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# Stress in the brain

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**Abstract**

Part I (first section) reports about research in the period 1964–1976, when the seminal observations were made on which today's concept of corticosteroid action on the brain is based. These key observations concern the discovery of nuclear corticosterone receptors in the limbic brain that mediate control over neuronal circuits underlying hypothalamic–pituitary–adrenal activity and behavioural adaptation. Part II (second section) covers the period of 1977–1989. It is about some aspects of the neuropeptide concept, the implementation of micro-neurochemistry using the "Palkovits punch", and the application of in vitro autoradiography. Vasopressin and oxytocin receptors were identified and their implication in behaviour was examined using the song control of the canary bird as a model system. Two distinct nuclear receptor types for corticosteroids were identified: mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) which mediate in a coordinate manner the steroid control of hypothalamus–pituitary–adrenal activity and behaviour. Part III (third section) is from 1990 up to 2000. Focus is on the balance of MR- and GR-mediated actions in control of homeostasis as a determinant of health and disease. MR operates in *pro-active* mode to prevent homeostatic disturbance, while additional GR activation promotes in *reactive* fashion recovery after stress. An imbalance in MR and GR underlies behavioural deficits and neuroendocrine disturbances increasing vulnerability for stress-related brain disorders. The complete hippocampal genome is screened for corticosteroid responsive genes, which are potential targets for drugs promoting restorative capacity still present in the diseased brain. 

*Keywords:* Stress; Corticosteroid; Hypothalamus–pituitary–adrenal activity

The *Leitmotiv* of this essay is the action of corticosteroid hormones in the central nervous system. The hormones are secreted in circadian fashion and after stress. As adrenocortical secretory product the stress hormone acts in concert with other components of the hypothalamic–pituitary–adrenal axis in order to facilitate coping with stress and behavioural adaptation, and to prepare the organism for the next encounter. In order to achieve this goal the corticosteroids exert long-term control over cell metabolism to allow sufficient fuel for energy demanding neural processes. They control neuronal excitability and the activity of specific neurotransmitter and neuropeptide systems. Corticosteroids are critical in programming brain function for life during early life experiences, and they are major vulnerability factors in the precipitation of stress- and age-related disorders.

Depending on the nature of a stimulus processing occurs in specific neuronal pathways, producing various cocktails of secretagogues stimulating pituitary adrenocorticotropin (ACTH) and adrenal corticosterone release. While ultimately the secretion of corticosteroids occurs in rather non-specific fashion in response to every stimulus, which disturbs or threatens to disturb homeostasis, their action on target organs such as the brain has an enormous diversity. This is because corticosteroid action depends on the type of nuclear receptor activated by the hormone, and on the (molecular and cellular) context hormone action is operating. This is an essential feature for the corticosteroids, since it requires a precise definition of environmental conditions — and thus, experimental design — for assessment of the corticosteroid effects. For instance, if corticosteroids are administered beyond the context of their physiological functions (such as in response to a stress), it is often reported that the hormone causes deficits in, e.g. memory. As was pointed out (de Kloet et al., 1999) behaviour under these "out of context conditions of stress

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and elevated hormone concentrations" shifts to a more opportune response, eliminating memory which is at that time no more relevant rather than that it disturbs memory.

The search for the site and mechanism of negative feedback action of corticosteroids is where my contribution to *David's Festschrift* begins. I will discuss three episodes. First, the period of *upbringing* from 1964 to 1976 starting with the initial observation by David de Wied that dexamethasone, a potent synthetic glucocorticoid, primarily acts on the pituitary level in blocking stress-induced ACTH release. It ends with characterization of brain and pituitary receptors for corticosteroids. The second period from 1977 to 1989 represents *coming of age* in the Rudolf Magnus Institute (RMI). During this period key observations on the two corticosteroid receptor types, mineralocorticoid (MR) and glucocorticoid receptors (GR) were made, while for the first time, oxytocin and vasopressin receptors were identified in brain. *Earning a living* features the period 1990–2000. It marks the development in Leiden of a multi-disciplinary research program from gene to behaviour aimed to resolve the action mechanism of corticosteroids and to identify novel gene targets for the hormone in the brain.

The current research program involves intensive collaboration with three former RMI colleagues. On the molecular and cellular level Marian Joëls, and in research on behaviour and autonomic functions Béla Bohus and Wybren de Jong. It also is embedded in collaborative research with friends like Bruce McEwen, Seymour Levine, Jonathan Seckl, William Rostene, Alex De Nicola and Florian Holsboer, among others. Last but not least it is inspired by one of the most creative contemporary Dutch neuro-endocrinologists: David de Wied.

## 1. 1964–1976. Corticosteroids: how do they act in brain and pituitary?

### *1.1. Glucocorticoid feedback: brain or pituitary?*

Who does *not* remember that during this episode the neuropeptide concept evolved from the notion that end products from large precursor molecules such as ACTH, vasopressin and oxytocin not only exerted classical endocrine effects, but also affected brain and behaviour? And what about peptide fragments devoid of endocrine effects that still maintained their activity in centrally regulated processes? And were not they common products for endocrine glands, neurons and the immune system? (reviewed in de Kloet and de Wied, 1980). Meanwhile, somewhat hidden under this avalanche of neuropeptide data, David de Wied also had established as a classical endocrinologist the pituitary as a primary site of action for dexamethasone in the suppression of stress-induced ACTH release (de Wied, 1964). That is where my story begins.

