



Non-genomic steroid signaling through the mineralocorticoid receptor: Involvement of a membrane-associated receptor?

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ABSTRACT

Corticosteroid receptors in the mammalian brain mediate genomic as well as non-genomic actions. Although receptors mediating genomic actions were already cloned 35 years ago, it remains unclear whether the same molecules are responsible for the non-genomic actions or that the latter involve a separate class of receptors.

Here we focus on one type of corticosteroid receptors, i.e. the mineralocorticoid receptor (MR). We summarize some of the known properties and the current insight in the localization of the MR in peripheral cells and neurons, especially in relation to non-genomic signaling. Previous studies from our own and other labs provided evidence that MRs mediating non-genomic actions are identical to the ones involved in genomic signaling, but may be translocated to the plasma cell membrane instead of the nucleus. With fixed cell imaging and live cell imaging techniques we tried to visualize these presumed membrane-associated MRs, using antibodies or over-expression of MR-GFP in COS7 and hippocampal cultured neurons. Despite the physiological evidence for MR location in or close to the cell membrane, we could not convincingly visualize membrane localization of endogenous MRs or GFP-MR molecules. However, we did find punctae of labeled antibodies intracellularly, which might indicate transactivating spots of MR near the membrane. We also found some evidence for trafficking of MR via beta-arrestins. In beta-arrestin knockout mice, we didn't observe metaplasticity in the basolateral amygdala anymore, indicating that internalization of MRs could play a role during corticosterone activation. Furthermore, we speculate that membrane-associated MRs could act indirectly via activating other membrane located structures like e.g. GPER and/or receptor tyrosine kinases.

1. Introduction

“The action of CRF on ACTH release may be inhibited by a rapid effect of corticosteroids at the cell membrane.” This phrase in a review of Keller-Wood and Dallman (1984) illustrates the long history of indications for the existence of a rapid, non-genomic mechanism through which corticosterone can affect brain function. In peripheral tissue, Ganong and Mulrow (1958) described rapid actions of mineralocorticoids even earlier, in experiments specifically designed to separate rapid from more chronic effects. In the past two decades more mechanistic insight into the non-genomic mode of action of corticosteroids was obtained.

The rodent stress hormone corticosterone (cortisol in humans) is

secreted from the adrenal gland. The cascade of corticosterone secretion starts with regulation of hypothalamic neurons, sequentially activating the hypothalamus-pituitary-adrenal (HPA) axis (De Kloet et al., 2005). The secretion of corticosterone follows a circadian rhythm, with a low level at the beginning of the inactive phase and peaking at the end of the inactive and start of the active phase (Qian et al., 2012; Engeland et al., 1977; Butte et al., 1976). The circadian rhythm overarches short-duration ultradian peaks, with inter-peak intervals of 1–2 h (Lightman et al., 2008). On top of the circadian and ultradian release pattern, stressful or arousing events cause a rapid rise of corticosterone, resulting in high corticosteroid levels in both the body and brain. Within 30–60 min HPA-axis activity is attenuated first via a rapid and next via a slow negative feedback mechanism, both depending on corticosteroid

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receptor activation in the hypothalamus, so that 2–3 h after stress onset corticosteroid levels are back to baseline (Herman et al., 2016; Herman and Tasker, 2016).

Corticosterone binds to corticosteroid receptors, more specifically the mineralocorticoid (MR) and glucocorticoid receptor (GR) (Reul and de Kloet, 1985; Evans and Arriza, 1989), which are both present in brain and peripheral tissues. Trough levels of corticosterone are sufficiently high to substantially occupy the MR, since this receptor has a high affinity for corticosterone. The GR has a 10-fold lower affinity, so it is activated only to a limited extent under basal conditions (Munck et al., 1984; Reul and de Kloet, 1985). The circadian and stress-induced peaks in the secretion of corticosterone, though, are normally high enough to activate GRs.

MR and GR were cloned in the late 1980's (Arriza 1987) and shown to belong to a family of nuclear transcription-factors. Upon binding of the hormone to the intracellularly localized receptor, a cascade is started resulting in nuclear translocation of the ligand-bound receptors where they initiate and participate in gene transcription through the recruitment of co-transcription factors and binding to specific DNA binding sites, eventually resulting in long lasting changes in - among many things - the membrane properties of neurons (see for review Revollo and Cidlowski, 2009). It is assumed that activation of this so-called nuclear GR (nGR) will protect and help recover the brain from a stressful event (De Kloet et al., 1999) and to adapt the individual to stress-related changes in the environment (McEwen, 2007). nGR activation causes among other things an increase of glutamate transmission in the dorsal hippocampus (Karst and Joëls, 2005), which hampers subsequent induction of long-term potentiation (LTP) (Shors et al., 1990; Pavlides et al., 1993; Joëls and Krugers, 2007; Joëls et al., 2012) a phenomenon that is important for memory formation (Lynch, 2004). Overall, consolidation of stress-related information is increased, while consolidation of other information reaching these circuits *after* stress is hampered (de Quervain et al., 1998, 2017; Vogel et al., 2016). Activation of MR transcription is thought to act in a complementary fashion, stabilizing brain networks under basal (i.e. non-stressed) conditions (Joëls et al., 2012). In general, conditions that are related to predominant activation of nuclear MRs (nMRs) are favorable for the survival and stability of neuronal networks.

The way in which corticosterone activates the brain via nuclear corticosteroid receptors has been intensively studied over the past 50 years. However, it is known that corticosteroids can also change neuronal activity and behavior in a rapid manner (Pfaff et al., 1971; McEwen, 1979; Gilad et al., 1987), which is incompatible with the slower transcriptional activities, but little was known about the nature of the rapid signaling routes. From the start of this century, a gradual increase in the number of publications appeared.

Di et al. (2003) was the first paper to describe the rapid corticosterone effects in the hypothalamus. They provided evidence for a non-genomic effect mediated most likely via membrane located GRs (mGRs), which is responsible for the rapid feedback mechanism of the HPA-axis after a stress response. The membrane location was supported by the fact that corticosterone-BSA, a membrane impermeable conjugate, could mimic the rapid effects by corticosterone or the GR-agonist dexamethasone. This was found to involve a G-protein coupled pathway, although there is also evidence that membrane actions of steroid receptors can subsequently potentiate nuclear transcription (Vasudevan and Pfaff, 2008).

These results were the start of a series of studies examining the role of non-genomic corticosteroid receptors in the brain. In this review, we will give an overview of the progress of our knowledge of the non-genomic corticosteroid receptors, especially the non-genomic, presumably membrane-associated mineralocorticoid receptors (mMRs) in the brain.

1.1. Membrane-associated corticosteroid receptors. An electrophysiological approach

Almost 20 years ago, dexamethasone and corticosterone were reported to affect the release of glutamate from terminals projecting to the parvocellular neurons of the hypothalamus (Di et al., 2003, 2005; Di and Tasker, 2008). This body of work from the lab of Jeffrey Tasker demonstrated a rapid decrease of miniature evoked postsynaptic AMPA currents (mEPSCs), each of which represents the postsynaptic (AMPA-receptor mediated) response to the spontaneous release of a single glutamate-containing synaptic vesicle. The decrease of the mEPSC frequency was mediated by a retrograde activation of endocannabinoid receptors. At that time, it remained unclear which receptor is responsible for the effect, since an antagonist of the MR, spironolactone, or of GR, RU38486, both could not prevent the effect. More recently, evidence was provided in favor of the involvement of the GR (Nahar et al., 2015).

