Heliyon 9 (2023) e15840

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Neurocytotoxicity of imidacloprid- and acetamiprid-based comercial insecticides over the differentiation of SH-SY5Y neuroblastoma cells

Lenin J. Ramirez-Cando [a], Marcelo S. Guzmán-Vallejos [a], Luis G. Aguayo [b], Fernando D. Vera-Erazo [b], Santiago J. Ballaz [c,*]

[a] School of Biological Sciences & Engineering, Yachay Tech University, Urcuquí, Ecuador
[b] Department of Physiology, School of Biological Sciences, Universidad de Concepcion, Chile
[c] Medical School, Universidad Espíritu Santo, Samborondón, Ecuador

ARTICLE INFO

Keywords:
Cell differentiation
Cell survival
Neurons
Neonicotinoids
Oxidative stress
Toxicity

ABSTRACT

Neonicotinoids are effective insecticides with specificity for invertebrate nicotinic acetylcholine receptors. Neonicotinoids are chemically stable and tend to remain in the environment for long so concerns about their neurotoxicity in humans do nothing but increase. Herein, we evaluated the chronic toxic effects of acetamiprid- and imidacloprid-based insecticides over the differentiation of human neuroblastoma SH-SY5Y cells, which were exposed to these insecticides at a concentration range similar to that applied to crop fields (0.01–0.5 mM). Both insecticides did not have acute cytotoxic effects in both non-differentiated and in staurosporine-differentiated SH-SY5Y cells cytotoxicity as measured by the MTT and vital-dye exclusion tests. However, after a chronic (7-day) treatment, only imidacloprid dose-dependently decreased the viability of SH-SY5Y cells ($F(4,39) = 43.05$, $P < 0.001$), largely when administered-during cell differentiation ($F(4,39) = 51.86$, $P < 0.001$). A well-defined dose-response curve was constructed for imidacloprid on day 4 ($R^2 = 0.945$, $EC_{50} = 0.14$ mM). During differentiation, either imidacloprid or acetamiprid dose-dependently caused neurite branch retraction on day 3, likely because of oxidative stress, to the extent that cells turned into spheres without neurites after 7-day treatment. Despite their apparent safety, the neurodevelopmental vulnerability of SH-SY5Y neurons to the chronic exposure to imidacloprid and to a lesser extent to acetamiprid points to a neurotoxic risk for humans.

1. Introduction

Neonicotinoids (NNIs) are the most widely used synthetic insecticides globally [1,2]. They are used to control a variety of crop pests and are also common in veterinary applications. Due to their water solubility and long persistence in the environment [3], NNIs can enter the human body by ingestion, inhalation or dermal contact [4,5]. Despite being considered safe for humans [6–9], concerns about their potential neurological effects have increased in the last four years [10–13]. Compelling evidence points to a meaningful

* Corresponding author. School of Biological Sciences and Engineering, Yachay Tech University, Hacienda San José s/n, Proyecto Yachay. 100115, Urcuquí. Ecuador.
E-mail address: sballaz@yachaytech.edu.ec (S.J. Ballaz).

https://doi.org/10.1016/j.heliyon.2023.e15840
Received 20 December 2022; Received in revised form 19 April 2023; Accepted 24 April 2023
Available online 28 April 2023

association between exposure to NNIs and mental diseases in rural populations [14,15]. Given their penetrability across the placenta [16] and blood-brain barrier [17], NNIs are likely to alter neurodevelopment in humans [18,19]. NNI-induced toxicity has already been probed in rodent neurons. The NNI insecticides imidacloprid and acetamiprid have excitatory effects on cerebellar neurons of neonatal rats that are blocked by nAChR inhibitors {20]. Prenatal exposure to acetamiprid alters neurogenesis in mouse neocortex [20], while its in utero and lactational exposure causes anxiety [21]. Acetamiprid induces cytotoxicity in mouse PC12 neurons [22]. Imidacloprid and acetamiprid cause metabolic interferences and oxidative damages in mouse Neuro.2a neuroblasts [23]. Only three studies have so far investigated *in vitro* toxicity of imidacloprid using human neurons [24–26].

The human SH-SY5Y neuroblastoma is a dopaminergic cell line that shows an excellent *in vitro* sensitivity to putative neurotoxins [24,27–29]. Interestingly, these cells express several nAChR subunits [30,31] and can mimic neuronal differentiation with the appropriate treatment [32]. Nicotinic Ach receptors mediate in the development of the human brain [33]. Therefore, this study was aimed to analyze both the acute and chronic *in vitro* neurotoxic effects of commercially-based Imidacloprid or IMI (1-[(6-Chloro-3-pyridyl) methyl)-4, 5-dihydro imidazole-2-yl) and acetamiprid or ACE (*N*-[(6-chloro-3-pyridyl) methyl]--*N*′-cyano-*N*-methylacetamide) insecticides in either undifferentiated SH-SY5Y cells or during their differentiation with staurosporine.

2. Material and methods

2.1. Reagents and chemicals

Dulbecco's modified Eagle minimum essential/Ham's F-12 (DMEM/F12) plus Glutamax media, Neurobasal medium, minimum essential medium (MEM), B-27 supplement, fetal bovine serum (FBS), sodium pyruvate, L-glutamine, penicillin G/streptomycin mix, and enzyme-free PBS-based cell dissociation buffer were purchased from Gibco (Carlsbad, CA). Staurosporine [ready-made 1 mM solution in dimethyl sulfoxide (DMSO)], L-glutamic acid, D-glucose, uridine, sodium orthovanadate, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma-Aldrich (St. Louis, MO). Commercially available NNI insecticides were acetamiprid or ACE 200 g/kg soluble powder (SP) in talcum (non-toxic mineral clay) and imidacloprid or IMI 20 g/100 mL of concentrated suspension (CS) in water. They were only used certified brands of insecticides that fulfilled purity standards.

2.2. Culture of SH-SY5Y cells

The human neuroblastoma cell line SH-SY5Y was a generous gift of Dr. Javier Saez-Castresana (University of Navarra, Spain). Cells were routinely grown to confluence (>80%, cultures passed once or twice per week) in "growth medium" [DMEM/F12 (1:1) medium Glutamax, 10% vol/vol fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin], and maintained at 37 °C in a humidified O_2 (95%) and CO_2 (5%) atmosphere. The medium was exchanged every 2–3 days during cell growth. Cells were harvested using an enzyme-free phosphate-buffered saline (PBS)-based cell dissociation buffer.

2.3. Cell differentiation protocol

Harvested SH-SY5Y cells were seeded in growth medium and after overnight incubation to let cells adhere, dividing SH-SY5Y cells were treated with a very low concentration (6 nM) of staurosporine or ST (a nonspecific inhibitor of phospholipid/calcium-dependent protein kinases) to differentiate them into mature neurons [34]. The ST regime was as follows: medium was exchanged for "differentiation medium" consisting of serum-free Neurobasal medium supplemented with B-27 (2% vol/vol), pyruvate (100 μg/mL), uridine (50 μg/mL), glutamine (5 mM), 100 U/mL penicillin G, and 100 μg/mL streptomycin, and 6 nM staurosporine (day 0). From then onward, staurosporine was added fresh in Neurobasal medium at every medium change (days 2, 4 and 6). Fully differentiated SH-SY5Y neurons were achieved on the seventh day of staurosporine treatment.

