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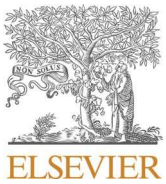

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Analysis of two guideline-compliant developmental neurotoxicity studies with imidacloprid to assist the interpretation of findings that impact global registrations

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ABSTRACT

Imidacloprid is a neonicotinoid insecticide that was first registered in the early 1990's, with global registrations that include agricultural, residential and veterinary uses. In 2001, Bayer reported a developmental neurotoxicity (DNT) study with imidacloprid that complied with U.S. EPA and OECD guidelines to support global registrations. The report concluded that there were slight effects in the offspring at the highest dietary level related to general or acute (neuro)-toxicity and no evidence of DNT at any dose; however, the European Food Safety Authority (EFSA) questioned certain findings at the high dose and whether the study established a clear NOAEL. More recently, the Jai Research Foundation (JRF) independently conducted a guideline-compliant DNT study with imidacloprid for other registrants, with elements incorporated into the study design to facilitate comparison with the Bayer study that could address these issues. This paper examines the findings from both studies, in the context of an updated literature review, to address regulatory uncertainty and persistent claims from non-governmental organizations that imidacloprid is a developmental neurotoxicant. The present analysis supports the interpretation that differences in brain measurements at the high dose in the Bayer study were incidental and unrelated to treatment and that imidacloprid is not a developmental neurotoxicant.

1. Introduction

Imidacloprid (CAS 138261-41-3) was the first neonicotinoid insecticide (neonic) registered for commercial uses and is the most important representative of the class (Sheets et al., 2016). Neonics are registered for a variety of agricultural, commercial, residential and veterinary uses, including the control of mosquitos that transmit malaria and other vector-borne diseases. The importance of neonics is due to their efficacy for various applications, favorable safety profiles, the restrictions placed on other insecticides and their use for resistance management (Jeschke et al., 2011; Simon-Delso et al., 2015).

The insecticidal activity of neonics is attributed to stimulation of postsynaptic nicotinic acetylcholine receptors (nAChR) in insects (Casida, 2018; Tomizawa and Casida, 2005). While related to nicotine, the neonics are much less toxic to vertebrate species, with lower activity for the nAChR isoforms expressed in vertebrate species than the isoform expressed in insects, rapid metabolism and poor penetration of the blood-brain barrier (Chao and Casida, 1997; Yamamoto and Casida, 1999; Tomizawa and Casida, 2003, 2005). Nevertheless, imidacloprid and other neonics have been thoroughly investigated for adverse effects on the developing nervous system, based on a nicotinic mode of insecticidal action, acute neurotoxic effects in mammals at high doses and recognition of nicotine as a developmental neurotoxicant in humans and laboratory animals (Slikker et al., 2005; Sheets, 2014; Sheets et al., 2016).

Imidacloprid has been evaluated for DNT (Sheets and Lake, 2001)

Abbreviations: DNT, (Developmental neurotoxicity); U.S. EPA, (United States Environmental Protection Agency); OECD, (Organization for Economic Co-operation and Development); EFSA, (European Food Safety Authority); GLP, (Good Laboratory Practice); NOAEL, (No-Observed Adverse Effect Level); nAChR, (nicotinic acetylcholine receptor).

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and other evidence of developmental and reproductive toxicity, using a full complement of studies conducted in accordance with U.S. EPA and OECD guidelines and Good Laboratory Practice (GLP) standards (U.S. EPA, 2017, U.S. EPA, 2019). Such guideline- and GLP-compliant studies are designed to provide information suited for hazard identification and quantitative risk assessments, with an appropriate sample size, complement of tests and range of defined doses, administered by a route that is relevant to human circumstances of exposure (Raffaele et al., 2010). Furthermore, guideline-compliant DNT studies include endpoints associated with the effects of nicotine in developing rodents, including measures of motor activity, cognitive function and a thorough microscopic examination of the brain (Slikker et al., 2005).

The DNT study reported by Bayer has been evaluated by regulatory agencies around the world and the principal elements of study design and associated findings were published in a comprehensive review of neonics for evidence of DNT (Sheets et al., 2016). This review examined the results from guideline-compliant DNT studies with imidacloprid and five other neonics, in the context of the published *in vitro*, *in vivo* and epidemiology studies, and concluded there were no common effects that are consistent with DNT or neurodevelopmental effects associated with nicotine or smoking during pregnancy.

The present paper provides a comprehensive analysis of the Bayer study (Sheets and Lake, 2001) and a DNT study that was conducted more recently with imidacloprid at JRF laboratory (Patel, 2010). While the review by Sheets et al. (2016) concluded imidacloprid does not affect neurodevelopment, EFSA cited uncertainty for a measure of caudate-putamen width in high-dose females in the Bayer study that prevented them from making a firm conclusion (EFSA, 2013). A contributing factor to the uncertainty was the absence of comparable brain measurements at lower doses to assist interpretation. The present paper examines the collective results from both guideline-compliant DNT studies, in the context of an updated literature review, to assist the interpretation and support global registrations.

