30 YEARS OF THE MINERALOCORTICOID RECEPTOR

The brain mineralocorticoid receptor: a saga in three episodes

Marian Joëls1,2 and E Ronald de Kloet3

1Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center, Utrecht, The Netherlands
2University of Groningen, University Medical Center, Groningen, The Netherlands
3Division of Endocrinology, Department of Internal Medicine, Leiden University Medical Center, Leiden, The Netherlands

Abstract

In 1968, Bruce McEwen discovered that 3H-corticosterone administered to adrenalectomised rats is retained in neurons of hippocampus rather than those of hypothalamus. This discovery signalled the expansion of endocrinology into the science of higher brain regions. With this in mind, our contribution highlights the saga of the brain mineralocorticoid receptor (MR) in three episodes. First, the precloning era dominated by the conundrum of two types of corticosterone-binding receptors in the brain, which led to the identification of the high-affinity corticosterone receptor as the ‘promiscuous’ MR cloned in 1987 by Jeff Arriza and Ron Evans in addition to the classical glucocorticoid receptor (GR). Then, the post-cloning period aimed to disentangle the function of the brain MR from that of the closely related GR on different levels of biological complexity. Finally, the synthesis section that highlights the two faces of brain MR: Salt and Stress. ‘Salt’ refers to the regulation of salt appetite, and reciprocal arousal, motivation and reward, by a network of aldosterone-selective MR-expressing neurons projecting from nucleus tractus solitarii (NTS) and circumventricular organs. ‘Stress’ is about the limbic-forebrain nuclear and membrane MRs, which act as a switch in the selection of the best response to cope with a stressor. For this purpose, activation of the limbic MR promotes selective attention, memory retrieval and the appraisal process, while driving emotional expressions of fear and aggression. Subsequently, rising glucocorticoid concentrations activate GRs in limbic-forebrain circuitry underlying executive functions and memory storage, which contribute in balance with MR-mediated actions to homeostasis, excitability and behavioural adaptation.

Introduction

On Tuesday April 14, 1987, while visiting Ron Evans and Jeff Arriza, we learned that they had cloned the mineralocorticoid receptor (NR3C2 or MR). At the time, one of us (M Joëls) was working next door, with George Siggins and Floyd Bloom at the Scripps Clinic. Evans’ and Arriza’s manuscript had just passed the Science review process and was waiting to be published on July 6th of the same year (Arriza et al. 1987). We were all excited, particularly as from our studies, evidence was mounting that there had to be an MR-like receptor...
co-existing with the classical glucocorticoid receptors (GRs) in the brain. This was discovered in seminal binding studies using $^3$H-corticosterone in the absence or presence of the unlabeled pure glucocorticoid RU28362 (Moguilewsky & Raynaud 1980). The San Diego visit in March 1987 gave us (E R de Kloet) a head start in combining new methods to measure MR with the knowledge gained from pharmacological and endocrine studies.

In this tribute to the 30 years anniversary of the cloning of the MR, we will try to capture the special feeling that MR scientists of this world share. This is because many card-carrying MR aficionados are old enough to remember the attempts to piece together what the peculiar aldosterone- and corticosterone receptor-binding profiles meant and still young enough to appreciate the technological breakthroughs for the sake of better MR understanding. Here, we will start with a brief account of the pre-MR cloning era, then highlight the excitement during the first 5 post-cloning years and conclude with a synthesis that is beginning to reveal the plot of an unfolding brain MR story.

Precloning era

When Bruce McEwen at Rockefeller University inspected the print out of the scintillation counter to learn about brain retention and uptake of $^3$H-corticosterone administered to an adrenalectomised (ADX) rat, he was shocked. Wisdom at the time predicted that the hormone would accumulate in the ‘hypophysiotropic’ hypothalamus that harboured the main regulator of the hypothalamic–pituitary–adrenal (HPA) axis: corticotropin-releasing hormone (CRH, cloned in 1981 by the late Wylie Vale) and vasopressin in the paraventricular nucleus (PVN). However, it was not in the PVN that corticosterone was retained; by far the highest uptake and retention of the tracer occurred in the hippocampus (McEwen et al. 1968, Gerlach & McEwen 1972). This observation matched with the behavioural and neuroendocrine effects of glucocorticoid implants in suprahypothalamic regions (Bohus & Lissak 1968). The impact was large: it became clear that brain regions beyond the hypothalamus had to be of crucial importance for the action of adrenal hormones on the processing of salient information (McEwen et al. 2015).

Tracer amounts of corticosterone are retained and accumulated by mineralocorticoid receptors in extrahypothalamic limbic brain regions.

Saturday November 30, 1968, the Nature publication on hippocampal corticosterone retention was published, and Monday December 2, I (E R de Kloet) started my PhD research. In those days, there was no distracting Internet. New research findings were read and discussed immediately after publication. Inspired by the new data, one could do right away a cutting-edge experiment in a Popperian attempt to reject a hypothesis as there were no time-consuming rules and regulations. For instance, in this corticosterone case, we assumed in The Netherlands that we would get better results with the much more potent synthetic glucocorticoid dexamethasone. However, whatever I (E R de Kloet) tried, dexamethasone did not mimic corticosterone binding in the rat brain but, rather, was retained in the pituitary (de Kloet et al. 1974). In 1975, when I (E R de Kloet) was a postdoc in Bruce McEwen’s lab, it was concluded that the $^3$H-dexamethasone tracer was indeed poorly retained in cell nuclei of hippocampus (de Kloet et al. 1975). About 20 years later, we discovered one of the two reasons: in contrast to corticosterone, the dexamethasone tracer poorly penetrates the brain because it is a substrate for multidrug resistance P-glycoproteins (mdr Pgp) encoded by the ABCBI gene in the blood–brain barrier (Meijer et al. 1998).

