Corticosteroid receptor dynamics : analysis by advanced fluorescence microscopy

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Chapter **2**

Rapid non-genomic effects of corticosteroids through the membrane-associated MR and GR and their role in the central stress response

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Corticosteroids affect brain functioning through both delayed, genomic and rapid, non-genomic mechanisms. The latter mode of action was long known but only in recent years the physiological basis in the brain is beginning to be unravelled. We now know that corticosteroids exert rapid, non-genomic effects on the excitability and activation of neurons in (amongst others) the hypothalamus, hippocampus, amygdala and prefrontal cortex. In addition, corticosteroids affect cognition, adaptive behaviour and neuroendocrine output within minutes. Knowledge on the identity of the receptors and secondary pathways mediating the non-genomic effects of corticosteroids on a cellular level is accumulating. Interestingly, in many cases an essential role for the 'classical' MR and GR in a novel membrane-associated mechanism is found.

Here, we systematically review the recent literature on non-genomic actions of corticosteroids on neuronal activity and functioning in selected limbic brain targets. We will discuss the relevance of these permissive effects for cognition and neuroendocrine control, and the integration of this novel mode of action into the complex balanced pattern of stress effects in the brain. Subsequently, we will review the knowledge regarding the underlying molecular pathways addressing the following questions: How do the MR and GR translocate to the membrane and what are their signalling partners?

Corticosteroids play a major role in the response of the brain to stress. For many years, they were believed to be only responsible for the delayed and prolonged effects of stress, as opposed to monoamines and neuropeptides which were thought to establish rapid effects (de Kloet et al., 2005). While this is generally true, the picture is actually more complex. For instance, corticosteroids influence a wide range of behaviors and endocrine outputs within minutes, a timeframe that is too rapid to be explained by genomic effects (de Kloet et al., 1999; Haller et al., 2008). In agreement, we and others recently established that corticosteroids rapidly alter neuronal activity and excitability in a number of brain areas, providing a physiological basis for the rapid effects on behavior (Tasker et al., 2006; de Kloet et al., 2008). Many rapid effects are still mediated by the classical corticosteroid receptors, the MR and the GR, but by a subpopulation of these receptors, anchored at the membrane (Karst et al., 2005, 2010). The existence of such a rapid mode of action raises many new questions. Where in the brain do these rapid effects take place? Which receptors and pathways are involved in these effects? What are the functional consequences for cognition and neuroendocrine control? How are these rapid corticosteroid actions integrated with other components of the stress response? Equally important are the remaining molecular questions. How strong is the evidence for a membrane-localization of the MR and GR and for other types of (novel) membrane receptors. Also, as steroid receptors do not have a transmembrane domain, how do MR and GR associate with the plasma membrane? And finally, are there common downstream pathways. In this chapter we discuss our current understanding of rapid actions of corticosterone, with emphasis on their function within the brain.

2.1 Rapid effects of corticosterone in the brain

The rapid effects of corticosterone on brain and cognition have been subject of several recent reviews (Dallman, 2005; Tasker et al., 2006; de Kloet et al., 2008; Haller et al., 2008; Prager and Johnson, 2009; Evanson et al., 2010a). However, over the last two years a number of new studies have emerged that extend and challenge the existing views on the function and nature of these rapid effects. Here, we focus on the integration of these new findings in the existing theories on rapid corticosteroid signalling. The findings are discussed per brain area; i.e. the hypothalamus, pituitary, hippocampus, amygdala and frontal cortical areas. In the following sections, the major findings in these four different brain areas are summarized (see for overview Table 2.1). In this review we restrict ourselves to the non-genomic roles of the MR and GR within neurons. Both receptors have vital functions in the periphery and also here many non-genomic actions have been observed. However, these are beyond the scope of this review and have been described elsewhere (Boldyreff and Wehling, 2003; Grossmann and Gekle, 2009; Funder, 2010).

Hypothalamus

The PVN is one of the core structures in the HPA-axis. PVN neurons express high levels of GR, but virtually no MR. Indeed, through GR activation in the PVN corticosterone negatively feeds back on the HPA-axis in a delayed, genomic fashion (de Kloet et al., 1998). However, corticosterone also regulates HPA-axis activity in a more rapid time frame, through non-genomic actions (Jones et al., 1972; Dallman, 2005). Importantly, a recent study showed that this rapid inhibition can be induced by local infusion of dexamethasone or a membrane-impermeable conjugate of dexamethasone with bovine serum albumine (dex-BSA) into the PVN (Evanson et al., 2010b). This effect can be prevented by co-administration of an antagonist of the cannabinoid receptor type 1 (CB1) (Evanson et al., 2010b). Thus, at the level of the PVN, corticosterone rapidly reduces HPA-axis activation in a non-genomic, membrane-associated manner, involving endocannabinoid signalling.

Insight in the neurobiological substrate of these fast effects was provided by Tasker and colleagues. This group was the first to carry out detailed studies on the frequency of miniature excitatory postsynaptic currents (mEPSCs) in the PVN and the nearby supraoptic nucleus (SON) (Di et al., 2003). An mEPSC reflects the postsynaptic current resulting from the spontaneous release of a single glutamatergic vesicle from a presynaptic terminal (Bekkers and Stevens, 1989). Importantly, the frequency of these events (particularly in the absence of changes in mEPSC amplitude) is considered to be determined by presynaptic properties, reflecting changes in either release probability of the vesicles or changes in the number of synaptic contacts. Tasker and colleagues established that a high dose of corticosterone (between 100 nM and 1 μM) or its synthetic analogue dexamethasone reduces the frequency of mEPSCs in PVN neurons (Di et al., 2003; Malcher-Lopes et al., 2006). This effect was detectable within 5 minutes and did not reverse when corticosterone was washed out. Effectively, the excitability of PVN neurons was reduced by application of corticosteroids in a rapid but prolonged manner. Rapid changes in mEPSC frequency induced by corticosterone could not be blocked by MR or GR antagonists (Di et al., 2003, 2009). In contrast, preliminary data shows that they are prevented by conditional knockout of the GR gene within the hypothalamus and thus will involve the (membrane-associated) GR (Haam et al., 2010; Tasker and Herman, 2011). How this new finding should be integrated with the lack of effect of antagonists remains unclear and awaits further clarification in a full study. The effects within the PVN were further shown to be non-genomic, membrane-initiated and to involve G-protein coupled signalling. Interestingly, rapid corticosteroid actions required retrograde endocannabinoid signalling and the CB1 receptor. The presumed cellular signalling pathway is visualized in Figure 2.1A. Since the CB1 receptor is also required for rapid inhibition of the HPA-axis (Evanson et al., 2010b), the rapid inhibition of mEPSC frequency (and thus excitability) of PVN neurons could provide the cellular substrate for this phenomenon.

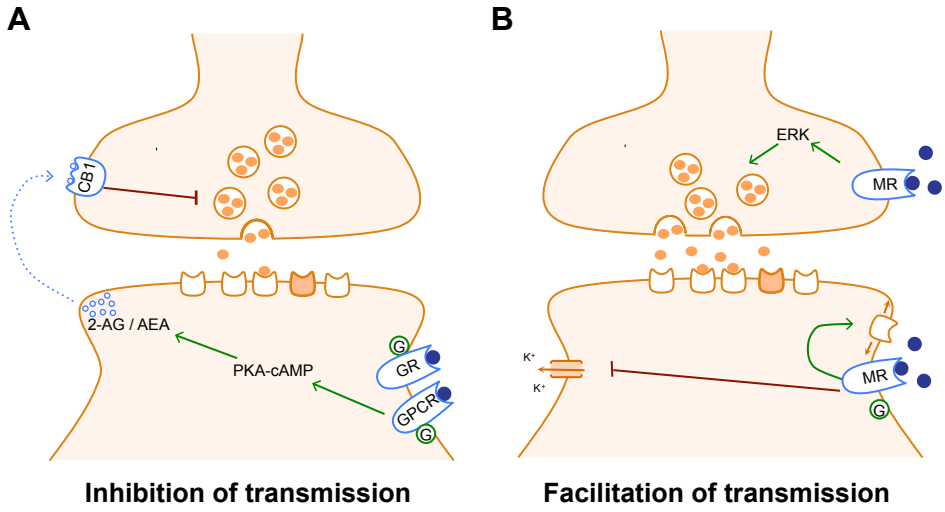

Figure 2.1: Schematic representation of the synaptic pathways of corticosterone-induced rapid effects on glutamatergic transmission
(A) Inhibition of glutamatergic transmission is initiated by postsynaptically located receptors; this can be either G-protein coupled receptors (hypothalamus) or membrane-localized GRs (amygdala). Activation of these receptors by corticosterone induces activation of G-proteins and the cAMP-protein kinase A (PKA) pathway, which eventually induces synthesis of the retrograde messengers anandamide (AEA) and 2-arachidonoylglycerol (2-AG). In a retrograde mode of action at the presynaptic terminal 2-AG and AEA activate the cannabinoid receptor type 1 (CB1), which in turn inhibits the release probability of glutamatergic vesicles. (B) Facilitation of glutamatergic transmission is initiated by both pre- and post-synaptically located membrane-MRs. Presynaptically, activation of the MR by corticosterone activates an extracellular signal-regulated kinase (ERK) pathway resulting in stimulation of the release probability of glutamate vesicles. At the same time, postsynaptic activation of a membrane-associated MR inhibits potassium I_A-currents, and stimulates membrane diffusion of AMPA receptors. All three effects together result in a facilitation of glutamatergic transmission.

