Corticosteroid receptor dynamics : analysis by advanced fluorescence microscopy

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Chapter 7

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7.1 Summary of main conclusions

Despite considerable knowledge regarding the molecular basis of corticosteroid actions within target cells, much still remains poorly understood. The molecular and cellular effects of corticosteroids ultimately determine their actions at the tissue and organism levels and a detailed understanding is required to understand how corticosteroids promote adaptation to stress. In this thesis I aimed to explore further finesses in the cellular dynamics of the MR and GR in both their membrane-associated and their nuclear subpopulations. I specified three aims.

1. To investigate the non-genomic effects of corticosteroids in different brain areas and to explore how these effects fit within the onset of the stress response.
2. To set up *in vitro* models to show the presence and function of a distinct membrane-associated population of the MR.
3. To characterize the chromatin binding dynamics of the MR and GR and to explore the effect of mutations within the receptors and of different ligands on their nuclear dynamics.

In *Chapter 2* I evaluated the current state of knowledge regarding the non-genomic actions of corticosteroids through membrane-associated receptors and their relevance for brain functioning. One of the most striking conclusions was that rapidly after stress, corticosteroids affect the excitability of multiple limbic brain areas, but in different response patterns over various time domains. These patterns include rapid non-genomic and slower genomic actions of the hormones that are mediated in a complementary manner by MR and GR. In addition, I discussed the rapid, non-genomic effects of corticosteroids on endocrine output and behavior and found that these effects correlate well with the observed patterns of neuronal excitability changes. Finally, I addressed the current state of knowledge regarding the underlying signaling cascades of these steroid effects and listed the main caveats in the current knowledge. For example, the regulation of MR and GR translocation to the membrane is still elusive, as is the proportion of the membrane population involved and its potential localization in specialized membrane compartments.

In *Chapters 3 and 4* we developed *in vitro* models to study the molecular pathways underlying the non-genomic effects of corticosteroids. In *Chapter 3* we showed that NS-1 cells have potassium A-type currents upon NGF-induced differentiation. These A-type currents are inhibited by corticosterone and cort-BSA within minutes, but only when the MR was present. We thus showed that the MR is required and sufficient for this rapid corticosteroid action. Moreover, this effect is specific for some subtypes of potassium channels. In N1E-115 cells, another type of potassium currents were observed: Kv3-generated slowly-inactivated currents. These currents were not affected by corticosterone. We also observed a remarkable instability of MR protein in our in vitro models. In *Chapter 4* we used TIRF in combination with

SMM to explore whether this combination of imaging techniques is a valid method to show and explore the membrane localization of YFP-tagged MR. One of our main findings here was that the MR shows a larger slow diffusing fraction near the membrane in two cell lines; CHO and COS-1 cells. As membrane-associated proteins are known to diffuse much slower than cytoplasmic proteins, this is a strong indication for the existence of a membrane-associated subpopulation of the MR. The mobility of the membrane-associated population was not affected by hormones.

In *Chapters 5 and 6* I explored the nuclear subpopulation of both GR (*Chapter 5*) and MR (*Chapter 6*) with a combination of SMM and FRAP. Here I found that the combination of both imaging techniques gave a detailed and reproducible quantification of the intranuclear dynamics of both receptors. Both GR and MR showed free diffusion within the nucleus interspersed with short (presumptively nonspecific) and long (presumptively specific) DNA-binding events. GR deletion mutants devoid of (most) DNA-binding showed a stark reduction of these DNA-binding events. The mobility pattern of the MR and GR were highly similar when bound to a high affinity agonist. When bound to an antagonist, both receptors showed less frequent nonspecific binding and less frequent and shorter specific DNA-binding events. Interestingly, intermediate patterns were seen for the GR and MR bound to less potent agonists, and this was correlated with steroid structure. Due to differences in their ligand-binding pocket, different steroid side-groups affected the DNA-binding of the MR and GR. This suggests that specific ligand-receptor interactions strongly affect the affinity for DNA binding in a receptor specific manner.

