NeuroToxicology 32 (2011) 31–37

Contents lists available at ScienceDirect

NeuroToxicology

The environmental pollutant endosulfan disrupts cerebral cortical function at low doses

Oscar U. Scremin [a,b,c,d,*], Dante R. Chialvo [a,b,d], Simona Lavarello [a], Hector H. Berra [a], Miguel A. Lucero [a]

[a] Instituto de Toxicologia Agraria de Rosario, Facultad de Ciencias Medicas, UNR, Santa Fe 3100, Rosario CP2000, Argentina
[b] Physiology Dept., David Geffen School of Medicine at UCLA, Los Angeles, CA, United States
[c] Research Service, VA Greater Los Angeles Healthcare System, Los Angeles, CA, United States
[d] Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET), Argentina

ARTICLE INFO

Article history:
Received 12 August 2010
Accepted 1 December 2010
Available online 7 December 2010

Keywords:
Organochlorine insecticides
Epilepsy
GABA-A receptors
Electroencephalogram
Evoked potentials
Cerebral cortex
Endosulfan

ABSTRACT

Endosulfan can induce convulsions that could lead to brain damage. The variability and lack of specificity of neurological signs and symptoms in the pre-convulsive stages makes early diagnosis difficult. We sought to determine if electrophysiological exploration of the cerebral cortex could yield objective signs of endosulfan intoxication at levels that do not elicit convulsions. Endosulfan was administered intravenously to Sprague–Dawley adult rats under urethane anesthesia at doses from 0.5 to 4 mg/kg. EEG power and the evoked potentials (EP) to forepaw electrical stimulation were studied over the contralateral (S1CL) and homolateral (S1HL) cortical somatosensory areas and the contralateral visual area (V1CL). At each area, five EP waves were measured. Arterial blood pressure, heart rate and body temperature were also recorded. Endosulfan induced a dose-related increase in EPs at all sites. At S1CL, EP peak amplitude was greater than baseline at 1, 2 and 4 mg/kg for the first negative, second positive and third negative waves, and at 2 and 4 mg/kg for the first and third positive waves. Similar but less marked trends were observed at S1HL and V1CL. A shift of EEG power to higher frequencies (alpha and beta EEG bands) was only present at 4 mg/kg. In conclusion, endosulfan induced a large increase of cortical evoked potentials amplitudes at doses that did not elicit convulsions. These responses could be used as a non-invasive diagnostic tool to detect low-level endosulfan intoxication in humans and to help establish the NOAEL and LOAEL levels of this pollutant.

Published by Elsevier Inc.

1. Introduction

Endosulfan (6,7,8,9,10,10-hexacloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide), an organochlorine compound of the cyclodiene group is an insecticide of widespread use in agriculture, in spite of partial restrictions to its use in the European Union and other areas. In Argentina alone, approximately two thousand metric tons of this compound are imported each year and are used on various commodity crops and fruits, grains and vegetables of local consumption (SENASA, 2009). Atmospheric spread of this toxicant has reached cities and areas far removed from sites of application, including the Arctic (Liu et al., 2009; Weber et al., 2006; Evans et al., 2005; Hageman et al., 2006; Stern et al., 2005; Jantunen and Bidleman, 1998; Pozo et al., 2009) and is found in body fluids and tissues of animals including humans within and outside of agricultural areas (Nag and Raikwar, 2008; Cerrillo et al., 2005; Ramesh and Vijayalakshmi, 2002; Maitre et al., 1994; Beck et al., 1966; Jergentz et al., 2004; Jofre et al., 2008; Cid et al., 2007; Lanfranchi et al., 2006).

Neurotoxicity of endosulfan has been studied experimentally in tissue cultures (Sunol et al., 2008; Wozniak et al., 2005), invertebrates (Ghiasuddin and Matsumura, 1982; Chen et al., 2006; Bloomquist, 1993) and vertebrates including mammals (Brunelli et al., 2009; Ballesteros et al., 2009; Paul et al., 1992; Cabaleiro et al., 2008; Banerjee and Hussain, 1986). In addition, a wide array of molecular targets of this insecticide have been discovered in the endocrine (Wozniak et al., 2005) and immunological systems (Aggarwal et al., 2008). The mechanism of neurotoxicity of endosulfan appears to be dominated by its capacity to inhibit non-competitively the GABA-A type of receptors (Cole and Casida, 1986; Chen et al., 2006) although other targets

Abbreviations: S1CL, primary somatosensory contralateral area; S1HL, primary somatosensory homolateral area; V1CL, primary visual contralateral area; ms, milliseconds; mV, millivolts; ED50, effective dose 50%; NOAEL, No Observed Adverse Effect Level; LOAEL, lowest Observed Adverse Effect Level; GABA, gamma amino butyric acid; EEG, electroencephalogram; MABP, mean arterial blood pressure; HR, heart rate; TEMP, rectal temperature; ANOVA, analysis of variance.

* Corresponding author at: VA Greater Los Angeles Healthcare System, Research Service, 11301 Wilshire Blvd, Los Angeles, CA 90073, United States.
Tel.: +1 310 268 3895; fax: +1 310 268 4209.
E-mail address: oscremin@ucla.edu (O.U. Scremin).

0161-813X/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.neuro.2010.12.001

are believed to exist (Sunol et al., 2008; Paul et al., 1994; Vale et al., 2003). GABA-A receptors are pentameric proteins formed by various combinations of 19 receptor subunits in mammals, each attributed to a different gene (Simon et al., 2004). These proteins function as chloride channels with the capacity to hyperpolarize and inhibit neurons in the central nervous system. The major subtype expressed in the adult brain has a $(\alpha 1)2(\beta 2)2(\gamma 2)1$ stoichiometry. Receptor diversity is generated predominantly by α or β variant receptors or by the replacement of the γ-subunit, and each subtype has characteristic functional and pharmacological properties (Olsen and Sieghart, 2008).

GABA-A receptors have been shown to be involved in both phasic, inhibitory synaptic transmission and tonic, perisynaptic inhibition (Macdonald et al., 2004). The GABA-A receptors have been involved in epileptogenesis both in animals and humans. Mutations in the $\gamma 2$ subunit of this receptor were found in families where the main phenotype was febrile seizures (Baulac et al., 2001) and in the $\alpha 1$ subunit in a common form of juvenile epilepsy (Cossette et al., 2002).

