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Low-dose cholinesterase inhibitors do not induce delayed effects on cerebral blood flow and metabolism

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Abstract

The acetylcholinesterase (AChE) inhibitors sarin and pyridostigmine bromide (PB) have been proposed as causes of neurobehavioral dysfunction in Persian Gulf War veterans. To test possible delayed effects of these agents, we exposed rats to low (subsymptomatic) levels of sarin (0.5 LD_{50} s.c. 3 times weekly) and/or PB (80 mg/L in drinking water) for 3 weeks. Controls received saline s.c. and tap water. At 2, 4 and 16 weeks after exposure, regional cerebral blood flow (rCBF) and glucose utilization (rCGU) were measured in conscious animals with the Iodo-^{14}C-antipyrine and ^{14}C-2 deoxyglucose methods, respectively.

Two weeks after exposure, PB+sarin caused significant rCBF elevations, but no changes in rCGU, in neocortex, with lesser effects on allocortex. Four weeks after exposure, the same general pattern was found with sarin. Only a few changes were found at 16 weeks post-treatment. The predominant effects of sarin or PB+sarin on rCBF at earlier times after treatment are consistent with the well known direct cerebral vascular effect of cholinergic agonists. The lack of changes in rCBF and rCGU observed at 16 weeks after treatment does not support the hypothesis that repeat exposure to low-dose cholinesterase inhibitors can generate permanent alterations in cerebral activity.

Keywords: Nerve agents; Sarin; Pyridostigmine bromide; Cerebral glucose utilization; Cerebrovascular circulation; Low dose cholinesterase toxicity

1. Introduction

Although the immediate and long term consequences of acute intoxication with organophosphorus (OP) AChE inhibitors are well known (Ecobichon and Joy, 1982; Sidell, 1974; Chambers, 1992), potential harmful delayed effects of repeated exposure to low (non-symptomatic) levels of these agents have attracted less attention. These effects may be of relevance, if they exist, to military personnel possibly exposed to non-symptomatic levels of sarin during the Persian Gulf War (McCauley et al., 2001), and to agricultural workers and the general population exposed to OP insecticides of widespread use.

Administration of AChE at low levels generates a number of physiological changes. Central AChE inhibition

Abbreviations: AChE, Acetylcholinesterase; BuChE, Butyrylcholinesterase; ChAT, Cholineacetyltransferase; DG, Deoxyglucose; IAP, Iodo-antipyrine; OP, Organophosphorus; PB, Pyridostigmine bromide; RBC, Red blood cells; rCBF, Regional cerebral blood flow; rCGU, Regional cerebral glucose utilization; Am, Amygdala; Au, Auditory; Au1, Primary auditory; BF, Barrel field; Ect, Ectorhinal; Ent, Entorhinal; Fa, Face area; FL, Forelimb area; HL, Hindlimb area; I, Insular; M1, Primary motor; M2, Secondary motor; PA, Parietal association area; Pir, Piriform; RS, Retrosplenial; S1, Primary somatosensory; S2, Secondary somatosensory; Te, Temporal; Tr, Trunk area; V1, Primary visual; V2, Secondary visual.

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enhances arterial blood pressure (Varagic, 1955; Buccafusco, 1996), and decreases cerebrovascular resistance (Scremin and Shih, 1991; Scremin et al., 1993; Scremin et al., 1988), decreases body temperature and elevates nociceptive threshold (Russell et al., 1986). AChE inhibition with pyridostigmine, a carbamate AChE inhibitor that does not cross the blood brain barrier, can induce dose-dependent bradycardia (Stein et al., 1997) or increased arterial blood pressure following a single intravenous dose of 2 mg/kg (Chaney et al., 2002). There were no effects on arterial blood pressure or heart rate but enhanced heart rate variability and baroreflex sensitivity during continuous administration with an osmotic minipump of up to 10 mg/kg mass/day (Bernatova et al., 2003; Joaquim et al., 2004). However, The persistence of these effects beyond the period of drug administration has not been explored.

Previous work on delayed effects of low dose OP AChE inhibition has led to contradictory results. While some authors have reported some effects (Burchfield and Duffy, 1982; Ecobichon and Joy, 1982), other studies have found no increase over the general population in the incidence of mental, neurological, hepatic, and reproductive pathology or cancer of subjects exposed in the work environment (Panel on Anticholinesterase Chemicals, 1982) or after accidental exposures (Coordinating Subcommittee, 1985; Moore, 1998).

The present study was designed to determine whether exposure to sarin and/or PB, in doses and times that presumably applied to Persian Gulf War veterans, could elicit long lasting alterations in the patterns of cerebral cortex activity of conscious animals, as assessed with measurements of cerebral blood flow and metabolism, and thus test whether the agents could elicit subtle delayed effects of cerebral function. Quantitative autoradiography with ^{14}C-labeled Iodo-antipyrine (IAP) and 2-deoxyglucose (2-DG) was used to trace regional cerebral blood flow (rCBF) and regional cerebral glucose utilization (rCGU) of the cerebral cortex, respectively. Spatial resolution of these techniques is on the order of 10 lines/mm (Gallistel and Nichols, 1983), allowing identification of the small, specialized areas of the rat cerebral cortex. These variables have been extensively used to reveal patterns of activity in the central nervous system (McCulloch, 1982; Sokoloff, 1981; Reivich et al., 1969; Sakurada et al., 1978; Holschneider et al., 2003). The somatosensory, auditory, motor, visual and association areas of the neocortex, as well as the various components of the allocortex were sampled in the treated animals and compared with age-matched controls.

Comparison of rCBF and rCGU on a regional basis is particularly suited to the present study. One well documented acute effect of AChE inhibitors, like sarin, that penetrate the blood–brain barrier is an increase in rCBF predominantly in the neocortex without a concomitant increase in rCGU at low (non-symptomatic) doses (Scremin et al., 1988; Scremin and Shih, 1991). A generalized increase in both variables was observed at convulsant doses (Shih and Scremin, 1992). Thus, the rCBF/rCGU ratio provides a sensitive indicator of the extent and intensity of the central nervous system effects of AChE inhibitors.

