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Multiple vaccine and pyridostigmine interactions: Effects on EEG and sleep in the common marmoset☆

K.E. Williams [a,*], T.M. Mann [a], S. Chamberlain [a], A. Smith [a], S. Wilson [b], G.D. Griffiths [a], A.P. Bowditch [a], E.A.M. Scott [a], P.C. Pearce [a]

[a] *Dstl Biomedical Sciences Department, Porton Down, Salisbury, SP4 0JQ, UK*
[b] *Psychopharmacology Unit, Dorothy Hodgkin Building, University of Bristol, Bristol, BS1 3NY, UK*

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Abstract

Following active service during the 1990/1991 Gulf conflict, a number of UK and US veterans presented with a diverse range of symptoms, collectively known as Gulf Veterans' Illnesses (GVI). The administration of vaccines and/or the pretreatment against possible nerve agent poisoning, pyridostigmine bromide (PB), given to Armed Forces personnel during the Gulf conflict has been implicated as a possible factor in the aetiology of these illnesses. The possibility that long-term health effects may result from the administration of these vaccines (anthrax, pertussis, plague, yellow fever, polio, typhoid, tetanus, hepatitis B, meningococcal meningitis and cholera) and/or PB, have been investigated using a non-human primate model, the common marmoset.

This paper reports the results from two aspects of the study, brain electrical activity (EEG, collected during performance of a touchscreen mediated discrimination task) and sleep.

There were no marked long-term changes in EEG or sleep patterns that could be attributed to vaccines and/or PB administration. The changes that were detected were predominantly time related and independent of treatment. Where statistical differences were detected between treatments, the magnitudes of the difference were relatively minor and therefore not regarded as having long term biological significance.

Keywords: Vaccines; Pyridostigmine bromide; Non-human-primates; EEG; Sleep

1. Introduction

Following active service in the Persian Gulf during the first Gulf conflict of 1990/1, a number of UK and US veterans presented with a diverse range of symptoms which have become collectively known as Gulf Veterans Illnesses (GVI). A document published by the Parliamentary Office of Science and Technology provides an overview of research relating to these conditions (Border and Norton, 1997), the majority of which have been epidemiological in nature. As well being exposed to a complex environment in which there were many potentially hazardous elements, UK personnel deployed in the Middle East were vaccinated with two anti-biological warfare agent vaccines (selected on the basis of contemporary military intelligence), an additional vaccine adjunct and a range of health and hygiene vaccines appropriate for deployment to that region. In addition, pyridostigmine bromide (PB) was taken as a pretreatment to help preserve life in the event of nerve agent poisoning.

The study reported here was part of a multi-stage programme of work commissioned by the Veterans Policy Unit Gulf Veterans' Illnesses (VPU GVI) of the Ministry of Defence with oversight from an independent, cross-disciplinary panel of experts who have advised on experimental design, conduct and interpretation. The work programme was designed specifically to assist in the interpretation of data emerging from the epidemiological studies and addressed the effects of multiple vaccines and PB. This included a mouse study undertaken at the National Institute for Biological

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* Corresponding author. Tel.: +44 1980613743; fax: +44 19980613741.
E-mail address: KEWILLIAMS@dstl.gov.uk (K.E. Williams).

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Standards and Control (Rijpkema et al., 2005) and a study of Dstl staff members who were vaccinated against anthrax in the course of their employment. The findings reported here are drawn from a study designed to assess whether administration of the range of vaccines administered to UK military personnel during the 1990/91 Gulf conflict, with and without PB, gives rise to long term adverse effects in a non-human primate model. Results from preparatory phases of this study in guinea pigs and marmosets, which identified dose levels and dose combinations of vaccines and PB that induced measurable responses without producing unacceptable short term effects, have been reported previously (Griffiths et al., 2001a,b, unpublished observations).

It was not the intention of the study to establish a model of GVI per se. The marmoset model was specifically developed to include measurement of indices that directly reflected the signs and symptoms most frequently reported by Gulf veterans; e.g. impaired cognition, sleep disturbances and fatigue (Coker et al., 1999; Unwin et al., 1999; Lee et al., 2001, 2002; Cherry et al., 2001). Thus, any changes in physiology, immune response and central function induced by vaccines/PB over 18 months following administration could be detected and interpreted. This paper reports the results of EEG and sleep analysis; other indices studied were general health, cognitive behaviour, muscle strength, pathology and immunology, which will be reported in separate publications.

A number of epidemiological studies have shown that sleep disturbance is one of the most prevalent symptoms reported by Gulf veterans both in the UK (Coker et al., 1999; Lee et al., 2001, 2002; Cherry et al., 2001) and in the US (Kang et al., 2000). Whilst sleep disturbances have been reported by veterans deployed to other conflicts (Hyams et al., 1996; Jones et al., 2002), their reported prevalence is higher in Gulf veterans (Proctor et al., 1998; Ismail et al., 2002).

There are relatively few studies which have investigated the effects of multiple or single vaccines on EEG or sleep. Those studies that have investigated EEG following vaccine administration did not report prolonged changes following Hempt (Timm and Wolter, 1971) or diphtheria–tetanus–pertussis (Topsis et al., 1996) vaccines. Studies that have investigated sleep disruption following immunisation have found that sleep disruption generally occurs as a result of physiological discomfort rather than because of an adverse immunological reaction (Sitzmann, 1987). Interactions between the immune system and sleep have, however, been more widely documented. In animal studies it has been demonstrated that cytokines, in addition to amplifying immune responses, can also influence sleep patterns (Krueger and Fang, 2000). Interleukin-1 (IL-1), in particular, has been widely studied and has been shown to promote slow-wave (deep) sleep (Krueger, 1990) and have an involvement in the regulation of non-rapid eye movement sleep (NREM) (Krueger and Fang, 1997). This particular cytokine is an endogenous pyrogen that stimulates acute-phase protein synthesis and promotes proliferation of Th2 $CD4^+$ cells (Benjamini et al., 1996). It has been hypothesised that the immune system of individuals exhibiting symptoms of GVI is biased toward a Th2-cytokine pattern as a result of exposure to multiple vaccinations that include pertussis (which is potently Th2 promoting) as an adjuvant (Rook and Zumla, 1997). Tumour Necrosis Factor (TNF-α) is also involved in NREM sleep regulation (Krueger et al., 1998). Significantly higher levels of TNF-α have been exhibited by Gulf veterans with chronic fatigue syndrome (CFS) when compared with a control group of civilians also suffering from CFS (Zhang et al., 1999). IL-1 and TNF-α are up-regulated following infection, sleep deprivation, excessive food intake and acute mild increases in ambient temperature (Krueger and Fang, 2000).

Acetylcholine (ACh) is known to play an important role in cortical activation during waking and rapid eye movement (REM) sleep (Jasper and Tessier, 1971; Jones, 1993). Furthermore, studies have shown that the EEG and sleep can be affected by accumulation of ACh resulting from the action of both irreversible and reversible acetylcholinesterase (AChE) inhibitors. Alterations of the EEG and/or sleep have been reported in humans (Metcalf and Holmes, 1969; Duffy et al., 1979), rhesus monkeys (Burchfiel et al., 1976) and marmosets (van Helden et al., 2004) following exposure to a low dose of the nerve agent sarin, although all of these studies lacked key control groups which would have enabled the functional significance of their findings to be interpreted. Moreover, the non-human primate studies did not utilise freely moving animals and so there were possibly complications of either physical or chemical restraint. In contrast, Pearce et al. (1999) and Muggleton et al. (2005), using telemetered freely moving marmosets and incorporating appropriate control group comparisons, concluded that there were no significant effects on the EEG over a 12–15 month period following either a similar low dose of sarin, or a range of doses of diazinon respectively.

