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Di (2-ethylhexyl) phthalate modulates cholinergic mini-presynaptic transmission of projection neurons in *Drosophila* antennal lobe

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ABSTRACT

Di (2-ethylhexyl) phthalate (DEHP) is one of the Phthalic acid esters which are added in polyvinyl chloride (PVC) products. Previous animal studies have showed that exposure to DEHP has a negative effect on the liver, kidneys, lungs, and reproductive system, particularly the developing testes of prenatal and neonatal males, but few can match the dramatic impact on the nervous system. *Drosophila* melanogaster as a model organism has been widely used in research of the nervous system. In order to examine the modulation of DEHP in excitatory cholinergic transmission, electrophysiological properties of spontaneous activities, spontaneous action potential (sAP), mini excitatory postsynaptic currents (mEPSCs), and calcium currents were measured in projection neurons (PNs) of *Drosophila* antennal lobe. In this study, DEHP (100 μM) was showed to influence the spontaneous activities of the PNs and DEHP (300 μM) significantly decrease the frequency of sAP. Meanwhile, DEHP (100 and 300 μM) also reduced the frequency and amplitude of mEPSCs. Furthermore, ion channel studies showed DEHP (100 and 300 μM) inhibited the peak current amplitude of calcium channel. These results indicated that the DEHP modulated the cholinergic mini-synaptic transmission of projection neurons in *Drosophila* antennal lobe, and this modulation might be mediated by inhibiting the calcium channel activities.

1. Introduction

Phthalates (phthalic acid esters, PAEs), a family of chemicals, which are used in plastics and many other products, have attracted researchers attention because of their hepatotoxic, teratogenic, and carcinogenic properties (Liang et al., 2008) besides their harm to pregnant women, infants and children (Jurewicz and Hanke, 2011). Therefore, San Francisco have enforced aforbiddenness of sale, distribution and manufacture of baby products which were made with any level of toxic chemical like PAEs.

Di-(2-ethylhexyl) phthalate, known as DEHP, is colorless, odorless liquid with a chemical formula of $C_{24}H_{38}O_4$, and is more fat-soluble than water-soluble (Shaz et al., 2011). DEHP, one of the most commonly used phthalates, has been added into the polyvinyl chloride (PVC) to soften and increase the flexibility of the plastic and vinyl products. As a common plasticizer, DEHP is widely used in cosmetics, personal care products, and consumer products such as food cans and food bags. It has been reported that the level of this toxic chemical will drop to 50% if people can avoid using plastic. DEHP and plastic components are not combined with tight covalent chemical bonds but Vander Waals' force, therefore the widespread use of this kind of products has made the exposure to DEHP unavoidable. It is harmful to the liver, kidneys, lungs, and reproductive system (Shea, 2003). What is more, it has been reported that at an appropriate concentration, DEHP has potential ability to affect the development of central nervous system (CNS) (Hokanson et al., 2006). Meanwhile, researches focused on rodent studies indicate negative effects on both neural development and behavior. DEHP has been shown to alter gene expression of G-protein-coupled receptors, affect the dopaminergic neurotransduction system in rat brain (Ishido et al., 2005), and decrease the activity of neuronal membrane Na^+–K^+ATPase (Dhanya et al., 2004).

The *Drosophila* melanogaster has been a model organism for about 100 years since the study of complex biological problems. Nowadays, it has been widely used as a system to study neurobiology, neuropharmacology, and neuropathologic mechanisms. This model organism represents a system where electrophysiology recordings, behavior and gene response can be readily examined. The antennal lobe projection neurons (PNs) of *Drosophila* are known to be cholinergic, and the nicotinic acetylcholine receptors (nAChRs) are reported to participate in most of the spontaneous excitatory drive in the circuit of normal sensory input. They receive the input signal from olfactory interneurons and transduct to higher brain center. PNs are interneuron, which together with other neurons establish a complex synaptic network in the antennal lobe.

