European Food Safety Authority

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## SCIENTIFIC OPINION

# Scientific Opinion on the developmental neurotoxicity potential of acetamiprid and imidacloprid[1]

**EFSA Panel on Plant Protection Products and their Residues (PPR)**[2, 3]
European Food Safety Authority (EFSA), Parma, Italy

This Scientific Opinion, published on 21 February 2014, replaces the earlier version published on 17 December 2013[*].

## ABSTRACT

The European Food Safety Authority asked the Panel on Plant Protection Products (PPR) and their residues to deliver a Scientific Opinion on the developmental neurotoxicity (DNT) potential of the neonicotinoid insecticides acetamiprid and imidacloprid. An *in vitro* study (Kimura-Kuroda et al., 2012) suggested that excitation and/or desensitisation of nicotinic acetylcholine receptors (nAChRs) by these compounds might affect developing mammalian nervous systems as occurs with nicotine. To evaluate the DNT potential of acetamiprid and imidacloprid, the PPR Panel scrutinised the open literature, the draft assessments reports and dossiers submitted for approval. The Panel concludes that both compounds may affect neuronal development and function, although several methodological limitations have been identified. Considering the available DNT studies for imidacloprid and acetamiprid, important uncertainties still remain and further in vivo studies following OECD test guideline (TG) 426 are required to robustly characterise a DNT potential and dose-response relationships, particularly for acetamiprid. The Panel considers that current ARfDs may not be protective enough for the possible DNT of acetamiprid and imidacloprid and no reliable conclusion can be drawn as regards the ADI for acetamiprid. More conservative reference values are proposed based on the analysis of the existing toxicological data. However, the current ADI for imidacloprid is considered adequate to protect against its potential developmental neurotoxic effects. Limitations of the in vitro system used by Kimura-Kuroda et al. (2012) prevent its current use as a screening tool in the regulatory arena. The PPR Panel encourages the definition of clear and consistent criteria at EU level to trigger submission of mandatory DNT studies, which could include development of an integrated DNT testing strategy composed of robust, reliable and validated in vitro assays and other alternative methods complementary to the in vivo TG 426 for assessing the DNT potential of substances.



[1] On request from the European Commission, Question No EFSA-Q-2012-00958, adopted on 21 November 2013.

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[3] Acknowledgement: The Panel wishes to thank the members of the Working Group on Acetamiprid and Imidacloprid Developmental Neurotoxicity: Antonio F. Hernández-Jerez, Karen Ildico Hirsch-Ernst, Alberto Mantovani, Colin D. Ockleford, Anna Bal-Price and Laura Ricceri for the preparatory work on this Scientific Opinion and EFSA's staff member Manuela Tiramani for the support provided to this Scientific Opinion.

[*] Minor changes of editorial nature were made. In addition, an amendment was done as the DNT study on imidacloprid was presented as two separate studies instead of only one as would have been more correct. However, the practical relevance of this is null as the opinion of the Panel is based on a detailed assessment of the findings of the study, which are not affected by this amendment. To avoid confusion, the original version of the Opinion has been removed from the website, but it is available on request, as is a version showing all the changes made.

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**KEY WORDS**
neonicotinoid, developmental neurotoxicity risk assessment, *in vitro* testing, nAChR agonists, pesticide approval regulations

## SUMMARY

Following a request from the European Commission, the EFSA Panel on Plant Protection Products and their Residues (PPR Panel) was asked to deliver a Scientific Opinion on the developmental neurotoxicity (DNT) potential of the neonicotinoid insecticides acetamiprid and imidacloprid.

Acetamiprid and imidacloprid have been evaluated under the criteria of Council Directive 91/414/EEC and approved in Regulation (EU) No 540/2011. In 2012, a paper was published (Kimura-Kuroda et al., 2012) describing *in vitro* experiments performed with acetamiprid and imidacloprid. It suggested that excitation or desensitisation or both of nicotinic acetylcholine receptors (nAChRs) by these neonicotinoids might affect the developing mammalian nervous system as is known to occur with nicotine.

To evaluate the DNT potential of acetamiprid and imidacloprid, the PPR Panel scrutinised the information available on the two neonicotinoid compounds in the open literature as well as in toxicological assessments for regulatory purposes, including the dossiers submitted for approval within the EU.

The following questions were identified by the European Commission and answered by the PPR Panel:

*Q1 Based on the available data, both in the scientific literature and in the toxicological dossier submitted for approval, do acetamiprid and imidacloprid exhibit developmental neurotoxic effects?*

Based on the available data, the PPR Panel concludes that both acetamiprid and imidacloprid show some indications of DNT potential. Evidence of effects on offspring from a developmental neurotoxicity study for imidacloprid in rats, submitted within the EU and the US-EPA assessment frameworks, reported decreased pup body weights, reduced motor activity level and changes in dimensions of brain structures (reduction in the thickness of corpus callosum and a decreased width of caudate/putamen). The DNT study for acetamiprid, carried out within the US-EPA assessment framework, showed decreased pup body weights, reduced pup pre-weaning survival and decreased maximum auditory startle response. The Panel therefore concludes that the two neonicotinoid compounds may affect neuronal development and function. However, there are limitations concerning the available evidence due to methodological issues and uncertainties as to whether or not neurotoxic effects in offspring occurred below doses eliciting maternal toxicity.

*Q2 Have acetamiprid and imidacloprid been assessed adequately for developmental neurotoxicity and if not, which further information would be required?*

Comprehensive toxicological databases are available for these two neonicotinoids. Toxicological *in vivo* studies include acute, subacute, subchronic, chronic, mutagenicity, carcinogenicity, reproduction and developmental studies carried out for the hazard characterisation of these compounds within the EU regulatory framework. However, available DNT studies show limitations. A DNT study on imidacloprid included data that may not be sufficient for a robust characterisation of dose-response, e.g. for changes in dimensions of brain structures. Regarding the DNT study performed for acetamiprid, important endpoints such as motor activity or learning and memory, which are expected to be the most sensitive DNT endpoints, could not be adequately assessed. Moreover, there are uncertainties with respect to the conclusion of the study report concerning the No Observed Adverse Effect Level (NOAEL) based on reduced pup auditory startle response. Overall, in the opinion of the Panel, the study can only provide supportive evidence, but is inadequate for a robust characterisation of effects and dose-response of acetamiprid-induced DNT. Thus, the PPR Panel concludes that further good quality *in vivo* studies following OECD TG 426 are required to more properly characterise a DNT potential as well as associated dose-response relationships, particularly for acetamiprid.

*Q3 Do the existing health-based guidance values provide adequate protection against any potential developmental neurotoxicity of acetamiprid and imidacloprid and if not what values would be necessary to provide such protection?*

Based on the indications provided by the available DNT studies and the associated uncertainties in establishment of the corresponding NOAELs, the Panel considers that the current ARfDs may not be protective enough for the possible developmental neurotoxicity of acetamiprid and imidacloprid. The same uncertainties do not allow to set a reliable ADI for acetamiprid. However, the ADI set for imidacloprid would provide adequate protection against its potential adverse effects on the developing nervous system. Accordingly, more conservative reference values are proposed based on the existing toxicological data.

The PPR Panel recommends that a more conservative NOAEL of 2.5 mg/kg bw per day for acetamiprid should be used as a point of departure for the derivation of ADI, ARfD and AOEL, which should all be set at 0.025 mg/kg bw per day. When new and more reliable DNT data are available, the point of departure can be revised.

As the current ARfD and AOEL for imidacloprid may not be protective enough for potential developmental neurotoxicity of this active substance, the Panel also recommends to conservatively lower these reference values to the same level as the ADI (0.06 mg/kg bw per day).

*Q4 Do the approved neonicotinoids need further investigation to clarify the mechanism of action of the nAChRs? Should all neonicotinoids be tested using the in vitro system mentioned in the publication of Kimura-Kuroda et al. (2012)?*

The PPR Panel considers that further research is warranted on the role of nAChRs in neuronal differentiation and maturation to fully understand the potential DNT induced by acetamiprid and imidacloprid. The *in vitro* system used in the study of Kimura-Kuroda et al. (2012) covers only very limited aspects of brain functions and its limitations currently prevent its use as a tool for screening developmental neurotoxicants in the regulatory arena. In particular, the *in vitro* system as proposed by this study requires considerable further characterisation and should be investigated to assess its relevance to the *in vivo* situation. To extend confidence in findings, provision of positive and negative controls and scrutiny of data for reliability and reproducibility are required. Only then should a test based on this system be considered as a possible screening tool for neurotoxicity potential. Moreover, the Panel recommends that further studies should be performed using the culture of other brain structures which also express nAChRs (hippocampus, entorhinal cortex, basal ganglia or thalamus) where the key developmental processes such as neuronal and glial proliferation, migration, differentiation, neurite outgrowth, synaptogenesis, networking, myelination and programmed cell death may be evaluated.

Based on the overall appraisal of existing data, revised in the context of the present Scientific Opinion, the PPR Panel considers that *in vitro* tests currently cannot substitute for *in vivo* DNT tests. Besides, no *in vitro* test can be used to date to set health-based reference values. The complexity of neurodevelopmental processes does not allow a proper assessment at the cellular level and behavioural outcomes cannot be assessed by *in vitro* models. However, *in vitro* assays may be regarded as complementary to animal testing because they may provide better understanding of the cellular/molecular mechanisms involved in developmental neurotoxicity. As such, *in vitro* tests could be incorporated into a DNT testing strategy to obtain mechanistic information or for purposes of screening/prioritisation. Following the provision included in section 5.6.2 ("Developmental toxicity studies") of the Commission Regulation n. 283/2013 on data requirements for active substances, the PPR Panel encourages the definition of clear and consistent criteria at EU level to trigger submission of mandatory DNT studies, which could include development of an integrated and cost-effective, tiered testing strategy composed of robust, reliable and validated *in vitro* assays and alternative methods complementary to the *in vivo* test guideline 426 for assessing the developmental neurotoxicity potential of substances.

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## BACKGROUND AND TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

Acetamiprid and imidacloprid are neonicotinoid insecticides evaluated under the criteria of Council Directive 91/414/EEC and approved in Regulation (EU) No 540/2011. Recently a paper has been published in the scientific literature[4] suggesting that excitation or desensitisation or both of nicotinic acetylcholine receptors (nAChRs) by neonicotinoids may affect the developing mammalian nervous system as is known to occur with nicotine. New data have now been evaluated by the Rapporteur Member States for these substances (Greece for acetamiprid and Germany for imidacloprid).

In their evaluation, Greece highlighted the need for further investigations to clarify the mechanism of action of acetamiprid and imidacloprid, while they accepted the conclusions reported in the study[4].
On the other hand, Germany concluded that the study merely contributes to the explanation of cellular mechanisms behind the neurotoxic effects of nicotine and neonicotinoids acetamiprid and imidacloprid. The German reviewers did not agree with the conclusions of the published study.

The following questions have been identified:

Question 1: Based on the available data both in the published scientific literature and in the toxicological dossier submitted for approval, do acetamiprid and imidacloprid exhibit developmental neurotoxic effects?

Question 2: Have acetamiprid and imidacloprid been assessed adequately for developmental neurotoxicity and if not, what further information would be of value in this assessment?

Question 3: Do the existing health-based guidance values provide adequate protection against any potential developmental neurotoxicity of acetamiprid and imidacloprid and if not what values would be necessary to provide such protection?

Question 4: Do the approved neonicotinoids need further investigation to clarify the mechanism of action of the nAChRs? Should all the approved neonicotinoids be tested using the *in vitro* system mentioned in the publication of Kimura-Kuroda et al. (2012)?

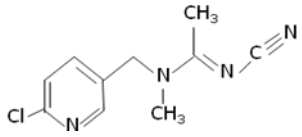
[4] Kimura-Kuroda J, Komuta Y, Kuroda Y, Hayashi Kawano H. Nicotine-like effects of the neonicotinoid insecticides acetamiprid and imidacloprid on cerebellar neurons from neonatal rats. PloS ONE 2012; 7 (2): e32432. doi: 10.1371/journal.pone.0032432

## ASSESSMENT

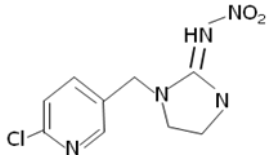
### 1. The active substances: general characterisation, mechanisms of action and metabolism.

Acetamiprid (ACE) and imidacloprid (IMI) belong to the first generation of systemic neonicotinoid insecticides. These compounds account for about 25 % of the current global insecticide market (Swenson and Casida, 2013). They are structurally similar to nicotine (see their chemical structure below):

(E)-$N^1$-[(6-chloro-3-pyridyl)methyl]-$N^2$-cyano-$N^1$-methylacetamidine (ACE)



(E)-$N^1$-1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitroimidazolidin-2-ylideneamine (IMI)



Nicotine

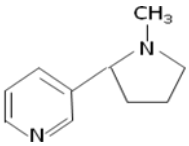


**Table 1:** Basic toxicological features of the three compounds

| | Nicotine | Imidacloprid | Acetamiprid |
|---|---|---|---|
| **Molecular Weight** | 162 (498*)<br>(Matta, 2007) | 255.7<br>(Gervais, 2010) | 222.68<br>(Abramovitch, 2002) |
| **Lethal/ near lethal dose for humans**[5] | 30-60 mg<br>0.5-1.0 mg/kg bw adults (Gosselin, 1988).<br>10 mg children (Arena, 1974) | < 44.6 ng/L (< 0.17 nM)<br>Plasma concentration 5 h post-ingestion in an intensive care survivor (Mohamed, 2009) | 2.3-59.8 mg/L<br>(10.3-268.7 µM)<br>Serum concentration after acute poisoning (Imamura, 2010; Todani et al., 2008) |
| **Oral $LD_{50}$ rat** | 50 mg/kg bw<br>(Okamoto, 1994) | 500 mg/kg bw male<br>380 mg/kg bw female<br>(Gervais, 2010) | 417 mg/kg bw male<br>314 mg/kg bw female<br>(Greece, 2001) |
| **Oral $LD_{50}$ mouse** | 3.34 mg/kg bw<br>(Okamoto, 1994) | 130 mg/kg bw male<br>170 mg/kg bw female<br>(Gervais, 2010) | 198 mg/kg bw male<br>184 mg/kg bw female<br>(Singh, 2012) |

*Nicotine hydrogen tartrate salt.

[5] No lethal doses for ACE or IMI have been identified in humans, but only plasma levels after acute poisoning. However, there are available lethal/near lethal dose of nicotine for human and children.
See: http://www.inchem.org/documents/pims/chemical/nicotine.htm#DivisionTitle:7.2.1.1 Adults

**Absorption, distribution, metabolism and excretion of ACE and IMI**

The neonicotinoids are moderately soluble in water, non-ionised, not readily hydrolysed at physiological pH and biodegradable, thus they do not accumulate in mammals or through food chains (Tomizawa and Casida, 2003).

*Acetamiprid*

ACE is rapidly ($C_{max}$ was reached at 0.5-7 h) and extensively absorbed after single (1 or 50 mg/kg bw) (~96 %) and repeated (1 mg/kg bw) (> 60 %) oral administration. It is then distributed reaching the highest concentrations in liver, kidneys, adrenals and thyroid. Excretion is rapid, mainly via urine during the first 24 h after treatment, with more than 90 % of the compound being excreted at 96 h.

After single oral administration in rodents, ACE is extensively metabolised, with 50-70 % of the dose being excreted as metabolites in both urine and faeces. After repeated administration, metabolites account for more than 90 % of the dose (Greece, 2001). The main metabolic pathway in rats is the demethylation to IM-2-1 (*N*1-[(6-chloro-3-pyridyl) methyl]-*N*2-cyanoacetamidine). This metabolite is further transformed to IC-O (6-chloronicotinic acid), with the release of IS-2-1 (N-cyanoacetamidine derivative) after cleavage from the side-chains (see Fig. 1). In lactating goats, less than 1 % of the radioactivity of the administered ACE was detected in milk, with the predominant residue being the IM 2-1 metabolite (JMPR, 2005).

