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Research report

Maternal separation enhances neuronal activation and cardiovascular responses to acute stress in borderline hypertensive rats

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Abstract

There is much evidence suggesting early life events, such has handling or repeated separations from the nest, can have a long-term effect on the biological and behavioral development of rats. The current study examined the effect of repeated maternal separation (MS) on the behavioral, cardiovascular, and neurobiological responses to stress in subjects vulnerable to environmental stressors as adults. Borderline hypertensive rats (BHR), which are the first generation offspring of spontaneously hyperternsive and Wistar-Kyoto rats, were separated from the dams for 3 h per day from postnatal day 1 through 14. Non-separated controls remained in the home cage. When allowed to explore the open field chamber for 60 min as adults, MS subjects had significantly greater locomotor activity compared to controls. All subjects were exposed to 30 min of restraint stress during which time mean arterial pressure (MAP) and heart rate (HR) were measured. Although both groups had comparable increases in MAP, MS animals displayed significantly higher HR throughout the stress period. Finally, MS subjects had significantly more stress-induced Fos positive cells, an estimate of neuronal activation, in the central nucleus of the amygdala (CeA), paraventricular nucleus of the hypothalamus (PVN), and the bed nucleus of the stria terminalis (BNST), each of which plays an important role in organizing the biobehavioral response to stress. These results suggest that maternal separation can further enhance stress reactivity in this model and may represent a useful approach for studying the relationship between early life events and future vulnerability to stressful situations.

Keywords: Maternal separation; Stress; Open field; Cardiovascular; Rats; Fos

1. Introduction

Chronic exposure to stress is associated with a variety of physical and psychological disorders. Although multifactorial in etiology, prolonged exposure to adverse experience is widely thought to be contributory to the development of such divergent conditions as ulcer, cardiovascular disease, immunosuppression, anxiety disorders and depression, among many others [4,13,17]. The biological pathways through which stressful events may lead to physiopathology include enhanced activation of the both the sympathoadrenal–medullary and hypothalamic–pituitary–adrenal (HPA) systems. This results in a constellation of responses that are normally adaptive during situations of acute challenge; increased adrenal release of catecholamines and glucocorticoids, elevation of blood pressure and heart rate, and enhanced cognitive processing of emotionally relevant stimuli. Although these events make good biological sense in the short term because they mobilize internal resources that increase the probability of successfully negotiating the stressful event, more long-term stress-induced activation of these systems can shift from being adaptive to pathogenic.

However, stress is not a unitary phenomenon and considerable variation exists in individual responses to chronic exposure to stress; indeed, a negative health outcome is not a certainty. In order to more clearly understand what factors influence individual variation in the response to stress, and therefore the potential vulnerability to disease, much attention has focused on understanding the roles of mediating variables such as genetics, gender, experience, and coping skills. Of particular interest for the current study is the extent to which alterations in early life experience can shape future responses to stress. Maternal separation, which typically involves isolating each pup individ-

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ually from the dam and littermates for at least 3 h per day, is a widely used procedure that produces an adult biobehavioral profile of increased behavioral and endocrine responses to stress [21,22,26]. On the other hand, separation for less time, usually 15 min per day (referred to as handling), produces a stress-immunization effect in adulthood characterized by substantial reductions in hypothalamic–pituitary adrenal axis and behavioral responses to stress [20,24]. The precise mechanisms by which these early life events translate into long-term changes in the offspring are unknown, but variations in the quality of maternal care, which may be exacerbated by the intervention, have received considerable attention [7,8]. More recently, evidence has emerged suggesting that early life experience can produce a structural change of the DNA that alters gene expression in certain brain areas that may in turn affect stress responsivity [25,37,38].

Experiential factors typically do not exert their effects independently, however, but rather interact with other existing organismal variables, such as genetic family history. In recent years, we have become interested in using the borderline hypertensive rat (BHR) as a model with which to examine these relationships. The BHR is the first generation offspring of the spontaneously hypertensive rat (SHR) and the normotensive Wistar-Kyoto (WKY) rat. Although resting blood pressure in the BHR remains in the borderline range (c. 140/90 mmHg), this model shows considerable sensitivity to environmental stressors in that either chronic behavioral stress or increased dietary sodium elevates resting pressure to hypertensive levels [18,32]. In addition, alterations in experience in the postnatal period are capable of affecting stress-induced cardiovascular responses in BHR. Specifically, cross-fostering BHR pups to WKY dams rather than leaving them with their natural SHR mothers, significantly reduces their blood pressure to acute stress [31]. Thus, whereas adult BHR demonstrate a susceptibility to stress-induced hypertension, there is also evidence suggesting that early life events can modify future reactivity to stressful situations. To further understand how early experience may affect adult responses to stress in this model, the present study was designed to examine the extent to which maternal separation affects behavioral, neurobiological, and cardiovascular responses to stressful and novel situations in the BHR.

