Neurotoxicology and Teratology 49 (2015) 81–90

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

Neurobehavioral impairments caused by developmental imidacloprid exposure in zebrafish

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ARTICLE INFO

Article history:
Received 7 October 2014
Received in revised form 16 April 2015
Accepted 24 April 2015
Available online 2 May 2015

Keywords:
Imidacloprid
Neonicotinoid
Nicotine
Neurodevelopment
Zebrafish
Behavior

ABSTRACT

Background: Neonicotinoid insecticides are becoming more widely applied as organophosphate (OP) insecticides are decreasing in use. Because of their relative specificity to insect nicotinic receptors, they are thought to have reduced risk of neurotoxicity in vertebrates. However, there is scant published literature concerning the neurobehavioral effects of developmental exposure of vertebrates to neonicotinoids.
Methods: Using zebrafish, we investigated the neurobehavioral effects of developmental exposure to imidacloprid, a prototypic neonicotinoid pesticide. Nicotine was also administered for comparison. Zebrafish were exposed via immersion in aqueous solutions containing 45 μM or 60 μM of imidacloprid or nicotine (or vehicle control) from 4 h to 5 d post fertilization. The functional effects of developmental exposure to both imidacloprid and nicotine were assessed in larvae using an activity assay and during adolescence and adulthood using a battery of neurobehavioral assays, including assessment of sensorimotor response and habituation in a tactile startle test, novel tank swimming, and shoaling behavior.
Results: In larvae, developmental imidacloprid exposure at both doses significantly decreased swimming activity. The 5D strains of zebrafish were more sensitive to both nicotine and imidacloprid than the AB* strain. In adolescent and adult fish, developmental exposure to imidacloprid significantly decreased novel tank exploration and increased sensorimotor response to startle stimuli. While nicotine did not affect novel tank swimming, it increased sensorimotor response to startle stimuli at the low dose. No effects of either compound were found on shoaling behavior or habituation to a startling stimulus.
Discussion: Early developmental exposure to imidacloprid has both early-life and persisting effects on neurobehavioral function in zebrafish. Its developmental neurotoxicity should be further investigated.
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1. Introduction

Compounds that disrupt normal cholinergic signaling such as the nicotinic cholinergic agonist nicotine are widely classified as neurotoxicants (Chao and Casida, 1997; Yamamoto and Casida, 1999). Nicotine has detrimental effects on behavioral function in many species (Eddins et al., 2009, 2010; Elliott et al., 2004; Levin and Chen, 2004), particularly with exposure during neurodevelopment when cholinergic systems play a morphogenic role. Despite this, concern over the developmental neurotoxicity associated with organophosphate (OP) pesticides (a once widely-used pesticide) use has led to the development of a new class of pesticides: the neonicotinoids, which share structural similarities to nicotine. The neonicotinoids are thought to have reduced toxicity compared to OP pesticides due to their presumed selectivity for insect over vertebrate nicotinic cholinergic receptors. An effective and widely used neonicotinoid pesticide is imidacloprid (1-(6-chloro-3-pyridylmethyl)-*N*-nitro-imidazolidin-2-ylideneamine) (Sheets, 2002).

Neonicotinoids are widely used and quite effective for control of sucking-insects on crops and for flea control on cats and dogs (Schenker et al., 2003). Like nicotine, the efficacy of neonicotinoids as pesticides (and therefore as neurotoxicants) comes from their ability to act as agonists at nicotinic acetylcholine receptors, an action they have in insects and mammals alike (Sheets, 2002; Tomizawa and Casida, 2000; Tomizawa et al., 1995). However, neonicotinoids are thought to selectively bind to insect nicotinic receptors with less action at vertebrate nicotinic receptors (Tomizawa and Casida, 2003, 2005). Thus, imidacloprid and other neonicotinoids are thought to have lowered toxicity profiles for mammals, birds, and fish, making them a popular alternative to organophosphates in commercial agriculture.

The effects of neonicotinoids on neurobehavioral development in vertebrates have not been well characterized, and assumptions regarding their safety have been made in the absence of thorough investigation. While some reports have failed to find overt morphological teratogenic

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http://dx.doi.org/10.1016/j.ntt.2015.04.006
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effects on embryogenesis following imidacloprid exposures up to 50 μM (Scheil and Kohler, 2009), the potential lasting behavioral effects of these exposures are largely unknown. Due to the structural and functional similarities between imidacloprid and nicotine (Kimura-Kuroda et al., 2012), and in some ways organophosphate pesticides such as chlorpyrifos (Fig. 1), it is possible that developmental imidacloprid exposure could similarly affect neurodevelopment and influence behavior later in life. Thus, we sought to clarify both the short and long-term effects of imidacloprid on neurodevelopment by investigating the effects of developmental exposure on behavior immediately after exposure and again during adolescence and adulthood.