The effects of reversible AChE inhibitors, such as the carbamates PB and physostigmine, on the EEG and sleep have also been investigated. Physostigmine, in the guinea pig, has been shown to increase the theta and alpha components of the EEG frequency spectrum at doses which resulted in 40–50% erythrocyte AChE inhibition (Philippens et al., 1996). Physostigmine has also been demonstrated to affect sleep architecture in humans (Sitaram et al., 1978) where reduction of the latency to REM onset was observed when physostigmine was infused prior to the first REM period. Additionally, if physostigmine was administered during the first REM period, wakefulness was induced. PB (a quaternary carbamate which, unlike physostigmine, does not readily cross the blood brain barrier) when administered to human volunteers at a dose relevant to its military usage (30 mg), caused no short-term effects on the EEG (Borland et al., 1985; Cook et al., 2002).

In recent years the more widespread availability of radiotelemetry equipment has enabled EEG, and in consequence sleep, to be monitored from non-restrained animals within their home cage environment (e.g. Pearce et al., 1998;

Crofts et al., 2001). Radiotelemetry provides a viable alternative to widely used methods employing at least some degree of restraint, such as primate chair systems (e.g. Adams and Barratt, 1974) or backpack and umbilical systems (Pearce et al., 1989), having neither permanent external connection points nor exit wounds.

2. Materials and methods

The study reported here was carried out in accordance with the Animals (Scientific Procedures) Act 1986.

2.1. Animals

Forty eight adult common marmosets (*Callithrix jacchus*) (24 vasectomised males and 24 females, weighing 331–565 g and aged 2–5.5 years at the start of the study) were used. All animals were housed in long-term, male–female pairings. Each pair of animals was housed in 4 interlinked cage units, each of 72(H)×47(W)×60(D) cm, connected by one vertical and two horizontal rigid linkers. Animal rooms were maintained at 25 °C on a 12-hour light/dark cycle with 30-minute 'dawn' and 'dusk' periods. The room light level during the light phase was 350–400 lx at 1 m above the floor. Animals were fed 20 g of primate pellets (Special Diet Services, Witham, Essex, UK) each day supplemented by fruit (orange, apple, banana). In addition, 1–2 tablespoons of forage mix consisting of cereal, raisins and sunflower seeds were given daily. Animals had unlimited access to water at all times.

2.2. Overview of experimental design

Behavioural, physiological and immunological parameters were monitored over 21 months. The experimental period was divided into seven 3-month periods (Fig. 1). Prior to the first (baseline) period, each animal was trained to perform a number of behavioural tasks and was implanted with a radiotelemetry transmitter (for monitoring the EEG — see below). At the end of this training period, animals were assigned to one of four treatment groups (Fig. 1; $n=12$ animals per group) on the basis of their gender (equal numbers of males and females), cognitive performance and bodyweight. Both animals in each pair received the same treatment. For logistical reasons, it was necessary to stagger the starting times of the study. Animals were monitored in 6 sets of 8 animals and each set consisted of 2 animals from each treatment group.

Over the first 51 days of Period 1, animals were vaccinated with 1/5th human dose, or administered vehicle, according to the schedule in Fig. 1.

The first day of vaccine administration was designated as day 0. On day 15, animals were anaesthetised (1.0 mg/kg i.m. diazepam (Valium, Roche) followed by 0.9 ml/kg 0.9%w/v alphaxalone and 0.3% w/v alphadalone acetate (Saffan, Glaxo) i.m.) and implanted subcutaneously below the right scapula with primed mini-osmotic pumps containing PB (500 μg/kg/day) (groups 2 and 4) or sterile saline (0.9%) (groups 1 and 3). Pumps were removed under anaesthesia on day 44. At the commencement of period 6, animals were challenged with a previously unseen antigen, keyhole limpet haemocyanin (KLH) in order to assess the immune competence of the animals. Animals were culled 18 months after the first vaccinations when pathology and a series of ex vivo electrophysiology studies were carried out. Throughout the study, all members of the research team were blind to the treatment administered to each animal.

2.3. EEG telemetry surgery

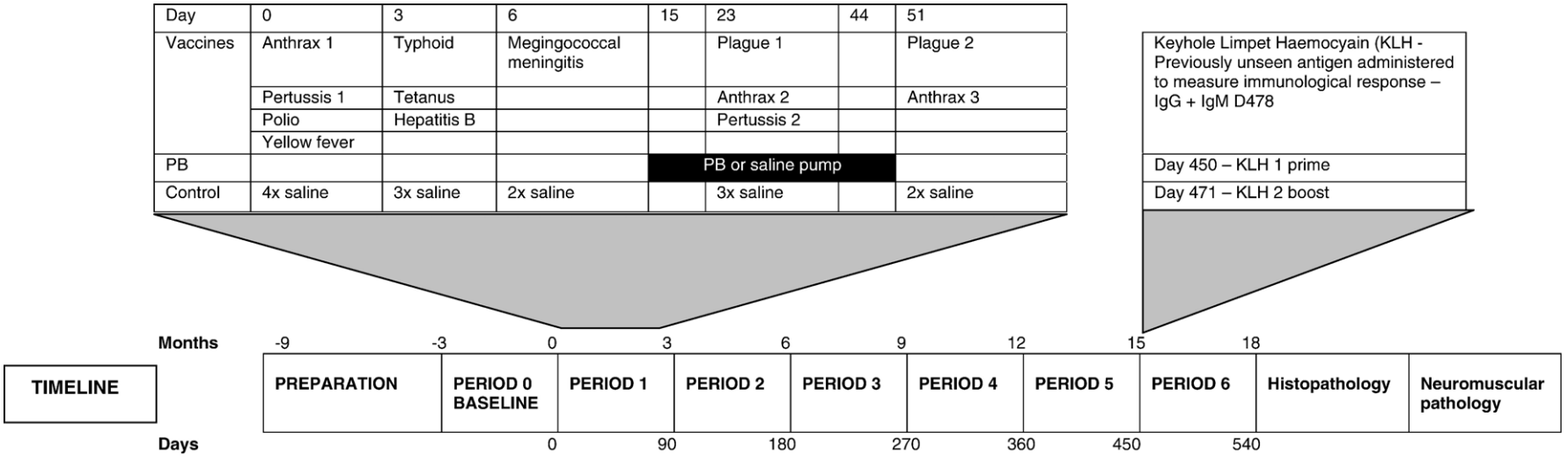
All animals were implanted with a single channel biopotential telemetry transmitter type TA11CTA-F40 (19×16×9 mm) (Data Sciences International (DSI), St Paul, MN, USA), with the transmitter body placed in the peritoneal cavity. Electrodes were placed on the dura, the first over the left side of the frontal cortex approximately 2 mm from the midline and 8 mm from bregma and the second positioned over the right parietal cortex approximately 2 mm right of the midline and 3 mm caudal to bregma. Full details of surgery are reported elsewhere (Pearce et al., 1998; Crofts et al., 2001). Following premedication with 1.0 mg/kg diazepam intramuscularly (i.m.), animals were anaesthetised with 0.9 ml/kg Saffan (Glaxo) i.m. Prophylactic antibiotic cover was provided by amoxycillin (Synulox, Pfizer), 0.1 ml i.m. at surgery, followed by 1×6.25 mg tablet daily (given with food) for 4 days post surgery. Postoperative analgesia was provided by carprofen (Rimadyl, Pfizer), 0.08 mg/kg s.c. at surgery with further doses at 24 h intervals if required.

