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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this effort was to re-monitor EEG-signals of vehicle- or pyridostigmine-pretreated marmosets during a 5 h air-exposure in Nov. 2001 under experimental conditions similar to those in Nov. 2000 when they were first 5 h exposed to air followed by 5 h exposure to low levels of GB vapor in air in a concentration range of 7.5 – 150 $\mu g/m^3$. The EEG-records were analysed by using Fast Fourier Transforms (FTT) in order to quantify the power spectra of the different frequency bands and by visual examination of the raw signals to qualify typical clinical effects. In Nov. 2000 there were significant ($p < 0.05$) changes in most of their EEG-bands during GB exposure compared to corresponding bands during previous air-exposure, the AChE-activity in blood samples was significantly inhibited, and one hour after exposure disturbances in memory and motor function were observed. The *LOAEL* (C.t) values for significant changes in EEG-bands were 0.2 (for d_2 and b_2 bands) and 0.1 $mg.min.m^{-3}$ (for t_1 band) for vehicle- or pyridostigmine-pretreated marmosets, respectively. During a 5 h air-exposure in Nov. 2001 all marmosets still demonstrated EEG-bands which differed significantly ($p < 0.05$) from corresponding bands during air-exposure one year earlier. In most vehicle-pretreated marmosets the energy (μV^2) per EEG-band was higher than that observed one year earlier. This phenomenon was less pronounced in pyridostigmine-pretreated animals.
Visual examination of the EEG-records revealed clear bursts of alpha frequencies (around 9 Hz), so-called sleep-spindles, which were more frequently present in pyridostigmine-pretreated GB exposed animals and in the vehicle-pretreated animals exposed to 7.5, 25 or 150 $\mu g/m^3$ GB. It was discussed that these late changes in spindle oscillation might be the result of changes in the cholinergic system, and concluded that one year after a 5 h low level exposure to GB vapor in air, abnormalities in the EEG-signal were still significant ($p < 0.05$).

14. SUBJECT TERMS
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INTRODUCTION

In our grant contract (DAMD17-97-1-7360) it is stated that we should investigate and report separately on possible late EEG effects in marmoset monkeys which were exposed to low levels of GB vapor in air one year earlier, after the final report (Van Helden 2001) would have been delivered. Five vehicle-pretreated and five pyridostigmine-pretreated monkeys are concerned which were first 5 h air-exposed followed by a 5 h exposure to low levels of GB in Nov. 2000. An interesting preliminary finding at that time was that during a 5 h lasting exposure to GB in a concentration range of 7.5 – 150 $\mu g/m^3$, the EEG seemed to be more sensitive for GB than the eye (by an order of magnitude), both in guinea pigs and marmosets. For vehicle-pretreated marmosets the *LOAEL* values for miosis and EEG changes were 2.5 and 0.2 $mg.min.m^{-3}$, respectively. For pyridostigmine-pretreated marmosets it was 3.1 and 0.1 $mg.min.m^{-3}$, respectively. Whereas in our study significant changes were found in all EEG-bands of all marmosets, Duffy and Burchfiel (1980), reporting on EEG-effects caused by GB intoxication in rhesus monkeys, had observed significant changes in a-, b_2- and d-bands 24 h after i.v. administration of a high dose of GB (5 $\mu g/kg$). Even one year later they still observed significant changes in the b_2-bands. The purpose of this effort is to record the EEG signals of our marmoset monkeys again in Nov. 2001 under experimental conditions similar to those during GB- or air exposure one year earlier.

MATERIALS & METHODS

Procedure

In Nov. 2000 EEG epochs of 5 vehicle-pretreated marmosets, recorded during a 5 h lasting exposure to air, were analyzed and the averaged amount of energy (μV^2) per EEG-band was calculated (see Fig 2). Then, these marmosets were GB-exposed (7.3, 14.6, 21.8, 49.7 or 137.7 $\mu g/m^3$, one animal per GB concentration) for 5 h during which period the EEGs were recorded and analyzed again. It was calculated which EEG-bands were significantly ($p< 0.05$) different from the corresponding bands in air-exposed animals (see Table 1). One year later (Nov. 2001) the same marmosets were again air-exposed for 5 h under similar experimental conditions while their EEG signals were recorded. According to the same analytical and statistical procedure it was determined which EEG-bands were still significantly ($p< 0.05$) different from the corresponding bands in air-exposed animals determined one year before. It should be emphasized that there were no time-matched marmosets available, i.e. animals which were only air-exposed one year earlier.

The same procedure was followed by analyzing EEG epochs of pyridostigmine-pretreated marmosets which were air-exposed and then GB-exposed one year earlier.

Assessment of EEG by telemetry

Each animal was provided with a transmitter (TL10M1-F50-W) (Data Sciences International, Minnesota, USA), while under halothane/N_2O anesthesia, for telemetric monitoring of EEG and VER-potentials. The transmitter was directly connected to the EEG electrodes implanted through the skull on the dura mater; bipolar recordings were taken from the visual cortex region (area 17). A general receiver converts telemetered data to a form readily accessible by a PCL812 AD card (ADVANTECH). Collection and analysis of telemetered data was performed by a PC. The EEG signals were amplified , filtered (0.3-30 Hz) and fed into the ADC of a PC. During a 5-h exposure to GB the EEG was routinely evaluated on-line by visual inspection. To avoid subjective bias and to permit a quantitative analysis, 5 epochs of 10 sec were chosen from a total recording period of 200 sec. EEG data were also preprocessed by spectral analysis using the Fast Fourier Transformation (FFT) technique. FFT

spectra were averaged per animal for statistical analysis. This analysis determines the EEG energies in each of the classical EEG frequency bands: Delta (d)1 = 0.8-2.0 Hz, Delta 2 = 2.0-3.5, Theta (t)1 = 3.5-5.5 Hz, Theta 2 = 5.5-7.5 Hz, Alpha (a)1 = 7.5-10 Hz, Alpha 2 =10-12.5, Beta (b)1 = 12.5-18 Hz, Beta 2 = 18-25 Hz. The total power (V^2) of the various frequency classes was used for the evaluation of the electrical brain activity.

Restrainment of the animals

The conscious marmoset is seated in a special metal chair (Fig 1), his arms and legs fixed on the chair, wearing a plastic helmet in order to fix the head of the animals to the chair in order to take photographs from both eyes every 10 min using a digital camera on a stand. The animals have learned to sit in this way in the exposure chamber for several hours while watching video-films featuring marmosets. The video-film appeared to be necessary to keep the animals awake.