The identification of corticosteroid feedback sites and mechanisms were "hot" topics in the sixties. One favourite approach was the implantation of minute amounts of crystalline corticosteroids at various places in the brain, and this approach used in rats unequivocally identified the medial basal hypothalamus as a prominent feedback site for synthetic and naturally occurring glucocorticoids, e.g. corticosterone in rodents and in humans also cortisol. de Wied's approach was to measure the effect of certified "stressors" in the dexamethasone acetate pre-treated median eminence lesioned rat. Circulating ACTH levels were measured by using the in vitro adrenal corticosteroid production as index. de Wied concluded that "the blocking action of dexamethasone is located in the anterior pituitary" suggesting that a "negative feedback action between adrenal steroid secretion and anterior pituitary function might operate under physiological conditions." de Wied also suggested that "circadian rhythms and common everyday environmental stimuli are regulated by the central nervous system by a feedback balance" (de Wied, 1964).

Unaware of these important novel findings in the corticosteroid field, I met David de Wied for the first time rather superficially in 1965, when I was a student with Kien Ebels engaged in the isolation of biologically active peptides from sheep pineal glands. Similar work was performed with brain extracts by one of de Wied's top ranked associates: Albert Witter. After a training at NV Organon under supervision of Johan van der Vies and the late Professor Marius Tausk it was on November 2, 1968, 9:00 a.m. in the professor's room at the RMI that I attended a discussion between David (de Wied) and Stefan (Szpilfogel) that decisively determined my (scientific and private) life. David called Stefan and arranged within a few minutes my PhD position at NV Organon (supervision Johan van der Vies) to study the mechanism underlying the dexamethasone suppression of stress-induced hypothalamus–pituitary–adrenal activity.

### *1.2. Hippocampus: prime target for corticosterone controlling stress in the brain*

My scientific development at Organon and the RMI then connects to the story of Bruce McEwen at the Rockefeller University. Just starting my PhD research with David de Wied in Utrecht and reading Bruce's Nature paper (McEwen et al., 1968), we assumed that the potent synthetic glucocorticoid dexamethasone would be even better retained in hippocampus than the naturally occurring glucocorticoid corticosterone. That assumption appeared incorrect. Dexamethasone was poorly retained in hippocampus irrespective a peripheral or an intracerebroventricular (i.c.v.) route of administration. As was concluded in my thesis: "the pituitary rather than the brain is the principal site of action of dexamethasone in the suppression of stress-induced ACTH release" (de Kloet et al., 1974).

Meanwhile, Bruce had become my mentor. In 1975 and 1976, we published a series of papers firmly demonstrating that dexamethasone poorly penetrated in brain (see Fig. 1). The small amounts of dexamethasone that did penetrate in brain were retained in a regional pattern that was distinctly different from corticosterone (de Kloet et al., 1975; McEwen et al., 1976). Also a steroid such as cortisol — not naturally occurring in the rat — was poorly retained in brain. However, the hippocampus and other limbic structures did accumulate and retain corticosterone, and also aldosterone. In autoradiograms, the pyramidal neurons and the dentate gyrus stand out as prime targets for these steroids. Corticosterone — the important stress hormone — retained in the hippocampus, an area with a critical function in cognition and affect. That could not be a coincidence!

The finding determined my scientific career. The localization of a stress hormone in the hippocampus had to be extremely important to understand stress in the brain. I have kept the correspondence with David about this challenging new concept of corticosteroid action in higher brain region. We agreed that: (1) there was for corticosterone more than a hypothalamus to act in the brain; (2) there are distinctly different modes of action of dexamethasone and corticosterone in the brain or, as David put it in his letter of January 7, 1974 to me at The Rockefeller University, New York City: “dexamethasone and corticosterone cannot be put in the same sack (kunnen niet over één kam geschoren worden).”

### *1.3. Why dexamethasone poorly penetrates in brain*

Dexamethasone poorly penetrates in brain. A tracer amount of [$^3$H]dexamethasone administered to adrenalectomized rats or mice is poorly retained by nuclear receptors in brain, while pituitary corticotrophs containing equivalent amounts of these receptors accumulate and retain large amounts of this synthetic steroid. It was only in the mid-nineties that we understood why. The reason was the multidrug resistance P-glycoprotein (mdr1a-) localized in the blood brain barrier, which extrudes xenobiotics including synthetic glucocorticoids (Schinkel et al., 1996). We showed that adrenalectomized mice with a genetic disruption of the mrd1a gene (the mutants were made available by Alfred Schinkel and Piet Borst of the Netherlands Cancer Institute) have a ten-fold increase of [$^3$H]dexamethasone uptake and retention in brain glucocorticoid target sites reaching levels observed in the pituitary (Meijer et al., 1998a). These data demonstrate that dexamethasone is extruded from brain by the mdr1a-P-glycoproteins. The data support the concept of a pituitary site of action of dexamethasone in the blockade of stress-induced ACTH release, which implies that chronic dexamethasone treatment does not replace the endogenous corticosteroids with respect to activation of in particular the MR in brain. Cortisol — not naturally occurring in the rat — is also extruded from the brain of rodents and surprisingly also from human brain (Karssen, unpublished). Interestingly, access of cortisol and corticosterone to the cell nucleus of pituitary corticotrophs is hampered by intracellular corticosteroid binding globulin (de Kloet et al., 1977).

