant increase of the mEPSCs frequency when co-applied with corticosterone at the time interval between 5 and 10 minutes (B). In C the change in the frequency over time is shown for 0.1 µM isoproterenol and 100nM corticosterone. It seems that isoproterenol is only affective at 5-10 minutes after the application.

Co-administration of isoproterenol and corticosterone

Corticosterone (100 nM) by itself increased the mEPSC frequency to a comparable extent as the most effective dose of isoproterenol (3 μM; see Figure 2A), while vehicle application was completely ineffective. When the two compounds were co-applied, the increase in mEPSC frequency was approximately doubled. One possibility is that corticosterone and isoproterenol act on different pathways, so that their effects will be summated. However, this appeared not to be the case. Thus, co-application of 100 nM corticosterone with lower doses of isoproterenol (i.e. 0.1 or 0.3 μM), which by themselves were ineffective in significantly enhancing the mEPSC frequency, led to a comparable overall increase in mEPSC frequency as seen with 3 μM isoproterenol. With none of the combinations, mEPSC amplitude or kinetic properties were altered (see Table I).

Although 0.1 μM isoproterenol was as effective as 3 μM when co-applied with 100 nM corticosterone in enhancing the mEPSC frequency, the effects of the former dose were only transient (Figure 2C). Thus, 10-15 minutes after co-application of the two compounds was commenced, mEPSC frequency was enhanced to a large extent. However, 5 minutes later the change in mEPSC frequency relative to baseline was doubled for the higher dose of isoproterenol, while the change in mEPSC frequency due to co-application of the lower doses of 0.3 or 0.1 μM with corticosterone did no longer differ from the effect of corticosterone alone. The data supports that low doses of isoproterenol transiently amplify the effect of100 nM corticosterone on spontaneous glutamatergic transmission in the BLA (or vice versa), while a higher dose causes more lasting effects.

Corticosterone administered with variable delay after isoproterenol

Earlier studies have shown that BLA neurons show metaplasticity in their response to corticosterone, i.e. they display enhanced mEPSC frequency when corticosterone is applied to cells that have not recently been exposed to corticosterone but decreased frequency in BLA cells to which corticosterone was already administered 1 hr earlier (Karst et al., 2010). We here replicated the phenomenon of metaplasticity and demonstrate that this shift in responsiveness to corticosterone occurs approximately 1 hr after termination of the first pulse (Figure 3A).

Given that 3 μM isoproterenol enhances mEPSC frequency similarly as corticosterone, we wondered if a comparable type of metaplasticity in responses to corticosterone occurs when slices have been pre-treated with the β-agonist. This is a very relevant question, because in intact animals corticosterone reaches BLA cells somewhat later than noradrenaline after exposure to stress(221). To mimic this situation, we applied (100 nM) corticosterone with various delays after the slice (and hence all cells) had been exposed to isoproterenol (3 μM, Figure 3B).

While corticosterone increased the mEPSC frequency by 50-60% in untreated slices (see Figure 2) prior exposure to 3 μM isoproterenol substantially reduced the effectiveness of corticosterone (Figure 3B). Cells responding with an increase in mEPSC frequency of >10% were only encountered within 40 minutes after isoproterenol administration was terminated. The majority of the cells, though, responded with a reduction or hardly any change in mEPSC frequency to corticosterone. The distribution and averaged responses over all cells was very different between slices pretreated with isoproterenol or with aCSF (Figure 4B). The data suggests that isoproterenol can affect subsequent responses to corticosterone, and does so with a shorter delay than corticosterone itself.

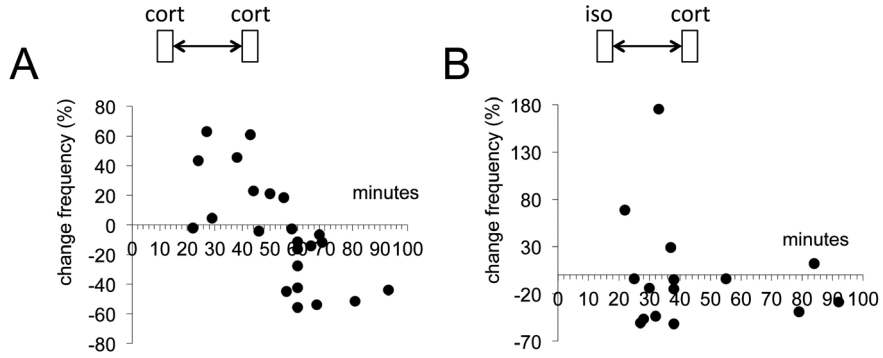


Figure 3. Here we show that metaplasticity of corticosterone occurs approximately from 60 minutes after the first application of corticosterone. After termination of 10 minutes corticosterone (100 nM) application at time 0 in A, a second application of 100 nM corticosterone causes again an increase in the frequency of BLA neurons when applied within one hour. Application after more than one hour after the first application causes a reduction of the mEPSC frequency. Surprisingly the same effect was obtained by a double application of isoproterenol. (B) On application of ISO (3 μM) for 10 minutes at time 0 followed by application of 100 nM corticosterone shortens the time window to less than 30 mins to cause an increase in mEPSC frequency.

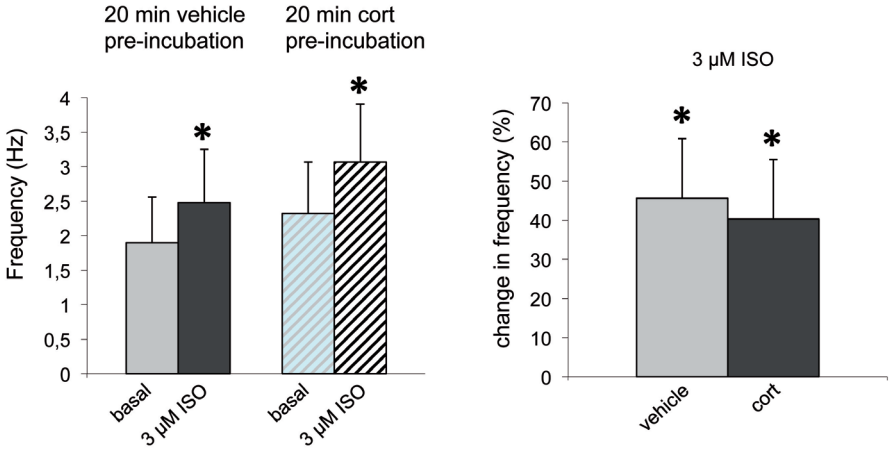


Figure 4. Delayed effect of corticosterone administered prior to isoproterenol doesn't change the effectiveness of isoproterenol. (A) In BLA neurons of slices pre-treated with 100 nM corticosterone for 20 minutes, an application of 3 μM isoproterenol after 1-4 hours cause the same increase in the mEPSC frequency compared to vehicle pre-treated slices. (B) Application of corticosterone (100 nM) has same effect if slices are pretreated with ISO (3 μM).

Corticosterone applied prior to isoproterenol

In view of the slow --presumably genomic-- influence of corticosterone on a second pulse of the corticosterone, we wondered if corticosterone exerts similar effects on isoproterenol. However, this appeared not to be the case. Application of corticosterone for 20 min resulted in a very comparable increase in mEPSC

frequency in response to a pulse of isoproterenol given 1-4 hrs later as pretreatment with the vehicle (Figure 4A).

Discussion

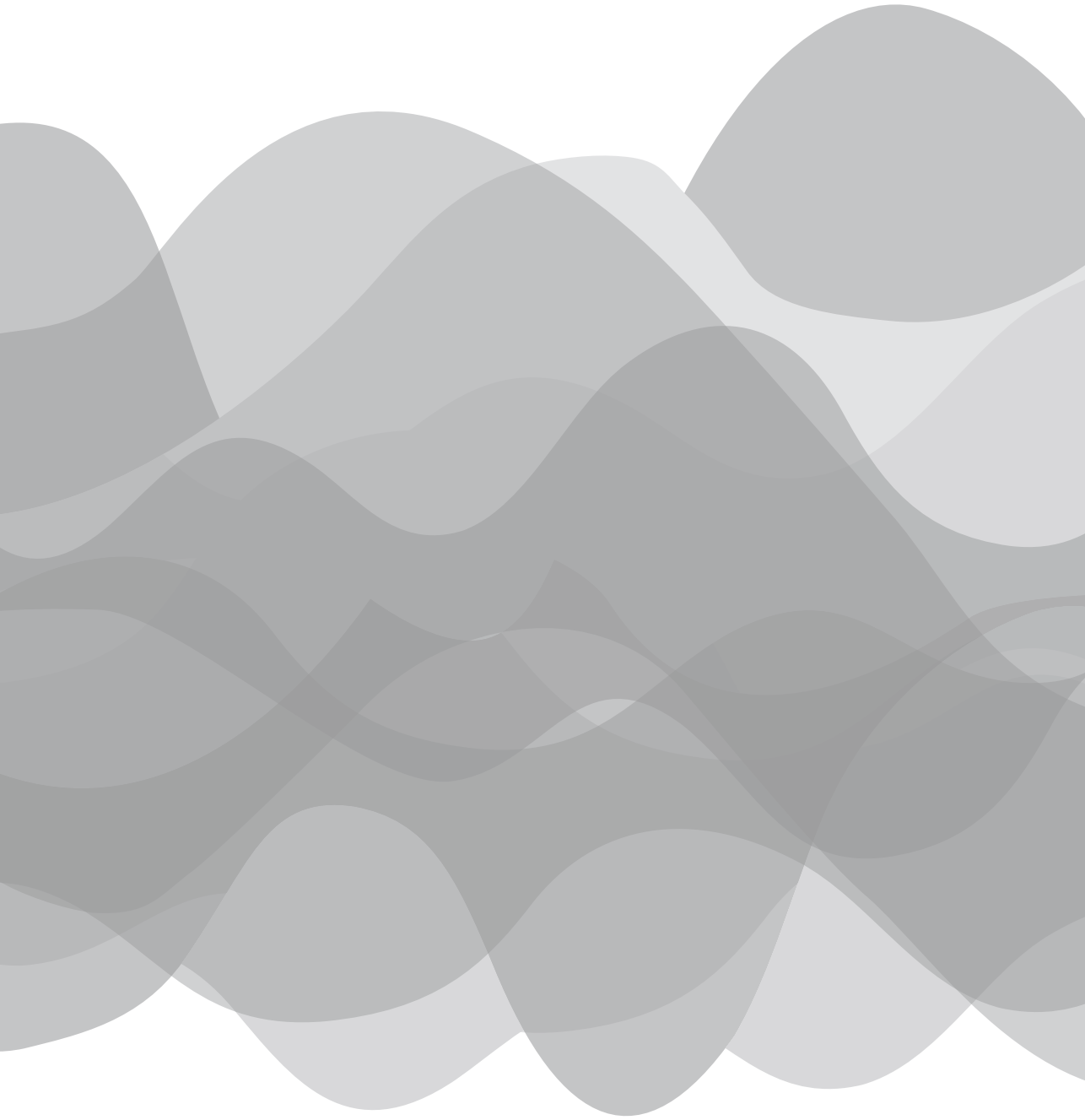
Previous studies have shown that corticosterone and isoproterenol in interaction affect evoked glutamatergic transmission in the mouse BLA(151)(153)(223). We here examined whether interactions also occur with respect to spontaneous glutamatergic transmission, and to this end focused on properties of mEPSCs. The main finding are that: *i*) isoproterenol, like corticosterone, quickly increases the frequency but not amplitude of mEPSCs in identified principal BLA neurons, with an inverted U-shaped dose-dependency; *ii*) the effects of the two compounds are not merely additive; and *iii*) isoproterenol determines the responsiveness to corticosterone but not vice versa. Collectively this suggests that isoproterenol initiates a pathway that alters the downstream effects of corticosterone.

At this moment we can only speculate about the intracellular pathways involved in the metaplasticity of BLA neurons to corticosterone. Previous experiments demonstrated that the presence of MR and GR, as well as protein synthesis and CB-R1 are all necessary for metaplasticity to occur (180). The delay with which metaplasticity occurs after a first application of corticosterone, as observed in the current study, i.e. 60 minutes after termination of the first pulse, is compatible with a genomic mechanism of action. Interestingly, recent observations by(226) showed that dendritic L-type calcium influx in the extended amygdala causes subsequent release of 2-arachidonoylglycerol (2-AG), one of two prevalent endocannabinoids in the brain. Corticosterone is known to slowly enhance L-type calcium currents in BLA neurons via the GR(227). Possibly, an enhanced pool of 2-AG induced via this genomic GR mediated pathway would make BLA cells more prepared for rapid CB-R1 dependent suppression of mEPSC frequency via membrane GRs, an effect also described for the paraventricular nucleus(228). This does not explain, though, why the rapid MR-dependent enhancement of mEPSC frequency is so much attenuated >1 hr after a pulse of corticosterone. Whether or not isoproterenol also converges on the calcium-dependent endocannabinoid pathway is not known. Electrophysiological investigations (151) (223)indicate that isoproterenol enhances P/Q-type calcium currents in BLA neurons. This would also cause a rise in intracellular calcium level, but whether this occurs in the dendritic compartment and affects 2-AG availability or release is unknown. However, convergence of corticosterone and isoproterenol on other intracellular signaling pathways, involving e.g. phospholipase C or protein kinase A, can also not be ruled out. This clearly requires dedicated follow-up experiments.

Interactive actions of noradrenaline and corticosterone on spontaneous transmission could be relevant for behavior. Interestingly, some aspects of the currently described findings were also observed in behavioral experiments. For instance, administration of noradrenergic (and steroid) compounds in the BLA affects emotional learning according to an inverted U-shaped dose-dependency(229) (230) (231), very similar to the dose-dependency here observed for isoproterenol. Also, the involvement of the CB-R1 in noradrenergic / corticosteroid modulation of emotional learning has been reported with respect to behavior (232). Finally, the functionality of corticosteroids on emotional learning was found to depend on noradrenergic signaling, but not vice versa (146). It should be kept in mind, though, that noradrenaline and corticosterone (and most likely other transmitters

and hormones) also affect evoked glutamatergic transmission (224) (225) (153). The characteristics of these interactions differed somewhat from those observed in the present study; the latter may be explained by the fact that mEPSC frequency is mostly determined by properties of the presynaptic compartment, while the amplitude of AMPA responses and synaptic plasticity (also) involves the postsynaptic compartment. Moreover, the functionality of other major transmitter systems in the BLA, such as GABA, is known to be altered by stress hormones (178). A direct link between phenomena at the level of mEPSCs and behavior should therefore be interpreted with great caution.

The observation that rapid corticosteroid actions become less effective in changing mEPSC frequency of BLA neurons after application of isoproterenol -and most distinctly with delays of >40 minutes- raises the question to what extent release of *endogenous* corticosterone released after stress is able to change spontaneous glutamatergic transmission in the BLA at all. Shortly after stress, BLA cells are expected to be exposed to high levels of noradrenaline and low levels of corticosterone, which may result in a short-lived strong increase in mEPSC frequency, although this particular combination of concentrations has not yet been tested. Based on the current findings it seems likely that later on (i.e. >40 minutes after stress onset), when low levels of noradrenaline circulate in combination with high levels of corticosterone; mEPSC frequency is not much altered anymore by the hormones. Clearly, this is a very reductionistic approach, not taking the putative contribution of other stress-related changes in transmitters and hormones e.g. CRF, into account. Nevertheless, future experiments, precisely mimicking the patterns of release for the main stress-related transmitters / hormones, should allow delineating exactly how spontaneous glutamatergic transmission in the BLA is affected in the aftermath of stress.



CHAPTER 6

Rapid responses to corticosterone change after adrenalectomy or chronic corticosterone treatment of mice

N Pasricha, M Joels, H Karst

1 Dept. Neuroscience & Pharmacology, Division of Neuroscience, Rudolf Magnus Institute, UMC Utrecht, The Netherlands

Submitted

Abstract

Corticosterone causes a fast increase in the release probability of glutamate-containing vesicles via non-genomic activation of membrane located corticosteroid receptors: With a dose of 10 nM and higher, the frequency but not amplitude of miniature excitatory postsynaptic currents (mEPSCs) in hippocampal neurons is increased. Previous studies have demonstrated that these rapid effects are mediated by mineralocorticoid receptors present in the membrane. We here elaborated these studies by investigating fast corticosteroid effects in dentate granule cells of mice exposed to rather extreme conditions of the stress system, 1) by strongly reducing corticosterone levels through adrenalectomy (ADX) or 2) by exposing animals to 25 µg/ml of corticosterone in the drinking water for 3 weeks. In ADX animals, the response to a normally sub-threshold dose (3nM) of corticosterone was substantial, compared to the effect seen in sham-operated controls; unexpectedly, this strong response seemed to be mediated by glucocorticoid rather than mineralocorticoid receptors. Conversely, no response at all was observed with 100 nM of corticosterone, which in the sham control group caused a large increase in mEPSC frequency. Mice treated for 3 weeks with a moderately high dose of corticosterone responded (compared to the vehicle treated controls) significantly less to *in vitro* administered 100nM corticosterone, with respect to mEPSC frequency. We conclude that periods of under- or over-exposure to corticosteroids alter rapid effects of corticosteroid hormones on dentate glutamatergic transmission.

Introduction

Short-term exposure to corticosteroid hormones (corticosterone in rodents) helps the organism to adapt to current challenges in the environment and thus is generally considered to be beneficial. By contrast, long-term exposure to stress/ corticosterone or corticosteroid deprivation is thought to be mal-adaptative and a risk factor for disease. For example, Cushing's disease, a condition characterized by hypersecretion of cortisol, is usually associated with symptoms of anxiety, depression and insomnia (233). Addison's disease and adrenal insufficiency -- conditions of extreme hypocortisolism-- are also linked to depression (234)and disturbances in mood, motivation and behavior (235). It is therefore of interest to examine how such extreme variations in circulating levels of corticosterone change the activity of neurons in the hippocampal formation, an area important for the etiology of mood and anxiety disorders.

Previous studies in rodents have already shown that such marked changes in corticosterone level induce molecular and cellular changes in the brain, including in the hippocampal formation (236). For instance, morphological studies revealed a decrease in dendritic complexity of dentate granular neurons in adrenalectomized (ADX) compared to control rats (237)(238). In addition, ADX caused apoptotic-like degeneration of dentate cells (239) (240) (241)associated with functional loss in the dentate network ((171), which may contribute to impairment in behavioral strategy and the ability to acquire new information (114) (173). Low doses of corticosterone, sufficient to activate mineralocorticoid but not glucocorticoid receptors (MR and GR respectively) appear to be important to prevent ADX-induced proliferation and apoptosis (242)(243)(241). In general, MRs are important to keep the hippocampal network viable ((174).

Chronic exposure to glucocorticoids or stress also permanently changes synaptic plasticity (244)(1) and increases glutamate levels (245). Similar effects may happen in the human brain, because dysregulation of glutamate transmission was recently reported in patients with mood disorders (245)(246). In the rat brain, binding levels of both MR and GR were found to be reduced after chronic stress (247) (248). Other papers showed that chronic over-exposure to corticosterone reduces neurogenesis (249) which involves GR activation, as the GR antagonist mifepristone was able to reverse these effects (250)(251).

Interestingly, long-term changes in circulating corticosteroid levels not only change basal cell properties but also affect the responses to acutely administered corticosterone (243)(166). For instance, evoked responses mediated by AMPA receptors in dentate granule cells are not affected by corticosterone in control animals but are strongly enhanced by corticosterone in rats earlier exposed to chronic stress (166). Similarly, corticosterone-induced effects on calcium influx in dentate neurons of chronically stressed rats are different from the effects seen in control animals (179).

These effects on glutamate transmission and calcium influx refer to slow, presumably gene-mediated corticosteroid actions. However, exposure to corticosterone also rapidly increases the mEPSC frequency but not amplitude of dentate neurons in a dose-dependent manner, most likely via membrane-located mineralocorticoid receptors (191). We therefore explored whether these *rapid* corticosteroid actions are affected by prior prolonged changes in circulating corticosteroid levels. To study this, we performed two series of experiments: 1) mice were studied 3-4 days after ADX or sham operation; 2) mice were exposed to

21 days of treatment with a moderately high dose of corticosterone (25 µg/ml) or vehicle in the drinking water. In slices prepared from young adult mice exposed to these conditions, we studied rapid effects on mEPSC properties induced by either a sub-threshold (3 nM) or a near-maximal (100 nM) dose of corticosterone(191), to be able to observe an enhancement or decrease respectively in the effectivity of corticosterone.

Materials and Methods

Animals

Male C57/Bl6 mice (Harlan, The Netherlands; n=53 in total) were group-housed, with food and water provided ad libitum and a 12h light / dark cycle (lights off at 19:00 h). All experiments were approved by the Animal Ethical Committee from Utrecht University. For every experiment, the mouse was decapitated within 2 minutes after being taken from the home cage and always before 10.00 am, to ensure low endogenous levels of corticosterone (181). Corticosterone levels were determined with a radioimmuno assay in trunk blood collected at the moment of decapitation.

Treatment groups

Adrenalectomy and Sham-operated groups

Mice were approx. 5-6 weeks of age at arrival; after approximately 2 weeks, they entered the experiment. Bilateral adrenalectomy (ADX) or sham operations were performed on male C57BL/6J control mice (25g, 7–8 wk old). Animals were anesthesized by administrating isoflurane in conjunction with O2 and NO2 for surgery. After operation, both Sham and ADX mice were single housed with the standard chow, ad libitum. ADX mice were given the option of tap water and a saline drinking-solution (in view of their compromised mineral balance), whereas sham mice were given tap water only.

For pain relief, animals were subcutaneously injected with 0.1 ml of Buprenorphine hydrochloride (10 times dilution of 300 µg/ml, Temgesic) during and 7-8 hours after the surgery. Animals were decapitated 3-4 days after surgery (at approximately 8 weeks of age). Trunk blood samples were collected to determine plasma corticosterone levels.