2. Methods

2.1. Subjects

Subjects used in this study were male borderline hypertensive rats (BHR). These animals were the first generation offspring of female spontaneously hypertensive rats (SHR) and male Wistar-Kyoto, both of which were purchased from Taconic Farms (Germantown, NY). Following harem breeding, pregnant SHR were housed individually in polyprophylene cages. Animals were housed in the vivarium with a 12:12 h light–dark cycle (lights on at 06:00 h). All animals had free access to food and water throughout the study. Day of birth was defined as pups being present by 16:00 h. All litters were culled within 24 h of birth to eight pups, four males and four females where possible. No more than one male pup from each litter was used for any given measure. Subjects used in the study were housed individually following weaning at 3 weeks of age. All procedures were conducted in accordance with National Institutes of *Health Guide for the Care and Use of Laboratory Animals.*

2.2. Maternal separation

Maternal separation (MS) was conducted daily from postnatal day (PD) 1 until PD14. Dams were removed first from the home cage and kept in a separate cage during the 3 h separation period. All pups were then removed and placed in individual plastic containers, maintained at 31 °C, with sawdust. Following the 3 h MS period, the pups were returned to the home cage followed by the dam. Non-maternal separated (N-MS) subjects were left undisturbed throughout the preweaning period. Cage changing was suspended during the 2-week maternal separation period.

2.3. Open field testing

At 6–8 weeks of age, subjects were tested for 60 min in the open field arena, a behavioral test used to assess general locomotor behavior in a novel environment. This chamber is 22.1 in. W × 21.2 in. L × 15 in. H and is interfaced to a PC data collection system (Hamilton-Kinder, San Diego, CA). Animals were transported from the animal facility to a holding room adjacent to the test room at least 2 h before the test. The arena was washed thoroughly between tests.

2.4. Cardiovascular recording

Arterial catheters were made from a 3 cm Micro-Renathane (.025 in. OD) tip (Braintree Scientific, Braintree MA) fused to tygon tubing. Animals were anesthetized with ketamine hydrochloride + acepromazine (100 mg/kg, 2 mg/kg, respectively, i.p.) and the catheter was introduced under aseptic conditions to the lower abdominal aorta via the right femoral artery. The catheter was tunneled up the back and exited dorsally at the neck. Each catheter was filled with heparinized saline (200 U/ml) and capped with a stainless steel plug. Animals were tested after a 48–72 h recovery period.

On the test day, each rat was transported in the home cage to the testing room and the arterial catheter was attached to a pressure transducer that was connected to PowerLab hardware and software (ADI, Grand Junction CO). Each subject was allowed to acclimate for at least 2 h prior to recording a blood pressure and heart rate for 30 min. Following this baseline period, the animal was transferred to a cylindrical Plexiglas restraint tube for 30 min. At the end of the 30 min restraint period, the subject was returned to the home cage for 90 min, at which time the brain was removed for immunohistochemistry.

