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Pharmacology Biochemistry and Behavior, Vol. 54, No. 1, pp. 249–253, 1996
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0091-3057/96 $15.00 + .00

SSDI 0091-3057(95)02177-9

Neuroendocrine Effects of Diazepam and Flesinoxan in the Stress-Induced Hyperthermia Test in Mice

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GROENINK, L., J. VAN DER GUGTEN, T. J. J. ZETHOF, J. A. M. VAN DER HEYDEN AND B. OLIVIER. *Neuroendocrine effects of diazepam and flesinoxan in the stress-induced hyperthermia test in mice.* PHARMACOL BIOCHEM BEHAV **54**(1) 249–253, 1996. – In the stress-induced hyperthermia (SIH) paradigm in mice, both a benzodiazepine receptor agonist, diazepam, and a 5-HT_{1A} receptor agonist, flesinoxan, reduced the stress-induced increase in rectal temperature. The SIH procedure itself enhanced plasma ACTH and corticosterone levels but not plasma glucose levels. Diazepam (3, 6, and 12 mg/kg PO) did neither affect basal plasma ACTH, corticosterone, or glucose levels, nor did it suppress the stress-induced rises in these parameters. Flesinoxan (1, 3, and 10 mg/kg PO) enhanced plasma ACTH and corticosterone concentrations under nonstress conditions but did not affect the stress-induced increases in ACTH and corticosterone secretion. No clear effects of flesinoxan on plasma glucose levels were found. Our results indicate that in mice the anxiolytic effects of diazepam and flesinoxan in the SIH paradigm are not paralleled by a blockade of stress-induced increases in plasma ACTH, corticosterone, and glucose levels.

Flesinoxan Diazepam ACTH Corticosterone Glucose Hyperthermia Anxiety Stress Mouse

THE STRESS-INDUCED hyperthermia (SIH) paradigm in mice is based on the phenomenon that psychological stress results in an acute increase in body temperature in several species (4,24). When group-housed mice are sequentially removed from their home cage with a 1-min interval, the rectal temperature of the mice removed last is higher than the temperature of the mice removed first. This phenomenon is called stress-induced hyperthermia (16,31). SIH can be blocked by benzodiazepines, viz. diazepam, alprazolam, and chlordiazepoxide, by 5-HT_{1A} receptor agonists, viz. flesinoxan, buspirone, and 8-OH-DPAT, and by specific 5-HT reuptake inhibitors such as fluvoxamine and fluoxetine. Antidepressants, neuroleptics, antipyretics, and muscle relaxants are not active in this model (15,31,32). We showed that SIH is accompanied by increases in plasma adrenocorticotropic hormone (ACTH), corticosterone, and glucose levels (11). The first two effects reflect activation of the hypothalamic-pituitary-adrenal (HPA) axis, whereas plasma glucose enhancement may well reflect activation of the sympathoadrenal medullary system (27,28).

In the present study we assessed whether the increases in plasma ACTH, corticosterone and glucose levels induced by the SIH-procedure in mice can be antagonized by anxiolytics that also antagonize SIH. We studied the effects of the benzodiazepine receptor agonist, diazepam, and of the selective 5-HT_{1A} receptor agonist, flesinoxan.

METHOD

Male NMRI-mice (Charles River, Sulzfeld, Germany), weighing 12–14 g on arrival at the laboratory, were housed in groups of 10 animals per cage (dimensions: 34 × 22 × 15 cm) for at least 1 week prior to testing. The animals were housed under nonreversed 12 L : 12 D cycle conditions (lights on from 0700 to 1900 h). The room temperature was kept at 21 ± 2°C and relative humidity at 60 ± 10%. The mice had free access to water and food, except during the experiments when no food was available. The experiments were carried out between 0800 and 1215 h.

Two separate experiments were performed. In the first experiment the effects of diazepam (3, 6, or 12 mg/kg PO) were

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measured, and in the second experiment the effects of flesinoxan (1, 3, or 10 mg/kg PO) were studied. The design of both experiments was identical. Diazepam (Brunschwig Chemie B.V., Amsterdam, The Netherlands) and flesinoxan-hydrochloride (synthetized by Solvay Duphar B.V., The Netherlands) were suspended in tragacanth (1%).

