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Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Characterization of the neurodevelopmental toxicity of the neonicotinoid insecticides Imidacloprid and Acetamiprid in human neuroblastoma SH-SY5Y cells

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Dedication

For every reader who finds it of utility.

Damián Estuardo Campoverde Chicaiza

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Resumen

Ecuador es un territorio ampliamente agrícola, en una región caracterizada por explotación y una pobre regularización, resultando en un descuidado contacto de la población con agroquímicos. Entre ellos tenemos pesticidas sistémicos neonicotinoides (NNs) caracterizados por su acción y alta especificidad sobre los receptores de acetilcolina de tipo nicotínico (nAChR´s) de insectos. Aunque han sido declarados prácticamente inocuos para humanos, impera caracterizar los efectos de estos pesticidas sobre el sistema nervioso sabiendo la importancia de los nAChR´s en el desarrollo de funciones cognitivas, y la liberación de neurotransmisores, además considerando las funciones y mecanismos aún desconocidos. Tres días después de iniciado el proceso de diferenciación de la línea celular de neuroblastoma humano SH-SY5Y y usando el método western blot para identificar alteraciones en la estructura o nivel de expresión de β-actina, y β-catenina se busca evaluar el efecto a diferentes concentraciones de Imidacloprid y Acetamiprid. Realizando cuatro muestras, se aplicó la prueba de Kruskal Wallis y su respectivo análisis post hoc, prueba de Dunn, para determinar si existía diferencia en la expresión de la proteína como efecto de la dosis aplicada. Se obtuvo para Imidacloprid($p < .05$) que a partir del tratamiento de 0.05 mM existe diferencia estadísticamente significativa, habiendo expresado entre el 50 - 73 % de β-actina respecto con el control. En cambio, para Acetamiprid ($p < .05$), no se pudo comprobar diferencia estadísticamente significativa de la expresión de β-actina entre los tratamientos. Para la β-catenina se realizó un western blot donde se captó dos bandas para el tratamiento con Acetamiprid evidenciando efectos de los neonicotinoides.

Palabras Clave:

Neonicotinoides, β-catenina, β-actina, western blot.

Abstract

Ecuador is a predominantly agricultural territory characterized by exploitation and poor regularization, resulting in neglected contact with agrochemicals by the population. Among them are systemic Neonicotinoids Insecticide (NNIs) characterized by their action and high specificity on insects' Nicotinic Acetylcholine Receptors (nAChR). Although they have been declared practically harmless for humans, it is imperative to characterize the effects of these pesticides on the nervous system, knowing the importance of nAChRs in the development of cognitive functions and the release of neurotransmitters, in addition to considering the functions and mechanisms still unknown. Three days after initiating the differentiation process of the human neuroblastoma cell line SH-SY5Y and using the western blot method to identify alterations in the structure or expression level of β-actin and β-catenin, we seek to evaluate the effect at different concentrations of Imidacloprid and Acetamiprid. The Kruskal Wallis test and its respective post hoc analysis, Dunn's test, were applied to determine if there was a difference in the protein expression as an effect of the applied dose. It was obtained for Imidacloprid ($p<.05$) that from the 0.05 mM treatment, there is a statistically significant difference, having expressed between 50 - 73 % of β-actin to the control. On the other hand, for Acetamiprid ($p<.05$), no statistically significant difference in β-actin expression between treatments was found. For β-catenin, a western blot was performed where two bands were detected for the Acetamiprid treatment, showing the effects of neonicotinoids.

Keywords:
Neonicotinoids, β-catenin, β-actin, western blot.

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Acronyms

(MAP-2) Microtubule-Associated Protein 2.

(VMAT-2) Vesicular Monoamine Transporter 2.

ACh Acetylcholine.

AChE Acetylcholinesterase.

Aß Amyloid beta.

BBB The Blood-Brain Barrier.

CaMKII Ca2+/calmodulin-dependent protein kinase II.

DA Dopamine, or dopaminergic neurons.

DDT Dichlorodiphenyltrichloroethane.

EFSA European Food Safety Authority.

EPA The US Environmental Protection Agency.

FAO The Food and Agriculture Organization of the United Nations.

FDA US Food and Drug Administration.

FLNa Filamin a.

FZ Receptor-ligand Frizzled.

Fz5 Frizzled Receptor 5.

GABA γ-Aminobutyric Acid.

GAP-43 Growth associated protein-43.

GMO Genetically Modified Organisms.

ICAMA Institute for the Control of Agrochemicals of China.

IRAC Insecticide Resistance Action Committee.

MARA Ministry of Agriculture and Rural Affairs of China.

MLA Methyllycaconitine.

MRL Maximum Residues Limit.

NA Noradrenaline.

NAc Nucleus Accumbens.

nAChR Nicotinic Acetylcholine Receptors.

nbM nucleus basalis of Meynert.

NNIs Neonicotinoids Insecticide.

PFC Prefrontal Cortex.

PI3K Phosphatidylinositol-3- kinase.

POPs Persistent Organic Pollutants.

RA Retinoic Acid.

RE Endoplasmic Reticulum.

ROS Reactive Oxygen Species.

SDGs Sustainable Development Goals.

sFRP1 Wnt-Secreted Frizzled Receptor 1.

SGZ Subgranular Zone.

SNpc Substantia Nigra pars compacta.

SvZ Subventricular Zone.

UAPP Unintentional Acute Pesticide Poisoning.

UN The United Nations.

VTA Ventral tegmental Area.

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Chapter 1

Introduction

1.1 Background

Agrochemicals management is an underestimated area of human health, considering its direct impact on food supply, an area that can change humanity's lifestyle. For centuries, with the development of agriculture in Neolithic times, monocultures promoted an imbalance favoring the proliferation of crops and their consumer animals, giving place to pests[1]. To control or to exterminate pests, the use of plant extracts such as chrysanthemum flower extracts used by Persians[2], or the extract of Nicotiana tabacum, which insecticides effects was announced by Darwin Erasmus in the 18th century. And also the use of minerals, which, with technological development, gave way to the massive use of arsenic salts. In 1940, with the chemical industry development, the pesticide revolution took place, producing massive quantities of synthetic toxic substances, and the use of fertilizer, pesticides, and genetic improvement of crops formed the Green Revolution, which is studied as a savior of humanity's starvation[1].

However, the massive use of pesticides and fertilizers with "miracle" results did not take long for side effects to appear related to human intoxication and environmental issues. Due to its application on the base of trophic levels, a range of species was affected, reaching large taxonomic groups at order or class level[3], including species with no relationship with agriculture[4]. Correct management of pesticides is essential to ensure food security and control disease vectors. However, traditionally, pesticides were used as a short-term

solution, and their results are closely related to water, soil, air, and food contamination. Also, consider the increasing cases of pesticide-resistant insects, which are accelerated by the irresponsible use of pesticides. Are pesticides becoming part of the problem?

On one hand, pests threaten human health, food, and property. To emphasize the importance of pest control, insects are the primary vectors for disease transmission and cause more than 700,000 deaths yearly [5]. In addition, crops are essential for human civilization, and it is estimated that more than 45% of the crops were lost because of pest infestation in developed countries[6]. Moreover, it is established that the apparition and increase of organic agronomic pesticides also increased crop yield from 42% in 1965 to 70% in the 1990s[7]. In contrast, in the next decades, the loss of the main crops worldwide reduced to between 10 to 41% in developed countries, but in developing countries, the loss due to pest infestation was between 40 to 70% [8], also would be considered that the 70% of pesticides were applied in the developed countries[9]. As the years went by, new pesticides were developed, including neonicotinoids in the 1990s, improving the situation. Moreover, it is set that one-third of agriculture products use pesticides[10, 11]. However, the harvest losses persist between 10 to 40% depending on the crop product[12]. However, it is estimated that the losses without the use of pesticides rise to 80% [13]. The contribution of pesticides is a determinant in crop production but also brings a myriad of environmental concerns.

On the other hand, the impact on the environment and human health due to the massive and often indiscriminate use of pesticides would alert the world. Around one-fifth of the land area is destined for agriculture, 12% for crops, and around 8% for pasture[14], an important fact considering that around 74% of pesticides are used in agriculture and livestock in many countries, and the remained is used in gardening, industry, and urban purposes[15]. In 2020, it was estimated that 3.5 million tons of pesticides were sprayed worldwide at a total cost of more than USD 60 billion[16]. Pesticide modes of application increase the damage exerted by pesticides because they are sprayed from planes, using ground machinery, dissolved in irrigation systems, or by handheld sprayers; some pesticides are applied as granules directly to the soil. It is estimated that only 25-50%

of pesticides sprayed from planes reach the crops, contrasting with 60 to 90% when they are applied since ground[17]. Moreover, as a reference in China, only 30% of the sprayed pesticides that reach the target crop are absorbed by the current crop[18].

The World Food Summit in 1996 states, "Food security exists when all people, at all times, have physical and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life ."[19] With this quote, the question emerges: Is the current management of pesticides, specifically neonicotinoids, a threat to human rights to food and clean water? It is only necessary to highlight 7 of 17 of the Sustainable Development Goals (SDGs) the Organization of The United Nations UN promotes to realize the high impact and low concern given to pesticide action in our society. It lists the goals that bad pesticide management threatens: life on land, life below the water, climate action, responsible consumption and production, clean water and sanitation, well-being and good health, and zero hunger.

Despite the existence of regulatory entities, the massive release of pesticides has been underestimated as a long-term problem. For decades, this was managed as a short-term decision, situational approvals, or prohibition after the damage was effected with significant impacts on arthropods' nontarget species, reducing soil biodiversity and productivity and reducing pest competitors[20]. Moreover, some pesticides can persist on soil for years, promoting the development of pest resistance. Measures are taken when the effects are evident over crop productivity or when severe intoxication-related cases emerge. As with neonicotinoids, regulation measures were taken when orchard production was affected due to pollinators' death. Neonicotinoids strongly affect pollinators, bees that pollinate 90% of main crops worldwide, and 75% of its population is affected by those pesticides[21]. Again, considering the evident effects, taking short-term solutions and assuming the low affinity of neonicotinoids to mammal's Nicotinic Acetylcholine Receptors as innocuousness. The risk is higher with systemic pesticides, which are absorbed and persist in every part of the plant for a couple of weeks; however, their availability in the soil varies from dozens to hundreds of days, inclusive for years in specific conditions[22], which increases the risk of food contamination and chronic exposure to pesticides.

Around the world, representative regulatory entities guide the decisions of agriculture departments in developing countries. Would be mentioned entities such as the European Food Safety Authority EFSA, US Environmental Protection Agency EPA, US Food and Drug Administration FDA, US Department of Agriculture, the Food and Agriculture Organization of the United Nations FAO, Institute for the Control of Agrochemicals ICAMA under the Ministry of Agriculture and Rural Affairs MARA in China, which take measures respective to agrochemicals such its prohibition, special regulations, doses of application, modes of application in order to reduce the environmental impact, also request or lead research to asses toxicity and pollution produced by agrochemicals. Correct pesticide management can reduce pesticide drift dramatically; an example is the measures taken over pesticide spray applications in the Netherlands, where the contamination of the irrigation system was reduced to 95%[23], and 21% of the impact of insecticides on non-target species[24]. While recognizing that environmental impact can be reduced, it does not mean zero impact, and again, long-term pesticide effects are being underestimated, especially those that affect the nervous system.

1.1.1 Farmers Impact

Europe Union practices a double standard with pesticides, some of them after being evaluated and categorized as extremely hazardous and prohibited in their territory due to the high impact on human health, it is not prohibited to produce and export to other countries such as Ecuador and others countries of the subcontinent, which import pesticides such as paraquat, acephate, atrazine, malathion, diuron from France, Germany, and other countries of the EU[25]. They did not come out unscathed because they imported many fruits and vegetables from those countries. Proof of this is the study carried out by Denmark, Estonia, Finland, Norway, and Sweden's national monitoring pesticide programs detected in the imported fruits from South America pesticide residues over the Maximum Residues Limit MRL between the detected pesticides was imidacloprid and Acetamiprid[26].

On the other hand, despite the regulation, developed countries are not free from neonicotinoid pollution, and it is not always related to agricultural activity. Such is the case

of imidacloprid residue found in water plant treatment in California at levels between 160 and 670 ng/L[27, 28]. The study points to pet flea and tick control treatments and protection components for outdoor structural materials such as polystyrene insulation, vinyl siding, adhesives, sealants, and treated wood as the pesticide sources[29]. Moreover, the low removal efficiency of imidacloprid in its liquid phase in wastewater treatment plants, where inclusive reported imidacloprid residues in drinking water, is reported[30, 31, 32].

Farmers play an important role in pesticide correct management, and in many cases, they are responsible for pesticide pollution. But also, they are the most vulnerable demographic group where the risk of intoxication increases due to the inherent proximity, and the risk of direct contact with pesticides increases influenced by socioeconomic factors such as culture and educational level. It is reported that 99% of deaths from unintentional pesticide poisoning occur in developing countries, even those countries that are not the higher consumers of agrochemicals[33]. Around the world, calculations have shown that about 385 million unintentional acute pesticide poisoning UAPP occur annually, where around 11,000 end up in fatalities. Considering the farming population is around 860 million, it implies that yearly, 44% of farmers are poisoned by pesticides[34].

Farmers' intoxication cases and proper pesticide management are linked with socioeconomic factors such as cultural values and educational level. Widely, farmers are stereotyped for their empirical knowledge but with a poor educational level; in that way, culture and norms, which depend on regulation institutions, directly impact their practices. In comparison, in the EU, more than 93% of farmers are older than 35 years, and only 8.5% have received full training [35]; however, their cultural values and regulation entities promote good practices related to pesticide management. On the other hand, in the United States of America, farmers have a higher educational level, where 41.6% have completed high school and 23.8% have earned a college degree[36]. In the case of Ecuador, it was estimated in 2003 that only 4.6% of farmers have complete secondary education[37]. A survey reveals the influence of education on Ecuadorian farmers, where less than 20% have course capacitation about pesticide management, where nearly the totality of small farmers dismiss the used active ingredient and its toxicity. Despite the lack of knowledge, nearly 70% of

them mix and formulate the cocktail of pesticides and the application dose. Moreover, 86% of people who have to buy pesticides ask for more toxic active ingredients[38]. Another influencing factor in Ecuador is the poverty levels and discrimination of indigenous communities, which makes difficult access to education and health[39]. It would consider the relevance of the agropecuarian sector for different countries; for example, for the United States, farms contribute 0.7% of their GDP and represent 1.2% of US employment[40]. In contrast, for Ecuador, the agricultural sector represents 9% of GDP and 55% of total exports, and the sector provides around 30% of employment in the country[41, 42].

1.1.2 Ecuador Background

Ecuador has a landmass area of 256,369 km2 and is the smallest of the 17 mega-diverse countries[43, 44]. The country has 83 natural protected areas comprising around 23% of the landmass; however, around 22% is designed as an agricultural area[41, 45]. Ecuador is also known for being the largest banana exporter and one of the biggest exporters of flowers, cacao, and shrimp[46]. Moreover, all those crops are characterized by an intensive farming system with a high environmental and population health impact.

The irresponsible management of pesticides in Ecuadorian banana plantations is known worldwide as a big ecological issue with a high impact on population health. It is common to see planes spraying pesticides every week, where the particles do not reach only banana crops but also workers, considering the reported child labor, local communities, and schools. The absence of waste treatment, where around 20% of pesticide waste is delivered directly to water channels or soil, is remarkable[38, 47].

Moreover, the effects of pesticide overpopulation are noticeable in the increased rate of reported disabilities around banana plantations, where workers' exposure to pesticides increases the level of acute genotoxic and cytotoxic effects, which increase the risk of cancer[48] and also can affect the nervous system resulting in neurological and behavioral deficits[49, 50, 51], affecting mainly during pregnancies where also is reported an altered birth weight[52, 53]. It would be taken into consideration that one of the most

used pesticides in banana plantations is Imidacloprid, especially to treat it against Back Sigatoka[54, 55]. However, despite banana plantation concerns, the flower workers in the highlands of the Andes are the most affected group by pesticide intoxication[56, 57].

On the other hand, Ecuador is the first country in the world to recognize the Rights of Nature in its constitution, where also states the prohibition of development production, commercialization, import, transport, store, and use of persistent organic pollutants POPs, internationally prohibited agrochemicals, and genetically modified organisms GMO which attend with population health, food sovereignty or ecosystems since 2008. However, contrary to its constitution, Ecuador has one of the highest application rates of pesticides worldwide, with an estimated 30% of its population living in areas with high pesticide application. Areas that overlap with many amphibian species point to the little importance given to biodiversity[58]. It is estimated that 6.35 kg of pesticides per habitant was spread in the fields in 2010[25]. The pesticide use in Ecuador reaches astonishing levels since the worldwide pesticide application per area is 1.8kg/ha[59], Manaby province presents a mean pesticide application of 64 kg/ha and a maximum of 81.4kg/ha[48]. Also 2010, Ecuador canceled the commercial permission for Extremely hazardous (IA) and highly hazardous (IB) pesticides. However, nowadays, some appear with a current register; between them are paraquat and glyphosate[60].

Ecuadorian farmers have been labor abused and suffered outrageous events such as those related by Jorge Acosta. During pesticide management training, a toxicologist told them that Mancozeb was innocuous, saying that it would be possible to drink a gallon and nothing would happen[61].

1.1.3 Neonicotinoids

Neonicotinoids NNIs were discovered in the 1980s but started to be commercialized until the 1990s. Their rapid popularization is primarily due to their systemic mode of action, high effectiveness at low dosages due to the selective neurotoxicity effects over nAChR of insects, adding its long-term protection, which results in lower pesticide bill costs[62, 63]. The NNIs comprise approximately 25% of the global market of pesticides, being the most

used[64]. Where imidacloprid represents 41% of the NNIs used[65].

As a way of offering evidence of its relatively high effectiveness, the LD50 of imidacloprid for a honeybee is 5 ng, while for a Dichlorodiphenyltrichloroethane (DDT), the LD50 is at least 10,000 times higher[66]. Moreover, its persistence in the environment, which results in prologued protection but also in a myriad of ecological concerns, is remarkable. Acetamiprid estimates its half-life to be between 31 and 450 days, and imidacloprid is in a range of 28 to 1250 days, even though it is reported to be 19 years for clothianidin[62, 67, 68, 69, 70, 71].

NNI mode of action is defined by its strong affinity for nAChR of insects; the binding induces a biphasic response, which increases the frequency of nerve impulses, which results in a blockage of nerve propagation, evidenced in the paralysis and death of insects at high concentrations. The inclusion of synergist factors inhibits the oxidative degradation of the agonist ligand, increasing the pesticidal effects of neonicotinoids[71, 72]. However, there is registered stimulation with no clear effects in low concentrations due to the different nAChR functions[71]. For example, imidacloprid at low doses in honeybees causes olfactory associative learning disabilities, alteration in orientation sense, and abnormal social behavior, registering alteration in gene expression in different maturation stages since larvae, pupae, and adults[73].

