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Disruption of mineralocorticoid receptor function increases corticosterone responding to a mild, but not moderate, psychological stressor

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Pace, Thaddeus W. W., and Robert L. Spencer. Disruption of mineralocorticoid receptor function increases corticosterone responding to a mild, but not moderate, psychological stressor. *Am J Physiol Endocrinol Metab* 288: E1082–E1088, 2005. First published January 25, 2005; doi:10.1152/ajpendo.00521.2004.—Glucocorticoid negative feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis is mediated by corticosteroid receptors. It is widely thought that during stress, glucocorticoid receptors (GR) are essential for this negative feedback. In contrast, mineralocorticoid receptors (MR) are associated with HPA axis regulation in basal, nonstress conditions. Notions about the relative roles of MR and GR for HPA axis regulation during stressor challenge may not be complete. Recent work in our laboratory suggests that previous estimates of MR occupancy at resting plasma levels of corticosterone (CORT) may be overestimated. It is possible that a significant number of MR may be available to mediate negative feedback during stressor challenge. We hypothesized that this may be especially the case during mild stressor challenge when the plasma CORT response is weak. In the present studies, adult male Sprague-Dawley rats were first treated systemically or centrally with the selective MR antagonist RU28318 (50 mg/kg sc or 500 ng·10 μl^{-1} ·2 h⁻¹ icv) or vehicle (300 μl propylene glycol sc or 10 μl /2 h sterile saline icv) and then challenged with 60-min novel environment or restraint. In vehicle controls, restraint resulted in a greater plasma CORT response than novel environment. Both systemic and central treatment with RU28318 significantly increased CORT responding to novel environment relative to vehicle controls. However, RU28318 treatment did not increase the CORT response to restraint. These data suggest that MR may be necessary for glucocorticoid regulation of HPA axis activity during mild stressors, but not during stressors that result in a more robust CORT response.

RU28318; glucocorticoid negative feedback; hypothalamic-pituitary-adrenal axis regulation

REGULATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) axis depends largely on glucocorticoid negative feedback (6). Circulating cortisol (in humans) or corticosterone (in rodents, CORT) inhibits the release of adrenocorticotropin hormone from the anterior pituitary (13), and CORT decreases the release and synthesis of corticotropin-releasing hormone (15, 20) and arginine vasopressin (15) from neurons located in the paraventricular nucleus (PVN) of the hypothalamus. The activity of PVN afferents may also be increased or decreased by glucocorticoids, with the effect of decreasing overall excitatory drive to the PVN (7). Importantly, CORT inhibition of the HPA axis is mediated by two subtypes of corticosteroid receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (6). Work over the last 3 decades suggests

that MR mediates CORT inhibition of HPA axis function during resting, or nonstress periods. GR, in contrast, is thought to mediate CORT regulation of stress-induced HPA axis activity (7). These differential roles of MR and GR are consistent with the finding that MR has a higher affinity for endogenous glucocorticoids than GR (22). Although it has also been recently suggested that regulation of the HPA axis during a stressor may require an interplay between MR and GR to mediate CORT negative feedback (7, 25), most researchers would agree that regulation of the HPA axis during a stressor would not be possible without normal GR function. As a result of recent studies in our laboratory examining 1) changes in MR protein levels after adrenalectomy (ADX) and 2) the expression of CORT response habituation to restraint after treatment with a selective MR antagonist, we believe that this conception may be incomplete. The goal of the present studies was to examine a primary role of MR to mediate CORT feedback during a stressor challenge, especially when that stimulus results in a weak CORT response and thereby minimal GR occupancy.

Understanding the relative roles of corticosteroid receptors begins by considering their binding characteristics. Although MR has a relatively high affinity for glucocorticoids ($K_d = 0.5\text{--}1.0$ nM), GR has a low affinity for ligand ($K_d = 2.5\text{--}5$ nM) (7). Subsequent investigations expand on these binding characterizations by examining *in vivo* MR and GR occupancies at various circulating levels of CORT. Early receptor binding studies indicated that nearly all MR are occupied in rats killed with very low circulating levels of CORT (i.e., 1 $\mu\text{g}/100$ ml plasma) (22). In contrast, the same studies reported that only ~10% of GR are occupied at the same circulating level of CORT. Only at higher plasma levels of CORT does GR occupancy begin to increase. On the basis of these profiles, Reul and deKloet concluded that, at nonstress levels of plasma CORT (i.e., basal levels), GR would have little interaction with CORT, whereas MR would be mostly occupied (22).