Prolonged corticosterone or vehicle treatment

Male mice (C57/BL6; 4-5 weeks old on arrival) were group-housed (n = 5/cage) for 7 days in standard cages. During the acclimation period, standard rodent chow and tap water were available *ad libitum*. After the 7-days acclimation phase, mice were single housed with chow *ad libitum* and given corticosterone (25 µg/ml) or vehicle in the drinking water for 3 weeks (252). Corticosterone was first dissolved in 100% ethanol (because of its hydrophobic nature), and then diluted in regular tap water to a final ethanol concentration of 0.1%; (nearly all) vehicle-treated controls had access to a 0.1% ethanol solution only. The water bottles were replaced every week and were also wrapped in aluminum foil to avoid corticosterone degradation. Experiments were conducted after 21 days of treatment. Adrenals and thymus were collected and weighed immediately after decapitation. Trunk blood samples were taken to measure corticosterone levels in plasma.

Slice Preparation and Recording

After decapitating the mouse, the brain was put in artificial cerebrospinal fluid (aCSF, 4o C) containing (in mmol/l): NaCl 120, KCl 3.5, MgSO4 1.3, NaH_2PO_4 1.25, $CaCl_2$ 2.5, D-glucose 10, and $NaHCO_3$ 25.0. Next, coronal slices (350 µm) of the hippocampus were made using a vibratome (Leica VT 1000S, Germany). The slices were incubated for at least 1 h at room temperature before the experiment was performed. We only used slices prepared from the dorsal half of the hippocampus.

Recording method

One hippocampal slice at a time was placed in the recording chamber. At least 10 minutes was allowed before patching of the neurons commenced. An upright microscope (Axioskop 2 FS plus; Zeiss, Oberkochen, Germany) with differential interference contrast and a water immersion objective (× 40) was used to identify the neurons. The slices were continuously perfused (flow rate 1.5 ml/min) with warm aCSF (temperature 30°C, pH 7.4) containing TTX (0.5 µM; Latoxan, Valence, France) to block sodium channels; and bicuculline (50 µM; Santa Cruz Biotechnology) to block GABAa receptors(182). A second perfusion line was installed for acute (in vitro) application of corticosterone.

Patch pipettes (borosilicate glass pipettes, inner diameter 0.86 mm, outer diameter 1.5 mm; Harvard Apparatus, Kent, UK) were pulled on a Sutter micropipette puller (Novato, California, USA) and had a tip resistance of 3-6 MΩ when filled with the pipette (intracellular) solution, containing (in mM): 120 Cs methane sulfonate, 17.5 CsCl, 10 Hepes, 2 MgATP, 0.1 NaGTP, 5 BAPTA; pH was 7.4, adjusted with CsOH. BAPTA was obtained from Molecular Probes (Leiden, The Netherlands), all other chemicals were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands).

The signals were amplified using an Axopatch 200B amplifier (Axon Instruments, USA) for whole cell recordings, operating in the voltage-clamp mode. The patch-clamp amplifier was interfaced to a computer via a Digidata (type 1322 A; Axon Instruments, USA) analog-to-digital converter.

Visually identified granular neurons in the dentate gyrus were selected for recording. After establishing a gigaseal, the membrane patch was ruptured and the cell was held at a holding potential of -70 mV. The liquid junction potential caused a shift of approximately 8 mV. We did not compensate for this potential shift. Recordings with an uncompensated series resistance of <2.5 times the pipette resistance were accepted for analysis. In view of the small current amplitudes, the recordings were not corrected for series resistance.

Miniature EPSC recording

The data was sampled at 5 kHz and stored on PClamp version 9.2 software, which uses a threshold-based event detection algorithm, with detection threshold levels set at 4 pA. The currents were identified as mEPSCs when the rise time was faster than the decay time(41). For all the recordings, the following mEPSC characteristics were determined: the frequency, peak amplitude, tau (τ) of rise time and tau of decay.

The whole-cell configuration becomes stable approximately 10 minutes after gigaseal formation; at that time, mEPSCs were recorded under baseline conditions, followed by recording during 20 minutes application of corticosterone (3 or 100 nM, Sigma). In this study we were primarily interested in the relative changes in

mEPSC properties under different physiological conditions like ADX or chronic corticosterone treatment. To probe the role of MR in ADX mice, we performed two experimental series, one in the presence of Spironolactone (MR antagonist; 100 nM dissolved in 1% Chloroform; Sigma Aldrich, The Netherlands) and the other in presence of Mifepristone (500 nM GR antagonist; 2.15 mg in 1 ml of ddH2O with few drops of 5 N HCl until dissolved; Sigma Aldrich).

Data analysis and statistics

Data was analyzed offline using ClampFit 9.2. Responses to exposure to different doses of corticosterone, as determined during the final 5 minutes of corticosterone application, were compared to the 5 minutes of baseline just preceding hormone treatment. Throughout the report, data is expressed as mean (determined for all cells in the group) ± standard error of the mean, unless stated otherwise. The relative changes in mEPSC properties were analyzed for each corticosterone dose (compared to the baseline just before hormone application), with a two-tailed paired Student's *t* test, significance set at $p < 0.05$. The cumulative distribution of mEPSC inter-event intervals during and prior to corticosterone application was compared with a Kolmogorov-Smirnov test. Comparison of corticosteroid actions on mEPSC properties between the various treatment-groups in the 1st experiment was performed with an ANOVA followed by a post-hoc multiple comparisons of the mean (Tukey). In the 2nd experiment, values obtained in vehicle and corticosterone treated animals were compared with an unpaired t-test.

Results:

Experiment 1: adrenalectomy

Blood samples were collected under rest and during the trough of the circadian rhythm, between 8.30 and 10.00 am. Accordingly, the mean plasma corticosterone levels in sham operated control mice were low, i.e. 17.41 ± 2.42 ng/ml (n=14). In 3 ADX mice, corticosterone levels were below the detection level (cut-off point at 6.5 ng/ml). In the remaining mice (n=11), corticosterone levels were very low (12.91 ± 0.95 ng/ml), though in the same range as in control mice.

Properties of mEPSCs were determined in 14 dentate granule cells from sham-operated control mice and in 14 cells from ADX animals (see typical examples in Figure 1A). Basal mEPSC characteristics (i.e. before corticosterone application) were not different between dentate cells from ADX and sham-operated control rats (unpaired t-test; frequency: p=0.53; amplitude: p=0.78; rise time: p=0.27; tau decay: p=0.79). In the sham-operated group, mEPSC frequency was slightly but not significantly (paired t-test; p=0.21) increased in the presence of 3nM corticosterone compared to baseline levels prior to corticosterone treatment (Figure 1B). In the presence of 100 nM corticosterone, the average mEPSC frequency did show a significant increase 15-20 minutes after onset of application, compared to the 5 minutes prior to hormone administration (p=0.001; Figure 1B). The log-transformed distribution of inter-event intervals was shifted to the left by 100 nM corticosterone (Figure 1C, left). The cumulative distribution of inter-event intervals was just not significantly altered by corticosterone treatment (p=0.08; Figure 1C, right). Corticosterone (3 or 100 nM) did not change mEPSC amplitude, mEPSC tau of rise time nor tau of decay (Table 1). These observations are largely in line with an earlier report on rapid corticosterone effects in dentate granule cells of naïve animals (191).

Table I. Relative changes in mEPSC properties (percentage change of baseline; mean±SEM) of mouse dentate granule cells after adrenalectomy (ADX) or sham operation, caused by 3 or 100 nM corticosterone. In part of the experiments, slices were pretreated with the GR-antagonist mifepristone (Mif) or the MR-antagonist spironolactone (Spiro). The mEPSC properties observed during corticosterone treatment were compared with properties prior to corticosterone application, using a paired t-test; significance indicated by *p<0.05, **p<0.01. Between group experiments were carried out with an ANOVA (significance p<0.05), and in case of significance followed up by a post-hoc Tukey multiple comparison of the means ($ p<0.05, compared to ADX).

Group	Δ frequency	Δ rise time	Δ decay	Δ amplitude
Sham 3 nM CORT (n=7)	18.4±9.9	-1.0±14.6	15.8±13.8	6.0±14.5
ADX 3 nM CORT (n=7)	48.9±9.9**	3.2±3.9	5.2±5.8	3.2±3.9
ADX 3 nM CORT + Mif (n=4)	-8.0±7.7$	-4.1±8.1	-4.0±5.3	-4.5±5.8
ADX 3 nM CORT + Spiro (n=6)	43.7±16.6*	9.4±9.3	13.8±9.9	19.3±11.5
Sham 100 nM CORT (n=7)	40.8±7.9**	-11.8±5.1	-8.5±10.2	-7.9±3.9
ADX 100 nM CORT (n=7)	10.5±10.8	-3.9±8.2	21.3±11.6	-7.2±4.1
ADX 100 nM CORT + Mif (n=6)	12.1±12.5	3.1±4.0	9.0±8.0	9.9±7.9
ADX 100 nM CORT + Spiro (n=4)	11.4±18.2	-15.5±4.4*	-11.6±5.7	-10.5±4.0

Unexpectedly, in ADX animals, 3 nM (p=0.002, paired t-test) but not 100 nM corticosterone (p=0.69) significantly increased the mEPSC frequency (see figure 1B). The cumulative distribution of mEPSC inter-event intervals recorded in the presence of 3 nM corticosterone compared to the period just prior to corticosterone application tended to be significant (p=0.09; Figure 1D, right). Both exposure to 3 nM and 100 nM corticosterone did not change amplitude, decay time nor the rise time of mEPSCs in ADX mice (Table I).

To follow up the unexpected result with 3 nM corticosterone in ADX mice, we investigated if these effects were due to MR or GR activation. It was earlier shown that the increase in mEPSC frequency induced in hippocampal dentate cells by 100 nM corticosterone acting via a non-genomic pathway is mediated by MRs rather than GRs (191), which is in line with findings in principal hippocampal CA1 and basolateral amygdala neurons(30)(180). This, however, did not appear to be the case for effects induced by 3 nM corticosterone in dentate cells of ADX mice. Thus, in ADX animals, 3 nM corticosterone in the presence of the MR-antagonist spironolactone caused an enhancement of mEPSC frequency (p=0.03, paired t-test; Figure 1B). Conversely, 3 nM corticosterone in the presence of the GR-antagonist mifepristone did not significantly change the frequency of mEPSCs (p=0.24). When comparing all four treatment groups, an overall significant difference in

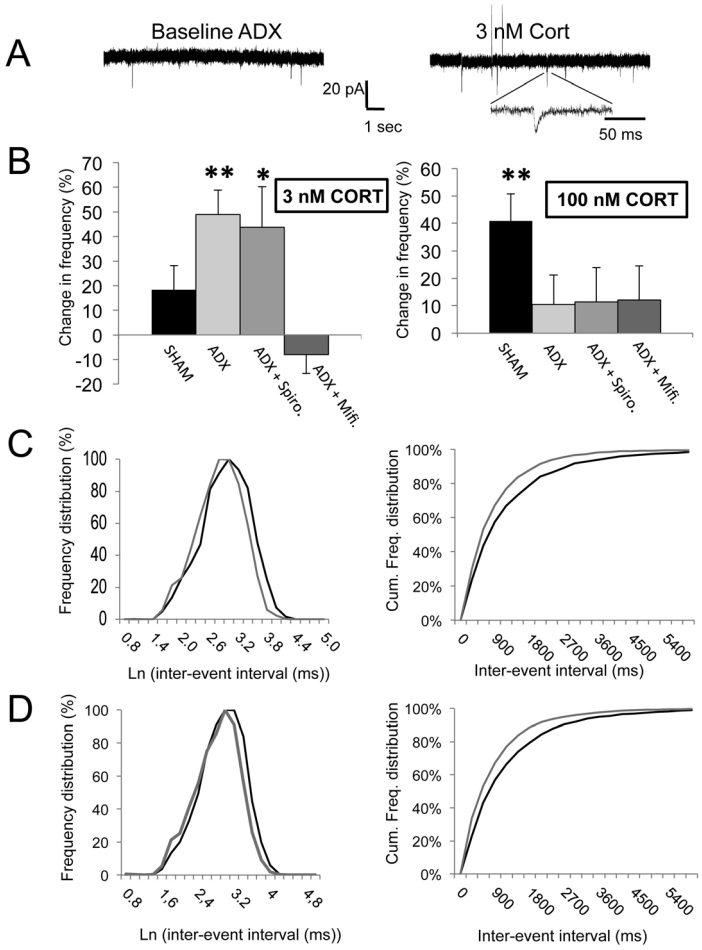


Figure 1. Corticosteroid actions in adrenalectomized and sham-operated mice.

(A) Typical traces showing mEPSCs in a dentate cell from an ADX mouse, prior to corticosterone (left) and in the presence of 100 nM corticosterone (right). The inset shows a mEPSC at a higher time resolution.

(B) Relative increase in mEPSC frequency by 3 nM corticosterone (left) or 100 nM corticosterone (right), in the various treatment groups. With 3 nM corticosterone, a significant increase in mEPSC frequency was observed in the ADX but not sham-operated control group. The increase was still visible in the presence of the MR-antagonist spironolactone (spiro) but not when the GR-antagonist mifepristone (mife) was administered. The higher dose of 100 nM corticosterone only caused a significant increase in mEPSF frequency in the sham-operated controls.

(C) Frequency distribution (left, log-transformed) and cumulative distribution (right) of the inter-event intervals before (black) and during 100 nM corticosterone (grey) in dentate cells from sham-operated control mice.

(D) Frequency distribution (left, log-transformed) and cumulative distribution (right) of the inter-event intervals before (black) and during 3 nM corticosterone (grey) in dentate cells from ADX mice.

The number of cells is mentioned in Table I and p-values in the main text.

* $p < 0.05$, ** $p < 0.01$.

% change in mEPSC frequency by 3 nM corticosterone was observed (ANOVA, p=0.02). Post-hoc comparisons revealed a significant difference between ADX / 3 nM CORT and ADX / 3 nM CORT + mifepristone treated cells (p=0.03, Tukey). The latter group also differed almost significantly from the ADX / 3 nM CORT + spironolactone treated cells (p=0.054). Altogether, this suggests that the increase in mEPSC frequency in ADX animals with 3 nM corticosterone is due to activation of GR rather than MR.

Experiment 2: chronic corticosterone treatment.

In this experiment, part of the animals (n=13) were treated with vehicle (0.1% ethanol) and the remaining mice (n=12) with corticosterone (25 μg/ml) in their drinking water for 3 weeks. In both groups, animals were weighed once every 7 days. Their weight did not differ significantly after 3 weeks of treatment with corticosterone compared to vehicle (Table II). The animals were decapitated on day 21, at which point in time adrenals and thymus were collected. Adrenal weight (corrected for body weight) was reduced (Table II) and there was complete thymus atrophy after 3 weeks of corticosterone treatment, as also reported by other groups (252). Basal corticosterone plasma levels of 21-days corticosterone treated animals were significantly increased ($p<0.01$, unpaired t-test) compared to vehicle treated animals (Table II).

Prior to *in vitro* administration of corticosterone, dentate granule cells from 3 weeks vehicle- and corticosterone-treated showed similar mEPSC properties (unpaired t-test; frequency: p= 0.39; amplitude: p=0.49; rise time: p=0.21), with the exception of the tau of decay which was significantly enhanced after 3 weeks of corticosterone compared to vehicle treatment (vehicle: 4.7±1.6 ms; 3 weeks corticosterone: 5.7±1.6 ms; $p<0.05$, unpaired t-test). In vehicle-treated control mice, dentate cells responded to *in vitro* administered corticosterone (100 nM) with an increased mEPSC frequency ($p<0.01$, paired t-test), while 3 nM was ineffective (Figure 2, Table III). In dentate cells from animals treated for 21 days with corticosterone in the drinking water, *in vitro* administered100 nM corticosterone did change the firing frequency (p=0.04 paired t-test), but to a significantly lower extent than in the vehicle treated controls (p=0.047, unpaired t-test; Table III). Application of a lower corticosterone concentration (3 nM) was ineffective. The mEPSC amplitude, decay and rise time were not affected by either dose of corticosterone, in mice treated with corticosterone or vehicle in the drinking water (Table III).

Table II. Effect of chronic stress on body weight, adrenal weight (absolute or relative to body weight), thymus weight and plasma corticosterone (CORT) levels. Corticosterone (n=12) and vehicle treated (n=13) mice were compared with an unpaired Student t-test (* $p<0.05$).

	Body weight (g)	Adrenal weight (g)	Relative adrenal weight	Thymus weight (g)	Plasma CORT level (ng/ml)
3 wks Vehicle	25.70±0.35	1.53±0.09	0.59±0.03	44.32±1.4	9.68±1.20
3 wks CORT	27.28±0.50	1.36±0.0	0.50±0.02*	undetectable	53.97±13.68*

Table III. Relative changes in mEPSC properties (percentage difference from baseline; mean±SEM) of mouse dentate granule cells after 3 weeks of vehicle or corticosterone treatment, caused by *in vitro* administered 3 or 100 nM corticosterone. The mEPSC properties observed during corticosterone treatment were compared with properties prior to corticosterone application, using a paired t-test; significance indicated by *p<0.05, **p<0.01. Between group experiments were carried out with an unpaired t-test ($ p<0.05, compared to vehicle treatment).

	Δ frequency	Δ amplitude	Δ decay	Δ rise time
Vehicle/3 nM CORT (n=4)	14.7±9.5	6.2±3.2	2.0±4.1	4.6±14.2
CORT/3 nM CORT (n=6)	12.9±10.5	-6.2±7.9	-2.2±6.1	1.4±6.8
Vehicle/100 nM CORT (n=4)	46.8±7.4**	-7.6±5.4	1.5±8.8	9.3±9.1
CORT/100 nM CORT (n=7)	17.4±9.5*$	7.2±8.7	8.4±8.3	0.2±6.3

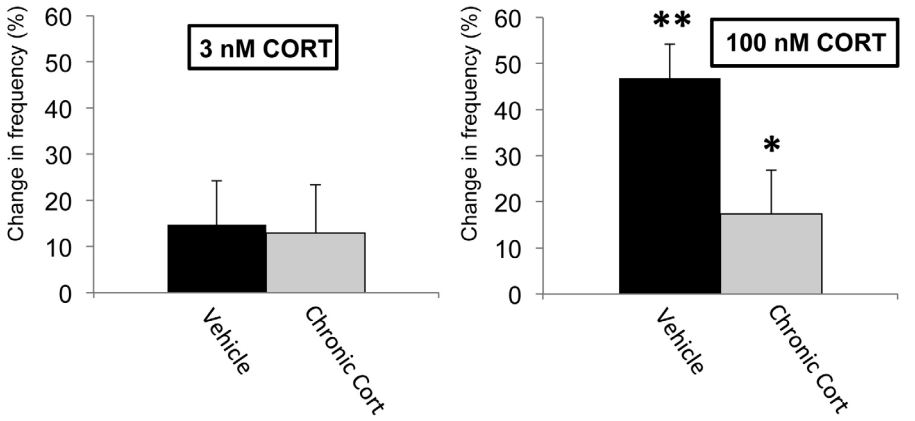


Figure 2. Change in mEPSC frequency induced by 3 nM corticosterone (left) or 100 nM corticosterone (right) in dentate cells from mice treated for three weeks with a moderately high dose of corticosterone or vehicle.
The number of cells is mentioned in Table III and p-values in the main text.
* p < 0.05, ** p< 0.01.

Discussion

Stress-related brain diseases, e.g. major depression, are often associated with aberrant levels of corticosteroid hormones (1) (253) (254). Prolonged over- or under-exposure to corticosteroids may alter neuronal function and thus contribute to the onset of disease symptoms. This may be true particularly for neurons in those areas that play an important role in the development of clinical symptoms, such as the hippocampus. Indeed, earlier studies have shown that neuronal properties of principal neurons in the CA1 area or dentate gyrus are markedly altered when corticosteroid hormone levels are long-lastingly very low (e.g. after removal of the adrenal glands) or very high, such as occurs after chronic stress or long-term

corticosterone administration (236). In some cases basal neuronal properties were altered after prolonged corticosterone over - or under-exposure, but more frequently the influence of these long-term changes in circulating corticosteroid level became only evident when cells were exposed to a renewed (*in vitro*) surge of corticosterone. For instance, in naïve rats corticosterone did not affect –in a slow genomic manner- the AMPA-receptor mediated synaptic transmission in dentate granule cells, but in animals exposed to 3 weeks of unpredictable stress *in vitro* corticosterone administration caused a slow enhancement of glutamatergic responses(166).

Over the past years it has become evident that corticosterone also affects hippocampal cell function in a *rapid* non-genomic manner (41) (31)(208). In this brief report we probed whether such *rapid* corticosteroid effects are also sensitive to the circulating levels of corticosterone in the days or weeks prior to recording. To this end we examined mEPSC properties, which are known to be affected in a rapid manner by corticosterone, of dentate granule cells. The data suggests that ADX does not affect basal properties of mEPSCs but does change the response to *in vitro* administered corticosterone. After ADX, neurons responded to a low dose of corticosterone, which in naïve or sham-operated mice is sub-threshold, while a high dose of the hormone -which in naïve or sham-operated animals near-maximally enhances mEPSC frequency - was ineffective in cells from ADX mice. Exposure to high doses of corticosterone for 3 weeks did not affect the basal properties of mEPSCs. Compared to vehicle-treated controls, dentate cells from mice treated for 3 weeks with corticosterone responded less effectively to a near-maximal dose of corticosterone, while no difference between the groups was seen with the lower (3 nM) corticosterone concentration.

Rapid corticosterone effects after ADX

Despite the removal of their adrenal glands, 3-4 days ADX mice still showed residual plasma levels of corticosterone at the circadian trough; these levels were not significantly different from those of adrenally intact mice. This is not unprecedented, since mice (compared to rats) have a relatively high amount of fat cells that are not removed upon ADX and produce corticosterone(255)(256). The remaining level of corticosterone is probably sufficient to still substantially activate MRs during most of the day (174) (242)(243)(241). Although we did not test corticosterone levels in ADX mice in situations other than under rest at the circadian trough, we assume that these animals (compared to sham operated controls) lacked the ability to raise circulating corticosterone levels at the circadian peak or after stress. Overall, ADX probably resulted in a condition in which during the day nuclear MRs were substantially activated while (in contrast to control animals) GRs were only marginally occupied. Whether this is also the case for membrane-located receptors is at this time hard to predict.