The uses of NNIs are focused on arthropod pest control in different fields, such as plant protection in horticulture, arable crops, fruit trees, flowers, timber, and gardens, in industries, as a prophylactic component for structural materials such as insulation, vinyl siding, sealants, pressure-treated wood decking. Also, for domestic pest control, such as cockroaches and ants, in the same way for pets treatment against ectoparasites such fleas and ticks, less common but also in chewable oral doses[62, 74, 75, 76, 77].

One of the main uses of neonicotinoids is seed coat protection, which persists on the plant for months, even years after sowing; as a systemic pesticide, they reach all parts of the crop[65]. Seed dressing offers better targeting of the crop than spray applications;

however, it is estimated that 80% to 90% of the active ingredient in seed dressing is not absorbed by the crop, which 2% is dispersed as dust[65, 78]. Moreover, even vertebrates are less susceptible to NNIs, it is observed alteration inclusive death of birds and mammals due to consuming small amounts of seed treated with NNIs[62].

In the beginning, the claim of the high specificity of neonicotinoids for insects and its negligible action over vertebrates was accepted and extensively reported[71, 72, 79]. Moreover, the presence of the blood-brain barrier BBB in mammals essentially points to neonicotinoids as almost innocuous for humans[63]. However, over the years, the impact on the environment was evident, encouraging deeper research to confirm the lower affinity of NNIs for some subtypes of nAChR on mammals, but also reported alterations in birds, fish, and mammals, including humans[62, 80, 81, 82, 83]. Recently, it was reported in vitro experiments that imidacloprid, as well as other neonicotinoids, can directly activate the human nAChR $\alpha 4$ $\beta 2$ subtype, demonstrating the gaps of knowledge on the range of impact of NNIs[62]. Nevertheless, there are concerns about the effect on the pollinator population due to their role as indicators of the environmental impact[62, 82], but also because pollinator decimation affects fruit crop yield. The weak decision of international entities against producer pressure is evident in the ban on some neonicotinoids in the EU and the United States of America, with episodic approval of use due to uncontrollable pest infestations, ignoring the long-term repercussions over soil organisms that reduce soil productivity[84, 20].

Even though NNIs have a lower effect on mammals, their negative influence on the human nervous system[81], female reproduction[83], genotoxic effects, reduction of the immune response, liver damage, and alteration in food intake, and weight loss have been proved[62, 81]. Highlighting the impact of NNis due to the importance of nAChR, in human nAChR are present in the central and peripheral nervous system, but also in bronchial epithelial cells, endothelial cells, lymphocytes, keratinocytes, cochlear hair cells, and chromaffin cells[62, 85]. Neonicotinoids have high solubility in water, adding their high persistence in the environment due to their resistance to hydrolysis and photodegradation; those characteristics increase the exposure of the population to NNIs through polluted water and food, but also via skin or inhalation in communities near sprayed areas such as the banana

plantation in Ecuador[86].

1.2 Problem statement

Imidacloprid and Acetamiprid are neonicotinoid pesticides extensively used worldwide over the last three decades due to their high effectiveness as systemic pesticides that stimulate insect Nicotinic Acetylcholine Receptors (nAChR). However, these receptors are present along the Animalia kingdom, with different affinities to those neonicotinoids. Moreover, some nontarget species have shown higher affinity, such as arthropods being the water ecosystem one of the hardest hit. The main use of those neonicotinoids is crop protection, but also against urban pests such fleas, ticks, and mites. Depending on conditions, those pesticides can persist in the applied environment for days to years. This property implies pesticide residues in food, water, air, and soil, resulting in considerable environmental concerns. An example of the spread range of neonicotinoids is that Imidacloprid residues have been found in nature reserves, water plant treatment, and drinking water due to their difficulty in removal. That happened in developed countries, which directs our attention to the scenario in developing countries like Ecuador.

Talking about pesticides in Ecuador implies considering soil, water, and food contamination due to irresponsible management caused by low or null instruction of farmers, as well as the economic priorities of the industry and, of course, a weak regulatory organism that allows pesticide spray from planes over communities near banana plantations.Talking about pesticides in Ecuador requires considering the importance of agriculture for its economy and the relevance of tropical diseases with insects as vectors. It is also essential to consider that we are talking about one of the most biodiverse countries in the world.

On the other hand, international pest control entities around the world, especially in the European Union since 2013, set off the alarm bell due to the high impact of neonicotinoids on pollinators, especially honeybees, which negatively affect fruit crop yield with a high economic impact. Considering the spread range of neonicotinoids, it is ironic that crop

yields call to action and not the environmental damage exerted for decades or the fact that people around the world are consuming food contaminated with pesticides that can not be metabolized. Pesticides that work over the nervous system, with an allosteric activation effect over nAChRs, participate in neuron signal transmission and demonstrate involvement in learning, motor memory, memory, and many other functions such as neurodevelopment. International entities dismiss the impact on big animals, especially mammals because it is believed to have a low affinity against neonicotinoids. However, it does not imply any effect, so it is mandatory to study the possible effects of neonicotinoids, considering the complexity of the nervous system and the frequent exposure of humanity to neonicotinoids. In that way, characterizing the effects of two of the most widely used neonicotinoids, Imidacloprid and Acetamiprid, are relevant in order to assess the possible role of neonicotinoids in neuro-system alteration and include implications in neurodegenerative diseases.

1.3 Objectives

1.3.1 General Objective

To identify the neurotoxic effects of the neonicotinoids Acetamiprid and Imidacloprid on the human neuroblastoma cell line SH SY5Y during the differentiation process by Western blot analysis of proteins.

1.3.2 Specific Objectives

- Compile the most relevant information to determine cornerstone proteins for characterizing the neonicotinoid neurotoxicity effect based on the antibody availability.
- To learn, optimize, and perform the western blot technique to measure alteration in protein expression, post-translational modifications, or protein deterioration as an effect of acetamiprid or imidacloprid treatment.
- Establish a hypothesis on the mechanism that may cause the variation of β-catenin and β-actin.

Chapter 2

Theoretical Framework

2.0.1 Neonicotinoids Insecticides (NNIs)

Neonicotinoids (NNIs) are the major group of pesticides used worldwide because of their selective toxicity and physicochemical properties, allowing them to provide prolonged protection[87]. Properties such as their high photostability, water solubility, and lipophilicity increase their half-life availability in the environment, allow them to reach every part of the plant, and improve their uptake[88]. The cornerstone of this study is related to NNis' affinity for nAChRs, which are present in organisms with nervous systems[89]; however, for decades, the NNIs' high selectivity for insect's nAChR subtypes has been disseminated, and even claiming the no toxicity for vertebrates dismissing the complexity of the nervous system and high diversity of nAChR[90, 91, 92, 93, 94, 72, 95, 96, 97].

The basis of the mode of action of NNIs consists of binding NAChR with the "problem" that Acetylcholinesterase (AChE) can not degrade NNIs as it does with Acetylcholine (ACh). This results in overstimulation and malfunctioning of the nervous system[98, 89]. This effect can be minimized with regulatory mechanisms or by reducing the affinity to NNIs through mutation on nAChR. Due to most of the arthropods' size and their highly expressed nAChR with high affinity to NNIs, they produce paralysis and death. However, in sub-lethal doses, there are many described effects, such as behavior alteration, genotoxicity, changes in gene expression, and others[99].

For decades were accepted the arguments for mammals' tolerance against NNIs such as

the low exposure dose due to the relatively larger size of mammals, the detoxication mechanisms, the BBB, and the low affinity of NNIs with the most studied nAChR present in mammals in preliminary experimental results[72]. However, more profound studies demonstrate that NNI pesticides can cross the BBB even more, detecting NNIs in mice's brain tissues since 0.8% to 5% of the administered amount and reach higher concentrations in serum when they were administered by intraperitoneal injection of imidacloprid, thiacloprid, or acetamiprid[100, 99, 101, 68, 102]. Nevertheless, it is not known how NNIs surpass the BBB, though nicotine shows the ability to change the BBB functioning if necessary[103].

On the other hand, the polarity of the nACHR subtype present in insects increases the affinity to NNIs, reinforcing the claimed selectivity of NNIs[104, 105]. Precisely, $\alpha 4\beta 2$ subunits were thought to be present in all nAChR in insects, but only the 8 to 10% nAChR subunits of vertebrates[89, 72]. Nevertheless, $\alpha 4\beta 2$ may not be highly expressed in vertebrates, but its role may be fundamental. In the case of the human brain, $\alpha 4\beta 2$ is one of the most expressed subtypes, being a relevant part of the dopaminergic pathways in the midbrain[106, 107, 108, 109]. Although NNIs have been believed to be irrelevant toxicity for mammals, researchers are finding different evidence, for example, the neurobehavioral and biochemical alterations observed in rats, which are related to derived metabolites of NNIs[102].

According to the mode of action classification of September 2023 from the Insecticide Resistance Action Committee IRAC, NNIs are classified in the subgroup 4A as nAChR competitive modulators, which mode of action implies binding on nAChRs orthosteric site, and causing hyper-excitation, lethargy, paralysis on insects[110, 111, 112]. Their toxicity level is classified by the EPA as II (moderately toxic) and III (slightly toxic)[89]. NNIs can act as partial or full agonists on the nAChR of insects, but in mammals, a weak activation has been observed[113, 114]. The affinity of ligands determines the provability to activate a total response of nAChR[115], which is determined by the ligand structure, in this case NNIs structure, as the nAChR subtype[116, 117, 118, 119].

Throughout evolution, nAChR shows little changes, conserving their ligand binding

site considerably; proposed it is due to their functions on the nervous system and muscle contraction, necessaries in escape and predation, their expression through the Animalia kingdom makes it an important target[120]. Despite this, in 1987, before neonicotinoid pesticides, only 1.5% of the pesticide market focused on nAChR, where nicotine sulfate was not viable due to being not profitable for the pesticide industry, their higher toxicity far mammals than insects, and the quick pest resistance development[89]. Nicotine was not commercially viable because burning tobacco leaves could easily obtain the insecticide action, the lethal dose was higher for flies than rats, and the low toxicity to insects promoted their resistance development, resulting in the ban of nicotine sulfate[65, 89, 121].

It has been reported that NNIs are inspired by nicotine structure[113]; however, their structural similarities are not obvious. In 1978, nithiazine was developed as the first neonicotinoid with a higher affinity for nAChR of insects, but with the problem of quickly losing its pesticide activity, it did not reach the market. Later it was reported that the photolability of nithiazine was the cause of pesticide action loss, where the resulting metabolites were unable to activate nAChR[122, 123, 124, 125]. From nithiazine structure and methodically changing its substituents, Shinzo Kagabu tells us how imidacloprid was discovered. Noting the importance of the ring, decreasing the toxic effect with a bigger ring size and losing its effect on non-cyclic structure. Testing introducing substituents to the five-membered ring, specifically the 2-nitromethylene imidazoline, which reports the best insecticide performance, through experimentation on grasshoppers determined that adding a benzil group improves pesticide action. Then, by changing the benzil group substituents, it was established that the insecticide action depends on the chemical species introduced and their position on the ring, which shows the importance of the structure's ability to interact with nAChR. p-Cloro and m-cyano demonstrate enhanced effects; however, they did not reach the effectivity of nithiazine. With those key facts, they restart from the nithiazine's structure, finding that nicotinyl or properly named 3-pyridylmethyl -2nitromehylene-imidazoline reaches insecticide effect higher than nithiazine, to finally probe 6-chloro-pyridylmethyl-2nitromethylene-imidazoline with an effect at least five times higher than nithazine. Finally, they found that changing the nitromethylene for a nitro imino derivative reaches an effect at least 125 times higher than nithiazine, and it is photo-

stable. Giving rise to imidacloprid N-1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-ylnitramide, and the NNis family. In the case of Acetamiprid, it was developed by modifying the imidazoline ring and nitro group, N-[(6-chloro-3-pyridyl)methyl]-N'-cyano-N-methyl-acetamidine[122, 125, 126, 87, 114, 93].

Imidacloprid and acetamiprid are from the first generation of NNIs, while imidacloprid is classified by its structure and moieties as neonicotinoids five-membered ring, N-nitroguanidine due to the oxygen atoms in the nitroguanidine makes them more reactive what is evidentiated on their toxicity. And acetamiprid is classified as a non-cyclical N-Cyanoamidine[127, 128, 129, 130, 131, 132, 65, 113].

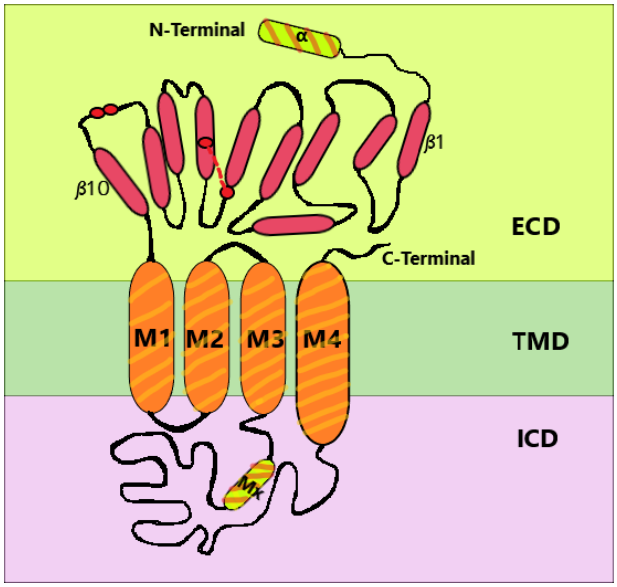
2.0.2 Nicotinic Acetylcholine Receptors (nAChRs)

The nAChR agonist binding site is the main target of NNIs. They are implied in a myriad of functions in the different nervous systems, from arthropods to mammals and, of course, including humans[73]. Would not be confused with the muscarinic acetylcholine receptors, which are also activated by the endogenous ligand ACh and, in the same way, are present in neuronal and non-neuronal tissues; however, they are metabotropic receptors liable for slow response in a range of milliseconds to seconds, mainly associated with parasympathetic functions[120]. Meanwhile, nAChR are cationic non-selective ionotropic receptors responsible for fast activation in a range of micro to sub-microseconds. In addition to being activated by ACh, they can also be activated by exogenous substances such as nicotine and NNIs with different affinities and response magnitudes and participate in a myriad of functions[120, 133, 134, 135, 136].

The nAChRs are members of the Cys-loop-gate ion channel superfamily neurotransmitter receptors[137]. The nAChR is formed by five subunits, which are highly variable in kinetics, electrophysiology, and pharmacology properties, which give the nAChR its affinity for the ligand and ion selectivity[136, 138]. Each subunit has a similar organization, an extracellular domain, a transmembrane domain, and an intracellular domain. At the extracellular domain, a major N-terminal or amino-terminal α-helix, ten β-strands forming

the called β-barrel, and a short carboxyl-terminus. All of them interact with the ligand, so it is where the orthosteric binding site domain, which regulates the open-close channel state, is located[120, 136, 139, 140]. In the transmembrane domain, four α helices are identified: M1, M2, M3, and M4. They span through both sides of the membrane. The M2 α-helix of each subunit conforms to the channel pore of the channel through the cell membrane, known as the inner ring. It is the narrowest section of the receptor pore. The other transmembrane domains encircled the pore, conforming to the outer ring, where M4 is the outermost, and M1 and M3 are perpendicular to M2-M4[141, 102, 139, 142]. A short intracellular loop is formed between M1 and M2, and a similar outer loop is formed between M2 and M3. The intracellular domain takes place between the M3 and M4 helices. It is large and variable, containing an α-helix. It is responsible for interacting with the inner cell signaling through protein interaction such as phosphorylation, redox state, or membrane fluidity, which controls the receptor desensitization, trafficking, and coherent response[120, 136, 143, 139, 144, 145, 146].

Figure 2.1: *nAChR Subunit*



The figure presents a general scheme of the nAChR subunit, where it is possible to differentiate its domains. Moreover, it could be defined as an α-subunit for the presence of two consecutive cyc-cys amino acids before $\beta 10$ on the extracellular domain.

The three domains participate in cation selectivity. The transmembrane and intracel-

lular domains alter the channel conductance[140]. It has been discovered that at both ends of the receptors, the extracellular domain and intracellular domain, there are vestibules that possess a strong negative charge, creating at least fourfold cation presence compared to anions[140]. It may be responsible for cation attraction. Although the electrostatic potential is not enough for cation selectivity, consequently, it is postulated that the polarity of residues in the intracellular domain highly contributes to the size and charge selectivity of the channel[147]. In addition, it is suggested that the ion gate is midway through the transmembrane domain, being the narrowest section, and their residues form a hydrophobic ring that can block ion permeation[147, 148, 149]. The high polarity on vestibules at both ends of the receptor, which increases channel conductance, a change on the hydrophobic ring at the narrow section of the channel formed by M2 transmembrane domain, with the receptor activation would promote a rapid ion flux[120, 147]. The nAChRs are not selective between cations, although the most frequently studied are sodium and potassium, and depending on the nAChR subtype, they can allow trespass calcium ions[120, 150, 151].

The nAChR subunits are classified as α and non-α. All subunits of receptors of the cys-loop family are characterized by forming a disulfide bond between two cysteines separated by 13 amino acids. However, the α subunits are also identified as having two cysteines adjacent, near the M1 transmembrane domain; this section highly influences the ligand binding site[141, 152, 102]. There are seventeen subunits of nAChR reported in vertebrate species α1-α10, β1-β-4, γ, σ, and ε. Although a myriad of nAChR could be formed, it is not observed, and only a few combinations result in functional receptors. Moreover, some receptors seem limited to take a specific position in the receptor or only be present in some species; for example, α8 are observed only in avians and γ subunit in fetal tissues[120, 137, 136, 153], and α5 and β3 are considered wild cards; they need at least two other types of subunits to form a receptor[137].

The nAChR subunits are also grouped by their function. Due to their prominent presence in the autonomic ganglia, brain, spinal cord, and muscles, they are classified as muscle nAChR, which are α1, β1, μ, σ, ε, and in the neuronal group, are α2-10, β2-4 subunits[102, 154, 155]. They are also classified depending on their subunit composition,

which can be homopentamers formed by the same α subunits; essentially, only $\alpha 7$, $\alpha 8$, $\alpha 9$, and $\alpha 10$ form homopentamers. However, $\alpha 9$ builds a homomeric receptor with modest activity, which is improved by forming the receptor with the $\alpha 10$ subunit; both subunits are lowly expressed[137]. On the other hand, heteropentamers, which are more abundant, are formed by α and non-α subunits[136, 137, 113]. It is studied that all nAChR comes from a common ancestor, which would be similar to the phylogenetically ancient $\alpha 7$ homopentamer receptor[136, 137]. The most studied nAChR are $\alpha 4\beta 2$, $\alpha 7$, $\alpha 2\beta 2$, $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 4$, $\alpha 6\beta 2$, and $\alpha 9\alpha 10$.