Several years ago, our laboratory (12) reported that hippocampal MR rapidly upregulate after ADX, as measured by Western blot. MR upregulation is evident 12 h after ADX and is complete by 24 h. This observation is relevant to the current topic when considering how the aforementioned receptor binding studies (22) were used to estimate relative occupancy of MR at various circulating levels of plasma CORT. In those studies, estimates of MR occupancy were made by comparing available hippocampal MR binding in adrenal intact animals with various levels of circulating CORT to hippocampal MR binding in ADX rats that had no circulating CORT. Because

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only unactivated corticosteroid receptors can be measured in these binding assays (4, 24), the ADX rat was used in these occupancy studies to provide an estimate of the total hippocampal MR available for binding. Because there are more MR in the ADX rat than in the adrenal-intact rat, binding measures obtained by Reul and de Kloet (22) probably overestimated the proportion of MR that are occupied by low levels of plasma CORT. In actuality, there is probably a portion of MR that is unoccupied, especially at the circadian trough of HPA axis activity.

In the present set of studies, we hypothesized that in situations of mild stress when the plasma CORT response is also minimal MR play a primary role to mediate suppressive glucocorticoid effects. This assumes that 1) during low basal CORT secretion a substantial proportion of MR remain unoccupied, and 2) during mild stressor situations plasma CORT levels never rise high enough to occupy a significant portion of GR while increasing the proportion of MR occupancy. To test this hypothesis, we first identified an acute psychological stressor that reliably results in low-intensity phasic HPA axis stress responses. We then further explored *in vivo* use of a receptor antagonist for MR (RU28318) by examining available corticosteroid receptor binding in the hippocampus of ADX rats. This allowed us to determine the extent and selectivity of RU28318 occupancy of MR and GR (14). We then observed the functional effects of MR blockade on HPA axis responding to both a mild stressor and a more intense stressor. Finally, we administered RU28318 into the cerebral ventricles to investigate the possibility that the MR mediating negative feedback inhibition of the HPA axis during stress are located centrally.

METHODS

Subjects

Male Sprague-Dawley Rats (Harlan Laboratories, Indianapolis, IN) weighing between 225 and 290 g were used for all experiments. Rats were allowed a 2-wk acclimation period after arrival to the animal facilities at the University of Colorado before any experimentation began. At least 1 wk of acclimation was allowed before any surgical procedures. Animals were housed two per polycarbonate tub with wood shavings and were allowed food (Purina Rat Chow) and tap water *ad libitum*. The colony room lights were regulated on a 12:12-h light-dark cycle, with lights on at 0700. Care and use of animals were in accordance with procedures approved by the University of Colorado Institutional Animal Care and Use Committee.

MR Antagonist

The MR antagonist RU28318 (Roussel Uclaf, now Hoescht Marion Roussel, Romainville, France) was administered subcutaneously (sc) or intracerebroventricularly (icv). For sc injection, a dose of 50 mg/kg in 100% propylene glycol (3 ml/kg) was given 1 h before tissue collection or stressor challenge. icv administration of RU28318 (total dose 500 ng/10 μ l sterile saline) took place over a 2-h period with 2- μ l slow infusions every 30 min, with completion of the injection immediately before stressor challenge. icv infusion of saline vehicle or RU28318 was performed in the home cage, but with a specialized cage top that allowed for free movement of the injection line. Infusion lines (C313C, Plastics One, Roanoke, VA) and microinjectors (C315I/SPC; 7mm, Plastics One) were affixed to the guide cannula and remained attached for the entire 2-h period during injection.

Surgical Procedures

For both ADX and icv guide cannula surgeries, rats were first fully anesthetized under ketamine (50 mg/kg) and xylazine (10 mg/kg) anesthesia. ADX was performed by dorsal approach. Rats were fitted with a unilateral steel guide cannula (C315G-5UP/SPC; 6 mm, Plastics One) in the right lateral ventricle (AP -0.8 mm from bregma, ML -1.4 mm, DV -3.8 mm from dura). Placement of guide cannulas was verified at the time of surgery by the saline drop technique. Briefly, a sealed 12-cm-long section of polyethylene tubing filled with sterile saline was attached to the top of each guide cannula before the start of surgery. If the tip of the guide cannula was placed correctly in the ventricle, the saline level would visibly drop when the line was held vertically and upon removal of the plug at the opposite end. If placement were off, such as in tissue surrounding the ventricle, the saline level would not drop. We have cross-validated this placement verification method with a functional angiotensin II drinking test and find a very high correspondence (unpublished observations). Placement was also verified after experimentation by dye injection immediately after animals were killed.

