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Mineralocorticoid and Glucocorticoid Receptor Antagonists in Animal Models of Anxiety

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KORTE, S. M., G. A. H. KORTE-BOUWS, G. F. KOOB, E. R. DE KLOET AND B. BOHUS. *Mineralocorticoid and glucocorticoid receptor antagonists in animal models of anxiety.* PHARMACOL BIOCHEM BEHAV **54**(1) 261–267, 1996. – The behavioral effects of intracerebroventricular (ICV) administration of a specific mineralocorticoid receptor (MR) antagonist [RU28318 (10–50 ng/2 μl)], a glucocorticoid receptor (GR) antagonist [RU38486 (1–50 ng/2 μl)], or both antagonists (50 ng/2 μl), were studied in two different animal models of fear and anxiety in rats. In the defensive burying paradigm simultaneous blockade of MR and GR increased immobility behavior, whereas a small decrease in defensive burying was seen. In the fear-potentiated startle test concurrent MR and GR blockade led to an increase in fear-potentiated startle at the highest loudness level (105 dB). In both tests the antagonists were not effective when given separately. The findings are discussed in terms of the involvement of GR and MR in neural mechanisms of fear and anxiety.

Mineralocorticoid receptor Glucocorticoid receptor Defensive burying Fear-potentiated startle Fear Anxiety

CORTICOSTEROIDS are of crucial importance for the regulation of adaptive behavior (8). Circulating corticosteroids enter the brain, where they bind to intracellular mineralocorticoid and glucocorticoid receptors (MR and GR). The MR and GR are both localized in high densities in, for example, amygdala, septum, and hippocampus (3,12,16,27,41,47). These brain structures are involved in emotional behavior, learning and memory (1,4,26,45).

The development of a selective mineralocorticoid antagonist (RU28318), and a glucocorticoid antagonist (RU38486) allowed to define receptor mediated behavioral effects in tests directed to novelty, learning, memory, and learned helplessness (7,18,30–32,33,35). Recently, we have observed that the GR antagonist given intracerebroventricularly (ICV) has anxiolytic-like effects on fear-enhanced behavior in the elevated plus-maze (22). In the evaluation of these findings it is of relevance that MR binds corticosterone with much higher affinity than GR. Accordingly, low circulating corticosteroid levels predominantly occupy MRs, whereas after stress and during the circadian peak both MR and GR are activated (42).

Animal models of fear and anxiety reflect different neural substrates (4,19,37). In the present study, two different animal models of fear and anxiety were used: namely, the defensive burying paradigm and the fear-potentiated startle test. Briefly, during exposure to the defensive burying paradigm, rats use, depending on the environment, an active behavioral strategy, namely, the pushing of bedding material towards and over the aversive stimulus, or a passive behavioral strategy, i.e., immobility behavior to avoid the stressor (6,10,20,38). The central amygdala and septum play an important role in the expression of immobility and burying (37,43). The acoustic startle response is a reflex contraction of the skeletal musculature in response to an intense acoustic stimulus, and can be increased when the reflex is elicited in the presence of a cue previously paired with shock (i.e., fear-potentiated startle) (2,4,14,17). The brain-circuit involved in fear-potentiated startle includes the central amygdala and the nucleus reticularis pontis caudalis (4). The limbic structures involved in the above-mentioned behaviors are known as loci of corticosteroid receptors, particularly GRs (8). The role of central MRs and GRs in fear and anxiety was investigated by administering their specific antagonists ICV to selectively block the function

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of each corticosteroid receptor type or both, using defensive burying and fear-potentiated startle as an index for fear and anxiety.

METHOD

Animals

Male Wistar rats (n = 46) (originally derived from Cpb, TNO, Zeist), weighing 300–340 g, were used for the defensive burying experiment. They were housed individually in clear Plexiglas cages (25 × 25 × 30 cm) on a 12 L : 12 D cycle (lights on between 0700–1900 h). All animals had free access to standard rat chow and tapwater. The experiments were carried out between 1000–1400 h. The fear-potentiated startle experiment was performed in the Scripps Research Institute, La Jolla, CA. In this experiment male Wistar rats (n = 39) (Charles River) were also used and housed in opaque cages (47 × 26 × 21 cm; l × w × h) on a 12 L : 12 D cycle (lights on between 0600–1800 h) group housed (two in a cage) on arrival. The experiments were carried out between 1900–2400 h.

