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Conditioned fear-induced tachycardia in the rat; vagal involvement

Marjoleen J.M.A. Nijsen [a,*], Gerda Croiset [a], Michaela Diamant [b], Ruben Stam [a], Dianne Delsing [a], David de Wied [a], Victor M. Wiegant [a]

[a] *Rudolf Magnus Institute for Neurosciences, Department of Medical Pharmacology, Utrecht University, P.O. Box 80040, 3508 TA Utrecht, Netherlands*
[b] *Department of Internal Medicine, Academic Medical Centre, Amsterdam, Netherlands*

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Abstract

The effects of conditioned fear on gross activity, heart rate, PQ interval, noradrenaline and adrenaline were studied in freely moving rats. Subcutaneous (s.c.) injections of atropine methyl nitrate (0.5 mg/kg) during rest resulted in a significant shortening of the PQ interval, indicating that the PQ interval can be used as a measure of vagal activity. Conditioned fear was induced by 10-min forced exposure to a cage in which the rat had previously experienced footshocks (5×0.5 mA $\times$ 3 s). In non-shocked controls, an increase in gross activity was found and a pronounced tachycardia, without changes in PQ interval. Conditioned fear rats showed immobility behaviour, associated with a less pronounced tachycardia and an increase in PQ interval. Noradrenaline was similarly increased in both groups, whereas adrenaline was increased in conditioned fear rats only. To further evaluate the role of the vagus, rats were exposed to conditioned fear after pre-treatment with atropine methyl nitrate (0.5 mg/kg, s.c.). Again, immobility was observed with a concomitant tachycardia, but without an increase in PQ interval. These results indicate that the autonomic nervous system is differentially involved in heart rate regulation in conditioned fear rats and in non-shocked controls: in non-shocked controls a predominant sympathetic nervous system activation results in an increase in heart rate, whereas in conditioned fear rats the tachycardiac response is attenuated by a simultaneous activation of sympathetic nervous system and parasympathetic nervous system.

Keywords: Stress; Autonomic nervous system; Electrocardiogram; Vagal outflow

1. Introduction

Abundant experimental evidence has demonstrated that stressful stimuli induce behavioural and autonomic responses (Bond, 1943; Mason, 1968; Bolles, 1970; Axelrod and Reisine, 1984). The behavioural responses to stress depend on the modality and intensity of the stressor as well as on the experience and possibility of the animal to cope with the stressor (Davis, 1970; De Boer et al., 1990, 1991; Diamant et al., 1991). These behavioural responses are accompanied by changes in autonomic activity (Wan et al., 1990; Diamant et al., 1991). Activation of the autonomic nervous system can be measured as changes in heart rate, blood pressure, body temperature and gastrointestinal motility, and represents the result of the complex interactions of the sympathetic, parasympathetic and enteric nervous system (Buwalda et al., 1990; Diamant and De Wied, 1993; Stam et al., 1995). In general, exposure to a stressor induces tachycardiac responses (Kirby and Johnson, 1990; Nyakas et al., 1990; Diamant et al., 1991; Roozendaal et al., 1991; Korte et al., 1992). Furthermore, in rats, stressful conditions induce increases in circulating catecholamines (Popper et al., 1977; Kvetnansky et al., 1978; McCarty and Baucom, 1982; De Boer et al., 1990; Korte et al., 1992), which have been demonstrated to correlate with sympathetic nervous system activity (Lake et al., 1976). Although a number of reports suggest the involvement of the sympathetic nervous system in acute tachycardiac responses to stress (Obrist et al., 1974; Cleroux et al., 1985; Overton, 1993), the parasympathetic nervous system also contributes to stress-induced cardiac responses (Porges, 1992; Wiersma et al., 1993; Bohus et al., 1996): an increase in heart rate may result from sympathetic activa-

* Corresponding author. Tel.: +31-30-2538845; fax: +31-30-2539032; e-mail: Nijsen@med.ruu.nl

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tion and/or vagal withdrawal or may involve coactivation of both divisions of the autonomic nervous system (Berntson et al., 1991). The exact role of both divisions of the autonomic nervous system in stress-induced cardiac responses has so far not been established. Efforts to distinguish sympathetic and parasympathetic contributions have been hampered by the lack of a reliable indicator of the vagal contribution (Wiersma et al., 1993; Hedman et al., 1995).

In previous pharmacological studies with rats that have intraperitoneally (i.p.) implanted electrocardiogram (ECG) transmitters, we showed that subcutaneously (s.c.) injected pilocarpine (muscarinic acetylcholine receptor agonist) increased the PR interval (atrioventricular transmission time) and isoprenaline (β-adrenoceptor agonist) decreased it (Croiset et al., 1994). These results agree with those of Levy and Zieske (1969), who reported that electrical stimulation of the vagus decreased heart rate and increased the PR interval, while electrical stimulation of the sympathetic outflow increased heart rate and decreased the PR interval. Prolongation of the PR or PQ interval in combination with tachycardia can only be attributed to a relative increase in vagal activity, as a possible reduction in sympathetic activity is implausible. Thomas and Randall (1983) showed that atropine treatment decreased atrioventricular transmission time significantly, whereas propranolol only slightly increased atrioventricular transmission time during rest, indicating that vagal influences predominate over sympathetic outflow in the control of atrioventricular conduction. It can be assumed that the PQ interval can be used as an index of vagal activity when a tachycardiac response is not accompanied by a reduction in PQ interval.

In more recent experiments, we found that an elongated PQ interval is reduced in rats by habituation to a novelty stressor (Nijsen et al., 1998). This suggests that vagal outflow becomes more important when the appraised intensity of the stressor is higher. To further test the latter hypothesis, the present study involves a stress model with a stronger intensity, i.e., forced exposure to the environment in which rats formerly experienced inescapable footshocks (conditioned fear) (Roozendaal et al., 1990; Korte et al., 1992). Using this test, Korte et al. (1992) showed that previously shocked rats display immobility accompanied by increases in adrenaline, whereas both shocked and non-shocked rats show similar increases in heart rate and noradrenaline. Using the same model, Roozendaal et al. (1990) reported immobility behaviour, associated with an attenuated tachycardia in previously shocked rats as compared to the response of non-shocked controls, suggesting the involvement of the parasympathetic nervous system in the heart rate response to conditioned fear. The aim of the present study was to clarify the respective contribution of the sympathetic and parasympathetic nervous system in cardiovascular responses to fear and novelty by means of measurements of heart rate, PQ interval, gross activity and plasma catecholamines.