Shortly after the first report from the Tasker group, more electrophysiological studies were published showing that a rapid, non-genomic mode of action plays a role in several other brain structures, including the hippocampus, the amygdala, and the prefrontal cortex (PFC) (Karst et al., 2005, 2010; Musazzi et al., 2010). This was in line with earlier indirect biochemical evidence (Venero and Borrell, 1999). From the electrophysiological and biochemical studies it appeared that not only the mGRs are responsible for rapid effects, but also mMRs. Thus, Karst and colleagues (Karst et al., 2005) showed that both corticosterone and the MR-agonist aldosterone within minutes cause an *increase* in the mEPSC frequency of hippocampal CA1 neurons, the dentate gyrus neurons, and in principal neurons of the basolateral amygdala (BLA) (Karst et al., 2010), all belonging to the limbic system. The rapid onset and short-lived effect is incompatible with a genomic pathway. Since the rapid increase of the mEPSC frequency by corticosterone and aldosterone was absent in forebrain specific MR-, but not GR-knockout mice and that it was entirely blocked by spironolactone, an antagonist of the nMRs, it was concluded that i) MRs rather than GRs are involved in this rapid increase of the mEPSC frequency; and ii) this might involve the same MR molecules as those mediating genomic actions. Corticosterone conjugated to BSA was also effective, indicating that the rapid non-genomic signaling pathway may involve a receptor located in the vicinity of the plasma membrane, in other words an mMR. However, in these physiological studies the affinity of the mMR for corticosterone appeared to be tenfold lower than that of the nMRs, suggesting that the mMRs, in contrast to the nMRs, that are already extensively occupied when corticosterone levels are low, are of importance and affect behavior during stressful events. Olijslagers and colleagues (Olijslagers et al., 2008) next unraveled the intracellular mechanism that is responsible for the increased vesicle release of glutamate during corticosterone activation. The release is accomplished via the MEK/ERK pathway by activating presynaptic synapsin.

The effect of mMRs in the hippocampus and other structures where they play a role in the release of glutamate involves receptors located on the presynaptic terminals. This contrasts with the mGRs described by (Di 2003), which are located postsynaptically. In other structures were non-genomic effects of mGRs were reported, the effects also originated from postsynaptically located mGRs. Of note, in limbic structures too postsynaptic effects have been described. For instance, Olijslagers et al. (2008) showed that activation of postsynaptic mMRs in the hippocampal CA1 area induced G-protein dependent phosphorylation, affecting voltage dependent potassium currents. A rapid, presumably postsynaptic effect of mGRs was observed in the amygdala, but this was only visible in stressed rats and mice and or in slices from naïve animals treated twice with a surge of corticosterone (Karst et al., 2010; den Boon et al., 2019). So far, in all structures known where glucocorticoids evokes a rapid response, mGR mediates a retrograde reduction of transmitter release via endocannabinoid receptors. This effect was not visible in forebrain-specific GR knockout mice (Karst et al., 2010).

The mMR-mediated increase of glutamate release during elevated levels of corticosterone is held responsible for a fast increase of LTP in the hippocampal CA1 and DG areas (Wiegert et al., 2006; Pu et al., 2007; Tse et al., 2011). A role for rapid non-genomic MR signaling in scanning and interpretation of the environment during learning and early phases of memory formation therefore seems likely. Such rapid effects on behavior during a stressful event are necessary to determine a strategy. The current consensus is that non-genomic mMR activation, at least in male subjects, will cause a shift toward simple learning strategies (Schwabe et al., 2007, 2010; Schwabe and Wolf, 2010)). Evidence for this notion has also been found in humans (Vogel et al., 2016, 2017). MRs are also important for the quick control of emotional arousal and adaptive behaviors, because this is lost in the absence of MRs in the forebrain, with the result that anxiety-related responses remain augmented (Brinks et al., 2009).

1.2. Visualization of membrane-associated corticosteroid receptors in the brain

Although physiological evidence for the existence of membrane-associated corticosteroid receptors mediating non-genomic effects is convincing, there is only limited visual evidence for such location at or near the membrane in mammalian neurons. The search for the existence of membrane corticosteroid receptors started in the 1990's. The group of Orchinik showed with a radio ligand binding assay the existence of cell-surface receptors on neurons of amphibians that rapidly modulated sexual behaviors (Orchinik et al., 1991; Moore and Orchinik, 1994; Orchinik et al., 1994). These receptors are located at the synaptic neuropil and not at the cell bodies. Attempts to identify membrane receptors in mammals were less successful. So far, two electron-microscopical studies demonstrated the existence of mGRs in the rat brain, more specifically in the hypothalamus and hippocampus (Liposits and Bohn, 1993) and in the amygdala (Prager et al., 2010); and another supplied evidence for mMRs in limbic areas (Prager et al., 2010). Indirect evidence for a dichotomy in the distribution of membrane versus cytosolic receptors came from a study of (Sase et al., 2014). In both fractions, membrane and cytosol, MR and GR were identified. Without doubt, efforts by other researchers was carried out trying to identify the membrane receptors, but this has not been reported. In part 2 of this manuscript we summarize our own attempts to date.

Whether membrane-associated receptors causing rapid changes in transmission are distinct from corticosteroid receptors translocating to the nucleus (and mediating genomic actions) is still a matter of debate. Based on the physiological studies, it could be argued that the non-genomic "membrane receptor" is mobilized (trafficked) from a pool of receptors in the nucleus or cytoplasm, and that both receptors have the same genetic background. This was indeed concluded from our previous experiments (Karst et al., 2005, 2010). The first indication for this mechanism was that rapid actions could also be induced by classical (transcriptional) MR agonists like corticosterone and aldosterone, and blocked by antagonists of the nMRs, e.g. spironolactone. The only difference we found in these pharmacological studies was that the affinity of corticosterone for mediating rapid effects seemed to be 10 fold lower than the slow effects mediated via the nMRs, which could be caused by steric hindrance. The second piece of evidence is that the rapid modulation of glutamate transmission is prevented in forebrain-specific MR knockout mice but not in forebrain-specific GR knockout mice (Karst et al., 2005).

In studies where the pharmacology of mGR was examined, the evidence is less clear. Di et al., 2005 demonstrated that antagonists of the nGRs do not always effectively block rapid glucocorticoid actions, presumably mediated via mGRs.

1.3. Membrane-associated corticosteroid receptors in peripheral cells

Rapid non-genomic effects of corticosterone were also described for

cells from peripheral tissues or cell lines. From work on these cells, much more is known about the properties of non-genomic (cortico)steroid membrane receptors. For instance, membrane receptors were found and described for vascular smooth muscle cells, endothelial cells, skeletal muscle cells, lymphocytes, cardiac myocytes, colonic epithelial and kidney cells (Hammes and Levin 2007). These receptors are among other things related to -but certainly not limited to-the regulation of blood pressure.