The other reason was that if $^3$H-dexamethasone had been able to penetrate the brain, the receptor binding in the hippocampus would still have differed from that reported by McEwen for $^3$H-corticosterone. Even in the mid-seventies, we were already entertaining this idea, i.e., the existence of two distinct populations of receptor sites for corticosterone and dexamethasone (de Kloet et al. 1975, McEwen et al. 1976). Meanwhile, $^3$H-aldosterone autoradiography and cytosol receptor binding showed a striking similarity with the pattern of corticosterone retention in the rat brain (Ermisch & Rühle 1978, de Nicola et al. 1981, Beaumont & Fanestil 1983). Moreover, inclusion of the glucocorticoid RU28362 appeared to discriminate between high- and low-affinity binding of corticosterone (Moguliewsky & Raynaud 1980, Veldhuis et al. 1982). Then, it was discovered by the late Zig Krozowski that cytosol of brain and kidney display similar binding properties for aldosterone, the certified mineralocorticoid (Krozowski & Funder 1983). Finally, the hippocampal cells expressing irGR (Fuxe et al. 1985) did not match the autoradiograms of the naturally occurring corticosterone (Gerlach & McEwen 1972). Notably, the hippocampal CA3 virtually lacked GR, whereas the very same area was known to
highly retain tracer amounts of $^3$H-corticosterone in adrenalectomised rats.

That was the moment the idea sparked that we probably missed the GR in the autoradiograms because its affinity was too low to produce a signal after the tracer doses of $^3$H-corticosterone. Thus, we administered in vivo graded doses of corticosterone to adrenalectomised animals, and then measured in vitro the unoccupied sites with $^3$H-corticosterone in the absence or presence of the pure glucocorticoid. This experiment demonstrated in micropunched discrete areas all over the brain, the presence of two populations of receptor sites that could bind $^3$H-corticosterone with a tenfold difference in affinity. The high-affinity site was termed Type 1 corticosterone preferring (the MR) to distinguish it from the lower affinity Type 2 sites (the GR) that required stress levels of corticosterone for activation and that also bound synthetic glucocorticoids (Reul & de Kloet 1985). From that time onward, the design of our studies was dictated by the properties and localisation of the Type 1 and Type 2 corticosterone receptor populations in the brain.

**Post-cloning period**

Armed with the new tools to study the brain corticosterone receptors, it was quickly established that the cloned MR was identical to the pharmacologically characterised rat brain Type 1 receptors in properties and neuro-anatomical localisation (Arriza et al. 1988, van Eekelen et al. 1988, Herman et al. 1989, Ahima et al. 1991). Highest expression of MR was found in the limbic structures, notably, the hippocampus of rats, mice, hamsters, birds and primates that was previously found to retain $^3$H-corticosterone (Rhees et al. 1972, Gerlach et al. 1976, Sutanto & de Kloet 1987, Patel et al. 2008). The naming of the receptor was ambivalent though as the soluble brain MR did bind aldosterone as well as the naturally occurring glucocorticoids and even progesterone, hence, its promiscuous nature (Funder 2016). The discovery in 1988 by John Funder and Chris Edwards shed light on this mystery: it was found that the enzyme 11β-hydroxysteroid dehydrogenase Type 2 (HSD-2) functions as gate keeper in (among others) the kidney by inactivating the bioactive natural glucocorticoids, thus rendering aldosterone selectivity of the MR (Edwards et al. 1988, Funder et al. 1988, Funder & Myles 1996). The 11HSD-2 is not only co-localised with MR in cells engaged in the regulation of the electrolyte balance such as the kidney tubular cells but also in discrete regions of the brain (see below). In contrast, the 11HSD-1 reductase regenerates bioactive glucocorticoids and is widely expressed in neurons and glial cells of the brain (Chapman et al. 2013).

In most brain cells, therefore, MR retains corticosterone with very high affinity. Consequently, this receptor is expected to be substantially occupied by corticosterone. Even in the morning free corticosterone (not bound to corticosteroid binding globulin (CBG) circulates in the rat in a 10- to 100-fold excess of corticosterone over aldosterone. Indeed measurement of immunoreactive steroid in purified cell nuclei of the hippocampus provided a rise of from 200 to 900pg corticosterone/mg DNA over the circadian cycle against a surprisingly steady 20pg aldosterone/mg DNA (Yongue & Roy 1987). Thus, the amount of aldosterone in the hippocampal cell nuclei is only 10% of corticosterone and started decreasing towards the circadian peak. At the start of the inactive period, corticosterone is mainly retained by MR in the nucleus, whereas at the start of the active period, the hormone is additionally bound to GR.

What is the significance of a brain MR that, even under basal conditions, is largely occupied by the natural ligand? An immediate response to that question is that the receptor rather than the ligand is the rate-limiting step, as John Funder once remarked when he visited the Rudolf Magnus Institute in Utrecht on one of his global excursions. This implies that one would like to know the turnover of the receptor rather than the level of expression as indication of receptor activity for instance by measuring its proteasome-dependent clearance (Conway-Campbell et al. 2007).

**Neuroendocrinology, volume regulation and spatial learning**

If the MR is always substantially occupied with ligand, we reasoned that this receptor should have something to do with the regulation of the set point of the HPA axis under basal conditions (de Kloet & Reul 1987). Using adrenalectomised animals that were replaced with corticosterone, more or less mimicking amounts required for differential occupancy of MR and GR Mary Dallman’s group indeed demonstrated that saturation of the MR was important for basal HPA axis activity (Dallman et al. 1989). Intracerebroventricular (i.c.v.) and intrahippocampal administration of the novel MR antagonist RU28318 increased basal am and pm HPA axis activity and potentiated the initial release of ACTH and corticosterone during stress exposure (Ratka et al. 1989, van Haarst et al. 1997). In humans, higher systemic
doses of the MR antagonist spironolactone also increased basal and stress-induced cortisol secretion (Cornelisse et al. 2011).

