SYMPATHETIC AND PARASYMPATHETIC CONTROL OF HEART RATE RESPONSE TO RESTRAINT STRESS DURING THE VULNERABLE PERIOD IN NEWBORN RATS

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Abstract

Newborn infants and animals undergo dramatic development of the autonomic nervous system ; however, there remains a lack of normative information concerning the vulnerable period. This study investigated the maturation of the heart rate (HR) and the autonomic regulation of HR responses to restraint stress during the first 2 postnatal weeks in rats. Autonomic blockade with atropine, metoprolol, and dual atropine/metoprolol blockade (2 mg/kg) at postnatal day (P) 4 and P12 demonstrated predominantly sympathetic effects on baseline HR. From birth to P9, rats restrained by electrocardiography electrodes for 5 min displayed slow cardiodeceleration, which changed into transient bradycardia (TB) during P9–14. Interestingly, this TB was abolished by autonomic blockade on P12 ; stable tachycardia (18% increase) and unstable HR were observed under parasympathetic and sympathetic blockade, respectively. In addition, HR under dual blockade increased by 25% ($p=0.009$) after the animals were restrained but not under dual blockade with a 10-fold higher dose. These results suggest that TB is mediated by a complex sympathetic–parasympathetic interaction, which is immature during the vulnerable period and can potentially cause life-threatening cardiac events.

Key words : newborn rats, heart rate, restraint stress, accentuated antagonism, sudden infant death syndrome

Introduction

Sudden infant death syndrome (SIDS) is defined as the sudden unexpected death of infants less than 12 months of age from a cause that remains unexplained after complete autopsies and death scene investigations[1-3)]. Age dependency is the hallmark of SIDS. The incidence of SIDS peaks at 2–4 months of age[3-5)], which is characterized as the vulnerable period of marked homeostatic (autonomic) instability when critical developmental changes occur in homeostatic systems, including autonomic control of heart rate (HR)[6-8)]. Rat/mouse studies also attributed SIDS during the period of autonomic instability (the first 2 postnatal weeks) to the loss of cardiorespiratory reflexes in the face of exogenous stressors such as hy-

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poxia, hyperthermia, and hypercapnia[1,9-11] or to immature functioning of the central/peripheral autonomic nervous system (ANS) with abnormalities in the β-adrenoceptor/muscarinic receptor-acetylcholine receptor balance in the heart and brainstem[12,13]. However, there remains a lack of information about the trajectory of ANS development during the vulnerable period in rats/mice as well as human infants. The paucity of normative developmental information is one reason that makes it difficult to study SIDS[14]. We previously reported changes in HR responses to restraint stress, ranging from slow cardiodeceleration (SCD) to transient bradycardia (TB), during the first 2 postnatal weeks in mice[15]. An age-dependent change in HR response pattern was also observed in rats in this study. However, we found that the autonomic control of TB differs between rats and mice. TB in rats is likely to be mediated by a complex interaction between sympathetic and parasympathetic nervous system activities, which was not observed in our previous experiment in mice[15]. In this study, we investigated developmental changes in baseline HR and autonomic control of HR responses to restraint stress during the first 2 postnatal weeks in rats.

Materials and methods

Pregnant Wistar rats (CLEA Japan, Tokyo ; Japan SLC, Shizuoka) were monitored with an infrared camera to determine the exact delivery time. Rat pups aged 0-14 days that were born in our laboratory were raised in a litter with their dams in a home cage (42 × 25 × 20 cm). The pups and their dams were housed at 25 ± 1°C under a 12-h : 12-h light-dark cycle with food and water provided ad libitum. In total, 393 rat pups and their dams were euthanized by CO_2 inhalation after the experiments were conducted as per the protocol, which was approved by the Akita University Institutional Committee for Animal Studies. The methods of this study are based on previously reported protocols[15].

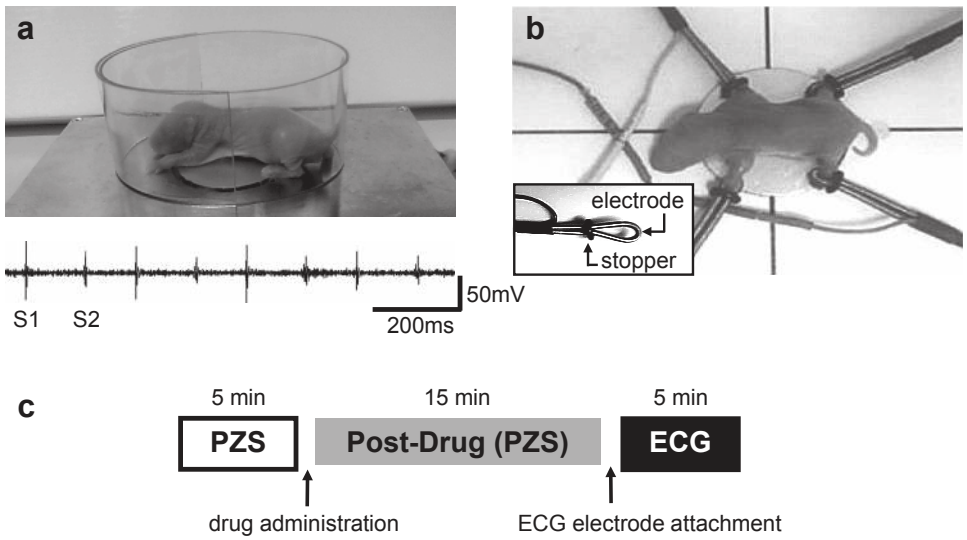

Fig. 1. Noninvasive baseline HR measurement by the PZS and measurement of HR responses to restraint stress by ECG

a Baseline HR of a rat was measured by the PZS, which converts vibrations (heartbeat, respiration, and body movements) into an electrical signal. Representative heart sounds (S1, S2) of a rat at P0 are shown in the lower panel.

b HR responses to restraint stress were measured by restraining the animals with ECG electrodes. The rat's legs were secured to the ECG electrodes during the ECG recording. The inset in B shows the structure of the ECG electrode.

c The protocol for measuring HR response to restraint stress under autonomic blockade is shown.