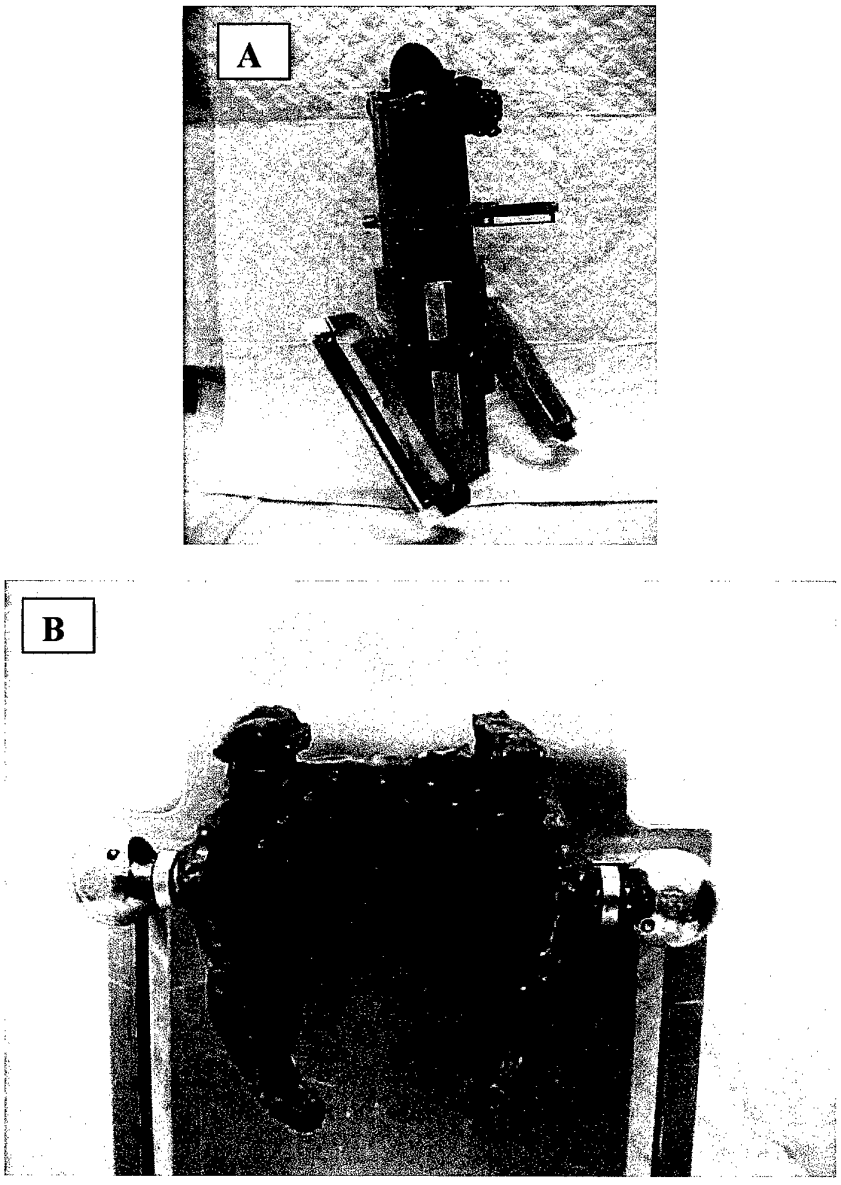

Fig 1. Special marmoset chair (panel A) and detail of helmet (panel B) to fix its head.

Statistical analysis

For statistical analysis the TNO Department for Applied Statistics (Head: Dr P Defize) was consulted. To compare the read-out parameter (the EEG signal) obtained per animal exposed to GB, with the averaged values of the corresponding parameters obtained from six air-exposed animals (controls), a two-sample t-test was used under the assumption that the variance in both the control group (n = 6) and the experimental group (n = 1) was equivalent (Montgomery 1991). The following equations were used: s*2 = SUM(xi - MEAN(x))/(n-1), and t = (y-MEAN(x))/(s*SQRT(1+1/n)), in which x = control value, y = value to be tested against control. The energies of the various EEG-bands at set time intervals were standardized as follows before statistical analysis: let A and B be the EEG-band energy within an animal at t = 0 and t = 30 min, respectively. Then standardized band-energy at t = 30 was set to A/B+B/A. The same standardized calculations were done at t = 60, 90 min. etc. Again a modified t-test was used to compare the standardized band-energies per animal exposed to GB with the averaged standardized band energies at the corresponding time intervals from the six air-exposed animals (controls). Animals provided with Alzet pumps containing saline and exposed to air for 5 h were compared with similarly pretreated animals exposed to various concentrations of GB for 5 h. Animals provided with Alzet pumps containing pyridostigmine bromide and exposed to air for 5 h were compared with similarly pretreated animals exposed to GB for 5 h.

RESULTS

Since the last repeated EEG-recordings in Nov. 2001 should be compared to those recorded in Nov. 2000, the earlier EEG-findings were taken from the final report of DAMD17-97-1-7360 and presented here again.

Vehicle-pretreated marmosets

The analysis of the online registered EEG epochs from vehicle-pretreated marmosets (n = 5) during a 5 h exposure to air in Nov. 2000, is demonstrated in Fig 2. The averaged amounts of energy per band (d_1, d_2, t_1 etc.) did not change significantly between t = 0 and t = 300 min of exposure.

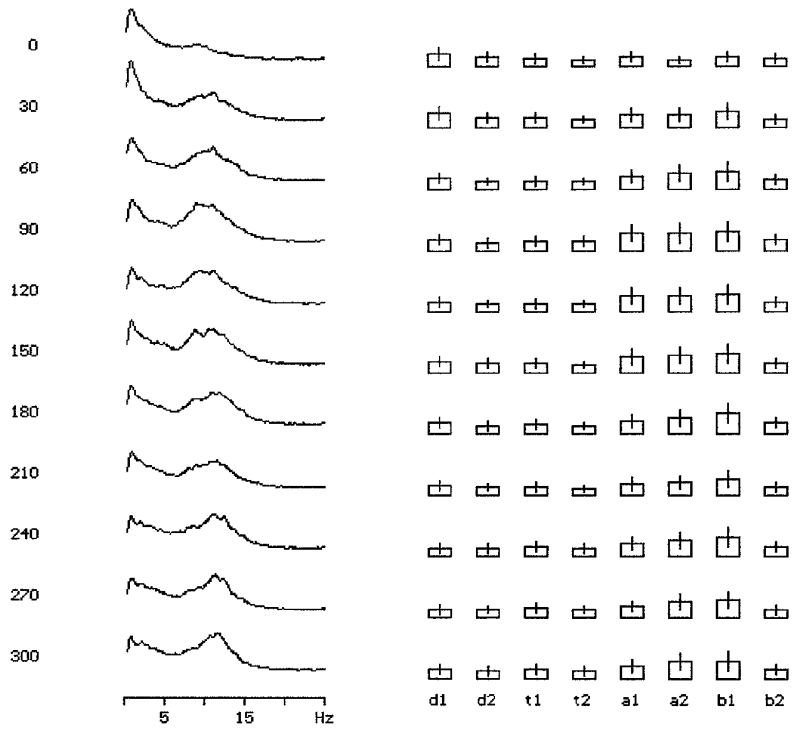

Fig 2. EEG analysis of marmosets (n = 5) provided with Alzet pumps containing vehicle and exposed to air for 5 h in Nov. 2000. Indicated are the exposure time intervals (min) at which the EEG-analysis was carried out, the averaged frequency spectrum (Hz) and the averaged energy (μV^2) per EEG-band.

