UNIVERSITY OF NOVA GORICA
SCHOOL OF ENVIRONMENTAL SCIENCES

ENVIRONMENTAL STRESSORS AND NEURODEGENERATION: EVALUATION OF THE EFFECTS OF APPLICATION OF PESTICIDES ON NEURONAL CELLS

DIPLOMA THESIS

Dane Lojen

Mentor: Dr. Giulietta Pinato

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STATEMENT

I declare that the present diploma thesis is the result of my own research. Results which were achieved in the frame of common research with other researchers or were contributed by other researchers (experts) are explicitly displayed or quoted (cited) in the diploma thesis.

Dane Lojen

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SUMMARY

Imidacloprid has been considered as safe for non-target organisms and the environment, but many studies show that its use is especially dangerous for bees, birds and aquatic life, and it might also influence the food webs and affect biodiversity. Its high binding affinity to nicotinic acetylcholine receptors in neurons of central nervous system, which influences Ca^{2+} homeostasis, makes it potentially dangerous for mammals and humans due to its possible involvement into neurodegeneration. In my thesis, imidacloprid's influence on Ca^{2+} homeostasis after acute and chronic exposure was investigated on hippocampal neurons and iMN9D cells, and compared with the effect of nicotine. Changes in cytosolic Ca^{2+} concentrations were measured with epifluorescence microscopy as relative fluorescence changes of Ca^{2+} probe Oregon Green 488 BAPTA-1, AM. Neurons acutely exposed to imidacloprid exhibited substantially increased amplitudes of cytosolic Ca^{2+} concentrations. When exposed to nicotine, Ca^{2+} homeostasis was comparable to the control. In neurons chronically exposed to either of the substances, spontaneous Ca^{2+} influxes were inhibited, but Ca^{2+} peaks were restored after the administration of gabazine (neuronal network synchronization). iMN9D cells seem to be inappropriate to explore imidacloprid's effects on Ca^{2+} homeostasis as they showed very insignificant Ca^{2+} peaks. A viability test showed statistically significant imidacloprid's neurotoxicity only during one experiment. Present results and literature review confirm imidacloprid's possible involvement into neurodegeneration and its definite environmental toxicity, so its use should be restricted by law.

Key words: ecosystems, environment, imidacloprid, neurodegeneration, toxicity

POVZETEK

Do sedaj je prevladovalo mnenje, da imidakloprid ni nevaren za organizme in okolje, novejše raziskave pa kažejo, da ogroža predvsem čebele, ptice in vodne organizme. Njegova uporaba lahko vpliva na prehranske splete in biodiverziteto. Ima visoko afiniteto vezave na nikotinske acetilholinske receptorje v nevronih centralnega živčnega sistema in povzroča depolarizacijo nevronov zaradi dotoka Ca^{2+} v citoplazmo. Zato je imidakloprid potencialno nevaren tudi za sesalce, med drugim ljudi, saj je morda povezan z razvojem nevrodegenerativnih bolezni. Za potrebe diplomske naloge sem preiskoval učinke imidakloprida na spremembe koncentracij Ca^{2+} v citoplazmi primarne kulture celic hipokampusa in na celice iMN9D ter jih primerjal z učinki nikotina. Spremembe koncentracij Ca^{2+} sem meril z epifluorescenčnim mikroskopom kot relativne spremembe fluorescence kalcijevega barvila Oregon Green 488 BAPTA-1, AM. Pri akutni izpostavitvi nevronov imidaklopridu je prišlo do občutnega povečanja amplitud koncentracij Ca^{2+}. Valovi Ca^{2+} pri izpostavitvi nikotinu so bili primerljivi s kontrolnim vzorcem. Pri nevronih, ki so bili kronično izpostavljeni imidaklopridu oz. nikotinu, je prišlo do inhibicije valov Ca^{2+}, ki so se pa ponovno pojavili po dodatku gabazina (sinhronizacija omrežja živčnih celic). iMN9D celice se niso izkazale kot primeren model za raziskave vpliva imidakloprida na valove Ca^{2+}. Test preživetja je pokazal nevrotoksičnost imidakloprida samo v enem poizkusu. Pričujoči rezultati in pregled literature kažejo, da je imidakloprid zelo verjetno vpleten v nevrodegenerativne procese, in je nevaren za okolje; njegovo uporabo bi morali omejiti.

Ključne besede: ekosistemi, imidakloprid, nevrodegeneracija, okolje, strupenost

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LIST OF ABBREVIATIONS

AChBP = acetylcholine binding protein
nAChR = nicotinic acetylcholine receptor
AD = Alzheimer's disease
AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMS = amyotrophic lateral sclerosis
AOX = aldehyde oxidase
APO = apochromatic objective
ARA-C = anti-mitotic cytabarine
AST = aspartate aminotransferaze
ATP = adenosine triphosphate
CCD = colony collapse disorder
CNS = central nervous system
CYP450 = cytochrome P450
DA = dopamine
DIC = differential interference contrast
DOC = dissolved organic carbon
DMEM = Dulbecco´s modified eagle medium
DT50 = dissipation time 50
DWLOC = drinking water level of comparison
EC50 = effective concentration 50
EFSA = European Food Safety Agency
EPA = Environmental Protection Agency
ER = endoplasmatic reticulum
FBS = fetal bovine serum
GABA = γ-aminobutyric acid
$GABA_A$ = ionotropic receptor and ligand-gated Cl^- channel
mGluR = metabotropic glutaminate receptor
HVA = high voltage activated
IFN = interferon
IMI = imidacloprid
IPM = integrated pest management
IP_3R = inositol triphosphate receptor
IUPAC = International Union for Pure and Applied Chemistry
LC50 = lethal concentration 50
LD50 = lethal dosis 50
LDH = lactate dehydrogenase
LOAEL = lowest observable adverse effect level
LOEC = lowest observed effective concentration
LVA = low voltage activated
MSA = multiple system atrophy
NCX = sodium calcium exchanger
ND = not detected
ND filter= neutral density filter
NMDA = N-methyl-D-aspartate receptor
NOAL = no observable adverse effect level
NOEC = no observed effect concentration
NOS = nitric oxide synthase
aPAD = acute population adjusted dose
cPAD = chronic population adjusted dose
PBS = phosphate buffer

PD = Parkinson’s disease
PMCA = plasma membrane calcium ATPase
PS = Parkinsonian syndromes
PSP = progressive supranuclear palsy
RCF = relative centrifugal force
RfD = reference dose
RNS = reactive nitrogen species
ROS = reactive oxygen species
RyR = ryanodine receptor
SERCA = sarco-/endoplasmatic reticulum calcium ATPase
SNC = substantia nigra pars compacta
TNF = tumour necrosis factor
TRPC = transient receptor potential type C
USDA = United States Department of Agriculture
VGCC = voltage gated calcium channel
WHO = World Health Organisation

1 INTRODUCTION

Through the last decades, the use of neonicotinoids and their intake in the environment has grown rapidly (Jeschke P. et al., 2011), so it is of great importance that we gain knowledge about their impact on environment and on humans. Many epidemiological studies have suggested linkage between chronic exposure to pesticides and neurodegeneration, resulting in lowered cognitive performance and increased prevalence of different types of dementia, such as Alzheimer's, Parkinson's and Huntington's disease, and amyotrophic lateral sclerosis (Baldi I. et al., 2003; Marambaud P. et al., 2009; Zaganas I. et al., 2013). Chao S.L. and Casida J.E. (1997) suggested, after only a few years of using them, that neonicotinoids could act on nAChRs receptors in mammals.

In most studies, the types of compounds and doses of exposure participators had been previously exposed to, have been nearly impossible to determine. However, the studies have shown chronic synergistic effects of all chemicals on people who were exposed to them. Also, exact toxicity mechanisms of many phytopharmaceutical compounds (acute as well as chronic) and their synergistic effects remain partially or completely unknown. Therefore, figuring out exact toxicity mechanisms of phytopharmaceuticals is highly relevant for the protection of the environment and public health. More targeted prospective studies are needed in order to find the links between pesticide exposure and dementia (Baldi I. et al., 2003; Zaganas I. et al., 2013).

One of today's commonly used pesticides whose safety has not been sufficiently explored is imidacloprid. Although imidacloprid (belonging to the group of neonicotinoids) is considered safe, it actually has a similar molecular structure as nicotine, which is known to affect nAChRs and adversely influence the development of the mammalian nervous system, especially the developing brain (Kimura-Kuroda J. et al., 2012).

In my thesis, I investigate the connections between the exposure to imidacloprid and the appearance of processes involved in the development of neurodegenerative diseases.

2 THEORETICAL BACKGROUND

2.1 Pesticides in the environment and neurodegenerative diseases

2.1.1 Xenobiotics and neurodegeneration

Currently, the incidence of neurodegenerative diseases is increasing. Pathogenesis of many of them remains partially or completely unknown but, many of those diseases are thought to be connected with environmental factors. A neurodegenerative disease means damage posed to developing or already matured neuronal system, which is expressed through distinctive gradual and progressive neuronal dysfunction and death. Neurons can be lost through programmed cell death—apoptosis—and also through uncontrolled cell death called necrosis. The most common neurodegenerative diseases are Alzheimer's disease, Parkinson's disease, Parkinsonian syndrome (multiple system

atrophy and progressive supranuclear palsy), and amyotrophic lateral sclerosis (Brown R.C. et al., 2005).

Known risk factors that can lead to the development of neurodegenerative diseases are genetic polymorphism and aging. Other possible factors, many of which are environmental, are gender, poor education, endocrine conditions, oxidative stress, inflammation, stroke, hypertension, diabetes, smoking, head trauma, depression, infection, tumours, vitamin deficiencies, immune and metabolic conditions, and chemical exposure (Brown R.C. et al., 2005).

To find connections between exposure to environmental factors and neurodegenerative diseases, epidemiological evidence is needed. To provide conclusive association and adequate statistical relevance, epidemiological studies should take into account the following factors: genetics, endocrine conditions, oxidative stress, infection and inflammation, nutrition, vascular conditions, depression, head trauma, tumours, lifestyle, and other potential risk factors such as age at the onset of the disease, socioeconomic status, gender, ethnicity, and level of education (Marder K. et al., 1998). Known confounders are also smoking, caffeine and alcohol consumption.

Ideally, epidemiological studies should be performed on a population that is exposed to known amounts of environmental stressors before the development of any neurodegenerative disease. Such a population should then be monitored to find the possible association between the exposure and the aetiology of the disease. Instead, more realistically, investigations are done in a retrospective way. Exposure of individual cases is examined after the disease has already been diagnosed. If this is the case, people may over-report the exposure to xenobiotics because of the desire to find answers for their condition, or they may have problems remembering (Brown R.C. et al., 2005).

It is very important to correctly identify the disease. This might be somewhat problematic because many diseases have very similar symptoms (co-existing conditions) and are therefore hardly distinguished and diagnosed. A reliable diagnosis can be stated in about 80 % of the cases; other cases can be reliably diagnosed only with an autopsy. A major problem is, in fact, determining a reliable diagnosis early on, which is currently attempted with neuropsychological tests (Mok W., 2004).

In most cases, it is very hard to find a clear connection between the neurological outcome and a particular chemical (agent) because people are simultaneously exposed to many different compounds. In many studies, pesticides are divided into subgroups, but these still contain too many examples to identify adverse effects of individual chemicals. There is also the problem of finding the right case-control group that has not been exposed to the object of the research (Baldi I. et al., 2003).

In occupational studies, the healthy worker effect has to be considered. This means that individuals who participate in the study are healthier than those who do not participate, because the study includes only those employees who have remained healthy and retained the job; those who fell ill before are not considered. In non-occupational studies, those having neurodegenerative diseases sometimes participate less due to their lower mobility and stunted communication ability (Brown R.C. et al., 2005).

Sometimes, it is important to find an early window of exposure, since later exposure could not be of such great importance for the pathogenesis of the disease. In

environmental exposure data, a peak exposure, or accumulative exposures under limit of detection, might not be captured in the average exposure data. In bio monitoring, capturing non-persistent exposures as well as finding the right sites in the tissues where compounds take their effects, can be a problem. Due to aforementioned reasons, it is hard to find the proper dose-response curve. The statistical significance of the results of the studies might be problematic because the numbers of study participants are small and the results usually connect the pathogenesis of the diseases with groups of chemicals and not with single compounds (Brown R.C. et al., 2005).

2.1.2 The most common neurological disorders

Alzheimer's disease (AD) is a progressive degenerative disease that affects the central nervous system (CNS). Distinctive symptoms are memory loss and severe deficits in one or more of the following domains: aphasia (language disturbance), agnosia (failure to recognize people or objects in presence of intact sensory function), apraxia (inability to perform motor acts in presence of intact motor system), or executive function (plan, organize, sequence actions, or form abstractions). Listed effect can be so severe as to completely change a person's personality and make normal life and work impossible (Selkoe D., 2001; Brown R.C. et al., 2005).

Known risk factors are increasing age, familial association, Down syndrome, and the apolipoprotein E4 allele (McCullagh C.D. et al., 2001). Environmental risk factors that might lead to AD are exposure to certain metals (aluminium, zinc, selenium, iron, mercury, copper, lead), pesticides (especially organophosphates and carbamates), defoliants, fumigants, and electromagnetic fields. Many epidemiological studies have examined possible links between environmental pollutants and development of AD. However, the results of the studies are conflicting, seeing as some of them found a link but others did not. So much still has to be done in the future to shed some light onto the risks of environmental agents on the development of AD (Selkoe D., 2001).

Parkinson's disease (PD) is, in contrast to AD, not characterized by cognitive and personal changes, but by abnormalities in motor control. Characteristic symptoms are resting tremor, bradykinesia (slowness in voluntary movements), rigidity in reflexes, the loss of facial expression, and walking with small steps (Moore D.J. et al., 2005).

PD is connected with a number of genetic polymorphisms (Checkoway H. et al., 1998), but in most cases, it could be linked to some environmental factors, too. Possible risk factors are exposure to pesticides—especially in relation to farming occupation and using water from the wells contaminated by pesticides—and exposure to various metals, such as zinc, aluminium, manganese, iron and mercury. Many epidemiological studies were made to find these relations. The results were contradictory, just like in the studies of AD. A few studies suggest an association of PD and exposure to organic hydrocarbon solvents and wood (Moore D.J. et al., 2005).

Similar to the PD is a disease called Parkinsonian syndrome (Drayer B.P. et al., 1986), which includes the following disorders:

- MSA type, which is a cluster of three related disorders, one of which is Parkinsonian that is characterized by low blood pressure resulting in dizzy spells (Wenning G.K. et al., 2004), and
- PSP type, which is a neurologic condition affecting the brainstem and also has symptoms similar to those of PD, characterized by movement and visual

abnormalities (Drayer B.P. et al., 1986). The aetiology of the Parkinsonian syndrome is unknown, but it is believed to be connected to environmental factors, such as exposure to metal dusts, fumes, plastic monomers and additives, organic solvents, and pesticides (Brown R.C. et al., 2005).

Amyotrophic lateral sclerosis (AMS) is a motor neuron disease also called Lou Gehrig's disease. It is very rare and is characterised by degeneration of motor neurons resulting in muscular weakness. Intellectual abilities and personality are usually maintained. A part of genetic cases is related to the mutation of superoxide dismutase 1 enzyme, but most cases are thought to be induced by environmental factors (McGuire V. et al., 1997), such as exposure to agricultural chemicals, exposure to metals (lead, mercury, iron, manganese, selenium and aluminium), and working in plastic manufacturing or electrical industry. The studies have limited power because of the small size of population with this disease (Brown R.C. et al., 2005).

2.2 Classification of pesticides

Ecotoxicological point of view treats insecticides in the environment as organic pollutants originating either from nature (mostly found in plants) or synthetically produced. According to their chemical composition, they can be classified as organochlorines, organophosphates, carbamates, pyrethroids, neonicotinoids, ryanoids, phenylpyrazoles, and others. Biochemical classification divides pesticides into different groups according to their target organisms: insecticides, herbicides, fungicides, rodenticides, acaricides, algicides, avicides, bactericides, molluscides and nematocides (EPA, 2012).

Marrs T.C. (2012) classified insecticides according to their target organic systems, organs, and different systems on a cellular level (receptors). Insecticides are divided based on those targeting systems in insects that are also present in mammals, and those targeting systems that are not present in mammals. Insecticides that target systems which are also present in non-target organisms, especially those targeting nervous systems, may be harmful for non-target organisms as well. Similarly, insecticides targeting systems that are not present in non-target organisms may also cause harmful effects to non-target organisms, since they can act as analogues (table 1).

2.2.1 Insecticides that interfere with systems non-specific to insects

Most of such insecticides selectively target the nervous system. Their selectivity mostly depends on the differences between insect and mammalian nervous systems. Mammalian nervous systems are physically more protected against noxious substances. Mammals also have a different metabolism and a different receptor distribution. Receptors in mammals are structured in a way that causes a lower binding affinity of specific pesticides, as opposed to the insects' receptors. There are two types of insecticides that target the nervous system: those that target Na^+-channels, and those that target neurotransmission and are mainly cholinergic and GABA-ergic (Marrs T.C., 2012).

Na^+-channel modulators— organochlorines (DDT), pyrethrins and synthetic pyrethroids —affect sodium channels of peripheral nerves, causing paraesthesia, confusion, malaise, and headache in mammals (DDT, 1999).

Insecticides targeting cholinergic neurotransmission bind strongly to nAChRs in CNS. In low concentration, they cause neuronal stimulation, but a high concentration would lead to receptor blockade, paralysis, and death. This class of insecticides can be further divided in two main groups: organophosphates and carbamates, and neonicotinoids (Marrs T.C., 2012).

Table 1 *Classification of insecticides regarding to their target systems in insects and mammals (Marrs T. C., 2012: 1333).*

Table 1. Main groups of insecticides

	Group	Subgroups	Examples
Substances that target systems in insects that have counterparts in mammals	Organochlorines (OCs)		DDT
	Anticholinesterases	Organophosphates (OPs)	Malathion
			Fenitrothion
			Diazinon
		Carbamates	Carbaryl
			Aldicarb
	Cholinergic group	Nicotine and the neonicotinoids	Nicotine
			Imidacloprid
	Pyrethrins and synthetic pyrethroids	Natural compounds	Pyrethrum
		Synthetic pyrethroids	Cypermethrin
			Flumethrin
			Permethrin
	Natural compounds, other than pyrethrins and nicotine		Abamectin
			Ivermectin
			Rotenone
	$GABA_A$ blockers	Phenylpyrazoles	Fipronil
		Cyclodiene organochlorines	Chlordane
			Endosulfan
Substances that interfere with systems specific to insects	Octopamine receptor agonists		Amitraz
	Juvenile hormone mimics		Methoprene
			Hydroprene
			Fenoxycarb
	Moulting disruptors	Ecdysone agonists	Tebufenozide
			Methoxyfenozide
		Precise mechanism not known	Cyromazine
			Dicyclanil
	Chitin synthesis inhibitors		Diflubenzuron
			Lufenuron
			Hexaflumuron
			Buprofezin

Organophosphates and carbamates kill insects with inactivation of acetylcholinesterase, which results in over-activation of muscarinic receptors (Gupta R. and Milatovic D., 2012).

The mode of action of neonicotinoids will be discussed in detail later in my thesis, so I will not describe them in this section.

$GABA_A$ blockers target the ɣ-aminobutyric acid (GABA) system. Examples of $GABA_A$ blockers are phenylpyrazoles and cyclodiene organochlorines. $GABA_A$ receptors are ligand-gated chloride channels involved in synaptic inhibition. Clinical signs of

intoxication with $GABA_A$ blockers in mammals are excitation and convulsions (Woodward K.N., 2012).

2.2.2 Insecticides that interfere with systems specific to insects

Insecticides targeting structures that are not present in non-target organisms can be divided into following groups: octopamine receptor agonists, insecticides targeting hormonal systems of insects, and insecticides inhibiting synthesis of chitin (Marrs T.C., 2012).

Octopamine receptor agonist is for example amitraz. Octopamine is an important neurotransmitter in insects that has analogues in mammals. Human poisoning causes CNS depression and can also disturb glucose homeostasis (Berry M.D., 2004).

Insecticides targeting insects' hormonal systems are juvenile hormone mimics, ecdysone agonists and other moulting interfering compounds, and chitin synthesis inhibitors.

Juvenile hormone mimics insecticides prevent successful development from larva to adult insect. A methoprene metabolite can bind to mammalian retinoid X receptors causing abnormalities in retinoid-responsive pathway (Harmon M.A. et al., 1995). Retinoids are teratogenic, but the only effects on male fertility in rats were observed after hydroprene poisoning. Fenoxycarb affects blood cells and liver and increases the incidence of tumours (Dhadialla T.S. et al., 1998).

Growth regulators ecdysone and 20-hydroxyecdysone are steroids, but ecdysone agonists are chemically not steroids. They bind to the ecdysone receptors in insects and interfere with moulting, therefore causing death. They have no notable anabolic function in mammals, and their low toxicity is expressed on blood parameters. There are no signs of embriotoxicity and teratogenicity in rats and mice (Dinan L. and Lafont R., 2006).

For two moult-interfering insecticides (cyromazine and dicyclanil), mechanisms of action on moulting are not precisely known. To mammals, dicyclyanil is more toxic than many other insecticides because of its influences on liver functions, and is carcinogenic (WHO, 2012).

Chitin synthesis inhibitors are chemically benzoylureas or thiadiazin. They cause insects' death after moulting because the new chitinic skeleton cannot be properly synthesized. For mammals, their toxicity is low, causing problems in blood, bone marrow, liver, and spleen (Marrs T.C., 2012).

2.3 Neonicotinoids market, properties and use

2.3.1 Market

Worldwide, the usage of insecticides enabled the increase in agricultural productivity in the twentieth century. Imidacloprid was the first insecticide of the wide group of neonicotinoids introduced to the market. Neonicotinoids were developed in the late

eighties and in early nineties as a response to the widespread insects' resistance to older insecticides, such as organophosphates, pyretroids and carbamates, and their health and environmental issues. Imidacloprid was first registered by United States Environmental Protection Agency (EPA) in 1994, followed by thiacloprid, thiamethoxam, nitenpyram, acetamiprid, clothianidin and dinotefuran. Neonicotinoids have been the most important and the fastest growing class of insecticides and are registered in more than 120 countries for use on over 140 different crops. In 2008, the total value of all pesticides used had reached 6.330 billion euros, and neonicotinoids represented 23.7 % of the total pesticides used. The total value of insecticides for seed treatment in 2008 was 957 million euros, and 80 % of the pesticides used for seed treatment were neonicotinoids (Jeschke P. et al., 2011).

Detailed data of neonicotinoids' market for most countries is not available, but figures for the UK for the last twenty years, shown in fig. 1, can illustrate the market in the developed countries (Goulson D., 2012). The increased use of clothianidin for seed treatment is responsible for the decrease of imidacloprid consumption in the UK.

