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Early maternal separation has mild effects on cardiac autonomic balance and heart structure in adult male rats

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Abstract
Early life adverse experiences have long-term physiologic and behavioral effects and enhance stress sensitivity. This study examined the effects of maternal separation (MS) on cardiac stress responsivity and structure in adulthood. Male Wistar rats were separated from the dams for 3 h per day from postnatal days 2 through 15. When exposed to 5-day intermittent restraint stress (IRS) as adults, MS, and control rats showed similar acute modifications of cardiac sympathovagal balance, quantified via heart rate variability analysis. In addition, MS had no effect on cardiac pacemaker intrinsic activity (as revealed by autonomic blockade with scopolamine and atenolol) and did not affect the circadian rhythmicity of heart rate, neither before nor after IRS. However, MS differed from control rats in cardiac parasympathetic drive following IRS, which was heightened in the latter but remained unchanged in the former, both during the light and dark phases of the daily rhythm. The evaluation of adult cardiac structure indicated that stress experienced during a crucial developmental period induced only modest changes, involving cardiomyocyte hypertrophy, increased density of vascular structures, and myocardial fibrosis. The mildness of these functional–structural effects questions the validity of MS as a model for early stress-induced cardiac disease in humans.

Keywords: *biological rhythms, heart rate variability, heart remodeling, maternal separation, parasympathetic nervous system, restraint*

Introduction

It is widely accepted that stressful events occurring during early postnatal life may alter neuroendocrine and behavioral stress responsiveness and lead to greater susceptibility for psychopathology throughout life (Heim and Nemeroff 2001; Cirulli et al. 2009).

Animal models based on the disruption of the mother–infant relationship have been used for a long time to better understand the short- and long-term effects of early adverse experiences (Lehmann and Feldon 2000; Levine 2001; Faturi et al. 2010). In rodents, one of the most commonly used experimental paradigms of early life stress is maternal separation (MS), in which pups are removed from the maternal nest repeatedly for a variable time period during the lactation phase. Brief MS (3–15 min per day for several days), also termed early handling (EH), has rather robust developmental effects and leads to attenuated adrenal and behavioral response to stress in adulthood (Levine 1957; Levine et al. 1967; Meerlo et al. 1999). However, prolonged MS (3 h or more per day, for several days) has long-term effects that appear to be highly variable, depending on details of the procedures and rat strains used (Lehmann and Feldon 2000). A number of studies indicate that animals subjected to MS develop a phenotype that is opposite to that of individuals exposed to EH (Plotsky and Meaney 1993; Meaney et al. 1996). In particular,

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maternally separated rats were reported to display greater hypothalamic–pituitary–adrenal (HPA) axis reactivity to acute challenges and higher levels of anxiety in adulthood (Huot et al. 2002; Plotsky et al. 2005; Aisa et al. 2007), potentially resulting in a larger vulnerability to stress-related disease. However, other studies have reported no major effects of MS on adult adrenocortical activity and anxiety-like behavior (Slotten et al. 2006; Hulshof et al. 2011).

In recent years, there has been a growing interest in the effects of early adverse experiences on cardiovascular system function and structure. Individual features of cardiovascular regulation result from a dynamic interaction between genetically programed developmental processes and environmental conditions (Tucker et al. 1984). During ontogeny, cardiac development and maturation partly depend on, and interact with, input from the autonomic nervous system (ANS; Claycomb 1976; Larson and Porges 1982; Tucker and Johnson 1984a; Tucker 1985). For these reasons, stress experienced in this crucial phase may interfere with normal autonomic fiber distribution to the myocardial tissue, which in turn might lead to persistent changes in the functional and morphological characteristics of the heart (Tucker et al. 1984; Tucker and Johnson 1984b).

In humans, epidemiological evidence indicates that unfavorable events experienced early in life are associated with an increased susceptibility to develop heart disease in adulthood. For instance, the Adverse Childhood Experiences Study reported that childhood abuse and neglect are closely associated with the most important risk factors, such as smoking, obesity, physical inactivity, and depression, for ischemic heart disease (Dong et al. 2004). However, such epidemiological studies provide no evidence for causal relationships and so far only few experimental studies with animal models have investigated the direct link between early adverse experiences and cardiovascular (dys-)function (Sanders and Anticevic 2007; Loria et al. 2010a,b). On the one hand, results of these studies suggest that MS does not influence baseline values of heart rate and blood pressure. On the other hand, in one study, MS increased heart rate responsivity to an acute stressor in adult borderline hypertensive rats, with no significant changes in blood pressure response (Sanders and Anticevic 2007). Furthermore, early life stress rendered adult rats more susceptible to angiotensin II-induced hypertension, tachycardia, and vascular inflammation, which may contribute to the pathogenesis of cardiovascular disease (Loria et al. 2010a,b). Altogether, the data collected so far indicate that MS may contribute to adult cardiovascular morbidity; nonetheless, it is still unknown whether the autonomic control of cardiac function is affected, at rest and during exposure to acute environmental stimuli and whether the early life adverse experience of MS may alter the development of adult heart morphology and tissue anatomy.

Studies in both humans and animals report that impaired cardiac autonomic regulation characterizes many pathologic conditions of cardiac (Brook and Julius 2000; Thayer et al. 2010) and non-cardiac origin (Ewing et al. 1985; Thayer et al. 1996). In humans, the analysis of heart rate variability (HRV), which describes the small beat-to-beat differences in heart rate is a non-invasive approach to gather information about the modulation of the two branches of the ANS to the heart (Task Force 1996). The same approach has also been successfully applied to rat electrocardiogram (ECG) recordings (Sgoifo et al. 1998; Aubert et al. 1999).

In this study, we tested the hypothesis that adverse events experienced early during postnatal life may interfere with the development of the heart and its autonomic neural control, possibly leading to an increased stress susceptibility in adulthood. In particular, we assessed whether MS in rats induces long-lasting modifications of: (i) cardiac autonomic regulation, both at rest and under challenging conditions, namely restraint stress and pharmacological autonomic blockade, and (ii) cardiac architecture, in terms of gross morphological changes, vascular density, and myocardial structural damage.

Materials and methods

Animals and housing

Twenty-four male Wistar rats were used in this study. All efforts were made to reduce animal pain or discomfort. Experiments were conducted in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the University of Parma Animal Welfare Committee. Female and male Wistar rats (Charles River, Calco, Italy) were paired for a period of 10 days, after which the males were removed. Subsequently, pregnant females were left undisturbed, except for a daily visual check for the presence of pups. The day of birth was defined as pups being present by 10:00 h and was designated as day 0. On postnatal day 1, the litters were culled to eight pups, four males and four females. At weaning (3 weeks of age), they were housed with same-sex siblings. No more than two male pups from each litter were used for any given measure. During the entire experiment, all rats were kept under controlled temperature (22 ± 2°C) and lighting (lights on from 07:00 to 19:00 h) conditions. *Ad libitum* access to food and water was provided throughout the study.

Maternal separation

MS, which consisted of daily separation of the litter from the dam for 3 h (09:00–12:00 h), was carried out

from postnatal Day 2 to postnatal Day 15, in accordance with previous studies (Plotsky and Meaney 1993; Hulshof et al. 2011). Each litter was removed from the nest and transferred to another room, to prevent vocal communication between mother and pups. During this 3-h period, the pups were placed with their siblings in glass beakers in a water bath set at 32–33°C, consistent with normal nest temperature (Schmidt et al. 1986), in order to prevent a decrease in body temperature. Indeed, a reduction in body temperature of the litter has been shown to increase maternal care upon reunion (Leon et al. 1978; Stern and Johnson 1990), which was found to reduce behavioral and neuroendocrine stress reactivity (Liu et al. 1997; Francis et al. 1999).

During the separation period, the dams remained in the home cage. Following the 3-h MS period, pups were returned to the home cage. Control pups belonged to other (independent) litters and were left undisturbed throughout the pre-weaning period in their mother's nest.