An experiment started with injecting the mice ($t = -60$ min). Drugs or vehicle were given orally by an intragastric gavage in a volume of 10 ml/kg body weight. All animals in a cage received the same treatment and were injected within 2 min. For each treatment eight cages were used. One hour after the injection mouse 1 was decapitated ($t = 0$ min) and its blood collected. Mice 2 to 9 were subsequently used for temperature measurement using a 1-min interval in which each mouse was measured sequentially according to Zethof et al. (30). The temperature of mouse 10 was not measured, but the animal was decapitated ($t = 10$ min) and its blood collected. The temperature of mouse 6 was remeasured 30 min after the first temperature measurement, and the temperature of mouse 8 was remeasured 60 min after the first temperature measurement. The measurements at $t = 0$ and $t = 1$ min will be indicated as basal, and the measurements at $t = 8$ and $t = 9$ min are indicated as end.

Mice were decapitated using scissors. Blood was collected in ice-cooled Eppendorf cups containing 0.21 M EDTA (50 μl/ml blood). Plasma was separated by centrifugation (3000 rpm for 10 min at 4°C) and stored at −80°C until assayed. Plasma ACTH concentrations were measured in duplicate without prior extraction using a commercially available RIA (Diagnostic Products Corporation, Los Angeles, CA). Corticosterone was extracted with dichloromethane from 25 μl plasma samples. Corticosterone plasma concentrations were measured in duplicate using a radioimmunoassay (RIA) with an antiserum raised against corticosterone-21-hemisuccinate bovine serum albumin (30). Plasma glucose concentrations were measured in duplicate using a commercially available hexokinase UV test (Hoffman-LaRoche Ltd., Diagnostica, Mijdrecht, The Netherlands).

Each cage was considered as a separate, experimental unit. Therefore, measurements of mice 1 (basal) and 10 (end) out the same cage (plasma levels) were regarded as repeated measurements in the statistical analyses of plasma levels. In the analyses of temperature effects, measurements of mice 2, 9, 6, and 8 out of one cage were regarded as repeated measurements.

To determine the effect of the SIH procedure on temperature, plasma ACTH, corticosterone, and glucose levels, the values obtained in the control group were evaluated separately, using a one-way analysis of variance (ANOVA) with time as a repeated measure within factor (two levels for hormonal parameters, basal and end; four levels, 0, 10, 30, and 60 min for temperature).

The effects of diazepam and flesinoxan under basal nonstress conditions were determined using the measures obtained at $t = 0$ min (basal), which is 60 min after injection. A one-way ANOVA with dose as between factor (four levels; 0, 3, 6, and 12 mg/kg for diazepam and 0, 1, 3, and 10 mg/kg for flesinoxan) was used to analyze these data.

The response patterns of rectal temperature, plasma ACTH, corticosterone, and glucose levels to diazepam or flesinoxan in the SIH paradigm were tested using a one-way ANOVA with time as a repeated measure within factor (two levels, basal and end). Posthoc comparisons were made using the paired Student's t-test. The level of significance was $p < 0.05$ for all tests.

RESULTS

Diazepam Experiment (Fig. 1A–C)

In vehicle-treated mice the normal SIH was found. The end temperature was significantly enhanced as compared to the basal temperature ($p < 0.001$). Subsequently, the rectal temperature declined and was back to baseline values 1 h after starting the SIH measurements (data not shown). The end plasma ACTH levels were significantly enhanced as compared to the basal plasma ACTH levels ($p < 0.05$). Plasma corticosterone levels were also significantly enhanced following the SIH measurement ($p < 0.005$). The vehicle-treated group showed no change in plasma glucose levels between basal and end measurements ($p = 0.82$; Table 1).

Basal Effects of Diazepam

The highest dose of diazepam significantly reduced the basal temperature compared to that of vehicle-treated animals ($p < 0.05$). None of the doses of diazepam affected the plasma ACTH levels, as measured 60 min postinjection, $F(3, 30) = 0.17$, $p = 0.91$. Diazepam dose-dependently enhanced plasma corticosterone concentrations 60 min after injection, $F(3, 31) = 2.71$, $p = 0.06$, although not significantly. Plasma glucose levels were not altered 1 h after diazepam injection, $F(3, 31) = 1.45$, $p = 0.25$.

Effects of Diazepam Following Stress

The SIH found in vehicle-treated mice was blocked in mice treated with 6 or 12 mg/kg diazepam (both $p < 0.05$), but not in mice treated with 3 mg/kg diazepam.