These averaged amounts of energy per EEG band of vehicle-pretreated and air-exposed animals were compared to the amounts of energy of the corresponding EEG bands of vehicle-pretreated plus GB-exposed animals (7.3, 14.6, 21.8, 49.7 or 137.7 $\mu g/m^3$, one animal per concentration). In Table 1 only the EEG changes are demonstrated which appeared to be significantly different ($p < 0.05$) from that in previously air-exposed animals. For all significant changes shown in Table 1, the *LOAEL* (C.t) values were calculated using the actual GB concentrations during the course of exposure (not shown) instead of the indicated

mean concentrations in the table. These *LOAEL* values are given in Fig 3. It appeared that the d_2 and b_2 EEG-bands were most sensitive to GB exposure, whereas the d_1, b_1 and a_2 bands were least sensitive. The lowest C.t-value established in this way was 0.2 $mg.min.m^{-3}$, representing the *LOAEL* for the first emerging EEG changes in vehicle-pretreated and GB-exposed marmosets.

In Nov. 2001 each of these vehicle-pretreated marmosets still demonstrated EEG-bands which were significantly ($p < 0.05$) different from the corresponding bands during air-exposure in Nov. 2000 (see Table 1). The time points at which EEG-bands became significantly different during the course of the 5 h exposure period were different between Nov. 2000 and Nov. 2001 (see Table 1). For example, during exposure to 7.5 $\mu g/m^3$ GB in Nov 2000, significant EEG-differences were observed at each time point, whereas in Nov 2001 the first significantly different bands appeared by the end of air-exposure. In contrast, during exposure to 49.7 $\mu g/m^3$ GB in Nov. 2000, there were EEG-differences at 180 and 240 min of exposure, whereas in Nov. 2001 there were significantly different bands at each time point during air-exposure. Whether this observation reflects late effects on EEG as a result of GB exposure 1 year earlier, is not clear.

Many of the EEG-bands during GB-exposure in Nov. 2000 which differed significantly from corresponding bands during air-exposure in Nov. 2000, were still significantly different during air-exposure in Nov. 2001 (compare for example the EEG-bands shown at 21.8 $\mu g/m^3$ GB, analyzed in Nov. 2000 and in Nov. 2001).

Remarkably, it was found that in most vehicle-pretreated animals the energy (μV^2) per EEG-band was higher than that observed during air-exposure one year earlier (Fig 4). This might indicate that neurons have become more sensitive for excitation. This phenomenon was less pronounced in pyridostigmine-pretreated and GB-exposed animals (Fig 7).

Table 1. Statistically analyzed differences between EEG-bands from vehicle-pretreated and GB-exposed (7.3, 14.6, 21.8, 49.7, or 137.7 μg/m³ GB, one animal per concentration) marmosets, and the corresponding EEG-bands from vehicle-pretreated and air-exposed (n = 5) animals. Indicated are the EEG-bands which were significantly different ($p<0.05$) from the corresponding bands in air exposed animals (Nov. 2000). The italicized bands (red) represent the significant ($p<0.05$) differences in EEG between air-exposure in Nov. 2001 and that in Nov. 2000.

Mean (± SEM)* GB conc. (μg/m³)	Exposure time (min)									
	30	60	90	120	150	180	210	240	270	300
7.3 ± 0.1	d_2 b_2	b_2	d_2 t_1 b_2	t_1	d_2 t_1 t_2	t_1 t_2 b_2	t_1 t_2 b_2 *b_2*	t_1 t_2 b_2 *b_2*	t_1 *b_2*	t_1 *b_2*
14.6 ± 0.2	*t_1*		*d_1*		*d_1*	*b_2*	*b_2*	b_2 *b_2*		t_1 b_2
21.8 ± 0.4	a_1 *a_1*	t_1 t_2 a_1 *$t_1 t_2 a_1$*	t_1 t_2 a_1 *$t_1 t_2 a_1$*	*a_1*	t_2 a_1 *$t_1 t_2 a_1$*	t_2 a_1 *$t_1 t_2 a_1$*	t_1 t_2 a_1 *$t_1 t_2 a_1$*	t_2 a_1 b_2 *$t_1 t_2 a_1$*	t_2 b_2 *$t_1 t_2 a_1$*	t_2 b_2 *$t_1 t_2 a_1$*
49.7 ± 0.6	*b_2*	*$t_1 t_2 a_1$* b_2	*$t_1 t_2 a_1 b_2$*	*$t_1 t_2$ b_2*	*t_2 b_2*	b_2 *t_2 b_2*	*$t_1 t_2 a_1$* *b_2*	b_2 *$t_1 t_2 a_1$* *b_2*	*b_2*	*t_1 b_2*
137.7 ± 1.7	*a_1*	d_1 t_1 t_2 b_1 *b_2*	t_1 *d_1 d_2 t_1 b_2*	t_1 t_2 *d_1*	d_1 t_1 t_2 *d_1*	t_1 t_2 b_2 *d_1 b_2*	t_1 t_2 a_1 a_2 *t_2 b_2*	*b_2*	*b_2*	b_2 *b_2*