This study also includes PB exposure, because this agent has been used as a pretreatment for nerve agent intoxication during the Persian Gulf War (Keeler et al., 1991), and there are also contradictory reports on the potential long-term undesirable effects of exposure to PB, with some authors reporting a delayed enhancement of the acoustic startle response in rats (Servatius et al., 1998) and others showing no significant effects on this variable (Scremin et al., 2003).

The experimental model used was developed previously for the evaluation of neurologic and cognitive delayed effects of sarin and PB (Scremin et al., 2003). It consists of PB administration in the drinking water at a concentration of 80 mg/L for 3 weeks, in combination with sarin ($0.5\times LD_{50}$, s.c.; three injections per week for 3 weeks) or an equivalent volume of saline s.c. at the same regime. These treatments induced no signs of intoxication, even though red blood cell AChE was inhibited between 60% and 70%.

2. Materials and methods

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 Labs (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 0600 hours. Laboratory chow and drinking water were freely available.

Experiments were conducted at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) and the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System. 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 the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). 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, proposed by the Committee to Revise the Care and Use of Laboratory Animals of 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. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs. (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 subcutaneously (s.c.). PB was purchased from Sigma-Aldrich (St. Louis, MO) and prepared twice weekly in tap water and provided as drinking water to experimental groups for a 3-week period.

2.1. Experimental groups

Separate sets of animals were studied at 2, 4, or 16 weeks after treatment. Within every set, animals were divided into four treatment groups. Group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline (Control group). Group 2 animals received PB in drinking water (80 mg/L), with an estimated dose, based on water consumption, of 10 mg/kg body mass/day (Scremin et al., 2003), approximately equivalent to the dose used in humans for prophylaxis of OP poisoning (1.2 mg/kg body mass/day), based on surface area dosage conversion between species (Freireich et al., 1966). These animals (PB group) were injected with saline s.c. Group 3 animals received tap water and were injected with sarin (62.5 μg/kg, s.c., equivalent to $0.5\times LD_{50}$) (sarin group). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above (PB+sarin group). PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 0800 hours. At 0900 hours that Monday morning, injection of either saline (0.5 mL/kg, s.c.) or sarin (62.5 μg/kg, s.c.) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week. PB in drinking water was terminated and switched to regular tap water at 1700 hours on Friday of the third week. Animal dosing procedures were performed at the USAMRICD laboratory. After a period of 1, 3, or 15 weeks following treatment, depending on the experimental sets, animals were transported by air-conditioned vans and air-freight to the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System, where they were allowed to recover for a minimum of one additional week before starting assessment of the outcome variables at 2, 4, or 16 weeks after control, PB, sarin, or PB+sarin treatments. Telemetry measurements of locomotor activity and heart rate performed in animals after they arrived at the VA Greater Los Angeles Healthcare System (data not shown), have indicated normal circadian rhythms in animals transported under the same conditions and studied at the intervals used in the present report. Moreover, in this experimental design all animals (treated and controls in each experimental set) were transported in the same way at the same time to control for any potential differences due to transportation stress.

The 12 groups of animals described above (4 treatments×3 times after treatment) were duplicated to measure rCBF and rCGU, since only one of these methodologies was used on a single animal.

2.2. Observation of signs of intoxication

Animals were observed for signs of cholinergic intoxication for at least 1 h following sarin injection. The signs, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination) were scored according to the rating schedule described elsewhere (Shih and Romano, 1988).

2.3. Blood AChE measurements

When animals were received at the USAMRICD laboratory, they were allowed to acclimate for a week. During this period blood was collected from the tail vein (Liu et al., 1999) on 2 separate days to establish baseline whole blood and red blood cell (RBC) AChE activity. After the experiment was started on the following Monday, subsequent blood collections were done on each Friday, at about 60 min after sarin or saline injections, during the 3-week exposure period and continued for 3 more weeks during the recovery period. Blood (0.25 mL) was collected into an Eppendorf 1.5 mL microtube containing 50 μL (1000 USP unit per mL) heparin sodium and mixed. Forty microliters of whole blood was transferred to another microtube containing 160 μL 1% Triton-X 100 (in saline) solution, mixed well and immediately flash frozen. The remaining blood was then centrifuged for 5 min at 14,000 RPM (20,000 RCF). Plasma was carefully aspirated off, and 20 μL RBC's was transferred into a microtube containing 180 μL 1% Triton-X 100 solution. The tube was tapped firmly until RBC's were lysed and dispersed. The tube was immediately flash frozen. Both the whole blood and RBC samples were stored at −75 °C until ChE analysis. At the time of analysis, samples were processed immediately after thawing to avoid spontaneous re-activation or additional inhibition of ChE activity. Whole blood and RBC AChE activity were determined by an automated method using a COBAS/FARA clinical chemistry analyzer (Roche Diagnostics, Nutley, NJ). The analytical procedure was based on the manual method of Ellman (Ellman et al., 1961) and modified for the COBAS/FARA system using acetylthiocholine as substrate.