Piezoelectric sensor (PZS) surface temperature control

We determined the surface temperature of the PZS (Fig. 1) as a noninvasive HR measurement[16] at 31.5°C after evaluating the body surface temperature of the rats on postnatal day (P) 6 for 5 min using a noncontacting infrared digital thermometer (SK-8940 ; skSATO, Japan). Their body surface temperature [36.5±0.8°C, n=6 ; mean±standard deviation (SD)] was stable for 5 min (−0.01±0.3°C, p=0.919) at the PZS surface temperature of 31.5°C, whereas it decreased (−1.03±0.7°C, p=0.017) and increased (0.73±0.3°C, p=0.003) at 25°C and 35°C, respectively. HR (327±28 beats/min, n=6) was also stable during 5 min at the PZS surface temperature of 31.5°C (1.0±3 beats/min, p=0.453), whereas it significantly decreased at 25°C (−28.9±12.2 beats/min, p=0.002) and 35°C (−29.6±27.8 beats/min, p=0.048). The room temperature was set at 25±1°C.

Baseline HR measurement

Baseline HR (PZS-HR) was calculated from beat-to-beat intervals of heart sounds recorded for 5 min by the PZS system[16]. To avoid the interference of the circadian rhythm with baseline HR, experiments were performed between 8 : 00 and 20 : 00 every 24 h (starting with a delay/advance of <3 h) from birth time. Pups were transported over approximately 80 cm from their home cage to the PZS in 3 s, and the PZS recording was initiated within several seconds. A plastic ring with an inner diameter of 60 mm and a height of 35 mm was placed around the PZS to prevent rats from escaping (Fig. 1a).

To evaluate the change in PZS-HR during 5 min, we measured the PZS-HR HR at 0 and 5 min in rats from P0 to P14. The PZS converts vibrations (e.g., heart sounds, respiration sounds) into an electrical signal when a rat's chest/abdomen is in contact i.e., during resting and sleeping states. Consequently, the baseline HR time course becomes discontinuous during active state. Therefore, the PZS-HR at 0 and 5 min was calculated from the average of 10 available consecutive beat-to-beat intervals that represented the trend of HR near 0 and 5 min, respectively. The beat-to-beat intervals over 5 min of measurement were automatically calculated from the PZS signal by the peak detection algorithm of Clampfit 9 software (Molecular Devices, Sunnyvale, CA). Because HR data calculation by the automatic analysis was often erroneous, we fixed the errors by observing the raw PZS signal and manually verifying HR at 0 and 5 min.

HR measurement using electrocardiography (ECG-HR)

ECG electrodes were composed of a spring steel coil loop that could be shrunk by a rubber stopper (Fig. 1b, inset). Because the ECG electrodes were fixed on the PZS board, the rats were inevitably restrained during the ECG recording for 5 min (Fig. 1b) ; therefore, a change in ECG-HR was considered to represent an HR response to restraint stress. ECG-HR at 0 and 5 min was calculated from R-R intervals using the same method used to calculate PZS-HR. PZS-HR and ECG-HR data for HR development analysis were separately recorded from different groups of rats. PZS temperature control and PZS-HR/ECG-HR measurements were performed using the ATC-402 PZS cardiorespiratory monitor (Unique Medical, Tokyo, Japan).

Autonomic blockade experiment

After baseline HR measurement, the muscarinic receptor blocker atropine, the β1-adrenoceptor blocker metoprolol, or both (dual blockade) were mixed with saline (vehicle) and administered to the pups on P4 and P12 to determine sympathetic and parasympathetic contributions to baseline HR. Atropine sulfate (Mylan Pharmaceuticals, Morgantown, WV) and/or metoprolol tartrate (MP Biomedicals, Santa Ana, CA) were dissolved in 0.9% saline solution before the experiments, and a 30-gauge insulin syringe (U-100 ; Becton-Dickinson, Franklin Lakes, NJ) was used for intraperitoneal injection (2 mg/kg). The drug dosages were selected on the basis of previous studies[17-19]. Postblockade PZS-HR was recorded for 5 min, starting 10 min after the autonomic blocker injections (Fig. 1c).

To evaluate HR responses to restraint stress under autonomic blockade, ECG-HR was recorded after the postblockade HR measurement as described above according to the time diagram shown in Figure 1c. Control ECG-

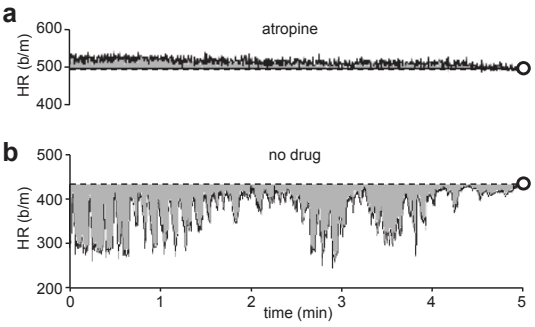

Fig. 2. AOC analysis
a An example of AOC analysis of HR response to restraint stress under atropine blockade. The AOC is indicated by the gray area, which is the sum of the difference between HR at every sampling point over 5 min and the HR at 5 min (open circle).
b The AOC of TB becomes negative as indicated by the gray area.

HR (no drug administration) was recorded for 5 min immediately after the 5 min of PZS-HR recording. To assess changes in HR response patterns induced by the autonomic blockers, we used area over the curve (AOC) analysis, which integrates the differences between instantaneous HR at every sampling point of the R-wave peak over 5 min (Figs. 2a and 2b ; gray area) and the HR at 5 min (Figs. 2a and 2b ; open circle). For the statistical evaluation, we then divided the value of the AOC by the total R-wave-detection time, which was different for each rat because the total number of failures in ECG detection due to struggling differed among individual rats, of the period when the ECG signal was successfully detected.

Data acquisition and analysis

PZS and ECG signal outputs from the ATC-402 monitor were stored in a computer using Clampex9 software via an analog-to-digital converter (digidata1322A ; Molecular Devices, Sunnyvale, CA) at a sampling interval of 0.2 ms. Changes in PZS-HR and ECG-HR over the 5-min time course were assessed using a paired Student's t-test. For multiple comparisons, the post-hoc Dunnett's test was used after one-way ANOVA and the Shapiro-Wilk normality test. An unpaired t-test and the Mann-Whitney U test were used as appropriate. Data are expressed as means ± standard errors unless otherwise stated. P values of <0.05 were considered statistically significant.

