Pharmacology Biochemistry and Behavior, Vol. 56, No. 3, pp. 507–513, 1997

0091-3057/97 $17.00 + .00

PII S0091-3057(96)00244-4

Hippocampal Mineralocorticoid, but Not Glucocorticoid, Receptors Modulate Anxiety-Like Behavior in Rats

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Received 9 February 1996; Accepted 5 July 1996

SMYTHE, J. W., D. MURPHY, C. TIMOTHY AND B. COSTALL. *Hippocampal mineralocorticoid, but not glucocorticoid, receptors modulate anxiety-like behaviour in rats.* PHARMACOL BIOCHEM BEHAV **56**(3) 507–513, 1997.—Stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis is regulated by negative-feedback mechanisms in the form of cytosolic and nuclear steroid receptors, sensitive to levels of circulating corticosterone (CORT). There are two types of steroid binding sites found in the brain: (i) mineralocorticoid receptors (MR); and (ii) glucocorticoid receptors (GR). The hippocampus expresses the highest density of both MR and GR relative to other brain regions, and has long been recognized as a principal component controlling HPA axis inhibition. Because hippocampal cholinergic blockade produced anxiety-like behaviour, and affected HPA axis function, we explored if the induction of anxiety might be attributable to changes in CORT. CORT also produced anxiety, although in a qualitatively unique manner than that produced by cholinergic blockade. In the present study, we have examined if CORT-induced anxiety occurs through an interaction with hippocampal MR or GR. Adult, male Lister Hooded rats were implanted bilaterally with hippocampal cannulae, and received infusions of either the MR antagonist, spironolactone (150 ng), or the GR antagonist, RU38486 (150 ng), either 10 min or 3 h prior to being tested in the Black-White box. MR blockade, 10 min prior to testing, led to a pronounced anxiolytic effect as revealed by the increased amount of time spent in the white compartment, and increased amount of intercompartmental exploration. There was no effect of MR blockade 3 h prior to testing, and GR antagonism produced no effects at either pretreatment time. These data are the first to show that hippocampal MR are directly involved in anxiety; moreover, the time course of the effect demonstrates that a non-genomic mechanism probably underlies this response. Stress may be an important predisposing factor in the development and expression of anxiety.

Anxiety Hippocampus Hypothalamic-pituitary-adrenal axis Corticosterone Mineralocorticoid Glucocorticoid Spironolactone RU38486 Black-White box

ACUTE activation of the hypothalamic-pituitary-adrenal (HPA) axis in rodents can be elicited by various stimuli, including footshock, restraint, blood loss, predator odour, endotoxins and hypothermia (25). The response of the HPA axis is initiated at the level of the paraventricular nucleus (PVN), which serves to integrate and compile afferent neural inputs conveying information about the type and magnitude of the stressor, and signals the appropriate level of pituitary output (29,30,32). Stress-induced PVN cell activity promotes the secretion of corticotrophin-releasing hormone (CRH) into the capillary bed of the median eminence from where it is transported to the anterior pituitary corticotrophs, and causes the release of adrenocorticotrophin (ACTH). ACTH reaches the adrenal cortex and stimulates the synthesis and release of corticosterone (CORT) (2,22). CORT is a highly catabolic steroid, essential for short term adaptation to stress, but toxic and damaging if plasma concentrations are elevated for protracted periods of time (25). As such, the HPA is modulated by negative-feedback systems in the brain and pituitary, sensitive to circulating CORT levels, that act to dampen further CRH and ACTH secretion, thereby inhibiting the stimulatory signal for CORT release (8,22).

There are two classes of CORT sequestering receptors located in the brain and pituitary; both are soluble, cytoplasmic

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proteins that form activated conjugates when bound to CORT and act to alter protein transcription (8,25,31). Whereas these genomic actions have been well documented it is obvious that rapid actions (e.g., membrane based) must also occur in order for there to be quick effects such as fast negative-feedback (19,24). The mineralocorticoid receptor (MR) binds with high affinity to aldosterone and CORT (Kd 0.5 –1.0 nM), while the glucocorticoid receptor (GR) binds with relatively lower affinity (Kd 2.0–5.0 nM). Under basal conditions where plasma CORT concentrations are approximately 2–8 ug/dl, MR are almost totally occupied, while upwards of 70% of GR are unoccupied and available (19,31). Thus stress-induced CORT release is largely regulated by CORT binding to GR since they appear to have dynamic range (although see (8).

