

**Functional neurotoxicity assays to assess
substance-induced signaling alterations of human
dopaminergic neurons**

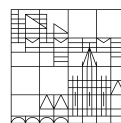
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Abbreviations

ACh	Acetylcholine
AChBP	Acetylcholine binding protein
Aceta	Acetamidiprid
ARfD	Acute reference dose
AMP	Amphetamine
AR-R	AR-R 17779
ATP	Adenosine 5'-triphosphate
AUC	Area under the curve
BMC	Benchmark concentration
$[Ca^{2+}]_i$	Intracellular free calcium concentration
cAMP	N6,2'-O-dibutyryl 3',5'-cyclic adenosine monophosphate
Ca _v channel	Voltage-gated calcium ion channel
Cloth	Clothianidin
DN-IMI	Desnitro-imidacloprid
DNT	Developmental neurotoxicity
DA	Dopamine
DAT	Dopamine transporter
DMSO	Dimethyl sulfoxide
Dino	Dinotefuran
EC ₂₅	Quarter maximal effective concentration
HS	High-sensitivity
IFD	Induced-fit docking
Imida / IMI	Imidacloprid
IMI-olefin	Imidacloprid-olefin
K _v channel	Voltage-gated potassium ion channel
LD ₅₀	Median lethal dose
LS	Low-sensitivity
LUHMES	LUND human mesencephalic
MEA	Microelectrode array
Mec	Mecamylamine
MLA	Methyllycaconitine
MM-GBSA	Molecular mechanics generalized Born surface area
Nav channel	Voltage-gated sodium ion channel
nAChR	Nicotinic acetylcholine receptor
NNC	NNC 55-0396
P2X	Ionotropic purinergic

P2Y	Metabotropic purinergic
pCTX	Pacific ciguatoxin
pEC ₂₅	Negative logarithm of the quarter maximal effective concentration
pEC ₅₀	Negative logarithm of the half maximal effective concentration
pIC ₅₀	Negative logarithm of the half maximal inhibitory concentration
PBTK	Physiology-based toxicokinetic
PLO	Poly-l-ornithine
PNU	PNU-120596
STX	Saxitoxin
TEA	Tetraethylammonium
Thiac	Thiacloprid
Thiam	Thiamethoxam
TTX	Tetrodotoxin
Tubo	Tubocurarine
VTD	Veratridine

Zusammenfassung

Die potentielle Toxizität einer Substanz für das humane Nervensystem wird immer noch durch Tierversuche ermittelt, die in manchen Fällen eine mangelnde Aussagekraft für Menschen aufweisen. Auf menschlichen Zellen basierende Ansätze haben das Potential die Limitationen der Tiermodelle zu überwinden. In **Manuskript 1** haben wir ein auf der humanen neuronalen Zelllinie LUHMES basierendes Testsystem etabliert, das eine Quantifizierung der freien intrazellulären Calcium Konzentration ($[Ca^{2+}]_i$) im Hochdurchsatz für die Beurteilung neuronaler Aktivität ermöglicht. Die Eignung dieses Ansatzes für die Detektion Substanz-induzierter Aktivitätsänderungen von ionotropen Rezeptoren, spannungsgesteuerten Ionenkanälen und elektrogenen Neurotransmittertransportern wurde am Beispiel von drei, für die neuronale Aktivität wichtigen, Toxinzielen gezeigt: Ionotrope purinerge Rezeptoren, spannungsgesteuerte Natriumionenkanäle und dem elektrogenen Dopamintransporter (DAT). Zudem stimulierte die Aktivierung des DAT synchrone $[Ca^{2+}]_i$ Oszillationen des neuronalen Netzwerks, die sensitiv für verschiedene Substanzen waren, welche die Aktivität des DAT oder spannungsgesteuerter Ionenkanäle beeinflussten. In **Manuskript 2** haben wir gezeigt, dass LUHMES Neuronen funktionale $\alpha 7$ und neuronale nicht- $\alpha 7$ nikotinische Acetylcholinrezeptoren (nAChR) haben. Zudem bestätigten unsere Daten die Anwendbarkeit unseres Ansatzes für die Detektion von agonistischen und antagonistischen Effekten auf nAChR vermittelte Signale. Auf dieser Basis haben wir die Effekte der sechs wichtigsten kommerziell erhältlichen Neonikotinoide untersucht, da es vermehrt Hinweise auf eine Wirkung dieser Insektizide auf Säugetiere gegeben hat. Dabei wurde entdeckt, dass vier von ihnen $[Ca^{2+}]_i$ Antworten auslösen, unter anderem über die Aktivierung des $\alpha 7$ nAChR Subtyps. Des Weiteren, wurde ein desensitisierender Effekt der aktiven Neonikotinoide detektiert, da eine Vorbehandlung der Zellen mit diesen Pestiziden eine darauffolgende Aktivierung der nAChR durch nAChR Agonisten negativ modulierte. Diese Daten weisen darauf hin, dass vier aktive Neonikotinoide LUHMES Neurone über ihre $\alpha 7$ und nicht- $\alpha 7$ nAChR beeinflussen. Da Neonikotinoide in der Umwelt und im Menschen transformiert werden und einige Metabolite eine höhere Toxizität für Säugetiere als für Insekten aufweisen, haben wir in **Manuskript 3** die Effekte des Imidacloprid (IMI) Metaboliten Desnitro-Imidacloprid (DN-IMI) mit unserem LUHMES basierten Testsystem erforscht, um dessen Effekte auf humane Neuronen umfassend zu untersuchen. DN-IMI aktivierte $\alpha 7$ und nicht- $\alpha 7$ nAChR auf LUHMES Neuronen und modulierte die durch nAChR Aktivierung ausgelösten Reaktionen negativ. Unsere Antagonistendaten bestätigten eine Aktivierung von nAChR durch DN-IMI, mit einer starken Beteiligung von nicht- $\alpha 7$ nAChR. Zudem haben wir die agonistischen

Effekte von DN-IMI auf mehrere humane nAChR Subtypen, die in *Xenopus laevis* Oozyten heterolog exprimiert wurden, untersucht, um die betroffenen Subtypen eindeutig zu identifizieren. Dabei stellte sich heraus, dass humane $\alpha 7$ und mehrerer nicht- $\alpha 7$ nAChR durch DN-IMI mit ähnlicher Potenzen wie Nikotin und einer deutlich höheren Effektivität verglichen mit der Ausgangssubstanz IMI aktiviert werden. Diese Ergebnisse zeigen die Wichtigkeit einer umfassenden Gefahrenbewertung für Ausgangssubstanzen und Metaboliten. Insgesamt ermöglicht das LUHMES basierte Testsystem die Begutachtung von nachteiligen Effekten auf viele bedeutsame neuronale Ziele mit einer hohen Reproduzierbarkeit und einer Übereinstimmung mit Literaturwerten und Ergebnissen, die mit anderen Methoden ermittelt wurden (Patch-Clamp und Oozyten Messungen). Diese Ergebnisse verdeutlichen den Wert von Testsystemen, die auf humanen neuronalen Zellen basieren, für die Bewertung potentiell nachteiliger Effekte auf die Funktion des humanen Nervensystems.

Summary

The potential toxic risk of a substance on the human nervous system is still mainly determined in animal experiments, which in some cases lack predictivity for humans. Approaches based on human neuronal cells have the potential to overcome the limitations of animal models. In **manuscript 1**, we established an alternative test system based on the human neuronal cell line LUHMES with a high-throughput quantification of free intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) to assess neuronal activity. The suitability of this approach to detect substance-induced activity alterations of ionotropic receptors, voltage-gated ion channels, and electrogenic neurotransmitter transporters was exemplified for three crucial neuronal toxicant targets: Ionotropic purinergic receptors, voltage-gated sodium ion channels, and the electrogenic dopamine transporter (DAT). Besides, the activation of the DAT stimulated synchronized $[\text{Ca}^{2+}]_i$ oscillations of the neuronal network that were sensitive to different compounds affecting the DAT or voltage-gated ion channels. In **manuscript 2**, we showed that LUHMES neurons have functional $\alpha 7$ and neuronal non- $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) and verified the applicability of our approach to detect agonistic and antagonistic effects on nAChR signaling. On this basis, we explored the effects of the six most important commercially available neonicotinoids, as some studies indicated effects of these insecticides on mammals, and discovered that four of them trigger $[\text{Ca}^{2+}]_i$ responses. Thereby, the activation of the $\alpha 7$ nAChR subtype by the four active neonicotinoids was observed. We also detected a desensitizing effect of the active neonicotinoids, as the pretreatment of the cells with these four neonicotinoids negatively modulated a subsequent activation of the nAChRs by nAChR agonists. These data suggest that several neonicotinoids affect LUHMES neurons via the activation of their $\alpha 7$ and non- $\alpha 7$ nAChRs. As neonicotinoids are transformed in the environment and in humans, and some metabolites exhibit a higher toxicity for mammals than insects, we explored in **manuscript 3** the effects of the imidacloprid (IMI) metabolite desnitro-imidacloprid (DN-IMI) with our LUHMES-based test system for a broad examination of its effects on human neurons. DN-IMI activated $\alpha 7$ and non- $\alpha 7$ nAChRs present on LUHMES neurons and negatively modulated the responses evoked by nAChR agonists. Our antagonist data verified an activation of nAChRs by DN-IMI with a strong contribution of non- $\alpha 7$ nAChRs. In addition, we studied the agonistic effect of DN-IMI on several human nAChR subtypes heterologously expressed in *Xenopus laevis* oocytes to clearly identify the affected subtypes. Our results showed the activation of human $\alpha 7$ and several non- $\alpha 7$ nAChRs by DN-IMI with similar potencies compared to nicotine and with significantly higher effectiveness than its parent compound IMI. These findings

demonstrate the importance of a comprehensive risk assessment for parent compounds and metabolites. In summary, our LUHMES-based test system allows the evaluation of adverse effects on multiple, important neuronal targets with high reproducibility and concurrence with literature data and results obtained with other methods (patch clamp and oocyte recordings). These findings highlight the value of human neuronal cell-based assays for assessing potential adverse effects on the function of the human nervous system.

List of manuscripts

Manuscripts included in this thesis

1. **Loser, D.**, Schaefer, J., Danker, T., Möller, C., Brüll, M., Suci, I., Ückert, A.-K., Klima, S., Leist, M., Kraushaar, U., 2021. Human neuronal signaling and communication assays to assess functional neurotoxicity. Arch. Toxicol. 95, 229–252. <https://doi.org/10.1007/s00204-020-02956-3>

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2. **Loser, D.**, Hinojosa, M.G., Blum, J., Schaefer, J., Brüll, M., Johansson, Y., Suci, I., Grillberger, K., Danker, T., Möller, C., Gardner, I., Ecker, G.F., Bennekou, S.H., Forsby, A., Kraushaar, U., Leist, M., 2021. Functional alterations by a subgroup of neonicotinoid pesticides in human dopaminergic neurons. Arch. Toxicol. 95, 2081–2107. <https://doi.org/10.1007/s00204-021-03031-1>
3. **Loser, D.**, Grillberger, K., Hinojosa, M.G., Blum, J., Haufe, Y., Danker, T., Johansson, Y., Möller, C., Nicke, A., Bennekou, S.H., Gardner, I., Bauch, C., Walker, P., Forsby, A., Ecker, G.F., Kraushaar, U., Leist, M., Potent neuromodulatory activity of the imidacloprid metabolite desnitro-imidacloprid via human nACh receptor agonism.
Submitted for publication to Archives of Toxicology.

Manuscripts not included in this thesis

Further manuscript

Brüll, M., Spreng, A.-S., Gutbier, S., **Loser, D.**, Krebs, A., Reich, M., Kraushaar, U., Britschgi, M., Patsch, C., Leist, M., 2020. Incorporation of stem cell-derived astrocytes into neuronal organoids to allow neuro-glial interactions in toxicological studies. ALTEX - Altern. Anim. Exp. 37, 409–428. <https://doi.org/10.14573/altex.1911111>

Published abstracts

Loser, D., Danker, T., Möller, C., Niedworok, A., Kraushaar, U., 2019. Development of a microelectrode array (MEA) based neurotoxicity assay for detecting the seizurogenic activity of novel drug candidates. Conference

Abstract: MEA Meeting 2018, 11th International Meeting on Substrate Integrated Microelectrode Arrays. <https://doi.org/10.3389/conf.fncel.2018.38.00016>

Izzi, F., **Loser, D.**, Cesare, P., 2019. Micro electrode arrays to investigate neuron-glia crosstalk in neuropathic pain in-vitro models. Conference Abstract: MEA Meeting 2018 | 11th International Meeting on Substrate Integrated Microelectrode Arrays. <https://doi.org/10.3389/conf.fncel.2018.38.00029>

Loser, D., Danker, T., Möller, C., Kraushaar, U., 2017. Pharmacological safety testing on iPSC derived cardiomyocytes using the patch clamp and the Microelectrode Array (MEA) technique. Published in: Abstracts of the 83rd Annual Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT) and the 19th Annual Meeting of the Association of the Clinical Pharmacology Germany (VKliPha) with contribution of the Arbeitsgemeinschaft für Angewandte Humanpharmakologie e. V. (AGAH). Naunyn-Schmiedeberg's Arch Pharmacol 390, 1–101 (2017). <https://doi.org/10.1007/s00210-017-1354-7>

1 General introduction

The detection and evaluation of adverse effects on the nervous system are still challenging during drug development (Cook et al., 2014; Walker et al., 2018), but also for chemicals from other industry sectors. During the development of new drugs, most candidate compounds that fail due to neurotoxicity issues are detected in the clinical phase (Cook et al., 2014; Walker et al., 2018), as these adverse effects on the nervous system function of humans are not well predicted by classical animal models (Mead et al., 2016; Olson et al., 2000). Thus, even after drug marketing, reported side effects are often linked to interferences with the function of the nervous system (Redfern et al., 2010). Undetected drug toxicity is still a serious reason for hospitalization and death in the EU (Giardina et al., 2018) and the US (Sonawane et al., 2018). The withdrawal of drugs from the market is often caused by toxic side effects and neurotoxicity alone accounts for around 20% of withdrawals (McNaughton et al., 2014; Onakpoya et al., 2016). Neurotoxic side effects may arise as sensory disturbances, pain, cognitive impairment, or seizures, which can be caused by alterations of neuronal functions without any morphological changes, as observed, e.g., for hepatotoxic effects. Classical preclinical neurotoxicity assessment lacks predictivity for humans and contributes, thereby, to a decline in new drug applications. Thus, better test systems for the evaluation of functional neurotoxicity for humans are needed.

1.1 Neuronal cell model system

For the establishment of a neurotoxicity assay, it is essential to develop a test system that exhibits a high reproducibility between technical and biological replicates and captures adverse effects on the individual cells and the coupled neuronal network.

There are mainly three options for the cellular basis for such an assay. First, primary neurons can be used to assess functional neurotoxicity, as they built functional networks with different neuronal cell types. These cells are often of rodent origin (Bradley et al., 2018; Forsby et al., 2009; Kreir et al., 2018; Sandström et al., 2017), which bears the risk of species-specific differences (Leist and Hartung, 2013).

Second, neuronal cells differentiated from human induced pluripotent stem cells (iPSC) can be utilized for test assays (Brownjohn et al., 2017; Kondo et al., 2017; Pei et al., 2016; Ryan et al., 2016; Sherman and Bang, 2018; Sirenko et al., 2019; Tukker et al., 2020; Xu et al., 2013). However, their generation is expensive (Bravery, 2015; Engle et al., 2018; Huang et al., 2019; McKernan and Watt, 2013) and particularly complex, causing variability between differentiations and a hard to control maturation state

(Handel et al., 2016; Little et al., 2019; Volpato et al., 2018; Volpato and Webber, 2020; Xia et al., 2016). These disadvantages are limiting factors for the establishment of reliable functional neurotoxicity assays that are based on such cells. Because high reproducibility of the differentiation with a homogenous maturation state of the neurons is required.

A third option is the use of neurons derived from neuronal cell lines (Forsby et al., 2009; Klima et al., 2020; Krug et al., 2013; Stiegler et al., 2011). These cells can be of human origin and can be produced with relatively low costs in high quantities, which is attractive for larger toxicity screenings. However, a disadvantage of some cell lines is their low ability to form functional networks, which is problematic for the investigation of substance-induced alterations of neuronal network activity. Nevertheless, they are interesting as a basis of functional neurotoxicity assays focusing on the assessment of substance-induced alterations of the function of neuronal ion channels, receptors, and transporters present on these neuronal cells. One example of an exciting neuronal cell line is the human neuronal precursor cell line LUHMES. These cells can be differentiated into post-mitotic dopaminergic neurons in a few days (Scholz et al., 2011), at relatively low costs and high quantities. Furthermore, these neuronal cells increase the amount of functional voltage-gated sodium ion channels (Nav channels) and the expression of synaptic markers during the differentiation process (Scholz et al., 2011). Therefore, LUHMES neurons are an interesting cell model for functional neurotoxicity assays. Moreover, these cells are already well-established as a model system to research toxic effects on human neurons using assays based on morphological, metabolical, and biochemical endpoints (Brüll et al., 2020; Delp et al., 2021, 2019, 2018b, 2018a; Harris et al., 2017; Krug et al., 2014, 2013; Lohren et al., 2015; Smirnova et al., 2016; Tong et al., 2017; Witt et al., 2017; Zhang et al., 2014). So far, very little was known about the electrophysiological characteristics of LUHMES neurons (Scholz et al., 2011) and their suitability for functional neurotoxicity testing.

1.2 Assessment of functional neurotoxicity

Several assays have been developed to detect compound-induced neuronal cell death or morphological changes (Barbosa et al., 2015; Forsby et al., 2009; Schultz et al., 2015; Wilson et al., 2014). Some have been optimized to assess damages to specific cell components, e.g., mitochondria (Delp et al., 2019, 2018a). However, such assays fail to detect several functional neurotoxicants that can alter the function of individual neurons or coupled neuronal networks but assessing these effects is an essential part of the safety evaluation of a compound.

A direct way to investigate changes of neuronal activity is the use of the patch clamp technique (Fig. 1A), which allows the evaluation of the neuronal membrane potential or the current flow through the cell membrane (Cummins et al., 2009; Kang and Kitai, 1993; Molleman, 2003; Smith et al., 1992). This approach gives high-resolution information on substance-induced functional changes on the level of individual neurons. It is also possible to investigate the connectivity of two neurons within a local network using paired patch clamp recordings (Hefft et al., 2002; Kraushaar and Jonas, 2000). However, these approaches have the limitation of a low throughput, which was addressed by the development of automated patch clamp devices. This technique is mainly based on chips for planar recordings of dissociated cells (Fig. 1B) (Obergrussberger et al., 2015). Thus, the investigation of adverse effects on an intact neuronal network would not be possible.

A different approach that allows the examination of spontaneously active neuronal networks is the microelectrode array (MEA) technique, which enables the recording of extracellular field potentials (Alloisio et al., 2015; Bader et al., 2017; Bradley et al., 2018; Hogberg et al., 2011; Kraushaar et al., 2017; Kreir et al., 2018; McConnell et al., 2012; Nicolas et al., 2014; Odawara et al., 2016; Tukker et al., 2020, 2018; Vassallo et al., 2016). Thus, this technique can be used to assess adverse effects on the averaged function of individual neurons (e.g., spiking and burst activity) and of the network activity (e.g., network burst activity) with medium throughput (Alloisio et al., 2015; Bader et al., 2017; Bradley et al., 2018; Kraushaar et al., 2017; Kreir et al., 2018; Tukker et al., 2020, 2018).

A complementary approach to assess the activity of individual neurons and neuronal networks is live-cell fluorescence imaging of the free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) with calcium-sensitive dyes (Brüll et al., 2020; Grunwald et al., 2019; Karreman et al., 2020; Sirenko et al., 2019). This technique allows a high-throughput quantification of $[\text{Ca}^{2+}]_i$ changes and, thereby, indirectly of the electrical activity of the neuronal culture (Fig. 1D, E). For example, the activation of ionotropic neurotransmitter receptors on neurons leads to a transient increase of the $[\text{Ca}^{2+}]_i$ either directly by an influx of Ca^{2+} through the receptor pore or indirectly by depolarizing the membrane potential, which activates voltage-gated Ca^{2+} channels (Ca_v channels). Thus, different types of signaling events can be measured using Ca^{2+} -imaging (Grienberger and Konnerth, 2012; Leist and Nicotera, 1998) and toxic effects, which disturb the normal signaling, can easily be detected and quantified (Brüll et al., 2020; Sirenko et al., 2019).

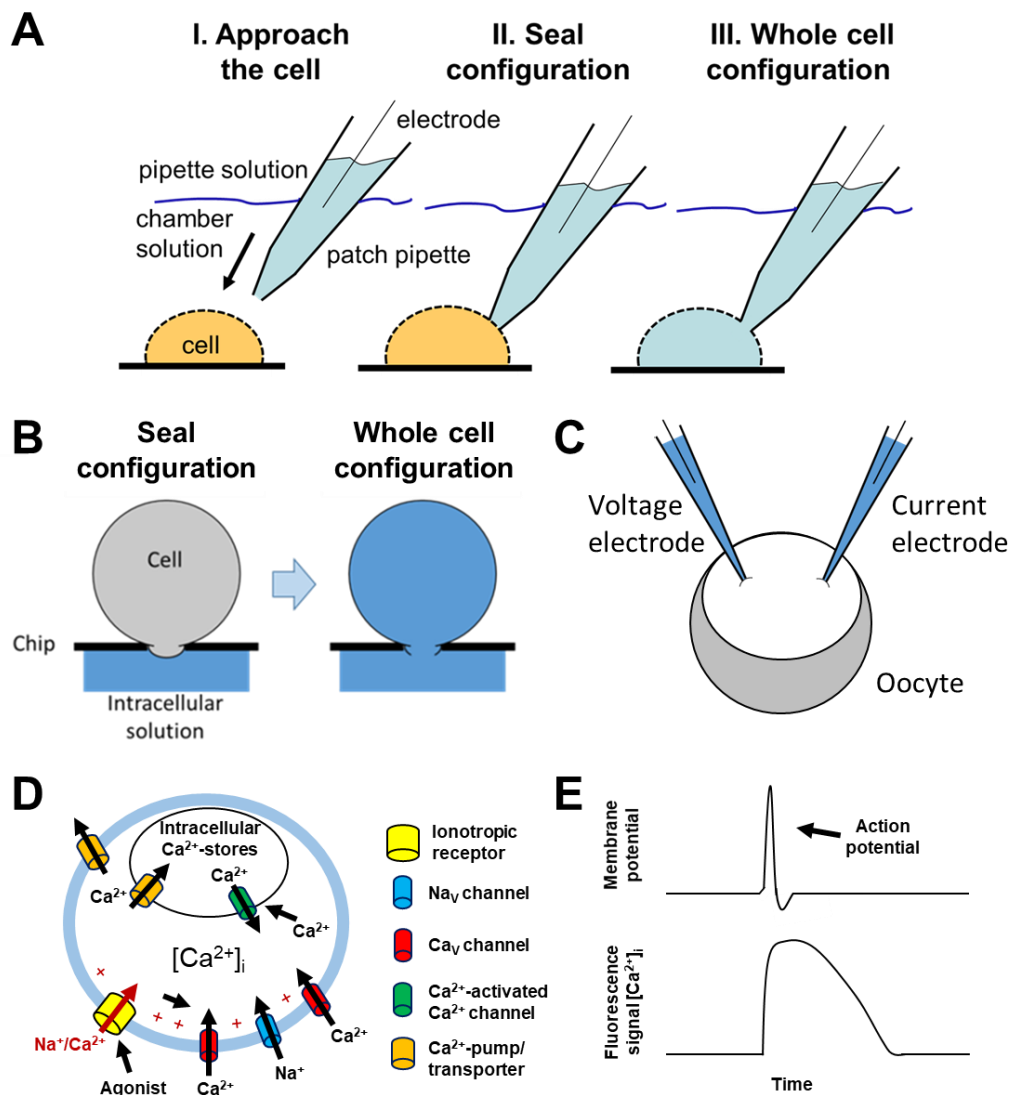


Fig. 1: Basic principles of utilized recording methods.

(A) The basic principle of the procedure of manual patch clamp is presented. (I) The adherent cell is surrounded by the chamber (extracellular) solution and the patch pipette, filled with the pipette (intracellular) solution, is moved to the cell. (II) The seal is generated by applying a gentle vacuum, which leads to the development of high electrical resistance ($\geq 1\text{G}\Omega$). (III) The whole-cell configuration is reached by a strong vacuum pulse to rupture the cell membrane at the tip of the patch pipette. The pipette solution fills the inside of the cell. **(B)** The principle of planar patch clamp is displayed. The procedure is similar as described before in (A). But in this approach, the cell is gently sucked into a hole in the chip to generate a seal. Afterward, a strong vacuum pulse will rupture the cell membrane to reach the whole-cell configuration. Finally, recordings in voltage-clamp or current-clamp mode can be performed. **(C)** The receptor information is injected into the *Xenopus laevis* oocyte before the recordings. After a few days of incubation, the recordings are performed in the two-electrode voltage-clamp configuration. **(D)** A schematic drawing of the principle behind the detection of physiological responses of a cell to an agonist of an ionotropic receptor using Ca^{2+} -imaging is shown. The agonist-triggered activation of the receptor can lead to the influx of $\text{Na}^+/\text{Ca}^{2+}$ into the cell, which depolarizes the cell membrane and activates, thereby, voltage-gated ion channels like voltage-gated Ca^{2+} channels (Ca_v channels). The increase of [Ca^{2+}]_i leads to an activation of intracellular Ca^{2+} stores, which also release Ca^{2+} into the cytosol. This process is followed by the transport of Ca^{2+} out of the cell and into the intracellular Ca^{2+} stores to regain the resting [Ca^{2+}]_i. **(E)** The electrical responses of a cell, e.g., an action potential (top), correlate with the [Ca^{2+}]_i changes (bottom) that can be detected via a Ca^{2+} -sensitive dye.

The assessment of neurotoxic effects on neurons can be challenging, as these cells are a complex model system that contains, e.g., various ion channels, receptors, and transporters. Therefore, the identification of the exact target (e.g., a certain nAChR subtype) of a toxicant or the assessment of toxicant potencies for different receptor subtypes can be challenging in Ca^{2+} -imaging experiments. For example, multiple nAChR subtypes can be present on a neuron, making it hard to determine to what extent the different nAChRs are affected by the toxicant. This challenge can be addressed by another method that enables the assessment of toxic effects on individual receptor subtypes or ion channels after they are heterologously expressed in *Xenopus laevis* oocytes. The defined genetic information of the subunits is injected into the oocytes, which produce the proteins and integrate the functional receptor or ion channel into their membrane. By using two-electrode voltage-clamp recordings (Fig. 1C), the current flux through the cell membrane can be recorded and the effects of toxicants can be assessed on an individual receptor subtype or ion channel (Benallegue et al., 2013; Bermudez and Moroni, 2006; Carbone et al., 2009; Harpsøe et al., 2011; Jonsson et al., 2006; P. Li et al., 2011; Mazzaferro et al., 2011; Mineur et al., 2009; Moroni et al., 2006).

1.3 Toxicant targets involved in neuronal function

The assessment of substance-induced adverse effects on the function of the nervous system is very important, as small alterations of the activity of voltage-gated ion channels, ligand-gated ion channels, or neurotransmitter transporters can have major effects on brain function. Acute toxic effects that directly affect the neuronal function can be caused by the interference of an exogenous substance with these cellular components, which are essential for the electrical activity of individual neurons and, thereby, for the finely tuned neuronal network activity. The resulting disturbances can lead to serious implications like seizures (Kreir et al., 2018; van der Linde et al., 2011) or acute poisonings (e.g., ciguatera (Dickey and Plakas, 2010; Skinner et al., 2011)). At the same time, adverse effects on neuronal function can negatively affect the developing nervous system, which can cause long-term consequences for neuronal connectivity (Grandjean and Landrigan, 2006, 2014; Slikker Jr et al., 2005; Smirnova et al., 2014). During brain development, various neurogenic processes are stimulated by neurotransmitters, e.g., by serotonin (Agrawal et al., 2019; Migliarini et al., 2013; Schaefer et al., 2013) or GABA, which induces synaptogenesis (Oh et al., 2016) and neuronal migration (Li et al., 2018). Another vital neurotransmitter is acetylcholine (ACh) (Hasselmo, 2006; Lauder and Schambra, 1999), which acts via metabotropic (so-called muscarinic) receptors (mAChR) and ionotropic (so-called nicotinic) nAChRs. The

latter can be affected by exogenous nicotine, a well-known developmental neurotoxicant of the mammalian nervous system (Aschner et al., 2017; Dwyer et al., 2009; LeSage et al., 2006; Levin et al., 1993; Slikker Jr et al., 2005; Slotkin et al., 2016). Thus, substances that interfere with neurotransmitter signaling might bear the risk of not only acute toxicity but also developmental neurotoxicity (Grandjean and Landrigan, 2006, 2014; Smirnova et al., 2014).

1.3.1 Voltage-gated ion channels

Voltage-gated ion channels are necessary for the electrical activity of neurons. Mainly voltage-gated sodium ion channels (Nav channels) and voltage-gated potassium ion channels (Kv channels) are involved in the generation of a neuronal action potential. Moreover, Cav channels are activated and contribute to various critical cellular processes (e.g., synaptic vesicle release (Dolphin and Lee, 2020; Ermolyuk et al., 2013)).

As Nav channels are involved in the depolarization phase of neuronal action potentials, they are crucial for signal transduction. During the firing of an action potential, Nav channels pass mainly three functionally different states (Catterall, 1992). At the onset of the action potential, the channels are activated (transition from closed to open state) by depolarization, followed by a relatively fast (few ms) inactivation. The transition from the inactivated state to the closed state (recovery from inactivation) is voltage-dependent and is promoted by the repolarization of the membrane potential. Finally, channels in the closed state can be opened again via depolarization.

Some drugs, e.g., the local anesthetic lidocaine (Clarkson et al., 1988; Huang et al., 2006; Leffler et al., 2007), prefer to bind to Nav channels in the inactivated state compared to the other states, which leads to a use-dependent inhibition of the channels. Moreover, the complex transition between the gating states can also be affected by compounds like the biotoxin veratridine (Catterall, 1992; Power et al., 2012; Tsukamoto et al., 2017; Zhang et al., 2018), causing disturbances of the neuronal activity.

1.3.2 Ligand-gated ion channels

There are many different types of ligand-gated ion channels present on neuronal cells, which exhibit varying gating kinetics, ion permeability and are activated by different endogenous agonists. An important receptor type is the purinergic (P2) receptor that can be split into metabotropic P2 (P2Y) receptors and ionotropic P2 (P2X) receptors (Abbracchio et al., 2006; Burnstock and Kennedy, 1985; Khakh et al., 2001). These receptors play a meaningful role in neurons and glia cells of the peripheral and central nervous systems, e.g., in dopaminergic neurons. They are involved in various

physiological and pathological conditions like Parkinson's disease. (Amadio et al., 2007; Khakh et al., 2001; Patrilli-Cram et al., 2021; Puchałowicz et al., 2014; Syed and Kennedy, 2012; Tóth et al., 2019; Verkhratsky et al., 2012). The P2X receptors are ligand-gated cation channels activated by ATP with high permeability for Ca^{2+} . These receptors can assemble in homo- or heteromers, formed by a total of seven subunits. The subunit composition determines the gating properties and the ligand affinities (Bianchi et al., 1999; Gevers et al., 2006; Khakh and North, 2012; Koshimizu et al., 2000).

Another type of ligand-gated ion channel is the ionotropic nAChR, which is activated by the neurotransmitter ACh. The nAChRs are widely expressed in the central nervous system, among others in dopaminergic neurons, where they affect the electrical activity and, thereby, the dopamine release. (de Kloet et al., 2015; Grady et al., 1992; Mameli-Engvall et al., 2006; Quirk and Kulak, 2002; Quirk and Wonnacott, 2011; Rapier et al., 1988). These receptors assemble to homo- or heteromeric pentamers, formed by either a single subunit (e.g., $\alpha 7$), two subunits (e.g., $\alpha 4$ and $\beta 2$), or more than two subunits. The subunit composition dictates the affinity and efficacy of a ligand and the gating properties (Capelli et al., 2011; Carbone et al., 2009; Jonsson et al., 2006; Mihalak et al., 2006; Nelson et al., 2003). For example, $\alpha 7$ nAChRs inactivate very fast compared to other nAChR compositions such as $\beta 2$ -containing receptors, which exhibit slower inactivation kinetics (Alijevic et al., 2020; Elliott et al., 1996; Mihalak et al., 2006; Nelson et al., 2003). These ligand-gated channels are permeable to Na^+ and/or Ca^{2+} to different extents (Fucile, 2004). Thus, their activation leads to a depolarization, which triggers the opening of voltage-gated ion channels such as Cav channels.

1.3.3 Neurotransmitter transporter

The uptake of neurotransmitters is a key process in the regulation of signal transduction. The different neurotransmitters are transported back into the releasing neurons via specific transporters, e.g., the dopamine (DA) transporter (DAT) on dopaminergic neurons. This monoamine transporter is an electrogenic symporter that transports dopamine, two Na^+ ions, and one Cl^- ion along their electrochemical gradient into the neurons (Gu et al., 1994; Harris and Baldessarini, 1973; Krueger, 1990; Kuhar and Zarbin, 1978; Schenk, 2002; Sonders et al., 1997). The transport of one dopamine molecule results in a net influx of one positive charge (Na^+) that depolarizes the membrane potential and can, thereby, lead to an activation of voltage-gated ion channels like Cav channels (Cameron et al., 2015; Robertson et al., 2009; Sitte et al., 1998; Sonders et al., 1997).