2.4. Measurement of cerebral glucose utilization

Regional cerebral glucose utilization (rCGU) was measured with the ^{14}C 2-DG autoradiographic technique (Sokoloff et al., 1977). One arterial and one venous catheter were implanted in the femoral vessels under halothane anesthesia. After surgery, animals were placed in a Bollman cage and allowed to recover from anesthesia for 1 h. In these cages the animals rest in prone position with their limbs hanging to the sides. Acrylic non-traumatic bars entrap the animal preventing locomotion but allowing limb and head movements. The cage was covered with a cloth in order to

prevent cooling of the animal and to eliminate visual contact with the environment. Rectal temperature was recorded with a BAT-12 thermocouple thermometer and maintained constant by means of a TCAT-1A (Physitemp, Clifton, NJ) temperature controller and a source of radiant heat. A sample of arterial blood was obtained for measurement of blood gases and pH in an ABL-5 blood acid-base system (Radiometer, Copenhagen, Denmark) and then ^{14}C 2-DG (Amersham, Arlington Heights, IL) dissolved in 0.5 mL of saline at a concentration of 100 μCi/kg body mass was administered intravenously at a rate of 1 mL/min for 30 s. Eleven arterial blood samples (70 μL) were then obtained over a period of 45 min for measurement of glucose concentration (glucose oxidase method) and radioactivity (liquid scintillation counting) to allow calculation of rCGU. After euthanasia (pentobarbital, 50 mg/kg with 3 M KCl i.v. bolus), performed immediately after obtaining the last blood sample (45 min after ^{14}C 2-DG infusion) the brain was removed, flash frozen in methylbutane chilled to −70 °C and embedded in OCT compound (Miles, Elkhart, Indiana) for later sectioning in a cryostat at −20 °C in 20 μm slices. These sections were heat-dried and exposed to Kodak Ektascan film in spring-loaded X-ray cassettes along with eight standards of known radioactivity to obtain a ^{14}C-2-DG autoradiograph. Tissue radioactivity was derived by densitometry of tissue and standards autoradiographs and rCGU values were obtained using the operational equation and values for the lumped and rate constants previously described (Sokoloff et al., 1977).

2.5. Measurement of cerebral blood flow

Regional cerebral blood flow (rCBF) was measured with the ^{14}C-IAP quantitative autoradiographic method (Sakurada et al., 1978). Two arterial and two venous catheters were implanted in the femoral vessels under halothane anesthesia. After surgery, animals were placed in a Bollman cage and allowed to recover from anesthesia for 1 h. Rectal temperature was recorded with a BAT-12 thermocouple thermometer connected to a TCAT-1A (Physitemp) temperature controller and a source of radiant heat. One arterial catheter was connected to a pressure transducer interfaced to a polygraph for continuous recording of arterial blood pressure; the other one was used for sampling of arterial blood. One of the venous catheters was connected to a motor driven syringe containing the radioactive tracer solution and the other one to a similar syringe containing the euthanasia solution (pentobarbital, 50 mg/kg with 3 M KCl i.v. bolus). A sample of arterial blood was obtained for measurement of blood gases and pH in a Radiometer ABL-5 blood acid-base system and then the infusion of ^{14}C-IAP (Amersham, Arlington Heights, IL) was started. Infusate volume was 0.6 mL, dose was 100 μCi/kg and infusion period was 30 s. Arterial blood samples (30 μL) were obtained every 3 s from a free flowing catheter. Circulation was arrested by the euthanasia solution delivered intravenously over the last 4 s of the ^{14}C-IAP infusion. The exact timing of circulatory arrest was determined from the polygraph record of arterial blood pressure. The brain was then rapidly removed and processed for autoradiography as described above for rCGU measurements. rCBF was calculated from film optical density of brain autoradiographs and standards, and arterial blood radioactivity as described previously (Sakurada et al., 1978).

Although pentobarbital was used in these experiments, the drug cannot affect rCBF or rCGU because it is administered after the uptake of the blood flow or metabolism tracers has taken place and after the last sample of blood has been collected (during the last 4 s of the rCBF experiments or after the last blood sample of the rCGU experiment).

2.6. Cerebral cortical regions sampled

The following regions, identified according to the Atlas of Paxinos and Watson (Paxinos and Watson, 1998) were sampled for measurements of rCBF and rCGU in 20 locations in each of 15 coronal planes spaced 0.4 mm from each other. The numbers of locations per region sampled in every animal are indicated in the following list after the abbreviation: Neocortex: auditory cortex (Au, 4), primary auditory cortex (Au1, 8), barrel cortical field (BF, 16), face cortical area (Fa, 26), forelimb cortical area (FL, 10), hindlimb cortical area (HL, 6), insular cortex (I, 22), primary motor cortex (M1, 22), secondary motor cortex (M2, 18), parietal association area (PA, 4), primary somatosensory cortex (S1, 2), secondary somatosensory cortex (S2, 8), temporal cortex (Te, 12), trunk cortical area (Tr, 4), primary visual cortex (V1, 20), and secondary visual cortex (V2, 20); allocortex and transitional areas: ectorhinal cortex (Ect, 6), entorhinal cortex (Ent, 24), piriform cortex (Pir, 48), retrosplenial cortex (RS, 12); amygdala (Am, 8).

2.7. Data analysis

Means of AChE activity for every treatment group were calculated. Statistical significance of differences between every treatment group and the respective controls at each time after treatment was assessed by analysis of variance (ANOVA) followed by multiple comparisons with the Bonferroni technique (Snedecor and Cochran, 1980). A probability of <0.05 (<0.016 after Bonferroni adjustment) was used to declare differences as significant. Mean values of rCBF and rCGU were calculated for every location sampled in all experimental groups and times after treatment. Statistical significance of location means of drug treatment groups against those of their respective controls at each time after treatment was assessed by multiple comparisons with the Bonferroni technique as described above.