Of all the brain regions involved in HPA negative-feedback, perhaps none has been more thoroughly characterized than the hippocampal formation (CA1-4, dentate gyrus, subiculum; popularly truncated to 'hippocampus'). Both MR and GR densities are highest in the hippocampus compared to other brain regions, and a substantial amount of empirical data has demonstrated that stimulation of hippocampal GR has a marked inhibitory effect on stress-induced ACTH and CORT release (25). Moreover, hippocampal lesions result in pronounced hypersecretion of ACTH and CORT in response to acute restraint stress, and hippocampectomized rats often show elevated basal CORT levels (13).

The septo-hippocampal cholinergic (ACh) projections to the hippocampus appear to be involved in HPA negative-feedback, as we have previously demonstrated that intrahippocampal injections of scopolamine potentiate stress-induced ACTH and CORT release (5). This finding was interesting because of the known relationship between hippocampal ACh projections and electrophysiological measures of central arousal; hippocampal theta activity driven by ACh systems, invariably accompanies fearful or stressful encounters (6,7). It has long been known that lesions of the septo-hippocampal regions can produce an anxiolytic effect (16), and that benzodiazepines increase stimulation intensities required for septal driving of theta activity (35,36). We investigated whether or not ACh activity was involved in mediating anxiety-like behaviour (hereafter called 'anxiety') as elicited by the Black-White box, and found that scopolamine administration produced relatively more anxiety than the control treatment (33). Thus, inhibition of ACh neurotransmission increases HPA stress responses and behavioural indices of fear, while blocking arousal-related theta activity. ACh activity may be essential for an animal to assess the degree of threat contained within any environment and initiate feedback control over the response. Given the relationship between ACh function, stressful environments and HPA activity (11,21,25,26,33), we then examined the effects of exogenous CORT administration on Black-White box-induced anxiety. We found that doses of CORT, designed to mimic stress-induced levels, increased anxiety in a time-dependent fashion, with the most efficacious pretreatment time at 5 min (34). This result was somewhat surprising since previous research using the social interaction test (14) and elevated plus maze (1) had shown CORT to be anxiolytic. These differences are difficult to reconcile, but may be due to differential activation of MR versus GR (19).

These data, taken together, show that HPA axis function influences anxiety, and suggest that the hippocampus is both an important regulator of the HPA axis and of anxiety. To date, we do not have definitive, empirical evidence showing that the hippocampus mediates the increase in anxiety produced by either scopolamine or CORT. Moreover, with the characterization of MR and GR, it remains unclear if one or both receptors is uniquely involved. Korte et al. (23) recently reported that intraventricular MR antagonists 'released' conditioned fear-induced immobility, and produced anxiolytic effects in rats tested in the elevated-plus maze following exposure to an aversive conditioned stimulus. However, they report no direct effect of MR or GR antagonists in the absence of pre-exposure to an aversive stimulus, but they do speculate that the hippocampus may be involved in the behaviours they observe. In the present study, we sought to ascertain if specific hippocampal MR and GR are directly involved in mediating anxiety elicited by the Black-White box, an objective made possible by testing rats following intrahippocampal injections of spironolactone (MR antagonist) or RU38486 (GR antagonist). The results reveal that hippocampal MR, but not GR, mediate anxiety and that the response occurs too rapidly to be genomically-mediated.

METHODS

Subjects

Adult, male Lister Hooded rats, weighing 350–450 g at the time of surgery were used in the study. They were obtained from the breeding unit at the Univ. of Bradford and housed at the testing facility when required. Rats were housed in polycarbonate cages with wire tops in groups of 4–5. Food (rat chow) and water were provided ad libitum. Cages were kept in quiet rooms, with ambient temperatures of 20–22°C. Cage maintenance was undertaken twice weekly, but never on the day of testing. The rats were maintained on a normal light cycle (on at 08:00 h. off at 20:00 h. All behavioural testing commenced at 11:00 h and never went beyond 17:00 h. This ensured that testing was done under low basal ACTH and CORT conditions, in order to minimize individual variation in basal HPA activity.

Surgery

Rats were anaesthetized with sodium pentobarbital (60 mg/kg ip) and placed into a stereotaxic frame with the plane between bregma and lambda held horizontally. An incision was made along the midline of the scalp, and the periosteum was retracted. Using bregma as a reference point, holes were drilled bilaterally over the hippocampal regions (A-P -3.5; M-L $\pm$ 2.5 mm). Guide tubes (25 gauge) were lowered into the stratum radiatum (D-V 2.4 mm) and cemented into position with dental acrylic, using 4 jeweller's screws as anchors. The injection stilette (30 gauge) protruded an additional 0.5 mm beyond the end of the guide tube; therefore, the infusion depth was actually aimed at the stratum moleculare (D-V 2.7 mm) of the dentate gyrus. Rats were permitted at least 3 wk to recover from surgery before being subjected to testing.