Radiotelemetry system

The radiotelemetry system employed in this study consisted of flat transmitters measuring 25 × 15 × 8 mm (TA11CTA–F40, Data Science International, St Paul, MN, USA) and platform receivers. At 4 months of age, the rats were anesthetized with tiletamine hydrochloride + zolazepam hydrochloride (Zoletil, 200 mg/kg, s.c.) and the transmitters chronically implanted according to a surgical procedure that guarantees high-quality ECG recordings also during sustained physical activity (Sgoifo et al. 1996). Immediately after surgery, rats were individually housed and injected for 2 days with gentamicin sulfate (Aagent, Fatro, 0.2 ml/kg, s.c.). The rats were allowed 10 days of recovery before the start of experimental recordings.

Outline of adult manipulations

An overview and timeline of the measurements that were carried out in adulthood is provided in Figure 1. Four-month-old MS ($n = 12$) and control ($n = 12$) rats were exposed to an intermittent restraint stress (IRS) protocol. Rats were subjected to restraint stress on 5 consecutive days. Each restraint session consisted of confinement in a wire-mesh tube for 15 min (inner diameter 6 cm, length 20 cm). Eight days before and after the IRS protocol, pharmacological blockade of the two branches of the ANS was carried out (see below for details). The weeks in between the pharmacological tests and the IRS protocol were used to assess the daily rhythmicity of heart rate. From the day of surgery, the rats were weighed on a weekly basis until the day of euthanasia.

Pharmacological autonomic blockade

The competitive muscarinic receptor antagonist methylscopolamine (0.05 mg/kg) and the sympathetic blocker atenolol (2 mg/kg; Sigma, St Louis, MO, USA; Ngampramuan et al. 2008) were injected s.c. to block vagal and sympathetic influences to the heart in MS and control rats. After baseline ECG recording, methylscopolamine was injected and the ECG recorded to evaluate the effect of parasympathetic blockade; 15 min afterwards, atenolol was administered to the same rats to determine intrinsic heart rate. Intrinsic heart rate is established when the cardiac ANS is completely blocked, which is supposed to take

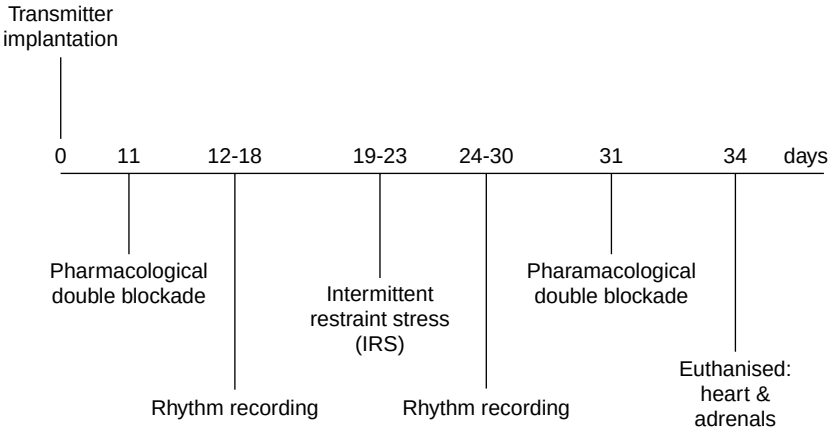

Figure 1. Schematic diagram of the experimental protocol applied to control and maternally separated rats in adulthood. Transmitter implantation = surgical chronic implantation of transmitters for radiotelemetric ECG recordings. Pharmacological double blockade = pharmacological blockade of the two branches of the ANS with methylscopolamine (muscarinic receptor antagonist) and atenolol (beta-adrenergic receptor antagonist). IRS = daily exposure to restraint stress for 5 consecutive days. Rhythm recording = around-the-clock, radiotelemetric ECG recording for heart rate rhythmicity evaluation. Euthanized, heart and adrenals = euthanasia of the rats and subsequent removal of hearts and adrenals.

place approximately 10–15 min after the sympathetic blocker injection (Safa-Tisseront et al. 1998; Souza et al. 2009; Sant'Ana et al. 2011). The pharmacological test was carried out twice, 8 days before and 8 days after IRS (Figure 1).

Electrocardiographic data collection and analysis

ECG waves were acquired on a personal computer via the ART-Silver 1.10 data acquisition system (Data Sciences Int., St. Paul, MN, USA) with 1000 Hz sampling frequency. Continuous ECG recordings were carried out during the first and fifth restraint stress episodes and the two pharmacological tests (Figure 1) according to the following schedule: (i) restraint stress: 30 min baseline, 15 min test, 30 min recovery and (ii) pharmacological double blockade: 30 min baseline, 15 min following scopolamine injection, 45 min following atenolol injection. Chart5 software (ADInstruments, Sydney, Australia) was employed to calculate the average R–R interval duration (RR, ms), which corresponds to the average inter-beat interval in a given time period. In addition, time- and frequency-domain parameters of HRV were quantified. The time-domain indexes used in this study were the root mean square of successive differences between adjacent R–R intervals (r-MSSD, ms) and the percentage of successive interval differences larger than 20 ms (pNN20, %). R-MSSD and pNN20 quantify short-term, high-frequency (HF) variations of RR and therefore estimate the activity of the parasympathetic nervous system (Stein et al. 1994). Frequency-domain (fast Fourier transform) indices were collected in accordance with the guidelines for frequency-domain computations of HRV (Task Force 1996). We considered only low-frequency (LF; 0.2–0.75 Hz) and HF (0.75–2.5 Hz) bands of the spectrum, and their power was quantified as normalized units (n.u.). The power of LF represents the activity of both branches of the ANS (Eckeberg 1991); the power of HF is due to the activity of the parasympathetic nervous system and includes respiration-linked oscillations of heart rate (Chess et al. 1975). The LF/HF ratio estimates the fractional distribution of power, which is taken as an indirect measure of sympathovagal balance (Task Force 1996). A stationary ECG signal is recommended to reliably carry out short-term frequency-domain HRV analysis and the presence of artifacts could significantly influence the results (Task Force 1996). For these reasons, those parts of ECG recordings that werenon-stationary and/or exhibited recording artifacts were excluded from the analysis.

Daily rhythm data collection and analysis

ECG waves were sampled around-the-clock for 60 s every 60 min in two different periods: (i) pre-IRS, for 7 days, starting on the day after the first pharmacological test, and (ii) post-IRS, for 7 days, starting on the day after the last restraint test (Figure 1). ECG recordings were analyzed by means of a software package developed in our laboratory (Sgoifo et al. 2001) and RR and r-MSSD values were quantified as means for the 12-h light (resting) phase and 12-h dark (activity) phase.

Post-mortem measurements

Three days after post-IRS pharmacological blockade (Figure 1), rats were euthanized. Under anesthesia (tiletamine hydrochloride + zolazepam hydrochloride s.c., Zoletil, 200 mg/kg), the heart was arrested in diastole with cadmium chloride solution (100 mM IV) and excised for subsequent morphological/morphometric analysis. Following heart removal, the adrenals were also excised and weighed. Adrenal glands were also obtained from 20 additional 5-month-old rats (MS, $n = 10$; control, $n = 10$), which were not exposed to adult experimental manipulation.

Cardiac remodeling. The two atria, the right ventricle (RV) and the left ventricle (LV) inclusive of the septum were separately weighed, fixed in 10% buffered formalin solution, and used for morphometric studies. The following parameters were determined: heart weight (HW), LV weight (LVW), RV weight (RVW), LVW/HW, and RVW/HW. LV free wall thickness and LV transverse diameters were morphometrically computed (Image Pro-plus). The LV chamber volume was calculated according to the Dodge equation (Dodge and Baxley 1969). From each heart embedded in paraffin, 5 μm-thick left ventricular sections were cut from the equatorial slice and used for subsequent analyses.