The implication of this work in the mid seventies was that suppression of pituitary ACTH and adrenal corticosterone release by dexamethasone created a state of “chemical adrenalectomy.” In other words: moderate amounts of dexamethasone were hampered in access to central targets and at the same time depleted the brain from its endogenous corticosteroids. About 20 years later,

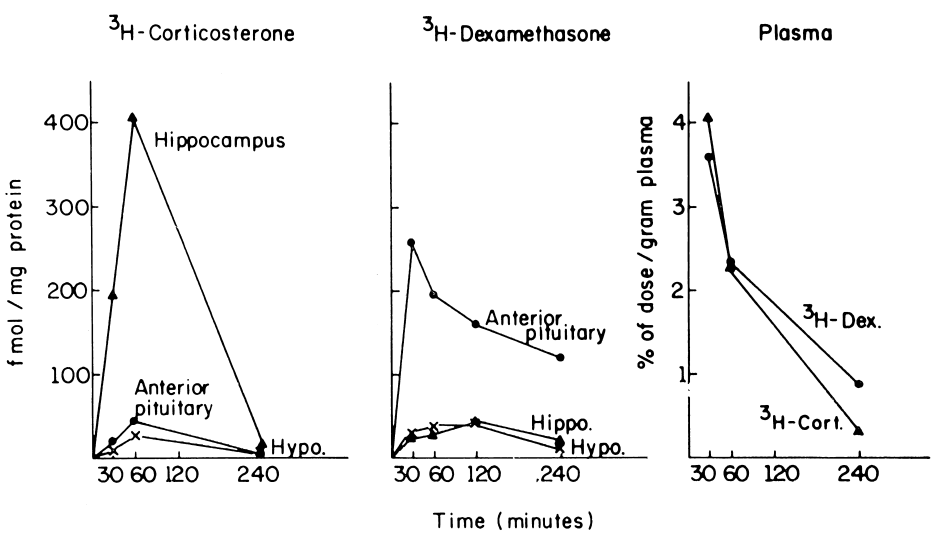


Fig. 1. Binding of [$^3$H]corticosterone and [$^3$H]dexamethasone in vivo to purified cell nuclei of hippocampus, hypothalamus and anterior pituitary, at various time intervals after intravenous injection of the [$^3$H]steroids in doses of 5 nmol. Binding is expressed as fmol steroid/mg protein. Radioactivity per ml of plasma is expressed as a percentage of the dose. Each data point is the average of results from three experiments. The standard error ($\pm$S.E.M.) of each data point is in the order of $\pm$5% of the indicated value, but is not shown in the figure (reprinted with permission from De Kloet et al., 1975).

we and others observed that the "chemical adrenalectomy" caused by dexamethasone treatment actually increased apoptosis of dentate gyrus neurons in the hippocampus (Hornsby et al., 1996), a process that previously had been described to occur after adrenalectomy. The induction of apoptosis appeared more pronounced if the synthetic steroid was given to aged animals, and it could be prevented by pretreatment with corticosterone (Hassan et al., 1996). Accordingly, deficits in brain function after low dexamethasone may be due to *under*-rather than *overexposure* to corticosteroids.

Around the time we first described the fate of [$^3$H]dexamethasone and its accumulation in the pituitary corticotrophs, dexamethasone just had become applied in the so-called dexamethasone suppression test (Carroll et al., 1976). This appeared to be a helpful test in the detection of a hyperactive hypothalamus–pituitary–adrenal axis common to about half of the depressive patients. The test has ever since been used as a test for hypothalamus–pituitary–adrenal dysfunction. For instance in our studies we were able to classify patients suffering from the fibromyalgia syndrome, a syndrome characterized by incapacitating pain in the tender points, which actually increases during exercise (Griep et al., 1998). Our findings provided firm evidence that fibromyalgia syndrome is a stress-related disorder characterized by pain amplification, adrenocortical hyporesponsiveness and disregulated corticosteroid feedback action in the brain. Likewise, the chronic fatigue syndrome also can be classified as a stress-related disorder characterized by apparent corticosteroid receptor supersensitivity in key locations of the stress system and reduced corticosteroid output (Visser et al., 1998). In view of the secondary adrenal deficiency observed in fibromyalgia syndrome and chronic fatigue syndrome, we are facing a disease state in which the symptoms resemble those observed after a lack of corticosteroids. The administration of corticosteroids does not ameliorate the condition, since the deficit is at the nuclear receptor level (theses of Jeroen Visser, 2000 and Ed Griep, 2000, Leiden University).

## 2. 1977–1989: Steroids, neuropeptides and the brain

After my return, the quest was to combine the steroid receptor research with the ongoing work on neuropeptides in the RMI. To "conquer a position" in the ongoing highly competitive neuropeptide research at the RMI two neuropeptide links were pursued. In addition, I was "promoted" in 1977 to scientific secretary in charge of the so-called "LPH program" funded by NV Organon with the goal to identify novel biologically active peptides. This program lasted 8 years. Meanwhile, the work on steroid receptors was secured by two grants (to Door Voorhuis and Dick Veldhuis 1978–1982) from the Netherlands Research Organization.

