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Do not hover always on the surface of things, nor take up suddenly, with mere appearances; but penetrate into the depth of matters, as far as your time and circumstances allow, especially in those things which relate to your profession.

Isaac Watts

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Chapter 1

General introduction

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1. STRESS, ALLOSTASIS AND ALLOSTATIC LOAD

The word 'stress' is commonly used to describe the straining force which the living organism experiences when it is required to respond to a certain challenge of homeostasis (1). These challenges are called 'stressors' and can be the result of threatening situations, ranging from being chased by a predator (2) to defending a scientific thesis.

The behavioural and physiological adaptations that the organism displays in response to a stressor are often referred to as 'allostasis', 'the allostatic response' or 'the stress response' (3). Allostasis literally means 'maintaining homeostasis through adaptive changes' and helps the organism to cope with the stressor by enhancing energy mobilization, immunity, attention and information storage and by repressing temporarily unnecessary processes such as reproductive physiology and digestion (4). However, if the stressor is not appropriately dealt with and, as a consequence, the stress response is not shut off properly, these initially beneficial effects of allostasis can become damaging for the organism, thereby turning into 'allostatic load' (5). Allostatic load thus refers to the negative effects of a malfunctioning stress response. Since allostasis is able to affect many different physiological processes, allostatic load can be associated with a wide variety of pathological conditions, among which cardiovascular disease, metabolic disease and affective disorders are prominent (6,7).

Two major players in the stress response are 1) the hippocampus, a brain structure involved in learning and memory formation, and 2) glucocorticoid hormones secreted by the adrenal glands in response to a stressor. The interaction between the glucocorticoid hormones and the hippocampus is involved in the fine tuning of the stress response. The studies described in the current thesis are focused on the molecular mechanism via which glucocorticoids acutely affect the function of neurons in the hippocampus.

2. MEDIATORS OF THE STRESS RESPONSE

Sympatho-adrenal-medullary system and the hypothalamic-pituitary-adrenal axis

The perception of a stressor is the key trigger that initiates the stress response. There are different kinds of stressors that can activate different brain circuits (8). In general, stressors can be divided into physical and psychological stressors. Physical stressors, such as infections and pain, activate aminergic cells in the brainstem (9,10). Psychological stressors are processed by limbic brain areas, including the amygdala, hippocampus and prefrontal cortex (8). These limbic brain areas mediate the cognitive and emotional processing of psychological stressors, thereby appraising the challenge and assessing its stressfulness (11,12). Both the brainstem and the limbic brain areas communicate to a deep brain structure called the hypothalamus which integrates the stressor-specific

information. Subsequently, the hypothalamus organizes the behavioural response and communicates to the peripheral organs by 1) activating the sympathetic nervous system and 2) activating a neuroendocrine signaling cascade that is called the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1) (13,14).

Activation of the sympathetic nervous system, together with the behavioural and cognitive responses, constitutes the so-called first wave of the stress response (15). The sympathetic nervous system stimulates the release of adrenalin from the adrenal medulla into the bloodstream and the physiological effects of the sympathetic nervous system and adrenalin develop almost immediately, increasing heart rate and cardiac output,

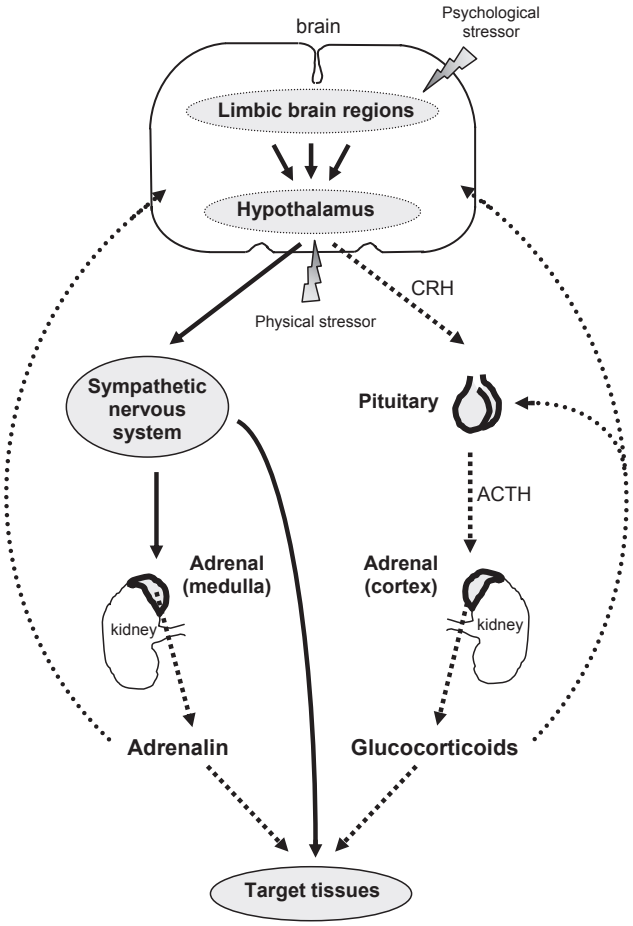

Figure 1. Simplified scheme of the sympatho-adrenal-medullary system and the hypothalamic-pituitary-adrenal (HPA) axis as described in the text. The rapid responding sympatho-adrenal-medullary system is displayed on the left whereas the slower responding HPA axis is displayed on the right. In the latter system, the hypothalamus activates the pituitary via secretion of corticotropin releasing hormone (CRH) which in turn secretes adrenocorticotrophic hormone (ACTH) into the bloodstream. ACTH stimulates the adrenal cortex to release glucocorticoids into the bloodstream.

diverting blood to the skeletal muscles, elevating blood glucose levels and suppressing the reproductive and digestive systems.

A second, delayed, wave of the stress response is responsible for modulating and fine tuning the physiological changes that were initiated in the first wave (15). Glucocorticoid hormones are the key players during this second wave and are released by the adrenal cortex in response to the activation of the HPA-axis. They constitute a class of structurally related hormones such as cortisol and corticosterone, which are the main naturally occurring glucocorticoids in humans and rodents respectively. Under basal, non-stressed, conditions HPA-axis activity is limited, resulting in the release of low amounts of glucocorticoid hormones in a circadian manner. In response to a stressor, this circadian control is overridden and glucocorticoid concentrations can rise in the course of minutes, leading to modulatory effects on the target tissues within the hour. Glucocorticoids also target the HPA-axis itself, exerting a negative feedback loop via the pituitary and hypothalamus. Additionally, the interaction between glucocorticoids and limbic brain structures regulates the activity of the HPA-axis as well, but indirectly by modulating the processing of stressful information (13).

Based on the modulation of the initial stress response the effects of glucocorticoids on their target tissues can be grouped into 1) *permissive effects*, in which basal concentrations of glucocorticoids affect the way the initial stress response is executed, 2) *stimulatory effects*, in which stressor-induced increased glucocorticoid concentrations enhance the effects of the initial stress response, 3) *inhibitory* effects, in which stressor-induced increased glucocorticoid concentrations inhibit the initial stress response and 4) *preparative effects* in which the response to a following stressor is modulated (15).

Receptors for glucocorticoids

In general, the effects of glucocorticoids on target tissues are mediated by the glucocorticoid receptor (GR) which is expressed throughout the body (16). However, in the brain as well as in certain other peripheral tissues, such as the kidney, an additional receptor, the mineralocorticoid receptor (MR) is also involved in relaying the glucocorticoid signal (13). The distribution throughout the brain differs for MR and GR and particular in limbic brain areas, such as the hippocampus, the MR is highly expressed (17). Compared to the GR this receptor has a 10-fold higher affinity for natural glucocorticoids, resulting in predominant MR-occupation under basal glucocorticoid concentrations and additional GR-occupation when glucocorticoid concentrations increase.

The two receptors play a role in the regulation of HPA-axis activity; the MR maintains basal activity of the axis under low concentrations of glucocorticoids whereas the GR facilitates the negative feedback under increasing glucocorticoid concentrations after the HPA axis has been activated by a stressor.

Both the MR and GR belong to the family of ligand-inducible transcription factors and are able to influence gene transcription (18). Many of the effects that these receptors

exert therefore seem to be the result of changes in gene expression and subsequent changes in protein levels of the target tissues and cells. The specific mode of action as well as the sets of genes these receptors target will be discussed in the section concerning the molecular mechanisms.

The hippocampus

The hippocampus, together with other limbic brain areas, plays an important role during the stress response since it is involved in the animals' reactivity to novelty (13) and mediates the formation and retrieval of declarative memories (19,20).

Anatomically, the hippocampus can be divided into two major regions that are interlocked with each other; the dentate gyrus, which contains granule cells and the cornu ammonis, which contains pyramidal cells (21) (Figure 2). The cornu ammonis can be further subdivided into four regions that are designated as CA1, CA2, CA3 and CA4. The different hippocampal subregions are interconnected with each other via the trisynaptic circuit. This circuit starts with the dentate gyrus receiving afferent projections from the entorhinal cortex and projecting mossy fibers to the CA4 and CA3 regions. These regions project Schaffer collaterals to the CA1 region. The CA1 region projects efferents out of the hippocampal region via the alveus. Additionally, also other internal and external afferents and efferents have been reported in the hippocampus. The pyramidal and granule cells use glutamate as their major neurotransmitter whereas the interneurons use GABA. Other neurotransmitters are present in the hippocampus as well since the neuropil is enriched with noradrenergic, serotonergic and cholinergic axon terminals.

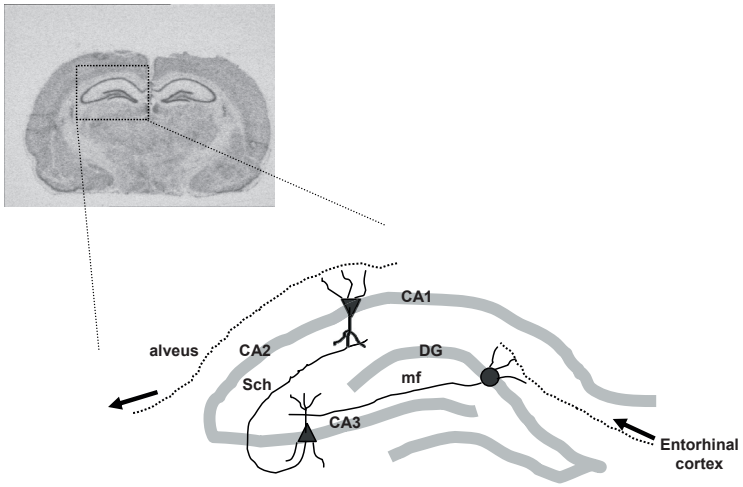

Figure 2. The hippocampus. The upper panel displays an autoradiogram of a whole brain section from rat. The lower panel displays a schematic enlargement of the right hippocampus in which the trisynaptic circuit, as described in the text, is shown. Mf; mossy fibers, Sch; Schaffer collaterals

Within the hippocampus, the different subregions differentially express MRs and GRs. The MR is expressed in the entire cornu ammonis (CA1-4) and dentate gyrus whereas GR is predominantly expressed in CA1, CA2 and dentate gyrus (22). In CA3 GR is expressed to a much lower extent (23) leading to a higher ratio of MR versus GR in this region. Additionally, there are large differences in the general transcriptomes between the different hippocampal subregions (24) and therefore the availability of coactivators and corepressors (25), transrepression partners and downstream pathways may be different. Due to these differences in MR / GR ratios and subregion-specific transcriptomes, glucocorticoids can display region-specific effects on hippocampal neuronal functioning (26,27,28).

3. GLUCOCORTICOIDS AND THE HIPPOCAMPUS

Glucocorticoids are able to modulate hippocampal neuronal properties, thereby influencing hippocampal behavioural and neuroendocrine output (13). The effects glucocorticoids exert on hippocampal neuronal function can be acute or chronic, depending on the exposure time. Acute exposure to glucocorticoids affects hippocampal neuroexcitability, synaptic plasticity and metabolism, whereas chronic exposure to glucocorticoids drives plasticity towards neurodegeneration and suppressed neurogenesis (13). The studies described in the current thesis focus on the molecular mechanisms that underlie the acute effects of glucocorticoids on hippocampal neuronal function and in the following sections these effects are discussed.

Neuroexcitability

The effects of glucocorticoids on hippocampal neuroexcitability have been well studied in explant hippocampal slices. These slices are directly produced from freshly dissected hippocampi and can be kept alive in artificial cerebrospinal fluid for up to 12 hours (29). In the slice preparation neuronal currents can be measured in all the different hippocampal subregions. The interaction between glucocorticoids and neuroexcitability has especially been studied in the CA1 region. This region projects to the subiculum, enthorinal cortex and several subcortical areas (21,30).

In general, neuroexcitability is determined by 1) voltage-gated ion conductances and 2) the neuron's ability to respond to neurotransmitters like serotonin, glutamate, acetylcholine and noradrenalin. Briefly, neurons fire action potentials when the membrane is depolarized and neuroexcitability can be defined as the number of action potentials the neuron is able to generate in a certain time window. The ability to generate action potentials, and thus neuroexcitability, can decrease when, for instance, the cells become hyperpolarized.

Particularly (L-type) voltage-gated calcium currents are under the control of glucocorticoids. Under basal levels of glucocorticoids, predominantly occupying MRs, these

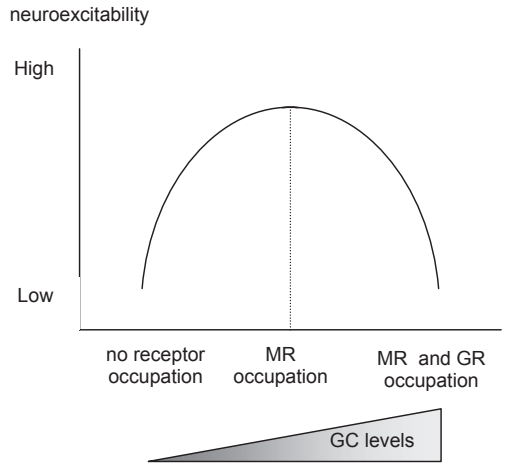

Figure 3. The effects of glucocorticoids (GCs) on hippocampal neuroexcitability. Neuroexcitability is low when glucocorticoids are absent and no MRs and GRs are occupied. Under basal concentrations of glucocorticoids in which MRs are occupied neuroexcitability is high whereas under high concentrations of glucocorticoids in which both MRs and GRs are occupied neuroexcitability decreases. The resulting reverse U-shaped dose dependent response is displayed.

calcium current amplitudes are small after the neurons are activated by depolarization of the membrane (31). When additionally GRs are activated by a brief, high concentration, corticosterone pulse the size of the amplitudes increases. This GR-mediated effect develops over a time period of 1-4 hours after initial GR-activation and is correlated with an increase in the transcription rate of calcium channel subunits (Y. Qin, unpublished observation). On the other hand, depleting all endogenous glucocorticoids by removing the adrenals (adrenalectomy) results in increased calcium current amplitudes as well. Hence, the effect of glucocorticoids on voltage-gated calcium current amplitude displays a U-shaped dose dependent response in which MR and GR play different roles (32). The regulation of these calcium currents affects the activation of slow calcium dependent potassium currents (33,34). These potassium currents hyperpolarize neurons after depolarization and this afterhyperpolarization results in decreased neuroexcitability. In line with the glucocorticoid effects on calcium currents, afterhyperpolarization is small under MR-occupation, resulting in high neuroexcitability, and large when GR is occupied or when glucocorticoids are removed, resulting in low neuroexcitability. Thus the effects of glucocorticoids on neuroexcitability follow a reverse U-shaped dose dependent response (Figure 3) and seem to be dependent on transcriptional changes (35).

Other voltage-gated ion channel currents are far less affected by glucocorticoids with only the inward rectifying potassium Q-current showing a clear U-shaped dose dependent response (36). This current also contributes to the overall reverse U shaped dose dependent response of neuroexcitability.

Beside the effects on ion currents, glucocorticoids also affect the cells' responses to neurotransmitters, especially the G-protein coupled serotonin 1A receptor which hyper-

polarizes the cell membrane (37,38). When glucocorticoids are removed (adrenalectomy) or when GRs are activated by a brief high concentration corticosterone pulse, serotonin receptor 1A mediated hyperpolarization is high whereas under basal levels of glucocorticoids (MR occupation) hyperpolarization is low. Modulation of the serotonin receptor 1A induced hyperpolarization contributes to the overall glucocorticoid dependent, reverse U-shaped, neuroexcitability pattern. The mechanisms underlying the effects of glucocorticoids on serotonergic transmission are currently unknown although the MR-induced reduction of the serotonin-response is dependent on *de novo* protein synthesis (39) whereas the GR-mediated induction develops in a delayed manner (13,32), both of which suggest a genomic mode of action.

Synaptic plasticity

The capacity of synaptic transmission to be modified is generally referred to as synaptic plasticity. Synaptic plasticity can be the result of the pattern of synaptic activity which either enhances or attenuates synaptic transmission. Long-lasting forms of synaptic plasticity are believed to underlie learning and memory formation. Two forms of long-lasting synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) have been thoroughly studied in the hippocampus, especially in the synaptic connections between the Schaffer collaterals and the cells of the CA1 (40). Normally, low frequency stimulation of the Schaffer collaterals results in moderate excitatory postsynaptic potentials (EPSPs) in the CA1 cells. However, when the Schaffer collaterals are stimulated with high frequencies of stimuli, the amplitudes of these EPSPs increase and remain increased upon subsequent stimulation, resulting in a state of LTP. On the other hand, the EPSP amplitudes decrease when the Schaffer collaterals are stimulated with a low frequency for a long period, resulting in a state of LTD. The mediators of LTP and LTD include glutamate (NMDA) receptors, calcium ions, calcium-dependent kinases and calcium-dependent phosphatases. Furthermore, changes in the morphology of the dendritic spines, where the synapses are formed, have been associated with changes in the induction of LTP, possibly due to the fact that these spines play a role in the compartmentalization of calcium and other LTP-involved molecules (41).

Glucocorticoid-activated GRs can reduce the induction of LTP and enhance the induction of LTD in the CA1 region of the hippocampus (42). There has been much debate about whether these effects of glucocorticoids are mediated via hippocampal receptors or via receptors which are located in brain structures that project to the hippocampus, such as the amygdala (43,44). The influence of these projections was removed in a study in which the explant hippocampal slice preparation was used (45). This study showed that mild, primed burst stimulation induced LTP is hampered 1-4 hours after a brief high concentration corticosterone pulse (occupying GRs). Additionally it was shown that in the same experimental setting also a more robust (10 Hz) stimulation induced LTP is re-

duced by corticosterone (46). Therefore, the effects of glucocorticoids on certain types of hippocampal LTP are directly mediated via hippocampal GRs. More specifically, this study revealed that modulation of hippocampal NMDA-receptor activity underlies the GR-mediated reduction in LTP induced by both primed burst and 10 Hz stimulation protocols. Since the GR-mediated effects develop in a delayed manner, changes in transcription may underlie these effects. However, there is evidence that NMDA-receptor mRNA expression is not changed 1-3 hours after GR-activation (46). This could indicate that other, channel function modifying proteins may be regulated. In addition, there have been strong indications that AMPA receptor subunit trafficking is affected by activated GRs and this may occur for NMDA receptors as well (O. Wiegert, unpublished observation).

Metabolism

Glucocorticoids got their name from their profound effects on glucose metabolism. Initially, after the organism has experienced a stressor, blood glucose concentrations rapidly rise to facilitate glucose transport to and utilization in the brain. This is mediated by the sympathetic nervous system which antagonizes the effects of insulin on muscle and adipose cells, thereby reducing the translocation of GLUT4 glucose transporters (responsible for glucose uptake) to the cell membrane (47). Additionally, glutaminergic innervation of astrocytes at the blood-brain-barrier stimulates GLUT1-mediated glucose uptake in the brain (47).

Delayed secretion of glucocorticoids further increases blood glucose concentrations by inhibiting glucose uptake and utilization in peripheral organs. Also in the brain glucocorticoids display inhibitory effects on glucose utilization (15). This may constitute a negative feedback system to adjust the effects of the initial stress response on neuronal glucose utilization. The effect of glucocorticoids on neuronal glucose utilization can be the result of either reduced glucose metabolism in the cells or reduced glucose transport into the cells (48). The latter is supported by two studies in which it was shown that glucocorticoids inhibit glucose transport in *ex vivo* cultured hippocampal neurons and glia cells in a delayed (> 4 hours) manner (49,50). Two glucose transporters that have been studied in this context are GLUT3 and GLUT8. Both are expressed in the pyramidal neurons and granule neurons of the hippocampus. In vivo application of acute restraint stress does not affect the mRNA levels of both transporters but does increase endoplasmic reticulum GLUT8-accumulation (51,52). The functional consequence of this increased GLUT8-accumulation on neuronal glucose metabolism however has not been clarified yet. There are indications that this stress-mediated effect on GLUT8-accumulation *in vivo* is dependent on insulin. However, the mechanism that underlies the glucocorticoid-induced effects in *ex vivo* preparations as well as whether these specific glucose transporters are involved is currently unknown.

4. MOLECULAR MECHANISMS

Transactivation and transrepression

Basal transcription of genes, which is mediated by general transcription factors at the gene promoters, can be modulated by sequence-specific transcription factors such as AP1, NFκB, CREB and nuclear receptors. These sequence-specific transcription factors bind to specific sequences on the DNA upstream of the gene promoters and enhance or inhibit basal transcription via direct or indirect (coregulator dependent) interactions with the general transcription factors (18).

MR and GR belong to the superfamily of nuclear receptors and show a similar structural organization: 1) an amino-terminal region containing a ligand-independent activation function (AF-1), 2) a DNA binding / dimerization region that is highly homologuous between the two receptors, 3) a linker region and 4) a ligand binding region which contains a second, ligand-dependent, activation function (AF-2). Both activation functions interact with coregulator proteins and mediate the effects of the receptors on transcription (16).

MR and GR are localized in the cell cytoplasm in the absence of ligand and translocate to the cell nucleus upon binding of ligand. Nuclear accumulation studies in primary neurons and hippocampal slices have indicated maximal nuclear uptake to take place in between 30 and 60 minutes after ligand activation (53,54). Nuclear localized MRs and GRs are able to modulate gene transcription in two ways (Figure 4). Firstly, the recep-

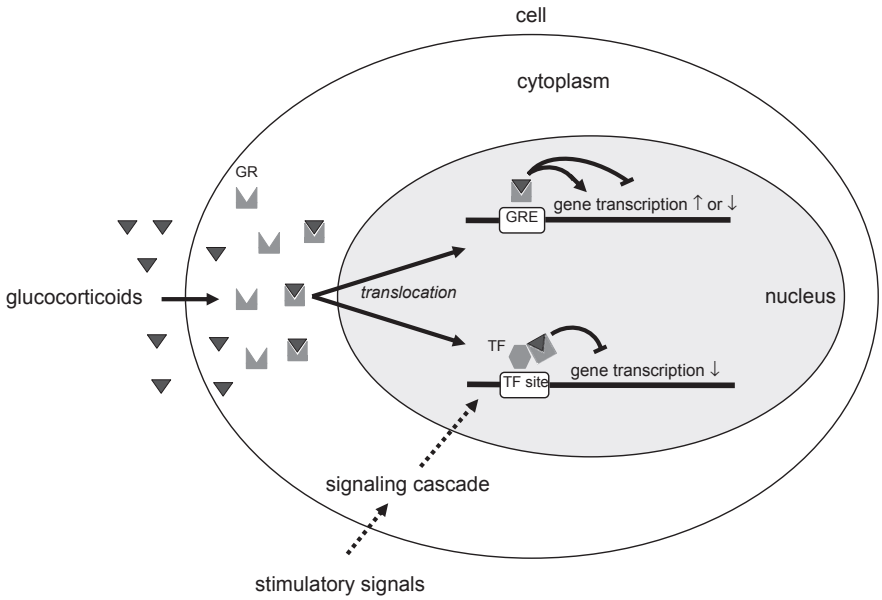

Figure 4. Molecular mechanisms underlying glucocorticoid actions. In transactivation, ligand-activated GRs or MRs bind to GREs and enhance or inhibit gene transcription. In transrepression, ligand-activated GRs or MRs bind to other activated transcription factors (TFs), thereby blocking TF-mediated transcription.

tors can dimerize to form homodimers and bind to glucocorticoid responsive elements (GREs) on the DNA in the proximity of gene promoters. Subsequently, the receptors interact directly or indirectly, via recruitment of coactivators or corepressors, with the basal transcription machinery, enhancing or inhibiting gene expression by increasing or decreasing the frequency of transcription (55,56). This mode of action is generally called *transactivation* and also includes transcriptional repression via binding of receptors to negative GREs (nGREs). Secondly, monomeric receptors can bind to sequence-specific transcription factors such as NFκB, AP1 or CREB which have been activated by other signaling cascades, thereby inhibiting their transcriptional activity (57,58,59,60). It is generally believed that this mode of action, which is called *transrepression*, accounts for many of the inhibitory effects glucocorticoids exert on stress-induced activation of the immune system (61). Additionally, there are indications that MR and GR can form GRE-binding heterodimers which may enhance the diversity of glucocorticoid action on gene transcription (62,63,64).

Receptor and tissue specific actions

Ligand-activated hippocampal MRs and GRs regulate the transcription of distinct, yet overlapping sets of genes. This was shown in a large-scale gene expression profiling study performed by Datson et al. (65) in which the majority of glucocorticoid-responsive genes was regulated either by MR or GR alone or displayed a different direction in transcriptional response. Since MRs and GRs recognize the same GREs (66,67), these differential transcriptional effects most likely can be explained by binding of different coactivators or corepressors to the receptors and / or differences in transrepressive capacity between the receptors (68).

Furthermore, the same receptor can also exert different effects in different tissues. For instance, the expression of the CRH gene is inhibited by GR in the hypothalamic cells whereas it is enhanced by GR in other cells (69) which may be explained by the availability of different coactivators / corepressors. Additionally, in a number of genes, the GREs are organized into glucocorticoid responsive units (GRUs) in which the GREs are flanked by other accessory transcription factor binding sites (16). For genes containing these GRUs the transcriptional response is dependent on binding of glucocorticoid receptor dimers and accessory transcription factors. Since expression of accessory transcription factors can be cell- or tissue-specific, GRUs can restrict the transcriptional response to certain tissues and cells. In this respect it is interesting to note that for a subset of hepatic genes involved in gluconeogenesis it has been shown that within their GRUs they share a number of binding sites for liver-enriched transcription factors C/EBP and FoxA (16,70). In addition, the repertoire of available transrepression partners for the MRs and GRs may also differ between different cells and tissues, resulting in transrepression of different sets of genes. Thus, the cellular context can be an important factor in determining the

effects of activated glucocorticoid receptors. However, the extent to which this cellular context determines the genomic response is currently unknown.

Glucocorticoid target genes

Glucocorticoids display differential effects on different tissues, thereby exerting a pleiotropic mode of action. This is also reflected in the different genes known to be regulated by glucocorticoids throughout different tissues. In the liver, glucocorticoids induce the expression of genes involved in gluconeogenesis (phosphoenolpyruvate carboxykinase), the urea cycle (carbamoylphosphate synthetase) and amino acid degradation (tyrosine aminotransferase) by binding to GRE sites.

In the adrenal medulla the phenylethanolamine N-methyltransferase (PNMT) gene, which is involved in the synthesis of adrenalin, is also induced by glucocorticoids binding to a GRE site.

Another well-known target is the immune system; glucocorticoids exert anti-inflammatory and anti-proliferative effects by inhibiting the expression of cytokines and adhesion molecules which most likely is mediated via transrepression of the transcription factor NFκB (71).

In the pituitary, glucocorticoids inhibit the synthesis of ACTH by inhibiting the expression of its precursor molecule proopiomelanocortin (POMC) via binding to an nGRE site upstream of the POMC promoter.

Finally, in the hippocampal large-scale gene expression profiling study performed by Datson et al. (65) genes involved in signal transduction, protein synthesis, protein trafficking, protein turnover and cellular metabolism were found to be responsive to glucocorticoids, illustrating the pleiotropic effects of glucocorticoids on gene expression. However, with respect to the effects of glucocorticoids on hippocampal cell function, currently no direct link has been established with the glucocorticoid-responsive genes and therefore the exact molecular mechanisms underlying these effects remain to be clarified.

Primary and downstream transcriptional effects

As previously discussed, glucocorticoid receptors directly modulate gene expression via transactivation and transrepression, exerting primary transcriptional effects. Subsequently, primary regulated genes may regulate gene transcription as well, causing secondary transcriptional effects which may lead to tertiary and further downstream genomic effects. Hence the primary glucocorticoid-responsive genes serve as master switches that determine the downstream transcriptional responses further in time (Figure 5). Although primary glucocorticoid-responsive genes have been characterized such as several of the genes mentioned in the previous section, information on the dynamics of glucocorticoid receptor mediated primary and downstream genomic responses in neuronal tissue remains sparse.

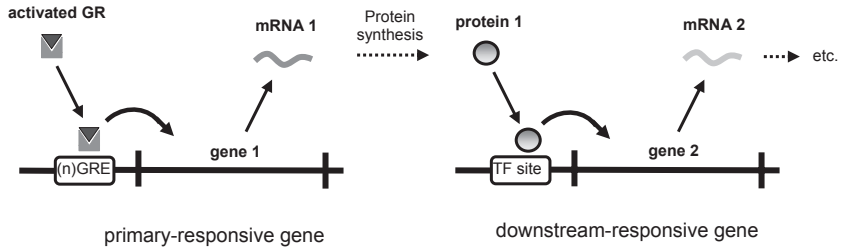

Figure 5. Primary and downstream glucocorticoid-responsive genes. On the left side a primary GR-responsive gene (gene 1) is induced by binding of activated GR to a GRE upstream of the promoter of gene 1. Gene 1 codes for mRNA 1 which after protein synthesis is translated into protein 1. This protein functions as a transcription factor (TF) at a TF-site upstream of the promoter of gene 2, inducing its expression. Hence, gene 2 is a downstream GR-responsive gene.

5. GENOMICS APPROACH

Functional and comparative genomics

Powerful large-scale gene expression profiling technologies have become available in recent years, allowing entire transcriptomes to be rapidly characterized in a quantitative manner, collectively known as 'genomics'. Genomics approaches have created new possibilities to understand the effects of glucocorticoids on neuronal functioning.

With respect to the molecular mechanisms that underlie the effects of ligand-activated glucocorticoid receptors on neuronal function, the *functional genomics* approach (Figure 6) constitutes a highly valuable methodology. In this approach the expression levels of several thousands of genes are measured using large-scale gene expression profiling techniques. In contrast to the candidate gene approach, which is a hypothesis-driven research strategy, the functional genomics approach is driven by the question which genes are regulated. Subsequently, the generated gene expression profiles are scanned for responsive gene patterns that are examined for their role in glucocorticoid-induced phenotypic changes.

The effects of glucocorticoids on the transcriptome are dependent on both the cell type and the activation status of the cells, i.e. the *cellular context*. The question to which extent the cellular context determines the glucocorticoid-induced genomic response can be dealt with by applying the *comparative genomics* approach. Using this approach, glucocorticoid-modulated gene expression profiles in different cell types or in similar cells which are activated by different environmental factors are compared with each other, thereby elucidating the degree of overlap of responsive genes. Subsequently, the number of genes with overlapping expression patterns between these different conditions can be used as an estimate of the context-specificity of the genomic response.

In addition, large-scale gene expression profiling provides a powerful tool to gain more insight into the *dynamics* of the genomic responses with respect to primary and downstream glucocorticoid-responsive genes. Time-dependent expression profiles of

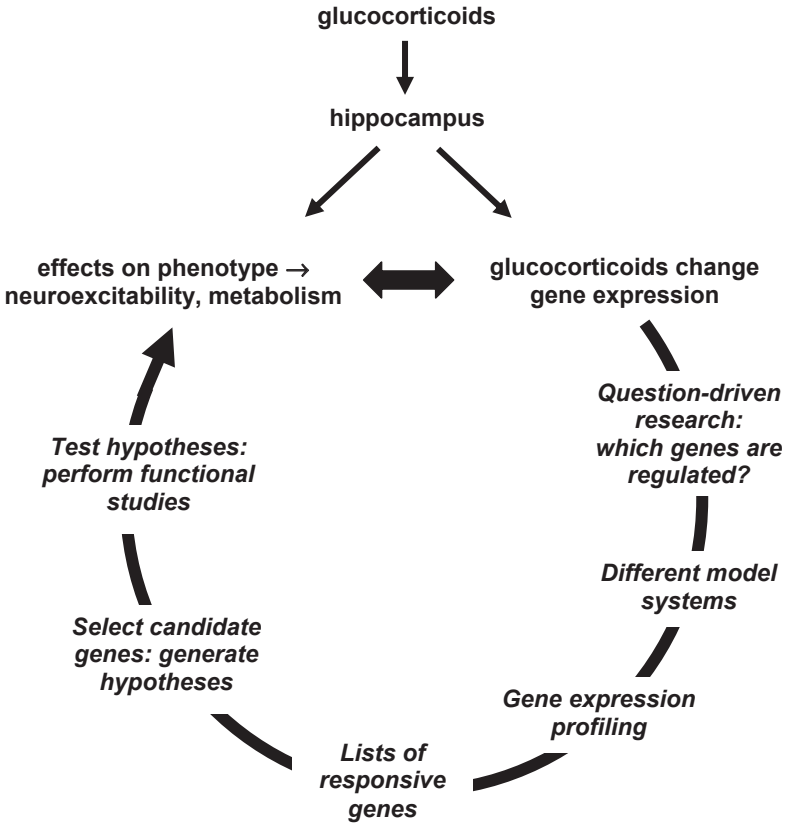

Figure 6. Outline of the functional genomics approach. The different steps are explained in the text.

primary-responsive genes can be generated in the presence of protein synthesis inhibitors which block the downstream actions of primary-responsive genes. In order to discriminate between primary and downstream-responsive genes throughout time, these profiles of primary-responsive genes can be compared with time-dependent expression profiles generated in the absence of protein synthesis blockers in which both primary and downstream-responsive genes are present. In this way, very specific information on the temporal patterns of primary and downstream-responsive genes can be obtained.

Model systems

Different model systems to study the effects of glucocorticoids on neuronal gene expression are available, ranging from *in vivo* animal models to *ex vivo* brain preparations and *in vitro* clonal cell lines with neuronal properties.

Regarding the *in vivo* animal models, pharmacological manipulation of glucocorticoid concentrations aimed at specifically activating MR and / or GR constitutes a very powerful approach to assess MR and GR-responsive genes. For example, more than 200 MR and GR-responsive genes were elucidated in the rat hippocampus by combining adre-

nalectomy (no receptor occupation) with the implantation of corticosterone pellets or corticosterone injections for differential occupation of MR and GR (65). In this respect, administration of agonists and antagonists for specific activation and blockade of MR or GR could be of major interest to further discriminate between the transcriptional actions mediated by the two receptors. This approach allows examining the effects of different ratios of MR and GR activity on the neuronal transcriptome, taking into account their specific pharmacokinetic and dynamic properties. Another interesting *in vivo* possibility to elucidate the effects of GR on the hippocampal transcriptome is the use of the transgenic GR-dimerization defective mouse line (72). In this mouse line, the GR contains a point mutation which impairs GR homodimerization and DNA-binding, leaving transrepression unaffected. In electrophysiological studies using these mice it was found that the glucocorticoid-mediated effects on calcium currents and serotonin-responses are dependent on GR homodimerization and DNA-binding (37).

Since many of the glucocorticoid-mediated effects have been observed in the *ex vivo* explant hippocampal slice preparation, these slices provide an ideal model system for profiling glucocorticoid-responsive genes. By using slices, the changes in gene expression can be correlated with altered hippocampal cell function. However, in spite of the progress in understanding cellular actions exerted by the steroids, the precise molecular mechanism underlying the electrophysiological effects still remains largely unknown. For instance, the GR-dependent increase in 5HT1A-receptor mediated hyperpolarization does not appear to be linked to an increase in expression of 5HT1A-receptor mRNAs. This could be due to the fact that the changes in mRNA levels precede the effects on cell function. Hence, assessing gene expression changes throughout a functionally relevant time interval in hippocampal slices would be of major interest.

Primary cultures of neurons and clonal cell lines with neuronal properties constitute very interesting *in vitro* model systems with respect to the assessment of 1) context-specificity of glucocorticoid-mediated changes in gene expression and 2) primary and downstream transcriptional responses. They provide an easy substrate for direct pharmacological manipulation and subsequent transcriptome analyses. However, since primary cultures besides neurons also contain glia and endothelial cells, they are very heterogeneous as compared to clonal cell lines. In addition, the preparation and maintenance of primary cultures is a very laborious task which is in sharp contrast with the use of clonal cell lines.

Several different neuronal cell lines are available which express glucocorticoid receptors (GRs). Mineralocorticoid receptor (MR) expressing cell lines however are less available and hence transfections of neuronal cell lines with MR-expression plasmids could present an alternative.

If neuronal cell lines are used for transcriptome analyses, it should be taken into account that they are derived from tumors and that tumor cells in general are genomically

unstable due to deletions, insertions and translocations. In this respect PC12 cells are very suitable for large-scale gene expression profiling since among neuronal cell lines they are unique in displaying a highly stable karyotype. PC12 cells are diploid and contain 40 chromosomes (38 autosomes plus an X and Y chromosome) whereas, for example, NIE-115 neuroblastoma cells contain 192 chromosomes, illustrating the relatively stable genomic constitution of PC12 cells. PC12 cells express the GR endogenously and can be differentiated into catecholaminergic neuron-like cells using nerve growth factor (NGF) (73,74). They reach a very high degree of differentiation with the generation of long neurites, the appearance of electrical excitability and expression of sodium, potassium and calcium channels as well as membrane receptors, including G-protein coupled receptors (75,76). Hence, since neuronal PC12 cells 1) are genomically very stable, 2) are phenotypically very different from hippocampal neurons and 3) express endogenous GRs, they provide a very suitable substrate for the comparison of context-specific GR-mediated transcriptional responses. They can also be used for assessing the dynamics of primary and down-stream responsive genes in a comparative genomics approach.

Techniques

Profiling gene expression on a large scale can be performed in multiple ways. Two of the most commonly used techniques are Serial Analysis of Gene Expression (77) (SAGE) and DNA microarrays. In SAGE, transcript levels are quantified by sequencing and counting 10 nucleotide long SAGE tags derived from the 3' untranslated regions of the transcripts. In principle these SAGE tags are long enough to uniquely identify the transcripts of origin. Subsequently, the tags are ligated into concatamers which can be cloned into plasmids. By sequencing these plasmids and counting the SAGE tags a gene expression profile is generated for each experimental sample.

DNA microarrays on the other hand are microscopic glass slides or chips onto which a large number of probes are printed or synthesized *in situ* in a high density, with each probe corresponding to a part of a certain transcript (78). Fluorescently labeled RNA, obtained from the experimental samples, is hybridized to the microarray, resulting in hybridization signals for each transcript. Gene expression levels for each experimental sample are measured by quantifying these hybridization signals. Currently, several kinds of DNA microarray systems are available and they can differ in multiple ways. Firstly, the probes that are printed on the array can differ in length. Originally, DNA microarrays were spotted with long cDNA probes. However, currently many microarray systems use shorter, more specific oligonucleotide probes which range in length from 25 to 60 nucleotides that are synthesized *in situ* on the array. Secondly, microarray systems can differ in the way hybridization of target RNA to the array is performed. In single-target hybridizations, experimental samples are hybridized to separate arrays. In dual-target

hybridizations, two experimental samples (i.e. treatment and control) which are labeled with two different fluorescent dyes are hybridized to the same array.

A very well known and widely used commercial microarray system is the Affymetrix GeneChip system (Figure 7). This system operates with single-sample hybridizations and uses probe sets that represent the transcripts. Per transcript the probe set consists of 11 to 20 probe pairs, each of which contains one 25 nucleotides long perfect match (PM) and one 25 nucleotides long mismatch (MM) oligo. The latter is designed to measure non-specific binding and cross-hybridization by changing the middle base of the PM-oligo. Subsequently, transcript abundancy is quantified by subtracting the MM-signal from the PM signal. Additionally, a statistical test (Wilcoxon Signed Ranks Test) is used to calculate whether the PM-signal is significantly higher than the MM-signal, thereby supplying a measure for the reliability of transcript detection.

In order to select the proper gene expression profiling technique some considerations can be made. Firstly, in terms of sensitivity, SAGE and microarrays seem to perform equally well in brain tissue since there is a strong correlation between the detectability of transcripts in both methods (79). Secondly, SAGE is a laborious time-consuming procedure whereas microarray procedures in general are easier and performed much faster. Finally, since only the expression levels of the genes that are present on the microarray are measured, using microarrays has been described as following a '*closed*' gene expression profiling strategy. Using SAGE on the other hand has been described as following an *'open'* gene expression profiling strategy since no selection of genes is made on forehand. Therefore, when using SAGE, new unexpected transcripts may be profiled.

Inherent to using large-scale gene expression profiling techniques for transcriptomes analyses is the generation of false positive and false negative results. Currently, different statistical tools are available for microarray analyses that estimate and control the number of false positives generated and minimize the number of false negatives, such as Significance Analysis of Microarrays (SAM) (80) and the BRB Array Tools package. Therefore, subsequent validation of the results obtained by application of these methodologies is needed and for this purpose a number of techniques are available. In this thesis two methods are used, i.e. real-time quantitative PCR and mRNA *in situ* hybridization, each of which has its own advantages. Real-time quantitative PCR is very rapidly performed and very useful for measuring gene expression in neuronal cell lines and explant hippocampal slice preparations. On the other hand, the use of mRNA *in situ* hybridization allows gene expression differences in different subregions of entire brain sections to be assessed. Both techniques therefore complement each other.

Functional follow-up studies

After gene expression profiling has been performed, candidate genes can be selected based on their putative roles in the effects of glucocorticoids on neuronal function (Fig-

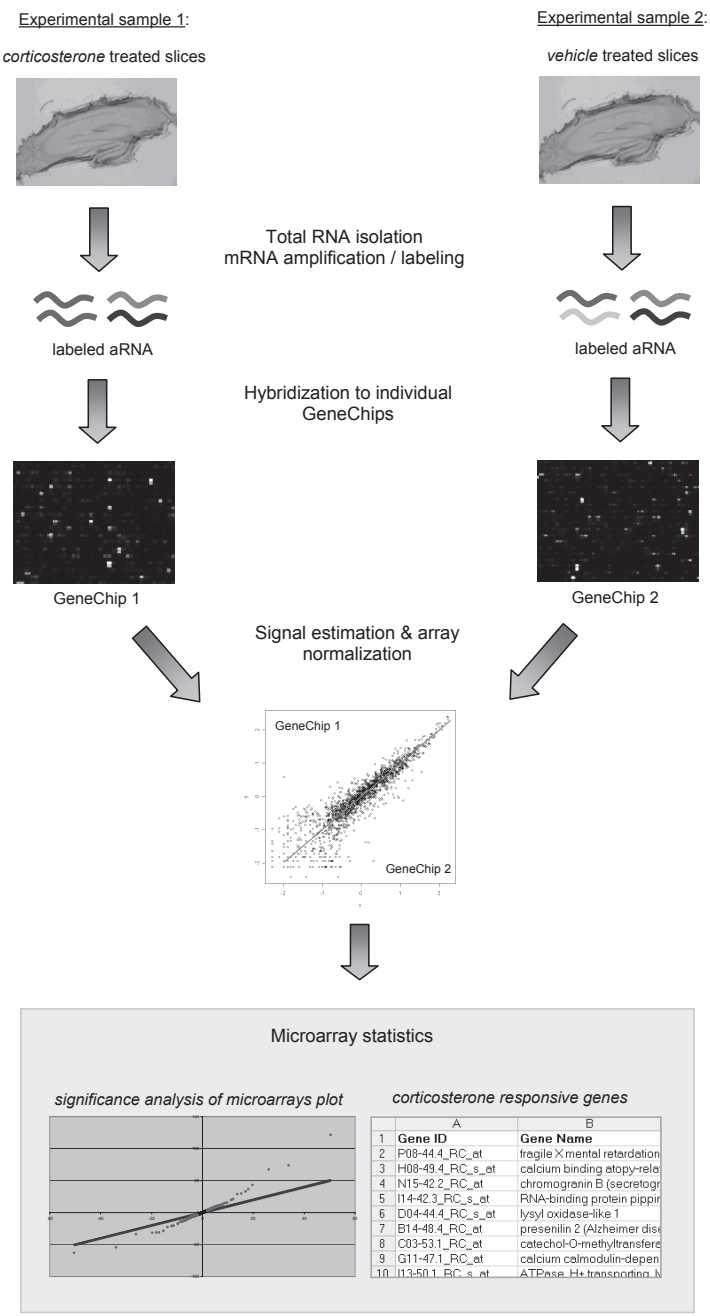

Figure 7. Affymetrix GeneChip technology. Total RNA is isolated from two experimental samples: corticosterone and vehicle treated hippocampal slices. For each sample the mRNA portion is amplified and labeled with biotin. Each labeled amplified RNA (aRNA) sample is fragmented and subsequently hybridized to an individual GeneChip that contains probe sets for several thousand transcripts. After hybridization, the GeneChips are scanned, resulting in expression signals for every probe set. Subsequent normalization and statistical testing is performed (as described in the text) to obtain a list of differentially expressed genes.

ure 6). Transgenic or knock-out animals which overexpress or lack the gene of interest respectively are very useful model systems in this respect. However, generating these animals is a very time consuming and laborious task. Lately, application of siRNA technology *in vivo* has emerged as an alternative tool to very locally inhibit the transcription of a target gene. However, targeting the right population of cells and establishing sufficient downregulation of the target gene still is a time consuming and laborious task and therefore integrating this approach in the laboratory remains difficult. As an alternative, clonal cell lines can be used to study the functional effects of transcriptional regulation of candidate genes. For instance, genes can easily be overexpressed by transfecting expression plasmids or inhibited by applying siRNA technology. Many different neuronal cell lines are available and the choice which cell line to use will predominantly depend on the endogenous expression of the genes of interest and the presence of (a) markers of the cellular processes under investigation.

6. SCOPE OF THE THESIS

Objectives

The central theme of this thesis was to determine which transcriptional changes underlie the glucocorticoid effects mediated by GRs on hippocampal neuronal function. The first objective therefore was to use a functional genomics approach to assess the time course of the GR-mediated transcriptional responses in the hippocampus and to identify candidate genes that could be linked to the changes observed in hippocampal cell function.

The second objective was to investigate to which extent the genomic response to acutely activated GRs is context-specific by using a comparative genomics approach in which the overlap in GR-mediated gene expression between different neuronal substrates, i.e. hippocampal slices and neuronal PC12 cells, was assessed. Additionally, the PC12 cells were used to gain more insight into the dynamics of the GR-mediated genomic response with regard to primary and downstream GR-responsive genes throughout time.

The third objective was to select a candidate gene from the obtained hippocampal gene expression profile and to test the functional consequences of its regulation by activated GRs.

Experimental approach

In order to study the GR-mediated changes in the hippocampal gene expression the *ex vivo* hippocampal slice preparation was used and GR-induced transcriptional changes were profiled throughout a defined time window, using Affymetrix GeneChips. **Chapter 2** describes the exact experimental setup and obtained results. Furthermore, to demonstrate the reliability of the data set obtained in the slice model, a subset of genes was

selected from the hippocampal slice expression profile and validated in an *in vivo* setting, using mRNA *in situ* hybridizations to pinpoint the hippocampal subregions in which gene expression changes took place **(chapter 3)**.

The extent to which the genomic response to acutely activated GRs overlaps between different neuronal substrates was elucidated in **chapter 4** by generating a time curve of GR-responsive genes in neuronal catecholaminergic PC12 cells, using Affymetrix GeneChips, and by comparing this expression profile with the hippocampal slice expression profile. Additionally, to gain more insight into the nature of the GR-mediated transcriptional response, primary and downstream responsive genes were assessed in these PC12 cells by blocking translation with the protein synthesis inhibitor cycloheximide.

The aim of **chapter 5** was to provide a systematic review of the current findings concerning the large-scale glucocorticoid-mediated genomic response in neural tissue.

Furthermore, LIM kinase 1 was selected from the hippocampal expression profile as a candidate gene that may underlie (part of) the effects of glucocorticoids on long-term potentiation (LTP) in the hippocampus via regulation of cytoskeletal configurations. Hence, a functional study was performed in **chapter 6** to correlate its expression levels to actin cytoskeletal configurational changes in an in vitro model system, i.e. neural NG108-15 cells.

Finally, in **chapter 7** all the currently generated experimental data are discussed in a broader context.

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Chapter 2

Acute activation of hippocampal glucocorticoid receptors results in different waves of gene expression throughout time

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ABSTRACT

Several aspects of hippocampal cell function are influenced by adrenal-secreted glucocorticoids in a delayed, genomic fashion. Previously, we used Serial Analysis of Gene Expression to identify glucocorticoid receptor (GR)-induced transcriptional changes in the hippocampus at a fixed time point. However, since changes in mRNA levels are transient and most likely precede the effects on hippocampal cell function, the aim of the current study was to assess the transcriptional changes in a broader time window by generating a time curve of GR-mediated gene expression changes.