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Nevertheless, few studies have been performed on the mechanism of neurotoxicity of DEHP. To achieve this goal, the present study was designed to record from PNs to evaluate DEHP in excitatory cholinergic transmission in neurons of *Drosophila* antennal lobe. By investigating the properties of spontaneous firing, spontaneous action potential (sAP), mini Excitement Postsynaptic Currents (mEPSCs) and calcium currents, we found that DEHP regulated cholinergic mini-presynaptic transmission of PNs in *Drosophila* antennal lobe.

2. Materials and methods

2.1. Fly trains

Drosophila stocks were reared on standard cornmeal agar medium supplemented with dry yeast at 24 °C and 60% relative humidity, and a previously developed method (Gu and O'Dowd, 2006, 2007) was used to study spontaneous activity, mEPSCs and calcium channel currents of PNs in the whole brain. In our lab, oratory *Drosophila* melanogaster produces new adults in 14 days. In order to record the flies at accurate time points, all experiments were performed on wild-type Canton-S female flies 2 days before eclosion which were identified by red eyes and transparent wings in the puparium.

2.2. Drugs and solutions

DEHP was bought from Sigma–Aldrich Chemicals Co. (St. Louis, MO). DEHP was formulated into stock solution with dimethyl sulfoxide (DMSO). The stock solutions were further diluted in the bath solution, to the appropriate concentrations of DEHP. After the establishment of a whole-cell configuration, the cells were allowed to stabilize for 3–5 min, and then to start a protocol. Each final concentration of DEHP was used once currents and potential were stable to investigate the influence of DEHP on PNs. DMSO and the chemical products used to prepare external and internal solutions were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) unless otherwise specified in the protocol.

The standard external solution contained (in mM) 101 NaCl, 1 $CaCl_2$, 4 $MgCl_2$, 3 KCl, 5 glucose, 1.25 NaH_2PO_4, and 20.7 $NaHCO_3$, pH 7.2, 250 Osm. An internal solution containing the followings (in mM): 102 K-gluconate, 0.085 $CaCl_2$, 1.7 $MgCl_2$, 17 NaCl, 0.94 EGTA, and 8.5 HEPES with pH of 7.2 and 235 mOsM.

2.3. Confocal images of Drosophila melanogaster brain

All brains were obtained from flies 2 days before eclosion. The entire brain, including optic lobes, was resected and prepared for recordings in standard external solution containing 20 units/ml papain with 1 mM l-cysteineas previously described (Gu and O'Dowd, 2006, 2007). Then the dissected brains were mounted in an RC-26 perfusion chamber (Warner Instruments, Hamden, CT, USA) containing the recording solution bubbled with 95% O_2 and 5% CO_2 (2 ml/min).

The soma and the terminals were injected with biocytin in the recording pipette in the whole cell configurations for at least 30 min. After electrophysiological recording, the brain was fixed in phosphate buffered 4% formaldehyde at 4 °C for 10 h and subjected to biocytin staining. Then, the brain was washed in 1% PBS three times, blocked and incubated in blocking buffer (0.1 MPBS, 0.1% Triton X-100, 1% BSA) containing streptavidin-CY3 (Molecular Devices) for 3 h at room temperature. After incubation, the brain was washed three times at 5-min intervals in PBS. A BX51WI microscope with a 40× objective and confocal camera was used to acquire photos of dendritic arborization of the visual projection neurons. Each representative image was randomly sampled 10 times and the counter was blinded to sample identities (fly genotype, age and other experimental conditions). Statistical significance was calculated using Student's *t*-test with two-tailed *P* values.

