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Circadian rhythms of heart rate and locomotion after treatment with low-dose acetylcholinesterase inhibitors

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ABSTRACT: This study tested the hypothesis that repeated exposure to low levels of sarin, pyridostigmine bromide (PB) or their combination, at doses equivalent to those possibly experienced by veterans of the 1991 Persian Gulf War, could lead to persistent or delayed autonomic effects and thus help to explain the cause of clinical findings in this population. Male Sprague-Dawley rats were treated for 3 weeks with: saline injection (0.5 ml kg⁻¹, s.c., 3 times weekly) with tap drinking water (control); saline injection with PB (80 mg l⁻¹ in drinking water); sarin injection (62.5 µg kg⁻¹, s.c., 0.5 × LD₅₀, 3 times weekly) with tap drinking water (sarin); or sarin injection with PB in drinking water (sarin + PB). At 2, 4 or 16 weeks post-treatment, heart rate (HR) and locomotor activity (LA) were studied by radiotelemetry. Two weeks post-treatment, HR in drug-treated animals was significantly lower than in controls. A decrease in low-frequency HR power spectrum (PS) was found at 00:00 h and 08:00 h with sarin + PB and at 00:00 h with sarin, while total power was enhanced with sarin + PB at 22:00 h. Minimal effects of drug treatments on HR and HR PS were detected at 4 and 16 weeks post-treatment. No significant differences in LA between control and other groups were found. Since no consistent long-term effects were found in any of the variables studied, these experiments do not support the hypothesis that repeated administration of low doses of PB and the nerve agent sarin can induce persistent or delayed alterations in autonomic function. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS: nerve agents; sarin; pyridostigmine bromide; heart rate variability; autonomic nervous system

Introduction

The possible effects of low-dose acetylcholinesterase (AChE) inhibitors on the function of the autonomic nervous system have recently attracted attention with regards to clinical findings in a population of veterans from the 1991 Persian Gulf War (PGW) (Haley *et al.*, 1997a; Fukuda *et al.*, 1998; Wolfe *et al.*, 1998). Exposure of PGW veterans to subsymptomatic levels of sarin has been documented by field studies and modeling analyses of environmental contamination by this agent (McCauley *et al.*, 2001; General Accounting Office, 2003), as well as in epidemiological studies (Wolfe *et al.*, 1998). Treatment of soldiers with PB as a preventive measure against nerve agents occurred during the PGW (Keeler *et al.*, 1991). By virtue of the prominent physiological

role of acetylcholine (ACh) as a central and peripheral neurotransmitter within the autonomic nervous system, exposure to AChE inhibitors has the potential of altering autonomic function. Indeed, some PGW veterans complain of a number of symptoms that suggest autonomic dysfunction (Haley *et al.*, 1997b), although the true significance and association with environmental exposure at the PGW theater of operations has been disputed (Doebbeling *et al.*, 2000; Ismail *et al.*, 1999). A recent report suggests alterations of circadian variations of heart rate (HR) in some PGW veterans (Haley *et al.*, 2004). Analyses of the relationships between exposure to low (subsymptomatic) doses of AChE inhibitors and delayed symptoms and signs associated with the exposure in PGW veterans are limited by the paucity of objective biomarkers of exposure of human subjects and by the limited information regarding controlled experiments in animal subjects. Administration of AChE inhibitors at low levels can induce a number of changes in the autonomic control of the cardiovascular system. Central AChE inhibition increases arterial blood pressure (Varagic, 1955; Buccafusco, 1996) and decreases cerebrovascular resistance (Scremin and Shih, 1991; Scremin *et al.*, 1993; Scremin *et al.*, 1988). AChE inhibition

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with pyridostigmine bromide (PB), a carbamate AChE inhibitor that does not cross the blood–brain barrier, can induce dose-dependent bradycardia (Stein *et al.*, 1997) or increase arterial blood pressure following a single intravenous dose of 2 mg/kg (Chaney *et al.*, 2002). Continuous administration of PB in the drinking water for 7 days at a rate of 31 mg kg⁻¹ day⁻¹ has been shown to enhance heart rate (HR) variability and baroreflex sensitivity when assessed acutely in conscious rats with indwelling catheters (Soares *et al.*, 2004). Similar results were observed after 3 days of continuous PB administration at 10 mg kg⁻¹ day⁻¹ with osmotic minipumps in mice, but only when the drug was associated with stress (Joaquim *et al.*, 2004). No effects on heart rate were reported in the same species with 1 or 3 mg kg⁻¹ day⁻¹ (Bernatova *et al.*, 2003). These effects have been reported during the administration of AChE inhibitors, but the existence of persistent or delayed cardiovascular effects beyond the period of drug administration, a condition that applies to the putative delayed effects in PGW veterans, has not been explored to date in experimental animals.

The heart rate is normally tightly coupled to locomotor activity (LA) (Basset *et al.*, 2004). AChE inhibitors are known to induce changes in exploratory activity resulting in decreased locomotion in a novel environment (Scremin *et al.*, 2003), as well as decreased spontaneous LA at night when rats are most active (Timofeeva and Gordon, 2002; Wang and Fowler, 2001). It is for these reasons that simultaneous recording of LA and HR is essential to determine if possible changes in HR caused by AChE inhibitors are due to direct modulation of parasympathetic innervation of the heart or by an indirect effect derived from changes in LA.