2. Literature review

A literature search using Chemical Abstracts, Medline, CABA, BIOSIS, EMBASE, SciSearch, Agricola, FSTA, PQSciTech, IPA and TOXCENTER was performed on January 15, 2025 to gather the relevant *in vitro, in vivo* and epidemiology studies published during the last 10 years that examined imidacloprid for effects on the developing nervous system. This search used the same terms included in the search performed on June 16, 2015 (Sheets et al., 2016), with the focus on imidacloprid. After removal of duplicates, 254 citations were identified and screened for relevance, considering the following criteria. For *in vivo* studies, original research publications were included if they involved a mammalian test model exposed to imidacloprid during gestation and/or lactation and the study measured neurodevelopmental endpoints. *In vitro* studies were included if the cells were from mammals, represented immature neural tissue and the findings were linked to DNT outcomes. *In vivo* and *in vitro* studies that described mixture exposures (e.g. commercial formulations), conference abstracts and papers not available in English were excluded. Epidemiology studies were included if the study examined exposure to imidacloprid during pregnancy or early childhood development and neurodevelopmental outcome. Studies that satisfied this relevance assessment were evaluated for reliability using a customized risk of bias (RoB) tool based on criteria from the ToxRTool (Schneider et al., 2009) and the National Toxicology Program (NTP) Office of Health Assessment and Translation (OHAT) RoB tool (NTP, 2019).[1] The outcome of this assessment, including the reliability conclusion and the main reasons for non-inclusion of studies with a high RoB, is summarized in Supplementary Material 1.

3. Materials and methods

The first DNT study with imidacloprid was conducted at Bayer's toxicology laboratory in the United States (Sheets and Lake, 2001) and the second was conducted at the JRF laboratory in India (Patel, 2010). Both labs are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the studies were conducted in compliance with OECD and U.S. EPA Principles for GLP and in accordance with OECD (TG 426) and U.S. EPA (OCCSP 870.6300) test guidelines and studies with reference chemicals (positive controls) were performed to support the sensitivity and reliability of the associated test methods (Table 1). Additional elements were included in both studies to assist interpretation and the JRF study incorporated critical design elements that were consistent with the Bayer study to facilitate comparison, including the same dietary concentrations, initi-

Table 1
Comparison of guideline developmental neurotoxicity study designs.

	Bayer Study[a]	JRF Study[b]
Rat Strain	Wistar (CRL:W(HAN)BR) (Charles River, US)	Wistar (HsdHAN: WIST) (JRF breeding facility, India)
Technical-grade Imidacloprid	98.4 % purity	99.42 % purity
Dietary Levels (minimum 20 litters/ dietary level)	0, 100, 250, 750 ppm	0, 100, 250, 750 ppm
Treatment Duration	GD 0 – LD 21	GD 0 - LD 21
Detailed Clinical Observations: Dams	GD 6, 13, 20; LD 4, 11, 21	GD 12, 18; LD 3, LD 9
Behavioral Ontogeny	Surface righting, response to acoustic stimulus, pupil response	Surface and air righting, negative geotaxis, wire maneuver, distance traveled
Physical Developmental Landmarks	Eye opening	Eye opening, pinna unfolding, incisor eruption and hair growth
Sexual Maturation	Daily, beginning PND 29 (F) and PND 38 (M)	Daily, beginning after weaning (PND 21)
Detailed Clinical Observations: Pups	PND 4, 11, 21, 35, 45, 60	PND 4, 11, 21, 35, 45, 60
Motor Activity	PND 13, 17, 21, 60 (16/ sex/dose) Figure-8 maze	PND 13, 17, 21, 60 (20/sex/ dose) Photobeam activity system
Auditory Startle Habituation	PND 22, 38 and 60 (16/ sex/dose)	PND 23 and 61 (20/sex/ dose)
Learning and Memory	PND 22/29: Passive Avoidance; PND 60/67: M-water maze (16/sex/ dose)	PND 24/28 and PND 62/66 (Morris water maze at both life stages) (20/sex/dose)
Microscopic Pathology and Brain Measurements	PND 11 and 75 (±5 days) (10/sex/dose at each age)	PND 11 and 70 (±5 days) (10/sex/dose at each age)

[a] Bayer Study (Sheets and Lake, 2001).
[b] JRF Study (Patel, 2010); PND (postnatal day), LD (lactation day), GD (gestation day), M-male, F-female.

[1] Methodology as applied by EFSA in their assessment of the insecticide, flupyradifurone (EFSA, 2022), and re-evaluation of starch sodium octenyl succinate (E 1450) as a food additive (EFSA, 2020). SciRAP sub-questions consistent with approaches utilized by EFSA were added to allow for a more granular assessment (https://ki.se/en/imm/scirap-science-in-risk-assessment-and-policy/scirap-tools).

ation of treatment on gestation day (GD) 0 vs GD 6 and collection of brain tissues on postnatal day (PND) 11 vs PND 21, based on the U.S. EPA guideline that was in force when the Bayer study was conducted and before the OECD guideline was finalized. Differences in the assessment of behavioral ontogeny and physical landmarks and the test devices and procedures used to evaluate the offspring are considered in the analysis.

3.1. Housing

Animal rooms were maintained on a 12h:12h light:dark cycle, with controlled temperature (19–25 °C) and humidity (30–70 %). Animals were provided rat pellet feed *ad libitum* and an unlimited supply of drinking water throughout the study. Feed samples were analyzed to verify homogeneity and stability of imidacloprid in the diet and to measure the dosage (mg imidacloprid/kg bwt/day) throughout gestation and lactation.

3.2. Parental (F0) generation

Mating was accomplished in both studies by co-housing one nulliparous female (minimum 12 weeks of age) with one adult male, with the day sperm was observed in the vaginal smear designated GD 0. On GD 0, females were randomly assigned to one of four dose groups and placed into individual cages with bedding material, while males were euthanized or allocated for other uses.

