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Sexual Segregation in Infancy and Bi-Directional Benzodiazepine Effects on Hot-Plate Response and Neophobia in Adult Mice

GIOVANNI LAVIOLA*[1] AND GIAMPIETRO LOGGI†

*Section of Behavioral Pathophysiology, Laboratorio di Fisiopatologia di Organo e di Sistema
Istituto Superiore di Sanità, viale Regina Elena, 299, I-00161 Rome, Italy
†Department of Mental Health RM 11, via di Val Cannuta 206, I-00166 Rome, Italy

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LAVIOLA, G. AND G. LOGGI. *Sexual segregation in infancy and bi-directional benzodiazepine effects on hot-plate response and neophobia in adult mice.* PHARMACOL BIOCHEM BEHAV **42**(4) 865-870, 1992.—In the present experiment, the hypothesis that rearing animals in conditions of sexual segregation in infancy (ISS) would affect their adult behavioral reactivity to drug or environmental challenges was tested. Outbred Swiss CD-1 mouse litters were reduced at birth to six pups according to three conditions: MM (all males), MF (sex-balanced composition), and FF (all females). At weaning (day 21), all mice were rehoused in unisexual groups. At adulthood (day 70), animals were challenged either with BDZ agonist chlordiazepoxide (CDP at 2.5- or 5.0-mg/kg dose) or BDZ receptor partial inverse agonist Ro 15-3505 (RO at 3-, 10-, or 30-mg/kg dose) and assessed in sequence for pain reactivity in a hot-plate apparatus (set at 55 ± 1°C), for locomotor activity in a Varimex apparatus, and finally for neophobia level by measuring the latency to first approach a novel object. As concerns the hot-plate test, lick latency was significantly shortened in MF females receiving CDP (5.0 mg/kg), while RO was either ineffective in MF females or induced a prominent dose-dependent analgesia in FF females. Activity was decreased by CDP (2.5 mg/kg) and enhanced by RO (3.0 mg/kg). For latency to approach a novel object, males as a whole exhibited shorter times than females. Mixed-sex animals of both sexes were less fearful, being also more explorative than their corresponding unisexually reared groups. In particular, MF males receiving either a 5.0-mg/kg CDP dose or a 3.0-mg/kg RO dose explored the object more often than MM males. Overall, these results a) support the involvement of BDZ-mediated processes in the modulation of sensory function and of behavioral reactivity to environmental stimuli and b) indicate that drugs acting at the level of the GABA-BDZ receptor complex in the CNS can bidirectionally modulate responses to painful stimulation and neophobia/exploratory patterns. In addition, subtle variations of social environment during the infantile period can exert long-term effects on drug-induced behavioral changes.

Infantile sexual segregation Social development Hot plate Locomotor activity Novel object Benzodiazepines Mouse

THERE is now substantial evidence for a role of the GABA-benzodiazepine (BDZ) receptor complex of the brain in the regulation of social responses (8,17,22). Furthermore, not only are these CNS systems probably activated during pregnancy and lactation in the dam (27,38) but with respect to pups they may be sensitized during critical stages of physical and social development (4,25,26,39).

Accordingly, GABA-BDZ systems are widely expressed in neural areas responsible for receiving and organizing incoming sensory information from which the social bond and emotions must be constructed. Within higher brainstem and forebrain areas, GABA-BDZ neurons richly innervate limbic areas known to be of importance in the governance of animals' social interactions (24,48,53). Such a selective distribution of BDZ binding sites, primarily in areas that participate in the sensory or emotional processing of environmental stimuli, has been suggested to reflect the involvement of endogenous BDZ processes in behavioral reactivity (6,52).

In rodents, the administration of nonsedating doses of chlordiazepoxide (CDP) and diazepam is very effective in reducing distress vocalizations—a behavioral index of the emotional state—in rat pups separated from the mother and litter-

[1] To whom requests for reprints should be addressed.

mates (22). In addition, several experiments have shown an influence of social environment on GABA-BDZ system functioning (21,23). In fact, prolonged isolation has been demonstrated to alter BDZ receptor density in the brain of adult mice (14). Isolated mice are also less sensitive than group-housed mice to diazepam impairment of rotarod performance (47).