**Time-based average of vapor concentrations measured at 2-5 min intervals.*

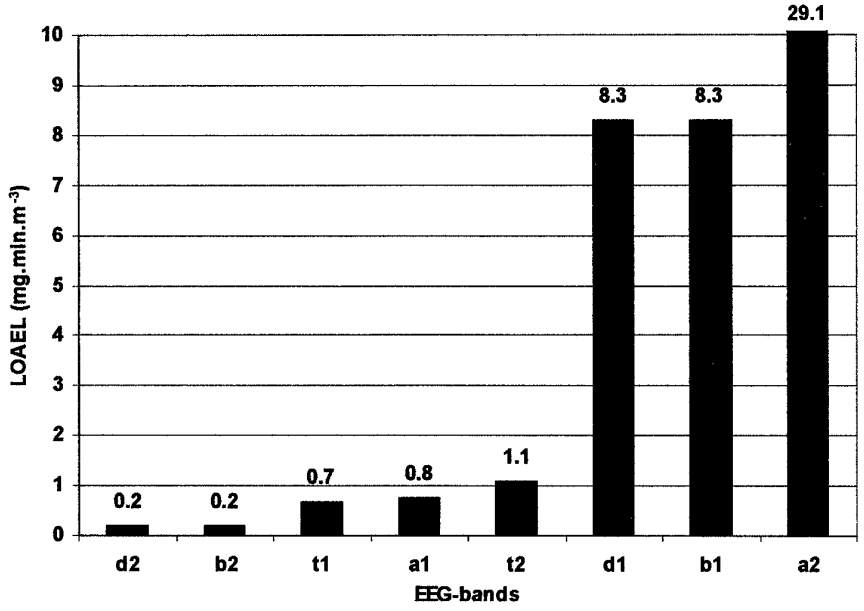

Fig 3. EEG-bands (horizontal axis) of vehicle-pretreated GB-exposed marmosets (Nov. 2000) which became first significantly ($p < 0.05$) different from the corresponding bands in air exposed animals, and the calculated corresponding LOAEL levels (vertical axis).

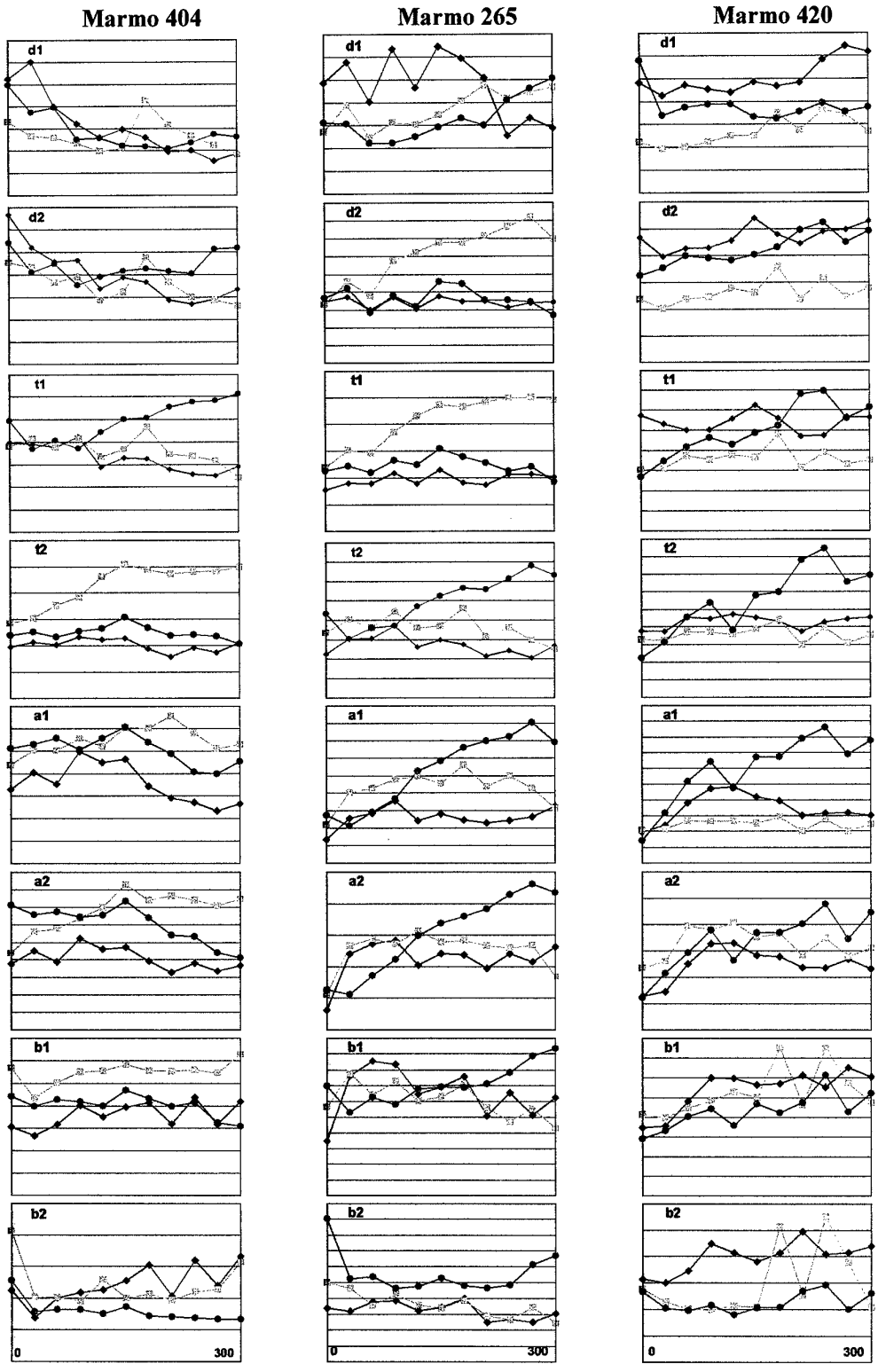

Fig 4. The course of the energy (μV^2) per EEG-band d_1, d_2, t_1, etc.(vertical axis) of vehicle-pretreated marmosets monitored first during a 300 min (horizontal axis) exposure to air in Nov. 2000 (blue curve, ◆), and then to GB vapor in air in Nov. 2000 (green curve, ■), and finally to air again in Nov. 2001 (red curve, ●). Marmo 404 was exposed to: 7.3 $\mu g/m^3$ GB; Marmo 265: 14.6 $\mu g/m^3$ GB; Marmo 420: 21.8 $\mu g/m^3$;