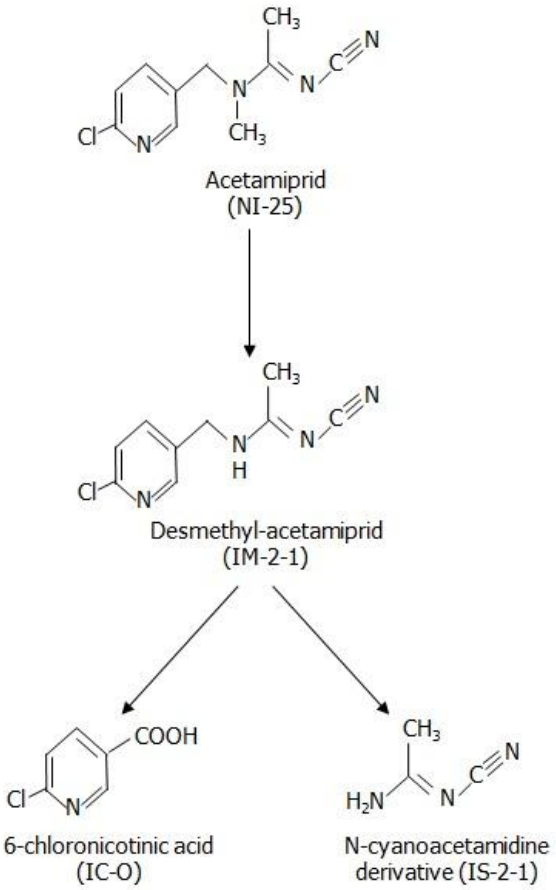



**Figure 1:** Proposed metabolic pathways of ACE (NI-25, parent compound) in rats **(**for more details see Joint FAO-WHO meeting on Pesticide residues in food - Evaluations 2011; http://apps.who.int/iris/bitstream/10665/75147/1/9789241665278_eng.pdf)

*Imidacloprid*

IMI is rapidly ($C_{max}$ was reached at 1-4 h) and almost completely absorbed after oral administration of 1 or 20 mg/kg bw in rats and then widely distributed in the body to all tissues with the exception of the central nervous system (CNS), fatty tissues and the mineral portion of bone following either oral or i.v. administration (Klein, 1987). Maximum concentrations are reached in kidneys and liver. It is excreted mainly via urine (75 %). By contrast, other studies have found both IMI and some metabolites, including desnitro-IMI, in the brain after intra-peritoneal administration of a single dose of the parent compound to mice (Ford and Casida, 2006).

After oral administration of single and repeated doses to rats IMI shows extensive metabolism, with metabolites accounting for about 78 % of the recovered radioactivity. Up to 90 % of the administered dose is metabolised in the liver through two pathways (WHO, 2001; USDA, Forest Service, 2005). In the first route, IMI undergoes aerobic nitro-reduction by either the microsomal CYP450s or the cytosolic molybdo-flavoenzyme aldehyde oxidase (AOX) to yield nitrosimine (M07, IMI-NO) and amino-guanidine (M08, IMI-$NH_2$) derivatives and desnitro-IMI (M-09, IMI-NH) (Dick et al., 2007; Swenson and Casida, 2013). Desnitro-IMI is further degraded by oxidative cleavage of the methylene bridge to imidazolidine, which is excreted in the urine, and 6-chloronicotinic acid that undergoes further metabolism via glutathione conjugation to form mercaptonicotinic acid and hippuric acid. In the second major pathway, IMI is metabolised by hydroxylation of the imidazolidine ring by CYP3A4 to form 4-hydroxy and 5-hydroxy metabolites (M01 and M02, respectively). This goes on to form glucuronide conjugates, and dihydroxy IMI (M03). The hydroxylated compounds may lose water to form the olefin metabolite (M06). Differences in AOX expression and nitroreduction may result in species differences in toxicity and residue dissipation (Swenson and Casida, 2013).

It is not known whether IMI can be converted to desnitro-IMI in the brain. However, this possibility might theoretically occur because the nitroreduction is primarily due to AOX (Swenson and Casida, 2013) and AOX has been found in liver, lung, brain and spinal cord (Kurosaki et al., 1999).

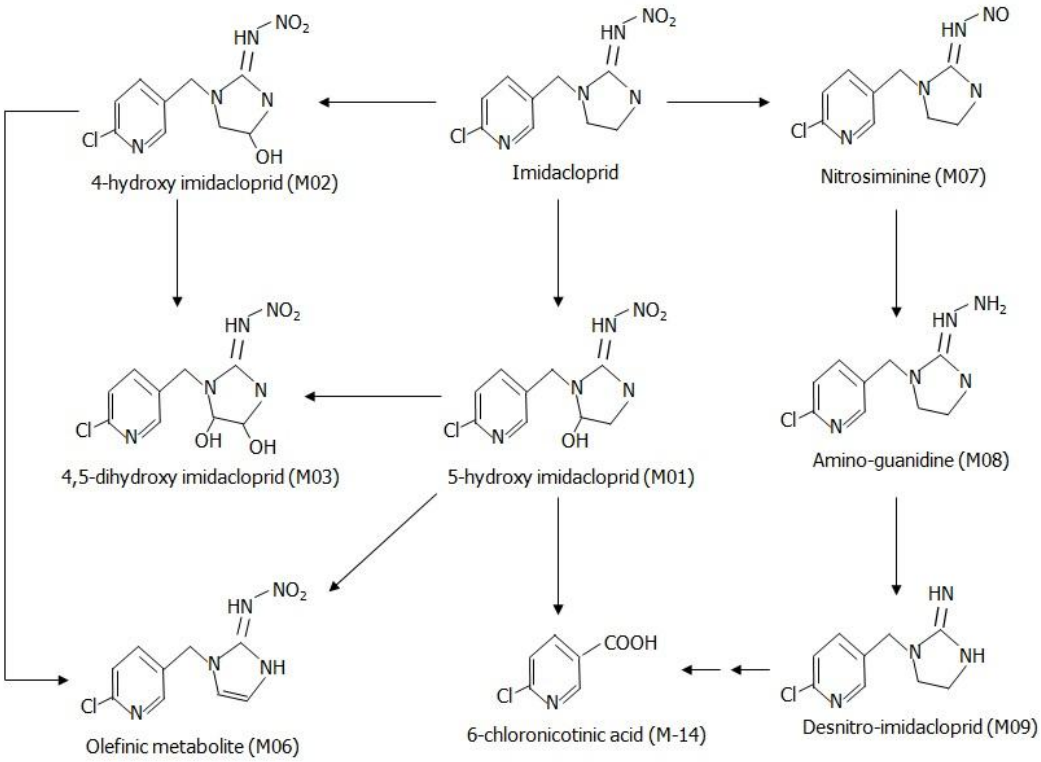



**Figure 2:** Proposed metabolic pathways of imidacloprid in the lactating goat FAO (adapted from http://www.fao.org/ag/AGP/AGPP/Pesticid/JMPR/Download/2002_eva/IMIDA_EVjjb.pdf).

Either ACE or IMI are cleaved to the same urinary metabolite (6-chloronicotinic acid) derived from the chloropyridinylmethyl moiety. The desnitro-IMI is more active on mammalian α4β2 receptors than the corresponding insect systems and more toxic to mammals than the parent insecticide. This metabolite shows high affinity to and/or agonist potency at the mammalian α4β2 nAChR subtype comparable to or greater than that of nicotine (Tomizawa and Casida, 2003). Accordingly, the desnitro metabolites can be considered as bioactivation products (Ford and Casida, 2006).

*Penetration of IMI and ACE through blood brain barrier (BBB)*

The evaluation of the actual relevance of the findings of Kimura-Kuroda et al. (2012) is hampered by the paucity of data concerning nicotinoids and neonicotinoids permeability to the BBB in adulthood and during development. IMI was initially thought to be excluded from vertebrate brain (Rose, 2012). In contrast, the study of Kimura-Kuroda et al. (2012) mentions that ACE and IMI "readily pass through the blood brain barrier", a statement based on data from Ford and Casida (2006) who report that at doses of 10 mg/kg intraperitoneally administered to adult albino mice, IMI and ACE penetrate into the brain reaching maximum tissue levels of 6 µg/g (15 min after dosing) and 3 µg/g (240 min after dosing), respectively. Comparatively, 18 and 12 µg/g were observed for liver and 8 and 6 µg/L for plasma. However, no information is currently available on permeability to IMI and ACE of the immature BBB. Any penetration is expected to be greater than at adulthood, though ontogeny of permeability to non-protein compounds is more heterogeneous and may depend on the nature of the agent considered (Saunders et al., 1999).

The mammal BBB and particularly its development are increasingly known (Ballabh et al., 2004; Liebner et al., 2012). The rates of development of the brain vary between human and laboratory animal species (Engelhardt, 2006, 2008) and the rat is not considered a good model for extrapolation to humans. There is insufficient data to estimate risks in the infant less than 4 months old (Webster, 2009) owing to the lack of knowledge on the efficiency of the BBB at various ages as well as to allometric considerations, which affect comparative dosage estimates and require refinement based on surface area rather than on mass. Accordingly, there is a need for further research to establish to what degree IMI and ACE and their metabolites cross into the human brain and to quantify any such capacity at different stages of the BBB development.

Nicotine, which is mostly protonated at physiological pH, is known to cross the adult human and rat BBB to become concentrated in the brain (Matta et al., 2007; Cisternino et al., 2013). Recent data on adult rats have found that an organic cation transport system (pyrilamine-sensitive) mediates blood-to-brain influx transport of nicotine at the BBB and this process is expected to play an important role in regulating nicotine-induced neural responses (Tega et al., 2013). In an *in situ* brain perfusion study using the micromolar concentrations found in the plasma, most (79 %) of the net transport of nicotine at the BBB was carrier-mediated by a proton-coupled antiporter that helps nicotine cross the BBB at a faster rate and to a greater extent than passive diffusion, which accounts for 21 % (Cisternino et al., 2013). This antiporter also transports and is competitively inhibited by other secondary or tertiary amine CNS compounds (André et al., 2009). Since desnitro-IMI behaves as a nicotinoid rather than a neonicotinoid compound, this metabolite might use the same transport system to reach the brain. In fact, the desnitro metabolite enters the brain following direct intraperitoneal administration in mice (Chao and Casida, 1997). As for permeability of nicotine into the developing brain, it is known that nicotine reaches the foetus through placenta and is concentrated in foetal blood at levels 15 % higher than in maternal blood (Luck et al., 1985). Nicotine is also transferred to the maternal milk with a milk/serum ratio of 2.9 (Luck and Nau, 1984). The effects of prenatal nicotine exposure on foetal brain have been extensively documented in rat models, including cell loss (in forebrain and cerebellum), up-regulation of nAChRs and consequent alterations also on catecholaminergic function (Slotkin, 1998).

It is known that nicotine can interact directly with nAChRs on brain endothelial cells. In both preclinical and clinical studies, nicotine produce BBB dysfunction through alterations in tight junction

protein expression and conformation, increased glial activation, increased enzyme activation related to BBB cytoskeleton remodelling, and induction of neuroinflammatory pathways (Kousik et al., 2012). Preclinical studies using rodent models of acute and chronic nicotine exposure reported compromised BBB integrity. *In vitro*, nicotine produces a dose-dependent (0.1–100 μM) decrease in transendothelial electrical resistance leading to increased BBB permeability, sufficient enough to produce a 60–150 % increased invasion of *Escherichia coli* across brain microvascular endothelial cells (Kousik et al., 2012). It is important to establish whether exposure to neonicotinoids such as ACE and IMI could affect BBB permeability.

### Mode of action as insecticides

Both nicotine and neonicotinoid insecticides bind to nicotinic acetylcholine receptors (nAChRs) and mimic the action of acetylcholine by opening the ion channels which allow the entry of $Na^{+}$ and $Ca^{2+}$ into cells. These compounds vary in their affinity for different nAChR subtypes, with nicotine showing selective toxicity for vertebrates whereas neonicotinoids are highly selective for insect nAChRs. The binding of neonicotinoids to insect nAChRs is virtually irreversible.

Despite the common structural features, neonicotinoids and nicotine have different protonation states at physiological pH. The basic nitrogen of nicotine will become positively charged in neutral aqueous solution because it is protonated by water. This positive charge gives nicotine a strong affinity for mammalian nAChRs as occurs with the ammonium head of ACh, which is an essential requirement to interact with the nAChRs (Tomizawa and Casida, 2003). At the same time, the charge on nicotine lowers its effectiveness as an insecticide, because the insect BBB reduces free access of ions through the central nervous barrier (Rose, 2012). The BBB does not prevent nicotine poisoning in mammals as it is well known that it crosses the BBB (Cisternino et al., 2013). In addition nicotine can interact with mammalian nAChRs located in the peripheral nervous system and necessary for vital functions such as breathing.

Neonicotinoids have, instead of an easily protonated nitrogen, the nitro or cyano or equivalent electronegative pharmacophore, crucial for optimum insecticide potency. The electronegative pharmacophore is believed to associate with a cationic subsite (possibly lysine, arginine, or histidine) in the insect nAChR (Tomizawa and Casida, 2003, 2005). The low mammalian toxicity of IMI and ACE can be explained in large part by the lack of a charged nitrogen atom at physiological pH. These compounds show weak affinity for mammalian nAChRs but strong affinity for insect nAChRs. Furthermore, the uncharged molecules can penetrate the insect BBB, while there is controversy about their ability to cross the mature human BBB. However, desnitro-IMI, which is formed in mammals during metabolism as well as in environmental breakdown, does have a charged nitrogen, and is therefore similar to nicotine, and shows high affinity for mammalian nAChRs. For this reason it is considered as a nicotinoid rather than a neonicotinoid compound (Tomizawa and Casida, 2005).

## 2. Role of nicotinic acetylcholine receptors in acetamiprid and imidacloprid toxicity

### Receptors & mode of action

The nAChRs are fast ionotropic receptors that form ligand-gated ion channels with unique localisation in pre-, post-, and extrasynaptic membranes. They are found in the CNS neurones of insects and in both the central and peripheral nervous systems of mammals as well as in neuromuscular junctions.

In the cell membrane nAChRs are made up of a combination of five subunits (α, ß, δ, γ and ξ) arranged symmetrically around a central pore. Each subunit comprises four transmembrane domains with both the N- and C-terminus located extracellularly. The signature cysteine loop is located in the N-terminal domain of the receptor. The assembly of differing combinations of subunits results in a large number of different receptors. Thus, the diversity of nAChR subunits is a major determinant of

the specialised properties and functions of the mature receptors. The neuronal nAChR subtypes are various homomeric or heteromeric combinations of twelve different subunits: α2 through α10 and β2 through β4. The expressed amounts of the various subtypes vary in different parts of the nervous system. The major population of vertebrate brain nAChRs appears to be α4ß2 and α7 subtypes (insensitive and sensitive to α-bungarotoxin, respectively). It is known that α7, α8 and α9 subunits are blocked by α-bungarotoxin, while α2 to α6 and ß2 to ß4 are resistant to α-bungarotoxin (Courjaret and Lapied, 2001).

Ion-channel receptors reside in a constant equilibrium between open and closed states. Therefore, these receptors are responsive to external compounds acting as activators (agonists), inhibitors (antagonists), or co-modulators of agonist/antagonist actions. When an agonist binds to the site, all present subunits undergo a conformational change and the channel (pore) is opened, promoting the influx of extra cellular sodium and efflux of intracellular potassium to disrupt the equilibrium status of the membrane potential (Tomizawa and Casida, 2003). Activation of nAChRs causes nervous stimulation but high and prolonged levels of activation lead to blockage of the receptors (desensitisation). Total blocking of muscular-type receptors causes paralysis and death.

Activation of nAChRs often results in increased intracellular free $Ca^{2+}$ levels through the direct passage of extracellular $Ca^{2+}$ across the receptor channels in neurons, in addition to $Na^{+}$ and $K^{+}$. The raised cytoplasmic calcium levels trigger a series of calcium-dependent intracellular processes. Among the different nAChR subtypes in the brain, the homomeric α7 subtype exhibits higher permeability to $Ca^{2+}$ than the other ligand-gated ion channels permeable to this divalent cation, such as the NMDA (N-methyl-D-aspartate) receptor (Takarada et al., 2012). This receptor subtype is also known to desensitise rapidly (Wu et al., 2011).