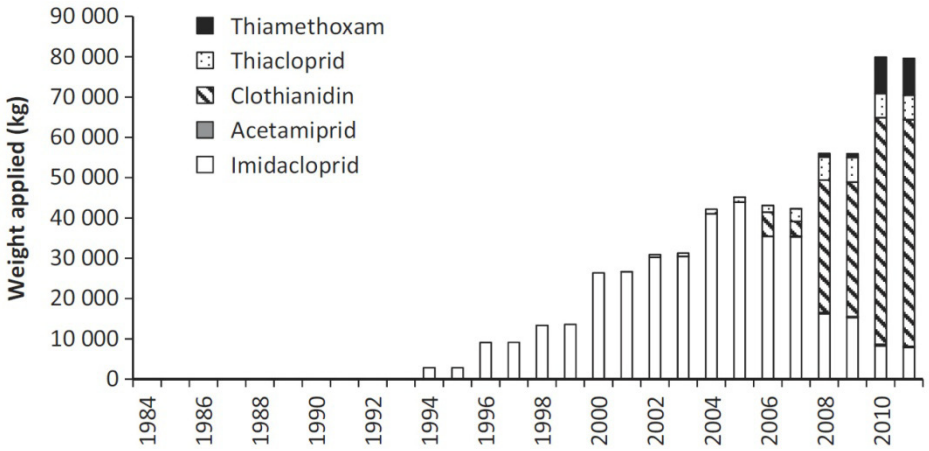

Figure 1: *Consumption of neonicotinoids in the UK (Goulson D., 2012: 978).*

The producers of neonicotinoids are Bayer CropScience (imidacloprid, thiacloprid and clothianidin), Syngenta (thiametoxam), Sumitomo Chemical Takeda Agro Company (nitenpyram, clothianidin), Nippon Soda (acetamiprid) and Mitsui Chemicals (dinotefuran) (Jescke P. et al., 2011).

Bayer's patent for imidacloprid expired in 2006 and the patents for some other neonicotinoids will also expire soon; this has already led to the production of generic products, mostly in India and China (Jeschke P. et al., 2011). Therefore, a higher production and consumption of neonicotinoids can be expected in the coming years, if they are not banned for some purpose. In April 2013 European Union legislation banned the use of thiametoxam, imidacloprid and clothianidin for two years. In two years EFSA has to prepare a new risk assessment report for their re-registration in the EU.

2.3.2 Physicochemical properties

Neonicotinoidic insecticides, also called neonicotinoids, are synthetic derivates of nicotine, the biological target of which is the neuronal system, especially the nicotinic

acetylcholine receptors (nAChRs). Nicotine is a natural alkaloid found in plants that belong to the nightshade family, for example tobacco. Its function is to protect the plants from insects. Aqueous tobacco extract was previously used as a natural insecticide. According to the International Union for Pure and Applied Chemistry (IUPAC), the chemical name of nicotine is (S)-3-(1-methylpirrolidin-2yl)pyridine (Ujvary I., 1999). The structural formula of nicotine is shown in fig. 2.

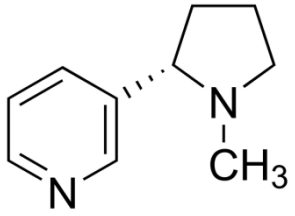

Figure 2: *Structural formula of nicotine (Sigma Aldrich, 2014)*

Until now, seven neonicotinoids have been introduced to the market: imidacloprid, thiacloprid, thiamethoxam, nitenpyram, acetamiprid, clothianidin and dinotefuran. They can be classified as N-nitroguanidines, nitromethylenes, or N-cyanoamidines. They are compounds with two heterocyclic six-membered rings (thiametoxam), or one six-membered and the other one five-membered ring (imidacloprid and thiacloprid), or one of the five- or six-membered rings and one of four noncyclic compounds bound to the ring. There are six-membered rings in nitenpyram and acetamiprid, and five-membered rings in clothianidin and dinotefuran (Jeschke P. et al., 2011). The structural formulas of these compounds are shown in Annex A.

Physicochemical properties of neonicotinoids are determined by their ring systems and noncyclic constituents. The said properties are photo-stability, liphophility and water solubility, metabolism in living organisms, and decomposition in the environment. Noncyclical neonicotinoids are less lipophilic than the cyclic ones (Jeschke P. and Nauen R., 2005).

An imidacloprid molecule (fig. 3), also classified as N-nitroguanidin, with the IUPAC name 1-(6-chloro-3-pyridyl)-N-nitroimidazolidin-2-ylideneamine, consists of a six-membered pyridine and a five-membered imidazoline ring and has the molecular weight of 255.7 g/mol.

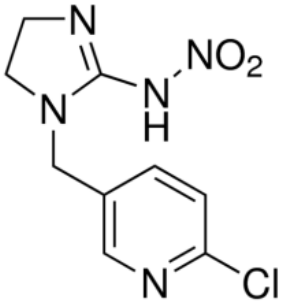

Figure 3: *Structural formula of imidacloprid (Sigma Aldrich, 2014)*

It forms colourless crystals with a slight but characteristic odour, its melting point is at 144 °C, its density is 1.54 g/cm^3 and its solubility in water is 0.61 g/L (both at 20 °C). Its octanol-water partition coefficient (log K_{ow}) is 0.57 at 21 °C. Caution words used for

imidacloprid range from caution to danger, reflecting combined toxicity of active and other ingredients in the commercial products. It is resistant to hydrolysis in acidic and neutral conditions; hydrolysis is stimulated by higher pH values and rising temperature (Gervais J.A. et al., 2010; Jeschke P. et al., 2011).

2.3.3 Application and use

Neonicotinoids are used for a variety of purposes, such as crop protection, as a compound in some consumer and professional products, and in products sold on animal health market—veterinary products. Neonicotinoids are systemic insecticides that are translocated and distributed through the whole plant, providing long lasting insect control. Insects are affected by contact with or through ingestion of the treated plants (Jeschke P. et al., 2011).

In crop protection, neonicotinoids are used to protect many different crops (plants) against most of the harmful insects. They can be used in different ways, such as foliar application (spraying plants), seed treatment, soil application (granules), and as an additive in soil drenching (compounds added in irrigation system—drip or drench system). When neonicotinoids are used for seed treatment, seeds are first processed with the insecticide and then covered by a one- or multi- layer polymer-coating to fix the active substance to the grain (Girolami V. et al., 2009; Jeschke P. et al., 2011). In the developed countries, 60 % of neonicotinoids are used for seed treatment, especially for oilseed rape, sunflower, grains, beets, and potatoes (imidacloprid, clothianidin and thiametoxam). Plants grown from treated seeds are protected against pests for months (vines, citrus trees) or even years (maple trees) (Goulson D., 2013).

For foliar application, neonicotinoid containing preparations can be prepared as oil dispersions, resulting in formation of small crystals covered by an oil film on the leaf surface. In this way, better retention is provided through minimizing the runoff in case of rain. Such application also provides better penetration of the active compound through leave's cuticule and translocation into the leaf tissue (Girolami V. et al., 2009; Jeschke P. et al., 2011).

Neonicotinoids are effective against a broad spectrum of sucking and chewing insect-pests reducing crops, including insects that are vectors of many viral plant diseases. The most common target insects are aphids, whiteflies, leaf- and planthoppers, thrips, some micro-Lepidoptera, a number of coleopteran pests, wireworms, rootworms, Colorado potato beetle, and some others (Jeschke P. et al., 2011).

Neonicotinoids are used to protect a lot of different plants, such as leafy and fruiting vegetables, sugar beet, cotton, pome fruit, grains, tobacco, rice, citruses, corn, oilseed rape, potatoes, stone fruit, soy beans, peanuts, artichoke, tea, sunflowers and some others (Jeschke P. et al., 2011).

Neonicotinoids containing products for consumer and professional market are used in households, lawns, and gardens to control termites, turf pests (white grubs, cockroaches) and ants (Jeschke P. and Nauen R., 2005).

In veterinary medicine, products on the basis of neonicotinoids are used for treatment of external parasites, like fleas and ear mite on cats, dogs, and other domestic animals (Jeschke P. et al., 2011).

2.3.4 Economic benefits of neonicotinoids

In the last 60 years, yields per hectare have increased as a result of many changes in agriculture, not only because of the wide use of artificial fertilizers and pesticides, but also due to new agronomic techniques and improved crop variety. But the yield increases in developed countries during the last twenty years (the “neonicotinoid era”) have been modest or even the same as before the neonicotinoids were available. This is the case when it comes to the yields of oilseed rape in the UK, even though close to 100 % of seeds are treated. Even in cases when yield increases during the last years were evident, it is not easy to disentangle the share of yield increase due to the usage of neonicotinoids from the share of changes in agronomic practices. Some studies suggest that the yield benefits are small (compared to unprotected control crops) and often exceed the cost of the insecticide. Therefore, further studies for different crops in different geographical regions are needed to establish in which cases the prophylactic use of neonicotinoids is cost-effective. As alternatives to neonicotinoids, pyrethroid sprays or IPM (integrated pest management) system of farming are available. IPM system means pest management that includes monitoring of pest populations, maximally using biological and cultural controls, and minimizing the use of chemical pesticides (Goulson D., 2013).

2.4 Neonicotinoids in environment

Neonicotinoids are systemic, environmentally persistent, highly water soluble and have a high level of mobility, so they tend to get washed into surface waters and leach into ground water. They also tend to bind with the soil (Gervais J.A. et al., 2010; Mineau P. and Palmer C., 2013: 9).

2.4.1 Imidacloprid in soil

Imidacloprid’s sorption to soil depends on the soil type, the organic matter content, and the concentration of imidacloprid (sorption is falling inversely proportional with rise of concentration of imidacloprid). The leaching from the soil is lower and the sorption is higher in soils with high organic matter content (Liu W. et al., 2006). Imidacloprid metabolites that remain after its metabolism and degradation can be strongly bound to the soil. The most common degradates found in soil are 6-chloronicotinic acid, two cyclic ureas, olefinic cyclic nitroguanidine, a cyclic guanidine, and its nitroso and nitro derivatives. In an experiment, imidacloprid was applied to the soil and after one year, up to 40 % of the residues could not be extracted. In calcareous soil, the sorption of imidacloprid depends on the content of dissolved organic carbon (DOC), and it decreases with the increasing content of DOC. Therefore, in calcareous soil with a high content of DOC, imidacloprid can be easily leached away into surface and ground waters (Gervais J.A. et al., 2010).

Imidacloprid’s half-life in the soil depends on the soil type, amendation of the soil (agricultural or non-agricultural), and the content of organic fertilizers. In non-agricultural soil, the half-life of imidacloprid was estimated to be 188-997 days, and in cropped soils, the DT50 was estimated to be 69 days (Gervais J.A. et al., 2010). Dissipation time DT50 of imidacloprid was determined to range from 28 (lateritic soil, laboratory test) to 1,230 days (loam soil, field test). Repeated applications of imidacloprid lead to accumulation in the soil. A 6-year study in the UK has shown that

the concentration in the soil was growing proportionally with years (up to 60 ppb), depending on the application rate. After six years, the experiment was completed (Goulson D., 2013).

2.4.2 Imidacloprid in water

Neonicotinoids can be detected in ground water, streams, and storm water ponds. In water environment, imidacloprid can be degraded by photolysis, by hydrolysis, and by microbes in sediments. Photodegradation and hydrolysis, although having different chemical pathways, mostly result in the formation of imidacloprid-urea. Photo degradation occurs under UV and visible light and is relatively fast. The half-life of imidacloprid photolysed by light from a xenon lamp was 57 minutes. In nature, the degradation in clear water by photolysis is very rapid. The most important products of the photolysis of imidacloprid are a cyclic guanidine derivative, a cyclic urea, an olefinic cyclic guanidine, and two fused ring products (Gervais J.A. et al., 2010). DT50 in outdoor experiments was 1.4 to 10 days and in a laboratory in the dark from 30 to 150 days (EFSA, 2008: 30).

Hydrolysis of imidacloprid occurs at alkaline pH, but not at neutral and acidic pH. The product of hydrolysis is the metabolite imidacloprid-urea with the IUPAC name 1-[(6-chloro-3-piridinyl)methyl]-2-imidazolidone. This can be further broken down by the oxidative cleavage of N-C bond between pyridine and imidazolidine, and the emerged products can be further degraded into CO_2, NO_3^- and Cl^- (Liu W. et al., 2006).

In the water sediments system, the half-life of microbial degradation of imidacloprid was in between 30 and 162 days. The main degradation product was the guanidine compound (Gervais J.A. et al., 2010).

In many places, the contamination of surface and ground water with neonicotinoids is already high and may cause long-lasting effects on terrestrial and aquatic food chains. Taking everything into account, the contamination of soil and water with imidacloprid should be monitored on a regional level in the scale of watersheds in order to protect the environment (Mineau P. and Palmer C., 2013: 8-9).

2.4.3 Uptake, metabolism, and effects on plants

Neonicotinoids are absorbed by plants through the roots when grown from treated seeds or applied to the soil, or through leaves at foliar application. Once absorbed, they are trans-located throughout plant tissues, including leaves, fruits, pollen, and nectar where the highest concentration is found in shoots. In plants, neonicotinoids are also metabolized, so in addition to the active compound, its different metabolites are also present (Gervais J.A. et al., 2010). Studies suggest that from 1.6 to 20 % of the active ingredients are absorbed by the plants from seed dressing. The concentrations in plant tissues and sap, ranging from 5 to 10 ppb are generally sufficient to protect the plant against insects (Goulson D., 2013).

A high concentration of neonicotinoids can be also found in guttation because plants bio-accumulate neonicotinoids in guttation in concentrations that are even higher than their concentrations in preparations for crops treatment. Guttation is a natural plant phenomena found in all vascular plants, most typical for grasses and young plants, and

represents the excretion of xylem fluid (sap) through the hydatodes at leaf tips and margins. Guttation occurs because of the slight osmotic pressure which causes water that enters the roots to rise. The phenomenon is the strongest at night when the stomata are closed and, therefore, the osmotic pressure is higher, but it is also present at day time. However, it is not geographically limited just to areas with humid air and moist soil and can easily be found in drier conditions, too (Girolami V. et al., 2009).

The contamination of guttation occurs when neonicotinoids were applied for seed treatment. The contamination of guttation drops is the most intensive in the first three weeks after the sowing. The concentrations also vary depending on humidity, temperature, and wind conditions. It has a high environmental relevance because guttation is a food source for some beneficial insects, including bees, and contaminated guttation therefore represents a direct way of intoxication of the beneficial insects (Girolami V. et al., 2009).

There are three main ways of metabolism in plants: hydroxylation and subsequent loss of water, reduction and/or loss of the $-NO_2$ group, and oxidative cleavage of the methylene bridge between the two rings, giving various metabolites (EFSA, 2008: 19).

In plants, imidacloprid can be metabolized into a guanidine metabolite, an olefin metabolite, urea derivative 1-[(6-chloropyridin-3-yl)methyl]imidazolidin-2-one and 6-chloronicotinic acid and 5-OH-imidacloprid. Three of the plant metabolites (imidazolin derivate, olefin metabolite, and nitroso derivative) are even more toxic to aphids than imidacloprid. However, the transformation to metabolites is only partial, so the pure compound is partially present in the plant for quite a long time (in sugar beet treated with imidacloprid by soil application, imidacloprid was still present in leafs after eighty days). The half-life of imidacloprid, applied by foliar treatment, in leaves ranged from three to five days. In addition, imidacloprid is transferred to guttation in an even higher concentration than other neonicotinoids, despite the fact that a lower amount is used for seed treatment, and can reach a concentration of up to 200 mg/L (Gervais J.A. et al., 2010).

Imidacloprid is also known to have the ability to change gene expression in plants. It delays the expression of drought stress marker genes and suppresses the expression of pathogenesis related genes, which stimulate photosynthesis and improve resistance of plants to draughts (Jeschke P. et al., 2011).

Experts of EFSA think that there is low risk of phytotoxic effects to non-target plants. Five dicots (dicotyledon) and six monocots (monocotyledon) were observed, and no phytotoxic effects were found (EFSA, 2008: 47).

2.4.4 Residues of imidacloprid in food and water

Imidacloprid is widely present in food. It is more commonly present in fresh, unprocessed food than in processed food. In one monitoring, carried out in 2006 by The United States Department of Agriculture (USDA) Pesticide Date Program, imidacloprid was detected in 80 % of all bananas and cauliflowers, in 72 % of spinach samples, 17.5 % of applesauce, and 0.9 % of raisin samples, 26.6 % of apple samples, and 18.1 % of grapes samples. However, the detected concentrations were under the limit of the EPA in all cases (Gervais J.A. et al., 2010).

The EPA stated the reference dose (RfD) for imidacloprid for humans to be 0.057 mg/kg/day. RfD is an estimate of the quantity of a certain chemical that a person could be exposed to every day for the rest of their life without risk of adverse health effects. The chronic population adjusted dose (cPAD) is 0.019 mg/kg/day and the acute population adjusted dose (aPAD) is 0.14 mg/kg/day (Gervais J.A. et al., 2010).

For imidacloprid in drinking water, the EPA stated the drinking water levels of comparison (DWLOCs), which determine the upper limits for imidacloprid concentration in drinking water. DWLOC for acute exposure is 3,625 ppb (3.625 mg/L) and 1,775 ppb (1.775 mg/L) for chronic exposure (Fossen M., 2006).

2.5 Neurotransmission and homeostasis of calcium

2.5.1 Neurotransmission

A typical neuronal cell consists of a cell body, many dendrites, and one axon, as shown in fig. 4. Dendrites have multiple branches ending with postsynaptic terminals (postsynaptic spines), on which different receptors controlling ion channels are located. The axon is a long extension of the cell that can also be branched. At the end of the axon are presynaptic terminals. The connection between presynaptic and postsynaptic terminals is called a synapse. Signals are transferred from the presynaptic terminal of one neuron to postsynaptic terminals of another neuron. The presynaptic terminal forms vesicles where neurotransmitters are stored. These vesicles are docked with the membrane, enabling the release of neuronal transmitters into the synapse (Ross W.N., 2012; EFSA, 2014: 12).

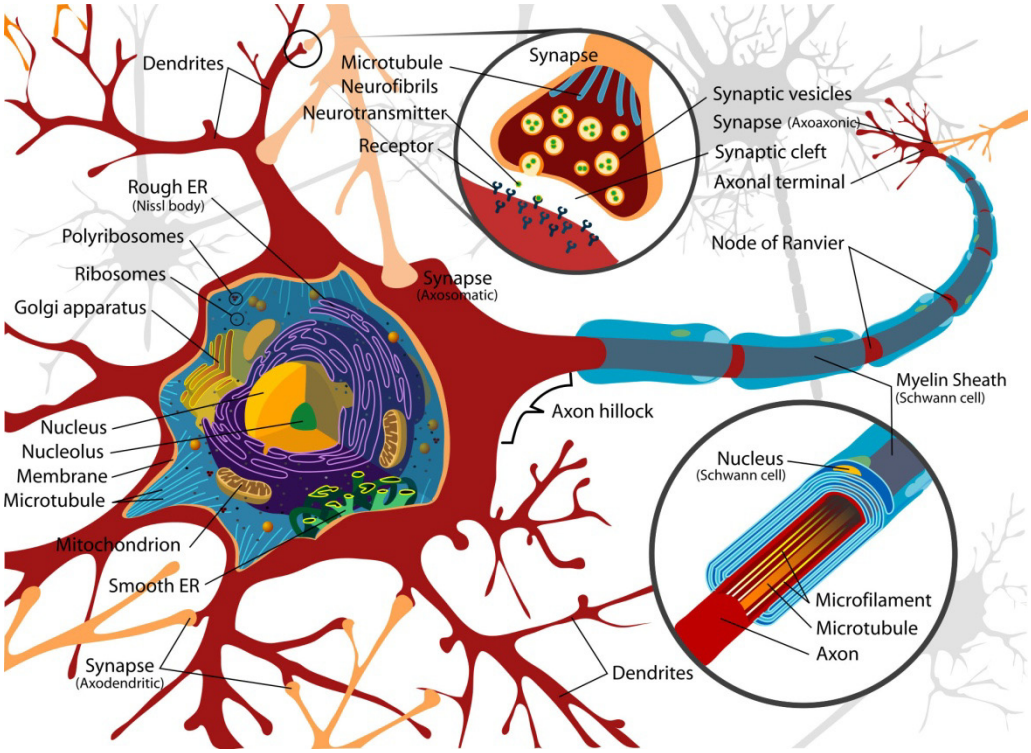

Figure 4: *Complete neuron cell diagram (Wikipedia, picture released in public domain)*

When action potentials invade presynaptic terminals, they cause the release of neurotransmitter in the intersynaptic space. Released neurotransmitter molecules bind to post-synaptic receptors, open the ligand-gated ion channels, which changes the membrane potential of the postsynaptic neuron, determining the transmission of electrical signals in the nervous system.

2.5.2 Homeostasis of calcium

Intracellular Ca^{2+} is an important intracellular messenger whose homeostasis is accurately regulated (Grienberger C. and Konnerth A., 2012). Most neurons have the resting concentrations of Ca^{2+} between 50 and 100 nM. During electrical activity, intracellular calcium concentrations can rise up to 10 to 100 folds (Berridge M.J., 2000). Cytosolic calcium influxes, effluxes, and its exchange with the intracellular compartments are kept in balance by several different types of channels. The cytosolic Ca^{2+} concentration is also regulated by the Ca^{2+}-binding proteins. Two main groups of calcium channels exist: the voltage gated calcium channels (VGCCs) and the ligand-gated calcium channels. When Ca^{2+} enters the cell membrane, the membrane potential is changed, which is called depolarization (Grienberger C. and Konnert A., 2012).

VGCCs represent a broad class of calcium channels that are highly selective for Ca^{2+}. Different types of VGCCs have a wide variety of voltage-dependent activations with different voltage thresholds depending on their biophysical, pharmacological, and molecular features, and are also present in different neurons and neuronal sub-compartments. VGCCs can be divided according to their activation potential; there are two groups, high-voltage activated (HVA) and low-voltage activated (LVA) calcium channels. They are effectively activated by the back propagation of action potential and by synaptically-mediated depolarization of dendritic spines. They are the main determinants of somatic Ca^{2+} signals. HVA are traditionally classified as L- (“long lasting”), P- (“Purkinje”)/Q-, N- (“neutral”), and R- (“residual”) type calcium channels. Certain types are present in certain types of neurons and also in different cellular compartments. LVA channels belong to the T-type (“transient”). VGCCs are also involved in calcium-mediated gene expression cascade (Catterall W.A., 2000).

Ligand-gated calcium channels represent an extensive group of calcium channels including ionotropic glutamate receptors NMDA (N-Methyl-D-Aspartate Receptors), metabotropic glutamate receptors mGluRs (7-transmembrane G protein-coupled receptors, broadly distributed within the nervous system), nicotinic acetylcholine receptors (nAChRs), and transient receptor potential C-type channels (TRPC). The two main groups of ionotropic glutamate receptors are NMDA and Ca^{2+} permeable AMPA receptors (α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptors) (Grienberger C. and Konnerth A., 2012).

NMDA receptors mediate a major part of postsynaptic Ca^{2+} influx. They are non-specific channels that permeate sodium, calcium, and potassium ions, where the fraction of Ca^{2+} varies between 6 and 12 %. They are involved in the process of long-term modification of synaptic strength. Specific properties of the receptors depend on the subunit composition, the status of phosphorylation of the receptor, and the membrane potential of the neuron (Burnashew N. et al., 2000).

Ca^{2+} permeable AMPA receptors are permeable to sodium, calcium, potassium, and zinc ions. They have fast kinetics and contribute to the mechanisms of the synaptic plasticity in aspiny neurons (Jonas P. et al., 1994).