The orthosteric binding sites take place between two neighboring subunits, where the N-terminal, the β-strands, and the α helix of each subunit conform the loops that give place to the hydrophobic ligand-binding pocket in the extracellular domain[136, 142, 139]. In the orthosteric binding site, an α subunit participates, which forms the front face, also called the positive side, and the other subunit provides the negative or back face for the ligand binding site. The front face forms the loops A, B, and C, and the back face provides the loops D, E, and F[136, 156]. Only $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 7$, $\alpha 8$, and $\alpha 9$ can work as the front face binding domain because of the characteristics of cysteine adjacent pair[120]. Although $\alpha 5$ and $\alpha 10$ are α subunits, they lost the residues necessary to hold the positive position[120]. The D, E, and F loops are provided by $\alpha 10$, $\beta 2$, $\beta 4$, sigma, gamma, and epsilon. In addition, the fifth subunit position, which does not directly participate in the orthosteric binding site in heteropentameric receptors, modulates the receptor properties[120, 157, 158]. It is observed in $2(\alpha 4\beta 2)^*$ that the fifth subunit varies the affinity for the ligand. As a demonstration of nAChR diversity, in the fifth position of $2(\alpha 4\beta 2)$ can be found $\alpha 4$,$\alpha 5$,$\alpha 6$,$\beta 2$,$\beta 3$[159].

Hydrophobic interactions between ligands and the binding pocket domain produce a rearrangement affecting the hydrogen bonds in the binding site, recruiting water molecules, and producing a rotational force of the loops toward the ligand due to its chemical nature. The rotational force over the β-barrel extracellular domain pushes the ligand inside, reaching deeper structures in the receptor, such as the Cys-cys pair and F loop, completing the ligand binding phase. It results in a higher torque which, when all orthosteric binding sites

are occupied, can rotate the subunits, altering the radios of the pore channel, rotating it from the hydrophobic-close channel state to a fully open state which presents a hydrophilic behavior, and facilitating the cross of cations through the pore for microseconds, depolarizing the membrane and exerting the signal[160, 120, 161, 162]. It is established that heteropentameric nAChR has two orthosteric binding sites, and homomeric would have five binding sites[163]. nAChRs are optimized to withstand rapid changes, pointing to the reason for being a highly preserved structure through evolution[120]. On the other hand, failures in the conductance are responsible for diseases such as myasthenia syndromes and some epilepsy cases.

Besides the orthosteric binding site, there are other binding sites, such as allosteric binding sites, whose function is related to receptor conformational changes and modulation of the signal response. Moreover, drugs with allosteric binding sites as targets are designed to treat neurological impairments such as Alzheimer's disease[164]. Allosteric binding sites present advantages on selectivity because they are more susceptible to changes through evolution, and being a less aggressive modulation reduces the adverse effects of a totally open, close response of the orthosteric binding site[165, 166, 167].

nAChRs are distributed through the body, implied in several functions with impressive breadth, not only limited to receiving and transmitting the response of cholinergic activation but also being part of important roles related to neurotransmitter release, neuron environment regulation, structuring and maintenance of neurites, and synapses, modulating neural viability depending their location and subunit composition[136, 168, 164]. nAChRs are present at postsynaptic, somatodendritic, and presynaptic regions in neurons and are also expressed in microglia cells, macrophages, and other immune cells[169, 170, 171, 172, 173, 174].

NAChRs are involved in a wide range of functions, including the immune system, muscle contraction, and a myriad of brain functions. Due to the scope of cholinergic neurons, nAChR acts as an orchestra conductor, triggering and modulating brain functioning. In the immune system, nAChRs are present in immune cells, where they can regulate inflamma-

tory mediators being part of the anti-inflammatory cholinergic pathway, and also involved in neuroprotection pathways where the Wnt pathway may take an important role mediating neuron viability and neuron plasticity, inclusive reveals protection against Amyloid beta (Aβ) plaques toxicity, which are a hallmark of Alzheimer disease[175, 176, 174, 177]. Also, they are involved in microglial inflammation response[174]. Furthermore, in the basal forebrain, the nucleus basalis of Meynert nbM presents a high density of cholinergic neurons, and projects its axons to the cortex, olfactory tubercle, amygdala, hypothalamus, and thalamus. Further, 90% of thalamus innervation from the brainstem are cholinergic neurons[178]. Another important group of cholinergic neurons is in the midbrain, specifically on the pedunculopontine nucleus and laterodorsal tegmental nucleus, which innervates with cholinergic neurons in the basal ganglia, thalamus, and other regions. The septal nucleus and vertical limb of the diagonal band of Broca innervates the amygdala and hippocampus with cholinergic neurons[178]. To clarify, the relevance of nAChR lies in the projection of cholinergic neurons, which modulates the activity in the close neurons in different brain regions, highlighting their influence through nAChR over dopaminergic, glutamatergic, and GABAergic neurons[106, 168]. The impact of nAChR on brain functions is linked with cognition, memory, working memory, reversal learning, behavior, emotions, reward, habit formation, motivation, selective attention, arousal state, motor control, and spatial navigation, where the participation of $\alpha4\beta2^*$ and $\alpha7$ subtypes are remarkable[179, 168, 96, 180, 181, 182, 183, 184, 185].

The activation of the nAChRs regulates the equilibrium of the called neuromodulators: ACh, catecholamines, and serotonin, as well as glutamate and γ-aminobutyric acid GABA, are essential for correct brain functioning[186, 179, 168, 187, 188, 189]. It is found that an imbalance in those neuromodulators is responsible for different neurologic and psychiatric disorders such as Alzheimer's disease, schizophrenia, epilepsy, Parkinson's Disease, and many other disorders[137, 190, 191, 192, 193, 194, 195]. One of the hallmarks of Alzheimer's disease is the loss of cholinergic neurons in the forebrain, regions with high activity of nAChR; it has been shown that AD progression is delayed by the replacement of the lost neurons with new neural stem cells[174]. That is why nAChR is highly studied as a therapeutic target, making us consider the diversity of nAChR subtypes and their

pharmacological properties[136].

Knowing the relevance of the dopaminergic system in different neurological disorders, highlighting Parkinson's Disease, would point out the importance of nAChR in the modulation of the dopaminergic system, responsible for dopamine DA release, and the landscape of the mesolimbic dopaminergic system is described[196].

The ventral tegmental area VTA innervates with dopaminergic neurons in the prefrontal cortex PFC, nucleus accumbens NAc, amygdala, and hippocampus, where presents $\alpha 7$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 6\beta 2$* nAChR subtypes[168, 187, 188, 189]. The VTA receives glutamatergic neurons from PFC and pedunculopontine tegmentum. Those presynaptic innervations abound in the nAChR $\alpha 7$ subtype. It also receives gabaergic projection from NAc and amygdala, which highly express the nAChR $\alpha 4\beta 2$* subtype in presynaptic neurons and interneurons. The VTA influences rewards, addictions, mood regulation, social interactions, stress, learning, and memory functions. The PFC is responsible for abstract thoughts involved in reasoning, high-order decision-making, planning, organization, working memory, and even emotions[179]. In the NAc, the glutamatergic innervation from PFC presents $\alpha 7$ nAChR subtype, the cholinergic interneurons present $\alpha 4\beta 2$* subtypes, and the innervation of dopaminergic neurons from VTA present $\alpha 6\beta 2$*, $\alpha 4\beta 2$*, $\alpha 6\alpha 4\beta 2\beta 3$* subtypes[168]. The PFC is responsible for abstract thoughts involved in reasoning, high-order decision-making, planning, organization, working memory, and even emotions[179]. It is registered that GABAergic non-fast spiking interneurons, dopaminergic, and glutamatergic neurons in PFC present $\alpha 7$, $\alpha 4\beta 2$ nAChRs subtypes; the pyramidal neurons in the V layer do not express nAChRs but are regulated by nAChR activity[168]. The hippocampus receives dopaminergic projection from VTA, and there is glutamatergic transmission with the amygdala and NAc where $\alpha 7$, $\alpha 4\alpha 5\beta 2$, $\alpha 4\beta 2$ nAChR were found[168]. The mode of function of those regions highly depends on the equilibrium of the neuromodulators, even being considered a pseudo-critical point system where different ratios of neurotransmitters allow proper learning, arousal, rest, attention, and other states[197, 198, 199].

Would notice the wide influence of nAChR, and moreover, increasing their complexity

by the diversity of its subtypes, which in many cases are poorly studied, such as $\alpha 3$ and $\alpha 6$ subtypes, and even more, not all the functions of the most studied subunits are known or understood[137, 200]. A short review of different nAChR subtype functions, $\alpha 7$ especially permeable to calcium ions, being involved in neurotransmitter release, neurites and synapses maintenance, and cell viability[137, 201, 202, 203]. In experiments, the inhibition of $\alpha 7$ functioning alters the memory work. $\alpha 7$ and $\alpha 4\beta 2^*$ are shown to be involved in neuroplasticity in pyramidal and gabaergic neurons in the hippocampus[204, 205, 206, 207], and $\alpha 7$ and $\alpha 3\beta 4^*$ show modulate glutamate release[208, 209, 210]. In VTA, $\alpha 7$ is present in at least 40% of GABAergic and dopaminergic neurons, which shows being involved in rewarding pathways that may be related to nicotine addiction[211, 212, 106]. On hippocampus $\alpha 7$, $\alpha 3\beta 2^*$ shows a strong influence on correct memory functioning; in a test, the agonist stimulation results in enhanced cognition, and antagonists impair this function[213, 214, 215]. On the other hand, $\alpha 4^*$ nAChR subtypes seem to be highly involved in addictions related to cholinergic stimulus, with a high affinity for nicotine when formed with $\beta 2$ or $\beta 4$, being subject to upregulation[136, 137, 216, 217]. Similar to $\alpha 4$, the $\alpha 6$ nAChR subunit has a high affinity and is upregulated by nicotine activation[218, 219].

2.0.3 SH-SY5Y Cell Line

Sh-SY5Y is a human neuroblastoma cell line derived from differentiated neurons that have been extensively used as a human disease model, reducing the issues inherent to using neuronal models from other species. In vitro models favor neurology research, allowing the evaluation of protein functioning, molecular mechanisms, and drug effects[220, 221]. The SH-SY5Y cell line comes from the third clonal selection of the SK-N-SH cell line. The parental cell line comes from the biopsy of a metastatic tumor on the bone marrow of a 4-year-old female, which was extracted and presented epithelial-like and neuroblast-like cells[222].

The SH-SY5Y cell line expresses considerable dopaminergic-like activity and minor cholinergic-like behavior, especially undifferentiated ones[222]. Due to the release of noradrenaline NA and tyrosine hydroxylase activity, Sh-SY5Y cells are categorized as a cate-

cholaminergic cell line and, more specifically, dopaminergic[223, 224, 225, 226]. However, Sh-SY5Y cells differentiated identity highly depends on substances used for differentiation protocol[227]. Retinoic acid RA promotes differentiated neurons with cholinergic-like behavior[228, 225, 229, 230, 231]. It is suggested to consider SH-SY5Y derived neurons as a neuronal entity and not classify it as cholinergic, dopaminergic, etc., because they do not resemble neuronal subtypes and can express, at times, different characteristics of the different subtypes[220].

On the other hand, it is a regular practice to probe different treatments on Sh-SY5Y-derived neurons, monitoring for up to 3 weeks and evaluating the differentiation on the third day [220, 232]. The cell line presents many advantages, being human-derived, and animal issues are avoided. It presents human brain-like phosphorylation, a high proliferation, and relatively low cultivation cost, and it is relatively easy to reach a homogeneous culture and neuronal maturity and fast differentiation. They are also available for genetic manipulation[220]. However, it is limited to probing the chronic effects of toxins or representing neuron-aged characteristics due to their being monitored for up to 21 days in most cases[220].

Chapter 3

State of the Art

NNIs are systemic pesticides that exert a toxic effect by overstimulating nAChRs in insects. They have been highly regulated, and some NNIs were banned due to their indiscriminate impact on pollinators. However, the effects on overall arthropod populations would also be considered, which, in the long term, implies a crop productivity reduction and environmental imbalance. NNIs were characterized by a high specificity against insects and rapidly gained popularity due to their high effectivity, long-time protection, and the need for a relatively lower dose, which were linked with lower production costs and environmental impact. However, their properties, such as high persistence in the environment and high solubility, induce insect resistance to NNI development and facilitate food, soil, and water contamination, which translates to population direct exposition to NNIs for around three decades and counting because some NNIs can persist in the environment for years as the case of clothianidin that can persist until 19 years[70].

Pesticide management has protagonist scenarios of a severe environment and human health deterioration worldwide, with critical effects highlighting genotoxicity, neurotoxicity, carcinogenicity, and parkinsonism inducement. Pesticide management would receive the same importance as any medicine brunch because of the high exposure of the overall population to them. A clear example is systemic pesticides, which can be administered to the population through contaminated food and water. This is the case of NNIs, which were found included in drinking water due to the difficulty of being removed from water treatment plants. The slow response time of regulatory entities to identify pesticide im-

pacts is a troubling indicator of bad control and unconcern. For example, since the start of imidacloprid commercialization, around ten years have passed for its approval in the EU, and eight years have passed to identify and take measures against NNIs due to their impact on pollinator population, event highly supported by the reduction in fruit production[84]. Moreover, eventual approval of NNIs use by a declared state of emergency gives an image that regulatory entities' loyalty is to crop yield and not to the population's health. How many years will it take to determine their impact on humanity?

For years, experiments have verified the low effect of NNIs on vertebrate nAChRs, especially on mammals, which need to dramatically increase the administered dose to produce acute toxic effects. It is shown in **Table 3.1**. Over the years, the dramatic scenario starring a high demand and spill of NNIs in the world has produced an increasing sense of menace for every organism with a nervous system, even more considering the function performed by nAChRs in the human brain, it became necessary to characterize the NNIs effect on human nAChRs. First of all, as checked before, NNIs are in direct contact with the overall human population, and it's proved that in different magnitudes, they can trespass the BBB[102].

Table 3.1: *NNIs Higher Affinity for Insects than Mammals*

Neonicotinoids	**Mammals Oral LC50**			**Insects Topical LC50**		
	Animal	**Dose**		**Animal**	**Dose**	
Acetamiprid	Rat	140 mg/kgbw	[233]	24 ng/insect	Leptinotarsa decemlineata	[234]
Imidacloprid	Mouse	131mg/kgbw	[62]	0.82 ng/insect	Nilaparvata lugens	[235]
				18 ng/insect It is 0.018 mg/kg	Honeybees	[236]
	Rat	443 mg/kgbw	[62]	39 ng/insect	Leptinotarza	[131]

This table presents the LC50 of acetamiprid and imidacloprid experimental reported for insects and mammals to compare the affinity of NNIs. If comparing the LC50 of imidacloprid for honeybees and mose, considering the dose in mg/kg BW, the dose required for the mice is more than seven thousand and two hundred times that for bees.

While NNIs can categorized by chemical structure similarities, a minimum structural variation shows dramatic differences in nAChR activation inclusive of being the same sub-

type. Adding the great diversity and indispensable function of nAChRs on the human nervous system would set the alarm bells ringing. However, it has a contrary effect: the gaps in knowledge of NNI interactions and the initial experimental results of almost innocuity of NNIs for vertebrates supply a comfortable environment to underrate the NNIs' impact. After declaring the end of the world, the question is why the impact of NNIs is not easily detectable on the population's health after decades of exposure.

Considering the complex and highly regulated process in which nAChRs are involved and the nonexistence of a control group, it is essential to report the experimental observations in order to identify possible camouflage effects, such as subtle psychological or psychiatric alteration, among which can be behavioral changes, addictions, attention, and cognition impairment. Another consideration is the limited research of chronic effects related to its cost in vivo and in vitro experiments, primarily to assess NNIs impact in aged brains, and the impossibility of reaching it on neural cell lines.

Observations

First, it would be pointed out that most studies, in vivo or in vitro, of NNI's toxicity are performed using the NNIs whole molecule it may be because the NNIs molecules have a high stability. However, in the field, where the insecticides persist for days to years their molecules will break, and the lack of knowledge of NNIS interaction did not allow us to see the potential effect of NNI-derived metabolites. Furthermore, it was discovered that some of the derived metabolites show higher affinity to mammal receptors depending on the nAChR subtype, registering an agonist activation at least as good as ACh and nicotine, where would be pointed the case of desnitro imidacloprid with $\alpha 4\beta 2$ nAChR human subtype demonstrates a high affinity and full response effect on LUHMES human neuronal cell line[113, 237, 230]. Remember that the $\alpha 4\beta 2$ nAChR subtype is highly expressed in the human brain and participates in different functions such as cognition, memory, and behavior.

The NNI effects can be camouflaged on the complexity of brain functions, especially

those that are difficult to monitor, such as behavior, even more so when the population was exposed for decades. On rats, thiamethoxam administration for seven days induced anxiety, which was correlated with a decrease in acetylcholinesterase(AChE)[238]. In another experiment on infant rats, clothianidin significantly reduced cognitive functions due to altered striatum dopaminergic release[239]. Other effects are registered in the **Table 3.2**.

Table 3.2: *NNI Reported Effects on Mammals*

NNI	Animal Model	Effects	
imidacloprid	mice	Immunosupresive effects.	[240]
		Impairment in learning and memory behavior.	[241]
	rats	Alteration on antioxidant defense.	[242]
		Alteration on ovarian weight, and female hormones release.	[243]
		Reduction on sperm motility, alteration on morphology.	[244]
		Increase germ cells apoptosis.	[244]
		Decrease pain threshold.	[245]
		Decrease locomotor activity.	[104]
		Calcium ion influx alteration induce cytotoxicity on cerebellar neurons.	[104]
acetamiprid	mice	At low and high doses, a significant reduction in anxiety levels.	[246]
	rats	Loss of learning, memorization, and locomotion.	[247]
		Neurotransmitters imbalance, adrenaline increase, and dopamine and serotonin decreases.	[247]
thiametoxan	rats	Increased anxiety behaviour.	[238]
clothianidin	rats	Deterioration of cognitive function.	[239]

This table presents different reported effects of NNIs on mammals. It is remarkable the behavioral anxiety alteration.

Particularly, acetamiprid in vivo and in vitro experiments revealed to cause damage to DNA, proteins, and lipids, which is related to Reactive Oxygen Species (ROS) and nitrogen reactive species causing oxidative stress, and also consider the role of lipids on synaptic vesicle membrane composition[248, 249, 250, 251, 113]. Acetamiprid represents 10.5% of NNIs' global sales[113, 252]. The danger of acetamiprid lies in its high solubility that facilitates ingestion through contaminated food. It is registered that acetamiprid can accumulate in different tissues, mainly damaging the liver and kidneys[113]. Also, it is reported that acetamiprid interacts with $\alpha 3\beta 4^*$ nAchR in adrenal medulla glands and stimulates epinephrine secretion[73]. Acetamiprid can downregulate $\alpha 3$, $\alpha 4$, and $\alpha 7$ nAChR subunit expressions in rats[113]. The impact of nAChR activation by acetamiprid is linked to calcium, sodium, potassium, and magnesium ions imbalance in cerebellar neu-

rons, which was evident in rat experiments[253], and the growing evidence that acetamiprid is genotoxic, reprotoxic, and neurotoxic[113]. However, it is unclear; opposite results were obtained in mice[254].

