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Postnatal Handling, Perinatal Flumazenil, and Adult Behavior of the Roman Rat Lines

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FERNÁNDEZ-TERUEL, A., P. DRISCOLL, R. M. ESCORIHUELA, A. TOBEÑA AND K. BÄTTIG. *Postnatal handling, perinatal flumazenil, and adult behavior of the Roman rat lines.* PHARMACOL BIOCHEM BEHAV 44(4) 783–789, 1993. – The effect of infantile handling stimulation and/or perinatal flumazenil (Ro 15-1788; a benzodiazepine receptor antagonist; 3.7 mg/kg/day) administration on exploratory and emotional-related behavior was investigated using adult females from the Roman high- and low-avoidance (RHA/Verh and RLA/Verh) lines. When rats (6 months old) were exposed to a hexagonal tunnel maze including an illuminated central arena, it was found that RHA/Verh rats were more active, explored more maze area, showed more outward preference, and more frequently entered the illuminated center than RLA/Verh rats. In addition, postnatal stimulation decreased emotional-related behavior in both lines of rats, as expressed by increased entry into, and time spent in, the central arena. Perinatal flumazenil treatment decreased entry into the maze central arena in both rat lines but this effect was counteracted by postnatal (handling) stimulation. Thus, the present study extends to adult RHA/Verh and RLA/Verh rats the positive long-lasting effects of postnatal handling and shows postnatal handling × flumazenil interactions in some behavioral parameters related to the pattern of exploration and exploratory efficiency.

Infantile stimulation Roman high- and low-avoidance rats Perinatal flumazenil (Ro 15-1788)
Exploration Emotional reactivity Benzodiazepine receptor

THE existence of a link between GABAergic transmission and anxiety was independently discovered by Costa et al. (1,8) and Haefely et al. (25,36) and was followed by the discovery of high-affinity recognition sites for the anxiolytic benzodiazepines [benzodiazepine receptors (BZRs)], which are coupled to $GABA_A$ receptors in the mammalian CNS (34,37). It is generally accepted at present that the $GABA_A$ receptor complex, coupled to the chloride ionophore ($GABA_A$/BZR/Cl-complex), plays a major role in anxiety and stress. This is supported by the following evidence: a) Anxiolytic/anticonvulsant benzodiazepines upregulate whereas anxiogenic/proconvulsant (benzodiazepine receptor inverse agonists) β-carbolines downregulate GABAergic transmission and b) the $GABA_A$/BZR/Cl- complex is also sensitive to the action of environmental stressful stimuli (2,5–7,38).

Benzodiazepine receptors in the rat brain are detectable 8 days before birth, their density reaching near maximal levels by 2 weeks after birth (4). It has been shown that BZR ontogeny can be manipulated by perinatally administering BZR ligands. Thus perinatal treatment with diazepam (average 5 mg/kg/day) or with the BZR antagonist flumazenil (3 mg/kg/day) lead, respectively, to enduring decreases or increases of BZR density in adult rats, which are paralleled, respectively, by chronic anxiety or "fearlessness" (29,30). Interestingly, behavioral manipulations (such as postnatal handling of rats) that enduringly decrease anxiety or reactivity to stressful situations (10,14,26–28,32) have also been shown to produce an increase in BZR density in adulthood (3), as well as (possibly related to that) changes in the behavioral effects of flumazenil (Ro 15-1788) 5 mg/kg in rats undergoing stressful learning tasks (15).