Other sources of Ca^{2+} influx are nAChRs and TRPC channels. A more detailed description of the nAChRs structure and function is given in subsection 2.5.3. The TRPC channels are non-selectively permeable to cations (Na^{+}, Ca^{2+}, Mg^{2+}). They can be activated by a broad variety of stimuli, for instance by phosphatase C, and can also open due to depletion of intracellular Ca^{2+} stores (Ramsey I.S. et al., 2006).

Fig. 5 summarizes the most important sources of neuronal Ca^{2+} signalling showing influxes and effluxes of Ca^{2+} and their release from the internal sources of the cell (Grienberger C. and Konnerth A., 2012).

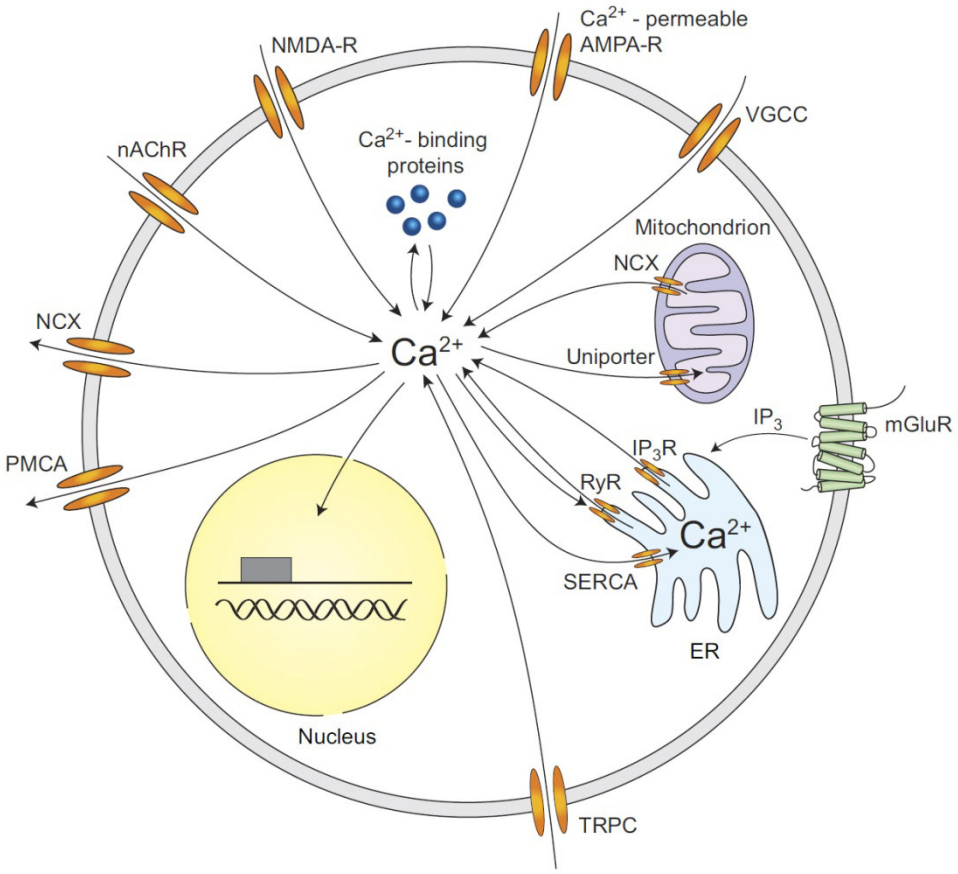

Figure 5: *Neuronal calcium signalling (Grienberger C. and Konnerth A., 2012: 863)*

Important regulators of cytosolic Ca^{2+} are the systems that regulate Ca^{2+} release from internal stores, such as endoplasmatic reticulum (ER) and mytochondria. The Ca^{2+} release from endoplasmatic reticulum, where the calcium concentration is very high, is mediated by inositol triphosphate receptors (IP_3Rs) or by ryanodine receptors (RyRs). IP_3 that binds to IP_3Rs is released by the activation of phospholipase C through mGluRs (Niswender C.M. and Conn P.J., 2010). The RyRs are activated by other sources of cytosolic Ca^{2+} and amplify the action started by other channels (Ca^{2+}-induced Ca^{2+} release). The transport of Ca^{2+} from the cytoplasm back to the

endoplasmatic reticulum is performed by sarco-/endoplasmatic reticulum calcium ATPase–SERCA (Berridge M.J., 1998).

The mytochondria can serve as a calcium buffering system, which can uptake cytosolic Ca^{2+} through the Ca^{2+} uniporter and release it through the sodium-calcium exchange NCX.

Cytosolic Ca^{2+} is also regulated by calcium-binding proteins (calcium buffers) such as parvalbumin, calbidin-D28k, calretinin (Duchen M.R., 1999; Schwaller B., 2010).

Ca^{2+} has to be transferred from cytosol to extracellular space. This is mediated by the plasma membrane Ca^{2+} ATPase (PMCA) and the sodium-calcium exchanger (NCX) (Berridge M.J. et al., 2003).

Different contributions in neuronal Ca^{2+} signalling are very difficult to monitor because different sources are active simultaneously, their activity overlapping with strong interactions (Grienberger C. and Konnerth A., 2012).

2.5.3 Nicotinic acetylcholine receptors (nAChRs)

nAChRs are fast, ligand-gated, ionotropic receptors that form channels located in pre-, post-, and extrasynaptic membranas. In insects, these channels are only found in the neurons of the central nervous systems, but in mammals, they are present in the central and peripheral nervous systems and in neuromuscular junctions (Fucile S., 2004; EFSA, 2014: 11).

nAChRs consist of combinations of subunits α, β, ɣ, δ, and ξ, arranged around the central pore. Each subunit has its transmembrane domain with N- (amino terminus) and C-terminal (carboxyl terminus) located extracellularly. The neuronal nAChRs subtypes are various homomeric or heteromeric combinations of 12 subunits—α2 to α10 and β2 to β4. Diversity in subunit composition influences the nAChRs properties, such as gating mechanism, single channel conductance, acetylcholine-affinity, and Ca^{2+} permeability, determining a high functional diversity. Ca^{2+}, in particular, permeability determines the activation of Ca^{2+}-dependent intracellular processes, including Ca^{2+} induced Ca^{2+} release and the activation of biochemical pathways. The combinations of subunits in nAChRs are specific in different regions of the central nervous system. In most vertebrates' brains, they appear to be combinations of α4β2 and α7 subtypes (Grienberger C. and Konnerth A., 2012; EFSA, 2014: 11-12). A scheme of two nAChRs is shown in fig. 6.

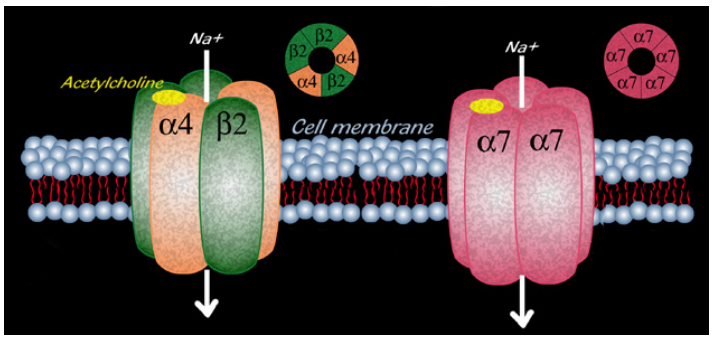

Figure 6: *nAChRs (Georgi S., 2005)*

For opening the central pore at least 2 molecules of ACh have to be bound on the 2 active sites that are located on the junctions of α4 subunits with other subunits (Georgi S., 2005).

2.6 Ecotoxicology of neonicotinoids

Neonicotinoids, their mixtures and residues in the environment, possess a moderate to a high level of toxicity to wildlife. Affected are invertebrates, such as beneficial insects and aquatic invertebrates, as well as vertebrates like fish, amphibians, birds, and mammals. Therefore neonicotinoids may affect food chains in the water and land ecosystems. Their toxicity to beneficial insects, such as bees, must be considered because a big share of the world's diet (one third of the diet in the US) depends on insect pollinators (Mineau P. and Palmer C., 2013: 3-9).

American Bird Conservancy accused EPA of greatly underestimating the risk neonicotinoids posed to the environment. American Bird Conservancy claimed that EPA had performed risk assessment using scientifically unsound and outdated methodology, did not consider indirect effects of neonicotinoids, did not properly review their effects on aquatic invertebrates, birds, and wildlife, and did not take the warnings of independent researchers, even of their own experts, into account. American Bird Conservancy highlighted the importance of development of biomarkers to be able to detect whether the deaths of birds and other wildlife were caused by intoxication with neonicotinoids. Such biomarkers do not exist yet (Mineau P. and Palmer C., 2013: 34, 67).

2.6.1 Biochemical action of the neonicotinoids

Neonicotinoids are potent agonists, selectively affecting the nicotinic acetylcholine receptors having the ability to bind to several types of nAChRs. nAChRs have a central role in mediation of fast excitatory synaptic transmission in the insects' central nervous system (CNS). A study of binding of neonicotinoids to insects' nAChRs was performed on crystal structures of two soluble homopentameric acetylcholine-binding proteins (AChBPs), which were used as an extracellular model of insects' nAChRs. It was found out that neonicotinoids were bound into the structure, built of two soluble homopentameric acetylcholine binding proteins. It was also discovered that imidacloprid is bound by formation of a N-H bond between the nitro group of imidacloprid and a tyrosine in the active site of the receptor (Jeschke P. et al., 2011).

Imidacloprid has a high affinity of binding but low binding efficacy to human α4β2 containing nAChRs. It can open a channel only to some degree and cannot induce a maximal response. Nicotine also has a high affinity for α4β2 containing nAChRs. When neonicotinoids bind to nAChRs, the binding is followed by a spontaneous discharge of nerve impulses, causing failure of neurons to propagate any signal. This happens because binding to nAChRs is irreversible resulting in sustained activation of nAChRs due to the fact that acetylcholine esterase cannot degrade neonicotinoids (Tomizawa M. and Casida J.E., 2003; EFSA, 2014: 11-13).

Imidacloprid can inhibit normal action of acetylcholine because of desensitization of the nAChRs. (Kimura-Kuroda J. et al., 2012).

The main problem of neonicotinoids is that they have a similar mode of action when it comes to target and non-target organisms, so they are very dangerous to beneficial insects. However, they have a much lower binding affinity to nAChRs of mammals and other vertebrates, including birds, because of a different structure and subunit composition of their nAChRs. In mammals, neonicotinoids cannot pass the blood-brain barrier. In addition to CNS, mammals also have, nAChRs present within the neuromuscular junction (Gervais J.A. et al., 2010).

In contrast to Gervais J.A. (2010), researchers of EFSA claim that imidacloprid and its metabolite, desnitro-imidacloprid, can penetrate through the blood-brain barrier. Both metabolites were found in the brain of the mice after the intra-peritoneal administration. Some studies have shown that desnitro-imidacloprid in mammals behaves similarly to nicotine and has a comparable or an even greater binding affinity for α4β2 containing nAChRs (Tomizawa M. and Casida J.E., 2002; EFSA, 2014: 11).

2.6.2 Imidacloprid - oxidative stress and inflammatory response

Oxidative damage is caused by free radicals, which are atoms, molecules or ions with unpaired valence electrons or an open electron shell. Common free radicals are reactive oxygen species (ROS), reactive nitrogen species (RNS), and H_2O_2. Oxidative stress occurs when the balance between antioxidants and oxidants is moved toward the oxidants. Free radicals are often generated by environmental agents, such as metals, pesticides, and other chemicals.

Many pesticides are known for being able to induce oxidative stress, thereby causing oxidative damage. Oxidative stress is considered to be one of the main mechanisms that link pesticide exposure and diseases, such as neurological disorders and cancer. The relationship between neonicotinoids, oxidative stress and inflammatory events is described by Tomizawa M. (2004) and by Duzguner V. and Erdogan S. (2010).

In a recent study (Duzguner V. and Erdogan S., 2012) the authors researched oxidative stress and inflammatory processes on rats exposed to imidacloprid. Pesticides can alter the formation of free radicals and the levels of enzymatic and non-enzymatic antioxidants. ROS and RNS can be generated during the metabolism of pesticides and also by pure compounds. The typical oxidative damage includes DNA damage, lipid per-oxidation and damage to the proteins, which can potentially lead to cell death with apoptosis or necrosis. A known cause of cell death is mitochondrial dysfunction due to the respiratory chain dysfunction caused by the oxidative damage. Neuro-degeneration can be associated with oxidative stress in the central nervous system, including the hippocampus, cortex, striatum and cerebellum.

It was observed that imidacloprid causes oxidative stress in brain and in liver where it induces a mRNA transcription of three isoforms of nitric oxide synthases (iNOS, eNOS and nNOS), which then leads to increased levels of NO synthases and consequently to an increased level of NO. NO is produced from the amino acid L-arginine by the enzymatic action of NOS. Nitric oxide is a diatomic free radical. Despite having an important role in the homeostatic regulation of cardiovascular, neuronal, and immune systems, it is also a well-known toxic agent. When its level becomes too high, it causes oxidative damage, including lipid per-oxidation, damage to proteins, and DNA damage which may lead to cell death.

In experiments on rats chronically exposed to imidacloprid (dose of exposure 1 mg/kg/day for 30 days), the level of NO increased by 23 % in brain and by 50 % in liver, causing a significant lipid per-oxidation in plasma, brain, and in liver.

In the same experiment, a significant depletion of glutathione was found. Imidacloprid also caused an elevated activity of xanthine oxidase in brain and liver. Xanthine oxidase generates superoxide. Imidacloprid caused decreased activity of liver enzymes aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) by 1.5 and 2.6 times respectively. It increased the activity of myeloperoxidase in liver and catalase in brain up to two times.

Another mechanism associated with pesticide neurotoxicity might be their ability to directly or indirectly modulate immune and inflammatory mechanisms, which is also connected with oxidative mechanisms. Exposure to various insecticides can induce accelerated synthesis of cytokines, such as interferon ɣ (IFN) and tumour necrosis factor-α (TNF). They can also reduce anti-inflammatory cytokine interleukin-10 (IL) and inhibit signal transduction correlated with their toxicity. Stimulation of the nervous system by acute or long-term exposure to these chemicals may lead to synaptic plasticity or attenuated neuronal functions. Chronic exposure of rats to imidacloprid resulted in the induction of chronic inflammation in brain and in liver that was induced by pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12 and IFN-ɣ (Duzguner V. and Erdogan S., 2012).

2.6.3 Toxicity to macro- and micro-organisms in soil

The risk of toxicity to earthworms is low when seeds treated with imidacloprid are used. After six years of field monitoring, no significant decline in number of earthworms was observed. Acute toxicity for earthworms LC50 is 10.7 mg/kg soil dw (dry weight) and reproductive toxicity NOEC≥ 0.178 mg/kg soil dw. Furthermore, the soil -dwelling predatory mite *Hypoaspis aculaifer* (G. Canestrini 1884), was not observed to be affected. For soil arthropod (order springtails) *Falsomia candida* Willem 1902, NOEC is 1.25 mg/kg soil and for the soil-dwelling myte *Hypoaspis aculaifer* NOEC is≥ 2.67 mg/kg soil dw. EFSA does not exclude the potential risk for soil-dwelling arthropods in orchards through the application of imidacloprid with spraying.

The risk to non-target microorganisms in soil is considered to be low when imidacloprid-treated seeds are used. No effects on nitrogen- and carbon-mineralisation were observed 28 days after applying of 2 kg imidacloprid/hectare (EFSA, 2008: 113-115).

2.6.4 Toxicity to terrestrial invertebrates

In recent years, bees colony collapse disorders (CCD) have been observed worldwide, which has resulted in major losses in pollination of plants. There have been cases when worker bees have not returned to their hives. A correlation between the time of corn sowing and the dying of bees was noticed. A hypothesis that connected the use of neonicotinoids with dying of bees was created, but the exact toxicity mechanism, including the way of intoxication, was not known. It was thought that wild flowers might be contaminated by dust and debris of seed coating lifted into the air by sowing mechanisation, but this was not confirmed at first. It was also found that the intoxication

of bees through plants' pollen and nectar is not an adequate cause of CCD because the concentrations of neonicotinoids in pollen and nectar were too low (bellow 10 ng/g=10 ppb) to cause severe damage to bees. It was further hypothesised that bees might be intoxicated by feeding or drinking guttation drops from the plants treated with neonicotinoids, which was later confirmed by a study (Girolami V. et al., 2009).

During the last years, researches have confirmed that bees can be intoxicated by dusting during the process of sowing treated seeds. This risk could be minimized only by permission to use only treated seeds of the highest quality, produced according to the latest available techniques, by using mechanisation that ensures minimal release of dust during storage, transport and sowing, and, at last, by ensuring a high degree of drilling into soil, minimisation of spillage and dust emission (EFSA, 2013: 2).

Latest reports confirm that risks of bees' intoxication by nectar, pollen and guttation exist (Goulson D., 2013). Bees are especially endangered if neonicotinoids are applied to plants in time of blooming. Avoiding foliar application of insecticides during crops and weeds flowering reduces the risk for bees (EFSA 2008: 42).

Acute intoxication with abnormal foraging behaviour of bees was found when the concentrations of neonicotinoids in their food source exceeded 40 µg/L (40 ppb). When the concentrations of neonicotinoids exceeded 0.5 mg/L (500 ppb), first missing bees were observed, and when food sources contained more than 3 mg/L (3,000 ppb), bees failed to return because they died (Girolami V. et al., 2009).

European Food Safety Authority (EFSA) gives data for acute and chronic toxicity to bees for imidacloprid. LD50 for acute oral toxicity is between 3.7 and 70.3 ng/bee and LD50 for acute contact toxicity between 42.2 and 129 ng/bee. In chronic tests, the NOEC value for acute oral toxicity at dietary exposure was 46 ppb, and sublethal chronic effects (learning behaviour) were observed at 50 ppb, chronic lethal effects at 24 ppb and behavioural impacts at 20 ppb. Not only imidacloprid but also some metabolic products of the compound are toxic for bees. (EFSA, 2008: 41-42).

Symptoms of intoxication were generic agitation after consumption of contaminated guttation, followed by jerky inward arching of the abdomen, often profuse regurgitation (at this stage flying capability was still retained) and uncoordinated movements. Then flying capability is blocked and death appears. When intoxication is so severe that flying capability is blocked, it becomes irreversible and death appears inevitably, usually in a few minutes. This happens because neonicotinoids cause paralysis of the thorax muscles.

Seeing as the concentration of neonicotinoids in leaf-guttation drops on plants grown from seeds treated with neonicotinoids are much higher than those that are toxic to bees, they can be easily poisoned and killed. The concentration of neonicotinoids in guttation drops on plants grown from treated seeds are constantly higher than 10 mg/L, with maxima up to 100 mg/L for thiamethoxam and clothianidin, and up to 200 mg/L for imidacloprid. It was also found that thiometoxam and clothianidin were more toxic to bees than imidacloprid, but imidacloprid tends to reach higher concentrations in guttation drops on treated plants (Girolami V. et al., 2009).

The usage of neonicotinoids for seed treatment may be the most problematic in the early spring when there are not many other water sources available and bees exploit guttation drops as a water source as they require intensive drinking activity. When available, bees prefer other water sources, such as nectar fluid. This became

especially relevant after the introduction of a new cold-resistant hybrid corn that can be sowed in the middle of March, when flowers are not yet available. However, sufficient reliable data on how much bees actually drink guttation is not yet available. Repellence of neonicotinoids to bees was not observed (Girolami V. et al., 2009).

One of the other beneficial arthropods that is very sensible to imidacloprid is parasitic wasp *Aphidius rhopalosiphi* De Stefani-Perez 1902 (EFSA 2008: 43). Typical LD50 for insects varies from 0.82 for brown planthopper *Nilaparvata lugens* (Stål 1854*)* to 88 ng/insect for Colorado potato beetle *Leptinotarsa decemlineata* Say 1824, which is 0.82 and 0.67 ng/mg of body weight, respectively (Goulson D., 2013).

Despite neonicotinoids being highly efficient insecticides, a tangible threat of resistance development exists. A high potential of resistance development has already been shown by some insects, such as whiteflies *Bemisia tabaci* (Gennadius 1889), transmitter of plant diseases and greenhouse whitefly *Trialurodes vaporariorum* Westwood 1856, the brown planthopper *Nilaparvata lugens*, the Colorado potato beetle *Leptinotarsa decemlineata*, mango leafhopper *Idioscopus clypealis* (Lethierry 1889) and a few others (Jeschke P. et al., 2011).

2.6.5 Toxicity to terrestrial vertebrates

2.6.5.1 Toxicity to birds

Toxicity of neonicotinoids to birds is lower than acute toxicity of many previously used pesticides, e. g. carbamates and organophosphates, but they are still reasonably toxic. However, their toxicity to birds was underestimated because tests have only been performed on a few avian test species, such as mallards and bobwhites, which is certainly not sufficient, since toxicity among individual species can vary in the range from 1.5 to 10 folds. It is assumed that the bird-population decline in Europe could be related to the use of neonicotinoids (Mineau P.and Palmer C., 2013: 5).

The most dangerous route of exposure for birds to get intoxicated is oral exposure, meaning that birds consume seeds treated with neonicotinoids. They have a high potential to affect birds in the chronic fashion with their strong ability to affect avian reproduction, and also in acute way as they can kill birds when the latter are exposed to high doses. Manufacturers claim that birds' access to treated seed is sufficiently prevented by the burying of seeds into the soil. However, some seeds almost always remain on the surface, and some birds also scrape and dig in soil to find seeds. With the use of current machinery, spills are very common, too. Manufactures also claim that neonicotinoids are repellent to birds, so they should not eat treated seeds, but this does not seem to be truth in terrain conditions (Mineau P. and Palmer C., 2013: 5-7).

Repellence was confirmed in laboratory conditions, but in field conditions birds often consume treated seeds. Repellence, if found in field conditions, strongly depends on the hesitation to consume food from new sources or post-ingestion intoxication and illnesses. Effects on altrical and precocial bird species also remain unclear. Farmland and grassland birds are expected to be the most impaired because they tend to be among the highly exposed to neonicotinoids (Mineau P. and Palmer C., 2013: 5-7).

The extent of exposure strongly depends on the time of availability of the seeds. In acute exposure, imidacloprid proved to be the most toxic, seeing as a single maize seed treated with imidacloprid can kill an average-sized bird. Somewhat less toxic are clothianidin and thiametoxam; a few treated seeds are needed to achieve lethality to birds. Long-term chronic exposure of birds to neonicotinoids can result in deliberation, partial paralysis, and other sub-lethal effects. Bird reproduction during egg-laying season is known to be affected if their diet includes only one tenth of corns treated with any known neonicotinoid (Mineau P. and Palmer C., 2013: 5-7). Sub-lethal effects in birds are hyporeactivity, ataxia, wing drop, diarrhoea, immobility, reduced reproduction, and some others (Goulson D., 2013).

Contrary to Mineau P. and Palmer C., (2013), the experts of EFSA claim that the risk to granivorous birds is low when treated seeds are properly buried into soil and the spills removed because a very low number of seeds remain on the surface of the field (0.17 %).