Morphometric analysis. Sections stained with Masson's trichrome were analyzed by optical microscopy (magnification 250 ×) in order to evaluate the total amount of interstitial and reparative fibrosis (Beltrami et al. 1994) in the LV myocardium. According to a procedure previously described (Costoli et al. 2004), quantitative evaluation of fibrotic tissue was carried out in 60 randomly selected fields from the sub-endocardium, mid-myocardium, and sub-epicardium, with the aid of a grid defining a tissue area of 0.160 mm^2 and containing 42 sampling points, each covering an area of 0.0038 mm^2. To define the volume fraction of fibrosis in the three layers of the ventricular wall, the number of points overlaying myocardial scarring was counted and expressed as a percentage of the total number of points explored. For reparative fibrosis, the numerical density of fibrotic foci per unit area of myocardium was also determined.

Myocyte size. The cross-sectional area (CSA) of myocytes was determined by measuring the cell diameter in transversely oriented myocytes, but only in those cells where the entire nuclear profile was clearly defined. To obtain CSA, two diameters were measured and their mean value was used to compute the area. For each LV, 120–250 cardiomyocytes were analyzed at a magnification of $\times 1000$.

Vessel density. The quantification of capillaries and venules was carried out in sections stained with polyclonal rabbit anti-vW factor (dilution 1:100, Dako), which recognizes endothelial cells, followed by fluorescein isothiocyanate-conjugated anti-rabbit secondary antibodies (Jackson Laboratory, Baltimore, PA, USA). Nuclei were recognized by bisbenzimide staining (Hoechst No. 33258, Sigma). Morphometric sampling at $\times 1000$ magnification consisted of counting the number of capillary and venule profiles in a measured area of tissue sections of both the epi-myocardium and endo-myocardium, in which myocytes are transversely oriented. Capillaries were distinguished according to their luminal diameter (range 4–6 μm) from venules (range 6–10 μm) in which vW factor positive profiles lack multiple layers of smooth muscle cells. The number of capillaries and venules per unit area of myocytes was computed. This approach was followed to eliminate the effects of variations caused by changes in the interstitial compartment. Sampling of vessel measurements involved a minimum of 20 and a maximum of 30 microscopic fields for the LV of each rat (Maestri et al. 2003).

Data analysis and statistics

Values of all parameters are expressed as mean ± SEM. Statistical analyses were carried out using the SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA) and statistical significance for all tests was set at $p < 0.05$.

In order to detect possible differences at rest between the two experimental groups, the values of R–R interval and HRV indices of 30 min baseline recordings preceding restraint and pharmacological challenges were statistically analyzed by means of two-way analysis of variance (ANOVA), with "postnatal treatment" as between-subject factor (two levels: MS and control rats) and "time" as within-subject factor (two levels: first and fifth restraint test; pre- and post-IRS autonomic blockade). In order to analyze the effects of postnatal treatment on cardiac response to restraint and autonomic blockade, we calculated delta values for each 5-min time point relative to the baseline and named them delta1, delta2, … delta9 for restraint tests, and delta1, delta2, …,delta12 for pharmacological challenges. Statistical analysis was then carried out on delta instead of absolute values to abolish possible group differences in stressor responsivity due to differences in baseline. Two-way ANOVA was applied to delta values, with "postnatal treatment" as between-subject factor (two levels: MS and control rats) and "time" as within-subject factor (9 levels for restraint and 12 levels for pharmacological challenge).

Average light and dark phase values of RR and r-MSSD on each day before and after IRS were calculated. Control and MS values of RR and r-MSSD in baseline conditions (pre-IRS) during the light and dark phases were compared via Student's *t*-test. Delta values between each post-IRS day and average pre-IRS value of light and dark phases were computed. Statistical analysis on delta values was carried out by means of two-way ANOVA, with "postnatal treatment" as between-subject factor (two levels: MS and control rats) and "time" as within-subject factor (7 days).

Following ANOVAs, post-hoc analysis on ECG data was applied where appropriate using Student's *t*-test, after checking for variance homogeneity by means of the Levene test.

Values of body weight were analyzed via two-way ANOVA for repeated measures, with "postnatal treatment" as between-subject factor (two levels: MS and control rats) and "time" as within-subject factor (6 weeks). The weight of adrenal glands at sacrifice was expressed as a ratio relative to the rat body weight (mg adrenal gland weight/100 g body weight) and statistically compared by Student's *t*-test. Statistical analysis of cardiac structural measures was also carried out via Student's *t*-test when two groups were analyzed, or via ANOVA followed by *t*-test for multiple comparisons.

Results

Electrocardiographic response to restraint

Two-way ANOVA applied to baseline values of RR and HRV parameters measured just before the first and fifth restraint episodes did not reveal significant effects of group, time, or group × time interaction. This indicates that MS did not induce clear changes in resting HRV indexes, neither at the beginning (first episode) nor at the end (fifth episode) of adult intermittent restraint period (Table I).

Table I also reports the absolute values of RR and HRV parameters for each 5-min time point during and after the first and fifth restraint episodes. Two-way ANOVA on delta values (values of each 5-min time point during restraint test and recovery relative to baseline) for the first restraint test revealed only a significant effect of time for RR ($F(8,176) = 109.5$, $p < 0.01$) and r-MSSD ($F(8,176) = 9.3$, $p < 0.01$). No significant effect of group was observed for any of

Table I. Values (mean ± SEM) of average R–R interval (RR), time-domain HRV parameters (r-MSSD, pNN20), frequency-domain HRV parameters (LF, HF, LF/HF) during baseline conditions (30-min average value), restraint test (15 min), and recovery phase (45 min), in maternally separated (MS; $n = 12$) and control ($n = 12$) rats, at restraint tests 1 and 5.

