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Pharmacology, Biochemistry and Behavior 78 (2004) 465–471

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Sexually dimorphic effects of postnatal allopregnanolone on the development of anxiety behavior after early deprivation

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Received 29 January 2004; received in revised form 18 March 2004; accepted 29 March 2004
Available online 17 June 2004

Abstract

Stress early in life exerts persistent detrimental effects on brain development. In this experiment, a rodent model of child neglect, early deprivation (ED), was used to investigate the role of the neurosteroid allopregnanolone [AlloP; 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP)] in the development of anxiety behavior. Subjects were either undisturbed controls or ED: separated individually for 6 h per day from postnatal day (PN) 2 to 6. Control and ED subjects were also either noninjected, vehicle-injected or injected with 5 mg/kg AlloP prior to the isolation. At PN 7, responses to 2.5 or 5 μg icv injections of AlloP were determined for separation-induced ultrasonic vocalizations (USVs). Tolerance to the USV-reducing effect of daily AlloP was seen in control but not ED pups, and daily AlloP reversed the expected ED suppression of USVs. As adults, controls treated with postnatal AlloP were less anxious than all other groups on the elevated plus maze. ED counteracted this effect. Male controls showed a reversal of the typical sex difference. There were no effects on open-field activity. These results suggest that the neonatal brain is responsive to alterations in AlloP levels, and that neuroactive progesterone metabolites may play a role in mediating the development of stress-related sex differences.

Keywords: Allopregnanolone; 3α-hydroxy-5α-pregnan-20-one; 3α,5α-THP; Stress; Maternal separation; Early deprivation; Sex differences; Plus maze; Ultrasonic vocalizations; USV; Anxiety

1. Introduction

The progesterone metabolite allopregnanolone [AlloP; 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP)] has been increasingly studied over the last decade as a possible "endogenous tranquilizer." AlloP is a potent positive modulator of the GABAa receptor, enhancing GABA-stimulated chloride uptake (Barbaccia et al., 1996; Paul and Purdy, 1992). In addition to peripheral endocrine sources, AlloP is synthesized in glial cells from cholesterol in response to stress and acts rapidly as an anticonvulsant, sedative and anxiolytic (Majeska, 1992; Molina-Hernandez et al., 2003). In animal models of anxiety behavior, AlloP has been shown to reduce ultrasonic vocalizations (USVs) after maternal separation (Zimmerberg et al., 1994), increase time on the open arms of the plus maze (Bitran et al., 1991), increase exploratory behavior in the light–dark transition test (Wieland et al., 1991), and reduce conflict behavior (Molina-Hernandez et al., 2003).

Recent studies have examined sex differences in response to AlloP in anxiety behavior paradigms (Frye et al., 2000; Reddy and Kulkarni, 1999). On several measures of anxiety behavior, females in proestrous were less anxious than diestrous females or males, and this sex difference was mirrored in levels of both plasma and hippocampal progesterone and AlloP (Frye et al., 2000). Furthermore, the diminution of anxiety behavior in proestrous females was reversed when an enzyme inhibitor of progesterone's metabolism to AlloP in the hippocampus was administered (Bitran et al., 1995; Rhodes and Frye, 2001).

AlloP is present in fetal brain in levels similar to adult males and proestrous and estrus females, although the levels of the parent progesterone are markedly lower (Kellogg and Frye, 1999). This dissociation indicates that brain-derived produced AlloP does serve a developmental function independent of progesterone. AlloP also shows a developmental time course prenatally (Pomata et al., 2000) and postnatally, with levels dropping during the first week of life and

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doi:10.1016/j.pbb.2004.03.021

peaking at the end of the second week (Grobin and Morrow, 2001). Sex differences do not appear until 25 days of age.

