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Application of multielectrode array (MEA) chips for studying the neurotoxicity of mixtures

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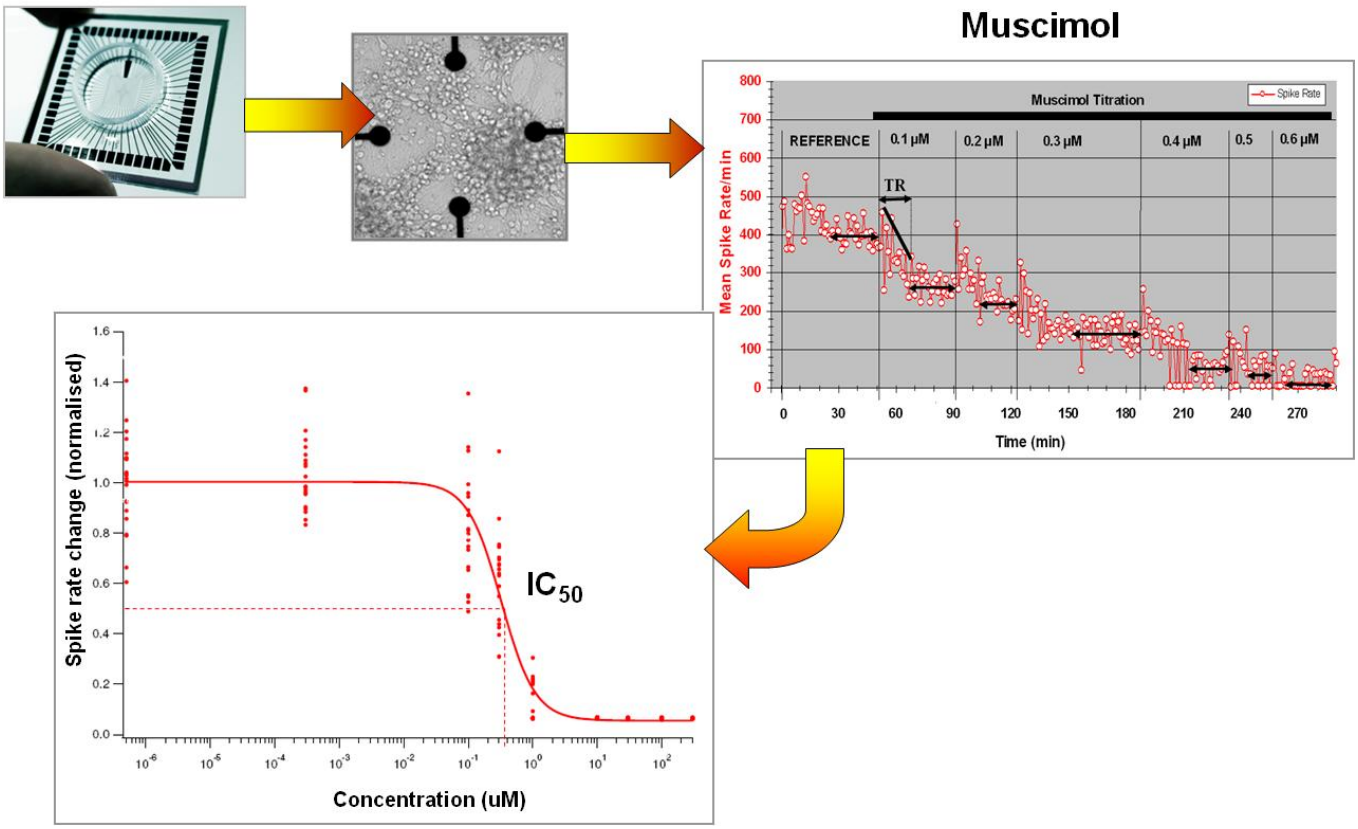

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EXECUTIVE SUMMARY

In vitro neuronal networks are a simplified and accessible model of the central nervous system. Moreover, they exhibit morphological and physiological properties and activity-dependent path-specific synaptic modification similar to the *in vivo* tissue. Cortical neurons grown on multi electrode array (MEA) chips have been shown to be a valuable tool to study fundamental properties of neuronal network activity, synaptic plasticity, learning *in vitro*, and functional pharmacological screening. The variation of spontaneous activity of *in vitro* neuronal networks coupled to MEAs has been studied using several binary mixtures (inhibitors with different mode of action: Verapamil and Muscimol, Fluoxetine and Muscimol; inhibitors with the same mode of action: Deltamethrin and Permethrin; and an excitatory and an inhibitory compound with different mode of action: Kainic acid and Muscimol) with the aim of characterize and assess their combined effects. Individual dose-response and binary mixtures curves have been generated. Concentration Addition (CA) and Independent Action (IA) frameworks have been used to compare calculated and experimental results. In addition, Nuclear Magnetic Resonance (NMR) spectroscopy has been employed to assess that no chemical reaction or complexation took place between mixtures components, as well as to monitor the presence of potential impurities and, in this case, to evaluate their relative amount in the tested samples. The results suggest that additivity: CA and IA are able to predict in most of the cases the total toxicity of the mixture. The variability of the results makes difficult to assess which of both approaches is the most accurate. The presence of both excitatory and inhibitory effects as in the case of Kainic acid may further complicate the analysis of the experimental datasets and biphasic concentration-dose response curves may be need to analyze the joint effect.

1. INTRODUCTION

The number, diversity and complexity of synthetic chemicals produced and released to the environment is overwhelming. Organic chemicals are ubiquitous and affect every possible aspect of modern life. Unfortunately, organic pollution of air, water, soils, sediments and biota is common and, therefore, humans and all other organisms are exposed to multi-component complex chemical mixtures comprising all the aspects of our normal life, e.g. food, consumer products, indoor pollution, etc. The parameters that influence the partitioning of the organic chemicals in the environment include the physical and chemical characteristics of a compound, its behaviour with respect to chemical reactions or microbial degradation, and other physical conditions such as temperature, availability of water, light and oxygen (Walker *et al*., 1996). The major processes responsible for distributing synthetic organic chemicals throughout the biosphere are volatilization and atmospheric transport, transport to waters in soluble form or adsorbed to particles or movement through the food chain.

As a consequence, we are rarely exposed to only one single contaminant, but typically to mixtures of numerous man-made-chemicals with varying constituents in varying concentrations and concentration ratios (Faust *et al*., 2003). However, in contrast to this exposure reality, the toxicological reality is that until recently about 95% of the resources in toxicology were devoted to studies on single chemicals (Groten, 2000). Nevertheless, toxicity data from laboratory tests with single pure chemicals provide essential input to scientific assessments of chemical risks to organisms. On the other hand, the behaviour of chemicals in a mixture may not correspond to that predicted from data on the pure compounds (Altenburger *et al*., 2004). However, the direct testing of all the potential combinations of contaminants is unfeasible, and thus we are confronted with the task of deriving valid predictions of multiple mixture toxicity from toxicity data on individual compounds (Faust *et al*., 2003). The experimental evidence on mixture effects leads to the issue of risk assessment of combined exposures even when each component is present below the individual threshold dose (concentration). Early pioneering studies have been conducted with bacteria (Bulich et al., 1990), daphnids (Barry et al., 1995) and fish (Ankley et al., 2006), and were followed up by additional experiments with populations and communities of unicellular organisms (Schmitt-Jansen et al., 2007). More recently, studies with endpoints relevant to endocrine disruption have been documented for receptor-binding, and receptor-activation assays (Blake et al., 2010), as well as tests with mammalian cell lines and higher organisms like rodends (Yangs and Dennison, 2007; Choi et al., 2010).

Therefore, combined exposure is a reality that dictates the necessity to pay a great deal of attention to hazard identification, exposure assessment and risk characterization of mixtures. However, the present approach provides threshold doses or concentrations of regulatory concern (such as acceptable daily intakes or predicted no effect concentrations) for individual chemicals and exposures below these

levels are usually considered safe. In addition, with a few exceptions, chemical risk assessment considers the effects of single substances in isolation, an approach that is only justified if the exposure to mixtures does not bear the risk of an increased toxicity.

In addition, the present situation is so that toxicity testing for hazard identification relies mostly on the use of animal models, but this approach is costly and time-consuming, and is not practical for hazard identification of the thousands of chemicals such as under the REACH directive or in the high production volume program. Thus, alternative approaches to risk assessment and hazard identification are needed that have higher throughput capability and are predictive of *in vivo* effects (Coecke et al., 2007; Lilienblum et al., 2008) even in the context of mixture toxicity.

In a recent review on the state of the art on mixture toxicity (Kortenkamp et al., 2009) it was concluded that there was a deficit on mixtures studies in, amongst others, the area of neurotoxicity and that it was difficult assessing, based on experimentally published data, the type of combination effect.

In a mixture, chemicals may basically behave in two ways from a toxicological point of view: they can have a joint action or they can interact. In the first case they may act through independent action (IA), also referred to as Loewe additivity and Bliss independence, when the toxicity of the individual chemical is independent of the other compounds in the mixture, or by concentration addition (CA) when the overall toxicity equal the sum of the toxicity of the mixture. CA and IA have been applied to describe the mixture of components having similar and dissimilar mode of action (MoA), respectively (Greco et al., 1995; McCarty and Bogert, 2006). In the second case, the effects of the interaction may be antagonistic or synergistic, decreasing or increasing the effects of the joint action, respectively. Furthermore in the last years theoretical models for the prediction of mixture toxicity have been developed and optimised by comparison with experimental data (Ra et al., 2006; Ferreira et al., 2008) providing encouraging results towards a fundamental role of predictive models to be used as a complementary gold standard for mixture toxicity assessment.

The objective of this work is to provide an assessment of the type of combined effect exerted by binary mixtures by measuring the spontaneous electrical activity of *in vitro* neural networks grown on multielectrode array (MEA) chips. *In vitro* neuronal networks are a simplified and accessible model of the central nervous system, exhibiting morphological and physiological properties (Kriegstein and Dicher, 1983) and activity-dependent path-specific synaptic modification similar to the *in vivo* tissue (Jimbo et al., 1999, Bi and Poo, 1999). Cortical neurons grown on MEA chips have been shown to be a valuable tool to study fundamental properties of neuronal network activity (Maeda et al., 1995; Gross et al., 1999; Beggs et al., 2003; van Pelt et al., 2005; Pasquale et al., 2008), synaptic plasticity (Maeda et al., 1998; Jimbo et al., 1999), learning *in vitro* (Shahaf and Marom, 2001; Eytan et al., 2003; Novellino et al., 2007), functional pharmacological screening (Morefield et al., 2000; Keefer et al., 2001; Gopal 20003; Chiappalone et al. 2003; Gramowski et al. 2006) and toxicological applications

(Streit, 1993; Gross et al., 1997; Gramowski et al., 2000; Shafer et al., 2008; Johnstone et al., 2010; Novelino et al., 2011; Defranchi et al., 2011; Hogberg et al., 2011).

There are few studies concerning the application of MEAs to study mixtures toxicity. Losa et al. (2009) and Johnstone et al. (2009) have studied the concentration-response relationships of a mixture of 5 different pyrethroid insecticides (permethin, cypermethrin, cyfluthrin deltamethrin and esfenvalerate), observing a decreased spontaneous spike rate in a manner that was not effect additive. However, no detailed calculation was performed.

In this work, the effects on spontaneous activity of in vitro neuronal networks coupled to MEAs has been studied using several binary mixtures (inhibitors with different mode of action: Verapamil and Muscimol, Fluoxetine and Muscimol; inhibitors with the same mode of action: Deltamethrin and Permethrin and and excitatory and an inhibitor with different mode of action: Kainic acid and Muscimol) with the aim of characterize and assess their combined effects. Individual dose-response and binary mixtures curves have been generated. Concentration Addition and Independent Action frameworks have been used to compare calculated and experimental results. In addition, Nuclear Magnetic Resonance (NMR) spectroscopy has been employed to assess that no chemical reaction or complexation took place between mixtures components, as well as to monitor the presence of potential impurities and, in this case, to evaluate their relative amount in the tested samples.

2. METHODS AND APPROACH

2.1. COMPOUNDS USED

For this study a total of six compounds were selected according to their mode of action (MoA), to their presence on the consumer's daily life and to the amount of data available from the literature concerning their effects on the nervous system. Three of them are molecules used in several drug preparations and drug testing for medical purpose (Fluoxetine, Verapamil and Kainic acid) and two of them (Deltamethrin and Permethrin) are from the most commonly used and best described pesticides (pyrethroids respectively of type II and I). The compounds used were:

1. R-(-)-Fluoxetine hydrochloride[1] (F, Sigma Aldrich – F1678), CAS: 114247-09-5. F is a serotonin reuptake inhibitor. In both vertebrates and invertebrates, serotonin functions as a neuromodulator to either facilitate or inhibit synaptic activity mediated by neurotransmitters (Fink and Gothert, 2007).