Repeated measurement ANOVA revealed a significant time effect on plasma ACTH, $F(1, 27) = 12.2$, $p < 0.005$, but no effect of diazepam treatment, $F(3, 27) = 1.86$, $p = 0.16$, nor an interaction, $F(3, 27) = 1.79$, $p = 0.17$, was found. This indicates that diazepam does not affect stress-induced ACTH secretion.

Diazepam treatment had no effect on plasma corticosterone levels per se, $F(3, 28) = 1.74$, $p = 0.18$. However, a significant time effect, $F(1, 28) = 10.2$, $p < 0.005$, and treatment × time interaction, $F(3, 28) = 94.4$, $p < 0.025$, was found. Figure 1C shows that with increasing doses of diazepam the differences between basal and end plasma corticosterone concentrations disappears.

The effect of diazepam treatment on plasma glucose concentrations, $F(3, 28) = 5.87$, $p < 0.005$, was similar in nonstressed (basal) and stressed (end) mice, as no time effect or a treatment × time interaction was found [resp. $F(3, 28) = 3.68$, $p = 0.15$; $F(3, 28) = 1.09$, $p = 0.38$].

Flesinoxan Experiment (Fig. 2A–C)

Vehicle-treated mice showed the expected hyperthermia ($p < 0.0001$). The body temperature was back to baseline values at $t = 60$ min (data not shown).

Following the SIH procedure, plasma ACTH levels were enhanced in the vehicle-treated control group, although not significantly at a two-tailed level ($p = 0.10$). The plasma corticosterone levels also increased following the SIH procedure ($p < 0.025$), whereas end plasma glucose levels were significantly reduced as compared to the basal plasma glucose levels ($p < 0.05$; Table 1).

Basal Effects of Flesinoxan

The highest dose of flesinoxan (10 mg/kg PO) significantly reduced the basal temperature ($p < 0.05$).