Previous work in this laboratory and others have shown that limiting maternal contact during the first weeks of life in a rat can lead to altered affective (McIntosh et al., 1999; Zimmerberg et al., 1999, 2003) and cognitive behaviors (Frisone et al., 2002; Lehmann et al., 1999; Pryce et al., 2003) as well as altered general arousal or locomotor activity (Kehoe et al., 1996; Matthews et al., 1996; Zimmerberg and Shartrand, 1992). This paradigm has alternatively been called maternal separation, early deprivation (ED) or social isolation (Lehmann and Feldon, 2000), and is useful as an animal model of child neglect (Pryce and Feldon, 2003; Teicher et al., 2003). We use the term ED to designate a paradigm in which the stress is focused on the individual pup, who is isolated alone, while its dam continues to have nonexperimental pups in the home cage during the isolation, as opposed to paradigms in which the dam is left alone and the pups are separated from her together (maternal separation; Sanchez et al., 2001). Pups isolated from their dams in this fashion emit fewer distress calls (USVs) after a brief maternal separation compared to controls whether isolated from postnatal day (PN) 2 to 6 or PN 7–11 (Zimmerberg et al., 2003b). A sonographic analysis of the USVs made by pups with a history of ED of 6 h per day from PN 2 to 6 revealed fewer call bouts, longer interbout intervals, fewer calls per bout and fewer calls with double shifts compared to controls (Zimmerberg et al., 2003a). As adults, rats with a history of neonatal isolation groom more after a stressor (Zimmerberg et al., 1999) and spend less time on the open arms of a plus maze (Patchev et al., 1997).

Because AlloP is an anxiolytic and present in neonatal brains, we hypothesized that it might play a role in the development of altered behaviors after ED. There were three hypotheses in the present experiment. First, would the neonatal stress of isolation alter the responsiveness of the GABAa receptor to the modulation by AlloP, as demonstrated in the dose-dependent response to AlloP on decreasing USVs? Secondly, would administering AlloP to pups during the isolation period act as a "treatment" for the stress of ED? And finally, would either ED or postnatal AlloP alter sexually dimorphic behavior in adults on the plus maze?

2. Methods

2.1. Subjects

Subjects were bred in this laboratory from female and male Long–Evans hooded rats (Harlan Sprague Dawley, Indianapolis, IN). Pregnant females, determined by presence of a vaginal plug, were individually housed in plastic cages (45 × 25 × 15 cm) in an isolated nursery on a 0700 to 1900 h light–dark cycle with the temperature maintained at 23 °C. Litters were culled to 12 pups on the day following their birth (PN 1), 6 males and 6 females. In each litter, one male and one female were randomly assigned to one of six conditions, and toe-clipped for numbering. The six conditions were control–control (C–C), control–vehicle (C–V), control–AlloP (C–A), early deprivation–control (ED–C), early deprivation–vehicle (ED–V), and early deprivation–AlloP (ED–A). Separate litters were used for neonatal and adult testing. Subjects from adult test litters were weighed again at PN 25 when they were weaned into pairs of same-sex siblings in standard hanging cages. All experimental protocols were approved by the Williams College Institutional Review Animal Care and Use Committee.

2.2. Materials and procedures

2.2.1. Early deprivation PN 2–6

For each litter at the appropriate day from PN 2 through 6, at 1000 h, the dam was removed to a holding cage and the pups placed on a heating pad in a 12-partitioned tray. Pups were quickly sorted into their treatment conditions reported above by looking at their toe number. Vehicle (V) pups received a subcutaneous injection of 20% 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals International, Natick, MA). AlloP (A) pups received a subcutaneous injection of 5 mg/kg of 3-alpha-hydroxy-5 alpha-pregnan-20-one (synthesized by Robert H. Purdy, UCSD, San Diego, CA) suspended in the vehicle. The three male and three female control (C) pups and the dam were quickly returned to the home nest. The three male and three female pups to be separated (ED) were placed individually in plastic containers (11.5 cm diameter) and the cups were placed in a circulating, heated water bath (99 × 74 cm) maintained at 34 °C. After 6 h of isolation, the ED pups were returned to the nursery, the dam again placed in a holding cage, and all the pups were then returned to the home cage with the dam. Rectal temperatures were taken on PN 2 after ED in the litters used for adult testing.

2.2.2. Experiment 1: AlloP dose–response curves in neonates

Testing for USVs took place at 1100 h on PN 7, the day following chronic social isolation from PN 2 to 6. The dam was removed to a holding cage, and all of the pups were taken in their home cage to an adjacent room. The cage was placed on a heating pad maintained at 32 °C. Pups were then marked on their backs with numbers designating injection condition: no injection, vehicle (2 μl of 20% 2-hydroxypropyl-β-cyclodextrin), 2.5 μg/2 μl of AlloP, or 5.0 μg/μl of AlloP. All injections were intracerebroventricular. Injections were staggered so that all pups were tested 15 min after injection.