In these ADX mice, 100 nM corticosterone did not induce any change in mEPSC frequency nor in any other mEPSC property. In dentate cells from sham operated controls, 100nM corticosterone did enhance the averaged value of the mEPSC frequency, although the cumulative inter-event distribution was just not significantly changed in the presence of corticosterone. This suggests that the rapid effects of corticosterone in sham operated animals were similar but possibly somewhat more subtle than seen in naïve (non-operated) mice of the same age. The complete lack of response to 100nM corticosterone in ADX mice may reflect

a down-regulation in the capacity or affinity of MRs on the plasma membrane, presumably linked to a condition in which a substantial number of (nuclear) MRs are continuously activated, unopposed by GRs. We refrained from testing a concentration higher than 100 nM, in view of the non-specific effects of steroids at such supra-physiological concentrations (257) (258).

An unexpected finding was the quite marked response to 3 nM corticosterone in cells from ADX mice, a dose that in sham-operated and naïve animals is sub-threshold. In ADX mice, this concentration caused a significant increase in the mEPSC frequency, which exhibited a pharmacological profile compatible with mediation via GRs rather than MRs. We currently have no explanation for this phenomenon. It should be pointed out, though, that we never tested the pharmacological profile of this concentration of corticosterone in adrenally intact mice, so that we cannot entirely exclude that this profile may also occur in dentate cells from intact animals.

Rapid corticosterone effects after 3 weeks of corticosterone over-exposure

We performed a brief survey to examine how the opposite condition, i.e. a period of corticosterone over-exposure, affects rapid corticosteroid effects on dentate mEPSC properties. Rather than administering corticosterone by injection for 3 weeks, which for mice (even with vehicle-injection) is a highly stressful procedure, we opted for administration of corticosteroids via the drinking water (259). Since drinking behavior mostly takes place during the first part of the active period (252), this mode of administration is thought to quite closely follow the circadian fluctuations in corticosterone level, so that the overall 24 hrs corticosteroid exposure is expected to be moderately elevated(260)(252), particularly during the circadian peak. However, we also observed a considerable difference in plasma corticosterone level at the moment of decapitation, which might reflect either drinking behavior shortly before decapitation or a build-up of earlier consumed corticosterone. In agreement with the presumably higher exposure to corticosterone in the experimental group, adrenal weight was significantly reduced and the thymus showed severe atrophy. We did expect to see a somewhat reduced gain in body weight over the 3 weeks' period of corticosterone administration, but this was currently not observed. This may relate to the fact that 11β-HSD1 is known to be enhanced with high glucocorticoid levels, as seen e.g. in Cushing's syndrome and aged animals (261)(262). The increase in enzyme levels led to an increase in body weight or obesity (263).

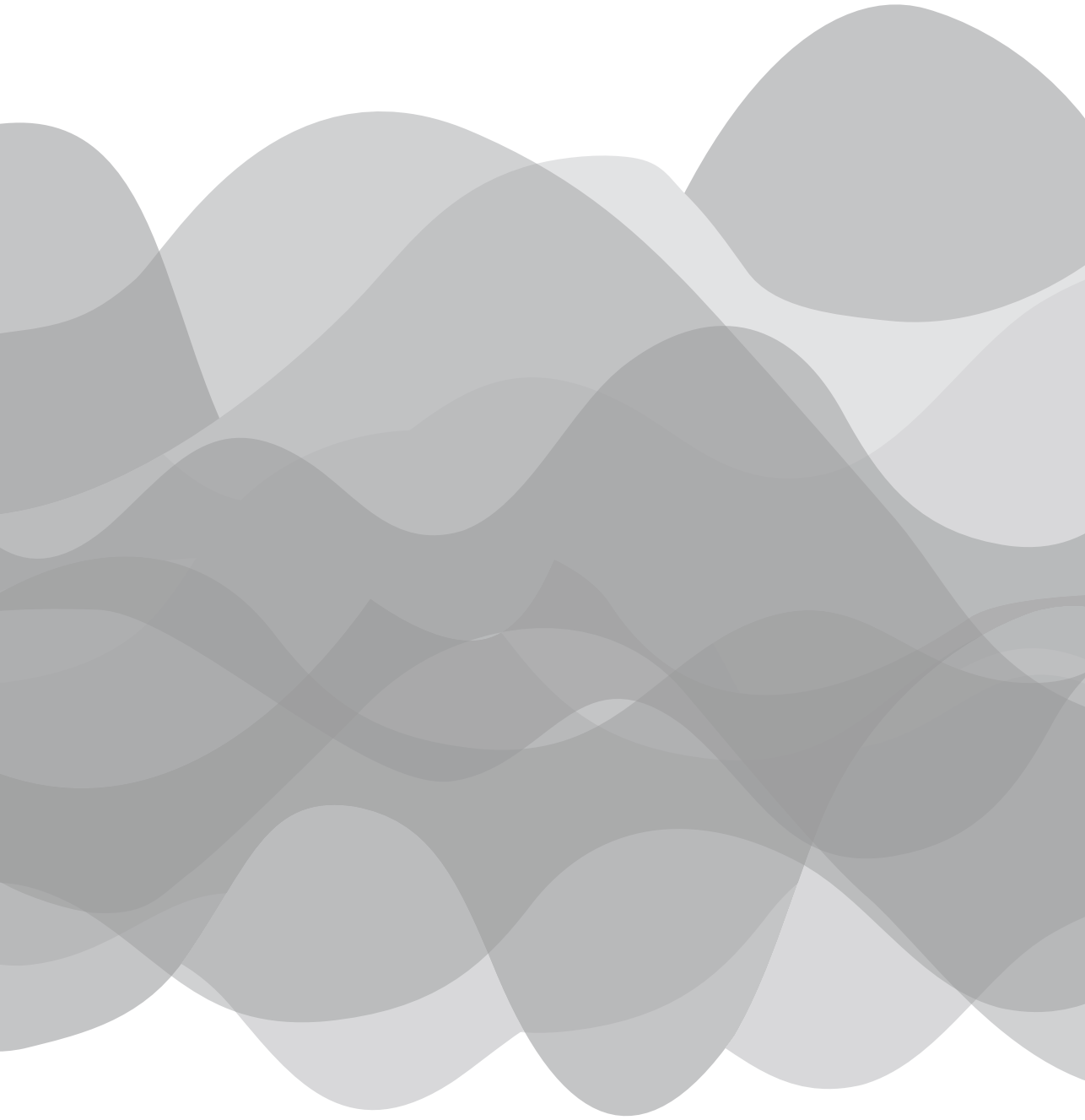
With the selected corticosterone application method and dose, one may assume that (nuclear) MRs are almost completely activated at all times, while GRs are activated to a large extent particularly during the active period. In response to this pattern, down-regulation of MR as well as GR mediated actions might occur (6)(264). In agreement with these expectations, we observed that the change in mEPSC frequency in response to 100 nM corticosterone was indeed significantly reduced in the chronically corticosterone- compared to the vehicle-treated groups as well as the earlier values reported for naïve mice(191). This suggests that chronic over-exposure to corticosterone may diminish rapid effects of the hormone on glutamatergic transmission.

In conclusion, we performed an explorative study to examine whether rapid corticosteroid effects in one particular limbic brain region, the dentate gyrus, are altered when circulating hormone levels during the days or weeks prior to recording

are profoundly changed, similar to what has been observed with regard to slow gene-mediated corticosteroid actions. The findings indicate that rapid effects to a high dose of corticosterone (100 nM) or most optimally induced under 'standard' conditions, i.e. without any intervention in the circulating hormone levels. Unexpectedly, a clear response to a normally sub-threshold dose of corticosterone was revealed when corticosteroid levels were greatly reduced, i.e. after ADX. The functional significance of these findings needs to be further explored.

Acknowledgement

This work was supported by NWO grant 817.02.017. Thanks to Angela Sarabdjitsingh for performing corticosterone radioimmunoassay.



CHAPTER 7

Discussion

1. Regional differences in rapid corticosteroid responses

The first question I addressed in my thesis regarded possible regional differences in rapid responses to corticosterone (Chapters 2 and 3). At the time I started, rapid effects had only been described in any detail for the CA1 hippocampal region (30) and the PVN in the hypothalamus (265).

After experiencing stress, the brain is exposed to corticosteroids as well as neuropeptides and catecholamines (5) (134). Previous electrophysiological studies showed that slow corticosteroid actions in brain are region-dependent, which is determined by various factors including the distribution of corticosteroid receptors. Thus, hippocampal subregions CA1 and DG highly express MR and GR, whereas the CA3 region is dominated by MR (1). However, receptor distribution is not the only factor determining the slow response to corticosterone. For instance, despite the abundance of both MR and GR in DG and CA1, neurons in these two areas show differences in response to corticosterone exposure (179). In CA1 neurons, Ca^{2+} current amplitude was found to be increased several hours after exposure to corticosterone, while this was not seen in dentate granule cells. It was concluded that, next to differential receptor distribution, local properties such as the nature of afferent fibers, the expression of intracellular proteins and e.g. micro-RNAs contribute to the overall regional differences.

The studies discussed above refer to the slow gene-mediated actions of corticosterone. Rapid corticosteroid actions, however, involve different signaling cascades than the genomic pathway, e.g. G-protein coupled signaling in both the pre- and post-synaptical compartment (31). In Chapter 2 of this thesis, I demonstrated rapid effects of corticosterone via a receptor with the pharmacological profile of the MR, most likely located in the plasma membrane, comparable to what had been shown for the CA1 area (30)and what was recently also reported for neurons in the BLA. Interestingly, though, BLA neurons showed sustained rapid effects. The sustained character of the response was found to involve GRs and protein synthesis (180). This underlines that rapid corticosteroid actions also show region-dependency, possibly linked to variation in receptor distribution although other explanations cannot be ruled out at this moment.

The existence of regional differences was confirmed and further explored in Chapter 3. In this chapter, we showed that rapid non-genomic effects can indeed, as hypothesized, translate the hourly short-duration pulses of corticosterone into functional output in CA1 and DG cells, although some attenuation was seen after 2 hours. This attenuation was abolished in presence of a protein synthesis inhibitor, showing the likely interference of genomic effects. Yet, BLA neurons did not follow the pulses of corticosterone exposure and rather showed inhibitory effects during the 2nd exposure and no response upon subsequent pulses. These experiments again emphasize the regional differences between rapid corticosteroid actions in various brain regions. **Overall, the data suggests that especially after repeated exposure to corticosterone, the spontaneous glutamatergic activity of BLA neurons versus neurons in the CA1 or DG regions may start to diverge; this is the first main finding of this thesis.** The divergence between BLA and hippocampus may be relevant in the light of ultradian pulses (at the circadian peak) but also when an organism is exposed to several stressors in rapid succession.

2. Corticosterone pulses: the mechanism underlying attenuated responses.

The second question of my thesis focused on the functional relevance of pulsatile corticosterone exposure for neuronal activity (Chapters 3 and 4). I here first summarize the findings and then speculate on the putative underlying mechanism. In Chapters 3 and 4 we exposed cells to four pulses of an identical (high) concentration of corticosterone (100 nM). This is unlike the situation of ultradian pulses, where circulating corticosteroid hormones show a gradual increase from low to high corticosterone levels or vice versa (196) (197) (187). To not complicate the interpretation of our findings with these gradual natural shifts in concentration, we first looked into the effect of corticosterone pulses of equal amplitude, similar to the paradigm used in a previous in vivo study (195).

The data in Chapter 3 indicates that principal neurons in the hippocampal CA1 area and DG quite reliably translate hourly shifts in corticosterone level into changes in mEPSC frequency, although a temporary attenuation was seen upon the 3rd exposure. With control experiments we excluded the possibility that this attenuation was caused by deterioration of the brain slices but rather reflects a reproducible phenomenon. In Chapter 4 we complemented these electrophysiological studies with *in vitro* neuroimaging data in (unidentified) cultured hippocampal neurons, studying rapid corticosteroid actions on surface diffusion of the postsynaptic GluA2 subunit. Interestingly, in this study too we found suggestive evidence that the response to a 3rd pulse of corticosterone differs from responses to earlier and even later pulses, although in Chapter 4 (unlike Chapter 3) the response to the 4th pulse of corticosterone was not quite comparable to that of the 1st and 2nd application. **Overall, the data suggests that repeated shifts in corticosterone level initially are well-translated into altered glutamatergic transmission, but that 2-3 hrs after the first pulse hippocampal neurons become less responsive.** This is of course a critical time-domain for consolidation of stress-related information (266) (112)and also coincides with the earlier reported appearance of gene-mediated attenuation in hippocampal information transfer (267) (268).

A second consistent observation of these chapters is that repeated exposure to corticosterone not only affects rapid effects but also the development of slow (presumably gene-mediated) actions of corticosterone. Thus, on application of a single pulse of corticosterone for 20 mins to CA1 pyramidal cells, AMPA-mediated mEPSC amplitude was reported to be increased several hours later, via a GR dependent mechanism (30). In Chapter 3 we report that on exposure to hourly corticosterone pulses, the amplitude did not change considerably over time. Corticosterone pulses given in the presence of cycloheximide showed a slight but not significant increase in baseline mEPSC frequency with each pulse but also no change in amplitude. Similarly, it had been shown that a single pulse of corticosterone increases GluA2 receptor trafficking rapidly and that this elevation of GluA2 receptor surface trafficking is maintained even after two hours (32). In Chapter 4, we showed that GluA2 surface trafficking is rapidly enhanced during the 1st and 2nd pulse and comes back to baseline after each pulse exposure. The baseline surface diffusion did not change throughout the four consecutive pulses. Moreover, the synaptic content of GluA2 subunits during the pulses remained comparable, unlike the enhanced synaptic content several hours after a single corticosterone exposure. It should be noted that the corticosterone application in Chapters 3 and 4 (10 min pulses) was not entirely comparable to the pulse duration used in earlier

studies (20 min). Alignment of these paradigms will be necessary before definite conclusions can be drawn. Also, in future it is necessary to investigate if these differences in response to single versus multiple corticosterone pulses exist when we gradually increase the dose with each pulse, which more closely mimics the natural conditions. Nevertheless, I tentatively conclude that **the gene-mediated effects initiated by a 1st pulse of corticosterone on hippocampal neurons seem to be normalized by exposure to a 2nd pulse of corticosterone approximately 1 hr after the 1st pulse. This putative contribution to homeostatic control may constitute another functional relevance of ultradian pulses.**

At this moment I can only speculate about an explanation of both observations. The fact that in the presence of cycloheximide corticosterone quite consistently increased mEPSC frequency (Chapter 3) indicates that the attenuation in response to the 3rd pulse critically depends on protein synthesis. It is tempting to assume that this involves a genomic action initiated by the 1st pulse of corticosterone and mediated by MR or GR, but at this moment we have no evidence to support this. This could be addressed by applying pulses of CORT-BSA rather than corticosterone itself, as was done in Chapter 4. The findings in Chapter 4 indicate that the difference in response to the 1st and the 3rd pulse is not necessarily caused by a genomic action affecting subsequent non-genomic responses, but can even occur in the absence of gene-mediated signaling. However, we cannot exclude that the altered (presynaptic) change in mEPSC frequency during the 3rd pulse of corticosterone may be caused by a different mechanism than the altered (postsynaptic) change in GluA2 subunit diffusion.

Regardless of the involvement of gene-transcription, the altered response to the 3rd pulse may be caused by changes in corticosteroid signaling, glutamate signaling or another pathway indirectly changing one of these. With regard to the first possibility, one could think of internalization of mMRs, caused by consecutive exposure of high doses of corticosterone (100 nM). Internalization has e.g. been described for G protein-coupled receptors, via phosphorylation and arrestin binding of the receptor(198). An old mathematical model of ligand-receptor interaction (269) showed that the rate of internalization for any particular ligand will be determined by ligand and receptor concentration, their affinities, rates of ligand synthesis and loss, receptor recycling, the rate of endocytosis, and the transport capacity of the endocytotic apparatus. In addition, maintaining the activity of receptors in the membrane depends on ligand concentration, number of receptors and ligand-receptor interaction. The receptors are recycled in a time dependent manner (269). Possibly, receptors were recycled during exposure to a 3rd high-concentration corticosterone pulse and this recycling was not possible in the presence of the protein synthesis inhibitor cycloheximide. It remains unclear, however, how mMRs would become available again upon the next exposure to corticosterone, as suggested by the findings in Chapter 3 (but not Chapter 4).

With regard to the second possibility (altered glutamate signaling), it is possible that AMPA receptors desensitize due to the AMPA-responses evoked by earlier pulses. This is based on the finding that AMPA receptors can indeed display reduced affinity to glutamate due to high levels of glutamate released pre-synaptically (270). In this model, the desensitization may be alleviated by the temporary attenuation of AMPA receptor activation during the 3rd corticosterone pulse; hence, the return of a rapid corticosterone response upon the 4th pulse.

The mechanism explaining ‘normalization’ of gene-mediated effects by

subsequent exposure to a corticosterone pulse is even more puzzling. Maybe the 2nd pulse starts a gene-mediated cascade that interrupts or at least alters the genomic actions initiated by the 1st pulse. If so, this may also hold true for the altered rapid response to the 3rd pulse discussed above; in that case, this altered response to the 3rd pulse would occur independent of genomic signaling, as is indeed suggested by the observations with cort-BSA in Chapter 4. Whether gene-mediated events are indeed affected by a second 'hit' of corticosterone requires dedicated experiments, examining patterns of gene transcripts after a single or multiple pulses of corticosterone. Such proof would be a necessary step before the underlying mechanism can be explored.

3. Interaction of hormones released directly after stress exposure

The third question of my thesis was instigated by the fact that after stress limbic cells are not only exposed to corticosterone but also to many other transmitters such as CRH and noradrenaline (134), so that interactions may occur. Especially the observation that BLA neurons are first exposed to a wave of noradrenaline and slightly later to a wave of corticosterone (271) inspired us to study the interactions between the two hormones. It has been hypothesized that these hormones collectively promote consolidation of relevant information (272). A suggested mechanism was that corticosterone enhances memory consolidation by potentiating β-adrenoceptor-cAMP/PKA signaling in the BLA (146).

Interaction of corticosterone and isoproterenol has earlier been shown to affect *evoked* glutamatergic transmission in the mouse BLA, though only through slow actions of corticosterone (223) (153). In Chapter 5, we have shown that: 1) isoproterenol, like corticosterone, rapidly increases the mEPSC frequency but not amplitude of BLA neurons with an inverted U-shaped dose-dependency; 2) co-application of both hormones did not simply cause additive effects but more complex synergistic responses; and 3) that isoproterenol applied before corticosterone alters the response to corticosterone while the reverse is not true. Altogether, the results from Chapter 5 indicate that **isoproterenol initiates a pathway which alters the downstream effects of corticosterone. This may have important implications, given that under physiological conditions BLA neurons are first exposed to noradrenaline and then to corticosterone.**

The complex synergistic influence on glutamatergic signaling seen with co-applied isoproterenol and corticosterone is not unprecedented. For instance, cultured hippocampal cells show an enhanced effect on mEPSCs upon co-application of both hormones, which was suggested to be associated with altered AMPAR surface expression and function (136). β-adrenoceptor activation by isoproterenol has also been found to induce phosphorylation of GluA1-AMPAR subtypes which facilitates AMPAR surface expression, enhances AMPAR-mediated mEPSCs and lowers the threshold for LTP induction (273)(274). In cultured hippocampal cells too, particularly moderately high doses of isoproterenol and corticosterone resulted in synergistic effects. In the BLA, putative synergistic effects have been studied for synaptically evoked responses involving glutamatergic transmission. Neither at the field potential level (225) nor in single neurons (153) did co-applied corticosterone change the effect of isoproterenol.

The element of timing between corticosteroid and noradrenergic signaling was never addressed before. Doing so was inspired by the rapid metaplastic

corticosterone actions reported for BLA neurons (180), which was published while I was in the middle of my PhD project. The metaplasticity of corticosteroid actions in the BLA is known to be dependent on MR, GR, CB-R1 as well as protein synthesis (180). The fact that corticosterone responses switch from enhancing to decreasing mEPSC frequency exactly 60 minutes after the first exposure to the hormone (Chapter 5) is indeed compatible with a gene-mediated pathway. One can think of a mechanism by which mEPSC frequency is quickly increased via MR and at the same time Ca-influx is slowly enhanced via GR (227). The latter may cause enhanced release of 2-AG (226). Enhanced 2-AG in turn was shown to rapidly suppress mEPSC frequency via CB-R1 in PVN neurons (228). Future experiments could test whether or not Ca-influx plays a critical role in this BLA metaplasticity. But what causes the metaplasticity when isoproterenol is given prior to corticosterone, a phenomeonon that seems to develop within 40 minutes? Possibly, Ca-influx plays a role here too. It was reported that isoproterenol causes an enhancement in P/Q-type calcium currents in BLA neurons (151) (223); this also is expected to cause a rise in intracellular calcium level. Whether this affects 2-AG availability or release is unknown. This needs to be addressed in future studies.

4. Functional relevance of rapid corticosteroid actions in health and disease

There are many examples of studies showing that very prolonged changes in circulating corticosteroid levels alter neuronal properties of hippocampal cells as well as the delayed response to acutely (in vitro) administered corticosterone (131). A recent extensive study using microarrays demonstrated that such conditions –e.g. chronic stress- are indeed associated with alterations in the transcriptome (167), although this analysis was restricted to expression patterns under rest, i.e. in the absence of additional acute exposure to corticosteroids. It seems likely that the functional changes described with electrophysiological recording after prolonged changes in circulating corticosteroid level are caused by altered gene expression, but a direct link between gene expression and electrophysiological properties has rarely been supplied (e.g. (275)).

The final question of my thesis was to determine if chronic over- or underexposure to corticosterone changes mEPSC properties and the rapid effects exerted by corticosterone on these properties, similar to what has been found for delayed corticosterone effects. In Chapter 6, I reported that this is indeed the case, but the effects followed a rather surprising pattern. Thus, rapid responses to *in vitro* administered corticosterone were generally attenuated when a moderately high dose of corticosterone was given for 3 weeks through the drinking water. This was not entirely unexpected because chronic over-exposure to corticosterone has been reported to down-regulate MR and GR expression (276) (277), although this was not consistently found (264) (278). Since all the available evidence so far indicates that rapid corticosterone actions require the presence of the MR gene and therefore are probably also mediated by 'classical' MR molecules which are inserted into the plasma membrane rather than located intracellularly, downregulation of this gene would also decrease the capacity by corticosterone to induce rapid increases in mEPSC frequency.

