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Acute *in vitro* neurotoxicity of some pyrethroids using microelectrode arrays

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ABSTRACT

Pyrethroid-mediated changes on microelectrode array (MEA) parameters, such as mean firing rate (MFR), mean burst rate (MBR), and number of active channels (nAC) were investigated by exposing neuronal networks to cumulative concentrations (0.01, 0.1, 1, 10, and 100 μM) of pyrethroids (Type-1, bifenthrin and permethrin; Type-2, beta-cyfluthrin, cypermethrin, deltamethrin, and lambda-cyhalothrin). The average MFR, MBR, and nAC increased for all pyrethroids (except nAC for deltamethrin) at lower concentrations and decreased at higher concentrations. The increase in the average MFR, MBR, and nAC was not statistically significant in most of the cases. Increase in MFR, MBR, and nAC was observed in 19/19, 18/19, and 12/19 individual experiments, respectively, at lower concentrations. The IC_{50}s calculated on MEA parameters were more or less similar. The relative potencies calculated on the IC_{50}s of MEA parameters had a strong positive correlation. These observations indicate that the MEA parameters MFR, MBR, and nAC follow the same trend for pyrethroid-mediated changes, and provide a similar outcome. The rank orders of relative potencies on the IC_{50}s of the MEA parameters distinguish type-1 pyrethroids from type-2 pyrethroids, with type-2 being more potent. As increase in MFR at the lower concentrations of pyrethroids was observed in all the individual experiments (19/19), it may be considered as the characteristic effect of pyrethroids on neuronal excitability.

1. Introduction

The current requirements on the neurotoxicity evaluation of a chemical are not standard among the regulatory authorities. While the United States Environmental Protection Agency (U.S. EPA) requires adult neurotoxicity studies for all pesticides (U.S. EPA, 2013), the Organization for Economic Co-operation and Development (OECD), and the European Union (EU) do not require a specific neurotoxicity study, or a specific additional neurotoxicity endpoint, over the initial standard toxicity studies until the available information necessitates it, or a possible neurotoxic effect is observed in the course of standard toxicity testing (acute toxicity studies, repeated dose toxicity studies) or in the developmental/reproductive toxicity testing (Bal-Price et al., 2015, EU, 2013; Llorens et al., 2012; OECD, 2004; Zuang et al., 2015). Despite the differences among the regulatory authorities, all the chemicals are being examined for neurotoxic effects, either as a separate study or as part of standard toxicity testing, as the neurotoxicity evaluation forms an important toxicological endpoint.

The neurotoxicity testing strategies at present involve toxicity determination by characterization of effects using animal models. However, the large number of compounds to be tested, cost, and time scale needed for testing makes testing challenging, and create the need for alternative methods. On the other hand, the development of alternative methods for neurotoxicity testing is challenging due to the complex nature and diverse functions of the nervous system, making it difficult for any single *in vitro* model to act as a complete alternative, capturing all the mechanisms of effects of neurotoxicity caused by a chemical. Hence, at present, no single *in vitro* method completely replaces the neurotoxicity testing *in vivo*, and no validated methods are available for neurotoxicity testing *in vitro* (Bal-Price et al., 2010; Coecke et al., 2006; Zuang et al., 2015).

While there are several endpoints proposed for neurotoxicity testing *in vitro* (Bal-Price et al., 2008; Bal-Price et al., 2010; Coecke et al., 2006), electrophysiology assessment has its own importance, as it captures the electrical excitability of neurons, which can be altered by a chemical rapidly, without genomic or proteomic involvement, and before the changes can show up morphologically. Given the fact that neuronal activity is the functional aspect of the nervous system, electrophysiology measurement determines the immediate functional alterations in the nervous system due to a chemical insult (Johnstone et al., 2010; Melani et al., 2005; Novellino et al., 2011). Several *in vitro* studies have shown the electrophysiological activity of neuronal cells getting affected, when exposed to the test chemicals (Alloisio et al., 2015; Defranchi et al., 2011; Johnstone et al., 2010; van Vliet et al., 2007).

