Metyrapone and fluoxetine suppress enduring behavioral but not cardiac effects of subchronic stress in rats

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IN HUMANS, CHRONIC STRESS is an established risk factor for heart disease. Indeed, chronically stressed individuals are often characterized by anomalies in the autonomic regulation of the heart, such as elevated sympathetic and reduced vagal tone, which, in turn, can induce tachycardia, reduction in heart rate variability (HRV), and altered baroreceptor reflex function (2, 19, 22, 34). With this growing body of clinical and epidemiological evidence of association between chronic stress and heart disease, it is now timely for the preclinical research to reveal the underlying pathogenetic mechanisms. In addressing this issue, we analyzed stress-induced changes in the heart rate (HR) of experimental rats exposed to a paradigm of subchronic foot shock stress.

In rats, HR varies significantly in association with the light-dark cycle (32, 35), being relatively low during the light phases, when animals are predominantly at rest, and relatively high during the dark phases, when animals are engaged in active behaviors (15, 32). Therefore, we believe that a detailed analysis of changes in HR, as well as in its circadian rhythmicity, may be advantageous when assessing stress-induced disturbances in the neural/autonomic control of the heart. As there is no established framework for such analysis, in this study, we examined the effects of subchronic foot shock stress on the HR using two different analytical approaches. We first evaluated the changes in HR following subchronic stress during both the light/inactive and dark/active phases of the circadian cycle and related them to the locomotor behavior of our animals. Secondly, we applied a new logistic curve fitting procedure recently developed by Head et al. (8) to evaluate the presence of stress-induced changes in the temporal organization and fluctuations of the circadian rhythm of HR.

The second objective of our study was to employ pharmacological treatments in an attempt to reveal the neural mechanisms responsible for the effects of subchronic stress. On the basis of the observation that the serotonergic system has been implicated in various cardiovascular disorders (33), as well as in circadian rhythm disturbances (31), we investigated whether chronic administration of the serotonin-selective reuptake inhibitor (SSRI) fluoxetine prevents stress-induced changes in HR and circadian rhythm. In the present study, fluoxetine was chosen based on 1) the drug’s frequent use in preclinical practice for reversing a wide range of physiological and behavioral effects of stress and 2) data demonstrating physiological effects on serotonergic transport in rats (18). In addition, we evaluated whether HR and circadian disturbances were mediated by stress-induced elevations of corticosterone, a stress hormone released upon activation of the hypothalamic-pituitary-adrenal (HPA) axis, by repeated treatment with an inhibitor of corticosteroid synthesis, the drug metyrapone. Metyrapone was chosen after demonstrating, in a pilot study, its efficacy in preventing the stress-induced rise in plasma corticosterone levels observed in control rats after a session of foot shock.

Finally, we attempted to obtain information about the effects of subchronic stress on the modulation of the autonomic
cardiac outflow using selective sympathetic and vagal blockade and HRV analysis.

**MATERIALS AND METHODS**

*Animals, Housing, and Preliminary Surgery*

Male adult Sprague-Dawley rats (10 wk of age; body wt: 250–300 g) were used in this study. Animals were individually housed in a temperature-controlled holding room (21 ± 1°C) for the duration of the study, and held on a 12:12-h light-dark cycle (lights on at 0700). Food and water were freely available. All experiments were approved by the University of Newcastle Animal Care and Ethics Committee and were conducted in accordance with the New South Wales Animal Research Act and the Australian Code of Practice for the use of animals for scientific purposes.

Radiotelemetric transmitters (TA11CTA-F40, Data Sciences International, St. Paul, MN) for recording HR and locomotor activity (LOC) were implanted at least 1 wk prior to the commencement of the experiments. The surgery was performed under isoflurane (2% in 100% oxygen) anesthesia. The transmitter body was placed in the abdominal cavity; one electrode was fixed to the dorsal surface of the xyphoid process, and another electrode was placed in the anterior mediastinum close to the right atrium, according to Sgoifo et al. (26). Such electrode location guarantees high-quality ECG recordings, even during vigorous physical activity.

**Experimental Protocol**

Experiments were carried out between 1000 and 1600 (i.e., in the inactive phase of the light-dark cycle). All rats were randomly assigned to seven groups (summarized in Table 1): 1) no foot shock group (NO-FS, n = 6), 2) foot shock with no drug group (FS-ND, n = 6), 3) foot shock with fluoxetine (FS-FLUOX, n = 7), 4) foot shock with vehicle (FS-VEH, n = 7), 5) foot shock with metyrapone (FS-MET, n = 7), 6) foot shock with atenolol (FS-ATEN, n = 7), and 7) foot shock with scopolamine (FS-SCOP, n = 7). Groups 1, 2, and 3 were used in experiment 1, groups 4 and 5 were used in experiment 2, and groups 6 and 7 were used in experiment 3. Each animal was used for just one experimental procedure. The FS-ND group served as a control for both the FS-FLUOX and the FS-ATEN group, as drugs were given in drinking water (see *Experiment 1: effects of subchronic foot shock on HR and LOC and an attempt to prevent these effects with SSRI treatment*).