However, rapid inhibitory effects of corticosterone in the PVN are not restricted to vasopressin- and CRH-containing parvocellular neurons, but they are seen in all neuronal populations (parvocellular and magnocellular) in the PVN (Di et al., 2003, 2005; Tasker et al., 2006). In the magnocellular neurons in the PVN and SON, a second effect was observed on the spontaneous release of *gamma-aminobutyric acid* (GABA), the main inhibitory neurotransmitter. The frequency of mIPSCs (miniature inhibitory postsynaptic currents) was rapidly increased by dexamethasone, but this required even higher concentrations (1 µM or more) (Di et al., 2005, 2009). Functionally, this suggests a more general coordinative role for the non-genomic effects of corticosterone in the hypothalamus, which requires further specification (Tasker et al., 2006).

Pituitary

Fast and delayed effects of corticosteroids have also been observed at the level of the anterior pituitary gland, where GR is abundantly expressed and MR levels are quite low (Reul et al., 1990). Already in the 1970's and 80's both rapid and delayed actions

of corticosteroids on pituitary ACTH release were reported (Jones et al., 1972; Widmaier and Dallman, 1984). Inhibition of ACTH release was seen as early as 1 minute and as late as 2 hours after corticosteroid administration. The latter is a genomic action mediated by GR-driven gene transcription, while the former action was insensitive to protein synthesis inhibitors and thus mediated by non-genomic pathways (Keller-Wood and Dallman, 1984). Interestingly, the rapid inhibition of ACTH release was only seen when corticosterone levels were rapidly rising and not when they were already high, suggesting that this feedback is rate-sensitive (Jones et al., 1972; Kaneko and Hiroshige, 1978).

The cellular basis of the rapid effects is not well established and controversy remains about the receptor mediating the effects. On the one hand, pretreatment with a GR antagonist did not prevent the rapid effects of corticosterone on CRH-induced ACTH secretion *in vivo* (Hinz and Hirschelmann, 2000). Also, in a pituitary-derived cell line a membrane binding place for dexamethasone and corticosterone was identified that did not have any affinity for the GR-antagonist RU486 (Maier et al., 2005). However, another line of evidence does suggest a role for the classical GR in mediating rapid feedback at the pituitary. Thus, a rapid and non-genomic translocation of annexin-I by dexamethasone was prevented by GR-antagonist treatment in a pituitary derived cell line (Solito et al., 2003). This translocation of annexin-I was required for rapid inhibition of ACTH release (Buckingham et al., 2003; Tierney et al., 2003). Thus, corticosterone rapidly inhibits ACTH release from the pituitary, but whether this is due to a novel receptor or to the classical GR is still controversial. This rapid inhibition is also seen in control human subjects, while it is absent in depressed patients, suggesting that the rapid negative feedback is somehow associated with disease (Young et al., 1991).

Hippocampus

Adaptation to a stressful situation is a coordinated effort mediated by the limbic system —the hippocampus and amygdala— in coordination with the prefrontal cortex (see Figure 2.2). This, among other things, involves projections of these areas to and hence control over the PVN (Ulrich-Lai and Herman, 2009). Collectively, these areas also facilitate the formation of a memory trace of a stressful emotional event. Processing of contextual information depends predominantly on hippocampal function. The hippocampus expresses high levels of both MR and GR in all subfields (except its cornu ammonus-3 (CA3) region that mainly expresses MR) (Reul and de Kloet, 1985). Corticosterone exerts strong genomic effects on the activity and plasticity of all hippocampal subfields as well as on hippocampus-dependent memory (McEwen, 2001; Kim and Diamond, 2002; Mirescu and Gould, 2006; Joëls, 2008). Low levels of corticosterone, through MR activation, facilitate plasticity and hippocampus-dependent memory (Diamond et al., 1992). By contrast, absence or very high levels of corticosterone inhibit plasticity; the latter is mediated through the GR (Alfarez et al., 2002; Kim et al., 2004).

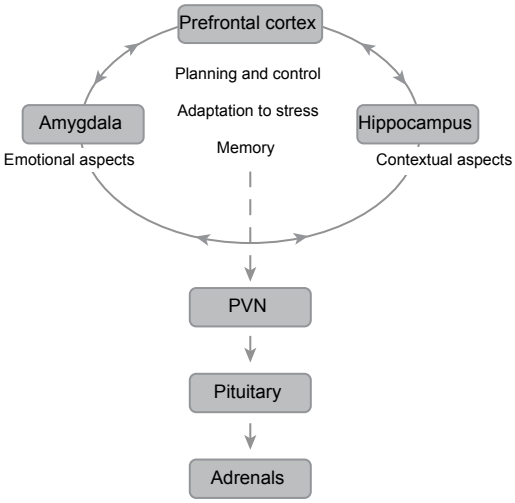

Figure 2.2: Brain circuitry of stress
The limbic system is implicated in adaptation, learning & memory processes, mood, and control of the HPA-axis. The hormones of the HPA-axis coordinate information processing and promote connectivity between amygdala, prefrontal cortex and hippocampus to facilitate behavioral adaptation. Projections from the limbic structures innervate the PVN network and regulate transsynaptically the activity of the HPA-axis.

Similar to neurons in the hypothalamus, hippocampal neurons spontaneously show mEPSCs. In a first study (Karst et al., 2005), the effect of corticosterone was examined on mEPSC frequency in the CA1 region of the hippocampus. It appeared that within 5 minutes of corticosterone administration, the frequency of mEPSCs is significantly *enhanced*, i.e. changed in a direction opposite to that observed in the PVN. The amplitude was unaffected (Karst et al., 2005; Olijslagers et al., 2008) (see Figure 2.3A,B). This effect was recently reproduced by other investigators (Qiu et al., 2010) and granule neurons in the dentate gyrus respond similarly to corticosterone as CA1 neurons (Pasricha et al., 2011). Similar to the corticosteroid effect in the hypothalamus, the rapid effect in the hippocampus does not depend on gene transcription and involves a membrane-located receptor (Karst et al., 2005). However, further studies established profound differences between rapid responses to corticosterone in the hippocampus compared to the PVN. The increased mEPSC frequency in hippocampus rapidly reversed when corticosterone was washed out. Also, in the CA1 the effect occurred at a 10-fold lower dose of corticosterone (10 nM or higher) than in the hypothalamus. Importantly, corticosterone efficiently enhanced mEPSC frequency in the hippocampus of wild type and GR knockout mice, but the effects were completely abolished in MR knockout mice, supporting that rapid effects in the hippocampus are mediated by MRs (Karst et al., 2005). This was confirmed with specific MR and GR antagonists. Importantly, the membrane-located MR appears to have a lower affinity than the cytosolic form (Karst et al., 2005), so that it potentially could play an important role when corticosteroid levels rise, shortly after stress (Joëls et al., 2008). Follow-up studies suggested that rapid corticosteroid effects involve MRs inserted into the presynaptic membrane (Olijslagers et al., 2008) (see Figure 2.1B). This is backed up by preliminary evidence that shows localization of the MR in the plasma membrane of hippocampal neurons, co-localized with the presynaptic marker synapsin I (Qiu et al., 2010).