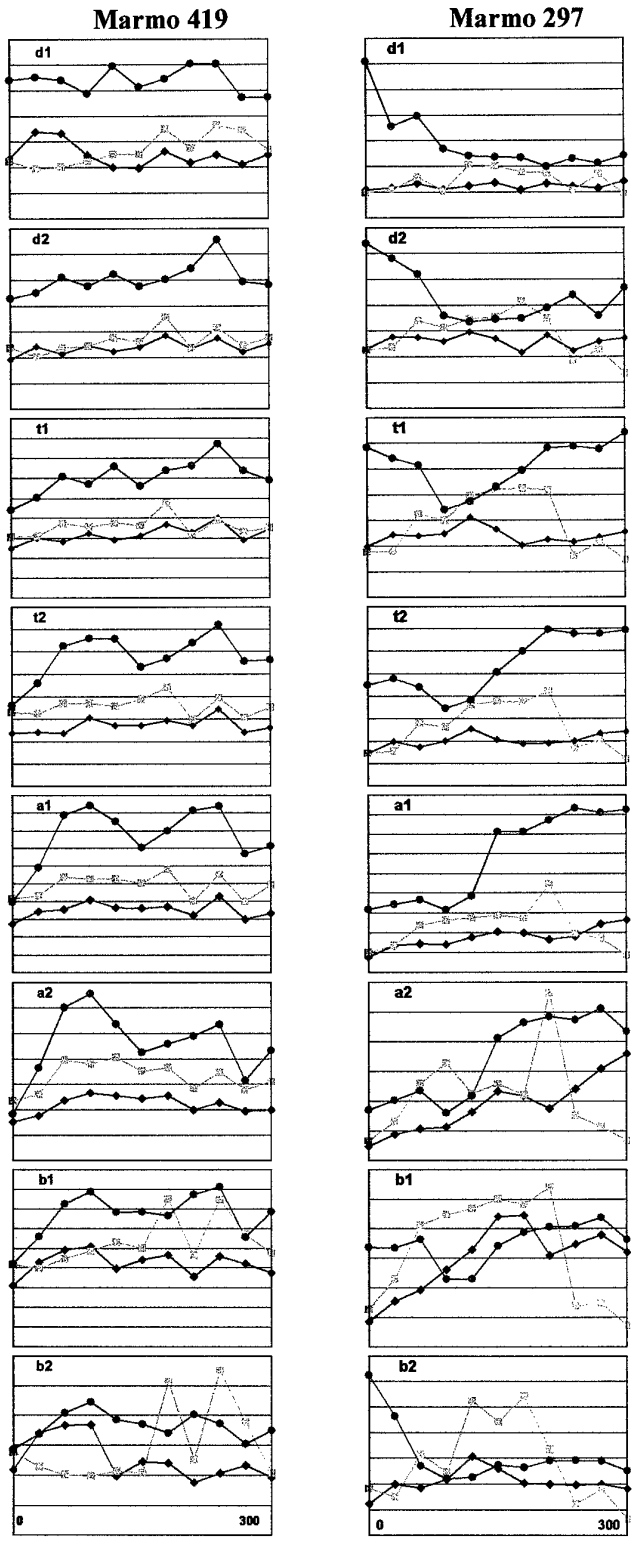

Fig 4 (continued). Marmo 419: 49.7 µg/m³; Marmo 297: 137.7 µg/m³. Note that the energy in most EEG-bands of all animals is higher during the recent air-exposure (red curve, ●) compared to that in Nov. 2000, i.e. before GB-exposure (blue curve, ◆).

Pyridostigmine-pretreated marmosets

The analysis of the online registered EEG epochs from pyridostigmine-pretreated marmosets (n = 5) during a 5 h exposure to air in Nov. 2000, is demonstrated in Fig 5. The averaged amounts of energy per band (d_1, d_2, t_1 etc.) did not change significantly between t = 0 and t = 300 min of exposure.

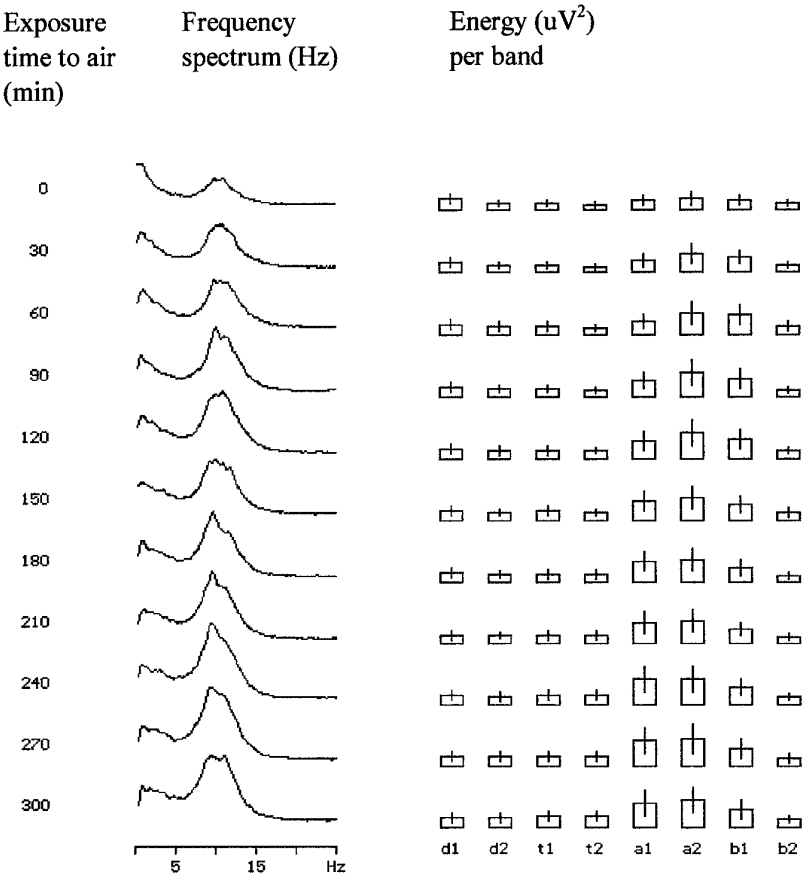

Fig 5. EEG analysis of marmosets (n = 5) provided with Alzet pumps containing pyridostigmine (0.04 mg/kg/h) and exposed to air for 5 h (Nov.2000). Indicated are the exposure time intervals at which the EEG-analysis was carried out, the averaged frequency spectrum and the averaged energy (uV^2) *per EEG-band per time interval.*

These averaged amounts of energy per EEG band of air-exposed animals were compared to the amounts of energy of the corresponding EEG bands of GB-exposed animals (7.2, 14.6, 24.2, 51.7 or 145.9 $\mu g/m^3$, one animal per concentration). In Table 2 only the EEG changes which appeared to be significantly different ($p < 0.05$) from that in air-exposed animals are given. For all significant changes given in Table 2, the *LOAEL* (C.t) values were calculated using the actual GB concentrations during the course of exposure (not shown) instead of the indicated mean concentrations in the table. These *LOAEL* values are given in Fig 6. It appeared that the t_1 and d_2 EEG-bands were most sensitive to GB exposure, whereas the b_1 band was least sensitive. The lowest C.t-value established in this way was 0.1 $mg.min.m^{-3}$, representing the *LOAEL* for the first emerging EEG changes in pyridostigmine-pretreated and GB-exposed marmosets.