The brain MR also appeared to affect autonomic outflow and the blood pressure response to a stressor. We found that i.c.v. administration of the MR antagonist RU28318 caused a slow and long-lasting suppression of the stress-induced pressor response (van den Berg et al. 1990). The stressor was actually the tail sphygmonometry procedure itself that was used as an indirect method of blood pressure measurement, which involved training of the animal to the restraint in a plexiglass container and exposure to a warm lamp to facilitate blood flow by releasing the tail sphincter. However, with direct telemetric recording, the depressor effect of the MR antagonist could only be measured if the tail-cuff conditions (warming and restraint stress) were applied (van den Berg et al. 1994, de Kloet et al. 2000). The depressor effect was more profound in the DOCA salt model and was abolished after denervation of the kidney (Rahmouni et al. 1999, 2001). Interestingly, Gomez-Sanchez had discovered previously increased blood pressure after i.c.v. administration of the agonist aldosterone (Gomez-Sanchez 1986, 2014). As will be pointed out below, these MR agonist and antagonist effects could possibly be explained by the manipulation of MR in the nucleus tractus solitarii (NTS), which would explain the long delay in effect after injection of the antagonist in the lateral ventricles.

At the behavioural level MR function was tested by either administering an MR antagonist i.c.v. or after replacement of adrenalectomised animals with doses of corticosterone that were just sufficient to occupy the MR but too low to activate GR. These experiments performed e.g. in the Morris maze revealed yet another aspect of MR function. Melly Oitzl discovered that the i.c.v.-administered MR antagonist was only effective when given briefly (15 min) prior to the memory retrieval task. In this task, the rats quickly learn to localise a hidden platform in a pool of opaque water using the spatial context, which is typical for the role of hippocampus in spatial learning. When the animal is tested for memory performance at 24 h after learning and the hidden platform is removed (the so-called probe trial), adrenally intact control rats will continue swimming in the area where the platform was previously located. An adrenally intact animal treated with the MR antagonist i.c.v. 15 min before the memory retrieval will initially visit the original platform location, but subsequently start to look for an alternative escape route. This observation led us to propose that the hippocampal MRs are involved in the selection of a behavioural strategy in search of an escape route (Oitzl & de Kloet 1992).

Subsequent studies showed that activation of MR not only rapidly promotes memory retrieval but also mediates with short delay the effect of corticosterone on selective attention, reactivity to novel situations and encoding of the experience for memory storage. The memory traces are encoded for appropriate retrieval at a later time in the same context. These rapid effects mediated by MR occur in rodents (Oitzl & de Kloet 1992, Oitzl et al. 1994, Khaksari et al. 2007), birds (Sandi & Rose 1994) and in response to cortisol in humans (Cornelisse et al. 2011). The effects of corticosterone mediated by the GR are complementary to MR. Thus, blockade of GR after learning prevented memory consolidation of the encoded experience and behavioural response (Oitzl et al. 1992). The GR-mediated effects are gene dependent and observed after systemic, i.c.v. and intra-hippocampal administration of the anti-glucocorticoids. The memory consolidation effect involves GR homodimers as it was abolished in mutants carrying a point mutation, which prevents dimerisation (Oitzl et al. 2001).

In neuroendocrine regulation, GR operates complementary to MR. Under basal am conditions, the occupancy of GR is too low and therefore the antiglucocorticoid mifepristone is ineffective. However, the initial HPA axis response after stress is rapidly attenuated by the GR antagonist rather than enhanced as is observed after the MR antagonist. Subsequently, circulating stress-induced corticosteroid levels remained high for a prolonged period because mifepristone interferes with glucocorticoid feedback (Ratka et al. 1989, van Haarst et al. 1997). Also, in volume regulation, the actions mediated via GR are complementary to those mediated by MR. For instance, a single i.c.v. mifepristone administration caused a longer-lasting and more prolonged increase in the systolic pressor response rather than the decrease after central MR blockade, when measured with the tail-cuff method (van den Berg et al. 1990).

Thus, glucocorticoids act as a double-edged sword in coordination of brain and behaviour. MR controls basal and stress-induced activity of the HPA axis, sympathetic outflow and selection of a behavioural (coping) strategy. GR subsequently suppresses stress-induced activations while promoting memory storage of the experience for future use.
MR and neuronal excitability

Although it was realised in the nineties that corticosterone binds to brain MR and thus influences neuroendocrine regulation and behaviour, the steps between binding and function were unclear. This was gradually revealed by electrophysiological experiments.

In a large series of experiments, it was found that corticosterone affects many properties of hippocampal CA1 pyramidal neurons, after a U-shaped dose-dependent curve (Joëls & de Kloet 1992, 1994, Joëls 2006, Joëls et al. 2012). As an example of corticosteroid actions on CA1, neurons can serve the hormone’s effect on serotonin (5-HT) transmission. Activation of 5-HT1A receptors in hippocampal CA1 cells is known to increase the conductance of an inwardly rectifying K-channel, causing the membrane to hyperpolarise. We reported that in the absence of corticosterone (i.e., in adrenalectomised rats), activation of 5-HT1A receptors leads to a large hyperpolarisation compared to the condition that low levels of corticosterone are present. Selective activation of MRs was indeed found to be associated with very small responses to 5-HT. If GRs were additionally activated (e.g., as occurs after stress), a slow enhancement in 5-HT1A receptor-mediated hyperpolarisation response was observed (Joëls et al. 1991). The latter depended on DNA binding of GR homodimers (Hesen et al. 1996, Karst et al. 2000).

Very similar principles were found to hold for the influx of calcium through L-type voltage-dependent calcium channels, as well as properties that are linked to this calcium influx such as the attenuation of firing frequency upon a steady depolarisation of cells (Joëls & de Kloet 1990, Chameau et al. 2007).

Dentate granule cells were also reported to be exquisitely sensitive to corticosterone acting via MR. Thus, in the absence of MR activation, the cell cycle of dentate cells accelerates, leading to both increased neurogenesis and apoptotic cell death (Wong & Herbert 2005). In addition, MR activation is a prerequisite to maintain the complexity of granule cell dendritic trees. Interestingly, in these cells, GR function is somehow not always translated from the transcriptional to the protein level, yielding a sigmoidal rather than a U-shaped dose dependency (Joëls 2006, 2007, van Gemert et al. 2009).

MR activation of hippocampal cells seems to be a necessary condition for viability of neurons and steady transfer of excitatory signals. GR-dependent actions develop against this background of MR signalling to restore transiently raised excitability.