Recording period	Group	RR (ms)		r-MSSD (ms)		pNN20 (%)		LF (n.u.)		HF (n.u.)		LF/HF	
		Restraint 1	Restraint 5	Restraint 1	Restraint 5	Restraint 1	Restraint 5	Restraint 1	Restraint 5	Restraint 1	Restraint 5	Restraint 1	Restraint 5
Baseline (min)	MS	210.8 ± 4.43	211.8 ± 5.14	6.87 ± 0.45	7.58 ± 0.83	1.10 ± 0.22	1.87 ± 0.57	30.63 ± 2.53	29.88 ± 3.06	69.37 ± 2.53	70.12 ± 3.06	0.48 ± 0.07	0.42 ± 0.05
	Control	209.9 ± 3.45	207.7 ± 5.90	5.84 ± 0.68	5.50 ± 0.77	1.19 ± 0.32	1.06 ± 0.54	32.88 ± 3.38	32.04 ± 3.35	67.12 ± 3.38	67.96 ± 3.35	0.57 ± 0.10	0.56 ± 0.09
Test													
1 (0–5)	MS	122.8 ± 0.97	125.5 ± 1.60	3.47 ± 0.77	2.24 ± 0.34	0.29 ± 0.16	0.22 ± 0.09	47.85 ± 2.85	47.89 ± 2.23	52.15 ± 2.85	52.11 ± 2.23	0.98 ± 0.11	0.96 ± 0.09
	Control	121.2 ± 1.08	122.1 ± 1.23	1.69 ± 0.17	2.07 ± 0.22	0.12 ± 0.08	0.10 ± 0.05	43.01 ± 1.84	48.06 ± 1.61	56.99 ± 1.84	51.94 ± 1.61	0.77 ± 0.06	0.90 ± 0.07
2 (5–10)	MS	122.3 ± 1.24	129.2 ± 1.92	1.88 ± 0.16	3.19 ± 0.30	0.06 ± 0.03	0.14 ± 0.04	54.01 ± 1.79	49.31 ± 2.36	45.99 ± 1.79	50.69 ± 2.36	1.15 ± 0.11	1.02 ± 0.10
	Control	123.2 ± 2.15	131.3 ± 2.97	2.12 ± 0.24	2.63 ± 0.29	0.20 ± 0.18	0.12 ± 0.06	51.02 ± 1.42	49.91 ± 2.01	48.98 ± 1.42	50.09 ± 2.01	1.01 ± 0.08	0.98 ± 0.10
3 (10–15)	MS	132.1 ± 1.98	139.1 ± 2.80	3.47 ± 0.31	4.09 ± 0.27	0.20 ± 0.07	0.29 ± 0.09	47.52 ± 2.46	46.76 ± 2.83	52.48 ± 2.46	53.24 ± 2.83	0.95 ± 0.09	0.93 ± 0.10
	Control	138.0 ± 2.24	145.4 ± 3.95	3.15 ± 0.37	3.45 ± 0.52	0.22 ± 0.07	0.36 ± 0.20	50.17 ± 2.33	47.29 ± 2.01	49.83 ± 2.33	52.71 ± 2.01	1.06 ± 0.11	0.80 ± 0.09
Recovery													
4 (15–20)	MS	143.6 ± 2.80	147.0 ± 2.14	5.09 ± 0.47	4.64 ± 0.26	1.12 ± 0.32	1.16 ± 0.30	48.73 ± 3.69	47.18 ± 2.12	51.27 ± 3.69	52.82 ± 2.12	1.09 ± 0.19	0.93 ± 0.09
	Control	142.6 ± 2.60	145.4 ± 2.16	3.66 ± 0.42	3.75 ± 0.42	0.55 ± 0.20	0.50 ± 0.16	52.67 ± 1.67	54.88 ± 1.79	47.33 ± 1.67	45.12 ± 1.79	1.14 ± 0.07	1.15 ± 0.13
5 (20–25)	MS	152.1 ± 3.33	153.6 ± 2.70	4.79 ± 0.45	4.03 ± 0.28	0.84 ± 0.22	0.45 ± 0.08	52.62 ± 3.04	51.95 ± 2.50	47.38 ± 3.04	48.05 ± 2.50	1.08 ± 0.11	1.14 ± 0.10
	Control	146.8 ± 2.64	152.4 ± 1.98	3.68 ± 0.38	3.93 ± 0.54	0.53 ± 0.17	0.60 ± 0.21	53.35 ± 2.31	54.67 ± 2.31	46.65 ± 2.31	45.33 ± 2.31	1.19 ± 0.11	1.16 ± 0.15
6 (25–30)	MS	152.6 ± 3.41	154.5 ± 2.51	5.00 ± 0.43	4.32 ± 0.39	0.99 ± 0.26	0.60 ± 0.17	46.96 ± 3.62	45.34 ± 2.45	53.04 ± 3.62	54.66 ± 2.45	0.99 ± 0.16	0.87 ± 0.09
	Control	147.1 ± 2.92	154.9 ± 1.52	3.51 ± 0.24	4.03 ± 0.62	0.37 ± 0.10	0.57 ± 0.25	53.44 ± 2.20	50.73 ± 1.14	46.56 ± 2.20	49.27 ± 1.14	1.09 ± 0.15	1.00 ± 0.06
7 (30–35)	MS	154.5 ± 3.79	151.8 ± 2.20	4.46 ± 0.46	4.39 ± 0.36	0.98 ± 0.26	0.88 ± 0.22	44.34 ± 3.32	43.75 ± 3.24	55.66 ± 3.32	56.25 ± 3.24	0.77 ± 0.08	0.85 ± 0.13
	Control	151.7 ± 1.96	154.7 ± 3.03	4.07 ± 0.40	3.49 ± 0.35	1.10 ± 0.32	0.50 ± 0.18	50.15 ± 1.98	46.60 ± 2.35	49.85 ± 1.98	53.40 ± 2.35	1.04 ± 0.08	0.91 ± 0.08
8 (35–40)	MS	157.5 ± 3.85	160.5 ± 3.77	4.05 ± 0.39	4.53 ± 0.24	0.48 ± 0.15	0.42 ± 0.11	47.45 ± 2.85	46.72 ± 1.77	52.55 ± 2.85	53.28 ± 1.77	0.97 ± 0.12	0.91 ± 0.06
	Control	149.7 ± 2.30	159.8 ± 3.66	3.64 ± 0.43	4.19 ± 0.51	0.54 ± 0.25	0.55 ± 0.22	50.57 ± 2.71	44.33 ± 2.02	49.43 ± 2.71	57.67 ± 2.02	1.09 ± 0.13	0.82 ± 0.06
9 (40–45)	MS	158.8 ± 4.75	168.4 ± 5.72	4.06 ± 0.36	4.09 ± 0.28	0.34 ± 0.14	0.33 ± 0.11	42.18 ± 3.83	41.92 ± 3.64	57.82 ± 3.83	58.08 ± 3.64	0.82 ± 0.14	0.71 ± 0.09
	Control	158.4 ± 3.63	174.1 ± 5.65	4.49 ± 0.55	4.14 ± 0.51	0.72 ± 0.38	0.54 ± 0.15	43.14 ± 3.19	41.13 ± 3.35	56.86 ± 3.19	58.87 ± 3.35	0.73 ± 0.09	0.76 ± 0.09

Abbreviations: HF, high frequency; HRV, heart rate variability; LF, low frequency; r-MSSD, root mean square of successive differences between adjacent R–R intervals; n.u., normalized units; pNN20, percentage of successive interval differences larger than 20 ms.

the parameters considered. Hence, MS did not affect the magnitude and temporal dynamics of the changes in sympathovagal balance recorded during and immediately after an acute stressor in adulthood. At the fifth (last) restraint test, the time course of RR and HRV parameters was again similar in MS and control rats (Table I). Two-way ANOVA on delta values revealed a significant effect of time for RR ($F(8,176) = 91.9$, $p < 0.01$), r-MSSD ($F(8,176) = 16.6$, $p < 0.01$), LF ($F(8,176) = 5.2$, $p < 0.05$), HF ($F(8,176) = 5.2$, $p < 0.05$), and LF/HF ($F(8,176) = 7.3$, $p < 0.05$). However, a significant effect of postnatal treatment was found only for r-MSSD ($F(1,22) = 5.1$, $p < 0.05$). Post-hoc analysis on r-MSSD delta values revealed only sporadic differences between the two experimental groups, which were limited to a few time points in the recovery phase ($t_{delta5}(22) = 2.06$, $p < 0.05$; $t_{delta6}(22) = 2.32$, $p < 0.05$; $t_{delta8}(22) = 2.61$, $p < 0.01$; $t_{delta9}(22) = 2.60$, $p < 0.05$).

Electrocardiographic response to pharmacological autonomic blockade

Two-way ANOVA applied to baseline values of RR and HRV parameters did not reveal any significant effect of group, time, or group × time interaction. MS did not induce changes in resting HRV indices, neither at pre-IRS nor at post-IRS pharmacological autonomic challenge (Table II).

Table II also reports the absolute values of RR and HRV parameters for each 5-min time point during the first and second pharmacological challenge. Two-way ANOVA on delta values applied to the first pharmacological challenge revealed a significant effect of time for RR ($F(11,242) = 401.6$, $p < 0.01$), r-MSSD ($F(11,242) = 7.9$, $p < 0.05$), LF ($F(11,242) = 21.7$, $p < 0.01$), HF ($F(11,242) = 21.7$, $p < 0.01$), and LF/HF ($F(11,242) = 16.8$, $p < 0.01$). When applied to the second pharmacological challenge, two-way ANOVA on delta values revealed a significant effect of time for RR ($F(11,242) = 915.2$, $p < 0.01$), LF ($F(11,242) = 14.1$, $p < 0.01$), HF ($F(11,242) = 14.2$, $p < 0.01$), and LF/HF ($F(11,242) = 14.9$, $p < 0.01$). However, significant effects of group were not observed; hence, MS did not affect cardiac autonomic responsiveness to vagal and sympathetic blockade, neither before nor after the IRS.