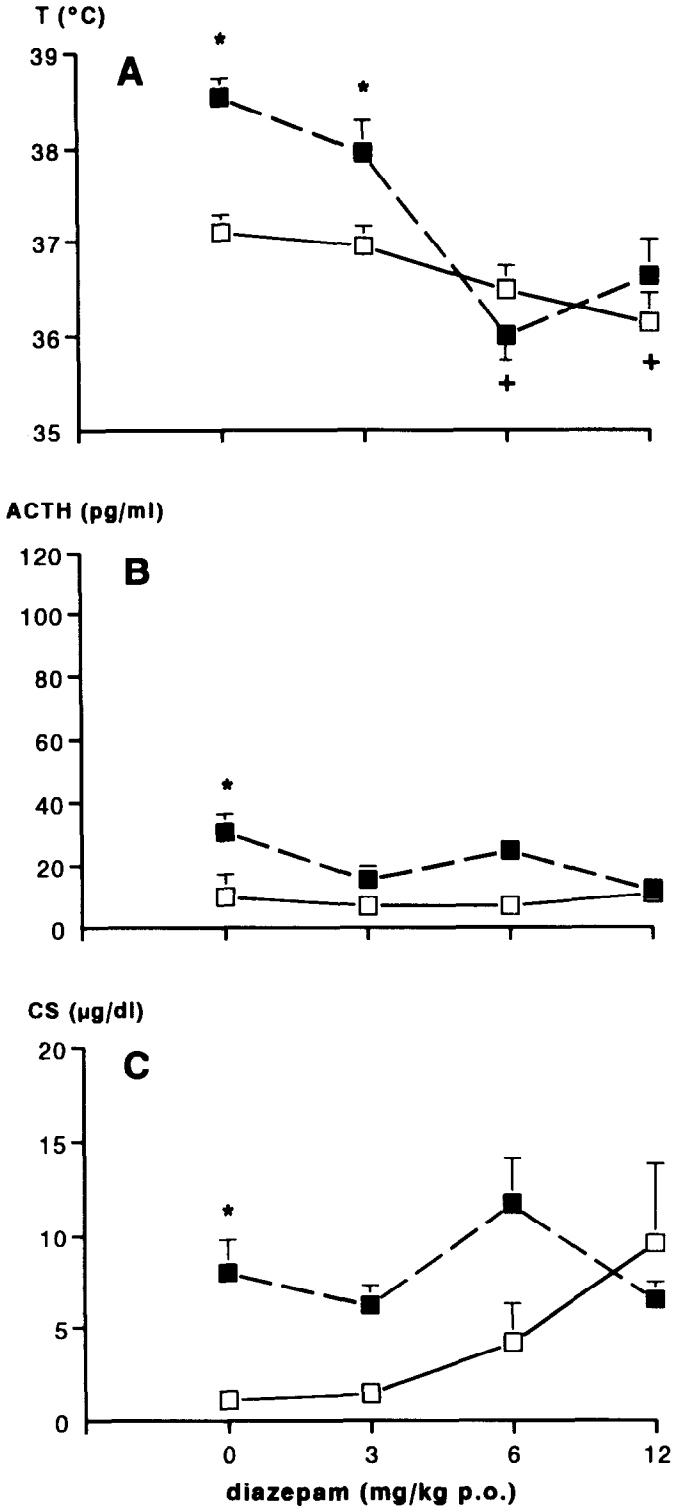

FIG. 1. The effects of diazepam on (A) rectal temperature (T), (B) plasma ACTH and (C) plasma corticosterone (CS) levels under basal nonstress conditions (open squares) and following the SIH-procedure (filled squares). Data are given as mean (±SEM). *$p < 0.05$ as compared to corresponding nonstressed mice, $^{+}p < 0.05$ as compared to corresponding vehicle mice.

TABLE 1

PLASMA GLUCOSE LEVELS 1 h AFTER DRUG TREATMENT (BASAL) AND FOLLOWING THE SIH PROCEDURE (END)

	Plasma Glucose Levels (mmol/l)	
	Basal	End
Diazepam		
Vehicle	12.3 ± 0.76	12.1 ± 0.43
3 mg/kg PO	12.7 ± 0.35	13.2 ± 0.56
6 mg/kg PO	12.9 ± 0.35	14.9 ± 0.73^{+}
12 mg/kg PO	13.9 ± 0.68	14.4 ± 0.40^{+}
Flesinoxan		
Vehicle	11.6 ± 0.35	10.9 ± 0.42*
1 mg/kg PO	10.1 ± 0.30^{+}	10.9 ± 0.27
3 mg/kg PO	11.0 ± 0.30	11.4 ± 0.33
10 mg/kg PO	11.0 ± 0.42	11.7 ± 0.49

Results are expressed as mean ± SEM.
*$p < 0.05$ as compared to corresponding $t = 0$ min (basal).
$^{+}p < 0.05$ as compared to corresponding vehicle.

One hour after flesinoxan administration plasma ACTH, $F(3, 30) = 6.23, p < 0.005$, and plasma corticosterone levels, $F(3, 31) = 15.5, p < 0.001$, were significantly enhanced. Plasma ACTH levels were only enhanced after the highest dose of flesinoxan, whereas plasma corticosterone concentrations were increased in all flesinoxan-treated mice. Plasma glucose levels in the 1 mg/kg flesinoxan-treated mice were significantly lower than the levels in vehicle-treated mice ($p < 0.05$).

Effects of Flesinoxan Following Stress

All doses of flesinoxan significantly reduced the stress-induced hyperthermia as compared to the temperature of stressed vehicle-treated mice ($p < 0.05$). However, compared to nonstressed mice, the temperature of stressed mice treated with 10 mg/kg flesinoxan was significantly increased.

Flesinoxan treatment affected the plasma ACTH levels, $F(3, 26) = 5.49, p < 0.005$, but did not suppress the stress-induced rises in plasma ACTH concentrations, as still a time effect was found, $F(1, 26) = 9.8, p < 0.005$. No interaction was found between flesinoxan treatment and time, $F(3, 26) = 2.46, p = 0.09$.

Flesinoxan treatment enhanced the plasma corticosterone levels both in stressed and nonstressed mice, $F(3, 28) = 25.7$, $p < 0.001$. The plasma corticosterone levels of stressed mice did not differ from those of nonstressed mice, as no time effect was found, $F(1, 28) = 0.37, p = 0.55$, nor a treatment × time interaction, $F(3, 28) = 1.68, p = 0.19$.

Plasma glucose concentrations were not affected by flesinoxan treatment, $F(3, 28) = 1.88, p = 0.15$, in the SIH procedure (see Table 1, $F_{time}(1, 28) = 1.53, p = 0.23$, $F_{treatment \times time}(3, 28) = 1.88, p = 0.16$.