The interaction of NNIs is complex and depends on NNIs' and nAChR's chemical structure. As is evident with imidacloprid, which strongly affects insects' nAChRs, even imidacloprid can block the ACh activation in specific nAChR subtypes present in insects[255]. However, in other subtypes, imidacloprid can not induce its toxic effect. However, it can strongly potentiate the ACh binding response. It is also observed that its metabolites can activate some nAChR subtypes, which the whole molecule can not do. That was reported in an experiment on chickens where the metabolite desnitro imidacloprid showed a high affinity to $\alpha 4\beta 2^*$ nAChRs. However, the imidacloprid whole molecule could not activate the same nAChR subtype[256]. However, co-applied with ACh, it can potentiate the agonist response[257]. The complexity of NNIs binding interaction can be evidential at insects' nervous system level; imidacloprid and thiacloprid have similar chemical structures, both having a pyridine ring and registering similar interaction with the binding pocket of $\alpha 4\beta 2$ nAChR of insects resulting in full agonist stimuli. However, tested on Pame $\alpha 7$, a cockroach nAChR subtype, imidacloprid can not activate the receptor, but thiacloprid induces a weak response despite the expected similar pharmacological action[73]. It shows that not all nAChR on insects are activated for NNIs; even being activated does not mean the same response level, which constitutes a complex scenario for mammals where the toxic effects are not easily detectable.

The impact of NNIs on the human brain has similarities with the registration of nicotine, both targeting nAChR, and some of them share similar components, such as the case of imidacloprid, acetamiprid, thiacloprid, and nitenpyram, which count with 3-pyridylmethyl-2-nitromethylene-imidazoline, also known as nicotinyl, in their structures. Therefore, it can be helpful to associate or compare NNIs effects with the known effects of nicotine stimulus, such as addiction, relaxation, upregulation, and desensitization of nAChR. It is also linked to cell maintenance roles such as proliferation, inflammatory response, cell viability, differentiation inhibition, and apoptosis inducement.

Despite the higher toxicity of nicotine for mammals than insects, its effects are not noticeable at first. Mammals developed metabolic mechanisms for degradation and rapid clearance of nicotine in order to protect neurons of greater concentration levels. Nicotine can trespass the BBB and accumulate in a lipophilic brain environment, reaching tenfold higher concentrations than in brain plasma[258, 120]. In the case of NNIs, they also present lipophilic characteristics and demonstrate the ability to cross the BBB. However, they are designed to permanently bind nAChRs to induce toxicity. It is not clear if NNIs permanently bind human nAChR subtypes, but the difficulty that presents to be degraded at the liver and the damage that NNIs produce on liver and kidney tissues is known[113, 259].

Nicotine has shown neuro teratogenic effects, altered BBB functioning, and chronic exposure in mice reveals neural proliferation inhibition. Moreover, nicotine ensures a toxic effect on microglia in doses above 10µM[103, 174, 260]. However, lower doses demonstrate reduced microglial inflammatory response[174]. It goes as far as to consider that nicotine also has positive effects, such as inducing defense mechanisms against Alzheimer's and Parkinson's diseases[261, 262]. However, the therapeutic mechanism remains unknown. It is observed that nicotine provides protection against amyloid beta (Aβ) oligomers, which are a hallmark of Alzheimer's disease. Aβ induces elevated levels of TNF-α, IL-1β, upregulation of axin-2, p-β-catenin, and down-regulating β-catenin, p-GSK- 3β, microtubule-associated protein-2, and choline acetyltransferase. However, with pretreatment of nicotine at 10µM, the protection effect appears to reduce the toxic impact of Aβ) oligomers, where a reduction in TNF-α and IL1-β is observed. The results indicate that nicotine reduces the inflammatory response and down-regulates axin-2 and also avoids β-catenin downregulation. Presumably, the participation of the Wnt-β catenin pathway in the neuroprotection mechanism presents a partial recovery of proliferation and differentiation ability, inhibiting the induced apoptosis[174, 263].

Besides, on the short review of nAChR subtypes, it was noticed that the α7 subtype is involved in neurotransmitter release, neurites and synapses maintenance, and cell viability[137, 201, 202, 203]. The interaction of α7 nAChR and nicotine is elusive; α7

shows lower affinity than others, such as $\beta 2^*$ nAChR subtypes[264, 168]. Nicotine seemingly does not bind the $\alpha 7$ subtype directly but shows modulator functions; for example, on the dopaminergic circuit, the selective inhibition of $\alpha 7$ nAChR reduces the reward effect of nicotine stimulus[212]. In another experiment, it was observed that infusions of nicotine in the amygdala enhance working memory and memory consolidation. However, testing with methyllycaconitine MLA, a selective $\alpha 7$ nAChR inhibitor, produced the contrary effects on memory[180]. It was also tested that nicotine induces $\alpha 7$-nAChR interactions with the phosphatidylinositol-3- kinase PI3K signaling pathway, which protects against Aβ oligomers toxicity[263].

The influence of nicotine in dopaminergic circuit. On the other hand, as previously reviewed, the importance of nAChR on the mesolimbic dopaminergic circuit, nicotine activation of $\alpha 4\beta 2$ nAChR subtypes enhance DA release on NAc, and the activation is followed by rapid desensitization[265, 266, 168]. Simultaneously, $\alpha 4\beta 2$ nAChR on GABAergic terminals induces a temporary inhibition of DA release. Again, it is followed by receptor desensitization, resulting in a loss of inhibitory action over DA release[264, 168]. On the other hand, $\alpha 7$ nAChRs on glutamatergic neurons of VTA increase the release of glutamate on NMDA-type glutamate receptors, modulating dopaminergic cell bodies that enhance excitatory postsynaptic currents. In that way, nicotine impacts the reward circuit, synaptic plasticity, and memory formation[266, 267, 168]. It is important to remark that the therapeutic effects of nicotine are related to the activation and desensitization of the receptors[264].

Desensitization of nAChR is essential to avoid extreme responses and control brain plasma chemical equilibrium for correct brain functioning. In the case of nicotine-induced desensitization, it is highly impacted by the nAChR subunit composition, nicotine concentration, and exposure time. Among the remarkable registered side effects of nicotine desensitization is the upregulation of certain nAChRs with high affinity to nicotine, such as the case of $\alpha 4\beta 2^*$ and $\alpha 6^*$ nAChR and others[268, 269, 270, 271]. The sensitivity to nicotine can be highly variable depending on the nAChR subtype; for example, the neuronal nAChR subtypes can show fifty-fold higher sensitivity to nicotine than muscle

subtypes[120]. In the nAChR subtypes with high affinity for nicotine, such as $\beta 2^*$ nAChR, nicotine activation increases ion permeability and desensitization rate[120]. Referent to concentration, it is observed that desensitization increases with nicotine concentration[272], but it is not so simple; it is observed that time of exposure has a higher impact than concentration, with different effects. It is described that a short exposure time, in a range of seconds to minutes, reversibly leads to functional receptor desensitization. However, permanent inactivation is obtained with a more prolonged exposure in a range of minutes to hours[273, 274, 275]. It is also observed in very low concentrations and chronic exposure in a range of days[273, 137]. The tricky thing is that with a lower concentration and a very short time of exposure, nicotine can induce desensitization, but the nAChR up-regulation effect is not reached[273].

Brain functioning is susceptible to neuromodulators, where cholinergic neurons are essential in coordinating their release and building brain work microenvironments. The presence of exogenous ligands can easily alter the equilibrium with adverse effects but also opens the possibility of voluntarily modulating brain development. As pointed out in the phrase credited to Paracelsus, "The dose makes the poison." adverse and positive effects in a dose-dependent manner are observed on nicotine administration; the question emerges: Would it be the same for NNIs? Understanding how nAChR influences neural proliferation, differentiation, and survival of neural stem cells opens the door to neuron replacement therapies[174]. It is known that in adulthood, mammals present niches of neurogenesis. However, glial cells, which support roles that direct neural networks, do not integrate new neurons into mature neural circuits[276]. It seems that microenvironments influence that behavior. It increases the interest in identifying NNIs and nACHR interaction mechanisms.

It is established that NNIs use the orthosteric binding site of nAChRs, sharing the binding site of ACh. As checked on the theoretical framework, the extracellular domain of nAChR is composed of the N terminal, followed by an α helix, and ten β strands, which are the extracellular tail of the M1 transmembrane domain and the C-terminal. Furthermore, heteromeric receptors present two orthosteric binding sites, and homomeric receptors have five binding sites. The binding site hydrophobic pocket is formed by three loops of two adjacent nAChR subunits; one of them must be an α subtype due to their structure where

the cysteine pair characteristic of α subunits seems to have an important role in binding. However, as seen before, some α subunits have lost important parts of their structure, which hamper them from being the main, also known as the positive or front face of the binding site. This subunit provides the A, B, and C loops. The other subunit, which can be α or nonα, aport the negative or back face on the binding domain and conforms the loops D, E, and F. Moreover, when two α subunits conform the binding pocket, it gives place to a G-loop formation.

NNIs, classified as agonist competitors of nAChR, have to interact with the binding loops. However, it is not necessary to interact with all of them. In order to have a better understanding of NNIs and nAChR chemical interaction, it is crucial to determine which are the key residues that participate in binding. Being the most used NNI, imidacloprid is used in studies to determine the residues that improve imidacloprid binding, using site-directed mutagenesis, molecular modeling, and also assessing the molecular charges surrounding imidacloprid using ionic liquids interaction technique, in this case, ammonium ion[142].

Electronic charges assessment on imidacloprid molecule shows electron deficiency on the imidazolidine ring, which would promote interaction with electron reach residues presumably present on insect nAChR subtypes[142, 114, 93]. An exciting discovery is that in contrast with cyclical NNIs, noncyclic NNIs form a ring when they bind nAChRs[65].

On the other hand, other experiments determine that the α subunits have residues that enhance NNIs action. It is observed that α-α binding sites interact with nitro or cyano groups of NNIs[142]. In the binding pocket, the front face provided by an α subunit has the loop C. This loop possesses the cysteine pair characteristic of α subunits that form a YXCC motif (tyrosine-amino acids-cysteine-cysteine). In an experiment using chicken $\alpha4\beta2$ nAChR subtype, it was found that exchanging proline residues for glutamate, the anionic form of glutamic acid, residues on the loop C of $\alpha4$ subunit enhance imidacloprid affinity supporting the observation of need reach electron residues. However, a similar experiment on Drosophila Melanogaster reduced the affinity of the $\alpha2$ subtype for imidacloprid[277]. Another study on the avian $\alpha7$ nAChR subtype reveals that Gln79 has a high impact on NNIs agonist effect; exchanging it with Arg enhances activation

response, but exchanging it with Glu reduces the response[278]. It reinforces the idea of complex binding and the importance of the nAChR subtype. Furthermore, mutation on loop D shows resistance development in insects; the exchange of Arg81 for a Thr confers resistance to Myzus persicae[142, 279]. Multiple changes were tested on loop D with similar results with the particularity of an imperceptible alteration of the ACh effect, which highlights the importance of the D loop on imidacloprid affinity[142]. Additionally, modeling experiments using Ls-AChBP as a model determines the binding interaction between Gln55 on loop D with the nitro group of imidacloprid, as well as the interaction between the F-loop and nitro group, but water intervention is needed. Conversely, water molecules facilitate the formation of hydrogen bonds between the pyridine ring of imidacloprid and loop E and the interaction of the imidazolidine ring with Tyr185 loop C. The N-terminus, which is found on the B-loop, also presents interaction with NNIs. However, the residues involved can not be determined[142, 280]. The influence of NNIs does not always lead to nAChR activation, and many times, it can work as a modulator of the agonist activation. It is attractive to consider allosteric binding sites, and precisely, there is an allosteric binding site near the N-terminal, experimentally discovered on $\alpha 7$ nAChR sutype[281].

It is highly established that NNIs act over the orthosteric binding sites; it is accepted and experimentally observed their agonist activity. Despite NNIs being classified as orthosteric nAChR competitors, the possible interaction with allosteric binding sites would not be dismissed. In the previously cited experiment with chicken nAChR subtypes, desnitro imidacloprid metabolite showed a high affinity and full activation of $\alpha 4\beta 2^*$ nAChRs, while imidacloprid whole molecule was unable to activate the same nAChR subtype[256]. However, imidacloprid co-applied with ACh has the power to strengthen the agonist response[257]. The objective of this chapter is to remark on the diversity and complexity of the binding interactions; there are allosteric binding sites that produce conformational changes characterized for modulating the response on channel open-close state. There are other binding sites, such as unorthodox binding sites on heteromeric nAChR present on nAChR with three α subunits. There are also noncanonical binding sites that can not interact with ACh, extracellular Ca+ binding sites, and binding sites not well studied near the C-terminus and N-terminus. At the transmembrane domain, there are intrasubunit trans-

membrane sites and inter-subunit transmembrane sites[282].

Having the panorama of NNIs characterizing their impact looks complex considering the diversity of NNIs structure and nAChR subtypes. Moreover, nAChRs are highly conserved through evolution, being an essential part of the nervous system. Both characteristics imply a well-adapted but also highly regulated mechanism. The inherent complexity of the human brain, which is full of gaps of knowledge, and the necessary continuous activation of nAChR makes it highly difficult to identify toxicity as an effect of NNIs. However, the functional sensitivity of the brain to microenvironment changes, which is mainly modulated by neurotransmitters, and the cascade of events that amplifies successful signals, the impact of NNIs would be evident on neuron cytoskeleton, neurotransmitters equilibrium, the abnormal metabolic activity as a result of NNIs agonist activation would results on reactive oxygen species and nitrogen reactive species that leads a oxidative stress toxicity consequences. NNI oxidative stress inducement was observed in many species and in vitro experiments, which can be one of the mechanisms of NNI toxicity[248].

In that way, it is viable to monitor alterations in the cytoskeleton as an indicator of neuron health and plasticity conservation. Additionally, it would be a good indicator of a healthy microenvironment, where wnt pathways seem a solid indicator being implied on cell viability pathways, differentiation, proliferation, inflammatory response, and neurites outgrowth, therefore in neuroplasticity. Also, it would be relevant to monitor nAChR upregulation and desensitization.

Chapter 4

Methodology

To better understand the neurotoxic effects of neonicotinoids, it is necessary to characterize Acetamiprid and Imidacloprid treatment effects on the SH-SY5Y human neuroblastoma cell line. The western blot technique was used to analyze changes in the protein expression level, protein post-translational modifications, or protein deterioration. The cell homogenates were extracted during the differentiation process when neuroblastoma cells showed higher sensitivity to the treatment of neonicotinoids. In addition, the test aims to determine alterations in metabolic pathways and suggest a mechanism of action that may be extrapolated and studied in vivo experiments.

4.1 Phases of Problem-Solving

4.1.1 Description of the Problem

People worldwide are in continuous contact with neonicotinoid insecticides through contaminated food, water, air, and soil. Exposure is higher in developing countries with high agricultural activity, where weak regulation, poor education, and lack of basic amenities are common. These factors increase the probability of direct and chronic exposure to neonicotinoids.

Neonicotinoids are systemic pesticides whose mode of action, according to IRAC, is classified as a competitive modulator of nAChR[110]. Knowing the high impact of neon-

icotinoids on nontarget species, especially pollinator populations decimating, forces their prohibition or stronger regulation. However, the effects on the nervous system of more complex species are dismissed. Moreover, neonicotinoids are systemic pesticides, and some of them can persist in soil for years, particularly 1 to 8 days for acetamiprid and 40 to 997 days for Imidacloprid[70]. Describing a scenario with systemic pesticides applied by farmers with poor education and weak regulation entities gives rise to farmers applying neonicotinoids at any time and in any amount, which results in commercially available food contaminated with neonicotinoids in markets and supermarkets, affecting the overall population.

Moreover, the claim of high specificity activity of neonicotinoids over insects nAChR, or almost innocuous for vertebrates, downplayed the importance of determining what neonicotinoids make to the human nervous system. Neonicotinoids' interaction with nAChR threatens essential brain functions such as memory creation and consolidation, learning, olfactory sensitivity, mood modulation, and movement control memory. Although neonicotinoids are believed incapable of crossing the BBB, or its effects on mammals or big vertebrates are negligible, in vitro experiments on human neuroblastoma cell lines, in vivo experiments on rodents, and the impact on environment observations demonstrate alteration as a result of neonicotinoid exposure.

This work uses the western blot technique to measure changes in protein expression as an effect of two of the most used neonicotinoids, Acetamiprid and Imidacloprid, over the human neuroblastoma cell line SH-SY5Y during its differentiation process.

4.1.2 Analysis of the Problem

It is essential to characterize the effects of neonicotinoids on the nervous system, even if they cannot penetrate our brain barriers, to increase our knowledge of functions of nAChR that are essential not only for their participation in learning, memory, and neurodevelopment but also for their importance as a therapeutic target for neurological disorders, including neurodegenerative diseases such as Parkinson's and Alzheimer's.

The name neonicotinoid means new nicotine due to its inspiration from nicotine molecules. On the other hand, nicotine presents dose-dependent effects in the human brain, both positive and adverse. Showing protective effects against Parkinsong's disease stimulating dopaminergic neurons that express different subtypes of nAChR, but also can hamper the growth of developing neurons and increased apoptosis rate in the mature nervous system[174].

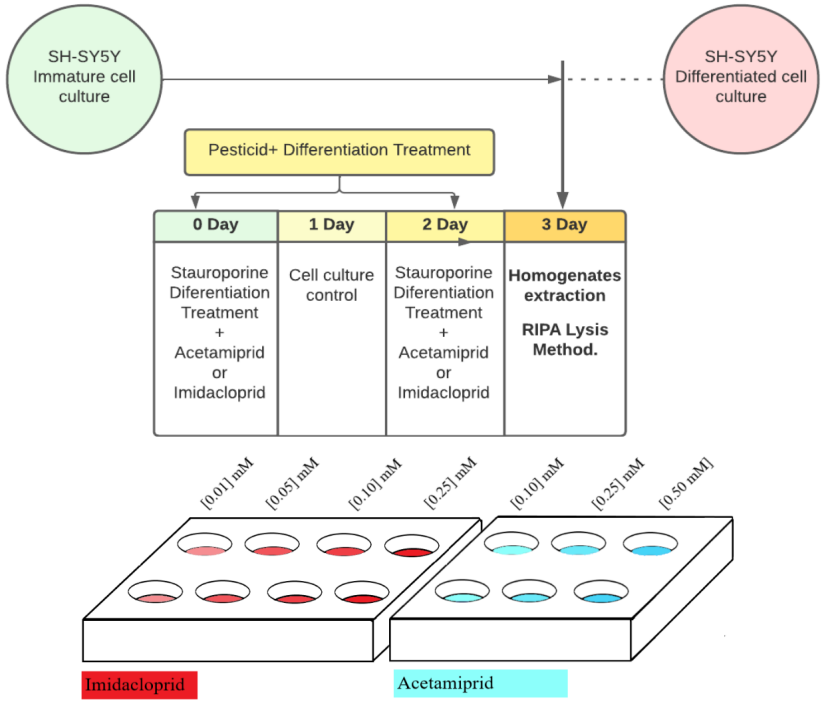
Like nicotine, the interaction of NNIs over nAChRs is described as a competitive agonist modulator. While the human brain counts with a mechanism for breakdown and rapid clearance of nicotine, it is not clear if that mechanism acts over NNIs, which were designed to permanently activate nAChRs on insects, exerting their toxic mechanism in that way. In normal conditions, ACh is degraded by AChE. However, some neonicotinoids can not be deactivated by the Acetylcholinesterase (AChE) action[89]. Even more, some NNIs cause an increment in AChE activity[115, 283, 253], but others inhibit the AChE activity even showing a selective action over it[284]. Again, this shows their effects in a dose-dependent manner. For example, it is reported that acetamiprid has inhibitory effects at 75.2μM and can enhance AChE activity at higher doses and short exposure time[253]. The current medicine to treat Alzheimer's disease is an AChE inhibitor[115]. In the case of permanent stimulation, without nAChR degradation, its influence downstream in different metabolic pathways would be revealed, probably causing nAChR desensitization and upregulation.

