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Social condition affects hormone secretion and exploratory behavior in rats

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Abstract

Studies of behavior, endocrinology and physiology have described experiments in which animals housed in groups or in isolation were normally tested individually. The isolation of the animal from its group for testing is perhaps the most common situation used today in experimental procedures, i.e., there is no consideration of the acute stress which occurs when the animal is submitted to a situation different from that it is normally accustomed to, i.e., group living. In the present study, we used 90 male 120-day-old rats (*Rattus norvegicus*) divided into 5 groups of 18 animals, which were housed 3 per cage, in a total of 6 cages. The animals were tested individually or with their groups for exploratory behavior. Hormones were determined by radioimmunoassay using specific kits. The results showed statistically significant differences between testing conditions in terms of behavior and of adrenocorticotrophic hormone (ACTH: from 116.8 ± 15.27 to 88.77 ± 18.74 when in group and to 159.6 ± 11.53 pg/ml when isolated), corticosterone (from 561.01 ± 77.04 to 1036.47 ± 79.81 when in group and to 784.71 ± 55.88 ng/ml when isolated), luteinizing hormone (from 0.84 ± 0.09 to 0.58 ± 0.05 when in group and to 0.52 ± 0.06 ng/ml when isolated) and prolactin (from 5.18 ± 0.33 to 9.37 ± 0.96 when in group and to 10.18 ± 1.23 ng/ml when isolated) secretion, but not in terms of follicle-stimulating hormone or testosterone secretion. The most important feature observed was that in each cage there was one animal with higher ACTH levels than the other two; furthermore, the exploratory behavior of this animal was different, indicating the occurrence of almost constant higher vigilance in this animal (latency to leave the den in group: 99.17 ± 34.95 and isolated: 675.3 ± 145.3 s). The data indicate that in each group there is an animal in a peculiar situation and its behavior can be detected by ACTH determination in addition to behavioral performance.

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- Group
- Stress
- Reproduction
- ACTH
- Luteinizing hormone
- Corticosterone
- Prolactin

Introduction

The social behavioral patterns of a species are usually complex and of great importance to survival. Most of these behaviors normally reflect the social organization of the species in terms of dominant/subordinate relationships. Group life has been studied for a long time, with pros and cons regarding this tendency. Particularly important, in this re-

spect, are the studies of Allee (1-3), since he was a pioneer researcher in the systematic study of this subject.

Group living can maximize the acquisition of food and reduce the possibility that any single individual may be preyed upon (4,5). Various parameters have been used to analyze the relationships between animal raised in isolation or in social groups, such as exploratory activity, social interactions, locomotor activity and reactivity, defecation score, vigilance behavior, hormones, blood cells, vocalizing, and others. Several factors influence the exploratory activity of animals. In addition, age, genetic predisposition and even sex exert their effects (6-9). An important consideration concerning animals kept in groups is that social interactions may result in increased interest in enriched environments due to the social facilitation promoted by the group (9). Another important aspect is that animals showing a higher success rate in response to challenges in these evaluations may yield results that interfere with those of other animals (10). Thus, the study of sociability in exploratory evaluations should be undertaken with caution. In addition, the meaning of the physiological and behavioral data obtained is still controversial (11), with conflicting results having been reported in the literature. Some investigators (12,13) have reported that isolated rats are more active or reactive in open-field tests, while others have reported that isolated rats show reduced spontaneous activity (14,15). However, most of these tests have been carried out on animals isolated from their original groups, both when they were raised in groups and isolated. In any case, in the standard situation, at the time of the test the animal is isolated from its group and its behavioral and hormonal variations are considered to be typical control situations, i.e., an animal evaluated when separated from its group will provide physiological and behavioral measurements that are acutely affected by the condition of isolation during the test. Thus, the information obtained in this way is considered to be a control and will be compared to any other situation in behavioral, physiological or pharmacological studies, etc. An important parameter used to compare the traditional situation is the variation in hormone secretion (16,17) with particular emphasis on hormones of the hypothalamus-pituitary-adrenal (HPA) axis such as corticotropin-releasing hormone, adrenocorticotrophic hormone (ACTH) and corticosterone (CT). However, other hormones could be used as markers of the stress condition to which an animal may be submitted, among them prolactin (Prl). This hormone also acts on the control of reproductive functions and behaviors. Therefore, it may be a marker of interactions between stress and reproductive changes.