2.4. EEG recording and analysis

The single channel EEG signals were collected by means of a cylindrical receiver (DSI model No. RLA3000) positioned in the forage medium below a rigid extension to the animal's home cage. This took place on four occasions during the first month of each 3 month period whilst the animals were performing a cognitive task at a touch screen computer placed in front of their home cage (Stevens et al., in press). In order to achieve consistency of recording conditions, EEG signals were not analysed outside these times. The raw signal was sampled at 1000 Hz, edited for movement, 'out of range' and alpha wave artefacts (resultant from eye closure during reward acceptance) and was then subjected to Fast Fourier Transform (FFT) analysis using 30×2-second blocks of raw signal for each time point. The relative mean percentage power occupancy in the frequency bands delta (1–3.5 Hz), theta (3.5–7.5 Hz), alpha (7.5–13 Hz), beta (13–40 Hz, beta 1=13–22 Hz, beta 2=22–40 Hz, total beta=13–40 Hz) and total power were calculated.

2.5. Sleep recording

Animals were housed in mixed-sex pairs and generally slept together in a polypropylene bucket suspended approximately



EEG	Collected during behavioural testing	Implantation of radiotelemetry transmitters	Collected during behavioural testing – summarised per period						
	Sleep		Collected monthly – summarised per period						
	Muscle function	Task training	Measured weekly – summarised per month						
	Behavioural testing	Task training	1 behavioural sequence per period						
Blood sampling	WBC phenotyping		3 samples	Day 57	Day 114 & 183	Day 264	Day 300	Day 364 & 420	
	Immunology		1 sample	Day 15, 43 & 71		Day 210		Day 390	
	Clinical Chemistry		1 sample	Day 84		Day 266			
Urine analysis	Cortisol		1 sample	Sample collected at end of each period					

TREATMENT GROUPS

1: S+S 2: S+P
3: V+S 4: V+P

n = 12 per group

Fig. 1. Outline of experimental protocol.

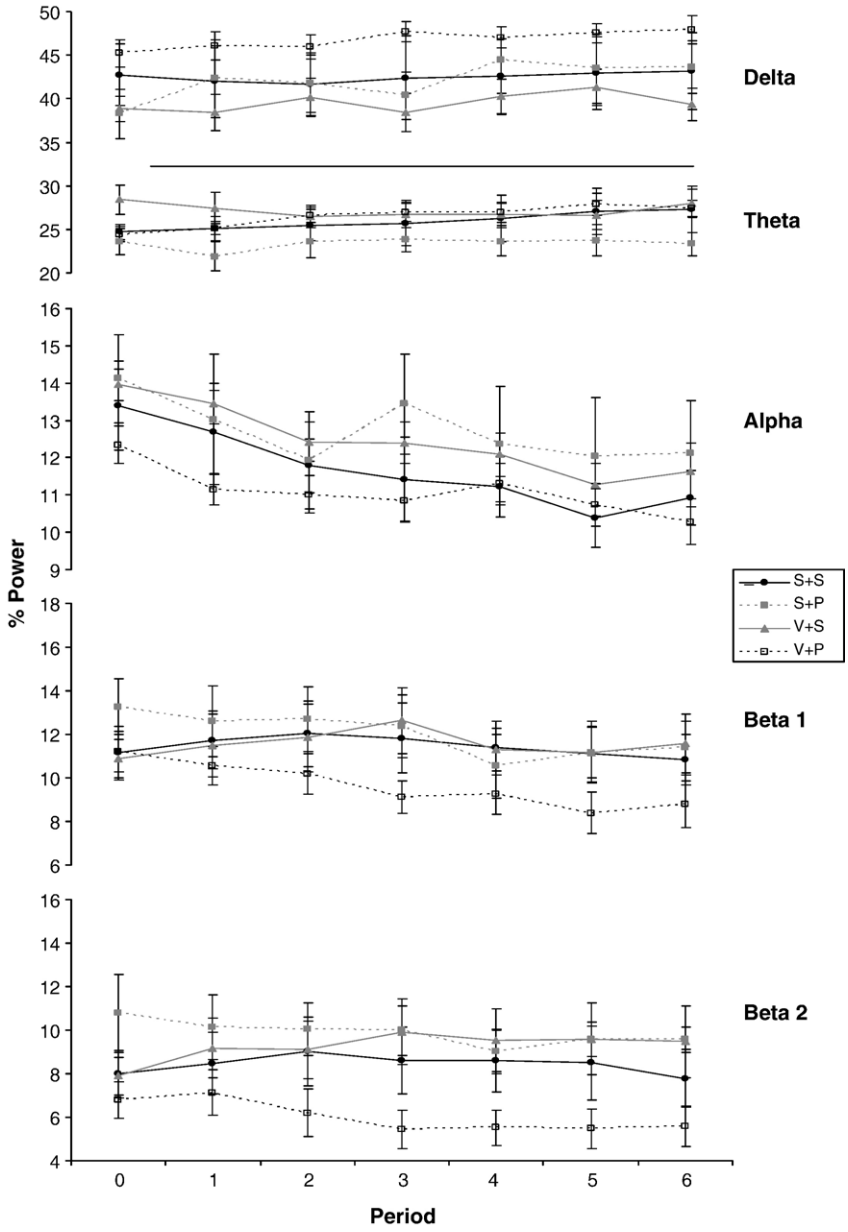


Fig. 2. Changes in percentage power contribution to the different EEG frequency bands of each treatment group over time.

25 cm below the roof of their home cage. A small number of pairs chose to sleep on the cage floor, this pattern of behaviour being established during the baseline period and did not change during the study period, and so receivers and cameras were appropriately positioned in order to collect the signals.

Sleep data were derived from EEG and video monitoring of animals during the 12-h dark phase. A sufficient number of recordings were made from each animal to ensure that three high quality traces per period were obtained. An infrared camera, connected to a time-lapse video recorder, was placed on top of the home cage, enabling each animal's position and movement throughout the night to be recorded.

A cylindrical receiver (DSI model No. RLA3000) was appropriately positioned, either secured by clips to the underside of the sleep bucket or placed under the cage floor. The receiver was connected to an Embla recorder and isolation unit via connections to a BCM 100 unit, a dedicated computer and an UA10 Universal Analogue Adaptor. The signal from the Embla recorder passed via an id-capsule to a laptop computer (digital HiNote VP). Off-line analysis was performed using Somonologica software (Flaga, Iceland) with support from video recordings.

2.6. Sleep staging

Sleep EEG was retrospectively staged, into light sleep, deep sleep, REM and wake, according to criteria established in a previous sleep study undertaken in the common marmoset (Crofts et al., 2001), which was based on the rules of Rechtschaffen and Kales (1968).

