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Monocrotophos, an organophosphorus insecticide, disrupts the expression of *HpNetrin* and its receptor *neogenin* during early development in the sea urchin (*Hemicentrotus pulcherrimus*)

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ABSTRACT

Netrins, chemotropic guidance cues, can guide the extension of serotonergic axons by binding to netrin receptors during neural development. However, little is known about whether disruption of netrin signaling is involved in the mechanisms by which organophosphorus pesticides affect serotonergic nervous system (SNS) development. In this study, we evaluated the effects of the pesticide monocrotophos (MCP) on the expression patterns of *HpNetrin* and its receptor *neogenin* as well as on the intracellular calcium ion (Ca^{2+}) levels in *Hemicentrotus pulcherrimus* (sea urchin) by exposing fertilized embryos to 0, 0.01, 0.10, and 1.00 mg/L MCP. The results showed that MCP disrupted *HpNetrin* and *neogenin* expression at different developmental stages in *H. pulcherrimus* and that Ca^{2+} appeared to be involved in the MCP-induced developmental neurotoxicity. Specifically, the lower concentrations of MCP elevated *HpNetrin* and *neogenin* transcription, resulting in higher intracellular Ca^{2+} levels during the early developmental stages in the sea urchin; this may affect netrin-directed cell migration/axon extension and subsequently disrupt serotonergic axon branching and synapse formation. In contrast, 1.00 mg/L MCP exhibited an inhibitory effect on *HpNetrin* and *neogenin* transcription. This finding implies that the regulatory roles of these factors may be diminished during early development, thereby causing developmental defects in the sea urchin. Collectively, our results provide a basis for exploring the involvement of netrin and neogenin in the organophosphate-induced disruption of the SNS during development.

1. Introduction

The serotonergic nervous system (SNS) emerges during embryogenesis and participates in the regulation of morphogenesis and swimming activity of sea urchin embryos and larvae (Bisgrove and Burke, 1986; Yaguchi and Katow, 2003). It is known that a ganglion in a small region of the apical most ectoderm and a serotonin receptor cell network in the blastocoel comprise the SNS in sea urchin larvae (Katow et al., 2004, 2007; Yaguchi et al., 2000). Serotonergic cells initially form around the oral ectoderm near the animal plate in late gastrula larvae and then form axons bilaterally in the animal plate ectoderm, which extend toward the middle-ridge to generate a neural plexus at the apical ganglion. Most of these axons extend towards the lateral ectodermal regions of the oral lobe until they reach their targets, and this extension of serotonergic axons is strictly directional during early development in the sea urchin (Katow, 2008). Furthermore, research shows that such directional extension of the axons occurs ubiquitously in both vertebrates and invertebrates and is regulated by several axon guidance cues including netrins, semaphorins, slits, and ephrins (Charron et al., 2003; Dickson and Senti, 2002; Fujisawa, 2004; Katow, 2008; Keynes and Cook, 1995; Kidd et al., 1999; Kolodkin et al., 1992; Wu et al., 1999).

Netrins were initially proposed as midline-derived axon guidance cues that direct axon migration towards the ventral midline during embryogenesis in all bilaterally symmetrical animals (Chisholm and Tessier-Lavigne, 1999; Dickson, 2002;

Abbreviations: SNS, serotonergic nervous system; Hp, *Hemicentrotus pulcherrimus*; DCC, deleted in colorectal cancer; UNC-5, uncoordinated-5; Ca2+, calcium ions; MCP, monocrotophos; hpf, hours post fertilization; qRT-PCR, quantitative real-time polymerase chain reaction; ANOVA, analysis of variance.

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Dickson and Keleman, 2002; Guthrie, 1997; Rajasekharan and Kennedy, 2009; Tessier-Lavigne and Goodman, 1996). Hpnetrin, a netrin homolog in the sea urchin (*Hemicentrotus pulcherrimus*), has been shown to mediate serotonergic axon guidance in this basal deuterostome (Katow, 2008). During the early developmental stages of the sea urchin, HpNetrin is expressed on the entire basal surface of the swimming blastula and mesenchyme blastula, and then, at and after the early gastrula stage, its expression increases at the animal plate but decreases in the rest of the ectodermal regions; by the pluteus stage, a gradient concentration pattern is formed. Katow (2008) further demonstrated strong HpNetrin expression in the dorsal aboral ectoderm and posterior ventroaboral ectoderm regions, whereas the most anterior oral ectoderm regions showed low HpNetrin immunoreactivity. Additionally, the study by Katow (2008) revealed that serotonergic cells form around a region between the above two areas with moderate HpNetrin intensity, and then extend their axons toward the middle-ridge, whereupon the axons move away from the HpNetrin-rich region after crossing the middle-ridge. Based on these findings, it is clear that HpNetrin initially functions as an attractant and subsequently as a repellent guidance cue for serotonergic axons during sea urchin development.