Surgery

The rats were secured in a stereotaxic frame for implantation of unilateral intracerebroventricular (ICV) cannulae, under halothane anesthesia. The guide cannulae were positioned 1.0 mm above the right lateral ventricle with the tooth bar set at +5.0 mm at AP 0.6 mm and L ± 2.0 from bregma and DV −3.2 mm from point of entry (15,34). The rats were allowed at least 1 week for postsurgery recovery.

Treatment

The steroid receptor antagonists were injected ICV at a dose of 10 and/or 50 ng/2 μl/rat 30 min before the exposure of the animals to the test situation. Similar drug treatment has shown to produce behavioral effects in animal models of learning and anxiety (22,30). The glucocorticoid antagonist (anti-GR; RU38486; 17β-hydroxy-11β-(4-dimethylamino-phenyl)17α-(1-propynyl)estra-4,9-diene-3-one) (13,39) and the mineralocorticoid antagonist (anti-MR; RU28318; 3,3-oxo-7-propyl-17-hydroxy-androstan-4-en-17yl-propionic acid-lactone) (36) were dissolved in ethanol and diluted in 0.9% NaCl solution to the required concentration. The final concentration of ethanol was 2%. The vehicle control contained the same ethanol concentration. Each animal received only one ICV injection. The observations were made by trained observers who were blind to the treatment order. According to a study of De Boer et al. (5), the plasma corticosterone peak is expected at 1730 h in the animals tested in the defensive burying paradigm and at 1630 h in the animals tested in the fear-potentiated startle. Testing in both test situations was randomized across time to allow for changing endogenous corticosterone levels.

Statistics

Comparisons between behavioral data of vehicle treated stressed and control animals were made by Student's t-tests. One-way analysis of variance (ANOVA) was performed on the raw data of vehicle and drug treated stressed animals. The Dunnett's post hoc t-test was used to compare the drug treated stressed animals to the vehicle treated stressed group. A probability level of $p \leq 0.05$ was taken as significant.

DEFENSIVE BURYING PARADIGM

Procedure

The shock-probe defensive burying test was performed in the animals' home cage. The floor was covered with wood shavings (height 2 cm). A removable Teflon probe (6.5 cm long, 1 cm in diameter) was positioned 2 cm above the bedding through a small hole in the center of the longest wall of the cage for 10 min. Two exposed wires (0.5 mm in diameter) were each wrapped (25 times) independently around the probe. Whenever the animal touched both wires simultaneously with some part of its body an electric current of 1.5 mA was delivered to the animal (23). This shock intensity was obtained by adjusting a variable resistor in series with a 1000 V shock source. During the entire 10-min period the shock circuit was left on, i.e., repeated shock probe procedure (44). Control animals received no shock. The following day the animals were reexposed to the now unelectrified probe for 5 min. During this period the duration of defensive burying and immobility behavior were measured. Defensive burying was defined as moving toward the probe and spraying or pushing the bedding material toward the probe with rapid movements of the snout or forepaws (38). Immobility behavior was considered if the animal was completely motionless while the body weight was supported by its limbs. The stress-induced immobility is characterized by the display of a freezing posture and alertness, which is indicated by the scanning behavior (i.e., head movements from side to side) The drug or vehicle was administered 30 min before the reexposure of the animal to the nonelectrified probe.

FEAR-POTENTIATED STARTLE

Procedure

Startle testing was performed in two SR-LAB (San Diego Instruments, San Diego, CA) test stations. The startle stimuli consisted of a broad band noise with a rise of 1 ms and a falltime of 1 ms, and were produced by a loudspeaker (Radio Shack Supertweeter) mounted 24 cm above the animals. Each ventilated chamber contained a stabilimeter of a 8.2 cm diameter Plexiglas chamber resting on a platform. A piezoelectric accelerometer beneath the platform detected movements of the animal, which were digitized (0–4095), rectified, and recorded by the computer as 100 ms readings starting at the onset of each dependent measure. The mean startle amplitude during this period was measured.