In Nov. 2001 two marmosets of the pyridostigmine-pretreated group appeared to have faulty EEG-electrodes and could therefore not be analyzed again. These were the two animals which had been exposed to 7.2 $\mu g/m^3$ and 24.2 $\mu g/m^3$ GB, respectively.

The remaining three pyridostigmine-pretreated marmosets demonstrated EEG-bands which were still significantly ($p < 0.05$) different from the corresponding bands during air-exposure in Nov. 2000 (see Table 2).

It should be emphasized, however, that the marmoset which was exposed to 51.7 $\mu g/m^3$ GB in Nov. 2000, did not show any significantly different bands during GB-exposure at that time, whereas during air-exposure in Nov. 2001 many EEG-bands differed significantly from the corresponding bands during air-exposure in Nov. 2000. Also, the marmoset which had been exposed 145.9 $\mu g/m^3$ hardly showed any significantly different bands during exposure one year earlier. Whether this observation reflects late effects on EEG as a result of GB exposure one year earlier, is not clear. In these animals the energy (μV^2) per EEG-band was not higher in Nov. 2001 than that observed during air-exposure in Nov. 2000 (Fig 7). During the EEG-recording of marmoset 406 in Nov. 2001 the receiver had been disconnected for about 2 hours due to a technical failure. Since animals are always tested in a separate room to prevent disturbance by human interaction on the EEG, a video camera was used to detect failures. However, this unexpected incident was not noticed by the video camera.

Table 2. Statistically analyzed differences between EEG-bands from pyridostigmine-pretreated and GB-exposed (7.2, 14.6, 24.2, 51.7, or 145.9 $\mu g/m^3$ GB, one animal per concentration) marmosets, and the corresponding EEG-bands from pyridostigmine-pretreated and air-exposed (n =5) animals. Indicated are the EEG-bands which were significantly different ($p<0.05$) from the corresponding bands in air exposed animals (Nov. 2000). The italicized bands (red) represent the significant ($p<0.05$) differences in EEG between air-exposure in Nov. 2001 and that in Nov. 2000.

Mean (± SEM)* GB conc. ($\mu g/m^3$)	Exposure time (min)									
	30	60	90	120	150	180	210	240	270	300
7.2 ± 0.1	t_1			b_2			b_2		b_2	
14.6 ± 0.2	d_2 t_1	d_2	d_2 t_1 *t_1 a_1*	d_2 t_1 t_2	d_1 d_2 t_1 t_2 a_1 a_2 *d_1 a_2*	d_1 d_2 t_1 t_2 a_1 a_2 *d_1 a_2*	d_1 d_2 t_1 t_2 a_1 a_2 *d_1*	d_2 t_1 t_2 a_2	d_2 t_1 t_2 *d_1 a_2*	d_1 d_2 t_1 a_1 a_2 *d_1 a_2*
24.2 ± 0.9	d_2	d_2 a_2	d_1 d_2 t_1 t_2	d_2 t_1 a_2	d_2 a_2	a_2	d_2 a_2	d_2 t_1 a_2	a_2	a_2 b_1
51.7 ± 1.2	*d_2 t_1*	*d_1 d_2*	*d_1 d_2*	*d_2*	*d_1*		*d_1*	*d_1*		*d_1*
145.9 ± 1.3	t_1		*d_1 d_2 t_1 b_2*	x	x	x	x			*d_1*

**Time-based average of vapor concentrations measured at 2-5 min intervals; x = not recorded (see text)*

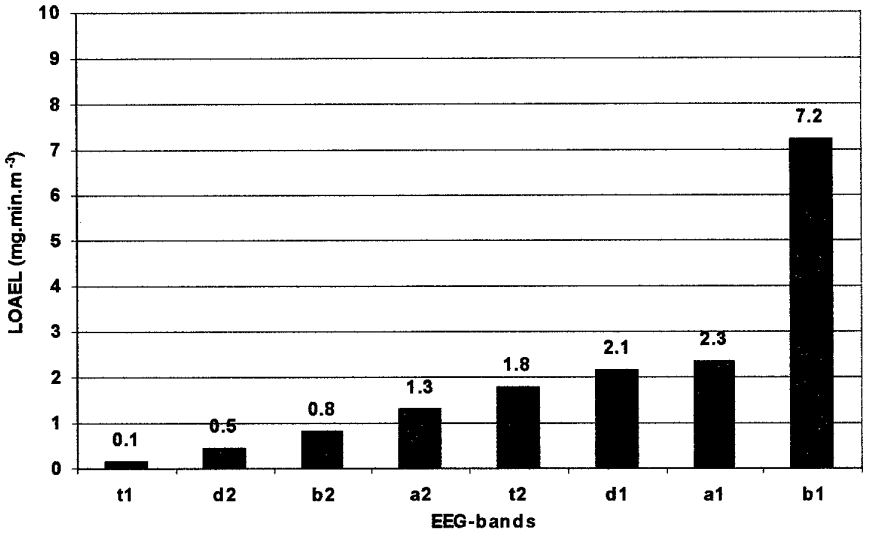

Fig 6. EEG-bands of pyridostigmine-pretreated GB-exposed marmosets (Nov. 2000) which became first significantly ($p < 0.05$) different from the corresponding bands in air exposed animals (horizontal axis), and the calculated corresponding LOAEL levels (vertical axis).

