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A Potential Gastrointestinal Link between Enhanced Postnatal Maternal Care and Reduced Anxiety-Like Behavior in Adolescent Rats

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Abstract

Early life experience impacts emotional development in the infant. In rat pups, repeated brief (i.e., 15 min) maternal separation (MS15) during the first 1–2 postnatal weeks has been shown to increase active maternal care and to reduce later anxiety-like behavior in the offspring. We hypothesized that the anxiolytic effect of MS15 is partly due to increased intestinal release of cholecystokinin (CCK) in rat pups as a result of increased maternal contact. We predicted that rats with a history of MS15 would display less anxiety in the elevated plus maze (EPMZ) and novelty-suppressed feeding (NSF) tests compared to non-separated (NS) controls, and that the anxiolytic effect of MS15 would be attenuated in rats in which daily MS15 was accompanied by systemic administration of a CCK-1 receptor antagonist (i.e., devazepide). Treatment groups included NS control litters, litters exposed to MS15 from postnatal days (P)1–10, inclusive, and litters exposed to MS15 with concurrent s.c. injection of devazepide or vehicle. Litters were undisturbed after P10 and were weaned on P21. Subsets of adolescent males from each litter were tested in the EPMZ on P40-41, while others were tested for NSF on P50-52. As predicted, rats with a developmental history of MS15 displayed reduced anxiety-like behavior in the EPMZ and NSF tests. The anxiolytic effect of MS15 was preserved in vehicle-treated rats, but was reversed in devazepide-treated rats. These results support the view that endogenous CCK-1 receptor signaling in infants is a potential pathway through which maternal-pup interactions regulate the development and functional organization of emotional circuits that control anxiety-like behavior in the offspring.

Keywords

cholecystokinin; devazepide; early life experience; visceral circuits; development

Introduction

In mammals, the duration and quality of early maternal care exerts pronounced physiological and behavioral effects in the developing offspring (cf. Koehnle and Rinaman, 2009, for a recent review). Although the specific aspects of maternal care that are most critical to the infant's development have yet to be defined, it seems likely that nursing/suckling-related gastrointestinal and somatosensory stimuli play a major role (Hofer, 1973; Uvnäs-Moberg, 1989; Uvnäs-Moberg, Widström, Marchini, & Winberg, 1987; Weller & Feldman, 2003). Visceral and somatosensory signals derived either directly or indirectly from suckling may synergize with gastrointestinal hormones such as cholecystokinin (CCK), which decreases in rat pups during maternal separation and increases after maternal reunion (Weller *et al.*,

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1992). CCK is released from intestinal mucosal secretory cells when nutrients, including those present in milk (Lewis & Williams, 1990), are transferred from stomach to small intestine (Liddle, 1995). CCK subsequently binds to CCK-1 receptors expressed in the gastrointestinal tract and along vagal afferent fibers (Moran, Baldessarini, Salorio, Lowery, & Schwartz, 1997; Schwartz & Moran, 1998) to increase the activity of vagal sensory inputs to the medullary dorsal vagal complex (Moran *et al.*, 1997). CCK-1 receptors are especially abundant and widely distributed in the upper gastrointestinal tract in neonatal rats (Robinson, Moran, Goldrich, & McHugh, 1987; Robinson, Moran, & McHugh, 1985), in which intragastric milk and exogenously administered CCK have calming effects (Blass & Shide, 1993; Weller & Blass, 1988; Weller & Dubson, 1998). Further, functional antagonism of CCK-1 receptors with systemically administered devazepide (also called L-364,718 or MK-329) counteracts the calming effects of milk infusion or normal suckling in rat pups (Blass & Shide, 1993; Weller & Dubson, 1998).

Ascending visceral sensory pathways from body to brain undergo a significant amount of synaptic assembly and refinement in rats during the first two weeks of postnatal life (Rinaman et al., 2000), a period during which the amount and quality of maternal care can exert life-long effects on stress responsiveness and emotionality in the offspring. In particular, brief (i.e., 15 min) maternal separation of rat pups (MS15) each day during the first 2 weeks postnatal leads to significantly reduced HPA axis responses to stress and reduced anxiety-like behavior when the offspring are tested later in life (Huot, Gonzalez, Ladd, Thrivikraman, & Plotsky, 2004; Ladd *et al.*, 2000; Plotsky & Meaney, 1993; Wigger & Neumann, 1999). Previous studies have reported observational evidence that MS15 evokes potentiated maternal care (i.e., licking/grooming, arch-backed nursing) after pup reunion (Liu, Diorio, Tannenbaum, Caldji, Francis, Freedman, Sharma, Pearson, Plotsky, & Meaney, 1997), thereby promoting increased tactile and viscerosensory stimulation of the pups. Hypothalamic and limbic forebrain systems that receive and process these sensory feedback signals overlap with those that regulate emotional arousal and stress responsiveness; thus, the long-lasting influence of MS15 on the offspring's later responses to stressful and emotionally evocative stimuli may be linked to altered activity within those neural systems as they assemble postnatally.