On the other hand, experiments in vitro point out the effect dependent on dosage over time that increases dendrite growth and accelerates differentiation at a low dosage and short time but results in loss of dendrite density and death due to oxidative stress at chronic and high dosage exposure. As the phrase attributes to Paracelsus, "The dose makes the poison." It is necessary to characterize the effects of neonicotinoids to dimension the hazard or opportunity they represent. There is a high probability of identifying the NNI's effects monitoring changes on nAChR expression level or cytoskeleton proteins and pathways involved in their maintenance.

4.1.3 Algorithm Design

The study departs from homogenate samples taken of SH-SY5Y cell ongoing differentiation. As described in the **Figure 4.1**, the cell homogenates were extracted with the RIPA lysis methods on the third day after being treated with staurosporine cell differentiation protocol at day 0 and day 2, also adding the respective pesticide treatment for each group of Acetamiprid [0.10] mM, [0.25] mM, [0.50] mM and the respective Imidacloprid treatment [0.01] mM, [0.05] mM, [0.10] mM, [0.25] mM.

Figure 4.1: *Homogenates Extraction*



As described in **Figure 4.2**, the experimental workflow starts with protein selection, which considers the characteristics of the sample, antibody availability, and a bibliography review to identify possible proteins impacted by NNIs toxicity.

In order to reduce the time of western blot, optimization would be algorithmically developed. However, limited sources compel us to skip steps. A system to follow can be as follows. First, to certify the protein sample quality, ensure the proper concentra-

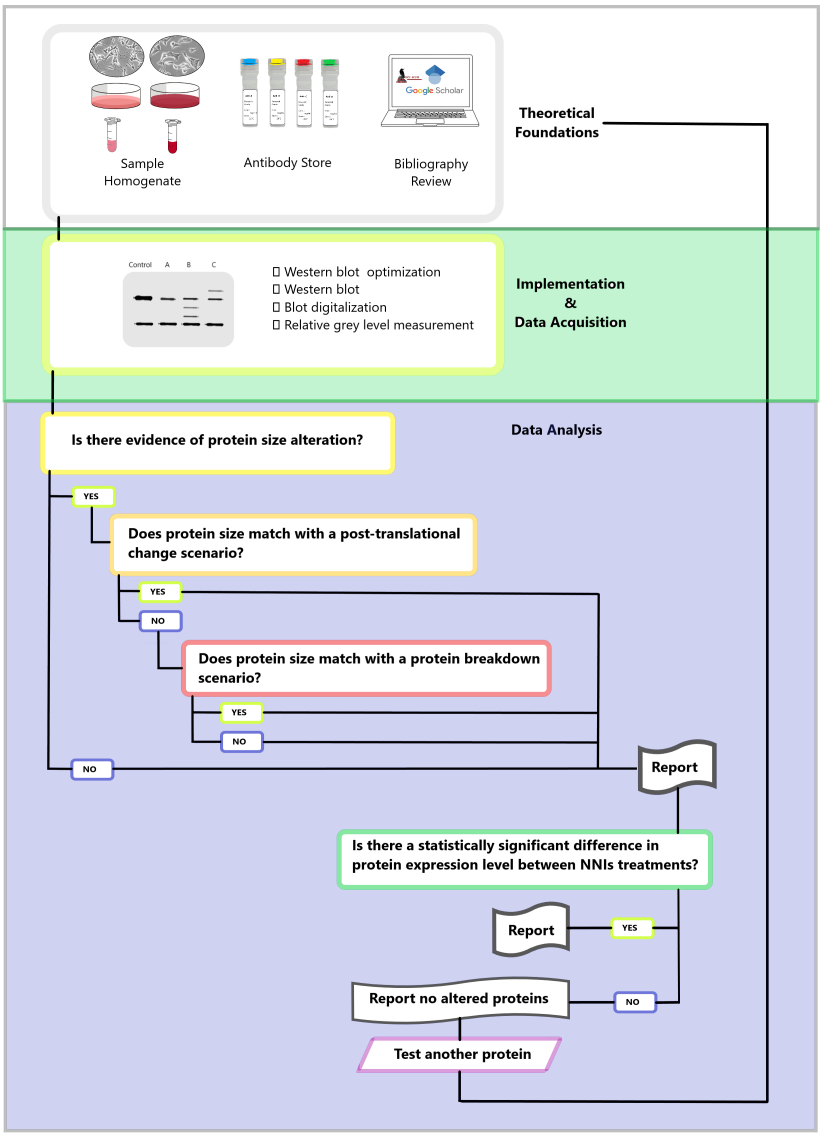
tion, perform electrophoresis and stain protein on the gel to verify that they were not degraded, and optimize running parameters to obtain a proper separation. After that, the protein transference to the membrane is performed and stained to ensure a correct transference. Bigger proteins take more time to transfer properly. Finally, determine the proper antibody for a highly expressed protein, optimize antibody concentration, mode of incubation, number of membrane washing needed, and evaluate the results after blot imaging. It is important to generate reliable and reproducible results, facilitate the identification of issues within the process, and estimate the viability of performing Western Blot.

It is a common practice to perform a reduced repetition of western blot for the protein of interest, between three to five repetitions. It is because of the cost and time required, but also because the western blot is not a quantitative test that rapidly dismisses inferential statistics use. The data resulting after normalization do not adjust to a probability distribution, which compels the use of a non-parametric test, adding a very small sample size, resulting in weak results and the loss of statistical power. Instead, descriptive statistics are used.

Western blot allows us to locate protein size modifications and quantify relative protein expression. Size modification may indicate post-translational changes or protein deterioration as an effect of pesticide treatment. On the other hand, the protein expression level facilitates the detection of dose-dependent effects. Western blot is mainly considered a pseudo-quantitative method, the protein expression level is measured as the light intensity emitted by the respective blot, then normalized to the selected loading control, and relativized to the control sample.

The necessity of inferential statistical analysis is to identify differences between treatments with statistical significance; due to the very small sample size (n=4) and not fill parametric conditions, the nonparametric Kruskal Wallis test with its respective post hoc Dunn's test with the sequential Bonferroni significance adjustment is used.

Figure 4.2: *Algorithm Design*



4.1.4 Implementation

Selection of the Protein of Interest

Before performing a western blot, the proteins to be monitored must be determined based on a bibliography review, antibody availability, and sample characteristics.

The primary mode of action of NNIss is through the agonist activation of nAChRs on insects. In the beginning, several experiments declared the negligible, even no effect of NNIs on the human nervous system. They believed no effect was due to the mam-

mal's detoxification mechanisms, brain barriers, and the low agonist effect over vertebrates nAChR[89]. However, other experiments reveal adverse effects related to synaptic plasticity, proliferation, apoptosis inducement, and oxidative stress toxicity[174].

This research aims to characterize the effects of Imidacloprid and Acetamiprid during the differentiation of the SH-SY5Y human neuroblastoma cell line. On the list of viable antibodies to be used were Anti-Microtubule-Associated Protein 2 (MAP-2), Dopamine D2 receptor, β-catenin, β-actin, Anti-Vesicular Monoamine Transporter 2 (VMAT-2), Anti-Growth Associated Protein-43 GAP-43, all of them were produced on rabbit, for which there was a functional secondary antibody. There was also Anti-Neuronal acetylcholine receptor $\alpha 7$, but it was produced in mice, and there was no Anti-mouse secondary antibody.

However, MAP-2 is more expressed in mature neurons than in the differentiation process neurons. GAP-43 is related to morphological changes during neuronal maturation; alteration of GAP-43 induces neuron apoptosis[285]. VMAT-2 is present on catecholamine vesicular transport, which may be influenced by nAChR activation. However, optimizing the western blot protocol for them was impossible. On the other hand, the Dopamine D2 receptor in in-vitro experiments may require high protein concentration on the sample or a specific differentiation protocol to improve its expression. Also, D2 is highly influenced by nAChR in a functional brain, but in an in-vitro test, there seems to be a lower influence[286]. The anti-$\alpha 7$ nAChR was a prominent candidate because NNIss would directly influence it but can not be used. There were also antibodies for proteins such as MAP-2, VMAT-2, and GAP-43, which are important indicators of neural morphology changes, alteration in homeostasis transmission, synaptic plasticity, and dendrite growth and regeneration.

β-Catenin is indirectly influenced by nAChR activation, being the protagonist of the canonical Wnt pathway, which is involved in receptor transport, neurite growth, neuroplasticity, proliferation, and neural differentiation. Alteration on that pathway has a high probability of being evident in the level of expression of cytoskeleton proteins, adding to the interest in neuroprotection against Parkinson's disease through Wnt pathways; this an-

tibody was selected. Finally, cytoskeleton proteins suffer significant changes during neuron differentiation and can be affected by NNIs; for that reason, they can be good indicators of variations as an effect of NNIs. It should be noted that β-Actin is used as a loading control; however, during neurodevelopment and NNIs effects, it does not work as a loading control because it may suffer alteration as the effect of NNIs.

Suitable Protein Concentration to Perform a Western Blot Analysis

Optimizing the amount of protein loading per well during electrophoresis is necessary. In contrast, a low amount is difficult to detect, and an excess will present a saturated signal, making detecting differences difficult. More importantly, fix the amount to ensure reliability and reproducibility.

Estimating the number of micrograms of proteins that will be added per well also depends on the level of expression of the protein of interest. For highly expressed proteins, it is studied that 1 to 3 μg of lysate per well has the best performance, medium level of expression proteins it is recommended until 10μg per well, and for low expressed proteins may use up to 40μg[287]. Therefore, the Bradford method was used for protein quantification. Considering the recommended loading volume per well for ten wells, 1.0 mm thickness mini gel is 25μl[288].

Table 4.1: *Homogenate Samples*

Treatment	Concentration[µg/ml]	Protein Loaded per well[µg]
Control	786	7.86
I [0.01 mM]	722	7.22
I [0.05 mM]	746	7.46
I [0.10 mM]	645	6.45
I [0.25 mM]	612	6.12
A [0.10 mM]	604	6.04
A [0.25 mM]	520	5.20
A [0.50 mM]	528	5.28

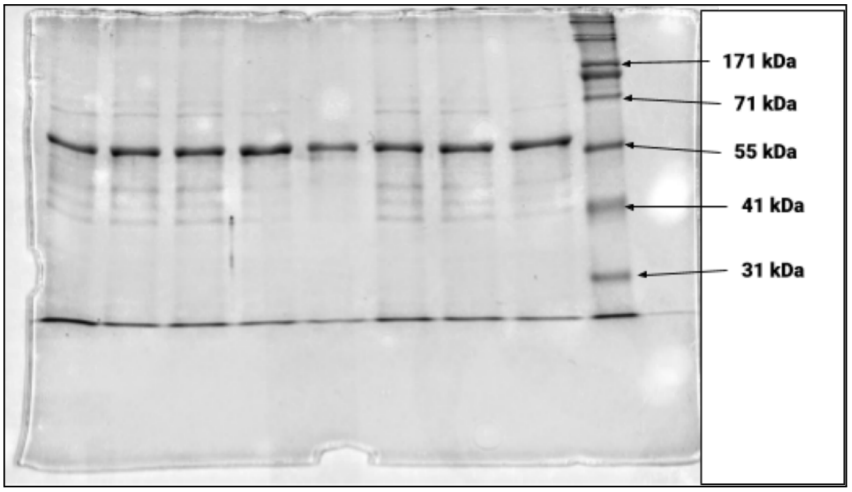
The table presents the initial protein concentration of the extracted homogenates, the total amount of homogenates present on 10µl added per well, considering the addition of the same volume of bromophenol blue as a color marker for electrophoresis, and in the column is present the amount of protein lysate running per line.

Table 4.1 shows that the protein concentration varies between 520μg/ml and 786μg/ml as neonicotinoid dose increases. The final amount of protein per well is between 5.20 μg and 7.86μg, which is suitable for highly expressed proteins such as cytoskeleton proteins but can be low for proteins with lower expression levels, such as MAP-2 on immature neurons, D2 dopamine receptor, or nAChRs.

Protein Integrity Assessment and Electrophoresis Parameters Optimization

Defective sample storage may result in protein degradation, so it is necessary to assess protein integrity. At the same time, electrophoresis parameters would be adjusted to obtain a proper band separation in the range of interest. Running gel electrophoresis and staining proteins with the Coomassie Brilliant Blue is valid for evaluating the proper band separation and protein integrity.

Figure 4.3: *Protein staining with Coomassie Blue R250*



The **Figure 4.3** shows that protein staining with Coomassie Brilliant Blue R250 shows proper protein separation on bands, especially for proteins with a molecular weight below 71kDa. If analyzing proteins with a higher molecular weight is necessary, the voltage can be increased from 150V to 200V while running the resolving gel, and the percentage of gel can be decreased from 10% to 8%.

Loading Control

A comparison without a framework, the function of loading control, does not make sense. The loading control would be a protein uninfluenced by treatments, different in size from the protein of interest, and with a significant expression level. In this case, finding an appropriate loading control was impossible because of antibody availability and the influence of NNIs treatments. In those cases when it is impossible to find a loading control, it is recommended to guarantee the loading of the same amount of protein per well on the polyacrylamide gel during the electrophoresis and supply the required details to be reproducible. However, the probability of experimental error increases, and the results can be easily dismissed.

Protein Transfer to Membrane

After transferring proteins from polyacrylamide gel to the PDVF membrane, staining the proteins with the Ponceau method works as a control point. It helps to ascertain a proper protein transfer, considering that bigger proteins take more time to pass into the membrane. In our case, there were no available Ponceau reagents. Instead, the pre-stained protein ladder was used as a reference.

Figure 4.4: *PDVF Membrane after Protein Transfer*

At the left of the membrane is the pre-stained protein ladder. Proper illumination makes proteins visible on columns when the membrane dries.

Antibody dilution

The antibodies used are Anti-β Catenin antibody($\sim$92kDa) produced in rabbits, whole antiserum from Sigma-Aldrich®at 1:1000 dilution, and Anti-β-Actin($\sim$45kDa), clone RM112 produced in rabbits from Sigma-Aldrich®, where the immunogen was a peptide corresponding to the N-terminus of β-Actin, also was used a dilution of 1:1000.

Chemiluminescence Western Blot Imaging

In the past, x-ray films were the gold standard for western blot imaging, but with the problems of limited dynamic range, needing a long time of exposure for low amounts of protein that results in loss of signal in the noise, or for high expressed proteins the over exposition to signal result in an over-saturation that preclude a quantitative measure of the signal. However, nowadays, digital imaging systems offer similar sensitivity and a higher linear range of detection that allows the performance of a quantitative study. Moreover, the digital system does not need a complete room, films, or dangerous developing reagents; it is faster, cheaper, and less aggressive with the membrane.

However, due to the lack of a digital system, the x-ray film membranes were used, and the chemiluminescence reactive was played for 2 minutes. The film was exposed for 10 minutes and followed the instructions of the x-ray imaging reagents; they were developed for 10 minutes and fixed by others for 10 minutes.

Digitization

For digitization, the smartphone Redmi 9C camera application was used, with the parameters set for light metering mode, matrix metering, ISO 100, and shutter speed priority 1/1000. A panel light of 3000K was used as the light source.

Data processing

For data processing, the free software used for image processing and analysis was ImageJ 64-bit Java 8. With this tool, the brightness, contrast, and image orientation were adjusted

for a proper mean grey value measurement without altering the relative grey level of each blot.

Relative Protein Quantification

To summarize the process, the result is the protein concentration as a measurement of the mean grey value of the areas of bands marked on x-ray films by light emitted by the reaction of the chemiluminescent substrate and the enzyme-conjugated with the secondary antibody. The resultant mean gray value is normalized to the loading control of each line and expressed as a ratio of the control.

$$(A/L_A)/(C/L_C) = Q \tag{4.1}$$

Where A is the grey value corresponding to the protein of interest.
C is the grey value corresponding to the control.
L_A, the grey value corresponding to the loading control of the protein of interest.
L_C is the grey value corresponding to the loading control of the protein of control.
Q is the relative amount of the protein of interest. Expressed as the ratio of the control after being normalized to the protein of interest.

This technique is considered pseudo-quantitative because the result represents the proportion of protein expression to control and not the amount of protein. The imager needs to measure the light emitted linearly to express a proportional relationship, and it is not reached with X-ray films as it is with digital systems.

4.1.5 Experimental Setup

4.1.6 Testing

Many problems related to limited laboratory sources were encountered during implementation, which affected the strength of the results. The biggest issue is the need for protein

loading control, which provides a reference framework for comparing the relative grey value of each blot. However, there was no suitable candidate for loading control between the limited antibodies available, especially without a functional secondary anti-mouse antibody. It is expected that nucleus proteins were not highly affected by NNIs treatment. A candidate for loading control would be the histone H3.

The second biggest problem is the lack of an appropriate imaging system. Without a digital imager, the X-ray film system was used. The problem worsens because the darkroom is a bathroom located at least 200 m from the laboratory. It is inevitable to arrive there without the risk of exposing the western blot membrane, photosensitive substrate, and reagents to light, dust, and other pollutants. Moreover, the lack of a functional chemiluminescent HRP substrate forced the use of an eight-year-expired substrate that, with the minimum movement, interrupts the chemiluminescent reaction. It precludes the direct exposition of the x-ray film to the membrane, making it necessary to place an acetate film to reduce the contact with the HRP substrate on the membrane.

Additionally, the hydrophobic nature of PVDF membranes requires accurate time management for each washing process, blocking, incubation, and imaging because if the membrane gets dry, the following applied solution will not perform a homogenous contact. It is necessary to emphasize that the membrane had to be transported from one laboratory to another due to the lack of an orbital shaker necessary for the process listed before and the 200 m needed to develop the imaging process. It repeatedly altered the time the membrane was immersed in the different reagents.