In general, DA signaling is involved in various physiological and pathophysiological processes, and the DAT is an important target of antidepressants and psychostimulants (Schmitt et al., 2013; Simmler et al., 2013). This monoamine transporter can be affected by two different classes of compounds. In the first class are substances that are substrates of the DAT and act as competitive antagonists of DA uptake, e.g., the psychostimulant drug amphetamine (Cameron et al., 2015; Schmitt et al., 2013; Sitte et al., 1998; Sonders et al., 1997). The second class comprises compounds that inhibit the DAT and abolish DA uptake but are no substrates of the DAT, e.g., nomifensine or the addictive drug cocaine (Andersen, 1989; Krueger, 1990; Schmitt et al., 2013; Sulzer et al., 1995). Thus, substance-induced alteration of the activity of the DAT and other neurotransmitter transporters can have severe implications on brain function.

1.4 Important evaluated chemicals and toxicants

1.4.1 Biotoxins affecting Nav channel function

Nav channels are crucial for neuronal electrical activity because of their contribution to the onset of action potentials. Thus, toxicants affecting their normal function can cause severe implications such as acute poisonings (e.g., ciguatera) (Anwar et al., 2018; Gaillard and Pepin, 2001; Lehane and Lewis, 2000; Llewellyn, 2009; Nicholson and Lewis, 2006; Vilariño et al., 2018). Several Nav channel toxins inhibit the channel's inactivation, which causes an overstimulation of the neurons, whereas other toxins block the Nav channels and, thus, the action potential generation. Both effects are equally problematic for normal neuronal function and signal transmission. Therefore, the assessment of Nav channel toxicity on human neurons is of major importance.

The biotoxin veratridine (VTD) is an alkaloid produced by plants of the Liliaceae family, e.g., *sabadilla* and *white veratrum* (Mohammed et al., 2017; Ulbricht, 1998; Wang and Wang, 2003; Zhang et al., 2018). It is a Nav channel modulator known to prolong Nav channel activation by shifting the activation threshold to more negative potentials and by inhibiting the inactivation (Catterall, 1992; Power et al., 2012; Tsukamoto et al., 2017; Zhang et al., 2018). The VTD-induced inhibition of Nav channel inactivation increases the persistent Nav channel current (Power et al., 2012; Tsukamoto et al., 2017; Zhang et al., 2018). This prolonged influx of Na⁺ results in a membrane potential depolarization that activates other voltage-gated ion channels, including Cav channels. This coupling allows the investigation of the effects of the Nav channel modulator VTD using the Ca²⁺-imaging technique (Mohammed et al., 2017; Vetter et al., 2012).

Another major group of Nav channel modulators consists of the ciguatoxins (CTXs). These marine biotoxins are polycyclic ethers synthesized by dinoflagellates (Lehane and

Lewis, 2000; Litaker et al., 2017, 2010; Nicholson and Lewis, 2006; Vilariño et al., 2018). They accumulate in the marine food chain and the consumption of CTX-contaminated fish can lead to a severe intoxication in humans, called ciguatera (Dickey and Plakas, 2010; Lehane and Lewis, 2000; Nicholson and Lewis, 2006; Skinner et al., 2011; Vilariño et al., 2018). The CTXs incorporate several analogs that can be found in the Caribbean Sea, the Indian Ocean, or the Pacific Ocean. The most potent analog is the pacific CTX-1 (pCTX-1), as demonstrated by a toxicity study with mice yielding a median lethal dose of 0.25 µg/kg compared to 2.3 µg/kg for pCTX-2 and 0.9 µg/kg for pCTX-3 (Lehane and Lewis, 2000; Lewis et al., 1991; Nicholson and Lewis, 2006). CTX-1 has been shown to modulate the Nav channels by reducing their activation threshold (Hogg et al., 2002; Inserra et al., 2017), resulting in a depolarization of the membrane potential and action potential firing of neurons (Bidard et al., 1984; Birinyi-Strachan et al., 2005; Hogg et al., 2002, 1998; Nicholson and Lewis, 2006).

Besides Nav channel modulators, biotoxins that inhibit Nav channels are of great interest. Tetrodotoxin (TTX) and its analogs are alkaloids that are produced by several genera of bacteria (Bane et al., 2014; Durán-Riveroll and Cembella, 2017; Yasumoto et al., 1986). TTX can lead to severe intoxications via the consumption of contaminated fish, e.g., pufferfish (Bane et al., 2014; Islam et al., 2011). This toxin is often used as a research tool to discriminate TTX-sensitive and TTX-resistant Nav channels, as TTX inhibits them in different concentration ranges (Durán-Riveroll and Cembella, 2017; England and de Groot, 2009; Lee and Ruben, 2008; Ogata and Ohishi, 2002; Zhang et al., 2013).

Another relevant group of marine biotoxins comprises saxitoxin (STX) and its analogs, which are alkaloids generated by certain marine dinoflagellates and cyanobacteria (Deeds et al., 2008; He et al., 2016; Westrick et al., 2010; Wiese et al., 2010). They accumulate in the marine food chain and the consumption of contaminated seafood can cause paralytic shellfish poisoning in humans (Deeds et al., 2008; Durán-Riveroll and Cembella, 2017; Wiese et al., 2010). Furthermore, they can contaminate water supplies after cyanobacteria blooms (Durán-Riveroll and Cembella, 2017; Negri et al., 1995; Wiese et al., 2010). The toxic effects of STX are caused by its inhibitory effect on Nav channels (Durán-Riveroll and Cembella, 2017; Llewellyn, 2009; Mattei and Legros, 2014; Noda et al., 1989; Terlau et al., 1991).

1.4.2 Neonicotinoids and metabolites

The nAChRs are important toxicant targets and affected by the well-known developmental neurotoxicant nicotine (Aschner et al., 2017; LeSage et al., 2006; Levin et al., 1993). Thus, other substances that exhibit an effect on the nAChR signaling might

also bear a potential risk to affect brain development negatively. On these grounds, the effects of neonicotinoids on human neurons are of toxicological interest. These insecticides, which disturb the insect nervous system by affecting nAChRs (Brown et al., 2006; Tan et al., 2007), have increasingly been used for pest management (Casida and Durkin, 2013; Goulson, 2013; Jeschke et al., 2011). They have been designed to bind with higher affinities to insect than to mammalian nAChRs (Casida, 2018; Tomizawa et al., 2000; Tomizawa and Casida, 2005). However, several studies indicate an impact of these insecticides on mammals (Abou-Donia et al., 2008; Berheim et al., 2019; Burke et al., 2018; Duzguner and Erdogan, 2012). In a study with neonatal rat neurons, the neonicotinoids acetamiprid and imidacloprid triggered responses in the low μM range by activating nAChRs (Kimura-Kuroda et al., 2012).

There are different ways how human can be exposed to neonicotinoids, as they are well soluble in water and exhibit relatively long half-lives in soil (Bonmatin et al., 2015; Goulson, 2013; Morrissey et al., 2015; Thompson et al., 2020), thus, they can get easily distributed in the environment and can be found, e.g., in drinking water (Klarich et al., 2017; Sultana et al., 2018; Thompson et al., 2020). Another way humans are exposed to neonicotinoids is through the consumption of food products like fruits and vegetables, where imidacloprid (IMI) has often been detected (Chen et al., 2014; Craddock et al., 2019; Thompson et al., 2020).

Furthermore, neonicotinoids can be transformed in the environment by abiotic (e.g., photolysis) or biological (e.g., microbial) processes (Simon-Delso et al., 2015; Thompson et al., 2020). In mammals, different enzymes (e.g., cytochrome P450 and aldehyde oxidase) can metabolize these pesticides, e.g., by a transformation of the nitro group of IMI (Dick et al., 2005; Ford and Casida, 2006; Schulz-Jander et al., 2002; Schulz-Jander and Casida, 2002; Shi et al., 2009; Simon-Delso et al., 2015; Swenson and Casida, 2013; Thompson et al., 2020). One of the generated metabolites of IMI is desnitro-imidacloprid (DN-IMI), which has been detected, e.g., in mice (Ford and Casida, 2006; Swenson and Casida, 2013) and in human urine samples together with the metabolite imidacloprid-olefin (IMI-olefin) (Wang et al., 2020) (Fig. 2). This suggests that these metabolites can be produced within mammals by endogenous metabolism. Besides the indirect exposure to neonicotinoid metabolites through the metabolism of ingested neonicotinoids, these metabolites can be directly taken up via food products. For example, in addition to the precursor imidacloprid (IMI), some of its metabolites (e.g., IMI-olefin) have been detected in honey (Codling et al., 2016; Thompson et al., 2020). Moreover, DN-IMI is a major IMI degradation product in the environment and is produced abiotically by photodegradation or biotically as a major metabolite in many plants (Anon, 2006; Koshlukova, 2006). It has been reported to be formed in various foods such as apples, tomatoes, eggplants, and potatoes, where it

accounted for around 10-30% of IMI degradation products (Anon, 2006). Furthermore, DN-IMI has also been found in drinking water (Klarich Wong et al., 2019; Wan et al., 2020).

Some neonicotinoid metabolites are more toxic to insects than their precursor, e.g., IMI-olefin (Codling et al., 2016; Dai et al., 2006; Thompson et al., 2020). In contrast, other metabolites exhibit lower activity on insect nAChRs and a higher potency for mammalian nAChRs, like DN-IMI (Tomizawa et al., 2000; Tomizawa and Casida, 2005). It has been reported that this metabolite exhibits binding affinities for mammalian nAChRs similar to nicotine (Chao and Casida, 1997; D'Amour and Casida, 1999; Tomizawa et al., 2000; Tomizawa and Casida, 1999), a well-known neurotoxicant (Dwyer et al., 2009; Grandjean and Landrigan, 2006; LeSage et al., 2006; Levin et al., 1993; Slikker Jr et al., 2005; Slotkin et al., 2016; Zahedi et al., 2019). These observations are in good agreement with reports about increased toxicity of DN-IMI in mice compared to its parent compound IMI (Chao and Casida, 1997; Tomizawa et al., 2000).

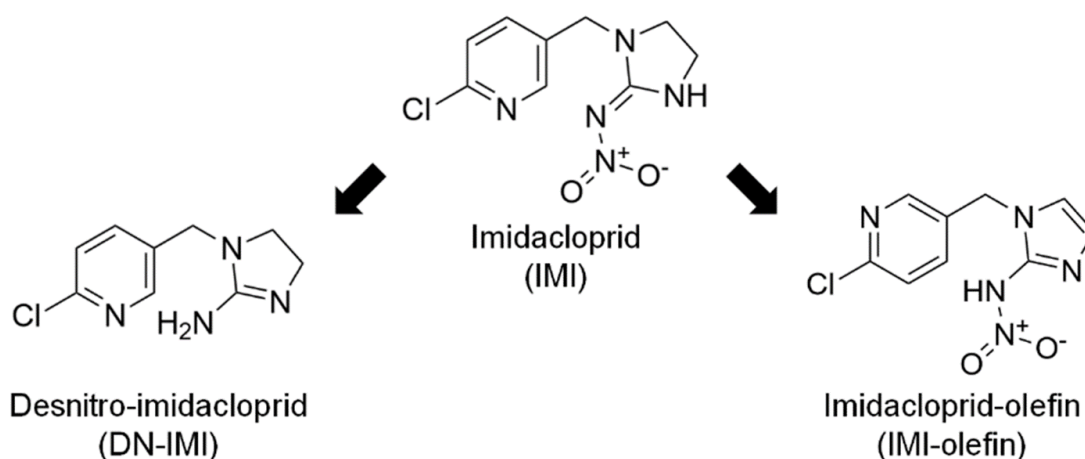


Fig. 2: Chemical structures of two imidacloprid metabolites.

The chemical structures of imidacloprid (IMI) and two of its metabolites desnitro-imidacloprid (DN-IMI) and imidacloprid-olefin (IMI-olefin) are presented. The nitro group of IMI can be transformed into the desnitro group. IMI-olefin possesses an imidazole ring (additional double bond in the imidazolidine ring) (manuscript 3, modified).

2 Aims

The general aim of this thesis was the electrophysiological characterization of LUHMES neurons and the exemplification of their applicability for the assessment of functional neurotoxicity for important neuronal toxicant targets, with a focus on the adverse effects of several common neonicotinoids and metabolites on human nAChR signaling.

The specific aims were:

- 1) To provide a detailed electrophysiological characterization of LUHMES neurons and to investigate their applicability for assessing adverse effects on crucial neuronal toxicant targets. P2X receptors were examined as an example for ionotropic receptors, Nav channels as an example for voltage-gated ion channels, and the DAT as an example for electrogenic neurotransmitter transporters.
- 2) To further characterize LUHMES neurons by investigating the signaling via nicotinic acetylcholine receptors (nAChR) and to explore the assay's capability for the detection of adverse effects on these receptors. As a use case, the effects of several neonicotinoids on human nicotinic signaling were researched.
- 3) To examine potential adverse effects of the imidacloprid (IMI) metabolite desnitro-imidacloprid (DN-IMI) on nAChR signaling of LUHMES neurons and to compare the effects with that of its parent compound and nicotine. Furthermore, an aim was to identify affected human nAChR subtypes.

3 Results

3.1 Manuscript 1: Human neuronal signaling and communication assays to assess functional neurotoxicity

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3.1.1 Abstract

Prediction of drug toxicity on the human nervous system still relies mainly on animal experiments. Here, we developed an alternative system allowing assessment of complex signaling in both individual human neurons and on the network level. The LUHMES cultures used for our approach can be cultured in 384-well plates with high reproducibility. We established here high-throughput quantification of free intracellular Ca^{2+} concentrations $[\text{Ca}^{2+}]_i$ as broadly applicable surrogate of neuronal activity and verified the main processes by patch clamp recordings. Initially, we characterized the expression pattern of many neuronal signaling components and selected the purinergic receptors to demonstrate the applicability of the $[\text{Ca}^{2+}]_i$ signals for quantitative characterization of agonist and antagonist responses on classical ionotropic neurotransmitter receptors. This included receptor sub-typing and the characterization of the anti-parasitic drug suramin as modulator of the cellular response to ATP. To exemplify potential studies on ion channels, we characterized voltage-gated sodium channels and their inhibition by tetrodotoxin, saxitoxin and lidocaine, as well as their

opening by the plant alkaloid veratridine and the food-relevant marine biotoxin ciguatoxin. Even broader applicability of $[Ca^{2+}]_i$ quantification as an end point was demonstrated by measurements of dopamine transporter activity based on the membrane potential-changing activity of this neurotransmitter carrier. The substrates dopamine or amphetamine triggered $[Ca^{2+}]_i$ oscillations that were synchronized over the entire culture dish. We identified compounds that modified these oscillations by interfering with various ion channels. Thus, this new test system allows multiple types of neuronal signaling, within and between cells, to be assessed, quantified and characterized for their potential disturbance.

3.1.2 Introduction

Assessment of adverse effects on the nervous system is still a challenge for the development of drugs and for many chemicals in other industry sectors (Schmidt et al., 2017; Walker et al., 2018). It is widely accepted that misleading outputs from traditional preclinical screenings contribute to the current decline in new drug applications, and therefore, more and better tests are required. The most frequent drug side effects observed after drug marketing are related to the disturbance of nervous system function (Redfern et al., 2010). In this context, it is important to note that the toxicity of excitable tissues like the nervous system (or the heart) differs from typical toxic effects observed, e.g., in the liver. For electrically active cells, pronounced impairment can occur in the absence of any morphological changes. The undetected toxicity of drugs is still a major cause of death in the EU (Giardina et al., 2018) and the USA (Sonawane et al., 2018). Neurotoxicity and cardiotoxicity account together for nearly 60% of drug rejections during trials or post-commercialization (McNaughton et al., 2014; Onakpoya et al., 2016; Walker et al., 2018). In the case of the nervous system, functional toxicants may lead to sensory disturbances, nausea, cognitive impairment or seizures. The occurrence of such adverse effects in man is not predicted well by classical animal models (Mead et al., 2016; Olson et al., 2000), and a large consortium of pharmaceutical industry has therefore initiated the NeuroDeRisk project within the innovative medicines initiative 2 (IMI2) of the Horizon2020 framework program (<https://cordis.europa.eu/project/id/821528>).

Several assays have been developed that assess the capacity of test compounds to kill neurons or to affect their morphology (Barbosa et al., 2015; Forsby et al., 2009; Schultz et al., 2015; Wilson et al., 2014). Some of them have proven useful also for larger screens, or were optimized to detect specific cell damage, e.g., to mitochondria (Delp et al., 2019, 2018a). However, such assays fail to detect several functional toxicants. It

is therefore important that also neurophysiological end points can be robustly assessed. One approach is to measure the effects on a large panel of known receptors, enzymes, transporters and channels that are required for neuronal function (Pottel et al., 2020). A more economic variant of this approach uses a physiological parameter that is easily measurable and relates to many of the above toxicant targets. The change of the free intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ is such an end point. The quantification can be performed at high throughput by using live-cell fluorescence imaging of neuronal cultures loaded with calcium-sensitive dyes (Brüll et al., 2020; Grunwald et al., 2019; Karreman et al., 2020; Sirenko et al., 2019).

Two major issues have to be addressed for establishment of an assay on this basis. First, a test system is required that is sufficiently robust to allow comparisons from cell to cell, from well to well, from plate to plate and also between biological replicates (= different cell preparations/assay days). Second, neuronal network features need to be captured. Testing of individual cells alone does not fully capture the neuronal physiology. It can assess many toxicant targets, but not the coupling of neurons with one another (the major functional feature of the nervous system).

Current attempts to develop improved neurotoxicity assays tackle these two issues in different ways (Schultz et al., 2015). The main options for test systems are rat primary neurons (Bradley et al., 2018; Forsby et al., 2009; Kreir et al., 2018; Millard et al., 2019; Sandström et al., 2017), cells differentiated from pluripotent stem cells (Brüll et al., 2020; Pei et al., 2016; Sherman and Bang, 2018; Sirenko et al., 2019; Tukker et al., 2020), and cell lines (Forsby et al., 2009; Klima et al., 2020; Krug et al., 2013; Stiegler et al., 2011). The latter often have the disadvantage that they do not form effective synapses. Neurons derived from iPSC have a large potential, as shown by some screen applications (Brownjohn et al., 2017; Kondo et al., 2017; Pei et al., 2016; Ryan et al., 2016; Sherman and Bang, 2018; Sirenko et al., 2019; Tukker et al., 2020; Xu et al., 2013), but their maturity and reproducibility are hard to control (Handel et al., 2016; Little et al., 2019; Volpato et al., 2018; Volpato and Webber, 2020; Xia et al., 2016), and costs are very high (Bravery, 2015; Engle et al., 2018; Huang et al., 2019; McKernan and Watt, 2013). Primary cells can form excellent networks and contain many cell types of interest. They have for a long time been the major model used for mechanistic studies (Gerhardt et al., 2001; Hansson et al., 2000). However, they are usually of rodent origin, and molecular epitopes, gene expression programs and physiological functions may differ between species (Leist and Hartung, 2013).

Several approaches have been developed to test for neuronal connectivity. For instance, paired patch clamp recordings of synaptically connected pre- and post-synaptic cells allow the investigation of neuronal transmission within a local network (Hefft et al., 2002; Kraushaar and Jonas, 2000). An approach that allows more throughput are

microelectrode arrays (MEA) that record extracellular field potentials of spontaneously active neuronal networks (Alloisio et al., 2015; Bader et al., 2017; Bradley et al., 2018; Hogberg et al., 2011; Kraushaar et al., 2017; Kreir et al., 2018; McConnell et al., 2012; Nicolas et al., 2014; Odawara et al., 2016; Tukker et al., 2020, 2018; Vassallo et al., 2016). By comparison of the firing patterns measured on various electrodes in a culture dish, network synchronization parameters can be derived. This system can thus assess toxicant effects both on the (averaged) function of individual neurons (e.g., spiking activity) or on network activity and allows therefore comprehensive screens (Alloisio et al., 2015; Bader et al., 2017; Bradley et al., 2018; Kraushaar et al., 2017; Kreir et al., 2018; Tukker et al., 2020, 2018). The use of rat neurons is most established for such MEA assays, but also human iPSC neurons are increasingly being used (Kraushaar et al., 2017; Odawara et al., 2018, 2016; Tukker et al., 2020, 2018). To this date, the cost and complexity of the latter approach have prevented larger screens, and only few studies requiring at least medium throughput (concentration–response curves with several replicates and assay conditions) have been published (Kraushaar et al., 2017; Odawara et al., 2018, 2016; Tukker et al., 2020).

We therefore investigated new strategies that will lead to an in vitro neurotoxicity assay using human cells, allowing for highly reproducible assay conditions at low cost and permitting measurements on single cell function as well as on network properties. As test system basis, we used LUHMES cells, which are conditionally immortalized, but non-transformed (Gutbier et al., 2018) human neurons that are well established for morphological, metabolic and biochemical neurotoxicity testing (Brüll et al., 2020; Delp et al., 2019, 2018a, 2018b; Harris et al., 2017; Krug et al., 2014, 2013; Lohren et al., 2015; Smirnova et al., 2016; Tong et al., 2017; Witt et al., 2017; Zhang et al., 2014). We established Ca^{2+} -signaling as the main end point, on the population level, as well as on the level of individual cells and confirmed their basic neuroexcitability parameters. Examples are provided for the assessment of toxicants affecting ion channels, receptors and transporters with high reproducibility and data accuracy. Finally, oscillations of $[\text{Ca}^{2+}]_i$ across the entire culture dish were identified and exemplified as readout for neuronal connectivity and as a measure to identify compounds modifying neuronal network features.

3.1.3 Materials and methods

Materials and chemicals

An overview of experimental tool compounds and toxicants is given in table MS1_S1. Pacific ciguatoxin (pCTX) isolated from a moray eel was provided by the laboratory of Richard Lewis, University of Queensland, Brisbane, Australia.

Cell culture

The cultivation of the LUHMES cells was performed as described earlier (Krug et al., 2013; Schildknecht et al., 2013; Scholz et al., 2011). In brief, LUHMES cells were cultured in standard cell culture flasks (Sarstedt) that were pre-coated with 50 µg/ml poly-L-ornithine (PLO) and 1 µg/ml fibronectin (Sigma-Aldrich) in H₂O overnight at 37 °C. The cells were maintained in proliferation medium containing advanced DMEM/F12 (Gibco) with 2 mM L-glutamine (Sigma Aldrich), 1 × N2-supplement (Gibco) and 40 ng/ml recombinant human basic fibroblast growth factor (FGF-2, R&D Systems). The cells were kept at 37 °C and 5% CO₂ and passaged three times a week, when the culture reached a confluency of 75–90%. Cells were used until passage 18. For differentiation, cells were cultured in differentiation medium consisting of advanced DMEM/F12 (Gibco) supplemented with 2 mM L-glutamine (Sigma Aldrich), 1 × N2-supplement (Gibco), 1 mM N₆,2'-O-dibutyryl 3',5'-cyclic adenosine monophosphate (cAMP) (Sigma Aldrich), 1 µg/ml tetracycline (Sigma Aldrich) and 2 ng/ml recombinant human glial cell-derived neurotrophic factor (GDNF, R&D Systems).

For automated patch clamp recordings, the cells were differentiated for 9 days. The medium was changed every other day, supplemented with 1 µg/ml laminin (Sigma Aldrich). For Ca²⁺-imaging, the cells were pre-differentiated for 48 h in cell culture flasks, detached and plated at a density of 20.000 cells per well on 0.1% PEI-coated 384-well plates (Greiner Bio-One), respectively. For manual patch clamp recordings, the cells were plated at a density of 750 cells/µl on 0.1% PEI-coated glass coverslips. The cells were further differentiated for another 7–8 days. 50% of the medium was exchanged every 2–3 days.

Dopamine uptake

LUHMES cells were differentiated for 6 days in a 24-well format and then treated with the indicated DAT inhibitors. After 15 min, the natural DAT substrate dopamine (DA, 10 µM) was added in radioactively-labeled (³H, 1.5 Bq/mol) form. After 10 min at 37 °C, the supernatant was removed and cells were washed with PBS. Then lysis buffer was added (PBS containing 0.2% Triton X-100). The amount of radioactive label taken up by the cells as well as the residual activity in the cell supernatant (plus washing buffer)

was measured on a scintillation counter. All inhibitor data were normalized to the uptake of cells only exposed to solvent (1.2 ± 0.4 nmol DA/ 10^6 cells).

Gene expression profiling

Five biological replicates were generated from LUHMES cells differentiated for 2, 3, 5, 6, 8, 10, and 11 days, as well as from undifferentiated LUHMES cells (day 0). These samples were prepared for transcriptome analysis considering genes of neurotransmitter receptors, ion channels, and calcium binding proteins. Samples were analyzed via the TempO-Seq assay, which is a targeted RNA sequencing method developed by BioSpyder Technologies Inc. (Carlsbad, CA, USA). The method is described in detail in House et al. (2017). For sample preparation, LUHMES grown in 96-well plates were lysed in 25 μ l 1 $\times$ BioSpyder lysis buffer according to the manufacturer's instructions. The lysate from ten wells was pooled for each sample. Samples were stored at -80 °C before shipping on dry ice to BioClavis (Bio-Clavis, Ltd., Glasgow, UK) for TempO-Seq analysis. The resulting FASTQ files were aligned using the STAR algorithm to a pseudo-transcriptome by BioClavis and eventually normalized and standardized to a data format of x gene specific counts per one million reads. Traditional whole genome RNA sequencing (RNAseq) was performed for comparison and validation. Cells were cultured in six-well plates. For sample preparation, the medium was removed and cells were lysed in TriFast reagent (Peqlab, VWR, USA). The lysate of six wells was pooled for each sample. Samples were stored at -20 °C until they were sent on dry ice to the department of toxicogenomics at the University Maastricht, The Netherlands, for RNAseq analysis.

Ca²⁺-imaging

Ca²⁺-imaging was performed using HT Functional Drug Screening System FDSS/ μ CELL (Hamamatsu Photonics) at nominal 37 °C. The FDSS/ μ Cell system enables the indirect recording of changes of intracellular Ca²⁺ [Ca²⁺]_i via a Ca²⁺-sensitive fluorescent dye. The fluorescence signal of a complete 384-well plate is acquired at once with a highspeed and high-sensitivity digital ImagEM X2 EM-CCD camera (Electron Multiplying Charge-Coupled Device, Hamamatsu Photonics), but with limited spatial resolution. Therefore, the software only determines the mean fluorescence signal of each well. The signals of individual cells could not be captured. For compound application, the integrated dispenser head with 384 pipette tips was used, which can add the test compound to all wells simultaneously. The cells were preincubated with Cal-520 AM (AAT Bioquest) at a concentration of 1 μ M for 1 h at 37 °C. For recording, the medium was exchanged by a buffer solution containing [mM]: 135 NaCl, 5 KCl, 0.2 MgCl₂, 2.5 CaCl₂, 10 HEPES and 10 D-glucose, pH 7.4. Test compound application was executed

after obtaining a 1.5 min baseline recording. Where applicable, a second application was executed 4.5 min after the first application. The total recording never exceeded 8 min.

Automated patch clamp recordings

Automated patch clamp recordings were performed on a QPatch (Sophion Bioscience) with 16X QPlates. After detachment, the cells were resuspended at a concentration of $3\text{--}4 \times 10^6$ cells per ml. The extracellular solution contained [in mM]: 145 NaCl, 4 KCl, 10 CaCl_2 , 10 HEPES, 20 TEA, 1 4-AP and 0.1 Cd^{2+} , pH 7.4. The intracellular solution contained [in mM]: 120 CsF, 20 CsCl, 5 NaCl, 10 HEPES and 10 EGTA, pH 7.2. Recordings were obtained at room temperature. Between test pulses, the cells were held in whole-cell clamp at a holding potential of -80 mV. For TTX and lidocaine experiments, cells were hyperpolarized to -120 mV for 200 ms, before they were depolarized with ten pulses to 0 mV for 10 ms with an interval of 100 ms between each pulse. For the recordings with ICA-121431, a selective voltage-gated sodium channel (Nav) antagonist, a pulse protocol was used which inactivated 50% of the Nav channels to achieve a selective inhibition of the Nav channels (McCormack et al., 2013). Therefore, a pre-pulse to -55 mV for 500 ms was applied corresponding to the half-inactivation voltage of the cells' Nav channels, followed by a test pulse to 0 mV for 10 ms. The holding potential between the pulses was -120 mV. For the biophysical characterization of the activation properties of Nav , the cells were kept at a holding potential of -90 mV. Prior to the test pulse, the cells were hyperpolarized to -120 mV for 200 ms. The test pulse had a duration of 100 ms and was increased from -70 mV to $+40$ mV in $+10$ mV steps. To examine the characteristics of the steady-state inactivation of Nav , the cells were stimulated with a test pulse to 0 mV for 10 ms after a pre-pulse with voltage steps from -110 mV to -10 mV in $+10$ mV steps, which lasted for 500 ms. The recovery from inactivation of Nav was investigated as follows: cells were held at a potential of -120 mV followed by a first reference test pulse to 0 mV for 200 ms, which was followed by a second test pulse to 0 mV for 20 ms. The time between the two test pulses was increased from 1 ms with a factor of 2.5 in nine steps.

Manual patch clamp recordings

Manual patch clamp was executed with an EPC 10 USB patch clamp amplifier and PatchMaster Software (version 2 x 90.5; HEKA Elektronik, Lambrecht, Germany). Extracellular solution contained [mM]: 140 NaCl, 4 KCl, 1 MgCl_2 , 1.8 CaCl_2 , 10 HEPES and 10 D-glucose, pH 7.4. Intracellular solution contained [mM]: 107 K-gluconate, 10 KCl, 1 MgCl_2 , 10 HEPES, 5 EGTA, 4 Na_2ATP and 0.2 NaGTP, pH 7.20. Recordings were obtained at room temperature. The cells were kept at a holding potential of -70 mV. In voltage clamp mode Nav and voltage-gated potassium (K_v) channels were activated

for 300 ms by voltage pulses ranging from – 70 to + 70 mV in + 10 mV steps after a hyperpolarizing step to – 120 mV for 200 ms. To investigate the firing behavior, cells were stimulated in current clamp mode by hyper- and depolarizing current pulses of 300 ms duration. The pulse protocol was executed at 0.2 Hz. For agonist tests in current clamp and voltage clamp mode, cells were kept at a holding potential of – 70 mV and the compounds were applied for 5 s.

Data analysis

After offset correction using the FDSS software (version 3.2), the Ca²⁺-imaging data were exported and further analyzed with scripts written in R (version 3.6.3) (R Core Team, 2020). The concentration–response curves were fitted using a log-logistic model described by Ritz et al. (2015), utilizing the R package *drc* with its function *drm()* and *LL2.2()* with the following equation: $f(x) = d/[1 + \exp(b(\log(x) - \tilde{e}))]$ (Ritz et al., 2015). The logarithm of the half-maximal effective concentration (logEC₅₀) between 0 and the upper limit (*d*), which was set to 1, is represented by $\tilde{e}$, *x* denotes the concentration and *b* stands for the slope parameter (Ritz et al., 2015). In cases with normalizations to responses induced by another compound, the function *LL2.3()* was used with a variable upper limit (*d*; Ritz et al., 2015). The same equation was used to determine the half-maximal inhibitory concentration (logIC₅₀). Then the logEC₅₀ and logIC₅₀ values were converted into the pIC₅₀ and pEC₅₀ values, which are the negative logarithms to base 10.

Automated patch clamp data were pre-processed using the QPatch Assay Software (version 5.0) (Sophion Bioscience, DK) for offset correction and to detect the peak currents of the Nav channels. For further analysis, scripts written in R were utilized. For the calculation of the voltage-dependent conductance ($G/G_{\max}$), the peak currents (*I*) were divided by the difference of the stimulation voltage (*V*) and the reversal potential (*V_{rev}*) using the equation: $G = I/(V - V_{\text{rev}})$ and then normalized to the maximal conductance ($G_{\max}$; (Cheng et al., 2011; Wang et al., 2015; Zhang et al., 2013)). For steady-state inactivation ($I/I_{\max}$), the peak currents were normalized to the maximal peak current. The analyzed data of the voltage-dependent conductance ($G/G_{\max}$) and the steady-state inactivation ($I/I_{\max}$) of Nav were fitted with the Boltzmann equation: $G/G_{\max}$ and $I/I_{\max} = 1/(1 + \exp[(V_{50} - V)/k])$, which was used to estimate the half-maximal voltage (*V*₅₀) and the slope factor (*k*; (Cheng et al., 2011; Wang et al., 2015; Zhang et al., 2013)). For the analysis of the recovery from inactivation, the peak current of the second test pulse (*I_{peak2}*) of each time interval was normalized to the peak current of the first reference test pulse (*I_{peak1}*) of the corresponding time interval. Data were fitted with a bi-exponential function resulting in a fast and a slow time constant using the equation: $I_{\text{peak2}}/I_{\text{peak1}} = A_1 [1 - \exp(-t/\tau_1)] + A_2 [1 - \exp(-t/\tau_2)]$ (Zhang et al.,

2013). The parameter t stands for the time interval, A_1 and A_2 are the amplitudes and τ_1 and τ_2 represent the recovery time constants.

The raw data of the manual patch clamp recordings were analyzed in scripts written in R. For leak subtraction, the P/4 algorithms of PatchMaster and QPatch Software were used in the voltage clamp recordings for manual and automated patch clamp experiments, respectively.