2.5. Immunohistochemistry

Animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and perfused transcardially with 200 ml normal saline followed by 400 ml of 4% paraformaldehyde prepared in 0.1 M phosphate buffer (PB), pH 7.4. Following removal, brains were postfixed overnight in 4% paraformaldehyde and then stored in cryoprotectant (30% sucrose). Coronal sections (40 μm) were taken using a freezing microtome/cryostat (Leica). Free-floating sections were washed several times in 0.01 M PB before being soaked in 0.3% H_2O_2 to quench endogenous peroxidase activity. Following several washes in PB, sections were incubated in PB + 0.1% Triton x-100 and 2% normal goat serum to block non-specific binding. Following another series of washes, sections were incubated in the primary antibody (diluted 1:10,000, Santa Cruz Biotechnology, Santa Cruz CA) for 48 h at 4 °C. Sections were then incubated for 2 h in the biotinylated secondary antibody (dilution 1:500, Vector), followed by several washes in PB before incubation for 2 h in the avidin–biotin complex (dilution 1:1000, ABC Elite, Vector). The reaction product was developed using diaminobenzidine tetrahydrochloride (DAB, 0.5 mg/ml) in the presence of 0.02% H_2O_2. Stained sections were washed several times in PB and mounted on gelatin coated slides, dried, and coverslipped using Permount. Control procedures for Fos immunoreactivity included processing sections with sequential omission of the primary antibody, biotinylated secondary antibody, and the ABC reagent. Cell counts were obtained from a 1 in 4 series of immunostained 40 μm coronal sections through the areas of interest (areas PVN = 0.06 mm^2, BNST = 0.36 mm^2, CeA = 0.36 mm^2) using a Nikon Eclipse 300 microscope and Image J software (NIH).

2.6. Statistical analyses

Total horizontal activity in the open field was collected for 60 min was analyzed using the student's *t*-test. These data were also binned into six 10 min blocks and analyzed using repeated measures analysis of variance (ANOVA) with time as the repeated measures factor and condition as the grouping factor. MAP and HR data collected during the 60 min restraint stress period were analyzed using repeated measures ANOVA with time six 10 min blocks) as the repeated measures factor and condition as the grouping factor. Significant interactions were followed up using Tukey's HSD test. Resting MAP and HR, total locomotor activity and Fos data were analyzed using the student's *t*-test. A significance level of .05 was applied to all tests. Data are expressed as the mean ± S.E.M.

3. Results

3.1. Open field behavior

Fig. 1 (top panel) shows the total spontaneous locomotor activity during the 1 h test. MS BHR ($n = 9$) displayed significantly more activity compared to N-MS ($n = 19$) subjects, $t(26) = 3.78$, $p < .001$. When analyzed as 10 min bins, a mixed model ANOVA revealed a significant main effect of time $F(5,130) = 54.86$, $p < .0001$, condition $F(1,26) = 22.71$, $p < .0001$ and a significant time × condition interaction $F(5,130) = 3.31$, $p < .0008$. As can be seen in Fig. 1 (bottom panel), although both groups had comparable decreases in activity over the 60 min test period, MS subjects were significantly more active at all but the 40 and 50 min time points.

Table 1
Resting MAP and HR (mean ± S.E.M.)

	MAP (mmHg)	HR (bpm)
MS ($n = 17$)	126.5 ± 2.0	327.3 ± 6.1
N-MS ($n = 10$)	123.1 ± 2.2	313.9 ± 7.6

3.2. Cardiovascular responses

Analysis of MAP and HR data collected during a home cage rest session, shown in Table 1, (MS $n = 17$; N-MN $n = 10$) revealed no difference between groups $t(25) = 1.07$, $p < .292$ and $t(25) = 1.37$, $p < .81$, respectively. Repeated measures ANOVA performed on HR during stress found a significant main effect of time $F(5,100) = 18.57$, $p < .001$ and condition $F(1,20) = 26.60$, $p < .001$ and a significant interaction of time × condition $F(5,100) = 4.67$, $p < .001$; MS subjects had higher heart rate at all but the first time point (Fig. 2, top panel). The same analysis of MAP (MS $n = 14$, N-MS $n = 10$) collected at six time points during the 30 min stress session (Fig. 2, bottom panel) indicated that both groups tend to return to baseline over time $F(5,100) = 11.52$, <p.001. There was not a significant main effect of condition $F(1,20) = 1.21$, $p < .284$ or of the time × condition interaction $F(1,100) = .29$, $p < .917$.

3.3. Immunohistochemistry

As seen in Fig. 3, subjects exposed to the maternal separation procedure displayed significantly greater stress-induced Fos expression in each of the three brain areas examined ($n = 9$–14