The present experiments were designed to test the hypothesis that repeated exposure to low levels of sarin, PB, or a combination of PB and sarin at doses equivalent to those possibly experienced by PGW veterans could lead to persistent or delayed effects on circadian variations in HR or LA.

Methods

Animals

Male Crl:CD(SD)IGSBR Sprague-Dawley rats, weighing 250–300 g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Laboratories (Kingston, NY) and housed individually in temperature (21 ± 2 °C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12 h light–dark full spectrum lighting cycle with lights on at 07:00 h and off at 19:00 h. Laboratory chow and water were freely available. Treatment of animals was conducted at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD). All animals were then shipped

by air conditioned vans and air-freight to the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System (VAGLAHS), where the planned studies in these animals were performed. Regarding possible effects on animals of travel between laboratories, it is generally agreed that re-entrainment after a time zone change requires approximately 1 day per hour difference for east bound travel and 1 day per 1.5 h difference for westbound travel (Eastman *et al.*, 2005; Benstaali *et al.*, 2001). In the case of our animals, travel was westbound and the time zone difference 3 h. Thus, re-entrainment should have been complete after 2 days. The animals were allowed a minimum of 1 week recovery on arrival to the Los Angeles laboratory before commencing experimentation, to avoid detrimental effects of travel-induced stress and time zone change. The HR and LA were recorded by telemetry starting 1 week after receiving the animals during a minimum of 1 week, and in some cases (data not shown) several weeks. No drifts in circadian rhythms of control animals were observed during the first week of recording that might indicate incomplete adaptation to the time zone change.

The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by AAALAC-International. The animals used in these studies were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, by the Institute of Laboratory Animal Resources, National Research Council, and published by National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

Materials

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs Inc. (Berkeley, CA). Sarin, obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Saline or sarin injection volume was 0.5 ml kg⁻¹ subcutaneously (s.c.). PB was purchased from Sigma-Aldrich (St. Louis, MO), and PB solution was prepared twice weekly in tap water and provided as drinking water to experimental groups for a 3 week period.

Experimental Groups

Separate sets of animals were studied 2, 4 and 16 weeks after completion of 3 weeks of PB and/or sarin exposure. Within every set, animals were divided into four treatment groups. The control animals received regular tap water as drinking water and were injected with saline

(0.5 ml kg⁻¹, s.c.). The PB group received PB in drinking water (80 mg l⁻¹) and was injected with saline. The sarin group received tap water and was injected with sarin (62.5 µg kg⁻¹, s.c., equivalent to 0.5 × LD₅₀). The sarin + PB group received PB in drinking water and was injected with sarin. PB in drinking water was provided continuously to the PB and sarin + PB groups starting on Monday morning at 08:00 h. At 09:00 h that Monday morning, injection of either saline (0.5 ml kg⁻¹, s.c.) or sarin (62.5 µg kg⁻¹, s.c.) was initiated. The injection was given three times (Mondays, Wednesdays and Fridays) per week for 3 weeks. PB in drinking water was terminated and switched to regular tap water at 17:00 h on Friday of the third week.

It has been determined earlier using these regimens that no sign of toxicity was found in rats drinking water containing PB (80 mg l⁻¹) for 3 weeks and that a 0.5 × LD₅₀ sarin was the highest dose that did not cause observable acute toxic effects when given alone or in combination with PB in drinking water for a period of 3 weeks (Scremin *et al.*, 2003; 2005).

The number of animals at 2 weeks post-treatment was control = 6, PB = 7, sarin = 6, sarin + PB = 7; at 4 weeks post-treatment, control = 8, PB = 8, sarin = 8, sarin + PB = 6; at 16 weeks post-treatment, control = 8, PB = 8, sarin = 8, sarin + PB = 6. The number of animals per group was uneven because data were discarded in some animals due to the inconsistent signal: noise ratio of the telemetry ECG recordings that compromised HR measurements.

Implantation of Telemetry Transducers

Animals were anesthetized by exposure to 2.5% halothane in air in a closed plexi-glass chamber with continuous flow of gas from an anesthesia machine. After 2–3 min the animal was transferred to a table provided with a heating pad. A maintenance concentration of halothane (1.5%) was given by mask throughout the surgical procedure. The concentration was raised if withdrawal to painful stimulation was observed. A scavenging system (Fluosorb) prevented excess halothane from reaching the environment. Radiotelemetry transmitters specifically designed for rats (Data Sciences International TL10M3 F50 EEE) were implanted subcutaneously on the back of the animal just below the shoulder blades using an aseptic technique. The transmitter weighs 11.5 g and has a volume of 5.5 cm³. A 2 cm skin incision was performed between the scapulae. The radiotelemetry implant was placed in a pocket fashioned by blunt dissection of the subcutaneous space at the site. One pair of leads was set up for recording of electrocardiogram (ECG) by suturing one of them with 5–0 polyvinyl material to the subcutaneous tissue over the right scapula and the other one at the level of the heart apex. The skin

incision over the radiotelemetry implant was closed with 5-0 polyvinyl suture material. Anesthesia was discontinued after surgical wounds were sutured. The condition of the animal was monitored frequently during the post-surgical period. An analgesic (buprenorphine, 0.05 mg kg⁻¹ s.c. twice daily) was administered during the first 24 h after surgery.