The following R packages were utilized for data handling: cowplot (Wilke, 2019), dplyr (Wickham et al., 2020), drc (Ritz et al., 2015), ephys2 (Danker, 2018), ggplot2 (Wickham, 2016), htmlwidgets (Vaidyanathan et al., 2019), lemon (Edwards, 2019), magick (Ooms, 2020), magrittr (Bache and Wickham, 2014), matrixStats (Bengtsson, 2020), miniUI (Cheng, 2018), modelr (Wickham, 2020), multcomp (Hothorn et al., 2008), plotrix (Lemon, 2006), proto (Grothendieck et al., 2016), shiny (Chang et al., 2020), shinyjs (Attali, 2020), shinyTree (Trestle Technology, LLC, 2017), tidyverse (Wickham et al., 2019).

The raw count tables of gene expression profiling with Tempo-Seq assay and traditional whole genome RNA sequencing (RNAseq) were analyzed with the R package DESeq2 (v1.24.0) (Love et al., 2014). RNAseq counts were normalized to the library size and the transcript length (Transcripts per kilobase million (TPM)) (Wagner et al., 2012). TempoSeq counts were normalized to total counts per sample [counts per million (CPM)]. Gene lengths were retrieved from the hg18 reference genome (NCBI Build 36.1) with the R package Goseq (v1.40.0) (Young et al., 2010). TPM/CPM were averaged over the five biological replicates.

Data handling and statistics

Unless mentioned differently, values are presented as mean $\pm$ SEM. If not indicated otherwise, experiments were performed with at least three technical replicates per condition. Detailed data on pEC₅₀, pIC₅₀ and n numbers are found in supplementary tables. Statistical significance was defined as $P < 0.05$ and was determined by one-way ANOVA with Dunnett's post hoc test as indicated.

3.1.4 Results and discussion

Implementation of high-throughput Ca²⁺-signaling and application to purinergic receptor profiling

A neurofunctional test should ideally be able to assess neuronal activity changes related to various effector systems that directly affect the membrane potential, and thus the neuronal firing properties. Some of the most important toxicant targets are voltage-

dependent ion channels, ligand-gated ion channels and electrogenic transporters. The classical neurotoxicological approach uses electrophysiological techniques. These are resource intensive, can only be performed with equipment not readily available in most cell culture laboratories, and require specialist knowledge to perform and interpret the experiments. We explored here whether an imaging-based approach could be used alternatively to capture various neuronal responses in a high-throughput fashion. Notably, the term “high throughput” is here not meant to imply assessment of very large compound collections. We rather see it as important to allow good in vitro toxicology practice, i.e., recording of full concentration–response curves with sufficient replicates (i.e., at least 20–30 data points per compound). Such data sets are still hard (or very expensive) to obtain with patch clamp approaches or microelectrode arrays (MEA). As readout, we used here the rapid changes (peaks) of $[Ca^{2+}]_i$, because this can be measured easily by imaging devices (in cells loaded with fluorescent indicators), and as this end point is sensitive to changes in the membrane potential that lead to action potentials in neurons.

Initially, we explored purinergic (P2) receptors as example for ligand-gated ion channels. They play an important role in neurons of the peripheral and central nervous system. The P2X family, the focus of our experiments, is present in different types of neurons, for example in dopaminergic neurons. They take various roles in pathological conditions like Parkinson’s disease and in pain mediation (Abbracchio et al., 2006; Amadio et al., 2007; Burnstock and Kennedy, 1985; Khakh et al., 2001; Puchałowicz et al., 2014; Syed and Kennedy, 2012; Tóth et al., 2019).

To obtain reference data, we performed manual patch clamp recordings to investigate the presence of P2X receptors on a single-cell level. LUHMES cells were plated on glass coverslips, where they adhered and extended long neurites (Fig. MS1_1a). The application of ATP triggered action potential firing (Fig. MS1_S1A), associated with fast-inactivating inward currents (Fig. MS1_S1B). The shape of the current curves indicates the presence of P2X1 or P2X3 receptors (Bianchi et al., 1999; Koshimizu et al., 2000; Li et al., 2013; North, 2002). More detailed follow-up experiments suggest that also some receptors with slow inactivation kinetics (e.g., P2X4 or P2X7) (Bianchi et al., 1999; Koshimizu et al., 2000; Li et al., 2013; North, 2002) may be present (Fig. MS1_S1C, D). Gene expression studies on the LUHMES cells confirmed the presence of mRNA of several P2X receptors (Fig. MS1_S2), with P2X3 appearing to be dominant.

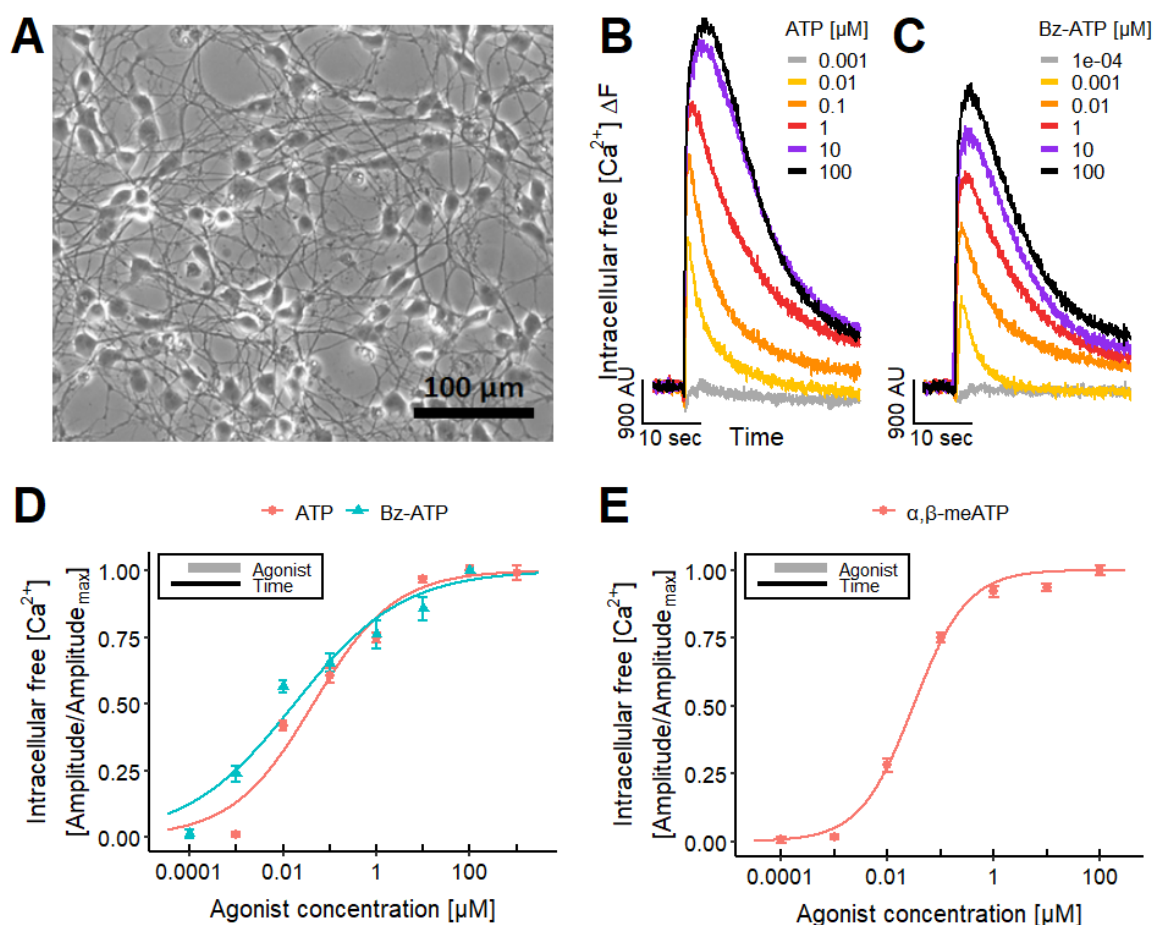


Fig. MS1_1 Effects of different P2 receptor agonists.

a LUHMES cells were differentiated for 7 days on a pre-coated glass coverslip before a phase contrast image was taken. **b, c** Ca^{2+} -imaging traces displaying the responses of the LUHMES neurons to the application of **b** ATP and **c** BzATP. **d** The concentration–response curves yield pEC_{50} s of 7.35 ± 0.08 for ATP and 7.76 ± 0.11 for BzATP in Ca^{2+} -imaging. **e** Concentration-dependent effect of $\alpha,\beta\text{-meATP}$ in Ca^{2+} -imaging, resulting in a pEC_{50} value of 7.52 ± 0.03 . The concentration–response curve depicts the mean of five differentiations of the LUHMES neurons. See Fig. MS1_S3D for more details on the curves of the single differentiations. Note the treatment schemes (upper left corner), illustrating the experimental design. Detailed data on n numbers are found in table MS1_S4

On this basis, we tried to assess the responses induced by the activation of P2X receptors using Ca^{2+} -imaging. LUHMES loaded with a $[\text{Ca}^{2+}]_i$ indicator dye were exposed to the endogenous P2X receptor agonist ATP (Fig. MS1_1b) and the synthetic ATP analog BzATP (Fig. MS1_1c) (Bianchi et al., 1999; Khakh and North, 2012). A strong signal peak was recorded, and the fast decrease of the fluorescence signal (half-life of ~ 8 s at $100 \mu\text{M}$ ATP) found here is typical for P2X1 and P2X3 receptors as reported previously in GT1-7 cells (He et al., 2003; Koshimizu et al., 2000) (Fig. MS1_1b). We obtained pEC_{50} values from seven-point concentration–response curves ($n = 42$ data points per compound) (Fig. MS1_1d), and these were well in line with published data for human P2X1 and P2X3 receptors.

As our results suggest a strong contribution of P2X1 and P2X3 to the responses induced by ATP, we used this as an example for a mechanistic follow-up of an observed effect. A specific agonist of these receptors (i.e., α,β -meATP) was employed to confirm their involvement (Abbracchio and Burnstock, 1994; Bianchi et al., 1999; Burnstock and Kennedy, 1985; Gever et al., 2006; Khakh and North, 2012). We first obtained reference data by manual patch clamp. The electrophysiological responses (Fig. MS1_S3A, B) suggested the activation of P2X1 and P2X3 receptor subtypes (Bianchi et al., 1999; Burgard et al., 2000; Koshimizu et al., 2000; Li et al., 2013; North, 2002). The selective agonist α,β -meATP also led to a cellular response in Ca^{2+} -imaging experiments (Fig. MS1_S3C). The recordings were performed for five differentiations at seven concentrations to illustrate the reproducibility of the test system and end point (Fig. MS1_S3D). The pEC_{50} value of 7.5 (Fig. MS1_1e) and the fast inactivation of the fluorescence signal (Fig. MS1_S3C) confirmed a functional expression of P2X1 and/or P2X3 receptors (Bianchi et al., 1999; Gever et al., 2006).

For a biological calibration of the α,β -meATP signal, we compared it to the signal triggered by an increase in the buffer K^+ concentration (maximal depolarizing stimulus). The response evoked by the purinergic agonist reached up to 28% of the K^+ -induced signal intensity. This is well in line with P2X receptor activation being a physiological response that does not reach the level of complete and irreversible cell depolarization (Fig. MS1_S3E, F).

As a next step to characterize the suitability of the test system, we studied interference with Ca^{2+} -signaling. The antagonistic effect of TNP-ATP (competitive antagonist) on the response to ATP and α,β -meATP was compared in Ca^{2+} -imaging experiments (Fig. MS1_2a). At high concentrations, we observed a complete block of signaling, and the concentration–response features of the antagonist were agonist dependent, as expected for a competitive inhibitor. The significant difference of pIC_{50} values (6.1 and 7.5) suggests that ATP stimulates a broad panel of P2X receptors (for some of which TNP-ATP has a relatively low affinity) (Gever et al., 2006; Virginio et al., 1998). To further explore how well such differential antagonist effects can be described and quantified, we used a selective antagonist of P2X3 receptors, A-317491 (Gever et al., 2006; Virginio et al., 1998). This compound potently and completely blocked the response to α,β -meATP, while it showed only a weak partial effect on ATP signals (Fig. MS1_2b).

It needs to be noted here that ATP, but not α,β -meATP, can also activate P2Y receptors (Abbracchio et al., 2006; von Kügelgen and Harden, 2011), for example P2Y1 (Palmer et al., 1998; Waldo and Harden, 2004) and P2Y11 receptors (Communi et al., 1999; Qi et al., 2001; White et al., 2003), which are also expressed in LUHMES neurons and which may theoretically act as response modifiers (Fig. MS1_S2).

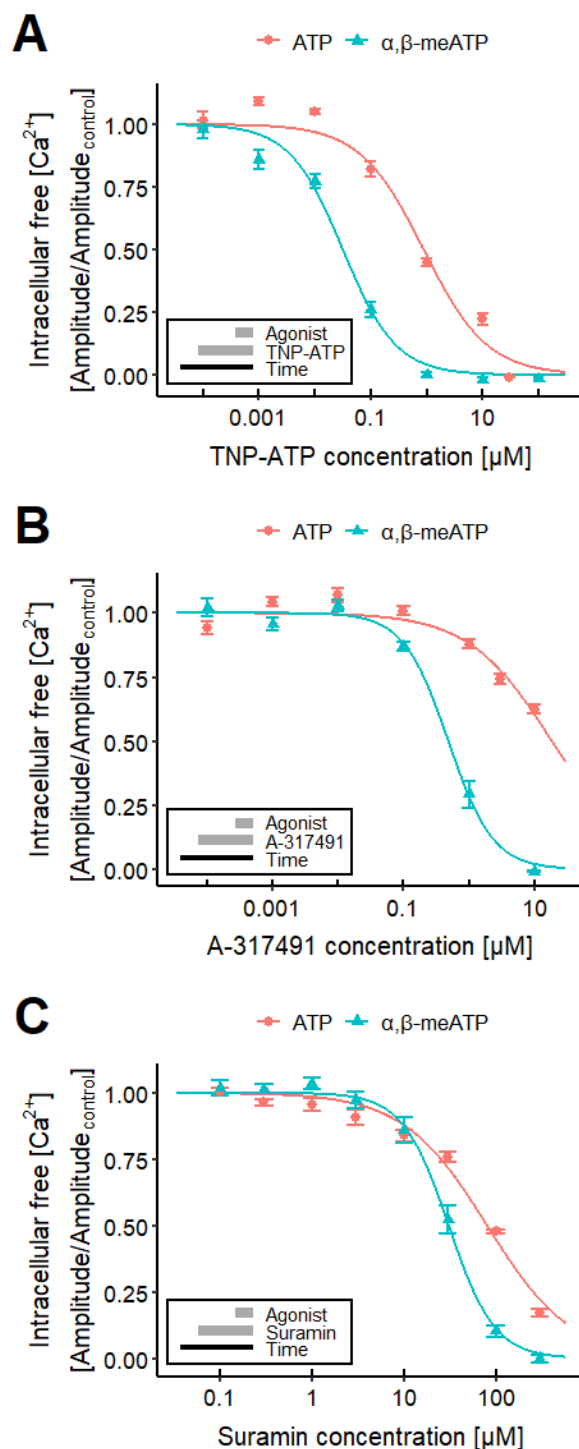


Fig. MS1_2 Characterization of P2X receptors.

Ca^{2+} -imaging experiments researching the effects of different P2X receptor antagonists on the response of LUHMES cells triggered by 1 μM ATP ($\sim EC_{75}$, Fig. MS1_1d) and 0.1 μM α,β -meATP ($\sim EC_{75}$, Fig. MS1_1e). **a** The responses were blocked concentration-dependently by TNP-ATP with pIC_{50} s of 6.05 ± 0.06 for ATP and of 7.50 ± 0.05 for α,β -meATP. The values are significantly different. **b** A-317491 blocked the responses of ATP up to 40% and yielded a pIC_{50} for α,β -meATP of 6.31 ± 0.05 . **c** An inhibitory effect of suramin could be detected for ATP and α,β -meATP, which resulted in pIC_{50} s of 4.09 ± 0.03 and 4.51 ± 0.03 , respectively. Note the treatment schemes (lower left corner), illustrating the experimental design. Detailed data on n numbers are found in table MS1_S4

Finally, we used the antiprotozoal agent suramin to show applicability of the LUHMES system to characterize xenobiotics potentially interfering with P2X receptors. One known side effect of suramin, a drug used for the treatment of sleeping sickness (Kennedy, 2013; Wéry, 1994), is the inhibition of P2X receptors (Coddou et al., 2011; Garcia-Guzman et al., 1997; Gevert et al., 2006; Khakh and North, 2012). The experiments yielded pIC₅₀s of 4.1 for ATP and 4.5 for the P2X₃ ligand α,β -meATP (Fig. MS1_2c). The data obtained with α,β -meATP agree with a previously reported pIC₅₀ of approximately 4.8 for human P2X₃ receptors (Garcia-Guzman et al., 1997).

In summary, these initial experimental system evaluations confirmed that measurement of [Ca²⁺]_i in LUHMES can capture (some) electrophysiological responses related to drug effects and toxicity. The data available at this stage indicate that the potency quantifications for agonists and antagonists are precise (little variation between cell preparations) and exact (very similar to reference systems).

Characterization of Nav channel toxicants

Next, we moved to the detection of potential channel modulators and checked whether agents affecting voltage-dependent sodium channels (Nav channels) could be characterized by Ca²⁺-imaging. Nav channels are essential for the onset of action potentials and thus are of critical importance for the neuronal electrical activity. An alteration of their function can lead to severe functional neurotoxicity, even with lethal consequences on the level of the organism (Anwar et al., 2018; Gaillard and Pepin, 2001; Lehane and Lewis, 2000; Llewellyn, 2009; Nicholson and Lewis, 2006; Vilariño et al., 2018; Wiese et al., 2010). Nav channels couple to [Ca²⁺]_i indirectly by triggering cell depolarization, which in turn leads to the opening of voltage-dependent calcium channels and thus an influx of Ca²⁺ into neurons (Mohammed et al., 2017; Vetter et al., 2012).

To check practical applicability, we studied the Ca²⁺-response of well-known Nav channel toxicants. First, veratridine (VTD), a plant alkaloid (Ulbricht, 1998; Wang and Wang, 2003) known to delay Nav channel inactivation (Catterall, 1992; Power et al., 2012; Tsukamoto et al., 2017; Zhang et al., 2018), was used. For background information, patch clamp data were obtained and VTD reversibly increased the action potential duration (Fig. MS1_3a). Imaging experiments then showed a concentration-dependent rise of [Ca²⁺]_i that resulted in a pEC₅₀ value of 5.4 for VTD (Fig. MS1_3b). These data are in good agreement with published data on human SH-SY5Y cells (Vetter et al., 2012). Also here, we moved on to also study antagonism: VTD-induced responses were blocked by the Nav channel antagonist tetrodotoxin (TTX, Fig. MS1_S4A) with a pIC₅₀ of 7.9 (Fig. MS1_3c). The inhibition of VTD-mediated effects by TTX is consistent with previously reported data from patch clamp recordings on rat hippocampal neurons

(Alkadhi and Tian, 1996), Ca^{2+} -imaging experiments on mouse DRG neurons (Mohammed et al., 2017) and human SH-SY5Y cells (Vetter et al., 2012).

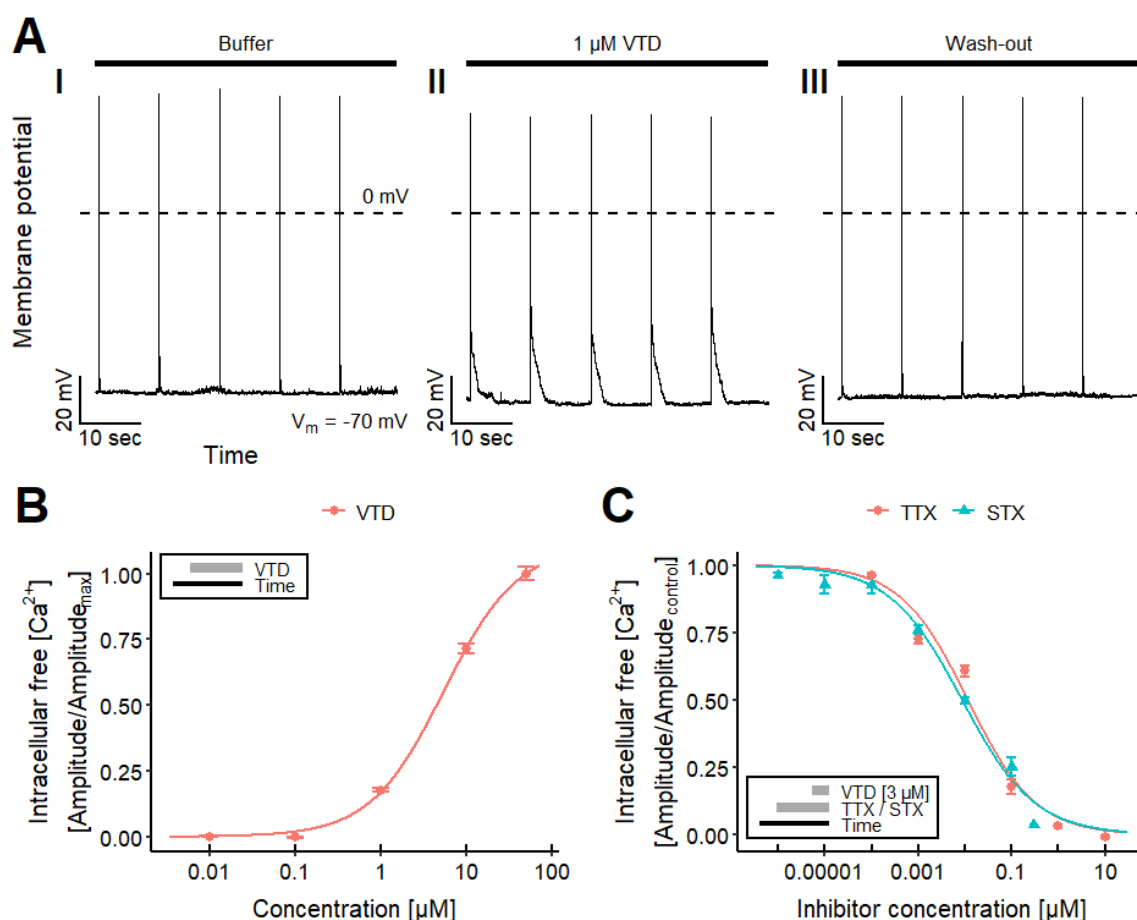


Fig. MS1_3 Nav channel toxicity—effect of veratridine (VTD).

a Traces of a manual patch clamp experiments with LUHMES neurons showing five action potentials, triggered by 10 ms depolarizing current pulses with 0.1 Hz, in untreated control (I), in the presence of 1 μM veratridine (VTD, II) and after wash-out (III, $n = 4$). VTD reversibly increased the action potential duration. **b** Ca^{2+} -imaging experiments demonstrating the concentration-dependent effects of VTD on LUHMES neurons, which yielded a pEC_{50} value of 5.38 ± 0.03 . **c** Concentration-dependent inhibitory effect of TTX and STX on the response triggered by 3 μM VTD, resulting in pIC_{50} values of 7.93 ± 0.06 for TTX and 8.06 ± 0.06 for STX. Note the treatment schemes, illustrating the experimental designs. Detailed data on n numbers are found in table MS1_S4

As additional proof-of-concept test compound, we used saxitoxin (STX), an alkaloid that is produced by certain genera of cyanobacteria and marine dinoflagellates (Deeds et al., 2008; He et al., 2016; Westrick et al., 2010; Wiese et al., 2010). It can contaminate water supplies and accumulate in the marine food chain, and thus cause paralytic shellfish poisoning in humans (Deeds et al., 2008; Durán-Riveroll and Cembella, 2017; Wiese et al., 2010). Like TTX, STX is known for its inhibitory effect on the Nav channels (Durán-Riveroll and Cembella, 2017; Llewellyn, 2009; Mattei and Legros, 2014; Noda

et al., 1989; Terlau et al., 1991). We tested the effect of STX on the response evoked by 3 μ M VTD to verify the capability of our assay to detect Nav channel-modulating biotoxins (Fig. MS1_S4B). The pIC₅₀ value of 8.1 (Fig. MS1_3c) found here is comparable to data previously described for recordings with rat Nav1.2 expressed in *Xenopus laevis* oocytes (Noda et al., 1989). These results demonstrate the capability of setting up a LUHMES cell-based assay for the detection of biotoxins, which affect Nav channel activity, and the quantification of their effects, using Ca²⁺-imaging.

To further evaluate the usability of LUHMES cells as relevant functional neurotoxicity model, we examined the effect of pacific ciguatoxin-2 (pCTX-2) on the LUHMES cells via Ca²⁺-imaging (Fig. MS1_S4C-E). The ciguatoxins are marine biotoxins synthesized by dinoflagellates (Lehane and Lewis, 2000; Litaker et al., 2017, 2010; Nicholson and Lewis, 2006; Vilariño et al., 2018). These polycyclic ethers accumulate in the marine food chain and can lead to intoxications in humans, called ciguatera, after consumption of CTX-contaminated fish (Dickey and Plakas, 2010; Lehane and Lewis, 2000; Nicholson and Lewis, 2006; Skinner et al., 2011; Vilariño et al., 2018). The CTXs comprise several analogs, with an acute toxicity (mice) of pCTX-2 of 2.3 μ g/kg (Lehane and Lewis, 2000; Lewis et al., 1991; Nicholson and Lewis, 2006). We found in LUHMES neurons that 15 nM pCTX-2 increased [Ca²⁺]_i. This response was followed by long-lasting oscillations of the Ca²⁺-imaging signal. The initial increase of the baseline Ca²⁺ level is likely explained by Na⁺ influx, followed by entry of Ca²⁺ into the cytosol through Cav channels (Molgó et al., 1993). The oscillation of the Ca²⁺-imaging signal is noteworthy as our data are not single-cell recordings, but measures of [Ca²⁺]_i in all cells of the whole neuronal network. It is thus a first indication of synchronized activity of the LUHMES neuron population (see more details below). Notably, it has been shown earlier that CTX-1 can induce oscillations of the membrane potential and repetitive firing of action potentials in other cells (Bidard et al., 1984; Birinyi-Strachan et al., 2005; Hamblin et al., 1995; Hogg et al., 2002, 1998).

Finally, we also explored the effects of a microcystin to test for the specificity of the LUHMES test system. Microcystins are cyclic peptides produced by a number of cyanobacteria genera (He et al., 2016; Sivonen and Jones, 1999) found for example in contaminated water and fish (Campos and Vasconcelos, 2010). They inhibit protein phosphatases, like PP1 and PP2A (Campos and Vasconcelos, 2010; MacKintosh et al., 1990), but are not known to affect ion channels. We therefore anticipated that microcystin-LF (a potent hepatotoxicant) would not alter the response of the LUHMES cells. We tested the effect of a high concentration of 2 μ M microcystin-LF, alone and on the response to 3 μ M VTD (Fig. MS1_S4F). The toxin did not show any effects in our Ca²⁺-imaging assay, as expected. This result illustrates the capability of our assay to

distinguish between biotoxins directly affecting the electrical activity of neurons and toxins that exhibit a different, cytotoxic mode of action.

In summary, our findings show the capability of the LUHMES test system to detect biotoxins that affect Nav channel activity in an agonistic or antagonistic way. Taken together, the experiments on P2X receptors and Nav channels demonstrated that whole-culture, high throughput Ca^{2+} -imaging is a suitable assay end point to broadly cover various types of functional neurotoxicants.

Electrophysiological characterization and pharmacological modulation of Nav

Before moving on with exploring further types of potential toxicant targets, we considered it important to provide a basic electrophysiological characterization of the LUHMES cultures. As the cells are derived from a cell line, it was important to confirm that all cells in culture consistently show genuine neuronal electrical properties (as assessed here by the generation of action potentials). A comprehensive investigation of firing behavior ($n = 274$ cells) showed that 45% of cells displayed phasic (Fig. MS1_S5A) and 51% a tonic firing pattern (Fig. MS1_S5B). Depolarization failed to induce action potentials in only 4% of the cells. Such a co-occurrence of phasic and tonic action potential firing behavior has also been described for primary dopaminergic neurons (Grace and Bunney, 1984a, 1984b). These findings support our earlier data on few selected cells (Scholz et al., 2011), and provide clear proof for functional expression of voltage-gated ion channels throughout the whole population.

When cells were exposed to depolarizing voltage pulses (voltage clamp recordings), rapid transient inward currents were observed when the membrane potential was raised to levels higher than -40 mV. This was followed by long-lasting outward currents (Fig. MS1_4a, b). The inward currents were blocked by TTX ($1 \mu\text{M}$). Outward currents were inhibited by a combination of TEA (10 mM intra- and extracellularly) together with the replacement of K^+ with Cs^+ in the intracellular solution (data not shown) to prevent current flow through K_v channels. Taken together, these data confirmed expression of functional Nav and K_v channels, a key feature of excitable neurons. To further provide a solid background description of the test system, we obtained gene expression data for many channel constituents and other genes involved in neuronal signaling. The distinct neuronal features of the cells were also confirmed here (Fig. MS1_S2).

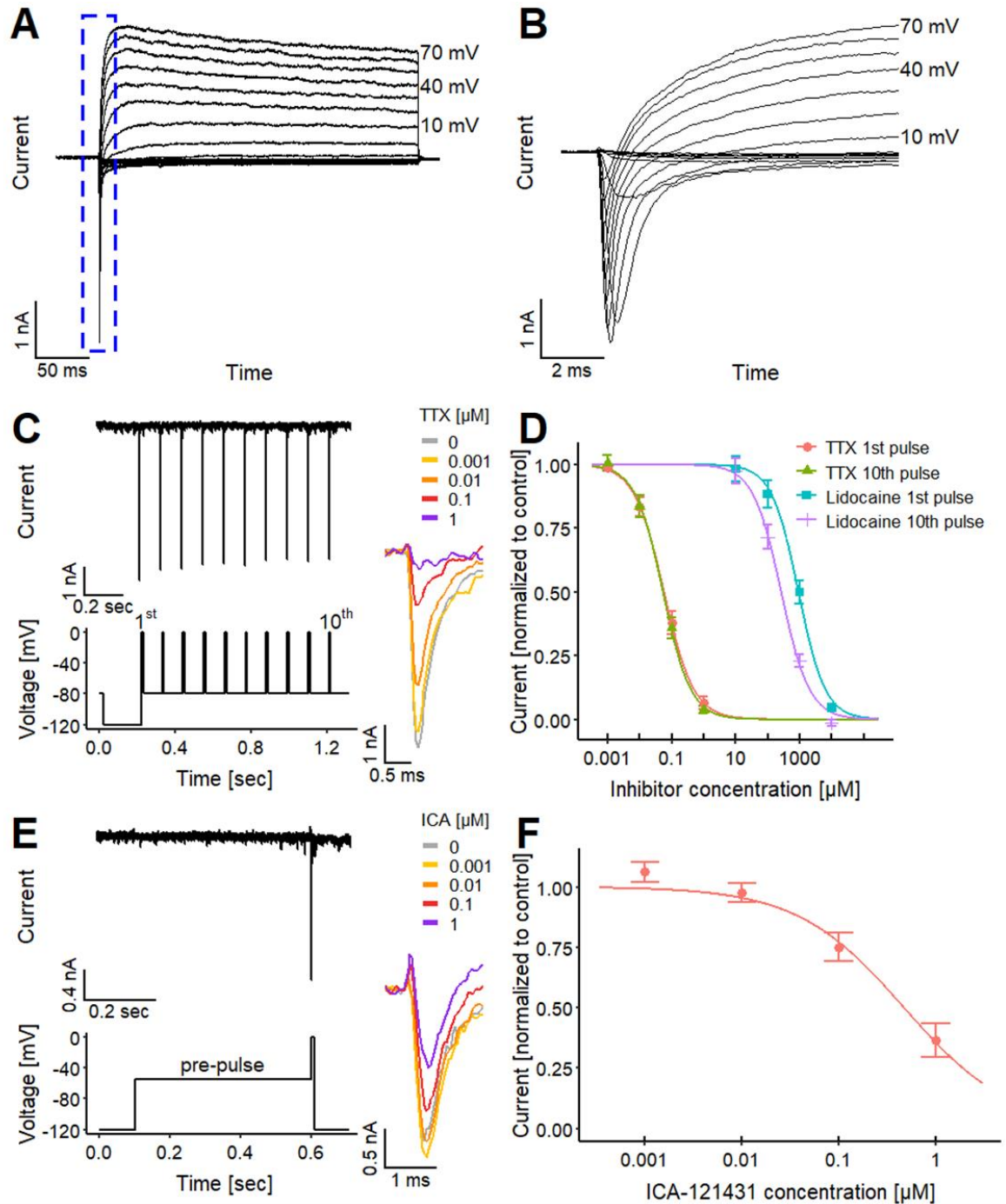


Fig. MS1_4 Electrophysiological characteristics of Nav channel.

a, b Manual patch clamp recordings of **a** activation of voltage-gated inward and outward currents stimulated by different voltage steps with **b** a magnification of the inward current (note: different time axis). **c–f** Automated patch clamp recordings for a pharmacological characterization of voltage-gated Na⁺ (Nav) channels, using **c, d** TTX and lidocaine to investigate the use-dependent and acute inhibitory effects and **e, f** ICA-121431 to narrow Nav channel subtypes down. **e** Exemplary trace of the inward current triggered by the pulse protocol with ten closely spaced test pulses. Traces on the right depict the concentration-dependent effect of TTX on the 1st pulse. **d** Concentration–response curves yielded pIC₅₀ values of 7.23 ± 0.05 (1st pulse) and 7.26 ± 0.05 (10th pulse). Lidocaine had pIC₅₀ values of 3.03 ± 0.07 (1st pulse) and 3.57 ± 0.06 (10th pulse). The pIC₅₀ values for lidocaine are significantly different, unlike the pIC₅₀ values of TTX. **e** To ensure a selective effect of ICA-121431, measurements need to be performed under conditions

where 50% of the Nav channels are inactivated (McCormack et al., 2013). This was achieved by the application of a prepulse to -55 mV before the stimulus, the determined V_{50} value for steady-state inactivation in these cells (Fig. MS1_S5F). Traces on the right depict the concentration-dependent effect of ICA-121431. **f** Effect of ICA-121431 with a pIC_{50} value of 6.33 ± 0.10 . Detailed data on n numbers are found in table MS1_S4

To better understand the functional implication of the expression of different Nav channel subtypes, we set out to explore their potential electrophysiological role in LUHMES. Because of the higher throughput than manual patch clamp, we used automated planar patch clamp recordings to obtain detailed biophysical data on the Nav channels. All features were in good agreement with data on Nav expression systems (Fig. MS1_S5C–F) (Cummins et al., 2001; McCormack et al., 2013; Oliva et al., 2014; Patel et al., 2016). This coherence also applied to the use dependence of the channels, a feature which can have important toxicological implications (Fig. MS1_4c). Lidocaine was studied here as a well-known example of a use-dependent blocker for Nav channels. It led to significantly different pIC_{50} values of 3.03 (for the 1st peak of a stimulation sequence) vs 3.57 (10th peak of the sequence, Fig. MS1_4d). The higher potency of lidocaine on the tenth Nav current peak indicates a use-dependent mechanism of the inhibition on Nav channels as reported previously (Clarkson et al., 1988; Huang et al., 2006; Leffler et al., 2007). The pIC_{50} values of lidocaine were similar to those obtained for rat hippocampal neurons (~ 3.4) (Kaneda et al., 1989).