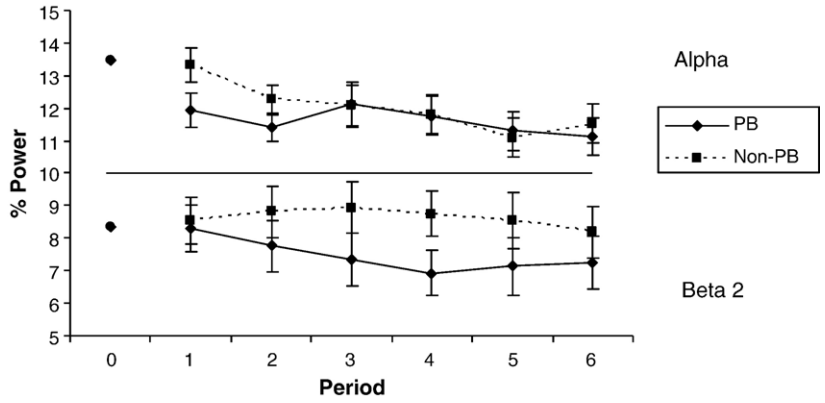


Fig. 3. Change in relative power of alpha and beta 2 wave activity (adjusted values) with time for animals treated with pyridostigmine (pooled data S+P and V+P) (n=20) compared with non-pyridostigmine treated animals (pooled data S+S and S+V) (n=2).

From the single channel EEG signal alone it was difficult to distinguish between waking and REM stages and all videotapes were scored to identify periods of REM. In previous studies, manual video analysis had suggested that periods of REM are characterised by irregular breathing movements and are often accompanied by fine movement and, on some occasions, very slow rolling head movement. In addition, the onset of REM is frequently accompanied by a gradual loss of posture as the animals roll out from their curled position, probably as a result of reduced muscle tone. Very short apparent wakenings were frequently observed at both the beginning and end of each period of REM. The characteristics of REM varied slightly between animals, for example, identification of REM was clearer in certain individuals than others because of uniformity of signal structure and events preceding the REM period, although within individuals the patterns observed were consistent.

In order to aid the discrimination between wake and REM sleep, once sleep characteristics had been established from the manual scoring of videotapes, an automated process was developed using a Digital Video Motion Detector (DVMD). This image analysis hardware utilised both animals as the detection zone, as movement of one animal affected the other. A threshold level of motion detection was determined for each recording such that all movements above the level of breathing and minor twitches were identified using software within the DVMD. Once the threshold level had been surpassed, a relay within the DVMD closed, causing a change in resistance which was recorded using a data logger (Dr DAQ, Pico Technology Ltd). All recorded movements of longer than 10 s duration were time matched to the EEG data and were used to aid the discrimination between wake and REM sleep.

Sleep parameters used for analysis were: total sleep time; sleep efficiency (percentage of time spent asleep during the sleep period); total duration of light, deep (slow wave) and REM sleep; amount of wake and number of wakenings; number of REM periods and REM latency.

A number of rules were applied to ensure consistency in these measurements:

1. The sleep period was judged to have started after at least 2 min of light sleep had been recorded (irrespective of lighting conditions). All previous periods of short sleeps i.e. dozing, were ignored.
2. REM-like periods of less than 1 min were scored as wake, even if no movement was detected.

2.7. Statistical analysis

The overarching strategy for statistical analysis was devised in collaboration with the Statistical Services Department of the University of Reading, who undertook the analysis on an independent consultancy basis. Individual variables were analysed using a mixed model approach for repeated measurements. The mixed model included fixed effects for set, gender, treatment group, time and the treatment × time interaction. An unstructured (i.e. completely general) variance–covariance structure was used to model the variances over time and the covariances amongst the timepoints. The baseline value of the variable was also included in the model. Set, treatment and gender were all factors, whereas the baseline value was a covariate. Where appropriate, if the profiles over time for a variable showed no evidence of non-linearity, time was incorporated in the model as a continuous variate rather than as a factor. The treatment main effect and the treatment × time interaction were of key interest, since together they investigate a null hypothesis of no difference between the treatment profiles over time.

In order to investigate the effects of the treatments in more detail, particularly the effect of PB and vaccines, the analysis also included the following two components which further explored the overall treatment effect (and the treatment × time interaction):

2.7.1. Factorial treatment structure (the effects for vaccine and PB and their interaction)

- the main effect of vaccines (V+S, V+P)
- the main effect of PB (S+P, V+P)
- the vaccines × PB interaction
  (plus their interactions with time,
  when the data were repeated measurements)

2.7.2. Comparisons with control (S+S)

- comparison of S+P versus S+S
- comparison of V+S versus S+S
- comparison V+P versus S+S
  (plus their interactions with time,
  when the data were repeated measurements).

The treatment groups were also compared at baseline to determine whether there was a difference amongst them prior to the start of treatment. Where data were not normally distributed, they were transformed prior to analysis and the natural log transformation was usually employed. The results of all analyses were presented as least squares means for the four treatments (over time), plus standard errors. If no main effect or interaction was apparent in the initial ANOVA analysis ($p<0.1$), any significant effects in the factorial analysis and comparisons with control were discounted. This prevented undue emphasis on effects which might occur by chance in a study where over 200 comparisons in total were being made.

Subsequently, a critical examination of the statistically significant differences observed was undertaken to determine whether any differences observed were of biological significance.

3. Results

For technical and logistical reasons, complete data sets were not obtained from all of the animals. Analysis of EEG was carried out on 41 animals (S+S=9, S+P=8, V+S=12, V+P=12) and on 39 animals for sleep (S+S=8, S+P=8, V+S=12, V+P=11).

3.1. EEG

No long-term effects on brain electrical activity of treatment with vaccines and PB, either alone or in combination, were detected (see Fig. 2 which uses mean data). A number of significant treatment-related transient

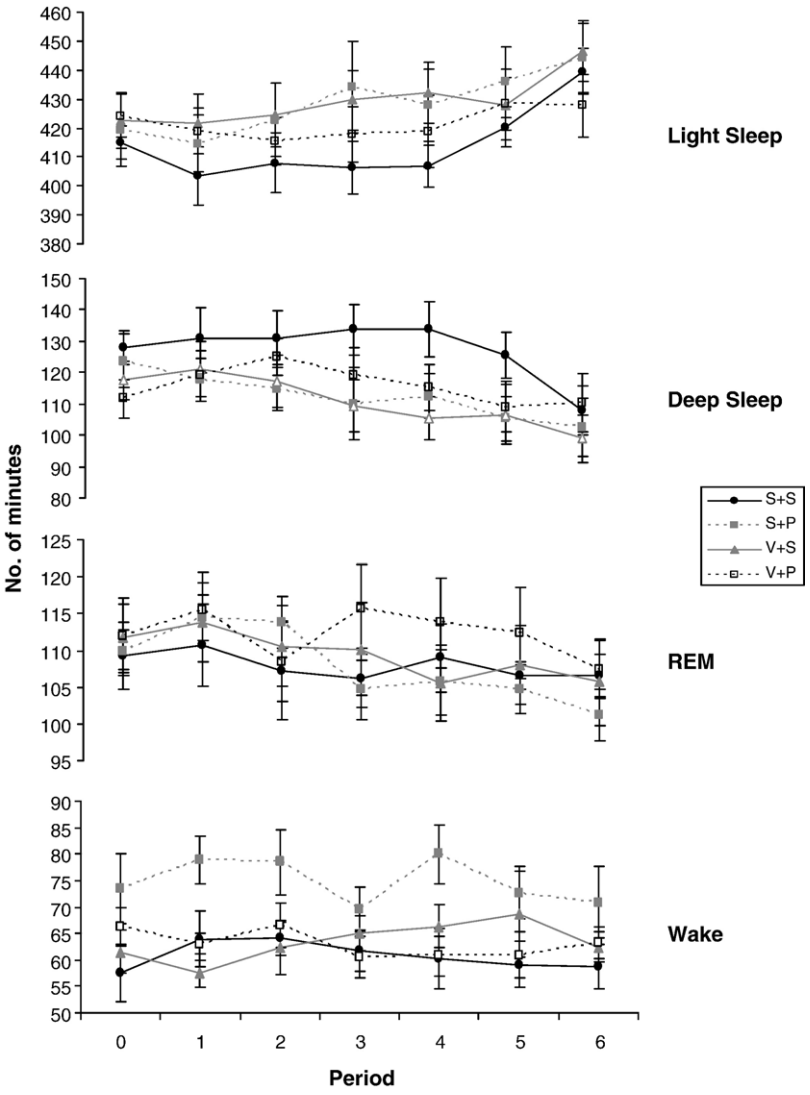


Fig. 4. Changes in light and deep sleep, REM and wake within the different groups over time.