Black-White Box Apparatus

The Black-White test was similar to that previously employed in our laboratory (10), although built larger to accomodate the increase in size of rats over mice. The overall dimensions of the box were 25 $\times$ 15 $\times$ 15 in (length, width, height). The bottom of the box was dissected by orthogonal lines creating a 5 in grid pattern. The box was further divided into two chambers, the Black (10 $\times$ 15 $\times$ 15 in) and the White (15 $\times$ 15 $\times$ 15 in) by a barrier possessing a doorway through which rats could traverse. The Black compartment was dimly illuminated with weak, red lights, while the White compart-

ment was intensely illuminated by bright, white lights. A video camera, connected to a VHS recorder and monitor was used to record each rats activities in the box. The entire Black-White box was surrounded by a black curtain to minimize any possibility of distraction created by experimenter movement and helped to minimize extraneous sounds.

Testing Procedure

On the day of testing, the rats were brought to the testing room and left for 3 h to acclimatize to the novel surroundings. They had continual access to food and water during this period and remained with their housing companions. Each rat received bilateral infusions of either spironolactone (50 ng/μl) or RU38486 (50 ng/μl) or vehicle (artificial CSF) at either 10 min or 3 h before behavioural assessment. Because of the insolubility of these compounds, a small amount of absolute ethanol was used to first dissolve the antagonists, and the volumes were then brought up with CSF. Total injection volumes were 3 μl. Individual rats were placed into the middle of the white compartment, facing away from the intercompartmental doorway, at the start of the trial and left for 5 min. The group order was counterbalanced according to a latin square design. At the conclusion of the 5 min test period, the rats were returned to their cages, and another animal was placed into the box. In between rats, the box was cleaned out with a 70% alcohol solution. Samples sizes for each group ranged from 9–11.

Behavioural Measures

The videotapes made on the day of testing were scored by an investigator who was blind to the pretreatment regime. Each animal was scored for the following measures: (i) time to exit from the white compartment to the black; (ii) time to re-enter the white compartment from the black; (iii) total time in the white compartment; (iv) activity (squares crossed per unit time) in the white compartment; (iv) activity (squares crossed per unit time) in the black compartment; and (vi) number of crossings between the black and white chambers. In order to control for changes in activity levels due to differences in time distribution (i.e. rats spending more time in the black chamber invariably cross more squares), measures of activity are expressed as squares crossed per 10 sec.

Drugs

Spironolactone was purchased from the Sigma Chemical Co. (UK), while RU38486 was supplied by Prof. M. J. Meaney, McGill Univ., Canada.

Histology

Following testing, each rat was transcardially perfused with 10% formalin and had their brains removed and stored in formalin + 20% sucrose. After 2 wk, brains were sectioned at 60 mm to confirm guide cannulae placements.

Statistical Analysis

All dependent measures were assessed using univariate analsyes of variance (ANOVA). Each drug was analysed independently from the other. Post hoc tests were performed using student's t-test, applying a Bonferroni correction procedure to maintain the pairwise comparison alpha level at .05 and minimize Type 1 errors (9).