Substantial evidence implies that the attraction or repulsion of serotonergic axons is mediated by the binding of netrin to its receptors on the axonal growth cone (Bashaw and Goodman, 1999; Fazeli et al., 1997; Serafini et al., 1996). These receptors probe the extracellular environment for guidance cues and transform the attractive or repulsive signals intracellularly to guide the neurons toward their targets (Dickson, 2002; Klagsbrun and Eichmann, 2005). A variety of different receptors are engaged by netrins; among them, deleted in colorectal cancer (DCC) receptors, including DCC and neogenin, mediate the chemoattractant responses to netrins, while uncoordinated-5 (UNC-5) receptors appear to be responsible for the netrin-dependent chemorepellent effect (Fazeli et al., 1997; Keino-Masu et al., 1996; Serafini et al., 1996; Vielmetter et al., 1994). In addition, axon guidance by netrin-1 has been shown to depend on localized calcium ion (Ca^{2+}) signals, an important second messenger, in the growth cone (Dent et al., 2004; Dent et al., 2004; Hong et al., 2000; Ming et al., 2002Dent et al., 2004Dent et al., 2004; Hong et al., 2000; Ming et al., 2002).

Increasing evidence suggests that exposure to several kinds of organophosphate pesticides during development can interfere with the SNS, which plays important roles in regulating multiple developmental events in the sea urchin (Marc et al., 2005; Qiao et al., 2003; Xu et al., 2012; Yao et al., 2010). For example, Yao et al. (2010) found that monocrotophos (MCP), an organophosphate pesticide, inhibited the outgrowth of serotonergic axons and decreased the serotonergic cell number, thereby perturbing SNS development. Although Xu et al. (2012) previously showed that MCP disrupts the SNS in sea urchin embryos and larvae by disturbing serotonin metabolism, little is known about the underlying mechanisms by which organophosphate pesticides affect the formation of the serotonergic system. Insight into the functions of netrin-mediated axon guidance during the formation of the sea urchin serotonergic system suggests that disruption of SNS development might be associated with disturbances in extracellular guidance cues such as netrin (Katow, 2008). However, limited information is available regarding whether organophosphate pesticides disturb SNS development by affecting the expression profiles of netrin and its receptor.

Here, we aimed to investigate the mechanisms underlying the effects of organophosphate pesticides, namely MCP, on the development of the SNS. According to the developmental progression of the SNS and the spatio-temporal expression of HpNetrin during SNS formation (Bisgrove and Burke, 1987; Katow, 2008; Yaguchi et al., 2000), we chose seven different developmental stages, namely the early blastula (12 h postfertilization [hpf]), swimming blastula (15-hpf), early gastrula (18-hpf), gastrulation completed (24-hpf), early prism (30-hpf), prism (36-hpf), and two-arm pluteus (48-hpf) stages, to estimate the effects of the MCP pesticide on the expression patterns of HpNetrin and its receptor neogenin, as well as the intracellular Ca^{2+} levels, during early development in *H. pulcherrimus*. Based on our findings, we make predictions regarding the signaling pathways by which organophosphate pesticides exhibit neurotoxicity in developing animals.

2. Materials and methods

2.1. Collection, culture, and exposure of sea urchin embryos

Gametes of the adult sea urchin (*H. pulcherrimus*) were obtained by intrablastocoeleic injection of 0.5 mol/L KCl, and the eggs were fertilized and incubated as described previously (Xu et al., 2012). Embryos were incubated in filtered seawater at approximately 20 °C and used for the following experiments.

We purchased the MCP pesticide (a 40% water-soluble preparation) from a pesticide factory in Qingdao, China. The pesticide exposure test was performed as described by Xu et al. (2012). Briefly, 1 L of embryo suspension containing ~150–200 embryos per mL was exposed to serial concentrations of MCP pesticide (0.01, 0.10, and 1.00 mg/L), while controls were reared in filtered seawater. The test concentrations were approximately 1/1000, 1/100, and 1/10 of the 96-h median lethal concentration values of MCP (11.18 mg/L). The experiments were performed in 2 L beakers, and the water was replaced every 24 h to keep the MCP concentration constant. Three replicates were performed for each condition. The embryos and larvae from each test concentration group were exposed for 12 h, 15 h, 18 h, 24 h, 30 h, 36 h, and 48 h, and then collected. Specifically, 3×10^4–4×10^4 embryos/larvae from each group were pooled as one sample, frozen in liquid nitrogen, and then stored at −80 °C for *HpNetrin* and *neogenin* mRNA quantification.

2.2. Gene expression analysis

We conducted isolation of the total RNA from the sea urchin embryos and larvae, first-strand cDNA synthesis, and quantitative real-time polymerase chain reaction (qRT-PCR) as described by Xu et al. (2012). Briefly, embryos and larvae (3×10^4–4×10^4) were homogenized and the total RNA from each sample was isolated using the reagent TRIzol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The prepared RNA samples were measured by spectrometry at 260 and 280 nm prior to treatment with DNase I (Promega, Madison, WI, USA) to remove any genomic DNA contamination. Equal amounts of RNA (1 μg) were used to synthesize first-strand cDNA by utilizing a reverse transcriptase kit according to the protocol provided by the manufacturer (Toyobo, Tokyo, Japan). The qRT-PCR was performed using the SYBR green mix kit (TaKaRa, Dalian, China) and analyzed on an Eppendorf Mastercycler® ep *realplex*^{2} real-time quantitative PCR System (Eppendorf, Wesseling-Berzdorf, Germany). The primer sequences of *HpNetrin*, *neogenin*, *actin*, and *18s rRNA* are shown in Table 1. The amplification protocol was as follows: 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and at 61 °C for 30 s, and finally a melting curve was generated. Two housekeeping genes including *actin* and *18 s rRNA* were used as internal controls and the expression level of each target gene was normalized to the geometric mean of the mRNA content of these two reference genes using the $2^{-D\Delta Ct}$ method.