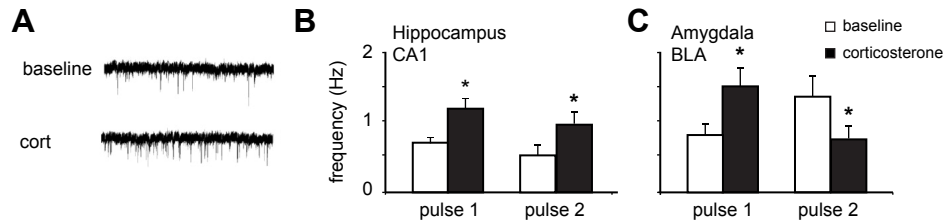

Figure 2.3: Effect of two pulses of corticosterone on mEPSC frequency in the CA1 region and the basolateral amygdala (BLA)
(A) Typical traces of mEPSC pulses recorded from hippocampal neurons before (white bars) and after (black bars) treatment with 100 nM corticosterone. (B) In hippocampal CA1 neurons exposure to two consecutive pulses of 100 nM corticosterone (1 hour apart) both induce a reversible increase in mEPSC frequency. (C) In amygdalar BLA neurons, the first pulse of corticosterone induces an increase in mEPSC frequency, this increase is not reversible. For the second pulse of corticosterone the basal mEPSC frequency is already elevated and the second pulse induces an irreversible decrease instead. mEPSC, miniature excitatory postsynaptic current. $* p < 0.05$ compared with baseline (paired t test). *Figure reprinted with permission from Karst et al. (2010).*

Corticosterone also affects two postsynaptic features of CA1 neurons through the MR. Firstly, corticosterone was found to inhibit postsynaptic I_A-currents, an effect that could be blocked with an MR-antagonist (Olijslagers et al., 2008). I_A-currents are potassium currents that are negative regulators of neuronal excitability and plasticity (Hoffman et al., 1997; Yuan et al., 2002). Consequently, the inhibition of these currents by corticosterone is expected to stimulate excitability and plasticity of hippocampal neurons. Secondly, corticosterone stimulated, within 5 minutes, lateral diffusion of *α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid* (AMPA) receptors in cultured hippocampal neurons (Groc et al., 2008). This effect also turned out to depend on membrane-localized MRs (Groc et al., 2008). Potentially, a more mobile pool of AMPA receptors facilitates the induction of synaptic plasticity. All of these studies support a membrane-localised form of the MR as main mediator of rapid corticosteroid signalling in the hippocampus. Overall, corticosterone seems to rapidly potentiate the excitability of hippocampal neurons via membrane-MRs located on both pre- and postsynaptic sites, thus priming the hippocampal circuit for subsequent stimulation by context-dependent factors.

However, not all rapid effects in the hippocampus involve the MR. First, a non-genomic increase in spine density of hippocampal neurons was found to depend on GRs rather than MRs (Komatsuzaki et al., 2005). Yet other rapid corticosterone effects occur independent of MR or GR and therefore could be mediated by a novel (so far not identified) membrane-localized receptor. This applies to rapid stimulatory corticosterone effects on inhibitory transmission (Hu et al., 2010), on levels of extracellular excitatory amino acid (Venero and Borrell, 1999), long-term potentiation (LTP) induction (Wiegert et al., 2006) and *N-methyl-D-aspartatic acid* (NMDA)-dependent neurotoxicity (Xiao et al., 2010). Some studies also reported inhibitory actions of corticosterone on NMDA signalling (Sato et al., 2004; Liu et al., 2007). Apparently, corticosterone affects hippocampal signalling in multiple ways, involving membrane-located MRs, GRs and other, still unknown receptors.

Amygdala

Stressful events invariably activate the amygdala, the brain's principal emotional centre (Roozendaal et al., 2009). The amygdala expresses both MR and GR in its various subnuclei (Reul and de Kloet, 1985) and amygdala-dependent memory, such as cued learning and emotional memory is very sensitive to stress and corticosteroids (Roozendaal et al., 2009). Interestingly, genomic effects of corticosterone on the amygdala are generally opposite to those seen in the hippocampus, with enhanced activity in the former (Duvarci and Pare, 2007; Mitra and Sapolsky, 2008) and reduced activity and plasticity in the latter (Alfarez et al., 2002, 2009; Kim et al., 2004). In addition, the amygdala is one of the main targets of the adrenergic system. Many corticosteroid effects on amygdala functioning in fact require adrenergic signalling (Roozendaal et al., 2009). This interaction might in part be mediated by non-genomic effects of corticosteroids. For instance, a systemic injection of corticosterone directly after a learning task rapidly (within 15 minutes) increased the levels of noradrenaline in the basolateral amygdala (BLA) and this was correlated to the later facilitation of fear memory by corticosterone (McReynolds et al., 2010).

An important finding that raised interest in non-genomic actions of corticosterone in the amygdala was the demonstration of MR and GR at the plasma membrane in amygdalar neurons. Johnson et al. used detailed electron microscopic analyses to study the subcellular distribution of the GR (Johnson et al., 2005) and MR (Prager et al., 2010) in the lateral amygdala. The GR was identified at the plasma membrane as well as in the nucleus and cytoplasm. GRs turned out to be present at both postsynaptic dendrites and presynaptic sites (Johnson et al., 2005). More recently the same was shown for the MR (Prager et al., 2010).

The functional consequences of corticosterone on mEPSC frequency in the BLA and the central nucleus of the amygdala (CeA) were recently revealed (Karst et al., 2010). In the CeA, corticosterone had no effect on either frequency or amplitude of the mEPSCs. However, in the BLA, corticosterone induced a significant increase in mEPSC frequency, comparable to the effects found in hippocampus albeit slightly slower in onset (Karst et al., 2010) (Figure 2.3C). Comparable to the hippocampus, this enhanced mEPSC frequency after corticosterone treatment was MR-dependent and non-genomic in nature (Karst et al., 2010). However, in contrast to the hippocampus, the effect in the amygdala was not only slower in onset, but also persistent after washout of the hormone. One hour after a pulse of corticosterone mEPSC frequency was still high. This lasting phase of the response was found to depend on protein synthesis and required the presence of both MR and GR (Karst et al., 2010).

The long-lasting effects of corticosterone were shown to also determine the responses of BLA neurons to subsequent pulses of the hormone. When BLA cells were exposed to a second pulse of corticosterone, mEPSC frequency was *reduced* (Karst et al., 2010) (see Figure 2.3C). Reduction in mEPSC frequency also occurred in tissue prepared from animals exposed to restraint stress prior to slice preparation. Interestingly, this rapid and non-genomic effect to renewed corticosteroid expo-

sure depended on the GR rather than the MR. Similar to the hypothalamus (but in contrast to the hippocampus), it was shown to involve a postsynaptically localized GR and subsequent retrograde endocannabinoid signalling (see Figure 2.1A). Thus, in a non-stressed animal corticosterone seems to have a stimulatory effect in the (basolateral) amygdala. However, due to the long-lasting nature of these effects, a second exposure to corticosterone induces opposite effects, suggesting metaplasticity of corticosteroid responses. These data suggests that the amygdala will respond differently to a stressor depending on the recent stress history of the organism.

Prefrontal cortex

The prefrontal cortex (PFC) is critically involved in complex behavioural control, such as behavioural inhibition, decision-making and working memory. It is extensively connected to the amygdala and receives afferents originating in the hippocampus (Arnsten, 2009). Despite its important function, the PFC is underrepresented concerning studies on the effects of corticosteroids and stress. A number of studies have examined the effect of chronic stress or corticosterone exposure on the PFC. Under these conditions, LTP, dendritic complexity and PFC-dependent working memory were reduced in a genomic fashion (Arnsten, 2009; Holmes and Wellman, 2009). On the contrary, exposure to acute stress or corticosterone increased glutamatergic transmission and improved working memory performance (Yuen et al., 2009, 2010). These effects occurred with a delay of several hours and were shown to require gene transcription (Yuen et al., 2010). Thus acute and chronic stress affect PFC plasticity and functionality in an opposite manner.

The only studies so far in the PFC that focused on rapid, non-genomic effects were performed in synaptosomes. In this preparation, corticosterone induced a rapid enhancement of glutamate uptake and of calcium-dependent calmodulin stabilization (Sze and Iqbal, 1994; Zhu et al., 1998). Unfortunately, the receptors or pathways involved were not examined. In a recent study, Roozendaal and colleagues reported a putative membrane-GR mediated effect of corticosterone in the insular cortex that is involved in memory acquisition. In this elegant study, administration of either corticosterone or cort-BSA directly into the insular cortex facilitated the acquisition of object recognition memory (Roozendaal et al., 2010). Although there are some concerns about the stability of cort-BSA *in vivo*, this is still indicative of a membrane-initiated effect. The effect was prevented by co-administration of a GR antagonist. The authors further proved that the facilitation of memory by membrane-GR activation was established through protein kinase A (PKA), cAMP response element-binding (CREB) and histone acetylation (Roozendaal et al., 2010). Taken together, rapid non-genomic actions of corticosterone are found in (some) prefrontal areas; so far they seem to be mostly excitatory (as are the sub-acute genomic effects) and could have implications for higher-order learning in complex tasks. However, the data is still very sparse.