3.3. Reproduction and litter parameters

F0 females were evaluated daily for evidence of delivery from GD 20 to the completion of delivery, which was designated lactation day (LD) 0 for the dam and PND 0 for the pups. Reproductive indices, including litter size and the "status" of pups at birth were recorded. On LD 4, litter size was standardized by random selection to yield, as near as possible, four males and four females per dam. At that time, one male and/or one female from each litter was randomly assigned to cohorts for neurobehavioral testing and one male or one female/litter (representing 20 litters) was assigned for neuropathology and brain morphometry on PND 11 or PND 70–75 (±5 days). Dams with litters that were used for postnatal assessment were euthanized when the pups were weaned on LD 21.

3.4. F1-generation

Surviving male and female offspring were observed (cage-side) for clinical signs at least once daily and detailed observations were performed once daily during the preweaning period and once weekly thereafter. Surviving pups were weighed on PND 0, 4 and at least once weekly thereafter until termination, as well as at the attainment of vaginal patency or preputial separation. Food consumption was measured weekly, from the time pups were placed into single housing until termination. F1 males and females from each litter were examined daily for the attainment of physical landmarks and reflex-based behaviors (Table 1).

3.5. Neurobehavioral assessment

The rooms used for neurobehavioral testing maintained the same conditions as the room where animals were housed in both studies. The order of testing and assignment of animals to specific devices was semi-random, to ensure groups were balanced across the devices and test sessions. Details of the test devices and procedures used to assess motor activity, acoustic startle habituation and learning and memory are provided in Supplementary Material 2.

3.6. Termination/gross pathology

Pups selected for microscopic examination and brain measurements on PND 11 or PND 75 (±5 days) (10/sex (representing 20 litters)/dose at each age) were euthanized and underwent a gross necropsy examination. On PND 11, brains were collected from non-perfused (Bayer) or perfused (JRF) animals, with additional tissues (spinal cord & peripheral nerves) collected in the JRF study. Both methods are well suited to preserve tissues for histopathologic and morphometric analysis (Garman et al., 2015); however, this difference could have an impact on the actual values and, therefore, limits the comparison of brain measurements on PND 11 between the studies. On PND 75 (±5 days), tissues were collected in both studies following *in situ* perfusion fixation using 10 % buffered formalin. For PND 11 (JRF) and PND 70–75 (±5 days) (both labs) offspring, brain, spinal cord, eyes, sciatic nerve and gastrocnemius (Bayer) or biceps femoris (JRF) were collected and stored in 10 % formalin.

3.7. Histology

The brains from PND 11 control and high-dose animals, and all tissues collected from control and high-dose animals on PND 70–75 (±5 days), were processed for microscopic examination and brain measurements in both studies. Sections of the brain, spinal cord, eyes, optic nerves and skeletal muscle from control and high-dose animals were embedded in paraffin and examined utilizing hematoxylin and eosin (H&E) stain. Dorsal root ganglia, gasserian ganglia and peripheral nerves were embedded in glycol methacrylate (GMA), sectioned at 2–3 μm and stained using a modified Lee's stain. Tissues from low- and mid-dose animals remained in formalin unless the study scientists determined that microscopic examination was warranted, based on findings at the high dose.

3.8. Brain measurements

After gross measurements of cerebrum and cerebellum were taken, brains were divided into eight (Bayer) or six (JRF) coronal sections for microscopic examination. These sections were processed according to standard procedures for paraffin embedding, sectioned at 5 μm and stained with H&E and/or luxol fast blue/cresyl violet stains. Microscopic measurements in both studies included frontal cortex thickness, corpus callosum width, caudate putamen width and hippocampal gyrus thickness. In addition, Bayer measured the thickness of the external germinal layer of the cerebellum from PND 11 animals and JRF measured olfactory lobe thickness and width, thalamus diameter and medulla oblongata height and width at both life stages. Additional details and photomicrographs that illustrate these measurements are provided in Supplementary Material 3. Measurements were taken for control and high-dose animals, with measures taken at the next lower dose if there was a treatment-related difference from control.

3.9. Statistical analysis

In general, continuous data were initially assessed for equality of variance using Bartlett's test. Group means with equal variances were analyzed using analysis of variance (ANOVA), followed by a Dunnett's test. In the event of unequal variances, these data were analyzed by Kruskal-Wallis ANOVA followed by Mann-Whitney *U* test. Micropathology frequency data were evaluated using a Chi-Square procedure, followed by a one-tailed Fischer's Exact test. Additional statistical tests to assess continuous and frequency data were applied when deemed appropriate. Additional details of the statistics are provided in Supplementary Material 4.

4. Results

4.1. Dietary concentration and stability analysis

The homogeneity and stability of imidacloprid in the diet was confirmed in both studies by analysis of feed samples. Measures of imidacloprid concentration (ppm) in the diet and maternal body weight and feed consumption confirmed the animals received comparable doses (mg imidacloprid/kg body weight/day) in the two studies throughout gestation and lactation (Supplementary Material 5). Based on these results, nominal dietary concentrations of 0, 100, 250 and 750 ppm imidacloprid resulted in mean analytically confirmed doses in the Bayer study of 8, 20 and 57 mg/kg/day, respectively, during gestation, and 17, 39 and 121 mg/kg/day, respectively, during lactation. The higher doses during lactation reflect increased consumption of the treated diet by the dam and offspring during lactation. By comparison, the mean analytically confirmed doses in the JRF study during gestation and lactation were 10, 23 and 60 mg/kg/day and 18, 43 and 123 mg/kg/day, respectively.