The involvement of hormones of the reproductive axis (luteinizing hormone - LH, follicle-stimulating hormone - FSH, and testosterone in males) in stress situations is well known, with the occurrence of both excitatory and inhibitory actions (18).

As pointed out by Misslin et al. (19) and Marchlewska-Koj (20), behavioral and/or social changes lead to hormonal alterations and vice versa, and the situation of acute isolation of an animal from its group culminates in differentiated exploratory performances (14).

The proposal of the present study was to relate behavioral alterations to possible hormonal alterations promoted by the acute stress situation caused by the isolation of an animal from its group. To investigate the potential variation of the main hormones of the reproductive and stress axis between rats tested individually and in group, we tested the animals in a particular environment in which the animal had the option to leave the den by itself (21,22). Since exploratory behavior is considered to be a form of spontaneous uncoerced behavior (23,24), it represents the most suitable parameter for use in this type of study. Thus, the objective of the

present investigation was to determine the differences in hormonal secretion and behavior between animals submitted to a stress situation (separation from their original group) and to a control situation (exploration together with the original group).

Material and Methods

Animals

We used 90 young adult male hooded Long Evans (*Rattus norvegicus*) rats from 30 females assigned to five different experimental conditions or 18 rats per experimental condition. After weaning, only male pups from litters of both sexes were selected at random and kept in groups of three males, with the group including no siblings. Each group was housed in a 31 x 46 x 17 cm wire mesh covered plastic cage lined with wood chip bedding. Food and water were available *ad libitum* and the light:dark cycle was 12:12 h, with lights on at 6:00 am. The highest possible standards for the humane treatment of animals were used, based on the National Institutes of Health Guide for the Care and use of Laboratory Animals.

Apparatus for the behavioral test

The animals were tested in a previously described apparatus (22). Briefly, the apparatus consisted of a den compartment, i.e., a plastic cage measuring 30 x 45 x 16 cm for the group tests or 18 x 27 x 14 cm for the individual tests. This compartment is connected through a tunnel (a 7.6-cm diameter and 10-cm long rigid plastic tube which could be maintained open or closed) to a complex environment consisting of five interconnected chambers. Each chamber is composed of two light blue-painted wooden compartments (20 x 20 x 25 cm) separated by a water tank (20 x 20 x 10 cm) and connected by runways of different widths. After each test the floor and walls of the system were cleaned with a cloth moistened with water, and then dried. In addition to the normal illumination of the room (six 40-W fluorescent lamps in a 20-m^2 room) a 100 W incandescent bulb was placed 1 m above the last chamber of the system.

Procedure

At 113 days of age all animals were placed in contact with the system (as a group, and as a single habituation trial) for 15 min without behavioral recording. At 120 days of age, each animal was tested individually or together with its home group. Eighteen animals (6 cages of 3 animals each) was the number for each experimental category, i.e., groups I to V. The tests were performed between 13:10 and 15:40 h, and the order of testing (individual or collective) was random. The animal or group of animals was allowed to adapt to the den compartment for a period of 30 min prior to the test, as previously described (21). The actual test started with the opening of the tunnel and lasted 15 min during which the behaviors of the animals were recorded on video tape and then fed to a computer for analysis. The three animals of each cage were marked with distinctive color marks on their fur 5 days before the tests. The animals were killed by decapitation and trunk blood was collected immediately after each experimental situation, as follows: group I, control, animals removed directly from their original cage and sacrificed. Group II, animals sacrificed after 30 min of collective adaptation to the experimental cage. Group III, animals sacrificed after 30 min of individual adaptation to the experimental cage. Group IV, animals sacrificed after 30 min of collective adaptation to the experimental cage followed by 15 min for the exploratory test. Group V, animals sacrificed after 30 min of individual adaptation to the experimental cage followed by 15 min for the exploratory test.