Therefore, we used rat hippocampal slices obtained from adrenalectomized rats, substituted in vivo with low corticosterone pellets, predominantly occupying the hippocampal mineralocorticoid receptors. To activate GR, slices were treated in vitro with a high (100 nM) dose of corticosterone and gene expression was profiled 1, 3 and 5 hours after GR-activation. Using Affymetrix GeneChips, a striking pattern with different waves of gene expression was observed, shifting from exclusively downregulated genes 1 hour after GR-activation to both up and down regulated genes 3 hours after GR-activation. After 5 hours, the response was almost back to baseline. Additionally, real-time qPCR was used for validation of a selection of responsive genes including genes involved in neurotransmission and synaptic plasticity such as the CRH receptor 1, monoamine oxidase A, LIM Kinase 1 and calmodulin 2. This allowed confirmation of GR-responsiveness of 15 out of 18 selected genes.

In conclusion, direct activation of GR in hippocampal slices results in transient changes in gene expression. The pattern in which gene expression was modulated suggests that the fast genomic effects of glucocorticoids may be realized via transrepression, preceding a later wave of transactivation. Furthermore, we identified a number of interesting candidate genes which may underlie the glucocorticoid-mediated effects on hippocampal cell function.

INTRODUCTION

Glucocorticoids, which are secreted by the adrenals in response to stress, control hippocampal cellular function (1). Acute activation of the hippocampal glucocorticoid receptor (GR) affects neuronal excitability and energy metabolism within hours, whereas chronic activation results in atrophy of dendrites of pyramidal neurons (2-4). The delayed onset of these changes and the fact that the GR belongs to the superfamily of ligand regulated nuclear receptors (5,6) suggests a genomic mode of action for these glucocorticoid-dependent effects.

We previously used Serial Analysis of Gene Expression to assess the GR-induced transcriptional changes in the hippocampus against an occupied mineralocorticoid receptor background and identified over 100 GR-responsive genes 3 hours after a single corticosterone injection in rats (7). However, with respect to neuronal excitability, the precise mechanisms underlying the GR-induced changes still remain unknown, most likely due to the fact that changes in mRNA levels do not necessarily have to coincide with the effects on cell function (8,9). For instance, the GR-dependent increase in 5HT1A-receptor mediated hyperpolarization has so far not been directly linked to an increase in 5HT1A-receptor mRNAs. This could suggest that 1) transient changes in receptor mRNA levels precede the effects on neuroexcitability and/or 2) other, receptor function modifying proteins, which are transcriptionally regulated by GRs, are responsible for these effects. Therefore, the aim of the current study was to elucidate GR-regulated genes throughout a defined time interval by large scale gene expression profiling, thereby identifying candidate genes possibly involved in the GR-mediated effects on hippocampal cell function.

The hippocampal slice preparation is an ideal model system for monitoring the transcriptional responses since the hippocampus has a laminated structure and therefore the architecture of the hippocampal network is largely maintained after slicing. As a result, the histology strongly resembles that of in situ preparations (10). Furthermore, the influence of projections from other brain areas to the hippocampus is removed and the GRs can be directly activated without the interference of peripheral effects. Additionally, the slice preparation can be maintained for up to 12 hours (11) and neuronal activity can be measured throughout this time period. In these slices the effects of GR-activation on cellular electrophysiology have been well described, allowing changes in gene expression to be directly correlated with the changes observed in the electrophysiological recordings.

Activation of GR by a 20 minute exposure to a high concentration of corticosterone results in increased calcium currents and serotonin mediated hyperpolarization with a delay of 1 to 4 hours, through a mechanism requiring DNA-binding of GR-homodimers (12,13). Additionally, nuclear accumulation studies (14,15) in primary neurons and hippocampal slices indicate maximal nuclear uptake 30 to 60 minutes after ligand activation.

Moreover, studies assessing glucocorticoid-responsive genes in vivo (16-18) have shown that hippocampal transcriptome modulation takes place between 1 and 6 hours after the initial GR-activation. Therefore, a time window of 1 to 5 hours was chosen to profile GR-mediated changes in gene expression.

We used hippocampal slices obtained from adrenalectomized animals replaced with low corticosterone pellets, occupying the high affinity mineralocorticoid receptors. In order to activate the GRs, slices were treated with a brief *in vitro* corticosterone pulse and gene expression was profiled 1, 3 and 5 hours afterwards.

MATERIALS & METHODS

Animals

All experiments were carried out with the approval of the Animal Care Committee of the Faculty of Medicine, Leiden, The Netherlands (DEC nr. 02095).

Young male Wistar rats (<200 g) obtained from Charles Rivers Laboratories Inc. (Germany) were used for the experiments and housed under a 12 h light : 12 h dark cycle (lights on at 7:00h.) with food and drinking water (and after adrenalectomy also a 0.9% NaCl drinking solution) available *ad libitum*.

In order to obtain constant basal plasma levels of corticosterone, 15 rats (5 per time point) were adrenalectomized under isoflurane anesthesia and substituted with a subcutaneous pellet releasing a low concentration of corticosterone (20 mg corticosterone / 80 mg cholesterol). After 3 days, the rats were decapitated and from each rat both hippocampi were immediately isolated and subjected to slicing.

Hippocampal slice preparation and treatment

Identical conditions were used as for previously performed electrophysiological recordings in order to facilitate comparison. Per time point, 5 rats (e.g.10 hippocampi) were used. Briefly, after isolation the hippocampi were sliced with the McIlwain Tissue Chopper™, generating approximately twenty 400 µm slices for each hippocampus. Per hippocampus, slices were collected in separate containers containing carbogenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 3.5 mM KCl, 2.0 mM $CaCl_2$, 1.5 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4 and 10 mM glucose).

Throughout the experiment all slices originating from the same hippocampus were always kept separately from the others. After an equilibration period of 1 hour in ACSF at room temperature all the slices obtained from the left or right hippocampus of each animal (n=5 per time point) were used either for GR-activation or as controls, resulting in a paired setup allowing within-animal comparisons between treatment and control groups.

GRs were activated by *in vitro* treatment with a high concentration (100 nM) 20 minute pulse of corticosterone applied to ACSF which was pre-heated to 32°C. The control slices were treated with a 20 minute 0.009% ethanol (vehicle) pulse in pre-heated ACSF.

Twenty minutes after corticosterone or vehicle treatment, slices were placed back into normal ACSF at room temperature. One, three or five hours after the initial addition of corticosterone, all the slices originating from the same hippocampus were pooled again and total RNA was isolated using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA preparation and Affymetrix array hybridization

After isolation, total RNA was purified using the QIAGEN RNEasy® Mini Kit RNA Cleanup procedure (QIAGEN Inc., Valencia, CA, USA). RNA quality was assessed with the LabChip® RNA 6000 Nano Assay on the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Per RNA sample, 10 µg was used as input into the Affymetrix procedure as recommended by Affymetrix (www.affymetrix.com). Briefly, total RNA was converted to double-stranded cDNA after which the mRNA portion was amplified and biotin-labeled using the ENZO BioArray HighYield RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA, USA). The amplified RNA was purified with the QIAGEN RNEasy® Mini Kit RNA Cleanup procedure and the quality was checked with the LabChip® RNA 6000 Nano Assay on the 2100 Bioanalyzer (Agilent Technologies). The RNA samples were hybridized to Rat Genome U34A GeneChips (Affymetrix) at the Leiden Genome Technology Center (LGTC®), Leiden University, The Netherlands.

Per time point, 10 Affymetrix arrays were used, 5 for the corticosterone-treated slices and 5 for the control slices. Each array included ~8000 probe sets, representing ~7000 full-length or annotated sequences and ~1000 EST clusters.

Affymetrix data analysis

Microarray Analysis Suite 5.0 (MAS5.0; Affymetrix) was used to estimate signal intensities plus signal reliabilities and to normalize the array signals by total intensity normalization (19).

Transcripts that generated present and/or marginal calls throughout all 10 arrays per time point were selected for further analysis, removing all transcripts that generated one or more absent calls.

Significance Analysis of Microarrays (SAM) (20) was used to identify responsive genes. Briefly, when using microarrays, a large number of hypotheses is tested in a single experiment (8000 genes), resulting in many false positives (multiple testing problem). SAM is a non-parametric test which allows the user to control the False Discovery Rate (FDR), i.e. the relative number of false positives generated. For all three time points SAM was applied to the paired data sets derived from left and right hippocampi and the lowest FDR was chosen to assess the most significant responsive genes.

Gene Ontology Biological Process classifications were obtained using the NetAffx Analysis Center (21) (www.affymetrix.com), allowing genes involved in similar biological processes to be grouped together.

Furthermore, the obtained data set was compared with our previously generated SAGE data set. To allow cross-platform comparisons, the Expression Analysis Systematic Explorer (22) was used to couple Affymetrix probes to UniGene clusters. The NCBI SAGEmap ftp-site was used to couple these UniGene clusters to SAGE tags (Figure 2). Both the Expression Analysis Systematic Explorer (EASE) and the NCBI SAGEmap used UniGene build 139.

Corticosterone

In order to measure corticosterone levels, trunk blood was immediately collected in EDTA-coated tubes after decapitation. Blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C after which the plasma was collected and stored at -20°C. Corticosterone levels were measured with the murine Corticosterone RIA Kit (ICN Biomedicals™, Costa Mesa, CA, USA).

Real-time quantitative PCR

A selection of responsive genes was validated in the same experimental RNA samples that were used for GeneChip analysis, using real-time qPCR on a DNA Engine Opticon® 2 Real-Time PCR Detection System (MJ Research, Inc., Waltham, Massachusetts, USA). Prior to cDNA-synthesis, all RNA samples were DNase-treated with DNase I (Invitrogen Life Technologies), according to the manufacturer's protocol. Synthesis of cDNA was performed in a total volume of 20 µl, using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). Per experimental sample, 50 ng of RNA was put into the cDNA-synthesis reaction (23). Standard curves were generated by performing cDNA-synthesis reactions on 5, 50, 100 and 500 ng of input RNA. As a control for genomic contamination, RT- samples were used. The PCR was performed in a total volume of 25 µl, consisting of 12.5 µl 2×PCR MasterMix with SYBR® Green I (qPCR™ Core Kit for SYBR® Green I, EUROGENTEC, Seraing, Belgium), 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer (= 5 pmol), 6.5 µl water and per primer pair either 5 µl cDNA-sample, RT- sample or water (no template control).

The following heating protocol was used: 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C + 1 minute at 60°C (for both annealing and extension). Afterwards, the temperature was gradually increased to 95°C in order to make dissociation curves.

Dissociation curves were examined for each primer pair and controlled for specificity of the reaction and genomic contamination by checking the RT- and no template control samples. Then, for each primer pair the standard curve was plotted and the PCR efficiency was estimated. All used primer pairs displayed reaction efficiencies between

80 and 100%. Target gene Ct-values ranged from 18 to 32 whereas RT- and no template control samples showed no products after 40 cycli.

Since the PCR efficiencies of the target and normalization genes were not equal, the standard curve method was used to quantify the expression differences (24). Expression levels of the target genes were normalized with the expression levels of beta-actin since 1) this gene is widely used as an internal control for real-time qPCR, 2) this gene was not found to be responsive to corticosterone in the current study and the previously performed SAGE study and 3) so far, no reports have been available in published literature indicating that beta-actin is a corticosterone-responsive gene.

In general, PCR-primers were designed in the regions where the probe sets used on the Affymetrix GeneChips were derived from.

The non-parametric Wilcoxon Signed Ranks Test was used to assess significant differential expression of GR-responsive genes.

Primer sequences:

The following primers were designed for validation of observed corticosterone-responsive genes:

Gene Title	Probe set ID	Forward primer (5' - 3')	Reverse primer (5' - 3')	amplicon (bp)
Mineralocorticoid receptor	M36074_at	CCCGCGTGGGAAGTGTT	TGGAAGCGGGAAGAAGCA	64
Shaw-related potassium channel 2	X62839mRNA_s_at	AGCTTCAAGAAATGCCCACAA	CTCTGTAATTTGCAGCAAAACCA	73
Atypical PKC-specific binding protein	AB005549_at	GGCCGGTCTATCCAGTCCTT	TTTCCTTGTCCCACTCTGTGC	75
Casein kinase II, alpha 1 polypeptide	L15618_at	GGACGTCCACCCTCTCCTTAA	GGTTCAGACACGGTGCTTCTG	78
Cytoplasmatic dynein intermediate chain	X66845_at	AGCCAGATATGATTGGGTGCA	AAACACCACATCTCAAGTCTTTGG	75
Metallothionein	M11794cds#2_f_at	ATGTGCCCAGGGCTGTGT	CGTCACTTCAGGCACAGCA	64
CRH receptor 1	U53486mRNA_s_at	GCCCTGCAGCCTCAATTTC	GCTTGTGGCCCAGAAGGTC	73
EST196031 Rn.4183	rc_AA892228_at	CAGGACATTAAGCAGCCTACTTACAG	GCATGCAGAGGCCACCTTAC	77
LIMK-1	D31873_at	GAGAGAGGTCCAGTCCCATGTG	GGCTTTGATCAGGAAATGAGATG	77
Calcineurin subunit A alpha	D90035_s_at	TGACCACTTCCTGTTCACTTTTTTT	GCAAGAACATCCAACTGCTGAG	80
Calmodulin 2	M17069_at	TTTAGGAACCGTCGGCATGT	GTACACGCTGTCGACTGTCCA	74
Apolipoprotein E	X04979_at	GCTGGGTGCAGACGCTTT	GTACCGTCAGTTCCTGTGTGACTT	74
Monoamine oxidase A	D00688_s_at	CGTCCAAGGTGTACAGAGGAAAAT	AAGGGTAGTGTGTATCACATGGAGC	95

Gene Title	Probe set ID	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)	ampli-con (bp)
EST190379 Rn.24136	rc_AA800882_g_at	ACAAACAGCTTGACACTTGAC	AACCTCAGAGGGCCCAAGA	69
Beta-chain clathrin associated protein	M77246_at	GTGTGGGAGGCAGGTGGTAT	CCAGGAATCGGCACCG	70
Enolase 2, gamma	X07729exon#5_s_at	TTGCCTGAACACCGGAACA	CTATGGCGGGTCGGGAC	77
Sodium channel, voltage-gated, type 2	M22254_at	TGGTGTCACTGGGTCCCTTAG	ATACAGCGGCATCAGCAAGA	69
BARS50	AF067795_at	TTGGCATGAACCCCTTGTTC	GCACTGCAGACACACCTCAGA	66
Beta-actine	V01217_at	TGACCGAGCGTGGCTACA	CAGCTTCTCTTTAATGTCACGCA	70

RESULTS

Hormone concentrations and RNA quality

Measurement of corticosterone concentrations demonstrated that all animals had basal levels of corticosterone (3-7 µg/dl), predominantly occupying the hippocampal MRs and partially occupying the hippocampal GRs, presumably leaving room for extensive GR-activation.

Since the effects of the slicing procedure and ACSF-storage on slice mRNA quality were unknown, we used the LabChip® RNA 6000 Nano Assay (Agilent Technologies) to assess integrity and amount of RNA. No evidence of RNA degradation was observed since the ratios of ribosomal 28S/18S intensities exceeded 1.5 for every sample.

GR-responsive genes: dynamic pattern

To ensure the reliability of the data set, all transcripts that generated one or more absent calls were removed from the data set, resulting in reliable detection of 3335 genes 1 hour after GR-activation, 3289 genes 3 hours after GR-activation and 3184 genes 5 hours after GR-activation. Using these stringent selection criteria, the resulting detection efficiencies were 37% for both the 1 and 3 hours time point and 36% for the 5 hours time point, resulting in a detection overlap of 2867 genes in every time point.

Significance Analysis of Microarrays (SAM) revealed moderate responses 1 and 3 hours after GR-activation with FDRs of 43% and 37% respectively, and a weak response 5 hours after GR-activation (FDR=53%). The magnitude of gene expression responses varied between 10 and 60%.

Strikingly, a clear pattern in gene regulation was observed, with waves of gene expression occurring at different time points, shifting from exclusive downregulation of 81 genes 1 hour after GR-activation to 161 both up or downregulated genes 3 hours after GR-activation (Figure 1, Table 1 and Table S1). After 5 hours the response was almost

Table 1. GR-responsive genes for all three time points. FC = fold change.

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*FC 5 hours*	*Gene Title*	*GO Biological Process Description*
Signal Transduction					
AF030358_g_at		0.76887		chemokine (C-X3-C motif) ligand 1	signal transduction /// chemotaxis
J05592_g_at	0.79214			protein phosphatase 1, regulatory (inhibitor) subunit 1A	signal transduction /// glycogen metabolism
L23219_at			1.18458	guanine nucleotide binding protein, gamma 7	signal transduction
D26180_at	0.71834			protein kinase N1	signal transduction
U90610_g_at		1.18337		chemokine (C-X-C motif) receptor 4	G-protein coupled receptor protein signaling pathway
rc_AA891916_g_at		0.84722		membrane interacting protein of RGS16	G-protein coupled receptor protein signaling pathway
U53486mRNA_s_at		1.42996		corticotropin releasing hormone receptor 1	G-protein coupled receptor protein signaling pathway
M12672_at		0.75180		guanine nucleotide binding protein, alpha inhibiting 2	G-protein coupled receptor protein signaling pathway
rc_AA859520_at		0.77783		Heterotrimeric guanine nucleotide-binding protein alpha q subunit	G-protein coupled receptor protein signaling pathway
M17069_at		0.88985		calmodulin 2	G-protein coupled receptor protein signaling pathway
J05677mRNA_s_at		1.22537		natriuretic peptide receptor 1	intracellular signaling cascade /// cGMP biosynthesis
AF026505_at		0.64875		Arg/Abl-interacting protein ArgBP2	intracellular signaling cascade
S74351_s_at		1.60266		dual specificity phosphatase 1	intracellular signaling cascade
U02553cds_s_at		1.56780		dual specificity phosphatase 1	intracellular signaling cascade
D31873_at		1.26354		LIM motif-containing protein kinase 1	intracellular signaling cascade
D78588_at		0.88000		diacylglycerol kinase zeta	intracellular signaling cascade
rc_AA875253_at		0.69295		ADP-ribosylation factor-like 1	small GTPase mediated signal transduction

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*FC 5 hours*	*Gene Title*	*GO Biological Process Description*
L12380_at		0.82114		ADP-ribosylation factor 1	small GTPase mediated signal transduction
S49491_s_at		1.34028		preproenkephalin, related sequence	neuropeptide signaling pathway /// immune response
M63901_g_at		0.82117		secretory granule neuroendocrine protein 1	neuropeptide signaling pathway /// protein folding
L07925_g_at		1.19847		ral guanine nucleotide dissociation stimulator	neuropeptide signaling pathway /// transport
M11794cds#2_f_at		1.26349		Metallothionein	nitric oxide mediated signal transduction
rc_AI102562_at		1.21683		Metallothionein	nitric oxide mediated signal transduction
U70050_at		1.30780		jagged 2	Notch signaling pathway /// cell fate determination
M12112mRNA#3_s_at	0.82293			angiotensinogen	cell surface receptor linked signal transduction
AB017140_g_at	0.83592			homer homolog 1 (Drosophila)	metabotropic glutamate receptor signaling pathway
M59814_at		0.85764		Eph receptor B1	transmembrane receptor protein tyrosine kinase signaling pathway
rc_AA893743_g_at		0.75135		Protein kinase inhibitor, alpha	negative regulation of protein kinase activity
L15618_at	0.78114			casein kinase II, alpha 1 polypeptide	Wnt receptor signaling pathway /// regulation of cell growth
rc_AA957896_s_at	0.90426			mitogen activated protein kinase kinase 2	protein amino acid phosphorylation
Regulation of transcription					
M36074_at	0.88568			mineralocorticoid receptor (MR)	regulation of transcription, DNA-dependent
rc_AI176710_at			1.28570	nuclear receptor subfamily 4, group A, member 3	regulation of transcription, DNA-dependent
D13309_s_at		0.79705		nuclease sensitive element binding protein 1	regulation of transcription, DNA-dependent
rc_AI227715_at		1.28016		retinoblastoma-like 2	regulation of transcription, DNA-dependent
U09228_at		0.67615		transcription factor 4	regulation of transcription, DNA-dependent
M34253_g_at		0.71646		interferon regulatory factor 1	regulation of transcription, DNA-dependent

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*FC 5 hours*	*Gene Title*	*GO Biological Process Description*
AF023087_s_at	0.85455	1.18488		nerve growth factor induced factor A	regulation of transcription, DNA-dependent
rc_AI014091_at		0.71600		Cbp/p300-interacting transactivator	regulation of transcription, DNA-dependent
rc_AI008639_at	0.77806			MAD homolog 4 (Drosophila)	regulation of transcription, DNA-dependent
rc_AI104524_s_at			1.32296	heterogeneous nuclear ribonucleoprotein A/B	regulation of transcription, DNA-dependent
D37951UTR#1_at	0.89545			human immunodeficiency virus type I enhancer binding protein 2	regulation of transcription, DNA-dependent
X84210complete_seq_s_at		1.40662		nuclear factor I/A	regulation of transcription, DNA-dependent
U91679_at		1.29938		FEV (ETS oncogene family)	positive regulation of transcription
rc_AI171268_at	0.74219			Inhibitor of DNA binding 3	negative regulation of transcription from Pol II promoter
U30381_at		0.74329		zinc finger protein 148	negative regulation of transcription from Pol II promoter
AB000491_at		0.76938		proteasomal ATPase (SUG1)	negative regulation of transcription
AF030091UTR#1_g_at	0.80233			cyclin L1 (ania-6a)	RNA processing
Regulation of translation					
X62671cds#1_s_at	0.88530	1.16128		hybrid protein (ubiquitin-like protein/rps30)	protein biosynthesis
X66370_at	0.85376			ribosomal protein S9	protein biosynthesis
rc_AA892123_at			1.08962	ribosomal protein L36	protein biosynthesis
rc_AI008641_at	0.84630			ribosomal protein L22	protein biosynthesis /// ribosome biogenesis
X51707cds_s_at	0.90951			ribosomal protein S19	protein biosynthesis /// ribosome biogenesis
rc_AA892895_r_at		1.25344		EST196698 Rn.3391	protein biosynthesis /// ribosome biogenesis
rc_AI178750_at		1.06600		eukaryotic translation elongation factor 2	protein biosynthesis /// translational elongation
X58465mRNA_g_at		1.08129		ribosomal protein S5	protein biosynthesis /// ribosome biogenesis

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*FC 5 hours*	*Gene Title*	*GO Biological Process Description*
X15096cds_s_at		1.15029		acidic ribosomal phosphoprotein P0	protein biosynthesis /// translational elongation
rc_AI012604_at		0.86462		eukaryotic translation initiation factor 5	protein biosynthesis /// translational initiation
Energy metabolism					
X56133_at		0.87735		F1-ATPase alpha subunit	ATP biosynthesis
X54510_at	0.87307			ATP synthase coupling factor 6	ATP synthesis coupled proton transport
U07181_g_at	0.82188			lactate dehydrogenase B	glycolysis
X07729exon#5_s_at	0.66408			enolase 2, gamma	glycolysis
D49653_s_at		1.20581		leptin	energy reserve metabolism /// signal transduction
U96130_at	0.84485			glycogenin 1	glycogen biosynthesis /// carbohydrate biosynthesis
Oxidative stress metabolism					
U86635_at	0.86520			glutathione S-transferase, mu 5	metabolism
U86635_g_at	0.91496			glutathione S-transferase, mu 5	metabolism
X02904cds_s_at		1.19923		glutathione-S-transferase, pi 1 /// glutathione S-transferase, pi 2	xenobiotic metabolism
X07365_s_at		0.78354		glutathione peroxidase 1	response to oxidative stress
L24896_s_at	0.88171			glutathione peroxidase 4	response to oxidative stress
Cholesterol metabolism					
U36992_at	0.83433			cytochrome P450, family 7, subfamily b, polypeptide 1	cholesterol metabolism /// electron transport
U53706_at		1.15474		mevalonate (diphospho) decarboxylase	cholesterol biosynthesis /// isoprenoid biosynthesis
Miscellaneous metabolism					
rc_AA997886_s_at		1.20486		cytochrome P450, family 2, subfamily d, polypeptide 22	electron transport /// drug metabolism

Probe Set ID	FC 1 hour	FC 3 hours	FC 5 hours	Gene Title	GO Biological Process Description
E01524cds_s_at		0.84675		P450 (cytochrome) oxidoreductase	electron transport
X02918_at			1.08883	prolyl 4-hydroxylase, beta polypeptide	electron transport
D37920_at	0.85716			squalene epoxidase	electron transport /// aromatic compound metabolism
D88364_at		1.26212		phosphatidylinositol glycan, class L	GPI anchor biosynthesis
U72497_at			1.24330	fatty acid amide hydrolase	fatty acid metabolism
rc_AA894296_at		0.85359		phosphoglucomutase 1	carbohydrate metabolism /// calcium ion homeostasis
AF034218_at		1.37420		hyaluronidase 2	carbohydrate metabolism /// hyaluronan catabolism
rc_AI228110_s_at		0.82375		UDP-glucuronosyltransferase 8	glycosphingolipid biosynthesis /// metabolism
rc_AI102868_g_at	0.88055			phosphoserine aminotransferase 1	L-serine biosynthesis
AF067795_at		1.27671		BFA-dependent ADP-ribosylation substrate BARS50	L-serine biosynthesis
rc_AA800745_at	0.86907			aminolevulinate, delta-, dehydratase	porphyrin biosynthesis /// heme biosynthesis
X55298_at	0.86873			ribophorin II	N-linked glycosylation /// N-linked glycosylation via asparagine
M57664_at		1.17637		creatine kinase, brain	phosphocreatine metabolism /// brain development
U95001UTR#1_s_at	0.73694	0.79797		developmentally-regulated cardiac factor (DRCF-5)	phosphoinositide metabolism
J03572_i_at		1.22560		alkaline phosphatase, tissue-nonspecific	metabolism
U81186_g_at	0.79409			smooth muscle-specific 17 beta-hydroxysteroid dehydrogenase	steroid biosynthesis /// metabolism
AF093773_s_at		1.08950		cytosolic malate dehydrogenase (Mdh)	tricarboxylic acid cycle
AB000717exons#1-8_s_at	0.82931			methionine adenosyltransferase II	S-adenosylmethionine biosynthesis
Cytoskeleton					
rc_AA892373_at		0.87678		syndecan binding protein	actin cytoskeleton organization and biogenesis

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*FC 5 hours*	*Gene Title*	*GO Biological Process Description*
rc_AA817887_at		0.87757		profilin 1	regulation of actin polymerization/depolymerization
M60666_s_at	0.84978			tropomyosin 1, alpha	muscle contraction /// muscle development
Ubiquitin pathway					
AB011369_s_at	0.90069			protein kinase C-binding protein Beta15	ubiquitin cycle /// protein ubiquitination
U11760_at	0.86057			valosin-containing protein	ubiquitin cycle /// transport
D10756_at	0.89500			proteasome (prosome, macropain) subunit, alpha type 5	ubiquitin-dependent protein catabolism
D17296_at	0.80517			ubiquitin C	ubiquitin-dependent protein catabolism
E03358cds_at		0.82688		proteasome (prosome, macropain) subunit, alpha type 2	ubiquitin-dependent protein catabolism
U50842_at		0.79584		developmentally down-regulated gene 4A	ubiquitin cycle /// protein modification
Transport miscellaneous					
AB003515_at		0.79733		GABA(A) receptor-associated protein like 2	transport /// intracellular protein transport
D12770_s_at		1.14520		solute carrier family 25 member 4	transport /// mitochondrial transport
X66494_at	0.91233			choline transporter	transport /// neurotransmitter transport
M94918mRNA_f_at		1.09914		hemoglobin beta chain complex	transport /// oxygen transport
X56325mRNA_s_at		1.18004		hemoglobin alpha, adult chain 1	transport /// oxygen transport
rc_AA892776_at	0.85158			solute carrier family 25 (mitochondrial carrier;), member 3	transport /// phosphate transport
AF031642_at		1.43529		solute carrier family 14 (urea transporter), member 2	transport /// response to water deprivation /// urea transport
X55572_at		0.90357		apolipoprotein D	transport /// tissue regeneration
M77246_at	0.88904	0.81598	1.18781	beta-chain clathrin associated protein complex AP-2	intracellular protein transport /// protein complex assembly
rc_AA799784_at		0.66435		RAB6, member RAS oncogene family	Golgi vesicle transport

Probe Set ID	FC 1 hour	FC 3 hours	FC 5 hours	Gene Title	GO Biological Process Description
Ion transport					
X62839mRNA_s_at	0.79486			potassium voltage gated channel, Shaw-related, member 2	potassium ion transport /// transport /// ion transport
X62840mRNA_s_at	0.81548			potassium voltage gated channel, Shaw-related, member 1	potassium ion transport /// transport /// ion transport
X12589cds_s_at			1.23072	potassium voltage-gated channel, shaker-related, member 1	---
M22254_at	0.84478			sodium channel, voltage-gated, type 2, alpha 1 polypeptide	sodium ion transport /// generation of action potential
rc_AI169005_at		0.81965		chloride channel, nucleotide-sensitive, 1A	chloride transport /// regulation of cell volume
rc_AA955388_s_at		0.74288		ATPase, Ca++ transporting, plasma membrane 2	calcium ion transport /// cation transport
U78977_at			1.09489	ATPase, class II, type 9A	cation transport
M58758_g_at			1.18012	ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	proton transport
Cell adhesion					
AB005549_at	0.75895			atypical PKC-specific binding protein	cell-cell adhesion /// cytokinesis /// cell cycle
S61868_at		1.18188		syndecan 4	cell adhesion /// cell-cell signaling
AB008538_at		0.82303		activated leukocyte cell adhesion molecule	cell adhesion /// signal transduction
L14851_at			1.33732	neurexin 3	cell adhesion
Synaptic transmission					
D00688_s_at	0.91274	0.85613		monoamine oxidase A	neurotransmitter catabolism /// electron transport
X97374exon_g_at		1.41825		prepronociceptin	synaptic transmission /// neuropeptide signaling pathway
rc_AI102031_g_at		1.23401		bridging integrator 1	synaptic transmission /// endocytosis /// cell differentiation

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*FC 5 hours*	*Gene Title*	*GO Biological Process Description*
X04979_at		1.09126		apolipoprotein E	synaptic transmission, cholinergic
U14398_g_at		0.69717		synaptotagmin 4	neurotransmitter secretion /// transport
Cell cycle/proliferation					
X63594cds_at		1.34022		nuclear factor of kappa light chain gene enhancer	regulation of cell proliferation
L26268_g_at	0.87512			B-cell translocation gene 1, anti-proliferative	negative regulation of cell proliferation /// cell proliferation
rc_AI177366_at	0.84442			Integrin beta 1	regulation of cell cycle /// G1/S transition of mitotic cell cycle
D90035_s_at		0.82695		calcineurin subunit A alpha	G1/S transition of mitotic cell cycle /// calcium ion transport
Immune response					
AF029240_g_at		1.19300		RT1 class Ib, locus S3	immune response
X16481_r_at		1.21903		parathymosin	immune response
rc_AI169802_at		0.89727		ferritin, heavy polypeptide 1	immune response /// iron ion transport /// iron ion homeostasis
rc_AA859645_at		0.80486		attractin	inflammatory response /// heterophilic cell adhesion
Regulation of apoptosis					
L00981mRNA#2_at	0.75489			tumor necrosis factor superfamily, member 2	apoptosis /// induction of apoptosis
U90261UTR#1_g_at	0.84423			SH3-domain kinase binding protein 1	regulation of apoptosis
Proteolyse					
U62897_at			1.23519	carboxypeptidase D	proteolysis and peptidolysis
D13907_g_at		0.67629		peptidase (mitochondrial processing) beta	proteolysis and peptidolysis
D29683_at		1.24531		endothelin converting enzyme 1	proteolysis and peptidolysis

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*FC 5 hours*	*Gene Title*	*GO Biological Process Description*
Protein folding					
rc_AI170613_g_at	0.87572			heat shock 10 kDa protein 1	protein folding /// response to unfolded protein
rc_AI170613_at			1.25684	heat shock 10 kDa protein 1	protein folding /// response to unfolded protein
rc_AA859806_at		0.82780		Cysteine string protein	protein folding /// synaptic transmission
Miscellaneous					
rc_AI232374_at	0.80048			H1 histone family, member 0	nucleosome assembly
D42137exon_s_at	0.89193			annexin A5	blood coagulation
S49400_at	0.91053			protein tyrosine phosphatase, non-receptor type 5	protein amino acid dephosphorylation
rc_AI180424_at	0.79537			14-3-3 protein beta-subtype	protein targeting /// protein-mitochondrial targeting
U08290_at		1.22286		neuronatin alpha	development
rc_AI175539_at		1.58511		parvalbumin	muscle development
rc_AA866276_at		0.80044		myeloid-associated differentiation marker	myeloid blood cell differentiation
rc_AI113289_s_at		0.86044		protein tyrosine phosphatase, non-receptor type 1	protein amino acid dephosphorylation
J03886_at		1.24701		myosin light chain kinase 2, skeletal muscle	protein amino acid phosphorylation
U32681_at		1.30676		deleted in malignant brain tumors 1	response to wounding /// cell differentiation /// tissue regeneration
M91597_s_at	0.92536			expressed in non-metastatic cells 2	GTP biosynthesis /// UTP biosynthesis /// CTP biosynthesis
rc_AA892832_at		0.79504		ELOVL family member 5	fatty acid elongation

From left to right are listed the GeneChip Probe Set IDs, the fold changes in the 1, 3 and 5 hours time point, the gene titles and the Gene Ontology Biological Process descriptions. Fold changes below 1 indicate downregulated genes; fold changes above 1 indicate upregulated genes. Please note that genes were placed into categories based on their first GO-component. Since this is an arbitrary division, many genes can be placed into multiple functional categories.

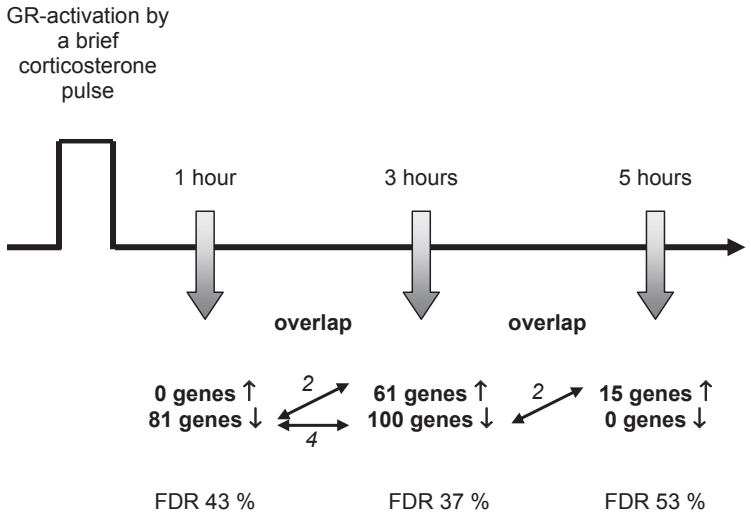

Figure 1. Different waves of gene expression after glucocorticoid receptor (GR)-activation according to a pattern of 81 exclusively downregulated genes 1 hour after GR-activation, 161 both up or downregulated genes 3 hours after GR-activation and 15 up regulated genes 5 hours after GR-activation. The arrows indicate overlap between the time points. The user-defined False Discovery Rate (FDR) from Significance Analysis of Microarrays (SAM) analysis is indicated.

back to baseline, with only 15 genes being upregulated. Furthermore, there was very little overlap between the time points (Table 2). Except for four genes at the 3 hours time point, all the other responsive genes at each time point generated reliable signals on the Affymetrix GeneChips at the other time points, thereby eliminating the possibility that these genes were not picked up as responsive genes in the other time points due to lack of signal on the Affymetrix GeneChips.

In order to determine whether changing the user-defined FDR would influence the observed pattern, the FDRs were increased to the highest possible values. This did not alter the observed pattern of 1) exclusive downregulation of genes 1 hour after GR-activation, 2) both up- and downregulated genes 3 hours after GR-activation and 3) the small overlap between the time points. However, increasing the FDR for the 5 hours time point resulted in the addition of downregulated genes to the list of responsive genes.

GR-responsive genes: functional classification

In order to assign functions to the responsive transcripts, Gene Ontology classifications were obtained using the NetAffx Analysis Center. Genes were grouped together into a large variety of functional classes. Activation of GR clearly affected the expression of genes involved in different types of cellular metabolism, transcription, translation, different aspects of signal transduction, protein/vesicle trafficking, ion transport, cell adhesion, the cytoskeleton and synaptic transmission. Hence, in general, the different waves of gene expression at different time points contained similar functional gene groups.

Table 2. Overlapping genes between the three time points. FC = fold change

Probe set ID	Gene title	FC 1 hour	FC 3 hours	FC 5 hours
AF023087_s_at	nerve growth factor induced factor A	0.85455	1.18488	
X62671cds#1_s_at	hybrid protein (ubiquitin-like protein/rps30)	0.88530	1.16128	
U95001UTR#1_s_at	developmentally-regulated cardiac factor (DRCF-5)	0.73694	0.79797	
rc_AA892851_g_at	EST196654 Rn.3616	0.74547	0.74772	
D00688_s_at	monoamine oxidase A	0.91274	0.85613	
M77246_at	beta-chain clathrin associated protein complex AP-2	0.88904	0.81598	1.18781
rc_AA800882_at	EST190379 Rn.24136		0.62222	1.36565

Genes that overlap between the three time points. From left to right are listed the GeneChip Probe Set IDs, the gene titles and the fold changes in the 1, 3 and 5 hours time point.

Some of the interesting responsive genes were classified as signal transduction components and included corticotropin releasing hormone (CRH) receptor 1, mitogen activated protein kinase kinase 2, LIMK1 and calmodulin 2. Additionally, genes from other functional groups such as ania-6 (RNA processing), monoamine oxidase A (neurotransmitter catabolism), two potassium ion channels and prepronociceptin (synaptic transmission) were also identified as hippocampal GR-responsive genes.

Overlap with the SAGE data set

We previously used adrenalectomy in combination with low corticosterone pellets (MR-occupation) and high corticosterone injections in vivo (GR occupation) to assess corticosteroid-regulated gene expression in rat hippocampus 3 hours after a high corticosterone injection, using Serial Analysis of Gene Expression (7). Briefly, in SAGE, gene expression profiles are established by sequencing and counting 10 base pairs long SAGE tags which are derived from a defined position within the 3' untranslated regions of the transcripts (25). In order to compare the current data set with the SAGE data set, we used both the Expression Analysis Systematic Explorer (EASE) and the NCBI SAGEmap ftp-site as described in the materials and methods section (Figure 2). Since 1) not every SAGE tag and Affymetrix probe set could be annotated with a UniGene cluster and 2) not every annotated SAGE tag was present on the Affymetrix GeneChip, only seventy of the 203 responsive SAGE tags could be annotated as Affymetrix probe sets. However, still 4 (6%) of the responsive genes found with SAGE were also regulated 1 hour after GR-activation in this study, whereas 7 genes (10%) were regulated 3 hours after GR-activation (Table 3). The majority of these genes showed the same direction of regulation (8 genes out of 11) providing good positive controls for the current data set. Two of these genes (malate dehydrogenase and F1-ATPase alpha subunit) play a role in cellular metabolism whereas another, apolipoprotein E, functions as a lipid transporter and is involved in synaptic

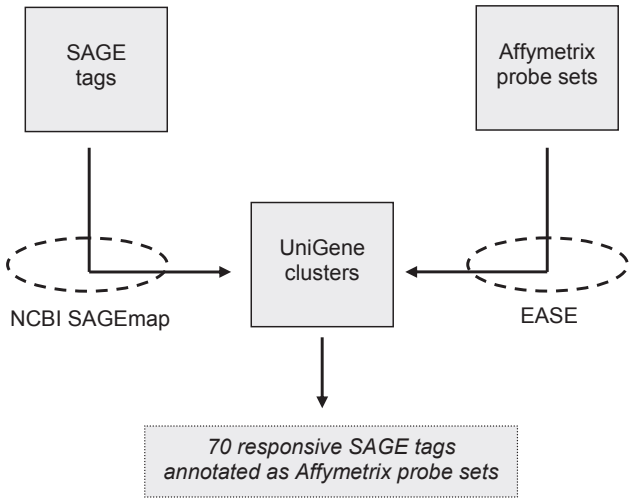

Figure 2. Cross-platform comparison between Serial Analysis of Gene Expression (SAGE) tags and Affymetrix probe sets. Affymetrix probe sets were annotated with UniGene clusters using Expression Analysis Systematic Explorer (EASE) whereas SAGE tags were annotated using the NCBI SAGEmap. Both annotation tools provide information on the UniGene builds used in the annotation process and therefore allow comparison of data sets via the same builds (build 139).

Table 3. Overlap with SAGE. FC = fold change

SAGE tag	Unigenes	Probe set ID	Gene title	FC 1 hour	SAGE change
GAACATATTT	Rn.11273	X66845_at	dynein, cytoplasmic, intermediate chain 1	0.79120	GR down
TGGTGGAATG	Rn.4231	L15618_at	casein kinase II, alpha 1 polypeptide	0.78114	MR down
TATAATCTGT	Rn.29774	M63485_at	matrin 3	0.80711	GR up
ACTTAGTTGT	Rn.5790	X54510_at	ATP synthase coupling factor 6	0.87307	MR up
SAGE tag	**Unigenes**	**Probe set ID**	**Gene title**	**FC 3 hours**	**SAGE change**
TTTGTGACTG	Rn.3946	AF067795_at	BFA-dependent ADP-ribosylation substrate BARS50	1.27671	MR up
ACGTAAAAAA	Rn.13492	AF093773_s_at	cytosolic malate dehydrogenase (Mdh)	1.08950	MR up
GAGAGCTAAC	Rn.5785	U08290_at	neuronatin alpha	1.22286	MR up
ACCAGCCAGG	Rn.32351	X04979_at	apolipoprotein E	1.09126	GR up
GTGGGTGTGT	Rn.3391	rc_AA892895_r_at	EST196698 Rn.3391	1.25344	GR down
AATAAAAGTT	Rn.40255	X56133_at	F1-ATPase alpha subunit	0.87735	GR down
TTGCTGTTGA	Rn.5968	M17069_at	calmodulin 2	0.88985	MR down

Genes that overlap between the current and the Serial Analysis of Gene Expression (SAGE) study. The upper panel displays the genes that overlap between the 1 hour time point and the SAGE study; the lower panel displays the genes that overlap between the 3 hours time point and the SAGE study. In the SAGE change column genes are responsive to the mineralocorticoid receptor or glucocorticoid receptor and the direction of regulation is designated as up or down.

transmission. Furthermore, calmodulin 2, which was downregulated in both data sets, plays a role in calcium channel functioning. Interestingly, 6 of the GR-regulated genes in the current data set were MR-regulated in the SAGE data set.

Validation of a subset of responsive genes

Since SAM revealed moderate gene expression responses with relatively high FDRs throughout the three time points, the GR-dependent change in expression of a subset of functionally interesting genes was validated with real-time qPCR.

Beta-actin, which is commonly used as normalization control and which was not responsive in the current and the SAGE data set, was used to normalize target gene expression values. In Table 4, the genes selected for validation and their validation results are listed. Furthermore, for some of the validated genes the expression pattern is illustrated in Figure 3.

Table 4. Real time qPCR validation results. FC = fold change.

Gene title	Experiment	FC affymetrix	FC qPCR	Wilcoxon	Validation
Mineralocorticoid receptor	slice 1 hour	0.9	0.5	P<0.05	true positive
Shaw-related potassium channel 2	slice 1 hour	0.8	0.8	P<0.05	true positive
Atypical PKC-specific binding protein	slice 1 hour	0.8	0.8	P<0.05	true positive
Casein kinase II, alpha 1 polypeptide	slice 1 hour/SAGE	0.8	0.7	P<0.05	true positive
Cytoplasmatic dynein intermediate chain	slice 1 hour/SAGE	0.8	0.9	P<0.05	true positive
Metallothionein	slice 3 hours	1.3	1.4	P<0.05	true positive
CRH receptor 1	slice 3 hours	1.4	1.5	P<0.05	true positive
EST196031 Rn.4183	slice 3 hours	0.7	0.7	P<0.05	true positive
LIMK-1	slice 3 hours	1.3	1.4	P<0.05	true positive
Calcineurin subunit A alpha	slice 3 hours	0.8	0.7	P<0.05	true positive
Calmodulin-2	slice 3 hours/SAGE	0.9	0.7	P<0.05	true positive
Apolipoprotein E	slice 3 hours/SAGE	1.1	1.1	P<0.05	true positive
Monoamine oxidase A	slice 1/3 hours	0.9/0.9	0.9/0.9	P<0.05	true positive
EST190379 Rn.24136	slice 3/5 hours	0.6/1.4	0.9/1.4	P<0.05	true positive
Beta-chain associated protein complex AP-2	slice 1/3/5 hours	0.9/0.8/1.2	0.7/0.7/1.5	P<0.05	true positive
Enolase 2, gamma	slice 1 hour	0.7	X	X	false positive
Sodium channel, voltage-gated, type 2	slice 1 hour	0.8	X	X	false positive
BARS50	slice 3 hours/SAGE	1.3	X	X	false positive

Validation of 18 genes. The fold changes obtained with Affymetrix GeneChip analysis and real-time qPCR are displayed as well as the result of statistical testing (Wilcoxon Signed Ranks Test). Genes were classified as true positives if the expression changes observed with Affymetrix GeneChips could be replicated with real-time qPCR.

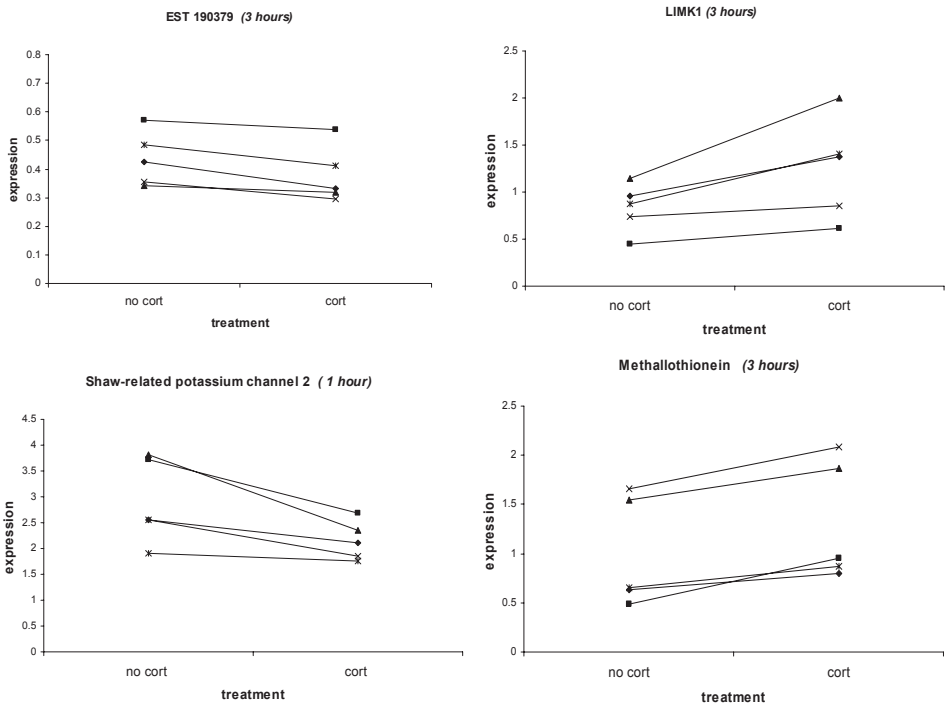

Figure 3. Real-time quantitative PCR results illustrated for four genes. Normalized gene expression levels obtained from corticosterone treated and vehicle treated hippocampal slices are plotted and for each separate animal connected with a line (paired setup).

Genes that were selected for validation belonged to interesting functional classes regulated throughout the time points. Additionally, a few genes that showed expression responses in multiple time points and some genes which overlapped with the SAGE data set were included for validation. In total 18 genes were selected and the GR-induced expression response of 15 genes could be confirmed ($P<0.05$, Wilcoxon Signed Ranks Test)

The fold-changes obtained by real-time qPCR were in good concordance with the fold-changes obtained by GeneChip analysis. Interestingly, despite the relatively high FDRs that were obtained by analyzing the GeneChip data with SAM, we could confirm GR-responsiveness of 15 out of 18 genes by real-time qPCR.

DISCUSSION

The aim of this study was to elucidate potential molecular mechanisms underlying the action of corticosterone on the hippocampus by generating a time curve of predominantly GR-responsive genes. We identified over 200 likely GR-responsive genes and found a clear pattern of moderate changes in gene expression in all three time points.

Validation of a subset of genes by real-time qPCR resulted in a remarkable consistency of fold-changes since the changes observed in qPCR validation nicely followed the changes observed in GeneChip analysis. Furthermore, 83% of the selected genes could be confirmed as GR-responsive, indicating an overall FDR of 17%. Since the overall GeneChip FDR estimated by SAM was considerably higher, less false positives were found in the selected subset than expected. Although the subset was not selected randomly, these results could indicate that SAM to a certain extent overestimated the FDR, suggesting a higher number of false negatives.

In order to assess how specific the profiled genes are for neurons, we checked all the genes that were validated by real-time qPCR and found that all 12 genes with a known localisation in brain are expressed in principal neurons of the hippocampus, indicating that the majority of the profiled genes are specific for neurons (The Allan Brain Atlas; www.brainatlas.com).