2. Materials and methods

2.1. Animals and housing

Naive male albino rats of an outbred Wistar strain (U:WU) weighing 250–280 g at the beginning of the experiments were used. The rats were housed individually in Macrolon cages ($23 \times 17 \times 14$ cm) containing a layer of wood shavings, under conditions of constant ambient temperature (21 ± 1°C), constant humidity and normal light/dark rhythm (with lights on from 0700 to 1900). After surgical implantation of a telemetric transmitter or cannulation of the jugular vein, the animals were housed individually in Plexiglas cages ($25 \times 25 \times 40$ cm) under presurgical conditions. For the forced exposure test a $30 \times 32.5 \times 38.5$ cm shock box was used, made of stainless steel walls with a Plexiglas door and a grid floor made of 2.5 mm brass rods spaced 1.0 cm apart. Food (complete laboratory chow: Hope Farms, Woerden, The Netherlands) and water were accessible ad libitum throughout the experiment.

2.2. Surgery

One group of rats was equipped with telemetric devices to study behavioural (gross activity) and autonomic (heart rate and PQ interval) responses. Another group of rats were provided with a cannula in the jugular vein to determine plasma catecholamine concentrations. Operations were performed under fentanyl/fluanisone anaesthesia (Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium; 0.1 ml/100 g body weight (BW), i.m.) and midazolam hydrochloride (Dormicum®, Hoffman–LaRoche, Mijdrecht, The Netherlands; 0.1 ml, i.p.) as a muscle relaxant. Before we injected the muscle relaxant, we tested the analgesic effect of fentanyl anaesthesia in the rat by checking its pedal and cornea reflexes. Total absence of the pain response normally appeared after 10 min and then the muscle relaxant was injected. Telemetric transmitters were implanted in the abdominal cavity, according to the procedure described by Wan et al. (1990). A small longitudinal incision was made on the linea alba anterior to the abdomen. Two electrodes originating from the top of the transmitter were guided subcutaneously on either side of the thorax; the electrode tips were sutured into position to obtain a bipolar ECG signal. Changes in position of this transmitter can be monitored by a receiver and reflect gross activity of the rat. The animals were cannulated according to the method of Steffens (1969) with some modifications. A sterile silicone cannula (Silastic®, Dow Corning, Midland, MI, USA), filled with a 50-UI/ml heparin solution (0.9% NaCl, containing 50 IU/ml heparin), was inserted into the external jugular vein and passed down near the entrance of the atrium. The cannula was guided under the skin towards the skull, connected to a stainless steel tube that was attached to the skull with

dental cement. The end of the cannula was filled with 0.05 ml PVP (50% PolyVinylPyrrolidonum in Millipore-filtered water, containing 50 IU/ml heparin). Postoperatively, the animals received 0.1 mg/kg of the long-acting opiate analgesic, Buprenorfine hydrochloride (Temgesic®, Reckitt & Colman, Kingston-upon-Hull, UK; 0.1 ml, s.c.). They were allowed to recover from surgery for 10 days in the experimental room. During the recovery period the animals were handled every day for weighing and habituation purposes. Cannulated rats were allowed to become accustomed to the conditions under which blood sampling would take place (twice before the experiment). After the experiment, all rats were killed by an overdose of pentobarbital (160 mg/ml), and the abdominal cavity visually inspected for infections. None of the animals showed any signs of infection.

2.3. Telemetry

The telemetry system consisted of small wireless transmitters, model TA11CTA-F40 (Data Sciences, St. Paul, MN, USA), and receivers, model RLA1020 (Data Sciences). Digital data, ECGs and gross activity, were transmitted from the receiver to a DataQuest IV data acquisition system (Data Sciences). The mean heart rate was automatically calculated from ECGs and gross activity was automatically averaged with the DataQuest IV system. The PQ interval is defined as the beginning of the P wave and the beginning of the QRS complex (see Fig. 1). The mean PQ interval was automatically analysed from ECGs by a special software program, PhysioStat PS1000 (Data Sciences). Before drug treatment, a 10-s sampling period with intervals of 3 min was used to record ECGs and gross activity during a 30-min recording period in the home cage (baseline). A 10-s sampling period with intervals of 30 s was used to record ECGs and gross activity after drug treatment for 30 min in the home cage. At $t = 60$ min a final 10-s sample period was recorded in the home cage. A 10-s sampling period with intervals of 3 min was used to record ECGs and gross activity during a 30-min recording period in the home cage just before (baseline) and during a 60-min recording period in the home cage just after (post treatment) exposure to the shock box. A 10-s sampling period with intervals of 30 s was used to record ECGs and gross activity during the 10 min of testing in the shock box. To simplify the figures, data points at 1-min instead of 30-s intervals are shown for effects of drug treatment or during the 10-min forced exposure test. The data from 2 samples taken at 30-s intervals were pooled to produce each data point in Figs. 2, 3 and 5.

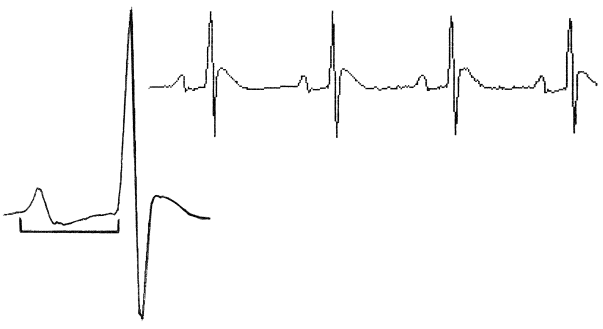

Fig. 1. The above tracing is an example of an original ECG recording in the rat. The ECG complex in front is the reference complex made with a special software program. Lines represent the start and end of the PQ interval, as defined by the software program.

2.4. Catecholamine determination

Catecholamines were determined by high performance liquid chromatography (HPLC) based on isocratic reversed-phase chromatographic separation in combination with amperometric detection. All determinations were performed on a Separations model GT-103 liquid chromatograph (Separations Analytical Instruments, H.I. Ambacht, The Netherlands) equipped with an automatic sampler and injector in conjunction with a peak column (Inertsil®, G.L. Sciences, Tokyo, Japan; packing: Inertsil® ODS-2, particle size: 5 μm) and an amperometric detection system consisting of auxiliary electrodes and an Ag–AgCl reference electrode. The resulting signal was processed by an Axiom Chromatography model 727 chromatography datasystem. Both the column and the detector cell were placed in the column oven of the liquid chromatograph.

