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BRIEF COMMUNICATION

Decreased Reactivity to Anxiolytics Caused by Early Protein Malnutrition in Rats

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ALMEIDA, S. S., L. M. DE OLIVEIRA AND F. G. GRAEFF. *Decreased reactivity to anxiolytics caused by early protein malnutrition in rats.* PHARMACOL BIOCHEM BEHAV 36(4) 997–1000, 1990.—In order to investigate whether early malnutrition causes lasting changes in the reactivity to anxiolytic drugs, rat dams during lactation (21 days) and pups after weaning until the 49th day of life were fed on 8% casein diet (M rats), while their well-nourished controls received 25% casein (W rats). From day 50 on all animals ate the same balanced diet. Experiments started on the 91st day. Rats deprived for 22 hours drank water containing either 1.8% or 2.7% sodium chloride for 30 min in a test chamber, total intake being measured. Dose-effect curves for diazepam (0.5–5.0 mg/kg, IP), as well as for the nonbenzodiazepine anxiolytics ipsapirone (0.5–5.0 mg/kg), ritanserin (0.05–1.0 mg/kg) and isamoltane (2.5–20.0 mg/kg) were determined in M as well as in W rats. Diazepam and ipsapirone dose-dependently released drinking suppressed by either salt concentration in W rats, but caused little or no effect in M rats. Ritanserin and isamoltane were ineffective in both groups. These and previously reported results show that early protein malnutrition markedly reduces anticonflict effects of anxiolytics, indicating long-lasting impairment of neuronal systems underlying emotional behavior.

Early protein malnutrition Hypertonic saline intake Diazepam Ipsapirone

MALNUTRITION during the period of rapid growth of the central nervous system results in morphological, neurochemical and behavioral changes that persist after a period of nutritional rehabilitation (17). In particular, an increased reactivity toward aversive or stressful situations, generated by the dietary insult has been suggested (11, 14, 21).

Experimental evidence reviewed elsewhere (12) supports the hypothesis that anxiolytic drugs affect brain neural systems commanding aversive behavior. If early protein malnutrition impairs these brain systems, an altered susceptibility to anxiolytic drugs would be a likely consequence. Indeed, experimental evidence obtained so far seems to confirm this prediction. Thus, in two animal models of anxiety, the light-dark transition and the Geller-Seifter punishment tests, the anticonflict effect of the benzodiazepine anxiolytic diazepam was far smaller in animals malnourished at an early age than in matched controls (5). Similarly, in a step-down inhibitory avoidance task a dose of chlordiazepoxide that markedly decreased response latency in well-nourished rats was ineffective in previously malnourished animals (2).

Nevertheless, further experimental evidence is necessary to establish whether the above differences are due to altered emotional reactivity or secondary to other changes brought about by early malnutrition. For instance, pain mechanisms are likely to be affected by early protein restriction (20), and in both the above studies (2,5) painful electrical shock was used. Also, differences in predrug baseline between malnourished and well-nourished animals may have been a confounding factor (2). In addition, it is interesting to know whether the changes in drug reactivity determined by malnutrition extend to recently developed nonbenzodiazepine anxiolytics (10, 15, 18).

For this purpose, a conflict test not involving pain, namely drinking suppressed by hypertonic salt solutions that proved sensitive to both benzodiazepine and nonbenzodiazepine anxiolytics (7–9) was used in the present study. A comparison of drug susceptibility between rats given a low-protein diet from birth until the 49th day of life and matched controls fed with a normal protein diet was made by determining dose-effect curves with the benzodiazepine anxiolytic diazepam, as well as with three nonbenzodiazepine putative anxiolytics: ipsapirone (24), ritanserin (6,15) and isamoltane (18).