Prior to testing, animals received 10 noise stimuli of 90, 95, and 105 dB in order to assign each rat into matched groups having equivalent startle levels at these noise intensities.

Fear conditioning was performed in a separate apparatus (96 × 42 × 96 cm) with background white noise (55 dB, A scale). Within the apparatus were four smaller cages (22 × 20 × 18 cm) on two shelves. The floor of these cages consisted of 2 mm stainless steel bars (a total of 20) spaced 1 cm apart. The unconditioned stimulus was shock generated by four constant-current (Coulbourn) shockers located outside the larger box. The conditioned stimulus was produced by 15 W light bulb located on the top of each cage. A total of 20 light footshock pairings were given on 2 consecutive days. A 3.700 ms light was paired with a 500 ms, 0.6 mA shock presented 3.200 ms after the light onset (4). The control group received the same amount of foot shocks, but these were not paired with light. Fear-potentiated startle testing was performed in

the SR-LAB stations. The conditioned stimulus was produced by a 15 W light bulb identical to that used in the shock cages.

Thirty minutes after the infusion of corticosteroid antagonist into the ventricle, animals were placed in the startle test cages followed by a 5-min acclimation period with 55 dB (A scale) background noise, whereafter the animals received 10 95-dB noise bursts. After these 10 stimuli, each animal received 20 noise bursts at each of the three intensities 90, 95, and 105 dB. Half of the stimuli at each of these three intensities were presented in darkness (noise-alone trial type) and half were presented 3200 ms after the onset of a 3700 ms light (light-noise trial type). All startle stimuli were presented at a 30-s interstimulus interval. The 10 occurences of each of the six trial types were presented in a balanced, irregular order across the test session. Every 10 noise stimuli were followed by a background noise (no stimulation). Mean startle amplitude in the presence of the light (light and noise), the absence of the light (noise alone), and the difference between the two, i.e., fear-potentiated startle, were measured.

RESULTS

Behavioral Effects of RU38486 and RU28318 in the Defensive Burying Paradigm

Figure 1A shows defensive burying and Fig. 1B shows the immobility behavior during reexposure to the nonelectrified probe. The defensive burying behavior was increased in stressed animals compared to control animals ($p = 0.0001$). The overall drug treatment effect almost reached the level of significance, $F(5, 34) = 2.358$, $p = 0.0611$. Simultaneous treatment with both MR and GR antagonist (both 50 ng) did not significantly reduce time spent on defensive burying behavior. Neither MR antagonist nor single GR antagonist treatment alone had any effect on burying behavior.

Furthermore, vehicle-treated stressed animals showed a slight but nonsignificant increase in immobility behavior compared to vehicle-treated control animals during reexposure to the nonelectrified probe. In the stressed animals the ANOVA revealed a significant treatment effect, $F(5, 34) = 3.397$, $p = 0.0135$. This can be ascribed to a substantial increase in time spent on immobility behavior in the RU28318 + RU38486 (both 50 ng)-treated group ($p < 0.05$).

Effects of RU38486 and RU28318 in the Startle Paradigm

Figure 2 shows no effects of RU38486, RU28318, or simultaneous administration on noise alone startle during presentation of 90, 95, and 105 dB.

Figure 3 shows the delta mean startle amplitude of control and stressed animals. A transformation of the data was used to facilitate the presentation. Vehicle control (i.e., no fear conditioning) animals reacted with a lower startle amplitude to the light-noise trial type compared to the noise-alone trial type. This means that the delta mean startle amplitude of the vehicle control group is a negative value (-6 for 90 dB, -34 for 95 dB, and -141 for 105 dB). To facilitate the presentation, the delta mean startle amplitude of all groups was adjusted by adding $+6$ to the 90 dB data, $+34$ to the 95 dB data, and $+141$ to 105 dB data.

Figure 3 shows no effect on fear potentiation at 90 and 95 dB. At 105 dB, however, the vehicle stressed animals showed significant higher fear-potentiation compared to vehicle-treated controls ($p = 0.0066$). ANOVA of these data (105 dB) of fear-potentiated startle of the stressed animals revealed a significant drug treatment effect, $F(3, 26) = 4.679$, $p = 0.0096$, due to a further increase in fear potentiation in the RU38486 + RU28318 (both 50 ng)-treated animals ($p < 0.05$).