2. Muscimol hydrobromide[2] (M, Sigma Aldrich – G019), CAS: 18174-72-6. M is a psychoactive alkaloid and it is a selective agonist of the $GABA_A$ receptor, thus enhancing the inhibitory

[1] http://www.chemspider.com/Chemical-Structure.3269.html
[2] http:// www.chemspider.com/RecordView.aspx?rid=a969e9d6-172a-40ff-8a47-fca9acc581db
[3] http://www.chemspider.com/Chemical-Structure.59223.html
[4] http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=24868901
[5] http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=24869115
[6] http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=10255

neurotransmission and suppressing spontaneous activity. GABAergic agonists, like muscimol, are employed mainly as antiepileptic drugs or in conjunction with antipsychotics (Bartholini, 1985)

3. (±)-Verapamil hydrochloride[3] (V, Sigma Aldrich – V4629), CAS: 152-11-4. V is an L-type voltage-dependent calcium channel antagonist. It blocks slow activating calcium channels modulating the neuronal excitability and reducing electrical activity (Rüschenschmidt et al., 2004).

4. Deltamethrin[4] (D, Sigma Aldrich – 45423), CAS: 52918-63-5. D is a widely used insecticide which belongs to the type II pyrethroids class of pesticides. D is a neurotoxin whose major target are sodium channels, the effect is the prolongation of sodium permeability during the recovery phase of the action potential in neurons. This causes a persistent depolarization of the membrane, which in turn lowers the action potential threshold and causes repetitive firing leading to paralysis (Bradberry et al., 2005)

5. Permethrin[5] (P, Sigma Aldrich - 45614) CAS: 52645-53-1. P is also a widely used insecticide belonging to the type I pyrethroids class. It acts on sodium channels as well as deltamethrin producing the same effects but with less potency (Shafer et al., 2008)

6. Kainic acid[6] (K, Sigma Aldrich – K0250) CAS: 58002-62-3. Is a glutamate analog originally isolated from a dried red alga (*Digenia simplex*), that binds selectively to a subset of glutamate receptors which serve as ligand-gated ion channels on neurons, and that is used as an anthelmintic and experimentally to induce seizures in laboratory animals (Swanson and Sakai, 2009). As a glutamate analog has an excitatory action inducing depolarisation of the neuronal cell membrane.

Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

2.2. NEURAL NETWORK

The experiments were performed on cryopreserved neurons from embryonic rat brain cortex (Lonza, R-CX-500). Once arrived vials containing 4 million cells were quickly thawed in a 37°C water bath with continuous gentle agitation.

Viable cells (trypan blue assessment) were plated at a density of 35000 per chip in a culture medium composed of PNBM, L-Glutamine, Gentamycine, AmphotericinB, and NSF-1 (Cryocell Bullet kit, Lonza CC-4461). The chips were then incubated at 37°C in 5%CO_2, 20%O_2 in order to let the neuronal network to grow and reach its mature state (3-4 incubation weeks). Starting from day in vitro (DIV) 3, half of the culture medium was changed twice a week under the laminar hood until the beginning of the experiment.

2.3. CHIP PREPARATION

60-electrode MEA chips have been employed with 30µm diameter electrodes, 200 µm inter-electrode spacing with an integrated reference electrode (Multichannel Systems GmbH, Reutlingen, Germany).

Prior to plating the cells, the MEA chip was sterilized (2 hours in oven at 122°C) and afterwards, to promote cell adhesion and neurite outgrowth, it was coated with laminin (Sigma L2020) and poli-D-Lysin (Sigma P6407).

2.4. RECORDING SYSTEM AND SIGNAL PROCESSING

The activity was recorded by the MEA120-2-System from Multi Channel Systems (MCS GmbH, Ruetlingen, Germany, http://www.multichannelsystems.com). In particular the MEA was fed into the MEA Amplifier (Gain 1000x) and data were recorded by MC_Rack software at a sampling rate of 10 kHz. A band pass digital filter (60Hz-4000Hz) was also applied. The system also includes a temperature controller (TC02, MCS GmbH) that allows heating the MEA chips and thus the medium from the bottom.

Spikes were detected when the amplitude of the neuronal electrical activity overcame a threshold set at -6.5 times the standard deviation of the mean square root noise. The recorded signals were then processed to extract parameters related to the spontaneous electrophysiology at both spike and burst level as previously described (Chiappalone et al., 2005).

2.5. ELECTROPHYSIOLOGICAL RECORDING

Neuronal cultures were recorded for spike activity from the third to the fifth week *in vitro*. The experiments were performed on different days using cultures from a minimum of two different isolations. At the beginning of the experimental session a medium change (50%) is performed to establish the "reference activity" and the spontaneous activity which was recorded for 40 minutes. The medium volume during the experiment is 1000µl. The experimental protocol is an "accumulative treatment", and it consists of the administration of 5 to 8 serial concentrations of each compound or mixture (see Table 1).

Binary mixtures were prepared with fluoxetine-muscimol (F-M), verapamil-muscimol (V-M), deltamethrin-permethrin (D-P) and kainic acid-muscimol (K-M) in three different concentration proportions: 2:8, 5:5, 8:2. For each binary mixture a 100 mM stock solution was prepared in water or DMSO depending on the solubility characteristics of the compounds. In the stock solution each compound was present at the concentration of: 80 mM, 20 mM or 50 mM depending on the proportions for the given mixture.

Each administration was performed by gentle manual pipetting. A volume of 100µl of medium was taken out of the chip and mixed with a small volume (1 to 10 µl) of the compound (or mixture) solution and gradually returned to the chip in order to avoid any synapse disruption. The electrophysiological activity was monitored and recorded for at least 40 minutes at the beginning of each experiment before the compounds administration and used as reference activity. After each

administration a time period varying between 5 to 10 minutes was allowed to reach a stable level of activity and then a 20 minute time window of recording was considered for the processing purpose (see Novellino et al., 2011).

Acceptance criteria basing on the quality of the recording were established as previously described (Novellino et al., 2011).

In a subset of experiments the treatment reversibility was also tested: at the end of the recordings the medium was washed out in two steps within 10 minutes: a) 50% medium change (i.e. 500µl), b) 100% medium change (1000µl). After the second medium change, the electrophysiological activity was recorded for further 40 minutes and recovery to the reference mean firing rate was assessed.

Table 1: Summary on compound solutions and concentrations

Compound	Solvent	Concentrations applied
Fluoxetine	water	10 nM, 100 nM, 1µM, 10µM, 100µM
Muscimol	water	10 nM, 100 nM, 1µM, 10µM, 100µM
Verapamil	water	10 nM, 100 nM, 1µM, 10µM, 100µM
Deltamethrin	DMSO	10 nM, 100 nM, 1µM, 10µM, 100µM
Permethrin	DMSO	10 nM, 100 nM, 1µM, 10µM, 100µM, 300, µM, 500 µM, 1mM*
Kainic acid	water	10nM, 50nM, 100 nM, 500 nM, 1 µM, 5 µM, 10 µM

* only in a subset of experiments

2.6. NMR ANALYSIS

Samples preparation. Sample of pure compounds and related mixtures in H_2O and DMSO at the concentration of 10mM and 100µM were added with 10% D_2O and deuterated DMSO respectively in order to stabilize the 'lock' for the NMR analysis.

Samples analysis. ^1H NMR spectra were registered on a Bruker (Rheinstetten, Germany) DRX-500 instrument operating at 500.13 MHz for ^1H observations using a Broadband Inverse (BBI) microprobe maintained at 298 K.

Suppression of the H_2O signal was obtained using pre-saturation experiment (pulse program zgcppr). In this case, ^1H NMR spectra were digitized into 16K data points over a spectral width of 20 ppm with an acquisition time of 1.8 s. An additional relaxation delay of 10 s was included, making a total recycling time of 11.8 s. A 90° pulse was used with 32 scans. Spectra were Fourier transformed applying a line broadening apodization function of 2.0 Hz.

Double suppression of the DMSO and the residual H_2O signals was obtained using pre-saturation experiment (pulse program wetdc). In this case, ^1H NMR spectra were digitized into 32K data points over a spectral width of 15 ppm with an acquisition time of 1.1 s. An additional relaxation delay of 5 s

was included, making a total recycling time of 6.1 s. A 90° pulse was used with 8 scans. Spectra were Fourier transformed applying a line broadening apodization function of 1.0 Hz.

All NMR spectra were processed in Bruker TopSpin 1.3. Chemical shifts are referenced to the internal standard TSP at 0.0 ppm present in each sample at the concentration of 0.58mM. All spectra were manually phased and baseline corrected.

2.7. MODELLING MIXTURES TOXICITY

Realistically, the testing of all chemical mixtures and possible concentrations is not viable. As a consequence, different models on mixture toxicity based on the toxicity of single compounds have been developed. The objective is to reduce the amount of experiments and to be able to predict mixtures toxicity. As we will show later on, the main drawback associated with this approach is the attribution of a correct mechanism/mode of action to the involved chemicals.

2.7.1. Modelling the toxicity of single compounds

One of the most important concepts used in toxicology is the dose-response relationship. In the past, the most used approach was to consider a linear function with or without threshold, i.e. at increasing concentrations there is an increase in the response and nonlinear with saturation at 100%, see Fig. 1. Actually, dose-response curves of single chemicals are fitted to sigmoidal shape curves with values between 0-1 (0-100%). Several models have been proposed in literature (Backhaus *et al*., 2004), between them:

- Weibull (W):

$$f(x) = exp[-exp(\theta_1 + \theta_2 \, log_{10} \, x)] \quad (1)$$

- Box-Cox transformed Weibull (BCW):

$$f(x) = exp\left[-exp\left(\theta_1 + \theta_2 \frac{x^{\theta_3} - 1}{\theta_3}\right)\right] \quad (2)$$

- Logit (L):

$$f(x) = 1 - \frac{1}{[1 + exp(-\theta_1 - \theta_2 \, log_{10} \, x)]} \quad (3)$$

- Generalized Logit (GL):

$$f(x) = 1 - \frac{1}{[1 + exp(-\theta_1 - \theta_2 \, log_{10} \, x)]^{\theta_3}} \quad (4)$$

- Morgan-Mercier Flodin (MMF):

$$f(x) = \frac{1}{1 + \theta_1 \cdot x^{\theta_2}} \quad (5)$$

where θ_1,θ_2,and θ_3 are parameters of the equations. As said before, normally the functions have a lower (L) and upper (U) asymptotes with values of 0 and 1 or the opposite in our case in which we

measure the decrease of neuronal electrical activity. However, in some cases, at low concentrations chemicals shown stimulating effects (hormesis effect) having a U-type shape in the lower part of the concentration-response relationship (Calabrese and Baldwin, 2003). In this case, it is possible to move along the y-axis the function using the following expression:

$$F(x) = L + (U - L) f(x) \quad (6)$$

However, the U-type shape form cannot be reproduced with this approach (Backhaus *et al*., 2004). Recently, a biphasic set of equations has been proposed by Beckon et al. (2008), which has the following form:

$$y = \left(\frac{1}{1 + (\varepsilon_{up} / x)^{\beta_{up}}} \right) \left(\frac{1}{1 + (\varepsilon_{dn} / x)^{\beta_{dn}}} \right) \quad (7)$$

with $\beta_{up}>0$ and $\beta_{dn}<0$. Following Beckon et al. (2008) the β-values represent the steepness, whereas ε–values represent the dose at the mid-point of the rising and of the falling respectively. This approach was introduced to consider biphasic relationships in dose-response curves and it can be extended to consider more than one positive and negative effect and therefore it is able to model hormesis.

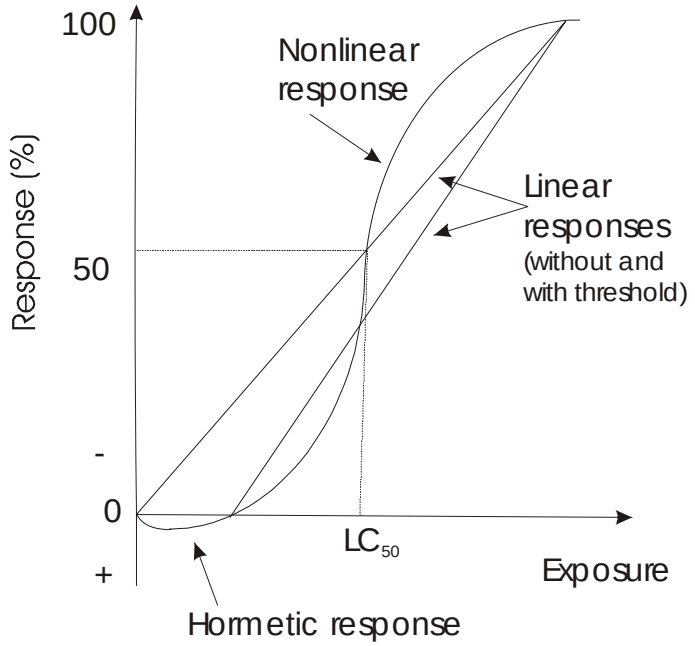


Figure 1. General dose-response functions: a/ linear with and without thresholds and nonlinear with hormesis.

