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Exposing imidacloprid interferes with neurogenesis through impacting on chick neural tube cell survival

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Abstract

As a neonicotinoid pesticide, imidacloprid is widely used to control insects in agriculture and fleas on domestic animals. However, it is not known whether imidacloprid exposure negatively affects neurogenesis during embryonic development. In this study, using a chick embryo model, we investigated the effects of imidacloprid exposure on neurogenesis at the earliest stage and during late-stage embryo development. Exposing HH0 chick embryos to imidacloprid in EC culture caused neural tube defects and neuronal differentiation dysplasia as determined by NF/Tuj1 labeling. Furthermore, we found that F-actin accumulation on the apical side of the neural tube was suppressed by exposure to imidacloprid, and the expression of BMP4 and Shh on the dorsal and ventral sides of the neural tubes, respectively, were also reduced, which in turn affects the dorsolateral hinge points during bending of the neural plate. In addition, exposure to imidacloprid reduced cell proliferation and increased cell apoptosis, as determined by pHIS3 labeling and TUNEL staining, respectively, also contributing to the malformation. We obtained similar results in late-stage embryos exposed to imidacloprid. Finally, a bioinformatics analysis was employed to determine which genes identified in this study were involved in neural tube defects (NTDs). The experimental evidence and bioinformatics analysis suggested that imidacloprid exposure during chick embryo development could increase the risk of neural tube defects and neural dysplasia.

Key words: imidacloprid, neural tube defect, neuronal differentiation, cell proliferation/apoptosis, BMP4/Shh.

Introduction

As one of two types of neonicotinoid pesticides, imidacloprid, which chemical name is

1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine with a molecular weight of 255.661, water solubility of 0.51 g/L(20℃), acute oral LD50 of 450 mg/kg and acute dermal LD50 of more than 5000 mg/kg for rats, half-life of 150d in soil, and is widely used to control sucking insects, termites, soil insects on crops or horses (Kilpatrick et al., 2005), and fleas on domestic animals (Rust et al., 2011; Rust et al., 2002). Imidacloprid is an insecticide that acts by mimicking nicotine, which naturally exists in many plants, such as tobacco. In the current market, imidacloprid products are sold in various forms, including liquids, granules, dusts and packages that can be dissolved in water. These imidacloprid products have been available for sale in the US since 1994. As a systemic insecticide, imidacloprid products are usually sprayed on soil and leaves and then spread to the plant's stems, leaves, fruit, and flowers (He et al., 2012; Tan et al., 2012). When insects consume the plants treated with imidacloprid products, their nervous systems are damaged, and they eventually die (Simon-Delso N. et al., 2015). Humans can be exposed to imidacloprid products via skin/eye contact or through consumption or inhalation when handling the pesticide or an animal that was recently exposed to imidacloprid (Wang et al., 2015). Because imidacloprid is used as a systemic insecticide, human exposure to imidacloprid may occur when eating the fruit, leaves, or roots of plants that were treated with imidacloprid. Once humans are exposed, imidacloprid products can cross the lining of the intestine and are transported to the whole body through circulation of the blood (Fahim Mohamed et al., 2009).

The toxicity of imidacloprid is embodied in neurotoxic action through disrupting neural signaling in adult insects (Tomizawa and Casida, 2005). However, little is known about its

potential toxic effects on early embryo development in spite of a few reports on human health, including reproductive ability (Elbert et al., 2008; Gu et al., 2013; Wamhoff and Schneider, 1999). Currently, more and more attention is being paid to the toxic effects of pesticides on embryo development, including neural dysplasia. And the public is concerned about the risk of congenital diseases caused by exposure to pesticides, such as imidacloprid. However, there is no direct evidence of toxicological effects on neurogenesis or corresponding mechanisms nowadays. In this study, we investigated whether or not imidacloprid could affect neurogenesis and explored the relevant mechanism of imidacloprid-induced NTDs during early chick neurogenesis.

As the primary rudiment for the central nervous system, the neural tube is divided into cranial and trunk regions; the cranial region develops into the brain, and the trunk region develops into the spinal cord. Neural tube formation describes a dynamic process during neurulation. It undergoes neural plate elevation/bending to the dorsal midline to eventually form a fluid-filled tube. Normal neural tube formation relies on a variety of physiological processes, such as cell proliferation, cytoskeleton establishment and cell viability, among others (Cearns et al., 2016). Several well-known secreted cell intrinsic signaling molecules, such as FGF, Wnt, Hh and TGF-beta, have been reported to control cell cycle progression during neurulation (Cayuso and Marti, 2005). NTDs refer to improperly developed neural tubes, where the neural tube remains open at birth, which may occur in the brain or spinal cord. Both genetics and the external environment can cause NTDs (Copp and Greene, 2010; Mitchell, 2005). Many transgenic mouse models of NTDs have an accompanying phenotype of short, blunt or absent tails, which include *T (Brachyury)*, Danforth's short tail and others

(Northrup and Volcik, 2000). NTDs can also be found with other birth defects. For example, some NTDs are associated malformations including anencephaly/exencephaly and spina bifida, which suggests that NTDs might be related to the abnormalities of neural crest development (Stevenson et al., 2004).

We used a classical chick embryo model, which has frequently been used to study embryonic development and the effect of numerous external compounds on embryonic development, and several *in ovo* or shell-less systems have been developed for these purposes (Datar and Bhonde, 2011; Scott-Drechsel et al., 2013). Therefore, in this study, we examined the potential harmful effects of imidacloprid exposure on neurogenesis and the corresponding mechanism using early chick embryos.