### Table 1. Experimental groups and outline of the procedures applied in this study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Subchronic Foot Shock</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO-FS</td>
<td>No</td>
<td>No treatment</td>
</tr>
<tr>
<td>FS-ND</td>
<td>Yes</td>
<td>No treatment</td>
</tr>
<tr>
<td>FS-FLUOX</td>
<td>Yes</td>
<td>Chronic treatment with fluoxetine</td>
</tr>
<tr>
<td>FS-VEH</td>
<td>Yes</td>
<td>Repeated treatment with vehicle before each session of foot shock</td>
</tr>
<tr>
<td>FS-MET</td>
<td>Yes</td>
<td>Repeated treatment with metyrapone before each session of foot shock</td>
</tr>
<tr>
<td>FS-ATEN</td>
<td>Yes</td>
<td>Chronic treatment with atenolol</td>
</tr>
<tr>
<td>FS-SCOP</td>
<td>Yes</td>
<td>Injection of scopolamine the day before the onset of subchronic foot shock and the first day after the last session of foot shock</td>
</tr>
</tbody>
</table>

The NO-FS group served as control for the FS-ND group. The FS-ND group served as control for both the FS-FLUOX and the FS-ATEN groups, as drugs were given in drinking water. The FS-VEH group served as control for the FS-MET group, as both vehicle and metyrapone were injected 2 h prior to stress exposure. In the FS-SCOP group, scopolamine was given acutely to avoid possible side effects of chronic administration and comparisons were only possible between prestress and poststress data due to the discrepancy in the protocol of administration of the drug.

with SSRI treatment for details). The FS-VEH group served as a control for the FS-MET group, as both vehicle and metyrapone were injected 2 h prior to stress exposure (see *Experiment 2: effects of subchronic foot shock on HR and LOC and an attempt to prevent these effects with an inhibitor of corticosterone synthesis* for details).

In each group, baseline HR and LOC recordings were performed in the home cages for 3 days. Subsequently, rats of the groups 2, 3, 4, 5, 6, and 7 were submitted to a subchronic stress protocol, consisting of a foot shock session on five consecutive days. In each session, the animal was placed into a Perspex cylinder (22-cm length × 9-cm diameter) located inside an enclosed box. Foot shocks (1.5 mA, 1 s) were delivered to the metal floor grid using a programmable animal shoker (San Diego Instruments, San Diego, CA). Each foot shock stress session lasted 1 h, during which the animals received six shocks, with a randomly allocated interval between each shock to avoid predictability. Following each session, the animals were returned to their home cages. During the same period, rats in group 1 remained undisturbed in their home cages. After the last session of foot shock, HR and LOC were recorded in all groups for 6 days.

**Experiment 1: effects of subchronic foot shock on HR and LOC and an attempt to prevent these effects with SSRI treatment.** Animals of the NO-FS and the FS-ND group received tap water throughout the study protocol. Animals of the FS-FLUOX group were given fluoxetine (a selective serotonin reuptake inhibitor, SSRI) in drinking water dose (10 mg·kg⁻¹·day⁻¹) starting a week before the start of baseline recordings until the end of the experiment. The dose was chosen on the basis of data in the available literature (3). Subsequently, rats of the FS-ND and the FS-FLUOX group were submitted to subchronic foot shock, whereas the rats of the NO-FS group remained undisturbed in their home cages for the whole duration of the subchronic stress protocol.

**Experiment 2: effects of subchronic foot shock on HR and LOC and an attempt to prevent these effects with an inhibitor of corticosterone synthesis.** Rats of the FS-VEH group were injected with vehicle (1 ml/kg ip), and rats of the FS-MET group were injected with metyrapone (inhibitor of corticosterone synthesis) at the dose of 50 mg/kg ip 2 h before being submitted to each session of foot shock. The dose and time of metyrapone injection were selected on the basis of the available literature data, and on the basis of our pilot experiment (see *Chemicals and Pilot Studies with Drug Trials*).

**Experiment 3: assessment of autonomic mechanisms responsible for the effects of subchronic foot shock on HR.** Animals of the FS-ATEN group received atenolol (ß1-adrenergic receptor antagonist) in drinking water dose (65 mg·kg⁻¹·day⁻¹; see sections, *Chemicals and Pilot Studies with Drug Trials*) starting a week before the start of baseline recordings until the end of the experiment. Subsequently, the rats were submitted to subchronic foot shock. For this experiment, the FS-ND group served as a control for the FS-ATEN group, as the drug was given in drinking water.