In conclusion, in this thesis I described a set of experiments that focuses on the function and the dynamics of the two phases of the (cellular) stress response: membrane-initiated / non-genomic and nuclear / genomic actions. The main results are graphically illustrated in Figure 7.1. In the next sections I will discuss a number of interesting observations we made in more detail.

7.2 The presence of the MR at the plasma membrane

In *Chapters 3 and 4* we set up two different *in vitro* models to study the role of the membrane-associated subpopulation of the MR in the rapid non-genomic actions of corticosterone. First, in *Chapter 3* we found that in differentiated NS-1 cells MR-transfection is required for a rapid reduction in potassium A-type current amplitudes by corticosterone. Importantly, we found here that a similar reduction in A-type amplitude is obtained with an equivalent dose of cort-BSA, which is membrane impermeable. These results thus strongly suggest that the MR is present at the cell membrane. In *Chapter 4* we studied the mobility of YFP-tagged MR in COS-1 and CHO cells with a combination of imaging techniques. Here we found that a larger fraction of YFP-MR molecules shows very slow diffusion when imaged near the membrane (with TIRF microscopy) than when imaged in the cytoplasm (with

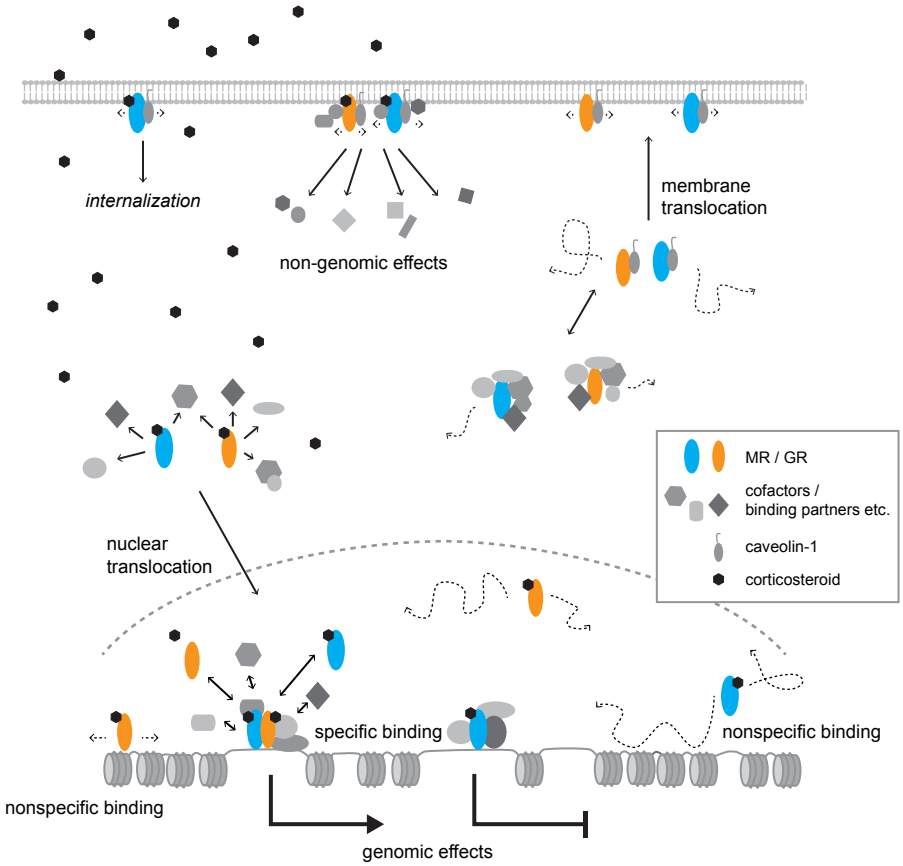

Figure 7.1: Cellular corticosteroid effects through the MR and GR
Without hormone, the bulk of the population of the MR and GR is present within the cytoplasm where it is bound to chaperones. Presumably, a small fraction of cytoplasmic receptors associate with caveolin-1, which induces membrane translocation. As discussed in *Chapters 2, 3, and 4*, at the membrane, hormone binding to the receptors attracts new binding partners and results in the activation of non-genomic signaling pathways. Putatively, ligand association also leads to receptor internalization. Ligand also reaches the cytoplasm where it results in the release of the receptors chaperones and nuclear translocation. Within the nucleus, the receptors interact with chromatin in either ultrashort, short or longer binding events. The first two likely represent nonspecific receptor-DNA interactions and aid in the search for specific binding sites. Also at specific binding sites the receptors interact only transiently with the DNA, as do all cofactors. Specific DNA-binding can occur as either homodimers (not shown), heterodimers or monomers. Ultimately, both MR and GR induce the transactivation and transrepression of a large set of responsive genes; i.e. genomic effects. The experiments presented in *Chapters 4, 5 and 6* suggest that the MR shows mostly very slow diffusion at the membrane, a mixture of slow and fast diffusion within the cytoplasm and both MR and GR show a mixture of fast diffusion and numerous immobilization due to chromatin binding within the nucleus. This is indicated by the dotted lines in the figure.