Synthesis

In the years after the post-cloning hype, brain MR research has produced new data to such an extent that synthesis is possible. Here, we will discuss the identification of a neuronal network responsive after aldosterone in brain, the discovery of non-genomic MR-mediated actions on neuronal excitability, the use of genetically modified and other MR-based animal models and the remarkable role of MR gene variations in resilience and health.

The aldosterone-responsive MR neuronal network

Epithelial cells in e.g. kidney, bladder, colon and sweat glands are targets for aldosterone in the regulation of salt retention. In these tissues, aldosterone activates the MR and its downstream target genes, i.e. serum and glucocorticoid-induced kinase (SGK-1), the ubiquitin ligase NEDD4-2 and the epithelial sodium channel (ENaC) to promote Na resorption. This mechanism also seems to underly sensory salt detection in the tongue, whereas it is thought to regulate a circumventricular organ-based circuit involved in detection of changes in osmotic balance by a hypothesised Na sensor (Fu & Vallon 2014). Some authors have postulated a role in salt appetite and volume regulation of local synthesis of aldosterone in the hypothalamus and circumventricular organs (Wang et al. 2016), but see a commentary by Funder (2005). The action exerted by aldosterone on salt appetite may occur in synergy with angiotensin II, as was observed in the salt-depleted animal model generated by furosemide administration (Sakai et al. 1986, Krause & Sakai 2007).

Recently, aldosterone-induced salt appetite was found to be under control of a small group of neurons in the caudal part of the NTS. The seminal finding to arrive at this conclusion was by Randall and coworkers (Randall et al. 1995) who observed that 11HSD-2 was expressed in a similar NTS area as previously observed for MR mRNA (Arriza et al. 1988). Then Geerling and coworkers (Geerling et al. 2006) demonstrated strong signals of colocalisation of 11HSD-2 and MR using immunocytochemistry in the caudal NTS. A weak colocalisation signal was also observed in some other circumventricular organs (subfornical and subcommissural organs) and in the ventromedial nucleus of the hypothalamus. The MR-NTS neurons connect to the nearby area postrema, a periventricular region that is readily accessible to peptides and other compounds in the 4th ventricle. i.c.v. infusion of the MR agonist aldosterone into the 4th, but not the lateral ventricles, increased salt
appetite (Formenti et al. 2013); and MR knockout in the NTS prevented this aldosterone-induced salt appetite (Koneru et al. 2014).

Mutant mice with a conditional knockout of the 11HSD-2 gene selectively in the NTS showed increased salt appetite, but only when offered saline. The salt uptake of these mutants was three-fold higher than that of the controls (Evans et al. 2016). Systemic spironolactone could not entirely block the increased salt appetite. This is in support with previous pioneering work of Alan Epstein and Randall Sakai showing that in salt-depleted animal, the increased salt appetite is fully blocked when in addition to spironolactone also an angiotensin antagonist is administered (Sakai et al. 1986). Salt appetite was not a consequence of a decreased detection threshold.

The 11HSD-2-mutant mice offered saline also showed increased systolic and diastolic blood pressure, particularly in the dark phase of the circadian cycle. The reasoning is that in the absence of 11HSD-2, the MR will be occupied by corticosterone, which circulates in much higher concentrations than aldosterone. Indeed, the rise in blood pressure is attenuated after suppression of corticosterone secretion by dexamethasone even though the synthetic steroid or excess corticosterone also exert a pressor effect by itself (van den Berg et al. 1990, Gomez Sanchez 2014). Mutants receiving a salt diet became hypertensive, whereas controls receiving the same diet remained normotensive and were thus salt resistant. Blood pressure normalised again when salt was removed. Renal sodium excretion was not affected in the mutants suggesting that volume expansion was not underlying the pressor effect. Rather, the pressor response to phenylephrine was enhanced, and the baroreflex was impaired in the mutants. Although blood pressure was not measured after spironolactone in this study, the salt-sensitive rise in blood pressure seems to involve an enhanced MR-dependent sympathetic drive (Evans et al. 2016). The data are thus consistent with the previous finding that spironolactone i.c.v. decreased the pressor response by attenuating sympathetic outflow (Rahmouni et al. 1999).

Aldosterone targets the MR-NTS neuronal network in the regulation of salt appetite and reciprocally sympathetic outflow, cardiovascular functions and behaviour.

MR-NTS neurons are innervated by afferent projections from the vagus nerve carrying microbiomarker information from the gut and the gastro-intestinal tract. There are also afferents from sensory neurons to the NTS that detect sodium in the taste buds of the tongue for further processing in the chorda tympani branch of cranial nerve VII.

Efferents of the MR-NTS neurons project to the parabrachial nucleus, central amygdala, paraventricular nucleus and hippocampus. There are also projections to the bed nucleus of the striae terminalis (BNST), which is a hub in the network from where efferents run to orexin neurons implicated in arousal. BNST neurons innervate the limbic brain and the nucleus accumbens underlying motivation and reward behaviours likely with the goal to still salt appetite. Pathways from the nucleus accumbens and paraventricular neurons subsequently signal salt satiation and then aversion (Geerling et al. 2006, Krause & Sakai 2007, Geerling & Loewy 2008, 2009). This neuronal network may explain why interventions in the frontal brain regions such as e.g. the amygdala, can also affect aldosterone regulation of salt appetite (Sakai et al. 2000).

Salt appetite is affected by mineralocorticoids in a U-shaped dose–response manner. After adrenalectomy, salt appetite rises, which is readily normalised by aldosterone replacement, but with higher doses of the mineralocorticoid, salt appetite increases again. Glucocorticoids increase salt appetite by inducing MR and by sustaining-through activation of the mesolimbic dopaminergic level, the motivation to ingest salt (Krause & Sakai 2007).

