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Mineralocorticoid receptor overexpression in forebrain decreases anxiety-like behavior and alters the stress response in mice

Aaron M. Rozeboom*, Huda Akil†, and Audrey F. Seasholtz*†‡

*Cellular and Molecular Biology Graduate Program and †Molecular and Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, MI 48109

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Although numerous stress-related molecules have been implicated in vulnerability to psychiatric illness, especially major depression and anxiety disorders, the role of the brain mineralocorticoid receptor (MR) in stress, depression, and affective function is not well defined. MR is a steroid hormone receptor that detects circulating glucocorticoids with high affinity and has been primarily implicated in controlling their basal level and circadian rhythm. To specifically address the role of MR in hypothalamic-pituitary-adrenal axis activity and anxiety-related behaviors, we generated transgenic mice with increased levels of MR in the forebrain (MRov mice) by using the forebrain-specific calcium/calmodulin-dependent protein kinase II α promoter to direct expression of MR cDNA. A mild but chronic elevation in forebrain MR results in decreased anxiety-like behavior in both male and female transgenic mice. Female MRov mice also exhibit a moderate suppression of the corticosterone response to restraint stress. Increased forebrain MR expression alters the expression of two genes associated with stress and anxiety, leading to a decrease in the hippocampal glucocorticoid receptor (GR) and an increase in serotonin receptor 5HT-1a, consistent with the decreased anxiety phenotype. These data suggest that the functions of forebrain MR may overlap with GR in hypothalamic-pituitary-adrenal axis regulation, but they dissociate significantly from GR in the modulation of affective responses, with GR overexpression increasing anxiety-like behavior and MR overexpression dampening it. These findings point to the importance of the MR:GR ratio in the control of emotional reactivity.

glucocorticoid receptor | 5HT-1a

The hypothalamic-pituitary-adrenal (HPA) axis controls the production and release of adrenal glucocorticoids (GC) across the daily circadian rhythm and in response to stress. The major functions of GC within the HPA axis are 2-fold: (*i*) to alter metabolic processes in a manner that provides the energy needed to combat the stressful stimuli, and (*ii*) to recover homeostasis. These functions are essential to the well being of the organism, and dysregulation of the axis can result in potentially deleterious consequences. Indeed, evidence suggests that multiple mood disorders, including anxiety conditions and major depression, are associated with chronic alterations in the circulating levels and circadian rhythm of GC (1–3). Furthermore, clinically efficacious antidepressant treatment is often accompanied by normalization of the HPA axis, underscoring the importance of HPA axis function in the control of affect (4).

The actions of GC are mediated by two corticosteroid receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). MR and GR function as ligand-activated transcription factors that reside in the cytoplasm, dimerize upon ligand binding, translocate to the nucleus, and exert transcriptional control (either positive or negative) over glucocorticoid-responsive genes. MR and GR have different affinities for corticosterone (K_d = 0.5 nM for MR and 5 nM for GR), and thus are differentially activated throughout the circadian rhythm and during times of stress. GR is widely expressed throughout the brain, whereas MR expression, although present in various brain regions, is found predominantly in the hippocampus in rodents. Interestingly, MR is highly expressed in the cerebral cortex of squirrel monkeys and humans, suggesting additional roles for cortical MR in primates (5, 6). In brain regions where MR and GR are coexpressed, they can heterodimerize, further increasing the complexity by which the receptors can signal (7). Although numerous studies have shown that MR and GR act synergistically in HPA axis inhibition, other work has suggested that the actions of MR and GR can be antagonistic. For example, MR and GR mediate opposite effects on ion conductances in hippocampal pyramidal neurons (8) and can interact with coactivators in distinct manners (9). These differential mechanisms of action allow for a diverse response to corticosterone even within the same cell. Because of the importance of corticosteroid-mediated activity in anxiety and depressive disorders, it is critical to understand the individual roles of forebrain MR and GR in HPA axis regulation and control of affect.

The traditional view posits that, because of its high affinity and low capacity, activation of MR is essential for maintenance of the basal circadian rhythm, whereas activation of GR is required for the stress response and the subsequent recovery of homeostasis via negative feedback (10). However, more recent studies suggest that MR activity is required in conjunction with GR at the peak of the circadian rhythm and during times of stress (11) or that MR alone can mediate negative feedback in response to various challenges (12–14). These results are consistent with MR antagonist studies in humans that demonstrate a clear role for MR in HPA regulation and increased MR function in depression (15, 16). In contrast, recent findings in forebrain-specific MR-deficient mice show no alterations in HPA axis activity under basal or stress-activated conditions (17). Clearly, additional studies are required to elucidate the role of MR in both HPA function and modulation of affective responses.