In peripheral cells with a functional nucleus, corticosteroid receptors mediating non-genomic effects can coexist with corticosteroid receptors involved in genomic signaling. Also, both GR and MR are co-expressed in many peripheral cells which, as in the CNS, interact functionally and probably transcriptionally. In the kidney and vascular system MR is responsible for rapid non-genomic modulation; this receptor has high affinity for both aldosterone and corticosterone. Due to the fact that corticosterone in the kidney is mostly inactivated by 11 β -HSD2 (Funder, 2005; Gomez-Sanchez and Gomez-Sanchez, 2014), aldosterone is the predominant steroid involved in MR signaling (Ackermann et al., 2010), an important mechanism to regulate blood pressure. Molecules interfering with protein synthesis, such as actinomycin D and cycloheximide, were not able to block the effect of aldosterone on the activity of the sodium-proton exchanger in kidney cells, supporting a non-genomic mechanism (Arima, 2006; Wehling et al., 1991, 1992; Christ et al., 1994).

However, the pharmacological profile of these peripheral MRs mediating rapid non-genomic effects was not exactly the same as that of nMRs. Already in 1991, Martin Wehling proposed the existence of a new membrane aldosterone receptor, based on the observation that MR antagonists could not block the effect of aldosterone on the activity of the sodium-proton exchanger, the rapid effect of aldosterone in human mononuclear leukocytes HML cells (Wehling et al., 1991). Still, to date no study succeeded to characterize and isolate this "new" receptor (Eisen et al., 1994; Lösel and Wehling, 2008; Hermidorff et al., 2017). In fact, in some studies evidence for a close relationship between the nMR and mMR was demonstrated. After transfecting ovary or human embryonic kidney cells, cells without endogenous MRs but with hMR, a non-genomic aldosterone activation was detected that could be blocked with spironolactone (Grossmann et al., 2005). However, if 'classical' MR antagonists are ineffective in blocking rapid corticosteroid actions, it doesn't necessarily mean that the mMR has a different genetic origin than the nMR. It is useful to always test more than one antagonist. For example, Alzamora et al., 2000; Mihailidou and Funder, 2005 and Wildling et al., 2009 showed that in the same cells spironolactone sometimes did not but other classical MR antagonists, like RU28313, did block the rapid effect of aldosterone.

Still, there is also some evidence for the existence of a separate class of mMR receptors. Even cells that do not express the nMR are still able to respond to aldosterone in a non-genomic fashion (Harvey and Higgings, 2000), actions that could not be blocked by aldosterone antagonists (Rossol-Haseroth et al., 2004). Aldosterone-BSA was reported to reproduce rapid aldosterone effects in cells where the nMR is not expressed (Le Moëllic et al., 2004; Grossmann et al., 2005; de Quervain et al., 2017; Ashton et al., 2015). From peripheral cell studies, we also know that non-genomic steroid actions occur in cells without functional nuclei (Friedman and Friedman, 1958; Blackmore et al., 1991). Examples of cells without functional nuclei that do respond to steroids are erythrocytes and sperm cells. The effect of steroids on these cells are rapid, within minutes, and are poorly inhibited by the genomic receptor antagonists (Blackmore, 1999). For instance, in sperm cells the sex hormone progesterone is bound by the b-face of the steroid C/D ring to the membrane receptor, whereas the genomic nuclear receptor binds to the a-face (Blackmore et al., 1996), which indicates that non-genomic receptors may act in a different way or with different affinity than nuclear receptors. Other studies too suggest that non-genomic steroid receptors may belong to a different pool than the genomic receptors (Alzamora et al., 2000; Lösel et al., 2004).

Exactly how MRs mediating rapid effects could be associated with the plasma membrane is unclear. Contrary to other steroid receptors, MRs lack a palmitoylation site, making it impossible for the MR to insert into the membrane. However, an alternative way for MR to be associated with the membrane is via the scaffolding proteins striatin and caveolin-1 (CAV1) (Coutinho et al., 2014; Ashton et al., 2015). If so, the MR would be located at the intracellular side of the membrane. Rapid MR signaling by this complex is thought to take place by transactivating receptor kinases, including EGFR, PDGFR and IGF1R (Ruhs et al., 2017). Another possibility for aldosterone and/or corticosterone is to activate another, hitherto unrevealed receptor. It has been hypothesized that receptors that belong to the GPCR family such as AT1 and GPER1 (=GPR30) are candidates for the non-genomic. In agreement, aldosterone can activate ERK1/2 phosphorylation, which is blocked by the MR antagonist, eplerenone, as well as by the GPER1 antagonist, G15 (Gros et al., 2007, 2011).

2. Subcellular localization of MRs, as identified with imaging techniques

As concluded from part 1, there is some evidence that at least in the brain the mMR may have the same genetic background as the nMRs, although the existence of a separate pool of (hitherto unknown) receptors cannot be excluded. Based on our own observations, we hypothesized that MRs may traffic from the cytoplasm to the membrane and vice versa, depending on the number of MRs and fluctuation of corticosterone levels. One of the indications for the existence of trafficking is that in BLA neurons, which contain a relatively low number of nMRs, corticosterone will only cause a rapid MR-mediated increase in the mEPSC frequency upon the first exposure to the hormone, while later pulses result in the opposite effect via mGRs (Karst et al., 2010). This may be due to internalization of mMRs, which would have clear consequences given the relatively low number of MRs. This line of reasoning is further elaborated in part 3 of this paper.

We first describe our attempts to visualize MRs in membranes. For that purpose we made use of fixed cell imaging and live cell imaging in COS7 cells or primary cultured hippocampal neurons (DIV:15). We used four different fluorescent anti-MR antibodies (#598, #599, #600, #601, see Table 1) to visualize MRs. In some experiments the number of endogenous MRs were reduced with MR shRNAs (#93, #94 and #95, see Table 2) in combination with overexpression of GFP-labeled MRs to eliminate interference by endogenous MRs and increase visualization of mMRs. We used N' and C' terminal GFP constructs, MR-N'-GFP and MR-C'-GFP, to investigate if different GFP fusions altered the localization or expression of MR. After we developed the tools to visualize endogenous MR and overexpressed MR-N'-GFP and MR-C'-GFP, we investigated the localization of MR in relation to the plasma membrane.

Table 1
List of MR-antibodies.

#	Name	Immunogen	Origin	Reactivity	Supplier, reference
598	Anti-MR 1D5	Synthetic Peptide 1-18	Mouse	rat, mouse, dog, human and rabbit	Gift, Gomez-Sanchez et al. (2006)
599	Anti-MR H3122	MR amino acid 2-99	Mouse	Mouse, Rat, Human, Bird	Abcam, Suh et al. (2006)
600	Anti-MR H10E4C9F	Aldosterone-3	Mouse	Human, Rat, Chicken, Rabbit	Thermo Fisher Scientific, Prager et al. (2010)
601	Anti-MR 4D6	Synthetic Peptide 365	Mouse	Rat, Mouse	DSHB, Gomez-Sanchez et al. (2006)

Table 2
Primer sequences of MRshRNAs

#	FW Sequence 5'-3'
MRshRNA #93	TCATATCCTGCATACAACA
MRshRNA #94	ATGGTGATGACTCTCAGAA
MRshRNA #95	GGATGTGTCTATCATCGTT

2.1. Materials and methods

2.1.1. Antibodies

A range of primary anti-MR antibodies (Ab) were used (Table 1). Also, rabbit anti-Homer (dilution: 1:1000 -REF), rabbit anti-Bassoon (dilution 1:1000- Stressgen), were used.