There is another aspect that also needs attention. This condition of high mineralocorticoid level and high salt uptake generates a phenotype with increased risk of inflammation. For instance, the deoxycorticosterone-2% salt treated rat is a classical model for hypertension, ischaemia and enhanced pro-inflammatory cytokine action (Rocha & Stier 2001, Young et al. 2010, Jaisser & Farman 2016). Glucocorticoids have a potent anti-inflammatory action. This led Hans Selye to postulate the ‘pendulum hypothesis’: actions of mineralocorticoid and glucocorticoid hormones are opposite in the maintenance of homeostasis as exemplified by their pro- and anti-inflammatory actions, respectively (Selye 1952). In retrospect, this pendulum hypothesis refers to MR and GR, which mediate opposing actions on inflammation by the naturally occurring glucocorticoids.

Non-genomic MR-mediated actions

In 2005, we observed that application of corticosterone to CA1 hippocampal cells quickly and transiently increases the frequency of miniature excitatory postsynaptic currents (mEPSC), each of which represents the postsynaptic response to the spontaneous release of one glutamate-containing vesicle. This increase is most likely caused by an enhanced release probability of the vesicles. The rapid
The rapid effects were abolished after pretreatment with MR antagonists and were absent in mutant mice with the forebrain MR deleted (Karst et al. 2005, Joëls et al. 2012). Relevant to this chapter, the membrane-mediated effects were obtained with concentrations of 10 nM corticosterone or aldosterone and higher. Apparently, the pool of readily available membrane-associated MRs has a lower affinity to corticosterone than the nuclear MR. As a consequence, unlike the nuclear MR, the membrane MR can quickly respond to a rise in corticosterone level such as occurs during stress. This would lend a new and important role to the membrane MR in mediating...
stress levels of corticosterone beyond that of nuclear MRs, which are already substantially occupied under rest (Joëls et al. 2008, Groeneweg et al. 2011). Activation of the membrane MR results in the downregulation of the presynaptic Type 2 metabotropic glutamate (mGlu2) receptor presumably in response to increased glutamate release, although also a direct regulatory effect has been suggested (Nasca et al. 2015).

Principal cells in the basolateral amygdala are also capable of developing these fast MR-dependent responses in glutamate transmission. The onset of the response was slightly more gradual than that in the hippocampus and, importantly, the frequency did not return to baseline upon washout of the hormone but stayed high. This prolonged effect is gene mediated and involves the GR. The long-term change in excitability reflects the fact that rapid-onset MR-mediated in cooperation with slow GR-mediated actions lastingly change the state of amygdala cells. The main consequence thereof is that these cells respond differently to a second ‘hit’ by corticosterone, i.e., by no response or even a decrease (as opposed to the increase) in mEPSC frequency. The latter rapid effect of the second hit depends on non-genomic actions via a GR and involves the endocannabinoid receptor 1 (Di et al. 2003). We dubbed this change in response to ‘the corticosterone metaplasticity’ (Karst et al. 2010).

Metaplasticity may play a role during the day because amygdala cells are hit by multiple corticosterone pulses in succession as part of the physiological ultradian release pattern of the hormone. However, also during the stress response, metaplasticity might be relevant. Recently, we reported that the β-adrenoceptor agonist isoproterenol causes enhanced mEPSC frequency in amygdala cells, similar to corticosterone. A wave of isoproterenol, through metaplasticity, also affected the response to subsequently administered corticosterone. With low-to-intermediate concentrations of isoproterenol and corticosterone (mimicking mild-to-moderate stress conditions) corticosteroid actions were subdued, so that the effects of isoproterenol prevailed. With high concentrations of both compounds, though, corticosterone actions were dominant, causing a long-lasting enhancement of excitability in the amygdala (Karst & Joëls 2016). This may explain why events associated with such high concentrations are usually extremely well retained in memory, particularly with regard to emotional aspects that are processed via the basolateral amygdala (Fig. 1).

The response of brain cells to glucocorticoids is not necessarily always the same. The recent history of the organism may greatly affect the final outcome, as demonstrated in the basolateral amygdala. To what extent this principle also holds for other brain areas requires more investigation.

**MR function in limbic-forebrain regions during behaviour**

**Pharmacology** Rats, when exposed to an unexpected stressor, commonly respond to a single stimulus (stimulus–response behaviour) rather than integrating multiple distal spatial cues in collecting a reward. This observation was made using a circular hole board where the presence of a reward was marked by a bottle (stimulus) as well as distal cues. By simply placing the bottle at the other side of the circular hole board, animals using the single stimulus or habit response will change to the new location of the bottle, whereas animals using a more complex spatial strategy will go to the previous (original) location. Non-stressed animals mostly use the flexible spatial hippocampal strategy, but if stressed, they engage in a cognitively less demanding intuitive habits linked to the striatum (Dias-Ferreira et al. 2009, Schwabe et al. 2010, Sousa 2016). Of note, sex differences were observed: Although males outperform females in spatial learning under basal conditions, the reverse is the case after stress (Ter Horst et al. 2013).

The stress-induced switch towards striatal-based habit learning can be blocked by i.c.v. application of the MR antagonist spironolactone. If the MR is blocked, the animals maintain the original spatial strategy (Schwabe et al. 2010, 2012, Arp et al. 2014). Similar behavioural observations were made in humans, in various tasks. Concomitant fMRI measurement revealed that tasks involving a trade-off between simple effective strategies and complex, flexible strategies indeed show an MR-dependent switch from hippocampal towards striatal circuits under stress (Schwabe et al. 2013, Vogel et al. 2015a,b, 2016a,b).

**Limbic MR activation promotes during stress the switch from deliberate hippocampal cognitive behaviour to a striatal-based habit response.**

**Genetic modification** Stress-induced HPA axis activity is attenuated in mice with conditional overexpression of MR in the forebrain, particularly when crossed with heterozygous (GR−/−) mutants. These mutants with an increased MR:GR ratio in the forebrain (MR up and GR down) are less prone to anxiety, whereas they show better cognitive performance and perseveration of acquired behaviour (Harris et al. 2013). This phenotype previously was observed in mutant mice with forebrain-specific MR overexpression (Rozeboom et al. 2007). MR-overexpressing
mice exposed daily for three weeks to unpredictable stressors were protected to deficits in low arousing learning tasks and showed reduction in hippocampal neurogenesis (Kanatsou et al. 2015). Similarly, rats with viral overexpression of MR in the rat dentate gyrus are resistant to impairment in an object recognition test (Ferguson & Sapolsky 2007, 2008).

