

REVIEW ARTICLE

## A critical review of neonicotinoid insecticides for developmental neurotoxicity

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### ABSTRACT

A comprehensive review of published and previously unpublished studies was performed to evaluate the neonicotinoid insecticides for evidence of developmental neurotoxicity (DNT). These insecticides have favorable safety profiles, due to their preferential affinity for nicotinic receptor (nAChR) subtypes in insects, poor penetration of the mammalian blood–brain barrier, and low application rates. Nevertheless, examination of this issue is warranted, due to their insecticidal mode of action and potential exposure with agricultural and residential uses. This review identified *in vitro*, *in vivo*, and epidemiology studies in the literature and studies performed in rats in accordance with GLP standards and EPA guidelines with imidacloprid, acetamiprid, thiacloprid, clothianidin, thiamethoxam, and dinotefuran, which are all the neonicotinoids currently registered in major markets. For the guideline-based studies, treatment was administered via the diet or gavage to primiparous female rats at three dose levels, plus a vehicle control ( $\geq 20$ /dose level), from gestation day 0 or 6 to lactation day 21. F1 males and females were evaluated using measures of motor activity, acoustic startle response, cognition, brain morphometry, and neuropathology. The principal effects in F1 animals were associated with decreased body weight (delayed sexual maturation, decreased brain weight, and morphometric measurements) and acute toxicity (decreased activity during exposure) at high doses, without neuropathology or impaired cognition. No common effects were identified among the neonicotinoids that were consistent with DNT or the neurodevelopmental effects associated with nicotine. Findings at high doses were associated with evidence of systemic toxicity, which indicates that these insecticides do not selectively affect the developing nervous system.

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## Introduction

The neonicotinoids are a commercially important class of insecticides, with increased usage in recent years due to their favorable safety profiles, the restrictions placed on other insecticides and their utility for resistance management (Jeschke et al. 2011; Simon-Delso et al. 2015). Representatives of this class are used to control insect pests in a variety of agricultural, commercial, residential, and veterinary settings, with insecticidal activity attributed to the activation of post-synaptic nicotinic acetylcholine receptors (nAChR) in insects (Buckingham et al. 1997; Nagata et al. 1998). Neonicotinoids registered in major markets are acetamiprid, imidacloprid, thiacloprid, clothianidin, thiamethoxam, and dinotefuran. These six neonicotinoids have distinct structural features compared with nicotine that result in enhanced selectivity for insect nAChRs (see Table 1 for a comparison of structures). Also, the amino nitrogen atom in nicotine is ionized, while in the neonicotinoids, the corresponding nitrogen atom is not ionized but bears a partial positive charge (Yamamoto et al. 1998). Given their mode of insecticidal activity and that nicotine is neurotoxic and generally recognized as a developmental neurotoxicant (Levin & Slotkin 1998; Ernst et al. 2001), it is important to understand the neurotoxic potential of neonicotinoids in humans at all life stages and ensure the information used for risk assessments is suited to protect infants and children.

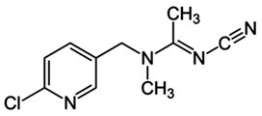
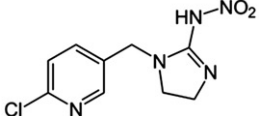
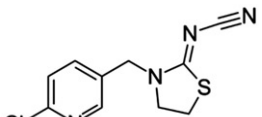
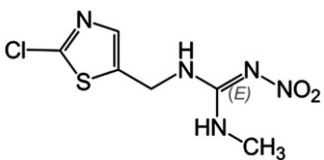
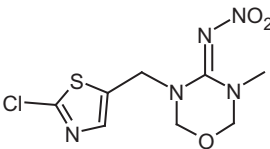
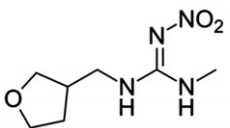
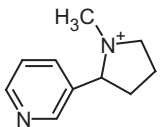
Yamamoto and Casida (1999) provide the most complete set of published mammalian toxicology information for the neonicotinoids, with chapters on imidacloprid, acetamiprid, thiamethoxam, and nitenpyram. These resource and studies cited by Chao & Casida (1997) and Sheets (2014) report that some neonicotinoids produce transient signs of nicotinic activity (e.g. tremors) in rats and mice following acute oral administration; however, there are considerable

differences in activity across the class, with no evidence of nicotinic activity in rodents treated with dinotefuran at any dose level (Akayama & Minamida 1999). For reference, the expression of “nicotinic signs” following exposure to nicotine varies by species but generally includes ataxia, tremor, and seizures, followed by evidence of CNS depression, muscular weakness, paralysis, or respiratory failure (Schep et al. 2009). Previous reviews with a limited number of neonicotinoids (Chao & Casida 1997; Yamamoto 1999; Sheets 2002) indicate that these compounds are not developmental or reproductive toxicants, but this is the first critical review to evaluate the neonicotinoids for evidence of developmental neurotoxicity.

The specificity of the neonicotinoid insecticides for the nAChR subtype that occurs in insects, combined with poor penetration of the mammalian blood–brain barrier, rapid metabolism, and low application rates (Chao & Casida 1997; Tomizawa & Casida 1999 2003 2005; Yamamoto et al. 1995) contributes to high margins of human safety associated with commercial uses (Sheets 2002). By comparison, nicotine is more toxic to mammals than insects, due to a higher affinity for mammalian nAChRs and greater penetration across the blood–brain barrier in mammals (Chao & Casida 1997). Demonstrated differences in nAChR subtype specificity are illustrated by the comparison of LD<sub>50</sub> values and receptor binding data in insects and mammals (Table 2). Based on acute LD<sub>50</sub> values, nicotine is 5-fold more toxic to the rat (53 mg/kg) than the housefly (272 mg/kg), whereas imidacloprid is 20-fold less toxic to the rat (450 mg/kg) than the housefly (22 mg/kg) (Yamamoto 1999). Accordingly, imidacloprid binds avidly to the insect nAChR, and relatively poorly to the mammalian nAChRs, including the major neuronal subtypes  $\alpha 4\beta 2$  and  $\alpha 7$ , while nicotine binds poorly to the insect nAChR and more avidly to the mammalian nAChRs (Matsuda et al. 2001).

A cursory literature search indicates limited published information that pertains to investigating the neonicotinoid insecticides for evidence of DNT. Examples include evidence of nicotinic activity (i.e. receptor-mediated Ca<sup>2+</sup> influx) with imidacloprid and acetamiprid in primary cell cultures of cerebellar granule cells from neonatal rats (Kimura-Kuroda et al. 2012) and findings in offspring from pregnant rats after a single intraperitoneal dose of imidacloprid (337 mg/kg, 0.75 × LD<sub>50</sub>) on gestation day (GD) 9 (Abou-Donia et al. 2008). In addition, there is a complement of previously unpublished DNT studies performed in accordance with EPA (1998) guidelines for all six neonicotinoid insecticides that are currently registered for commercial uses in major markets. Finally, there is a

**Table 1.** Neonicotinoid insecticides examined in guideline developmental neurotoxicity studies and structural comparison to nicotine<sup>a</sup>.

	Sponsor	IUPAC name/CAS #	Structure
Acetamiprid (>99%)	Nippon Soda (Tokyo, Japan)	<i>N</i> -[(6-chloro-3-pyridyl)methyl]- <i>N'</i> -cyano- <i>N</i> -methyl-acetamide 135410-2-7	
Imidacloprid (98.4%)	Bayer CropScience (Monheim am Rhein, Germany)	<i>N</i> -[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl] nitramide 138261-41-3	
Thiacloprid (99.2%)	Bayer CropScience (Monheim am Rhein, Germany)	{(2 <i>Z</i> )-3-[(6-Chloropyridin-3-yl) methyl]-1,3-thiazolidin-2-ylidene} cyanamide 111988-49-9	
Clothianidin (95.5–95.9%)	Sumitomo Chemical Co. (Tokyo, Japan)	( <i>E</i> )-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine 210880-92-5	
Thiamethoxam (98.8%)	Syngenta Crop Protection (Greensboro, NC)	3-[(2-Chloro-1,3-thiazol-5-yl) methyl]-5-methyl- <i>N</i> -nitro-1,3,5-oxadiazinan-4-imine 153719-23-4	
Dinotefuran (99.5%)	Mitsui Chemicals Agro, Inc. (Tokyo, Japan)	2-methyl-1-nitro-3-[(tetrahydro-3-furanyl) methyl] guanidine 165252-70-0	
Nicotine	–	( <i>S</i> )-3-[1-Methylpyrrolidin-2-yl]pyridine 54-11-5	

<sup>a</sup>Study designs based on EPA (1998).

considerable body of published studies that implicate nicotine as a developmental neurotoxicant for reference (Slikker et al. 2005; Slotkin 1998 2004).

The present review provides a critical evaluation of all the available *in vitro* and *in vivo* studies that tested neonicotinoid insecticides for DNT in mammals or other relevant test systems, as well as epidemiology studies that evaluated the relationship between evidence of exposure to neonicotinoids during pregnancy, infancy and/or early childhood, and neurodevelopmental outcomes. This includes findings identified in a comprehensive literature review for the entire class and previously unpublished studies performed in accordance

with EPA (1998) guidelines. These guideline studies were performed to complement developmental toxicity and two-generation reproduction studies for each neonicotinoid insecticide, in order to satisfy EPA requirements to investigate potential effects on the developing nervous system and determine whether an additional safety factor is needed to protect infants and children under the US Food Quality Protection Act (FQPA 1996). With limited information available in the literature, publishing these guideline DNT studies will significantly add to the body of information that is available to the broader scientific community. The collective results are then examined to determine whether the neonicotinoid

**Table 2.** Comparative acute toxicity and nicotinic receptor binding of substrates in insects and mammals.

Class	Compound	Housefly LD <sub>50</sub> (µg/g) without synergist	Housefly LD <sub>50</sub> (µg/g) with synergist <sup>b</sup>	Insect nAChR binding <sup>a</sup>		Mammalian nAChR binding (neuronal)	
				IC <sub>50</sub> (nM)	Rat oral LD <sub>50</sub> (mg/kg)	IC <sub>50</sub> α4/β2 type (nM) <sup>c</sup>	IC <sub>50</sub> α7 type (nM) <sup>d</sup>
Neonicotinoid	Thiamethoxam	ND	ND	5000 <sup>e</sup>	1563	>100 000	>300 000
	Clothianidin	ND	ND	2.2	>5000	3500	190 000
	Acetamiprid	17	ND	8.3	182	700	290 000
	Imidacloprid	22	0.05	4.6	450	2600	270 000
	Thiacloprid	ND	0.03	2.7	640	860	100 000
	Dinotefuran	ND	ND	900	2400	>100 000	>1 000 000
Nicotine	(-)-Nicotine	272	>50	4000	53	7.0	21 000
References	Yamamoto (1999)	Yamamoto (1999)	Yamamoto (1999) and Tomizawa and Casida (2003)	Tomizawa and Casida (2005)	Tomizawa and Casida (2005)	Tomizawa et al. (2000) and Tomizawa and Casida (2005)	Tomizawa et al. (2000) and Tomizawa and Casida (2005)

ND = not determined

<sup>a</sup>IC<sub>50</sub> values for displacing [<sup>3</sup>H]imidacloprid binding to the house fly (*Musca domestica*) (acetamiprid), aphid (*Myzus persicae*) (thiamethoxam) and fruit fly (the other neonicotinoids) receptor.

<sup>b</sup>Intrathoracic injection with synergist *O*-propyl *O*-(2-propynyl) phenylphosphonate (CYP450 inhibitor) pre-treatment.

<sup>c</sup>IC<sub>50</sub> values for displacing [<sup>3</sup>H]nicotine binding to the vertebrate α4/β2 nAChR.

<sup>d</sup>IC<sub>50</sub> values for the vertebrate α7 nAChR subtype (assayed by [<sup>125</sup>I]α-BGT binding).

<sup>e</sup>Thiamethoxam shown to have much greater affinity for insect nAChR (fruit fly) as its desmethyl metabolite (IC<sub>50</sub> = 20 nM) and after further metabolism to form clothianidin (IC<sub>50</sub> = 2.2 nM) (Zhang et al. 2000).

insecticides produce evidence of DNT under conditions that are relevant to human health.

## Methods

A comprehensive review of the literature was performed to identify all the published studies that might be relevant to the subject of the neonicotinoid insecticides and developmental neurotoxicity. The resulting collection of *in vitro*, *in vivo*, and epidemiology studies was then evaluated for relevance to the subject using established inclusion criteria. Previously unpublished DNT studies with the neonicotinoids that were performed in rats in accordance with EPA guidelines (EPA 1998) were also identified. Finally, the published and guideline-based studies that satisfied inclusion criteria were evaluated using criteria that have been developed to assess human relevance.

## Literature review

A comprehensive literature search using PubMed was performed on 24 September 2014 and again on 16 June 2015 to gather the relevant studies that examined neonicotinoids for effects on the developing nervous system. This search used the following terms for development, neurotoxicity, and neonicotinoids, respectively: (1) offspring OR neonatal OR *in utero* OR development OR developmental OR pregnancy OR pregnant OR gestational OR newborn OR prenatal OR perinatal OR teratology OR fetus OR fetal OR age-dependent OR age dependent OR age sensitivity) AND (2) brain OR neuron OR nervous OR neurotoxic\* OR neurolog\* OR

neurobehavior\* OR neurodevelopment OR developmental neurotoxic\* OR motor OR cognition OR cognitive OR behavior OR receptor OR neurotransmitter OR cerebellum OR hippocampus OR striatum OR cortex) AND (3) neonicotinoid OR imidacloprid OR acetamiprid OR clothianidin OR thiacloprid OR dinotefuran OR thiamethoxam. This search identified 236 citations that were screened for relevance to this review. A search performed on 25 June 2015 using ToxNet identified 284 citations that were also screened for subject relevance.

Additional MEDLINE (PubMed) searches were performed on 24 September 2014 and again on 16 June 2015 with the following terms and results: developmental neurotoxicity AND imidacloprid (OR acetamiprid) – one citation; developmental neurotoxicity AND thiacloprid (OR clothianidin, thiamethoxam, dinotefuran) – 0 citations; neurotoxicity AND neonicotinoid – seven citations; neurodevelopmental toxicity AND neonicotinoid – one citation; neurodevelopmental toxicity AND (imidacloprid, acetamiprid, thiacloprid, clothianidin, thiamethoxam, OR dinotefuran) – 0 citations; chloronicotinyl insecticides AND developmental neurotoxicity – 0 citations; brain development AND neonicotinoid – eight citations. A broader search of nervous system AND neonicotinoid provided 52 citations, while nervous system AND neonicotinoid NOT insect – identified 11 citations. Reference lists in recent articles on the subject were also cross-checked for relevant papers that may have been missed by the aforementioned searches. A search performed using ToxNet on 25 June 2015 identified no additional citations that pertained to the subject.

## **Inclusion criteria**

### **In vivo studies**

Studies from the literature reviews were included if exposure involved a single neonicotinoid insecticide that was delivered during gestation and/or lactation and the study measured neurodevelopmental endpoints. Inclusion criteria used by the EPA (2012) were applied to determine whether the study warranted inclusion and detailed evaluation for reliability and relevance to human health. Those criteria consisted of the following: results are presented in English as a full article or as an EPA (including joint review with PMRA) evaluation, with data (i.e. not a secondary source or only an abstract); exposure to a single neonicotinoid, not a mixture; the test article characterization, chemical concentration, and dose levels are clearly reported; the duration of exposure during gestation and/or lactation before weaning is clearly defined; studies with exposure continuing after weaning (e.g. one- or multi-generation studies) are included; treatment group(s) is compared with appropriate concurrent controls. If testing a commercial formulation, this would include a group treated with the formulation minus the active ingredient; the nature of the test organism and sample size are defined; and findings are considered relevant to assess human health risks for DNT outcomes, including functional, neurologic, morphologic or pharmacologic changes.

### **In vitro studies**

*In vitro* studies were included if published in English and the investigators link the findings to DNT outcomes with exposure to a single neonicotinoid.

### **Epidemiology studies**

The search criteria identified studies that evaluated the relationship between exposure to neonicotinoids during pregnancy, infancy and/or early childhood, and neurodevelopmental outcomes. Studies that ascertained pesticide exposure by questionnaires, environmental monitoring, or biomarkers were eligible for inclusion, provided that exposure to specific neonicotinoids or to neonicotinoids in general was measured or queried directly.

### **Guideline studies**

A search for guideline DNT studies was performed by identifying all the neonicotinoid insecticides that are registered for commercial uses in the US, where this study is conditionally required for registration, and by contacting the principal registrants, as these studies

were not published. Reviews of the associated study reports performed by the EPA or jointly with Health Canada Pesticide Management Regulatory Agency (PMRA) were used, along with determinations from the study scientists, to identify laboratory-specific study design and procedures and findings that were attributed to treatment.

## **Criteria to evaluate suitability to support human health risk assessment**

### **In vitro studies**

Standardized criteria based on EPA (2012) were used to evaluate the utility of *in vitro* studies for identifying a potential hazard or understanding a mode of action relevant to DNT. These criteria included adequate descriptions of the test system/test method, purity, composition, and origin of the test substance, dose-concentrations tested, solubility, impurities and pH, the presence or absence of metabolic activation, appropriate negative and positive controls for reference, and a clear description of statistical methods used to analyze the data.