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METHOD

Animals

Eighty male Wistar rats from the animal house of the Campus of Ribeirão Preto of the University of São Paulo were used. During the lactation period (21 days), each litter was randomly culled to six male pups on the first day of birth. From the same day on, half the animals were suckled by mothers maintained on 8% casein diet (malnourished, M) and the other half by mothers maintained on 25% casein diet (well-nourished, W). The diets were prepared as described by Barnes *et al.* (3). After weaning, animals were housed individually and received the same diet of the respective foster mother until the 49th day of age. From day 50 on, all rats were fed a balanced lab chow diet. Between days 70 and 91, the animals were tested on an elevated plus-maze under the same drug regimen used in the present experiment (Almeida, de Oliveira and Graeff, in preparation). During the experimental phase, the rats were maintained under 12-hour light–12-hour dark cycle (lights on at 7 a.m.) and the room temperature was kept at 23–25°C. Body weight at the 91st day of age was 372 ± 45 g and 237 ± 24 g for W and M rats, respectively. The difference was statistically significant, $t(78) = 21.90$, $p < 0.001$.

Apparatus

The test chamber was a $24 \times 18 \times 19$ cm stainless steel box, identical to the home cage, except that food was removed and a 50 ml calibrated cylinder was clipped to the frontwall, allowing measurement of fluid intake.

Drugs

The following drugs were used: diazepam (Roche), ipsapirone hydrochloride (Bayer), ritanserin (Janssen) and isamoltane hydrochloride (Ciba-Geigy). Diazepam was suspended in distilled water containing 2% Tween 80. Ritanserin was dissolved in distilled water acidulated with acetic acid to pH 4.0. The remaining drugs were dissolved in 0.9% NaCl solution. All compounds were injected IP in concentrations to give an injection volume of 1 ml/kg.

Procedure

A procedure similar to that described by Cooper and Desa (7) was followed. During the first week, animals were adapted to a 22-hr water-deprivation schedule and to obtaining tap water in daily 30-min sessions in the test chamber. Following the drinking sessions, the animals were returned to their home cages for a further 90-min access to water. In the home cage, food was always available. Along the following week rats were adapted to drinking 1.8% NaCl solution in the test chamber, and in the next three weeks drugs were administered. Each animal was injected with every dose of a single drug, the dose sequence being randomized. Injections were made at intervals of at least 3 days. Ten M and 10 W rats were randomly allocated to each of the following treatments: diazepam (0.5–5 mg/kg), ritanserin (0.05–1 mg/kg), ipsapirone (0.5–5 mg/kg) and isamoltane (2.5–20 mg/kg). One week from the last injection, the whole experiment was repeated using a 2.7% NaCl solution. The interval between drug administration and the test session was 15 min for diazepam and 30 min for the remaining drugs. During this period, the rats were kept in the test chamber devoid of the drinking tube.

Analysis of Results

The data submitted to statistical analysis were the liquid intake during 30 min, measured in the last day of each adaptation period as well as following injections. Split-plot ANOVA and Duncan's Multiple Range Test were used.

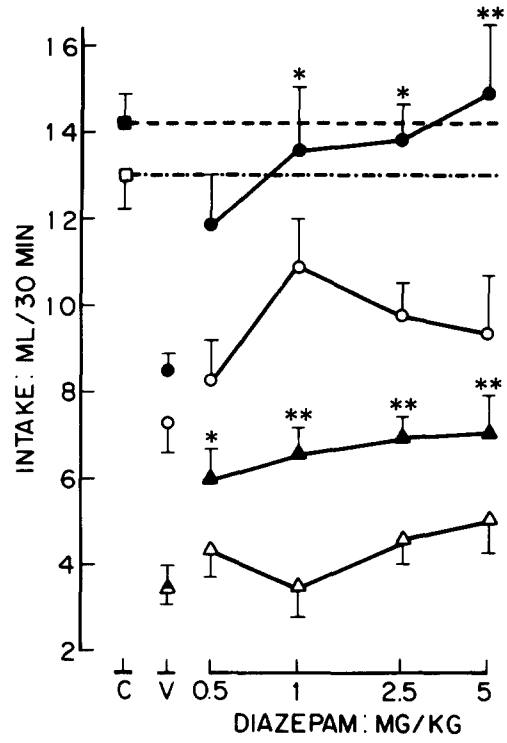

FIG. 1. Effect of diazepam on drinking suppressed by either 1.8% (circles) or 2.7% (triangles) NaCl concentrations in well-nourished (filled symbols) and in previously protein malnourished rats (empty symbols). Points in the figure represent the mean and bars the SEM of 10 rats. C and dashed horizontal lines indicate nonsuppressed water intake. V: vehicle injection. $*p<0.05$, $**p<0.01$ compared to vehicle by the Duncan's test.