Testing Procedures

Experiment 1: validation of novel environment as a low-intensity stressor compared with restraint. Restraint consisted of placing a rat in a clear, well ventilated, cylindrical Plexiglas tube with adjustable length (15.5 ± 2.5 cm length) atop a table next to the home cage room. Novel environment consisted of placing a single rat in an empty polycarbonate tub ($47 \times 23 \times 20$ cm) with a metal grate lid in an isolated room with twice the home cage room lighting intensity, adjacent to the rats' home cage room. Rats were exposed to either 30 min of restraint or novel environment or remained in home cages ($n = 4$). After this period, blood was rapidly sampled via the tail clip method for later plasma CORT analysis. In cases of novel environment, rats were quickly moved into a restrainer for blood sampling. For the no-stress control group, previously undisturbed rats were taken directly from home cages and blood sampled via tail clip.

Experiment 2: validation of RU28318 as a selective MR, but not GR, antagonist. Rats were adrenalectomized (described above) 48 h before testing and then allowed to recover in home cages with 0.9% saline and food available *ad libitum*. On the test day, animals were injected sc with RU28318 or vehicle ($n = 6$) and then returned to home cages for a period of 1 h. After that time, rats were killed by guillotine, and tissue was processed as described below for measurement of available MR and GR in the hippocampus with a corticosteroid receptor binding assay.

Experiment 3: effect of systemic RU28318 treatment on CORT responding to novel environment or restraint. Rats were injected systemically with RU28318 or vehicle in the manner and dose described above and then returned to home cages. One hour after injection, rats were exposed to novel environment or restraint for 1 h (different cohorts of rats were used for either stressor). After 30 and 60 min of novel environment or restraint, rats were blood sampled by the tail clip method. Animals challenged with novel environment were rapidly transferred to restrainers for blood sampling at 30 min and then returned to novel-environment chambers. The same rats were blood sampled in restrainers again at 60 min. Animals were then returned to home cages for a period of 1 h afterward, at which time a final blood sample was taken to measure recovery of plasma CORT after stress. A no-stress home cage control group was included in the cohort challenged with novel environment, with one blood sample collected 1 h after injection with RU28318 or vehicle ($n = 6$) at the same time restraint or novel environment challenge began for other rats.

Experiment 4: effect of icv RU28318 treatment on CORT responding to novel environment or restraint. Two weeks before testing, all rats were fitted with an icv guide cannula (described above). On the test day, rats received microinjections of either saline vehicle or RU28318, after which time they were challenged with novel environ-

ment ($n = 11$) or restraint ($n = 6$) for 1 h (different cohorts of rats were used for either restraint or novel environment challenge). Blood sampling took place immediately after the beginning of stressor challenge for basal plasma CORT measurement (restraint group only), after 30 and 60 min of stressor challenge, and 1 h after cessation of the stressor (novel environment cohort only), with the same sampling methods employed in *experiment 3*. As in *experiment 3*, different cohorts of rats were used on different test days for either novel environment or restraint challenge.

Corticosteroid Receptor Binding Assay

Available corticosteroid receptor binding procedures followed methods previously described (14). This procedure measures only the portion of MR and GR that are unactivated at the time of tissue processing. Thus a decrease in available receptor binding after acute ligand treatment indicated receptor activation by ligand (14, 24). Rats were decapitated, at which time the hippocampus was rapidly dissected on ice and immediately frozen at -80°C until homogenization.

CORT Radioimmunoassay

Measurement of plasma CORT was conducted with radioimmunoassay (RIA) procedures as previously described by Spencer et al. (25), except for the use of a different antiserum (B3-163; Endocrine Sciences, Calabasas Hills, CA). For assays in the given experiments, intra- and interassay coefficients of variability were 7 and 9%, respectively.

Statistical Analysis

Data were initially analyzed for each experiment with separate one- or two-way analysis of variance (ANOVA) tests, followed by post hoc tests (Fisher's least significant difference test). An α -level of $P \leq 0.05$ was used to determine statistical significance, and, where relevant, exact significance values are reported. A computer statistical package (Statview v. 4.51; Abacus Concepts, Berkeley, CA) was used to perform the analyses. Data presented in the figures represent group means \pm SE.

RESULTS

Experiment 1: Comparison of Novel Environment and Restraint

Plasma CORT levels after 30 min of novel environment or restraint were analyzed with a one-way ANOVA for independent measures. Rats displayed different CORT responses to situations of no stress, novel environment, or restraint, $F(2,9) = 37.71$, $P < 0.01$. Post hoc analysis revealed that rats exposed to novel environment had significantly lower CORT levels compared with rats challenged with restraint, whereas rats challenged with novel environment tended to have CORT levels that were higher than no-stress controls (Fig. 1).