Previous analysis showed that animals

tested independently of adaptation condition (groups IV and V) did present two different plasma ACTH profiles as well as different behavioral performances, i.e., one animal in each box showed higher plasma ACTH levels than its partners. Then, we divided the animals into two subgroups which we called "stressed" (S, showing higher plasma ACTH levels) and "partners" (P).

Hormone measurements

Blood samples were centrifuged at 2000 *g* at 4ºC and plasma was separated and frozen at -20ºC until the time for the hormone measurement. Plasma CT, testosterone, Prl, ACTH, FSH, and LH were determined by radioimmunoassay (RIA) (25,26) using specific kits provided by the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK/NIH-USA). All results are reported in terms of NIH-RP_2 (reference preparation) standards. The lower detection limits were 0.04 ng/ml for LH, 0.19 ng/ml for FSH and 0.19 ng/ml for Prl. In order to avoid inter-assay variation, all samples were measured in the same RIA at the same time. The intra-assay coefficients of variation were 4, 3 and 4% for LH, FSH and Prl, respectively.

Plasma ACTH was measured by double antibody RIA (27) using iodinated ACTH-I_{125} from NEN Life Science Products (Boston, MA, USA) and antibody and reference standards provided by the NIDDK/NIH. The lower limit of detection was 1.5 pg/ml. In order to avoid inter-assay variation, all samples were measured in the same RIA at the same time. The intra-assay coefficient of variation was 7.8%.

Testosterone was determined by RIA (28) using ^{3}H-testosterone from NEN Life Science Products. The specific antibody was provided by Dr. J. Antunes-Rodrigues (Universidade de São Paulo, Ribeirão Preto, SP, Brazil). The lower limit of detection was 1.6 ng/ml. The intra-assay coefficient of variation was 5.4%.

Plasma CT was measured using extraction with ethanol (29). The specific antibody was provided by Dr. J. Antunes-Rodrigues. All samples were measured in the same assay using ^{3}H-corticosterone from NEN Life Science Products. The lower limit of detection was 50 pg/ml. The intra-assay coefficient of variation was 5.8%.

Statistical analysis

The performance (mean ± SEM) for the animals tested individually or with their group was evaluated by "time outside the den compartment" (sum of the durations of all episodes in which the animal remained in the environment), "total traveled distance" (sum of all displacements of the animal between compartments of the system), "number of rearing episodes" (every time the animal stood up on its hindlimbs, leaning or not on the wall with its forelimbs), and "latency to leave the den". Values of each behavioral variable were compared by ANOVA, followed by the Tukey test when pertinent.

The values (means ± SEM) of the plasma hormones (ACTH, LH, FSH, Prl, CT and testosterone) for each group and the behavioral data were analyzed statistically by one-way ANOVA followed by the Tukey test whenever appropriate, using the Prism 2.01 program, with the level of significance set at $P < 0.05$.

Groups IV and V were subdivided into two subgroups: stressed (S, the most stressed animal always having highest ACTH) and partners (P, the two cage mates).

Results

The animals of the "S" and "P" subgroups in each group exhibited a similar pattern for time in the environment, distance covered, latency to leave the den and rearing (Figure 1). Males housed together (group IV) spent more time out of the den compartment than males housed singly during the

test, thus having a better opportunity to explore the environment. Their latencies to leave the den compartment were also more reduced and their rearings were more numerous. Thus, grouped animals explored the environment more, in terms of all the items studied, when compared to group V animals (animals submitted to the exploratory test separate from their group mates). Statistically significant differences ($P < 0.05$) were observed between group IV/S *vs* V/S (b) and group IV/P *vs* V/S (d).

In each cage one animal had significantly higher circulating ACTH levels than cage mates. This difference was not observed for the other hormones tested. Figure 2 shows plasma ACTH for the "S" (the most stressed animal in the cage) and "P" (the other two partners) subgroups in each experimental group. There was a significant difference between subgroups "S" and "P" within each experimental group (I, II, III, IV and V; $P < 0.05$) and between group I and groups II, III, IV and V. Plasma ACTH was lower ($P < 0.05$) in group IV (collective test) than in group V (individual test).