Materials and Methods

Chick manipulations

Fertilized leghorn eggs were acquired from the Avian Farm of South China Agriculture University (Guangzhou, China). Two approaches were employed to carry out the imidacloprid exposure in this study. For imidacloprid exposure at the early embryonic stage, Hamburger-Hamilton (HH) stage 0 chick embryos (Hamburger and Hamilton, 1951) from fertilized eggs were incubated with DMSO (control) or concentration gradient of imidacloprid (62.5 μM, 125 μM, 250 μM, 500 μM) in early chick (EC) culture medium in a humidified incubator (Yiheng Instruments, Shanghai, China) at 38°C and 70% humidity until the chick embryos developed to the HH10 stage (Chapman et al., 2001). For imidacloprid

exposure at a later embryonic stage, HH10 chick embryos were exposed to 200 μl DMSO (control) or 500 μM imidacloprid through injection into windowed eggs *in vivo* and then further incubated for 4.5 days (HH25) (Chapman, et al., 2001). The experiments were performed in triplicate with 20 eggs assigned to each group, and surviving embryos were fixed in 4% PFA overnight at 4°C to be harvested for further assessment.

Histology

Whole-mount embryos were fixed in 4% PFA overnight at 4°C. Following immunofluorescence staining, HH10 chick embryos were sectioned into 12 μm or 16 μm thick slices using a cryostat microtome (Leica CM1900). The 4.5-day-old chick embryos were dehydrated, embedded in paraffin wax and serially sectioned at 4 μm using a microtome (Leica RM2126RT); the sections were dewaxed in xylene, rehydrated and stained with hematoxylin and eosin dye (H&E) or immunofluorescence stained.

Immunohistochemistry

Immunostaining of whole-mount embryos or sections was performed using the following antibodies: neurofilament (NF, 1:100, Life Technologies, USA), Tuj1 (1:200, Neuromics, USA), Pax7 (1:300, DSHB, USA), phospho-histone3 (pHIS3; 1:300, Santa Cruz, USA), cleaved caspase3 (c-caspase3; 1:300, Cell Signaling Technology, USA), and PCNA (1:100, DSHB, USA). Briefly, the fixed chick embryos or sections were incubated with primary antibody at 4℃ overnight on a shaker. After extensive rinsing in PBST (0.1% Tween-20), samples were incubated with the corresponding Alexa Fluor-555 or 488 secondary antibody

(1:1000, Invitrogen, USA) at 4℃ overnight (embryos) or at room temperature for 2 hours (sections) on a shaker for immunofluorescence staining. All embryos or sections were later counterstained with DAPI (1:1000, Invitrogen) at room temperature for 1 hour.

In situ hybridization

Whole-mount *in situ* hybridization of chick embryos was performed with a standard *in situ* hybridization protocol (McShane et al., 2015). Digoxigenin-labeled probes were synthesized for *BMP4* and *Shh* (Wang et al.). Briefly the embryos were fixed with 4% PFA overnight at 4°C, dehydrated in a graded series of methanol, and stored at −20°C (overnight). The next day, the embryos were hybridized with *BMP4* or *Shh* digoxigenin-labeled antisense probe overnight at 65°C. After hybridization, the bound RNA probe was visualized by incubation with alkaline phosphatase–conjugated anti-digoxigenin antibodies, and the color was developed in NBT/BCIP (Roche, Basel, Switzerland). The whole-mount stained embryos were photographed and then prepared for cryosectioning to a thickness of 16 μm on a cryostat microtome (Leica CM1900).

TUNEL analyses

The extent of apoptosis in the neural tubes was determined using an In Situ Cell Death Detection Kit (Roche). The TUNEL staining was performed according to the instructions provided by the manufacturer. The presence of $TUNEL^+$ cells was determined using an Olympus microscope with image analysis software (Olympus, Japan). We quantified the percentage of $TUNEL^+$ cells relative to all the neural tube cells in each histological slide. We

compared the percentage of TUNEL^{+} neural tube cells in the control and experimental neural tubes.

Western blot

Chick embryos (HH10) were collected and lysed with CytoBuster^{TM} Protein Extraction Reagent (#71009, Novagen). The total protein concentration was established using a BCA quantification kit (BCA01, DingGuo BioTECH, CHN). Samples containing equal amounts of protein were resolved by SDS-PAGE and then transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5% Difco^{TM} skim milk (BD) and then incubated with primary and secondary antibodies. The antibodies used were Smad1/5/8 (Santa Cruz, USA); HRP-conjugated anti-rabbit IgG (Cell Signaling Technology, USA). All primary and secondary anti-bodies used were diluted to 1:1000 and 1:2000 in 5% skim milk, respectively. The protein bands of interest were visualized using an ECL kit (#34079, Thermo Fischer Scientific Inc..) and GeneGnome5 (Syngene). The staining intensity of the bands was determined and analyzed using Quantity One software (Bio-Rad). The western blot results were produced from 3 independent sets of experiments.