Data Acquisition and Analysis

To analyze circadian variations of HR and LA, as well as HR variability, ECG and LA were recorded every 30 min for an interval of 300 s, for 7 consecutive days, starting 4 days after implantation of telemetry units. Using the Data Sciences software, the time of occurrence of each heartbeat was extracted from the raw ECG and a 300 s time series of consecutive inter-beat intervals (RR intervals) was constructed to allow subsequent time domain and frequency domain measurements.

Heart rate variability was studied by power spectrum analysis of HR fluctuations. To this effect, the time series of the periods between two consecutive R waves of the ECG (RR intervals) was re-sampled at a rate of 10 Hz, and subsequently the frequency spectrum was calculated using a Fast Fourier Transform. Data are presented as cumulative power over the following frequency bands: total (between 0.05 and 5 Hz), low frequency (between 0.26 and 0.75 Hz) and high frequency (between 0.76 and 5 Hz) (Pereira de Souza Neto *et al.*, 2001). The ratio of power in low to high frequency bands was considered an index of sympathovagal balance (Malliani *et al.*, 1991).

The HR and LA were averaged every hour. A database consisting of 7 days of HR and LA hourly averages for every animal was analyzed by performing separate repeated measures ANOVA at each of the three intervals after treatment (2, 4 and 16 weeks), with within factor 'day within recording series' (seven levels) and between factor 'treatment' (four levels) for every hour of the day. Power spectrum of HR (HRPS) was calculated for the third day of recording at an interval of every 2 h. Statistical analysis consisted of ANOVA with factor 'treatment' (four levels) for every 2 h interval. Significance level for F ratios and multiple comparisons among treatment groups was set at 0.05 to establish significance.

Results

The analysis of HR and LA dynamics was conducted for a period of 1 week by averaging telemetry measurements every hour each day with room lights turned on at 07:00 and off at 19:00 h. The results indicated wide fluctuations of both variables between day and night. In general, changes in HR paralleled those in LA in synchrony with regards to the light/darkness cycle. Since

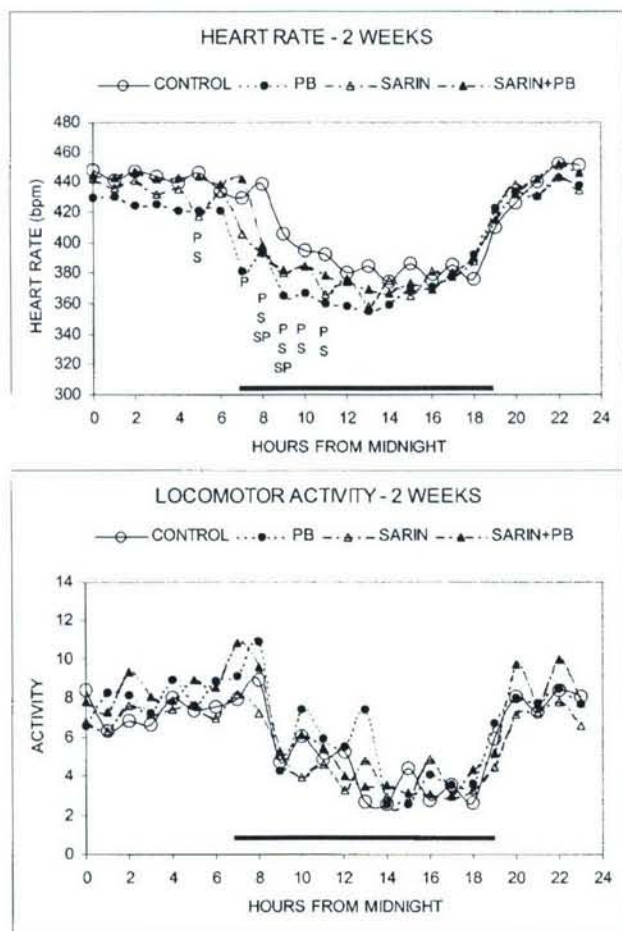


Figure 1. Means of HR (top panel) and LA (bottom panel) as a function of time of day (midnight = 0) measured by telemetry in rats in their home cages for a period of 1 week every hour for 24 h each day. Measurements were performed for 7 days, starting at 2 weeks after treatments were discontinued. Repeated measures ANOVA was performed at every hour with day of the week as the within factor (7 levels) and treatment group as the between factor (4 levels). Significant differences in HR from controls (Fisher LSD multiple comparisons procedure, $P < 0.05$) are indicated for PB (P) at hours 5, 7, 8, 9, 10, and 11; sarin (S) at hours 5, 8, 9, 10 and 11; and sarin + PB (SP) at hours 8 and 9. No significant differences between controls and treatments were found for LA at any hour. Black bars at the bottom of the figure represent the period during which lights were on

rats are nocturnal animals, LA and HR were maximal during the dark period (Figs 1–3). The changes induced by treatments on the magnitude of these variables were not similar, however.