The present study was designed to test the hypothesis that the ability of daily MS15 to reduce later anxiety-like behavior is at least partly due to increased gastrointestinal CCK release and signaling at CCK-1 receptors. We predicted that rats with a developmental history of MS15 would display reduced anxiety-like behavior, as previously reported, and that the anxiolytic effect of MS15 would be attenuated or reversed in rats in which MS15 was accompanied by systemic administration of devazepide to antagonize endogenous CCK-1 receptors.

Methods

Animals

Experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The progeny of 24 multiparous pregnant Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) were used in this study. Pregnant rats arrived at our animal facility at a gestational stage between embryonic days 13 and 18, and were housed singly in opaque polyethylene tub cages (72 × 22 × 20 cm high) with soft woodchip bedding and a wire lid in a controlled environment (20–22°C) with a 12-hour light dark cycle (lights on at 0700 hr) and *ad libitum* access to water and pelleted rat chow (Purina #5001, Bethlehem, PA). Two or three pregnant dams arrived at our facility in each shipment from the animal supplier, over a 15 month period. Litters were assigned sequentially to the four treatment groups based on the day/time that newborn pups were found, and no more than one litter from each shipment was assigned to the same treatment group. Tub cages were checked each morning and evening to accurately determine the date of parturition, designated postnatal day (P)0. All

litters were culled to 8 pups and then weighed *en masse* on P1. Every litter contained at least 2 pups of each sex; however, behavioral results reported here include data collected only from males. Each litter was transferred together with the dam to a clean tub cage with fresh bedding on P7, and again on P14. Litters were assigned sequentially and semi-randomly to one of four postnatal treatment groups (5–8 litters/group), described below.

Postnatal Treatment Groups

Non-separated (**NS**) control litters (n=5 litters) were left undisturbed after litter culling and weighing on P1, apart from the two weekly cage changes mentioned above. The NS condition represents our typical animal-facility reared environment.

Pups in **MS15** litters (n=5 litters) were separated from their dam for 15 min/day from postnatal day 1 (P1) through P10, inclusive, at approximately the same time each day (~0900–1000 hr). For this purpose, the dam was briefly removed from the home tub cage and placed into an adjacent clean tub. Using gloved hands, all of the pups were removed from their home cage along with a handful of home cage bedding and were placed together into a smaller polyethylene tub (26.7 × 15.2 × 12.7 cm high). The dam was then returned to her home cage and the small tub containing the pups was brought into an adjacent room. Pups were placed into an incubator (34°C, ~60% humidity). The entire litter was weighed *en masse* on P5 and P10 just before being placed into the incubator. Incubator conditions were controlled to approximate the home cage "nest" environment (~36°C, ~30–50% humidity). Pups remained in the incubator for 15 minutes and then, using gloved hands, the entire litter was returned simultaneously to their dam in the home cage.

Pups in **devaz+MS15** litters (n=8 litters) were treated as described above for MS15 litters, except each pup received an injection of the CCK-1 receptor antagonist devazepide (2.3 nmol in 100 nl vehicle, s.c.) each day at the onset of MS15 from P1 through P10. This dose was selected based on previous work demonstrating the efficacy of devazepide to block the behavioral effects of exogenously administered and endogenously released CCK in rat pups (Blass & Shide, 1993; Weller & Dubson, 1998; Weller, Smith, & Gibbs, 1990).

Pups in **veh+MS15** litters (n=6 litters) were treated as described above, but each pup received an injection of vehicle solution alone (5% DMSO, 5% Tween 80 in 0.15M NaCl, s.c.) each day at the onset of MS15 from P1 through P10.

