The effects of stress and corticosterone on synaptic plasticity and neuronal morphology in the rodent hippocampus

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Publication date
2006

Document Version
Final published version

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De totstandkoming van dit proefschrift werd financieel ondersteund door:
Financial support for printing this thesis was kindly provided by:

Accountantskantoor Alfarez en Partners
J.E. Jurriaanse Stichting
Jur International B.V.
Molecular Probes/Invitrogen
Nederlandse Vereniging voor Microscopie
Van Leersumfonds KNAW

Cover design and layout Dominique van Lanschot
Cover illustration confocal image of dendritic spines from a pyramidal neuron of the hippocampal CA1 area, visualized with the fluorescent dye Alexa, recorded by the author of this thesis.

Printed by DPP Utrecht

The effects of stress and corticosterone on synaptic plasticity and neuronal morphology in the rodent hippocampus

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus prof.mr. P.F. van der Heijden
ten overstaan van een door het college voor promoties ingestelde commissie, in het openbaar
te verdedigen in de Aula der Universiteit

op vrijdag 3 maart 2006, te 12.00 uur
door
Deborah Natascha Alfarez
geboren te Amsterdam

Promotiecommissie:

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Nothing shocks me. I'm a scientist.

Harrison Ford, as Indiana Jones

Abbreviations

ACSF	artificial cerebrospinal fluid
ACTH	adrenocorticotrophin hormone
AMPA	a-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	analysis of variance
Ca2+	calcium
CORT	corticosterone
CRH	corticosteroid releasing hormone
fEPSP	field excitatory post-synaptic potential
GR	glucocorticoid receptor
HPA	hypothalamus-pituitary-adrenal axis
Ih	half maximum stimulus intensity
LA	lateral amygdala
LTP	long-term potentiation
MANOVA	analysis of variance for repeated measure
MR	mineralocorticoid receptor
NMDA	N-methyl-D-aspartate
PBS	primed-burst stimulation
PSA	population spike amplitude
PSD	postsynaptic density
PTSD	posttraumatic stress disorder
sfEPSP	slope of the field excitatory postsynaptic potential
VTA	ventral tegmental area

Contents

CHAPTER 1
General Introduction

General introduction

The aim of this thesis was to examine how acute and prolonged exposure to stress and the stress hormone corticosterone affects synaptic plasticity (part I) and neuronal morphology (part II) in the rodent hippocampal formation.

I THE STRESS SYSTEM, CORTICOSTERONE AND ITS RECEPTORS

Stress and the hypothalamic-pituitary-adrenal axis

Coping with the everyday hassle of modern life is a prerequisite for individuals to survive. Anyone who feels that he or she is sometimes experiencing something strenuous and wearing is probably familiar with the term "stress". The original term "stress" was first used by Hans Selye when he described the process of adaptation to the "stresses and strains" of everyday existence (for a short overview on the work and ideas of Hans Selye see the intermezzo on page 183).

If the body experiences a challenging situation that in one way or another leads to disruption of homeostasis, animals react to this threat with a general discharge of the sympathetic nervous system, described by Walter Cannon in the 1920s as the flight-or-fight response. This response was later recognized as the first stage of coping with stressful situations resulting in a final common pathway in the stress response: the hypothalamic-pituitary-adrenal (HPA)-axis (see box 1). Adrenaline and noradrenaline are released from the adrenal medulla and corticosterone from the adrenal cortex which both in turn increase heart rate, respiration, blood pressure and gluconeogenesis. In this thesis the effects of corticosterone, being the main effector of the HPA axis, are studied on synaptic strengthening as well as neuronal morphology of the hippocampus.

Under non stress conditions, secretion of adrenocorticotropin hormone (ACTH) and corticosterone exhibits a circadian rhythm, with peak levels just before the active period (early morning in humans and late afternoon in nocturnal animals like rats) and a trough towards the end of the active period, giving the characteristic rhythm where mean plasma ACTH and corticosteroid concentrations during the active period exceed plasma concentrations during the inactive period by approximately 2 fold (Bradbury et al., 1994; Kalsbeek et al., 1996). In addition to the production of corticosteroids, the adrenal cortex produces mineralocorticoid hormones (aldosterone) for regulating the salt and water balance and androgen hormones involved in the maturation and maintenance of the reproductive organs. In response to stress, adrenal production of corticosterone may rise as much as 10 fold. Physiologically, corticosterone mediates an adaptive response to stress. Corticosteroids act as an "antagonist" of insulin by promoting breakdown of proteins into carbohydrates so mobilizing energy reserves, while enhancing glycogen storage in the liver for future energy resources. This adaptive response favors survival: metabolic energy is reserved in order to endure the stressful situation. At the same time, stress is also known to inhibit the response of immune cells and pro-inflammatory mediators, thus lowering the immune response which in the long run can lower the chances of survival of the organism.

Mineralocorticoid receptors and Glucocorticoid receptors

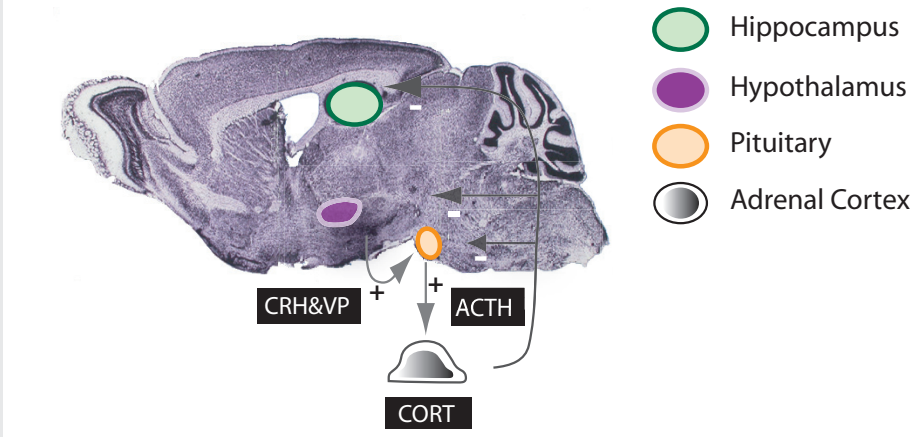
After secretion into the circulation, steroid hormones are bound to transporter proteins; in the case of corticosterone 90% is bound to corticosteroid-binding globulin (CBG or transcortin) when circulating levels are low (e.g. during the trough of the circadian cycle or under non stressful conditions). Work by Seckl and others has shown that another determinant for the level of circulating corticosterone is the enzyme 11?-hydroxysteroid dehydrogenase (11ß-HSD). Type 1 of 11ß-HSD converts inactive corticosterone to the active state, while type II oxidizes active corticosterone to the inactive form (Seckl, 1997).

BOX 1

Hypothalamic-pituitary-adrenal (HPA) axis

When an organism is exposed to a stressful or aversive event the hypothalamic-pituitary-adrenal (HPA) axis is activated. The HPA axis maintains its own homeostatic set point unless driven by environmental signals (i.e. stress) received by the nervous system. Central control of glucocorticoid secretion is regulated by a select population of neurosecretory neurons in the hypothalamic paraventricular nucleus (PVN). Upon stimulation by stress, these neurons secrete corticotrophin-releasing hormone (CRH) and vasopressin (VP) into the pituitary-hypophyseal portal circulation. VP acts synergistically with CRH and potentiates the CRH induced release of ACTH. The anterior lobe of the pituitary responds by secreting adrenocorticotrophin hormone (ACTH) which is released into the blood and subsequently drives the synthesis and secretion of glucocorticoids by the adrenal cortex.

The glucocorticoid hormone (cortisol in man and corticosterone in most rodents) inhibits synthesis and secretion of CRH within the hypothalamus and suppresses ACTH synthesis in the pituitary. Circulating via the blood stream, corticosterone is delivered widely over the body, including the central nervous system (Dallman et al., 1994). In this thesis the effects of corticosterone, as being the main effector of the HPA axis, are studied upon learning, memory and morphology of a limbic structure, the hippocampus.



The rhythm of free corticosterone in the brain can be assessed via microdialysis since the extracellular space of the brain is devoid of CBG. Mice brains show low corticosterone levels in the morning (0-0.2 nM), which increase toward the dark period (0.75-1.0 nM). Exposure to a rat increases corticosterone levels significantly (1.50-2.00 nM) for approximately 60 minutes after which it returns to baseline levels (Linthorst et al., 2000). Recovery of corticosterone via the microdialysis probe used in this study is ~12.5-15%, so that in reality the concentrations are probably at least 6 to 8-fold higher (Linthorst, personal communication).

Glucocorticoids bind to two different receptors in the brain, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (Reul and de Kloet, 1985). GR density is highest in the parvocellular paraventricular nucleus (PVN)(Herman et al., 2003). MR and GR appear to be co-localised in abundance in the limbic system which is responsible for emotional function and the formation of memory (see further paragraph II The Hippocampal Formation). Using immunocytochemistry, in situ hybridisation and more recently confocal microscopy, the precise co-localisation of MR and GR in the hippocampal CA1 area and dentate gyrus, amygdala and medial prefrontal cortex has been shown (Han et al., 2005; Marlier et al., 1996; Rosenfeld et al., 1988; Van Eekelen and De Kloet, 1992; van Steensel et al., 1995).

MRs and GRs belong to the family of nuclear receptors, which are ligand-activated transcription factors (Carson-Jurica et al., 1990). Alternative transcription of the gene sequence coding for the MR and GR generates different isoforms of these receptors. In addition, variation in the translation initiation sites and post-translational modifications give rise to many receptor variants. In particular the latter will largely affect the possibilities for protein-protein interactions, which may partly explain the mechanism via which the diverse biological functions of these receptors are mediated (Pascual-Le Tallec and Lombes, 2005; Zhou and Cidlowski, 2005). The MR displays a high affinity to corticosterone (Kd = 0.5 nM), which is about 10 fold higher than the affinity of the GR to corticosterone (Kd = 5.0 nM)(Reul and de Kloet, 1985). This means that the MR is occupied for up to 90% with basal levels of corticosterone that are circulating during the circadian trough, while the low affinity GR is particularly activated during the peak of the circadian rhythm or after exposure to and during stress (de Kloet et al., 1990; Reul and de Kloet, 1985).

Corticosteroid receptor action

The classical view is that steroid hormones enter the cell and bind to the MR and GR that are present in the cytoplasm. Upon binding, the receptor-ligand complex is translocated to the nucleus, where it acts as a transcription factor and modifies transcription by activation of the so called glucocorticoid response elements (GREs) in the promoter region of target genes or by interacting with other transcription factors such as AP1 or NFkB (Beato et al., 1995; Diamond et al., 1990; McKay and Cidlowski, 1998; Reichardt and Schutz, 1998; Schoneveld et al., 2004). Activation of gene transcription can occur through homodimerization but also via heterodimerization. Repression of GR function is usually mediated through monomers via protein-protein interactions with other transcription factors (Liu et al., 1995; Reichardt et al., 2000; Trapp et al., 1994). Recent advances in gene expression profiling have boosted the knowledge concerning genes that are regulated by corticosterone and MR and GR activation (Datson et al., 2001; Vreugdenhil et al., 2001; Wintermantel et al., 2004). Gene profiling of mouse lines, genetically selected for aggressive behavior, that display differences in the HPA axis, show differential expression of cytoskeleton and signal transduction genes that may be related to differences in the hippocampus of these mice (Feldker et al., 2003).

Recently, new ideas have emerged proposing membrane bound steroid receptors (Falkenstein and Wehling, 2000; Losel and Wehling, 2003). For estrogen it has been established that rapid actions of estrogen are mediated (amongst others) through a membrane bound receptor (Levin, 2001; Revankar et al., 2005). A membrane receptor that mediates glucocorticoid action has been found in the amphibian brain

(Evans et al., 1998; Evans et al., 2000; Orchinik et al., 1991) as well as in the avian brain (Breuner and Orchinik, 2001). In addition, using electron microscopy, it was shown that principal neurons and GABAergic interneurons in the lateral nucleus of the amygdala contain GRs located in the membrane of postsynaptic densities (Johnson et al., 2005). Indications for rapid, non-genomic effects by corticosterone involving a membrane receptor have recently been supplied for the PVN (Di et al., 2005) and hippocampal CA1 area (Karst et al., 2005).

The MR is implicated in the appraisal process and the onset of the stress response. Activation of the GR terminates the stress response, mobilizes the energy resources required for this purpose and facilitates recovery. GRs regulate the activity of the HPA axis directly via the PVN, but also indirectly via stimulatory pathways originating in the amygdala and neuronal inhibitory pathways, for instance coming from the hippocampus (Herman et al., 1989; Jacobson and Sapolsky, 1991; Sapolsky et al., 1991) and the prefrontal cortex (Diorio et al., 1993).

Balance in the expression of the two receptors is important and disparity can cause disruption of homeostasis (De Kloet and Derijk, 2004). A naturally occurring form of altered MR/GR expression is shown in aging where an exaggerated HPA response to stress is linked to a decrease in GR mRNA and binding capacity (Bodnoff et al., 1995a; De Kloet et al., 1991; Morano et al., 1994; Murphy et al., 2002; van Eekelen et al., 1991b). Also maternal deprivation during the first post-natal week can permanently influence stress regulation and GR expression untill senescence (de Kloet et al., 1990; Vazquez et al., 1996). Changes in MR/GR balance are also prevalent in depressive disorders and seems to be coupled to enhanced stress response (Holsboer, 2000; Meyer et al., 2001).

II THE HIPPOCAMPAL FORMATION

Hippocampal formation

The hippocampus, one of the major targets for corticosterone in the brain, is a structure involved in memory storage. The hippocampus is a large banana shaped structure between the thalamus and the cerebral cortex. A major axon tract, the fornix, links the hippocampus with the hypothalamus. The hippocampus is formed by two interlocking sheets of cortex and in cross-section has defined laminar structure with layers visible where rows of pyramidal cells are arranged. The formation comprises of six cytoarchitectonically distinct regions: the dentate gyrus (DG), the hippocampus proper, subdivided into area CA3, CA2, and CA1, the subicular complex (subiculum, presubiculum, parasubiculum), and the enthorinal cortex (Amaral and Witter, 1989; Lopes da Silva et al., 1990; Scharfman et al., 2000). More details about the hippocampal formation and neuronal connections are given in box 2.

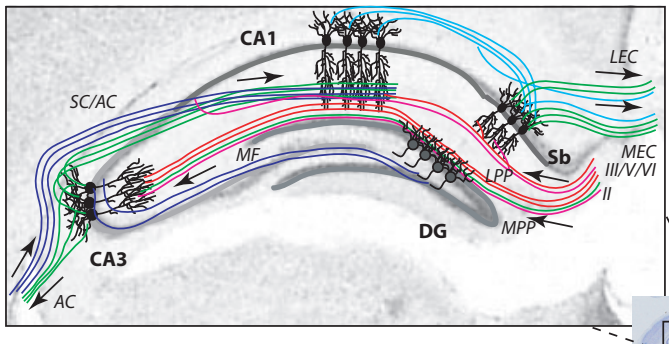
The hippocampus and memory

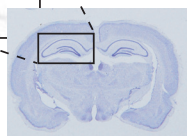
Brenda Milner - using the case study of patient H.M.- showed that the hippocampus is essential for declarative memory (Milner, 1959). In addition to the hypothesis that the hippocampus is critical for declarative memory (Squire, 1992), animal studies have shown that the hippocampus is important for spatial memory (Olton et al., 1978; Winson, 1978). In humans the spatial component of hippocampal functioning became for instance clear in a study using PET scans on London taxi drivers. They showed a heightened hippocampal activity when answering questions regarding routes through the city (Maguire et al., 1997). In rats, hippocampal damage impairs performance on the Morris water maze, a task in which a rat must swim through murky water to find a rest platform that is just under the surface. A rat with hippocampal damage finds the platform, but unlike the control rats, it has trouble remembering the

BOX 2

The Hippocampal Formation

The connections within the hippocampus generally follow the tri-synaptic pathway: a well-characterized loop that originates mainly in the adjacent entorhinal cortex. The different cell layers and sections are defined by the series of connections made. The main principal cell layers are encountered in the CA1-3 regions and the dentate gyrus. The perforant path is the major input to the hippocampus arising from the entorhinal cortex. Axons from layers II project to dentate gyrus neurons and CA3 pyramidal cells via the Lateral Perforant Path (LPP) and Medial Perforant Path (MPP). Projections from layer III and indirectly from deeper layers of the entorhinal cortex project to the pyramidal cells of the CA1 and subiculum (Sb). CA3 pyramidal cells also receive input from the DG via the mossy fibers (MF). CA3 axons project to the CA1 pyramidal cells via the Schaffer Collaterals (SC) and to CA1 cells in the contralateral hippocampus via the Associational Commisural pathway (AC). The SC pathway is utilized very extensively to study NMDA receptor-dependent LTP and LTD (see paragraph II Hippocampal long-term potentiation and long-term depression). CA1 pyramidal cells send axons to the subiculum, which project back to the lateral and medial entorhinal cortex, thus forming a loop.





Lateral Entorhinal Cortex: LEC
Medial Entorhinal Cortex: MEC
Lateral Perforant Path: LPP
Medial Perforant Path: MPP
Dentate Gyrus: DG
CA3 pyramidal neurons: CA3
Mossy Fibers: MF
Schaffer Collateral pathway: SC
Assocational Commisural Pathway: AC
CA1 pyramidal neurons: CA1
Subiculum: Sb

location from one trial to the next and therefore does not improve the latency of finding the platform (Morris et al., 1982). Of all the hippocampal areas, the CA1 region, forming connections with the adjacent cortical regions (entorhinal cortex) appears to be crucial in the consolidation of spatial memory (Eichenbaum, 2000; Morris et al., 1982; Remondes and Schuman, 2004).

Hippocampal long-term potentiation and long-term depression

Before I will discuss the effects of exposure to stress and corticosteroid hormones on hippocampal synaptic plasticity, I will highlight two phenomena that may represent cellular changes underlying mechanisms of learning and memory (Bliss and Collingridge, 1993; Bliss and Lomo, 1973) i.e. long-term potentiation (LTP) and long-term depression (LTD).

Hebbian plasticity

Donald Hebb proposed in 1949 that connections between neurons increase in efficacy in proportion to the degree of correlation between pre- and postsynaptic activity (D.O. Hebb, 1949 "The organization of behavior"). Such alterations in synaptic efficacy like LTP and LTD are believed to underlie learning and memory processes. Since the seminal work of Bliss and Lomo it is now standard to define LTP as an experimental phenomenon which can be used to demonstrate modifications of synaptic strength. LTP can be induced by high-frequency stimulation. It reflects a strengthened or potentiated connection between a presynaptic and postsynaptic neuron (See also box 3).

Characteristics of LTP

LTP reveals characteristics that make it an interesting candidate mechanism for aspects of learning and memory. First, it is long-lasting: hippocampal in vivo recordings show potentiation lasting hours until a year (Abraham, 2003; Abraham et al., 2002; Staubli and Lynch, 1987). Second, it can be induced by physiologically relevant patterns (Larson and Lynch, 1986) and third, it is specific for activated synapses (Engert and Bonhoeffer, 1999; Schiller et al., 1998). LTP can be dissociated in two phases: early-LTP (lasting tens of minutes) and late-LTP (persisting for hours to weeks); the latter requires protein synthesis and heterosynaptic activity during its induction (Deadwyler et al., 1987; Frey et al., 1988; Krug et al., 1984; Stanton and Sarvey, 1984). Furthermore, an important functional relevance was shown by studies where early-LTP could be transformed protein synthesis-dependently into late-LTP by subjecting rats to arousing novel environmental stimuli prior to LTP induction (Straube et al., 2003) or during the consolidation of spatial memory (Uzakov et al., 2005). Also, disruption of LTP can lead to impairments in spatial working memory (Reisel et al., 2002; Schmitt et al., 2005), while manipulations that facilitate or enhance LTP can lead to improved performance (Bach et al., 1995; Morris et al., 1986; Tang et al., 1999; Uzakov et al., 2005).

Mechanisms underlying synaptic plasticity

The initial observation that the NMDA receptor antagonist AP-5 prevents the induction of LTP in hippocampal slices was a crucial step towards the unraveling of the mechanisms behind LTP (Harris et al., 1984). Seminal work by Richard Morris provided the first evidence that NMDA receptors are required for the formation of memories. Rats, that received the NMDA receptor blocker APV intraventricularly, had impaired ability in the Morris water maze. Moreover, when slices of the hippocampus were taken from APV-receiving and control rats, LTP could not be induced in APV-treated rats in contrast to the control group (Morris et al., 1986).

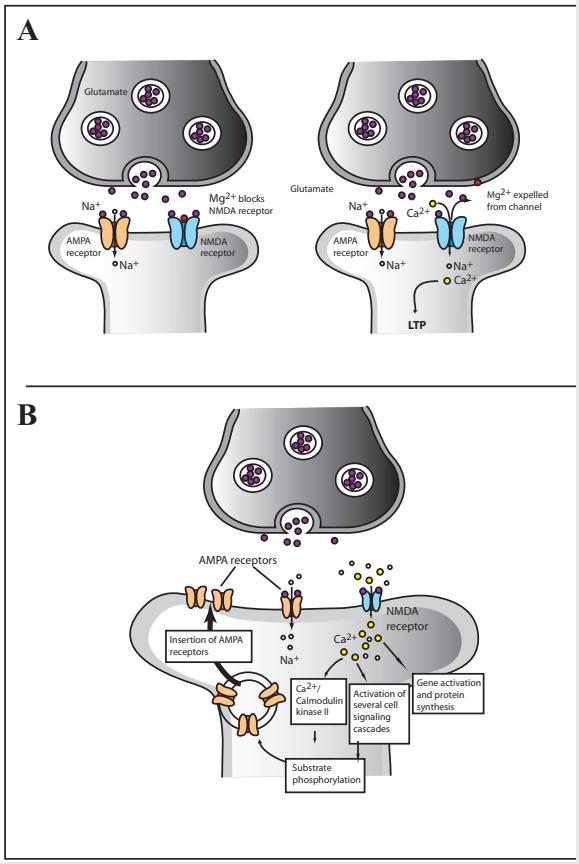
Although different stimulation patterns can be used, a high-frequency tetanus is most commonly used to elicit LTP. At rest, the NMDA receptor calcium channel is blocked by magnesium; the blockade is relieved only after high-frequency stimulation which induces strong postsynaptic depolarization. Calcium

BOX 3

Long Term Potentiation

The most studied and understood form of LTP is NMDA-receptor dependent LTP (occurring after high frequency stimulation of the Schaffer collaterals in the CA1 area). During postsynaptic depolarization the Mg2+ block of voltage-dependent NMDA receptors is removed, which leads to the influx of Ca2+ through the NMDA receptor channel and a rise in Ca2+ within the dendritic spine. The influx of Ca2+ leads to activation of calcium/calmodulin-dependent protein kinase II (CaMKII), linking calcium signals to neuronal plasticity. One way in which CaMKII increases the synaptic sensitivity to glutamate is via phosphorylation of the GluR1 subunit of AMPA receptors that are already resident at the post synaptic surface, resulting in an increase in the single channel conductance of AMPA receptors (figure 3A). Other signaling cascades activated by induction of LTP include cAMP which leads to activation of transcription factors such as CREB, activation of several kinases such as PKC, PKA and tyrosine kinase and the MAPK/ERK pathway which is linked to activation of phosphatidylinositol 3-kinase (PI 3-kinase). Induction of the late phase of LTP also involves activation of immediate early genes and protein synthesis. Another mechanism related to the induction of LTP is the trafficking of AMPA receptors to the postsynaptic site, occurring in about one third of instances of LTP (figure 3B). The appearance of AMPA receptors occurs via insertion of AMPA receptors into the postsynaptic membrane.

Box 3, Figure 3
Schematic overview of Long-term Potentiation



adapted from Neuroscience, edited by Purves et al. 1997

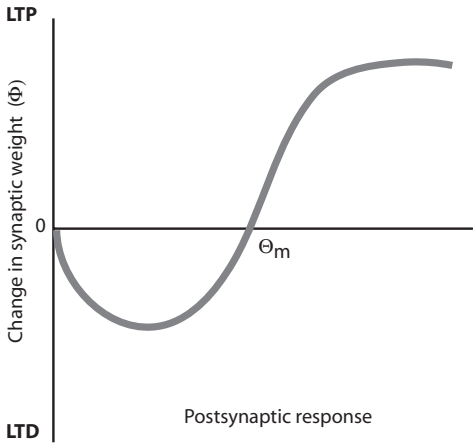
A

During low-frequency synaptic transmission, glutamate binds to both AMPA and NMDA receptors. If the postsynaptic neuron is at resting membrane potential, NMDA channels will be blocked by Mg2+ ions and no current will flow through the receptor. High-frequency stimulation will cause a prolonged depolarization that results in the Mg2+ block being removed from the NMDA channel. This allows Ca2+ to enter the postsynaptic neuron and the resulting increase in Ca2+ concentration within the dendritic spine of the postsynaptic cell will trigger LTP.

B

The postsynaptic response upon LTP induction involves Ca2+ ions that enter the cell upon depolarization which in turn activate many postsynaptic protein kinases. CaMKII, an abundant protein in the postsynaptic site increases the sensitivity to glutamate via phosphorylation but is also associated with the process of inserting AMPA receptors into the postsynaptic membrane.

adapted from Neuroscience, edited by Purves et al.1997



figuur 4

Bienenstock, Cooper and Munro (BCM) model for activity-dependent variation in the threshold for LTP. Relation between postsynaptic cell firing and changes in synaptic weight at the active synapses is described by the function F. Activity above the modification threshold (Θm) leads to LTP, while activity below results in LTD.

signals the beginning of long-term potentiation (Chittajallu et al., 1998; Dunwiddie and Lynch, 1979; Melchers et al., 1988), and the rise in intracellular calcium leads to downstream molecular events resulting in the expression and maintenance of LTP.

There is considerable evidence that LTP induction depends on the activity of several protein kinases, including calcium/calmodulin-dependent protein kinase II (CaMKII) (Lisman et al., 2002; Malinow et al., 1988), protein kinase C (PKC)(Lovinger et al., 1987; Matthies and Reymann, 1993; Muller et al., 1991), protein kinase A (PKA)(Matthies and Reymann, 1993), mitogen-activated protein kinase (MAPK)(English and Sweatt, 1997; Thomas and Huganir, 2004), and tyrosine kinases (O'Dell et al., 1991). Studies in transgenic mice have been frequently used to elucidate the role of the hippocampus in learning and memory focussing on the role of NMDA receptors and calcium influx and the activity of several protein kinases. The group of Alcino Silva using transgenic mice mutants proposed a relationship of autophosphorylation of CaMKII in the hippocampal formation to obtain adequate hippocampal LTP and spatial learning (Giese et al., 1998). Autophosphorylation of CaMKII has been proposed to be the key event in memory storage. However, recently this hypothesis was tested using the passive avoidance task and fear conditioning in transgenic mice lacking autophosphorylation of CaMKII. They showed impaired learning but extended training enabled normal memory (Irvine et al., 2005) suggesting that for long term memory CaMKII might be dispensable.

Next to mechanisms involved in the strengthening of synaptic strength by high frequency stimulation, prolonged inhibition of synaptic transmission (LTD) could be evoked by low frequency stimulation (Ito, 1989; Sejnowski, 1991). The successful search for a mechanism which could induce 'depression of synaptic strength' started with the observation that in the juvenile visual cortex synaptic depression could occur after a period of monocular deprivation. This led to the theory published by Bienenstock, Cooper, and Munro (BCM theory, see next paragraph). Although differences in the method of LTD induction exist (Kerr and Abraham, 1996), a typical protocol for inducing LTD in the CA1 involves prolonged low frequency stimulation at 0.5-5 Hz. LTD requires activation of NMDA receptors (Dudek and Bear, 1992), a rise in postsynaptic calcium concentration (Mulkey and Malenka, 1992), activation of a serine-threonine protein phosphatase cascade (Mulkey et al., 1993) but which can result in internalization of AMPA receptors (Man et al., 2000).

The Bienenstock, Cooper and Munro (BCM) theory made several assumptions about how synapses modify plasticity. Bienenstock, Cooper and Munro designed a model of experience-dependent synaptic plasticity, to account for the plasticity of visual cortex synapses during development (Bienenstock et al., 1982). This model has two main features. First, synaptic modification varies as a non-linear function of postsynaptic activity: low levels of afferent activity result in LTD, while higher levels of activity lead to LTP. The point when LTD is changing into LTP is called the modification threshold, Θm. A second feature of the model is that Θm is not fixed, but changes according to prior postsynaptic activation, dubbing Θm a "sliding" modification threshold (Bear et al., 1987). Candidate mechanisms underlying metaplasticity range from changes in NMDA receptor mediated synaptic transmission, CaMKII activation to protein synthesis (Abraham and Bear, 1996). (see figure 4).

"AMPAfication"

The identification of "silent synapses" leads to the suggestion that insertion of receptors is involved in synaptic plasticity (Malinow and Malenka, 2002). (see box 3, figure 3B).

Synapses are postsynaptically silent if they show an NMDA but no AMPA receptor response. Immunogold labeling of AMPA and NMDA receptors supported that these synapses exist (Nusser et al., 1998). Strong synaptic stimuli lead to NMDA receptor activation and an increase in calcium which triggers the synaptic insertion of AMPA receptors into the postsynaptic site (Isaac et al., 1995; Liao et al., 1995; Luscher et al., 2000; Malinow and Malenka, 2002). Another component next to the trafficking is the phosphorylation of subunits of the AMPA receptor, GluR1 and GluR4 (Boehm and Malinow, 2005). GluR1 undergoes a regulated phosphorylation by CaMKII during LTP (Barria et al., 1997) which leads to an increase in conductance. Next to changes in conductance also AMPA receptor surface expression and synaptic clustering are found to be linked with CaMKII and PKC activity which interacts via the protein stargazin (Chen et al., 2000; Schnell et al., 2002; Turetsky et al., 2005). So during LTP phosphorylation of GluR1 by CaMKII enhances its conductance, while phosphorylation of stargazin controls trafficking to the synapse. Lateral mobility for the AMPA receptor is activity dependent: increasing neuronal activity increased AMPA receptors lateral diffusion (Groc et al., 2004; Tardin et al., 2003).

Although the evidence for AMPA receptor insertion during LTP is vast, some questions still remain. First, in adult animals 75% of the synapses show colocalization of NMDA and AMPA receptors, only relatively small synapses are silent as shown by immunogold labeling (Nusser et al., 1998; Takumi et al., 1999). The low number of synapses lacking AMPA receptors could not possibly account for the large number of silent synapses found with electrophysiological recordings. Also, the temporal pattern of AMPA receptor insertion (several minutes) does not match with the time course of LTP induction which takes place over a few seconds (Hayashi et al., 2000; Shi et al., 1999).

In conclusion, LTP (and LTD) are attractive mechanisms to represent changes in synaptic weight which can be involved in learning and memory processes. In addition to the activation of protein kinases also trafficking or functional modifications of AMPA receptors in the postsynaptic membrane are believed to be involved.

III STRESS AND THE HIPPOCAMPAL FORMATION

Neurons of the hippocampal formation are very sensitive to the effects of stress, due to the high amount of MRs and GRs. The effects on learning and memory processes range from being facilitating (retaining memories for stressful events very well (Debiec et al., 2002; Milekic and Alberini, 2002)) to inhibiting (hampering the retrieval of learned information (de Quervain et al., 1998; de Quervain et al., 2000)). In this chapter I will review effects of stress on hippocampal-dependent memory, synaptic functioning (LTP

and LTD) and neuronal morphology.