For testing, a pup was brought into the adjacent USV testing room, which was maintained at 23 °C, and tested by an investigator blind to the injection or postnatal treatment conditions. The pup was placed in a 20 × 20 × 17 cm white plastic box marked into nine equal squares. Microphones attached to bat detectors (Model S-25, Ultrasound Advice, London) were suspended 12 cm above the bottom of the box. The bat detectors were set at 45 kHz (sensitivity range

of 40–50 kHz). The duration and number of selected behaviors were counted using a behavioral observation software, LabTimer, and included inactivity, sniffing, head turning, pivoting, locomoting, twitching and crossing squares. After testing, rectal temperatures and body weights were recorded. The subjects were rapidly decapitated and their brains removed and weighed.

2.2.3. Experiment 2: Plus maze and activity in adults

Activity was assessed in a wooden box ($60 \times 60 \times 15$ cm) with a wire mesh cover. The number of lateral movements were automatically recorded when the subject crossed a photoiodide beam. The plus maze was made of wood and painted black. It contained two open and two closed arms (20×4 in.) extending from a central platform (4×4 in.) and raised 36 in. from the ground. Lights focused upon the maze maintained the open arms at approximately 200 lx and the closed arms from 0 to 10 lx. A VHS camera was suspended from the ceiling above the maze and connected to a video recorder and monitor.

At 125–135 days of age, a subject was removed from the colony room and brought to an adjacent testing room. It was placed in the activity box for 10 min both to determine baseline locomotor activity and to acclimate the subject to the testing room. Between each subject, the box was wiped clean. After activity testing, the investigator reentered the room to place the subject on the plus maze in the center square. Subjects were alternately placed facing a closed or open arm. Between subjects, the plus maze was also wiped clean. An investigator blind to the postnatal treatment condition viewed the subject's behavior on a monitor in another room, and recorded the time the subject spent on the open arms, closed arms and center square using LabTimer. Entrance into an arm or the center was determined by the movement of the rat's head and front paws into that arm. All testing was conducted between 1300 and 1600 h.

2.3. Data analysis

Data were analyzed using an ANOVA multivariate analysis with postnatal condition (control or ED) and postnatal treatment (control, vehicle or AlloP) as between-subject factors. For the neonates, Day 7 drug treatment was also a between-subject factor (no injection, vehicle, Dose 1 or Dose 2 of AlloP). Significant main effects were analyzed by Fisher's LSD tests and significant interactions were analyzed with post hoc multiple means comparison tests (significance level set at $P < .05$).

3. Results

3.1. Experiment 1: AlloP dose–response curves in neonates

The subjects ($n = 144$) tested for USVs were from 12 litters, resulting in 24 subjects per the six postnatal treatments and 6 subjects from each of these six treatments in each drug dose group. Body weight on PN 7 was significantly affected by the postnatal condition, $F(1,120) = 54.20$, $P = .001$, with ED pups weighing less than controls (13.1 ± 0.2 versus 15.2 ± 0.2 g). Postnatal daily injections of vehicle or AlloP did not affect body weight. Brain weight was similarly affected, $F(1,120) = 14.82$, $P = .002$, with ED brains weighing less than controls (0.666 ± 0.006 versus 0.697 ± 0.005 g) and no effect of postnatal daily injections. There were no significant differences among any of the six postnatal treatment groups or PN 7 test day injections on rectal temperature after the USV test (mean of 29.14 ± 0.26 °C for ED and 29.38 ± 0.17 °C for controls).

There was a significant three-way interaction between postnatal condition, postnatal treatment, and PN 7 drug injection condition, $F(6,120) = 4.06$, $P = .001$ on USVs. Fig. 1 shows the dose–response curves for control and ED subjects. There are several significant findings from the post hoc means comparisons. In control subjects, AlloP at 2.5 or 5.0 μg significantly reduced vocalizations compared to no injection or vehicle in the control and vehicle postnatal treatment groups, but only 5 μg significantly reduced vocalizations in the pups who had received AlloP daily from PN 2 to 6. In comparison, in the ED subjects, both doses of AlloP significantly reduced vocalizations in all groups. Secondly, among subjects who did not receive AlloP on the test day (no injection and vehicle groups), control subjects that had received daily AlloP had significantly fewer vocalizations compared to their respective control groups. In contrast, ED subjects that had received daily AlloP had significantly greater vocalizations compared to their respective control groups.