2.4. Neonicotinoid treatment

The reference to set up a range of decreasing concentrations was based on the amount of product applied to crops, which was equivalent to a concentration of 0.5 mM of sheer insecticide according to the percentage (W/W) of the active ingredient specified by the manufacturer. Insecticides were freshly dissolved in either "growth medium" or in "differentiation medium," and refreshed in every medium change (days 0, 2, 4 and 6). Cells were exposed to the following treatments from 1 to 7 days when convenient: vehicle (control), ACE (0.01–0.5 mM), and IMI (0.01–0.5 mM). Cytotoxic effects of IMI and ACE over the SH-SY5Y cell line were then evaluated considering four endpoints: i) Mitochondrial function, ii) Membrane integrity, iii) Cell morphology, and iv) Oxidative stress.

2.5. Neuronal-viability assessment

The reduction of the thiazolyl blue tetrazolium bromide, or MTT dye, to formazan was taken as the initial indicator of cell viability [35]. For MTT experiments, cells were seeded in 96-well microplates at a density of 2.5×10^4 cells per well (100 μL). A stock (5 mg/mL in PBS) of MTT salt was diluted tenfold in the culture medium. After a 2 h-incubation at 37 °C, the yellow MTT salt was reduced to purple formazan by active mitochondrial reductase enzymes of living cells. The medium was aspirated and the formazan crystals solved in 100 μL of pure DMSO. Optical density (OD) was read at 560 nm with the appropriate amount of DMSO as a blank using a microplate reader (Rayto RT-2100C Microplate Reader, Rayto Life and Analytical Sciences Co. Ltd., China). Absorbance of wells with

vehicle (control) was set equal to 100 to normalize absorbance values of treated wells. As a positive control of cell death, wells were treated with 10% DMSO. Each treatment condition was replicated eight times per independent experiment.

Cell viability was further evaluated by the vital-dye (Trypan blue or TB) exclusion test to detect membrane integrity. Cells were seeded at a density of 2.5×10^3 cells well (200 μL) in 48-well plaques. Trypan blue was dissolved directly into the culture medium (0.02% vol/vol) to evaluate the fraction of dead (Trypan blue-tangible) cells versus total cell counts at 200-fold magnification under the light of an inverted microscope (Motic AE31E, Motic China Group Co., Hong Kong, China). Pictures were taken using a coupled digital camera and MotiConnect software (Motic China Group Co., Hong Kong, China). Cell counts were conducted on a minimum of 500 cells found in multiple fields using the free ImageJ software (http://imagej.nih.gov/ij). Each treatment condition was replicated three times.

2.6. Neurite outgrowth and cell morphology

The impairment of key neurodevelopmental processes such as neurite outgrowth inhibition were used as endpoints for the screening of developmental neurotoxicity [36]. SH-SY5Y cells were seeded at a density of 2.5×10^3 cells well (200 μL) in 48-well plaques and visual appearance of cells analyzed in phase-contrast images on ImageJ. Digital photographs were taken from each well and stored on a PC. Neurite-like structures (referred to as neurites) were measured and analyzed on days 1, 3, 5, and 7 (four replicates of approximately 500 cells per treatment condition). Neurite outgrowth was quantified by counting the number of cells

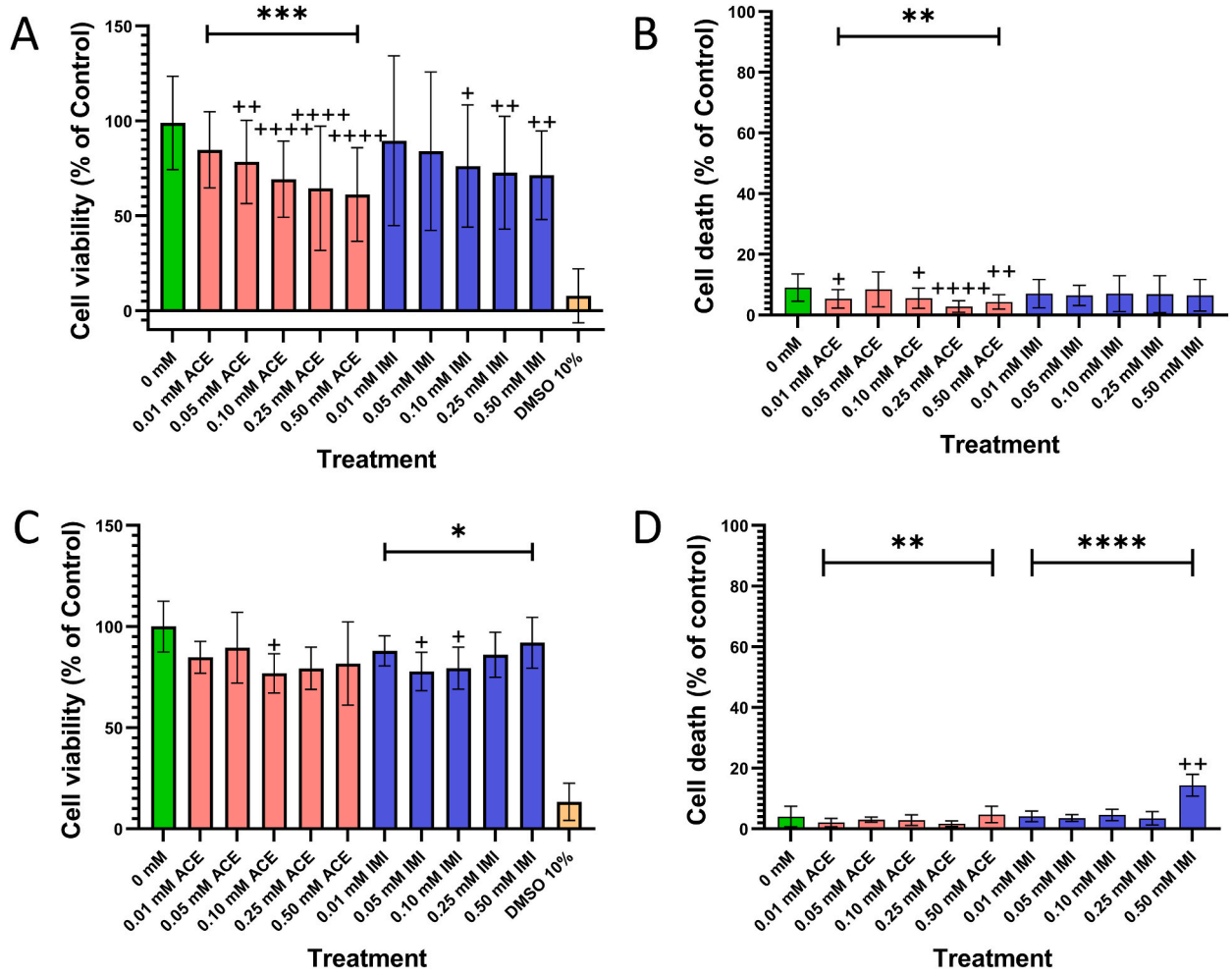

Fig. 1. Toxicity of acetamiprid and imidacloprid in Non-Differentiated or in Differentiated SH-SY5Y neurons after a 24-h treatment, Fig. 1.- Toxicity of acetamiprid and imidacloprid in Non-Differentiated or in Differentiated SH-SY5Y neurons after a 24-h treatment. Insecticides (0.01–0.5 mM) were administered to either Non-differentiated (immature) cells (A, B) or to cells matured after the differentiation with staurosporine (C, D). Approximately 500 cells were counted in each sample in the TB test. Values are expressed as the mean ± SEM of one (B, C, D) to three (A) independent experiments. Statistical significance is marked as follows: *P < 0.05), **P < 0.01, ***P < 0.001, and ****P < 0.0001 for differences between treatments; and +P < 0.05, ++P < 0.01, +++P < 0.001, and ++++P < 0.0001 for differences between each treatment and control (0 mM as vehicle).

exhibiting neurites that were two times longer than the cell body diameter in length. Neurite length averaged per cell was evaluated relative to control (0 mM as vehicle) to exclude artifacts from different cell numbers [36]. Cell morphology was analyzed qualitatively.