The importance of genetic factors on the effects of environmental manipulations such as postnatal handling-stimulation have also been demonstrated (17,26,28). It is still an open question, however, as to whether both the early pharmacological (especially perinatal BZR ligand administration) and environmental (postnatal handling) manipulations can influence adult behavior in psychogenetically selected rat lines, such as Roman high- and low-avoidance rats, which have been selected and bred for rapid acquisition (RHA/Verh) vs. nonacquisition (RLA/Verh) of active, two-way avoidance. Adults of the latter line show a lower density of BZRs in several

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brain regions (23), as well as lower GABA-stimulated chloride uptake (24), and are more emotional in behavioral tests (9, 11,18,20,22,31,42). Possibly related, both rat lines differ in their response to drugs acting at the $GABA_A$/BZR/Cl- complex (12,16,31). In addition, decreased reactivity has been noted in weanling rats of both lines after postnatal handling (17) and differential handling vs. flumazenil effects have been seen, also in weanlings (18).

The aim of the present study was to determine if postnatal handling and/or perinatal flumazenil treatment are also able to induce enduring changes in the activity, exploration, and emotional reactivity of adult RHA/Verh and RLA/Verh rats.

METHOD

Animals

Fourteen pregnant RHA/Verh and 16 pregnant RLA/Verh female rats (80th generation of selective breeding) were used in the present study. Animals were maintained with food (Nafag 890) and tapwater available ad lib, and lights were on from 0900–2300 h. On gestation day 15, they were randomly distributed across the eight experimental groups (four groups from each line) to which their offspring would be assigned (see below). From these females, 74 female offspring were used for the present experiment; 37 RHA/Verh and 37 RLA/Verh. The experimental groups were as follows: RHA/CON, 7 RHA/Verh offspring that had been exposed to vehicle (see the Drug Treatment section) from prenatal day 15 to postnatal day 14, receiving no postnatal handling; RHA/FLU, 14 RHA/Verh rats that had received perinatal flumazenil (average dose 3.7 mg/kg/day) from prenatal day 15 to postnatal day 14, without being handled postnatally; RHA/HAN, 6 RHA/Verh pups that had received no flumazenil treatment but were postnatally handled from day 1 to day 21 (see below for procedure); RHA/HAN-FLU, 10 RHA/Verh pups that had received both treatments, perinatal flumazenil plus postnatal handling. The respective RLA/CON (n = 7), RLA/FLU (n = 12), RLA/HAN (n = 8), and RLA/HAN-FLU (n = 10) groups received the corresponding treatments. All pups were weaned at postnatal day 22. They were group housed in macrolon cages with food (Nafag 890) and water continuously available and received no further treatment(s) until the experiment was carried out when they were 6 months old. The different experimental groups each consisted of rats from at least three different litters (which had 10–12 pups each).

Perinatal Flumazenil Treatment

From gestation day 15 to the 14th day after giving birth, mothers (from both lines) assigned to that treatment were given drinking water containing flumazenil (prepared fresh daily, dissolved in ethylene glycol plus water; final concentration 0.5%), whereas the remaining mothers received only water plus the same concentration of vehicle. Every 24 h (in the morning), the consumed volumes were determined. The average daily dose of flumazenil was 3.7 mg/kg (relative to the weight of dams plus the litter).

It is reasonable to assume that fetuses and pups will be exposed to flumazenil (during the critical period of BZR ontogenesis; see the introductory section) because: a) it has been shown that some benzodiazepines cross placenta and are excreted in milk (13), and pre- or perinatal treatments with BZR agonists or antagonists (i.e., flumazenil) induce long-lasting changes in BZR density in the brain (21,29); b) the direction of these changes depend upon the type of ligand (agonist or antagonist) and the dose used (21,29,30); and c) the enduring behavioral effects and upregulation of BZR induced by perinatal exposure to low doses of flumazenil [3 mg/kg/day on average; (29,30)] are similar to those produced by chronic administration of flumazenil 4 mg/kg to adult Sprague-Dawley rats (29,33,39–41), indicating, therefore, that perinatally administered flumazenil reaches the fetuses and pups and interacts with BZR in a way similar to that of chronic flumazenil administered to adult rats.