Results

Developmental changes in baseline HR

The baseline HR of newborn rats showed a steep increase from P0 to P3 (218±4 to 363±9 beats/min, $n=9$), followed by a smaller increase until P14 (467±5 beats/min, $n=9$). There was no significant change in PZS-HR during the 5 min of baseline HR measurement in all rats from P0 to P14 ($p>0.057$, $n=188$; Fig. 3a). Baseline HR data on P4 and P12 ($n=33$ and 34, respectively) were combined with the PZS-HR data before the autonomic blockade experiments.

Effects of autonomic blockers on baseline HR

Baseline HR was significantly decreased by metoprolol (P4 : 372±4 to 258±4 beats/min, $p=0.000$; P12 : 395±11 to 266±9 beats/min, $p=0.000$) and dual blockade with metoprolol and atropine (P4 : 361±9 to 229±2 beats/min, $p=0.000$; P12 : 401±6 to 273±10 beats/min, $p=0.000$), whereas atropine (P4 : 373±8 to 376±11 beats/min, $p=0.814$; P12 : 461±9 to 436±2 beats/min, $p=0.112$) and saline (P4 : 345±13 to 365±10 beats/min ; P12 : 404±4 to 399±5 beats/min) had no effect on baseline HR ($n=6$ each, Dunnett's test, Figs. 3b and 3c). This finding demonstrates that baseline HR is dominated by sympathetic regulation. In contrast, the tonic parasympathetic contribution to baseline HR may be absent or negligible in newborn rats, consistent with the findings observed in mice[15].

Developmental changes in HR response to restraint stress

Figures 4a and 4b show the representative time courses of PZS-HR and ECG-HR, respectively (P4 : upper, P12 : lower). Detectable heartbeats mostly continued for several to tens of seconds during the first postnatal week (Fig. 4a, upper). As the animals aged, heartbeats were often sparsely detected and mostly continued for only several seconds because of the vigorous activity and occasional escape of the animals over the plastic ring ; this caused fragmentation of PZS-HR (Fig. 4a, lower). In response to ECG electrode attachment, the rats mostly displayed SCD (Fig. 4b, upper) on P4, where-

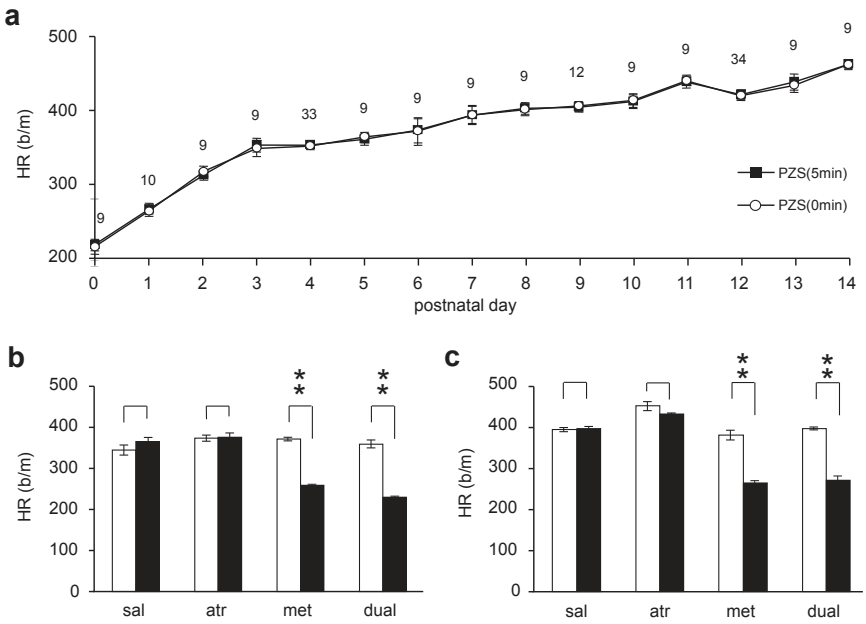

Fig. 3. Development of baseline HR and its autonomic regulation during the first 2 postnatal weeks in rats
a Baseline HR at 0 (open circles) and 5 (filled squares) min during P0–14 in rats are plotted. Numbers of data are indicated above each marker.
b and c Effects of autonomic blockers on baseline HR [sal = saline (vehicle), atr = atropine, met = metoprolol, dual = atr + met] in rats at P4 (b) and P12 (c) are shown. $^{**}p<0.01$

as on P12, 63% (5/8) rats exhibited TB accompanied by rapid phasic HR fluctuations (up to 110 beats/min change in 2.1±0.6 s, $n=19$) that repetitively appeared at 4–12-s intervals (Fig. 4b, lower). Figure 4c shows age-dependent HR responses to restraint stress from the attached ECG electrodes. Rats on P0–8 mostly exhibited SCD, which changed to TB on P9 in one rat ; subsequently, >30% rats displayed TB until P14, with a maximum of 88% displaying TB on P13 (Table 1). Individual changes from SCD to TB occurred between P9 and P14 in rats, which is a shorter period than that observed in mice (P5–P13).

Only P0 rats during the first postnatal week exhibited an initial decrease in ECG-HR immediately after ECG electrode attachment (0 min : 181±6 beats/min, $n=9$) relative to PZS-HR at 5 min (218±4 beats/min, $n=9$, unpaired t-test, $p<0.0001$; Mann-Whitney U test, $p=0.0005$). This was followed by SCD of −9±3 beats/min ($p=0.028$). The initial decrease in ECG-HR may be mediated by neutrally controlled sympathetic withdrawal as observed in a mouse experiment[15] where mice exhibited an initial decrease in ECG-HR from P1 to P13, but not on P0. During the second postnatal week, simultaneous recording of PZS and ECG signals on P12 (Fig. 5a) revealed that the rapid phasic fluctuations in HR mostly appeared concurrently with short periods of struggle that were detected by the PZS (arrows). Detailed analysis of PZS and ECG signals over an expanded time scale (Figs. 5b and 5c) further illustrated that the rapid HR fluctuations (increases) were mostly preceded by the onset of struggle.