RNA isolation and RT-PCR

Total RNA was isolated from HH10 or 4.5-day-old chick embryos using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized to a final volume of 25 μL using SuperScript RIII first-strand (Invitrogen, USA). Following reverse transcription, PCR amplification of the cDNA was performed as described

previously (Dugaiczyk et al., 1983; Maroto et al., 1997). The sets of primers used for RT-PCR are provided in the Supplementary Fig. S1. The PCR reactions were performed in a Bio-Rad S1000TM Thermal cycler (Bio-Rad, USA). The final reaction volume was 50 μL composed of 1 μL of first-strand cDNA, 25 μM forward primer, 25 μM reverse primer, 10 μL PrimeSTARTM Buffer ($Mg2^+$ plus), 4μL dNTPs Mixture (TaKaRa, Japan), 0.5 μL PrimeSTARTM HS DNA Polymerase (2.5U/μL TaKaRa, Japan), and RNase-free water. cDNA was amplified for 30 cycles. One round of amplification was performed at 94°C for 30 s, 30 s at 58°C, and 30 s at 72°C. The PCR products (20 μL) were resolved using 1% agarose gels (Biowest, Spain) in 1× TAE buffer (0.04 M Trisacetate and 0.001 M EDTA) and 10,000x GeneGreen Nucleic Acid Dye (TIANGEN, China) solution. The resolved products were visualized using a transilluminator (SYNGENE, UK), and photographs were captured using a computer-assisted gel documentation system (SYNGENE). Gene expression was normalized to *GAPDH* (a housekeeping gene). The PCR results were produced at least 3 independent sets of experiments.

Photography

Whole-mount embryos and regions of interest were photographed using a stereo-fluorescence microscope and processed using the Olympus software package Image-Pro Plus 7.0. Whole-mount sections were photographed using an Olympus IX51 epi-fluorescence microscope (at 20x and 40x) and analyzed using CW4000 FISH Olympus software.

Bioinformatics analysis

For the protein–protein interaction (PPI) network analysis, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) was used (Hallmann et al., 2014). GO (Gene Ontology) enrichment analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis were performed using the DAVID (Database for Annotation, Visualization, and Integrated Discovery) tools (Simon-Delso N., et al., 2015).

Data analysis

Data analyses and generation of statistical charts were performed using GraphPad Prism 5 software (GraphPad Software, CA, USA). The results are presented as the mean value ($\bar{x} \pm$ SD). All data were analyzed using an independent samples t-test, which was employed to assess the difference among the experimental groups. $P<0.05$ was considered to be statistically significant.

Results

Imidacloprid exposure causes neural tube defects and neural dysplasia in chick embryos.

To investigate the effect of imidacloprid exposure on neurogenesis at an early stage, we exposed HH10 chick embryos to DMSO (control) or 62.5 μM, 125 μM, 250 μM and 500 μM imidacloprid dissolved in EC culture medium as shown in Fig. 1A. We found both of embryo mortality and neural tube defect incidence rose with the increase of the imidacloprid concentration (Fig. 1B), and then we performed NF and Tuj1 immunofluorescent staining, the

HH10 chick embryos exposed to either to DMSO (control) or 500 μM imidacloprid to investigate the effect of imidacloprid exposure on neural differentiation. When the chick embryos developed to HH10, the neural tubes at both the cranial and trunk levels (3^{rd} to 6^{th} pairs of somites) were completely closed, as shown in the control embryos (Fig. 1C, E, G, I, K). However, we found that some neural tubes of cranial and/or trunk level were not completely closed in the imidacloprid-treated HH10 chick embryos (Fig. 1D, F, H, J, K). Since NF belongs to type IV intermediate filaments, it is the most abundant cytoskeletal components in mature neurons and myelinated axons, and also an essential determinant of axon caliber (Paul N. Hoffman et al., 1987). And Tuj1 (class III β-tubulin) is expressed in neurons of the central and peripheral nervous system (Janice E. Alexander et al., 1991), and is expressed early in the neural differentiation (Shim et al., 2004) so that it has been regarded as a phenotypic marker of neural cells.. In our study, lower expression of neurofilament (NF) was observed in imidacloprid-treated embryonic neural tubes compared to untreated controls at both the cranial and trunk levels ($P < 0.05$; control: Mean ± SD = 5.00 ± 0.82; imidacloprid: Mean ± SD = 3.50 ± 0.58; n = 4 for each groups; Fig. 1L). Similar phenotypes were found in the HH10 chick embryos exposed to imidacloprid when Tuj1 immunofluorescence staining was performed ($P < 0.05$; control: Mean ± SD = 188.70 ± 23.03; imidacloprid: Mean ± SD = 69.00 ± 23.43; n = 3 for each groups; Fig. 1M). These results suggest that exposure to imidacloprid indeed suppresses neuronal differentiation and cause neural tube defects in early chick embryos.

Apical constriction is inhibited by exposure to imidacloprid during neural tube formation

of gastrulating chick embryos

As shown in Fig. 2A, F-actin is involved in regulating the apical constriction of the neural tube during embryonic neurulation (Moreau et al., 1999; Morita et al., 2010). We carried out F-actin fluorescent staining in the embryos exposed to either DMSO (control) or 500 μM imidacloprid and demonstrated that F-actin accumulated at the apical side (indicated by arrows) of neural tubes in control chick embryos (Fig. 2A-A2'), while apical F-actin accumulation was dramatically reduced in imidacloprid-treated neural tubes (Fig. 2B-B2'). The arrows showed that the expression in control group was lower than that in the imidacloprid group on the luminal side of neural tube. Circumferential bands of contractile microfilaments, composed of actomyosin, serve to constrict the apical portion of the cell, producing a wedge-like shape that could drive epithelial bending (Cearns, et al., 2016). As the neural folds elevate, cell numbers increased to a greater extent in the dorsolateral neural plate that contacted the surface ectoderm (McShane, et al., 2015). Additionally, abnormality of connexin43 (Cx43), a gap junction protein, is interrelated with neural tube defects (Boot et al., 2006; Yong-zhen, 2011), Previous researches have shown that, whether overexpressed or knocked out the Cx43, it would be associated with neural tube defects (J. L. Ewart et al., 1997), RT-PCR data showed that connexin43 expression increased at mRNA level in imidacloprid-treated neural tubes in comparison to controls in HH10 embryos ($P < 0.01$; control: Mean ± SD = 0.51 ± 0.06; imidacloprid: Mean ± SD = 0.93 ± 0.11; n = 3 for each groups; Fig. 2C-C1).