The moderate changes in gene expression that were found in the current study are in agreement with previous reports on corticosteroid-responsive genes in the hippocampus, showing fold changes of less than 2 for the majority of genes in different hippocampal subregions by *in situ* hybridization (7,18,26,27). Although the observed differences in expression are remarkably consistent with what is reported in literature, it can be expected that by using whole hippocampus the expression differences are most likely diluted by hippocampal subregions and non-neuronal cell types which are not or less responsive to activated GRs (28,29). However, by using a paired study design we were able to enhance the detection of these subtle expression differences, increasing statistical power by observing very consistent changes of gene expression within the individual animals.

The current data set contained a number of genes that were already known to be responsive to corticosteroids in the hippocampus, such as metallothionein 1A (30), glutathione peroxidase (31,32) and the mineralocorticoid receptor (33). Therefore, these genes constitute good positive controls. Furthermore, we performed cross-platform comparisons with the previously generated SAGE data set and found 8 overlapping genes which showed similar changes in direction of expression. These 8 genes constituted approximately 10% of the responsive SAGE tags that could be detected on the GeneChips used in the current study. The current experiment differed from the SAGE experiment in the fact that in the current experiment prior to corticosterone treatment the hippocampus was excised from its surroundings. This resulted in loss of input from extra-hippocampal regions. Since these inputs may also be steroid responsive, the current data set does not reflect the total GR influence on hippocampal gene expression. Furthermore, since the corticosterone treatment was restricted to a 20 minute 100nM pulse whereas in the SAGE experiment GRs were activated with an (1 mg/kg bodyweight) in vivo injection, this most likely will lead to differences in hippocampal corticosterone exposure as well

as additional peripheral effects of the injections. Finally, by comparing different gene expression platforms, a lot of genes are omitted from the actual comparison due to annotation differences. We believe that these experimental differences and the restrictions of cross-platform comparison can explain the relatively small overlap of 8 genes as well as the change in direction of expression regulation of 3 genes.

In conclusion, the reliability of the current data set was demonstrated by the validation results, the observed fold-changes, the presence of positive controls and the overlap with the SAGE study.

By generating a time curve of GR-responsive genes a striking dynamical pattern was revealed. We observed different waves of gene expression throughout the time frame, with only downregulated genes 1 hour after GR-activation. Since no protein synthesis inhibitors were used in the current study, the question whether these identified genes are primary GR-responsive genes or responsive to downstream GR-induced changes cannot be answered. Activated GRs can modulate gene expression either via transrepression, by interacting as a monomer with other transcription factors thereby inhibiting transcription, or via transactivation, by binding as a homodimer to glucocorticoid responsive elements (GREs) on the DNA, thereby inhibiting or stimulating transcription. The finding that all the genes which were responsive 1 hour after GR-activation were downregulated may indicate that at this time point these genes are primary GR-responsive genes and regulated via transrepression. Hence, this would mean that in general transrepression precedes and/or is faster than transactivation. In agreement with this, Almon and co-workers (34) found a similar dynamical pattern of transcription regulation by corticosteroids in liver, in which the majority of rapidly modulated genes (45 out of 50) were downregulated after injection of corticosteroids, whereas a robust, delayed wave of upregulated genes followed 2 hours later. Interestingly, some of the responsive genes which were regulated in more than one time point changed their direction of expression throughout the time points. A similar observation was found by Fujikawa and co-workers, showing a biphasic regulation of hippocampal mRNAs coding for growth hormone receptor, GR and MR throughout time during acute stress (35). This biphasic regulation could be the effect of downstream regulatory mechanisms, indicating that these genes change from primary responsive genes to downstream responsive genes throughout time. Therefore, the 3 and 5 hours time points would each contain at least 2 downstream responsive genes since both time points contain two genes which have changed their direction of expression in comparison with the previous time point (Figure 1). Additionally, one gene, metallothionein, for which the presence of a GRE has been reported (36) was also found to be regulated after 3 hours, indicating that at this time point most likely both primary and downstream responsive genes are present.

The different waves of gene expression at the different time points contained similar functional groups. Responsive genes were grouped into functional classes such as

signal transduction, regulation of transcription, regulation of translation, metabolism, cell adhesion and synaptic transmission. Classification of GR-responsive genes into these functional groups is in agreement with previous studies showing similar functional categories in hippocampus and other tissues (7,34,37).

In terms of hippocampal neuronal excitability, both calcium channels and 5HT1A receptors comprise interesting genes. Glucocorticoid hormones have a clear effect on excitability of neurons in the CA1 area of the hippocampus, especially on voltage-gated calcium currents and 5HT1A receptor-mediated hyperpolarization (8). However, in the current study, the 5HT1A receptor was undetectable on the GeneChips used, which is consistent with several of our previous GeneChip studies in hippocampus (38-40). This is most likely due to expression levels below the detection limit of GeneChips. Therefore expression of the 5HT1A receptor could not be assessed using GeneChips. Furthermore, no GR-mediated transcriptional effects on calcium channels were found. Since stress-induced increases in calcium currents in vivo have been shown to be preceded by transient increases in calcium channel subunit mRNAs in the hippocampal CA1 region (41), the absence of calcium channels in the current study may indicate that 1) putative GR-mediated effects on calcium channel transcription in the CA1 subregions are undetectable due to the dilution of the response by other hippocampal subregions and non-neuronal cell types, or 2) calcium channel subunit expression is regulated at a different time point than currently profiled. Furthermore, it should be noted that by using GeneChips or microarrays in general, the possibility exists that false negative data is generated due to the large amount of hypotheses tested in a single experiment. This example underpins that a link between the corticosterone-induced changes in hippocampal neuronal excitability in the CA1 subregion and changes in gene expression using expression profiling is complex, even when the experimental protocols are matched as closely as possible within the current technical constraints. The same line of reasoning may hold true for other genes that are known to be responsive to corticosteroids such as brain-derived neurotrophic factor (BDNF) which was detectable on the GeneChips but not found to be regulated in the SAM analysis. BDNF is known to be downregulated in vivo 3 hours after a single corticosterone injection in both dentate gyrus and CA1 regions and in this case the differences between in vivo treatment and in vitro hippocampal slice treatment as previously described with respect to the comparison with the SAGE study should also be taken into account.

Our current expression profile corresponds with the well-documented effects of glucocorticoids on brain metabolism. For instance, it has been reported that corticosterone inhibits glucose utilization by neurons and astrocytes (42). In the current data set, lactate dehydrogenase B, which is involved in glucose utilization, is downregulated, possibly underlying (part of) this effect. Other downregulated genes playing a role in energy metabolism included the F1-ATPase alpha subunit and ATP synthase coupling factor 6

which are both involved in ATP synthesis. Since it is known that corticosterone accelerates ATP-loss after a metabolic insult (43) these two genes constitute interesting putative candidate genes for the glucocorticoid effects on ATP-synthesis. Beside the effects on energy metabolism, glucocorticoids also play a role in oxidative stress metabolism (44) and in the present study we observe a number of regulated genes which may be involved.

Strikingly, a number of genes playing a role in hippocampal neurotransmission and synaptic plasticity were found to be responsive to corticosterone. The functional implication of regulation of four of these genes is described in more detail below.

Firstly, monoamine oxidase A, which is involved in the catabolism of serotonin, dopamine and noradrenaline (45), was found to be downregulated at two time points, i.e. 1 and 3 hours after GR-activation. All three neurotransmitters have been associated with certain aspects of hippocampal functioning. The hippocampus is known to play a role in learning and memory formation and dopamine and noradrenaline have been associated with mnemonic processing and memory retrieval respectively (46,47). The hippocampus also plays an important role in anxiety and the dorsal hippocampal serotonergic system has been associated with anxiogenic responses (48). Hence, downregulation of monoamine oxidase A could modify these aspects of hippocampal function by increasing the availability of these neurotransmitters. To our knowledge, such a direct effect of corticosteroids on hippocampal neurotransmitter availability has not been observed before.

Secondly, CRH receptor 1 was found to be upregulated by corticosterone 3 hours after GR-activation. CRH is a neuropeptide that is released after stress by the hypothalamus and modulates many neuroendocrine and behavioral responses (49-51). In the hippocampus, the majority of the pyrimidal cells in CA1 and CA3 express the CRH receptor 1 (52). Since the GR is predominantly expressed in CA1, CA2 and DG, it may be that the effect of activated GRs on CRH receptor1 takes place in the CA1 area. Interestingly, administration of exogenous CRH into the hippocampus affects long-term potentiation (LTP) (53). Additionally, CRH-producing neurons have been found in interneurons in the pyramidal layers of the hippocampus (54) and there is evidence that stress-induced CRH of hippocampal origin activates hippocampal pyramidal cells via the CRH receptor 1 (55). Therefore, transient upregulation of the hippocampal CRH receptor 1 by stress-released corticosterone constitutes a very interesting phenomenon which could lead to an enhancement of the CRH-induced effects on hippocampal functioning.

Thirdly, calmodulin 2 was found to be downregulated both in the current study and the SAGE study. The calmodulins constitute a unique gene family in which all three different members (calmodulin 1, 2 and 3) code for exactly the same protein (56). Calmodulin has been hypothesized to function as a central regulator of synaptic plasticity (57) and, similar to CRH, to play a role in LTP, implying that corticosterone may influence this phenomenon via the regulation of expression of multiple genes.

Finally, LIMK1 was found to be upregulated 3 hours after GR-activation. In vitro it has been shown that this gene, via phosphorylation of ADF/cofilin, can enhance polymerization of actin filaments (58,59). Furthermore, studies with LIMK1 knock-out mice have shown that this gene is involved in actin cytoskeleton-dependent regulation of dendritic spine morphology and synaptic function (60). Hence, the transient upregulation of this gene by corticosterone could be a means to temporarily modify synaptic function. In this respect it is interesting to note that calcineurin A was downregulated 3 hours after GR-activation. This gene is known to be enriched in the dendritic spines of hippocampal neurons and to be involved in destabilizing both actin filaments and dendritic spine structure (61,62). Furthermore, Tojima and co-workers (59) demonstrated that calcineurin can inhibit protein expression of LIMK1 in neuronal NG108-15 cells, which would suggest that the corticosterone-induced downregulation of this gene could increase LIMK1-protein expression.

In conclusion, by profiling gene expression after GR-activation in hippocampal slices throughout a time window of 1 to 5 hours, we have found a very interesting dynamic pattern of gene regulation, with several subsequent waves of gene expression and only downregulated genes 1 hour after GR-activation. Furthermore, we have identified interesting candidate genes that may underlie the glucocorticoid-mediated effects on hippocampal cell function. However, due to 1) the resolution of the time frame profiled, 2) the technical difficulties with the detection of low abundant genes such as the 5HT1A receptor, 3) the use of whole hippocampi and 4) the transient changes observed in gene expression, establishing a correlation between changes in mRNA levels and changes in hippocampal function still remains complex. On the other hand, in the current study new unexpected genes such as LIMK1, calmodulin 2, monoamine oxidase A and CRH receptor 1 were found to be GR-responsive, thereby raising the question what the functional consequences are of GR-regulation of these genes in the hippocampus. Hence, the current study can be used to formulate new hypotheses about the effects of corticosterone on hippocampal cell function. Therefore, future studies need to be focused on the functional consequences of GR-regulation of presently identified interesting hippocampal genes.

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SUPPLEMENTARY MATERIAL

Table S1. From left to right are listed the GeneChip Probe Set IDs, the fold changes in the 1, 3 and 5 hours time point, the gene titles and the Gene Ontology Biological Process descriptions. Fold changes below 1 indicate downregulated genes; fold changes above 1 indicate upregulated genes.

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*FC 5 hours*	*Gene Title*	*GO Biological Process Description*
AB016489_s_at	0.88106			jumping translocation breakpoint	---
AF034582_g_at	0.87415			SEC31-like 1 (S. cerevisiae)	---
C06598_at	0.92833			similar to binding protein	---
L26292_g_at	0.70512			Kruppel-like factor 4 (gut)	---
M31322_g_at	0.86328			sperm membrane protein (YWK-II)	---
M63485_at	0.80711			matrin 3	---
M81639_at	0.78973			stannin	---
rc_AA799726_at	0.82732			Similar to mKIAA1737 protein	---
rc_AA800549_at	0.80255			Transcribed locus	---
rc_AA800693_at	0.81134			Transcribed locus, moderately similar to XP_488563.1	---
rc_AA800693_g_at	0.85774			Transcribed locus, moderately similar to XP_488563.1	---
rc_AA874928_g_at	0.86164			sorting nexin 4 (predicted)	---
rc_AA891314_at	0.89191			poly(rC) binding protein 4 (predicted)	---
rc_AA946313_s_at	0.80394			secreted acidic cysteine rich glycoprotein	---
rc_AA964320_at	0.95849			NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8	---
rc_AI009132_at	0.82736			similar to chromosome 13 open reading frame 12 (predicted)	---
rc_AI010371_at	0.84454			Transcribed locus	---
rc_AI112237_at	0.77218			NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	---
rc_AI232012_at	0.87777			NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8	---
rc_H33149_at	0.84493			similar to RIKEN cDNA 1810047C23	---
X52733cds_s_at	0.88393			ribosomal protein L27a (predicted)	---
X66845_at	0.79120			dynein, cytoplasmic, intermediate chain 1	---
X76985_at	0.85592			latexin	---
rc_AA892851_g_at	0.74547	0.74772		EST196654 Rn.3616	---
AF069525_at		0.84834		ankyrin 3, epithelial isoform g	---
AF095741_g_at		1.12291		Mg87 protein	---
D13966_at		1.31674		insulin receptor-related receptor	---

Probe Set ID	FC 1 hour	FC 3 hours	FC 5 hours	Gene Title	GO Biological Process Description
L40364_f_at		1.44847		MHC class I RT1.O type 149 processed pseudogene	---
M13100cds#6_f_at		0.75022		similar to ORF2 consensus sequence encoding endonuclease	---
rc_AA799369_at		0.83915		similar to RIKEN cDNA 1190002L16 (predicted)	---
rc_AA799473_at		0.71189		Similar to R31449_3	---
rc_AA799550_at		0.81196		T-cell activation protein	---
rc_AA799570_at		0.80322		Similar to mmDj4	---
rc_AA799607_at		0.69066		Transcribed locus	---
rc_AA799824_at		0.87664		Similar to Vacuolar ATP synthase subunit C (V-ATPase C subunit)	---
rc_AA800029_at		0.79104		pam, highwire, rpm 1 (predicted)	---
rc_AA800296_at		0.79944		Similar to poly(A) polymerase V	---
rc_AA800456_at		0.71941		Transcribed locus	---
rc_AA800513_at		0.79192		Similar to transformation/transcription domain-associated protein	---
rc_AA800753_at		0.71393		Similar to importin 7	---
rc_AA800882_g_at		0.66441		EST190379 Rn.24136	---
rc_AA858600_at		0.88112		leucine-zipper-like transcriptional regulator, 1 (predicted)	---
rc_AA866291_at		0.78168		similar to cornichon-like protein (predicted)	---
rc_AA866432_at		0.84821		LOC363015	---
rc_AA866459_at		0.84950		Cyclin D binding myb-like transcription factor 1	---
rc_AA874832_at		0.80373		anaphase-promoting complex subunit 5 (predicted)	---
rc_AA874848_s_at		0.86137		Thymus cell antigen 1, theta	---
rc_AA875084_at		0.70353		transducin-like enhancer of split 1, homolog of Drosophila E(spl)	---
rc_AA875171_at		0.75744		N-acetylglucosamine-1-phosphotransferase, gamma subunit	---
rc_AA891069_at		0.74698		serine/arginine-rich protein specific kinase 2 (predicted)	---
rc_AA891161_at		0.66544		Transcribed locus	---
rc_AA891476_at		0.87216		Transcribed locus	---
rc_AA891824_at		0.88196		serine/arginine-rich protein specific kinase 2 (predicted)	---
rc_AA891864_at		0.68641		ATP/GTP binding protein 1 (predicted)	---
rc_AA892014_s_at		0.79215		HLA-B-associated transcript 1A	---
rc_AA892228_at		0.74292		EST196031 Rn.4183	---

Probe Set ID	FC 1 hour	FC 3 hours	FC 5 hours	Gene Title	GO Biological Process Description
rc_AA892270_g_at		1.10753		similar to DNA polymerase epsilon p17 subunit	---
rc_AA892376_at		0.82120		protein associated with PRK1	---
rc_AA892394_at		0.67246		Similar to CUG triplet repeat RNA-binding protein 1 (CUG-BP1)	---
rc_AA892394_g_at		0.76976		Similar to CUG triplet repeat RNA-binding protein 1 (CUG-BP1)	---
rc_AA892422_at		0.77592		mitochondrial ribosomal protein L11	---
rc_AA892465_at		1.18084		helicase with zinc finger domain (predicted)	---
rc_AA892500_at		0.66947		Similar to mKIAA0623 protein	---
rc_AA892506_at		0.76250		coronin, actin binding protein 1A	---
rc_AA892548_at		0.69869		Transcribed locus	---
rc_AA892666_at		1.22762		galactose mutarotase (aldose 1-epimerase)	---
rc_AA892831_s_at		0.82520		proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	---
rc_AA892842_at		0.72104		similar to capping protein alpha 2 subunit	---
rc_AA893173_at		0.81139		vacuolar protein sorting 29 (S. pombe) (predicted)	---
rc_AA893515_at		0.72324		translocation protein 1 (predicted)	---
rc_H31887_at		1.28709		similar to RIKEN cDNA 1700037H04 (predicted)	---
rc_H33656_at		0.92096		Transcribed locus	---
U25264_at		0.93048		selenoprotein W, muscle 1	---
U48288_at		0.69457		A kinase (PRKA) anchor protein 11	---
X53581cds#5_f_at		0.75750		similar to RIKEN cDNA 9330196J05 (predicted)	---
X83231_at		1.22802		inter-alpha trypsin inhibitor, heavy chain 3	---
rc_AA800882_at		0.62222	1.36565	EST190379 Rn.24136	---
rc_AA891842_at			1.34095	Similar to death receptor 6	---
rc_AI639381_at	0.81049			---	---
X02412_at	0.88790			---	---
X51536cds_at	0.90839			---	---
U75404UTR#1_s_at		1.18993		---	---
X14210cds_at		1.07495		---	---
S46798cds#1_s_at		1.14169		---	---
U11071_f_at		1.14464		---	---
rc_AA965147_at		0.76936		---	---
rc_AI639002_i_at		1.36076		---	---
rc_AI639102_g_at		0.77318		---	---

Probe Set ID	FC 1 hour	FC 3 hours	FC 5 hours	Gene Title	GO Biological Process Description
rc_AI639427_at		0.80273		---	---
rc_H31610_at		0.82677		---	---
rc_AA892860_at		0.90096		---	---
rc_AA892864_at		0.75562		---	---
rc_AA875630_at		0.71008		---	---
rc_AA891054_at		0.66438		---	---
M61177_s_at		1.10081		---	---

Chapter 3

Rapid glucocorticoid effects on the expression of hippocampal neurotransmission-related genes

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ABSTRACT

We previously assessed corticosterone mediated gene expression in acute explant hippocampal slices and found over 200 responsive genes 1, 3 and 5 hours after glucocorticoid receptor (GR) activation by a brief corticosterone pulse. Interestingly, 1 hour after GR activation all genes were downregulated, many of which are involved in hippocampal neurotransmission and plasticity.

The aim of the current experiment was 1) to measure the expression of several of these neurotransmission-related genes that were corticosterone-responsive 1 hour after GR-activation in an *in vivo* setting, 2) to elucidate in which hippocampal subregion these expression changes take place and 3) to assess the specificity of regulation by activated GRs. For this purpose, rats were subcutaneously injected with vehicle, corticosterone or corticosterone pretreated with GR-antagonist RU38486. One hour after the corticosterone injections, mRNA expression levels of 5 selected genes were measured using in situ hybridization.

The mineralocorticoid receptor (MR), MAO-A, casein kinase 2 and voltage dependendent potassium mRNA's, but not dynein mRNA, were rapidly downregulated *in vivo* after corticosterone administration in hippocampal subregions. Furthermore, RU38486 pretreatment reversed in all cases these effects, illustrating the GR-specificity of transcriptional regulation by corticosterone. The results are important for understanding the role of GR in pleiotropic control of hippocampal neurotransmission and plasticity, which is characterized by recovery of function transiently raised by excitatory input.

INTRODUCTION

Hippocampal neurotransmission is influenced by glucocorticoids (1,2,3). For example, high concentrations of glucocorticoids secreted during stress increase the neuron's responses to serotonin, reduce β-adrenergic receptor mediated effects, enhance calcium influx and impair long-term potentiation (LTP) ((4). These effects on hippocampal plasticity exerted by the glucocorticoids likely involves transcriptional changes, since they have a slow onset and persist for at least 4 hours (2,3,5). Such genomic effects of glucocorticoids in high concentrations are mediated by the glucocorticoid receptor (GR), which upon ligand binding can modulate gene transcription via transactivation or transrepression.

Glucocorticoids are secreted in an ultradian rhythm with pulses lasting for about 20 minutes which are sufficient to substantially activate the GR (6,7). In neurophysiological studies over the past two decades using the hippocampal slice this corticosterone pulse was mimicked and as a result a pattern of GR-mediated effects occurred aimed to suppress excitability that was transiently raised by excitatory stimuli (4,8). With the acute explant hippocampal slice, we previously assessed using Affymetrix GeneChips the transcriptional response to a 20 minute corticosterone pulse and found more than 200 corticosterone-responsive genes 1, 3 and 5 hours post-injection (9). One hour after GR-activation all responsive genes were downregulated, suggesting that under this condition in the hippocampus transrepression may be the prevailing pathway mediating the transcriptional response to corticosterone. Naturally gene expression regulation via negative GREs could also be involved.

This study was designed to examine if these responsive genes identified *in vitro* also were altered *in vivo* one hour after administration of a high dose of corticosterone activating the GR. This is an important validation since the data from hippocampal slices were obtained from whole hippocampi and therefore the exact hippocampal subregions in which gene regulation took place could not be pinpointed. Different hippocampal subregions display different levels of GR-expression; GR is predominantly expressed in CA1, CA2 and dentate gyrus whereas in CA3 GR is expressed to a much lower extent (10,11). Furthermore, the different hippocampal subregions have entirely different transcriptomes (12) and hence the availability of transrepression partners and downstream pathways may differ from one subregion to another, giving rise to subregion-dependent differences in corticosterone-mediated gene regulation. Also, since in hippocampal slices the input from extra-hippocampal regions (which could also be steroid-responsive) as well as the peripheral effects of increasing concentrations of corticosterone are lost, this *in vitro* generated data set may not reflect the full extent of GR-mediated effects on hippocampal gene expression in the living brain.

In the current study genes were selected for further study that were representative for the rapid transcriptional effects of glucocorticoids on neurotransmission and plastic-

ity. This included first of all the mineralocorticoid receptor (MR), which is an established target for GR. The GR and its high affinity congener the mineralocorticoid receptor (MR) are postulated to mediate in a coordinate and balanced manner the effects of glucocorticoids. Hence, their mutual interaction is of crucial importance to test the role of the MR/GR balance in neuro-excitability (2). The second responsive gene included was monoamine oxidase A (MAO-A) because of its role in neurotransmitter catabolism. Since MAO is a target for antidepressants its regulation by glucocorticoids is of obvious importance. Third, voltage-gated potassium channel Kv3.2 since this gene directly affects action potential propagation (13). Fourth, casein kinase 2 plays a role as a downstream mediator of NMDA-dependent LTP (14). Finally, cytoplasmic dynein because it possibly plays a role in (GR) receptor translocation (15).

To assess GR specificity of the corticosterone effects the rats were pretreated with the GR-antagonist RU38486. One hour after corticosterone injection, the rats were decapitated and the expression levels of the selected genes were assessed by using mRNA *in situ* hybridization. The data support the concept that the expression patterns in vitro hippocampal slice system are an appropriate model system for generating hypotheses that are testable under *in vivo* conditions, even though these genomic effects are often limited to hippocampal subregions.

MATERIALS & METHODS

Animals

All experiments were carried out with the approval of the Animal Experiment Committee of the University of Amsterdam (protocol number DED108).

Young male Wistar rats of approximately 200 g were used for the experiments and were housed individually on a 12 h light/dark cycle (lights on at 8.00 a.m.) with food and drinking water *ad libitum*. On the day of the experiment, at 9.30 a.m., the animals were subcutaneously injected with 800 µl (arachide) oil (vehicle group; n = 8) or corticosterone (Sigma, the Netherlands; 10 mg / animal) dissolved in oil (cort group; n = 8). An additional group of animals received a subcutaneous injection with 800 µl GR-antagonist RU38486 (Sigma, The Netherlands; 25 mg / kg body weight) at 8:30 a.m., followed one hour later by an injection with corticosterone (RU + cort group; n=8). One hour after the injections (at 10:30 a.m.), the animals were decapitated. Brains were immediately isolated, frozen on dry ice, and stored at -80°C.

Corticosterone

In order to measure plasma corticosterone concentrations, trunk blood was collected in EDTA-coated tubes immediately after decapitation. Blood samples were centrifuged

at 3000 r.p.m. for 10 minutes at 4°C, after which the plasma was collected and stored at -20°C. Corticosterone concentrations were measured with the murine corticosterone RIA kit (ICN Biomedicals™, Costa Mesa, CA, USA).

Oligonucleotides

Where possible, 45-mer oligonucleotide probes were designed in the 3' untranslated regions where the probe sets used on the Affymetrix GeneChips (9) were derived from. The mismatch oligos were identical to the perfect match oligos except for six point mutations (transversions), evenly spaced at approximately seven nucleotides distance. Perfect match and mismatch probes are listed in Table 1.

Table 1. Oligonucleotide probes for mRNA *in situ* hybridization.

Gene title	Probe set ID	Perfect match oligo (5'-3')	Mismatch oligo (5'-3')
casein kinase 2	L15618_at	cac-gac-agt-gta-gaa-gta-agg-gtg-ctc-cat-ggc-ctc-tct-tgc-agt	cac-Tac-agG-gta-gaa-Tta-agg-Ttg-ctc-caG-ggc-ctc-Gct-tgc-aTt
dynein, cytoplasmic, intermediate chain 1	X66845_at	tag-gac-tac-gcc-aga-agt-aca-tgt-aag-cac-aaa-ttc-aac-cag-agg	tCg-gac-taA-gcc-aga-Cgt-aca-Ggt-aag-caA-aaa-ttc-Cac-cag-aTg
mineralocorticoid receptor (MR)	M36074_at	ttc-gga-ata-gca-ccg-gaa-acg-cag-ctg-acg-t	Gtc-gga-Cta-gca-Acg-gaa-Ccg-cag-Atg-acg-G
monoamine oxidase A	D00688_s_at	aac-cat-ctt-gaa-gac-aca-ggt-aga-ctt-aga-gat-cta-atc-ctc-tga	aCc-cat-Att-gaa-gac-Cca-ggt-aTa-ctt-agC-gat-cta-Ctc-ctc-tTa
potassium voltage gated channel (Kv3.2)	X62839mRNA_s_at	tac-aca-gtt-tca-act-aca-gca-gtg-atg-aag-aca-aac-aca-cgc-ttc	Gac-aca-gGt-tca-acG-aca-gca-gGg-atg-aaT-aca-aaA-aca-cgc-Gtc

From left to right are listed the gene titles, probe sets on the Affymetrix Rat Genome U34A GeneChips, sequences of the perfect match oligos and sequences of the mismatch oligos. The point mutations (transversions) in the mismatch oligos are displayed in capital letters.

mRNA *in situ* hybridization

Using a CM1900 cryostat (Leica Microsystems, Wetzlar, Germany) twenty micrometer coronal brain sections were prepared from the isolated brains, thaw mounted on poly-L-lysine-coated slides and stored at -80°C. *In situ* hybridizations were performed as described previously (16), using specific 45-mer oligonucleotide probes. These probes were labeled with α-^{33}P-dATP (Perkin Elmer Life Sciences) at the 3'-terminals, using terminal transferase (Amersham), which resulted in 50 – 75% incorporation efficiency (10 -15 A-residues per oligonucleotide). Per gene 4 sections were hybridized with the perfect match oligo for each animal. Additionally, per gene 4 sections were hybridized with the mismatch oligo. These sections were subsequently exposed to a Kodak X-OMAT MR film (Rochester, NY, USA) for 4 - 9 days, depending on signal intensity. The signals were in the linear range of gray values according to the [^{14}C] RPA 504 microscales (Amserham, Aylesbury, UK). Per gene, the radioactive labeled slides from the different treatment groups were distributed onto the same film. The optical density was quantified using an Olympus image analysis

system (Paes, The Netherlands) equipped with a Cue CCD camera. In order to obtain a specific expression signal for each gene the mismatch signal was used as a measure for background intensity and subtracted from the perfect match signal. The expression levels were measured in the CA1, CA2, CA3 and dentate gyrus (DG) regions. The scoring of the gene expression measurements was performed blindly, after which the experimental conditions were re-assigned to the differently coded groups. One-way ANOVA and post-hoc LSD tests were performed to assess statistically significant gene expression changes ($P < 0.05$).

RESULTS

Corticosterone values

As expected, plasma corticosterone concentrations were significantly elevated ($P < 0.05$) in corticosterone injected animals (66 +/- 12 µg / dl) and corticosterone + RU38486 injected animals (96 +/- 44 µg / dl) compared to the vehicle injected animals (2.0 +/- 1 µg / dl).

In situ hybridizations

Sections hybridized with the perfect match probes yielded strong specific hybridization signals whereas sections hybridized with the mismatch probes displayed no specific hy-

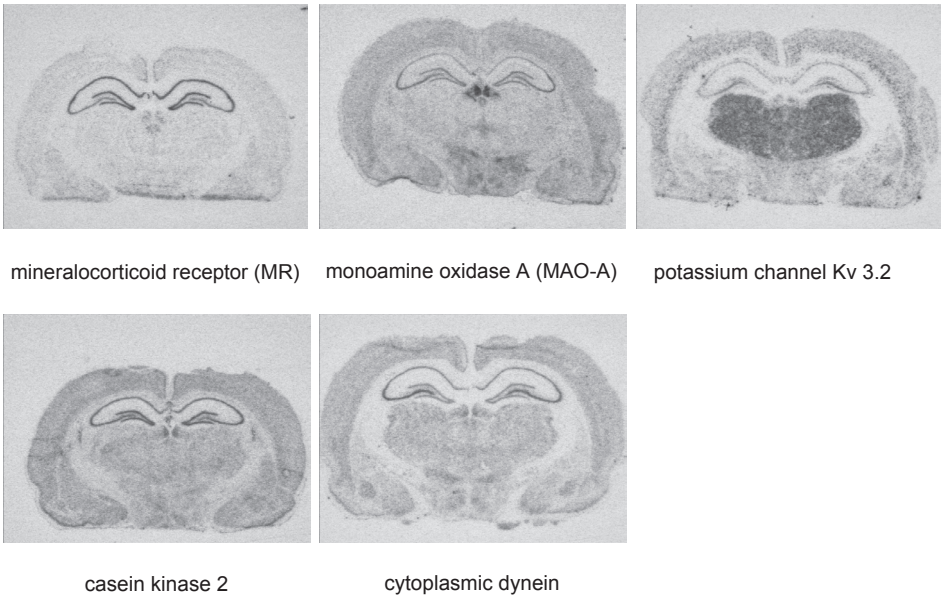

Figure 1. Representative mRNA *in situ* hybridization autoradiographs of frontal hippocampal sections hybridized with different oligonucleotide probes.

bridization signal, illustrating the binding-specificity of the oligonucleotide probes. The expression of each gene displayed a characteristic distribution pattern throughout the brain and throughout the different hippocampal subregions (Figure 1) with for instance monoamine oxidase A (MAO-A) showing a lower expression level in the CA1 subregion compared to the other subregions and a strong signal in the habenula. Potassium channel Kv 3.2, conversely, showed lowest expression in the dentate gyrus and very high expression levels in the thalamus region. Additionally, expression could be observed in and just above the pyramidal cell layer. Furthermore, for MAO-A and the mineralocorticoid receptor (MR) the CA2 region was clearly distinguishable from the surrounding regions and therefore the expression levels in this region were also measured.

Strikingly, four out of five genes displayed a significant downregulation ($P < 0.05$) 1 hour after corticosterone administration in at least 1 hippocampal subregion (Figure 2). Additionally, for each corticosterone-responsive gene the pretreatment with the GR-antagonist RU486 significantly ($P< 0.05$) reversed the corticosterone-mediated effect in nearly all cases.

More specifically, for the MR mRNA, a significant decrease occurred in the CA3 region after corticosterone; RU486 pretreatment significantly increased expression in all hippocampal subregions compared to the animals treated only with corticosterone. Monoamine oxidase A expression decreased in CA1, CA3 and DG subregions after corticosterone treatment. This decrease was fully reversed in animals receiving corticosterone + RU486 treatment in the CA1 subregion. Potassium voltage-gated channel Kv3.2 displayed significant reductions in gene expression in the CA1 and DG after corticosterone, which both were blocked by RU486 administration. Finally, casein kinase showed significant transcriptional downregulation in the CA1, CA3 and DG subregions after corticosterone treatment, which in all three areas was reversed by RU486 pretreatment. Interestingly, with all of these 4 genes the pattern of transcriptional regulation in the corticosterone and corticosterone + RU486 treatment groups was very similar for the different subregions.

Cytoplasmic dynein did not show a significant downregulation in any hippocampal subregion after corticosterone administration. Also the combined corticosterone and RU486 treatment did not have any effect on the expression level of this gene.

DISCUSSION

The present study showed that 4 out of 5 selected genes, previously identified as corticosterone-responsive 1 hour after corticosterone administration to explant hippocampal slices, also were reduced in expression at 1 hour after administration of high amounts of corticosterone to intact animals. All effects appeared to be GR-mediated. Importantly, our data are not a mere validation of the earlier microarray observations (9) with *in situ*

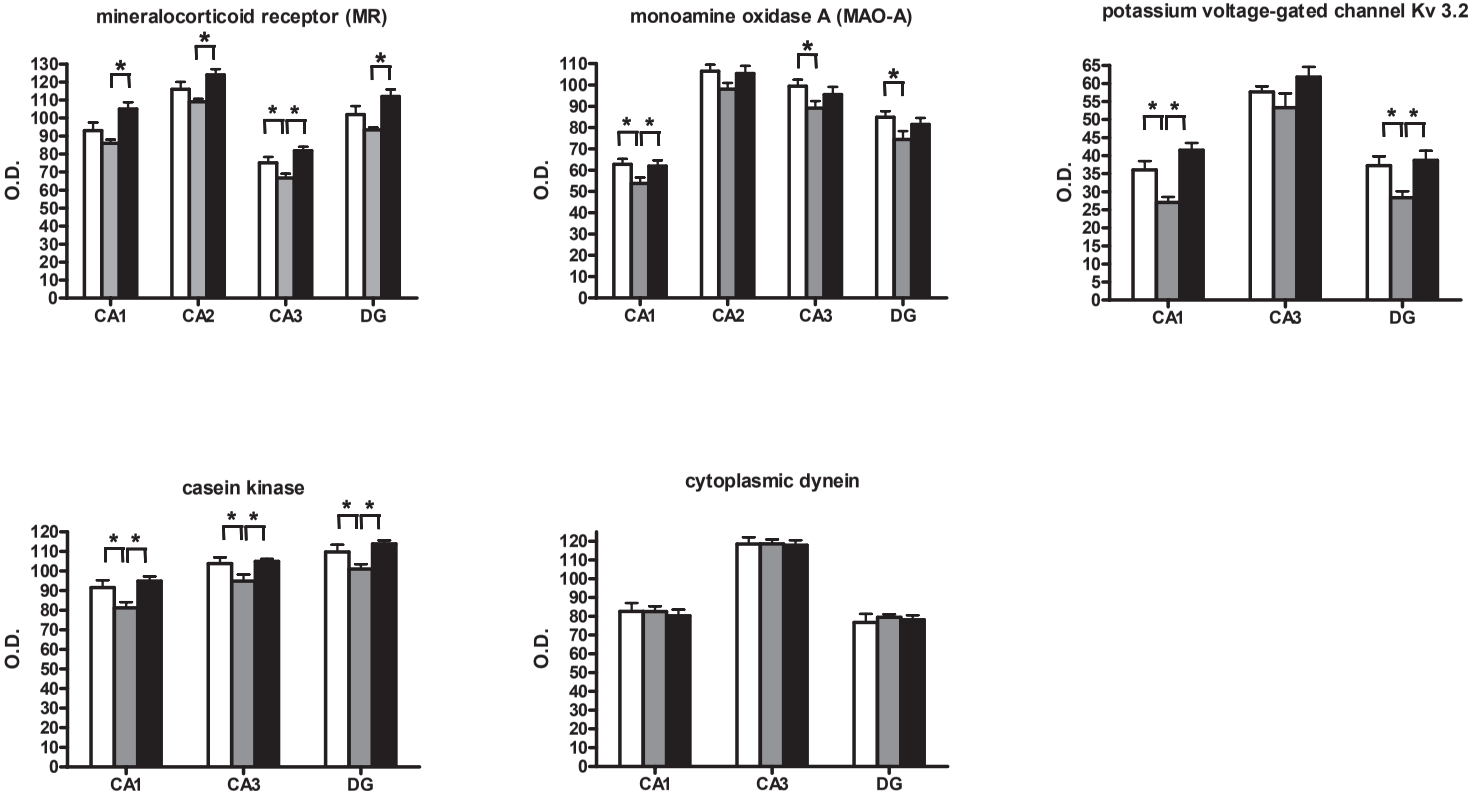

Figure 2. Expression levels of 5 genes in different hippocampal subregions after vehicle (white bars), corticosterone (grey bars) or corticosterone plus GR-antagonist (RU38486) treatment (black bars). Expression levels were measured using mRNA *in situ* hybridization. The significance level (*) was set at $P < 0.05$. O.D.; optical density.

hybridization, as the earlier study used *in vitro* application of corticosterone to a reduced hippocampal preparation whereas here corticosterone was applied to the intact animal; the latter comes close to physiologically relevant situations (e.g. after stress), allowing peripheral effects of corticosterone as well as effects in hippocampal ánd non-hippocampal brain regions. Given the highly comparable outcome for 4 out of 5 genes in the two studies, we conclude that the influence of projections from peripheral organs and extra-hippocampal brain structures expressing GR (17) did not affect the *in vivo* GR-mediated transcriptional response. This observation also further reinforces the notion that we studied primary responsive genes. The effects sizes displayed values as low as 10%, which is in line with hormone-induced gene expression changes observed in other studies (9,18-22). In fact, small reliable changes in gene expression in the order of 10 – 30% are commonly observed in the effects of glucocorticoids on hippocampal neuronal functioning (23).

Generally, the 4 responsive genes showed a significant downregulation of expression (or a trend towards downregulation) by corticosterone in every hippocampal subregion. Consequently, pooling hippocampal subregions -as was done in the earlier microarray study- is not expected to dilute transcriptional changes induced by corticosterone. Conversely, genes that do show regional differences in GR-dependent transcriptional regulation might not be picked up as significant in an approach using material pooled from all hippocampal subareas. The fact that 4 out of 5 genes tested in the present study displayed comparable GR-induced expression changes for all hippocampal regions, despite profoundly different local profiles including for the genes under study themselves (12), is interesting. Again, this supports that these 4 genes form primary targets for corticosterone.

A number of functional implications can be inferred from our findings. First, the hippocampal MR was downregulated in CA3, while blockade with the antagonist enhanced MR expression in all regions. This suggests that GR-mediated downregulation had occurred by corticosterone. Corticosterone binds with high affinity to the MR. The finding is, therefore, of interest since it points to heterologous rather than homologous downregulation of MR. Moreover, although corticosterone-induced MR downregulation is known, it has not been reported to occur already within the 1 hour time interval (24). Since many GR-mediated effects are known to counteract those induced via MR, this rapid downregulation of MR could constitute an important regulatory control in the balance of MR- and GR mediated effects thought to be important for neuroexcitability (2).

Second, the synaptic input from mono-aminergic neurons may be modulated via downregulation of MAO-A, which is involved in the catabolism of aminergic neurotransmitters such as serotonin, dopamine and noradrenalin in synapses. Therefore downregulation may lead to higher pre-synaptic levels of these neurotransmitters, thereby specifically enhancing the efficiency of synaptic aminergic input. The rapid GR-mediated action of

corticosterone is of interest, since it provides an additional substrate for its pre-synaptic stimulatory actions on aminergic transmission in addition to the corticosterone enhanced amine biosynthesis. This GR-mediated effect on MAO-A expression occurs fast suggesting it participates in the recovery process from stress promoted by the glucocorticoids.

Third, increased activity of casein kinase 2 was shown to be correlated with the induction of NMDA-receptor mediated long-term potentiation (LTP) (14,25). Downregulation of casein kinase 2 by corticosterone in the hippocampus may therefore inhibit NMDA-receptor mediated LTP in a delayed genomic fashion. This would fit with earlier observations that GR activation indeed suppresses the induction of NMDA-type LTP (26) and that this is most likely not caused by a downregulation of NMDA receptor subunits (9 and Qin and Joëls, unpublished observation). Therefore, regulation of other, NMDA function modifying proteins –like casein kinase 2– may underlie the effect of corticosterone on LTP.

Finally, voltage-gated potassium channel Kv3.2 functions as a delayed rectifier-type potassium channel that is activated by large membrane depolarizations. Downregulation by activated GR may therefore result in enhanced action potential propagation along the axons due to decreased hyperpolarization of the membrane potential.

One gene, dynein, did not display corticosterone-responsiveness in the current design which could indicate that the expression of this gene may be under the control of hippocampal afferents. Alternatively, since in the current experiment injections of corticosterone occurred in intact animals, the time frame of corticosterone action does not necessarily have to coincide with that in the hippocampal slice experiment due to differences in hormone kinetics and tissue condition. Hence, further study is required to examine the corticosterone-response of dynein, also given the role of the protein in GR-receptor translocation (15).

With the 4 genes representative for distinct rapid genomic processes underlying hippocampal neurotransmission and plasticity novel aspects of GR-mediated corticosterone physiology emerge. Some genes such as MAO-A are suppressed, which may give rise to an enhanced aminergic input. Depending on whether the amines involved exert excitatory or inhibitory effects –which is determined both by the ligand and the receptor subtype mediating its actions- reduced levels of MAO-A could thus increase or decrease the local excitability. Other genes such as MR and casein kinase are suppressed and if translated in reduced functional proteins would suppress excitability. Conversely, suppression of the voltage gated potassium channel would probably result in enhanced neuroexcitability (Figure 3).

Hence, the presently studied glucocorticoid-dependent transcriptional responses potentially could change hippocampal neurotransmission. Most though not all effects would favor a suppression of the local excitability. However, some caution is required since the genes constitute only a small fraction of a transcriptional profile which contains

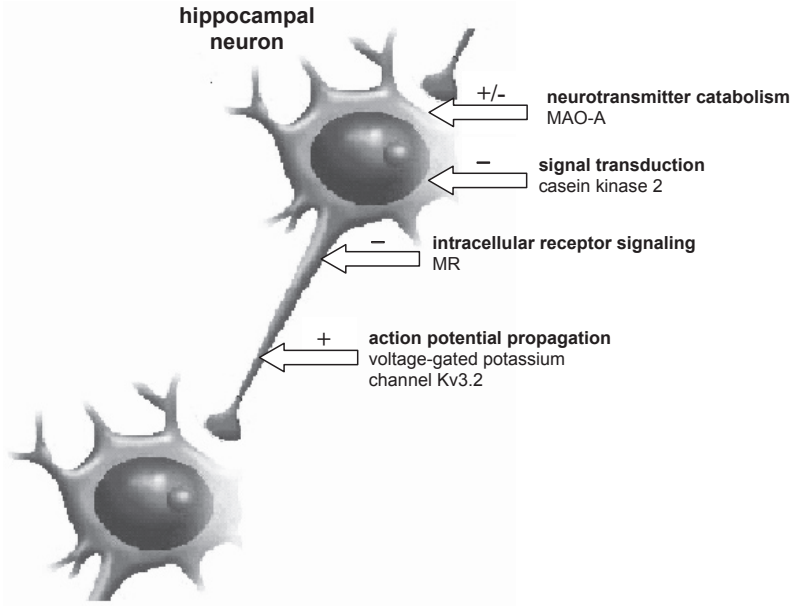

Figure 3. Corticosterone-mediated downregulation of the currently in vivo profiled genes affects neurotransmission in hippocampal neurons. The different cellular levels via which corticosterone exerts effects on neurotransmission are signal transduction, intracellular receptor signaling, action potential propagation and neurotransmitter catabolism. The + and – symbols indicate the enhancing and inhibiting effects respectively of glucocorticoid-mediated gene expression regulation on neurotransmission.

more than 200 GR-responsive genes. Therefore, the overall picture on how corticosterone influences neuronal functioning in different hippocampal subregions is far more complex.

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Chapter 4

The dynamic pattern of glucocorticoid receptor-mediated transcriptional responses in neuronal PC12 cells

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ABSTRACT

The aim of the current study was 1) to examine the overlap in the pattern of GR-mediated transcriptional responses between different neuronal substrates and 2) to assess the nature of these responses by differentiating between primary and downstream GR-responsive genes. For this purpose, NGF-differentiated catecholaminergic PC12 cells were used in which endogenous GRs were activated briefly with a high dose of corticosterone followed by gene expression profiling 1 and 3 hours afterwards using Affymetrix GeneChips.

The results revealed a strikingly similar temporal pattern to what was reported previously in hippocampus, with only downregulated genes 1 hour after GR-activation and the majority of genes upregulated 3 hours after GR-activation. Real-time qPCR of transcripts in cycloheximide treated cells showed that all 5 GR-responsive genes selected from the 1 hour time point were primary-responsive, whereas all 4 GR-responsive genes selected from the 3 hours time point were downstream-responsive. At the level of individual genes, the overlap with the previously generated hippocampal data sets was small, illustrating the cell-type specifity of GR-mediated genomic responses. Finally, we identified a number of interesting genes, such as SWI/SNF, SNAP-25 and certain Rab proteins which may play a role in the effects of glucocorticoids on catecholaminergic neuronal functioning.

INTRODUCTION

Glucocorticoids modulate neuronal functional properties such as excitability and energy metabolism (1-3). Since glucocorticoid receptors (GRs) are ligand-inducible transcription factors, changes in gene expression may underlie these effects. Activated GRs can modify gene transcription via transactivation and transrepression (4,5). In transactivation, GRs bind to glucocorticoid responsive elements (GREs) on the DNA, thereby either enhancing or repressing (negative GREs) gene transcription. In transrepression, GRs bind via protein-protein interactions to other transcription factors such as AP-1 and NF-κB, thereby inhibiting their transcriptional activity.

We have previously assessed time dependent GR-responsive genes in the explant hippocampal slice preparation after a single corticosterone pulse, using Affymetrix GeneChips. The design mimicked the one previously used in electrophysiological studies (6). At 1 hour after brief GR-activation we first found exclusively down regulated genes and then both up and downregulated genes 3 hours after GR-activation (7). Since so far only a limited number of genes repressed via negative GREs have been identified, these data suggest that in hippocampal tissue the mechanism of transrepression via protein-protein interactions may precede transactivation in time after acute activation of GRs. However, currently no additional data exist that permit generalization of this highly characteristic time-dependent, neuronal genomic response to corticosterone mediated by GR. Moreover, also the nature of these temporal transcriptional responses regarding primary and downstream responsive genes is unknown.

The aim of the current study was 1) to assess to which extent the genomic response to acutely activated GRs overlaps between different neuronal substrates and 2) to gain more insight into the pattern of GR-mediated primary and downstream transcriptional responses by blocking protein synthesis with cycloheximide. We hypothesize that many primary responsive genes will be found at an early stage in time after GR-activation, which will influence gene expression of other genes later on.

For this purpose, clonal cell lines with neuronal properties constitute interesting model systems since they can be easily manipulated by direct pharmacological treatment. PC12 cells are unique among clonal cells in the fact that they contain a near-diploid chromosome number and display a highly stable karyotype, making them very suitable for large-scale gene expression profiling. PC12 cells are derived from rat adrenal pheochromocytoma cells and display a catecholaminergic phenotype. Upon stimulation with nerve growth factor (NGF) the cells develop long branching neurites and become electrically excitable, thereby obtaining a neuronal phenotype (8). In comparison to other neuronal cell lines, PC12 cells come closest to mimicking a specific population of differentiated neuronal cells, expressing sodium, potassium and calcium channels as well as membrane receptors, including G-protein coupled receptors (9).

We used neuronal PC12 cells to characterise gene expression profiles after GR-activation. Therefore, PC12 cells were differentiated to obtain a neuronal phenotype by exposure to NGF for 10 days (8). Similar to the previously performed hippocampal slice study, GRs were activated by treating the cells with a brief corticosterone pulse and gene expression was profiled 1 and 3 hours afterwards using Affymetrix GeneChips. Additionally, the experiment was repeated to distinguish primary responsive and downstream responsive genes by blocking protein translation with cycloheximide, after which the expression of a subset of selected genes was measured using real-time qPCR. Furthermore, in order to assess the generality and specificity of the GR-mediated transcriptional responses on the level of the individual genes, the currently obtained GR-responsive genes were compared to 1) GR-responsive genes found in the explant hippocampal slice preparation (7) and 2) hippocampal corticosterone-responsive genes obtained by using Serial Analysis of Gene Expression (SAGE) 3 hours after a single in vivo injection of corticosterone (10).