2.7.2. Joint Action: Non-interactive and interaction models

Even though early toxicological studies were devoted to the characterization on single chemicals, Bliss defined in 1939 several categories of chemical action, which are still relevant (Dybing *et al.*, 2002). These are: Concentration Addition (CA), Independent Action (IA) and interactions.

a/ Concentration Addition (CA): Assumes that the components in the mixture have a similar action but differ only with respect to their individual potency. Introduced by Loewe and Muischnek (1926), it is also known as Loewe additivity, simple joint action or dose addition. This may be expressed in terms of toxic units (TUs) which are the ratio of the concentration *i–th* substance in the mixture to the concentration needed to provoke a certain effect (Backhaus *et al*., 2004):

$$TU_i = \frac{C_i}{ECx_i} \tag{8}$$

whereas C_i is the concentration of toxicant *i* in the mixture producing *x*% effect (e.g. EC_{50}). Therefore the overall toxic unit, for a mixture with n components, is equal to:

$$TU_{mix} = \sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{C_i}{ECx_i} = \frac{C_{mix}}{ECx_{mix}} = 1 \tag{9}$$

Individual concentrations can be expressed as constant proportions p_i of the total concentration C_{mix}, with $p_i=C_i/C_{mix}$. In order to calculate the ECx_{mix}, this equation can be re-written as:

$$ECx_{mix} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{ECx_i}} \tag{10}$$

The concentration addition is the most common approach to risk assessment of mixtures and it is applicable over the whole range of exposure levels from low non-toxic levels when all chemicals in the mixture act in a similar way (Feron and Groten, 2002).

Some well analysed examples of this approach are the study on algal toxicity by s-triazine mixtures reported by Faust *et al*. (2001) and (2003) or for the application of toxic equivalency factors (TEF) used to describe the combined toxicity of isomers or structural analogues such as dioxins or PCBs (Birnbaum and DeVito, 1995; Dybing *et al.*, 2002) where the total potency of the combined occurrence is calculated as the sum of the concentration of each individual congener multiplied by its specific potency. Also toxicity of PAHs and phototoxic PAHs (Calamari and Vighi, 1992; Ankley *et al.*, 1996; Swartz *et al*. 1997; Erickson *et al*., 1999; Fent and Batscher, 2000).

However, it is important to considerer that the mode of action of a certain group of chemicals may only be the same for a particular species and therefore it may be not possible to generalize to other organisms.

b/ Independent Action (IA): IA, also known as Bliss independence (Bliss, 1939) and response addition (Greco *et al*., 1995), is based on a the concept of statistically independent distribution of the sensitivities of the individuals towards the toxicants. In this case, it is assumed that the joint probability, p_{mix}^{s}, that an individual survives a concentration, $C_{mix} = \sum_{i=1}^{n} C_i$, is given by:

$$p^{s}_{mix} = 1 - \prod_{i=1}^{n} [1 - p^{d}(C_i)] \quad (11)$$

whereas the probability of dying p^d is the complementary of the survival probability, i.e. $p^d = 1 - p^s$. Although, originally it was formulated for mortality/survival analysis, it can be applied in dose-response analysis as:

$$ECx_{mix} = 1 - \prod_{i=1}^{n} (1 - ECx_i) \quad (12)$$

IA predicts that a mixture of chemicals will not exert an adverse effect when individual chemicals in that mixture are present below their individual No Observable Adverse Effect Level (NOAEL). According to US EPA (2000), IA should be used for mixtures of chemicals that produce the same toxic effect in the same target organ, but which do so by dissimilar mechanisms of action (Borgert *et al*., 2004).

Both approaches have shown their validity (Faust *et al*., 2001; Faust *et al*., 2003;Vighi *et al*., 2003, a.o.), CA when used for chemical mixtures with similar action and IA when used for chemical mixtures with dissimilar action. Combination of both approaches has been also attempted (Altenburger *et al*., 2004). Although both models (CA, IA) involve summing, either the component doses or their toxic effects, differences between models may produce large differences in the risks estimated for a particular mixture. However, with a regulatory perspective, i.e. worst case scenario, CA may be defendable as a pragmatic assumption by default since normally high mixture toxicity is predicted. Alternatively, the use of QSAR criteria was proposed by Vighi *et al*. (2003) to classify the substances as supposedly similarly or dissimilarly acting when no information is available.

c/ Interactions: In any case, both proposed approaches (CA, IA) to evaluate joint toxicity are "non-interaction" approaches, that is, they assume that chemicals are simply additive, and neither synergistic nor antagonistic, when combined in mixtures (Borgert *et al*., 2004). Several approaches have been proposed to take into account the interactions between chemicals to describe their combined effect that may result in a stronger effect (synergism, potentiation) or weaker effect (antagonism, inhibition) than expected on the basis of either CA or IA.

Antagonistic effects were explained by Escher *et al*. (1996), at the molecular level, by competition for sites in the membrane that may decrease toxicity. Synergistic effects can be explained by damage in the cell membrane. Organic solvents, in particular, will affect the membrane permeability and cause proton leak leading to uncoupling (Lewis *et al*., 1994; Escher *et al*., 1999). To study these effects mechanistic studies have shown (Andersen and Jennison, 2004) that interactions should be described at the level of target tissue dose and are best categorized as either pharmacokinetic (PK) or pharmacodynamic (PD). PK interactions occur when the presence of other chemical alters the relationship between the applied dose and the target tissue dose of a compound, whereas PD

interactions occur when the presence of a second chemical alters the relationship between target tissue dose and tissue response.

Joint or interactive effects of a mixture observed at a clearly toxic-effect-levels of the individual chemicals in the mixture do not predict the joint or interactive effects of the mixture that might occur at exposure levels of the mixture similar to or lower than the highest no-toxic-effect-levels of the individual chemicals. This conclusion is highly relevant for designing further toxicity studies of mixtures as well as for low dose extrapolation of mixture toxicity data (Feron and Groten, 2002).

All three basic principles of joint action and interaction are theoretical. In reality, however, it is likely to have to deal with these concepts at the same time, especially when mixtures consist of more than two compounds and when the targets (individuals rather than cells) are more complex (Groten, 2000).

A frequent goal in mixture toxicology is primarily to determine situations where the effects of combinations of chemicals differ from the additive effects of the chemicals given individually. A great deal of effort has focused on creating various statistical methods for assessing when differences from additivity become significant and on identifying potentially important interactions that would change perceptions of the risks of mixtures of chemicals (Andersen and Dennison, 2004).

Effects of mixtures at low concentrations are a controversial issue (Faust *et al*., 2003). Under the assumption of Concentration Addition any concentration of any mixture component is expected to contribute to the overall toxicity of a mixture; there would be no threshold concentration other than zero. Under the Independent Action the situation is different. Only those concentrations of individual toxicants that cause individual effects greater than zero are expected to contribute the overall toxicity.

2.7.3. Calculating mixture's toxicity from individual components

Concentration response curves for single substances describe the intensity of a defined effect as a function of the toxicant concentration.

For the case when the assumed action mechanism is CA and we are interested in calculating the total effect caused by a mixture there is an iterative procedure where the function:

$$error = \left(1 - \sum_{i=1}^{n} \frac{C_i}{f_i^{-1}(E(C_{mix}))}\right)^2 \qquad (13)$$

has to be minimised. The procedure consists on defining an effect (E) and a mixture concentration C_{mix}, then calculate the individual concentrations that will produce this effect using the inverse of Eqs. (1-5). For example for the Box-Cox-Weibull (BCW), we will have:

$$f_i^{-1}(E(C_{mix})) = \left[1 + \frac{\theta_3}{\theta_2}(\ln[-\ln(1-E)] - \theta_1)\right]^{1/\theta_3} \qquad (14)$$

Then the Eq. (13) is calculated and the procedure repeated by changing the mixture concentration until the error is minimized.

The procedure in the case of IA also requires iteration. In this case the error to minimize is:

$$error = \left[x\% - 1 + \prod_{i=1}^{n} (1 - f_i(p_i(ECx_{mix}))) \right]^2 \quad (15)$$

whereas the total effect is x%. In this case one defines a total effect and a mixture concentration, then calculates the individual effects of each component in the mixture at their specific concentration and evaluates Eq. (15). The procedure is repeated until the appropriate mixture concentration is obtained.

Ii is generally accepted that for dissimilarly acting toxicants, IA will produce a better fit of the mixture toxicity (Backhaus *et al*; 2000; Faust *et al*., 2003; a.o.), whereas in the case of similarly acting chemicals CA will adjust more accurately the experimental results (Calamari and Vighi, 1992; Altenburger *et al*. 2000; Faust *et al*., 2001; a.o.). However, with a regulatory perspective, i.e. worst case, CA by predicting higher toxicity seems a more pragmatic option (Vighi *et al*., 2003). In any case, no-interactions have been assumed to occur in these two approaches. Thus although the additivity models are mathematically simple, they require assumptions about the mechanisms of action (only similar or dissimilar) and the high to low dose extrapolation. Therefore theoretical considerations in risk assessment of chemical mixtures should be verified by simple case studies (Groten, 2000).

3. RESULTS AND DISCUSSION

Several experiments were carried out on the MEA chip using two pyrethroids: Permethrin (P), and deltametrhin (D); Muscimol (M) and Verapamil (V); Muscimol and Fluoxetine (F); and Muscimol and Kainic Acid (K). First the pure compounds were examined and concentration-Normalized firing rate (NFR) curves obtained. Afterwards three mixtures of molar percentage: 20-80, 50-50 and 80-20 were examined. The concentrations have been indicated in Table 1 and they depend on the inhibitory potency of the compound of the mixture.

3.1. NMR ANALYSIS

^{1}H NMR analyses were performed in order to obtain several information on the status of the samples including confirmation of the expected chemical structure in the sample, quantitative data of the real concentration, stability in solution during a period of time, possible presence of impurities and related proportion, and possible formation of new products or abducts in the case of mixtures.

All expected chemical structures were confirmed by ^{1}H NMR experiments and comparison with literature data. Examples on Muscimol, Fluoxetin, and Verapamil are shown in Figure 2.

The absolute concentration of the samples was calculated by comparison of the internal standard NMR signal with a known signal of each compound. In general, [TSP] x 9 x A_2/N_2 = [compound], were [TSP] = concentration of TSP (mM) in the NMR tube; 9 = number of protons of TSP; A_2 = integral of a know signal from each compound; N_2 = number of protons giving rise to the known signal; [compound] = concentration of the compound (mM) in the NMR tube. The majority of the cases

showed the real concentration of the samples within the expected ranges. However, this was not the case of few samples due to possible manual preparation mistakes. In these cases, the possibility to monitor the actual concentration of the samples by NMR analysis was extremely useful in order to prevent possible misinterpretation of the pharmacological results.