Dorsolateral hinge points (DLHPs) appear to be the mechanism for the bending of bilateral neural plates for neural tube closure during neural tube formation (McShane, et al.,

2015). DLHP-mediated neural tube morphogenesis is regulated by both BMP and Shh signaling (Stottmann et al., 2006; Ybot-Gonzalez et al., 2007) as shown in Fig. 3A. BMP4 *in situ* hybridization was performed, and the results showed that BMP4 expression at the dorsal side of neural tubes exposed to imidacloprid was suppressed in comparison to the controls at both the cranial and trunk levels (Fig. 3B-C'), which was further confirmed by RT-PCR data ($P < 0.05$; control: Mean ± SD = 0.09 ± 0.01; imidacloprid: Mean ± SD = 0.08 ± 0.01; n = 4 for each groups; Fig. 3D-D1). And we detected the smad1/5/8 using western blot. The result showed that the expression of Smad1/5/8 protein was decreased in imidacloprid-treated group compared to control group ($P < 0.01$; control: Mean ± SD = 0.77 ± 0.06; imidacloprid: Mean ± SD = 0.54 ± 0.03; n = 3 for each groups; Fig. 3E-E1). Meanwhile, we also demonstrated that both cranial and trunk Shh expression was reduced in the imidacloprid-treated embryos (Fig. 3H-I, H1-I1) compared to controls (Fig. 3F-H, F1-H1) using Shh *in situ* hybridization. The transverse sections were treated identically, as indicated by dotted lines in F-I, so that gene expression differences due to distinct levels in different sections could be avoided. Additionally, Pax7 immunofluorescence staining was performed in the same embryos after Shh *in situ* hybridization had been carried out (Fig. 3F'-I'), which showed that neural tube defects occurred and that Pax7 expression on the dorsal side of neural tubes decreased in imidacloprid-treated embryos compared to controls at both the cranial and trunk levels ($P < 0.01$; control: Mean ± SD = 37.67 ± 8.23; imidacloprid: Mean ± SD = 27.33 ± 4.01; n = 12 for each groups; Fig. 3F'-I', J). Moreover, we detected the expression of gli1 using RT-PCR assay, and showed the gli1 expression was lower at mRNA level in the imidacloprid-treated group than in the control group in HH10 embryos ($P < 0.01$;

control: Mean ± SD = 0.06 ± 0.00; imidacloprid: Mean ± SD = 0.04 ± 0.00; n = 3 for each groups; Fig. 3K-K1). These results suggest that the inhibition of BMP4 and Shh expression in neural tubes might be responsible for the neural tube defects.

Imidacloprid exposure restricts cell proliferation and enhances apoptosis in the developing neural tubes.

Appropriate neural tube fusion requires precisely regulated cell apoptosis, proliferation, and differentiation in developing neural tubes (Tang et al., 2005; Wei et al., 2012). Using pHIS3 as a cell proliferation marker, we assessed the cell proliferation of neural progenitor cells in the developing neural tube after exposure to either DMSO (control) or 500 μM imidacloprid (Fig. 4). There were fewer $pHIS3^{+}$ cells in imidacloprid-treated embryos compared to controls at both the cranial and trunk levels ($P < 0.0001$**;** control: Mean ± SD = 14.11 ± 0.84; imidacloprid: Mean ± SD = 5.56 ± 1.27; n = 6 for each groups; Fig. 4E), suggesting that cell proliferation was suppressed by the exposure to imidacloprid during neurulation.

Using the same strategy, we investigated whether cell apoptosis was affected by imidacloprid exposure through determining c-caspase3 and caspase9 expression (Fig. 5). The results showed increased c-caspase3 in the imidacloprid-treated neural tubes compared to controls at both the cranial and trunk levels ($P < 0.0001$**;** control group: n = 14, Mean ± SD = 9.86 ± 3.78; imidacloprid group: n = 17, Mean ± SD = 32.47 ± 13.02; Fig. 5A-E), and RT-PCT assays also confirmed that caspase9 expression was higher in the imidacloprid group than in the control group ($P < 0.05$**;** control: Mean ± SD = 0.19 ± 0.02; imidacloprid: Mean ±

SD = 0.42 ± 0.05; n = 3 for each groups; Fig. 5F-F1), indicating that cell apoptosis is activated by exposure to imidacloprid.

Neural tube defects and neural dysplasia occur when late-stage chick embryos are exposed to imidacloprid.