Figure 2 also shows plasma Prl, LH and CT for the five groups studied. There were several differences among groups, with the hormonal responses differing between experimental situations. Plasma Prl increased during the procedure (adaptation for the first 30 min and the 15 min of the test), while LH showed the opposite response. Finally, CT showed a significant rapid increase followed by a decrease. No difference in plasma FSH or testosterone was observed between groups (data not shown).

Discussion

The most important difference of the present experiment compared to others in the current literature is that the animals were tested at the same time with their group or isolated. We observed two important results: 1) the animals showed greater exploratory behavior when tested together with their original group than in isolation, and 2) one animal in each cage was in a state of constant alert even when the group was not stressed (group I) by the experimental procedures.

The present model provides a unique opportunity to study the variation of hormone secretion and behavior in animals tested in isolation or together with their group. Analysis of Figure 1 shows the higher ex-

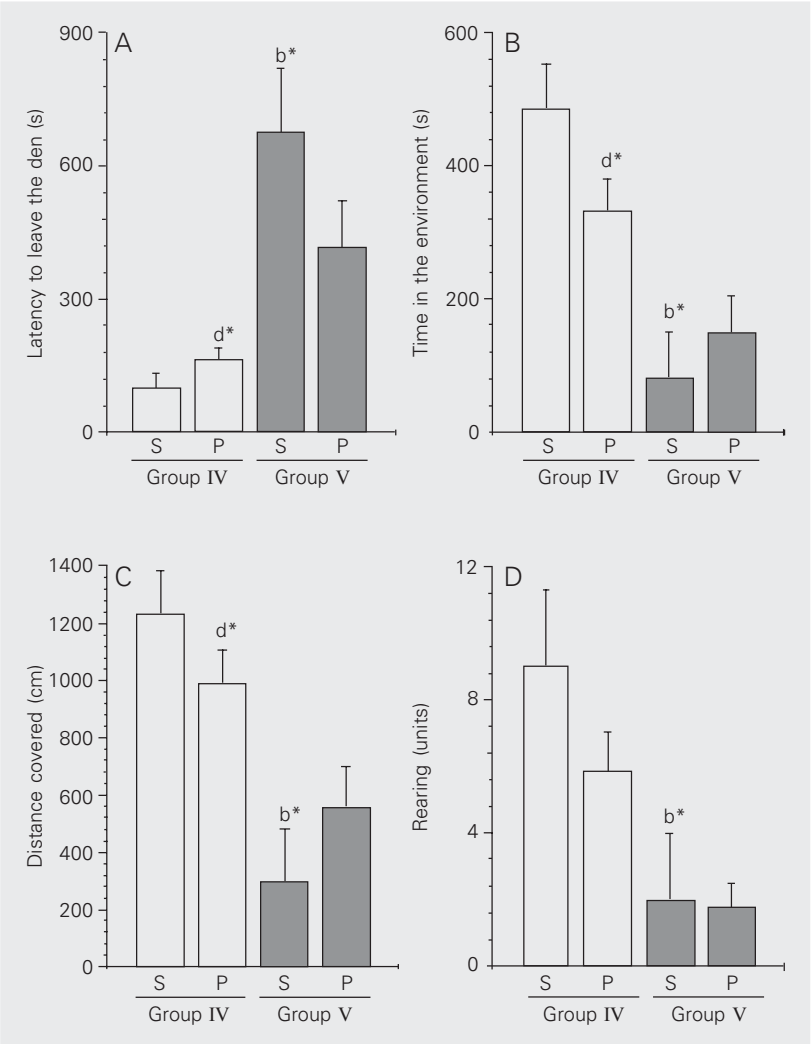

Figure 1. Behaviors studied in the exploratory tests applied to grouped (IV) or isolated (V) animals submitted to 30 min of adaptation to specific conditions (grouped or isolated) and then to 15 min of effective exploratory test. *A*, Latency to leave the den; *B*, time in the environment; *C*, distance covered; *D*, rearing. Both groups (collective - IV and isolated - V) were divided into two subgroups ("S" - most stressed animal in the cage, and "P" - the other two animals in the cage). Significant differences were observed between group IV/S and V/S (b) and group IV/P and V/S (d) (*$P < 0.05$, ANOVA). The number of animals was 6 for subgroup "S" and 12 for subgroup "P", both for groups IV and V.