In addition to the roles of MR and GR in HPA axis regulation, research over the last decade has suggested a potential role of these molecules in anxiety modulation. The evidence is clearest for GR because genetic manipulation has shown that decreased GR signaling in the brain results in decreased anxiety-like behavior (18, 19), whereas overexpression of GR in the forebrain

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Abbreviations: EPM, elevated plus maze; GC, glucocorticoids; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; ISH, *in situ* hybridization; MR, mineralocorticoid receptor; PVN, paraventricular nucleus.

‡To whom correspondence should be addressed at: University of Michigan, Basic Science Research Building, MBNI, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200. E-mail: aseashol@umich.edu.

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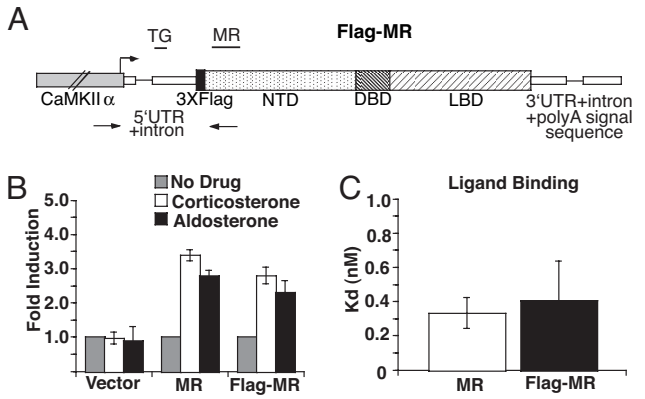

Fig. 1. Transgene construct and properties of Flag-MR. (*A*) Schematic of transgene construct. The CaMKIIα promoter directs expression of a 3×Flag-tagged mouse MR cDNA. Arrows represent the location of the primer pair used for PCR genotyping. The two bars represent the locations of the two probes used for ISH analysis (TG, transgene specific; MR, endogenous MR + transgene directed MR). NTD, *N*-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain. (*B*) Transactivation of glucocorticoid responsive element (GRE)-luciferase by MR vs. Flag-MR. CV-1 cells were transfected with MR, Flag-MR or vector alone, GRE-luciferase, and CMV-βgal; 24 h later, cells were treated with drugs for an additional 24 h (corticosterone = 100 nM, aldosterone = 10 nM). Normalized luciferase activity (luciferase activity/βgal activity) for samples was divided by their respective no drug control to result in fold induction of reporter gene activity. The data are presented as mean ± SEM and represent three to five independent experiments done in duplicate. (*C*) Ligand-binding properties of MR vs. Flag-MR. Cos-1 cells were transfected with MR or Flag-MR. Cells were harvested after 40 h, and [^{3}H]corticosterone was used to determine dissociation constants (K_d) for MR and Flag-MR in five independent Scatchard analyses. Data represent mean ± SEM. Experimental details are in *SI Methods*.