Experiment 2: Validation of RU28318 as a Selective MR, but Not GR, Antagonist

RU28318 treatment decreased the amount of detectable MR, but not GR, in the hippocampus. Hippocampal receptor-binding measures were expressed as percentage of total binding compared with vehicle-treated rats and were initially analyzed by a two-way ANOVA for mixed measures (corticosteroid receptor subtype \times RU28318 treatment). There was an overall effect of RU28318 treatment to decrease corticosteroid receptors detectable in the cytosol [$F(1,10) = 24.35$, $P = 0.0006$]. Importantly, treatment with RU28318 was found to differentially affect MR and GR [$F(1,10) = 19.62$, $P = 0.0013$; Fig.

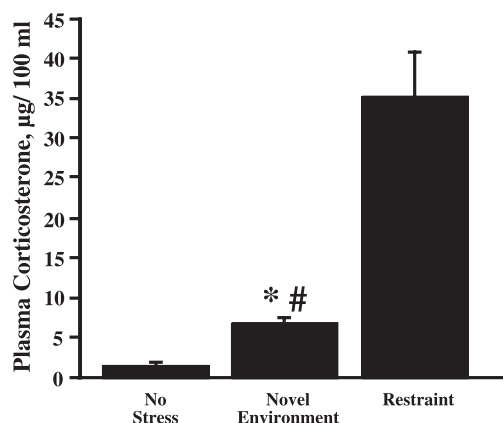


Fig. 1. Novel environment results in a smaller corticosterone (CORT) response than restraint. Rats were challenged with novel environment or restraint for 30 min, at which time all animals were blood sampled via tail clip method. A blood sample was taken from rats that were not previously disturbed (no-stress group). * $P < 0.0001$ vs. restraint group; # $P = 0.07$ vs. no-stress group.

2]. Post hoc analysis revealed that levels of available GR binding did not differ between vehicle- and RU28318-treated rats, whereas levels of available MR binding were dramatically reduced as a result of drug treatment.

Experiment 3: Effect of Systemic RU28318 Treatment on CORT Responding to Novel Environment or Restraint

Rats displayed plasma CORT responses to both novel environment and restraint. Interestingly, RU28318 treatment resulted in a larger CORT response to novel environment compared with vehicle injection. However, RU28318 treatment had no effect on restraint-induced plasma CORT levels.

Data from either the novel environment cohort or the restraint cohort were initially analyzed with separate three-way ANOVAs for mixed measures (stress exposure \times drug treatment \times time point), and relevant comparisons were examined. On the whole, rats exposed to novel environment had significantly greater plasma CORT levels than home cage controls, $F(1,20) = 35.97$, $P < 0.0001$. In addition, across all time points, animals treated with RU28318 had higher CORT levels compared with vehicle-treated rats, $F(1,20) = 12.84$, $P = 0.0019$. Plasma CORT levels were higher at 30 and 60 min in general, $F(2,40) = 185.47$, $P < 0.0001$. Post hoc analysis revealed that RU28318 treatment resulted in significantly higher plasma CORT after 30 min of novel environment exposure (Fig. 3).

In contrast to the significant increase in plasma CORT responding to novel environment as a result of RU28318 treatment, the same drug manipulation did not result in elevated plasma CORT responding to restraint, regardless of time of sampling, $F(1,15) = 0.012$, $P > 0.05$. However, post hoc analysis indicated a very strong trend ($P = 0.055$) for increased basal (0 min of restraint) CORT levels in animals treated with RU28318 compared with vehicle animals.

Experiment 4: Effect of icv RU28318 Treatment on CORT Responding to Novel Environment or Restraint

As in *experiment 3*, RU28318 treatment increased the plasma CORT response to novel environment challenge but not

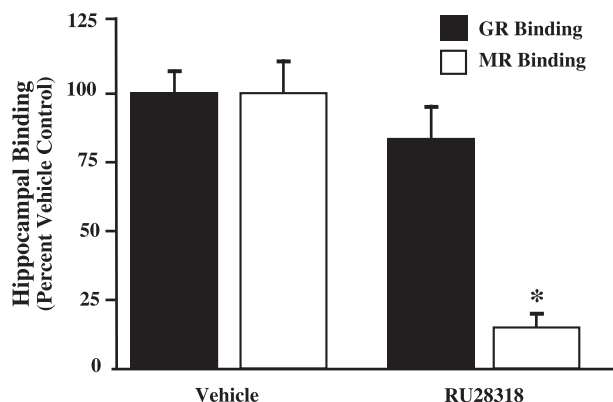


Fig. 2. Validation of RU28318 as a selective mineralocorticoid receptor (MR) antagonist (systemic administration). GR, glucocorticoid receptor. Rats were adrenalectomized 2 days before testing. On the test day, animals were injected with vehicle (3 ml/kg propylene glycol sc) or RU28318 (50 mg/kg sc) and returned to home cages for 1 h, and then tissue was collected (see METHODS). * $P < 0.0001$, vs. MR binding after vehicle injection.

to restraint challenge. In this experiment, RU28318 was administered centrally instead of systemically.