4.2. P-generation females

No treatment-related clinicals signs or mortality were noted in either study at any dietary level. In both studies, food consumption was decreased during gestation and lactation at the high dose, but not at lower doses, with a greater reduction in the JRF study (max. 36 % and 15 %, respectively) than the Bayer study (max. 9 % and 14 %, respectively). Decreased food consumption at the high dose was associated with a maximum 6–7 % decrease in body weight during gestation (Supplementary Material 6) and lactation (Supplementary Material 7) in the JRF study and no effect on body weight in the Bayer study.

4.3. Reproduction and litter parameters

There was no treatment-related effect on any reproductive parameter in either study at any dietary level. The only litter parameter affected was decreased live birthweight for high-dose males (−7.8 %) and females (−6.6 %) in the JRF study, with a non-statistically significant 5 % mean lower birthweight for high-dose males and females, relative to controls, in the Bayer study (Supplementary Material 8).

4.4. F1 males and females

There were no treatment-related deaths or clinical signs at any dose and no difference from control for any developmental landmark or reflex-based behavior in either study (Supplementary Material 8). Body weight was significantly reduced for high-dose males and females on PND 4–21 in the Bayer study and PND 0–21 in the JRF study, with an average 11–13 % less than control on PND 4–21 in the Bayer study and an average 11–12 % less than control on PND 4 and a maximum 17–19 % less than control on PND 21 in the JRF study (Table 2). In the JRF study, pup body weight was also significantly less than control at the mid-dose on PND 17 (males) and PND 21 (males and females). The differences from control decreased after weaning in both studies, with term body weight for high-dose males and females 4 % and 1 % less than control, respectively, in the Bayer study and 6–7 % less than control for both sexes in the JRF study.

4.5. Motor activity

In the Bayer study, motor activity averaged 31–38 % less than control for high-dose F1 males and females on PND 17 and 37 % less than control for high-dose females on PND 21 (Fig. 1A and B). These differences from control were not statistically significant, but they were attributed to treatment based on the magnitude and consistent occurrence at the high dose in both sexes. There were no differences from control at any dose on PND 13 and PND 60 and no differences from control at lower doses at any age. In the JRF study, there was no treatment-related effect on motor activity at any dose or age (Fig. 1C and D).

4.6. Acoustic startle habituation

There were no treatment-related effects on the amplitude of the startle response or habituation in either study (Supplementary Material 9). In the Bayer study, the response amplitude for PND 60 females at 100 ppm (total session and intervals 2–5) and 250 ppm (interval 5 only) were statistically greater than controls; however, these differences from control are considered incidental and unrelated to treatment, based on the lack of dose-response or difference in males at any dose. In the JRF study, there were no statistical differences from control at any dietary

Table 2
Body weight (g) for F1 Males and Females from Birth until Weaning.

Bayer Study	Sex	Control	100 ppm	250 ppm	750 ppm
Birthweight[1]	M	5.9 ± 0.1	5.8 ± 0.1 (−1.7)	5.9 ± 0.1 (0)	5.8 ± 0.1 (−1.7)
	F	5.7 ± 0.1	5.5 ± 0.1 (−3.5)	5.6 ± 0.1 (−1.8)	5.5 ± 0.1 (−3.5)
PND 4	M	9.5 ± 0.2	9.4 ± 0.2 (−1.0)	9.4 ± 0.2 (−1.0)	8.6 ± 0.3* (−9.5)
	F	9.3 ± 0.2	9.0 ± 0.2 (−3.2)	9.2 ± 0.2 (−1.1)	8.2 ± 0.2** (−11.8)
PND 11	M	22.1 ± 0.5	22.5 ± 0.4 (+1.8)	22.1 ± 0.5 (0)	19.4 ± 0.5** (−12.2)
	F	21.6 ± 0.6	22.0 ± 0.4 (+1.9)	21.6 ± 0.5 (0)	18.7 ± 0.5** (−13.4)
PND 17	M	36.2 ± 0.7	36.7 ± 0.6 (+1.4)	36.0 ± 0.7 (−0.5)	31.6 ± 0.7**(-12.7)
	F	35.2 ± 0.7	35.7 ± 0.5 (+1.4)	35.1 ± 0.6 (−0.3)	30.9 ± 0.7** (−12.2)
PND 21	M	46.2 ± 1.0	46.4 ± 0.7 (+0.4)	45.2 ± 1.0 (−2.2)	41.1 ± 1.0** (−11.0)
	F	44.8 ± 1.1	45.1 ± 0.6 (+0.7)	44.0 ± 0.9 (−1.8)	40.0 ± 0.9** (−10.7)

[1]Mean ± SE (% difference from control); n = 23–25 litters; *p ≤ 0.05; **p ≤ 0.01.

JRF Study	Sex	Control	100 ppm	250 ppm	750 ppm
Birthweight[1]	M	6.4 ± 0.6	6.2 ± 0.3 (−3.9)	6.2 ± 0.4 (−3.9)	5.9 ± 0.5** (−7.5)
	F	6.1 ± 0.5	5.9 ± 0.5 (−3.3)	5.9 ± 0.4 (−3.3)	5.7 ± 0.5* (−6.6)
PND 4	M	11.0 ± 1.4	10.7 ± 0.9 (−2.7)	10.4 ± 0.8 (−5.5)	9.7 ± 1.2**(-11.8)
	F	10.7 ± 1.3	10.5 ± 1.1(-1.9)	10.0 ± 0.9 (−6.5)	9.5 ± 1.2** (−11.2)
PND 11	M	25.8 ± 2.4	25.5 ± 1.7 (−1.2)	24.7 ± 2.2 (−4.3)	21.8 ± 2.0** (−15.5)
	F	25.0 ± 1.9	25.0 ± 1.8 (0)	23.8 ± 2.0 (−4.8)	21.6 ± 2.2** (−13.6)
PND 17	M	42.1 ± 3.3	41.3 ± 2.7 (−1.9)	39.4 ± 3.9* (−6.4)	34.7 ± 3.5** (−17.6)
	F	39.6 ± 2.9	39.9 ± 3.2 (+0.8)	38.2 ± 3.7 (−3.5)	34.1 ± 3.4** (−13.9)
PND 21	M	56.1 ± 4.3	54.6 ± 3.4 (−2.7)	52.3 ± 4.0** (−6.8)	45.7 ± 4.7** (−18.5)
	F	52.6 ± 3.5	51.8 ± 3.9 (−1.5)	49.5 ± 3.7* (−5.9)	43.8 ± 4.6** (−16.7)