Embryo development is a dynamic process. To determine whether the harmful effect of imidacloprid exposure could extend to late-stage embryo development, we exposed the chick embryos to imidacloprid at the HH10 stage *in vivo* through injection of 200 μl DMSO (control, final concentration - 0.1%) or imidacloprid (final concentration - 500 μM) into windowed pre-incubated eggs as shown in Fig. 6A. From the intact view of the chick embryo head and trunk regions, we observed neural tube defects in imidacloprid-treated embryos (Fig. 6D-E, F), while no similar phenotypes were found in control embryos (Fig. 6B-C, F). The weight of 4.5-day chick embryo was significantly decreased in imidacloprid group compared with control group ($P < 0.05$**;** control: Mean ± SD = 69.10 ± 19.46; imidacloprid: Mean ± SD = 47.00 ± 11.09; n = 10 for each groups; Fig. 6G). The malformation of neural tubes induced by imidacloprid exposure could be more distinctly observed in their corresponding transverse sections, especially at the trunk level, in which the neural tube still remained open. Moreover, we carried out Tuj1 and Pax7 immunofluorescence staining in the same population of embryos to investigate the effect of imidacloprid exposure on neuronal differentiation. The results showed that Tuj1-labeled area in the imidacloprid group at the trunk level (Fig. 6I-I1) was much less than the one in the control group (Fig. 6H-H1), indicating that neural differentiation (labeled by Tuj1 expression) is inhibited by imidacloprid exposure. And a

similar phenotype was found in the Pax7-labeled dorsal area of neural tubes (Fig. 6J-K1), and RT-PCR assays also showed that Pax6 and Neurogenin2 expression was lower in the imidacloprid group than in the control group ($P < 0.001$**;** control: Mean ± SD = 0.20 ± 0.01; imidacloprid: Mean ± SD = 0.16 ± 0.00; n = 3 for each groups; Fig. 6L-L1; $P < 0.05$**;** control: Mean ± SD = 0.07 ± 0.00; imidacloprid: Mean ± SD = 0.06 ± 0.00; n = 3 for each groups; Fig. 6L-L2), suggesting that imidacloprid exposure during late-stage embryonic development could also impair the normal development of the neural tube in chick embryos. Meanwhile, TUNEL assays demonstrated that cell apoptosis in imidacloprid-treated embryos was also increased in the late-stage chick embryos in comparison to controls ($P < 0.01$**;** control: Mean ± SD = 38.35 ± 2.01; imidacloprid: Mean ± SD = 56.78 ± 4.20; n = 4 for each groups; Fig. 7A-C). PCNA immunofluorescence staining showed that cell proliferation in imidacloprid-treated embryos was dramatically reduced in comparison to controls ($P = 0.0785$**;** control: Mean ± SD = 58.64 ± 5.99; imidacloprid: Mean ± SD = 50.27 ± 6.83; n = 5 for each groups; Fig. 7D-F). RT-PCR data showed that imidacloprid exposure suppressed the mRNA expression of cyclin D1 and p21 (Cyclin D1: $P < 0.05$**;** control: Mean ± SD = 0.89 ± 0.06; imidacloprid: Mean ± SD = 0.70 ± 0.12; n = 3 for each groups; p21: $P < 0.001$**;** control: Mean ± SD = 0.63 ± 0.03; imidacloprid: Mean ± SD = 0.39 ± 0.03; n = 3 for each groups; Fig. 7G-G1). These results indicate that the disturbance in cell apoptosis and proliferation contributes to the malformation of neural tubes induced by exposure to imidacloprid.

Protein–protein interactions and pathway analysis of the genes which were assessed in this study

The genes used in this study were mapped using a STRING database to construct a protein-protein interaction (PPI) network (Fig. 8A). SHH, BMP4, PAX7 PCNA, cyclin D1, p21 and caspase3 can form PPI pairs (Fig. 8A). A summary of the results based on the DAVID analysis showed that p21, PCNA, cyclin D1 and SHH are involved in the cell cycle and can induce NTDs (Fig. 8B). Caspase3, p21, SHH and BMP4 are involved in apoptosis and can induce NTDs (Fig. 8B). SHH, BMP4 and PAX7 are involved in cell fate commitment and can induce NTDs (Fig. 8B). p21 and PCNA can inhibit cyclin D1 directly; SHH can also directly inhibit BMP4 in the neural tube. Finally, we assessed the effect of imidacloprid on these genes as shown in Fig. 8.

Discussions

As one type of pesticide, imidacloprid has been used worldwide to control insects in agriculture and on domestic animals (Kilpatrick, et al., 2005). Therefore, the biological safety of imidacloprid products has attracted much attention (Elbert, et al., 2008; Gu, et al., 2013; Wamhoff and Schneider, 1999). It is known that imidacloprid toxicity varies among different species. For example, imidacloprid is very toxic to honeybees and other beneficial insects, but it is not very toxic to birds and is slightly toxic to fish (Crosby et al., 2015; Hallmann, et al., 2014; Rondeau et al., 2014). However, the risk for humans is that the accumulation of this pesticide on plants and animals, including pets, will inevitably be transferred to humans through close daily contact with treated plants or animals. Generally, toxic effects of the pesticide in adults are more noticeable and obvious, and the toxic effects of imidacloprid on developing embryos and fetuses are ignored.