If this principle is applicable in general, one would expect the opposite effect after chronic reduction in circulating corticosterone level e.g. after ADX: increased surface expression of MRs and hence a higher sensitivity to corticosterone. To some

extent my observations in Chapter 6 are compatible with this notion. Administration of 3 nM corticosterone, which in adrenally intact mice is a sub-threshold dose, did enhance the mEPSC frequency in dentate granule cells from ADX mice, pointing to super-sensitivity to corticosterone. However, unexpectedly this effect turned out to be mediated by GRs rather than MRs, based on the pharmacological profile. Of course, this needs substantiation in genetically modified animals. Assuming for now that the pharmacological tools give reliable information –and they have done so in all studies on rapid corticosterone effects so far (41) (180) (191)- it seems puzzling why dentate cells revert to GRs for their rapid actions after ADX. In adrenally intact mice, rapid effects by 100 nM corticosterone were found to depend entirely on MRs (Chapter 2). Although not specifically investigated, it seems straightforward to assume that the receptor involved in mediation of lower corticosterone concentrations is also the MR. Conversely, none of the studies so far on rapid corticosteroid actions in hippocampal cells has supplied any evidence for involvement of GRs, contrary to reports for the BLA (180) and hypothalamus (265). Nevertheless, the data shows that **under the conditions where corticosteroid levels are very low for a prolonged period of time (as in ADX-ed mice), rapid corticosterone actions in the dentate involve GR rather than MR.**

Possibly, very low levels of corticosterone –which are expected to primarily activate nuclear MRs- specifically downregulate the expression of MR, while GR expression is upregulated. This would not only alter gene-dependent corticosteroid signaling, but also rapid effects, at least if these two pathways involve the same pool of receptor molecules. Why such conditions of low levels of membrane-located MRs and high levels of membrane-located GRs would result in increased rather than decreased mEPSC frequency is at this time hard to explain. This is only possible if 1) membrane-located GRs in the hippocampus exert an opposite effect from that seen in the BLA and hypothalamus or 2) ADX completely reverses the signaling pathways of membrane-located GRs. Assuming that such a shift in MR/GR balance indeed occurs (also for rapid effects), this is expected to cause even stronger responses with a higher dose of corticosterone. However, 100nM corticosterone was completely ineffective in granule cells from ADX mice, when activating MRs, GRs or both.

While the mechanism giving rise to these complex modulations in corticosterone effectiveness after chronic corticosteroid over- or under-exposure remains elusive at this moment, the consequences are nevertheless important. Prolonged aberrations in corticosteroid level have been reported in association with many diseases (53) (1) (54). The findings in my thesis underline that **under conditions associated with HPA dysfunction, hippocampal cells respond very differently to corticosteroid hormones, both directly and some hours after stress exposure.**

5. Importance of timing for corticosteroid actions

The overarching finding of this thesis is that timing is very important in determining the consequences of corticosteroid exposure for neuronal functional. Below I will highlight four examples of the relevance of timing.

First, the results from Chapter 5 indicate that corticosteroid actions in BLA neurons depend on the exposure to other stress mediators -in this case the β-adrenoceptor agonist isoproterenol- in **a period of <40 minutes** before corticosterone hits the cells. Most likely, similar interactions can occur with other stress mediators, but this has not yet been investigated. The collective pattern of

transmitter and hormone waves in the BLA will thus determine the overall change in neuronal activity.

Second, corticosteroid actions have generally been examined after one acute stress situation. However, daily stress situations are often characterized by several homotypic or heterotypic stressors in rapid succession. Even experiencing a single stressor may result in reliving the experience several times, e.g. by relating the situation to oneself or others. Others have already shown that multiple complex as opposed to simple stressors affect the brain in a different manner, involving CRH (279)(280). In Chapters 3 and 4, I report that the result of exposure of hippocampal and BLA cells to multiple pulses of corticosterone cannot be derived from a mere extrapolation of a single exposure. The development of gene-mediated corticosteroid effects is clearly affected by pulses of corticosterone >1 hr after the initial exposure. Moreover, BLA and hippocampal cell activity starts to deviate upon multiple pulses of corticosterone **over the course of several hours**. Whether these findings also bear relevance to the consequences of ultradian pulses needs further investigation (see section 6).

Third, the circulating corticosterone levels in the **days to weeks** prior to recording do determine the response to acutely administered corticosterone. Apparently, long-term aberrations in circulating hormone levels initiate effects that change the cell or network properties for a prolonged period of time.

Finally, many studies have shown that stress exposure early in life has **life-long** consequences for brain function as well as for the functionality of the HPA-axis, including neuronal responses to corticosterone (281) (282) (283). This was shown to involve epigenetic programming (284)(285). Recently, it was shown that such early life conditions do affect NMDA/AMPA signaling in the hippocampus ((286) under basal conditions, but also following corticosterone exposure. More extensive investigations of the consequences of early life exposure on rapid corticosteroid signaling in the adult brain may reveal the mechanism by which early life adversity promotes vulnerability to disease in susceptible individuals (287).

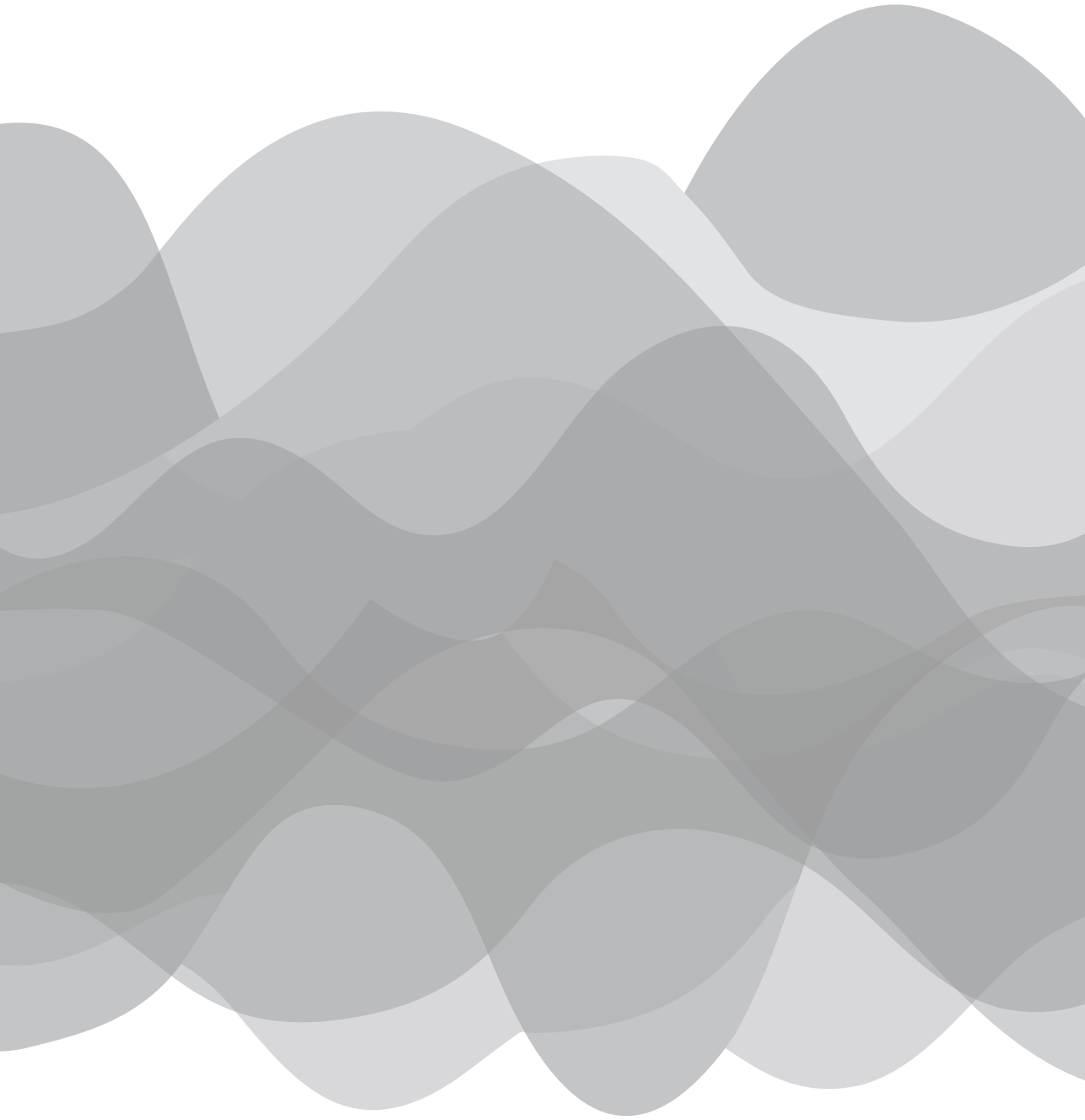
6 Future perspectives

While I have highlighted in the previous sections several new insights that were obtained based on my studies, there are still many questions left unanswered. I have summarized some of these questions in Box 1.

Box 1. Questions for future experiments

1. Neurons in the PFC or CA3 region of the hippocampus have different MR/GR expression patterns than cells in the CA1 area, DG or BLA. What is the rapid response of these cells to corticosterone?
2. How do hippocampal and BLA neurons respond to ultradian pulses that mimic the pattern seen during 24 hrs, i.e. increasing or decreasing in amplitude over time?
3. Is transcriptional regulation initiated by a pulse of corticosterone indeed interrupted by a second pulse of corticosterone 1 hr later?
4. Does pulsatile exposure to corticosterone not only affect spontaneous glutamatergic transmission, but also evoked glutamatergic transmission, LTP and ultimately behavior that depends on LTP?
5. Is GluA1 surface diffusion similarly affected by rapid pulses of corticosterone as GluA2 diffusion?
6. How do BLA and hippocampal neurons respond to physiologically relevant waves of noradrenaline and corticosterone?
7. How are the expression and membrane location of MR and GR affected by ADX in mice?

Obviously, the questions in Box 1 are not exhaustive; there are many more questions that are relevant to fully understand the importance of rapid corticosteroid signaling for brain function. However, already now it is clear that these rapid effects are complex, relevant and contribute importantly to the full spectrum of corticosteroid actions in the healthy but certainly also in the diseased brain. This potentially offers possibilities to define novel targets for treatment strategies of such diseases.



References

1. **de Kloet ER, Joels M, Holsboer F** 2005 Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6:463-475
2. **McEwen BS** July 2007 Physiology and Neurobiology of Stress and Adaptation: Central Role of the Brain. Physiological Reviews 87:873-904
3. **Foote SL, Bloom FE, Aston-Jones G** 1983 Nucleus locus ceruleus: new evidence of anatomical and physiological specificity. Physiological Reviews 63:844-914
4. **Stanford SC** 2006 Adrenaline and Noradrenaline: Introduction. eLS: John Wiley & Sons, Ltd.
5. **Ulrich-Lai Y, Herman JP** 2009 Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci 10:397-409
6. **de Kloet ER, Vreugdenhil E, Oitzl MS, Joëls M** 1998 Brain Corticosteroid Receptor Balance in Health and Disease. Endocrine Reviews 19:269-301
7. **Dallman MF, Pecoraro N, Akana SF, la Fleur SE, Gomez F, Houshyar H, Bell ME, Bhatnagar S, Laugero KD, Manalo S** 2003 Chronic stress and obesity: A new view of "comfort food". Proceedings of the National Academy of Sciences 100:11696-11701
8. **Herman J, Schafer M, Young E, Thompson R, Douglass J, Akil H, Watson S** 1989 Evidence for hippocampal regulation of neuroendocrine neurons of the hypothalamo-pituitary-adrenocortical axis. The Journal of Neuroscience 9:3072-3082
9. **Jacobson L, Sapolsky R** May 1991 The Role of the Hippocampus in Feedback Regulation of the Hypothalamic-Pituitary-Adrenocortical Axis. Endocrine Reviews 12:118-134
10. **Rubin R, Mandell A, Crandall P** 1966 Corticosteroid responses to limbic stimulation in man; localization of stimulus sites. 153:767-768
11. **Knigge K** 1961 Adrenocortical response to stress in rats with lesions in hippocampus and amygdala. Proc Soc Exp Biol Med 108:18-21
12. **Fendler K, Karmos G, Telegdy G** 1961 The effect of hippocampal lesion on pituitary-adrenal function. Acta Physiol Acad Sci Hung 20:293-297
13. **Allen JP, Allen CF** 1974 Role of the amygdaloid complexes in the stress-induced release of ACTH in the rat. 15:220-30.
14. **Dunn JD, Whitener J** 1986 Plasma corticosterone responses to electrical stimulation of the amygdaloid complex: cytoarchitectural specificity. 42:211-7
15. **Figueiredo HF, Bodie BL, Tauchi M, Dolgas CM, Herman JP** 2003 Stress Integration after Acute and Chronic Predator Stress: Differential Activation of Central Stress Circuitry and Sensitization of the Hypothalamo-Pituitary-Adrenocortical Axis. Endocrinology 144:5249-5258
16. **Rao RP, Anilkumar S, McEwen BS, Chattarji S** 2012 Glucocorticoids Protect Against the Delayed Behavioral and Cellular Effects of Acute Stress on the Amygdala. Biol Psychiatry 72:466-475
17. **McEwen BS, Weiss JM, Schwartz LS** 1968 Selective retention of corticosterone by limbic structures in rat brain. 220:911-2
18. **Reul JMHM, de Kloet ER** 1985 Two Receptor Systems for Corticosterone in Rat Brain: Microdistribution and Differential Occupation. Endocrinology 117:2505-2511
19. **Lightman SL, Wiles CC, Atkinson HC, Henley DE, Russell GM, Leendertz JA, McKenna MA, Spiga F, Wood SA, Conway-Campbell BL** 2008 The significance of glucocorticoid pulsatility. Eur J Pharmacol 583:255-262
20. **Patel PD, Lopez JF, Lyons DM, Burke S, Wallace M, Schatzberg AF** 2000 Glucocorticoid and mineralocorticoid receptor mRNA expression in squirrel monkey brain. J Psychiatr Res 34:383-392
21. **Seckl JR, Dickson KL, Yates C, Fink G** 1991 Distribution of glucocorticoid and mineralocorticoid receptor messenger RNA expression in human postmortem hippocampus. Brain Res 561:332-337

22. **Joëls M** 2006 Corticosteroid effects in the brain: U-shape it. Trends Pharmacol Sci 27:244-250
23. **Gomez-Sanchez EP, Ganjam V, Chen YJ, Liu Y, Clark SA, Gomez-Sanchez CE** 2001 The 11β hydroxysteroid dehydrogenase 2 exists as an inactive dimer. Steroids 66:845-848
24. **Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM** 1987 Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237:268-75
25. **Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG, Evans RM** 1985 Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318:635-41
26. **Miesfeld R, Rusconi S, Godowski PJ, Maler BA, Okret S, Wikström A, Gustafsson J, Yamamoto KR** 1986 Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. Cell 46:389-399
27. **Lu NZ, Cidlowski JA** 2006 Glucocorticoid receptor isoforms generate transcription specificity. Trends Cell Biol 16:301-307
28. **Datson NA, Morsink MC, Meijer OC, de Kloet ER** 2008 Central corticosteroid actions: Search for gene targets. Eur J Pharmacol 583:272-289
29. **Song I, Buttgereit F** 2006 Non-genomic glucocorticoid effects to provide the basis for new drug developments. Mol Cell Endocrinol 246:142-146
30. **Karst H, Joels M** 2005 Corticosterone Slowly Enhances Miniature Excitatory Postsynaptic Current Amplitude in Mice CA1 Hippocampal Cells. J Neurophysiol 94:3479-3486
31. **Olijslagers JE, Kloet ERd, Elgersma Y, van Woerden GM, Joëls M, Karst H** 2008 Rapid changes in hippocampal CA1 pyramidal cell function via pre- as well as postsynaptic membrane mineralocorticoid receptors. Eur J Neurosci 27:2542-2550
32. **Groc L, Choquet D, Chaouloff F** 2008 The stress hormone corticosterone conditions AMPAR surface trafficking and synaptic potentiation. Nat Neurosci 11:868-870
33. **Komatsuzaki Y, Murakami G, Tsurugizawa T, Mukai H, Tanabe N, Mitsuhashi K, Kawata M, Kimoto T, Ooishi Y, Kawato S** 2005 Rapid spinogenesis of pyramidal neurons induced by activation of glucocorticoid receptors in adult male rat hippocampus. Biochem Biophys Res Commun 335:1002-1007
34. **Xiao L, Feng C, Chen Y** 2010 Glucocorticoid Rapidly Enhances NMDA-Evoked Neurotoxicity by Attenuating the NR2A-Containing NMDA Receptor-Mediated ERK1/2 Activation. Molecular Endocrinology 24:497-510
35. **Sato S, Osanai H, Monma T, Harada T, Hirano A, Saito M, Kawato S** 2004 Acute effect of corticosterone on N-methyl-D-aspartate receptor-mediated Ca2+ elevation in mouse hippocampal slices. Biochem Biophys Res Commun 321:510-513
36. **Liu L, Wang C, Ni X, Sun J** 2007 A rapid inhibition of NMDA receptor current by corticosterone in cultured hippocampal neurons. Neurosci Lett 420:245-250
37. **Orchinik M, Matthews L, Gasser PJ** 2000 Distinct Specificity for Corticosteroid Binding Sites in Amphibian Cytosol, Neuronal Membranes, and Plasma. Gen Comp Endocrinol 118:284-301
38. **Di S, Malcher-Lopes R, Halmos KC, Tasker JG** 2003 Nongenomic Glucocorticoid Inhibition via Endocannabinoid Release in the Hypothalamus: A Fast Feedback Mechanism. The Journal of Neuroscience 23:4850-4857
39. **Tasker JG, Herman JP** 2011 Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic–pituitary–adrenal axis. Stress 14:398-406
40. **Qi A, Qiu J, Xiao L, Chen Y** 2005 Rapid activation of JNK and p38 by glucocorticoids in primary cultured hippocampal cells. J Neurosci Res 80:510-517
41. **Karst H** 2005 Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. . Proceedings of the National Academy of Sciences 105 (52): 19204-7

42. **Johnson LR, Farb C, Morrison JH, McEwen BS, Ledoux JE** 2005 Localization of glucocorticoid receptors at postsynaptic membranes in the lateral amygdala. Neuroscience 136:289-299
43. **Prager EM, Brielmaier J, Bergstrom HC, McGuire J, Johnson LR** 2010 Localization of mineralocorticoid receptors at mammalian synapses. PLoS One 5:e14344
44. **Hammes SR, Levin ER** 2007 Extranuclear Steroid Receptors: Nature and Actions. Endocrine Reviews 28:726-741
45. **Pedram A, Razandi M, Sainson RCA, Kim JK, Hughes CC, Levin ER** 2007 A Conserved Mechanism for Steroid Receptor Translocation to the Plasma Membrane. Journal of Biological Chemistry 282:22278-22288
46. **Razandi M, Pedram A, Levin ER** July 1, 2010 Heat Shock Protein 27 Is Required for Sex Steroid Receptor Trafficking to and Functioning at the Plasma Membrane. Molecular and Cellular Biology 30:3249-3261
47. **Groeneweg F, Karst H, de Kloet E, Joëls M** 2011 Rapid non-genomic effects of corticosteroids and their role in the central stress response. Journal of Endocrinology 209:153-167
48. **Plant TM** 2008 Hypothalamic Control of the Pituitary-Gonadal Axis in Higher Primates: Key Advances over the Last Two Decades. J Neuroendocrinol 20:719-726
49. **Ebling FJP, Barrett P** 2008 The Regulation of Seasonal Changes in Food Intake and Body Weight. J Neuroendocrinol 20:827-833
50. **de Kloet ER, Sarabdjitsingh RA.** 2008 Everything has rhythm: focus on glucocorticoid pulsatility. Endocrinology 149(7): 3241-3
51. **Panda S, Hogenesch JB, Kay SA** 2002 Circadian rhythms from flies to human. Nature 417:329-335
52. **Reppert SM, Weaver DR** 2002 Coordination of circadian timing in mammals. Nature 418:935-41
53. **Young E, Abelson J, Lightman S** 2004 Cortisol pulsatility and its role in stress regulation and health. Front Neuroendocrinol 25:69-76
54. **Herbert J, Goodyer IM, Grossman AB, Hastings MH, De Kloet ER, Lightman SL, Lupien SJ, Roozendaal B, Seckl JR** 2006 Do Corticosteroids Damage the Brain? J Neuroendocrinol 18:393-411
55. **Jasper MS, Engeland WC** 1991 Synchronous ultradian rhythms in adrenocortical secretion detected by microdialysis in awake rats. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 261:R1257-R1268
56. **Windle RJ, Wood SA, Shanks N, Lightman SL, Ingram CD** 1998 Ultradian rhythm of basal corticosterone release in the female rat: dynamic interaction with the response to acute stress. Endocrinology 139:443-450
57. **Holaday JW, Martinez HM, Natelson BH** 1977 Synchronizedultradian cortisol rhythms in monkeys: persistence during corticotropin infusion. Science 198:56-8
58. **Tapp WN, Holaday JW, Natelson BH** 1984 Ultradian glucocorticoid rhythms in monkeys and rats continue during stress. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 247:R866-R871
59. **Cook CJ** 2001 Measuring of extracellular cortisol and corticotropin-releasing hormone in the amygdala using immunosensor coupled microdialysis. J Neurosci Methods 110:95-101
60. **Weitzman ED, Fukushima D, Nogeire C, Roffwarg H, Gallagher TF, Hellman L** 1971 Twenty-four Hour Pattern of the Episodic Secretion of Cortisol in Normal Subjects. Journal of Clinical Endocrinology & Metabolism 33:14-22
61. **Lewis JG, Bagley CJ, Elder PA, Bachmann AW, Torpy DJ** 2005 Plasma free cortisol fraction reflects levels of functioning corticosteroid-binding globulin. Clinica Chimica Acta 359:189-194

