

25. ASSESSING THE EFFECTS OF LOW DOSE EXPOSURE TO ANTICHOLINESTERASES

Leah Scott and Peter Pearce
Biomedical Sciences Department,
CBD Porton Down, Salisbury, SP4 0JQ.

ABSTRACT

The effects of exposure to low doses of cholinesterase inhibitors are still poorly understood. Reliance upon human epidemiological evidence to assess the effects of such exposure is unsatisfactory because of the uncertainty associated with exposure levels and the potential complication of a plethora of pre-disposing factors. There is, therefore, a requirement to conduct focused research in relevant animal models which optimise the confidence with which animal-derived data can be extrapolated to man. Such an approach will provide a scientifically sound context for the interpretation of epidemiological investigations.

Burchfiel et al (1976) suggested that, in non-human primates, a sign-free dose of sarin, induced small but statistically significant changes in aspects of the electroencephalogram (EEG) although the functional significance of these changes was not known. Recent studies at CBD have led to the development of a sophisticated, multifaceted non-human primate model, which revisits and extends these earlier studies.

The model has already been used to investigate the effects of a similar dose of sarin on EEG, sleep and cognitive function in marmosets. Pearce et al, (1999) did not replicate the previously reported EEG changes and found no decrement in cognitive performance over the 15-month study duration. The model offers considerable potential for studying the effects of a wider dose range of sarin and other compounds of interest.

The approach adopted and the results of the study will be described in detail as well as related work in progress and opportunities for the future.

BACKGROUND

There is widespread concern in both military and agricultural communities as to whether acute or chronic exposure to low doses of organophosphate (OP) anticholinesterase compounds, at doses which do not produce marked clinical signs, give rise to adverse long term effects. While the acute toxicity of OP compounds is well characterised (e.g. McLeod, 1985) long term effects relating to central functioning are poorly understood. Many studies have suggested that administration of OPs produce effects in the central nervous system that persist for many weeks or months (e.g. Grob & Harvey, 1953; Steenland, 1996). Typical symptoms which have been reported include tension, anxiety, difficulty in concentrating, slowness of recall, mental confusion and sleep disturbances (e.g. Sherman, 1995). The majority of these studies, however, have lacked precise information on the level of OP exposure, the degree of acetylcholinesterase (AChE) inhibition and the time course of sequelae. For example, Bowers et al (1964) investigated 93 Army or Air Force personnel exposed to OP and found changes in a variety of psychological parameters. However, no quantitative testing of cognitive function was carried out. Follow up of victims of the sarin attack on the Tokyo underground in 1995 has suggested long term effects on behaviour may have occurred in some people (Yokoyama et al 1998).

The rapid onset of signs and symptoms of poisoning following OP exposure can be explained in terms of acetylcholine accumulation following cholinesterase inhibition but no mechanism has been identified for the induction of long term effects. Experimental studies in non-human primates have suggested that administration of clinically sign free doses of the OP sarin (isopropyl methylphosphonofluoridate) can give rise to subtle but statistically significant changes in brain electrical activity, as gauged by electroencephalography (EEG). Burchfiel, Duffy & Sim (1976) demonstrated a significant increase in the fast beta component (13-50 Hz) of the EEG spectrum in response to both a large acute and repeated small doses of sarin in rhesus monkeys. In this study however, EEG was monitored only at 24 h and 12 months following dosing and cholinesterase assessments were not reported. The functional significance of these changes is not known.

The object of the study recently conducted at CBD (Pearce et al, 1999) was to ascertain whether administration of an acute low dose of sarin would give rise to long-term EEG changes in common marmosets (*Callithrix jacchus*). In order to assess the functional significance of any EEG changes which might be observed, the animals were trained to perform complex behavioural test sequences. Regular monitoring of AChE activity was also incorporated into the study design which was based upon, and represents a refinement of, earlier unpublished preliminary studies conducted in a small number of rhesus monkeys at CBD. Marmosets were selected for the present study because of their increasingly widespread use in neuropsychological investigations (e.g. Roberts et al, 1988; Crofts et al, 1995; Pearce et al, 1998; Ridley et al, 1996). Their small size and species characteristics make them particularly suitable for long-term studies.

