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Effects of an Environmentally-relevant Mixture of Pyrethroid Insecticides on Spontaneous Activity in Primary Cortical Networks on Microelectrode Arrays

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Abstract

Pyrethroid insecticides exert their insecticidal and toxicological effects primarily by disrupting voltage-gated sodium channel (VGSC) function, resulting in altered neuronal excitability. Numerous studies of individual pyrethroids have characterized effects on mammalian VGSC function and neuronal excitability, yet studies examining effects of complex pyrethroid mixtures in mammalian neurons, especially in environmentally relevant mixture ratios, are limited. In the present study, concentration response functions were characterized for five pyrethroids (permethrin, deltamethrin, cypermethrin, β -cyfluthrin and esfenvalerate) in an in vitro preparation containing cortical neurons and glia. As a metric of neuronal network activity, spontaneous mean network firing rates (MFR) were measured using microelectrode arrays (MEAs). In addition, the effect of a complex and exposure relevant mixture of the five pyrethroids (containing 52% permethrin, 28.8% cypermethrin, 12.9% β -cyfluthrin, 3.4% deltamethrin and 2.7% esfenvalerate) was also measured. Data were modeled to determine whether effects of the pyrethroid mixture were predicted by dose-addition. At concentrations up to 10 μ M, all compounds except permethrin reduced MFR. Deltamethrin and β -cyfluthrin were the most potent and reduced MFR by as much as 60 and 50%, respectively, while cypermethrin and esfenvalerate were of approximately equal potency and reduced MFR by only ~20% at the highest concentration. Permethrin caused small (~24% maximum), concentration-dependent increases in MFR. Effects of the environmentally relevant mixture did not depart from the prediction of dose-addition. These data demonstrate that an environmentally-relevant mixture caused dose-additive effects on spontaneous neuronal network activity in vitro, and is consistent with other in vitro and in vivo assessments of pyrethroid mixtures.

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1. Introduction

Pyrethroids insecticides are widely used for agricultural, industrial and residential pest control. Although these compounds have been used for over fifty years in the United States, their use has increased significantly in recent years as a result of cancellations in uses of other classes of insecticides (Casida et al, 1998; Amweg et al., 2005; Williams et al., 2008; Spurlock and Lee, 2008). Pyrethroid-containing products often contain more than one pyrethroid, due to differing insecticidal properties among this class of compounds. Furthermore, the increasing use of pyrethroids in general increases the probability that exposure will be to multiple, not individual compounds (Tulve et al., 2006; Stout et al., 2009) either simultaneously or sequentially. Thus, understanding their interactions in mixtures is an important toxicological and human health issue.

Pyrethroids disrupt the kinetics of voltage-gated sodium channels (VGSCs) in insect and mammalian neurons, in part by prolonging VGSC inactivation and thereby increasing the amount of time the channel is open. This in turn disrupts membrane excitability leading to alterations in neuronal activity and is the basis for the insecticidal and toxicological effects of pyrethroids (For review see Narahashi 1996; Narahashi et al., 1998, Narahashi 2000). Exposure to high doses of pyrethroids causes two different syndromes that are generally dependent on the chemical structure of the compound. Type I syndrome, characterized by hyperexcitability and tremor, is caused by pyrethroids that lack a cyano group as part of the chemical structure. Type II syndrome, characterized by choreoathetosis, dyskinesia and salivation, is caused by pyrethroids that contain a substituted cyano group attached to the alcohol portion of the molecule (Verschoyle and Barnes 1972; Verschoyle and Aldridge 1980; Lawrence and Casida, 1982; for review, see; Soderlund et al., 2002). Exposure to some compounds, such as esfenvalerate, causes some symptoms of both syndromes, and are referred to as “mixed” type compounds (Breckenridge et al., 2009). These two different clinical syndromes correlate with pyrethroid effects at the VGSC level, where Type II pyrethroids delay channel inactivation and deactivation for a longer period of time as compared to Type I pyrethroids (Ray and Forshaw, 2000). This difference in effect at the channel contributes to repetitive action potential firing (Type I) or depolarization-dependent block of action potentials (Type II), which are key events contributing to the differential clinical responses. Recently, an Adverse Outcome Pathway has been proposed that catalogs the scientific evidence linking pyrethroid-induced changes in VGSC function to the clinical syndromes (Bal-Price et al., 2015). Although the actions of many individual pyrethroids have been examined at the ion channel and cellular level, studies examining effects of mixtures of pyrethroids on function at the ion channel, cellular and neural network level, at environmentally-based exposure ratios, are lacking.