In this study, we utilized zebrafish (*Danio rerio*) as a model organism for determining the effects of imidacloprid on neurobehavioral function. Zebrafish are a useful preclinical vertebrate model due to their capacities for complex behavior and higher throughput data collection than mammalian models (Levin and Cerutti, 2008). The small size and relatively easy maintenance of zebrafish also offers economic and logistic benefits over mammalian models. Zebrafish spawn within approximately 30 min of morning light, hatch in 2–3 days post fertilization (dpf), develop complex behavior within the first week, and reach sexual maturity in 2–3 months. Thus, the effects of exposure to a compound can easily be observed over the entire lifespan of the organism (e.g., Levin et al., 2006). Additionally, their ability to absorb compounds through the water facilitates pharmacological and toxicological studies, particularly for water soluble chemicals. Due to the popularity of zebrafish, well-established protocols exist for a number of different behavioral tests, allowing us to assess elements of sensorimotor plasticity, emotional function, social behavior, and cognition (Bailey et al., 2013; Levin and Cerutti, 2008). The goal of the present study was to investigate behavioral endpoints across the lifespan, with larval, adolescent and adult testing times. We used two zebrafish strains (AB* and 5D) for larval assessments. For adolescent and adult assessments we used the type of zebrafish most frequently described in the literature, the AB* strain.

2. Methods

2.1. Design

All zebrafish, in each testing age group (i.e. larval, adolescent and adult), were treated with imidacloprid, nicotine or vehicle control for 0–5 dpf (with daily renewal of solutions). Larval activity levels, assessed at 6 dpf, quantified swimming behavior as well as reactivity to bright and dark environments (Ahmad et al., 2012). A separate cohort was dosed and raised identically and tested at 1.5 months of age to examine neurobehavioral function (sensorimotor response and habituation, social behavior, and novel tank swimming) during adolescence. A third cohort was dosed and raised identically and tested at 3 months of age, at sexual maturity, to examine sensorimotor response and habituation, social behavior, novel tank swimming, and predator avoidance during adulthood. Nicotine exposures were included to permit comparisons between two nicotinic cholinergic receptor acting compounds: nicotine (which has been widely studied) and imidacloprid (of primary interest here).

2.2. Subjects

The procedures were approved by the Duke University Institutional Review Committee for the use of animal subjects. Zebrafish (*D. rerio*) from two wild-type strains, AB* and 5D, were maintained in a colony room at approximately 28.5 °C on a 14:10-h light/dark cycle. Tanks used de-ionized H_2O, sea salt (Instant Ocean, 9.0 g/5 gal H_2O), and neutral regulator (Seachem, 2.5 g/5 gal H_2O) to maintain a pH of 7, and were kept with continual aeration and filtration. Fish were fed twice daily with lab-grown brine shrimp (45 g salt, 5 g eggs/2 L deionized H_2O) and ground flake fish food (TetraMin Tropical Flakes). All behavioral testing of larvae was completed between 4:00 PM and 6:00 PM during the light phase. Due to the time-course of the adolescent and adult behavioral tests, this testing took place throughout the day (9:00 AM to 6:00 PM), with subjects from each exposure group counterbalanced among testing times to eliminate any potential confounding effects of time of day on behavior.

All subjects used in the study were bred in the lab from AB* and 5D progenitors. Zebrafish embryos were collected at the beginning of the 14-h light cycle on the morning following the pairing of same strain adult breeders. Embryos were inspected before use, and those unfertilized or showing obvious malformations were excluded. All subjects used in the larval activity analyses and adolescent/adult tests were without overt physical malformation at the beginning of exposures. Several adolescent or adult zebrafish across all exposure groups exhibited a mild mini-fin mutant phenotype (Connors et al., 1999), which was only apparent as the fish increased in size. This is a genetic malformation of the tail that can alter swimming; consequently, all mini-fin subjects were excluded from behavioral testing. As this occurred across all groups, mini-fin exclusions did not disproportionately affect any group, and there was no difference in N among any of the exposure groups or control at adolescent or adult time points (p's > 0.05).

2.3. Chemical exposures

Animals destined for larval, adolescent or adult testing were dosed separately but identically: approximately 2 h post fertilization (hpf), eggs were inspected under a microscope, and placed in glass Petri dishes at a density of 35-eggs/50 mL aqueous solution and maintained in an incubator (28 °C, 14 h light/10 h dark cycle) for 5 d. Solutions were renewed daily (including control) using normal aquarium water and nicotine hydrogen tartrate salt (Sigma Aldrich, St. Louis, MO, USA) or imidacloprid (Sigma Aldrich, St. Louis, MO, USA). The five doses included a control (home aquarium water), low dose nicotine (45 μM nicotine solution), high dose nicotine (60 μM nicotine solution), low dose imidacloprid (45 μM) and high dose imidacloprid (60 μM), each renewed daily from 0–5 dpf. This dose range was selected based on prior (unpublished) pilot, dose-ranging studies.

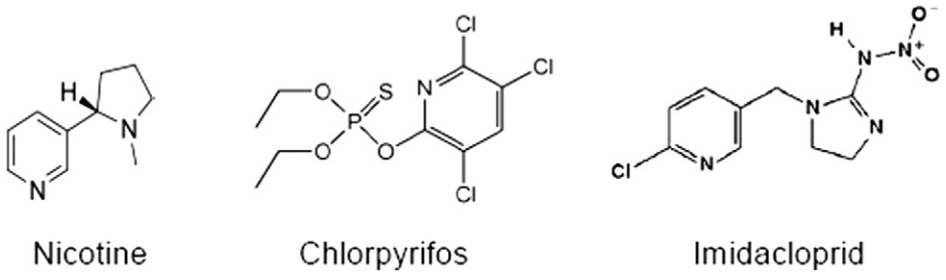

Fig. 1. Molecular structures of nicotine, chlorpyrifos, and imidacloprid.

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