The behavioural test employed was modified from human and animal studies and is based upon one element of the

Cambridge Neuropsychological Test Automated Battery (CANTAB). It involves the presentation of a number of visual discriminations on a touch sensitive screen and is derived from the Wisconsin Card Sort Test (Grant & Berg, 1948). A number of rule changes are incorporated which are differentially sensitive to a range brain lesions (e.g. Roberts et al, 1992) and pharmacological interventions (Sahakian & Coull, 1993). Tests from this battery are suitable for presentation to both humans and non-human primates and the sequence of stages was adapted from previous human (e.g. Sahakian et al, 1990) and primate (Roberts et al, 1988) studies to facilitate long term repeated presentation. A home cage approach to testing was employed, which has previously been shown to be practicable (Crofts et al, 1995, 1996) and conducive to rapid training and task acquisition (Muggleton et al, 1997).

Traditionally, EEG has been monitored in primates by methods which include the use of restraint chairs (Adams & Barrett, 1974) or backpack and umbilicals (Pearce et al, 1989). In the recent study, EEG measurement was carried out remotely by making use of implantable radiotelemetry which enables acquisition of high quality data over long periods of time with minimal disruption to the animal. The feasibility of this approach has previously been reported elsewhere (Pearce et al, 1996, 1998) and offers considerable advantages over 'traditional' methods of EEG recording, especially in the context of long term studies.

METHODS AND MATERIALS

Animals and diet: Seventeen common marmosets (8 male and 9 female, bred at CBD Porton Down) weighing 319-516g at the beginning of the study were used. All animals were pair housed either in single sex pairs or in mixed sex pairs in which the males were vasectomised. Their daily diet, given after testing, consisted of 20g pellets (Complete primate diet E, Special Dietary Services, Witham, Essex, UK) with supplements of orange segments. On non-testing days, alternative supplements included banana, apple and egg. A sawdust filled tray, in which a small amount of preferred foods (e.g. Rice Krispies, sunflower seeds) was dispersed, allowed the animals to freely engage in foraging behaviour. No form of food deprivation was employed during the study and water was available ad-libitum.

Housing: Housing for each pair consisted of 4 stainless steel cage units measuring H72 x W47 x D60 cm connected together by 2 horizontal external extensions and 1 vertical extension (H18 x W71 x D23 cm and H105 x W 17 x D 23 cm respectively) in order to allow full use of all 4 units. Various items of cage furniture, including hanging wooden dowels, buckets and other playthings, were also placed in the cages. During behavioural training and testing the pairs were separated so that each had use of a single upper unit of its home cage with a rigid extension unit (H18 x W17 x D30 cm) attached to the front. Illumination was provided by sodium lighting, at a level of 350-400 lux at 1 m above the ground, and maintained on a 12 hour light/dark cycle with dusk and dawn effects over 1 hour periods. Temperature was maintained at 25 °C with 40% humidity.

Drugs: Sarin (isopropyl methylphosphonofluoridate) was synthesised at CBD Porton Down to 95% purity and was stored as 5.0 mg.mL⁻¹ solutions in isopropanol. Solutions were diluted to appropriate concentrations with 0.9% saline to give an injection volume of 0.5 mL. 0.9% saline was used for control injects, again in a volume of 0.5 mL.

Overview of experimental design: The experimental design was centred upon successive presentation of behavioural test sequences over months. The sequence of procedures was conducted in all subjects although the precise time course of the study was dictated by the behavioural performance of individual animals, which served as their own controls.

The first behavioural test sequence was presented after stable baselines of performance had been established. This was followed by surgical implantation of a radiotelemetry transmitter and electrodes which enabled EEG to be recorded at intervals throughout the study. After recovery from surgery, the second and third behavioural test sequences were presented. Control blood samples were taken at intervals to determine baseline levels of erythrocyte cholinesterase activity before administration of sarin or control vehicle. Subsequent to dosing, behavioural test sequences were presented as appropriate.

Administration of either saline or 2.5 or 3.0 µg.kg⁻¹ sarin by i.m. injection occurred following completion of the third behavioural sequence. The sarin dose was adjusted to 3.0 µg.kg⁻¹ following cholinesterase estimations from the first two subjects dosed which received a dose of 2.5 µg.kg⁻¹. Data from these subjects were included in the analysis as they did not fall outside the range of inhibitions seen for subjects receiving the higher dose. Subsequent to dosing, further behavioural test sequences were presented.