There was added to the plasma samples (100 μl), 10 mg aluminium oxide (Recipe Chemicals and Instruments, München, Germany), 11 μl internal standard (3,4-dihydroxybenzylamine (Sigma®, Brunschwig chemie, H.I. Ambacht, The Netherlands); 5 μM dissolved in water) and 300 μl 2 M Tris buffer D (Recipe Chemicals and Instruments). After mixing, the supernatant (110 μl) was removed and the aluminium oxide was washed three times with 400 μl Tris buffer (Recipe Chemicals and Instruments). Glacial acetic acid solution (1% volume/volume, Merck, Darmstadt, Germany; 110 μl) was used to elute the catecholamines from the aluminium oxide. From this eluate, 90 μl was injected on HPLC. Each run included standard samples (100 μl) containing noradrenaline and adrenaline (Sigma®, Brunschwig chemie, Amsterdam; 5 μM dissolved in water) and 3,4-dihydroxybenzylamine. Data from unknowns were calibrated to the standards, and expressed as pg/ml plasma. The sensitivity of the assay was 1 pg/ml sample.

2.5. Drug treatment

Atropine methyl nitrate (Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands) was dissolved in saline. Atropine was injected s.c. 30 s before the forced exposure test or during rest in a dose of 0.5 mg/kg BW and constant volume of 0.25 ml. Atropine methyl nitrate is a peripherally acting agent, which does not cross the blood-brain barrier (Brown and Taylor, 1995).

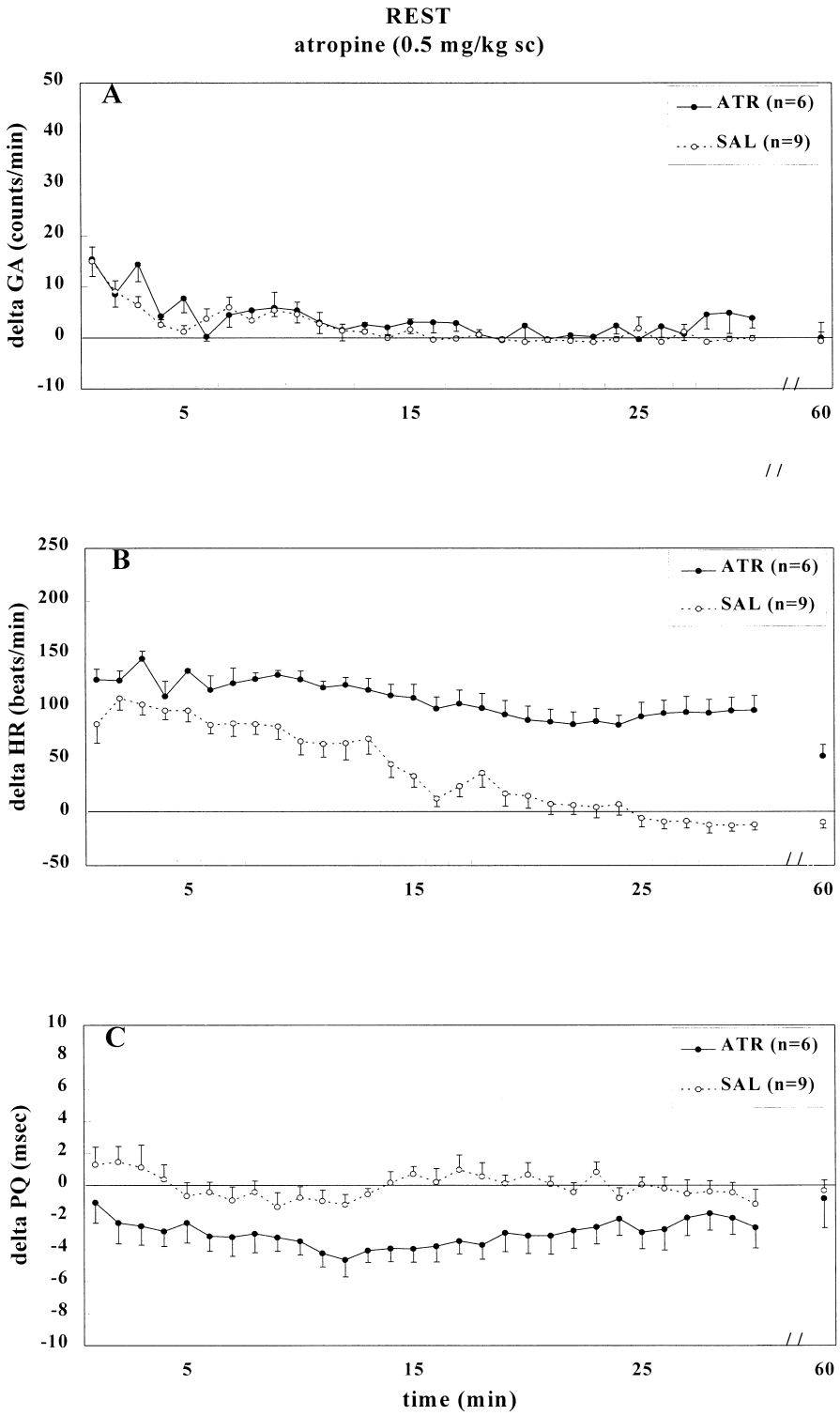

Fig. 2. Changes in gross activity (GA) (A), heart rate (B) and PQ interval (C) induced by treatment with atropine methyl nitrate (ATR) and saline (SAL) during rest. Atropine methyl nitrate (0.5 mg/kg) or saline was injected at $t = 0$ min. Data are presented as means ± S.E.M.

2.6. Experimental design

All experiments were performed during the light phase of the lighting cycle between 0900 and 1300.