### **In vivo studies**

The following standardized criteria were used as guiding principles to evaluate the reliability and suitability of studies to identify potential neurodevelopmental effects on human health. These are consistent with criteria used by others to review neurodevelopmental toxicology in the peer-reviewed literature (Adams 2010; Maurissen 2010; Neurotoxicology and Teratology Guide for Authors 2015) and those articulated by EPA's Office of Pesticide Program Guidance for considering and using open literature toxicity studies to support human health risk assessment (EPA 2012).

- Litter of origin is accounted for in study design and statistical analyses; maternal influences with exposure during gestation or lactation may exert significant impact on the offspring (DeSesso et al. 2009; Holson & Pearce 1992; Holson et al. 2008).
- A minimum of six animals per treatment condition is needed to provide minimal confidence in the results (Chapin et al. 2008, p. 235). For reference, the DNT guideline requires a minimum 10 per sex for neurobehavioral endpoints (EPA 1998).
- Route of administration should be relevant to human exposure conditions.
- Dose-response evaluations include more than two dose levels; studies with only one dose level are considered to have very limited utility.



- Studies conducted only at dose levels associated with significant systemic toxicity or lethality, or designed to compare LD50s, were considered to be of low utility.
- The time of testing needs to be balanced across dose groups and other factors if data are pooled in the statistical analyses (Maurissen 2010). For example, if sex is a factor in the analysis, the time of testing should be balanced with respect to sex.
- Contemporary historical control data from the same laboratory using identical methods can be used to assess reliability of the methodology, variability inherent in the measure, and whether the difference from control represents a treatment effect (EPA 2014; Raffaele et al. 2008).

### Epidemiology studies

The suitability of the epidemiology studies is based on the following questions developed by EPA Office of Pesticide Programs (EPA 2012; Li et al. 2012):

- Was the study conducted primarily in a hypothesis-generating or a hypothesis-testing mode? Studies with no specific *a priori* hypothesis are more likely to generate false-positive results.
- Were the methods used for assessing exposure and outcomes valid, reliable and adequate? Was a wide range of exposures sampled?
- Was appropriate information collected on both cases and controls for potentially confounding factors, such as sociodemographic, behavioral, and dietary factors, and were they appropriately controlled in the analyses of the data?
- Did the study sample the population or individuals of interest? Was selection bias minimized and generalizability optimized? How does the study population relate to the universe of potentially exposed populations?

- Did the study have adequate statistical power to detect meaningful differences for outcomes between the different groups of exposed and unexposed or less exposed individuals, while controlling for important confounding factors?

### Results

The numbers of publications identified in the literature using various search terms are provided in the Methods. Only six of these publications, which report *in vivo* or *in vitro* studies with imidacloprid, acetamiprid, and clothianidin, satisfied the inclusion criteria used to identify the relevant literature (Table 3). The conformance of these studies with criteria used to establish human relevance is shown in Table 4, with the results and experimental design summarized in Table 5. This review provides a summary of key results of published studies and a discussion of their strengths and limitations, relative to an evaluation of developmental neurotoxicity and human relevance. The reader is referred to the original articles for the actual data. Publications that did not meet inclusion criteria include reports of studies that examined neurotoxicity in insects or adult vertebrates or developmental toxicity but without any neurodevelopmental endpoints. Two epidemiology studies that are relevant to this subject were also identified. Yang et al. (2014) did not satisfy the inclusion criteria that pertain to exposure but it was included to provide additional perspective.

### Publications: *in vitro* DNT studies

Kimura-Kuroda et al. (2012) examined the effects of imidacloprid (>98% pure), acetamiprid (>98% pure), and nicotine (>99% pure) on primary cell cultures of cerebellar granule cells that were collected from newborn rats and cultured for 14 d. All three compounds were

Table 3. Inclusion criteria used to evaluate publications in this review.

	Abou-Donia et al. (2008)	Tanaka (2012a)	Tanaka (2012b)	Ozdemir et al. (2014)	Crosby et al. (2015)	
<i>In vivo</i> studies	English; full article with data tables	Yes	Yes	Yes	Yes	
	Single chemical: Not a mixture or formulation	Yes	Yes	Yes	Yes	
	Chemical conc. and dose levels reported	Yes	Yes	Yes	No/Yes	Yes
	Explicit duration of exposure	Yes	Yes	Yes	Yes	Yes
	Distinguish DNT from acute neurotoxic effect	Yes	Yes (GD0-LD21)	No (repro study)	No (PND 7 to term)	Yes
	Appropriate concurrent control for reference	Yes	Yes	Yes	Yes	Yes
	Test organism and sample size defined	Yes	Yes	Yes	Yes	Yes
	Relevant to human risk for DNT	Yes	Yes	Yes	Yes	Unknown
<i>In vitro</i> studies	Kimura-Kuroda et al. (2012)					
	English; full article with data tables	Yes				
	Findings relevant to DNT outcomes	Yes				

Table 4. Criteria used to evaluate the strength and relevance of studies included in this review.

	Abou-Donia et al. (2008)	Tanaka (2012a)	Tanaka (2012b)	Ozdemir et al. (2014)	Crosby et al. (2015)	Guideline DNT studies
<i>In vivo</i> studies Based on Adams (2010), Maurissen (2010), and EPA/OPP (2012)	Account for litter as the experimental unit for analysis Minimum 6/dose group (10 for behavior) Route of administration relevant to humans >2 dose levels Effects at a moderate dose (limited toxicity) Test article characterization is adequate Balance time of testing across dose levels	Yes <sup>a</sup> Yes (10) Yes (dietary) Yes (3) Yes Yes (>99.0%) No <sup>b</sup>	Yes <sup>a</sup> Yes (10) Yes (dietary) Yes (3) Yes Yes (>99.0%) No <sup>b</sup>	No (6 M/4 litters) No (4 litters) Yes (oral) Yes (3) No No No <sup>b</sup>	Not relevant (zebrafish) Yes (15–18 or 30–34) Unclear (immersion) No (2) Yes No No (purity not defined) Yes	Yes Yes (10–20) Yes (dietary) Yes (3) Yes Yes Yes (96% to >99%) Yes
<i>In vitro</i> studies from: EPA (2012)	Description of test system/method Test material purity/composition Dose-concentration tested Solubility/impurities and pH Metabolic activation (+/–) Appropriate negative & positive controls Clear description of method of analysis	Yes Yes (>98%) Yes No N.A. Yes (nicotine) Yes				

N.A., not applicable.

<sup>a</sup>Yes for measures after weaning based on experimental design of 1 pup/sex/litter and analysis conducted on each sex separately. Prior to weaning, all pups in the litter were tested and the authors do not indicate whether the litter was the experimental unit of analysis.

<sup>b</sup>No<sup>b</sup> indicates the paper does not mention the observations were conducted blind or balanced across dose groups.

reported to produce an excitatory Ca<sup>2+</sup> influx at concentrations greater than 1 μM in small neurons that expressed mRNA of α3-, α4- and α7-nAChR subunits that was inhibited by nAChR antagonists (α-bungarotoxin, dihydro-β-erythroidine, and mecamylamine). This excitatory response to acetamiprid and imidacloprid was shown to occur at lower concentrations than predicted based on their binding affinities (Tomizawa & Casida 2005). The firing patterns, proportion of excited neurons, and peak excitatory Ca<sup>2+</sup> influxes with acetamiprid and imidacloprid differed from nicotine, which was attributed to different effects on nAChRs by the neonicotinoids.

These results suggest that acetamiprid and imidacloprid express greater potency for nAChRs that are present in the neonatal rat than expected based on binding affinities for nAChRs expressed in adult tissues (Table 2). However, this model system has severe limitations to assess the potential for neonicotinoids to produce acute neurotoxicity or DNT *in vivo*. For example, the neurons had reached an advanced stage of maturation, since proliferation, migration, and differentiation had already occurred (Bal-Price et al. 2010) and metabolic degradation and a blood–brain barrier are critical components that do not exist in this system. Also, the method used to examine nAChR activity was not specific to a single mode of action, which may explain inhibition with a variety of nAChR antagonists and the similar reaction of the cells to nicotine, acetamiprid, and imidacloprid at equimolar concentrations, despite considerable differences in relative potency (Table 2). Further, the antagonism of the Ca<sup>2+</sup> influx by probes that selectively affect either α7 or α4β2 receptors had the same effect on nicotine and both neonicotinoids at equimolar concentrations, which the authors acknowledge, was unexpected. For example, the effect of all three chemicals was completely blocked by the homomeric nAChR antagonist α-bungarotoxin, suggesting an α7-mediated effect, whereas the heteromeric nAChR antagonist dihydro-β-erythroidine (DhβE) also blocked their effects completely. Finally, alternative (non-cholinergic) reactions and pathways may have been involved, as this system used an indirect measurement of a cholinergic reaction (by excluding acetylcholine as a positive control). In any case, evidence of nAChR activity as a molecular-initiating event in this model system must be evaluated in the context of *in vivo* results to support DNT as an adverse outcome (Bal-Price et al. 2015).

### Publications: *in vivo* DNT studies

Abou-Donia et al. (2008) investigated the effects of imidacloprid (~99.5% pure) administered as a single







intraperitoneal (i.p.) dose in corn oil to pregnant Sprague–Dawley rats on gestation day (GD) 9, with groups consisting of five vehicle control and five treated animals (Table 5). The treated group received 337 mg imidacloprid/kg body weight, which was cited as 75% of the acute oral LD<sub>50</sub> in rats; however, the relative potency by intraperitoneal versus oral administration is unknown. Inclined plane, beam-walking, and forepaw grip performance were evaluated on post-natal day (PND) 30, with motor cortex, septal hippocampus, and cerebellum examined for histopathology.

Differences from control in F1 (first-generation offspring) animals that were attributed to treatment included deficits in beam-walk time without a difference in beam-walk score. Deficits in the inclined plane test and grip time were also noted, along with increased plasma cholinesterase activity (F1 males only), and brain region-specific acetylcholinesterase activity in F1 males and females. Glial fibrillary acidic protein (GFAP) immunostaining was increased in motor cortex (layer III), CA1 layer, CA3 layer, and dentate gyrus of the hippocampus of F1 animals, compared with vehicle control, without evidence of histopathology in any brain region examined.

Key aspects of the experimental design and analysis present challenges with respect to data reliability and human relevance, which is consistent with the author's conclusion that the findings require additional investigation for support. This includes the use of a small sample size and a single high dose administered by a route of uncertain relevance to human exposures. The reported differences from control, including beam-walk time and inclined plane performance, were also minimal, with littermates treated as independent subjects ( $N = 20$  pups versus  $N = 5$  litters), which can severely inflate alpha levels (i.e. the false declaration of an effect) (Holson et al. 2008). Further, having only one dose group to compare with controls precludes establishing a dose–response relationship. The exposure window and corresponding effects observed also raise questions that pertain to the experimental design and known biology. In particular, GFAP immunostaining, which represents an active glial process, was increased in specific brain regions ~50 d after a single dose. The mechanism that would mediate such a response is unclear, especially since only rudimentary elements of the CNS (i.e. neural plate and early neural folds; DeSesso 2012) are present when the dose was administered.

Tanaka (2012a,b) reports two studies with clothianidin (>99% pure)-treated CD-1 mice (Table 5). In Tanaka (2012a), parental (P)-females received clothianidin in the diet at 0, 20, 60, or 180 ppm during gestation and lactation, and all groups received control diet after

weaning. In Tanaka (2012b), P-generation male and female mice received clothianidin at constant dietary levels of 0, 30, 60, or 120 ppm from 5 weeks of age until the F1 offspring were 11 weeks old. Measures in F1 animals in both studies included surface righting, negative geotaxis, cliff avoidance, swimming behavior, olfactory orientation, cognitive function (in a Biel multiple-T water maze), exploratory behavior (3 and 8 weeks old; 10-min session), and spontaneous behavior (9–10 weeks old; 120 min session with twelve 10-min intervals). Both studies used the same equipment and measures of exploratory and spontaneous behavior: the total distance (cm), number of horizontal activities, movement time(s), number of rearings, rearing time(s), average time of movement(s), average speed (cm/s), and average time of rearing(s). One pup/sex/litter was tested for all neurobehavioral measures, except for the spontaneous behavior and Biel maze, where all pups were reportedly tested. Multiple comparisons between treated and controls were conducted for each 10-min interval using the Steel–Dwass test. In addition, the longitudinal pattern over the 120-min session was statistically analyzed. Furthermore, a number of the differences ascribed to treatment were only supported by trend tests; not by the statistical analyses cited in the methods. The results from both studies are compared below to evaluate the consistency of effects on neurodevelopmental endpoints, with a more detailed and comprehensive comparison of results provided in the discussion.

Clothianidin had no effect on reproduction or body weight in P-generation males or females in either study at any dietary level. Further, measures of cognition in the Biel maze were not affected in F1 males or females in either study at any dose. Increased body weight was reported in F1-animals at PND 4–21 (Tanaka 2012a) or PND 4–7 (Tanaka 2012b), but these differences from control were not dose-related. Further, the group mean body weight of control F1 males (9.80 g) and females (9.34 g) on PND 21 in Tanaka (2012a) was notably greater than control F1 males (8.28 g) and females (8.00 g) in Tanaka (2012b). Treatment-related changes in some neurodevelopmental landmarks were noted during lactation, and movement parameters showed some differences at 3, 8, and/or 9–10 weeks of age; however, relatively few parameters were altered considering the large number of group comparisons that were analyzed for each endpoint, time point, and/or time interval. In addition, the pattern of statistical differences from control was somewhat inconsistent across these two studies, in terms of the endpoints that were involved or the sex and age associated with the finding, and differences from control occurred most often at the

low- or mid-dose, not the high dose (Table 5). Therefore, it is unclear whether these differences in outcome indicate inconsistent results or are due to differences in study design.

These studies satisfy criteria used to judge data reliability and human relevance; however, comparison of results for control and treated animals between the two studies suggests variable data and modest differences for a few of multiple comparisons that were not consistently dose-related. Furthermore, the differences in neurodevelopmental parameters in treated F1 mice typically represented increases in performance, relative to control, that may be confounded by unexplained differences in body weight (i.e. higher body weights in treated groups) during lactation. A more detailed evaluation of these studies is provided in the Discussion section.

Ozdemir et al. (2014) examined clothianidin (purity not reported) for potential effects on learning and memory in infant Wistar rats and the expression of related genes in the hippocampus. Doses of 0, 2, 8, and 24 mg/kg clothianidin were administered once daily by gavage on PND 7 through PND 97 to six F1 males per litter (4 litters per dose level). Separate groups of six adult males received the same complement of daily doses to establish whether findings were specific to developmental exposure. Cognitive function was evaluated using the Morris water maze, and mRNA expression levels of *N*-methyl *D*-aspartate 1 (GRIN 1), muscarinic receptor M1, synaptophysin (SYP), and growth-associated protein 43 (GAP-43) were measured in the hippocampus on PND 97. Acquisition (learning) was measured as the time required for animals to locate the submerged platform on each of five consecutive days, with memory consolidation measured in a probe trial on the following day, based on the time spent in the correct quadrant with the platform removed. The treatment had no effect on acquisition (learning phase) of the Morris water maze or gene expression at any dose. However, a significant reduction in time the high-dose animals spent in the target quadrant for the probe test suggests a treatment effect on the consolidation of memory. By comparison, clothianidin had no effect on performance in the Morris water maze in males that were treated only as adults for a similar duration.

This study evaluated multiple dose levels to establish a dose-response relationship and testing clothianidin for effects in separate groups of males treated for 3 months helps to support this finding being associated with exposure during development. However, this study does not satisfy important criteria used to judge data reliability and human relevance, including an adequate sample size, balancing testing across treatment groups to

account for diurnal variation, consideration of the litter (rather than the individual pups) as the experimental unit and characterization of the test article. The use of different vehicles (corn oil or DMSO) to treat separate groups of adult males at the same doses also raises questions concerning the suitability (e.g. solubility) of water as a vehicle in the developmental study.

Crosby et al. (2015) examined imidacloprid (purity not reported) for developmental neurobehavioral effects in zebrafish (*Danio rerio*) as a model organism. Zebrafish larvae were exposed via immersion in aqueous solutions containing 45 or 60  $\mu$ M imidacloprid or 45 or 60  $\mu$ M nicotine from 4 h through 5 d post-fertilization. Neurobehavioral testing was conducted on day 6 post-fertilization (larvae) or at 1.5 mo old (adolescent) or 3 mo (adult) of age. The battery of tests included activity measure, sensorimotor response and habituation in a tap-elicited startle test, novel tank swimming, and shoaling behavior. Both imidacloprid and nicotine decreased larval activity during dark phases (a time when control activity is higher compared to light phases). Persistent neurobehavioral changes were noted in adolescent ( $n = 15\text{--}18/\text{dose group}$ ) and adult ( $n = 30\text{--}34/\text{group}$ ) zebrafish. Developmental exposure to imidacloprid significantly increased the startle response in the adolescent but not the adult. Nicotine had similar effects, except that the startle response was increased only at the low (45  $\mu$ M) dose for the adolescent fish, not at the high dose or adults at either dose. Developmental exposure to imidacloprid decreased novel tank exploration in both the adolescent and the adult, whereas decreased novel tank exploration following nicotine exposure was noted only for the adult. Neither treatment affected shoaling behavior or habituation to the startle response at either dose or age.