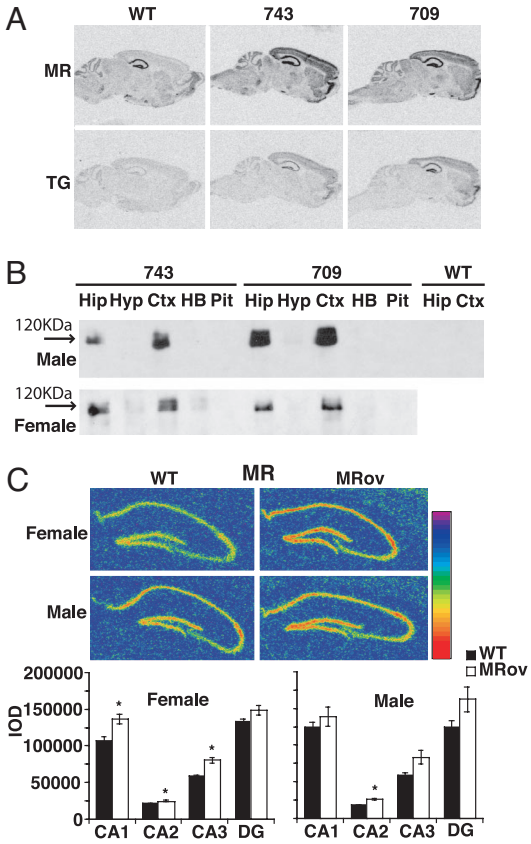

Fig. 2. Forebrain-specific transgene expression in MRov mice. (*A*) ISH with MR and transgene-specific (TG) probes on sagittal sections from wild type (WT) and two different MRov founder lines reveals forebrain specificity of transgene expression. (*B*) Western blot analysis with anti-Flag antibody confirms expression of Flag-MR in forebrain regions of both male (*Upper*) and female (*Lower*) mice. Regions include hippocampus (Hip), hypothalamus (Hyp), cortex (Ctx), hindbrain/cerebellum (HB), and pituitary (Pit). (*C*) Representative pseudocolored ISH images with an MR-specific cRNA probe show overexpression of MR mRNA in subregions of the hippocampus (blue = low expression, red = high expression). Quantitation represents integrated optical density (IOD) analysis of 4–5 animals/genotype/sex and 9–14 sections/animal. Data are presented as mean ± SEM. *, $P < 0.05$ by *t* test.

results in increased anxiety-like behavior (20). MR antagonist application directly into the hippocampus (21, 22) or the brain in general (23) suggests a similar anxiogenic role for MR either exclusively or in conjunction with GR. However, these effects are transient and do not address whether sustained alterations of MR would be anxiogenic or anxiolytic.

To specifically examine the role of MR in corticosteroid-mediated basal and stress-induced feedback regulation of the HPA axis and/or anxiety-like behaviors, we generated transgenic mice that overexpress forebrain MR (MRov). In this system, the calcium/calmodulin-dependant protein kinase IIα (CaMKIIα) promoter is used to direct expression of MR specifically to forebrain regions. This promoter is not active prenatally, preventing overexpression of MR during development. In contrast to the forebrain-specific MR-deficient mice, we find that overexpression of MR in the forebrain does impact stress-induced regulation of the HPA axis in female mice and results in a reduction in anxiety-like behavior in both male and female mice. These data highlight the differential roles for MR and GR in the modulation of anxiety-related behaviors and suggest a more prominent role for MR in HPA axis regulation.

Results

Generation of Forebrain-Specific MR Overexpressing Mice. To generate transgenic mice that overexpress MR specifically in the forebrain (MRov), we used the previously described CaMKIIα promoter (24) to direct expression of a 3×Flag-tagged MR cDNA. The 3×Flag epitope tag was added at the N terminus of MR to allow detection of transgene-directed MR (Fig. 1*A*). The addition of the 3×Flag epitope did not significantly alter the transcriptional activation properties of MR (Fig. 1*B*), and MR and Flag-MR showed similar transactivation profiles across corticosterone concentrations from 10 pM to 100 nM (data not shown). The binding properties of MR and Flag-MR expressed *in vitro* were also not significantly different (Fig. 1*C*). The transgene construct was then used for production of transgenic mice.

In situ hybridization (ISH) analysis with both MR and transgene-specific probes (delineated in Fig. 1*A*) was used to determine the sites of transgene expression in 10 different founder lines. Of these 10 lines, only 7 expressed the transgene. Transgene-directed MR expression was localized largely to the hippocampus and cortex, with varying intensities of expression in these regions seen between the different lines (data not shown). Because expression patterns were similar in most lines, lines 743 and 709 were selected for further analysis. ISH with sagittal sections demonstrated that MR transgene mRNA was detected specifically in forebrain regions (Fig. 2*A*). RT-PCR experiments with RNA from brain and peripheral tissues revealed no transgene expression outside of the forebrain (data not shown). Although transgene mRNA was widely expressed in cortex and all subfields of the hippocampus, ISH experiments with a transgene-specific probe showed no expression of the transgene in either the paraventricular nucleus (PVN) [see supporting information (SI) Fig. 6] or the amygdala of MRov mice, additional sites of endogenous MR expression (25–27). Western analysis with tissue prepared from different brain regions and a

transgene-specific anti-Flag antibody confirmed the presence of transgene-directed Flag-MR protein in hippocampal and cortical regions for both lines (Fig. 2*B* and SI Fig. 7), consistent with ISH results. The very low levels of Flag-MR protein seen in the hypothalamus and hindbrain/cerebellum lanes for line 743 females were not observed in additional analyses, consistent with the lack of transgene-specific mRNA in these regions.