Data from either the novel environment cohort or the restraint cohort were analyzed with separate three-way ANOVAs for mixed measures (stress exposure \times drug treatment \times time point). On the whole, treatment with RU28318 in the context of novel environment resulted in higher levels of plasma CORT, $F(1,21) = 8.39$, $P = 0.009$. Post hoc analysis revealed that the icv injection of RU28318 resulted in significantly higher CORT responses to novel environment after both 30 and 60 min relative to vehicle controls (Fig. 4). In contrast, central treatment with RU28318 did not increase restraint-induced plasma CORT levels [$F(1,20) = 0.071$, $P > 0.05$]. In addition, RU28318 failed to elevate basal levels of CORT (Fig. 4).

DISCUSSION

In the present study, we demonstrate that treatment with an MR antagonist shortly beforehand increases the CORT response to novel environment. This suggests that suppressive glucocorticoid effects (i.e., CORT negative feedback) that take place during challenge with novel environment are mediated, at least in part, by MR. We also show that the same drug treatment does not increase the CORT response to restraint.

Thus HPA axis negative feedback regulation is not as dependent on MR during restraint challenge as it is during novel-environment challenge. By comparison of the CORT responses of rats challenged with restraint or novel environment after RU28318 treatment, it appears that MR may be important for mediating the suppressive CORT effects that limit HPA axis responding to milder stressors. During more intense stressors, normal CORT negative feedback requires GR input. This role of GR for HPA axis regulation has been identified by previous studies (for a review, see Ref. 7).

Before conducting functional pharmacological studies in the present work, we first verified that RU28318 selectively targets MR and not GR. ADX rats treated with RU28318 systemically 1 h before being killed had cytosolic MR levels (as detected by the radioligand binding assay) that were reduced by 86%. The same drug treatment did not alter GR binding relative to vehicle controls. The loss of MR binding on short-term treatment with RU28318 probably reflects dissociation of MR from accessory proteins [i.e., heat shock protein (HSP)90, HSP70], and translocation to the nucleus. Other studies have demonstrated that this activation process occurs in response to corticosteroid receptor antagonists as well as agonists (14). Activated corticosteroid receptors are not detected by the receptor-binding procedure (4, 16, 27).

In addition to validating the selectivity of RU28318 for MR before functional studies, we also compared novel environment to restraint in an experiment not involving injections or surgery (*experiment 1*). We determined that novel environment results in a much weaker plasma CORT response than restraint. As a result, novel environment appears to be a mild stimulus compared with restraint stress. On the basis of the comparison of plasma CORT levels in undisturbed rats to that of animals challenged with a stressor, we expect that the MR occupancy would be greater after novel-environment challenge than in unstressed (home cage control) animals. However, we would not expect the CORT responses to novel environment to occupy a significant portion of GR. During restraint the situation is probably different; as many as 50% of GR may become occupied along with the greater plasma CORT response that accompanies restraint (26, 27).

In the first of the two functional studies (*experiment 3*), animals were treated systemically with RU28318 before either novel environment or restraint. We observed an increase in the