As Nav channel subtypes play an important role in toxicology and pharmacology, we used here TTX as tool to distinguish two major classes potentially expressed on LUHMES. The pIC_{50} values found here for TTX were in the low nM range (~ 7.2), indicating the presence of TTX-sensitive Nav channels. To compare these inhibition data to the lidocaine data set, we explored whether TTX effects were use dependent. In contrast to lidocaine, the pIC_{50} for the first and the tenth pulse were identical for TTX (Fig. MS1_4d). Based on these data, the Nav subtypes of LUHMES may be Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.6 or Nav1.7 (all reported to be TTX-sensitive, (England and de Groot, 2009; Lee and Ruben, 2008; Ogata and Ohishi, 2002; Zhang et al., 2013)). However, the subtypes Nav1.4 and Nav1.7 are mainly present in skeletal muscle and in the peripheral nervous system, respectively (England and de Groot, 2009; Lee and Ruben, 2008; Ogata and Ohishi, 2002; Zhang et al., 2013). The remaining subtypes Nav1.1, Nav1.2, Nav1.3 and Nav1.6 are strongly expressed in the CNS (England and de Groot, 2009; Lee and Ruben, 2008; Ogata and Ohishi, 2002; Zhang et al., 2013). To further narrow down Nav channel subtypes, the selective Nav channel antagonist ICA-121431 was used. It has been shown to exhibit high potency for Nav1.1 and Nav1.3 ($IC_{50} = 0.023$ μ M and 0.013 μ M, respectively), an intermediate potency for Nav1.2 ($IC_{50} = 0.240$ μ M) and low potency for Nav1.6 and Nav1.7 ($IC_{50} = 13$ μ M and 10 μ M), respectively (McCormack et al., 2013). We found a pIC_{50} value of 6.3, (Fig. MS1_4e, f), characteristic

for Nav1.2 (McCormack et al., 2013). This implies that the mainly active Nav channel subtype in the LUHMES neurons is Nav1.2. These findings are consistent with the expression levels of Nav channels (Fig. MS1_S2). Data on mRNA levels suggest that Nav1.2 and Nav1.9 show a time-dependent expression, reaching a maximum on d9. Other channels (e.g., Nav1.3 and Nav1.8) are also expressed on d9, but do not show the typical developmental up-regulation.

Assessment of agents interfering with the dopamine transporter (DAT)

As LUHMES are dopaminergic cells, we chose the dopamine transporter (DAT) to exemplify measurements of electrogenic effects of neuronal transporters. This was intended as basis to explore functional neurotoxicity of transporter modulating drugs. To confirm the expression of functional DAT, we examined the uptake of radioactively-labeled DA ($[^3\text{H}]\text{DA}$) into LUHMES cells. There was a fast and specific uptake, as expected (Fig. MS1_S6). The uptake of $[^3\text{H}]\text{DA}$ was significantly reduced by the DAT inhibitors AMP, GBR12935 (Andersen, 1987; Rothman et al., 1993), cocaine (Han and Gu, 2006; Schmitt et al., 2013) and nomifensine.

The DAT acts as a symporter of dopamine, two Na^+ and one Cl^- ion, and depends on the electro-chemical gradient of the two ions to transport dopamine into the neurons (Gu et al., 1994; Harris and Baldessarini, 1973; Krueger, 1990; Kuhar and Zarbin, 1978; Schenk, 2002; Sonders et al., 1997). This transport can lead to a depolarization of the membrane potential and thereby to an activation of voltage-gated ion channels (Cameron et al., 2015; Robertson et al., 2009; Sitte et al., 1998; Sonders et al., 1997). Cameron et al. (2015), showed recently that the activation of the DAT by dopamine (DA) and amphetamine (AMP) leads to the activation of L-type Ca_v channels.

Measurements of Ca^{2+} showed that this end point can be used to monitor DAT activity in LUHMES cultures: The addition of DA evoked an increase in $[\text{Ca}^{2+}]_i$ (Fig. MS1_5a). Furthermore, the psychostimulant drug amphetamine (AMP), which acts as a substrate of the DAT (Cameron et al., 2015; Fleckenstein et al., 2007; Jones et al., 1998; Robertson et al., 2009; Schmitt et al., 2013; Siciliano et al., 2014; Sitte et al., 1998; Sonders et al., 1997), induced also signals in Ca^{2+} -imaging experiments (Fig. MS1_5b) (Cameron et al., 2015). The pEC_{50} of 6.1 and 7.0 for DA and AMP, respectively (Fig. MS1_5c), are in accordance with published findings (Cameron et al., 2015).

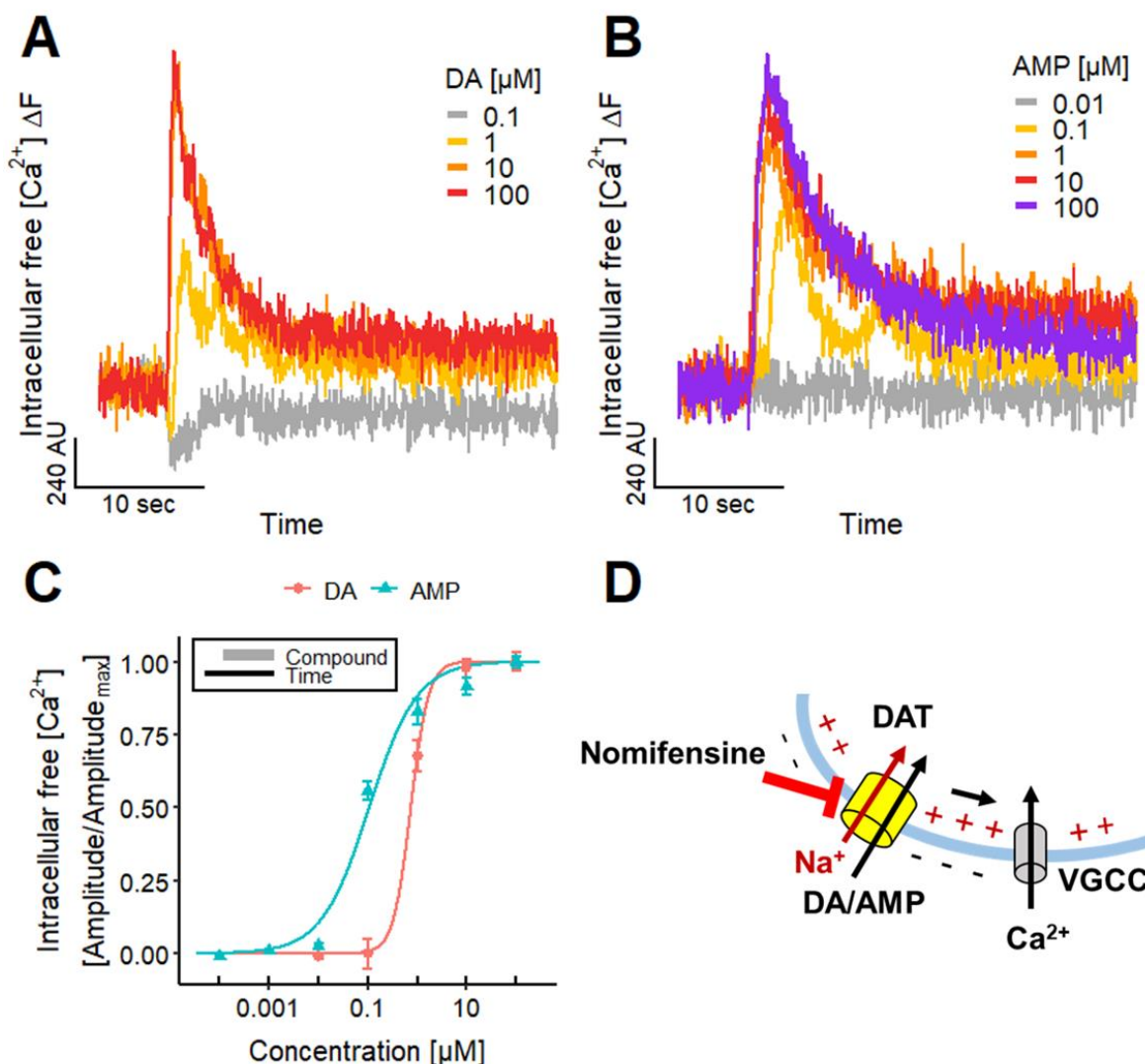


Fig. MS1_5 Altered neuronal signaling by DAT substrates.

a, b Traces of Ca^{2+} -imaging experiments illustrating the responses of the LUHMES neurons evoked by different concentrations of **a** dopamine (DA) and **b** amphetamine (AMP). **c** Concentration-response curves for DA and AMP with pEC_{50} values of 6.13 ± 0.12 and 6.98 ± 0.06 , respectively. Note the treatment scheme (upper left corner), illustrating the experimental design. Detailed data on n numbers are found in table MS1_S4. **d** Schematic illustration of the underlying mechanisms of Ca^{2+} -imaging signals evoked by DA and AMP. The transport of DA and AMP via the DAT into the cell leads to a net influx of one positive charge (Na^+) that can activate voltage-gated ion channels, like Ca_v ion channels. The DAT can be blocked by nomifensine

To make use of the fact that measurements of $[Ca^{2+}]_i$ can give very exact information on compounds affecting the DAT (Fig. MS1_5d), we explored this approach for characterization of antagonists. The DAT blocker nomifensine (Andersen, 1989; Krueger, 1990; Sulzer et al., 1995) inhibited the responses evoked by DA and AMP (Fig. MS1_6a) with a pIC_{50} value of 7.6–7.7 (Fig. MS1_6b). This is similar to data on the uptake of radioactively labeled DA in rat synaptosomes (Randrup and Bræstrup, 1977).

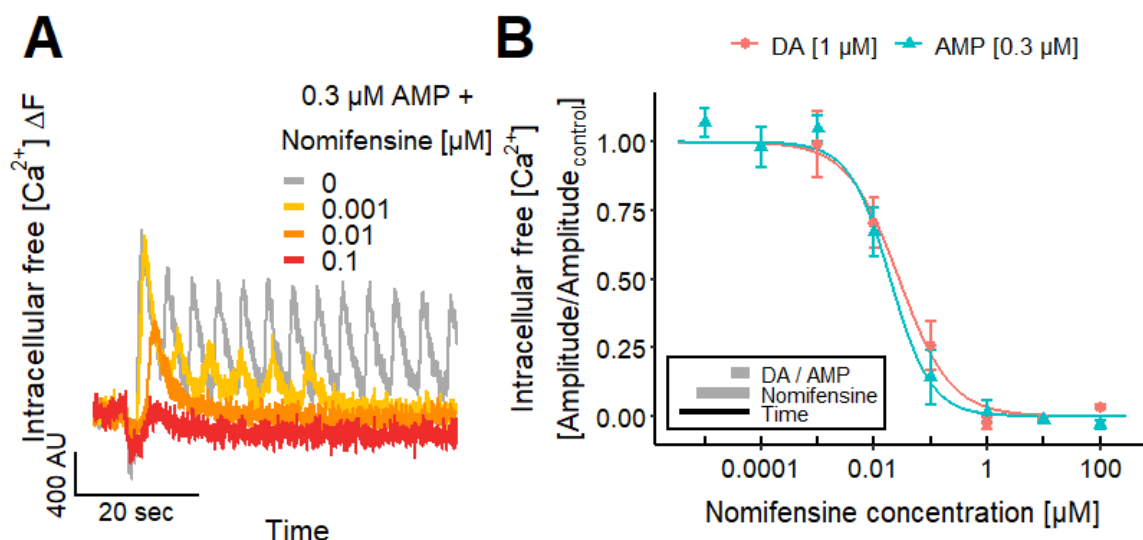


Fig. MS1_6 Altered neuronal signaling by blocking the DAT.

a Ca^{2+} -imaging traces of the inhibitory effect of nomifensine on the response of LUHMES neurons to 0.3 μM AMP. **b** The concentration-dependent inhibition by nomifensine resulted in pIC_{50} values of 7.55 ± 0.12 for DA and 7.71 ± 0.10 for AMP. Note the treatment scheme (lower left corner), illustrating the experimental design. Detailed data on n numbers are found in table MS1_S4

These results demonstrate the usability of our LUHMES cell-based test system for the assessment of substance-induced changes in DAT activity. The use of high-throughput Ca^{2+} -imaging allows the direct detection of DAT-mediated signal changes without requiring special reagents (radioactive labeling to measure $[^3\text{H}]\text{DA}$ uptake).

Ca^{2+} -oscillations as indicator of coupled neuronal networks

Addition of DA (Fig. MS1_7a) and AMP (Fig. MS1_7b) triggered not only an increase of $[\text{Ca}^{2+}]_i$, but also prominent and long-lasting oscillations of the Ca^{2+} -imaging signal. This observation is insofar remarkable, as the $[\text{Ca}^{2+}]_i$ -signal was derived from thousands of cells at the same time, and from an area having a diameter of $> 2000 \mu\text{m}$ (for comparison: LUHMES cell bodies are about $20 \mu\text{m}$ wide). As non-coordinated oscillations of individual cells would cancel out under our measurement conditions (recording of the average signal of all cells), the measurable oscillations indicate that all cells change $[\text{Ca}^{2+}]_i$ in a synchronized way, and that LUHMES cultures must therefore form a functionally coupled network.

We explored how consistent this phenomenon occurred across several wells and cell differentiations: in 62% ($n = 125$) of cases, DA ($1 \mu\text{M}$) triggered oscillations. When AMP ($0.3 \mu\text{M}$) was used, oscillations were observed in 48 of 50 experiments. The oscillation frequency was consistent throughout the experiments in a range of 0.2–0.23 Hz (Fig. MS1_7c, d). This means that the entire culture in a well increased the average $[\text{Ca}^{2+}]_i$ about every 5 s in a coordinated way. In summary, these findings suggest that whole

culture measurements of $[Ca^{2+}]_i$ allow assessment of neuronal network properties. An example of a drug triggering network oscillations is given here with AMP.

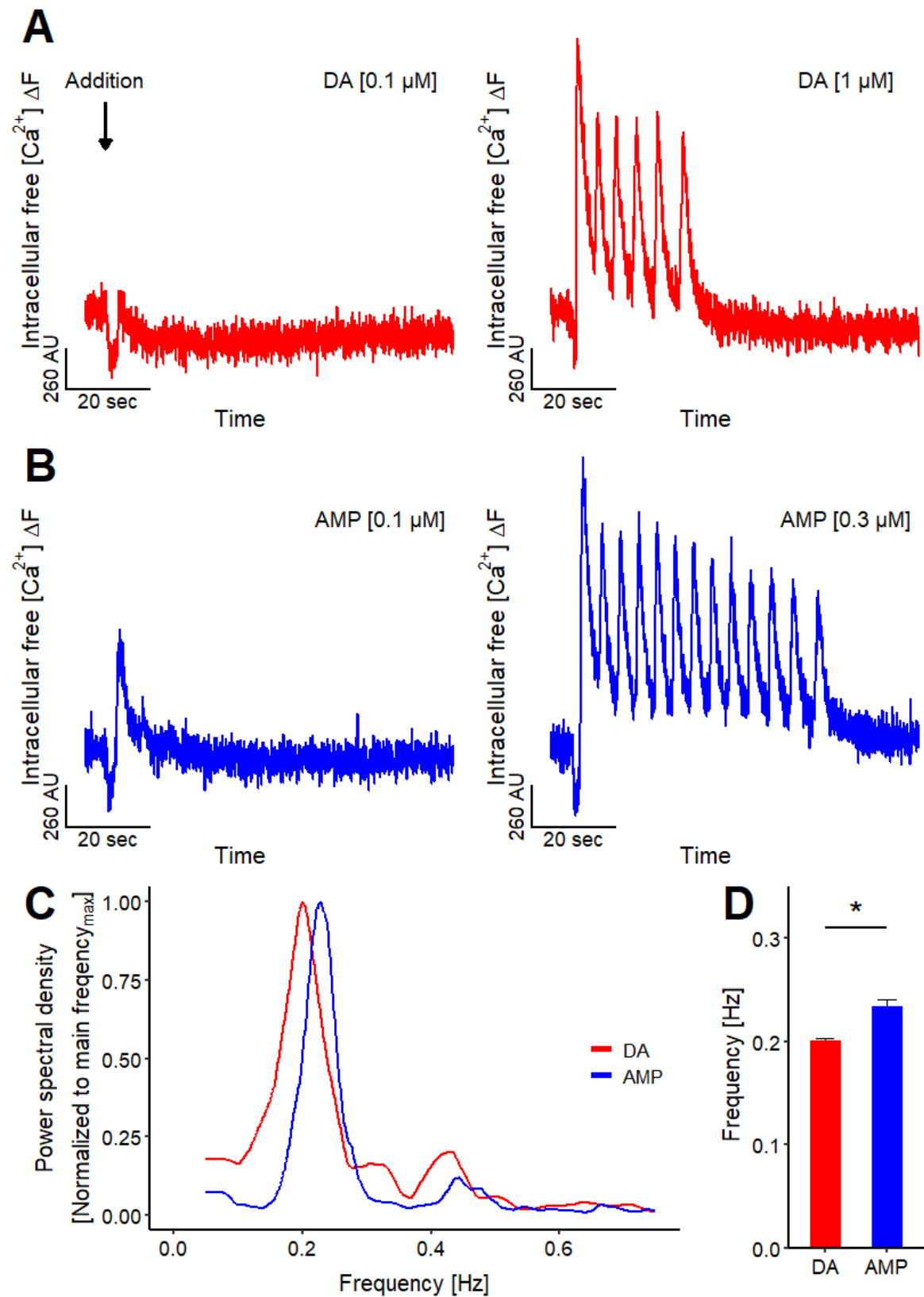


Fig. MS1_7 Oscillation of Ca^{2+} -imaging signal.

a Traces of Ca^{2+} -imaging signals of the application of 0.1 and 1 μM DA. The latter concentration induced in 61.6% ($n = 125$) of the recordings oscillations of the Ca^{2+} -imaging signal. **b** Exemplary traces of the addition of 0.1 and 0.3 μM AMP. Oscillations of the Ca^{2+} -imaging signal were induced by the application of 0.3 μM AMP in 94.0% ($n = 50$) of the recordings. **c** Periodogram based on the mean results of FFT analysis of the oscillations induced by 1 μM DA ($n = 6$) and 0.3 μM AMP ($n = 10$) highlighting a main oscillation frequency. **d** Main oscillation frequency for 1 μM DA and 0.3 μM AMP are 0.201 ± 0.002 Hz ($n = 6$) and 0.234 ± 0.007 Hz ($n = 10$), respectively. Statistical significance was determined between DA and AMP (*, significant)

Modulation of $[\text{Ca}^{2+}]_i$ oscillations in functionally coupled neuronal cultures

To test the hypothesis that DAT activity is required for the oscillations, we used the DAT blocker nomifensine. The oscillations induced by AMP and DA were indeed blocked by this drug (Fig. MS1_6a). Nomifensine thus exemplifies possible modes of action of drugs that dampen or break network synchronization. However, it was important to test whether oscillations driven by the DAT may also be modified by drugs with other neuronal targets. We therefore asked which types of channels may be involved in ensuring coordinated oscillatory activity in LUHMES cultures, and whether drugs interfering with such channels would affect network oscillations as potential neurofunctional end point.

First, we examined the involvement of Nav channels in Ca^{2+} -oscillations by using TTX to block action potential generation and propagation along the neurites. The signal amplitudes induced by DA (1 μM) and AMP (0.3 μM) were significantly reduced to 69 and 21%, respectively (Fig. MS1_8a–d). Although these effects indicate a contribution of Nav channels, they also suggest that there are additional components mandatory for the observed oscillations. We therefore examined the participation of L-type Cav channels in Ca^{2+} -oscillations: the selective L-type Cav channel antagonist nifedipine (Helton et al., 2005) reduced the oscillation amplitude to 70% for DA and to 51% for AMP. These findings suggest that functional L-type Cav channels are involved in Ca^{2+} -oscillations. Their presence is in line with the expression levels of L-type Cav channel mRNA (Fig. MS1_S2), indicating a high expression of Cav1.2. As the presence of functional T-type Cav channels is supported by high mRNA levels of Cav3.2 (Fig. MS1_S2), we investigated the impact of T-type Cav channels on the DA/AMP induced oscillations: the selective T-type Cav channel blocker NNC 55–0396 (NNC, (Huang et al., 2004)) caused a strong reduction of the amplitude to 2–3% (> 95% inhibition) (Fig. MS1_8a–d). This indicates a major contribution of T-type Cav channels in Ca^{2+} -oscillations.

One may ask why T-type Cav channels take such an important role here (Fig. MS1_8e): These Cav channels have a lower activation threshold compared to L-type Cav channels (Helton et al., 2005; Lieb et al., 2014). This higher voltage sensitivity enables a stronger

electrical coupling with the DAT (Cameron et al., 2015), but it may also have important pathophysiological (e.g., epilepsy (Cain and Snutch, 2013; Huc et al., 2009)) and toxicological implications.

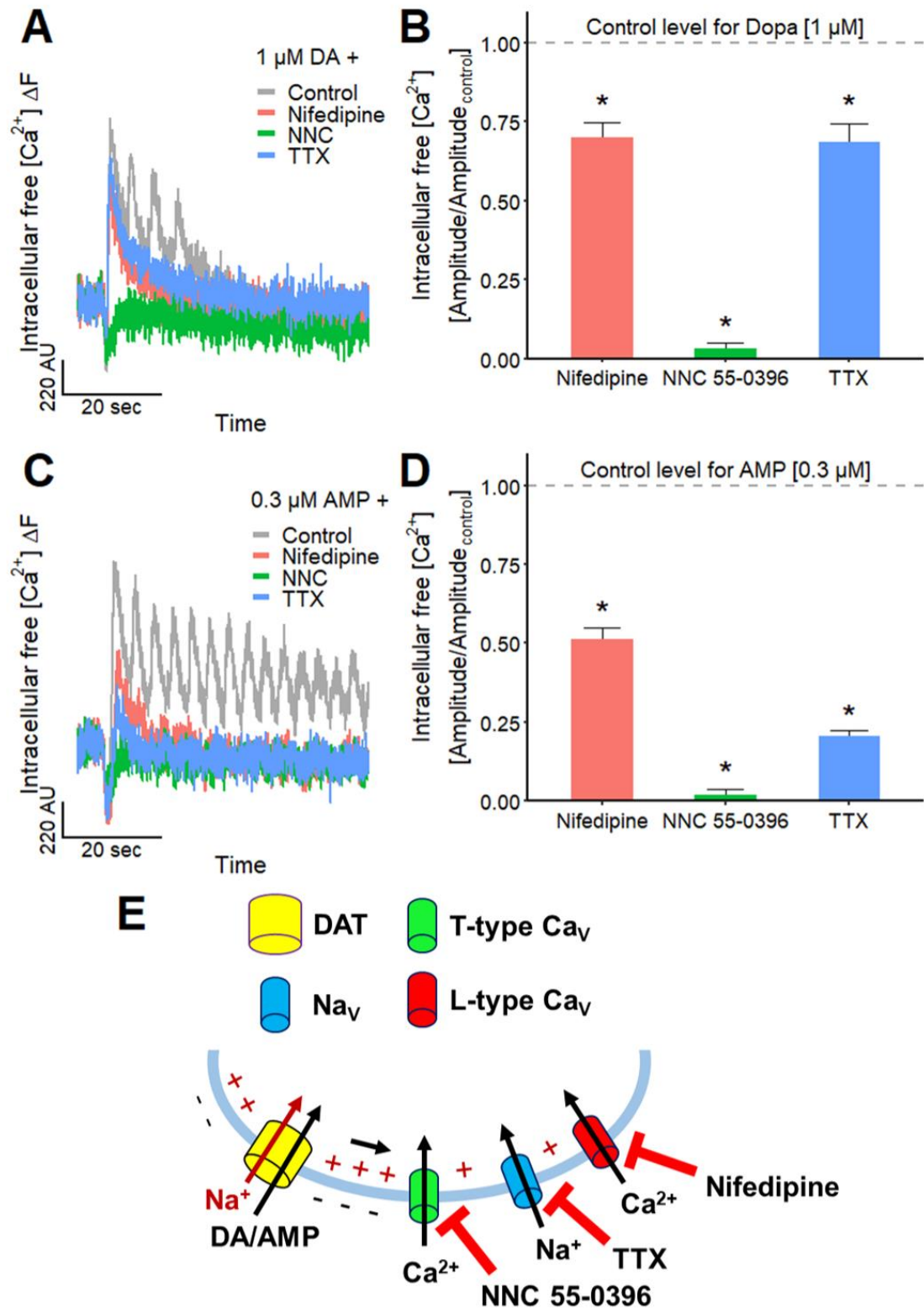


Fig. MS1_8 Substance-induced modulation of Ca^{2+} -imaging signal oscillations.

a Traces of a Ca^{2+} -imaging experiment with LUHMES cells displaying the responses triggered by 1 μM DA during control and in the presence of 10 μM nifedipine (L-type Ca_v channel inhibitor), 30 μM NNC 55-0396 (NNC; T-type Ca_v channel inhibitor) and TTX (1 μM). Note the Ca^{2+} -imaging signal oscillations during control. **b** Corresponding mean inhibitory effects of the three antagonists on the signal evoked by 1 μM DA. The amplitude was reduced compared to control ($n = 5$) to $70.2 \pm 0.05\%$ by nifedipine ($n = 6$), to $3.0 \pm 0.02\%$ by NNC 55-0396 ($n = 6$) and to $68.6 \pm 0.06\%$ by TTX ($n = 6$). Statistical significance was determined against negative control recordings (*, significant). **c** Ca^{2+} -imaging traces showing the effect of 10 μM nifedipine, 30 μM NNC 55-0396 (NNC) and 1 μM TTX on the responses of the LUHMES neurons stimulated by the addition of 0.3 μM AMP. Note the Ca^{2+} -imaging signal oscillations during control. **d** Mean inhibitory effects of the antagonists on the response triggered by 0.3 μM AMP. The amplitude was reduced compared to control ($n = 6$) to $51.4 \pm 0.03\%$ by nifedipine ($n = 6$), to $2.1 \pm 0.02\%$ by NNC 55-0396 ($n = 5$) and to $20.5 \pm 0.02\%$ by TTX ($n = 6$). Statistical significance was determined against negative control recordings (*, significant). **e** Schematic illustration of the results and the underlying context. The transport of DA and AMP by the DAT results in a net influx of one positive charge (Na^+) into the cell which can activate voltage-gated ion channels via a depolarization of the membrane potential (Fig. MS1_5)

In summary, this final set of experiments showed that LUHMES cultures, assessed by whole-well Ca^{2+} -imaging as an end point not only can be used to investigate modulations of ion channels, neurotransmitter receptors, and transporters that affect individual neurons, but also for identification of chemicals that alter synchronous activity in this test system.

3.1.5 Conclusion and outlook

LUHMES have in the past been used as toxicity test system with biochemical and morphological end points (Brüll et al., 2020; Delp et al., 2019, 2018b; Scholz et al., 2011; Smirnova et al., 2016). To the best of our knowledge, we provide here for the first time an extensive overview of neurophysiological changes triggered by external chemicals in LUHMES neurons. We show a broad panel of such responses to exemplify the functioning and performance of LUHMES as test system of functional neurotoxicity. Some of the results yield further neurobiological characterization of the test system. For instance, our results indicate the functional expression of P2X3 receptors, as demonstrated by the inhibitory effect of A-317491 on the response evoked by α,β -meATP. We also illustrated the high reproducibility of the differentiation of LUHMES neurons by the low standard deviation of 0.16 of five pEC_{50} values (mean of 7.52) determined for the responses of five differentiations to α,β -meATP. A use case was given by the characterization of suramin and by showing how the system can be used to provide exact quantitative data on agonist and antagonist potencies and specificities. In the future, the identification of side effects of antiepileptic and anti-inflammatory drugs addressing purinergic receptors could be of interest, due to their wide distribution in the

nervous system (Burnstock and Verkhratsky, 2012; Di Virgilio and Vuerich, 2015; Riquelme et al., 2020).

In a further step, we showed that high-throughput Ca^{2+} assays can substitute patch clamp for many applications, and provide a central toxicological platform to investigate diverse neurofunctional modulators/toxicants. This method enables the utilization of adherent cells in an intact neuronal network compared to automated patch clamp, where cells need to be detached. This assay served, e.g., as a useful tool for the detection of marine neurotoxins such as TTX, STX and CTX. It may be used in the future also for, e.g., cyanobacterial toxins, like kalkitoxin (LePage et al., 2005). The possibility of examining use-dependent effects on this Nav channel is also meaningful for the research of anticonvulsants, like phenytoin (Brodie, 2017; Goldenberg, 2010), and the detection of side effects of local anesthetics or pyrethroids on CNS Nav channels (Cao et al., 2011; Casida and Durkin, 2013; Groban, 2003; Mather et al., 2005; Neal et al., 2010).

Scholz et al. (2011) mentioned the presence of TTX-sensitive Nav channels in this cell model. We went further by identifying Nav1.2 as the major functionally active sodium channel (Fig. MS1_4f). By establishing a procedure to utilize the LUHMES neurons in automated patch clamp, we overcame the low throughput of manual patch clamp.

A major outcome of our study, besides the broad test system description as necessary basis for further work, was the demonstration that LUHMES neurons are functionally coupled over long distances (entire well). The finding of oscillations of activity was very clear for different stimuli such as DA, AMP and CTX, and we provided a description of the robustness of the phenomenon. A thorough investigation of the underlying biology was out of the scope of this study, but it is an important goal for the future. To substantiate our findings, and to ensure they are not strange random observations, we provided some mechanistic links: DAT and T-type Cav were found to be major players in such culture $[\text{Ca}^{2+}]_i$ oscillations, and also Nav contributed to them. Potential applications for this assay could be the identification of all agents that disturb neuronal network functions, such as antipsychotics, seizurogenic substances and antiepileptic drugs that do not solely affect the GABA-glutamate system.

In summary, we highlighted the suitability of LUHMES neuronal cultures as powerful tool for high-throughput neuronal toxicity screening using industry-applicable automated patch clamp and Ca^{2+} -imaging. Furthermore, we revealed the presence of several meaningful targets on the LUHMES neurons for the assessment of neurotoxicity and exemplified this in several case studies. In future studies, it would be worthwhile investigating the effects of the tested neurotoxicants on 3D models and co-culture systems with astrocytes (Brüll et al., 2020).

3.1.6 Acknowledgments

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3.1.7 Conflict of interest

The authors declare no conflict of interest.

3.1.8 Author contributions

DL: conceived of or designed the study, performed research, analyzed data and wrote the paper. JS, IS, SK: performed research and analyzed data. TD, CM: conceived of or designed the study, contributed new methods or models and analyzed data. MB, AÜ: performed research, analyzed data and wrote the paper. ML, UK: conceived of or designed the study and wrote the paper.

3.1.9 Supplementary information

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00204-020-02956-3>) contains supplementary material, which is available to authorized users.