EEG telemetry:The apparatus and surgical procedures used have been fully described elsewhere (Pearce et al, 1998). EEG was collected at least weekly during periods when subjects were undergoing behavioural testing (see below).

BEHAVIOURAL TRAINING AND TESTING

The equipment, training and behavioural test sequences have been described in detail elsewhere (Pearce et al, 1998, Muggleton et al, 1997). After preliminary training, animals were presented with simple discriminations involving pairs of stimuli which consisted of either blue filled 'shapes' or white 'lines' followed by successive presentations of more complex discriminations and reversals. Testing sessions occurred daily Monday-Friday and the maximum session duration was 30 minutes with a maximum of 60 trials per session. At the end of the training phase, subjects were assigned as either 'shape' or 'line' responders. Subsequent test sequences each consisted of a nine stage series of discriminations based on that used by Roberts et al (1988). A criterion of eight successive correct responses determined progression between stages.

Each sequence began with a simple discrimination stage (SD) followed by a simple reversal (SR). Shapes and lines were then combined to form the first compound discrimination (CD) and animals were rewarded for attending to the dimension, shape or line, to which they had initially been assigned. Following presentation of a reversal of this compound discrimination (CR), a pair of novel compound stimuli were presented and animals were rewarded for attending to the same dimension as previously (IDS, intra dimensional shift). Following a reversal (IDR) another pair of novel compound stimuli were presented. At this stage the animals were rewarded for attending to the previously unrewarded dimension (EDS, extra dimensional shift). The sequence finished with a reversal (EDR). This sequence was repeated for the duration of the study using novel stimuli.

Animals underwent one test sequence before the EEG implantation procedure, followed by another two sequences before administration of sarin or saline. No behavioural testing was conducted for one month after surgery or for one week periods between test sequences thereafter. Eight subjects received saline injections and nine received sarin. Groups were balanced on the basis of training performance as well as for other factors, such as gender, as far as possible.

Acetylcholinesterase Determination: Acetylcholinesterase activity in whole blood and plasma was determined by the method of Ellman et al (1961). Three control samples were obtained, one sample taken 3 hours following administration of sarin or saline and subsequent samples taken at approximately monthly intervals.

DATA HANDLING AND ANALYSIS

EEG: The approaches to EEG sampling and analysis have been reported elsewhere (Pearce et al 1998). EEG results were calculated as amplitude (μV) per Hz in the frequency bands delta (1-3.5 Hz), theta (3.5-7.5 Hz), alpha (7.5-13 Hz), beta 1 (13-22 Hz), beta 2 (22-40 Hz), beta (13-40 Hz) and total amplitude. Median frequency was also calculated. In order to make between animal comparisons as well as within animal comparisons, all data were normalised such that post dose data were calculated as a change from a mean of at least 10 recordings made pre-dose. Mixed model analyses of variance, including time as a linear covariate, were used to analyse the above 8 outcomes separately and a \log_{10} transformation of the data was used to satisfy assumptions of normality (the Watson statistic) and equal variances (Bartlett's test).

Behaviour: Mixed model analyses of variance with five fixed factors were used to compare the effects of Stimulus type (shape or line), Stage (SD,CD,IDS,EDS), Reversal, Period and Dose on the number of errors made in achieving criterion at each stage. Period was defined as period 1 (averages of sequences 4-6), period 2 (sequences 7-9) and period 3 (sequences 10-12), each expressed relative to the average of baseline sequences 1-3. The presence of any interaction between any of these effects was considered. Normality assumptions were checked using the Watson statistic and Bartlett's test was used to check for equal variances. As the residuals from the analysis of total number of errors data violated the above assumptions, a \log_e (No. of errors + 1) transformation was used. Post-hoc analyses of significant effects was carried out using appropriate contrasts which involved investigating ratio of ratios.

RESULTS

EEG: Details of statistical interactions are given in Table 1. Figure 1 displays the mean amplitude per Hz for frequency bands theta, alpha, beta 1 and beta 2, expressed as a percentage change from pre-exposure values.