These results indicate persistent effects in zebrafish associated with exposure to imidacloprid via immersion during the first 5 d of development and that these effects are somewhat consistent with the findings in zebrafish associated with developmental exposure to nicotine. This study satisfies criteria used to judge data reliability, including multiple (two) dose levels, adequate sample size, and balancing testing across treatment groups. The purity of the test material was not stated, but high purity is expected based on the source of the material cited. No measures of body growth of the zebrafish were provided. Such information would help determine whether the reported findings were associated with a treatment-related effect on growth and development. There was no mention as to whether the tests were performed in a "blinded" fashion to avoid potential bias, although the tests were largely automated. The human relevance for the dosage and route

of exposure (i.e. immersion of the larvae) in this screening model is uncertain and it is unclear how the findings from zebrafish relate to mammalian/human neuronal processes. For example, Papke et al. (2012) report differences in the response of nAChRs in zebrafish to some nicotinic compounds, compared with mammals. Crosby et al. (2015) acknowledge the need to extend the research from zebrafish to mammals.

### **Publications: epidemiology studies**

Yang et al. (2014) examined whether residential proximity to pesticide applications during early pregnancy was associated with an increased risk of anencephaly, spina bifida, or other anatomical anomalies (e.g. cleft palate), based on population-based data and information from maternal interviews for the San Joaquin Valley of California (1997–2006). This study was hypothesis generating in nature, with a “sizable” number of comparisons made. Interviews were conducted with mothers of 71% of eligible cases ( $n = 763$ ) and 69% of controls ( $n = 974$ ). Seventy-three cases of anencephaly and 123 cases of spina bifida were identified in the study population, with imidacloprid the only neonicotinoid among 257 chemicals and 52 chemical groups that were identified. The study established that there was a general lack of association between proximity to agricultural pesticide use and risks of selected birth defects, as the associated odds ratios for the birth defects examined may have arisen by chance alone. This paper failed to satisfy the inclusion criteria because pesticide exposure was based on residential proximity to pesticide use during early pregnancy, not evidence of actual exposure to any particular pesticide or chemical class.

Keil et al. (2014) examined whether there was an association between autism spectrum disorder (ASD) and the use of products that contain approximately 9% imidacloprid as a flea and tick treatment on household pets from 3 months prior to conception through breastfeeding, as recalled by the mother when the children averaged 3.6–3.8 years of age. The dataset included information from 262 typically developing controls and 407 children assigned the status of ASD by study personnel. Children previously identified as having ASD were recruited from an administrative database of the California Department of Developmental Services, which contract 21 Regional Centers to coordinate services for persons with developmental disabilities, health and service providers, other research studies, and self-referrals. Exposure and confounder data were collected through a phone interview with the mother, with no biomonitoring information to establish or estimate exposure.

The adjusted odds ratio (OR) with 95% confidence interval (CI) was 1.3 (0.79–2.2). More specifically, the OR was 0.69 (CI 0.27–1.8) for occasional users (less than once a month during pregnancy) and 2.0 (CI 1.0–3.9) for mothers identified as consistent (at least once a month during pregnancy) users of these products. The models were adjusted for the child’s sex, regional center of birth, age, maternal education, race/ethnicity and parity, and pet ownership during the prenatal period. Keil et al. (2014) caution that based on sensitivity analysis, the apparently higher ORs for exposure among consistent users could be a result of recall bias if mothers of children with ASD disproportionately report consistent (rather than occasional) use. Further, the paper cautions that analysis of subgroups (e.g. occasional versus consistent users) can result in loss of precision, due to the smaller numbers of individuals. Accordingly, Keil et al. (2014) conclude the association between ASD and prenatal imidacloprid exposure in this study was weak and could result from exposure misclassification alone.

### **Guideline DNT studies**

Guideline DNT studies with imidacloprid, acetamiprid, clothianidin, thiacloprid, thiamethoxam, and dinotefuran are included in this review. The only neonicotinoid insecticide with active registrations that is not included is nitenpyram, which has limited registrations only outside the US. The structures of the six neonicotinoids and purity of the test materials used in DNT studies are shown in Table 1.

### **Compliance**

These studies were conducted in accordance with EPA OCSPP guideline 870.6300 (EPA 1998) and US (40 CFR Part 160) and OECD (ENV/MC/CHEM (98) 17) Good Laboratory Practice (GLP) guidelines. The principal elements of the experimental design are shown in Table 6, with differences in test procedures among the four laboratories that performed the studies listed in Table 7. Certain differences in experimental design, compared with this EPA guideline, were applied to these studies, to assist interpretation and to support compliance with anticipated requirements for OECD TG 426 (OECD 2007), which was not finalized when several of these studies were performed. These differences in design included (1) exposure from GD 0 or 6 – PND 21 (rather than GD 6 – PND 10); (2) the sample size for neuropathology and brain morphometry was 10 F1 animals/sex/dose, representing 20 litters at each age (rather than 6/sex/dose, representing 12 litters at each

**Table 6.** Principal elements of EPA and OECD guideline developmental neurotoxicity study<sup>a</sup>.

P-generation females (number per dose level)		
No. females assigned <sup>b</sup>	GD 0 = the day of insemination	25–30
Detailed clinical observations	≥2 × each gestation and lactation	10
F1 males and females (no. males and females; ≥20 litters/dietary level) <sup>c</sup>		
Detailed clinical observations	PND 4, 11, 21, 35, 45, and 60	10–20
Body weight	PND 4, 11, 17, and 21 and at least once every 2 wks thereafter	All pups/litter
Sexual maturation (preputial separation and vaginal patency)	Daily from before initiation until landmark is achieved	All pups/litter
Motor activity	PND 13, 17, 21, and ~PND 60–70	10–20 <sup>d</sup>
Acoustic startle	Around the time of weaning and ~PND 60–70	10–20
Cognition	Post-weaning and PND 60–70	10–20
Brain measurements	PND 11 or 21–22	10
	Term (~PND 70)	10
Neuropathology <sup>e</sup>	PND 11 or 21–22	10
	Term (~PND 70)	10

<sup>a</sup>Test materials administered via the diet or by gavage (acetamiprid) to P-females from GD 0 or 6 to LD 21 (see text for details).

<sup>b</sup>25–30 untreated female rats per dietary level were co-housed with untreated males to obtain ≥20 litters per dose group.

<sup>c</sup>F1 males and females from each litter were randomly assigned to a test set, with each litter represented by at least one male or one female (≥20 litters per dose level).

<sup>d</sup>EPA (1998) requires one male OR one female per litter (minimum 20 litters) and OECD (TG 426) requires one male AND one female per litter (minimum 20 litters) for neurobehavioral endpoints.

<sup>e</sup>An example of neuropathology procedures used in these studies is provided in Supplementary material (Appendix I).

age); and (3) additional brain morphometry measurements were taken.

### Test material

Technical-grade test materials (95.5–99.5% purity) were provided by the Sponsors (Table 1) and the identity of the active ingredient and stability under the conditions of storage were verified by appropriate analytical methods. Acetamiprid was formulated in corn oil and administered once daily by gavage to P-generation females, while other test materials were mixed in the diet for *ad libitum* consumption. Homogeneity and stability in corn oil or the diet were verified, and dose or dietary levels were periodically analyzed to verify the concentration of test substance given to the animals at each level and to determine the associated dose levels during gestation and lactation.

### Study design

Test materials were administered at a constant concentration (ppm) in the diet or a constant dosage (mg/kg/d) administered by gavage from GD 0 or GD 6 through day 21 of lactation and postnatal development (Table 7). By convention, the day on which insemination was evident is designated GD 0 and the day of parturition is PND 0. Treatment via the diet or by gavage of the dam is used to model dietary exposure in humans during pregnancy and lactation, with the offspring exposed to test substance and/or toxicologically active metabolite(s) that passes through the placenta, into the milk, and (with dietary administration) by consuming progressively greater amounts of treated feed from ~PND 15 to PND 21. Pharmacokinetic data, evidence of offspring

toxicity, or changes in biomarkers provide evidence the offspring were exposed through the milk to the test substance or active metabolite(s).

P-females were randomly assigned to one of the three dose levels or a vehicle (corn oil or blank diet, as appropriate) control group (20–30/dose level). On specific days after parturition, F1 animals from each litter were randomly selected to provide the minimum number of males and females needed for neurobehavioral and neuropathologic assessments (Table 6). Details regarding the test procedures and associated measures, as well as requirements to verify the suitability of the various measures to identify treatment-related effects with reference chemicals, are provided in the test guideline (EPA 1998). Additional details of the procedures for neuropathology and brain measurements are also provided in Supplementary materials (Appendix I). In accordance with the test guideline (EPA 1998), the high dose was selected with the objective to induce evidence of toxicity in the dam or pups without precluding a meaningful evaluation of the results, with the mid- and low-doses selected to induce less toxicity than the high dose and no evidence of toxicity, respectively. Also in accordance with EPA guidelines, tissues from control and high-dose F1 males and females were examined for neuropathology and brain morphometry, with step-down analysis of lower dose groups if there was evidence of a treatment effect at the high dose. Additional details that are specific to the individual studies are provided in Table 1, 6 and 7.

### Statistical analysis

The statistical analyses that were used in the guideline studies for each neonicotinoid are summarized in



Table 7. Laboratory-specific test procedures for guideline developmental neurotoxicity studies.

	Laboratory	Strain and treatment period	Motor activity	Acoustic Startle habituation <sup>a</sup>	Cognition	Brain neuropathology <sup>b</sup>	Brain morphometry <sup>c</sup>
Acetaminophen	WIL Laboratories	Sprague-Dawley GD6 – LD21	60-min open-field (18.5 × 9.5 × 8 in.) photobeam SDI-System	SDI SR-Lab; accelerometer; 115 dB noise burst	Biel maze: multi-phase water-filled 8-unit T-maze	PND 11 (perfusion) PND 70 (perfusion) 5 coronal sections	PND 11 and ~PND 70: hemisphere height, FC, PC, three measures of hippocampus, height caudal pons, CB
Imidacloprid	Bayer Laboratory	Wistar GD0 – LD21	60-min figure-8 maze photobeam Coulbourn	Coulbourn; load-cell force transducer; 120 dB noise burst	Passive avoidance & M-water maze	PND 11 (immersion) PND 72 (perfusion) 7 coronal sections	PND 11 and ~PND 70: FC, PC, CP, CC, HG, CB
Thiacloprid	Charles River (formerly Argus)	Sprague-Dawley GD0 – LD21	60-min open-field (40.6 × 25.4 × 17.8 cm)	Coulbourn; load-cell force transducer; 120 dB noise burst	Passive avoidance & M-water maze	PND 11 (immersion) PND 70 (perfusion) 11 coronal sections;	PND 11 and ~PND 70: FC, PC, CS, CC, HG, CB
Clothianidin	Charles River (formerly Argus)	Sprague-Dawley GD0 – LD21	60-min open field (40.6 × 25.4 × 17.8 cm)	Coulbourn; load-cell force transducer; 120 dB noise burst	Passive avoidance and M-water maze	PND 11 (immersion) PND 83-87 (perfusion)	PND 11 and ~PND 85: FC, PC, CS, CC, HG, CB
Thiamethoxam	Syngenta Central Toxicology Laboratory	Wistar GD6 – LD21	50-min open-field (8.5 × 6 × 5 cm IR sensors)	SDI SR-Lab accelerometer; 110 dB noise burst	“Y”-shaped water maze	11 coronal sections PND 11 (immersion) PND 62 (perfusion) 7 coronal sections	PND 11 & ~PND 62: FC, DC, PIC, CC, thalamus, HW, DG, CB
Dinotefuran	Charles River (formerly Argus)	Sprague-Dawley GD6 – LD21	60-min open field (40.6 × 25.4 × 17.8 cm)	Coulbourn; load-cell force transducer; 120 dB noise burst	Passive avoidance and M-water maze	PND 21 (perfusion) PND 69 (perfusion) 9 coronal sections	PND 21 and ~PND 70: FC, CS, CC, HG, CB

<sup>a</sup>Acoustic startle response amplitude measured as g (load cell) or  $V_{max}$  (accelerometer).

<sup>b</sup>In addition, neuropathology examination of peripheral nervous system tissues taken at ~ PND 70–80 was conducted (including muscle, eye, spinal cord, proximal sciatic nerve, proximal tibial nerve and distal tibial nerve). See Supplementary Appendix 1 for example.

<sup>c</sup>FC, frontal cortex; PC, parietal cortex; PIC (piriform cortex; DC, dorsal cortex; CP, caudate-putamen width; CS, corpus striatum; maximum diagonal measure of caudate-putamen & globus pallidus; CC, corpus callosum, HG hippocampal gyrus; HW, hippocampal width; DG, dentate gyrus; CB, cerebellum; C, cerebrum.

Table 8. In all cases, the litter was the experimental unit of analysis. In general, body weight, motor activity, acoustic startle response, and maze testing results were analyzed separately for each sex and age, using two-way repeated-measures analysis of variance (RANOVA) and/or one-way ANOVA, with *post-hoc* tests including Dunnett's or Student's *t*-test. For thiamethoxam, Y-maze performance data were initially transformed using Freeman and Tukey's double arcsine transformation prior to one-way ANOVA for each intersession and total session measure. For acetamiprid, behavioral data for both sexes were combined (1 male or female per litter) and analyzed using a three-way RANOVA mixed model approach. Approaches used to analyze continuous data with unequal variance included Kruskal–Wallis followed by Dunn's or Fisher's-Exact Test. For passive avoidance results with imidacloprid, latency-to-cross and trials-to-criterion data were analyzed using Kruskal–Wallis, Wilcoxon, and Fisher's exact tests. The incidence of neuropathology findings was analyzed using Fisher's exact or Kolmogorov–Smirnov tests. Brain morphometry data were analyzed using ANOVA (essentially *t*-test for two groups). For thiamethoxam, morphometry data were analyzed using an analysis of covariance, with final body weight as a covariate.

### Findings in guideline studies

The following analysis is based on reviews of the study reports and supporting data conducted by EPA or through joint review with Health Canada PMRA (Table 9). This approach provides the reader with a consistent and objective evaluation of the available information for all six neonicotinoids, performed by an authoritative body with the responsibility to evaluate and apply these results for hazard identification and risk assessments. For additional details, the reader is referred to the associated data evaluation records prepared by these Agencies (cited below). The interpretation of the scientists who performed the studies and/or analyzed the results is also included to provide additional information and perspective.

Decreased body weight associated with decreased weight gain during gestation or lactation was the most common treatment-related finding in P-females (Table 9); however, it should be understood that the evaluation of P-females is limited in this study, with the emphasis on evaluating the F1 generation for evidence of DNT. For F1 males and females, decreased body weight and weight gain during lactation were also the most common findings, with birth weight decreased only with the high dose of acetamiprid and thiamethoxam. None of the neonicotinoids affected

reproduction or produced clinical signs in P-females or F1-animals at any dose. Further, there was no effect on habituation (motor activity or the acoustic startle response) or evidence of cognitive deficits or neuropathology in F1 males or females in any of these studies.

### Acetamiprid

Acetamiprid (>99% purity) was administered daily by gavage to P-females on GD 6 to LD 21 at dose levels of 0, 2.5, 10, or 45 mg/kg/d. The NOAEL for P-females and F1 offspring was 10 mg/kg/d, based on the findings at the high dose of 45 mg/kg/d (EPA 2013a). Body weight was decreased (4–5%;  $p < 0.05$ ) from GD 9 to 20 in P-females at 45 mg/kg/d, with a mean body weight loss of 3 g from GD 6 to 9, compared with a mean 13 g gain in controls. For the entire gestation period, mean body weight gain in pregnant females at 45 mg/kg/d was 15% lower ( $p < 0.05$ ) than the control group. Food consumption was decreased 40% ( $p < 0.05$ ) during the first week of exposure (GD 6–12) and 18% ( $p < 0.05$ ) from GD 6 to 20.

Treatment-related effects in high-dose F1 animals included decreased post-natal survival (PND 0–1; 3 dams had total litter loss on PND 1), decreased birth weight (–7% in F1 males (NS) and –9% in F1 females;  $p < 0.05$ ), decreased body weight (–5 to –10% in F1 males and females) and decreased body weight gain (–9 to –21%, primarily in F1 males) after weaning to PND 72, and decreased acoustic startle response in F1 males on PND 20 (–42%) and PND 60 (–53%). There were no treatment-related macroscopic or microscopic findings for brain, spinal cord or peripheral nerves or for brain morphometry measurements. EPA (2008) deferred drawing conclusions for the learning and memory and motor activity data (see below), but has since concluded “the toxicology database is complete for acetamiprid and acceptable guideline studies for developmental, reproductive toxicity, neurotoxicity (including DNT) and immunotoxicity are available” (EPA 2013b).

The acoustic startle response test in the DNT guideline is primarily designed to evaluate habituation (EPA 1998), which is a primitive learning process in which the organism decreases its response to redundant, non-significant stimuli (Davis 1984). On both PND 20 and PND 60, there was no effect on acoustic startle habituation (no significant interaction of dose with trial;  $p > 0.05$ ). On PND 20, there was a significant dose ( $p = 0.003$ ) effect for peak startle amplitude, but no significant dose  $\times$  sex interaction. In *post-hoc* comparisons with control, only the high dose was decreased significantly when males and females were combined ( $p = 0.002$ ) or when males were analyzed alone ( $p = 0.023$ ). On PND 60, males and females were

Table 8. Statistical analyses used in guideline developmental neurotoxicity studies.

