Chapter **1**

General introduction

1.1 The stress response

Stress is broadly defined as a disruption of homeostasis, be it real or anticipated. The response to stress has two faces. On the one hand, it is a highly adaptive response to disturbances in homeostasis. On the other hand, if the stress response is dysregulated it is a potential risk factor for a large number of diseases, ranging from peripheral illnesses such as obesity and heart and cardiovascular problems to psychiatric disorders including major depression, schizophrenia, drug addiction and posttraumatic stress disorder (de Kloet et al., 2005; McEwen, 2008; Yehuda, 2009). Stress-related disorders can occur when the balance between the multiple players, phases and responsive tissues in the stress system is disturbed, so the adaptive stress response converts into a maladapted, detrimental chain of events (de Kloet et al., 1998; McEwen, 2001). Individual variations in this balance, due to genetic or environmental factors, determine whether an individual is resistant or sensitive to stress-related disorders (Kaffman and Meaney, 2007; Oitzl et al., 2010). Key to understanding what causes the balance to shift from adaptive towards detrimental effects of stress is a comprehensive understanding of the different players and phases involved in the stress response and their interactions with each other.

Two stress systems: the ANS- and HPA-axis

The body rapidly responds to a stressor by a combined activation of two stress systems in order to deal with the stressor and reinstate homeostasis as rapidly as possible. These two stress systems are the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis. The autonomous nervous system responds within seconds via sympathetic and parasympathetic innervations throughout the body. This system results in a rapid release of adrenaline and noradrenaline and a number of peripheral effects, such as an increase in blood pressure and heart rate, combined with a number of rapid effects in the brain. Together this results in an increased state of alertness, vigilance, fear or aggression also called the "fight-or-flight" response (de Kloet et al., 2005; Ulrich-Lai and Herman, 2009).

On a slightly slower time scale (minutes to hours) the HPA-axis is activated (see Box I). This hormonal response system culminates in the release of corticosteroid hormones in the blood stream. Cortisol is the main corticosteroid in humans, while rodents produce mainly corticosterone. Corticosteroids enable a long-term response to the stressor and affect tissues throughout the periphery as well as major response sites within the brain. Their most pronounced actions include the regulation of glucose, fat and protein metabolism, anti-inflammatory actions and effects on mood, memory and cognition (de Kloet et al., 2005; McEwen, 2008; Ulrich-Lai and Herman, 2009; Silverman and Sternberg, 2012; McGaugh, 2013). Throughout this thesis I will use the term corticosteroids in reference to the naturally occurring glucocorticoids: cortisol and corticosterone and their endogenous metabolites. Mineralocorticoids, such as aldosterone, are officially also part of the family of cor-

Box I The HPA-axis

The release of corticosteroids is regulated by the HPA-axis (Figure 1.1). Perception of a potentially threatening situation activates the paraventricular nucleus (PVN) of the hypothalamus. In the PVN, corticotrophin releasing hormone (CRH) and vasopressin-containing neurons are activated and stimulate the pituitary to release adrenocorticotropin hormone (ACTH). In turn, this hormone induces the release of corticosteroids from the adrenal glands. Corticosteroids circulate in the blood stream and thus reach every organ in the body, including the brain. Corticosteroids signal back to the HPA-axis at all levels to inhibit further release, thus giving a negative feedback and preventing overexposure (Figure 1.1). Activation of the HPA-axis is also affected by higher brain areas, most notably by the limbic system. Corticosteroids are released in hourly pulses that are highest in amplitude during the active period, thereby causing an overall circadian release pattern (Young et al., 2004). This pattern of pulsatility is essential to keep tissues responsive to stress-induced peaks in corticosteroid release (Lightman and Conway-Campbell, 2010; Sarabdjitsingh et al., 2012). Superimposed on this ultradian and circadian rhythm is the response to a stressor, which (depending on the severity of the perceived stressor) results in high circulating corticosteroid levels for several hours.

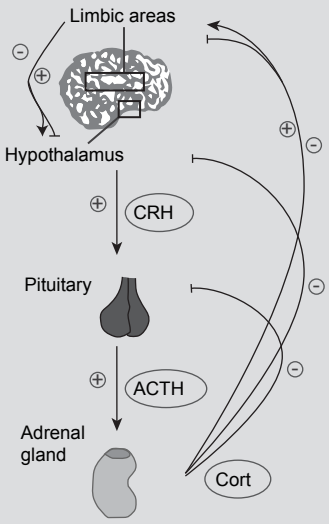

Figure 1.1: The HPA-axis
Schematic overview of the HPA-axis. Secreted cortisol or corticosterone exerts a negative feedback on all levels to prevent overstimulation of the axis. Through limbic areas, corticosteroids exercise an additional level of feedforward and feedback regulation.

ticosteroids, but are different in both their function and release regulation. I will always name these separately when including them in my discussions. (see Box II)

Two corticosteroid receptors: MR and GR

The actions of corticosteroids are mediated by two receptor types, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Reul and de Kloet, 1985). Both belong to the family of nuclear receptors, a family of hormone-activated transcription factors. The dual receptor system gives corticosteroids a versatile response pattern. The MR has a ∼10-fold higher affinity for the naturally occurring corticosteroids (corticosterone and cortisol) than the GR (MR: Kd of 0.5 nM and GR of 5 nM). This implies that both receptors are activated at different time points during a stress response: the MR is already activated to a large extent by basal corticosteroid levels, while the GR becomes gradually activated when corticosteroid

levels rise, for example after a stressful event, during the circadian rise in corticosteroids (Reul and de Kloet, 1985; Kitchener et al., 2004) or during the peak of ultradian pulses (Conway-Campbell et al., 2007). Of note, the membrane-associated subpopulation of the MR (to be discussed later in this introduction) has reduced corticosteroid affinity and requires higher hormone levels. The GR is expressed in virtually every cell of the body. Within the brain, the GR is expressed ubiquitously as well, both in glia cells and neurons, with highest levels in the PVN and in the hippocampus (Reul and de Kloet, 1985). The expression of the MR, on the other hand, is more restricted. High levels of MR are found in limbic areas, with moderate levels in the (prefrontal) cortex and the amygdala and high levels in the hippocampus (Reul and de Kloet, 1985). In addition, MR expression is found in circumventricular organs and the nucleus of the solitary tract (Geerling et al., 2006) and peripherally within the kidneys and throughout the cardiovascular system. But, in these latter tissues the MR acts as aldosterone receptor as corticosterone is metabolized into an inactive form in these tissues (see Box II).