62. **Henley DE, Russell GM, Douthwaite JA, Wood SA, Buchanan F, Gibson R, Woltersdorf WW, Catterall JR, Lightman SL** 2009 Hypothalamic-Pituitary-Adrenal Axis Activation in Obstructive Sleep Apnea: The Effect of Continuous Positive Airway Pressure Therapy. Journal of Clinical Endocrinology & Metabolism 94:4234-4242
63. **Conway-Campbell BL, McKenna MA, Wiles CC, Atkinson HC, de Kloet ER, Lightman SL** 2007 Proteasome-dependent down-regulation of activated nuclear hippocampal glucocorticoid receptors determines dynamic responses to corticosterone. Endocrinology 148:5470-5477
64. **Waxman DJ, Ram PA, Park S, Choi HK** 1995 Intermittent Plasma Growth Hormone Triggers Tyrosine Phosphorylation and Nuclear Translocation of a Liver-Expressed, Stat 5-related DNA Binding Protein: Proposed role as an intracellular liver gene transcription. Journal of Biological Chemistry 270:13262-13270
65. **Gevers EF, Wit JM, Robinson IC** 1996 Growth, growth hormone (GH)-binding protein, and GH receptors are differentially regulated by peak and trough components of the GH secretory pattern in the rat. Endocrinology 137:1013-1018
66. **Belchetz PE, Plant TM, Nakai Y, Keogh EJ, Knobil E** 1978 Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone. Science 202:631-3
67. **Rothman MS, Wierman ME** 2007 The role of gonadotropin releasing hormone in normal and pathologic endocrine processes. Current Opinion in Endocrinology, Diabetes and Obesity 14: 306-10
68. **Wildt L, Häusler A, Marshall G, Hutchison JS, Plant TM, Belchetz PE, Knobil E** 1981 Frequency and Amplitude of Gonadotropin-Releasing Hormone Stimulation and Gonadotropin Secretion in the Rhesus Monkey. Endocrinology 109:376-385
69. **Matthews DR, Naylor BA, Jones RG, Ward GM, Turner RC** 1983 Pulsatile insulin has greater hypoglycemic effect than continuous delivery. Diabetes 32:617-21
70. **Hauffa BP** 2001 Clinical implications of pulsatile hormone signals. Growth Horm IGF Res. Suppl A: S1-8.
71. **Sarabdjitsingh RA, Isenia S, Polman A, Mijalkovic J, Lachize S, Datson N, de Kloet ER, Meijer OC** 2010 Disrupted Corticosterone Pulsatile Patterns Attenuate Responsiveness to Glucocorticoid Signaling in Rat Brain. Endocrinology 151:1177-1186
72. **Ixart G, Barbanel G, Conte-Devolx B, Grino M, Oliver C, Assenmacher I** 1987 Evidence for basal and stress-induced release of corticotropin releasing factor in the push-pull cannulated median eminence of conscious free-moving rats. Neurosci Lett. 74:85-9
73. **Mershon JL, Sehlhorst CS, Rebar RW, Liu JH** 1992 Evidence of a corticotropin-releasing hormone pulse generator in the macaque hypothalamus. Endocrinology 130:2991-2996
74. **Oster H, Damerow S, Kiessling S, Jakubcakova V, Abraham D, Tian J, Hoffmann MW, Eichele G** 2006 The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. Cell Metab 4:163-173
75. **Son GH, Chung S, Choe HK, Kim H, Baik S, Lee H, Lee H, Choi S, Sun W, Kim H, Cho S, Lee KH, Kim K** 2008 Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. Proceedings of the National Academy of Sciences 105:20970-20975
76. **Watanabe K, Hiroshige T** 1981 Phase relation between episodic fluctuations of spontaneous locomotor activity and plasma corticosterone in rats with suprachiasmatic nuclei lesions. Neuroendocrinology 33:52-9
77. **Buijs RM, Kalsbeek A, van der Woude TP, van Heerikhuize JJ, Shinn S** 1993 Suprachiasmatic nucleus lesion increases corticosterone secretion. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 264:R1186-R1192

78. **Walker JJ, Terry JR, Lightman SL** 2010 Origin of ultradian pulsatility in the hypothalamic–pituitary–adrenal axis. Proceedings of the Royal Society B: Biological Sciences 277:1627-1633
79. **Keller-Wood ME, Dallman MF Winter** 1984 Corticosteroid Inhibition of ACTH Secretion. Endocrine Reviews 5:1-24
80. **Ma X, Levy A, Lightman SL** 1997 Rapid changes in heteronuclear RNA for corticotrophin-releasing hormone and arginine vasopressin in response to acute stress. Journal of Endocrinology 152:81-89
81. **Gupta S, Aslakson E, Gurbaxani B, Vernon S** 2007 Inclusion of the glucocorticoid receptor in a hypothalamic pituitary adrenal axis model reveals bistability. Theoretical Biology and Medical Modelling 4:8
82. **Spiga F, Harrison LR, Wood SA, Atkinson HC, MacSweeney CP, Thomson F, Craighead M, Grassie M, Lightman SL** 2007 Effect of the Glucocorticoid Receptor Antagonist Org 34850 on Basal and Stress-Induced Corticosterone Secretion. J Neuroendocrinol 19:891-900
83. **Abelson JL C,G.C.** 1996 Hypothalamic-pituitary-adrenal axis activity in panic disorder: 24-hour secretion of corticotropin and cortisol. Arch Gen Psychiatry 53:323-331
84. **Hartmann A, Veldhuis JD, Deuschle M, Standhardt H, Heuser I** 1997 Twenty-Four Hour Cortisol Release Profiles in Patients With Alzheimer's and Parkinson's Disease Compared to Normal Controls: Ultradian Secretory Pulsatility and Diurnal Variation. Neurobiol Aging 18:285-289
85. **Aziz NA, Pijl H, Frölich M, van der Graaf AWM, Roelfsema F, Roos RAC** 2009 Increased Hypothalamic-Pituitary-Adrenal Axis Activity in Huntington's Disease. Journal of Clinical Endocrinology & Metabolism 94:1223-1228
86. **Deuschle M, Schweiger U, Weber B, Gotthardt U, Körner A, Schmider J, Standhardt H, Lammers C, Heuser I** 1997 Diurnal Activity and Pulsatility of the Hypothalamus-Pituitary-Adrenal System in Male Depressed Patients and Healthy Controls. Journal of Clinical Endocrinology & Metabolism 82:234-238
87. **Holsboer F** 2000 The Corticosteroid Receptor Hypothesis of Depression. Neuropsychopharmacology 23:477–501
88. **Windle RJ, Wood SA, Kershaw YM, Lightman SL, Ingram CD, Harbuz MS** 2001 Increased Corticosterone Pulse Frequency During Adjuvant-Induced Arthritis and its Relationship to Alterations in Stress Responsiveness. J Neuroendocrinol 13:905-911
89. **Bleakman D, Alt A, Witkin JM** 2007 AMPA receptors in the therapeutic management of depression. CNS & neurological disorders drug targets 2:117-26
90. **Alt A, Nisenbaum ES, Bleakman D, Witkin JM** 2006 A role for AMPA receptors in mood disorders. Biochem Pharmacol 71:1273-1288
91. **Groc L, Heine M, Cognet L, Brickley K, Stephenson FA, Lounis B, Choquet D** 2004 Differential activity-dependent regulation of the lateral mobilities of AMPA and NMDA receptors. Nature Neuroscience 7:695-6
92. **Zhang Y, Sheng H, Qi J, Ma B, Sun J, Li S, Ni X** 2012 Glucocorticoid acts on a putative G protein-coupled receptor to rapidly regulate the activity of NMDA receptors in hippocampal neurons. American Journal of Physiology - Endocrinology And Metabolism 302: E747-E758
93. **Li N, Liu R, Dwyer JM, Banasr M, Lee B, Son H, Li X, Aghajanian G, Duman RS** 2011 Glutamate N-methyl-D-aspartate Receptor Antagonists Rapidly Reverse Behavioral and Synaptic Deficits Caused by Chronic Stress Exposure. Biol Psychiatry 69:754-761
94. R**osenmund C, Stern-Bach Y, Stevens CF** 1998 The tetrameric structure of a glutamate receptor channel. Science 280:1596-9
95. **Hollmann M, Heinemann S** 1994 Cloned glutamate receptors. Annual review of Neuroscience 17:31-108

96. **Tanabe Y, Masu M, Ishii T, Shigemoto R, Nakanishi S** 1992 A family of metabotropic glutamate receptors. Neuron 8:169-179
97. **Wisden W, Seeburg PH** 1993 Mammalian ionotropic glutamate receptors. Current opinion in neurobiology 3:291-8
98. **Wenthold R, Petralia R, Blahos J I, Niedzielski A** 1996 Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. The Journal of Neuroscience 16:1982-1989
99. **Zhu JJ, Esteban JA, Hayashi Y, Malinow R** 2000 Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. Nature Neuroscience 11:1098-106
100. **Sheng M, Sala C** 2001 PDZ domains and the organization of supramolecular complexes. Annu Rev Neurosci 24:1-29
101. **Malinow R, Malenka RC** 2002 AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci 25:103-126
102. **Barry MF, Ziff EB** 2002 Receptor trafficking and the plasticity of excitatory synapses. Curr Opin Neurobiol 12:279-286
103. **Kapitein LC, Schlager MA, Kuijpers M, Wulf PS, van Spronsen M, MacKintosh FC, Hoogenraad CC** 2010 Mixed Microtubules Steer Dynein-Driven Cargo Transport into Dendrites. Curr Biol 20:290-299
104. **Passafaro M, Piëch V, Sheng M** 2001 Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. Nature Neuroscience 4:917-26
105. **Gerges NZ, Backos DS, Rupasinghe CN, Spaller MR, Esteban JA** 2006 Dual role of the exocyst in AMPA receptor targeting and insertion into the postsynaptic membrane. The EMBO Journal 25:1623-34
106. **Triller A, Choquet D** 2005 Surface trafficking of receptors between synaptic and extrasynaptic membranes: and yet they do move! Trends Neurosci 28:133-139
107. A**desnik H, Nicoll RA, England PM** 2005 Photoinactivation of Native AMPA Receptors Reveals Their Real-Time Trafficking. Neuron 48:977-985
108. **Ashby MC, Maier SR, Nishimune A, Henley JM** 2006 Lateral Diffusion Drives Constitutive Exchange of AMPA Receptors at Dendritic Spines and Is Regulated by Spine Morphology. The Journal of Neuroscience 26:7046-7055
109. **Ehlers MD, Heine M, Groc L, Lee MC, Choquet D** 2007 Diffusional trapping of GluR1 AMPA receptors by input-specific synaptic activity. Neuron 54:447-460
110. **Gerrow K, Triller A** 2010 Synaptic stability and plasticity in a floating world. Curr Opin Neurobiol 20:631-639
111. **Heine M, Groc L, Frischknecht R, Beique JC, Lounis B, Rumbaugh G, Huganir RL, Cognet L, Choquet D** 2008 Surface mobility of postsynaptic AMPARs tunes synaptic transmission. Science 320:201-205
112. **Joëls M, Sarabdjitsingh RA, Karst H** 2012 Unraveling the Time Domains of Corticosteroid Hormone Influences on Brain Activity: Rapid, Slow, and Chronic Modes. Pharmacological Reviews 64:901-938
113. **Martin S, Henley M, Holman D, Zhou M, Wiegert O, van Spronsen M, Joëls M, Hoogenraad C, Krugers H** 2009 Corticosterone Alters AMPAR Mobility and Facilitates Bidirectional Synaptic Plasticity. PLoS ONE 4:e4714
114. **Oitzl MS, de Kloet ER** 1992 Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. Behav Neurosci 106:62-71
115. **Sandi C, Rose SP** 1994 Corticosteroid receptor antagonists are amnestic for passive avoidance learning in day-old chicks. Eur J Neurosci 6:1292-7
116. **Roozendaal B, de Quervain DJ-, Ferry B, Setlow B, McGaugh JL** 2001 Basolateral Amygdala–Nucleus Accumbens Interactions in Mediating Glucocorticoid Enhancement of Memory Consolidation. The Journal of Neuroscience 21:2518-2525

117. **Jin X, Lu Y, Yang X, Ma L, Li B** 2007 Glucocorticoid receptors in the basolateral nucleus of amygdala are required for postreactivation reconsolidation of auditory fear memory. Eur J Neurosci 25:3702-3712
118. **Roozendaal B, McEwen BS, Chattarji S** 2009 Stress, memory and the amygdala. Nat Rev Neurosci 10:423-433
119. **Roozendaal B, Hernandez A, Cabrera SM, Hagewoud R, Malvaez M, Stefanko DP, Haettig J, Wood MA** 2010 Membrane-associated glucocorticoid activity is necessary for modulation of long-term memory via chromatin modification. J Neurosci 30:5037-5046
120. **Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A** 1984 Magnesium gates glutamate-activated channels in mouse central neurones. Nature 307:462-465
121. **Kim JJ, Foy MR, Thompson RF** 1996 Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. Proc Natl Acad Sci U S A 93:4750-4753
122. **Armanini MP, Hutchins C, Stein BA, Sapolsky RM** 1990 Glucocorticoid endangerment of hippocampal neurons is NMDA-receptor dependent. Brain Res 532:7-12
123. **Krugers HJ, Koolhaas JM, Bohus B, Korf J** 1993 A single social stress-experience alters glutamate receptor-binding in rat hippocampal CA3 area. Neuroscience Lett. 154:73-7
124. **Bartanusz V, Aubry JM, Pagliusi S, Jezova D, Baffi J, Kiss JZ** 1995 Stress-induced changes in messenger RNA levels of N-methyl-D-aspartate and AMPA receptor subunits in selected regions of the rat hippocampus and hypothalamus. Neuroscience 66:247-252
125. **Kole MHP, Swan L, Fuchs E** 2002 The antidepressant tianeptine persistently modulates glutamate receptor currents of the hippocampal CA3 commissural associational synapse in chronically stressed rats. Eur J Neurosci 16:807-816
126. **McEwen BS** 1999 Stress and hippocampal plasticity. Annu Rev Neurosci 22:105-22
127. **Weiland NG, Orchinik M, Tanapat P** 1997 Chronic corticosterone treatment induces parallel changes in N-methyl-D-aspartate receptor subunit messenger RNA levels and antagonist binding sites in the hippocampus. Neuroscience 78:653-662
128. **Yashiro K, Philpot BD** 2008 Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. Neuropharmacology 55:1081-1094
129. **Kim MJ, Dunah AW, Wang YT, Sheng M** 2005 Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron 46:745-760
130. **Czyrak A, Maćkowiak M, Chocyk A, Fijał K, Wedzony K** 2003 Role of glucocorticoids in the regulation of dopaminergic neurotransmission. Pol J Pharmacology 55:667-74
131. **Joels M, Krugers HJ** 2007 LTP after stress: up or down? Neural Plast 2007:93202
132. **Hill MN, McEwen BS** 2010 Involvement of the endocannabinoid system in the neurobehavioural effects of stress and glucocorticoids. Prog Neuro-Psychopharmacol Biol Psychiatry 34:791-797
133. **Haj-Dahmane S, Shen R** 2011 Modulation of the serotonin system by endocannabinoid signaling. Neuropharmacology 61:414-420
134. **Joels M, Baram TZ** 2009 The neuro-symphony of stress. Nat Rev Neurosci 10:459-466
135. **Mora F, Segovia G, del Arco A, de Blas M, Garrido P** 2012 Stress, neurotransmitters, corticosterone and body–brain integration. Brain Res 1476:71-85
136. **Zhou M, Hoogenraad CC, Joëls M, Krugers HJ** 2012 Combined β-adrenergic and corticosteroid receptor activation regulates AMPA receptor function in hippocampal neurons. Journal of Psychopharmacology 26:516-524
137. **Roozendaal B, Nguyen BT, Power AE, McGaugh JL** 1999 Basolateral amygdala noradrenergic influence enables enhancement of memory consolidation induced by hippocampal glucocorticoid receptor activation. Proceedings of the National Academy of Sciences 96:11642-11647

138. **Joëls M, Fernandez G, Roozendaal B** 2011 Stress and emotional memory: a matter of timing. Trends Cogn Sci 15:280-288
139. **Cahill L, Mcgaugh JL** 1996 The neurobiology of memory for emotional events: adrenergic activation and the amygdala. Proceedings of Western Pharmacology society 39:81-4
140. **Adolphs R, Cahill L, Schul R, Babinsky R** 1997 Impaired declarative memory for emotional material following bilateral amygdala damage in humans. Learn Mem 4:291-300.
141. **Hamann SB, Ely TD, Grafton ST, Kilts CD** 1999 Amygdala activity related to enhanced memory for pleasant and aversive stimuli. Nature Neuroscience 2:289-93
142. **Canli T, Zhao Z, Brewer J, Gabrieli JDE, Cahill L** 2000 Event-Related Activation in the Human Amygdala Associates with Later Memory for Individual Emotional Experience. The Journal of Neuroscience 20:RC99-RC99
143. **Quirarte GL, Galvez R, Roozendaal B, McGaugh JL** 1998 Norepinephrine release in the amygdala in response to footshock and opioid peptidergic drugs. Brain Res 808:134-140
144. **Droste SK, de Groote L, Atkinson HC, Lightman SL, Reul JMHM, Linthorst ACE** 2008 Corticosterone Levels in the Brain Show a Distinct Ultradian Rhythm but a Delayed Response to Forced Swim Stress. Endocrinology 149:3244-3253
145. **Quirarte GL, Roozendaal B, McGaugh JL** 1997 Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala. Proceedings of the National Academy of Sciences 94:14048-14053
146. R**oozendaal B, Quirarte GL, McGaugh JL** 2002 Glucocorticoids interact with the basolateral amygdala β-adrenoceptor-cAMP/cAMP/PKA system in influencing memory consolidation. Eur J Neurosci 15:553-560
147. **McReynolds JR, Donowho K, Abdi A, McGaugh JL, Roozendaal B, McIntyre CK** 2010 Memory-enhancing corticosterone treatment increases amygdala norepinephrine and Arc protein expression in hippocampal synaptic fractions. Neurobiol Learn Mem 93:312-321
148. **Ferry B, Magistretti PJ, Pralong E** 1997 Noradrenaline modulates glutamate-mediated neurotransmission in the rat basolateral amygdala in vitro. Eur J Neurosci 9:1356-64
149. **DeBock F, Kurz J, Azad SC, Parsons CG, Hapfelmeier G, Zieglgänsberger W, Rammes G** 2003 ?2-Adrenoreceptor activation inhibits LTP and LTD in the basolateral amygdala: involvement of Gi/o-protein-mediated modulation of Ca2+-channels and inwardly rectifying K+-channels in LTD. Eur J Neurosci 17:1411-1424
150. **Buffalari DM, Grace AA** 2007 Noradrenergic Modulation of Basolateral Amygdala Neuronal Activity: Opposing Influences of α-2 and β Receptor Activation. The Journal of Neuroscience 27:12358-12366
151. **Huang YY, Nguyen PV, Abel T, Kandel ER** 1996 Long-lasting forms of synaptic potentiation in the mammalian hippocampus. Learning & Memory 3:74-85
152. **Wang S, Cheng L, Gean P** 1999 Cross-Modulation of Synaptic Plasticity by β-Adrenergic and 5-HT1A Receptors in the Rat Basolateral Amygdala. The Journal of Neuroscience 19:570-577
153. **Liebmann L, Karst H, Joëls M** 2009 Effects of corticosterone and the ?-agonist isoproterenol on glutamate receptor-mediated synaptic currents in the rat basolateral amygdala. Eur J Neurosci 30:800-807
154. **Brown ES, Varghese FP, McEwen BS** 2004 Association of depression with medical illness: does cortisol play a role? Biol Psychiatry 55:1-9
155. **Lupien SJ, de Leon M, de Santi S, Convit A, Tarshish C, Nair NP, Thakur M, McEwen BS, Hauger RL, Meaney MJ** 1998 Cortisol levels during human aging predict hippocampal atrophy and memory deficits. Nature Neuroscience 1:69-73
156. **Blackburn-Munro G, Blackburn-Munro RE** 2001 Chronic Pain, Chronic Stress and Depression: Coincidence or Consequence? J Neuroendocrinol 13:1009-1023