To determine whether transgene expression in the hippocampus of MRov mice resulted in overexpression of total MR mRNA relative to wild-type littermates, ISH experiments with an MR-specific probe that detects both endogenous and transgene-directed MR were performed by using both wild-type and transgenic male and female mice (Fig. 2*C*, line 743). ANOVA reveals a significant effect of genotype ($P < 0.05$) in all subregions of the hippocampus (CA1, CA2, CA3, and dentate gyrus), with MRov mice exhibiting greater levels of expression and no gender effects in any of the subregions. If the data are analyzed separately to compare either male or female transgenics with their respective wild-type littermates, a significantly higher expression of total MR mRNA in CA1, CA2, and CA3 ($P < 0.05$ for each subregion) in female MRov mice was seen. In male transgenic mice, a significant increase in MR mRNA was seen only in CA2 ($P < 0.05$), with a trend toward increased MR mRNA in CA3 ($P = 0.06$). In a second set of ISH studies, where males and females were analyzed in separate experiments, similar results were obtained. The mean gray levels (not corrected for area) from this experiment (SI Fig. 8) show that MR mRNA levels for transgenic and wild-type mice are greatest in CA2 and DG.

Together, these data demonstrate that transgenic MRov mice overexpress hippocampal MR mRNA by ≈20–25% relative to wild-type littermate controls, representing a physiologically relevant increase in expression *in vivo*. Moreover, female mice tend to show more areas of hippocampal overexpression than males. As shown above, the MRov mice also express significant levels of MR in the cerebral cortex, a site of increased MR expression in primates, allowing potential functional interactions of MR alone or with GR in additional cortical sites.

Normal Basal HPA Axis Activity, but Sexually Dimorphic Corticosterone Release in Response to Restraint Stress in MRov Mice. The traditional view of MR function in HPA axis control suggests a primary involvement in the maintenance of basal tone with little to no role in the response to stress. To assess the effects of forebrain MR overexpression on basal HPA axis activity, circadian trough and peak corticosterone levels were determined. Plasma samples from transgenic line 743 and wild-type littermates were collected 1–2 h after lights on or 30–60 min before lights off. There were no differences between transgenics and wild-type littermate controls for either sex at the nadir of the circadian rhythm (Fig. 3*A*). In two independent experiments, corticosterone levels were 20–27% lower in both male and female transgenic mice at the peak of the circadian rhythm; however, this result did not reach significance (Fig. 3*B*, experiment 1 shown, 20 and 22% reductions, respectively). Because all anxiety-like behavior tests constitute mild stressors, we also assessed corticosterone levels in MRov mice under mild stress conditions. Plasma samples were collected 10 min after a 5-min exposure to the elevated plus maze (EPM); no significant differences between transgenics and wild types were observed for either sex (data not shown). To assess HPA axis function in response to a more intense stressor, male and female transgenic and wild-type mice were subjected to 30 min of restraint stress. Plasma samples were collected at 0, 20, 40, and 60 min (Fig. 3*C*). There was no significant difference in corticosterone levels between male transgenic and wild-type mice. Female transgenic mice, however, exhibited a moderate suppression of the corticosterone response to stress throughout the stress and recovery

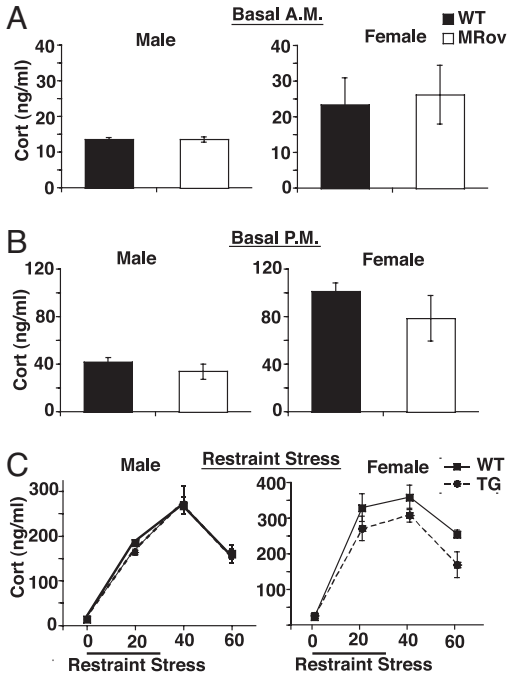

Fig. 3. Normal basal HPA axis activity, but sexually dimorphic suppression of corticosterone release in response to restraint stress. (*A*) Basal a.m. corticosterone levels ($n = 4–5$/genotype/sex). (*B*) basal p.m. corticosterone levels ($n = 5–6$/genotype/sex). (*C*) Restraint stress-induced corticosterone release in male and female MRov (TG) and wild-type (WT) mice in the a.m. Female MRov mice exhibit a moderate suppression of the corticosterone response to stress over the stress and recovery time course. ANOVA reveals a significant difference due to genotype ($P = 0.029$, $n = 4–10$/genotype/time point).

time points compared with wild-type littermates ($P < 0.05$); no significant differences were observed in corticotropin levels. These results demonstrate a sexually dimorphic corticosterone response to restraint stress in the MRov mice.