DISCUSSION

The vehicle-injected mice showed increases in body temperature, plasma ACTH, and corticosterone levels following the SIH procedure, as described previously for noninjected mice (11). In the flesinoxan experiment, the basal plasma ACTH levels in vehicle animals are high as compared to those of mice in the diazepam experiment. This high level may account for

the absence of a two-tailed significant SIH-induced effect on plasma ACTH levels.

The increase in plasma glucose levels induced by the SIH procedure as found in a previous study (11) could not be replicated in the present study. However, the stressor-decapitation interval (10 min) may have influenced the results, as a previous study showed that maximal glucose responses are reached 30 min after starting the SIH procedure (11). The effects of diazepam and flesinoxan on plasma glucose levels following stress are rather complex, which may also be due to the time point of measurement. Therefore, the results on plasma glucose levels will not be discussed extensively.

Under basal nonstress conditions diazepam reduced the body temperature but had no significant effects on plasma ACTH, corticosterone, and glucose levels. Although the plasma corticosterone levels of the 12 mg/kg diazepam-treated group were markedly enhanced, this tendency was not reflected in the plasma ACTH levels, but this may be due to the injection-test interval. Plasma ACTH levels are probably back to baseline values 1 h after the injection. Nevertheless, our results indicate that basal effects of diazepam on the activity of the HPA axis and on plasma glucose levels in mice are not as robust as in rats (21,23).

Diazepam reduced SIH, as reported previously (3,30). Diazepam did not reduce the stress-induced elevations in plasma ACTH. Moreover, diazepam did not seem to suppress the SIH-induced corticosterone secretion either. Although the differences in plasma corticosterone concentration between nonstressed and stressed mice disappeared with higher doses, this is not due to a decrease of the corticosterone levels in stressed mice, but rather to an increase in plasma corticosterone levels in nonstressed mice.

The inability of diazepam to suppress the stress-induced HPA axis activation is remarkable, as in rats benzodiazepines do suppress stress-induced increases in plasma ACTH and corticosterone concentrations [for review, see (7)]. This lack of effect cannot be attributed to the dose range used, as the higher doses of diazepam (6 and 12 mg/kg PO) effectively reduced SIH. Our results may, therefore, indicate a difference in benzodiazepine receptor involvement in stress regulation between mice and rats.

Flesinoxan enhanced plasma ACTH and corticosterone levels under nonstress conditions. Similar effects in mice have been described for 8-OH-DPAT, another 5-HT_{1A} receptor agonist (19,22). These results are in agreement with effects of 5-HT_{1A} receptor agonists in rats (1,10). No clear effects of flesinoxan on basal plasma glucose levels were found, whereas flesinoxan has been reported to enhance plasma glucose levels in rats (10).

Flesinoxan blocked SIH, as described previously (30). This anxiolytic effect was not paralleled by a reduction of the stress-induced rises in plasma ACTH levels. The plasma corticosterone concentrations of flesinoxan-treated stressed mice did not differ from those of nonstresssed mice. This might indicate that flesinoxan blocks the stress-induced HPA axis activity. However, it is more likely that the corticosterone-enhancing effects of flesinoxan mask the stress-induced corticosterone enhancement.

Although flesinoxan has no clear suppressant effects on stress-induced rises in plasma ACTH and corticosterone, the effects of flesinoxan and stress on the HPA axis activity are certainly not additive. In rats, additive effects of stress and intrinsic effects of 5-HT_{1A} receptor agonists on HPA axis activity have been reported (6,10,14,18), although suppressant effects at lower doses have also been suggested (28). This may