In a separate group of rats (FS-SCOP), parasympathetic blockade was performed using methyl-scopolamine (muscarinic receptor antagonist). Animals of this group received a subcutaneous injection of scopolamine (50 μg/kg) during the last day of prestress period and during the first day after the last session of foot shock, at the same time of the day (1500). We have chosen to use a double acute administration (as opposed to chronic administration of atenolol) to avoid numerous side effects of muscarinic blockade, such as sensorimotor disturbances and cognitive impairment (10, 23). Given the discrepancy in the protocols for the administration of the two drugs, no direct comparisons between FS-SCOP and FS-ATEN data were made. However, for the FS-SCOP group, comparisons were possible between prestress and poststress data. In both instances, the acute effect of scopolamine on HR was evaluated during the 15 min that followed the injection.
Chemicals

Fluoxetine (Sigma, St. Louis, MO) was dissolved in water to result in the daily dose of 10 mg/kg. Fluid intake was monitored daily for 7 days before and throughout the entire experiment, and bottles were weighed always at the same time of the day (1200). On the basis of the daily water intake, drugs were dissolved in 30 ml of drinking water to guarantee intake of the total dose for each animal. This volume of drinking water was consumed by the rats all day long. Body weight was recorded in 1-wk intervals for drug adjustments. Metyrapone (Sigma) was dissolved in a mixture of dimethyl sulfoxide (50%) and Ringer (50%) solution to result in the dose 50 mg/kg (see below in *Pilot Studies with Drug Trials* for details). Vehicle was a mixture of dimethyl sulfoxide (50%) and Ringer (50%). Atenolol (Sigma) was dissolved in water to result in the daily dose of 65 mg/kg (see below in *Pilot Studies with Drug Trials* for details). Fluid intake was monitored as described above. Scopolamine methyl-bromide (Sigma) was dissolved in distilled water to result in the dose of 50 μg/kg.

Pilot Studies with Drug Trials

For atenolol and metyrapone, drug trials were performed in pilot studies using separate groups of rats. In the first experiment, we determined the oral dose for atenolol: one group of rats (n = 5) received atenolol in drinking water at a dose of 30 mg·kg−1·day−1 during the first week and 65 mg·kg−1·day−1 during the second week. On the 7th and 14th day, a bolus intraperitoneal injection of atenolol (2 mg/kg) was administered. We found that this bolus drug injection provoked a small reduction in HR after 1-wk treatment with a lower, but not the higher, dose of the drug. Thus, we concluded that the daily dose of 65 mg/kg is optimal for complete sympathetic blockade and used it for further experiments. In the second experiment, we validated the dose and time course of metyrapone injection. At the dose of 50 mg/kg, metyrapone has been shown to produce a greater than 50% reduction in plasma corticosterone levels that reaches a maximal effect within the first hours and gradually returns to baseline within 20–24 h (11, 21). To test the efficacy of this metyrapone dose to suppress stress-induced elevation in corticosterone, two separate groups of rats (n = 6 each) were injected with either the metyrapone dose (50 mg/kg ip) or vehicle (1 ml/kg ip) 2 h prior to a single session and then immediately after, and 1 h after it. Blood was collected in chilled tubes from the tail nick before the foot shock stress session and stored at 20°C. Plasma corticosterone levels were assayed using a commercial radioimmunoassay kit (MP Biomedicals, Costa Mesa, CA). We demonstrated that this dose and time course of metyrapone injection was efficient in preventing the stress-induced rise in plasma corticosterone levels in response to foot shock that was observed in the rats treated with vehicle (Fig. 1).

Recording and Analysis of HR and LOC Data

HR (bpm) and LOC (expressed as cpm) were sampled continuously before (3 days) and after (6 days) the subchronic stress period. Initially, for each individual rat, HR and LOC were quantified as a means of 12-h inactive (light) phase (HRi, LOCi), and 12-h active (dark) phase (HRa, LOCa) of the circadian cycle (Fig. 2, A and C). Subsequently, average inactive and active phase group values were calculated for prestress and poststress periods. Also, for each individual rat, delta values of HR and LOC were calculated by subtracting prestress mean values from corresponding poststress mean values. Subsequently, average group delta values were calculated.

Analysis of HR nonrelated to LOC (HRLOCa) was accomplished by selecting for analysis only HR values during specific periods of time (between 0800 and 1800 for light phases and between 2000 and 0600 for dark phases to avoid transition periods) when animals had no LOC (Fig. 2B). Animals were considered immobile when they were not making any movement for at least 1 min. Initially, for each individual rat, HRLOC- was quantified as the mean of 4 or 5 locomotion-free periods during inactive (light) (HRiLOC-), and active (dark) phases (HRaLOC-) (Fig. 2B). Subsequently, average inactive and active-phase group values were calculated for prestress and poststress periods.

HRV analysis was conducted on continuous data sets during the last 2 days prestress and the first 2 days poststress using Power Lab (ADInstruments, Sydney, Australia). ECGs were band pass filtered (2–300 Hz) and, after R wave peak detection, 120-s ECG segments were generated for every hour. In the time domain, the following indexes were obtained from each ECG segment: SDNN (standard deviation of RR intervals) and RMSSD (square root of the mean squared differences of successive RR intervals). For spectral (frequency-domain) analysis of HRV, power spectrum was obtained with a fast Fourier transform-based method (Welch’s periodogram: 256 points, 50% overlap, and Hamming window), and high-frequency (1–4 Hz) was determined for each ECG segment. Subsequently, for each time- and frequency-domain index, average active and inactive phase group values were calculated for prestress and poststress periods.