### *2.1. Neuropeptide link I: amine biochemistry, retrograde transport and peptide metabolism*

Encouraged by David, I further developed my expertise in neuroanatomy and brain dissection. In the early seventies I used dissection of the brain in 16 neuroanatomically defined regions. This elementary knowledge served as basis to acquire the Palkovits punch technique in which more than 100 nuclei with a diameter between 200 and 1000 μm were punched out of frozen brain sections for various assays (at a rate of 1200 punches/h). This research proceeded among others, in collaboration with the groups of Dirk Versteeg, Gabor Kovacs and Wybren de Jong interested in peptide effects, on markers for catecholamine and serotonin turnover. The 1-month stay in Budapest led to a collaborative project with Eva Mezey. At that time it was debated whether pituitary peptides could also modulate brain function by retrograde transport higher up through the pituitary stalk to the pericapillary space in the median eminence. The answer is unambiguously yes! Pituitary peptides can reach the pericapillary space from where they are capable to freely diffuse into the cerebroventricles and to synaptic terminals (Mezey et al., 1978; Dorsa et al., 1979; de Kloet et al., 1986).

Peter Burbach was my first student (PhD in 1980), and I was fortunate enough to obtain an institutional grant to pursue with Peter the metabolism of endorphins and neurohypohyseal hormones in the brain (see Burbach, this volume) (de Wied, 1969; de Wied et al., 1993). His research was fundamental for the neuropeptide concept, since he showed that peptide fragments, devoid of classical endocrine activity, still had maintained activity to modulate centrally regulated functions underlying the behavioural performance of rats in passive and active avoidance test conditions. Peter demonstrated that some of these peptides, e.g. the α and γ endorphins, vasopressin-(4-9), were generated as stable intermediates and we characterized the enzymes involved. Supported by an NWO grant, Jos Lebouille (PhD in 1986) developed routine assays for the measurement of endopeptidase activities involved in generating these neuropeptides.

### *2.2. Neuropeptide link II: from brain receptors to the canary song control system*

While the genes encoding pro-opiomelanocortin (POMC)-related peptides and neurohypophyseal hormones were cloned, we identified in 1983 for the first time high affinity binding sites for vasopressin and oxytocin in the rat brain using in vitro autoradiography (Biegon et al., 1984). The distribution of the labelled sites appeared highly discrete in limbic–midbrain areas, and the sites were indicated as "receptor hot spots." The receptor dense areas were concentrated in small areas with high density, which gradually spread with a decreasing density mostly in a frontal caudal direction.

Jack Elands (PhD 1989) characterized the binding sites for vasopressin and oxytocin. He used vasopressin and oxytocin analogs with a tyrosin amide residue at position 9, which could be iodinated allowing detection and quantitation of very low amounts of receptor (Elands et al., 1988). Thus, V1a receptors were found in lateral septum, ventral hippocampus, central amygdala and n tractus solitarii. OT receptors in olfactory nucleus*, olfactory tubercle*, bed nucleus of the stria terminalis*, central amygdala*, ventral hippocampus and ventromedial nucleus** (* marks estrogen responsiveness, de Kloet et al., 1986a). We also found that the V1a receptor binds vasopressin with high affinity, while the oxytocin receptor is apparently non-selective showing similar affinity for oxytocin and vasopressin. The localization and binding specificity of the receptor sites has provided important criteria for the study of functional responses to these peptides. We also identified high affinity binding sites for the vasopressin-(4-9) fragments in circumventricular and periventricular brain areas, which may explain why these peptides were still active in behaviour when administered i.c.v. (de Kloet et al., 1985).

The discrete localization of the vasopressin and oxytocin receptors ensures selective control of the peptides over the efficiency of information handling in the limbic-midbrain circuitry. Based on this remarkable property of the central oxytocin and vasopressin receptors we postulated that these peptides promote coordination and synchronization of the chain of events, and thus the transition of behavioural states, following stress as well as during the circadian and reproductive cycle.

Together with David de Wied and Door Voorhuis (PhD 1990) we have tested this hypothesis by targeting the song control system of the canary with a vasotocin analog. The singing behaviour of the canary may be considered as an expression of male reproductive behaviour. With song the male attracts a female, competes with another male and claims territorial ownership. Singing has been demonstrated to require a learning process. These basic facts were the basis of a series of pioneering experiments by Door on the role of the central vasotocin system of the male canary in singing behaviour. The canary is an "open-ended learner," which implies that every autumn the song repertoire of the bird can be changed until a new stable song repertoire is achieved in the spring. The acquisition of new syllables to the song repertoire coincides with a dramatic increase in volume of two nuclei of the motor control system, i.e. the nucleus hyperstriatum and the nucleus robustus archistriatalis. Testosterone facilitates neurogenesis, growth and connectivity in these nuclei and the steroid is necessary for transition from unstable plastic song to full song.

The findings of Door Voorhuis were generated in three phases.