Stress, hippocampus and memory

The present view is that glucocorticoids, released within the context of a task, promote long-term consolidation of information (Akirav et al., 2004; Bartolomucci et al., 2002; Conrad et al., 1999; de Kloet et al., 1999; Lupien and Lepage, 2001b; McGaugh and Roozendaal, 2002; Oitzl and de Kloet, 1992; Roozendaal, 2002; Wolf, 2003). Thus, in various species, including man, and in different training paradigms, acute post-training administration of corticosterone (enough to activate the GR) enhances memory consolidation, whereas administration of selective GR antagonists impairs consolidation. Accordingly, a point mutation in the mouse GR, which selectively prevents dimerization and DNA-binding of the GR, impaired spatial memory performance (Oitzl et al., 2001). By contrast, exogenous corticosteroids, GR activation or additional stressors applied before and out of the context of acquisition training or retention testing, impair acquisition and retrieval of spatial information respectively (de Quervain et al., 1998; Diamond et al., 1996; Diamond et al., 1999; Kim and Diamond, 2002; Kuhlmann et al., 2005). Taken together, these studies indicate that exposure to stress affects different stages of learning and memory processes; effects that critically depend on the moment of corticosterone release in concert with the learning process. Moreover, prolonged exposure to elevated corticosterone levels hampers the acquisition and storage of spatial information (Bodnoff et al., 1995a; Conrad et al., 1996; Krugers et al., 1997).

Stress, hippocampus and synaptic plasticity

Electrophysiology

The activity of hippocampal cells largely depends on amino acid-mediated transmission. Since Pfaff showed in 1971 that cortisol suppresses single unit firing of hippocampal cells in vivo, major developments in electrophysiological recording techniques have taken place. These have allowed a more in-depth investigation of corticosteroid actions on hippocampal neuronal function (Joëls et al., 2002).

Electrophysiological recordings in adrenalectomized animals indicates that the presence of adrenal steroids- and particularly MR activation- is critical for synaptic stability (Diamond et al., 1992; Pavlides et al., 1996; Rey et al., 1987; Shors et al., 1990b). Also rises in corticosterone levels after stress, leading to the activation of GR in addition to MR, can affect synaptic functioning (Foy, 1987; Shors et al., 1990a; Shors et al., 1989); for an overview see (Joëls, 2001; Joëls et al., 1997). One of the most persistent findings is that GR activation enhances calcium current amplitude (and thus influx) through voltage-activated calcium channels (Karst, 2000; Karst, 1994; Kerr et al., 1992).

The relation between corticosteroid hormones and their effect shows a U-shaped or inverted U-shaped dose dependency. For instance, calcium currents are low in amplitude with predominant MR activation compared to the situation where hormones are absent; additional GR activation (plus MR) increases calcium influx (Karst, 1994). Similar to the effects found on the calcium currents, Rey et al. (1987) found that activation of MRs causes an increase in population spike amplitude compared to when MRs (and GRs) are unoccupied, while higher concentrations of corticosterone result in a decrease of the population spike amplitude (Rey et al., 1987).

Corticosteroids and stress also affect the AMPA/NMDA ratio as is shown by patch-clamp studies. Focusing on the slow gene-mediated effects of corticosterone (as in experiments when signals are recorded 1-4 hours after exposing hippocampal slices to high corticosterone levels), CA1 neurons showed an increase in amplitude of AMPA currents (Karst and Joëls, 2005). In the dentate gyrus, AMPA and NMDA receptor currents turned out to be unaltered by application of corticosterone to the slice. Yet, in tissue from chronically stressed rats, AMPA -but not NMDA- mediated currents were increased by corticosterone (Karst and Joëls, 2003). In contrast, CA3 pyramidal neurons of chronically stressed rats showed an increase in NMDA receptor-mediated currents that was dependent upon phosphorylation of the

NMDA receptor (Kole et al., 2002b). The onset latency was shortened while input resistance increased (Kole et al., 2004). Studies in another area, i.e. the ventral tegmental area (VTA), confirm that elevation of corticosterone levels, in this case by a swim stress, slowly increases the AMPA/NMDA ratio. This effect is mediated via GRs and involves the GluR1 receptor subunit (Dong et al., 2004; Saal et al., 2003).

Corticosterone and LTP or LTD

Corticosteroid hormones have profound modulatory effects on synaptic plasticity in the hippocampal formation. Since the orginal paper by Foy et al. where rats exposed for 30 minutes to restraint stress showed impaired hippocampal LTP, numerous different stress paradigms and their influence on hippocampal LTP and LTD have been examined (Foy, 1987). In order to show that the effect of corticosterone on hippocampal synaptic plasticity is due to direct activation of steroid receptors in the hippocampal formation (e.g. without any influence from other brain areas), hippocampal slices were exposed in vitro to high levels of corticosterone. Slices from intact mice treated in vitro with corticosterone showed reduced potentiation of the population spike amplitude in the CA1 area after high frequency afferent stimulation (Rey et al., 1994). Although very high doses of corticosterone were used in this study (5 mM) the effect of corticosterone could be blocked by a GR antagonist suggesting that corticosterone inhibits synaptic plasticity via activation of GR. In addition, the authors showed that treatment of slices with low levels of corticosterone (0.5 nM) or aldosterone enhanced LTP. The conclusion was that activation of GRs appears to result in inhibition of hippocampal synaptic plasticity, while activation of MRs promotes synaptic plasticity. Work by David Diamond (1992) showed that in rats with controlled corticosterone levels (adrenalectomized rats with corticosterone pellets) the balance between MR and GR occupation exerts a concentration-dependent biphasic influence on the expression of hippocampal plasticity (the aforementioned inverted U-shape relationship). Primed burst potentiation showed a positive correlation with low levels of corticosterone (0-10 µg/dl), a peak response at intermediate levels (11-20 µg/dl), and a negative correlation at high levels (21-93 µg/dl) of corticosterone (Diamond et al., 1992). The negative correlation between high levels of corticosterone and potentiation in the CA1 was also found after GR activation in vitro of hippocampal slices (Rey et al., 1994; Zhou et al., 2000), elevated corticosteroid levels during neonatal life (Domenici et al., 1996), GR activation in vivo during 1 day or 1 week (Karten et al., 1999) or application of a GR agonist (Pavlides et al., 1996).

Studies using in vivo recordings of field potentials revealed similar effects of corticosteroids on hippocampal synaptic plasticity: both acute and prolonged exposure to elevated corticosteroid levels impair primed burst potentiation in the CA1 area (Bodnoff et al., 1995a) and LTP in the dentate gyrus (Pavlides et al., 1993; Smriga et al., 1996). Moreover, activation of the MR can promote synaptic transmission both in the dentate gyrus and CA3 area (Pavlides et al., 1995a; Pavlides and McEwen, 1999; Pavlides et al., 1995b). In addition to the suppressive effect of corticosteroid hormones on long-term potentiation, corticosterone has been reported to promote LTD in the dentate gyrus (Pavlides et al., 1995a; Pavlides et al., 1995b) as well as LTD in the CA1 area using a GR agonist (Coussens et al., 1997).

Stress and LTP or LTD

A large number of studies has demonstrated that exposure to stressful events impairs subsequent induction of hippocampal LTP in the CA1 area of hippocampal slices (Foy, 1987; Garcia, 1997; Kim et al., 1996; Shors et al., 1990b; Shors et al., 1990c; Shors and Thompson, 1992). In addition, Mesches et al. (1999) demonstrated that psychosocial stress impairs primed burst potentiation in vitro, while LTP was not affected. The authors suggested that psychosocial stress influences hippocampal plasticity only when threshold level, physiologically patterned stimulation is used (Mesches et al., 1999).

The effects of stressful events on synaptic plasticity in vitro are supported by studies in vivo. Relatively mild stressful events impair hippocampal LTP (Korz and Frey, 2003; Xu et al., 1998a). More severe

Table 1. Effects of learning, LTP and LTD on hippocampal morphology

reference	learning paradigm	method	brain area	animal	result
learning **in vivo**					
Wenzel 1980	brightness discrimination	Golgi	hippocampus CA1^{apical}	adult rat	↑ spine density
Moser 1994	water maze	Golgi	hippocampus CA1^{basal}	adult rat	↑ spine density
Kleim, 1996	motor learning	Golgi	motor cortex	adult rat (fem)	↑ spine density
Kleim 1997	motor learning	Golgi	cerebellar cortex	adult rat	↑ spine density
Leuner 2003	eye blink	Golgi	hippocampus CA1^{basal}	adult rat	↑ spine density
Van Reempts 1992	avoidance task	EM	hippocampus DG	adult rat	synaptic remodelling ↑length PSD
Bourgeois 1994	development	EM	prefrontal cortex	rhesus monkey	↓ spine density in adulthood
Rusakov 1997	water maze	EM	hippocampus CA1^{basal} DG	adult rat	nc spine density & ultrastructure
Geinisman 2000	eye blink	EM	hippocampus CA1^{apical}	adult rabbit	nc spine density & ↑ PSD area
LTP **in vivo**					
Desmond 1986	LTP	EM	hippocampus DG	adult rat	nc spine density, change spine type ratio
Desmond 1990	LTP	EM	hippocampus DG	adult rat	change in ultrastructure
Geinisman 1991	LTP	EM	hippocampus DG	adult rat	change spine type ratio & ↑ PSDs
Geinisman 1996	L-LTP	EM	hippocampus DG	adult rat	change spine type ratio
Trommald 1996	LTP	EM	hippocampus DG	adult rat	↑ spine density
Rusakov 1997	LTP	Golgi	hippocampus DG	adult rat	↓ spine density
Sorra 1998	L-LTP	EM	hippocampus CA1^{apical}	adult rat	nc spine density & spine type ratio
Popov 2004	L-LTP	EM	hippocampus DG	adult rat	↑ PSD & change spine type ratio
LTP in vitro					
Hosokawa 1995		LTP	confocal acute slice CA1^{apical}	PND 21-30 rat	↓ spine length
Buchs 1996	LTP	EM	organotypic CA1^{apical}	-	↑ PSD length & number
Engert 1999	LTP	confocal	organotypic CA1^{apical}	-	no change spine density
Toni 1999	LTP	EM	organotypic CA1^{apical}	-	change spine type ratio & ↑ MSB
Ostroff 2002	LTP	EM	acute slice CA1	PND 15 rat	↑ polyribosomes & ↑synapse size
Zhou 2004	LTD	2-photon	acute slices CA1^{apical}	PND 14-18 rat	↓ spine volume
Stewart 2005b	LTP	EM	acute slice CA1^{apical}	adult mouse	↑ PSD & ultrastructure change

↑ = increase; ↓ = decrease; nc = no change

stressful paradigms also impair synaptic plasticity in the dentate gyrus (Shors and Dryver, 1994). In addition, stressful experiences not only suppress LTP but also facilitate the induction of LTD (Kim et al., 1996; Manahan-Vaughan, 2000; Manahan-Vaughan and Braunewell, 1999; Xu et al., 1997; Xu et al., 1998b).

In line with a suppression of synaptic efficacy by stressful events it has been reported that placing animals in a novel cage (novelty stress) prevents the induction of primed burst potentiation in the CA1 area (Diamond et al., 1994). Interestingly, exposure to novel (stressful) conditions was even found to reverse once established LTP in the CA1 area (Xu et al., 1998a). Moreover, Xu et al., (1998b) reported that the GR antagonist RU486 prevents the effects of novelty on LTP and LTD, in a protein-synthesis-dependent and RNA-dependent way. In addition to the suppressive effects of stressful experiences and elevated corticosterone levels on hippocampal synaptic plasticity, some recent studies have reported that exposure to novelty or stressful experience can also facilitate hippocampal synaptic plasticity, depending on the timing of stress, novelty and LTP induction. Thus when high frequency stimulation and exposure (in the context of the event) to novelty (Li et al., 2003) or swim stress (Korz and Frey, 2003) are closely linked in time facilitation of LTP is reported, shifting short-term plasticity into long-term synaptic plasticity. In addition, several lines of evidence indicate that the amygdala is involved in mediating stress effects on hippocampal LTP and memory. More specifically, amygdala lesions or inactivation have been shown to block or attenuate stress-induced impairments in hippocampal long-term potentiation and spatial memory (Akirav, 1999; Kim et al., 2005; Kim, 2001; Korz and Frey, 2005). At the time that studies of this thesis were started it was not clear however, to what extent stress and corticosterone can still change hippocampal function regardless of the connections with the amygdala (see further chapter 2).

The number of studies addressing the effects of chronic exposure to stress or corticosterone on synaptic plasticity is limited. It was shown that prolonged treatment with elevated corticosteroid levels impairs LTP in the dentate gyrus (Smriga et al., 1996) and that chronic stress impairs LTP measured in vivo in the CA3 area and dentate gyrus (Pavlides, 2002). When the experiment for this thesis were started it was not known if LTP in the CA1 area is modulated by chronic stress, under basal corticosterone conditions or after short exposure to high levels of corticosterone (see chapter 3).

IV NEURONAL AND SYNAPTIC MORPHOLOGY

Synaptic morphology

Remodelling of synaptic networks through an activity-dependent formation or elimination of synaptic connections or a remodelling of spine shape is believed to contribute to synaptic plasticity as well as long-term memory (Geinisman, 2000; Segal, 2005; Yuste and Bonhoeffer, 2001). The belief that spines are the main sites of excitatory synaptic transmission stimulated research to investigate the role of spine number and morphology in learning and memory processes or more cellular mechanisms like LTP. This field of research has produced interesting observations but also conflicting results.

Early studies using electron microscopy or Golgi staining reported an increased spine density after exposing animals to behavioural learning tasks (Kleim et al., 1996; Kleim et al., 1997; Leuner et al., 2003; Moser et al., 1994; Wenzel et al., 1980). In contrast, other electron microscopic studies, some using the serial sectioning technique, did not replicate these findings (Bourgeois et al., 1994; Geinisman, 2000; Rusakov et al., 1997a; Van Reempts et al., 1992). Studies examining the structure of synaptic contacts associated with behavioral learning have found more consistent results. These studies reported for instance increased enlargement of the postsynaptic density (PSD) (Geinisman et al., 2000) or clustering of synaptic active zones (Rusakov et al., 1997a) after learning.

Similar discrepant results came from groups reporting that spine density is stable after LTP in vivo, in animals (Desmond and Levy, 1986; Desmond and Levy, 1990; Geinisman et al., 1991; Geinisman et al., 1996; Rusakov et al., 1997b; Sorra and Harris, 1998) or in vitro, in hippocampal slices (Engert and Bonhoeffer, 1999; Hosokawa et al., 1995). However, some groups did find LTP-associated increases in synapse density in vivo (Andersen and Soleng, 1998; Trommald et al., 1996). Yet, the reshaping of spine shape and structure after LTP has been replicated in numerous studies (Buchs and Muller, 1996; Geinisman et al., 1991; Ostroff et al., 2002; Popov et al., 2004; Stewart et al., 2005b; Toni et al., 1999; Toni et al., 2001; Zhou et al., 2004). Time-lapse video confocal microscopy provided the first evidence that activation of glutamate receptors modulates spine motility (Fischer et al., 1998; Fischer et al., 2000). For an overview of data concerning the effects of behavioral learning and LTP on synaptic morphology see table 1.

To address the question if these processes are associated with changes in experience-dependent plasticity, two groups repeatedly imaged individual pyramidal neurons in the mouse barrel cortex in vivo (Grutzendler et al., 2002; Trachtenberg et al., 2002). In order to investigate the influence of sensory experience, some whiskers of these animals were trimmed. Even though roughly the same approach was used, the authors reached different conclusions about the stability of spines in adult animals. Trachtenberg found that among the different classes of spines, most spines disappeared after 3 months. Grutzendler et al. on the other hand found that 96% of spines were stable. Moreover they showed a half-life of more than 13 months.

Although there are discrepancies, it is clear from these studies that changes in spine density, spine shape ratio and ultrastructural changes of the synapse are associated with different forms of learning and may contribute to synaptic plasticity.

Stress, hippocampus and neuronal morphology

Changes in hippocampal neuronal structure have been implicated in synaptic plasticity. Changes in synaptic and dendritic structure after exposure to stressful events and corticosteroid hormones could therefore have implications for hippocampal function. Such changes have to some extent been described for stress and corticosterone treatment, particularly in the CA3 hippocampal region. For a overview of the most relevant data concerning the effects of stress on dendritic remodeling see table 2.

Effects of brief exposure to stress or corticosterone are barely investigated. One study investigating the effects of two days subordination stress in rats found an increase in basal dendrites of CA3 pyramidal cells with a concomittant decrease in the apical tree, when studied 3 weeks after the stressor (Kole et al., 2004). Much more is known about the effects of prolonged stress exposure. Rats treated with supraphysiological doses of corticosterone for 3 weeks show shrinkage of the apical tree of hippocampal CA3 neurons (Fuchs et al., 2001b; Magarinos and McEwen, 1995a; Magarinos et al., 1996; Watanabe et al., 1992c), and altered synaptic terminal structure in mossy fibres (Magarinos and McEwen, 1995a). Exposure of rats and tree shrews to prolonged stress induced similar morphological alterations (Magarinos and McEwen, 1995a; Magarinos and McEwen, 1995b; Magarinos et al., 1996; Magarinos et al., 1997). The observation that cyanoketone, which prevents the stress-induced rise in plasma corticosterone levels, blocked the stress-induced atrophy, suggests that corticosterone released during stress mediates the stress-induced atrophy (Magarinos and McEwen, 1995a). In addition, excitatory amino acids may be involved in the stress-induced atrophy since phenytoin (Magarinos and McEwen, 1995a) and a NMDA receptor antagonist (Magarinos et al., 1997) both blocked CA3 shrinkage. The stress-induced dendritic atrophy in apical dendrites of CA3 neurons seems to be a reversible phenomenon (Sousa et al., 2000). No morphological changes are found in the basal dendritic tree. More detailed studies indicated that rats stressed for 3-4 weeks display synaptic changes in the CA3 area, including alterations in mossy fibre terminals (Magarinos et al., 1997; Sandi et al., 2003), a loss of synapses and a

reduction in the surface area of postsynaptic densities (Stewart et al., 2005a), as well as a loss of simple perforated synapses in stratum lucidum of CA3 (Kole et al., 2004) and retraction of thorny excrescences (Stewart et al., 2005a). Exposure of rats and primates to even longer periods of high levels of corticosterone and prolonged stress may reduce CA3 volume and induce loss of hippocampal cells (Sapolsky et al., 1985; Sapolsky et al., 1990).

Studies on the effects of exposure to (prolonged) stress and elevated corticosteroid hormone levels on dendritic structure and spine number in the hippocampal CA1 area are relatively sparse. Shors et al. (2000) reported that exposure to stress increases spine density in male, but reduces spine density in female rats after brief exposure to stress, when examined 24 hours after the stressor. No effect was observed immediately after the stressor (Sousa et al., 2000). In contrast, evidence for a putatively more rapid action of corticosterone on dendritic spines comes from a study using acute hippocampal slices exposed to dexamethasone, a specific agonist of the GR. In this study spinogenesis was observed in CA1 pyramidal neurons within one hour after application (Komatsuzaki et al., 2005). The only study describing effects of chronic stress on adult hippocampal CA1 dendritic morphology is by Sousa et al. (2000) who reported a 13-20% reduction in apical dendritic length (which did not reach statistical significance), together with a reduction in mean length of terminal dendritic segments. Taken together, the structural changes in the hippocampal CA3 area are consistently investigated while not much is known about effects of the acute or chronic stress in the CA1 area, an essential brain structure for the process of memory formation and consolidation (see further chapter 4 and 5).

V OUTLINE OF THIS THESIS

Stress and corticosteroid hormones are strong modulators of learning and memory processes. Stress can be beneficial for successful consolidation of memory but can also hamper this process when the stressful event occurs prior to and out of the learning context. Chronic stressors are thought to interfere with succesful learning. This relationship becomes apparent in human studies where hypersecretion of corticosteroids or depressive illness is associated with impaired memory function and shrinkage of the hippocampal formation.

Animal studies have shown that there is an intricate relationship between the level of corticosteroids and synaptic plasticity supporting the notion of an inverted U-shaped function between these two parameters. In this thesis, the effects of brief and chronic stress on a cellular model for learning and memory are examined in the CA1 area of the hippocampal formation (part I). I next focused on the morphological changes in the same area after brief exposure to corticosteroids in vitro or exposure to chronic unpredictable stress (part 2).

Research Questions

PART I

Both in vivo and in vitro recordings have demonstrated that elevations of corticosteroid hormone levels or stress can modulate hippocampal LTP and LTD. However, it is much debated whether the modulation of stress is primarily caused by effects of corticosterone via hippocampal MRs and GRs, or that other brain areas are critically involved. To address this issue, in chapter 2, the effect of in vitro corticosterone application is investigated on synaptic potentiation in a reduced preparation, i.e. mouse hippocampal slices. Synaptic potentiation, evoked by two different stimulation protocols, was studied in both the pyramidal cell layer as well as the dendritic layer. We compared these effects with effects seen after exposing mice to a brief psychological stressor which in addition to activation of hippocampal GRs also activates GRs in other brain areas and elevates non-steroid stress-related factors.

Table 2. Effects of stress on hippocampal morphology

	Stressor	Finding
Magarinos & McEwen, 1995	21 days restraint	CA3c: ↓ length apical dendrites
Sousa et al., 2000	chronic unpredictable stress paradigm & chronic corticosterone injections	DG: ↓ total dendritic length; CA3: ↓ total dendritic length, ? length terminal segments; ↓ length terminal segments
CA1:		
Shors et al., 2001	24 hours after 30 sec restraint tail shock	CA1: ↑ spine density apical dendrites
Vyas et al., 2002-2004	10 days immobilization stress & 10 days unpredictable stress	CA3: ↓ total dendritic length and branching points by immobilization; in lesser extent after unpredictable stress. A 21 day recovery period did reverse CA3 atrophy
Kole et al., 2004	22 days after 1 hours social defeat or 21 days	2 days CA3: ↓ apical dendritic length and ? length basal den drites 21 days: CA3: ↓ apical dendritic length
Stewart et al., 2005	21 days restraint stress	whole hippocampus lower volume; CA3: retraction thorny excrescences, ↑ perforated PSDs

Exposing rats for 3 weeks to unpredictable stressful events is believed to induce HPA-axis disturbances and can serve as an animal model for depression. Changes in HPA-axis associated with depressive disorders have been implicated in the cognitive symptoms prevalent in depression. In chapter 3 of this thesis, the effects of prolonged stress on hippocampal synaptic function under controlled corticosteroid levels were examined. We first recorded, after chronic stress, hippocampal synaptic plasticity in vitro in the CA1 and dentate gyrus hippocampal subfields, when plasma corticosterone levels are low. Second, we studied the effect of an additional challenge, i.e. elevated corticosteroid hormone levels which activate GRs in addition to MRs, on synaptic plasticity.

PART II

In part II the putative role of stress was examined on hippocampal CA1 dendritic morphology and spine density, in two different substrates.

As discussed in paragraph IV, remodelling of synaptic networks and changes in spine shape and density are involved in synaptic plasticity. In order to assess putative changes on hippocampal CA1 morphologyfr brief in vitro elevation of corticosteroid levels, we examined CA1 pyramidal neuronal morphology in hippocampal organotypic slice cultures (chapter 4). In these experiments, we examined the effect of two doses of corticosterone, on dendritic morphology and complexity as well as on spine density after reconstructing neurons that were filled with the fluorescent dye Alexa. Also, in order to establish the role of the glucocorticoid receptor in this process, RU486, a glucocorticoid receptor antagonist, was used to see if the corticosterone induced morphological changes could be prevented.

Several studies using magnetic resonance imaging have reported reduced hippocampal volume in patients with depression or other stress-related disorders (Bremner et al., 2000; Sapolsky, 2000; Sheline et al., 1996). Interestingly, cognitive impairments seem to occur in parallel with this structural damage (O'Brien et al., 2004; Starkman et al., 1992; Steffens et al., 2000; Vermetten et al., 2003). Animal studies have shown that one of the most consistent effects of chronic stress on morphology is a reduction in the branching and length of CA3 pyramidal apical dendrites. Since the CA1 area plays an important role in the consolidation of memory processes, we therefore questioned in chapter 5, whether chronic stress could also induce effects on CA1 pyramidal neuronal morphology. To this end, CA1 pyramidal neurons were reconstructed, using confocal microscopy, in slices from control and chronically stressed animals. In order to mimic acute stress exposure against a background of chronic stress or control treatment, neurons were examined under basal low and raised corticosteroid levels. Also, in order to assess whether GR blockade could normalize the effects of chronic stress, animals were treated with the GR antagonist RU486 during a 4 day period, similar to that reported to successfully suppress symptoms of depression (Belanoff et al., 2001).

CHAPTER 2

Corticosterone and stress reduce synaptic potentiation in mouse hippocampal slices with mild stimulation

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Neuroscience, 2002 Vol. 115 (4): 1119-1126

Abstract

Elevation of circulating corticosterone levels, either through exogenous administration of the hormone or following stress exposure, is known to reduce hippocampal synaptic potentiation in rodents. It is presently debated whether this reduction is due to activation of hippocampal glucocorticoid receptors or is primarily caused in other brain structures projecting to the hippocampus. To address this issue, we examined whether synaptic potentiation in hippocampal slices from mice with low basal corticosterone levels was altered 1-4 hrs after a brief in vitro administration of 100 nM corticosterone. Population spike and field EPSP were recorded in the cell and dendritic layers respectively of the CA1 area, in response to Schaffer-commissural fiber stimulation. Basal characteristics of the stimulus-response relationship were not affected by corticosterone treatment, except that after corticosterone treatment the maximal field EPSP slope was reduced while the excitability ratio was increased. For studies on potentiation of the field EPSP and population spike, stimulus intensities were chosen to evoke half maximal responses before potentiation; this intensity was significantly lower for the field EPSP than for the population spike. Primed burst potentiation of the field EPSP but not population spike was significantly attenuated after corticosterone treatment. When using a more rigorous stimulation paradigm, i.e. theta burst potentiation; synaptic potentiation was not affected by corticosterone. Raising corticosterone levels in mice by exposure to a psychosocial stressor led to comparable results in subsequent in vitro experiments; stress reduced primed burst potentiation only of the field EPSP. These data support that corticosterone affects synaptic potentiation in the mouse via direct activation of hippocampal glucocorticoid receptors but only when using mild stimulation conditions.

Introduction

Corticosteroid hormones are released from the adrenal glands in a circadian rhythm and elevated circulating levels are often found after exposure to stressful events (De Kloet, 1991). Due to their lipophilic character corticosteroids readily cross the blood-brain barrier and bind to both thus far characterized receptors in the brain: the mineralocorticoid-receptor (MR) and glucocorticoid-receptor (GR) which are enriched in the hippocampal formation (McEwen et al., 1986).

Over the past decade, in vivo as well as in vitro recording of synaptic plasticity have demonstrated that elevation of of corticosteroid hormones or stress influence hippocampal synaptic plasticity. Diamond et al. (1992) demonstrated that in vivo elevated corticosteroid levels (presumably occupying both MR and GR) impair primed burst potentiation in the CA1 hippocampal area of the rat, while low levels of corticosterone (predominantly occupying MRs) facilitate primed burst potentiation (Diamond et al., 1992). The role of the two corticosteroid receptors was strengthened by subsequent experiments in which selective MR- and GR-ligands were applied prior to induction of synaptic potentiation (Pavlides et al., 1995a). A similar involvement of the corticosteroid receptors was also proposed for other hippocampal subfields (Pavlides and McEwen, 1999; Pavlides et al., 1996; Pavlides et al., 1995b). Importantly, not only exogenously applied corticosterone but also endogenous release of the hormone due to stressful conditions was shown to suppress hippocampal long-term potentiation in vitro (Foy, 1987; Kim et al., 1996; Mesches et al., 1999; Shors et al., 1989) and in vivo (Diamond et al., 1994; Shors and Dryver, 1994; Smriga et al., 1996; Xu et al., 1997; Xu et al., 1998b). The suppressive effects mediated by the GR on synaptic potentiation were observed both after acute and prolonged elevation of corticosteroid levels (Bodnoff et al., 1995b; Domenici et al., 1996; Karten et al., 1999; Pavlides et al., 1993; Smriga et al., 1996).

Although there seems to be general consensus on the finding that stress modulates hippocampal synaptic plasticity, it is much debated whether this modulation is primarily caused by effects of corticosterone via hippocampal MRs and GRs. Thus, recent studies have shown that in vivo the amygdalar complex is critical for the development of stress-induced effects on hippocampal synaptic plasticity (Akirav, 1999; Kim, 2001). This may imply that stress-induced activation of amygdala rather than hippocampal GRs is

the primary event in the earlier observed stress-related suppression of hippocampal synaptic potentiation. Also, earlier studies raised the possibility that stress-induced factors other than corticosterone, e.g. endogenous opioids, may play a role in the suppression of synaptic potentiation following exposure to unpredictable stressors (Shors et al., 1990b; Shors et al., 1990c).
The aim of the present study was to examine the effect of in vitro corticosterone application on synaptic potentiation in a reduced preparation, i.e. hippocampal slices from mice, thus precluding the influence of the amygdala and non-steroid factors released after stress. Slices were prepared from mice under rest at the trough of their circadian corticosterone release, a situation that presumably results in predominant activation of MRs (De Kloet, 1991). Synaptic potentiation was investigated after in vitro vehicle treatment (20 min) or perfusion with 100 nM corticosterone -sufficient to fully activate MRs as well as GRs in the hippocampus (Karst, 2000)- 1-4 hrs before induction of synaptic potentiation. Synaptic potentiation was studied in the pyramidal cell layer of the hippocampal CA1 area (population spike amplitude, PSA) as well as the dendritic layer (slope of the field excitatory postsynaptic potential, sfEPSP). In a second series of experiments using the same recording protocol, we compared these effects of in vitro GR activation on hippocampal synaptic potentiation with effects seen after exposure of the mice to a psychological stressor, which presumably activates hippocampal GRs but also activates GRs in other brain areas and elevates non-steroid stress related factors.

Experimental procedures

All experiments were approved by the local committee on Animal Bioethics of the University of Amsterdam.

Animals

Male C57/Bl6 mice (± 6 weeks of age) were obtained from Harlan CPB. Upon arrival the animals were individually housed with food and water ad libitum available. Lights were on from 8:00 a.m. till 20:00 p.m.

Corticosterone treatment

Animals were decapitated between 9 and 10 a.m. when plasma corticosterone levels are low. After decapitation the brain was dissected and chilled in ice-cold (4°C) artificial cerebrospinal fluid (aCSF, containing (in mM) NaCl (120), KCl (3.5), NaH2PO4 (1.25) NaHCO3 (25), CaCl2*2H2O (2.5), MgSO4*7H2O (1.3), Glucose (10)) gassed with 95 % O2 and 5 % CO2. Trunk blood was collected and plasma corticosterone levels were later determined with I125-corticosterone radioimmunoassay for mice (ICN Pharmaceuticals NY, USA). Transverse hippocampal slices (400 mm) were prepared using a tissue chopper (Krugers, 1997). Sixty minutes following slice preparation, slices were treated in aCSF either with vehicle or corticosterone (100 nM in 0.01 % ethanol) for 20 minutes at 32 oC. Previous studies have shown that this dosage elicits alterations in cellular properties that require dimerization of the glucocorticoid receptor (Karst, 2000). After at least one hour, one slice at a time was transferred to the recording chamber and fully submerged in warm (32oC), gassed (95 % O2 and 5 % CO2) aCSF (perfusion rate: 2-3 ml/min).