Body Weight Assessments and Weaning

The effects of postnatal treatment on litter weights (i.e., growth curves) was monitored by weighing each MS15 litter *en masse* on P1, P5, and P10. Pups in NS control litters were weighed *en masse* after litter culling on P1, but were not weighed again until weaning (P21), at which time individual body weights were obtained from male weanling rats in all litters. Litter and individual body weight data were tabulated in Excel spreadsheets and later analyzed by ANOVA using SPSS statistical software, with postnatal treatment group as the independent variable. Male rats were group housed with male littermates after weaning, and remained undisturbed (except for weekly transfer to a clean cage with fresh bedding) until the behavioral studies described below were initiated.

Elevated Plus Maze (EPMZ)

On P40 or P41, one to three male rats from each litter were tested in the EPMZ during the early light phase of the photoperiod, between 0900–1000 hr. The EPMZ was located in a quiet, isolated behavioral testing room adjacent to the animal housing room. Testing room lighting was moderate, with even illumination of both open arms (brighter) and both closed arms (darker). A video camera was positioned to film both open arms and the center of the maze.

For testing, each rat was placed into the maze center with its nose pointing away from the camera. The experimenter then left the testing room. After 5 min, the experimenter re-entered the room, removed the rat from the maze, and returned it to its home cage in the adjacent housing room. The EPMZ was cleaned with a mild odor-neutralizing cleanser and dried thoroughly between rats.

Videotapes were reviewed without knowledge of each rats' developmental group. Scored variables included (1) number of open arm entries, (2) number of closed arm entries, (3) time spent in open arms, and (4) time spent in closed arms. The ratio of time spent in open vs. closed arms (O:C ratio) was computed for each rat. Higher O:C ratios indicate a less anxious phenotype, and vice-versa; the number of arm entries provides additional indices of locomotor activity (Pellow, Chopin, File, & Briley, 1985). Data were analyzed by multivariate ANOVA using SPSS statistical software, with postnatal treatment group as the independent variable. To avoid the inflated sample size that accompanies data collection from several pups per litter, behavioral data were analyzed after averaging values from individual pups within each litter, so that n = number of litters (5–7) per postnatal treatment group. When f values indicated a significant effect of postnatal group on EPMZ results, the ANOVA was followed up with post-hoc t-tests, with Bonferroni correction for multiple comparisons. Group differences were considered significant when $P < 0.05$.

Novelty-Suppressed Feeding (NSF)

On P50-52, food was removed from the home cages of group-housed littermates between 0900–1000 hr, and bedding was replaced to remove hidden chow. Water was not removed. After 24 hrs of food deprivation, 1 or 2 male rats from each litter (different from those previously tested in the EPMZ) were tested in the NSF paradigm in an adjacent room. For this purpose, rats were placed, one at a time, into a novel square arena (3 ft × 3 ft white plastic floor, upward-sloping black sides) with a familiar food pellet fixed at the center. Testing room lighting was moderate, with even arena illumination. The latency for each rat to begin eating (i.e., contacting the food pellet with its teeth) in the center of the arena was recorded. Latency to feed was recorded by an experimenter sitting quietly several feet away from the arena. Cages were coded so that the experimenter had no knowledge of the rats' developmental group history. The arena was cleaned with a mild odor-neutralizing cleanser and dried thoroughly between rats, and a fresh food pellet was used for each rat.

Littermates not used in the NSF test were instead tested for latency to begin eating in their home cage when food was returned after the 24 hr deprivation period. For this purpose, latency to feed was defined as the time taken for the first rat within each home cage to begin eating.

Feeding latency data were analyzed by two-way ANOVA using SPSS statistical software, with postnatal treatment group and testing environment (i.e., novel arena vs. home cage) as independent variables. Behavioral data were analyzed after averaging values from individual pups within each litter, so that n = number of litters per postnatal treatment group. When f values indicated a significant effect of postnatal group on latency to eat in either environment, the ANOVA was followed up with post-hoc t-tests, with Bonferroni correction for multiple comparisons. Group differences were considered statistically significant when $P < 0.05$.

Results

Body Weights

Litter weights on the day of culling (P1) did not differ among the 4 postnatal treatment groups [ANOVA $F(3,20) = 0.78$, $P = 0.52$]. On P1, NS control litters weighed 53.8 g ± 0.5, similar to P1 litter weights in the three MS15 conditions (Fig. 1). As shown in Figure 1, there also was

no significant effect of treatment group (i.e., MS15 with or without daily s.c. injection of vehicle or devazepide) on litter weights assessed on P5 and P10 [repeated measures ANOVA for P1, P5, and P10; $F(2,16) = 0.61$, $P = 0.55$]. As mentioned in the methods, litter weights were not assessed for NS controls after P1. However, the average body weights (i.e., average per litter) of weanling males from all 4 postnatal treatment groups were similar on P21 [$F(3,20) = 1.9$, $P = 0.15$], evidence that postnatal growth curves did not differ as a function of treatment group.