The seedlings grown from treated seeds represent a potential high risk to herbivorous birds such as wood pigeons *Columba palumbus* Linnaeus 1758. A potential risk to insectivorous birds in orchards cannot be excluded due to the possibility of poisoned insects consumption and the lack of insects (EFSA, 2008: 40).

Goulson D. (2013) agrees with Mineau P. and Palmer C. (2013: 3) that seeds treated with imidacloprid are a threat to birds, seeing as they can be exposed to lethal doses. The US EPA estimated that approx. 1 % of drilled seeds remain accessible to granivorous vertebrates as they are not buried into the soil. Typical rates (50,000 seeds maize/ha and 800,000 seeds oilseed rape/ha, 1 % of seeds) deliver an LD50 to 100 partridges (LD50 for grey partridge is 14 mg/kg body weight).

Water- and coastal- birds can also be affected by neonicotinoids through their effects on aquatic and terrestrial food chains. Neonicotinoids lower the population of insects and aquatic invertebrates that represent the food source for birds, consequently causing a decline in the bird population. However, this linkage may be hard to prove because other factors, such as habitat loss, food supply during winter and migration, and predation, are involved (Mineau P. and Palmer C., 2013: 3-7).

2.6.5.2 Toxicity to mammals

Imidacloprid is efficiently absorbed through the gastrointestinal tract. In rats, the absorption efficiency was 92 % of an unspecified dose and the peak concentration in plasma was achieved after 2.5 h (WHO, 2004). In human intestinal cells, the absorption was also very high, and it was established that active transport system was involved. Dermal absorption of pets was low. Imidacloprid's toxicity is high in case of lung exposure, when it is inhaled in form of aerosols, but very low when inhaling dust. After the rats' absorption of imidacloprid, it was distributed throughout the whole body except the fatty tissues, the central nervous system (CNS), and the mineral portion of bones (Gervais J. A. et al., 2010).

According to EFSA (2014), in mammals (test on rats) imidacloprid is predicted to be metabolized primarily in liver (up to 90 %) in two major different pathways:

- aerobic nitro-reduction catalized by microsomal cytochrome P450 (CYP 450) or by cytosolic molibdo-flavoenzyme aldehyde oxidase (AOX) followed by

oxidative cleavage of the methylene bridge (the bond between the two rings of imidacloprid), and

- hydroxylation of imidacloprid's imidazolidine ring by cytochrome CYP 3A4.

Metabolites formed during, aerobic nitro-reduction are nitrosamine, aminoguanidine derivatives and desnitro-imidacloprid, which is further degraded by oxidative cleavage to imidazolidine and 6-chloronicotinic acid, which is metabolized via glutathione conjugation to form mercaptonicotinic acid and hippuric acid.

In the process of hydroxylation metabolites, 4-hydroxy-, 5-hydroxy- and 4,5-dihydroxy-metabolites are formed. The hydroxylated metabolites can lose water and form olefin metabolite. Some other products of metabolism are also formed, but in minor concentrations. No information on specific metabolism of imidacloprid in the human body exists (EFSA, 2014: 9).

The scheme of imidacloprid metabolism in the lactating goat is shown in fig. 7.

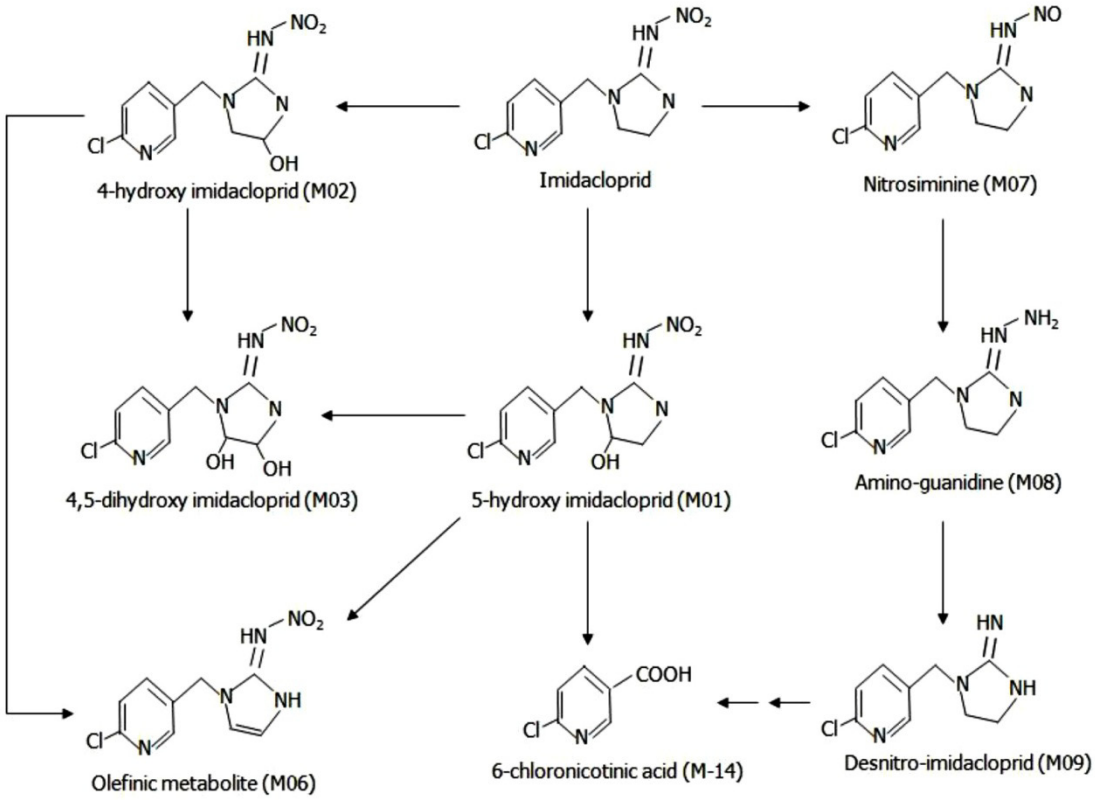

Figure 7: *Proposed metabolic pathways of imidacloprid in the lactating goat (EFSA, 2014: 9)*

Metabolites and unmetabolized imidacloprid are excreted in feces (5-hydroxy-imidacloprid and olefin derivatives) and 6-chloronicotic acid, imidazolidine, 5-hydroxy-imidacloprid and olefin derivatives in urine (Gervais J.A. et al., 2010).

Oral LD50 value for imidacloprid intoxication is 380 mg/kg for female and 500 mg/kg for male rats. For male mice, LD50 is 130 mg/kg and for female mice170 mg/kg (WHO, 2004). The symptoms after oral exposure are salivation and vomiting. In case of a very high oral exposure, the symptoms include lethargy, vomiting, diarrhea, salivation,

muscle weakness and ataxia, which are all indicative for imidacloprid's action on nicotinic receptors. In such a case, death appears in 24 hours. Other signs of exposure to high doses are uncoordinated gait, tremors, and reduced activity (Thyssen J. and Machemer L., 1999). Signs of intoxication appear within 15 min after administration and if intoxication is not too high, they disappear in 24 hours, with the exception of lacrimation and urine staining, which may persist up to 4 days (Sheets L.P., 2002).

In humans, signs of intoxication noticed in case studies are disorientation, agitation, incoherence, sweating, breathlessness, drowsiness, dizziness, vomiting and fever; they are the result of the synergistic effect of imidacloprid and other compounds included in commercial preparations (Deepu D. et al., 2007).

After chronic exposure of mammals to higher doses, the following symptoms were observed: reductions of body weight gain, liver damage, reduced blood clotting function, and platelet counts at 61 mg/kg/day in male and 420 mg/kg/day in female rats, increase in plasma cholesterol, reduced food intake, increased liver weight, and increased activity of cytochrom P 450 (Eiben R. and Rinke M., 1989). It was found that imidacloprid is not carcinogenic and does not change DNA (Thyssen J. and Machemer L., 1999; WHO, 2004).

Adverse effect to mammals, including human, is the effect of nicotine and neonicotinoids on developing brain. Subtypes α4β2 and α7 of the nAChRs are implicated in neuronal proliferation, apoptosis, differentiation, migration, synapse formation and neural circuit formation of mammals. Experiments have been done on rats, but adverse effects on humans may be expected too, as it is known that newborn rats are equivalent to the human embryo from the aspect of brain development (Kimura-Kuroda J. et al., 2012). Passing of imidacloprid through blood-brain barrier (Ford K.A. and Casida J.E., 2006) and high affinity of some metabolites of the neonicotinoids to nAChRs (Tomizawa M. and Casida J.E., 2005) represent a real danger to mammals.

Although neonicotinoids are relatively low toxic to vertebrates, for granivorous mammals exists a high acute risk of consuming high doses of treated seeds spilled during sowing. The same risk exists for herbivorous mammals in orchards and fields (EFSA, 2008: 40). With typical sowing rates and approximately 1 % of seeds remaining accessible to small granivorous mammals the quantity of seeds per hectare can deliver an LD50 to 167 mice. Three treated seeds of maize deliver more than LD50 for a mouse (Goulson D., 2013).

2.6.6 Toxicity to aquatic life

Neonicotinoids proved to be very toxic to freshwater, estuarine, and marine invertebrates in acute and in chronic exposure. The marine invertebrates are even more sensitive than the freshwater ones. Numerous studies have been conducted, but the results are difficult to compare and interpret because of the wide variability of the toxicity values even when standardized methods are used. Comparability of the results obtained from different studies can be especially problematic because of the different exposure times, mostly being 24, 48 or 96 hours, and the ways of reporting the toxicity; it is mostly expressed as lethal or effective dose (LD50 or EC50). Sometimes is it hard to define weather the organism is death or incapacitated. The preferred value to be used is EC50, which is defined by incapacitation of the organism. Criterion of an

organism to be sufficiently incapacitated is that it fails to respond if gently prodded and it detaches from the substrate, being carried away by the current (Mineau P .and Palmer C., 2013: 40-41).

When determining the acute toxicity of imidacloprid, it is important to take into account that the difference between the EC50 (immobilisation) and LC50 (the concentration that kills 50 % of the individuals) for aquatic organisms is much higher than that of other insecticides. For imidacloprid the ratio LC50/EC50 is between 100 and 600. Therefore, the EC50 values should be used. The most sensitive insect families are the harlequin fly *Chironomus riparius* Meigen 1804 and the mayflies *Baetidae*. The toxicity values for *Chironomidae* stated by EFSA (EFSA, 2008) are the following:

- acute toxicity: EC50 value is 55.2 µg/L (55.2 ppb) for 24 h
- 28 days chronic toxicity value: EC5 is 1.9 µg/L (1.9 ppb),
- Community risk: NOEC of 0.6 µg/L (0.6 ppb), given DT50 of 5.8-13 d, LOEC was 1.5 µg/L (1.5 ppb) (at the end of the experiment at this concentration no recovery was observed).

EFSA suggested a safety factor 1-3, which gives the NOEC value of 0.2 µg/L (0.2 ppb) as the highest permissible concentration of imidacloprid for macrozoobenthos (Mineau P. and Palmer C., 2013: 41-42).

The LC50 values for aquatic insects vary from 0.65 to 44 ppb. Differences in LC50 for the same taxa exist because of the different duration of exposure. For example, the LC50 for mayfly *Epeorus longimanus* Eaton 1885 is 2.1 ppb at 24 h, and 0.65 ppb at 96 h exposure. Most crustaceans and annelids are less sensitive than insects. There are only a few studies about the toxicity of imidacloprid to those organisms. For instance, chronic toxicity value for 28 days exposure is 7.1 ppb for the amphipod *Hyalella azteca* Saussure 1858 and acute toxicity 48 h for the brine shrimp *Artemia sp.* Leach 1819 is 361,000 ppb (Goulson D., 2013).

Beside the concentration, the toxicity of imidacloprid is influenced by the specie's sensitivity, organism's mass to surface ratio, and determined by its size, and the developmental stage of an organism. The caddis fly's sensitivity to imidacloprid, for example, varies greatly, up to five times, in dependence on the larval instars (the period of postembryonic growth between moults) (Mineau P. and Palmer C., 2013: 44).

Another factor influencing the sensitivity is the chemical composition of the preparation. Technical active substances contain 95-99 % of the pure compound, while its content in formulated commercial products is usually 40-80 %. The formulants (difference to 100 %) are not always inert substances and some of them, or some of their metabolites, can influence the toxicity of the preparation. Additional toxicity may also be contributed by the metabolites of the active compound (Mineau P. and Palmer C., 2013: 44).

Toxicity also depends on the temperature and the light conditions and is seasonally related. It was found out that the 96 h EC50 for imidacloprid, affecting the amphipod *Gammarus roeseli* Gervais 1835, varied from 1.9 to 129 µg/L (1.9 to 129 ppb), with the highest values in field test observed in spring. The illumination enables and accelerates the chemical degradation of imidacloprid by photolysis. Laboratory tests showed that the toxicity was even twice as high in light than it was in the dark. This could be caused by the toxicity of the metabolites. Therefore, the reactions of imidacloprid in turbid and

strongly coloured water are expected to differ from those in clear, deeply illuminated water (Mineau P. and Palmer C., 2013: 45).

Fish are distinctly less sensitive to neonicotinoids than the insects. LC50 values can differ greatly, ranging from 16 to 177 ppm or more (Goulson D., 2013). The toxicity of imidacloprid to fish also can differ greatly. The LC50 for a 96 h exposure is 237 mg/L (237 ppm) for golden orfe *Leuciscus idus* Linnaeus 1758, and for rainbow trout *Oncorhyncus mykiss* (Wallbaum 1792) the LC50 for a 96 h exposure is 21 mg/L (Gervais J.A. et al., 2010).

The risk of the bioaccumulation of imidacloprid and its metabolites is considered to be low in water ecosystems (EFSA, 2008: 41).

3 EXPERIMENTAL PART

The aim of the experiments was to compare the effects of imidacloprid and nicotine on neuronal cells. It is known that both compounds have a high nAChRs-binding affinity in mammalian neuronal cells, but the difference between them is that nicotine has a higher receptors'-activating efficiency than imidacloprid. The activation of the nAChRs is closely related to the intracellular Ca^{2+} homeostasis and to the excitability of the neuronal cells.

The effects of the imidacloprid and the nicotine on intracellular Ca^{2+} homeostasis in cells were tested on the primary cultures of the hippocampal neurons and on the iMN9D cell line. Their impact on viability of the neuronal cells was also tested on hippocampal neuron primary cultures. Intracellular Ca^{2+} was monitored with an epifluorescent microscope using fluorescent Ca^{2+} probes. Imidacloprid's and nicotine's effects on the viability of the neurons were determined by a cell-counting technique with pre-exposed cells stained by protein-specific antibodies and fluorescent dyes.

3.1 Plating cells

3.1.1 Plating hippocampal neurons

3.1.1.1 Hippocampal neurons primary cultures

Primary neuron cultures can be prepared from hippocampus, cerebral cortex, or other brain regions. They can be prepared from fetal, neonatal, or adult brain derived neurons. The primary culture of rat and mouse neurons cultures are widely used in the areas of neurotoxicity and neuropharmacology. They are also used in the validation of various drug targets and their influence on the central nervous system disorders, such as Parkinson's and Alzheimer's disease, and amyotrophic lateral sclerosis (Ray B. et al., 2009). Protocols for preparing the cultures from cortex or hippocampus are described in literature.

3.1.1.2 Cleaning of coverslips

For plating the cells, round coverslips with a diameter of 18 mm and a surface of 254 mm^2 were used. 3.6 mL of the cell suspension is sufficient for preparing around 16 coverslips. For the incubation of coverslips with cells, 4 Petri dishes with volume of 5 mL were used.

First the coverslips were cleaned and sterilized according to the following protocol: all coverslips from one box were placed into a 50 mL Falcon tube containing 30 mL of HCl 0.5 M. The acid was prepared by diluting 37 % HCl (Acids Shelf), first 1:10, and then once more 1:2, with deionized water. The coverslips were separated by gently twisting of the Falcon tube, which was then positioned horizontally and left overnight at room temperature. HCl was taken away and the coverslips were rinsed three times with water (each time the tube was twisted). Water was removed, as quantitatively as possible, with a plastic Pasteur pipette, and 30 mL of absolute EtOH (ethanol) were added. The Falcon tube was twisted, laid in a horizontal position, and left for one hour at room temperature. Ethanol was then removed, the coverslips were transferred into a glass Petri dish and left in an oven at 150 °C for 1-1.5 hours (dry sterilization).

3.1.1.3 Preparation of growth medium

An initial medium for growing of the neuron culture was prepared as follows: to 500 mL of MEM w / Glutamax, 3 g of D-glucose, 1.8 g of Hepes (buffer), 50 mg of Apo-Transferrin, 15 mg of insulin (dissolved in 10 mL of H_2O, 1-2 drops of 1 M NaOH added), 0.05 mg of biotin (stock solution 1 mg/mL), 0.75 mg of vitamin B12, 125 µL of Gentamicin and dialized 5-10 % FBS (fetal bovine serum, added just before each use), were added. Then pH was adjusted to 7.3 and the medium was filtered through a filter 0.22 (pore size 0.22 µm).

Before the experiments two aliquots of the medium with different additives were prepared. For the first aliquot 2 mL of FBS (10 % solution) were added to 18 mL of the initial medium and the solution was filtered. The second aliquot was prepared from 19 mL of initial medium by adding 1 mL of FBS and 6.6 µL of Ara-C (anti-mitotic cytarabine that blocks proliferation of glia cells), dilution 1:3,000. The solution was filtered before use.

3.1.1.4 Sample preparation for microscopy

Rats have two hippocampi, one in each half of the brain. The hippocampi are suspended, giving 3.6 mL of the cell suspension. The sample contains about 50,000 to 100,000 cells (neurons and glia cells) per 100 µL of suspension.

The sterilized coverslips were coated with polyornithine, which is a synthetic aminoacid chain. The polyornithine coating is used to provide an enhanced cells' adhesion to the glass (or plastic) surfaces. Each of the 16 coverslips was coated with 200 µL of polyornithine solution and incubated over-night. The stock solution of polyornithine (concentration 5 mg/ml) was diluted with pure water in a ratio of 1:30 (133 µL polyornithine and 3,867 µL of water). In all experiments, 4 mL of the polyornithine solution were prepared. Before the treatment of the coverslips, the polyornithine solution was filtered through a filter 0.22 (pore size of 0.22 µm).

Before plating the cells, each polyornithine-coated slide was treated with a drop of matrigel for a few minutes.

250 µL of the cell suspension were administered onto the coated coverslips. The coverslips were put into Petri dishes, and the medium (from the first aliquot) was added. The cells were incubated at 37 °C in a controlled CO_2 atmosphere (5 % CO_2). After three days, half of the medium was replaced by the medium from the second aliquot.

3.1.2 Plating iMN9D cells

The MN9D-Nurr1Tet-On (iMN9D) cell line was derived by fusion of the mouse embryonic ventral mesencephalic cells and the mouse neuroblastoma cells. iMN9D cells are widely used as a model of dopaminergic (DA) neurons derived from mouse substantia nigra pars compacta (SNc), because of their ability to synthesize tyrosine hydroxylase and release dopamine (Choi H.K. et al., 1991). They are used to test the mechanisms of loss of DA neurons in Parkinson's disease (Holtz W.A. and Malley K.L., 2003). They can be used in an undifferentiated or in a differentiated form, but better resemble properties of DA neurons if used in differentiated form. However, their electrophysiological properties are not exactly the same as with DA model and largely depend on the method of the differentiation (Rick C.E. et al., 2006).

The most similar properties of the iMN9D cells to the DA neurons can be achieved by a differentiation method, where the iMN9D cells are first treated with GDNF (glial cell line-derived neurotrophic factor) followed by butyric acid. But even after this differentiation method the cells still lack the A-type potassium currents and feature smaller Ca^{2+} and Na^{+} currents than the DA neurons (Rick C.E. et al., 2006).

3.1.2.1 Growth medium

The iMN9D cells grow in a medium prepared from a mixture of 50 % of DMEM + Glutamax (Dulbecco's Modified Eagle Medium) and 50 % of F12 (nutrient mixture). To this mixture 10 % of the fetal bovine serum, 100 µg/mL penicillin, 10 µg/mL streptomycin and 200 µg/mL G418 (to add fresh) are added.

For growing the cells DMEM/F12 medium (Life technologies), fetal bovine serum, penicillin and streptomycin (Sigma Aldrich) were used.

3.1.2.2 Thawing cells

Vial with the frozen cells is thawed in a water bath. Then the content (about 1 mL) is pipetted into a centrifuge tube, 9 mL of medium are added and gently mixed. After spinning at 500 RCF (relative centrifugal force) for 5 min the supernatant is removed. The cells are re-suspended in 10 mL of the medium (without G418), plated into a 10 cm dish and incubated for 24 h at 37 °C in a humidified CO_2 atmosphere. After 24 h the cells are checked for adherence and potential contamination. When the cells recover from thawing, supplemental medium (with G418) is added.

3.1.2.3 Splitting cells

To split the cells the medium is first removed from the plate and the cells are washed with the PBS 1X (phosphate buffer). Subsequently, 1 mL of sterilized trypsin is sloshed gently over the cells and the cells are incubated at 37 °C for 5 min. 5 mL of the medium are added and the liquid with cells is pipetted upward and downward for a few times to break clamps of the cells. After this, the cells are transferred into a centrifuge tube and centrifuged at 500 RCF for 5 min. The supernatant is removed and 2 mL of the medium are added. Then the cells are triturated, using a 1 mL pipette tip, to obtain a well mixed single cell solution. Then the cells are counted and plated onto new plates.

3.1.2.4 iMN9D differentiation

For differentiation, to the cells incubated in medium as described above, neomycin 250 mM (Life Technologies) and 3 µg/mL of doxycycline (Sigma Aldrich) were added. The medium, with the additives for differentiation, was changed every 48 hours.

The iMN9D cells grow in a medium prepared as described in 3.1.2.1.

3.1.3 Fixation of cells

After imaging the samples can be fixed for the later analyses. The fixation is done with a solution of paraformaldehyde (4 %) in phosphate buffer (PBS). To achieve the fixation of the cells, the sample has to be kept in the solution for 30 min and then to be washed with the PBS.

3.2 Basics of calcium imaging

In general, the equipment required for calcium imaging consists of a computer-supported microscope equipped with suitable excitation and emission filters, a light source, and a CCD camera (Lichtman J.W. and Conchello J.A., 2005). A typical scheme of an epifluorescent microscope is shown in fig. 8.