Great strides have been made in the characterization of the interactions of endosulfan with GABA-A receptors *in vitro* in cultures of cortical neurons (Sunol et al., 2008). Intracellular recordings from neurons in tissue slices maintained viable *in vitro* have established in detail the effects of GABA on cellular excitability (McCormick, 1989). Studies of neurotoxicity *in vivo* in mammals have addressed the threshold for convulsive activity and lethal dose 50% (LD50) or observations of open field behavior and locomotion (Paul et al., 1993; Castillo et al., 2002) but few have been focused on electrophysiological variables (Anand et al., 1986).

Clinical short term endosulfan toxicity is dominated by the potential of this pesticide to induce convulsions and other central nervous system signs and symptoms (Durukan et al., 2009; Karatas et al., 2006). Permanent neurological damage has been reported following endosulfan-induced convulsions (Brandt et al., 2001; Aleksandrowicz, 1979). Clinical manifestations of endosulfan poisoning are difficult to detect in the absence of convulsions, which may only appear 24–48 h after exposure (Reigart and Roberts, 1999). The non-specific nature of signs and symptoms induced by endosulfan make diagnosis difficult during pre-convulsive stages. Availability of non-invasive clinical tools to detect the central nervous system abnormalities in this condition would be helpful in order to treat the intoxication before convulsions start.

The present work was designed to describe the electrophysiological events that exist at low levels of endosulfan toxicity and to provide early objective signs of sub-clinical stages of endosulfan intoxication, possibly translating in the future to non-invasive evaluation of this condition. Experiments were conducted under anesthesia to avoid restraint stress and discomfort that would have resulted by recording somatosensory evoked potentials with electrical stimulation over several hours in non-anesthesized animals. The ED50 for endosulfan convulsions was determined in animals anesthetized with urethane without instrumentation, and incremental doses below the convulsive ED50 were administered to animals under the same anesthesia instrumented for recording of cerebral cortical electrical activity and peripheral (forepaw) electrical stimulation.

2. Materials and methods

Male Sprague–Dawley adult rats, 300–350 g body mass were used. All procedures were approved by the Bioethics Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Animals were anesthetized with urethane (1.5 g/kg by intraperitoneal route). One femoral artery and vein were cannulated for arterial blood pressure recording and drug infusion respectively. Two platinum needles were inserted into the right forepaw and connected to an Accupulser Signal Generator and A385 Stimulus Isolator (World Precision Instruments, Inc., Sarasota, FL). The skull surface was exposed through a skin incision and the galea was dissected away. Three Ag–AgCl electrodes were positioned with micromanipulators touching the bone surface and were paired with Ag–AgCl electrodes in the subcutaneous space of the head. A small drop of conductive medium (OmniPrep, D.O. Weaver & Co., Aurora, CO, USA) on each electrode was used to facilitate electrical contact with bone. Cranial electrodes were positioned at the projections of forepaw primary somatosensory area on the left (S1CL, Bregma 0 mm, Left Lateral 4 mm) and right cortex (S1HL, Bregma 0 mm, Right Lateral 4 mm) and the primary visual area on the left cortex (V1CL, Bregma −5 mm, Left Lateral 4 mm) (Paxinos and Watson, 1998).

The right forepaw was stimulated with 0.3 ms duration and 0.5 Hz frequency pulses, supramaximal for elicitation of the S1 contralateral evoked response. The electro-cortical activity during the 500 ms that followed the stimulation pulse was amplified with an Iso-4 low noise amplifier (World Precision Instruments, Inc., Sarasota, FL) and digitized at 1 kHz with a PowerLab data acquisition system (ADInstruments, Inc., Colorado Springs, CO). Epochs containing evoked cortical activity were processed with MATLAB scripts to obtain averaged evoked potentials of 30 consecutive samples.

EEG power spectrum for the EEG frequencies 1–30 Hz was obtained from 1 min segments of recording in the absence of forepaw stimulation using a Cosine-Bell windowing function and 1024 window size with 50% overlap (Labchart 7 Spectrum Module, AD-Instruments). EEG power was computed in the following frequency bands: delta, 1–4 Hz; theta, 4–8 Hz; alpha, 8–14 Hz, and beta, 14–30 Hz. The data was normalized by dividing power in each frequency band by the sum of the powers of all four frequency bands for each animal and endosulfan dose (Fractional Power).

Arterial blood pressure was continuously sampled from the arterial catheter with a carrier amplifier and transducer (World Precision Instruments, Inc., Sarasota, FL). Mean arterial blood pressure (MABP) and heart rate (HR) were calculated on line from the arterial blood pressure recordings by Labchart 7 software (ADInstruments, Inc., Colorado Springs, CO). Rectal temperature TEMP was recorded with a T-type thermocouple thermometer (BAT 12 Electronic Thermometer, Physitemp Instruments, Clifton, NJ).

Endosulfan (Thiodan©, CHEM SERVICE, Inc., West Chester, PA) of purity: I (alpha) 67%; II (beta) 33%, was dissolved in propylene glycol and administered intravenously through a cannulated femoral vein with total volume infused between 0.05 and 0.1 ml. These doses of propylene glycol did not induce changes in the variables under study (data not shown). Endosulfan was administered at 0.5, 1, 2, and 4 mg/kg with an interval of 2 h between doses.

The up and down method (Dixon, 1965) was used to estimate the endosulfan ED50 for convulsions under urethane anesthesia in experiments without instrumentation. A convulsion was defined as tonic–clonic limb and trunk contractions continuing for 15 min after which period animals were euthanized with an overdose of urethane.

2.1. Data analysis

The peak latencies from the trigger pulse (ms) and amplitudes (mV) of the following cortical electrical activity stimulus-evoked waves were recorded: first positive (P1), first negative (N1), second positive (P2), second negative (N2) and third positive (P3). Latency and amplitude of evoked potentials waves were analyzed by ANOVA with factor endosulfan dose (0.5, 1, 2, and 4 mg/kg) and compared, if the ANOVA F-ratio was statistically significant ($P < 0.05$) to the control condition (no endosulfan) by post hoc

Dunnet's multiple comparisons tests. The same statistical analysis was performed for Fractional Power, MABP, HR and TEMP data.

3. Results

3.1. ED50 for endosulfan induced convulsions

Convulsive activity on a separate group of anesthetized animals without instrumentation was usually initiated by trunk and tail brief tonic dorsiflexion, followed by tonic–clonic convulsions affecting trunk and limbs. This convulsive activity continued unabated until euthanasia, which was performed after 15 min of initiation. Salivary hypersecretion was also observed during this period.