Elevated Plus Maze

Multivariate one-way ANOVA revealed a significant effect of postnatal treatment on time spent in the open arms of the EPMZ [Fig. 2A, open bars; $F(3,20) = 18.71$, $P < 0.01$], time spent in the closed arms [Fig. 2A, closed bars; $F(3,20) = 15.52$, $P < 0.01$], and the open:closed arm time ratio [Fig. 2B; $F(3,20) = 18.00$, $P < 0.01$]. For each of these three variables, post-hoc t-tests (with Bonferroni correction for multiple comparisons) demonstrated significant differences between the NS and MS15 groups, between the NS and veh+MS15 groups, between the MS15 and devaz+MS15 groups, and between the veh+MS15 and devaz+M15 groups ($P < 0.01$ for each comparison, for each variable; Fig. 2A, B). Thus, adolescent male rats with a developmental history of MS15 demonstrated decreased anxiety-like behavior in the EPMZ, as evidenced by increased open-arm time and decreased closed-arm time compared to NS controls, and devazepide (but not vehicle) treatment reversed the MS15 effect. There was no significant effect of postnatal treatment on the total number of arm entries during the EPMZ test [$F(3,20) = 1.56$, $P = 0.23$; Fig. 2C].

Novelty-Suppressed Feeding

One-way ANOVA revealed a significant effect of postnatal treatment on latency to begin eating in the center of the open field [Fig. 3; $F(3,19) = 12.32$, $P < 0.01$]. Post-hoc t-tests (with Bonferroni correction for multiple comparisons) demonstrated significant differences between the NS and MS15 groups, between the NS and veh+MS15 groups, between the MS15 and devaz+MS15 groups, and between the veh+MS15 and devaz+M15 groups ($P < 0.01$ for each comparison; Fig. 3). Conversely, there was no significant effect of postnatal treatment group on the latency of littermates to feed in their home cage when food was returned [range 6–34 secs across litters; $F(3,19) = 0.28$; $P = 0.86$].

Discussion

Our results in devazepide-treated rats support the novel hypothesis that the effect of MS15 to attenuate later anxiety-like behavior involves CCK-1 receptor signaling during postnatal development. Litters exposed to MS15 with or without daily s.c. injections of devazepide or vehicle displayed similar body weight growth curves (i.e., litter weights) during the course of MS15 from P1-P10. Further, the individual body weights of rats in all four treatment groups (including NS controls) were similar at weaning, arguing against any effect of postnatal treatment to alter the overall health of the developing rats. Our results confirm that adolescent male rats with a developmental history of MS15 display reduced anxiety-like behavior in the EPMZ and NSF tests, and demonstrate that this effect is maintained in MS15 rats that received concurrent vehicle administration, arguing against any general effect of daily s.c. injections to counteract the anxiolytic effects of MS15. Instead, our results support the view that enhanced CCK release in infant rats is a potential pathway through which maternal care can regulate the postnatal development and functional organization of central visceral/emotional circuits that underlie anxiety-like behavior. It should be noted that these results in male rats may not generalize to females. We did not include female rats in the present study due to concerns regarding the earlier onset of puberty in females compared to males, and the known effects of estrous cycle on EPMZ behavior (Marcondes, Miguel, Melo, & Spadari-Bratfisch, 2001; Sadeghipour *et al.*, 2007). The foremost goal of the present study was to test the hypothesis

that devazepide treatment would attenuate the anxiolytic effects of MS15. Now that positive results have been obtained in male rats, it will be important to determine their generalizability to females.