Several studies also suggest that endogenous pain-inhibiting systems interact with the social environment. Social agonistic interactions may promote analgesia, which is reversed by the BDZ antagonists (40). This undirectly suggests an involvement of BDZ regulatory mechanisms. Differential effects of Ro 15-1788 (RO) in either individually housed or grouped animals have been reported for male mice assessed in agonistic confrontation tests (41).

Overall, these data are consistent with the propositions that brain GABA-BDZ systems modulate social emotions and behaviors, social environment sustains the functional activity of the brain GABA-BDZ receptor complex, and some environmental factors may be important in the genesis of anxiety-related disorders (13,17,33,49).

As concerns (in mice and other laboratory animals) the influence of social environment in infancy on subsequent behavioral development, the literature on effects of sex of littermates is scattered (2,7,11-12,18,46,51) (see also the Discussion section). A large proportion of this work has been directed toward an examination of the effects of individual housing (social isolation) on adult behavior. Surprisingly, there have been few investigations on the effects of sexual segregation in infancy upon subsequent behavioral development despite the fact that all-male or all-female litters occur regularly in laboratory rats and mice.

The following experiment tested the hypothesis that rearing animals in conditions of sexual segregation in infancy would affect their adult behavioral response to environmental challenges. Adult mice were tested in behavioral procedures commonly used to assess reactivity to noxious and nonnoxious stimuli and motor activity and challenged with drugs acting on the GABA-BDZ receptor complex—a neural substrate considered an important participant in an organism's response to environmental challenge (6,35,52).

METHOD

Animals, Breeding, and Rearing Conditions

Mice of an outbred Swiss CD-1 strain purchased from Charles River Italia (Calco, Italy) were used. Upon arrival, animals were housed in standard conditions (temperature 21 ± 1°C, relative humidity 60 ± 10%) with lights on from 9:30 p.m.-9:30 a.m. Males and nulliparous females were housed separately in groups of eight in 42 × 27 × 15 cm Plexiglas boxes with sawdust as bedding and a metal top. Pellet food (enriched standard diet purchased from Piccioni, Brescia, Italy) and water were continuously available.

After 2 weeks, breeding pairs were formed and housed in 33 × 18 × 14 cm boxes. Females were inspected daily at 10:00 a.m. for the presence of vaginal plug (pregnancy day 0) and for delivery (postnatal day 1). The stud was removed 10 days after finding of the plug. Pregnant mice were randomly assigned to each of three experimental conditions, with litters reduced at birth to six pups and culled to all males (MM), three males plus three females (MF), or all females (FF). At weaning (day 21), all mice were rehoused in 42 × 27 × 15 cm Plexiglas boxes in same-sex groups.

Drug Preparation

An acqueous suspension of CDP or RO was prepared fresh daily by mixing the compound in distilled water containing two drops of Tween-80 per 10 ml solution. To maintain an even suspension, it was stirred at low speed until the time of injection. All mice were injected IP with either CDP, RO, or saline (NaCl 0.9%)-Tween 80 vehicle in a volume of 0.01 ml/g body weight.

Apparatus and Procedures

When adults (day 70 ± 1), one mouse in each litter was randomly assigned to one of the following treatments: vehicle, CDP (at either 2.5 or 5.0 mg/kg), or RO (at 3, 10, or 30 mg/kg). The range of doses and the time interval lasting between injection and testing were selected according to literature data (5,10,52) and on the basis of pilot experiments.

Nociception assessment. After injection, each animal was returned to the home cage for 15 min (in the case of RO) or for 30 min (in the case of CDP), then placed on a hot-plate apparatus (Model-DS37, Socrel Basile, Comerio, Italy) set at 55 ± 1°C, used to assess reactivity to a noxious thermal stimulus. The time from the beginning of the test to the first licking of a hindpaw (latency time) was recorded (cutoff time 60 s).

Locomotor activity test. Immediately after, single animals were introduced in a clean box of the same type as the home box. The box was placed on a Varimex Activity Meter apparatus (four units, Columbus Instruments, Columbus, OH), set at a standard level. Only the horizontal sensor systems were used, and the recording session lasted 20 min.

Assessment of neophobia level. At the end of the activity test, a stimulus object (a 4.9 × 3.0 cm Plexiglas black cylinder) was placed by the experimenter at one side of the box and the latency to make the first sniffing contact with it (cut off 180 s) and the number of approaches to it during the following 2 min recorded.