Postnatal Stimulation (Handling) Treatment

At postnatal day 1, the handling treatment started, consisting of first removing the mother and then pups from the nest twice daily (first time between 1030 and 1300 h and second time between 2000 and 2230 h). The first daily handling session consisted of first separating and weighing each mother and then weighing pups and placing them gently and individually in plastic cages lined with paper towels for a period of 10 min. After 5 min in this situation, each pup was individually (and gently) handled for 5 s and returned to the respective cage for the remaining 5 min. At the end of the 10-min period, each pup was gently handled for 3 s and then returned to its home cage. When all pups from one litter were back in their home cage, the mother was also returned to it. The same procedure, without weighing animals, was done at the evening (second time) handling session. Handling treatment finished at postnatal day 21 (1 day before weaning).

Litters pertaining to nonhandled groups (i.e., CON and FLU groups; see the Animals section) were left undisturbed until weaning.

Behavioral Testing Procedure

A hexagonal tunnel maze with a diagonal diameter of 1.4 m and containing concentric interconnected alleys was used [see the "complex maze" (31) and Fig. 1]. A total of 42 infrared photocell units, indicated in Fig. 1, which were interfaced to an IBM-XT personal computer were uniformly distributed

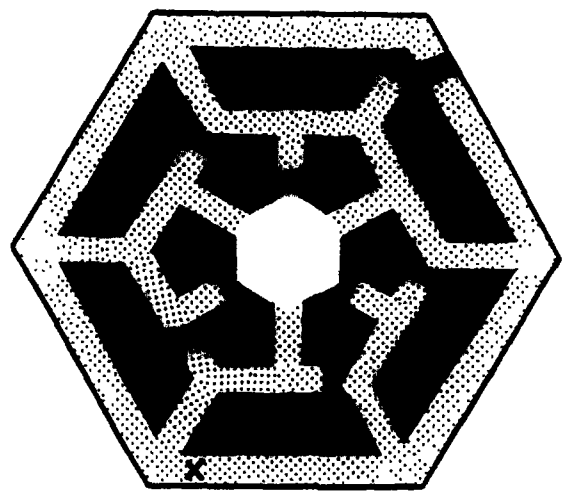

FIG. 1. The alley configuration in the hexagonal tunnel maze, including a brightly illuminated central arena and unlit alleys and nine strategically placed barriers. The starting point is indicated by the "X."

within the maze alleys. Entry into and exit from the central, 750 cm^2 (brightly illuminated) arena could be measured. The ceiling and side walls were fitted together to form a unit that could be lifted from the floor to permit easy removal of a subject and subsequent cleaning. The maze contained eight barriers, arranged in such a way as to allow exploration of the maze with or without entering the central arena. The central open arena was illuminated by a 60-W incandescent light bulb suspended 43 cm above the center. The rest of the maze was not illuminated and contained several blind alleys, as shown in Fig. 1.

Behavioral testing was carried out between 1030 and 1700 h. Each rat received a single 6-min test in the maze, each test beginning with the placement of the rat into the outer maze alley through a door in the ceiling of the maze apparatus (indicated by the X in Fig. 1). At the conclusion of each test, the floor of the maze was thoroughly wiped clean.

Before testing, animals were habituated to the experimental room for at least 7 min. The order of testing was arranged so that approximately equal numbers of rats from each treatment condition and genetic line were tested during each portion of the light cycle. The parameters scored during the maze testing were: a) total activity (TA, number of photobeam interruptions); b) explored area (EA, number of different photobeam activated); c) percentage of outward activity (%OA, percentage of the number of photobeam interruptions in the outer part of the maze adjusted for TA); d) percentage of inward activity (%IA, percentage of the number of photobeam interruptions in the inner part of the maze adjusted for TA); e) entries into the central illuminated arena (EC); f) percentage of entries into the illuminated arena adjusted for TA (%EC = EC/TA × 100); g) time in the center (TC); and h) the average time per entries in the center (TC/EC).