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A significant *in vitro* method for assessing the electrophysiology of neurons is microelectrode arrays (MEAs). Johnstone et al. (2010) discussed the advantages of the MEA technique over other electrophysiological techniques, its predictive capacity, and its high throughput nature. From the *in vitro* neurotoxicity perspective, the functional neural activity assessed using MEAs has been used to detect acute, sub-chronic, and chronic neurotoxicity, and certain neurobehavioral parameters, such as learning and memory (Gopal et al., 2007; Hogberg et al., 2011; Marom and Shahaf, 2002; Robinette et al., 2011; Shahaf and Marom, 2001).

In this study, the acute *in vitro* neurotoxicity of some pyrethroids was determined. Pyrethroids are neurotoxic insecticides with agricultural, industrial, commercial, institutional, and household uses. Pyrethroids are classified as Type-1 or Type-2 based on their chemical structure and the toxic syndrome they produce in rodents. Type-1 pyrethroids cause aggressive sparring and tremors (T-syndrome) and lack the cyano group in their structure. Type-2 pyrethroids cause choreoathetosis and salivation (CS syndrome), and have a cyano group in their structure. However, some type-2 pyrethroids produce signs involved in both these syndromes (Soderlund et al., 2002; Soderlund, 2012). Bifenthrin and permethrin that were used in this study, are type-1 pyrethroids, while beta-cyfluthrin, cypermethrin, deltamethrin, and lambda-cyhalothrin are type-2 pyrethroids. Although Losa et al. (2009) investigated the concentration response of pyrethroids using MEA, the report was not elaborate and, moreover, the concentration-response was investigated inhibiting the GABAergic activity of neurons (GABAergic inputs, which have inhibitory effects, form the normal and essential part of cortical activity, either during the spontaneous or sensory evoked activity (Okun and Lampl, 2008)). On the other hand, McConnell et al. (2012) investigated some pyrethroids with a different approach, using single dose exposure that did not involve evaluating the concentration response relationships. Other reports that have investigated the concentration response relationships of pyrethroids using MEA, have either used one or two pyrethroids, or have inhibited the GABAergic activity of neurons (Alloisio et al., 2015; Meyer et al., 2008; Scelfo et al., 2012; Shafer et al., 2008; Valdivia et al., 2014; Vassallo et al., 2017). Recently Johnstone et al. (2017), without inhibiting the GABAergic inputs, investigated the concentration response of five pyrethroids and their mixture by determining the percent changes in the mean network firing rate from the baseline. However, the study by Johnstone et al. (2017) did not involve the statistical analysis of pyrethroid mediated changes in the mean network firing rate and did not calculate the specific inhibitory concentrations (such as IC_{50} values) of pyrethroids on mean network firing rate. In the current study, the concentration response of some pyrethroids were investigated on MEA parameters, such as mean firing rate, mean burst rate, and the number of active channels, and their IC_{50} values were determined. The changes caused by the pyrethroids on these MEA parameters were also investigated.

2. Materials and methods

2.1. Pyrethroids

Six pyrethroids were used in the study: beta-cyfluthrin (Sigma Aldrich-46003; CAS# 68359-37-5; purity-99.8%) bifenthrin (Sigma Aldrich-34314; CAS# 82657-04-3; purity-98.6%), cypermethrin (Sigma Aldrich-36128; CAS# 52315-07-8; purity-94.3%), deltamethrin (Sigma Aldrich-45423; CAS# 52918-63-5; purity-99.6%), lambda-cyhalothrin (Sigma Aldrich-31058; CAS# 91465-08-6; purity-97.8%) and permethrin (Sigma Aldrich-45614; CAS# 52645-53-1; purity-98.3%). The stock solutions of these pyrethroids were prepared in their original containers by adding DMSO (Sigma Aldrich-D 2650), and were stored at − 20 °C in the dark.

