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**Increased spontaneous activity and progressive suppression of adult neurogenesis in the hippocampus of rat offspring after maternal exposure to imidacloprid**

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**Highlights**

* Imidacloprid progressively suppressed hippocampal neurogenesis throughout adulthood.
* Imidacloprid increased spontaneous activity in open field tests of adult offspring.
* Imidacloprid persistently affected cholinergic signaling in the hippocampus.
* Imidacloprid induced neuroinflammation and oxidative stress in the hippocampus.
* The NOAEL of imidacloprid for offspring neurotoxicity was 83 ppm in diet.

**Abstract**

Imidacloprid (IMI) is a widely used neonicotinoid insecticide that poses risks for developmental neurotoxicity in mammals. The present study investigated the effects of maternal exposure to IMI on behaviors and adult neurogenesis in the hippocampal dentate gyrus (DG) of rat offspring. Dams were exposed to IMI (83, 250, or 750 ppm in diet) from gestational day 6 until day 21 post-delivery on weaning, and offspring were maintained until adulthood on postnatal day 77. In the neurogenic niche, 750-ppm IMI decreased numbers of late-stage neural progenitor cells (NPCs) and post-mitotic immature granule cells by suppressing NPC proliferation and ERK1/2–FOS-mediated synaptic plasticity of granule cells on weaning. Suppressed reelin signaling might be responsible for the observed reductions of neurogenesis and synaptic plasticity. In adulthood, IMI at ≥ 250 ppm decreased neural stem cells by suppressing their proliferation and increasing apoptosis, and mature granule cells were reduced due to suppressed NPC differentiation. Behavioral tests revealed increased spontaneous activity in adulthood at 750 ppm. IMI decreased hippocampal acetylcholinesterase activity and *Chrnb2* transcript levels in the DG on weaning and in adulthood. IMI increased numbers of astrocytes and M1-type microglia in the DG hilus, and upregulated neuroinflammation and oxidative stress-related genes on weaning. In adulthood, IMI increased malondialdehyde levels and numbers of M1-type microglia, and downregulated neuroinflammation and oxidative stress-related genes. These results suggest that IMI persistently affected cholinergic signaling and induced neuroinflammation and oxidative stress during exposure. Thereafter, it increased sensitivity to oxidative stress in the hippocampus, causing hyperactivity and progressive suppression of neurogenesis throughout adulthood. The no-observed-adverse-effect level of IMI for offspring behaviors and hippocampal neurogenesis was determined to be 83 ppm (5.5–14.1 mg/kg body weight/day).

**Introduction**

Imidacloprid (IMI) is a type of chlorinated nicotine belonging to neonicotinoid pesticides. Due to its structural similarities to nicotine, IMI can specifically bind to the alpha subunits of the nicotinic acetylcholine receptor (nAChR) in the same way as nicotine to attack the nervous system of exposed insects. Ultimately, this causes accumulation of acetylcholine by blocking neuronal nerve impulse propagation to initiate paralysis and then death.1 Moreover, IMI is considered a highly effective and low-toxicity insecticide due to its higher binding affinity to insect nAChR compared with vertebrates.1 Thanks to these factors, IMI has become widely used worldwide as an agricultural and pet insecticide.1 However, additional properties of IMI (e.g., high water solubility, non-volatility, and difficult biodegradation) cannot be ignored because they result in its persistence in water, soil, crops, pets, and even humans.2 There is increasing evidence of IMI toxicity in mammals, including genotoxicity, reproductive toxicity, developmental toxicity, and developmental neurotoxicity (DNT).1

The placenta and breast milk are the primary means of exposure to toxicants for offspring. Even at the lowest exposure levels in humans, IMI and its metabolites can permeate the mammalian blood-brain barrier and placenta, leading to accumulation in the brain.3,4 Lactational transfer of orally administered IMI has also been reported in a goat.5 Moreover, IMI is one of the most frequently detected neonicotinoid insecticides in breast milk; in China, IMI has been detected in 62.0% of human breast milk samples.6 Given the vulnerability and delayed consequences of the developing mammalian brain,7 as well as the importance of nAChR in brain development,8 the possibility of neurodevelopmental effects cannot be excluded even if no adverse effects of maternal IMI exposure have been observed or there is little or no effect on survival or morphology of offspring in lifetime cases.9 Furthermore, several studies have suggested that IMI may exert DNT. Experimentally, IMI can exert excitatory effects on nAChRs similar to nicotine (a neurotoxin during brain development) and alter brain development-related transcriptome expression in cultured rat neonatal neurons.10 Maternal exposure to IMI by infusion using an implanted osmotic pump from gestational day (GD) 4 to postnatal day (PND) 21 caused behavioral changes of mouse offspring in adulthood.3 Moreover, repeated oral exposure of adolescent rats to IMI causes oxidative brain damage accompanying depression-like behaviors and less exploratory activity.2 These animals also display induced pro-inflammatory cytokine production and damaged antioxidant systems in the brain.2 Therefore, the hazards and effects of IMI on brain development and function of mammalian offspring in utero and during lactation need to be examined in relation to neuroinflammation and oxidative stress.