First, HH0 chick embryos were incubated in EC culture containing either DMSO (control) or concentration gradient of imidacloprid (such as 62.5 μM, 125 μM, 250 μM, 500 μM) until HH10 (1.5 days) to study the possible effects of imidacloprid at the early embryonic developmental stage (Fig. 1A-B). The concentration of imidacloprid (500 M) employed in this study was same as previously reported (Gu, et al., 2013). Frequent neural tube defects (Fig. 1K-L) were observed after imidacloprid exposure (Fig. 1C-J). Moreover, the expression of NF and Tuj1, markers of neuronal differentiation, were dramatically reduced in imidacloprid-treated embryos in comparison to controls. Therefore, we detected NF and Tuj1 for neurogenesis in this study. The results suggest that imidacloprid exposure at high concentration or for long-term indeed negatively affects embryonic neurulation, and the effects are observed not only in the morphogenesis of neural tube formation but also on neuronal differentiation. Acetylcholinesterase (AChE) plays an important role for the cholinergic neurotransmission since it can catalyze the hydrolysis of the neurotransmitter acetylcholine (ACh). And because of this, AChE is the target of many toxins such as insecticides, chemical weapons, and snake venoms. Either excess AChE inhibition or bankrupt of AChE results in an ACh accumulation and the consequent overstimulation of the muscles, which leads to spasms and myopathy-like phenotypes. Previous reports showed that both acetylcholinesterase and acetylcholine receptors are associated with neurogenesis, and could cause neural tube defects if they are barely functional (Nils Klü ver, et al., 2011; Rogers, et al., 2012; Stephen Brimijoin and Koenigsberger., 1999). We detected the expressions of acetylcholinesterase and acetylcholine receptors by RT-PCR, and the results showed that expressions of both acetylcholinesterase and acetylcholine receptors were

inhibited by imidacloprid treatment (Wang et al., 2016). The results suggest that imidacloprid treatment might lead to neural tube defects or affect neurogenesis by inhibiting the both important gene expressions mentioned-above.

The bending of the neural plate and neural tube closure are closely correlated with the apical constriction of apical-side neural plates/tubes (Moreau, et al., 1999; Morita, et al., 2010), which is in turn related to cytoskeleton function in neural plates/tubes. In this study, the reduction of F-actin accumulation at the apical side of the neural tubes in imidacloprid-treated embryos (Fig. 2) indicates that the imidacloprid-induced attenuation of F-actin might contribute to the failure of normal neural plate bending and neural tube formation.

DLHPs have been shown to be the morphological mechanism for bending of neural plates and neural tube closure through an imbalance in proliferation between the dorsolateral and ventromedial neural plate (McShane, et al., 2015). These cellular processes are principally regulated by Wnt signaling on the dorsal side of the neural tube and Shh signaling on the ventral side of the neural tube (Stottmann, et al., 2006; Ybot-Gonzalez, et al., 2007). Imidacloprid-treated embryos suggests that imidacloprid interferes with crucial gene expression in developing neural tubes and may be responsible for the morphological dysplasia of the neural tube. As described above, BMP and Shh regulate the cell cycle of neural projector cells through DLHPs. Here, we compared with $pHIS3^{+}$ cell proliferation and TUNEL-labeled cell apoptosis between control and imidacloprid-treated embryos, and demonstrated that the imidacloprid-treated group significantly suppressed cell proliferation (Fig. 4) and increased apoptosis (Fig. 5) in neuronal progenitor cells.

Embryonic development is a dynamic process. During the developmental period, the embryo is sensitive to the external environment, and differences in susceptibility may be due to the different developmental stages for each structure or organ. For nervous system development, neurulation occurs in the early stage of embryo development, and neuronal differentiation proceeds at relatively late stage. Therefore, based on this consideration, we carried out an experiment in which the embryos were exposed to imidacloprid at a relatively late stage of embryo development, HH10 (Fig. 6A). We found malformation of the neural tube at both the cranial and trunk levels with high frequency (Fig. 6B-F). Neuronal differentiation and Pax7 expression were likewise suppressed by imidacloprid in late-stage embryos (Fig. 6H-K), and RT-PCR data showed that Pax6 and Neurogenin2 were suppressed by imidacloprid too (Fig. 6K-K1), indicating that the negative effect of imidacloprid on neurogenesis is not time dependent, and it can occur at any developmental period. One question raised here is whether the malformation of the neural tube was due to the failure of neural tube closure or re-exposure of the neural tube after closure. In normal embryonic development, neural tube should be closed at HH10 at the level of the 3^{rd} to 6^{th} pairs of somites, which has been shown in control group. For the later stage of embryonic experiment, we injected imidacloprid to the chick embryos at HH10, in which the neural tube has been closed normally. However, we found the neural tube of this level re-open in the experimental groups on the 4.5 day. This phenotype is similar with the high-salt treated chick embryos in our previously study (Jin et al., 2015). Thus, we speculated that the opening of the neural tubes was related to the abnormal cell proliferation and apoptosis, so that we performed PCNA and Tunel staining. From the results, we could see that the $Tunel^{+}$ cells were

significantly increased in the experimental group, while $PCNA^+$ cells did not change significantly compared to control. It is suggested that the opening of the neural tube in imidacloprid treated group might be associated with the increase of cell apoptosis.

In recent years, bioinformatics analysis has become very popular because it is an effective way to identify interactions between DNA and proteins *in vivo* (Rondeau, et al., 2014). The protein-protein interaction (PPI) network, GO enrichment analysis and KEGG pathway enrichment analysis indicated that the genes in our study were indirectly involved in NTDs (Fig. 8). Taken together, we revealed that imidacloprid exposure to developing embryos could lead to a higher risk for neural tube defects and neural dysplasia. The cellular mechanism may be impairment of the cytoskeleton of neuronal progenitor cells, which in turn affects the normal bending of the neural plate through interfering with DLHPs. Additionally, the imidacloprid-induced suppression of BMP4 and Shh in the developing neural tube could contribute to the phenotypes described above. Furthermore, abnormal gene expression could increase cell apoptosis and inhibit cell proliferation, which aggravates the malformation of early neural tubes during embryogenesis. It is obvious that more precise molecular experiments are required to completely reveal the toxicological mechanism for imidacloprid exposure to embryonic neurogenesis.