Exposure to stress

Part of the animals were exposed to a psychosocial stressor. Before the experiment, the mouse was placed overnight in one half of an experimental cage which was divided in two halves separated by a wire netting. The next morning a rat was placed in the other part of the cage providing a non-physical stressful situation for the mouse. One hour after interaction between the animals was initiated, the mouse was decapitated, trunk blood was collected and slices were prepared as described above. Control animals were taken directly from their home cage.

Electrophysiological recordings

Electrophysiological experiments started at least one hour following preparation of hippocampal slices. A bipolar stimulation electrode (stainless steel, insulated except for the tip) was placed in the Schaffer collateral/commissural fibers. Low resistance glass microelectrodes (2-5 MW, filled with aCSF) were placed in either the pyramidal cell layer or the dendritic layer of the CA1 area. Upon stimulation (pulse duration: 150 ms) we recorded either the amplitude of the population spike amplitude (PSA) in the cell layer or slope of the field EPSP (sfEPSP) in the dendritic layer (figure 1A).

In each experiment we first determined the maximal field EPSP amplitude or population spike amplitude by gradually increasing the stimulus intensity (interstimulus interval 30 seconds) until the responses saturated. The relationship between stimulus intensity and the evoked response was fit by a sigmoidal function: $R(i)=Rmax/(1+exp((i-ih)/(-S))$, where R(i) is the response at intensity (i), Rmax is the maximal response, ih is the intensity at which half maximal response is observed, and slope factor S represents an index proportional to the slope of the stimulus-response curve (figure 1C). The stimulus intensity that evoked the half maximal response was used throughout the experiment, including during repetitive stimulation. Importantly, the half maximal stimulus intensity of the fEPSP is in general lower than that of the PSA (figure 1B). Since only one stimulation intensity could be used during repetitive stimulation (either Ih of the PSA or the sfEPSP), this precluded simultaneous investigation of synaptic potentiation of the PSA and sfEPSP in one slice.

After establishing the input-output relationship and determining the half-maximal stimulation intensity, we monitored baseline synaptic transmission for at least 10 minutes. Next primed burst stimulation was applied, which consisted of a single stimulus followed 180 ms later by a burst of 4 stimuli at 200 Hz (Fig 1D, (Diamond et al., 1992; Krugers, 1997). Subsequently we recorded synaptic responses every minute during a period of 40 or 50 minutes, still using the Ih established at the start of the experiment. Finally, an input-output curve was established to verify whether the stimulus-response relationship was changed during the course of the experiment. In one series of experiments (after administration of corticosterone or vehicle) we applied theta burst instead of primed burst stimulation. Theta burst stimulation consisted of a train of 4 pulses at 100 Hz, repeated 200 ms later by another 4 pulses at 100 Hz. This set was repeated to a total of 5 times, at an interval of 30 seconds (Krugers, 1997). Next, we recorded synaptic responsiveness every minute during a period of 60 minutes and determined the input-output relationship at the end of the experiment.

Table 1. Baseline electrophysiological parameters of the PS-amplitude (PSA) and sfEPSP of vehicle and corticosterone treated slices.

Treatment	n	Max. PSA (mV)	Max. sfEPSP (mV/ms)	Excitability Ratio	I_h PSA (mA)	I_h sfEPSP (mA)	S_{PSA}	S_{sfEPSP}
Veh	33	6.31 ± 0.41	-3.23 ± 0.42	2.54 ± 0.24	85.9 ± 10.9	59.9 ± 8.2$^{\$}$	17.7 ± 2.4	22.1 ± 3.8
Cort (100 nM)	30	5.87 ± 0.39	-1.98 ± 0.18*	3.45 ± 0.29$^{\#}$	89.4 ± 13.4	52.4 ± 6.7$^{\$}$	17.1 ± 3.0	23.0 ± 3.9

*After corticosterone treatment the maximal slope of the fEPSP was reduced ($P<0.01$), and the $^{\#}$excitability ratio was increased ($P<0.05$). $^{\$}$The stimulus intensity that evokes half maximum sfEPSP (I_h) was significantly lower when compared to Ih of the PSA. Slope factor S for both PS-amplitude and sfEPSP was comparable between vehicle and corticosterone treated slices.

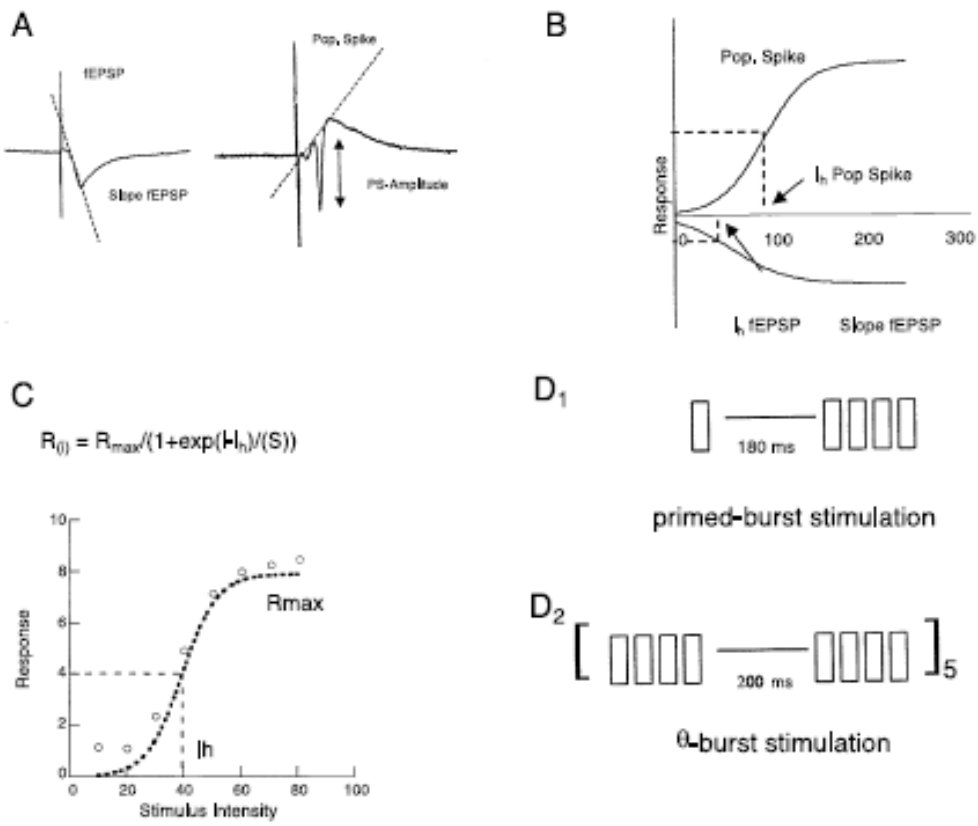

Figure 1.

Summary of data analysis and stimulation paradigms. PSA and sfEPSP were determined as shown in A. Figure B shows that the half maximal stimulation intensity (Ih) of the sfEPSP was lower than Ih of the PSA. Input-output curves were fitted using a Bolzmann equation and maximum response and Ih were calculated (C). Primed burst stimulation (D1) and theta-burst stimulation (D2) were used to evoke synaptic potentiation (see text for details).

Statistical analysis

Values for the maximal response and half maximal stimulus intensity were compared between control and treatment groups, using ANOVA. Comparison of synaptic responses over time (before and after repetitive stimulation) between control and treatment groups were performed with analysis of variance for repeated measures (MANOVA). In both cases, $p < 0.05$ was accepted as indicating significant differences.

Results

Corticosterone and synaptic plasticity

We first addressed the question whether corticosterone (100 nM), administered to hippocampal slices for 20 minutes modulates synaptic plasticity in the CA1 area later on. When all animals in this series were

taken together, basal plasma corticosterone levels after decapitation were comparable between mice from which slices were treated either with vehicle (39.0 ± 9.6 ng/ml, n=28) or corticosterone (36.9 ± 13.1 ng/ml, n=23). This supports that in slices treated with vehicle mostly MRs were occupied, whereas in slices treated with 100 nM of corticosterone MRs as well GRs were fully activated (De Kloet, 1991).
After treatment with corticosterone, the maximal sfEPSP was significantly reduced compared to vehicle treated slices (Table 1: vehicle: -3.23 ± 0.42 mV/ms; corticosterone: -1.98 ± 0.18 mV/ms), although the stimulus intensities and slope factor of the input-output relationship were comparable. The stimulus intensity required to induce half maximal responses for the PSA was significantly higher than for the sfEPSP, both in the control and the corticosterone treated groups. In contrast to the maximal sfEPSP, the maximal PSA was not altered after corticosterone treatment. Consequently, the excitability ratio (defined as amplitude of the population spike divided by the slope of the fEPSP) was increased after corticosterone treatment (Table 1). Input-output data for both the PSA and sfEPSP were comparable between stress and naive animals (data not shown).
Primed burst stimulation was applied at two different stimulus intensities: half maximal stimulation intensity of the sfEPSP (relatively weak stimulus, figure 2A) or half maximal stimulation intensity of the PSA (relatively strong stimulus, figure 2B). In both cases primed burst stimulation enhanced synaptic efficacy, although the magnitude of potentiation was larger when using a relatively strong stimulus compared to weaker stimulus (amount of potentiation at t=50 min. of 146% ± 10 and 114% ± 7, respectively). Corticosterone treatment significantly ([F(1,18)=13.861; p<0.01]) reduced primed burst potentiation when recording the sfEPSP, i.e. when a relatively weak stimulus was applied throughout the experiment (figure 2A). In contrast to the sfEPSP, primed burst potentiation of the PSA -using a stronger stimulus intensity- was not affected by corticosterone ([F(1,11)=0.053; p=0.82], figure 2B).

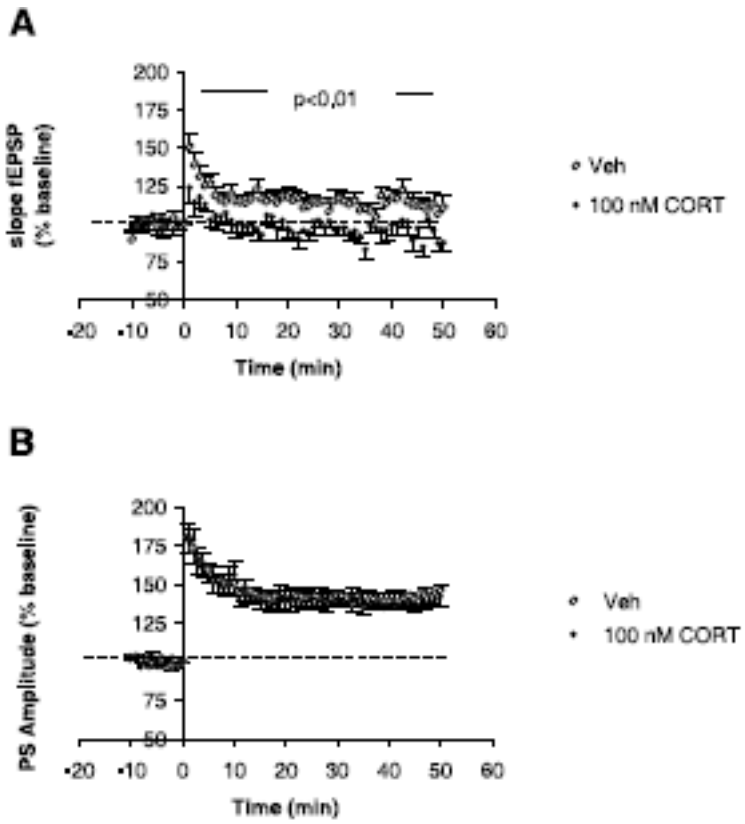

Figure 2.
Potentiation of the sfEPSP (A) and PS-amplitude (B) after primed burst stimulation. Corticosterone treatment significantly reduced primed burst potentiation when recording the sfEPSP (A) but not when recording the PS-amplitude (B). P<0.05 was considered as significantly different. Recordings sfEPSP: vehicle n=10 and corticosterone n=11; recordings PSA: vehicle n=8 and corticosterone n=5.

In an additional set of experiments, we recorded synaptic potentiation using a more rigorous stimulation paradigm- i.e. theta burst stimulation. In accordance with the more rigorous stimulation paradigm, theta burst stimulation robustly increased synaptic plasticity in the hippocampal CA1 area of both the slope of the fEPSP (figure 3A) and population spike amplitude (figure 3B). Although potentiation of the sfEPSP was somewhat reduced shortly after repetitive stimulation in the corticosterone treated group, no significant effects of corticosterone on synaptic plasticity were observed when looking over the entire 1 hr period after theta burst stimulation ([F(1,15)=2.387;p=0.14], figure 3A). Also, corticosterone did not modulate synaptic potentiation of the population spike amplitude ([F(1,10)=1.086; p=0.32], figure 3B).

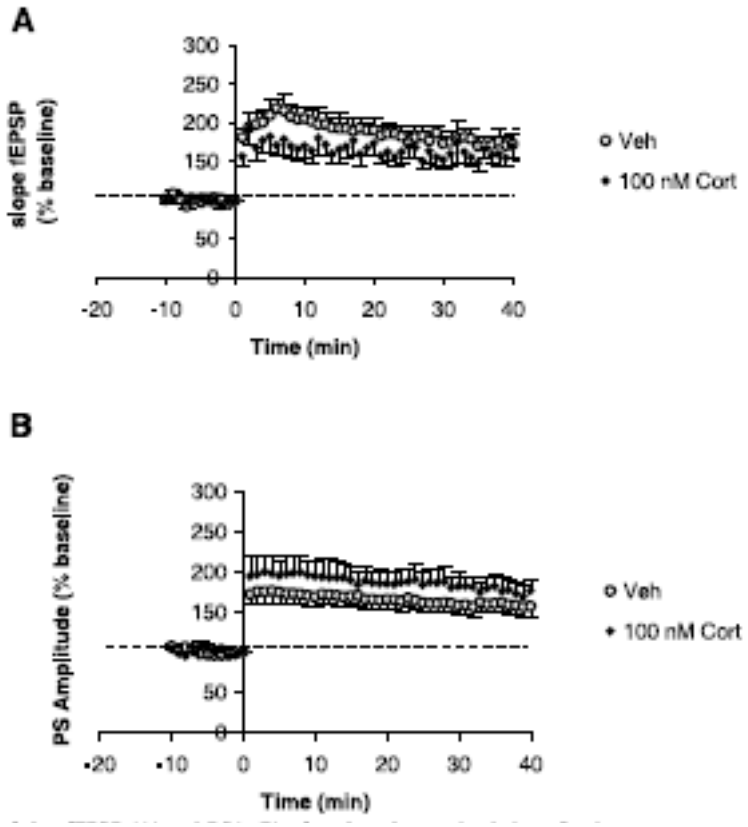

Figure 3

Potentiation of the sfEPSP (A) and PS-amplitude (B) after theta-burst stimulation. Corticosterone treatment did not influence synaptic potentiation in CA1 dendritic field (A, vehicle n=10 and corticosterone n=8) or cell layer (B, vehicle n=6 and corticosterone n=6).

Stress and synaptic plasticity

In order to compare the effect on synaptic potentiation of rises in corticosterone concentration from exogenous and endogenous sources, we performed experiments in which mice were subjected to a psychosocial stressor prior to in vitro recording. The psychosocial interaction between the rat and mouse resulted in a significant elevation of plasma corticosterone levels in the stressed mice (167.8 ± 21.0 ng/ml, n=11) when compared to control animals (27.4 ± 7.9 ng/ml, n=13). In contrast to the effects of corticosterone, no effects of stress were seen on maximal sfEPSP (naive animals -4.3 ± 0.9; stressed animals –3.2 ± 0.57).

As in the earlier set of experiments in which corticosterone was administered exogenously, two stimulus intensities were applied in slices from stressed or control mice: 1) a stimulus intensity that evoked the half maximum PSA (relatively strong stimulus intensity) or 2) a stimulus intensity that evoked the half

maximum sfEPSP (weaker stimulus intensity). Figure 4A shows that following primed burst potentiation, the sfEPSP was increased in the control group (141% ± 9 at t= 50 min.). A significant reduction ([F(1,12)=5.055; p<0.05]) of the primed burst potentiation was observed after prior exposure to a psychosocial stressor (111% ± 9 at t= 50 min.). By contrast, no significant changes in primed burst potentiation were observed when recording the PSA, i.e. using a stronger stimulus intensity (F(1,11)=2.326; p=0.16], figure 4B).

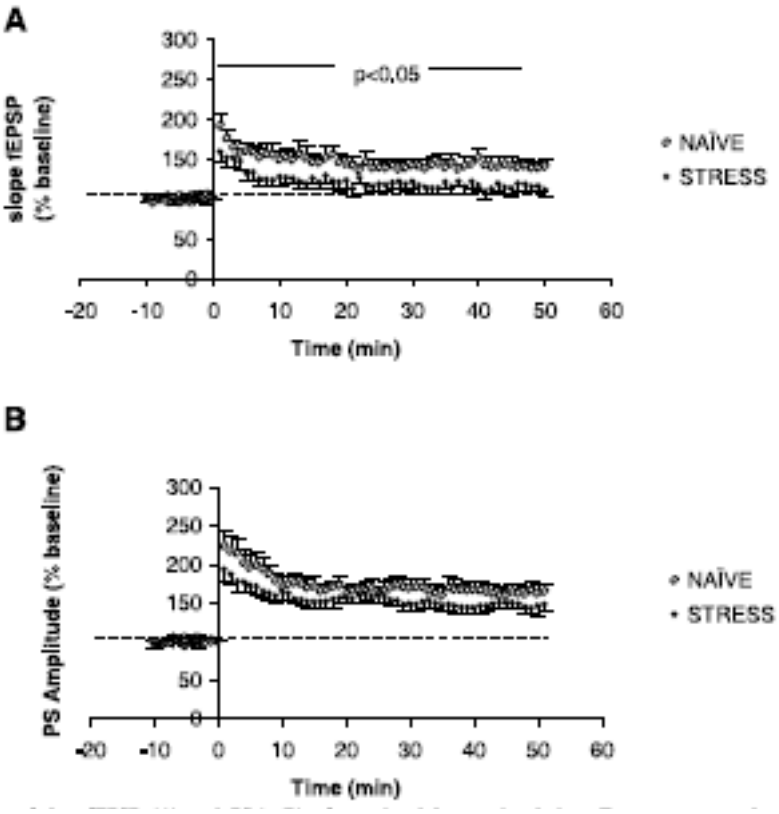

Figure 4

Potentiation of the sfEPSP (A) and PSA (B) after primed burst stimulation. Exposure to psychosocial stress significantly reduced primed burst potentiation when recording the sfEPSP (A) but not when recording the PSA. P<0.05 was considered as significantly different.

Recordings sfEPSP: vehicle n=7 and stress n=7; recordings PSA: vehicle n=7 and stress n=6.

Discussion

Stress is known to reduce synaptic potentiation in the CA1 hippocampal area of rodents (Diamond et al., 1994; Foy, 1987; Kim et al., 1996; Mesches et al., 1999; Shors et al., 1989). Although stress results in elevation of corticosteroid levels, it is not unequivocal whether corticosterone -and more specifically GRs- mediate the stress-induced attenuation of synaptic potentiation. Thus, earlier studies indicated that non-steroid factors released after stress, i.e. opioid hormones, are involved in this phenomenon (Shors et al., 1990b; Shors et al., 1990c). In support for a role of corticosterone, and GRs in particular, in modulating hippocampal synaptic efficacy a more recent study, using a different stress paradigm, showed that a stress-induced shift in the propensity to evoke synaptic depression versus potentiation requires protein synthesis and is blocked by the GR-antagonist RU 38486 (Xu et al., 1998b). Another matter of debate is whether the stress-induced attenuation of synaptic potentiation is directly aimed at the hippocampus or critically involves other brain regions which in turn project to the hippocampus. Recently, it was

shown that the integrity of the amygdalar complex is important for the development of stress effects on hippocampal synaptic plasticity (Akirav, 1999; Kim, 2001). This opens the possibility that stress exposure primarily affects amygdala rather than hippocampal function. We hypothesized that if stress-induced modulation of hippocampal synaptic plasticity is mostly due to corticosterone acting at the level of the hippocampus, it should be possible to mimic this modulation by directly applying the hormone to hippocampal slices. So far, only one study reported such direct effects, but this involved an extremely high dose of corticosterone (5 µM) which is likely to act through a non-genomic pathway; accordingly, relatively rapid effects were reported (Rey et al., 1994).

In the present study in adrenal intact mice we therefore tested the hypothesis that corticosterone hampers hippocampal synaptic plasticity by directly acting at the level of the hippocampal formation. The dosage of corticosterone (100 nM) applied to the hippocampal slices is sufficient to activate both MRs and GRs (Karst, 2000) and the genomic nature of effects by thus applied corticosterone is supported by the long delay between corticosterone treatment and recording (1-4 hrs). Moreover, exogenous application of 100 nM corticosterone to slices from adrenal intact mice earlier changed hippocampal calcium currents through a mechanism involving binding of GR-homodimers to the DNA (Karst, 2000).

We first established the effect of acute in vitro corticosterone treatment on basal input-output characteristics. It appeared that the maximal sfEPSP but not the PSA was significantly reduced after corticosterone treatment. Although few earlier studies specifically addressed this issue, some studies indeed found similar results after corticosterone application (Karten et al., 1999); in one study, though, 100 nM corticosterone was found to also reduce the PSA (Vidal et al., 1986). The reduction of the fEPSP by corticosterone is important, since it may indirectly influence the apparent hormonal effects on synaptic potentiation. Thus, recording baseline synaptic transmission with a relatively (on the input-output curve) high stimulus intensity (relative large signals) is expected to result in less pronounced potentiation than when using a lower baseline stimulus intensity. Since we found that corticosterone reduces the maximal sfEPSP, it is therefore pivotal to record baseline synaptic transmission at half maximum stimulus intensities in order to get potentially the same amount of potentiation in vehicle and corticosterone-treated slices. The corticosterone-evoked change in basal input-output relationships may therefore partly explain the earlier reported attenuation of synaptic plasticity. In contrast to corticosterone treated slices, no differences in input-output data (including maximum slope of the sfEPSP) were found after exposure to stress. This may indicate that corticosteroid reductions in baseline synaptic transmission are compensated by other factors that accompany exposure to aversive stimulation. In this respect it is very interesting that CRH has recently been demonstrated to increase baseline synaptic transmission in the hippocampal formation (Blank et al., 2002). However, even when stimulating with half maximal intensity, we presently found that corticosterone reduces synaptic potentiation of the sfEPSP, though not of the PSA. The latter was not caused by a general inability to influence synaptic potentiation of the PSA, since modulation of the PSA using the same protocol was earlier demonstrated e.g. in transgenic mice with a comparable C57/Bl6 background (Krugers et al., 1997). The dissociation between corticosterone effects at the level of dendritic and somatic potentials may signify that these potentials are differentially affected by the hormone. This cannot be excluded presently. Alternatively, the findings can be explained by the fact that synaptic potentiation of the sfEPSP involves a lower absolute stimulus intensity than the PSA. In this view, only mild stimulation conditions allow appearance of corticosteroid effects on synaptic potentiation. Indeed, many studies demonstrating a suppressive effect of corticosterone or stress on synaptic plasticity used a relative low stimulus intensity to induce potentiation (Diamond et al., 1992; Diamond et al., 1994; Mesches et al., 1999). This interpretation is supported by our observations that corticosterone was less effective in attenuating synaptic potentiation when using a more rigorous stimulus paradigm, i.e. theta versus primed burst stimulation. The lower effectiveness of high frequency stimulation (i.e. 100 Hz for 1 s) versus primed burst stimulation was also found in an earlier study, addressing effects of psychosocial stress

on synaptic potentiation in rats (Mesches et al., 1999). To fully prove the view that corticosterone only affects synaptic potentiation when using mild stimulus conditions, it will be necessary to compare corticosterone effects on PS-amplitude as well as sfEPSP at several stimulation intensities. Unfortunately, this is not feasible with extracellular recording: with half-maximal stimulus intensity for the sfEPSP, the PSA is too small and variable for reliable analysis, while half-maximal stimulation intensity for the PSA already evokes a near-maximal sfEPSP.

In conclusion, the present study shows that corticosterone directly applied to mouse hippocampal slices can indeed attenuate synaptic potentiation, though probably only under mild stimulation conditions. The cellular mechanism underlying this phenomenon needs further examination in the future with whole cell recording. However, the notion that corticosteroids only modulate hippocampal synaptic responsiveness with mild stimulation may indicate that corticosteroids influence post-synaptic properties that determine whether summation of post-synaptic currents can occur. One way corticosteroids could reduce synaptic potentiation is by increasing the K+-mediated Ca2+- dependent afterhyperpolarization (Joëls and de Kloet, 1989; Kerr et al., 1989). Moreover, one may speculate that corticosteroids reduce glutamate-receptor (e.g. NMDA-) mediated currents thereby diminishing Hebbian synaptic plasticity. Alternatively, corticosteroids may reduce presynaptic release properties of hippocampal CA1 synapses (Zhou et al., 2000). Intracellular or whole cell recordings are required to resolve these issues.

Interestingly, we noted that synaptic potentiation was attenuated to a comparable degree after psychosocial stress exposure, indicating that the influence of other brain regions or stress-released factors other than corticosterone is limited. Under the present recording conditions stronger forms of repetitive stimulation, e.g. bursts at theta frequency, may nevertheless involve other parts of the brain, which explains the critical role of the amygdala for hippocampal potentiation during stress exposure as described recently (Akirav, 1999; Kim, 2001).

CHAPTER 3

Chronic unpredictable stress impairs long-term potentiation in rat hippocampal CA1 area and dentate gyrus in vitro

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European Journal of Neuroscience, 2003 Vol. 17: 1928-1934

Abstract

Rises in corticosteroid levels e.g. after acute stress impair synaptic plasticity in the rat hippocampus when compared to the situation that levels are basal i.e. under rest. We here addressed the question whether basal and raised levels of corticosterone affect synaptic plasticity similarly in animals that experienced chronic stress prior to corticosterone application. To this end, rats were exposed to a 21-days variable stress paradigm. Synaptic plasticity was examined in vitro in the dentate gyrus and CA1 hippocampal region, 24 hours after exposure to the last stressor i.e. when corticosterone levels are basal (low). First we observed that long-term potentiation was greatly impaired in both CA1 and dentate gyrus after 3 weeks of exposure to variable stress, when recorded under conditions where plasma corticosterone levels are low. Second, administration of 100 nM corticosterone in vitro reduced synaptic plasticity in CA1 of control rats, but induced no further impairment of synaptic plasticity in chronically stressed rats. Third, in the dentate gyrus, corticosterone incubation did not affect synaptic plasticity in slices from both control and stressed animals. We conclude that i) exposure to chronic variable stress per se reduces synaptic plasticity both in CA1 and dentate gyrus and ii) acute rises in corticosterone level induce no additional impairment of synaptic plasticity in the CA1 region of chronically stressed rats. It is tempting to speculate that the stress-induced reduction of hippocampal efficacy provides a cellular substrate for cognitive deficits in hippocampus-dependent learning tasks seen after prolonged exposure to stressful events.

Introduction

Stressful events are generally remembered well, but exposure to an aversive experience has also been reported to impair the acquisition, storage and retrieval of novel information (Bodnoff et al., 1995a; de Kloet et al., 1999; Lupien and Lepage, 2001a; McEwen, 2000; Roozendaal et al., 2001). Related to the latter, acute exposure to an aversive event was found to impair hippocampal long-term potentiation (LTP) (Diamond et al., 1994; Foy, 1987; Mesches et al., 1999; Shors and Dryver, 1994) which may serve as a cellular model for learning and memory (Bliss and Collingridge, 1993).

There is considerable evidence that the stress-induced effects on hippocampal synaptic potentiation are mediated, at least in part, by the stress-evoked release of corticosteroid hormones from the adrenal glands (Dachir et al., 1993; Diamond et al., 1992; Pavlides et al., 1996; Xu et al., 1998b) and subsequent activation of the low affinity glucocorticoid receptors (GR) in this area (Reul and de Kloet, 1985). Although the involvement of other hormones and/or brain regions also may play a role (Akirav, 1999; Blank et al., 2002; Kim, 2001), we recently showed that high levels of corticosterone can indeed hamper hippocampal synaptic potentiation by directly acting at the level of the hippocampal formation (Alfarez et al., 2002).

While the effects of exposure to acute stressful events and elevated corticosteroid hormone levels on synaptic efficacy are well described, little is known about the effects of prolonged stress on hippocampal synaptic function. Thus, while chronic stress is known to alter hippocampal morphology (McEwen, 1999) and produces deficits in cognitive performance in hippocampus-dependent learning tasks (Krugers et al., 1997; Luine et al., 1996), it remains to be addressed whether rises in corticosteroid levels also impair hippocampal synaptic efficacy in animals that experienced a prolonged period of stress earlier on. Although recent studies suggest that chronic stress indeed modulates hippocampal synaptic plasticity (Gerges et al., 2001; Pavlides, 2002), the above question was not answered in these studies since synaptic plasticity could not be examined with basal corticosteroid levels due to the experimental design. Therefore, we presently examined whether exposure to chronic stress alters hippocampal synaptic plasticity later on i) when corticosteroid levels are low and ii) when steroid levels are raised in vitro. To address these questions we first recorded hippocampal synaptic plasticity in vitro in various hippocampal subfields from control and chronically stressed animals twenty-four hours after exposure to the last stressor. At this time point plasma corticosterone levels are low and occupy almost exclusively mineralocorticoid receptors (MRs; De Kloet, 1991). Second, we studied whether elevated corticosteroid-hormone

levels (activating GRs in addition to MRs) affect synaptic plasticity in slices from control and chronically stressed animals. Therefore slices were treated in vitro with corticosterone levels high enough to activate GRs in addition to MRs.

Materials and Methods

Animals

The local committee on Animal Bioethics of the University of Amsterdam approved all experiments. Male Wistar rats (Harlan CPB, The Netherlands), weighing 100-150 g at the start of the stress regime or handling procedure were used. The bodyweight increased to approximately 260 gr at the end of the experimental procedure, and animals were considered adult (approximately 11 weeks of age) at the moment of electrophysiological recordings. All animals were housed in pairs for at least 7 days before experimental procedures started. Food and water were provided ad libitum, lights were on from 8:00 a.m. until 8:00 p.m. and the temperature was kept between 20-22°C.