2.6.1. Validation of the PQ interval as a measure of vagal activity

Baseline heart rate, PQ interval and gross activity of individual, single-housed rats were telemetrically recorded

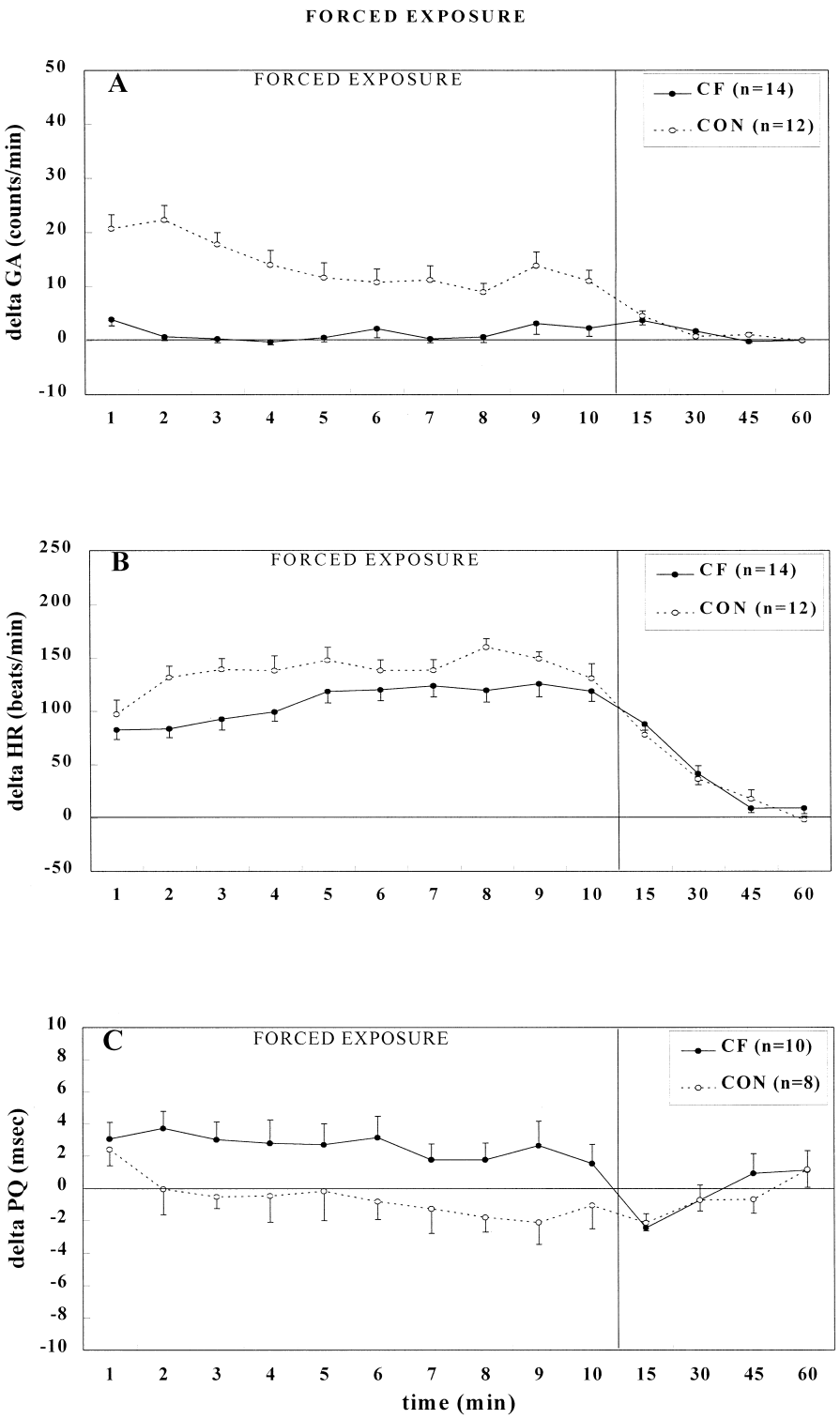

Fig. 3. Changes in gross activity (GA) (A), heart rate (HR) (B) and PQ interval (C) in conditioned fear (CF) and control (CON) rats during and after the 10-min forced exposure test. The vertical line marks the end of the forced exposure test. Data are presented as means ± S.E.M.

in the home cage for 30 min. Subsequently, the rats were s.c. injected with saline ($n = 9$) or atropine methyl nitrate ($n = 6$) and immediately returned to their home cages, where heart rate, PQ interval and gross activity were recorded for another 60-min period.

2.6.2. Behavioural and autonomic responses to conditioned fear

On day 1 of the experiment, individual, single-housed animals with implanted transmitters were transferred one by one to a novel shock box where they remained for 10

min. A group of 14 rats received 5 × 0.5 mA × 3 s footshocks given at random intervals throughout the 10-min period starting within 30 s of entry (shocked group), while a group of 12 rats received no shock (control group). On day 2, baseline heart rate, PQ interval and gross activity of rats were recorded in the home cage for 30 min under resting conditions and then the procedure from day 1 was repeated, except that none of the animals received any shock (conditioned fear or control group). During this period, heart rate, PQ interval and gross activity were recorded. After testing, the rats were returned to their home cages where heart rate, PQ interval and gross activity were recorded for another 60 min (post treatment).

2.6.3. Catecholamine responses to conditioned fear

On day 1 of the experiment, individual, single-housed cannulated animals were transferred one by one to a novel shock box where they remained for 10 min. A group of 8 rats received 5 × 0.5 mA × 3 s footshocks given at random intervals throughout the 10-min period starting within 30 s of entry (shocked group), while another group of 9 rats received no shock (control group). On day 2, 1 h prior to testing, the animals were connected to the sampling polyethylene cannula. At $t = 0$ min the rat was picked up and moved from the home cage to the shock box. None of the animals received any shock (conditioned fear or control group). At $t = 10$ min the rat was returned to the home cage. Blood samples were drawn at $t = -5$, 1, 5, 15, 35 and 65 min, using the method described by Wiersma and Kastelijn (1985), with some modifications. The samples (350–400 μl) were collected in small cups containing heparin (10 μl of 500 IU/ml) and antioxidant (10 μl of EDTA, 44 mg/ml) and centrifuged (10 min, 4000 rpm, 4°C). Plasma was separated and stored at −80°C for catecholamine determination. The volume of sampled blood was replaced by saline, in order to avoid volume changes. Before the start of the experiments, similar amounts of blood were collected during rest, but no changes in plasma NA and A were found. This indicates that the procedure of repeated blood sampling itself does not result in sympathetic activation, as has also been reported by others (Wiersma and Kastelijn, 1985).