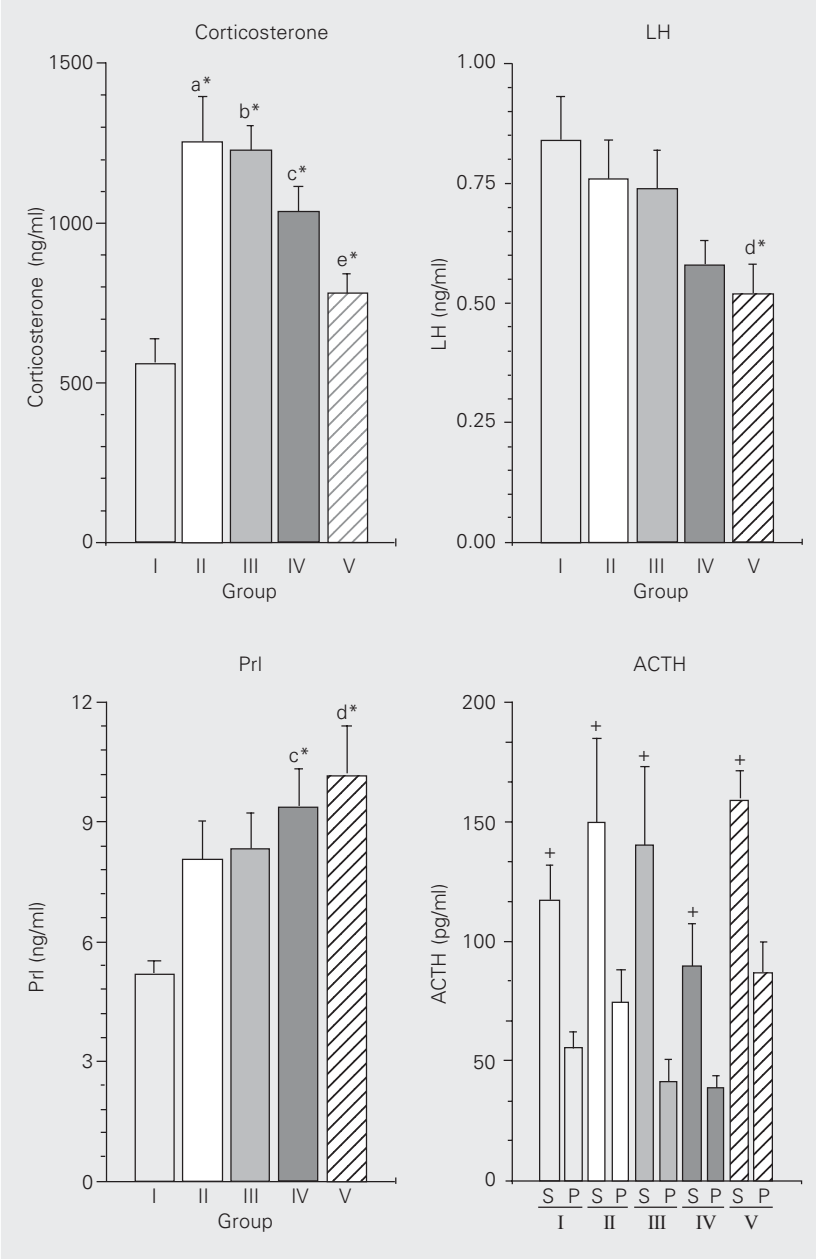

Figure 2. Plasma hormone levels: prolactin (Prl), luteinizing hormone (LH), corticosterone (CT), and adrenocorticotropic hormone (ACTH) in the following groups: I, no manipulation; II, 30 min for adaptation of the animals of the same original group to a new cage; III, 30 min for adaptation of the animal isolated from the group and placed in a new cage; IV, 30 min for adaptation and 15 min for the collective exploratory test; V, 30 min for adaptation and 15 min for the individual exploratory test. For plasma ACTH determination, the groups were divided into two subgroups: "S" (the most stressed animal in the cage) and "P" (the other two partners). *P < 0.05, significant differences were observed between a) group I *vs* II, b) group I *vs* III, c) group I *vs* IV, d) group I *vs* V, e) group III *vs* V (one-way ANOVA). $^{+}$P < 0.05, significant differences were observed between subgroups "S" and "P" within each experimental group for ACTH. The number of animals was 17 for the determination of CT, 17-18 for LH, and 15-17 for Prl. For ACTH, the number of animals was 6/12 for subgroups "S" and "P" in groups I and II, 6/9 for group III, 6/10 for group IV, and 6/11 for group V.

ploratory behavior of grouped animals in terms of the four items studied, i.e., distance covered, rearing, time in the environment, as well as latency to leave the den. This situation elicited an ideal performance (a situation nearest the natural behavior of this species) by all members of the group, even the most alert ("S") one, whereas an animal tested separately became more alert and explored less.

Social interactions are complex phenomena, substantially related to endocrine responses (20). Acute disturbances of standard conditions are quickly reflected in plasma hormone concentrations. In other words, hormone secretion rapidly changes in animals submitted to new maintenance conditions (19), reflecting social or behavioral alterations. For this reason, under free living conditions the animals perform their behaviors at the "periphery or in the central area" of their group both as a function of their behaviors and hierarchy and as a function of their hormonal profiles (intimately related factors) and hormonal and behavioral evaluations complement each other in an important manner.

Most of the studies in this area approach the alteration of the HPA axis as a function of social situation, with special emphasis on litter-mother relationships, and have been mainly conducted on primates (16), monogamous species such as prairie voles (*Microtus ochrogaster*) (30), or species kept in pairs, such as hamsters (*Phodopus sungorus*) (31). However, other types of social relationships among adult animals eventually can affect the HPA axis (32). Relationships among adult animals, especially rats, represent the most usual situation in scientific research, although at present the number of publications in this area is small. The understanding of these endocrine and social parameters in adult rats is of paramount importance since this species is used in several studies of behavior and the animals are frequently isolated for analysis.