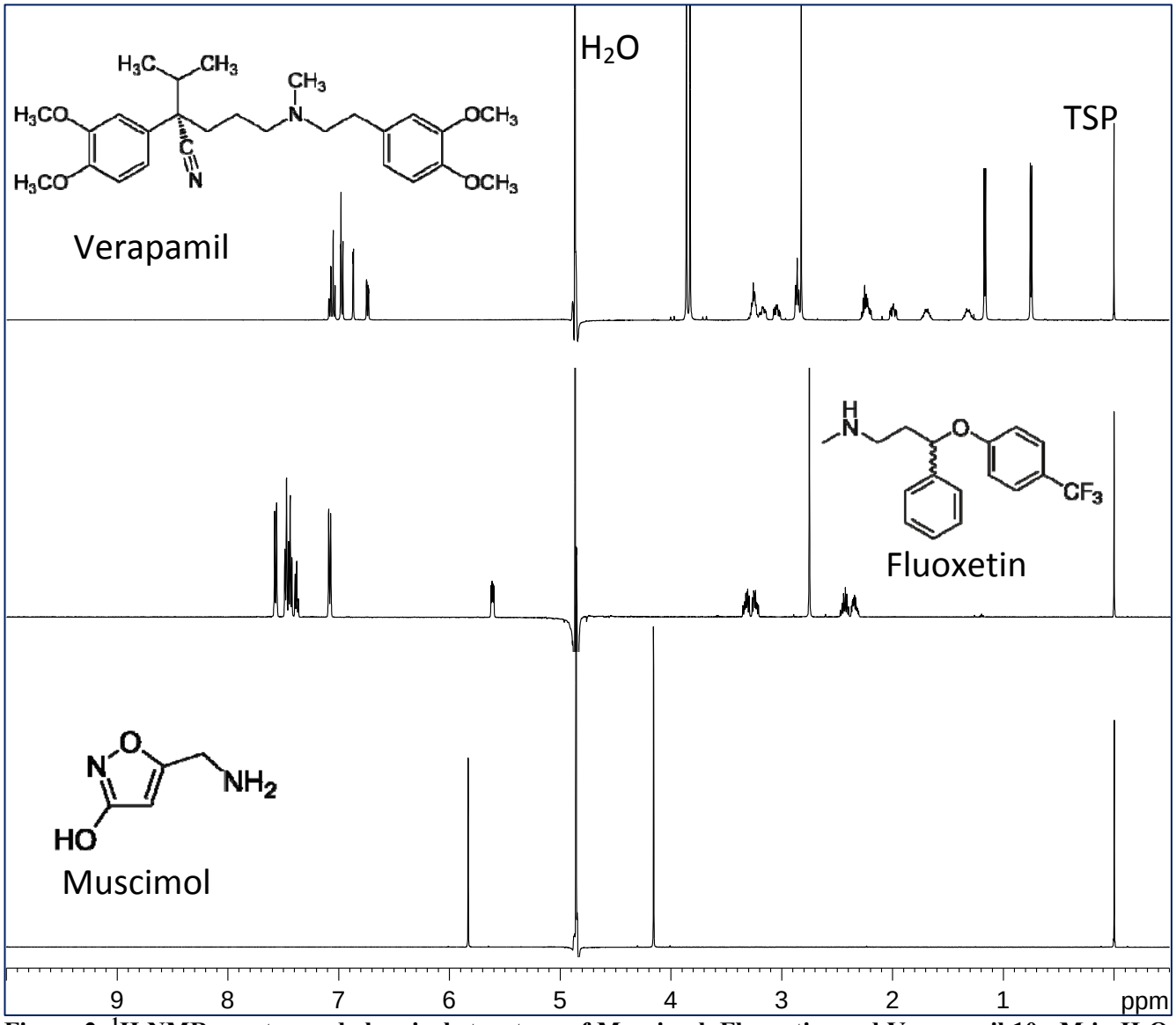


Figure 2. ^{1}H NMR spectra and chemical structure of Muscimol, Fluoxetin, and Verapamil 10mM in H_2O with 10% D_2O and TSP as internal standard (0.58mM).

The stability of the samples was monitored along a period of three weeks by repeating the ^{1}H NMR experiment every three days for each sample. No degradation was observed; this result guaranteed the possibility to perform the pharmacological analysis during this period of time without the need to prepare every time a new batch sample.

Impurities were observed in most of the cases. Their relative concentration were higher in more diluted samples, indicating that such impurities derived from the sample preparation process rather than from the sample dried materials itself. The example of Muscimol is shown in Figure 3.

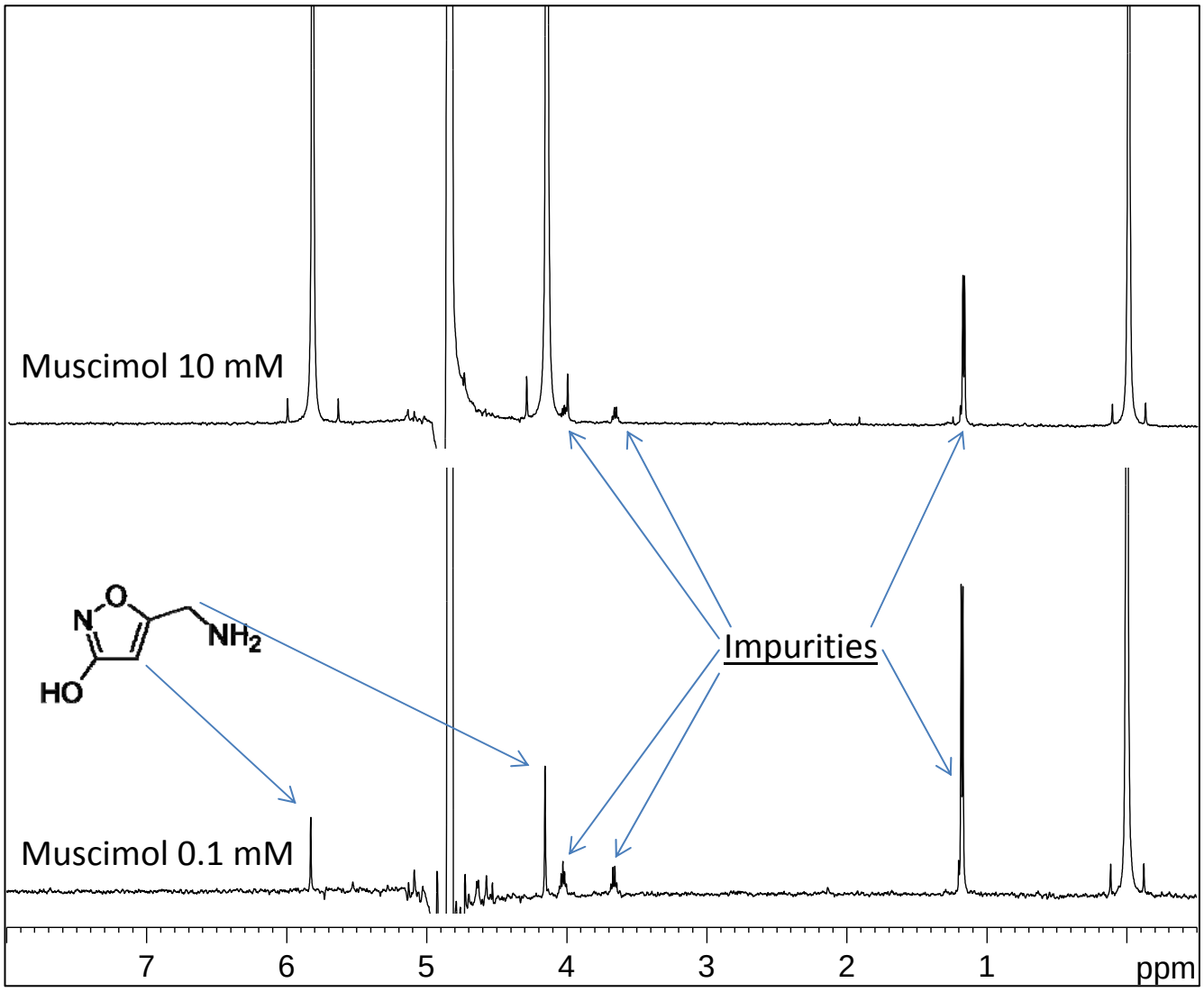


Figure 3. ^{1}H NMR spectra of Muscimol at 10mM and 0.1mM. Impurities are more abundant in less concentrated Muscimol.

In the case of mixtures, the relative amount of both components for each mixture analyzed was in the expected range. Moreover, no formation of new products or abducts was observed as shown in the example of Muscimol/Fluoxetin 1/1 mixture in Figure 4.

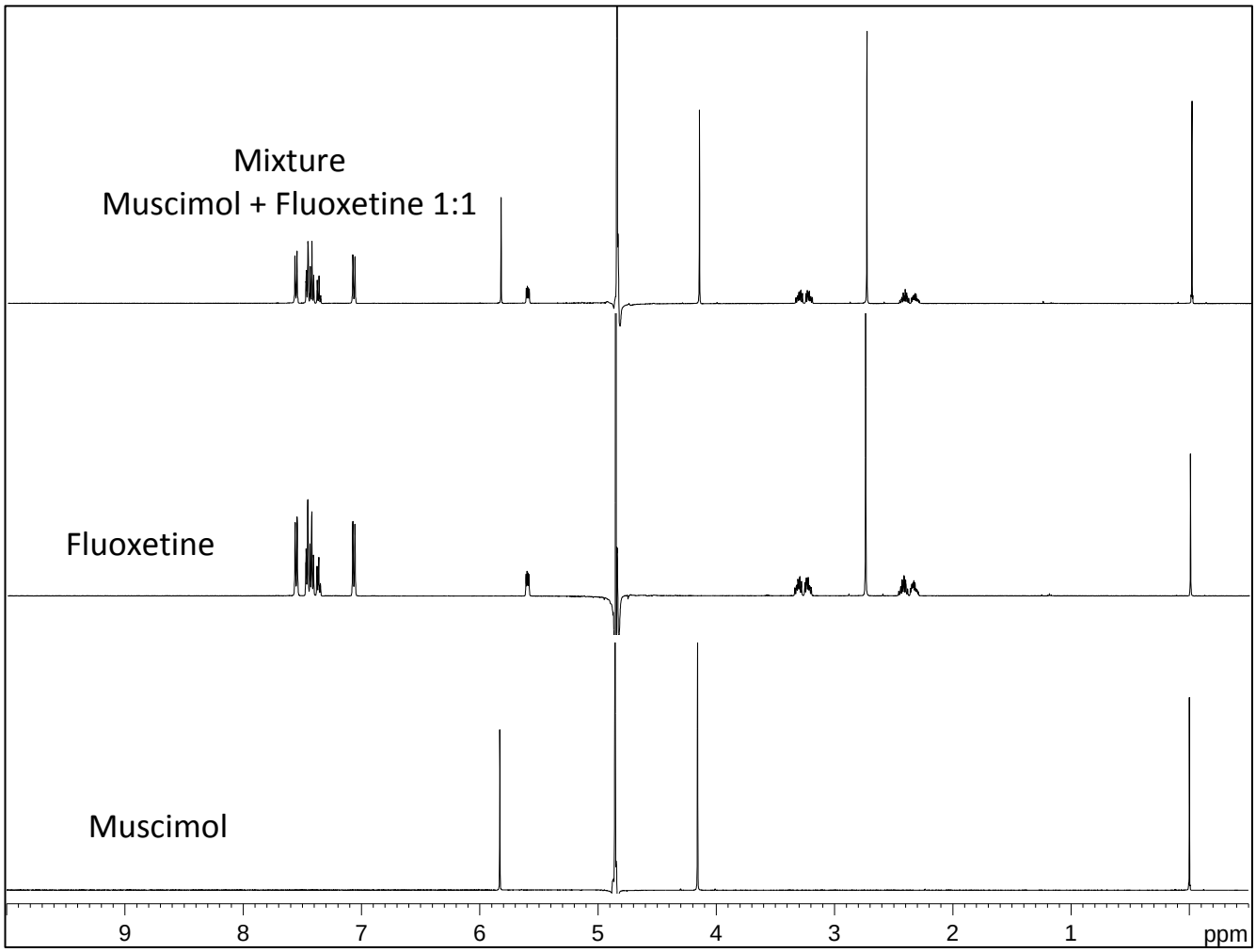


Figure 4. ^{1}H NMR spectra Muscimol, Fluoxetin, and their 1:1 mixture. No complexes or new derivatives are observed.

3.2. FITTING CONCENTRATION-RESPONSE CURVES FOR PURE COMPOUNDS AND MIXTURES

Experimental concentration-response (Normalized firing rate) curves were fitted for the pure compounds as well as for the studied mixtures using Eqs. (1)-(5). The fitting parameters obtained are summarized in Table 2, whereas Figure 5 shows the shape of the different obtained curves for the single compounds.

Table 2. Parameters of the Concentration-response (Normalized firing rate) fitted curves using Eqs. (1)-(5).