No significant changes between sarin and control groups as a pattern over time (dose \times time interaction) in either theta ($p=0.65$) or alpha ($p=0.22$) frequency bands were noted, although there was a divergence between control and treated animals after 12 months recording in the latter.

In the beta 1 frequency band, there was no change in treated animals despite a non significant fall in amplitude in control animals (dose \times time, $p=0.22$).

In the beta 2 frequency band, there was an increase in amplitude in treated animals when compared with both baseline values and control animals. Over time, the effect of sarin approached statistical significance ($p=0.07$). The estimated difference in slopes between control and sarin subjects was found to be 0.005 (confidence interval -0.8% , 1.8%), representing an extra increase in beta 2 of 0.5% per month in sarin subjects. However, within the group of treated

animals, amplitude increased by over 40% in one animal. If this animal was excluded from the results analysis, the dose \times time interaction was found to be $p=0.13$ and the difference in slope of 0.08% per month (confidence interval -1.2% , 1.4%).

Neither delta, total amplitude nor median frequency parameters showed any differences approaching statistical significance. No significant differences between subjects assigned to respond to shapes and those assigned to lines (stimulus type) arose due to sarin administration.

Behaviour: The long-term performance profile obtained was comparable for the control and dose groups. Figure 2 illustrates the changes in the number of errors made in reaching criterion at each stage of each sequence corrected to a mean of the three sequences performed pre sarin or saline administration. Results of the behaviour analysis of sarin effects are shown in Table 2.

Three significant three way interactions were found for type \times dose \times stage ($p = 0.015$), type \times reversal \times stage ($p < 0.001$) and type \times stage \times period ($p < 0.001$). The three way interaction involving dose was investigated further, results of which are shown in Table 3, as this analysis would encompass factors seen in the two way interactions. The four-way interaction was not analysed further as the important aspects were considered in evaluation of the three way interactions.

On one component of the test, the first compound discrimination (CD), the sarin treated groups of animals performed significantly better than control animals for shapes, but for lines they were similar ($p<0.001$) (see Table 4). In addition, superior performance of the sarin treated group was seen at the simple discrimination stage (SD).

Acetylcholinesterase activity: Three hours following sarin administration mean erythrocyte cholinesterase inhibition was 51.3% (range 36.4 - 67.1%) and this returned to baseline levels 4-45 weeks later. When average cholinesterase activity three months after dosing was compared with average pre-dosing controls no significant difference was seen ($p=0.50$) so, on average, levels had returned to baseline by three months. No changes over time were seen in control subjects.

DISCUSSION

A single low dose of the organophosphorous compound sarin, leading to acetylcholinesterase inhibition of 51%, produced no significant changes in either EEG, as measured by radiotelemetry, and no decrement in cognitive behaviour, as measured by an attentional set-shifting task, in the common marmoset.

This lack of effect on EEG is inconsistent with previous studies (e.g. Burchfiel et al 1976) and unpublished work at CBD which showed small changes in the beta 2 frequency band in a limited number of rhesus monkeys 7-9 months following a comparable dose of sarin ($2.5\mu\text{g}\cdot\text{kg}^{-1}$). There are significant methodological differences between these earlier rhesus monkey studies and the present marmoset study. In the present study, no anaesthesia or restraint was used immediately prior to EEG monitoring. Using this alternative approach, a noteworthy consistency in EEG within subjects was seen over time, possibly because of improved consistency of data capture conditions as EEG was recorded whilst the animals were responding to the behavioural test. Additionally, no loss of signal quality occurred over time. This illustrates the acceptability of this method of EEG collection and its ability to provide a good baseline for comparison with data obtained following pharmacological intervention. Administration of sarin did not result in a significant effect on any of the EEG measures recorded here. However there was a trend towards significance in the beta 2 band ($p=0.07$) which is worthy of note because it is in the same frequency range and direction that changes were seen in previous studies. The statistical analysis revealed that this trend was due to one outlying subject showing a larger progressive increase in beta 2 activity over the course of the study, which could be indicative of a threshold for effect which was reached in this subject alone. Additionally, visual inspection of the data initially indicated a downward trend in the beta 1 band for the control group, which was not seen in the exposed subjects. Again a single outlying subject accounted for this trend. There was no evidence of a behavioural change in these individuals. It does, however, raise the possibility of the existence of a species specific threshold for effect. This could be addressed in future studies involving a larger dose range.