A detailed analysis of circadian variations of HR was performed by means of the double-logistic curve fitting procedure described by Head and colleagues (8), according to the equation:

\[ y = P_1 - P_2 \left( 1 + e^{P_3 (x - C_1)} \right) + \frac{P_2}{1 + e^{P_3 (x - C_2)}} \]

where the parameters were P1, the “active” phase plateau (data obtained during dark phases); P2, the circadian amplitude; (P1–P2), the “inactive” phase plateau (data referring to light phases); P3 and P5, the rates of the transitions from inactive to active and from active to inactive plateaus, respectively; P4 and P6, the time points at which the respective transitions reach 50% (Fig. 2A). From each individual rat, we analyzed 48 h of continuous data sets, both during prestress (last 2 days) and poststress periods (first 2 days) using CIRCAD software, as described previously (8).

Statistical analysis was performed using SPSS 11.5 software package (SPSS, Chicago, IL). Statistical significance for all tests was set at \( P \leq 0.05 \). All parameters in figures and tables are expressed as means ± SE. Two-way ANOVA for repeated measures was applied on HR and LOC data for comparisons between J) NO-FS vs. FS-ND groups, 2) FS-ND vs. FS-FLUOX groups, 3) FS-VEH vs. FS-MET groups, and 4) FS-ND vs. FS-ATEN groups, with between-subject
locomotor activity (LOC) in a representative rat of the foot shock-no treatment (FS-ND) group. A: calculation of mean values of HR during the inactive (HRI) and active (HRA) phases of the circadian cycle and scheme of double-logistic fitting circadian analysis. P1 represents the active phase plateau, P1–P2 corresponds to the inactive phase plateau, and P2 is the circadian amplitude. P3 and P5 are rates, with P3 being the rate for the transition between the active and inactive phase plateau and P5 being the rate for the transition between the inactive and active phase plateau. P4 and P6 represent the time of day at which the curve reaches the midtransition between plateaus as indicated. B: assessing of mean values of HR during period of not locomotion (Loc-) (Fig. 4, C and D) and the rate for the transition between the two phase plateaus (P3, P5) of circadian rhythm of HR between prestress and poststress periods (Table 3). In the FS-ND rats, LOC values during the active phase of poststress period compared with the prestress period (Fig. 4, E and F).

**RESULTS**

**Experiment 1: Effects of Subchronic Foot Shock on HR and LOC and Attempt to Prevent These Effects With SSRI Treatment**

In rats not subjected to subchronic foot shock stress (NO-FS group), no differences were observed in HR and LOC indices between periods corresponding to prestress and poststress time points in other groups (data not shown).

In rats exposed to subchronic foot shock (FS-ND), we found a significant reduction in HR during both poststress active and inactive phases compared with the prestress period (Fig. 3 and Fig. 4, A and B). This stress-induced fall in HR persisted when changes in HR were analyzed in the absence of any locomotion (Loc-) (Fig. 4, C and D). After subchronic foot shock, no changes were observed in any of the HRV indices between prestress and poststress periods (Table 2). In the FS-ND rats, LOC values during the active phase of the circadian cycle were lower after subchronic foot shock compared with the prestress period (Fig. 4, E and F). Consequently, the circadian amplitude of LOC was reduced after stress (prestress: 3.6 ± 0.2 cpm vs. poststress: 2.9 ± 0.3 cpm, t = −2.5, P < 0.05). In rats of the FS-ND group, circadian fitting analysis confirmed the stress-induced fall in HR after subchronic foot-shock both during the active (P1) and inactive (P1–P2) phases (Table 3) and showed a significant reduction in the rate of the inactive-to-active transition (P5) during poststress period compared with the prestress value (Table 3). No changes were found in the daily amplitude (P2) of the circadian rhythm of HR between prestress and poststress periods (Table 3).

In the fluoxetine-treated rats (FS-FLUOX), HR values were significantly lower after subchronic foot shock compared with the prestress period during both the active and inactive phases (Fig. 4, A and B and Table 3). The magnitude of this stress-induced fall in HR did not differ from that observed in the FS-ND rats (Table 4). In the FS-FLUOX rats, the stress-induced reduction in HR values was also present after analysis of HR during periods of no locomotion (Loc-) (Fig. 4, C and D) with the magnitude of this fall being in this circumstance larger during the inactive phase compared with the FS-ND group (Table 4). No changes were observed both in the amplitude (P2) and in the transitions between the two phase plateaus (P3, P5) of circadian rhythm of HR between prestress and poststress periods (Table 3). In the FS-FLUOX rats, subchronic foot shock did not provoke any effect on LOC (Fig. 4, E and F).