2.7. Oxidative stress assessment

The probe 2′,7′-dichlorodihydrofluorescein diacetate or H_2DCF-DA was used to evaluate the generation of reactive oxygen species (ROS) in treated cells [37]. Briefly, cells were seeded at 2.5×10^4 on coverslips placed in 12-well plates (1.5 mL) and exposed to the insecticide for four days while differentiating with staurosporine. After treatment, cells were incubated 30 min with 10 μM of DCF-DA dissolved in Neurobasal medium, fixed with 3.7% formaldehyde in ice-cold PBS for 10 min, washed three times with PBS, dried on air, and mounted on glass slides with Entellan™ rapid mounting medium (Merck-Millipore, Germany). Cells were imaged in six fields per experimental condition at 200× magnification using a Leica DM4000 B imaging fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). Fluorescence intensity was estimated with excitation and emission filters at 485 ± 10 nm and 530 ± 12.5 nm respectively with the aid of a blue filter (450–490 nm) and the Leica Application Suite X software for Life Science. Images finally were analyzed on ImageJ.

2.8. Data analysis

Statistical analysis was conducted using GraphPad Prism 9.0.2v for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) and R studio software (https://www.rstudio.com). In the experiments, replicates per endpoint in the experimental design consisted of three to five measurements conducted in four to eight wells per treatment condition. Results were expressed as mean ± SEM of these replicates.

MTT data from long-term exposure were obtained from experiments carried out with eight replicates. MTT results were statistically evaluated by performing one-way ANOVA followed by Tukey's test for each time point. When convenient, dose-response curves (DRC) were generated using the log(agonist) vs. concentration – Find EC Anything model, as the best-fitted model, which was selected based on the goodness of fit and residuals analysis, from GraphPad Software [38]. For the rest of the tests, statistical analysis was performed on data from four replicates per treatment using one-way ANOVA followed by Dunnett's test to compare the concentration– response curves of the SH-SY5Y cell line exposed to NNIs at each time period. For all data analysis, a *P*-value lower than 0.05 was considered statistically significant.

To better understand the correlation between Neuronal Morphology (Y) and NNI exposure (X), it was carried out two different tests: (i) the Pearson test to establish the relation between the variance (Y) and covariance (XY); and (ii) the Kendall test to verify whether the two variables may be regarded as statistically dependent.

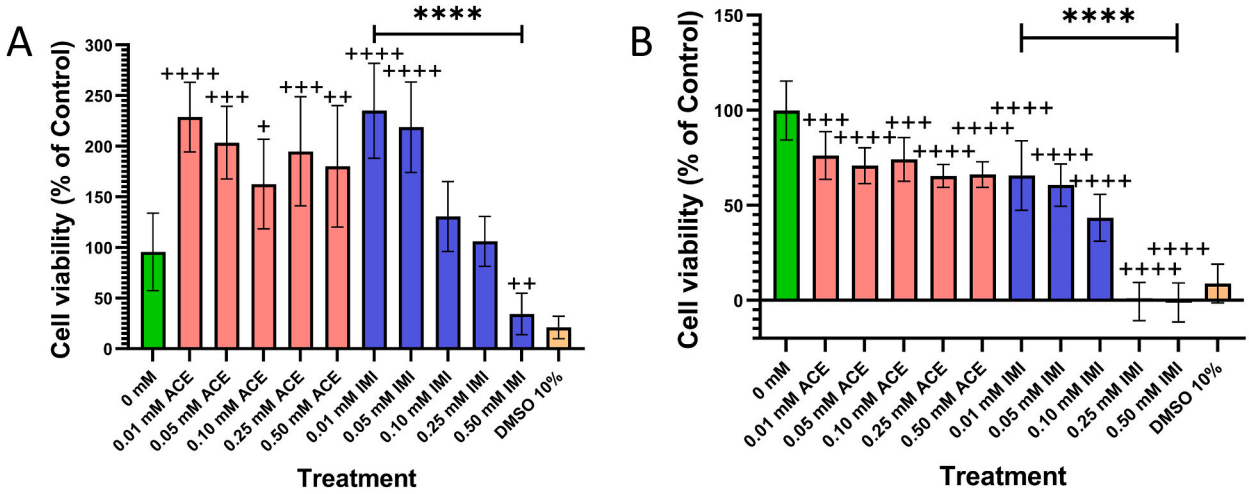

Fig. 2. Toxicity of acetamiprid and imidacloprid in either Non-Differentiated or in Differentiating SH-SY5Y neurons after a 7-d (168-h) treatment. Viability of the last treatment day according to the MTT test. Insecticides (0.01–0.5 mM) were administered to either Non-differentiated cells (Fig.2A) or Differentiating cells (Fig.2B). Values are expressed as the mean ± SEM of two to three independent experiments. Statistical significance is marked as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ for differences between treatments; and $+ P < 0.05$, $++P < 0.01$), $+++P < 0.001$, and $++++P < 0.0001$ for differences between each treatment and control (0 mM as vehicle).

3. Results

3.1. Viability of SH-SY5Y cells after acute (24-h) treatment with NNI insecticides

We first wanted to know whether a short (24-h) exposure to increasing concentrations of NNI insecticides altered cellular viability. Fig. 1 represents the viability, as determined by MTT assay (Fig. 1A and C) and vital dye exclusion tests (Fig. 1B and D), of Non-Differentiated (Fig. 1A and B) and Differentiated (Fig. 1C and D) SH-SY5Y cells. Data show that in the MTT test using Non-differentiated cells (Fig. 1A), the one-way analysis of variance revealed a significant treatment effect for ACE ($F(4,159) = 5.15$, $P < 0.001$), but not for IMI ($F(4,159) = 1.56$, $P = 0.184$). ACE concentrations higher than 0.1 mM only caused a mild reduction (<40%) of the control mitochondrial activity ($P < 0.001$). In the case of cells treated just after differentiation (Fig. 1C), only the effects of IMI treatment were found to be significant ($F(4,39) = 2.64$, $P = 0.0499$) while ACE had no effects ($F(4,39) = 0.98$, $P = 0.432$). Furthermore, living cell cultures were examined for the presence of Trypan blue-tangible (dead) cells (Fig. 1B and D) to corroborate MTT data (Fig. 1A and C), indicating that the ratio of dead cells caused by increasing insecticide was kept in the same low basal range as the control. In-house experiments (data not shown) using lab-grade IMI and ACE compounds corroborated the lack of acute neurotoxicity at concentrations as high as 5 mM.

3.2. Viability of SH-SY5Y cells after chronic (168-h or 7-day) exposure to NNI insecticides

Fig. 2A represents cell viability of non-differentiated SH-SY5Y cells after a 7-day treatment with NNIs. Even if chronic IMI ($F(4,39) = 43.05$, $P < 0.001$) dose-dependently decreased mitochondrial activity of immature cells over time (Fig. 2A, EC50 = 0.23 mM, Fig. 3b), low doses (0.01–0.05 mM) promoted cell proliferation ($P < 0.001$ compared to control). ACE did not significantly increase cell proliferation ($F(4,39) = 2.26$, $P = 0.082$). Because differentiation is likely to modify cellular susceptibility to neurotoxins, the same treatment regime was investigated during neuronal differentiation with staurosporine (Fig. 2B). Chronic IMI ($F(4,39) = 51.86$, $P < 0.001$), but not ACE ($F(4,39) = 1.96$, $P = 0.122$), compromised the viability of Differentiating SH-SY5Y cells (EC50 = 0.12 mM, Fig. 3).