The hippocampus is the primary location for both memory formation and antidepressant effects in the brain.11 The hippocampal dentate gyrus (DG) recapitulates all the processes of embryo-fetal neurogenesis in a process called “adult neurogenesis”, beginning with self-renewal of neural stem cells (NSCs; type-1 cells) in the subgranular zone (SGZ). Proliferative neural progenitor cells (NPCs; type-2a, type-2b, and type-3 cells in developmental sequence) are differentiated from type-1 cells. Actively mitotic type-3 NPCs produce postmitotic immature granule cells that wmigrate and integrate into the granule cell layer (GCL) in the DG, finally becoming mature granule cell lineages.12 Because they undergo myelination, synaptic exuberance and pruning, networking, and function building, newborn neurons play crucial roles in hippocampus-dependent brain functions such as learning and memory, anxiety regulation, and locomotor activity after stimuli.13 In addition, neurogenesis in the DG is regulated by various types of neurotransmitter inputs and the internal environment. For example, γ-aminobutyric acid (GABA)-ergic interneurons can promote and modulate the axonal branching and synaptogenesis of newborn neurons, as well as the differentiation and migration of NSCs and NPCs into the correct locations by secreting reelin or expressing parvalbumin, a calcium-binding protein.14,15 Similarly, the inputs of cholinergic and glutamatergic neurons regulate the differentiation of granule cell lineages and numbers of newborn granule cells.15 Notably, accumulating evidence suggests that nAChRs are involved in neurodevelopment by modulating release of several kinds of synaptic neurotransmitters, including GABA, acetylcholine, glutamate, serotonin, and dopamine.8

Because the DG encapsulates the whole process involved in DNT of the developing brain, we propose that monitoring the DG is a valuable and effective tool for detecting target cell populations of DNT and its irreversibility in vivo using rodents. For example, we found that developmental exposure of rats to aflatoxin B1 targets GABAergic interneurons expressing nAChR in the DG to suppress proliferation of type-3 NPCs. Similarly, ethanol suppressed synaptic plasticity by over-activating microglia, while glycidol targeted the newly generating nerve terminals of immature granule cells, resulting in suppression of late-stage hippocampal neurogenesis.16–18

This study was carried out to disclose potential target cells and mechanisms involved in the disruption of neurodevelopment in rat offspring after maternal IMI exposure from GD 6 to weaning day, as well as its potential reversibility with the exposure scheme provided by the Organization for Economic Co-Operation and Development (OECD) Guidelines for the Testing of Chemicals (Test No. 426: Developmental Neurotoxicity Study).19 For these purposes, the hippocampal neurogenesis of offspring was examined as an endpoint along with hippocampus-related behaviors including the distribution, population, proliferation, apoptosis, and synaptic plasticity of granule cell lineages in the SGZ/GCL; population changes of GABAergic interneurons in the DG hilus; and oxidative stress and acetylcholinesterase (AChE) activity levels in the hippocampus.

**Discussion**

In response to 750-ppm IMI exposure in the present study, maternal rats displayed decreased food consumption during gestation, decreased water consumption during the first and third weeks of gestation, and decreased water consumption during the second and third weeks of lactation. Regarding exposure effects on offspring, following exposure to 750-ppm IMI, male pups had decreased BW from PND 9 to PND 21 that subsequently recovered. However, there were no observed clinical signs for gait or other behaviors of dams or offspring. Therefore, 750-ppm IMI in diet was considered a reasonable highest test dose to show slight effects on dams or offspring for detecting DNT, in accordance with the recommendation in the OECD Guidelines for the Testing of Chemicals (Test No. 426: Developmental Neurotoxicity Study).19