Daily rhythms in cardiac activity

MS and control rats exhibited similar light and dark phase values of heart rate (RR) and vagal activity (r-MSSD) under baseline conditions, i.e. before the IRS period (RR_{light}: $t(22) = 0.72$, $p =$ n.s.; RR_{dark}: $t(22) = 1.20$, $p =$ n.s.; r-$MSSD_{light}$: $t(22) = 0.51$, $p =$ n.s.; r-$MSSD_{dark}$: $t(22) = 1.45$, $p =$ n.s.; Figure 2A,C).

Two-way ANOVA on RR delta values (value at each post-IRS day relative to the pre-IRS reference value; Figure 2B) did not reveal any significant effect of group, time or group × time interaction, neither for the light phase nor for the dark phase. Hence, MS did not affect daily heart rate rhythmicity, neither before nor after the IRS.

However, two-way ANOVA on delta values of r-MSSD (Figure 2D) revealed a significant effect of postnatal treatment for both the light and dark phases of the daily rhythm (r-$MSSD_{light}$: group $F(1,22) = 10.8$, $p < 0.01$; r-$MSSD_{dark}$: group $F(1,22) = 36.5$, $p < 0.01$). Control rats exhibited higher delta values of r-MSSD in the active (dark) and passive (light) phases of the circadian rhythm, with significant differences compared to MS counterparts on all days (1–7) following IRS for the dark phase ($2.6 \leq t(22) \leq 4.6$, $p < 0.05$) and on Days 1–4 and 6 following IRS for the light phase ($2.7 \leq t(22) \leq 4.4$, $p < 0.05$; Figure 2D).

Body weight and adrenal gland weight

Body weight values were similar in control and MS rats at the time of transmitter implantation (466 ± 11 vs. 462 ± 13 g) and euthanasia (457 ± 9 and 460 ± 11 g). Two-way ANOVA on body weight values revealed a significant effect of time ($F(5,110) = 8.44$, $p < 0.01$) due to post-surgery weight loss, which was similar in the two groups of rats. However, no differences were found between MS and control rats across the experimental protocol. Hence, MS stress did not produce changes in body weight temporal dynamics, which were similar in control and MS rats both before and after IRS. However, MS rats at euthanasia had significantly heavier adrenals compared to controls (MS: 20.99 ± 1.18 mg/100 g; control: 16.50 ± 1.27 mg/100 g; $t(22) = 2.60$, $p < 0.05$). In addition, adrenals excised from 10 additional rats exposed only to early life manipulation were significantly larger than those of controls ($n = 10$; MS: 17.58 ± 1.46 mg/100 g; control: 13.38 ± 1.13 mg/100 g; $t(18) = 2.27$, $p < 0.05$).

Cardiac anatomy and myocardial structure

As shown in Table III, slight differences were observed between MS and control rats with respect to LVW and LV linear parameters. Actual LVW and weight relative to HW were significantly lower in the MS group, whereas RVW was similar in MS and control rats (Table III). As a result of modest reductions in both LV length (−3%) and equatorial diameter (−6%), chamber volume was significantly smaller (−14%) in MS rats compared to control rats (Table III). To determine whether these changes in gross cardiac

Table II. Values (mean ± SEM) of average R–R interval (RR), time-domain (r-MSSD, pNN20) and frequency-domain HRV parameters (LF, HF, LF/HF) in baseline conditions (30-min average value) and after scopolamine (15 min) and atenolol (45 min) injections, in maternally separated (MS; $n = 12$) and control ($n = 12$) rats, at pharmacological challenges 1 and 2 (tests 1 and 2).