MRov Mice Exhibit Decreased Anxiety-Like Behavior. Previous studies with intracerebroventricular MR antagonist administration in rats suggest that temporary blockade of MR in the brain results in decreased anxiety (22, 23), suggesting that increased MR signaling would be anxiogenic. To further address the role of constitutive forebrain MR overexpression in anxiety-like behavior, MRov mice were analyzed in a series of behavioral paradigms, including general locomotor activity and time in the center of the open field, in addition to the EPM. Transgenic and wild-type mice of both sexes were initially tested for general locomotor activity for 15 min in the open field for 3 consecutive days. These data were collected in five 3-min bins per day. Because subsequent behavioral tests are 5-min tests, only the first two bins (6 min) are presented. For line 743, the results show that MRov and wild-type mice both habituate normally to the open field and do not reveal any changes in general locomotor activity over the testing period (Fig. 4*A*). Thus, forebrain MR overexpression does not result in any generalized locomotor alterations that could obscure further behavioral analyses.

To determine the consequences of forebrain overexpression of MR on anxiety-like behaviors, the time spent in the center of the open field was determined for male and female MRov and wild-type mice. MRov mice of both sexes exhibited increased time in center on days 2 and/or 3 of testing, an indicator of decreased anxiety-like behavior (Fig. 4*B*). Male and female MRov mice and wild-type controls were also tested on the EPM, a widely used test of anxiety-like behavior. Male MRov mice

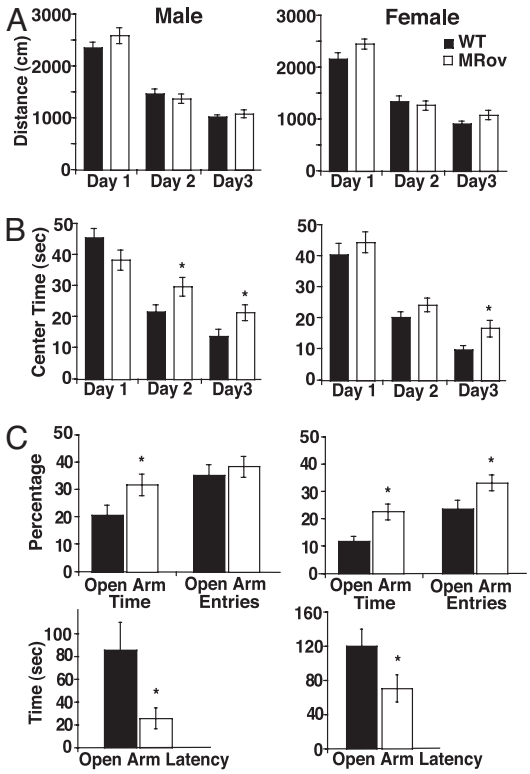

Fig. 4. Reduced anxiety-like behavior in forebrain MRov mice. (*A*) Three days of open field exposure show normal habituation and no changes in locomotor activity in either male or female MRov mice (first 6 min of activity shown). (*B–C*) Anxiety-like behavior measures in male and female MRov mice. (*B*) Open field activity reveals increased center time on days 2 and/or 3. (*C*) MRov mice show decreases in measures of anxiety-like behavior on the EPM, with significant changes in percentage of open arm time, percentage of open arm entries (females only), and open arm latency. Data represent mean ± SEM of 19–40 animals/genotype/sex. *, $P < 0.05$.

spent a significantly greater percentage of time in the open arms of the EPM compared with wild-type controls ($P < 0.05$, Fig. 4*C*). The latency to the first entry on the open arm was significantly less in male MRov mice compared with wild type ($P < 0.05$). In a second group of male animals, significant differences were observed in these two measures, in addition to showing a significant increase in percent open arm entries (data not shown). Over two rounds of testing, female MRov mice spent a significantly greater percentage of time in the open arms of the EPM compared with wild-type controls ($P < 0.05$, Fig. 4*C*). They also showed a significant increase in the percent of open arm entries ($P < 0.05$) and a significant decrease in the latency to the first entry on an open arm ($P = 0.05$).

To rule out the possibility of insertion site effects, most of these behavioral experiments were repeated in another line of MRov mice (line 709). Although the phenotype was not as pronounced in line 709 as it was in line 743, there were many similarities, suggesting that the decreased anxiety-like behavior is not due to an insertion site effect (SI Fig. 9).