	W			BCW				L			GL				MMF		
	θ_1	θ_2	r^2	θ_1	θ_2	θ_3	r^2	θ_1	θ_2	r^2	θ_1	θ_2	θ_3	r^2	θ_1	θ_2	r^2
Permethrin (P)	-1.007	0.6088	0.973	-1.056	0.2296	0.06134	0.977	-0.7538	0.8587	0.958	-148.7	48.3	0.008003	0.989	0.4706	0.3729	0.958
Deltamethrin (D)	-0.6067	1.683	0.982	-0.6429	3.029	1.888	0.996	-0.2739	2.724	0.976	-4.26	5.826	0.2107	0.983	0.7604	1.183	0.976
Muscimol (M)	1.079	1.031	0.9868	1.408	0.7759	0.1982	0.9998	2.345	1.598	0.9766	0.3656	26.94	0.01903	0.9954	10.43	0.6942	0.9766
Verapamil (V)	-18.49	19.5	0.9231	-2.388	0.0002882	14.14	0.9476	-18.29	20.96	0.9231	-2.456	7.205	7.799	0.9231	-	-	-
Fluoxetine (F)	-1.063	2.07	0.9867	-1.15	0.6626	0.3229	0.9978	-0.977	3.724	0.9813	-98.87	89.57	0.01207	0.9909	0.3763	1.618	0.9813
Kainic Acid (K)	-0.4914	3.177	0.9685	-0.4168	1.113	-0.4609	0.9742	-0.1216	3.805	0.9716	1.779	2.65	4.534	0.9736	0.8855	1.653	0.9716
20P-80D	-0.1109	0.7384	0.9975	-0.06916	0.3146	-0.04984	0.9983	0.5431	1.155	0.9955	-0.6245	1.523	0.4729	0.9969	1.721	0.5017	0.9955
50P-50D	-0.6221	0.9318	0.9922	-0.7729	0.405	0.1941	0.9969	-0.2577	1.389	0.9865	-3.791	2.994	0.2402	0.9942	0.7726	0.6032	0.9865
80P-20D	-0.6974	0.6264	0.9851	-0.76	0.2458	0.06369	0.9892	-0.3329	0.9317	0.9678	-7.958	3.313	0.1229	0.9913	0.7168	0.4048	0.9678
20M-80V	0.9665	3.28	0.9988	0.9632	7.1	-14.37	0.9933	2.547	4.786	0.999	4.435	3.602	6.454	0.999	12.77	2.079	0.999
50M-50V	0.554	2.597	0.9982	0.5523	10.28	-12.59	0.9883	1.541	3.496	0.9984	3.597	2.508	7.176	0.9985	4.668	1.518	0.9984
80M-20V	1.299	2.066	0.9993	1.488	0.2655	-0.9711	0.995	3.272	3.803	0.996	2.701	8.227	0.1833	1	26.54	1.655	0.996
20M-80F	0.4612	1.901	0.9885	0.4905	8.537	-12.25	0.9598	1.32	2.686	0.9867	-8.821	37.64	0.03119	0.9896	3.744	1.166	0.9867
50M-50F	0.1363	1.09	0.9963	0.1256	0.57	0.13	0.9991	0.8612	1.602	0.9864	-2.759	5.644	0.1384	0.9987	2.366	0.6958	0.9864
80M-20F	0.6141	1.584	0.9977	0.6472	0.9334	0.2101	1	1.622	2.378	0.9943	-8.173	46.34	0.02189	0.9991	5.066	1.033	0.9943
20M-80K	0.1757	1.429	0.992	0.1933	0.7465	0.1312	0.9937	0.8883	2.015	0.9805	-9.399	29.24	0.03643	0.9953	2.431	0.875	0.9805
50M-50K	0.5121	2.383	0.9812	0.7373	1.932	0.4841	0.9929	1.244	3.01	0.9693	-12.6	69.4	0.02004	0.9853	3.469	1.308	0.9693
80M-20K	1	2.457	0.9933	1.299	1.827	0.3747	0.9986	2.151	3.463	0.9858	2.043	26.74	0.06219	0.998	8.596	1.504	0.9858

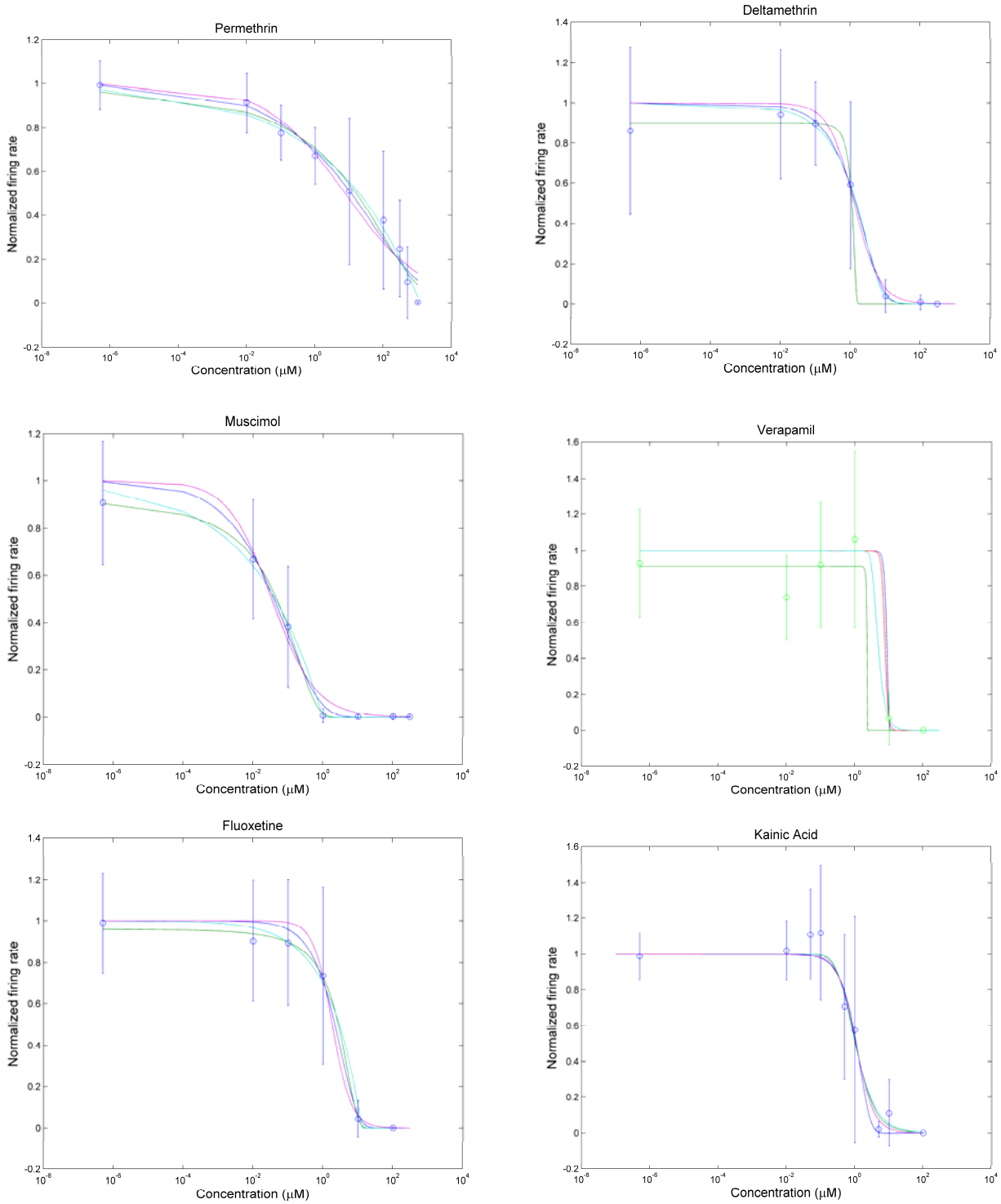


Figure 5. Experimental normalized firing rate (average ± standard deviation) and fitted concentration-response curves, Eqs. (1)-(5), for the six pure compounds: Permethrin, Deltamethrin, Muscimol, Verapamil, Fluoxetine and Kainic acid.

As mentioned in the previous section, the problem with this type of functions, i.e. Eqs. (1)-(5), is that they only consider one type of effect. However, as it can be seen for the case of Kainic acid there are two consecutive effects: at the beginning, at low concentrations, there is an excitatory effect and after, when concentrations start to increase, there is an inhibitory effect. In this case, it is possible to use the

functions developed by Beckon et al. (2008), Eq. (7). Figure 6 shows the fit obtained with this type of function that is able to capture both effects.

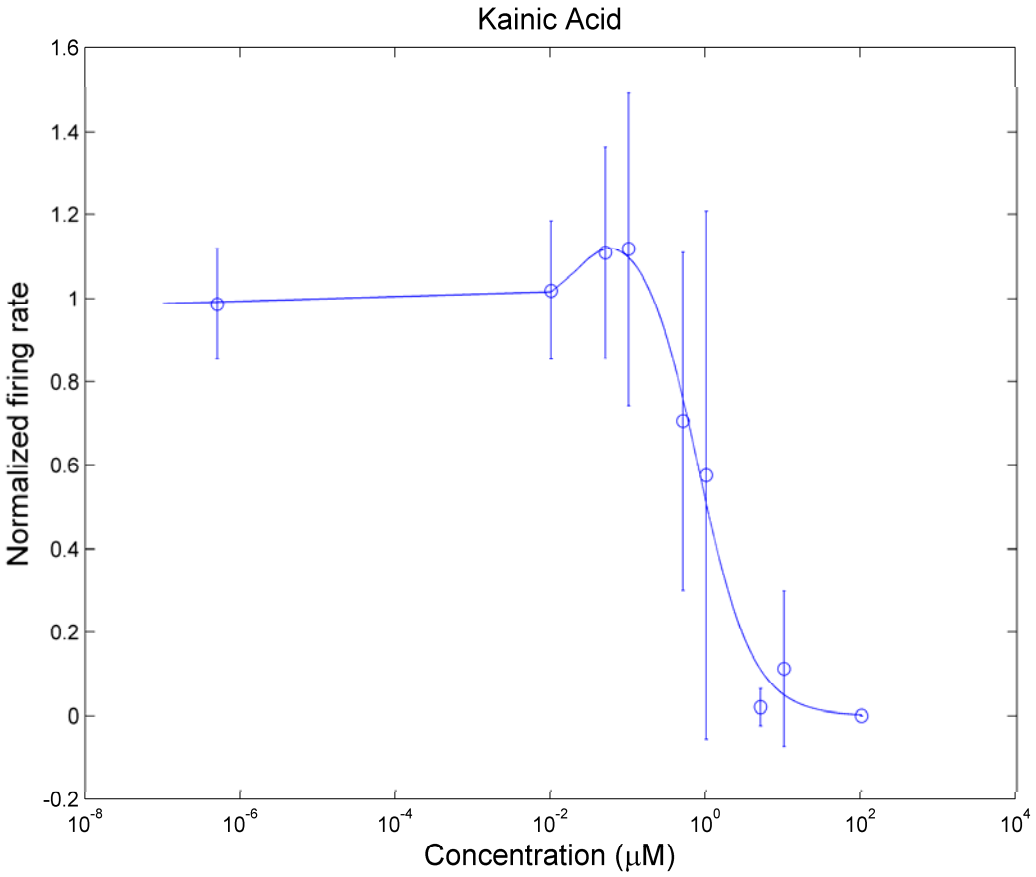
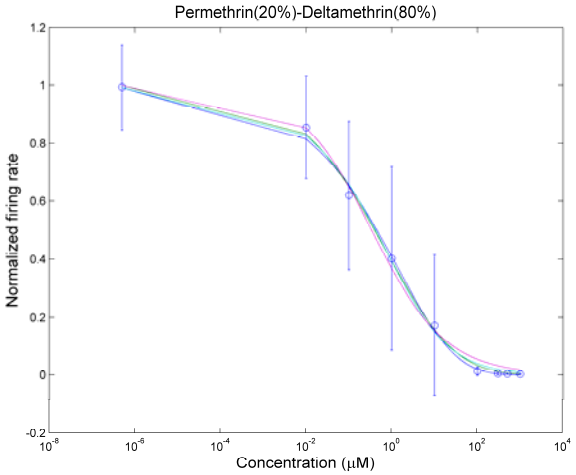
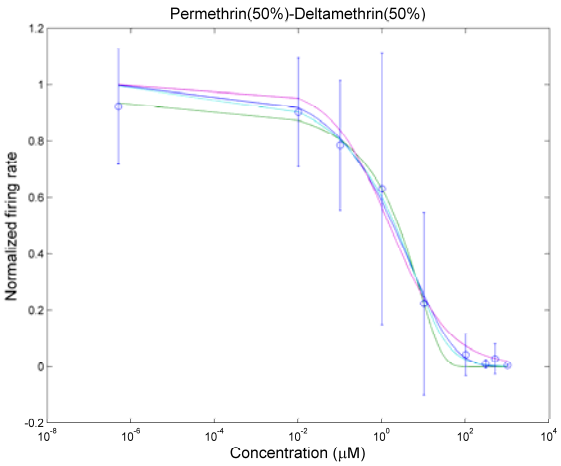
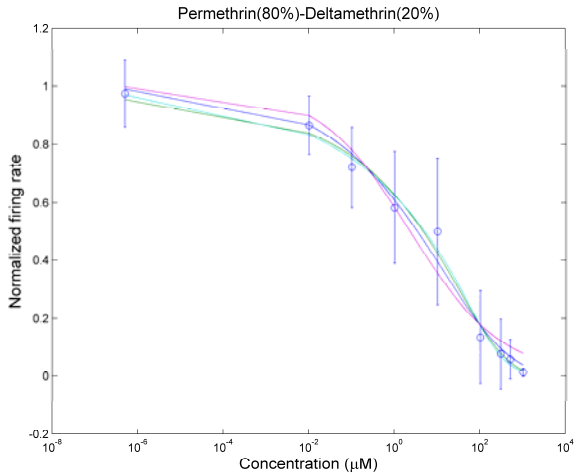
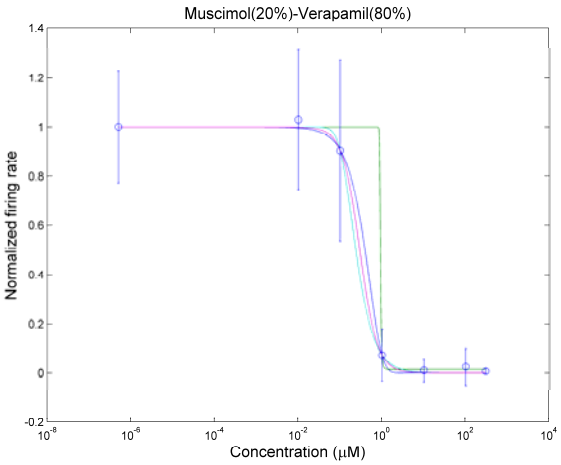