wide-field microscopy). Membrane-bound proteins generally show a much lower mobility than cytoplasmic proteins (Owen et al., 2009), thus also these findings support the notion of a membrane-bound subpopulation of the MR.

Our findings are in line with the available literature. Numerous studies have shown that cort-BSA was effective and intracellular corticosterone ineffective in mimicking rapid MR-dependent corticosterone effects on glutamate transmission in the brain (Karst et al., 2005; Olijslagers et al., 2008). The presence of a membrane-associated MR has also been convincingly demonstrated using synaptosome extracts and at neuronal membranes using electron microscopy (Prager et al., 2009; Qiu et al., 2010). Within neurons, the MR appeared to be enriched at the presynaptic and postsynaptic membranes. However, the how and why of membrane-association of the MR remain largely unknown. Regulation of membrane translocation of the ERα has been studied in considerable detail. At the membrane, ERα has been found primarily in caveolae, and binding to HSP27, palmitoylation and association with caveolin-1 were all shown to be essential for translocation to the plasma membrane (Razandi et al., 2002, 2010; Acconcia et al., 2005). The MR also binds caveolin-1 directly and seems to associate with lipid rafts (potentially caveolae) (Grossmann et al., 2010; Pojoga et al., 2010b). This suggests that the MR is transported towards caveolae by caveolin-1 association as well, but direct evidence is still lacking. The motif required for palmitoylation of the ERα is conserved among many steroid receptors and was shown to be required for membrane translocation of the AR, PR and ERβ (Pedram et al., 2007). However, the MR lacks the essential cysteine and can therefore not be palmitoylated at this sequence. Consensus palmitoylation sequences are found elsewhere in the MR (Ren et al., 2008), but the MR was never shown to be palmitoylated. Alternatively, the MR could use another pathway for translocation to the membrane.

A characterization of the essential steps in MR membrane association is thus still pending. Moreover, many questions exist regarding the signal partners the MR may associate with at the membrane and it is still incompletely understood whether the MR (and other steroid receptors) is located in the outer or the inner leaflet of the membrane (see *Chapter 2* for further discussion on these issues). Finally, for the ERα it is predicted that 5–10 % of the population exists at the membrane (Chambliss et al., 2000). A similar or smaller percentage is expected for the MR, but remains to be established and will probably depend on ligand binding and cell context as well (Wang and Wang, 2009; Karst et al., 2010). With the combination of TIRF and SMM we were already able to see a distinct diffusion of the MR near the membrane. In future studies, these experiments could be supplemented with disruptions of caveolin-1, palmitoylation or membrane compartments (lipid rafts). This would shine further light on the membrane translocation pathway of the MR.