The differences in affinity and expression pattern between the MR and GR are also reflected in their respective functions within the stress response. Due to its high affinity for corticosteroids, the MR plays a proactive role in maintaining homeostasis. Within the brain, low levels of corticosteroids, activating the MR, are required to maintain basal firing frequency and stability of limbic circuits (Joëls et al., 2008). Probably through input of higher brain regions to the PVN, the MR modulates HPA-axis activation (Reul et al., 2000). In cognition, the MR is involved in appraisal of novel situations, learning strategies, response selection and emotional reactivity (Oitzl and de Kloet, 1992; Schwabe et al., 2010; Zhou et al., 2010, 2011; Kruk et al., 2013; Souza et al., 2014). Part of these MR-mediated actions are likely due to non-genomic signaling (to be discussed further in *Chapter 2*). Conversely, the GR, which is only activated when corticosteroid levels rise, plays a reactive role in the stress response. For one, through the GR, corticosteroids inhibit their own release in order to prevent corticosteroid overexposure (de Kloet and Reul, 1987). Within the brain, GR activation generally suppresses transiently raised excitatory transmission for instance by enhancement of calcium dependent K+ afterhyperpolarization (Joëls and de Kloet, 1989) and by increasing serotonin dependent K+hyperpolarization (Joëls and de Kloet, 1990; Joëls et al., 1991). GR activation can also stimulate recruitment and mobility of AMPA receptors into the post-synaptic membrane (Groc et al., 2008; Popoli et al., 2011). As a result LTP, the cellular form of memory, is occluded by these GR-mediated effects (Alfarez et al., 2002; Kim and Diamond, 2002). One of the main function of the GR within cognition is to promote consolidation of stress-related information and to facilitate behavioral adaptation (Oitzl and de Kloet, 1992; de Kloet et al., 1999; de Quervain et al., 2009; Zhou et al., 2010). In addition, the GR exerts important functions within the periphery, for example regarding glucose metabolism and suppression of immune activation during stress (de Bosscher and Haegeman, 2009).

Box II The promiscuity of the mineralocorticoid receptor

The MR is an odd receptor in the sense that it binds multiple families of functionally different hormones. Thus, both the naturally occurring glucocorticoids (corticosterone or cortisol) and mineralocorticoids (aldosterone and deoxycorticosterone) bind the MR with similar affinity, and also progesterone is bound with high affinity (Joëls et al., 2008; Funder, 2010; Gomez-Sanchez, 2010). In the epithelial cells of the kidneys and distal colon, the MR acts as the prime receptor for aldosterone and is essential for the regulation of the body's salt and fluid balance (Gomez-Sanchez, 2011). However, the circulating plasma level of corticosterone/cortisol is 100 to 1000-fold higher than that of aldosterone, so how does the MR in the kidney retain its mineralocorticoid sensitivity? This enigma was solved with the discovery of the key enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (Edwards et al., 1988; Funder et al., 1988). This enzyme converts cortisol and corticosterone into inactive metabolites (see Figure 1.2) and thus strongly reduces the excess of these glucocorticoids in aldosterone-sensitive tissues. Indeed, 11β-HSD2 is highly expressed in aldosterone-sensitive tissues, such as the kidneys, skin and colon and is absent from classical glucocorticoid target tissues as the liver, brain and immune system (Wyrwoll et al., 2011). In the brain its expression is limited to some nuclei in the brainstem, most notably the nucleus of the solitary tract, an important nucleus in salt homeostasis regulation (Geerling et al., 2006). In addition to regulating the hormone accessibility of the MR, 11β-HSD2 also acts to protect tissues against glucocorticoids. For example, there is widespread expression of 11β-HSD2 throughout the placenta during gestation and the developing fetus shows 10–100 fold lover glucocorticoid levels as the mother (Wyrwoll et al., 2011). Deficiencies in 11β-HSD2 expression during late pregnancy generally decrease the birth weight and may be associated with cognitive and affective deficits later in life (most notably increased anxiety and a dysregulation of the HPA-axis) (Cottrell et al., 2014). In classical glucocorticoid target tissues such as the liver and the brain, another type of enzyme is expressed: 11β-HSD type 1 (Agarwal et al., 1989). The enzyme 11β-HSD1 has predominant reductase activity. It converts inactive cortisone and 11β-dehydrocorticosterone into the active 11β-hydroxy form (see Figure 1.2) and thus potentiates glucocorticoid effects (Jamieson et al., 1995).

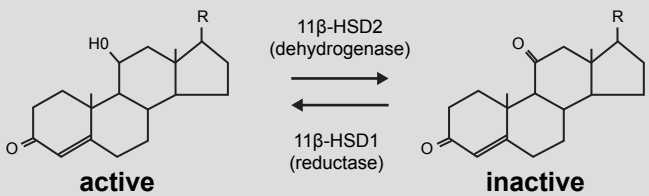

Figure 1.2: 11β-HSD1 and 2
The active corticosteroids, cortisol and corticosterone are metabolized into their inactive counterparts, cortisone and 11-dehydrogenated corticosterone by 11β-HSD2 and vice versa regenerated by 11β-HSD1.