These results demonstrate that the period of change from SCD to TB in rats, which is shorter than that observed in mice[15], may represent the dynamic developmental change in ANS in the second postnatal week.

HR responses to restraint stress under autonomic blockade

In rats on P4, restraint stress under atropine, metoprolol, and dual blockade induced similar responses of SCD.

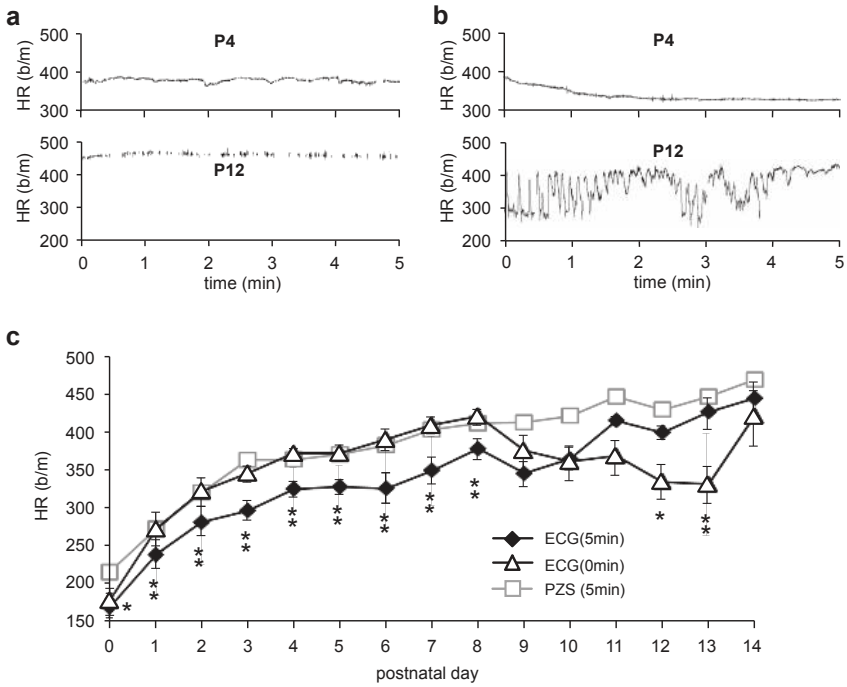

Fig. 4. HR responses to restraint stress in rats at P4 and P12
a Representative time courses of baseline HR before restraint on P4 and P12 are shown.
b Representative HR traces show responses to restraint stress after ECG electrode attachment on P4 (SCD) and P12 (TB). The TB at P12 is composed of a slowly recovering component and rapid HR fluctuations.
c Age-dependent HR responses to restraint stress induced by ECG electrode attachment at 0 (open triangles) and 5 (filled rhombuses) min at each age are plotted. Baseline HR is shown as a gray line with open squares. Numbers of data are indicated in Table 1. $*p<0.05$, $**p<0.01$

Table 1. Patterns of HR response to restraint stress in rats during the first two postnatal weeks

Age, d	SCD, beats/min	(*p*)	(*n*)	TB, beats/min	(*p*)	(*n*)	unstable, *n*	stable, *n*	total, *n*
P0	−23±7	0.001**	6			0	0	2	8
P1	−39±18	0.002**	7			0	0	2	9
P2	−45±16	0.000**	8			0	0	1	9
P3	−51±8	0.000**	8			0	1	0	9
P4	−40±12	0.003**	5			0	1	0	6
P5	−63±23	0.001**	7			0	1	1	9
P6	−61±27	0.002**	7			0	1	0	8
P7	−68±28	0.001**	7			0	2	0	9
P8	−62±27	0.009**	5			0	4	0	9
P9	−72±24	0.004**	5	+136±0		1	2	0	8
P10	−68±9	0.001**	3	+101±19	0.019**	3	3	0	9
P11	−43±6	0.012*	3	+138±23	0.000**	5	0	0	8
P12	−46±8	0.112	2	+129±20	0.000**	5	0	1	8
P13			0	+114±41	0.000**	7	1	0	8
P14			0	+120±37	0.189	2	1	3	6

SCD and TB are the changes in ECG-HR between 0 and 5 min ; (p) and (n) are statistical significance and the number of rats exhibited SCD or TB, respectively. Number of rats who showed other HR-response patterns, unstable or stable, are listed in the right columns in addition to the total number of rats examined at each age. $*p<0.05$, $**p<0.01$.

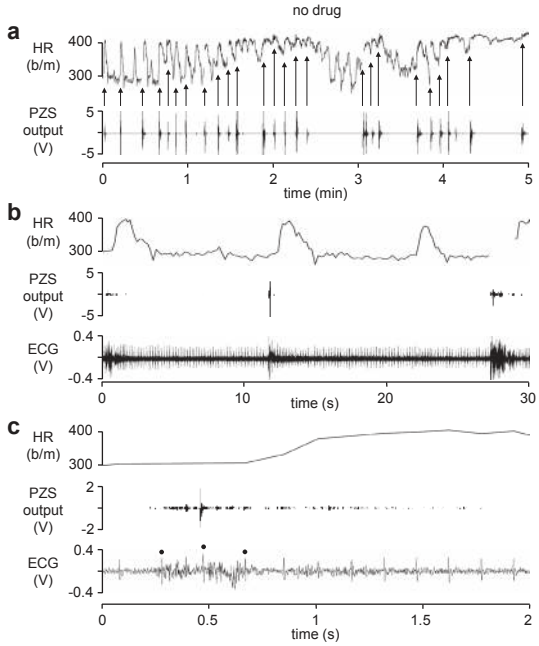

Fig. 5. Analysis of rapid HR fluctuations
a TB (same as in Fig. 3b) is illustrated with a simultaneously recorded PZS signal that indicates struggling activity as a large-amplitude vibration that appears concurrently with a rapid HR fluctuation (arrows).
b Expanded 30-s trace from the beginning of the trace in a is shown with the ECG trace. Beat-to-beat intervals were mostly detectable throughout the recording, except during sizeable struggling activities at approximately 28 s.
c Expanded traces for 2 s from the trace in b with ECG traces. Low struggling activity did not affect the detection of beat-to-beat intervals (small dots).