MATERIALS & METHODS

Cell culture and treatment

PC12 cells were cultured in DMEM medium (4500mg/l glucose, Invitrogen Life Technologies, Carlsbad, CA, USA) substituted with 10% horse serum, 10% FBS, penicillin (20 U / ml) and streptomycin (20 µg / ml) on 150 mm × 25 mm plastic culture dishes (Corning Incorporated, NY 14831, USA) which were coated with 5 µg / cm^2 rat tail collagen (Roche).

For the large-scale gene expression profiling experiment, PC12 cells were cultured until 10 (150 × 25 mm) culture dishes were obtained (passage 15). Per time point 5 dishes were used and from each culture dish the cells were divided over two new dishes at approximately 20% confluency, resulting in 5 pairs of dishes per time point which were kept paired throughout the entire experiment. All the cells were differentiated into a neuronal phenotype by maintaining them in serum deprived and NGF-β (50 ng / ml; Sigma®, MO, USA) substituted medium for 4 days, followed by 5 additional days in low serum (2% horse serum + 2% FBS) and NGF (50 ng / ml) substituted medium (8). On the last day of differentiation, per culture dish pair, one dish was used for GR-activation by treatment with a 100 nM 20 minute pulse of corticosterone, whereas the other dish was used as a vehicle (0.009% ethanol) treated control, resulting in a paired experimental setup (n=5 per time point). The corticosterone pulse was produced by replacing the differentiation medium with differentiation medium containing 100 nM corticosterone. Twenty minutes later, the corticosterone containing medium was removed and the original differentiation medium was reinstated onto the cells. One or three hours after the initial addition of corticosterone (or vehicle) total RNA was isolated from each culture dish using TRIzol® (Invitrogen Life Technologies) according to the manufacturer's instructions.

For the cycloheximide experiment exactly the same culture protocol and paired experimental design were used (n=5 per time point). Twenty minutes prior to GR-activation or vehicle treatment, cycloheximide (10 μM) was applied to the NGF-containing culture medium and was kept there throughout the rest of the experiment.

RNA preparation and Affymetrix array hybridization

After isolation, total RNA purification was performed with the QIAGEN RNeasy® Mini Kit RNA Cleanup procedure (QIAGEN Inc. Valencia, CA, USA). RNA quality was assessed using the LabChip® RNA 6000 Nano Assay on the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and the Degradometer Software (Auer et al 2003). Per RNA sample, 10μg was used for cDNA synthesis and in vitro transcription, according to the procedure described by Affymetrix (www.affymetrix.com). Briefly, total RNA was used to generate double-stranded cDNA after which the mRNA portion was amplified and biotin-labeled using the ENZO BioArray HighYield RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA, USA). The QIAGEN RNEasy® Mini Kit RNA Cleanup procedure was used to purify the amplified RNA. Quality of the RNA was checked with the LabChip® RNA 6000 Nano Assay on the 2100 Bioanalyzer (Agilent Technologies). The amplified RNA samples were then hybridized to Rat Expression Array 230A GeneChips (Affymetrix) at the Leiden Genome Technology Center (LGTC®), Leiden University, The Netherlands.

Per time point, 6 Affymetrix arrays were used, 3 for the corticosterone-treated culture dishes and 3 for the control culture dishes. Each array included ~15000 probe sets, representing ~5000 full-length or annotated sequences and ~10000 EST clusters.

Affymetrix data analysis

GeneChip® Operating Software 1.2 (GCOS; Affymetrix) was used to normalize the array signals by total intensity normalization (11) and to calculate signal intensities plus signal reliabilities. Only transcripts which generated present and/or marginal calls throughout all 6 arrays per time point were included for further analysis, removing the transcripts that generated one or more absent calls. In order to identify responsive genes, Significance Analysis of Microarrays (SAM version 2.21) (12) was used. When analyzing microarrays, many false positives can be expected due to the multiple testing problem. SAM is a permutation based test that allows the user to control the False Discovery Rate (FDR), i.e. the relative number of false positives generated. For both time points SAM was applied to the paired data sets derived from corticosterone treated and vehicle treated culture dishes and the lowest FDR was chosen to assess the most significant responsive genes. In order to verify the results obtained by SAM, the class comparison analysis from the BRB ArrayTools package (version 3.3.0, developed by Dr. Richard Simon and Amy Peng Lam) was used. Additionally, genes involved in similar biological processes were grouped

together using Gene Ontology Biological Process classifications that were obtained from the NetAffx Analysis Center (13).

Furthermore, the obtained data set was compared with our previously generated hippocampal SAGE and slice data sets. In the hippocampal SAGE experiment, adrenalectomized rats were substituted with low corticosterone pellets to assess MR-responsive genes and injected with a high corticosterone injection to assess GR-responsive genes 3 hours afterwards. Subsequently, gene expression profiles were established using the SAGE procedure in which 10 base pairs long SAGE tags derived from a defined position within the 3' untranslated regions of the transcripts were sequenced and counted. In the hippocampal slice experiment, we used explant hippocampal slices obtained from adrenalectomized, low corticosterone pellet substituted rats (MR occupation). These slices were treated with a brief *in vitro* corticosterone pulse to activate GRs and gene expression was profiled 1, 3 and 5 hours afterwards using Affymetrix GeneChips. To allow cross-platform comparisons between SAGE tags and GeneChip probe sets, the Expression Analysis Systematic Explorer (EASE) (14) was used to couple Affymetrix probes to UniGene clusters whereas the NCBI SAGEmap ftp-site was used to couple these UniGene clusters to SAGE tags. Both EASE and the NCBI SAGEmap used UniGene build 139. The NetAffx Analysis Center was used to link the probe sets from the Rat Expression 230A GeneChips (PC12 cell experiment) with the Rat Genome U34A GeneChips (hippocampal slice experiment).

Real-time quantitative PCR

A selected subset of responsive genes was validated in all the obtained experimental samples (n=5 per time point), including the RNA samples that were used for the GeneChip analysis (n=3 per time point). Additionally, the same genes were also validated in the cycloheximide treated samples (n=5 per time point).

Validation was performed by real-time qPCR on a DNA Engine Opticon® 2 Real-Time PCR Detection System (MJ Research, Inc., Waltham, Massachusetts, USA). All RNA samples were subjected to DNAse treatment with DNAseI (Invitrogen Life Technologies) according to the manufacturer's protocol. Subsequently, cDNA was synthesized using M-MuLV Reverse Transcriptase RNaseH$^-$ (Finnzymes Oy, Espoo, Finland) and random primers (150 ng; Invitrogen Life Technologies) in a total volume of 20µl. Per experimental sample, 100 ng of RNA was used for the cDNA-synthesis reaction using 150 ng random primers whereas standard curves were made with 5, 50, 100, 500 and 1000 ng RNA. In order to control for genomic contamination, RT$^-$ samples were generated. The PCR was performed in a total volume of 25 µl, consisting of 12.5 µl 2×PCR MasterMix with SYBR® Green I (qPCR™ Core Kit for SYBR® Green I, EUROGENTEC, Seraing, Belgium), 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 6.5 µl water and per primer pair either 5 µl cDNA-sample, RT$^-$ sample or water (no template control). The PCR conditions were as follows:

Table 1. Primers for validation of corticosterone-responsive genes

Gene Title	Probe Set ID	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon (bp)
SWI/SNF related	1373565_at	CCTGTCTGTTGGCCCTGGT	GTCGGCCCTGTCCAGTAAGATA	171
beta-actine	1398835_at	TGACCGAGCGTGGCTACA	CAGCTTCTCTTTAATGTCACGCA	70
lactate dehydrogenase A	1367586_at	CACACTGCCAACTGCATGC	TGGTGAGGGTGCGTAGCA	163
ribosomal protein S6	1367573_at	ACCAAAGCGCCCAAGATTC	AGCAATACGTCGGCGTTTGT	68
actin related protein 2/3 complex, subunit 1B	1386925_at	GGCTAAGGGCTGCTTTGCT	CTTCCTCTTCCCCTCTTTGGA	86
synaptosomal-associated protein 25 (snap 25)	1387073_at	GGGTTTGTCGAATGCTTTTGA	CAACAAGAGCCAGACTTAGA AGATCTT	86
RAB7, member RAS oncogene family	1387797_at	AGTCCTTCACAGACCAAGA ACACAC	TCACGTTCTGGTCTGTTTAGA GGAG	73
tubulin, beta 5	1370290_at	GCCTCACACACTCCCCAGAG	GGAATGACCAAGCCAAGGAA	72
calpactin	1386890_at	CCCTGAACTCCTCCCTGTGA	TGACGACAGAGCTGCCGACCCA	67
translation elongation factor 1-delta subunit	1388134_at	CCCACAGACCCAACATGTCTC	CTGCTGGTGTGGCTCCTTTC	70
18S rRNA	accession nr. M11120	CCCTGCCCTTTGTACACACC	CGATCCGAGGGCCTCACTA	66

10 minutes at 95°C and 40 cycles of 15 seconds at 95°C + 1 minute at 60°C (for both annealing and extension). Afterwards, the temperature was gradually increased to 95°C in order to make dissociation curves.

Dissociation curves were used to control for the specificity of the reaction and genomic contamination whereas the standard curves were used to quantify the expression differences. Expression levels of the target genes were normalized to the expression levels of 18S ribosomal RNA since this transcript displays very high and stable expression levels. The non-parametric Wilcoxon Signed Ranks Test was used to assess significant differential expression of GR-responsive genes.

In general, PCR primers were designed in the regions where the probe sets used on the Affymetrix GeneChips were derived from (Table 1).

Corticosterone

In order to measure corticosterone levels in low serum NGF-containing culture medium and corticosterone-treated culture medium, the murine Corticosterone RIA Kit (ICN Biomedicals™, Costa Mesa, CA, USA) was used according to the manufacturer's instructions.

Western Blotting for the detection of the glucocorticoid receptor (GR)

In order to verify expression of the glucocorticoid receptor in differentiated PC12 cells, Western Blotting was performed. For this purpose, two separate culture dishes with PC12 cells were differentiated into the neuronal phenotype as previously described. Additionally, in order to generate a positive control sample for GR-detection, COS-1 cells were transfected with 2 µg rat GR plasmid (6RGR) (15) and 3 µg pSP64 carrier plasmid (Promega Corporation, Madison, WI, USA) using SuperFect® Transfection Reagent (QIAGEN Inc.) according to the manufacturer's instructions. Non-transfected COS-1 cells and rat MR-transfected COS-1 cells (2 µg rat MR plasmid (16) and 3 µg pSP64 carrier plasmid) were used as negative controls.

Cells were lysed in ice-cold ½ RIPA lysis buffer (20 mM Triethanolamine, 0.14 M NaCl, 0.05% deoxycetant, 0.05% SDS, 0.05% Triton X-100) substituted with protease inhibitors (cØmplete Protease Inhibitor Cocktail Tablets; Roche Applied Science, Penzberg, Germany). Subsequently, the cell lysates were centrifuged for 30 minutes at 13000 rpm at 4°C after which the supernatants were collected. Protein content was quantified using the BCA™ Protein Assay (Pierce Biotechnology, Rockford, IL, USA) and from each sample 25 µg was loaded onto a 10% SDS-PAGE gel. After electrophoresis, the samples were blotted overnight onto an Immobilon P membrane (Millipore Corporation, MA, USA). GR was detected using 4 µg mouse monoclonal anti-GR (MA1-510; Affinity BioReagents Inc., IL, USA) as a primary antibody and 0.4 µg goat-anti-mouse IgA conjugated with horse radish peroxidase (Santa Cruz Biotechnology Inc., CA, USA) as a secondary antibody. Luminol sodium salt (Sigma®) substituted with p-Coumaric acid (Sigma®) was used as substrate for the peroxidase reaction.

Immunocytochemistry for the detection of GR-translocation

In order to assess translocation of the GR into the nucleus after an increase in corticosterone concentrations from 12 nM (background) to 100 nM (corticosterone pulse) an additional experiment was performed. Differentiated PC12 cells were stimulated with a 20 minute 100 nM corticosterone or vehicle pulse similarly to the original experiment, on top of a 10 nM corticosterone background (mimicking the 12 nM background concentration in the original experiment) and 30 minutes, 1 and 3 hours afterwards the cells were processed for immunocytochemistry. The control groups consisted of cells cultured in corticosterone-free (stripped) medium and stimulated with a vehicle pulse. PC12 cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4 for 15 min. To block non-specific staining, cells were treated with 5% NGS in PBS including 0.3% TX-100 for 30 minutes at room temperature. Rabbit polyclonal anti-GR (M-20 1:500, Santa Cruz Biotechnology) was diluted in PBS containing 1% BSA and 0.1% TX-100 for 60 min at room temperature. Control sections were incubated with equal amounts of normal rabbit IgG, which were used as substitute for the primary antibody. After washing,

cells were incubated with AlexaFluor-488 labeled goat-anti-rabbit IgG (1:750, Molecular Probes, USA) in PBS containing 0.1% TX-100 and 1% BSA for 45 min at room temperature. Finally, cells were washed and nuclei were visualized with Hoechst 33258 (1:1000, Molecular Probes, USA) in PBS for 10 min. Before slides were mounted with Aqua Polymount (Polysciences, Inc), cells were washed again. The following day, samples were analyzed and images were taken at a 630x magnification using a Leica DM 6000B fluorescence microscope (Leica Microsystems). All images were taken using identical microscope and filter settings. Image analysis was performed using ImageJ 1.32j software (NIH, USA). To assess differences in GR translocation, changes in optical density of nuclear GR immunoreactivity were measured in a semi-quantitative manner. Hoechst staining was used to identify the nuclear surface of individual cells and a circular mask was applied with the analysis software. These "masks" served as a template and were pasted onto the corresponding GR images to measure the optical density within the nucleus. Non-specific binding (normal rabbit IgG) was also measured and subtracted from the total signal to obtain the specific signal. Per experimental group, nuclear GR-immunoreactivity of approximately 30 randomly selected cells was measured and statistical significance was calculated using analysis of variance (ANOVA) and post-hoc Fisher's LSD testing.

Putative transcriptional elements in the DNA of primary responsive genes

In order to scan the genomic sequence of the primary responsive genes for the presence of transcription factor binding sites, rat, mouse and human sequences, starting at 2500 bp upstream of the first exon, were downloaded from the Ensembl website (www.ensembl.org). The TRANSFAC® 7.0 database matrices were used to define the consensus binding sequences for AP1 (NNTGASTNMNN), NFκB (GGGRMTYYCC) and CREB (NNTGACGTNNNN) whereas the consensus site for the nGRE was obtained from Schoneveld et al. (17) (ATYACNNTNTGATCN). Both the promoter region (starting at 2500 bp upstream of the first exon) and the intronic regions of the rat genes were scanned for the presence of these consensus sequences. In order to assess whether the obtained binding sites were conserved between species, they were compared with the mouse and human sequences for location and nucleotide similarity.

RESULTS

Endogenous GR expression in NGF-differentiated PC12 cells

The presence of endogenous GR in NGF-differentiated PC12 cells was demonstrated in two independent samples using Western Blotting. COS-1 cells transfected with plasmid containing rat GR were used as positive controls whereas empty COS-1 cells were used as a negative controls. Both the NGF-differentiated PC12 cells and the GR-transfected

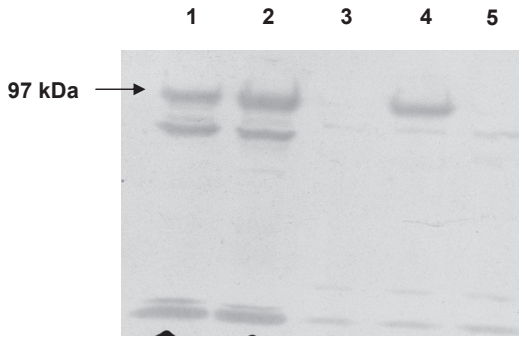

Figure 1. Western Blotting analysis for the detection of the glucocorticoid receptor (GR) in neuronal PC12 cells. Lanes 1 and 2: two independently differentiated PC12 samples, both showing a 97 kDa band corresponding to the GR. Lane 3: non-transfected COS-1 cells showing no band (negative control). Lane 4: COS-1 cells transfected with rat GR-plasmid showing a band corresponding to the GR (positive control). Lane 5: COS-1 cells transfected with rat MR, showing no band (negative control). The additional bands below the 97 kDa band most likely reflect aspecific binding of the antibody to other proteins.

COS-1 cells showed the expected 97 kDa band that corresponded to GR (Figure 1). The non-transfected and rat MR-transfected COS-1 cells lacked this band, demonstrating the specificity of the GR-signal in the PC12 cells. The additional bands that are observed in the PC12 cell samples most likely reflect a-specific binding of the antibody to other proteins.

Corticosterone concentrations and RNA quality

Measurement of corticosterone concentrations revealed a concentration of 12 nM in low serum NGF-containing culture medium, partially occupying the endogenous GRs though leaving room for additional GR-activation. The corticosterone-treated medium showed a concentration of 110 nM, most likely resulting in nearly complete occupation of the endogenous GRs.

Total RNA quality was assessed using the LabChip® RNA 6000 Nano Assay (Agilent Technologies) in combination with the Degradometer Software (18), showing no indications of RNA breakdown since the ratios of ribosomal 28S/18S intensities exceeded 1.5 for every sample and the Degradometer analyses did not generate any quality alerts.

GR-translocation into the cell nucleus 30 minutes, 1 and 3 hours after GR-activation

In order to verify whether there is an increase in GR-activation by application of the 100 nM corticosterone pulse over the tonic level of activation resulting from the 12 nM corticosterone in the culture medium, GR-translocation from the cytosol into the nucleus was assessed 30 minutes, 1 and 3 hours after the beginning of the 20 minute corticosterone or vehicle pulse, using semi-quantitative immunocytochemistry analysis. For this purpose 10 nM corticosterone was used as background concentration to mimic the 12 nM background concentration and the 100 nM corticosterone pulse was produced similarly to

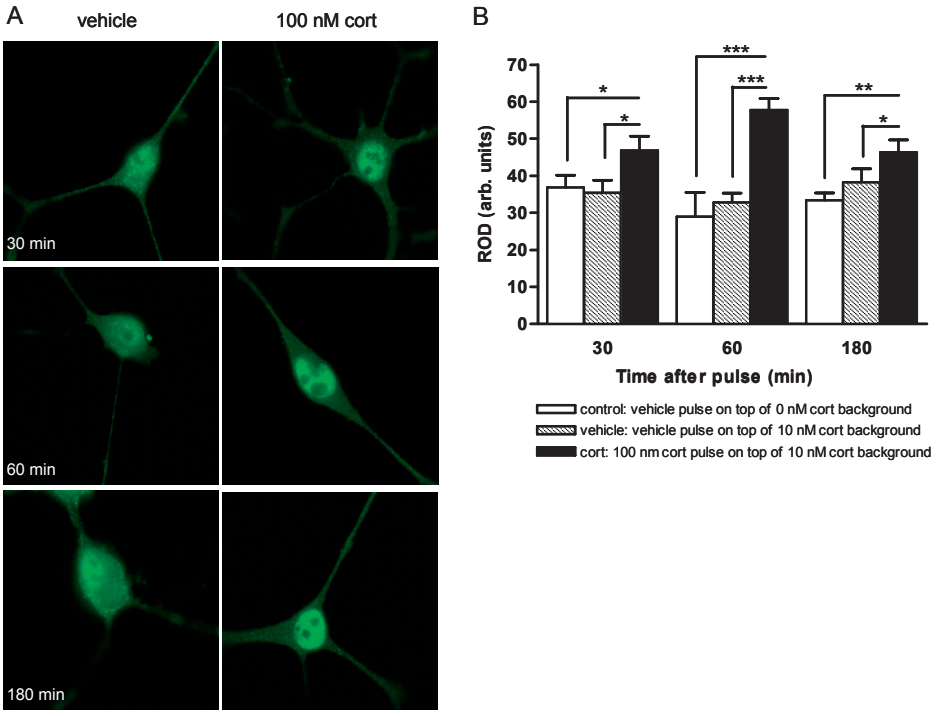

Figure 2. Nuclear distribution of GR in differentiated PC12 cells at 30, 60 and 180 min after administration of a 100 nM corticosterone or vehicle pulse on top of a 10 nM corticosterone background. A) Representative fluorescence images of GR immunoreactivity (ir) at 30, 60 and 180 minutes after corticosterone or vehicle pulse on top of a 10 nM cort background. The GR-ir distribution after the vehicle pulse mimics the distribution in the control groups (vehicle pulse on top of 0 nM background, pictures not shown). B) Semi-quantitative analysis of nuclear optical density. The white bars correspond to the control groups, the grey bars to the vehicle treated cells and the black bars to the 100 nM corticosterone treated cells. Bars represent GR-ir expressed as mean relative optical density (ROD) ± SEM. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ANOVA and post hoc Fisher's LSD testing).

the first experiment. As illustrated in Figure 2, under basal tonic levels (10 nM) of corticosterone, GR-immunoreactivity was evenly distributed over the nucleus and cytoplasmic compartment after vehicle administration at all three time points. This distribution under basal tonic levels was highly similar to that of the control groups exposed to a vehicle. Application of the 100 nM corticosterone pulse on top of the tonic corticosterone background level markedly increased nuclear localization of GR 30 minutes, 1 and 3 hours afterwards when compared to the vehicle-stimulated groups. At all three time points the corticosterone pulse showed a significantly higher GR-immunoreactivity in the cell nucleus compared to their corresponding vehicle-treated groups and the control groups. Clearly, nuclear GR-immunoreactivity was highest 60 minutes after the corticosterone pulse whereas 3 hours afterwards GR-immunoreactivity was returning to baseline. There were no significant differences between the vehicle-treated groups.

GeneChip detection efficiency

After the RNA samples, obtained 1 and 3 hours after the beginning of the corticosterone pulse, were hybridized to the GeneChips, GCOS software (Affymetrix) was used to measure the transcript signals and to estimate the reliability of the transcript signals. In order to obtain a reliable data set, stringent selection criteria were used in which all the transcripts that generated one or more absent calls were removed from the data set. For both time points, this selection resulted in the detection of 51% of the transcripts that were present on the GeneChips. Detection of low abundant transcripts such as neurotransmitter receptors, neurotrophic / growth factors and ion channels was poor and similar to the detection level of the previously performed GeneChip experiment on hippocampal tissue (7). For example, fibroblast growth factor receptor 1 and fibroblast growth factor 2 are known to be expressed in PC12 cells (19,20) but were not detectable on the GeneChips used. On the other hand, transcripts involved in catecholaminergic metabolism such as tyrosine hydroxylase, dopa decarboxylase and catechol-O-methyltransferase as well as one dopamine receptor (D3) and three cholinergic receptors (nicotinic alpha 3, alpha 5 and muscarinic 4) were reliably detected. These catecholaminergic metabolic genes and nicotinic alpha 3 receptor were undetectable on the GeneChips used for the hippocampal slices, indicating cell type-specific expression of these genes in neuronal PC12 cells.

GR-responsive genes 1 and 3 hours after GR-activation: temporal pattern and functional classification

After selection of the transcripts that generated reliable signals, Significance Analysis of Microarrays (SAM, version 2.21) was used to assess the responsive genes. In order to

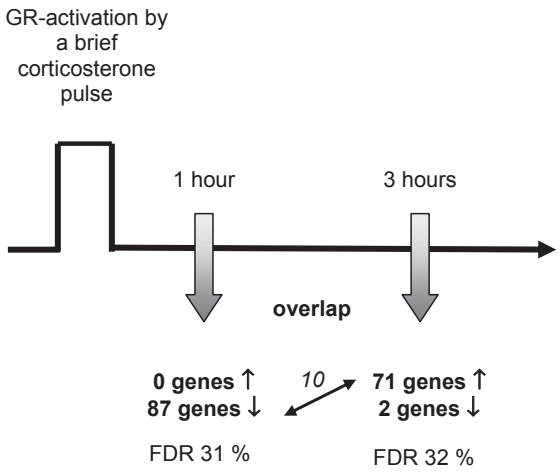

Figure 3. Dynamic pattern of gene expression changes 1 and 3 hours after corticosterone induced GR-activation. Strikingly, one hour after GR-activation, only 87 downregulated genes were found whereas 3 hours after GR-activation the majority of genes was upregulated. The arrow indicates the 10 genes that overlap between the two time points. The SAM estimated FDRs are indicated.

obtain the most significant responsive genes, the FDR was set at 31% for the 1 hour and 32% for the 3 hours time point since these are the lowest possible FDRs that were reliably estimated. This resulted in 87 exclusively downregulated genes 1 hour after GR-activation and 71 upregulated and 2 downregulated genes 3 hours after GR-activation (Figure 3, Table 2, Supplementary Table). Ten genes overlapped between the two time points, showing downregulation at the 1 hour time point and upregulation at the 3 hours time point (Figure 2).

In order to examine the effects of different FDRs on the dynamic pattern of expression regulation, the FDRs were increased in both time points. At the 1 hour time point upregulated genes appeared after increasing the FDR to 38%, resulting in 137 downregulated and 10 upregulated genes. Further increasing the FDR to 67% resulted in 1091 genes called significant of which 786 were downregulated, thereby indicating that 1 hour after GR-activation the most significant genes were downregulated. Increasing the FDR in the 3 hours time point to 68% resulted in 464 upregulated and 18 downregulated genes, illustrating that 3 hours after GR-activation the most significant genes were upregulated. In order to verify these results obtained by SAM, the class comparison analysis from the BRB ArrayTools package was used. This yielded similar results, with predominantly downregulated genes 1 hour after GR-activation and the majority of genes being upregulated 3 hours after GR-activation.

Additionally, the NetAffx Analysis Center was used to annotate the responsive genes (FDR of 31% 1 hour and 32% 3 hours after GR-activation) with Gene Ontology Biological Process classifications. In general, both time points contained similar functional groups such as signal transduction components, transcription and translation regulators, energy metabolism components, cytoskeleton related transcripts and synaptic transmission components.

Validation of a subset of responsive genes

A subset of 10 functionally interesting genes obtained from the GeneChip experiment, including SWI/SNF, beta actin, SNAP25 and RAB7, was selected for validation by real-time qPCR. Of this subset, 8 genes were changed in expression 1 hr after corticosterone administration and 4 after 3 hrs, 2 being altered both at 1 and 3 hrs after treatment. The same subset of genes was tested in the samples obtained from the independent cycloheximide experiment. Normalization was performed using expression levels of 18S ribosomal RNA, which in general is highly and stably expressed. The non-parametric Wilcoxon Signed Ranks Test was used to assess the significance of the observed expression changes. The results are displayed in Table 3 (left panel).

Out of the 10 selected genes, the change in expression of 7 genes was confirmed by real-time qPCR ($P < 0.05$) and could therefore be designated as true positives. The observed fold-changes obtained by real-time qPCR nicely followed the observed fold-changes obtained by the GeneChip experiment.

Table 2. Corticosterone-responsive genes 1 and 3 hours after GR-activation. FC = fold change

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*Gene Title*	*GO Biological Process Description*
Signal Transduction				
1398296_at	0.898396229		membrane interacting protein of RGS16	G-protein coupled receptor protein signaling pathway
1370041_at	0.85571571		stathmin-like 2	intracellular signaling cascade /// neuron differentiation
1368505_at	0.747573368		regulator of G-protein signaling 4	inactivation of MAPK activity /// signal transduction
1370042_at	0.849599258	1.125937728	stathmin-like 2	intracellular signaling cascade /// neuron differentiation
1369897_s_at		1.146930646	GNAS complex locus /// XLas protein	G-protein coupled receptor protein signaling pathway
1367590_at		1.090487579	RAN, member RAS oncogene family	small GTPase mediated signal transduction
1367618_a_at		1.235443954	discs, large homolog 5 (Drosophila) (predicted)	intracellular signaling cascade
1386857_at		1.07285566	stathmin 1	intracellular signaling cascade /// microtubule depolymerization
Regulation of transcription				
1386868_at	0.84552525		ribosomal protein S10	regulation of transcription, DNA-dependent
1398860_at	0.866735675		neural precursor cell expressed, developmentally down-regulated gene 8	regulation of transcription from RNA polymerase II promoter
1371490_at	0.850513144		heat shock factor binding protein 1	negative regulation of transcription
1373565_at	0.814717477		SWI/SNF related, matrix associated, actin dependent regulator of chromatin	chromatin remodeling
1379550_a_at		1.211316579	general transcription factor II I repeat domain-containing 1	regulation of transcription, DNA-dependent /// development
1387028_a_at		1.056258303	Inhibitor of DNA binding 1, helix-loop-helix protein (splice variation)	negative regulation of transcription
Regulation of translation				
1367560_at	0.877629979		acidic ribosomal phosphoprotein P0	protein biosynthesis /// translational elongation
1370866_at	0.852178052		ribosomal protein L41	protein biosynthesis /// translation

Probe Set ID	FC 1 hour	FC 3 hours	Gene Title	GO Biological Process Description
1367640_at	0.893609664		ribosomal protein S12	protein biosynthesis /// ribosome biogenesis
1398882_at	0.887497192		ribosomal protein S5	protein biosynthesis /// ribosome biogenesis
1367639_a_at	0.868892503		ribosomal protein S2	protein biosynthesis
1371344_at	0.835085885		ribosomal protein L27a (predicted)	protein biosynthesis
1388296_at	0.885813179		ribosomal protein S18	regulation of translation /// ribosome biogenesis
1371761_at	0.85983393		ribosomal protein L34 (predicted)	protein biosynthesis
1367573_at	0.93453468	1.091909342	ribosomal protein S6	protein biosynthesis /// ribosome biogenesis
1368211_at	0.905390085	1.070886109	ribosomal protein S14	protein biosynthesis /// ribosome biogenesis
1398885_at	0.830248519	1.11610072	ribosomal protein L23	protein biosynthesis /// ribosomal protein-nucleus import
1367645_at	0.906035201	1.066071865	ribosomal protein S17	protein biosynthesis
1399047_at	0.77454905	1.181521022	mitochondrial ribosomal protein L27 (predicted)	protein biosynthesis
1367606_at		1.098490333	ribosomal protein S3a	protein biosynthesis /// ribosome biogenesis
1371573_at		1.119213877	ribosomal protein L36a (predicted)	protein biosynthesis /// ribosome biogenesis
1388303_at		1.11618509	ribosomal protein L26 (predicted)	protein biosynthesis /// ribosome biogenesis
1367610_at		1.105031172	ribosomal protein L19	protein biosynthesis
1367630_at		1.169589483	ribosomal protein S11	protein biosynthesis
1371297_at		1.176008712	ribosomal protein L7a (predicted)	protein biosynthesis
1370109_s_at		1.094419677	eukaryotic translation elongation factor 1 alpha 1	protein biosynthesis /// translational elongation
1373040_at		1.086208023	eukaryotic translation initiation factor 3, subunit 5 (epsilon) (predicted)	protein biosynthesis /// regulation of translational initiation
1398775_at		1.14993028	ribosomal protein S15a	protein biosynthesis

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*Gene Title*	*GO Biological Process Description*
Ion transport				
1367814_at	0.896041699	1.100837774	ATPase, Na+/K+ transporting, beta 1 polypeptide	potassium ion transport /// sodium ion transport
Energy metabolism				
1386880_at	0.727483923		acetyl-Coenzyme A acyltransferase 2	fatty acid metabolism
1389964_at	0.846588107		NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1 (predicted)	fatty acid biosynthesis
1398781_at	0.911292023		ATPase, H+ transporting, V1 subunit F	ATP biosynthesis
1367586_at	0.853958697		lactate dehydrogenase A	glycolysis
1398755_at		1.101826445	ATPase, H+ transporting, V0 subunit C	ATP biosynthesis
1367622_at		1.123917516	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	ATP synthesis coupled proton transport
1369940_at		1.194471989	transaldolase 1	carbohydrate metabolism
1370893_at		1.086603875	acetyl-coenzyme A carboxylase alpha	fatty acid biosynthesis /// metabolism
1388358_at		1.156451023	electron-transfer-flavoprotein, beta polypeptide	fatty acid beta-oxidation
1370918_a_at		1.14149688	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	generation of precursor metabolites and energy
1367620_at		1.096299197	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c isoform 3	generation of precursor metabolites and energy
1367617_at		1.118484049	aldolase A	fructose metabolism /// glycolysis
1388318_at		1.075913027	phosphoglycerate kinase 1	glycolysis
Oxidative stress metabolism				
1367578_at	0.902195085		peroxiredoxin 2	response to oxidative stress
1367641_at	0.842029777		superoxide dismutase 1	response to oxidative stress

Probe Set ID	FC 1 hour	FC 3 hours	Gene Title	GO Biological Process Description
Miscellaneous metabolism				
1373918_at	0.800275161		retinol dehydrogenase 11 (predicted)	metabolism /// retinol metabolism
1372310_at	0.877078201		similar to tumor-related protein (predicted)	metabolism
1388960_at	0.898944049		pyrophosphatase	metabolism
1368365_at	0.920121934		aldehyde dehydrogenase family 3, subfamily A2	lipid metabolism /// central nervous system development
1370295_at	0.879667997		expressed in non-metastatic cells 1	GTP biosynthesis /// UTP biosynthesis /// CTP biosynthesis
1367766_at	0.894432198		expressed in non-metastatic cells 2	GTP biosynthesis /// UTP biosynthesis /// CTP biosynthesis
1373664_at	0.877781113		phosphatidylinositol glycan, class C (predicted)	GPI anchor biosynthesis
1370163_at	0.83901326		ornithine decarboxylase 1	polyamine biosynthesis
1367750_at	0.784851744		phosphoribosyl pyrophosphate synthetase-associated protein 1	nucleoside metabolism /// nucleotide biosynthesis
1367979_s_at	0.751529858		cytochrome P450, subfamily 51	electron transport /// proteolysis and peptidolysis
1398839_at	0.860086572		thioredoxin 1	electron transport /// electron transport
1370888_at	0.859316097		cytochrome c oxidase, subunit Va	electron transport
1367667_at		1.075311475	farensyl diphosphate synthase	cholesterol biosynthesis
1367932_at		1.166772041	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	acetyl-CoA metabolism
1367687_a_at		1.1660217	peptidylglycine alpha-amidating monooxygenase	peptide amidation /// peptide metabolism
1388301_at		1.103865891	ubiquinol-cytochrome c reductase core protein I	mitochondrial electron transport, ubiquinol to cytochrome c
Cytoskeleton				
1370290_at	0.825749859		tubulin, beta 5	microtubule-based process /// microtubule-based process
1387892_at	0.811413813		tubulin, beta 5	microtubule-based process /// microtubule-based process
1398835_at	0.865652187		actin, beta	cell motility /// cytoskeleton organization and biogenesis

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*Gene Title*	*GO Biological Process Description*
1386890_at	0.802652577		calpactin	regulation of cell growth /// regulation of cell differentiation
1388566_at	0.880669848		LIM and SH3 protein 1	cortical cytoskeleton organization and biogenesis
Ubiquitin pathway				
1373820_at	0.738072041		similar to constitutive photomorphogenic protein 1 (predicted)	ubiquitin cycle /// protein ubiquitination
1371638_at	0.841625734		ring finger protein 7 (predicted)	ubiquitin cycle /// caspase activation
1398778_at		1.203375213	proteasome (prosome, macropain) subunit, alpha type 1	ubiquitin-dependent protein catabolism
1371348_at		1.073932081	proteasome (prosome, macropain) subunit, beta type 5	ubiquitin-dependent protein catabolism
1372873_at		1.276565652	F-box protein 38 (predicted)	ubiquitin cycle
Cell adhesion				
1367681_at	0.902083925		CD151 antigen	cell adhesion
1367628_at	0.806829407		lectin, galactose binding, soluble 1	heterophilic cell adhesion
1379252_at	0.873467612	1.084353621	Immunoglobulin superfamily, member 4A (predicted)	cell adhesion /// synaptogenesis
1384132_at		1.058020549	immunoglobulin superfamily, member 4A (predicted)	cell adhesion /// synaptogenesis
Synaptic transmission				
1398780_at	0.933919565		Rab acceptor 1 (prenylated)	vesicle-mediated transport
1373646_at	0.773418332		RAB15, member RAS onocogene family	neurotransmitter secretion
1387797_at		1.076803948	RAB7, member RAS oncogene family	small GTPase mediated signal transduction /// transport
1387073_at		1.098731918	synaptosomal-associated protein 25	neurotransmitter uptake /// synaptic transmission
Miscellaneous				
1388163_at	0.893638708		solute carrier family 25, member 5	transport /// mitochondrial transport
1367584_at	0.757443524		annexin A2	skeletal development /// angiogenesis

Probe Set ID	FC 1 hour	FC 3 hours	Gene Title	GO Biological Process Description
1369991_at	0.880858304		signal peptidase complex 18kD	signal peptide processing /// proteolysis and peptidolysis
1374767_at	0.664297175		similar to hypothetical protein FLJ14675 (predicted)	proteolysis and peptidolysis
1370785_s_at	0.944161077		translocase of outer mitochondrial membrane 20 homolog (yeast)	protein targeting /// protein-mitochondrial targeting
1370304_at	0.902112826		translocator of inner mitochondrial membrane 17a (yeast)	protein targeting /// protein-mitochondrial targeting
1398850_at	0.816440278		peptidylprolyl isomerase A	protein folding /// regulation of viral genome replication
1371530_at	0.870667899		keratin complex 2, basic, gene 8	protein amino acid phosphorylation
1372779_at	0.78218232		UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 1 (predicted)	protein amino acid glycosylation
1386859_at	0.844598864		transketolase	fertilization (sensu Metazoa) /// regulation of growth
1371304_a_at	0.850692326		similar to myosin, light polypeptide 6, alkali	cytoskeleton organization and biogenesis
1371511_at	0.888479604		actin related protein 2/3 complex, subunit 2 (predicted)	cell motility /// regulation of actin filament polymerization
1386925_at	0.885051546	1.076950162	actin related protein 2/3 complex, subunit 1B	cell motility
1370243_a_at		1.164654606	prothymosin alpha	regulation of cell cycle /// transcription
1388417_at		1.081912539	anaphase-promoting complex subunit 5 (predicted)	regulation of cell cycle /// G2/M transition of mitotic cell cycle
1367992_at		1.11256137	secretory granule neuroendocrine protein 1	protein folding /// transport /// intracellular protein transport
1367709_at		1.101164607	CD63 antigen	positive regulation of endocytosis
1386979_at		1.179892596	developmentally regulated protein TPO1	myelination
1398757_at		1.07007276	nucleophosmin 1	intracellular protein transport /// nucleocytoplasmic transport

From left to right are listed the GeneChip Probe Set IDs, the fold changes in the 1 and 3 hours time point, the gene titles and the Gene Ontology Biological Process descriptions. Downregulated genes are indicated by fold changes below 1, upregulated genes by fold changes above 1. Genes were arbitrarily placed into categories based on their first GO-component.

Primary and downstream responsive genes

In order to distinguish primary responsive genes from downstream responsive genes an independent experiment was performed, using the same conditions but in the presence of the protein synthesis inhibitor cycloheximide. The results are displayed in Table 3 (right panel).

Strikingly, 5 out of 5 downregulated genes 1 hour after GR-activation also showed downregulation in the presence of cycloheximide at this time point ($P < 0.05$), indicating that these genes are primary GR-responsive genes that do not require protein synthesis

Table 3. Real time qPCR validation results. FC = fold change.

	Original experiment (no cycloheximide added)					Cycloheximide experiment		
Gene title	Experiment	FC affymetrix	FC qPCR	Wilcoxon	Validation	FC qPCR	Wilcoxon	Response type
SWI/SNF related	PC12 1 hour	0.8	0.7	P<0.05	true positive	0.8	P<0.05	primary
actin, beta	PC12 1 hour	0.9	0.6	P<0.05	true positive	0.7	P<0.05	primary
lactate dehydrogenase A	PC12 1 hour	0.9	0.8	P<0.05	true positive	0.5	P<0.05	primary
ribosomal protein S6	PC12 1/3 hours	0.9/1.1	0.7/1.4	P<0.05	true positive	0.5/no response	P<0.05	primary/ downstream
actin related protein 2/3 complex, subunit 1B	PC12 1/3 hours	0.9/1.1	0.7/1.3	P<0.05	true positive	0.6/no response	P<0.05	primary/ downstream
synaptosomal-associated protein (snap-25)	PC12 3 hours/SAGE	1.1	1.7	P<0.05	true positive	no response	X	downstream
RAB7, member RAS oncogene family	PC12 3 hours	1.1	1.2	P<0.05	true positive	no response	X	downstream
tubulin, beta 5	PC12 1 hour	0.8	X	X	false positive			
calpactin I	PC12 1 hour	0.8	X	X	false positive			
translation elongation factor 1-delta subunit	PC12 1 hour	0.9	X	X	false positive			

Validation of 10 GR-responsive genes. On the left side the results of the validation in the original experiment are displayed with from left to right the fold changes observed with Affymetrix GeneChip analysis, fold changes observed with real-time qPCR, the result of statistical testing and the validation result depicted. Genes were classified as true positives if the expression changes could be replicated by real-time qPCR. On the right side, the results of the cycloheximide experiment are displayed with from left to right the fold change obtained by real-time qPCR, the result of statistical testing and the response type depicted. Genes were classified as being primary responsive if in the presence of cycloheximide they showed a similar transcriptional regulation as observed in the original experiment. Genes were classified as being downstream responsive if the observed transcriptional change in the original experiment could not be replicated in the cycloheximide experiment. X indicates that the observed changes in gene expression on the GeneChips could not be reproduced by real-time qPCR.

for their regulation. In contrast, 4 out of 4 genes that were validated for the 3 hours time point in the original experiment could not be validated in the cycloheximide experiment, strongly suggesting that these genes are downstream GR-responsive genes. Additionally, two genes overlapped between the 1 and 3 hours time points, showing a primary response 1 hour after GR-activation and a downstream response 3 hours after GR-activation in the cycloheximide experiment.

Putative transcriptional elements in the DNA of primary responsive genes

Five out of five genes that were responsive 1 hour after GR-activation displayed a primary transcriptional response which could be the result of binding of activated GRs either to negative glucocorticoid responsive elements (nGREs) or other transcription factors like AP1, NFκB- and CREB, inhibiting their actions. Therefore, a search was performed on the presence of AP1-, NFκB- and CREB-binding sites, as well as on the presence of nGREs in the promotor regions and intronic regions of these 5 rat genes, focusing on sites that were conserved in mouse and human. This resulted in many hits, of which only a few were conserved. These conserved sites are displayed in Table 4. Two genes, lactate dehydrogenase A and beta actin, contained conserved AP1-sites. For beta actin this AP1-site was located 103 nucleotides upstream of the first exon and highly conserved between rat, mouse and human, all displaying the same sequence (GGTGAGTGAGC). Lactate dehydrogenase contained an AP1-site 265 nucleotides upstream of ATG-containing exon 2 which was highly conserved between rat (GATGAGTAAGA) and mouse (GATGAGTAAGT). Additionally, lactate dehydrogenase A also contained a CREB-site 43 nucleotides upstream of the first exon that was also highly conserved (identical sequence) between rat and mouse (TCTGACGTCAGC). For the other 3 genes no conserved AP1- and / or CREB-sites nor any of the other selected binding sites were found.

Table 4. Putative transcriptional elements in the DNA of 5 primary responsive genes.

primary GR-response gene	AP1 site	CREB site	NFkappaB site	nGRE site
lactate dehydrogenase A	265 nt upstream ATG containing exon 2	43 nt upstream exon 1	x	x
beta actin	103 nt upstream exon 1	x	x	x
SWI/SNF component	x	x	x	x
ribosomal protein S6	x	x	x	x
actin related protein	x	x	x	x

Conserved transcriptional elements. Both lactate dehydrogenase A and beta actin contain an AP1-site with sequences GATGAGTAAGA and GGTGAGTGAGC respectively. Lactate dehydrogenase A contains a CREB-site with sequence TCTGACGTCAGC. X indicates that no binding site is present.

Overlap between GR-responsive genes in PC12 cells and hippocampal tissue

Previously, we profiled GR-responsive genes in hippocampal tissue using both Serial Analysis of Gene Expression (SAGE) and Affymetrix GeneChips (7,10)in two different experimental setups that are described in the materials and methods section. The overlap between GR-responsive genes in the current PC12 data set and the previous hippocampal data sets was assessed using the NetAffx Analysis Center, the Expression Analysis Systematic Explorer (EASE) and the NCBI SAGEmap.

The results of this meta-analysis are displayed in Figure 4 and Table 5, clearly showing that the overlap between hippocampal slice and hippocampal SAGE experiments was significantly higher than the overlap between the PC12 experiment and the hippocampal experiments.

Very little overlap was found between the PC12 cell data set and the hippocampal data sets. Firstly, out of 203 responsive SAGE tags, 160 could be annotated as detectable probe sets on the Affymetrix GeneChips. Out of these 160 transcripts only 7 were found responsive in both data sets of which 6 showed a similar expression change direction. Secondly, out of 160 responsive genes found in the PC12 data set 153 could be detected on the hippocampal slice GeneChips whereas out of 242 responsive genes found in the hippocampal data set 211 could be detected on the PC12 GeneChips. Five genes were found to be responsive in both data sets of which only 3 showed a similar expression change direction. Hence, the overlap between both hippocampal and PC12 data sets ranged between 2 and 4%.

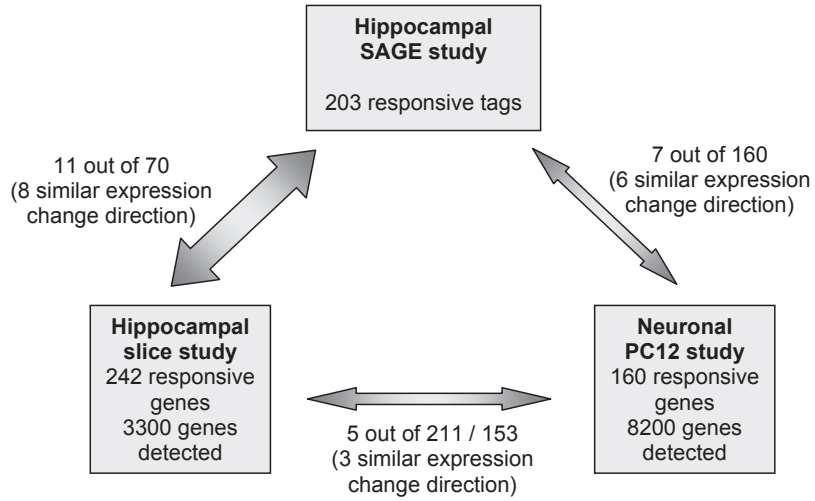

Figure 4. Overlapping gene sets between the current neuronal PC12 study and the previously performed hippocampal slice and SAGE studies. The arrows indicate the amount of overlap between the three studies. The thick arrow indicates a significantly higher overlap between the hippocampal slice study and the hippocampal SAGE study than between the neuronal PC12 study and the hippocampal studies ($P < 0.05$ Chi Square Test). The numbers are explained in the text.

Table 5. Overlap with hippocampal slice and SAGE experiments

	Affymetrix GeneChips		Neuronal PC12 cells		Hippocampal slice preparation		Hippocampal SAGE study	
Gene Title	Probe set ID RAE230	Probe set ID U34A	1 hour	3 hours	1 hour	3 hours	regulation	SAGE tag
expressed in non-metastatic cells 2	1367766_at	M91597_s_at	0.8944322	-	0.92536	-	-	-
latexin	1367768_at	X76985_at	0.8157009	-	0.85592	-	-	-
ribosomal protein L27a (predicted)	1371344_at	X52733cds_s_at	0.8350859	-	0.88393	-	-	-
acidic ribosomal phosphoprotein P0	1367560_at	X15096cds_s_at	0.87763	-	-	1.15029	-	-
anaphase-promoting complex subunit 5	1388417_at	rc_AA874832_at	-	1.081912539	-	0.80373	-	-
transketolase	1386859_at	U09256_at	0.8445989	-	-	-	down	GGATGCCGGG
polyubiquitin	1386852_x_at	D16554_at	0.8811138	-	-	-	down	GTAAGCATAA
ribosomal protein S14	1368211_at	rc_AA945806_at	0.9053901	1.070886109	-	-	down	TTGGCTGCCC
ribosomal protein L19	1367610_at	rc_AA800054_at	-	1.105031172	-	-	up	GATCAGTCAT
aldolase A	1367617_at	M12919mRNA#2_at	-	1.118484049	-	-	down	CCTACTAACC
synaptosomal-associated protein 25 (snap25)	1387073_at	no probe set	-	1.098731918	-	-	up	TATATTAAAT
ATP synthase, H+ transporting	1367620_at	rc_AI180425_at	-	1.096299197	-	-	up	GAAATATGTG
cytoplasmic dynein 74 kD intermediate chain	1387025_at	X66845_at	-	-	0.79120	-	up	GAACATATTT

	Affymetrix GeneChips		Neuronal PC12 cells		Hippocampal slice preparation		Hippocampal SAGE study	
Gene Title	Probe set ID RAE230	Probe set ID U34A	1 hour	3 hours	1 hour	3 hours	regulation	SAGE tag
casein kinase II alpha subunit (CK2)	1387170_at	L15618_at	-	-	0.78114	-	down	TGGTGGAATG
matrin 3	1368151_at	M63485_at	-	-	0.80711	-	up	TATAATCTGT
ATP synthase coupling factor 6	1370230_at	X54510_at	-	-	0.87307	-	up	ACTTAGTTGT
BFA-dependent ADP-ribosylation substrate	1370029_at	AF067795_at	-	-	-	1.27671	up	TTTGTGACTG
cytosolic malate dehydrogenase (Mdh)	1367653_a_at	AF093773_s_at	-	-	-	1.08950	up	ACGTAAAAAA
neuronatin alpha	1369999_a_at	U08290_at	-	-	-	1.22286	up	GAGAGCTAAC
apolipoprotein E	1370862_at	X04979_at	-	-	-	1.09126	up	ACCAGCCAGG
EST196698 Rn.3391	no probe set	rc_AA892895_r_at	-	-	-	1.25344	down	GTGGGTGTGT
F1-ATPase alpha subunit	1370378_at	X56133_at	-	-	-	0.87735	down	AATAAAAGTT
calmodulin 2	1370246_at	M17069_at	-	-	-	0.88985	down	TTGCTGTTGA

Genes that overlap between the currently generated neuronal PC12 cell data set and the previously generated hippocampal slice (Morsink 2006) and hippocampal SAGE (Datson 2001) data sets. From left to right are listed the gene titles, Probe Set IDs on the RAE230 GeneChip, Probe Set IDs on the U34A GeneChip, fold changes in the 1 and 3 hours time point in the hippocampal slice data set, fold changes in the 1 and 3 hours time point in the neuronal PC12 cell data set, regulation (up- or downregulation) in the hippcampal SAGE data set and the corresponding SAGE tag.