[1] Mean ± SD (% difference from control); n = 23–25 litters; *p ≤ 0.05; **p ≤ 0.01, M-males, F- females.

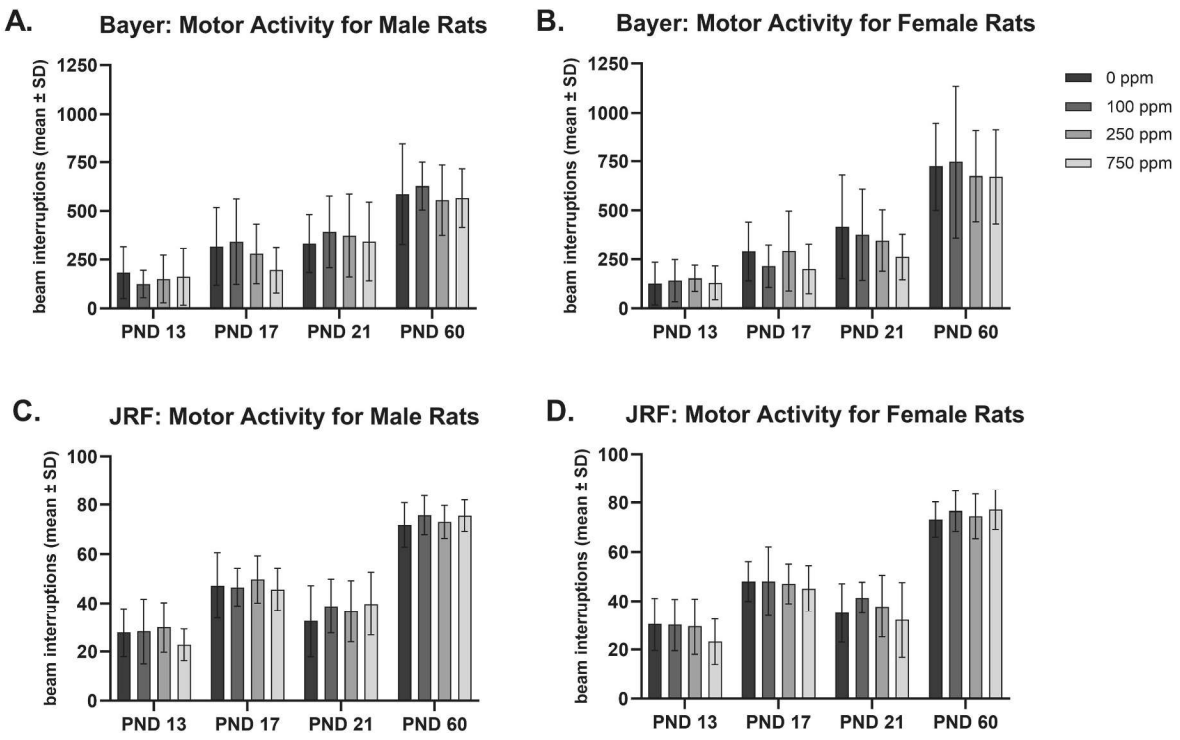

Fig. 1. Measures of motor activity from the Bayer study in F1 male (A) and female (B) rats and the JRF study in F1 male (C) and female (D) rats treated via the diet with imidacloprid from GD 0 to PND 21. The results are depicted as the sum of beam interruptions during a 60 min (Bayer) or 15 min (JRF) session on PND 13, 17, 21 and 60 ± 2 (Bayer study: n = 14–16/sex/dose; JRF study: n = 24–25/sex/dose; representing at least 20 litters/dose level in both studies).

level.

4.7. Learning and memory

Bayer study: There was no effect on measures of acquisition and retention in juvenile (passive avoidance) or adult (M-maze) males or females at any dietary level. All groups demonstrated learning by avoiding the aversive stimulus (foot shock), as shown by increased latency following Trial 1 of the acquisition phase and memory by increased latency for Trial 1 of the retention phase (Supplementary Material 10). In the M-maze, statistical differences from control were limited to the first trial of acquisition in males, with an increased number of errors (100 and 750 ppm) and duration to reach the goal (Supplementary Material 11). These findings are attributed to the exceptionally small number of errors and short duration of the first trial for the control males, as shown by comparing the results for control males (Trial 1 vs Trial 2) to control females.

JRF study: There was no evidence of a treatment-related effect on performance in the Morris water maze in juvenile or adult F1 males or females at any dietary level (Supplementary Material 12). Acquisition and retention were evident at all dietary levels by decreased total distance traveled and latency to locate the submerged platform with experience (days 1–5). The total distance traveled on the first test occasion was significantly less than control for high dose females on PND 24, but was not attributed to treatment, since there was no difference from control on subsequent test days and no difference in weanling males on any day.