The HR initially increased by +31±11, +16±11, and +12±11 beats/min under atropine, metoprolol, and dual blockade, respectively, after ECG electrodes were attached, following which it gradually decreased over 5 min (SCD) by −57±3, −53±5, and −44±4 beats/min, respectively (n=5 ; Figs. 6a and 6b). The amplitudes of these SCDs were comparable to those under the no drug condition (−41±8 beats/min ; p>0.128, Dunnett's test). This finding appears to indicate that SCD is mediated by factors other than ANS control. However, dual blockade at a 10-fold higher dose (20 mg/kg) diminished SCD (−6±3 beats/min, n=5 ; Fig. 6a, lower : thick gray line), which may implicate the ANS control of SCD and suggest that the drug dosage was insufficient for the stress condition.

In rats on P12, TB induced by restraint stress was alleviated by autonomic blockade. The restraint stress-induced HR response under parasympathetic blockade was stable tachycardia (507±6 beats/min at 5 min, n=6 ; Figs. 7a, upper and 7b) of approximately 75 beats/min above the postblockade HR before restraining (431±7 beats/min, p<0.001) ; this indicates strong and continuous sympathetic activity during the period of restraint. On the other hand, HR responses of individual rats to restraint stress under sympathetic blockade were unstable, particularly during the first half of the 5-min period, although the average restraint stress-induced HR responses at 0 and 5 min (283±15 and 271±18 beats/min, n=6) did not significantly differ from postblockade HR (267±9 beats/min, p>0.432 ; Figs. 7a, middle and 7b). Several temporal HR augmentations were observed (Fig. 7a, middle, arrows), which increased from relatively stable ECG-HR (263±13 beats/min, n=11 from 6 rats ; horizontal gray bars) near the level of intrinsic HR (cardiac pacemaker rhythm under the blockade of autonomic control). Under dual blockade, the restraint stress significantly elevated HR to 339±4 beats/min (n=6, p=0.001) from the intrinsic (postblockade) HR of 274±10 beats/min (Figs. 7a, lower and 7b). This HR elevation was diminished by dual blockade with a 10-fold higher dose of a β1-blocker (metoprolol 20 mg/kg + atropine 2 mg/kg ; 256±12 beats/min, n=5, p=0.068 ; Fig. 7a, lower : thick gray line) ; the dose of atropine was not changed to exclude possible changes in sympathetic-ganglionic blockade that may affect HR and determine whether the HR elevation was due to an insufficient β1-blocker dose. AOC analysis demonstrated the obvious HR response pattern change from TB in the three aforementioned autonomic blockade conditions (p<0.001, Dunnett's test ; Fig. 7c). The positive small bars in Figure 7c denote slight decreases in HR over 5 min (positive AOC values : gray area in Fig. 2a) in the P12 rats, while the negative bar denotes a negative AOC value (gray area in Fig. 2b). These slight decreases in HR responses to restraint stress in the P12 rats under autonomic blockade may be due to sympathetic excitation accompanied by struggling activity, which is more vigorous

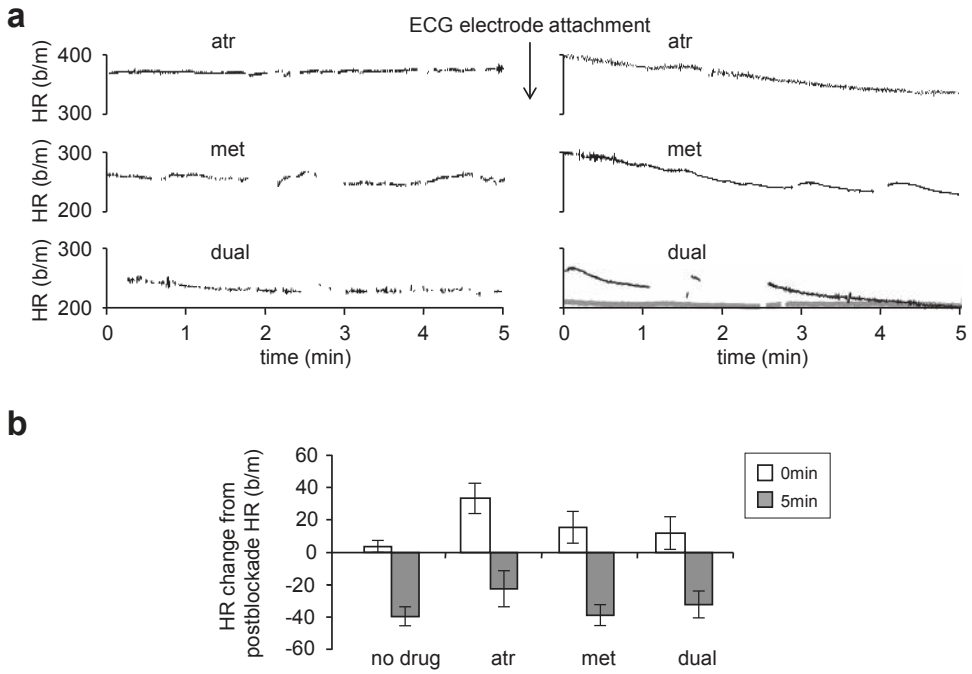

Fig. 6. HR responses to restraint stress under autonomic blockade in rats on P4
a Representative traces of postblockade HR (left) and HR responses (right) to restraint stress under atropine (upper), metoprolol (middle), and dual (lower) blockade (2 mg/kg each) are shown. The struggling of the animals often made the ECG-HR detection discontinuous. HR response under dual blockade with a 10-fold higher dose is indicated by a thick gray line in the dual blockade trace.
b HR changes at 0 (open square) and 5 min (filled square) from each postblockade HR at 5 min are illustrated. The difference in HR between 0 and 5 min is similar among the autonomic blockers.

at the beginning of restraint than during the latter half of the measurement, as indicated by the PZS signals in Figure 8 (a-c, lower panels). Several HR augmentations were observed concurrently with struggling activities (Fig. 8b, open circles), and rapid phasic HR increases of small amplitudes also appeared concurrently with struggling activities (Fig. 8b, arrows).