The overlap between both hippocampal data sets was considerably larger since out of 70 SAGE tags, 11 overlapped and 8 showed similar expression change directions, resulting in a 16% overlap.

When compared to the overlap between the hippocampal slice study and the hippocampal SAGE study, the overlap between the PC12 data set and both hippocampal data sets was significantly smaller ($P < 0.05$ Chi Square Test). The overlap between the PC12 study and hippocampal slice study was not significantly different from the overlap between the PC12 study and the hippocampal SAGE study.

DISCUSSION

The present study revealed a dynamic temporal pattern of genomic responses to acutely activated GRs in neuronal PC12 cells that were constantly stimulated with NGF. At one hour after a brief exposure to corticosterone the genes that were most significantly changed all appeared downregulated, whereas after 3 hours the majority was upregulated. This pattern is very similar to the results we previously obtained in the hippocampus slice preparation, showing that downregulation preceded the upregulation of genes observed two to four hours later, if exposed to a corticosterone pulse (7). This striking temporal pattern actually may be due to the mode of action of corticosterone involving primary and downstream GR-responsive genes.

Activated GRs can modulate gene transcription in two ways. Firstly, in transrepression monomeric GRs can bind to other transcription factors, thereby inhibiting their transcriptional activity. Secondly, in transactivation GRs dimerize and bind to so called glucocorticoid responsive elements (GREs) on the DNA in the proximity of gene promoters, thereby either enhancing or repressing (via negative GREs) gene transcription (4,5). The terms transactivation and transrepression have been derived from the effects glucocorticoids exert on the physiological stress-response. Glucocorticoids are known to restrain the stress response from overshooting via inhibition of stress-induced transcription factors (1) and therefore the term transrepression is used to designate this process. Binding of GRs to GREs often is implicated in the restorative functions glucocorticoids exert following a stress-response for instance on energy metabolism. Since many of the genes involved are upregulated by glucocorticoids the term transactivation is used to describe the process of GR binding to GREs, not withstanding the fact that some prominent genes (e.g. POMC) are regulated by negative GREs. Hence, the dynamic pattern of gene regulation observed in the present study may indicate that transrepression precedes transactivation in time, resulting in downregulated genes at early time points. Some caution, though, is required with the interpretation of these data. The current technique is not very suitable to study low-abundant gene products, such as some of the ion channel subunits. Earlier physi-

ological studies in hippocampal cells have shown that GR-dependent changes in calcium channel conductance, seen as early as 1 hr after steroid administration, depend on transactivation (6). In accordance, calcium channel subunit expression in rats was found to be upregulated in the same timeframe (Y.Qin, unpublished observation). This indicates that upregulation after short intervals is possible, although it does not seem to be the prevailing pathway.

Very little is known about the rate at which the different steps involved in transactivation and transrepression proceed. However, it seems that since on average mRNA decay is not faster than mRNA elongation (21,22), blocking the actions of other transcription factors via protein-protein interactions may occur more rapidly than transactivation due to the fact that in the latter process cofactor recruitment and chromatin remodeling is required (17,23-25). Additionally, it should be noted that direct interactions between the GR and the general transcription machinery have been reported as well, influencing gene transcription without the need for cofactor recruitment and / or chromatin remodeling (26). This could constitute a mechanism that would facilitate rapid regulation of gene transcription prior to the currently profiled 1 hour time point. Since there have been examples in literature in which glucocorticoids rapidly upregulate gene expression (27), assessing gene expression changes at shorter time intervals (< 1 hour) in the current setting would be of major interest.

Translocation of GR into the nucleus under the present experimental conditions was confirmed by immunocytochemistry, showing significant increases in nuclear localization after application of the corticosterone pulse. These results therefore suggest the functional activation of GR by the 20 minute corticosterone pulse on top of the basal tonic levels of corticosterone and are in line with other reports in literature (28,29).

Activation of GR by a 20 minute corticosterone pulse is an established model to study the neurophysiology of corticosterone action. This pulse mimics the peak in the ultradian secretion pattern of corticosterone. Hence, the relationship between the kinetics of corticosterone application and dynamics of the GR-mediated transcriptional response can be informative with regard to the sensitivity of the transrepression and transactivation mechanisms. In this respect the finding of Jonat and coworkers (30) that transrepression occurs at lower concentrations than transactivation after 12 hours of dexamethasone incubation is very interesting and may indicate that also in the current experimental setup transrepression is more sensitive for GR-ligand than transactivation.

When compared to our previously performed GeneChip experiment (7) overall transcript detection was improved from 37% to 51% in the current data set. This increase can most likely be attributed to the fact that in the current study the Rat Expression Array 230A GeneChips were used in which the quality of the probe sets is improved and which also contains more genes. Additionally, a less complex biological substrate such as PC12 cells could result in enhanced detection of low abundant gene classes such as

neurotransmitter receptors and growth factors. However, in the current setting the detection of these low-abundant transcripts was not improved. This most likely reflects a technical constraint constituted by the GeneChips which can be illustrated by the fact that both fibroblast growth factor receptor 1 and fibroblast growth factor 2 are known to be expressed in neuronal PC12 cells (19,20) but were not detectable on the currently used GeneChips. This result is in line with previous findings in laser microdissected hippocampal subregions in which little detection improvement was gained for specific classes of low abundant gene transcripts (31).

The GR-induced fold-changes in gene expression were very moderate. Using a paired experimental design, statistical power was greatly enhanced since very consistent changes in gene expression were observed within each pair. Additionally, 75% of the selected genes could be validated by real-time qPCR, demonstrating the reliability of the currently generated data set. The observed moderate changes are in contrast to the amplitude of glucocorticoid-mediated responses observed in PC12 cells in previous studies (32-35). However, major differences exist in glucocorticoid treatment of the cells between the current and previously published studies. In the current study, a short, 20 minute 100 nM corticosterone pulse was administered, which is in sharp contrast to the longer (1 - 48 hours) continuous exposure to higher (1 μM) concentrations of the highly potent synthetic GR-agonist dexamethasone. Furthermore, the currently obtained moderate changes in gene expression are in good concordance with the GR-mediated effects on gene expression reported *in vivo* (10,36-38).

Strikingly, the cycloheximide experiment revealed that the 5 validated genes for the 1 hour time point were all primary responsive, indicating that activated GRs either bound to negative GREs or to other transcription factors, inhibiting their transcriptional actions. Therefore, both the promoter region and the intronic regions of these genes were checked for the presence of consensus negative GRE (nGRE) sites (17). No nGRE sites were present, supporting the hypothesis that 1 hour after GR-activation gene transcription may be inhibited by protein-protein interactions between GRs and other transcription factors instead of GR-binding to the DNA. Since the cells were constantly stimulated with NGF, which supposedly leads to activation of CREB, AP1 and NFκB (39,40), the same regions were also examined for the presence of binding sites for these transcription factors and for two genes (lactate dehydrogenase A and beta actin) clearly conserved AP1-sites and CREB-sites were found, pointing towards a transrepression mechanism mediated by activated GRs. However, for the 3 remaining primary responsive genes the mechanism of action still remains speculative and other, so far unknown interacting pathways could be involved.

Among these primary responsive, downregulated genes, SWI/SNF related, matrix associated, actin dependent regulator of chromatin (SNF2) constitutes a very interesting transcript. This gene is a component of the SWI/SNF complex which is involved in GR-

mediated transactivation (4,41). In yeast, mutating the components of this complex results in loss of GR-induced transactivation. Hence, transient downregulation of the SNF2 component which may already have taken place prior to the 1 hour time point could be part of the mechanism which temporarily hampers transactivation, resulting in the currently observed dynamic pattern.

Four genes that were tested (ribosomal protein S6, actin related protein 2/3 complex, RAB7 and synaptosomal-associated protein 25) displayed a downstream response in the 3 hours time point. These results could indicate that 3 hours after GR-activation only downstream responsive genes are present in the current experimental setup. It should be realized, though, that our sample size was limited, so that the results cannot be simply generalized. In agreement, in the previous hippocampal data set (Morsink 2006), metallothionein was found to be upregulated 3 hours after GR-activation. Since there is clear evidence that this gene contains two functionally active GREs upstream of the promoter sequence (42), it most likely is a primary responsive gene, implying that the 3 hours time point would be constituted both by primary and downstream responsive genes. In this respect, profiling gene expression 2 hours after GR-activation and assessing primary and downstream-responsive genes at this time point would be of major interest.

In order to gain more insight into the mechanism that underlies the transcriptional response 1 hour after GR-activation, expanding the currently performed limited search for transcription binding sites on the DNA by looking at other transcription factor binding sites and using a combination of a large-scale bioinformatics and chromatin immunoprecipitation approach would be of use. Identifying commonly shared transcription factor binding sites in the proximity of promoter regions and assessing the GR-transrepressive capability on these elements could provide a definitive answer to the question whether 1 hour after GR-activation transrepression via protein-protein interactions underlies the transcriptional response. Since PC12 cells are relatively easily to manipulate, they would provide a good neuronal substrate for performing these follow up studies.

The overlap of GR-responsive genes between the neuronal PC12 data set and both previously generated hippocampal data sets was very small. Although a large number of hippocampal GR-responsive genes was detectable on the GeneChips used for the neuronal PC12 cells only 12 genes in total overlapped between PC12 cells and hippocampus, of which 9 showed similar changes in expression direction. Since NGF-differentiated PC12 cells are known to display a catecholaminergic phenotype, the small overlap with hippocampal tissue clearly demonstrates a high degree of tissue / cell specificity of the GR-mediated gene expression response at the level of the individual genes. Additionally, the overlapping 9 genes point towards a, albeit very limited, cell type independent transcriptional response and hence these genes constitute good positive controls, demonstrating the reliability of the current data set.

The currently performed experiment therefore can be used as a model to study the cell type specific transcriptional actions of acutely activated GRs on dopaminergic / noradrenergic neuronal functioning. Glucocorticoids are known to display profound effects on both dopaminergic and noradrenergic systems. For instance, in the nucleus accumbens, acute administration of corticosterone or dexamethasone increases dopamine release from these neurons 1 to 4 hours later (3,43). Glucocorticoids can also enhance noradrenergic signaling in brain (44). In this respect it is interesting to note that in the current and previously performed SAGE experiment an upregulation of synaptosomal associated protein 25 (SNAP-25) was observed after corticosterone administration. This gene plays a role in calcium-dependent exocytosis and an increased expression could therefore lead to increased neurotransmitter release (45), resulting in enhanced neurotransmission. Furthermore, the current data set contained 3 genes involved in Rab-mediated endocytosis, Rab 1 acceptor, Rab 15 and Rab 7. Rab proteins are small GTPases which coordinate vesicular membrane transport. Given the fact that glucocorticoids enhance exocytosis-mediated dopamine and noradrenalin release, endocytosis-mediated recycling of membrane should also be enhanced to restore membrane balance. Therefore, these Rab proteins may provide an additional molecular mechanism by which glucocorticoids regulate the process of enhanced neurotransmitter release.

Interestingly, beta actin appeared to be downregulated 1 hour after GR-activation. Beta actin is a constituent of the actin cytoskeleton, which plays a role in dendritic spine morphology and synaptic transmission (46). Furthermore, LIM/SH3 protein also was downregulated 1 hour after GR-activation and this protein is strongly concentrated at synaptic sites (47). LIM/SH3 protein can associate with actin and possibly recruits signaling molecules to the actin cytoskeleton, thereby playing a role in cytoskeletal organization. Hence, downregulation of both beta actin and LIM/SH3 protein indicates that synaptic plasticity of catecholaminergic neurons may be modulated by corticosterone.

In conclusion, by using neuronal PC12 cells for gene expression profiling after acute activation of GRs we have demonstrated that the temporal genomic response to acutely activated GRs overlaps between different neuronal substrates. Furthermore, the results obtained in the cycloheximide experiment suggests that the 1 hour time point consists of primary responsive genes which, as master switches, may regulate the transcriptional response of genes further in time. Additionally, by assessing the overlap between different data sets we have illustrated the cell type specificity of the GR-mediated transcriptional response. Finally, we have identified a number of functionally interesting genes that may increase the current understanding how glucocorticoids affect dopaminergic and noradrenergic neuronal functioning.

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Supplementary Table. Corticosterone-responsive genes 1 and 3 hours after GR-activation. FC = fold change

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*Gene Title*	*GO Biological Process Description*
1367744_at	0.636244709		melanoma antigen, family D, 2	---
1367768_at	0.815700921		latexin	---
1369930_at	0.894899283		---	---
1371390_at	0.83159745		tubulin, beta, 2	---
1371487_at	0.773520241		SH3 domain binding glutamic acid-rich protein-like 3 (predicted)	---
1371542_at	0.852804258		similar to Tubulin alpha-4 chain (Alpha-tubulin 4)	---
1371653_at	0.873037255		---	---
1371782_at	0.818809859		NIPSNAP-related protein	---
1371783_at	0.904408339		heat shock protein	---
1372104_at	0.74527328		---	---
1372169_at	0.696559784		similar to RIKEN cDNA 4121402D02	---
1372293_at	0.757432555		Transcribed locus	---
1374758_at	0.712830556		Transcribed locus	---
1376052_at	0.87620947		similar to hypothetical protein FLJ20512	---
1386852_x_at	0.88111382		polyubiquitin	---
1386867_at	0.861815594		brain protein 44-like	---
1387770_at	0.950184704		putative ISG12(a) protein	---
1388134_at	0.920150897		eukaryotic translation elongation factor 1 delta	---
1388297_at	0.884867241		eukaryotic translation elongation factor 1 gamma	---
1388468_at	0.862872319		Similar to hypothetical protein	---
1388588_at	0.853421092		mammary tumor virus receptor 2 (predicted)	---
1386891_at	0.893989405	1.088585138	phosphatidylethanolamine binding protein	---
1370193_at		1.08000597	protein tyrosine phosphatase 4a1	---
1370803_at		1.086458818	ZW10 interactor	---
1371419_at		1.137432297	spectrin beta 2	---
1371507_at		1.103355176	Similar to 4921517L17Rik protein (predicted)	---
1371883_at		1.092022171	monocyte to macrophage differentiation-associated	---

Probe Set ID	*FC 1 hour*	*FC 3 hours*	*Gene Title*	*GO Biological Process Description*
1372003_at		1.193571359	Transcribed locus	---
1372394_at		1.083304633	similar to HECT domain containing 1	---
1372568_at		1.115249893	Transcribed locus	---
1374898_at		0.701578764	similar to CG8841-PA (predicted)	---
1375049_at		1.124390516	---	---
1375181_at		1.182918504	similar to 60S ribosomal protein L12	---
1375631_at		1.058215641	pleckstrin homology domain containing, family B member 2 (predicted)	---
1377310_at		1.239280582	Transcribed locus	---
1382200_at		1.30736587	similar to Prr6 protein	---
1387016_a_at		1.091065761	stromal cell derived factor receptor 1	---
1388477_at		1.127486641	similar to RAN-binding protein 3 (RanBP3)	---
1389820_at		1.123294362	---	---
1389840_at		1.153754562	splicing factor 3b, subunit 1	---
1390048_at		1.159571262	serine/arginine repetitive matrix 2 (predicted)	---
1390153_at		1.109047637	Similar to hypothetical protein BC019095 (predicted)	---
1390234_at		0.835331391	splicing factor 3b, subunit 1	---
1398326_at		1.119310264	similar to Nur77 downstream protein 2	---
1399012_at		1.123475984	similar to RIKEN cDNA 1110001M20 (predicted)	---

From left to right are listed the GeneChip Probe Set IDs, the fold changes in the 1 and 3 hours time point and the gene titles. These genes could not be annotated with Gene Ontology Biological Process descriptions. Downregulated genes are indicated by fold changes below 1, upregulated genes by fold changes above 1.

Chapter 5

Glucocorticoid control of gene transcription in neural tissue: methodological and functional implications

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ABSTRACT

Glucocorticoid hormones, which are released by HPA-axis activation in response to stress, may exert modulatory effects on neural function in a delayed genomic fashion. The two receptor types that can bind glucocorticoids, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), are ligand-inducible transcription factors. Therefore, changes in gene expression most likely underlie glucocorticoid-mediated genomic effects on neuroexcitability, synaptic plasticity and energy metabolism. In the past 5 years large scale gene expression profiling has evolved as a powerful tool to assess glucocorticoid-mediated transcriptional changes within the brain, contributing to the generation of new hypotheses on the molecular mechanism underlying glucocorticoid-mediated effects. The application of several gene expression profiling techniques in different biological model systems has led to interesting findings with regard to receptor specificity, dynamics and context-specificity of glucocorticoid-mediated transcriptional regulation. These findings have revealed the enormous diversity in glucocorticoid mediated transcriptional responses assigned to various functional gene classes. At present, the focus is shifting towards experimental and technical refinement to overcome some of the limitations which are currently encountered in gene expression profiling of discrete brain regions.

This review aims to provide an overview of the results that were obtained using different gene expression profiling techniques in various biological model systems for assessing glucocorticoid-mediated transcription in neuronal tissue. Additionally, future prospects concerning the experimental and technical refinement in particular in relation to expression profiling in brain are discussed.

1. INTRODUCTION

Glucocorticoid hormones, which are released by the adrenals in response to stress, may exert modulatory effects on many different organs and tissues in a delayed genomic fashion. While actions of glucocorticoids in the liver and immune system have been studied to a much greater extent, here we focus on the neural transcriptional effects of glucocorticoids. So far, studies addressing the neural effects have been sparse and from the few large-scale gene expression profiling studies that were performed in brain the view has emerged that measuring glucocorticoid-induced transcriptional changes in neural tissue is complicated by several factors. These include the small glucocorticoid-induced transcriptional effects, the cellular heterogeneity that exists in brain and the large transcriptomes which are present in neural cells.

The current review aims to provide an overview of the results that were obtained assessing acute glucocorticoid-mediated transcriptional responses in neural tissue using different large-scale gene expression profiling techniques and biological model systems. Furthermore, several new strategies are presented which can be applied to overcome the current problems and to further investigate glucocorticoid-mediated transcriptional effects in brain.

In **section 2**, a short overview of glucocorticoids, their effects and the underlying molecular mechanisms are given. In **section 3**, two of the more commonly used techniques for large-scale gene expression profiling in neuronal tissue, i.e. SAGE and DNA microarrays, are described. The currently obtained findings using this approach with regard to receptor specificity **(section 4)**, dynamics **(section 5)** and context-specificity **(section 6)** of glucocorticoid-mediated transcriptional regulation as well as the functional implications of glucocorticoid-mediated transcriptional regulation **(section 7)** is discussed. Issues concerning technical refinement are addressed in **section 8** and finally, future prospects are presented in **section 9**.

2. GLUCOCORTICOIDS

The hypothalamic-pituitary-adrenal (HPA)-axis plays a key role in mediating stress responses in the organism and under normal conditions its activity is limited, resulting in the release of basal amounts of glucocorticoid hormones from the adrenals in an ultradian and circadian fashion. When the organism experiences a stressor, the pulsatile secretion increases in frequency and magnitude (1) and glucocorticoid concentrations will rise in a time course of several minutes, leading to glucocorticoid-induced transcriptional changes in target tissues within an hour. Additionally, glucocorticoids also exert negative

feedback on the HPA-axis itself via the pituitary, hypothalamus and hippocampus (2,3), shutting down the stress-induced HPA-axis response.

Many different organs and tissues are affected by glucocorticoids and some of the effects glucocorticoids exert include increasing blood glucose concentrations (hence their name), stimulation of lung development and inhibition of the immune system. Furthermore, glucocorticoids are able to pass the blood brain barrier and affect various brain functions such as cognition, behaviour and mood (4). The hippocampus, a brain structure involved in learning, memory formation and HPA axis control, is one of the key brain structures affected by glucocorticoids. Several aspects of hippocampal function, such as neurotransmission and metabolism, are affected by glucocorticoids. For instance, in the hippocampus increased concentrations of glucocorticoids inhibit β-receptor mediated effects, impair long-term potentiation (LTP), enhance calcium influx and increase the neuron's responses to serotonin (5).

Since glucocorticoids affect many different organs and tissues, chronic overexposure has been associated with a wide variety of pathological conditions. Correlations between chronically elevated levels of glucocorticoids and cardiovascular disease, metabolic disease and immunity-related disorders have been shown to exist (4,6,7). Moreover, high concentrations of glucocorticoids most likely exert pathological effects on brain function as well. For instance, there is a clear association between affective disorders and high concentrations of glucocorticoids, since 50% of the patients suffering from depression have hypercortisolism (3) and patients with Cushing's disease, in which glucocorticoid levels are high due to pituitary or adrenal tumors, often display depression as well.

Glucocorticoid receptors

Many of the glucocorticoid-induced effects on target tissues are mediated via the glucocorticoid receptor (GR) which is expressed throughout the entire body (8). However, in brain there is a second receptor for glucocorticoids, the mineralocorticoid receptor (MR) with a much more restricted expression, which binds glucocorticoids with a 10-fold higher affinity than GR, resulting in predominant MR-occupation under basal glucocorticoid concentrations and additional GR-occupation when glucocorticoid concentrations rise (3). Both receptors belong to the family of ligand-inducible transcription factors and can modulate gene transcription in the cell nucleus (9). Therefore, many of the effects that glucocorticoids exert are the result of changes in gene expression.

MR has a much more restricted expression than GR. While GR is ubiquitously expressed throughout the body, MR expression is restricted to specific tissues including the kidney and limbic brain regions such as the hippocampus (10). Also within the hippocampus, different ratios of MR / GR exist in different hippocampal subregions (CA1, 2, 3, 4 and dentate gyrus) and since they are transcription factors it is quite possible that different sets of target genes are transcriptionally regulated in these subregions.

Both receptors have a different function in the regulation of HPA-axis activity; MR maintains basal activity of the axis under basal concentrations of glucocorticoids whereas GR facilitates the negative feedback under rising glucocorticoid concentrations after HPA-axis activation by a stressor (3).

Molecular mechanisms

Upon ligand-binding, both MR and GR translocate to the cell nucleus where they can modulate gene transcription via *transactivation* and *transrepression* (9).

In transactivation, ligand-bound MRs and GRs form homodimers that bind to glucocorticoid-responsive elements (GREs) on the DNA in the proximity of gene promoters. Subsequently, cofactors (coactivators and / or corepressors) are recruited to the receptors and via interactions with the general transcription machinery gene transcription can either be enhanced (positive GREs) or repressed (negative GREs). Examples of genes upregulated by glucocorticoids via positive GREs include the phenylethanolamine N-methyltransferase (PNMT) gene in the adrenal and genes involved in gluconeogenesis and the urea cycle in the liver (8). In the pituitary, glucocorticoids are known to inhibit the expression of ACTH by inhibiting the transcription of its precursor molecule proopiomelanocortin (POMC) via binding to a negative GRE site. The 5HT1A-receptor is another well known gene that contains a negative GRE-site.

In transrepression, monomeric receptors inhibit gene expression by binding to transcription factors like NFκB, AP1 and CREB that were activated by other signaling cascades (11,12,13,14). It is known that this mode of action accounts for many of the inhibitory effects glucocorticoids exert on immune system functioning (15) and several immune-related genes that are downregulated via transrepression include cytokines and adhesion molecules (16). The availability of different transrepression partners is most likely determined by the cellular context in which glucocorticoids operate.

Finally, there are indications that MR and GR can form GRE-binding heterodimers which may enhance the diversity of glucocorticoid action on gene transcription (17,18,19).

3. LARGE-SCALE GENE EXPRESSION PROFILING TECHNOLOGY

Powerful large-scale gene expression profiling technology has become available in recent years, allowing entire transcriptomes to be rapidly characterized in a quantitative manner, also known as *genomics*.

By profiling the expression levels of several thousands of genes glucocorticoid-regulated genes can be identified, allowing new hypotheses to be generated as to how transcriptional regulation of selected candidate genes may underlie the glucocorticoid-mediated effects on neural function. In addition, issues concerning receptor and context

specificity and dynamics of the genomic response to glucocorticoids can be addressed by generating and comparing expression profiles under different ratios of activated MR and GR, in different cell types, under diverse conditions and throughout time.

Several techniques, such as Serial Analysis of Gene Expression (SAGE), DNA microarray technology, differential display and subtractive hybridization, are available for performing large-scale gene expression profiling. So far, SAGE (20) and DNA microarrays (21) have been most widely used techniques in the field of glucocorticoid interactions with the (neural) transcriptome. Both techniques are constituted by their own unique methodological procedures and subsequently require highly specific data analysis tools. More detailed information on SAGE and DNA microarray procedures as well as their specific data analysis tools can be found in sections 3.1, 3.2 and Figures 1 and 2. Additionally, a methodological comparison between SAGE and DNA microarrays is provided in section 3.3.

Serial Analysis of Gene Expression (SAGE)

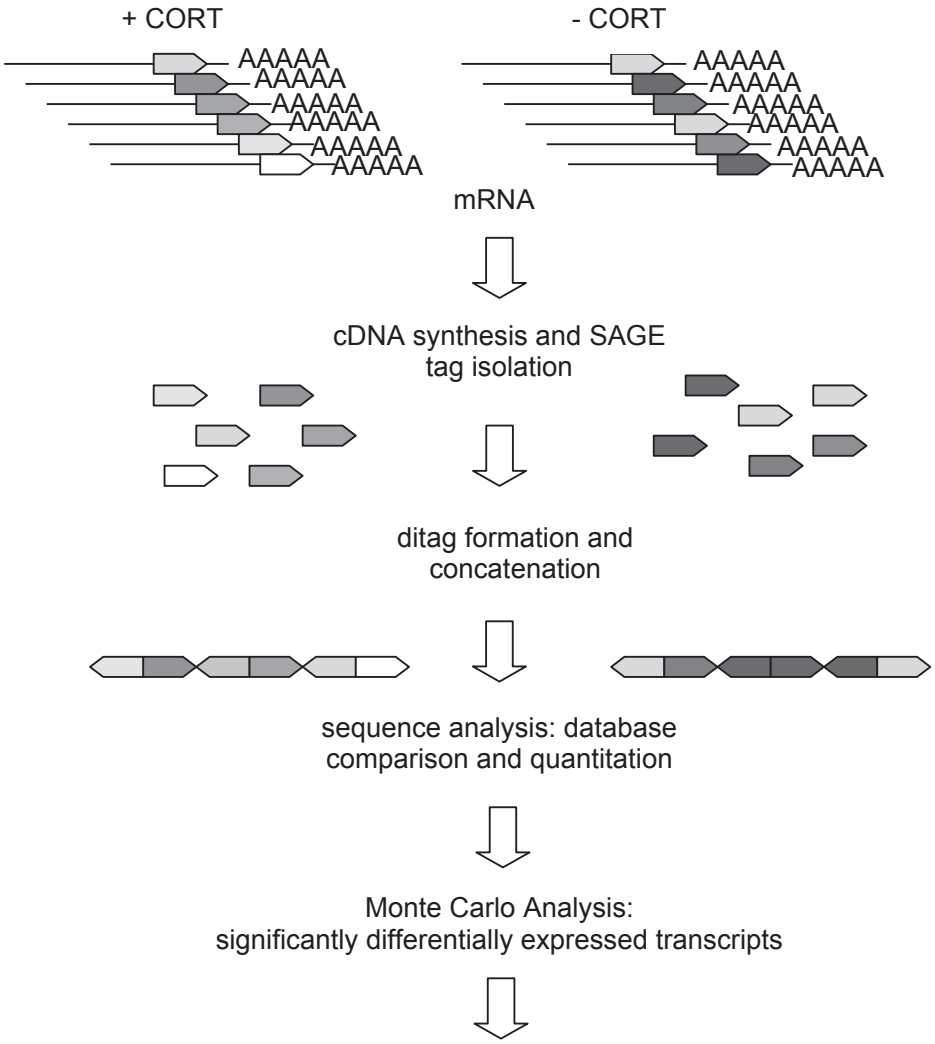
In brief, when using SAGE, mRNA transcript levels are measured by counting so-called SAGE tags. These 10 nucleotide long, transcript-specific SAGE tags are derived from the transcript's 3' untranslated regions and contain sufficient information to uniquely identify the corresponding transcripts. Subsequent sequencing and counting of the generated SAGE tags results in a gene expression profile for each experimental sample (Figure 1).

SAGE procedure

In SAGE, mRNA is isolated from the experimental samples and double stranded cDNA is generated using biotinylated oligo-dT primers directed against the polyA-tails of the mRNA transcripts. Subsequently, SAGE tags are isolated from the cDNA via two endonuclease reactions.

In the first endonuclease reaction, a restriction endonuclease (anchoring enzyme) which recognizes a 4 bp sequence (e.g. NlaIII, CATG) is used to cleave the cDNA, after which the 3' parts of the cleaved cDNAs are isolated by binding of the biotinylated oligo-dT primers to streptavidin beads. Prior to the second endonuclease reaction, linkers which contain recognition sites for the second endonuclease are attached to the cleaved cDNAs via the anchoring restriction sites.

In the second endonuclease reaction, a type II S restriction endonuclease (tagging enzyme), which typically cleaves DNA at a distance approximately 20 bp away from its recognition site on the linkers, is used to generate the short, 10 bp SAGE tags. Subsequently, the resulting SAGE tags are ligated into long multimers called concatemers, which are then cloned into plasmids and sequenced. Using these concatemers greatly enhances the efficiency of the sequencing step because they minimize the number of required sequencing reactions.



Tag sequence	*Tag count + CORT*	*Tag count - CORT*	*P value*	*Gene name*
ATAACACATA	593	312	0	ATP synthase subunit
AAATAAAACT	50	14	0	NADH dehydrogenase
TAACTTTAAG	30	9	0.00041	neuronal olfactomedin
CTCCAATAAT	10	0	0.001	ESTs (Rn.8002) Pkia
TCCAATAAAG	26	8	0.001	ESTs highly similar to

Figure 1. Overview of the Serial Analysis of Gene Expression (SAGE) procedure. Details are described in the text (section 2.1). + CORT: glucocorticoid-treated sample, - CORT: control sample.

After a sufficient number of tags have been sequenced, the obtained tag sequences are used to estimate tag abundancies which are representative for mRNA abundancies in the original pool.

Subsequently, a special SAGE software package is used to assess significantly differentially expressed genes. The SAGE software uses Monte Carlo Analysis to determine statistical significance by performing simulations on the data set and calculating the likelihood for each transcript to obtain a difference in expression equal or greater than the observed difference due to chance alone (22). A P-value cutoff is set (taking an acceptable, low, false-positive rate into account) which results in a list of significantly expressed SAGE tags. Finally, the SAGE software matches the tags against the GenBank and / or UniGene databases in order to identify the corresponding transcript for each tag.

In addition to the standard SAGE software, presently an alternative software package is available, called eSAGE (23), which further streamlines the analysis of SAGE data. Among the extra features included in the eSAGE package is the ability to follow SAGE sequencing efficiency.

SAGE websites

A clear advantage of SAGE is that absolute SAGE tag counts are used to represent transcript abundancies, which allows data comparison between different experiments performed at different locations, under the condition that the total number of sequenced SAGE tags is taken into account. At the SAGEmap website (www.ncbi.nlm.nih.gov/SAGE/) (24), a public SAGE database has been constructed which contains over 300 SAGE libraries obtained from human tissue and over 200 libraries obtained from mouse tissue. Furthermore, it contains libraries obtained from several other organisms as well, such as for example rat and *C. elegans*.

Another well known website in which tags derived from different organs and tissues from human and mouse are collected and can be compared is the SAGE Genie website which is available at 'http://cgap.nci.nih.gov/SAGE' (25).

Modified SAGE procedures

Presently, several modifications of the general SAGE procedure exist, each improving different limitations of the general SAGE procedure.

One of the limitations of the SAGE procedure is the relatively high input of RNA that is required. Therefore, several modifications to the procedure have been developed which require lower amounts of input RNA, including PCR-SAGE (26), SAGE-Lite (27), SAGE adaptation to downsized extracts (SADE; (28), miniSAGE (29) and microSAGE (30). MicroSAGE, for example, is more easily performed in comparison to SAGE due to the fact that several steps are performed in a single tube, thereby preventing the loss of material. In addition, several extra PCR cycles are performed before the concatemers are generated, resulting in the use of very limited amounts of starting material.

Another limitation of the SAGE procedure is the fact that the tags generated are often located several hundred bp upstream of the 3' ends (internal tags), which makes it difficult to specify where the corresponding transcripts start and end on the genome. A modified SAGE procedure called longSAGE generates 5' and 3' tags derived from the first and last 20 bp of the transcripts respectively, thereby allowing the mapping of the transcription initiation site and the polyadenylation site of each transcript on the genome and thus providing a more precise genomic localization of the transcripts (31).

DNA microarrays

DNA microarrays are microscopic glass slides or silica chips onto which a large number of probes are printed or synthesized *in situ* at a high density. RNA which has been obtained from the experimental samples is labeled and hybridized to the microarray. The resulting hybridization signals for each transcript are quantified, resulting in a gene expression profile for each experimental sample.

DNA microarray systems

Several kinds of DNA microarray systems are currently available and they are different in multiple ways. First, different microarray systems use probe sequences of different lengths. For example, the first DNA microarrays were spotted with long (in general > 300 bp), PCR-amplified cDNA probes whereas currently most microarray systems use shorter, more specific oligonucleotide probes which range in length from 25 to 60 nucleotides that are synthesized *in situ* on the array. Second, there are differences in the way hybridization of target RNA to the array is performed. In single-target hybridizations, separate arrays are used to hybridize separate experimental samples whereas in dual-target hybridizations, one array is used to hybridize two experimental samples (i.e. treatment and control) which are labeled with two different fluorescent dyes (typically Cy3 and Cy5). When using the dual-target hybridization microarrays, the fact that two samples can be hybridized onto one microarray at once and expression levels can immediately be compared between the two samples constitute an advantage. However, compared to single-target hybridizations a clear disadvantage is the need for dye-swap experiments and more complicated normalization methods.

A very well known and widely used commercial microarray system which operates with single-sample hybridizations and uses probe sets that represent the transcripts is the Affymetrix GeneChip system. Each probe set consists of 11 to 20 probe pairs and each probe pair contains one 25 nucleotides long perfect match (PM) and one 25 nucleotides long mismatch (MM) oligo. The MM-oligo contains a single point mutation in the middle compared to the PM-oligo and is used to measure non-specific binding and cross-hybridization.

Affymetrix signal estimation and normalization

After the arrays have been hybridized with labeled mRNA obtained from the experimental samples, the analysis software normalizes the arrays to correct for technical variation and estimates the transcript signal intensities, which is followed by statistical testing (Figure 2).

The Affymetrix software (GeneChip Operating Software; GCOS, formerly known as MAS) performs total intensity normalization (32) in which the total intensities of each array are scaled towards the same value. Subsequently, the expression levels of each transcript are calculated by subtracting the MM-signal from the PM-signal for each probe pair and by subsequently calculating a robust mean of the probe pairs in the probe set. Additionally, a statistical test is performed to assess whether the probe set PM-signals are significantly higher than the probe set MM-signals, allowing transcripts that are reliably detected to be selected for further analyses.

In recent years, other Affymetrix GeneChip analysis tools have been developed, among which Robust Multi-chip Analysis (RMA) (33). Instead of normalizing each array separately, RMA fits a mathematical model through the entire set of arrays and takes into account both transcript abundancy and probe affinity (34). RMA analysis has been shown to reduce variance for the lower intensity signals in comparison to MAS (33,35), possibly resulting in improved detection of lower abundant signals. However, currently the RMA tool ignores the MM-signals, using only the PM-signals, and therefore no reliability estimate is generated (34).

Subsequent analysis using diagnostic plots is crucial in order to verify whether processing and normalization of the arrays went properly. Two of the more commonly used plots are the *scatterplot*, in which the gene intensities of two (paired) arrays are plotted, and the *box-and-whisker plot*, in which median intensities and intensity quartiles of unpaired arrays are displayed. Erroneous normalization can be visualized in the scatterplot as any deviation of the data cloud from the line y = x, whereas in the box-and-whisker plot unequal medians and variances indicate improper normalization.

Microarray statistics

After array normalization, signal estimation and optional pre-filtering of the data, a multitude of statistical tests developed for analyzing microarrays can be applied to assess differentially expressed (groups of) genes. The most commonly used tests so far have been 'gene level tests' in which individual genes are tested separately. Well known gene level tests include Significance Analysis of Microarrays (SAM) (36), the class comparison tool in the BRB ArrayTools package (developed by Dr. Richard Simon and Amy Peng Lam; http://linus.nci.nih.gov/BRB-ArrayTools.htm) and ANOVA (37).

However, recently a number of 'global tests' have been developed in which *a priori* defined sets of genes, which are for instance based on biological process classifications,

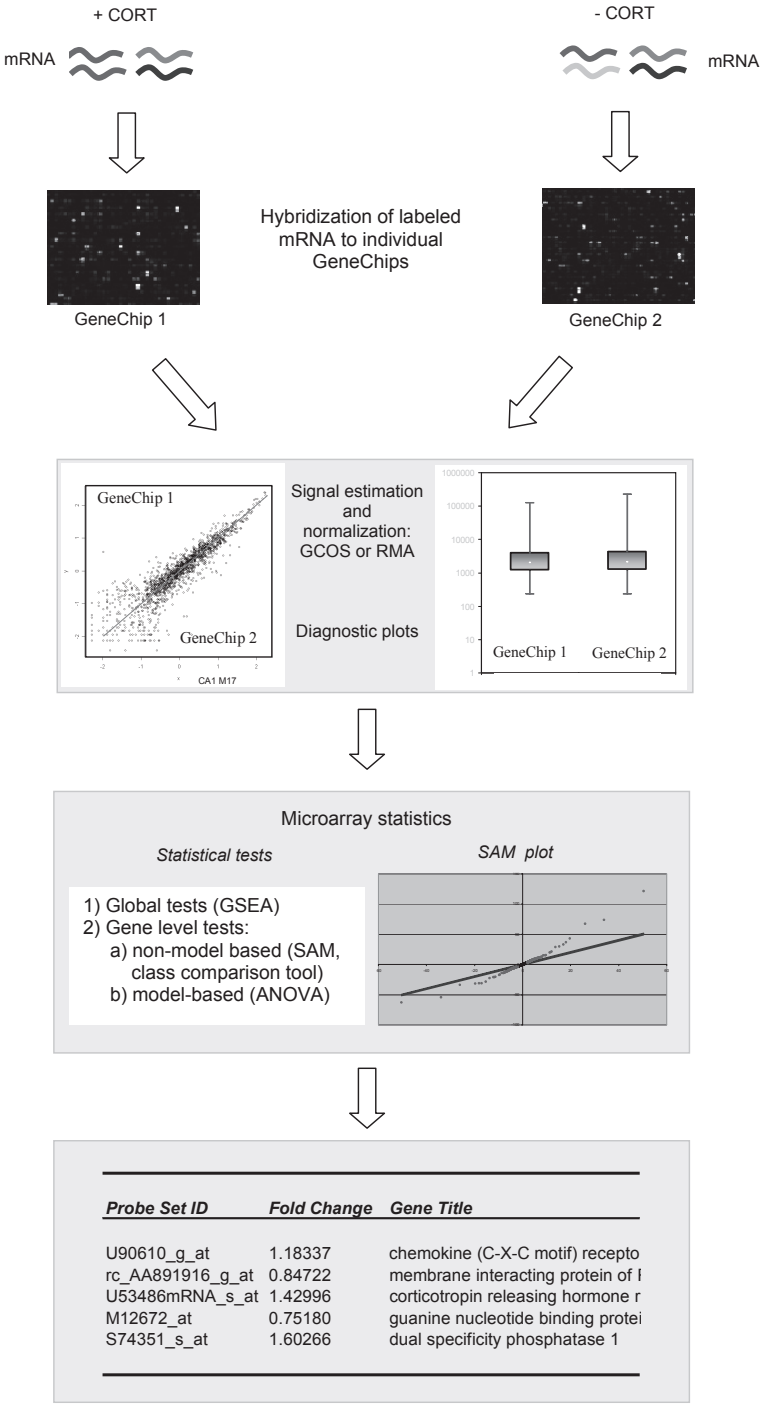


Figure 2. Overview of the DNA microarray procedure. Details are described in the text (section 2.2). + CORT: glucocorticoid-treated sample, - CORT: control sample.

are tested. Several examples of global tests include Gene Set Enrichment Analysis (GSEA) (38) and Parametric Analysis of Gene Set Enrichment (PAGE) (39). In comparison to gene level tests, global tests are believed to be more suitable for detecting subtle gene expression changes due to the fact that the statistical significance of a group of coregulated genes will be greater than the separate statistical significances of each individual gene in that group (39).

When using microarrays, a large number of genes and thus hypotheses are tested in a single experiment, resulting in many false positives due to the multiple testing problem. A generally accepted solution for this problem has been the application of so-called False Discovery Rates (FDRs) (40). Using this methodology, lists of differentially expressed genes are generated in association with an FDR estimate which expresses the percentage of genes present in the list due to chance alone. FDR estimates are generally provided in many statistical tests developed for microarray analysis, including all of the previously mentioned tests.

Beside statistical tools, clustering tools are also being applied to microarray data such as hierarchical and K-means clustering. These tools perform grouping of data based on calculated 'distances' between genes (41), but since no formal statistical model is applied these methods in general only provide a visual tool, facilitating graphical representation of the data.

SAGE versus DNA microarrays

Using either SAGE or DNA microarrays for gene expression profiling in the brain depends on several considerations that can be made.

First, brain is a highly complex heterogeneous tissue containing numerous nuclei, subnuclei, neuronal and non-neuronal cells in which many different genes are expressed. Therefore the detection of brain region-specific, low-abundant transcripts can be difficult due to dilution of these signals by cells in other brain regions (42). Both SAGE and Affymetrix GeneChips seem to perform equally well in brain tissue with respect to detectability, since a strong correlation between the detection of transcripts was observed in the hippocampus (43). Both methods reliably measured gene expression of medium to high abundant transcripts, whereas low-abundant transcripts were poorly detected. These results indicate that at the transcript detection level there is no reason to prefer either SAGE or DNA microarrays

Second, the use of microarrays in general has been described as following a *closed* gene expression profiling strategy since only the expression of genes that are present on the microarray is measured. In contrast, the use of SAGE has been designated as following an *open* gene expression profiling strategy since no selection of genes is made on forehand, thereby allowing the discovery of novel transcripts. Therefore, if the detection of novel transcripts is desired, using SAGE is a necessity. Additionally, SAGE generates

digital expression profiles, allowing easy comparisons between different SAGE libraries generated in different laboratories. In order to provide some sort of a standard annotation for microarray experiments, the Minimum Information About a Microarray Experiment (MIAME) protocol was developed, which authors can use for unambiguous interpretation of microarray data and reproduction of the experiment (44). However, comparison of microarray data is still very much complicated by the many different microarray platforms that are used.

Third, DNA microarray procedures are relatively fast and easily performed in comparison to SAGE, which constitutes a more laborious and time-consuming procedure. Therefore, when using SAGE, the number of experimental groups usually can only be limited whereas the use of DNA microarrays practically allows more elaborate experimental designs. However, developments in single molecule sequencing (45) are likely to contribute to faster and more cost-economic SAGE procedures in the future.

In conclusion, at present the issue of choosing a suitable gene expression profiling technique therefore logically depends 1) on the fact whether gene discovery is desired and 2) on the number of experimental groups that are under investigation.

4. MR AND GR-SPECIFICITY OF THE GENOMIC RESPONSE

In brain, the hippocampus is one of the regions in which the highest expression of MR and GR is found (10) and its neuronal activity is profoundly modulated by naturally occurring glucocorticoids cortisol and corticosterone (46). Therefore, the vast majority of studies which assess glucocorticoid-mediated changes in neural gene expression have focused on the hippocampus and in particular on GR-mediated expression. Under basal concentrations of glucocorticoids, predominantly occupying MRs, calcium currents and 5HT1A (serotonin)-receptor mediated hyperpolarization are low in the hippocampal CA1 subregion whereas under additional GR occupation due to increasing glucocorticoid concentrations, calcium currents and 5HT1A-receptor mediated hyperpolarization are high (46). The effects that are observed under high glucocorticoid concentrations are also observed under glucocorticoid depletion, for instance after adrenalectomy, in which no receptors are occupied. Therefore, the effects of glucocorticoids follow a U-shaped dose-dependency. There are strong indications that these glucocorticoid-mediated effects on calcium currents and 5HT1A-receptor mediated hyperpolarization are dependent on changes in gene transcription since they develop in a delayed manner which is dependent on DNA-binding of the receptors and *de novo* protein synthesis (3,46, 47,48).

One of the first studies to address the glucocorticoid-induced genomic changes in the hippocampus in response to MR and / or GR-activation at a large-scale was performed on pooled rat hippocampal lysates which were subjected to SAGE (49). Exposure of the

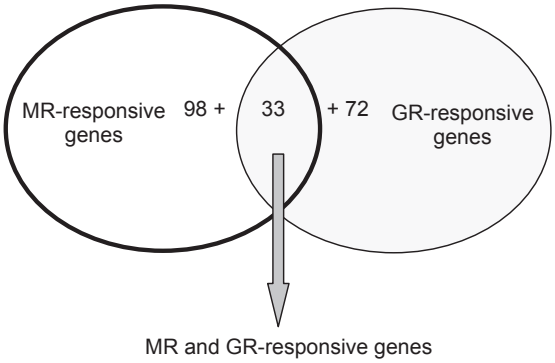


Figure 3. MR- and GR-specificity of the genomic response in hippocampus, assessed by using Serial Analysis of Gene Expression (SAGE). MR and GR transcriptionally regulate distinct, yet overlapping sets of genes (49). Details are described in the text (section 4).

hippocampus to glucocorticoids was manipulated *in vivo* using adrenalectomy in combination with implantation of low corticosterone pellets (MR occupation) and additional corticosterone injections (MR + GR occupation). SAGE-expression profiles, encompassing over 20.000 SAGE tags per experimental group revealed that in the hippocampus more than 80% of the glucocorticoid-responsive genes were regulated by either MR or GR alone, whereas the remaining genes were regulated by both MR and GR. Thus, in the hippocampus under conditions of selective MR and / or GR-activation, both receptors appear to regulate specific, though partially overlapping sets of genes (Figure 3). These results therefore strongly indicated that the differential effects of glucocorticoids on hippocampal neuronal properties are mediated by largely different signaling pathways.

Since MRs and GRs share a common DNA binding domain and recognize the same GRE-sequences on the DNA which they can bind to (50,51) the question is how to explain these differences in genomic action of the two receptors. Currently the idea is that the differential effects of MR and GR on gene expression are most likely mediated via binding of different coactivators / corepressors to the receptors and/or by differences in transrepressive capacity between the receptors (52,53). MR and GR share a relatively low level of homology within their N-terminal domains and therefore these domains are interesting with regard to binding of MR and GR-specific coregulators and transrepression partners (53). Additionally, differential effects of coregulators have been demonstrated in an *in vitro* study in which the coactivator SRC-1e enhanced the transcriptional response mediated by the MR N-terminal domain (54) but not by the GR N-terminal domain. More strikingly, the coregulator ELL was found to exert a completely opposite effect on MR versus GR-mediated gene transcription *in vitro* by acting as a coactivator for MR and as a corepressor for GR (52). Furthermore, with regard to differences in transrepressive capacity between MR and GR, Pearce and Yamamoto (55) demonstrated that activated GRs were able to transrepress AP1 stimulated transcription from a composite response element whereas under the same conditions MRs were inactive. A specific sequence in the

N-terminus of the GR was shown to be responsible for this transrepression. Additionally, several members of the protein inhibitor of activated STAT (PIAS) family were found to specifically interact with MRs but not with the GRs in a neuronal cell line, most likely via specific interactions with the N-terminal domain (56).