RESULTS

Significant line effects appeared in TA, $F(1, 72) = 65.0$, $p < 0.001$ (Fig. 2a), EA, $F(1, 72) = 51.41$, $p < 0.001$ (Fig. 2b), %OA, $F(1, 72) = 47.27$, $p < 0.001$ (Fig. 2c), and EC, $F(1, 72) = 4.32$, $p < 0.05$ (Fig. 3a), indicating that RHA/Verh rats were more active and explored more area, their outward activity was greater, and they made overall more entries to the center than the RLA/Verh line. Conversely, the percentage of inward activity [%IA, $F(1, 72) = 51.80$, $p < 0.001$; Fig. 2d] and the average duration of entries to the center [TC/EC, $F(1, 72) = 11.01$, $p < 0.01$; Fig. 3d] was increased in RLA/Verh females with respect to RHA/Verh.

Interestingly, the RLA/HAN-FLU group showed the highest TC/EC values, being significantly different from RLA/CON rats ($p < 0.05$, Duncan's test) and leading to a nearly significant line × flumazenil interaction in that measure, $F(1, 72) = 3.41$, $p = 0.07$ (Fig. 3d).

Postnatal handling induced overall increases in EC, $F(1, 72) = 9.09$, $p < 0.01$ (Fig. 3a), %EC, $F(1, 72) = 6.43$, $p < 0.02$ (Fig. 3b), and TC, $F(1, 72) = 12.25$, $p < 0.001$ (Fig. 3c). Flumazenil decreased %EC, $F(1, 72) = 4.28$, $p < 0.05$ (Fig. 3b) and a similar (however nonsignificant) tendency was observed in EC and TC. Such flumazenil effects were nevertheless reversed by postnatal handling, especially in the RLA/Verh line ($p < 0.05$ between RLA/FLU and RLA/HAN-FLU groups, Duncan's test; Figs. 3a–3c).

Analysis of variance showed significant postnatal handling × flumazenil interactions in %OA, $F(1, 72) = 12.41$, $p < 0.01$; (Fig. 2c) and in %IA, $F(1, 72) = 11.57$, $p < 0.01$ (Fig. 2d), probably reflecting the fact that either treatment increased %OA and decreased %IA in both rat lines when administered alone, while the combination of both treatments in the RLA/HAN-FLU group led to values similar to these of RLA/CON rats in both parameters (Figs. 2c and 2d).

DISCUSSION

The present results on activity, explored area, and entries into the center replicate previous studies that consistently found that adult RHA/Verh rats are more active and go more readily through the illuminated center than do RLA/Verh rats (11,31,35). Moreover, comparing the activity of the outer and inner parts of the maze between both lines, the percentage of photobeam interruptions in the outer alley was greater for RHA/Verh animals with respect to RLA/Verh, with the contrary being the case with regard to the inner part of the maze. This agrees with other studies (11,35) and has been interpreted as being indicative of a more efficient exploratory behavior by the RLA/Verh line, as, although they explored a smaller maze area (as shown by the explored area index), their activity in the inner and outer alleys was more similar (42–54% in the outer and 41–56% in the inner, depending upon the treatment groups; Fig. 2c–d) than in the RHA/Verh line (53–67% and 26–43%, respectively).

Despite a lack of significant handling effects on activity and explored area, it is worth mentioning that the tendency to an increase in such parameters was clear, as indicated by the fact that RHA/Verh controls were more active and explored more maze area than RLA/Verh controls, but that this significant difference was not present when comparing RHA/Verh controls to RLA/Verh-HAN animals (due to the relative increase in activity and explored area observed in the last group; see Figs. 2a and 2b).