Two modes of actions: genomic and non-genomic

Both MR and GR are ligand-driven transcription factors. They predominantly reside in the cytoplasm in their unbound state. Upon hormone binding, the receptor-ligand complex translocates to the nucleus and affects gene transcription (Beato and Sanchez-Pacheco, 1996). Although the MR and GR share almost identical DNA-binding domains, they do not regulate the same sets of genes. For example, the set of genes that are over- or under-expressed after MR versus GR activation show only limited (less than 30 %) overlap (Datson et al., 2001).

However, over the last decades an alternative mode of action was found for both receptors that does not involve gene transcription. Genomic effects are slow in onset and the first physiological responses are expected after a delay of at least 15 minutes, and often in the order of hours (Haller et al., 2008). This is in sharp contrast to the reality of some of the corticosteroid effects, of which the fastest have been observed within seconds to minutes. Thus, there must be an alternative mode of action of corticosteroids that does not involve the genomic MR and GR mediated pathways. Indeed, a small portion of the population of the MR and GR is localized at the plasma membrane where they interact with multiple kinase signaling pathways and exert rapid, non-genomic effects (Di et al., 2003, 2009; Johnson et al., 2005; Karst et al., 2005, 2010; Prager et al., 2009). As such, both the MR and GR affect neuronal excitability and behavior over a wide time range: from a few minutes after corticosteroid exposure until many hours thereafter (Joëls et al., 2012).

To understand the response of a tissue or an organism to stress it is important to understand what happens at the lower levels; i.e. at the cellular level. These cellular actions are the result of a complex interplay between the effects of corticosteroids and other stress hormones, and effects mediated by the two corticosteroid receptors (MR and GR). The existence of two corticosteroid responsive pathways (genomic and non-genomic) adds to the complexity. For the cellular basis of both modes of action much has already been discovered but more remains unknown. In the next two sections, I will introduce the state of knowledge for both the genomic and the non-genomic mode of action of corticosteroids.

1.2 Non-genomic corticosteroid signaling

For many years rapid actions of corticosteroids on neurotransmission and behavior were noted but their cellular basis remained ill understood. For one, corticosteroid application can inhibit neuroendocrine output of the brain within a few minutes (Evanson et al., 2010b). On the level of behavior, depending on the context, corticosterone application results rapidly in increased locomotion (Sandi et al., 1996a), aggression (Mikics et al., 2004; Kruk et al., 2013) or risk assessment (Mikics et al., 2005). Also in humans corticosteroids affect learning and emotional processing in a non-genomic manner (Henckens et al., 2012; van Ast et al., 2013).

The Tasker group (Di et al., 2003, 2005, 2009; Malcher-Lopes et al., 2006) performed a set of pioneering experiments that opened the field to understanding the cellular basis of these rapid effects. They demonstrated that a high dose of corticosterone (from 100 nM) irreversibly reduces the frequency of miniature excitatory postsynaptic currents (mEPSCs) within 5 minutes within the PVN of the hypothalamus. This effect did not require gene transcription or protein synthesis. The frequency of mEPSCs is a measure of the excitability of neurons, reflecting either the release probability of glutamate vesicles or the number of synaptic contacts. In subsequent years, the Joëls group (Karst et al., 2005, 2010; Pasricha et al., 2011) found rapid effects of corticosterone on mEPSC frequency in the hippocampus (both CA1 and DG) and the basolateral amygdala. They observed a rapid reduction of mEPSC frequency in the hippocampus, which is opposite to what was found in the PVN. Moreover, within the hippocampus, the rapid reduction was reversible and induced by lower doses of corticosterone (from 10 nM). Over recent years many more effects have been identified in stress responsive areas in the brain, I will summarize these in *Chapter 2*.

Membrane-associated MR and GR

A large part of the non-genomic actions of corticosteroids require the presence of the MR or GR. For instance, the enhanced mEPSC frequency in the hippocampus is absent in MR-/- mice and prevented by pretreatment with MR antagonists (Karst et al., 2005). Similarly, in the amygdala rapid GR-dependent effects were observed (Karst et al., 2010). Rapid behavioral effects have been shown to depend on the GR (Barsegyan et al., 2010) and MR (Khaksari et al., 2007) as well. Thus, the MR and GR appear to have an alternative function as mediators of non-genomic corticosteroid signaling. Intriguingly, in this role the MR and GR seem to be accessible at the outside of the plasma membrane. Rapid effects can still be induced with a membrane-impermeable conjugate of corticosterone (cort-BSA) (Karst et al., 2005, 2010; Groc et al., 2008; Roozendaal et al., 2010) and cannot be induced by intracellularly infused corticosterone (Liu et al., 2007; Olijslagers et al., 2008). Additionally, the presence of the MR and GR at the (synaptic) membrane was demonstrated in membrane extracts and synaptosomes (Komatsuzaki et al., 2005; Wang and Wang, 2009; Qiu et al., 2010) and with electron microscopy imaging (Johnson et al., 2005; Prager et al., 2009). Part of the downstream signaling cascades has also been unraveled. One of the most common signaling partners are the G-proteins, which bind to the receptors at the membrane. Inhibition of G-protein activation abolishes the rapid effects of corticosterone on a variety of neuronal functions (Zhu et al., 1998; Di et al., 2003, 2009; Olijslagers et al., 2008; Hu et al., 2010). More downstream, the cAMP-PKA and ERK1/2 pathways have been implicated in non-genomic corticosteroid actions (Liu et al., 2007; Olijslagers et al., 2008; Di et al., 2009; Barsegyan et al., 2010).

The regulation of membrane translocation for the MR and GR remains incompletely understood. For the related estrogen receptor α (ERα), presence within caveolae, association with caveolin-1 and palmitoylation have been established as vital steps in its membrane targeting (Levin, 2009). However, only association with caveolin-1 has been shown for the MR and GR (Matthews et al., 2008; Pojoga et al., 2010b). Moreover, the fraction of the receptors associated with the membrane, their local binding partners and the effect of ligand treatment on membrane trafficking and internalization have not yet been addressed.