The concentration-response curves of IMI treatment in the MTT assay (see methods, Fig. 3A) followed the familiar symmetrical sigmoidal shape in the Differentiating SH-SY5Y cells (Fig. 3A) since the third day (Day 3: $R^2 = 0.668$, EC50 = 0.39 mM; Day 4: $R^2 = 0.945$, EC50 = 0.14 mM; Day 5: $R^2 = 0.847$, EC50 = 0.23 mM; Day 6 $R^2 = 0.800$; EC50 = 0.12 mM; Day 7: $R^2 = 0.846$, EC50 = 0.12 mM). Fig. 3B shows the response surface (MTT-based cell viability versus time period and concentration) models, which shows the patterns of the dose-dependent cytotoxicity (MTT test) for IMI in Differentiating SH-SY5Y cells. In Non-differentiated SH-SY5Y cells, however, only moderate R-squared values were obtained for IMI at the end treatment regime (Day 6: $R^2 = 0.680$, EC50 = 0.35 mM; Day 7: $R^2 = 0.785$, EC50 = 0.23 mM; graphs not shown).

3.3. NNI insecticide effects on neurite outgrowth and cell morphology

Fig. 4 shows the time course of neurite outgrowth per cell in Differentiating SH-SY5Y neurons during 7-d treatment with ACE analyzed every other day. Even if ACE treatment did not significantly induce death in SH-SY5Y cells, it significantly reduced by 50% the neurite outgrowth of Non-differentiated cells over time (Day 1: $F(4,19) = 3.09$, $P < 0.05$; Day 3: $F(4,19) = 26.12$, $P < 0.001$; Day 5: $F(4,19) = 3.28$, $P < 0.05$; Day 7: $F(4,19) = 10.98$, $P < 0.001$). Interestingly, the reduction of neurite outgrowth was more accentuated during the day 5 and 7 of differentiation (Day 1: $F(4,18) = 2.32$, $P = 0.108$; Day 3: $F(4,18) = 3.33$, $P < 0.05$; Day 5: $F(4,18) = 2.09$, $P = 0.14$; Day 7: $F(4,18) = 72.53$, $P < 0.001$). Compared to control, cell morphology changed in that they turned into spheres without neurites. This effect could be clearly seen after 24 h of treatment (Fig. 5). A similar pattern of branch retraction occurred in the case of

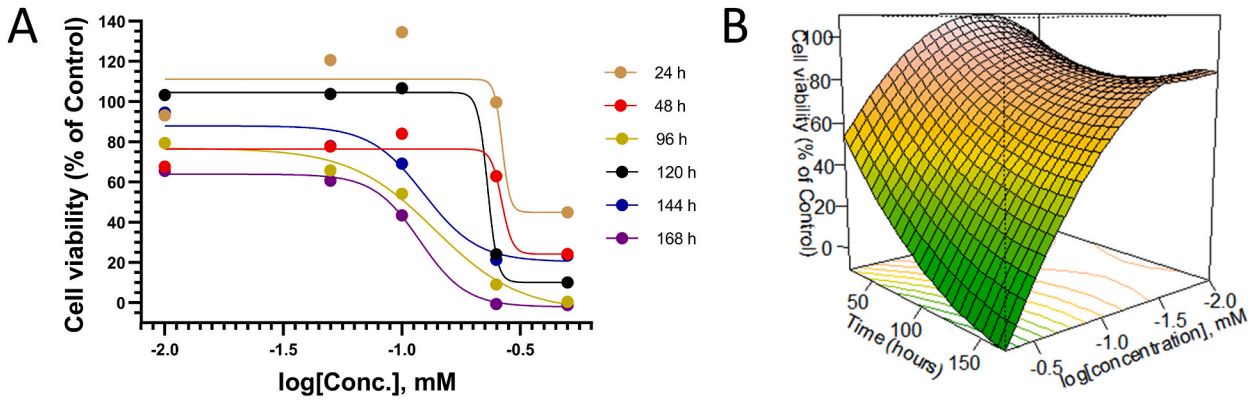

Fig. 3. Toxicity of 7-d (168h) treatment with imidacloprid in Differentiating SH-SY5Y neurons. (A) Different dose-response curves were generated according to the time of exposure: 72 h, 96 h, 120 h, and 144 h (X = log10[concentration]; Y = cell viability). In this model equation, F is constrained to a constant value of 50 to fit the model to EC_{50}. (B) MTT assay three-dimensional graph (surface response models) of Differentiating SH-SY5Y cells generated by polynomial regression analysis (See methods).

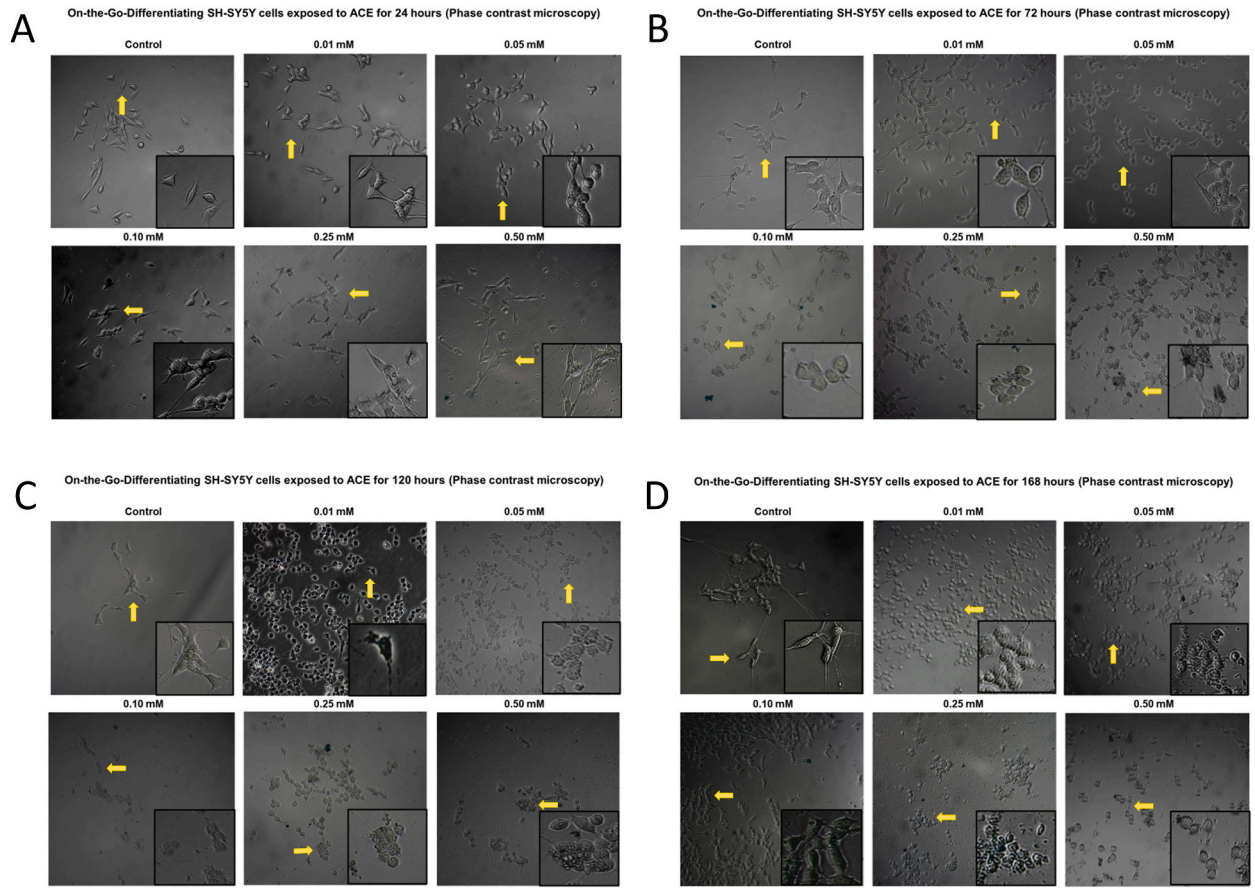

Fig. 4. Representative phase contrast micrographs of Differentiating SH-SY5Y neurons over a time course of 7-d treatment with acetamiprid. Images of typical fields of SH-SY5Y cells viewed under phase-contrast microscopy (200× magnification). **(A)** 24 h, **(B)** 72 h, **(C)** 120 h, and **(D)** 168 h treatment. Yellow arrows show zoomed regions (Scale bar = 100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