157. **Willner P, Mitchell PJ** 2002 The validity of animal models of predisposition to depression. Behav Pharmacol. 13:169-88
158. **Holsboer-Trachsler E, Stohler R, Hatzinger M** 1991 Repeated administration of the combined dexamethasone-human corticotropin releasing hormone stimulation test during treatment of depression. Psychiatry Res 38:163-171
159. **Schmider J, Lammers C, Gotthardt U, Dettling M, Holsboer F, Heuser IJE** 1995 Combined dexamethasone/corticotropin-releasing hormone test in acute and remitted manic patients, in acute depression, and in normal controls: I. Biol Psychiatry 38:797-802
160. **van Praag HM** 2004 Can stress cause depression? Prog Neuro-Psychopharmacol Biol Psychiatry 28:891-907
161. **McEwen BS** 2004 Protection and Damage from Acute and Chronic Stress: Allostasis and Allostatic Overload and Relevance to the Pathophysiology of Psychiatric Disorders. Ann N Y Acad Sci 1032:1-7
162. **McEwen BS** 2000 The neurobiology of stress: from serendipity to clinical relevance. Brain Res 886:172-189
163. **Heine VM, Maslam S, Zareno J, Joëls M, Lucassen PJ** 2004 Suppressed proliferation and apoptotic changes in the rat dentate gyrus after acute and chronic stress are reversible. Eur J Neurosci 19:131-144
164. **Joëls M, Karst H, Alfarez D, Heine VM, Qin Y, Riel Ev, Verkuyl M, Lucassen PJ, Krugers HJ** 2004 Effects of Chronic Stress on Structure and Cell Function in Rat Hippocampus and Hypothalamus. Stress 7:221-231
165. **Lucassen PJ, Heine VM, Muller MB, van der Beek EM, Wiegant VM, De Kloet ER, Joels M, Fuchs E, Swaab DF, Czeh B** 2006 Stress, depression and hippocampal apoptosis. CNS & neurological disorders drug targets 5:531-46
166. **Karst H, Joëls M** 2003 Effect of Chronic Stress on Synaptic Currents in Rat Hippocampal Dentate Gyrus Neurons. Journal of Neurophysiology 89:625-633
167. **Datson NA, Speksnijder N, Mayer JL, Steenbergen PJ, Korobko O, Goeman J, de Kloet ER, Joëls M, Lucassen P**J 2012 The transcriptional response to chronic stress and glucocorticoid receptor blockade in the hippocampal dentate gyrus. Hippocampus 22:359-371
168. **Gunnar MR, Vazquez DM** 2001 Low cortisol and a flattening of expected daytime rhythm: potential indices of risk in human development. Development and psychopathology 13:515-38
169. **Yehuda R, Seckl J** 2011 Minireview: Stress-Related Psychiatric Disorders with Low Cortisol Levels: A Metabolic Hypothesis. Endocrinology 152:4496-4503
170. **Sloviter RS, Valiquette G, Abrams GM, Ronk EC, Sollas AL, Paul LA, Neubort S** 1989 Selective loss of hippocampal granule cells in the mature rat brain after adrenalectomy. Science 243:535-8
171. **Stienstra CM, Van der Graaf F, Bosma A, Karten YJG, Hesen W, Joëls M** 1998 Synaptic transmission in the rat dentate gyrus after adrenalectomy. Neuroscience 85:1061-1071
172. **Fransén E** 2005 Functional role of entorhinal cortex in working memory processing. Neural Networks 18:1141-1149
173. **Oitzl MS, Fluttert M, de Kloet ER** 1994 The effect of corticosterone on reactivity to spatial novelty is mediated by central mineralocorticosteroid receptors. Eur J Neurosci. 6:1072-9
174. **Woolley CS, Gould E, Sakai RR, Spencer RL, McEwen BS** 1991 Effects of aldosterone or RU28362 treatment on adrenalectomy-induced cell death in the dentate gyrus of the adult rat. Brain Res 554:312-315
175. **Hashimoto H, Marystone JF, Greenough WT, Bohn MC** 1989 Neonatal adrenalectomy alters dendritic branching of hippocampal granule cells. Experimental neurology 104:62-7

176. **Lightman SL, Windle RJ, Ma X-, Harbuz MS, Shanks NM, Julian MD, Wood SA, Kershaw YM, Ingram CD** 2002 Hypothalamic-Pituitary-Adrenal Function. Arch Physiol Biochem 110:90-93
177. **Funder J** 1994 Corticosteroid receptors and the central nervous system. J Steroid Biochem Mol Bio. 49(4-6):381
178. **Duvarci S, Paré D** 2007 Glucocorticoids Enhance the Excitability of Principal Basolateral Amygdala Neurons. The Journal of Neuroscience 27:4482-4491
179. **van Gemert NG, Carvalho DMM, Karst H, van der Laan S, Zhang M, Meijer OC, Hell JW, Joels M** 2009 Dissociation between rat hippocampal CA1 and dentate gyrus cells in their response to corticosterone: effects on calcium channel protein and current. Endocrinology 150:4615-24
180. **Karst H, Berger S, Erdmann G, Schütz G, Joëls M** 2010 Metaplasticity of amygdalar responses to the stress hormone corticosterone. Proceedings of the National Academy of Sciences 107:14449-14454
181. **Karst H, Karten YJG, Reichardt HM, de Kloet ER, Schutz G, Joels M** 2000 Corticosteroid actions in hippocampus require DNA binding of glucocorticoid receptor homodimers. Nat Neurosci 3:977-978
182. **Zhou X, Dong X, Priestley T** 2006 The neuroleptic drug, fluphenazine, blocks neuronal voltage-gated sodium channels. Brain Res 1106:72-81
183. **Lee G GS** 2009 GABAA receptor signaling in the lateral septum regulates maternal aggression in mice. Behav Neurosci. 123(6):1169-77
184. **Lösel RM, Falkenstein E, Feuring M, Schulty A, Tillmann H, Rossol-Haseroth K, Wehling M** 2003 Nongenomic Steroid Action: Controversies, Questions, and Answers. Physiological Reviews 83:965-1016
185. **Liebmann L, Karst H, Sidiropoulou K, van Gemert N, Meijer OC, Poirazi P, Joëls M** February 2008 Differential Effects of Corticosterone on the Slow Afterhyperpolarization in the Basolateral Amygdala and CA1 Region: Possible Role of Calcium Channel Subunits. Journal of Neurophysiology 99:958-968
186. **Windle RJ, Wood SA, Lightman SL, Ingram CD** 1998 The Pulsatile Characteristics of Hypothalamo-Pituitary-Adrenal Activity in Female Lewis and Fischer 344 Rats and Its Relationship to Differential Stress Responses. Endocrinology 139:4044-4052
187. **Lightman SL, Conway-Campbell BL** 2010 The crucial role of pulsatile activity of the HPA axis for continuous dynamic equilibration. Nat Rev Neurosci 11:710-718
188. **de Kloet ER, Oitzl MS, Joëls M** 1999 Stress and cognition: are corticosteroids good or bad guys? Trends Neurosci 22:422-426
189. **Joëls M, Krugers HJ, Lucassen PJ, Karst H** 2009 Corticosteroid effects on cellular physiology of limbic cells. Brain Res 1293:91-100
190. **Stavreva DA, Wiench M, John S, Conway-Campbell BL, McKenna MA, Pooley JR, Johnson TA, Voss TC, Lightman SL, Hager GL** 2009 Ultradian hormone stimulation induces glucocorticoid receptor-mediated pulses of gene transcription. Nat Cell Biol 11:1093-1102
191. **asricha N, Joëls M, Karst H** 2011 Rapid Effects of Corticosterone in the Mouse Dentate Gyrus Via a Nongenomic Pathway. J Neuroendocrinol 23:143-147
192. **Joëls M, Karst H, DeRijk R, de Kloet ER** 2008 The coming out of the brain mineralocorticoid receptor. Trends Neurosci 31:1-7
193. **Hawley DF, Leasure JL** 2012 Region-specific response of the hippocampus to chronic unpredictable stress. Hippocampus 22:1338-49
194. **Maggio N, Segal M** 2007 Striking Variations in Corticosteroid Modulation of Long-Term Potentiation along the Septotemporal Axis of the Hippocampus. The Journal of Neuroscience 27:5757-5765

195. **Sarabdjitsingh RA, Conway-Campbell BL, Leggett JD, Waite EJ, Meijer OC, de Kloet ER, Lightman SL** 2010 Stress Responsiveness Varies over the Ultradian Glucocorticoid Cycle in a Brain-Region-Specific Manner. Endocrinology 151:5369-5379
196. **Linthorst ACE, Reul JM** 2008 Stress and the brain: Solving the puzzle using microdialysis. Pharmacology Biochemistry and Behavior 90:163-173
197. **Linthorst A, Flachskamm C, Muller-Preuss P, Holsboer F, Reul J** 1995 Effect of bacterial endotoxin and interleukin-1 beta on hippocampal serotonergic neurotransmission, behavioral activity, and free corticosterone levels: an in vivo microdialysis study. The Journal of Neuroscience 15:2920-2934
198. **Marchese A, Paing MM, Temple BRS, Trejo J** 2008 G Protein-Coupled Receptor Sorting to Endosomes and Lysosomes. Annu Rev Pharmacol Toxicol 48:601-629
199. **Bondar G, Kuo J, Hamid N, Micevych P** 2009 Estradiol-Induced Estrogen Receptor-α Trafficking. The Journal of Neuroscience 29:15323-15330
200. **Dominguez R, Micevych P** 2010 Estradiol Rapidly Regulates Membrane Estrogen Receptor α Levels in Hypothalamic Neurons. The Journal of Neuroscience 30:12589-12596
201. **Buchanan TW, Lovallo WR** 2001 Enhanced memory for emotional material following stress-level cortisol treatment in humans. Psychoneuroendocrinology 26:307-317
202. **van Stegeren AH, Roozendaal B, Kindt M, Wolf OT, Joels M** 2010 Interacting noradrenergic and corticosteroid systems shift human brain activation patterns during encoding. Neurobiol Learn Mem 93:56-65
203. **Wirth MM, Scherer SM, Hoks RM, Abercrombie HC** 2011 The effect of cortisol on emotional responses depends on order of cortisol and placebo administration in a within-subject design. Psychoneuroendocrinology 36:945-954
204. **Droste SK LS** 2009 The ultradian and circadian rhythms of free corticosterone in the Brain are not affected by gender: An In Vivo Microdylysis Study in Wistar Rats. J Neuroendocrinol. 21:132-40
205. **e Kloet ER, Van Acker S,A.B.E., Sibug RM, Oitzl MS, Meijer OC, Rahmouni K, De Jong W** 2000 Brain mineralocorticoid receptors and centrally regulated functions. Kidney Int 57:1329-1336
206. **De Kloet ER, Sutanto W, Rots N, van Haarst A, van den Berg D, Oitzl M, van Eekelen A, Voorhuis D** 1991 Plasticity and function of brain corticosteroid receptors during aging. Acta Endocrinol (Copenh) 125 Suppl 1:65-72
207. K**rugers HJ, Alfarez DN, Karst H, Parashkouhi K, van Gemert N, Joels M** 2005 Corticosterone shifts different forms of synaptic potentiation in opposite directions. Hippocampus 15:697-703
208. **Dorey R, Pierard C, Shinkaruk S, Tronche C, Chauveau F, Baudonnat M, Beracochea D** 2011 Membrane mineralocorticoid but not glucocorticoid receptors of the dorsal hippocampus mediate the rapid effects of corticosterone on memory retrieval. Neuropsychopharmacology 36:2639-2649
209. **Maggio N, Segal M** 2012 Cellular basis of a rapid effect of mineralocorticosteroid receptors activation on LTP in ventral hippocampal slices. Hippocampus 22:267-275
210. **Tse YC, Bagot RC, Hutter JA, Wong AS, Wong TP** 2011 Modulation of synaptic plasticity by stress hormone associates with plastic alteration of synaptic NMDA receptor in the adult hippocampus. PLoS One 6:e27215
211. **Zamanillo D, Sprengel R, Hvalby O, Jensen V, Burnashev N, Rozov A, Kaiser KM, Köster HJ, Borchardt T, Worley P, Lübke J, Frotscher M, Kelly PH, Sommer B, Andersen P, Seeburg PH, Sakmann B** 1999 Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. Science 284:1805-11

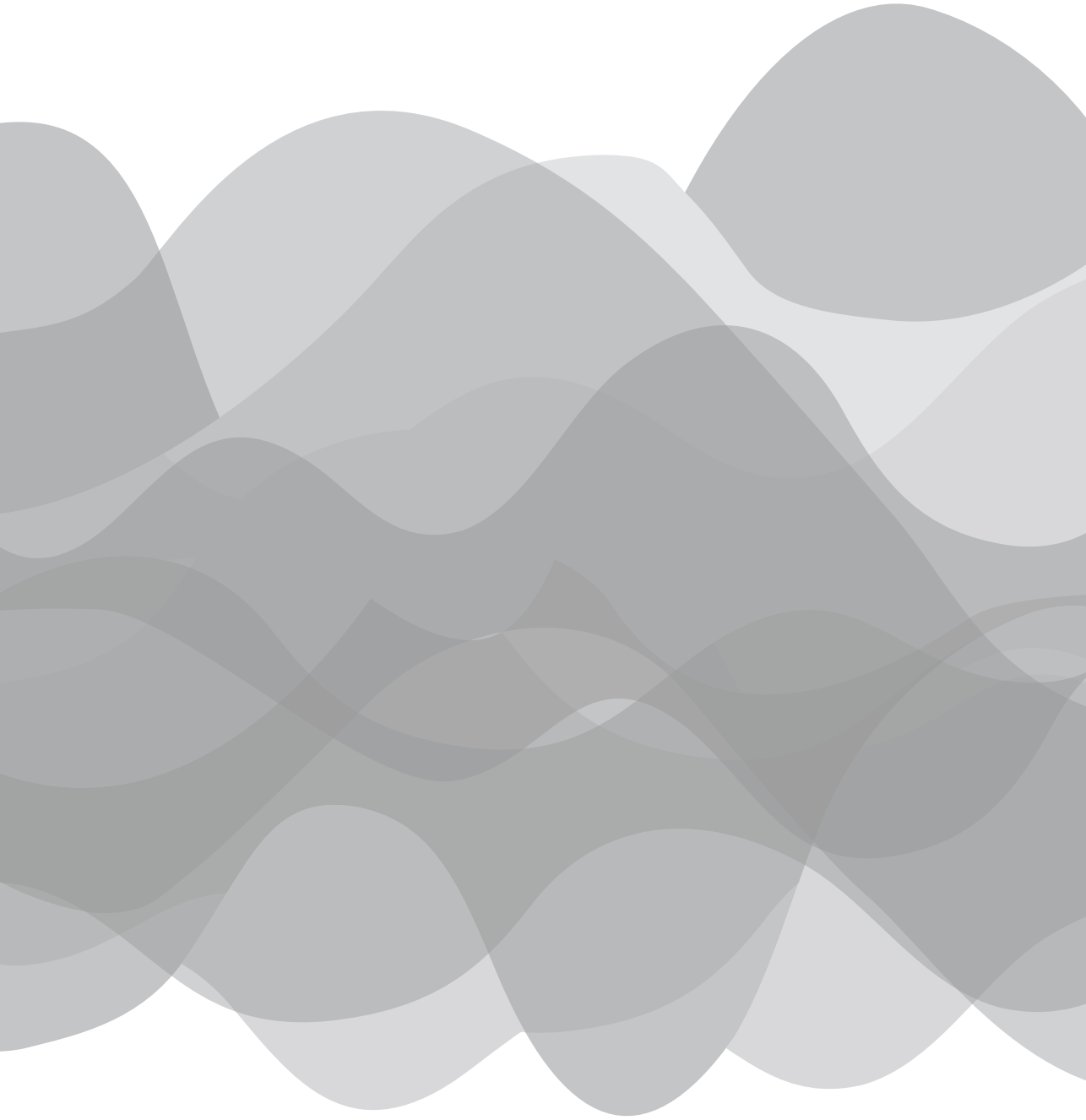
212. **Jensen V, Kaiser KMM, Borchardt T, Adelmann G, Rozov A, Burnashev N, Brix C, Frotscher M, Andersen P, Hvalby Ø, Sakmann B, Seeburg PH, Sprengel R** 2003 A juvenile form of postsynaptic hippocampal long-term potentiation in mice deficient for the AMPA receptor subunit GluR-A. The Journal of Physiology 553:843-856
213. **Czondor K, Mondin M, Garcia M, Heine M, Frischknecht R, Choquet D, Sibarita JB, Thoumine OR** 2012 Unified quantitative model of AMPA receptor trafficking at synapses. Proc Natl Acad Sci U S A 109:3522-3527
214. **Geinisman Y, Disterhoft JF, Gundersen HJ, McEchron MD, Persina IS, Power JM, van der Zee EA, West MJ** 2000 Remodeling of hippocampal synapses after hippocampus-dependent associative learning. J Comp Neurol 417:49-59
215. **Kow LM, Pfaff DW** 2004 The membrane actions of estrogens can potentiate their lordosis behavior-facilitating genomic actions. Proc Natl Acad Sci U S A 101:12354-12357
216. **Vasudevan N, Kow LM, Pfaff DW** 2001 Early membrane estrogenic effects required for full expression of slower genomic actions in a nerve cell line. Proc Natl Acad Sci U S A 98:12267-12271
217. **Carrasco GA, Van de Kar LD** 2003 Neuroendocrine pharmacology of stress. Eur J Pharmacol 463:235-272
218. **Tsigos C, Chrousos GP** 2002 Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53:865-871
219. **McGaugh JL, Roozendaal B** 2002 Role of adrenal stress hormones in forming lasting memories in the brain. Curr Opin Neurobiol 12:205-210
220. **Valentino RJ, Van Bockstaele E** 2008 Convergent regulation of locus coeruleus activity as an adaptive response to stress. Eur J Pharmacol 583:194-203
221. **Bouchez G, Millan MJ, Rivet J, Billiras R, Boulanger R, Gobert A** 2012 Quantification of extracellular levels of corticosterone in the basolateral amygdaloid complex of freely-moving rats: A dialysis study of circadian variation and stress-induced modulation. Brain Res 1452:47-60
222. **Roozendaal B, Okuda S, Van der Zee EA, McGaugh JL** 2006 Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. Proceedings of the National Academy of Sciences 103:6741-6746
223. **Lin H, Wang S, Luo M, Gean P** 2000 Activation of Group II Metabotropic Glutamate Receptors Induces Long-Term Depression of Synaptic Transmission in the Rat Amygdala. The Journal of Neuroscience 20:9017-9024
224. **Pu Z, Krugers HJ, Joëls M** 2007 Corticosterone time-dependently modulates β-adrenergic effects on long-term potentiation in the hippocampal dentate gyrus. Learning & Memory 14:359-367
225. **Pu Z, Krugers HJ, Joëls M** 2009 β-Adrenergic facilitation of synaptic plasticity in the rat basolateral amygdala in vitro is gradually reversed by corticosterone. Learning & Memory 16:155-160
226. **Puente N, Cui Y, Lassalle O, Lafourcade M, Georges F, Venance L, Grandes P, Manzoni OJ** 2011 Polymodal activation of the endocannabinoid system in the extended amygdala. Nature neuroscience14:1542-7
227. **Karst H, Nair S, Velzing E, Rumpff-van Essen L, Slagter E, Shinnick-Gallagher P, Joëls M** 2002 Glucocorticoids alter calcium conductances and calcium channel subunit expression in basolateral amygdala neurons. Eur J Neurosci 16:1083-1089
228. **Di S, Tasker JG** 2008 Rapid synapse-specific regulation of hypothalamic magnocellular neurons by glucocorticoids. Progress in brain research 170:379-88

229. **Huff NC, Wright-Hardesty KJ, Higgins EA, Matus-Amat P, Rudy JW** 2005 Context pre-exposure obscures amygdala modulation of contextual-fear conditioning. Learning & Memory 12:456-460
230. **Berlau DJ, McGaugh JL** 2006 Enhancement of extinction memory consolidation: The role of the noradrenergic and GABAergic systems within the basolateral amygdala. Neurobiol Learn Mem 86:123-132
231. **Roozendaal B, Castello NA, Vedana G, Barsegyan A, McGaugh JL** 2008 Noradrenergic activation of the basolateral amygdala modulates consolidation of object recognition memory. Neurobiol Learn Mem 90:576-579
232. **Carvalho A, Reyes A, Sterling R, Unterwald E, Bockstaele E** 2010 Contribution of limbic norepinephrine to cannabinoid-induced aversion. Psychopharmacology 211:479-491
233. **Pereira AM, Tiemensma J, Romijn JA** 2010 Neuropsychiatric Disorders in Cushing's Syndrome. Neuroendocrinology 92:65-70
234. **Iwata M, Hazama GI, Shirayama Y, Ueta T, Yoshioka S, Kawahara R** 2004 A case of Addison's disease presented with depression as a first symptom. Seishin Shinkeigaku Zasshi. 106:1110-6
235. **Anglin RE, Rosebush PI, Mazurek MF** 2006 The neuropsychiatric profile of Addison's disease: revisiting a forgotten phenomenon. J Neuropsychiatry Clin Neurosci. 18:450-9
236. **Joëls M, Karst H, Krugers HJ, Lucassen PJ** 2007 Chronic stress: Implications for neuronal morphology, function and neurogenesis. Front Neuroendocrinol 28:72-96
237. **Gould E, Woolley CS, McEwen BS** 1990 Short-term glucocorticoid manipulations affect neuronal morphology and survival in the adult dentate gyrus. Neuroscience 37:367-375
238. **Wossink J, Karst H, Mayboroda O, Joëls M** 2001 Morphological and functional properties of rat dentate granule cells after adrenalectomy. Neuroscience 108:263-272
239. **Gould E, Cameron H, Daniels D, Woolley C, McEwen B** 1992 Adrenal hormones suppress cell division in the adult rat dentate gyrus. The Journal of Neuroscience 12:3642-3650
240. **Cameron HA, Gould E** 1996 Distinct populations of cells in the adult dentate gyrus undergo mitosis or apoptosis in response to adrenalectomy. J Comp Neurol. 369:56-63
241. **Krugers HJ, van der Linden S, van Olst E, Alfarez DN, Maslam S, Lucassen PJ, Joëls M** 2007 Dissociation between apoptosis, neurogenesis, and synaptic potentiation in the dentate gyrus of adrenalectomized rats. Synapse 61:221-230
242. **Stienstra CM, Joëls M** 2000 Effect of Corticosteroid Treatment In Vitro on Adrenalectomy-Induced Impairment of Synaptic Transmission in the Rat Dentate Gyrus. J Neuroendocrinol 12:199-205
243. **Joëls M** 2007 Role of corticosteroid hormones in the dentate gyrus. Progress in Brain Research. 166:355-370
244. **Tata DA, Anderson BJ** 2010 The effects of chronic glucocorticoid exposure on dendritic length, synapse numbers and glial volume in animal models: Implications for hippocampal volume reductions in depression. Physiol Behav 99:186-193
245. **Zarate CA, Du J, Quiroz J, Gray NA, Denicoff KD, Singh J, Charney DS, Manji HK** 2003 Regulation of Cellular Plasticity Cascades in the Pathophysiology and Treatment of Mood Disorders. Ann N Y Acad Sci 1003:273-291
246. **Feyissa AM, Woolverton WL, Miguel-Hidalgo JJ, Wang Z, Kyle PB, Hasler G, Stockmeier CA, Iyo AH, Karolewicz B** 2010 Elevated level of metabotropic glutamate receptor 2/3 in the prefrontal cortex in major depression. Prog Neuro-Psychopharmacol Biol Psychiatry 34:279-283
247. **Sapolsky RM, Krey LC, McEwen BS** 1984 Stress Down-Regulates Corticosterone Receptors in a Site-Specific Manner in the Brain. Endocrinology 114:287-292