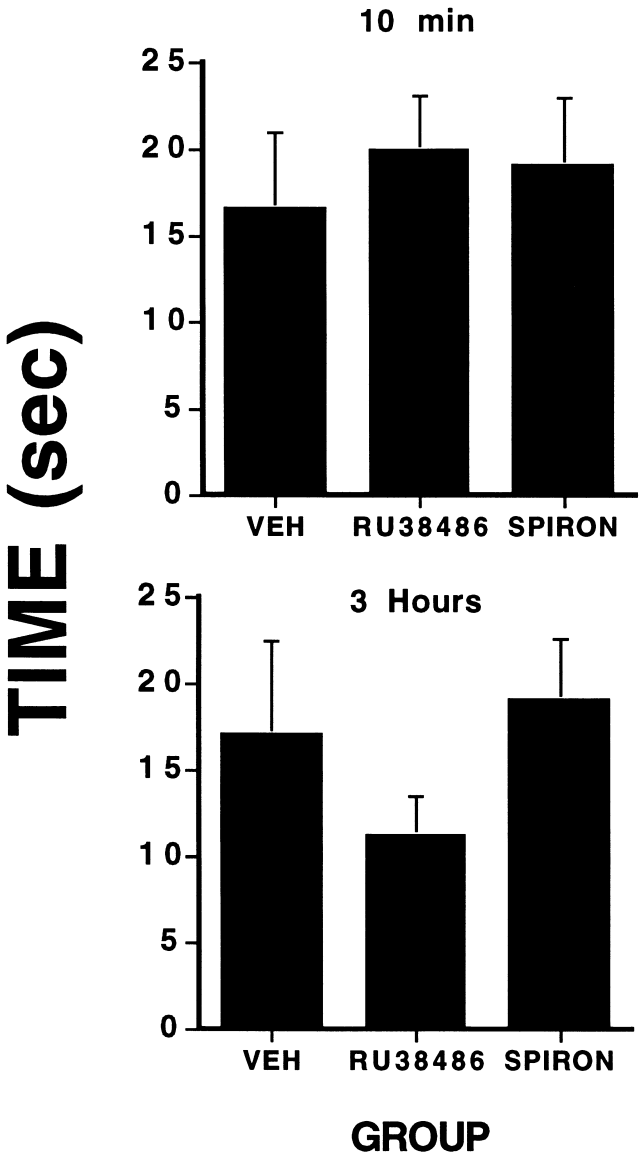

FIG. 1. Latencies to exit from the white chamber to the black chamber following initial placement in the black-white box. There was no effect of drug at either pretreatment time, as all rats generally exited the white chamber at approximately the same time. Values shown are means ± SEM.

RESULTS

Histology

Histological assessment of all rat brains confirmed that the guide cannulae were located in the dorsal hippocampal region. Infusion sites ranged from 2.7 mm to 3.3 mm in depth. No animal had to be excluded on the basis of poor placements.

Time to Enter Black Compartment

ANOVA revealed no significant effects at either the 10 min pretreatment time point, $F(2, 28) = 0.19$, ns, or the 3 h time point, $F(2, 25) = 1.32$, ns, on latency to exit the white compartment. These data are illustrated in Fig. 1.

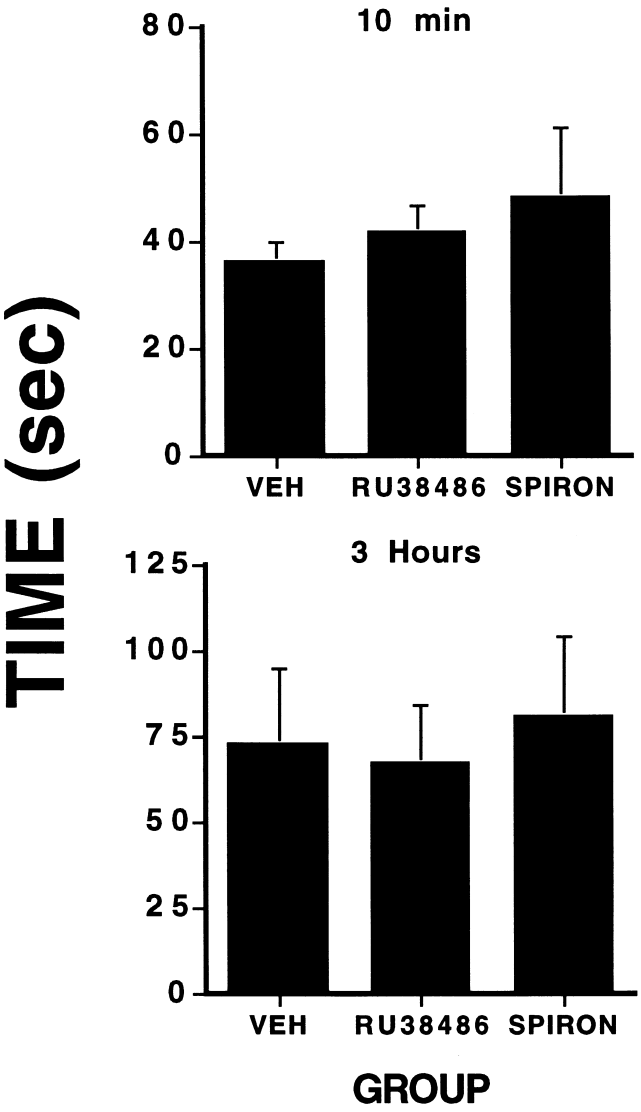

FIG. 2. Mean latencies before rats returned to explore the white chamber following their initial entry into the black compartment. Most animals returned to explore the white compartment within a short span of time, and there were no group differences evident amongst these data. There was also no effect of pretreatment time. Values shown are means ± SEM.