2.1.2. Cloning MR GFP fusion constructs

The MR plasmids were constructed by cloning the rat MR sequence from the GFP-MR plasmid into GFP containing GW2 and B-actin plasmids. The rat MR was isolated using PCR amplification with addition of Kozak-atg/stop codon and AscI and Sall (Thermo Scientific) restriction sites to enable ligation into the backbone of the GW2 and B-actin plasmid. Short hairpin RNA (shRNA) sequences targeting rat MR (n = 3) were created by annealing oligos with addition of HindIII and BglII (Thermo Scientific) sticky ends to enable ligation into the backbone of the pSuper vector. All produced constructs were sequence confirmed.

2.1.3. Cell culture and transfection

COS7 cells were cultured in DMEM + Ham's F10 (50/50%) medium containing 10% FCS and 1% penicillin/streptomycin. Two days before transfection, cells were plated on 19 or 24 mm diameter coverslips (24 mm in 6 well plate for live cell imaging and 19 mm in 12 well plate for immunocytochemistry). Cells were transfected using Fugene 6 (Roche). DNA (1 µg/well, for a 6 wells plate) was mixed with 3 µl Fugene 6 in 200 µl DMEM, incubated for 30 min, and then added to the COS7 cells and incubated overnight at 37 °C in 5% CO₂. Primary hippocampal cultures were prepared from embryonic (day 18) rat brains. Cells were plated on coverslips and grown in neurobasal medium (NB) supplemented with B27, 0.5 µM glutamine, 12.5 µM glutamate and penicillin/streptomycin. After approximately fourteen days, hippocampal neurons were transfected using Lipofectamine 2000 (Invitrogen). DNA (1.8 µg/well, for a 12 wells plate) was mixed with 3.3 µl of Lipofectamine 2000 in 200 µl NB, incubated for 30 min, and then added to the neurons in NB with glutamine at 37 °C in 5% CO₂ for 90 min. Next, neurons were washed with NB and transferred in their original medium supplemented with 50% fresh NB with B27, 0.5 µM glutamine, 12.5 µM glutamate and penicillin/streptomycin at 37 °C in 5% CO₂ for two days when transfecting MR GFP fusion constructs and four days for shRNA constructs. For overnight treatment experiments with Isopr or Cort, one day prior to fixation or live cell imaging, Isopr with an end concentration of 3 µM and Cort with an end concentration of 100 nM was added to the cells and incubated at 37 °C in 5% CO₂ overnight.

2.1.4. Fixed cell imaging

Cells were fixed with 4% formaldehyde in PBS for 10 min, permeabilized with 0.5% Triton-x100 in PBS for 10 min s, and blocked with 0.5% BSA in PBS for 30 min at RT. Next, cells were incubated overnight at 4 °C with an appropriate dilution of primary antibodies in 0.5% BSA in PBS and then for 1 h at room temperature in an appropriate dilution of secondary antibody in 0.5% BSA in PBS. The cells were mounted on a slide using Vectashield or Vectashield with DAPI (Vector Laboratories). Imaging of shRNA transfected/stained neurons and stained COS7 fixed samples was

Performed on Nikon Eclipse 80i (Nikon) upright microscope using 100x (Plan Apo NA 1.40 oil), 40x (Plan Fluor NA 1.30 oil) and 10x (Plan Fluor NA 0.30) objectives and a 9 CoolSNAP HQ2 CCD camera (Photometrics), a mercury lamp was used for excitation (Osram). Nikon NIS-

Elements Basic Research imaging software was used to obtain images, these were analyzed using ImageJ (Fiji) and quantified using Neuron J and the Sholl analysis plugin. Imaging of MR localization in neurons was performed using the AxioObserver Z1 (Zeiss) Confocal microscope using the 63x (Plan Apochromat NA 1.40 oil) objective. Controlled by ZEN 2011 software, we acquired a z stack of 6–20 images (averaged two times) for each cell. Using ImageJ, the images were stacked into a single image by either creating a maximum projection image or a single plane was selected. Graphs were produced using Graphpad Prism 5.0.

2.1.5. Live cell imaging

Time-lapse live-cell imaging was performed using a Nikon Ti perfect focus microscope with an incubation chamber at 37 °C and 5% CO₂ (Tokay Hit; INUG2-ZILCS-H2) mounted on a motorized stage (AS1). A 24 mm coverslip containing cells was mounted using metal rings. 0.6 ml incubation medium (neurons) or fresh culture medium (COS7) was added to submerge the cells. Each run, 5 cells were selected and imaged every 20 s for a total of 20 min using 40x objective (Plan Fluor NA 1.30 oil) and a Coolsnap camera (Photometrics). A mercury lamp was used for excitation (Osram). Before each run, Isopr with an end concentration of 3 μM and cort with an end concentration of 100 nM was added to the sample at t = 0. MicroManager software was used to acquire images and control the microscope.

2.2. Results

2.2.1. MR localization in the soma and dendrites

First, we verified whether the MR in the MR-N'-GFP and MR-C'-GFP construct is still intact and recognized by antibodies for MR. Previously validated anti-MR Ab #599 and Ab #598, #600 and #601 were used to stain MR-N'-GFP and MR-C'-GFP transfected COS7 cells. MR observed with antibodies #598, 599 and 601 strongly resembled MR-N'-GFP and MR-C'-GFP. Ab #600 did not recognize overexpressed MR. Antibodies #598, 599 and 601 were then used to stain MR-N'-GFP or MR-C'-GFP transfected neurons. With the most optimal acting antibody, we also examined the effectiveness of the antibody for the MR-N'-GFP and MR-C'-GFP constructs. In these cells the endogenous MRs were reduced with shRNAs (Table 2). No difference in localization of MR-N'-GFP or MR-C'-GFP was observed in both neurons and COS7 cells.

The localization of MR-N'-GFP or MR-C'-GFP in neurons was enriched in the soma and was found to be diffuse throughout the neurites. In COS7 cells, MR expression varied between cells: in some cells MR was enriched in the nucleus but in other cells MR was observed in the cytoplasm throughout the entire cell. No fluorescent signal was observed in non-transfected cells, confirming that COS7 cells do not express MR endogenously (Guiochon-Mantel et al., 1991). Based on literature, hippocampal neurons were expected to show a strong MR expression inside the soma, therefore the nuclear enrichment of MR-N'-GFP or MR-C'-GFP and the antibodies was investigated. MR was observed to be enriched inside the nucleus of all neurons as evidenced by both MR-N'-GFP and MR-C'-GFP and anti-MR antibody staining. This can be explained by corticosterone activating MR, since low concentrations of corticosterone were present in the incubation medium supplement B27 (ThermoFisher, Scientific Inc.). This most likely caused MR to translocate to the nucleus prior to fixation (Galigniana et al., 2010). The antibody staining strongly resembled the MR-N'-GFP and MR-C'-GFP expression. Interestingly, Ab #601 showed a punctae-like appearance. Although this might indicate the existence of MR clusters, it is yet unclear why this was only seen with Ab #601.

2.2.2. MR localization in membrane

To investigate whether MR is enriched in or localized on the plasma membrane, membrane bound red fluorescence protein tag (tagRFP) (Merzlyak et al., 2007) was transfected in neurons and endogenous MR was subsequently stained with anti-MR antibodies. Confocal microscopy enabled dendritic cross-sections to be analyzed by selecting single

optical sections, visualizing the plasma membrane on both sides of the dendrite (Fig. 1A). By measuring the fluorescent intensity across a dendrite, a fluorescent intensity profile could be generated (Fig. 1B). MR was not observed at the same localization as the peaks representing the membrane tagRFP, but rather in-between the peaks, arguing against membrane enrichment of MR. This could be explained by a low abundance of MR in dendrites, caused by MR activation via corticosterone present in the incubation medium. This is supported by the observation that MR was enriched in the nucleus of all neurons. The punctae-like appearance observed in the dendritic cytoplasm (Fig. 1A), could be explained by the MR's presence in subcellular fractions, where it interacts with partners, e.g. receptor tyrosine kinases, like EGFR, PDGFR and IGF1R.