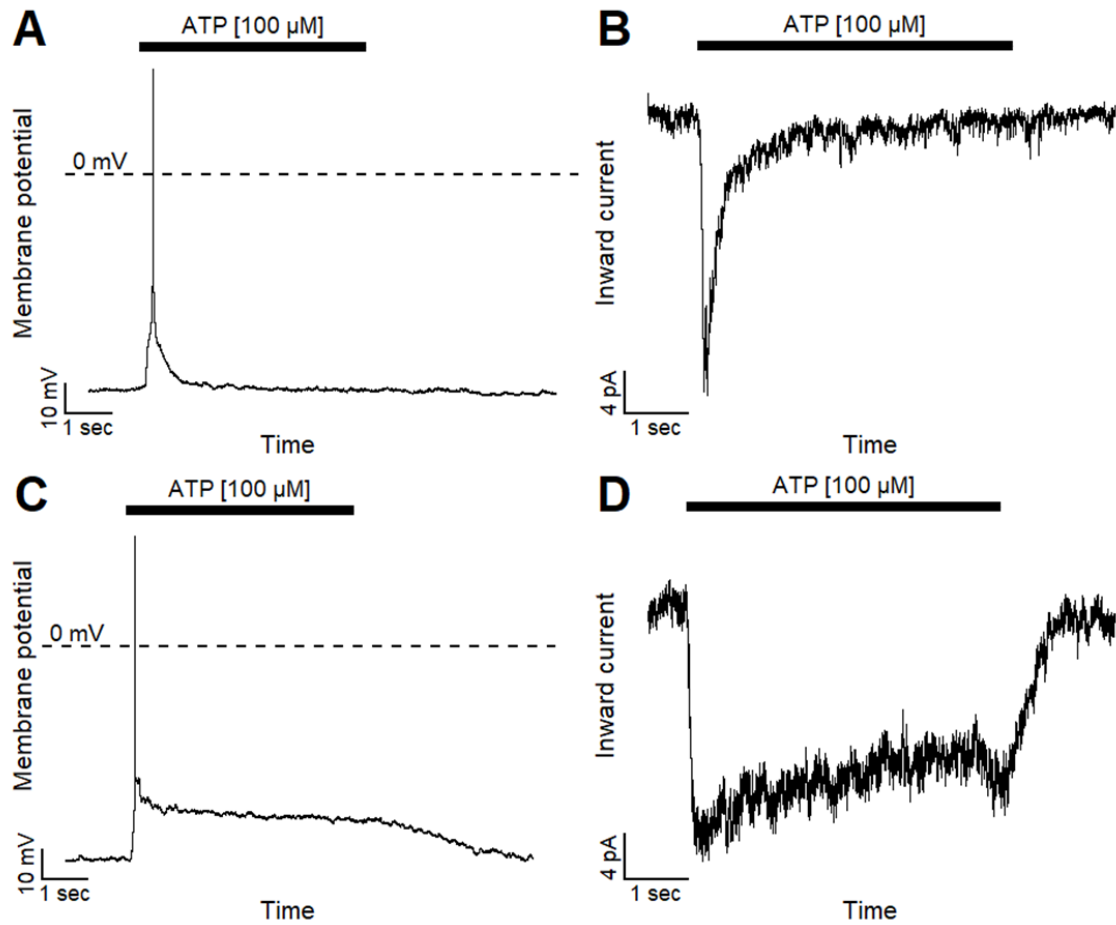


Fig. MS1_S1. Functional P2X receptor expression in LUHMES neurons.

Manual patch clamp recordings of the responses of LUHMES neurons evoked by the application of 100 μ M ATP for 5 sec. (A) Action potential firing in current-clamp recording with a short depolarization ($n = 7$). (B) Voltage-clamp recording with a fast inactivating inward current ($n = 15$). Besides the fast kinetics, (C) a slow repolarization of the membrane potential was also recorded in current-clamp ($n = 4$). (D) This finding was substantiated by voltage-clamp recordings that showed slowly inactivating inward currents ($n = 13$).

Five biological replicates were generated from LUHMES cells differentiated for 2, 3, 5, 6, 8, 10, and 11 days, as well as from undifferentiated LUHMES cells (day 0). The heatmaps visualize the normalized and logarithmic counts for each gene (rows) of neurotransmitter receptors, ion channels, and calcium binding proteins, and day of differentiation (columns, indicated in blues). Darker red indicates high expression, orange indicates low expression, and white indicates no expression. The genes are clustered by the gene group (e.g. receptor or ion channel subtype). Gene groups are indicated by color in the first column. Samples were analyzed via (A) the TempO-Seq assay and via (B) traditional whole genome RNA-sequencing (RNAseq).

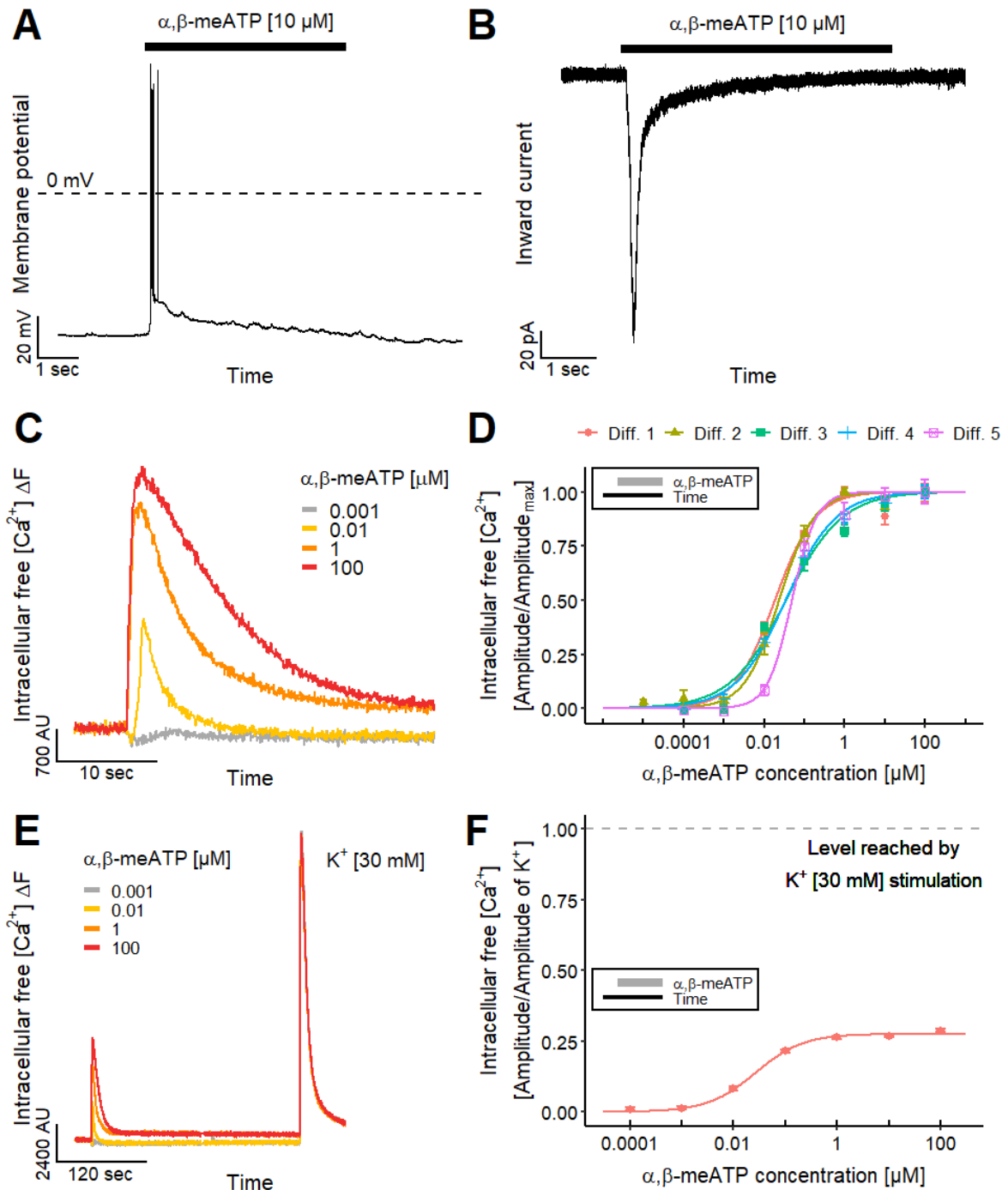


Fig. MS1_S3. Effect of P2X receptor agonist α, β -meATP on LUHMES neurons.

(A-B) Traces of manual patch clamp recordings of the application of 10 μ M α, β -meATP for 5 sec. (A) In current clamp, the cells responded either with tonic ($n = 21$) or phasic firing behavior ($n = 25$, not shown) due to a transient depolarization of the membrane potential. (B) In voltage clamp, an inward current with fast activation and inactivation kinetics was measured in all recorded cells ($n = 16$). (C) Traces of Ca^{2+} -imaging signals produced by the addition of different concentrations of α, β -meATP on LUHMES neurons. (D) Comparison of concentration-response curves recorded from five differentiations (Diff. 1-5) for the effect of α, β -meATP. The resulting pEC₅₀ values show a very low variability between the differentiations. (E) Exemplary traces of Ca^{2+} -imaging displaying the responses evoked by different concentrations of α, β -meATP (1st peak) followed by the second application of 30 mM K^+ (2nd peak). (F) The concentration-response curve showing

the mean over 5 differentiations, for which the amplitude evoked by α,β -meATP was normalized to the corresponding amplitude of the K^+ -application. The maximal amplitude of α,β -meATP reached 27.6% of the K^+ amplitude with a pEC_{50} value of 7.58 ± 0.04 . Note the treatment schemes, illustrating the experimental design. Detailed data on n numbers are found in table MS1_S4.

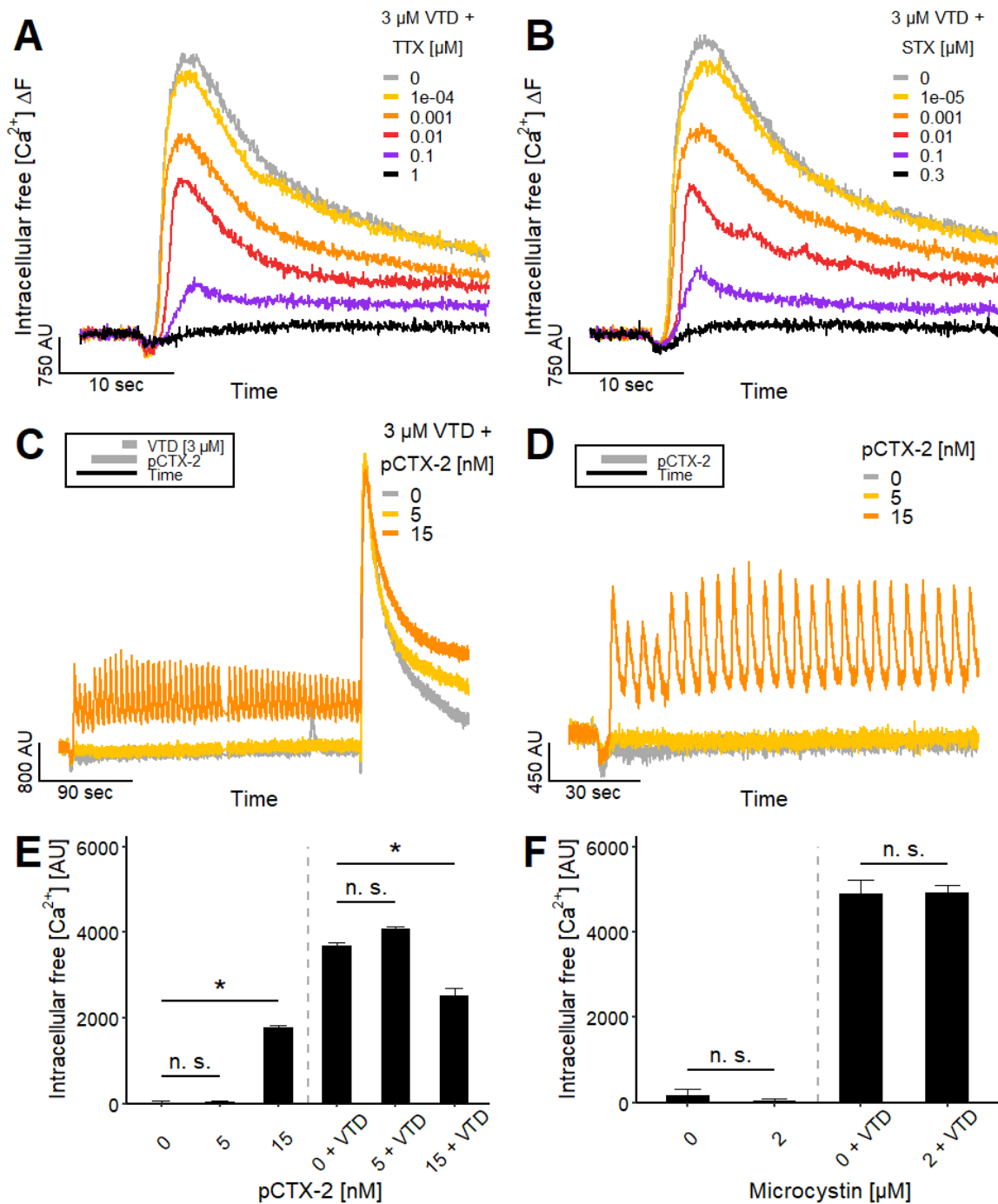


Fig. MS1_S4. Effects of marine biotoxins on LUHMES neurons.

(A-B) Traces of Ca^{2+} -imaging experiments showing the responses of LUHMES cells evoked by the application of 3 μM VTD in the presence of different concentrations of (A) TTX and (B) saxitoxin

(STX). (C-D) Ca^{2+} -imaging signals displaying the effect of (C) pacific ciguatoxin-2 (pCTX-2), a Na_v channel modulator, followed by the application of 3 μM VTD on LUHMES neurons. 15 nM pCTX-2 induced an increase of the baseline and oscillations of the Ca^{2+} -imaging signal. (D) Magnification of the effects of pCTX-2. Note the oscillations triggered by 15 nM pCTX-2. (E) The response evoked by 15 nM pCTX-2 ($n = 6$) reached an amplitude of 1779 ± 50 AU. At 5 nM pCTX-2 ($n = 4$) no effect was detected compared to control ($n = 6$). The amplitude induced by 3 μM VTD, after the addition of pCTX-2, was significantly reduced by 15 nM pCTX-2 taking the baseline increase into account. Otherwise the effect of 15 nM pCTX-2 on the VTD response would not be significant. (F) The biotoxin microcystin-LF was tested in a concentration of 2 μM followed by the application of 3 μM VTD. The results show no significant effect of 2 μM microcystin-LF ($n = 6$) compared to control ($n = 5$), as well as on the response triggered by 3 μM VTD. Note the treatment scheme (upper left corner), illustrating the experimental design. (E-F) Statistical significance was determined against negative control recordings (*, significant; n. s., not significant).

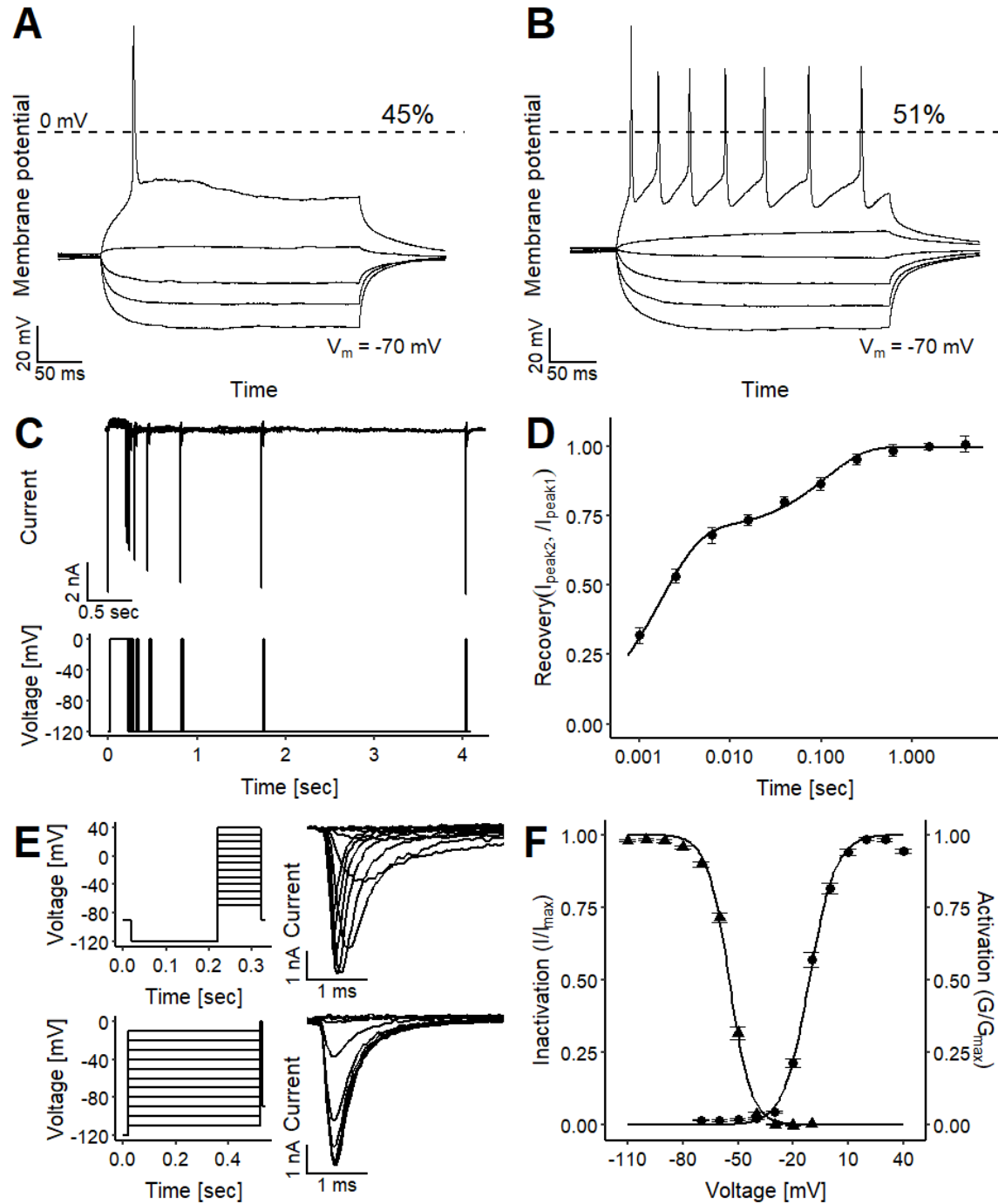


Fig. MS1_S5. Firing behavior and biophysical properties of Na_v channel currents of LUHMES neurons.

(A-B) Investigation of the firing behavior of LUHMES neurons using manual patch clamp. The cells were stimulated with 300 ms hyper- and depolarizing current pulses from holding potential (V_m ; $n = 274$). (A) 45% of the cells showed phasic and (B) 51% tonic firing behavior. Depolarization failed to induce action potentials in only 4% of the cells. (C-F) Automated patch clamp recordings of voltage-gated sodium (Na_v) channels expressed in LUHMES neurons. (C) Exemplary traces of the Na^+ current triggered by the pulse protocol illustrated below. (D) The recovery from steady-state inactivation of the Na_v channels was fitted biexponentially, resulting in a slow component with $A_1 = 0.3$ and $\tau_1 = 112.5$ ms and a fast component with $A_2 = 0.7$ and $\tau_2 = 1.7$ ms ($n = 6$). (E) On the left side, pulse protocols for the investigation of the activation (top) and the steady-state inactivation (bottom) kinetics of the Na_v channel currents are presented. On the right side,

the corresponding exemplary traces of the Na^+ currents evoked by the test pulses are displayed. (F) Mean voltage-dependent conductance (G/G_{max}) for the activation kinetics ($\bullet$) with a half maximal activation voltage (V_{50}) of -11.3 mV and a constant (k) of 7.0 ($n = 13$) and steady-state inactivation (I/I_{max}) ($\blacktriangle$) with $V_{50} = -54.9$ mV and $k = -5.9$ ($n = 21$). The curves were fitted with the Boltzmann equation.

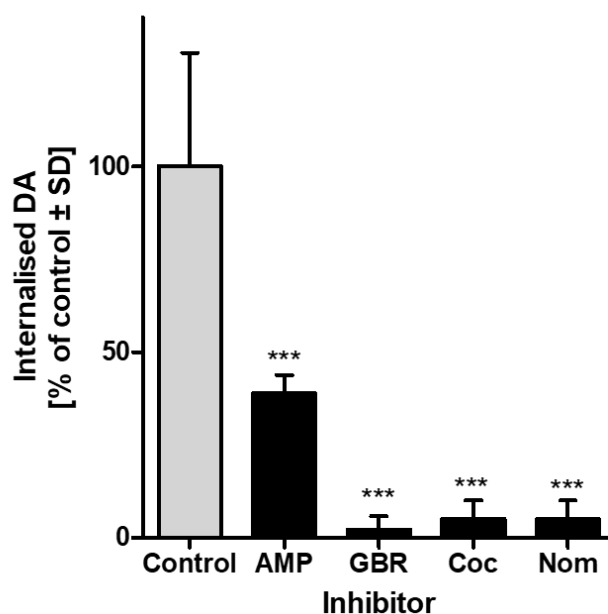


Fig. MS1_S6. Inhibition of dopamine transporter (DAT) activity in LUHMES cells.

The uptake of radioactively-labelled DA ($[^3\text{H}]\text{DA}$) by LUHMES cells was inhibited by the DAT antagonists amphetamine (AMP, 10 μM), GBR12935 (GBR, 1 μM), cocaine (Coc, 100 μM) and nomifensine (Nom, 40 μM). All data are means $\pm$ SD of triplicate data. *** $p < 0.0001$ according to ANOVA, followed by Dunnett's post-hoc test. Parallel time course measurements showed that at 10 minutes, 50% of the maximum uptake had occurred. Altogether, cells accumulated about 6% of all DA in the culture medium. Inhibition studies performed for 30 and 60 min (not shown) yielded essentially similar data.

Table MS1_S1. Compound list.

Chemical	Supplier	Order number	Function	Reference
4-Aminopyridine	Sigma Aldrich	275875	K _v channel blocker	
α,β -Methyleneadenosine 5'-triphosphate trisodium salt (α,β -meATP)	Tocris	3209	P2 receptor agonist	Bianchi et al., 1999; Gevert et al. 2006
A-317491 sodium salt hydrate	Sigma Aldrich	A2979	selective P2X ₃ & P2X _{2/3} antagonist	Jarvis et al., 2002
Advanced DMEM/F12	Gibco	12634010		
($\pm$)-Amphetamine	Sigma Aldrich	A-011	competitive antagonist and substrate of DAT, monoamine releaser,	Cameron et al., 2015; Fleckenstein et al., 2007; Siciliano et al., 2014; Sitte et al., 1998; Sonders et al., 1997
Boric acid	Sigma Aldrich	B7660		
BzATP triethylammonium salt	Tocris	3312	P2 receptor agonist	Bianchi et al., 1999
Cal-520 AM	Biomol	ABD-21130		
Cadmium chloride	Sigma Aldrich	202908		
Cesium chloride	Sigma Aldrich	289329		
Cesium fluoride	Sigma Aldrich	198323		
Dibutyl cyclic-AMP sodium salt (cAMP)	Sigma Aldrich	D0627		
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D8418		
Dopamine chloride	Sigma Aldrich	H8502	Neurotransmitter	
Recombinant human FGF basic (FGF-2)	R&D Systems	4114-TC		
Fibronectin	Sigma Aldrich	F1141		
Recombinant human GDNF protein (GDNF)	R&D Systems	212-GD		
L-glutamine solution	Sigma Aldrich	G7513		
ICA-121431	Tocris	5066	Na _v channel blocker	McCormack et al., 2013
Lidocaine hydrochloride monohydrate	Sigma Aldrich	L5647	non-selective Na _v channel antagonist	Kaneda et al., 1988; Leffler et al., 2007
N-2 supplement	Gibco	17502048		
Na-ATP (ATP)	Sigma Aldrich	A6419	non-selective P2 receptor agonist	Bianchi et al., 1999
Nomifensine maleate salt	Sigma Aldrich	N1530	DAT antagonist	Andersen 1989; Krueger 1990; Sulzer et al. 1995
Nifedipine	Sigma Aldrich	N7634	selective L-type Ca _v channel blocker	Helton et al., 2005
NNC 55-0396 dihydrochloride	Tocris	2268	selective T-type Ca _v channel blocker	Huang et al., 2004
Pluronic F-127	Invitrogen	P3000MP		
Polyethyleneimine solution (PEI)	Sigma Aldrich	P3143		
Poly-L-ornithine (PLO)	Sigma Aldrich	P3655		
Saxitoxin	Sigma Aldrich	93665	Na _v channel blocker	Durán-Riveroll and Cembella, 2017; Noda et al., 1989; Terlau et al., 1991
Sodium tetraborate	Sigma Aldrich	221732		
Tetraethylammonium chloride (TEA)	Sigma Aldrich	T2265	K _v channel blocker	
Tetracycline hydrochloride	Sigma Aldrich	T7660		
Tetrodotoxin citrate (TTX)	Tocris	1069	Na _v channel blocker	Kaneda et al., 1988; Tsukamoto et al., 2017
TNP-ATP triethylammonium salt	Tocris	2464	selective P2X receptor antagonist	Virginio et al., 1998; Gevert et al. 2006
Trypsin-EDTA (0.05 %)	Gibco	25300062		
Veratridine	Tocris	2918	Na _v channel modifier	Alkadhi and Tian, 1996; Mohammed et al., 2017; Vetter et al., 2012

Table MS1_S2. Overview of pEC₅₀ values for agonist experiments.

Compound	pEC₅₀ ± SEM	Pharmacology	Figure
ATP	7.35 ± 0.08	P2R agonist	1D
Bz-ATP	7.76 ± 0.11	P2R agonist	1D
α,β-meATP	7.52 ± 0.03	P2R agonist	1E
α,β-meATP (normalized to K⁺ peak [30 mM])	7.58 ± 0.04	P2R agonist	S3F
Effect of α,β-meATP		P2R agonist	
Diff. 1	7.73 ± 0.06		S3D
Diff. 2	7.63 ± 0.05		S3D
Diff. 3	7.46 ± 0.07		S3D
Diff. 4	7.46 ± 0.04		S3D
Diff. 5	7.31 ± 0.06		S3D
VTD	5.38 ± 0.03	NaV channel modulator	3B
DA	6.13 ± 0.12	Neurotransmitter	5C
AMP	6.98 ± 0.06	DAT substrate/antagonist	5C

Table MS1_S3. Overview of pIC₅₀ values for antagonist experiments.

Compound	pIC ₅₀ ± SEM	Pharmacology	Figure
Effect on response to 1 μM ATP			
TNP-ATP	6.05 ± 0.06	P2X receptor antagonist	2A
Suramin	4.09 ± 0.03		2C
Effect on response to 0.1 μM α,β-meATP			
TNP-ATP	7.50 ± 0.05	P2X receptor antagonist	2A
A-317491	6.31 ± 0.05	P2X3 and P2X2/3 antagonist	2B
Suramin	4.51 ± 0.03		2C
Effect on response to 3 μM VTD			
TTX	7.93 ± 0.06	Na _v channel blocker	3C
STX	8.06 ± 0.06	Na _v channel blocker	3C
Effect on Na _v channel currents		Interpretation	
TTX 1 st pulse	7.23 ± 0.05	Acute inhibition	4D
TTX 10 th pulse	7.26 ± 0.05	Use-dependent inhibition (-)	4D
Lidocaine 1 st pulse	3.03 ± 0.07	Acute inhibition	4D
Lidocaine 10 th pulse	3.57 ± 0.06	Use-dependent inhibition (+)	4D
ICA-121431	6.33 ± 0.10	Na _v 1.2 antagonist	4F
Effect on response to 1 μM DA			
nomifensine	7.55 ± 0.12	DAT antagonist	6B
Effect on response to 0.3 μM amphetamine			
nomifensine	7.71 ± 0.10	DAT antagonist	6B

Table MS1_S4. Overview of concentrations and replicates.

Compound	Concentration (n numbers)	Figure
ATP	0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 100 μ M (6), 1000 μ M (6)	1D
Bz-ATP	0.0001 μ M (6), 0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 100 μ M (6)	1D
α,β -meATP	0.00001 μ M (6), 0.0001 μ M (22), 0.001 μ M (23), 0.01 μ M (28), 0.1 μ M (26), 1 μ M (26), 10 μ M (27), 100 μ M (21)	1E
α,β -meATP (normalized to K ⁺ peak [30 mM])	0.00001 μ M (6), 0.0001 μ M (22), 0.001 μ M (23), 0.01 μ M (28), 0.1 μ M (26), 1 μ M (26), 10 μ M (27), 100 μ M (21)	S3F
Effect of α,β-meATP		
Diff. 1	0.01 μ M (5), 0.1 μ M (5), 1 μ M (5), 10 μ M (5), 100 μ M (5)	S3D
Diff. 2	0.00001 μ M (6), 0.0001 μ M (5), 0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6)	S3D
Diff. 3	0.0001 μ M (5), 0.001 μ M (5), 0.01 μ M (5), 0.1 μ M (5), 1 μ M (5), 10 μ M (5), 100 μ M (5)	S3D
Diff. 4	0.0001 μ M (6), 0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 100 μ M (6)	S3D
Diff. 5	0.0001 μ M (6), 0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (4), 1 μ M (4), 10 μ M (5), 100 μ M (5)	S3D
Effect on response to 1 μM ATP		
	Control for TNP-ATP (12), A-317491 (12) and Suramine (34)	2
TNP-ATP	0.0001 μ M (6), 0.001 μ M (5), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 30 μ M (6)	2A
A-317491	0.0001 μ M (6), 0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 3 μ M (6), 10 μ M (6)	2B
Suramin	0.1 μ M (7), 0.3 μ M (7), 1 μ M (7), 3 μ M (7), 10 μ M (7), 30 μ M (7), 100 μ M (7), 300 μ M (7)	2C
Effect on response to 0.1 μM α,β-meATP		
	Control for TNP-ATP (8), A-317491 (4) and Suramin (14)	2
TNP-ATP	0.0001 μ M (6), 0.001 μ M (5), 0.01 μ M (5), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 100 μ M (6)	2A
A-317491	0.0001 μ M (6), 0.001 μ M (5), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6)	2B
Suramin	0.1 μ M (7), 0.3 μ M (7), 1 μ M (7), 3 μ M (7), 10 μ M (6), 30 μ M (6), 100 μ M (5), 300 μ M (7)	2C

(Table MS1_S4 continues on the next page.)

Table MS1_S4. Overview of concentrations and replicates. (continued)

VTD	0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 50 μ M (6)	3B
Effect on response to 3 μM VTD	Control for TTX (6) and STX (6)	3C
TTX	0.0001 μ M (6), 0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6)	3C
STX	0.000001 μ M (6), 0.00001 μ M (6), 0.0001 μ M (6), 0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 0.3 μ M (6)	3C
Effect on Na_v channel currents		
TTX 1st pulse	0.001 μ M (4), 0.01 μ M (4), 0.1 μ M (4), 1 μ M (4)	4D
TTX 10th pulse	0.001 μ M (4), 0.01 μ M (4), 0.1 μ M (4), 1 μ M (4)	4D
Lidocaine 1st pulse	10 μ M (5), 100 μ M (5), 1000 μ M (5), 10000 μ M (5)	4D
Lidocaine 10th pulse	10 μ M (5), 100 μ M (5), 1000 μ M (5), 10000 μ M (5)	4D
ICA-121431	0.001 μ M (4), 0.01 μ M (4), 0.1 μ M (4), 1 μ M (4)	4F
DA	0.01 μ M (9), 0.1 μ M (11), 1 μ M (12), 10 μ M (15), 100 μ M (15)	5C
AMP	0.0001 μ M (6), 0.001 μ M (4), 0.01 μ M (6), 0.1 μ M (5), 1 μ M (6), 10 μ M (6), 100 μ M (6)	5C
Effect on response to 1 μM DA	Control for nomifensine (8)	6B
nomifensine	0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 100 μ M (6)	6B
Effect on response to 0.3 μM AMP	Control for nomifensine (12)	6B
nomifensine	0.00001 μ M (6), 0.0001 μ M (6), 0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 100 μ M (6)	6B

3.2 Manuscript 2: Functional alterations by a subgroup of neonicotinoid pesticides in human dopaminergic neurons

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3.2.1 Abstract

Neonicotinoid pesticides, originally developed to target the insect nervous system, have been reported to interact with human receptors and to activate rodent neurons. Therefore, we evaluated in how far these compounds may trigger signaling in human neurons, and thus, affect the human adult or developing nervous system. We used SH-SY5Y neuroblastoma cells as established model of nicotinic acetylcholine receptor (nAChR) signaling. In parallel, we profiled dopaminergic neurons, generated from LUHMES neuronal precursor cells, as novel system to study nAChR activation in human post-mitotic neurons. Changes of the free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were used as readout, and key findings were confirmed by patch clamp recordings. Nicotine triggered typical neuronal signaling responses that were blocked by antagonists, such

as tubocurarine and mecamylamine. Pharmacological approaches suggested a functional expression of $\alpha 7$ and non- $\alpha 7$ nAChRs on LUHMES cells. In this novel test system, the neonicotinoids acetamiprid, imidacloprid, clothianidin and thiacloprid, but not thiamethoxam and dinotefuran, triggered $[Ca^{2+}]_i$ signaling at 10–100 μM . Strong synergy of the active neonicotinoids (at low micromolar concentrations) with the $\alpha 7$ nAChR-positive allosteric modulator PNU-120596 was observed in LUHMES and SH-SY5Y cells, and specific antagonists fully inhibited such signaling. To provide a third line of evidence for neonicotinoid signaling via nAChR, we studied cross-desensitization: pretreatment of LUHMES and SH-SY5Y cells with active neonicotinoids (at 1–10 μM) blunted the signaling response of nicotine. The pesticides (at 3–30 μM) also blunted the response to the non- $\alpha 7$ agonist ABT 594 in LUHMES cells. These data show that human neuronal cells are functionally affected by low micromolar concentrations of several neonicotinoids. An effect of such signals on nervous system development is a toxicological concern.