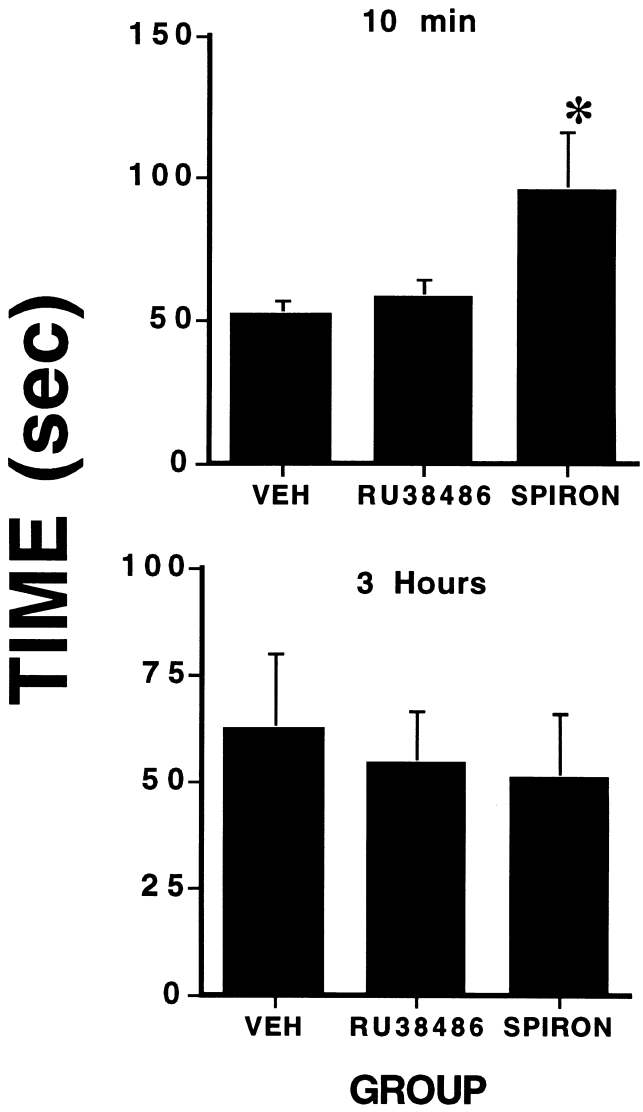

FIG. 3. Amount of time spent in the white chamber for the 300 sec test trial. Spironolactone-treated rats spent almost 100% more time in the white compartment compared to either the VEH or RU38486-treated animals, but only following the 10 min pretreatment time. By 3 h post-injection this effect no longer occurred. Values shown are means ± SEM. *Significantly different from VEH and RU38486 groups ($p < .05$).

Time to Re-enter White Compartment

There was no significant effect of pretreatment at 10 min, $F(2, 28) = 0.51$, ns, nor was there any effect at 3 h $F(2, 25) = 0.11$, ns. These data are depicted in Fig. 2.

Time Spent in White Compartment

At the 10 min pretreatment time, ANOVA revealed a significant effect of drug $F(2, 28) = 3.51$, $p < .04$. Post hoc comparisons showed that sprironolactone-treated rats spent longer in the white compartment than did the VEH or RU38486-treated rats (p's $< .05$). The magnitude of the increase was almost 100% relative to the VEH group. RU38486 treated rats were not significantly different from the VEH control group. At the 3 h time point, there was no effect of drug pretreatment $F(2, 25) = 0.13$, ns. These data are presented in Fig. 3.

Number of Intercompartmental Crossings

There was a significant effect of drug pretreatment at the 10 min time point with $F(2, 28) = 5.44$, $p < .01$. As shown in Fig. 4, sprironolactone-treated rats exhibited greater intercompartmental exploration compared to both the VEH ($p < .01$) and RU38486 ($p < .005$) groups. Interestingly, ANOVA on the data from the 3 h pretreatment also showed a significant effect of drug pretreatment $F(2, 25) = 3.73$, $p < .04$. Post hoc comparisons here, however, revealed that spironolactone