Two studies have examined the response *in vivo* to exposure to environmentally relevant mixtures, and reported that effects are dose-additive, and that differences in the neurotoxicity of pyrethroids appear to be driven by toxicodynamic rather than toxicokinetic factors (Starr et al., 2012; 2014). In vitro, effects of a binary (Scelfo et al., 2012) and an equimolar mixing ratio mixture of 11 (Cao et al., 2011) pyrethroids have been reported to be dose-additive. This is similar to the dose-addition reported following exposure an equi-effects based ratio to the same 11 pyrethroids in vivo (Wolansky et al., 2009). However, real life exposures to

pyrethroids are not likely to be binary or equimolar mixtures. Instead, exposure to complex mixtures of pyrethroids will be based on use patterns of the individual compounds in the mixture (c.f., Tolve et al., 2006).

Primary cortical cultures obtained from postnatal rodents form functional, spontaneously active neuronal networks in which excitatory (glutamatergic) and inhibitory (GABAergic) pathways are present. Microelectrode array (MEA) technology makes it possible to record extracellular action potential spikes and groups of spikes (bursts) from networks of primary neurons in vitro (Pine 2006; Nam and Wheeler, 2011). The present experiments examined the actions of five pyrethroids, and a mixture of them on spontaneous network activity in rat cortical neural networks in order to assess whether pyrethroid effects are “dose-additive” in nature. For this work, the relative ratios of each compound in the mixture were based on their detection rates in environmental monitoring studies.

2. Materials and Methods

2.1 Chemicals

Chemicals used in the present experiment were purchased from ChemServices (West Chester, PA; Table 1) and were dissolved in a 1:1 (vol:vol) mixture of DMSO:ethanol at 1000 fold above the desired final concentration.

2.2 Cell Culture

All animal protocols were reviewed and approved by the NHEERL Institutional Animal Care and Use Committee and complied with all required animal use guidelines. Cell cultures were prepared from 0–24 hr old Long-Evans rats as described in Valdivia et al. (2014). Briefly, cortical tissue was minced, digested with DNAase and pelleted by centrifugation. The pellet was resuspended and filtered through a 100 µm pore Nitex filter into a sterile beaker to remove the meninges, debris, and large clumps of tissue. Cells were plated (1.5×10^5 cells in a 25 µl drop of medium) onto the surface of multi-(48) well MEA (mwMEA; Axion Biosystems, Atlanta, GA) plates that had been pre-coated with polyethyleneimine (PEI) and laminin as previously described (Valdivia et al., 2014). After allowing 2 hrs for cells to attach, 500 µl of Neurobasal-A Medium containing B-27 supplement, GlutaMax and penicillin-streptomycin was added to each well. This culture protocol results in a mixed culture that contains glutamatergic and gabaergic neurons and glia (Mundy and Freudenrich, 2000).

2.3 Microelectrode Array (MEA) Recording

Acquisition of spontaneous network activity from cortical cultures utilized an Axion Biosystems (Atlanta, GA) Maestro 768-channel amplifier, a Middle-Man data acquisition interface, and the Axion Integrated Studios (AxIS) v1.9 (or later) software. Channels were sampled simultaneously with a gain of 1200× and a sampling rate of 12.5 KHz/channel. On day in vitro (DIV) 12 or 13, spontaneous activity of neuronal cells on 48 well mwMEA plates was recorded and inspected to determine the usability of each individual well. An electrode with an average of 5 spikes/min was considered active. Wells that did not exhibit spontaneous activity levels of 10 active electrodes were deemed unusable, and not treated

with a compound in subsequent experiments. Experiments were conducted on DIV 14 or 15. Any electrodes with root mean square (rms)-noise levels $>10 \mu\text{V}$ were grounded prior to data recording; data are not collected from grounded electrodes. A Butterworth band-pass filter (300 – 5000 Hz) was utilized along with a variable threshold spike detector (Biffi et al., 2010) set at $8\times$ standard deviation of the rms noise on each channel during recordings.