It is especially worth noting, in addition, that postnatal handling still had significant positive effects on EC, %EC, and TC in 6-month old animals (5 months after such treatment finished) and that such effects were apparently greater in RLA/Verh than in RHA/Verh rats (although there was no significant line × handling interaction; see Figs. 3a–c). That trend was similar to what we observed previously in weanling RHA/Verh and RLA/Verh animals (17,18). Because the degree of entry into the illuminated arena is likely related to anxiety or emotional reactivity (11,17,18,31,35), our results appear to reflect a long-lasting (slight but overall significant) reduction of anxiety-like behavior as a consequence of postnatal handling. Conversely, perinatal flumazenil treatment appeared to have enduring detrimental effects in anxiety-related parameters (i.e., reductions of EC, %EC, and TC in flumazenil-treated groups). Such an effect of perinatal flumazenil treatment was in the opposite direction of that found by Marczynski et al. (29) but is unlikely to be due to dosage differences because our present dose (3.7 mg/kg/day) was similar to that used by those authors (3 mg/kg/day). On the other hand, they reported anti-anxiety-like activity even when using a 4-mg/kg/day dose (in adult Sprague-Dawley rats), whereas anxiogenic-like effects were obtained only when using a quite higher dose of 6.6 mg/kg/day (29). Thus, the disagreement between our results and Marczynski et al.'s findings is more likely accounted for by differences between the strains of rats used in each study (RHA/Verh and RLA/Verh vs. Sprague-Dawley), which further indicates that genetic (as well as other predispositional) factors must be taken into consideration when studying the effects of drugs.

An interesting finding from the present study, also reported for the first time, was that flumazenil detrimental ef-