The functional diversity of nAChRs allows channels to take part in two major types of neurotransmission. Classical synaptic transmission (wiring transmission) involves the release of high concentrations of neurotransmitter, acting on immediately postsynaptic neighbouring receptors. Binding of acetylcholine to these receptors is a key step in the physiology of nerve to nerve communication at the post-synaptic membrane where they give rise to trans-membrane currents and excitatory post-synaptic potentials. In contrast, paracrine transmission (volume transmission) involves neurotransmitters released by synaptic boutons, which then diffuse through the extracellular medium until they reach their receptors, which may be distant (Albuquerque, 2009). Agonists also bind to presynaptic sites where they cause neurotransmitters of other types to be released (Wonnacott, 1997).

During brain development the toxicity depends on the window of the exposure due to the different stage of cell differentiation/maturation. Regarding the α7 nAChR subtype, two features of this homomeric receptor have to be taken into account from a toxicological perspective. First, the high levels of expression during brain development as compared to adults in both humans (Falk et al. 2002) and rodents (Zhang et al. 1998; Tribollet et al. 2004) support the important role of nAChRs for the development of the CNS and are indicative of a potential vulnerability of developing mammals to early neonicotinoid exposures. Secondly, the occurrence of α7 nAChR in non-cholinergic neurons and in non-neuronal cells, e.g. astrocytes, microglia, T-lymphocytes and endothelial cells (Wu et al., 2011; Olofsson et al. 2012; Taly and Charon, 2012) suggests that target-cell populations of the neonicotinoids may also include those of the immune system and/or those that mediate neuroinflammatory mechanisms, and angiogenesis.

**Interspecies differences in affinity for nAChRs**

Vertebrates and insects have structural differences in nAChRs, both in the subunit composition and in the structure of the receptors themselves. Both these may affect the receptor binding capacity of specific molecules. Structural variations between the insect and mammalian nAChRs produce quantitative differences in the binding affinity of the neonicotinoids to these receptors. Most neonicotinoids show high affinity for insect nAChRs but low affinity for mammalian receptors. This in turn confers the notably greater selective toxicity towards insects compared to mammals (US-EPA,

2012). However, not only affinity for receptors but also efficacy contributes to the insecticidal activity of neonicotinoids (Nishiwaki et al., 2003, Ihara et al., 2004). Efficacy describes the way that agonists vary in the response they produce when they occupy the same number of receptors. High efficacy agonists may produce their maximal response even while occupying a relatively low proportion of the total receptor population. Lower efficacy agonists do not activate receptors to the same degree and may not be able to produce the maximal response (http://www.tocris.com/pharmacologicalGlossary.php). In this regard, IMI is a high affinity but a low efficacy agonist of the human α4ß2 receptor (Li et al., 2011).

Each receptor subtype has distinct electrophysiological and pharmacological properties; for instance, the α4ß2-containing nAChRs have the highest nicotine-binding affinity in mammalian cells, representing more than 90 % of the high affinity nicotine binding sites in mammalian adult brain.

Neonicotinoids have little or no effect on the vertebrate peripheral neuronal nAChR subtype and on some central nAChR subtypes, including α4ß2 and α7 (Tomizawa and Casida, 2003). Binding experiments have shown that IMI and ACE have low to moderate potency at the α3 and α4ß2 receptors and are essentially inactive at the α1 and α7 receptors (Tomizawa and Casida, 1999). However, more recent studies have shown that IMI is significantly weaker compared to acetylcholine at activating chicken α7 receptors and is essentially inactive at chicken α4ß2 receptors (Li et al., 2011). From the Schild plot analysis[6], Li et al. (2011) estimated that the affinity of IMI for the human α4ß2 receptor expressed in HEK (human embryonic kidney) cells is 18 µM.

As regards the specificity of nicotine ACE, IMI and some IMI metabolites for vertebrate α4β2 nAChRs, the following $IC_{50}$ (nM) have been reported (Tomizawa and Casida, 2005):

**Table 2:** Comparative $IC_{50}$ (nM) of nicotine, acetamiprid, imidacloprid and metabolites for the α4β2 nAChRs

| Compound | Vertebrate | Insect |
|---|---|---|
| Nicotine | 7 | 4000 |
| ACE | 700 | 8.3 |
| IMI | 2600 | 4.6 |
| 1. IMI-NH (desnitro IMI, M09) | 8.2 | 1530 |
| 2. IMI-NNO (nitrosimine metabolite, M07) | 850 | n.a. |
| 3. IMI-NH2 (amio-guanidine, M08) | 42 | n.a. |
| 4. IMI-olefin (M06) | 1700 | n.a. |

n.a.: not available

Minor structural changes of neonicotinoids may confer differential selectivity for different nAChR subtypes. These include those that occur in the metabolic pathways of the IMI parent compound within different mammal tissues, including brain (Ford and Casida, 2006). In particular, the desnitro IMI metabolite has affinity levels comparable to nicotine for the α4β2 receptor, thus much higher than IMI (Chao and Casida, 1997; Tomizawa and Casida, 1999 and 2002), as well as for α1 and α3 receptors (Tomizawa and Casida, 1999). In *ex-vivo* binding studies in mouse brain membranes, desnitro IMI is a competitive inhibitor of nicotine binding (Chao and Casida, 1997). Another example of the spectrum of the effects of these "minor" structural changes of the IMI molecule that confer differential selectivity to nAChR has been reported with nitromethylene IMI analogues. Although these molecules are not IMI metabolites, their affinity for α7 receptor subtype is higher than that of nicotine and IMI (Tomizawa and Casida, 1999).

[6] The Schild plot is a pharmacological method of receptor classification. To construct a Schild plot, the dose-effect curve for an agonist is determined in the presence of various concentrations of a competitive antagonist.

In experiments on mouse fibroblast M10 cells (stably expressing α4β2 receptors) after 3 day exposure to IMI, nAChR binding was up-regulated by five- to eightfold. In contrast, the desnitro-IMI metabolite led to up-regulation by eightfold. Data on potency for *in vitro* nAChR up-regulation indicated that binding to the α4β2 receptor initiates the up-regulation (Tomizawa and Casida, 2000).

**Physiological role of nAChRs during brain development**

Neurogenesis appears to be controlled by ionotropic signal inputs mediated by nicotinic cholinergic, GABAergic and glutamatergic systems in developing brains. Thus, the nAChRs play a key role in mammalian brain development and function (Role and Berg, 1996). The developmental role of these receptors in mammal brain is extensively understood, especially for presynaptic modulation of CNS synapses (Sher et al., 2004). Here, nAChRs play a role in formation of rudimentary neural and sensory circuits, neuronal pathfinding and neuronal survival during the early prenatal period (Dwyer et al. 2009).

The nAChRs are expressed in several brain structures such as cerebellum, hippocampus, entorhinal cortex, basal ganglia and thalamus (Court et al., 2000). The α7 nAChR subtype is believed to be involved in the regulation of neuronal growth, differentiation, synapse formation and synaptic plasticity during the development of the human brain (Falk et al., 2002). The α7 receptors, which mediate the effects of developmental neurotoxicants, increase glutamate release onto postsynaptic NMDA receptors (Mao et al., 2007). Overactivation of α7 nAChRs during development results in neuronal cell death, whereas stimulation in adult animals is neuroprotective (Laudenbach et al., 2002) In the murine hippocampal dentate gyrus, α7 subunit appears to be essential for normal survival, maturation and integration of adult-born neurons (Campbell et al. 2010).

Moreover, high levels of α4 subunit expression in human foetal brain are consistent with the hypothesis of a morphogenic role of nAChRs during the early CNS development (Agulhon et al., 1998). Functional α4β2 nAChRs are constitutively expressed in undifferentiated neural progenitor cells from foetal mouse neocortex where they play a pivotal dual role in the proliferation toward self-replication and the commitment/differentiation into particular progeny lineages during brain development (Takarada et al., 2012).

Disruption of cholinergic innervation during early postnatal development (e.g. neonatal lesions to basal forebrain cholinergic afferents in rats) results in delayed cortical neuronal development, permanent changes in cortical cytoarchitecture, and altered cognitive function (reviewed in Hohmann and Berger-Sweeney, 1998). Furthermore, the impaired cholinergic transmission during ontogenesis may be associated with some developmental brain disorders such as autism, Rett syndrome and attention deficit and hyperactivity disorder (ADHD), with muscarinic receptors playing also a key role in cortical morphogenesis (Hohmann and Berger-Sweeney, 1998; Court et al., 1997, 2000). Nevertheless, the administration of nicotine and nicotine agonists has shown to reduce ADHD symptoms severity (reviewed in Gizer et al., 2009). On the other hand, genes encoding nAChR α4 (CHRNA4) and α7 (CHRNA7) subunits may represent candidate genes for autism, Rett syndrome and ADHD, although the evidence is limited (Gizer et al., 2009; Yasui et al., 2011; Williams et al., 2012).

## 3. Comparative Morphology and Development of Rat and Human Cerebellar Cortex

The paper under consideration (Kimura-Kuroda et al., 2012) used cultured rat neonatal cerebellar granule cells exposed to ACE and IMI and this Opinion addresses potential DNT of these active substances in humans. Thus, this section provides a comparative study of morphology and development between rat and human cerebellar cortex.

The cerebellum is composed of three major histological subdivisions: the cortex, the underlying white matter and the deep cerebellar nuclei (Biran et al., 2012). The basic histological layering of the cerebellar cortex is similar in rodents and primates and consists of five different types of neurons organised in three distinct cellular layers (see Fig. 3 and 4):

- *Molecular layer*, the most superficial one, is composed of stellate and basket cells, two types of inhibitory interneurons that form synapses onto Purkinje cell dendrites. The axons of the granular cells penetrate to the molecular layer where they divide in a T-shaped manner to form parallel fibers that make excitatory contacts with dendrites of Purkinje cells.
- *Purkinje cells layer* (also referred to as the piriform layer). This is a thin row of cell bodies of Purkinje cells and climbing fiber terminals, which make excitatory synapses with the dendritic tree of a Purkinje cell. Purkinje cell axons penetrate the granular cell layer and most of them terminate in deep cerebella nuclei, where they exert an inhibitory function.
- *Granular layer*, the most internal one, is comprised by the small and thickly populated granular cells, inhibitory interneurons (Golgi type II cells) and excitatory mossy fibers that synapse onto granule cell dendrites and stimulate them. Cerebellar granule cells send parallel fibers up through the Purkinje layer into the molecular layer where they branch out and spread through Purkinje cell dendritic arbors. These parallel fibers form thousands of excitatory granule cell-Purkinje cell synapses onto the intermediate and distal dendrites of Purkinje cells.

All of these cell types, with the exception of the granule cells, use the inhibitory neurotransmitter GABA. Granule cells use glutamate as their neurotransmitter, and therefore exert excitatory effects on their targets. Both stellate and basket cells form GABAergic synapses onto Purkinje cell dendrites. The Purkinje cell is the only neuronal type that has an axon that leaves the cerebellar cortex. There are only two types of axons that enter the cerebellum, mossy fibers and climbing fibers. Climbing fibers arise only from the inferior olivary nucleus, while mossy fibers comprise all of the other cerebellar inputs. Both of these fiber types release excitatory neurotransmitter, glutamate in the case of the mossy fibers and aspartate in the case of climbing fibers (Swenson, 2006).

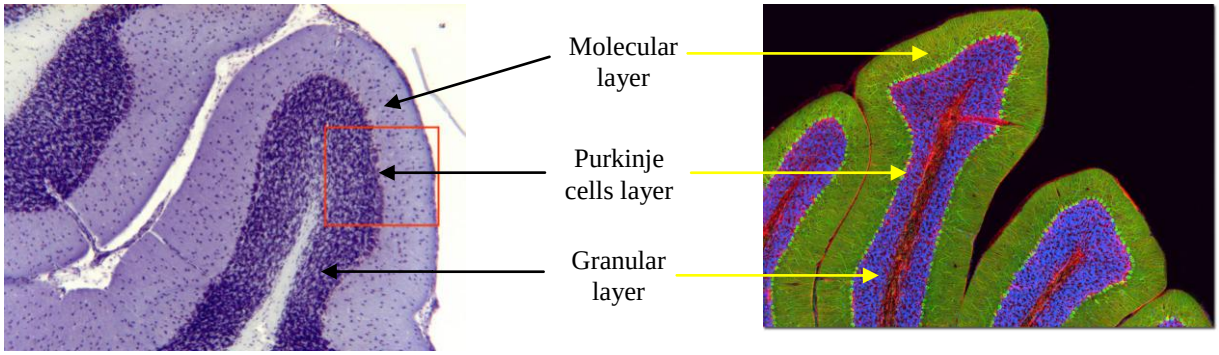



**Figure 3:** Histological sections of the cerebellar cortex layers.
Left: human cerebellum (Department of Experimental Biology, University of Jaen, Spain; http://virtual.ujaen.es/atlas/cerebelo/cerebelo10x.htm).
Right: rat cerebellum (http://biofinity.org/images/Rat%20Cerebellum%20Micrograph.jpg)

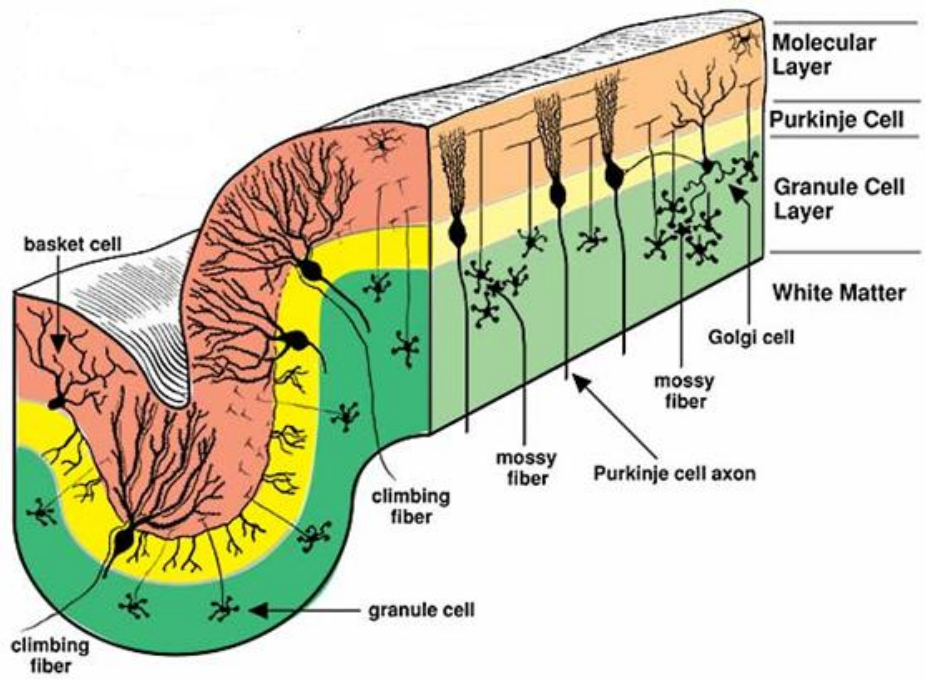



**Figure 4:** Schematic representation of the cerebellar cortex layers (Atlas Catalog.Veterinary Neurohistology. Central Nervous system http://vanat.cvm.umn.edu/neurHistAtls/cataPages/cataCNS.html).

During cerebellum ontogenesis, in contrast to other regions of the CNS, cerebellar neurons are generated in two germinative neuroepithelia in two waves of proliferation and migration processes that occur in similar order but at different rates in rodents and primates (Biran et al., 2012):

- The first neurons to be generated are the deep nuclear neurons and all the Purkinje cells that migrate immediately thereafter to the cerebellar plate. In parallel, the first granular cell precursors are generated and then migrate tangentially to cover the superficial zone of the cerebellar plate forming the extragranular layer.