248. **Wang C, Wang S** 2009 Modulation of presynaptic glucocorticoid receptors on glutamate release from rat hippocampal nerve terminals. Synapse 63:745-751
249. **Brummelte S, Galea LAM** 2010 Chronic high corticosterone reduces neurogenesis in the dentate gyrus of adult male and female rats. Neuroscience 168:680-690
250. **Mayer JL, Klumpers L, Maslam S, De Kloet ER, Joëls M, Lucassen PJ** 2006 Brief Treatment With the Glucocorticoid Receptor Antagonist Mifepristone Normalises the Corticosterone-Induced Reduction of Adult Hippocampal Neurogenesis. J Neuroendocrinol 18:629-631
251. **Oomen CA, Mayer JL, De Kloet ER, Joëls M, Lucassen PJ** 2007 Brief treatment with the glucocorticoid receptor antagonist mifepristone normalizes the reduction in neurogenesis after chronic stress. Eur J Neurosci 26:3395-3401
252. **Karatsoreos IN, Bhagat SM, Bowles NP, Weil ZM, Pfaff DW, McEwen BS** 2010 Endocrine and Physiological Changes in Response to Chronic Corticosterone: A Potential Model of the Metabolic Syndrome in Mouse. Endocrinology 151:2117-2127
253. **Vreeburg SA, Hoogendijk WG, van,Pelt J.** 2009 Major depressive disorder and hypothalamic-pituitary-adrenal axis activity: Results from a large cohort study. Arch Gen Psychiatry 66:617-626
254. **Herbert J** 2013 Cortisol and depression: three questions for psychiatry. Psychol Med 43:449-69
255. **Kershaw EE, Flier JS** 2004 Adipose Tissue as an Endocrine Organ. Journal of Clinical Endocrinology & Metabolism 89:2548-2556
256. **Seckl JR, Morton NM, Chapman KE, Walker BR** 2004 Glucocorticoids and 11beta-Hydroxysteroid Dehydrogenase in Adipose Tissue. Recent Prog Horm Res 59:359-393
257. S**wain LD, Schwartz Z, Caulfield K, Brooks BP, Boyan BD** 1993 Nongenomic regulation of chondrocyte membrane fluidity by 1,25-(OH)2D3 and 24,25-(OH)2D3 is dependent on cell maturation. Bone 14:609-17
258. **Falkenstein E, Tillmann H, Christ M, Feuring M, Wehling M** 2000 Multiple Actions of Steroid Hormones - A Focus on Rapid, Nongenomic Effects. Pharmacological Reviews 52:513-556
259. **Bowles NP, Hill MN, Bhagat SM, Karatsoreos IN, Hillard CJ, McEwen BS** 2012 Chronic, noninvasive glucocorticoid administration suppresses limbic endocannabinoid signaling in mice. Neuroscience 204:83-89
260. **Gourley SL, Taylor JR** 2009 Recapitulation and Reversal of a Persistent Depression-like Syndrome in Rodents. Current Protocols in Neuroscience. Chapter 9: Unit 9.32
261. **Morton NM** 2010 Obesity and corticosteroids: 11β-Hydroxysteroid type 1 as a cause and therapeutic target in metabolic disease. Mol Cell Endocrinol 316:154-164
262. **Sooy K, Webster SP, Noble J, Binnie M, Walker BR, Seckl JR, Yau JLW** 2010 Partial Deficiency or Short-Term Inhibition of 11β-Hydroxysteroid Dehydrogenase Type 1 Improves Cognitive Function in Aging Mice. The Journal of Neuroscience 30:13867-13872
263. **Morton NM, Seckl JR** 2008 11beta-hydroxysteroid dehydrogenase type 1 and obesity. Front Horm Res. 36:146-64
264. **Meyer U, van Kampen M, Isovich E, Flügge G, Fuchs E** 2001 Chronic psychosocial stress regulates the expression of both GR and MR mRNA in the hippocampal formation of tree shrews. Hippocampus 11:329-336
265. **Tasker JG, Di S, Boudaba C** 2002 Functional synaptic plasticity in hypothalamic magnocellular neurons. Prog Brain Res. 139:113-9
266. **Meeter M, Murre JMJ** 2004 Consolidation of Long-Term Memory: Evidence and Alternatives. Psychol Bull. 130:843-857
267. J**oëls M, de Kloet ER** 1989 Effects of glucocorticoids and norepinephrine on the excitability in the hippocampus. Science 245:1502-5

268. **Joëls M, Velzing E, Nair S, Verkuyl JM, Karst H** 2003 Acute stress increases calcium current amplitude in rat hippocampus: temporal changes in physiology and gene expression. Eur J Neurosci 18:1315-1324
269. **Bajzer Z, Myers AC, Vuk-Pavlović S** 1989 Binding, internalization, and intracellular processing of proteins interacting with recycling receptors. A kinetic analysis. Journal of Biological Chemistry 264:13623-13631
270. **Robert A, Howe JR** 2003 How AMPA Receptor Desensitization Depends on Receptor Occupancy. The Journal of Neuroscience 23:847-858
271. **Krugers HJ, Karst H, Joëls M** 2012 Interactions between noradrenaline and corticosteroids in the brain: from electrical activity to cognitive performance. Frontiers in Cellular Neuroscience 6:15
272. **Joëls M, Pu Z, Wiegert O, Oitzl MS, Krugers HJ** 2006 Learning under stress: how does it work? Trends Cogn Sci 10:152-158
273. **Hu H, Real E, Takamiya K, Kang M, Ledoux J, Huganir RL, Malinow R** 2007 Emotion Enhances Learning via Norepinephrine Regulation of AMPA-Receptor Trafficking. Cell 131:160-173
274. **Joiner ML, Lisé MF, Yuen EY, Kam AY, Zhang M, Hall DD, Malik ZA, Qian H, Chen Y, Ulrich JD, Burette AC, Weinberg RJ, Law PY, El-Husseini A, Yan Z, Hell JW** 2010 Assembly of a beta2-adrenergic receptor--GluR1 signalling complex for localized cAMP signalling. EMBO J. 29:482-95
275. **Qin Y, Karst H, Joëls M** 2004 Chronic unpredictable stress alters gene expression in rat single dentate granule cells. J Neurochem 89:364-374
276. **Kitrakil E, Karandrea D, Kittas C** 1999 Long-lasting effects of stress on glucocorticoid receptor gene expression in the rat brain. Neuroendocrinology 69:331-8
277. **Zhan L, Zhang J, Sun H, Liu H, Yang Y, Yao Z** 2011 Exposure to enriched environment restores the mRNA expression of mineralocorticoid and glucocorticoid receptors in the hippocampus and ameliorates depressive-like symptoms in chronically stressed rats. Curr Neurovasc Res. 8:286-93
278. **Chen J, Tang Y, Yang J** 2008 Changes of Glucocorticoid Receptor and Levels of CRF mRNA, POMC mRNA in Brain of Chronic Immobilization Stress Rats. Cell Mol Neurobiol 28:237-244
279. **Brunson KL, Avishai-Eliner S, Hatalski CG, Baram TZ** 2001 Neurobiology of the stress response early in life: evolution of a concept and the role of corticotropin releasing hormone. Mol Psychiatry 6:647-56
280. **Chen Y, Andres A, Frotscher M, Baram TZ** 2012 Tuning synaptic transmission in the hippocampus by stress: The CRH system. Frontiers in Cellular Neuroscience 6:13
281. **Mirescu C, Peters JD, Gould E** 2004 Early life experience alters response of adult neurogenesis to stress. Nature Neuroscience 7:841-6
282. **Oomen CA, Soeters H, Audureau N, Vermunt L, van Hasselt FN, Manders EMM, Joëls M, Lucassen PJ, Krugers H** 2010 Severe Early Life Stress Hampers Spatial Learning and Neurogenesis, but Improves Hippocampal Synaptic Plasticity and Emotional Learning under High-Stress Conditions in Adulthood. The Journal of Neuroscience 30:6635-6645
283. **Baker L, Williams L, Korgaonkar M, Cohen R, Heaps J, Paul R** 2012 Impact of early vs. late childhood early life stress on brain morphometrics. Brain Imaging and Behavior Epub
284. **Fish EW, Shahrokh D, Bagot R, Caldji C, Bredy T, Szyf M, Meaney MJ** 2004 Epigenetic Programming of Stress Responses through Variations in Maternal Care. Ann N Y Acad Sci 1036:167-180
285. **Szyf M, McGowan P, Meaney MJ** 2008 The social environment and the epigenome. Environ Mol Mutagen 49:46-60

286. **Bagot RC, Tse YC, Nguyen H, Wong AS, Meaney MJ, Wong TP** 2012 Maternal Care Influences Hippocampal N-Methyl-D-Aspartate Receptor Function and Dynamic Regulation by Corticosterone in Adulthood. Biol Psychiatry 72:491-498
287. **Sterley T, Howells F, Russell V** 2011 Effects of early life trauma are dependent on genetic predisposition: a rat study. Behavioral and Brain Functions 7:11



Summary

Stress is part of our daily life. Appropriate coping strategies with stressful situations are necessary for successful adaptation. By contrast, uncontrollable stress, especially when experienced over a longer period of time, can form a considerable risk factor for disease and is indeed known to be causally involved in the etiology of many disorders, including major depression.

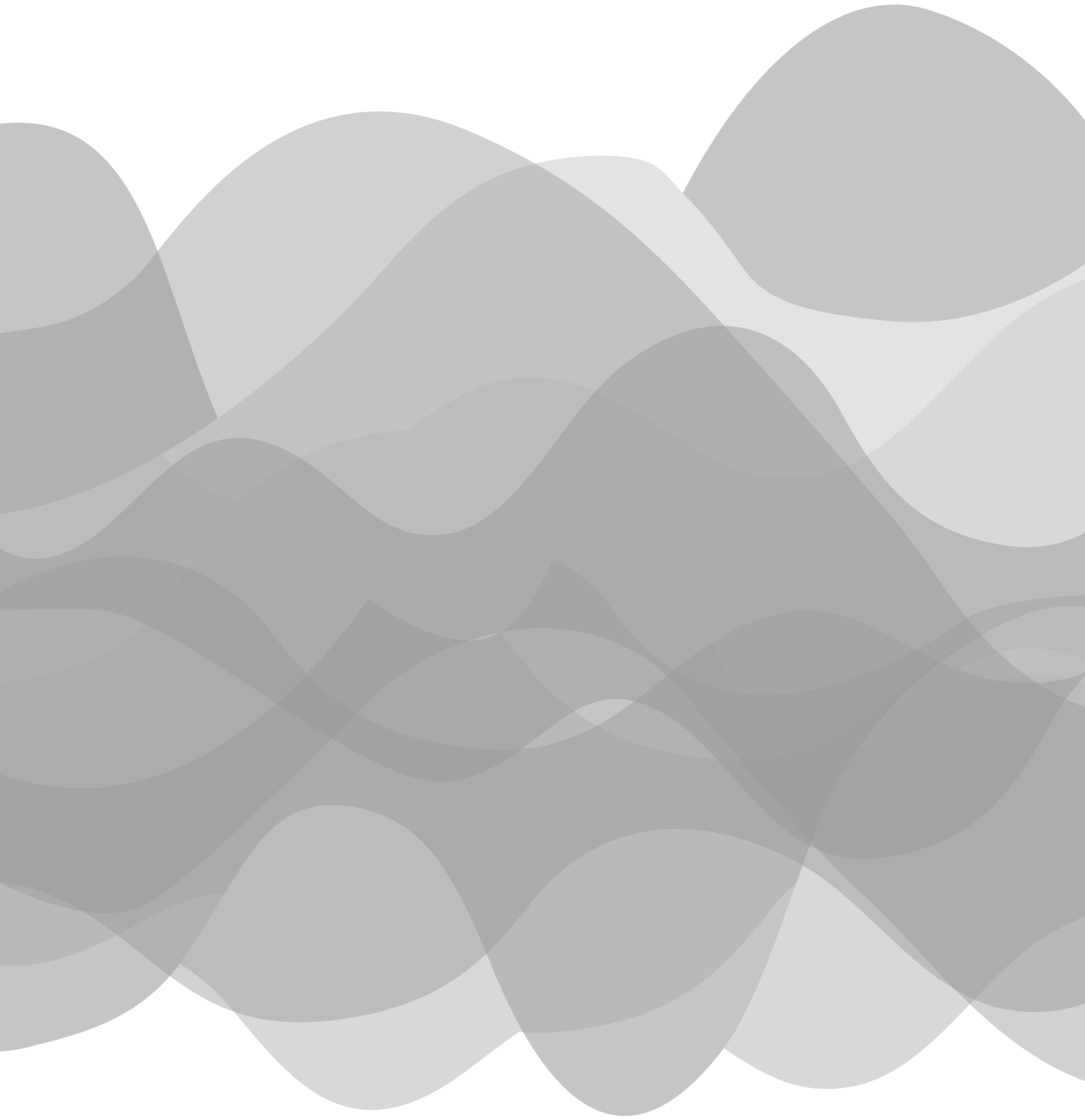
This project focuses on the mechanisms underlying processing of stressful situations. It is important that these situations are remembered for the future, so that renewed exposure will lead to meaningful anticipation and correct interpretation of the situation. It is equally important, though, that after exposure to stressful events the brain activity is normalized, so that the event is not remembered to the extent that it pervades the life of the individual, such as occurs in post-traumatic stress disorder. Understanding the basic mechanisms involved in the processing of stressful information is therefore of great relevance to the well-being of the individuals and may help to reduce the risk on developing stress-related pathology.

We here investigated that following stress exposure, the rodent brain is exposed to a cocktail of hormones, such as the adrenal hormone corticosterone, catecholamines like noradrenaline and neuropeptides. In concert these hormones enable the animal to face the challenge, adapt its behavior and remember the information about the stressful event for future use. Up till now it was generally thought that noradrenaline and peptides are the main actors in the initial phase of the stress response, enhancing alertness, vigilance and attention. Corticosteroids were thought to be important later on, for normalization of brain activity and consolidation of the event, via a genomic pathway.
This project shows that corticosteroids can also exert rapid effects that do not involve gene transcription, in various parts of the brain. We demonstrated these rapid effects (among other areas) in the hippocampus, an area important for memory formation. The effects take place via mineralocorticoid receptors that are positioned in or close to the plasma membrane, which is unexpected, because so far these receptors were only observed within the cell, i.e. in the cytoplasm or nucleus. We showed that rapid corticosteroid actions increase the chance that glutamate –the main excitatory transmitter in the brain- is released. At the same time, the receptors that mediate effects of glutamate move to a site where they are more active.

What is the relevance of these rapid corticosteroid actions? Our hypothesis was that through these rapid effects, the brain can quickly adapt its function to fluctuations in hormone levels. Such fluctuations happen after stress, but also during the active period of the day, when corticosterone is (spontaneously) released in hourly pulses. We indeed demonstrated that the membrane mineralocorticoid receptors are the means for hippocampal cells to quickly and accurately translate the hourly fluctuations of corticosterone into changes in glutamate transmission.
A second hypothesis was that –because corticosterone acts in the same rapid time-domain as noradrenaline- stress hormones affect each other's function. This is indeed the case, at least in the amygdala, an area important for emotion. Noradrenaline quickly increases the activity of amygdala cells. This is very much amplified when corticosterone is present at the same time. However, surprisingly, if corticosterone reaches amygdala cells some time after noradrenaline, it is no longer able to affect amygdala cell activity.

Finally, we examined if chronic stress and adrenalectomy (removal of adrenals/ corticosterone from the system) limits the potential of hippocampal cells to respond to corticosterone with rapid non-genomic effects. We observed that in both of these rather extreme conditions, responses to a high dose of corticosterone are largely suppressed.

These studies greatly advance our current understanding of how stress affects the brain especially hippocampus and amygdala.



Nederlandse Samenvatting

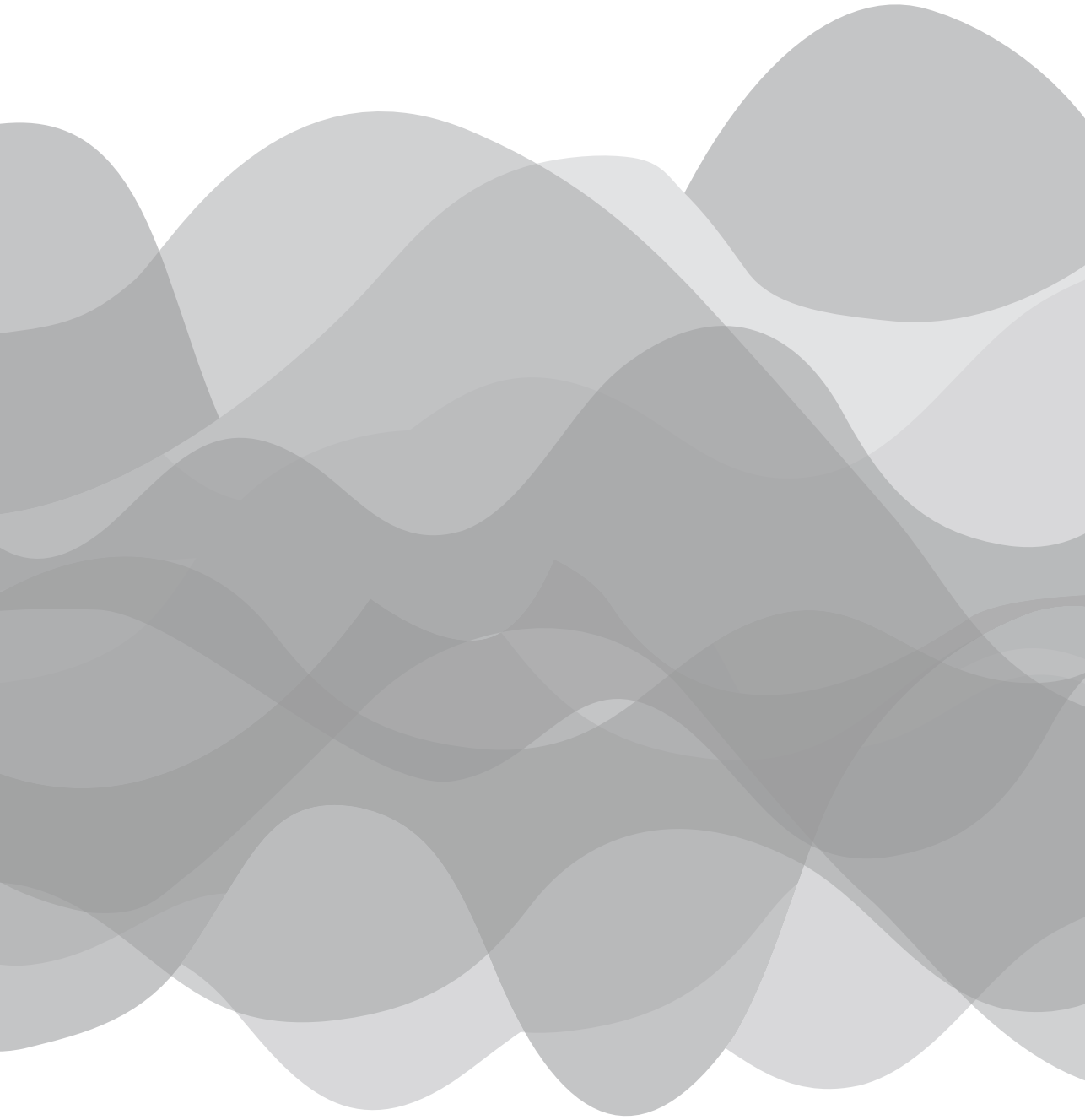
Stress maakt deel uit van ons dagelijkse leven. Het is belangrijk om daar op een adequate manier mee om te gaan, zodat het lichaam zich telkens herstelt en in daardoor balans blijft. Wanneer de stress niet meer controleerbaar is en als het gedurende een lange tijd wordt ondervonden, kan dat een risicofactor zijn voor het ontstaan van allerlei ziektebeelden, bijvoorbeeld ernstige depressiviteit.

Met dit onderzoek hebben we gekeken naar de mechanismen die betrokken zijn bij het verwerken van stressvolle situaties. Het is belangrijk dat die situaties goed onthouden worden, zodat we in de toekomst tijdens een vergelijkbare gebeurtenis een goede inschatting van de situatie kunnen maken en daar op juiste manier op kunnen reageren. Ook is het belangrijk, dat de hersenactiviteit zich normaliseert na een stressvolle gebeurtenis. Dat gebeurt bijvoorbeeld niet in post traumatische stress syndroom patiënten, met het gevolg dat die stressvolle gebeurtenis het leven gaat overheersen. Het begrijpen van de mechanismen die bij het verwerken van stressvolle informatie plaatsvinden, is daarom dan ook van groot belang voor het welbevinden en kan helpen om het risico voor het ontwikkelen van stress gerelateerde ziektebeelden te verminderen.

In dit proefschrift hebben we beschreven wat de effecten zijn van een cocktail van hormonen, zoals het bijnierschorshormoon corticosteron, catecholaminen, zoals noradrenaline en neuropeptiden, op een aantal hersenstructuren van de muis. Een samenspel van deze hormonen zorgt er voor dat een individu een stressvolle situatie kan inschatten, het gedrag kan aanpassen en dat de gebeurtenis wordt opgeslagen in het geheugen. Tot voor kort werd aangenomen dat, voornamelijk noradrenaline en peptiden de belangrijkste rol speelden gedurende de eerste fase van een stress respons. Zij zouden verantwoordelijk zijn voor het verhogen van de alertheid, waakzaamheid en aandacht. Van corticosteron dacht men, dat het pas in een later stadium, na de stressvolle gebeurtenis een rol zou spelen. Corticosteron zou voornamelijk belangrijk zijn voor het normaliseren van de hersenactiviteit en het consolideren, vastleggen, van de gebeurtenis. Men dacht dat corticosteron alleen zou verlopen via gen transcriptie. Een mechanisme dat traag (uren) verloopt en lang aan kan houden. In dit proefschrift beschrijven we dat corticosteron ook via een snelle route de hersencellen kan beïnvloeden. Een mechanisme dat niet verloopt via de gen transcriptie. We toonden onder anderen aan dat dit snelle effect van corticosteron in de hippocampus, een structuur die belangrijk is voor het vastleggen van geheugen. De effecten vinden plaats na activatie van de in of nabij de membraan gelegen mineralocorticoid receptoren (MR), een van de twee corticosteroid receptoren. De andere is de glucocorticoid receptor (GR). Dit was onverwacht, want er werd verondersteld dat de receptoren zich alleen in het cytoplasma en de kern zouden bevinden. De membraan geassocieerde receptoren zijn verantwoordelijk voor het snelle effect van corticosteron. Zij zorgen er voor dat, na activatie, glutamaat gemakkelijker wordt afgegeven van de presynaptische eindigingen, waardoor een verhoogde activiteit ontstaat. Glutamaat is de belangrijkste excitatoire neurotransmitter., die een belangrijke rol speelt bij leer- en geheugenprocessen. Ook zorgt corticosteron er voor dat er meer glutamaatreceptoren naar de synapsen worden getransporteerd, waardoor de exciteerbaarheid nog verder toeneemt.