2.6.4. Effects of vagal blockade on behavioural and autonomic responses to conditioned fear

On day 1 of the experiment, individual, single-housed animals with implanted transmitters were transferred one by one to a novel shock box for 10 min, where they received 5 × 0.5 mA × 3 s footshocks given at random intervals throughout the 10-min period starting within 30 s of entry. The following day, baseline heart rate, PQ interval and gross activity of rats were recorded in the home cage for 30 min under resting conditions and then a group of 11 rats were s.c. injected with saline and a group of 13 rats was s.c. injected with atropine methyl nitrate. Immediately after injection, the rats were re-exposed to the shock box for 10 min without receiving any shock (conditioned fear). During this period, heart rate, PQ interval and gross activity were recorded. After testing, the rats were returned to their home cages where heart rate, PQ interval and gross activity were recorded for another 60 min (post treatment).

The experiments were approved by the ethical committee for animal experimentation of the Medical Faculty (Dierexperimentele Commissie), Utrecht University, The Netherlands.

2.7. Statistics

Values for behavioural (gross activity) and autonomic (heart rate and PQ interval) parameters are presented as mean changes (±S.E.M.) in comparison to their baseline value. Catecholamine concentrations are presented as means pg/ml (±S.E.M.). All data were analysed by a two-factor multiple analysis of variance (MANOVA) with repeated measures, with one between-subjects factor (treatment) and one repeated measures within-subjects factor (time). The treatment factor had two levels (atropine methylnitrate and saline; conditioned fear and control) and the time factor had 31 levels (drug treatment during rest), 10 levels (behavioural and autonomic response to conditioned fear) or 5 levels (catecholamine response to conditioned fear). A Spearman correlation test was used to determine the correlation between heart rate and gross activity in individual rats during 10 min of conditioned fear, using pairs of heart rate and gross activity for every 30-s sample after drug treatment or during exposure to the test cage. P values of < 0.05 were considered significant.

Due to the poor quality of the telemetric signal, the PQ interval data from 4 conditioned fear and 4 control rats were excluded from further analysis (Fig. 3C). As there were missing values for adrenaline, the data from 1 conditioned fear rat were excluded from further analysis (Fig. 4B).

3. Results

3.1. Validation of the PQ interval as a measure of vagal activity

3.1.1. Gross activity

Mean baseline gross activity levels were 0.7 ± 0.1 counts/min in rats prior to saline treatment and 0.6 ± 0.4 counts/min prior to atropine methyl nitrate treatment. Both atropine methyl nitrate and saline treatment increased baseline gross activity in rats in the home cage for 10 min because of handling (Fig. 2A). Three out of 6 atropine methyl nitrate-treated rats were extremely active at $t = 3$ and 5 min, which resulted in peaks of gross activity at these time points. There was a significant effect of treatment (MANOVA: $F(1,13) = 6.0$, $P < 0.05$) and time ($F(30,390) = 8.0$, $P < 0.001$).

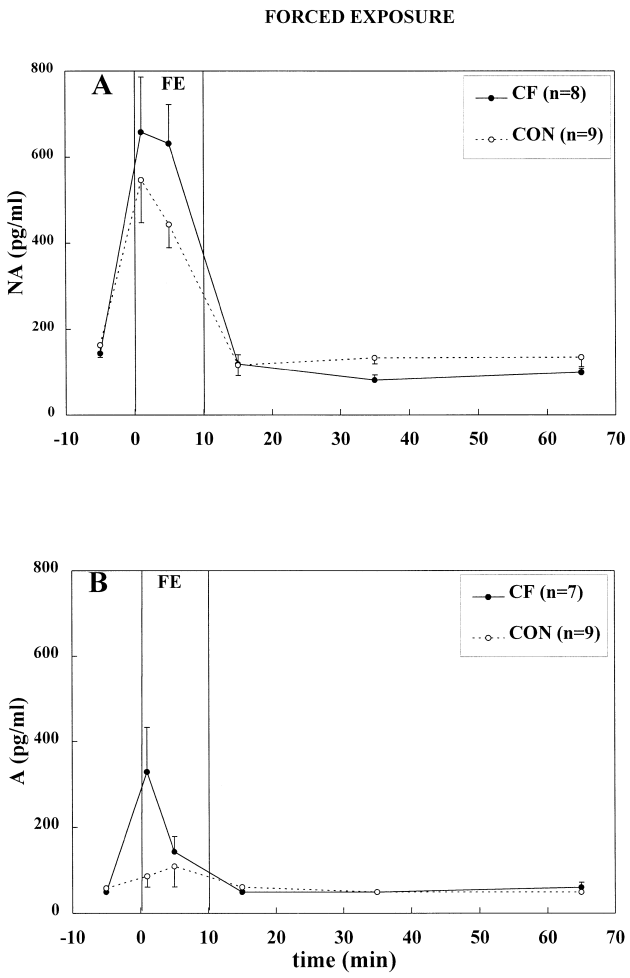

Fig. 4. Noradrenaline (NA) (A) and adrenaline (A) (B) levels in conditioned fear (CF) and control (CON) rats before, during and after the 10-min forced exposure test (FE). The vertical lines mark the start and end of FE. Data are presented as means ± S.E.M.

3.1.2. Heart rate

Mean baseline heart rate levels were 362 ± 6 beats/min in rats prior to saline treatment and 364 ± 4 beats/min prior to atropine methyl nitrate treatment. Both atropine methyl nitrate and saline treatment initially increased heart rate in rats in the home cage (Fig. 2B). In atropine methyl nitrate-treated rats, the heart rate remained high for the entire test period (60 min), whereas the heart rate of saline-treated rats return to baseline levels within 15 min. There was a revealed significant effect of treatment (MANOVA: $F(1,13) = 45.3$, $P < 0.001$), effect of time ($F(30,390) = 4.3$, $P < 0.001$) and an interaction of treatment by time ($F(30,390) = 21.5$, $P < 0.001$). In 11 out of 15 rats a significant positive correlation was found between heart rate and gross activity; in 2 out of 15 rats a tendency to a positive correlation was found ($P = 0.06$ and 0.07).