DISCUSSION

The present study shows in two paradigms for fear and anxiety, effects of ICV administration of the MR and GR antagonist, when administered in combination.

In the defensive burying paradigm combined blockade of MR and GR resulted in an enhanced immobility response, without a significant change in defensive burying. Decreases in defensive burying behavior evoked by the classical benzodiazepines (6,44) or novel 5-HT_{1A} receptor agonists (20,46) are often interpreted as anxiolytic actions. Although a moderate suppression of defensive burying was found, the major effect seemed to be a shift from an active (burying) to a passive behavioral strategy (immobility) after combined MR and GR blockade. Both burying and immobility can be seen as different behavioral strategies to face a threat (21). However, the neuroendocrine consequences of these strategies differ substantially. Burying behavior is accompanied by neurosympathetic activation as reflected by increased plasma norepinephrine levels and tachycardia (6,9,20). In contrast, immobility behavior is accompanied by a relatively increased activation of the adrenocortical, adrenomedullary, and neurosympathetic system as reflected by pronounced elevations of plasma corticosterone, and epinephrine, in addition to norepinephrine (6,20). Therefore, the passive (immobility) strategy is often regarded as a more emotional state, which is apparently promoted by simultaneous blockade of MR and GR.

Combined blockade of MR and GR led to a further increased fear-potentiated startle response, without changing the noise alone startle. Drugs considered to be anxiogenic, for example, yohimbine and DMCM, also produce a further increased fear-potentiated startle response (2,4,14,17). Therefore, the increased fear-potentiated startle response after combined MR and GR blockade is regarded as an increase in anxiety levels, which is confirmed by the finding that defecation was increased after combined MR and GR blockade in rats (Douma et al., unpublished results) but not after separate drug treatment. The enhanced fear-potentiated startle response, however, also can be interpreted as a shift to a more passive behavioral strategy. It was observed that the amplitude of the fear-potentiated startle response was directly related to the percentage of freezing/immobility that preceded the response (24). It would be of interest to compare the results after combined blockade of MR and GR with data of adrenalectomized (ADX) rats. In these rats the lack of circulating corticosterone obviously eliminates the receptor-mediated effect. We have no data on performance of ADX rats in the defensive burying paradigm or fear-potentiated startle test. Removal of the adrenals appeared, however, anxiogenic in the social interaction test. Conversely, ADX rats that had been given replacement corticosterone therapy did not differ from the sham-operated controls (11).

Corticosterone action may be associated with individual differences in the search for strongly activating situations (sensation seeking). Rats with a high preference for novelty when given the choice between a familiar and novel environment show a higher predisposition to self-administer corticosterone (40). Interestingly, corticosterone in the range of stress-induced levels (binds to both MR and GR) possesses reinforc-