ED50 for convulsions calculated with the up and down method (Dixon, 1965) was 5.7 mg/kg ($n = 6$).

3.2. Cortical evoked potentials

Following i.v. injection of endosulfan, a rapid increase in amplitude of evoked potential waves was observed that peaked within the first hour of observation (Fig. 1) declining slowly thereafter to reach levels close to those recorded prior to drug administration at the low doses. In the first analysis, the average latency and amplitude of the evoked potential waves over the 2 h observation period for every dose was recorded (Tables 1 and 2). The potential evoked by stimulation of the contralateral forepaw in the absence of endosulfan consisted of a positive deflection (S1CLP1) with an average peak latency of (mean ± S.E.) 14.3 ± 0.6 ms, followed by a negative deflection (S1CLN1) with a latency of 22.1 ± 1.8 ms (Table 1). Amplitude of the S1CLN1 wave (−0.06 ± 0.01) was smaller than the S1CLP1 wave (0.15 ± 0.01 mV). Peak latency and average amplitude of the slower waves are described in Tables 1 and 2 respectively. The most notable change observed after administration of 0.5 mg/kg of endosulfan i.v. was an increase in the amplitude of S1CLN1 that grew as a function of dose to achieve a maximum at the 4 mg/kg dose (Table 2). A dose-related increase, although of a lesser magnitude, was also observed in the first positive wave (S1CLP1) and the second negative wave (S1CLN2). No statistically significant changes in wave peak latencies were observed, except for an increase in the second positive wave (S1CLP2) at 2 and 4 mg/kg.

A second analysis performed using the maximal value of waves amplitudes recorded for every dose is presented in Fig. 2. A dose-related trend was found in the S1CL area, with statistical significance difference from control values achieved at 2 and 4 mg/kg for the S1CLP1 and S1CLP3 waves and 1, 2 and 4 mg/kg for the rest of the waves. Similar trends were observed for the EPs in S1HL and V1CL areas (Fig. 2).

Table 1
Means ± S.E. peak latencies (ms) of waves in cortical evoked potentials elicited by forepaw stimulation in the somatosensory contralateral (S1CL) or homolateral (S1HL) S1 areas and the visual contralateral (V1CL) area ($n = 8$).

Waves	Dose (mg/kg)				
	0	0.5	1	2	4
S1CLP1	14.3 ± 0.6	14.2 ± 0.4	14.2 ± 0.4	13.8 ± 0.5	15.2 ± 0.3
S1CLN1	22.1 ± 1.8	21.9 ± 0.8	22.1 ± 0.8	21.4 ± 0.6	24.4 ± 0.9
S1CLP2	33.7 ± 4.0	38.3 ± 3.4	46.1 ± 3.8	47.4 ± 1.4†	52.2 ± 1.6†
S1CLN2	98.5 ± 18.7	94.8 ± 8.1	108.9 ± 12.7	117.5 ± 6.1	124.7 ± 4.7
S1CLP3	203.9 ± 18.4	212.2 ± 10.3	224.6 ± 22.2	219.2 ± 11.9	221.2 ± 12.5
S1HLP1	17.9 ± 1.6	19.0 ± 1.1	18.8 ± 1.3	17.4 ± 2.4	22.2 ± 0.5
S1HLN1	31.3 ± 2.7	31.8 ± 0.5	29.6 ± 0.9	31.3 ± 1.4	35.3 ± 1.6
S1HLP2	55.6 ± 4.2	51.9 ± 3.0	50.5 ± 3.8	57.7 ± 2.1	59.7 ± 1.6
S1HLN2	173.0 ± 39.1	104.3 ± 8.0	106.6 ± 11.5	119.2 ± 6.8	131.2 ± 12.5
S1HLP3	265.7 ± 44.8	220.3 ± 9.4	227.1 ± 21.2	217.7 ± 16.0	257.4 ± 24.2
V1CLP1	14.0 ± 4.1	16.5 ± 7.8	10.6 ± 2.5	12.3 ± 2.2	15.4 ± 3.3
V1CLN1	32.3 ± 6.2	35.9 ± 5.3	28.2 ± 3.2	32.1 ± 3.7	34.4 ± 2.1
V1CLP2	58.1 ± 10.1	58.1 ± 5.6	53.4 ± 6.4	60.4 ± 5.9	60.1 ± 5.1
V1CLN2	108.7 ± 10.9	104.6 ± 17.5	97.9 ± 11.2	118.9 ± 6.1	114.5 ± 4.4
V1CLP3	183.2 ± 27.5	169.8 ± 30.2	173.3 ± 30.8	206.6 ± 20.4	209.9 ± 20.2

The last 2 characters in the abbreviations refer to the first positive (P1), first negative (N1), second positive (P2), second negative (N2), and third positive (P3) waves. Values represent the average over the 2 h observation period that followed endosulfan pulse administration. Significance of peak latency differences of each dose level from the control condition (dose = 0) was tested for every wave by ANOVA followed, if significant, by Dunnet's t tests.

† $P < 0.05$.

3.3. Power spectrum analysis

An increase in Fractional Power in the alpha and beta bands was found in all areas at 4 mg/kg. There was a trend for a decrease in the delta band at doses greater than 1 mg/kg that was not statistical significant until a dose of 4 mg/kg was reached. EEG theta bands did not show significant differences from controls at any dose (Fig. 3).

3.4. Muscular manifestations

There was no muscle activity observed at the doses of 0.5 and 1 mg/kg. At 2 mg/kg, out of eight animals, only one showed twitching of vibrissae and ear muscles 13 min after endosulfan administration that ceased 35 min later while two animals showed only transient vibrissa movements 22 and 30 min after drug administration. At 4 mg/kg all eight animals showed vibrissae twitching between 3 and 13 min and ear twitching between 13 and