Stress paradigm

A 21-day variable stressor paradigm was used to induce chronic stress (Herman et al., 1995; Karst and Joëls, 2003). The following stressors were used: (1) Immobilization: rats were placed in plastic immobilization cages for 1h; (2) Cold immobilization: rats were placed in plastic immobilization cages in a cold room (4 °C) for 1h; (3) Vibration: rats in group-cages were placed on top of an orbital shaker and shaken for 1 h at 30 rev/min.; (4) Isolation: rats were moved to individual cages for a 24-hour period; (5) Crowding: rats were housed 4-6 per cage for a 24-hour period; (6) Swim: rats were placed in a plastic container filled with water (25-30°C) for 30 min.; (7) Cold-water swim: rats were placed in a plastic container filled with water (10-15°C) for 5 minutes. The different stressors were distributed randomly. Two different stressors were administered per day, one in the morning and one in the late afternoon. Rats were not exposed to cold twice on the same day. Control rats were removed from their cages, handled briefly and weighed, for 21 days.

Slice preparation and corticosterone treatment

Twenty-four hours after exposure to the last stressor, rats were decapitated between 9 and 10 a.m. when plasma corticosterone levels are low. Trunk blood was collected for plasma corticosterone level analysis by means of a radio immunoassay (RIA). Adrenals and thymus gland were collected, cleaned, and weighed.
Immediately after decapitation, the brain was dissected from the skull and chilled (at approximately 4 °C) in artificial cerebrospinal fluid (ACSF) containing (in mmol/L): NaCl 120, KCl 3.5, MgSO4 5.0, NaH2PO4 1.25, CaCl2 0.2, D-Glucose 10, and NaHCO3 25.0, gassed with 95% O2 and 5% CO2. Hippocampal slices, 400 µm thick, were prepared with a vibroslicer (Leica VT 1000S). Briefly, frontal lobes and cerebellum were removed and the dorsal side of the brain was glued on the slicing plateau. Coronal slices were prepared and incubated at room temperature in recording 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. After 1 hour, slices were treated in recording-ACSF with vehicle or corticosterone (100 nM in 0.01% ethanol) for 20 min at 32 °C. Previous studies have shown that this dosage elicits alterations in synaptic plasticity (Alfarez et al., 2002) and modulates cellular properties that require homodimerization of the GR (Karst, 2000). After at least one hour, slices were transferred to a standard recording chamber and perfused with oxygenated recording-ACSF (2.3 ml/min) at 32 °C.

Electrophysiology

Bipolar nichrome stimulating electrodes were either placed in the Schaffer collateral/commissural fibers or in the molecular layer of the dentate gyrus to activate the perforant path. Upon stimulation (pulse

duration: 150 ms), field EPSPs (fEPSPs) were recorded using a glass microelectrode (2-5 MW filled with ACSF) that was placed in the stratum radiatum of the CA1 area or in the middle third of the molecular layer of the dentate gyrus respectively. In both areas, the slope and amplitude of the fEPSP were calculated (Fig. 1A). For recordings in the dentate gyrus g-aminobutyric acid-mediated activity was blocked with 10 µM (-)-bicuculline methiodide (Sigma chemicals, (Wang et al., 2000).

Experimental protocol

In each experiment we first determined the maximal fEPSP amplitude and fEPSP slope by gradually increasing the stimulus intensity (interstimulus interval 30 seconds) until the responses saturated. The relationship between stimulus intensity and the evoked response was fit by a sigmoidal function: R(i)=Rmax/(1+exp((i-ih)/(S)), where R(i) is the response at intensity (i), Rmax is the maximal response, ih is the intensity at which half maximal response is observed, and slope factor S represents an index pro-

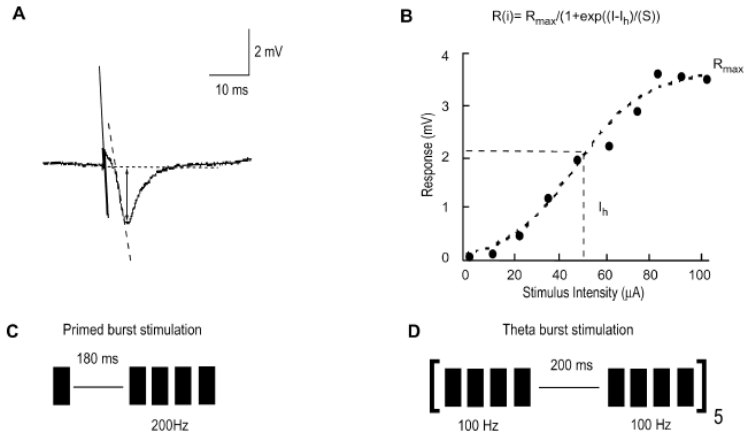

Figure 1

Twenty-four hours after the last stressor, hippocampal sections were cut. Field potentials were recorded in the hippocampal CA1 and dentate gyrus after stimulating the Schaffer collateral/commissural fibers and perforant path respectively. Amplitude of fEPSP and slope of initial downward phase of fEPSP were determined as shown in A. Input-output curves were fitted using a Bolzmann equation: R(i)=Rmax/(1+exp((i-ih)/(S)). Maximum response (Rmax), half maximal stimulus intensity (Ih) and slope factor S (S) were calculated (B). Primed burst stimulation (C) and theta-burst stimulation (D) were used to evoke synaptic potentiation (see text for details).

portional to the slope of the stimulus-response curve (Fig. 1B). After determining the half-maximal stimulation intensity of the amplitude, paired-pulse responses were tested at this intensity with interstimulus intervals (isi) of 20, 40 and 50 ms for CA1 and 20, 40 and 60 ms for dentate gyrus (Bronzino et al., 1997; Zhou et al., 2000). Next, we recorded baseline synaptic transmission with half-maximal stimulation intensity for at least 10 minutes. Slices in which the baseline was not stable or where the fEPSP amplitude was < -1.0 mV (CA1) or < -2.0 mV (dentate gyrus) were rejected. After baseline recording, high frequency stimulation was elicited in CA1 or dentate gyrus. Synaptic potentiation in CA1 was evoked using primed-burst stimulation (PBS) consisting of a single stimulus followed 180 ms later by a burst of 4 stimuli at 200 Hz (Alfarez et al., 2002); Fig. 1C). In the dentate gyrus theta-burst stimulation was applied which consisted of a burst of 4 pulses at 100 Hz, repeated 200 ms later by another burst of 4 pulses at 100 Hz. This train was repeated to a total of 5 times, with an intertrain interval of 30 seconds (Fig. 1D).

After high frequency stimulation synaptic responsiveness was recorded for 50 minutes in both CA1 and dentate gyrus.

Statistical analysis

Synaptic potentiation after high frequency stimulation was represented as percentage change from baseline. Comparison of synaptic responses over time between control and chronic stress group was performed with analysis of variance for repeated measures (MANOVA). Values for the input-output curves were compared using ANOVA and student t-test. All data are expressed as average ± standard error of the mean (SEM). P values <0.05 were considered significantly different.

Results

Chronic stress parameters

Body weight values and organ weights of control and stressed animals are shown in Table 1. While control animals gained considerable body weight during the course of the experiment, chronically stressed rats showed less body weight gain during the experiment when compared to the control animals [$F(1,21)=5.11$; $P<0.05$].

In addition, stressed animals displayed adrenal hypertrophy when corrected for body weight [$F(1,21)=7.77$; $P<0.05$] while thymus weight was reduced in chronically stressed animals [$F(1,21)=5.88$; $P<0.05$]. Taken together, these data strongly indicate that animals included in the present study were chronically exposed to elevated corticosteroid levels during the 21 days stress paradigm. One day after the last stressor, plasma corticosterone levels were low again and not significantly elevated when compared to the control rats (controls: 2.2 mg/ml ± 1.3, chronic stress: 4.7 mg/ml ± 2.5).

Table 1. Effects of chronic stress on body weight gain, adrenal weight and thymus weight

	Control	Chronic Stress
Body weight gain (% initial body weight)	93.3 ± 8.3	73.3 ± 6.8 *
Adrenal weight (mg)	39.0 ± 2.0	45.0 ± 2.7
Adrenal weight (mg/100 g body weight)	14.4 ± 0.6	17.9 ± 1.0 *
Thymus weight (mg)	658.7 ± 8.2	548.7 ± 22.3 *
Thymus weight (mg/100 g body weight)	249.2 ± 18.6	217.6 ± 7.5

Exposure to various unpredictable stressors for 21 days attenuated bodyweight gain, increased adrenal weight corrected for body weight and reduced thymus weight. Plasma corticosterone levels were not significantly different between control and stressed animals 24 hours after the last stressor. * indicates p< 0.05 vs. the control group.

Input-output curves

Input output curves were created in order to verify whether exposure to the 21-day variable stress paradigm and/or brief application of corticosterone influenced basal circuitry properties in CA1 area and dentate gyrus. Parameters of the input-output curves of the CA1 and dentate gyrus are shown in Table 2.

In our current experimental set-up we found that in control animals basal characteristics of synaptic circuitry were different between the CA1 area and the dentate gyrus of control animals: the maximal response (Rmax) was higher in dentate gyrus when compared to CA1 [$F(1,56)=17.6$; $P<0.01$]; half maximal stimulus intensity (ih) higher in CA1 area when compared to the dentate gyrus [$F(1,58)=4.36$; $P<0.05$] and slope factor S, higher in CA1 compared to the dentate gyrus [$F(1,56)=10.15$; $P<0.05$].

Table 2. Basal synaptic characteristics for hippocampal CA1 area and dentate gyrus

	CA1				dentate gyrus		
Group	N (rats)	Rmax (mV/ms)	Ih (mA)	S-factor	N (rats)	Rmax (mV/ms)	lh (mA)	S-factor
Control	6	-1.5 ± 0.2	95.0 ± 12.0	14.4 ± 1.7	7	-2.0 ± 0.3	57.0 ± 9.9	9.3 ± 1.1
Stress	10	-1.4 ± 0.2	97.8 ± 9.7	13.6 ± 1.7	8	-2.2 ± 0.1	67.6 ± 20.3	6.4 ± 0.7
Control/corticosterone	8	-1.2 ± 0.2	89.4 ± 14.7	11.8 ± 1.6	7	-2.7 ± 0.3	54.6 ± 13.7	10.3 ± 2.3
Stress/corticosterone	6	-1.7 ± 0.2	90.8 ± 23.3	13.4 ± 2.4	6	-2.0 ± 0.3	56.5 ± 10.5	10.5 ± 0.7

Maximal slope of the fEPSP (Rmax), half maximal stimulus intensity (Ih) and the slope of the input output curve (slope factor S) in CA1 area and dentate gyrus. No differences in basal characteristics were found in CA1 area or dentate gyrus between the different groups.

However, both in CA1 and dentate gyrus, basal characteristics were not different after exposure to the 21-day variable stress paradigm when compared to slices from control animals. Also slices from control and chronically stressed animals that were treated with corticosterone compared to vehicle-treated slices did not exhibit significantly different basal characteristics.

Paired pulse stimulation

In the CA1 area, paired pulse stimulation in slices from control animals treated with vehicle resulted in facilitation of the second response (when compared to the first response) at all interstimulus intervals that were tested (20, 40 and 50 ms; Fig. 2A). In chronically stressed animals we found that paired pulse facilitation was significantly enhanced at interstimulus interval 40 ms, when compared to control animals (Fig. 2A). Corticosterone treatment did not affect paired pulse responsiveness significantly, neither in the control nor chronically stressed group. The observed reduction of paired pulse facilitation at 50 ms of the control group treated with corticosterone compared to the untreated control group did not reach statistical significant difference.

In the dentate gyrus, the paired pulse ratio in control slices was approximately 1. In addition, the paired pulse ratio was comparable between the control group and all experimental groups (Fig. 2B). This indicates that both the medial and lateral perforant path were stimulated during recordings in the dentate gyrus (Bronzino et al., 1997; Colino and Malenka, 1993).

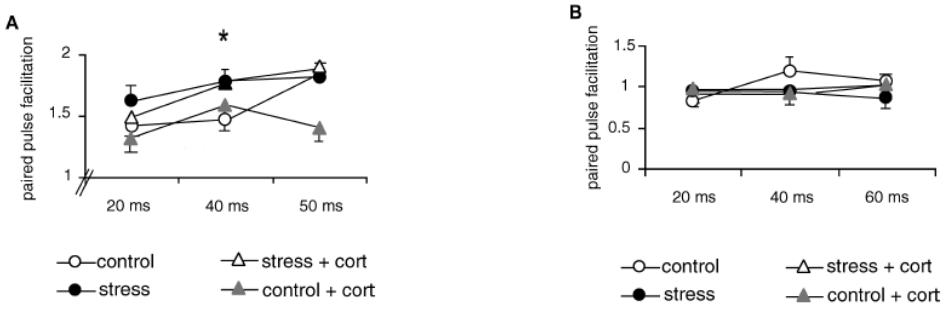

Figure 2

*Paired pulse responses in CA1 area (A, interstimulus intervals 20, 40, 50 ms) and dentate gyrus (B, interstimulus interval 20, 40, 60 ms). Chronic stress facilitated paired pulse responsiveness in CA1 area significantly at interstimulus interval 40 ms compared to slices from control animals, * indicates P< 0.05.*

Synaptic plasticity

In the CA1 area, synaptic potentiation was evoked by primed-burst stimulation. This resulted in a stable potentiation over time in slices from control animals. By contrast, synaptic potentiation was completely blocked in slices from chronically stressed animals ([$F(1,10)=7.004$; $p<0.05$]; Fig. 3). As reported before (Alfarez et al., 2002), we found at present that brief administration of corticosterone to hippocampal slices reduced synaptic potentiation in the CA1 area later on ([$F(1,8)=5.93$; $p<0.05$], Fig. 3B). By contrast, corticosterone treatment of slices from chronically stressed animals did not modulate synaptic efficacy when compared to vehicle-treated slices of chronically stressed animals (Fig. 3).

In the dentate gyrus, theta burst stimulation evoked long-term synaptic potentiation in slices from control animals (Fig. 4B). Slices from chronically stressed animals displayed potentiation of the fEPSP amplitude immediately after high frequency stimulation (Fig. 4A). Synaptic potentiation then declined rapidly to that observed before high frequency stimulation levels ([$F(1,8)=14.32$; $P<0.01$]; Fig. 4B).

Corticosterone treatment of slices from both control and chronically stressed animals did not influence synaptic potentiation (Fig. 4).

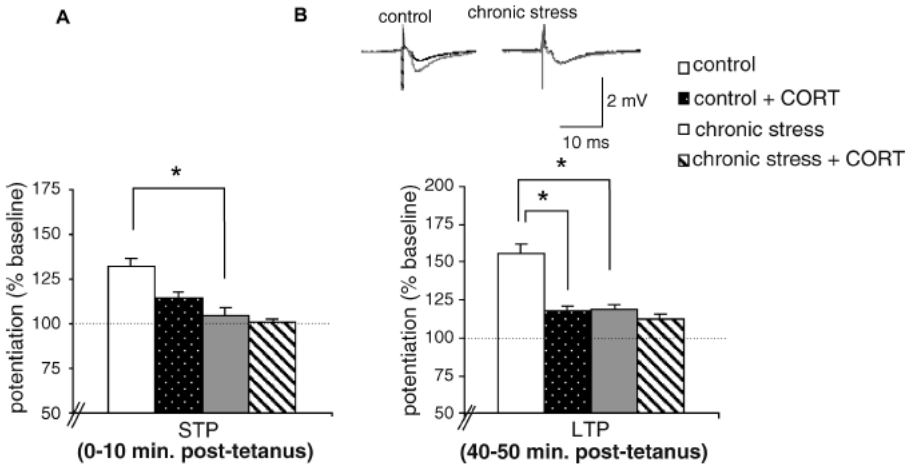

Figure 3

*Potentiation of the amplitude of the fEPSP after Primed Burst Stimulation in area CA1. A Short term potentiation (potentiation recorded over the first 10 minutes after primed-burst stimulation) is significantly reduced in stressed animal, and slices from stressed animals treated with corticosterone. B Moreover, long-term potentiation (potentiation recorded over the last 10 minutes) is significantly reduced in corticosterone treated slices as well as slices from chronically stressed animals with and without corticosterone treatment. (control n=6; chronic stress n=10; control + corticosterone n=8; stress + corticosterone n=6) * indicates P< 0.05 Inset: representative fEPSPs from control and chronic stress group. Baseline response and response 40-50 min. post-tetanus are shown for both groups.*

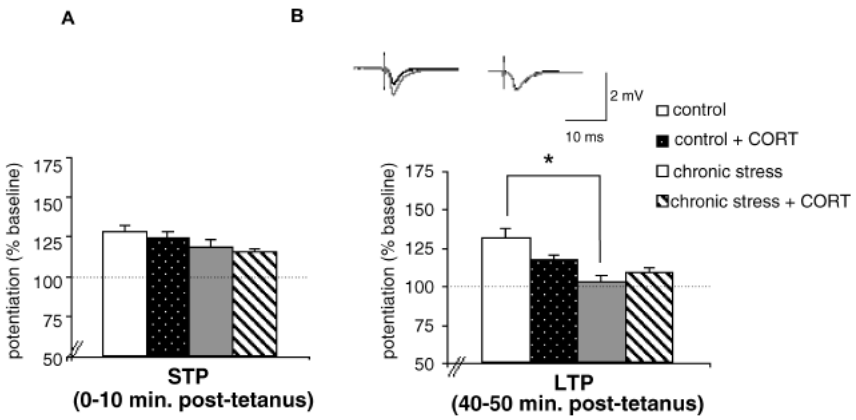

Figure 4

Potentiation of the amplitude of fEPSP after theta-burst stimulation in the dentate gyrus A Neither corticosterone, nor exposure to stress influenced short-term synaptic potentiation B However, long-term potentiation (potentiation recorded over the last 10 minutes) was significantly impaired in slices from animals exposed to prolonged stress. (Control n=7; Chronic stress n=8; control + CORT n=7; Stress + CORT n=6)

** indicates P< 0.05 Inset: representative fEPSPs from control and chronic stress group. Baseline response and response 40-50 min. post-tetanus are shown for both groups.*

Discussion

In this study we examined hippocampal synaptic plasticity in slices from animals that were exposed to various mild stressors twice a day for 21 days. We found that exposure to this chronic stress paradigm drastically reduced synaptic plasticity in both the CA1 area and the dentate gyrus twenty-four hours after exposure to the last stressor (when plasma corticosterone levels are low). Acute administration of corticosterone to slices from control animals reduced synaptic plasticity in the CA1 area but not in the dentate gyrus. Brief administration of corticosterone had no apparent effect on synaptic plasticity in the CA1 area or dentate gyrus of chronically stressed animals.

Twenty-one days of exposure to various mild stressors induced classical features of prolonged elevations of circulating corticosterone levels. Stressed animals showed reduced body weight gain, increased adrenal weight and a decrease in thymus weight. In chronically stressed animals basal corticosterone levels were slightly though not significantly elevated when compared to control animals. The latter implies that synaptic function and plasticity were investigated under conditions where in both control and stressed animals MRs but not GRs were extensively activated (De Kloet, 1991). Importantly, there is presently no experimental evidence to support that the relative MR occupation was largely altered in animals subjected to unpredictable stressors for 21 days. Thus, no changes in MR- and GR-mRNA expression and binding in hippocampus were observed in other studies (Herman and Spencer, 1998; Paskitti et al., 2000) as well as in tissue from the animals included in the present study (E. van Riel & M. Joëls, unpublished observation). The current investigation of synaptic plasticity under conditions of predominant MR activation differs from that in other recent studies (Gerges et al., 2001; Pavlides, 2002), where GRs were likely to be extensively activated due to the anesthetics used during the in vivo experiments.

The fact that synaptic potentiation under conditions of predominant MR activation was markedly impaired after prolonged exposure to aversive events can be explained in several ways. First, it is feasible that basal membrane properties (e.g. resting membrane potential and input resistance) were changed, which could have large consequences in view of the voltage-dependency of the NMDA-receptor that is essential for synaptic potentiation (Bliss and Collingridge, 1993). This seems unlikely though, since synaptic function before high frequency stimulation as established with input-output relations was not significantly affected by chronic stress in either the CA1 area or dentate gyrus. Yet, a more in depth investigation, also at the single cell level, would be necessary to exclude this possibility. Second, the data could be explained by a reduced capacity or functionality of AMPA receptors. Again, this is unlikely since basal transmission -which is largely mediated by AMPA receptors- was not significantly depressed by chronic stress. Furthermore, whole cell recording of dentate granule cells (Karst and Joëls, 2003) as well as CA3 pyramidal neurons(Kole et al., 2002a) also showed no changes in AMPA receptor mediated responses one day after chronic stress exposure, under comparable experimental conditions. Furthermore, AMPA receptor subunits in hippocampal subfields were either not changed or even increased in expression after chronic stress (Schwendt and Jezova, 2000; Watanabe et al., 1995). Third, reduced capacity or function of NMDA receptors could be considered as explanation of the present findings. Earlier data do not support this. Whole cell recording in CA3 pyramidal neurons revealed enhanced rather than reduced NMDA-receptor function after chronic stress (Kole et al., 2002b), while in the dentate no changes were observed (Karst and Joëls, 2003). Data about CA1 pyramidal cells, though, are presently lacking. As with the AMPA receptors, NMDA receptor subunit expression was found to be enhanced or not changed after chronic stress (Schwendt and Jezova, 2000; Watanabe et al., 1995; Weiland et al., 1997). Interestingly, the stress-induced enhancement of NMDA-responses in CA3 neurons suggests the possibility that chronic stress and synaptic potentiation converge onto the same pathways. If this also occurs in CA1 neurons, this could result in occlusion of synaptic potentiation after a period of chronic stress, according to the principles of the sliding-threshold theory (Abraham and Bear, 1996; Bienenstock et al., 1982). The most likely explanation for the reduced synaptic potentiation after chronic stress is that processes downstream

of the glutamate receptor activation are impaired. This could for example relate to altered Ca2+-homeostasis of CA1 pyramidal cells. Thus, corticosteroids are known to strongly modulate Ca2+-influx through voltage gated Ca2+-channels (Karst, 2000). In GR knock-out mice -which are chronically exposed to very high corticosteroid levels due to dysfunctional negative feedback- Ca2+-currents of CA1 pyramidal neurons were markedly enhanced (Hesen et al., 1996). Ca2+-influx through voltage-gated Ca2+-channels is known to play a role in synaptic potentiation (Grover and Teyler, 1990; Magee and Johnston, 1997) but could also interfere with NMDA receptor properties (Li et al., 2002). Also, changes at the level of second messenger systems could interfere with the efficacy to induce synaptic potentiation. These changes are not necessarily linked to rises in corticosteroid level during chronic stress exposure, but could also depend on alterations in the central CRH system (Blank et al., 2002). In depth investigation of this possibility is necessary in future studies.

While GR activation by 100 nM corticosterone in vitro reduced synaptic potentiation in the CA1 area of control slices -in agreement with earlier findings (Alfarez et al., 2002)-, administration of the same dose of corticosterone had no apparent effect in tissue from chronically stressed rats. This may have been caused by a reduced GR capacity or function. The former explanation seems unlikely, since data about GR binding and mRNA expression from others (Herman and Spencer, 1998; Paskitti et al., 2000) as well tissue from the animals presently investigated (E. van Riel & M.Joëls; unpublished observation) show little change after chronic stress. Moreover, GR functionality after chronic stress seems to be enhanced instead of reduced, due to prolonged DNA-interactions of GRs (Kitchener et al., 2004). It seems most likely that no additional effect of GR activation on synaptic potentiation could be seen, on top of the strong impairment already induced by prior chronic stress exposure. If so, this would largely hamper the potential of CA1 neurons to respond to novel stressors in animals that earlier experienced a prolonged period of stress. A similar lack of effect of in vitro administered corticosterone was observed in the dentate gyrus, but here the effects in control animals already did not reach significance. This underlines that GR-dependent effects on synaptic transmission in dentate gyrus may differ from those in the CA1 area, in line with earlier observations (Stienstra and Joëls, 2000).

One of the current views about memory formation is that neurons are activated in temporal patterns, thereby changing the strength of synaptic connections (Chrobak et al., 2000). Based on our findings that prior exposure to prolonged stress largely hampers the potential to strengthen synaptic connections under basal rest conditions and thereby precludes additional effects of acute GR activation in the CA1 area, one would predict that exposure to chronic stress hampers the acquisition of novel, hippocampus-dependent information. Chronic stress has indeed profound effects on cognitive performance: chronic stress was found to inhibit the acquisition of novel spatial, hippocampus-dependent information (Krugers, 1997; Luine et al., 1996). Our current results may therefore provide a cellular substrate for the cognitive impairment in hippocampus-dependent learning tasks as seen after prolonged exposure to aversive events.

CHAPTER 4

Corticosterone rapidly induces dendritic atrophy of hippocampal CA1 neurons in organotypic slices

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To be submitted

Abstract

Numerous studies have shown that chronic exposure to high levels of corticosterone can affect dendritic morphology of hippocampal cells. Less information is available about the effects of brief corticosterone administration. To examine this, we studied the effect of corticosterone on CA1 pyramidal neuronal morphology in hippocampal organotypic slice cultures. Two doses of corticosterone (30 and 100 nM) were tested, which both activate the low affinity glucocorticoid receptor. Dendritic morphology and spine density of CA1 neurons was established by imaging neurons filled with the fluorescent dye Alexa. Application of 100 nM corticosterone for 20 minutes induced atrophy of the apical dendritic tree. Fractal analysis showed that total neuronal complexity was reduced two-fold when compared to vehicle treated neurons. Exposing organotypic slices to 30 nM corticosterone affected apical length in a time dependent way: neurons filled more than 2 hours after exposure to corticosterone showed atrophy of the apical dendritic tree. Blocking the glucocorticoid receptor by treating the slices prior the corticosterone treatment with RU486 prevented dendritic atrophy caused by both corticosterone concentrations. In addition to the effects on dendritic morphology, we examined spine density after corticosterone treatment. Neither 30 nor 100 nM of corticosterone affected spine density significantly. Our results suggest that high levels of corticosterone, via activation of the glucocorticoid receptor, induce dendritic atrophy. These findings may at least in part explain the suppressive effects of corticosterone on hippocampal synaptic plasticity.

Introduction

Glucocorticoid hormones which are released from the adrenals in a circadian rhythm and after exposure to stressful events have profound effects on hippocampal synaptic plasticity as well as on hippocampal dependent learning and memory processes. In general, there is considerable evidence that the release of corticosteroid hormones during a (moderate) stressful learning task facilitates the consolidation of the memory for that event (Akirav et al., 2001; Cordero et al., 1998; Diamond et al., 1992; Sandi et al., 1997), while suppressing learning processes that take place some time after the stress-related rise in corticosteroid levels (Kirschbaum et al., 1996; McGaugh and Roozendaal, 2002; Roozendaal et al., 1996; Wolf, 2003). Accordingly, when synaptic plasticity is studied, stress and elevated corticosteroid hormone levels have been reported to facilitate hippocampal synaptic potentiation when given around induction of potentiation (Korz and Frey, 2003), but to impair synaptic plasticity induced by high frequency stimulation one to four hours after application of corticosterone (Alfarez et al., 2002; Kim and Diamond, 2002; Pavlides et al., 1995a).

In the brain, corticosteroid hormones act via the high affinity mineralocorticoid receptor (MR) and the lower affinity glucocorticoid receptor (GR), which both are abundantly present in the adult hippocampal formation (de Kloet et al., 2005; McEwen et al., 1968; Reul and de Kloet, 1985). Upon binding to corticosterone, these receptors influence brain function by modulating gene transcription, either as homodimers or as monomers via protein-protein interactions with other transcription factors (Beato et al., 1995; Reichardt et al., 1998). Both MRs and GRs have been implicated in the effects of corticosterone on learning and memory processes and synaptic plasticity (de Kloet et al., 1999; Joëls, 2001).

An important question that remains to be addressed is exactly how exposure to stressful events and elevated corticosteroid hormone levels affect hippocampal synaptic plasticity. Remodelling of synaptic networks through an activity-dependent formation or elimination of synaptic connections is believed to contribute to synaptic plasticity, information processing as well as long-term memory (Geinisman, 2000; Jourdain et al., 2003; Muller et al., 2002; Nikonenko et al., 2002). Prolonged exposure to stress and elevated corticosteroid hormone levels have been reported to cause dendritic atrophy of hippocampal CA3 pyramidal cells (Magarinos et al., 1996; Sousa et al., 2000), along with suppressed learning performance (Sousa et al., 2000). In contrast, it has recently been demonstrated that exposure to two days of social defeat increases rather than decreases dendritic arborization of CA3 neurons with a concomitant impair-

ment of LTP even when observed with a 21 days delay after the stressful event (Kole et al., 2004). Taken together these data indicate that hippocampal CA3 neurons undergo structural plasticity after stress and corticosterone exposure, related to changes in learning and synaptic plasticity.

Surprisingly, far less is known about how exposure to stressful events and elevated corticosteroid hormone levels affect hippocampal CA1 dendritic structure. This is remarkable since the hippocampal CA1 area is closely involved in learning and memory processes (Remondes and Schuman, 2004; Riedel and Micheau, 2001). Both lesioning studies and studies in transgenic mice with genetic disruptions in the hippocampal CA1 area point to an important role of this region in acquisition and consolidation of long-term spatial memory (Giese et al., 1998; Remondes and Schuman, 2004; Winder et al., 1998). Only few studies have reported that exposure to stressful events alters hippocampal CA1 structural properties. One study reported that exposure to stress increases spine density in male, but reduces spine density in female rats after brief exposure to stress when examined 24 hours after the stressor. No effect was observed immediately after the stressor (Shors et al., 2001). In contrast, evidence for a putatively more rapid action of corticosterone on dendritic spines comes from a study using acute hippocampal slices exposed to dexamethasone, a GR agonist. In this study spinogenesis was observed in CA1 pyramidal neurons within one hour (Komatsuzaki et al., 2005). Taken together, these studies point out that elevated corticosteroid hormone levels may have differential and dynamic effects over time on hippocampal structure.

In the present study we addressed therefore the question whether a brief exposure to elevated corticosteroid hormone levels alters hippocampal CA1 neuronal morphology and dendritic spine density. For this purpose we used a viable and stable preparation, i.e. hippocampal organotypic slice cultures. First, we used immunocytochemistry to determine the presence of the MR and GR in this preparation. Next, we examined the effect of corticosterone at concentrations which activate the glucocorticoid receptor, i.e. as found after exposure to a stressful event, on dendritic morphology and spine density over time. Finally, we examined whether the effects of corticosterone on dendritic structure were mediated via the low affinity glucocorticoid receptor.

Materials and Methods

Preparation of organotypic slices

All work was carried out in approval of the UK Home Office regulations and local committee on Animal Bioethics of the University of Amsterdam. Organotypic slices were prepared using the method of Stoppini et al. (1991). In brief, under sterile conditions in a flow cabinet, 300 ?m thick parasagittal slices were prepared from 5-day-old male Sprague-Dawley rat pups. To avoid excess sprouting of granule cell axons in the dentate gyrus a portion of the entorhinal cortex was left attached to the hippocampal slice (De Simoni et al., 2003). Each slice was immediately placed on confetti at the bottom of a Millicell culture plate (Millipore) such that each of six chambers of the plate contained three slices. The slices were kept at the interface of a serum culture medium: 25% horse serum, 50% minimal essential medium, 23% Earle´s balanced salt solution (all from Gibco BRL), 5000 u/100 ml penicillin, 1200 u/100 ml nystatin (both from Sigma-Aldrich). The medium was changed three times per week. When required at 14-21 days in vitro (DIV, DIV14-21); individual slices were removed from the incubator. For the GR staining, organotypic slices were exposed to 100 nM corticosterone for 20 min which is known to induce accumulation of the GR into the nucleus (Nishi et al., 2001).