7.3 *In vitro* MR expression

In *Chapter 3* we came across some important issues regarding the stability of the MR in *in vitro* settings.

i) First, we used MR transfection in the (non MR-expressing) cell line NS-1. Strikingly, despite successful DNA transfection, we could not detect MR protein in this cell line. MR mRNA was expressed in NS-1 cells after transfection but this did not result in detectable MR protein levels, as assessed by numerous biochemical approaches: Western blot, immunofluorescence staining, detection of YFP-tagged MR and transactivational assays. Functionally, we did observe an MR-specific effect of corticosterone on potassium A-type currents in a large, well-controlled data set at first, but failed to reproduce these effects in a second smaller data set. This discrepancy we cannot explain. In *Chapters 3, 4 and 6* we used a number of other non MR-expressing cell lines: COS-1, CHO and N1E-115. In these three cell lines expression of the MR or YFP-MR was detected after transfections. The lack of MR protein expression thus appears cell line specific.

ii) Secondly, we encountered problems with MR expression during stable transfection in another cell line: CHO cells. We attempted to induce stable expression of YFP-tagged MR in CHO cells. While the procedure led to successful protein expression in a control experiment (transfection of YFP-YFP) in 3 out of 4 clones, for YFP-MR all YFP-positive clones only had fragments of the MR attached to the YFP. This strongly suggests that MR expression is selected against in these cells.

iii) Thirdly, we tested MR protein expression in a cell line that had been stably transfected with MR by the Grossmann-Gekle group (Krug et al., 2002; Grossmann et al., 2005). However, we could not detect MR protein within these cells and also the original investigators had noted a regression of MR levels in these cells even while grown in selection media (personal communication with C. Grossmann), again suggestive of selection against MR expression during cell division.

iv) In line with our observations, unexpected instability of the MR has been published by the Gomez-Sanchez group as well (Gomez-Sanchez et al., 2006). While testing an array of new MR-antibodies, this group found that the MR is easily degraded during *in vitro* handling. Thawing tissue samples (under protection of protease inhibitors and while kept cold) resulted in partially degraded MR, while such procedures kept other steroid receptors intact. Together, these and our observations suggest a remarkable instability of the MR protein and a selection against MR expression within (dividing) cell lines. One could expect that these types of observations have been made by others as well and more publicity on this issue would be valuable for the field. Our current studies were not directed towards this question and at present we can therefore merely speculate about underlying biochemical or functional mechanisms.

Regarding the lack of (detectable) MR protein expression in NS-1 cells in the light of detectable mRNA expression, a biochemical explanation remains elusive. The failure to detect a protein while mRNA is expressed could be caused by either an inhibition of mRNA translation or by protein degradation. First, microRNA induced inhibition of MR mRNA seems unlikely as most microRNA recognition sites for

the MR are found on its 3′ UTR (de Kloet et al., 2009; Sõber et al., 2010), which is not included in the MR plasmids used. Evidence for putative enhanced protein degradation of the MR is lacking as well. Steroid receptors are normally protected from degradation by chaperones, and therefore enhanced MR degradation is seen when the key chaperone HSP90 is inhibited (Faresse et al., 2010). However, HSP90 is expressed in NS-1 cells and the chaperone complex is highly homogenous between steroid receptors. Thus a lack of (common) chaperones is not expected in NS-1 cells in the light of functional expression of the GR, PR and ERs in these cells (MacLusky et al., 2003; Morsink et al., 2006).

Regarding the selection against MR expression during the generation and maintenance of stable cell lines, some speculations can be made. Functionally, MR is found to be protective and anti-apoptotic rather than detrimental, at least in neurons (Gass et al., 2000; Gomez-Sanchez and Gomez-Sanchez, 2012; Munier et al., 2012). However, MR expression is generally restricted to well-differentiated tissues and associated with cellular differentiation (Le Menuet et al., 2012). In line with this, within dividing cell lines endogenous expression of the MR is rare and mostly restricted to a small number of renal derived cell lines (Faresse et al., 2012; Hori et al., 2012). Of note, this is in contrast to other steroid receptors which do show widespread expression in commonly used cell lines (see for example Horwitz et al., 1975; Kao et al., 2009; Polman et al., 2012). Hypothetically, MR expression could be incompatible with undifferentiated, fast dividing cells and therefore selected against in (some) cell lines.