EPMZ and NSF tests

The elevated plus maze (EPMZ) is well-validated and widely used for eliciting and assessing anxiety-like behavior in laboratory rats (Fernandes & File, 1996; Hogg, 1996; Pellow *et al.*, 1985), in which EPMZ behavior is sensitive to drugs that increase or decrease anxiety in humans (Baldwin, Johnston, & File, 1989; Charney, Heninger, & D.E. Redmond, 1983; Pellow, Johnston, & File, 1987; Szemeredi *et al.*, 1991). Previous work has shown that EPMZ behavior is altered by MS15 and other experimental paradigms that alter the amount and/or quality of maternal care received postnatally (Daniels, Pietersen, Carstens, & Stein, 2004; Kalinichev, Easterling, Plotsky, & Holtzman, 2002; Lehmann & Feldon, 2000; Slotten, Kalinichev, Hagan, Marsden, & Fone, 2006). The present results confirm that MS15 increases the amount of time that rats spend in the open arms of the EPMZ, and demonstrate that this anxiolytic effect is reversed in rats in which MS15 was performed concurrent with systemic devazepide to antagonize CCK-1 receptors.

Variations in postnatal maternal care also have been reported to alter novelty-induced suppression of appetitive behavior (i.e., NSF) in rats (Caldji *et al.*, 1998). Similar to behavior in the EPMZ, NSF in rodents also is modified by clinically effective anti-anxiety drugs (Bodnoff, Suranyi-Cadotte, Quirion, & Meaney, 1989). After a period of food deprivation, longer latencies to begin feeding in the center of an unfamiliar open field are interpreted as more anxious behavior, whereas shorter latencies to begin feeding are interpreted as less anxious behavior. Our results confirm that MS15 decreases the amount of time it takes for food-deprived rats to begin eating in the center of a novel open arena, and demonstrate that this anxiolytic effect is reversed in rats that received devazepide treatment concurrent with MS15. Conversely, there was no significant effect of postnatal treatment group on the latency of food-deprived rats to feed in their home cage, arguing against any effect of postnatal treatment on general appetitive responses to food.

MS15 effects on neural system development

It seems likely that repeated daily MS15 from P1-P10 affects the postnatal development of central somatosensory and/or viscerosensory neural pathways. Indeed, neonatal rearing conditions were reported to have a significant impact on stress-evoked release of norepinephrine release in the paraventricular nucleus of the hypothalamus (PVN), associated with altered alpha2 autoreceptor binding in the medullary dorsal vagal complex (DVC) (Liu, Caldji, Sharma, Plotsky, & Meaney, 2000). Nor/adrenergic neurons within the DVC are recruited by vagal sensory inputs, and are activated in neonatal and adult rats after CCK administration (Rinaman *et al.*, 1995; Rinaman, Hoffman, Stricker, & Verbalis, 1994). A subset of DVC nor/adrenergic neurons (including those that respond to systemic CCK) project directly to the PVN (Rinaman *et al.*, 1995) to activate corticotropin-releasing hormone-positive neurons at the apex of the HPA axis (Rinaman & Dzmura, 2007), and this projection pathway undergoes significant maturation in rats during the first two weeks postnatal (Rinaman, 2001; Rinaman *et al.*, 1995). Conceivably, increased or decreased CCK signaling during this developmental period could alter nor/adrenergic projection pathways from the DVC to the PVN, which could contribute to experience-dependent differences in later stress responsiveness and affective behavior.

Devazepide and endogenous CCK

It is unclear how long each daily dose of devazepide (2.3 nmol) remained effective in blocking CCK-1 receptors in rat pups. Based on published (Chang & Lotti, 1986; Lotti, Pendleton,

Gould, Hanson, & Chang, 1987) and unpublished data (T. Moran and A. Weller, personal communication), we assume that the effect persisted for at least one and perhaps a few hours after injection. Devazepide selectively antagonizes CCK-1 and is ineffective at CCK-2 receptors (Lotti *et al.*, 1987). However, devazepide readily crosses the blood-brain barrier (Pullen & Hodgson, 1987), thereby antagonizing CCK-1 receptors located both in the periphery and in the brain after systemic administration. We hypothesize that the devazepide effects observed in our study are due to blockade of peripheral CCK-1 receptors associated with gastrointestinal tissue and vagal afferents, but we cannot rule out the possibility of effects at different peripheral or central sites.