- During postnatal life, the second wave of proliferation occurs in the extragranular layer. The secondary germinal zone gives rise to the granular cells which migrate radially inward to their final destination in the internal granular layer. In the molecular layer, the onset of synaptic inputs of the axons of the granular cells (parallel fibers) is concomitant with the onset of the final postsynaptic dendritogenesis of the Purkinje cells. In the rat, although the extension of the lateral domain of the dendritic tree of the Purkinje cells is achieved at postnatal day 15 (PND 15), its final adult size is reached at PND 30 (Altman and Bayer, 1985). In humans, the adult number of narrow, leaflike gyri of the cerebellar cortex (referred to as folia or cerebellar laminae) is achieved around two months postnatally (Loeser et al., 1972) and the extragranular layer disappears around the 7th postnatal month (Sidman and Rakic, 1973).

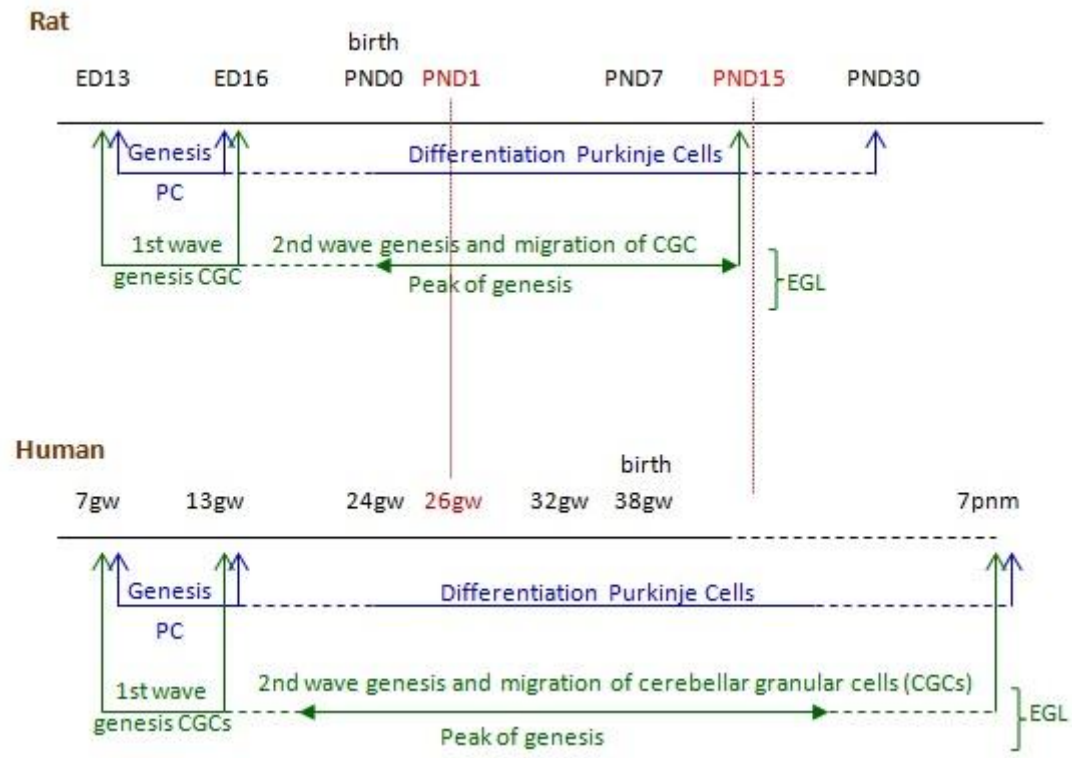



**Figure 5:** Comparison of timing of development of the Purkinje cells (PC, in blue colour) and cerebellar granular cells (CGC, in green colour) in the cerebellar cortex in rat and human. EGL: external granular layer; Embryonic day (ED), postnatal day (PND), gestational weeks (gw), postnatal months (pnm) (adapted from Biran et al., 2012). Vertical dotted red lines indicate the time interval that elapses between when CGCs were taken from neonatal rats in the Kimura-Kuroda et al. study (PND 1) and the end of the *in vitro* culture time (14 additional days). At this time, CGCs are already mature.

## 4. Review of the paper by Kimura-Kuroda et al. (2012)

### 4.1. Background and Scope

Studies of four main types were quoted by the authors on effects of neonicotinoids on the vertebrate nervous system:

i) *in vitro* data, ranging from nAChR modulation in rat PC12[7] cells to whole-cell patch-clamp recording in stellate cells of mouse cochlear nucleus after IMI exposure at doses of ≥10 µM (Nagata et al. 1998; Bal et al. 2010);
ii) *in vivo* data from intracranial (striatal) neonicotinoids exposure and subcutaneous repeated injections of thiamethoxam in adult rats (de Oliveira et al. 2010);
iii) *in vivo* data from developmental exposure studies where a single intraperitoneal injection of 337 mg/kg bw (75 % $LD_{50}$) was administered to rats on gestational day 9 (Abou-Donia et al. 2008);
iv) *in vitro* direct activation of the human α4ß2 receptor (measured by patch clamp techniques) after IMI treatment (Li et al., 2011).

[7] PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla that can be used as a model system for neuronal differentiation.

Based on these studies, the authors investigated potential developmental neurotoxic effects of the widely-used neonicotinoid insecticides IMI and ACE using an *in vitro* system of neuronal cells, the cerebellar granule cells (CGCs) of neonatal rats. Nicotine was used as a "positive control" because of its known effects on mammalian nAChRs.

### 4.2. Methodology

*Experimental design*

The *in vitro* system consisted of primary cell cultures of rat CGCs isolated from PND 1 pups that were cultured for 14 days *in vitro* (DIV). The cell proportion was CGCs (90 %), Purkinje cells (1 %) and astrocytes (5 %). In this system, CGCs expressed several types of nAChR (α3, α4 and α7), which were confirmed by measuring mRNA expression of these receptor subunits at 14-16 DIV.

The cultures were exposed to 0.5 to 100 µM solutions of the test substances using continuous perfusion by means of a peristaltic pump for up to 10 minutes. A subset of perfusions was followed up by exposure to 100 mM KCl 500 seconds thereafter in an attempt to stimulate the neurons. Another subset included prior application of selective antagonists for different types of nAChRs (α-bungarotoxin for α7 receptors, dihydro-β-erythroidine for α4β2 receptors and mecamylamine, as a relatively selective antagonist for α3β4 receptors). The parameter studied was the $Ca^{2+}$ influx measured using a Fluo-4-based assay and the related excitatory patterns in cell cultures and single cells. The peak intracellular concentrations of $Ca^{2+}$ and the proportion of the excited neurons were measured. The influence of prior administration of the antagonists was also assessed.

### 4.3. Interpretation of the results by the authors

Kimura-Kuroda et al. (2012) suggest that IMI and ACE, similarly to nicotine, exert excitatory effects on nAChRs of CGCs in culture at low concentrations (starting from 1µM) and therefore may adversely affect the developing human brain.

The authors considered the readout of the Fluo-4-based assay as an 'index of neuronal physiological activity'. Application of ACE, IMI or nicotine led to a marked transient increase in Fluo-4 fluorescence intensity that was interpreted as a transient increase in calcium influx. This intracellular $Ca^{2+}$ mobilisation in CGCs revealed that ACE and IMI have agonist activity on mammalian nAChR since a characteristic excitatory pattern was induced. This effect appeared to conform to an all-or-none response pattern, as concentrations of 1, 10 and 100 µM of the individual test substances all essentially elicited the same magnitude of response (peak fluorescence), while doses of 0.5 µM did not have any detectable effect.

### 4.4. Comments of the PPR Panel

Since cerebellar development takes place much later than that of other brain structures, and as it continues postnatally in both humans and rats, primary CGC culture is a suitable and widely used system for *in vitro* DNT studies (Bal-Price et al., 2010a).

Although a number of mechanistic aspects remain to be clarified, the data clearly show consistent and treatment-related effects and a no-effect concentration was identified for both neonicotinoid compounds. However, the toxicological relevance of these observations remains to be defined as the applied assay covers only one narrow aspect of neuronal function and/or development. The information provided by the study cannot be directly extrapolated to predict the outcome of *in vivo* exposures in adult or developing humans or other mammals.

*Experimental design*

In the study, exposure to IMI and ACE was performed acutely at 14 DIV. At this time, the culture of CGCs is expected to have already reached advanced maturation since proliferation, migration and some stages of differentiation should have already taken place (Bal-Price et al., 2010b). Therefore, the results relate to mature neurons (adult neurotoxicity rather than to developmental neurotoxicity). Moreover, additional DNT specific endpoints should have been studied to determine if key developmental processes, such as neuronal migration, differentiation (e.g. neurite outgrowth, synaptogenesis), glial proliferation and maturation, might be affected by the exposure to IMI and ACE at non-cytotoxic concentrations during early stages of cell culture, prior to the time when neurons are fully differentiated (approximately 10 DIV).

*Decreased content of glial cells*

In the paper under study, the culture medium was replaced at 2 DIV by serum-free synthetic medium to prevent growth of astrocytes. As a result, the culture contained around 5 % of glial fibrillary acidic protein (GFAP) positive cells (astrocytes), which is less than in the culture with serum, where the glial proportion is around 18 % (~13 % astrocytes and ~5 % microglia; Hogberg, 2010). In the Kimura-Kuroda paper there is no data on the content of microglia and oligodendroglial cells. The presence of glia is important as these cells, especially astrocytes, protect neurons against toxic insults. The content of glia differs in different brain structures (Azevedo et al., 2009). Neurotoxicity is generally lower in mixed (neuronal/glial) cultures which have greater numbers of astrocytes. In addition, the presence of astrocytes is important for cell-cell interaction as these cells secrete factors that modulate synaptic transmission and enhance the formation of neuronal interconnections (Hatton, 2002). Overall, the PPR Panel considers that *in vitro* systems with a low content of glial cells have less direct relevance to the *in vivo* situation.

*CGCs culture medium without KCl*

A protocol with KCl free medium was previously used by Kimura-Kuroda et al. (2002) for culture of cerebellar Purkinje cells in mice. However, it is not known whether it is optimal for CGCs as it could affect the process of neuronal maturation as well as the response of the CGCs to the applied treatments.

KCl causes membrane depolarisation due to the increase in the equilibrium potential of potassium and, thus, is widely used for studying electrical activity-dependent changes in neurons, muscle, and endocrine cells. However, the medium used for the CGCs culture did not contain KCl solution. The classical method for measuring activity-dependent responses in granule neurons in culture was based on a method that uses membrane-depolarising concentrations of extracellular KCl. KCl should be present in the medium of CGCs at high concentration (on average 25 mM) (Cold Spring Harbor Protocols: Culture of cerebellar granule cells, 2008). This high concentration is critical as membrane depolarisation-induced transmembrane $Ca^{2+}$ flux is essential for the maintenance of the granule cells. It mimics the influence of the *in vivo* physiological stimulation through excitatory amino acid receptors including NMDA receptors, involving $Ca^{2+}$ entry and the activation of $Ca^{2+}$/calmodulin-dependent protein kinase (Yan et al., 1984; Gallo et al., 1987; Sée et al., 2001; Sato et al., 2005; Suzuki et al., 2005).

Additionally, calcium-dependent signalling pathways regulate the gene expression profile of CGCs (Kramer et al. 2003; Sato et al. 2005). An interesting example is the developmental switch that occurs in subunit composition for $GABA_A$ and NMDA neurotransmitter receptors as granule neurons mature (Watanabe et al., 1992; Farrant et al., 1994; Mathews et al., 1994). Upon hyperpolarisation, the α6 subunits of the $GABA_A$ receptor and the NR2C subunit of the NMDA receptor are up-regulated (Mellor et al., 1998; Suzuki et al., 2005; for review see Nakanishi and Okazawa, 2006).

*Developmental role of nAChR in brain*

Since IMI and ACE are nAChR agonists, it is important to characterise their effects well in the context of developmental neurotoxicity. To investigate the role of nAChRs during neuronal development/maturation under *in vitro* conditions it would be necessary first to conduct kinetic studies of nAChR expression to define at what developmental stage these receptors are expressed and to define what role they play in neuronal differentiation (e.g. using specific receptor antagonists).

*Evaluation of the applied methodology*

The imaging of calcium influx into cells is a common and useful technique for measuring calcium signals in functional neurons in culture and provides a useful indirect measure of action-potential generation within individual neurons. The key advantage of the technique is that it allows monitoring the activity of a large population of neurons with single-cell resolution.

Calcium imaging techniques take advantage of calcium indicator dyes, which are BAPTA[8]-based organic molecules that change their spectral properties in response to the binding of $Ca^{2+}$ ions. Kimura-Kuroda et al. (2012) measured the calcium ($Ca^{2+}$) influx into cells by using the $Ca^{2+}$-sensitive fluorescent dye Fluo-4. This increases calcium-dependent fluorescence excitation at 488 nm by argon ion laser and gives higher signal levels and ease of use for confocal microscopy without requiring the use of a background quencher dye. The method is widely used and highly sensitive. The choice of Fluo-4 was considered by the PPR Panel to be appropriate and relevant as it was expected to allow measurement of the response of the cells to further treatments after the application of neonicotinoids.

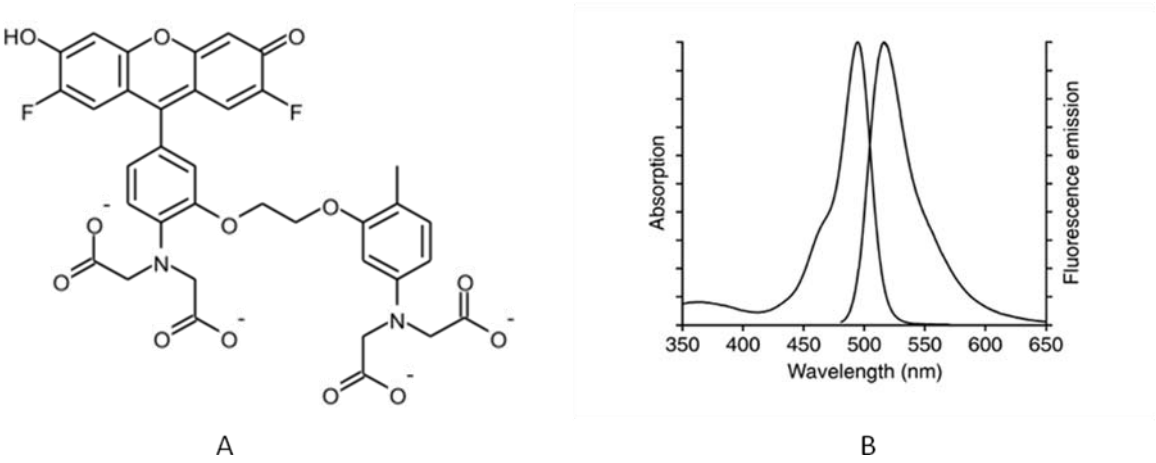



**Figure 6:** Fluo-4 chemical structure (A) and absorption and fluorescence emission spectra (B).

In the Kimura-Kuroda et al. study, CGC cultures were preloaded with Fluo-4 acetoxymethyl ester (Fluo-4 AM, the precursor of Fluo-4) prior to treatment with different doses of IMI, ACE and nicotine. This precursor is generally expected to easily penetrate the cell membrane. Once within the cell, it is

[8] 1,2-bis(o-aminophenoxy)ethane-N,N,-N',N'-tetraacetic acid.

assumed to be degraded by esterases yielding Fluo-4, whose fluorescence increases dramatically upon binding of divalent cations (e.g., $Ca^{2+}$).

Both nicotine and the two neonicotinoids studied produced a significant transient increase in Fluo-4-dependent fluorescence, which may be interpreted as an increase in $Ca^{2+}$ influx. However, no plausible explanation was provided for the mechanism(s) underlying this apparently dose-independent all-or-none response. It is not clear to which extent various difficulties that might occur when working with indicator dyes have been taken into account within the study, e.g. leakage of dye from the cells (which might lead to an attenuated response after prolonged cellular perfusion) or compartmentalisation of the dye.