- First, a sexual dimorphic testosterone- and season-dependent vasotocin immunostaining was discovered in the lateral septum and the bed nucleus stria terminalis, which are areas involved in motivational aspects of reproductive behaviour. Vasotocin immunoreactive fibers also innervate the region encapsulating the robustus archistriatalis, the song control nucleus (Voorhuis et al., 1988).
- Second, one population of low affinity testosterone-sensitive neurohypophyseal hormone binding sites is widely distributed in the canary brain including the robustus archistriatalis. High affinity vasotocin receptors exclusively surround the robustus archistriatalis. Thus, the morphological features of the central vasotocin system in the bird strongly favoured our hypothesis of vasotocin control of singing behaviour (Voorhuis et al., 1990).
- The third phase was most intriguing: How to test the hypothesis on the role of vasotocin in singing? We have intuitively chosen a brief treatment with the desglycinamide vasotocin analog, which we had previously used successfully in the experiments on male preference and sexual behaviour of the female rat. The desglycinamide vasotocin analog treatment was given the first 3 days (0.7 μg, s.c., three times daily) to castrated birds which had received on day 0 a silastic implant containing testosterone to ensure exposure to this steroid for 4 weeks. The testosterone treatment was chosen to ensure that the birds would all have comparable levels of the steroid. The quality of the song was analyzed with time frequency sound spectrography. For further experimental details see Voorhuis et al. (1991). Birds were tested for 30 min in singing behaviour in the first 30 min immediately following lights on. We found that the short-term desglycinamide vasotocin analog treatment influenced the amount of singing behaviour measured between 1 and 4 weeks after onset of the experiment. The song duration (seconds of song/30 min) was affected in a dual mode. In early autumn the desglycinamide vasotocin analog enhanced song duration of testosterone-primed canaries, but the same vasotocin analog decreased song duration in the period November/January (see Fig. 2). These results suggest that the neuropeptide vasotocin is implicated in control of seasonal changes in singing behaviour. The peptide seems to advance the seasonal chain of events underlying the development of singing behaviour in the canary (Voorhuis et al., 1991).

### *2.3. Mineralocorticoid (MR) and glucocorticoid receptors (GR) in hippocampal neurons*

In those years, I was also focused on the further characterization of corticosteroid receptor function in the brain. Before 1976, knowledge unanimously predicted the paraventricular nucleus in the hypothalamus as the major site of the central action of corticosterone and receptors were expected to be localized in the corticotrophin-releasing hormone producing cells. Therefore, the abundant and highly selective retention of corticosterone in pyramidal

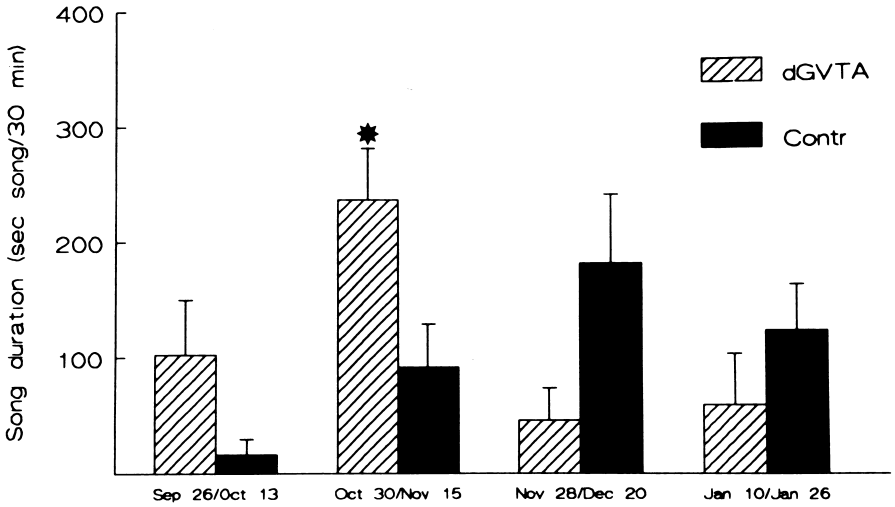


Fig. 2. Mean song duration (30 min in control canaries (contr.)) and canaries treated with desglycinamine vasotocin analog in four different experiments carried out at different times of the year.

and granular neurons of the hippocampus (McEwen et al., 1968) was an unexpected finding and led to much speculation about their function. In view of their localization in hippocampus, the corticosterone receptors were considered to mediate effects on learning and memory processes, mood and affect, while dysfunction of these receptors was thought to be related to cognitive failure, depressed mood and damage.

In 1980, selective glucocorticoids of Roussel-Uclaf had become available. Dick Veldhuis (NWO, PhD 1982) discovered that in cytosol of hippocampus mineralocorticoid (MRs) and glucocorticoid receptors (GRs) were present (Veldhuis et al., 1982). The tracer amounts of corticosterone retained so abundantly in hippocampus of adrenalectomized rats actually were bound with high affinity to MRs, and not to GRs (Reul and de Kloet, 1985). The affinity of corticosterone to GRs in hippocampus appeared to be too low for a detectable signal using these tracer doses. Thus, while before 1985 corticosteroid effects in hippocampus were solely ascribed to GRs, the field had to readjust its focus to two corticosteroid receptor types, i.e. MRs and GRs. Hans Reul (NWO, PhD 1987) pioneered on the discrimination between MR and GR, continued working with John Funder and after his return to the Netherlands he first examined MR and GR in the aging dog before becoming group leader at The Max Planck Institute for Psychiatry in Munich in 1991. The immunocytochemical and in situ hybridization studies by Anke van Eekelen (NWO grant, PhD 1991) clearly demonstrated the localization of MR and GR in rat brain (Van Eekelen et al., 1988), and co-localized in the same clusters in the cell nucleus.