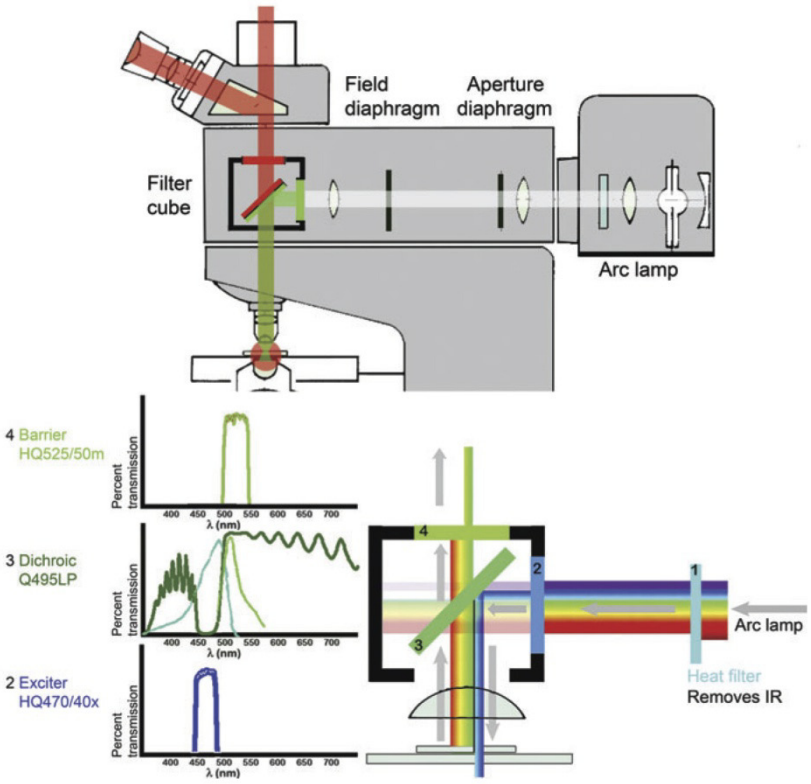

Figure 8: *Fluorescence microscope (Lichtman J. W. and Conchelo J.-A., 2005: 915)*

3.2.1 Bases of digital imaging

Bases of the digital imaging are adopted from the publication Nikon MicroscopyU (Spring et al., 2013).

In microscopic images, and in each monochromatic digital image, the values of the light intensity are defined as the relative, discrete grey values with limited dynamic range and bit depth.

The dynamic range defines the limits of the detection of the camera's sensor. It represents the difference between the lowest and the highest light intensity that the sensor can detect. If the light intensity is under the lower threshold of the sensor it is captured as black, and if it exceeds the upper threshold it is captured as white.

The tonal range is defined by the bit depth and tells how many different grey values in the gradient between black and white can be captured by the sensor's A/D converter (analogue to digital converter), representing the greyscale gradation. In practice it tells what is the smallest difference between two light intensities that sensor is able to capture as two different grey values.

Most experimental setups have a monochromatic camera that produces microscopic images with a bit depth of 12 or 16 bits. This means that each pixel, integrated into the sensor, represents a given intensity of the light with one of 4,096 or 65,536 possible

discrete grey values, where 1 represents pure black and 4,096 or 65,536 represent pure white on the greyscale.

In theory, 16 bit images captured by today's sensors, should provide a sufficient dynamic range and a fine enough greyscale gradation for an adequate representation of the sample's fluorescent intensities. But there are many factors causing that only a part of the theoretically available colour scale can be used to represent the real sample's fluorescence values as grey values, which results in the limited accuracy, and the lowered dynamic range of the measurements (Spring K. R. et al., 2013).

One of the major factors affecting the range of the greyscale, that the camera´s sensor can percept, is a low fluorescence intensity of the dyes, that cannot be avoided, because high concentrations of the dye would interfere with intracellular Ca^{2+} homeostasis and the Ca^{2+} signalling pathways (Neher E., 2000).

When acquiring a single image, this may not be a problem because one can make good use of the dynamic range that the camera allows by adjusting the exposure time and the sensitivity of the light sensor.

3.2.2 Background fluorescence

When performing the imaging with fluorescent dyes one definitely has to take into account the possible appearance of the background fluorescence. The background fluorescence can appear from many reasons such as fluorescent fixatives, or sample medium, refraction mismatch among the immersion medium, the coverslip and the objective, or by the cellular debris in the sample (fluorescent proteins or other compounds). In case of a sample with a fluorescent background, the measured fluorescence value consists of the sum of the background fluorescence and the fluorescence of the object (cell). To obtain a real fluorescence value of the object of interest there are two different methods to exclude the error of the background. The first method is to measure the fluorescence of the background image without the sample (blind probe) and then subtract the measured values from the fluorescent values in other images. The second method is to measure an average fluorescent value of the background, in an empty area of the current field of view, and subtract that value from the values in the area of interest (Lichtman J. V. and Conchello J.-A., 2005; Bootman M. D. et al., 2013).

3.2.2.1 Live imaging and sampling-rate

To follow the fast changes in fluorescence intensity it is necessary to acquire a fast, continuous sequence of the images. This means that exposure times have to be short to allow a fast shooting rate. Because of this, images might appear underexposed, resulting in the fact that only a part of the available dynamic range is used to record the sample's fluorescence.

3.2.3 Calcium dyes

The concentration of the intracellular Ca^{2+} can be monitored by the optical microscopic technique called calcium imaging. It relies on using the fluorescent calcium dyes, such

as Fura-2, Fluo4, Oregon Green-BAPTA and others that change their fluorescent properties upon binding of Ca^{2+}.

The calcium dyes are fluorescent molecules which can be excited with monochromatic light (λ_{ex}) corresponding to the absorption maximum and emit light of longer wavelength (λ_{em}) than the excitation one (Bootman M. D. et al., 2013).

Calcium dyes are available in cell permeable or impermeable forms. The cell permeable dyes can be loaded into the cells by applying them to the solution and leaving them to penetrate the cell membrane (Oregon Green, Fura-2-AM). The cell impermeable dyes must be directly injected into the cells with microinjectors, electroporation, or through diffusion from a patch clamp pipette.

Under exposure to the light, most dyes undergo photo-bleaching. The process occurs due to the reaction of indicator with oxygen causing generation of non-fluorescent molecules of the dye. A decrease of the fluorescence intensity can also occur because of intercellular compartmentalisation of the dye. Such decrease of the fluorescence may severely affect the measurements' accuracy. When using definite dyes, it is possible to reduce the loose of ability to fluoresce by photo-bleaching (Bootman M. D. et al., 2013).

3.2.3.1 Single-wavelength (non-ratiometric) Ca^{2+} indicators

The fluorescence intensity of the dye, present in a cell, depends on the number of the dye molecules (intracellular dye concentration), illumination intensity, dye's absorption coefficient, and dye's quantum yield. However, only a part of the dye-emitted light can be detected by the sensor of the microscope camera, due to the fact that the photon collection efficiency of the optical setup and the quantum efficiency of the sensor are always lower than 1. Almost all indicators of this class are excited in the visible wavelength range. There is no sufficient shift in the excitation and/or the emission wavelength, when Ca^{2+} is bound, to allow a ratiometric measurement. Graph a) in fig. 9 shows an example of the emission spectra for a single-wavelength indicator. In graph a) there are fluorescence intensities for Ca^{2+}-bound (dashed line) and for Ca^{2+}-free (solid line) of the indicator (Helmchen F., 2000).

When using the single-wavelength Ca^{2+} indicators it is not clear if the changes in the fluorescence intensity are a result of the differences in Ca^{2+} concentrations or the differences arise from different dye absorption of the individual cells (in the same field), or the bleaching and dye compartmentalisation. Therefore, the single-wavelength indicators are mostly used for qualitative measurements where the results are expressed as relative changes in Ca^{2+} concentrations (Bootman M. D. et al., 2013). An example of such indicator is Oregon Green BAPTA-1, AM.

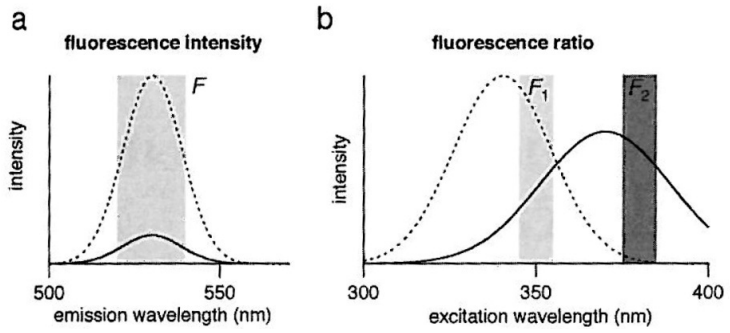

Figure 9: *Changes in fluorescence properties of calcium indicators (Helmchen F., 2000: 32.2)*

3.2.3.2 Dual-wavelength (ratiometric) Ca^{2+} indicators

The dual-wavelength indicators have two absorption and emission optimums. When Ca^{2+} is bound, the excitation and emission optimums are shifted. An example of the shift of the emission optimums for a typical dual-wavelength indicator is shown in graph b) in fig. 9. In the graph the dashed line represents the fluorescence intensity for Ca^{2+}-bound (saturated) and the solid line the intensity for Ca^{2+}-free (zero) form of an indicator (Helmchen F., 2000).

The ratio between both emission optimums (with background correction) depends only on Ca^{2+} concentration and is independent of changes in dye concentration, optical path length and light intensity (Helmchen F., 2000).

The disadvantage of the dual-wavelength indicators is that their dynamic range (difference between emission light intensities of Ca^{2+}-free and Ca^{2+}-bound form of the indicator) is often low, and can be even lower than at the single wavelength indicators, which reduces the ability to detect small changes in Ca^{2+} concentration (Bootman M.D., 2013).

To achieve live imaging with good time resolution rapid switching of the wavelengths is essential, so a light microscopic setup with a fast filter switching system is required (Helmchen F., 2000).

Dual-wavelength indicators, such as Fura-2-AM, are mostly used for quantitative measurements of Ca^{2+} concentrations (Bootman M. D. et al., 2013).

3.2.4 Image analysis software

Most imaging software enables controlling of the microscope and camera, as well as analysing the microscopic images and time-lapse images (video). A very useful feature that is usually provided is the monitoring of the dynamic range, when performing imaging, using a histogram. In the imaging software, a histogram is a real time updated graph showing the distribution of the grey values on the colour scale from black to white. If the distribution of the grey values is uniform from black to white, this indicates a good dynamic range of an image, but if the majority of grey values is shifted to one (e.g. the dark) side of the histogram, this results in a reduction of the dynamic range.

Image analyses are usually performed by special image-analysing software, like ImageJ (free), Axio Vision or SlideBook, and many others that can be used to read the grey values of specific areas of interest in an image and to calculate their average values. By acquiring multiple images, one can also monitor the time course of the fluorescence within a specific area in the sample (cell or a specific part of the cell). Grey values of individual pixels represent fluorescence intensities, which enable to determine the average fluorescence values within specific areas of interest in an image.

From the fluorescence values, concentrations of Ca^{2+} can be calculated by using a calibration curve. In most cases, relative values of fluorescence, which only enable to compare the fluorescence of different areas, are obtained because the determination of absolute values requires excessive and complicated calibration process.

3.2.5 Calibration of measurements

To determine the concentration of the intracellular Ca^{2+} it is essential to prepare a calibration curve. It requires a formula that mathematically describes the relation between the by Ca^{2+} induced fluorescence value of the dye and the belonging grey value at the given experimental conditions. The calibration has to be performed with at least three (optimally five) different, pre-defined concentrations of Ca^{2+}. The calibration can be done in vivo (inside the cell), using the patch clamp technique, or in vitro, with microslides or capillaries filled with a solution closely imitating the cytoplasmic conditions during the experiment (Helmchen F., 2000).

3.3 Microscopic setup

The epifluorescent live calcium imaging was performed on an Olympus IX 81 microscopic system equipped with a Lambda DG-4 fluorescent light from Sutter Instruments featuring, built-in fluorescent filters and a system for rapid filter switching, a high sensitive camera Hamamatsu ORCA-R2 and the SlideBook 5 image analysis software (fig. 10). For the Ca^{2+} imaging an apochromatic objective (APO) with a 40x magnification was used. The fluorescence microscopy was performed with an epifluorescence design, meaning that the excitation light was focused onto the specimen through the objective lens. The coverslips with cells were inserted in the imaging chamber which was fully integrated with the table of the microscope and enabled usage of a constant cells' perfusion system and regulation of the temperature. Volume of the imaging chamber was 750 µL.

Figure 10: *Epifluorescence microscopic setup ready for imaging*

A simple and reliable, self-composed perfusion system setup based on the principle of gravity, was used. The perfusion setup was composed of a laboratory stand and clamp, a syringe housing, plastic tubes, nozzle holders, nozzles, a three way- and a one-way valve, Erlenmeyer flasks and a vacuum pump. The syringe housing was mounted on the stand and served as a reservoir of the perfusion solution. Closing the influx of the perfusion solution was enabled by a valve built-in under the reservoir, and the removal of bubbles was enabled by the three-way valve under the reservoir. The flux was regulated by adjusting the height of the reservoir. To provide the perfusion solution influx as laminar as possible, without formation of waves and vortexes, the nozzles were placed at a slight angle of approximately 30 degrees. This was very important because waves could cause distortions of the microscopic image, local changes of illumination and image aberrations due to different refractions of the light with different wavelengths. These distortions could cause huge errors because the value of Ca^{2+} content in the sample depends on the fluorescence intensity of the dye. Nozzles for the setup were improvisational, made from pipette tips, which lengths were adjusted simply by scissors. Then they were bent and their size was adjusted by thermo-mechanical treatment.

To minimize the interference of the light from the surroundings, the space between the microscope table and the objective was shaded.

Fluorescent images of the fixed samples, stained with fluorescent dyes, for the purposes of the morphological evaluations of cells and determination of the cells' survivability rates were photographed with Carl Zeiss Axio Observer Z 1 microscope.

The microscopic system was equipped with AxioCamMR3 12-bit camera, and for imaging EC Plan-Neofluar 10x/0.30 Ph 1 objective was used.

3.4 Cell functionality evaluation

3.4.1 Cell perfusion solution – Ringer's solution

The Ringer's solution is used for perfusion of the cells under the microscope. It consists of definite salts and glucose, as the source of energy for the cells. Its composition is given in the table below. Amounts of the reagents in the table are sufficient for the preparation of 0.5 L of solution.

Table 2: *Composition of Ringer's solution*

Compound	Concentration [mM]	Volume [mL] or mass [g]
NaCl	145	14.5 mL, [5 M]
KCl	3	1.5 mL, [1 M]
$CaCl_2$	1,5	0.75 mL, [1 M]
$MgCl_2$	1	0.5 mL, [1 M]
Glucose	10	0.9 g
Hepes	10	5 mL [1 M]

The reagents are dissolved in water, the solution diluted close to 0.5 L and the pH value is adjusted to 7.4 with NaOH. Then the solution is diluted to 0.5 L.

3.4.2 Cell staining with Oregon Green BAPTA-1, AM

Oregon Green BAPTA-1, AM is a non-ratiometric dye, excited by visible light at the wavelength of 488 nm, and emitting the light of wavelength 510 nm. In the experiments, 488 nm filters were used for the excitation-, and 450 nm filters for the emission-light. Using visible light for the dye excitation has the advantage of avoiding the UV-induced toxicity; on the other side the disadvantage is that non-ratiometric measurements do not allow to compare quantitatively the light intensity values for long periods of time due to the dye bleaching. Values that are comparable are only the ones measured in short time frames where bleaching is not significant (Oregon Green 488 BAPTA-1, AM).

Oregon Green BAPTA-1, AM solution for staining cells was prepared from a stock solution with a concentration of 4 mM to obtain a final concentration of 2 μM in the bath. For staining the cells were exposed to the dye for 30 min. After that, the coverslips were washed with Ringer's solution and placed into the imaging chamber. The intracellular concentration of the dye was not known and was different for individual cells.

3.4.3 Drugs administration and live imaging of the samples

After staining the cells with Oregon Green BAPTA-1, AM, the coverslips with the cells were inserted into the imaging chamber, Ringer's solution was added and the chamber was installed into the table of the microscope. Insertion of a coverslip with the cells is shown in fig. 11.

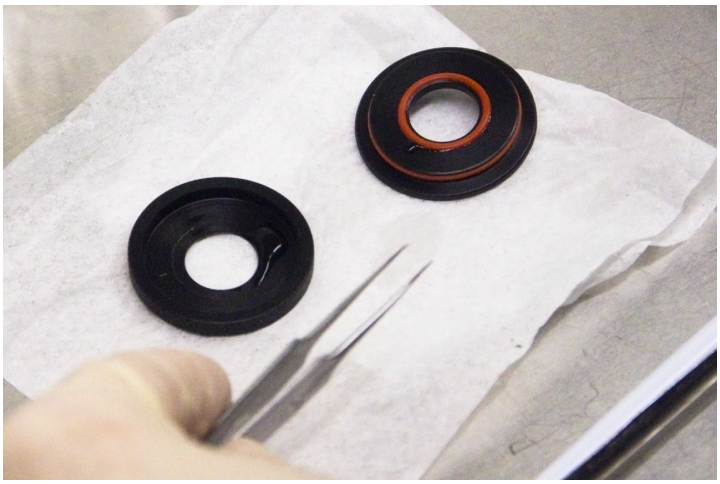

Figure 11: *Insertion of the sample into the imaging chamber (imaging chamber disassembled)*

Then the perfusion system with Ringer's solution was established (fig. 12). The solution was heated in a thermo block to 37 °C before use.

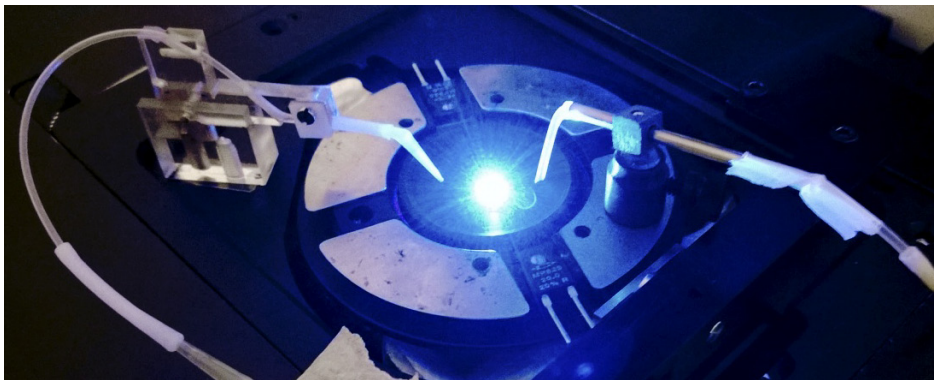

Figure 12: *Imaging chamber with the perfusion system*

During imaging the microscope was controlled by SlideBook 5 program. Then the sample was quickly viewed through the ocular of the microscope and a field of view with an appropriate arrangement of the cells was chosen. Afterwards, a differential interference contrast (DIC) image with the visible light of the chosen field was captured.

This process was followed by the fluorescence live-imaging, showing the changes of Ca^{2+} concentration inside the cells. For the Ca^{2+} imaging experiments it is of great importance to properly set the exposure time in order to avoid clipping of the highest fluorescence signal at cells' depolarisation and the appearance of a too low signal to noise ratio toward the end of the experiment, as the dye gradually bleaches. The

exposure time was set using the live-image histogram in Slidebook 5. It was set in the way that the brightest highlights (grey values) were approximately in the middle of the camera's dynamic range in the histogram. This provided a good starting point as it enabled capturing Ca^{2+} peaks (exposure increase) and enabled also capturing the signals when the fluorescent intensity gradually decreased because of the bleaching.

In different sets of experiments different settings of the fluorescent lamp intensities were used. In the first experiments the fluorescent light was set to 50 %, but this caused a too high bleaching therefore the fluorescent light intensity was lowered by changing the alignment of the galvanometric mirrors in the lamp system. In the last set of experiments the bleaching was further reduced by use of the 25 % neutral density (ND) filter.

At most recordings the exposure time was around 100 ms which enabled a capturing frequency around 10 frames per second. The exact values depended on the cells absorption of the dye, the lamp settings and the camera's sensitivity-gain. At all recordings the camera's binning was set to 8x8 to improve the signal to noise ratio.

Generally, it was observed that the iMN9D cells showed a lower absorption of the dye (Oregon Green Bapta-1, AM) than the hippocampal neurons, so longer exposures and higher gain settings were needed to obtain a proper exposure. This resulted in recordings with a slightly worse signal to noise ratio and a lower accuracy of the relative values of calcium or in a decreased frame rate because of the longer exposure settings (time accuracy of the measurement).

In each set of the experiments first a control recording, showing spontaneous Ca^{2+} concentration changes inside the cells, was captured. After this, the tested compound was administered through the perfusion system, or with a pipette added directly into the imaging chamber. Some samples were pre-exposed to the chemicals before the microscopy.

When adding the compound directly into the imaging chamber, perfusion had to be stopped that the compound was not washed away before the cells could respond to it. In this case, the upper column of the microscope had to be lifted to provide an easier access to the imaging chamber. This caused some huge artefacts in the recordings because of the vibrations and changes in the illumination.

In some experiments the tested compound was added through the perfusion system (acute exposure). If this was the case, it took around 20 to 30 s for it to reach the imaging chamber.

3.5 Cell recovery

3.5.1 Cells' exposure

3.5.1.1 Overnight exposure

To evaluate the viability and morphology of neurons and glia cells at a long term exposure to imidacloprid and nicotine the following experiment was designed. Four

coverslips with the hippocampal neurons were exposed to the nicotine and imidacloprid overnight in an incubator. One of the coverslips was the control, the second was exposed to imidacloprid of a concentration of 10 μM, the third to a concentration of 100 nM and last one to nicotine at a concentration of 10 μM. After the exposure, the cells were fixed and stained with the fluorescent dyes for imaging. The cells were stained with DAPI (staining of nuclei), anti-β-tubulin (staining of cytoskeleton) primary rabbit antibody (1:1,000, Sigma) with secondary goat anti-rabbit antibody with bound Alexa Fluo R' 488 (1:500, Sigma) and Neu-N (staining of somata of neurons) primary mouse antibody (1:50, Millipore) and secondary anti-mouse antibody with bound Ms Alexa Fluo 594 (1:500, Millipore).

3.5.1.2 Calcium homeostasis evaluation after short term exposure

Eight coverslips with the neurons were first exposed to the drugs for 2 hours. Two of them were controls, two were exposed to imidaclopid at a concentration of 50 μM, two to nicotine at a concentration of 50 μM, and two to rotenone of a concentration of 100 μM. All coverslips were incubated for two hours.

After this, the Ca^{2+} homeostasis of the cells on each of the coverslips was evaluated. The spontaneous activity of the neurons by monitoring of intracellular Ca^{2+} was evaluated before and after the addition of gabazine.

After the Ca^{2+} live imaging the cells were fixed and stained. In this experiment the cells were stained with DAPI and with anti-β-tubulin primary rabit antibody (1:1,000, Sigma) and with secondary goat anti-rabbit antibody with bound Alexa Fluo R' 488 (1:500, Sigma).

3.5.2 Photographing samples

In the first experiment (overnight exposure) after staining 15 multi-layer images of each coverslip were photographed at 10x magnification on the Zeiss and 10 images at 40x magnification on the Olympus microscope. Each multi-layer image contained three images in individual layers photographed at the wavelengths determined by the excitation and emission maximums of the dyes and an image photographed at visible light. Other images in individual layers were photographed at 470 nm (emission) for DAPI (excitation 350 nm), at 525 nm (emission) for Alexa Fluo 488 (excitation 490 nm) and at 603 nm (emission) for Alexa Fluo 594 (excitation at 578 nm).

In the second experiment only 15 images for each coverslip at a 10x magnification on the Zeiss microscope were photographed.