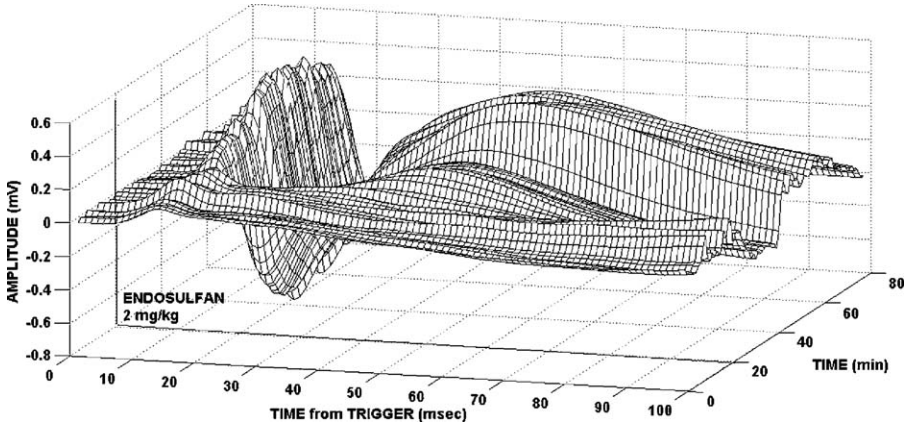

Fig. 1. Representative results of an experiment with baseline recording during 20 min, followed by 60 min of recording after a single i.v. injection of 2 mg/kg endosulfan. Every wave represents the average of 30 consecutive sweeps. The right forepaw was stimulated with 0.3 ms, supramaximal pulses at 0.5 Hz frequency. The cortical evoked activity was recorded from the skull surface overlying the contralateral forepaw primary somatosensory area of the cerebral cortex.

Table 2
Means ± S.E. amplitude (mV) of waves during the 2-h observation period in cortical evoked potentials elicited by forepaw stimulation in the somatosensory contralateral (S1CL) or homolateral (S1HL) S1 areas and the visual contralateral (V1CL) area ($n = 8$).

Waves	Dose (mg/kg)				
	0	0.5	1	2	4
S1CLP1	0.15 ± 0.01	0.18 ± 0.02	0.25 ± 0.05	0.31 ± 0.05†	0.46 ± 0.08†
S1CLN1	−0.06 ± 0.01	−0.12 ± 0.07	−0.52 ± 0.17†	−0.58 ± 0.13†	−0.73 ± 0.16†
S1CLP2	0.10 ± 0.02	0.11 ± 0.02	0.17 ± 0.03	0.15 ± 0.02	0.13 ± 0.03
S1CLN2	−0.09 ± 0.02	−0.16 ± 0.04	−0.22 ± 0.04†	−0.20 ± 0.02†	−0.24 ± 0.05†
S1CLP3	0.11 ± 0.02	0.14 ± 0.03	0.19 ± 0.04	0.20 ± 0.02	0.21 ± 0.05
S1HLP1	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.02	0.07 ± 0.04	0.19 ± 0.05†
S1HLN1	−0.02 ± 0.01	−0.02 ± 0.01	−0.03 ± 0.01	−0.15 ± 0.11	−0.24 ± 0.12
S1HLP2	0.05 ± 0.01	0.07 ± 0.03	0.11 ± 0.05	0.09 ± 0.03	0.10 ± 0.03
S1HLN2	−0.03 ± 0.01	−0.05 ± 0.02	−0.16 ± 0.05†	−0.11 ± 0.03	−0.18 ± 0.04†
S1HLP3	0.03 ± 0.01	0.07 ± 0.01	0.11 ± 0.04	0.10 ± 0.03	0.15 ± 0.02†
V1CLP1	0.02 ± 0.02	0.04 ± 0.03	0.01 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
V1CLN1	−0.03 ± 0.02	−0.04 ± 0.03	−0.03 ± 0.02	−0.09 ± 0.03	−0.15 ± 0.09
V1CLP2	0.05 ± 0.03	0.06 ± 0.05	0.06 ± 0.03	0.07 ± 0.03	0.06 ± 0.02
V1CLN2	−0.05 ± 0.02	−0.07 ± 0.04	−0.08 ± 0.04	−0.13 ± 0.04	−0.12 ± 0.05
V1CLP3	0.03 ± 0.02	0.05 ± 0.03	0.07 ± 0.04	0.11 ± 0.03	0.09 ± 0.03

The last 2 characters in the abbreviations refer to the first positive (P1), first negative (N1), second positive (P2), second negative (N2) and third positive (P3) waves. Values represent the average over the 2 h observation period that followed endosulfan pulse administration. Significance of latency differences of each dose level from the control condition (dose = 0) was tested for every wave by ANOVA followed, if significant, by Dunnet's t tests
† $P < 0.05$.

29 min of endosulfan administration with two animals progressing to clonic trunk movements.

3.5. Physiological variables

Mean arterial blood pressure increased as a function of dose, reaching values significantly different from baseline at 2 and 4 mg/kg. No significant changes from baseline were found for heart rate and rectal temperature (Table 3).

4. Discussion

To our knowledge, this is the first study that characterized the cerebral cortex electrophysiological changes associated with low, subconvulsive doses of endosulfan using time and frequency domain techniques. The short latency somatosensory cortical evoked response (first positive (P1) and first negative (N1) waves in the present recordings) show considerable analogy between rats and primates (Allison and Hume, 1981). There is consensus that the positive short latency wave represents depolarization of pyramidal neurons in layer IV and deep layer III of the cerebral cortex, while the following negative deflection corresponds to the apical dendritic depolarization of the same cells (Jellema et al., 2004). Thus, the balance between the two waves is related to the extent of depolarization of pyramidal cells. The large increase in the amplitude of the short latency negative wave (N1) induced by endosulfan argues for a facilitation of the spread of activation towards the superficial layers of the cerebral cortex. It is noteworthy that a significant increase in amplitude of most somatosensory EP waves was present at 1 mg/kg, which represents 0.18 ED50 for convulsions to a single dose of endosulfan. In the experiments with incremental doses of this agent, clonic movements first appeared at 4 mg/kg, possibly due to some effect summation, since the EP waves amplitudes did not return to baseline two hours after the 2 mg/kg dose when the 4 mg/kg dose was administered (data not shown).