When all the other behaviors were analyzed, there were no effects of postnatal condition (controls versus ED) or postnatal daily drug treatment (control, vehicle or AlloP). While the number or duration of sniffing, pivots, head turns or locomotions did not differ between injection groups, there were some significant main effects of injection condition on PN 7. The percent time that the pups remained inactive was significantly increased by both doses of AlloP, $F(3,120) = 2.84$, $P = .04$, with both doses of AlloP differing from the no injection and vehicle groups but not from each other ($45.5 \pm 4.6 \pm$ for controls and $62.80 \pm 4.2\%$ for AlloP groups). The number of twitches observed differed by injection condition, $F(3,120) = 4.01$, $P = .009$, with pups receiving AlloP making more twitches than no injection or vehicle groups (1.9 ± 0.4 versus 0.3 ± 0.1 and 0.4 ± 0.2 twitches, respectively). The number of squares crossed was less in the AlloP groups than the no injection group, but no different than the vehicle group, $F(3,120) = 2.72$, $P = .05$.

3.2. Experiment 2: Plus maze and activity in adults

The subjects ($n = 76$) were from seven litters. There were no significant differences between postnatal condition or

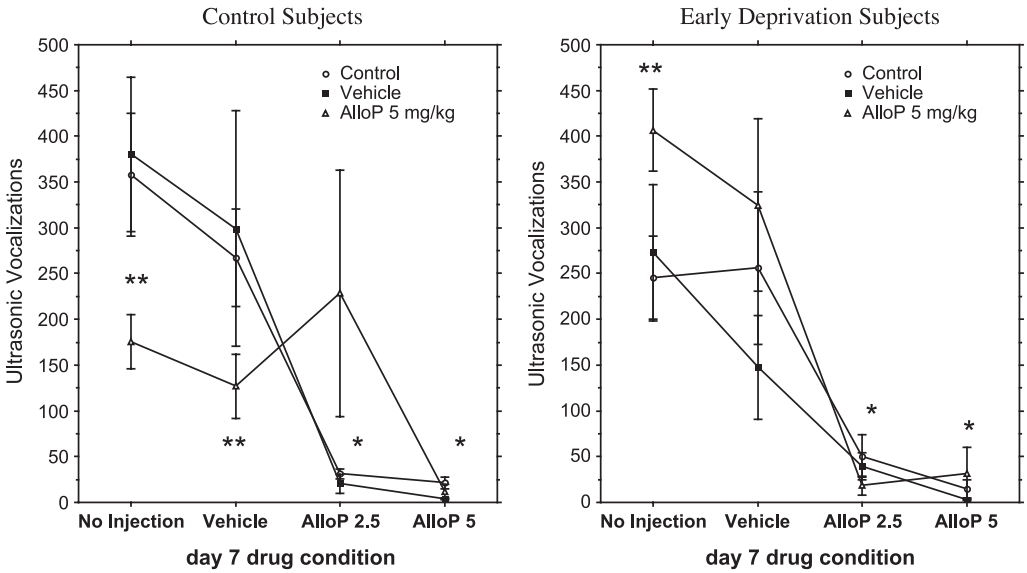

Fig. 1. Mean number of USVs (± S.E.M.) in 7-day-old control and ED subjects from three postnatal daily treatment groups (control, vehicle and AlloP 5 mg/kg) tested after no injection, vehicle injection, or one of two doses of AlloP (2.5 or 5 μg/2 μl. * AlloP intracerebroventricular injections on test day significantly different from respective control groups; * * Postnatal daily AlloP group significantly different from postnatal control and vehicle groups and postnatal daily AlloP–control and AlloP–ED groups significantly different from each other.

drug treatment groups in body weight at 25 or 125 days of age; males were heavier than females.