1.3 Genomic corticosteroid signaling

As discussed, a small fraction of the MR and GR are anchored at the cell membrane, but the bulk of the receptor population is present within the cytoplasm. In the absence of ligand, these cytoplasmic receptors are kept in an inactive conformation by association with a large chaperone complex. Most chaperones are shared by all steroid receptors, these include heat shock proteins (HSP) 90, 40 and 70, p23, p70, and cochaperone proteins, such as the immunophilins FKBP51, FKBP52, and protein phosphatase 5 (PP5) (Picard, 2006). Association with chaperones also serves to keep the nuclear localization sequence hidden and prevents protein degradation (Faresse et al., 2010). Corticosteroids are lipophilic and as such easily pass the cell membrane and reach their receptors within the cytoplasm. Ligand binding to the ligand-binding pocket (LBP) results in a massive conformational shift that exposes the nuclear localization signal. Next, the ligand-receptor complex is actively transported into the nucleus by importins (primarily importin α (Tanaka et al., 2003)).

DNA binding

Through the two zinc fingers in their DBD (see Box III), the MR and GR bind directly to GREs on the DNA. Direct DNA-binding of the MR and GR to GREs is mostly associated with enhanced gene transcription or transactivation. The receptor binds the DNA as a dimer, forms a complex with coactivators and attracts the general transcription machinery to induce increased transcription of the related gene (Datson et al., 2008). A cofactor is defined as a protein that affects the interaction of a transcription factor with the DNA, but that does not act as a transcription factor itself. Cofactors have diverse functions and affect the stability of the transcriptional machinery, (de)acytelate histones or recruit other cofactors or transcription factors (Zalachoras et al., 2013). Cofactors are divided into coactivators and corepressors depending on their role in gene transcription. The GR also induces transrepression of genes. This can occur through direct binding to negative GREs, but it is mostly associated with indirect DNA-binding. For example, the GR has been well described to bind the transcription factors AP-1 and NFκB and inhibit transcription of their

Box III Functional domains of the GR and MR

All steroid receptors share a modular structure encompassing four functional domains. The N-terminal domain is least conserved between steroid receptors (around 15 % overlap) and contains the activating factor-1 (AF-1) domain. Adjacent is the highly conserved (90 % homology among steroid receptors) DNA-binding domain (DBD), which recognizes the hormone response elements in the DNA by its two zinc finger structures. Due to the almost perfect sequence homology between this region in the MR and GR they recognize and bind the same DNA sequences, dubbed glucocorticoid response elements (GREs) (Datson et al., 2008). C-terminally located is the ligand-binding domain (LBD). The sequence homology of the LBD is also generally quite high between steroid receptors (~60 % between the MR and GR), but the structures lining the ligand-binding pocket show less overlap and determine ligand specificity (Bledsoe et al., 2002; Huyet et al., 2012). The conformation of the LBD is highly affected by ligand binding, it exposes chaperone binding sites in its unbound conformation, while ligand binding leads to exposure of the nuclear localization signal and AF-2 domain (Pascual-Le Tallec and Lombès, 2005). For most steroid receptors, helix-12 which contains the AF-2 domain refolds into an open conformation only when agonists are bound and not upon binding of antagonists (Fagart et al., 1998; Bledsoe et al., 2004). Both the AF-1 and AF-2 mediate cofactor binding and are affected by ligand-induced conformation changes. While the AF-2 domain is highly conserved, the AF-1 is very variable and determines receptor specific and cellular context-dependent cofactor binding patterns (Fuse et al., 2000; Simons and Kumar, 2013). In between the DBD and LBD is a small hinge region that enables folding and also contains a dimerization sequence and a nuclear localization signal.

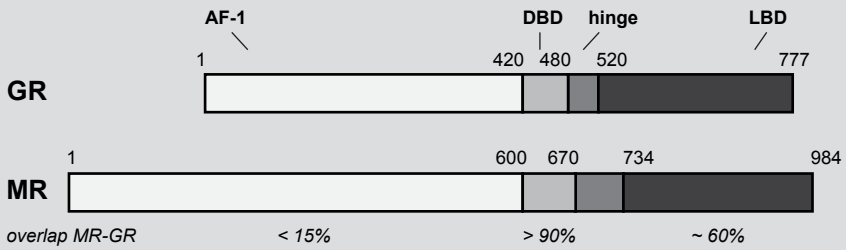

Figure 1.3: Functional domains of the GR and MR
Schematic overview of the modular structure of the GR and MR. Below the average sequence homology between the two receptors is given. Image adapted from Pippal and Fuller (2008).

target genes (Nixon et al., 2013). This is functionally important for GR's immunosuppressant actions. Indirect binding does not necessarily have to entail transrepression though. Co-binding of the GR and other transcription factors can also induce gene transcription (Kassel and Herrlich, 2007; Ratman et al., 2013).

The MR and GR show a large overlap in their DBD and activating function domains (see Figure 1.3) and as result their genomic actions overlap. In tissues where the MR and GR are coexpressed they also form heterodimers (Nishi et al., 2004) and DNA transactivation will thus occur through a mixture of GR-GR homodimers, MR-MR homodimers and GR-MR heterodimers. The extent of overlap between MR and GR regulated genes is still debated. Early gene expression profiling studies found only limited overlap in MR- and GR- responsive genes (Datson et al., 2001, 2008), while a recent study investigating direct DNA binding using chromatine immuno precipitation (ChiP) did find MR binding for all examined GR-target sequences (Polman et al., 2013). The MR and GR are known to share many cofactors (also with other steroid receptors), but each receptor uses a distinctive set as well (Yang and Young, 2009; Zalachoras et al., 2013). In regards to transrepression though indirect binding to the DNA, this has classically been associated with only the GR and not the MR. Indeed, the MR lacks immunosuppressant efficacy (Pippal and Fuller, 2008). However, *in vitro* transrepression of AP-1 activity by the MR have been shown by some (Africander et al., 2013), albeit with much reduced potency compared to the GR (Pearce and Yamamoto, 1993). This has not yet been validated *in vivo*. An overview of MRs and GRs cellular actions is represented in Figure 1.4.