Recording period	Group	RR (ms)		r-MSSD (ms)		pNN20 (%)		LF (n.u.)		HF (n.u.)		LF/HF	
		Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
Baseline (min)	MS	203.6 ± 3.89	205.9 ± 5.41	6.47 ± 0.70	6.01 ± 0.61	1.39 ± 0.38	1.72 ± 0.57	36.29 ± 3.50	31.55 ± 2.35	63.71 ± 3.50	68.45 ± 2.35	0.57 ± 0.07	0.50 ± 0.06
	Control	205.3 ± 4.23	200.7 ± 5.76	5.38 ± 0.56	5.60 ± 0.57	0.93 ± 0.26	1.57 ± 0.44	36.86 ± 1.96	38.78 ± 1.83	63.14 ± 1.96	61.22 ± 1.83	0.61 ± 0.05	0.64 ± 0.07
Scopolamine													
1 (0–5)	MS	136.8 ± 2.51	138.5 ± 1.09	1.47 ± 0.17	1.69 ± 0.16	0.09 ± 0.04	0.05 ± 0.02	47.28 ± 2.39	48.34 ± 2.62	52.72 ± 2.39	51.66 ± 2.62	0.94 ± 0.09	0.99 ± 0.11
	Control	136.7 ± 3.65	137.1 ± 2.39	1.66 ± 0.20	1.73 ± 0.19	0.06 ± 0.02	0.08 ± 0.04	50.28 ± 1.95	48.36 ± 3.26	49.72 ± 1.95	51.64 ± 3.26	1.05 ± 0.08	1.01 ± 0.11
2 (5–10)	MS	133.5 ± 2.14	134.6 ± 2.05	0.78 ± 0.06	0.87 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	48.48 ± 2.02	46.36 ± 2.21	51.52 ± 2.02	53.64 ± 2.21	0.98 ± 0.08	0.89 ± 0.08
	Control	135.8 ± 3.26	131.6 ± 2.22	0.90 ± 0.08	1.10 ± 0.13	0.01 ± 0.08	0.04 ± 0.02	49.05 ± 1.86	54.45 ± 2.94	50.95 ± 1.86	45.55 ± 2.94	0.94 ± 0.08	1.29 ± 0.13
3 (10–15)	MS	131.9 ± 1.70	135.8 ± 2.52	0.99 ± 0.14	0.76 ± 0.06	0.01 ± 0.01	0.00 ± 0.00	48.69 ± 3.29	48.49 ± 3.73	51.31 ± 3.29	51.51 ± 3.73	1.03 ± 0.12	0.94 ± 0.12
	Control	137.4 ± 3.15	133.5 ± 2.75	0.96 ± 0.16	0.95 ± 0.12	0.05 ± 0.04	0.00 ± 0.00	53.95 ± 2.32	58.59 ± 1.67	46.05 ± 2.32	41.41 ± 1.67	1.16 ± 0.13	1.37 ± 0.11
Atenolol													
4 (15–20)	MS	155.2 ± 1.42	154.7 ± 1.28	1.79 ± 0.24	2.76 ± 0.45	0.28 ± 0.09	0.50 ± 0.19	54.32 ± 2.45	55.67 ± 2.34	45.68 ± 2.45	44.33 ± 2.34	1.18 ± 0.12	1.32 ± 0.12
	Control	155.2 ± 2.22	155.6 ± 1.77	1.93 ± 0.34	1.72 ± 0.19	0.26 ± 0.12	0.12 ± 0.03	48.24 ± 2.90	50.23 ± 3.27	51.76 ± 2.90	49.77 ± 3.27	1.00 ± 0.11	1.01 ± 0.12
5 (20–25)	MS	174.8 ± 2.69	173.6 ± 1.12	1.22 ± 0.14	1.57 ± 0.15	0.03 ± 0.02	0.02 ± 0.01	46.61 ± 1.56	42.17 ± 3.42	53.39 ± 1.56	57.83 ± 3.42	0.84 ± 0.07	0.72 ± 0.08
	Control	177.2 ± 2.26	174.0 ± 2.65	1.10 ± 0.07	1.15 ± 0.09	0.01 ± 0.01	0.00 ± 0.00	41.01 ± 2.30	42.91 ± 4.25	58.99 ± 2.30	57.09 ± 4.25	0.72 ± 0.06	0.84 ± 0.12
6 (25–30)	MS	185.5 ± 2.09	181.4 ± 1.87	1.25 ± 0.14	1.48 ± 0.15	0.04 ± 0.02	0.04 ± 0.04	46.43 ± 2.77	38.13 ± 3.16	53.57 ± 2.77	61.87 ± 3.16	0.91 ± 0.09	0.66 ± 0.08
	Control	185.2 ± 2.55	180.1 ± 2.86	1.38 ± 0.09	1.50 ± 0.15	0.02 ± 0.01	0.01 ± 0.01	38.28 ± 1.94	39.88 ± 4.84	61.72 ± 1.94	60.12 ± 4.84	0.64 ± 0.05	0.68 ± 0.11
7 (30–35)	MS	189.6 ± 2.62	185.5 ± 2.31	1.82 ± 0.30	1.60 ± 0.16	0.10 ± 0.05	0.08 ± 0.04	36.43 ± 3.86	37.53 ± 3.76	63.57 ± 3.86	62.47 ± 3.76	0.56 ± 0.07	0.66 ± 0.10
	Control	185.8 ± 1.68	182.1 ± 3.24	2.19 ± 0.21	1.66 ± 0.20	0.14 ± 0.05	0.07 ± 0.06	44.25 ± 3.22	42.95 ± 3.54	55.75 ± 3.22	57.05 ± 3.54	0.86 ± 0.11	0.76 ± 0.12
8 (35–40)	MS	191.0 ± 2.73	186.9 ± 2.42	1.65 ± 0.28	1.52 ± 0.13	0.07 ± 0.04	0.00 ± 0.00	41.67 ± 4.27	38.89 ± 4.01	58.33 ± 4.27	61.11 ± 4.01	0.83 ± 0.15	0.63 ± 0.08
	Control	187.5 ± 1.49	185.9 ± 3.22	1.50 ± 0.17	1.54 ± 0.16	0.04 ± 0.02	0.02 ± 0.01	33.21 ± 3.80	36.98 ± 2.52	66.79 ± 3.80	63.02 ± 2.52	0.49 ± 0.07	0.61 ± 0.05
9 (40–45)	MS	193.3 ± 2.63	190.1 ± 2.75	1.88 ± 0.24	1.42 ± 0.20	0.05 ± 0.02	0.01 ± 0.01	38.30 ± 4.36	33.74 ± 3.42	61.70 ± 4.36	66.26 ± 3.42	0.69 ± 0.10	0.55 ± 0.08
	Control	189.6 ± 2.20	188.2 ± 3.05	1.37 ± 0.11	1.34 ± 0.16	0.01 ± 0.01	0.00 ± 0.00	38.46 ± 2.59	35.26 ± 2.74	61.54 ± 2.59	64.74 ± 2.74	0.61 ± 0.07	0.58 ± 0.07
10 (45–50)	MS	196.5 ± 3.20	191.8 ± 2.39	1.68 ± 0.17	1.32 ± 0.14	0.09 ± 0.06	0.03 ± 0.03	45.18 ± 1.56	32.19 ± 5.57	54.82 ± 1.56	67.81 ± 5.57	0.78 ± 0.08	0.48 ± 0.11
	Control	191.7 ± 3.13	189.9 ± 3.22	1.44 ± 0.12	1.31 ± 0.11	0.08 ± 0.04	0.03 ± 0.02	41.89 ± 2.75	37.24 ± 5.12	58.11 ± 2.75	62.76 ± 5.12	0.64 ± 0.08	0.73 ± 0.17
11 (50–55)	MS	194.9 ± 2.93	193.6 ± 2.27	2.38 ± 0.23	1.29 ± 0.11	0.12 ± 0.04	0.01 ± 0.01	42.70 ± 5.19	31.07 ± 4.93	57.30 ± 5.19	68.93 ± 4.93	0.86 ± 0.13	0.55 ± 0.13
	Control	193.5 ± 3.53	189.5 ± 3.58	1.46 ± 0.14	1.37 ± 0.17	0.04 ± 0.02	0.00 ± 0.00	38.57 ± 3.61	42.77 ± 5.54	61.43 ± 3.61	57.23 ± 5.54	0.60 ± 0.08	0.79 ± 0.15
12 (55–60)	MS	196.7 ± 2.65	195.4 ± 2.76	2.10 ± 0.28	1.37 ± 0.10	0.11 ± 0.05	0.00 ± 0.00	34.98 ± 4.35	34.00 ± 5.00	65.02 ± 4.35	66.00 ± 5.00	0.70 ± 0.10	0.60 ± 0.12
	Control	196.9 ± 3.04	189.2 ± 3.77	1.27 ± 0.16	1.09 ± 0.14	0.00 ± 0.00	0.00 ± 0.00	29.37 ± 3.42	39.00 ± 5.22	70.63 ± 3.42	61.00 ± 5.22	0.46 ± 0.08	0.82 ± 0.19

Abbreviations: HF, high frequency; HRV, heart rate variability; LF, low frequency; r-MSSD, root mean square of successive differences between adjacent R–R intervals; n.u., normalized units; pNN20, percentage of successive interval differences larger than 20 ms.