Basal Expression of Genes Associated with HPA Axis Activity. We hypothesized that the constitutive overexpression of MR in forebrain regions might result in altered expression of genes associated with HPA axis activity and anxiety-like behaviors. To address this hypothesis, the basal or stress-induced expression patterns of corticotropin-releasing hormone (CRH) and hnCRH in the PVN, GR in the hippocampus and PVN, and 5HT-1a in the hippocampus were analyzed by ISH in MRov mice. Region CA1 of the hippocampus showed a significant decrease in the expression of GR mRNA in male MRov mice ($P < 0.05$), whereas in female MRov mice, this same region showed a nonsignificant trend toward decreased GR mRNA ($P = 0.11$, Fig. 5*A*). This effect was selective to the hippocampus because no significant changes in GR mRNA levels were observed in the PVN (SI Table 1). There were also no significant changes in the expression of steady-state CRH mRNA levels in the PVN of male and female transgenic mice and no changes in hnCRH in the PVN of transgenic mice after 20 min of restraint stress for either sex (SI Table 1). Interestingly, both male and female MRov mice showed a significant increase in the expression of 5HT-1a mRNA in region CA1 of the hippocampus (Fig. 5*B*, $P < 0.05$). The transgenic mice also showed an increase in [^{3}H]8-OH-DPAT binding in CA1 (Fig. 5*C*, wild type = 0.71 ± 0.003 and transgenic = 0.76 ± 0.008, $P = 0.0004$), consistent with elevated levels of 5HT-1a protein. The relative change in 5HT-1a receptor binding was less than the change in mRNA levels, consistent with previous studies (28). Together these results demon-

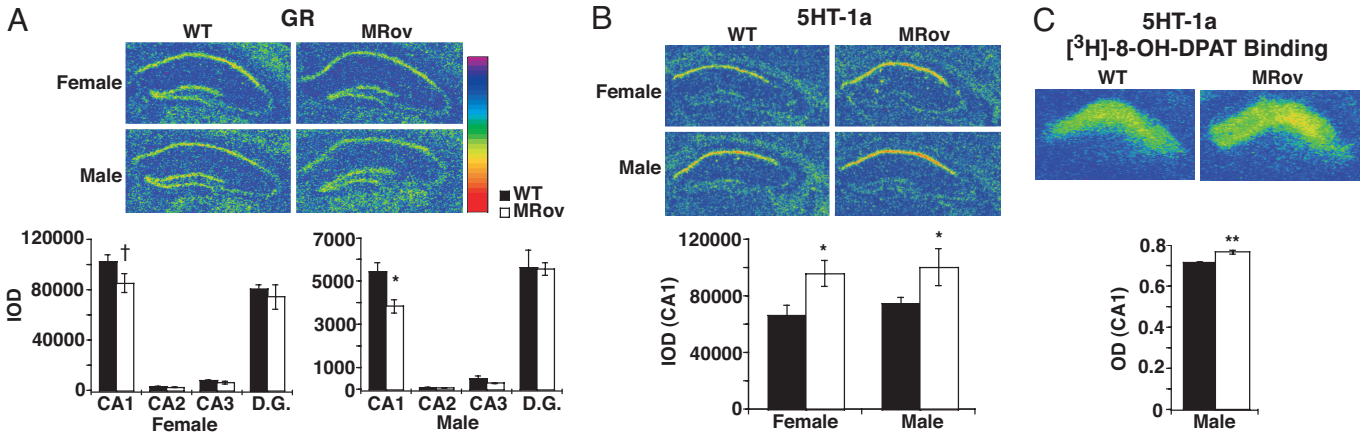

Fig. 5. Altered expression of GR and 5HT-1a in MRov mice. (*A*) ISH analyses with a GR-specific probe show a decrease in GR mRNA in region CA1 of the hippocampus of male MRov mice and a trend toward decreased GR in region CA1 of female MRov mice (*, $P < 0.05$, †, $P = 0.11$) ($n = 5$ animals/genotype/sex, and 12–16 sections/animal for males and 7–9 sections/animal for females). (*B*) ISH with a 5HT-1a-specific probe shows significantly increased levels of the receptor mRNA in the CA1 of male and female MRov mice ($n = 4–5$ animals/genotype/sex, and 9–12 sections/animal). (*, $P < 0.05$.) (*C*) 5HT-1a receptor binding with [^{3}H]8-OH-DPAT shows significantly increased levels of binding (**, $P = 0.0004$) ($n = 6$ males/genotype and 8–10 sections/animal). Representative pseudocolored images are shown.

strate that overexpression of MR in the forebrain alters the basal mRNA expression patterns of GR and 5HT-1A, genes involved in both HPA axis function and anxiety-related behaviors.