2.2 Functional implications of rapid corticosteroid effects in the brain

Taking all results into account, we can distinguish some interesting general features of the rapid effects of corticosteroids in the brain.

i) It is important to notice that all non-genomic effects are permissive or conditional effects. In none of the studies corticosteroids induced any activity on their own, instead they facilitate or inhibit signalling of ion channels, receptors and neurotransmitters. In short, they increase or decrease the threshold for activation of these neurons by context-dependent factors. Therefore, it will depend on the context which effects (in which brain areas) will be most pronounced during a stressful encounter.

ii) We see a distinctive pattern with a general increase in excitability for some areas (hippocampus, amygdala and potentially the prefrontal cortex) and a decrease in others (the hypothalamus).

iii) While some responses are transient (mostly in the hippocampus), other effects are prolonged (hypothalamus, pituitary and amygdala). The brain circuitry activated by stress will thus be different depending on the delay after the stressor.

iv) In general, the inhibitory effects on hypothalamic functioning seem to require a higher dose of corticosterone than most effects in other brain areas. If so, the set of responses seen after a mild stressor may be different from that of a more severe stressor, the latter having an additional negative effect on PVN-related responses (Prager and Johnson, 2009).

v) Finally, a number of rapid corticosteroid effects require the presence of classical MR and GR inserted in or attached to the plasma membrane, while other effects are mediated through yet unknown (G-protein coupled) receptors. In general, MR-mediated effects tend to stimulate excitation, while GR-mediated effects can also be inhibitory (see Figure 2.1).

We will refer to these five general points when we next consider the potential functional consequences of rapid corticosteroid actions in the brain for HPA-axis regulation and cognition, also taking the ultradian release pattern into consideration. Finally we will address the integration of these rapid effects with the rest of the brain's response to stress.

Regulation of the HPA-axis

Corticosteroids exert rapid, as well as delayed, inhibitory feedback at the core structures of the HPA-axis; the PVN of the hypothalamus (Evanson et al., 2010b) and the pituitary gland (Jones et al., 1972; Hinz and Hirschelmann, 2000). In the pituitary this seems to be caused by both GR-dependent (Buckingham et al., 2003) and GR-independent (Hinz and Hirschelmann, 2000) rapid signalling pathways. In the PVN, the rapid suppression of glutamatergic transmission by corticosterone could well underlie (amongst others) fast suppression of the HPA-axis in a GR-independent

manner (Tasker, 2006). As mentioned earlier, this hypothesis is backed up by the effectiveness of intra-PVN infusions of dexamethasone or dex-BSA on HPA-axis activity in a rapid time frame (Evanson et al., 2010b).

In addition, extra-hypothalamic structures also control the activity of the HPA-axis. For instance, the hippocampus and prefrontal cortex exert negative feedback on the HPA-axis through (indirect) projections to the PVN, while the amygdala has a stimulatory influence on the PVN and thus HPA-axis (Ulrich-Lai and Herman, 2009). Rapid non-genomic corticosteroid actions in these areas may affect this limbic control over the HPA-axis. This also enables a role for the MR, absent from the hypothalamus, in the regulation of HPA-axis activation. Indeed, MRs in the hippocampus are important to determine the threshold of the stress response (Reul et al., 2000; Joëls et al., 2008). In agreement, treatment of rats with MR agonists induced a rapid suppression of both ACTH and corticosterone release (Atkinson et al., 2008). Thus, not only can corticosterone inhibit HPA-axis activation directly through its genomic and non-genomic effects at core structures of the axis, it also provides a second layer of control at limbic areas that enables a subtler and context-dependent rapid trans-synaptic regulation of the HPA-axis.

Adaptation of behaviour and cognition

In addition to regulation of the HPA-axis through (trans-synaptic) connections to the PVN, the limbic circuitry is vital for adaptation to stressful events and the formation of memory of these events (Figure 2.2). Many actions of corticosteroids, for example facilitation of memory consolidation, are dependent on gene transcription, through activation of the genomic GR (and MR) (Oitzl et al., 2001). However, corticosteroids also affect behaviour and memory in a rapid and presumably non-genomic manner. Thus, rapid effects of corticosteroids have been described for a number of adaptive behaviours, including rapid facilitation of novelty-induced locomotion (Sandi et al., 1996a,b), context-dependent aggression (Mikics et al., 2004) and risk assessment behaviour (Mikics et al., 2005). These effects were all observed within 7 minutes and the latter two were proven to be independent of gene transcription, see also Table 2.1. In all cases, an injection with corticosterone rapidly increased a specific type of behaviour that is seen as adaptive in that context (i.e. aggression towards an intruder, or locomotion and risk assessment in a novel environment). Interestingly, the MR has been repeatedly reported to be involved in these types of behaviour, involving novelty reactivity, coping strategies and aggression (Oitzl and de Kloet, 1992; Sandi and Rose, 1994; Berger et al., 2006; Joëls et al., 2008; Brinks et al., 2009; Kruk et al., 2013). As these behavioural effects are rapidly induced and by stress-doses of corticosterone, they always seemed incompatible with the constitutively active genomic MR. The lower affinity membrane-MR could prove to be the logical substrate for these effects. Unfortunately, this role of the membrane-MR has not been studied directly yet. There is circumstantial evidence for involvement of MRs in novelty behaviour. This comes from a study using knock-

out mice for the limbic system-associated membrane protein (LSAMP). These mice showed increased novelty reactivity and impaired learning (Catania et al., 2008; Qiu et al., 2010), and associated with this, a reduction in non-genomic MR function in the hippocampus (Qiu et al., 2010).

In behavioural studies on the regulation of memory, the GR is reported to have a predominant function in memory consolidation, while the MR is mostly involved in memory retrieval and learning strategies (Oitzl and de Kloet, 1992; de Kloet et al., 1999). A similar convergence of functions is seen in the rapid domain. Firstly, a rapid facilitation of memory consolidation by corticosterone was shown to depend on the (presumably membrane localized) GR in the cortex (Roozendaal et al., 2010). Secondly, application of antagonists for endocannabinoid signalling in the amygdala was reported to block corticosterone-induced effects on memory consolidation (Campolongo et al., 2009). Together, this suggests that the membrane-GR mediated and endocannabinoid-dependent inhibition of neuronal excitability (see Figure 2.1A and Karst et al. (2010)) might be implicated in memory consolidation. In contrast, corticosterone effects on memory retrieval seem to be MR-mediated. Administration of corticosterone 30 minutes before a memory retrieval task impaired retrieval of information in a non-genomic, hippocampal-dependent and MR-mediated manner (Khaksari et al., 2007; Sajadi et al., 2007). Finally, acute stress or cort-BSA infusion into the hippocampus induced a shift in memory retrieval tested 5 or 15 minutes later, although this study did not investigate the receptor involved (Chauveau et al., 2010). Rapid —in addition to delayed— corticosteroid effects thus seem to be involved in all phases of the memory process, i.e. acquisition, consolidation and retrieval. In general, the GR seems to potentiate consolidation via both rapid and delayed (genomic) pathways. Conversely, the MR seems to have a specific (non-genomic) role during memory retrieval, possibly as a mechanism to focus attention to a new stressor. Taken together, in its role as rapid corticosteroid sensor, the MR facilitates adaptive behaviour in the context of the stressor while inhibiting behaviours that are no longer relevant.

Implication of ultradian pulses

Corticosteroids do not only reach the brain in high amounts during a stressful situation, but also during ultradian peaks (Droste et al., 2008). Rapid non-genomic corticosteroid actions might have an additional function in translating these pulses into ultradian alterations in brain function. Indeed, both rapid feedback on the HPA-axis (Windle et al., 1998), aggressive behaviour (Haller et al., 2000) and novelty reactivity (Sarabdjitsingh et al., 2010) depend on the phase of an ultradian pulse the animal is in. In a recent study by Sarabdjitsingh et al., ultradian pulses were manipulated experimentally. Exposure to noise stress induced a stronger ACTH release and higher behavioural reactivity when animals were stressed during the rising phase of an ultradian corticosterone pulse compared to animals exposed to the same stressor during the falling phase (Sarabdjitsingh et al., 2010).