Discussion

HR development

Sympathetic and parasympathetic tones will mature at different rates in infancy[20]. We observed these differences in maturation rates between the sympathetic and parasympathetic autonomic nervous systems in the present study. Tonic sympathetic tone may progressively increase during the first 3 postnatal days in parallel with the steep increase in baseline HR. This was followed by a gentle increase until P14 in our study (Fig. 3a). Baseline HR in newborn rats is continuously elevated by sympathetic regulation as per our results of autonomic blockade experiments (Figs. 3b and 3c) and previous investigations[15,17,21,22]. On the other hand, our data indicate that tonic parasympathetic tone is negligible and may appear after a couple of postnatal weeks[21,23]. These results are consistent with those of our previous study in mice[15]; therefore, the dose (2 mg/kg) of autonomic blockers appears to be sufficient to block ANS control of baseline HR at rest or in a freely moving condition in newborn rats. We often detected HR momentarily (approximately 1-3 s) during rest and sparsely during active states. However, this may not result in an increased baseline HR because HR during active states is not significantly higher than that at rest in newborn rats[17].

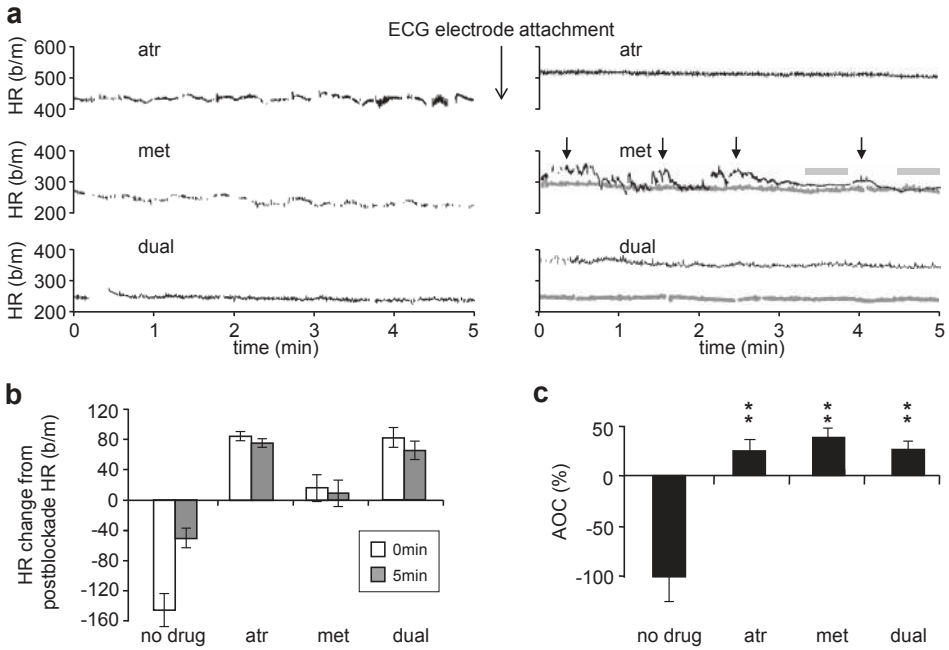

Fig. 7. HR responses to restraint stress under autonomic blockade in rats on P12

a Representative traces of postblockade HR (left) and HR responses (right) to restraint stress under atropine (upper), metoprolol (middle), and dual (lower) blockade (2 mg/kg each) are shown. The HR responses under atropine (upper) and dual blockade (lower) were relatively stable and higher than postblockade HR. Conversely, HR response under metoprolol (middle) was unstable with several temporal augmentations (arrows) from the relatively stable HR (horizontal gray bars) near the postblockade HR. HR response under sympathetic and dual blockade with 20 mg/kg of metoprolol is shown as a thick gray line in the middle and lower trace, respectively.

b HR changes at 0 (open square) and 5 min (filled square) from each postblockade HR are illustrated.

c Results of AOC analysis are shown to clarify changes in the HR response pattern after autonomic blockade. Negative and positive bars denote TB and SCD, respectively. The minor SCD observed in each autonomic blockade condition may be due to struggling activity, which was strongly observed at the beginning of restraint by ECG electrode attachment (see PZS signal in Fig. 8). $^{**}p<0.01$

The stepwise HR developmental curve in rats was reported in 1965[24]. However, this result may have been affected by the invasive protocols used for measurements, such as restraining the rats' paws with tape and stabbing ECG wires into their armpits. Therefore, our result obtained noninvasively using the PZS system may accurately represent genuine HR development in newborn rats. This study is the first, as per our knowledge, to delineate the precise daily developmental curve for baseline HR during the early postnatal weeks in rats.

Autonomic blocker dose

The temporal increases in ECG-HR on P12 under postsynaptic β1-adrenoreceptor blockade by 2 mg/kg of metoprolol could be due to sympathetic excitation during struggling (Fig. 7a, middle). The temporal increase in HR may be mediated by the increase in norepinephrine (NE) release from sympathetic nerve terminals because of struggling, which may be coupled with unblocked β1-adrenoceptors under insufficient β1-adrenoceptor blockade with 2 mg/kg of metoprolol. As expected, by increasing the drug dose by 10-fold (20 mg/kg metoprolol), the temporal HR augmentation was diminished by both metoprolol and dual blockade (Fig. 7a, middle and lower), suggesting that although a higher dose is required for the stress condition, the dose of 2 mg/kg is sufficient for autonomic blockade under resting or freely moving conditions. Indeed, some researchers have recently used high-dose

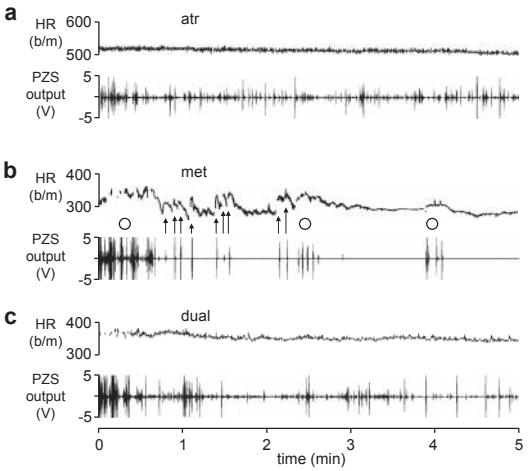

Fig. 8. Analysis of autonomic regulation of rapid HR fluctuations
Representative HR responses to restraint stress under autonomic blockade (2 mg/kg) with atropine (upper), metoprolol (middle), and dual (lower) blockade are shown. HR responses to restraint stress under atropine and dual blockade were relatively stable, regardless of struggling activities. In contrast, HR was unstable under metoprolol blockade ; temporal HR augmentations appeared coincidently with struggling bouts (open circles), and rapid small-amplitude HR fluctuations were observed concurrently with sparse struggling activity (arrows).