To mitigate the effect of corticosterone binding to endogenous receptors, MR was overexpressed using the MR-N'-GFP and MR-C'-GFP construct and compared with membrane tagRFP in a dendritic cross-section (Fig. 1). After overexpression, the punctae-like appearance of MR was no longer observed (Fig. 1C). Probably they are masked by the high amount of GFP-MRs. However, MR-N'-GFP and MR-C'-GFP (like endogenous MR) was observed primarily in-between the peaks of membrane tagRFP, indicating that even with large amounts of MR plasma membrane enrichment of MR is not observed.

2.2.3. Synaptic MR localization

Previous studies hypothesized mMR to be present in the pre- and postsynaptic regions of neurons, which was confirmed by electron-microscopy (Prager et al., 2010). Also in the amphibian brain, mGRs are located mainly over synaptic neuropil and not over the cell bodies (Orchinik et al., 1991). Because of electron-microscopy limiting labeling possibilities, we investigated the localization of mMR in the pre- and postsynaptic zone, using the validated anti-MR Ab #599 in combination with fluorescent confocal microscopy.

To investigate whether the localization of mMR changes when corticosterone or Isoproterenol were introduced, neurons were treated 1 day before fixation with these compounds and subsequently co-stained with the pre- and postsynaptic markers Bassoon and Homer, respectively. Isoproterenol is a β-adrenoceptor agonist, which was shown to be able to move MR from the cytosol to the nucleus; this could contribute to enhanced MR transcriptional activity (Victorio et al., 2016). MR was observed throughout the dendrites, spines (Fig. 2A) and axons (Fig. 2B) in its distinctive punctae-like appearance. No co-localization was observed between MR and Bassoon or MR and Homer indicating that MR is not enriched in the pre- or postsynaptic zone but, rather, is present in the cytoplasm throughout the neurites. Treatment with corticosterone or Isoproterenol did not change the localization of MR.

In conclusion, we were not able to observe MR on the membrane of dendrites nor pre- or postsynaptically with confocal microscopy, examining endogenous receptors, or when endogenous MR was down-regulated with shRNA and an GFP-MR complex was overexpressed. Potentially, the low concentration of corticosterone in the incubation medium may have caused internalization of the receptor (see section 3), hampering the visualization of mMRs. This seems unlikely, though, given the relatively low affinity of the mMR for corticosterone demonstrated in electrophysiological studies. Thus, despite the fact that in hippocampal cultured neurons rapid MR-mediated effects (Mikasova et al., 2017; Sarabdjitsingh et al., 2014) with corticosterone as well as with corticosterone-BSA can be demonstrated, the localization of the receptor in the membrane remains elusive.

3. Metaplasticity due to internalization of mMR?

Although we were not able to detect mMRs with the fixed cell and live cell imaging techniques, it cannot be excluded that these receptors do exist. As mentioned, it is possible that due to the background concentration of corticosterone in the incubation medium mMRs are internalized, masking their presence (Grossmann et al., 2010).

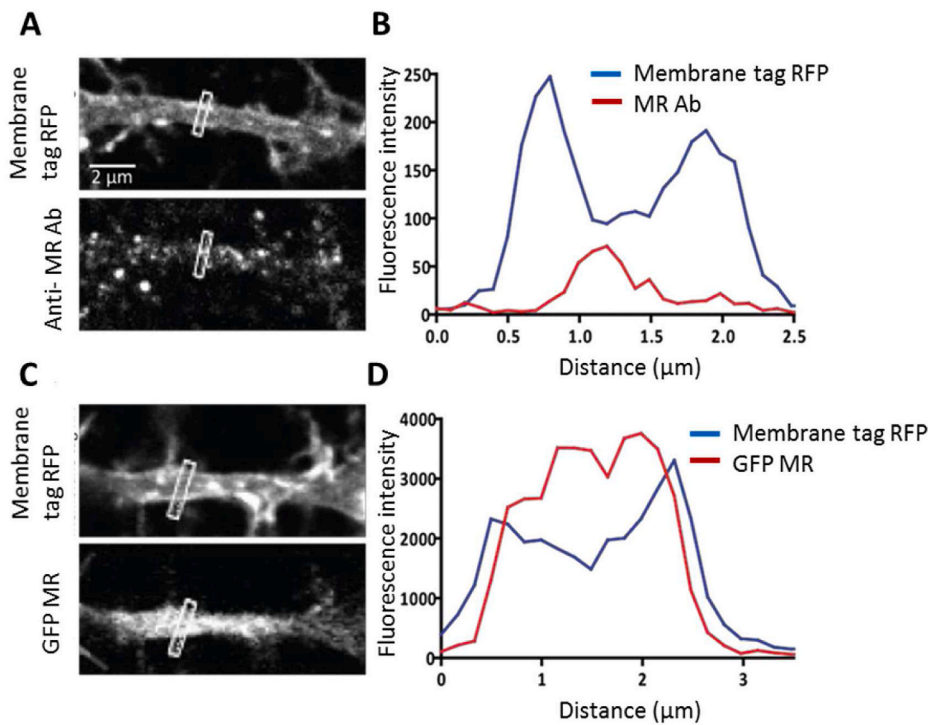


Fig. 1. MR does not localize or enrich on the plasma membrane.

A. Confocal microscopy single plain showing a cross-section of a dendrite from a neuron (DIV: 16) expressing membrane tag RFP, stained with anti-MR Ab. Fluorescent intensity was measured inside the white box. **B.** Quantification of measured fluorescence, intensity vs distance along the area. **C.** Confocal microscopy single plain showing a cross-section of a dendrite from a neuron (DIV: 16) expressing membrane tag RFP and MR-N'-GFP or MR-C'-GFP. Fluorescent intensity was measured inside the white box. **D.** Quantification of measured fluorescence, intensity vs distance of the line.

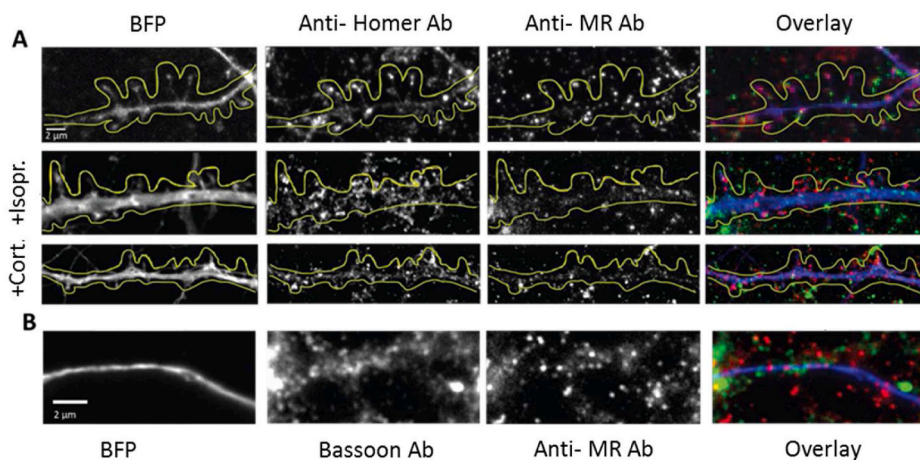


Fig. 2. MR localization in neurons upon treatment. Fluorescent Microscopy: **A.** Dendrites of neurons (DIV: 16) stained with blue fluorescent protein (BFP), to visualize neuronal morphology, with the postsynaptic marker anti-Homer Ab and with the anti-MR Ab. Homer is used as a postsynaptic marker and Bassoon as a marker of presynaptic terminals. The neurons were treated with corticosterone or isoproterenol (isopr.) The control received no treatment. The yellow lines indicate the outline of the neurons to clarify spine and dendrite morphology (overlay: Blue = BFP, Green = anti-MR Ab, Red = Anti-Homer Ab). **B.** Axon of neuron (DIV: 16) stained with anti-Bassoon Ab and anti-MR Ab (overlay: Blue = BFP, Green = anti-Bassoon Ab, Red = Anti-Homer Ab).