Regarding the effects of IMI on adult hippocampal neurogenesis of offspring in the present study, numbers of DCX+ and TUBB3+ cells were decreased and accompanied by downregulation of *Dpysl3* and *Tubb3,* without alterations in numbers of TBR2+ cells at 750 ppm on PND 21. In granule cell lineages of the hippocampal DG, DCX is mainly expressed in cell populations from type-2b NPCs to post-mitotic immature granule cells.29 Because TBR2 is expressed in type-2b NPCs12 and TUBB3 is a marker of newly generated immature post-mitotic granule cells,24 the obtained results suggest that type-3 NPCs and postmitotic immature granule cells were decreased following IMI exposure at 750 ppm. Considering the observed decrease in numbers of PCNA+ proliferating cells in the SGZ at this dose, the decline in DCX+ and TUBB3+ cells is thought to be caused by suppressed proliferation of late-stage NPCs. However, after cessation of ≥ 250 ppm IMI exposure from PND 21, no persistent effects on DCX+ or TUBB3+ cell populations were observed in adulthood on PND 77, and numbers of GFAP+ type-1 NSCs and NeuN+ postmitotic granule cells were decreased. Following cessation of 750-ppm IMI, these features were accompanied by downregulation of the NSC marker gene *Nes* in the DG*.* Considering numbers of TUBB3+ cells were unchanged, IMI might have induced progressive disruption of hippocampal neurogenesis to cause reductions in type-1 NSCs and mature granule cells at the adult stage. In addition, following exposure to 750-ppm IMI, we observed increased numbers of TUNEL+ apoptotic cells in the SGZ but not GCL, as well as downregulation of the proliferation marker *Pcna* and anti-apoptosis-related *Bcl2l1*. These findings suggest a delayed effect of IMI to decrease numbers of NSCs by inducing apoptosis and suppressing proliferation. Because the maturation process of neurons in hippocampal adult neurogenesis takes approximately around 7 weeks in rodents, a net increase in mature granule cells results from recruitment from the immature neuronal cell pool.29,30 Therefore, it is reasonable to consider that the observed decrease of mature granule cells on PND 77 was a delayed consequence of the reduction of type-3 NPCs and immature granule cells on PND 21.

Reelin, a type of large glycoprotein, is especially secreted by GABAergic interneurons distributed in the hippocampus during adult neurogenesis, whereby it plays important roles in various aspects of neurogenesis, including neural proliferation, differentiation, migration, and maturation.14 Experimentally, loss of reelin causes a decrease in the number and maturation rate of surviving immature granule cells, which display decreased dendritic complexity.31 Moreover, when reelin was excluded from the culture medium, NSC differentiation was retarded, causing a reduction in immature neurons.32 In the current study, 750-ppm IMI significantly decreased numbers of RELN+ interneurons in the DG hilus on PND 21. Therefore, we considered IMI-induced reductions in immature granule cells and type-3 NPCs on PND 21 to result from suppressed proliferation and delayed differentiation of mitotic NPCs caused by disrupted reelin signaling. In addition, the hippocampus plays critical roles in processing and control of learning and memory formation, which mainly depend on neuronal plastic changes by rapidly and selectively upregulating the expression of IEGs.33 Previous studies have demonstrated important roles of reelin signaling on synaptic strength and plasticity. There are reports showing that a lack of reelin can lead to the inhibition of reelin-dependent ERK1/2 phosphorylation, resulting in suppressed expression of ERK1/2-dependent IEG proteins including FOS and ARC in mature neurons.34 In this study, numbers of p-ERK1/2+ and FOS+ granule cells were significantly decreased on PND 21 following exposure to 750-ppm IMI. In the hippocampus, downregulation of IEGs leads to abnormalities in hippocampal-dependent learning behaviors that can be detected by Y maze, novel environment exposure, and contextual fear conditioning tests.33,35 Following exposure to 750-ppm IMI, we observed a slight but non-significant decrease in Y-maze spontaneous alternation rates on PND 27 that occurred in parallel with decreased populations of cells immunoreactive for RELN, p-ERK1/2, and FOS during the weaning period. However, in adulthood, with the restoration of reelin signaling and IEG expression, IMI exposure did not alter parameters in the contextual fear conditioning test. These results suggest that IMI-induced disruptions of reelin signaling at the end of IMI exposure (750 ppm) on weaning might also be associated with suppressed synaptic plasticity of granule cells at this time point.

In the present study, we found that IMI exposure at 750 ppm downregulated *Chrnb2* in the DG on both PND 21 and PND 77. During hippocampal adult neurogenesis, neurons mainly express two types of nicotinic receptors, α7-nAChRs and β2-nAChRs, which are encoded by *Chrna7* and *Chrnb2*, respectively.36 They are critical for cholinergic signaling-based modulations of neurogenesis. For example, *Chrnb2*-knockout mice show a significant decrease in the proliferation of granule cells.37 Therefore, the downregulation of *Chrnb2* observed on PND 21 and PND 77 following 750-ppm IMI exposure might be involved in suppressing NPC proliferation at these ages.