7.4 Advanced imaging methods to examine protein function and localization

In *Chapter 4* I presented a novel approach to test for membrane presence of the MR by a combination of SMM and TIRF microscopy. TIRF microscopy is an adaptation to wide field fluorescence microscopy. In TIRF, the excitation laser is redirected to exit the objective at a large angle relative to the optical axis and is totally internally reflected at the glass-medium interface. As a result an evanescent wave field is created that excites fluorophores in a very small (60–100 nm) section above the glass-medium interface (Axelrod et al., 1983; Axelrod, 2001; Martin-Fernandez et al., 2013). As such, TIRF is the method of choice to image membrane-associated molecules in the scope of unwanted cytoplasmic background. TIRF has been successfully combined with FRAP, FCS and especially SMM (see for an overview Axelrod, 2008). We were the first to utilize a combination of TIRF / wide field microscopy and SMM to distinguish between membrane-associated and cytoplasmic proteins. With this combination of techniques we found that a larger fraction of YFP-MR molecules shows slow diffusion when imaged in TIRF (imaging both membrane-associated and cytoplasmic MRs) than when imaged in wide field (negligible contribution of

membrane-associated MR). Importantly, we found that the shift in population distribution was not due to the smaller Z-depth in TIRF mode. This is strongly suggestive of the existence of a membrane-associated subpopulation. However, we found that short-term hormone treatment did not affect the dynamics of the MR when imaged in TIRF, indicating that ligand activation of the putative membrane-associated population of MRs does not change its mobility. However, our pioneering study does show great potential of combining imaging techniques to deduce protein localization and function.

In *Chapters 5 and 6* we used SMM in combination with quantitative modeling of FRAP with Monte Carlo simulations to quantify the DNA-binding dynamics of the GR (*Chapter 5*) and the MR (*Chapter 6*). More than a decade ago FRAP studies first demonstrated the high dynamics of steroid receptors and other transcription factors within the nucleus (McNally et al., 2000; Stenoien et al., 2000; Schaaf and Cidlowski, 2003; Farla et al., 2004). However, quantification of this dynamic behavior has been a major challenge. Quantitative analysis of FRAP is possible, but requires *a priori* predictions, careful control for laser properties and complicated mathematical models (van Royen et al., 2009b; Mueller et al., 2010, 2013). In addition, FRAP is not very accurate at predicting fast protein diffusion. SMM has an important advantage over FRAP and associated techniques in that the quantitative analysis requires fewer *a priori* assumptions. In addition, SMM has a very high temporal and spatial resolution and is therefore more accurate in describing the dynamics of fast diffusing proteins. A disadvantage of SMM is its shorter maximal time length (up to several hundred milliseconds maximally), which makes the combination with FRAP even more valuable (see for a comparison of imaging techniques: Table 1.1, *Chapter 1*). A combination of two or more independent imaging techniques is widely recognized as the most powerful approach to overcome modeling errors (Mueller et al., 2013; Voss and Hager, 2014). We found that the combination of these two independent quantitative models gave a very extensive and, most importantly, consistent quantification of the DNA-binding dynamics of both receptors. Throughout our studies we tested 18 experimental groups (including the MR and GR bound by a variety of ligands and multiple GR (deletion) mutants). For these 18 groups the quantification of the (combined) DNA-bound fraction was performed independently by SMM and FRAP and we found that the two approaches were on average within 6.5 ± 1.1 % accuracy of each other. In addition, the combination of SMM and FRAP is valuable as it gave us a very complete overview of the dynamics of the MR and GR within the nucleus, from the millisecond to the minute time range. SMM has the temporal and spatial resolution to accurately predict diffusion coefficients of the freely diffusing fraction, while FRAP provides information over an extended time range to predict average DNA-binding times. In another study (van Royen et al., 2014), the accuracy of SMM to predict diffusion coefficients was compared to FCS. FCS is very sensitive for fast diffusing proteins and FCS analysis replicated the diffusion coefficient of the diffusing subfraction with high accuracy for the AR (aver-

age difference of 0.4 ± 0.04 μm²/s,vanRoyenea2014). In addition to our current work a number of recent studies have been published that used combinations of FRAP with SMM and/or FCS (Stasevich et al., 2010a; Mazza et al., 2012; van Royen et al., 2014). For example, Mazza et al. (2012) used SMM to guide the choice of modeling parameters for FCS and FRAP and thereby restrict the degrees of freedom that made the analysis of these imaging approaches so variable in the past.