ANOVA of HR values indicated significant effects for the factors 'treatment' and 'hour of day' at all intervals after treatment. Maximal levels of HR were observed during the night and minimal levels observed during daylight hours (Figs 1–3, top panels). In animals tested 2

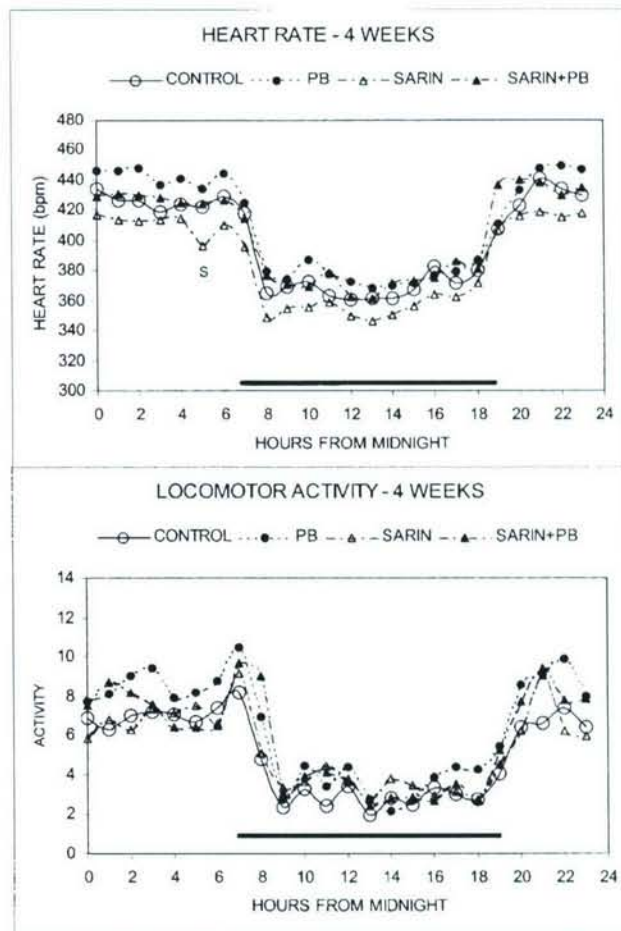


Figure 2. Means of HR (top panel) and LA (bottom panel) as a function of time of day (midnight = 0) measured by telemetry in rats in their home cages for a period of 1 week every hour for 24 h each day. Measurements were performed for 7 days, starting at 4 weeks after treatments were discontinued. Repeated measures ANOVA was performed at every hour with day of the week as the within factor (7 levels) and treatment group as the between factor (4 levels). Significant difference from controls (Fisher LSD multiple comparisons procedure, $P < 0.05$) is indicated for sarin (S) at hour 5. No significant differences between controls and treatments were found for LA at any hour. Black bars at the bottom of the figure represent the period during which lights were on

weeks after treatment, HR was lower than in controls at hours 05:00, 07:00, 08:00, 09:00, 10:00 and 11:00 in the PB group, at hours 05:00, 08:00, 09:00, 10:00 and 11:00 in the sarin group, and at hours 08:00 and 09:00 in the sarin + PB group. Four weeks after treatment, only the sarin group was significantly lower than the controls at 05:00 h, while 16 weeks after treatment only the PB group was higher than the controls at 20:00 h.

Since HR correlates under most circumstances with the magnitude of physical activity, in this case estimated by the radiotelemetry signal level that codes for rate of

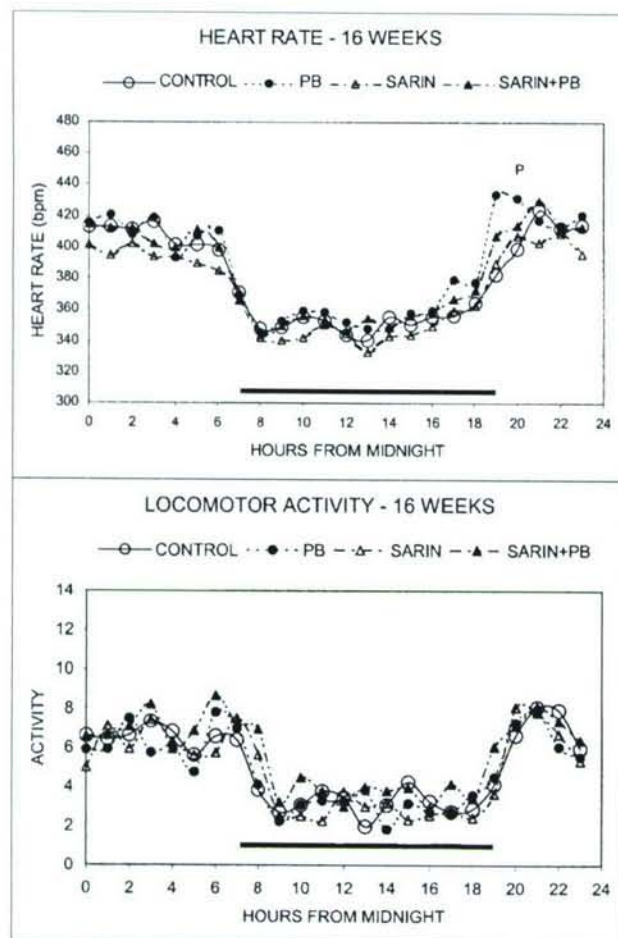


Figure 3. Means of HR (top panel) and LA (bottom panel) as a function of time of day (midnight = 0) measured by telemetry in rats in their home cages for a period of 1 week every hour for 24 h each day. Measurements were performed for 7 days, starting at 16 weeks after treatments were discontinued. Repeated measures ANOVA was performed at every hour with day of the week as the within factor (7 levels), and treatment group as the between factor (4 levels). Significant difference from controls (Fisher LSD multiple comparisons procedure, $P < 0.05$) is indicated for PB (P) at hour 20. No significant differences between controls and treatments were found for LA at any hour. Black bars at the bottom of the figure represent the period during which lights were on