**Selected lines** Studies with genetically selected mouse or rat lines generally support the role of MR shown by pharmacology and genetic modification in neuroendocrinology and behaviour. Not only Lewis rats, but also spontaneous hypertensive Wistar Kyoto and apomorphine-susceptible rats, have elevated hippocampal MR expression and reduced circulating corticosterone levels (Sutanto et al. 1992, Oitzl et al. 1995). Rats selected in an elevated plus maze and open field behaviour for low anxiety (LA) show better performance in spatial memory performance, lower circulating corticosterone levels and a higher hippocampal MR expression than high anxiety (HA) animals (Herrero et al. 2006). Locomotor activity in a novel environment also has been a selection criterion. The high-reactive rodents are highly susceptible to a chronic unpredictable stressor at midlife and show cognitive deterioration during the ageing process, whereas the low-reactive rats are protected to midlife stress and show elevated hippocampal MR expression (Oitzl et al. 2000, Sandi & Touyarot 2006).

Another example is the selection of wild mice for short-attack and long-attack latency (SAL vs LAL). The SAL mice are dominant and show a non-flexible control in the home cage with high hippocampal MR expression as opposed to the less-aggressive mice that show a flexible behavioural response and higher corticosterone levels, when faced with a novel challenge (Veenema et al. 2003). Collectively, these studies suggest that high hippocampal MR expression is associated with low anxiety, an active coping style, superior cognitive performance and low corticosterone levels, provided such animals are in control and in their own territory.

Acute stress increases hippocampal MR expression (Gesing et al. 2001, Bigio et al. 2016), but also a decrease has been reported for MR hRNA, whereas the effects appeared to depend on strain and methodology (Herman et al. 1999). Exposure to synthetic glucocorticoids also increases hippocampal MR (Reul et al. 1987, 1989) as do tricyclic antidepressants (Seckl & Fink 1992), whereas aged, chronically stressed and/or depressed individuals have a suppressed brain MR expression (van Eekelen et al. 1995, Klok et al. 2011b). Adult rodents that have experienced extensive maternal care as pups have higher expression of hippocampal MR than their littermates that have received poor care (Champagne et al. 2008). Handling increases, whereas adverse experiences during perinatal life cause MR downregulation in later life (de Kloet et al. 2005b).

*High limbic MR is characteristic for a dominant individual that shows resilience if in control, but increased vulnerability to psychopathology during loss of control.*

**Genetic variation in humans** Common genetic variants of the MR have been functionally characterised. The rs5522 (A/G) SNP is located in exon 2 and encodes the amino acid change isoleucine 180 to valine; the G-allele has *in vitro* a lower transactivation capacity for cortisol. In humans, it predicts enhanced HPA axis and autonomic reactivity, increased susceptibility for depression, impaired reward learning and increased amygdala reactivity during chronic stress (DeRijk et al. 2006, Bogdan et al. 2010, 2012). The other functional SNP is the rs2070951 (C/G), located at two nucleotides from the translation initiation site. The G-allele results in higher activity of the renin–angiotensin–aldosterone system and a higher blood pressure, which would be related to the lower MR activity measured for this variant (van Leeuwen et al. 2010).

*Common and functional MR haplotypes might relate to the extent of MR activation conferring inter-individual variability in susceptibility for psychopathology.*

The rs5522 and rs2070951 SNPs have been studied with a haplotype approach: haplotype 1 (hap 1, GA, frequency 49%), haplotype 2 (hap 2, CA, frequency 41%), haplotype 3 (hap 3, CG, frequency 9%) and haplotype 4 (hap 4, GG, which is rarely observed). Hap 2 showed the highest expression and transactivation potency in COS-1 and neuroblastoma cells (DeRijk et al. 2011, van Leeuwen et al. 2011). School teachers homozygous for hap 2 showed highest resilience on the Trier Inventory for Chronic Stress subscales ‘excessive demands at work’ and ‘social overload’ and displayed a brisk HPA axis and heart rate response in the Trier Social Stress test (van Leeuwen et al. 2011).

In females, the hap 2 carriers showed more dispositional optimism, less rumination, fewer thoughts of hopelessness and lower risk of depression (Klok et al. 2011a). In line with this is the higher implicit happiness score of MR-haplotype 2 homozygotes. Hap 2 carriers...
appeared less sensitive to variations in female hormone exposure during the reproductive cycle and to the depressinogenic side effects of oral contraceptives. The MR genotype moderates the influence of oestrogen and progesterone on emotional information processing (Hamstra et al. 2015, 2016, 2017). The MR gene variants were found to sex-dependently moderate the effect of early life trauma on vulnerability to depression in a population-based cohort (n=665) and an independent clinical cohort from the Netherlands Study of Depression and Anxiety (n=1639). The findings of these studies suggest that MR haplotypes associate with personality traits, stress reactivity and risk for depression in a sex-dependent manner in the face of early life trauma (Vinkers et al. 2015).

The MR:GR balance concept

Molecular studies

Ron Evans and Jeff Arriza (Arriza et al. 1988) predicted that ‘composite gene networks of MRs and GRs may be partly overlapping and partly distinct.’ They envisioned a ‘binary glucocorticoid response system, which would provide a continuum of control and thus expands the range of physiological response by synergistic or competitive interactions’, provided both receptors are expressed in the same cells. In hippocampus, MR and GR are co-expressed in abundance as shown by confocal microscopy (Van Steensel et al. 1996, Han et al. 2005).

The predicted composite gene networks were identified using expression profiling of the hippocampus of ADX animals replaced with a low dose of corticosterone providing predominant MR occupation. A second group of corticosterone-replaced animals receiving an additional corticosterone injection mimicking the stress response was killed 3h later. The study revealed that only 20% of the genes were responsive to MR as well as GR (Datson et al. 2001, 2008). In a subsequent study, animals were first exposed to a chronic stress procedure and then received corticosterone injections. The gene patterns examined in laser-dissected hippocampal regions revealed that a history of chronic stress had profound consequences for the subsequent response to acute corticosterone challenge, differentially affecting the expression of several hundreds of genes as compared to challenged non-stressed control animals. The difference concerned an overrepresentation of genes involved in chromatin reorganisation and epigenetic processes in the stress group (Datson et al. 2013).