In conclusion, advanced fluorescence microscopy techniques have shown their merits for the study of many classes of proteins, including membrane-bound or nuclear proteins. New approaches such as SMM enable more precise quantifications of protein dynamics with a high temporal and spatial resolution. As each analysis method has its biases, a combination of multiple functional imaging approaches limits these biases to skew the outcome and should be common procedure in quantitative studies on protein dynamics.

7.5 Towards a unifying model of steroid receptor DNA-binding dynamics

In *Chapter* 5 we used a combination of SMM and FRAP to quantify the intranuclear dynamics of the GR, and in *Chapter 6* we used the same experimental approach to study the dynamics of the MR. Here, we found that the diffusion behavior of both receptors in their ligand-activated state was best described by the existence of a single freely diffusing and multiple DNA-bound states. For example, GR bound to a potent agonist (such as Δ-fludrocortisone or dexamethasone), spends $\sim 50\,\%$ of the time diffusing freely through the nucleus, intermitted by DNA binding for either ~ 0.5 second ($\sim 30\,\%$) or 2 to 3 seconds ($\sim 20\,\%$). A highly similar pattern of DNA-binding events was identified for the MR bound to corticosterone, cortisol or aldosterone.

Nonspecific and specific DNA-binding

As expected, GR mutants deficient for DNA-binding (the ΔDBD and ΔLBD mutants) had much reduced frequency and duration of the DNA-binding events, but in addition they also showed a higher effective diffusion rate. Whenever we observed a less mobile receptor (GR and MR bound by less potent agonists, antagonists or deletion mutants), this was always accompanied by both less frequent and shorter DNA-binding events and a higher diffusion coefficient. These observations led us to postulate that a further DNA-binding event was hidden in the diffusing fraction of the receptor: ultra-short DNA-binding for < 6.25 ms (our imaging time interval). Such ultra-short (millisecond) interactions with the DNA have been recognized for other transcription factors as well (Elf et al., 2007; Hammar et al., 2012). We thus presume that the dynamics of the MR and GR within the nucleus is characterized

by (1) free diffusion, (2) ultra-short DNA interactions (< 6 milliseconds), (3) short DNA interactions (∼0.5 second) and (4) long DNA interactions (2–3 seconds).

There is still active debate regarding the proportion of specific and nonspecific DNA binding events for transcription factors (extensively discussed in Mueller et al., 2013). There are several indications to suggest that the two shorter DNA binding events identified for the MR and GR represent predominantly nonspecific binding to chromatin. Van Royen et al. (2014) examined the dynamics of the AR by SMM and FRAP in a similar design as described in this thesis and reported the same three (putative) DNA-binding events. In these studies an AR point mutation that disrupted specific DNA-binding abolished only the longest binding event, while the shorter DNA-binding events were largely unaltered (van Royen et al., 2014). This finding is reminiscent to observations for other transcription factors showing preservation of a large fraction of DNA-binding events when only specific DNA-binding was inhibited (Elf et al., 2007; Sekiya et al., 2009; Mazza et al., 2012). Transcription factors, including steroid receptors, face the complicated task of finding their few target sites within the bulk of DNA (Hager et al., 2009). It is thought that frequent nonspecific binding events aid in this targeting task. Indeed, *in vitro* studies supported by theoretical modeling approaches have suggested that frequent low-affinity interactions with DNA increase the efficiency of transcription factor target finding, because such interactions may keep the transcription factor in close proximity to open DNA (Gowers et al., 2005; Elf et al., 2007; van den Broek et al., 2008; Hager et al., 2009). For this purpose two complementary modes of trafficking may occur. These include both intersegmental jumps with frequent binding and unbinding to the DNA as well as 50–100 bp scanning events where the transcription factor moves over the DNA (Gowers et al., 2005). We present a model that includes the set of DNA-interactions that fit our experimental observations in Figure 7.2A.