Rat pups should not have experienced any significant decrease in endogenous CCK levels during daily 15 min separations. However, we posit that upon maternal reunion, increased somatosensory-related stimulation promotes increased gastric emptying and delivery of existing milk from the stomach into the duodenum (i.e., similar to maternal licking-induced micturition and defecation reflexes), contributing to an initial increase in plasma CCK levels. This idea is supported by a report that dog puppies exhibit increased plasma CCK levels when they are returned to their littermates after a period of isolation (Uvnäs-Moberg *et al.*, 1987), suggesting that somatosensory and other types of sensory stimulation provided by physical contact is sufficient to promote CCK release even in the absence of suckling. Plasma CCK levels also rapidly increase in human babies (Uvnäs-Moberg, Marchini, & Winberg, 1993) and in infant rats during suckling (Blass & Shide, 1993). The rapid time course suggests that tactile and/or visceral stimulation contributes to the initial elevation of plasma CCK during suckling onset, whereas milk itself later promotes and sustain CCK release during the nursing bout and after its conclusion (Uvnäs-Moberg *et al.*, 1993). In this regard, plasma CCK levels increase more quickly and reach higher peak levels in calves that are allowed to suckle non-nutritively after a milk meal (Passille, Christopherson, & Rushen, 1993), evidence for a role of continued tactile and/or visceral sensory-motor factors in promoting nutrient-induced CCK release. In human infants fed milk via a nasogastric tube, CCK levels increased by a markedly greater amount when infants were maintained in skin-to-skin contact with a caregiver during and after the feeding, compared to CCK levels in infants fed through a nasogastric tube without concurrent contact (Tornhage, Serenius, Uvnas-Moberg, & Lindberg, 1998). If the MS15 paradigm as used in our study promotes increased or focused maternal care received by pups, this could increase their total exposure to endogenous CCK, and thus periodically increase gastrointestinal vagal sensory activation over the course of early postnatal development. Exploration of the potential consequences of such increased activation on the developmental assembly and functional organization of central visceral/emotional circuits is an area that is ripe for exploration using experimental paradigms that manipulate early maternal-infant interactions.

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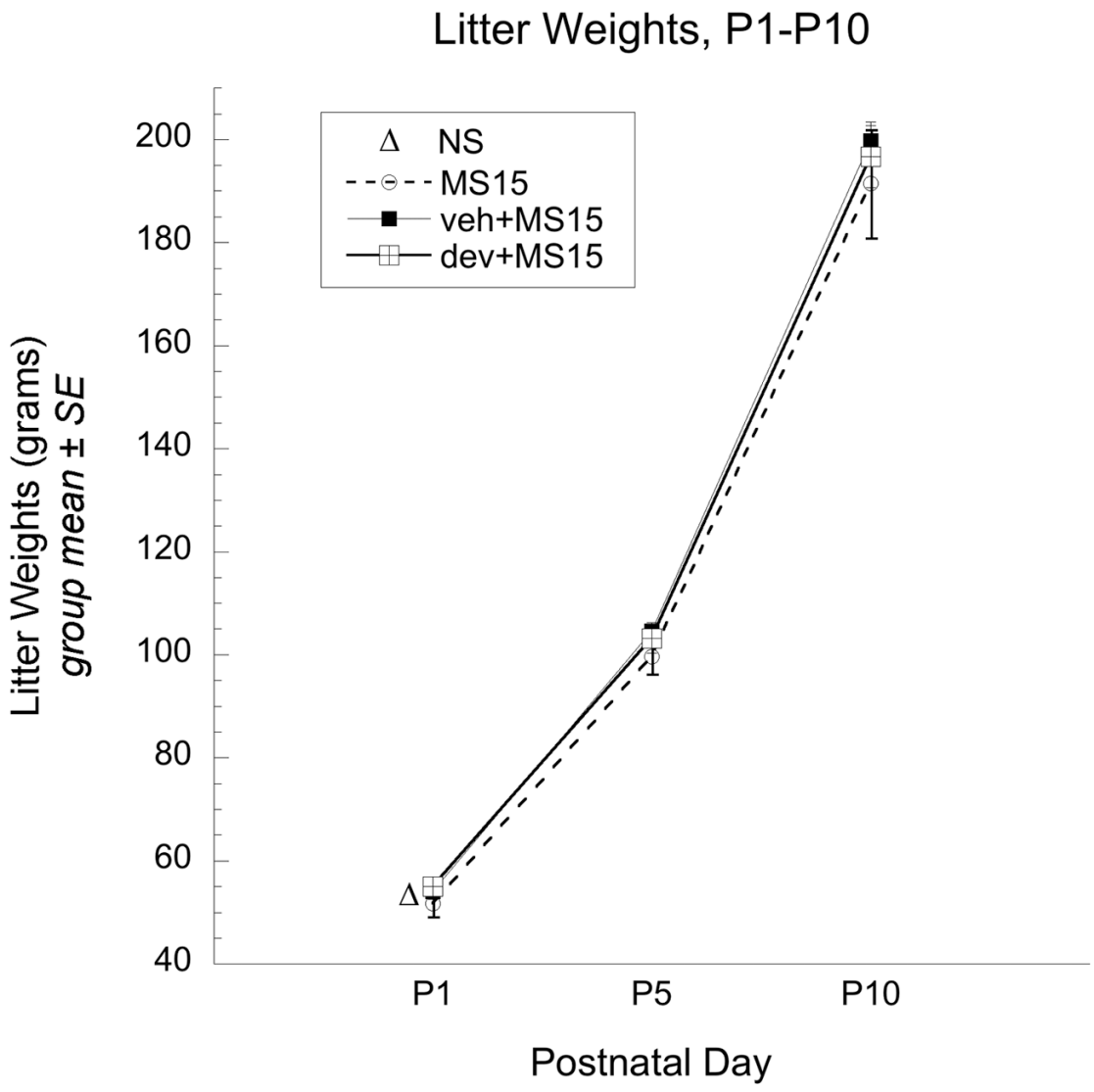