	Acetaminiprid – WIL Laboratories	Imidacloprid – Bayer Laboratory	Thiacloprid, Clothianidin & Dinotefuran – Charles River (formerly Argus) Laboratories	Thiamethoxam – Syngenta Central Toxicology Laboratory
Body weight	One-way (dose, interval) ANOVA followed by Dunnett's	Bartlett's test for equal variance ( $\alpha=0.001$ ) One-way ANOVA and Dunnett's (equal variance) Kruskal-Wallis and Mann-Whitney (unequal variance) tests	Bartlett's test for equal variance ( $\alpha=0.05$ ) ANOVA followed by Dunnett's (equal variance) Kruskal-Wallis (unequal variance) followed by Dunn's ( $\leq 75\%$ scores tied) or Fisher's exact ( $>75\%$ scores tied)	One-way ANOVA and ANCOVA (with initial body weight as covariate), followed by Student's t-test
Variables with graded or count scores	Not applicable	Not applicable	Kruskal-Wallis and Dunn's ( $\leq 75\%$ scores tied) or Fisher's exact ( $>75\%$ scores tied)	For proportions of males or females affected, Fisher's exact test.
Motor activity	One-way (dose, interval) ANOVA for total and intersex for each sex followed by Dunnett's; three-way (dose, interval, sex) RANOVA (Proc Mix SAS using AR(1) or CS models). If dose $\times$ sex interaction is significant then males and females are analyzed separately. If dose $\times$ interval interaction is significant then ANOVA for each interval followed by Dunnett's. If dose $\times$ interval interaction is not significant and dose effect is significant then Dunnett's test is conducted on the pooled cumulative session data	Two-way RANOVA (dose, interval) and Dunnett's tests	Two-way RANOVA (dose, interval). If dose $\times$ interval interaction was significant, then ANOVA for each interval followed by Dunnett's. If there was a significant dose effect then Dunnett's was conducted on data pooled across intervals	One-way ANOVA followed by Student's t-test. Conducted for each time interval and overall
Acoustic Startle	Same as motor activity except factors included dose, trial and sex	Two-way RANOVA (dose, interval) and Dunnett's tests	Same as motor activity except the factors included trial instead of interval	One-way ANOVA followed by Student's t-test, for maximum amplitude and time to maximum amplitude
Biel Maze	Same as motor activity except factors included dose, trial and sex and each path was analyzed separately	Not applicable	Not applicable	Not applicable
M-Maze or Y-Maze	Not applicable	ANOVA and Dunnett's tests	Same as for body weight	ANOVA following double arcsine transformation of Freeman and Tukey's, then Student's t-test
Passive avoidance (latency, trials-to-criterion)	Not applicable	Kruskal-Wallis, Wilcoxon and Fisher's exact tests	Same as for body weight	Not applicable
Neuropathology	Kolmogorov-Smirnov test	Chi-square and Fisher's exact tests	Fisher's exact (one-tailed) test	Fisher's exact test
Brain morphometry	ANOVA followed by Dunnett's	ANOVA or T-test (two groups)	ANOVA (continuous data)	ANOVA and ANCOVA using final body weight as covariate

Table 9. Summary of results for guideline developmental neurotoxicity studies.<sup>a</sup>

Exposure	Acetaminophen		Imidacloprid		Thiacloprid		Clothianidin		Thiamethoxam		Dinotefuran	
	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure
Nominal dietary conc. (ppm)												
Achieved dose (mg/kg/d)												
Gestation:	0, 2.5, 10, 45	0, 8.0, 19.4, 54.7	0, 4.4, 25.6, 40.8	0, 4.4, 25.6, 40.8	0, 12.9, 42.9, 142	0, 4.3, 34.5, and 299	0, 12.9, 42.9, 142	0, 4.3, 34.5, and 299	0, 12.9, 42.9, 142	0, 4.3, 34.5, and 299	0, 79.4, 237, 784	0, 79.4, 237, 784
Lactation:	0, 2.5, 10, 45	0, 12.8, 30.0, 80.4	0, 8.2, 49.4, 82.8	0, 8.2, 49.4, 82.8	0, 27.3, 90.0, 299	0, 8.0, 64.0, and 594	0, 27.3, 90.0, 299	0, 8.0, 64.0, and 594	0, 27.3, 90.0, 299	0, 8.0, 64.0, and 594	0, 158, 501, 1643	0, 158, 501, 1643
LOAEL												
Parental (P):	45	55	25.6	25.6	142	299	142	299	299	299	N.A.	N.A.
Offspring (F1):	45	55	25.6	25.6	42.9	299	42.9	299	299	299	N.A.	N.A.
NOAEL												
Parental (P):	10	20	4.4	4.4	42.9	34.5	42.9	34.5	34.5	34.5	784	784
Offspring (F1):	10	20	4.4	4.4	12.9	34.5	12.9	34.5	34.5	34.5	784	784
↓ BWT or BWG	Gestation	Lactation	25.6 mg/kg: gestation 40.8 mg/kg: gestation and lactation	25.6 mg/kg: gestation 40.8 mg/kg: gestation and lactation	Gestation and lactation	Gestation and lactation	Gestation and lactation	Gestation and lactation	Gestation and lactation	Gestation and lactation	-	-
Treatment-related findings <sup>b</sup> : P-generation												
Detailed clinical observations	-	-	-	-	-	-	-	-	-	-	-	-
↓ BWT or BWG	M and F at birth, lactation and post-weaning	M and F during lactation	M and F during lactation and post-weaning	M and F during lactation and post-weaning	42.9 mg/kg: F during lactation 142 mg/kg: M and F (lactation and post-weaning)	M and F at birth, during lactation and post-weaning	42.9 mg/kg: F during lactation 142 mg/kg: M and F (lactation and post-weaning)	M and F at birth, during lactation and post-weaning	M and F at birth, during lactation and post-weaning	M and F at birth, during lactation and post-weaning	-	-
Treatment-related findings <sup>b</sup> : F1-generation												
Detailed clinical observations	-	-	-	-	-	-	-	-	-	-	-	-
Sexual maturation	-	-	-	-	-	-	-	-	-	-	-	-
Motor activity	-	-	-	-	-	-	-	-	-	-	-	-
Acoustic startle response	↓ M on PND 20 and 60	-	- <sup>c</sup>	- <sup>c</sup>	42.9 mg/kg: ↓ F on PND 21 142 mg/kg: ↓ M and F on PND 21 42.9 mg/kg: ↓ F on PND 22 (NS); 142 mg/kg: ↓ M (NS) and F on PND 22	Delayed: M and F	42.9 mg/kg: ↓ F on PND 21 142 mg/kg: ↓ M and F on PND 21 42.9 mg/kg: ↓ F on PND 22 (NS); 142 mg/kg: ↓ M (NS) and F on PND 22	42.9 mg/kg: ↓ F on PND 21 142 mg/kg: ↓ M and F on PND 21 42.9 mg/kg: ↓ F on PND 22 (NS); 142 mg/kg: ↓ M (NS) and F on PND 22	42.9 mg/kg: ↓ F on PND 21 142 mg/kg: ↓ M and F on PND 21 42.9 mg/kg: ↓ F on PND 22 (NS); 142 mg/kg: ↓ M (NS) and F on PND 22	Delayed: M only	-	-
Cognition	↑ number of errors in M on PND 22-27 (NS)	-	Altered passive avoidance in F on PND 23-25 (↑ latency in Trial 2 at 25.6 and 40.8 mg/kg of 4 total trials, no effect on trials to criterion)	Altered passive avoidance in F on PND 23-25 (↑ latency in Trial 2 at 25.6 and 40.8 mg/kg of 4 total trials, no effect on trials to criterion)	PND 11: ↓ corpus striatum and corpus callosum in M; Term: ↓ corpus striatum and dentate gyrus in M	-	-	-	-	-	-	-
Brain measurements	-	↓ caudate-putamen F on PND 72	PND 11: ↓ corpus striatum and corpus callosum in M; Term: ↓ corpus striatum and dentate gyrus in M	PND 11: ↓ corpus striatum and corpus callosum in M; Term: ↓ corpus striatum and dentate gyrus in M	142 mg/kg: PND 11: ↑ hippos. gyrus and cerebellum in F; PND 85: ↓ hippos. gyrus in F	↓ absolute brain wt. and gross measures M and F	142 mg/kg: PND 11: ↑ hippos. gyrus and cerebellum in F; PND 85: ↓ hippos. gyrus in F	142 mg/kg: PND 11: ↑ hippos. gyrus and cerebellum in F; PND 85: ↓ hippos. gyrus in F	↓ absolute brain wt. and gross measures M and F	↓ absolute brain wt. and gross measures M and F	-	-
Neuropathology	-	-	-	-	-	-	-	-	-	-	-	-

<sup>a</sup>Study design based on EPA (1998).  
<sup>b</sup>Findings noted by EPA or PMRA reviewers at the high-dose level only except for thiacloprid and clothianidin, for which findings at the mid- and high-dose are provided. NS = Not statistically-significant, compared to control.  
<sup>c</sup>“-” = No effect at any dose or dietary level.  
<sup>d</sup>Non-statistical differences from control EPA considered “suggestive” of a potential effect (EPA 2002b).

analyzed separately, due to a significant dose  $\times$  sex interaction ( $p=0.038$ ). There was a significant dose effect in males ( $p=0.046$ ) but not females, with high-dose males significantly different from control ( $p=0.015$ ).

There were numerical decreases in startle amplitude at 10 mg/kg on PND 20 ( $-27\%$ ) and PND 60 ( $-40\%$ ) that EPA did not consider were treatment related. This interpretation was based on the differences from control not being statistically significant and the group means for treated males being near the 50th percentile or within the inter-quartile range (IQR) of the historical control (HC) data (19–22 studies using identical test procedures), which indicates a startle response consistent with controls for the laboratory. Furthermore, these non-statistically significant differences in startle amplitude at 10 mg/kg/d are within the variability for control animals of 30% at weaning and 51% as adults for peak startle amplitude published by EPA scientists for smaller-scale studies (Supplementary Table 1). Benchmark dose–response levels of 25% and 40% (lower than EPA's average CV ( $SD \times 100/\text{mean}$ )) approximate the benchmark dose levels (1 SD) of 12.6 and 13.1 mg/kg/d, respectively, for PND 20 and 60 (PROAST software Version 38.9; RIVM 2014) using best fit and lowest values from Exponential and Hills models. This analysis provides additional support for the NOAEL of 10 mg/kg/d.

As mentioned above, EPA (2008) indicated that further evaluation of the motor activity and Biel maze tests would rely on a retrospective analysis for comparison of the variability of results in this early study (one of the first submitted to EPA) with other DNT study results. EPA's initial concerns were related to variability and control behavior for motor activity on PND 13 and 17 and variability in learning and memory data. It is now recognized that there is high biological variability for motor activity data in this study design, especially at PND 13, when the animals have limited mobility and sensory function, as well as PND 17, when sensorimotor skills and function are rapidly developing (Makris et al. 2009). The mean CVs (males and females) for the acetamiprid DNT study were 73, 70, 51, and 36% for motor activity for PND 13, 17, 21, and 61, respectively (EPA 2008 2013a). These values are within the range (73–87; 46–75; 29–55; 13–38%) and comparable with the means  $\pm$  SD ( $83 \pm 3$ ;  $59 \pm 12$ ;  $40 \pm 13$ ;  $25 \pm 9\%$ ) for the CV values of motor activity control data from five other neonicotinoid studies (EPA 2002a b 2007 2013b; Health Canada PMRA 2002).

Repeated measures ANOVA analysis of motor activity with male and female data combined increased the sample size from 10 to 20 per dose group and resulted in

a significant effect over intra-session time ( $p<0.001$ ) at PND 17, 21, and 61, which reflects habituation. There were no statistically significant dose effects or dose interactions with time at any age, indicating no difference in habituation for any dose group (Figure 1). These results support the determination that acetamiprid did not affect motor activity at any age or dose level.

The Biel Maze test used in this study is one of the most challenging learning and memory tests used in studies performed in accordance with EPA (1998) guidelines, as it involves sequential (Path A) and reverse (Path B) learning through a multiple-T water maze. EPA (2008) noted non-statistically significant increases in the number of errors for trials 5–10 in Path B only in high-dose F1 males on PND 22, but not on PND 62 (Figure 2), and no effect in females at either age. On PND 22, there was a significant effect of trial ( $p<0.001$ ) for both latency and the number of errors for Path A, Path B (path reversal), and Path A-probe (see Figure 2 for details), indicating learning in all groups. However, there were no significant dose  $\times$  trial interactions ( $p=0.146$ – $0.993$ ), indicating no effect of acetamiprid on learning (Figure 2). There was a marginally significant overall dose effect ( $p=0.053$ ) for the number of errors for Path A, with only the high dose level marginally significant ( $p=0.060$ ; Dunnett's). Graphical presentation of the data indicates that the most consistent learning behavior occurs during Path B (Figure 2), after the animal was introduced to the maze in Path A, and that there is no consistent dose-related pattern of effects on Path B.

In summary, the high dose of 45 mg/kg/d acetamiprid decreased body weight gain in P-females and F1 animals, decreased the acoustic startle response in F1 males and was associated with a marginally significant increase in the number of errors in the Biel maze in F1 males just after weaning. The biological significance of this numerical increase is uncertain, because it was not seen in F1 males for Path B or Path A-probe or in females for either path. EPA concluded the study is acceptable and established a NOAEL of 10 mg/kg/d for P-females and F1 offspring (EPA 2013a).

### **Imidacloprid**

Imidacloprid (98.4% purity) was administered on GD 0 to LD 21 at constant dietary levels of 0, 100, 250, or 750 ppm. Treatment-related effects in P-females and F1 animals were evident at 750 ppm but not at lower dietary levels (EPA 2002a). Based on these results, the EPA selected the dose equivalents of 55 and 20 mg/kg/d for the LOAEL (750 ppm) and NOAEL (250 ppm), respectively, pending morphometric measurements for caudate-putamen in F1 females at 250 ppm (Table 10).