2. 4. Exposure

Each experiment consisted of three mwMEA plates, and was repeated three times using separate cell preparations. Individual wells were considered the statistical unit, giving an “n” of 9 for most treatment conditions (some wells were excluded based on the criteria above for active electrodes). Each plate contained complete concentration-response curves for all 5 pyrethroids and the mixture. On the day of the experiment, baseline activity was recorded for 1 hr; directly after baseline recording, compounds were added to each well at the desired concentration (one concentration/well). The final concentration of DMSO:ethanol was 0.1% (vol/vol), and did not alter mean firing rate (MFR, see results). An hour of spontaneous activity was recorded following compound treatment. MFR data from each well were expressed as a percent of pre-exposure control values from the same well. Immediately after the experiment, cell viability was examined in each MEA well using the CellTiter Blue and lactate dehydrogenase (LDH) assays as described by Wallace et al. (2015).

2.4 Statistical analysis and Determination of Additivity

To determine whether the compounds tested in the environmental mixture altered MFR in a concentration (dose)-additive manner, a nonlinear logistic additivity model was used (Crofton et al., 2005; Wolansky et al., 2009; Cao et al., 2011). Since this model has been utilized several times, details of the model and statistical approach are provided in the supplemental materials. Two notable assumptions were made for the current data set. First, by definition, the model requires that changes occur in a single direction (e.g., all compounds decrease the parameter of interest). For the case of the present data set, the permethrin effect was assumed to cause no change in MFR for the sake of the model, even though slight increases were observed. An assumption of a non-decreasing association was made for permethrin in order to build an additivity model that satisfies the definition given in supplemental equation (1). Second, because effects on MFR did not reach a maximum, the estimated concentration-response curves were extrapolated outside of the range of tested concentrations for some chemicals and was assumed to be common across the set of chemicals. The additivity model (supplemental equation 2) was fit to the single chemical data and the mixture model (supplemental equation 4) was fit to the mixture data simultaneously. This allows for a common estimate for the scale parameter, the intercept, and the maximum effect parameter, α .

3. Results

3.1 Effects of individual pyrethroids on spontaneous network activity

All five pyrethroid compounds altered network MFR; the four Type II compounds decreased, while the Type I compound permethrin increased MFR in a concentration-dependent manner (Figure 1). Of the Type II compounds, deltamethrin and β -cyfluthrin were

the most potent, with cypermethrin and esfenvalerate being of approximately equal potency and producing only small (~20%) decreases in MFR at the highest concentration tested. Permethrin increased MFR by ~24% at the highest concentration tested (10 μ M). None of the compounds nor the mixture caused any cytotoxicity at the concentrations tested (data not shown).

3.2 Effects of a 5 pyrethroid mixture on spontaneous network activity in cortical networks

The results from the additivity model including parameter estimates and corresponding p values are provided in Table 2. The slope parameters (designated by β s) associated with four of the five single chemicals (all but permethrin) and for the fixed-ratio mixtures (designated by θ_{mix}) were negative and significant, indicating that as the concentration of the chemical or total concentration of the mixture increases, the mean MFR (% control) decreases. Supplemental Figure 2 provides plots of the individual chemical concentration-response data with curves fit from Supplemental equation 2. From these figures, the concentration response curves for the single chemicals do not appear to have parallel shapes. However, parallelism is not required for the general form of dose additivity.