Immunocytochemistry

To examine the presence and distribution of MRs and GRs in the organotypic slices at DIV 14-21 immunocytochemistry was used and compared with the distribution of MRs and GRs in the hippocampus of male Sprague Dawley rats at postnatal day (PND) 5, PND 19 and at adult age (± 6 weeks old). Organotypic slices DIV 14-21 were fixed overnight with 4% paraformaldehyde in 0.1 M phosphate

buffer (PFA-PB, pH 7.4) by immersion fixation. Whole brains from PND 5, PND 19 and adult rats were quickly removed and immersion-fixed at 4 °C for 24–72 h in 4% PFA-PB. The material was cryoprotected with a 30% sucrose solution in PB overnight at 4 °C. Coronal sections at 40 µm thickness were obtained using a sliding microtome. Sections and organotypic slices were stored at 4 °C in PB, pH 7.4, containing 0.01% sodium-azide until needed.

Glucocorticoid receptor staining

After removal of endogenous peroxidase activity (coronal sections 1% H2O2 in PB; organotypic slices 0.5 % H2O2 in PB for 15 minutes), tissue was pre-incubated in 2% normal goat serum in 0.12 M phosphate buffer saline containing 0.3% Triton (PBS-Tx) for 1 hour (h) at room temperature (RT). The tissue was then left in primary antibody rabbit anti-GR serum (Morimoto et al., 1996), diluted in PBS-Tx (1:5000) for 42 h at 4°C. After rinsing in PBS (6x 5 minutes), sections were incubated with biotinylated goat anti-rabbit IgG (1:200) for 2 h and avidin–biotin–peroxidase (ABC 1:1000) in 0.5% bovine serum albumin (BSA) and 0.01% PBS-Tx for 2 h. A 3,3'diamino-benzidine (DAB) solution, reinforced with 0.02% Nickel, was used to visualize the antibody (0.50 mg/ml DAB/0.01% H2O2). Next, sections were mounted, dehydrated, passed through xylene, and coverslipped with Entellan (Merck).

Mineralocorticoid receptor staining

Mineralocorticoid positive nuclei were identified with a protocol similar to the glucocorticoid receptor staining. Endogenous peroxidase activity was removed in sections and organotypic slices similar to the GR protocol (see above). Tissue was incubated with anti-MR primary antibody (MRN 1D5, generous gift from C.G.Gomez-Sanchez; University of Mississippi Medical Centre, USA; (Gomez-Sanchez et al., 2005) diluted in 0.5 M Tris buffer (TB) (1:500) for 48 hr at 4 °C. After rinsing in 0.5 M Tris-buffered saline (TBS, 6x 5min), sections were incubated with biotinylated sheep anti-mouse IgG (1:200) in 0.3% TBS-Tx for 2 h and ABC (1:800) TBS-Tx for 2 h. A 3,3'diamino-benzidine (DAB) solution was used to visualize the antibody (0.50 mg/ml DAB/0.01% H2O2). Next, sections were mounted, dehydrated, passed through xylene, and coverslipped with Entellan (Merck).

Corticosterone and RU486 treatment

On the day of the experiment an organotypic slice was taken from the incubator and transferred to a recording chamber containing ACSF at 32 °C (in mM: NaCl 125, KCL 2.4, NaHCO3 26, NaH2PO4 1.25, glucose 25, CaCl2 2, MgCl2 1, oxygenated with 95% O2-5% CO2). In order to establish the effects of a brief exposure to corticosterone, slices were treated for 20 min at 32°C with vehicle or two different doses of corticosterone (Sigma-Aldrich, Steinhoven, Germany, 30 and 100 nM in ≤ 0.01% ethanol). Previous studies have shown that these dosages activate MR and GR, elicit alterations in synaptic plasticity and modulate cellular properties that require homodimerization of the GR (Alfarez et al., 2003; Alfarez et al., 2002; Karst, 2000; Stienstra and Joëls, 2000). In order to verify the role of the GR, half of the slices were incubated in RU486 (mifepristone, Sigma-Aldrich, 500 nM) for 10 minutes before and during exposure to corticosterone. At least 1 h after the treatment with corticosterone or RU486, slices were transferred to a standard recording chamber and perfused with oxygenated recording ACSF (2.3 mL/min) at RT.

Imaging

At different time intervals after incubation with corticosterone individual neurons were filled passively using the whole-cell patch-clamp technique (De Simoni et al., 2003). Intracellular solution contained (mM): CsCl 140, Hepes 5, EGTA 10, Mg-ATP 2; with pH adjusted to 7.4 with CsOH. Alexa Red (Alexa Fluor 568, Molecular Probes) 0.2 mg/ml was included in the intracellular solution for morphological

analysis. After a neuron was filled, the electrode was carefully removed and the slice was fixed in 4 % paraformaldehyde or imaged straight away for subsequent analysis. Dendritic morphology and spine density of neurons in the organotypic slices was evaluated using an Olympus Fluoview confocal microscope (generously supplied by Olympus, London, UK) with a Omnichrome series 43 KrAr laser on an upright Olympus BX50W1 with the following Olympus objectives: 20? water immersion, numerical aperture (NA) 0.5, 60 x water immersion, NA 0.9, or 60 ? oil immersion, NA 1.4.

Neuronal reconstruction and morphometric analysis

The morphological analysis was performed using Image J (National Institute of Health, USA, http://rsb.info.nih.gov/ij/) in combination with the Neuron _Morpho plugin (Giampaolo D'Alessandro, University of Southampton, UK) and LMeasure (Ruggero Scorcioni, George Mason University, Krasnow Institute, USA). The overall dendritic morphology of CA1 neurons was analysed using a low magnification objective on the confocal microscope, acquiring an image every 1.5 or 2 ?m step in the z-direction. Using Image J software together with the macro Neuromorpho and the program LMeasure three parameters of the basal or apical tree were measured: branch points, width and total dendritic length. The branch point indicates a node where the dendrite splits into two. Width of the neuron is calculated after orienting the dendritic tree along the primary axis. Length is calculated as the total length of all dendritic segments. LMeasure was also used to make Sholl plots (Sholl, 1953) reflecting the complexity and exact differences between cells.

The fractal dimension of the neurons was calculated using Flok´s border-dilatation method (Jelinek and Fernandez, 1998) and NIH Image software. For this analysis, neurons were projected at the same scale, converted to binary files and skeletonized so that throughout the profile of the neuron all processes were one pixel in diameter. Low intensity pixels representing background signal were manually removed. The calculation of the fractal dimension has been validated for assessment of the morphology of neurons (De Simoni et al., 2003) but also see: (Cannon et al., 1999).

Spine analysis

The spine density was calculated in images from high-resolution confocal scans (60 x water objective) using the method of DeSimoni et al. (2003). Briefly, spines were counted double-blind in each frame at high (2.5-3) zoom and the number was divided by the length of the dendrite within the frame. Images were taken at least 20 µm from the soma, excluding the primary apical tree which is shown to have different spine densities compared to higher order dendrites (De Simoni et al., 2003). In each section of dendrite chosen, all the spines present were included in the analysis. The basal and apical department of the neuron was subdivided according to the order of dendrite considered. Of each cell 5-14 compartments were recorded and analyzed for spine density. In total 1863 µm of dendrite was recorded (ranging between 100 – 390 µm dendrite per neuron) and analyzed.

Statistical analysis

All data were analyzed using the statistics package SPSS 11.0 (SPSS for Windows). Results are expressed as means ± S.E.M. The numbers quoted throughout refer to the number of cells analyzed. Values for the total dendritic length and Sholl plot data were compared using ANOVA and Student's t-test. To evaluate spine density, ANOVA with post hoc analysis was applied to evaluate group differences. P values < 0.05 were considered significantly different.

Results

Immunocytochemistry

First, specificity of the anti-MR antibody was tested in adult brain-specific MR knockout mice (MR -/-;

MRCamKIICre) and control mice (MR+/+; MRloxP/loxP) both generously provided by Stefan Berger and Günther Schütz; German Cancer Research Institute, Heidelberg, Germany (Karst et al. 2005 in press). Figure 1 shows MR-positive cells in the CA1 pyramidal area of the hippocampus in the MR+/+ brain (figure 1A) while no MR reactivity is observed in the CA1 area of MR-/- mice (B) indicating that the presently applied antibody is highly specific for MRs. At PND 5 (similar age as used to prepare the organotypic slice cultures), few MR positive cells were present in the hippocampal CA1 area (figure 1C). However, at both PND 19 (the age comparable to developmental stage DIV 14-19 of the organotypic cultures) and in adult tissue most CA1 pyramidal cells were found to be immunopositive for MR (figure 1E, G).

The antibody that was currently applied to detect GR positive cells has previously been shown to be specific for the glucocorticoid receptor (Morimoto et al., 1996). GR immunoreactive cells were present in hippocampal CA1 pyramidal cells at all ages tested (figure 1D, F, H).

Neuronal structure of the organotypic cultures was assessed using the Nissl method (figure 2 A, B). In organotypic slices MR positive cells were present in different hippocampal regions with positive nuclei in the CA1 pyramidal cell layer (figure 2C, D). GR positive cells were also abundantly present in the organotypic slice culture and the CA1 area (figure 2E, F). Organotypic slice cultures are surrounded by a glial cover during development in vitro (del Rio et al., 1991). GR-postive glial cells can be responsible for the dense staining. For all experiments, in absence of the primary antibody no positive immunoreactivity was observed.

Taken together, these studies indicate that both MRs and GRs are present in hippocampal CA1 pyramidal cells in organotypic cultures (DIV 14-21), at the age of the slices when the effects of corticosterone on dendritic morphology were tested.

Figure 1

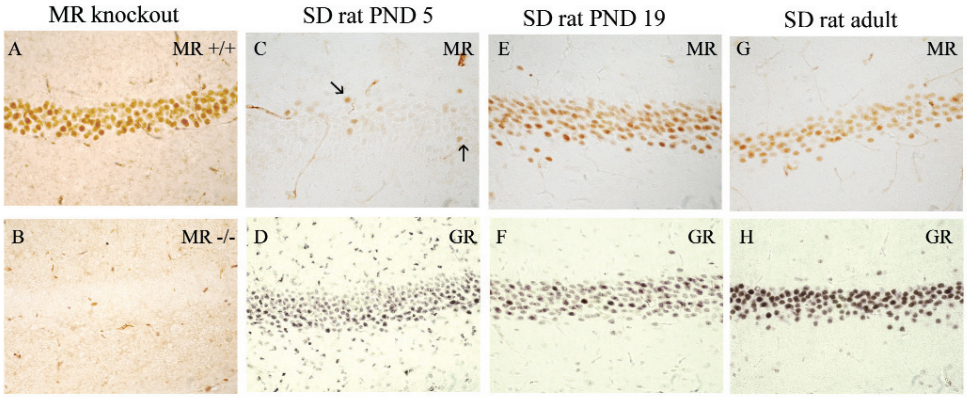

Figure 2

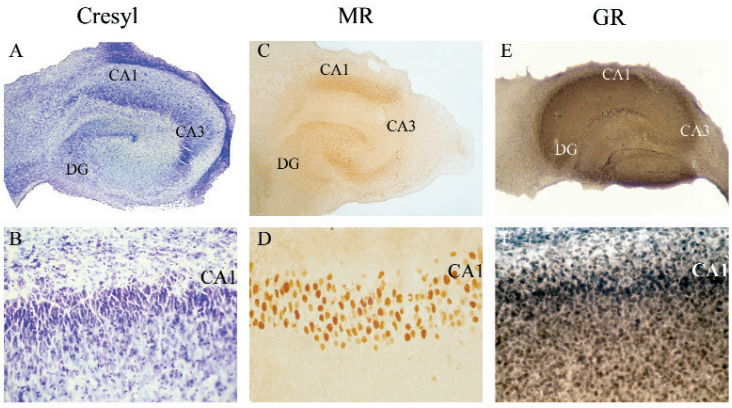

Figure 1

To show the specificity of the antibody (MRN 1D5) it was tested on MR+/+ and MR-/- adult mouse tissue. Hippocampal CA1 area is shown. A) MR immunoreactive cells are found in CA1 pyramidal layer of MR +/+ mice. B) By contrast, no MR immunoreactive cells are present in the CA1 area of MR-/- mice, indicating the specificity of the presently applied antibody. Representative examples of immunocytochemistry for MR (A, E, G) and GR (D, F, H) reactivity in CA1 pyramidal cells in PND 5 (C, D), PND 19 (E, F) and adult (G, H) Sprague-Dawley (SD) rat. In the CA1 area in PND 5 animals there is a low amount of MR positive cells in the CA1 area (C). At PND 19, the amount of MR immunoreactive cells is comparable to that present in adult animals (E, G). All three ages show comparable amount of GR positive cells in the CA1 area (D, F, H).

Figure 2

Organotypic hippocampal slice cultures were used for immunocytochemistry after 14 days in culture. A) Cresyl violet stained sections illustrate clearly defined neuronal regions CA1, CA3 and dentate gyrus. B) Higher magnification of the CA1 pyramidal layer showing pyramidal cells C) MR immunolabeling is present in all hippocampal regions D) Higher magnification showing strongly positive cells in the CA1 pyramidal region E) GR immunolabeling is present in all hippocampal regions except for the CA3 area. F) Higher magnification of CA1 area shows positive pyramidal nuclei with surrounding positive glia. CA1: CA1 layer, CA3: CA3 layer, DG: dentate gyrus.

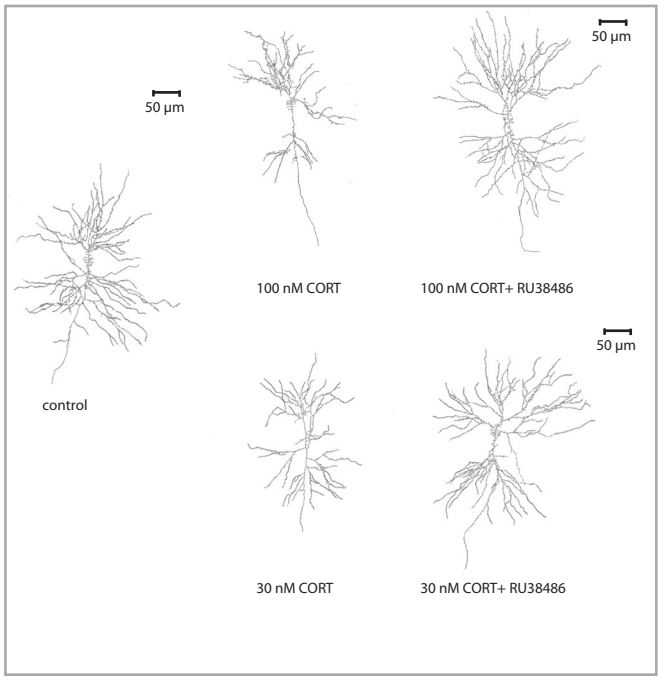

Figure 3 Projection of cells after treaatment

Exposure of organotypic slices to 30 and 100 nM corticosterone affects CA1 neuronal dendrite structures. Shown are representative confocal microscopy projections from z-stack images of the different treatments: vehicle, 30 nM corticosterone > 120 min. after incubation, 100 nM corticosterone, RU486+30 nM corticosterone and RU486+100 nM corticosterone. Note the reduction in dendritic length on the apical side in the 30 and 100 nM corticosterone group. For further numerical details see Table 1.

Table 1. Morphological analysis of the dendritic trees of CA1 hippocampal neurons

	Basal dendrites			Apical dendrites		
	number branch points	width (µm)	length (µm)	number branch points	width (µm)	length (µm)
Vehicle	17.0 ± 2.2	261.1 ± 23.7	1733 ± 240	19.2 ± 1.6	234.7 ± 17.8	1937 ± 201
100 nM CORT	18.2 ± 3.3	270.7 ± 6.0	1507 ± 96	13.2 ± 2.2	242.5 ± 7.4	998 ± 131
30 nM CORT	21.6 ± 2.9	307.7 ± 10.8	1890 ± 207	21.8 ± 3.9	251.7 ± 19.2	1917 ± 266
100 nM CORT+RU486	18.5 ± 1.5	287.2 ± 12.6	1947 ± 405	22.6 ± 2.7	256.9 ± 13.9	1908 ± 385
30 nM CORT+RU486	23.5 ± 1.9	257.4 ± 21.1	2081 ± 245	19.8 ± 2.4	245.3 ± 17.1	1804 ± 261
RU486	19.7 ± 1.9	302.8 ± 7.9	1700 ± 244	26.4 ± 6.4	228.7 ± 31.8	3103 ± 253

Values represent the mean ± S.E.M. Treatment of slices with 100 nM corticosterone induces strong atrophy of the apical tree (** P = 0.01) with a concomitant decrease in number of bifurcations (* P = 0.05). Incubating slices for 10 minutes in RU486 induced apical hypertrophy († P = 0.004). No differences were found for parameters of the basal dendritic tree. Vehicle: n = 8; 100 nM CORT: n = 4; 30 nM CORT: n = 8; 100 nM CORT + RU486: n = 5; 100 nM CORT + RU486: n = 6; RU486: n = 5.

CA1 neuron morphology

For an overall view of the hippocampal cells, projections were made of low resolution scans (20 x water immersion objective). Figure 3 shows typical examples of pyramidal neurons receiving different treatments. Results of the morphological analysis are shown in Table 1. While no effects of corticosterone treatment were found on the total length, width and number of branch points of basal dendrites, the apical dendritic tree showed remarkable alterations upon exposure to corticosterone. Exposure of slices to 100 nM corticosterone induced a significant decrease in the number of nodes in the apical dendritic tree ($P < 0.05$) and reduced the total length of apical tree segments when compared to the vehicle group ($P < 0.05$). Treatment with RU486 prevented the effect of corticosterone on dendritic morphology (Fig 4A, Table 1).

Next, we compared total apical dendritic length of vehicle and 30 nM corticosterone treated slices. Exposure of slices to 30 nM corticosterone did not change apical dendritic length when compared to vehicle treated slices (P=0.9, data table 1). However, plotting the time-delay of filling a neuron after corticosterone treatment against the apical length, we found a time-dependent decrease in apical dendritic length: with increasing time after application of corticosterone, dendritic structures reduced significantly (Figure 4B; regression analyses F=0.006). Importantly, slices treated with vehicle or RU486+30 nM corticosterone showed no significant regression (data not shown). This indicates that 30 nM corticosterone also induces hippocampal CA1 neuronal dendritic atrophy but with a much longer delay. To emphasize the time-dependent effect of corticosterone on dendritic length, we divided the 30 nM corti-

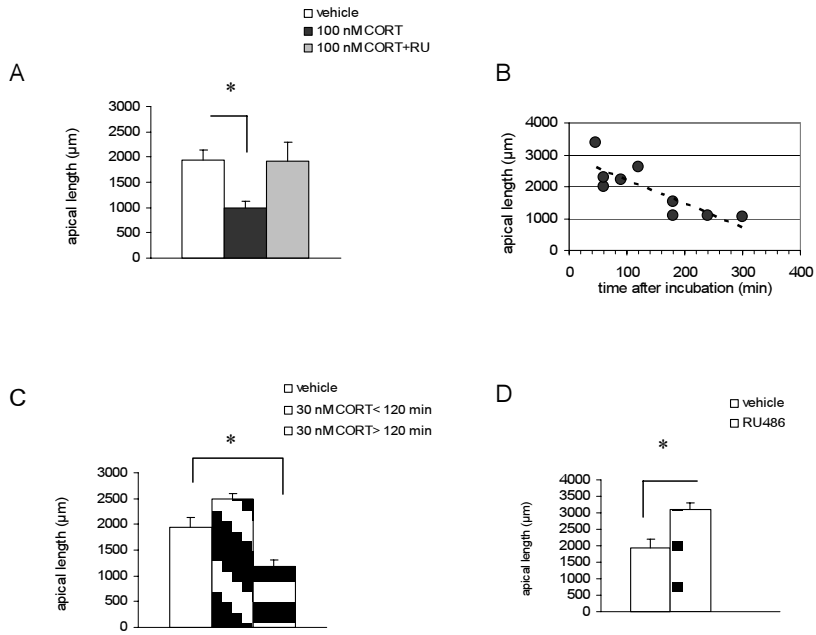

Figure 4

A) Apical dendritic length in 100 nM CORT treated slices differs significantly from the vehicle-treated group (unpaired t-test p=0.01); RU468 + 100 nM CORT treatment restored the average length to the level of the vehicle control group. B) 30 nM CORT treated slices showed a time-dependent effect. Slices imaged > 120 minutes after incubation showed shorter dendrites (unpaired t-test p<0.05). C) regression analysis showed that the length of apical dendrites decreases significantly over time (F=0.05). Regression was not significant for the vehicle group (data not shown, F=0.5). D) The length of apical dendrites is significantly increased in RU486 treated slices (p<0.01)

costerone group in two groups: neurons filled within 120 min. after corticosterone exposure and neurons filled longer than 120 minutes after exposure. Accordingly, total apical dendritic length was significantly reduced at > 2 hours after the corticosterone treatment when compared to the vehicle group ($P < 0.05$; Figure 4C). Finally, slices treated with RU486 without corticosterone show a significant increase in apical length when compared to vehicle treated slices ($P < 0.01$, Figure 4D).

Sholl plot and fractal analysis of the dendritic tree

In order to evaluate the distribution of dendritic processes across the various sublayers of the CA1 region, we created Sholl plots (Sholl, 1953). Summed dendritic length is expressed as a function of distance from the soma in radial bins of 36 µm (Figure 5A, B). Apical atrophy in neurons exposed to 100 nM corticosterone was restricted to dendrites at ~100 and 200 µm distance from the soma (vehicle n = 7; 100 nM corticosterone n = 4; ring 107 $P < 0.05$; ring 143 and 179 $P < 0.01$; MANOVA; $P = 0.001$ Figure 5A). Pretreatment with RU486 clearly blocked the corticosterone-induced atrophy, no differences in Sholl plot values were seen when compared with the vehicle group. Comparing the RU486 group with the 100 nM corticosterone group shows significantly increased apical length proximal to the soma in the RU486 group (100 nM corticosterone + RU486; n = 5; ring 36 and 71 $P < 0.05$; Figure 5A). Sholl plot analysis from cells filled more than 2 hours after exposure to 30 nM corticosterone showed a significant decrease of dendritic length compared to vehicle (30 nM corticosterone > 120 min. n = 4; ring 143 and 180 $P < 0.01$; Figure 5B). No changes were observed in the other groups.

In order to compare the complexity of dendrites in the experimental groups, we used fractal analysis, a technique that has been used to measure the complexity of pyramidal neurons (De Simoni et al., 2003). The average fractal dimension (D) of CA1 pyramidal neurons in all groups is shown in Figure 5C. There was a significant difference between the fractal dimension of the vehicle group and 100 nM corticosterone group group ($P < 0.05$) which indicates that the complexity of the dendritic tree was reduced more than two-fold by corticosterone.

Spine density

To study the effects of corticosterone on excitatory synaptic connections in more detail we analyzed spine density after the different treatments. Data from higher order dendritic branches was pooled since previous research in the same substrate showed that there is no influence of branch order upon spine density (De Simoni et al., 2003). The results are shown in table 2. No significant effect of corticosterone treatment was found on spine density of the basal or apical dendritic tree.

Table 2. Spine density basal and apical dendritic tree CA1 pyramidal neurons

	spines µm-1 basal tree	spines µm-1 apical tree
Vehicle	0.60 ± 0.03	0.64 ± 0.06
30 nM CORT	0.67 ± 0.03	0.77 ± 0.09
100 nM CORT	0.65 ± 0.06	0.49 ± 0.07
30 nM CORT + RU486	0.69 ± 0.03	0.72 ± 0.02
100 nM CORT+RU486	0.59 ± 0.04	0.68 ± 0.07
RU486	0.64 ± 0.05	0.60 ± 0.06

Values represent the mean ± S.E.M. There was no effect of corticosterone treatment when basal and apical dendritic spine density was tested separately. Vehicle: n = 6; 100 nM CORT: n = 3; 30 nM CORT: n = 4; 100 nM CORT + RU486: n = 4; 100 nM CORT + RU486: n = 5; RU486: n = 5.

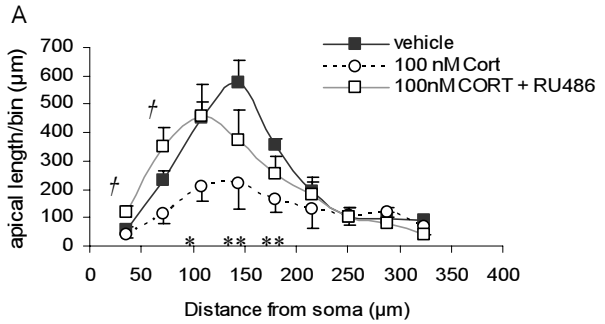

Figure 5

*Sholl analysis was used to evaluate the distance-dependent distribution of the apical dendrites. Dendritic length was summed per radii at 36 µm distance, with the middle of the soma set at zero. A) Comparing vehicle with 100 nM CORT shows that there is a reduction in dendritic length in the stratum radiatum for rings at 107, 143 and 180 µm distance from soma (unpaired t-test * P < 0.05 ** P < 0.01). Comparing the RU486+100 nM CORT group with 100 nM CORT shows a significant difference in dendritic length at 36 and 71 µm from the soma (unpaired t-test † P < 0.05). B) Comparing vehicle with 30 nM CORT > 120 min shows that there is a reduction in dendritic length for the rings at 143 and 180 µm distance from the soma (unpaired t-test * P < 0.01). C) Fractal dimension D of CA1 neurons in the different experimental groups. There is a significant reduction in dendritic complexity between vehicle and 100 nM corticosterone treated groups (unpaired t-test * P < 0.05).*

Discussion

Elevated corticosteroid hormone levels have been reported to exert structural effects on hippocampal neuronal morphology. In particular hippocampal CA3 pyramidal cells show dynamic changes: 2 days of mild stress enhanced dendritic arborization of hippocampal CA3 pyramidal cells when examined 21 days later (Kole et al., 2004) while prolonged exposure to stress reduced dendritic complexity of hippocampal CA3 pyramidal cells (Magarinos et al., 1997; McEwen and Magarinos, 1997; McKittrick et al., 2000; Sousa et al., 2000). We here show that the effects of corticosteroid hormones on cell morphology are not exclusive for the CA3 area. We report that a brief exposure of organotypic cultures to corticosteroid hormones, at dosages enough to activate glucocorticoid receptors in addition to mineralocorticoid receptors, causes a rapid atrophy of apical dendrites of hippocampal CA1 pyramidal neurons while leaving basal dendrites unaffected. Our study shows that 100 nM (high concentration) corticosterone induces loss of apical dendritic length and dramatic changes in morphology; exposure to 30 nM corticosterone (moderately high concentration) reduces apical dendritic length in a time dependent way. Electrophysiological studies show that another consequence of GR activation after exposure of rats to acute stress is a decrease in capacitance of CA1 neurons time-dependently after the stressor (Joëls et al., 2003). Importantly, these changes were accompanied by increased L-type calcium current amplitude and a decrease in the T-type currents in a similar time dependent way. The decrease in capacitance and the specific decrease of T-type currents which are located on distal dendrites could point towards atrophy of the distal dendrites (Christie et al., 1995).

The only in-depth study on the effects of stress and corticosterone on hippocampal CA1 dendritic morphology comes from Sousa et al. (Sousa et al., 2000). These authors reported significant reduction in terminal segment length after 21 days of chronic unpredictable stress and corticosterone administration, but no significant reduction in total apical dendritic length. Although we examined effects of corticosterone in a different substrate (i.e. organotypic slices), we presently report that short exposure to corticosterone can induce atrophy of apical dendrites in a more rapid way. The changes in dendritic morphology were not accompanied by spinogenesis in either the apical or basal dendrites. Others have observed spinogenesis after subjecting male rats to a brief stressor or exposing hippocampal brain slices to dexamethasone (Komatsuzaki et al., 2005; Shors et al., 2001). The reported spinogenesis after dexamethasone application was limited to an increase in thin and mushroom spines and occurred within one hour after dexamethasone application, which is faster than the time-scale that we studied in the present study. Spine shape has been related to functional aspects of spines, and thin spines are thought to fulfill some of the criteria for silent synapses (Geinisman, 2000; Kasai et al., 2003; Segal, 2005). Possibly, the rapid modulation of spine shape could be related to rapid changes observed in synaptic plasticity after stress.

CA1 pyramidal cells in early postnatal tissue and organotypic slice cultures of a comparable developmental age were presently shown to express both mineralocorticoid receptors and glucocorticoid receptors. MR expression is low in CA1 pyramidal neurons at PND5 when compared to tissue later in development (PND 19 and adult). For the GR we replicated the finding that GRs are present at early postnatal stages, with progressively stronger immunoreactivity towards adulthood (van Eekelen et al., 1991a). The organotypic slice cultures from DIV 14-21 express both MR and GR in the CA1 pyramidal cell layer. An important question that has to be addressed is the role of the GR in the effects of corticosterone on dendritic remodelling. Studies using similar dosages of corticosterone as well as the period of incubation that were used at present have been reported to alter hippocampal CA1 cellular properties via genomic actions (Karst et al., 2000). Although it has to be established whether the presently observed structural effects involve transcriptional regulation after GR activation, the time interval that is required to cause these effects fits within such a putative genomic action. Evidence points towards a role for the GR in the presently observed structural effects of corticosterone. First, the dosages of corticosterone that were used at present activate GRs in addition to MRs. Moreover, co incubation with the GR antagonist RU486 pre-

vented the effects of corticosterone on dendritic morphology, although it should be noted that RU486 itself already induced effects on dendritic morphology.

Several studies suggest that MR/GR balance (i.e. ratio of MR versus GR) is important for cellular function and viability. For example: activation of the MR maintains neuronal activity and integrity while additional GR activation suppresses neuronal activation (Diamond et al., 1992; Joëls et al., 2002; Krugers et al., 1994). Furthermore, a reduction in MR/GR balance have been postulated in depressive disorders (Johren et al., 1994; Meyer et al., 2001) and aging (Peiffer et al., 1991; van Eekelen et al., 1992). The shift in MR/GR balance due to the abundant GR positive immunoreactivity (increased level of GR, presumably in glial cells) observed in the organotypic slices can be an important factor for the strong effects upon dendritic morphology currently presented. It remains to be investigated whether the reported change in balance between MR and GR activity in cultures and acute tissue from the same developmental stage would indeed result in a different effect of corticosterone treatments on dendritic morphology.

Taken together, we find at present that the apical dendritic tree of pyramidal neurons in the CA1 area shows atrophy after exposure to elevated corticosteroid hormone levels. The reduction of dendritic elements is not occurring equally across the dendritic tree but affects only the apical part, in a confined spatial domain: between approximately 100 and 180 µm distance from the soma, the area where most excitatory input comes from axons of CA3 neurons (Schaffer collaterals) as well as the associational commissural pathway from the contra-lateral side. Since the dendritic tree and dendritic spines are the postsynaptic sites of excitatory input in the mammalian brain one may predict that these structural alterations are accompanied by altered network function. Indeed we have demonstrated before that, in acute slices rather than organotypic slices, a brief exposure to corticosterone, at dosages that were used at present, hampers synaptic plasticity (Alfarez et al., 2003; Alfarez et al., 2002; Wiegert et al., 2005). These results stress that the different hippocampal subfields are subject to substantial structural plasticity. It is tempting to speculate that these structural alterations are relevant for network function, although this remains to be proven.