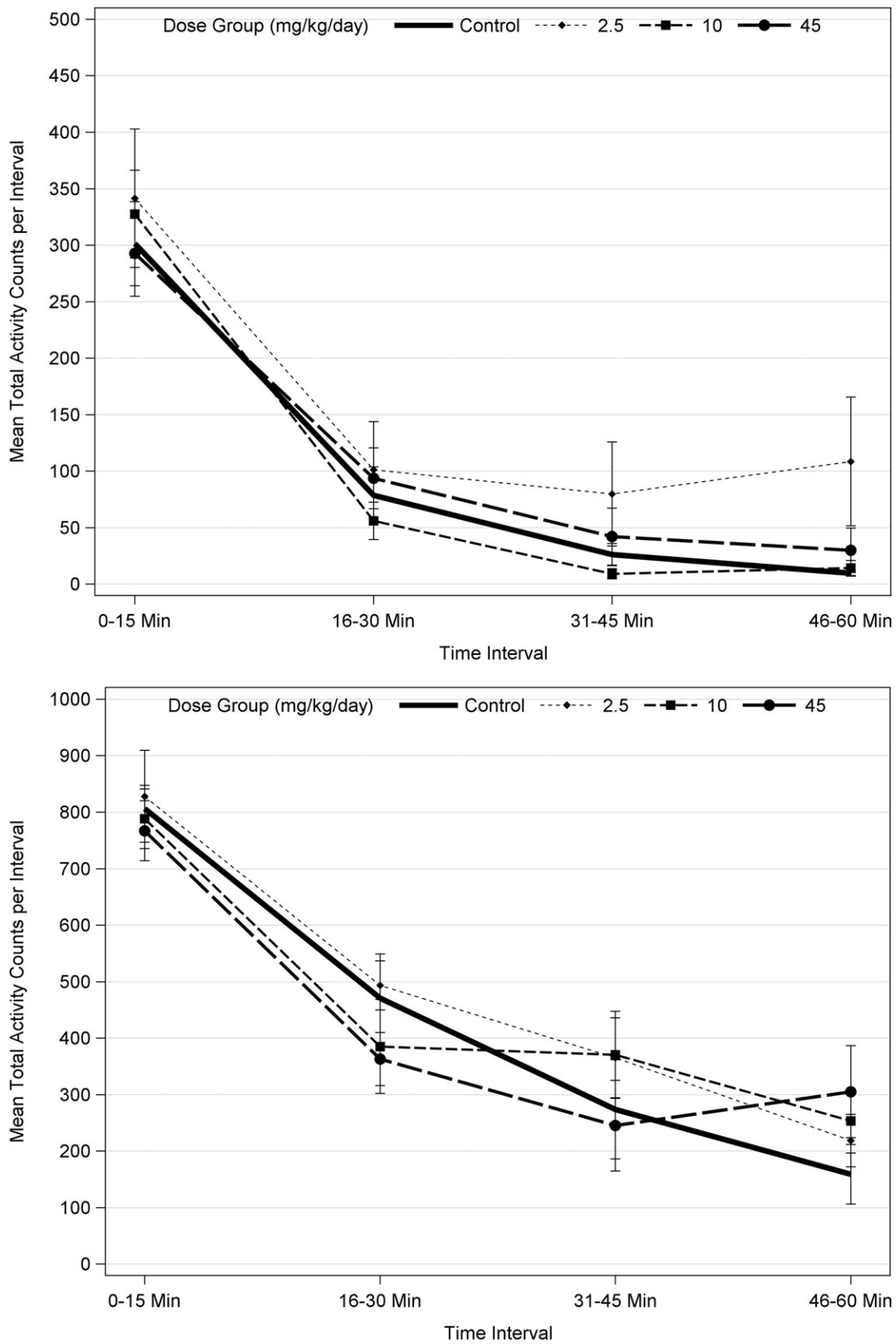
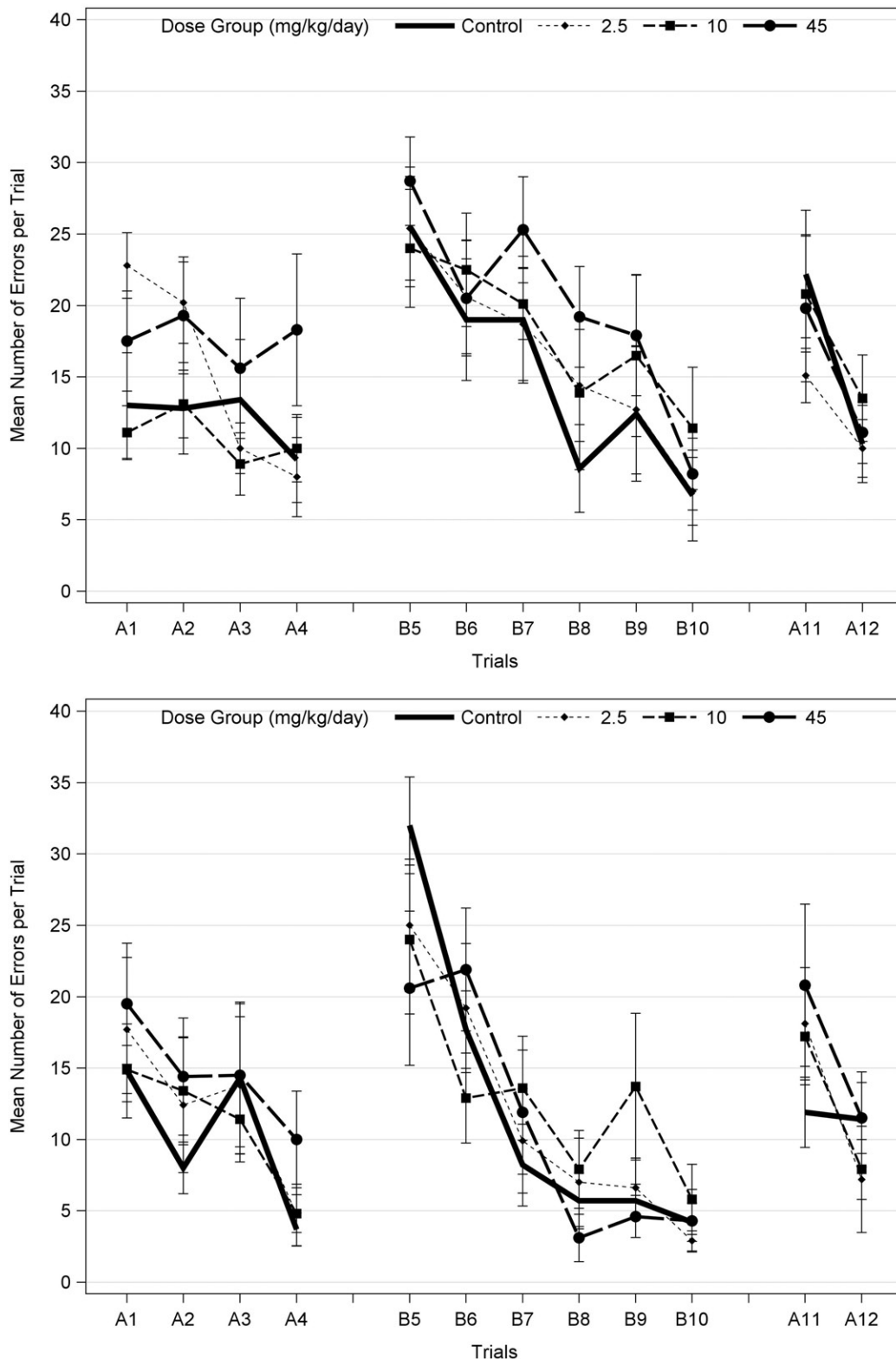


Figure 1. Motor activity for PND 21 (top) and PND 60 (bottom) F1 male rats ( $n=10$ /sex/dose level) exposed to acetaminophen via gavage administration to the dam (GD 6 to LD 21). There were no statistically significant results based ANOVA for each trial for males or females, separately, or when data were combined for males and females and analyzed using repeated measures ANOVA and Dunnett's (see text).



**Figure 2.** Mean ( $\pm$ SEM) number of errors per trial on Biel water maze (8 unit T-test) in F1 male offspring (10/sex) on PND 22 (top) and PND 62 (bottom), following developmental exposures to acetaminophen. Both males and females were tested for 7 d with four swim trials on a straight channel on the first day (no effect; data not shown); 2 trials/day on Path A for 2 d (A1–A4), 2 trials/d on path B (reverse of path A; B5–B10) for 3 d; and two trials on path A (A11–A12) on the last day (probe test). A different set of animals was tested on PND 22 and PND 62. The number of errors (deviation from correct channel with all four paws) and the time required for the animal to escape (data not shown) were recorded. EPA (2008) identified possible trend in errors only in high-dose males at PND 22, but also noted there were no effects on latency. There was no statistical significance at either age when males and females were analyzed separately (ANOVA and Dunnett's for each trial;  $\alpha = 0.05$ ). When males and females were combined ( $n = 20$  litters/dose group) and analyzed using repeated measures ANOVA with trials as the repeated measures, there was a significant effect of trials ( $p < 0.018$  or  $p < 0.001$ ) but no significant dose effects or dose interactions with sex or trial for each age ( $\alpha = 0.05$ ).

**Table 10.** Caudate-putamen width (mm) in F1 rats treated with imidacloprid<sup>a</sup>.

Dietary level	PND 11		PND 72	
	Male	Female	Male	Female
Control	2.70 ± 0.05	2.77 ± 0.02	3.66 ± 0.01	3.75 ± 0.01
750 ppm	2.71 ± 0.05 (+0.3%)	2.62 ± 0.05 (−5.4%)	3.70 ± 0.004 (+1.0%)	*3.68 ± 0.004 (−1.9%)
HC, range of mean values <sup>b</sup>	2.70–2.81	2.67–2.77	3.12–3.76	3.21–3.75

<sup>a</sup>Mean ± SD (percent difference from control); \* $p \leq 0.05$ ;  $n = 10$ /sex/dose group for 20 litters/dose (EPA 2002a).

<sup>b</sup>Historical control (HC) based on two (PND 11) and 19 (PND 72) guideline DNT studies in the same laboratory.

In high-dose P-females, feed consumption was decreased during the third week of gestation (−9%; NS) and the first week of lactation (−14%,  $p < 0.05$ ), with an associated decrease in body weight gain during LD 0–7 (−33%,  $p < 0.05$ ). Treatment-related effects in high-dose F1 animals consisted of decreased body weight in males and females, averaging 11–13% less than control from PND 4–21, decreased motor activity (NS; ANOVA/Dunnett's) on PND 17 (−31 to −38% in males and females) and PND 21 (−37% in females only). EPA (2002a) also considered decreased caudate-putamen width (−1.9%;  $p = 0.03$ ; Chi-square/Fisher's exact test) in term females (PND 72), compared with controls, a treatment effect. Litter parameters, including litter size and birth weight, clinical signs, acoustic startle response, or measures of cognition were not affected at any dose, and there was no evidence of neuropathology in high-dose F1 males or females.

The modest (−1.9%; 70  $\mu$ m) difference in caudate-putamen width in high-dose F1 females on PND 72, compared with controls, was considered a treatment effect, based on statistical significance and a non-statistical (−5.4%;  $p = 0.07$ ) difference from control on PND 11, which suggested a consistent pattern (EPA 2002a). Measures at the mid-dose were requested to assist interpretation; however, shrinkage associated with the continued storage of those tissues in formalin (Garman et al. 2015; Tsuji & Crofton 2012) precluded morphometric comparison with tissues from control and high-dose animals. Those tissues were not processed and measured along with control and high-dose tissues because the study scientists considered the differences in caudate-putamen width at the high dose spurious and unrelated to treatment, given the minimal difference from control and the unusually high measure for the controls. In support of this interpretation, the group mean for the female concurrent control group at PND 72 set the upper limit value for 19 studies in the historical control database (3.21–3.75 mm), while the group mean for 750 ppm treated females (3.68 mm) was above the average for historical controls at PND 72 (Table 10). The lack of effect in high-dose males on PND 11 ( $p = 0.55$ ) or

on PND 72 ( $p = 0.25$ ) also supports the conclusion that this difference in females was unrelated to treatment.

The study scientists attributed the decreased motor activity for high-dose F1 males (PND 17) and females (PND 17 and 21) to acute toxicity associated with the pups consuming high doses of imidacloprid in the treated feed, in addition to the milk. This interpretation is supported by the pattern of occurrence in both sexes at the highest dietary level, specifically during late lactation when the pups are consuming the treated feed, with no effect on habituation at any age or any effect on any measure of activity on PND 13 or PND 60.

In summary, the high dose of 750 ppm imidacloprid decreased body weight and weight gain in P-females and F1 animals and reduced motor activity in F1 males and females during the period of peak exposure associated with consuming the treated diet before weaning. There were no treatment-related clinical signs, and no effect on the acoustic startle response or cognition at any dose level or evidence of neuropathology at the high dose. The evidence supports the difference in caudate-putamen width in high-dose F1 females, compared with controls, was a spurious finding but measures at lower doses are not available to assist the interpretation.

### Thiacloprid

Thiacloprid (99.2% purity) was administered on GD 0 to LD 21 at constant dietary levels of 0, 50, 300, or 500 ppm. The EPA (2002b) determined the NOAEL for P-females and F1 animals was 50 ppm (4.4 mg/kg/d), based on findings at 300 and 500 ppm (25.6 and 40.8 mg/kg/d, respectively). Body weight gain and food consumption were reduced in P-females during the first week of exposure (GD 0–6) at 300 and 500 ppm and LD 1–4 at 500 ppm. In F1 males and females, body weight was decreased on PND 7–21 and after weaning at 300 and 500 ppm, with delayed preputial separation at both dietary levels and delayed vaginal opening at the high dose (EPA 2002b).

Decreased body weight in mid- and high-dose F1 males and females was evident beginning on PND 7,

with statistical differences from control in both sexes on PND 11 (−9 to −15%) at 300 ppm and on PND 21 (−13 to −15%) at 500 ppm. After treatment was discontinued, body weight remained less than control at both doses, with a mean weight 8–10% less than control on PND 37 and 3–7% less than control on PND 64. Associated with the decreased body weight and weight gain during growth and development, preputial separation was delayed at the mid- and high-dose an average 1.5 d, compared with controls (PND 48.2 versus 46.7), and vaginal patency was delayed an average 1.3 d (PND 34.7 versus 33.4) at the high dose.

The EPA (2002b) noted “altered performance” in the passive avoidance task in F1 females at 300 and 500 ppm, including a significant increase in Trial 2 latency for the first test session and an adverse effect on retention (Table 11). For brain morphometry measurements in high-dose F1 males, measures of the corpus striatum (−4%) and corpus callosum (−14%) were significantly ( $p < 0.05$ ) less than control on PND 11 and measures of corpus striatum (−4%) and dentate gyrus (−5%) were significantly less than control at term, with non-significant differences from control in high-dose F1 females (corpus callosum (−3%) on PND 11 and corpus striatum (−2%) at term) (Table 12) cited as evidence to support a pattern of effects (EPA 2002b). Offspring survival, autonomic function, M-water maze performance, brain weight, and qualitative histopathology were not affected by treatment in F1 males or females at any dietary level.

For motor activity, both the mean number of movements and the time spent in movement were comparable with control F1 animals on all test days at all dietary levels (EPA 2002b); however, the EPA noted a “suggestive” effect that involved a higher number of movements for test intervals 2–4 (of six intervals), compared with controls, for 300 and 500 ppm males and females on PND 17 and 21. EPA (2002b) also considered non-statistical ( $p > 0.05$ , ANOVA/Dunnett’s) increases in the acoustic startle response for multiple testing blocks in PND 60 F1 males (300 and 500 ppm) and females (500 ppm) suggestive evidence of a treatment effect. The results show the mean response amplitude for males on PND 60 was 34% and 27% greater than control at the mid- and high-doses, respectively, while the mean response amplitude for high-dose females was 15% greater than control. The relationship between the motor activity and acoustic startle findings and treatment is unclear, as the differences from control were modest, inconsistent by sex and were generally not dose-related. For these reasons, the study scientists considered these differences from control were spurious and unrelated to treatment.

**Table 11.** Trials-to-criterion and latency (sec) for passive avoidance in F1 Sprague–Dawley rats treated with thiacloprid<sup>a</sup>.

	0 ppm	50 ppm	300 ppm	500 ppm
<b>Females</b>				
Session 1 (acquisition)				
Trials-to-criterion	4.8 ± 1.9	4.1 ± 0.7	4.0 ± 0.9	3.7 ± 1.0
Trial 1	10.2 ± 8.7	10.9 ± 9.6	11.0 ± 5.0	16.0 ± 14.5
Trial 2	29.2 ± 22.5	33.8 ± 23.3	<b>43.3 ± 21.9*</b>	<b>51.6 ± 14.7**</b>
Session 2 (retention)				
Trials-to-criterion	2.8 ± 0.7	3.1 ± 0.8	3.0 ± 0.7	3.1 ± 0.4
Trial 1	27.2 ± 26.2	20.8 ± 20.4	26.6 ± 21.9	23.6 ± 20.0
<b>Males</b>				
Session 1 (acquisition)				
Trials-to-criterion	4.3 ± 1.2	5.0 ± 2.8	4.2 ± 1.0	3.9 ± 1.3
Trial 1	8.2 ± 7.4	7.8 ± 5.2	11.2 ± 11.1	16.2 ± 15.9
Trial 2	32.8 ± 22.1	27.0 ± 22.8	32.9 ± 19.7	44.6 ± 19.5
Session 2 (retention)				
Trials-to-criterion	3.0 ± 0.8	3.0 ± 0.5	3.3 ± 1.1	3.0 ± 0.5
Trial 1	31.4 ± 26.3	18.5 ± 13.8	24.4 ± 21.5	18.9 ± 19.4

\* $p \leq 0.05$ . \*\* $p \leq 0.01$ ;  $n = 19$ –20/sex/dietary level (one pup/sex/litter) (EPA 2002b).

Acquisition was measured on PND 23–25 as the latency to cross from a lighted compartment where the animal was placed to the adjacent darkened compartment in a shuttle box. Upon crossing, a mild shock was delivered via the metal grid floor as an aversive stimulus. Acquisition was measured as an increase in latency to cross to the darkened side, compared with previous trials, and the number of trials to achieve criterion performance (failure to cross within the maximum 180 s trial duration on two consecutive trials). Retention was evaluated 7 d later, based on latency to cross for Trial 1, compared with Trial 1 of acquisition. The values shown in bold are the principal findings discussed in the text.

<sup>a</sup>Mean ± SD.

For passive avoidance, the neurobiological or toxicological significance of increased latency for Trial 2 of acquisition in F1 females is also unclear, as it would indicate more rapid, not impaired, acquisition and there was no difference in the number of trials-to-criterion for acquisition or retention at any dose. For this reason, and because males showed no similar difference from control, the study scientists considered this a spurious finding that was unrelated to treatment. For retention, the study scientists did not consider the numerically lower latency to cross for Trial 1 that EPA (2002b) noted for treated males was an effect, because it was not statistically significant or dose related and there was no other indication of a difference in performance in males to support this as a treatment effect.

Statistical and non-statistical differences in brain measurements noted above were generally modest (4–5%) and group means for treated animals (with only one exception) were within the historical control range (Table 12). The 14% lower measure of corpus callosum in PND 11 males was statistically significant but was not accompanied by a difference at PND 67–78 or in females at either age. This relatively large difference from control on PND 11 is attributed to greater variability in brain measures at that age,

Table 12. Brain measurements ( $\mu\text{m}$ ) in F1 rats treated with thiacloprid<sup>a</sup>.

	Males			Females		
	Control	500 ppm	Historical control <sup>b</sup>	Control	500 ppm	Historical control <sup>b</sup>
PND 11						
Corpus striatum	<b>2286 ± 69</b>	<b>2205 ± 94*</b> (-4%)	2052–2488	2265 ± 672	2253 ± 86	1938–2530
Corpus callosum	<b>293 ± 37</b>	<b>253 ± 44*</b> (-14%)	272–312	278 ± 34	269 ± 27	251–331
Termination (PND 67–78)						
Corpus striatum	<b>3156 ± 95</b>	<b>3018 ± 117*</b> (-4%)	2920–3624	3084 ± 110	3012 ± 93	2834–3379
Dentate gyrus	<b>1659 ± 82</b>	<b>1578 ± 75*</b> (-5%)	1552–1819	1458 ± 55	1491 ± 38	1420–1602

The values shown in bold are the principal findings discussed in the text.

\* $p < 0.05$ ;  $n = 10/\text{sex}/\text{dose level}$  (20 litters/dose) (EPA 2002b).

<sup>a</sup>Mean  $\pm$  SD (percent difference from control).

<sup>b</sup>Based on six guideline DNT studies (range of mean values).

when the brain is relatively small and growing rapidly, with less distinct surface markers for reference or myelination to help achieve consistent section homology (Bolon et al. 2006; Garman et al. 2001; Kaufmann & Groters 2006).