(10 mg/kg) autonomic blockers to monitor stress-induced responses[25].

HR responses to restraint stress

The age-dependent HR response pattern shift from SCD to TB (Fig. 4) is similar between rats and mice[15]. However, differences in the onset (mice : P4, rats : P9) and incidence of TB were observed ; almost 100% of the mice exhibited TB on P9-14, whereas in rats, a maximum of 88% animals displayed TB on P13. TB appears to disappear after P14 (Fig. 4c, Table 1). A similar finding was reported in a previous study ; the magnitude and incidence of TB (duration of <2 s : different HR response pattern from our finding) during an inactive (resting) state increased dramatically at P12 but decreased and disappeared by P20[17].

The HR response of SCD may be mediated by ANS control, though the experiments performed to assess SCD by using only two doses of autonomic blockers in this study may have been insufficient. In addition, there might be a possible involvement of humoral factors other than circulating catecholamines as discussed in our previous study in mice[15]. More precise research is needed to clarify the effects of sympathetic/parasympathetic control on HR. However, ANS control of TB appears to differ between rats and mice when analyzed under autonomic blockade conditions. The magnitude of TB was decreased by both sympathetic and parasympathetic blockade in mice. In contrast, TB was completely eliminated by any administered autonomic blocker in rats (Fig. 7a). This finding suggests that TB in rats is not merely an algebraic sum of the activity levels of the sympathetic and parasympathetic nervous systems ; rather, it is mediated by a complex interaction between these systems.

Rapid phasic HR fluctuations

The pattern of TB in rats was not similar to that in mice. Rapid phasic HR fluctuations frequently appeared in rats (Fig. 4b, lower), but it was seldom observed in mice. The difference in behavior after ECG electrode attachment may explain the difference in the TB pattern ; rats exhibited alternating periods of struggling and resting, whereas mice exhibited freezing behavior (feigning death) after an initial struggle for several seconds[15]. Struggling precedes most rapid HR fluctuations (Figs. 5b and 5c) ; therefore, ANS activity accompanied by struggling may be responsible for the fluctuations observed. A rapid HR fluctuation comprises frequent and rapid HR increases for several seconds ; these increases are likely to be mediated by vagal control (parasympathetic withdrawal) because they were completely eliminated by parasympathetic and dual blockade, but not by sympathetic blockade (Fig. 8). Several small-amplitude rapid HR fluctuations appeared concurrently with struggling activity (Fig. 8b, arrows). Some rapid HR fluctuations were not preceded by struggling (Figs. 5a and 5b), but further detailed investigation is needed to clarify whether the PZS did not detect all instances of struggling or whether some rapid HR fluctuations were actually unaccompanied by struggling behavior.

Accentuated antagonism

Accentuated antagonism is a vagal-sympathetic interaction that occurs when vagal activity becomes more pronounced with an increase in sympathetic activity, which may be mediated at both the prejunctional and postjunctional levels[26]. A series of experimental studies on intrinsic cardiac ganglion (ICG) functions demonstrated that acetylcholine released from vagal nerve terminals depresses NE release from adrenergic nerve terminals to the β1-adrenoceptor on cardiac muscle cells via muscarinic receptors on adrenergic nerve terminals (prejunctional inhibition)[26,27]. This mechanism of prejunctional inhibition makes it possible to explain the restraint stress-induced elevation in HR at P12 under dual blockade in rats (Fig. 7a, lower) because NE release from adrenergic nerve terminals may be increased by the disabling of prejunctional inhibition by dual blockade compared with metoprolol blockade alone. This postulation is supported by the absence of HR elevation in the presence of dual blockade with a 10-fold higher dose of metoprolol (Fig. 7a, lower), which also indicates that β1-adrenoceptor blockade using 2 mg/kg of metoprolol is insufficient for stress conditions. However, it remains to be elucidated whether muscarinic receptors in ICGs mature before or simultaneously with those on atrial/cardiac muscle cells during the first 2 postnatal weeks. In other words, the extent to which prejunctional or postjunctional muscarinic inhibition matures and contributes to the sympathetic-parasympathetic interaction in newborn rats remains unknown.

In in vivo animal studies, accentuated antagonism was observed in the diving reflex in muskrats in 1995-1996[28,29] and in rats in 2010[30], and we are the first to report the possible occurrence of accentuated antagonism in neonatal rats, which may be induced by restraint stress and may be responsible for TB. These in vivo studies discuss accentuated antagonism on the basis of the following understanding : "accentuated antagonism means that in the autonomic control of HR, even relatively weak parasympathetic activity can overpower strong sympathetic stimulation"[26].

Considering the results of this study, TB elicited by restraint stress may be mediated by a strong sympathetic-parasympathetic interaction because it disappeared when one side of ANS was interrupted. In addition, we infer that TB per se can be labeled as evidence of the establishment of accentuated antagonism because during TB vagal control, which decreases HR, predominates over the strong sympathetic activity that increases from the level at baseline HR and persists during 5 min of restraint (Fig. 7a).

Furthermore, the small-amplitude rapid phasic HR fluctuations observed under metoprolol (sympathetic) blockade (Fig. 8b, arrows) can also be evidence explaining the feature of accentuated antagonism because the appearance of small-amplitude rapid phasic HR fluctuations under metoprolol blockade (but not under atropine nor dual blockade) indicates the existence of prejunctional vagal control of NE release from sympathetic nerve terminals.