Figure 1.
Line graph showing group mean litter weights (± SE) assessed on P1, P5, and P10 in the three MS15 postnatal treatment groups (n=5–8 litters per group). There was no significant effect of devazepide or vehicle treatment on litter weights. Only a single point at P1 is shown for the NS group, as NS litters were not weighed on P5 or P10.

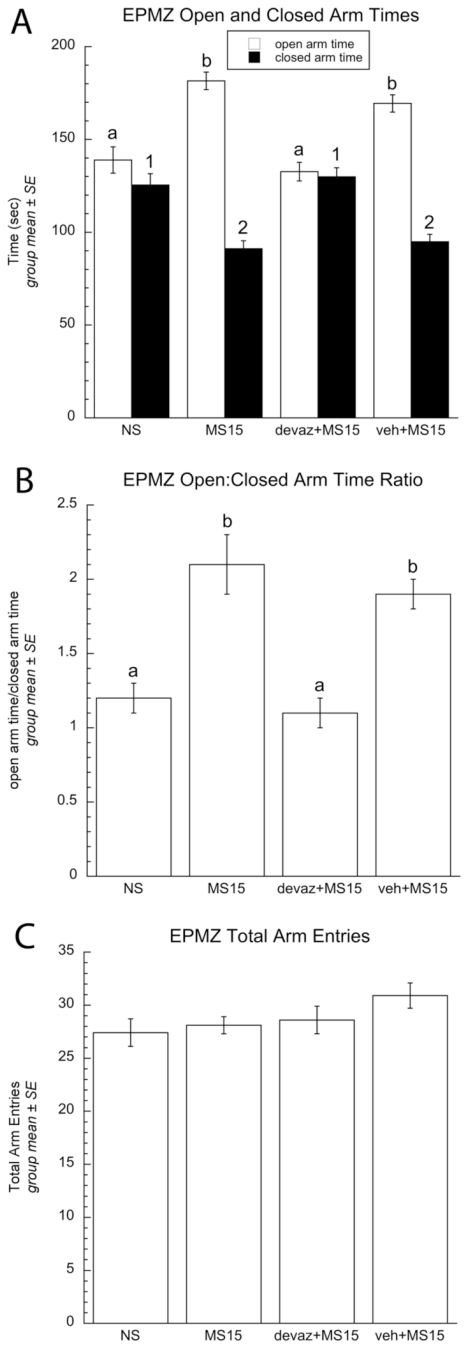

Figure 2.

Bar graphs depicting results in the elevated plus maze (EPMZ) in adolescent male rats (group means ± SE; n=5–8 litters/group). A, open and closed arm times. Open bars with different letters (a,b) and closed bars with different numbers (1,2) are significantly different ($P < 0.05$). MS15 increased open arm time and decreased closed arm time compared to results in NS controls. The MS15 effect was reversed in rats that received concurrent devazepide treatment (devaz+MS15), but was maintained in rats that received vehicle treatment (veh+MS15). B, ratio of open:closed arm times in NS controls and the 3 MS15 groups. The anxiolytic effect of MS15 (i.e., higher ratio) was reversed in devaz+MS15 rats, but was maintained in veh+MS15 rats. Bars with different letters (a,b) are significantly different ($P < 0.05$). C, there was no

significant effect of postnatal treatment on the total number of arm entries during the EPMZ test.