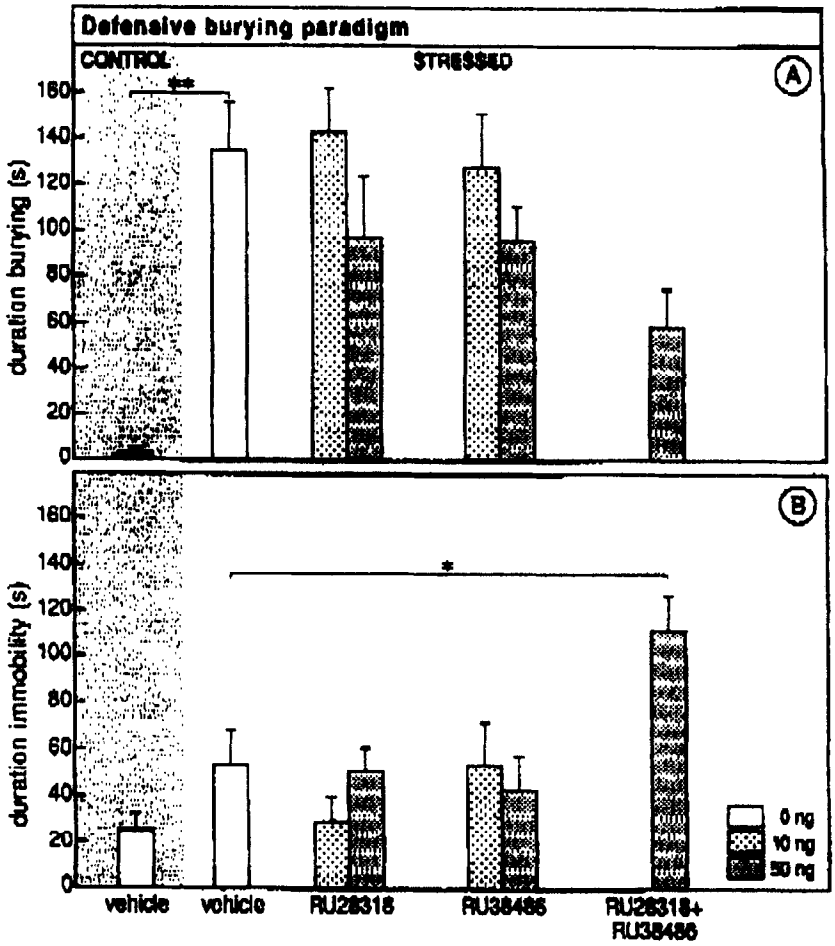

FIG. 1. Time spent (mean ± SEM) on defensive burying behavior (A) and immobility behavior (B) during a 5-min reexposure to a deenergized probe 30 min after ICV-administered vehicle (controls, $n = 6$; stressed, $n = 9$), antimineralocorticoid (RU28318; 10 ng, $n = 6$; 50 ng, $n = 6$), antiglucocorticoid (RU38486; 10 ng, $n = 6$; 50 ng, $n = 7$), or RU28318 + RU38486 (both 50 ng, $n = 6$). $^*p \leq 0.05$; $^{**}p \leq 0.01$, significantly different from control.

ing properties (40). Studies from the same group previously had shown that rats predisposed for amphetamine self-administration had prolonged corticosterone secretion, following exposure to novelty (39). Accordingly, it seems that increased corticosterone coincides with novelty preference, suggesting that such rats are less anxious. Besides the level of plasma corticosterone, the relative amount of MRs and GRs in the limbic system is assumed to play a crucial role in emotional behavior (8). For instance, postnatal handling produces increased hippocampal GR concentrations and attenuates fearfulness in the adult animal (e.g., decreased freezing, increased exploration) (28).

Seemingly in contrast with the above-suggested role of corticosterone we observed in earlier experiments anxiolytic-like effects after separate treatment with the MR antagonist RU28318 on fear-motivated immobility and with the GR antagonist RU38486 on fear-enhanced behavior in the elevated plus-maze (22). In addition, inhibition of the synthesis of corticosterone by metyrapone resulted in a similar anxiolytic-like effect in the elevated plus-maze (Roozendaal et al., submitted). We did not find anxiolytic effects of the corticosteroid antagonists in the defensive burying paradigm or fear-potentiated startle paradigm. It cannot be excluded that the dose-response curve of the corticosteroid antagonists is different for the various animal models of anxiety like defensive burying paradigm, fear-potentiated startle, and the plus-maze. In addition the neurosubstrate for fear enhancement in the plus-maze, burying behavior, and immobility or fear-potentiated startle, however, may be different. Lesions of septum decreased burying behavior and increased open-arm exploration in plus-maze, whereas amygdaloid lesions produced neither of these anxiolytic effects (45). However, lesions of the amygdala blocked fear-potentiated startle (4) and conditioned freezing/immobility (25,43). Thus, we suggest as a possible explanation for the seemingly contradicting results in the different animal models of fear and anxiety that corticosterone either via GR and/or MR modulates the function of discrete brain areas that are involved in the control of different aspects of anxiety and fear. Further studies are needed to investigate a possible U-shaped relation between the behavioral stress re-