In conclusion, both MR and GR exert receptor-specific effects on hippocampal neuronal properties and these receptor-specific effects are also present at the transcriptional level. The transcriptional differences between the receptors are currently believed to occur via binding of different coregulators and transrepression partners to MR and GR.

5. DYNAMICS OF THE GENOMIC RESPONSE

The previously mentioned application of SAGE to assess hippocampal MR and GR-induced transcriptional changes resulted in the identification of over 200 MR and GR-responsive genes (49). However, with respect to the neuronal properties, the precise molecular mechanisms underlying the glucocorticoid-induced effects still have been difficult to elucidate. This is illustrated by the fact that the GR-mediated increase in serotonin-response has so far not been directly linked to increases in serotonin receptor transcripts. Since in the SAGE study responsive genes were assessed at a fixed time point after receptor activation (in the case of GR-responsive genes three hours after receptor activation by a corticosterone injection) transient changes in transcript levels that possibly precede the effects on neuronal properties could have been missed.

Transcriptional responses to acute glucocorticoid administration have been measured in the hippocampus at different points throughout time. In one study, the transcriptional response of brain derived neurotrophic factor (BDNF) and the TrkB-receptor to glucocorticoids was measured *in vivo* in the hippocampus after a corticosterone injection. This study revealed transient downregulation of BDNF in the dentate gyrus at 3 hours and upregulation in the CA1 subregion 12 hours afterwards (57). Other studies have examined the effects of *acute* stress on hippocampal gene expression levels in which rising glucocorticoid concentrations constitute only one of several factors contributing to the stress response. One such study examined hippocampal expression levels for MR, GR and growth hormone receptor (GHR) in a time frame of 0.5 – 12 hours after acute restraint stress in the water (RSW). A striking biphasic regulation of all 3 genes was observed in which an initial rapid (≤ 1 hour) transcriptional downregulation was succeeded by upregulation 2 hours afterwards in the dentate gyrus (58).

In order to gain more insight into the hippocampal transcriptional response to acute administration of glucocorticoids throughout time, a large-scale gene expression profiling study was conducted in *ex vivo* hippocampal slices. These slices were obtained from rats which were adrenalectomized and replaced with low corticosterone-secreting pellets,

occupying endogenous MRs (59). This preparation was chosen since many of the effects of activated GRs on neuroexcitability have been described in this preparation (48,60,61), allowing direct comparison between gene expression profiles and phenotypic effects. GRs were activated by a 20 minute corticosterone pulse and gene expression was profiled 1, 3 and 5 hours afterwards, which is the time frame in which GR-induced changes take place ((48,61-63). Using Affymetrix GeneChips and subsequent Significance Analysis of Microarrays (SAM), an unexpected dynamic pattern of transcriptional responses was found throughout the profiled time window, which shifted from exclusively downregulation of genes 1 hour after GR-activation towards both up and downregulation of genes 3 hours afterwards. After 5 hours the response was almost back to baseline (Figure 4). Since so far only a limited number of genes have been identified which are repressed via negative GREs, this dynamic pattern suggested that in hippocampal tissue acute activation of GRs results in transrepression involving interactions with other transcription factors followed by a later wave involving transactivation. Hence, the obtained time-dependent profiles led to the question how general this highly characteristic dynamic response is for different neural subtypes.

In order to answer this question, the effects of acutely activated GRs on neural transcription were studied in a completely different neural substrate, i.e. clonal PC12 cells. These cells differentiate into catecholaminergic neuron-like cells when exposed to nerve

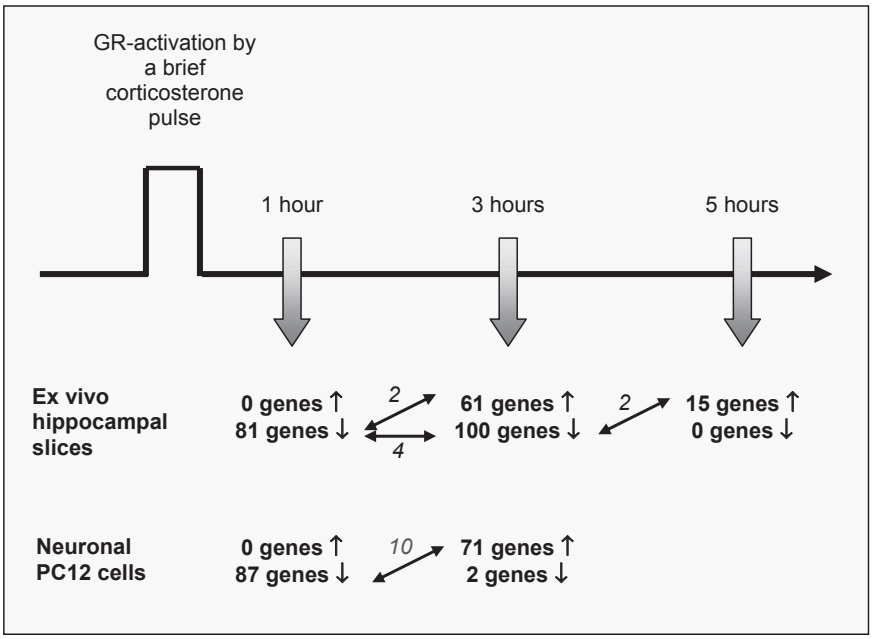


Figure 4. Dynamics of the genomic response in the hippocampal slice preparation and neuronal PC12 cells, showing a shift from downregulated genes 1 hour after GR-activation towards upregulated genes 3 and 5 hours afterwards (59,77). Details are described in the text in section 5.

growth factor (NGF), thereby generating long neurites, developing electrical excitability and expressing sodium, potassium and calcium channels as well as membrane receptors such as G-protein coupled receptors (64, 65, 66, 67). Furthermore, PC12 cells are known to express GR endogenously (68) and to display a highly stable karyotype (for which they are unique among clonal cell lines), making them very suitable for expression profiling of glucocorticoid-responsive genes. Hence, a similar experimental setup was chosen as in the hippocampal slice study and GRs were activated in differentiated PC12 cells by application of a 20 minute corticosterone pulse.

Translocation of GR into the nucleus after application of the corticosterone pulse was confirmed by immunocytochemistry and Affymetrix GeneChips were subsequently used to measure gene expression. Strikingly, SAM-analysis revealed a highly similar dynamic pattern of exclusively downregulated genes at 1 hour and the majority of genes upregulated at 3 hours after GR-activation (Figure 4). These data suggested that also in neuronal PC12 cells transrepression may precede transactivation in time which results in downregulated genes at early time points. Hence, in both neural tissues tested, acutely activated GRs mediated a very similar time-dependent genomic response.

The obtained transcriptional patterns were in line with the pattern observed in another large-scale gene expression profiling study that was performed in the liver (69). Here, endogenous GRs were activated by acute administration of the synthetic glucocorticoid methylprednisolone, after which changes in gene expression were assessed in a time window of 15 minutes to 72 hours using Affymetrix GeneChips. Subsequently, three different clustering tools were used to define clusters of genes which displayed a similar dynamic pattern and these analyses revealed that the vast majority of early modulated genes were downregulated whereas a delayed wave of upregulated genes followed 2 hours later, mimicking the observed dynamic pattern in hippocampal slices and neuronal PC12 cells.

Since all three large-scale gene expression profiling studies measured the transcriptional response after acute activation of GR by application of a single glucocorticoid pulse (which mimics the peak in the ultradian secretion pattern), the observed dynamic pattern may be specific for this particular situation.

However, a shortcoming of the current technology is that expression levels of low-abundant transcripts are not reliably measured and therefore caution with the interpretation of these data is required. Upregulation of genes by activated GRs after short time intervals is possible and has been demonstrated in hippocampal slices for calcium channel subunit expression (Y.Qin, unpublished observation). In addition, there are strong indications that the rapid upregulation of the phenylethanolamine-N-methyltransferase (PNMT) gene in the adrenals after acute stress is mediated via its upstream GRE (70). Hence, upregulation of gene transcription can occur after short time intervals, although the prevailing pathway seems to be downregulation. Currently, enhancement of the

detection of low-abundant transcripts is desired to obtain a complete picture of the transcriptional response.

Information regarding the rate at which the different steps involved in transactivation and transrepression proceed is sparse. There are strong indications that overall mRNA decay is not faster than mRNA elongation (71,72). Therefore, it does not seem likely that an imbalance in decay versus elongation is responsible for the observed wave of downregulation. Hence, the rapid transcriptional downregulation of genes at early time points could be explained by the fact that blocking the ongoing effects of other transcription factors during transrepression may occur more rapidly than transactivation in which recruitment of cofactors and chromatin remodeling needs to be initiated only at the time of nuclear receptor activation (8,53,73,74). Interestingly, GRs have been shown to directly interact with the general transcription machinery as well (75), possibly resulting in rapid upregulation of gene transcription. Hence, expanding the time window with shorter time intervals (< 1 hour) would be of major interest.

Conducting experiments separating primary from downstream transcriptional responses would greatly enhance our current understanding of the mechanisms that underlie the temporal pattern of glucocorticoid-mediated transcriptional responses and the role of activated GR as a component of the transcriptional regulatory complex. One of the first studies to assess genes which are directly under the transcriptional control of activated GRs at a large scale was performed in human lung carcinoma cells, using cycloheximide to block protein synthesis and subsequent downstream transcription (76). Using microarrays, many transcriptionally responsive genes, both up and downregulated were found after constant treatment of the cells with dexamethasone for 6 hours. Subsequently, the promoter regions of a small subselection of 11 GR-responsive genes were screened using chromatin immunoprecipitation (ChIP; see section 9), resulting in the identification of GR-binding regions for 8 genes, including glucocorticoid-induced leucine zipper (GILZ; upregulated) and serum inducible kinase (SNK; downregulated). Hence, this study clearly demonstrated the strength of using both large-scale gene expression profiling against a cycloheximide background and application of ChIP to identify primary GR-responsive genes.

The issue of primary GR-responsive genes was also briefly addressed in the PC12 study (77) and for a limited number of responsive genes the transcriptional response was profiled in an additional experiment in which cycloheximide was used to block downstream transcriptional responses. It was found that the genes which were selected from the 1 hour time point were primary responsive whereas the genes that were selected from the 3 hours time point were downstream responsive.

Additionally, a closer look at the genes found to be GR-responsive in hippocampal slices revealed that metallothionein was upregulated 3 hours after GR-activation. Since there are indications that this gene contains two functionally active GREs (78) it most

likely is a primary responsive gene. This would therefore indicate that in the time-dependent profile the 1 hour time point is constituted by mainly primary responsive genes while at the 3 hours time point both primary and downstream responsive genes are present. Therefore, in order to gain more conclusive data, the analysis should be expanded by increasing the number of time points after GR-activation and using large-scale gene expression profiling methods to maximize the number of genes measured.

Finally, the question whether transrepression is the primary event 1 hour after GR-activation could be approached by identifying commonly shared transcription factor binding sites in the proximity of promoter regions of transcriptionally regulated genes and subsequent testing of these putative binding sites for binding of GR and transrepression partners in a large-scale fashion.

In conclusion, acute activation of neural GRs results in a highly characteristic time-dependent expression profile. This profile suggests that transrepression may be the prevailing pathway by which GRs regulate neural gene transcription at early time points although currently more conclusive data are needed to support this idea. The nature of the genes that are regulated by GRs throughout time and the functional categories to which they belong are discussed in section 7.

6. CONTEXT-SPECIFICITY OF THE GENOMIC RESPONSE

The nature of the genomic response to glucocorticoids not only depends on which receptor (MR or GR) is activated but also on the cell type *(cellular context)* and activation status of the cells *(environmental context)*.

Cellular context

Activated GRs have been shown to exert different effects in different (neural) substrates. For example, GR-activation in the hypothalamic paraventricular nucleus (PVN) inhibits CRH neurons whereas extrahypothalamic CRH neurons are stimulated by activated GRs (2,79). Additionally, expression of the monoamine oxidase A (MAO-A) gene was found to be inhibited by activated GRs in the hippocampus (59) and enhanced in skeletal muscle cells (80).

The extent to which this cellular context determines the glucocorticoid-mediated genomic response in neural tissue was addressed by comparing the expression profile obtained in neuronal PC12 cells with those obtained in the hippocampal SAGE study (49) and the hippocampal slice study (59). This meta-analysis revealed that the extent of overlap between glucocorticoid-responsive genes identified in PC12 study and both hippocampal studies was very small, between 2 to 4% of the responsive transcripts, in comparison to the overlap between both hippocampal data sets (16%) (77). Thus, at the

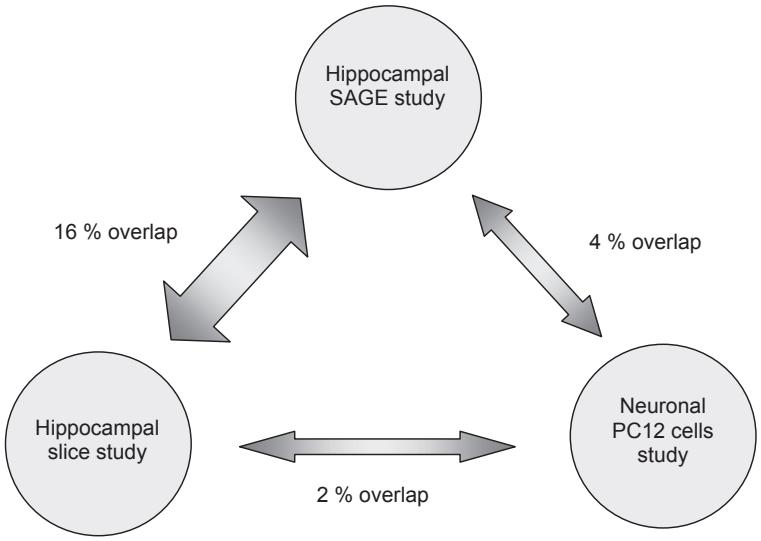


Figure 5. Cellular context-specificity of the genomic response to glucocorticoids in neural tissue. Meta-analysis showing a significant larger overlap between the hippocampal SAGE and the hippocampal slice data set than between the neuronal PC12 and hippocampal data sets (77).

level of the individual genes, the genomic response to acute administration of glucocorticoids appeared to be highly dependent on cellular context.

Differences in cellular context may occur due to differences in the availability of cofactors in different cell and tissue types or even between brain subregions. In this respect it is interesting to note that the genomic response to glucocorticoids is determined by the interaction between promoter composition and available cofactors. As a consequence, different genes with different kinds of promoters display different glucocorticoid-induced transcriptional responses depending on the availability of cofactors. This was illustrated by *in vitro* experiments in which different splice variants of steroid receptor cofactors (SRCs) were shown to be recruited to promoters containing different numbers of GREs (54,81). Thus, transcriptional modulation by activated GRs clearly depends on the GRE composition of the target gene's promoter region and the availability of cofactors.

Furthermore, for several genes it has been shown that the GREs are organized into so-called glucocorticoid responsive units (GRUs) in which GREs are flanked by other accessory transcription factor binding sites (8). The transcriptional response of GRU-containing genes to glucocorticoids not only depends on activated and GRE-bound GRs, but also on binding of these accessory transcription factors. Since the expression of accessory transcription factors can be cell and tissue-specific, GRUs may differentially mediate the transcriptional response in different tissues and cells. An interesting example in which this principle may occur includes a subset of hepatic genes involved in gluconeogenesis which share a number of binding sites for liver-enriched transcription factors C/EBP and FoxA within their GRUs (8,82).

Additionally, the availability of transrepression partners may differ between different cell and tissue-types, resulting in transrepression of different sets of genes. Although so far no differential expression of NFκB, AP1 and CREB has been observed in for instance hippocampal subregions, other transcription factors which could potentially interact with glucocorticoid receptors (NF1, NGFI-B, HES-1 and the New England Deaconess transcription factor) were found to be differentially expressed in the hippocampal CA3 and dentate gyrus subregions (42).

Environmental context

Beside the effect of differences in neural cell type on the glucocorticoid-mediated transcriptional response, the activation status of the neural cells most likely also exerts an effect. For instance, there are several afferents (for instance the perforant path, alvear path or brain stem afferents) which affect the activation status of the hippocampus, thereby modulating the activity of intracellular pathways and thus affecting the availability of cofactors and transrepression partners for glucocorticoid receptors in hippocampal cells. Different experimental conditions (such as different stressors) will differentially activate these afferents, resulting in a condition-specific activation status of the hippocampal cells. This, in turn, will most likely determine the set of genes that is transcriptionally modulated by glucocorticoids.

Thus, in contrast to the dynamics of the genomic response to glucocorticoids, the nature of the response seems to be largely determined by the cellular and environmental context, resulting in a tissue and activation status specific pattern of glucocorticoid-responsive genes. Most likely, differences in availability of cofactors, accessory transcription factors and transrepression partners underlie this context-specificity.

7. FUNCTIONAL IMPLICATIONS

In the previous three sections, the general characteristics of the glucocorticoid-mediated transcriptional response have been discussed. The current section will deal with the functional implications of glucocorticoid-mediated transcriptional regulation.

Functional gene classes affected by glucocorticoids

One aspect that has become evident from genomics studies into glucocorticoids is that they can modulate transcription of genes involved in a wide variety of different cellular processes. This has been shown in many different studies, demonstrating *pleiotropic* effects of glucocorticoids on several different transcriptomes. Functional categories of glucocorticoid-responsive genes overlap to a large extent between different tissues and

well known examples include (energy) metabolism, signal transduction, oxidative stress, regulation of transcription, regulation of translation and cell adhesion (49,59,69,83).

With respect to neural tissue, several interesting observations have been made. First, in the hippocampus MR and GR were shown to regulate genes which can be grouped into similar functional categories (49). Second, also in hippocampal slices and neuronal PC12 cells similar functional categories were affected by glucocorticoids throughout different time intervals after GR-activation (59,77). Hence, at the level of functional categories affected by glucocorticoids in neural tissue, so far no clear receptor, context or time-specificity has been observed, although often these functional categories are represented by different individual transcripts.

So far not many functional studies have been conducted with these different glucocorticoid-responsive genes. Therefore, providing an answer to the question what it means for the neuron that glucocorticoids affect so many functional gene groups remains difficult. However, some speculation is possible when considering the sequence of events that occur throughout the general stress-response. After an organism has experienced a stressor, limbic brain areas, among which the hippocampus, are rapidly activated to mediate the cognitive and emotional processing of the stressor. Glucocorticoids are released by the HPA-axis in a delayed manner and are responsible for modulating and fine

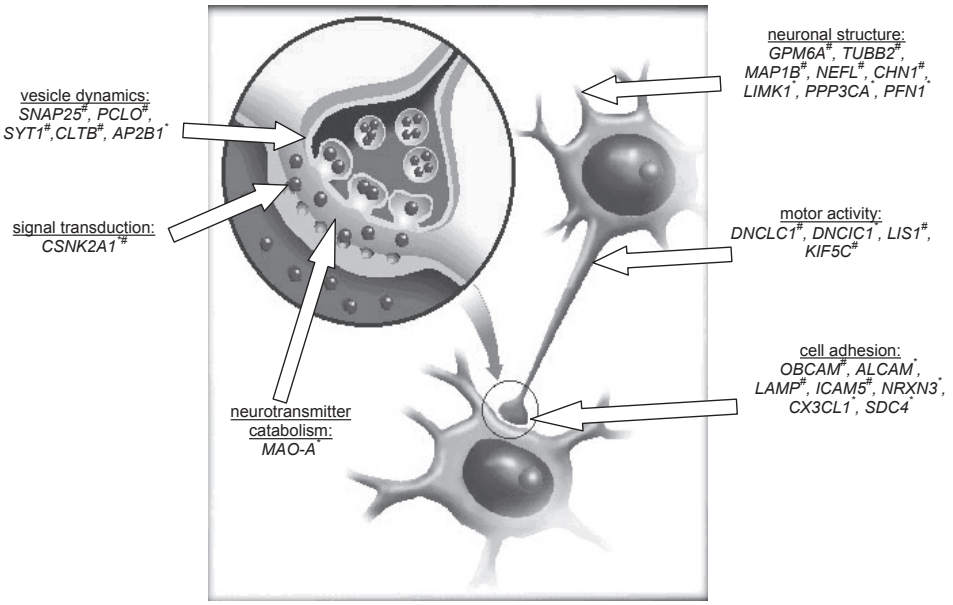


Figure 6. Glucocorticoids exert direct transcriptional effects on different components of the hippocampal neurotransmission cascade. These components include 1) signal transduction, 2) neuronal structure, 3) vesicle dynamics, 4) neurotransmitter catabolism, 5) motor activity and 6) cell adhesion. *; the gene was found to be glucocorticoid- responsive in the hippocampal slice study (59). #; the gene was found to be glucocorticoid-responsive in the hippocampal SAGE study (49): renewed annotation of hippocampal SAGE tags obtained in 2001 five years after the original publication allowed functional classification of several previously unknown corticosterone-responsive genes (unpublished data).

tuning of the initial stress-response, facilitating learning and memory formation (84,85). This is reflected by the different functional gene groups that are affected by glucocorticoids and that can be considered to be interconnected with each other. For instance, the neurotransmissive capacity of a neuron logically depends on the amounts of energy and protein available, whereas signal transduction between neurons depends on synaptic strength and cell adhesion. Thus, transcriptional regulation by glucocorticoids of many different, though interconnected functional processes could therefore provide a mechanism by which the initial neural stress-response in limbic brain areas is optimally adjusted in an overall balanced, adaptive manner.

Interestingly, when assessing different lists of glucocorticoid-responsive genes it becomes clear that several different gene ontology groups transcriptionally affected by glucocorticoids may be grouped into a larger functional group, i.e. hippocampal neurotransmission. Very clearly, glucocorticoids exert direct transcriptional effects on many different components of the hippocampal neurotransmission cascade (Figure 6). These components include patterns of gene expression underlying 1) signal transduction, 2) neuronal structure, 3) vesicle recycling, 4) neurotransmitter catabolism and 5) motor activity and 6) cell adhesion (49,59), all of which providing numerous interesting candidate genes for future follow-up studies.

Candidate genes

Glucocorticoids & signal transduction

One of the main hippocampal properties that are affected by acute administration of glucocorticoids is long-term potentiation (LTP), a property which directly affects synaptic transmission. It has been shown that activated GRs inhibit LTP in the CA1 subregion (3,46,47,48) and since this effect occurs in a delayed manner, the underlying mechanism most likely includes changes in gene expression. The currently obtained gene expression profiles contain several putative candidate genes which may underlie this effect of glucocorticoids on LTP.

In this respect, *casein kinase 2* (CSNK2A1) is a very interesting gene as it was found to be downregulated in both hippocampal SAGE (49) and hippocampal slice (59) studies after acute administration of glucocorticoids. This gene is part of the downstream signal transduction cascade of the NMDA-receptor (86) and enhanced casein kinase 2 activity was found to be correlated with the induction of NMDA-receptor mediated LTP (87). Since 1) activated GRs inhibit NMDA-receptor mediated LTP and 2) there is no evidence for transcriptional regulation of NMDA-receptor mRNA (59 and Qin and Joëls unpublished observation), downregulation of casein kinase 2 after GR-activation could in part underlie this effect. Hence, casein kinase 2 constitutes a putative candidate gene involved in glucocorticoid-modulated long-term potentiation (Figure 6).

Glucocorticoids & neuronal structure

Renewed annotation of hippocampal SAGE tags obtained in 2001 (49) 5 years after the original publication (unpublished data) allowed functional classification of several previously unknown corticosterone-responsive genes. Combining the newly annotated SAGE tags with the other functionally annotated corticosterone-responsive genes pointed to a role of glucocorticoids in regulating neuronal structure. Both genes which are structural components of the cytoskeleton (*beta-tubulin (TUBB2),* neurofilament*, light polypeptide (NEFL))* as well as genes that mediate neurite outgrowth (*chimerin 1 (CHN1)* (88), *glycoprotein M6A (GPM6A)* (89)) or are associated to the microtubuli (*microtubule associated protein 1B (MAP1B)* (90)) were identified as being corticoisteroid-responsive. In Figure 6, these genes are integrated in the overall picture of the effects of glucocorticoids on neurotransmission.

Strikingly, many genes specifically involved in the dynamics of the actin cytoskeleton were found in both hippocampal and PC12 gene expression profiles. Clearly, acute administration of glucocorticoids affects this system by modulating mRNA levels of genes such as *LIMK1, calcineurin subunit a (PPP3CA), profilin 1 (PFN1), beta-actin* and *LIM/SH3 protein*. The actin cytoskeleton is involved in the morphology of dendritic spines and changes in actin cytoskeletal configurations have been connected to LTP, affecting synaptic transmission (91, 92). LIMK1 has been shown to be necessary for proper accumulation and distribution of actin filaments in hippocampal dendritic spines and to be involved in the regulation of spine size (92,93) whereas calcineurin has been shown to inhibit LIMK1 protein expression (94) and to be involved in destabilization of actin filaments and hippocampal dendritic spines (95). Profilin is known to inhibit the polymerization of actin filaments and plays a role in (activity-dependent) remodeling of hippocampal synaptic structure (96-98). Furthermore, profilin was also found to be differentially expressed when comparing the hippocampal transcriptomes of two genetically selected mouse strains (i.e. short attack latency and long attack latency mice) that differ in aggressive behaviour, HPA-axis reactivity and morphology of mossy fiber terminal fields (99,100). Thus, glucocorticoid-mediated transcriptional upregulation of LIMK1 and downregulation of calcineurin and profilin in the hippocampus possibly affects dendritic spine morphology, most likely resulting in altered synaptic plasticity (Figure 6 and Figure 7).

Additionally, in the gene expression profile generated in PC12 cells, both *beta actin* and *LIM/SH3 protein* were found to be downregulated after GR-activation, indicating that also in these (catecholaminergic-like) cells glucocorticoids affect actin cytoskeletal configuration which possibly results in altered synaptic plasticity.

Glucocorticoids & vesicle dynamics

From the obtained gene expression profiles it becomes clear that glucocorticoids exert an effect on both exocytosis and endocytosis by affecting vesicle recycling. For instance,

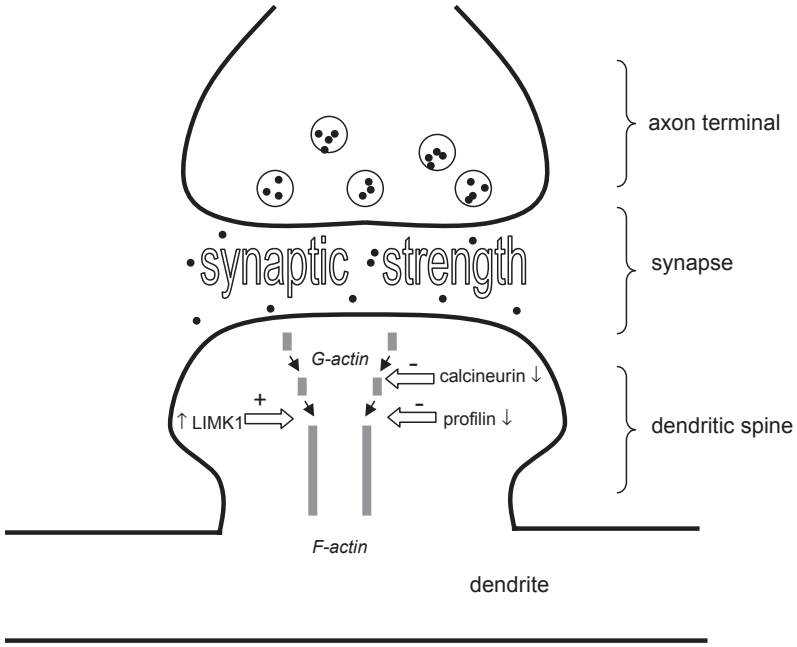


Figure 7. Regulators of hippocampal dendritic spine morphology which are transcriptionally affected by activated GRs. LIMK1 enhances the formation of filamentous (F-) actin from actin monomers (G-actin) whereas calcineurin and profilin destabilize F-actin. ↑; upregulated by activated GRs. ↓; downregulated by activated GRs. LTP; long-term potentiation.

in the PC12 and hippocampal SAGE expression profiles, *synaptosomal-associated protein 25 (SNAP-25)* was found to be upregulated after acute glucocorticoid administration. This gene affects calcium-dependent exocytosis and therefore an increase in mRNA expression could lead to increased neurotransmitter release (101) which would result in enhanced neurotransmission. Other exocytois-related genes included *presynaptic cytomatrix protein (Piccolo; PCLO),* which is involved in the assembly and function of pre-synaptic active zones (102) and *synaptotagmin I (SYT1),* which is important for vesicle trafficking to the active zones (103,104). Furthermore, several endocytosis-involved genes were also identifed as being corticosteroid-responsive among the (re-annotated) SAGE tags and in the hippocampal slice data set, including *clathrin light chain (CLTB)* (105), which is a component of coated vesicles, and *adaptor-related protein complex 2, beta 1 subunit (AP2B1)*, which serves to link clathrin to receptors in coated vesicles (106) (Figure 6).

Furthermore, in PC12 cells several genes involved in Rab-mediated endocytosis *(Rab 1 acceptor, Rab 15 and Rab 7)* were found to be GR-responsive. Since enhanced exocytosis disrupts membrane balance, enhancement of endocytosis and thus membrane recycling by glucocorticoid-mediated transcriptional regulation of several Rab proteins may constitute a mechanism to restore membrane balance in these cells.

Glucocorticoids & neurotransmitter catabolism

Another property that affects hippocampal neurotransmission is the availability of neurotransmitters in the synaptic cleft. The availability of neurotransmitters serotonin, dopamine and noradrenalin is under the control of the catabolic enzyme *monoamine oxidase A (MAO-A)*. Strikingly, this gene was found to be downregulated in the hippocampus after acute activation of GR (59). Hence, this would suggest that glucocorticoids are able to increase the availability of neurotransmitters in the hippocampal synaptic clefts, thereby influencing hippocampal neurotransmission (Figure 6).

Glucocorticoids & cell adhesion

Among the hippocampal glucocorticoid-responsive genes, many neural cell adhesion molecules were present, including *opioid binding cell adhesion molecule (OBCAM)*, *activated leukocyte cell adhesion molecule (ALCAM)*, *limbic system associated membrane protein (LAMP)*, *intracellular adhesion molecule 5 (ICAM 5)*, *neurexin 3 (NRXN3)*, *chemokine C-X3-C motif ligand 1 (CX3CL1)* and *syndecan 4 (SDC4)*. Interestingly, there is growing evidence that cell-adhesion molecules not only play a role in the development of synaptic structures but also in regulating synaptic plasticity and learning and memory formation (107). Thus, regulation of cell adhesion gene expression may constitute an additional molecular mechanism by which glucocorticoids affect hippocampal synaptic plasticity (Figure 6).

Glucocorticoids & autoregulatory loop of MR / GR signaling

Glucocorticoids clearly affect motor activity and axonal transport by regulating transcription levels of *dynein light chain LC8 type 1 (DNCLC1)*, *dynein cytoplasmic 1 intermediate chain 1 accessory subunit polypeptide (DNCIC1)* (108), *lissencephaly 1 protein (LIS1)* (109) and *kinesin family member 5c (KIF5C)* (110) (Figure 6). Since cytoplasmic dynein has been shown to be involved in GR-receptor trafficking (111), regulation of this motor protein may affect glucocorticoid receptor translocation itself, thereby affecting glucocorticoid receptor signaling. Interestingly, there are indications that glucocorticoid receptor signaling may additionally be modulated by changes in gene expression which affect 1) receptor binding affinity and 2) receptor levels. First, glucocorticoid-mediated transcriptional regulation of glucocorticoid receptor chaperone *Hsp90* and co-chaperones *FKBP1a* and *DNACJ5* was observed (49), which could affect the binding affinity of MR and / or GR for ligand (112-115). Second, glucocorticoid receptor levels may be under the influence of glucocorticoids since 1) *MR* itself was found to be transcriptionally responsive to activated GRs and 2) activated GRs regulate the expression of *NGF-IA* which is most likely involved in GR-expression (116,117). Therefore, these data suggest that GCs affect their own signaling at multiple levels via an autoregulatory loop, which may play an important role in their negative feedback.

Chronic exposure to glucocorticoids

Beside the genes described above which were found in the gene expression profiles generated after *acute* administration of glucocorticoids, several interesting candidate genes were found in two other large-scale gene expression profiling studies in which the effects of *chronic* glucocorticoid exposure on the hippocampal transcriptome were analyzed.

In the first study, tree shrews were subjected to chronic administration of cortisol in the drinking water for 28 days after which a gene expression profile was generated using subtractive hybridization (118). Several genes were found to be transcriptionally regulated and downregulation of four of those genes *(NGF, membrane glycoprotein 6A, CLK-1 and G-protein alpha q)* was confirmed in a chronic psychosocial stress experiment (119). Since the effect on transcription of all genes except NGF was prevented by treatment with the antidepressant clomipramine, these genes constitute highly interesting candidate genes that may play a role in the pathophysiology of stress-related depression.

In the second study, mice genetically selected for long-attack latency (LAL mice) were exposed to a sensory contact stressor for 25 days after which Affymetrix GeneChips were used to analyze the changes in hippocampal gene expression (120). Strikingly, a number of genes involved in the NFκB signaling cascade *(CHUK/IKKα, several ras-family members and several NFκB-responsive genes)* were found to be downregulated after chronic stress. Hence, it was proposed that transcriptional modulation of this cascade could be the underlying mechanism by which the vulnerability of hippocampal neurons is increased after chronic stress.

At present, the obtained candidate genes very clearly demonstrate the fact that the current challenge is to design functional studies to test the generated hypotheses. Furthermore, beside the large quantity of glucocorticoid-responsive genes which have presently been identified, the expression profiles of many low abundant genes are still unavailable due to the fact that large-scale gene expression profiling techniques have a limited detection capacity. For instance, both SAGE and DNA microarrays so far have been unable to reliably detect several ion channels, neurotransmitter receptors, growth factor receptors and transcription factors in hippocampal tissue (49,59,121). Therefore, technical refinement will be necessary to also include those genes in future large-scale gene expression profiling of glucocorticoid-responsive genes.

8. TECHNICAL REFINEMENT

Currently, there are two main complicating factors hampering large-scale expression profiling of glucocorticoid-responsive genes in neural tissue. First, both SAGE and DNA microarrays have been unable to detect low abundant genes. Second, glucocorticoids

induce very subtle changes in gene transcription (less than twofold), making them difficult to detect.

Concerning the detection limits of large-scale gene expression profiling techniques, several options for improving the detection of transcripts are available. Since brain tissue in general consists of many different cell types, each expressing different repertoires of genes, cell-type specific transcripts may be masked due to the complexity of the heterogeneous tissue. Therefore, reducing the complexity of the tissue by isolating the subregion of interest, constituting a more homogenous neuronal population, most likely will result in the enhanced detection of cell-type specific transcripts. With regard to the hippocampus, a first subregion-specific expression profile was generated using a modified SAGE-procedure, called microSAGE, in combination with a punch which contained part of the hippocampal dentate gyrus subregion (30). Additionally, in order to be able to more specifically isolate brain regions, a second feasibility study was performed in which laser microdissection (LMD) was applied to isolate hippocampus subregions and DNA microarrays were used to generate subregion-specific expression profiles (42). In this study, hippocampal dentate gyrus and CA3 subregions were isolated and good quality total RNA was obtained from the fragments. Subsequently, the mRNA portions of the total RNA samples were amplified, labeled and hybridized to Affymetrix GeneChips (Figure 8). Using standard Affymetrix GCOS software in combination with SAM, an extensive inventory of genes differentially expressed between CA3 and dentate gyrus was obtained. Additionally, when compared to the expression profiles that were obtained in a study in which the hippocampus was crudely dissected into 3 pieces using a scalpel (122), the

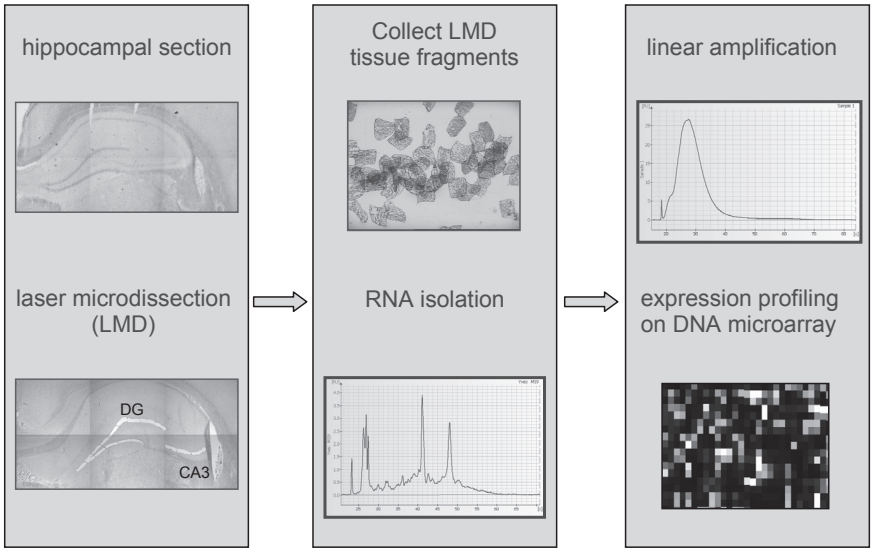


Figure 8. Laser-microdissection (LMD) procedure applied to the hippocampus. First, the hippocampal subregions of interest are excised after which total RNA is isolated. Then, the mRNA portion is amplified, labeled and subsequently hybridized to DNA microarrays (42).

LMD-expression profile proved to be more specific for principal hippocampal neurons. Hence, this clearly demonstrated that the use of LMD in gene expression profiling results in more subregion-specific expression profiles. However, since still no neurotransmitter receptors were reliably detected in both CA3 and dentate gyrus, technical refinement should not only include reduction in tissue complexity and enhanced specificity of the selected subregions but also application of more sensitive microarray detection algorithms and / or platforms.

With regard to the first, an interesting option would be the implementation of Robust Multi-chip Analysis (RMA) methodology (33) for analyzing Affymetrix GeneChip transcript signals instead of using standard Affymetrix GCOS software. As described in section 2, RMA has been shown to reduce variance for the lower intensity signals in comparison to GCOS, most likely resulting in the detection of lower abundant transcripts. Additionally, since changes in gene expression which occur only in one or two subregions may not be detected due to dilution by other, non-responsive subregions, isolating separate brain subregions using LMD could also greatly enhance the detection of subtle glucocorticoid-induced changes in gene expression.

Besides the application of more sensitive microarray detection algorithms, enhancing the detection of transcripts could also be achieved by using chemiluminescence-based microarray platforms, such as for instance the Applied Biosystems Expression Array System (https://products.appliedbiosystems.com). These platforms are believed to display higher detection ranges in comparison to the fluorescence-based platforms due to the fact that chemiluminescence enables the detection of only a few molecules (123).

At the level of statistical testing, enhancement of detection of glucocorticoid-responsive genes using microarrays may be achieved as well. So far, only *gene level* statistical tests, such as SAM, have been used to assess glucocorticoid-induced transcriptional changes. However, recent research in the field of microarray statistics has resulted in the development of *global tests* such as Gene Set Enrichment Analysis (GSEA) (38) and Parametric Analysis of Gene Set Enrichment (PAGE) (39). As described in section 2, in global testing pre-defined sets of genes, based on functional classification or biological pathways, instead of individual genes are tested and therefore these tests are believed to enhance the detection of subtle transcriptional changes.

Therefore, the reduction of neural tissue complexity by using LMD and application of more sophisticated microarray algorithms and / or platforms will most likely enhance the detection of low-abundant transcripts as well as the detection of subtle glucocorticoid-induced changes in gene transcription.

9. FUTURE PROSPECTS

The currently obtained results using large-scale profiling of glucocorticoid-responsive genes in neural tissue have revealed some remarkable features of the genomic response concerning receptor and context-specificity as well as time-dependency. Elaborating on these features may enhance our understanding on how glucocorticoid-receptors interact with the transcriptional machinery.

One of the main challenges is to establish a link between gene expression profiles and the corresponding DNA-binding sites to which glucocorticoid receptors bind after activation, thereby identifying primary target genes. These binding sites can be assessed by using chromatin immunoprecipitation (ChIP). Briefly, in ChIP, occupation of DNA sequences by certain proteins is quantified by first cross-linking proteins to the DNA and DNA sonication into small fragments which is followed by immunoprecipitation with the antibody against the protein of interest (for example GR or MR) and quantitative PCR with primers against the DNA sequence of interest (certain GREs for instance).

Interestingly, a modification of ChIP, called re-ChIP (124), is available which can be used to identify the coregulators and transrepression partners that bind to activated MRs and GRs on the DNA. Therefore, by using re-ChIP, new converging pathways can be elucidated. In re-ChIP, two immunoprecipitation steps are performed with two different antibodies, after which the DNA binding site is identified. Using different antibodies, binding of different combinations of glucocorticoid receptors and cofactors / transrepression partners to the DNA can thus be assessed.

Furthermore, large-scale identification of binding sites to which activated GRs bind throughout time would be of major interest in order to understand the observed dynamic pattern of the genomic response to glucocorticoids. In both the hippocampus and neuronal PC12 cells, one hour after GR-activation all the responsive genes were downregulated (59, 77), implying that *transrepression* is the prevailing pathway at this time point. Therefore, no direct binding of activated GRs to the DNA would be expected at 1 hour after GR-activation. On the other hand, at 3 hours after GR-activation both up- and downregulated genes were found, indicating that transcription may be modulated via the process of *transactivation*. Thus, assessing the binding sites to which activated GRs bind at this time point would allow the identification of primary target genes affected by transactivation as well as new GREs and nGREs to which activated GRs can bind.

Currently, there are two modifications of the ChIP-procedure available that allow large-scale identification of transcription factor binding sites. First, in ChIP-on-chip (125), the PCR step is omitted and instead the isolated DNA fragments are hybridized to a microarray which contains sequences for known transcription binding sites. Second, in Serial Analysis of Chromatin Occupancy (SACO) (126), both ChIP and SAGE are combined. The SACO procedure starts with precipitation of the transcription factor of interest that is

attached to the DNA, which is followed by generation and sequencing of tags derived from the immunoprecipitated DNA. Thus, in contrast to ChIP-on-chip, SACO allows the identification of new transcription factor binding sites, thereby constituting an *open approach*.

So far, many glucocorticoid-responsive genes have been profiled in neural tissue and therefore selecting potentially interesting candidate genes for functional follow-up studies is one of the major tasks to perform in order to understand the molecular mechanisms that underlie glucocorticoid effects on neuronal function. In this respect, application of siRNA-methodology to knock-down the transcription of candidate genes in specific model systems is of major interest. Furthermore, overexpression of candidate genes can be achieved by using viruses which allow transfection of differentiated, non-dividing neuronal cells. With respect to the hippocampus, using organotypic hippocampal slices would be of major interest to study the effects of inhibiting or enhancing the transcription of candidate genes. These slices can be cultured for several weeks and measurement of mRNA levels, protein levels and phenotypic analyses such as electrophysiology and morphology are relatively easily performed. Furthermore, at present an exciting development is the application of siRNA molecules into specific brain regions of intact animals, allowing *in vivo* measurements to be linked to decreased mRNA expression of candidate genes.

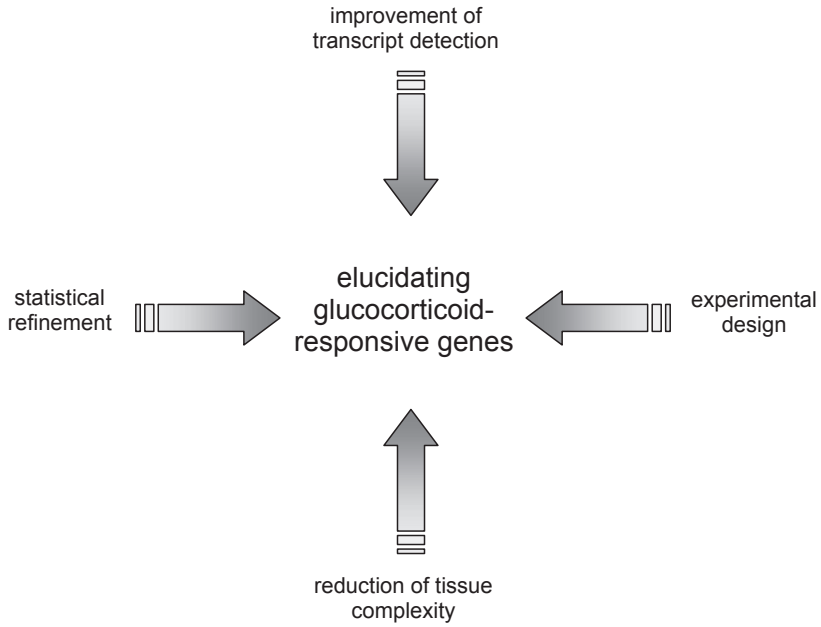


Figure 9. Elucidating glucocorticoid-responsive genes in neural tissue using microarrays can be optimized by 1) improvement of transcript detection using more sensitive detection algorithms or microarray platforms, 2) reduction of tissue complexity by more specifically isolating the brain subregion of interest using laser capture microdissection, 3) statistical refinement using more sensitive global tests and 4) applying experimental designs that take into account the environmental context, i.e. activation status of the brain region under investigation.

Finally, as mentioned previously, glucocorticoids actions are time and context dependent and therefore display an enormous diversity. This is a very important aspect to account for when assessing the role of a certain brain area, such as the hippocampus, in the stress response. Therefore, in future experiments concerning the elucidation of glucocorticoid-responsive genes, beside technical refinement as described in the previous section, refinement of the *experimental design* must be taken into account as well (Figure 9). This means that the currently used models in which neural gene expression is profiled after acute administration of glucocorticoids are replaced by models in which manipulation of glucocorticoid concentrations is combined with acute stressors that affect neural activation status. For instance, the hippocampus can be activated by application of certain (stressful) learning tasks which can be combined with adrenalectomy and / or glucocorticoid injections or antagonist administration. Hence, by measuring the effects of glucocorticoids on neural gene transcription under a certain stress-induced activation status, a more refined view on how glucocorticoids modulate different types of stress-responses in the brain will be obtained.

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Chapter 6

Towards an *in vitro* model to study the effects of GR-enhanced LIMK1 mRNA expression on the actin cytoskeleton

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ABSTRACT

Previously, using large-scale gene expression profiling performed in *ex vivo* hippocampal slices, LIMK1 was found to be upregulated 3 hours after GR-activation whereas calcineurin and profilin were downregulated at this time point. All three proteins accumulate in hippocampal dendritic spines and play different roles in dendritic spine morphology by differentially affecting actin cytoskeletal dynamics.

The aim of the current study was to focus on one of these genes, i.e. LIMK1, and to develop an *in vitro* model system to test the hypothesis that activated GRs can influence actin cytoskeletal dynamics via upregulation of LIMK1 expression levels.

For this purpose, NG108-15 cells were treated for 5 days with vehicle, GR-agonist dexamethasone, cAMP or a combination of cAMP and dexamethasone. Each day, neuritogenesis was measured as a marker for changes in actin cytoskeletal configuration and total RNA was isolated in order to measure the expression levels of LIMK1 mRNA. Exposure of these cells to vehicle or dexamethasone alone did not induce neuritogenesis whereas exposure to cAMP significantly increased neuritogenesis throughout time. The cAMP-mediated increase in neuritogenesis was further enhanced by combined exposure to dexamethasone. However, the increase in cAMP and combined cAMP / dexamethasone mediated neuritogenesis was not associated with (preceding) increases in LIMK1 mRNA levels.

Hence, in the currently used experimental design the cAMP-induced changes in actin cytoskeletal configuration which are enhanced by dexamethasone did not correlate to an increase in LIMK1 mRNA expression. Therefore, other experimental designs and / or model systems have to be considered in order to further explore the hypothesis that a GR-mediated increase in LIMK1 mRNA expression is involved in modifying the actin cytoskeleton.

INTRODUCTION

In a previously performed large-scale gene expression profiling experiment, several genes involved in actin cytoskeletal configuration were found to be regulated 3 hours after GR-activation in *ex vivo* hippocampal slices (1). These genes included *LIMK1* (upregulated), *calcineurin* (downregulated) and *profilin* (downregulated). Hence, the question was raised what the possible consequences are of GR-mediated transcriptional regulation of these genes for hippocampal neuronal function.

All three genes have been shown to accumulate at hippocampal dendritic spines, postsynaptic structures on which the majority of synapses are formed in the brain (2,3) (4,5,6,7). There have been strong indications that these genes play a role in dendritic spine morphology via regulation of actin cytoskeletal dynamics (3,4,8,9,10). Spine morphology is believed to be dependent on a stable pool of filamentous (F-) actin in the spine's core and a more dynamic pool of F-actin in the spine's periphery and is able to rapidly change in response to multiple physiological stimuli (9). Additionally, changes in hippocampal spine morphology have been associated with changes in synaptic transmission, i.e. long-term potentiation (LTP) (10).