2.2. Primary cortical neuronal culture

The experimental protocol involving animals was approved by the Animal Ethics Committee of IIBAT (Approval No. 1/105/IAEC/2013). The primary cortical neuronal culture was prepared from gestational day-18 Wistar rat fetuses (animals were obtained from the animal house facility of IIBAT). Briefly, the timed-pregnant Wistar rats were euthanized by CO_2 exposure, and the fetuses were collected in cold Hank's balanced salt solution (HBSS [$-Ca^{2+}$, $-Mg^{2+}$], with 1% penicillin-streptomycin). Under sterile conditions, 8–9 fetuses were dissected in cold HBSS and the cortical tissues (extracted using microsurgical forceps without removing the whole brain from the skull) were collected in cold Hibernate E medium (with 1% penicillin-streptomycin). The collected tissues were then transferred in to a petridish containing cold Hibernate E medium for the removal of meninges and blood vessels using microsurgical forceps under a dissection microscope. The cortical tissues were then enzymatically digested using TrypLE Express solution (10 min at 37 °C) and triturated in Hibernate E medium 8 times using a fine tipped Pasteur pipette with 1 mm diameter. Then, the supernatant containing dispersed cells was centrifuged at 1100 rpm at room temperature for 2 min. The resulting pellet was re-suspended and triturated (4–5 times each using fine tipped Pasteur pipette with 1 mm and ½ mm diameter) in pre-warmed Neurobasal medium (supplemented with 2% B27, 1% Gultamax-I, and 1% penicillin-streptomycin) and then plated on to the pre-coated MEA chips (Pacifici and Peruzzi, 2012; Xu et al., 2012).

2.3. Preparation of the MEA chips and plating the neuronal cells

The standard glass MEA Chips [60MEA 200/30iR-Ti-gr (glass ring 12 mm), with internal reference, Multichannel Systems MCS GmbH, Reutlingen, Germany] were sterilized by soaking in 70% ethanol (30 min) and exposing to UV light (60 min). The chips were then coated with poly-ethyleneimine (PEI) (0.05% PEI in 50 mM HEPES buffer) and laminin (0.02 mg/mL in plating media). The cell suspension prepared was counted for the number of cells using an automated cell counter (Invitrogen), and seeded as 50 µL droplets ($4.0–6.5 \times 10^6$ live cells/mL, viability ranged between 73 and 93%) on to the electrode field of pre-coated chips. The cells were allowed to adhere to the MEA chips for 1 h (approximately), and then 1 mL of the Neurobasal medium (supplemented with 2% B27, 1% Gultamax-I, and 1% penicillin-streptomycin) was added to the chips. The MEA chips were covered with the lids having ethylene-propylene membranes (ALA Scientific instruments Inc, USA) to maintain sterility and prevent evaporation. The cells were maintained in Neurobasal medium supplemented with 2% B27, 1% Gultamax-I, and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Half of the medium was changed twice a week.

2.4. Experiment outline

Each pesticide was tested 3–4 times using six neuronal isolations, with no pesticide being tested more than twice using the same isolations. The MEA experiments were performed between 24 and 35 days *in vitro* (DIV). The cultures were fed with fresh medium at least 24 h before the experiment to allow the culture to stabilize for its activity. The amount of medium in the MEA chip was 1 mL before 24 h of start of the experiment. Five dilutions of the compound (100, 10, 1, 0.1, and 0.01 mM) were freshly prepared on the day of the experiment by serially diluting the stock solution using DMSO in a sterile amber colored glass vials. Before the addition of the pesticide concentration, the reference activity (baseline activity) of the network was recorded until the activity was found to be stable for at least 10 min. For adding the pesticide concentrations to the culture, 200 µL of the media from the MEA chip was pipetted out into a sterile glass vial, to which 1.0 or 0.9 µL of the corresponding pesticide dilution was added, vortexed for 5–10 s using the vortex shaker (Spinix, Tarsons), and then replaced