*AChR antagonist assays*

Pretreatment/concomitant treatment of cell cultures with different antagonists of nAChRs either suppressed or prevented the nicotine-, IMI- or ACE-dependent transient increase in Fluo-4 fluorescence, suggesting that $Ca^{2+}$ influx was due to activation of nAChRs. Two antagonists believed to be selective for a particular receptor subtype were used: α-bungarotoxin for the α7 subtype and dihydro-ß-erythroidine (DHbE) for α4ß2 and α3ß4 subtypes. However, results did not allow specification of the predominant receptor subtype responsible for the observed effect. The IMI-, ACE- and nicotine-induced $Ca^{2+}$ influxes in neurons were completely blocked by the homomeric antagonist α-bungarotoxin, indicating that the effect was mediated by α7 receptor subtype. However, the heteromeric nAChR antagonist DHbE also blocked the response. The involvement of α7 receptor in mediating IMI and ACE effects could be important because they are particularly abundant in developing brains of rodents (Zhang et al, 1998; Tribollet et al., 2004) and humans (Falk et al., 2002) from very early phases of prenatal development (Dwyer et al. 2009). Presynaptic α7 receptors are responsible for generating very fast calcium currents and, when they do so, causing neurotransmitters release (Dani 2001; Rousseau et al., 2005), but in other cases non-α7 subtypes are also involved (Nagumo et al., 2011).

*Extracellular KCl challenge*

Kimura-Kuroda et al. (2012) also tested whether treatment with either nicotine, IMI or ACE might affect the response of cells to an increase in extracellular KCl (100 mM). KCl is expected to cause membrane depolarisation and thus an increase in calcium influx via voltage-dependent calcium channels. Lack of response to KCl (100 mM) was observed after application of ACE or IMI (even after washing the compounds out), indicating that neurons were in a non-conducting, inactivated state. This is an important demonstration of functional damage caused by these compounds to the neurons, which might not be able to respond correctly to the physiological stimulus. It is noted, however, that the time frames between application of the individual test substances and the KCl challenge appeared to differ between the experiments with different test substance concentrations. Moreover, a normal response to KCl was observed for nicotine at the highest dose tested (100 µM) eliciting a somewhat stronger calcium influx than IMI and ACE. Neither 10 nor 1 µM produced this effect. This finding was not properly discussed by the authors but it deserves a scientific explanation.

Additionally, the fact that CGCs were cultured for 14 DIV without KCl in the medium could affect the process of neuronal maturation as well as the response of the neurons to KCl treatment.

*Lack of biotransformation under in vitro conditions*

The metabolic activity of the cultured neuronal and glial cells is low or absent (Bal-Price 2010a), therefore it is important to know whether *in vivo* toxicity of IMI and ACE is mediated by metabolites, by the parent compound, or by both. The low (or the absence of) metabolism under *in vitro* conditions could affect and likely underestimate the interpretation of results obtained in cell culture studies. As

has been mentioned above, the desnitro metabolite of IMI exhibits higher affinity for vertebrate nAChR and causes higher acute toxicity. On the other hand all ACE metabolites are of lower toxicity than the parent compound (JMPR, 2005).

*Evaluation of IMI and ACE concentrations applied to the in vitro system*

The PPR Panel cannot rule out some methodological uncertainties in reporting the effective concentrations of nicotine, ACE and IMI. For instance: a) the volume of the medium surrounding the exposed cells is unknown; b) it is not clear whether the concentrations reported were measured in the pressure pipette, or whether they were the diluted ones collected after application (in this last case, a lower concentration than the ones in the pipette would be expected); c) each administration was effected locally, and pressure was applied to a syringe whose tip was 100 µm from the neurons imaged; d) the stability of the compound in culture medium was not evaluated; e) the binding of ACE and IMI to plastic and culture medium was not determined so the free concentration of the compounds were not defined.

*Dose-response relationship*

Difficulties in interpreting the value of the paper under study for risk assessment, apart from the methodological uncertainties outlined above, stem from the claim that neonicotinoid concentrations showing effects in the *in vitro* assay corresponded to dose levels much lower than the NOAEL determined in the regulatory *in vivo* tests (e.g., 50 times lower in the case of IMI). Accordingly, it is hard to interpret the reliability and usefulness of the study for risk assessment. The dose-response relationship, though often linear for active substances, may not always be so. There is some evidence that nonlinearity may be the case for ligands binding to the nAChRs, which exhibit an inverted U-shaped dose-response curve (Matta et al., 2007). It is regrettable that a larger number of intermediate concentrations (e.g., between 0.5 and 1 µM) of IMI and ACE was not tested as this could have contributed to a more robust and comprehensive set of data. Such information would be valuable to find the limit concentration for the response *in vitro* and suggests that further research is warranted.

*Comparison of concentrations of actives used in vitro with reported human toxicity cases*

Three cases are reported in the published literature of acute ACE poisoning. After 120 min. from a subcutaneous administration of about 8 ml of a formulation containing 18 % of ACE, a 58 year-old male showed a concentration level of 2.39 µg/ml (10.7 µM), whereas a 74 year-old woman had a serum concentration of 59.83 µg/ml (268 µM) after ingestion of about 100 ml of a solution containing 2 % of ACE (Imamura et al., 2010). The third case involved a 79-year-old man who showed a blood concentration of 21.1 µg/ml (94.7 µM) some time after the ingestion and was discharged from hospital without complication on the next day (Todani et al., 2008).

In a prospective human case series, of 56 patients with acute self-poisoning due to a commercial IMI formulation, who had been admitted to a hospital, 54 had symptoms such as nausea, vomiting, headache, dizziness, abdominal pain, and diarrhoea during the hospital stay. These symptoms were largely self-resolving. The median Glasgow coma score[9] on presentation was 15 (IQR: 10–15). There were no deaths, but two patients developed more severe symptoms requiring management in an intensive care unit. The median volume amount ingested was 15 mL (IQR 10–50 mL). Exposure was confirmed in 28 patients, with a median admission plasma concentration of 10.58 ng/L (0.047 nM); IQR: 3.84–15.58 ng/L and range: 0.02–51.25 ng/L (Mohamed et al., 2009). Owing to the time gap between ingestion and blood sampling, these concentrations do not reflect the maximum plasma

[9] Glasgow Coma Scale (GCS) is a neurological scale that aims to give a reliable and objective way of recording the conscious state of a person. GCS values ranges from 3 (deep coma state) to 15 (normal).

levels. As some patients were treated with atropine and oximes because of suspected organophosphate intoxication, the effects of IMI poisoning might have been enhanced.

*Comparison of concentrations of active substances used in vitro with in vivo animal studies*

It is useful to try to link the data in the Kimura-Kuroda et al. (2012) experiments with the extensive literature on nicotine to establish whether the doses administered are likely to be of pharmacological, physiological, behavioural or toxicological relevance. The rise in concentration of nicotine in a smoker's blood that has not been abstaining is maximal just after smoking a cigarette, variously estimated as reaching a peak concentration of 10.9 ng/ml (0.067µM) (Patterson et al., 2003) or 25-50 ng/ml corresponding to 0.154-0.307 µM (Benowitz et al., 1988). Plasma concentrations retrieved from rat plasma following injections to the femoral artery of 1 mg/kg of nicotine peaked at 0.0021 µM after 10 minutes, falling to 0.0007 µM after 20 minutes (Sastry et al., 1995). In contrast, the range of concentrations of nicotine used by Kimura-Kuroda et al. (2012) was considerably greater, ranging from 1-100 µM.

From the results of kinetic studies conducted with single doses of IMI in rats (Klein, 1987), the association between *in vivo* dose levels (mg/kg bw) and the plasma concentrations at $T_{max}$ (approximately 1 h after dosing) was as follows:

- Rat: 1 mg/kg bw (oral) caused a $C_{max}$ plasma of 0.72 µg/ml, equivalent to 2.8 µM
- Rat: 20 mg/kg bw (oral) caused a $C_{max}$ plasma of 13 µg/ml, equivalent to 50 µM

These results demonstrate approximate dose linearity. Taking into account these data, the estimated NOAEL for DNT reported for IMI (5.5 mg/kg bw, see section 5.3) would result in 15.4 µM, which falls within the dose range assayed by Kimura-Kuroda et al. (2012) that induced adverse effects on CGCs (1-100 µM). Thus, a clearly effective *in vitro* concentration corresponds to a dose level *in vivo* devoid of any neurotoxic effects in the available DNT studies.

DNT effects are usually induced by low doses of environmental chemicals and chronic exposure. However, Kimura-Kuroda et al. (2012) focused only on the acute effects. Acute exposure *per se* might be relevant to developmental effects when it occurs at a critical phase *in utero* or during lactation. In addition, it would be relevant to study the *in vitro* effects induced by lower concentrations after repeated/prolonged exposure as the effect pattern may be different.

*Relevance of the rat in vitro results to the in vivo situation*

A crucial point is the exposure regimen used and its comparability to *in vivo* systemic exposures to IMI and ACE. The *in vitro* situation may differ as a result of ADME (absorption, distribution, metabolism, excretion) differences *in vivo*, differences between species and among individuals of one species due to lifespan, gender, ill-health and other factors (Matta et al., 2007).

The experiments recorded in the Kimura-Kuroda et al. (2012) study were performed on an exposed population of neurones treated with neonicotinoids that have direct access to the cell surfaces where the nAChRs are available. The results indicated that IMI and ACE have agonist activity on mammalian nAChRs at a concentration of 1 µM, which is much lower than the concentrations predicted from their binding affinities performed on isolated cell membrane fractions. For a better interpretation of this finding, there is a need to know how relevant the *in vitro* concentrations of IMI and ACE are to the concentrations that may reach the brain *in vivo*. It should also be considered that *in vivo* toxicity of IMI is triggered by both the parent compound and its active (toxic) metabolites.

Additional studies are required to determine whether the results obtained by Kimura-Kuroda et al. (2012) are reproducible. Not only CGC culture but also other brain structures that express nAChRs (e.g. cortex and hippocampus) should be investigated. Experiments are also required to determine

whether the observed effects are reversible. Interpreting the lack of response to 100 mM KCl would be helped by such studies.

It is of note that a single analytical parameter was measured in the Kimura-Kuroda study, specifically changes in intracellular Fluo-4 fluorescence as an indicator of changes in intracellular calcium. In order to better relate the results of this study to the *in vivo* situation and to define a cellular response, parameters other than intracellular calcium levels should be tested. Additional positive control experiments using other nAChR ligands (particularly acetylcholine) and further negative control experiments should be carried out.

Overall, uncertainties limiting the ability to extrapolate from these results to human risk assessment arise from: a) knowledge gaps and limited value of animal models, and b) difficulties in identifying *in vitro* endpoints suitable for establishing regulatory reference values. The first ones include:

- Species differences between rat and human.
- Data gaps regarding permeability of the immature blood brain barrier to IMI and ACE.
- The lack of knowledge of concentrations of IMI and ACE at the adult cerebellum *in vivo* that should be used for relevant concentration ranges for *in vitro* studies. In Kimura-Kuroda et al. (2012) experiments the free concentrations of ACE and IMI that reach the cells are unknown, and only nominal concentrations are available *in vitro*.
- The role of metabolism regarding the toxicity of the metabolites on the developing brain.
- The levels of partition of IMI and ACE into the foetal brain throughout development and any concentration/dilution effects in human breast milk following exposure of the mother.
- The rates of metabolism and excretion in adults and at the relevant developmental stages, including the role of maturation of the detoxifying organs such as the liver.
- The incomplete investigation of toxicity at each developmental stage, and in particular the stage(s) of development at which the developing cerebellum is most vulnerable.
- The permanence of any changes and the extent of any ability of the developing brain to compensate the toxic effects by adaptation or defence mechanisms.

Difficulties in identifying *in vitro* endpoints suitable for establishing regulatory reference values include:

- The simplicity of the CGCs cultures may not reflect the more complex *in vivo* situation.
    - Susceptibility of CGCs may depend on the proportion of glial cells within the culture.
    - The tested cells represent a small population of the brain cells and other CNS structures may be more sensitive.
    - There is a lack of knowledge about the responses of the other structures of the CNS.
- The lack of a clear linkage between the effects measured on intracellular free calcium, the neural responses and the neurodevelopmental outcomes of those responses.

While the findings cannot be ignored, further research is definitely needed to establish to what extent measurements of *in vitro* neurotoxicity are relevant to human risk assessment.

## 5. The Guidelines for Developmental Neurotoxicity (DNT) Testing: Comments of the PPR Panel

Developmental neurotoxicity (DNT) studies according to the OECD TG 426 or the corresponding USA EPA guideline (USA EPA, 1998) are designed to provide information on the potential functional, behavioural and morphological effects on the developing nervous system after *in utero* and early life exposure to chemical compounds. More recently, OECD has published the extended one-generation reproductive toxicity study (TG 443) that also allows to assess the potential impact of

chemical exposure on the developing nervous system (OECD, 2011). This new guideline is intended to evaluate all developing life stages, both prenatal and post-natal up to puberty, and the extended impact of developmental hits up to adulthood. Testing guideline 443 would cover in a detailed way specific endpoints, such as nervous system or immune function, according to the indications by previous studies. However, systematic testing for DNT is not routinely required by most regulatory agencies and becomes obligatory only if it has been triggered by the observations during organ toxicity testing (Bal-Price et al., 2010 and 2011).

As early as 1998, the US-EPA published the test guidelines OPPTS 8706300 on DNT. This was the basis for further development of the OECD TG 426, which was endorsed in 2007. The TG 426 addressed important issues and incorporated improvements recommended by expert consultation meetings held between 1996 and 2005. Both US-EPA and OECD guidelines are based entirely on *in vivo* studies in which the test substance is administered to rodents during gestation and lactation to cover critical pre- and postnatal periods of nervous system development. Then, gross functional, behavioural and neuroanatomical abnormalities are assessed during postnatal development and adulthood (Figure 4). These assessments encompass physical development, behavioural ontogeny, motor activity, motor and sensory function, learning and memory, brain morphometry and neuropathology. However, for learning and memory assessment the guidelines methodology is flexible and its sensitivity varies, which may lead to some deficit in these domains and subtle effects to remain undetected (Raffaele et al., 2010; OECD TG 426).

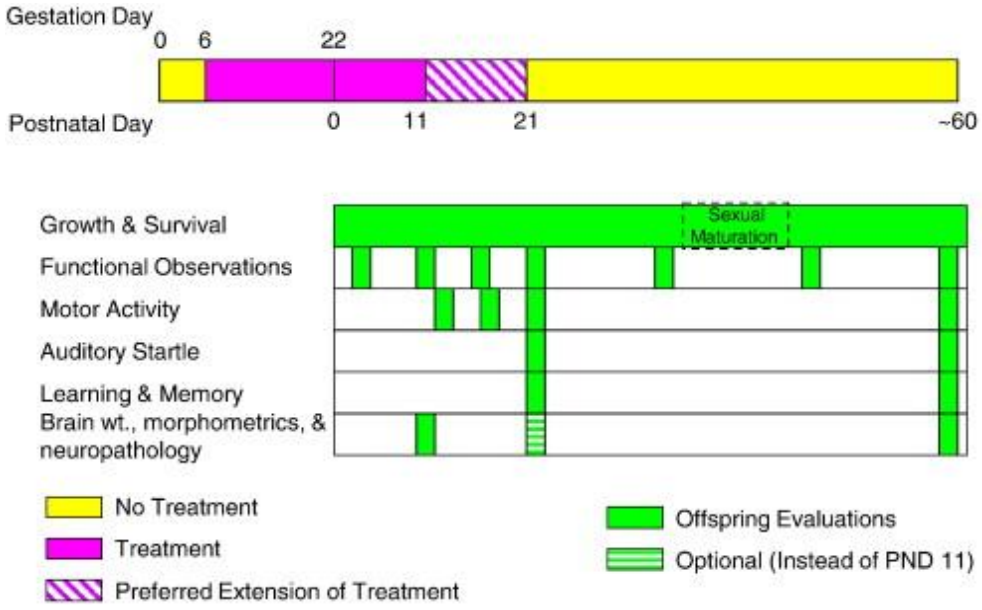



**Figure 7:** DNT study design. A schematic representation of the exposure period and the parameters evaluated in the EPA guideline DNT study (Raffaele et al., 2010).