CHAPTER 5

Increased hippocampal CA1 dendritic arborization after chronic stress is reversed by glucocorticoid receptor activation

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To be submitted

Abstract

Exposure to double social defeat and chronic stress has been reported to cause atrophy of hippocampal CA3 pyramidal neurons. In the present study we examined the effects of chronic stress and acute elevations of corticosteroid hormone levels on neuronal morphology of hippocampal neurons in the CA1 area, a region that is critically involved in learning and memory processes. To assess this, rats were exposed to a regime of 21 days of unpredictable stress. Handled animals served as controls. The day after the last stressor or control treatment, individual CA1 pyramidal neurons in hippocampal slices of these animals were filled with a fluorescent dye and reconstructed using confocal microscopy. We examined the morphological changes both with elevated and basal corticosteroid hormone levels by treating hippocampal slices briefly with high levels of corticosterone (100 nM for 20 minutes) or vehicle respectively 1-4 hours before filling of the cells. We found that exposure to 21 days of unpredictable stress increases apical dendritic length of CA1 pyramidal cells. Moreover, brief exposure of slices from control animals to elevated corticosterone levels resulted in hypertrophy of apical CA1 dendrites that was comparable to the hypertrophy in stressed animals. In contrast, a similar treatment of hippocampal slices from chronically stressed animals reversed the hypertrophy that was observed under basal corticosterone levels. These morphological alterations were confined to the apical dendrites and absent in basal dendrites. In vivo administration of the glucocorticoid receptor antagonist RU486 at days 17-21 of the stress or control treatment prevented the effects of acute corticosterone administration, but not those of exposure to chronic stress. The dynamic structural alterations elicited by corticosterone and chronic unpredictable stress were confined to specific regions of the dendritic tree. We therefore speculate that the morphological alterations result in altered network function and may have functional implications.

Introduction

Exposure to stressful events activates the hypothalamo-pituitary-adrenocortical axis resulting in enhanced corticosteroid hormone levels. These hormones easily cross the blood brain barrier and bind to the high affinity mineralocorticoid receptor (MR) and the lower affinity glucocorticoid receptor (GR), which are abundantly present in the hippocampal formation (McEwen et al., 1968; Reul and de Kloet, 1985). By targeting many genes through these two receptor types, corticosteroid hormones function in a binary fashion, and serve as a master switch in the control of neuronal and network responses that underlie behavioural adaptation upon a stressful event (de Kloet et al., 2005; Diamond et al., 1992; Pavlides et al., 1995b).

However, exposure to prolonged periods of stress and the concomitant prolonged exposure of brain cells to elevated corticosteroid hormone levels may have rather adverse consequences at the cognitive and physiological level, both in experimental animals and humans (Bodnoff et al., 1995b; Conrad et al., 1996; Dachir et al., 1993; Lupien et al., 1998; Seckl and Olsson, 1995). In genetically predisposed humans, exposure to prolonged stress can trigger psychiatric disorders, such as depression (Holsboer, 2000). Particularly strong evidence for a role of the GR in depression comes from studies reporting that short term treatment with the antiglucocorticoid RU486 is effective in reducing psychotic symptoms in depressed patients (Belanoff et al., 2001; Belanoff et al., 2002; Simpson et al., 2005) as well as cognitive deficits (Young et al., 2004).

Imaging techniques have allowed examination of neurobiological correlates of depression. In particular, structural changes in the brain have been reported in patients suffering from depressive illness. Thus, structural brain imaging studies in depressed patients and humans exposed to enhanced HPA-axis activity have reported reduction in hippocampal volume (Bremner et al., 2000; Sheline et al., 2003; Videbech and Ravnkilde, 2004). The volume loss correlated with the duration of the depression (MacQueen et al., 2003; Sheline et al., 1996), with antidepressant medication having a neuroprotective effect on the volume changes (Sheline et al., 2003). The current hypothesis implies a link between patients suffering from

long-term depressive illness and Cushing's syndrome on the one hand and atrophy of the hippocampus and prefrontal cortex on the other hand (Bremner et al., 2000; Sheline et al., 1996; Starkman et al., 1992). These effects are reversible by treating the disorder (Starkman et al., 1999; Vermetten et al., 2003). Detailed studies in experimental animals revealed that prolonged exposure to stress causes reversible dendritic atrophy of hippocampal CA3 pyramidal cells both in rats and primates (Magarinos and McEwen, 1995a; Sapolsky et al., 1990; Sousa et al., 2000). Interestingly, the alterations in dendritic atrophy occur in parallel with impairments in learning and memory performance (Arbel et al., 1994; Fuchs et al., 2001a; McEwen, 2005; Sousa et al., 2000) and are reversed by a recovery period following the stress treatment, which allegedly reduced HPA-axis activity (Sandi et al., 2003; Sousa et al., 2000).

While there is substantial evidence that chronic stress reduces hippocampal CA3 pyramidal cell arborization it is remarkable that there is little known about how prolonged exposure to stress affects the structure of hippocampal CA1 pyramidal cells. This is surprising since both lesioning studies and transgenic mice models reveal that the hippocampal CA1 area is relevant in particular for learning and memory processes (Giese et al., 1998; Otmakhov et al., 2004; Remondes and Schuman, 2004; Silva et al., 1992), which are disturbed in chronically stressed animals as well as in patients suffering from depressive illness.

The aim of the present study was therefore to examine whether exposure to chronic unpredictable stress alters hippocampal CA1 neuronal morphology. To address this question, the fluorescent dye Alexa was injected via the patch clamp method into individual CA1 pyramidal neurons from control and chronically stressed animals, 24 h after the last stressor. In order to mimic acute stress exposure against a background of chronic stress or control treatment, slices from both control and chronically stressed animals were examined under 2 conditions, i.e. (i) basal low corticosteroid levels and (ii) raised corticosteroid levels. Also, in order to assess whether GR blockade could prevent the effects of chronic stress, animals were treated with the GR antagonist RU486 during a 4 day period, similar to a regime reported successfully to suppress symptoms of depression (Belanoff et al., 2001).

Experimental Procedures

Animals

The local committee on Animal Bioethics of the University of Amsterdam approved all experiments. Male Wistar rats (Harlan CPB, The Netherlands), weighing 175-250 g at the start of the stress regime or handling procedure were used. All animals were housed in pairs for at least 7 days before experimental procedures started. Food and water were provided ad libitum, lights were on from 8:00 a.m. until 8:00 p.m. and the temperature and humidity were kept between 20-22 °C and 55 ± 15 % respectively.

Stress paradigm

At the start of the experiment, rats were randomly assigned to the following experimental groups: handled control (HC); chronic unpredictable stress (CUS); handled control treated with RU486 (HC_RU) and chronic unpredictable stress treated with RU486 (CUS_RU). Control rats were removed from their cages, handled briefly and weighed, for 21 days. To assess the effects of chronic stress on dendritic morphology animals were exposed to a 21-day period of chronic unpredictable stress as reported before (Alfarez et al., 2003; Herman et al., 1995). The following stressors were used: (1) Immobilization: rats were placed in plastic immobilization cages for 1h; (2) Cold immobilization: rats were placed in plastic immobilization cages in a cold room (4 °C) for 1h; (3) Vibration: rats in group-cages were placed on top of an orbital shaker and shaken for 1 h at 30 rev/minute; (4) Isolation: rats were moved to individual cages for a overnight period; (5) Crowding: rats were housed 4-6 per cage for a overnight period; (6) Swim: rats were placed in a plastic container filled with water (25-30 °C) for 30 minutes; (7) Cold-water swim: rats were placed in a plastic container filled with cold water (10-15 °C) for 5 minutes. The different stressors were distributed randomly. Two different stressors were administered per day, one in the

morning and one in the late afternoon. Rats were not exposed to cold twice on the same day.
During the last 4 days of the 21 day period, CUS and HC animals received RU486 (5 mg/100 mg body weight dissolved in 1.5 ml milk) or milk (1.5 ml milk) via a gastro–oesophageal tube directly into the stomach (Karst et al., 1997). On the last day of the 21-day stress period, a blood sample was collected in the early afternoon when plasma corticosterone levels are elevated due to diurnal rhythm. The next day rats were decapitated between 9 and 10 a.m. when plasma corticosterone levels are low. Trunk blood was collected for plasma corticosterone level analysis by means of a Radio Immunoassay (RIA). Adrenals and thymus gland were collected, cleaned, and weighed.

Slice preparation and corticosterone treatment
Immediately after decapitation, the brain was removed from the skull and chilled (at approximately 4 °C) in artificial cerebrospinal fluid (aCSF) containing (in mmol/L): NaCl 120, KCl 3.5, MgSO4 5.0, NaH2PO4 1.25, CaCl2 0.2, D-Glucose 10, and NaHCO3 25.0, gassed with 95% O2 and 5% CO2. Next, hippocampal slices, 400 µm thick, were prepared with a vibroslicer (Leica VT 1000S). Briefly, frontal lobes and cerebellum were removed and the dorsal side of the brain was glued on the slicing plateau. Coronal slices were prepared and incubated at room temperature in recording 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. After 1 hour, half of the slices from all experimental groups were treated in recording aCSF with vehicle, the other half with corticosterone (100 nM in 0.01% ethanol) for 20 minutes at 32 °C (HC_CORT, HC_RU_CORT; CUS_CORT and CUS_RU_CORT). Previous studies have shown that this dosage elicits alterations in synaptic plasticity (Alfarez et al., 2003; Alfarez et al., 2002) and modulates cellular properties that require homodimerization of the GR (Karst, 2000).

Imaging
One slice at a time was placed in a recording chamber mounted on an upright microscope (Nikon Optiphot-2). Slices were continuously perfused with aCSF (32°C, 2-3 ml/s) and kept fully submerged. Tetrodotoxin (0.5 M), tetraethylammonium chloride (10 mM), 4-aminopyridine (5 mM) and CsCl (5 mM) were added to the medium to block voltage-gated K+ and Na+ currents. Patch-clamp electrodes for recording (borosilicate glass, 1.5 mm outer diameter; impedance approximately 3-4 ΩM) were pulled on a Sutter micropipette puller and placed above the slice. The intracellular pipette solution contained (in mM): 141 Cs-methane sulfonate, 10 HEPES, 5 1,2-bis(2-aminophenoxy)ethane-N,N,N?,N?-tetra-acetic acid, 2 MgATP, 0.1 NaGTP; pH 7.4, 300 mOsm. To this solution we added Alexa hydrozin 568 (Molecular Probes, 0.2 mg/ml). Whole cell voltage clamp recordings were made 1-4 hours after corticosterone treatment using an Axopatch 200A amplifier (Axon Instruments, USA). After diffusion of the dye into the cell, the slices were fixed in 4% phosphate-buffered paraformaldehyde at 4°C for 30 min and rinsed three times with phosphate buffer. Slices were mounted on glass slides under a coverslip, with Vectashield (Vector Laboratories).
Immunofluorescent cells in fixed sections were evaluated using a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) confocal laser-scanning device equipped with a dry Plan-Neofluar 20x/0.75 lens using the 568 ArKr laser. The morphological analysis was performed using Image J (National Institute of Health, USA, http://rsb.info.nih.gov/nih-image/ (De Simoni et al., 2003) in combination with the Neuron_morpho plug-in (Giampaolo D'Alessandro, University of Southampton, UK) and LMeasure (Ruggero Scorcioni, George Mason University, Krasnow Institute, USA). LMeasure was used to define the following parameters: number of branch points (node where dendrite splits into two), width of the apical or basal tree (the width of the neuron is determined after reorientation along its primary axis) and total length of all dendritic segments (evaluated using 3D z-stacks). In order to establish the structural effects of the chronic stress treatment, RU486 treatment and in vitro GR activation, different morphological characteristics

of CA1 pyramidal cells were measured and compared. Basal and apical dendritic tree were analyzed separately.

Statistical analysis

Results are expressed as means ± s.e.m. Body weight gain and Sholl plots were compared using analysis of variance for repeated measures (MANOVA). All other results were analyzed for statistical significance ($P < 0.05$) using a one-way ANOVA or Student's t test.

Results

Chronic stress parameters

Body weight gain and organ weights of animals in the 4 experimental conditions are shown in Table 1. During the course of the 21 day period HC animals gained significantly more body weight than CUS animals ($P = 0.01$). Treatment of CUS animals with RU486 for the last 4 days of the experiment did not restore body weight gain to levels observed in HC_RU animals. Other parameters that were measured to examine HPA axis function were adrenal and thymus weight. CUS animals displayed adrenal hypertrophy when corrected for body weight compared to HC animals ($P = 0.005$). A similar hypertrophy was seen in CUS rats treated with RU486, the CUS_RU group exhibited adrenal hypertrophy in comparison to the CUS group ($P = 0.01$). Thymus weight when corrected for body weight did not reveal any difference between the four experimental groups. One day after the last stressor, plasma corticosterone levels were low again and not significantly different between groups (HC: 0.44 ± 0.1 µg/dl, CUS: 1.55± 0.7 µg/dl; HC_RU: 0.74 ± 0.4 µg/dl, CUS_RU: 2.90± 1.6 µg/dl).

Effects of chronic unpredictable stress and corticosterone on dendritic morphology of CA1 neurons

Projected confocal stacks of typical fluorescent CA1 pyramidal neurons give an impression of the overall morphology after the control treatment and chronic stress treatment, under basal and elevated corticosterone levels (Figure 1). While no effects of CUS and elevated corticosterone levels were found on the total length, width and number of branch points of basal dendrites (see table 2), the apical dendritic tree showed remarkable alterations upon exposure to chronic stress and in vitro corticosterone administration (Figure 2A-D, Table 2). First, the total apical dendritic length of CUS animals showed a 35% increase in comparison to HC animals ($P = 0.004$), with a simultaneous increase in number of branch points ($P = 0.02$; Figure 2A and Table 2). The width of the dendritic tree was not affected by prolonged exposure to stress. Second, exposing slices from HC animals to high corticosterone levels for 20 minutes showed a comparable 35% increase in total apical length ($P = 0.003$) with a simultaneous increase in branch points ($P = 0.05$; Figure 2A and Table 2). Remarkably, treatment of slices from CUS animals with corticosterone resulted in a reduction of apical dendritic length ($P = 0.04$) to a degree comparable to the length of HC animals (Figure 2A).

To evaluate in detail the distribution of dendritic processes across the CA1 layer stratum radiatum, we created Sholl plots (Sholl, 1953) in which summed dendritic length is expressed as a function of distance from the soma in radial bins of 60 µm (Figure 2B-D). Exposure to 21 days of stress increased branch-length proximal from the soma, approximately at 120 µm distance ($P = 0.01$) as well as more distal from the soma, i.e. at approximately 200-400 µm from the soma ($P = 0.002$; Figure 2B). Application of corticosterone to slices from HC animals increased branch-length distal from the soma at approximately 300-400 µm ($P < 0.03$; Figure 2C), while no effects more proximal to the soma were found. Administration of corticosterone to slices from CUS animals reduced branch-length at 450 µm ($P < 0.01$, Figure 2D).

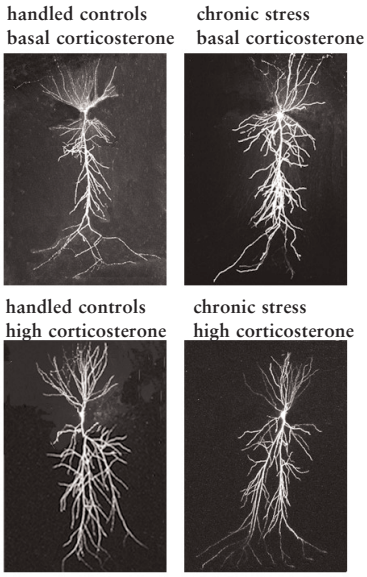


Figure 1

Morphology of CA1 neurons of the HC and CUS group under basal and elevated corticosterone levels.

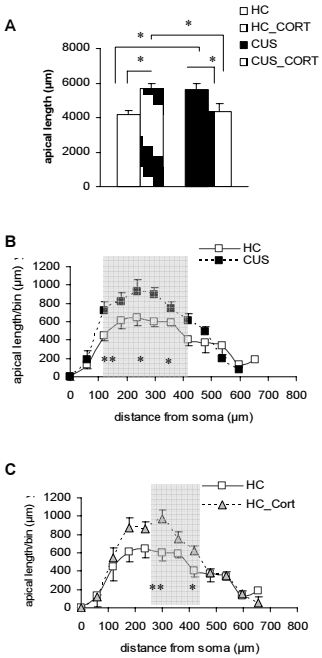
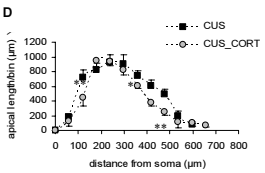


Figure 2 Influence of 21 days CUS and in vitro high corticosterone Sholl plot distribution of apical dendrites



*Total apical length of CA1 pyramidal cells was measured in slices from HC and CUS animals, either treated with vehicle or high corticosterone. A Total apical dendritic length of CUS animals differs significantly from HC animals (P = 0.004); treating slices from HC animals with corticosterone increased apical length significantly compared to untreated slices (P = 0.003); treating slices from CUS animals with corticosterone reversed apical hypertrophy to HC levels (CUS compared to CUS_CORT P = 0.04; HC_CORT compared to CUS_CORT P = 0.04). Sholl analysis was used to evaluate the distance-dependent distribution of the apical dendrites. Dendritic length was summed per radii at 60 µm distance, with the middle of the soma set at zero. Grey area shows significant difference between groups tested with Manova; individual points were tested with a Student t-test * < P 0.05; ** < P 0.01 B Comparing plots of HC and CUS animals shows an overall increase in dendritic length from proximal to soma approximately at ring 60 to more distal in the stratum radiatum area (Wilks labda = 0.03, F = 16.5, P = 0.008) and specifically for ring 120 P = 0.01; ring 240 P = 0.05; ring 420 P = 0.03. C Comparing HC and HC_CORT Sholl plots shows an overall increase in dendritic length 300-420 µm from the soma in the medial part of stratum radiatum (Wilks labda = 0.3, F = 4.7, P = 0.03); ring 300 and ring 420 showed significant differences (ring 300 P = 0.02; ring 420 P = 0.004). D Comparing CUS and CUS_CORT Sholl plots showed a decrease in dendritic length between 400-500 µm from the soma in the distal part of the stratum radiatum (Wilks labda = 0.3, F = 6.8, P = 0.02); ring 480 showed significant differences (ring 480 P = 0.01).HC: n = 7; HC_CORT: n = 5; CUS: n = 5; CUS_CORT: n = 5.*

Table 1. Effects of chronic stress on body weight gain, adrenal weight and thymus weight

	HC	CUS	HC_RU	CUS_RU
Body weight gain (% of initial body weight)	118 ± 4.7	79.4 ± 4.1	106 ± 5.5	83.2 ± 5.0
Adrenal weight (mg)	39.5 ± 2.2	43.6 ± 1.5	43.0 ± 2.2	53.7 ± 3.7
Adrenal weight (mg/100g body weight)	11.8 ± 0.6	14.9 ± 0.6	13.4 ± 0.7	18.2 ± 1.1
Thymus weight (mg)	669.2 ± 52.7	589.0 ± 32.0	647.5 ± 23.3	546.8 ± 32.5
Thymus weight (mg/100g body weight)	198.9 ± 14.2	201± 9.9	201.2 ± 7.4	186.1 ± 9.4

Values represent the mean ± S.E.M. Exposure to 21 days of stress attenuated body weight gain (MANOVA: HC vs CUS, $P = 0.01$) and increased adrenal weight corrected for body weight (HC vs CUS $P = 0.005$). RU486 treatment induced adrenal hypertrophy in CUS animals (CUS vs CUS_RU $P = 0.01$). * = $P < 0.05$ CUS group compared to HC group. † = $P < 0.05$ CUS_RU compared to untreated group.
N = rats: HC: N = 6; HC_RU: N = 6; CUS: N = 6;CUS_RU: N = 6.

Effects of RU486 treatment on stress induced apical hypertrophy

To test the hypothesis that the GR is involved in the stress induced apical hypertrophy, the GR antagonist RU486 was administered to HC and CUS animals during the last 4 days of the experimental procedures. We found that RU486 did not reverse the stress induced apical hypertrophy in CUS animals. RU486 treatment by itself also did not affect dendritic morphology of HC animals (Figure 3A). In agreement, the other morphological characteristics were not influenced by RU486 treatment (Table 2). However, when evaluating the Sholl plot it appears that treating HC animals for 4 days with RU486 increased dendritic length proximal to the soma compared to untreated HC animals (P = 0.01, Figure 3B). Sholl plots of the CUS group shows no difference with the CUS_RU group (Figure 3C).

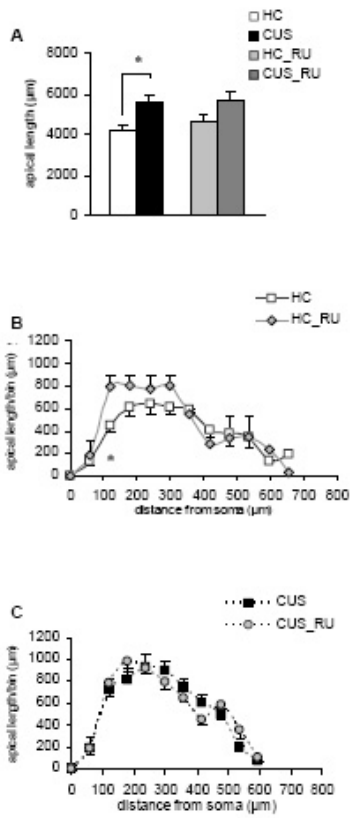


Figure 3c

Effects of RU38468 on stress induced apical hypertrophy

Total apical length of CA1 pyramidal cells was compared between HC and CUS animals that were treated for 4 days with RU38468. Treatment with RU38468 did not change total dendritic length of CUS animals compared to the untreated CUS animals (P = 0.9); RU38468 treatment also did not affect apical length in HC animals (P = 0.2). B Although the total apical dendritic length shows no differences, comparing Sholl plots of HC and HC animals treated with RU38468 reveals an increase in dendritic length close to the soma at ring 120 (P = 0.01) C Comparing Sholl plots of CUS and CUS animals treated with RU38468 shows no significant difference in dendritic organization. HC: n = 7; HC_RU: n = 6; CUS: n = 5; CUS_RU: n = 5.

Effects of RU486 treatment on in vitro corticosterone treatment

While apical dendritic length was found to be significantly increased when slices from HC animals were treated with corticosterone, this effect was prevented when animals were treated for 4 days with RU486 (Table 2, Figure 4A). Evaluating the Sholl analyses of HC_CORT and HC_RU_CORT slices shows that there is a specific effect of RU486 distal from the soma (ring 300: P = 0.007 ring 420: P = 0.01; Figure 4B) i.e. exactly at those parts of the dendritic tree that are increased by corticosterone administration. A similar effect of RU38486 was seen in CUS animals: exposing slices from these animals to high corticosterone reduced apical dendritic length. This effect was prevented by in vivo treatment for four days with RU486 (Table 2, Figure 4C). In this case though, no significant differences were seen for a particular bin in the Sholl plot analysis (data not shown).

Discussion

In this study we examined the effects of 21 days of variable and unpredictable stress on CA1 pyramidal neuron morphology under two conditions: basal (low) and elevated corticosteroid hormone levels. First, we show that under basal conditions chronic stress induces hypertrophy of the apical dendritic tree of hippocampal CA1 cells. A similar increase in dendritic length was found after exposing slices from control animals to corticosterone. However, exposing slices from chronically stressed animals to elevated corticosterone levels reversed the hypertrophy observed in chronically stressed animals under basal corticosterone levels. The structural effects of acute corticosterone administration, but not those of chronic stress could be prevented by treating animals with the glucocorticoid receptor antagonist RU486 for the last 4 days during the 21 day experimental period.

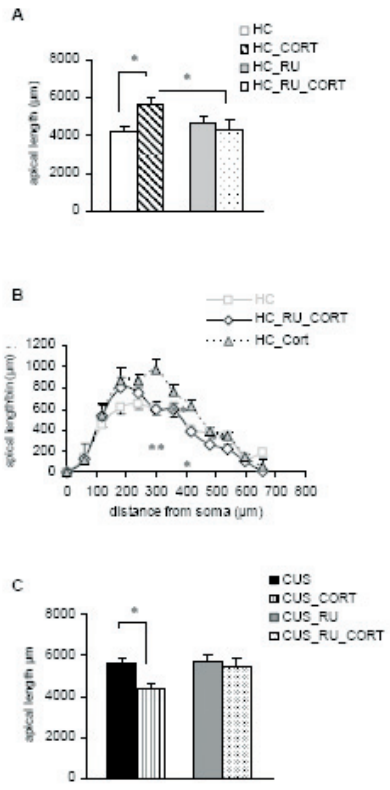


Figure 4

Effects of RU38468 on in vitro corticosterone administration

Total apical length of CA1 pyramidal cells was compared between HC and CUS animals with and without RU38468 treatment, under basal and high corticosterone conditions.

A The corticosterone induced apical hypertrophy was abolished by in vivo treatment with RU38468 in HC animals (P = 0.4); apical length compared between HC_CORT and HC_RU_CORT slices showed a significant difference (P = 0.01). B RU treatment shows a specific reversal of the corticosterone induced effects in HC slices. Comparing Sholl plots of HC_CORT and HC_RU_CORT slices shows a significant reduction in dendritic length at ring 300 P = 0.007 and ring 420 P = 0.01. HC Sholl plot is shown in plot as reference. C The corticosterone induced apical atrophy in CUS_CORT slices was not observed in CUS_RU_CORT slices (P = 0.7).

HC: n = 7; HC_CORT: n = 5; HC_RU: n = 6; HC_RU_CORT: n = 6; CUS: n = 5; CUS_CORT: n = 5; CUS_RU: n = 5; CUS_RU_CORT: n = 4.

Table 2. Morphological analysis of the dendritic trees of CA1 hippocampal neurons

	Basal dendrites			Apical dendrites		
	Number branch points	width (µm)	length (µm)	Number branch points	width (µm)	length (µm)
HC	19.4 ± 4.3	205.6 ± 55.5	2333 ± 349	37.2 ± 2.9	545.3 ± 16.1	4190 ± 241
HC_CORT	20.2 ± 2.0	210.3 ± 55.9	2555 ± 194	47.4 ± 3.5	551.8 ± 9.4	5652 ± 316
HC_RU	19.0 ± 3.3	297.6 ± 56.2	2502 ± 255	41.6 ± 4.2	525.4 ± 24.1	4678 ± 366
HC_RU_CORT	15.5 ± 5.5	381.6 ± 27.3	2668 ± 458	37.3 ± 2.6	532.3 ± 13.4	4343 ± 277
CUS	19.7 ± 2.0	308.6 ± 42.2	2292 ± 197	48.2 ± 2.6	533.0 ± 22.9	5636 ± 427
CUS_CORT	18.5 ± 1.5	275.2 ± 25.3	2262 ± 108	40.6 ± 3.5	529.2 ± 7.8	4355 ± 428
CUS_RU	20.2 ± 2.0	291.4 ± 31.9	2537 ± 197	45.6 ± 4.2	583.0 ± 4.8	5668 ± 493
CUS_RU_CORT	22.0 ± 1.6	222.7 ± 41.0	2369 ± 283	45.7 ± 3.1	551.7 ± 20.9	5462 ± 149

Values represent the mean ± S.E.M. Total dendritic length: HC vs HC_CORT, P = 0.003; HC vs CUS, P = 0.004; CUS vs CUS_CORT, P = 0.04. Number of branch points: HC vs HC_CORT, P = 0.05, HC vs CUS, P = 0.02. No differences were found for parameters of the basal dendritic tree. * = P < 0.05 CUS group compared to HC group. † = P < 0.05 compared to untreated group.
N = cells: HC: n = 7; HC_CORT: n = 5; HC_RU: n = 6; HC_RU_CORT: n = 6; CUS: n = 5; CUS_CORT: n = 5; CUS_RU: n = 5; CUS_RU_CORT: n = 4.

Chronic unpredictable stress and brief exposure to corticosterone induce apical hypertrophy in CA1 pyramidal neurons

Considerable evidence indicates that exposure to chronic stress causes atrophy of apical dendrites in hippocampal CA3 pyramidal cells. These effects of exposure to prolonged stress were mimicked by chronic administration of supraphysiological doses of corticosterone and could be prevented when corticosteroid production was reduced (Magarinos and McEwen, 1995b; Sousa et al., 2000). Recent evidence shows that in contrast to the effects found after chronic exposure to stress, two consecutive days of social defeat in rats can induce CA3 pyramidal hypertrophy of the basal cone (Kole et al., 2004). The structural effects of stress are dynamic and not restricted to the hippocampal formation. Stress-induced dendritic atrophy in CA3 neurons and pyramidal neurons of the medial prefrontal cortex is reversible upon a recovery period (Radley et al., 2005; Sousa et al., 2000) while neurons of the amygdala complex show hypertrophy after a prolonged period of stress (Vyas et al., 2002) with a concomitant increase in spines (Mitra et al., 2005).

In addition to these structural effects of exposure to stress in the hippocampal CA3 area we report at present that exposure to chronic stress results in hypertrophy of the apical dendritic tree of CA1 pyramidal cells. Importantly, these effects appear to be specific for the apical cone. Brief exposure to a high dose of corticosterone (sufficient to occupy the GR) induces a similar increase in dendritic length in animals from the HC group. The only study describing effects of chronic stress in adult age upon hippocampal CA1 dendritic morphology is by Sousa et al. (2000) who reported a 13-20% reduction in apical dendritic length (which did not reach statistical significance), together with a reduction in mean length of terminal dendritic segments (Sousa et al., 2000). The differences between this and our current result may be related to differences in experimental procedures. First, these authors, like most other studies on the structural effects of stress, used the Golgi-impregnation technique to visualize cells. This technique has a selective affinity for cells that have been subject to trauma (van den Pol and Gallyas, 1990) meaning that data obtained with Golgi staining could contain a high number of damaged cells. This might be partly reflected by the mean total length of apical CA1 dendrites in the Sousa study which is 1/3 of the mean total length we found using the patch-clamp approach combined with confocal microscopy to image the cells; differences in fixation procedures may have added to the discrepancy in total dendritic lengths. Variation in the "state" of neurons selected by the Golgi approach may also explain why relatively high numbers of cells are usually necessary to show differences after experimental treatment. This seems less of a problem using the present staining techniques in live tissue, which accordingly exhibit quite low standard errors, even with relatively small numbers of cells. This is supported by the observation that data from the handled controls and handled controls treated with RU486 showed very comparable dendritic lengths. Secondly, differences in handling procedures of the animals, potentially affecting circulating corticosteroid levels at the moment of decapitation, could give rise to discrepancies, as underlined by our present data. Thus, our data indicate that reduction in dendritic length may be seen, when dendritic length is examined in slices from chronically stressed animals that are exposed to elevated corticosterone levels prior to decapitation, as opposed to the situation that corticosterone levels are low. These data emphasize the importance of standardized experimental protocols since high circulating corticosteroid levels can for example be induced when animals are sacrificed in the afternoon or moderately stressed prior to decapitation e.g. by transport or anesthesia.