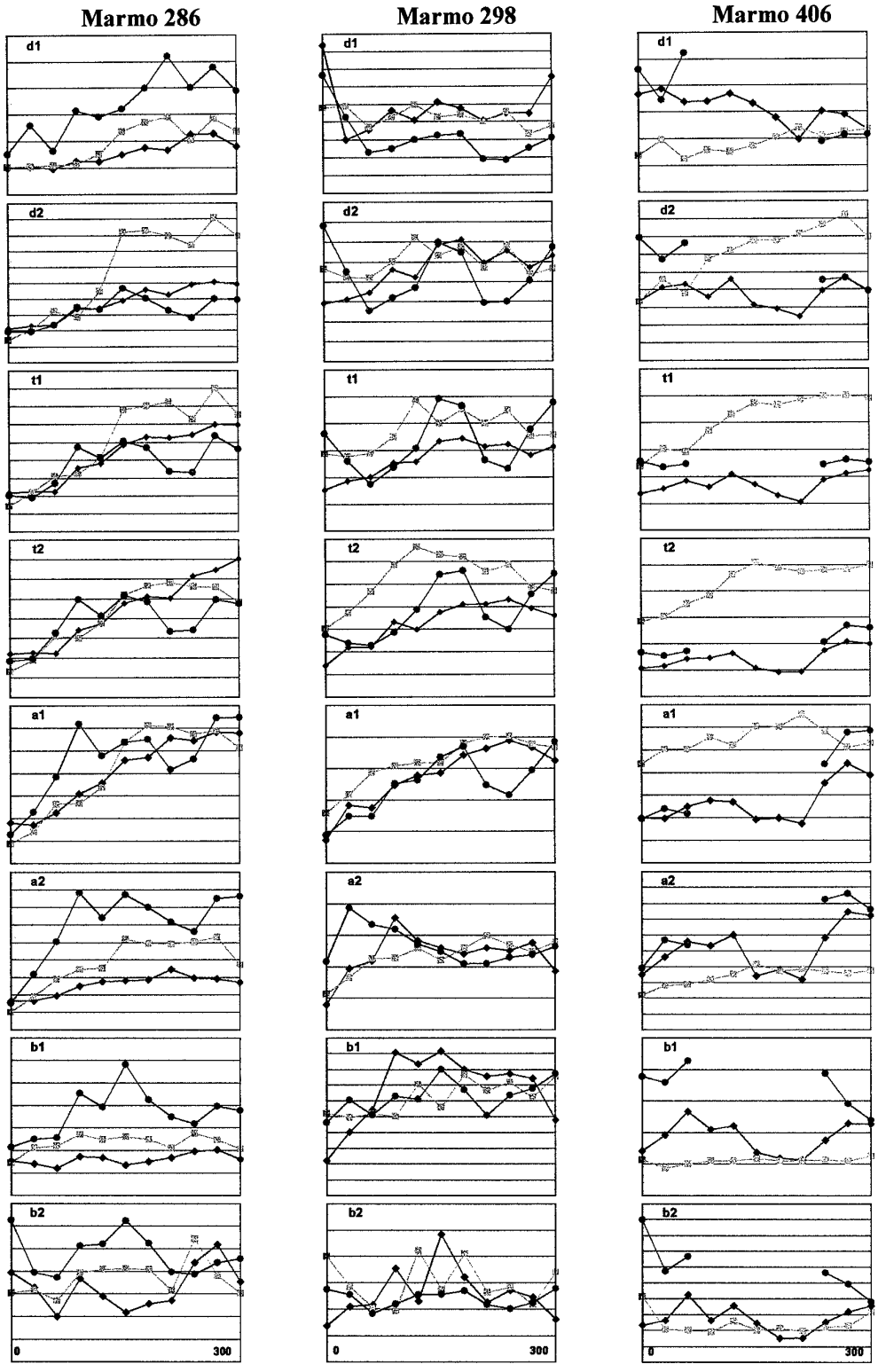

Fig 7. The course of the energy (μV^2) per EEG-band d_1, d_2, t_1, etc.(vertical axis) of pyridostigmine-pretreated marmosets monitored during a 300 min (horizontal axis) exposure to air in Nov. 2000 (blue curve, ◆),and then to GB vapor in air in Nov. 2000 (green curve, ■), and finally to air again in Nov. 2001 (red curve, ●). Marmo 286 was exposed to: 14.6 µg/m³; Marmo 265: 14.6 µg/m³ GB; Marmo 298: 51.7 µg/m³; Marmo 406: 145.9 µg/m³. *Note that the energy in most EEG-bands of all animals is not higher during air-exposure in Nov. 2001 (red curve, ●) compared to that in Nov. 2000 (blue curve, ◆).*

As mentioned, the EEG was analysed by using fast Fourier transforms (FTT) which makes it possible to quantify the effects on the power spectra of different frequency bands, and by visual observation of the raw EEG-signals to qualify typical clinical effects on the EEG.
For visual observation the EEG-recordings before GB exposure (baseline EEG) (Nov. 2000) were compared to the EEG-recordings in Nov. 2001. From these baseline EEG-recordings it appeared that the amplitude of the signals varied between the animals, which is presumably due to differences in thickness of the skulls between the different animals and to possible small differences in electrode placement. Therefore, each EEG signal was compared with the previous registration during air-exposure of the same animal (its own control).
In the baseline EEG registration a random signal was found in which all frequencies were present. Beside this random signal, clear bursts of alpha activity (around 9 Hz) with a duration of 1-3 sec each were present (Fig 8). In sleep-EEG research such bursts are indicated as sleep-spindles which represent the electrographic landmark for the transition from waking to sleep which is associated with loss of perceptual awareness (Steriade et al., 1990). These spindle oscillations seem to be generated in reticular thalamic neurons and are transferred along the thalamocortical pathways to neocortical neurons (Steriade et al., 1985; Steriade et al., 1990).

Fig 8: Example of spindles (arrows) in a marmoset monkey EEG-recording lasting 30 sec.

One year after exposure to low levels of GB vapor in air these spindles were more frequently present in all the pyridostigmine-pretreated GB-exposed animals and in the vehicle-pretreated animals exposed to 7.5, 25 or 150 $\mu g/m^3$ GB (see Table 3). The duration of the spindles in some cases was also increased to 4-8 sec. At concentrations of 15 and 50 $\mu g/m^3$ GB there was a decline in the spindle activity observed in vehicle-pretreated marmosets.

Table 3: Percentage increase (+) or decrease (-) in spindle numbers compared to baseline values.

Pretreatment	GB dose range (µg/m3)				
	7.5	15	25	50	150
Vehicle	+ 130	- 16	+ 131	- 16	+ 6
Pyridostigmine	NT	+ 765	NT	+ 26	+ 32

NT: not tested due to faulty electrodes.

DISCUSSION

The present findings show that in marmoset monkeys there are still statistically significant EEG abnormalities present one year after a 5 h whole-body exposure to low levels of GB vapor in air. These findings are consistent with those of previous studies demonstrating disorders in the central nervous system in subjects exposed to sarin, as revealed by the persistence of an abnormal EEG (Duffy et al 1979; Burchfiel and Duffy 1982) or an abnormal evoked potential (Murata et al 1997). Disorders in the central nervous system of rescue workers about 3 years after the Tokyo subway sarin attack, are also consistent with our present findings. These rescue workers suffered from a chronic decline of memory function (Nishiwaki et al 2001).