4.8. Neuropathology and brain morphometry

There were no gross or microscopic lesions attributed to treatment in either study. In the Bayer study, absolute brain weight for low-dose females was significantly greater than control on PND 75 (±5 days) (Supplementary Material 13). This finding is not attributed to treatment, based on the occurrence at only the low dose and in only one sex. In the JRF study, absolute brain weight for high-dose females was significantly less than control on PND 11 (−8 %), while terminal body weight was significantly less than control in both sexes (−16 % and −17 %) and relative (to body weight) brain weight was increased (+14 % and +10 %) on PND 11 in both sexes. As such, the difference in absolute brain weight for PND 11 females and the increases in relative brain weight in both sexes are considered secondary to decreased body weight. On PND 70 (±5 days), there was no difference from control for absolute or relative brain weights in males or females at any dietary level.

For brain measurements, there were no differences from control that were attributed to treatment in either study, nor were there common differences from control for both studies. In the Bayer study, the thickness of the external germinal layer of the cerebellum was significantly less than control in PND 11 females (not measured in the JRF study) and the width of the caudate-putamen in high-dose females was less than control on PND 75 (±5 days) (Table 3a). This difference from controls is not attributed to treatment since the Study Pathologist noted there were considerable regional differences in thickness of the caudate-putamen associated with rapid development on PND 11 and there was no corresponding difference in males. The difference in caudate-putamen width was not attributed to treatment, based on the minimal difference from control (−1.9 %), lack of occurrence in males, and because the mean for the high-dose females (3.677 mm) was well within the range of values for control animals from Bayer's laboratory (mean range: 3.213–3.750 mm for 19 DNT studies), while the mean for concurrent controls in this study (3.750) was the highest value in the historical control database (Supplementary Material 14). In the JRF study, on PND 70 (±5 days) the corpus callosum width in males and females was greater than controls and the thickness of neocortex in females was greater than controls (Table 3b). The lack of consistency by gender for the neocortex and lack of consistency relative to the results in the Bayer study (corpus callosum) indicate these differences from control are incidental and unrelated to treatment.

Table 3a
Brain measurements (mm) in Control and High-Dose F1 Male and Female Rats on PND 11.

PND 11	Bayer Study						JRF Study					
	Males			Females			Males			Females		
	Control	750 ppm	%	Control	750 ppm	%	Control	750 ppm	%	Control	750 ppm	%
Brain weight (g)	1.333	1.346	+1.0	1.358	1.290	−5.0	1.09	1.05	−3.7	1.09	1.00**	−8.3
Cerebrum length	12.40	12.44	+0.3	12.53	12.16	−3.0	12.9	12.5	−3.1	12.8	12.4	−3.1
Cerebellum length	6.49	6.50	+0.2	6.39	6.40	+0.2	6.0	5.8	−3.3	6.0	5.9	−1.7
Frontal cortex	1.676	1.619	−3.4	1.658	1.590	−4.1	NM			NM		
Neocortex	NM			NM			1.192	1.267	+6.3	1.254	1.185	−5.5
Parietal cortex	1.646	1.655	+0.5	1.690	1.676	−0.8	NM			NM		
Caudate putamen	2.700	2.708	+0.3	2.769	2.617	−5.5	2.285	2.234	−2.2	2.262	2.200	−2.7
Corpus Callosum	0.537	0.534	−0.6	0.602	0.436	−27.6	0.377	0.400	+6.1	0.390	0.402	+3.1
Hippocampal gyrus	1.325	1.328	+0.2	1.393	1.336	−4.1	1.063	1.137	+7.0	1.155	1.097	−5.0
Thalamus	NM			NM			5.744	5.688	−1.0	5.922	5.417	−8.5
Cerebellum height	3.637	3.838	+5.5	3.834	3.885	+1.3	3.562	3.600	+1.1	3.717	3.771	+1.5
Cerebellum (ext. germinal layer)	0.092	0.088	−4.3	0.075	0.068*	−9.3	NM			NM		

NM = Not measured or measures are not comparable; % = Percent difference from control; *p ≤ 0.05; **p ≤ 0.01, n = 10/sex (20 litters)/group.

Table 3b
Brain measurements (mm) in Control and High-Dose F1 Male and Female rats on PND 70–75 (±5 days).

PND 70–75 (±5 days)	Bayer Study						JRF Study					
	Males			Females			Males			Females		
	Control	750 ppm	%	Control	750 ppm	%	Control	750 ppm	%	Control	750 ppm	%
Brain weight (g)	1.902	1.837	−3.4	1.737	1.742	+0.3	1.94	1.86	−4.1	1.82	1.79	−1.6
Cerebrum length	14.74	14.33	−2.8	14.36	14.24	−0.8	15.5	15.5	0.0	15.2	15.2	0.0
Cerebellum length	8.27	8.09	−2.2	8.29	8.14	−1.8	7.9	7.8	−1.3	7.8	7.8	0.0
Frontal cortex	1.968	1.993	+1.3	2.018	1.983	−1.7	NM			NM		
Neocortex	NM			NM			1.392	1.443	+3.7	1.348	1.544*	+14.5
Parietal cortex	1.985	2.021	+1.8	2.025	1.990	−1.7	NM			NM		
Caudate putamen	3.665	3.703	+1.0	3.75	3.677*	−1.9	3.080	3.116	+1.2	3.056	3.105	+1.6
Corpus Callosum	0.499	0.529	+6.0	0.536	0.498	−7.1	0.352	0.420*	+19.3	0.393	0.445*	+13.2
Hippocampal gyrus	1.871	1.719	−8.1	1.830	1.809	−1.1	1.534	1.550	+1.0	1.585	1.698	+7.1
Thalamus	NM			NM			7.755	8.182	+5.5	7.918	7.623	−3.7
Cerebellum height	5.688	5.507	−3.2	5.553	5.537	−0.3	5.757	5.773	+0.3	5.682	4.836	−14.9

NM = Not measured or no comparable measure; % = Percent difference from control; *p ≤ 0.05, n = 9–13/sex (20 litters)/group.