Internalization is a phenomenon that could also explain metaplasticity in the BLA (Karst et al., 2015); we here define metaplasticity as the phenomenon that the response to a compound, in this case corticosterone, differs from an earlier (recent) response to the same compound (at the same concentration), due to changes in the cellular context. Thus, treatment of brain slices containing the BLA of non-stressed mice with corticosterone causes a rapid increase in the release of glutamate, represented by an increase in the frequency of mEPSCs. Comparable effects were found in CA1 and DG neurons. However, when a second surge of corticosterone was applied, >1 h after the first application, corticosterone was found to decrease the mEPSC frequency in the BLA neurons, a phenomenon we dubbed ‘metaplasticity’ (Karst et al., 2010). The implication for behavior could be that this mechanism prevents the amygdala –important for emotional processing– from being over-activated. This was not observed in CA1 and DG neurons (den Boon et al., 2019). We hypothesized that since BLA neurons have a considerably lower amount of MRs than hippocampal neurons, most of the mMRs are internalized after the first surge of corticosterone, enabling

the mGRs, which might be more abundant, to dominate over the mMRs effect.

Internalization of steroid receptors is a phenomenon already described for the estrogen receptor (Bondar et al., 2009): Internalization was shown to be increased by estradiol in primary hypothalamic astrocyte cultures, with a time course of 5 min. One of the mechanisms that could be responsible for internalization is the interaction of steroid receptors with Beta-arrestin. Beta-arrestin’s interaction with several non-GPCR receptors, for instance the insulin receptor (Luan et al., 2009) and the estrogen receptor α (Dominguez et al., 2009), is well-documented. However, beta-arrestins are particularly known to couple to many different GPCRs, most notably rhodopsin and the beta-adrenoceptor, to regulate receptor internalization, G-protein coupling and signal transduction

Two forms of beta arrestins, beta arrestin 1 (Arrb1) and beta arrestin 2 (Arrb2), are ubiquitously expressed throughout the rodent brain, including the limbic system (Breivogel 2013). In general, Arrb1 levels are higher than Arrb2 levels. Arrb1 and Arrb2 bind agonist-occupied,

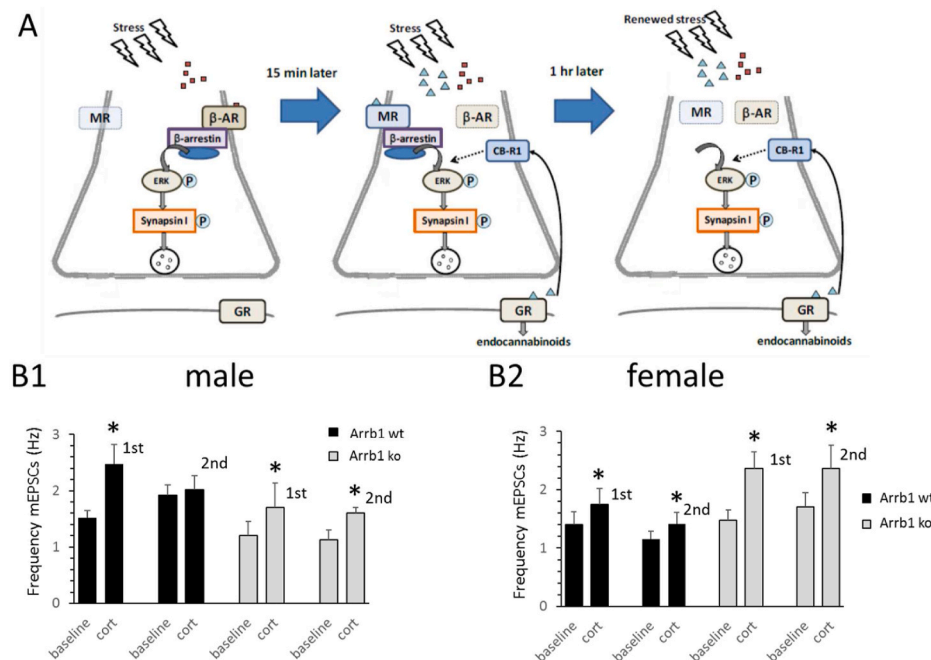
phosphorylated GPCRs and thereby block receptor-G protein interaction as well as target GPCRs for endocytosis. Beta-arrestins can form transient complexes that dissociate near plasma membranes or stable complexes that undergo internalization as a unit (Shenoy et al., 2006; Casella et al., 2011; Han et al., 2013). The interaction between GPCRs and arrestins forms the basis for attenuation of GPCR signaling, as well as downregulation and sensitization (DeWire et al., 2007). In addition to their role in downregulation, arrestins also can bind directly to proteins involved in signal transduction (ERK, JNK (c-Jun N-terminal kinase) and p38 MAPK) and mediate the interaction between GPCRs and downstream signaling pathways (Shenoy and Lefkowitz, 2003). It was reported that memory retrieval induce β 1-adrenergic Arrb signaling leading to postreactivation memory stabilization, via ERK signaling and protein synthesis (Liu et al., 2015). A study investigating Arrb1 KO mice suggested that Arrb1 may have a protective role against increased alcohol consumption in females and hyperactivity in both genders (Robins et al., 2018).

We hypothesized that upon stress, MR in the BLA are internalized in a beta-arrestin-dependent way, leaving only postsynaptic membrane glucocorticoid receptors (mGR) to respond to increased levels of corticosterone (Fig. 3). Of note, an interaction between beta arrestins and the mineralocorticoid receptor has not yet been described. To test our hypothesis we performed experiments on mice homozygous for a Arrb1 mutation, in which Arrb1 is lacking completely. If the proposed mechanism is indeed taking place, we expect that in Arrb1 KO mice, the response to renewed stress is similar to the response to the initial stress, in other words that metaplasticity is absent. Since in the rodent brain the amount of expression of Arrb1 is higher than that of Arrb2 (Breivogel et al., 2013), we here only report on the former.

3.1. Materials and methods

All animal procedures were approved by the local ethics committee (2014.I.06.036).

In the current study, we used young-adult male and female Arrb1 and



100 nM Cort. A second application again increased the mEPSC frequency in both groups.