chronic IMI in Non-differentiated (Day 1: $F(4,19) = 14.61$, $P = 0.279$; Day 3: $F(4,17) = 5.17$, $P < 0.05$; Day 5: $F(4,19) = 3.19$, $P < 0.05$; Day 7: $F(4,19) = 6.44$, $P < 0.01$) and Differentiating cells (Day 1: $F(4,19) = 14.61$, $P < 0.001$; Day 3: $F(4,19) = 33.91$, $P < 0.001$; Day 5: $F(4,19) = 6.94$, $P < 0.001$; Day 7: $F(4,15) = 3.67$, $P < 0.05$).

3.4. NN-induced oxidative stress

Given that almost no cells were left after 7-d treatment with the highest doses of imidacloprid, we looked at the middle of differentiation (4-d treatment) when its cytotoxic effect began to be significant to construct a more accurate dose response for oxidative stress. The DCHF-DA reagent exhibited noticeable increasing fluorescence in cells treated with NNIs at lower doses. The results obtained after 4 days of exposure of Differentiating SH-SY5Y cells to NNIs revealed that NNI insecticides prompted a significant increase in reactive oxygen species (ROS) levels of both treatments, yet only the oxidative stress induced by IMI seemed to be dose dependent. Kendall's test shows from the control until 0.10 mM a positive correlation ($t = 0.895$; $P < 0.05$). The latter points out the dose-response dependency of ROS production at low doses. Furthermore, coupling these results with cell viability is evident that concentration over this threshold kills cells, consequently, ROS is similar to the control as shown in Table 1.

4. Discussion

This work showed how the human SH-SY5Y neuroblastoma, a gold standard *in vitro* cellular model for neurotoxicity, was unaffected by acute treatment with acetamiprid- and imidacloprid-based commercial insecticides. While imidacloprid was cytotoxic when SH-SY5Y cells were chronically treated with the insecticide during their differentiation into mature neuron-like cells, chronic acetamiprid only provoked neurite retraction and oxidative stress without a significant increase of cell death toll. These findings revealed a hidden developmental neurotoxicity of NNI-type insecticides.

Although NNIs are classified as moderately hazardous, sub-millimolar concentrations of IMI produced a significant drop of cell

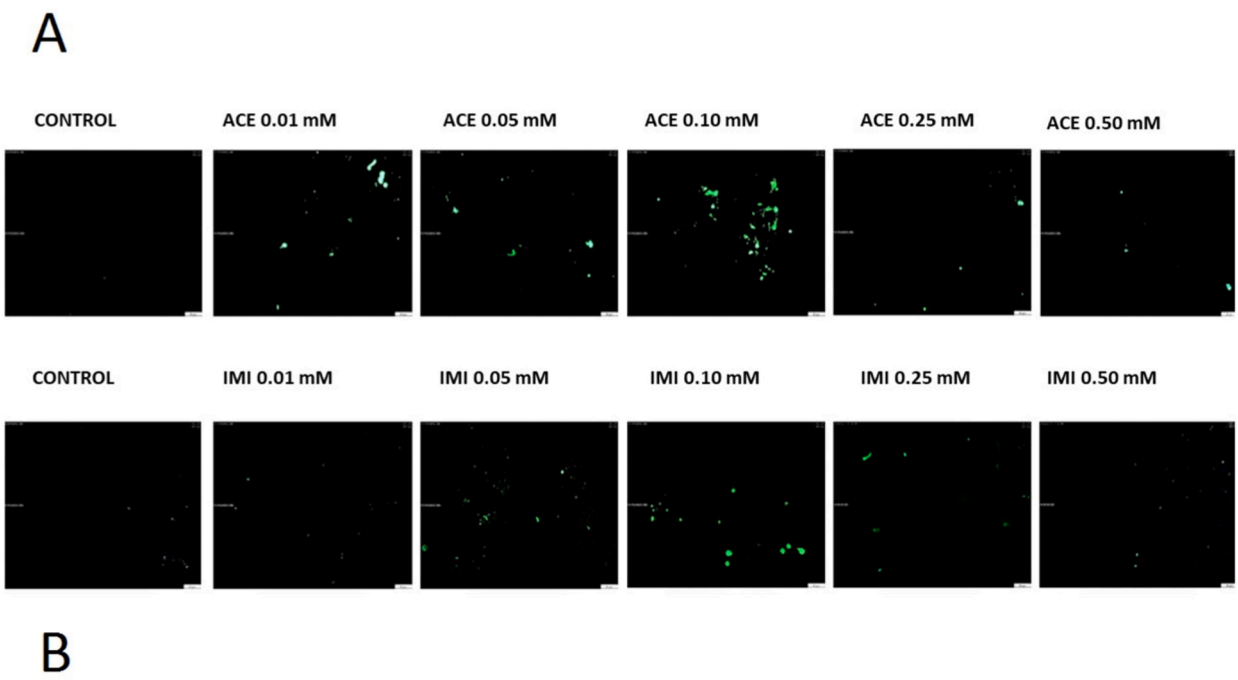

Fig. 5. Representative micrographs of Differentiating SH-SY5Y neurons over the time course of 4-d treatment with and acetamiprid and imidacloprid. Images of typical fields of SH-SY5Y cells viewed under fluorescence microscopy. **(A)** Acetamiprid or ACE and **(B)** imidacloprid or IMI. The excitation and wavelengths were 485 nm and 530 nm respectively (200× magnification).

Table 1
Reactive Oxygen Species analysis. Data is presented as geometric mean and variance of intensity index.

	IMI		ACE	
[mM]	Intensity index	VAR (index)	Intensity index	VAR (index)
0	0,0452	0,0099	0,0404	0,0087
0,01	0,0837	0,0019	0,1054	0,0024
0,05	0,2094	0,0002	0,2107	0,0001
0,1	0,3271	0,0004	0,2813	0,0003
0,25	0,3802	0,0001	0,2146	0,0001
0,5	0,1686	0,0005	0,1561	0,0006

viability, an increase of oxidative stress, and neurite branch retraction in human neuroblastoma SH-SY5Ycells. The toxic stress imposed to SH-SY5Y cells by a chronic (3 days) treatment with imidacloprid was not new [24], with the only difference that the cytotoxic effects in our study occurred after the 3rd of neuronal differentiation with staurosporine (Fig. 3B). IMI shows nicotine-like activity in mammalian neurons *in vitro* [39], which may have an impact in neural development [33,40–42] and synaptic transmission [43]. Although neuroblastoma cell lines could differentiate using alternative mechanisms than those from normal cells, consistent evidence suggests that SH-SY5Y cells can mimic key features of neuronal development and differentiation [32]. Our results therefore suggest a hidden developmental neurotoxicity of these NNIs in human neurons. Further studies are required to confirm the lack of differentiation in presence of IMI by either employing immunoblotting (e.g., MAP2 protein) or microscopy (e.g., phalloidin staining).