Corticosteroids reach a multitude of tissues where they execute very variable functions, on a cellular level this means they require the capacity for tissue specific patterns of gene transcription. Indeed, Chip-Seq studies and microarray studies have both shown that there is only a moderate overlap in GR target genes and bound sequences between different cell types (Datson et al., 2008; Reddy et al., 2009; Yu et al., 2010; John et al., 2011; Polman et al., 2012, 2013). For example, the set of sequences bound by GR in a neuronal-like cell line overlapped for only 7 % with those found in an alveolar-derived cell line and for 11 % with an adipose-derived line (Polman et al., 2012). Differences in chromatin structure and cofactor availability are important determinants of this tissue specificity. The DNA sequence of the GR binding site (GBS) represents another level of complexity and regulation of GR's (and MR's) genomic output. Identified GR binding sites show a certain level of sequence homology and thus a consensus GBS (Strähle et al., 1987). However, there are also quite some variations to the consensus sequence possible. In an elegant study, the Yamamoto group showed that single nucleotide mutations to identified GBS's affected the conformation of the GR when bound and resulted in severe shifts in (*in vitro*) transactivation capacity (Meijsing et al., 2009). This effect was found to depend on cofactor recruitment. Within the brain an *in silico* search for preserved GR binding sequences resulted in the identification of a brain-specific consensus GBS (Datson et al., 2011) and also found considerable variation within this sequence and its flanking regions between different GR-regulated genes. The exact correlation between binding sequences and GR's conformation and capacity to induce gene transcription is still only partly understood but it is clear that this represents another important level of orchestrating GR's (and MR's) genomic actions.

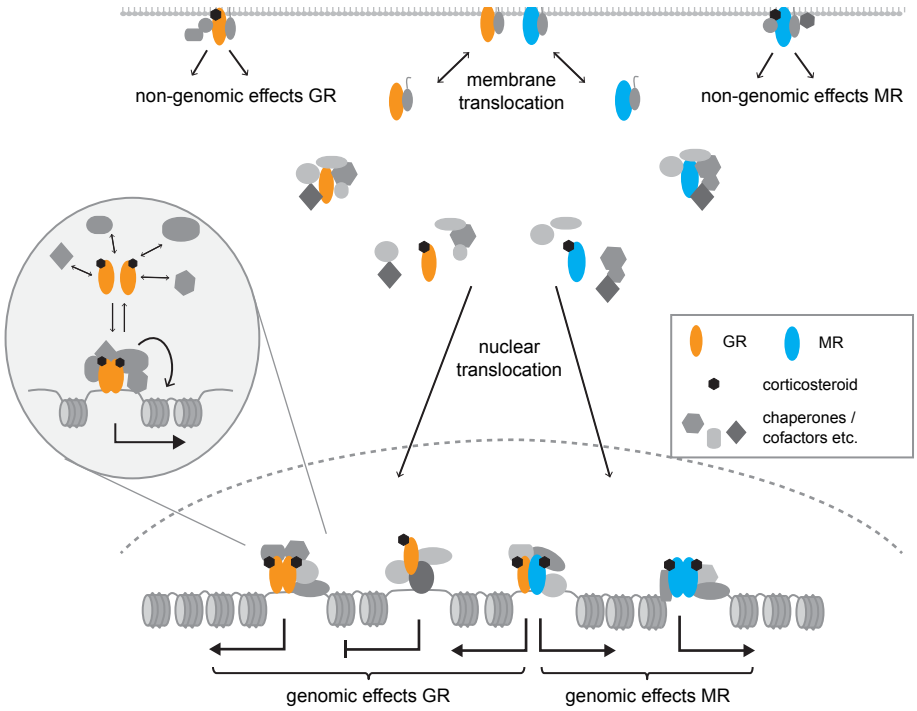

Figure 1.4: Cellular MR- and GR-mediated corticosteroid actions
A small fraction of MR and GR translocate (through unknown pathways) to the plasma membrane, where they associate with caveolin-1 and other binding partners and induce non-genomic corticosteroid effects. The largest fraction of both MR and GR resides within the cytoplasm while unbound and is bound here by chaperones. Hormone binding releases the chaperones and enables nuclear translocation. Within the nucleus both receptors bind the DNA in a dynamic fashion (see insert). The GR can bind its target genes through direct receptor-DNA interactions as dimer and indirectly through binding to / with other transcription factors as monomer. The first is predominantly associated with transactivation and the latter with transrepression, but both modes of DNA binding can lead to both stimulation and inhibition of gene transcription. Indirect DNA binding by the MR is less established and its genomic actions are thus mostly restricted to direct binding as dimer and transactivation. When coexpressed, the MR and GR also form heterodimers on the DNA.

Steroid receptors show highly dynamic DNA-binding

For many years the binding of steroid receptors (and other transcription factors) to their DNA-target sites was envisioned as a static event: the transcription factor binds, recruits cofactors and RNA polymerases and gene transcription is initiated (Perlmann et al., 1990). However, since the start of the new millennium all components of the transcriptional machinery have been found to be highly dynamic. In this new era many advanced (functional) imaging techniques have been developed, improved and used that enable the observation of protein dynamics on a seconds to even milliseconds timescale. I will discuss some of the most important advances in microscopy in the next section of the introduction. First the current view on steroid receptor DNA-binding dynamics will be discussed.