RESULTS

In spite of the above difference in body weight, drinking volume of either water or concentrated saline was similar in both M and W rats. In all treatment groups two-factor ANOVA evidenced a significant effect of salt concentration [$F(2,36) = 146.11$, $p<0.001$; $F(2,36) = 139.26$, $p<0.001$; $F(2,36) = 183.38$, $p<0.001$ and $F(2,36) = 198.15$, $p<0.001$ for diazepam, ritanserin, ipsapirone and isamoltane groups, respectively], but no effect of diet nor a significant diet × salt concentration interaction.

Figure 1 shows that diazepam dose-dependently increased saline intake by W rats at both NaCl concentrations used, but was ineffective in M rats. ANOVA evidenced significant effects of drug at both 1.8%, $F(4,72) = 5.98$, $p<0.001$, and 2.7%, $F(4,72) = 6.13$, $p<0.001$, NaCl concentrations as well as significant effects of diet, $F(1,18) = 20.28$, $p<0.001$ and $F(1,18) = 9.45$, $p = 0.006$, respectively. However, drug × diet interactions were nonsignificant.

As shown in Fig. 2, a similar picture was determined by ipsapirone, except for the highest dose of the drug which caused significant increases in saline intake by M rats. ANOVA evidenced significant influences of drug, $F(4,72) = 11.11$, $p<0.001$; $F(4,72) = 13.65$, $p<0.001$, and diet, $F(1,18) = 7.00$, $p = 0.01$;

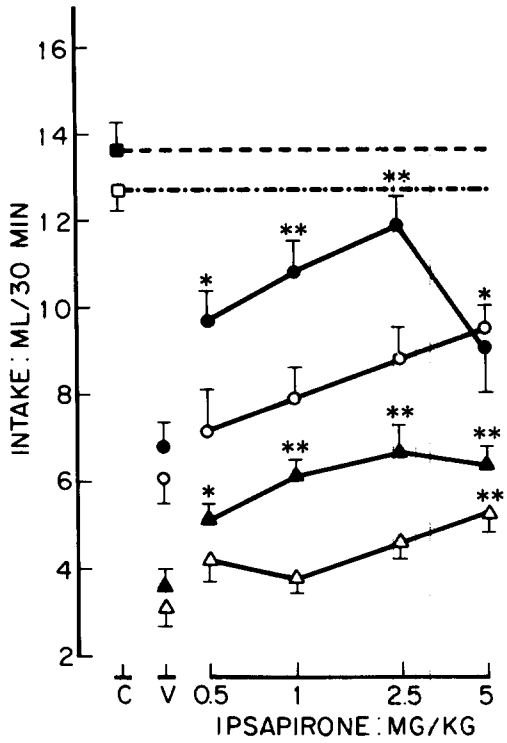

FIG. 2. Effect of ipsapirone on drinking suppressed by high NaCl concentrations. Specifications are in the legend of Fig. 1.

$F(1,18) = 10.56$, $p = 0.004$, in addition to significant drug × diet interactions, $F(4,72) = 3.34$, $p < 0.01$; $F(4,72) = 2.49$, $p = 0.05$.

Ritanserin and isamoltane did not produce any significant changes in drinking for either the M or W rats.