**Experiment 2: Effects of Subchronic Foot Shock on HR and LOC and Attempt to Prevent These Effects With an Inhibitor of Corticosterone Synthesis**

In the FS-VEH group, HR values were significantly lower after subchronic foot shock stress compared with the prestress period during both the active and inactive phases of the circadian cycle (Fig. 4, A and B). This stress-induced reduction in HR persisted when HR analysis was performed during a period of no locomotion (Loc-) (Fig. 4, C and D). Also, the FS-VEH rats showed lower values of LOC during the active phase of poststress period compared with the prestress values (Fig. 4, E and F), and a consequent reduced circadian amplitude of LOC after subchronic stress (prestress: 4.0 ± 0.2 cpm vs. poststress: 2.9 ± 0.2 cpm, t = −3.1, P < 0.05). In the FS-VEH rats, circadian fitting analysis confirmed the stress-induced fall in HR after subchronic foot shock, both during the active (P1) and inactive (P1–P2) phases (Table 3). The circadian amplitude of HR (P2) was not affected by subchronic stress, whereas after subchronic stress, the locomotor activity was significantly reduced (Fig. 4, C and D).

**Factor “group” (two levels), within subject factor “time” (two levels: prestress and poststress) and interaction factor “group × time”. After ANOVAs, post hoc analyses were applied when appropriate using Student's t-tests, after controlling for homogeneity of the variance via a Levene test. Student's t-tests, again after controlling for homogeneity of variances via a Levene test, were applied on 1) HRV and 2) FS-SCOP group data for comparisons between prestress and poststress values.
foot shock, we observed a significant reduction in the rate of the inactive-to-active transition (P5) compared with the prestress period (Table 3).

In metyrapone-treated rats (FS-MET), subchronic foot shock provoked a significant fall in HR values compared with the prestress period during both the active and inactive phases of the circadian cycle (Fig. 4, A and B and Table 3), which was also evident during periods of no locomotion (Loc-) (Fig. 4, C and D) and whose magnitude did not differ from that observed in the FS-VEH rats (Table 4). In the FS-MET rats, no changes were observed in the circadian amplitude of HR (P2), whereas, after subchronic foot shock, the rate of the transition between the inactive to the active phase plateaus was higher than the prestress period (P5).

Fig. 3. HR recordings before (3 days) and after (6 days) the subchronic foot shock stress. Arrows indicate foot shock daily sessions (1-h duration). A: example from a single rat of the FS-ND group. B: mean ± SE dark-light group data for HR (FS-ND; n = 6).

Fig. 4. Effects of subchronic foot shock on HR (A and B), HR nonrelated to locomotion (Loc-) (C and D), and LOC (E and F), during the active (left) and the inactive (right) phases of the circadian cycle. Data are presented as means ± SE.

* and † Significantly different from corresponding prestress values (P < 0.01 and P < 0.05, respectively). § Significantly different from corresponding FS-ND group value (P < 0.01). Results of ANOVA: 1) for FS-ND vs. chronic treatment with fluoxetine group (FS-FLUOX) significant effects of time for HR (active phase: F = 89.36, P < 0.01; inactive phase: F = 89.95, P < 0.01), HR(Loc-) (active phase: F = 94.36, P < 0.01; inactive phase: F = 91.95, P < 0.01), and LOC (active phase: F = 17.93, P < 0.01); 2) for rats that received repeated treatment with vehicle before each session of foot shock (FS-VEH group) vs. rats receiving repeated treatment with metyrapone before each session of foot shock (FS-MET group), significant effects of time for HR (active phase: F = 39.36, P < 0.01; inactive phase: F = 102.08, P < 0.01), HR(Loc-) (active phase: F = 94.36, P < 0.01; inactive phase: F = 89.36, P < 0.01), and LOC (active phase: F = 8.97, P < 0.05); 3) for FS-ND vs. rats receiving chronic treatment of atenolol (FS-ATEN group), significant effects of time for HR (active phase: F = 70.21, P < 0.01; inactive phase: F = 133.97, P < 0.01) and HR(Loc-) (active phase: F = 99.36, P < 0.01; inactive phase: F = 89.36, P < 0.01).
Table 2. HRV indices in the FS-ND group (n = 6) before and after subchronic foot shock stress exposure during the active and the inactive phases of the circadian cycle

<table>
<thead>
<tr>
<th>FS-ND group</th>
<th>SDNN, ms</th>
<th>RMSSD, ms</th>
<th>HF, ms²</th>
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<tbody>
<tr>
<td></td>
<td>Active</td>
<td>Inactive</td>
<td>Active</td>
</tr>
<tr>
<td>Prestress</td>
<td>2.5 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Poststress</td>
<td>3.1 ± 0.3</td>
<td>2.5 ± 0.3</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. SDNN, standard deviation of RR intervals; RMSSD, square root of the mean squared differences of successive RR intervals; HF, high frequency.

(Table 3). In the FS-MET rats, subchronic foot shock did not provoke any effect on LOC (Fig. 4, E and F).