The marmosets were successfully trained to perform an attentional set-shifting paradigm in their home cages. Analysis of behavioural performance over the 12-15 month period following sarin administration revealed no effects on any aspect of the multistage sequence. The behavioural sequence employed throughout the study was used because it contained elements which would be expected, on the basis of previous work, to be sensitive to detrimental effects caused by cholinesterase inhibitors. For example, Roberts et al (1992) showed a decrement in discrimination reversal performance following cholinergic lesions. Other elements in the test have been shown to be sensitive to other manipulations e.g. dopaminergic lesions affect intradimensional versus extradimensional shift performance with comparison of these two stages, giving a measure of set-shifting performance (Roberts et al 1994).

Results from the paradigm presented do not indicate any detrimental effects associated with the administration of sarin at any point post administration. The significant dose interaction was seen to reflect an improvement in the CD stage in 'shape' subjects at a single time point. The significance of this improvement is unclear. Most of the other interactions seen in the analysis of behavioural performance revealed by the ANOVA were predicted at the start of the study. Reversal elements were consistently found to be more difficult than the preceding discriminations. On the set-shifting element of the sequence, 'shape' subjects found the intradimensional shift easier than the extradimensional shift, a result consistent with findings reported elsewhere (e.g. Roberts et al 1988). This pattern of performance is consistent with the predictions of two stage theories of attention and may be taken as being indicative of learning set formation. It has been suggested that the ID and ED elements of this type of behavioural test are of equal complexity but require different abilities (Roberts & Sahakian 1993). This contrasts with the performance of subjects assigned to the line group which found the ED shift easier than the ID shift. This effect has been reported previously (Crofts et al 1996) and may represent interaction between the saliency of the dimensions, the test sequence employed and the learning criterion. Thus, for this paradigm, in future studies it will be necessary to treat the groups assigned to the line and shape dimensions separately. Over the course of the experiment there was a trend towards improved performance of the behavioural sequence in all groups. Overall, there was no deleterious effect of sarin on either the shape or the line group, on any element of the test sequence or on the rate of improvement in performance over the year.

This study offers further validation of the approaches and techniques employed. Home cage behavioural testing of marmosets on tests from CANTAB has previously been shown to be viable (Crofts et al 1995, 1996, Pearce et al 1998) and collection of behavioural data over a period of more than 24 months illustrates that this method is suitable for the sort of long term study reported here. Additionally, the collection of EEG by telemetry has illustrated that electrophysiological data can also be collected over a long period without adverse effects due to the presence of the transmitter. Use of the telemetry techniques also enables common marmosets to be used in studies where electrophysiological measures are desirable. The use of a small implantable transmitter means that this is a much more attractive option than those previously available and may mean that it could be possible to employ this small, new world primate in studies where previously larger species, for example, the old world rhesus macaque, may have been used. Furthermore, the opportunity for remote electrophysiological monitoring in the recent study has also enabled the characterisation of sleep patterns. These data which are currently undergoing analysis, may further elucidate the sequelae of exposure to low doses of OP compounds.

The results of this study illustrate no significant effects on EEG and no deleterious effects on cognitive performance following a dose of sarin that inhibited erythrocyte cholinesterase by 51%. The degree to which peripheral measures of cholinesterase activity reflect central enzyme activity is not clear but in vivo central cholinesterase measurement techniques are limited. Suggestions of a 'threshold for effect' mean further studies are required to investigate the dose-response for sarin and other OP compounds.