Effect		Receptor	Conc	Area	Delay	Preparation	Signalling pathways	Reference
mEPSC freq	↓	other or mGR	100 nM	PVN & SON	5 min	rat, *ex vivo*	Gαs, cAMP-PKA, ECB, CB1	[1–5]
mIPSC freq	↑	other	1 μM	PVN & SON	5 min	rat, *ex vivo*	Gβγ, NO release	[2,4]
eEPSC freq ↓ eIPSC amplitude	↑	unknown	1 μM	SON	7 min	rat, *ex vivo*		[4]
2-AG and AEA levels	↑	unknown	1 μM	hypoth	10 min	rat, *ex vivo*	PKA	[3–4]
Vasopressin release	↓	mGR	100 nM	hypoth	20 min	rat, *ex vivo*	Ca^{2+}, PLC	[6–7]
HPA-axis	↓	unknown	10 ng local	PVN	15 min	rat, *in vivo*	CB1	[8]
mEPSC freq	↑	mMR	10 nM	CA1 & DG	5 min	mouse, *ex vivo*	ERK1/2	[9–12]
I_A current	↓	MR	100 nM	CA1	5 min	mouse, *ex vivo*	G-proteins	[10]
AMPAR mobility	↑	mMR	50 nM	CA1	2 min	rat, culture		[13]
mIPSC freq	↑	MR	30 nM	Ventral CA1	not known	rat, *ex vivo*		[14]
MR at membrane		mMR		hippoc		mouse, culture		[11]
spine density	↑	mGR	100 nM	CA1	60 min	rat, *ex vivo*		[15]
GR at membrane		mGR		hippoc		mouse, *ex vivo*		[15]
aspartate and glutamate levels	↑	other	600 ng/ml local	hippoc	20 min	rat, *in vivo*		[16]
NMDA-dependent neurotoxicity	↑	other	10 nM	hippoc	15 min (+24 h)	rat, culture	ERK1/2, NR2A	[17]
LTP induction	↑	other	100 nM	CA1	10 min	mouse, *ex vivo*		[18]
sIPSC freq	↑	other	25 nM	CA1	5 min	rat, *ex vivo*	G-proteins, NO	[19]
JNK & p38 phosphorylation	↑	other	1 nM	hippo	5 min	rat, culture	G-proteins, PKC	[20]
NMDA-dependent current	↓	not GR	100 nM	hippoc	seconds	rat, culture	cAMP-PKA	[21]
NMDA-dependent current prolonged		not GR	1 μM	hippoc	seconds	rat, culture		[22]
AEA levels	↑	unknown	3 mg/kg sc	hippoc	10 min	rat, *in vivo*		[23]
NMDA-dependent current	↓	unknown	400 nM	CA1	seconds	mouse, *ex vivo*		[24]
Ca^{2+}-currents	↓	unknown	10 pM	CA1	4 min	guinea pig, *ex vivo*	G-proteins, PKC	[25]
Memory retrieval altered		unknown	0.3 nmol local	dorsal hippoc	10 min	mouse, *in vivo*		[26]

GR in membrane		mGR		BLA		mouse		[27]
MR in membrane		mMR		BLA		mouse		[28]
mEPSC freq	↑	mMR	100 nM	BLA	15 min	mouse, *ex vivo*		[29]
mEPSC freq	↓	mGR	100 nM	BLA	15 min	mouse, *ex vivo*	CB1	[29]
AEA levels	↑	unknown	3 mg/kg sc	amygdala	10 min	rat, *in vivo*		[23]
glutamate uptake	↑	unknown	10 nM	frontal cortex	5 min	rat, synaptosomes	G-proteins	[30]
calmodulin dynamics	↑	unknown	30 nM	cortex	15 min	rat, synaptosomes		[31]
Memory consolidation	↑	mGR	3 ng local	insular cortex / mPFC	24 h	rat, *in vivo*	βAC, cAMP, PKA, CREB	[32–33]
Working memory	↓	mGR	3 ng local	mPFC	60 min	rat, *in vivo*	βAC, cAMP, PKA	[33]
ATP-induced currents	↓	GR	10 nM	DRG	seconds	rat, culture	PKA	[34]
memory retrieval	↓	MR	1 mg/kg		30 min	rat, *in vivo*	opioids	[35–36]
risk assessment	↑	unknown	0.5 mg/kg		7 min	rat, *in vivo*		[37]
aggressive behaviour	↑	unknown	0.5 mg/kg		7 min	rat, *in vivo*		[38]
locomotion	↑	other	2.5 mg/kg		7 min	rat, *in vivo*	NO	[39–40]

Table 2.1: Rapid effects of corticosterone on neuronal functioning in the hypothalamus, hippocampus, amygdala and prefrontal cortex
'**unknown**' receptor was not examined, '**other**' not the MR or GR, **DRG** dorsal root ganglion, **eIPSC/EPSC** evoked IPSC/EPSC, **AEA** anandimide, **2-AG** 2-arachidonoylglycerol, **sIPSC** spontaneous IPSC, **mMR/mGR** membrane-associated MR/GR, **sc** subcutaneous, **ECB** endocannabinoids, **CB1** cannabinoid receptor type 1, **NO** nitric oxide, **NR2A** NMDA receptor 2A subunit, **PKC** protein kinase C, **βAC** β-adrenoceptor, **WB** western blot, **IFM** immunofluorescent microscopy, **EM** electron microscopy.
[1] (Di et al., 2003), [2] (Di et al., 2005), [3] (Malcher-Lopes et al., 2006), [4] (Di et al., 2009), [5] (Haam et al., 2010), [6] (Liu et al., 1995), [7], (Liu and Chen, 1995), [8] (Evanson et al., 2010b), [9] (Karst et al., 2005), [10] (Olijslagers et al., 2008), [11] (Qiu et al., 2010), [12] (Pasricha et al., 2011), [13] (Groc et al., 2008), [14] (Maggio and Segal, 2009), [15] (Komatsuzaki et al., 2005), [16] (Venero and Borrell, 1999), [17] (Xiao et al., 2010), [18] (Wiegert et al., 2006), [19] (Hu et al., 2010), [20] (Qi et al., 2005), [21] (Liu et al., 2007), [22] (Takahashi et al., 2002), [23] (Hill et al., 2010), [24] (Sato et al., 2004), [25] (Ffrench-Mullen, 1995), [26] (Chauveau et al., 2010), [27] (Johnson et al., 2005), [28] (Prager et al., 2010), [29] (Karst et al., 2010), [30] (Zhu et al., 1998), [31] (Sze and Iqbal, 1994), [32] (Roozendaal et al., 2010), [33] (Barsegyan et al., 2010), [34], (Liu et al., 2008), [35] (Khaksari et al., 2007), [36] (Sajadi et al., 2007), [37] (Mikics et al., 2005), [38] (Mikics et al., 2004), [39] (Sandi et al., 1996b), [40] (Sandi et al., 1996a)

These responses were seen within minutes, so that non-genomic mechanisms must have been involved. In the brain, these effects were associated with *in*creased activity of the amygdala and *de*creased activity of the PVN (recorded by c-fos expression) during the rising compared to the falling phase (Sarabdjitsingh et al., 2010), reminiscent of the corticosteroid effects seen for mEPSC frequency in PVN and amygdala. Hypothetically, during the rising phase of an ultradian pulse, non-genomic pathways are activated in limbic areas, which in turn could affect stress-related behaviour.

Integration of non-genomic and genomic effects

In several cases, rapid non-genomic corticosteroid actions were shown to transgress into more lasting effects, integrating two temporal domains (rapid and delayed) which up till recently were each linked to different classes of stress hormones, i.e. monoamines (and to some extent neuropeptides) on the one hand and corticosteroids on the other hand. For example, rapid effects in the hypothalamus are long lasting (Di et al., 2003) and thus HPA-axis feedback will be inhibited over a long period of time. Indeed, dexamethasone infusions in the PVN exert both rapid and delayed negative feedback actions on the HPA-axis activity (Dallman et al., 1994; Dallman, 2005). Similarly, the increased excitability in the BLA starts as a non-genomic MR-dependent phenomenon and eventually evolves into a genomic phenomenon that also requires the GR (Karst et al., 2010). At a cognitive level, the facilitation of memory consolidation by cort-BSA injections in the insular cortex is evoked by a membrane-associated effect that evolves into a genomic effect through activation of the transcription factor CREB (Roozendaal et al., 2010). Finally, rapid corticosterone effects on aggressive and risk assessment behaviour are independent of gene transcription immediately after corticosterone injection but develop into transcription-dependent effects later on (Mikics et al., 2004, 2005). Thus, many non-genomic effects of corticosterone are tightly linked to later genomic actions. At least in one case (Karst et al., 2010), the initial non-genomic action is required for the subsequent genomic phase, suggesting that both phases work in coordination.

However, non-genomic and genomic actions can also be integrated if they occur independent from each other. In the hippocampus, the initial enhanced mEPSC frequency is quickly reversed: when corticosteroid levels drop, the effects are immediately lost (Karst et al., 2005). Supposedly, a brief period of enhanced excitability is followed by a refractory period with an increased threshold for the induction of new signals, the latter depends on genomic GR signalling (Alfarez et al., 2002, 2009; Krugers et al., 2010). A similar dichotomy was seen with respect to LTP induction in the hippocampus. Corticosterone given immediately before LTP induction stimulated LTP induction (Wiegert et al., 2006), while corticosterone applied hours earlier inhibited the induction of the same type of LTP (Diamond et al., 1992; Pavlides et al., 1993). The initial rapid facilitation of signalling might help the organism to

appraise the novel situation; gradually the genomic phase will take over and restore the activity of the circuits to regain homeostasis (Joëls et al., 2006).