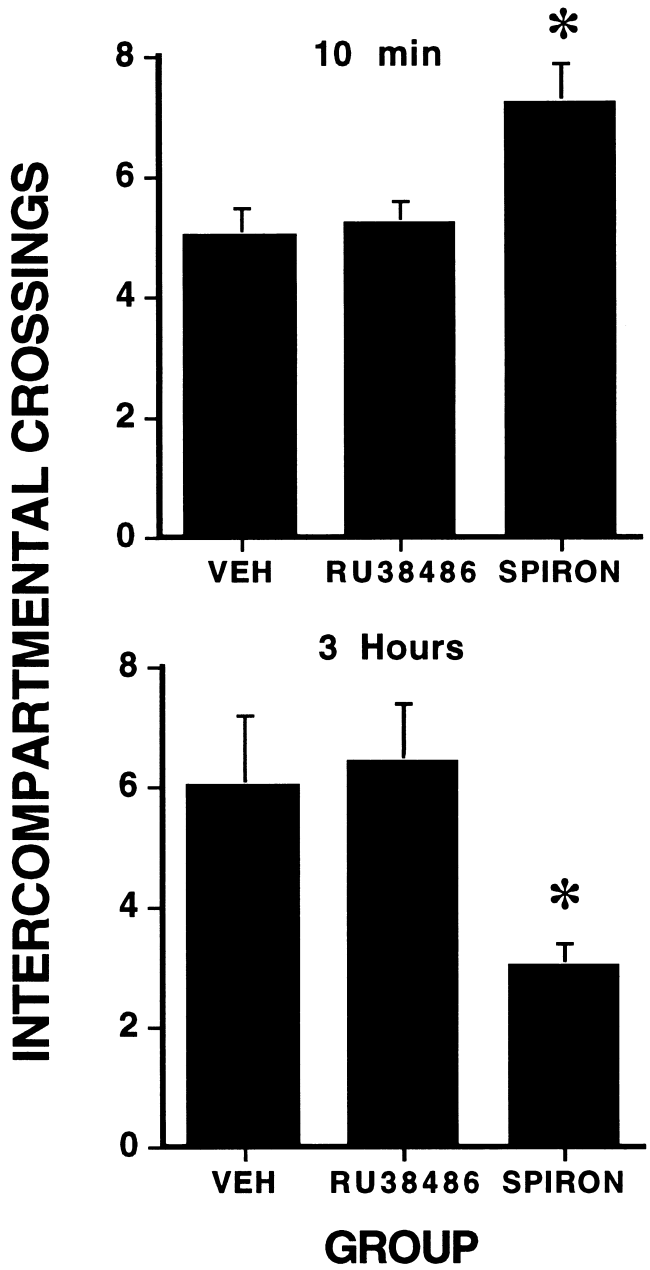

FIG. 4. Total number of intercompartmental crossings occurring in the 300 sec test trial. At the 10 min pretreatment time, spironolactone significantly increased intercompartmental exploration compared to the other 2 groups. However, at 3 h post-spironolactone administration, rats exhibited significantly less crossing behaviour. Values shown are means ± SEM. *Significantly different from VEH and RU38486 groups ($p < .05$).

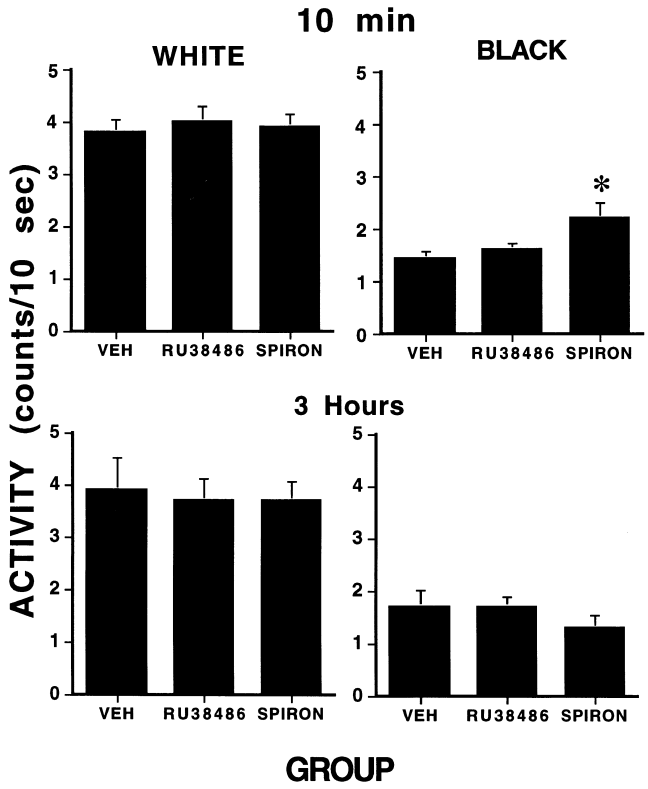

FIG. 5. Activity scores for all groups in each of the black and white compartments. Measures are expressed as activity counts per 10 sec time period in order to determine rate of activity and correct for the unequal time distribution between the differently coloured chambers. Means ± SEM are depicted. *Significantly different from VEH and RU38486 groups ($p < .05$). Hippocampal mineralocorticoid receptors modulate anxiety.