The first to study the dynamics of the GR at the DNA was the Hager group. They developed a tandem repeat of a naturalistic GR binding site, the mouse mammary tumor virus (MMTV) promoter, incorporated into the native DNA. This repeat consists of 800–1200 GR binding sites and as such binding of GFP-GR to this site can be visualized directly (Walker et al., 1999; McNally et al., 2000). With this model system, the Hager group used fluorescence recovery after photobleaching (FRAP) to show very rapid exchange of the GR at its binding site. The half maximum of recovery was obtained within 5 seconds at the site (McNally et al., 2000; Stavreva et al., 2004). A multitude of follow up studies on the GR and related steroid receptors (Stenoien et al., 2000; Schaaf and Cidlowski, 2003; Farla et al., 2004; Stavreva et al., 2004; Mueller et al., 2008; Stasevich et al., 2010b), irrevocably showed that activated steroid receptors remain very mobile within the nucleus. Similarly, a rapid exchange at the DNA was found for all transcription factors and also for cofactors and RNA polymerases (Dundr et al., 2002; Gorski et al., 2008; Johnson et al., 2008). Only a few structural chromatin components (core histone proteins) were found to be stably associated with the DNA (Kimura and Cook, 2001). An important observation was that mobility of steroid receptors is negatively correlated to their transcriptional activity (Schaaf and Cidlowski, 2003; Stavreva et al., 2004, 2009). This suggests that DNA-binding is either less frequent or shorter in duration for less potently activated receptors. In addition, a large fraction of the observed immobilizations are presumed to be due to nonspecific DNA binding (Mueller et al., 2013). Nonspecific DNA-binding likely aids a transcription factor in its search for specific binding sites, but the exact mechanisms remain debated. *In vitro* transcription factors show 1D sliding across the DNA (i.e. remains associated with the DNA), in addition to 3D hopping (rapid association and dissociation to the DNA), but whether this also occurs on nascent chromatin remains unclear (Gowers et al., 2005; Hager et al., 2009).

The highly dynamic behavior of steroid receptors at the second and millisecond level must further be integrated with additional levels of dynamics at longer time frames. For both the GR and the ERα spontaneous oscillations of receptor binding to responsive genes are observed within single cells in 20–40 minute cycles (Becker, 2002; Métivier et al., 2003). Id est: a responsive gene will cycle through 20–40 minute periods of high occupancy (by dynamically interchanging receptors) and similar periods with very low occupancy. These types of asynchronous oscillations are likely caused by local chromatin remodeling. Chromatin remodeling precedes recruitment of the GR to a DNA sequence and displacement of the chromatin remodeling complex is soon followed by GR displacement from the sequence (Nagaich et al., 2004). In addition to these oscillations, in the organism also the hormone secretion oscillates and also this induces oscillations in gene expression (Stavreva et al., 2009). Thus, oscillating pulses of corticosterone also result in oscillations of GR-DNA binding, RNA polymerase binding and mRNA transcript levels of endogenous GR-target genes in cells and even in an intact rat (Stavreva et al., 2009).

Taken together, these findings paint a picture of a highly dynamic transcriptional complex at gene promoters (and enhancers) with multiple components dynamically exchanging. This process has been dubbed the "hit-and-run" mode of transcription (Rigaud et al., 1991). This is a field still in development and theories about what types of DNA interactions occur and what their relevance to transcription is are still being developed (recent reviews on this topic include (Mueller et al., 2008; Biddie and Hager, 2009; Hager et al., 2009; Voss and Hager, 2014). More recently, the relevance of transcription factor dynamics for local chromatin remodeling and complex formation has received more attention and added another level of complexity to the view on transcription factor-DNA interplay (Voss and Hager, 2014). One of the largest challenges remains to accurately quantify the dynamics at sufficient spatial and temporal resolution and therefore the field is very dependent on the development of novel sophisticated imaging methods.

1.4 The analysis of protein dynamics using advanced fluorescence microscopy techniques

The use of fluorescently tagged receptors has revealed much about the dynamics of the MR and GR within the cell and within the nucleus. Over the last fifteen years a number of live imaging and advanced microscopy techniques have been developed to enable the tracking of fluorescent proteins within living cells. In the next sections I will introduce the three mostly used techniques and discuss their advantages and disadvantages. I will also briefly discuss some imaging approaches directed towards specific subcellular regions.

Fluorescence Recovery After Photobleaching

All fluorescent proteins experience photobleaching, i.e. the irreversible loss of fluorescent capacity due to (prolonged) excitation. This phenomenon can be utilized to measure protein dynamics with FRAP (van Royen et al., 2009a). In FRAP a target area is exposed to a high intensity laser pulse to effectively bleach all fluorescent molecules within that area. Subsequently, the recovery of fluorescence within that area is monitored. As photobleaching is a permanent state, any recovery of fluorescence is due to fluorescent proteins moving from unbleached areas into the target area and replacing the bleached molecules that are moving out of this area. Thus, the faster the recovery, the more mobile the fluorescent protein (Figure 1.5A). Advantages of FRAP are that it is relatively simple to implement and that it records protein dynamics over a large time range (from a few 100 milliseconds to several minutes; Table 1.1). Its main disadvantages are that it is not accurate in tracking fast diffusion (< 100 milliseconds) and that there is no consensus on how to quantify the FRAP recovery curves. A review of all quantitative FRAP studies showed that

	FRAP	FCS	SMM
Spatial resolution (in XY)	diffraction limited	diffraction limited	20 nm
Time domain	$\sim$100 ms – 10^2 sec	µs – 10^1 sec	$\sim$5 ms – 10^0 sec
Population modeling	yes	yes	yes/no
Tracking of fast diffusing proteins	poor	good	good
Assessment DNA-binding times	yes	no	yes/no*

Table 1.1: Comparison of advanced imaging techniques
* attenuations to the illumination schedule enable a quantification of DNA-binding times for SMM (Gebhardt et al., 2013).

differences in modeling parameters and a failure to correct for laser irregularities severely affected the acquired parameters (Mueller et al., 2008).

Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) is a fluorescence imaging technique that can be used for accurate assessment of the mobility of fast diffusing proteins (Chen et al., 1999; Ries and Schwille, 2012). In FCS, the fluorescent signal in a relatively small ($\sim$200 nm diameter) area is monitored over time (Figure 1.5B). This measured fluorescence fluctuates as fluorescent proteins move in and out of the volume. The faster a protein diffuses, the shorter it will reside within this measuring volume. Therefore, the correlation of fluorescence over increasing time frames is recorded and the rate of change of the correlation curve over time is a measure of the diffusion rate of the protein. The main advantage of FCS is that it can reliably quantify relatively rapid diffusion. Its main disadvantages are that FCS can only be applied for a limited time domain (microseconds to several seconds) and that the size of the imaging volume is restricted by diffraction of light and can thus not become smaller than $\sim$200 nm in XY (depending on the objective and laser wavelength) (Table 1.1).

Single-Molecule Microscopy

In single-Molecule Microscopy (SMM), a wide-field fluorescence microscopy setup is used. Fast, ultra-sensitive imaging of samples with very low levels of fluorescence enables the visualization of single fluorescent molecules (Figure 1.5C). The fluorescent signal can be fitted with a Gaussian curve, of which the center represents the location of the fluorescent molecule. This approach results in a positional accuracy of 20–40 nanometer. The positional accuracy depends only on the signal-to-noise ratio and can therefore be a lot smaller than the diffraction-limited resolution of conventional fluorescence microscopy. A further advantage of SMM is its high temporal resolution (several milliseconds). Quantification of SMM data has been achieved by two methods: single-molecule tracking (SMT) or Particle Image Corre-

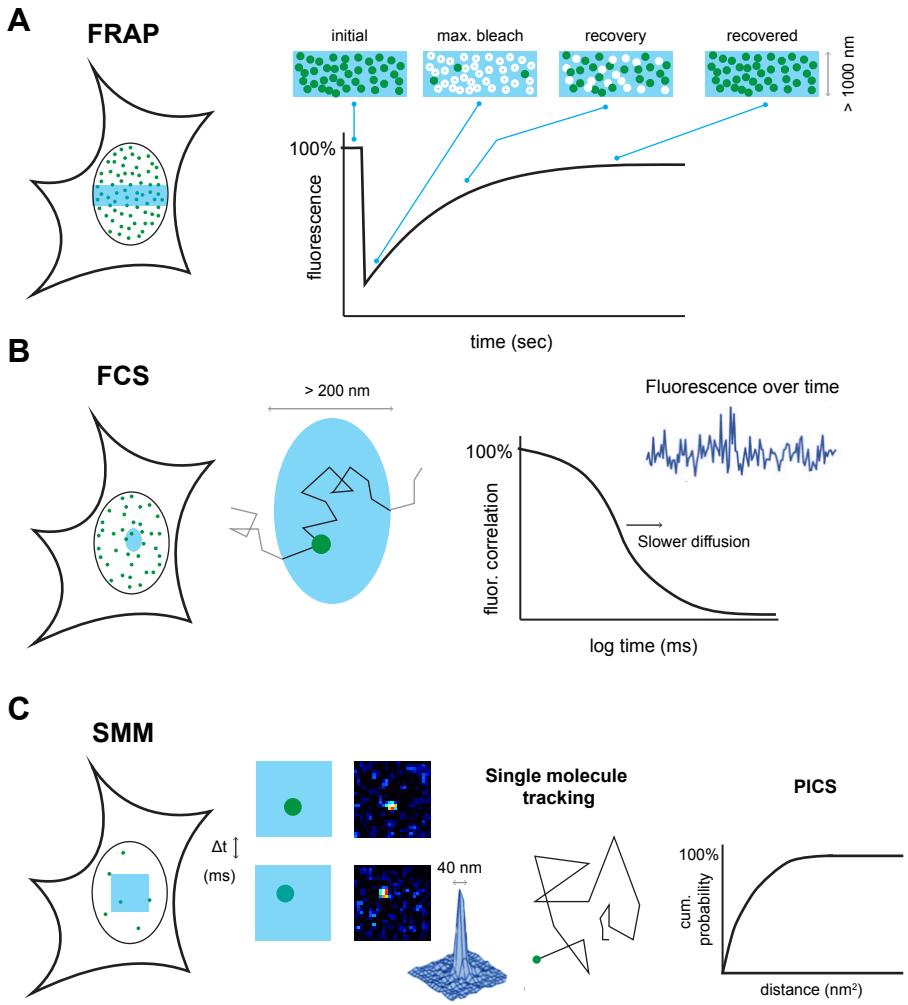

Figure 1.5: Functional imaging approaches
A schematic overview of the three main functional fluorescence imaging methods. (A) FRAP. In FRAP a > 1000 nm diameter area is bleached by a high-energy laser pulse and subsequently the recovery of fluorescence within this area is observed. Quantification of protein dynamics is obtained from modeling of the recovery curve. (B) FCS. In FCS a > 200 nm diameter area is observed and fluorescence within this area is observed. Quantification of protein dynamics is obtained by autocorrelation of the fluorescence over time. (C) SMM. In SMM a region of interest (ROI) is chosen within a lightly fluorescent cell. Within this ROI, single fluorescent molecules are observed and as their fluorescence is fitted with a Gaussian curve a positional accuracy of ∼40 nm is obtained. Quantification of protein dynamics occurs either through tracking of single molecules or by PICS where for each time lag all distances of molecules between the two images is fitted. FRAP: Fluorescence Recovery After Photobleaching, FCS: Fluorescence Correlation Spectroscopy, SMM: Single-Molecule Microscopy.

lation Spectroscopy (PICS). In SMT, a very low density of fluorescent molecules is required and the trajectories of single molecules are constructed over several frames (Persson et al., 2013). The length of these trajectories is restricted by blinking and

photo-inactivation (bleaching) of the used fluorophore. Population data can be extracted by population modeling or non-averaged data can be analyzed (Elf et al., 2007). In PICS, higher densities of fluorophores are allowed and blinking is less of a problem (Semrau and Schmidt, 2007). PICS measures the correlation of distances of each single fluorescent molecule to all existing fluorescent molecules in a subsequent time frame. From the overall data the uncorrelated distances are subtracted and the remaining correlated distances are fitted with population models. The main disadvantage of SMM is that it is not easily applied to long time ranges (Table 1.1). SMT is hampered by the stability of fluorophores over longer time delays and both SMT and PICS become less accurate over long time delays due to escaping of the faster diffusing molecules from the imaging volume (van Royen et al., 2014). Labelling of proteins with gold particles or quantum dots enables long-term imaging and is very promising, but has so far been applied in only a few occasions due to labeling issues (Cognet et al., 2014).