Overall, this implies that the temporal pattern of activation by corticosterone is different for the various areas. As summarized in Figure 2.2, both the hippocampus and amygdala, are more sensitive for incoming signals during stress or corticosterone exposure, while activity in the PVN is rapidly inhibited. In a delayed fashion, the hippocampus will switch to a state where the threshold for activation is elevated, while activation thresholds in the amygdala and hypothalamus do not differ between the two time-domains. Hypothetically, this can have consequences for the cognitive functions associated with these brain areas. For example, as the amygdala is involved in emotional memory formation, the prolonged activation in this area might support efficient encoding of emotional aspects of a stressful event, which could explain the preferential memory of emotional over neutral, hippocampal-dependent information (Buchanan and Lovallo, 2001; Karst et al., 2010). Finally, it seems that a second exposure of corticosterone switches amygdalar excitability back to its pre-stress state (Karst et al., 2010). This mechanism could protect the amygdala from inappropriately prolonged activation (McEwen, 2001; Karst et al., 2010). For the PFC, the limited data so far, suggest that its sensitivity is elevated by corticosterone in both an acute and more prolonged manner. However, as the data for the PFC is still sparse, we have not included it in Figure 2.2.

2.3 Molecular aspects of non-genomic corticosterone actions

The quest for a better understanding of the role of non-genomic corticosteroid signalling is paralleled by another quest: that for a better understanding of the cellular basis of these non-genomic effects. Here we will summarize the current state of understanding of the membrane localization, and translocation, of the MR and GR as well as that of their downstream signalling partners. We will, again, focus mostly on corticosteroid signalling in neural tissues but we will also use knowledge from the periphery and of related steroids and their receptors where necessary.

Presence of MR and GR at the plasma membrane, critical evaluation of the evidence

For many years the membrane localization of the MR and GR has been controversial, however, over the last years evidence of their membrane presence has culminated. (i) Intracellular applied corticosterone cannot induce rapid non-genomic effects; therefore it is unlikely that the receptors are located inside the cells. (ii) Membrane impermeable corticosterone-BSA (cort-BSA) and dex-BSA conjugates induce the same rapid effects as free corticosterone or dexamethasone. Moreover, they do so with equal (Xiao et al., 2010) to slightly reduced (Karst et al., 2005; Qi et al., 2005)

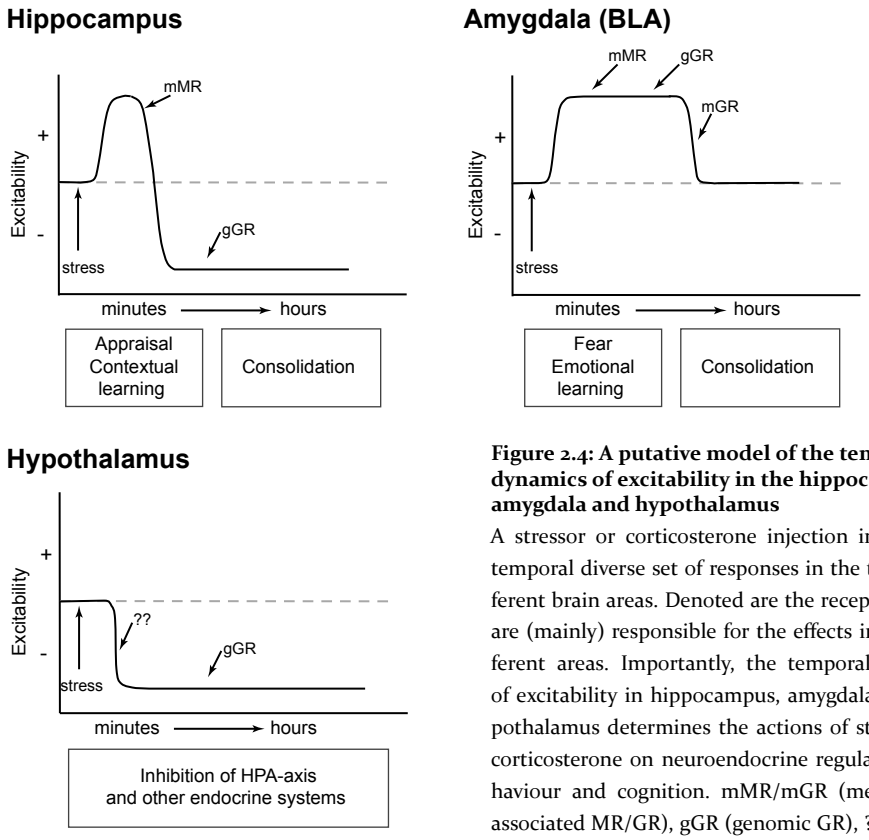

Figure 2.4: A putative model of the temporal dynamics of excitability in the hippocampus, amygdala and hypothalamus

A stressor or corticosterone injection induces a temporal diverse set of responses in the three different brain areas. Denoted are the receptors that are (mainly) responsible for the effects in the different areas. Importantly, the temporal pattern of excitability in hippocampus, amygdala and hypothalamus determines the actions of stress and corticosterone on neuroendocrine regulation, behaviour and cognition. mMR/mGR (membrane-associated MR/GR), gGR (genomic GR), ?? (receptor unclear).

efficacy. (iii) Most convincingly, the presence of MR and GR has been shown in synaptosome extracts (Komatsuzaki et al., 2005; Wang and Wang, 2009; Qiu et al., 2010) and at neuronal membranes using electron microscopy (Johnson et al., 2005; Prager et al., 2010). (iv) Finally, the MR and GR are by no means unique in their association with the plasma membrane. Membrane localization has been shown for most, if not all, steroid receptors including the ER α and β, AR and PR (Hammes and Levin, 2007).

Not all rapid corticosteroid effects can be attributed to the MR or GR though. Multiple non-genomic actions of corticosteroids on neurotransmission (Wiegert et al., 2006; Di et al., 2009), HPA-axis regulation (Evanson et al., 2010b) and behaviour (Sandi et al., 1996b) remain in the presence of MR and GR antagonists and are thus postulated to require a novel membrane-associated receptor. However, the identity of this receptor has proved very difficult to resolve; as yet, none have been cloned. The most likely candidates are G-protein coupled receptors (GPCR), because inhibitors of G-proteins can prevent many —though not all (Orchinik et al., 1997)— MR/GR independent corticosteroid effects (Di et al., 2003, 2005). Multiple non-MR/GR corticosteroid binding sites have been identified in the membrane of

neuronal substrates in a number of species (Orchinik et al., 1991, 1992, 1997, 2000; Guo et al., 1995; Maier et al., 2005; Breuner and Orchinik, 2009; Schmidt et al., 2010). However, the affinity and selectivity of these binding sites is very variable, making it unlikely that they all stem from a single type of evolutionary conserved receptor.

The association of steroid receptors at the plasma membrane

How is the membrane association of receptors mediating rapid corticosteroid actions accomplished and how is this process regulated? Unfortunately, there is little known about this subject regarding MR and GR. However, much more results have been obtained on the membrane translocation of ERα. Since ERα and corticosteroid receptors may share some of the pathways involved in membrane localization, we will first evaluate the available insights in the ERα and next compare this with what is presently known about corticosteroid receptors.

The estrogen receptors ERα and ERβ can both be targeted to the cell membrane (Gorosito et al., 2008; Micevych and Dominguez, 2009) where they primarily exist in caveolae (Razandi et al., 2002). Caveolae are invaginations of the plasma membrane formed by caveolins, scaffolding proteins that bind and bring together a large number of signalling molecules including GPCRs, G-proteins, c-Src and other kinases; this facilitates rapid signal transduction (Anderson, 1998; Cohen et al., 2004). The most ubiquitously expressed caveolin is caveolin-1. Ablation of caveolin-1 severely diminished ERα membrane localization (Sud et al., 2010). Moreover, mutation of a single amino acid (S522A) in the ligand binding domain of the ERα resulted in a 60 % reduction of caveolin-1 binding, membrane localization and rapid signalling of ERα (Razandi et al., 2003). Caveolin-1 binding is also required for membrane translocation of the ERβ, AR and PR (Lu et al., 2001; Salatino et al., 2006; Gilad and Schwartz, 2007). Mutation of another amino acid, cysteine477 (C477A), resulted in an almost complete reduction of ERα membrane localization, while its genomic functions were left undisturbed (Acconcia et al., 2005). This mutation was shown to be essential for palmitoylation of the receptor. Palmitoylation is a post-translational modification where a lipid tail is attached to the receptor, thus enabling insertion into the plasma membrane. ERα palmitoylation is essential for caveolin-1 binding, membrane translocation and rapid signalling (Acconcia et al., 2005; Pedram et al., 2007). A final component of the ERα membrane translocation pathway was identified recently: disruption of heat shock protein (HSP) 27 prevented palmitoylation, caveolin-1 binding, membrane localization and rapid signalling of ERα (Razandi et al., 2010). Together this leads to a model where ERα associates with HSP27, this interaction enables ERα to get palmitoylated, and due to the palmitoylation the receptor can bind caveolin-1 which facilitates transport to the plasma membrane (see Figure 2.5A).