The third most significant problem was the laboratory availability. To perform a western blot from gel preparation, electrophoresis with mini-Protean tetra cell of BIO-RAD, the transfer to the membrane with the mini transfer module for the mini-Protean tetra cell of BIO-RAD, until primary antibody incubation, and the laboratory limitations require at least 6 hours of laboratory disponibility being performed two western blot per week at best.

Other problems were registered, such as the contaminated pH-meter calibration solu-

tions, considering the importance of the pH of buffer solutions during electrophoresis to ensure a homogeneous displacement of proteins. The alteration in pH leads to faster heating of electrophoresis buffer and gel, where proteins on the warmer region of the gel will run faster. Resulting in a smile effect or burning the gel near the electrodes. There were also storage problems with homogenates, antibodies, HRP chemiluminescent substrate, ladder, and Laemmli buffer, which has a recommended storage temperature that can not be respected. Due to the limited space, some were not correctly stored for months, reducing their efficacy. Also, some of them were expired. The recommended antibody incubation system, overnight antibody incubation, is performed at 4℃ and shaken at 60 rpm. Unfortunately, there is no machine or tool to do it. In some cases, it leads to low-quality antibody binding or unspecific binding; it is evident after blot imaging as a lack of signal or a blotched background.

On the other hand, the preliminary homogenates protein concentration may need to be increased to detect low-expressed proteins. With all those obstacles, naked eyes evidenced differences between control and imidacloprid or acetamiprid treatments for membrane proteins such as β-actin.

4.2 Analysis Method

The first step in characterizing the neurodevelopmental toxicity of neonicotinoids is to identify whether there are statistically significant differences in protein expression between control and NNIs treatments. In that way, a comparison between treatments was carried out, where the control corresponded to a 0 mM NNIs dose. That allows us to infer the protein prejudices during neurodevelopment as an effect of the dose-dependent manner of Imidacloprid and Acetamiprid.

The nature of the western blot, not being a quantitative test, makes it challenging to use inferential statistics. Moreover, to perform the "quantitative western blot," control over variables, especially the parameters involved in imaging and digitization must be ensured. As mentioned before, it is necessary from the measures in the linear dynamic

range; in another way, the proportions do not represent a dimensional relationship, turning the variable into an ordinal variable more than a numerical one. It is evident in the quantification of the control protein, where the measured grey value will always result in 1 after being normalized to the loading control and its posterior standardization to be expressed as a proportion of itself. The data resulting after normalization do not adjust to a probability distribution, which compels the use of a non-parametric test. In addition, a very small sample size precludes meeting the requirements of almost any statistical test to compare groups.

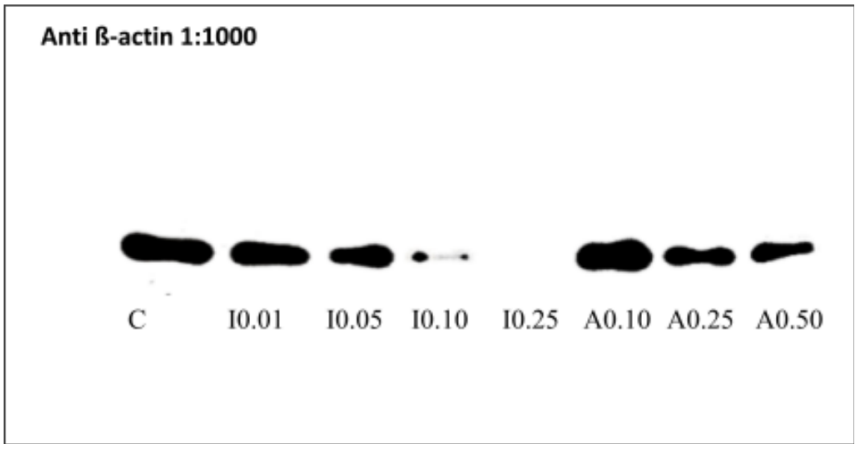
However, the necessity to identify differences between treatments with statistical significance; due to the very small sample size (n=4) and not fill parametric conditions, the nonparametric Kruskal-Wallis test with its respective post hoc Dunn's test with the sequential Bonferroni significance adjustment is used. Kruskal-Wallis test needs a sample size of at least 5, a condition which is not fulfilled, producing weak results and the loss of statistical power. Unless inferential statistics are requested, descriptive statistics are used for western blot.

Chapter 5

Results

The **Figure 5.1** shows the result of digitization after contrast and orientation parameters adjustment. For all the blots, the sample arrangement was Control, I[0.01]mM, I[0.05]mM, I[0.10]mM, I[0.25]mM, A[0.10]mM, A[0.25]mM, and A[0.50]mM. In the case of β-actin, the Control sample mainly presented the highest values, and the second highest was for A[0.10]mM. To the naked eye, it is observed that imidacloprid treatments produce a higher fall of β-actin than acetamiprid at the same doses. Moreover, for the I[0.25]mM, β-actin could not be detected if there is any.

Figure 5.1: *Blot of β-Actin Protein Analysis*

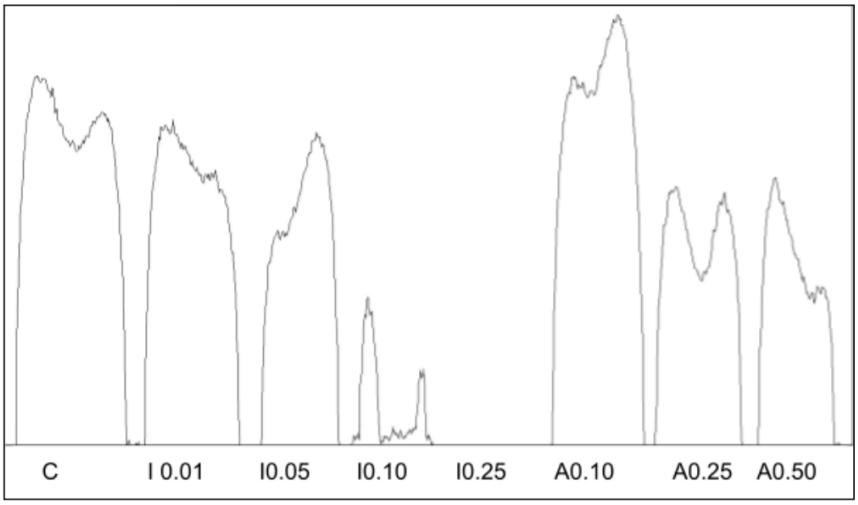


The figure presents one of the four blots analysis of ß-Actin protein for the control, Acetamiprid, and Imidacloprid treatments. For the analysis, the primary antibody concentration was 1:1000, the higher concentration recommended by the manufacturer.

In the **Figure 5.2**, the histogram curve represents the mean greyscale of each band

on the western blot; this area is computed, and the resultant value represents the amount of studied protein. In this case, it is the ß-actin protein. The area value has arbitrary units, so it is necessary to establish a reference framework to compare them. A frequent practice is to express the area of the band of interest as a ratio of the area associated with the Control band. The data of each of the four repetitions are compiled in **Table 5.1** for imidacloprid treatments and **Table 5.2** for acetamiprid treatments.

Figure 5.2: *Histogram of the grey scale values of the bands of β-Actin Blot*



Each curve represents each band's mean greyscale, and the curve's area represents the sum of the mean grey value of the whole band area. For that reason, the area is used to quantify the protein expression.

As mentioned and evidenced in **Table 5.1** and **Table 5.2**, imidacloprid treatments have a higher impact on β-actin with a strong descending tendency and lower standard deviation. The effect of acetamiprid is different, with a no market decrease of β-actin for the two lower doses and a higher standard deviation than imidacloprid treatments.

Table 5.1: *β-Actin Relative Concentration for Imidacloprid Treatments*

# Blot	Control	I [0.01 mM]	I [0.5 mM]	I [0.10 mM]	I [0.25 mM]
1	1.00	0.91	0.74	0.65	0.00
2	1.00	0.77	0.50	0.18	0.00
3	1.00	0.73	0.53	0.09	0.00
4	1.00	0.74	0.67	0.32	0.00
Mean	1.00	0.79	0.61	0.31	0.00
SD	0.00	0.07	0.10	0.21	0.00

Table 5.2: *β-Actin Relative Concentration for Acetamiprid Treatments*

# Blot	Control	A [0.10 mM]	A [0.25 mM]	A [0.50 mM]
1	1.00	2.01	2.14	1.24
2	1.00	0.44	0.13	0.01
3	1.00	0.93	0.52	0.41
4	1.00	0.51	0.53	0.34
Mean	1.00	0.97	0.83	0.50
SD	0.00	0.63	0.77	0.45

It was observed in the **Table 4.1** that protein concentration on homogenates decreases as the NNI dose increases; the ß-actin reduction can be attributed to a general protein affection and not a specific alteration of cytoskeleton proteins. The **Figure 5.3** shows the bar plot representing the relative protein concentration of samples beside the bar plot of relative β-actin amount for the respective NNI treatment; the relative protein concentration is obtained by dividing the homogenate concentration of each treatment by the concentration of the control homogenate, and the β-actin protein amount is computed from the western blot. The figure also shows that for imidacloprid treatments, a drastic decrease of β-actin is inversely proportional to imidacloprid dose, an effect that is unclear for relative homogenates protein concentration. Moreover, the relative β-actin amount is always lower than the relative homogenates protein concentration. On the other hand, even if the reduction of both relative β-actin amount and relative homogenates protein concentration is conserved, the effect on acetamiprid treatments has two particularities. First, the relative β-actin amount is higher than the relative homogenates protein concentration for the two lower acetamiprid doses. Second, acetamiprid treatments present a lower relative homogenates protein concentration than imidacloprid treatments, and the reduction is less market.

In the **Figure5.4**, the box plot shows how the mean of β-actin decreases as NNI treatment increases. Despite the dispersed values, fifty percent of the values of every treatment do not intersect. Also, the values are more dispersed for the I [0.10]mM treatment, and there is no detected β-actin for the I [0.25]mM treatment.

It is helpful to determine a difference in the NNI effect in a dose-dependent manner; it may allow finding the threshold dose to trigger an effect over a specific protein that may

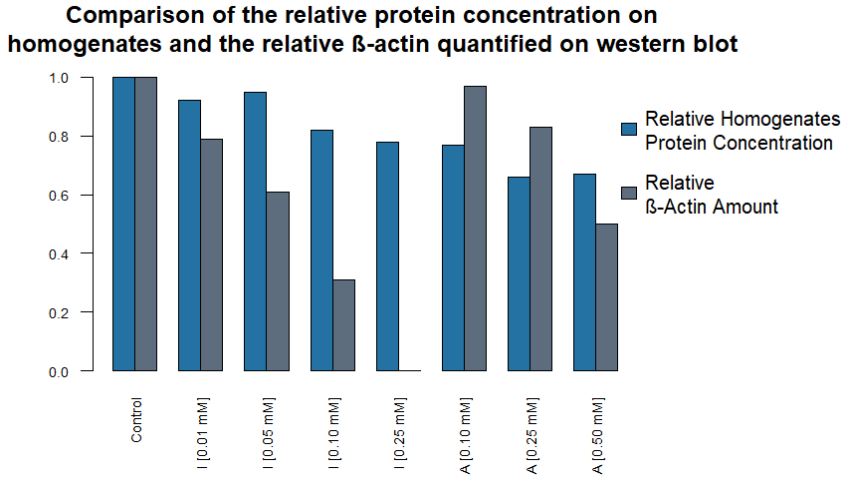


Figure 5.3: *Comparison of Protein Concentration and Amount of β-Actin*

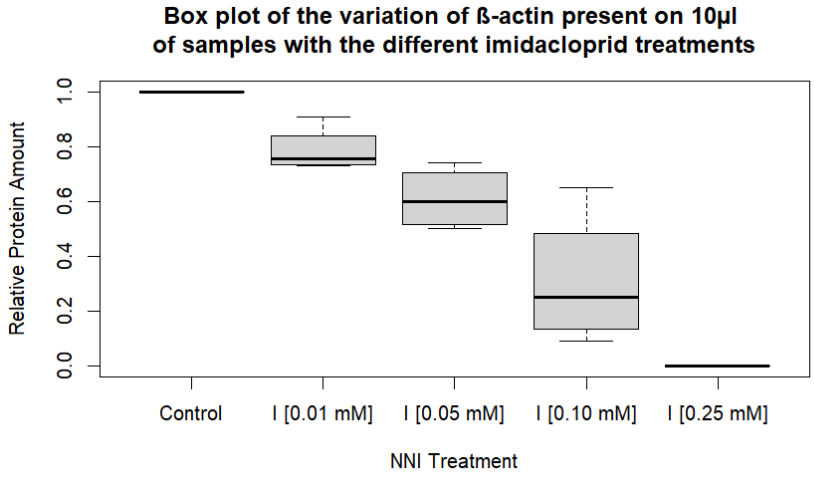


Figure 5.4: *Blox Plot of β-Actin on Imidacloprid Treatments*

draw the landscape of the NNI toxic mechanism. In the specific case of β-actin, a reduction is observed as an effect of NNI treatments, but other proteins may increase its expression, as is observed with β-catenin in the **Figure 5.6b**. The dose-dependent response of different proteins gives clues to the mechanism of action of NNIs. Due to the data characteristics, it is appropriate to perform ANOVA; it does not meet normality distribution or similarity variance treatments. Despite ANOVA robustness when there is no normal distribution, and with the Welch correction applied when the sample presents variance heterogeneity, there is nothing to do with the very small sample size.

On the other hand, Kruskal-Wallis is the nonparametric test for ANOVA; it is assumed that the data of each treatment has a similar shape distribution, the observations are independent, the dependent variable can be represented as groups, and the dependent variable is an ordinal or continuous scale variable. This nonparametric test allows measuring if there is a significant difference in the distribution between groups based on the median parameter. In general, nonparametric tests show a lower statistical power than parametric ones. The power dramatically decreases when conditions are not met; in this case, the minimum sample size per treatment required is five.

Moreover, it is not recommended to use inferential statistics for western blot due to the nature of the data, which has a low sample size. Nevertheless, the Kurskall-Wallis test was used to estimate if there is a statistically significant difference in β-actin protein amount as an effect of NNI treatment.

In the case of imidacloprid, the Kruskal-Wallis test results on the rejection of the null hypothesis, with a statistically significant difference between treatments where the critical value for a significance level of $alpha$: 0.01 is $H = 11.070$, and the computed is $H = 17.91, p = 0.001$.

The test was followed by the post-hook Dunn's test with the sequential Bonferroni significance level correction to counteract the family-wise error. As shown in the **Table 5.3**, there is not a statistically significant difference between the Control and I[0.01]mM treatment, nor between I[0.01]mM, I[0.05]mM, and I[0.10]mM treatments, nor I[0.05]mM, I[0.10]mM, and I[0.25]mM treatments. However, there is a statistically significant difference between the Control and I[0.05]mM, I[0.10]mM, and I[0.25]mM treatments, and also between I[0.01] mM and I[0.25]mM.

In the **Figure 5.5**, the box plot shows how the mean of β-actin decreases as acetamiprid treatment increases. Fifty percent of the values of every treatment intersect, making it difficult to find a statistical difference between treatments. The effect of acetamiprid over cytoskeleton proteins seems more subtle than imidacloprid. Also, the values are more

Table 5.3: *Dunn's post hook for Imidacloprid Treatments*

	Control	I [0.01 mM]	I [0.5 mM]	I [0.10 mM]	I [0.25 mM]
Control		0.306	0.047	0.006	0.000
I [0.01 mM]	0.306		0.335	0.081	0.005
I [0.05 mM]	0.047	0.335		0.434	0.062
I [0.10 mM]	0.006	0.081	0.434		0.278
I [0.25 mM]	0.000	0.005	0.062	0.278	

dispersed compared with imidacloprid results.

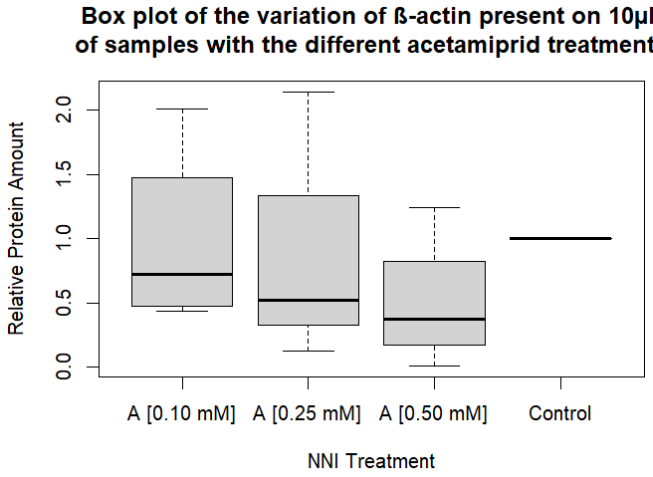


Figure 5.5: *Blox Plot of β-Actin on Acetamiprid Treatments*

In the case of acetamiprid, on the Kruskal-Wallis test, it was impossible to reject the null hypothesis, interpreted as there is not enough evidence of difference in β-actin amount as an effect of acetamiprid treatments. The critical value for a significance level of $alpha : 0.05$ is $H = 8.333$, and the computed is $H = 3.246, p = 0.355$.

No differences were found between treatments of acetamiprid, so the post-hoc Dunn's test was performed to observe the magnitude of acetamiprid effects. As shown in the **Table 5.4**, none treatment presented a statistically significant difference, but the highest dose, p:0.07, gives an idea that the significance level can be reached with a more controlled procedure.

The **Figure 5.6b** shows the effect of the different doses of imidacloprid and acetamiprid over β-catenin amount. Finding the increment of catenin expression for every NNI treat-

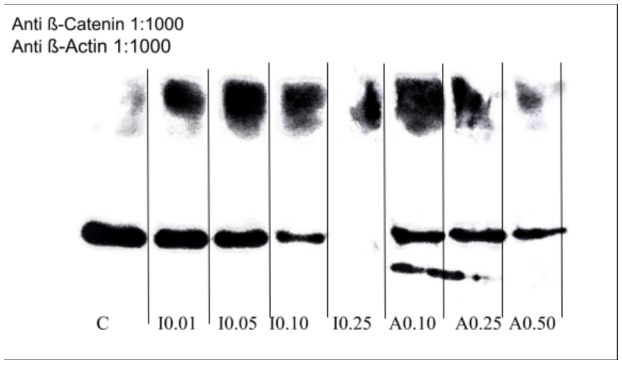
Table 5.4: *Dunn's post hook for Acetamiprid Treatments*

	Control	**A [0.10 mM]**	**A [0.25 mM]**	**A [0.50 mM]**
Control		0.411	0.331	0.073
A [0.10 mM]	0.411		0.881	0.331
A [0.25 mM]	0.331	0.881		0.411
A [0.50 mM]	0.073	0.331	0.411	

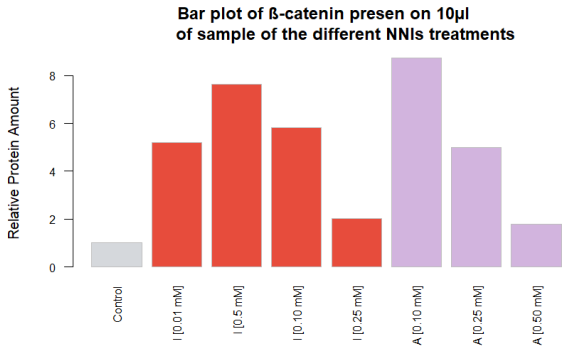
ment even reaches 8.74 folds β-catenin amount of control for the A[0.10]mM treatment. The **Figure 5.6a** shows the one repetition performed of a western blot for β-catenin protein. It would be said that the resultant x-ray film presented a high contrast and, after image processing, may result in loss of the signal.