displacement of animals inside their home cages (a measure of LA), it is important to examine the fluctuations in this variable to aid in the interpretation of circadian HR changes and the effects of treatments. Changes in LA recorded by telemetry simultaneously with HR are shown in Figs 1–3 (bottom panels).

In the case of the groups studied at 2 weeks after treatment, the differences in HR among treatments were not paralleled by LA. In fact, no significant difference between means of LA of drug-treated animals and controls was found at any hour of the day (Fig. 1, bottom

panel). The same was true for the 4- and 16-week post-treatment times (Figs 2 and 3, bottom panels, respectively). Thus, it appears that the treatment-induced changes in HR were due to alterations in the autonomic control of this variable rather than to changes in LA.

HRPS analysis indicated that 2 weeks post-treatment (Fig. 4), low frequency HRPS was lower than in controls at hours 00:00 and 08:00 with sarin + PB and at hour 00:00 with sarin, while total power was enhanced at hour 22:00 and reduced at hour 08:00 with sarin + PB. Lesser changes were observed in the PB group 4 weeks post-treatment, when low frequency power and the low to high power ratio decreased at 10:00 and 12:00 h and at 12:00 h, respectively (Fig. 5). At 16 weeks post-treatment, the ratio of low to high frequency power decreased at 14:00 h in the PB group and high frequency power increased in the sarin + PB group at 12:00 h (Fig. 6).

Discussion

The treatment regimen used in this study was designed to model the exposure of PGW veterans to AChE inhibitors. The dose of PB was equivalent, after adjusting for relative dose between species (Freireich *et al.*, 1966), to that received by soldiers as prophylactic against nerve agents (Keeler *et al.*, 1991). The dose of sarin was designed to be the highest devoid of acute manifestations of toxicity either alone or in combination with PB in drinking water for a 3 week period (Scremin *et al.*, 2003). In our previous use of this model, AChE inhibition in red blood cells was present during treatment, but by 3 weeks post-treatment the inhibition was reduced considerably in magnitude for sarin and sarin + PB groups, while it had recovered to normal levels in the case of PB treatment (Scremin *et al.*, 2003; 2005).

The aim of this study was to look for delayed effects, i.e. beyond the period of low-level exposure to AChE inhibitors. A lack of any acute toxic effects during 3 weeks of sarin and PB administration, either alone or in combination, fulfilled the conditions required to model the potential low-level exposure of PGW veterans. This model was, however, the 'worst case' model for PGW exposure scenario where veterans did not report any symptom of miosis, an initial sign of inhalation exposure.

Alterations in HR dynamics are possible with this treatment regimen because cholinergic mechanisms play a crucial role in the autonomic control of this variable both centrally (Brezenoff and Giuliano, 1982) and peripherally (Higgins *et al.*, 1973). The sinoatrial node, the heart pacemaker, is innervated by parasympathetic and sympathetic post-ganglionic neurons. Only the parasympathetic postganglionic neurons release ACh at their sinoatrial terminals, but both types of neurons are activated by cholinergic synapses from pre-ganglionic