Regarding the effect on glial cell populations in the present study, 750-ppm IMI increased numbers of GFAP+ astrocytes and Iba1+ microglia/macrophages, and upregulated transcript levels of their encoding genes (*Gfap* and *Aif1*) on PND 21. In the brain, microglial activation is heterogeneous and can be categorized into two opposing phenotypes: M1 pro-inflammatory and M2 anti-inflammatory.17,25 In the present study, numbers of CD68+ cells, representing activated M1- and M2-type microglia/macrophages, were increased with IMI exposure at ≥ 83 ppm on PND 21; whereas, the number of CD163+ M2-type microglia/macrophages was unchanged with IMI exposure at this age. These results suggest that maternal IMI exposure induced pro-inflammatory responses by activating M1-type microglia/macrophages at the end of exposure, even with the lowest dose. This finding is consistent with upregulation of *Il6* and *Tnf*, the two major pro-inflammatory cytokine genes induced by M1-type microglia,38 following exposure to 750-ppm IMI. Upregulation of interleukin (IL)-6 and TNF-α contributes to the reduced proliferation and differentiation of NPCs,39 suggesting that these factors may influence the observed suppression of NPC proliferation on PND 21. In contrast, we also observed upregulation of *Il4* and *Tgfb1* with 750-ppm IMI. TGF-β1 is an anti-inflammatory and neuroprotective cytokine that can be induced by activated astrocytes.40 IL-4 is also an anti-inflammatory cytokine that can induce neuroprotective activation of astrocytes, and these alternatively activated astrocytes may release IL-4, IL-10, and TGF-β.41 Therefore, although we observed microglia/macrophage populations polarized toward the M1 phenotype after exposure to 750-ppm IMI, the increase of GFAP+ astrocytes on PND 21 might be a neuroprotective response to the induced neuroinflammatory responses.

On PND 77 after cessation of IMI exposure, numbers of GFAP+ astrocytes and Iba1+ microglia/macrophages in the DG were restored to normal levels in the present study. Although the observed increase of CD68+ M1-type microglia/macrophages was sustained at 750 ppm, transcript levels of both pro-inflammatory (*Il1b*, *Il6*, *Tnf*) and anti-inflammatory (*Il10*, *Il4*, *Tgfb1*) cytokine genes were decreased or tended to decrease at this time point. This finding suggests that suppression of both pro-inflammatory and anti-inflammatory responses occurred, leading to a compromised immune system at the adult stage. In agreement with the present study results, immunosuppressive effects such as suppression of phagocytic activity, chemotaxis, and cytokine gene expression, as well as increased oxidative stress, have been reported after repeated IMI exposure in rodents.42,43 Moreover, developmental exposure of rats to IMI caused age-dependent suppressive effects on their developing immunity.44 Meanwhile, ACh and nicotine can inhibit lipopolysaccharide-induced TNF-α production by microglia through binding with microglial α7-nAChR as a main target receptor of IMI.45 Although the affinity of IMI to mammalian nAChR is much lower than that of insects, it has been demonstrated that IMI could bind to α7-nAChR of rat neurons to exert excitatory effects.39 Although the transcript level of *Chrna7* (encoding α7-nAChR) did not change in the DG on PND 21 or PND 77 in the present study following exposure to 750-ppm IMI, the transcript level of *Chat* (encoding choline *O*-acetyltransferase, an ACh-synthesizing enzyme) was increased on PND 21. In addition, AChE activity was persistently suppressed in the hippocampus with this exposure, suggesting sustained accumulation of ACh. We speculate that sustained ACh accumulation after developmental IMI exposure might suppress cytokine expression on PND 77.