Importantly, this membrane translocation process seems to be a common pathway for all steroid receptors. The group of Levin (Pedram et al., 2007) identified a conserved sequence surrounding the palmitoylation site of ERα and this same

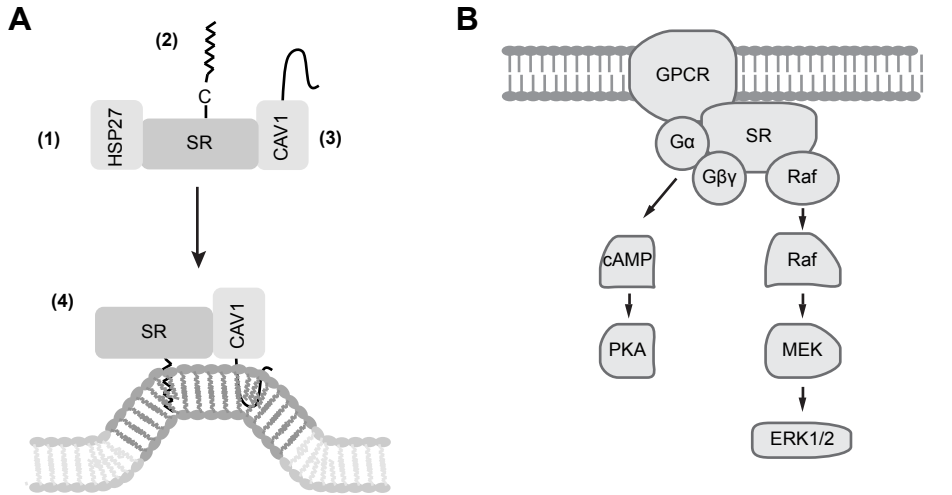

Figure 2.5: Steroid receptor membrane association and downstream signalling
(A) The putative common pathway for membrane translocation of steroid receptors is shown with the ERα as example. Translocation of the ERα requires the association of heat shock protein 27 (HSP27) (step 1), subsequently the receptor is palmitoylated at cysteine 477 (step 2), this facilitates association of the adaptor protein caveolin-1 (CAV1) (step 3). Finally, the ERα is transported to the plasma membrane, where it is localized in caveolae (step 4). (B) Model of the downstream signalling pathways implied in non-genomic corticosteroid signalling in neurons.

sequence was identified in the AR, PR, ERβ and other receptors. Mutation of key amino acids in this sequence abolished membrane localization and rapid signalling for all steroid receptors tested (Pedram et al., 2007). Similarly, association of HSP27 is required for membrane translocation of ERα, PR and AR (Razandi et al., 2010). Thus, so far the data suggest that there is a common membrane translocation pathway for all (or most) steroid receptors involving caveolin-1, palmitoylation and HSP27.

Membrane translocation of MR and GR

Now the question remains whether the MR and GR are transported to the membrane in a similar way. For these receptors only a few studies have been reported and none in brain cells. In peripheral models an association between both MR and GR to caveolin-1 has been demonstrated. In epithelial cells, dexamethasone induced rapid binding of GR to c-Src and subsequent activation of the PI3K-Akt pathway (Matthews et al., 2008). Transfection of a double-negative form of caveolin-1 disrupted all aspects of this signalling cascade, as did disruption of caveolae. In addition, a direct interaction between the GR and caveolin-1 was seen with co-immunoprecipitation (Matthews et al., 2008). In contrast, in hepatic cells no colocalization of membrane-associated GR and caveolin-1 could be found with conventional confocal microscopy (Spies et al., 2006).

For MR, a similar association was studied in caveolin-1 knockout (cav1-/-) mice (Pojoga et al., 2010a,b). First of all, a direct association between the MR and caveolin-1 (but not caveolin-2) was shown with co-immunoprecipitation in heart homogenates from both rat and mouse as well as in cultured human endothelial cells (Pojoga et al., 2010a). As expected, this association was lost in cav1-/- mice. Secondly, these mice showed heightened vascular responses to treatment with the MR antagonist eplerenone (as compared to wild type mice) and a reduced sensitivity to aldosterone treatment on myocardial damage (Pojoga et al., 2010b). Thus, not only is the MR associated with caveolin-1 in vascular tissues, but a loss of caveolin-1 also alters the vascular responses to MR agonists and antagonists. The precise consequences of the loss of caveolin-1 for MR-associated functioning seem to depend strongly on the context of the response.

Additional supporting evidence for the membrane localization of the MR comes from the group of Grossmann and Gekle (2008, 2010). In an initial study, they showed that transfection of only the ligand binding domain of the MR was sufficient for aldosterone to rapidly activate the ERK1/2 pathway in Chinese hamster ovary cells (Grossmann et al., 2008). This is similar to the ERα, where the ligand binding domain suffices for membrane translocation and signalling (Razandi et al., 2002). More recently, they studied the colocalization between the MR and the EGF receptor. This colocalization was lost when lipid rafts (including caveolae) were disrupted (Grossmann et al., 2010). This strongly suggests that the MR is localized in caveolae, since the EGF receptor is known to be associated with caveolae.

Finally, regarding the conserved palmitoylation motif, an interesting picture emerges. The palmitoylation motif of the GR contains all essential groups and would be predicted to be a palmitoylation site (although the GR was not tested in the original study) (Pedram et al., 2007). The MR, by contrast, lacks the essential cysteine residue. As this cysteine provides the thiol group to which the palmitate tail is transferred, the MR cannot be palmitoylated at this sequence. The MR could be palmitoylated at another motif or could translocate to the membrane through an alternative pathway.

Regulation of membrane translocation and place in the membrane

Why does only part of the receptor population translocate to the membrane while the bulk remains in the cytoplasm and nucleus, and what determines the proportion of these pools? For the ERα, most studies estimate that approximately 5–10 % of the receptor population is localized at or in the membrane, which leaves 90–95 % of the population in the cytoplasm and nucleus (Chambliss et al., 2000). Caveolin-1 overexpression was found to elevate the proportion of membrane ERα (Sud et al., 2010), suggesting that this protein has a regulatory effect.

It is known that ligand binding affects membrane translocation. Most studies show that treatment with (high concentration of) ligands reduces palmitoylation, association with caveolin-1 and membrane expression (Razandi et al., 2002; Ac-

concia et al., 2005; Micevych and Dominguez, 2009). In contrast, other studies report an *increased* membrane translocation with steroid treatment (Razandi et al., 2002; Gorosito et al., 2008; Bondar et al., 2009). Clearly, the timing, concentration and duration of ligand exposure will influence these effects. GR expression in hippocampal synaptosomes was slightly decreased after 3 weeks of daily corticosterone injections and increased by adrenalectomy (which abolishes endogenous corticosterone) (Wang and Wang, 2009), suggesting that the GR also traffics from the membrane by ligand treatment. Interestingly though, in amygdalar neurons acute stress or corticosterone treatment abolished MR-mediated non-genomic signalling, while it actually allowed GR-mediated actions to take place (Karst et al., 2010).

It is still unclear how steroid receptors are integrated into the plasma membrane. The effectiveness of impermeable hormone conjugates (such as estradiol-BSA or cort-BSA) suggests that the receptors are accessible from the outside of the plasma membrane. In addition, biotinylation studies (for ERα) provide evidence for an extracellular recognition site of the receptors (Bondar et al., 2009). This would suggest that the receptors are integrated in the outer sheet of the membrane with their palmitate tail. However, this seems in contradiction with studies showing a direct interaction of steroid receptors with caveolin-1 (Razandi et al., 2002; Sud et al., 2010) and second messenger molecules such as c-Src and G-proteins (Sanchez et al., 2011), which suggest that receptors are inserted into the inner sheet of the membrane, where they are able to interact with the cytoplasmic molecules. Possibly the steroid receptor shuttles to the inside of the membrane upon activation, but at present this is mere speculation.

A general model of steroid downstream signalling

As a final point we will evaluate the secondary pathways of steroid receptors. Surprisingly, although the physiological functions of steroids are very diverse (ranging from sexual differentiation to electrolyte balance) the non-genomic signal pathways show a large overlap. We will discuss the very basics of steroid receptor downstream signalling in order to come to general characteristics.