Work with hamsters (31) has shown that plasma cortisol increases ($P < 0.05$) in isolated animals, whereas testosterone is unaffected. Other studies have shown hormonal variations in animals submitted to different maintenance conditions (13,16). However, these studies always focus on support conditions, without considering an acute situation such as exploratory tests.

Rats form social groups gradually during development, with social structure being usually complete at about 150 days of age (33). Rats raised in isolation have been reported to have higher basal CT levels and lower resistance to restraint stress than rats raised in small groups (34). It is possible that even the acute isolation such as that used in our experiment may have an alerting effect leading to a reduction in exploratory motivation. Isolated animals when exploring a new environment are thus more prone to alert reactions (35) and tend to have reduced exploratory behavior.

Work with capybaras (*Hydrochaeris hydrochaeris*) (5) has demonstrated that not all individuals of a social species benefit equally from the advantage of living in groups. Among these animals living in large groups, females show reduced vigilance, whereas subordinate males living under more isolated conditions spend more time in vigilance.

In the present study, significant physiological differences in hormone release were observed in the alert "S" animal (Figure 2), especially in terms of ACTH concentration, which was determined in animals sacrificed immediately after the test. Changes in the release of other hormones, with lower fluctuations, probably also occurred after a longer period of time. Plasma CT (Figure 2) also showed differences, probably as a consequence of general procedures, and not necessarily due to isolation since the increase in this hormone did not depend on the social or isolated situation.

The present results show that plasma Prl increased while plasma LH decreased during stress, with the highest plasma Prl values and the lowest plasma LH values being detected in group V. It has been shown that stress stimulates Prl release (36-38) and inhibits LH release (39). Thus, the present results agree with previous reports and describe other markers (like isolation of the original group) of the stress condition in animals of group V.

Taken together, our results indicate the importance of the association of endocrinologic and behavioral studies and emphasize the necessity for a collective approach to social species such as the rat, as well as the importance of the social environment for the motivational regulation of behavior in this species.

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