changes and treatment-independent time-effects were detected. Where specific treatment-related outcomes were found they are illustrated, using adjusted values (least squares means), in Fig. 3.

3.1.1. Delta wave activity

There was a statistically significant increase in the relative magnitude of delta wave activity over time (F (1,29)≡=6.22, P≡0.019) independent of treatment.

3.1.2. Theta wave activity

There was a statistically significant increase in theta wave activity over time (F (1,29)≡=5.95, P≡0.0211) independent of treatment.

3.1.3. Alpha wave activity

Pyridostigmine (pooled data S+P and V+P) treated animals had significantly less alpha wave activity, a difference of approximately 1–2% during the first 2 periods, compared with non-pyridostigmine (pooled data S+S and S+V) treated animals (F (5,29)≡=2.89, P≡0.03; Fig. 3). No other changes were apparent.

3.1.4. Beta 1 wave activity

There was a statistically significant decrease in beta 1 wave activity over time (F (1,29)≡=6.04, P≡0.02), independent of treatment.

3.1.5. Beta 2 wave activity

There were individual time points in the middle of the study where animals treated with pyridostigmine (pooled data S+P and V+P) had significantly less beta 2 activity than non-pyridostigmine (pooled data S+S and V+S) treated animals (F (5,29)≡=2.99, P≡0.027: Fig. 3).

3.2. Sleep

Figs. 4 and 5 illustrate means values of sleep parameters across time. Where specific outcomes were obtained for each of the measures of sleep they are illustrated using adjusted (least

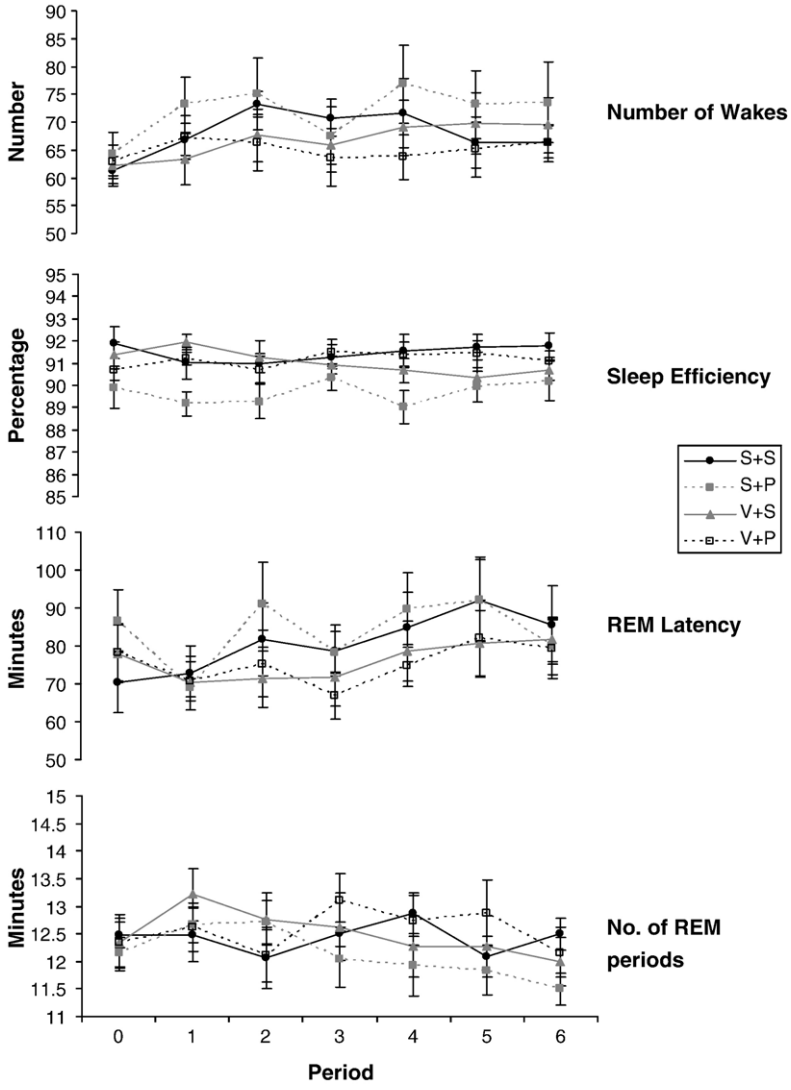


Fig. 5. Number of wakes, sleep efficiency, REMI latency and the number of REM periods within the different treatment groups over time.

squares means) values in Figs. 6 and 7. There were no significant differences between treatment groups at any measure taken at baseline.

3.2.1. Light sleep

There was an overall increase in light sleep over time that was independent of treatment (F (1,28)≡=43.88, P≡<0.0001).

3.2.2. Deep sleep (SWS)

There was an overall reduction in SWS over time, independent of treatment (F (1,28)≡=24.1, P≡<0.0001).

3.2.3. REM

Animals treated with pyridostigmine (S+P) showed significantly decreased amounts of REM sleep during the latter part of the study, compared to controls (S+S) (F1,28)≡= 5.68, P≡0.024).

3.2.4. Wake

In the early part of the study animals treated with vaccine (pooled data V+S and V+P) had less wakefulness than non-vaccine treated animals (pooled data S+S and S+P) (F (1,28)≡ =5.32, P≡0.029).

3.2.5. Number of wake periods

Animals from vaccinated groups (pooled data V+S and V+P) showed fewer waking periods during the early and middle parts of the study than non-vaccinated groups (pooled data S+S and S+P) (F (1,28)≡4.52, P=0.042). In addition, animals treated with vaccines only (V+S) showed fewer waking periods than controls (S+S) during these parts of the study (F (1,28)≡=5.83, P≡0.023).

3.2.6. Sleep efficiency

Animals from vaccinated groups (pooled data V+S and V+P) showed an increased level of efficiency during the first half the study compared with non-vaccinated animals (pooled data S+S and S+P) (F (1,28)≡=5.43, P≡0.027). In addition, animals treated with vaccines only (V+S) showed an increased level of efficiency in the early part of the study but a decreased level of efficiency in the latter part of the study compared with controls (S+S) (F (1,28)≡=6.4, P≡0.017).

3.2.7. REM latency

There was a significant increase in REM latency in all groups over time (F (5,28)≡=5.55, P≡0.001), which was independent of treatment.