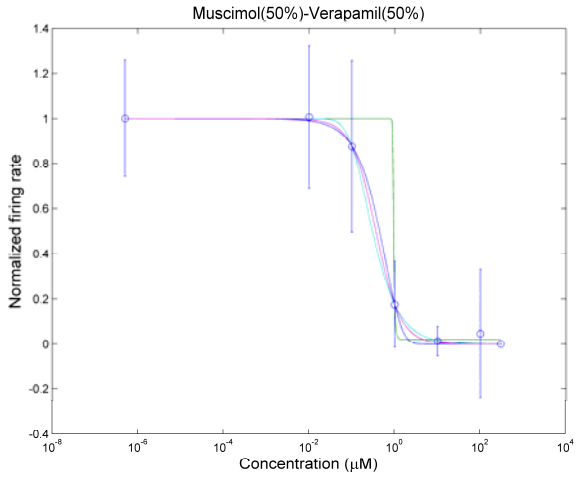
Figure 6. Curve fitted with the biphasic dose-response, Eq. (7), relationship.

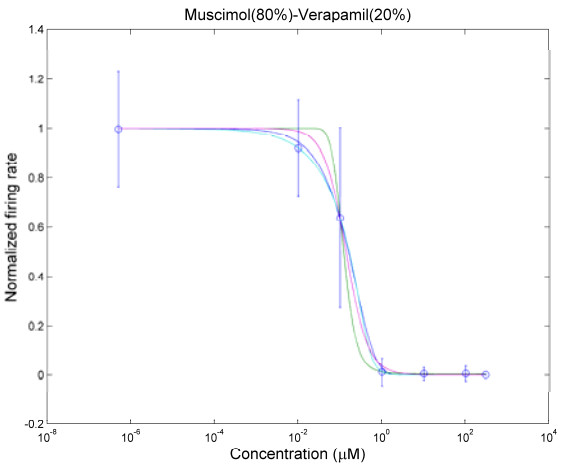


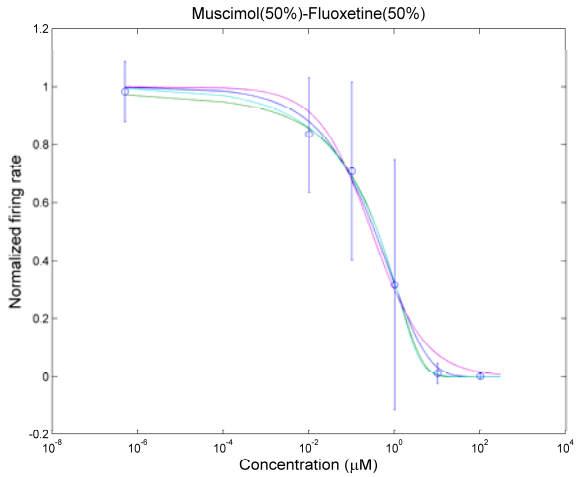


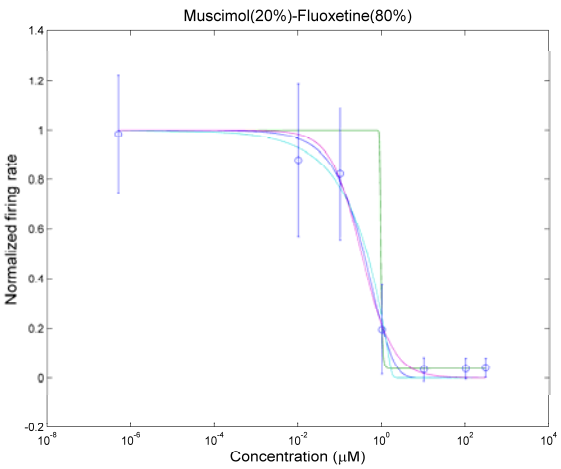

Permethrin(80%)-Deltamethrin(20%)
Normalized firing rate
Concentration (μM)


Muscimol(20%)-Verapamil(80%)
Normalized firing rate
Concentration (μM)


Muscimol(50%)-Verapamil(50%)
Normalized firing rate
Concentration (μM)


Muscimol(80%)-Verapamil(20%)
Normalized firing rate
Concentration (μM)


Muscimol(50%)-Fluoxetine(50%)
Normalized firing rate
Concentration (μM)


Muscimol(20%)-Fluoxetine(80%)
Normalized firing rate
Concentration (μM)

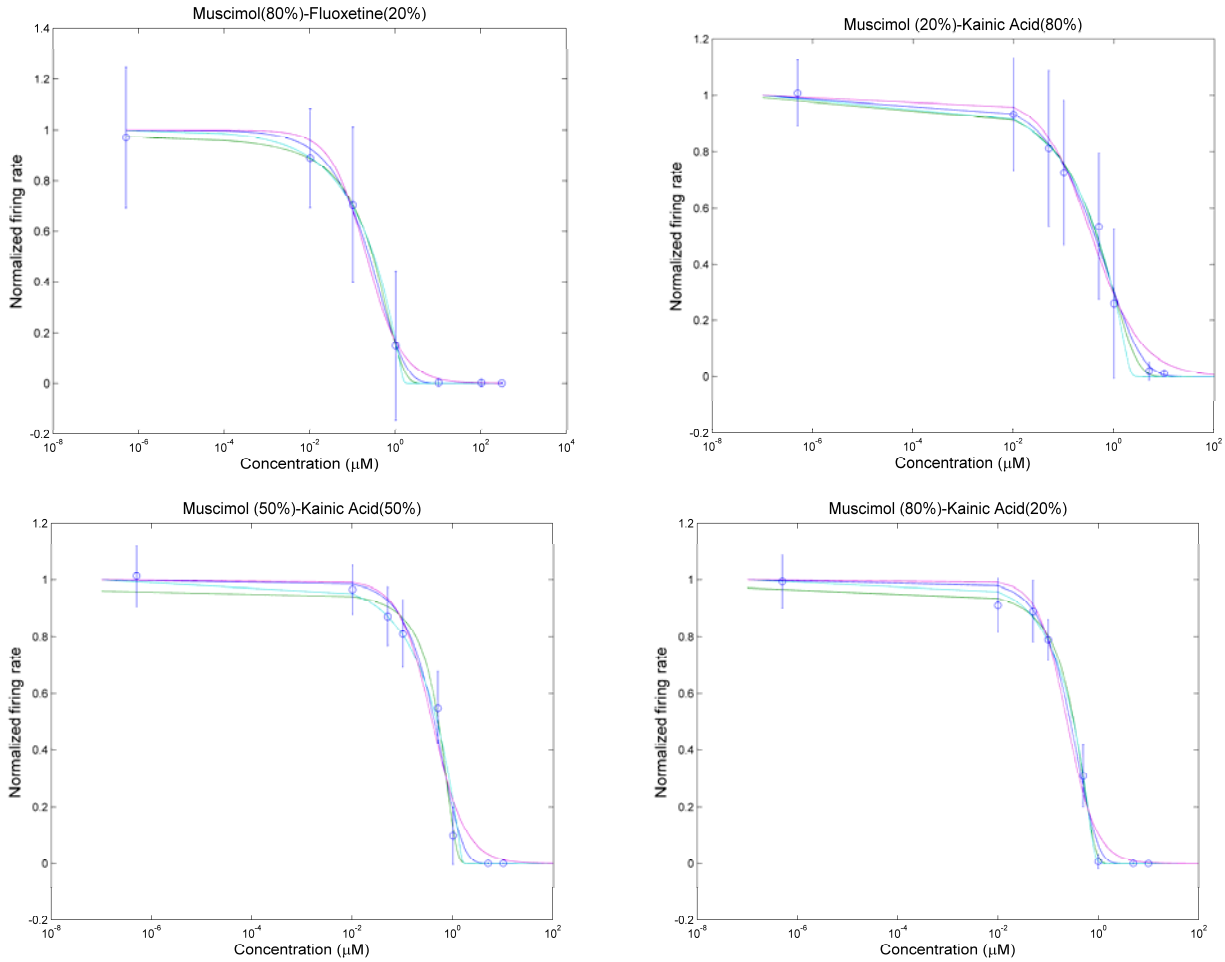


Figure 7. Experimental normalized firing rate (average ± standard deviation) and fitted concentration-response curves, Eqs. (1)-(5), for the twelve mixtures: 20P-80D, 50P-50D, 80P-20D, 20M-80V, 50M-50V, 80M-20V, 20M-80F, 50M-50F, 80M-20F, 80M-20V, 20M-80K, 50M-50K, 80M-20K.

The calculated values of EC_{50} using the different correlations, Eqs. (1)-(5), and the parameters summarized in Table 2 are shown in Table 3. In principle, one should expect that the values would be between those of the single compounds and change accordingly with the proportion of those in the mixtures. This is the case only for Muscimol-Fluoxetine in all the mixtures and using all the correlations (see Table 3). For Permethrin-Deltamethrin, the order is correct, i.e. the IC_{50} increases as the percentage of Permethrin increases in the mixture. However, IC_{50}s for 20P-80D are lower than pure Deltamethrin which would suggest some synergism in this mixture. For the mixture Muscimol-Verapamil the IC_{50} values are between those of the pure compounds, but the order is not respected in the case of the 50-50 mixture and the same occurs in the case of Muscimol-Kainic acid. However, it is not clear if these differences are due to the high standard deviations found in the experimental data sets or are a property of the toxicity of the mixtures. Probably the ordering concerning the 50-50 mixture is due to the high dispersion of the experimental data sets. One should notice that the results are the average of several experiments and that each experiment use a different MEA chip in which a neuronal network has growth establishing different connections.

Table 3. IC_{50} values obtained with the different fitted equations.

	IC_{50} (μM)				
	W	**BCW**	**L**	**GL**	**MMF**
Permethrin (P)	11.2732	15.7412	7.5481	19.2969	7.5482
Deltamethrin (D)	1.3890	1.0878	1.2605	1.4896	1.2605
Muscimol (M)	0.0396	0.0475	0.0341	0.0431	0.0341
Verapamil (V)	8.4998	2.2561	7.4579	4.6839	-
Fluoxetine (F)	2.1700	2.7225	1.8296	2.9019	1.8295
Kainic Acid (K)*	1.0947	1.0467	1.0764	1.0190	1.0763
20P-80D	0.4506	0.3971	0.3387	0.4169	0.3389
50P-50D	1.8806	2.5013	1.5330	2.0968	1.5337
80P-20D	3.3747	4.5923	2.2767	5.0207	2.2762
20M-80V	0.3923	0.9131	0.2936	0.2361	0.2937
50M-50V	0.4421	0.9419	0.3624	0.3008	0.3624
80M-20V	0.1563	0.1209	0.1379	0.1640	0.1379
20M-80F	0.3669	0.9366	0.3225	0.440**5**	0.3223
50M-50F	0.3457	0.4002	0.2900	0.4006	0.2900
80M-20F	0.2404	0.2915	0.2079	0.3112	0.2079
20M-80K	0.4174	0.4541	0.3624	0.4685	0.3623
50M-50K	0.4279	0.5123	0.3861	0.4821	0.3864
80M-20K	0.2779	0.3278	0.2393	0.3212	0.2392

*For Kainic acid the biphasic curve produced an IC_{50} =1.0214 μM.

3.3. PERMETHRIN AND DELTAMETHRIN MIXTURES

Pyrethroids are synthetic chemicals whose structures mimic the natural insecticide pyrethrin. They are widely used in and around households, including on pets, in pests control, and in agriculture. They constitute a major proportion of the insecticide market and are common in commercial products such as household insecticides. The primary target site of this class of neurotoxic pesticides is the voltage-dependent sodium channel in excitable membranes. The interaction of pyrethroids with the sensitive fraction of the sodium channels results in a prolongation of the inward sodium current during excitation, as pyrethroid-modified sodium channels stay open much longer than normal (Shafer and Meyer, 2004). The prolonged sodium current induced by the pyrethroids results in pronounced repetitive activity, notably in sense organs, but — depending on pyrethroid structure — also in sensory nerve fibers, motor nerve terminals, and skeletal muscle fibers. Besides repetitive firing, membrane depolarization results in enhanced neurotransmitter release and eventually block of excitation (Vijverberg and Bercken, 1990) leading to paralysis and death.