Discussion

In this study, we characterize anxiety-like behaviors and the HPA axis of transgenic mice with chronic forebrain-specific overexpression of MR. We show that chronic elevations of forebrain MR result in decreased anxiety-like behaviors in both male and female transgenic mice. We also demonstrate that increased levels of forebrain MR result in a moderate suppression of the stress response in female transgenic mice, suggesting that, at least in females, MR contributes to negative feedback information during a stressful event. Furthermore, MR overexpression alters mRNA levels of two genes associated with stress and anxiety, increasing hippocampal 5HT-1A and decreasing hippocampal GR expression. This latter change results in a significantly altered MR:GR ratio in certain areas of the hippocampus. Our findings suggest that the functions of forebrain MR overlap with GR in HPA axis regulation, but counterbalance GR in modulation of anxiety-related behaviors.

Anxiety-Like Behavior. Both male and female MRov mice exhibit a decreased anxiety-like behavior phenotype. These data are at odds with a series of antagonist studies that suggest that MR activation plays an anxiogenic role either alone or in combination with GR. There are, however, two prominent changes at the molecular level in the MRov mice that may account for this result. First, forebrain GR plays an anxiogenic role in several genetic models of altered GR levels (18–20), and it is down-regulated in certain hippocampal areas of the MRov mice, consistent with MR-mediated down-regulation of GR (29). Thus, it is possible that overexpression of MR in the forebrain of MRov mice reduces anxiety behavior through a decrease in GR function or through alterations of the GR:MR ratio, which has been proposed to have functional import (2). Second, decreased anxiety-like behavior in the MRov transgenic mice may result from the increased expression of 5HT-1a in CA1 of both male and female MRov mice. Multiple genetic manipulation studies of 5HT-1a suggest that this receptor plays an anxiolytic role, and that postsynaptic sites of 5HT-1a, such as the hippocampus, mediate the anxiolytic effects (30).

The increased expression of the 5HT-1a receptor in the hippocampus of MRov mice was not predicted because 5HT-1a receptors are under tonic inhibition by corticosterone *in vivo* (31), and studies by de Kloet (32) suggest that MR is the primary inhibitor of 5HT-1a expression. In addition, *in vitro* studies show direct repression of 5HT-1a promoter activity by binding of MR and GR as both homo- and heterodimers to a novel negative glucocorticoid responsive element in the rat 5HT-1a promoter (33). Multiple hypotheses can be proposed to explain the observed increase in hippocampal 5HT-1a in MRov mice. First, adrenalectomy studies are short term. In contrast, MRov mice experience chronically elevated levels of MR for most of their lives. Hence, although the decrease in corticosterone levels seen at the p.m. time point in male and female MRov mice is not statistically significant, it may, over the course of the animals' lives, represent a significant biological decrease in glucocorticoid signaling that allows for enhanced 5HT-1a expression in the hippocampus. In addition, there may be a critical developmental time window during which MR overexpression may result in lifelong changes in serotonin receptor expression. Moreover, the change in hippocampal MR:GR ratio in MRov mice may affect the expression pattern of 5HT-1a through altered signaling on the 5HT-1a promoter directly or through an indirect mechanism such as changes in expression of other transcription factors. Although the mechanism remains to be elucidated, it is evident that chronic MR overexpression results in downstream changes that have different molecular and behavioral outcomes from short-term alterations in MR signaling. Of note is that the change in MR expression is relatively subtle and may well occur in the context of normal variability in gene expression. That such a modest and localized change in gene expression can result in lifelong changes in responsiveness to affective stimuli underscores the role of MR, and its partner GR, in fine-tuning reactivity to environmental stimuli.

HPA Axis Regulation. Several extrahypothalamic sites have been shown to inhibit HPA axis activity in a corticosterone-dependent manner, including the hippocampus (34, 35) and the cingulate cortex (36), both of which express MR and GR. Although it is clear that GR in these forebrain sites is important for HPA axis inhibition (37), the contribution of MR to HPA axis feedback poststress or at the peak of the circadian rhythm is currently under debate. Female MRov mice, which have increased levels of MR compared with wild-type controls in the cortex and all subregions of the hippocampus, do not exhibit significant alterations in basal corticosterone levels or an altered endocrine response to mild stress. However, they do show a small but significant attenuation in their response to restraint stress.

These data suggest that as MR becomes progressively more occupied in female MRov mice, it is capable of inhibiting the stress response and, to a lesser degree, the p.m. corticosterone levels. Recent data from Kalman *et al.* (38) support the potential biological relevance of this overexpression model. They find that MR occupation at the nadir of the circadian rhythm is closer to 50% rather than the previously thought 90%, suggesting a more dynamic capability of MR signaling in response to stress (38).

