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Effects of an environmentally-relevant mixture of pyrethroid insecticides on spontaneous activity in primary cortical networks on microelectrode arrays☆

Andrew F.M. Johnstone[a,1], Jenna D. Strickland[b,1], Kevin M Crofton[c], Chris Gennings[d], Timothy J. Shafer[a,*]

[a] National Health and Environmental Effects Research Laboratory (NHEERL), US EPA, Research Triangle Park, NC, United States
[b] Axion Biosystems, Atlanta GA, United States
[c] NCCT, US EPA, Research Triangle Park, NC, United States
[d] Population Health and Science Policy, Mt Sinai Hospital, NY, NY, United States

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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 microelectorde 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 μM, 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 and Quistad, 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

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* Corresponding author at: Integrated Systems Toxicology Division, U.S. Environmental Protection Agency, MD B105-05, Research Triangle Park, NC, United States.
E-mail address: shafer.tim@epa.gov (T.J. Shafer).

[1] These authors contributed equally to this manuscript

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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., Tulve 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 to 24 h 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 h 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 1200x 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 $\geq$5 spikes/min was considered active. Wells that did not exhibit spontaneous activity levels of $\geq$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 μV 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 8x 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

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