In 1986, Win Sutanto joined the group strengthening the corticosteroid research line. His task was to further characterize the MR and GR. In a first series of experiments, he found that in a cortisol secreting species: the hamster, MR displayed a much higher affinity to cortisol than to corticosterone (Sutanto and de Kloet, 1987). Subsequently, he found that the tracer cortisol was retained very well in vivo in the hamster hippocampus, while little cortisol penetrated in the rat brain (Sutanto et al., 1988). In industry supported programs the MR was screened for potential novel ligands; potent ligands were progesterone and 11-OH-progesterone. The action of gonadal steroids on affinity and capacity of in particular hippocampal MRs appears related to the sex differences in hippocampal regulation of the hypothalamus–pituitary–adrenal axis (Carey et al., 1995). MR shows a high degree of plasticity in response to neurotrophic peptides. For instance, behaviourally active superagonist ACTH-(4-9) analog induced MRs in the hippocampus of aged rats, in which this receptor is dramatically reduced in number (Reul et al., 1988); vasopressin-related peptides upregulate MRs (Veldhuis and de Kloet, 1982) and interleukin 1 decreases its affinity (Schöbitz et al., 1994).

The enzyme 11β-hydroxysteroid dehydrogenase converts bioactive corticosterone and cortisol to their inactive metabolites. The enzyme is co-localized with MR in epithelial cells in kidney and periventricular brain, where it confers aldosterone selectivity. The hippocampal neurons lack the enzyme and thus retain both aldosterone and corticosterone (Edwards et al., 1988).

### *2.4. Function of MR and GR in neuroendocrine regulation and behaviour*

Corticosterone maintains via MR in hippocampal neurons a high excitatory tone over an inhibitory GABAergic network surrounding the CRH-producing neurons in the PVN (Joëls and de Kloet, 1992). Accordingly, MR antagonist i.c.v. disinhibit basal and stress-induced hypothala-

mus–pituitary–adrenal activation via these receptors. GR antagonists i.c.v. interfere with negative feedback and prolong stress induced hypothalamus–pituitary–adrenal activation (Ratka et al., 1989). Chronic administration of the GR antagonist i.c.v. enhanced the amplitude of the circadian hypothalamus–pituitary–adrenal activity and the response to stress (Van Haarst et al., 1996). Chronic administration of the MR antagonist caused only a small increase in adrenocortical output.

Four types of behavioural studies were performed.

*First*, with Béla Bohus the stringent specificity of corticosterone in the so-called forced extinction paradigm was discovered. Rats were exposed to a passive avoidance test situation and 3 h later exposed to the space where the aversive experience had occurred. If animals were adrenalectomized 1 h previously this so-called forced extinction procedure did not work. Only when a low dose of corticosterone was administered at the time of adrenalectomy forced extinction was effective and the animals displayed only small latencies at 24-h retest. Since dexamethasone was ineffective, this effect was ascribed to the hippocampal MR (Bohus and de Kloet, 1981).

*Second*, also together with Béla Bohus we found that learning a passive avoidance response was enhanced by systemic administration of adrenaline and that the efficacy of adrenaline was reduced one million-fold after treatment with a low dose of corticosterone, but not with dexamethasone, which further points to an MR selective effect (Borrell et al., 1984). The link of corticosteroids with emotional behaviour was further pursued in the mid nineties, when Mechiel Korte performed experiments showed that both MR- and GR-mediated effects modulated distinct aspects of anxiety: MR activation appeared anxiolytic, while via GR cognitive components were included in fear conditioning (Korte et al., 1995).

*Third*, with Anna Ratka corticosteroids were found to exert a long lasting effect in the anti-nociceptive effect of morphine and β-endorphin. We found that adrenalectomy dramatically decreased the efficacy of morphine in the so-called "hot-plate" test, but only under conditions that the pre-surgical corticosterone levels were low. If corticosterone concentrations were elevated by stress or corticosterone injection latency times to respond to the high temperature were dramatically reduced and this effect of corticosterone was even detectable up to 2 weeks after adrenalectomy (Ratka et al., 1988).

*Fourth*, with Dick Veldhuis we found that blockade of GR by a selective glucocorticoid antagonist i.c.v. interfered with retention of acquired immobility in the Porsolt swim test. This effect was also observed after local administration of a few nanograms of the antagonist in the dorsal hippocampus, while administration of the antagonist in the midbrain or PVN was not effective (de Kloet et al., 1988). The latter injection in the PVN enhanced ACTH and corticosterone secretion. In subsequent studies Mechiel Korte demonstrated with a GR anti-sense approach also the role of hippocampal GRs in retention of an acquired immobility response (Korte et al., 1996).

## 3. 1990–2000: Hormones and brain function in Leiden

After David's retirement, I was appointed as Professor and Head of the Division of Medical Pharmacology at the Leiden/Amsterdam Center for Drug Research. This section will summarize some new research lines and highlights of the past decade. A complete account is given in de Kloet (1991) and de Kloet et al. (1998).

### *3.1. Electrophysiology*

The observations by Marian Joëls on the cellular actions of corticosteroid hormones in hippocampus have opened a complete new field of research. Our 1989 paper (Joëls and de Kloet, 1989, 1990, Joëls et al., 1991; See Joëls in this volume) initiated this research by demonstrating that via MRs corticosterone maintains excitability and therefore a high excitatory tone in the hippocampal circuitry. GR-mediated effects suppress excitability of hippocampal neurons transiently raised by excitatory input. Accordingly, maximal stability and optimal viability are achieved under a condition of predominant MR and little GR occupation corresponding to the receptor occupancy achieved by the average corticosteroid level circulating over a 24 h period (Joëls and de Kloet, 1994). Mice homozygous for GR gene disruption further demonstrated the critical significance of MR.GR interaction for excitability (Hesen et al., 1996).