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FIGURES AND TABLES

Figure 1. Changes in EEG frequency bands from baseline over time

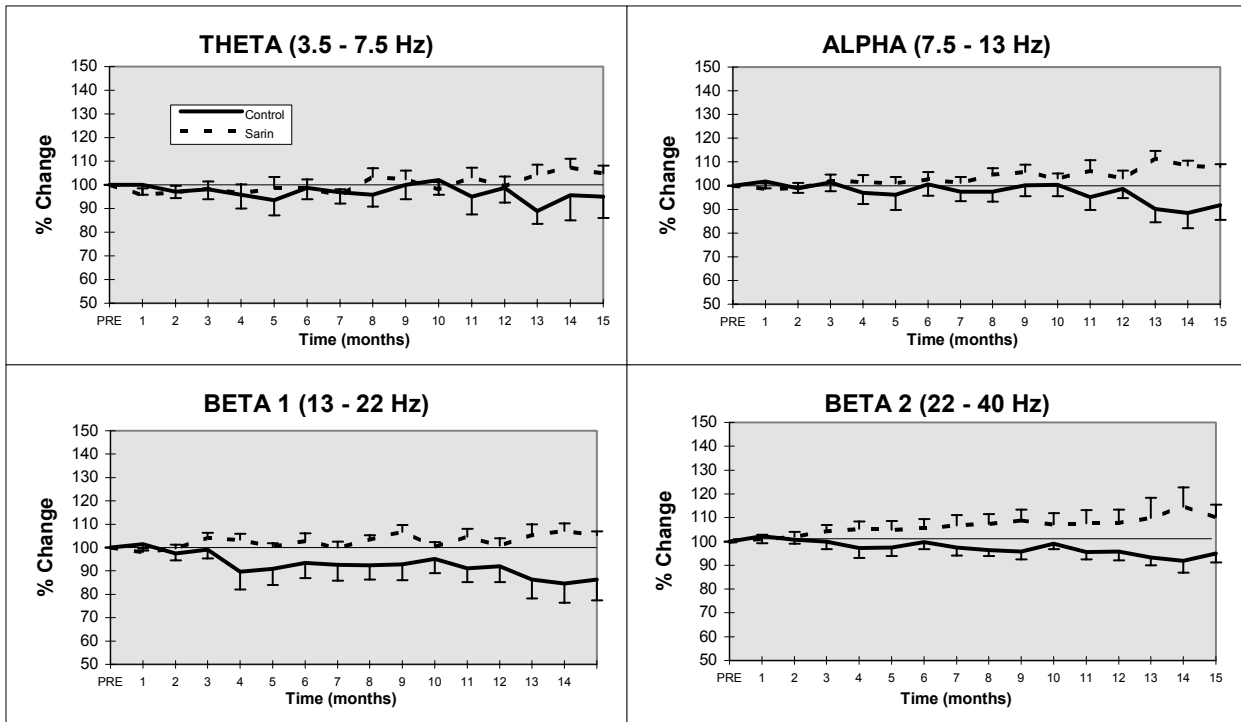


Figure 2: Changes in percent mean errors made in reaching criterion on each behavioural sequence presented