In summary, dietary levels of 300 and 500 ppm thiacloprid decreased maternal body weight gain and decreased body weight in F1 animals on PND 7–21 and after weaning, with delayed preputial separation at both doses and delayed vaginal opening at the high dose. EPA (2002b) noted “suggestive effects” on motor activity and acoustic startle and “altered performance” for passive avoidance at these doses, with differences from control for certain brain measurements in high-dose F1 males on PND 11 (corpus striatum and corpus callosum) or at term (corpus striatum and dentate gyrus), concluding the study is acceptable with an overall NOAEL of 50 ppm (4.4 mg/kg/d) for P-females and F1 offspring. The study scientists associated the delayed sexual maturation with decreased body weight during lactation and after weaning and considered the other findings represented spurious differences from control that were unrelated to treatment.

### Clothianidin

Clothianidin (95.5–95.9% purity) was administered on GD 0 to LD 21 at constant dietary levels of 0, 150, 500, or 1750 ppm. The NOAEL for P-females was 500 ppm (43 mg/kg/d) based on decreased body weight, body weight gain, and food consumption at the highest dietary level of 1750 ppm (PMRA 2002). The NOAEL for F1 animals was 150 ppm (13 mg/kg/d) based on decreased body weight, body weight gain, motor activity, and the acoustic startle response in females at 500 ppm (Table 9).

Food consumption was significantly reduced during gestation and lactation at 1750 ppm, with P-female body weight reduced as much as 8% ( $p < 0.05$ ) during both gestation and lactation. Body weight gain was markedly reduced, compared with controls, shortly after the introduction of treated feed on GD 0 (–63%, GD 0–3,  $p < 0.01$ ) and during early lactation (–67%, PND 4–7,  $p < 0.05$ ), whereas body weight gain was increased (+215%,  $p < 0.01$ ) on PND 14–22 of lactation, such that overall lactational body weight gain was similar to controls (PMRA 2002).

Body weight gain from PND 0 to 21 was decreased in F1 males and females by 18% ( $p < 0.01$ ) at 1750 ppm and by 7% ( $p < 0.05$ ) in 500 ppm females, with a maximum 16% ( $p < 0.05$ ) decrease in body weight at the high dose before weaning. Body weight was also decreased 6–7% ( $p < 0.05$ ) on PND 13–21 in F1 females at 500 ppm. After weaning, body weight remained significantly less than control in 1750 ppm F1 males and females on PND 23–37 (from –3 to –15%) and in F1 males on PND 72 (–4%), and body weight gain was reduced 21% ( $p < 0.05$ ) in F1 females on PND 65–72, compared to controls. A statistically significant decrease in the mean percent of high-dose F1 animals achieving criterion performance for surface righting on PND 3 was considered equivocal, and the onset of pinna unfolding, response to an acoustic stimulus, preputial separation or vaginal patency was not delayed at any dietary level (PMRA 2002). There were no treatment-related clinical signs; however, five high-dose F1 animals were found dead on PND 24–26, in spite of receiving only untreated feed since weaning on PND 21.

For motor activity, there was no effect on habituation or statistically significant difference in total session activity count or time spent in movement (PMRA 2002). However, on PND 21 there were non-statistical decreases in total motor activity (count) in



**Table 13.** Number of movements (counts) in F1 animals treated with clothianidin<sup>a</sup>.

Postnatal day	Dietary level (ppm)			
	0	150	500	1750
<b>Males</b>				
13	147 ± 160	163 ± 121	219 ± 164	169 ± 117
17	472 ± 241	368 ± 246	399 ± 301	448 ± 243
21	558 ± 172	490 ± 212	542 ± 224	<b>422 ± 237</b> (-24%) <sup>b</sup>
60 (±2 d)	723 ± 175	710 ± 128	759 ± 97	707 ± 140
<b>Females</b>				
13	220 ± 129	260 ± 200	200 ± 182	212 ± 155
17	475 ± 308	470 ± 242	476 ± 317	475 ± 310
21	579 ± 154	564 ± 195	<b>457 ± 229</b> (-21%) <sup>b</sup>	<b>519 ± 203</b> (-10%) <sup>b</sup>
60 (±2 d)	749 ± 136	663 ± 123	708 ± 113	651 ± 150 (-13%)

<sup>a</sup>Mean ± SD (percent difference from control);  $n = 20/\text{sex}/\text{dietary level}$  (1 pup/sex/litter); PMRA (2002).

<sup>b</sup>The values shown in bold are the principal findings discussed in the text.

F1 females at 500 ppm (-21%) and in 1750 ppm F1 males (-24%) and females (-10%), as well as decreased time (s) spent in movement for females at 500 ppm (-24%) and 1750 ppm (-19%) and for F1 males at 1750 ppm (-27%). The results for the number of movements are shown in Table 13 to illustrate these findings. Finally, there were statistically significant decreases in activity counts or time spent in movement in one-to-four of 12 5-min testing blocks on PND 21 for 1750 ppm males and females and the time spent in movement (-36%) in 500 ppm females for testing block 4. PMRA (2002) noted the interpretation of motor activity data is "made difficult due to the large coefficients of variation on PND 13, 17, and 21, which is a likely reason for lack of significance for some testing blocks".

PMRA (2002) attributed differences in activity count for F1 females at 500 ppm to treatment, based on a similar reduction in time spent in movement. The study scientists also concluded motor activity was decreased in 1750 ppm males and females, but concluded the differences in activity count for F1 females at 500 ppm were not a treatment effect since statistical significance was limited to only one test interval (block 4) and not the total session, there was no difference in F1 males at 500 ppm and the difference from control was generally not dose related.

The mean peak acoustic startle response was 48% ( $p < 0.01$ ) less than controls on PND 22 in 1750 ppm F1 females and 27% (NS) less than control in 500 ppm F1 females; the difference from control in 500 ppm females was statistically significant for only block 2 (-36%,  $p < 0.05$ ) of five 10-trial blocks. The values for control females at this age were high, relative to historical control; however, PMRA (2002) noted historical control

data were only available for two studies when this review was performed.

PMRA (2002) considered increased thickness of the hippocampal gyrus (+9%,  $p < 0.05$ ) and cerebellum height (+10%,  $p < 0.05$ ) on PND 11 and decreased thickness of the hippocampal gyrus (-5%;  $p < 0.05$ ) on PND 82-86 for 1750 ppm F1 females represented treatment-related effects. No morphometric findings were noted in 1750 ppm F1 males or 500 ppm F1 females at either age. The study scientists considered the aforementioned differences in 1750 ppm females spurious and unrelated to treatment, based on a lack of consistency (e.g. increased hippocampal gyrus and cerebellum measures on PND 11 and decreased hippocampal gyrus without a difference in cerebellum measures at term). An increased cerebellum height in high-dose F1 females on PND 11 (+10%) was also considered an isolated and spurious finding because there was no difference from control for males on PND 11 or either sex at term. Moreover, as noted for thiacloprid, it is especially difficult to achieve highly standardized coronal sections of the cerebellum in rats as young as PND 11 (Garman et al. 2001); consequently, the Society of Toxicologic Pathologists recommends that morphometric measures be conducted at weaning, rather than PND 11 (Bolon et al. 2006 2011).

In summary, the high dose of 1750 ppm clothianidin produced treatment-related effects on body weight, body weight gain, and food consumption in P-females and decreased body weight, body weight gain, motor activity, and acoustic startle in F1 males and females. In F1 females, transient and slight decreases in body weight, body weight gain, and the acoustic startle response were evident at 500 ppm. Increased measures of the hippocampal gyrus and cerebellum at PND 11 and decreased thickness of the hippocampal gyrus at term, relative to control groups, are considered to represent spurious findings that are unrelated to treatment.

### Thiamethoxam

Thiamethoxam (98.8% purity) was administered on GD 6 to LD 21 at constant dietary levels of 0, 50, 400, or 4000 ppm. The NOAEL for P-females and F1 animals was 400 ppm (34.5 mg/kg/d), based on treatment-related effects at the highest dietary level of 4000 ppm (298.7 mg/kg/d) (EPA 2007). Reduced body weight, body weight gain, and food consumption were evident in high-dose P-females during gestation and lactation, with a maximum 5% and 7% lower body weight during gestation and lactation, respectively, compared with controls. In high-dose F1 males and females, body

weight gain during lactation was 13–20% ( $p < 0.05$ ) less than control, with body weight less than controls at birth (–7 to –9%,  $p < 0.01$ ) and during lactation (–9 to –12%,  $p < 0.01$ ) and after weaning. The mean terminal body weight for high-dose animals selected for brain morphometry was 12–15% less than controls on PND 11 and 8–9% less than controls on PND 62 and absolute brain weight for F1 high-dose males and females was 4–5% less than controls ( $p < 0.05$ ) on PND 11 and PND 62 (EPA 2007). Preputial separation in high-dose males was also delayed by an average 1.5 d, compared with controls ( $p < 0.01$ ).

Associated with decreased brain weight at the high dose, brain measurements were generally reduced compared to control values. On PND 11, the cerebellum molecular layer thickness (–12%,  $p < 0.01$ ) and cerebellum length (–7%,  $p < 0.05$ ) were reduced in high-dose F1 males and thalamus width was reduced (–6%,  $p < 0.01$ ) in high-dose F1 females, compared to controls. Several other brain measurements averaged 3–5% less than control on PND 11, but the differences from control were not statistically significant. On PND 62, high-dose F1 males had significant reductions in dorsal cortex (–6 to –11%,  $p < 0.01$ ), piriform cortex (–9%,  $p < 0.05$ ) and corpus callosum thicknesses (–20%,  $p < 0.01$ ), thalamus height (–11%,  $p < 0.01$ ) and width (–7%,  $p < 0.01$ ), thalamus cortex overall width (–5%,  $p < 0.05$ ), and hippocampus width (–9%,  $p < 0.01$ ), compared with controls. On PND 62, high-dose F1 females had significant reduction in dorsal cortex thickness (–6 to –9%,  $p < 0.01$ ), thalamus width (–5 to –8%,  $p < 0.01$ ), thalamus/cortex overall width (–7%,  $p < 0.01$ ), and hippocampus width (–6%,  $p < 0.01$ ), compared with controls (EPA 2007). At lower doses, brain weight and morphometry were not different from control in males or females at either age. Moreover, there were no effects on functional or neurobehavioral parameters, as assessed by the functional observational battery, motor activity, acoustic startle response or learning, and memory tests at any dose (EPA 2007).

The study scientists concluded that the delay in the onset of preputial separation was secondary to reduced body weight during early growth and development. They also considered the differences in absolute brain weight and brain morphometry measures in the high-dose F1 animals were secondary to lower body weight during development, not a selective effect on the developing brain. To evaluate the relationship further, brain weight data were analyzed relative to terminal body weight (ANCOVA) and morphometric measurements were adjusted based on terminal body weight (ANCOVA) to determine whether the differences were related to decreased offspring growth resulting from

poor nutrition (Supplementary Table 2). When brain weight is adjusted for body weight, there is no statistically significant difference from control for any dose group. Similarly, there were no statistically significant differences for any brain measurement on PND 11, when adjusted for body weight. For the PND 62 measurements, small decreases in dorsal cortex thickness, thalamus width and/or hippocampus width, relative to controls, fell within the historical control range, while the concurrent control values were high, compared with the historical controls (Supplementary Table 3). The few statistically significant differences in PND 62 brain measurements that remained after adjustment for terminal body weight were also considered to be secondary to decreased body weight during brain growth and development.

In summary, the high dose of 4000 ppm thiamethoxam produced decreased food consumption and body weight in P-females throughout gestation and lactation, and significant decreases in F1 offspring body weights at birth, during lactation and associated growth and development. As a result, absolute brain weights and brain measurements were lower in the F1 offspring at 4000 ppm, compared to control, and the males had a 1.5-d delay in onset of preputial separation. The reduced brain weight and size in the F1 offspring is consistent with prior studies, which show large deficits in maternal nutrition reduced both body weight and absolute brain weight of the offspring (Garman et al. 2001; West & Kemper 1976).

### Dinotefuran

Dinotefuran (99.5% purity) was administered on GD 6 to LD 21 at constant dietary levels of 0, 1000, 3000, or 10 000 ppm. There were no treatment-related effects in the P-females or F1 males or females at any dietary level (EPA 2013b); therefore, a NOAEL for all toxicological effects in this study was established as 10 000 ppm (784 mg/kg/d). A transient decrease in body weight gain (–45%,  $p < 0.01$ ) from GD 6 to 9 in the P-female 10 000 ppm dose group was not considered adverse, since it was not sustained (e.g. no effect on GD 9–12, 12–15, 15–18, and 18–20) and there was no difference in body weight among dose groups during gestation, lactation, or post-weaning.

### Discussion

This review identified six studies reported in the literature that tested three neonicotinoid insecticides for evidence of DNT, as well as previously unpublished DNT studies conducted in accordance with EPA (1998)

guidelines for all six neonicotinoid insecticides that are registered for commercial uses in major markets. The published DNT studies used an assortment of *in vivo* and *in vitro* test systems and study designs with various research objectives. By comparison, the guideline-based studies are standardized and designed for use to identify evidence of DNT and for risk assessments, with a relatively large sample size, complement of tests and selection of doses administered by a route that is relevant to human circumstances of exposure with commercial uses (Raffaele et al. 2010). The guideline-based studies were not specifically tailored for the neonicotinoids, but the study design lends itself to identify evidence of DNT by any potential mode of action. The consistent study design used in these studies also serves to facilitate comparisons of findings across the class, in spite of the studies being performed at four different laboratories. Since clothianidin is a principal metabolite of thiamethoxam, evaluating the results from these two independent studies offers an excellent opportunity for comparison.

Following a brief summary of DNT effects with nicotine below, the results of epidemiology and DNT studies with neonicotinoid insecticides are discussed in terms of biological significance, human relevance, and consistency of effects across the different neonicotinoids and in comparison with the effects of nicotine.

### **Evidence of developmental neurotoxicity with nicotine**

Potential concern that the neonicotinoid insecticides could affect the developing nervous system is associated with their nicotinic mode of insecticidal action and recognition of nicotine as a likely developmental neurotoxicant in humans and laboratory animals. A detailed review of the evidence from individual DNT studies with nicotine is beyond the scope of this paper; therefore, the findings cited here are primarily taken from reviews on the subject (Abbott & Winzer-Serhan 2012; Bruin et al. 2010; Dwyer et al. 2008 2009; Pauly & Slotkin 2008; Slikker et al. 2005; Slotkin 1998 2004; Tiesler & Heinrich 2014).

In humans, the adverse effects associated with cigarette smoking during pregnancy on fetal and postnatal development have been the subject of many epidemiology studies. Adverse health consequences associated with smoking during pregnancy include low birth weight, sudden infant death syndrome, attention deficit hyperactivity disorder (ADHD), and various cognitive/behavioral disorders. However, components in cigarette smoke other than nicotine may contribute to these effects. For example, some studies using nicotine

replacement (e.g. transdermal patches) during pregnancy indicate nicotine *per se* does not adversely affect newborn body weight (Clark & Nakad 2011; Wisborg et al. 2000).

Animal studies have also described adverse developmental effects following exposure to nicotine during gestation and/or lactation. In these studies, nicotine was generally administered to pregnant rats or mice via minipumps, repeated subcutaneous injections, or the drinking water, to achieve and maintain dose levels approximating moderate-to-high tobacco usage. The results from these studies suggest that developmental nicotine exposure impacts a variety of functions in the developing fetus/neonate, including altered growth, control of respiratory and/or cardiovascular systems, motor activity levels, and/or cognitive function. Nicotine is believed to interfere with neuronal development, with adverse effects on various brain regions, including neocortex, hippocampus, cerebellum, and/or the limbic system (Dwyer et al. 2009). Developmental exposure to nicotine has been associated with many biochemical alterations in the brain, including decreased DNA synthesis, increased ornithine decarboxylase and *c-fos* activity, and changes in neurotransmitter levels and adenylyl cyclase activity (see reviews by Dwyer et al. 2009; Slikker et al. 2005). Quantitative morphometric and structural abnormalities have also been shown in various brain structures, including the hippocampus, cerebral cortex and nucleus accumbens, following gestational exposure to nicotine. In several brain regions, including the nucleus accumbens, Muhammad et al. (2012) noted changes in the number of dendritic branch bifurcations, dendritic length, and/or spine density of distal dendrites of the offspring (PND 21) of rats exposed to nicotine prenatally. Roy et al. (2002) noted increased numbers of glia in the hippocampus and somatosensory cortex of the offspring (PND 21–30) of rats exposed to nicotine during gestation. These investigators also noted decreased neuronal cell size and reductions in the thickness of the CA1, CA3, and dentate gyrus layers in the hippocampus. In both studies, these findings were noted in the absence of any effect of nicotine on offspring body weight.

### **Comparison of nicotine and neonicotinoid modes of action**

Binding of nicotine to mammalian nAChRs during development is a necessary step leading to developmental neurotoxicity (Slikker et al. 2005). One likely consequence of nAChR stimulation during gestational/neonatal CNS development is the premature onset of cell differentiation, resulting in neuronal death and/or

structural alterations in regional brain areas. In contrast to nicotine, the neonicotinoid insecticides exhibit nicotinic activity (e.g. tremors) in adult rats only at high dose levels, due to their low affinity for nAChR subtypes in vertebrates and poor penetration of the blood–brain barrier (Chao & Casida 1997; Tomizawa & Casida 2005; Yamamoto et al. 1995) (Table 2). Based on the differences in binding affinity to nAChR subtypes of different species, the toxicological properties of nicotine in insects and mammals are substantially different from the neonicotinoids.