The linear regressions of mean rCBF on mean rCGU for every region studied were calculated for every experimental

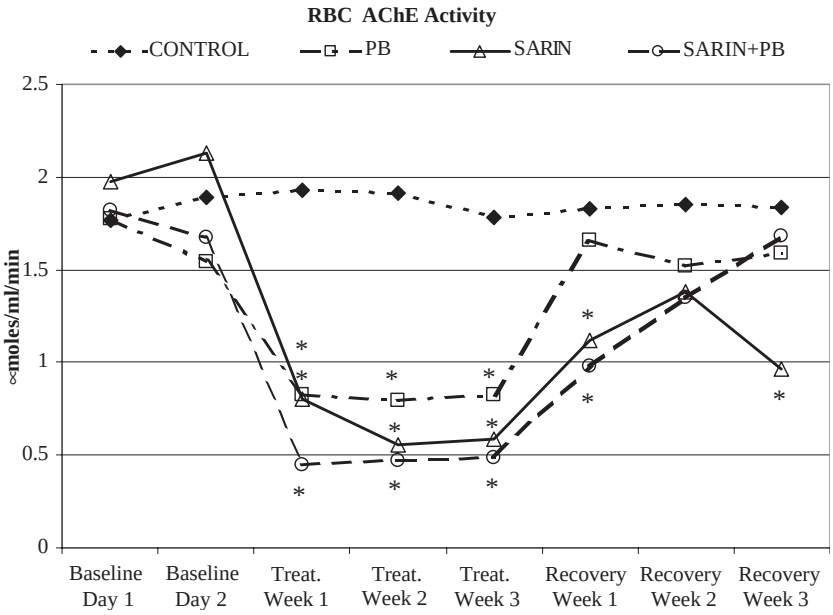

Fig. 1. RBC AChE activity was measured before (baseline), during treatment (treatment weeks 1–3) and the immediate recovery period (recovery weeks 1–3). Data (Means and SE) are in μmol/mL/min. *=significant vs. controls ($P<0.05$, Bonferroni adjusted for three contrasts).

group. Statistical significance of differences between slopes of the three drug treated groups against their respective controls for every time after treatment was assessed with the F ratio of the residual mean squares obtained when separate regressions were fitted for each condition to that obtained from a model in which a single pooled slope was fitted (Snedecor and Cochran, 1980). The significance level was set at 0.01 to compensate for the multiple comparisons performed.

2.8. Number of animals

Number of animals in rCBF groups, 2 weeks after treatment: Control=12; PB=10; Sarin=12; PB+Sarin=10; 4 weeks after treatment: Control=11; PB=8; Sarin=11; PB+Sarin=10; 16 weeks after treatment: Control=11; PB=7; Sarin=8; PB+Sarin=11. Number of animals in rCGU groups, 2 weeks after treatment: Control=9; PB=6; Sarin=5; PB+Sarin=8; 4 weeks after treatment: Control=7; PB=7; Sarin=8; PB+Sarin=8; 16 weeks after treatment: Control=5; PB=6; Sarin=7; PB+Sarin=5.

3. Results

3.1. Blood cholinesterase activity

Measurements of RBC AChE during drug treatment and the immediate recovery period are shown in Fig. 1. PB induced a pronounced decrease in enzymatic activity to

Table 1
Physiological variables in all rCBF and rCGU experiments

Treat.	Weeks	Blood pH -log [H^+]	Pa CO_2 (mmHg)	PaO_2 (mmHg)	Body mass (g)	Body temp (°C)	MABP (mm Hg)
Control	2	7.453±0.003	40.80±0.57	86.62±1.15	448.1±8.6	37.7±0.1	118.0±4.1
PB	2	7.456±0.008	41.15±0.86	85.08±1.36	455.6±9.5	38.0±0.1	110.2±3.4
Sarin	2	7.446±0.006	40.11±0.60	84.33±1.68	464.9±8.6	37.8±0.1	122.5±2.8
Sarin+PB	2	7.461±0.007	40.43±0.80	87.36±1.14	454.8±8.0	37.9±0.1	120.4±3.2
Control	4	7.452±0.004	40.28±0.80	85.89±1.27	482.5±10.6	37.7±0.1	119.5±2.4
PB	4	7.448±0.006	41.03±0.68	86.36±0.79	510.3±10.8	37.9±0.2	121.4±6.4
Sarin	4	7.446±0.004	39.00±0.57	84.63±0.94	483.9±10.0	37.8±0.2	117.5±3.8
Sarin+PB	4	7.451±0.004	40.24±0.49	89.19±1.41	491.4±11.1	37.8±0.1	126.2±3.3
Control	16	7.441±0.005	40.50±0.57	86.75±1.20	609.8±10.4	37.6±0.1	110.6±4.0
PB	16	7.437±0.005	40.61±0.52	84.36±1.25	634.6±23.1	37.9±0.2	120.4±3.5
Sarin	16	7.446±0.004	41.18±0.71	87.97±2.84	608.6±13.9	37.5±0.1	116.1±4.0
Sarin+PB	16	7.442±0.008	41.85±0.76	86.82±1.09	601.8±11.8	37.5±0.1	108.7±4.9

Body mass was measured before animals were anesthetized for the rCBF or rCGU procedures. Mean blood pH and gases ($PaCO_2$; PaO_2), body temperature, and arterial blood pressure (MABP) were measured immediately before injection of the radioactive tracer. Statistical comparisons between treatment groups within a given time after treatment (weeks) indicated no significant differences.