3.5.3 Image analysis in Image J

The image analysis was the same for both of the experiments described above. There were only minor differences as the cells from the second experiment were not stained with Alexa Fluo 594. Images photographed at the 10x magnification from the first experiment were analysed in Image J. With a cell counter plug-in, the number of all cells and the number of neurons in each image were counted. All cells were counted in

the layer showing nuclei (DAPI). The neurons were counted in the layer showing somata of neurons (Alexa Fluo 594).

The only difference when analysing images from the second experiment was the method of counting the neurons. Neurons were visually identified and counted by inspection of visible, Alexa Fluo 488 and DAPI layers. This was important because DAPI and Alexa Fluo 488 non-selectively stain all cells. In some images, the cells were very dense and overlapping. Areas of cells' overlapping also appeared overexposed in some layers. This meant that the neurons were much harder to identify than in the first experiment where they were selectively stained with Alexa Fluo 594.

3.6 Data analysis

3.6.1 Analyses of live images (movies)

The image sequences (movies) were analyzed in SlideBook 5 and in SlideBook Reader 5.5. Within the SlideBook program, areas of three to ten cells and the background area were selected from the first frame of the sequence. The program then automatically calculated the average grey value of each selected area for each frame in the sequence. The SlideBook also draws a graph showing the fluorescence dependence on the time, expressed as the number of time points, for each cell. Then the fluorescence values in arbitrary units (au) versus time, for all the cells and the selected background area, were exported into the Excel.

The background fluorescence was automatically subtracted from the fluorescence of the cell during exporting the data. Then the graphs showing dependence of the fluorescence from the time were drawn. This was followed by calculating the curve of bleaching. The bleaching followed approximately an exponential curve and was predictable. In typical measurements increases of the Ca^{2+} concentration, representing the cells' depolarisation measured as fluorescence intensities, are shown as small peaks (spikes) on the approximately exponential curve approaching the x-axis. The curve represents the resting intracellular Ca^{2+} concentration. The resting intracellular Ca^{2+} concentration is approximately constant so in theory it should result in the constant fluorescence intensity of the dye giving a straight line in the graph. However, as the dye is bleaching, despite the concentrations of Ca^{2+} remain constant, the fluorescence intensity is constantly falling with the time which is resulting in a curved line in the graph.

After the polynomial equation of the bleaching curve was found (an approximation of the real bleaching rate), the data were numerically corrected for bleaching and normalized. This was done by equation (1) which corrects the bleaching and transforms the fluorescence values from arbitrary units to relative fluorescence changes:

$$\frac{\Delta F}{F}(t) = \frac{F(t) - F_0(t)}{F_0(t)} \quad (1)$$

where: $\Delta F/F(t)$ = relative normalized fluorescence value, $F(t)$ = fluorescence in every time-point (t) and $F_0(t)$ = value from the curve of bleaching in every time-point.

Correction of the bleaching enables that one can see the changes in Ca^{2+} concentration more clearly and the conversion of the fluorescence in arbitrary units to relative changes of fluorescence from 0 to 1 makes it possible to compare the data for different cells from the same microscopic field of view.

However, in some cases the bleaching correction was not possible, because of very strong, long-lasting Ca^{2+} signals and a too short measuring time, which resulted in a too small number or no data-points representing the resting Ca^{2+} concentration, to find the curve of bleaching. If this happened, the fluorescence intensity was only transformed into relative fluorescence changes from 0 to 1 by the equation (2):

$$\frac{\Delta F}{F}(t) = \frac{F(t) - F_0(t_0)}{F_0(t_0)} \quad (2)$$

where: $\Delta F/F(t)$ = relative fluorescence value, $F(t)$ = fluorescence in every time-point (t) and $F_0(t_0)$ = value of the fluorescence at the start of the measurement of the intracellular Ca^{2+} concentration.

A problem with finding $F_0(t_0)$ can occur if the measurement starts with a peak of Ca^{2+}. If the peak is small the calculation can be started after the peak.

For acute exposure, average maximum amplitudes $\Delta F/F(t)$ of Ca^{2+} peaks were calculated. The same was done with the data from the pre-treatment experiments. Results are presented in graphs.

3.6.2 Cell density

The data were analysed in Excel by the same method for both of the experiments. The total number of cells and the number of neurons were determined by counting, and the number of glia cells was calculated by subtraction the number of neurons from the number of all cells in each image. The results are presented as graphs.

Then the share of neurons in percentages in each image was calculated followed by the calculation of the average share of neurons from all 15 images from each coverslip. After this, standard deviation was calculated. The procedure was the same for all coverslips. The shares of the glia cells were calculated by the same method as above. Finally, a graph showing shares of the neurons and glia cells in each coverslip was made.

Statistical significance of the results was determined by the application of the T-test. The result is considered to be statistically significant if the T-test value is 0.05 or lower.

4 RESULTS

4.1 Hippocampal neurons

4.1.1 Acute exposure of hippocampal neurons to imidacloprid and nicotine

Graphs (fig. 13, 15 and 16) show the acute responses of hippocampal neurons intracellular Ca^{2+} to the delivery of Ringer's solution (control, fig 13), imidacloprid (fig. 15) and nicotine (fig.16). All stimuli were administered directly, with a pipette. The final concentrations of imidacloprid and nicotine were 10 µM. In each graph, Ca^{2+} signals of three representative cells, marked on the DIC image next to the graph, are shown. The curves showing these signals are drawn with different colours that correspond to the colours of the circles marking the cells in the DIC image. The times at which the tested compounds were administered are marked with purple squares on the x-axes.

4.1.1.1 Control sample

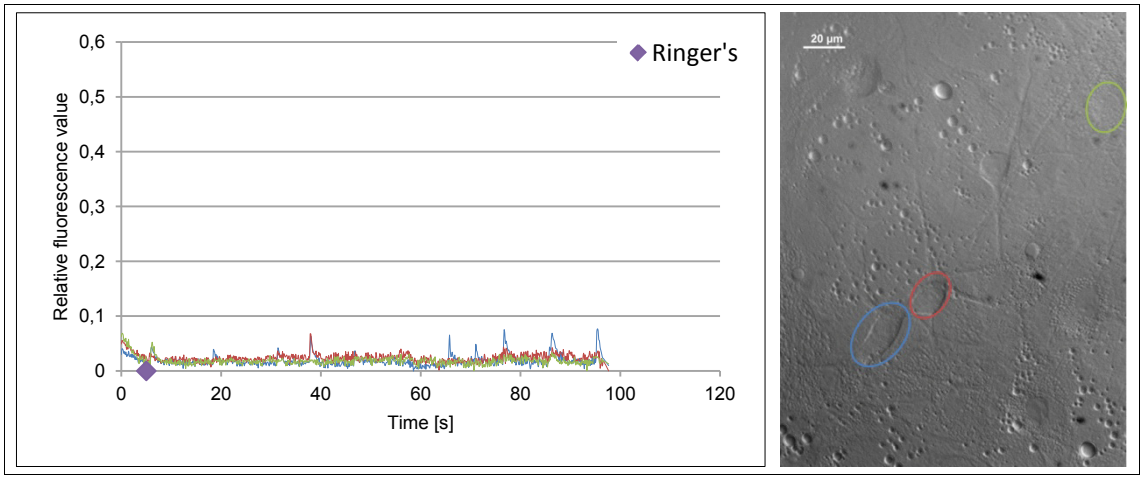

Figure 13: *Spontaneous Ca^{2+} signalling of the control sample*

Graph with its corresponding DIC image in fig. 13 shows Ca^{2+} signals of three hippocampal neurons that were treated with Ringer's solution.

One of our cultures of the hippocampal neurons (control sample) is shown in the microscopic composite image (fig. 14).

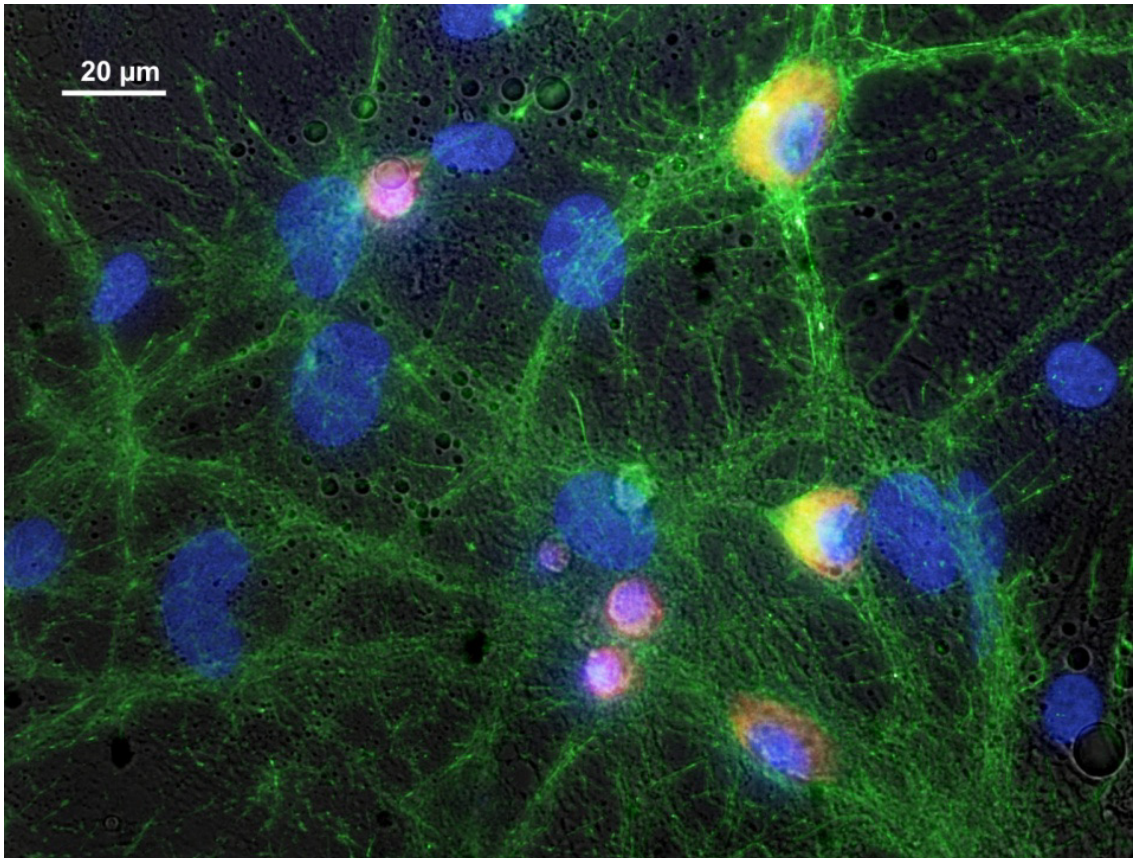

Figure 14: *Culture of hippocampal neurons*
Nuclei – blue, cytoskeleton – green, somata of neurons – yellow

The cells are stained with DAPI (staining of nuclei), anti-β-tubulin combined with the secondary antibody with bound Alexa Fluo R' 488 (staining of cytoskeleton), and Neu-N combined with secondary antibody with Ms Alexa Fluo 594 (staining of somata of neurons).

4.1.1.1 Imidacloprid

Graphs a) and b) with their corresponding DIC images in fig. 15 show Ca^{2+} signals of three representative hippocampal neurons in the two samples that were acutely treated with imidacloprid in concentration of 10 μM.

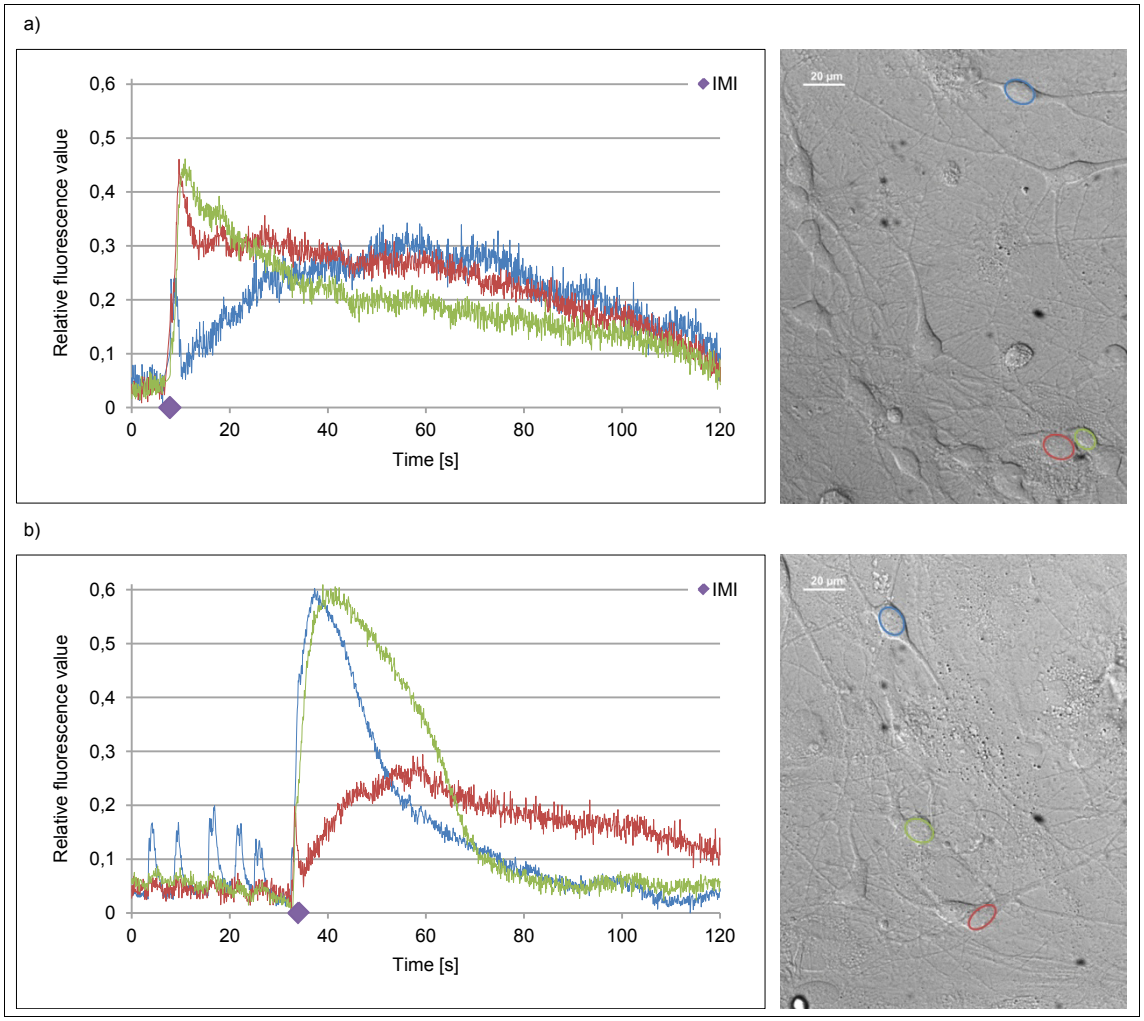

Figure 15: *Acute responses of hippocampal neurons to imidacloprid.*

An increase of cytoplasmic Ca^{2+} concentration, following the IMI delivery to hippocampal neurons, is clearly seen in the graphs above.

4.1.1.2 Nicotine

Graphs a) and b) in fig. 16 with their corresponding DIC images present Ca^{2+} signals of three hippocampal neurons in the two samples that were directly treated with nicotine in concentration of 10 μM.

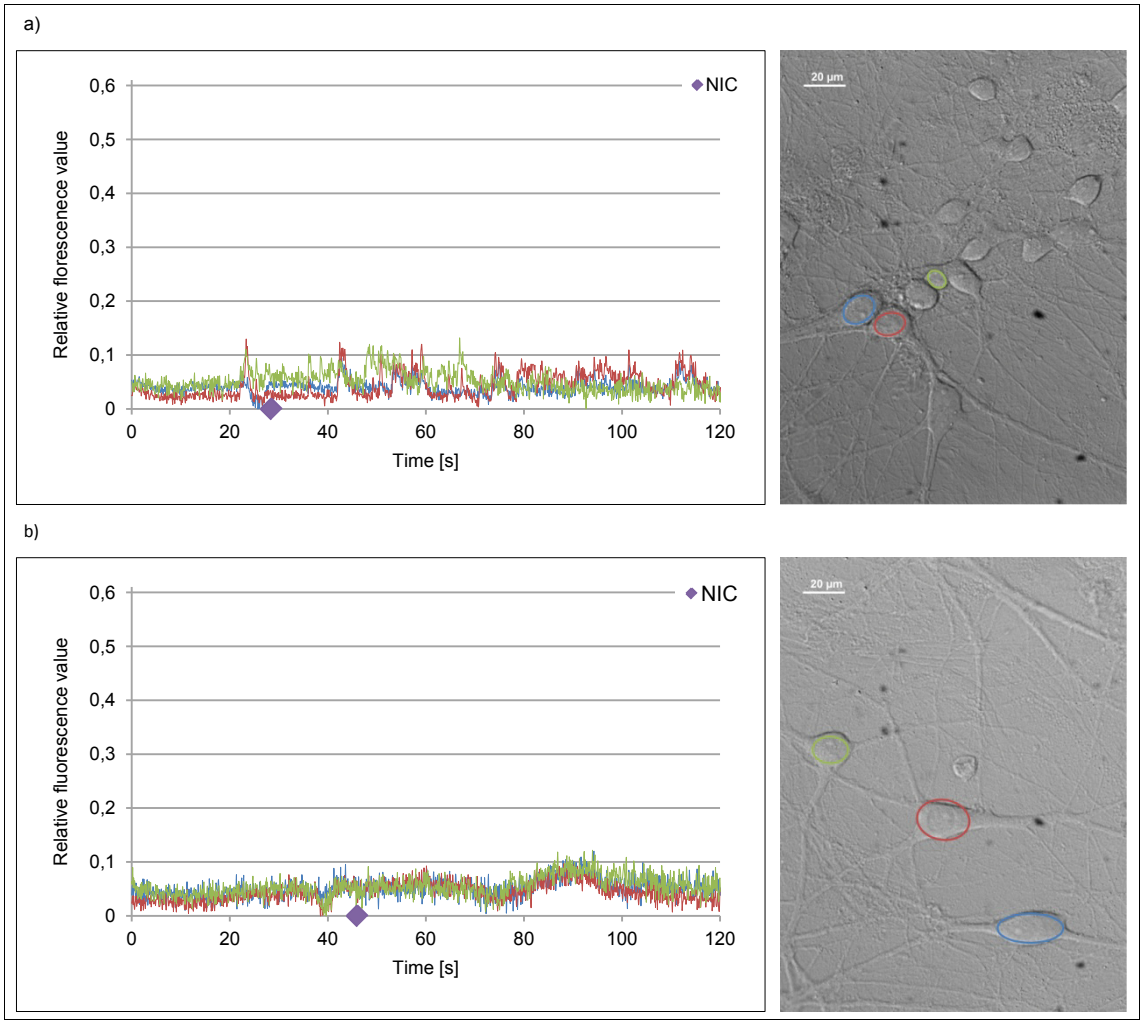

Figure 16: *Acute responses of hippocampal neurons to nicotine*

Graphs in fig. 15, especially graph b), show that nicotine does not significantly increase the influxes of Ca^{2+} into the cytoplasm in hippocampal neurons. Changes of cytosolic Ca^{2+} after adding nicotine are similar to those before addition of the drug except for an increase in the frequency of spontaneous events shown in graph b).

4.1.1.3 Average maximum amplitudes of Ca^{2+} peaks

Average maximum amplitudes of the relative Ca^{2+} changes of two control samples and two samples acutely exposed to imidacloprid (10 µM) are presented in fig. 17. In each of the two samples of the same type responses of 6 cells were measured, which gives 12 measurements per sample type (n = 12).

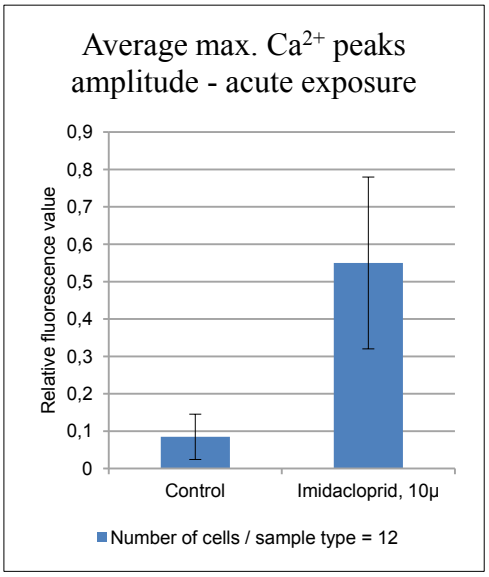

Figure 17: *Average max. Ca^{2+} peaks amplitude - acute exposure*

Acute exposure to nicotine (10 µM) did not seem to produce single peak responses. In one over the two experiments performed, it was observed a significant change in spontaneous Ca^{2+} events frequency.

4.1.2 Pre-exposure of hippocampal neurons to imidacloprid and nicotine

Graphs (fig. 18 to 20) show effects of imidacloprid and nicotine on the spontaneous Ca^{2+} events of hippocampal neurons recorded after two hours of exposure to the compounds and 24 hours of recovery. For comparison, a control sample was recorded. The concentration of imidacloprid and nicotine in both pre-exposure solutions was 50 µM.

After recording the spontaneous activity, gabazine was administered to each sample and the samples were recorded further. Gabazine blocks the inhibitory signals in the network formed by hippocampal neurons, resulting in a synchronized depolarization of all neurons in the sample. Gabazine was added to the samples to test the network connectivity after the pre-exposure to imidacloprid and nicotine.

In each graph, Ca^{2+} signals of three representative cells are shown. The cells are marked on the DIC image next to the graphs. The curves showing these signals are drawn with different colours that correspond to the colours of the circles marking the cells in the DIC image.

4.1.2.1 Control sample

Spontaneous Ca^{2+} signalling of hippocampal neurons from the control sample is shown in fig. 18. Graph a) with its corresponding DIC image shows spontaneous Ca^{2+} signalling of the three marked neurons (control). Graph b) shows the same area after the addition of gabazine (control with gabazine).