Along the specified fixed-ratio mixture of the chemicals, the slope parameter under

additivity is $\sum_{i=1}^c \beta_i a_i = \theta_{\text{add}}$. Using the fixed-ratios given in the supplemental methods section and the parameter estimates in Table 2, the slope estimate under additivity along the fixed-ratio ray of the 5 chemicals was estimated as -0.51 ($\text{SE}=0.16$; Table 2); the slope estimated from the mixture data was -0.53 ($\text{SE}=0.19$). Following Gennings et al (2002) and Casey et al (2004), the hypothesis of additivity is an hypothesis of coincidence:

$$H_0:\text{additivity} \iff H_0:\theta_{\text{mix}}=\theta_{\text{add}}$$

which was not rejected for these data ($p=0.834$). Figure 2 illustrates model-predicted fit to the empirical data (blue line) as well as the response curve predicted by dose addition (dashedline). These curves are essentially coincident, and therefore provide clear evidence that the effects of the mixture on MFR in cortical networks can be predicted from the theory of additivity.

4. Discussion

The present results demonstrate that an environmentally-relevant mixture of pyrethroids alter the spontaneous activity of neural networks in a dose-additive manner. This adds to a growing list of studies that indicate dose-addition pyrethroid mixtures, both in vitro and in vivo (Wolansky et al., 2009; Cao et al., 2011). Collectively, these data support the conclusion that effects of pyrethroids on neural activity and behavior are dose-additive, even when Type I and II compounds are present in the mixture at differing ratios.

Previously, it has been demonstrated that mixtures of pyrethroids alter sodium flux via voltage-gated sodium channels in a concentration-additive manner (Cao et al., 2011) and that motor function is similarly altered in a concentration-dependent manner by pyrethroids

(Wolansky et al., 2009). Although the present study made an assumption that the slope of permethrin effects was zero in order to satisfy the model, the results were consistent with additivity, and consistent with these previous studies (Cao et al., 2011; Wolansky et al., 2009) using the same exact mixture ratio. The result of pyrethroid concentration-additive effects at the network level *in vitro* is also consistent with this level of assessment being intermediate between effects on VGSC and behavior. The observation of concentration-additivity at the network level is important relative to proposed molecular sites of action for pyrethroids. Although it is generally accepted that VGSCs are a primary target for pyrethroid toxicity (for review see Soderlund et al., 2002; Bal-Price et al., 2015), alternate sites of action have been proposed to contribute to pyrethroid toxicity, particularly for type II compounds. Function of GABA_A receptors, voltage-gated calcium channels (VGCCs), a high conductance chloride channel and delayed rectifying potassium channels have been reported to be altered *in vitro* at pyrethroid concentrations similar to those that alter VGSC function (Hildebrand et al., 2004; Clark and Symington, 2007; 2008; Neal et al., 2010; Burr and Ray, 2004, Forshaw et al., 2000, Tian et al., 2009). Because VGSCs, VGCCs, chloride and potassium channels all play significant roles in membrane excitability and synaptic transmission, it is reasonable to hypothesize that pyrethroids may in fact, affect multiple channels in concert. Actions on multiple targets could contribute to non-additivity of pyrethroid effects on cellular excitability. Neural network activity measured on MEAs can be altered by broad classes of pharmaceutical and toxicological agents (for review, see Johnstone et al., 2010), and as such are well suited to measure the apical result of effects of alterations to multiple ion channels. The present results indicate that even if pyrethroids do alter function of multiple ion channels in cortical networks, the combined result of these alterations is concentration-additive with respect to effects on network excitability. This is consistent with previously observed additivity at the level of *in vitro* VGSC-mediated responses (Cao et al., 2012) and *in vivo* motor function (Wolansky et al., 2009). Finally, other studies have reported apparent less-than-additive effects of pyrethroids on VGSC (Song et al., 1996) function and hippocampal synaptic transmission (Ray et al., 2006). While they appear inconsistent with the present results, it should be noted that these studies examined only binary mixtures at a single concentration, and were not designed to be a rigorous test of additivity.