Activation of the contralateral visual cortex in response to forepaw stimulation was barely detectable in the absence of endosulfan and grew progressively in amplitude as doses were increased. The evoked electrical activity in the homolateral

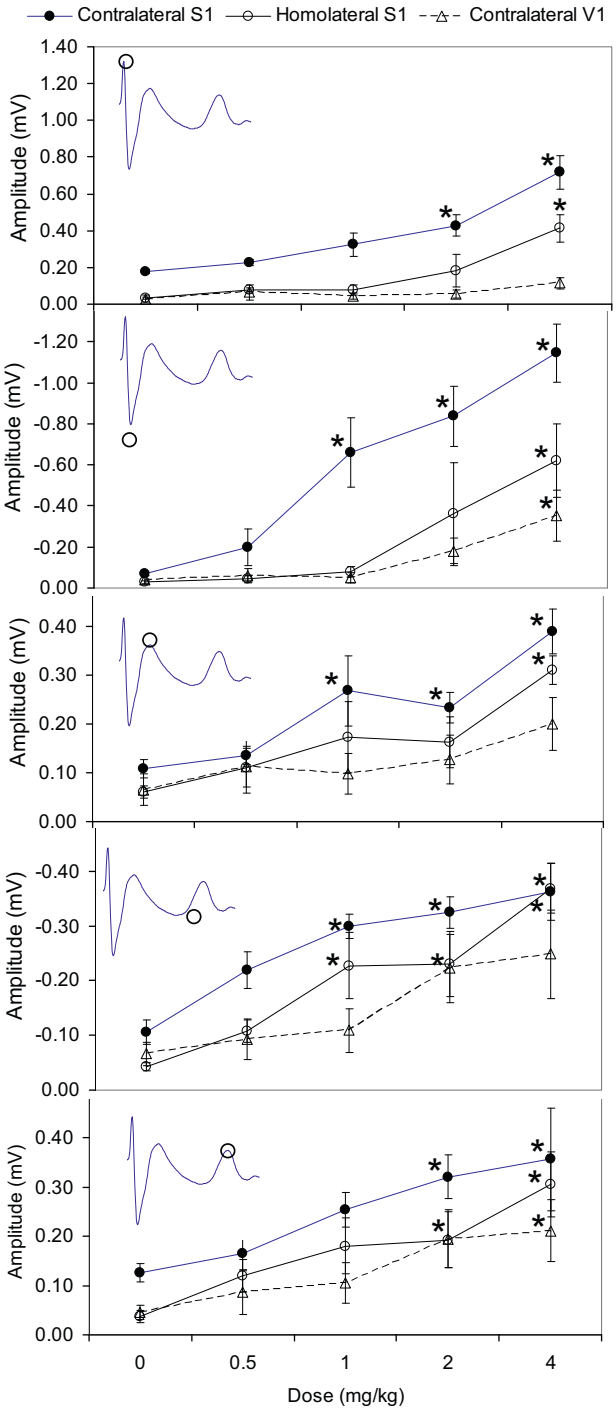

Fig. 2. Peak amplitude of contralateral S1 (solid circles, full lines) homolateral S1 (open circles, full lines) and contralateral V1 (open triangles, dashed lines) cortical evoked activity (average of 30 consecutive samples) obtained during the 2-h observation period at each dose level of endosulfan. Circles in the evoked potential inserts at the top left corner of each panel were drawn to indicate the wave from which values were obtained. From top to bottom, first positive, first negative, second positive, second negative and third positive waves. *Significantly different from the control condition (dose = 0) by ANOVA and Dunnet's multiple comparison tests, $P < 0.05$, $n = 8$.

representation of the forepaw was minimal in the absence of endosulfan in the present experiments. This phenomenon is in line with recent fMRI observations that while detecting activation of the cerebral cortex in the same contralateral area to the stimulated side used by us for recording of evoked potentials, did not show activation of the homolateral area with forepaw (Peeters et al.,

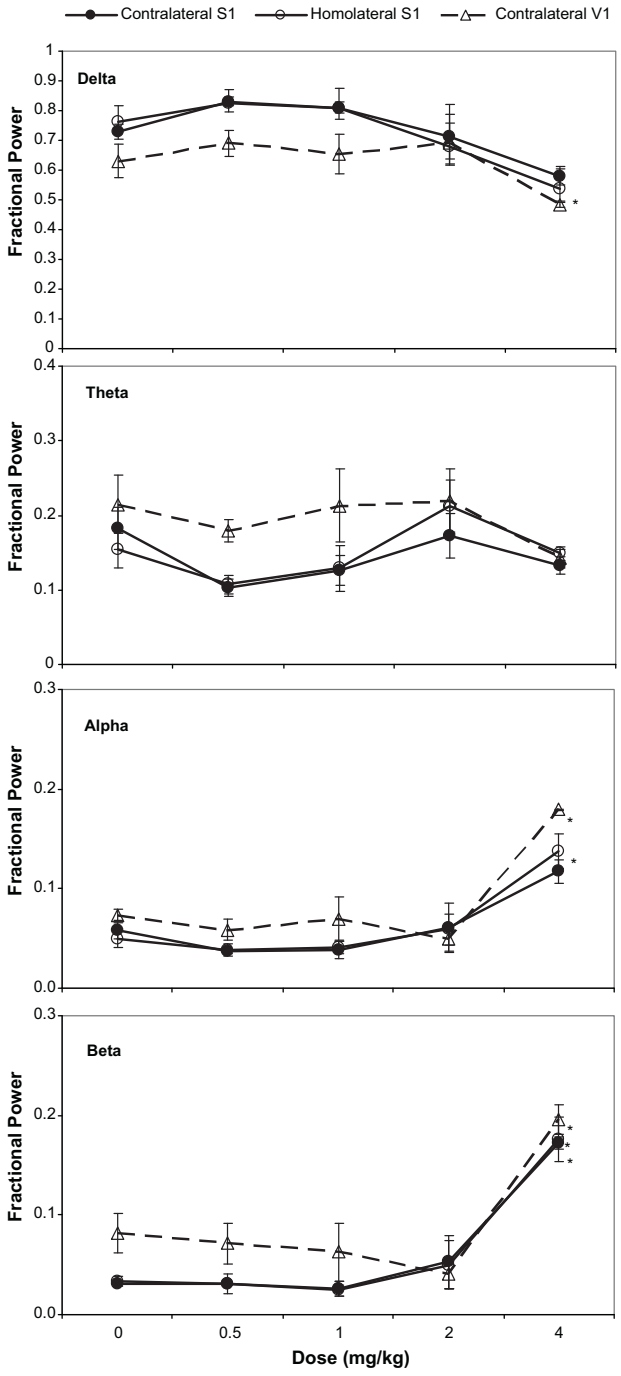

Fig. 3. EEG frequency bands relative powers of contralateral S1 (solid circles, full lines) homolateral S1 (open circles, full lines) and contralateral V1 (open triangles, dashed lines) as a function of endosulfan dose. From top to bottom, delta band (1–4 Hz), theta band (4–8 Hz), alpha band (8–14 Hz) and beta band (14–30 Hz). *Significantly different from the control condition (dose = 0) by ANOVA and Dunnet's multiple comparison tests, $P < 0.05$, $n = 8$.