Using the fitted curves from the pure compounds we have compared the predicted CA and IA mixture toxicity with the experimental values. Figure 8 shows the results obtained for the three curves using fitted Weibull curves for the pure compounds. The IC_{50} obtained with CA and IA are: 1.6960 μM and 0.9795 μM for 20P-80D; 2.4283 μM and 1.1919 μM for 50P-50D; and 4.3401 μM and 1.9901 μM 80P-20D, respectively. The results for 50P-50D and 80P-20D are in agreement with the values

obtained by the experimental fit of the data whereas for the case of 20P-80D the predicted values are slightly higher. As it can be observed in this case, contrarily with most frequent results, the values obtained by CA are always higher than those obtained with IA.

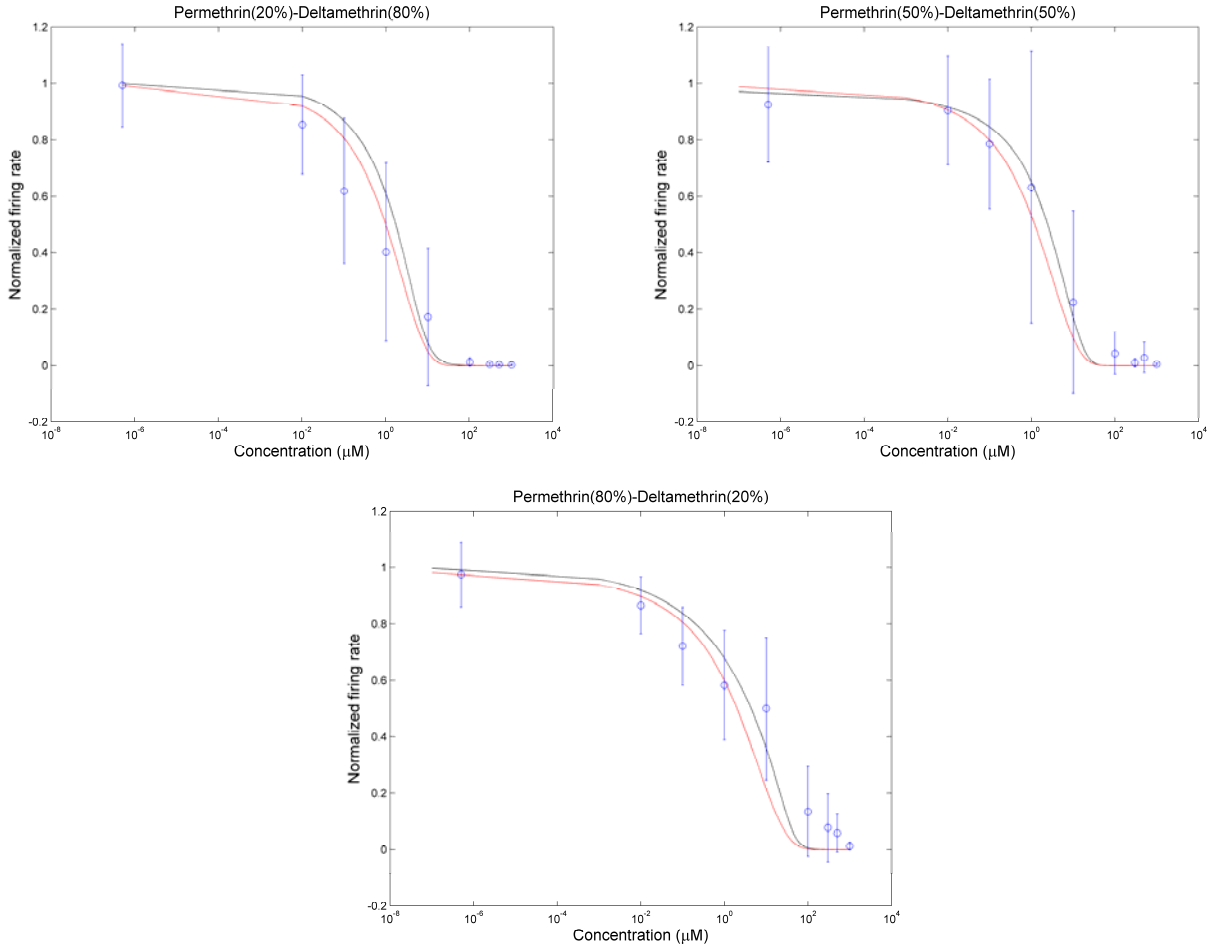


Figure 8. Experimental and calculated -based on pure compounds data- concentration-response curves using concentration addition (black) and independent action (red) for Permethrin and Deltametrin mixtures.

3.4. MUSCIMOL AND VERAPAMIL MIXTURES

Verapamil is an L-type calcium channel blocker of the phenylalkylamine class. It is a common drug used in the treatment of hypertension, angina pectoris, cardiac arrhythmia (Harder et al., 1993) and most recently, cluster headaches (Leone et al., 2000). Verapamil's mechanism in all cases is to block voltage-dependent calcium channels reducing neuronal and muscular excitability.

Muscimol is a $GABA_A$ receptor agonist thus mimicking the effect of the most widely distributed inhibitory neurotransmitter in the central nervous system: GABA. The effects of Muscimol on neuronal activity both *in vitro* and *in vivo* have been well characterized (Zivkovic et al., 1983; Avoli et al., 1994; Bosman et al., 2005). $GABA_A$ agonists reduce neuronal excitability by generating and influx of Cl^- ions which hyperpolarizes the cell membrane. As a consequence neuronal activity is quenched and they are said to have an inhibitory effect.

Very recently our group has led an interlaboratory study where the reproducibility of MEA data obtained on neuronal activity of Muscimol and Verapamil has been demonstrated (Novellino et al., 2011). Furthermore both Muscimol and Verapamil have been characterized on *in vitro* neuronal cultures for their effects on electrical activity (Keith et al., 1994; Novellino et al., 2011) thus providing a good set of chemicals for studying the effect of mixed inhibitory compounds with different mode of action.

Using the fitted curves from the pure compounds we have compared the predicted CA and IA mixture toxicity with the experimental values. Figure 10 shows the results obtained for the three curves using fitted Weibull curves for the pure compounds. The IC_{50} obtained with CA and IA are: 0.1955 µM and 0.1987µM for 20M-80V; 0.0790µM and 0.1987µM for 50M-50V; and 0.0495µM and 0.0427µM 80M-20V, respectively. In all cases the predicted results are lower that the fitted experimental data, implying that the real toxicity is lower than the calculated using additivity. In this case CA and IA produce nearly identical results, with the exception of the 50M-50V where CA predicts higher toxicity than IA.

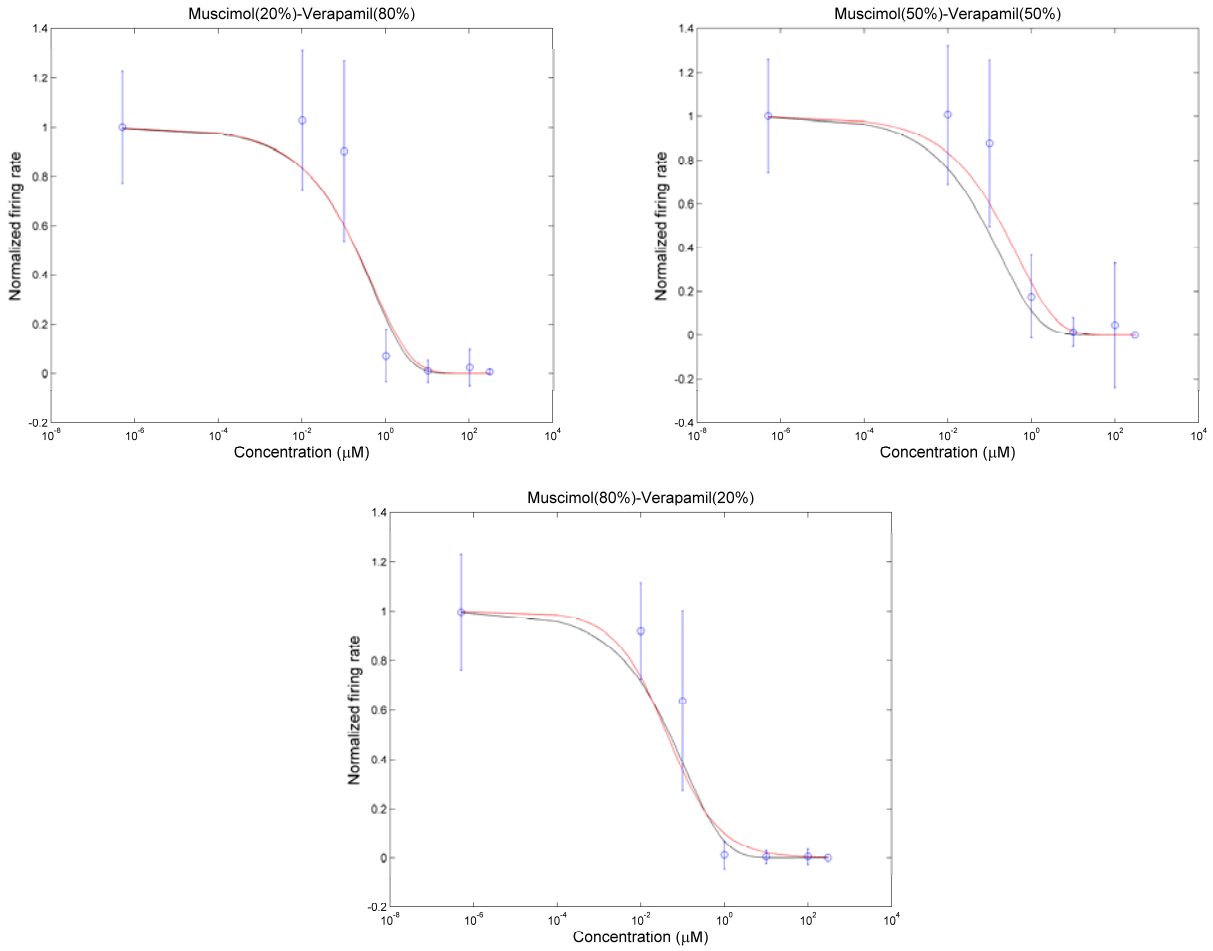


Figure 9. Experimental and calculated -based on pure compounds data- concentration-response curves using concentration addition (black) and independent action (red) for Muscimol and Verapamil mixtures.

3.5. MUSCIMOL AND FLUOXETINE MIXTURES

Fluoxetine acts on the serotonergic system by inhibiting the serotonin (5-HT) reuptake thus enhancing its the effect on the central nervous system. It is one of the most diffused drugs for the treatment of major depression and some psychiatric disorders like panic and bipolar disorders and bulimia (Mayer and Walsh, 1998; Shelton, 2003). Its effect on neuronal activity in vitro has been already characterised with the MEA (Xia et al., 2003, Novellino et al., 2011) thus, together with muscimol it provides another good set of data to test the effect of compounds having the same effect (inhibitory), but with different mode of action.

Using the fitted curves from the pure compounds we have compared the predicted CA and IA mixture toxicity with the experimental values. Figure 10 shows the results obtained for the three curves using fitted Logit curves for the pure compounds. The IC_{50} obtained with CA and IA are: 0.1588 µM and 0.1661 µM for 20M-80F; 0.0669µM and 0.0688 µM for 50M-50F; and 0.0424 µM and 0.0428 µM 80M-20F, respectively. In all cases the predicted results are lower that the fitted experimental data, implying that the real toxicity is lower than the calculated using additivity. In this case CA and IA produce nearly identical results.