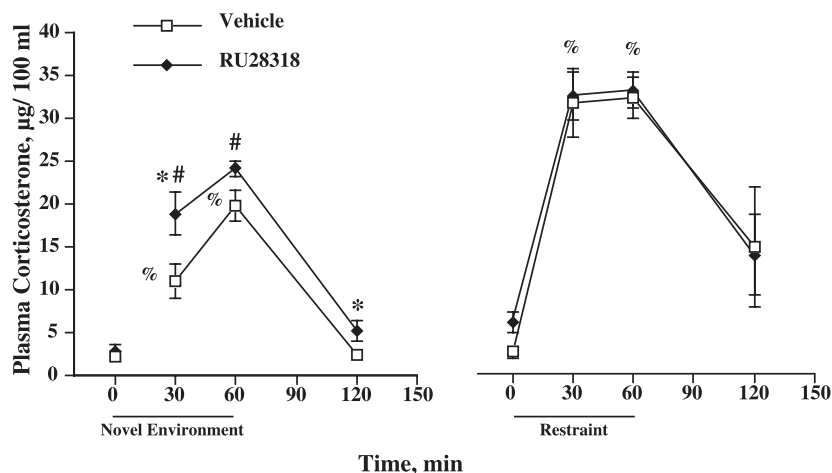


Fig. 3. Plasma CORT levels after 30 and 60 min of novel environment or restraint and after treatment with RU28318 (50 mg/kg sc) or vehicle (3 ml/kg propylene glycol). Blood was collected via tail clip method. Drug injection took place 1 h before stressor onset. A no-stress control group was included for both vehicle and RU28318 treatments in the novel-environment cohort. In the restraint cohort, animals were blood sampled immediately upon start of restraint for a no-stress, basal CORT measurement, and then restraint continued for 60 min with blood sampling. A CORT recovery measure taken 1 h after the end of novel environment or restraint is also included for both stressor groups. * $P < 0.02$ vs. vehicle group at same time point and same stressor treatment. # $P < 0.02$ vs. RU28318 group/home cage at 0 min. % $P < 0.02$ vs. vehicle group at 0 min.

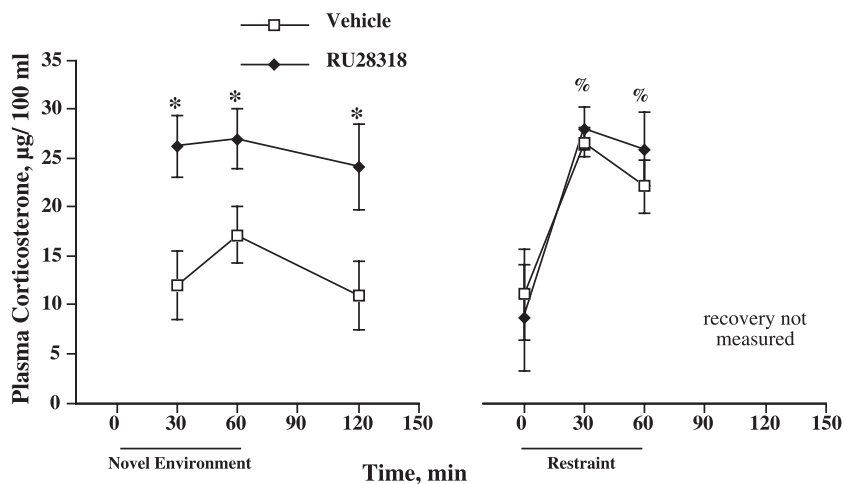


Fig. 4. Effect of RU28318 ($500 \text{ ng} \cdot 10 \mu\text{l}^{-1} \cdot 2 \text{ h}^{-1}$ icv) or vehicle (sterile saline) on plasma CORT levels after 30 and 60 min of novel environment or restraint. A stress recovery measure was taken (1 h after the end of stress) for rats challenged with novel environment only. Blood was collected via tail clip method. $*P < 0.03$ vs. vehicle group at the same time point. $\%P < 0.02$ vs. vehicle group at the no-stress (0 min) time point.

CORT response after 30 min of novel-environment challenge as a result of RU28318 treatment. A similar (but not statistically significant) effect was seen after 60 min of novel environment, but this was probably confounded by the stress of the blood sampling. The plasma CORT level after only 30 min of novel environment is the best indicator of RU28318's effect on the CORT response to this stressor, because subjects had not yet been blood sampled. Regardless, a similar effect of RU28318 was not seen in animals challenged with restraint at any point. We interpret this as evidence that MR may mediate suppressive effects of glucocorticoids during and immediately after stressor challenge, but such a role is likely only when that stressor is mild or when a stressor results in a mild response. We do not believe that the lack of an effect of RU28318 on the CORT response to restraint was due to a ceiling level of CORT secretion. We (25) have previously shown that pretreatment of rats with a combined MR and GR antagonist before restraint leads to an even greater CORT response than that present in vehicle or MR antagonist alone treated rats in *experiment 3*.