WT littermates (6–9wk old; The Jackson Laboratories (Kohout et al., 2001)), decapitated under rest in the morning, when corticosteroid levels are very low. Immediately after decapitation, the brain was removed from the skull and chilled (4 °C) in artificial cerebrospinal fluid (aCSF) containing 120 mmol/L NaCl, 3.5 mmol/L KCl, 5.0 mmol/L MgSO₄, 1.25 mmol/L NaH₂PO₄, 0.2 mmol/L CaCl₂, 10 mmol/L D-glucose, and 25.0 mmol/L NaHCO₃ (gassed with 95% O₂ and 5% CO₂). Coronal slices (350 μ m thick) containing the BLA and/or dorsal hippocampus were prepared with a vibroslicer (Leica VT 1000S). Slices were stored at room temperature until use in recording aCSF containing 120 mmol/L NaCl, 3.5 mmol/L KCl, 1.3 mmol/L MgSO₄, 1.25 mmol/L NaH₂PO₄, 2.5 mmol/L CaCl₂, 10 mmol/L D-glucose, and 25 mmol/L NaHCO₃. One slice at a time was placed in a recording chamber mounted on an upright microscope (Axioskop 2 FS plus; Zeiss) with differential interference contrast, water-immersion objective (63 \times), and 10 \times ocular. The slices were continuously perfused with aCSF (flow rate 2–3 mL/min, temperature 32 °C, pH 7.4) consisting of 120 mM NaCl, 3.5 mM KCl, 1.3 mM MgCl₂, 2.5 mM CaCl₂, 25 mM NaHCO₃, 1.25 mM KH₂PO₄, and 10 mM D-glucose; bicuculline methchloride (20 μ M; Tocris) and tetrodotoxin (0.5 μ M; Latoxan) were added to block GABA_A receptor-mediated signals and action potentials, respectively. Whole-cell voltage-clamp recordings were made with an Axopatch 200B amplifier (Axon Instruments) using borosilicate glass electrodes (impedance 4–6 M Ω , 1.5-mm outer diameter; Clark) pulled with a micropipette puller (Brown/Flaming P-87; Sutter Instruments). For mEPSC recordings, the intracellular pipette solution contained 120 mM Cs methane sulphonate, 7.5 mM CsCl, 10 mM Hepes, 5 mM BAPTA, 2 mM MgATP, and 0.1 mM Na GTP (295 mOsm, pH 7.4 adjusted with CsOH). BAPTA was obtained from Molecular Probes; all other chemicals were purchased from Sigma. Neurons in the BLA were selected for recording if they displayed a pyramidal-shaped cell body. All mEPSCs were recorded with a holding potential of –70 mV. If the neuron under study displayed stable mEPSC properties during baseline recording (at least 10 min), 100 nM corticosterone (Sigma) was applied for \approx 20 min via the perfusion medium. Corticosterone was prepared weekly in a

Fig. 3. A. Hypothetical model explaining homotypic and heterotypic metaplasticity by stress hormones in the BLA. Upon stress exposure, BLA neurons are first exposed to noradrenaline (left). This is thought to activate a cascade involving β -adrenoceptors, β -arrestin and ERK phosphorylation, which through Synapsin I phosphorylation results in enhanced release probability of glutamate (Shenoy et al., 2006). After appr. 15 min (middle) β -adrenoceptors are internalized. At that time, corticosterone has reached BLA neurons and activates both MR and GR located in the membrane. MRs tap into the same mechanism as β -adrenoceptors, which initially causes enhanced release probability of glutamate, but after appr. 1 hr (right) results in MR internalization. If BLA cells at that time are exposed to corticosterone e.g. by renewed stress exposure, only membrane-located GRs are still available for binding, which via endocannabinoids retrogradely suppress glutamate signaling.

B1. Arrb1 WT and KO male respond to 100 nM Cort. In WT mice, the mEPSC frequency was quickly increased by the application of 100 nM Cort. However, probably due to internalization of the MR's no effect after a second application of Cort on the mEPSC frequency was observed anymore. In Arrb1 KO males both the first and the second application of Cort (100 nM) increased the mEPSC frequency.

B2. In WT and Arrb1 KO females the mEPSC frequency was quickly increased by the application of

stock solution (1 mM in 95% ethanol) and diluted to their final concentration in aCSF just before application. Series resistance and capacitance were monitored during the whole recording. Responses were filtered at 5 kHz and digitized at 10 kHz (Digidata 1322A; Axon Instruments). All data were acquired, stored, and analyzed on a PC using pClamp 9.0 and Clampfit 9.2 (Axon Instruments). Minimal cutoff for mEPSC analysis was 6 pA.

3.1.1. Data analysis

We used paired t tests to compare mEPSC properties between the last 5-min baseline recordings and the last 5 min of recordings in the presence of CORT.

3.2. Results

We performed experiments on both male and female Arrb1 KO mice and WT controls. As expected, there was an increase of the mEPSC frequency in response to a brief application of corticosterone in WT male littermates of Arrb1 KO mice. A second application of corticosterone (1 h later) did not cause an increase in mEPSC frequency (Fig. 3B1). In male Arrb1 KO mice, though, both the first and the second application of corticosterone resulted in an increase in the mEPSC frequency (Fig. 3B1). In WT and Arrb1 KO females, both applications of corticosterone were able to increase mEPSC frequency (Fig. 3B2).

These experiments in Arrb1-WT mice partially confirm earlier data on stress metaplasticity in the BLA: In Arrb1 WT males, the first application of corticosterone increased glutamate transmission, while the second application did not. In previous experiments in naïve male C57Bl/6 mice, the second pulse of corticosterone actually *decreased* mEPSC frequency. The difference with earlier findings regarding the second pulse may be related to the difference in genetic background of the animals. The lack of metaplasticity in WT female mice, thus where two subsequent applications of corticosterone both caused an increase in glutamate release, is a novel finding and needs further attention in future experiments. Differences between males and females in their response to stress mediators is not unprecedented, though (Bangasser et al., 2018, 2019). Overall, we carefully conclude that some degree of internalization of mMR via Arrb1 may take place in BLA cells of WT males, which could contribute to the observed metaplasticity.

4. Discussion

For many years now, MRs mediating rapid effects are hypothesized to have a plasma membrane localization (Meinel et al., 2014; Di et al., 2003; Gekle et al., 2001; Welberg and Seckl, 2001; Groeneweg et al., 2011). This is supported by electron-microscopy experiments (Prager et al., 2010), and e.g. by a Western blot study where the membrane and cytosolic fraction were dissociated (Sase et al., 2014). Also studies in peripheral tissues or cell lines are in line with this notion. Moreover, electrophysiological experiments using membrane impermeable corticosterone-BSA conjugates provided suggestive though indirect support. In the current paper we describe experiments with fixed cell and live cell imaging that could have given direct prove for the membrane localization of MRs. However, we did not find compelling evidence for MR localization in the plasma membrane of dendrites or soma, nor in the pre- or postsynaptic membrane zone.

How can these findings be reconciled? The first possibility is that the abundance of mMRs is very low compared to that of nMR, as was e.g. earlier found for the estrogen receptor (Milner et al., 2001); and that the method is simply not sensitive enough to demonstrate a small fraction of receptors in the membrane. For this reason we overexpressed a MR-N'-GFP and MR-C'-GFP construct (in cells deprived of the endogenous MR). Although this resulted in very clear visualization of intracellularly localized MRs, it didn't allow us to identify MRs in the membrane.