In accord with such differences, the Insecticide Resistance Action Committee (IRAC 2015) Mode of Action Classification Scheme makes the following distinctions in sub-classes of insecticides acting as nAChR agonists (Table 14). Regarding the assignment of nicotine and other chemicals to sub-groups (4B, 4C, and 4D) separate from the neonicotinoids (4A), the IRAC made the following comment: “Although these compounds are believed to have the same target site, current evidence indicates that the risk of metabolic cross-resistance between subgroups is low” (IRAC 2015). The IRAC sub-grouping of nicotine separately from the neonicotinoids, along with the literature studies that highlight the large inter-species differences in affinity for binding to nAChR’s of mammals or insects (Table 2), point to very different biological and toxicological properties of the neonicotinoids, compared to nicotine.

Sulfoxaflor and the butenolides are sub-grouped separately from either nicotine or the neonicotinoids; accordingly, chemicals from these two subgroups (4C and 4D) are not evaluated in detail in this review. Sulfoxaflor is distinguished from the other sub-groups by the manner in which it interacts with the  $(\alpha_1)_2\beta_1\delta\gamma$  nAChR expressed in the muscle of the fetal rat, which produces forelimb flexure, hind-limb rotation, bent clavicles and decreased neonatal survival as a result of sustained muscle contraction (Rasoulpour et al. 2012). In the rat, the fetal nAChR  $(\alpha_1)_2\beta_1\gamma\delta$  is replaced shortly after birth with the adult form  $(\alpha_1)_2\beta_1\delta\epsilon$ , which does not appear to interact with sulfoxaflor. Given the fundamental qualitative differences in sulfoxaflor agonism on the rat versus the human muscle nAChR, this mode of action is not relevant to, or at least very unlikely to occur, in

humans (Ellis-Hutchings et al. 2014). Therefore, sulfoxaflor is a developmental toxicant in the rat but it does not result in neurotoxicity in the adult rat or other mammalian species, due to its specificity in interaction with the fetal nAChR.

### Neonicotinoid epidemiology studies

Two epidemiology studies were identified that examined the potential relationship between maternally reported history of residential proximity to pesticide applications during pregnancy and neurodevelopmental defects (Yang et al. 2014) or maternally reported history of using imidacloprid products for flea and tick treatment on pets and ASD (Keil et al. 2014). Yang et al. (2014) showed a general lack of association between proximity to agricultural pesticide use and risks of selected birth defects. Keil et al. (2014) reported a “weak imprecise positive association” (the lower bound estimates for the 95% CI was less than or equal to 1) between product use during pregnancy and increased risk of ASD that could result from exposure misclassification alone, due to potential bias of mothers with autistic children and reliance on recall from ~4 years prior to the survey. Exposure estimates associated with post-application contact with cats and dogs treated with products containing 10% imidacloprid, similar to the conditions experienced by users in the study by Keil et al. (2014), are 3.4–6.6  $\mu\text{g}/\text{kg}/\text{d}$  (Lunchick 2010; Supplementary Appendix II), which indicate very low risk associated with such uses. In fact, these exposure estimates are ~3000–6000-fold lower than the NOAEL of 20 mg/kg/d for the DNT study with imidacloprid in rats and 1400–2700-fold lower than the NOEL of 9.3 mg/kg/d that is used for intermediate-term human risk assessments by EPA (2012b).

### Comparison of neonicotinoid and nicotine: in vitro study

Kimura-Kuroda et al. (2012) reported excitatory  $\text{Ca}^{2+}$  influx with nicotine, imidacloprid, and acetamiprid that was possibly associated with nAChR activation in primary cultures of cerebellar granule cells from

Table 14. IRAC classification of chemicals interacting with the nAChR<sup>a</sup>.

Main group and primary site of action	Chemical sub-group or exemplifying active ingredient	Active ingredients
(4) Nicotinic acetylcholine receptor (nAChR) agonists	(4A) Neonicotinoids	Acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam
	(4B) Nicotine	Nicotine
	(4C) Sulfoxaflor	Sulfoxaflor
	(4D) Butenolides	Flupyradifurone

<sup>a</sup>IRAC (2015).



newborn rats at 1–100  $\mu\text{M}$ . Differences in firing patterns, proportion of excited neurons, and peak excitatory  $\text{Ca}^{2+}$  influxes that were evident with imidacloprid and acetamiprid, relative to nicotine, were attributed to different effects on nAChRs. It is unclear whether evidence of  $\text{Ca}^{2+}$  influx in this system is associated with nAChR activity or whether this would lead to DNT, due to the transient nature of the exposure and advanced stage of cell maturation. Moreover, systems that limit access to sensitive tissues *in vivo* (e.g. metabolic degradation and the blood–brain barrier) did not exist in this test system; therefore, such findings identify a potential molecular-initiating event *in vitro*, but do not necessarily represent an adverse outcome for DNT. Previously, these neonicotinoids have been shown to express marked differences in nAChR activity and lower potency than nicotine, which was not the case in this study. Therefore, these findings should be confirmed and must be interpreted in the context of the results from *in vivo* studies.

### **Comparison of neonicotinoids and nicotine: in vivo DNT studies**

#### **General indices of growth (P-females and F1 animals)**

Nicotine has anorexic properties in mature animals that have been associated with central cholinergic-linked metabolic processes (Jo et al. 2002). Several studies have shown decreased body weight gain with post-natal exposure to doses of nicotine as low as 0.5 mg/kg/d (Abbott & Winzer-Serhan 2012). The effect of prenatal nicotine exposure on pup birth weight is not as clearly defined (Winzer-Serhan 2008), although a more recent study in rats suggests that prenatal nicotine reduces birth weight (Schneider et al. 2011). While evidence supports nicotine having a specific, direct effect on processes that mediate body weight gain, decreased birth weight and decreased body weight gain commonly represent generalized non-specific effects or are associated with reduced feed consumption with treatments administered at high dietary concentrations.

Decreased body weight gain, body weight, and/or food consumption occurred in P-females at the high dose in five of the six guideline DNT studies, with decreased body weight gain and food consumption also occurring with clothianidin at the next lower dietary level. With dinotefuran, a transient decrease in body weight gain in high-dose P-females was attributed to palatability at an approximate limit dose of 10 000 ppm in the diet, rather than an adverse or toxic effect. Decreased body weight and body weight gain were also the most common effects in F1 animals. Sustained

decreases in body weight during development were associated with delayed sexual maturation in high-dose males (thiacloprid and thiamethoxam) and females (thiacloprid). By comparison, Tanaka (2012b) reported increased body weight and weight gain in F1 mice treated with clothianidin during lactation and evidence of accelerated development at all dose levels. The differences from control in this and a related study (Tanaka 2012a) show a possible treatment-related increase in weight gain in treated mice during early development and maturation, but the observed differences were not dose related and therefore may not be robust or represent a treatment-related effect.

#### **Neuropathology and brain morphometry (F1 animals)**

An investigation of neonicotinoids for DNT should include a thorough microscopic examination of the brain, including regions associated with morphologic effects following exposure to nicotine during development or that contain cholinergic neurons. In this context, exposure to nicotine during gestation has been reported to produce structural abnormalities in the hippocampus and somatosensory cortex in rats (Roy et al. 2002) and the cerebellum, hippocampus, basal ganglia, and thalamus are rich in nAChR (Court et al. 2000). Abou-Donia et al. (2006) have also reported that exposure of P-females to 3.3 mg/kg/d nicotine via minipump on GD 4–20 decreased surviving Purkinje neurons in the cerebellum of F1 animals and increased GFAP immunostaining in the cerebellum (white matter and granular cell layer) on PND 90.

As per EPA (1998) guidelines, the guideline DNT studies with the neonicotinoids included a rigorous microscopic evaluation of brain, spinal cord, peripheral nerves, and other neural tissues for neuropathology, along with gross and microscopic measurements of several brain regions. The results from these studies identified no evidence of neuropathology in any neural tissue, including brain regions cited for morphologic changes following developmental exposure to nicotine (Roy et al. 2002) or that are rich in nAChR (Court et al. 2000).

Abou-Donia et al. (2008) reported increased GFAP immunostaining by 16–45% in specific areas of the motor cortex and hippocampus of rats on PND 30, without evidence of histopathology, following a single high-dose of imidacloprid (337 mg/kg; 75% of the acute oral  $\text{LD}_{50}$  value) delivered i.p. on GD 9. However, the authors note that these findings require additional investigation to support, due to the limited scope of this study. Moreover, it is difficult to devise a mode of action to explain the occurrence of an active glial



response in specific brain regions 7 weeks after a single dose that was administered when only rudimentary elements of the CNS existed (implantation of the blastocyst on GD 6). In terms of human relevance, i.p. administration of a high dose in corn oil has unknown relevance to human exposure circumstances, as this route bypasses first-pass catabolism in the gut and liver and the absorption kinetics are unknown. The guideline DNT study with imidacloprid administered at dietary levels equivalent to 55 mg/kg/d during gestation and 80 mg/kg/d during lactation also reported no evidence of neuropathology in cerebral cortex or hippocampus (EPA 2002a). GFAP activity was not measured in this study, but there was no evidence of inflammation (e.g. microglia) associated with activated astrocytes on PND 11 or PND 72.

While brain weight and associated brain tissue are typically conserved in adult rats that experience decreased food consumption and body weight with various treatments, decreased maternal nutrition during gestation and lactation has been shown to decrease offspring body weight and brain measurements independent of chemical exposure (Garman et al. 2001; West & Kemper 1976). Where large deficits in maternal nutrition reduce offspring body weight and absolute brain weight, the brains of undernourished pups may never achieve the age-appropriate developmental size of controls (Garman et al. 2001). Moreover, in such cases, the effect may be greater on brain regions that mature after birth (e.g. cerebral cortex, cerebellum, and hippocampus) than on subcortical structures that develop *in utero* (Garman et al. 2001). Garman et al. (2001) noted the need for additional published results on brain weight adjusted for body weight in DNT studies for a wide array of compounds to allow a systematic determination of whether alterations in these measurements are predictive of neurotoxicity or are instead an indicator of malnutrition.

The DNT study with thiamethoxam provides a useful example of compound-induced decreases in birth weight, lactational body weight, absolute brain weight, and brain size that appears to be secondary to lower maternal nutrition. Among the studies cited in this review, the high dose of thiamethoxam had the greatest effect on body weight in F1 animals during development, which was associated with a decrease in brain weight and several brain measurements (EPA 2007). With thiamethoxam, the pattern of a consistent decrease in multiple brain measurements (without adjustment for body weight), the decrease in absolute brain weight and F1 offspring body weight, and the 15–20% decrease in P-female food consumption throughout gestation and lactation and no specific indicators of DNT, supports the

conclusion that decreased absolute brain weight and brain measures at the high dose resulted from malnutrition during gestation and lactation, rather than a direct effect on the developing brain.

Differences in microscopic brain measurements are more problematic to interpret in the absence of neuropathology, as a decrease in such measurements may result from non-specific malnutrition during development (e.g. as seen with thiamethoxam) or represent biological variability or variability associated with methods for selection of homologous sections and morphometry measures, rather than a specific effect on brain development. There is higher biological variability in younger animals (e.g. PND 11) and curved brain structures (e.g. hippocampus and cerebellum). In addition, 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 interpretation, it is useful to determine whether differences from control are dose related or consistent by age and gender and to compare measures for control and treated animals to historical control values that are suited for comparison. The interpretation is also more problematic when measures are only available for control and high-dose animals, as measures for other dose groups would otherwise help illustrate biological variability among groups of 6–10 rats per sex within the same study and a dose-response relationship. As in this review, it is also useful to compare positive and negative findings among representatives of the same chemical class or mode of action to determine whether there is a consistent pattern of effects, but it is important to verify whether the measures from different studies are suited for comparison.

Treatment with imidacloprid was associated with a slight ( $-1.9\%$ ,  $p \leq 0.05$ ) decrease in caudate-putamen thickness in high-dose females on PND 72, with a numerically lower ( $-5.4\%$ ) but non-statistical difference from control in high-dose females on PND 11 (EPA 2002a). A decrease in the same measurement at both ages may be considered consistent over time; however, disruption of morphologic development would alter growth and differentiation, which would be expected to produce a greater effect on tissue size later in development (Bolon et al. 2006; Garman et al. 2001). In the present case, one would expect a greater (not lesser) effect on PND 72 than PND 11, since this measure of the caudate-putamen increased by 35% from PND 11 (2.77 mm) to PND 72 (3.75 mm) in control females. Measures at lower doses are not available to determine whether this difference was dose related or to establish a clear NOAEL, as the tissues from lower dose groups were

not measured, due to shrinkage from continued storage of those tissues in formalin (Garman et al. 2015; Tsuji & Crofton 2012). To avoid this situation, the timely processing of all tissues reserved for morphometry to paraffin or slides at all dose levels is now standard laboratory practice. In the present case, it is relevant to note measures for high-dose males were not different from control at either age and the modest differences from control in high-dose females were associated with a particularly high group mean for the concurrent control group (i.e. this control group set the upper limit for 19 DNT studies that constituted the laboratory HC database), while the group mean for high-dose females was well within the HC range. Therefore, it is reasonable to accept the study scientists' conclusion that this finding in one sex is not a robust or treatment-related effect.

With thiacloprid, statistical differences in measures of corpus striatum and corpus callosum in high-dose F1 males on PND 11 and corpus striatum and dentate gyrus in high-dose F1 males at term were attributed to treatment (EPA 2002b). As with imidacloprid, measures were not taken at lower doses to assist interpretation and establish a clear NOAEL. However, there were no similar differences in females at either age and the magnitude of the difference from control did not increase with further growth and development of the tissues.

The only evidence of potential overlap among the morphometric findings involves the measures of caudate-putamen and corpus striatum with imidacloprid (EPA 2002a) and thiacloprid (EPA 2002b). However, precise comparisons cannot be made, due to differences in the measurements that were taken. With imidacloprid, the caudate-putamen width was measured at the level of the optic chiasm, whereas with thiacloprid the corpus striatum was measured as the diagonal width of the caudate-putamen and globus pallidus combined at the level of the septal nuclei and anterior commissure. Further, this finding was evident in females with imidacloprid and in males with thiacloprid, and in both cases, the differences were slight (−1.9% to −5.4% for imidacloprid and −4% for thiacloprid) and the finding with imidacloprid was associated with an exceptionally high mean value for the concurrent female control group (Table 10).

Thus, there are no reports of neuropathology with any of the neonicotinoids evaluated, which includes brain regions identified as rich in nAChR (Court et al. 2000), regions reported to express structural abnormalities following developmental exposure to nicotine (Roy et al. 2002) and regions that showed an excitatory response to neonicotinoids in primary cell cultures

(Kimura-Kuroda et al. 2012). Among the brain measurements used to investigate DNT, differences from control were either associated with decreased body weight during development, including lower birth weight (thiamethoxam), or were evident in only one sex for a given neonicotinoid (imidacloprid and thiacloprid) or inconsistent among representatives of the class. Moreover, we are not aware of any gender-specific brain lesion associated with exposure to a neuroactive chemical during sexually-immature stages of development. For the guideline study with imidacloprid (EPA 2002a), the differences in caudate-putamen and corpus striatum measurements are likely spurious and unrelated to treatment, given the biological variability and unusually high group mean value for female controls.

### *Clinical signs and motor function (P-females and F1 generation)*

The expression of acute “nicotinic signs” with nicotine varies to some extent by species. In humans, nicotinic signs generally consist of an excitatory phase that may include nausea and vomiting, excessive salivation, sweating, hypertension, tachycardia, ataxia, tremor, and seizures, and a later phase with hypotension and bradycardia, central nervous system depression, coma, muscular weakness, paralysis, or respiratory failure (Schep et al. 2009).

None of the DNT studies in this review reported nicotinic signs of toxicity in P- or F1 animals treated with a neonicotinoid insecticide. With imidacloprid, tremor has been reported to occur in adult rats and mice at high doses administered by gavage, but not at lower doses or with dietary administration (Sheets 2014), as the target tissue concentrations required to produce such effects are only achieved under such circumstances. In the guideline DNT studies, the neonicotinoids were generally administered via the diet. Although acetamiprid was administered by gavage to the P-females in the guideline DNT study, nicotinic signs were not evident in the P-females or F1 animals at any dose. The lack of nicotinic signs in these studies reflects the low activity of the neonicotinoids toward nAChR subtypes in mammals, poor penetration of the blood–brain barrier and the low tissue levels associated with sustained exposures, which are generally relevant to human circumstances of exposure.

Evidence of neurologic or behavioral effects with neonicotinoid insecticides includes slight deficits associated with beam-walk time, inclined plane test and grip time in 30-d-old rats treated with a single intraperitoneal dose of 337 mg/kg imidacloprid on GD

9 (Abou-Donia et al. 2008). However, additional investigation is required to determine whether these findings are reproducible and represent treatment related effects, based on the widely accepted criteria used to judge data reliability. A more thorough investigation, as suggested by the study authors, has not been reported and other studies in rats did not use the same tests or administer the treatment at a similar dose or route. For reference, deficits in motor coordination were not evident in the guideline DNT study with imidacloprid, at dietary levels that achieved a minimum 55 and 80 mg/kg/d during gestation and lactation, respectively (EPA 2002a). However, subtle deficits in motor function may have contributed to decreased activity in high-dose F1 males and females on PND 17 and 21 when they were exposed to high doses of imidacloprid via the diet.