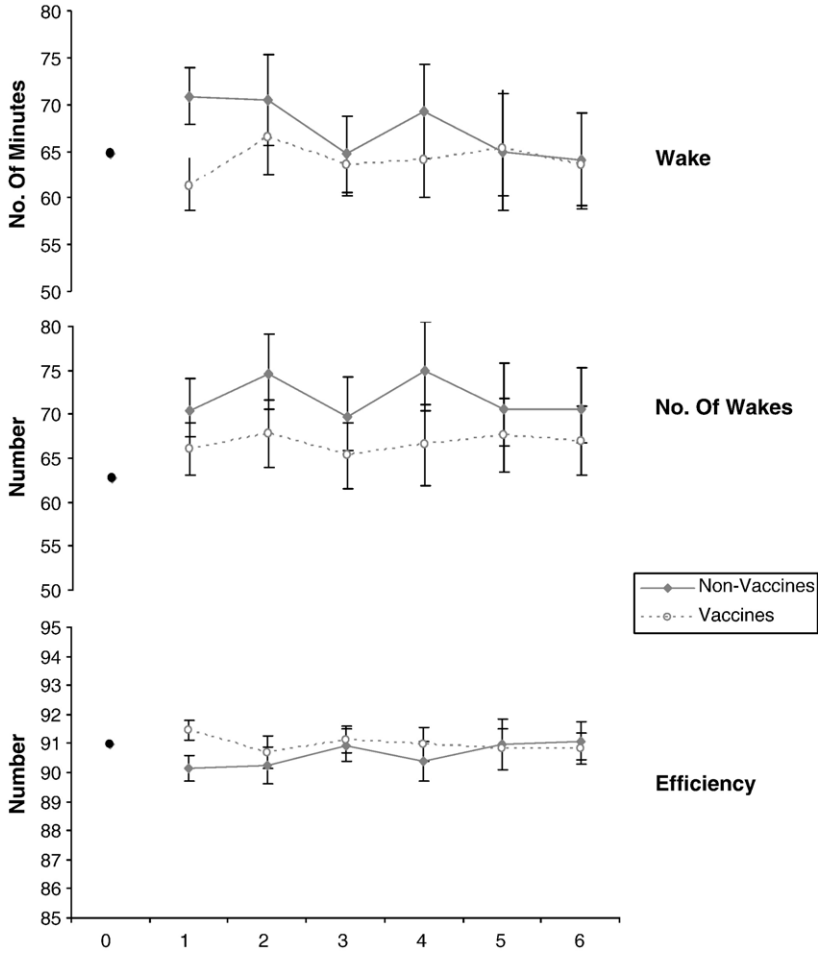


Fig. 6. Changes in amount of wake, number of wakes and sleep efficiency (adjusted values) for different sets of pooled data.

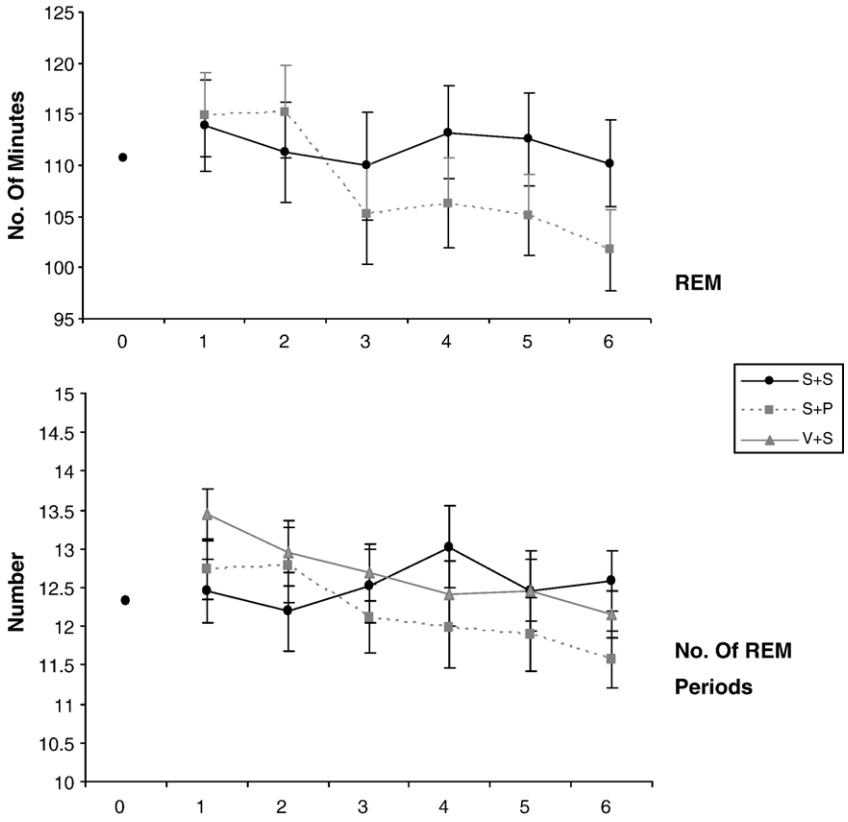


Fig. 7. Changes in the amount of REM sleep and number of REM periods (adjusted values) for different sets of pooled data.

3.3. Number of REM periods

Over the period of the study there were fewer REM periods in the pyridostigmine treated group (S+P) than the control group (S+S) (F (1,28)≡=7.65, P=0.01). In the vaccine treated group (V+S) there were more REM periods in the early part of the study compared with controls (S+S) (F (1,28)≡=8.31, P≡0.008).

4. Discussion

There were no marked long-term changes in brain electrical activity that could be attributed to vaccines and/or PB administration. The changes that were detected were predominantly time related and independent of treatment. Overall, the EEG activities of all animals remained remarkably stable over the 21 months of the study. Where statistical differences were detected, the low relative magnitude of the changes (<2% of total power) and the lack of correlation with any functional changes, do not suggest that they are of biological significance. In the analysis of EEG data, the increases in relative amounts of delta and theta and decreases in beta 1 activities with time may have been due to the animals' increase in age, the study lasted the equivalent of 10% of the life span of an average marmoset, or could reflect changes in electrode properties over the course of the study.

Animals treated with PB displayed less alpha activity than those that did not receive PB. The difference in this frequency band between the control and PB alone groups (~1.0% of total power) lessened over time, with no apparent difference seen from period 3 onwards. When considering the pooled data (PB treated compared to non-PB) the difference within the beta 2 band was more apparent with time (~0.8% increasing to ~1.5% of total power) but resolved towards the end of the study. In the study reported here, the pyridostigmine was administered over 28 days and produced a mean erythrocyte inhibition of approximately 30%. The changes detected in EEG were not accompanied by behavioural changes (Stevens et al., in press) or long term alterations in sleep patterns that were considered likely to be biologically significant. These results are in agreement with studies carried out in human subjects investigating the effects of pyridostigmine on EEG (Borland et al., 1985, Cook et al., 2002). Studies in humans and animals investigating other AChE inhibitors which did show changes in EEG all used compounds that cross the blood brain barrier readily e.g. physostigmine (Philippens et al., 1996) and sarin (Metcalf and Holmes, 1969; Duffy et al., 1979; Burchfiel and Duffy, 1982). Pyridostigmine, however, does not cross this barrier readily and this may be an explanation as to why no changes were seen. Where studies, albeit in non-primate species, have suggested that pyridostigmine can induce central effects (Friedman et al., 1996; Kaufer et al., 1999; Abdel-Rahman et al., 2004), the presence of either stress or other chemicals is a factor. Others, however, have been unable to find evidence for this in their studies (Lallement et al., 1998; Sinton et al., 2000; Grauer et al., 2000).