3.1.3. PQ interval

Mean baseline PQ intervals were 50.0 ± 0.7 ms in rats prior to saline treatment and 49.0 ± 1.5 ms prior to atropine methyl nitrate treatment. Fig. 2C shows the changes in mean PQ interval after saline and atropine methyl nitrate treatment in rats in the home cage. Atropine methyl nitrate treatment resulted in a reduction of the PQ interval for 30 min, whereas the PQ interval in saline-treated rats did not change. There was a revealed significant effect of treatment (MANOVA: $F(1,13) = 14.1$, $P < 0.005$) and time ($F(30,390) = 1.6$, $P < 0.05$).

3.2. Behavioural and autonomic responses to conditioned fear

3.2.1. Gross activity

Mean baseline gross activity levels were 0.4 ± 0.1 counts/min in non-shocked controls and 0.8 ± 0.2 counts/min in conditioned fear rats. Fig. 3A shows the changes in mean gross activity in controls and conditioned fear rats during the forced exposure test. The controls showed an increase in gross activity, whereas conditioned fear rats remained immobile. There was a revealed significant effect of treatment (MANOVA: $F(1,24) = 59.8$, $P < 0.001$), effect of time ($F(9,216) = 5.7$, $P < 0.001$) and an interaction of treatment by time $F(9,216) = 4.7$, $P < 0.001$).

3.2.2. Heart rate

Mean baseline heart rate levels were 364 ± 5 beats/min in non-shocked controls and 361 ± 5 beats/min in conditioned fear rats. The changes in mean heart rate in controls and conditioned fear rats during the forced exposure test are depicted in Fig. 3B. The control group had a more pronounced tachycardia than did the conditioned fear group. There was a revealed significant effect of treatment (MANOVA: $F(1,24) = 5.3$, $P < 0.05$), effect of time ($F(9,216) = 12.8$, $P < 0.001$) and an interaction of treatment by time ($F(9,216) = 2.6$, $P < 0.01$). No correlation between heart rate and gross activity was found in control and conditioned fear group.

3.2.3. PQ interval

Mean baseline PQ intervals were 51.7 ± 1.1 ms in non-shocked controls and 52.2 ± 1.3 ms in conditioned fear rats. Fig. 3C shows the changes in mean PQ interval in controls and conditioned fear rats during the forced exposure test. An increase in PQ interval was found in conditioned fear rats, whereas the PQ interval in the control group remained at its baseline. There was a revealed significant effect of treatment (MANOVA: $F(1,16) = 5.3$, $P < 0.05$) and time ($F(9,144) = 2.2$, $P < 0.05$).

3.3. Catecholamine responses to conditioned fear

3.3.1. Noradrenaline

The mean baseline noradrenaline levels were 163.5 ± 28.4 pg/ml in non-shocked controls and 144.1 ± 20.5

pg/ml in conditioned fear rats. In both conditioned fear and control rats noradrenaline levels were markedly increased during forced exposure (see Fig. 4A). There was a revealed significant effect of time (MANOVA: $F(4,60) = 36.6$, $P < 0.001$).

3.3.2. Adrenaline

The mean baseline adrenaline levels were 55.6 ± 8.6 pg/ml in non-shocked controls and 51.0 ± 0.3 pg/ml in conditioned fear rats. Fig. 4B shows the dynamics of adrenaline levels in control and conditioned fear rats be-