2.4. Electrophysiological recording from PNs in isolated whole brain

All brains were obtained from female flies 2 days before eclosionas previous. Pipettes were targeted to PNs in the dorsal neuron cluster in the antennal lobe. Whole-cell recordings were performed with pipettes (10–15 MΩ) filled with internal solution. The pipettes were pulled using a micropipette puller. Cholinergic mEPSCs were recorded using the same internal solution as that used for whole-cell recording (Gu et al., 2009), and were performed in the presence of TTX to block sodium channels and PTX to block GABA receptors. The majority of mEPSCs recorded in these conditions could be blocked by curare. APs were recorded in the whole-cell patch–clamp configuration at the holding potential of −70 mV. The number of action potentials was counted, and only overshooting action potentials more positive than 0 mV were included, meanwhile peak amplitude was measured before and after drug applications. Current-clamp and voltage-clamp recordings were performed using patch–clamp electrodes. Giga ohm seals were achieved before recording in on-cell configuration, followed by whole-cell configuration while in voltage-clamp mode. Slow and fast capacitance compensation was automatically performed. Access resistance was continuously monitored during the experiments. Recordings were made at room temperature, and only a single projection neuron was examined in each brain.

All electrophysiological recordings were carried out using a BX51WI upright microscope (Olympus, Lehigh Valley, PA). Signals were acquired with EPC10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany), and were filtered at 5 kHz using a built-in filter and digitized at 5 kHz. Data analysis was performed by the pClamp10 Clampfit software (Molecular Devices).

2.5. Analysis of synaptic currents

The mEPSCs were detected using Mini analysis software (Synaptosoft, Decatur, GA). Events were accepted for analysis only if they were asymmetrical with a rising phase faster than 3 ms and a more slowly decaying phase. In addition, the threshold criterion for inclusion was 3pA.

2.6. Statistics

Statistical comparison the magnitude of spontaneous action potential, mEPSCs and calcium currents before and after application of DEHP was made with one-way ANOVA. Results are presented as mean ± SEM, and differences were considered significant if $P < 0.05$.

3. Results

3.1. Confocal image: the morphology of PNs of Drosophila melanogaster pupae 2 days before eclosion

In order to study the effects of DEHP on PNs, we first demonstrated the morphology properties of PNs. Fig. 1 showed the detailed morphology of PNs in the isolated brain, which are members of the neural circuits. Previous studies have reported the PNs that come from antennal lobe to be cholinergic and cholinoceptive and have closely connection with cholinergic input to Kenyon cells in *Drosophila* (Bicker, 1999; Yasuyama et al., 2002). In this study, Spontaneous activities, sAP, mEPSCs and calcium currents are recorded from this kind of PNs in the prepared brains of fly pupae 2 days before eclosion.

3.2. Electrophysiological recording: effects of DEHP on spontaneous extracellular activity of PNs in the isolated pupae brains 2 days before eclosion

To investigate the effects of DEHP on the electrophysiology properties of brain neurons in detail, spontaneous firing, an initial

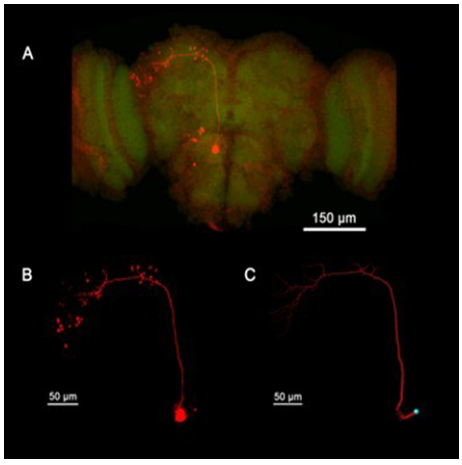

Fig. 1. Confocal images demonstrated the detailed morphology of the recorded projection neurons (PNs). Image of the fruit fly brain with biotin labeled olfactory PNs, showing the detail morphology of the recorded neurons (A). A single neuron has been labeled (B). There is one major branch of the soma stalk of the visual projection neuron, and this branch curves dorso-medially, giving off several small collaterals. 3D reconstruction of the projection neurons, using 3D imaging software (C).