The TB pattern composed of slow and rapid phasic HR components (Fig. 2a) may be mediated by vagal control because the parasympathetic nervous system can regulate both short- and long-term HR variations[31]. In addition, because a gently recovering TB was invoked by stimulating the nonmyelinated vagal fibers in rabbits[32], the TB pattern composed of slow and rapid phasic HR components may be mediated by nonmyelinated and myelinated vagal fibers, respectively.

In addition, because TB appeared temporarily for only a few days and appeared to dissipate after P14, it may merely be a byproduct of maturation of the immature central/peripheral ANS. This phenomenon may be helpful to understand sympathetic-parasympathetic interactions, including accentuated antagonism, in intact newborn rats. Our method may provide a novel in vivo approach for evaluating sympathetic-parasympathetic interaction including accentuated antagonism in neonatal animals, unlike a number of previous studies that used cardiac tissues[33], cardiac neurons[34], whole-heart preparations[27], and other models. In addition, signs of accentuated antagonism have been observed in children[35], adults[26], and animals (dogs[36] and rats[30]), but not in newborn mice[15] and infants. Therefore, the rat is useful for studying ANS control of HR, including accentuated antagonism, and its physiological/pathophysiological role during the early postnatal period.

Cardiac theory, sleep, vulnerable period, accentuated antagonism, and SIDS

The cardiac theory proposes that SIDS results from the sudden appearance of a lethal arrhythmia (or sinus arrest)[20,37], and there is direct evidence of bradycardia during the final hour of SIDS[38,39]. SIDS is also known as sleep-related sudden death. Because the autonomic system becomes unstable and sinus pauses occur in infants with augmented vagal tone during rapid eye movement (REM) sleep[40], newborn infants who spend much of their time in REM sleep are inevitably exposed to a potentially life-threatening situation.

Many studies depicted autonomic changes such as appearance of cardiac vagal tone[23], increase in baroreflex sensitivity[41], and a change in the properties of ICG neurons[34] during the second postnatal week in rats. Therefore, in addition to our finding that dramatic ANS changes occur during the second week in parallel with the changes in HR responses to restraint stress from SCD to TB, the second postnatal week may be a critical developmental period for the establishment of stress-induced vagal control of negative chronotropic function in rats.

Accentuated antagonism in humans affects ventricular refractoriness[42], the shortening of which can lead to ventricular arrhythmias[43], while nerve activities (sympathetic-parasympathetic interactions) in ICGs are common triggers of atrial fibrillation[34,44]. Accentuated antagonism amplifies negative chronotropic (parasympathetic) effects as a function of sympathetic activity level. However, the accentuated antagonism induced by ANS during early developmental stages may be substantially unstable and may adversely affect HR control, even in a healthy infant with no particular impairment. Another possible mechanism of sudden cardiac death in infants with SIDS is lethal arrhythmia, i.e., ventricular tachycardia, which can be elicited by increased sympathetic nervous system activity during REM sleep. However, we did not obtain any data to clarify the connection between tachycardia and SIDS in this study.

Taken together, it may be possible to infer that sympathetic-parasympathetic interactions, which may be immature accentuated antagonism, elicited by some extrinsic stressors at immature stages can trigger lethal arrhythmias (bradycardia or tachycardia) and result in SIDS in infants.

Limitations

We focused on the second postnatal week as the critical developmental period because TB appeared and was controlled by ANS during this period ; therefore, we concentrated on understanding the mechanism of ANS control of TB, with less attention being devoted to SCD that appeared in the first postnatal week.

Our methods for HR measurement are vulnerable to body movement. The HR trace was discontinuous when measured by the PZS as well as ECG because of the struggling behavior of the rats. This makes it difficult to assess sympathetic-parasympathetic balance using heart rate variability (HRV) analysis because it requires continuous HR data for at least 1 min. In addition, we should use caution when applying the HRV estimation to a signal that lacks stationarity because it is recommended that HRV should be estimated while the animal exhibits stable physical activity. On the other hand, beat-to-beat HR analysis for several seconds was efficient for analyzing rapid HR fluctuations in this study, which cannot be achieved by HRV analysis. This demonstrates that our method for instantaneous HR analysis, which tracks rapid HR fluctuations, is useful and a prerequisite for understanding the swift control of HR by the parasympathetic nervous system.

One disadvantage of our protocol is that it cannot alter sympathetic postganglionic nerve activity, which is required to evaluate the prejunctional function of sympathetic-activity-level-dependent vagal control (accentuated antagonism). Therefore, another method that enables a more precise evaluation of accentuated antagonism in newborn rats is needed.

Because restraining experiments in human infants is ethically challenging, it is difficult to examine whether they exhibit accentuated antagonism. However, we may need to investigate whether infants respond to various extrinsic stressors such as sounds, vibrations, ambient temperature, smoke, hypoxia, and dream content during REM sleep during the vulnerable period and whether these responses trigger accentuated antagonism.

We are aware that it is not sufficient to verify accentu-

ated antagonism using only two doses of autonomic blockers, and we may need to further examine the autonomic regulation of HR responses to restraint stress using a more precise dose-response relationship. The observation of relationship between TB and SIDS is another unaccomplished examination, however, it seems to be difficult to observe SIDS in rat because it requires thousands of rat experiments as the incidence of SIDS is very low and even if one rat dies, the death may not necessarily be of SIDS.

In addition, we observed that the conventional dose of autonomic blockers may be insufficient ; however, we should be careful when using high doses because it is not clear whether and to what extent high-dose autonomic blockers pass through the blood-brain barrier and affect autonomic regulation of HR.

Conclusions

This study revealed precise developmental changes in the baseline HR in rats during the first 2 postnatal weeks. The HR response to restraint stress changed from SCD to TB during the second postnatal week, indicating the dramatic development of ANS during the vulnerable period. We also observed a complex TB pattern and complicated HR responses to restraint stress under autonomic blockade, which could not be explained by any sympathetic-parasympathetic interactions other than accentuated antagonism, which enhances the negative chronotropic effect on the strong sympathetic activity. We speculate that immature sympathetic-parasympathetic interactions, which can be immature accentuated antagonism, may uncontrollably exaggerate vagal-mediated negative chronotropic effects on the heart, leading to severe cardiac dysfunction.

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