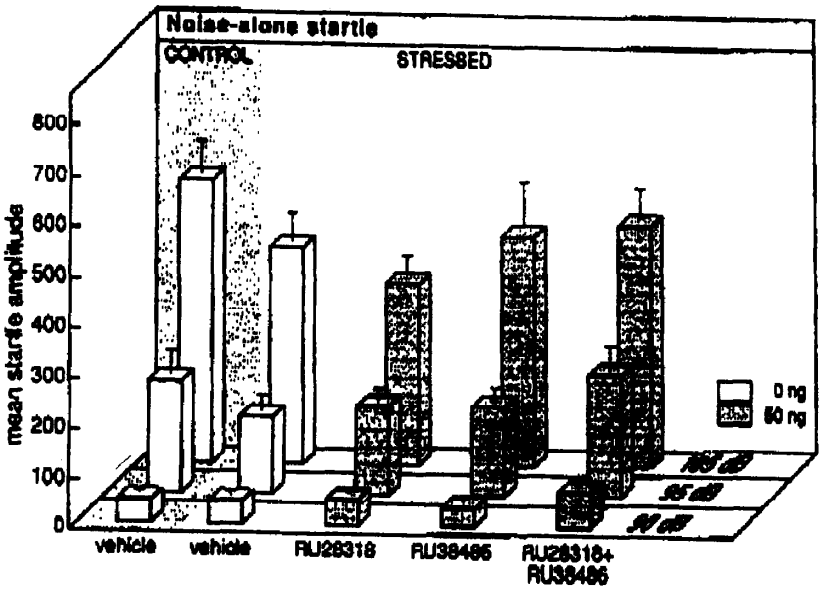

FIG. 2. Amplitude (mean ± SEM) of noise-alone startle at 90, 95, and 105 dB 30 min after ICV-administered vehicle (controls, $n = 9$; stressed, $n = 9$), antimineralocorticoid (RU28318; 50 ng, $n = 7$), antiglucocorticoid (RU38486; 50 ng, $n = 7$), or RU28318 + RU38486 (both 50 ng, $n = 7$). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, significantly different from control.

sponses and the circulating level of corticosterone. This might give further insight into the mechanisms involved in the earlier reported anxiolytic-like effect of the GR antagonist and the anxiogenic-like effect after combined MR and GR blockade.

In summary, simultaneous blockade of MR and GR promoted immobility behavior in the defensive burying paradigm and increased fear-potentiated startle. These results are interpreted as anxiogenic. Conversely, binding of corticosterone to both MRs and GRs may produce a less emotional state.

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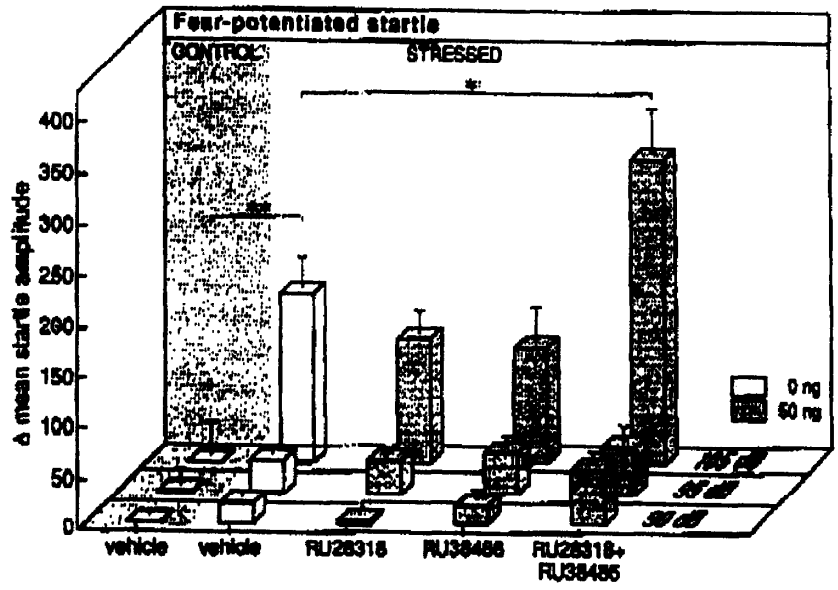

FIG. 3. Fear-potentiated startle (i.e., noise and light startle minus noise-alone startle) at 90, 95, and 105 dB. For further explanations, see Fig. 2.

gifts of the RU38486 and RU28318 (Roussel-UCLAF Pharmaceutical Co., Romainville, France). This study was supported in part by the Netherlands Organization for Scientific Research (NWO, Grant No. 900-551-057).

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