All tests took place between 2:00-5:00 p.m. All females were in diestrus on the day of testing. The experimental design was counterbalanced to equate the representation of various groups at different test times. In the case of activity tests, the designs were also counterbalanced for assignment of animals to different Varimex units.

Statistical Analysis

The data were analyzed by mixed-model analyses of variance (ANOVAs) considering all variables, namely, the litter random variable (1,9) nested under the infantile sexual segregation (ISS), sex, and treatment administered before test. All ANOVAs on activity data also considered the within-subjects (repeated measures) variable. Posthoc comparisons within logical sets of means were performed using Tukey's HSD test.

RESULTS

Effects on Nociception

As shown in Fig. 1, a significant sex difference emerged, $F(1, 44) = 15.72$, $p < 0.001$, with females showing a shorter pain reactivity than the corresponding male group. Moreover, a main effect of treatment, $F(5, 22) = 4.93$, $p < 0.001$, and a sex × ISS interaction also appeared, $F(5, 220) = 2.64$, $p < 0.05$. Posthoc comparisons excluded any significant difference in the male group, but indicated a hyperalgesia profile upon a 5.0-mg/kg CDP dose in MF females ($p < 0.05$) and a

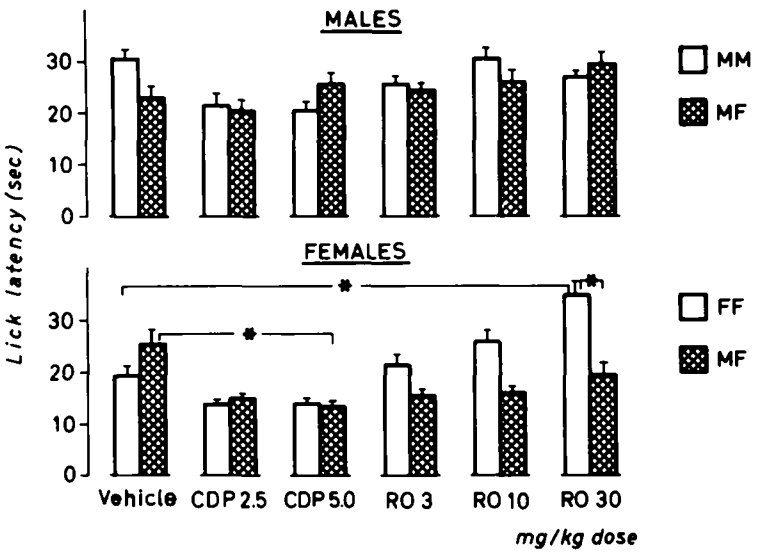

FIG. 1. Mean latency (SEM) to lick a hindpaw in a hot-plate test of male and female adult mice reared in all male (MM), all female (FF), or male and female (MF) litters. One animal from each litter was injected IP with either vehicle, CDP 2.5 and 5.0 mg/kg, or with RO 3, 10, and 30 mg/kg. (n = 12). *$p < 0.05$.

prominent dose-dependent analgesia in FF females following RO administration. In particular, the 30-mg/kg dose group was significantly different from corresponding FF controls ($p < 0.05$) and from similarly treated MF females ($p < 0.05$).

Effects on Locomotion

In the absence of significant main effects or interactions of sex and ISS variables, a significant main effect of treatment was evident, $F(5, 220) = 4.55$, $p < 0.001$. In particular, as shown in Table 1, CDP (2.5 mg/kg) depressed, while RO (3.0 mg/kg) increased, activity over the 20-min session ($p < 0.01$ in both comparisons with the corresponding control groups).

Effects on Neophobia Level

A significant sex difference for latency to first approach to a novel stimulus object was evident, $F(1, 44) = 4.66$, $p < 0.05$, with males showing a shorter latency than females. When considering the frequency of approaches in the subsequent 2-min period (see Fig. 2), a significant main effect of ISS was also evident, $F(1, 44) = 4.71$, $p < 0.05$, with MM and FF animals (i.e., mice reared in a unisexual condition) showing a significantly lower number of sniffing contacts than the corresponding MF group. A higher-level interaction between sex, ISS, treatment, and repeated measures also appeared, $F(5, 220) = 4.02$, $p < 0.001$. Exploration of data and successive multiple comparisons excluded significant differences in the female group, but indicated (see Fig. 2, bottom) that CDP at both doses significantly increased the number of approaches to the object in the MF male group ($p < 0.05$ or less vs. corresponding controls). Interestingly, both drugs were unable to affect the performance of MM animals. As a consequence, a significant difference also emerged between MM and MF males upon 5.0-mg/kg CDP and 3.0-mg/kg RO doses ($p < 0.01$, for both).