No short or long term changes in EEG were found which were dependent on the administration of vaccines. This is consistent with the findings of others (Shokeir and Zohdy, 1968; Timm and Wolter, 1971; Topsis et al., 1996).

Following sleep staging, measures of waking (amount and number of periods), sleep efficiency and sleep time were analysed as measures of 'quality' of sleep, as changes in these parameters may reflect the general disruption of sleep and form the basis of the sleep related complaints reported by some Gulf veterans. Additional analysis of NREM sleep (light and deep sleep) enabled full characterisation of sleep profiles. Other variables analysed in the study were selected on the basis of likely effects pyridostigmine may have had on the cholinergic control of sleep and possible effects of immune system interactions on sleep. These were REM latency, number of REM periods and time spent in REM sleep.

The sleep profiles of the marmosets during baseline recordings were qualitatively and quantitatively similar to those obtained from marmosets in previous studies (Crofts et al., 2001; Muggleton et al., 2005). Muggleton used the same approach employed in this study to investigate the effects of the organophosphate diazinon on sleep. In these studies, alterations in the amount of REM in the short term (over 1 week post dosing) were identified but no other changes were seen in either the short or long term. Levels of erythrocyte cholinesterase inhibition in the latter study were up to 82%, and recovery of enzyme took place over a 3-month period. The level of peripheral inhibition, however, may not necessarily reflect levels of central inhibition.

In the present study, there were some overall effects of time, which were unrelated to treatment e.g. the relative amount of light sleep, as compared with deep sleep, increased over the course of the study. These changes may have been a result of age-related effects or have been a function of electrode contact with the dura, where subtle changes in electrode properties may have taken place. There have been no other reported studies of sleep measurements by means of radiotelemetry over such a long study period.

There were no changes in sleep behaviour. The majority of animals slept in their pairs, curled up in their sleeping buckets hanging from the cage tops. Those that chose to sleep on the cage floors did so before the study started and this pattern did not change over the duration of the study.

Changes in sleep architecture which were related to treatment suggest that the presence of vaccines led to a relative increase in sleep efficiency and decrease in waking within the sleep period during the early part of the study. Analysis would suggest that, although these changes were statistically significant, they were quantitatively small and in a direction that suggested improved quality of sleep. This is in contrast to the reported changes in studies of Gulf veterans which imply long term difficulties with sleep. In the same study (Hornby et al., unpublished observations), no significant changes in immunological status were observed which could have been responsible for the short term NREM sleep promoting effects.

Effects of treatment on REM sleep were variable, with measures suggesting that pyridostigmine reduced the overall amount of REM sleep, but vaccines had a short-term effect in increasing the number of REM periods. Again, the small degree of change in these parameters, less than 30%, is unlikely to have clinical significance, especially as there was no correlation with treatment effects on other REM parameters such as a reduced latency to onset.

In conclusion, this study has shown that there were no marked long-term changes in brain electrical activity or sleep architecture, in marmosets, that could be attributed to the administration of the vaccines and/or PB administration. The changes that were detected were predominantly time related and independent of treatment. Where statistical differences were detected between treatments, the magnitudes of the difference were relatively minor; they were therefore not regarded as having long term biological significance.

Whilst the EEG and sleep outcomes must be considered alongside those from other elements of the study, (e.g. behaviour (Stevens et al., in press) and immunology (Hornby et al., unpublished observations), on the basis of the results reported here, sleep deficits reported by Gulf conflict veterans may not be attributable to the pretreatments administered.

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References

Abdel-Rahman A, Abou-Donia S, El Masry F, Shetty A, Abou-Donia M. Stress and combined exposure to low doses of pyridostigmine bromide, DEET and pymethrin produce neurochemical and neuropathological alterations in cerebral cortex, hippocampus and cerebellum. J Toxicol Environ Health 2004;A67:163–92.

Adams PM, Barratt ES. Nocturnal sleep in squirrel monkeys. Electroencephalogr Clin Neurophysiol 1974;36(2):201–4.

Benjamini E, Sunshine G, Leskowitz S. Activation of T and B cells by antigen. In: Benjamini E, Sunshine, Sunshine S, editors. Immunology a short course. 3rd ed. New York: Wiley-Liss; 1996. p. 199–226.

Border P, Norton M. Gulf War illnesses — dealing with the uncertainties. Parliamentary Office of Science and Technology; 1997.

Borland RG, Brennan DH, Nicholoson AN, Smith PA. Studies on the possible central and peripheral effects in man of a cholinesterase inhibitor (pyridostigmine). Hum Toxicol 1985;4:293–300.

Burchfiel JL, Duffy FH. Organo-phospahate neurotoxicity — chronic effects of sarin on the electroencephalogram of monkey and man. Neurobehav Toxicol Teratol 1982;4(6):767–78.

Burchfiel JL, Duffy FH, Sim VM. Persistent effects of sarin and dieldrin upon the primate electroencephalogram. Toxicol Appl Pharmacol 1976;35:273–8.

Cherry N, Creed F, Silman A, Dunn G, Baxter D, Smedley J, et al. Health and exposures of United Kingdom Gulf War veterans. Part I: the pattern and extent of ill health. Occup Environ Med 2001;58:291–8.

Coker WJ, Bhatt BM, Blatchley NF, Graham JT. Clinical findings for the first 1000 Gulf War veterans in the MOD's medical assessment programme. Br Med J 1999;318:290–4.

Cook MR, Graham C, Sastre A, Gerkovich MM. Physiological and performance effects of pyriostigmine bromide in healthy volunteers: a dose–response study. Psychopharmacology (Berl) 2002;162:186–92.

Crofts HS, Wilson S, Muggleton NG, Nutt DJ, Scott EAM, Pearce PC. Investigation of the sleep electrocorticogram of the common marmoset (*Callithrix jacchus*) using radiotelemetry. Clin Neurophysiol 2001;112: 2265–2273.

Duffy FH, Burchfiel JL, Bartels MG, Sim VM. Long-term effects of an organophosphate upon the human electroencephalogram. Toxicol Appl Pharmacol 1979;47:161–76.

Friedman A, Kaufer D, Shemer J, Hendler I, Soreq H, Tur-Kaspa I. Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. Nat Med 1996; 2:1382–5.

Grauer E, Alkalai D, Kapon J, Cohen G, Raveh L. Stress does not enable pyridostigmine to inhibit brain cholinesterase after parenteral administration. Toxicol Appl Pharmacol 2000;164:301–4.

Griffiths GD, Stevens D, Hornby R, Williams K, Pearce PC, Bowditch AP. Investigation of the effects of multiple vaccine and pyridostigmine administration in a non-human primate model. Conference on Illnesses among Gulf War Veterans: a Decade of Scientific Research. Jan 24–26 Alexandria, USA; 2001a.

Griffiths GD, Hornby RJ, Stevens DJ, Scott LA, Upshall DG. Biological consequences of multiple vaccine and pyridostigmine pretreatment in the guinea pig. J Appl Toxicol 2001b;21(1):59–68.

Griffiths GD, Hornby RJ, Jagger CP, Brown AP, Stoten A, Pearce PC et al. Development of methods to measure humoral immune responses against selected antigens in the common marmoset (*Callithrix jacchus*). Unpublished observations.