Figure 5.6: *β-Catenin Blot Analysis*

(a)



(b)



a)Presents the blot analysis of ß-Actin [1:1000] and ß-Catenin [1:1000] for the control, acetamiprid, and imidacloprid treatments. b)Presents the bar Chart of β-Catenin amount for each treatment.

Chapter 6

Discussion

Here, there is an extensive topic, the toxicity effect of pesticides that agonist binding nAChR. The subject needs to be carefully addressed. For the experimentation, we used the SH-SY5Y human neuroblastoma cell line, which is an established in vitro model for assessing neurotoxicity. The cell culture use stauroporine protocol to induce cell differentiation; simultaneously, samples were treated with imidacloprid and acetamiprid at different concentrations. Finally, homogenated samples were extracted on the third day during the differentiation process. The protein concentration on the homogenates shows a reduction with the increment of NNI treatment, but it is not strongly flagged.

In order to evaluate the reported neurotoxic effects of NNIs on the bibliography and find a key alteration in neuron functioning that allows tracking the mechanism of action, the necessity to screen protein expression anomalies was established. For that job, the western blot technique was used. The study had to start from the basis, considering the roles of nAChR and the known effects of similar reagents such as nicotine, and the reported alterations of NNIs such as neurite outgrowth inhibition, the dichotomy effect of proliferation promotion or inhibition[289], also considering calcium flux inducement, and others nicotinic like effects that suggest a dysregulation nAChR expression levels.

It was necessary to simplify the landscape to nAChR overstimulation depending on the subtype, and considering the most studied subtypes, which may lead to neurotransmitter release alterations such is the case of $\alpha 4\beta 2$[168], or neuron functioning, mainly related to

structure impact $\alpha 7$[137, 201, 202, 203]. Also, considering that, like with nicotine, the promising effects are evident at low doses, nAChR expression probably would be upregulated at the time of persistent desensitization for NNI treatment, the unusual binding. In normal conditions, AChE is commissioned to degrade ACh, which results in temporary functional nAChR desensitization, but with NNIs, the scenario is more complex. It is reported that AChE can not degrade them[89]. In other cases, some NNIs cause an increment in AChE activity[115, 253, 283]. Even more, others inhibit the AChE activity, even showing a selective action over it[284]. Their effect shows a dose-dependent character. It would be considered that the current medicine to treat Alzheimer's disease is an AChE inhibitor[115]. For clarity of understanding, it is summarized as detecting alteration in nAChR expression, proteins downstream related to nAChR activation, and cytoskeleton protein alterations where the downstream cascade of events would amplify the effects.

Due to the limited sources and time, only β-actin, a cytoskeleton protein, and a glimpse of β-catenin alteration were monitored. The results of western blot analysis show aggressive effects over β-actin expression, and as generally reported, imidacloprid induces a higher impact, with statistically significant dose influence. The effect of acetamiprid does not have statistical significance at any studied dose, but experimentation conditions can be responsible for the lack of significance. The highest dose, A[0.50]mM, promises to reach a statistically significant impact over β-actin expression.

Acetamiprid shows a different activity than imidacloprid. Acetamiprid is a non-cyclical NNI, and it is reported that non-cyclical NNIs tend to complete their ring through binding nAChRs structure. Imidacloprid, being a ring NNI, is reported to have higher insecticidal activity than acetamiprid, but the mild effect can be more dangerous, especially for chronic effects. While overstimulation on a strongly regulated and, through evolution, a highly conserved mechanism must have a mechanism to control it, such as desensitization, a mild effect promises a more complex response. Moreover, it should be noted that acetamiprid effects over cytoskeleton β-actin protein present higher variability, evidenced by the dispersal of its measurements.

Another case is the β-catenin. It is clear that data obtained from β-catenin is not reliable. That is because high saturation and the noise presented on the only western blot performed for β-catenin alters the measurement and produces misleading results. Even so, there is a clear increment of β-catenin expression with important implications for neurodevelopment, neuroplasticity, and oxidative stress. It is suggested that the involvement of Wnt pathways on β-catenin alterations, with important neuron functioning implications, opens the door to an extensive research field that will be aborded superficially later.

6.1 Previous Studies

Differentiation cells were used because previous studies reported no acute cytotoxicity effects of acetamiprid and imidacloprid for SH-SY5Y nondifferentiated and differentiated cells, even at concentrations as high as 5 mM[289]. In chronic exposure, for acetamiprid, a statistically significant negative effect on cell viability of nondifferentiated cells was observed, and for imidacloprid after differentiation. Also, mitochondrial activity reduction was reported for acetamiprid and imidacloprid. On the other hand, a chronic treatment during differentiation presents a reduction in cell viability in a dose-dependent manner for Imidacloprid treatments and neurite branch retraction for acetamiprid and imidacloprid[289]. Oxidative stress is extensively reported for chronic exposure to NNIs, and it is suggested to be responsible for the loss of neurites. The most interesting effect was imidacloprid proliferation inducement at low doses with statistical significance, but acetamiprid did not reach the statistical significance.

The whole study corroborates that neuronal differentiation varies cellular susceptibility to NNIs. Another relevant report was the drastic increment in neurite retraction at the end of the differentiation process, cells becoming sphere shapes with no neurites, and the positive correlation between NNI treatment and ROS production before killing the cells[289]. The energetic alteration as an effect of NNIs may lead to a proliferation inhibition[290].

6.2 Inferring Mechanism of Action

It is relevant to recapitulate the NNI scenario. They were intentionally modified for specific binding insect receptors, claiming to be almost innocuous for vertebrates despite being based on nicotine molecules[62]. However, over the years, several experiments have provided evidence of NNIs' effects on vertebrates and their effect on mammals that were believed immune to NNIs for having detoxification mechanisms and barriers that hinder NNIs from reaching the central nervous system.

From now on, the scenario will be built to support a possible pathway for NNIs activation. For that, departing from some key facts mentioned before is necessary. First, the higher susceptibility to NNIs during neurodevelopment was mentioned. Second, the results of this study present a downstream effect of the cytoskeleton β-actin protein alteration and the flagged β-catenin up-regulation. Third, the ability of NNIs to modulate calcium ion currents, presumably by α7 nACh receptors interaction[291, 292], influences neurodevelopment[293, 294].

Considering the higher susceptibility of the nervous system to exogenous toxins occurs during neuron differentiation. In humans, the brain is formed mostly around six years, and the neuron differentiation process occurs mainly in the early stages of neurodevelopment. The adult brain has two recognized active neurogenesis zones: the subgranular zone SGZ in the hippocampus and the subventricular zone SvZ in the forebrain[295, 296, 297]. The menace of NNIs is framed for the reported capacity of NNIs to pass through the human placenta[10] and the BBB[68, 99, 100, 101, 102], adding the ensured low-dose chronic exposure to NNIs due to contaminated food and water that affect to the overall population.

Moreover, to recall the nAChR relevance, the nAChR signaling downstream is implicated in processes such as neurodevelopment, neurogenesis, memory formation and consolidation, neuroprotective effects due to its stimulatory functions, participation in synaptogenesis[298], also related to learning, olfactory sensitivity, mood modulation, and motor memory[297]. On the other hand, dysregulation of the nAChR signaling is related to

diseases such as schizophrenia during neurodevelopment, Alzheimer's disease, and Parkinson's Disease[299, 300, 262]. To be more specific, the subtypes $\alpha 7$ and $\alpha 4\beta 2$ are implied in neurotransmission regulation and the effects downstream in cell signalings, such as the regulation of monoamine neurotransmitter release with significant influence on cell viability and morphological changes, since neurites outgrowth to vesicle traffiking[301].

Second, the β-catenin dysregulation. It would be clarified that β-catenin results show an excessive alteration, probably due to the experimentation conditions. However, it caught the attention and invoked the relevance of β-catenin as a master regulator on the canonical Wnt pathway that shares similar functions to nAChR. They are also widely distributed in the animal kingdom and remain active throughout life. Basically, Wnt ligands are secreted lipid-modified glycoprotein that binds to cell surface receptor-ligand Frizzled FZ, triggering a metabolic response.

For our study, the relevance of Wnt pathways lies in their morphogen characteristics mediating neuronal circuit formation. Wnt pathways are the right-hand man of nAChR[302], responsible for exerting many functions attributed to nAChR. To sum up, Wnt pathways are involved in cell-cell signaling, synaptic transmission, vesicle transport, and localization along the axon[303], coordinate the neuron cytoskeleton structure during neurodevelopment[304, 305], neuron differentiation and survival[302, 306, 304], synaptogenesis[307], and dendrite conformation[308]. Moreover, Wnt during embryonic development regulates cell fate in the blastula stage, also orchestrates morphogenetic movement during gastrulation, and extensively participates in neurodevelopment since neural tube formation, where it impulses the proliferation of neural progenitors pool[297].

On the one hand, NNIs present a higher effect on neurons during development. On the other hand, Wnt pathways are involved in the tropic properties of stem cells and the maintenance of neuronal stem cells[309, 310]. Wnt pathways are extensively complex. For understanding, there are at least nine other noncanonical Wnt pathways. Moreover, for example, nineteen Wnt ligands and ten Fz receptors have been reported in mice. Wnt pathways exhibit overlapping in their function and frequently antagonist effect [309, 311, 312, 313, 314].

Wnt/β-catenin is the canonical pathway and the most studied, which is evident in the influence of β-catenin for cellular development; however, among the noncanonical pathways, calcium ions signaling stands out due to its crucial intervention in neurodevelopment, cancer, inflammatory response, and signaling in the nervous system[309].

Moreover, it is stated that calcium signaling is the central signal mediator during early neurodevelopment, enhancing the relevance of noncanonical pathways[315]. Calcium ions, through the noncanonical signaling pathways, are involved in axon pathfinding[316]; neurite outgrowth depends on Ca2+/calmodulin-dependent protein kinase II (CaMKII), whose expression is mediated by calcium gradient[317]. It is important to recall that calcium ion concentration can be modulated by nAChR, which can mediate cytoplasmic calcium influx through the activation of nAChR permeable to calcium subtypes and also participate in voltage-dependent calcium channel activation that influences calcium-induced calcium release from the Endoplasmic Reticulum (RE)[318]. Furthermore, calcium influx peak response is between the reported effects of NNIs. A study on rat cerebellar neurons shows that a calcium influx peak response presents a switch characteristic; if the threshold is reached, the calcium influx occurs; otherwise, there is no response[104, 319].

The postulated pathway is complex, with many participants, and covers a range of functions that overlap, and without testing effects on each stage, it seems only a complex illusion. However, it is a promising illusion.

For example, if it follows the pathway of activation of Wnt5a/Ror, it results in Siah2 and calpain expression. While Siah2 down-regulates the level of β-catenin, the activation of calpain, which depends on calcium ions, leads to the degradation of cytoskeleton proteins such as filamin and spectrin.

At this point, NNIs trigger calcium peak responses, which have important implications, such as calmodulin activation, which in turn modulates the activity of calpain. In deeper review, calmodulin regulates filamentous actin-binding with filamin. Filamin is a non-muscle actin filament crosslinking protein, encharged, among other things, for the fiber conforma-

tion of F-actin. F-actin can be conformed between others by β-actin isoform. Moreover, F-actin is a key cytoskeletal component of dendritic filopodia and spines[320]. In humans, no expression or overexpression of filamin-a FLNa impairs neuronal migration within the cerebral cortex[321, 322], where presumably F-actin alterations are involved[320]. In addition, it would be considered that CaMKII activity works on synaptic modifications that are necessary for memory formation, neurotransmitter synthesis, neurotransmitter release, modulation of ion channel activity, cellular transport, cell morphology, neurite extension, and, in turn, synaptic plasticity, learning, and memory[323].

Considering that wnt5a activation is through the Ror pathway, downstream effects result in calcium-dependent degradation of filamin. This pathway matches a possible down-regulation of β-actin as an effect of the NNIs calcium peak effect.

6.3 Wnt and nAChR in Neuroprotection

Recalling the relevance of characterizing the NNIs effects, it is not only by the toxicity induced but also by possible selective interactions to nAChR subtypes, which are studied as therapeutical targets to treat Alzheimer's disease, Parkinson's disease, pain modulation, and other neurological disorders.

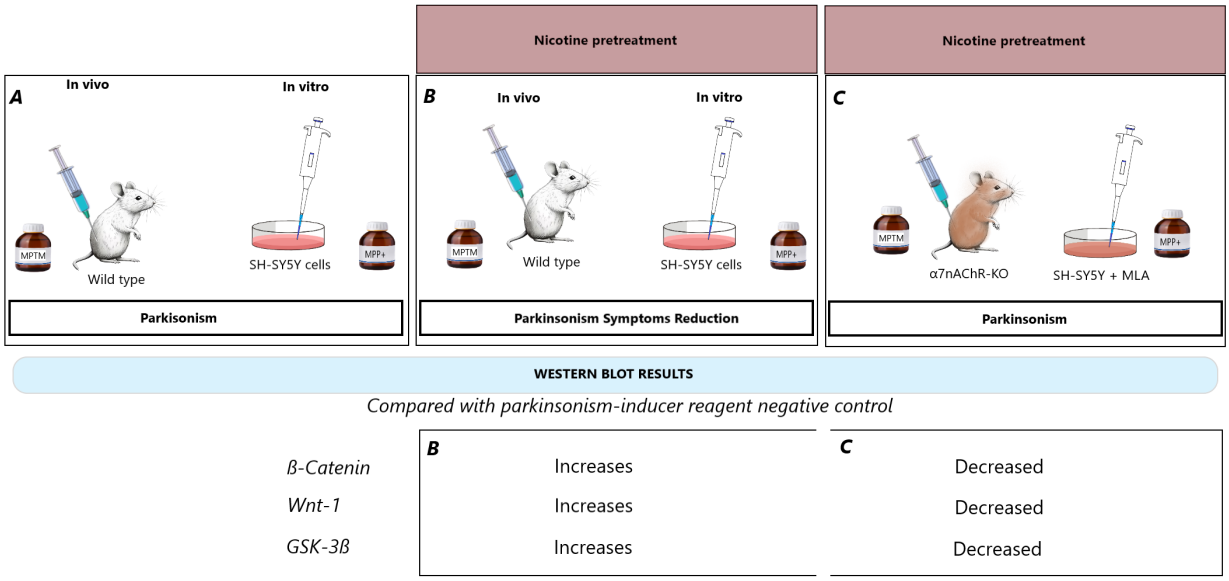
The most accepted interaction between nAChR and Wnt pathways is the receptor transportation function[302]. For example, Wnt7a, which is a ligand of the Wnt pathways, is responsible for the location of α7 nAChR[309, 324]. There is also evidence of Wnt-pathways participation in nAChR distribution in hippocampus neurons[324]. Nevertheless, there are other interactions implied on neuron protection that are less studied, and their mechanisms persist elusive[325], with relevant characteristics to face neurodegenerative diseases due to showing features to contrast chronic effects with relatively low progression[302].

As evidence of that fact, Wnt pathways and nAChR were tested for neuroprotection of DA neurons in vivo in a mouse model and in vitro with SH-SY5Y cells. First, they were

treated with MPTM and MPP+, respectively, reagents characterized and widely used to induce Parkinson's symptoms in dopaminergic neurons as a positive control group. On the other hand, before Parkinsonims inducement reagents application, nAChRs were activated through 10μM of nicotine pretreatment, which shows a significant reduction of the symptoms. However, when the experiments were treated with α7-siRNA to inhibit α7-nAChR expression for in vivo experiment and with MLA, which is recognized as a strong-selective antagonist of α7-nAChR, for SHSY5Y cell culture, the protective effect disappeared. Furthermore, when for the same experiment, a pretreatment with nicotine is performed, resulting in reestablishing the neuroprotection effects. A western blot analysis was carried out, revealing that β-catenin and other proteins that are important in the Wnt pathway, such as GSK-3β and wnt1 ligand, significantly increase their levels of expression and decrease by half concerning the control for the treatment of α7-siRNA, or MPTM in vivo and in vitro respectively. However, the pretreatment of nicotine almost completely reestablished the protein expression level[325].The experiment described can be better understood with the **Figure 6.1**.

Deeper studies also report indirect cooperation of the Wnt canonical pathway and nAChR activation in a functional brain, which results in neuroprotective effects over dopaminergic neurons in Substantia nigra pars compacta SNpc[325]. The Wnt canonical pathway is known to promote DA neuron proliferation[325]. The mechanism is entirely unknown and shows subtle effects. however, in vitro experiments reveal that the wnt5 ligand, a subtype of Wnt ligands, reduces DA neuron's progenitor proliferation, neurogenesis, and dendrite density, increasing differentiation and impulsing morphological changes[326, 327]. Furthermore, Wnt5a is also implied in cytoskeleton formation and the induction of calcium/calmodulin-dependent protein kinase[328].

Figure 6.1: *Protection effects of nicotine through Wnt/β-Catenin pathway*



	B	C
ß-Catenin	Increases	Decreased
Wnt-1	Increases	Decreased
GSK-3ß	Increases	Decreased

It is known that nicotine can provide neuroprotection against Parkinson's in a dose-dependent manner, possibly through stimulation of nAChR on the dopaminergic circuits. The figure represents the experiment by Liu et al.[325], where β-catenin and other elements of the Wnt canonical pathway increase their expression level. After being evident that NNIs also alter the levels of β-catenin, this phenomenon would be considered with promising selective properties due to the variability of NNIs molecules.