Plus maze behavior was significantly affected by the interaction of postnatal condition and drug treatment, $F(2,64)=3.66$, $P=.03$ (see Fig. 2). Control subjects spent more time in the open arms plus the middle square if they had received an injection of AlloP on PN 2 through 6. This effect, however, appears to be primarily due to the males in the control–AlloP group (see Fig. 3). In addition, although females spend significantly more time in the open arms in both no injection control and ED groups, males and females no longer differ in the control–AlloP group. The total number of entries into the open arms was not affected by any factor, nor were the number of total arm entries. There were also no significant effects of sex, postnatal

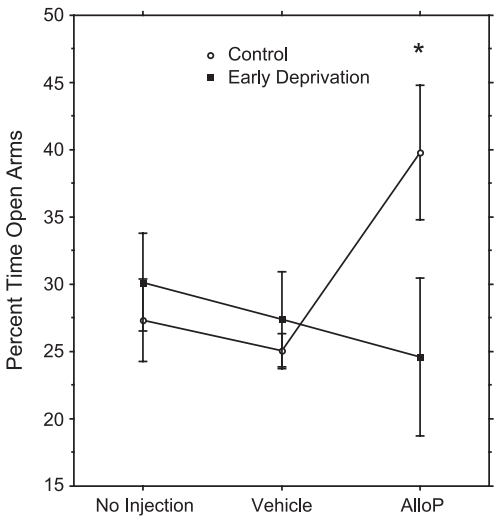

Fig. 2. Percent time in the open arms of the elevated plus maze (± S.E.M.) from one of two postnatal conditions (control or ED) and three treatments (control, vehicle and AlloP). * Significantly different from all other groups.

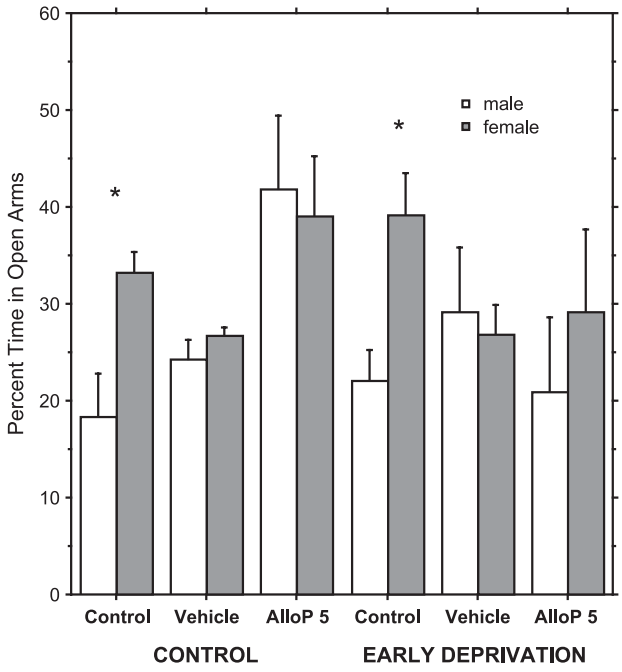

Fig. 3. Percent time in the open arms of the elevated plus maze (± S.E.M.) for male and female subjects from one of two postnatal conditions (control or ED) and three treatments (control, vehicle and AlloP). * Significant male versus female difference.

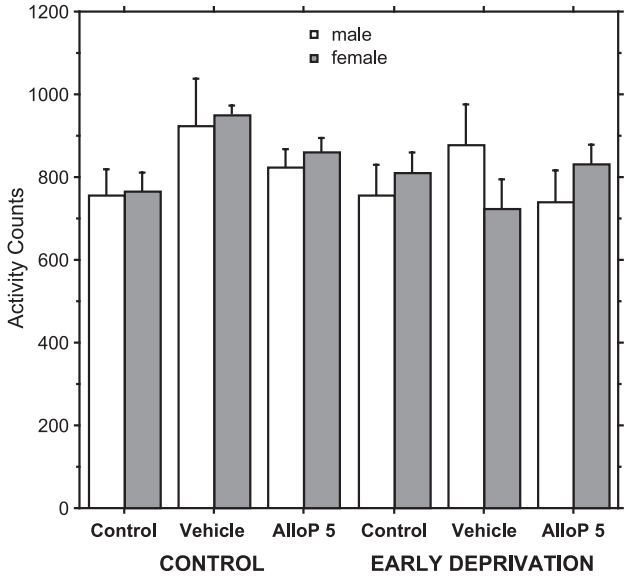

Fig. 4. Mean activity counts (± S.E.M.) in adult male and female subjects from one of two postnatal conditions (control or ED) and three treatments (control, vehicle and AlloP).

condition or drug treatment on activity in the activity boxes (see Fig. 4).