The OECD TG 426 states that "the Guideline allows various approaches with respect to the assignment of animals exposed *in utero* and through lactation to functional and behavioural tests, sexual maturation, brain weight determination and neuropathological evaluation. Other tests of neurobehavioural function (e.g., social behaviour), neurochemistry or neuropathology can be added on a case-by-case basis, as long as the integrity of the original required tests is not compromised". Accordingly, there is considerable flexibility in TG 426 concerning the study design, such as the choice of behavioural tests included in the study, and also the design of the individual tests, e.g., strength of stimulus, intervals between testing trials and sessions, number of trials per day, etc. It is up to expert judgment of the investigator to design on a case-by-case basis the most sensitive and appropriate test relevant for the exposure and toxicity of the compound under investigation. Although

this flexibility introduces potential sources of variability in DNT study design, the integrity of the original tests should not be compromised. Therefore, the studies conducted for regulatory testing require a high level of transparency and a justified and detailed approach concerning the study design and methods used.

A substantial amount of expertise is also required to interpret DNT study results, as well as to evaluate the reliability and relevance of DNT data for risk assessment. Expert judgment represents an integral and an important part of DNT testing and risk assessment. Therefore, to ensure proper use and the reliability of the DNT study it is evident that good guidance documents are essential to assist investigators both within research and regulatory testing in the designing of DNT studies as well as in the interpretation of the results from different DNT tests.

Notwithstanding this flexibility, according to some authors the DNT TG 426 represents so far the best available *in vivo* science-based testing protocol for assessing DNT potential to predict human health risk (Makris et al., 2009). The methodology used has been subjected to an extensive international validation, peer review and evaluation over the time. The reproducibility, reliability and sensitivity of the methods used have been demonstrated by using a wide variety of test substances. The results from DNT studies are used for hazard/risk assessment purposes because they meet the regulatory needs. In cases where data from a DNT study are not presented, safety factors may be employed to address the need for DNT data from a regulatory point of view (OECD Guideline Test No. 426, 2007; Raffaele et al., 2010).

From 1998 to early 2006, approximately 114 DNT studies have been performed using either the US-EPA guideline or the draft OECD guidelines. As of December 2008, 78 DNT studies were submitted to the EPA Office of Pesticide Programs (OPP) in support of pesticide registration. Although most of them evaluated organophosphorus insecticides, ACE and IMI were also tested (Raffaele et al., 2010).

DNT guidelines are complex, time consuming, costly and not suitable for routine testing of high numbers of chemicals. Some concerns in terms of feasibility and animal welfare have been raised in the scientific literature. Although the protocol of the guidelines is well designed and covers a broad window of exposure, the critical phase for some effects might be missed and not all effects would be found. Furthermore, the interpretation of results is difficult because of knowledge gaps concerning normal brain development on the functional, structural and molecular levels, thus complicating risk assessment of compounds (Beronius et al., 2013). A number of issues related to the interpretation of DNT studies have been raised, e.g. excessive variability may mask treatment-related effects and conversely minor statistically significant changes can be considered as treatment-related when in fact they might fall within the normal range (Raffaele et al., 2008). All findings should be considered in the context of the study and interpreted in conjunction with other findings. Even statistically significant findings should be consistent with a pattern of effects (Tyl et al., 2008).

It is not completely clear whether a negative DNT test is sufficient to exclude a DNT potential of a chemical compound. Although there are considerable data supporting the validity and sensitivity of the current US-EPA and OECD test guidelines, a number of activities have been and are underway to develop more rapid and efficient screening tools to predict developmental neurotoxicity of chemicals. *In vitro* and non-mammalian alternative systems-based models, along with *in silico* approaches could be scientifically robust methods suitable for the initial screening of chemicals for their potential to cause DNT. The data produced by alternative approaches may facilitate prioritisation of any further *in vivo* testing. However, these methods will need to be formally validated to meet regulatory requirements and offer possible alternative options for certain endpoints of DNT TG 426.

The *in vitro* and other alternative approaches could be used for a preliminary evaluation and for an initial chemical prioritisation to identify "the alerts" that may have the potential to induce developmental neurotoxicity. Additionally, since the understanding of toxicity mechanisms becomes increasingly important in risk assessment, *in vitro* studies should be considered to obtain information on the cellular/molecular mechanisms involved in DNT that are very difficult to achieve using only *in*

*vivo* approaches (Bal-Price et al., 2010, 2011). The relevance of the *in vitro* DNT testing for the *in vivo* situation (both toxicokinetics and toxicodynamic) should be carefully evaluated exploiting the available scientific evidence, but in general, the PPR Panel believes that *in vitro* tests currently used cannot substitute for *in vivo* DNT tests. To date, no *in vitro* test can be used to set health-based reference values.

Following the provision included in section 5.6.2 ("Developmental toxicity studies") of the Commission Regulation 283/2013, on data requirements for active substances, the PPR Panel encourages the definition of clear and consistent criteria at EU level to trigger submission of mandatory DNT studies. These could include development of an integrated DNT testing strategy composed of robust, reliable and validated *in vitro* assays and other alternative methods complementary to the *in vivo* TG 426 for prioritisation or screening of the DNT potential of substances. Although this goal is probably beyond the remit of this Opinion, the PPR Panel however encourages the work in the field to reach international agreement on how DNT testing should be addressed.

One of the most widely used *in vitro* systems in neurobiology is CGCs culture. The model is well characterised through its extensive use in studies of neuronal development, function and pathology. This *in vitro* system mainly consists of granule neurons (85 %), glial cells (15 %) and a few Purkinje cells. The primary cultures of CGCs are isolated from 1-10 days old pups as neurogenesis for the granule cells in cerebellum takes place postnatally, approximately between PND 0 and 10. The cells can be kept in culture for several weeks and are considered mature approximately after 10-12 days in culture. For DNT studies the cells should be exposed to the test chemical before they reach morphological and physiological maturation (up to approximately 8-10 DIV) to cover critical developmental processes (Bal-Price et al., 2010).

Further development and improvement of the OECD TG 426 guidance documents should be carried out on periodic basis as new knowledge becomes available, leading to the improved tests based on the most recent science and technology.

## 6. Toxicological Assessments of Acetamiprid and Imidacloprid for Regulatory Purposes

Both ACE and IMI were assessed under Directive 91/414/EEC for their inclusion into Annex I. Discussions between the Rapporteur Member States (RMS), Co-RMS and other Member States were organised to review the Draft Assessment Reports (DARs) and the comments received on each section (identity and physical/chemical properties, fate and behaviour in the environment, ecotoxicology, mammalian toxicology, residues and analytical methods).

For ACE Greece was designated to be the RMS and France the Co-RMS in the procedure. The EU Review report for this active substance was finalised in 2004 and ACE was authorised for use in the EU. As for IMI, Germany was designated as the RMS and submitted to EFSA the report of its detailed scientific examination. The EU review report was finalised in the meeting of the Standing Committee in July 2008 and the active substance was authorised for use in the EU.

The toxicological data reported in section 5.1 and 5.2 belong to the assessment reports considered for the peer review of ACE and IMI in the EU.

### 6.1. Acetamiprid

ACE is harmful by the oral route of administration; it has low acute dermal and inhalation toxicity; it is non irritant to the skin and the eyes of rabbits. It does not have sensitising potential.

The oral subacute and subchronic toxicity of ACE was studied in rats, mice and dogs. In a subchronic study in rats, the following doses were tested: 3.1, 6.0, 12.4, 50.8 and 99.9 mg/kg bw per day for males and 3.7, 7.2, 14.6, 56.0 and 117.1 mg/kg bw per day for females. NOAELs were identified for decreased body weight (12.4 mg/kg bw per day) and for altered liver function parameters (14.6 mg/kg bw per day) based on increased total cholesterol and decreased glucose levels. ACE was not found to be genotoxic.

The long term toxicity and carcinogenicity of ACE was studied in rats and mice. In the rat, the target organs were liver in males and females and kidney in males. Body weight was reduced and histo-pathological findings were observed in liver and kidney. In a 2-year chronic study in rats, the dose levels were: 7.1, 17.5 and 46.4 mg/kg bw per day for males and 8.8, 22.6 and 60.0 mg/kg bw per day for females. The NOAEL was set at 7.1 mg/kg bw per day based on centrilobular hypertrophy at 17.5 and 60 mg/kg bw per day in males and females, respectively. A NOAEL of 8.8 mg/kg bw per day was identified in female rats based on body weight reduction at 22.6 mg/kg bw per day. Microconcretions were observed in the renal papillae at 46.4 mg/kg bw per day in male rats. In the mouse study, the target organ was the liver. Body weight was reduced and hepatic histopathological findings were observed. However, NOAELs found for mice were higher than those for rats. ACE did not show a carcinogenic potential in rats and mice.

In pharmacological studies of the autonomic nervous system, ACE caused a decrease in blood pressure, mydriasis and inhibition of gastrointestinal movement. Concerning the somatic nervous system, muscle relaxation effects were observed. In the CNS, tremor and a decreased spontaneous activity were noted. An antidiuretic effect was observed in the urinary system.

A number of toxicity tests were performed with ACE metabolites. None were genotoxic in a number of assays and they had oral $LD_{50}$ levels higher than that of ACE. The same applies to 4 compounds that are present as impurities in technical ACE (WHO 2011).

### 6.1.1. Reproductive toxicity studies

The reproductive toxicity of ACE was studied in rats throughout two generations. The doses tested were 0, 6.5, 17.9 and 52 mg/kg bw per day. *In utero* growth and post-natal growth throughout lactation of F1 and F2 pups were significantly decreased at the highest dose (a NOAEL of 17.9 mg/kg bw per day was identified based on a decreased postnatal survival at 52 mg/kg bw per day). A delay in preputial separation and vaginal opening, as well as reduced litter size, decreased pup viability and weaning indices were also observed. As regards to maternal toxicity, the NOAEL was set at 6.5 mg/kg bw per day based on decreased body weight at 17.9 mg/kg bw per day. The body weight of F2 pups during lactation, as well as viability and weaning indices were significantly decreased at 17.9 mg/kg bw per day, so the offspring NOAEL was set at 6.5 mg/kg bw per day. Overall, ACE did not show potential for reproductive toxicity.

### 6.1.2. Developmental toxicity studies

Oral administration of ACE in pregnant rats and rabbits during the organogenesis period did not indicate any specific potential for developmental toxicity. The dose levels tested in the rat developmental study were 0, 5, 16 and 50 mg/kg bw per day, with a maternal NOAEL of 16 mg/kg bw per day based on reduced body weight and food intake. The dose levels tested in the rabbit study were 0, 7.5, 15 and 30 mg/kg bw per day and the maternal NOAEL was 15 mg/kg bw per day based on decreased body weight and food intake. In summary, no teratogencity and foetotoxicity was observed with rats and rabbits, and the foetal NOAEL was set at 50 and 30 mg/kg bw per day, respectively.

### 6.1.3. Neurotoxicity studies

ACE was studied for acute and subchronic behavioural neurotoxicity and neuromorphological effects in rats. The dose levels tested in the acute neurotoxicity study were 0, 10, 30 and 100 mg/kg bw. Clinical signs (tremors, walking and postural abnormalities, decreased grip strength and reduced locomotor activity) were observed in both sexes at the highest dose. These effects were also shown by males at an intermediate dose on the day of treatment. In a subchronic study, the doses tested were 0, 7.4, 14.8, 59.7 and 118 mg/kg bw per day for males and 0, 8.5, 16.3, 67.6 and 134 mg/kg bw per day for females. While decreased body weight and food intake were observed up to the top dose level, no evidence of irreversible neurotoxic effects was observed in the dose range assayed. The NOAEL for acute behavioural neurotoxicity was set at 10 mg/kg bw based on decreased locomotor activity observed at 30 mg/kg bw, while the respective subchronic NOAEL was set at 14.8 and 16.3 mg/kg bw per day for males and females, respectively, based on non-neurotoxic endpoints.

All together, neurotoxic effects occurred at approximately the same dose level as liver effects and general toxicity observed in the subchronic and reproduction studies. Also, there appears to be no difference in the dose ranges causing acute and prolonged neurotoxicity.

### 6.1.4. Developmental neurotoxicity (DNT) studies

No DNT studies were submitted or available for the authorisation of ACE at EU level.

**Table 3:** Summary of the toxicological assessment of ACE (Greece, 2001).

| | | | |
|---|---|---|---|
| **Acute toxicity** | | | |
| Rat $LD_{50}$ oral | in water: 417 mg/kg bw (male) R22<br>314 mg/kg bw (female) | | |
| Rat $LD_{50}$ dermal | > 2000 mg/kg bw | | |
| **Short term toxicity** | | | |
| Target / critical effect | Liver | | |
| Lowest relevant oral NOAEL | 12.4 mg/kg bw per day (200 ppm), 90 day rat study | | |
| **Long term toxicity and carcinogenicity** | | | |
| Target/critical effect | Liver and kidney | | |
| Lowest relevant NOAEL | 7 mg/kg bw per day (160 ppm), 2 year rat study | | |
| Carcinogenicity | No carcinogenic potential, treatment related mammary glands hyperplasia at 1000 ppm | | |
| **Reproductive toxicity** | | | |
| Reproduction target / critical effect | Reduced postnatal survival and decreased pup weight at parental toxic doses | | |
| Lowest relevant reproductive NOAEL | 6.5 mg/kg bw per day (100 ppm) in rats | | |
| Developmental target / critical effect | No teratogenicity or foetotoxicity was observed at the tested doses | | |
| Lowest relevant developmental NOAEL | 15 mg/kg bw per day in rabbits | | |
| **Neurotoxicity / Delayed neurotoxicity** | | | |
| NOAEL acute | 10 mg/kg bw, based on reduced locomotor activity in the rat at high and medium dose | | |
| NOAEL subchronic | 200 ppm (14.8 and 16.3 mg/kg bw per day for males and females respectively) based on reduced body weight and food consumptions | | |
| NOAEL developmental neurotoxicity | Not available | | |
| **Summary** | Value | Study | Safety factor |
| ADI | 0.07 mg/kg bw per day | 2 year rat study and 2-generation rat reproductive study | 100 |

| | | | |
|---|---|---|---|
| AOEL short term (Oral and systemic) | 0.124 mg/kg bw per day | 13-week rat study | 100 |
| AOEL long term (Oral and systemic) | 0.07 mg/kg bw per day | 2 year rat study and 2-generation rat reproductive study | 100 |
| ARfD (acute reference dose) | 0.1 mg/kg bw | Acute neurotoxicity rat study | 100 |

## 6.2. Imidacloprid

IMI is moderately acutely toxic in rodents with oral $LD_{50}$ values in the range of 380-650 mg/kg bw in rats and 130-170 mg/kg bw in mice. IMI has a low dermal and inhalation acute toxicity. It is neither a skin nor an eye irritant, and has no skin sensitising properties.

In subchronic toxicity studies in rodents (98-day, 96-day, 91-day neurotoxicity studies in rats and a 107-day study in mice) the decreased body weight or body weight gain was the most sensitive parameter and the liver was the main target organ in dogs (28-day, 90-day, 1-year) and rats. Decreased body weight, hepatotoxicity (clinical chemistry changes and histopathology findings) and histopathological degenerative changes in the testes were recorded. A NOAEL of 9.3 mg/kg bw per day was agreed for the 91-day neurotoxicity in rats based on decreased body weight. In some studies in dogs, liver and thyroid effects occurred (hepatocellular hypertrophy and follicular atrophy of the thyroid) as well as ataxia, tremors and/or mortalities were observed at the high dose only (180 mg/kg bw per day). The occurrence of ataxia and tremor suggested an effect on the CNS (NOAEL of 7.8 mg/kg bw per day).

In long term toxicity studies, the thyroid was the main target organ in rats, whereas in mice effects included reduction in body weight, weak hepatotoxicity and more frequent mineralisation of the thalamus. No evidence of an oncogenic potential of imidacloprid was found in both species. In a chronic toxicity study in rats, the following doses were tested: 0, 5.7, 17.1 and 50.6 mg/kg bw per day for male rats and 0, 8.3, 24.9 and 74.7 mg/kg bw per day for female rats. The lowest NOAELs identified were 5.7 and 24.9 mg/kg bw per day, based on thyroid effects (increased incidence of colloid mineralisation) at 17.1 and 74.7 mg/kg bw per day in males and females, respectively.