Taken together, our data indicate that corticosteroid hormone levels have profound effects on structural plasticity of CA1 hippocampal neurons. While the nature and underlying mechanisms of these changes remain to be resolved, they imply that neuronal functioning and reaction upon GR activation in chronically stressed animals is altered by the chronic stress regime thereby allowing corticosterone to reverse dendritic hypertrophy.

Role of the glucocorticoid receptor in structural effects of chronic stress and corticosterone.
Several studies have shown that the effects of chronic stress on hippocampal CA3 atrophy could be prevented when treating the animals with substances known for their antidepressant effects (Watanabe et al., 1992a; Watanabe et al., 1992b; Wood et al., 2004) or by treating animals with a steroid synthesis blocker (Magarinos and McEwen, 1995b). The structural changes in CA3 observed in rats given exogenous corticosterone implies that elevated corticosteroid hormone levels, at least in part, contribute to the structural effects seen after chronic stress (Sousa et al., 2000). Having established the effect of chronic unpredictable stress and brief corticosterone exposure on CA1 morphology, we investigated the role of GR activation in these effects. For this purpose we treated animals for the last 4 days of the experimental period with the glucocorticoid receptor antagonist RU486, an approach that was found effective in treating patients with psychotic depression (Belanoff et al., 2001). Our study shows that in vivo administration of RU486 resulted in endocrine and physiological effects. RU486 treatment in chronically stressed animals resulted in adrenal hypertrophy, which was comparable to a study where icv injections of RU486 resulted in inhibition of the HPA axis with simultaneously increased peripheral circulating corticosterone levels and increased adrenal weight (van Haarst et al., 1997; van Haarst et al., 1996). On the physiological level, our results reveal that four days of treatment with RU486 did not prevent the structural alterations elicited by chronic stress. This was not due to insufficient levels of RU486 in the brain, since in the same animals RU486 effectively reversed other properties: both CA1 hippocampal calcium currents (Karst et al. unpublished observations) and the degree of long-term potentiation (Krugers et al. unpublished observations) were restored in stressed animals treated with RU486. Possibly preventing the dendritic hypertrophy found after chronic exposure to stress by blockade of the GR might need longer treatment at an earlier stage of the stress protocol; indicating that stress in an early phase alters neuronal morphology. Conversely, we found in our present study that in vivo treatment of handled controls with RU486 and stress animals prevented morphological changes after in vitro exposure to corticosterone. This suggests a possible change of intracellular and molecular components of the CA1 neurons by the treatment with RU486 thereby preventing corticosterone being effective.
In conclusion, it remains to be resolved what the underlying mechanisms are behind the observation that chronic stress causes atrophy of the CA3 area while inducing hypertrophy in the CA1 area. One clear difference between these two hippocampal subfields is the absence of GRs in pyramidal neurons of the CA3 area (Han et al., 2005) so the effects of stress on the CA3 area are dependent upon activation of MR and not GR. Different areas of the hippocampus exhibit diverse sensitivity to corticosteroid hormones as demonstrated by the dentate gyrus which shows corticosteroid-induced cell death and decreased neurogenesis. The observed atrophy of the CA3 area after stress could be an indirect effect (since no GR is involved) caused by decreased input arising from the dentate gyrus (Gould et al., 1997; Gould et al., 1998; Sousa and Almeida, 2002). It is tempting to speculate that the effects of stress on the CA1 area are mediated by brief and long-term elevated corticosteroid hormone levels via a process that requires activation of the glucocorticoid receptor.

Functional implications of structural alterations
While our overall data indicates that exposure to chronic stress and briefly elevated corticosteroid hormone levels increase apical dendritic length, Sholl plot analyses reveal that the alterations under the various conditions do not involve the same regions of the dendritic tree. Chronic stress was found to induce apical hypertrophy in a large area from proximal to the soma, including the stratum radiatum area to more distal parts; changes by brief corticosterone administration were restricted to parts distal from the soma.
These structural alterations imply that exposure to chronic stress largely increases the ability of CA1 pyramidal cells to receive synaptic input. Counter intuitively, these structural alterations elicited by exposure to chronic stress are accompanied by a reduction in synaptic plasticity (Alfarez et al., 2003).

However, it is important to take into account that these structural alterations after exposure to chronic stress may yield a dendritic tree that receives different input not only in terms of quantity but also of quality, which can have consequences for the electrotonic properties of neurons. Proximal dendrites of CA1 neurons serve an entirely different role than dendrites more distal to the soma. More specifically, the proximal part of the dendritic tree has a low density input with a high inhibitory ratio (98% GABAergic) whereas distal dendrites have a high density of excitatory input (Halasy et al., 1996; Megias et al., 2001; Papp et al., 2001). Different classes of interneurons produce different forms of inhibition (e.g. interneurons that primarily target the perisomatic regions activate feedforward inhibition) or are triggered by different types of activity (e.g. interneurons targeting the soma are triggered by onset of activity, while those targeting distal dendrites are recruited by sustained activity) (Pouille and Scanziani, 2001; Pouille and Scanziani, 2004). Recently it was proposed that a shift from somatic to dendritic inhibition might be important for the induction of dendritic calcium spikes and synaptic plasticity (Mittmann et al., 2004; Pouille and Scanziani, 2004). We propose that the presently observed shift in input could impair synaptic plasticity as reported before after exposure to chronic stress (Alfarez et al., 2003).

Conclusion

In conclusion, our data reveal that exposure to chronic unpredictable stress increases apical dendritic length of hippocampal CA1 apical dendrites. This increment in dendritic complexity does not stand on its own: exposure to chronic stress has also been reported to increase dendritic arborization in the basolateral amygdala (Vyas et al., 2002) while brief stress increases the basal cone of CA3 pyramidal cells (Kole et al., 2004). When taking the studies on structural effects of (chronic) stress and elevated corticosteroid hormone levels together, there is considerable evidence now that exposure to stress may have bi-directional effects on dendritic complexity, which is at least in part dependent on the brain region that is examined. In addition, our data show that elevated corticosteroid hormone levels may have bi-directional effects on dendritic complexity depending on the history of the animal: brief elevation of corticosterone levels such as could occur after an acute stressor reduces dendritic arborization in chronically stressed animals, but increases dendritic complexity in naïve animals. These observations stress the necessity to perform experiments under very well controlled experimental conditions.

The functional consequences of changes in dendritic arborization after exposure to chronic unpredictable stress and elevated corticosteroid hormone levels are difficult to interpret. Yet, such alterations presumably alter both quality and quantity of synaptic input. Detailed understanding of the functional consequences of the presently observed structural alterations requires additional and detailed studies on synaptic input, synaptic properties and electrotonic coupling.

CHAPTER 6

Summary, general discussion and concluding remarks

I Summary

PART I

In part I of this thesis we investigated the effects of stress on hippocampal synaptic plasticity in both mice and rats. The stressful situations used were: exposure of animals to acute (1 hour) or chronic stress (21 days) and in vitro exposure of hippocampal slices to elevated levels of corticosterone (20 minutes). The following results were obtained:

Chapter 2
- Incubating slices with 100 nM corticosterone reduced the maximal slope of the fEPSP.
- Primed burst potentiation of the fEPSP was impaired after corticosterone treatment. Primed burst potentiation of the population spike was not impaired.
- Theta burst potentiation of both fEPSP and population spike was not impaired.
- Exposing mice briefly to a stressor impaired primed burst potentiation of the fEPSP.

Chapter 3
- Long-term potentiation of the fEPSP was impaired in both CA1 and dentate gyrus after exposure to chronic stress when recorded under conditions where plasma corticosterone levels are low.
- Long-term potentiation of the fEPSP was impaired in the CA1 area after in vitro exposure to high levels of corticosterone. No further decrease was observed after corticosterone treatment in stressed rats.
- Long-term potentiation in the dentate gyrus was not affected by in vitro exposure to high levels of corticosterone in both control and stressed rats.

PART II

In part II we investigated the effects of short term exposure to corticosterone and chronic stress on dendritic morphology and spine density of hippocampal CA1 neurons. Two different substrates of the hippocampal formation were used: organotypic slice cultures and acute slices. The following results were obtained:

Chapter 4
- Hippocampal organotypic slice cultures contain both mineralocorticoid and glucocorticoid receptors.
- Exposing organotypic slice cultures to 100 nM of corticosterone induced atrophy of the CA1 pyramidal apical dendritic tree.
- Atrophy of apical dendrites was induced in slices when imaged ≥2 hours following incubation with 30 nM corticosterone.
- The effects of corticosterone on dendritic length could be prevented with the GR antagonist RU486.

Chapter 5
- Rats exposed to 21 days of unpredictable stress showed increased apical dendritic length of CA1 pyramidal cells.
- Exposure of slices from control animals to elevated corticosterone levels resulted in hypertrophy of CA1 apical dendrites.
- Exposing slices of stressed animals to 20 minutes of 100 nM corticosterone reversed the hypertrophy.
- Treating control and stressed rats with the GR antagonist RU486 during the last 4 days of the stress experiment prevented the effects of acute corticosterone administration, but not the effects of expo sure to chronic stress.

PART I

Synaptic plasticity

In this thesis it was shown that exposing rats to chronic stress and mice to acute stress impairs some forms of synaptic plasticity (i.e. primed burst potentiation) while other forms of synaptic plasticity (i.e. theta burst potentiation) remain unaffected. Similarly, it was shown that exposure of hippocampal slices to elevated corticosteroid hormone levels also reduces primed burst potentiation while theta burst potentiation remains unaffected. These results clearly show that corticosterone can modulate hippocampal synaptic efficacy –at least in part- directly, without input from other brain areas being necessary in order to exert these effects.

An important question that remains to be addressed is how stressful situations and elevated corticosteroid hormone levels modulate synaptic efficacy.

Two issues will be discussed: 1) NMDA-receptor dependent LTP and 2) the role of intracellular calcium, AMPA receptors and its putative consequences for the threshold for LTP.

Stress and corticosterone reduce NMDA-receptor dependent LTP

One possible explanation for the different effects of corticosterone on PB and theta-burst potentiation may be that very robust stimulation paradigms elicit different forms of synaptic plasticity which are differently modulated by corticosteroid hormones and stressful events. Indeed robust stimulation paradigms like theta burst potentiation or pairing protocols involve activation of voltage-dependent calcium channels (VDCCs) in addition to NMDA receptors (Grover and Teyler, 1990; Magee and Johnston, 1997). Recent work by our group has shown that in particular the NMDA receptor dependent form of synaptic plasticity is reduced by exposure to elevated corticosteroid hormone levels (Krugers et al., 2005). This

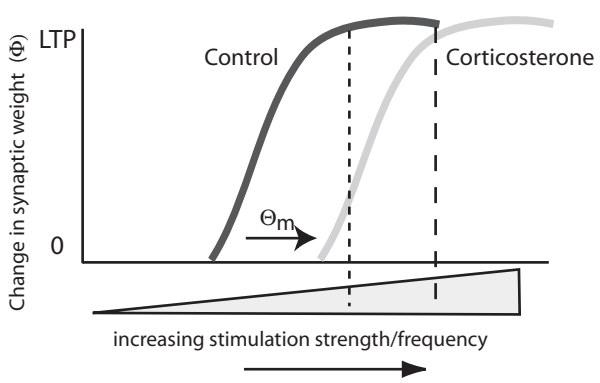
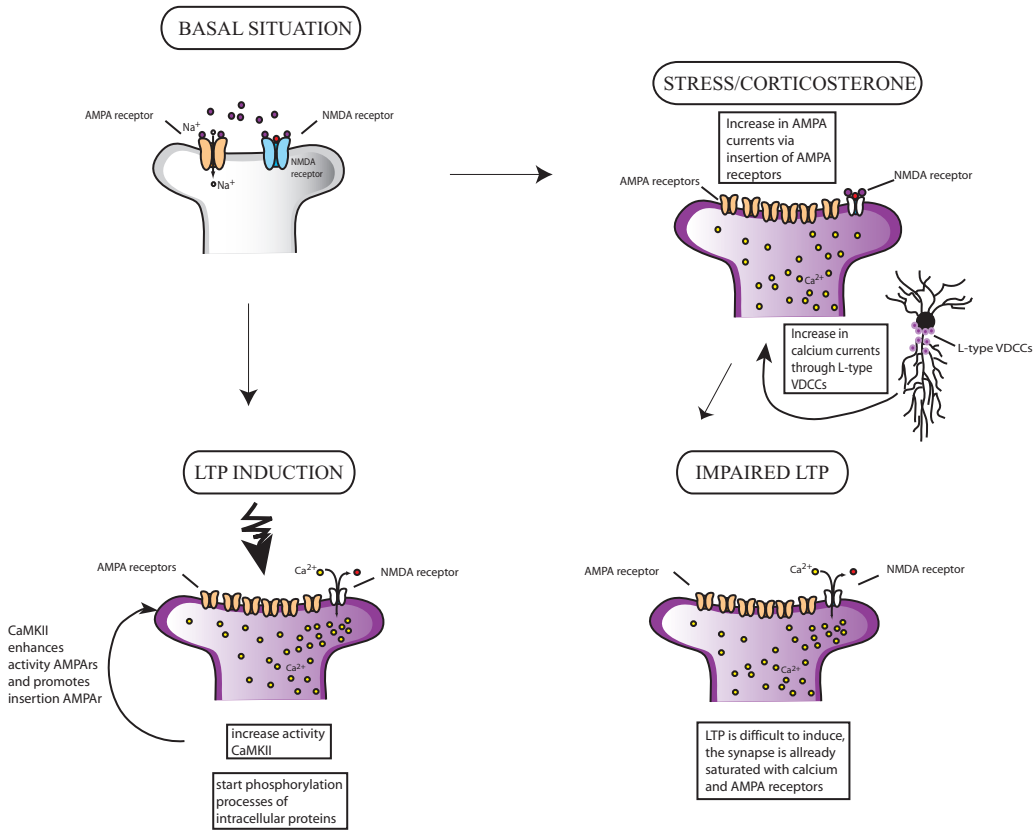


Figure 1

The effect of stress and corticosterone on the threshold for LTP

An adapted version of the Bienenstock, Cooper and Munro (BCM) model showing the relation between change in synaptic weight and afferent activity (in this thesis stimulation paradigm PB and θ-burst are described) resulting in a Θm shifted to the right. After stress or corticosterone treatment, induction of LTP is still feasible when robust stimulation protocols are used.



is in agreement with findings in this thesis. PB potentiation, which exclusively elicits NMDA receptor dependent LTP (Wiegert et al., 2005), is suppressed by elevated corticosteroid levels and after exposure to acute and chronic stress.

Mechanisms by which stress and corticosterone could alter the threshold for LTP

Two mechanisms are proposed which may be involved in the modification of synaptic plasticity after stress and corticosterone.

Calcium currents

Stress and corticosterone are known to modulate calcium currents. Exposing hippocampal slices to a GR agonist increases Ca2+ action potentials and both N-type and L-type voltage-sensitive Ca2+ currents (Kerr et al., 1992). Additional research showed that GR activation increases the amplitude of the Ca2+ currents, presumably the L-type Ca2+ currents, via a process that requires homodimerization of the glucocorticoid receptor (Joëls et al., 2003; Karst, 2000; Karst, 1994). Gene expression profiling showed that after in vitro corticosterone treatment of amygdala slices, genes coding for the A1 subunit constituting the pore of the L-type VDCC show increased expression, demonstrating a way in which corticosterone can target calcium regulating genes (Karst et al., 2002). Such changes also occur in the rat hippocampal CA1 area, though modulation of Ca2+ currents in the mouse hippocampus seems to involve other mech-

anisms (P. Chameau and Y. Qin, personal communication). Also the extrusion of Ca2+ appears to be slower after corticosterone exposure (Joëls et al., 2002; Takahashi et al., 2002), possibly through suppression of Ca2+-ATPases (Bhargava et al., 2002; Bhargava et al., 2000). Importantly, corticosterone increases the calcium-dependent afterhyperpolarization (Joëls and de Kloet, 1990; Kerr et al., 1989) which as a result increases the threshold for NMDA receptor dependent LTP (Sah and Bekkers, 1996).
In conclusion, numerous studies show the link between stress and corticosterone on calcium homeostasis in hippocampal neurons. The observed intracellular increase in calcium concentration may be involved in the increased threshold for induction of LTP. Calcium imaging in parallel with studying synaptic plasticity at different time points after corticosterone incubation could provide more insight into the role of calcium in synaptic plasticity changes after stress and corticosterone.

AMPA receptors

Addition of AMPA receptors into the postsynaptic membrane and particularly receptors containing the GluR1 subunit is known to mediate synaptic strengthening observed during LTP (Grosshans et al., 2002; Sheng and Lee, 2001). Studies using GluR1-GFP showed that after high-frequency stimulation GluR1 containing receptors can be delivered to synapses during LTP (Lissin et al., 1999; Lissin et al., 1998; Shi et al., 1999). An increased synaptic AMPA channel density follows the induction of synaptic potentiation (Andrasfalvy and Magee, 2004) and also changes in subunit composition are found after synaptic activity (Liu and Cull-Candy, 2000). Importantly, changes in AMPA receptor function have also been found in relation to stress. For instance, CA1 neurons show enhanced AMPA currents (both spontaneous mEPSCs and evoked responses) when tested 1-4 hours after application of 100 nM corticosterone in hippocampal slices (Karst and Joëls, 2005). No effect was found on the amplitude or kinetics of NMDA receptors. Also in other brain areas glutamatergic currents are affected by stress: dopaminergic neurons of the ventral tegmental area (VTA) show an increased AMPA/NMDA ratio one day after an acute stressor (Saal et al., 2003). These effects could be blocked using a GR antagonist indicating that elevated corticosteroid hormone levels mediate these effects. Furthermore, these effects were absent in GluR1 knockout mice, indicating that the GluR1 subunit is essential for the increase in AMPA receptor mediated synaptic transmission (Dong et al., 2004).
Chronic exposure to stress can also influence the AMPA/NMDA ratio. In hippocampal dentate gyrus cells an increase in AMPA current amplitude was reported after chronic stress when recording under elevated corticosterone conditions (Karst and Joëls, 2003). Next to the increase in AMPA currents, chronic stress leads to increased expression of the AMPA GluR1 subunit (Schwendt and Jezova, 2000).
Activation of AMPA and NMDA receptors is a dynamic process necessary for adequate induction of LTP. The ratio of AMPA and NMDA currents are constant during homeostatic synaptic scaling. However, recordings of AMPA-to-NMDA ratio soon after LTP induction showed that this ratio is altered. (Watt et al., 2004) showed that this perturbed ratio is controlled and reset during LTP in neocortical synapses. The rapid and long-lasting potentiation of AMPA currents is followed by a delayed but also long-lasting potentiation of NMDA currents which restores the original NMDA-to-AMPA ratio within 60 minutes after LTP induction. Importantly, blocking AMPA currents during LTP inhibited the potentiation of NMDA currents, implicating that potentiation of NMDA currents requires previous AMPA activation (Watt et al., 2004). The increase of AMPA currents after LTP induction was also found for CA1 pyramidal neurons (Andrasfalvy and Magee, 2004). So, although LTP initially perturbs the AMPA-to-NMDA ratio, NMDA potentiation follows AMPA potentiation albeit on a slower time scale. It is possible that the increase in AMPA currents observed after stress and corticosterone interferes with AMPA and NMDA receptor homeostasis and also with accurate induction of LTP. The corticosterone induced increase of AMPA currents could hamper synaptic plasticity when recorded long after the stressful situation. Thus, the stress induced changes in AMPA currents may be involved in the impairment of synap-

tic plasticity described in this thesis, although this remains to be investigated.
To elucidate the mechanisms further by which stress or corticosterone impairs LTP it will be necessary:
1) to examine the time-dependency of stress and corticosterone effects on LTP. Are there differences in the modulation of LTP when examined immediately after the stressor or when examined long (more than 2 hours) after exposure to stress or corticosterone?
2) to examine whether stress and corticosterone induce trafficking of GluR1 subunits to the postsynaptic membrane and
3) to examine the role of AMPA receptors in the effects of stress and corticosterone on hippocampal synaptic plasticity (e.g. by using an antagonist or GluR1 knockout mice)
In summary, the results in this thesis show that elevated corticosteroid hormone levels and exposure to stressful events increase the threshold to evoke LTP. There seems to be a strong relation between stress or elevated corticosterone hormone levels on the one hand and changes in AMPA current and receptor subunit insertion on the other hand, which are important factors for synaptic plasticity (Wheal et al., 1998). Also the timing of LTP induction after exposure to stress or to elevated corticosterone may determine whether LTP is reduced.
AMPA (and NMDA) receptor activity is closely associated with the ability to induce synaptic plasticity. It was demonstrated that increased levels of synaptically active AMPA receptors reduce the ability to elicit LTP, while facilitating the occurrence LTD (Stein et al., 2003). This may form the mechanistic basis for the BCM theory (Bienenstock et al., 1982) described in the General Introduction. It is also very reminiscent of what has been found for the effects of stress or novelty on LTP and LTD: stress and novelty increase the threshold for induction of LTP while promoting the chances of LTD (Kim et al., 1996; McEwen, 1994; Mesches et al., 1999; Pavlides et al., 1996; Xu et al., 1997). Maybe some form of 'synaptic plasticity' already occurs after corticosterone application making it more difficult to elicit LTP again (Diamond et al., 2004). In figure 1, a mechanism is proposed: LTP induction is hampered by occlusion of the synapse via a corticosterone-mediated increase of AMPA receptors and calcium saturation in the synapse. We tentatively propose that stress / corticosterone drives AMPA receptors into the synaptic density, thereby enhancing the threshold for subsequent LTP. This is depicted in an adapted BCM schedule shown in figure 2.

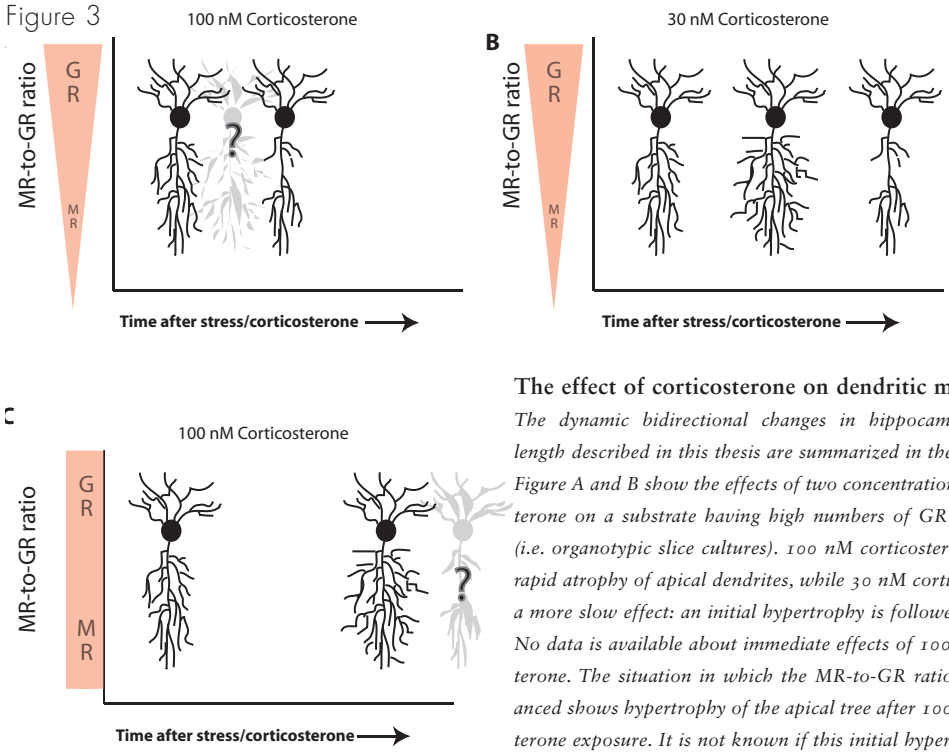
II GENERAL DISCUSSION OF THE RESULTS IN THIS THESIS

PART II
Hippocampal morphology

As described in the previous chapter, stress and corticosterone impair the induction of LTP. We proposed that stress and corticosterone evoke synaptic processes that are very similar to those elicited by LTP, so that occlusion will raise the threshold for LTP induction subsequent to stress / corticosterone. Morphological changes associated with LTP have gained much interest. In chapter 1, the relation between LTP and morphological changes is reviewed. In the second part of this thesis we examined if stress / corticosterone also induce morphological alterations in CA1 pyramidal neurons that resemble those seen in association with LTP. In this section, I will first briefly reiterate the morphological effects of LTP. Next, I will discuss the morphological effects seen after acute or chronic elevations in corticosterone level. Finally, in the concluding remarks I will compare the morphological characteristics after LTP with those seen after acute or chronic changes in corticosterone level and consider if morphological changes indeed are informative about the functionality of the circuit.

The association between morphology, functional plasticity and neuronal function

Figure 3



The effect of corticosterone on dendritic morphology

The dynamic bidirectional changes in hippocampal dendritic length described in this thesis are summarized in these figures. Figure A and B show the effects of two concentrations of corticosterone on a substrate having high numbers of GR positive cells (i.e. organotypic slice cultures). 100 nM corticosterone induces a rapid atrophy of apical dendrites, while 30 nM corticosterone has a more slow effect: an initial hypertrophy is followed by atrophy. No data is available about immediate effects of 100 nM corticosterone. The situation in which the MR-to-GR ratio is more balanced shows hypertrophy of the apical tree after 100 nM corticosterone exposure. It is not known if this initial hypertrophy is also followed by atrophy similar as in figure B.

The relation between changes in neuronal processes (dendrites) or sites where excitatory synaptic transmission takes place (spines) and learning and memory processes has been the focus of numerous studies. Table 1 of Chapter 1 gives an overview of key studies investigating the relationship between behavioural learning, LTP and synaptic morphology. Learning in general seems to increase the spine density. Less clear results were obtained with regard to LTP. Most studies pointed to enhanced synaptic contacts after LTP (expansion of spine heads and length, changes in spine type ratio and increase in the length and volume of PSDs), but other studies found either no changes or even the opposite effects.

Studies where laboratory animals are raised in a complex enriched environment showed not only increased spatial learning and memory performance (Leggio et al., 2005) but also increased dendritic arborization, enhanced both spine density and the size of synaptic contact areas (Diamond et al., 1987; Kozorovitskiy et al., 2005; Nithianantharajah et al., 2004; Rosenzweig and Bennett, 1996) and increased neurogenesis (van Praag et al., 2000). Moreover, placing animals in a complex environment can attenuate or prevent amyloid deposition (symptoms related to Alzheimer), CNS insults and lesions and even developmental disorders like the fragile X syndrome (Dong and Greenough, 2004; Johansson, 2003; Lazarov et al., 2005; Lewis, 2004; Restivo et al., 2005). Most of these studies have focussed on the relationship between changes in spine shape or density that occur after environmental enrichment. Although there are discrepancies concerning the magnitude of LTP and the relative changes in spine density, shape or spinogenesis (Segal, 2005) it is clear that spines shape, size and remodelling of the postsynaptic density can be modulated after experience and cellular synaptic plasticity, generally increasing the possibility for synaptic contacts.

Efforts to understand the relationship between dendritic architecture and the function of neurons have been numerous. Experience-dependent plasticity of dendrites of olfactory bulb mitral and tufted cells seems to be strongly controlled and limited (only a 2 to 3% change in dendritic length after odour enrichment) implicating that despite learning processes the neuronal network maintains stability of dendritic processes (Mizrahi and Katz, 2003). Consequently, factors that alter structural network stability may have functional implications. Much can be learned from studies which investigated the modulation of dendritic stability by hormones, growth factors or stress. For example, elevated levels of androgens were found to correlate with increased dendritic length of neurons in the amygdala while mental retardation is connected with a decrease in the length and complexity of dendritic branches in the cortex (Cooke and Woolley, 2005; Newey et al., 2005).

Acute rises in corticosterone level and morphology

The effect of chronic stress on CA3 neuron morphology has been extensively reviewed and discussed in chapter 1. In this thesis we extended the observation showing that CA1 neuronal morphology can be modulated by corticosterone and stress by showing that brief exposure to high levels of corticosterone decreased apical dendritic length of CA1 neurons in organotypic slice cultures while similar corticosterone treatment induced an increase in CA1 apical length in acute slices from adult rats. Imaging of CA1 neurons in slices from animals subjected to chronic unpredictable stress showed opposite effects: under basal conditions there seems to be an increase of apical dendritic length which is reversed by short exposure to corticosterone.

The different results after short exposure to corticosterone in hippocampal organotypic slices and acute hippocampal slices of adult rats may be related to differences in the nature of corticosteroid receptors which could be age – or preparation- dependent. In chapter 4 the role of the MR-to-GR ratio is discussed concerning the strong effects on dendritic remodelling after exposure of the organotypic slices to 100 nM corticosterone. In particular we compared the MR-to-GR ratio of the organotypic slices with the MR-to-GR ratio of tissue from a similar developmental stage (i.e. PND 19), which showed that the MR-to-GR ratio in the organotypic slices was shifted towards more GR compared to acute slices.

Both concentrations of corticosterone used in this study were sufficient to activate the GR in addition to the MR. Several studies show that MR activation maintains synaptic plasticity as well as neuronal structural integrity (Cameron and Gould, 1996; Conrad and Roy, 1993; Diamond et al., 1992; Jaarsma et al., 1992; Pavlides et al., 1996). Additional GR activation in both hippocampal primary and organotypic cell culture causes cytosolic Ca2+ accumulation (Elliott and Sapolsky, 1993; Mulholland et al., 2005) and inhibits hippocampal and glial glucose uptake (Horner et al., 1990; Virgin et al., 1991). We cannot exclude that the latter could also affect the transport of the fluorescent dye used in chapter 4 and 5. We speculate that calcium or glucose-dependent mechanisms may be involved in the effects of corticosterone on dendritic structure. The atrophy of apical dendrites could be related to the high amount of GR positive pyramidal neurons and glial cells in organotypic slices thereby exacerbating the effect of corticosterone. Results from chapter 4 indicate that acute slices from the same developmental age as the organotypic slice cultures contain less GRs (and consequently a different MR/GR ratio) which may protect these cells from deleterious effects of corticosterone. Future studies should be done to evaluate the exact role of MR-to-GR ratio in morphological changes. For instance, what are the consequences for neuronal morphology when not GR, but MR expression is upregulated? Organotypic slice cultures allow experiments in which the expression of the receptors can be manipulated with the use of viral infection techniques or siRNA. Following this manipulation of MR or GR expression the effects of corticosterone can be established using (time-lapse) imaging of neuronal morphology.

Critical evaluation of the data where organotypic slices are exposed to 30 nM corticosterone shows a decrease in dendritic length when examined more than 2 hour after corticosterone exposure. However,

when examining apical dendritic length less than 2 hour after exposure to corticosterone, an increase in apical length was observed from ± 1900 µm to ± 2500 µm. Although these differences were not statistically significant probably due to the small number of observations (n = 8 and n = 5 respectively), it is clear from this data that timing is essential in determining the (dynamic) effects of corticosterone on structure. The changes in dendritic structure after corticosterone incubation are rapidly induced in organotypic slices after exposure to 100 nM corticosterone, but of a slower nature in the organotypic slices after exposure to 30 nM corticosterone. In acute slices similar dose-dependent differences in delay were found in modulation of the serotonin response (Joëls and De Kloet, 1992), although under such circumstances the effective concentration range may have been shifted to the right, so that slowly developing GR effects are seen with 100 instead of 30 nM corticosterone (Joëls et al., 2003). Timing may therefore be essential in determining the effects of corticosterone on structure. (figure 3).