1999) or radial nerve stimulation (Cho et al., 2007). The enhancement of the homolateral cortical evoked response induced by endosulfan was most likely mediated by the transcallosal route that is prominent in this region of the cerebral cortex (Akers and Killackey, 1978). This idea is supported by the finding that the homolateral first positive wave (S1HLP1) peak latency was delayed with regards to the contralateral response (S1CLP1) (Table 2) by approximately the latency of the transcallosal response (Okuyama and Aihara, 1988). The participation of homolateral talamo-cortical projections in this phenomenon cannot be discarded however, since it is known that some cells in the somatosensory area have bilateral receptive fields (Iwamura, 2000). Elucidation of this point will require further experimentation with measurements of the effect of endosulfan on the transcallosal evoked potentials.

The role of GABA in the causation of motor phenomena associated with cyclodienes actions has been known since Ghiasuddin and Matsumura described the blockade by heptachlor epoxide and γ-hexachlorocyclohexane of the enhanced $^{36}Cl^-$ permeability induced by GABA in the coaxal muscle of the American cockroach (Ghiasuddin and Matsumura, 1982). Later studies have supported this interpretation for endosulfan in particular (Sunol et al., 2008). Glycine receptors, which also mediate neuronal inhibition, are known to be inhibited non-competitively by endosulfan but with one order of magnitude less potency than GABA-A receptors (Vale et al., 2003). GABA is considered the main inhibitory transmitter in the forebrain of mammals, although glycine receptors have also been described in many areas, including the cerebral cortex (Hernandes and Troncone, 2009). Antagonism of GABA-A receptors with iontophoretic local application of bicuculline enhanced receptor fields in rat barrel cortex (Li et al., 2002) and cat somatosensory cortex (Hicks et al., 1986). The GABAergic influences on the cat visual cortex appear to be region specific (Jirmann et al., 2009). Intracellular studies in human cerebral cortex slices have indicated that fast inhibitory synaptic potentials are blocked by the GABA-A antagonist bicuculline, are sensitive to Cl^- ions injections and are mimicked by the GABA-A agonist muscimol (McCormick, 1989). Thus, antagonism of GABA-A receptors by cyclodienes is a plausible hypothesis for the causation of the enhancement of somatosensory evoked activity reported here.

Regarding the localization of the GABA-A receptors associated with these effects, we cannot reach any conclusions from the available data. These effects could take place at the cortical level where, as discussed above, the substratum exists for such actions but they could also take place at sub-cortical levels, such as the thalamus.

It is important to note that the significant increase of evoked potential activity described in this work first occurred at 1 mg/kg (with a trend already at 0.5 mg/kg) in the absence of any motor manifestations and well below the ED50 for convulsive activity (5.7 mg/kg). It is then tempting to associate these phenomena with symptoms reported by patients prior to or in the absence of motor signs and convulsions, such as hyperactivity and anxiety (Durukan et al., 2009) (Reigart and Roberts, 1999). Decreased cortical

Table 3
Means ± S.E. peak values of heart rate (HRATE), mean arterial blood pressure (MABP) and rectal temperature (TEMP.) recorded at each dose level of intravenous endosulfan administration as a single pulse ($n = 8$).

	Dose (mg/kg)				
	0	0.5	1	2	4
HRATE (bpm)	378.4 ± 8.8	388.9 ± 11.2	388.8 ± 20.3	408.2 ± 10.6	405.6 ± 14.3
MABP (mm Hg)	86.7 ± 2.8	86.6 ± 2.0	98.5 ± 2.3	105.6 ± 5.0†	127.3 ± 5.4†
TEMP. (°C)	37.0 ± 0.1	37.3 ± 0.1	37.2 ± 0.1	37.5 ± 0.1	37.6 ± 0.3

Significance against the control condition (dose = 0) was tested by ANOVA followed, if significant, by Dunnet's t tests.
† $P < 0.05$.

inhibition correlates with anxiety, as demonstrated with transcranial magnetic stimulation (Wassermann et al., 2001). The findings with cortical evoked potentials in anxiety are not uniform and appear to be related to the conditions eliciting this behavioral state (Clark et al., 2009).

The distribution of power among EEG bands was considerably less sensitive to endosulfan, with a shift to higher frequencies observed only at 4 mg/kg.

The fact that arterial blood pressure was sensitive to endosulfan suggests that this variable might be helpful in recognizing early events induced by this agent.

Regulatory agencies establish reference doses and tolerances for the various exposure risks of endosulfan based on the No Observed Adverse Effect Level (NOAEL) and Lowest Observed Adverse Effect Level (LOAEL) for this toxicant. The endpoints used for NOAEL and LOAEL threshold are incidence of convulsions seen in female rats for acute dietary exposure and reduced body weight gain, enlarged kidneys, increased incidences of marked progressive glomerulonephrosis and blood vessel aneurysms in male rats (US Environmental Protection Agency, 2007). In light of the present observations, it might be worthwhile to add cortical evoked potentials as an endpoint for neurotoxicity in the establishment of NOAEL and LOAEL thresholds. The NOELs from studies considered in the EPA risk assessment range from 0.7 mg/kg to greater than 2.0 mg/kg. While the current NOEL of 0.5 mg/kg is lower, suggesting that cortical evoked potentials might be a more sensitive indicator of endosulfan toxicity than the ones currently used, further studies using oral, dermal and inhalation routes preferably in animals without anesthesia will be necessary to confirm this concept.

Non-invasive recording of cortical evoked potentials is a standard clinical neurophysiology technique used in human subjects for diagnostic purposes of peripheral and central neurological pathology. The current results do suggest that evoked potentials may be useful in characterizing effects of low dose longer term exposure and thus they do warrant characterization of effects of subchronic and chronic exposure (e.g. oral route of exposure) to endosulfan. The extension of this technique to the assessment of suspected endosulfan toxicity could be a useful tool in epidemiological studies of this neurotoxicant.

5. Conclusions

We found that the dose-related increases in the amplitude of somatosensory evoked potentials are sensitive indices of low level endosulfan intoxication. These measurements could be implemented non-invasively in human subjects and help in the diagnosis of low level intoxication with this agent or of the initial (pre-convulsive) stages in more severe cases.

Cortical evoked potentials could be used as an endpoint for neurotoxicity in the establishment of NOAEL and LOAEL thresholds when more information becomes available regarding other routes of administration and chronic or subchronic exposure. Further investigation of other modalities of cerebral evoked activity in conscious animals (e.g. auditory and photic) during acute and chronic endosulfan administration seems warranted.

Competing financial interests declaration

No competing financial interests exist.