In another experiment using corticosterone replacement of ADX animals, chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) identified 2470 specific binding sites of which 39% were localised within in genes (Fig. 2) (Polman et al. 2013). Of these binding sites in total 918 and 1470 were exclusive for MR and GR, respectively, whereas 475 sites were overlapping and did bind both receptors (van Weert et al. 2017). Motif analysis revealed a 100% similarity with GRE’s on every binding site. In addition, an additional AtOH/NeuroD consensus sequence

**Figure 2** Genomic distribution of glucocorticoid receptor-binding sites in rat hippocampus genome. (A) Distribution of GBS relative to the nearest gene, resulting in regions that lie within or outside genes. The black bar represents a gene, showing that 39% of the GBS are located within genes. The GBS that are located upstream or downstream from the nearest gene are divided into 3 bins: within 10kb, between 10 and 100 kb and more than 100 kb from a gene. (B) Pie chart showing the location of intragenic GBS within annotated RefSeq genes, divided into 5′-UTR (exon or intron), intron, exon, intron/exon overlap, and 3′-UTR (exon or intron) regions. Reproduced, with permission, from Polman JA, de Kloet ER & Datson NA, *Endocrinology*, volume 154, pages 1832–1844, 2013, Two populations of glucocorticoid receptor-binding sites in the male rat hippocampal genome. Copyright 2013, The Endocrine Society. See also van Weert et al. (2017).
was found at 400bp from the GRE for all MR-specific binding sites only, and which was thus absent at the MR–GR overlapping and GR-binding sites. In vivo, one of the members of this family of proteins, NeuroD2, was found at MR-, but not at GR-exclusive binding sites (van Weert et al. 2017). The NeuroD2 proteins have a role in neuronal differentiation and studies with NeuroD2 mutants revealed their role in stress susceptibility (Bagot et al. 2016).

Two lines of evidence suggest that under stressful conditions, other layers of complexity are added to MR and GR functioning. Thus, Gray and coworkers (Gray et al. 2014) compared the effect of corticosterone exposure with that of acute stressor exposure of the chronically stressed animals and found distinctly different profiles of responsive genes such as e.g. the NFκB pathway (see also Datson et al. 2012). Furthermore, a recent sequential tandem ChIP analysis by Mifsud and Reul (2016) using tissue of stressed adrenally intact intact animals provided further evidence for the possible existence of overlapping MR- and GR-binding sites indicative of MR:GR heterodimers as had been previously suggested by in vitro experiments (Liu et al. 1995, Trapp & Holsboer 1996). For the ultimate proof of heterodimers, molecular imaging approaches are necessary (for the process, see Groeneweg et al. 2014) (Fig. 3).

Previously, binding experiments were performed under basal conditions in ADX animals replaced with various doses of corticosterone and killed at 1h after administration to obtain gradual activation of first MRs and then additional GRs. These experiments were originally used for discrimination of MR from GR in hippocampus cytosol, which led to the notion that nuclear MR is largely occupied already under basal conditions, whereas nuclear GR requires stress levels of corticosterone. In the ChiP-seq experiments, this experimental approach led to the distinction of two populations of genomic-binding sites for MR and GR (Polman et al. 2013). One population became saturated with low corticosterone concentrations containing mostly MR, whereas a second population required high concentrations with a much higher GR:MR ratio. However, under adrenally intact stressed conditions, high receptor occupancy did not predict the extent of DNA binding, whereas circadian variations also did not translate unequivocally to occupancy of genomic-binding sites on the three genes investigated (Mifsud & Reul 2016). The complexity likely is caused by NeuroD, AP-1, NFkB and other transcription factors or coregulators (Zalachoras et al. 2013, van Weert et al. 2017).

Thus, NeuroD2 factor interaction seems to confer MR over GR-binding specificity to nearby GREs.

Cellular and behavioural studies

The genomic expression and binding studies reveal an enormous diversity in MR- and GR-mediated molecular

Figure 3

Ligand structure determines the nuclear mobility of the MR. A range of natural and synthetic agonists (black bars) and antagonists (red bars) were tested for their effect on the intranuclear mobility of the MR by both SMM (A) and FRAP (B and C) analysis. The MR and GR share several agonists, but the binding and functional characteristics differ. Indeed, a weak agonist for the GR, corticosterone (cort), which gave a very mobile GR, instead leads to a low mobility for the MR. A large bound fraction (SMM; white bars and FRAP; white and light grey bars combined) a low diffusion coefficient (in µm²/s, written within its corresponding bar in A) and long immobilization times (C). Of all functional steroid side groups, only the 18-keto (18=O) group appears to affect the mobility of the MR. SMM: n=20, FRAP: n=30. Data represented as total fit ± s.e.m. (of 3 separate PICS analyses) for SMM and as average of top 10% fits ± s.e.m. for FRAP. Aldo, aldosterone; csol, cortisol; dex, dexamethasone; DOC, deoxycorticosterone; epler, eplerenone; spiro, spironolactone. Reproduced under the terms of the original Creative Commons Attribution License, from Groeneweg FL, van Royen ME, Fenz S, Keizer VI, Geverts B, Prins J, de Kloet ER, Houtsmuller AB, Schmidt TS & Schaaf MJ, 2014, Quantitation of glucocorticoid receptor DNA-binding dynamics by single-molecule microscopy and FRAP. PLoS ONE, volume 14, e90532.
effects. At the neuronal level, these diverse actions appear translated to U-shaped or sigmoidal dose–response curves demonstrating the role of MR in maintaining high excitability and that of GR to normalise this transiently raised excitability (Joëls 2006). In hippocampal pyramidal neurons, MR and GR mediate opposing actions of corticosterone, whereas in the basal amygdala cells, concomitant β-adrenergic stimulation and a genomic GR-mediated action appears to profoundly potentiate and prolong the initial fast MR-mediated rise in excitability via enhanced glutamatergic transmission. This extended period of increased excitability will promote encoding of emotionally loaded information.