3.2.2 Introduction

Neonicotinoids are a class of insecticides that trigger nervous system disturbances by activation of the nicotinic acetylcholine (ACh) receptor (nAChR) (Brown et al., 2006; Tan et al., 2007). The compounds are widely used in agriculture for pest control (Casida and Durkin, 2013; Goulson, 2013; Jeschke et al., 2011), and they have been optimized to display a high species specificity. In general, they show a high potency on insect receptors, while they have been designed for low affinities on mammalian receptors (Casida, 2018; Tomizawa et al., 2000; Tomizawa and Casida, 2005, 2003). In regulatory studies, they have been proven to be relatively well-tolerated by rats, but some studies indicate an impact of neonicotinoids on mammals (Abou-Donia et al., 2008; Berheim et al., 2019; Burke et al., 2018; Duzguner and Erdogan, 2012; Gibbons et al., 2015; Thompson et al., 2020).

Some doubts on the extent of the species selectivity have been raised by experiments on neonatal rat neurons, where acetamiprid and imidacloprid triggered signaling effects at concentrations as low as 1–10 μM (Kimura-Kuroda et al., 2012). As nicotine signaling can affect the development of the mammalian nervous system as typical developmental neurotoxicant (Aschner et al., 2017; LeSage et al., 2006; Levin et al., 1993), concerns have been voiced that exposure of humans to neonicotinoids may bear the risk of developmental neurotoxicity (DNT). Clarification of neonicotinoid signaling in human

neurons is, therefore, of high toxicological interest (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013).

The endogenous neurotransmitter ACh affects neuronal activity via the activation of (so-called muscarinic) metabotropic receptors (mAChR) and (so-called nicotinic) ionotropic nAChR. The latter are a class of homo- or heteromeric ligand-gated channels with variable permeability for Na⁺ and/or Ca²⁺ (Fucile, 2004). Their activation leads to cell depolarization, to the activation of voltage-dependent Ca²⁺ channels, and thus, to an increase of the intracellular free Ca²⁺ concentration ([Ca²⁺]_i). A large number of genes coding for subunits of nAChRs have been identified, and all functional receptors are pentamers formed from either a single subunit (e.g., $\alpha 7$), two subunits (e.g., $\alpha 4$ and $\beta 2$) or > 2 subunits. The subunit composition determines the ligand affinities and the gating properties (Capelli et al., 2011; Carbone et al., 2009; Jonsson et al., 2006; Mihalak et al., 2006; Nelson et al., 2003). For instance, $\alpha 7$ receptors exhibit fast inactivating kinetics (Alijevic et al., 2020; Elliott et al., 1996; Mihalak et al., 2006), while, e.g., $\beta 2$ -containing receptors show slower inactivation properties (Alijevic et al., 2020; Elliott et al., 1996; Mihalak et al., 2006; Nelson et al., 2003). Both types of receptors are expressed in the central nervous system. E.g., nAChRs are expressed on dopaminergic neurons, where they regulate the release of dopamine (de Kloet et al., 2015; Grady et al., 1992; Mameli-Engvall et al., 2006; Quik and Kulak, 2002; Quik and Wonnacott, 2011; Rapier et al., 1988).

Substances that alter neurotransmitter signaling transiently (while they are present) in the adult nervous system may have long-term consequences (even for years after their actual exposure) in the developing nervous system (Grandjean and Landrigan, 2006, 2014; Slikker Jr et al., 2005; Smirnova et al., 2014). This is not only due to the often-cited high vulnerability of the developing brain (Fritsche et al., 2018; Rice and Barone Jr, 2000), but rather related to the fact that neurotransmitter signaling takes different roles in the developing and adult nervous system (Nguyen et al., 2001). While the brain is formed, GABA does not only serve short-term communication, but it also triggers synaptogenesis (Oh et al., 2016), synapse pruning (Wu et al., 2012), neuronal migration (Li et al., 2018), stem cell fate (Song et al., 2012) and neurogenesis (Kriegstein, 2005; Tozuka et al., 2005). Other neurotransmitters take similar roles, such as spine growth triggered by glutamate (Kwon and Sabatini, 2011), and various neurogenic processes affected by serotonin (Agrawal et al., 2019; Migliarini et al., 2013; Schaefer et al., 2013), or nicotine (Dwyer et al., 2009; Slikker Jr et al., 2005; Slotkin et al., 2016; Zahedi et al., 2019). The toxicological consequence is that disturbance of neurotransmitter signaling during development can result in an altered brain connectivity in later life. Therefore, compounds showing acute interferences with adult

neurotransmission need to be considered as potential developmental neurotoxicants (Grandjean and Landrigan, 2006, 2014; Smirnova et al., 2014).

Hundreds of signaling molecules and alterations in the nervous system converge to very few cellular changes, such as alterations of the neuronal membrane potential and modulation of ion channels. A traditional method to assess this is to pre-load cells with radioactive $^{86}\text{Rb}^+$ and to quantify the efflux upon stimulation. Using this method, SH-SY5Y neuroblastoma cells have been established as model to study nAChR signaling (Lukas et al., 1993; Tomizawa and Casida, 1999). Cell membrane depolarization activates voltage-dependent Ca^{2+} channels, leading to a transient increase of the $[\text{Ca}^{2+}]_i$, and some nAChRs in neurons are directly permeable for Ca^{2+} (reviewed by Fucile 2004). Therefore, Ca^{2+} -imaging can be used to capture many different types of signaling events. The detection can be performed at high throughput using live-cell fluorescence imaging of neuronal cultures loaded with calcium-sensitive dyes (Brüll et al., 2020; Grunwald et al., 2019; Karreman et al., 2020; Leist and Nicotera, 1998; Sirenko et al., 2019). A more direct, but lower throughput method to assess the neuronal membrane potential is patch clamp electrophysiology. This approach can directly measure the voltage or the current flow over neuronal membranes (Cummins et al., 2009; Kang and Kitai, 1993; Molleman, 2003; Smith et al., 1992).

The neuronal precursor cell line LUHMES is a widely used model to study biochemical and pharmacological effects on the human nervous system, as well as various types of toxicities (Brüll et al., 2020; Delp et al., 2021, 2019, 2018a, 2018b; Harris et al., 2017; Krug et al., 2014, 2013; Lohren et al., 2015; Smirnova et al., 2016; Tong et al., 2017; Witt et al., 2017; Zhang et al., 2014). The cells can be differentiated into post-mitotic dopaminergic neurons (Scholz et al., 2011), and we have recently shown their usefulness for functional neurotoxicity assessment, including the measurement of effects on ionotropic (purinergic) receptors (Loser et al., 2021b). The SH-SY5Y neuroblastoma cells are another human model, which has been used for studies on Ca^{2+} signaling in neuronal function such as mAChR and voltage-dependent Ca^{2+} channel activation (Gustafsson et al., 2010). The cells express several nAChR subunits (Gould et al., 1992; Peng et al., 1994) and nAChRs have been the target for neurotoxicity studies (Ring et al., 2015) and investigations on the role in amyloid processing (Mousavi and Hellström-Lindahl, 2009) using the SH-SY5Y cell model.

Here, we characterized functional nAChRs on LUHMES neurons, and we asked whether SH-SY5Y and LUHMES cell cultures can be utilized for the assessment of xenobiotic effects on nAChRs. After confirming that the test systems indeed delivers robust and literature-congruent data on nicotinic signaling, we evaluated the six neonicotinoids with the highest market share (Bass et al., 2015; Jeschke et al., 2011), namely acetamiprid (Aceta), imidacloprid (Imida), clothianidin (Cloth), thiacloprid (Thiac), thiamethoxam

(Thiam) and dinotefuran (Dino). Our studies' overall goal was to identify potential modulatory effects of these pesticides on human neuronal $[Ca^{2+}]_i$ homeostasis. As it is known that disturbed cholinergic signaling, e.g., by exposure to nicotine, may trigger developmental neurotoxicity, the underlying rationale for this study was to evaluate the propensity for neonicotinoids to replicate nicotine toxicity in preterm or neonate infants.

3.2.3 Materials and methods

Materials and chemicals

An overview of experimental tool compounds and toxicants is given in table MS2_S1. Chemical structures of acetylcholine (<https://pubchem.ncbi.nlm.nih.gov/compound/187#section=2D-Structure>), cytosine (<https://pubchem.ncbi.nlm.nih.gov/compound/10235#section=2D-Structure>), nicotine (<https://pubchem.ncbi.nlm.nih.gov/compound/89594#section=2D-Structure>) and varenicline (<https://pubchem.ncbi.nlm.nih.gov/compound/170361#section=2D-Structure>) were obtained from PubChem and visualized in ChemDraw JS (version 19.0.0-CDJS-19.0.x.9 + da9bec968, PerkinElmer).

LUHMES cell culture

The cultivation of the LUHMES cells was performed as described earlier (Krug et al., 2013; Schildknecht et al., 2013; Scholz et al., 2011). In brief, LUHMES cells were cultured in standard cell culture flasks (Sarstedt) that were pre-coated with 50 µg/ml poly-L-ornithine (PLO) and 1 µg/ml fibronectin (Sigma-Aldrich) in H₂O overnight at 37 °C. The cells were maintained in proliferation medium containing advanced DMEM/F12 (Gibco) with 2 mM L-glutamine (Sigma-Aldrich), 1 × N2-supplement (Gibco) and 40 ng/ml recombinant human basic fibroblast growth factor (FGF-2, R&D Systems). The cells were kept at 37 °C and 5% CO₂ and passaged three times a week, when the culture reached a confluency of 75–90%. Cells were used until passage 18. For differentiation, cells were cultured in differentiation medium consisting of advanced DMEM/F12 (Gibco) supplemented with 2 mM L-glutamine (Sigma-Aldrich), 1 × N2-supplement (Gibco), 1 mM N6,2'-O-dibutyryl 3',5'-cyclic adenosine monophosphate (cAMP) (Sigma-Aldrich), 1 µg/ml tetracycline (Sigma-Aldrich) and 2 ng/ml recombinant human glial cell-derived neurotrophic factor (GDNF, R&D Systems).

For Ca^{2+} -imaging, the cells were pre-differentiated for 48 h in cell culture flasks, detached and plated at a density of 20,000 cells and 30,000 cells per well on 0.1% PEI-coated 384-well and 96-well plates (Greiner Bio-One), respectively. For manual patch

clamp recordings, the cells were plated at a density of 750 cells/ μ l on 0.1% PEI-coated glass coverslips. The cells were further differentiated for another 7–8 days. 50% of the medium was exchanged every 2–3 days.

Cell culture of SH-SY5Y cells

SH-SY5Y cells (passage 50–70) were cultured as previously described (Attoff et al., 2016) and monthly screened for mycoplasma contamination (Lonza MycoAlert Mycoplasma Detection Kit). Briefly, they were cultured in MEM supplemented with 10% fetal bovine serum (Gibco, 31330095), 1% non-essential amino acid solution (Gibco, 11140035), 2 mM L-glutamine (Gibco, 25030024), 100 μ g/ml streptomycin and 100 U/ml penicillin (Gibco, 15140122). For maintenance culture, SH-SY5Y cells were seeded at 27,000 cells/cm² in 75 cm² cell culture flasks (Corning). The cells were passaged once a week using TrypLE Express Enzyme (Gibco). SH-SY5Y cells were differentiated into a neuronal-like phenotype by exchanging the maintenance medium with differentiation medium consisting of DMEM/F12 (Gibco, 31330095) supplemented with 1 mM L-glutamine (Gibco, 25030024), 100 μ g streptomycin/ml, 100 U penicillin/ml, 1 $\times$ N2-supplement (Gibco, 17502048) and 1 μ M all-trans retinoic acid (RA, Sigma, R2625) 24 h after seeding. The cells were incubated in 100% humidity at 37 °C in air with 5% CO₂. For determination of cell viability, 35,000 SH-SY5Y cells/well (109,375 cells/cm²) were seeded in maintenance culture medium in clear 96-well plates (Corning, 3599). 24 h after seeding, maintenance medium was replaced with differentiation medium and incubated for 3 days before exposure with compounds. Cell viability was determined after 24-h exposure with nicotine or neonicotinoids by the conversion of resazurin (Sigma, R2625) to resorufin in metabolically active cells (O'Brien et al., 2000). A 20 $\times$ resazurin stock solution was prepared by dissolving 11 mg resazurin salt in 1 ml 0.1 M NaOH and adjusting to 50 ml with PBS^{-/-} (pH set to 7.8 with 0.1 M HCl). The 20 $\times$ resazurin stock solution was sterile filtered and stored at 4 °C protected from light. After exposure with compounds, 150 μ l medium was removed leaving 50 μ l in each well and subsequently, 50 μ l of 2 $\times$ resazurin solution dissolved in DMEM/F12 was added. The plate was incubated for 3 h at 37 °C with 5% CO₂. Resorufin fluorescence was measured at excitation 540 nm and at emission 590 nm using a FlexStation II fluorometer (Molecular Devices).

Gene expression profiling

Five biological replicates were generated from LUHMES cells differentiated for 2, 3, 5, 6, 8, 10, and 11 days, as well as from undifferentiated LUHMES cells (day 0). Samples were analyzed via the TempO-Seq assay, which is a targeted RNA-sequencing method developed by BioSpyder Technologies Inc. (Carlsbad, CA, USA). The method is described

in detail in House et al. (2017). For sample preparation, LUHMES grown in 96-well plates were lysed in 25 μ l 1 $\times$ BioSpyder lysis buffer according to the manufacturer's instructions. The lysate from 10 wells was pooled for each sample. Samples were stored at -80°C before shipping on dry ice to BioClavis (BioClavis, Ltd., Glasgow, UK) for TempO-Seq analysis. The resulting FASTQ files were aligned using the STAR algorithm to a pseudo-transcriptome by BioClavis and eventually normalized and standardized to a data format of x gene specific counts per 1 million reads. Traditional whole-genome RNA-sequencing (RNAseq) was performed for comparison and validation. Cells were cultured in 6-well plates. For sample preparation, medium was removed and cells were lysed in TriFast reagent (Pisclab, VWR, USA). The lysate of six wells was pooled for each sample. Samples were stored at -20°C until they were sent on dry ice to the department of toxicogenomics at the University Maastricht, Netherlands, for RNAseq analysis.

Changes in SH-SY5Y nAChR subunit mRNA expression after exposure with nicotine or neonicotinoids were analyzed by whole-genome TempO-Seq by BioClavis (BioClavis, Ltd., Glasgow, UK) as described for the LUHMES cells. Cells (35,000 cells/well) were plated in 96-well plates (Corning) and differentiated for 3 days as described above. After 6 and 24 h of exposure with nicotine and neonicotinoids, 50 μ l lysis buffer (BioClavis) was added per well according to the manufacturer's instructions, and the plates were stored at -80°C . Before shipment, the plates were thawed on ice and two identical samples/situations were pooled to give 100 μ l in one well in a new 96-well plate kept on ice. The plate with pooled samples was stored at -80°C until shipment for TempO-Seq analysis.

The time course of expression in control cells was analyzed by RNA-sequencing. For this, SH-SY5Y cells were sampled at 0, 3, 6, and 9 days of differentiation. The mRNA extraction and RNA-sequencing experimental setup including data analysis has been described in Attoff et al. (2020).

Neurite outgrowth assay

LUHMES cells were differentiated for 2 days in differentiation medium in PLO–fibronectin pre-coated cell culture flasks. On day 2 of differentiation (d2 neurons), cells were seeded at a density of 100,000 cells/cm² into PLO–fibronectin pre-coated 96-well plates. After 1 h of attachment, cells were treated for 24 h with nicotine and neonicotinoids spanning a concentration range of 5 nM–100 μ M. Cells were stained with H-33342 (1 μ g/ml) and calcein-AM (1 μ M) and high-content imaging was performed. Live cells and neurite area were assessed in parallel using an automated algorithm as described previously (Krug et al., 2013; Stiegler et al., 2011).

LUHMES Ca²⁺-imaging

Ca²⁺-imaging was performed using HT Functional Drug-Screening System FDSS/μCELL (Hamamatsu Photonics) at nominal 37 °C. The FDSS/μCell system enables the indirect recording of changes of [Ca²⁺]_i via a Ca²⁺-sensitive fluorescent dye. The fluorescence signal of a complete 384-well plate is acquired at once with a high-speed and high-sensitivity digital ImagEM X2 EM-CCD camera (Electron Multiplying Charge-Coupled Device, Hamamatsu Photonics), but with limited spatial resolution. Therefore, the software only determines the mean fluorescence signal of each well rather than of individual cells. For compound application, the integrated dispenser head with 384 pipette tips was used, which can add the test compound to all wells simultaneously. Cells were preincubated with Cal-520 AM (AAT Bioquest) at a concentration of 1 μM for 1 h at 37 °C. For recording, the medium was exchanged by a buffer solution containing [mM]: 135 NaCl, 5 KCl, 0.2 MgCl₂, 2.5 CaCl₂, 10 HEPES and 10 D-glucose, pH 7.4. Test compound application was executed after obtaining a 1.5 min baseline recording. Where applicable, a second application was executed 4.5 min after the first application. The total recording never exceeded 8 min.

For Ca²⁺-imaging experiments with a higher resolution on the single-cell level, the Cell Observer (Carl Zeiss Microscopy, GER) was used. The Ca²⁺-sensitive dye, the cell handling before the experiment and the buffer were the same as described above for the experiments with the high-throughput FDSS/μCELL system. The compounds were applied after an initial baseline recording of the fluorescence intensity of at least 10 s.

Ca²⁺ measurements in SH-SY5Y

To measure acute changes in the average [Ca²⁺]_i of a population, SH-SY5Y cells were examined in the 96-well plate fluorescence reader FlexStation II (Molecular Devices) using the fluorophore Fura-2AM. SH-SY5Y (35,000 cells/well; 109,375 cells/cm²) were seeded in maintenance culture medium in black 96-well plates with clear bottom (Corning, #3603). 24 h after seeding, maintenance medium was replaced with differentiation medium. After 72 h of differentiation, Fura-2AM diluted in KRH buffer (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.0 mM CaCl₂, 6.0 mM D-glucose, and 25 mM HEPES (free acid), pH adjusted to 7.4 by 1.0 M NaOH) was added to the medium to a final concentration of 4 μM (Gustafsson et al., 2010). The plates were incubated for 30 min at 37 °C, before cells were washed once with 200 μl KRH buffer. 90 μl of KRH buffer without or with 10 μM PNU ± 125 μM mecamylamine (Mec) was added to the Fura-2AM-loaded cells. The plate was again incubated for 20 min to allow full hydrolysis of the AM group before the experiment. The fluorescence was assessed at 37 °C in the fluorescence plate reader (FlexStation II; Molecular Devices) at two different excitation wavelengths, 340 nm for Ca²⁺-bound Fura-2 and 380 nm for

free Fura-2, and at 510 nm emission every 3.1 s using bottom read settings. After 26–29 s of initial baseline recording of the fluorescence intensity, 10 μ l of the compound dilution (10 times higher than the final concentration to the cells) was transferred automatically by the FlexStation II ("Flex mode"), column wise to the cell plate wells (five wells per concentration) and the fluorescence intensity was monitored for another 150 s. The ratio of fluorescence intensity at 340/380 nm was determined and the mean values from the baseline recording before addition of test compounds was set to zero. The acute change in the Ca^{2+} influx after addition of the compounds was quantified as the area under the curve using the SoftMax Pro 4.8 software (Molecular Devices). All test compounds were dissolved in DMSO and stored as 100 mM stock solutions at -20°C . At the day of experiments, compounds were diluted in KRH buffer in 1:3 series, with 100 μM as the highest concentration. The DMSO concentration was kept to 0.1% in all dilution steps and 0.1% DMSO in KRH buffer was also used as a negative control. KCl (30 mM) in KRH was used as a positive control. To check the implication of the $\alpha 7$ nAChR isoform in the cholinergic response, the $\alpha 7$ nAChR allosteric modulator PNU (10 μM) was used to evaluate the effect on Ca^{2+} influx triggered by nicotine and the six neonicotinoids. For desensitization studies, the cells were pre-exposed for 20 min with Thiam, Dino or Thiac (final concentration range 0.046–100 μM) together with 10 μM PNU before addition of 11 μM nicotine. The nicotine-induced Ca^{2+} influx was normalized to the Ca^{2+} response triggered by 30 mM KCl. The Ca^{2+} influx induced by the neonicotinoids was normalized to the response to 11 μM nicotine.

Manual patch clamp recordings

Manual patch clamp experiments were performed in the whole-cell mode (Hamill et al., 1981) with an EPC 10 USB patch clamp amplifier and PatchMaster Software (version 2 $\times$ 90.5; HEKA Elektronik, Lambrecht, Germany). Extracellular solution contained [mM]: 140 NaCl, 4 KCl, 1 MgCl_2 , 1.8 CaCl_2 , 10 HEPES and 10 D-glucose, pH 7.4. Intracellular solution contained [mM]: 107 K-gluconate, 10 KCl, 1 MgCl_2 , 10 HEPES, 5 EGTA, 4 Na_2ATP and 0.2 NaGTP , pH 7.2. Recordings were executed at room temperature. For agonist tests in current-clamp and voltage-clamp mode, cells were kept at a holding potential of -70 mV and the compounds were applied for 5 s.

Physicochemical properties and molecular docking studies

Based on the specific ChEMBL-ID of the substances, AlogP values as a descriptor for lipophilicity were extracted from the ChEMBL-database (<https://www.ebi.ac.uk/chembl>) (Davies et al., 2015; Mendez et al., 2019). The values provided by ChEMBL were calculated using RDKit (<https://www.rdkit.org>, 2018) based on the method described by Wildman and Crippen (Wildman and Crippen, 1999). Polar surface areas (PSA values)

were calculated using the maestro 2020-2 software (Schrödinger Release 2020-2, 2020) suite (Ertl et al., 2000).

The cryo-EM-resolved 3D-structures for the human nAChR $\alpha 4\beta 2$ -subtype were extracted from the protein data bank (rcsb.org (Berman et al., 2000)). Structure PDB-ID: 6cnj was used for the $\alpha 4\beta 2$ -binding site because of its superior resolution compared to structure 6cnk which was used for the $\alpha 4\alpha 4$ -binding site (Walsh et al., 2018). For the $\alpha 4\beta 2$ -isoform, the box center of the grid for the induced fit docking (IFD) run was chosen to be the centroid of the cocrystallized ligand nicotine between chain A and B: [A:402] in 6cnj and [A:405] in 6cnk (Walsh et al., 2018), respectively.

The structure of the human nAChR $\alpha 7$ -isoform has not been experimentally resolved yet, but there are published homology models of the ligand-binding domain (LBD) available that were used for docking studies on this subtype (Ng et al., 2018). A recent paper from 2020 provided the information for the 3D-structure of this constructed extracellular LBD, that consists of two chains of the protein (Sakkiah et al., 2020). Previous studies characterized key ligand-binding residues on the human nAChR $\alpha 7$ -subtype as follows (Ng et al., 2018; Sakkiah et al., 2020): Tyr32, Phe33, Ser34, Trp55, Leu56, Gln57, Met58, Ser59, Trp60, Thr77, Arg79, Trp107, Val108, Leu109, Val110, Asn111, His115, Cys116, Gln117, Tyr118, Leu119, Pro120, and Pro121 from the complementary subunit, and Ser148, Trp149, Ser150, Tyr151, Gly152, Arg186, Phe187, Tyr188, Cys190, Cys191, Lys192, Glu193, Pro194, and Tyr195 from the principal subunit. Therefore, the centroid of these amino acids around the $\alpha 7$ -binding site was used as center of the grid for docking studies on this isoform.

Both, proteins and ligands have been prepared prior to the IFD protocol with extended sampling at pH 7 ± 0.5 using LigPrep and Protein preparation Wizard with default settings and an OPLS3e force field (Harder et al., 2016) that allowed the binding grid to adapt the residues around the ligand poses within 5 Å. The box size was set to 12 Å in Maestro (Schrödinger Release 2020-2, 2020).

An induced fit docking protocol as implemented in the Schrodinger Software Suite was applied. The protocol comprises a Glide initial docking run where the ligands are docked to the previously defined grid rigidly after a constrained minimization (Prime) of the receptor has been performed (Schrödinger Release 2020-2, 2020; Sherman et al., 2006). A large number of poses per ligand was generated by this first docking run, from which only a set was passed on to the next steps by applying energy filters (Schrödinger Release 2020-2, 2020; Sherman et al., 2006). This was followed by Prime side-chain prediction, another Prime minimization, Glide redocking and eventually the Scoring State. The resulting poses were then used as input for interaction fingerprint clustering with the average linking method (Schrödinger Release 2020-2, 2020).

Data analysis and statistics

For the high-throughput Ca^{2+} -imaging data obtained in LUHMES cells, an offset correction using the FDSS software (version 3.2) was performed. Afterwards, the data were exported and further analyzed with scripts written in R (version 3.6.3) (R Core Team, 2020). The concentration–response curves were fitted using a log-logistic model described by Ritz et al. (2015), utilizing the R package *drc* with its function *drm()* and *LL2.2()* with the following equation: $f(x) = d/[1 + \exp(b(\log(x) - \tilde{e}))]$ (Ritz et al., 2015). The logarithm of the half-maximal effective concentration ($\log\text{EC}_{50}$) between 0 and the upper limit (d), which was set to 1 is represented by $\tilde{e}$, x denotes the concentration and b stands for the slope parameter (Ritz et al., 2015). In cases with normalizations to responses induced by other compounds, the function *LL2.3()* was used with a variable upper limit (d ; Ritz et al., 2015). The same equation was used to determine the half-maximal inhibitory concentration ($\log\text{IC}_{50}$). Then, the $\log\text{EC}_{50}$ and $\log\text{IC}_{50}$ values were converted into the pIC_{50} and pEC_{50} values, which are the negative logarithms to base 10.

The single-cell Ca^{2+} -imaging recordings were exported and analyzed in Fiji ImageJ (version 1.52i) to get the average fluorescence signal of each cell. These signals were further analyzed in R, where a threshold detection was performed to detect responding cells. For this, the offset was corrected by subtracting the mean of 20–65% of the fluorescence signal of the pre-application period from the recording, to be robust against spontaneous activity. The threshold was defined as $\text{mean} + 3 \times \text{SD}$ of the negative control recordings, during the detection phase of 6.5 s.

The raw data of the manual patch clamp recordings were analyzed in scripts written in R. For leak subtraction, the P/4 algorithms of PatchMaster were used in voltage-clamp recordings. The following R packages were utilized for data handling: *cowplot* (Wilke, 2019), *dplyr* (Wickham et al., 2020), *drc* (Ritz et al., 2015), *ephys2* (Danker, 2018), *ggplot2* (Wickham, 2016), *htmlwidgets* (Vaidyanathan et al., 2019), *lemon* (Edwards, 2019), *magick* (Ooms, 2020), *magrittr* (Bache and Wickham, 2014), *matrixStats* (Bengtsson, 2020), *miniUI* (Cheng, 2018), *modelr* (Wickham, 2020), *multcomp* (Hothorn et al., 2008), *plotrix* (Lemon, 2006), *proto* (Grothendieck et al., 2016), *shiny* (Chang et al., 2020), *shinyjs* (Attali, 2020), *shinyTree* (Trestle Technology, LLC, 2017), *tidyverse* (Wickham et al., 2019).

Concentration–effect responses in the SH-SY5Y $[\text{Ca}^{2+}]_i$ were analyzed by the GraphPad Prism8.0 software using the four-parameter sigmoidal curve fit settings and the concentrations giving 50% (BMC_{50}) increase in $[\text{Ca}^{2+}]_i$ in relation to the nicotine response were estimated.

The raw count tables of gene expression profiling with TempO-Seq assay and traditional whole-genome RNA-sequencing (RNAseq) were analyzed with the R package DESeq2

(v1.24.0) (Love et al., 2014). RNAseq counts were normalized to the library size and the transcript length [Transcripts per kilobase million (TPM)] (Wagner et al., 2012). TempoSeq counts were normalized to total counts per sample [counts per million (CPM)]. Gene lengths were retrieved from the hg18 reference genome (NCBI Build 36.1) with the R package Goseq (v1.40.0) (Young et al., 2010). TPM/CPM were averaged over the five biological replicates.

Unless mentioned differently, values are presented as mean $\pm$ SEM. If not indicated otherwise, experiments were performed with at least three technical replicates per condition. Detailed data on pEC₅₀, pIC₅₀ and n numbers are found in supplementary tables. Unless mentioned differently, statistical significance was defined as $P < 0.05$ and was determined by one-way ANOVA with Dunnett's post hoc test as indicated. To determine benchmark concentrations, and their upper and lower 95% confidence intervals (BMCL, BMCU), the BMC online software of UKN was used (Krebs et al., 2020).

3.2.4 Results and discussion

Suitability of LUHMES neurons to study human nAChR responses

We used general gene expression profiling data to check the suitability of LUHMES as a model for an ACh signaling target cell. Two transcriptomics approaches based on RNA sequencing suggested that the cells express several subunits of the nAChR. Particularly high signals were obtained in differentiated cells for the $\alpha 4$, $\alpha 7$, and $\beta 2$ chains, but also some other cholinergic components showed gene expression. For instance, ACh esterase (AChE) and the muscarinic AChR4 were up-regulated during the differentiation process (Fig. MS2_S1). Our data support the hypothesis that multiple nAChR subtypes transmit neurotransmitter signals to LUHMES cells, but gene expression data alone do not reveal information about protein expression and function. Therefore, we investigated the effect of the selective nAChR agonist nicotine by performing measurements of the $[Ca^{2+}]_i$ as functional endpoint. Application of nicotine resulted in a rapid and concentration-dependent transient rise in $[Ca^{2+}]_i$ (Fig. MS2_1a). A quantification of the nicotine signaling yielded a pEC₅₀ value of 5.9 (Fig. MS2_1b). This finding is in line with other published datasets on human nicotinic receptors determined by other techniques, confirming the applicability of Ca²⁺-imaging used here: e.g., pEC₅₀ values of 5.5–6.1 (EC₅₀: 0.9–3.5 μ M) have been reported for human $\alpha 4\beta 2$ nAChRs using patch clamp (Buisson et al., 1996; Chen et al., 2018; Wu et al., 2006), Ca²⁺-imaging (Capelli et al., 2011; Chavez-Noriega et al., 2000) and membrane potential fluorescence (Fitch et al., 2003). Patch clamp recordings with human $\alpha 6/3\beta 2\beta 3$ (Armstrong et al., 2017; Chen et al., 2018) or $\alpha 4\beta 4$ (Wu et al., 2006) nAChRs showed pEC₅₀ values of $\sim$ 5.9. In this

context, it is noteworthy that muscular (nonneuronal) nAChR ($\alpha 1\beta 1\delta\gamma/\epsilon$) have clearly lower nicotine affinities (Capelli et al., 2011; Fitch et al., 2003) of around 25 μM (pEC_{50} : ~ 4.6).

After these encouraging initial experiments on nicotinic signaling, we used cytisine as a second, well-established agonist. This compound exhibits high affinity and low efficacy for $\beta 2$ -containing nAChRs (like $\alpha 4\beta 2$) (Coe et al., 2005; Rollema and Hurst, 2018). The compound-induced Ca^{2+} -signals were comparable in both kinetics and sensitivity to nicotine (Fig. MS2_1c). The potency of cytisine (pEC_{50} : 6.0) was also in the 1 μM range. A similar set of data (pEC_{50} : 6.1) was obtained for a third nicotinic agonist, varenicline (Fig. MS2_1b) (Mihalak et al., 2006). Again, these data were in good agreement with data obtained by several other functional test platforms (Arias et al., 2015; Chatterjee et al., 2011; Chavez-Noriega et al., 2000; Coe et al., 2005; Fitch et al., 2003; Wu et al., 2006).

As fourth agonist, we used the neurotransmitter ACh itself. The pEC_{50} of 6.0 (Fig. MS2_1b) is in accordance with a large set of literature data on the signaling effects of this endogenous ligand (Armstrong et al., 2017; Benallegue et al., 2013; Bermudez and Moroni, 2006; Buisson et al., 1996; Carbone et al., 2009; Harpsøe et al., 2011; Kuryatov et al., 1997; Mineur et al., 2009; Nelson et al., 2003). Finally, pilocarpine was used as mAChR agonist (Šantrůčková et al., 2014). Concentrations of up to 30 μM ($n \geq 7$, data not shown) did not trigger any $[\text{Ca}^{2+}]_i$ changes. In summary, the quantitative data on four diverse nAChR agonists (Fig. MS2_1d) indicate a functional expression of neuronal nAChRs, and the suitability of LUHMES cells to study compounds affecting the nicotinic signaling.

To investigate the electrophysiological impact on LUHMES neurons, manual patch clamp recordings were performed. Application of nicotine (10 μM) to a total of 42 neurons held in current-clamp evoked either tonic ($n = 28$, Fig. MS2_1e) or phasic ($n = 14$, data not shown) action potential firing. Furthermore, voltage-clamp recordings revealed that cells responded with a fast-activating and slowly inactivating inward current ($n = 12$, Fig. MS2_1f). These findings confirm the presence of functional nAChRs on LUHMES neurons as shown by Ca^{2+} -imaging. Moreover, the observed long-lasting depolarization of the membrane potential over the entire application period indicates a strong contribution of non- $\alpha 7$ nAChRs (e.g., $\alpha 4\beta 2$ or $\alpha 4\beta 4$ subtypes) (Alijevic et al., 2020; Elliott et al., 1996; Mihalak et al., 2006; Rollema et al., 2007; Wu et al., 2006).