Acknowledgements

This work was supported by funds and equipment donations from Capacitacion e Investigacion para la Medicina Argentina-Asociacion Civil, a non-profit academic organization.

References

Aggarwal M, Naraharisetti SB, Dandapat S, Degen GH, Malik JK. Perturbations in immune responses induced by concurrent subchronic exposure to arsenic and endosulfan. Toxicology 2008;251:51–60.
Akers RM, Killackey HP. Organization of corticocortical connections in the parietal cortex of the rat. J Comp Neurol 1978;181:513–37.
Aleksandrowicz DR. Endosulfan poisoning and chronic brain syndrome. Arch Toxicol 1979;43:65–8.
Allison T, Hume AL. A comparative analysis of short-latency somatosensory evoked potentials in man, monkey, cat, and rat. Exp Neurol 1981;72:592–611.
Anand M, Agrawal AK, Gopal K, Sur RN, Seth PK. Endosulfan and cholinergic (muscarinic) transmission: effect on electroencephalograms and [3H]quinuclidinyl benzilate in pigeon brain. Environ Res 1986;40:421–6.
Ballesteros ML, Durando PE, Nores ML, Diaz MP, Bistoni MA, Wunderlin DA. Endosulfan induces changes in spontaneous swimming activity and acetylcholinesterase activity of Jenynsia multidentata (Anablepidae, Cyprinodontiformes). Environ Pollut 2009;157:1573–80.
Banerjee BD, Hussain QZ. Effect of sub-chronic endosulfan exposure on humoral and cell-mediated immune responses in albino rats. Arch Toxicol 1986;59:279–84.
Baulac S, Huberfeld G, Gourfinkel-An I, Mitropoulou G, Beranger A, Prud'homme JF, et al. First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. Nat Genet 2001;28:46–8.
Beck EW, Johnson JC Jr, Woodham DW, Leuck DB, Dawsey LH, Robbins JE, et al. Residues of endosulfan in meat and milk of cattle fed treated forages. J Econ Entomol 1966;59:1444–50.
Bloomquist JR. Toxicology, mode of action and target site-mediated resistance to insecticides acting on chloride channels. Comp Biochem Physiol C 1993;106:301–14.
Brandt VA, Moon S, Ehlers J, Methner MM, Struttmann T. Exposure to endosulfan in farmers: two case studies. Am J Ind Med 2001;39:643–9.
Brunelli E, Bernabo I, Berg C, Lundstedt-Enkel K, Bonacci A, Tripepi S. Environmentally relevant concentrations of endosulfan impair development, metamorphosis and behaviour in Bufo bufo tadpoles. Aquat Toxicol 2009;91:135–42.
Cabaleiro T, Caride A, Romero A, Lafuente A. Effects of in utero and lactational exposure to endosulfan in prefrontal cortex of male rats. Toxicol Lett 2008;176:58–67.
Castillo CG, Montante M, Dufour L, Martinez ML, Jimenez-Capdeville ME. Behavioral effects of exposure to endosulfan and methyl parathion in adult rats. Neurotoxicol Teratol 2002;24:797–804.
Cerrillo I, Granada A, Lopez-Espinosa MJ, Olmos B, Jimenez M, Cano A, et al. Endosulfan and its metabolites in fertile women, placenta, cord blood, and human milk. Environ Res 2005;98:233–9.
Chen L, Durkin KA, Casida JE. Structural model for gamma-aminobutyric acid receptor noncompetitive antagonist binding: widely diverse structures fit the same site. Proc Natl Acad Sci USA 2006;103:5185–90.
Cho YR, Pawela CP, Li R, Kao D, Schulte ML, Runquist ML, et al. Refining the sensory and motor ratunculus of the rat upper extremity using fMRI and direct nerve stimulation. Magn Reson Med 2007;58:901–9.
Cid FD, Anton RI, Caviedes-Vidal E. Organochlorine pesticide contamination in three bird species of the Embalse La Florida water reservoir in the semiarid midwest of Argentina. Sci Total Environ 2007;385:86–96.
Clark CR, Galletly CA, Ash DJ, Moores KA, Penrose RA, McFarlane AC. Evidence-based medicine evaluation of electrophysiological studies of the anxiety disorders. Clin EEG Neurosci 2009;40:84–112.
Cole LM, Casida JE. Polychlorocycloalkane insecticide-induced convulsions in mice in relation to disruption of the GABA-regulated chloride ionophore. Life Sci 1986;39:1855–62.
Cossette P, Liu L, Brisebois K, Dong H, Lortie A, Vanasse M, et al. Mutation of GABRA1 in an autosomal dominant form of juvenile myoclonic epilepsy. Nat Genet 2002;31:184–9.
Dixon WJ. The up-and-down method for small samples. J Am Stat Assoc 1965;60:967–78.
Durukan P, Ozdemir C, Coskun R, Ikizceli I, Esmaoglu A, Kurtoglu S, et al. Experiences with endosulfan mass poisoning in rural areas. Eur J Emerg Med 2009;16:53–6.
Evans MS, Muir D, Lockhart WL, Stern G, Ryan M, Roach P. Persistent organic pollutants and metals in the freshwater biota of the Canadian Subarctic and Arctic: an overview. Sci Total Environ 2005;351–352:94–147.
Ghiasuddin SM, Matsumura F. Inhibition of gamma-aaminobutyric acid (GABA)-induced chloride uptake by gamma-BHC and heptachlor epoxide. Comp Biochem Physiol 1982;73C:141–4.
Hageman KJ, Simonich SL, Campbell DH, Wilson GR, Landers DH. Atmospheric deposition of current-use and historic-use pesticides in snow at national parks in the western United States. Environ Sci Technol 2006;40:3174–80.
Hernandes MS, Troncone LR. Glycine as a neurotransmitter in the forebrain: a short review. J Neural Transm 2009;116(12):1551–60.
Hicks TP, Metherate R, Landry P, Dykes RW. Bicuculline-induced alterations of response properties in functionally identified ventroposterior thalamic neurones. Exp Brain Res 1986;63:248–64.
Iwamura Y. Bilateral receptive field neurons and callosal connections in the somatosensory cortex. Philos Trans R Soc Lond B Biol Sci 2000;355:267–73.
Jantunen LM, Bidleman TF. Organochlorine pesticides and enantiomers of chiral pesticides in Arctic Ocean water. Arch Environ Contam Toxicol 1998;35:218–28.
Jellema T, Brunia CH, Wadman WJ. Sequential activation of microcircuits underlying somatosensory-evoked potentials in rat neocortex. Neuroscience 2004;129:283–95.
Jergentz S, Mugni H, Bonetto C, Schulz R. Runoff-related endosulfan contamination and aquatic macroinvertebrate response in rural basins near Buenos Aires, Argentina. Arch Environ Contam Toxicol 2004;46:345–52.