Total internal reflection fluorescence (TIRF) microscopy

Total internal reflection fluorescence (TIRF) microscopy provides a means to selectively excite fluorophores in a thin layer close to the glass-medium interface (Axelrod et al., 1983; Axelrod, 2008; Toomre, 2012; Martin-Fernandez et al., 2013). In short, in TIRF the excitation laser is redirected so that it exits the objective at a large angle relative to the optical axis. Once a critically large angle is achieved, the excitation light is totally internally reflected at the glass-medium interface. As a result a low energy electromagnetic field is generated from this interface, which is dubbed the evanescent wave field. The evanescent wave field is capable of exciting fluorophores, but its energy decreases exponentially, which results in an excitation field of 60–100 nm in depth. The biggest advantage of TIRF microscopy is that it produces wide-field images with very low background fluorescence from out-of-focus planes and thus a very high signal-to-noise ratio. TIRF microscopy can be combined with advanced imaging techniques as FCS, FRAP and SMM. Especially, the combination of SMM and TIRF has been used in a wide variety of studies to study the kinetics of membrane-bound proteins, near membrane structures and docking vesicles (Axelrod, 2008). In a variation of TIRF, entitled highly inclined and laminated optical sheet (HILO) microscopy, a thin sheet of excitation light penetrates the sample at an angle, thus exciting a thin section, but within the cell. HILO can thus be used to image within the cytoplasm or nucleus, with improved signal-to-noise ratio (Tokunaga et al., 2008). Similarly, reflected light sheet microscopy (RLSM) also enables imaging of a thin section within the cell and thus reducing out of focus light. RLSM has been successfully combined with single-molecule tracking (Ritter et al., 2010; Gebhardt et al., 2013).

1.5 Objective and outline

As the stress system is such a tightly regulated system, it is of vital importance to understand the finesses of the underlying molecular pathways, which ultimately determine the responses on the tissue and organism levels. Within a target cell corticosteroids can bind both the MR and GR and through these receptors it will activate both non-genomic and genomic pathways. Although much has been learned regarding the mode of action of both receptors, many questions still remain. In this thesis I will explore further finesses of the cellular actions of corticosteroids mediated by MR and GR in both their membrane-associated and nuclear subpopulations. I have three specific aims:

1. To investigate the multitude of non-genomic effects of corticosteroids in different brain areas and explore how these fit within the coordinated (rapid phase of the) stress response (*Chapter 2*).

2. To set up *in vitro* models to show the presence of a distinct membrane-associated population of the MR and further characterize its function, structure-function relationship and dynamics (*Chapter 3 and 4*).

3. To quantify the chromatin binding dynamics of the MR and GR within the nucleus and explore the effect of mutations within the receptors and different ligands on their DNA-binding dynamics (*Chapter 5 and 6*).

Thesis outline and approach

To investigate these three aims I will use a number of different experimental approaches.

In **Chapter 2** we will investigate available knowledge concerning the multitude of rapid non-genomic corticosteroid actions within different brain areas. We focus on the timing of the effects in each brain area, their interaction with delayed genomic effects and explore what this implies for the known behavioral and hormonal effects known to be associated with these brain areas.

Corticosteroids inhibit potassium A-type currents in a rapid, non-genomic and membrane-MR dependent fashion in hippocampal neurons. In **Chapter 3**, we will explore the potential of using neuronal-like cell lines, NS-1 and N1E-115 cells, as an *in vitro* setting to study a similar rapid potentiation of potassium currents by corticosterone in cells with or without MR protein expression. We show that the potassium A-type currents in NS-1 cells are inhibited by corticosterone in a membrane-initiated and MR-dependent fashion. The slow-inactivating potassium currents in N1E-115 cells are not affected by corticosterone. We further describe an instability of MR protein in *in vitro* settings.

Next, in **Chapter 4** we describe a novel approach to deduce whether a subpopulation of the MR is associated with the cell membrane. In these experiments we

use TIRF microscopy to specifically image fluorescently tagged MR at the cell membrane. Furthermore, we analyze the dynamics of this membrane-associated MR subpopulation with single-molecule analysis. Using these approaches, we find that near the membrane a distinct MR subpopulation is present, with dynamics substantially different from the cytoplasmic MR population.

In **Chapter 5** we use SMM and combine this with FRAP to derive a comprehensive characterization of the dynamics of nuclear GR. With this combined approach we obtain a reproducible quantification of the DNA-binding behavior of activated GR. When bound to potent agonists, GR molecules undergo frequent but transient immobilization due to DNA binding. Both the frequency and duration of DNA-binding are reduced when GR is bound by antagonists or, more surprisingly, by less potent agonists. We find evidence for specific ligand-receptor interactions to underlie differences in the GR's affinity for DNA. Furthermore, we study the effect of deletions of functional domains of the GR to show that receptors devoid of (direct) DNA-binding capacity indeed show reduced DNA binding.

In **Chapter 6** we build upon the approach and findings from **Chapter 5** and study the dynamics of nuclear MR with the same combination of SMM and FRAP. As for the GR, we find that also the MR shows more and longer DNA-binding when activated by its most potent agonists and reduced frequency and duration of these interactions when bound by antagonists or less potent agonists.

Finally, all results are discussed in a broader context in **Chapter 7**.