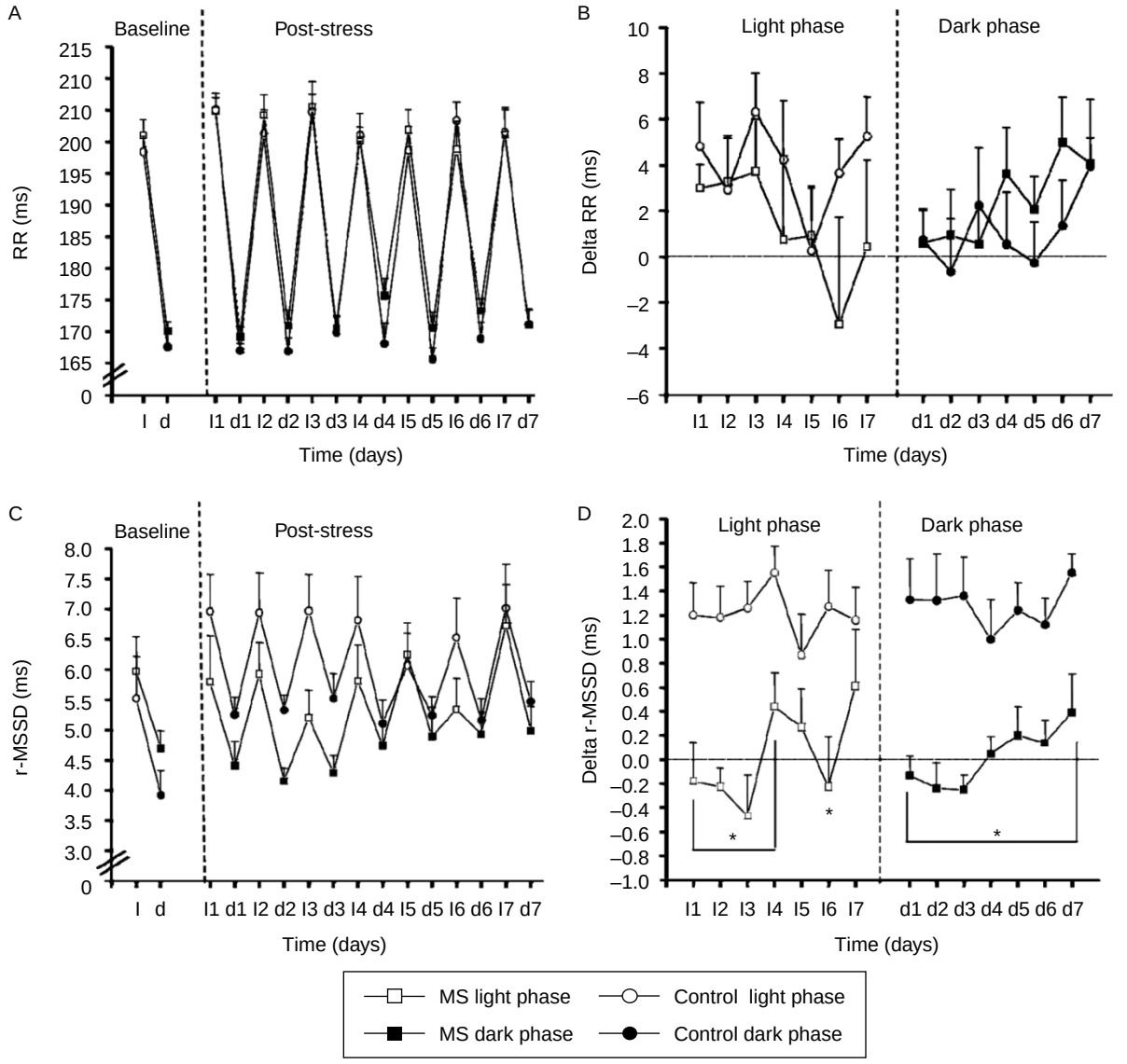

Figure 2. Daily rhythmicity of heart rate before and after the intermittent restraint protocol, in maternally separated and control rats. Average RR values (panel A) and r-MSSD values (panel C) for the 12 h light (open symbols) and dark phases (solid symbols) before (baseline: l and d) and after (post-stress: l1-l7 and d1-d7) the IRS. Delta values between each post-IRS day and the average pre-IRS value of light and dark phases are also reported (panels B and D), for both control ($n = 12$) and maternally separated (MS, $n = 12$) rats. RR, average R–R interval duration (ms). r-MSSD, root mean square of successive differences between adjacent R–R intervals (ms). Data are mean ± SEM. $\star p < 0.05$, significant differences between MS and control animals (Student's t-test).

anatomy were accompanied by changes in cardiomyocyte dimensions, myocyte CSA was measured. In the MS group, cardiomyocyte CSA was 5% larger than that in the controls (294.09 ± 3.92 vs. 278.79 ± 2.28 μm^2; $t(22) = 2.34$, $p < 0.05$), indicating that MS induced a mild hypertrophy of the individual cells.

The morphometric analysis of the myocardium documented no significant differences between the two groups of rats with respect to total amount of myocardial fibrosis in the LV myocardium (MS: 0.468 ± 0.077% vs. control: 0.474 ± 0.080%, n.s.). The volume fraction of myocytes was also unaffected by the experimental conditions (MS: 91.41 ± 0.55% vs. control: 91.38 ± 0.70%, n.s). Perivascular fibrosis and interstitial fibrosis, including small foci of collagen accumulation distributed in the myocardium, were present in both MS and control hearts. Interestingly, these forms of collagen accumulation were differently expressed in the experimental rats. Interstitial fibrosis was slightly larger in MS rats when the ventricular wall was considered as a whole (Table III) and also when the three different layers where separately examined, i.e. endo-myocardium, mid-myocardium, and epi-myocardium (MS: 0.12 ± 0.06%, 0.42 ± 0.16%, and 0.18 ± 0.07%, respectively) compared to

control group (0.02 ± 0.02%, 0.35 ± 0.12%, and 0.10 ± 0.04%, respectively). Perivascular fibrosis was more abundant in the mid-myocardium and epi-myocardium of control hearts (0.29 ± 0.10% and 0.76 ± 0.14% vs. MS: 0.19 ± 0.06% and 0.44 ± 0.09%, respectively), resulting in an overall larger amount of this form of collagen deposition in these rats (Table III).

The morphometric evaluation of vascular structures in the LV myocardium (Table III) showed that capillary density was significantly larger in MS rats compared to control counterparts. Although venule density was increased 1.5-fold in MS rats, this value did not reach statistical significance (Table III).

Discussion

The aims of this study were to investigate the long-term effects of early life adverse experience on (i) autonomic neural regulation of heart rate and (ii) cardiac morphological/morphometric characteristics in male rats.

The results obtained indicate that MS occurring in the first 2 weeks of neonatal life did not substantially alter adult cardiac sympathovagal balance, neither at rest nor under acute challenging conditions, namely repeated restraint stress and pharmacological autonomic blockade. However, MS rats did not show the enduring vagal rebound exhibited by control counterparts in the days following adult intermittent stress, which involved both the night and light phases of the circadian rhythm. In addition, MS induced a few minor changes in cardiac anatomy, involving modest degrees of cardiomyocyte hypertrophy, increased angiogenesis, and myocardial damage.

In this study, early MS was combined with a stress protocol based on intermittent restraint episodes in adulthood. MS did not change in a significant manner the acute response to a single restraint test and the habituation profile of cardiac autonomic response through repeated restraint episodes. Interestingly, in an experimental procedure resembling the present one (i.e. a 2-week daily MS followed by IRS in adulthood), Hulshof and colleagues (2011) reported a similar lack of differences between control and MS rats in HPA axis stress reactivity and habituation (i.e. similar plasma adrenocorticotrophic hormone and corticosterone levels).

In this paper, cardiac autonomic balance was assessed by means of a large number of heart variability indices, belonging to both time and frequency domains. During restraint tests 1 and 5, MS and control rats exhibited a similar increase in cardiac chronotropism and an incomplete return to baseline heart rate values during the immediate post-test period. Concomitant reduced values of time-domain HRV indices and HF power point to a withdrawal of the parasympathetic drive to the heart (Kleiger et al. 1993), which did not recover to initial values until the end of the test. Accordingly, the increase in LF power and LF/HF ratio during both restraint and recovery period points to a shift of autonomic regulation toward a sympathetic prevalence (Aubert et al. 1999). Notably, these HRV data also indicate that the dynamics of cardiac autonomic balance were similar across restraint episodes in MS and control rats, i.e. both groups did not exhibit habituation-like effects.

As expected (Japundzic et al. 1990; Aubert et al. 1999), the injection of a cholinergic muscarinic blocker (methylscopolamine) induced a robust increase in heart rate, accompanied by a clear reduction in the values of r-MSSD and pNN20. The decrease in HF power was accompanied by an increase in LF power and LF/HF ratio. Subsequent injection of a beta-blocker (atenolol) caused the return of heart rate and spectral indices of HRV to their basal values, whereas time-domain parameters remained substantially suppressed. Overall, the results obtained with the double pharmacological autonomic blockade indicate that MS had no substantial effects on cardiac pacemaker intrinsic activity, neither before nor after the IRS.

So far few studies have investigated the long-lasting consequences of MS on cardiovascular function and, to our knowledge, the present work is the first to explore long-term cardiac autonomic effects through HRV parameters. The lack of differences between MS and control rats, both at rest and during acute environmental and pharmacological challenge, supports the idea that MS *per se* does not bring about a cardiac autonomic pathophysiologic phenotype. In this regard, our findings are in agreement with a number of other studies that did not find any clear effect of MS on adult baseline heart rate and arterial

Table III. Values (mean ± SEM) of gross cardiac characteristics, myocardial fibrosis in the LV and vascular distribution in the LV, in maternally separated (MS; $n = 12$) and control ($n = 12$) rats.