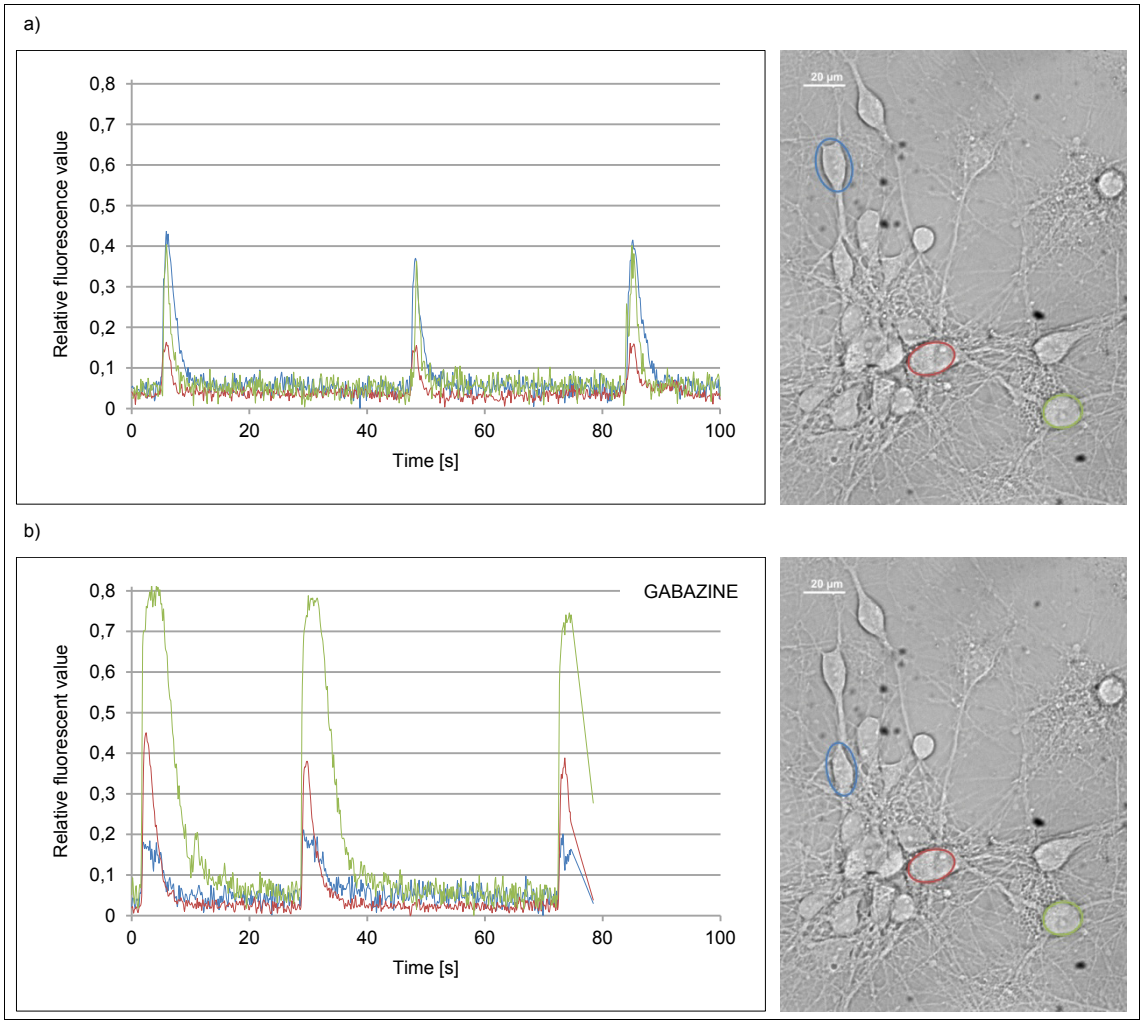

Figure 18: *Spontaneous Ca^{2+} signalling of hippocampal neurons without a) and with gabazine b)*

Graph b) in fig. 18 shows the effect of gabazine on relative fluorescence values in the control sample. If we compare graph b) to graph a) in the same figure (control without gabazine), we can see that gabazine increases the influxes of Ca^{2+} into the cytoplasm during the depolarization, seeing as the peaks in graph b) have approximately twice as high amplitudes than the ones in graph a). This increase of Ca^{2+} peaks reflects the full synchronization over the entire network.

4.1.2.2 Imidacloprid

Graph a) in fig. 19 with its corresponding DIC image shows spontaneous Ca^{2+} signalling of three neurons pre-exposed to the imidacloprid in concentration of 50 μM. Graph b) shows the same after the addition of gabazine.

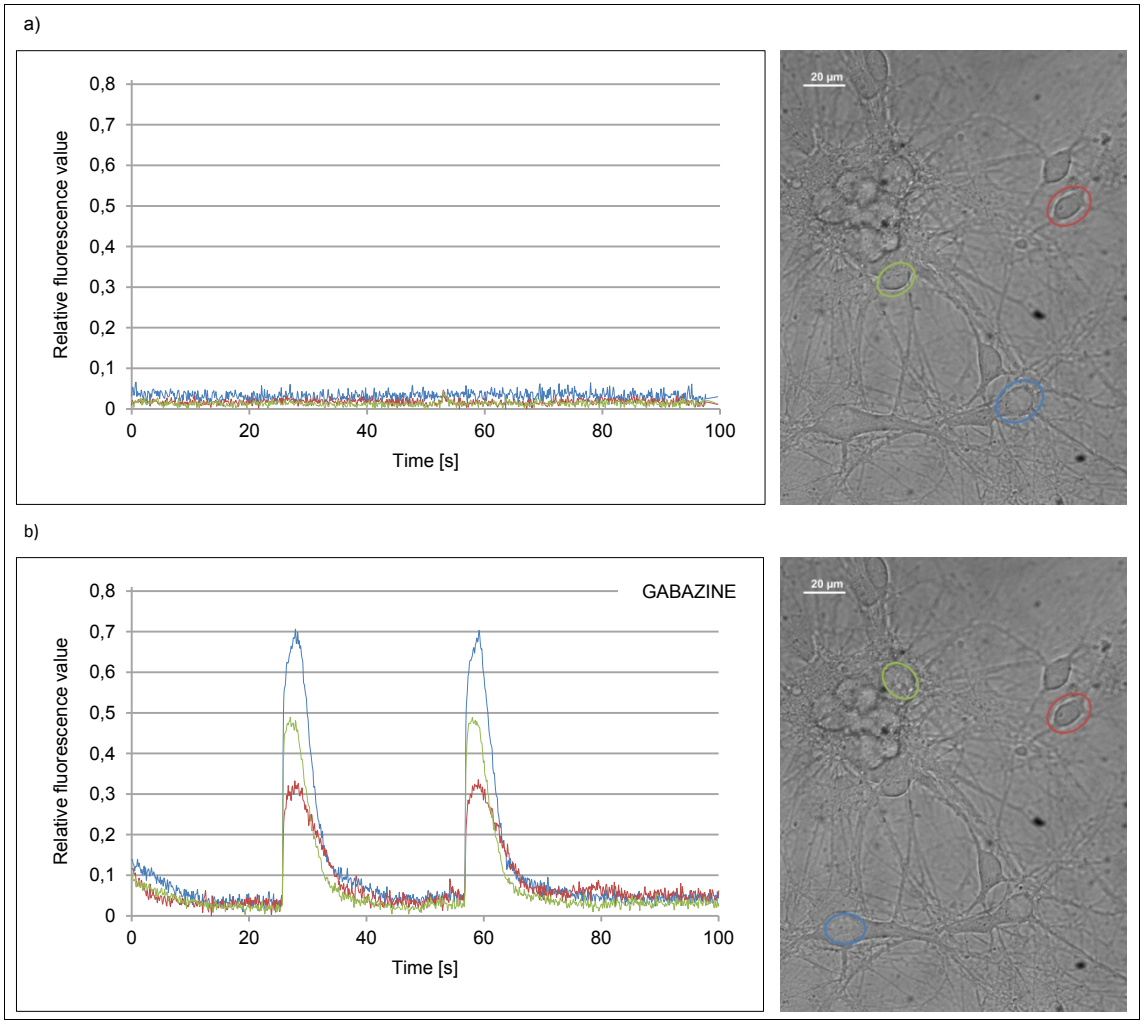

Figure 19: *Spontaneous Ca^{2+} signalling of hippocampal neurons pre-exposed to imidacloprid*

Graph a) in fig. 19 shows that pre-exposure of neurons to imidacloprid caused reduced spontaneous activity of the culture. However, after the administration of gabazine, as shown in graph b) in the same figure, the depolarization of cells was synchronous and the relative increases in cytosolic Ca^{2+} concentration were of similar amplitude as those in the control (fig.18 b).

4.1.2.3 Nicotine

Graph a) in fig. 20 with its corresponding DIC image shows spontaneous Ca^{2+} signalling of three neurons pre-exposed to the nicotine in concentration of 50 μM. Graph b) shows the same after the addition of gabazine.

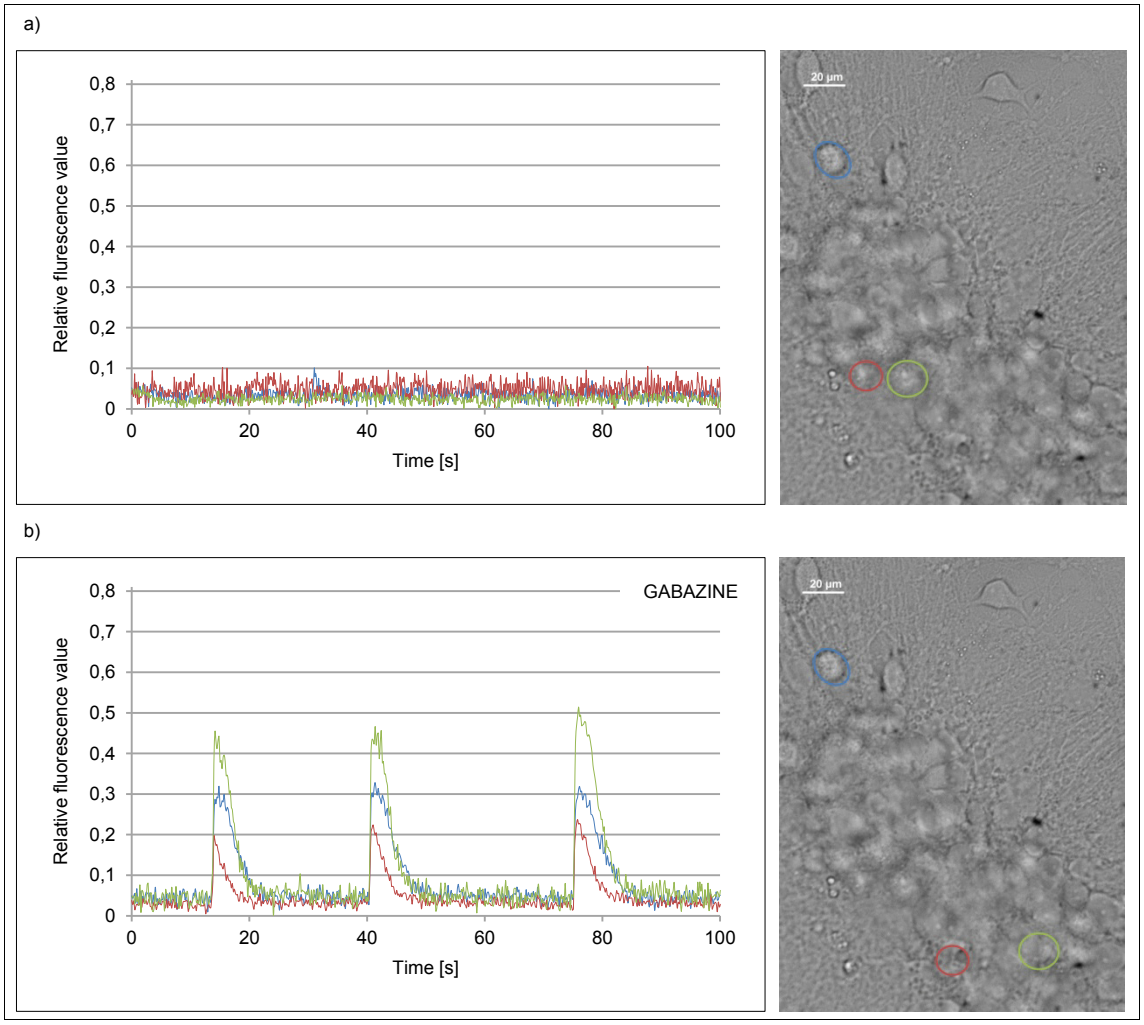

Figure 20: *Spontaneous Ca^{2+} signalling of hippocampal neurons pre-exposed to nicotine*

Graph a) shows that nicotine decreases the spontaneous activity in the culture. When gabazine is added, the amplitudes of changes in cytosolic Ca^{2+} concentration become similar to the ones in the control sample without addition of gabazine (fig. 18 a).

4.1.2.4 Average maximum amplitudes of Ca^{2+} peaks

The average maximum amplitudes of relative Ca^{2+} changes of samples pre-exposed to imidacloprid (50μM) and nicotine (50μM), with and without gabazine, are presented in the fig. 21. Numbers of cells included into the statistics, within each sample type, are given in the graph below.

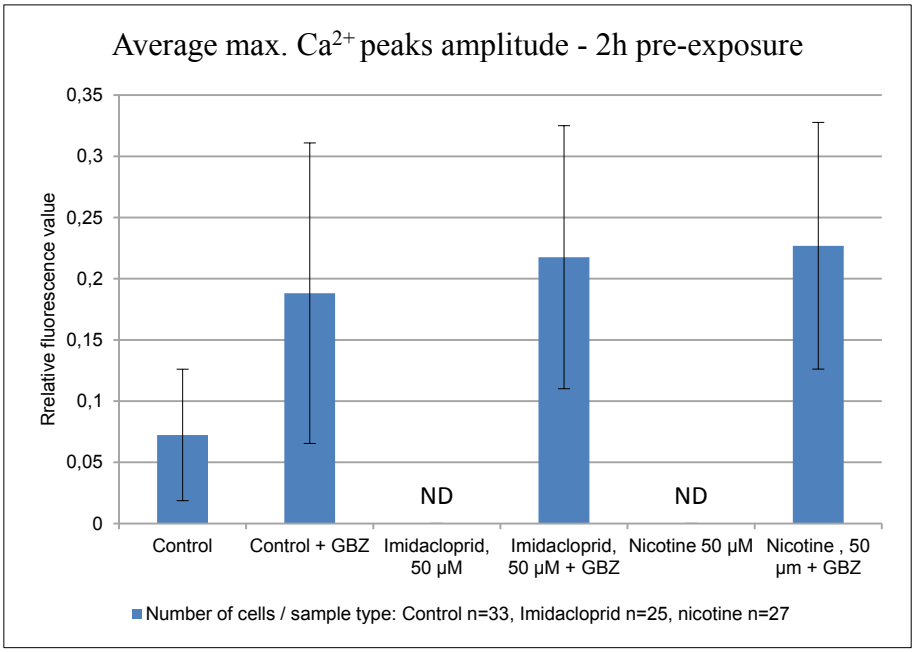

Figure 21: *Average max. Ca^{2+} peaks amplitude - 2h pre-exposure*
ND = not detected

There were no distinct Ca^{2+} peaks detected at the samples pre-exposed to nicotine and imidacloprid without gabazine which is marked in the graph as ND – not detected.

The average Ca^{2+} peak amplitudes of the samples pre-exposed to the imidacloprid and nicotine and of the control (fig. 21) are much lower than the amplitudes in fig. 19 b), 20 b) and 18 b). This is because for the graphs, mentioned above, only three representative cells were chosen and, therefore, the amplitudes' representativeness is limited.

4.1.3. Cell density

Cell density assay bases on counting neurons and nuclei of all cells, after exposure to the drugs. The number of glia cells was determined as the difference between the total number of the cells and the number of neurons.

Fig. 22 shows microscopic images of a control sample of hippocampal neurons (the same field of view), photographed at a 10x magnification. The cells are stained with DAPI (staining of nuclei), anti-β-tubulin combined with the secondary antibody with bound Alexa Fluo R' 488 (staining of cytoskeleton), and Neu-N combined with secondary antibody with Ms Alexa Fluo 594 (staining of somata of neurons).

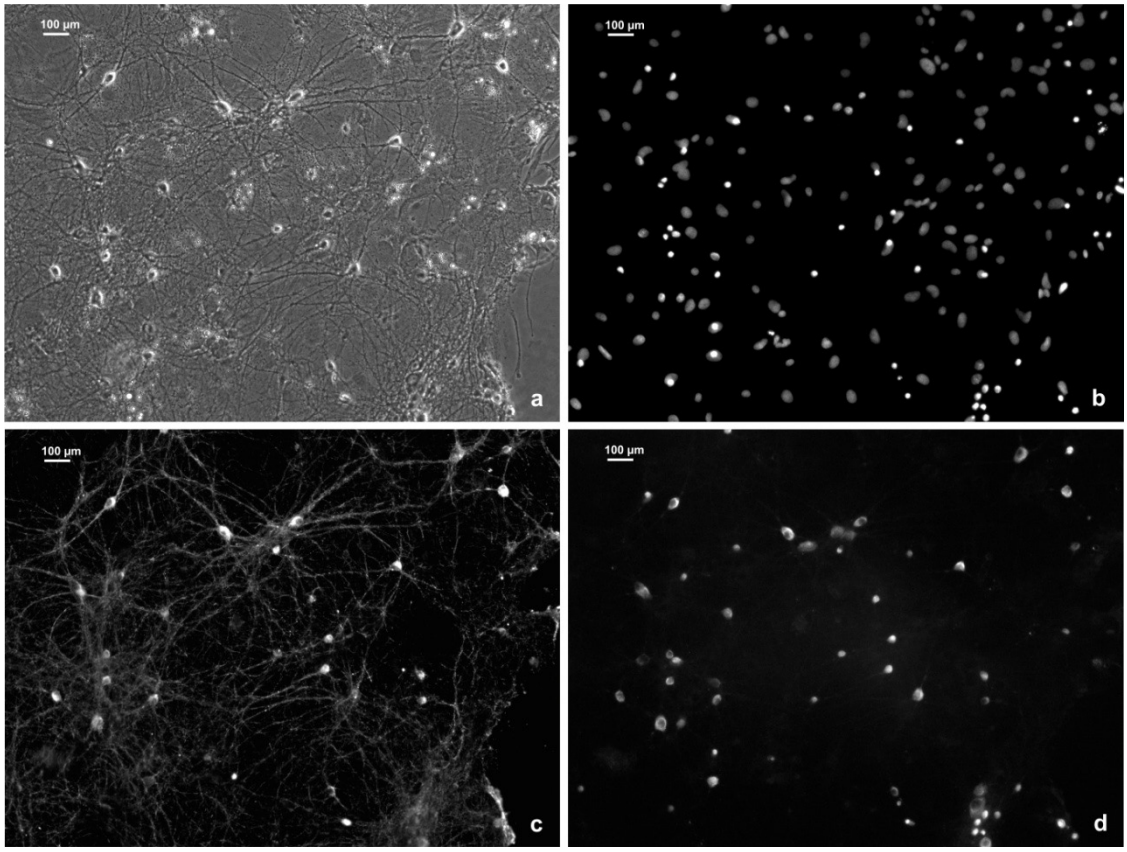

Figure 22: *Culture of hippocampal neurons*
a - visible image, b - nuclei, c – cytoskeleton and d - somata of neurons

4.1.3.1 Two-hours exposure

Fig. 23 shows the average number of neurons and glia cells within an average field of view within the sample type. The samples were exposed to imidacloprid, nicotine and rotenone for two hours and then left to recover for 24 hours. The average number of neurons and glia cells in a control sample was also determined.

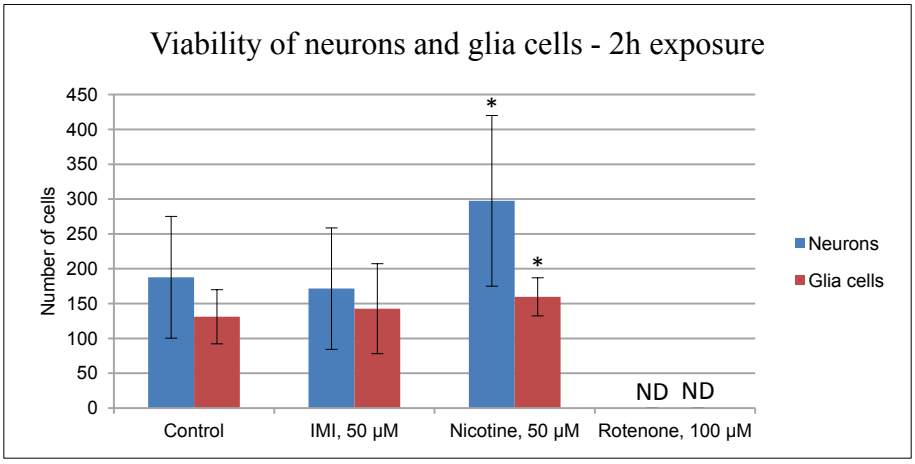

Figure 23: *Viability of neurons and glia cells – 2h exposure*

Fig. 24 shows the average share of neurons and glia cells within an average field of view within the sample type.

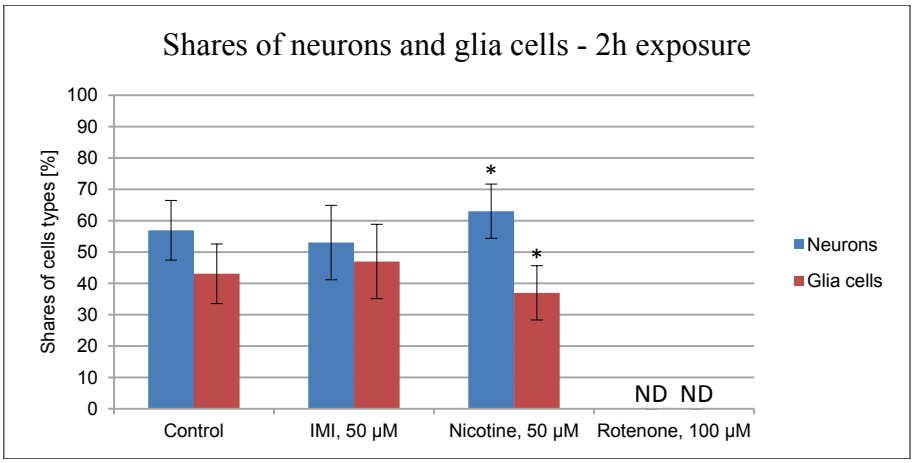

Figure 24: *Shares of neurons and glia cells – 2h exposure*

From the fig. 23 it can be seen that exposure to the imidacloprid in concentration of 50 µM for two hours resulted in a small decrease of the average number of neurons per field, and a small increase of the average number of glia cells per field. However, according to the T-test, the decreased viability of the neurons and increased viability of the glia cells is not statistically significant (P>0.4).

The average share of the neurons exposed to imidacloprid (fig. 24) decreased a little, and the average share of the glia cells increased a little in comparison with the control. According to the T-test, these differences were not statistically significant (P>0.1).

The hippocampal neurons after 2-h exposure to imidacloprid (50µM) and rotenone (100 µM) at a 10x magnification are presented in fig. 25. Staining of the neurons was the same as described at the fig. 22.

Nicotine, after a two-hour exposure in concentration of 50 µM (fig. 23), showed protective action as neurons and glia cells showed statistically significant higher viability than in the control (P<0.05). In the sample exposed to nicotine, there was also a statistically significantly higher share of the neurons and a statistically significantly lower share of the glia cells (fig. 24).

After a two-hour exposure to rotenone in concentration of 100 µM all neurons as well as all glia cells undergo cells death.