5. Discussion

The results for the two *in vivo* DNT studies with imidacloprid (Sheets and Lake, 2001; Patel, 2010) were very similar, despite being conducted in different laboratories approximately 10 years apart. Treatment-related effects for the dams and offspring in both studies were limited to the highest dietary concentration of 750 ppm, with no effect in either generation at 250 ppm. As such, the NOAEL for P- and F1-generations in both studies is approximately 20 mg/kg/day during gestation and 40 mg/kg/day during lactation.

No clinical signs of nicotinic activity, such as tremor, were evident in maternal animals or the offspring at any dose in either study. Furthermore, treatment-related effects in P-generation females were limited to decreased feed consumption at the high dose in both studies and an associated decrease in body weight gain at the high dose in the JRF study, with no effect on reproduction at any dose in either study. Treatment-related effects in F1 animals were also limited to the high dose, with decreased body weight in males and females in both studies and a slight decrease in motor activity in males and females during lactation in the Bayer study. There was no evidence of delayed development at any dose in either study and no treatment-related effects on cognition (passive avoidance, M-water maze or Morris water maze), neuropathology or brain measurements at any dose in either study.

The Bayer study identified a modest (non-statistical) decrease in motor activity in high-dose male (PND 17) and female (PND 17 and 21) offspring, without an effect on habituation or any latent or persistent effect on PND 60. Evidence of decreased activity occurred before weaning, when the offspring were consuming the treated diet as well as imidacloprid present in the milk, which is consistent with decreased activity as a sensitive measure of acute toxicity with imidacloprid in adult rats (Sheets, 2014). The lack of a corresponding effect on motor activity in the JRF study could be explained by the use of a different test device in that study (open field vs. figure-8 maze) and the relatively small differences from control in the Bayer study, which were not statistically significant.

Both DNT studies included an extensive microscopic evaluation of the brain, spinal cord and other tissues for pathology, along with gross and microscopic measurements of several brain regions. This included brain regions that are associated with morphologic effects in rats following exposure to nicotine during development (hippocampus and somatosensory cortex) or that are rich in nAChR (cerebellum, hippocampus, basal ganglia and thalamus) (Dwyer et al., 2009; Roy et al., 2002; Court et al., 2000). Neither DNT study found evidence of neuropathology in any tissue.

Quantitative morphometric and structural abnormalities have been reported in the hippocampus, cerebral cortex and nucleus accumbens of rodents following gestational exposure to nicotine (Roy et al., 2002). By comparison, there was no consistent difference in these or any other brain measurement in either DNT study with imidacloprid. In the Bayer study, the decreased caudate-putamen width in high-dose females on PND 75 (±5 days), relative to controls, was perceived as problematic for risk assessment purposes, because it was not feasible to take corresponding measurements at lower doses to assist interpretation and establish a clear NOAEL. This was because tissues from low- and mid-dose pups were subject to a shrinkage artifact from storage in formalin for an extended period before those measurements were requested. This case supports the recommendation to embed tissues collected for morphometric analysis in appropriate media at all dose

levels at the same time to avoid such issues (Li et al., 2017). For quantitative risk assessment, the U.S.EPA ultimately concluded a measurable difference would not be expected to occur at the next lower dose, given the minimal (−1.9 %) difference from control at the high dose and considering that the next lower dose was 3-fold lower than the high dose (US EPA, 2017). In the JRF study, there was no difference in caudate-putamen width in males or females, compared to controls, at either age but there was a statistically significant increase in corpus callosum width (both sexes) and neocortex thickness (males) on PND 70 (±5 days). The collective results indicate the differences from control in the Bayer study [decreased caudate-putamen width in females on PND 75 (±5 days)] and in the JRF study [increased corpus callosum width (both sexes) and neocortex (males) on PND 70 (±5 days)] are incidental and unrelated to treatment, as no consistent changes were observed across tissues or sex between the two studies.

Differences in brain measurements, in the absence of decreased brain weight or neuropathology, are problematic to interpret, as they may represent biological variability or variability associated with the methods used to collect homologous sections and measurements, rather than a treatment-related effect on brain development (Li et al., 2017). Variability can be associated with methods for processing tissues and defining anatomic landmarks as anchor points for selection of homologous sections and morphometry measurements (Garman et al., 2015). To assist the interpretation, it is useful to determine whether differences from control are consistent (e.g., by age and gender) and to compare measures for control and treated animals to the laboratory's historical control database. In the present case, the collective evidence indicates there was no effect on any brain measurement in either study.

Sass et al. (2024) claims to report "the first comprehensive assessment of unpublished rodent DNT studies on five neonicotinoids that were submitted to the U.S. EPA by neonicotinoid manufacturers" and concluded "Statistically significant shrinkage of brain tissue was observed in high-dose offspring for five neonics: acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam." In fact, Sheets et al. (2016) provided a comprehensive review of these guideline DNT studies with these neonics and noted the laboratory's and EPA's interpretation of the findings show no consistent pattern of decreased brain measurements across the class, while there were various isolated measurements that were either less than or greater than control with other neonics, in only one sex and generally at the juvenile or adult life stage. Such inconsistency supports the conclusion that these differences from control are incidental and unrelated to treatment. The brain measurements from the second DNT study with imidacloprid (Patel, 2010) also supports this conclusion.