	Control	MS
LVW (mg)	889.5 ± 22.08	818.8 ± 20.17⋆
RVW (mg)	201.9 ± 7.01	201.8 ± 8.11
LVW/HW (mg/mg)	0.908 ± 0.005	0.802 ± 0.006⋆
RVW/HW (mg/mg)	0.192 ± 0.005	0.198 ± 0.006
LV chamber length (mm)	14.58 ± 0.27	14.20 ± 0.23
LV chamber equatorial diameter (mm)	5.32 ± 0.18	5.02 ± 0.15
LV wall thickness (mm)	2.11 ± 0.06	2.14 ± 0.06
LV chamber volume (mm^3)	220 ± 15.67	189 ± 11.90⋆
Perivascular fibrosis (%)	0.316 ± 0.047	0.225 ± 0.043⋆
Interstitial fibrosis (%)	0.158 ± 0.060	0.243 ± 0.056⋆
Capillary density (n/mm^2)	132.2 ± 16.61	178.8 ± 11.42⋆
Venule density (n/mm^2)	6.31 ± 1.02	9.13 ± 2.54

Notes: LV, left ventricle; LVW, left ventricular weight; RVW, right ventricular weight; HW, heart weight; ⋆$p < 0.05$: significant differences between MS and control rats (Student's *t*-test).

blood pressure (Sanders and Anticevic 2007; Loria et al. 2010a,b). However, in the study by Sanders and Anticevic (2007) on borderline hypertensive rats, MS rats showed higher heart rate responsivity to restraint stress compared to control rats, despite blood pressure response was similar in the two groups. Although the MS protocol in the latter study was similar to ours, it may well be that borderline hypertensive rats are more susceptible to environmental stressors and that different blood pressure phenotypes play a pivotal role in mediating the differences in autonomic regulation of cardiac stress response.

The recording of the daily rhythms of heart rate and cardiac vagal activity allowed further exploration of the long-lasting effects of MS on cardiac chronotropy and its autonomic neural regulation, before and after the period of adult intermittent stress. MS and adult exposure to five restraint episodes did not affect the light–dark oscillation of heart rate. However, while autonomic control of cardiac chronotropy remained largely unchanged in MS rats, control counterparts showed a prolonged increase in cardiac parasympathetic drive following IRS, involving both the dark and light phases of the circadian rhythm. As the average night and day values of heart rate were similar before and after the stress period, we hypothesize that the increase in parasympathetic drive observed in control rats following IRS was accompanied by a concomitant enhancement of sympathetic tone.

This peculiar phenomenon, which was observed in control but not in maternally separated rats, might be called "enduring vagal rebound" or "persistent vagal rebound." In the literature, "vagal rebound" usually has a rather different meaning; it refers to short-term, brief vagal hyperactivity following a stressor, a sympathetic overdrive or reperfusion after acute myocardial infarction (Chiladakis et al. 2001; Mezzacappa et al. 2001). In this paper, it was a relatively persistent, long-term consequence of an intermittent stressor and can be viewed as an adaptive response of the organism. Indeed, the increase in vagal activity that often occurs to counterbalance stress-induced sympathetic activation is known to be associated with a reduced risk of cardiovascular disease and mortality (La Rovere et al. 1998).

If this point of view is correct, one may infer that cardiac autonomic neural control of adult rats that experienced early life challenge was less stress-responsive (i.e. perhaps less flexible) compared to control counterparts, and thus less favorable in terms of resilience. However, one may also maintain that the lack of changes in autonomic modulation observed in MS rats was the consequence of a shift of the regulatory range, which implied a reduced sensitivity to adult intermittent stress exposure (Koolhaas et al. 2011). Indeed, several studies support the view that early maternal environment prepares the regulatory range of the offspring for the conditions it may have to cope with in adulthood (Kaiser and Sachser 2005; Gluckman et al. 2007; Champagne et al. 2008). An example of this is given in a recent study in mice by Heiming and co-workers (2009): they showed that mice that were raised in a threatening environment exhibited less anxiety and more exploratory behavior as adults when confronted with challenging situations than animals that were raised in a stable environment.

One may question that the data collected following scopolamine administration do not support the observed raise in vagal activity emerging from post-stress rhythm analysis. Indeed, one may expect a somewhat larger tachycardic response to scopolamine in control rats after IRS. The likely reason for this discrepancy is that the second pharmacological challenge was carried out during the light phase of the day after the end of rhythm recordings (Day 31 in Figure 1). Figure 2 (panel D) shows that r-MSSD values of control rats in the last light phase (l7) were not significantly different anymore from corresponding MS values. Hence, the pharmacological challenge was carried out when the "vagal difference" between control and MS rats was already gone.

In the current study, animals exposed to MS and adult intermittent stress had heavier adrenal glands compared to control rats, which may reflect either enhanced steroidogenesis in the cortex or an increased catecholamine biosynthetic activity in the medulla (or both). However, increased adrenal weight in MS rats that were not exposed to adult manipulations suggests that MS *per se* is able to induce adrenal enlargement. Indeed, available data from the literature on the effects of postnatal stress on adrenal gland weight are not consistent. Some authors showed that 24-h maternal deprivation induces significantly heavier adrenals (Rots et al. 1996; Husum et al. 2002), while others reported no adrenal enlargement (Wigger and Neumann 1999) or even lighter glands (Slotten et al. 2006). We did not determine plasma corticosterone concentrations in this study, so we cannot relate any cardiac changes to increased corticosterone secretion.

The evaluation of the effects of MS on cardiac architecture indicated a moderate reduction in LVW and chamber dimensions in the absence of clear changes in myocardial structural composition. Myocardial fibrosis was minimally affected by MS and only interstitial deposition of collagen was moderately increased. These gross anatomical changes, in association with modest signs of cellular hypertrophy, likely reflect a physiologic adaptation to maternal stress of the growing heart during early postnatal development. The possibility cannot be excluded that a stressor imposed during a delicate phase of cardiac development in which remarkable cellular changes occur may structurally affect the heart. It is well established that a switch from a prevailing hyperplastic to a predominant hypertrophic growth (Anversa et al. 1980) as well as apoptotic death of cardiomyocytes

(Kajstura et al. 1995) all rapidly interplay within 3 weeks after birth in the rat heart. Whether MS-driven neurohormonal factors, implicated in physiologic and pathologic processes in the heart, may alter cardiomyocyte turnover resulting in cardiac structural remodeling following maturation is unknown. Slight hypertrophy of cardiomyocytes was observed here in the LV in association with a reduced mass and modest collagen deposition, indicating that precocious cell loss and reactive hypertrophy may have occurred early during post-natal cardiac development. Interestingly, all these events are known to be evoked in the heart by the renin–angiotensin system (Sadoshima and Izumo 1993), which in turn has been implicated in MS (Loria et al. 2010b).

The early postnatal period is also characterized by a dramatic growth of the coronary vascular bed and the functional capacity of the myocardium reaches adult values at approximately 16 days of age (Hopkins et al. 1973). The mean transmural number of capillaries undergoes a fourfold increase in the LV from postnatal Days 1–11 and the aggregate ventricular capillary length more than doubles between postnatal Days 5 and 11 (Olivetti et al. 1980). Our data point to an increased capillarization of the left ventricular myocardium in maternally separated animals. The detected increase in the density of vascular structures is relevant under the contention that enlargement of cardiomyocytes, as shown in the myocardium of MS rats, would result in the reduction in the number of capillaries per unit area (n/mm^2) according to morphometric principles. However, why promotion of angiogenesis or lack of regression of capillary formation should occur in the heart under these experimental conditions requires further investigation.

In conclusion, this study on rats shows that daily 3-h MS during the first 2 weeks of postnatal life did not affect in a significant manner adult intrinsic heart rate and acute cardiac autonomic responsivity to a psychological stressor. However, maternally separated rats did not exhibit the enduring vagal rebound observed in control counterparts after adult IRS, which might be viewed as an adaptive response aiming at favoring cardiovascular stress resilience. Moreover, MS induced some, although minor, changes in cardiac anatomy, involving modest degrees of cardiomyocyte hypertrophy, increased angiogenesis, and myocardial damage. Whether these functional and structural changes could have evolved to a more pathologic cardiac phenotype later in life is plausible, but remains to be determined.

The overall mildness of cardiac functional and structural effects of MS reported in this paper indicates that this approach might not be the best preclinical tool for modeling early age, stress-induced cardiac disease in humans.

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