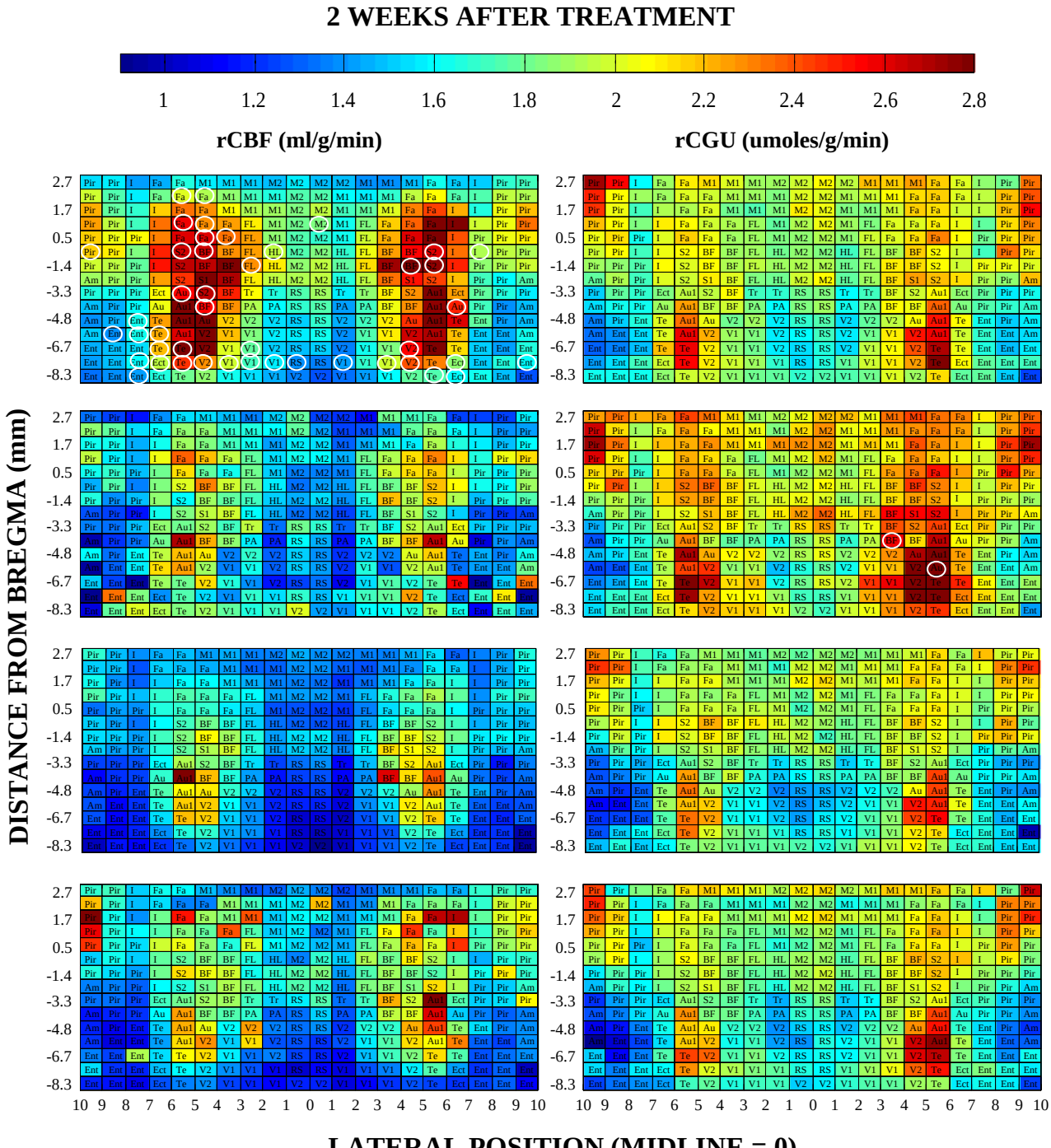

Fig. 2. Cerebral cortical rCBF (left panels) and rCGU (right panels) of animals studied 2 weeks after discontinuation of treatment are displayed in three-dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control=12; PB=10; Sarin=12; PB+Sarin=10. Number of animals in rCGU groups: Control=9; PB=6; Sarin=5; PB+Sarin=8. Regions are named according to Paxinos and Watson (Paxinos and Watson, 1998). Abbreviations: Am, amygdala; Au, auditory; Au1, primary auditory; BF, barrel field; Ect, ectorhinal; Ent, entorhinal; Fa, face area; FL, forelimb area; HL, hindlimb area; I, insular; M1, primary motor; M2, secondary motor; PA, parietal association area; Pir, piriform; RS, retrosplenial; S1, primary somatosensory; S2, secondary somatosensory; Te, temporal; Tr, trunk area; V1, primary visual; V2, secondary visual.

about 51% of baseline, which remained stable during the weeks of treatment and recovered after treatment ceased. Sarin produced a decrease in RBC AChE activity to about 33% of baseline, which remained stable during the treatment period and recovered following an irregular pattern with significantly lower values than controls during the third recovery week. The combination of PB and sarin also induced a significant depression of RBC AChE activity (27% of baseline), which persisted until the second week after treatment (Fig. 1).