administration decreased intercompartmental exploration relative to the VEH ($p < .04$) and RU38486 ($p < .02$) treated groups. These data are shown in Fig. 4.

Locomotor Activity

ANOVA on the 10 min pretreatment data showed no effect of drug on motor activity in the white compartment $F(2, 28) = 0.05$, ns. However, ANOVA did reveal a significant effect of drug on activity in the black chamber with $F(2, 28) = 4.51$, $p < .02$. Post hoc comparisons showed that spironolactone-treated rats exhibited significantly greater motor activity compared to the VEH ($p < .01$) and RU38486 ($p < .04$) treated groups. Separate ANOVAs on the locomotor data from the 3 h pretreatment revealed no drug effects in either the white chamber $F(2, 25) = 0.06$, ns, nor in the black chamber $F(2, 25) = 0.61$, ns. Data for all locomotor activity are depicted in Fig. 5.

DISCUSSION

The results of the present investigation implicate hippocampal MR in the modulation of anxiety as measured by the Black-White box test, and demonstrate that MR involvement reflects a non-genomic site of action rather than any effect on mRNA induction/inhibition. Intrahippocampal infusions of spironolactone had an anxiolytic effect as evidenced by a number of behavioural changes; MR antagonsim produced increased intercompartmental transfers, and increased total time spent in the white compartment. These behaviours are indicative of an anxiolytic action, that is, these animals exhibit less anxiety (10,33). This anxiolytic effect of MR blockade was only manifested at 10 min following drug infusion; infusions made 3 h prior to behavioural testing were largely ineffective, although intercompartmental crossing behaviour was

significantly lower in spironolactone-treated rats. Whether this reversal reflects an axiogenic response is unknown, and perhaps is unlikely given the absence of effect on other behavioural measures. It is intriguing to consider that MR blockade may initially elicit a period of reduced anxiety, followed by a period of rebound, heightened anxiety at a later time point. In contrast to the effect of MR antagonism, the GR antagonist RU38486 was completely inert and did not affect any behavioural measure at either time point. Our previous data showing that CORT elicits anxiety in the Black-White box (34), seen in the context of the present study, support the contention that CORT-induced anxiogenesis is most likely mediated by MR. By blocking these receptors, we observe 'released' behaviour and reduced anxiety. These results are in general agreement with those of Korte et al.(23) who reported that MR blockade effectively produced an anxiolytic effect in stressed rats tested in the elevated plus maze. One important difference between their study and the present one is that Korte et al. did not observe a direct anxiolytic effect of MR blockade, while our effect occurs in the absence of a prior stressor. The source of this difference is difficult to explain although logic would dictate that the differences in anxiety tasks might be an important factor.

It is important to note that there were no obvious motor disturbances that could account for the present data. While spironolactone infusions 10 min prior to testing significantly enhanced locomotor activity in the Black chamber, there was no effect of this agent on activity in the White compartment. Moreover, if the enhanced activity observed following MR blockade was a factor in the amount of intercompartmental crossings, it is surprising that other variables such as time to enter the black chamber, and time to re-enter the white chamber would not be similarly affected. Other researchers have shown that MR stimulation enhances exploration of novel items in an open field (28); certainly the Black-White box presents as a novel environment, but here we observe that MR blockade leads to greater exploration. Presumably, this reflects that the novelty response to the Black-White box represents only a fraction of the potential behaviours that can be induced in rats placed therein. Anxiety may be elicited in addition to the response to novelty, and these two behaviours may compete with each other. Thus in an open field, MR stimulation elicits more exploration, while in the Black-White box, MR antagonism elicits more exploration.