### **Motor activity**

Motor activity reflects the general status or condition of the animal and is sensitive to a variety of neurotoxic and non-neurotoxic mechanisms (Reiter 1983), including disturbances of sensory, motor, reactivity, excitability, cognitive, motivational states, perturbation of regulatory states, or general malaise. As such, automated measures of motor activity performed under well-controlled conditions are suited to screen chemicals for toxicity but not to characterize the underlying cause unless there is additional information to assist interpretation. For this reason, an automated measure of activity is included in neurotoxicity screening studies with pesticidal active ingredients (OCSP 870.6200 and OECD TG 424) using adult rats, in combination with detailed clinical observations, a functional observational battery (FOB) and neuropathology.

Mixed results have been reported regarding the effect of gestational and/or postnatal exposure to nicotine on motor activity. While many studies report increased activity or no effect in rodents exposed prenatally to nicotine, others have reported decreased activity (see review by LeSage et al. 2006). Schneider et al. (2012) speculated that repeated daily testing is necessary to detect an increase in activity following gestational exposure to nicotine (Supplementary Table 4), presumably to allow the animals to habituate to the activity chambers. However, Tizabi et al. (2000) did not detect an increase in activity on the fourth consecutive day of testing to high daily doses of nicotine. Furthermore, Thomas et al. (2000) and Huang et al. (2007) both report changes in activity without prior experience in the chamber, but the effects were not consistent (an increase and decrease, respectively) (Supplementary Table 4).

The reason(s) for these mixed results with nicotine is unknown. Contributing factors could involve differences among the studies in the daily nicotine dose, route of administration (including gradual increments of dose levels), period of exposure, control for hypoxia and weight loss, postnatal time point of testing, and/or some aspect of the testing procedures (LeSage et al. 2006; Schneider et al. 2012). For most studies, motor activity was tested after exposure to nicotine was discontinued, looking for persistent or latent effects. While a transient decrease in activity during the exposure period is a sensitive measure of acute toxicity with many chemical agents (Reiter 1983), a persistent change in activity, hyperactivity or decreased habituation after discontinuation of exposure are more clearly associated with DNT (Norton 1976), especially if such effects occur at doses that do not produce maternal toxicity.

Motor activity is measured in guideline-compliant (EPA 1998) DNT studies during the period of exposure on PND 13, 17, and 21, as well as on PND 60 to test for latent or persistent effects (Tyl et al. 2008). The effects noted in the guideline studies with neonicotinoids consisted of decreased motor activity at the highest dietary level with imidacloprid on PND 17 and 21 (EPA 2002a) and with clothianidin on PND 21 (PMRA 2002), without an effect on habituation in either case (Table 9). Decreased activity in F1 animals during the peak period of exposure to the test substance is consistent with the nature of the effect (e.g. decreased activity without an effect on habituation) and the sensitivity of this assay to detect acute toxic effects. With dietary exposure during lactation, F1 animals receive substantial dosages (mg/kg/d) of the test substance by consuming the treated diet during the last several days before weaning on PND 21, in addition to exposure through the milk.

Tanaka (2012a,b) evaluated mice treated during development with clothianidin for effects on eight activity parameters measured for 10 min each at 3 and 8 weeks of age and for 120 min (statistically analyzed for each 10-min interval) at 9–10 weeks of age (Table 5). In Tanaka (2012a), dietary exposure to clothianidin (0, 20, 60, and 180 ppm) during gestation and lactation did not result in any statistically significant difference from control for any of the movement parameters measured at 3 or 8 weeks of age, with the exception of decreased average time of rearing at 8 weeks in mid-dose F1 females only (Table 5). Tanaka (2012a) reported an increased average speed of movement (cm/s) in 3-week-old F1 males based on a trend test, but there were no statistical differences based on multiple comparisons between treated and control groups. These isolated findings are not consistent with an adverse effect on

motor activity, considering the lack of a consistent pattern across dose levels, sex and time, and the small number of statistical differences out of many analyzed parameters. In 9–10 week old mid-dose F1 males, several inter-related activity parameters were statistically increased in one or two of the 10-min intervals during the last 30 min of the 120-min session. However, there was no significant difference from control in the longitudinal pattern for any of the eight activity parameters at any dose level (Table 5). There were also no statistically significant differences in the low- or high-dose male groups or any of the female F1 groups at 9–10 weeks of age.

In Tanaka (2012b), mice received dietary levels of 0, 30, 60, and 120 ppm clothianidin from 5 weeks of age (P generation) through gestation, lactation and post-lactation, to 11 weeks of age. At 3 weeks, there was an increased trend for the number of rearings in F1 female mice but no statistical differences in pairwise comparisons and no effects in any of the eight parameters for males including inter-related measures of rearing time, average time of rearing and movement time (Table 5). In the F1 mice at 8 weeks, mid-dose males showed a statistically decreased average rearing time, whereas males had a significantly increased trend for movement time. Other measures in treated F1 male mice had no differences from control or significant trends, and none of the parameters was affected in F1 females at 8 weeks. At 9–10 weeks of age, all movement variables measured were parallel in a longitudinal pattern, except for movement time in females. Three statistically significant decreases in one parameter or one treatment group (low or mid-dose) were reported for an isolated 10-min interval (e.g. significantly lower average speed (cm/s) in females only at 70 min), but the overall pattern across the entire 120 min session showed no effect on any of these parameters (Table 5). Although Tanaka (2012b) considered the findings to represent adverse effects, the pattern was not consistent when considering the negative and positive results for the complement of movement parameters measured across age, sex, dose, and test intervals.

In summary, the effects of clothianidin on motor activity reported by Tanaka (2012a,b) were inconsistent within each study and across both studies, without a coherent pattern of increased or decreased activity measures in treated mice. The author attributes the different results across studies to differences in the two study designs, but the findings are not consistent across endpoints or sex, nor are they consistently dose related. Further, the statistical analysis did not address the multiplicity problem associated with the large number of statistical comparisons (e.g. dose levels, sex,

parameters, age of testing, and test intervals), which inflates the chance of Type I (false positive) errors (Holson et al. 2008; Maurissen 2010). In the guideline DNT study, dietary exposure to clothianidin from GD 6 to PND 21 was associated with decreased activity on PND 21 in F1 male and female rats at 142 mg/kg/d (both sexes) and females at 42.9 mg/kg/d (PMRA 2002). This profile of effects is consistent with decreased motor activity in high-dose F1 rats on PND 17 and 21 in the guideline DNT study with imidacloprid. The occurrence of a similar effect during a period of high exposure before weaning is also consistent with the interpretation that this represents an acute toxic effect, which is a very common outcome in acute neurotoxicity studies with a variety of treatments, including imidacloprid (Sheets 2014).

### *Acoustic startle*

Sustained exposure to nicotine during gestation has been reported to increase the startle response in F1 rats on PND 14, 18, and 70 (Lacy et al. 2011). The exposure used in this study was designed to simulate tobacco use during pregnancy, with the administration of 0.05 mg/kg nicotine three times daily on GD 8–21 via IV catheterization to the dam. While the test procedure investigated the effects of nicotine on pre-pulse inhibition, the effect on the startle response can be derived from trials without a pre-pulse stimulus. However, these results cannot be used to evaluate the effects of nicotine on startle habituation, since the variable pre-pulse conditions that were delivered among the trials may impede habituation (Koch 1999).

The startle response was increased in adolescent (1.5 months) zebrafish at 45  $\mu$ M but not at 60  $\mu$ M nicotine and there was no effect in adults (3 months) at either concentration (Crosby et al. 2015). Habituation of the startle response was not affected at either age. Similarly, developmental exposure of zebrafish to imidacloprid at 45 or 60  $\mu$ M increased the startle response in adolescents and not in adults, without affecting habituation of the response at either age (Crosby et al. 2015).

For guideline-compliant studies, the acoustic startle response test is designed and primarily included to evaluate the effects of treatment on habituation (EPA 1998), a primitive learning process in which the organism decreases its response to redundant, non-significant stimuli (Davis 1984). None of the neonicotinoids affected startle habituation in weanling or adult F1 animals at any dose level. Acetamiprid and clothianidin decreased total session startle response amplitude in F1 males and/or females without affecting habituation, but the pattern of effects with these two treatments differed. With acetamiprid, differences from control were evident in

males only, on both PND 20 and 60, whereas with clothianidin, the effect was greater in females, with differences from control in both sexes only on PND 22. These results also differ from the reported effect of nicotine to increase the startle response following gestational exposure in rats (Lacy et al. 2011) or the reported increase in startle response in adolescent (but not adult) zebrafish exposed to nicotine or imidacloprid during early development (Crosby et al. 2015). For both acetamiprid (EPA 2008) and clothianidin (PMRA 2002), the effects on startle amplitude in guideline DNT studies occurred only at doses that decreased body weight, body weight gain, and pup survival.

### *Cognition (learning and memory)*

There is wide-spread concern for reduced academic achievement and impaired intellectual abilities in the children of mothers who smoke during pregnancy (Clifford et al. 2012) but the results from investigations to determine whether there is a clear relationship are inconclusive (Tiesler & Heinrich 2014). The results from animal studies with nicotine are mixed, potentially due to differences in the model used (species, gender, nicotine dose, and critical period of exposure) and/or the task used to assess performance. For example, prenatal exposure of rats to nicotine has been associated with deficits in a spontaneous alteration task or radial arm maze performance (Levin & Slotkin 1998; Levin et al. 1993). These reports note the treatment-related effects with nicotine are more apparent with more complex tasks. While some investigators have demonstrated persistent cognitive deficits from prenatal nicotine exposure based on findings from two-way active avoidance tasks (Vaglenova et al. 2008), others report no effect of prenatal nicotine exposure in the acquisition of choice accuracy in a radial arm maze (Cutler et al. 1996). Eppolito and Smith (2006) evaluated the effects of nicotine in the Morris water maze of young-adult offspring of rat dams continuously infused with nicotine (~1 mg/kg/d) from GD 4 to PND 11. At PND 60, swim speed was significantly reduced in F1 males but not in females. The distance and the latency for Path A were significantly increased in F1 females (not in males) when collapsed across all 5 d of testing, but not for individual days. The longer latencies in nicotine-exposed females, which appeared to decrease after test days 2 and 3, were considered a deficit in reference memory. Abbott and Winzer-Serhan (2012) reviewed the collective results for these and other animal studies and concluded that developmental exposure to nicotine has only a minor effect on cognitive performance.

Male rats dosed daily by gavage with 24 mg/kg of clothianidin from PND 7 until PND 97 spent

significantly less time than controls in the target quadrant of the Morris water maze (Table 5). On one hand, the occurrence of this difference at the high dose suggests a treatment effect on consolidation of memory and the absence of this finding in a separate group of males that were treated for a similar duration beginning as adults suggests an effect associated with exposure during development. On the other hand, the deficiencies in this study that pertain to data reliability and human relevance (Table 4) should be considered in evaluating the weight-of-evidence for clothianidin having an effect on cognition.

Three other DNT studies have investigated clothianidin for effects on cognition and determined it had no effect. Each of these studies administered clothianidin in the diet, while Ozdemir et al (2014) treated the F1 males by gavage. Treatment of mice at dietary levels as high as 29 and 100 mg clothianidin/kg/d during gestation and lactation, respectively, did not affect performance at 7 weeks of age in a Biel-type water maze (Tanaka 2012a,b), which involves learning to escape a relatively challenging multiple-T water maze. In the guideline study, dietary exposure to clothianidin from GD 6 to PND 21 did not affect acquisition, retention or other measures of performance in a passive avoidance conditioning test performed after weaning or a water maze task at PND 62–63 at any dose, including the high dose of 142–299 mg/kg/d administered via the diet (PMRA 2002; Table 9). Therefore, the finding reported at 24 mg/kg/d with clothianidin (Ozdemir et al. 2014) is the only report of any neonicotinoid affecting cognition.

Acetamiprid was evaluated for potential cognitive effects using the complex Biel water maze testing paradigm, with sequential (Path A) and reverse (Path B) learning (EPA 2008). EPA noted an increased number of errors for trials 5–10 in Path B (NS) for the high-dose PND 22 F1 males, compared to controls, but no similar trend in F1 females or in Path A for males or females (Figure 2). Treatment with acetamiprid had no effect on acquisition of the task in PND 22 males or females at any dose, and there were no effects on PND 62 in males or females at any dose. Based on the lack of consistency for the two paths and ages in males, and no effect on latency or any measure in females, the study scientists concluded that acetamiprid had no effect on learning or memory.

In the guideline study with thiacloprid, the high dose (40.8 mg/kg/d) increased latency for Trial 2 of passive avoidance for the F1 females at weaning, compared to controls (EPA 2002b). In this test, the rats are shocked if they move from the lighted chamber in which they are placed to an adjacent darkened chamber. The biological significance of this finding is unclear, as there was no



effect on the number of trials-to-criterion for acquisition or retention at any dose and increased latency to cross for Trial 2 is consistent with more rapid, not impaired, task acquisition. There was also no effect on measures of learning or memory in the M-water maze, when the F1 offspring were tested as adults, and this finding occurred in only one sex, with no associated behavioral (e.g. motor activity) or neuropathology findings. Therefore, it is reasonable to accept the study scientists' conclusion that this is a spurious finding, unrelated to treatment.

In summary, there were inconsistent findings on cognitive tests following gestational and/or lactational exposure to clothianidin (24 mg/kg/d), acetamiprid (45 mg/kg/d), and thiacloprid (25.6 and 40.8 mg/kg/d). Although the mixed results for clothianidin could be due to the different exposure duration and assessment paradigms, the only study that reported an effect (Ozdemir et al. 2014) had the greatest deficiencies, based on the criteria used to evaluate strength and human relevance (Table 4). For acetamiprid and thiacloprid, the study scientists did not consider the differences from control to represent an effect of treatment on cognitive behavior. In addition, these findings were only seen in weanling pups at dose levels that substantially increased neonatal pup death (acetamiprid), decreased body weight in dams during gestation and lactation (acetamiprid and thiacloprid) or decreased pup weight prior to weaning (thiacloprid) (Table 9). The guidelines for a DNT study require that the highest dose level induce "overt signs of maternal toxicity" (EPA 1998). This can cause a combination of stress and nutritional deficits to dams and/or their offspring, which can affect cognitive test results (Markham et al. 2010; Weinstock 2008).

### Conclusions/weight of evidence

This review determined that there are relatively few studies published on the subject of DNT for the neonicotinoid insecticides and those studies generally have notable limitations relative to conformance criteria used to establish human relevance. A major challenge in translating the mode-of-action findings from published literature is associated with differences in metabolism and distribution (e.g. the lack of a blood-brain barrier in *in vitro* models); thus, these studies may be informative in certain respects but may not inform *in vivo* DNT responses. Other published studies are generally limited in critical aspects that pertain to human relevance, such as the selection of endpoints, sample size, and number of dose levels. By comparison, the sample size, route and duration of exposure, the selection of dose levels and the complement of tests used in the previously-

unpublished DNT studies performed in accordance with EPA (1998) guidelines are designed for hazard identification and use in risk assessments. Although these studies were not tailored to specifically investigate DNT associated with a nicotinic mode of action, the published literature with nicotine indicates the complement of tests in the study design is well suited for this purpose.

The collective information that is available shows no consistent pattern of effects among the six neonicotinoids or for any of these insecticides compared with nicotine. Dinotefuran produced no adverse effects in the guideline-compliant study at any dose level (EPA 2013b). The principal effects with the other neonicotinoids are associated with decreased body weight of F1 animals during development (e.g. delayed sexual maturation, decreased brain weight, and morphometric measurements) and acute toxicity (decreased motor activity associated with peak exposure via the diet and milk). Other findings among the individual studies, including differences in various brain measurements or performance in a neurobehavioral test, are inconsistent with a selective effect on the developing nervous system. These results may be useful for risk assessment purposes but the collective evidence indicates the neonicotinoid insecticides are not developmental neurotoxicants. This outcome is consistent with the relatively low activity of neonicotinoid insecticides toward nAChR subtypes that are expressed in mammals and other vertebrates and the absence of any other effect to suggest these compounds would be developmental neurotoxicants.

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### Declaration of interest

The authors are employed or funded by companies that manufacture and sell the neonicotinoid insecticides that were evaluated in this review. The authors are experts on the toxicology of these insecticides, with responsibility for addressing regulatory toxicology issues and reviews. RHC is involved in litigation for dinotefuran; however, the subject of litigation

does not pertain to DNT and EPA concluded dinotefuran had no effect in the DNT study, as reflected in this review. None of the other authors is involved in litigation for the neonicotinoid insecticides reviewed in this paper. This review is premised upon the publically-available literature, the publically-available EPA reviews of the DNT studies and the DNT study reports and data analysis. All authors contributed to the conception, design, data analysis and interpretation, drafting and critically revising this review paper, including sections unrelated to the insecticide produced by the company for which they worked. At the end of the review and drafting process and immediately prior to submission of the manuscript, a copy of the final draft was sent to company representatives for comment. Revisions made in response to comments and suggested edits were minor and did not alter or contribute to the opinions and conclusions. Thus, the analysis, interpretation and conclusions presented herein are solely those of the authors.

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