TABLE 1

LOCOMOTOR ACTIVITY ASSESSED IN A VARIMEX APPARATUS DURING A SINGLE 20-min SESSION IMMEDIATELY AFTER THE HOT-PLATE TEST

Vehicle	89.34 (±5.24)
CDP 2.5	74.59 (±6.36)*
CDP 5	100.63 (±5.55)
RO 3	105.42 (±4.64)*
RO 10	91.89 (±4.72)
RO 30	92.50 (±4.98)

Animals are the same as in Fig. 1. (n = 48).
*$p < 0.01$ vs. vehicle.

DISCUSSION

The results clearly show that:

1. Adult, female mice raised during infancy within litters with a balanced gender composition showed the expected hyperalgesia profile in response to CDP, while females reared in all-female litters exhibited a prominent dose-dependent analgesia in response to RO.
2. Mixed-sex-reared males and females were less fearful and showed more exploratory behavior than unisexually reared males and females. Both RO and CDP were able to reduce neophobia only in MF males, but not in the MM group.

The present results confirm and extend previous observations concerning the influence of social environment on GABA-BDZ system functioning. The experimental manipulation here adopted was quite different from those used in previous reports, which were mainly limited to the comparison of the different effects of group and individual housing (see the introductory section). Moreover, the previous results were obtained by testing animals shortly after the end of the period of enforced condition. In the present study, the interval between the end of exposure to different social conditions at testing

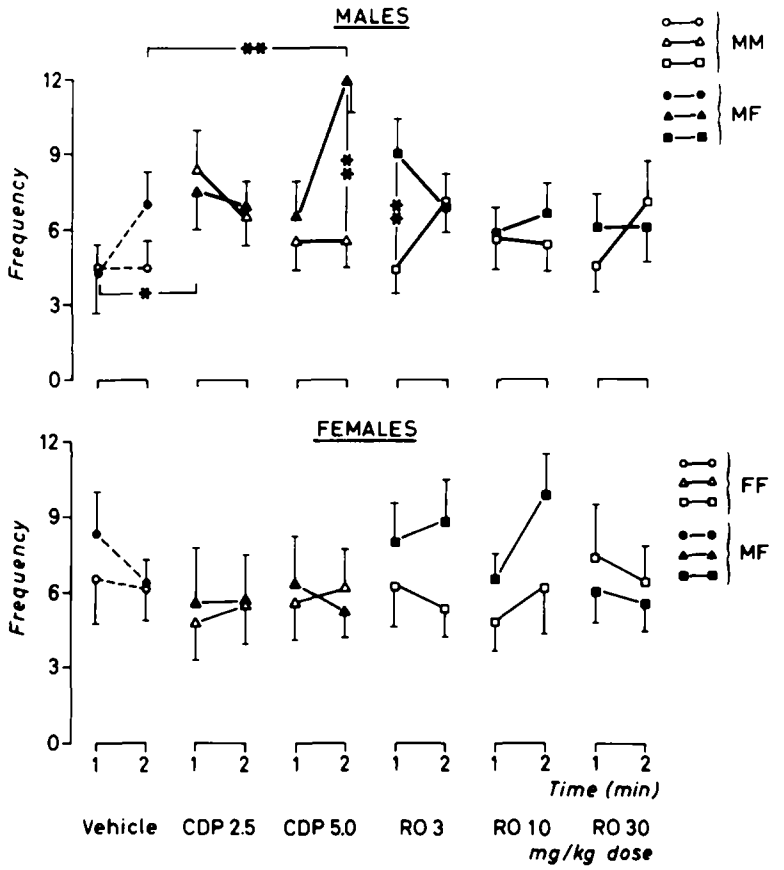

FIG. 2. Mean frequency of approaches to the novel object during a 2-min test as assessed at the end of the locomotor activity session. Animals are the same as in Fig. 1. (n = 12). *$p < 0.05$; **$p < 0.01$.

was much longer (approximately 50 days). Therefore, the results suggest that manipulation of social milieu during infancy has a long-term influence on systems that serve exploratory pattern and pain sensitivity by modulating response to painful stimulation.