The updated literature review identified one *in vivo* and two *in vitro* studies since the review reported by Sheets et al. (2016) that were considered relevant for the assessment of the DNT potential of imidacloprid and satisfied acceptance criteria for reliability. Studies that failed to satisfy acceptance criteria for relevance and reliability were not considered in this assessment, regardless of the reported findings. The *in vivo* study by Zou et al. (2024) reported maternal exposure to imidacloprid from GD 6 to LD 21 persistently affected cholinergic signaling, induced neuroinflammation and oxidative stress during exposure and increased sensitivity to oxidative stress in the hippocampus of the offspring, causing hyperactivity and progressive suppression of neurogenesis in adulthood at a dietary level of 750 ppm. These findings are not supported by the results of the present studies, which showed no morphological changes in the hippocampus or hyperactivity in the offspring under very similar circumstances of exposure. The two *in vitro* publications reported no biologically relevant interaction of imidacloprid with neuronal development. Although imidacloprid showed a slight dose-dependent inhibitory effect on neurite outgrowth at a high concentration in PC12 cells, it was not statistically significant, and the authors concluded that, based on their model, neonics in general are not identified as potential neurodevelopmental toxicants (Christen et al., 2017). In the neural network formation (NNF) assay, imidacloprid altered only one of 17 parameters (Frank et al., 2017). Considering that most of the NNF parameters are highly correlated, the change in only one parameter is of questionable biological relevance. Moreover, it is unclear whether the study design can distinguish between transient acute neurotoxic effects and DNT, as there was no wash-out of test substances before the measurements were collected. Interestingly, the lack of *in vitro* bioactivity of imidacloprid in both the NNF assay and an acute multi-electrode assay was recently reported by the same group (Martin et al., 2024).

These results are in alignment with the comprehensive DNT screening assessment by Masjosthusmann et al. (2020), in which imidacloprid was compared with two other neonics and nicotine in a battery of 17 *in vitro* DNT assays. The results from this assessment showed no common pattern of effects, which indicates individual neonics might act via mechanisms other than nAChR modulation on distinct neurodevelopmental endpoints. Imidacloprid showed specific activity in only two assays (oligodendrocyte differentiation and NNF) at around 10 μM. In this context, it is noted that approximately 40 % of the pesticides tested were positive in the oligodendrocyte differentiation assay and that there were no microscopic changes in myelination/white matter evident in the Guideline DNT studies. Furthermore, imidacloprid was no longer identified as specific hit in the oligodendrocyte differentiation assay when the same data set was re-analyzed with an improved data analysis pipeline (Blum et al., 2023).

More recently, an OECD integrated approaches to testing and assessment case study (OECD, 2022) examined imidacloprid and desnitro-imidacloprid for effects in a battery of *in vitro* DNT tests anchored to a postulated adverse outcome pathway, as well as nuclear receptor activation. *In vitro* toxicodynamic data were also contextualized with internal exposure predictions by physiologically based pharmacokinetic modelling and the authors confirmed imidacloprid has similar but less activity on nAChRs mediated Ca^{2+} influx in neuronal systems compared to nicotine. However, further testing established that imidacloprid/desnitro-imidacloprid and nicotine did not affect DNT-specific endpoints *in vitro*, nor did they show agonism or antagonism on various nuclear receptors or activate stress pathways. The authors concluded that significant uncertainties remain to relate the modulation of the nAChR activity to an adverse DNT outcome. Moreover, reverse modelling revealed that a brain concentration of ~1 μM imidacloprid would be reached after an intake of 0.16 mg/kg body weight in adults or children (OECD, 2022). Considering the authority set the ADI of 0.06 mg/kg/day, the *in vitro* point of departures derived in most *in vitro* studies are considered non-relevant for human exposure.

For the epidemiology literature, only one of 17 studies published since the review conducted by Sheets et al. (2016) examined prenatal and childhood exposure and neurodevelopment and concluded childhood exposure to neonics may affect neurodevelopment. This outcome was not specific to imidacloprid and only applied to boys; as such, the authors concluded a further study, with a larger sample size, is required to confirm a gender-specific difference in outcome (Wang et al., 2024). The authors of this study in a young Taiwanese cohort also concluded that exposure to neonics during the third trimester does not affect neurodevelopment. Three additional epidemiology studies concluded there was either 1) no consistent association with prenatal proximity to agricultural areas that reported having used imidacloprid and neurodevelopment (Gunier et al., 2022) or 2) associations of use with fewer maternal- and youth-reported behavioral and emotional problems (Zahid et al., 2024; Hyland et al., 2021). As such, the epidemiology literature does not support an association between exposure to imidacloprid and adverse effects on neurodevelopment.

6. Conclusions

The collective evidence from DNT studies conducted at Bayer and JRF laboratories that comply with U.S. EPA and OECD test guidelines, and our assessment of the published literature, supports the conclusion

that imidacloprid is not a developmental neurotoxicant, as it does not disrupt neurodevelopment in mammals. Furthermore, the JRF study addresses regulatory uncertainty associated with the Bayer study and supports a NOAEL of 250 ppm (20 mg/kg/day), based on decreased food consumption and body weight gain in maternal animals and decreased body weight, body weight gain and motor activity in F1-males and females at 750 ppm (57 mg/kg/day), with no morphological effect on the brain at the highest dietary level.

CRediT authorship contribution statement

L.P. Sheets: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **M. Patel:** Writing – review & editing, Methodology, Investigation. **L. Zorrilla:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **K. Bothe:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2026.106040.

Data availability

Data will be made available on request.

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