Another key finding in the present work is that specific hippocampal infusions of spironolactone produced an anxiolytic response. This supports previous data on the role of the hippocampus in anxiety and arousal (16,21,33), and suggests that HPA-hippocampal interactions are important regulators of anxiety. This finding can be extended to mean that stress may modulate anxiety, or may even be a major precipitating factor. The involvement of the hippocampus in anxiety is also supported by electrophysiological data obtained from this structure. Field activity recordings from the stratum moleculare of the dentate gyrus have revealed a cholinergically-mediated waveform (Type 2 theta) that is only elicited by arousing or stressful stimuli (6). It has been argued that the septo-hippocampal cholinergic projections are components of a forebrain arousal/attention system and that Type 2 theta represents sensory processing of threatening/fear-evoking stimuli (7,11,16). Thus alterations of hippocampal ACh activity should affect arousal and thus influence anxiety. This was the subject of our previous research (33). It should be noted that in the present study, spironolactone infusions were aimed at the stratum moleculare, the same dendritic layer that serves to generate theta activity. Whether this means that MR are involved in the regulation of hippocampal theta activity is unknown, although adrenalectomy does alter aspects of theta generation (3). The specific involvement of the hippocampus in the reduction of anxiety following spironolactone, seen in the Black-White box, may also be a factor in why we observe a direct effect of MR blockade, while Korte et al. (23) only see an indirect response. Their infusions were made intraventricularly and unilaterally, while ours were performed bilaterally and into the hippocampus. While MR are located principally in the septo-hippocampal corridor (31), it is possible that Korte et al.'s effects are affected by some incongruity between septal and hippocampal actions of MR blockade, a limitation circumvented in our study. It would be worthwhile to examine the effects of direct septal MR blockade to see if the anxiolytic response is qualitatively altered.

The rapid anxiolytic response to spironolactone cannot be reasonably accounted for by an action at the genome, i.e. that MR blockade prevents a rapid genomic response elicited by CORT. To date there is no empirical evidence that CORT binds membrane receptors in mammalian brain, but it has been logically proffered that rapid steroid effects can occur that do not require changes in protein transcription (19,24). The rapid anxiogenesis induced by CORT (34) is blocked by spironolactone (this study) but the mechanism via which this is achieved remains elusive. Extracellular recordings made from hippocampal neurons show that rapid depolarizations can occur in response to CORT iontophoresis, but intracellular records have failed to reveal any changes in ion conductances or membrane resistance occurring until 30-40 min post-CORT exposure (reviewed in (18). Thus rapid effects of CORT are most likely due to an enabling or permissive effect on ongoing neurotransmission signalled by other transmitter systems (19).

There are other behavioural effects mediated through MR-GR mechanisms, including learning and memory (27), Primed Burst (PB) Potentiation (4,12,), neuronal excitability (18,20) and reactivity to novelty (28). In these studies there is usually some degree of cooperativity evident between MR and GR. For instance, stimulation of MR enhances PB potentiation, while stimulation of GR inhibits PB potentiation (12). Thus we might have expected to see different MR-GR effects on anxiety, however only MR appear to be involved. It is possible that chronic stress effects might be required before GR involvement becomes evident. However, it is interesting to speculate that acute stress effects, reliant principally on GR, and acute anxiety reliant on MR, reflect that anxiety is a modified form of stress response (perhaps a minimal stressor). Cooperativity between MR and GR may be represented by a continuum in which anxiety (MR-mediated) is at one end, and stress (GR-mediated) is at the opposite end.

In summary, intrahippocampal MR blockade produced an anxiolytic response that was evident at 10 min but not 3 h post-infusion, while GR blockade was without effect at either time point. These data support and extend previous work showing that the hippocampus is an important regulator of stress, arousal and anxiety; furthermore, they suggest that stress may be a predisposing influence on the expression of anxiety. The rapid effect of MR antagonism probably precludes a genomic site of action for this anxiolytic response, and it is likely that MR blockade interferes with some CORT-mediated effect on ongoing neurotransmitter action. Given previous data on hippocampal ACh systems and HPA negative-feedback, the role of ACh in anxiety, and the ACh induction of arousal-related theta activity, it is very probable that

MR interact with ACh projections to the hippocampus. This suggestion has received some empirical support (15,17). The further characterization of such an interaction would provide useful insights into the relationship between stress and anxiety, and how hippocampal activity may separately regulate these behaviors.

ACKNOWLEDGEMENTS

This research was supported by the Costall-Naylor research fund, and an establishment grant from the Univ. of Bradford to JWS. RU38486 was generously donated by Prof. M. J. Meaney, Dept. Psychiatry, McGill Univ., Montreal, Canada. The authors thank Susan J. McLaughlin for editorial assistance with the manuscript.

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