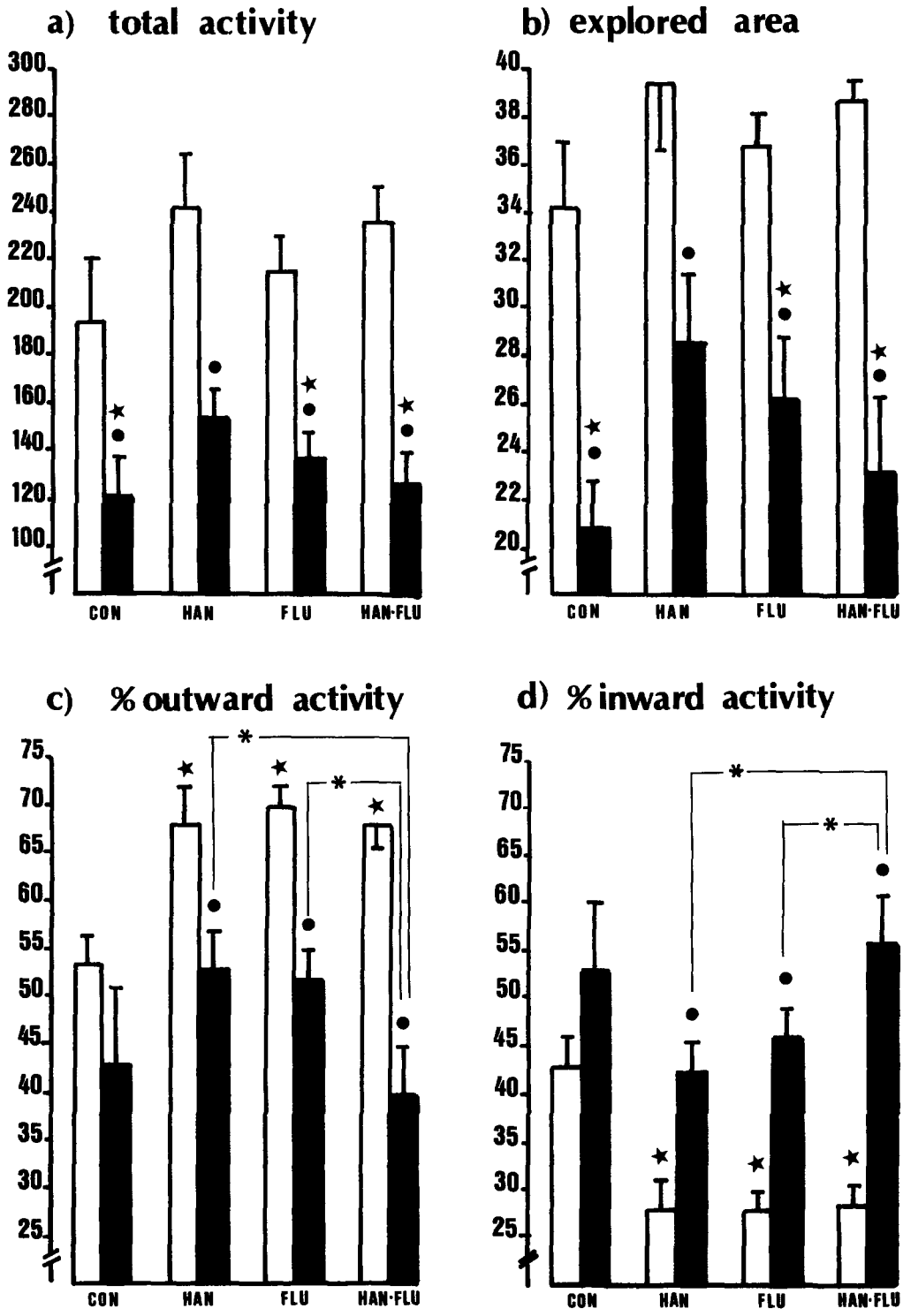

FIG. 2. Effects of 21 days of postnatal stimulation (HAN; handled groups) and perinatal flumazenil treatment (FLU; flumazenil-treated groups) on total activity (a), explored area (b), % outward activity (c), and % inward activity (d) in the hexagonal tunnel maze test in Roman low- and high-avoidance (dark and open bars, respectively) female rats. Means (±SEM) are represented. ★$p < 0.05$ vs. RHA/Verh-CON rats; ●$p < 0.05$ vs. the respective RHA/Verh group with the same treatment; *$p < 0.05$ between the groups indicated by the lines (Duncan's test).