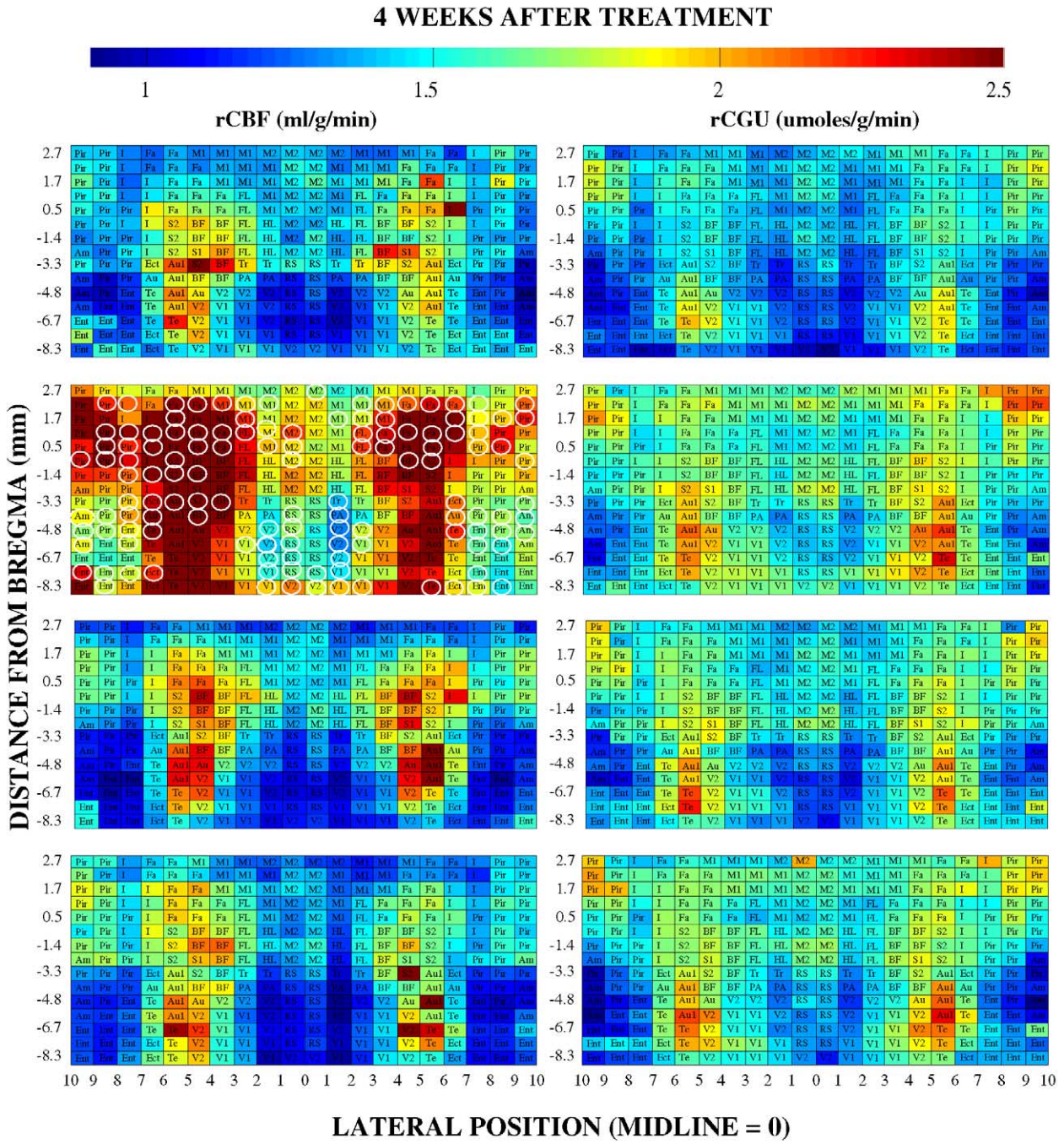

Fig. 3. Cerebral cortical rCBF and rCGU of animals studied 4 weeks after discontinuation of treatment are displayed in three-dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control=11; PB=8; Sarin=11; PB+Sarin=10. Number of animals in rCGU groups: Control=7; PB=7; Sarin=8; PB+Sarin=8. Regions are named according to Paxino and Watson (Paxinos and Watson, 1998). See abbreviations in Fig. 1 legend.

3.2. Arterial blood gases and pH, body temperature and mean arterial blood pressure

These variables, measured at the time of rCBF or rCGU measurements, did not show any significant differences with regard to controls for any of the experimental groups (Table 1). The expected increase in body mass with age was found between the three different times when rCBF and rCGU were measured, but no differences among groups were detected within a given time after treatment.

3.3. Cerebral blood flow and glucose utilization

Figs. 2–4 show, in three dimensional maps, the means of rCBF (left panels) and rCGU (right panels) of every location