The cellular actions of the glucocorticoids mediated by MR and GR can be translated to behavioural performance. Initially, the membrane MR mediates the rapid effects on information processing underlying selective attention and memory retrieval and allocation of energy to circuits underling the selected coping strategy (Vogel et al. 2016b). GR-mediated actions subsequently are concerned with executive functions and memory storage in preparation for future life.

How these new findings on different levels of biological complexity can be incorporated in the MR:GR balance hypothesis is a challenge. This hypothesis was inspired by Selye’s pendulum hypothesis involving opposing mineralocorticoid and glucocorticoid effects on inflammation. The ‘balance hypothesis’ refers to the complementary MR- and GR-mediated actions of cortisol or corticosterone that coordinate physiological regulations aimed to maintain homeostasis and to promote behavioural adaptation:

‘The MR:GR balance hypothesis predicts that upon imbalance of these receptor functions threats to homeostasis are less well communicated and coordinated among the various glucocorticoid targets. At a certain threshold this may lead to a condition of neuroendocrine dysregulation and impaired behavioural adaptation, which potentially can aggravate stress-related deterioration and promote susceptibility to stress-related disease for which the individual is genetically predisposed’ (de Kloet & Reul 1987, de Kloet 1991, 2014, de Kloet et al. 1998, 2005a).

The hypothesis has implications for pharmacotherapy with synthetic glucocorticoids. Dexamethasone, for instance, potently suppresses HPA axis activity leading to depletion of the MR ligands cortisol and corticosterone and thus decreased MR occupancy in the brain. As a consequence, brain functions dependent on MR may deteriorate, and this has been demonstrated in rodents (Karssen et al. 2001, 2005). Indeed, dexamethasone-treated mice showed neurological and memory deficits that could be ameliorated with corticosterone substitution (Liston & Gan 2011, Liston et al. 2013). The finding that corticosterone acts via changes to hippocampal neuronal spines during the sleep–wake cycle received the Society for Endocrinology’s 2015 award for the best article published in Journal of Endocrinology (Ikeda et al. 2015).

In humans, dexamethasone reduced slow wave sleep and caused, upon prolongation, dysphoric effects. Co-administration of cortisol restored slow wave sleep and caused an euphoric mood likely via activation of MR (Born et al. 1991, Plihal et al. 1996, Groch et al. 2013). Finally, a recent clinical trial demonstrated that dexamethasone therapy of young patients suffering from acute lymphoblastic leukaemia caused in about 30% of these patients severe adverse neuropsychological effects and sleep disturbances, which were ameliorated by cortisol add-on in doses used for replacement of adrenally deficient patients (Warris et al. 2016). The peripheral metabolic effects are not ameliorated or aggravated by the additional low dose of cortisol. The benefit of this refill for the brain MR supports the validity of the MR:GR balance concept and demonstrates its clinical utility (Meijer & de Kloet 2017).

### Perspectives

Today, 30 years after cloning, we know that the brain harbours aldosterone-selective and corticosterone/cortisol-preferring networks that deal with processing of salty and stressful information, respectively. These steroid effects mediated by MR proceed in a complementary fashion with GR-mediated actions. The effects of corticosteroids in the brain therefore depend on a fine-tuned balance between MR and GR that upon activation shifts energy resources to circuits and alter their connectivity underlying arousal, emotional expressions of fear and aggression, cognitive performance, motivation, reward and aversion (Korte et al. 1995, Kruk et al. 2013, Hermans et al. 2014, Sousa 2016, Vogel et al. 2016a,b). Thus, aldosterone not only evokes salt appetite but also reciprocally affects limbic-forebrain functions, via MR-based NTS and circumventricular networks (Geerling & Loewy 2008, 2009, Evans et al. 2016); the limbic-forebrain circuits themselves express abundantly MRs, which prefer corticosterone and cortisol. Aldosterone and the glucocorticoids are thus capable to affect the limbic-forebrain circuitry underlying cognitive and emotional functions (de Kloet & Joëls 2017).

The forebrain cortisol/corticosterone preferring MR network controls autonomic, neuroendocrine and
behavioural responses as part of a repertoire involving selective attention, memory retrieval, appraisal of salient information, selection of an appropriate coping strategy and encoding of the experience for memory storage. Thus, the MR can operate as a key regulator in a mechanism underlying resilience to stress. Such a crucial role of the brain MR in human resilience is underscored by variations in MR genotype, which were found to predict stress coping style and protection to depression, and by epigenetic modifications of the MR induced by (early) adversity (see Joëls et al. 2012, Bigio et al. 2016, de Kloet et al. 2016).

In years to come, we will learn much more about neuronal connectivity changes upon MR activation in different stressful contexts, at a time that our understanding of the human connectome is rapidly increasing. It is at present uncertain to what extent MR genetics contribute to circuit bias and how environmental stressors can modify MR function by epigenetic mechanisms. How precisely MR and GR interact on the genomic level as homodimers and heterodimers is also still poorly understood although progress is being made by the identification of the NeuroD co-activator conferring MR selectivity in hippocampus. On the cellular level, the coordinate membrane and genomic actions provide another challenge for translation to behaviour and physiological regulations (Karst & Joëls 2016).

This essay was focused on neuronal MR and GR. The same receptors are also however expressed in brain endothelial cells and glial cells with a prominent role in e.g. vascular function, cellular defence and energy metabolism. In these cells, the MR can drive inflammatory processes in case of vascular damage and promote atherosclerosis enhancing the risk for ischaemia. To what extent this pro-inflammatory action mediated by MR underlies co-morbidity of brain, heart and metabolic disorders is an important question for new research (see Jaisser & Farman 2016 for review).

The knowledge gained in these forthcoming studies is essential to understand the role of MR in mental health and disease multi-morbidity, to predict how pharmacological manipulation of the MR can contribute to therapeutic approaches and to appreciate that MR- and GR-mediated actions complement each other in a fine-tuned balance from gene to behaviour.

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