Wat is de relevantie van deze snelle modulatie van corticosteron? Onze hypothese was dat het brein zich snel aan zou passen aan fluctuaties van dit hormoon. Fluctuatie van corticosteron vindt plaats na stress, maar ook gedurende de actieve periode van de dag. Corticosteron wordt dan namelijk in verhoogde mate afgegeven. De afgifte vindt plaats in de vorm van pulsen die ongeveer ieder uur een piek vormen. Met deze studie hebben we inderdaad aangetoond dat de glutamaat transmissie snel en accuraat volgt op het pulsatiel aanbieden van corticosteron via de membraan geassocieerde MRs. Een tweede hypothese was, dat het snelle effect van corticosteron met die van noradrenaline zou kunnen interfereren. Dat bleek inderdaad het geval. In de amygdala, een hersenstructuur die belangrijk is voor de verwerking van emotionele gebeurtenissen, bleek gevoelig voor beide hormonen. Niet alleen corticosteron, maar ook noradrenaline veroorzaken beide een toename van de glutamaat afgifte. Wanneer ze beide tegelijkertijd worden aangeboden, wordt dit effect nog eens versterkt. Een opmerkelijke waarneming was echter, dat wanneer corticosteron na noradrenaline wordt toegediend, het effect van corticosteron niet meer plaatsvindt. Tot slot onderzochten we of chronische stress (voortdurend hoge corticosteron spiegel) of verwijdering van de bijnieren (geen/lage corticosteron spiegel) het effect van de snelle effecten van corticosteron kan beïnvloeden. In beide, extreme, situatie vonden we, dat de effecten die normaal gesproken in niet gestreste dieren plaatsvinden, niet meer optreden.

Met deze studie hopen we een bijdrage te hebben geleverd aan een beter begrip van de mechanismen die een rol spelen bij het verwerken van stress in het brein en dan in het bijzonder in de hippocampus en amygdala.



Acknowledgements

Many thanks to everyone who came along and became part of this wonderful journey of my 4 years Ph.D.

I started my Ph.D. project in Amsterdam with Prof. Marian Joels and Dr. Henk Karst. Our group was Charlotte, Felisa, Sigal, Micheal, Zhou Ming, Lutz, Femke and Els.

I am grateful to **Marian** for giving me an opportunity to work with her and for her support throughout. I had developed not only scientifically but also as a person with you. You are a woman of substance. After being with Marian, who is so organized and efficient with her time, every other place seems like a chaos. You know very well about the importance of 'timing', not only with hormone action but also when to push and when to motivate me during my ups and downs during last 4 years.

My sincere gratitude to **Henk** for his presence in the lab. You made life so relaxed and effortless most of the times. I remember you were telling me in the beginning, not to push myself when I got frustrated and rather see the progress I make everyday. I learnt to accept if things go wrong, let it go and then work on it constructively next day. In my last year, when I was planning experiments tightly packed on my schedule, I can not believe any supervisor would say what Henk said to me, "PhD should be your pleasant time and not stressful time". Thanks for keeping me realistic that I could hold on till the end. I appreciate your kindness and patience you kept with me.

I started with a wonderful team in Amsterdam. Charlotte being enthusiastic; Felisa, fun-filled, we had great times in Greece for a summer school, together with Rose; Sigal, very relaxed and chilled out; Micheal, enjoying every bit of science and his stay in Europe; **Zhou Ming**, dedicated scientist who is found behind his desk even in the late evenings. If asked to talk something other than science than the wisdom overflows from other aspects as well. It's always fun philosophying with you. Lutz, sitting next to my set-up giving advice for patching and telling me that in hippocampus, do a blind patch! Els, thanks for being around. Your presence made everything smooth in our rough schedules. **Femke** joined us from Leiden for a short project. That was the beginning of animal handling for me, when Femke helped me many times. Thanks for the training. She also followed us to Utrecht, where she got spot next to my set-up. During our coffee breaks, I learnt from you to be more environmental friendly than I used to be.

Meanwhile, Anup and Sandra also joined Marian's group. Then, I moved to Utrecht. I met new people from the group of Geert; Frank, Edwin and Bart. I learnt to enjoy science more with you guys whether it was dry ice rocket or combining cool stuff like optogenetics or in vivo electrophys. Many master students came by and added lot of fun to our routine lives of experimentation. All the students of **Anup** were so fascinated in 1st week of their internship, just by the fact how the amplifier works. I always wished I could make my own program to analyze my data like you, sounds so cool.

Sandra, from being room-mates in ENP meeting in 2009 and all the conferences thereafter until today we have been growing as friends. I remember how much we

cherished when we reached hotel in New Jersey from New York airport without getting lost, such an achievement! Our trip to Barcelona is memorable. We enjoyed Sangria in Barcelona everyday with great pastas or tapas, which relaxed us so much that we were laughing when my bag was stolen with my passport and visa. Height of relaxation and having bigger perspective on life was when instead of going to Indian embassy to arrange travel documents; we went on hiking trip to enjoy the sun without any doubts (we had trust in Dutch system to get us back anyway!). You were almost leaving to US after your PhD, which never felt right and you decided not to go. With lot of tea and our discussions on life, ideal job led us to great plans. I wish we both fulfill it when it's right time.

Ela, you became my officemate few months after I came to Utrecht and later, housemate. We spent so much quality time together. I always liked doing yoga but first time I started lessons together with you in Utrecht which were so much fun 'coz end of the lesson we both had irresistible smiles on our face. I appreciated how you enjoy life looking for things that are close to your nature weather it is African dance, singing lessons or making creative collages. Wish you wholesome life you are aiming for in coming years.

After a year in Utrecht, Angela joined as a postdoc and Marloes for second half of her PhD. **Angela**, I highly appreciate how calmly and efficiently you work: traveling from Den Haag everyday and doing great research with taking out time for socializing every now and then. It's amazing that being pregnant and having a baby means having sleepless nights didn't change anything of it. Good Luck with grant applications! **Marloes**, I enjoyed having you next to my set-up. Sometimes when you were frustrated with electrophys, you went back to your fMRI data and started working on that, to feel good. I liked your scientific thinking and the way you are open and determined to learn more everyday.

The coffee room chit-chat was fun with Henk and Leo. The great way to bring smile on **Leo's** face is help him cleaning the coffee room :-) He likes it 'clean'!

Thanks to Sandra, Ria, Vicky and Mariken for their help in translating Dutch documents and solving all the puzzling paper work for me with a 'smile'(Yes, they do smile when they look away from the computer!). Special thanks to **Vicky**, for being my secret source of cookies :)

Lot of renovation missions were carried out during 4 year stay. On one hand it was bit annoying and on the other hand we enjoyed all our different offices. Thanks to my officemates, Ela, Edwin, Arjen! Cookie box saved our lives many times, kept us going without complaining. Thanks to the cookie monster, **Edwin**. Ela and Edwin introduced the tradition of getting plants in the office which turned out to be a big venture that year. Now, the office is almost like a small garden in itself. **Ela** did the best thing ever by getting a sit-sack in the office, where we cherished reading papers and enjoying our short 5 minute breaks. **Arjen** is the nerd with chaotic desk but well arranged with his students always working next to him.
Also, with moving we had different office neighbors. I got acquainted with people from other groups. It was good times with Teresa,Woutjan, Rahul, Kaushik, Asheeta, Manila, Esther, Margarite, Eneda and Anna.

Teresa's charming smile caught my attention when one day I saw her in the coffee room. Finally, somebody who understood why writing poems is like an urge, it flows through to the paper and you feel relieved. You are a strong woman with soft heart. I learnt a lot from you weather it is about management or economics or philosophers of all times. A quote for you from Baruch Spinoza, "The highest activity a human being can attain is learning for understanding, because to understand is to be free".

Woutjan, from my lunch buddy, every Tuesday to a 'monthly tea' buddy. It was really nice to have you around in Utrecht when we could meet more often. When you were next to our house, we also had time to have dinners together or play games. Unfortunately, now your life is more in Amsterdam and my life is more in Nijmegen but we should not forget to be in touch.

For short period of collaboration when I went to Bordeaux, I had very nice time with people there. I got a warm welcome by my officemates especially Laurent Ladepeche. **Laurent Groc** gave me an opportunity to work in his group. I loved his enthusiasm and positivity. I appreciated his out of the box thinking when data comes out to be unexpected. **Lenka** supervised me with the project and I learnt from her to be critical to your own work. She took care of me when I got severe viral fever there where I didn't know anyone from taking me to the doctor to getting me food. Thanks for everything. Thanks to **Dolors** who encouraged me to join her for walking to the institute every morning at 7 am. I really enjoyed it. Unfortunately, most of the weekends I had to work but I could still take out time for some evenings to spend with **Katalin**. We had great chit-chat time, nice walks and some shopping time together. As there was a lot more to share with you, we are now connected through skype and I am so happy that you visited me in Nijmegen.

Apart from work life, I also fully enjoyed my home life with my great fun-filled housemates. When I came in, I met Isa, Eduardo, Eneda. Later on, Wouter, Amila, Anibal, Pradeep, Anup, Mette, Emrah, Marie, Magda joined in. Here, was when fun time started in our house. **Mette**, thanks for the wonderful baking recipes that made me interested into baking. Even when I was on a restricted diet, you managed to bake cookies that I could eat. **Wouter**, always came up with discussion topics in the house during our evening tea but I would never forget our endless discussion on 'Hope' which lasted several weeks until we all were fed up. We have to come back to it again. After 2 years of being in the house, **Isa** finally convinced me to try Salsa and I am glad I did. Unfortunately, I am not as passionate as you are otherwise it was a good offer to be your assistant in your upcoming Salsa school ;) Few people left the house soon. Other new people came in, Susana, Saskia, Susanne, Thomas, Amin and Joana. Here, was the time when everyday was a celebration. Thanks for being like a family, for being a support system in my tough times and for understanding me. We shared our happiness, sorrows and frustrations. Thanks for wonderful surprises and great dinners, cakes and cookies we shared in our kitchen; for nice parties in our living room. I enjoyed our maniac times, when we spend every evening on solving a puzzle or playing Frisbee or just being lazy and watching movies and once in a while getting health conscious, trying to go running whenever possible - crazy us! The lovely dinners on the street in summer days

was like small holidays. I am going to miss our house and my funny and lovely housemates. But, I warn you - I will keep visiting.

Susana, we partied hard, screamed on heavy metal music and then, relaxed in Sulphur bath in Budapest when we wanted to be off. Thanks for bringing the spark back in me.

I moved to Nijmegen to **Hanneke's** house. I was bit worried about my moving, working on thesis and working at new place all at the same time but you made it super-easy. Plants and flowers in my room already felt like home and in no time I was comfortable which helped me to go through the busy times with ease. Thanks for being considerate and helpful.

In Nijmegen, I joined a wonderful group full of enthusiastic scientists, **Piray, Erika and Hassiba**. Thanks for the support when I started working and finishing writing of my thesis at the same time. Thanks for understanding when you hardly knew me. Success with your fascinating projects, girls! **Areg**, you always make things sound easy and funny with your witty jokes. I like your dedication to the extent that you are synchronized with your rats. Good Luck finishing up! **Benno**, you being in charge of all these crazy scientists, it is incredible how you keep track of everything. Perhaps 'coz you never lose the focus - Amygdala!

I love my meditation buddies. Meditation evening was charmed by **Ela and Esther's** singing and calm, peaceful, smiling faces of **Connor and Marjolijn**. Of course, I can not miss listening to great adventures of **Cordula** whether in Holland or in Nepal. Keep rocking girls!

My gratitude to all the friends who came in my life and walked along with me. Thanks to my lovely friends in India: P**ooja, Arita, Maninder and Rohini**, for all time encouragement and support.

First time when I stepped out of home was my step in Germany. I found very fun-filled friends group: Bindu, Namrata, Bhadresh, Shailesh and Sabani. I learnt with you how to fly when I got wings and take responsibility of my life. Thanks 'Funtoosh family'.

When I came to Holland I never thought that here, I will find my love, **Wouter** who made this journey even more beautiful. Thanks for being there.

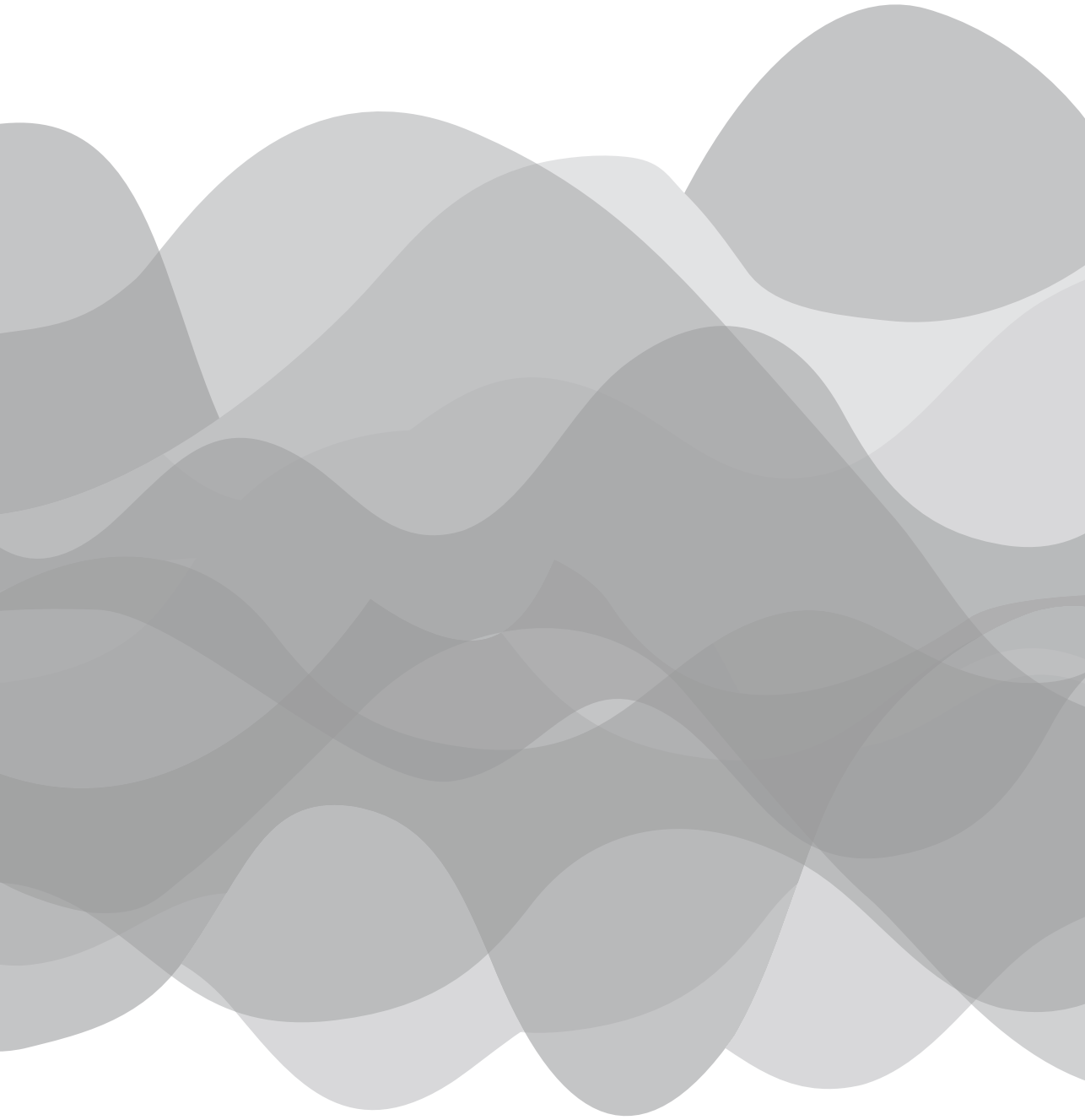
Thanks to **Koning's family** for warm welcome in their family - Jopie & Jacques with their love, Rutger & Lieke for always being kind, June for giving me Dutch lessons while making drawings with her, Joris & Mandy for their affection. Family get-togethers are fun with all of you.

Lastly and most importantly, thanks to my family. In one sentence I would say 'without you, I would not have been what I am today.' The big credit goes to invaluable advice and all time support of my brother, **Mandeep**, who kept me going at all times, in life or at work. Thanks for being there always. The great addition to our family is my sister-in-law, also called **Natasha**. Thanks for your love and

support, **Bhabhi**. I would like to convey my sincere gratitude to my parents. My father gave me a simple Mantra 'Don't worry, be happy' and Mom always said '..if your smile is with you, the whole world is with you'. This helped me to keep my smile on and never frown. ***Thank you, Papa and Mummy***.

Keep smiling always :-)

Love & regards,
Natasha



Curriculum Vitae

List of Publications

Pasricha N, Joels M, Karst H. Rapid effects of corticosterone in the mouse dentate gyrus via a nongenomic pathway. Journal of Neuroendocrinology 2011, Feb.;23(2):143:47

Pasricha N, Joels M, Karst H. Amygdala but not hippocampal cells become gradually resistant to repetitive corticosterone exposure. *In preparation*

Pasricha N, Joels M, Karst H. GluA2 surface diffusion in response to pulses of corticosterone. *In preparation*

Joels M, Pasricha N, Karst H. Rapid responses to corticosterone change after adrenalectomy or chronic corticosterone treatment of mice.
Submitted for publication

Abstract Publications

1. **Pasricha N, Mikasova L, Joels M, Karst H, Groc L.** Glucocorticoid Pulsatility, Oral presentation, 17th ONWAR Annual Ph.D. Retreat, Nov. 2011
2. **Pasricha N, Mikasova L, Joels M, Karst H, Groc L.** Influence of corticosterone pulsatility on presynaptic glutamate release and postsynaptic AMPA receptor surface trafficking in hippocampal neurons, SfN, November 2011
3. **Pasricha N, Mikasova L, Joels M, Karst H, Groc L.** Influence of repetitive corticosteroid pulses on glutamate release and AMPA receptor surface trafficking in hippocampal neurons. Endo-neuro-Psycho Meeting, 31st May- 1st June 2011
4. **Pasricha N, Joels M, Karst H.** Rapid effects of corticosterone in the mouse dentate gyrus via a non-genomic pathway. Journal of Neuroendocrinology, Feb. 2011
5. **Pasricha N, Joels M, Karst H.** Effects of corticosterone pulsatility on the excitability (mEPSCs) of granular neurons of dentate gyrus. 16th ONWAR Annual Ph.D. Retreat, Nov. 2010
6. **Pasricha N, Joels M, Karst H.** Rapid effects of corticosterone on mouse dentate gyrus via a non-genomic pathway. NCUtrecht, September 2010
7. **Pasricha N, Joels M, Karst H.** Rapid Non-genomic effects of corticosterone on mouse hippocampus. 7th World Congress on Stress, 25-27 August 2010
8. **Pasricha N, Joels M, Karst H.** Rapid effects of corticosterone on dentate gyrus of mouse hippocampus via non-genomic pathway. FENS conference, June 2010
9. **Pasricha N, Joels M, Karst H.** Effects of corticosterone pulsitility on the excitability (mEPSCs) of granular neurons of dentate gyrus. 16th ONWAR Annual Ph.D. Retreat, November 2009
10. **Pasricha N, Joels M, Karst H.** Effects of corticosterone pulsitility on the excitability (mEPSCs) of granular neurons in the mouse Dentate Gyrus. PENS summer school, September 2009
11. **Pasricha N, Joels M, Karst H.** Non-genomic rapid effects of corticosterone on the functioning of dentate gyrus of mouse hippocampus. Endo-neuro-Psycho Meeting, 3-5 June 2009
12. **Pasricha N, Joels M, Karst H.** The physiological role of membrane MRs in Hippocampal function. 15th ONWAR Annual Ph.D. Retreat, 27-28 November 2008

Curriculum Vitae

Natasha Pasricha was born on August 17, 1984 in Yamuna Nagar, India.

She started her scientific career in Department of Biophysics in Panjab University, India, where she completed her bachelors in 2005. October 2005, she moved to Germany where she pursued Masters in Life science informatics from University of Bonn. In July 2006, she worked as a research assistant in Life & Brain GmbH, Bonn where she worked with Dr. Thoralf Opitz on "Assessing physiological properties of human embryonic stem-cell derived neurons using patch clamp technique". In May 2007, she continued in Life & Brain GmbH with Prof. Oliver Brüstle and Dr. Thoralf Opitz, on her Master's thesis project: "Evaluating physiological properties of stem-cell derived neurons using patch clamp technique and computational modeling using NEURON simulation environment". In April 2008, she moved to The Netherlands and joined as a Ph.D. candidate in University of Amsterdam with Prof. Marian Joels and Dr. Henk Karst, where she started her work on "Rapid corticosteroid actions in the hippocampus and amygdala", which is described in this thesis. In September 2009, she moved to Rudolf Magnus Institute, University Medical Center Utrecht together with the group and completed her Ph.D. in UMC Utrecht in 2013. During her Ph.D., she also worked with Dr. Laurent Groc and Dr. Lenka Mikasova on "AMPAR trafficking on exposure to hourly corticosterone pulses" in University of Bordeaux, France. Currently, she is working as a post doctorate fellow in the lab of Prof. Benno Roozendaal in Department of Cognitive Neuroscience, St Radboud UMC Nijmegen.