4. Discussion

ED appears to have a selective effect on the rate of USVs after a brief maternal separation while not altering motor behaviors in general. USVs are thought to contribute to the formation of the maternal–infant bond; infant mammals vocalize when separated from their dams to elicit protection, nourishment and warmth. In this study, both male and female ED rats emitted fewer USVs than controls, replicating previous studies (Zimmerberg et al., 2003a, 2003b). Control (nonisolated) pups who had received daily AlloP, however, emitted fewer USVs. Because plasma and cortical concentrations of AlloP are increased by ED (Frisone et al., 2002; Kehoe et al., 2000; Zimmerberg et al., 2002), it is possible that the daily injections had the same effect as higher endogenous levels in nontreated ED subjects in this group. However, if ED subjects had received daily injections of AlloP, the effect was reversed. ED pups with daily AlloP, regardless of sex, vocalized more than their comparative noninjected or vehicle-injected groups. This reversal suggests that there may be dose-dependent effects of chronic AlloP exposure in the neonatal brain, and the combination of endogenous and exogenous AlloP effectively suppressed the stress of ED and reversed the "depressive pattern."

AlloP injection on PN 7 reduced USVs in all groups at the higher doses, 5 μg/2 μl icv. All but one group also had USV suppression after the lower dose, 2.5 μg/2 μl. This was the nonisolated control group who had received daily AlloP on the five previous days. Although a fuller dose–response curve will be explored, this results suggests that 5 days of AlloP administration produced some degree of tolerance to the neurosteroid. This tolerance may have been subserved by alterations in the GABAa receptor subunit composition, as suggested by other chronic studies (Follesa et al., 2001; Gulinello et al., 2001). The effects of AlloP on vocalization were not due to any anesthetic effects of the drug, because there were no marked differences in behavior. Although AlloP-injected subjects spent more time inactive, they also twitched more, had a similar number of body movements and were no different from vehicle in the number of squares crossed.

When tested for persistent effects of either postnatal isolation or injection condition 4 months later, adult subjects revealed no differences in activity either in the activity boxes or in total entries on the plus maze. There were also no differences in anxiety behavior as measured by time on the open arms between ED and control females or males, in contrast to previous studies (Patchev et al., 1997; Zimmerberg et al., 1999). However, daily AlloP administration in the first week of life in nonisolated subjects did cause a persistent change in anxiety behavior on the plus maze. As adults, controls with chronic postnatal AlloP spent more time on the open arms, and showed a reversal of the typical sex difference in the plus maze (females less anxious than males). As discussed below, chronic postnatal AlloP may have feminized the control males but not the ED males. There was also an effect of neonatal vehicle injections which eliminated the expected sex differences in both control and ED subjects. It is possible that the "daily handling" of these subjects had an effect independent of any neurosteroid effect.

A parallel study reported that THDOC, a similarly acting neuroactive steroid derived from corticosterone, administered in conjunction with a different maternal separation paradigm, reduced several of the persistent sequela in adults, namely, increased anxiety on the plus maze, decreased sensitivity of the HPA axis to glucocorticoid feedback and increased transcription of CRH mRNA in the hypothalamus (Patchev et al., 1997). In contrast, we did not find any differences in plus maze behavior between Controls and ED subjects. This discrepancy may be due to our use of an earlier neonatal age (PN 2–6 versus PN 5–10), longer isolation time (6 h versus 2 h) or age of adult testing (we tested at about 130 days of age, while they tested at 75 days of age). However, our effects of postnatal AlloP in decreasing anxiety on the plus maze in adulthood do support their findings with THDOC.

Maternal separation has been linked to changes in the GABAa receptor (Caldji et al., 2000; Insel, 1989). Interestingly, isolation for 30 days after weaning at PN 30 had the opposite effect than isolation during the preweaning period: cortical, hippocampal and plasma levels of AlloP were decreased (Serra et al., 2003). A subsequent stressor increased the AlloP cortical response in these social isolates compared to controls, at the same time resulting in a persistent decrease in several measures of GABAa receptor function.