At the present stage of the work, it is impossible to determine which factor was responsible for the differences related to early social environment condition. An individual variation in behavioral response to challenge at the adult stage with β-carbolines (BDZ inverse agonist and putative anxiogenic agents) has been shown in primates (23). This effect has been interpreted in terms of long-term influence of early life events on the function of the GABA-BDZ receptor system. The BDZ receptor matures early in ontogeny and may have an important physiological role in the mediation of affiliative bonds early in development (22,23). Therefore, one working hypothesis with respect to such a "sibling effect" (18) may be that the nature of the interactions between pups of the same litter [e.g., different levels of social grooming or playful interactions in infancy (32,50)] can affect the development and subsequent function of this system (35-37). On the other hand, differential postnatal maternal effects due to variation of the dam's behavior depending upon litter gender composition must also be taken into consideration (2,3).

These data also demonstrate that CDP administration at nonsedating doses induces a hyperalgesia profile, a response usually reported for other BDZs (42), while sedating ones are associated with analgesic effects (52). The BDZ receptor partial inverse agonist Ro 15-3505 has been found here to be active in a number of behavioral tests and also to exert an antinociceptive effect. However, the mechanisms by which RO increased hot-plate latencies is not clear. It is unlikely that the RO-induced antinociception was the result of a nonspecific decrease in motor activity or due to motor incoordination since RO administration in a dose range from 10-30 mg/kg had no effect on spontaneous motor activity (see Table 1). On the other hand, the ability of RO to induce analgesia is especially interesting in light of the very recent literature on stress-induced analgesia (30,43). RO may be viewed as a pharmacological stressor activating these same systems. Supporting this hypothesis, it has been shown that just as increases in stress severity can increase analgesia RO too can potentiate stress-induced analgesia (see the dose-dependent response of FF females in Fig. 1).

The different individual responses of animals to the hyperalgesic action of CDP or to the analgesic effect of RO apparently reflect the individual variation in sensitivity of central mechanisms mediating the bidirectional pain-modulating action of BDZ agents. An intriguing possibility that both BDZ agents can also affect in vivo the release of the putative endogenous ligand for BDZ binding sites cannot be excluded. It has been recently assumed by several authors that the putative

endogenous ligand for BDZ binding sites may exert a pharmacological activity opposite this of classical BDZ tranquillizers (19). Therefore, a tentative hypothesis is that in RO responders the concentration of the putative endogenous ligand (in CNS) is higher than in RO nonresponders. If it is the case or not, MM, as do as FF animals, or in general the ISS procedure, offer an additional tool for analyzing the functional significance of the relation between different ligand sites at the GABA-BDZ receptor complex.

In the present study, sexual segregation in infancy was apparently responsible for the increased neophobia exhibited by unisexually reared animals. It also altered function of the GABA-BDZ receptor complex – a neural substrate considered an important participant in an organism's responding to environmental challenge (6,35,52) – as revealed by the restriction to MF males of the facilitative effect of both CDP and RO in the test with the approach to a novel object. In this view, Primus and Kellogg (36) reported that castration of male rats as juveniles (postnatal day 19) contrasted the facilitative effect of diazepam on the adult response to an anxiogenic situation. An influence from gonadal steroids (or their metabolites) on function of the GABA-BDZ system has been extensively demonstrated (15,20,29,31,34). Taken together these reports indirectly suggest that interferences by sexual segregation in infancy (12,28,44,45,51) with gonadal hormones or their metabolites on pubertal development of the receptor complex should be considered.

The fact that artificial manipulation of social environment in infancy can qualitatively affect some aspects of the animal's behavioral repertoire in adulthood raises the question of whether comparable effects might occur naturally in view of the large differences in sex ratio of natural mouse litters. Perhaps, quantitative and/or qualitative variation in social interaction with the opposite sex in infancy is a contributor to the wide interindividual variation in the capacity of coping with environmental challenges, including drug administration (16) or toxicant exposure.

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