First, *in vitro* studies have shown that LIMK1, which is activated by the Rac (RhoGTPase) pathway, phosphorylates cofilin, thereby inhibiting the depolymerization of F-actin into monomeric (G-) actin mediated by unphosphorylated cofilin (11,12,13). Furthermore, examination of LIMK1 knock-out mice revealed that LIMK1 is necessary for proper accumulation and distribution of F-actin in hippocampal dendritic spines, maintaining normal spine morphology and LTP (10). Second, profilin is known 1) to inhibit actin polymerization at the growing ends of the filaments and 2) to be involved in activity-dependent remodeling of synaptic structure in hippocampal neurons (4). Third, calcineurin has been suggested to play a role in destabilizing F-actin and hippocampal dendritic spine structure (3,8,9). However, so far the targets for calcineurin in dendritic spines have not been elucidated.

In the hippocampus, activated GRs have been shown to inhibit LTP in a delayed, genomic fashion. Hence, GR-mediated transcriptional regulation of LIMK1, calcineurin and profilin, possibly leading to changes in actin cytoskeletal dynamics and dendritic spine morphology, may be part of the underlying molecular mechanism.

Therefore, the overall aim of the current study was to focus on LIMK1 and to address the hypothesis that activated GRs can mediate changes in the actin cytoskeleton via transcriptional upregulation of LIMK1. In order to test this hypothesis, cholinergic NG108-15 cells (mouse neuroblastoma and rat glioma hybridoma cells) may constitute a very suitable model system. These cells originate from neural cells and have been shown to endogenously express LIMK1 protein (14). Furthermore, exposure to the GR-agonist dexamethasone results in the induction of neuritogenesis in these cells, resulting in the development

of long neurites (15). Since in general neuritogenesis is dependent on actin cytoskeletal dynamics, NG108-15 cells thus provide a convenient neural substrate to study the association between GR-activation and the actin cytoskeleton. Additionally, an interesting observation in these cells has been the fact that cAMP also induces neuritogenesis which is associated with an increase in LIMK1 protein levels (14). Furthermore, the cAMP-induced protein synthesis of LIMK1 is inhibited by activated calcineurin (14). Finally, combined

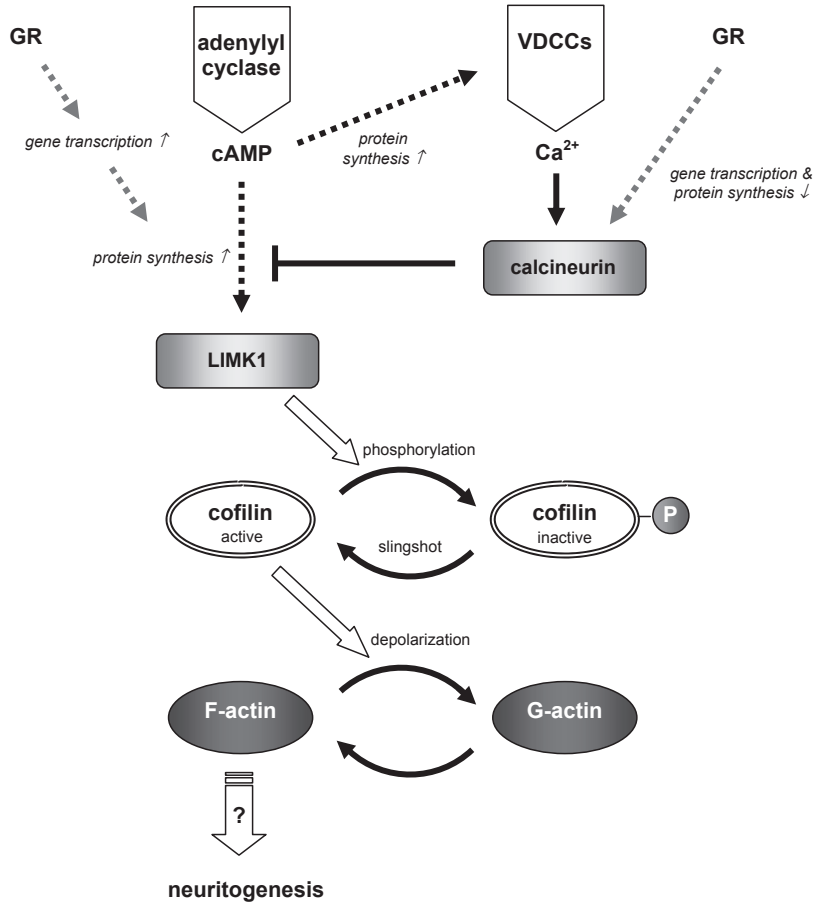

Figure 1. Schematic overview of the model on actin dynamics in NG108-15 cells postulated by Tojima et al 2003 combined with the hypothesis that activated GRs affect the actin cytoskeleton via transcriptional regulation of LIMK1 and calcineurin. This scheme has been adapted from (14). In this model, a rise in intracellular cAMP increases the protein synthesis of LIMK1 and VDCCs. Increased LIMK1 protein induces enhanced cofilin phosphorylation, resulting in a smaller amount of active cofilin. Subsequently, more F-actin accumulates in the cells, resulting in increased neuritogenesis. In contrast, enhanced expression of VDCCs allows the cells to accumulate more Ca2+ after membrane depolarization, leading to enhanced suppression of LIMK1 expression by calcineurin, resulting in the inhibition of neuritogenesis. The current hypothesis is that activated GRs may interfere with these mechanisms by transcriptionally upregulate LIMK1 and downregulate calcineurin, thereby enhancing the accumulation of F-actin and neuritogenesis. VDCC: voltage-dependent Ca2+ channel, P-cofilin: phosphorylated cofilin, F-actin: filamentous actin, G-actin: globular actin.

treatment with cAMP and dexamethasone has been shown to enhance the cholinergic phenotype of these cells (16,17). Figure 1 summarizes these findings in a molecular model postulated by (14), in combination with the currently proposed hypothesis.

Hence, the current goal was to first find an association between dexamethasone-induced neuritogenesis and increased LIMK1 transcription in NG108-15 cells. Therefore, the cells were exposed to vehicle, dexamethasone, cAMP or a combination of cAMP and dexamethasone for 5 days. Subsequently, each day, neuritogenesis was measured and total RNA was isolated for measuring LIMK1 mRNA expression levels.

MATERIALS AND METHODS

Cell culture and treatment

NG108-15 cells (passage 10) were cultured in DMEM medium (4500 mg / l glucose, Invitrogen Life Technologies, Carlsbad, CA, USA) substituted with 2% FBS (Invitrogen Life Technologies), 1 × hybridoma (HAT) mix (Invitrogen Life Technologies), penicillin (20 U / ml) and streptomycin (20 µg / ml) on 100 mm × 20 mm plastic culture dishes (Corning Incorporated, NY 14831, USA).

Prior to the experiment (on day 0), cells were transferred into 6-well plates (Corning Incorporated) in a density of 20.000 cells / well. For each experimental day twelve 6-wells plates were used which were equally divided over 4 experimental groups: vehicle, dexamethasone, cAMP and cAMP + dexamethasone. Hence, per day, each experimental group consisted of three 6-wells plates, i.e. 18 wells.

Three hours afterwards, the cells were exposed to medium containing vehicle (0.009% ethanol), dexamethasone (0.1 µM), cAMP (0.2 mM) or dexamethasone (0.1 µM) + cAMP (0.2 mM) and left to stand (37°C,5% CO_2) until the day that they were assigned for making photographs and isolating total RNA. The concentrations of cAMP and dexamethasone in the treatment medium were identical to those used by (16).

On day 1, the plates designated for this day were used for making photographs and isolating total RNA 24 hours after the treatment started. Per well, one picture was taken randomly in order to measure neuritogenesis and the average neuritogenesis value of 3 consecutive wells was used as one biological replicate, resulting in 6 biological replicates per experimental group (18 wells). Afterwards, for each experimental group, cells of the same 3 consecutive wells were pooled for RNA isolation, resulting in 6 total RNA samples per experimental group.

The same procedure was repeated on day 2, 3, 4 and 5, resulting in an n = 6 setup per experimental group per day.

On days 2 and 4 the treatment medium for all experimental groups was renewed for the days to follow.

Measuring neuritogenesis

In order to assess neuritogenesis pictures were taken from the cells using an inverted phase-contrast microscope (Zeiss / P.A.L.M. Laser Dissection Microscope, Carl Zeiss AG, Germany). The Neuron J Image Software (18) was used to 1) count the number of neurites per cell and 2) measure the length of the neurites in pixels and 3) measure the fraction of cells displaying neurites. Neurites were measured only if their length exceeded that of the cell body's diameter. Furthermore, for each branched neurite, only the longest branch was measured. For each experimental group approximately the same number of cells was counted per day.
Additionally, analysis of variance (ANOVA) and *post hoc* LSD tests were used to assess statistical significance.

Total RNA isolation and purification

On days 1, 3 and 5, total RNA was isolated 24 hours after the treatment medium was applied or renewed. On days 2 and 4, total RNA was isolated 3 hours after the treatment medium was applied or renewed in order to mimick the original setting in which LIMK1 mRNA transcription was found to be GR-responsive (i.e. 3 hours after GR-activation).

Per well, cells were homogenized in 330 µl TRIzol® (Invitrogen Life Technologies). Subsequently, cell homogenates were pooled for 3 consecutive wells and RNA isolation was performed according to the manufacturer's instructions.

Additionally, after isolation, total RNA purification was performed using the QIAGEN RNeasy® Mini Kit RNA Cleanup procedure (QIAGEN Inc. Valencia, CA, USA).

Real-time quantitative PCR for LIMK1 expression

LIMK1 expression levels were measured by using real-time qPCR on a DNA Engine Opticon® 2 Real-Time PCR Detection System (MJ Research, Inc., Waltham, Massachusetts, USA). All RNA samples were subjected to DNAse treatment with DNAseI (Invitrogen Life Technologies) according to the manufacturer's protocol. Subsequently, cDNA was synthesized using M-MuLV Reverse Transcriptase RNaseH$^-$ (Finnzymes Oy, Espoo, Finland) and random primers (150 ng; Invitrogen Life Technologies) in a total volume of 20µl. Per experimental sample, 100 ng of RNA was used for the cDNA-synthesis reaction using 150 ng random primers whereas standard curves were made with 5, 50, 100, 500 and 1000 ng RNA. In order to control for genomic contamination, RT$^-$ samples were generated. The PCR was performed in a total volume of 25 µl, consisting of 12.5 µl 2×PCR MasterMix with SYBR® Green I (qPCR™ Core Kit for SYBR® Green I, EUROGENTEC, Seraing, Belgium), 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 6.5 µl water and per primer pair either 5 µl cDNA-sample, RT$^-$ sample or water (no template control). The PCR conditions were as follows: 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C + 1 minute at 60°C (for

both annealing and extension). Afterwards, the temperature was gradually increased to 95°C in order to make dissociation curves.

Dissociation curves were used to control for the specificity of the reaction and genomic contamination whereas the standard curves were used to quantify the expression differences. Expression levels of the target genes were normalized to the expression levels of 18S ribosomal RNA since this transcript displays very high and stable expression levels.

Finally, analysis of variance (ANOVA) and *post hoc* LSD tests were used to assess statistical significance.

The following primers were used for LIMK1 (forward GAGAGAGGTCCAGTCCCATGTG, reverse GGCTTTGATCAGGAAATGAGATG) and 18S ribosomal RNA (forward CCCTGCCCTTTGTACACACC, reverse CGATCCGAGGGCCTCACTA).

RESULTS

Neuritogenesis

In this study three different parameters were used to assess the level of neuritogenesis, i.e. 1) the average number of neurites per cell, 2) the average neurite length and 3) the fraction of cells with neurites. In Figure 2 an example of NG108-15 cells in different experimental groups at day 5 is displayed.

Unexpectedly, the number of neurites per cell did not increase throughout time in the dexamethasone-treated group compared to the control group (Figure 3). In contrast, the number of neurites per cell significantly increased in the cAMP-treated groups. Strik-

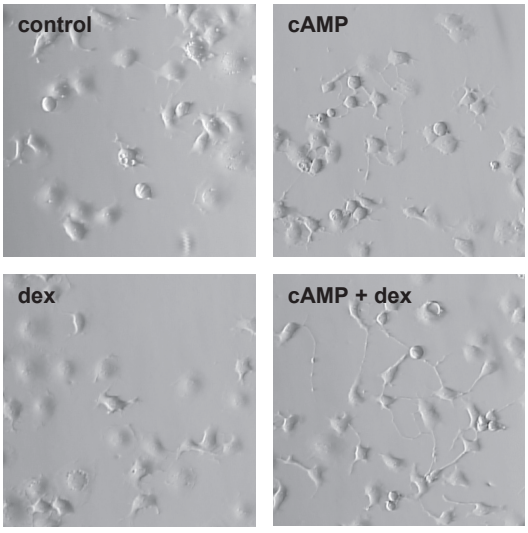

Figure 2. Images of cells on day 5 exposed to vehicle (control), cAMP, dexamethasone or cAMP + dexamethasone.

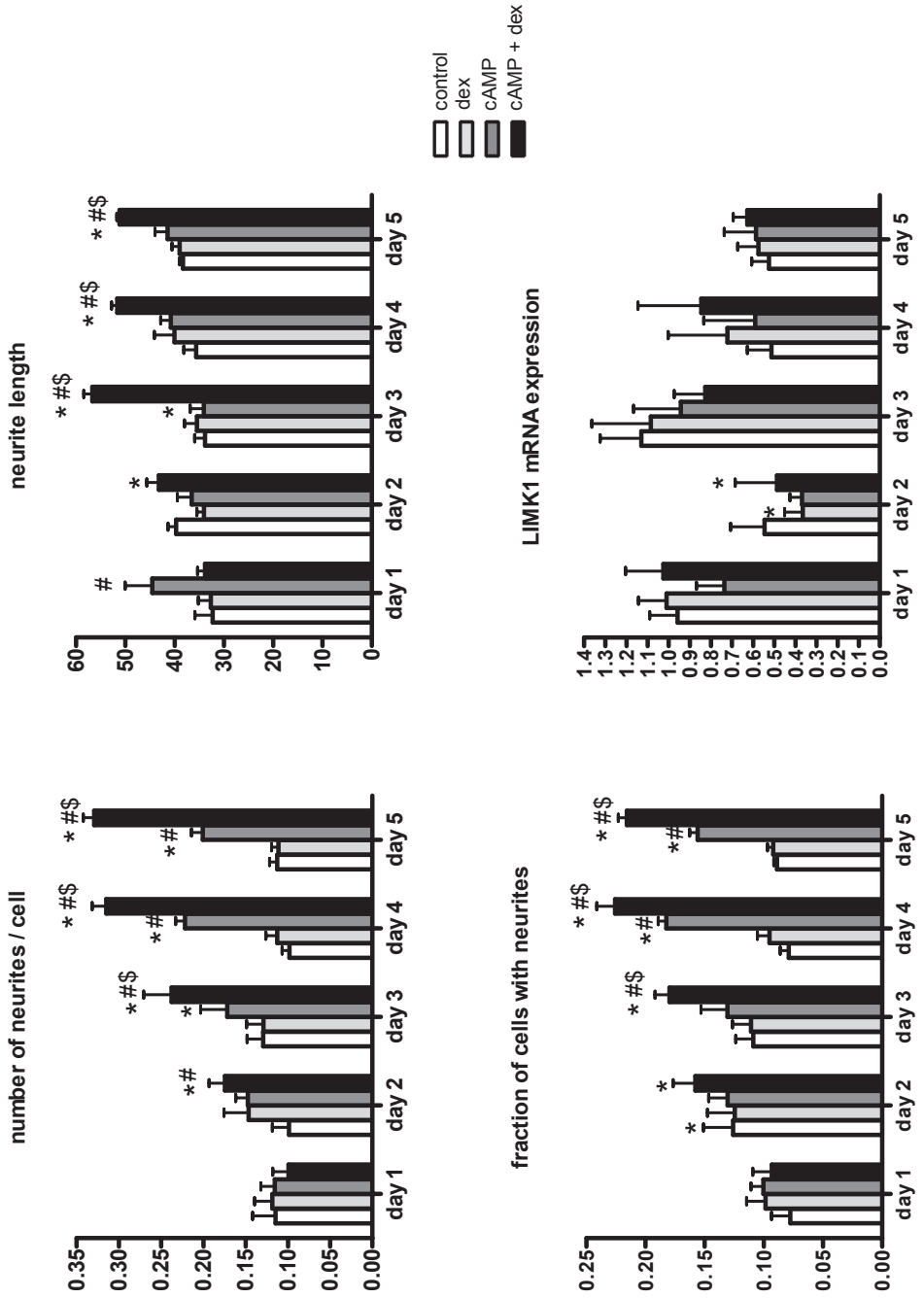

Figure 3. Quantification of 1) number of neurites per cell, 2) neurite length in pixels, 3) fraction of cells with neurites and 4) LIMK1 mRNA expression normalised to 18S rRNA. On days 1, 3 and 5 LIMK1 expression was measured 24 hours after treatment medium renewal on days 0, 2 and 4 respectively. On days 2 and 4, LIMK1 was measured 3 hours after treatment medium renewal (* $p < 0.05$ compared to day 1 counterpart; # $p < 0.05$ compared to control; $ $p < 0.05$ compared to cAMP).

ingly, the combined treatment of cAMP and dexamethasone even further increased the number of neurites per cell compared to the cAMP groups in an additive manner.

However, with respect to neurite length, a different pattern was observed (Figure 3). On day 1 the cAMP-group showed significantly higher neurite length compared to all the other groups whereas on day 2 this effect had disappeared. From day 3 till day 5 only the combined treatment group of cAMP and dexamethasone showed a significantly higher neurite length which seemed to decrease towards day 5.

Finally, the fraction of cells containing neurites more or less mimicked the pattern observed in number of neurites per cell, showing no effect of dexamethasone-treatment alone and an increasing effect by cAMP which was significantly enhanced by combined treatment with dexamethasone (Figure 3).

Hence, dexamethasone alone did not have any effect on the number of neurites per cell, length of neurites and fraction of cells with neurites whereas cAMP clearly increased the number of neurites per cell and the fraction of cells with neurites. Combined treatment of cAMP and dexamethasone enhanced these effects and markedly increased the length of the neurites as well, especially at day 3.

LIMK1 mRNA expression

Unfortunately, no clear effect of dexamethasone, cAMP or cAMP + dexamethasone on LIMK1 mRNA expression was found throughout time (Figure 3). The different treatment groups did not show any significant differences within the days. Additionally, some significant differences were obtained between the days for several groups although without any clear pattern. Furthermore, the standard errors of the mean were relatively large, indicating high levels of variation.

DISCUSSION

Several aspects of the currently performed experiment are in contrast to what has been shown before.

First, there seems to be a discrepancy with a report by Kim et al. (15) in which exposure to dexamethasone alone for 24 hours markedly increased both neurite length and number of neurites per cell. In this study, a much higher concentration of dexamethasone was used (2 μM) in comparison to the current study (0.1 μM). However, the different ligand concentrations probably do not underlie the different outcomes since a concentration of 0.1 μM dexamethasone has been shown to already fully occupy GRs (19). On the other hand, differences in cell culture conditions or passage number of the cells, resulting in a high variability among NG108-15 cells throughout different laboratories, may be the causative factor.

Second, previously it was found that increased LIMK1 protein levels are associated with increased neuritogenesis (14). In the current study however, no such association was found with LIMK1 mRNA. If mRNA predicts protein levels a number of issues may explain the discrepancy. Differences in cAMP-concentrations between the studies (0.2 mM versus 1 mM) may underlie this discrepancy as well as different culture conditions or cell passage numbers. Additionally, the increase in LIMK1 protein expression was observed after 7 days of cAMP-exposure (14), indicating that the exposure time used in the current study could have been too short to measure an increase in LIMK1 mRNA expression. Hence, the current data may suggest that the cAMP-mediated increase in neuritogenesis is independent of increased levels of LIMK1 mRNA during the first 5 days of treatment.

LIMK1 mRNA expression was measured on days 1, 3 and 5 twenty-four hours after the treatment medium was applied or renewed. On days 2 and 4 the expression was measured three hours after refreshing the treatment medium, thereby mimicking the original experimental design in which LIMK1 was found to be GR-responsive three hours after application of a corticosterone pulse (1). However, dexamethasone alone or in combination with cAMP did not show any clear effect on LIMK1 mRNA transcription on any of the days, either 3 or 24 hours after treatment medium renewals.

This result could indicate that either LIMK1 is not GR-responsive in NG108-15 cells or that the transcriptional response takes place at a different time point in NG108-15 cells compared to the hippocampal slice preparation. Additionally, since dexamethasone was constantly present in the treatment medium, chronic exposure to dexamethasone may affect downstream transcriptional mechanisms which counteract the initial GR-mediated increase in LIMK1 mRNA expression. Furthermore, the lack of a transcriptional response of LIMK1 after dexamethasone-treatment could be due to a ligand-specific transcriptional effect of corticosterone on LIMK1 mRNA transcription. Finally, the variability in LIMK1 gene expression was high, resulting in relatively large standard errors of the mean (SEM). An explanation for this variability could be the fact that LIMK1 mRNA expression was measured in the total set of cells present in the wells. In these wells, a large proportion of cells did not show neuritogenesis at all, as can been seen in Figure 3 (the largest fraction of cells with neurites being slightly less than 25% on day 4 with cAMP and dexamethasone). Hence, these undifferentiated cells may constitute a very variable population with a highly variable expression pattern of LIMK1, thereby interfering with the current gene expression measurements.

Interestingly, combined with dexamethasone, the effects of cAMP on the number of neurites per cell and the fraction of cells with neurites were enhanced. This is a very interesting observation since it is known that combined exposure to dexamethasone enhances the effects of cAMP on the cholinergic phenotype of NG108-15 cells as well (16,17). Therefore, also at the level of cellular morphology, dexamethasone and cAMP act in concert, enhancing the level of total cellular differentiation.

The effects of cAMP on neuritogenesis in NG108-15 cells are believed to occur via PKA-mediated phosphorylation of cAMP response element binding protein, i.e. CREB (20). With regard to the interaction between GR and the cAMP pathway, it has been shown that activated GRs can exert an inhibitory effect on the actions of CREB (21). However, there have also been reports in which cAMP-dependent protein kinase (PKA) has been shown to phosphorylate GR and to enhance GR-dependent transcription (22,23). Hence, administration of cAMP may result in both the activation of the cAMP pathway and, in parallel, the phosphorylation of GR. This phosphorylation of GR may constitute an extra regulatory step that is necessary for GR to exert an effect on neuritogenesis, providing an explanation for the fact that a combination of cAMP and dexamethasone enhances neuritogenesis whereas administration of dexamethasone alone does not induce neuritogenesis. In this respect, scanning the promoter sequences of genes involved in the configuration of the actin cytoskeleton for the presence of CREs and GREs would be of major interest to see whether the interaction between GRs and cAMP could be mediated via transcriptional regulation of these genes.

In the current experimental design, NG108-15 cells exposed to cAMP and/or dexamethasone did not show an increase in LIMK1 mRNA expression levels. At this point several options are available to follow-up on the presently conducted study. NG108-15 cells could be treated with corticosterone instead of dexamethasone and the analysis of LIMK1 mRNA expression could be extended over multiple time points. In addition, LIMK1 protein could be quantified. Furthermore, instead of continuous administration, GR-ligand could be applied in pulses, thereby mimicking the original experimental setup in hippocampal slices in which LIMK1 was found to be transcriptionally responsive to glucocorticoids.

As mentioned before, the discrepancies between the current and several previously performed studies may be caused by differences in passage number of the cells between different laboratories. Therefore, switching to another biological model system that is less dependent on the age of the substrate may be considered. Since the original gene expression study in which LIMK1, calcineurin and profilin were found to be GR-responsive was conducted in *ex vivo* hippocampal slices, organotypic slice cultures may constitute a more suitable model system (24). Organotypic tissue slices can be cultured for several weeks after their isolation and are easily manipulated by way of pharmacological treatments and / or siRNA transfections. Furthermore, exposure of hippocampal organotypic slices to a 30 nM corticosterone pulse has been shown to reduce the dendritic tree in the CA1 subregion in a delayed manner (Joëls, unpublished observation). This effect could be blocked by co-treatment with GR-antagonist RU486. Hence, with respect to actin dynamics, reduction of dendrites in these slices by acutely activated GRs is a very exciting observation which could be linked to GR-mediated transcriptional regulation of LIMK1, calcineurin and profilin.

In conclusion, unfortunately no association between dexamethasone-enhanced cAMP-induced neuritogenesis and LIMK1 mRNA expression was found in the current study performed in neural NG108-15 cells. However, interesting alternative experimental designs and biological model systems are available to further explore the hypothesis that increased mRNA expression of LIMK1 is involved in modifying the actin cytoskeleton.

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Chapter 7

General discussion

OUTLINE

1. Characteristics of the genomic response
2. Model systems
3. Functional gene classes
4. Candidate genes & hypothesis generation
5. Future prospects
6. Conclusions

The objectives of the studies described in the current thesis were 1) to determine which transcriptional changes underlie the effects of activated GRs on hippocampal cellular function throughout time, 2) to assess the extent to which this response is dependent on the cellular context, and 3) to establish a model in which the functional consequences of GR-mediated transcriptional regulation of a candidate gene, i.e. LIMK1, can be studied.

1. CHARACTERISTICS OF THE GENOMIC RESPONSE

Dynamics of the GR-mediated genomic response

In **chapter 2**, *ex vivo* hippocampal slices obtained from adrenalectomized rats that were replaced with low corticosterone pellets (occupying hippocampal MRs) were used to profile GR-mediated transcriptional responses. Acute activation of GRs by *ex vivo* exposure to a brief high concentration corticosterone pulse revealed a highly characteristic time-dependent genomic response that shifted from exclusively downregulated genes 1 hour after GR-activation towards upregulated genes 3 and 5 hours afterwards. This is a very interesting observation since so far only a limited number of genes have been identified which are repressed by binding of GRs to negative GREs. Therefore, the obtained temporal pattern may suggest that in hippocampal tissue transrepression via interactions with other transcription factors precedes transactivation at early time points.

The *in vivo* validation experiment described in **chapter 3** showed that 4 out of 5 selected genes which were responsive 1 hour after GR-activation in hippocampal slices displayed a similar transcriptional response in intact rats 1 hour after a single corticosterone injection. Hence, the influence from extra-hippocampal regions (which could also be steroid-responsive) and the peripheral effects of increasing concentrations of glucocorticoids, both of which are lacking in the hippocampal slice preparation, probably do not influence the GR-mediated transcriptional responses in the hippocampus to a great extent under non-stressed conditions. Therefore, the obtained transcriptional profile in **chapter 2** most likely mimics the transcriptional response to acutely activated GRs *in vivo*. In this respect it is interesting to note that also at the level of electrophysiology, *in vivo* activation of GRs by exposure to acute novelty stress increases calcium currents in a similar fashion to what has been observed in hippocampal slices in which GRs were activated by *ex vivo* exposure to a corticosterone pulse (1).

In order to determine how general the time-dependent genomic response to activated GRs is for neuronal tissue, an additional time curve of GR-mediated transcriptional responses was generated in a completely different neuronal substrate, i.e. NGF-differentiated PC12 cells, **in chapter 4**. Differentiated neuronal PC12 cells were shown to express endogenous GRs that translocate to the nucleus upon activation by a brief high concentration corticosterone pulse on top of low, basal concentrations of corticosterone

through a time window of 0.5 to 3 hours. Maximal nuclear retention was observed 1 hour afterwards. Strikingly, the corresponding time-dependent transcriptional profile was highly similar to the one obtained in *ex vivo* hippocampal slices, shifting from exclusively downregulated genes 1 hour after GR-activation towards almost exclusively upregulated genes 3 hours afterwards. Therefore, these data strongly suggested that also in neuronal PC12 cells transrepression may precede transactivation throughout time.

The large-scale transcriptional effects of acutely activated GRs throughout time have not been studied to a great extent yet. However, in liver, a similar time-dependent pattern of transcriptional responses was found after acute *in vivo* activation of GRs by injection of the synthetic GR-agonist methylprednisolone (2). Large-scale gene expression profiling of a time window of 15 minutes to 72 hours after GR-activation revealed the majority of genes to be downregulated at early time points (45 out of 50) whereas a robust delayed wave of upregulated genes followed 2 hours later. Since neuronal PC12 cells, hippocampal neurons and liver cells display completely different phenotypes, the observed similarity in the time-dependency of the GR-mediated transcriptional response may be indicative for a general mode of action of acutely activated GRs.

However, some caution with the interpretation of these data is required. The currently applied large-scale gene expression profiling methods are not suitable for measuring low abundant transcripts and therefore the expression patterns of these transcripts throughout time are unavailable. Additionally, GRs have been shown to very rapidly upregulate transcription of calcium channel subunits in *ex vivo* hippocampal slices (Y.Qin, unpublished observation). Furthermore, the rapid upregulation of the phenylethanolamine-N-methyltransferase (PNMT) gene after stress most likely is mediated via its upstream GRE (3) and therefore, upregulation at short time intervals after GR-activation is possible, although the prevailing pathway seems to be downregulation.

With regard to the rate at which the different steps involved in transactivation and transrepression proceed little information is currently available. In general, it seems that mRNA decay is not faster than mRNA elongation (4,5). Therefore, it is not likely that an imbalance in decay versus elongation is responsible for the observed wave of downregulation. Hence, the rapid transcriptional downregulation of genes at early time points could be explained by the fact that blocking the effects of other transcription factors during transrepression may occur more rapidly than transactivation in which recruitment of cofactors and chromatin remodeling is required (Figure 1) (6,7,8,9). However, there are indications that GRs can also directly interact with the general transcription machinery, modulating gene transcription without the need for cofactor recruitment and chromatin remodeling (6,10). Possibly, rapid upregulation of gene expression is mediated via this pathway (Figure 1). In this respect, expanding the currently profiled time window with shorter time intervals (< 1 hour) would therefore be of major interest.

Concerning the recruitment of steroid receptors to the DNA, an interesting observation has been the cyclic manner in which the estrogen receptor (ER) translocates to its response element after ligand-activation (11). Chromatin immunoprecipitation (ChIP) performed on an endogenously expressed ER-responsive gene in ERα-positive human breast cancer cells revealed that each cycle takes about 20 minutes and RNA polymerase is recruited to the DNA in the second cycle after the initial ligand activation, indicating that the first cycle is transcriptionally 'silent'. Such cyclic behaviour of steroid receptors would be interesting in light of the currently obtained time-dependent pattern of transcriptional regulation by activated GRs. However, one study, using ChIP, has shown that activated GRs remain loaded up to 2 hours after ligand activation on the MMTV-promoter

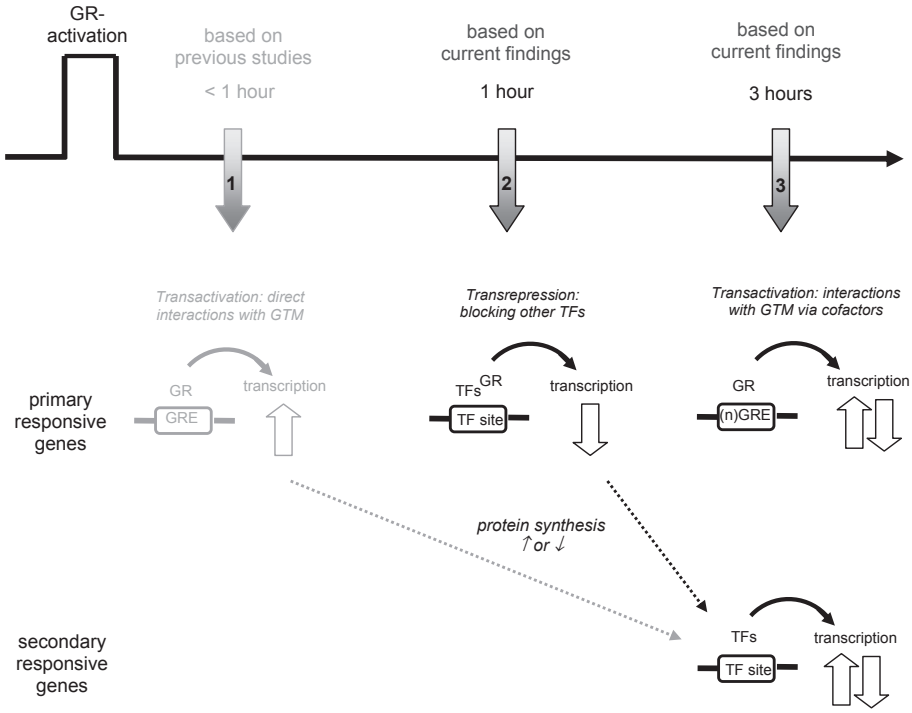

Figure 1. Hypothetical scheme of the molecular mode of action by which activated GRs modulate gene transcription throughout time. 1) Less than 1 hour after GR-activation by a short, 100 nM corticosterone pulse, rapid upregulation of genes takes place via direct interactions between GR and the general transcription machinery (GTM). Furthermore, genes that are downregulated via transrepression may also be present. 2) One hour after GR-activation, transrepression is the prevailing pathway via which GRs downregulate gene transcription by inhibiting other transcription factors (TFs). 3) Three hours afterwards, GRs modulate gene transcription by interacting with the GTM via recruitment of cofactors (coactivators and / or corepressors) resulting in up- (GRE) or downregulation (nGRE) of gene transcription. Additionally, at this time point secondary GR-responsive genes which are regulated by primary GR-responsive TFs or other transcription-modulating proteins are present in the expression profile. Furthermore, transrepression could also still take place at this time point.

(12) and therefore so far cyclic recruitment to the GRE has not been demonstrated yet for the GR. It should be taken into account however that in life cell imaging the receptors are very dynamic, showing rapid exchange between chromatin and the nucleoplasmic compartment on a time scale of seconds ('hit and run' model: 13,14).

Primary / downstream GR-responsive genes and transcription factor binding sites

Some of the genes that were regulated in two or more time points in chapters 2 and 4 changed their direction of expression throughout the time points in both hippocampal slices and neuronal PC12 cells. Such a 'biphasic' effect on gene expression has been previously demonstrated by Fujikawa et al. (15) for growth hormone receptor, GR and MR in the hippocampus after exposure to an acute stressor. This biphasic regulation of gene expression could possibly be the result of a downstream transcriptional mechanism that follows the initial primary response to activated GRs.

Therefore, in **chapter 4**, protein synthesis inhibitor cycloheximide was used to differentiate between primary and downstream transcriptional responses in neuronal PC12 cells, showing that 5 out of 5 selected genes from the 1 hour time point displayed primary transcriptional responses. This indicated that for these 5 genes activated GRs either inhibit transcription via binding to nGREs (transactivation) or via inhibition of other transcription factors (transrepression). Since no consensus nGRE-sites (6) were found upstream of the promoter regions, transrepression presumably is the mediating pathway. This was strengthened by the observation that for two of these primary responsive genes clearly conserved AP1-sites and CREB-sites were found upstream of the promoters. However, for the remaining 3 primary responsive genes no conserved transcription factor binding sites were found, indicating that other, unknown interacting pathways could be involved.

Furthermore, 4 out of 4 selected genes from the 3 hours time point displayed a downstream transcriptional response. This could indicate that at this time point only downstream-responsive genes are profiled. However, in the hippocampal slice data set metallothionein was found to be upregulated 3 hours after GR-activation and since this gene contains two functionally active GREs (16), implying that it is a primary responsive gene, this would mean that the 3 hours time point is constituted by both primary and downstream responsive genes (Figure 1). Hence, in this respect it would be interesting to also profile primary and downstream-responsive genes for instance 2 hours after GR-activation. There has been one study in literature in which GRs that were transfected into human osteosarcoma cells were activated by 2 hours of continuous exposure to dexamethasone and cycloheximide, resulting in the identification of both upregulated and downregulated primary responsive genes (17). However, due to the differences in biological substrates and glucocorticoid-treatment exposures, i.e. brief pulse of corticosterone versus continuous dexamethasone exposure, the results obtained in this study

cannot be easily extrapolated to the time-dependent expression profiles which were obtained in **chapters 2 and 4**.

Interestingly, two of the selected genes which were originally responsive in both time points revealed a primary transcriptional response 1 hour after GR-activation and a downstream transcriptional response 3 hours afterwards, supporting the view that the biphasic regulation of gene expression is the result of downstream transcriptional responses following primary transcriptional responses.

However, due to the small selection of tested genes the obtained results concerning primary and downstream transcriptional responses cannot be simply generalized. An alternative explanation for GR-mediated biphasic transcriptional responses for instance could be a transition from early transrepression towards transactivation later in time, both of which are primary transcriptional events. Therefore, expanding the cycloheximide experiment performed in **chapter 4** by enlarging the time window and using large-scale gene expression profiling could provide more insight into the dynamics of primary and downstream-mediated transcriptional changes.

Context-specificity of the GR-mediated genomic response

The nature of the genomic response to glucocorticoids not only depends on which receptor (MR or GR) is activated but also on the cell type and activation status of the cells, i.e. the *cellular context* and *environmental context* respectively.

Previous experiments have shown that activated GRs mediate different transcriptional effects in different cell-types. A well known example is the expression of the CRH gene which is inhibited by activated GRs in hypothalamic cells and enhanced in other cell-types (18).

The extent to which activated GRs act in different contexts was assessed in **chapter 4** by comparing the expression profile obtained in hippocampal slices with the profile obtained in neuronal PC12 cells, showing very little overlap. In contrast, the overlap between the hippocampal SAGE experiment (19) and hippocampal slice experiment was considerably larger. Hence, this response seems to be highly dependent on the cellular context.

Differences in cellular context may be the result of differences in the availability of cofactors in different cell and tissue types or even between brain subregions. *In vitro* experiments showed that different splice variants of SRCs were recruited to promoters containing different numbers of GREs (20,21). This illustrates the fact that the genomic response to glucocorticoids is dependent on the interaction between promoter composition, i.e. GRE composition, and available cofactors. As a consequence, different genes with different kinds of promoters display different glucocorticoid-induced transcriptional responses depending on the availability of cofactors.

Additionally, different cell-types express different sets of genes (22) and therefore the repertoire of available transrepression partners may also be different, resulting in the transrepression of different sets of genes.

Finally, GREs can be flanked by other accessory transcription factor binding sites, thereby constituting glucocorticoid-responsive units (GRUs) (6) in which the glucocorticoid-mediated transcriptional response is also dependent on the availability of these accessory transcription factors. These transcriptional responses can therefore remain limited to the cells / tissues which express the accessory transcription factors (6,23).

Amplitude of GR-induced transcriptional changes

In both hippocampal slices and neuronal PC12 cells, the amplitudes of the transcriptional responses to activated GRs were very moderate, showing fold changes of less than two. With regard to the hippocampus, these moderate GR-induced transcriptional changes are in agreement with previous studies, performed both with large-scale expression profiling techniques (19) and single gene *in situ* hybridizations (24-26). Apparently, small changes in gene expression underlie the effects of glucocorticoids on hippocampal neuronal functioning. This was nicely illustrated in a study in which the expression levels of several α1 calcium channel subunits were correlated to the stress-induced changes in calcium current amplitudes (1). Transcriptional changes in each α1 calcium channel subunit separately showed to be moderate, whereas there was a clear overall effect on calcium current amplitude.

In contrast, with regard to neuronal PC12 cells, there have been several studies in which glucocorticoid treatment induced larger gene expression fold changes (27,28,29,30). However, a direct comparison is complicated by the fact that in these studies much longer exposure times (1 – 48 hours) to higher concentrations (1 μM) of very potent synthetic glucocorticoids (dexamethasone) have been used. In contrast to these more robust treatment regimes, in the current design a relatively mild, physiological treatment was applied mimicking the peak in the ultradian secretion pattern of corticosterone.

From a methodological point of view, the gene expression profiling studies performed in **chapters 2 and 4** proved to be very successful in detecting subtle transcriptional changes. This was achieved by using a paired experimental design which greatly enhanced statistical power. Implementation of such a design in future studies therefore would be clearly beneficial in improving the detection of subtle transcriptional changes.

2. MODEL SYSTEMS

Ex vivo hippocampal slices

Several hippocampal properties such as neuroexcitability, synaptic transmission (long-term potentiation; LTP) and energy metabolism are affected by glucocorticoids in a delayed, genomic fashion (31,32,33,34). Therefore, in **chapter 2**, the *ex vivo hippocampal slice preparation* was used to assess potentially underlying GR-mediated transcriptional changes throughout a defined time window. This *ex vivo* preparation is a well established model to study the effects of glucocorticoids on hippocampal neuronal function (35,36) and therefore changes in gene transcription can directly be correlated to changes in neuronal function.

However, since in the hippocampal slice preparation input from extra-hippocampal regions (which could also be steroid-responsive) as well as the peripheral effects of increasing concentrations of corticosterone is lost, the data set may not reflect the full extent of GR-mediated effects on hippocampal gene expression. Therefore, in **chapter 3** a small selection of potentially interesting candidate genes involved in hippocampal neurotransmission were tested in an *in vivo* setting. Strikingly, the transcriptional responses for 4 out of 5 selected genes were confirmed *in vivo* 1 hour after GR-activation by a corticosterone injection. Thus, the hippocampal slice preparation constitutes a valid model to assess glucocorticoid effects on hippocampal gene expression since the influence of projections from extra-hippocampal brain structures does not seem to affect the GR-mediated transcriptional response to a large extent after a glucocorticoid injection *in vivo,* at least if tested with the 5 selected genes.

The currently obtained transcriptional profile in hippocampal slices contained a number of positive controls that were already known to be regulated by glucocorticoids, such as for instance metallothionein and glutathione peroxidase as well as several genes found in the hippocampal SAGE experiment (19). Furthermore, a number of interesting new candidate genes was obtained that could possibly be involved in the effects glucocorticoids exert on hippocampal metabolism and neurotransmission. Several of these candidate genes will be discussed further on.

Neuronal PC12 cells

Long-term exposure of PC12 cells to nerve growth factor (NGF) results in a profound change in phenotype of the cells in which proliferation is stopped, long neurites are generated and electrical excitability is gained (37). Furthermore, these cells synthesize and store large quantities of the neurotransmitters noradrenalin and dopamine, thereby displaying a catecholaminergic, sympathetic-neuron like phenotype. However, in contrast to sympathetic neurons, differentiated PC12 cells contain more dopamine than noradrenalin (38).

In **chapter 4**, differentiated PC12 cells were used to assess the transcriptional responses mediated by acutely activated GRs in order to elucidate general characteristics of the neuronal genomic response. This resulted in the identification of 160 GR-responsive genes distributed over 2 time points. Since this is the first study to assess glucocorticoid-responsive genes in neuronal PC12 cells in a large-scale manner, the obtained genes constitute potentially very interesting targets which can be used to gain more insight into the effects of acutely activated GRs on sympathetic, dopaminergic and / or noradrenergic neuronal function.

The interaction between glucocorticoids and the sympathetic nervous system has been well studied (39,40,41). However, in these studies, glucocorticoids have been administered in a chronic fashion which is in sharp contrast to the acute administration of a corticosterone pulse as performed in **chapter 4**. This pulse mimics the peak in the ultradian secretion pattern of corticosterone and therefore the currently obtained PC12 data set provides more interesting candidate genes with regard to the acute effects of glucocorticoids on dopaminergic and noradrenergic neuronal systems. Several of these candidate genes will be discussed further on.

3. FUNCTIONAL GENE CLASSES

In order to study the molecular mechanisms underlying the effects of activated GRs on neural function, in the current thesis large-scale gene expression profiling studies were performed to assess GR-responsive genes. Strikingly, more than 200 GR-responsive genes were found which could be grouped into many different functional classes, ranging from energy metabolism to vesicle dynamics (Figure 2). This finding is in concordance with previous reports on GR-responsive genes (2,19,42), demonstrating *pleiotropic* effects of glucocorticoids on several different transcriptomes.

Interestingly, in both hippocampal slices and neuronal PC12 cells similar functional gene categories were affected by glucocorticoids throughout different time intervals after GR-activation. Figure 3 displays the distribution of these functional gene categories throughout time in hippocampal slices and neuronal PC12 cells. Comparison of hippocampal slices and neuronal PC12 cells shows that activated GRs display a large effect on genes involved in signal transduction and gene transcription whereas in neuronal PC12 cells activated GRs affect genes involved in protein synthesis, energy metabolism and the cytoskeleton. In contrast, the other functional categories show a more or less similar distribution pattern between slices and PC12 cells. This is a very interesting observation since it shows that differences in cellular context determine the distribution of several functional gene categories that are transcriptionally affected by activated GRs. Therefore, from these results the view emerges that in the hippocampus signal transduction and

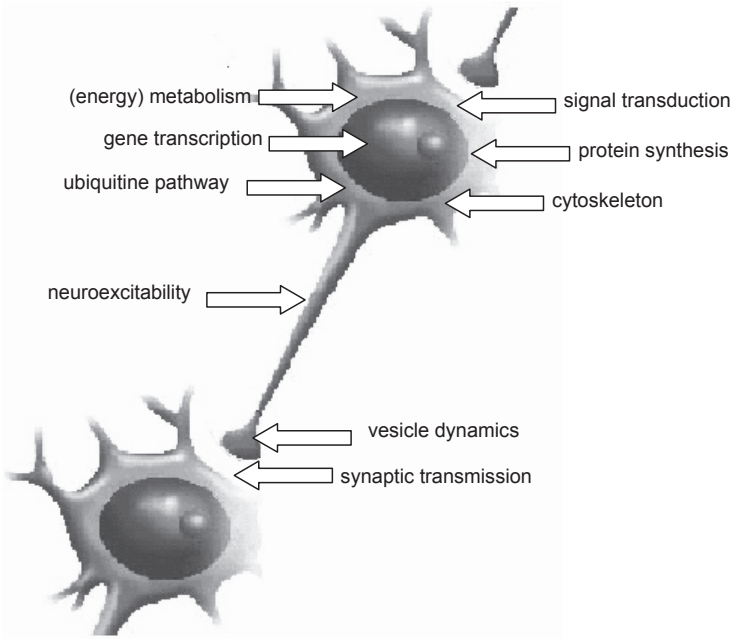

Figure 2. Pleiotropic effects of activated GR on gene expression in hippocampal slices and neuronal PC12 cells. Glucocorticoids affect transcription of diverse functional processes in a coordinate manner.

gene transcription are among the main targets of activated GRs whereas in neuronal (catecholaminergic) PC12 cells protein synthesis, energy metabolism and the cytoskeleton are more prominently affected.

Additionally, in each neural substrate, the distribution of functional categories differs to some extent at different time points as well. Figure 3 shows that in hippocampal slices the signal transduction group increases at the 3 hours time point whereas the oxidative stress metabolism and ubiquitine pathway categories decrease. In neuronal PC12 cells on the other hand, protein synthesis and energy metabolism increase at the 3 hours time point whereas oxidative stress involved genes and cytoskeleton-related genes disappear at this time point. Thus, also a time-dependent effect on the proportion of a number of functional categories that are affected by activated GRs is present in both neural substrates.

However, some caution is required with the interpretation of these functional distributions since the genes were placed into the functional categories based on their first Gene Ontology component. Therefore, many genes can be placed into multiple functional categories which overlap with each other, hampering complete discrimination between different categories.

Since so many different functional gene groups are affected by activated GRs, providing an answer to the question what the consequences are for neuronal function there-

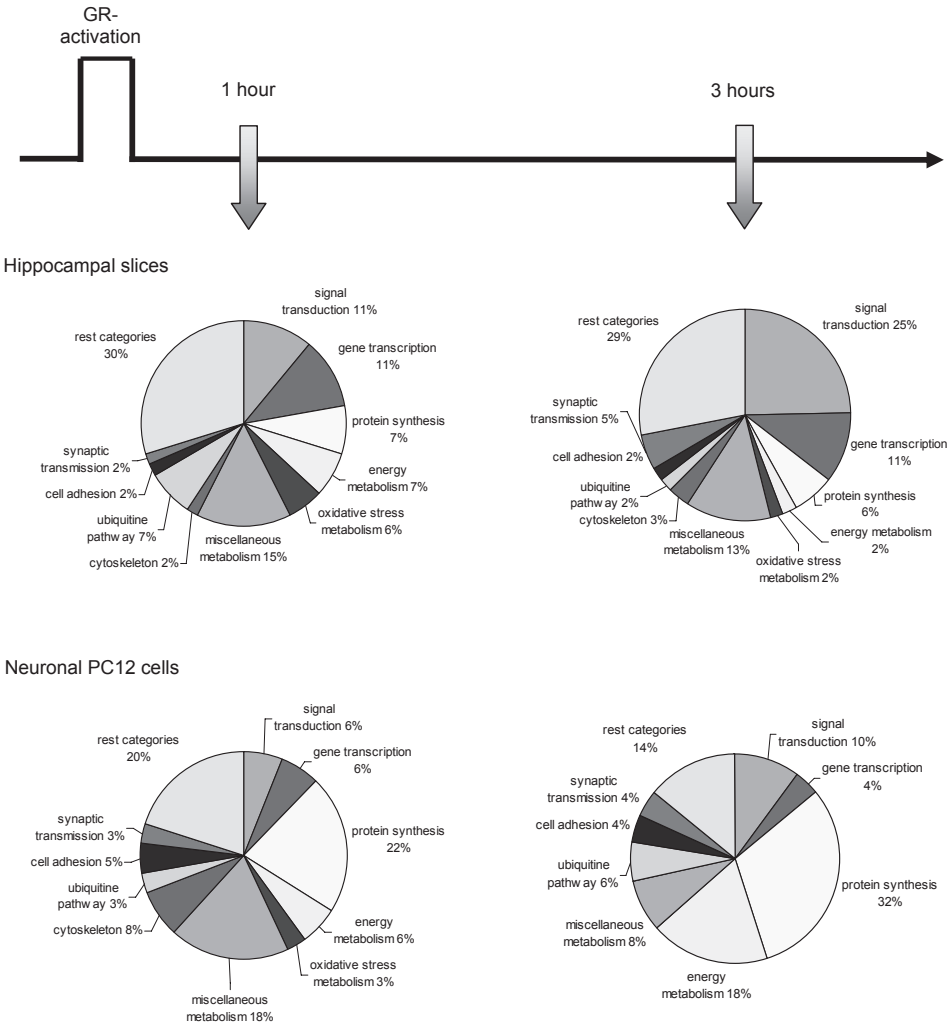

Figure 3. Functional gene categories regulated by activated GRs throughout time in hippocampal slices (upper panel) and neuronal PC12 cells (lower panel). Per time point the percentages indicate the proportion of responsive genes in the particular functional gene category obtained from the total number of genes that could be annotated with functional gene groups.

fore remains difficult. However, when considering the sequence of events that occur throughout the general stress-response some speculation is possible. In response to a stressor, limbic brain areas such as the hippocampus are rapidly activated to mediate the cognitive and emotional processing of the stressor. Subsequently, glucocorticoids are secreted in a delayed manner and are responsible for modulating and fine tuning of the initial stress-response, thereby facilitating learning and memory formation (43,44). In addition, the different gene groups that are affected by glucocorticoids can be considered to be functionally interconnected with each other. For instance, signal transduction

between neurons logically depends on cell adhesion and synaptic strength whereas neurotransmissive capacity of neurons is dependent on the amount of energy and protein that is available. Hence, the transcriptional regulation of many different, although interconnected functional gene groups may constitute a highly balanced and effective way by which the initial neuronal stress-response in the brain is coordinated, adjusted and fine tuned by activated GRs.