### *3.2. Behaviour: spatial learning and memory*

Using the Morris water maze, Melly Oitzl demonstrated how MR and GR activation contributed to the efficiency of the various stages in processing of spatial information. This finding of Melly Oitzl is considered the hallmark for understanding the behavioural implications of corticosteroid receptor diversity in brain (Oitzl and de Kloet, 1992). MR-mediated effects are critical during exposure to a novel environment, which can be a learning experience. The animal explores, interprets and chooses a particular behavioural repertoire to deal with this new experience. Also during retrieval of information an MR responsive network in limbic structures, including the hippocampus, operates in behavioural reactivity and response selection. GR-mediated effects induced by the stress of the novelty and/or learning experience dominate during the consolidation phase and promote information storage (Oitzl and de Kloet, 1992; Oitzl et al., 1994). Naturally, when GR-mediated effects occur out of context, or when the steroid is chronically elevated, or the receptor (i.e., transcription machinery) is deficient, ongoing behaviour is changed, and

shifts to a more opportune response (Oitzl et al., 1997a,b; de Kloet et al., 1999),.

### 3.3. Central cardiovascular regulation

In experiments with Wybren de Jong and Désirée Van den Berg (PhD, 1993), the role of corticosteroid receptors in central regulation of blood pressure was investigated. We demonstrated that MR antagonist i.c.v. suppressed after a delay of several h the pressor response to the stress of measuring blood pressure using the systolic blood pressure using the tail cuff. This procedure involved daily repeated exposure to restraint and warming. While the effect of MR antagonists (i.c.v.) lasted about 24 h, and even longer when deoxycorticosterone acetate (DOCA) salt-hypertensive rats were used, GR antagonist (i.c.v.) had the opposite effect. Blockade of central GRs enhanced the pressor response and this effect lasted 3 days (Van den Berg et al., 1990, 1994). MR antagonists (i.c.v.) also enhanced diuresis, which was obliterated after denervation of the kidney (Rahmouni et al., 1999). Vasoactive peptides such as vasopressin (Saravia et al., 1999) and angiotensin II (van Acker and Sibug, unpublished) are involved.

### 3.4. The serotonin link

In the hippocampus, we found that a low dose of corticosterone enhanced serotonin (5-HT) synthesis rate and release. Aldosterone blocked this effect demonstrating implication of MR (de Kloet et al., 1982, 1983; Korte-Bouws et al., 1996). 5-$HT_{1A}$ receptor expression (Onno Meijer, PhD in 1996) was down-regulated in selective hippocampal regions, particularly in dentate gyrus (de Kloet et al., 1986b; Meijer et al., 1998b). Meanwhile, together with Hans Sijbesma (PhD in 1991) the signalling of eltoprazine (a mixed 5-$HT_{1A}$ and 5-$HT_{1B}$ receptor agonist) in brain was investigated.

In the electrophysiological studies by Marian Joëls, it was shown that MRs also suppressed the 5-$HT_{1A}$ hyperpolarizarion response in hippocampal neurons (Joëls et al., 1991). However, occupation of GR with higher concentrations of corticosterone counteracted and obliterated the MR-mediated suppression of the 5-$HT_{1A}$ response revealing a U-shaped dose response curve. It seems therefore that MR and GR control in a coordinate, but opposite manner, a common denominator in post-synaptic 5-HT signalling. Moreover, if the corticosterone concentration is chronically elevated, the GR-mediated effect desensitizes and the neurons become less responsive to 5-HT (Karten et al., 1999). Collectively, these data demonstrate that corticosterone suppresses post-synaptically 5-HT signal transduction in site-specific fashion at low concentrations and at chronically elevated concentrations. The latter observation in the pioneering studies of the Joëls group has important implications for depression, since it suggests that the corticosterone-5-HT down-regulatory action precedes onset of the disease.

### 3.5. Programming the stress system by genotype and early life events: implications for the aging process

Several lines of investigation have merged in this theme. *First*, our studies on the role of the genotype which were performed by Nynke Rots (PhD 1995) with Alexander Cools using rat lines genetically selected for extreme differences in apomorphine-induced gnawing behaviour. The rat line with the highest dopaminergic activity shows a strong attenuation of the prolactine and corticosterone response to stress (Rots et al., 1996). Dopaminergic activity and corticosterone responses were also both affected by a traumatic early life experience.

*Second*, how the hypothalamus–pituitary–adrenal axis develops and what the effect of early experience is. These studies were inspired by Seymour Levine leading to a collaboration, which started in 1986 via research conducted by Rosenfeld et al. (1993) (PhD in 1989). She focused on the careful mapping of corticosteroid receptor types in the developing brain and the role of maternal behaviour. It evolved in the PhD theses of Helga van Oers (1998) and Judith Workel (1999) focusing on the immediate and persistent effects of maternal deprivation on the stress system and cognitive functions (see below and the paper by Levine).

*Third*, aging research, which involved the thesis research of Anke van Eekelen (1991), (Van Eekelen et al., 1995) the post-doctoral research of Hans Reul with the aging dog, and the outcome of the effect of early life experience for the aging process studies by Marcel Schaaf (PhD 1999), Judith Workel and Melly Oitzl from the perspective of central growth factors, corticosterone and cognitive function. These studies have in common that they demonstrate a critical role for corticosterone in cognitive aging.