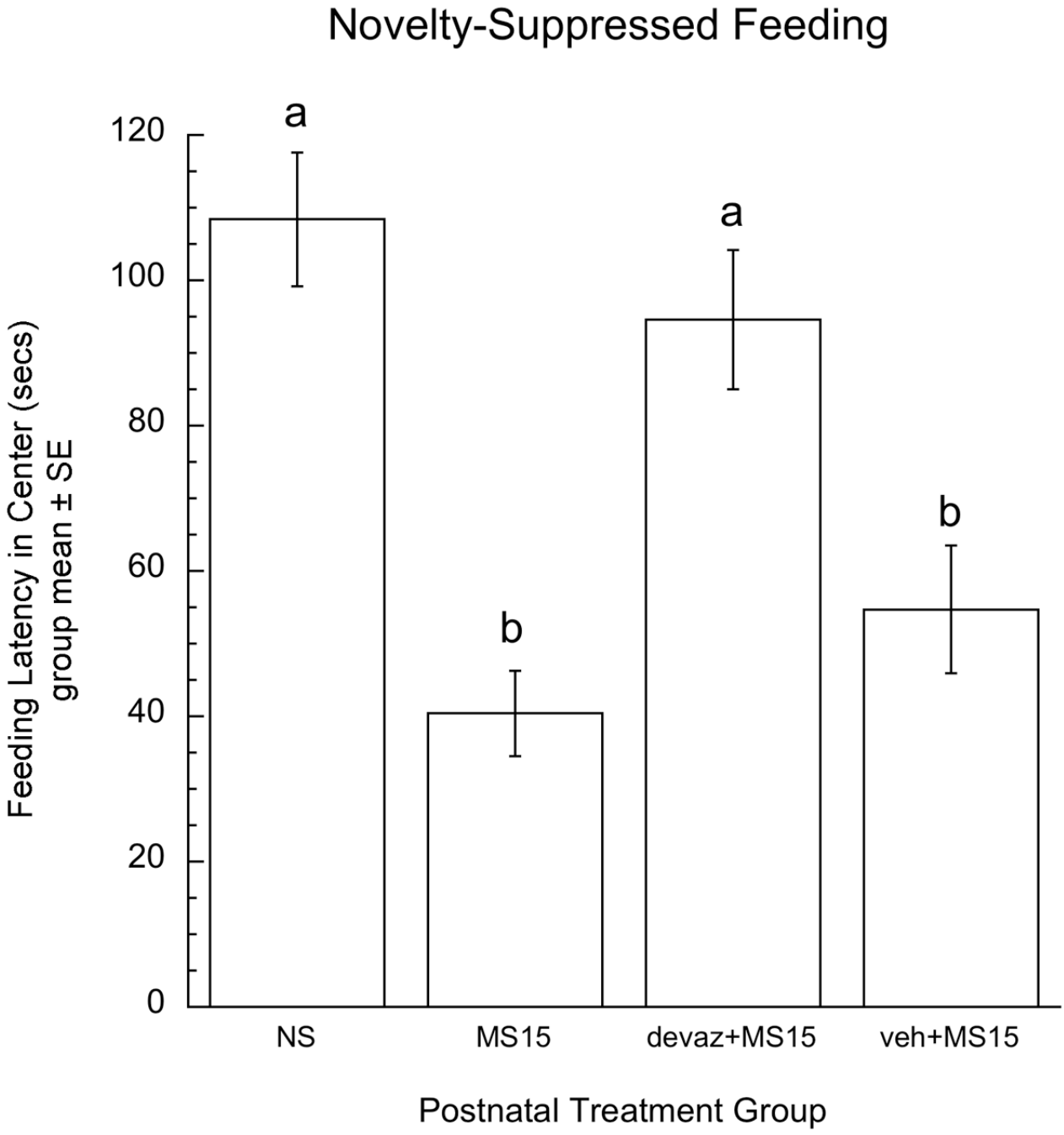

Figure 3.
Bar graphs depicting results in the novelty-suppressed feeding (NSF) test in adolescent male rats (group means ± SE; n=5–7 litters/group). Bars with different letters (a,b) are significantly different ($P < 0.05$). Compared to feeding latencies in NS controls, MS15 decreased the latency to begin eating in the novel arena. This effect was reversed in rats that received devazepide treatment concurrent with MS15 (devaz+MS15), but was maintained in rats that received vehicle treatment (veh+MS15).