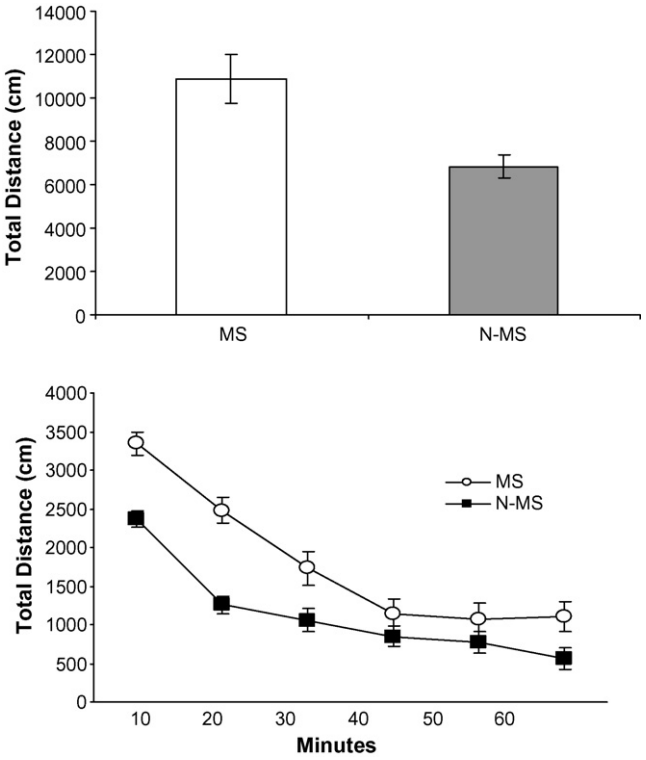

Fig. 1. Total exploratory activity in open field (top panel) during 60 min test and binned in 10 min blocks (bottom panel) for MS ($n = 9$) and N-MS ($n = 19$) subjects. Data are expressed as mean ± S.E.M.

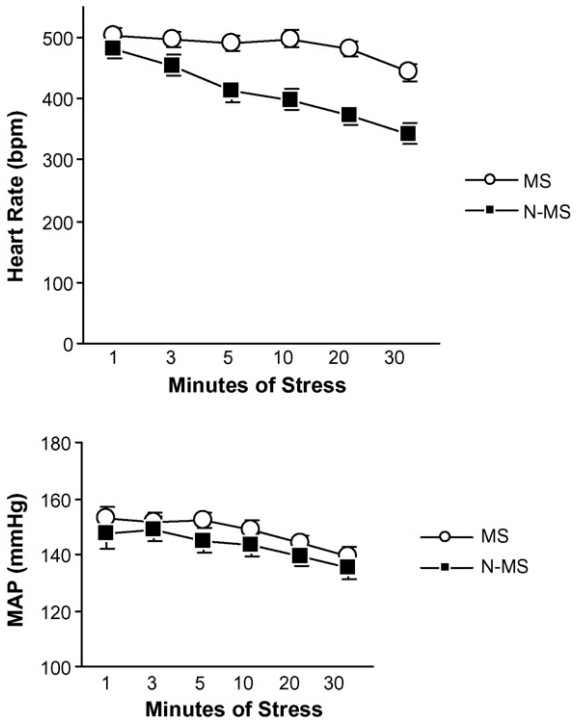

Fig. 2. HR (top panel) and MAP (bottom panel) during 30 min of restraint stress in MS ($n = 14$, open circle) and N-MS ($n = 10$, closed squares) BHR. Data are expressed as mean ± S.E.M.