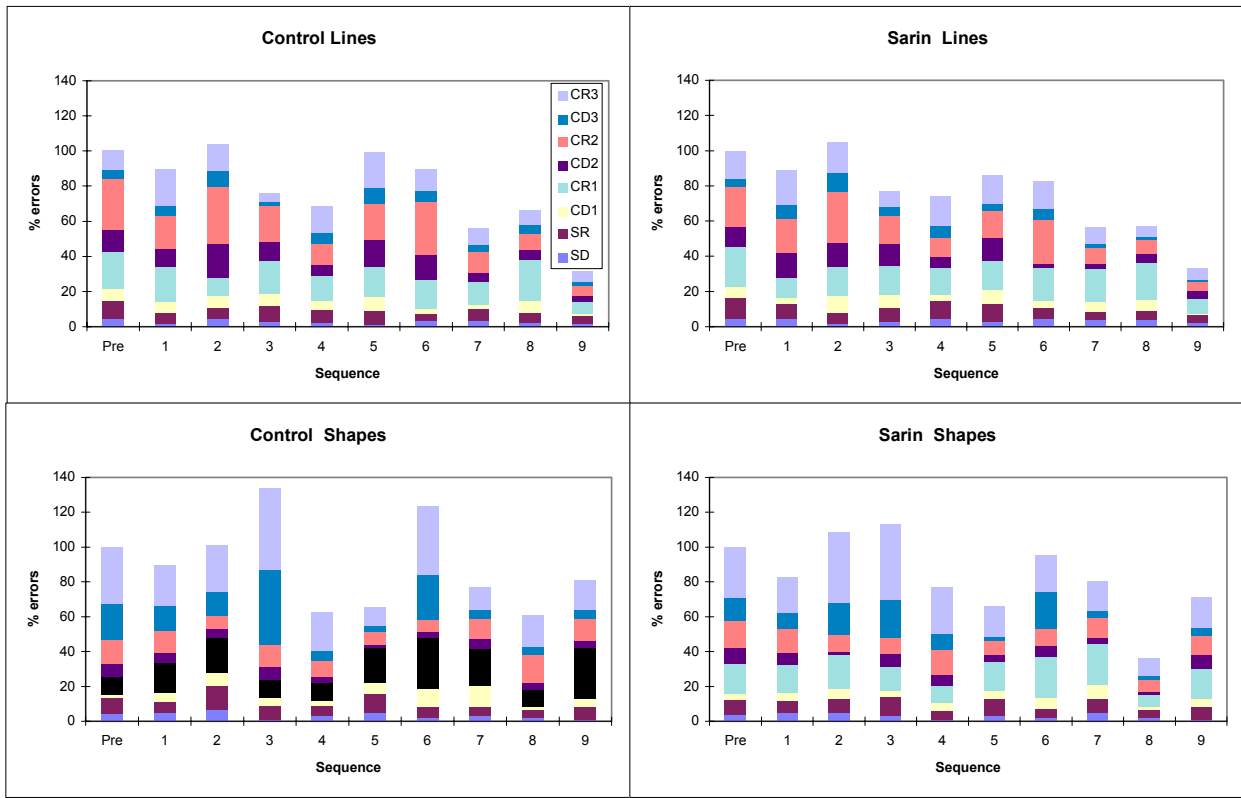


Table 1: Results from EEG ANOVA

Effect	Median	Delta	Theta	p values			Beta	Total Amp
				Alpha	Beta 1	Beta 2		
Stimulus type	0.21	0.11	0.32	0.58	0.53	0.53	0.53	0.18
Time	0.17	0.32	0.44	0.40	0.75	0.65	1.00	0.53
Dose	0.21	0.29	0.75	0.34	0.11	0.06	0.07	0.29
Type × Time	0.44	0.75	1.00	0.65	0.58	0.32	0.50	0.75
Type × Dose	0.58	0.75	0.75	1.00	0.44	0.58	0.65	1.00
Dose × Time	0.25	0.21	0.65	0.22	0.22	0.07	0.16	0.37
Type × Dose × Time	0.37	0.75	0.75	0.37	0.25	0.50	0.27	0.58

Table 2: Results of ANOVA analysis of behaviour

Effect	p value
type	0.65
dose	0.75
rev	0.44
stage	<0.001
period	<0.001
type*dose	0.29
type*rev	0.13
dose*rev	0.65
type*stage	<0.001
dose*stage	0.08
rev*stage	0.044
type*period	0.037
dose*period	0.067
rev*period	0.047
stage*period	<0.001
type*dose*rev	0.65
type*dose*stage	0.015
type*rev*stage	<0.001
dose*rev*stage	0.87
type*dose*period	0.65
type*rev*period	0.64
dose*rev*period	0.99
type*stage*period	<0.001
dose*stage*period	0.66
rev*stage*period	0.22
type*dose*rev*stage	0.61
type*dose*rev*period	0.86
type*dose*stage*period	0.05
type*rev*stage*period	0.46
dose*rev*stage*period	0.99
type*dose*rev*stage*period	0.72

Table 3: Post-hoc analysis of dose effects by stage (ratio of stimulus type (shape : line) for sarin treated animals relative to control animals)

Stage	ratio	95% Confidence Intervals	p
SD	0.69	0.46, 1.04	0.07
CD	0.47	0.31, 0.69	0.001
ID	0.74	0.49, 1.10	0.14
ED	1.21	0.81, 1.81	0.36

Table 4: Comparison of the mean errors on CD1 made by control and sarin dosed 'shape' subjects (untransformed data)

	Baseline	Post dose
Control mean	6.083	26.75
SEM	1.46	4.51
Dose mean	16.133	23.211
SEM	9.14	2.87