As steroids are lipophilic and easily penetrate the plasma membrane, their receptors do not need to be located at the plasma membrane. More likely, membrane-association of steroid receptors is required for binding to signalling partners that are present only at the membrane. In fact, caveolae are well known signalosomes that bring receptors, adaptor molecules and kinases together (Anderson, 1998). Indeed, the ERα was shown to assemble a multi-protein complex consisting of other membrane-spanning receptors (most often growth factor receptors) and multiple small adaptor molecules like G-proteins (both Gα and Gβγ subtypes) (Kumar et al., 2007), c-Src (Sanchez et al., 2011) and PI3K (Simoncini et al., 2000). Through this signalosome a variety of kinase pathways are activated (Hammes and Levin, 2007; Vasudevan and Pfaff, 2007; Micevych and Dominguez, 2009). Most commonly, activation of the phospholipase C - protein kinase C (PLC-PKC), cAMP-PKA (protein

kinase A), PI3K-Akt and Ras-ERK pathways have been found (Figure 2.1). Importantly, activation of components of these three general pathways has been reported for the ERα, ERβ, AR, PR, MR and GR. For example, ERK1/2 phosphorylation can be seen within minutes of stimulation with aldosterone, corticosterone, estradiol, androgens or vitamin D (Qiu et al., 2001; Pedram et al., 2007; Grossmann et al., 2008) and reviewed in Hammes and Levin (2007) and Grossmann et al. (2010).

The initial event, i.e. the composition of the signalosome, seems to determine which downstream pathway is recruited. For example, in hippocampal neurons estradiol can activate two distinctive pathways in a single cell; on the one hand activation of ERK1/2 leads to subsequent genomic effects through activation of the transcription factor cAMP response element binding (CREB), on the other hand inhibition of PKA induces a decrease in Ca^{2+}-currents (Boulware et al., 2005). These two effects originate from two separate pathways; one involves ERα bound to caveolin-1 and attracts the metabotrophic glutamate receptor GluR1A and G_q resulting in the activation of ERK1/2 and CREB, while the other effect originates from an ERα/β heterodimer bound to caveolin-3, GluR2/3 and G_{io}, this pathway results in the inhibition of PKA and Ca^{2+}-currents (Boulware et al., 2007). Also interesting in this regard is the role of the coreceptors in the signalosomes; multiple studies showed that inhibition of growth factor signalling prevented the non-genomic effects of steroids. For example, phosphorylation of ERK1/2 by either aldosterone (Grossmann et al., 2005) or estrogen (Razandi et al., 2003) could be prevented by inhibitors of the EGF receptor. Direct interactions between the MR (Grossmann et al., 2010) and ERα (Song et al., 2010) with growth factor receptors were also shown. In fact, some people opt for a GPCR hypothesis for rapid steroid signalling; the activation of a membrane steroid receptor activates a growth factor receptor and this enables further signalling (see Micevych and Dominguez, 2009). Whether this is just *one* mechanism of action or *the* mechanism of action remains to be determined.

Ultimately, activation of the cellular pathways affects the physiology of cells and tissues. Depending on the precise composition of the signalosome and the cellular context a wide variety of effects are obtained. These are too diverse to discuss in full here, but we will give a few examples for rapid aldosterone signalling in the periphery. In kidney cells, aldosterone rapidly enhances sodium transport through an ERK pathway, this results in a rapid effect on sodium absorption in these cells which eventually also regulates blood pressure (Gekle et al., 2001). In the vascular system, activation of the enzyme nitric oxide (NO) synthase by aldosterone (through a PI3K-Akt pathway) results in an increased release of NO which attracts immune cells and affects constriction of vascular smooth muscle cells (Hafezi-Moghadam et al., 2002; Mutoh et al., 2008).

The signal partners of central non-genomic corticosteroid signalling

The cellular pathways involved in neuronal non-genomic corticosteroid actions have not been studied in detail yet, however, many studies did examine the involve-

ment of some signal partners (see Table 2.1) and we can fit these within the general model of non-genomic steroid signalling. As for ERα and other steroid receptors, the most obvious effectors of the rapid effects are G-proteins. Inhibition of G-protein activation abolished the rapid effects of corticosterone on (i) inhibition of mEPSCs in the hypothalamus (Di et al., 2003), (ii) facilitation of mIPSCs in the hypothalamus (Di et al., 2005), (iii) facilitation of mEPSC's in the hippocampus (Olijslagers et al., 2008) (iv) inhibition of potassium currents in the hippocampus (Olijslagers et al., 2008), (v) inhibition of calcium currents in the hippocampus (Ffrench-Mullen, 1995) and (vi) activation of glutamate uptake in frontal neurons (Zhu et al., 1998) (see also Table 2.1). Interestingly, as for the estradiol effects in the hypothalamus, corticosterone can activate two different signalling pathways in single neurons in the hypothalamus. Through activation of Gαs, corticosterone induces the release of endocannabinoids and an inhibition of glutamate release, while Gβγ activation leads to the release of NO and the facilitation of GABA release in the same neuron (Di et al., 2009). It remains to be investigated whether different GPCRs or caveolin subtypes are also involved.

More downstream, corticosterone rapidly activates both the cAMP-PKA pathways and the ERK1/2 pathway in neurons. cAMP-PKA signalling is required in the hypothalamus (Malcher-Lopes et al., 2006) and for one effect in the hippocampus (Liu et al., 2007). Activation of the ERK1/2 pathway is seen after corticosteroid exposure in some cases (Xiao et al., 2005, 2010; Roozendaal et al., 2010) and is required for other effects (Olijslagers et al., 2008). Evidence for the involvement of the PI3K pathway has not yet been studied in the brain. Thus, although still very limited, non-genomic corticosteroid signalling in neurons follows similar kinase pathways as their peripheral counterparts and as that of other steroid receptors. Likely, the regional variation in the precise signalling cascades activated will prove to be crucial for understanding the more subtle difference between the actions in different neurons and under changing conditions. As examples from related fields show, this variation could well arise from the recruitment of different proximal adaptor molecules and interactions with signalling of other (neurotransmitter) receptors. In Figure 2.5B we show a very general model of the downstream signalling partners in central non-genomic corticosteroid signalling.

2.4 Concluding remarks

The existence of rapid effects of corticosterone has been known for over 50 years; however, it is only in the last 10 years that these effects have been studied in more detail. Yet, there are still many unanswered questions.

First, we cannot appreciate the consequences of non-genomic effects of corticosteroids when they are studied in isolation, instead we must view these effects in the context of the complete stress response. Exactly how rapid non-genomic and

genomic actions are integrated to collectively accomplish the behavioural response to stress awaits further investigation, as discussed in the previous section.

Secondly, through its non-genomic effects corticosterone acts in the same time-domain as other transmitters and hormones released after stress, e.g. catecholamines or CRH. This gives ample opportunities for cross-talk between the various stress hormones (Alfarez et al., 2009). For example, activation of the noradrenergic system in the amygdala is required for effects of corticosterone to take place (Roozendaal et al., 2002, 2006). However, at this time relatively little is known about the mechanism by which corticosteroids alter responsiveness to other stress factors and if non-genomic corticosteroid signalling is involved.

Thirdly, only a few signalling partners for rapid effects have been discovered. A comparison of the available data (see Table 2.1) suggests that many pathways are shared across brain areas. For example, multiple studies have proven involvement of G-proteins and the ERK-CREB pathway. Importantly, these same pathways are also activated by rapid signalling of other steroid receptors (Hammes and Levin, 2007; Vasudevan and Pfaff, 2007; Levin, 2008). Information gathered in these related fields could serve as an important guideline for investigation of the signalling partners of corticosteroids in the brain. For instance, both rapid corticosterone (Di et al., 2009) and estradiol signalling (Boulware et al., 2007) in neurons suggests that the specific type of G-protein that is engaged in the hormonal actions is an important determinant of the subsequent signalling cascade and the physiological outcome.

Fourthly, regulation of membrane translocation of the MR and GR in neurons is still undiscovered. Caveolin-1 is required for membrane translocation of all steroid receptors including the MR and GR (Matthews et al., 2008; Pojoga et al., 2010b). However, this has yet to be shown for the MR and GR in neurons. All three types of caveolins are expressed in the brain and they are known to be required for ERα and ERβ non-genomic signalling (Boulware et al., 2007). Interestingly, neurons do not have caveolae (Head and Insel, 2007), instead caveolins seem to be associated with synaptic markers and interact with multiple types of glutamate receptors. Thus, it is likely that caveolin association enables the enrichment of MR and GR at synaptic sites in the membrane (Johnson et al., 2005; Prager et al., 2010) and places the receptors well in reach to regulation of synaptic transmission.

Finally, the conserved palmitoylation motif found in many steroid receptors, including the ERα and the GR, is presumably ineffective in the MR. This motif is absolutely required for palmitoylation and membrane expression of ERα, ERβ, PR and AR (Pedram et al., 2007) and it thus remains unclear if and how the MR could be palmitoylated, possibly at another sequence. Consensus palmitoylation sequences were identified in the MR with the online CSS-Palm tool (Ren et al., 2008), however, this still needs conformation *in vivo*. Alternatively, the MR could use another pathway for translocation to the membrane.