The *in vitro* neurotoxicity of ACE, which also interacts with mammal neurons [39], has only been analyzed in mouse PC12 neurons [22]. We could not replicate ACE cytotoxicity in our SH-SY5Y cell preparation. Toxic stress of ACE was just limited to the arrest of neurite outgrowth and oxidative stress. In agreement with previous research [44], oxidative stress was a common toxic endpoint of the chronic treatment with NNIs during cell differentiation. Even if the oxidative stress triggered by chronic ACE was not enough to cause significant cell death, it was likely to interfere with changes in bioenergetics necessary for cell differentiation [45]. Starting from this, it may be deducted that nAChR-mediated [Ca^{2+}] signaling in SH-SY5Y cells [25,26] could take part in the neurodevelopmental effects induced by ACE [12] and IMI [13].

Some evidence supports the contention that the homopentameric α7 nitocitinic Ach (α7 nACh) receptors may mediate NNI-induced disturbances of cell differentiation [33]. The α7 nACh receptor, which is expressed in SH-SY5Y cells [31], evokes the highest Ca^{2+} transients in neurons to keep the intracellular concentration of the cation to an optimum [46]. IMI and ACE differently modulate ACh-induced Ca^{2+} currents on α7 nACh receptors [47,48], which would explain why IMI was more cytotoxic than ACE, while only IMI

at low concentrations promoted cell proliferation in immature SH-SY5Y cells [49]. In addition, ACE and IMI may also act like "orthosteric" agonists producing a rapid desensitization of α7 nACh receptors (unpublished observations), which is likely to produce oxidative stress [50,51] in differentiating SH-SY5Y cells.

Mounting evidence suggests that the general population is extensively exposed to NNIs present in the environment [52]. The occurrence of imidacloprid and acetamiprid and their metabolites has been demonstrated in paired urine and blood samples [53] as well as in paired urine and house dust [54]. Interestingly, NNIs can pass through the human placenta [16]. Although the Environmental Protection Agency (EPA) classifies these NNI insecticides as II (moderate) and III (slight) class toxic agents [55], our results suggest they could pose a real risk for the development of the human nervous system.

5. Conclusions

In summary, the present study did not detect any acute neurotoxic effects of commercial-based acetamiprid and imidacloprid insecticides, but a hidden developmental neurotoxicity following a chronic exposure to them. It casts doubts on their apparent weak toxicity, especially when the human nervous system shows more vulnerability to their developmental effects.

Relevance statement

Neonicotinoids are widely used insecticides that tend to linger and remain in the environment from 1 day to almost 19 years. This research represents the preliminary evidence of the developmental neurotoxicity of the neonicotinoid-class acetamiprid and imidacloprid insecticides for the first time ever using a well-established *in vitro* model of human neurons.

Author contribution statement

Lenin J. Ramirez-Cando; Santiago Ballaz: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Marcelo S. Guzmán-Vallejos: Performed the experiments; Wrote the paper.

Luis G. Aguayo: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Fernando D. Vera-Erazo: Performed the experiments.

Data availability statement

Data will be made available on request.

Funding

Santiago Ballaz and Lenin Ramirez received a grant (Ref. PIC-18-INE-YACHAY-002) from the *Secretaria Nacional, Ciencia, Tecnología e Innovación* (SENESCYT) of the Ecuadorian Government. Luis Aguayo received a grant (Ref. FONDECYT-ANID 1221080) from the *Fondo Nacional de Desarrollo Científico y Tecnológico-Agencia Nacional de Investigación y Desarrollo* of the Chilean Government.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to the *Programa de las Naciones Unidas para el Desarrollo* (PENUD) of ECUADOR for the sponsorship of the SENESCYT grant (PIC-18-INE-YACHAY-002).

References

[1] C. Bass, L.M. Field, Neonicotinoids, Curr. Biol. 28 (2018) R772–R773, https://doi.org/10.1016/j.cub.2018.05.061.
[2] S.M. Ensley, Neonicotinoids, third ed, Elsevier Inc, 2018, https://doi.org/10.1016/B978-0-12-811410-0.00040-4.
[3] D. Goulson, REVIEW: an overview of the environmental risks posed by neonicotinoid insecticides, J. Appl. Ecol. 50 (2013) 977–987, https://doi.org/10.1111/1365-2664.12111.
[4] K.H. Harada, K. Tanaka, H. Sakamoto, M. Imanaka, T. Niisoe, T. Hitomi, H. Kobayashi, H. Okuda, S. Inoue, K. Kusakawa, M. Oshima, K. Watanabe, M. Yasojima, T. Takasuga, A. Koizumi, Biological monitoring of human exposure to neonicotinoids using urine samples, and neonicotinoid excretion kinetics, PLoS One 11 (2016), e0146335, https://doi.org/10.1371/journal.pone.0146335.
[5] Q. Zhang, Z. Li, C.H. Chang, J.L. Lou, M.R.- Zhao, C. Lu, Potential human exposures to neonicotinoid insecticides: a review, Environ. Pollut 236 (2018) 71–81, https://doi.org/10.1016/j.envpol.2017.12.101.
[6] M. Tomizawa, J.E. Casida, Neonicotinoid insecticide toxicology: mechanisms of selective action, Annu. Rev. Pharmacol. Toxicol. 45 (2005) 247–268, https://doi.org/10.1146/annurev.pharmtox.45.120403.095930.
[7] L.P. Sheets, A.A. Li, D.J. Minnema, R.H. Collier, M.R. Creek, R.C. Peffer, A critical review of neonicotinoid insecticides for developmental neurotoxicity, Crit. Rev. Toxicol. 46 (2016) 153–190, https://doi.org/10.3109/10408444.2015.1090948.

[8] J.N. Houchat, A. Cartereau, A. Le Mauff, E. Taillebois, S.H. Thany, An overview on the effect of neonicotinoid insecticides on mammalian cholinergic functions through the activation of neuronal nicotinic acetylcholine receptors, Int. J. Environ. Res. Publ. Health 17 (2020) 3222, https://doi.org/10.3390/ijerph17093222.

[9] D.A. Thompson, H.-J. Lehmler, D.W. Kolpin, M.L. Hladik, J.D. Vargo, K.E. Schilling, G.H. LeFevre, T.L. Peeples, M.C. Poch, L.E. LaDuca, D.M. Cwiertny, R. W. Field, A critical review on the potential impacts of neonicotinoid insecticide use: current knowledge of environmental fate toxicity and implications for human health, Environ. Sci. Process Impacts 22 (2020) 1315–1346, https://doi.org/10.1039/C9EM00586B.

[10] A. Anadón, I. Ares, M. Martínez, M.R. Martínez-Larrañaga, M.A. Martínez, Chapter Four - Neurotoxicity of Neonicotinoids, in: M. Aschner, L.G. Costa (Eds.), Advances in Neurotoxicology, Academic Press, 2020, pp. 167–207, https://doi.org/10.1016/bs.ant.2019.11.005.

[11] C. Costas-Ferreira, L.R.F. Faro, Neurotoxic effects of neonicotinoids on mammals: what Is there beyond the activation of nicotinic acetylcholine receptors? A Systematic Review, Int. J. Mol. Sci. 22 (2021) 8413, https://doi.org/10.3390/ijms22168413.

[12] A. Hirai, S. Sugio, C. Nimako, S.M.M. Nakayama, K. Kato, K. Takahashi, K. Arizono, T. Hirano, N. Hoshi, K. Fujioka, K. Taira, M. Ishizuka, H. Wake, Y. Ikenaka, Ca^{2+} imaging with two-photon microscopy to detect the disruption of brain function in mice administered neonicotinoid insecticides, Sci. Rep. 12 (2022) 5114, https://doi.org/10.1038/s41598-022-09038-7.