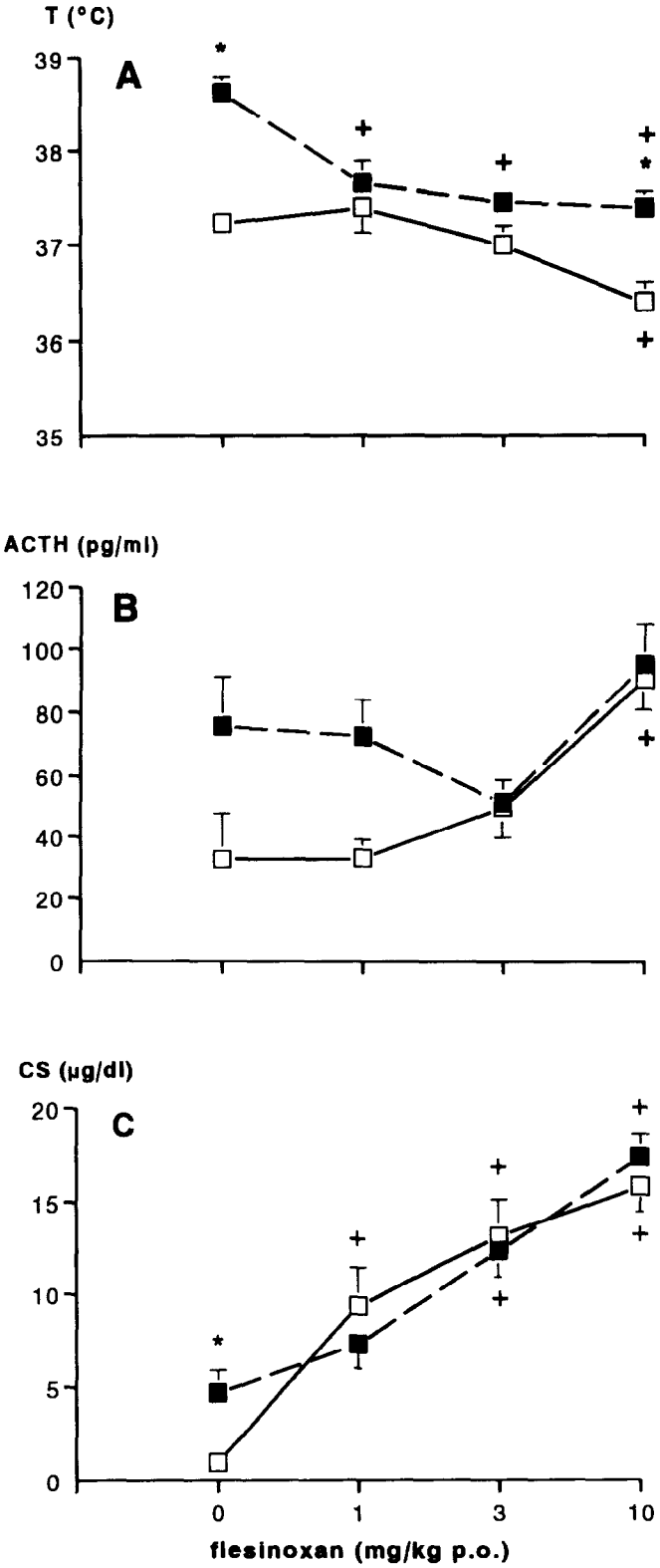

FIG. 2. The effects of flesinoxan on (A) rectal temperature (T), (B) plasma ACTH, and (C) plasma corticosterone (CS) levels under basal nonstress conditions (open squares) and following the SIH-procedure (filled squares). Data are given as mean (±SEM). *$p < 0.05$ as compared to corresponding nonstressed mice, $^{+}p < 0.05$ as compared to corresponding vehicle mice.

suggest a difference in HPA axis regulation between rats and mice. PCPA studies in rats support the idea of postsynaptic 5-HT_{1A} receptor involvement in ACTH and corticosterone secretion (9). In mice, the involvement of presynaptic 5-HT_{1A} receptors in corticosterone secretion has been suggested (19). A difference in 5-HT regulation of HPA-axis activation would not be that surprising, as for example, thermoregulation in rats and mice seems also differently regulated. In mice, hypothermic effects of 5-HT_{1A} receptor agonists are mediated presynaptically, whereas in rats they seem to be mediated postsynaptically (2). Further research is needed to examine whether the 5-HT involvement in HPA-axis activation really differs between rats and mice. Chronic treatment with either diazepam or flesinoxan could be an interesting approach for further research, as both in the diazepam and the flesinoxan experiment, the effects on basal ACTH and corticosterone levels may have confounded the effects following stress. Chronic drug treatment may reduce these basal effects.

The results of this study also indicate that the HPA axis is not directly involved in mediating the stress-induced hyperthermia in mice. CRF, the first released factor after stimulation of the HPA axis, has been reported to possess thermogenic effects, which are dependent on sympathetic activation of brown adipose tissue (25). As the HPA axis is stimulated during the SIH procedure [this study, (25)], it could be possible that the increase in rectal temperature is a direct effect of HPA axis stimulation. However, this seems not to be the case, as the HPA axis remains activated, whereas the SIH is reduced following diazepam or flesinoxan treatment.

ACKNOWLEDGEMENTS

We thank J. G. van de Kieft, R. van Oorschot, and W. E. van der Wal for their technical support.

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