We also attempted to determine whether MR responsible for this effect are centrally located (*experiment 4*). icv treatment with RU28318 elevated CORT responding to novel environment stress, suggesting that MR in the central nervous system may be crucial for CORT negative feedback during this stressor. Previous work indicates that MR is nearly absent from the PVN. MR binding is almost undetectable in the hypothalamus (27), and MR immunoreactivity or MR mRNA is not found in the PVN (1, 10, 19). On the other hand, MR is very abundant in the hippocampus, and this may be the critical site of RU28318 action. The effects of MR antagonism in *experiment 4* might represent disruptions of indirect negative feedback of the HPA axis (glucocorticoid effects exerted on areas of the brain outside the HPA axis), as opposed to interruptions in direct negative feedback (CORT effects exerted on components within the HPA axis). However, recent work in our laboratory indicates that icv administration of steroids may result in diffusion of such compounds to the pituitary. Pituitary GR activation was observed by immunohistochemical methods after icv administration of RU28362, a GR agonist (9). icv injection of RU28318 in *experiment 4* might therefore be antagonizing MR in the pituitary as well as in the hippocampus. This prevents us from concluding absolutely that central MR participate in control of HPA axis activity that occurs as a

result of novel environment challenge. Even if RU28318 is acting primarily at the pituitary in *experiment 4*, such observations still support the notion that MR are important for regulation of the HPA axis in the context of stressor challenge.

It has been shown by a number of studies that MR are important mediators of permissive glucocorticoid effects that are essential for maintenance of basal HPA axis activity. This is the case for regulation of CORT secretion at the circadian trough (2, 8, 11, 21, 25, 31) and the circadian peak (18, 29, 31). Interestingly, permissive regulation of HPA axis activity probably results from an interplay between MR and GR, and this is especially evident at the circadian peak (3, 25). However, MR may also be important for HPA axis regulation in situations other than the normal circadian rhythm. Cole et al. (5) demonstrated that RU28318 treatment shortly beforehand increased the CORT response to restraint, but only in rats that had been challenged with restraint on five previous occasions. Given the relatively mild CORT stress response that develops after chronic restraint challenge (i.e., habituation) compared with the more intense response observed in an animal exposed to restraint for the first time, the effect of MR antagonism in this chronic-stressor paradigm may be partially related to the mild stress response to chronic, repeated restraint. Normally, suppressive glucocorticoid effects may be mediated by portions of MR that are unoccupied at the time of stressor challenge, especially if that stimulus is mild. MR blockade may then disrupt the normal CORT regulation of HPA axis output during mild stressors but not during stimuli that have relatively greater intensities. In addition to the work of Cole et al., a more recent study reported that spironolactone treatment 1 h beforehand increases the peak plasma CORT response to restraint (17). Normal recovery of the CORT response to a stressor also requires the suppressive effects of glucocorticoids, and blockade of MR with spironolactone (17) and RU28318 (Pace TW and Spencer RL, unpublished observations, and Ref. 21) has been found to disrupt normal termination of the CORT response after a stressor has ended.

Interestingly, in several studies that report an effect of MR antagonists on HPA axis stress function (peak responding or recovery), the CORT stress responses of vehicle controls during and after those challenges is rather mild (5, 21). This contrasts with other studies, in which the CORT response to a stressor was more intense ($>26 \mu\text{g}/100 \text{ ml}$ plasma, as is



usually observed in restraint) and does not change as a result of MR blockade. Instead, antagonism of both MR and GR is required to disrupt negative feedback during such a stressor challenge (25). This suggests that, in situations of more intense stimuli, both MR and GR are needed to mediate the normal suppressive effects of glucocorticoids on HPA axis function. When a stimulus is weaker and the CORT response to that stimulus is low, the importance of MR in the negative feedback process is greater (Fig. 5).

Although this model of corticosteroid receptor function and suppressive glucocorticoid effects is supported by a number of studies, it does fail to explain the observations of others. Weidenfeld and Feldman (30) saw no effect of MR antagonism after they treated rats with ~ 300 ng of RU28318 centrally 1 h before photic stress. The lack of an effect with an MR antagonist but an increase in the CORT stress response with a GR antagonist is taken as support for the notion that MR are important for permissive (basal) CORT effects but not for suppressive (reactive) effects. What may account for the disagreement between these results and the studies presented in this paper? In *experiment 4* (Fig. 4), RU28318 is also administered via icv guide cannula, but at a much higher dose than used by Weidenfeld and Feldman (30). Although the kinetics of RU28318 have been described through systemic administration routes (unpublished document Roussel Uclaf), the metabolism and clearance of the compound has never been thoroughly investigated after injection into the brain. It may be essential that all MR are blocked throughout the stressor exposure, and to accomplish this a certain minimum dose is required when the compound is administered directly into the brain. In the case of our experiments that administer RU28318 centrally, this is the main reason why we chose to use a higher dose and why we delivered it over the course of 2 h. In effect,

we attempted to saturate the brain with the compound for the duration of the stressor challenge. Of course, it is also possible that MR located in the pituitary must be disrupted before hyperresponding of the HPA axis response to a stressor is observed. As indicated above, significant antagonism of MR in *experiment 4* may be taking place in the pituitary, as steroids administered into the brain ventricles have been previously observed to occupy GR in the pituitary (9).