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Competing interests

The authors declare no competing financial interests.

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Figure legends

Fig. 1. Imidacloprid exposure leads to abnormal neurogenesis during chick embryo development.

HH0 chick embryos were exposed to a concentration gradient of imidacloprid (62.5 μM, 125 μM, 250 μM and 500μM) imidacloprid or DMSO (control) in EC culture medium until the embryos reached HH10 as schematically shown in **A**. **B**: A bar chart showing the embryo

mortality and ratio of the neural tube defects in control and imidacloprid-treated embryos. **C-F**: NF immunofluorescent staining was performed on whole-mount HH10 chick embryos exposed to either DMSO (control, C, E) or 500 μM imidacloprid (D, F), and the cranial regions (C-D) and trunk regions (E-F) were assessed. **C1-D2, E1-F2**: The transverse sections are indicated by dotted lines in B-E (white arrows indicated the NF+ cells). **G-J**: Tuj1 immunofluorescent staining was performed on whole-mount HH10 chick embryos exposed to either DMSO (control, G, I) or 500 μM imidacloprid (H, J), and the cranial regions (G-H) and trunk regions (I-J) were assessed. **G1-H2, I1-J2**: Transverse sections are indicated by dotted lines in G-J (white arrows indicated the Tuj+ cells). **K**: A bar chart showing the incidence of neural tube defects in chick embryos exposed to DMSO (control) or imidacloprid. **L**: A bar chart showing NF expression in the trunk level of chick embryos exposed to DMSO (control) or imidacloprid. **M**: A bar chart showing Tuj1 expression in the trunk level of chick embryos exposed to DMSO (control) or imidacloprid. Scale bars =500 μm in C-D, G-H, 100 μm in E-F, I-J and 100 μm in C1-J2.

Fig. 2. Imidacloprid exposure suppresses the F-actin expression at the luminal side of the neural tube.

A-B: Schematic drawings indicating the process of neural tube closure in control (A) and imidacloprid-treated (B) embryos. The boxes in the schematic stand for several layers of neural tube cells, and the black arrows indicate the sites that F-actin is highly expressed in luminal side of neural tube. Moreover, the difference of fluorescent intensity between a full arrow and broken arrow is that the F-actin is expressed higher (full arrows) in control than the

one in imidacloprid-treated (broken arrows). **A1-A2**: Transverse sections of F-actin immunofluorescence-stained neural tubes (A1) with DAPI staining (A2) in the control group. **A1'-A2'**: High magnification images from the sites indicated by dotted squares in A1-A2. **B1-B2**: Transverse sections of F-actin immunofluorescence-stained neural tubes (B1) with DAPI staining (B2) in the imidacloprid-treated group. **B1'-B2'**: High magnification images from the sites indicated by dotted squares in B1-B2. **C**: RT-PCR data showing the mRNA expression of connexin43 in control and imidacloprid-treated embryos. **C1**: A bar chart showing the ratio of connexin43 expression to GAPDH in control and imidacloprid-treated embryos from RT-PCR data. Scale bars =100 μm in A1-B2 and 10 μm in A1'-B2'.

Fig. 3. Imidacloprid exposure inhibits BMP4 and Shh expression in the chick neural tube.

A: Schematic drawing indicating the dorsolateral hinge points (DLHP) and BMP4 and Shh expression patterns in developing neural tubes. **B-B1, C-C1**: Transverse sections of the cranial level (B-C) and trunk level (B1-C1) after whole-mount *in situ* hybridization was performed against BMP4 in the embryos exposed to DMSO (control, B-B1) or imidacloprid (C-C1). **D**: RT-PCR data showing the mRNA expression of BMP4 in the control and imidacloprid-treated embryos. **D1**: A bar chart showing the ratio of BMP4 expression to GAPDH in the control and imidacloprid-treated embryos using RT-PCR data. **E**: Western blotting data showing the protein expression of Smad1/5/8 in the control and imidacloprid-treated embryos. **E1**: A bar chart showing the ratio of Smad1/5/8 expression to β-actin in the control and imidacloprid-treated embryos from Western blotting data. **F-I**: Cranial (E-G) and trunk (H-I) images of chick embryos were taken after whole-mount *in situ*

hybridization was performed against Shh in the embryos exposed to DMSO (control, F, H) or imidacloprid (G, I). **F1-I1**: Transverse sections showing Shh *in situ* hybridization are indicated by the dotted lines in F-I. **F'-I'**: Cranial (F'-G') and trunk (H'-I') images of chick embryos were taken after whole-mount Pax7 immunofluorescence staining was performed in the embryos exposed to DMSO (control, F', H') or imidacloprid (G', I'). **F2_4-I2_4**: Transverse sections from the Pax7 immunofluorescence-stained embryos are indicated by the dotted lines in E'-H', in which F2-I2 show DAPI staining, F3-I3 show Pax7 immunofluorescent staining and F4-I4 show merged images. **J**: A bar chart showing the $Pax7^+$ cell numbers in the transverse sections of the control and imidacloprid-treated embryos. **K**: RT-PCR data showing the mRNA expression of gli1 in the control and imidacloprid-treated embryos. **K1**: A bar chart showing the ratio of gli1 expression to GAPDH in the control and imidacloprid-treated embryos from RT-PCR data. Scale bars = 500 μm in F-I, F'-I' and 100 μm in B-B', C-C', F1-I1, F2-I2, F3-I3, F4-I4.