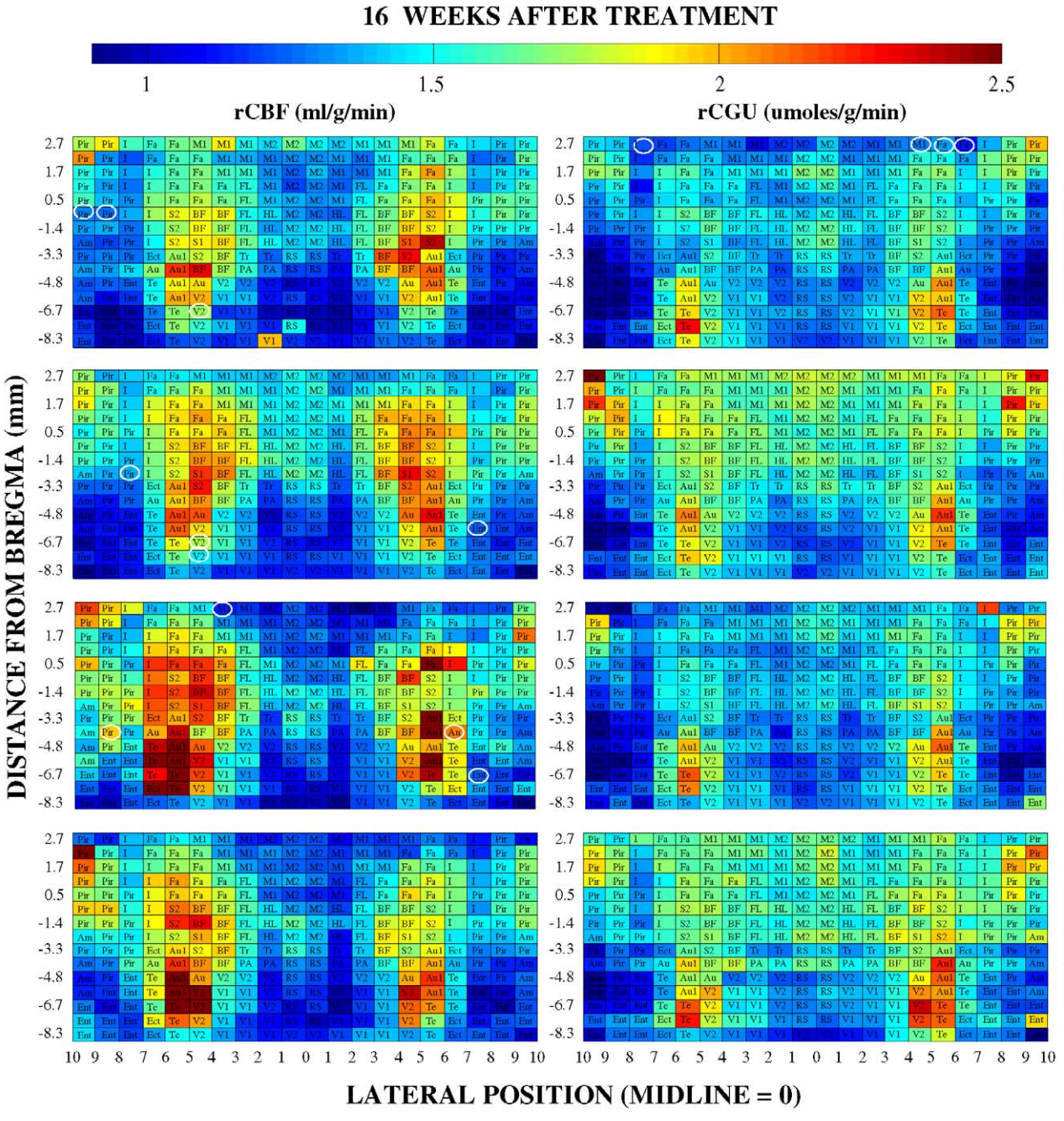

Fig. 4. Cerebral cortical rCBF of animals studied 16 weeks after discontinuation of treatment are displayed in three-dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control=11; PB=7; Sarin=8; PB+Sarin=11. Number of animals in rCGU groups: Control=5; PB=6; Sarin=7; PB+Sarin=5. Regions are named according to Paxinos and Watson (Paxinos and Watson, 1998). See abbreviations in Fig. 1 legend.

sampled. The ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions (mm) relative to the midline. Mean rCBF of every region is represented on a color scale. Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts).

Analysis of rCBF and rCGU in the control condition (no drug administration) indicated marked regional variations among locations within the cerebral cortex. In the case of rCBF cortical maps (Figs. 2, 3 and 4, bottom left panels) two rostral and one caudal clusters of locations with high rCBF were identified. The rostral paramedian high rCBF cluster included the face area (Fa), primary motor (M1), barrel field (BF) and secondary sensory (S2) areas, and the rostral lateral cluster was limited to the piriform region (Pir). The caudal high rCBF cluster included the primary auditory (Au1), temporal (Te), and secondary visual (V2) regions.

Cortical maps of rCGU resembled closely their rCBF counterparts in the caudal locations, but the rostral locations lacked a distinct high rCGU paramedian cluster resembling that described above for rCBF, while preserving a high rCGU cluster in the piriform region.

At 2 weeks after treatment (Fig. 2), significant changes in rCBF were only observed in animals treated with the combination of sarin+PB. The regions affected were located mostly on the neocortex (Fa, M2, S2, BF, FL, HL, Te, Au, Au1, V1, V2), with a few on Ent and Ect and only one on Pir. At 4 weeks after treatment (Fig. 3), the same general pattern was found in animals treated with sarin, with more significant locations in Pir, RS, and Am. Only few changes were found at 16 weeks post-treatment in the three experimental groups (Fig. 4).

In the case of rCGU cortical maps, very few and inconsistent statistically significant changes between experimental groups were found at each time after treatment (Figs. 2, 3 and 4, right panels).

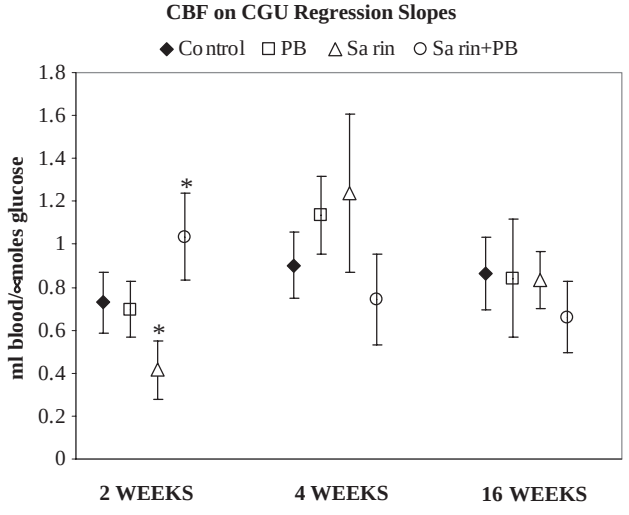

Fig. 5. The linear regressions of mean rCBF on mean rCGU for every region studied were calculated for every experimental group. The regression coefficients (slopes) and their 99% confidence intervals are shown. Statistical significance of differences between slopes of the three drug-treated groups against their respective controls for every time after treatment was assessed with the F ratio of the residual mean squares obtained when separate regressions were fitted for each condition to that obtained from a model in which a single pooled slope was fitted. The probability level at which differences were declared significant was set at 0.01 to compensate for the multiple comparisons performed. Asterisks indicate the groups in which the slopes differed significantly from controls.

3.4. Regression of rCBF on rCGU

Regression of rCBF on rCGU indicated slopes that were highly significantly different from zero with values ranging between 0.73–0.90 mL blood/μmol glucose in the control groups (Fig. 5). Comparisons of slopes of these regressions between drug treatment groups and controls indicated significant differences 2 weeks after treatment with an enhanced slope in animals treated with the combination of PB+sarin (1.04 mL blood/μmol glucose) and a decreased slope in the sarin group (0.41 mL blood/ μmol glucose). No statistically significant differences between slopes of drug treatment and control groups were found at 4 and 16 weeks after treatment.