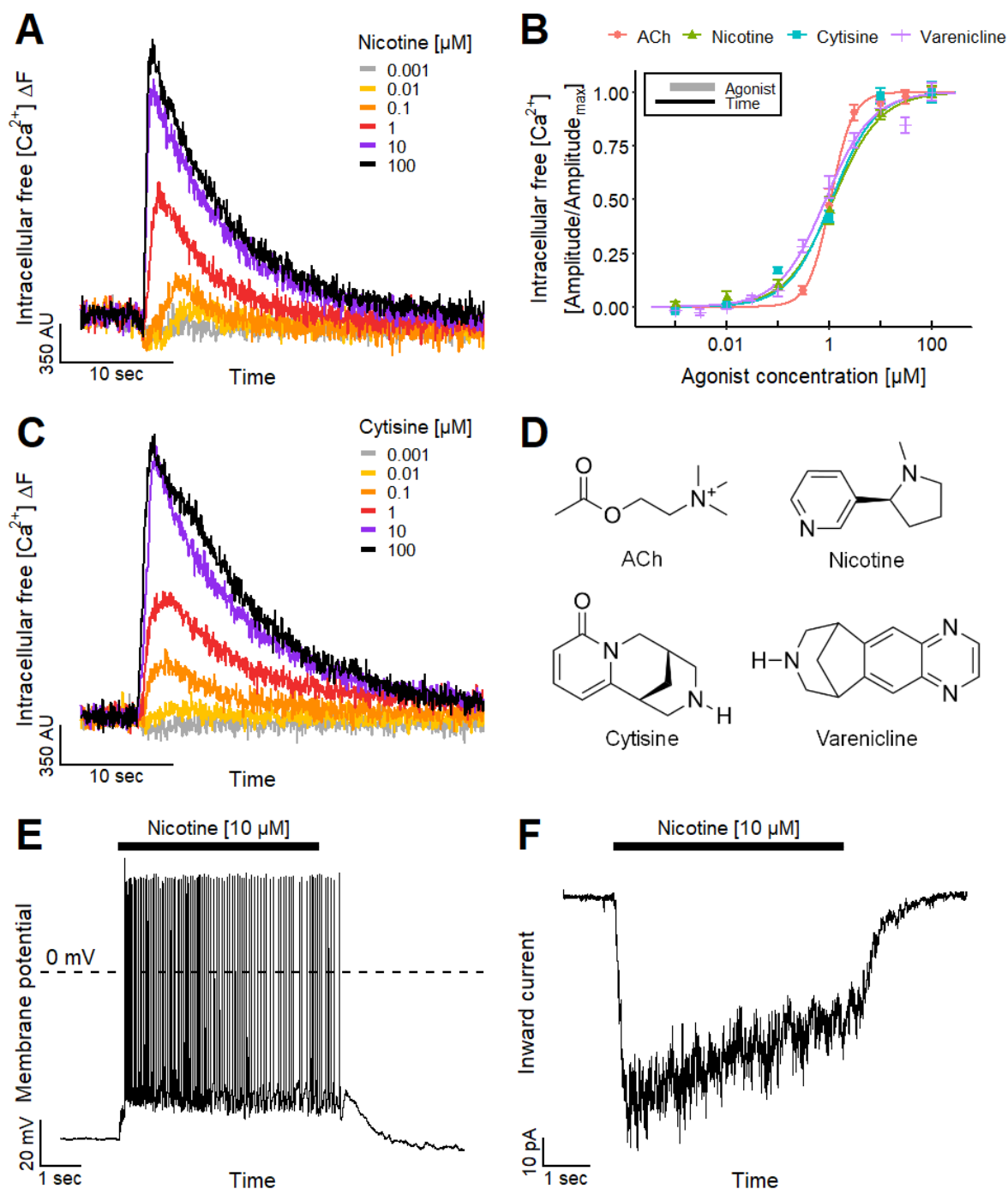


Fig. MS2_1 Identification of functional nicotinic acetylcholine receptors (nAChRs) on LUHMES neurons.

a Traces of Ca^{2+} -imaging recordings show the concentration-dependent effects of the nAChR agonist nicotine on LUHMES neurons. **b** Concentration-response curves for the effects of nAChR agonists acetylcholine (ACh), nicotine, cytosine and varenicline with pEC_{50} values of 5.98 ± 0.03 , 5.93 ± 0.05 , 5.95 ± 0.05 and 6.08 ± 0.04 , respectively. Note the treatment scheme (upper left corner), illustrating the experimental design. Detailed data on n numbers are found in table MS2_S4. **c** Ca^{2+} -imaging signals evoked by the addition of cytosine. **d** Chemical structures of the tested nAChR agonists. **e, f** Manual patch clamp recordings of the responses of LUHMES neurons evoked by the application of $10 \mu\text{M}$ nicotine for 5 s. **e** Firing of multiple action potentials with a long-lasting depolarization of the membrane potential ($n = 28$) recorded in current-clamp. **f** Slowly inactivating inward current ($n = 12$) measured in voltage-clamp

Investigation of nAChR subtype composition in LUHMES cells

To profile LUHMES neurons for functional neurotoxicity studies, we used additional pharmacological tools to shed light on the nAChR subtype composition. First, we tested whether antagonists of nAChRs modulated the Ca^{2+} -responses. The non-selective nAChR antagonist tubocurarine (Tubo) (Jonsson et al., 2006) has a long history in toxicology and is one component of an arrow poison for hunting. Application of Tubo antagonized the responses evoked by nicotine (Fig. MS2_2a), ACh (Fig. MS2_2b) and varenicline concentration-dependently (pIC_{50} values ~ 6.1) (Fig. MS2_2c). Tubo completely blocked the responses to all three agonists at concentrations $> 10 \mu\text{M}$, indicating that the entire agonist-triggered Ca^{2+} -signaling was mediated by nAChRs.

To further dissect the nAChR subtypes involved in LUHMES responses, we researched the effects of other antagonists. Methyllycaconitine (MLA) is more potent (low nM range) on the $\alpha 7$ nAChR than on other receptor combinations (Buisson et al., 1996; Capelli et al., 2011; Gopalakrishnan et al., 1995; Palma et al., 1996; Puchacz et al., 1994). Application of MLA (Fig. MS2_2d) yielded a pIC_{50} of 6.3 in our cell system (Fig. MS2_2f). This low affinity indicates that mainly non- $\alpha 7$ nAChRs are responsible for the $[\text{Ca}^{2+}]_i$ response. The LUHMES pIC_{50} was similar to previously reported pIC_{50} values for human $\alpha 4\beta 2$ expressed in cell lines (Buisson et al., 1996; Capelli et al., 2011), while it was different from the reported pIC_{50} values for $\alpha 7$, $\alpha 3\beta 4$, $\alpha 1\beta 1\delta\epsilon$ and $\alpha 6$ -containing ($\alpha 6/3\beta 2\beta 3$) nAChRs (Capelli et al., 2011). This points towards a major contribution of $\alpha 4\beta 2$ to the response evoked by nicotine in LUHMES.

To further substantiate the findings resulting from the MLA experiments, we applied the non-competitive nAChR antagonist mecamylamine (Mec) (Capelli et al., 2011; Papke et al., 2008). This drug shows higher potency for $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs compared to the $\alpha 7$ nAChR (Capelli et al., 2011). The pIC_{50} value of 6.2 (Fig. MS2_2f) is comparable to literature data of ~ 6.1 for human $\alpha 4\beta 2$ (Capelli et al., 2011; Chavez-Noriega et al., 2000) and 6.6 for human $\alpha 3\beta 2$ (Chavez-Noriega et al., 2000), while it was different from reported pIC_{50} values for $\alpha 7$, $\alpha 3\beta 4$, $\alpha 1\beta 1\delta\epsilon$ and $\alpha 6$ -containing ($\alpha 6/3\beta 2\beta 3$) nAChRs (Capelli et al., 2011).

Finally, we confirmed our previous results by applying MG 624, a slightly more potent nAChR antagonist (Capelli et al., 2011; Gotti et al., 2000). The LUHMES pIC_{50} of 6.8 (Fig. MS2_2e, f) is comparable to literature pIC_{50} values of 6.8 for $\alpha 4\beta 2$, 6.6 for $\alpha 3\beta 4$, 6.9 for $\alpha 7$ and 7.3 for $\alpha 1\beta 1\delta\epsilon$ (Capelli et al., 2011).

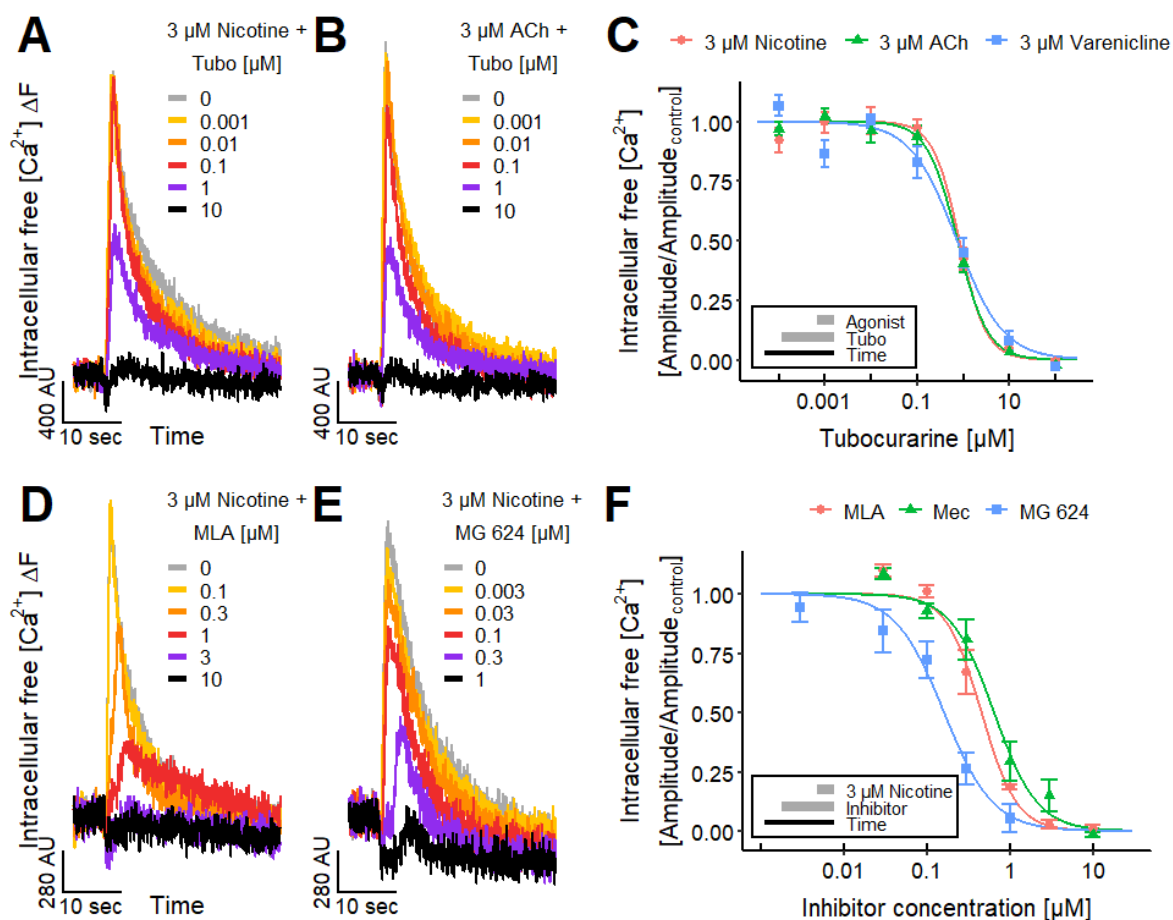


Fig. MS2_2 Characterization of the nAChRs.

a, b Ca²⁺-imaging signals of the effects of the pre-applied non-selective nAChR antagonist tubocurarine (Tubo) on the responses of LUHMES neurons triggered by **a** 3 μM nicotine and **b** 3 μM ACh. **c** Inhibitory effect of Tubo on the signals evoked by the acute exposure to 3 μM of nicotine, ACh and varenicline. The resulting pIC₅₀ values were 6.08 ± 0.04 for nicotine, 6.13 ± 0.04 for ACh and 6.13 ± 0.09 for varenicline. **d, e** Traces of the effects of **d** MLA and **e** MG 624 on the Ca²⁺-imaging signals stimulated by 3 μM nicotine. **f** Concentration–response curves for the effects of MLA, mecamylamine (Mec) and MG 624 on the responses triggered by the acute exposure to 3 μM nicotine. The pIC₅₀ values were 6.33 ± 0.04 for MLA, 6.17 ± 0.05 for Mec and 6.80 ± 0.07 for MG 624. Note the treatment schemes (lower left corner), illustrating the experimental design. Detailed data on n numbers are found in table MS2_S4

These antagonist data provided indirect evidence for a contribution of neuronal non-α7 nAChRs, e.g., α4β2 and/or α3-containing nAChRs, to the [Ca²⁺]_i responses of LUHMES evoked by nicotine. To address this issue more directly, we made use of the neuronal non-α7 nAChRs agonist ABT 594 (Donnelly-Roberts et al., 1998; Michelmore et al., 2002). We found a pEC₅₀ value of 8.4 (Fig. MS2_3b, c), which strongly suggests a presence of functional non-α7 nAChRs on LUHMES. In fact, the potency of this drug in the LUHMES system was even higher than previously reported for other cells (Donnelly-Roberts et al., 1998; Michelmore et al., 2002). This might be explained by the presence of multiple nAChR subtypes on LUHMES neurons, because the potency of ABT 594

depends on the nAChR subtype (Michelmore et al., 2002), as also described for other nAChR agonists (Capelli et al., 2011; Chavez-Noriega et al., 2000; Jonsson et al., 2006). As internal consistency check, we blocked ABT 594 responses with the nAChR antagonists Mec (Fig. MS2_3d) and Tubo (Fig. MS2_3e). The pIC_{50} values of 6.1 for Mec and 5.7 for Tubo (Fig. MS2_3f) are similar to the values that we obtained before for both antagonists for nicotine (Fig. MS2_2f).

In summary, our data demonstrate a functional expression of neuronal non- $\alpha 7$ nAChRs on LUHMES neurons and highlight the capability of this test system for detecting agonistic and antagonistic effects on nAChRs.

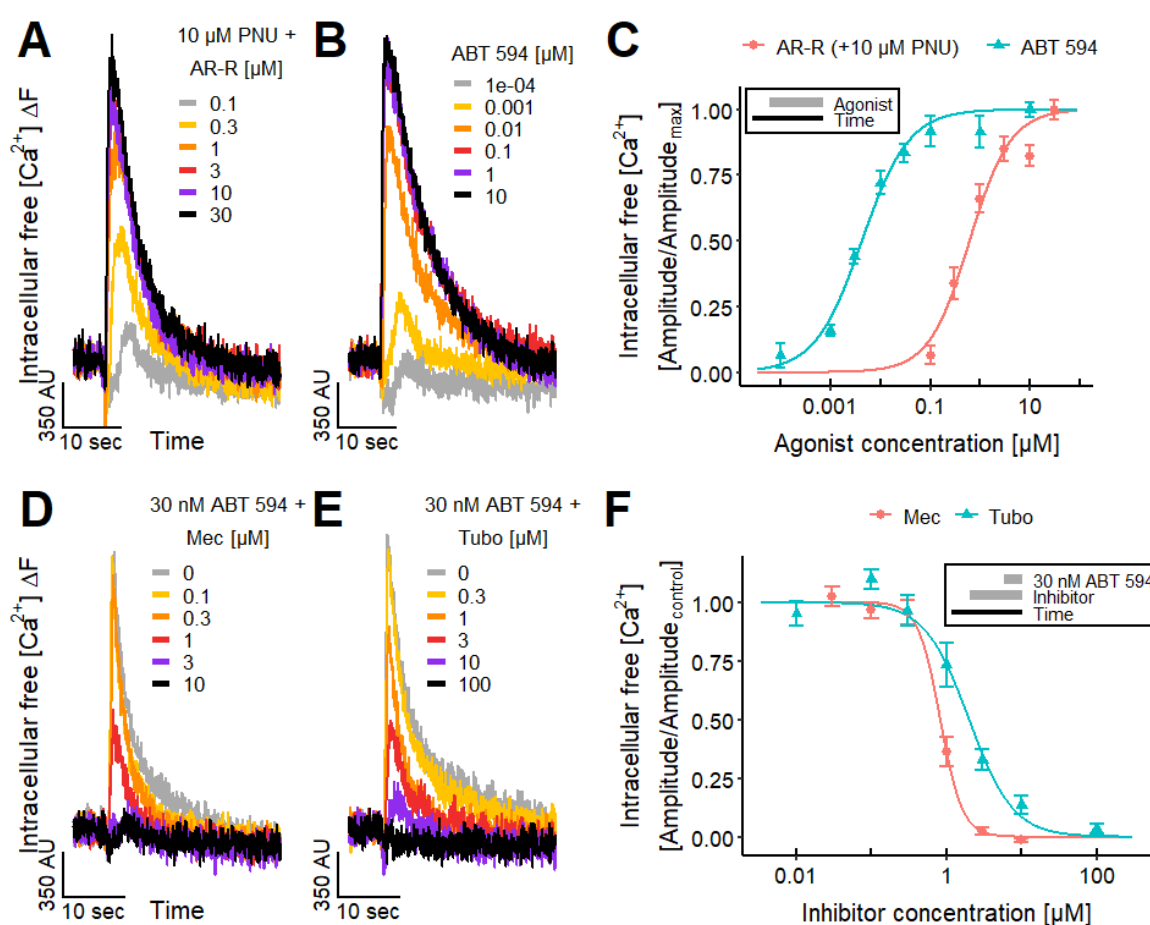


Fig. MS2_3 Differential agonist responses on nAChRs.

a, b Signals of Ca^{2+} -imaging triggered by the selective $\alpha 7$ nAChR agonist **a** AR-R 17779 (AR-R), after the pretreatment with 10 μM PNU-120596 (PNU), a positive allosteric modulator of $\alpha 7$ nAChR, and the selective non- $\alpha 7$ nAChR agonist **b** ABT 594. **c** Agonistic effect of AR-R in presence of 10 μM PNU and ABT 594 yielded pEC_{50} values of 6.20 ± 0.05 and 8.36 ± 0.05 , respectively. **d, e** Ca^{2+} -imaging traces of the effects of **d** Mec and **e** Tubo, which were preincubated for 4.5 min, on the response of the LUHMES neurons evoked by 30 nM ABT 594. **f** The concentration–response curves of the effects of Mec and Tubo on the response evoked by 30 nM ABT 594 resulted in pIC_{50} values of 6.08 ± 0.03 and 5.70 ± 0.05 , respectively. Note the treatment schemes, illustrating the experimental design. Detailed data on n numbers are found in table MS2_S4

Identification of $\alpha 7$ nAChR on LUHMES

As the gene expression data suggested that $\alpha 7$ nAChRs are highly expressed in LUHMES (Fig. MS2_S1), we investigated the functional role of this receptor subtype. First, we checked the effect of the selective $\alpha 7$ nAChR agonist AR-R 17779 (AR-R) (Micheltore et al., 2002; Mullen et al., 2000; Papke et al., 2004) on $[Ca^{2+}]_i$. We failed to detect a response ($n \geq 13$, data not shown). The most likely reason is a very fast inactivation of the $\alpha 7$ nAChR (Elliott et al., 1996; Mihalak et al., 2006). This has also been described for other $\alpha 7$ nAChR agonists in Ca^{2+} -imaging experiments (Chatzidaki et al., 2015; Dickinson et al., 2007; Gill et al., 2013; Larsen et al., 2019). As receptor desensitization can lead to technical challenges when investigating nicotinic agonists, several positive allosteric modulators including PNU-120596 (PNU) have been developed to counteract this phenomenon. We used here the selective positive allosteric modulator of the $\alpha 7$ nAChR, PNU-120596 (PNU). This compound can slow the $\alpha 7$ nAChR inactivation, and it, therefore, enables the detection of the $\alpha 7$ nAChR-mediated response (Chatzidaki et al., 2015; Dickinson et al., 2007; Grønlien et al., 2007; Hurst et al., 2005; Larsen et al., 2019; Ng et al., 2007; Papke et al., 2009; Williams et al., 2011). Under these conditions (presence of PNU), the cells responded concentration-dependently to the stimulation with AR-R (Fig. MS2_3a). The pEC_{50} value of 6.2 (Fig. MS2_3c) indicates the presence of functional $\alpha 7$ nAChRs. The LUHMES pEC_{50} value for AR-R is high, compared to a previously reported value for rat $\alpha 7$ expressed in *Xenopus laevis* oocytes ($pEC_{50} = 5$; Papke et al., 2004). This difference is most likely due to the increased agonist potency induced by the allosteric enhancer PNU, as previously described (Grønlien et al., 2007; Hurst et al., 2005).

To control for the $\alpha 7$ specificity of PNU in the LUHMES system, we tested, whether it would also enhance signaling of other receptors. In control experiments, cells were stimulated with 1 μM α, β -meATP (P2X receptor agonist (Bianchi et al., 1999; Khakh and North, 2012; Loser et al., 2021b)) and different concentrations of ABT 594 (non- $\alpha 7$ nAChR agonist). In both cases, no differences between recordings with and without PNU were detected (Fig. MS2_S2). We, therefore, conclude that PNU did not enhance $[Ca^{2+}]_i$ responses in general, but only those of the $\alpha 7$ nAChR.

In summary, the $\alpha 7$ nAChR-selective tool compounds showed consistent responses and interactions, thereby showing functional expression of $\alpha 7$ nAChRs on LUHMES neurons.

Direct effect of neonicotinoids on otherwise untreated LUHMES cultures

After demonstrating the presence of functional nAChRs on LUHMES and establishing the test system's suitability to study nAChR-based toxicity, we investigated a subset of six

neonicotinoids, namely acetamiprid (Aceta), imidacloprid (Imida), clothianidin (Cloth), thiacloprid (Thiac), thiamethoxam (Thiam) and dinotefuran (Dino), using Ca^{2+} -imaging as endpoint (Fig. MS2_4a, b). All compounds, except for Thiam and Dino, evoked responses, which we calibrated to the maximum effect observed at 10 μM nicotine (Fig. MS2_4c). We determined pEC_{25} values, as the responses triggered by the neonicotinoids did not reach the 50% response level in the tested concentration range ($\leq 100 \mu\text{M}$). A comparison of the responses of the four active compounds to those of nicotine based on pEC_{25} values indicated that the pesticides had a two orders of magnitude lower potency, but triggered clear responses at 10–100 μM concentrations (Fig. MS2_4d). Our data also indicate that the four active pesticides have lower potencies than nicotine or the endogenous neurotransmitter ACh. One straightforward explanation for the potency data observed may be the different affinities of the compounds for the set of nAChRs expressed on LUHMES cells. Binding assays using $\alpha 4\beta 2$ nAChR have suggested such potency differences (Tomizawa and Casida, 2005).

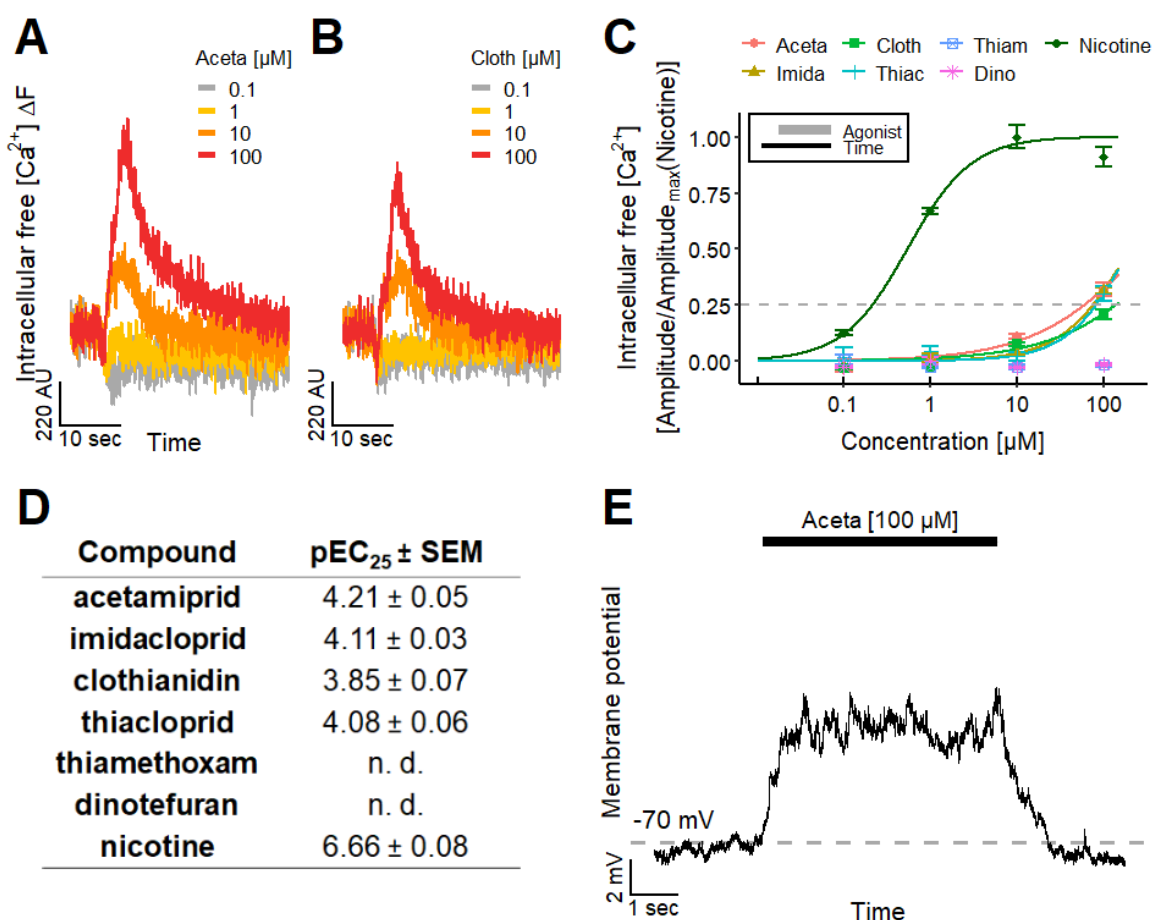


Fig. MS2_4 Effect of neonicotinoids on LUHMES neurons.

a, b Traces of Ca^{2+} -imaging show the effects of the neonicotinoids **a** Aceta and **b** Cloth on LUHMES neurons. **c** Concentration-dependent effect of the neonicotinoids Aceta, Imida, Cloth, Thiac, Thiam and Dino and the positive control nicotine. Amplitudes were normalized to the maximal amplitude

evoked by nicotine. Note the treatment scheme (upper left corner), illustrating the experimental design. **d** Table with corresponding pEC₂₅ values for the tested neonicotinoids and nicotine. Detailed data on n numbers are found in table MS2_S6. **e** Manual patch clamp recording of a long-lasting depolarization of the membrane potential during the application of 100 μ M Aceta for 5 s ($n = 4$). The Aceta-induced depolarization from a holding potential of -70 mV was not sufficient to evoke action potential firing

Our data show that also human cells react to neonicotinoids with changes of $[Ca^{2+}]_i$, as has been shown earlier for Aceta and Imida in other mammalian cells, i.e., primary rat neuronal cultures (Kimura-Kuroda et al., 2012). Effects of neonicotinoids on human neurons may have remained hitherto relatively unnoticed, as many tests only focus on endpoints related to cell viability and cell morphology. For instance, we did not observe any effect of neonicotinoids on the neurite outgrowth of LUHMES cells (Fig. MS2_S3). Our finding that human neuronal cells react to neonicotinoids demonstrates the importance of functional assays to assess potential adverse effects on neurons.

To confirm the important findings from Ca^{2+} -imaging experiments, we additionally performed manual patch clamp recordings (Fig. MS2_4e). Aceta (100 μ M) clearly depolarized the cells, but not to a sufficient extent to trigger action potential firing ($n = 4$). The time course of depolarization gives some indication on the types of receptors involved: the long-lasting effect suggests that mainly non- $\alpha 7$ nAChRs contributed to the current (Alijevic et al., 2020; Mihalak et al., 2006; Rollema et al., 2007). With $\alpha 7$ nAChR activation as main mode of action, a rapid depolarization followed by a timely repolarization would have been observed during the application. However, it cannot be excluded from the data available that $\alpha 7$ nAChRs at least partially were co-activated, together with non- $\alpha 7$ nAChRs.

Neonicotinoids effects on Ca^{2+} -signaling of individual LUHMES neurons

After our initial findings of neonicotinoid effects on human neuronal cultures (LUHMES), it was important to confirm $[Ca^{2+}]_i$ changes on the level of single cells. We used a Ca^{2+} -imaging approach with high spatial resolution, and found that the positive control nicotine triggered a rapid rise in $[Ca^{2+}]_i$ in most neuronal cell bodies as well as in the neurite network (Fig. MS2_5a). Aceta triggered a response only in a subset of cell bodies and in parts of the neurite network. Some cells clearly did not respond (Fig. MS2_5a, b). The concentration-dependency of the responding cells was quantified for nicotine, Aceta and Imida (Fig. MS2_5c). The curves looked similar to the concentration–response curves obtained with the high-throughput system (Fig. MS2_4c). At 1 μ M nicotine, the relative number of responsive cells reached its maximum of about 80%. For Aceta and Imida, cell responses were significantly increased at 10 μ M, and they comprised about one-quarter of all cells at the highest test concentration of 100 μ M. We also detected

responses of the LUHMES neurons to the application of Cloth and Thiac, but not for Thiam and Dino at their highest tested concentration (Fig. MS2_5d). To further confirm our findings from high-throughput imaging, we used Tubo also in single-cell recordings. The nAChR antagonist concentration-dependently reduced the number of cells responding to Aceta (100 μ M, Fig. MS2_S4). In summary, these findings confirm the data obtained with high-throughput Ca^{2+} -imaging measurements. They suggest an impact of Aceta, Imida, Cloth and Thiac on Ca^{2+} -signaling of LUHMES neurons; they also confirm the low/absent effectiveness of Thiam and Dino. It remains unclear, why the otherwise quite homogeneous LUHMES cultures show response heterogeneity at the single cell level. Since LUHMES form an interactive network, different cells in the network differ by their interconnection, their neighborhood and their firing history, and all this may contribute to differential responsiveness to nicotine or neonicotinoids (Loser et al., 2021b).

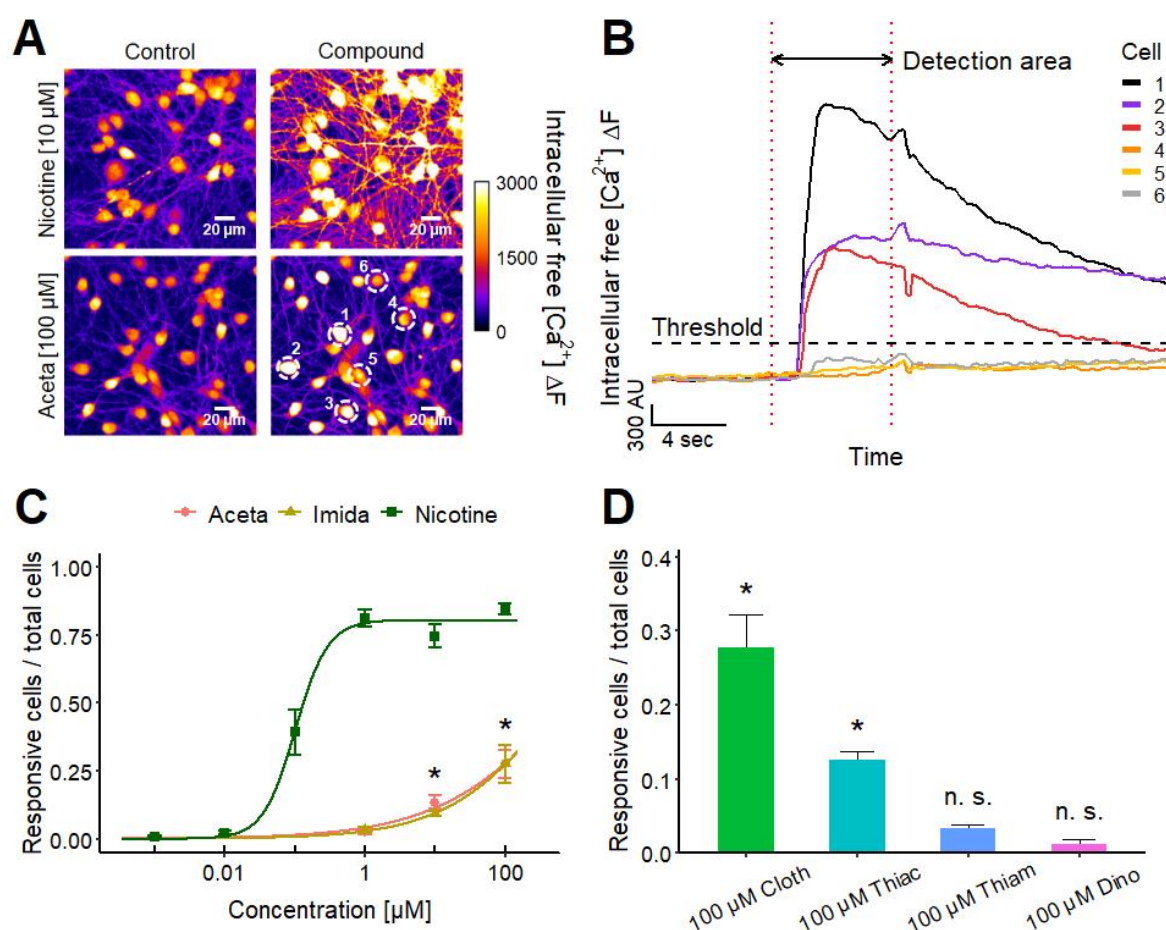


Fig. MS2_5 Effects of neonicotinoids on Ca^{2+} -signaling on the level of individual neurons.

a LUHMES neurons during control and during the application of 10 μ M nicotine and 100 μ M Aceta. Pictures of single-cell Ca^{2+} -imaging recordings were taken with the Cell Observer (Carl Zeiss Microscopy). Images are shown in false color to enhance the interpretability. **b** Traces of single-

cell Ca^{2+} -imaging recordings of six cells (marked in **a**) with and without response to the application of 100 μM Aceta. Threshold for response detection is defined as mean + 3 $\times$ SD of negative control recordings. **c** Percentage of cells that responded to the application of nicotine, Aceta and Imida in single-cell Ca^{2+} -imaging recordings. Changes are significant ($*p < 0.05$, t test) for 10 μM Aceta and Imida. Using more stringent ANOVA with Dunnett's post hoc test, there was a significant difference for 100 μM , but only a trend ($p > 0.05$ for 10 μM). This range of effect significance agrees well with calculations of the Imida benchmark concentration ($\text{BMC}_{10} = 11.2 \mu\text{M}$) and its upper 95% confidence limit ($\text{BMCU}_{10} = 26 \mu\text{M}$). **d** Fraction of cells reacting to Cloth, Thiac, Thiam and Dino at a concentration of 100 μM . Note the enlarged y -axis. **c**, **d** Statistical significance was determined against negative control recordings (*, significant by ANOVA; n. s., not significant). Detailed data on n numbers and percentages of responsive cells are found in table MS2_S7 and MS2_S8, respectively

The role of $\alpha 7$ nAChR for responses to neonicotinoids in LUHMES and SH-SY5Y neurons

The $\alpha 7$ nAChR is widely distributed in the central nervous system and is involved in the modulation of neurotransmitter release (Alkondon et al., 1999; Gotti et al., 2006; Gray et al., 1996; Levin et al., 2006; McGehee et al., 1995; Zoli et al., 2015). To address the role of $\alpha 7$ nAChRs in neonicotinoid effects, we performed Ca^{2+} -imaging experiments in the presence of PNU. While PNU had no effect by itself on non-stimulated cells, it drastically enhanced the $[\text{Ca}^{2+}]_i$ -increase (peak and duration) triggered by, e.g., Cloth or Thiac (Fig. MS2_6a, b). A systematic comparison of all six compounds used in our study showed that PNU significantly enhanced the responses to Aceta, Imida, Cloth and Thiac. Thiam and Dino had no effect, independent of the presence or absence of PNU (Fig. MS2_6c). The enhancing effect of PNU indicates an activation of human $\alpha 7$ nAChRs by Aceta, Imida, Cloth and Thiac, but not Thiam and Dino. These data are in good agreement with a study by Cartereau et al. (2018). An agonist activity of Imida on $\alpha 7$ nAChRs has also been described in other systems (Ihara et al., 2003; Yamamoto et al., 1998).