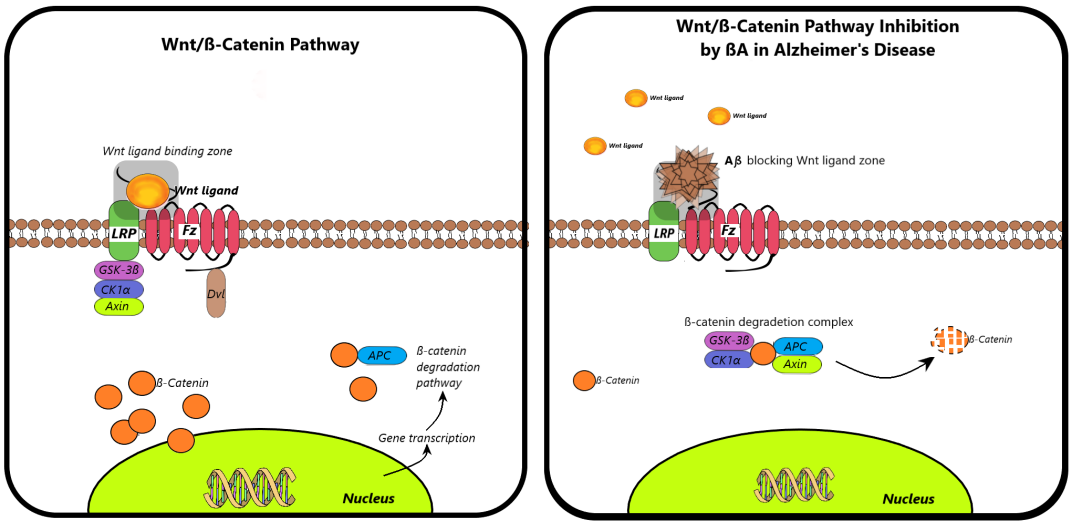
6.4 Wnt-pathways Impairment Involved in Neurological Diseases

AD hallmarks are extracellular Aβ neurotoxic plaques and intracellular neurofibrillary tangles made of hyperphosphorylated TAU protein[329]. On the other hand, it is reported that the Wnt/β-catenin pathway is altered, even inhibited, during AD. This is because toxic amyloid β protein binds Frizzled receptor 5 Fz5 near the Wnt ligand binding site and blocks the downstream signaling cascade[330, 331, 332]. Also, it is reported that the Wnt-pathway inhibition reduces neurogenesis in the adult SGZ region[333]; the loss of long-term retention memory abilities was probed in rats[334].

It is suggested that Wnt5a indirectly ameliorates the cytotoxic effects of Aβ protein, causing the inactivation of GSK3β which reduces the phosphorylation of Tau and leads to the expression of β-catenin, which in turn regulates neuroprotective genes[329]. Also, it is

observed that Dvl and PKC, which are participants of the wnt-canonical pathway, reduce the deposition of Aβ by promoting α-secretase activity[309]. The α-secretase is encharged to break down amyloid precursor protein, precluding amyloid beta formation. Moreover, in an experiment where Aβ and Wnt5A ligand were incubated with the receptor Fz5, the effect of Aβ was imperceptible compared with the control, attributed as an effect of the Wnt5A ligand. Even more, the protective effect was not evident when treated with an antagonist to Fz, the Wnt-secreted Frizzled related protein 1 sFRP1[335].

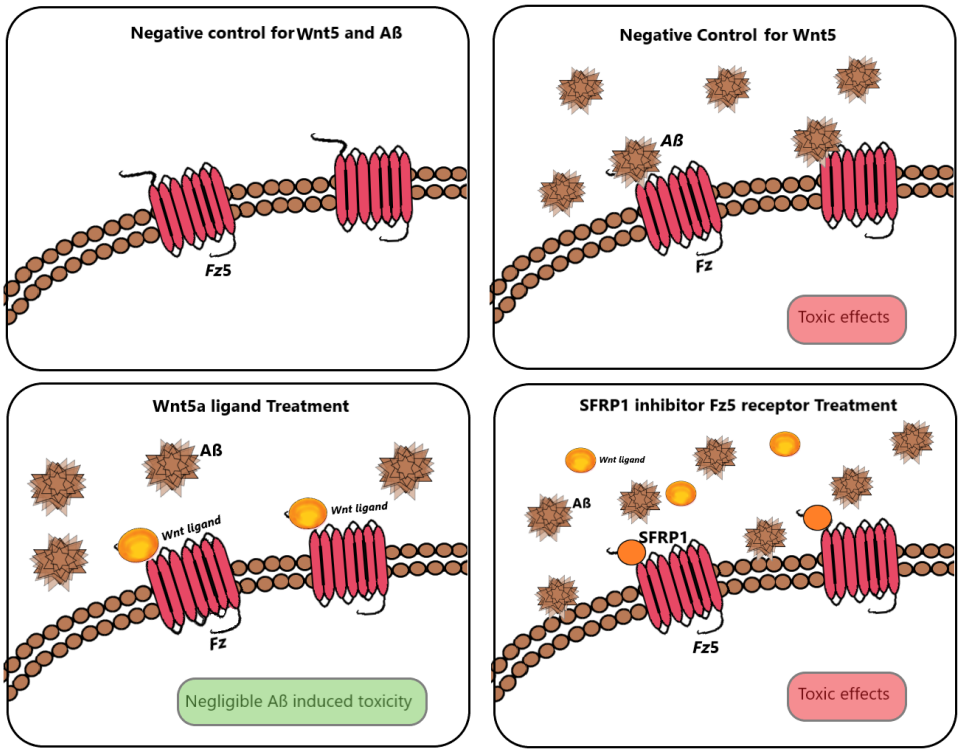
Figure 6.2: *Wnt Canonical Pathway Inhibited on AD*



It is observed that the Wnt/β-Catenin pathway can be inhibited during Alzheimer's disease. It is because Aß plaques block the Fz -Wnt ligand binding site, resulting in β-catenin degradation and preventing gene transcription. Moreover, it is known that Wnt-pathways present neuroprotection against Aß[309]. This recalls the importance of studying NNIs' mechanism of action, thinking about adverse effects, and considering a better understanding of nAChR for therapeutic purposes.

Considering the effects of altering the normal functioning of nAChR and, subsequently, Wnt pathways, neuron death seems to be the end due to oxidative stress, but before reaching that final, other subtle impairments would be led by NNI's exposure. Moreover, it is known that wnt/β-catenin presents specific activity against ROS toxicity[336, 337]. In addition to the Wnt pathways' specific functions of the nervous system during development, Wnt pathways can generally act as a proto-oncogene or a tumor suppressor depending on the conditions[338], and an alteration of their pathways is a risk factor. Furthermore, Wnt signaling also mediates inflammatory response in macrophage and T cells[309].

Figure 6.3: *Wnt-Pathway Neuroprotection Against AD*



The figure represents the reported neuroprotection against Aß effects of wnt5a-Fz5 binding[335].

On the other hand, NNIs at low doses may promote protective stimulation, but it would depend on nAChR desensitization if it is functional, temporary, or permanent. Also, increased proliferation is evidenced. However, with chronic exposure and higher doses, cell death by oxidative stress[289]. Oxidative stress may result from an altered metabolic rate due to the abnormal nAChR excitation, altering downstream pathways such as Wnt-pathways. The characteristics of reported NNI effects, such as higher activity over in development neurons and the possible alteration downstream, take into consideration NNIs participation or inducement of neurodegenerative diseases and other psychiatric impairments due to the intrinsic risk of being adult neurogenesis regions and during nervous system development, the more susceptible regions and stage respectively. However, nAChRs are ancient mechanisms and, in turn, highly regulated.

Neurons may be prepared for similar events, which is why they are susceptible during

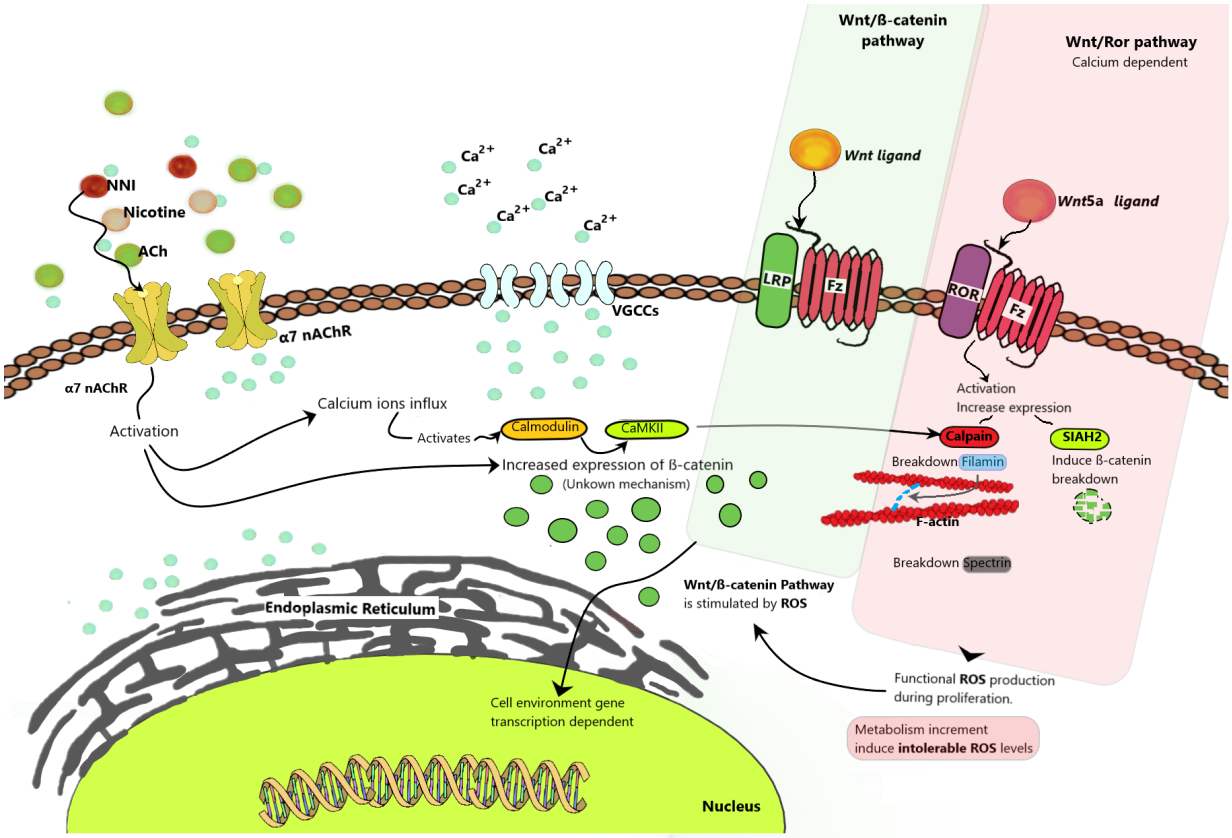
neuron development when a morphological change is required, a function in the jurisdiction of both the nAChR and Wnt pathways. Moreover, in mouse intestinal cells, the increased production of oxygen reactive species (ROS), which even more results necessary to induce cell proliferation, β-catenin stabilization, and expression of regulators of cell cycle progression such as Cyclin D1[339]. However, it can also be implied in cancer development. It puts forward that NNIs may alter the normal functioning of these mechanisms, reaching unsustainable ROS levels.

It could be suggested that in a functional system, NNIs would stimulate nAChR calcium permeable subtypes, resulting in calcium peak response, increasing calpain activity, and subsequently, cytoskeleton filaments breakdown such us β-actin, but also results in contrary disequilibrium effects such us β-catenin stabilization, that induces cell cytoskeleton formation. If it is true that a new equilibrium can be reached, it can also be considered that an increment in metabolic rate results in a ROS increment. However, its effects highly depend on the sensitivity of nAChR to result in desensitization and upregulation. Considering the limitations of in vitro experiments, such as the absence of specific regulatory mechanisms that can reduce the toxic effects, is essential.

On the other hand, considering the discrete-time and punctual regions where neurons are sensitive in the adult brain and the exposure to low concentrations of pesticide in order of μM to nM for the general population make us believe that only chronic effects would be visible, similar to some neurodegenerative diseases. The biggest problem of NNIs lies in their uncontrollable dose administration and time of exposure. There is no community that could be used as a control to evaluate the effects of NNIs after nearly thirty years of overall population exposure. However, it would be considered the existence of communities exposed to pesticides at higher rates, such as farmers and persons living in neighboring plantations.

Apart from the toxicity effect of NNIs on cells, behavioral alterations in vivo experiments are reported. Due to NNIs' chemical nature, they could be expected to affect addiction, attention alterations, and even more concentration. Once more, this appeals to

Figure 6.4: *NNIs Neurotoxicity Induced Through Calcium Imbalance*



NNIs through calcium permeable subtypes nAChRs stimulation induce calcium influx, which increases downstream metabolic activity, resulting in higher levels of ROS. In normal conditions, ROS induces proliferation through Wnt/β-catenin pathway activation. However, abnormal calcium influx can trigger intolerable ROS levels, producing no viable neurons or their death. This effect also would depend on nAChR desensitization and up-regulation-specific reactions against NNIs.

the problem of the unknown dose to which the population is exposed, and contrasting with nicotine, the NNI intake is unconscious and precludes people from reporting such subtle symptoms. All things considered, the presence of NNIs could be analyzed as food with nicotine content. And, of course, considering the molecular and affinity differences.

Boarding the dose problem of NNIs, a study in a Chinese community detected 0.02ng/ml of acetamiprid and 0.05ng/ml of imidacloprid on periodontal blood samples as the median concentration level[340], which is around one thousand two hundred to two thousand two hundred times less than a cigarette nicotine concentration level induced, 25-45ng/ml[341]. Moreover, the lethal dose for nicotine in plasma is 2μg/ml[341], while for imidacloprid,

2.05μg/ml results in acute intoxication[340], being similar to the nicotine lethal dose.

Considering that the promising effects of NNIs are closely related to α7 nAChR and calcium ions influx, it is important to understand the possible physiological effects by examining the role of this receptor and the tissues where it is found.

The α7 nAChR is widely expressed across numerous cell types, including neurons and glial cells in the nervous system, osteoclasts and osteoblasts in bone, smooth and skeletal muscle cells, immune cells (such as macrophages, T and B lymphocytes, and dendritic cells), as well as epithelial cells in the cardiovascular, respiratory, and gastrointestinal systems[342, 343, 344, 345]. They are also present in the liver, skin, and other tissues. However, this discussion will focus on their role in the nervous system. Notably, in regions like the hippocampus and prefrontal cortex, which are critical for cognition, working memory, learning, and association functions, α7 nAChR is highly expressed[346, 347, 348, 349]. Because of this, α7 nAChR is a key target for studying neurological disorders, addiction, pain response, and potential pharmacological therapies[346, 350].

The activation of α7 nAChR triggers a cascade of physiological processes, including calcium influx, neurotransmitter release, synaptic plasticity, and excitatory transmission. Additionally, it influences downstream events such as inflammation, autophagy, necrosis, transcription, and apoptosis[351]. The α7 nAChR is a homopentameric receptor composed of five identical subunits, each providing a binding site. Interestingly, the occupancy of a single site is sufficient to trigger a maximal response, while binding of inhibitors like α-bungarotoxin to one site can entirely prevent channel opening[344, 352, 353]. Desensitization depends on agonist concentration and exposure duration, leading to either temporary functional desensitization or permanent inactivation. Recovery from desensitization may take 15 to 30 seconds[345, 354]. Rapid desensitization minimizes potential cellular toxicity from excessive calcium influx during overstimulation[355]. On the other hand, allosteric modulators can delay, accelerate, or extend the desensitization time and even reactivate desensitized receptors, offering potential therapeutic strategies for sustained α7 nAChR activation[347, 355].

α7 nAChR agonists, such as nicotine, can upregulate receptor expression[356, 346]. While the underlying mechanism is not fully understood, this upregulation is believed to involve post-translational changes, increased receptor trafficking to synapses, and extended receptor functional life, rather than changes in gene expression[346]. Variations in α7 nAChR expression can result in dysregulations of its functions, such as immune regulation, particularly its anti-inflammatory effects, which are often associated with compensatory or neuroprotective mechanisms[356]. Notably, α7 nAChR activation has also been implicated in cell proliferation and may have links to cancer development[357]. Additionally, the activation and dysregulation of α7 nAChR may be involved in abnormal calcium ion influx. When calcium ions in mitochondria exceed normal levels, they can activate enzymes that initiate cell death pathways, stimulate nitric oxide production, and lead to the generation of reactive oxygen species, as well as promote calcium efflux[343].

Chapter 7

Conclusions

In summary, when talks of NNIs consider the menace implied by overall population exposure, especially in developing countries characterized by the absence of proper pesticide management, farmers and communities near plantations are the most affected. The exposure to NNIs can be described as chronic and at low doses. The understanding of NNIs' mechanism of action lies not only on the reported effects of NNIs, which can be summarized as cell viability deterioration, behavioral alterations, with possible implications on neurodegenerative diseases or psychiatric disorders, organ destruction such as liver damage, and cancer but also for the opportunity of a better understanding of nAChR with therapeutic purposes. This was evident in the altered AChE activity as an effect of NNIs, recalling that its activity modulation is the target of the current Alzheimer's disease treatment. On the other hand, although NNIs adverse effects were reported in vitro and in vivo experiments could not be denied their specificity to insects, which was evidenced by comparing the $mg/kgbw$ required to reach the $LC50$ in honey bees contrasting to mice, the latter needs more than seven thousand times higher dose. However, it increases curiosity about the effects on vertebrates, which can easily be camouflaged but visible with time after chronic exposure.

On the other hand, based on insect nAChR interaction, it is expected an orthosteric activity of NNIs over nAChR, but would not discard another type of interaction such as allosteric ones, especially considering the vast diversity of nAChR subunits and molecular variability of NNIs, which increases considering their metabolites which have demonstrated

affinity to human nAChR even higher or equal to nicotine or ACh such as the case of desnitro-imidacloprid. Furthermore, the stimuli of nAChR have a wide range of functions and influence downstream on essential pathways such as Wnt-pathways.

Although the effect of NNIs depends on the affinity of nAChR and NNIs, the dose and time of exposure, which modulate the nAChR desensitization and up-regulation, are also protective mechanisms that can be involved in the higher susceptibility during neuron development. Moreover, recalling the nAChR subunit diversity, it was only necessary for subtle mutation of Arg81 for a Thr on amino acid sequence on the subunit of D-loop on Myzus persicae for resistance development against NNIs.

Although complex experimental conditions weakly support the mechanism of action explanation, it is possible to suggest, based on the evident alterations in cytoskeleton proteins such as β-actin and possible dramatic alteration in β-catenin expression, that the most significant adverse effect of NNIs would be related to the structural formation of neurons, with important implications on neuroplasticity, neurodevelopment, neurite outgrowth, and in general, neuron cell viability. That makes us think about α7 nAChR, which is reported to be responsible for promoting those structural changes and cell viability. Even more, it is a calcium-permeable nAChR subtype that supports the suggestion of participation of dependent calcium-activation mechanisms such as calpain, calmodulin, and some non-canonical Wnt-pathways that influence receptor transportation, neuron motion, migration, and formation, but also cytoskeleton degradation, influencing neuroplasticity, neuroprotective effects, where alteration on such mechanisms can trigger neurodegeneration.

Furthermore, it is challenging to make evident adverse effects of NNIs after nearly thirty years of NNIs exposure on the overall population, even more so when their effects are visible on chronic exposure during neuron development, which is translated as short windows of higher susceptibility on adulthood, adding the diversity of NNIs molecules, and the diversity of nAChR subunits and their wide range of function, with an even wider downstream influence on diverse pathways, where a subtle change on amino acid sequence can convert a complete response to no response. Despite this, the amplified effects of NNIs

at the end of metabolic pathways on cytoskeleton proteins during neurodevelopment are undeniable. Although NNIs may increase cell proliferation at specific doses, ROS, and cell viability reductions are more commonly reported.

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Appendices

.1 Appendix 1.