4. CANDIDATE GENES & HYPOTHESIS GENERATION

Large-scale gene expression studies can be regarded as hypothesis generating studies since they allow new hypotheses to be generated as to how transcriptional regulation of the glucocorticoid-responsive genes may underlie the glucocorticoid-mediated effects on neural function. At this point, the obtained data sets concerning GR-responsive genes in the hippocampus and neuronal PC12 cells provide many potentially interesting candidate genes that may be used to formulate new hypotheses on how glucocorticoids affect neural function.

Glucocorticoid effects on energy metabolism

Energy metabolism is one of the functional gene categories that are very often present among the glucocorticoid-dependent gene expression profiles. Currently, a large number of genes involved in energy metabolism were among the GR-responsive genes in both the hippocampal and PC12 data sets. In the hippocampus, *lactate dehydrogenase B*, which plays a role in glycolysis, and *leptin*, which is involved in energy reserve metabolism were part of this functional gene group. Strikingly, 1 and 3 hours after GR-activation all the genes involved in energy metabolism, except leptin, were transcriptionally downregulated. This is a very interesting finding since the inhibitory effects that glucocorticoids exert on neuronal glucose utilization (45,46,47) could very well occur due to GR-mediated transcriptional downregulation of these genes. On the other hand, glucocorticoid-mediated upregulation of leptin could be part of a mechanism to restore energy balance in hippocampal cells after a stress response has been elicited.

In neuronal PC12 cells, the situation seems to be more complex. Here, all the energy metabolism related genes are downregulated 1 hour and upregulated 3 hours after GR-activation. *Lactate dehydrogenase A* (involved in glycolysis) is among the downregulated genes and the transcriptional effect on this gene could possibly lead to a decreased glucose utilization 1 hour after glucocorticoid administration. However, the situation seems to be reversed 3 hours after GR-activation since at this time point *aldolase A* and *phosphoglycerate kinase 1*, both of which are also involved in glycolysis, are upregulated.

Thus, a clear difference in the effects of glucocorticoids on energy metabolism is seen between hippocampal cells and neuronal PC12 cells on the transcriptional level throughout time.

Rapid glucocorticoid effects on hippocampal neurotransmission in vivo

The currently generated expression profiles of GR-responsive genes in the hippocampus contain a large set of genes that can affect neurotransmission at multiple cellular levels. Interestingly, several of these genes were found to be responsive 1 hour after GR-activation, thereby possibly exerting a rapid genomic effect on hippocampal neurotransmission. In order to validate a small selection of these genes *in vivo*, their expression levels were measured 1 hour after a glucocorticoid injection into intact animals in **chapter 3**, resulting in the confirmation of transcriptional downregulation of *MAO-A, casein kinase 2, MR and potassium channel Kv3.2* at this time point.

First, the GR-mediated transcriptional response of MAO-A is a very interesting observation since it indicates that activated GRs can manipulate the availability of the neurotransmitters serotonin, dopamine and noradrenalin in the hippocampal synaptic clefts by reducing the catabolism of these neurotransmitters. Second, the transcriptional response of casein kinase 2 may be involved in the glucocorticoid-mediated effects on hippocampal LTP, which will be discussed in the following section. Third, downregulation of the MR could impair neurotransmission since this receptor is known for its ability to facilitate neuroexcitability. Finally, the transcriptional response of voltage-gated potassium channel Kv3.2 could affect action potential propagation along the axons of hippocampal neurons, influencing hippocampal output. Since the changes in transcription were small it is likely that the membrane potential is less hyperpolarized, facilitating activation of sodium channels and allowing the neuron to fire more easily.

Thus, transcriptional downregulation of most of these genes, though not all, would favor a suppression of local neurotransmission 1 hour after GR-activation *in vivo*. However, some caution with the functional interpretation is required since these genes were selected from an expression profile containing over 200 genes. Therefore, the overall picture on how activated GRs affect hippocampal neurotransmission *in vivo* still remains complex.

Glucocorticoids and long-term potentiation (LTP)

As mentioned previously, long-term potentiation (LTP) is one of the main hippocampal properties that are affected by glucocorticoids. In the CA1 subregion, activated GRs inhibit LTP in a delayed manner (34), thereby reducing synaptic transmission and thus neurotransmission. In the current thesis several interesting candidate genes were found in chapter 2 that could possibly be involved in mediating these effects.

One of these genes, i.e. *casein kinase 2*, may directly be involved in the glucocorticoid-mediated effects on hippocampal LTP. This gene was found to be downregulated by

activated GRs in **chapter 2** and was validated *in vivo* in **chapter 3**. Casein kinase 2 has been shown to enhance NMDA-receptor function in hippocampal neurons, being part of the downstream signaling cascade (48). Furthermore, enhanced activity of casein kinase 2 was found to be correlated with the induction of NMDA-receptor mediated LTP (49). Since 1) activated GRs inhibit NMDA-receptor mediated LTP and 2) there is no evidence for transcriptional regulation of NMDA-receptor mRNA (chapter 2) (50), downregulation of casein kinase 2 one hour after GR-activation could in part underlie this effect at an early time point.

Additionally, there have been strong indications that AMPA receptor subunit trafficking is affected by activated GRs and this may occur for NMDA receptors as well (O. Wiegert, unpublished observation). Interestingly, the current data set contains one gene involved in intracellular protein transport, *beta-chain clathrin-associated protein complex AP-2*, that could possibly be mediating AMPA or NMDA-subunit trafficking. However, an exact hypothesis on how GR-mediated regulation of this gene may underlie receptor-trafficking is difficult to formulate and therefore remains open for speculation.

Glucocorticoids and the actin cytoskeleton

Interestingly, glucocorticoids affect the transcription of several genes involved in the configuration of the *actin* cytoskeleton in both hippocampal cells and neuronal PC12 cells. Transcriptional modulation of *LIMK1, calcineurin, profilin, beta actin and LIM/SH3 protein* by activated GRs may thus affect cellular properties associated with the actin cytoskeleton such as dendritic spine morphology.

Concerning the hippocampus, LIMK1, calcineurin and profilin were found to be GR-responsive 3 hours after GR-activation. These genes are known to play a role in the morphology of hippocampal dendritic spines. The morphology of hippocampal spines is connected to hippocampal LTP and can change very rapidly in response to different stimuli (51,52).

Using LIMK1 knock-out mice it was shown that LIMK1 is necessary for proper accumulation and distribution of F-actin in hippocampal dendritic spines and for normal hippocampal dendritic spine morphology (51). Furthermore, calcineurin has been shown to inhibit LIMK1 protein expression (53) and to be involved in destabilization of F-actin and hippocampal dendritic spines (54). Profilin is known to inhibit the polymerization of actin filaments at the growing ends (55,56) and seems to be involved in (activity-dependent) remodeling of hippocampal synaptic structure (57). Interestingly, profilin was also found to be differentially expressed in the hippocampus when comparing two genetically selected mouse strains (i.e. short attack latency and long attack latency mice) that differ in aggressive behaviour, HPA-axis reactivity and morphology of mossy fiber terminal fields (58,59). Hence, transcriptional regulation of LIMK1 (upregulation), calcineurin (downregulation) and profilin (downregulation) 3 hours after GR-activation could possibly cause a

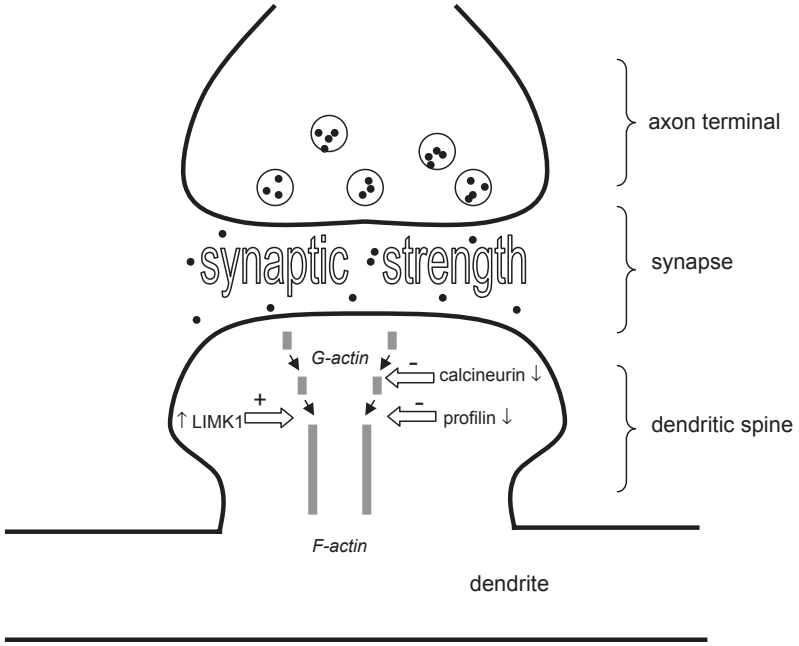

Figure 4. Regulators of hippocampal dendritic spine morphology which are transcriptionally affected by activated GRs. LIMK1 enhances the formation of filamentous (F-) actin from actin monomers (G-actin) whereas calcineurin and profilin destabilize F-actin. ↑; upregulated by activated GRs. ↓; downregulated by activated GRs. LTP; long-term potentiation.

shift in F-actin dynamics and the subsequent alterations in dendritic spine morphology could alter hippocampal LTP (Figure 4).

In LIMK1 knock-out mice, the aberrant spine morphology was associated with alterations in hippocampal LTP, showing a reduction in 5 or 10 Hz induced LTP and an induction of 50 or 100 Hz induced LTP (51). From this observation one could deduce that a GR-induced increase in LIMK1 would enhance 5 or 10 Hz induced LTP which would be in contrast to the observed inhibiting effect of activated GRs on 10 Hz induced LTP (50). However, complete and constant absence of LIMK1 in knock-out mice most likely does not equal the exact opposite situation of moderately and transiently increased LIMK1 expression levels in normal animals. Additionally, spine morphology is also dependent on activity of calcineurin and profilin, both of which are regulated by activated GRs as well as was shown in the current studies.

Unfortunately, the functional study performed in **chapter 6** did not result in an *in vitro* model to test the hypothesis that activated GRs via transcriptional regulation of LIMK1 can modulate the actin cytoskeleton. However, as discussed in **chapter 6**, other model systems remain open for exploration such as for instance the use of *ex vivo* organotypic slices or *in vivo* models.

Interestingly, also in neuronal (catecholaminergic) PC12 cells, the actin cytoskeleton seems to be a target for glucocorticoids. Activated GRs transcriptionally downregulate beta actin, which is part of the actin structural component, and LIM&SH3 protein, which plays a role in actin cytoskeletal organization. Thus similar to hippocampal cells, also in catecholaminergic cells glucocorticoids may affect synaptic plasticity by affecting the actin cytoskeleton.

Glucocorticoids and vesicle dynamics

In brain, glucocorticoids have been shown to affect catecholaminergic neuronal function. As discussed in **chapter 4**, acute exposure to corticosterone or dexamethasone increases dopamine release in the nucleus accumbens and enhances noradrenalin signaling in brain (60,61,62).

The currently obtained expression profile in neuronal catecholaminergic PC12 cells contains several interesting target genes that may be involved in glucocorticoid effects on catecholaminergic signaling. These genes affect attachment of (neurotransmitter containing) vesicles to the synaptic membrane as well as recycling of these vesicles. Interesting candidate genes that may be involved include *synaptosomal associated protein 25 (SNAP-25)*, which is involved in exocytosis (63) and several proteins involved in Rab-mediated endocytosis *(Rab 1 acceptor, Rab 15 and Rab 7)*. Enhancement of neurotransmitter secretion in general requires both exocytosis and endocytosis-mediated recycling of the cell membrane and hence GR-mediated transcriptional regulation of these genes may contribute to the secretion process of neurotransmitters in these cells. Hence, this may be a good example which illustrates the previously mentioned balanced and effective way by which activated GRs affect cellular function.

Glucocorticoid effects on protein synthesis in neuronal PC12 cells

One of the most striking observations in neuronal PC12 cells that were made in the current thesis is the large effect activated GRs exert on the transcription of genes involved in protein synthesis. One hour after GR-activation 22% of the downregulated genes (Figure 3) is comprised of genes involved in protein synthesis whereas 3 hours after GR-activation this percentage increases to 32%. At this time point all the genes involved in protein synthesis are upregulated. Hence, a massive shift from downregulation of protein synthesis towards upregulation takes place throughout time in these cells, with several genes overlapping between the two time points. When considering the effects glucocorticoids exert on catecholaminergic neurotransmitter release, the regulation of protein synthesis could constitute a mechanism by which first synthesis of proteins unrelated to neurotransmitter synthesis and release is downregulated. This is then followed in time by a re-allocation of the requisites for protein synthesis towards the production of enzymes necessary for neurotransmitter production. This way, neurotransmitter production is en-

hanced and in combination with the glucocorticoid-mediated transcriptional effects on exocytosis and endocytosis described previously, neurotransmitter release from these cells may be sustained over a longer period of time.

Candidate genes: current status

In summary, the currently obtained hippocampal and neuronal PC12 expression profiles of GR-responsive genes contains a large number of very interesting candidate genes with regard to energy metabolism, neurotransmission, hippocampal LTP, the actin cytoskeleton, vesicle-mediated neurotransmitter release and protein synthesis. The present task therefore is to design and perform functional follow-up studies with these genes in suitable model systems. Several options that are available for performing these functional studies will be discussed in the next section. However, it should also be taken into consideration that the expression profiles are not complete yet due to the fact that the current expression profiling techniques are unable to detect transcription of low abundant genes (such as for instance the 5HT1A-receptor). Therefore, although at present many glucocorticoid-responsive genes have been profiled the generation of new hypotheses may be hampered by the absence of these low abundant genes.

5. FUTURE PROSPECTS

In the current thesis several remarkable features of the genomic response to glucocorticoids in neuronal tissue have been observed regarding time-dependency and context-specificity. Elaborating on these features may deepen our current understanding on how glucocorticoids interact with the neuronal transcriptome at the level of molecular mechanism.

In order to obtain a more conclusive view on the molecular mechanisms that underlie the transcriptional responses after GR-activation throughout time, assessing transcription factor binding sites upstream of the GR-responsive genes would be of major interest. This way, primary target genes and transrepression partners that interact with activated GRs can be identified, thereby providing a more in depth view of the (potentially) different modes of action of activated GRs at the different time points. Furthermore, novel GREs and interacting pathways (other than AP1, NFκB and CREB) may be discovered. An interesting example of recently identified GR-interacting cofactors is the class of FOXO transcription factors. These FOXO cofactors seem to cooperate with activated GRs to induce the expression of the pyruvate dehydrogenase kinase-4 gene by binding to insulin response sequences that are in close proximity to the GRE-site that is present in the promoter region of this gene (64).

Therefore, a large-scale bioinformatics approach would be of use in which the promoter regions of all the GR-responsive genes are screened for commonly shared sequences, either known transcription factor binding sites or, using an open search approach, new sequences. Subsequently, binding of activated GRs and transrepression partners to these elements should be assessed. For this purpose, chromatin immunoprecipitation (ChIP) can be used. Briefly, in ChIP, occupation of DNA sequences by certain proteins is quantified by first cross-linking proteins to the DNA and DNA sonication into small fragments. This is followed by immunoprecipitation with the antibody against the protein of interest (GR or transrepression partner) and quantitative PCR with primers against the DNA sequence of interest (GREs). Moreover, a variant on ChIP, called re-ChIP (11) is available which can be used to assess the binding of cofactors and / or transrepression partners to the activated GRs on the DNA. Using re-ChIP, two immunoprecipitation steps are performed with different antibodies prior to the PCR step. In this way, binding of two proteins (in a protein complex) to the DNA can be measured.

An interesting alternative approach to identify the DNA elements to which activated GRs bind throughout time could be the use of genome wide ChIP-procedures such as ChIP-on-chip (65) and Serial Analysis of Chromatin Occupancy (SACO) (66). First, in ChIP-on-chip, the PCR step is omitted from the ChIP-procedure and instead the isolated DNA fragments are hybridized to a microarray which contains sequences for known transcription binding sites. Second, in SACO, a combination between ChIP and SAGE is performed, starting with precipitation of the transcription factor of interest that is attached to the DNA. Subsequently, binding site-specific tags are generated which are derived from the immunoprecipitated DNA. These tags are then sequenced, resulting in the identification of the DNA binding sites. Thus, in contrast to ChIP-on-chip, SACO allows the identification of new transcription factor binding sites, thereby constituting an *open approach*.

Furthermore, in order to elaborate on the context-specificity of the genomic response, using these ChIP and / or SACO procedures would be of major help as well. Application of these techniques could result in the identification of different transrepression partners and coregulators for activated GRs in 1) different neuronal cell types and 2) cells with different activation statuses.

In order to assess whether 1 hour after GR-activation the genes are transcriptionally downregulated via transrepression, the currently performed large-scale gene expression profiling experiment in hippocampal slices could be repeated with slices obtained from transgenic GR-dimerization defective mice (67). When performed in the presence of cycloheximide, primary genes which are transcriptionally mediated by GRs without the need for direct binding of GR-dimers to the DNA, as occurs in transrepression, can be identified.

With respect to the effects of glucocorticoids on cellular functioning, in the current thesis many different glucocorticoid-responsive genes have been profiled in both hip-

pocampal and neuronal PC12 cells. Several new hypotheses have been generated with respect to the control glucocorticoids exert on energy metabolism, neurotransmission, hippocampal LTP, the actin cytoskeleton, vesicle-mediated neurotransmitter release and protein synthesis. Hence, as mentioned previously, the challenge now is to test these hypotheses and to find causal connections between glucocorticoid-mediated changes in the expression of candidate genes and the neural phenotype. In order to perform these follow-up studies, several suitable model systems and techniques for gene expression manipulation can be considered.

Regarding the GR-responsive genes found in hippocampus, organotypic hippocampal slices constitute a very interesting substrate to perform functional studies with. As discussed in **chapter 6**, these slices can be cultured for several weeks and measurement of mRNA levels, protein levels and even phenotypic analysis such as electrophysiology and morphology are relatively easily performed. Although organotypic slices do show some re-wiring of the neuronal network after they are generated, they still have the advantage of containing such a neuronal network which to a certain extent is similar to that of the normal hippocampus. Several techniques are available to manipulate gene expression levels in these slices, such as siRNA and viral transfection tools. Using siRNA, transcription of the gene of interest is decreased by a specific siRNA molecule, leading to loss of function. Furthermore, the use of viruses allows transfection of differentiated (non-dividing) neuronal cells and hence siRNA molecules and / or overexpressing gene plasmids can be efficiently transfected into these cells. Since organotypic slices can directly be treated, they provide a very convenient model system for functional follow-up studies.

The latter also holds true for neuronal PC12 cells. As discussed previously, these cells mimic catecholaminergic neurons and can therefore be used as a model system to study the interactions between glucocorticoids and catecholaminergic function. Manipulation of these cells is relatively easily performed and therefore they are very suited for conducting functional experiments with candidate genes. However, logically more caution is needed to make appropriate extrapolations of this *in vitro* model system to the *in vivo* situation of catecholaminergic neurons in the brain.

As mentioned before, glucocorticoids play a modulatory role in the organism's stress response and thus operate in a certain stress-induced 'activation status'. The activation status determines the availability of different transcription cofactors and transrepression partners that can interact with activated GRs. Therefore, when assessing the role of brain regions in the stress response and elucidating the stress-induced pathways with which activated GRs interact, the experimental design should combine the manipulation of glucocorticoid concentrations with the exposure to acute stressors, affecting neuronal activation status. This could be achieved for example by applying certain (stressful) learning tasks which activate the hippocampus in combination with adrenalectomy and / or glucocorticoid injections or antagonist administration.

Finally, the currently available gene expression profiling techniques are not suited for detecting low abundant transcripts and therefore the transcriptional responses of these genes to glucocorticoids still remain largely unknown. Thus, the overall view on which genes are responsive to glucocorticoids is not complete yet. Therefore, enhancing the detection capacity of the profiling techniques as described in **chapter 5** is one of the future challenges in order to be able to improve the selection of candidate genes for functional follow-up studies.

6. CONCLUSIONS

From the studies performed in the current thesis, the following conclusions can be drawn:

1) Acute activation of GRs results in a very characteristic time-dependent genomic response in *ex vivo* hippocampal slices and neuronal PC12 cells. This time-dependent profile suggests that transrepression is the prevailing pathway 1 hour and transactivation is the prevailing pathway 3 hours after GR-activation.

2) The cellular context in which glucocorticoids operate conveys an enormous diversity in GR-mediated transcriptional effects to be detected by large-scale gene expression profiling.

3) Glucocorticoids exert pleiotropic effects on gene expression, thereby affecting diverse functional processes in a coordinate manner. These processes include signal transduction, gene transcription, protein synthesis, energy metabolism, cytoskeletal-controlled cellular properties, ubiquitine pathway, cellular adhesion and synaptic transmission.

4) In both *ex vivo* hippocampal slices and neuronal PC12 cells, many new candidate genes were found that could potentially underlie (part of) the effects glucocorticoids mediate on hippocampal and catecholaminergic neuronal function. These include genes involved in energy metabolism *(lactase A and B, leptin, aldolase A and phosphoglycerate kinase 1)*, neurotransmission *(MAO-A, potassium channel Kv3.2 and the MR)*, hippocampal LTP *(casein kinase 2 and beta-chain clathrin-associated protein complex AP-2)*, the configuration of the actin cytoskeleton *(beta actin, LIMK1, LIM/SH3 protein, calcineurin and profilin)*, vesicle-mediated neurotransmitter release *(SNAP25 and several Rab proteins)* and protein synthesis *(a large number of ribosomal proteins)*.

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Summary

One of the physiological systems that play a major role in mediating the organism's stress response is the hypothalamic-pituitary-adrenal (HPA) axis. Upon exposure to a stressor the HPA-axis triggers the adrenals to release glucocorticoids into the bloodstream.

Many different organs and tissues are affected by glucocorticoids, among which several brain areas. Especially the hippocampus, a brain structure involved in learning and memory formation, is affected by glucocorticoids. Here, two receptors are involved in transduction of the glucocorticoid signal; the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). In comparison to the GR, the MR has a 10-fold higher affinity for glucocorticoids, resulting in predominant MR-occupation under basal glucocorticoid concentrations and GR-occupation under rising glucocorticoid concentrations during the circadian rhythm and / or exposure to stress.

Both receptors are ligand-inducible transcription factors and therefore many of the effects glucocorticoids exert are the result of changes in gene expression. After ligand-binding, MR and GR translocate to the cell nucleus and via two mechanisms, i.e. *transactivation* and *transrepression*, regulate gene expression. In transactivation, ligand bound receptors form homodimers that bind to glucocorticoid-responsive elements (GREs) on the DNA. Subsequently, cofactors can be recruited to the receptors and via interactions with the general transcription machinery gene transcription can be enhanced (positive GREs) or repressed (negative GREs). In transrepression, monomeric receptors inhibit gene expression by binding to other transcription factors like NFκB, AP1 and CREB, thereby inhibiting their transcriptional effects.

Acutely activated GRs affect several hippocampal properties within several hours including inhibition of long-term potentiation (LTP), enhancement of calcium influx, inhibition of glucose uptake and an increase in the neuron's response to serotonin. Since these changes develop in a delayed genomic fashion, applying a *genomics approach* is of major interest in order to determine the underlying molecular mechanisms. Using this approach, expression levels of thousands of genes are measured in a single experiment, thereby aiming to elucidate which genes are regulated by activated GRs and to generate new hypotheses as to how transcriptional regulation of selected candidate genes may underlie the effects of activated GRs on hippocampal neuronal function.

Hence, the central theme of the current thesis was to gain more insight into the transcriptional changes that underlie the effects of acutely activated GRs on hippocampal neuronal function. Furthermore, issues concerning context-specificity and dynamics of the genomic response to acutely activated GRs were addressed as well.

Previously, Serial Analysis of Gene Expression (SAGE) was used to assess GR-induced transcriptional changes in the hippocampus *in vivo* against an occupied MR-background and over 100 genes were found to be responsive 3 hours after GR-activation. However, since changes in mRNA levels do not necessarily have to coincide with the effects on neuronal function, a large-scale profile of GR-responsive genes was generated in an expanded time window of 1 to 5 hours after GR-activation..

In **chapter 2**, for this purpose experiments are described with *ex vivo* hippocampal slices obtained from animals in which the MRs were occupied and an acute, 20 minute 100 nM corticosterone pulse was applied to activate the GRs. Strikingly, a highly characteristic pattern of transcriptional changes was observed throughout time, shifting from exclusively downregulated genes 1 hour after GR-activation to both up and downregulated genes 3 hours afterwards. Five hours after GR-activation the response was almost back to baseline. This time-dependent pattern suggested that the fast genomic effects of glucocorticoids may be realized via transrepression, preceding a later wave of transactivation. A similar pattern of transcriptional regulation by activated GRs has previously been found in liver, showing the majority of genes to be downregulated at early time points.

In order to assess the reliability of the *ex vivo* hippocampal slice model, five functionally interesting genes that were found to be responsive 1 hour after GR-activation were validated *in vivo* in **chapter 3**. For this purpose, rats were injected with a high concentration of corticosterone and 1 hour afterwards gene expression was measured using *in situ* hybridizations. Four out of five genes showed similar GR-induced transcriptional changes as observed in the slices. This is a very interesting observation since it demonstrates the reliability of the hippocampal slice data set and it shows that apparently projections to the hippocampus, which could also be GR-responsive, do not exert a large effect on the GR-mediated transcriptional response under the current conditions. Furthermore, every hippocampal subregion measured showed downregulation or a trend towards downregulation after GR-activation.

In **chapter 4** the aim was to elucidate how general the obtained time-dependent GR-induced transcriptional response in hippocampal slices was for neuronal tissue, the effects of acutely activated GRs on transcription were studied in a completely different neuronal substrate, i.e. neuronal catecholaminergic PC12 cells. Endogenous GRs were activated by exposure to a 20 minute 100 nM corticosterone pulse on top of a tonic 10 nM corticosterone background which resulted in a strikingly similar time-dependent pattern of transcriptional changes as observed in hippocampal slices, shifting from exclusively

downregulated genes 1 hour after GR-activation to both up and downregulated genes 3 hours afterwards. Furthermore, by repeating the experiment in the presence of the protein synthesis blocker cycloheximide and by assessing putative upstream promoter regions for transcription factor binding sites, more indications were found that trans-repression may be the prevailing pathway at early time points. In this respect, using a combination of bioinformatics and large-scale chromatin immunoprecipitation (ChIP) / Serial Analysis of Chromatin Occupancy (SACO) procedures would be of major interest to test this hypothesis in a follow-up study.

With regard to the context-specificity of glucocorticoid-mediated effects it has previously been shown that the same receptor can exert completely opposite effects in different types of neurons. In order to assess the extent to which GR acts context- specific on the transcriptome, the data sets obtained from the previously performed hippocampal SAGE, hippocampal slice and neuronal PC12 experiments were compared with each other, showing very little overlap between the hippocampal and PC12 data sets. Hence, acutely activated GRs elicit a highly context-specific genomic response in different neuronal substrates.

In **chapter 5** systematic overview concerning the currently obtained results using large-scale expression profiling of GR-responsive genes in relation to what is known about the mechanisms by which glucocorticoid receptors affect gene transcription was provided. The functional implications of GR-mediated transcriptional regulation of several candidate genes for neural function were discussed as well. Additionally, some methodological shortcomings and possibilities for technical refinement of the genomics procedure were addressed.

Large-scale gene expression profiling can be regarded as a hypothesis generating approach in an attempt to explain how transcriptional regulation of the glucocorticoid-responsive genes may underlie the glucocorticoid-mediated effects on neural function. Strikingly, in the current thesis more than 200 GR-responsive genes were described in **chapters 2 and 4** which could be grouped into many different functional classes, demonstrating the pleiotropic effects glucocorticoids exert on gene transcription. Interestingly, in both hippocampal slices and neuronal PC12 cells similar functional gene categories were affected by glucocorticoids throughout different time intervals after GR-activation. However, the distribution of genes over these functional gene classes differed between hippocampal and neuronal PC12 cells as well as between the different time points, showing both time and cellular context dependency.

Besides several genes that were already known to be glucocorticoid-responsive, many new interesting candidate genes were found in these expression profiles that may be used to formulate new hypotheses on how glucocorticoids affect neural function. The functional implications of transcriptional regulation of these genes are discussed throughout the different chapters. From the current thesis it becomes clear that glucocorticoids af-

fects the transcription of genes involved in energy metabolism *(lactate dehydrogenase A and B, leptin, aldolase A and phosphoglycerate kinase 1)*, neurotransmission *(MAO-A, potassium channel Kv3.2 and the MR)*, hippocampal LTP *(casein kinase 2 and beta-chain clathrin-associated protein complex AP-2)*, actin cytoskeleton-controlled cellular properties *(beta actin, LIMK1, LIM/SH3 protein, calcineurin and profilin)* and vesicle-mediated neurotransmitter release *(SNAP25 and several Rab proteins)*. Several hypotheses were postulated in this thesis and at present the challenge is to test these newly generated hypotheses, thereby elucidating causal connections between glucocorticoid-mediated changes in the expression of these candidate genes and the neural phenotype.

In order to perform these follow-up studies, several suitable model systems and techniques for gene expression manipulation can be considered. In **chapter 6**, the hypothesis was tested that GR-mediated transcriptional regulation of LIMK1 could be involved in rearrangements of the actin cytoskeleton. Changes in actin cytoskeletal conformation have been related in vivo to changes in dendritic spine morphology and hippocampal LTP. Hence, in this chapter an attempt was made to demonstrate that activated GRs via transcriptional regulation of LIMK1 can modify actin cytoskeletal dynamics. Neuronal NG108-15 cells were chosen as a model system since these neuronal cells express LIMK1 (as well as another GR-responsive actin-modifying protein, i.e. calcineurin) and they seem to respond to the specific GR-agonist dexamethasone by extending their neurites. Unfortunately, no clear relation between GR-activation, actin dynamics and LIMK1 mRNA expression was obtained, indicating that the NG108-15 cells did not constitute a proper model for studying the functional consequence of transcriptional regulation of this gene. However, as discussed in this chapter, hippocampal organotypic slices could constitute a highly interesting alternative model system to further investigate this hypothesis.

Finally, in **chapter 7** all the findings in the current thesis are discussed. The major conclusions are that

1) acute activation of GRs results in a very characteristic time-dependent genomic response in *ex vivo* hippocampal slices and neuronal PC12 cells which suggests that transrepression is the prevailing pathway 1 hour after GR-activation followed by a wave of predominant transactivation, resulting in both up and downregulated genes, at 3 hours.

2) the cellular context in which glucocorticoids operate conveys an enormous diversity in GR-mediated transcriptional effects to be detected by large-scale gene expression profiling.

3) glucocorticoids exert pleiotropic effects on gene expression, thereby affecting diverse functional processes in a coordinate manner.

4) in both *ex vivo* hippocampal slices and neuronal PC12 cells, many new candidate genes were found that could potentially underlie (part of) the effects glucocorticoids mediate on hippocampal and catecholaminergic neuronal function.

Samenvatting

Eén van de fysiologische systemen die een hoofdrol spelen tijdens de stress-respons is de hypothalamus-hypofyse-bijnier (HHB) as (Engels: hypothalamic-pituitary-adrenal (HPA) axis). Deze HHB-as wordt geactiveerd wanneer een organisme wordt blootgesteld aan een stressor met als gevolg dat de bijnieren glucocorticoïden afgeven aan de bloedbaan.

Verschillende organen en weefsels worden beïnvloed door glucocorticoïden, waaronder een aantal hersengebieden zoals de hippocampus. De hippocampus is onder andere betrokken bij leren en geheugenvorming en brengt twee receptoren voor glucocorticoïden tot expressie: de mineralocorticoïd receptor (MR) en de glucocorticoïd receptor (GR). In vergelijking met de GR heeft de MR een tienvoudig hogere affiniteit voor de natuurlijk voorkomende glucocorticoïden: cortisol (mens) en corticosteron (rat). Door dit verschil in affiniteit is bij basale concentraties van cortisol en corticosteron de MR voornamelijk bezet, terwijl bij hogere concentraties, tijdens het circadiane ritme of gedurende een periode van stress, de GR additioneel wordt bezet.

Beide receptoren behoren tot de groep van de ligand-geactiveerde transcriptiefactoren en veel van de effecten die door glucocorticoïden worden bewerkstelligd zijn het gevolg van veranderingen in genexpressie. Nadat de receptoren zijn gebonden door het ligand vindt er translocatie plaats naar de nucleus alwaar genexpressie via 2 mechanismen kan worden beïnvloed, te weten *transactivatie* door beide receptoren en *transrepressie* dat exclusief door GR geregeld wordt. Tijdens transactivatie vormen de ligand-gebonden receptoren homodimeren die kunnen binden aan zogenaamde glucocorticoïd-responsieve elementen (GREs) die zich bevinden in het promotergebied van sommige responsieve genen. Vervolgens worden co-factoren gebonden door de receptoren en via interacties met de algemene transcriptie machinerie kan transcriptie van de betreffende GRE-bevattende genen worden gestimuleerd (positieve GREs) of geremd (negatieve GREs). Tijdens transrepressie binden enkelvoudige receptoren aan andere transcriptiefactoren zoals NFκB, AP1 en CREB, wat resulteert in remming van door deze factoren aangestuurde gentranscriptie.

Acute activatie van GR kan binnen enkele uren verschillende hippocampale functies beïnvloeden. Zo onderdrukken ze bijvoorbeeld long-term potentiation (LTP), versterken ze de influx van calcium, remmen ze het glucose verbruik en versterken ze de respons van

neuronen op serotonine. Aangezien deze veranderingen plaatsvinden in een tijdsbestek van enkele uren, kan worden verondersteld dat ze afhankelijk zijn van veranderingen in genexpressie. Met het gebruik van een zogenaamde *genomics* aanpak kan derhalve veel inzicht worden verkregen in de onderliggende moleculaire mechanismen. Met deze aanpak worden de expressie-niveaus van duizenden genen in een enkel experiment gemeten (m.b.v. large-scale gene expression profiling technieken) met als doel vast te stellen welke genen transcriptioneel worden gereguleerd door de ligand-geactiveerde GR. Doorgaans leidt dit tot interessante nieuwe hypothesen betreffende de rol van het nieuw geïdentificeerde gen in de regulatie van hippocampus functie.

Het centrale thema van dit proefschrift was derhalve om meer inzicht te verkrijgen in de transcriptionele veranderingen die mogelijkerwijs de effecten van GR op de hippocampus kunnen verklaren. Daarnaast werden zaken als context-specificiteit en dynamiek van de genomische respons op acuut geactiveerde GR bekeken.

In een eerdere studie is de Serial Analysis of Gene Expression (SAGE) techniek gebruikt om GR-responsieve genen te vinden *in vivo* in de hippocampus in ratten waarin de MR van tevoren reeds bezet was met ligand. In deze studie werden 3 uur na GR-activatie meer dan 100 GR-responsieve genen gevonden. Echter, aangezien veranderingen in mRNA niveaus niet noodzakelijkerwijs gelijk hoeven te lopen met de effecten van GR op neuronale functie wordt in **hoofdstuk 2** een grootschalig expressie profiel van GR-responsieve genen gegenereerd in een tijdsbestek van 1 tot 5 uur na GR-activatie. Hiervoor werden *ex vivo* hippocampale plakken gebruikt die waren verkregen uit dieren waarin de MR vooraf reeds bezet was. Vervolgens werden de GR geactiveerd door deze plakken bloot te stellen aan een 20 minuten durende 100 nM corticosteron puls. Dit resulteerde in een opmerkelijk karakteristiek patroon van transcriptionele veranderingen waarbij 1 uur na GR-activatie de transcriptie van alle responsieve genen geremd werd, terwijl er 3 uur na GR-activatie sprake was van zowel stimulatie als remming van transcriptie van responsieve genen.

Vijf uur na GR-activatie was de genomische respons zo goed als voorbij. Dit tijdsafhankelijke patroon van transcriptionele regulatie suggereerde dat de snelle genomische effecten van glucocorticoïden tot stand komen via transrepressie en dat pas later in de tijd transactivatie plaatsvindt. Een vergelijkbaar tijdsafhankelijk genomisch patroon werd al eerder gevonden in de lever, met voornamelijk remming van gentranscriptie in de vroege tijdspunten.

Om de betrouwbaarheid van het *ex vivo* hippocampale plak model te bepalen werden er 5 mogelijk interessante responsieve genen uit het 1-uurs tijdspunt geselecteerd en vervolgens gevalideerd *in vivo* in **hoofdstuk 3**. Hiervoor werden ratten geïnjecteerd met een hoge dosis corticosteron waarbij 1 uur later de genexpressie van de geselecteerde genen werd gemeten met behulp van *in situ* hybridizaties. Vier van de vijf genen lieten *in vivo* een zelfde transcriptionele verandering zien als in de hippocampale plakken. Dit

validatie-experiment benadrukte derhalve de betrouwbaarheid van het *ex vivo* hippocampale plak model en liet tevens zien dat in ieder geval voor de 4 gemeten genen de respons niet afhankelijk is van (mogelijkerwijs ook GR-responsieve) neuronale projecties naar de hippocampus. Een andere interessante observatie was dat in iedere hippocampale subregio die gemeten werd, transcriptionele remming of een trend richting transcriptionele remming werd gevonden en dat deze veranderingen werden geblokkeerd door de GR-antagonist RU486.

Om te bepalen hoe algemeen het gevonden tijdsafhankelijke patroon van GR-gemedieerde transcriptie in hippocampale plakken is voor neuronaal weefsel wordt in **hoofdstuk 4** een nieuw experiment uitgevoerd waarin neuronale catecholaminerge PC12 cellen werden gebruikt om de effecten van acuut geactiveerde GR op genexpressie te bepalen. De GR in deze cellen werd geactiveerd met behulp van een 20 minuten durende 100 nM corticosteron puls bovenop een 10 nM corticosteron achtergrond. Dit resulteerde in een zelfde tijdsafhankelijk patroon van transcriptionele veranderingen zoals eerder was gevonden in hippocampale plakken, met 1 uur na GR-activatie alleen remming van transcriptie en zowel remming als stimulatie van transcriptie 3 uur na GR-activatie. Door het experiment te herhalen in de aanwezigheid van eiwitsyntheseremmer (cycloheximide) en door te kijken naar potentiële transcriptiefactor bindingsplaatsen in de promoter-regio's van enkele genen, werd de hypothese, dat transrepressie het voornaamste mechanisme is waarmee transcriptie wordt beïnvloed in de vroege tijdspunten, verder ondersteund. In deze context zou het dan ook zeer interessant zijn middels een combinatie van bioinformatica en technieken als chromatine immunoprecipitatie (ChIP) en Serial Analysis of Chromatin Occupancy (SACO) deze hypothese verder te testen in een vervolgstudie.

Met betrekking tot de context-specificiteit van glucocorticoïd effecten is bekend dat dezelfde receptor een compleet tegenovergesteld effect kan bewerkstelligen in verschillende typen neuronen. Om nu een inschatting te maken van de mate waarin de GR context-specifiek werkt, werden de genexpressie profielen van de hippocampale SAGE, hippocampale plak en PC12 cel studies systematisch met elkaar vergeleken. De mate van overlap tussen de hippocampale en de PC12 data sets was zeer gering, hetgeen betekent dat het effect van acuut geactiveerde GR op genexpressie in neuronaal weefsel in hoge mate afhankelijk is van de cellulaire context.

In **hoofdstuk 5** worden de via de genomics aanpak verkregen resultaten m.b.t. GR-responsieve genen besproken en gerelateerd aan wat er uit de literatuur bekend is betreffende de mechanismen waarmee glucocorticoïd receptoren genexpressie beïnvloeden. Verder wordt de functionele betekenis van GR-gemedieerde transcriptie in neuraal weefsel van enkele kandidaatgenen nagegaan. Daarnaast worden de methodologische tekortkomingen van de huidige gebruikte genomics technieken evenals enkele opties voor technische verbetering geanalyseerd.

Het gebruik van 'large-scale gene expression profiling' technieken kan worden beschouwd als een hypothese-genererende aanpak waarin wordt getracht een verband te leggen tussen transcriptionele regulatie van glucocorticoïd-responsieve genen enerzijds en de glucocorticoïd effecten op neurale functie anderzijds. In dit proefschrift staan in **hoofdstukken 2 en 4** de meer dan 200 gevonden GR-responsieve genen beschreven. Deze genen konden worden verdeeld over een groot aantal verschillende functionele categorieën. Dit wijst erop dat niet alleen wat werking betreft, maar ook op het niveau van het transcriptoom glucocorticoïden pleiotrope effecten bewerkstelligen. In zowel de hippocampale plakken als de neuronale PC12 cellen werden in de verschillende tijdspunten gelijksoortige functionele gencategorieën gevonden. Hierbij dient te worden opgemerkt dat de verdeling van genen over deze categorieën wel verschillend was tussen de tijdspunten alsmede tussen de hippocampale plakken en PC12 cellen. Dit betekent dus dat de effecten van glucocorticoïden op genexpressie afhankelijk zijn van zowel tijd als celtype.

De in dit proefschrift gevonden sets van genen bevatten ondermeer een aantal genen waarvan uit de literatuur al eerder bekend was dat ze glucocorticoïd-responsief zijn. Daarnaast is er een groot aantal interessante nieuwe kandidaatgenen gevonden waarmee nieuwe hypothesen kunnen worden geformuleerd omtrent de moleculaire mechanismen die ten grondslag liggen aan de effecten van glucocorticoïden op de functie van neurale cellen. De functionele consequentie van transcriptionele regulatie door glucocorticoïden van een aantal van deze genen wordt besproken in de verschillende hoofdstukken van dit proefschrift. Een aantal opvallende functionele categorieën die gevonden zijn in dit proefschrift zijn onder meer energie metabolisme *(lactaat dehydrogenase A en B, leptine, aldolase A en fosfoglyceraat kinase 1)*, hippocampale LTP *(caseïne kinase 2 en beta-chain clathrin-associated protein complex AP-2)*, actine-cytoskelet geassocieerde cellulaire eigenschappen *(beta-actine, LIMK1, LIM/SH3-eiwit, calcineurine en profiline)* en door synapsblaasjes gemedieerde neurotransmitter secretie *(SNAP25 en een aantal Rab-eiwitten)*. In dit proefschrift worden met behulp van een aantal van deze kandidaatgenen enkele potentieel interessante hypothesen gepostuleerd. Deze hypothesen maken de weg vrij voor verder functioneel onderzoek in de richting van het vaststellen van causale verbanden tussen de glucocorticoïd-geïnduceerde transcriptionele veranderingen enerzijds en de effecten van glucocorticoïden op het neurale fenotype anderzijds.

Voor het uitvoeren van deze functionele vervolgonderzoeken is een aantal interessante modelsystemen beschikbaar. Zo werd in **hoofdstuk 6** de hypothese getest dat GR-afhankelijke transcriptionele regulatie van LIMK1 betrokken zou zijn bij de structurele veranderingen van het actine cytoskelet. Het is bekend uit de literatuur dat veranderingen in de conformatie van het actine cytoskelet zijn geassocieerd met veranderingen in de morfologie van hippocampale dendritische 'spines' en LTP. In dit hoofdstuk wordt derhalve geprobeerd een verband te leggen tussen transcriptionele regulatie van LIMK1 door

geactiveerd GR en veranderingen in het actine cytoskelet. Om deze studie uit te voeren werd gekozen voor neuronale NG108-15 cellen als biologisch substraat vanwege het feit dat 1) deze cellen LIMK1 tot expressie brengen (alsmede een ander GR-responsief eiwit dat betrokken is bij de conformatie van het actine cytoskelet, nl. calcineurine) en 2) deze cellen lijken te reageren op GR-agonist met het uitgroeien van de neurieten. Helaas werd in deze studie geen duidelijke associatie gevonden tussen activatie van de GR, transcriptionele regulatie van LIMK1 mRNA en de conformatie van het actine cytoskelet, hetgeen suggereert dat mogelijkerwijs de NG108-15 cellen geen goed substraat zijn voor het testen van bovengenoemde hypothese. Echter, zoals ook wordt betoogd in **hoofdstuk 6**, is een aantal interessante alternatieven voorhanden om deze specifieke hypothese te testen, waaronder het gebruik van hippocampale organotypische plakken.

Tot slot worden in **hoofdstuk 7** alle bevindingen besproken die in dit proefschrift zijn gedaan. De belangrijkste conclusies die kunnen worden getrokken zijn dat:

1) acute activatie van GR leidt tot een uitermate kenmerkende tijdsafhankelijke genomische respons in zowel *ex vivo* hippocampale plakken als neuronale PC12 cellen, hetgeen suggereert dat transrepressie de voornaamste route is via welke genexpressie wordt beïnvloed 1 uur na GR-activatie. Deze transrepressie wordt gevolgd door voornamelijk transactivatie 3 uur na GR-activatie, resulterend in zowel stimulatie als remming van gentranscriptie.

2) de cellulaire context waarin glucocorticoïden werken een enorme diversiteit in de transcriptionele respons bewerkstelligt die vastgesteld kan worden met 'large-scale gene expression profiling' technieken.

3) glucocorticoïden pleiotrope effecten op genexpressie tot stand brengen met als gevolg dat verschillende functionele processen op een gecoördineerde manier worden beïnvloed.

4) in zowel *ex vivo* hippocampale plakken als neuronale PC12 cellen een groot aantal kandidaatgenen is gevonden die ten grondslag liggen aan het moleculaire werkingsmechanisme van glucocorticoïden in de hippocampus en in catecholaminerge neuronale cellen.

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Masterclass 'Stress and the resilient brain' with Bruce S. McEwen, Oegstgeest 2006. Title: Molecular mechanisms underlying corticosteroid actions on neuronal function.

4th Dutch Endo-Neuro-Psycho Meeting, Doorwerth 2005.Title: Identification of corticosteroid-responsive genes in hippocampus: from whole tissue to specific subregions.

Masterclass 'Nuclear receptors' with Jan-Åke Gustafsson, Oegstgeest 2004. Title: Identification of GR-responsive genes in rat hippocampal slices.

Poster presentations

2nd Annual Center for Medical Systems Biology (CMSB) Symposium, Amsterdam 2005. Title: Direct activation of glucocorticoid receptor reveals subtle and transient changes in gene expression.

LACDR Spring Symposium, Amsterdam 2005. Title: Direct activation of GR reveals subtle and transient changes in gene expression.

Society for Neuroscience 34th Annual Meeting, San Diego 2004. Title: Direct activation of GR reveals subtle and short-lived changes in hippocampal gene expression.

4th Forum of European Neuroscience (FENS), Lisbon 2004. Title: Identification of GR-responsive genes in rat hippocampal slices.

LACDR Spring Symposium, Amsterdam 2004. Title: Identification of GR-responsive genes in the rat hippocampus.

6th ULLA Summer School for Postgraduate Training and Research, Paris 2003. Title: Identification of corticosterone-responsive genes in the rat hippocampus.

1st Dutch Endo-Neuro-Psycho Meeting, Doorwerth 2002. Title: Molecular mechanisms underlying corticosteroid action on hippocampal cell function.

Elba Summer School and Workshop on Neurobiology of Stress in Health and Disease, Elba 2002. Title: Molecular mechanisms underlying glucocorticoid action on hippocampal cell function.

Curriculum vitae

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