DISCUSSION

The dose-dependent increase in hypertonic saline intake caused by diazepam and ipsapirone in W rats confirms previously reported results showing that drinking suppressed by high concentrations of salt is released by benzodiazepine as well as by nonbenzodiazepine anxiolytics (7–9). In particular, the marked effects of ipsapirone and analogs in this test shown by the present as well as by Cooper and co-workers' results (7,8) are remarkable, considering that most animal models of anxiety are far less sensitive to buspirone-like drugs than to benzodiazepine anxiolytics (10). Whether released drinking reflects the drug anxiolytic action is nevertheless disputable, since a few weeks of repeated administration are needed before buspirone-like drugs significantly improve pathological anxiety (24).

Concerning the influence of early malnutrition on the reactivity of rats to benzodiazepine anxiolytics, the present results confirm and extend recently reported observations made in two different laboratories. In one of these studies, Brioni and Orsingher (5) showed that doses of diazepam which were clearly effective on W rats failed to increase the number of transitions between the lighted and the darkened compartment of a black-white box as well as to release lever-pressing behavior punished by electric foot-shock in a Geller-Seifter conflict test in M rats. In the other study (2), performed in our laboratory, a dose of 5 mg/kg of chlordiazepoxide, IP, significantly shortened the latency of W rats to step down from a wooden platform onto a metal grid floor where they had been given a painful electric shock, but was completely ineffective in M rats.

Nevertheless, in the last experiment it was not clear whether the observed difference was primarily due to a lowered reactivity to the drug, since step-down latencies were much longer in M as compared to W rats under control conditions (2). Furthermore, when these latencies were made similar by increasing shock intensity in W rats, chlordiazepoxide was no longer active. Also, in the aforementioned light-dark transition test, M rats made considerable more transitions than W rats in the nondrugged condition, leaving little room for an anxiolytic drug effect (5). Although these results indicate that predrug baseline differences may be important, this factor could not have influenced either the present results or the Geller-Seifter experiment in Brioni and Orsingher's study (5), since in both cases nonsuppressed as well as punishment-suppressed behavioral baselines of M and W rats were strictly comparable. Therefore, a true hyporeactivity to diazepam is likely to be generated by protein malnutrition early in life.

In addition, the present results show that the lower doses of ipsapirone used did not change saline drinking in M rats, in contrast to the dose-dependent increases in saline intake determined by the same drug in W animals (Fig. 2). Only the highest dose of ipsapirone was effective in the former dietary group. Therefore, M rats apparently became less reactive to ipsapirone, a drug that primarily interferes with 5-HT neurotransmission by directly stimulating 5-HT_{1A} receptors (24). Therefore, the hyposensitivity to benzodiazepines caused by early protein malnutrition is likely to extend to anxiolytics that do not act through the GABA receptor complex.

The dose-response curves drawn from the present results (Figs. 1 and 2) indicate that maximum drug effects are decreased by malnutrition, more clearly in regard to diazepam. This finding points to differences in pharmacological efficacy rather than merely in potency. In addition, an increased reactivity to the hypothermic effect of diazepam in M rats has been reported (4). Therefore, although pharmacokinetic factors cannot be entirely ruled out, malnutrition seems to affect neural mechanisms involved in anxiolytic drug action.

As to the neurochemical changes induced by early malnutrition that may underlie hyporeactivity to anxiolytic drugs, there is reported evidence for both altered GABA- (1, 19, 23, 25) and 5-HT- (13, 16, 21, 22) mediated neurotransmission. However, precise correlations between known neurochemical changes and pharmacological response cannot be established yet. Because 5-HT neurons regulated by GABA have been implicated in benzodiazepine anxiolytic action (10,24), the most parsimonious hypothesis would be that persistent changes in 5-HT neurotransmission caused by early undernutrition are responsible for the decreased reactivity to both benzodiazepines and drugs like ipsapirone, that act directly on 5-HT mechanisms. But, of necessity, this hypothesis should be submitted to experimental test before it can be accepted.

In conclusion, the present as well as previously reported results show that rats malnourished during the period of growth of the central nervous system later become less responsive to anxiolytic drugs and in many cases react differently from well-nourished rats to aversive situations. Therefore, long-lasting changes in neuronal systems commanding emotional behavior may result from early malnutrition.

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