Hornby RJ, Pearce PC, Scott L, Griffiths G.D. Multiple vaccine and pyridostigmine bromide interactions in the common marmoset (*Callithrix jacchus*): immunological and endocrinological effects. Unpublished observations.

Hyams KC, Wignall FS, Roswell RH. War syndromes and their evaluation: from the U.S. Civil War to the Persian Gulf War. Ann Intern Med 1996;125(5): 398–405.

Ismail K, Kent K, Brugha T, Hotopf M, Hull L, Seed P, et al. The mental health of UK Gulf War veterans: phase 2 of a two phase cohort study. Br Med J 2002;325:576–9.

Jasper HH, Tessier J. Acetylcholine liberation from cerebral cortex during paradoxical (REM) sleep. Science 1971;172:601–2.

Jones BE. The organisation of central cholinergic systems and their functional importance in sleep-waking states. In: Cuello AC, editor. Cholinergic function and dysfunction. Progress in brain research. Amsterdam: Elsevier; 1993. p. 61–71.

Jones E, Hodgins-Vermaas R, McCartney H, Everitt B, Beech C, Poynter D, et al. Post-combat syndromes from the Boer War to the Gulf War: a cluster analysis of their nature and attribution. Br Med J 2002;324:321–4.

Kang HK, Mahan CM, Lee KY, Magee CA, Murphy FM. Illnesses among United States veterans of the Gulf War: a population-based survey of 30,000 veterans. J Occup Environ Med 2000;42:491–501.

Kaufer D, Friedman A, Soreq H. The vicious circle of stress and anticholinesterase responses. Neuroscientist 1999;5(3):173–82.

Krueger JM. Somnogenic activity of immune response modifiers. Trends Pharmacol Sci 1990;11:122–6.

Krueger JM, Fang JD. Cytokines in sleep regulation. In: Hayaishi O, Inoue S, editors. Sleep and sleep disorders: from molecule to behaviour. Academic Press; 1997. p. 261–77.

Krueger JM, Fang JD. Host defence. In: Kryger MH, Roth T, Dement WC, editors. Principles and practice of sleep medicine. W.B. Saunders Company; 2000. p. 255–64.

Krueger JM, Fang JD, Ping TS, Chen ZT, Kushikata T, Gardi J. Sleep — a physiologic role for IL-1 beta and TNF-alpha. Ann N Y Acad Sci 1998;856:148–59.

Lallement G, Foquin A, Baubichon D, Burckhart MF, Carpentier P, Canini F. Heat stress, even extreme, does not induce penetration of pyridostigmine into the brain of guinea pigs. Neurotoxicology 1998;19: 759–766.

Lee HA, Gabriel R, Bale AJ, Bolton P, Blatchley N. Clinical findings of the second 1000 UK Gulf War veterans who attended the Ministry of Defence's Medical Assessment Programme. J R Army Med Corps 2001; 147:153–60.

Lee HA, Gabriel R, Bolton P, Bale AJ, Jackson M. Health status and clinical diagnoses of 3000 UK Gulf War veterans. J R Soc Med 2002;95:491–7.

Metcalf DR, Holmes JH. EEG, psychological and neurological alterations in humans with organophosphate exposure. Ann N Y Acad Sci 1969;160 (1):357–65.

Muggleton NG, Smith AJ, Scott EAM, Wilson SJ, Pearce PC. A long term study of the effects of diazinon on sleep, the electrocorticogram and cognitive behaviour in common marmosets. J Psychopharmacol 2005;19:455–66.

Pearce PC, Halsey MJ, Ross JA, Luff NP, Bevilacqua RA, Maclean CJ. A method of remote physiological monitoring of a fully mobile primate in a single animal cage. Lab Anim 1989;23(2):180–7.

Pearce PC, Crofts HS, Muggleton NG, Scott EAM. Concurrent monitoring of EEG and performance in the common marmoset: a methodological approach. Physiol Behav 1998;64(4):591–9.

Pearce PC, Crofts HS, Muggleton NG, Ridout D, Scott EAM. The effects of acutely administered low dose sarin on cognitive behaviour and the electroencephalogram in the common marmoset. J Psychopharmacol 1999; 13(2):128–35.

Philippens IH, Wolthuis OL, Busker RW, Langenberg JP, Melchers BPC. Side effects of physostigmine as a pretreatment in guinea pigs. Pharmacol Biochem Behav 1996;55(1):99–105.

Proctor SP, Heeren T, White RF, Wolfe J, Borgos MS, Davis JD, et al. Health status of Persian Gulf War veterans: self-reported symptoms, environmental exposures and the effect of stress. Int J Epidemiol 1998;27: 1000–1010.

Rechtschaffen A, Kales A. A manual of standardized terminology techniques and scoring system for sleep stages of human subjects. Los Angeles: UCLA Brain Information Service/Brain Research Institute; 1968.

Rijpkema SG, Adams T, Rigsby P, Xing DK, Corbel MJ. Investigation in a model system of the effects of combinations of anthrax and pertussis vaccines administered to service personnel in the 1991 Gulf War. Hum Vaccines 2005;1:165–9.

Rook GAW, Zumla A. Gulf War syndrome: is it due to a systemic shift in cytokine balance towards a Th2 profile? Lancet 1997;349(9068):1831–3.

Shokeir AA, Zohdy AM. Changes in the central nervous system after experimental rabies infection and immunisation. 1 — an electroencephalographic study. Act Nerv Super (Praha) 1968;10:136–47.

Sinton CM, Fitch TE, Petty F, Haley RW. Stressful manipulations that elevate corticosterone reduce blood–brain barrier permeability to pyridostigmine in the rat. Toxicol Appl Pharmacol 2000;165:99–105.

Sitaram N, Mendelson WB, Wyatt RJ, Gillin JC. Physostigmine alters onset but not duration of REM sleep in man. Psychopharmacology (Berl) 1978;58 (1):111–4.

Sitzmann FC. Contraindications and complications in vaccinations. Wien Med Wochenschr 1987;137(24):564–8.

Stevens D, Scott EAM, Bowditch AP, Griffiths GD, Pearce PC. Multiple vaccine and pyridostigmine interactions: effects on cognition, muscle function and health outcomes in marmosets. Pharmacol Biochem Behav 2006;84:207–18.

Timm H, Wolter M. EEG changes following rabies immunisation with Hempt vaccine. JAMA 1971;218(5):738.

Topsis J, Kandall S, Weinstein J, Wilets I. Tolerance of initial diphtheria–tetanus–pertussis immunisation in preterm infants. J Perinatal 1996;16 (2,1):98–102.

Unwin C, Blatchley N, Coker W, Ferry S, Hotopf M, Hull L, et al. Health of UK servicemen who served in Persian Gulf War. Lancet 1999;353 (9148):169–78.

van Helden HPM, Vanwersch RAP, Kuijpers WC, Trap HC, Philippens IHC, Benschop HP. Low levels of sarin affect the EEG in marmoset monkeys: a pilot study. J Appl Toxicol 2004;24(6):475–83.

Zhang QW, Zhou XD, Denny T, Ottenweller JE, Lange G, LaManca JJ, et al. Changes in immune parameters seen in Gulf War veterans but not in civilians with chronic fatigue syndrome. Clin Diagn Lab Immunol 1999; 6:6–13.