IMI did not show genotoxic potential. The available data on the acute toxicity to rats show that its metabolites 5-hydroxy IMI, nitrosimine and IMI 2-ketone are of lower toxicity than IMI. However, desnitro IMI, which is considered the most abundant metabolite, is more acutely toxic than IMI by about a factor of 1.4 ($LD_{50}$ 280 and 500 mg/kg bw, respectively). The remaining metabolites were less toxic than the parent compound, showing $LD_{50}$ levels above 1000 mg/kg. No genotoxic potential of these metabolites has been reported. As occurs with nicotinoids, the desnitro metabolite is protonated under physiological pH, yielding a charged nitrogen moiety and raising its affinity for mammalian nAChRs. Since this metabolite is not more than twice as toxic as IMI, the risk to mammals from it is likely to be covered by the risk assessment for IMI.

### 6.2.1. Reproductive toxicity studies

The effects of IMI on the reproductive parameters were studied in a one-generation (range-finding) and 2-generation rat studies. IMI did not show any potential for reproductive toxicity. The dose levels tested in the 2-generation study were 0, 8, 20 and 50 mg/kg bw per day. The offspring NOAEL was set at 20 mg/kg bw per day based on decreased preweaning body weight gain. The parental NOAEL was set at 20 mg/kg bw per day based on decreased body weight gain and food intake at 50 mg/kg bw per day.

### 6.2.2. Developmental toxicity studies

The embryotoxic effects of IMI were investigated in rats and rabbits. In the rat study, the dose levels tested were 0, 10, 30 and 100 mg/kg bw per day. The NOAEL was set at 30 mg/kg bw per day for both maternal and developmental effects based on increased incidence of wavy ribs (offspring) and decreased body weight gain and food intake (dams) at 100 mg/kg bw per day. In the rabbit study, the dose levels tested were 0, 8, 24 and 72 mg/kg bw per day and the offspring NOAEL was set at 24 mg/kg bw per day based on decreased foetal weight, retarded ossification and increased prenatal litter loss at 72 mg/kg bw per day. The maternal NOAEL was set at 8 mg/kg bw per day based on decreased body weight gain and food intake at 24 mg/kg bw per day. Overall, there was no indication of a specific embryotoxic or foetotoxic potential.

### 6.2.3. Neurotoxicity studies

Neurotoxic effects of IMI were investigated in acute and subchronic tests. Also a DNT study was performed in rats. The acute NOAEL was 42 mg/kg bw per day based on clinical signs and clearly reduced motor/locomotor activity at 151 mg/kg bw (dose level tested were 0, 42, 151 and 307 mg/kg bw per day). Most clinical signs appeared related to acute receptor-mediated cholinergic toxicity of IMI. In the 13-week neurotoxicity study, the dose levels tested were 0, 9.3, 63.3 and 196 mg/kg bw per day (rat males) and 0, 10.5, 69.3 and 213 (rat females). The NOAEL was 9.3 and 10.5 mg/kg bw per day (for rat male and females, respectively) based on a decreased body weight. Although minor effects were observed in the functional observational battery (FOB) at the highest dose tested, there was no convincing evidence of neurotoxicity. The subchronic NOAEL was 196 and 213 mg/kg bw per day for rat males and females, respectively. In the 28- and 90-day dog studies, tremor and trembling were observed as relevant acute effects with a NOAEL set at 7.8 mg/kg bw per day.
Acute and prolonged neurotoxicity seem to occur at dose levels above those producing other toxic effects in the subchronic study. Moreover, acute neurotoxic effects seem to be more evident than those occurring upon prolonged exposure.

### 6.2.4. Developmental neurotoxicity (DNT) studies

In a DNT study, summarised in the DAR prepared by Germany, the following dose levels were tested: 0, 8, 20 and 56 mg/kg bw per day during gestation and approximately 0, 13, 30 and 80 mg/kg bw per day for the offspring. The NOAEL for pups was 30 mg/kg bw per day based on reduced preweaning body weight gain and decreased motor/locomotor activity at 80 mg/kg bw per day. A small but statistically significant decrease in the caudate/putamen width was noted in female pups at the highest dose tested. The NOAEL for maternal toxicity was set at 56 mg/kg bw per day based on a transient decrease in food intake at the end of gestation and beginning of lactation.

**Table 4:** Summary of the toxicological assessment of IMI (Germany, 2005; EFSA 2008[10]).

| | |
|---|---|
| **Acute toxicity** | |
| Rat $LD_{50}$ oral<br>Rat $LD_{50}$ dermal | ~ 500 mg/kg bw Xn, R22<br>> 5000 mg/kg bw |
| **Short term toxicity** | |
| Target / critical effect | Body weight gain (rat, dog), liver (clinical chemistry and histopathological findings: rat, dog), trembling/tremor (dog) |
| Lowest relevant oral NOAEL | 90-d rat: 14 mg/kg bw per day<br>1-yr dog: 15 mg/kg bw per day |
| **Long term toxicity and carcinogenicity** | |
| Target/critical effect | Body weight gain (rat, mouse), liver (blood chemistry: |

[10] http://www.efsa.europa.eu/en/efsajournal/pub/148r.htm

| | |
|---|---|
| | rat, mouse; organ weight decrease: mouse ); thyroid (mineralisation in the follicular colloid: rat) |
| Lowest relevant NOAEL | 2-yr rat: 5.7 mg/kg bw per day<br>2-yr mouse: 65.6 mg/kg bw per day |
| Carcinogenicity | No evidence for carcinogenicity |
| **Reproductive toxicity** | |
| Reproduction target / critical effect | Parental bw gain decreased; fertility parameters unaffected; pup weight gain reduction at parentally toxic doses |
| Lowest relevant reproductive NOAEL | Rat, 2-generation:<br>parental: 20 mg/kg bw per day<br>reproduction: 50 mg/kg bw per day<br>offspring: 20 mg/kg bw per day |
| Developmental target / critical effect | Rat: maternal bw gain and food consumption decreased; developmental toxicity indicated by increase in skeletal variant (wavy rib).<br>Rabbit: maternal bw gain and food consumption decreased, mortality; increased prenatal litter loss, reduced foetal weight, delayed ossification and skeletal anomalies at maternally toxic doses |
| Lowest relevant developmental NOAEL | Rat, maternal: 30 mg/kg bw per day<br>Rat, developmental: 30 mg/kg bw per day<br>Rabbit, maternal: 8 mg/kg bw per day<br>Rabbit, developmental: 24 mg/kg bw per day |
| **Neurotoxicity** | |
| NOAEL (acute neurotoxicity study)<br>NOAEL (subchronic neurotoxicity study)<br>NOAEL (developmental neurotoxicity study) | 42 mg/kg bw<br>9.3 mg/kg bw per day<br>20 mg/kg bw per day (during gestation) |

| **Summary** | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0.06 mg/kg bw per day | Rat, 2-year study | 100 |
| AOEL | 0.08 mg/kg bw per day | Dog, 28 and 90 days (acute effects) | 100 |
| ARfD (acute reference dose) | 0.08 mg/kg bw | Dog, 28 and 90 days (acute effects) | 100 |

## 6.3. The assessments of DNT studies by US-EPA

*a) Acetamiprid*

In a DNT study compliant to US-EPA guideline OPPTS 870.6300 (Aug 1998), acetamiprid was administered to 25 mated female rats dosed by gavage at doses of 0, 2.5, 10 and 45 mg/kg/day from gestation day (GD) 6 through lactation day (LD) 21. The maternal NOAEL was 10 mg/kg per day based on decreased body weight and body weight gain during gestation at 45 mg/kg bw per day. At this high dose level the offspring showed treatment-related decreased body weights and body weight gains in males and females post-weaning, decreased pre-weaning survival (PND 0-1), and decreased maximum auditory startle response in males. Treatment had no adverse effects on clinical signs, developmental landmarks, FOB, brain weight or brain morphology. No conclusions could be made on motor activity evaluation due to the low confidence in the data because of problems with the control data (i.e, the normal developmental pattern was not seen in control animals).The maximum auditory startle response amplitude was decreased 27 % (PND 20) and 40 % (PND 60) at 10 mg/kg bw per day, and it was decreased 42 % (PND 20) and 53 % (PND 60) at 45 mg/kg bw per day. However, only in the latter case the endpoint was considered as treatment-related. No conclusion was made on the effects of ACE on learning and memory because of the high variability in the data.

*b) Imidacloprid*

The same study summarised in the IMI DAR, and compliant to US-EPA guideline OPPTS 870.6300 (Aug 1998), was available to US EPA; however, differences in the assessment (e.g., dietary conversions, evaluation of some effects) were present. The average daily intake of IMI (administered to 30 parent female rat from GD 0 through PND 21) was 0, 8, 19.4, 54.7 mg/kg bw per day during gestation, and 12.8-19.5, 30.0-45.4 and 80.4-155.0 mg/kg bw per day during lactation. Treatment-related effects for maternal animals were a decrease in food consumption for dams in the high dose group compared to controls during the third week of gestation and the first week of lactation. There was also a decrease in body weight gain during lactation days 0-7. The maternal NOAEL was 19.5 mg/kg bw per day based on decreased food consumption and decreased body weight gain during lactation.

Treatment-related effects for offspring were limited to the high dose group. Body weights of high-dose males and females were significantly decreased by 11-13 % ($p < 0.05$) prior to and after weaning, with recovery (in females back to control levels by PND 50, in males to a 4 % difference that persisted to study termination). Body weight gains were also decreased 12-23 % during lactation, with recovery by PND 17. Overall motor activity was decreased on PND 17 in high-dose males (38 %) and females (31 %) and on PND 21 in females (37 %), although the differences were not statistically significant. The effects on motor activity were deemed as treatment related because of their magnitude and the occurrence at the high dose in both sexes during the period of exposure. High dose females at PND11 had a 5.5% decrease in thickness of the caudate/putamen in comparison to controls (2.617 *vs.* 2.769 mm). These females also had a 27.6% reduction in the thickness of corpus callosum (0.436 *vs.* 0.602 mm). The decrease in the caudate/putamen width persisted in high dose female animals at study termination (3.677 vs. 3.750 mm, $p<0.05$). The offspring NOAEL was 19.5 mg/kg bw per day based on decreased body weight and body weight gain, and decreased motor activity. The NOAEL for neuropathological findings in females was estimated to be 5.5 mg/kg bw per day based on the application of a x10 safety factor to the LOAEL (54.7 mg/kg bw per day) since neuropathology was not performed at lower doses.

## 6.4. Comments of the PPR Panel on developmental neurotoxicity studies on acetamiprid and imidacloprid

### 6.4.1. Acetamiprid

The DNT study on ACE was carried out within the US EPA assessment framework. The PPR Panel considers that, notwithstanding the claimed guideline compliance of this study, the data do not allow any firm conclusion to be drawn since important endpoints such as motor activity, learning and memory evaluation could not be properly assessed. Moreover, the PPR Panel notes that insufficient arguments seem to support the straight conclusion of the study reporting that (seemingly dose-related) reduced auditory startle responses in offspring first noted at 10 mg/kg bw was not related to treatment. Overall the study can only provide supportive evidence, but is inadequate for a proper characterisation of effects and dose-response relationship between ACE and developmental neurotoxicity. The PPR Panel recommends that, based on these uncertainties and methodological drawbacks, the NOAEL for DNT should be conservatively set at 2.5 mg/kg bw per day.

### 6.4.2. Imidacloprid

The maternal NOAELs of the DNT study were 20 (EU) and 19.5 (US-EPA) mg/kg bw per day, based on transient effects on food intake (EU study only) and weight gain. The NOAEL for DNT in the EU study was 20 mg/kg bw per day based on reduced preweaning body weight gain and decreased motor/locomotor activity at 56 mg/kg bw per day. Since the NOAEL and LOAEL for DNT are based

on the dose levels calculated during gestation, the PPR Panel cannot rule out that such effects were at least partly induced by prenatal exposure.

The NOAEL was estimated by California EPA at 5.5 mg/kg bw per day (from applying a 10-fold safety factor to the LOAEL) based on reduced thickness of the caudate/putamen and corpus callosum in females only. The PPR Panel notes that neuropathological examination at dose levels lower than 54.5 mg/kg bw per day would have facilitated a more accurate assessment of the NOAEL for neuropathological effects. The PPR Panel further notes that in the EU assessment a significant decrease in the caudate/putamen width was also present in females at 56 (prenatal exposure) mg/kg bw per day at study termination and it is unknown whether lower dose groups were analysed for neuropathology. Thus, this finding in particular is consistent over time and could be considered as a specific effect. Accordingly, the NOAEL should be conservatively set at 5.5 mg/kg bw per day.

The pathological changes observed in basal ganglia (caudate and putamen) and in corpus callosum may be associated with motor function control. In particular, putamen is connected with the *globus pallidus* and *substantia nigra* through various nervous pathways. Since putamen is involved in movement regulation and influence various types of learning, a decrease in thickness of this structure could be due to decreased number of neurons/glia ultimately leading to decreased motor activity. The neuronal nAChRs may be involved in some of this neuropathology, thus a possible link between morphological and functional changes should be taken into account. Since neuropathological assessment was first performed on PND 11, the timeline of the IMI developmental neurotoxicity could not be determined. Therefore, evidence from the DNT study in rats suggests that IMI may affect development of the brain structures, although the current data may be insufficient for a proper characterisation of effects and dose-response of IMI developmental neurotoxicity during pre- and postnatal phases.

The US-EPA deemed the study results of low concern because effects in pups are well characterised with a clear NOAEL and occurred in the presence of maternal toxicity (with the same NOAEL); thus, according to the US-EPA, no residual uncertainties for prenatal/postnatal toxicity were identified. However, the PPR Panel considers that the neuropathological findings may point out a specific susceptibility of pups since the "estimated NOAEL" in pups is more than three-fold lower than the maternal NOAEL. Thus, a better characterisation of IMI developmental neurotoxicity effects and NOAEL is recommended.

## 7. The reference values (Acceptable Daily Intake, Acute Reference Dose and Acceptable Operator Exposure Level) currently agreed in the EU

As regards ACE, neurotoxic effects occurred at approximately the same dose level as liver effects and general toxicity observed in the subchronic and reproduction studies. Also, no differences appear in the dose ranges causing acute and prolonged neurotoxicity, indicating that single exposure to ACE is neurotoxic. In contrast, for IMI acute and prolonged neurotoxicity seem to occur at dose levels above those producing other toxic effects in the subchronic study. Moreover, acute neurotoxic effects appear to be more evident than those occurring upon prolonged exposure. Overall, the two neonicotinoids seem to be somewhat different as far as neurotoxicity is concerned.

As regards the current reference values for ACE, the ADI and long-term AOEL are 0.07 mg/kg bw per day based on the NOAEL of 2 year rat study and 2-generation rat reproductive study with an uncertainty factor of 100. The short-term AOEL is 0.124 mg/kg bw per day based on a NOAEL of the 90-day study in rats (12.4 mg/kg bw) with an uncertainty factor of 100. The ARfD is 0.1 mg/kg bw based on the NOAEL of 10 mg/kg bw in an acute rat neurotoxicity study with an uncertainty factor of 100. Only the ARfD is based on a NOAEL for neurotoxicity.

The US-EPA DNT study clearly shows DNT effects of ACE (decreased maximum auditory startle response amplitude in males by 27 % at PND 20 and by 40 % at PND 60) at 10 mg/kg bw per day in the absence of maternal toxicity, although such effect was not considered as treatment related. However, notwithstanding the claimed guideline compliance, this study has limited reliability since examination of motor activity, learning and memory was not adequately performed; thus, no reliable NOAEL values are available. The PPR Panel considers that, based on the indications provided by the US-EPA DNT study (no EU DNT study was performed), the current ARfD and short term AOEL may not be protective enough for the possible DNT of ACE. Moreover, given the uncertainties aforementioned about the NOAEL setting in the one DNT study available for ACE, no conclusions can be reached as regards the ADI and long term AOEL. The PPR Panel recommends that, based on these uncertainties, the NOAEL for DNT should be conservatively set at 2.5 mg/kg bw per day instead of the originally considered 10 mg/kg bw per day. The occurrence in rat males only does not represent a weakness as the ontogeny of cholinergic enzymes is different in males and females (Loy and Sheldon, 1987). This more conservative NOAEL should be used as a point of departure for the derivation of ADI, ARfD and AOELs, which should all be set at 0.025 mg/kg bw per day until new and more reliable DNT data are available. Then, the point of departure can be revised.