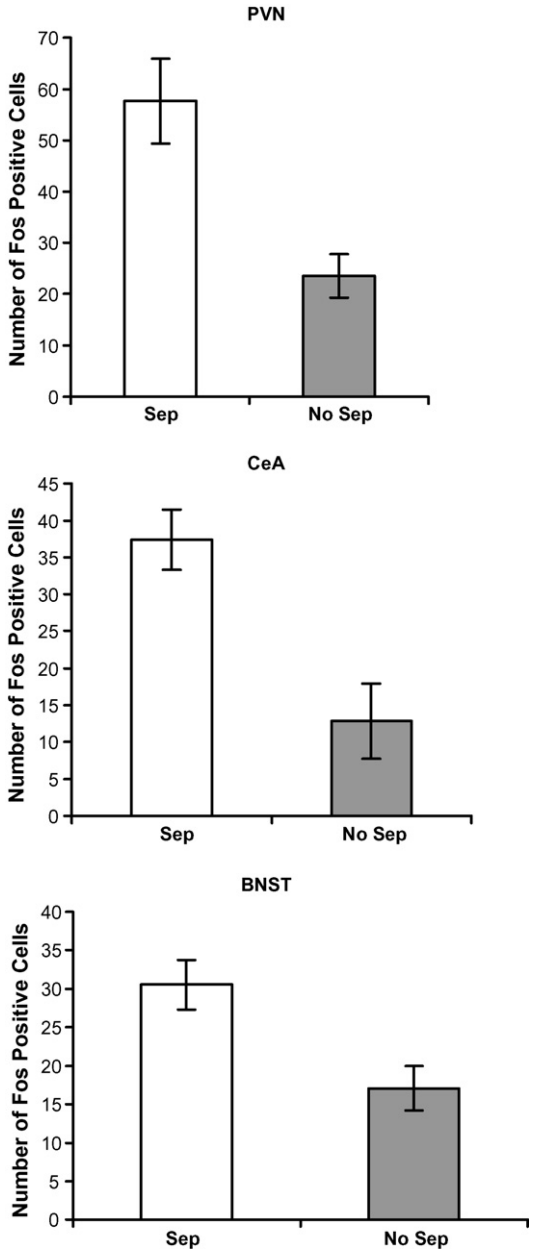

Fig. 3. Fos immunoreactive cells in PVN (top panel), CeA (middle panel), and BST (bottom panel) in MS and N-MS BHR ($n = 9$–14 per group). Data are expressed as the mean ± S.E.M.

per group); BNST $t(24) = 3.01$, $p < .006$, PVN $t(17) = 3.8$ $p < .001$, and CeA $t(18) = 6.02$ $p < .001$.

4. Discussion

The purpose of the present study was to examine the effect of an early environmental challenge on the biobehavioral response to stress in rats that show enhanced stress reactivity as adults. As mentioned previously, this model is sensitive to several environmental stimuli as adults, leading us to questions regarding whether events early in life can modify this susceptibility. Since, we were primarily interested in examining the extent to which daily maternal separation modulated adult responses in BHR, which is vulnerable to stress-induced cardiovascular disease as adults, rather than an analysis of varying genetic susceptibilities across strains, we did not examine WKY subjects in this study. Several lines of previous research suggest that WKY, as well as other normotensive strains of rats, are resistant to long-term perturbations in blood pressure caused by exposure to noxious environmental stimuli. For example, studies employing sensory stimulation/withdrawal [35], psychosocial stress [14] and classical [34] and operant [15] conditioning paradigms have all found little or no permanent effect of blood pressure and heart rate in normotensive strains of rodents. In addition, it has been reported that cross-fostering either WKY or Sprague-Dawley pups to SHR dams does permanently alter their adult blood pressure, suggesting resistance to the effects of a hypertensive maternal phenotype [9]. Thus, given the range of studies failing to find any substantive cardiovascular effect of environmental intervention in normotensive strains, we used daily pup separation from the dam as a postnatal challenge to examine the effects of early adverse experience on BHR.

Animals placed in a novel environment typically display initially high levels of exploratory activity that drops off as the subject becomes more familiar with the environment. Differences in the level of total locomotion and the pattern of behavior across time have been used as indices of an organism's level of emotionality [11] as well as ability to process new information from a novel environment [19]. Spontaneous motor activity was assessed in this experiment during a 60 min trial in the open field chamber. These data were analyzed in two ways. First, BHR subjects exposed to maternal separation had significantly higher levels of total locomotor activity compared to non-separated BHR. In addition, when examined in 10 min bins across time, MS subjects start the trial at higher levels of activity that are maintained throughout most of the test. Although this study did not utilize a normotensive strain of rat, previous reports suggest that MS has little or no effect on these subjects. For example, Wistar rats separated for 6 h on postnatal days 1–21 showed no differences in behavior in the open field or elevated plus maze compared to controls [27]. In addition, Wistar rats that are separated once for 24 h postnatal days 4, 9, or 18 show no evidence increased locomotor activity, although there is a tendency for females to be more active than males [19]. Thus, our data suggest a level of hyperactivity produced by MS not seen in normotensive Wistar rats, although differences in MS procedures used in other studies (age, duration, etc.) make precise comparisons difficult. Given that BHR are genetically predisposed toward heightened stress reactivity, the current data may suggest that MS further enhances this tendency, leading to increased escape behavior and hyperactivity in a novel environment.