Experiment 3: Assessment of Autonomic Mechanisms Responsible for the Effects of Subchronic Foot Shock on HR

As expected, in the FS-ATEN rats, chronic treatment with β-blocker atenolol reduced prestress values of HR during both active and inactive phases compared with the FS-ND group (Fig. 4, A–D). After sympathetic blockade, subchronic foot shock still provoked a full in HR (Fig. 4, A and B) that persisted when HR was analyzed during periods of no locomotion (Loc-) (Fig. 4, C and D) and whose magnitude did not differ from that observed in the FS-ND rats (Table 4). Interestingly, in the FS-ATEN group, no changes were observed in LOC values between prestress and poststress periods (Fig. 4E).

In the FS-SCOP rats, preinjection HR values during the inactive phase of the poststress period were significantly lower from corresponding prestress values (Fig. 5). Administration of scopolamine caused a rapid rise in HR; the peak HR reached during the poststress period was similar to that of the prestress period, but the increment in HR was larger during the poststress period (Fig. 5).

DISCUSSION

Our principal and novel finding is that subchronic psychophysiological stress provokes a substantial and enduring decrease in HR that lasts well beyond the duration of the stressor and is mediated via an increase in cardiac vagal tone-vagal rebound. The term “vagal rebound” is thoroughly used in the literature, and systematically refers to short-term vagal hyperactivity following a stressor, a sympathetic overdrive or reperfusion following acute myocardial infarction (4, 16, 25). In this paper, it is a relatively persistent, long-term effect of subchronic stress. These features allow naming this novel phenomenon “enduring vagal rebound”. The second important observation that we report here is that, in contrast to the motor-behavioral effects (hypolocomotion) of subchronic stress, this enduring vagal rebound is serotonin- and corticosterone-independent.

The bradycardia observed in our rats after subchronic stress during both the active and the inactive phases of the light/dark cycle is a new, interesting, and unexpected phenomenon, as it is widely accepted that stress induces tachycardia (9). On the basis of the observation that subchronic foot shock also led to a reduction in locomotion, our first hypothesis was that the bradycardic effect of subchronic stress was secondary to this reduction. However, because poststress bradycardia was also present during locomotion-free periods, we concluded that the effect was locomotor-independent.

Subchronic foot shock stress provoked only a partial disorganization of the circadian rhythm of HR. Indeed, after subchronic stress, changes in neither phase shifts nor circadian amplitude were observed, whereas the stress-induced slowing of the transition from the inactive to the active phase may be considered the most sensitive marker of this rhythm disorgan-

Table 3. Double-logistic curve fitting analysis of 48-h data series of circadian rhythms of HR during prestress and poststress periods

<table>
<thead>
<tr>
<th></th>
<th>Active Plateau P1</th>
<th>Amplitude P2</th>
<th>Inactive Plateau P1-P2</th>
<th>Rate P3</th>
<th>Transition Time P4</th>
<th>Rate P5</th>
<th>Transition Time P6</th>
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<td>FS-ND</td>
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<tr>
<td>Prestress</td>
<td>424 ± 4</td>
<td>70 ± 5</td>
<td>354 ± 6</td>
<td>-199 ± 72</td>
<td>8.1 ± 4.8</td>
<td>30 ± 4</td>
<td>12.9 ± 0.7</td>
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<tr>
<td>Poststress</td>
<td>402 ± 4</td>
<td>70 ± 6</td>
<td>332 ± 6</td>
<td>-183 ± 64</td>
<td>8.2 ± 4.9</td>
<td>19 ± 3†</td>
<td>13.4 ± 0.3</td>
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<td>FS-FLUOX</td>
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<tr>
<td>Prestress</td>
<td>438 ± 4</td>
<td>74 ± 2</td>
<td>364 ± 6</td>
<td>-188 ± 72</td>
<td>10.1 ± 4.6</td>
<td>28 ± 2</td>
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<td>Poststress</td>
<td>412 ± 4</td>
<td>79 ± 4</td>
<td>332 ± 4</td>
<td>-244 ± 69</td>
<td>10.3 ± 4.8</td>
<td>27 ± 4</td>
<td>12.9 ± 0.1</td>
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<tr>
<td>FS-VEH</td>
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<tr>
<td>Prestress</td>
<td>435 ± 3</td>
<td>79 ± 6</td>
<td>357 ± 5</td>
<td>-207 ± 72</td>
<td>7.1 ± 4.3</td>
<td>28 ± 5</td>
<td>13.3 ± 0.5</td>
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<tr>
<td>Poststress</td>
<td>427 ± 4</td>
<td>104 ± 12</td>
<td>323 ± 10*</td>
<td>-135 ± 59</td>
<td>10.4 ± 4.8</td>
<td>14 ± 2†</td>
<td>11.0 ± 1.4</td>
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<tr>
<td>FS-MET</td>
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<tr>
<td>Prestress</td>
<td>445 ± 5</td>
<td>90 ± 5</td>
<td>357 ± 6</td>
<td>-68 ± 42</td>
<td>7.3 ± 4.0</td>
<td>21 ± 3</td>
<td>13.4 ± 0.2</td>
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<tr>
<td>Poststress</td>
<td>423 ± 7†</td>
<td>90 ± 10</td>
<td>342 ± 3†</td>
<td>-15 ± 6</td>
<td>13.4 ± 4.7</td>
<td>31 ± 3†</td>
<td>12.2 ± 0.3</td>
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</tr>
</tbody>
</table>