Previously, some studies on pyrethroid effects on neural network activity have reported only decreases in MFR following treatment with permethrin and deltamethrin (Meyer et al., 2008; Shafer et al., 2008). In those studies, effects of pyrethroids were also more potent than in the current study. There are differences in the culture protocol between this and the previous study, as well as important differences in the experimental protocol. One important difference is that GABA_A receptors were not blocked in the current study. In addition, the previous (Meyer et al., 2008; Shafer et al., 2008) studies utilized a cumulative concentration-response treatment protocol, wherein each network was treated with sequentially increasing concentrations of compound, until the maximum concentration was reached. In the present study, each network received only a single concentration of compound, and the concentration-response was comprised from several networks on the same mwMEA plate. The use of a cumulative design in previous studies may contribute to differences in the overall potency observed in the present study. Similarly, because GABA receptors were

blocked in previous studies, the slight increase caused by permethrin may not have been observed due to an already high firing rate in the network. By contrast, if deltamethrin caused a depolarization-dependent block of action potential firing, a decrease in MFR would still be observed whether or not GABA receptors were blocked. From the standpoint of assessing the effect of the mixture, there are both advantages and disadvantages to the protocol used in the present study. The advantage is that by not blocking GABA_A receptors, the response reflects the summed effects of the compounds on all transmitter systems present in the culture, including the typically predominant glutamatergic and GABAergic networks. The disadvantage was that because of the slight increase observed with permethrin, an assumption was made that this compound had “no effect” in order to satisfy the requirements for the additivity model (see Supplemental).

In conclusion, the present results confirm dose-addition of pyrethroid insecticides, and extend the data supporting this effect to an environmentally-relevant mixture acting at the level of neural networks. Collectively, the results of this and other studies indicate that pyrethroid effects on neurological processes are dose-additive at the cellular, network and behavioral levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Concentration-response relationships for individual pyrethroid compounds and an environmentally-relevant mixture of pyrethroids. Effects of pyrethroids on Mean Firing Rate (MFR) as a percent of MFR is the same well prior to treatment with the indicated pyrethroid, mixture dilutions or vehicle are plotted. Each compound and the mixture were included on each plate (mean $\pm$ SEM, $n=7-9$). Vehicle-treated control wells were included on each plate and are shown on the graph for permethrin. Supplemental figure 2 provides fit curves for each compound. As shown in Table 1, all of the individual compounds except permethrin had significant slope values.

Figure 2. Effects of the pyrethroid mixture are consistent with dose additivity

In this figure, the observed empirical data points for the 5 pyrethroid mixture are indicated by the asterisks (*) and model predicted MFR (% control) for the empirical data is shown with the blue solid curve. The response as predicted under the model of dose additivity is shown by the green dashed curve. Since the two curves are essentially coincident, the response to the mixture is consistent with dose additivity.

Table 1

Compounds tested and components of the 5 chemical mixture

Compound	CAS#	Type	% Purity	% of Mixture
Permethrin	52645-53-1	Type I	99.9	52.2
Cypermethrin	52315-07-8	Type II	98.4	28.8
β -Cyfluthrin	68359-37-5	Type II	99.5	12.9
Deltamethrin	52918-63-5	Type II	>99	3.4
Esfenvalerate	66230-04-4	Mixed	99.5	2.7

Table 2

Estimated model parameters from the additivity model given in equation (2) and the mixture model given in equation (4) fit simultaneously with MSE=376. The estimated slope under additivity for the 5 pyrethroid mixture is $\hat{\theta}_{add(5)} = -0.51$ (SE=0.16; p=0.002). The test of the hypothesis of additivity (i.e., $H_0 : \theta_{add} = \theta_{mix}$) was not rejected (F=0.04 compared to $F_{1,409}$; p=0.834).

Parameter	Estimates	SE	Pvalue
α	42.4	4.2	<0.001
β_0	6.1	1.8	0.001
β_1 (β -Cyf)	-1.9	0.62	0.002
β_2 (Cyp)	-0.55	0.19	0.004
β_3 (Delta)	-3.0	0.97	0.003
β_4 (Esfen)	-0.54	0.19	0.004
θ_{mix5}	-0.53	0.19	0.005