Exposure to novel or threatening stimuli produces a complex pattern of endocrine, autonomic and behavioral activation designed to better enable the organism to negotiate the challenge. This study used the expression of the immediate early gene *c-fos* to estimate neuronal activation in brain regions important in mediating the response to stress. Although Fos expression is minimal under basal conditions, a variety of psychological and physiological challenges are capable of producing a significant elevation in Fos mRNA and the Fos protein product

[5,29], thus making it a useful and frequently used method to map neural circuitry. We assessed stress-induced Fos expression in the BNST, CeA, and PVN because of the well-known role these structures play in orchestrating an organism's response to stress. The PVN has long been recognized as pivotal in producing stress-induced changes in the neuroendocrine profile [16,33]. Stress-relevant information is received in the PVN by neurons that secrete corticotropin releasing hormone, setting in motion the HPA response. Successfully negotiating a stressful challenge, however, requires autonomic and behavioral responses in addition to neuroendocrine adjustments. Thus, limbic, cortical, brainstem, and hypothalamic regions become differentially involved depending on the characteristics of the stressor. For example, challenges that involve a psychological or emotional component, such as the open field or restraint, may be associated with more activation of hypothalamic and amygadoid nuclei compared with more physical stressors, such as ether inhalation or hemmorhage [1,12]. In addition to stimulus-dependent activation of particular neural circuits, connections also exist that support specific biological adjustments, such as the CeA and BNST signaling the dorsal–vagal complex to adjust cardiovascular functioning, thereby providing a way for higher brain centers to exert influence over blood pressure and heart rate [3].

The current study found that repeated maternal separation produced significant stress-induced increases in Fos immunoreactivity in the BNST, CeA, and PVN. This pattern of neural activation is consistent with several lines of relevant research. First, several studies have found that a single episode of acute stress produces substantial increases in Fos expression in the PVN, CeA, BNST as well as other areas [2,10]. Also, the magnitude of neuronal activation has been shown to be sensitive to briefer periods of maternal separation. Specifically, handling, which involves 15 min of daily separation during the first 2 postnatal weeks, results in a reduction in Fos immunoreactivity in the PVN, BNST, CeA, as well as other areas [1]. This is in line with an extensive body of research showing that handling generally produces a dampening of many endocrine and behavioral measures of stress [21,23,25]. Finally, a recent study, employing a structural rather than functional approach, found that both brief (15 min) and long (3 h) periods of maternal separation produced a significant reduction in the number of preautonomic limbic forebrain neurons [6]. Using retrograde transport of the pseudorabies virus to map postnatal neural circuit development, these investigators found that daily separation led to significantly fewer neurons in the CeA and BNST on PD 8 and 10; an effect not observed in the PVN, probably because these circuits are formed earlier in development [28]. Our results taken together with previous findings support the contention that early life events can substantially modify both structural and functional aspects of the neural circuits involved with processing emotionally relevant stimuli.

Since the BHR shows increased cardiovascular responses to both acute and chronic stress [30,32], we had hypothesized that repeated maternal separation would further enhance this tendency. In response to restraint stress we observed that while both groups had comparable increases in MAP, MS rats displayed a significant tachycardia during the stress period. Although this study did not examine how maternal separation may affect autonomic balance or cardiovascular regulation, several previous reports provide some context for understanding this observation. As previously described, maternal separation changes the brain circuitry in areas that have the potential to alter the central neural control of autonomic outflow [6], perhaps influencing parasympathetic and/or sympathetic tone. Of specific interest to the current study is a report that examined the ontogeny of autonomic control of heart rate in BHR, SHR, and WKY neonates [36]. Using pharmacological blockade and surgery to compare the development of heart rate control in strains of rats with differing sensitivities to stress, it was found that at weaning BHR and SHR displayed enhanced adrenal medullary drive as well as greater parasympathetic restraint. Based on these observations, it is possible that superimposing maternal separation on the autonomic profile of the BHR, which resembles that of its hypertensive parent, further disrupts heart rate control making it more sensitive to environmental stress.

In summary, we observed that daily maternal separation for the first 14 days of life produces increases in behavioral, neurobiological, and cardiovascular responses to stressful or novel stimuli in BHR. These results contribute to a larger body of work suggesting that early life events are capable of producing enduring effects in the offspring. Moreover, given that the BHR is sensitive to environmental challenges, the current study provides a potentially useful approach studying the interaction between early life events and future stress reactivity.

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