Data are presented as means ± SE of group data. P1, P2 and (P1–P2) are expressed as beats per minute (bpm); P3 and P5 are range-independent rates of change with units of 1/h. P4 and P5 are shown as times of the 24-h day. * and †Significantly different from corresponding prestress values (P < 0.01 and P < 0.05, respectively). Results of ANOVA: 1) for FS-ND vs. FS-FLUOX significant effects of time for P1 (F = 57.13, P < 0.01), P1–P2 (F = 121.45, P < 0.01) and P5 (F = 7.68, P < 0.05); 2) for FS-VEH vs. FS-MET significant effects of time for P1 (F = 14.27, P < 0.01) and P1–P2 (F = 18.84, P < 0.01), and a “group × time” interaction for P5 (F = 18.88, P < 0.01).
organization. Several studies reported that persistent circadian disturbances in HR develop after stressful events. Changes in circadian cardiac rhythmicity were found after a single episode of social defeat (15, 24) and after repeated social conflicts (30), and were generally described as a reduction or dampening of the circadian amplitude. It may be that in our study the overall lack of circadian effects of subchronic foot shock was due to the relatively mild impact of our stress paradigm compared with the more potent stressor of social defeat (13).

In an attempt to reveal which autonomic component(s) mediated the bradycardia induced by subchronic stress, we used pharmacological blockades to assess the contributions of sympathetic and parasympathetic (vagal) cardiac influences.

**Table 4. Effects of pharmacological treatment on bradycardia and hypolocomotion elicited by subchronic stress**

<table>
<thead>
<tr>
<th></th>
<th>ΔHR, bpm</th>
<th>ΔHR (Loc), bpm</th>
<th>ΔLOC, cpm</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Active</td>
<td>Inactive</td>
<td>Active</td>
</tr>
<tr>
<td>FS-ND</td>
<td>−23 ± 4</td>
<td>−20 ± 2</td>
<td>−34 ± 2</td>
</tr>
<tr>
<td>FS-FLUOX</td>
<td>−31 ± 4</td>
<td>−29 ± 4</td>
<td>−31 ± 3</td>
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<tr>
<td>FS-VEH</td>
<td>−19 ± 4</td>
<td>−18 ± 3</td>
<td>−24 ± 5</td>
</tr>
<tr>
<td>FS-MET</td>
<td>−13 ± 2</td>
<td>−15 ± 2</td>
<td>−12 ± 4</td>
</tr>
<tr>
<td>FS-ATEN</td>
<td>−16 ± 3</td>
<td>−20 ± 2</td>
<td>−25 ± 1*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE of group data; in each group, Δ = mean poststress value − mean prestress value. * and †Significantly different from corresponding FS-ND group values (P < 0.01 and P < 0.05, respectively).

We first performed a sympathetic blockade, using the β-adrenergic receptor blocker atenolol. As expected, chronic treatment with atenolol reduced basal HR compared with the respective control group (FS-ND). However, after sympathetic blockade, subchronic foot shock still provoked a significant fall in HR compared with the prestress period. Therefore, it seemed unlikely that the bradycardia after subchronic stress was related to a decreased sympathetic cardiac drive. During this experiment, we also made an interesting observation that atenolol treatment totally prevented the reduction in locomotion observed in the respective control group (FS-ND) after subchronic stress. This finding (i.e., that bradycardia was present in animals with unaffected locomotor activity) provides additional support to our idea that the bradycardia observed after subchronic stress was locomotor-independent. Additionally, this behavioral effect of atenolol also puts in doubt the common view that the drug does not cross the blood-brain barrier. In fact, some reports have demonstrated just the opposite in canine studies (1). Given the evidence that centrally administered beta-blockers prevented stress-induced cardiac arrhythmias in pigs (27), we hypothesize that atenolol may also have had a central anxiolytic effect and that this was the mechanism underlying the suppression of the locomotor-behavioral changes in our stressed rats.