Chronic stress and morphology

Exposure to chronic stress has previously been reported to reduce the apical dendritic length of hippocampal CA3 pyramidal neurons (see also chapter 1). In this thesis we show that chronic stress can induce hypertrophy of the apical dendritic tree of hippocampal CA1 cells. Not only the CA1 area shows hypertrophy after exposure to chronic stress, similar effects have been reported in the amygdala after 3 weeks of restraint stress (Vyas et al., 2002).

Our data from the animals that received the chronic stress treatment were compared with a group of animals that were handled for 21 days. In order to fully comprehend the functional implications of the observed dendritic remodelling as observed in chapter 5 it is necessary to think about the possible effects of 21 days handling or unpredictable stress on animals. Thus, in chapter 5, four experimental groups were investigated. The control animals were handled for 21 days and received the last 4 days of the experimental period milk via a gastro-oesophageal tube directly into the stomach. It will be necessary to assess the exact consequences of handling and 4 days of milk administration prior to the assessment of dendritic morphology.

This is important since some studies indicate that handling indeed can have effects on synaptic plasticity or morphology. Focussing on handling and synaptic plasticity, Korz and Frey (2003) found that handling of rats for 2 minutes significantly decreased LTP in the dentate gyrus in vivo measured immediately after the handling procedure. Evaluation of the circulating corticosterone levels indicated that these were elevated when measured 15 minutes after handling (Korz and Frey, 2003). Also at the structural level, handling was found to have effects: Seib and Wellman reported that handling increased spine density of medial-prefrontal cortex neurons close to the soma (Seib and Wellman, 2003).

With regard to chapter 5, even though corticosterone levels on the day of the experiment were comparable between groups, the handling procedure or the administration of milk via the gastro-oesophageal tube may have induced a daily increase in corticosterone levels of these animals. Even though dendritic morphology was recorded one day after the last handling-milk treatment, this delay may still have had effects on our results. A study by Garcia et al. (1997) showed an effect of an acute stressor (1 hour tail shock) on hippocampal LTP in vitro recorded 1 hour, 24 hour or even 48 hour after the stress. At 1 hour after the acute stressor, LTP was found to be impaired by 67%, and even when recorded 24 hour after the last stressor an impairment of 32% was observed. This indicates that effects of an acute stressor can be observed even 24 hours after the stressor. As a consequence, the data on dendritic hypertrophy should be regarded with caution and the use of an unhandled, untreated control group will be essential to understand the data entirely.

Mechanisms of stress and corticosteroid action on dendritic structure

The cytoskeleton of dendrites is composed mainly of microtubules with an outer layer of actin underly-

ing the plasma membrane and a dense actin matrix at the end of processes. In search of regulators of dendritic growth and development, the Rho GTPases have been proposed as key integrators between environmental cues and regulation of the dendritic cytoskeleton (Van Aelst and Cline, 2004). Studies trying to link neuronal activity and Rho GTPases indicated that blocking NMDA and AMPA receptors decreased dendritic arbour growth rate in Xenopus tectal neurons. Rho GTPase is also implicated in dendritic branch additions and stabilizations and extensions (Sin et al., 2002). Furthermore, changes in intra-cellular calcium also affect the dendritic cytoskeleton (Wong and Ghosh, 2002). A study in cortical slice cultures showed a strong role of L-type VDCCs and subsequent activation of CaMKIV and cyclic-AMP-responsive-element-binding protein (CREB)-mediated signalling; calcium-induced dendritic growth was suppressed by inhibition of CaMKIV and CREB activity (Redmond et al., 2002).
A study by Alfonso et al. (2005) investigated the role of M6a, a stress-responsive gene, in relation to changes in dendritic morphology. M6a is present in the hippocampal dentate gyrus, CA1 and CA3 cells and over-expression increases neurite outgrowth and filopodium and spine density in primary hippocampal cultures (Alfonso et al., 2005). Interestingly, expression levels of M6a are decreased in hippocampal tissue of tree shrews subjected to chronic stress and this down-regulation is prevented by the antidepressant clomipramine (Alfonso et al., 2004).
To conclude, further research should investigate mediators of dendritic remodeling and their relation to stress and corticosterone. Similar as with M6a, other stress-responsive genes might be involved in the process of dendritic remodeling. Also stress and corticosterone effects on Rho GTPases can support ideas about the intracellular mechanisms that are involved in the effects on hippocampal dendritic remodeling.

III CONCLUDING REMARKS

The dendritic atrophy seen after acute corticosterone exposure is not accompanied by a decrease in spine density, as was predicted based on the findings regarding LTP. However, the total reduction in dendritic length may cause a decrease in the number of spines, which in turn may contribute to the impairment in LTP. Clearly, essential information about the size, shape and localization of synaptic contacts after corticosterone treatment is lacking at this moment. Studies examining the effects of stress and LTP on synaptic morphology show that not only changes in spine density but also changes of the postsynaptic membrane are relevant. Detailed evaluation of the possible effects of stress and corticosterone on remodelling of spine synapses (with the use of EM) is necessary. No data is yet available on this issue, neither concerning the effect of corticosterone on synaptic plasticity in organotypic cultures nor concerning the effect of corticosterone on CA1 morphology in acute slices of a similar developmental age. This information is essential to assess if the morphological changes after corticosterone are linked to the changes in synaptic plasticity.
The dendritic hypertrophy observed after chronic stress demands a different approach. Next to the in this thesis described effects of chronic stress on dendritic remodelling, the effect of chronic stress on spine density, shape and size of CA1 cells needs to be investigated. If chronic stress causes a decrease in spine density, the observed dendritic hypertrophy might function as a rescue mechanism for the cell to recover from the decrease in synaptic input. This hypertrophy might restore the decreased input of the cell under basal/resting potential conditions while under conditions of learning (i.e. LTP) the changes in morphology hamper this process. Also stress-induced changes in the ratio of spine shape and size may be related to the observed decrease in LTP. In order to understand the consequences of the dendritic remodelling after stress on electrotonic functioning the use of neuronal modelling will be necessary.

Is bigger always better and smaller always worse?

Combination of two techniques, i.e. electrophysiological recordings and neuronal imaging, can complement each other. Yet, the results from part I and II in this thesis illustrate that it may also lead to paradoxical findings. This is not unprecedented as is evident from the literature on e.g. stress related morphological changes in the hippocampus. Pavlides et al. (2000) reported impaired LTP of both the CA3 and dentate gyrus area after chronic stress. Current source density analysis supported the idea that the impairment in LTP of the CA3 could be related to apical dendritic atrophy of CA3 pyramidal neurons. For dentate granule cells however, no evidence was or is found that dendritic atrophy occurs after chronic stress. So in the CA3 area the impairments in synaptic plasticity seem to be correlated to dendritic atrophy while in the dentate gyrus this association can not be made.

Also gender can 'overrule' the effects of hippocampal atrophy after chronic stress. Spatial memory is enhanced after 21 days of chronic stress in female rats tested in the radial arm maze, while a similar stress treatment impairs spatial memory in male rats (Bowman et al., 2001). Furthermore, despite CA3 dendritic retraction, female rats perform better on the Y-maze after chronic stress. The reported contribution of ovarian hormones (e.g. estrogens) to spatial memory was not interfering since the females were ovariectomized and treated with vehicle or estrogens (McLaughlin et al., 2005).

To conclude, changes in hippocampal morphology, synaptic functioning and behaviour do not always coincide. Further multidisciplinary research is necessary to elucidate the exact relationship between form and function.

Consequences for behavior

From the results of this thesis it is clear that acute stress and corticosterone impair LTP induction and alter the morphology of CA1 neurons. Behavioral studies show that mechanisms necessary for LTP induction (NMDA receptor activation or VDCC receptor activation) are involved in formation of spatial and emotional memories (Borroni et al., 2000; Woodside et al., 2004). It is tempting to speculate that similar mechanisms are involved in the effects of stress and corticosterone seen in our studies as well as behavioral studies where stress prior to and out of the learning context interferes with subsequent learning and memory processes (de Kloet et al., 1999; Roozendaal, 2003; Shors, 2004).

Exposure to chronic stress also impairs LTP. The prolonged exposure to high levels of corticosterone might be causing the impaired LTP, and possibly result in similar occlusion of the synapse as after acute stress or corticosterone, although now of a more persistent nature. Experiments in which the GR is antagonized during chronic stress may give more insight in the GR dependency of the mechanisms that underlie the impaired synaptic plasticity. To evaluate the behavioral relevance, it will be informative to combine these electrophysiological experiments with behavioral investigations.

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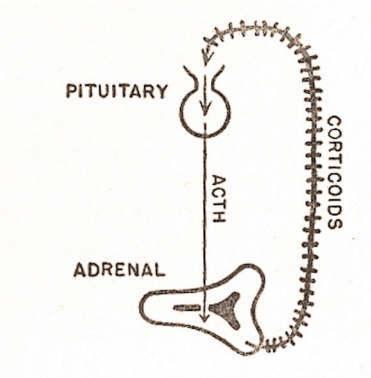
Intermezzo

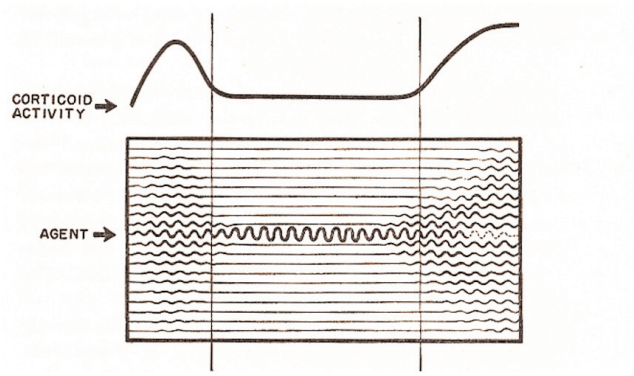
Hans Selye (1907-1982)
Discovery is the realization that something new exists

Hans Selye (born Selye Jànos in Hungary) devoted a scientific career spanning half a century investigating the subject of stress. As a young medical student at the University of Prague, Selye already created a starting point that would eventually lead to his theory of the stress syndrome. During this introduction to clinical medicine he examined various patients that "felt and looked ill". They generally "had fever, enlarged spleen or liver and a skin rash" and a number of other general symptoms. Selye, unbiased by ignorance and lack of knowledge, wondered why individual diseases were treated but not "the syndrome of just being sick". It was 1934 before he could pursue his hypothesis. At that time he was a promising young endocrinologist at McGill University's Biochemistry Department in Montreal searching for a new hormone. He did this by injecting rats with ovarian extract. The rats developed a triad of symptoms: enlargement of the adrenal cortex, atrophy of the thymus, spleen, and lymph nodes, and ulcers in the stomach. Selye thought that he was on the verge of finding a new hormone that caused these symptoms. This proved to be an illusion, when placental extract and, later, injections of kidney, spleen and numerous other organs produced the same effect. When formalin also produced these symptoms he knew he had failed to discover a new hormone.
This experiment, together with the memory of patients exhibiting symptoms of being sick drove him to formulate an idea that fitted this data together. Selye suggested that there was a general response elicited by a variety of external agents, later named "stressor". Through years of research he identified this complicated internal stress-processing mechanism, which came to be known as the hypothalamus-pituitary-adrenal system although he was never able to define stress (figure 1; adapted from "The stress of life"; Hans Selye, 1956). The scientific analysis of the stress response begins with Hans Seyle's Nature



ACTH stimulates the corticoid secretion of the adrenals; the corticoids inhibit the ACTH secretion of the pituitary

paper in 1936 titled "A syndrome produced by diverse nocuous agents". In this paper he discloses his ideas about a process called the General Adaptation Syndrome (G.A.S., later referred to as "the stress syndrome"), the process under which the body is confronted with 'stress'. The G.A.S. describes the body's response to demands placed upon it, and details how stress induces hormonal autonomic responses and how, over time, these hormonal changes can lead to ulcers, high blood pressure, arteriosclerosis,



Alarm Reaction
Auxiliary mechanisms are mobilized to maintain life so that the reaction spreads to large territories. No organ-system is as yet specially developed to cope with the task at hand.

Stage of Resistance
Adaptation is acquired due to optimum development of most appropriate specific channel of defense. Spacial concentration of the reaction makes corticoid production unnecessary

Stage of Exhaustion
Reaction spreads again due to wear and tear in the most appropriate channel. Corticoid production rises, but can maintain life only until even auxiliary channels are exhausted.

arthritis, and allergic reactions.
The body passes through three stages of coping during G.A.S.:
Firstly: alarm - the initial response to a challenge, the body prepares for a "fight or flight". This initial response is followed by the second stage of resistance to the stressor. After prolonged resistance adaptation is lost and the body eventually enters the third stage: exhaustion, a sort of aging "due to wear and tear" (figure 2; adapted from "The stress of life"; Hans Selye, 1956)
He also realized that there is great variance in individual reactivity towards stressors and that the same stressor could be pleasurable to one individual and very stressful to another.
Selye was a highly disciplined and creative scientist, teacher, and writer. Visiting scientists to his department were often addressed in their own language; he was fluent in Hungarian, German, English, French and Italian and also competent in Spanish, Czech and Russian. His research resulted in 33 books and over 1600 scientific articles on what he called the "diseases of adaptation". As a scientist he was aware of the importance of originality when it comes to science; as he states in his popular philosophical book "In vivo" that:" Discovery is the realization that something new exists".

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Special thanks to Luke Beeharry.

Nederlandse samenvatting

In dit proefschrift is beschreven hoe stress en het stresshormoon corticosteron de communicatie tussen hersencellen van de hippocampus en de vorm van deze zenuwcellen beïnvloeden. Op deze manier is getracht ook inzicht te krijgen in de onderliggende mechanismen van geheugenproblemen die vaak voorkomen bij mensen met depressieve klachten. In hoofdstuk 2 en 3 worden experimenten beschreven waarin de effecten van acute (kortdurende) stress of blootstelling aan corticosteron en chronische (langdurige) stress op een cellulair model voor leer en geheugenprocessen worden getest. In hoofdstuk 4 en 5 zijn experimenten gedaan die informatie geven over de veranderingen in vorm van hersencellen in de hippocampus.

Omgaan met stressvolle omstandigheden is in de moderne maatschappij noodzakelijk geworden om te overleven. Als een organisme wordt blootgesteld aan een stressvolle gebeurtenis dan wordt (ondermeer) de hypothalamus-hypofyse-bijnier as (HHB-as) geactiveerd. Na stimulatie door de stressor worden neuronen van de paraventriculaire kern in de hypothalamus geactiveerd en secreteren de hormonen corticoreline (corticotrophine releasing hormone, CRH) en vasopressine (VP). Deze hormonen stimuleren de afgifte van het adrenocorticotroop hormoon (ACTH) vanuit de hypofyse. Via de bloedbaan bereikt ACTH de bijnieren waar het de afgifte van het stresshormoon corticosterone in knaagdieren stimuleert (zie figuur 1 in hoofdstuk I). Corticosteron circuleert in het lichaam via de bloedbaan, passeert de bloed-hersen-barrière en heeft via een terugkoppelingsmechanisme negatieve invloed op de activiteit van de HHB-as.

In de hersenen zijn er twee receptoren voor corticosteron, de mineralocorticoïd receptor (MR, hoge affiniteit voor corticosteron) en de glucocorticoïd receptor (GR, lage affiniteit voor corticosteron; wordt alleen na een stressvolle situatie geactiveerd). Het hersengebied dat centraal staat in dit proefschrift, de hippocampus, bezit veel van deze receptoren. De hippocampus is sterk betrokken bij leer- en geheugenprocessen. Uit eerder onderzoek is gebleken dat zowel langdurige als korte stress leerprocessen kan faciliteren maar ook remmen. Dit effect is afhankelijk van de timing van de stressvolle gebeurtenis tijdens leerprocessen. Om het effect van stress en corticosteron op leren en geheugen te bestuderen is er gekozen voor een veelgebruikt cellulair model voor leer en geheugenprocessen: long-term potentiation (lange-termijn potentiatie, LTP).

In hoofdstuk 2 werd onderzocht of LTP, gemeten in hippocampus plakjes van de muis, beïnvloedt wordt door in vitro blootstelling aan een hoge dosis corticosteron. De mate waarin synaptische verbindingen in het CA1 gebied van de hippocampus kunnen worden versterkt werd daarna onderzocht door gebruik te maken van twee verschillende stimulatie protocollen. In het tweede deel van deze experimenten werd een muis blootgesteld aan een rat om korte psychologisch relevante stress op te wekken. De twee resultaten werden met elkaar vergeleken om de invloed van andere hersengebieden buiten de hippocampus te onderzoeken. Uit deze studie bleek dat zowel hoge plasma corticosteroid spiegels als een korte stressor LTP reduceren.

Voor de studie van hoofdstuk 3 werden ratten langdurig gestresst. Dit hield in dat de ratten voor 3 weken lang elke dag aan 2 onvoorspelbare psychologische stressoren werden blootgesteld. Uit eerder onderzoek is gebleken dat dit verstoring van de HHB-as veroorzaakt en kan dienen als een diermodel voor depressie. De effecten van de langdurige stress werden onderzocht in twee hippocampale gebieden, het CA1 gebied en de gyrus dentatus (DG). In deze twee gebieden werd LTP gemeten onder condities van basale en verhoogde corticosteroid spiegels. In zowel het CA1 gebied als de gyrus dentatus werd LTP onderdrukt in dieren die bloot waren gesteld aan chronische stress.

In het tweede gedeelte van dit proefschrift werd onderzocht of kortdurende blootstelling aan een hoge dosis corticosteron of langdurige stress invloed heeft op het aantal synaptische contacten en de morfologie van hippocampale CA1 neuronen. Veranderingen in neuronale netwerken en in synaptische contact-

punten (spines) zijn betrokken bij leer- en geheugenprocessen en LTP. Tabel 1 van hoofdstuk 1 toont een overzicht van de literatuur op dit gebied. Oude studies rapporteerden een toename bij dieren die een gedragstaak moesten leren, echter dit effect is in meer recente studies niet gerepliceerd. Een meer uniform beeld komt uit onderzoek naar effecten op de vorm en ultrastructuur van spines na het leren van een bepaalde taak. Ook de parallellen tussen spine dichtheid op de dendritische uitlopers en LTP zijn verwarrend; sommige onderzoeken rapporteren een stabiele spine dichtheid na LTP terwijl anderen een toename vinden. Wel lijkt er na LTP consistent een verandering in de vorm en ultrastructuur van spines op te treden. Opvallend is de proportionele verschuiving in de aantallen spines van een bepaalde vorm.

In hoofdstuk 4 van dit proefschrift werd onderzocht of een korte hoge dosis van corticosteron een effect heeft op de vorm van de dendrietboom en het aantal spines van CA1 pyramidaal neuronen. Het substraat voor deze experimenten bestond uit gekweekte hippocampale plakken die waren verkregen van jonge ratten. De resultaten tonen aan dat de hoge dosering corticosteron ervoor zorgt dat de apicale dendrieten korter worden (atrofie): dit effect treedt op na korte blootstelling aan 100 nM corticosteron. Er werd geen effect gevonden aan basale zijde van de dendrietboom. Fractaal analyse toonde aan dat de totale complexiteit van deze neuronen tweevoudig was afgenomen. De rol van de GR werd onderzocht door gebruik te maken van een GR antagonist. Toevoegen van een GR antagonist aan de gekweekte plakken voordat deze blootgesteld werden aan corticosterone blokkeerde het effect van corticosteron. Experimenten gedaan met een lagere dosis corticosteron (30 nM) veroorzaakte atrofie van de apicale dendrietboom op een tijdsafhankelijke manier. De experimenten gedaan om het effect van zowel de 30 als de 100 nM corticosterone op spine dichtheid te onderzoeken toonden aan dat er geen effect was. De resultaten van deze experimenten tonen aan dat ook korte periodes van hoge corticosteron niveaus een modulerend effect hebben op de morfologie van CA1 neuronen.

Aangezien in de literatuur vaak is beschreven dat chronische stress krimping veroorzaakt van CA3 neuronen, bestudeerden wij in hoofdstuk 5 het effect van chronische stress op de morfologie van CA1 neuronen. Dit is gedaan onder zowel basale als verhoogde corticosteron niveaus. Uit het (verrassende) resultaat van deze studie bleek dat na chronische stress de lengte van de apicale dendrietboom van de CA1 neuronen was toegenomen. Ook wanneer hippocampus plakjes van controle dieren kort werden blootgesteld aan een hoge dosis corticosterone trad deze vergroting van de dendrietboom op. Een identieke behandeling van hippocampus plakjes van gestresste ratten veranderde de dendritische hypertrofie in atrofie. Om ook in deze studie de rol van de GR vast te stellen werden de gestresste dieren aan het einde van de stressperiode behandeld met een GR antagonist. Deze behandeling blokkeerde niet de effecten van chronische stress maar wel de effecten van de korte blootstelling aan corticosteron. Deze resultaten tonen aan dat corticosteroïden verschillende effecten kunnen hebben op dendritische morfologie afhankelijk van de geschiedenis van het dier. De functionele implicaties zijn moeilijk te interpreteren maar onderzoek met behulp van neuronale modellen zal daar een belangrijke rol in kunnen spelen.

De resultaten die in dit proefschrift worden gepresenteerd maken duidelijk dat neuronen van de hippocampus door stress worden gemoduleerd. De resultaten van deel I laten zien dat zowel lang- als kortdurende stress de mate waarin synaptische verbindingen kunnen worden versterkt (cellulair leerproces) onderdrukt. Deel II toont aan dat parallel aan deze veranderingen in geheugenprocessen ook morfologische veranderingen optreden. De uitdaging in verder onderzoek zal zijn om deze resultaten te koppelen, om zo te begrijpen hoe vorm en functie van hersencellen elkaar beïnvloeden.

Dankwoord

Met een korte tussenpauze van 2 jaar loop ik al 13 jaar mee op de Anna´s Hoeve. Vroeger was alles beter, al is de in mijn tijd als eerstejaars student beloofde openbaar vervoersverbinding met de hoeve er nog steeds niet. Maar van het een kwam het ander en uiteindelijk resulteerde dat in het proefschrift dat nu voor je ligt. Nu het proefschrift bijna af is en de verdediging nadert, wordt het tijd om de mensen te bedanken die gewild of ongewild hebben bijgedragen aan de voltooiing van dit werk.

Allereerst wil ik Harm Krugers, mijn co-promotor, bedanken. Harm(-elito), ik prijs mijzelf erg gelukkig dat jij mijn begeleider bent geweest. Mijn niet zo positieve ervaring als promovenda in Utrecht is driedubbeldik gewist door mijn leuke tijd in Amsterdam. Jouw positieve levenshouding kon af en toe mijn negatieve spiraal omdraaien waardoor ik er weer 100 % zin in had. Als ik de hele dag nog niets had gemeten kwam je met een peptalk waardoor ik toch nog dat laatste plakje erin legde, en HOERA toch nog een meting had. Bedankt hiervoor!

Marian Joëls, mijn promotor, wil ik ook bedanken voor haar scherpe inzicht en de vrijheid die ik heb gekregen om een deel van mijn onderzoek in Londen uit te voeren. Ook in de eindfase van dit proefschrift ben je zeer onmisbaar geweest.

In tegenstelling tot het bij veel mensen heersende idee is promoveren niet een kwestie van in je eentje zitten mijmeren in een ivoren torentje. De handen moeten uit de mouwen, er moeten experimenten gedaan worden. Iemand die mij vaak, heel vaak, een helpende hand toestak in mijn torentje is Henk Karst. Beste Henk, zonder overdrijven kan ik zeggen dat ik een dunner proefschrift zou hebben gehad als jij mij niet zou hebben geholpen met het verkrijgen van data. Niet alleen voor mij maar voor het hele lab sta je altijd klaar, je bent echt de spil van het lab. Aan de andere kant van de gang is er nog een spil die ook erg belangrijk voor mijn proefschrift is geweest. Els Velzing, de duizendpoot die altijd alles voor je regelt. Hoe doe je dat toch ?! Jij bent van onschatbare waarde geweest bij het doen van de immuno´s, van het snijden tot het fotograferen. Ook was er vaak tijd voor een beetje afleiding met foto´s van leuke plantjes en diertjes. Natuurlijk zijn er nog heel veel andere collega´s in gebouw II die mij geholpen hebben, zo niet wetenschappelijk dan wel door het bieden van de broodnodige gezelligheid. Klaas en Dick, bedankt voor het verzorgen van de dieren. Suharti, jij hebt mij ingewijd in de kunsten van de histologie, bedankt hiervoor. Een deur verder kon ik altijd aankloppen bij Willem. Dank voor je enthousiasme, hulp bij microscoperen en voor je boeiende levenshouding. Monique, altijd leuk om met jou over jonge-meiden-mode te praten. Ik wil ook Wijnand Takkenberg en Erik Manders van de confocale microscoop bedanken voor hun hulp. Wijnand, live fast, die young. Jouw passie voor motoren is je vorig jaar fataal geworden. Je stond altijd klaar voor mij om te helpen, en geduldig drukte je dan de knop in die ik was vergeten…. Tijdens het scannen kon ik altijd leuk met jou kletsen over motoren, katten, geschiedenis of vrouwen. Werken bij de confogel is niet meer zo leuk als toen jij er was.

Mijn junior jaren als promovenda heb ik doorgebracht in een klein kamertje in goed gezelschap. Martin, de vrijdagmiddag acties waren altijd prima om stoom af te blazen. Sommige acties pakten alleen iets beter uit dan wij dachten….maar ja…weten wij veel!!?! Qinny, the chinese meals at your place (always the host, never the cook) were special events with lots and lots of food. Those were memorable evenings. En toen, Martin moest zijn proefschrift afmaken en ik ging maar eens boven kijken. Maar kamer 4.22 was eigenlijk ook best gezellig. Amos (de man die altijd gelijk heeft (shit)....maar ook de kamer vulde met muziek), Vivi (ik mis je openhartige en komische verhalen), Els (jij was altijd zo georganiseerd), Karin (veel succes met de laatste loodjes!), Solange (naast het brengen van gezelligheid kon ik veel van jou leren: wat moet ik lezen en hoe kan ik het koken), Neeltje (wat kun jij goed zingen!). Alle andere collega's: bedankt!

I also want to express my gratitude towards Frances Edwards for making it possible to do part of the work in this thesis in her lab at UCL, London. Thank you for your valuable discussions about my project. I also want to thank all the grrls from the lab, Anna (you are a very special person, not only did you teach me how to patch but also how to make cappucino!!!), Filomina, Roberta (I miss your hugs), Lily, Kana, David (Finally a boy!) and Stephanie (hanging around with you was so much fun, hope your new life will bring you love and happiness). I also want to thank all the other people for the nice evenings with beer and late night kebabs (Noah, you could always guide me to the best kebab-shop in town!).
Op de dag van de verdediging zullen er twee mensen naast mij staan die erg belangrijk voor mij zijn. Allereerst op links Tibor Brunt. Tiebje, ik ben erg blij dat jij mijn paranimf wilt zijn. Sinds jij mijn rekenmachine leende bij statistiek hebben we onafscheidelijk onze studietijd doorgebracht als een soort Batman en Robin, Snip en Snap, Peppie en Kokkie, Suske en Wiske, Bassie en Adriaan, Gert en Hermien etc etc.. Samen veel gekuierd in het Amsterdamse uitgaansleven...en dat blijven we hopelijk nog vaak doen! Mijn rechterflank wordt verdedigd door mijn andere paranimf Olof Wiegert. Lieve O., ik kwam je op dag 1 tegen toen ik op Anna's hoeve ging werken. Sindsdien werk ik met heel veel plezier met je samen. Je bent een brok amusement, het is nooit saai als jij er bent. Ik wens je veel succes in Bristol!
Als een echte socio-paat omring ik mij vaak met vrienden. Omdat biologen zo leuk zijn duikel je er daar veel op (Ja Joris, wij verplaatsen ons in groepen en komen altijd te laat) maar ook besturen (Chrisje, ik kan altijd erg lachen om je verhalen) en oud collega's (Celien, wanneer gaan we weer op vakantie?) kunnen vriendschappen opleveren. Ik wil jullie allemaal heel erg bedanken voor de afleiding en gezelligheid!
Al heb ik een kleine familie, die is mij toch zeer dierbaar: Mam, jij hebt mij geleerd om zelfstandig te zijn, en dat als je iets wilt je dat altijd kunt bereiken. Een levenshouding die heel belangrijk is! Lieve Erik, bedankt voor de leuke tripjes op je boot en je hulp met alles wat er maar voorbij komt. En nu het moeilijkste stukje van mijn dankwoord. Familie van Lanschot bedankt voor jullie liefdevolle ontvangst van mij in het gezin. Tot mijn grote verdriet zal Marten niet bij mijn verdediging zijn, en dat terwijl bij hem de grootste kans aanwezig was dat hij er wel iets van begreep. Marten bedankt voor al je interesse in mijn onderzoek, we missen je nog steeds heel erg.
En last but not least wil ik mijn lieve Derk bedanken. Bij mislukte experimenten moest jij vaak leiden onder mijn chagrijnigheid en probeerde je mij op te beuren door te zeggen dat ik het gewoon "de volgende dag toch nog een keer kon proberen?". Bedankt dat je er voor mij bent en hopelijk gaan we samen nog heel veel avonturen beleven!

DEBORAH

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Curriculum Vitae

Deborah Natascha Alfarez werd geboren op 30 juni 1975 in Amsterdam. Na het behalen van haar VWO diploma aan het St. Ignatius College te Purmerend, begon zij in 1993 met de studie Medische Biologie aan de Universiteit van Amsterdam. In de specialisatie fase van de studie deed zij onderzoekservaring op bij het Nederlands Instituut voor Hersenonderzoek in de groep van Prof. Dr. R. Buijs en bij de Faculteit Diergeneeskunde, afdeling proefdierkunde onder leiding van Dr. Z.L. Haberham en Prof. Dr. L.J. Hellebrekers. Ook heeft zij een educatieve stage gelopen bij science centrum NEMO in Amsterdam. De studiefinanciering was nog niet op en om zichzelf toch een beetje te kunnen redden in het veld heeft zij in 1997 haar propedeuse Biologie gehaald. In 1998, na het behalen van het doctoraal examen, begon zij als promovenda bij Kinder- en Jeugdpsychiatrie aan het UMC van de Universiteit Utrecht. Na een korte kennismaking met de psychiatrie werd deze loopbaan toch verruild voor een promotieplek bij het Swammerdam Institute for Life Sciences, Centre for NeuroScience, Faculteit Natuurwetenschappen, Wiskunde en Informatie aan de Universiteit van Amsterdam. Onder leiding van Dr. H.J. Krugers en Prof. Dr. M. Joels rondde zij in maart 2006 haar promotieonderzoek af, waarvan het resultaat nu voor u ligt. Naast haar werkzaamheden als onderzoeker heeft zij zich ook ingezet voor de belangen van jonge onderzoekers en is zij co-auteur van het populair wetenschappelijke boek " In de toekomst is alles fantastisch" verkrijgbaar mei 2006 bij Fontaine uitgevers.

A little nonsense now and then,
is cherished by the wisest men.

Roald Dahl, Charlie and the Chocolate Factory