The duration of time between central injection of RU28318 and stressor challenge may also be important when a suppressive role for MR is being investigated. Ratka et al. (21) also investigated the effects of MR blockade on responding to novel environment. Although they observed a significant effect of RU28318 long after the initial exposure to the stressor, they failed to observe a change in the peak CORT response with icv RU28318 treatment. Rats were injected with RU28318 only 15 min before novel-environment stress. Although their data are supportive of a stress recovery role for MR, the rather short amount of time between injection and novel environment may have negated their ability to observe an effect of MR antagonism on peak stress levels of CORT. In the current study icv injection is spread over a 2-h period, and this procedure may be significant to the changes in the CORT response to novel environment as a result of RU28318 treatment.

Identifying a regulatory role of MR during phasic HPA axis activity becomes even more appealing when one considers the types of stressors humans are subjected to on a daily basis. The low-intensity stressor of novel environment might be more like the low-level acute stressors that humans encounter routinely. Instead of producing large cortisol responses, most of the situations we encounter throughout the day produce only minor rises in plasma cortisol (28). The negative feedback control of stress cortisol secretion might be largely MR mediated in humans, especially in everyday situations. Interestingly, a study has found that the hippocampus in rhesus monkeys is abundant in MR but relatively devoid of GR (23). Inasmuch as this pattern of MR and GR expression may be representative of other primates, including humans, it may amplify the relative significance of MR dynamic regulation of the HPA axis.

Although much work still needs to be done to develop a more complete understanding of the role that MR plays in HPA axis control, we believe that this report highlights new considerations. The most important contribution of the current study is the demonstration that RU28318 increases peak CORT responding to a mild stressor, but not to a more moderate-intensity stressor. Armed with this notion, the effects of MR antagonism should be further investigated in studies utilizing not only novel environment, but other stressors of low and high intensity. Stressors of the same relative intensity but involving different sensory modalities should also be compared. In addition, the impact of MR blockade on control of the HPA axis response to stress is likely to be greater at the circadian trough instead of at the circadian peak, and future studies should investigate the effects of MR antagonism during mild stress in the morning compared with the evening.

In conclusion, we provide evidence to suggest that MR may be important not only for mediating permissive glucocorticoid regulation of the HPA axis, but also for suppressive regulation as well. After verifying the selectivity of RU28318 to antagonize MR, and after identifying a relatively weak stressor challenge according to HPA axis hormonal responses, we show

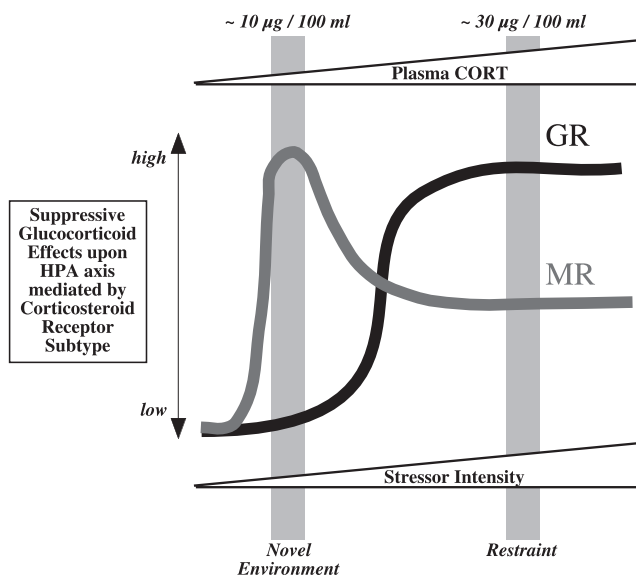


Fig. 5. A theory about stressor intensity and suppressive glucocorticoid effects mediated by corticosteroid receptors. MR may play an important role to mediate suppressive glucocorticoid effects during stressor challenge, especially when intensity of that stressor is mild. An exclusive role for MR is more likely in situations when intensity of the stressor is relatively weak or when the phasic CORT stress response is of lesser magnitude. In situations of higher-intensity stressor exposure or when the magnitude of hypothalamic-pituitary-adrenal (HPA) axis response is greater, MR likely plays a facilitatory role for GR.

that MR antagonism (both systemically and centrally) leads to CORT response dysregulation during the mild stimulus of novel environment. We use this evidence to suggest that MR may have an important role to mediate suppressive negative feedback during mild stressor events.

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