### 3.6. Current research

Thus, corticosteroid hormones secreted by the adrenal cortex readily enter the brain, bind to nuclear receptors and modify gene transcription either directly (transactivation) or indirectly via protein–protein interaction with other transcription factors. MR and GR are colocalised in neuronal circuits (limbic circuitry) with a critical function in behavioural adaptation, learning and memory processes. MR binds corticosteroid with high affinity and appear involved in the process underlying behavioural reactivity towards novel objects and plays a role in appraisal of this information (Oitzl et al., 1994). MR operates, therefore, in *pro-active* fashion and activates gene products with a function in the *maintenance* of homeostasis. GR binds corticosteroid with lower affinity and is occupied by cor-

ticosteroid only after stress. GR activation in hippocampus promotes behavioural adaptation (Oitzl and de Kloet, 1992). It operates in *reactive* mode and its responsive gene products promote *recovery* of disturbed homeostasis after stress.

The *objective* of the Leiden neuroscience research program is to identify novel molecular targets as leads towards treatment of stress-related brain disorders. For this purpose we conduct multidisciplinary studies from gene to behaviour focused on the action mechanism of the corticosteroid stress hormones in the brain mediated by MR and GR. These two nuclear receptors operate in coordinate fashion to control the expression of gene products with a critical function in neuronal plasticity and behavioural adaptation. The corticosteroid-responsive genes are now being identified using gene expression profiling technology developed by Nicole Datson and Erno Vreugdenhil, and subjected to in-depth functional analysis. The underlying notion is that a fundamental property of brain and behaviour is to change and to adapt. The corticosteroid responsive genes we are analysing qualify as candidate plasticity genes. Exposure to aberrant corticosteroid concentrations will reveal genes that become disregulated, and are considered at the same time potential targets to promote the restorative capacity still present in the diseased brain.

### 3.7. Hypothesis

We hypothesise that shifts in the balance of MR/GR-mediated actions in limbic brain alter the set point of stress system activity. Once an imbalance in MR/GR has occurred, the individual loses its ability to maintain homeostasis, if challenged by an adverse event. This leads to a condition of neuroendocrine disregulation and an impaired behavioural adaptation, which, when surpassing a certain threshold, may enhance vulnerability and trigger the onset of a stress-related neurological or psychiatric disorder to which the individual is genetically predisposed (de Kloet, 1991; de Kloet et al., 1998).

### 3.8. Some recent highlights

#### 3.8.1. Gene expression profiling in hippocampus: screening of 30,000 genes

Using the Serial Analysis of Gene Expression (SAGE) technique we have generated an expression profile of rat hippocampus. By April 15, 2000 approximately 80,000 SAGE tags have been analysed, corresponding to 28,000 different genes. At present, we have identified a few hundred genes that are potentially regulated by corticosteroids (Datson et al., submitted).

#### 3.8.2. New signalling route in maintenance of $Ca^{2+}$ homeostasis

Using SAGE a novel Calmoduline kinase (CaMK6) and a homologous N-terminal peptide (CaMK-related peptide or CARP) were discovered in hippocampus The genes encoding both products seem to be regulated by stress and corticosteroid hormones. This CARP-CaMK6 feedback loop may be critical under conditions of severe challenge of $Ca^{2+}$ homeostasis (Vreugdenhil et al., 1999).

#### 3.8.3. Coactivators and corticosteroid receptors

Proteins downstream of ligand-activated receptors are critical in determining the nature and magnitude of corticosteroid action. An example is the p160 steroid receptor coactivator family of proteins. The steroid receptor coactivator proteins have a discrete neuro-anatomical distribution pattern and colocalize with corticosteroid receptors in brain (Meijer et al., 2000).

#### 3.8.4. Genetic background and psychotropic effects of corticosteroids

Chronic stress and chronically elevated levels of corticosteroids impair rats in performance of a spatial memory task. Apolipoprotein E knockout mice (apoE 0/0), developed as model for study of Alzheimer's Disease, are also deficient in this test on cognitive performance. If, however, apoE 0/0 are exposed to stress and corticosteroids their performance improves dramatically. This observation demonstrates the importance of genetic background in the effect of stress in the brain (Oitzl et al., 1997a; Grootendorst and Oitzl, submitted).

#### 3.8.5. Early life events program neural stress circuitry

Stress system activity is programmed by genotype, but can be reset by early life events. In a collaborative project with Seymour Levine we have demonstrated that the separation of infants from maternal care has immediate and lasting effects on the expression of molecular and hormonal stress markers in body and brain, which can be restored by brief periods of tactile stimulation mimicking maternal care (Van Oers et al., 1998).

#### 3.8.6. Memory requires DNA-binding of the glucocorticoid receptor

In mutant mice with a defect of the DNA-binding-dependent mechanism ($GR^{dim/dim}$ mice) we found a selective impairment of spatial navigation in the water maze. Locomotion and anxiety-related parameters were comparable to wild type animals. Therefore, the facilitating effects of corticosterone on spatial learning and memory depend on DNA binding of the GR rather than protein–protein interaction with other transcription factors (Oitzl et al., submitted).

#### 3.8.7. Early life stress selects for extremes of cognitive aging

Individual differences in brain aging are related to life long patterns of stress system reactivity programmed by genotype and perinatal experience. We have demonstrated

that separation of infant rats from maternal care drives spatial learning ability of senescent rats to the extremes at the expense of average performance. Performance is not correlated to glucocorticoid levels at senescence. We propose that during midlife glucocorticoids activate a brain mechanism selecting for either successful aging or senility (Workel, thesis 1999; Oitzl et al., submitted; Schaaf et al., 1999).

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