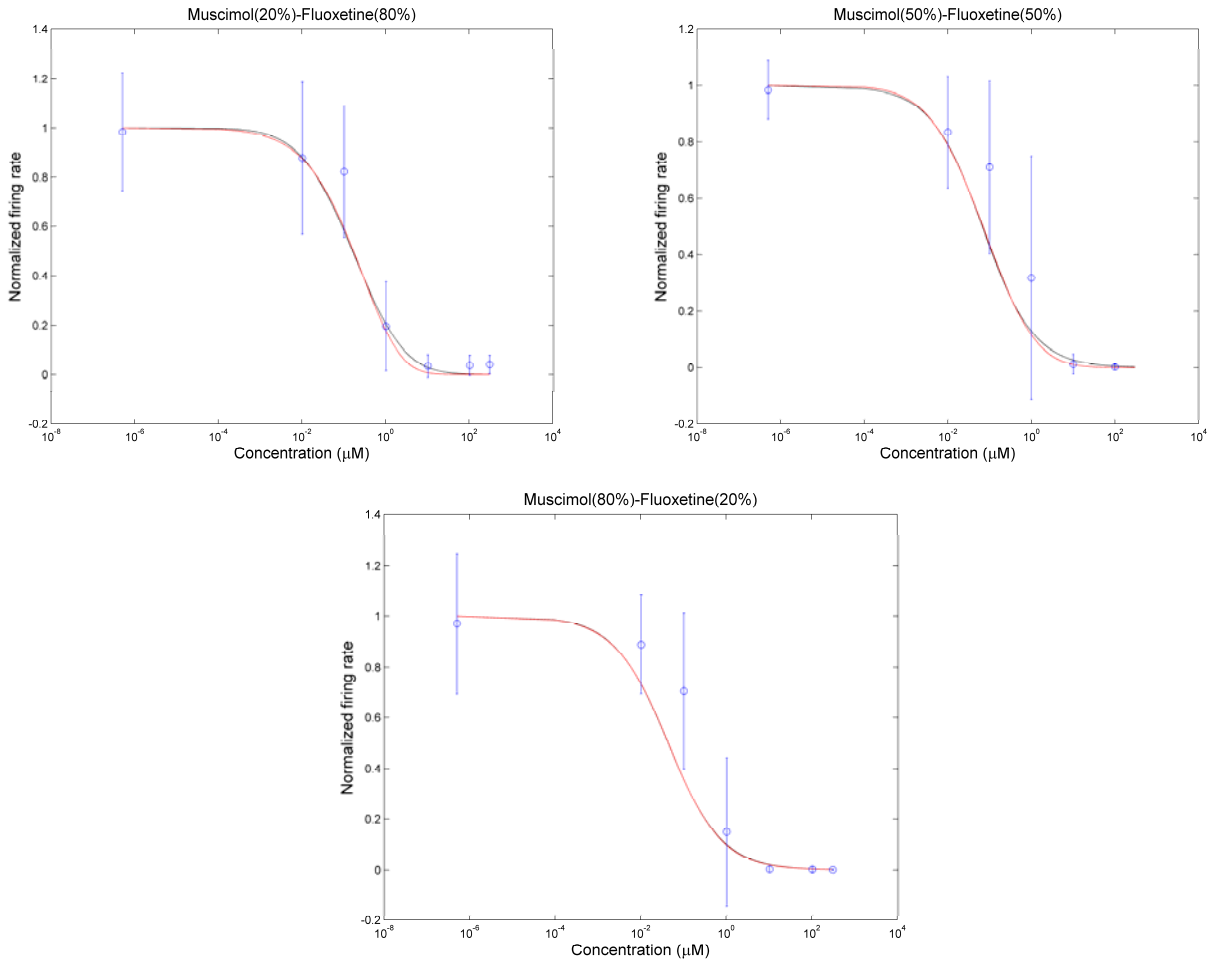


Figure 10. Experimental and calculated -based on pure compounds data- concentration-response curves using concentration addition (black) and independent action (red) for Muscimol and Fluoxetine mixtures.

3.6. MUSCIMOL AND KAINIC ACID MIXTURES

Kainic acid is a natural molecule present in some seaweed. It is a specific agonist for the ionotropic glutamate receptor which mimics the effect of glutamate, the major excitatory neurotransmitter on the central nervous system (Moloney, 2002). Kainic acid is a potent central nervous system stimulant, has neuroexcitotoxic and epileptogenic effects and has been developed as the gold standard neuroexcitatory amino acid for the induction of seizures and the study of neurodegenerative diseases in experimental animals (Moloney, 2002; see Vincent and Mulle, 2009, for a review). Its effect on neuronal activity and mechanism of action has been well described both *in vivo* and *in vitro* (Vincent and Mulle, 2009). Therefore, together with Muscimol it provides a good set of compounds to study binary mixtures where the two compounds have opposite effects (excitatory for Kainic acid and inhibitory for Muscimol) and different mode of action.

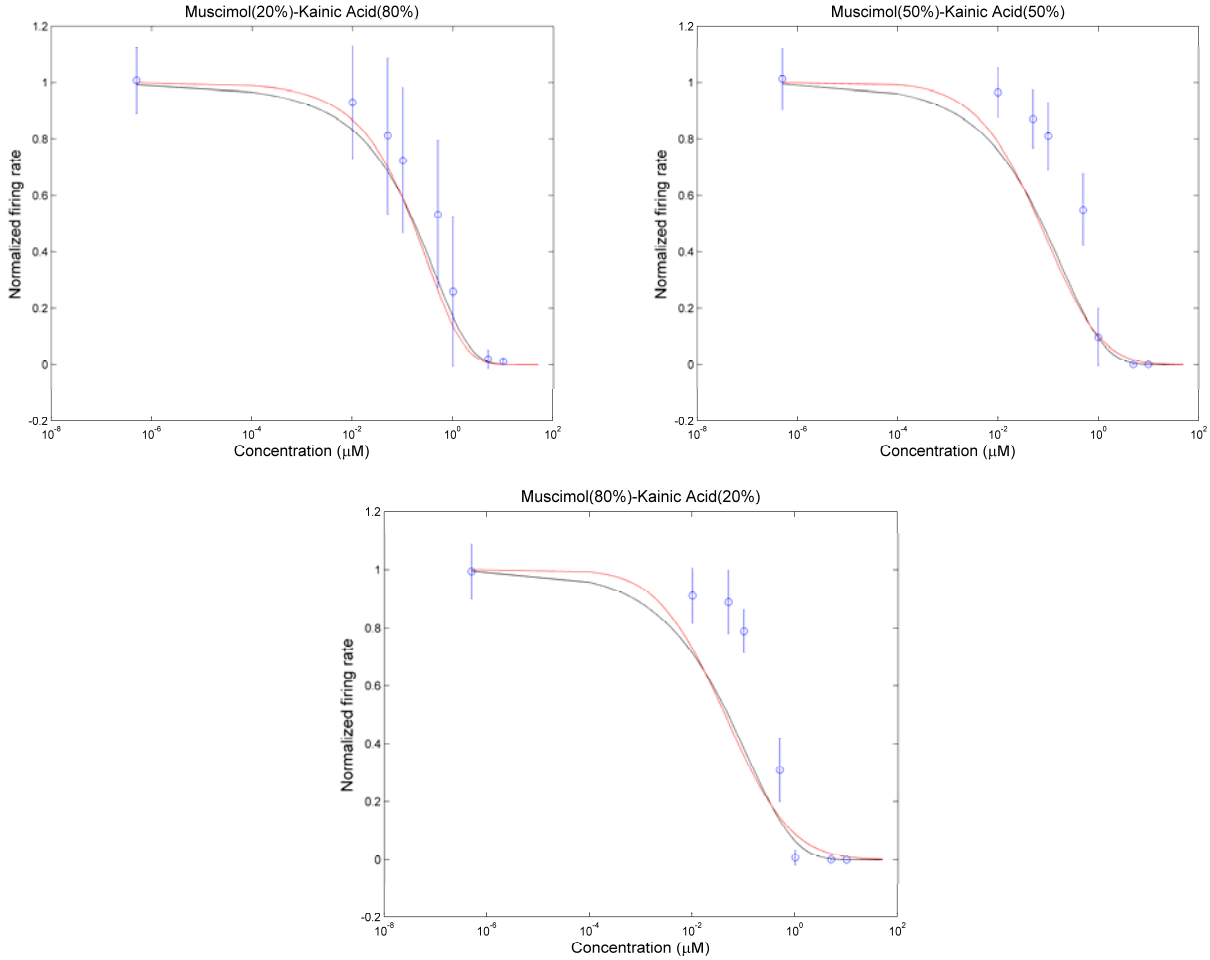


Figure 11. Experimental and calculated -based on pure compounds data- concentration-response curves using concentration addition (black) and independent action (red) for Muscimol and Kainic acid mixtures.

Using the fitted curves from the pure compounds we have compared the predicted CA and IA mixture toxicity with the experimental values. Figure 11 shows the results obtained for the three curves using fitted Logit curves for the pure compounds. The IC_{50} obtained with CA and IA are: 0.1732 μM and

0.1608 µM for 20M-80K; 0.0766 µM and 0.0688 µM for 50M-50K; and 0.0492 µM and 0.0429 µM 80M-20K, respectively. In all cases the predicted results are lower that the fitted experimental data, implying that the real toxicity is lower than the calculated using additivity. Also in this case, CA and IA produce nearly identical results.

4. CONCLUSIONS

Neurotoxicity assessment represents a major challenge within the mixtures context, because regulatory testing guidelines rely exclusively upon *in vivo* observations (see U.S. EPA Guidelines for Neurotoxicity Risk Assessment: FRL 6011-3 and OECD TG481, TG419, TG424 and TG426), and so far no *in vitro* methods for evaluating the neurotoxic hazard of a chemical have yet been validated. Novellino et al., (2011) have recently published the results of an interlaboratory study where the reproducibility of neurotoxicity data based on the measurement of neuronal activity was demonstrated with *in vitro* neuronal cultures on MEAs. This is an important step towards the validation process of the technique as standard tool for neurotoxicity assessment. Still neurotoxicity prediction with *in silico* methods remains an open issue of critical urgency.

In this study we have obtained concentration-response curves of the mean firing rate of neuronal cells cultured on MEA chips at different concentrations of single compounds and their binary mixtures and we have compared the predicted CA and IA mixture toxicity with the experimental data considering the IC_{50} values obtained with the two approaches.

The mixtures studied here include inhibitory compounds on electrical activity with similar mode of action (pyrethroids) and with different mode of action (Muscimol, Verapamil and Fluoxetine) as well as compounds with opposite effects on neuronal activity (excitatory effect: Kainic acid and inhibitory effect: Muscimol).

The obtained results show that for the mixtures where the compounds had different molecular target sites (i.e. different modes of action) the IA and the CA predictive models led to similar results indicating that the two models describe the behaviour of the mixture with comparable efficacy. Concerning the mixtures with the two pyrethroids (same mode of action) the results show that the IC_{50} obtained with the CA and IA models are quite similar when compared with the experimental variability and, hence, it is not possible to conclude that CA produces better results as one could expect. The same is also true for the other binary mixtures where one would expect better predictions using IA.

A recent published work (Qin et al., 2011) proposes an alternative approach where CA and IA are integrated through multiple linear regression (ICIM). By using two training sets of chemicals, it demonstrates that the ICIM approach has a strong predictive power than CA and IA where the two

models deviate from the concentration–response data of the mixtures. It would be worth exploring the ICIM approach with the binary mixtures used in this work.

In conclusion this work has demonstrated that neurotoxicity of mixtures, when electrical activity is considered as an end point, can be predicted using additivity, i.e., with the IA and CA approaches, at least for the binary mixtures analyzed. Therefore our results seem to confirm that the prediction of the neurotoxicity of a mixture from that of their single components is also feasible in this case. However, further experiments and an increasing number of components in the mixtures are necessary to address the issue if contrasting effects may be better predicted using other approaches.

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Abstract.
In vitro neuronal networks are a simplified and accessible model of the central nervous system. Moreover, they exhibit morphological and physiological properties and activity-dependent path-specific synaptic modification similar to the *in vivo* tissue. Cortical neurons grown on multi electrode array (MEA) chips have been shown to be a valuable tool to study fundamental properties of neuronal network activity, synaptic plasticity, learning *in vitro*, and functional pharmacological screening. The variation of spontaneous activity of *in vitro* neuronal networks coupled to MEAs has been studied using several binary mixtures (inhibitors with different mode of action: Verapamil and Muscimol, Fluoxetine and Muscimol; inhibitors with the same mode of action: Deltamethrin and Permethrin; and an excitatory and an inhibitory compound with different mode of action: Kainic acid and Muscimol) with the aim of characterize and assess their combined effects. Individual dose-response and binary mixtures curves have been generated. Concentration Addition (CA) and Independent Action (IA) frameworks have been used to compare calculated and experimental results. In addition, Nuclear Magnetic Resonance (NMR) spectroscopy has been employed to assess that no chemical reaction or complexation took place between mixtures components, as well as to monitor the presence of potential impurities and, in this case, to evaluate their relative amount in the tested samples. The results suggest that additivity: CA and IA are able to predict in most of the cases the total toxicity of the mixture. The variability of the results makes difficult to assess which of both approaches is the most accurate. The presence of both excitatory and inhibitory effects as in the case of Kainic acid may further complicate the analysis of the experimental datasets and biphasic concentration-dose response curves may be need to analyze the joint effect.
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