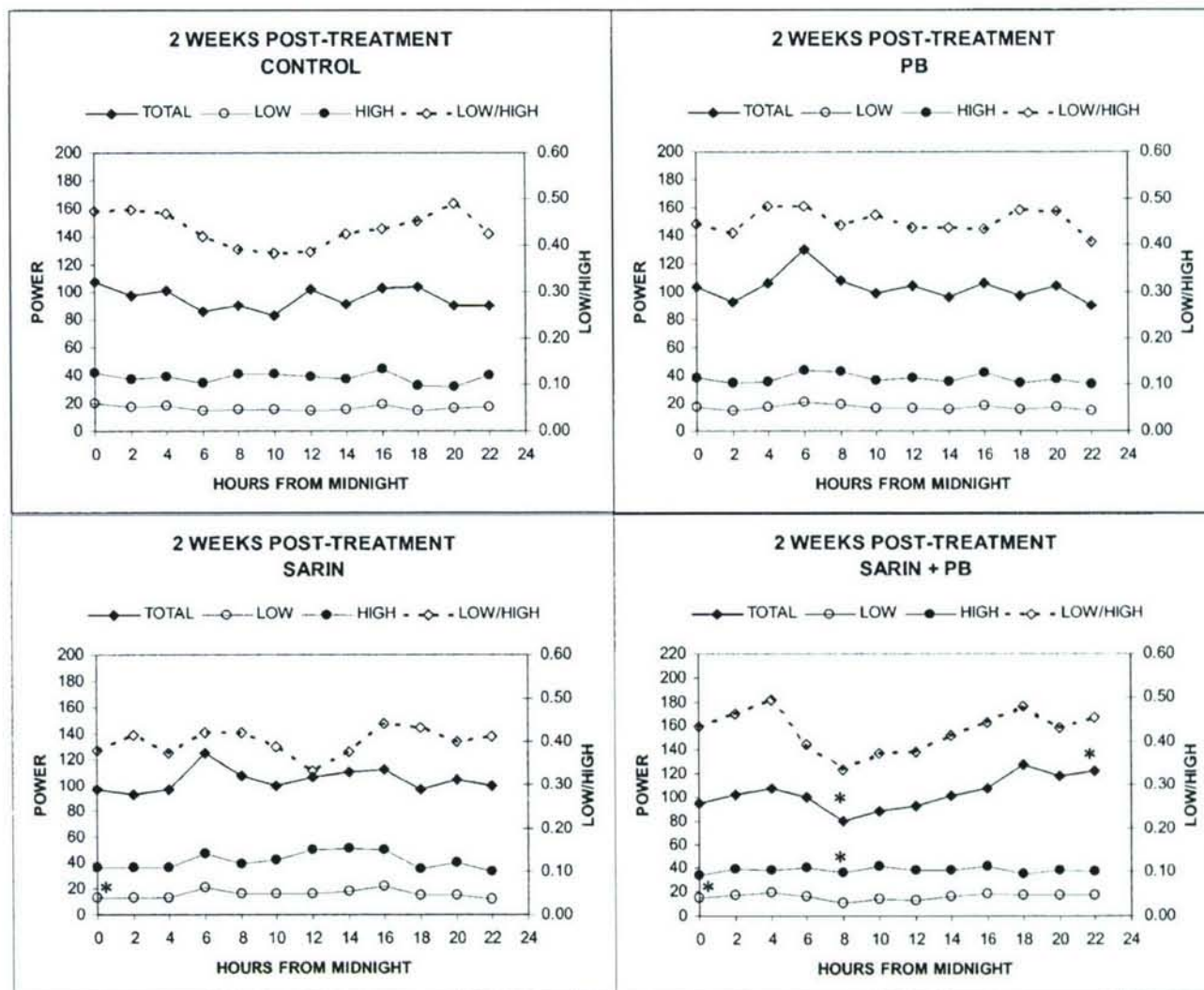


Figure 4. Means of HR cumulative power over the spectrum frequency bands TOTAL (between 0.05 and 5 Hz), LOW (between 0.26 and 0.75 Hz) and HIGH (between 0.76 and 5 Hz), and the ratio of power in low to high frequency bands (LOW/HIGH). HR was calculated from the electrocardiogram recorded every 30 min by radiotelemetry for 7 days. Means of the third day of measurements were averaged for each animal over 2 h intervals, and these values in turn were used to calculate group means. Data shown correspond to animals studied 2 weeks after discontinuation of treatment with PB (top right, number of animals (n) = 7), sarin (bottom left, n = 6) and sarin + PB (bottom right, n = 7). Statistical significance (P < 0.05) of differences from the control group (top left, n = 6) are indicated by an asterisk

neurons (Higgins *et al.*, 1973). Thus, facilitation of cholinergic transmission by AChE inhibitors has the potential to affect both parasympathetic and sympathetic influences, which exert opposite effects on HR. This may explain the diversity of effects of AChE inhibition reported in the literature. HR has been reported to decrease (Stein *et al.*, 1997; Cook *et al.*, 2002; Sant'anna *et al.*, 2003) increase (Yamamoto *et al.*, 1996; Soares *et al.*, 2004; Petroianu *et al.*, 1998), or remain constant (Joaquim *et al.*, 2004; Soares *et al.*, 2004) with acute AChE inhibition. As far as HR variability is concerned, AChE inhibition has been reported to either decrease (Cook *et al.*, 2002) or increase (Soares *et al.*, 2004) the high frequency component of HRPS, generally believed

to be a good index of parasympathetic cardiac drive (Pereira de Souza Neto *et al.*, 2001). The majority of reports point to enhanced HR variability with administration of AChE inhibitors. Given that reduced HR variability is an independent risk factor in heart disease (Routledge *et al.*, 2002), it has been suggested that cholinergic stimulation may become a therapeutic option for cardiac protection (Soares *et al.*, 2004).

In the present experiments PB, sarin, and their combination induced significant changes in the circadian variations of mean HR at 2 weeks post-treatment. In the case of PB, which showed the most pronounced effects, the bradycardia was very likely related to residual inhibition of AChE at peripheral sites, since this carbamate