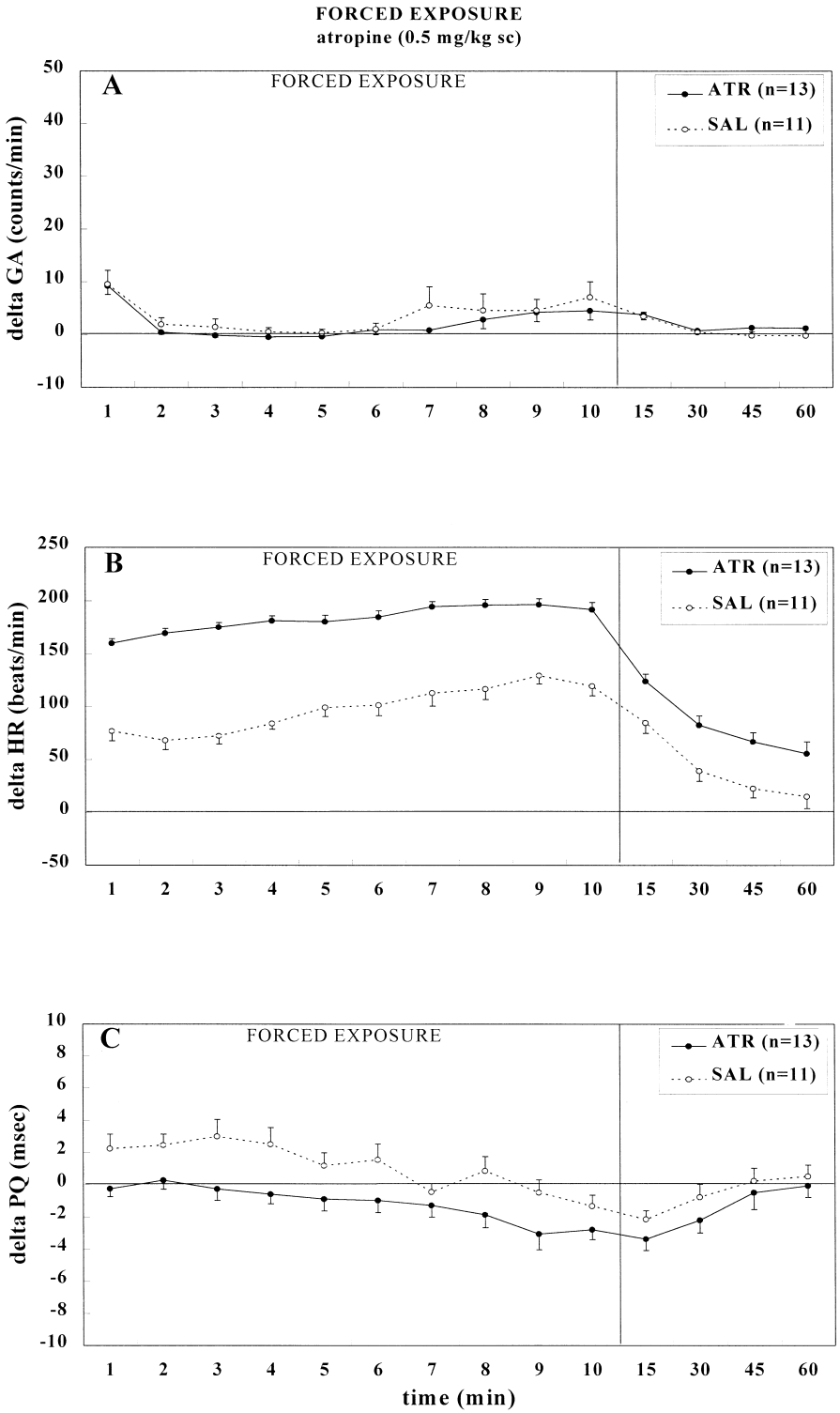

Fig. 5. Changes in gross activity (GA) (A), heart rate (HR) (B) and PQ interval (C) in atropine methyl nitrate-treated (ATR) and saline-treated (SAL) conditioned fear rats during and after 10-min forced exposure test. Atropine methyl nitrate (0.5 mg/kg) or saline was injected at $t = 0$ min. The vertical line marks the end of the forced exposure test. Data are presented as means ± S.E.M.

fore, during and after the forced exposure test. A marked increase in adrenaline was found in conditioned fear rats during the first minute of forced exposure, whereas adrenaline in the controls remained at its baseline. There was a revealed significant effect of treatment (MANOVA: $F(1,14) = 4.8$, $P < 0.05$), effect of time ($F(4,56) = 7.5$, $P < 0.001$) and an interaction of treatment by time ($F(4,56) = 4.5$, $P < 0.005$).

3.4. Effects of vagal blockade on behavioural and autonomic responses to conditioned fear

3.4.1. Gross activity

The mean baseline gross activity levels were 0.6 ± 0.2 counts/min in rats prior to saline treatment and 0.5 ± 0.1 counts/min prior to atropine methyl nitrate treatment. Both groups of rats remained largely immobile during conditioned fear (see Fig. 5A). There was a revealed significant effect of time (MANOVA: $F(9,198) = 9.6$, $P < 0.001$).

3.4.2. Heart rate

The mean baseline heart rate levels were 378 ± 3 beats/min in rats prior to saline treatment and 368 ± 4 beats/min prior to atropine methyl nitrate treatment. The changes in mean heart rate in saline and atropine methyl nitrate-treated rats during conditioned fear are shown in Fig. 5B. In atropine methyl nitrate-treated rats the tachycardia was twice as high as in saline-treated rats. There was a revealed significant effect of treatment (MANOVA: $F(1,22) = 111.7$, $P < 0.001$), effect of time ($F(9,198) = 27.2$, $P < 0.001$) and an interaction of treatment by time ($F(9,198) = 3.6$, $P < 0.001$). No correlation between heart rate and gross activity was found in saline and atropine methyl nitrate-treated rats during conditioned fear.

3.4.3. PQ interval

The mean baseline PQ intervals were 49.4 ± 0.9 ms in rats prior to saline treatment and 48.7 ± 0.7 ms prior to atropine methyl nitrate treatment. The increase in PQ interval in saline-treated rats during conditioned fear was completely blocked by atropine methyl nitrate treatment (see Fig. 5C). There was a revealed significant effect of treatment (MANOVA: $F(1,22) = 6.6$, $P < 0.05$) and time ($F(9,198) = 15.2$, $P < 0.001$).

4. Discussion

In the present study we show that heart rate is not simply proportional to the intensity of the stressor. A marked tachycardia was found in non-shocked controls, exposed for the second time to the test cage. A less pronounced tachycardia occurred in conditioned fear rats, that had previously experienced inescapable footshocks in this cage. Our results indicate that this non-linear relation between the intensity of the stressor and changes in heart rate may be ascribed to differential contributions of sympathetic nervous system and parasympathetic nervous system activation. In non-shocked controls only the sympathetic nervous system was activated, whereas evidence for activity of both branches of the autonomic nervous system was found in conditioned fear rats.

In order to examine the contribution of both branches of the autonomic nervous system to conditioned fear-induced responses, we measured plasma noradrenaline as a measure of sympathetic nervous system activity, adrenaline as a measure of sympathoadrenal medullary response, the PQ interval of the electrocardiogram as an index of parasympathetic nervous system activity, and heart rate as a parameter of both sympathetic nervous system and parasympathetic nervous system activity (Table 1) (Lake et al., 1976; Croiset et al., 1994). The involvement of autonomic nervous system activity during emotional stress was studied in a well-established model for conditioned fear, i.e., forced exposure to the environment in which rats had experienced inescapable footshocks (Fanselow, 1980). Direct evidence for an enhanced sympathetic nervous system activity was found in both conditioned fear and control rats, which showed similar increases in plasma noradrenaline during exposure to the test box. Although plasma noradrenaline concentrations are similar in both groups, it is still possible that differences in release between control and conditioned fear rats were masked by altered metabolism of noradrenaline in pre shock rats. The noradrenaline response in control rats may be partly caused by a transfer/novelty-in-

Table 1
Degree of change in heart rate, PQ interval, gross activity, plasma noradrenaline and plasma adrenaline in home cage rats (basal), non-shocked controls and conditioned fear rats, treated with saline or atropine methyl nitrate (0.5 mg/kg s.c.)

Group	Heart rate	PQ interval	Gross activity	Noradrenaline	Adrenaline
Basal/saline	+	0	+	nd	nd
Basal/atropine	+ +	− −	+	nd	nd
Non-shocked controls	+ +	−	+ +	+	0
Conditioned fear	+	+	0	+	+
Conditioned fear/saline	+	+	0	nd	nd
Conditioned fear/atropine	+ + +	−	0	nd	nd

The '+' represents an increase, '−' represents a decrease, '0' represents no change, and 'nd' stands for 'not determined'.

duced increase in somatomotor activity (Scheurink et al., 1989), whereas during conditioned fear, when somatomotor activity remains low, the increase in noradrenaline levels is probably due to conditioned emotional stress (De Boer et al., 1990). Our findings are in agreement with those of Korte et al. (1992), who showed that conditioned fear-induced tachycardia is accompanied by increases in plasma noradrenaline of the same magnitude as in control rats. In addition, a marked sympathoadrenal medullary response (increase in plasma adrenaline) was found in conditioned fear rats, which was absent in control rats, suggesting that adrenaline is a measure of the degree of emotional stress, in particular fear. These results are in agreement with those of De Boer et al. (1990), who showed a clear adrenaline response during exposure to a stressor in rats without coping possibilities. Although our results show a clear sympathetic nervous system and sympathoadrenal medullary response to conditioned fear, an attenuated tachycardia was found in comparison to the control group as has been reported by Roozendaal et al. (1990). This suggests that parasympathetic nervous system activity could play an important role during conditioned fear.