As regards the current reference values for IMI, the ADI is 0.06 mg/kg bw per day based on the NOAEL of a 2-year rat study of 6 mg/kg bw with an uncertainty factor of 100. The ARfD and AOEL are 0.08 mg/kg bw based on the NOAEL of both 28- and 90-day dog studies (8 mg/kg bw) with an uncertainty factor of 100. Only the ARfD and AOEL are based on a NOAEL for neurotoxicity.

The IMI DNT study showed a clear NOAEL for DNT effects (decreased motor activity), around 20 mg/kg bw per day; the same NOAEL was also observed for maternal toxicity, with the LOAEL for both effects being ~55 mg/kg bw per day. The PPR Panel considers that, based on this NOAEL, the current ARfD may be protective enough for the possible DNT of IMI. However, in the US-EPA assessment, a NOAEL for neuropathological findings could not be determined and by assuming a LOAEL of 54.7 mg/kg bw per day, the NOAEL was estimated by California EPA as being 5.5 mg/kg per day by applying a 10-fold default factor. This "estimated no effect level" (ENEL) might be applicable to repeated exposures to IMI to all population subgroups. Because decreases in brain structure dimensions could theoretically result from a single exposure *in utero*, the ENEL of 5.5 mg/kg bw per day could be used to estimate the risk of acute exposure to IMI in women of childbearing age. In this case, the current ARfD and AOEL may not be protective enough for potential DNT of IMI and should be conservatively lowered to the same level than the ADI (0.06 mg/kg bw per day). The ADI for IMI was estimated based on the chronic NOAEL of 5.7 mg/kg per day for thyroid effects in rats, which is sufficiently close to the ENEL of 5.5 mg/kg bw per day for decrease in the thickness of brain structures. Therefore it would be adequate for protection against the potential effects of IMI on the developing nervous system (California EPA, 2006).

## CONCLUSIONS AND RECOMMENDATIONS

### CONCLUSIONS

The PPR Panel concludes that both ACE and IMI show some indications of DNT based on the available information, but further good quality *in vivo* data are needed following OECD TG 426, to more properly characterise a DNT potential and associated dose-response relationships. This would mainly apply to ACE, but should also be considered for IMI. New studies would benefit from a wider range of test concentrations in order to discriminate between direct and indirect effects. In addition, all dosed animals should be subjected to neuropathological analysis (including histology of the brain, in accordance with OECD TG 426).
The data underlying the NOAEL used to set the reference values for ACE and IMI currently applied in the EU need to be further analysed and discussed, and a comparison with current risk assessment has to be taken into account to verify if there are uncertainties left.

**Question 1**: *Based on the available data both in the published scientific literature and in the toxicological dossier submitted for approval, do acetamiprid and imidacloprid show developmental neurotoxic effects?*
Evidence from a DNT study of IMI in rats has revealed decreased body weights, reduced motor activity level and changes in dimensions of brain structures (reduction in the thickness of corpus callosum and a decreased width of caudate/putamen). The DNT study on ACE has showed decreased body weights, reduced pup viability and decreased maximum auditory startle response. However, no conclusion can be drawn for ACE owing to non-compliance with test guidelines (problems with control data and high variability of some data) concerning the behavioural tests such as motor activity as well as learning and memory evaluation, which are recognised to be the most sensitive DNT endpoints. Therefore, it is concluded that these two neonicotinoid compounds may affect neuronal development and function. However evidence is limited as a result of methodology issues and because neurotoxic effects were observed at the same dose levels eliciting maternal toxicity.

**Question 2**: *Have acetamiprid and imidacloprid been assessed adequately for developmental neurotoxicity and if no, what further information would be of value in this assessment*?
Comprehensive toxicological databases are available for both neonicotinoids. Acute, subacute, subchronic, chronic, mutagenitic, carcinogenity, reproduction, developmental and DNT studies have been carried out for the hazard characterisation of both compounds within regulatory frameworks. One DNT study has been performed for IMI and one for ACE (not complete and not adequately assessed for DNT); in both cases, studies were considered acceptable for regulatory purposes although they were not necessarily guideline compliant. However, the PPR Panel considers that the available data do not allow a full characterisation of developmental neurotoxicity of the two neonicotinoids. In the case of ACE, important neurodevelopmental endpoints such as motor activity and learning and memory have not been properly assessed. For IMI, data on neuropathology is available only for the highest dose tested and there is uncertainty about whether neuropathological changes occur at lower doses. The latter is of utmost importance since these changes may appear after a single dose of the active substance administered prenatally.

**Question 3**: *Do the existing health-based guidance values provide adequate protection against any potential developmental neurotoxicity of acetamiprid and imidacloprid and if not what values would be necessary to provide such protection?*
Based on the indications provided by the available DNT studies and the associated uncertainties in establishment of the corresponding NOAELs, the Panel considers that the current ARfDs may not be protective enough for the possible developmental neurotoxicity of acetamiprid and imidacloprid. The same uncertainties preclude from reaching any reliable conclusion as regards the ADI for acetamiprid. In turn, the ADI set for imidacloprid would be adequate for protection against its potential adverse effect on the developing nervous system.

The PPR Panel recommends that a more conservative NOAEL of 2.5 mg/kg bw per day for ACE should be used as a point of departure for the derivation of ADI, ARfD and AOELs, which thus should be set at 0.025 mg/kg bw (per day). When new and more reliable DNT data are available, the point of departure can be revised.
As the current ARfD and AOEL may not be protective enough for potential DNT of IMI, the Panel also recommends to conservatively lowering these reference values to the same level as the ADI (0.06 mg/kg bw per day).

**Question 4**: *Do the approved neonicotinoids need further investigation to clarify the mechanism of action of the nAChRs? Should all the approved neonicotinoids be tested using the in vitro system mentioned in the publication of Kimura-Kuroda et al (2012)?*
Since the impaired cholinergic transmission during ontogenesis may be a component of some developmental disorders, additional studies with complementary DNT specific endpoints should be evaluated to have more solid information as to the potential DNT of neonicotinoids. The data provided by Kimura-Kuroda et al. (2012) indicate that further research on the action of neonicotinoids in the

cerebellum may be warranted to fully understand the DNT potential of ACE and IMI. However, the assay presented by these authors covers only a very limited aspect of brain function, and its limitations prevent a current use as a screening tool in the regulatory context. However, further studies should be performed using the culture of a wider range of brain structures besides those derived from cerebellar cortex, which also express nAChRs, to thoroughly evaluate key developmental processes such as neuronal and glial proliferation, migration, differentiation, neurite outgrowth, synaptogenesis, networking, myelination and programmed cell death.

The PPR Panel considers that it is necessary to develop mechanistic understanding of underlying molecular mechanisms and time frames of treatment with test substances to evaluate whether the observed effects in the study by Kimura-Kuroda et al. (2012) are reversible, to explain the meaning of the responses to the challenge by KCl and to define the more accurate estimation of concentrations of the test substance at the receptor site. To extend confidence in findings, provision of appropriate positive and negative controls and scrutiny of data for reliability and reproducibility are required. Accordingly, the *in vitro* system as proposed by Kimura-Kuroda et al. (2012) is not adequate for use as a tool for screening developmental neurotoxicants from a regulatory point of view. Considerable further characterisation is required before an improved protocol based on the one proposed by these authors can be used to assess its relevance for *in vivo* exposure. An integrated DNT testing strategy under a tiered approach, starting from validated *in vitro* assays to *in vivo* test, can be used to screen for pesticide DNT potential, including all neonicotinoids and their active (toxic) metabolites, provided that their toxicity is the consequence of binding to nAChR.

## RECOMMENDATIONS

The PPR Panel proposes several recommendations for early consideration by risk managers, although they do not have to be necessarily implemented from a regulatory standpoint. These recommendations are related to both the knowledge gaps of the neonicotinoids and the DNT testing framework.

- The PPR Panel supports the requirements for new *in vivo* studies compliant with OECD TG 426, in particular for acetamiprid, in order to clarify current uncertainties and set robust dose-response relationships for DNT effects.
- New studies are needed to clarify whether or not neonicotinoids, and their active (toxic) metabolites, can enter the brain across the BBB and to quantify any such capacity at all stages of development. The PPR Panel encourages the definition of clear and consistent criteria at EU level to trigger specific mandatory DNT studies, according to the provision included in section 5.6.2. ("Developmental toxicity studies") of the Commission Regulation No. 283/2013 on data requirements for active substances. This provision emphasises that "When indicated by observations in other studies or the mode of action of the test substance, supplementary studies or information may be required to provide information on the postnatal manifestation of effects such as developmental neurotoxicity".
- The PPR Panel supports the development of an integrated *in vitro* neurotoxicity testing strategy complementary to *in vivo* assays included into OECD TG 426 in order to screen the DNT potential of pesticides and other chemicals entering food chains, provided that the defined criteria to trigger mandatory DNT studies are met. The battery should be composed of robust, reliable and validated assays, relevant to the prediction of human toxicity, exploiting the wide range of potential alternative methods (not only *in vitro*), and should support a tiered and cost-effective hazard characterisation process tailored to the different knowledge bases for regulated chemicals. *In vitro* and non-mammalian alternative systems-based models, along with *in silico* approaches, could provide scientifically robust methods suitable for the initial screening or prioritisation of chemicals for their potential to cause DNT.

- The PPR Panel notes that the great interspecies differences observed in $LD_{50}$ for acetamiprid and imidacloprid, as well as in plasma levels after non-lethal acute poisonings by these active substances, could indicate the need to use human cells in culture for *in vitro* testing.
- Based on the reported subtle molecular changes in the structure of neonicotinoids that can lead to differential activation of nAChRs subtypes, more information would be needed on the potential developmental neurotoxic effects caused *in vitro* and *in vivo* by the active (toxic) metabolites of neonicotinoids, particularly those showing higher affinity for nAChRs than the parent compound.
- The role of nAChRs in neuronal differentiation and maturation should be evaluated in order to provide a mechanistic basis for the characterisation of possible developmental neurotoxic effects of neonicotinoids. Recommended experimental approaches include activating/inactivating or blocking nAChR expression and using the natural agonist compound acetylcholine as a positive control.

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## GLOSSARY

**ADI (Acceptable Daily Intake):** estimate of the amount of substance in food expressed on a body weight basis, that can be ingested daily over a lifetime, without appreciable risk to any consumer on the basis of all known facts at the time of evaluation, taking into account sensitive groups within the population (e.g. children and the unborn).

**AOEL (Acceptable Operator Exposure Level)**: the reference value against which non-dietary exposures to pesticides are currently assessed. It is intended to define a level of daily exposure throughout a spraying season, year on year, below which no adverse systemic health effects would be expected. The AOEL is normally derived by applying an uncertainty factor (most often 100) to a No Observed Adverse Effect Level (NOAEL) (corrected if appropriate for incomplete absorption) from a toxicological study in which animals were dosed daily for 90 days or longer. Less often, the critical NOAEL comes from a study with a shorter dosing period (e.g. a developmental study).

**ARfD (Acute Reference Dose)**: estimate of the amount of substance in food and/or drinking water, expressed on a body weight basis, that can be ingested over a short period of time, usually during one day, without appreciable risk to the consumer on the basis of the data produced by appropriate studies and taking into account sensitive groups within the population (e.g. children and the unborn).

**Auditory startle reflex (ASR):** it is a short-latency change in behaviour elicited by a sudden and intense acoustic stimulus. The ASR is noted as a sudden flinch or cessation of ongoing movement following the auditory stimulus.

**Basal ganglia**: are a group of nuclei in the brains of vertebrates situated at the base of the forebrain and strongly connected with the cerebral cortex, thalamus and other brain areas. The basal ganglia are associated with a variety of functions, including voluntary motor control, procedural learning relating to routine behaviours or "habits," eye movements, and cognitive, emotional functions.

**Caudate/putamen**: components of the basal ganglia.

**Corpus callosum**: great band of commissural fibers uniting the cerebral hemispheres of higher mammals including humans.

**Developmental neurotoxicity**: adverse effects of pre- and postnatal exposure on the development and function of the nervous system.

**Draft assessment report (DAR)**: initial evaluation of the dossier of an active substance prepared by a designated Member States.

**Desensitisation (of a receptor)**: Long-time response to receptor agonists due to high and prolonged levels of activation that produce a shift in receptor conformational equilibrium resulting in blockage of the receptor. The most common feature is a time-dependent change in ion current.

**Entorhinal cortex (Brodmann's area 28)**: it constitutes the main interface between the hippocampal formation and the neocortex. It is located in the medial temporal lobe and together with perirhinal cortex (Brodmann's area 35) and hippocampus plays an important role in spatial learning and memory.

**Excitation** (of a receptor): When an impulse arrives at a presynaptic terminal it causes the release of a neurotransmitter and its subsequent diffusion across the synaptic cleft where it activates postsynaptic receptor sites opening specific ion channels. At an excitatory synapse, ionic fluxes through these channels tend to depolarize the membrane, while different patterns of ionic flux hyperpolarize the membrane at inhibitory synapses.

**Glial proliferation**: Following a peripheral nerve injury, the satellite glia cells around the damaged nerve (e.g., Schwann cells, astrocytes and oligodendrocytes) start to proliferate for repairing the damaged tissue and restoring its function.

**Hazard characterisation:** Qualitative and, wherever possible, quantitative description of the inherent property of an agent or situation having the potential to cause adverse effects.

**Health-based reference values**: level of a substance that consumers and operators can be exposed to in the short- or long-term without an appreciable risk to their health. It is established on the basis of toxicological data showing the absence of an adverse effect with the correction by an uncertainty factor, conventionally of 100, to account for the differences between test animals and humans (factor of 10) and possible differences in sensitivity between humans (another factor of 10). It serves as reference for comparison with exposures occurring under realistic conditions over the short- or long-term.

**Hippocampus**: brain structure that plays an important role in the limbic system. It consists of gray matter covered on the ventricular surface with white matter, and that is involved in forming, storing, and processing memory and is also associated with learning and emotions.

**Myelin** is an electrically insulating phospholipid layer that surrounds the axons of many neurons.

**Myelination**: The acquisition, development, or formation of a myelin sheath around a nerve fiber.

**Neonicotinoid insecticides**: class of insecticides with chemical structures similar to nicotine that share a common mode of action by binding to nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects.

**Neurite outgrowth**: process during development, in which neurons make connections by growing in response to axon guidance cues; neurite outgrowth during nervous system development results in a network of synaptic connections between participating neurons.

**Networking** (neuronal): connections and circuits between neurons.

**Nicotinic acetylcholine receptors (nAChRs)**: receptors that form ligand-gated ion channels with unique localisation in pre-, post-, and extrasynaptic membranes. They are found in the central nervous system (CNS) neurones of insects and in both the central and peripheral nervous systems of mammals as well as in neuromuscular junctions.

**OECD TG**: the Organisation for Economic Co-operation and Development (OECD) Test Guidelines (TG) are a collection of the most relevant internationally agreed test methods used by government, industry and independent laboratories to determine the safety of chemicals and chemical preparations, including pesticides and industrial chemicals.

**Programmed cell death** (or apoptosis): it is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area. Apoptosis plays a crucial role in developing and maintaining the health of the body by eliminating old cells, unnecessary cells, and unhealthy cells.

**Synapse**: structure at which a nerve impulse is relayed from the terminal portion of a neuron (axon) to the (dendrites of an) adjacent neuron.

**Synaptogenesis**: formation of synapses between neurons.

**Thalamus**: is a midline paired symmetrical structure within the brains of vertebrates, including humans. It is situated between the cerebral cortex and midbrain and it is involved in the relaying of

sensory and motor signals to the cerebral cortex, and in the regulation of consciousness, sleep, and alertness.