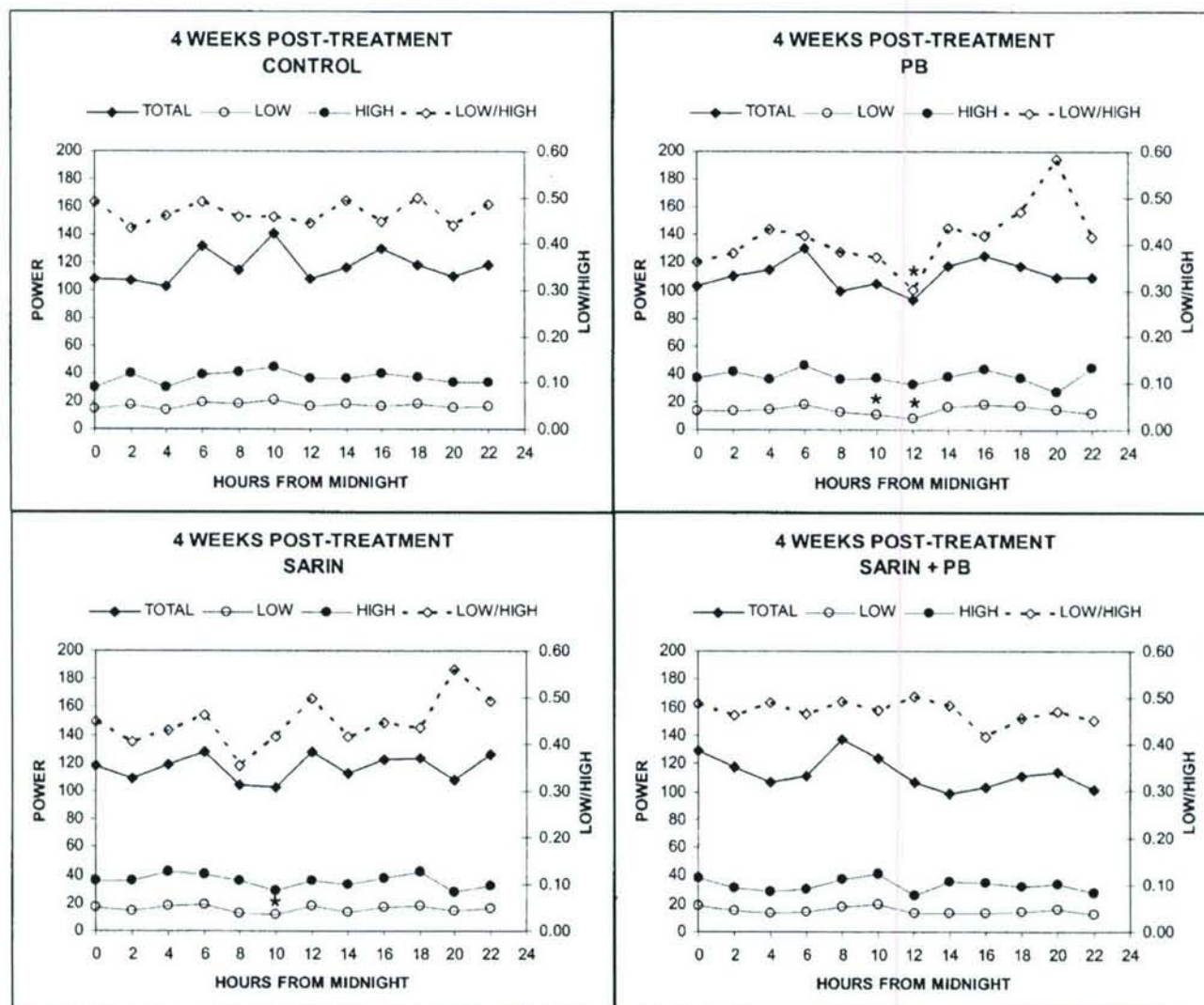


Figure 5. Means of HR cumulative power over the spectrum frequency bands: TOTAL (between 0.05 and 5 Hz), LOW (between 0.26 and 0.75 Hz) and HIGH (between 0.76 and 5 Hz), and the ratio of power in low to high frequency bands (LOW/HIGH). HR was calculated from the electrocardiogram recorded every 30 min by radiotelemetry for 7 days. Means of the third day of measurements were averaged for each animal over 2 h intervals, and these values in turn were used to calculate group means. Data shown correspond to animals studied 4 weeks after discontinuation of treatment with PB (top right, number of animals (n) = 8), sarin (bottom left, n = 8) and sarin + PB (bottom right, n = 6). Statistical significance ($P < 0.05$) of differences from the control group (top left, n = 8) are indicated by an asterisk

quaternary compound does not cross the blood brain barrier under the experimental conditions used in this report (Grauer *et al.*, 2000). Although the occurrence of sleep was not recorded in our experiments, the fact that AChE inhibitors only induced bradycardia during the light hours suggests that the phenomenon may be related to the higher incidence of sleep during that period in the rat. It has been reported that in rats, based on the analysis of HR variability, the decrease in HR associated with quiet sleep may be due to decreased sympathetic activity, while that associated with paradoxical (REM) sleep may be due to enhanced parasympathetic activity (Kuo and Yang, 2004). The observed daytime bradycardia may

then be explained by a heightened vagal effect on the heart pacemaker due to peripheral AChE inhibition, in combination with a decreased LA and sympathetic activity, all of which will tend to slow down HR. The absence of a bradycardiac effect of AChE inhibitors during the dark phase of the cycle may be explained by a decrease in vagal tone (Hicks *et al.*, 1998; Oosting *et al.*, 1997; Benessiano *et al.*, 1983) and an increase in LA, as the latter tends to accelerate the HR. All of these considerations are hypothetical, however, and the influence of non-cholinergic mechanisms in the causation of the observed bradycardia cannot be discarded.