Fig. 4. Imidacloprid exposure suppresses pHIS3 expression in chick neural tubes.

A-D: Cranial (A-B) and trunk (C-D) images of chick embryos were taken after whole-mount pHIS3 immunofluorescence staining was performed in the embryos exposed to DMSO (control, A, C) or imidacloprid (B, D). **A1-D1, A2-D2**: Transverse sections from the pHIS3 immunofluorescence-stained embryos are indicated by the dotted lines in A-D, in which A1-D1 show pHIS3 immunofluorescence staining, and A2-D2 are merged images of A1-D1+DAPI staining. **E**: A bar chart showing $pHIS3^+$ cell numbers in the transverse sections of control and imidacloprid-treated embryos. Scale bars = 500 μm in A-D and 100

μm in A1-D1, A2-D2.

Fig. 5. Imidacloprid exposure increases c-caspase3 and caspase9 expression in chick neural tubes.

A-D: Cranial (A-B) and trunk (C-D) images of chick embryos were taken after whole-mount c-caspase3 immunofluorescence staining was performed in the embryos exposed to DMSO (control, A, C) or imidacloprid (B, D). **A1-D1**: Transverse sections from the c-caspase3 immunofluorescence-stained embryos are indicated by the dotted lines in A-D, and DAPI staining was completed in each section. **A2-B2**: High magnification images of the sites indicated by dotted squares in A1-B1. **E**: A bar chart showing c-caspase3^{+} cell numbers in the transverse sections of control and imidacloprid-treated embryos. **F**: RT-PCR data showing the mRNA expression of caspase9 in control and imidacloprid-treated embryos. **F1**: A bar chart showing the ratio of caspase9 expression to GAPDH in control and imidacloprid-treated embryos using RT-PCR data. Scale bars = 500 μm in A-D, 100 μm in A1-D1 and 50 μm in A2-B2, C1-D1.

Fig. 6. Imidacloprid exposure also causes later neural tube defects and abnormal differentiation of neural tubes.

The HH10 chick embryos were exposed to DMSO (control) or 500 μM imidacloprid and harvested at 4.5 days. **A**: A schematic drawing indicates the experimental procedure. **B-E**: Representative intact head (B-C) and trunk (D-E) images from control (B, D) and imidacloprid-exposed (C, E) embryos. **B1-E1**: Representative H&E-stained transverse

sections indicated by dotted lines in B-E. **F**: The bar chart shows the neural tube malformation rates between control and imidacloprid-treated groups. **G**: The bar chart shows a comparison of the 4.5-day-old chick embryo weights between control and imidacloprid-treated groups. Abbreviation: NT, neural tube; DM, dermomyotome. **H-I, H1-I1**: Representative Tuj1 immunofluorescence-stained transverse sections from the trunk region from control (H-fluorescent, H1-fluorescent+DAPI) and imidacloprid-exposed (I-fluorescent, I1-fluorescent+DAPI) embryos. **J-K, J1-K1**: Representative Pax7 immunofluorescence-stained transverse sections from the trunk region from control (J-fluorescent, J1-fluorescent+DAPI) and imidacloprid-exposed (K-fluorescent, K1-fluorescent+DAPI) embryos. **L**: RT-PCR data showing the mRNA expression of Pax6 and Neurogenin2 in the control and imidacloprid-treated embryos. **L1-L2**: A bar chart showing the ratio of Pax6 (L1) and Neurogenin2 (L2) expressions to GAPDH in the control and imidacloprid-treated embryos using RT-PCR data. Scale bars = 10^6 μm in B-E, 100 μm in B1-E1 and 100 μm in H-K, H1-K1.

Fig. 7. Imidacloprid exposure enhances TUNEL staining and suppresses PCNA expression in the developing chick neural tubes.

A-B: Representative TUNEL-stained images of transverse sections from control (A) and imidacloprid-exposed (B) embryos. **A1-B1**: High magnification images of the sites indicated by dotted squares in A-B. **C**: Quantification of cell death between control and imidacloprid-treated embryos. The apoptotic rate was quantified in chick neural tube sections using the TUNEL assay. **D-E**: Representative PCNA immunofluorescence-stained images of

transverse sections from control (D) and imidacloprid-exposed (E) embryos. **D1-E1**: High magnification images of the sites indicated by dotted squares in D-E. **F**: A bar chart showing $PCNA^+$ cell numbers in the neural tubes of control and imidacloprid-treated embryos. **G**: RT-PCR data showing the mRNA expressions of cyclin D1 and p21 in control and imidacloprid-treated embryos. **G1**: A bar chart showing the ratio of cyclin D1 and p21 expression to GAPDH in control and imidacloprid-treated embryos using RT-PCR data. Scale bars = 100 μm in A-E and 20 μm in A1-E1.

Fig. 8. Bioinformatics analysis depicts the PPI and pathway in this study and the potential mechanisms for imidacloprid exposure leading to chick NTDs.

A: The genes used in this study were mapped with the STRING database to construct the PPI network. **B:** The results from the GO enrichment analysis and KEGG pathway enrichment analysis showed that the genes affected were mainly related to cell cycle, apoptosis and cell fate commitment and can induce NTDs. We noted that imidacloprid exposure affects the potential target genes and leads to chick NTDs.

Supplementary Fig. S1. Primer sets used in the RT-PCR study.