[13] B.D. Tonietto, A.O.M. Laurentino, M.T. Costa-Valle, L.V. Cestonaro, B.P. Antunes, C. Sates, N.G. Dos Santos, E. Dallegrave, S.C. Garcia, M.B. Leal, M.D. Arbo, Imidacloprid-based commercial pesticide causes behavioral, biochemical, and hematological impairments in Wistar rats, Environ. Toxicol. Pharmacol. 94 (2022) 103924, https://doi.org/10.1016/j.etap.2022.103924.

[14] B. van Wendel de Joode, A.M. Mora, C.H. Lindh, D. Hernández-Bonilla, L. Córdoba, C. Wesseling, J.A. Hoppin, D. Mergler, Pesticide exposure and neurodevelopment in children aged 6–9 years from Talamanca, Costa Rica, Cortex 85 (2016) 137–150, https://doi.org/10.1016/j.cortex.2016.09.003.

[15] C. Hyland, P.T. Bradshaw, R.B. Gunier, A.M. Mora, K. Kogut, J. Deardorff, S.K. Sagiv, A. Bradman, B. Eskenazi, Associations between pesticide mixtures applied near home during pregnancy and early childhood with adolescent behavioral and emotional problems in the CHAMACOS study, Environ. Epidemiol 5 (2021) e150, https://doi.org/10.1097/EE9.0000000000000150.

[16] H. Zhang, X. Bai, T. Zhang, S. Song, H. Zhu, S. Lu, K. Kannan, H. Sun, Neonicotinoid insecticides and their metabolites can pass through the human placenta unimpeded, Environ. Sci. Technol. 56 (2022) 17143–17152, https://doi.org/10.1021/acs.est.2c06091.

[17] C. Fuke, T. Nagai, K. Ninomiya, M. Fukasawa, Y. Ihama, T. Miyazaki, Detection of imidacloprid in biological fluids in a case of fatal insecticide intoxication, Leg. Med. (Tokyo) 16 (2014) 40–43, https://doi.org/10.1016/j.legalmed.2013.10.007.

[18] G. Ichikawa, R. Kuribayashi, Y. Ikenaka, T. Ichise, S.M.M. Nakayama, M. Ishizuka, K. Taira, K. Fujioka, T. Sairenchi, G. Kobashi, J.M. Bonmatin, S. Yoshihara, LC-ESI/MS/MS analysis of neonicotinoids in urine of very low birth weight infants at birth, PLoS One 14 (2019), e0219208, https://doi.org/10.1371/journal.pone.0219208.

[19] B. Laubscher, M. Diezi, R. Renella, E.A.D. Mitchell, A. Aebi, M. Mulot, G. Glauser, Multiple neonicotinoids in children's cerebro-spinal fluid, plasma, and urine, Environ. Health 21 (2022) 10, https://doi.org/10.1186/s12940-021-00821-z.

[20] N. Kagawa, T. Nagao, Neurodevelopmental toxicity in the mouse neocortex following prenatal exposure to acetamiprid, J. Appl. Toxicol. 38 (2018) 1521–1528, https://doi.org/10.1002/jat.3692.

[21] K. Sano, T. Isobe, J. Yang, T.T. Win-Shwe, M. Yoshikane, S.F. Nakayama, T. Kawashima, G. Suzuki, S. Hashimoto, K. Nohara, C. Tohyama, F. Maekawa, In utero and lactational exposure to acetamiprid induces abnormalities in socio-sexual and anxiety-related behaviors of male mice, Front. Neurosci. 10 (2016) 228, https://doi.org/10.3389/fnins.2016.00228.

[22] E. Annabi, I. Ben Salem, S. Abid-Essefi, Acetamiprid, a neonicotinoid insecticide, induced cytotoxicity and genotoxicity in PC12 cells, Toxicol. Mech. Methods 0 (2019) 1–7, https://doi.org/10.1080/15376516.2019.1624907.

[23] X. Wang, J. Qiu, Y. Xu, Y. Pan, H. Chen, Q. Jia, Y. Qian, Different cellular mechanism of imidacloprid and acetamiprid by a combined targeted lipidomics and metabolomics approach in Neuro-2a cells, Toxicol. In Vitro 83 (2022), 105426, https://doi.org/10.1016/j.tiv.2022.105426.

[24] D. Skandrani, Y. Gaubin, B. Beau, J.C. Murat, C. Vincent, F. Croute, Effect of selected insecticides on growth rate and stress protein expression in cultured human A549 and SH-SY5Y cells, Toxicol. In Vitro 20 (2006) 1378–1386, https://doi.org/10.1016/j.tiv.2006.06.001.

[25] D. Loser, K. Grillberger, M.G. Hinojosa, J. Blum, Y. Haufe, T. Danker, Y. Johansson, C. Möller, A. Nicke, S.H. Bennekou, I. Gardner, C. Bauch, P. Walker, A. Forsby, G.F. Ecker, U. Kraushaar, M. Leist, Acute effects of the imidacloprid metabolite desnitro-imidacloprid on human nACh receptors relevant for neuronal signaling, Arch. Toxicol. 95 (2021) 3695–3716, https://doi.org/10.1007/s00204-021-03168-z.

[26] D. Loser, M.G. Hinojosa, J. Blum, J. Schaefer, M. Brüll, Y. Johansson, I. Suciu, K. Grillberger, T. Danker, C. Möller, I. Gardner, G.F. Ecker, S.H. Bennekou, A. Forsby, U. Kraushaar, M. Leist, Functional alterations by a subgroup of neonicotinoid pesticides in human dopaminergic neurons, Arch. Toxicol. 95 (2021) 2081–2107, https://doi.org/10.1007/s00204-021-03031-1.

[27] H. Gustafsson, J. Runesson, J. Lundqvist, H. Lindegren, V. Axelsson, A. Forsby, Neurofunctional endpoints assessed in human neuroblastoma SH-SY5Y cells for estimation of acute systemic toxicity, Toxicol. Appl. Pharmacol. 245 (2010) 191–202, https://doi.org/10.1016/j.taap.2010.02.018.

[28] L.J. Ramírez-Cando, U. De Simone, T. Coccini, Toxicity evaluation of iron oxide (Fe_3O_4) nanoparticles on human neuroblastoma-derived SH-SY5Y cell line, J. Nanosci. Nanotechnol. 17 (2017) 203–211, https://doi.org/10.1166/jnn.2017.13046.

[29] M. Şenyildiz, A. Kilinc, S. Ozden, Investigation of the genotoxic and cytotoxic effects of widely used neonicotinoid insecticides in HepG2 and SH-SY5Y cells, Toxicol. Ind. Health 34 (6) (2018) 375–383, https://doi.org/10.1177/0748233718762609.

[30] M.L P.J. Groot Kormelink, W.H. Luyten, Cloning and sequence of full-length cDNAs encoding the human neuronal nicotinic acetylcholine receptor (nAChR) subunits β3 and β4 and expression of seven nAChR subunits in the human neuroblastoma cell line SH-SY5Y and/or IMR-321, FEBS Lett. 400 (1997) 309–314, https://doi.org/10.1016/s0014-5793(96)01383-x.

[31] J. Zhao, Y. Li, Y. Li, S. Xu, T. Tao, Y. Hua, J. Zhang, Y. Fan, Activation of α7-nAChRs promotes the clearance of α-synuclein and protects against apoptotic cell death induced by exogenous α-synuclein fibrils, Front. Cell Dev. Biol. 9 (2021) 637319, https://doi.org/10.3389/fcell.2021.637319.