A second possibility is that MRs traffic from the nucleus or cytoplasm

to the plasma membrane and vice versa; and that our experimental conditions were such that MRs were mostly internalized. Our experiments with the Arrb1-KO mice support the possibility of internalization. Also in line with this possibility, we detected punctae of MR-labeling throughout the neuron: in the nucleus, cytoplasm of the soma, dendrites and in spines. These punctae could be an artifact caused by PFA fixation or MR clustering, but they may also signify MR clusters that are trafficked to or from specific cell compartments. Removal of MRs from the membrane was indeed demonstrated in a study of (Grossmann et al., 2010), following exposure to aldosterone. This study showed that a small fraction of MRs are colocalized with EGFR at the cell membrane of HEK-293 cells, to form a signaling module. After 24 h exposure to aldosterone (10 nM), however, the MR-EGFR colocalized complexes in the membrane were no longer observed, and MR was completely translocated to the nucleus. To address this possibility, we treated the cultured cells with corticosterone or isoproterenol for 24 h but this did not affect the localization of MR in any way. Possibly, the fact that corticosterone is present in very low concentrations in the incubation medium might have caused pre-activation and internalization of MR, resulting in nuclear localization of the receptor at the time of the imaging experiment. This can be considered a limitation of the current experiments. However, the influence of such low corticosterone concentrations on the results seems very unlikely, given the rather low affinity of the mMR in electrophysiological experiments. Being able to grow neurons without corticosterone would allow investigating this directly. Unfortunately, MR plays an important role in the maturation of neurons and hence corticosterone cannot be absent during early stages in vitro. Nevertheless, it would be worthwhile in future experiments to apply over-night incubation with charcoal-stripped media to eliminate steroids and minimize steroid-mediated MR trafficking, although such manipulations of the cultures in turn may disturb the parameter under investigation.

At this stage we can certainly not exclude that other mechanisms than internalization of the mMRs contribute to metaplasticity. The observation that the mMR-mediated increase in glutamate release does not reverse in the BLA after the first surge of corticosterone may suggest that MR activation triggers a signaling switch that stays on well past the activation of the receptor, or that the receptor stays activated (i.e., enters and stays in the activated state). The latter mechanism is what was described by Di et al. (2016) at inhibitory synapses in the BLA, albeit via a different mechanism mediated by retrograde endocannabinoid signaling. A second surge of corticosterone might then induce a mGR dependent increase in the retrograde endocannabinoid release resulting in a reduction of the mEPSC frequency.

The localization observed as punctae could also indicate a third possibility, i.e. that MR after having bound corticosterone interacts with partners, e.g. receptor tyrosine kinases: EGFR, PDGFR and IGF1R (Fig. 4). In this theory MR would have a local signaling function, even though MR itself has no transmembrane domain (Pedram et al., 2007; Groeneweg et al., 2012). For instance, through EFGR signaling endosomes, cytoplasmic MR could bind corticosterone and start non-genomic actions or move throughout the cell. Such signaling pathways have been demonstrated for the estrogen receptor, which interacts with caveolin-1 and targets caveolae (Cohen et al., 2004). These caveolae are invaginations of the plasma membrane formed by scaffolding proteins like caveolin-1. Caveolin recruits signaling proteins like G-protein. Possibly MR is present in these caveolae. MR has indeed been found in association with caveolin-1 in human endothelial cells (Pojoga et al., 2010), but as yet this has not been demonstrated in brain tissue. To find and prove interacting partners of MR, mass spectrometry experiments should be performed. Co-staining MR with caveolin-1 markers could also reveal whether MR is located in caveolae. Still, localization of MR e.g. in caveolae is difficult to reconcile with the effectiveness of corticosterone-BSA in electrophysiological experiments. With regard to the latter, though, it cannot be excluded that these conjugates partly dissociate and thus 'free' corticosterone to pass the plasma membrane

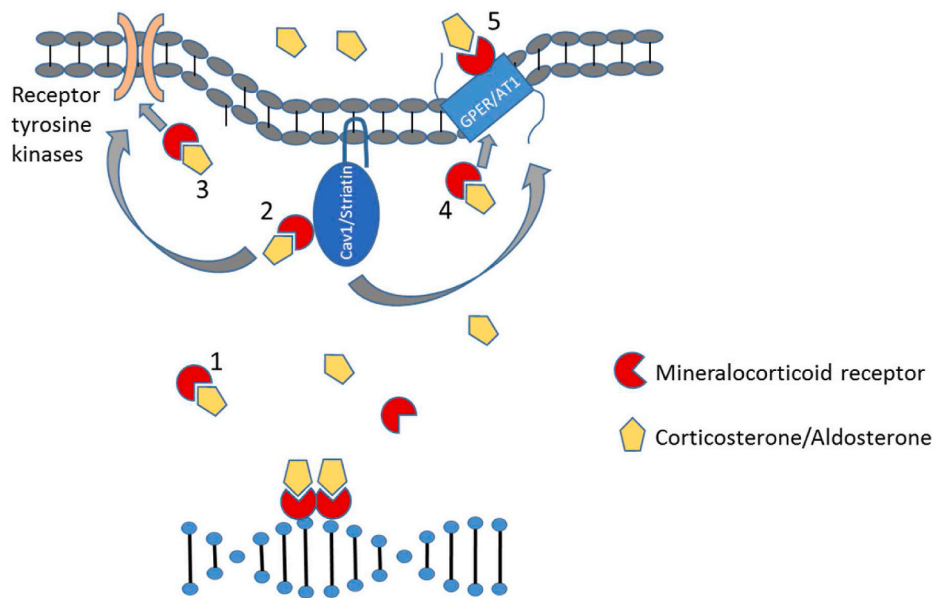


Fig. 4. The mineralocorticoid receptors have been identified with several different signaling functions. Corticosterone or aldosterone can bind to nuclear MRs and move to the nucleus where it affects gene transcription after binding as homodimers to the DNA (1). The MR-corticosterone/aldosterone complex can transactivate receptor tyrosine kinases (e. g. EGFR, PDGFR and IGF1R) via a direct interaction (3) or via CAV1 or Striatin coupled interaction (2). Transactivation can also take place via an interaction with GPER or AT1 receptors (4). Also direct coupling with the GPER receptor is possible (5). There was no transmembrane receptor type of MR identified yet.

and reach receptors in caveolae or other cell compartments. However, Weiss et al. (2019) showed that the dissociation of another steroid–BSA conjugate, dexamethasone-BSA, although present, was minimal and did not affect intracellular signaling, such that any effect of the conjugate was constrained to the membrane.

Lastly, the reason why we could not detect a membrane localized MR is that it involves a novel receptor, with a binding site for corticosterone and aldosterone that is not recognized by MR antibodies. Postulated candidates are molecules like GPER or AT1 (Fig. 4). In several studies, it was demonstrated that aldosterone affects GPER, indirectly via intracellular transactivation, or directly by binding to GPER. An antagonist of GPER, G15, prevented the effects of aldosterone, as did the MR antagonist plerenone (Gros et al., 2007, 2011). The existence of such a novel receptor would support the explanation that the membrane impermeable corticosterone-BSA conjugate is able to mimic the rapid effect of corticosterone (Karst et al., 2005).

In conclusion, the existence of rapid non-genomic actions via corticosteroid receptors has been observed for many decades, supposedly mediated by membrane localized receptors. However, visualization of these membrane-associated receptors, particularly MR, has proved to be difficult and remains a matter of debate. Despite this fact, the contribution of rapid MR- (and GR-) mediated effects to the repertoire of corticosteroids vastly expands their role in the organism's adaptive response to stressful events.

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