Interestingly, the antioxidant system in the hippocampal DG underwent the same shift as the immune system in male offspring after developmental IMI exposure at 750 ppm in this study. On PND 21, IMI upregulated expression levels of oxidative stress-related genes *Nfe2l2*, *Hmox1*, *Mt1*, *Mt2a*, and *Gpx4*. Metallothionein-I/II (encoded by *Mt1* and *Mt2a*) and glutathione peroxidase 4 (encoded by *Gpx4*) can respond to oxidative stress by scavenging free radicals and preventing their formation.46,47 Upregulation of these genes indicates operation of the anti-oxidant system to prevent MDA accumulation in the hippocampus on PND 21. In contrast, hippocampal MDA levels were increased and transcript levels of *Nfe2l2*, *Hmox1*, and *Gpx1* were decreased in the DG on PND 77, suggesting increased vulnerability to oxidative damage in the hippocampus due to suppression of antioxidant capacity. Among them, *Nfe2l2* encodes NFE2-like bZIP transcription factor 2 (Nrf2), a transcription factor extremely sensitive to oxidative stress that controls the expression of various antioxidant enzymes and proteins responsible for neuroprotection against oxidative stress.48 *Hmox1* is one of the important downstream genes regulated by Nrf2.48 Under various stimuli, upregulated heme oxygenase 1 (encoded by *Hmox1*) participates in not only protection against oxidative injury but also biological processes including anti-apoptotic responses, regulation of proliferation, and modulation of inflammation.48 There is no doubt that downregulation of *Nfe2l2*, *Hmox1*, and *Gpx1* is key to causing MDA accumulation in the present study. In addition, we found that numbers of TUNEL+ apoptotic cells were increased in the SGZ, accompanying downregulation of *Bcl2l1* on PND 77. *Bcl2l1* encodes BCL2-like 1, an anti-apoptotic protein responsible for protecting mitochondria from pro-apoptotic proteins.49 Oxidative stress induces mitochondrial dysfunction and predisposes cells to apoptosis. However, it has been demonstrated that deficiency of *Hmox1* increases the sensitivity of cells to oxidative stress-induced apoptosis.50 Expression of *Bcl2l1* decreases with increasing oxidative stress.51 Therefore, it is reasonable that IMI induced apoptosis in type-1 NSCs by inhibiting the mitochondrial anti-apoptotic protein BCL2-like 1 through excess production of reactive oxygen species and suppression of the antioxidant pathway. These results are consistent with a previous report showing that IMI administration to rats reduced antioxidant capacity in the brain and decreased expression of anti-apoptotic *Bcl2*.2

AChE is a critical component of the cholinergic system and disturbances in its homeostasis always lead to deficits in behaviors.52 Attention-deficit/hyperactivity disorder (ADHD) is one of the most common neurodevelopmental disorders and hyperactivity is a core feature, referring to excessive motor activity and difficulty staying still.53 Although the etiology of ADHD is not yet known, evidence suggests that dysregulation of the neonatal cholinergic system is a sufficient factor for the development of ADHD.54 For example, maternal nicotine exposure leads to the development of ADHD symptoms in adolescents, resulting from alterations in the ACh pathway.55 Consistent with the present study results, administration of IMI to pregnant mice increased the locomotor activity of offspring in adulthood.3 In addition, we found that following exposure to 750-ppm IMI, adult offspring had higher moving speeds and a trend for longer distances in open field tests, accompanied by sustained suppression of AChE activity and decreased *Chrnb2* expression. It is therefore speculated that disruption of the cholinergic system by IMI triggered hyperactivity in adult offspring. Furthermore, imbalances in oxidative stress status and disturbances of the immune system have been associated with ADHD.56 Therefore, further study is necessary to identify the specific mechanisms of IMI-induced hyperactivity in offspring.

According to a previous DNT study in rats, the no-observed-adverse-effect-level (NOAEL) of IMI is 20 mg/kg BW/day (during gestation), based on the reduction of preweaning body weight gain and decreased motor/locomotor activity at 80 mg/kg BW/day.57 In the present study, we determined the NOAEL of IMI for offspring neurogenesis after developmental exposure to be 83 ppm, equivalent to a maternal exposure of 5.5–14.1 mg/kg BW/day in rats, based on decreased numbers of NSCs and post-mitotic granule cells in adulthood at 250 ppm. According to a report detecting IMI in well water in California, the highest IMI residue was 5.97 ppb and detection levels higher than 283 ppb might be considered a health concern.58 Therefore, doses used to cause developmental neurotoxicity in experiments of the present study are not generally available in life.

1. **Conclusion**

Obtained results suggest that maternal IMI exposure suppresses hippocampal neurogenesis in offspring by targeting late-stage differentiation and ERK1/2–FOS-mediated synaptic plasticity of granule cells during exposure. Reductions of reelin signaling might be responsible for the observed suppression of neurogenesis and synaptic plasticity. At the adult stage, IMI bidirectionally decreased NSC and mature granule cell populations. Behavioral tests reveal increased spontaneous activity in adulthood. Maternal IMI exposure also persistently affected cholinergic signaling and induced both neuroinflammation and oxidative stress during exposure. Subsequently increased sensitivity to oxidative stress in the adult hippocampus might be responsible for the progressive suppression of neurogenesis and increased incidence of hyperactivity observed in adulthood. The NOAEL of IMI for offspring during neurogenesis was determined to be 83 ppm (5.5–14.1 mg/kg body weight/day).

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