[32] J. Kovalevich, D. Langford, Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology, 2, Methods Mol. Biol. 1078 (2013) 9–21, https://doi.org/10.1007/978-1-62703-640-5.

[33] K.T. Dineley, A.A. Pandya, J.L. Yakel, Nicotinic ACh receptors as therapeutic targets in CNS disorders, Trends Pharmacol. Sci. 36 (2015) 96–108, https://doi.org/10.1016/j.tips.2014.12.002.

[34] S. Ballaz, I. Morales, M. Rodríguez, J.A. Obeso, Ascorbate prevents cell death from prolonged exposure to glutamate in an in vitro model of human dopaminergic neurons, J. Neurosci. Res. 91 (2013) 1609–1617, https://doi.org/10.1002/jnr.23276.

[35] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 53–63, https://doi.org/10.1016/0022-1759(83)90303-4.

[36] J. Lee, B.I. Escher, S. Scholz, R. Schlichting, Inhibition of neurite outgrowth and enhanced effects compared to baseline toxicity in SH-SY5Y cells, Arch. Toxicol. 96 (2022) 1039–1053, https://doi.org/10.1007/s00204-022-03237-x.

[37] H. Wang, J.A. Joseph, Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader, Free Radic. Biol. Med. 27 (1999) 612–616, https://doi.org/10.1016/s0891-5849(99)00107-0.

[38] A. Malyutina, J. Tang, A. Pessia, drda: an R package for dose-response data analysis, bioRxiv (2021), https://doi.org/10.1101/2021.06.07.447323.

[39] J. Kimura-Kuroda, Y. Komuta, Y. Kuroda, M. Hayashi, H. Kawano, Nicotine-like effects of the neonicotinoid insecticides acetamiprid and imidacloprid on cerebellar neurons from neonatal rats, PLoS One 7 (2012) e32432, https://doi.org/10.1371/journal.pone.0032432.

[40] A. Abdel-Rahman, A.M. Dechkovskaia, J.M. Sutton, W.-C. Chen, X. Guan, W.A. Khan, M.B. Abu-Donia, Maternal exposure of rats to nicotine via infusion during gestation produces neurobehavioral deficits and elevated expression of glial fibrillary acidic protein in the cerebellum and CA1 subfield in the offspring at puberty, Toxicology 209 (2005) 245–261, https://doi.org/10.1016/j.tox.2004.12.037.

[41] M. Liu, G. Wang, S.Y. Zhang, S. Zhong, G.L. Qi, C.J. Wang, M. Chuai, K.K.H. Lee, D.-X. Lu, X. Yang, Exposing imidacloprid interferes with neurogenesis through impacting on chick neural tube cell survival, Toxicol. Sci. 153 (2016) 137–148, https://doi.org/10.1093/toxsci/kfw111.

[42] J.R. King, N. Kabbani, Alpha 7 nicotinic receptors attenuate neurite development through calcium activation of calpain at the growth cone, PLoS One 13 (2018), e0197247, https://doi.org/10.1371/journal.pone.0197247.
[43] J.G. Zhang, D.D. Ma, Q. Xiong, S.-Q. Qiu, G.-H. Guang, W.-J. Shi, G.-G. Ying, Imidacloprid and thiamethoxam affect synaptic transmission in zebrafish, Ecotoxicol. Environ. Saf. 227 (2021) 112917, https://doi.org/10.1016/j.ecoenv.2021.112917.
[44] S. Yan, Z. Meng, S. Tian, M. Teng, J. Yan, M. Jia, R. Li, Z. Zhou, W. Zhu, Neonicotinoid insecticides exposure causes amino acid metabolism disorders, lipid accumulation and oxidative stress in ICR mice, Chemosphere 246 (2020) 125661, https://doi.org/10.1016/j.chemosphere.2019.125661.
[45] L. Schneider, S. Giordano, B.R. Zelickson, M.S. Johnson, G.A. Benavides, X. Ouyang, N. Fineberg, V.M. Darley-Usmar, J. Zhang, Differentiation of SH-SY5Y cells to a neuronal phenotype changes cellular bioenergetics and the response to oxidative stress, Free Radic. Biol. Med. 51 (2011) 2007–2017, https://doi.org/10.1016/j.freeradbiomed.2011.08.030.
[46] V.V. Uteshev, α7 nicotinic ACh receptors as a ligand-gated source of Ca(2+) ions: the search for a Ca(2+) optimum, 27, Adv. Exp. Med. Biol. 740 (2012) 603–638, https://doi.org/10.1007/978-94-007-2888-2.
[47] A. Cartereau, C. Martin, S.H. Thany, Neonicotinoid insecticides differently modulate acetylcholine-induced currents on mammalian α7 nicotinic acetylcholine receptors, Br. J. Pharmacol. 175 (2018) 1987–1998, https://doi.org/10.1111/bph.14018.
[48] D. Xiang, X. Xu, Q. Zhou, R. Yan, M. Chen, Y. Guo, G. Zhu, The expression of soluble functional α7-nicotinic acetylcholine receptors in *E. coli* and its high-affinity binding to neonicotinoid pesticides, Pestic. Biochem. Physiol. 164 (2020) 237–241, https://doi.org/10.1016/j.pestbp.2020.02.002.
[49] C.-H. Wu, C.-H. Lee, Y.-S. Ho, Nicotinic acetylcholine receptor-based blockade: applications of molecular targets for cancer therapy, Clin. Cancer Res. 17 (2011) 3533–3541, https://doi.org/10.1158/1078-0432.CCR-10-2434.
[50] E. Parada, J. Egea, A. Romero, L. del Barrio, A.G. García, M.G. López, Post stress treatment with PNU282987 can rescue SH-SY5Y cells undergoing apoptosis via α7 nicotinic receptors linked to a Jak2/Akt/HO-1 signaling pathway, Free Radic. Biol. Med. 49 (2010) 1815–1821, https://doi.org/10.1016/j.freeradbiomed.2010.09.017.
[51] E. Navarro, I. Buendia, E. Parada, R. León, P. Jansen-Duerr, H. Pircher, J. Egea, M.G. Lopez, Alpha7 nicotinic receptor activation protects against oxidative stress via heme-oxygenase I induction, Biochem. Pharmacol. 97 (2015) 473–481, https://doi.org/10.1016/j.bcp.2015.07.022.
[52] T. Zhang, S. Song, X. Bai, Y. He, B. Zhang, M. Gui, K. Kannan, S. Lu, Y. Huang, H. Sun, A nationwide survey of urinary concentrations of neonicotinoid insecticides in China, Environ. Int. 132 (2019) 105114, https://doi.org/10.1016/j.envint.2019.105114.
[53] M. Xu, Z. Zhang, Z. Li, S. Kan, Z. Liu, D. Wang, Q. Liu, H. Zhang, Profiles of neonicotinoid insecticides and characteristic metabolites in paired urine and blood samples: partitioning between urine and blood and implications for human exposure, Sci. Total Environ. 773 (2021) 145582, https://doi.org/10.1016/j.scitotenv.2021.145582.
[54] H. Zhang, K. Shen, R. Wu, Z. Li, X. Wang, D. Wang, M. Zhan, W. Xu, Y. Gao, L. Lu, Occurrence and distribution of neonicotinoids and characteristic metabolites in paired urine and indoor dust from young adults: implications for human exposure, Environ. Res. 199 (2021) 111175, https://doi.org/10.1016/j.envres.2021.111175.
[55] B. Buszewski, M. Bukowska, M. Ligor, I. Staneczko-Baranowska, A holistic study of neonicotinoids neuroactive insecticides-properties, applications, occurrence, and analysis, Environ. Sci. Pollut. Res. Int. 26 (2019) 34723–34740, https://doi.org/10.1007/s11356-019-06114-w.