In order to obtain direct evidence for parasympathetic nervous system activity we used the PQ interval. Our findings show that parasympathetic nervous system blockade with atropine methyl nitrate during rest in rats resulted in a significant shortening of the PQ interval. This extends previous results and emphasises that elongation of the PQ interval represents an increase in parasympathetic nervous system activity (Croiset et al., 1994). Our results further show that activation of the parasympathetic nervous system was only present in conditioned fear rats and not in non-shocked controls which were only exposed to the novel cage for the second time. We had reported an increase in somatomotor activity, heart rate and PQ interval in rats, which were exposed to a novel cage for the first time (Nijsen et al., 1998). This elongation of the PQ interval was abolished after the fifth exposure to the cage, indicating vagal habituation. No recordings were made during the second, third and fourth exposure to novelty. It is possible that vagal habituation had already occurred during the second exposure to the novel cage as was found in the present study. In the present study, the conditioned fear rats had an increase in PQ interval concomitant with the tachycardiac response. Pre-treatment with atropine methyl nitrate completely blocked the increase in PQ interval during conditioned fear and led to a further increase in heart rate as compared to that in saline-treated conditioned fear rats. Thus, these results provide the first direct evidence for activation of the parasympathetic nervous system during conditioned fear. They further suggest that there is only a contribution of parasympathetic nervous system activation to stress-induced cardiac responses when the intensity of the stressor is high. This is in agreement with the suggestions of Porges (1992, 1995), that the parasympathetic nervous system, rather than, or in addition to, the sympathetic nervous system is an index of stress reactivity and stress vulnerability. Hilton and others have shown that cardiovascular changes in response to emotional stress, referred to as the 'defence reaction', result from stimulation of sympathetic preganglionic neurones in the spinal cord and inhibition of vagal cardioinhibitory neurones in the brainstem (Hilton, 1982; Hilton et al., 1983; Spyer, 1990; Lovick, 1996). This 'defence reaction' can also be elicited by electrical stimulation of 'defence areas' in the amygdala, hypothalamus and midbrain periaqueductal gray. We found an increase in both sympathetic nervous system and parasympathetic nervous system outflow during conditioned fear, suggesting that other brain areas are responsible for excitation of the vagus nerve in the brainstem. Lesion studies by Roozendaal et al. (1990), Roozendaal (1992) showed that the central nucleus of the amygdala is involved in the organisation of conditioned vagal responses.

The tachycardia in the vagally blocked, conditioned fear rats was even higher than that in non-shocked controls, whereas the increase in PQ interval was absent in both groups (see Table 1), indicating that sympathetic nervous system activity is more pronounced during conditioned fear than in the control situation. Whereas plasma noradrenaline was similarly increased in conditioned fear and control rats during exposure to the test box, plasma adrenaline was increased in conditioned fear rats only. Several lines of evidence indicate that stressors increase the levels of enzymes involved in adrenaline biosynthesis in the adrenal medulla (Kvetnansky et al., 1995). Hypophysectomy reduces this stress-induced increase in enzyme levels, while administration of glucocorticoids reinstates this effect, suggesting that glucocorticoids stimulate sympathoadrenal activity during stress (Wurtman and Axelrod, 1966; Viskupic et al., 1994). Thus, it is possible that our finding of increased levels of plasma adrenaline in conditioned fear rats is caused by a glucocorticoid-induced alteration of catecholamine biosynthesis in the adrenal medulla. The additional rise in heart rate in these rats may be due to a direct action of circulating adrenaline on the heart (Gilman et al., 1990). It may well be that parasympathetic nervous system activation normally plays a protective role against an exaggerated response to emotional stressors (Obrist, 1981), resulting in attenuation of the tachycardia in conditioned fear rats as compared to control rats. In addition, conditioned fear rats showed freezing behaviour, whereas non-shocked controls explored the test cage for 10 min. This freezing behaviour is not the type of behaviour that is characteristic of the stress-induced 'defence response', described by Hilton and others (Hilton, 1982; Hilton et al., 1983; Lovick, 1996). These authors reported that emotional stress in animals induces tachycardiac responses, which are accompanied by somatomotor and sympathetic nervous system activation, either to escape, from and/or to fight the threatening situation (Hil-

ton, 1982). It is possible that, in the present study, conditioned fear rats showed a mixture of two patterns of response: the sympathetic nervous system-mediated 'defence response', which tends to lead to tachycardia and the parasympathetic nervous system-mediated 'freezing response', which tends to lead to bradycardia.

Wan et al. (1990) reported that heart rate and somatomotor activities are correlated under non-emotional test conditions in the home cage. This is in agreement with our results showing that heart rate and gross activity are positively correlated in rats in the home cage after s.c. injection of saline. In these rats, the tachycardia, induced by handling and by the injection procedure was not accompanied by changes in PQ interval. The lack of changes in parasympathetic nervous system activity and the positive correlation between heart rate and gross activity in saline-treated rats, suggest that the handling and injection procedure induced an adaptive, sympathetic nervous system-mediated response partly as a result of increased physical activity. However, in the forced exposure experiment no correlation was found between heart rate and gross activity in the conditioned fear or the control group. Thus, the stress-induced tachycardia in these rats was not simply an adaptive response to increased somatomotor activity, but was related to cognitive–emotional processes activated during stress (Obrist et al., 1974; Wan et al., 1990).

Although results of several studies already have suggested a possible role of the vagus in mediating stress-related cardiac responses (Roozendaal et al., 1990; Buwalda et al., 1992, 1993; Wiersma et al., 1993), the present study provides the first direct evidence that the heart rate response during conditioned fear is the result of coactivation of the parasympathetic nervous system (increased PQ interval), the sympathetic nervous system (increased plasma noradrenaline) and the sympathoadrenal medullary systems (plasma adrenaline). The observation that, in control rats, the handling/novelty-induced tachycardia was predominantly associated with an increase in sympathetic outflow indicates that the heart rate response depends on the quality and/or intensity of the stress stimulus and the extent to which each of the autonomic systems is activated.

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