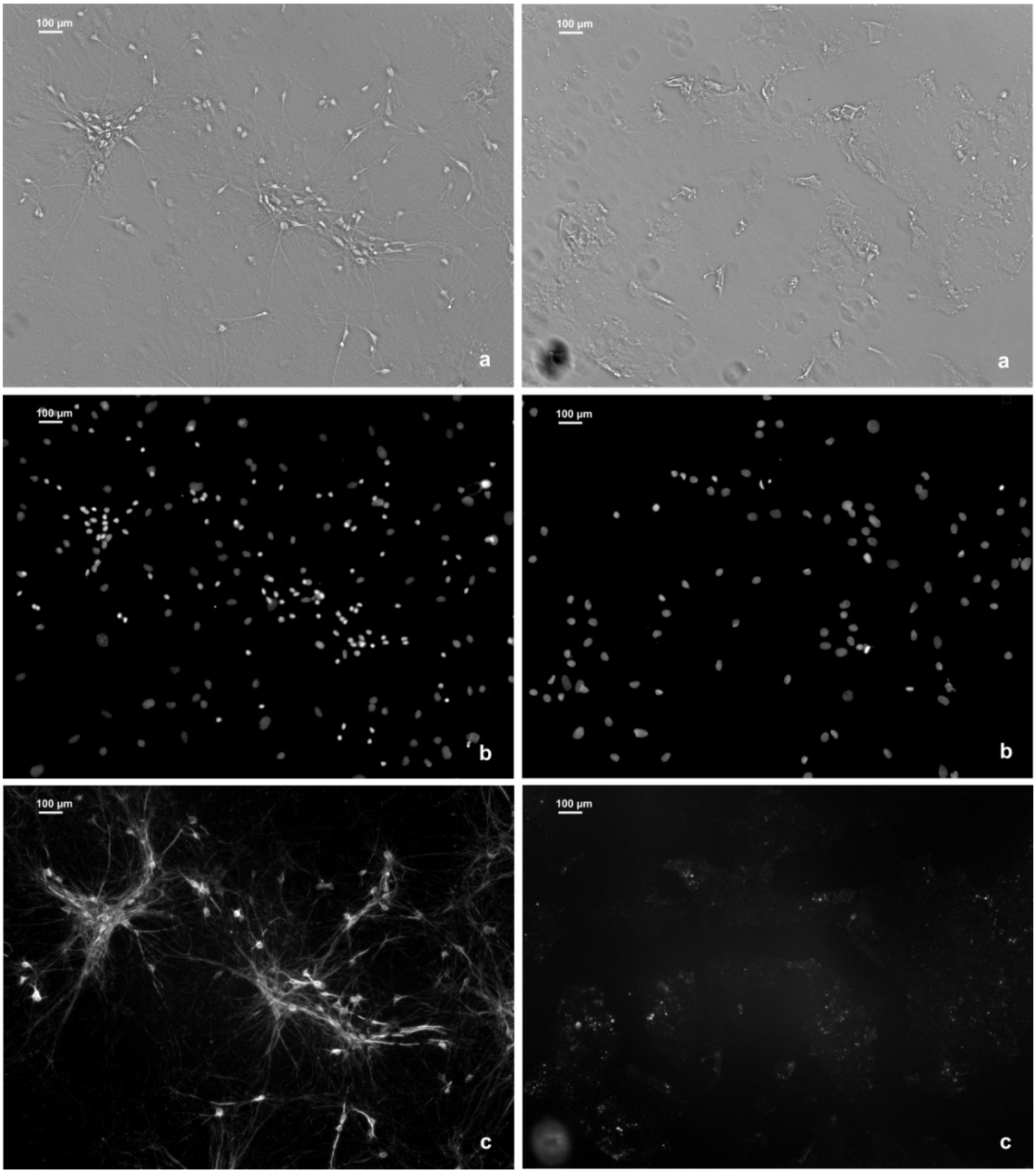

Figure 25: *Hippocampal neurons after 2-h exposure to IMI (left) and rotenone (right) a - visible image, b – nuclei, c - cytoskeleton*

4.1.3.2 Overnight exposure

Average numbers of the neurons and glia cells per field of view are shown in fig. 26. The concentrations of the imidacloprid and nicotine can be seen in the graph below.

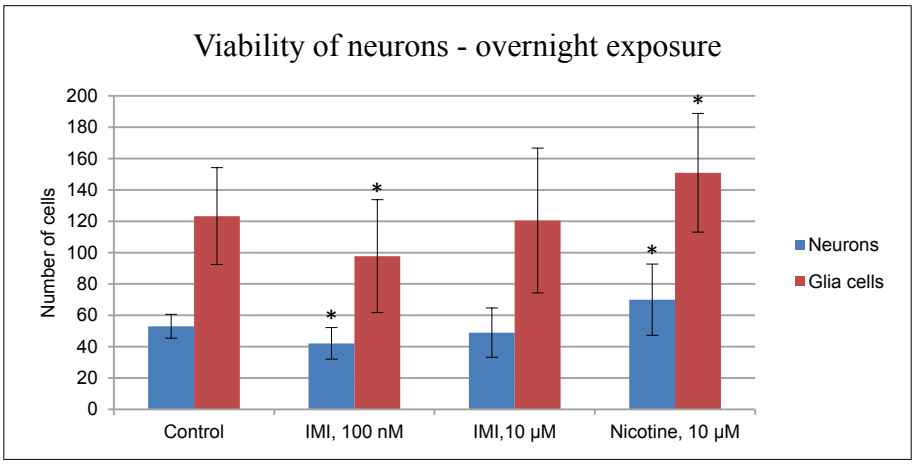

Figure 26: *Viability of neurons – overnight exposure*

The average shares of the neurons and glia cells are shown in fig. 27.

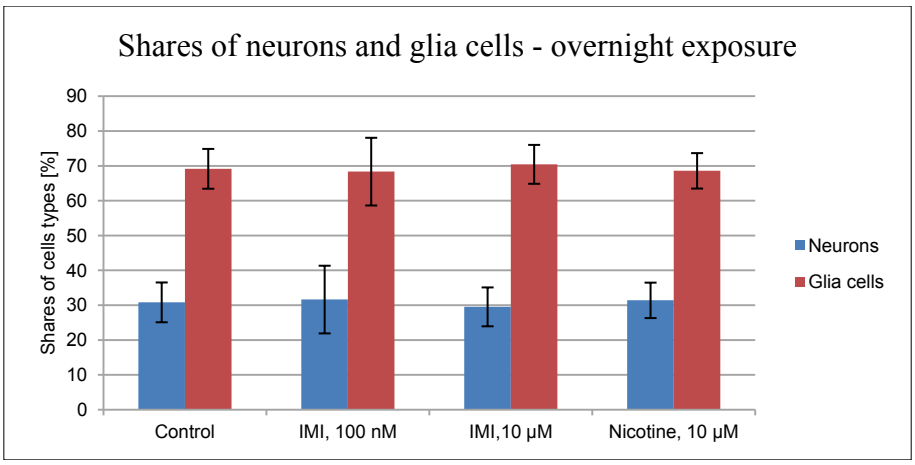

Figure 27 *Shares of neurons and glia cells – overnight exposure*

It is clear from the fig. 26 that the overnight exposure to imidacloprid in concentration of 100 nM resulted in a statistically significantly decreased average number of the neurons and glia cells per field. When using the imidaclopride's concentration of 10 µM, the decrease in the average number of neurons and glia cells per field was lower than when using a concentration of 100 nM and was not statistically significant.

The average share of the neurons and glia cells per field at both concentrations of imidacloprid were not statistically significantly different from the control sample (fig. 27).

After an overnight exposure of the cells to nicotine in concentration of 10 µM, the drug showed protective effects, making the viability of the neurons and glia cells statistically significantly higher than in the control sample (fig. 26). The average share of neurons and glia cells per field did not statistically significantly differ from the control (fig. 27).

4.2 iMN9D cells

Morphology of iMN9D cells was very different from that of the hippocampal neurons. The morphology can be seen in fig. 28. Even after 7 days of differentiation, most cells had round shapes (a) and only a few of them developed axons (b).

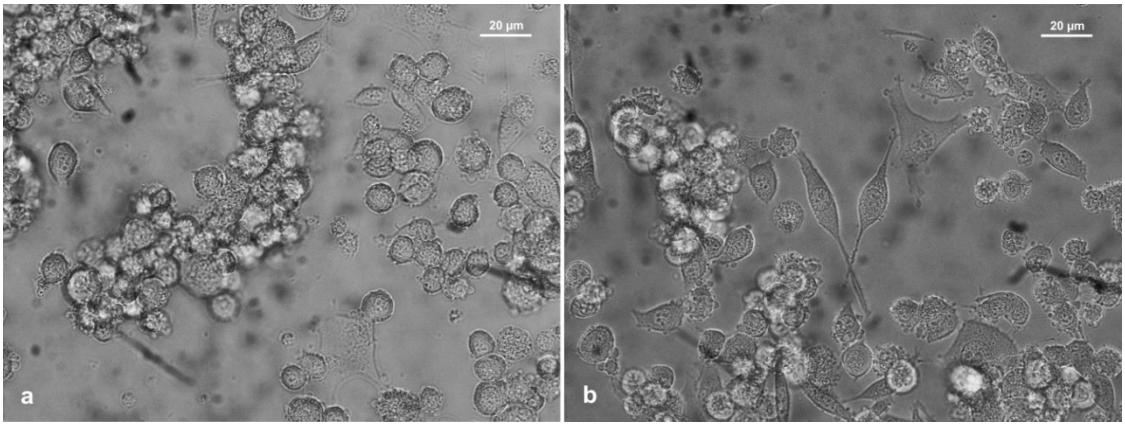

Figure 28: *iMN9D cells*

Several experiments were performed with iMN9D cells to follow their Ca^{2+} homeostasis after addition of imidacloprid and KCl, but the cells gave very little or no Ca^{2+} signals. Even after more than five days of differentiation, only very few cells gave very modest spontaneous Ca^{2+} signals.

All experimental procedures were the same as at the acute exposure of hippocampal neurons to imidacloprid (section 4.1.1).

Most of the cells did not show any Ca^{2+} signals after the administration of imidacloprid. Only very few and very small signals of rare cells were seen after the administration of KCl. From the performed experiments it can be considered that there was no response to imidacloprid in term of Ca^{2+} signalling. Fig. 29 shows spontaneous Ca^{2+} signalling of a control sample (one of the very rare samples with cells giving Ca^{2+} peaks).

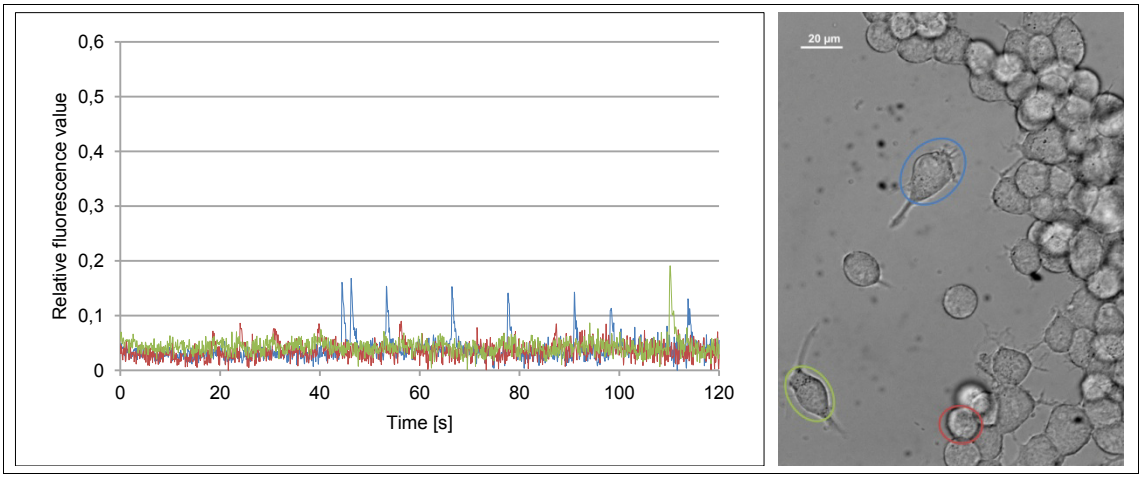

Figure 29: *Spontaneous Ca^{2+} signalling of iMN9D cells*

5. DISCUSSION

5.1 Possible uncertainties in measurements

Factors that may affect the accuracy and reliability of the measurements of cytosolic Ca^{2+} concentrations are: great variability in the responses of the tested cells and their absorption of the dye, interference of the light from the surroundings with the measurements (all efforts were made to sufficiently shade the microscope), camera noise, and a not very high sampling rate. Small errors in the measurements of Ca^{2+} could also arise in the phase of background subtraction because of uneven illumination of the background and from inaccuracies in the correction of the dye bleaching.

Mistakes made during cell density assays could arise from inaccuracies in counting, which could appear in areas with very high cell densities that were sometimes overexposed. In such areas in samples that were not stained with NEU-N, it was hard to distinguish neurons from glia cells.

At measurements of maximum average Ca^{2+} peak amplitudes at acute exposure to imidacloprid and nicotine a relatively low number of cells was included which could have influenced the representativeness of the samples.

5.2 Discussion of results

5.2.1 Hippocampal neurons

It is interesting that imidacloprid caused higher peaks of Ca^{2+} in the hippocampal neurons than nicotine which induced only very small peaks. This is in contrast to results of Kimura-Kuroda J. et al. (2012) who found that nicotine in concentrations from 1 to 100 μM consistently produced somewhat higher peaks of Ca^{2+} than imidacloprid. This difference might, at least partially, be caused by the use of cerebellar cultures from neonatal rats which could have acted differently than our cultures of hippocampal neurons. However, in both types of cells, α4β2 subtype of nAChRs is present, so there must be some other explanation. Kimura-Kuroda J. et al. (2012) explain identical amplitudes of Ca^{2+} peaks at different concentrations of imidacloprid by the fact that imidacloprid first induces Ca^{2+} influx through nAChRs, which is relatively small, but is sufficient to activate VGCCs, which provide much higher influx of Ca^{2+} into the cells. Increase in cytosolic Ca^{2+} acts as a negative feedback signal, eliciting VGCCs back to non-conductive, inactive state.

The shapes of Ca^{2+} peaks caused by imidacloprid and nicotine (at acute exposure) are consistent with the findings of Kimura-Kuroda J. et al. (2012). The slower, gradual fall of the Ca^{2+} peaks induced by imidacloprid, and the rapid fall of Ca^{2+} peaks induced by nicotine could probably be explained by the difference in the desensitization potentials of imidacloprid and nicotine to nAChRs.

Another interesting finding that deserves further investigation is that both imidacloprid and nicotine caused the inhibition of neuronal spontaneous activity (no peaks of Ca^{2+} were seen) after two hours of exposure and 24 hours of recovery at the concentration

of 50 μM. This is likely to be due to a shift in the balance between excitatory and inhibitory synapses toward inhibition.

Furthermore, it is hard to explain that imidacloprid showed higher toxicity at viability test after overnight exposure at concentration of 100 nM than at 10 μM and also at 50 μM after a two-hour exposure. More experiments would be necessary to asses this issue.

5.2.2 iMN9D cells

Taking everything into account, iMN9D cells differentiated with neomycin and doxycycline, as described in section 3.1.2.4, did not prove themselves to be an appropriate model for testing effects of imidacloprid to mammalian nervous systems.
The reason why iMN9D cells showed only very small changes in concentrations of intracellular Ca^{2+} could be found in an inappropriate method of differentiation. Fluxes of Ca^{2+} were much lower than those in hippocampal neurons because iMN9D cells probably did not develop all of the ionic channels that are found in neurons. This is consistent with the conclusions of Rick C.E. et al. (2006) that iMN9d cells only partially match the properties of dopaminergic neurons.

6 CONCLUSIONS

6.1 Key findings

It is obvious from the measurements of the intracellular Ca^{2+} concentrations in hippocampal neurons acutely exposed to imidacloprid and nicotine, both in concentrations of 10 μM, that imidacloprid produces higher influxes of Ca^{2+} into the cytoplasm than nicotine. This can be due to higher activation of nAChRs or due to additional effects determining intracellular Ca^{2+} increase. Relative fluorescence changes representing intracellular Ca^{2+} concentrations after acute exposure to imidacloprid, in concentration of 10 μM, were substantially different among different cells in the same sample and among cells from different samples, and were three to six times higher in comparison with the control.

Monitoring of the spontaneous calcium signalling of the hippocampal neurons, pre-exposed to imidacloprid and nicotine for two hours, both in concentration of 50 μM, shows that imidacloprid and nicotine have inhibitory effect on the spontaneous activity of the neuronal culture. However, peaks of Ca^{2+} in cytosol of neurons pre-exposed to imidacloprid as well as to nicotine were restored after the addition of gabazine, and were similar to the control sample.

The cell density assays show that imidacloprid has mild cytotoxic effects. Numbers of hippocampal neurons and glia cells after overnight exposure to imidacloprid in concentration of 100 nM were slightly reduced. However, cytotoxicity of imidacloprid to hippocampal neurons was not statistically significant after overnight exposure of cells to the drug's concentration of 10 μM and after two-hour exposure to the concentration of 50 μM.

The same assay also showed protective effect of nicotine. After overnight exposure of hippocampal neurons to nicotine in concentration of 10 μM and after two-hour

exposure in concentration of 50 μM, it can be seen that the number of neurons and glia cells was statistically significantly higher than in the control sample. It was also found that in hippocampal neurons, exposed to nicotine in concentration of 50 μM for two hours, the ratio between neurons and glia cells has statistically significantly changed in favour of neurons in comparison with the control.

The measurement of calcium homeostasis on iMN9D cell line, differentiated with neomycin and doxycycline, with application of imidacloprid and KCl, clearly showed that this is not an appropriate cell model to test the effects of imidacloprid on mammalian nervous systems.

6.2 Final verdict on neonicotinoids

The main properties of neonicotinoids are photo-stability, water solubility and lipophilicity. Therefore they are highly persistent and mobile in the environment and can accumulate in soil and leach into the surface as well as in the ground water. Their degradation in soil and in ground water is slow and they can persist there for several decades. Neonicotinoids can be present in drinking water.

Depending on their method of application, they are present in plant tissues as well as on plant surfaces. Their degradation in plants is slow and during metabolic processes they can be bio-activated. Neonicotinoids and their metabolites are widely present in food.

Imidacloprid acts on nicotinic acetylcholine receptors in central nervous system and causes paralysis and death of insects. Even though it was claimed that it is specific to the nAChRs of insects, it was found that it has a relatively high binding affinity to nAChRs of other species, including mammals, and is acutely and chronically toxic to these species as well.

Imidacloprid is highly toxic to beneficial insects, such as bees and other pollinators, and to other terrestrial and aquatic invertebrates. In invertebrates, imidacloprid has a cumulative and irreversible mode of action. By affecting the populations of the organisms on the bottom of the food chain, neonicotinoids have the potential to indirectly affect other organisms, such as birds, fish, reptiles, etc.
Birds are seriously affected by ingestion of the treated seeds and young plants growing from them because the highest concentrations of neonicotinoids are found in treated seeds. Imidacloprid is toxic for smaller birds, especially for songbirds in acute as well as in chronic exposure and also shows reproductive toxicity.

Higher doses of imidacloprid in mammals chronically exposed to it affect liver (imidacloprid is mainly metabolized in liver), blood, weight, and reproduction. However, chronic effects on the nervous system at lower doses of exposure through food and water might be much more severe.

Recently, it has been found that imidacloprid can pass through the mammalian blood-brain barrier and activate mammalian subtypes α2β4 and α7 of nAChRs. This means that imidacloprid can affect calcium homeostasis in neurons, causing undesired depolarisation of neurons. It was also found that imidacloprid can change the response of nAChRs to neurotransmitter acetylcholine by desensitization resulting in a decreased response to acetylcholine and changes in depolarisation patterns of

neurons. This is especially problematic in developing brain because imidacloprid acts on nAChRs subtypes which are involved in many processes in mammals, such as neuronal proliferation, apoptosis, differentiation, migration, synapse formation and neuronal circuit formation. Furthermore imidacloprid was found to be cytotoxic, causing oxidative stress in brain and liver.

My experiments confirmed that imidacloprid can induce Ca^{2+} influxes that cause the depolarisation of neurons and change spontaneous firing patterns by reducing Ca^{2+} peaks in neurons. However, the experiments gave very limited evidences of imidacloprid's induced neurotoxicity, probably because of the relatively short duration of the cell density assays.

Because of wide application of neonicotinoids in farming, the majority of people in developed countries is chronically exposed to them since they are present in food (especially fruit and vegetable that are not processed before consumption) and in drinking water.

Taking everything into account there are many indices pointing out that imidacloprid could cause neurodegenerative processes in mammals and in humans at long-term exposure to low concentrations. It might also be involved into the pathogenesis of neurodegenerative diseases such as Parkinson and Alzheimer.

From the environmental point of view, neonicotinoids can, in longer periods of time, cause changes in food chains because of the different interspecies sensitivity, and can affect the biodiversity in many of terrestrial and freshwater ecosystems.

As it was initially considered that neonicotinoids are relatively safe for mammals, they were easily registered for a broad variety of uses by governmental agencies worldwide. They overlooked the warnings of the environmental and health protection agencies and scientists about possible harmful effects on the environment and on human health; neonicotinoids have been registered and re-registered throughout the world.

To conclude, in order to protect the human health and environment, the use of imidacloprid and other neonicotinoids should be restricted to the minimal possible quantities. The best solution would be implementation of more environmental friendly farming practices, such as pyrethroid sprays and Integrated Pest Management (IPM), wherever possible.

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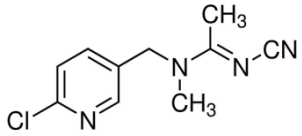
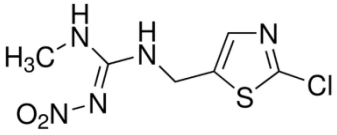
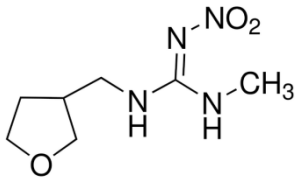
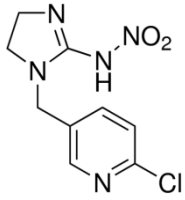
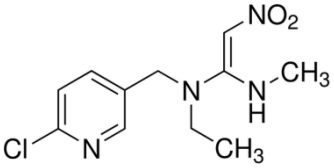
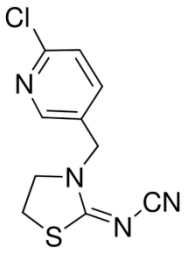
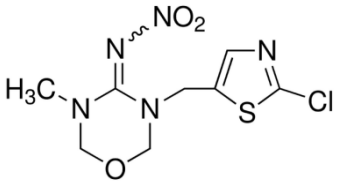
ANNEX A

NEONICOTINOIDS

Name	IUPAC formula	Structural formula
Acetamiprid	(E)-N-(6-Chloro-3-pyridylmethyl)-N′-cyano-N-methylacetamidine	
Clothianidin	(E)-1-(2-Chloro-5-thiazolylmethyl)-3-methyl-2-nitroguanidine	
Dinotefuran	(RS)-N-Methyl-N′-nitro-N″-[(tetrahydro-3-furanyl)methyl]guanidine	
Imidacloprid	N-{1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl}nitramide	
Nitenpyram	(E)-N-(6-Chloro-3-pyridylmethyl)- N-ethyl-N'-methyl-2-nitrovinylidenediamine	
Thiacloprid	[3-(6-Chloro-3-pyridinylmethyl)-2-thiazolidinylidene]cyanamide	
Thiamethoxam	3-(2-Chloro-5-thiazolylmethyl)tetrahydro-5-methyl-N-nitro-4H-1,3,5-oxadiazin-4-imine	

All formulas in the table are taken from the web page of the Sigma Aldrich (16.6.2014).

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