Jirmann KU, Pernberg J, Eysel UT. Region-specificity of GABAA receptor mediated effects on orientation and direction selectivity in cat visual cortical area 18. Exp Brain Res 2009;192:369–78.
Jofre MB, Anton RI, Caviedes-Vidal E. Organochlorine contamination in anuran amphibians of an artificial lake in the semiarid midwest of Argentina. Arch Environ Contam Toxicol 2008;55:471–80.
Karatas AD, Aygun D, Baydin A. Characteristics of endosulfan poisoning: a study of 23 cases. Singapore Med J 2006;47:1030–2.
Lanfranchi AL, Menone ML, Miglioranza KS, Janiot LJ, Aizpun JE, Moreno VJ. Striped weakfish (Cynoscion guatucupa): a biomonitor of organochlorine pesticides in estuarine and near-coastal zones. Mar Pollut Bull 2006;52:74–80.
Li CX, Callaway JC, Waters RS. Removal of GABAergic inhibition alters subthreshold input in neurons in forepaw barrel subfield (FBS) in rat first somatosensory cortex (SI) after digit stimulation. Exp Brain Res 2002;145:411–28.
Liu X, Zhang G, Li J, Yu LL, Xu Y, Li XD, et al. Seasonal patterns and current sources of DDTs, chlordanes, hexachlorobenzene, and endosulfan in the atmosphere of 37 Chinese cities. Environ Sci Technol 2009;43:1316–21.
Macdonald RL, Gallagher MJ, Feng HJ, Kang J. GABA(A) receptor epilepsy mutations. Biochem Pharmacol 2004;68:1497–506.
Maitre MI, de la SP, Lenardon A, Enrique S, Marino F. Pesticide residue levels in Argentinian pasteurised milk. Sci Total Environ 1994;155:105–8.
McCormick DA. GABA as an inhibitory neurotransmitter in human cerebral cortex. J Neurophysiol 1989;62(5):1018–27.
Nag SK, Raikwar MK. Organochlorine pesticide residues in bovine milk. Bull Environ Contam Toxicol 2008;80:5–9.
Okuyama S, Aihara H. Action of nootropic drugs on transcallosal responses in rats. Neuropharmacology 1988;27:67–72.
Olsen RW, Sieghart W. International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Pharmacol Rev 2008;60:243–60 (update).
Paul V, Balasubramaniam E, Kazi M. The neurobehavioural toxicity of endosulfan in rats: a serotonergic involvement in learning impairment. Eur J Pharmacol 1994;270:1–7.
Paul V, Balasubramaniam E, Sheela S, Krishnamoorthy MS. Effects of endosulfan and aldrin on muscle coordination and conditioned avoidance response in rats. Pharmacol Toxicol 1992;71:254–7.
Paul V, Sheela S, Balasubramaniam E, Kazi M. Behavioural and biochemical changes produced by repeated oral administration of the insecticide endosulfan in immature rats. Indian J Physiol Pharmacol 1993;37:204–8.
Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 4th ed. San Diego: Academic Press; 1998.
Peeters RR, Verhoye M, Vos BP, Van Dyck D, Van Der Linden A, De Schutter E. A patchy horizontal organization of the somatosensory activation of the rat cerebellum demonstrated by functional MRI. Eur J Neurosci 1999;11:2720–30.
Pozo K, Harner T, Lee SC, Wania F, Muir DC, Jones KC. Seasonally resolved concentrations of persistent organic pollutants in the global atmosphere from the first year of the GAPS study. Environ Sci Technol 2009;43:796–803.
Ramesh A, Vijayalakshmi A. Environmental exposure to residues after aerial spraying of endosulfan: residues in cow milk, fish, water, soil and cashew leaf in Kasargode, Kerala, India. Pest Manag Sci 2002;58:1048–54.
Reigart JR, Roberts JR. Recognition and management of pesticide poisonings. 5th ed. Washington, DC: U.S. Environmental Protection Agency; 1999.
SENASA. Importacion Agroquimicos 2006., 2009 [Ref Type, Internet Communication] http://www.senasa.gov.ar/contenido.php?to=n&in=524&ino=524&io=7331.
Simon J, Wakimoto H, Fujita N, Lalande M, Barnard EA. Analysis of the set of GABA(A) receptor genes in the human genome. J Biol Chem 2004;279:41422–35.
Stern GA, Macdonald CR, Armstrong D, Dunn B, Fuchs C, Harwood L, et al. Spatial trends and factors affecting variation of organochlorine contaminants levels in Canadian Arctic beluga (Delphinapterus leucas). Sci Total Environ 2005;351–352: 344–68.
Sunol C, Babot Z, Fonfria E, Galofre M, Garcia D, Herrera N, et al. Studies with neuronal cells: from basic studies of mechanisms of neurotoxicity to the prediction of chemical toxicity. Toxicol In Vitro 2008;22:1350–5.
US Environmental Protection Agency. The Health Effects Division's Addendum and Update to the 2002 Risk Assessment. DP Number D345935, November 13, 2007. http://www.regulations.gov.EPA-HQ-OPP-2002-0262 [11-13-2007, Ref Type, Electronic Citation].
Vale C, Fonfria E, Bujons J, Messeguer A, Rodriguez-Farre E, Sunol C. The organochlorine pesticides gamma-hexachlorocyclohexane (lindane), alpha-endosulfan and dieldrin differentially interact with GABA(A) and glycine-gated chloride channels in primary cultures of cerebellar granule cells. Neuroscience 2003;117:397–403.
Wassermann EM, Greenberg BD, Nguyen MB, Murphy DL. Motor cortex excitability correlates with an anxiety-related personality trait. Biol Psychiatry 2001;50: 377–82.
Weber J, Halsall CJ, Muir DC, Teixeira C, Burniston DA, Strachan WM, et al. Endosulfan and gamma-HCH in the arctic: an assessment of surface seawater concentrations and air-sea exchange. Environ Sci Technol 2006;40:7570–6.
Wozniak AL, Bulayeva NN, Watson CS. Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-alpha-mediated Ca^{2+} fluxes and prolactin release in GH3/B6 pituitary tumor cells. Environ Health Perspect 2005;113:431–9.