4. Discussion

The experimental results yielded values of blood AChE inhibition consistent with this rat model, previously used to assess behavioral and neurological effects of sarin and PB (Scremin et al., 2003). Although brain AChE was not measured, previous data indicates that at the levels of blood AChE observed, significant brain AChE activity inhibition could be safely assumed (Shih, 1983; Shih et al., 1990; Roberson et al., 2001). The reason we measured AChE activity during treatment and shortly after is because the administration of the drugs did not continue after the initial 3 weeks. The aim of the study was to look for delayed effects, i.e. beyond the period of AChE inhibition. 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 Persian Gulf War veterans. This model was, however, the "worse case" model for Persian Gulf War exposure scenario where veterans did not report any symptom of miosis, an initial sign of aerosol exposure.

Although it is generally assumed that rCBF and rCGU are valid correlates of brain function, it is important to measure both variables because there is ample evidence to indicate that rCBF, under the influence of vasoactive neurotransmitters, can be regulated independently from the levels of cerebral energy exchange (Scremin, 2003; Gulbenkian et al., 2001). Under both physiological and pathological conditions, many instances have been documented of a lack of correlation between rCBF and rCGU or

oxygen consumption (Gsell et al., 2000; Fox and Raichle, 1986). This is indeed the case in the current experiments. Large increases in rCBF were detected at 2 weeks in animals that received a combination of sarin and PB, and at 4 weeks in animals that received only sarin. In contrast, very few changes in rCGU were observed with these treatments and times after exposure.

The dissociation of rCBF and rCGU observed in the present experiments is similar to that observed immediately after administration of carbamate or OP cholinesterase inhibitors, known to enhance cerebral blood flow without a concomitant increase in rCGU or oxygen consumption, a phenomenon attributed to an excess of ACh at central sites with stimulation of muscarinic receptors (Scremin et al., 1982, 1988; Scremin, 1991; Blin et al., 1997). There are several possible causes for this phenomenon: 1) cholinergic stimulation primarily dilates cerebral blood vessels by a direct action on vascular smooth muscle without affecting neuronal function or metabolism, 2) cholinergic stimulation affects neuronal function with a very low (undetectable) metabolic cost, and the increase in rCBF is mediated by a neuronal non-metabolic mechanism, and 3) cholinergic stimulation affects neuronal function and enhances metabolism, but substrates other than glucose are used as fuels. The fact that neither glucose utilization nor oxygen consumption are enhanced by cholinergic agonists that induce large increases in rCBF argues against the last possibility (Scremin, 1991, 1993). It is well known that at appropriate doses, cholinergic agonists do affect the brain electrical activity and function (Lucas-Meunier et al., 2003). It is then possible that option 2 is more likely to be true. However, it is also possible that at low dose levels, a direct cerebrovascular effect of cholinergic agonists may be present without effects on nerve cells function. From the point of view of the objectives of this investigation, the important fact to consider is that the changes in nerve function, if any, were of a relatively transient nature since rCBF and rCGU changes were minimal at 16 weeks post-treatment. It is tempting to speculate that the early effects might have been due to residual inhibition of AChE in neurovascular compartments, that would dissipate at later times to explain the lack of effects at 16 weeks. However, this is merely speculative since we don't have any direct evidence of AChE inhibition in such compartments. It is by no means sure that the effects of these cholinesterase inhibitors observed after treatment, when AChE inhibition may be low or absent, are related to an immediate effect on the enzyme. We and other authors have observed delayed effects of low dose administration of AChE inhibitors beyond the period of enzyme inhibition. Moreover, similar blood AChE inhibition does not necessarily mean similar brain tissue inhibition. Sarin can cross the blood brain barrier but pyridostigmine does not, so the effects on blood AChE do not necessarily parallel changes in brain AChE.

The question remains as to why the effect of sarin when administered by itself was present at 4 weeks after treatment and not at 2 weeks. One possible explanation may be that in spite of residual AChE inhibition at 2 weeks, muscarinic receptor downregulation may have prevented the vascular effect from being expressed at this time. In support of this interpretation, we have previously detected significant downregulation of QNB binding 2 weeks after treatment with sarin but not with sarin+PB (Scremin et al., 2003). The difference in the effects of these two treatments may be related to the kinetics of central AChE inhibition, since occupation of peripheral AChE sites by PB may have displaced sarin towards central sites and enhanced ACh levels with regards to sarin alone, leading to the proposed muscarinic receptor downregulation at short times after treatment. These considerations are purely speculative, however, and elucidation of the mechanism of these late changes in rCBF with sarin alone or in combination with PB will require further experimentation.

Analysis of the regressions of rCBF on rCGU was carried out because the dependence of rCBF on rCGU levels is a well-known phenomenon that reflects the adjustments of blood flow, and hence of nutrients and oxygen supply, to the local levels of energy utilization. This is, however, not a constant, with variations known to occur following pharmacological interventions. Inhibition of AChE within the central nervous system is associated with enhancement of the slope of the rCBF/rCGU relationship (Scremin et al., 1993), while cholinergic muscarinic blockade with scopolamine has the opposite effect (Scremin and Jenden, 1996). The ratio of rCBF to rCGU may have significance in controlling the composition of the internal milieu of the brain and, thus, the excitability of nerve centers (Scremin, 2003). In the present experiments, animals that received sarin+PB manifested a significant enhancement in the rCBF/ rCGU slope 2 weeks after treatment, a phenomenon consistent with the hypothesis of residual AChE inhibition at this time. At the same interval after treatment, animals that had received sarin alone showed a significant decrease in the rCBF/rCGU slope, also in line with a downregulation of muscarinic receptors previously observed with sarin, but not sarin+PB at the same time after treatment in this experimental model (Scremin et al., 2003). The differential effect of the two treatments could thus be explained by the predominance of receptor downregulation that may have prevented the effect of excess ACh due to residual AChE inhibition, as discussed above for the differential effect on rCBF of both treatments.

In conclusion, the changes in rCBF and rCGU observed in the present experiments are consistent with a combination of residual AChE inhibition and downregulation of muscarinic receptors. The changes were not present at 16 weeks after treatment, a fact that does not support the hypothesis that low-level sarin or PB could elicit permanent changes in the central nervous system.

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