The mechanism of EEG abnormalities and memory disturbance due to GB exposure remains unclear. Whether these disorders are truly caused by a direct neurotoxicity of GB, remains to be established. It should be emphasized that the marmosets in our present study showed significant inhibition of AChE-activity in their blood and disturbances in memory and motor function about one hour after the exposure to GB. As AChE-activity will recover to normal levels in relatively short time, the EEG-abnormalities observed in the present study may have been caused by unknown mechanisms other than ChE-inhibition. However, the changes observed in spindle oscillation can be the result of changes in the cholinergic system caused by GB. Activation of the cholinergic brainstem-thalamic pathways can lead to a decrease of spindle oscillations (Steriade et al., 1990). Furthermore, activation of cholinergic forebrain-thalamic projections can lead to a similar effect (Buzsaki et al., 1988a, Buzsaki et al., 1988b). Therefore, activation of the inhibitory GABA-containing axons of the reticular thalamic nucleus, which is considered as the spindle pacemaker, is presumably responsible for the increase of the spindle oscillations. Spindle increase is related to an inhibition of sensory input and strongly associated with doze off activity, whereas a decrease in spindle number is correlated with increased activity. Murata et al (1997) suggested that abnormal event-related potentials which were detected in subjects exposed to GB may imply a consequence of lasting hippocampal pathology induced by GB. According to an interesting recent study using the patch-clamp technique, GB appears to inhibit the evoked release of GABA at low concentrations in rat hippocampal neurons, which is believed to be closely associated with memory function (Rocha et al 1998; Chebabo et al 1999).

Although there have been hardly any comprehensive studies of the effects of GB poisoning on the central nervous system, epidemiological studies on organophosphate (OP) pesticide intoxication can provide valuable information. Rosenstock et al (1991) reported significant differences between subjects exposed to OP pesticides and age-matched controls in the digit span test and the Benton visual retention test.

In animals repeatedly exposed to the OPs disulfoton or DFP, memory deficits have been reported (McDonald et al 1988; Bushnell et al 1991). Such cognitive impairment and altered EEG were also observed in some studies of occupationally GB exposed workers one year or more after the last exposure (Gershon and Shaw 1961; Metcalf and Holmes 1969). Spectral analysis of the EEG indicated significant increases in beta activity (12-30 Hz) in the exposed group, and sleep EEGs revealed significantly increased rapid eye movement (Gershon and Shaw 1961). Following a 2-week exposure to disulfoton, the density of muscarinic receptors was decreased to a similar degree in hippocampus and cerebral cortex (Costa et al 1990).

During the Tokyo subway event, as well as during the exposure of our conscious marmosets to GB vapor in air, a strong mental impact (stress) by itself can not be excluded. Kaufer et al (1998) demonstrated that both stress and AChE inhibition can lead to dramatic and persistent upregulation of AChE production in the central nervous system. This upregulation of AChE may lead to a shortage in synaptic ACh and might therefore result in a progressive deterioration in cognitive and neuromotor functions. It has been known for some time that exposure to stressors can result in long-term neuronal hypersensitivity, but the molecular mechanisms underpinning this response are poorly understood. Soreq and colleagues recently

showed that when mice were exposed to various types of experimental stress the production of AChE mRNA switched from the commonly prevalent form of the enzyme that is bound to the synaptic membrane (AChE-S) in favour of mRNA for a normally rare soluble form (AChE-R). This change persisted for weeks (Meshorer et al 2002). Moreover, this change compromised the capacity to cope with intensified cholinergic stimuli. One month after exposure to stress, mice hippocampal neurons showed strinkingly stronger responses to electrical stimulation *in vitro* in the presence of an anticholinesterase, than neurons from control mice. In this connection, a remarkable finding in our present study is of interest. We observed that in most vehicle-pretreated marmosets exposed to GB in Nov. 2000 the energy per EEG-band was higher during a 5 h air-exposure in Nov. 2001, than that observed one year earlier (Fig 4), which might indicate that neurons have become more sensitive for excitation. It should be emphasized that the marmosets were conscious and under restrainment (stressor) during the 5 h lasting exposure to GB in Nov. 2000. This observation which may be relevant to Gulf War and 'post-traumatic-stress-disorder' patients. However, the outcome of the present investigations should be considered as preliminary because of the limited number of monkeys used, and therefore require further effort for confirmation.
An interesting preliminary finding of the DAMD17-97-1-7360 study was that during exposure to GB, the EEG-signal seemed to be more vulnerable to GB than the eye (about an order of magnitude) both in guinea pigs and marmosets. This finding is of particular interest since the National Advisory Committee for Acute Exposure Guideline Levels for Hazardous Substances (NAC/AEGL Committee) expressed recently (May, 2001) that a major concern associated with exposures to anti-ChE agents such as the G-agents, is the possibility of chronic neurological effects even after asymptomatic exposures.

KEY RESEARCH ACCOMPLISHMENTS

One year after a 5 h low level whole-body exposure of conscious marmosets to GB vapor in air, in a concentration range of 7.5-150 $\mu g/m^3$, abnormalities in their EEG-signals were still significant ($p < 0.05$).

REPORTABLE OUTCOME

Conscious vehicle- or pyridostigmine-pretreated marmoset monkeys demonstrated significant ($p < 0.05$) changes in most of their EEG-bands during a 5 h lasting whole-body exposure to GB concentrations in the range of 7.5-150 $\mu g/m^3$ (low level exposure) as compared to the corresponding bands during a previous 5 h lasting air-exposure. The AChE-activity in blood samples taken during GB-exposure was significantly inhibited and one hour after the exposure disturbances in memory and motor function were observed. One year later, during a 5 h lasting air-exposure, all marmosets still demonstrated EEG-bands which differed significantly ($p < 0.05$) from corresponding bands during air-exposure one year earlier. In most vehicle-pretreated marmosets the energy (μV^2) per EEG-band was higher than that observed one year earlier. This phenomenon was less pronounced in pyridostigmine-pretreated animals.
Visual examination of the EEG-records revealed clear bursts of alpha frequencies (around 9 Hz), so-called sleep-spindles, which were more frequently present in pyridostigmine-pretreated GB exposed animals and in the vehicle-pretreated animals exposed to 7.5, 25 or 150 $\mu g/m^3$ GB. It was discussed that these late changes in spindle oscillation might be the result of changes in the cholinergic system, and concluded that one year after a 5 h low level exposure to GB vapor in air, abnormalities in the EEG-signal were still significant ($p < 0.05$).

Van Helden HPM, Vanwersch RAP, Kuijpers WC, Philippens IHC, Langenberg JP, Benschop HP (2001). Supplement to Final Report of Grant Agreement DAMD17-97-1-7360: Low Level exposure to GB vapor in air: Diagnosis/Dosimetry, Lowest Observable Effect Levels and performance-incapacitation.

CONCLUSIONS

It is concluded that one year after a 5 h whole-body exposure of conscious marmoset monkeys to low levels of GB vapor in air, in a concentration range of 7.5-150 $\mu g/m^3$, abnormalities in their EEG-signals are still significant ($p < 0.05$).

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