Three doses of AlloP (5 mg/kg) spaced over 2 days to adult and juvenile rats also resulted in enhanced anxiety behavior on the plus maze (Gulinello and Smith, 2003). In the acoustic startle task, however, only females given the AlloP showed an enhanced startle. These authors propose that up-regulation of the alpha4 subunit of the GABAa receptor mediates these behavioral effects (Gulinello et al., 2001). In our control subjects, 5 days of once daily 5 mg/kg AlloP had the opposite effect (fewer USVs), but did increase anxiety (more USVs) in the ED group. If the GABAa receptors of the ED group were experiencing an effectively prolonged or heightened exposure to AlloP (injected plus endogenously produced due to the isolation), then these results are consistent with those of the adult studies.

Although there were no sex differences in anxiety behavior in the neonates, as expected from previous studies (Zimmerberg et al., 1994, 1995), males appeared to be affected more by the postnatal AlloP treatment. Johnston and File (1990) first reported that female rats made a greater percentage of their entries into the open arms of the plus maze than male rats, and females had a tendency to spend a greater percentage of time on the open arms than males, indicating less anxiety behavior. Both organizational and activational influences of female gonadal hormones can be responsible for the differences on the plus maze (Zimmerberg and Farley, 1993). Male gonadal hormones do not appear to affect anxiety behavior in adult male rats because there were no apparent effects of either neonatal treatment with the antiandrogen flutamide or pubertal orchiectomy. In females, in contrast, both neonatal tamoxifen administration and prepubertal ovariectomy reduced the time spent on the open arms, with the most dramatic reduction observed in the group with both treatments. In the present study, females were less anxious than males in the noninjected control groups, as expected, but control males with a postnatal history of AlloP exposure displayed behavior similar to females.

Although the testosterone surge which has such dramatic effects on organizing sex differences in the brain is ending at PN 2 (Baum, 1990), it is possible that 1 day of AlloP in the context of the increased brain estradiol (testosterone's active metabolite) had an interaction that feminized the brains of the male control subjects. Patchev's laboratory has also shown that daily isolation for 2 h between PN 5 and 10 does not affect adult plus maze behavior, and males were more anxious than females (Mitev et al., 2003). Administration of 2 mg/kg of AlloP prior to the daily isolation reduced the level of anxiety, similar to the results in our study. While females in that study were not affected by maternal separation per se, ovariectomy at 60 days of age masculinized the females who had been isolated as seen in an increase in time in the closed arms of the plus maze, and this masculinization was reversed by concomitant AlloP treatment. This result supports our earlier study showing that ovariectomized females will be more anxious on the plus maze than intact females while males are unaffected by gonadectomy (Zimmerberg and Farley, 1993), but the behavioral results in ED females do not mirror the results in that study. Although there are procedural differences between our studies and theirs, in retrospect, it would have been advantageous for both laboratories to be aware of the estrus stage of female subjects (Frye et al., 2000).

It is possible that the neonatal AlloP administration led to a down-regulation of testosterone metabolism thus demasculinizing as opposed to feminizing the males (Romeo et al., 1999). There are definite plastic interactions between the two systems; for example, females with neonatal testosterone exposure and males with neonatal castrations do not exhibit anxiolysis in either burying or plus maze behavior when administered AlloP as adults (Fernandez-Guasti and Picazo, 1999). In our laboratory, we did not see any effect on adult plus maze behavior after neonatal flutamide administration, which would argue against this mechanism (Zimmerberg and Farley, 1993). However, others do see a demasculinizing effect of perinatal castration on plus maze behavior (Lucion et al., 1996), so further studies which measure adult levels of gonadal hormones might shed more light on the mechanism.

Although there are still some unresolved issues, in general these results do indicate that the neonatal brain is responsive to alterations in AlloP levels, whether endogenous or exogenous. The neonatal stress of isolation did not alter the responsiveness of the GABAa receptor to the modulation by AlloP when measured by the dose-dependent response to AlloP on decreasing USVs. However, administering AlloP to pups during the isolation period did serve act as a "treatment" for the stress of ED. Control pups (whose brains experienced periods of elevated AlloP perhaps analogous to ED) exhibited "depressive" behavior similar to that seen in ED pups. Finally, our indication that males were feminized (or demasculinized) subsequent to postnatal AlloP exposure suggest that neuroactive progesterone metabolites may play a role in mediating the neural organization of stress-related sex differences.

Acknowledgements

This research was supported in part by grants from the National Science Foundation, No. 0074627 (BZ) and IBN-0344103.

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