In addition, part of the longer-lasting binding events we identified will represent specific binding of the MR and GR to their target sequences. We found that both the ligand-activated MR and the GR spend ∼20 % of their time being bound to the DNA in a more prolonged fashion (approximately 2 to 3 seconds). This fraction of prolonged DNA binding was almost completely lost in DNA-binding deletion mutants. Antagonist-bound MR and GR do still show a fraction of < 1 second bound molecules, albeit at reduced frequency (6–10 %). Moreover, agonist structure affects both the frequency and the duration of the longest DNA-bound state for both the GR and MR. The exact relationship between steroid receptor DNA residence time and transcriptional output is not known. Gene transcription requires many subsequent events. The receptor dimerizes, attracts many co-factors and RNA polymerases and induces gene transcription as well as chromatin remodeling (Datson et al., 2008). It seems reasonable to hypothesize that formation of a more stable co-factor complex will lead to prolonged DNA-binding of a transcription factor, which will then affect local chromatin remodeling.

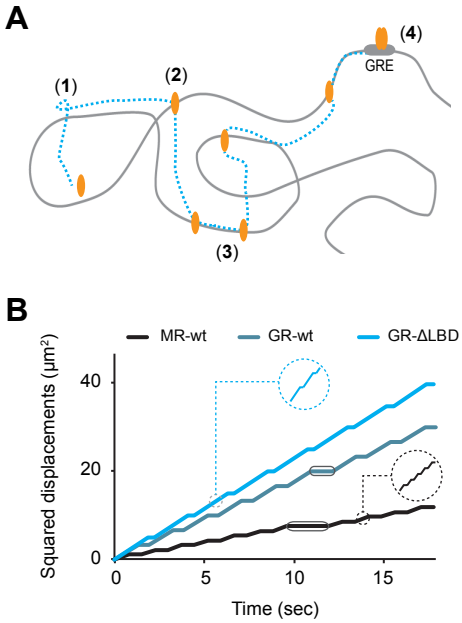

Figure 7.2: Dynamic interactions of the MR and GR at chromatin
(A) A schematic representation of the types of receptor-DNA interactions that are supported by our observation. The receptor shows a combination of (1) free diffusion, (2) ultrashort (< 6 ms) interactions with the DNA, (3) short (~0.5 s) interactions with the DNA that could represent 1D sliding along the DNA strands and least frequent (4) longer (> 2 s) interactions with the DNA that could represent specific binding to target genes. (B) Squared displacements of "perfectly average" MR and GR molecules (i.e. with the use of the average D_{fast}, binding times and fraction distributions as represented in Table 5.1, *Chapter 5* and Table 6.1, *Chapter 6*. This illustrates the difference in intranuclear dynamics of the MR and GR when bound by corticosterone and the GR-ΔLBD mutant. Corticosterone-bound GR has fewer nonspecific interactions (both ultrashort and short) with chromatin resulting in a larger distance traveled before finding a specific binding site (encircled) as compared to corticosterone-bound MR. The specific binding event is also shorter in duration. The GR-ΔLBD mutant lost most capacity for DNA-binding and only preserved a low frequency of ultrashort and short DNA-interactions while prolonged specific binding is lost altogether. Ultrashort DNA-interactions are 'hidden' within the diffusing fraction and result in a lower effective diffusion.

Steroid-receptor interactions determine the affinity for DNA

A main finding from our sets of experiments was that subtle differences in agonist structure have a profound effect on the frequency and duration of DNA-binding events of the MR and GR. For the GR, we identified two steroid side groups (the 17-hydroxyl and the 9-fluor groups) that, when present, induced more frequent and more stable GR-DNA interactions. For MR ligands, the 17-hydroxyl group is without effect, while presence of the 11-hydroxyl group is associated with a higher frequency of MR-DNA binding. We presumed that the more potent agonists make more connections to the amino acids lining the LBP and can therefore induce a stronger conformational shift of the receptor. Indeed, for the GR we found that mutation of a single amino acid in GR's LBP prevented the effect of the 9α-fluoro group (*Chapter 5*). Likely differences in ligand-induced receptors conformational shifts affect dimerization or cofactor binding and ultimately affinity for the DNA. We found that binding of a less potent agonist generally affects both the frequency of nonspecific DNA-interactions and the frequency and duration of binding to specific target sites. We expect that fewer nonspecific binding events could result in a longer search time for target sequence binding. A shorter duration of specific binding likely affects gene transcription and modulation. What the effect of such different chromatin-binding