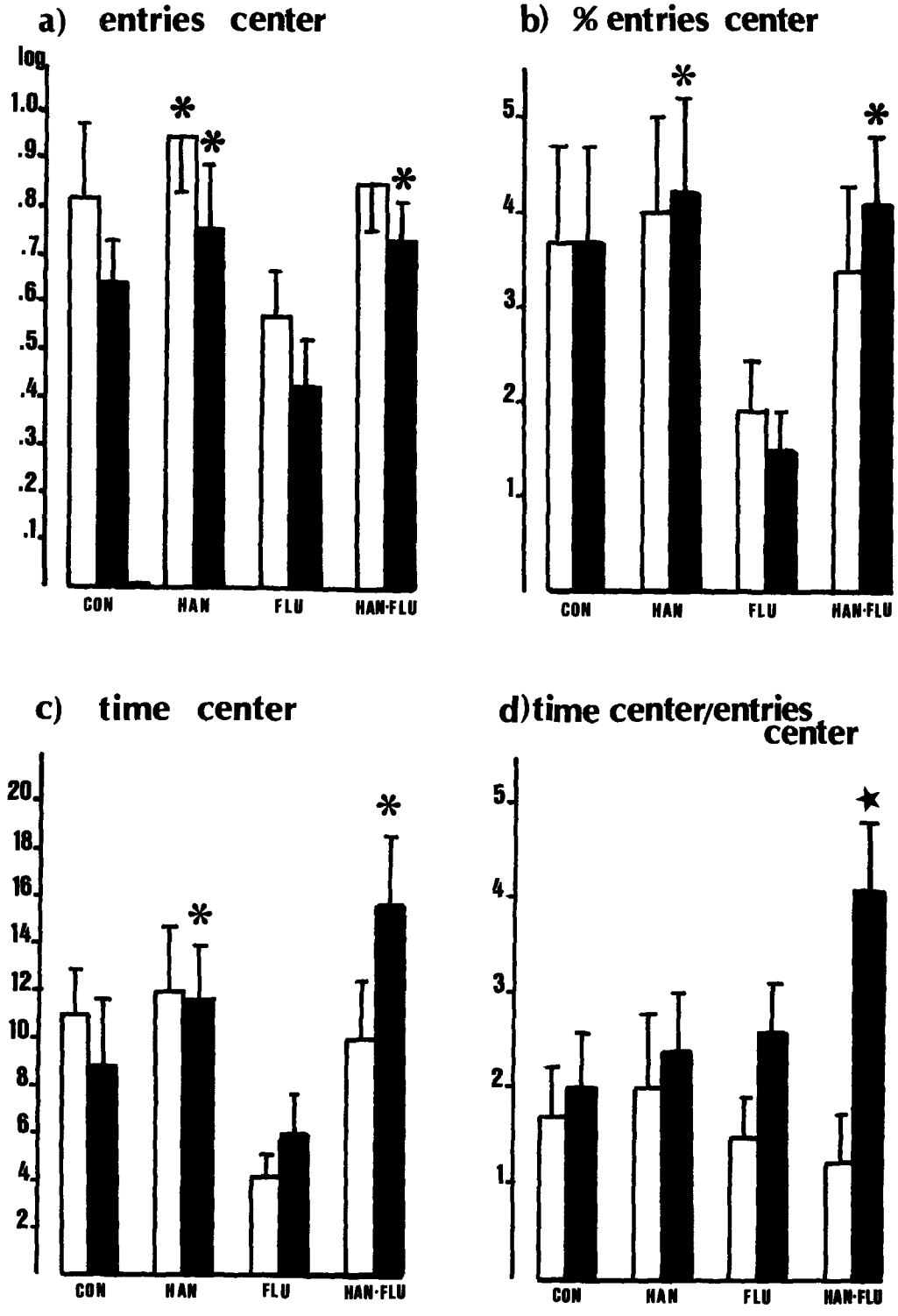

FIG. 3. Effects of 21 days of postnatal stimulation (HAN; handled groups) and perinatal flumazenil treatment (FLU; flumazenil-treated groups) on entries into the center (a), % of entries into the center (b), time in the center (c), and time in the center/entries into the center (d) in the hexagonal tunnel maze test in the same Roman low- and high-avoidance (dark and open bars, respectively) rats as in Fig. 2. Means (±SEM) are represented. *$p < 0.05$ vs. FLU group of the same rat line; ★$p < 0.05$ vs. RLA/Verh-CON group (Duncan's test).

fects were prevented by postnatal handling treatment in both rat lines (see HAN-FLU groups, Fig. 3). This is partially in agreement with what we found previously in weanling RHA/Verh and RLA/Verh rats treated with postnatal handling and/or perinatal flumazenil (18). In fact, when tested in a miniature and less complex (because there were no barriers in the alleys) version of the same tunnel maze used in the present study a detrimental effect of perinatal flumazenil (especially in EC and %EC) was observed only in RHA/Verh weanlings (but not in RLA/Verh pups, leading thus to line × flumazenil interactions in EC and %EC in that previous study), and this effect was partially counteracted by postnatal handling (18).

Summarizing the present results and those obtained in our previous studies, the main conclusions are: a) Postnatal handling has long-lasting (slight but significant) positive effects on emotional behavior in RHA/Verh and RLA/Verh rats (as indicated by the analysis of variance's overall significance of postnatal handling treatment on EC, %EC, and TC measures); b) the effects of perinatal flumazenil on emotivity-related behavior appear to vary across different strains of rats (14,18,29); c) the animal's age at the time of testing can also be important [(18) and the present study]; d) and the interaction between perinatal flumazenil and postnatal stimulation effects can also vary depending upon the strain, sex, testing situation, and the age of animals [(14,18,19) and the present study].

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