The ISH data show that, relative to wild-type mice, female transgenic mice show a greater percent increase in MR mRNA than the male mice. This result suggests that a threshold mechanism may be in place, where the percent change in MR may be important for its relative contribution toward negative feedback during a stressful event. This difference may account, in part, for the sexually dimorphic alteration in stress response observed in the MRov model.

Comparison with MR Knockout Models. In 1998, mice were generated that lack MR globally, but these mice die of dehydration by postnatal day 10 due to renal sodium and water loss, not allowing for behavioral analyses (39). Very recently, a forebrain-specific MR-deficient mouse was created by using the CaMKIIα promoter to direct Cre-recombinase activity only in forebrain regions ($MR^{CamKCre}$) (17). No alterations in corticosterone levels were seen either under basal a.m. or p.m. conditions or after 40 min of restraint stress. Also, contrary to the findings in MRov mice, no differences were seen in anxiety-like behaviors. There are, however, several significant differences between these two genetic models of increased or decreased forebrain MR. First, the respective mice are on slightly different genetic backgrounds. Second, although behavioral testing in MRov mice was performed during the nadir of the circadian rhythm (early in the light period), all of the behavioral testing for the $MR^{CamKCre}$ mice was performed during the dark cycle when corticosterone levels are higher. Testing during the dark cycle may result in a greater proportion of activated GR, whereas the testing in MRov mice is under predominant MR activation. Third, the loss of MR in $MR^{CamKCre}$ mice appears to be more widespread than the overexpression of MR in MRov mice. For example, MR is lost in the amygdala in $MR^{CamKCre}$ mice, but MR expression is not altered in the amygdala of MRov mice. Fourth, the MRov mice exhibit increased expression of MR in cortical regions that normally express low or nondetectable levels of MR in mice. Combined, these differences may result in a differential expression of compensatory mechanisms leading, overall, to altered patterns of behavior or HPA axis function in the $MR^{CamKCre}$ and MRov mice.

In conclusion, we have created a transgenic mouse with

physiologically relevant increases in MR expression in the forebrain to assess the role of MR in anxiety-like behavior and HPA function. The data demonstrate a significant role for MR in the reduction of anxiety-related behaviors and suggest important roles for MR signaling in HPA axis activity beyond maintenance of basal tone. These data also highlight the complex nature of MR and GR signaling, which may work together in the regulation of some pathways, but exhibit distinct activities in the modulation of others.

Materials and Methods

Generation and Characterization of MRov Mice. A 3×Flag epitope tag was added to the N terminus of mouse MR by a PCR-based cloning strategy. The transactivation and binding properties of Flag-MR were assessed by transactivation and steroid receptor binding assays. The Flag-MR cDNA was subcloned into pMM403, which contains an 8.5-kb fragment of the CaMKIIα promoter (24), resulting in pMM403-FlagMR, which was used to create transgenic mice. Ten transgenic founders were bred to C57BL/6J mice, and seven lines were established. Transgenic mice were maintained as hemizygotes, and wild-type littermates were used as controls in all experiments. Immunoprecipitation and Western blotting were used to determine the expression pattern of the transgene. These techniques, as well as cloning of the mouse MR cDNA, generation of the 3×Flag-tagged MR transgene construct, transactivation and binding assays, and genotyping are described in the *SI Methods*.

ISH. ISH was performed as previously described (40). More information regarding the construction of the riboprobes can be found in the *SI Methods*. Semiquantitative analysis of autoradiogram images was performed by using the MCID image analyses system and Image J (National Institutes of Health). Optical density measures were corrected for background to yield mean gray levels, which were multiplied by the area sampled to produce an integrated density measurement.

Behavioral Testing. All behavioral tests were performed with an $n = 14–20$ animals/genotype/sex, and all tests were repeated with a second cohort of mice. Male and female mice were individually housed for at least 7 days before the beginning of behavioral testing. All testing was performed between 0630 and 1300. The following testing order was used: open field (3 consecutive days) and EPM 3 days later. All behavior was scored manually by investigators blinded to the phenotype of the animal. Detailed descriptions of testing apparatuses can be found in the *SI Methods*.

Hormone Assays and Receptor Autoradiography. Detailed descriptions can be found in the *SI Methods*.

Statistical Analysis. All statistical analyses were performed by *t* tests or ANOVA. Two-factor ANOVA was performed on *in situ assays* for MR and 5HT-1a (genotype × sex), as well as on the stress corticosterone levels (genotype × time).

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