dynamics entails on the level of gene regulation by the MR and GR remains to be established. Assessment of the chromatin-binding patterns (e.g. by ChiP studies) after stimulations with a set of different MR and GR agonists would be very valuable and could elucidate the relevance of nonspecific and specific DNA-binding for gene regulation.

When both receptors are bound to a potent agonist, the DNA-binding patterns of the GR and MR are very similar. This is in correspondence to the high sequence homology of their DNA-binding domains, which results in both receptors recognizing the same response elements (GREs). Of note, the longest binding event of agonist-bound MR and GR was much shorter in duration as what was observed for the AR. Van Royen et al. (2014) found a fraction of AR bound for $\sim$8 seconds, whereas we found $\sim$3 seconds as longest binding event for the MR and GR. The AR also binds to GREs, however differences known to exist in the groups of cofactors bound and in the conformation of the receptors could underlie the observed prolonged DNA residence times of the AR (Centenera et al., 2008; van de Wijngaart et al., 2012).

Notably, as the natural ligands corticosterone and cortisol have a lower affinity for the GR than for the MR, within physiological conditions the chromatin binding pattern of the two receptors will be very different. In response to its physiological ligand, the MR will be DNA-bound more often and for longer periods than the GR (illustrated in Figure 7.2B). This observation is in in agreement with *in vivo* uptake of tritium labelled corticosterone that shows a much longer retention in the nucleus of MR-expressing than GR-expressing tissues (Reul and de Kloet, 1985). These findings were corroborated with an immunohistochemical study showing also a longer retention of MR than GR in the nucleus (Conway-Campbell et al., 2007) and of dexamethasone-bound GR than corticosterone-bound GR (Stavreva et al., 2009). However, whether such a difference also exists on the level of DNA-binding itself still needs to be established. Interestingly, in one recent study, binding of the GR and MR was assessed for 10 genes (Polman et al., 2013). Here, a lower occupancy rate was found for the MR then for the GR (contrary to our predictions), but this set of genes was initially selected based on GR-binding, thus biased.

Box I Future questions

From the experiments presented in this thesis many new questions came up and some old ones remained partly unanswered. Here, I list a few of the most interesting questions that sprang up from the current studies.

From Chapter 2

1. *Within the scope of neuronal non-genomic effects of corticosteroids, how do these integrate with rapid signaling of other stress hormones; i.e. catecholamines and CRH?*
2. *How are non-genomic and genomic actions of corticosteroids integrated? This question remains to be answered on both the cellular and the organism level.*

From Chapters 2 to 4

3. *What steps are required for membrane translocation of the MR and GR? Is the MR palmitoylated as are other steroid receptors?*
4. *What fraction of both receptors associates with the membrane, and are they located in specific membrane subdomains?*
5. *Is the larger slow diffusing fraction of YFP-MR caused by enrichment for membrane-associated MR in TIRF?*
6. *Is the MR incompatible with cell division? If so, what is the best strategy to create stable MR-expression in vitro?*

From Chapter 5 and 6

7. *What is the result of changes in the frequency of nonspecific DNA interactions of the MR or GR on gene regulation?*
8. *What are the effects of differences in chromatin dwell time on the recruitment of cofactors, the stability of the RNA polymerase cycle and on chromatin modifications?*
9. *How do differences in the binding of agonists to the ligand-binding pocket affect the conformation of the receptor's functional domains?*
10. *With regard to the observed difference in the DNA-binding dynamics of the MR and GR when bound to endogenous corticosteroids. What would be the effect of MR and GR co-expression on their respective DNA-binding dynamics in the presence of endogenous corticosteroids. What is the role of MR-GR heterodimers?*