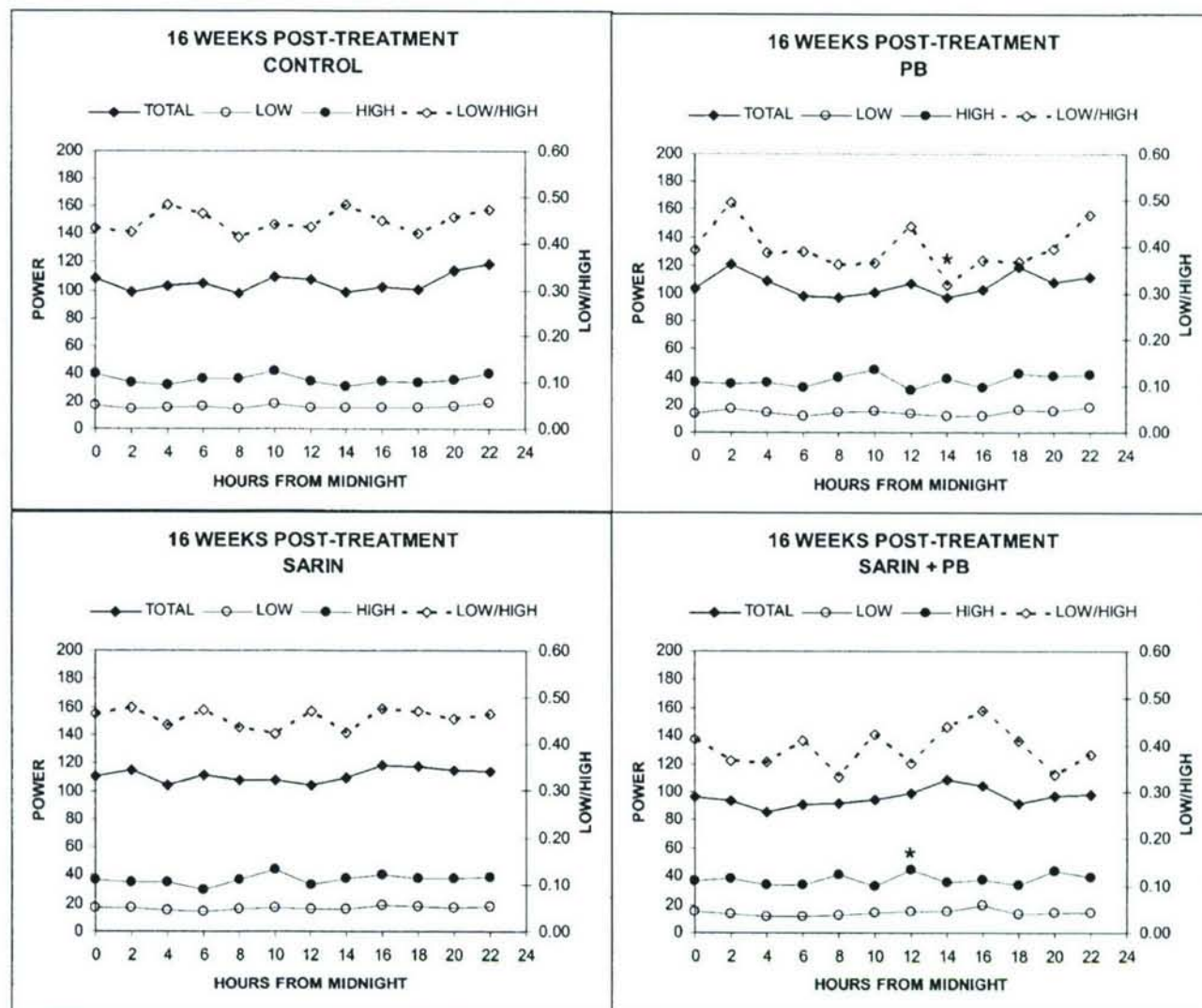


Figure 6. Means of HR cumulative power over the spectrum frequency bands: TOTAL (between 0.05 and 5 Hz), LOW (between 0.26 and 0.75 Hz) and HIGH (between 0.76 and 5 Hz), and the ratio of power in low to high frequency bands (LOW/HIGH). HR was calculated from the electrocardiogram recorded every 30 min by radiotelemetry for 7 days. Means of the third day of measurements were averaged for each animal over 2 h intervals, and these values in turn were used to calculate group means. Data shown correspond to animals studied 16 weeks after discontinuation of treatment with PB (top right, number of animals (n) = 8), sarin (bottom left, n = 6) and sarin + PB (bottom right, n = 7). Statistical significance ($P < 0.05$) of differences from the control group (top left, n = 7) are indicated by an asterisk

In the case of HRPS, few effects of drug treatments were detected and they did not follow a systematic pattern. In contrast with the case of HR, no consistent circadian variation in power was detected for any of the frequencies studied, with the single exception of the low to high ratio in control animals at 2 weeks after exposure, in which case a decrease of this ratio during the light hours could be demonstrated.

In conclusion, although marked changes in HR and HRPS have been detected 2 weeks post-exposure to the AChE inhibitors PB and sarin, no consistent changes were observed at longer periods after treatment. Thus,

these experiments do not support the hypothesis that repeated administration of low doses of PB and the nerve agent sarin can induce persistent or delayed alterations in autonomic function. However, since this study has been limited to adult male animals, further experimentation including females and younger animals may be warranted to rule out possible sex and age differential effects.

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