The effect of vagal blockade on the HR before and after subchronic stress was assessed by administration of M-cholinoreceptor blocker methyl-scopolamine. Similar to the FS-ND group, basal HR of the FS-SCOP rats during the poststress period was significantly lower than during the prestress period. As expected, after vagal blockade HR increased significantly, with the absolute values of tachycardia reached being identical in both instances despite the difference in baseline. Consequently, scopolamine-induced tachycardia (delta HR) was significantly larger after subchronic stress compared with the prestress period. This finding (vagal blockade transiently prevented the subchronic stress-induced fall in HR) clearly indicates that the poststress vagal tone was higher compared with the prestress level, suggesting that a vagal activation is the predominant mechanism mediating subchronic stress-induced bradycardia. Clearly, this vagal hyperactivity after subchronic stress is somewhat paradoxical, as sympathetic hyperactivity has been shown to characterize the stress response (7, 17, 25). Poststress vagal rebound has been described in one human study (16). However, in this study, vagal activity was assessed immediately after stress exposure, and we cannot be sure that this phenomenon would be enduring in the absence of stress. Interestingly, this stress-induced enduring vagal rebound was not sustained by changes in vagal HRV indices, which were
SUSTAINED BRADYCARDIA AFTER SUBCHRONIC STRESS

similar between prestress and poststress periods and showed an opposite trend in active and inactive phases. Thus, it appears that subchronic stress exposure affected cardiac vagal tone without altering the mechanisms modulating it (e.g., respiratory sinus arrhythmia). Indeed, as the quantification of HRV is influenced by the duration of the cardiac cycles, one would expect a significant increase in vagal HRV indices in the presence of a significant reduction in HR. However, the complexity of the relationship between HR and HRV, and the known influence of respiration on HRV indices, led us to hypothesize that these discrepant results may be due to a weakness in the application of HRV when applied to studies with long-term data collection and without the use of controlled respiration. The available data from the literature on the effect of stress on HRV in animals are controversial, and the effects are usually assessed during or shortly after stress; on the other hand, long-term HRV disturbances are described in chronically stressed humans (12, 29). We speculate that the sustained vagal activation observed after subchronic foot shock exposure may be a transient compensatory phenomenon that initially overcomes the commonly observed stress-induced sympathetic hyperactivity, and thus is a sign of adaptation. It may be that after a more prolonged exposure to stress, this adaptation might fail and the organism reaches a maladaptive phase of sympathetic dominance.

A further objective of our study was to reveal the neural mechanisms responsible for the effects of subchronic stress on HR and its circadian rhythmicity, as well as the effects on locomotor activity. The neurotransmitter serotonin is involved both in mood and cardiovascular control (5, 14). To test serotonin involvement in cardiac and motor-behavioral effects of subchronic stress, we chronically treated a separate group of rats with fluoxetine (selective serotonin reuptake inhibitor). Also, to test whether enduring cardiac and motor-behavioral effects of subchronic stress were mediated by corticosterone, a stress hormone released upon activation of the HPA axis, we treated another group of rats with metyrapone (inhibitor of corticosterone synthesis) prior to stress exposure. We found that neither fluoxetine nor metyrapone treatment prevented subchronic stress-induced bradycardia, suggesting that the vagal rebound observed after subchronic stress was not mediated by serotonergic or HPA axis mechanisms. On the other hand, fluoxetine treatment abolished the stress-induced slowdown in the transition from the inactive to the active phase of circadian rhythm, whereas in metyrapone-treated rats, this transition was faster during poststress period, compared with the prestress period. Therefore, it follows that serotonergic and HPA axis mechanisms are differently involved in the cardiac rhythm disorganization elicited by subchronic stress. In addition, we found that metyrapone treatment prevented the motor-behavioral changes (hypolocomotion) observed after subchronic stress, suggesting that the effects of subchronic foot shock on locomotion were mediated by stress-induced rises of corticosterone. The finding that a serotonin reuptake inhibitor (fluoxetine) was also efficient in preventing the reduction in locomotion induced by subchronic stress, suggests the possibility of functional interactions between the HPA and the serotonergic systems. Glucocorticoid release from the adrenal cortex is stimulated by ACTH secretion from the anterior pituitary gland, which, in turn, is controlled mainly by corticotropin-releasing factor (CRF) release from the hypothalamus. It has been long demonstrated that drugs that enhance the body’s serotonin levels, such as serotonin reuptake inhibitors, increase the release of corticosterone, ACTH, and CRF in rats (6). Also, chronic fluoxetine treatment in rats does not inhibit neuroendocrine responses to stress (36). Thus, it appears that a serotonergic influence on the HPA axis is unlikely to be the mechanism, whereby fluoxetine prevented subchronic stress-induced motor-behavioral changes. The efficacy of fluoxetine treatment could be instead attributed to an anxiolytic effect of enhanced serotonin levels in brain areas implicated in processing behavioral responses to stressful stimuli, such as the hippocampus, the hypothalamus, and the amygdala (28). On the other hand, metyrapone has been recently shown to increase the level of serotonin in the rat frontal cortex, although the underlying mechanisms are still not well understood (20). It may be speculated that, after metyrapone treatment, serotonin function increases also in the above-named brain areas that mediate behavioral responses to stress. Inhibition of corticosterone synthesis may thus well be the first crucial step in the sequence of events leading to prevention of the locomotor changes observed after subchronic stress.

Perspectives and Significance

This study presents the first demonstration of stress-induced enduring increase in cardiac vagal tone-vagal rebound. This effect seems to be corticosterone- and serotonin-independent. Given the importance of vagal tone for cardiac health, the evaluation of further details of the mechanisms mediating this enduring vagal rebound might have substantial clinical relevance.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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