As the stimulation of human neuronal nAChRs has important toxicological implications, we checked main findings in a second, entirely independent test system: SH-SY5Y human neuroblastoma cells. Such cultures have been used earlier to prepare cell membranes containing nAChRs and also to measure the efflux of $^{86}\text{Rb}^+$ (Lukas et al., 1993; Tomizawa and Casida, 1999). Here, we characterized the receptor subunit expression profile during retinoic acid-induced differentiation, and we found particularly high and consistent (over time) levels of $\alpha 7$ (Figs. MS2_7a and MS2_S5A). Upon exposure of 3-day differentiated SH-SY5Y cells to nicotine, a clear, but relatively low increase of $[\text{Ca}^{2+}]_i$ was observed, with a pEC_{50} value of about 7.0. This response was drastically augmented by the $\alpha 7$ stabilizer/allosteric modulator PNU. The increase in $[\text{Ca}^{2+}]_i$ triggered by nicotine in the presence of PNU was similar or even higher than the

very strong response evoked by 30 mM KCl (Figs. MS2_7b and MS2_S5C). In addition, the $[Ca^{2+}]_i$ responses stimulated by the neonicotinoids Aceta, Imida, Cloth and Thiac were significantly increased (in efficacy and in potency) in the presence of PNU (Fig. MS2_S5D). Under these conditions, the neonicotinoids Aceta, Imida, Cloth and Thiac triggered $[Ca^{2+}]_i$ responses with pEC_{50} values in the low μM range (Fig. MS2_7c). Thiam and Dino had no effect, which fully confirmed our previous findings in LUHMES cultures (Fig. MS2_6c). Cytotoxicity or major changes of receptor expression were not observed for nicotine, Aceta or Imida (Fig. MS2_S5A, B). In summary, the SH-SY5Y data provided clear evidence that a subclass of the tested neonicotinoids are agonists on the $\alpha 7$ nAChR, triggering a $[Ca^{2+}]_i$ response in human cells at low μM concentrations (Fig. MS2_7c). The high potency of the neonicotinoids in the neuroblastoma test system might be explained by a high contribution of the $\alpha 7$ nAChR in these cells, and by the known left-shifting effect of the concentration–response curve by the positive allosteric modulator PNU. Such a potency shift was also observed in LUHMES stimulated with AR-R (Fig. MS2_3c), and it is also known for other agonists (Grønlien et al., 2007; Hurst et al., 2005).

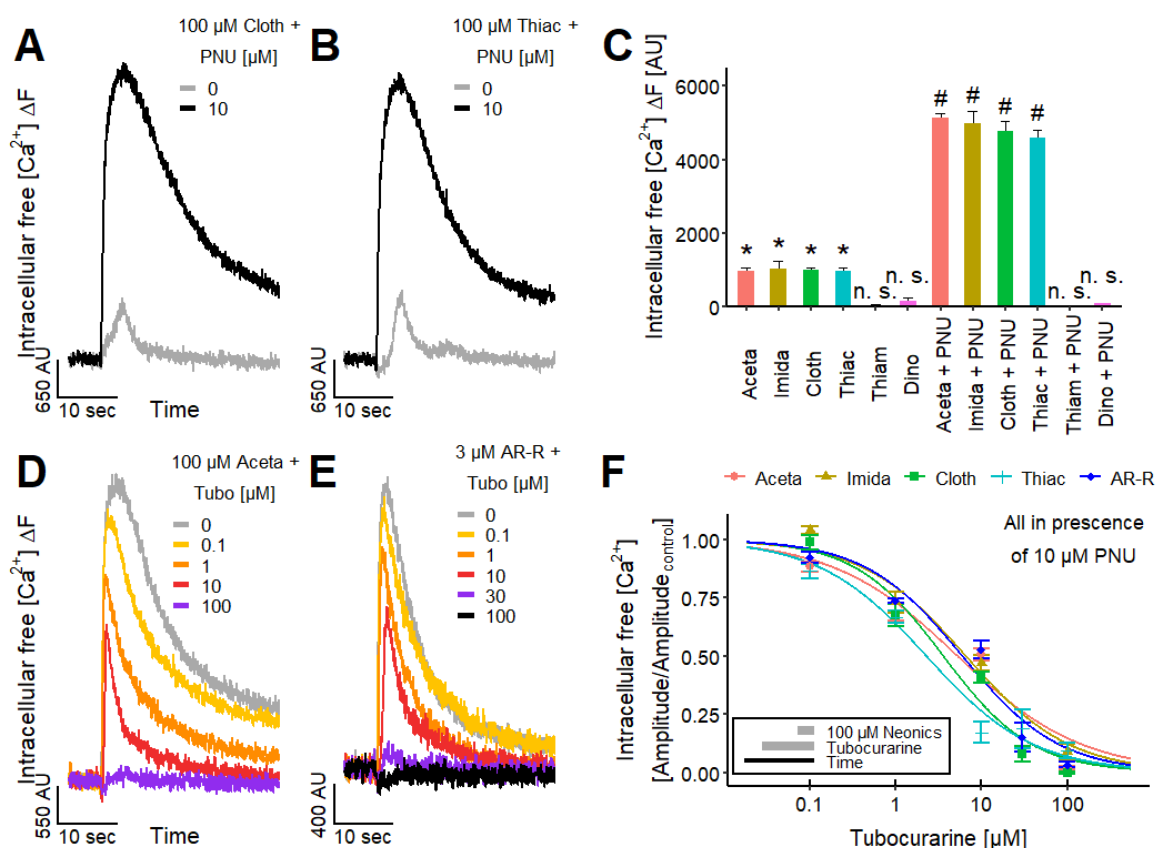


Fig. MS2_6 Effect of neonicotinoids on $\alpha 7$ nAChR.

a, b Ca^{2+} -imaging traces of the effects of the neonicotinoids **a** Cloth and **b** Thiac during control and in the presence of 10 μM PNU, which was preincubated for 4.5 min. **c** Effects of the

neonicotinoids Aceta, Imida, Cloth, Thiac, Thiam and Dino (100 μ M) on the Ca^{2+} -imaging signal of LUHMES neurons in absence and presence of 10 μ M PNU. Statistical significance was determined between the recordings of the neonicotinoids without PNU and negative control recordings (*, significant; n. s., not significant) and between the recordings of each neonicotinoid without and with PNU (#, significant; n. s., not significant). **d, e** Traces of Ca^{2+} -imaging showing the effect of pre-applied tubocurarine (Tubo) on the signals evoked by **d** 100 μ M Aceta and **e** 3 μ M AR-R, both in the presence of pre-applied 10 μ M PNU. **f** The concentration–response curves illustrate the effects of Tubo on the response of the LUHMES neurons to the acute exposure to the neonicotinoids Aceta, Imida, Cloth and Thiac (100 μ M) and the $\alpha 7$ agonist AR-R (3 μ M). All recordings were performed in the presence of 10 μ M PNU, which was preincubated for 4.5 min. The resulting pIC_{50} values were 5.28 ± 0.10 , 5.18 ± 0.09 , 5.45 ± 0.08 , 5.64 ± 0.08 and 5.22 ± 0.09 for Aceta, Imida, Cloth, Thiac and AR-R, respectively. Note the treatment scheme (lower left corner), illustrating the experimental design. Detailed data on n numbers are found in table MS2_S6

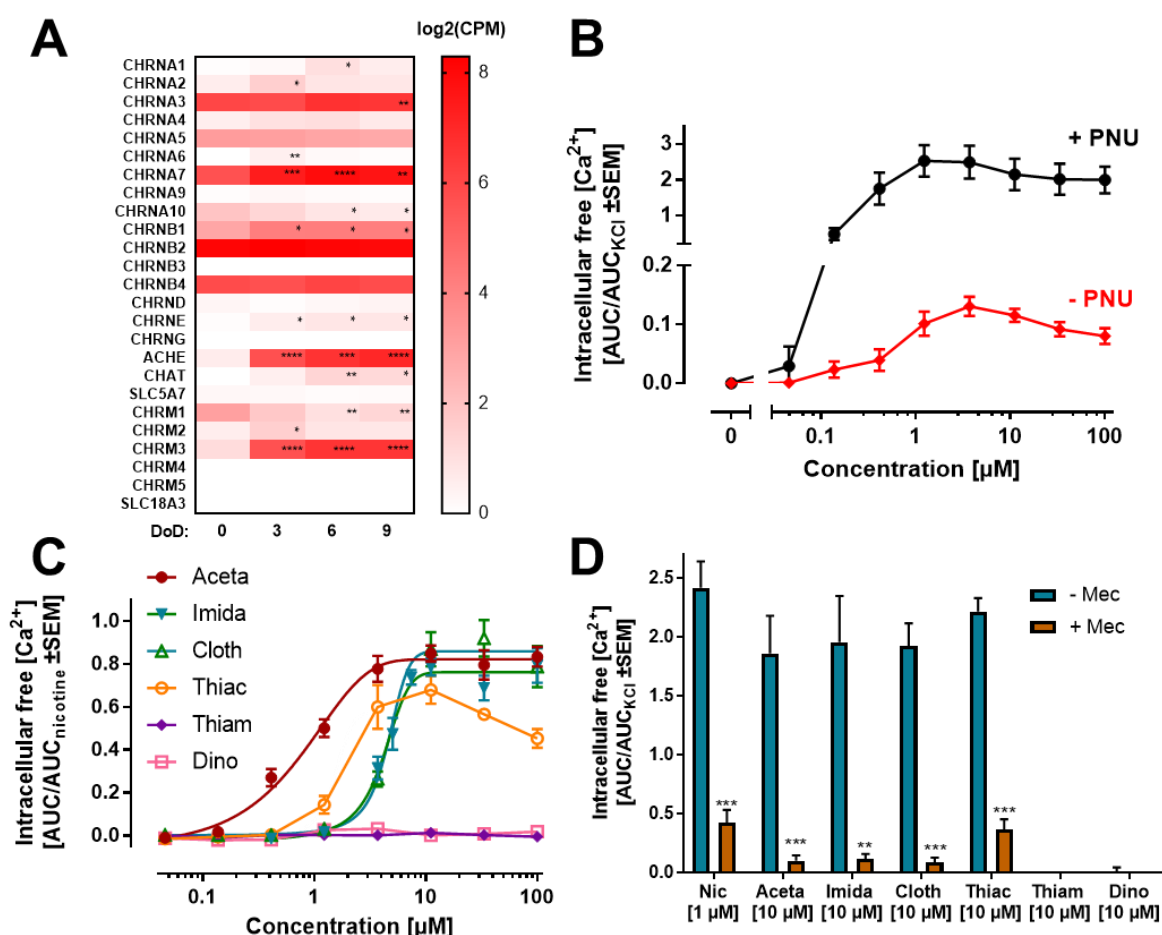


Fig. MS2_7 Nicotine signaling in SH-SY5Y cells.

a The expression of genes coding for nAChR subunits was determined by whole-transcriptome RNA-sequencing during differentiation of SH-SY5Y cells. The raw counts were normalized to counts per million total counts (CPM) and log2-transformed. Significance of changes between day of differentiation (DoD) zero and DoD3-9 was evaluated by ANOVA with Dunnett's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. **b** The SH-SY5Y cells were differentiated for 72 h, and used on DoD3 for Ca^{2+} -imaging experiments. The increase of the $[\text{Ca}^{2+}]_i$ triggered by nicotine was measured in the presence or absence of 10 μ M PNU. The responses were evaluated as the area under the curve (AUC) of the increased fluorescence of the

calcium-sensitive dye Fura-2 for 0–150 s after compound addition. An example of an original recording is shown in figure MS2_S5C. The responses were normalized to the $[Ca^{2+}]_i$ response of SH-SY5Y cells after depolarization with 30 mM KCl (AUC_{KCl}) ($n = 4-6$). **c** The $[Ca^{2+}]_i$ response of SH-SY5Y cells triggered by Aceta, Imida, Cloth, Thiac, Thiam and Dino was measured in the presence of 10 μ M PNU. The AUC of the response (0–150 s) was normalized to the AUC of the response evoked by the treatment of the cells with 11 μ M nicotine ($AUC_{nicotine}$) ($n = 3$). The estimated pEC_{50} values were 6.10 ± 0.07 , 5.38 ± 0.03 , 5.33 ± 0.01 and 5.73 ± 0.01 for Aceta, Imida, Cloth and Thiac, respectively. **d** The $[Ca^{2+}]_i$ responses of SH-SY5Y cells triggered by nicotine, Aceta, Imida, Cloth, Thiac, Thiam and Dino were measured in the presence of 10 μ M PNU, and the presence or absence of Mec (125 μ M). The AUC of the responses was normalized to the AUC_{KCl} ($n = 3-5$). Significance was evaluated by multiple t tests. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

As specificity control for the above-described experiments, we checked whether the responses of nicotine and the four active neonicotinoids were blocked by Mec. The elimination of $[Ca^{2+}]_i$ responses under this condition confirms nAChRs as mediators (Fig. MS2_7d). A similar control experiment was also performed in LUHMES cultures. The responses of all four active neonicotinoids, as well as AR-R, were blocked in the presence of PNU by nAChR antagonism (Fig. MS2_6d–f). Thus, the data from two different experimental systems showed that a subgroup of neonicotinoids triggered Ca^{2+} signaling in human neurons.

Molecular docking studies in support of observed functional effects

As there was a clear subgrouping of neonicotinoids concerning their functional effect on neurons, we explored whether structural commonalities/differences would support such activity differences. In a first step, some physicochemical parameters relevant to receptor interaction were compared. It was conspicuous that Dino is clearly the most hydrophilic compound of the set and that Thiam has a particularly high polar surface area (Fig. MS2_S6A). These extreme features may contribute to the fact that the two compounds behaved differently than the other four neonicotinoids, but the explanatory value of these data is quite limited.

Therefore, we took a more comprehensive approach to identify a possibly differential interaction of the compounds with the receptor. For this purpose, receptor models were established for the $\alpha 7$ nAChR and the $\alpha 4\beta 2$ nAChR, and molecular docking was performed for all six compounds. The docked poses for each compound were ranked and clustered according to their protein–ligand interaction fingerprints. Then, the highest populated clusters were analyzed (Fig. MS2_S6B). This revealed that Aceta, Imida, Cloth and Thiac align well in the known nicotine-binding site. The chloropyridine- and chlorothiazol-moieties of the pesticides pointed towards loops D and E from the complementary subunit and the electronegative nitro and cyano groups reaching out to the tip of the loop C (Fig. MS2_S6C). This binding behavior has also been described as “common binding mode”, and it has been described earlier (Tomizawa et al., 2008) for both Imida and nicotine, based on co-crystallization of these ligands with the

homopentameric ACh binding protein complex (AChBP: the most established model for $\alpha 7$ -related structures) (Ihara et al., 2008). Our docking study showed that Thiam almost exclusively exhibited an “inverted” binding mode with the electronegative nitroguanidine group placed close to the area where the other compounds position the chloroheteroaryl substructure, and vice versa (Fig. MS2_S6D). Dino showed a binding mode different from all the other compounds. This is most probably due to the lack of a chloroheteroaryl group (Fig. MS2_S6E). Thus, the molecular docking experiments indicate that the subgrouping might be determined by the predominant orientation of the compounds in the binding site. The set of high-quality (i.e., more likely) docking poses for Aceta, Imida, Cloth, and Thiac contained many solutions that correspond to the so-called common binding mode (similar to nicotine), but some also suggested an inverted binding mode. In the case of Thiam, only the inverted mode was observed. This might explain the low affinity of Thiam, as the inverted mode has been linked to lower binding affinity (Tomizawa et al., 2008). As Dino binding seemed to differ entirely from that of nicotine or the other compounds, a lowered affinity may be the consequence. These observations give a molecular rationale for the observed functional differences. However, it is clear that they will require more detailed follow-up by dynamic docking models, including entropy considerations and binding energy calculations. Nevertheless, the preliminary findings presented here give already some potential explanations and better define future research needs.

Modulation of cholinergic responses by neonicotinoids

An important feature of nAChR signaling is tachyphylaxis (self-inactivation of the receptor during the signaling process). Under such conditions, signaling may stop even in the presence of a ligand, and signaling cannot be repeated within a certain period after an initial stimulation. This complex behavior is also termed desensitization (Arias et al., 2015; Campling et al., 2013; Capelli et al., 2011; Eaton et al., 2014; Fenster et al., 1997; Lester, 2004; Marks et al., 2010; Papke et al., 2011; Paradiso and Steinbach, 2003; Quick and Lester, 2002; Rollema et al., 2010; Rollema and Hurst, 2018). We investigated tachyphylaxis to obtain further evidence for the action of neonicotinoids via the nAChRs. In our experimental setup, the neonicotinoids were added at various concentrations to LUHMES cultures, and thereafter, the $[Ca^{2+}]_i$ responses triggered by nicotine were recorded. In cells pretreated with the neonicotinoids, a strong attenuation of the nicotine signaling was observed (Fig. MS2_8a, b). A quantification of the concentration-dependency of the down-modulation resulted in pIC_{50} values of ~ 5.4 for Aceta, Imida, Cloth and Thiac (Figs. MS2_8c, MS2_S7A). Thiam and Dino did not show a negative modulation on the nicotine-induced response of LUHMES neurons, again

indicating/confirming that they do not interact with nAChRs. Experiments performed in SHSY5Y cells confirmed these results (Fig. MS2_S5E).

Another set of desensitization experiments was performed using the endogenous neurotransmitter ACh as stimulus. The resulting pIC_{50} values for response attenuation were ~ 5.5 for Aceta, Imida, Cloth and Thiac. Thiam and Dino had no effect (Fig. MS2_8d-f). For Imida, a similar desensitizing effect on ACh-evoked responses has been reported for insect nAChRs (Oliveira et al., 2011) earlier, and such tachyphylaxis phenomena are wide-spread and well documented for ionotropic cholinergic receptors in general (Arias et al., 2015; Campling et al., 2013; Capelli et al., 2011; Eaton et al., 2014; Fenster et al., 1997; Lester, 2004; Marks et al., 2010; Papke et al., 2011; Paradiso and Steinbach, 2003; Quick and Lester, 2002; Rollema et al., 2010; Rollema and Hurst, 2018). The different effects of the neonicotinoids Aceta, Imida, Cloth and Thiac in comparison with Thiam and Dino on the nicotine- and ACh-triggered responses are fully consistent with the differences in agonist activity.

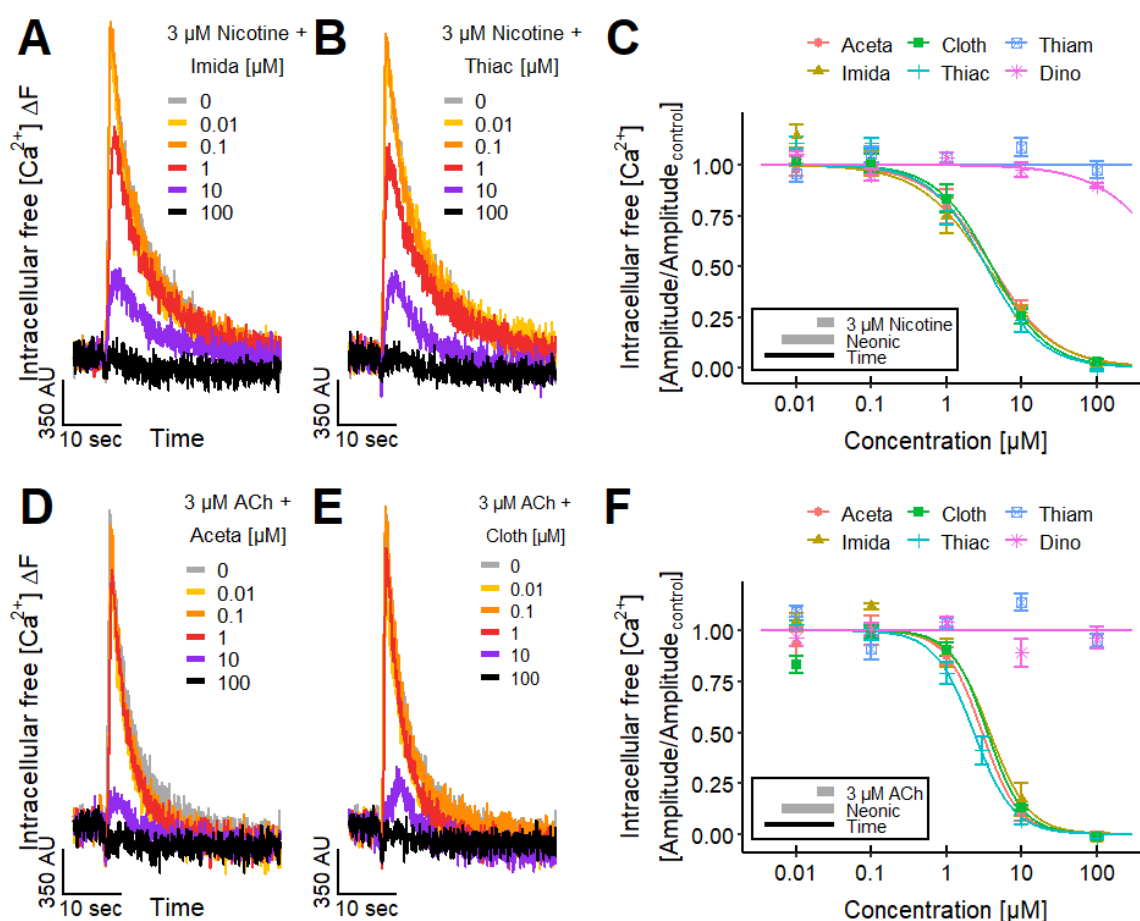


Fig. MS2_8 Effect of neonicotinoids on responses evoked by nicotine and ACh.

a, b Ca^{2+} -imaging traces displaying the responses of LUHMES neurons to the acute exposure to 3 μM nicotine in the presence of the neonicotinoids **a** Imida and **b** Thiac, which were preincubated for 4.5 min. **c** Concentration-dependent effects of the pre-applied neonicotinoids Aceta, Imida, Cloth, Thiac, Thiam and Dino on the nicotine-evoked responses. The resulting pIC_{50} values were

5.40 ± 0.08, 5.47 ± 0.10, 5.41 ± 0.07 and 5.48 ± 0.08 for Aceta, Imida, Cloth and Thiac, respectively. Thiam and Dino did not show an effect. **d**, **e** Ca²⁺-imaging traces showing the effects of the neonicotinoids **d** Imida and **e** Thiac, which were preincubated for 4.5 min, on the signals evoked by the application of 3 µM ACh. **f** Effects of the pre-applied neonicotinoids Aceta, Imida, Cloth, Thiac, Thiam and Dino on the response of LUHMES neurons triggered by 3 µM ACh. The pIC₅₀ values were 5.53 ± 0.09, 5.43 ± 0.08, 5.46 ± 0.07 and 5.64 ± 0.04 for Aceta, Imida, Cloth and Thiac, respectively. Thiam and Dino had no effect. Note the treatment schemes (lower left corner), illustrating the experimental design. Detailed data on n numbers are found in table MS2_S6

We also explored the issue of a potential cross-tolerance with other receptor systems. This might occur by, e.g., affecting intracellular processes related to Ca²⁺-signaling. We tested the impact of neonicotinoid pretreatment on signaling via the ionotropic purinergic receptor subtype P2X3, which has been shown to be functionally expressed in LUHMES (Loser et al., 2021b). The response evoked by the agonist α,β -meATP (Bianchi et al., 1999; Khakh and North, 2012; Loser et al., 2021b) was not affected by the pre-applied neonicotinoids or nicotine (Fig. MS2_S7B). This suggests that the tolerance mediated by the neonicotinoids for nicotine is triggered directly on the level of nAChRs.

The desensitizing activity of an agonist occurs typically at concentrations that can activate the receptor, as tachyphylaxis is a typical consequence of receptor activation. However, it is also possible that low concentrations, not sufficient to trigger significant receptor activation may desensitize a receptor. In the latter case, a ligand can have a higher potency for desensitization than for activation of the nAChR (Arias et al., 2015; Capelli et al., 2011; Fenster et al., 1997; Lester, 2004; Paradiso and Steinbach, 2003; Rollema et al., 2010; Rollema and Hurst, 2018). Here, we observed indeed a left-shift of potency in the concentration–response relationship. We determined the lowest active concentration, assuming a benchmark response of 10% (BMC₁₀). These BMC₁₀ values were obtained from the concentration–response curves using a log-logistic model. They ranged between 0.37 and 0.62 µM for the four active compounds. The upper end of their 95% confidence interval (the BMCU₁₀), i.e., concentrations having a high likelihood to trigger a biological effect, were at 0.87–3.6 µM (Table MS2_S9). These experiments confirm that effects of Imida and related compounds on human neuronal nAChRs are likely to occur in the low µM range.

For confirmation of the desensitizing effect of the neonicotinoids on nAChRs on single cells, we performed Ca²⁺-imaging experiments on a microscope stage. High or low concentrations of Aceta (10 µM or 100 µM) were applied prior to a nicotine (10 µM) stimulus. The voltage-gated Na⁺ channel modulator veratridine (VTD) was used as a positive control at the end of the stimulation series, to verify unaltered overall cell excitability and to exclude possible unspecific effects of Aceta on the electrical activity of the cells. Aceta (100 µM) significantly reduced the percentage of responding cells to

the ensuing addition of nicotine, whereas the response to VTD was not affected (Fig. MS2_S8). These observations support our previous results from high-throughput imaging (Figs. MS2_8C, MS2_S7A).

Tolerance triggered by neonicotinoids against the selective agonist ABT 594

In a final set of experiments, we asked whether the desensitizing effect of the neonicotinoids necessarily involved $\alpha 7$ nAChR. Therefore, cell pretreated with the neonicotinoids were exposed to the non- $\alpha 7$ nAChR agonist ABT 594. The pIC_{50} values for response attenuation were ~ 5.1 for Aceta, Imida, Cloth and Thiac (Fig. MS2_9a-c). When given at a concentration of 100 μM , the compounds blocked the response induced by 30 nM ABT 594 completely. As seen in the previous experiments, Thiam and Dino did not show any modulatory effect. These results further confirm our previous findings, as the pIC_{50} values were in a similar range as the values obtain for stimulations with nicotine and ACh (Fig. MS2_8).

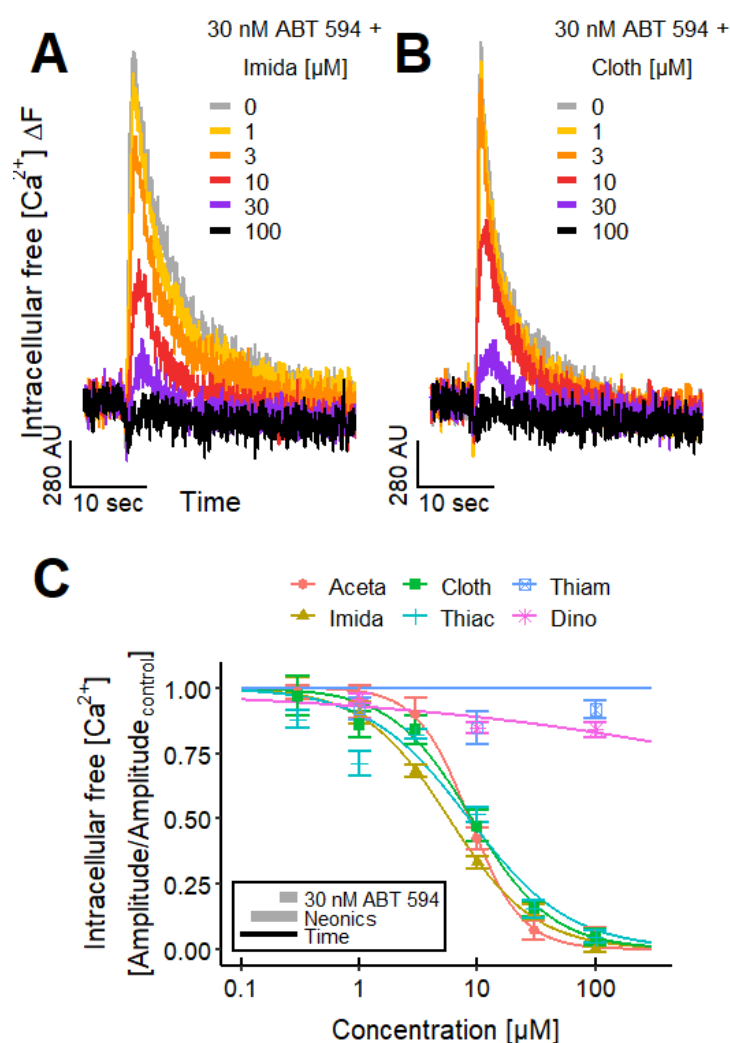


Fig. MS2_9 Effect of neonicotinoids on responses evoked by ABT 594.

a, b Ca^{2+} -imaging traces of the effects of the neonicotinoids **a** Imida and **b** Cloth, which were preincubated for 4.5 min, on the responses stimulated by the acute exposure to 30 nM ABT 594. **c** The concentration-dependent effects of the pre-applied neonicotinoids Aceta, Imida, Cloth, Thiac, Thiam and Dino on the ABT 594-induced response resulted in pIC_{50} values of 5.07 ± 0.03 , 5.24 ± 0.03 , 5.05 ± 0.05 and 5.08 ± 0.06 for Aceta, Imida, Cloth and Thiac, respectively. No pIC_{50} values could be determined for Thiam and Dino. Note the treatment scheme (lower left corner), illustrating the experimental design. Detailed data on n numbers are found in table MS2_S6

Exposure considerations and in vitro-to-in vivo comparisons

When exposure to neonicotinoids is considered, at least three scenarios need to be distinguished (Cimino et al., 2017): intentional exposure, occupational (non-intentional) exposure, normal dietary exposure. Several studies demonstrate a wide-spread use of the compounds for suicidal attempts, and under such conditions, several grams of active pesticide are ingested (Mohamed et al., 2009). While occupational exposure is usually low under most conditions in Europe due to restricted use (closed processes for seed treatment implemented), there are reports on hundreds of symptomatic cases (Marfo et al., 2015). For the general population, acute reference doses (ARfD) have been set by EFSA to, e.g., 25 $\mu\text{g/kg/day}$ for Aceta and 80 $\mu\text{g/kg/day}$ for Imida. Monitoring studies (e.g., in 2018 in Europe (European Food Safety Authority (EFSA) et al., 2020)) found for Aceta a maximal acute exposure of 57 $\mu\text{g/kg/day}$. Thus, assuming that it is realistic that the ARfD can be exceeded twice, a realistic maximal exposure on a single day may be 114 $\mu\text{g/kg}$ (Aceta) to 160 $\mu\text{g/kg}$ (Imida). In rodent studies, the NOAELs for chronic endpoints was 7–15 mg/kg for Aceta and this point-of-departure was used also for setting the ARfD due to uncertainties/limitations related to the regulatory developmental neurotoxicity study (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013).

The internal exposure data are relatively limited. For mice, treated with NOAEL levels of Imida and Aceta (10 mg/kg), the brain levels were around 3–6 ppm, and plasma concentrations were in a similar range (15–30 μM) (Ford and Casida, 2006). Human data are mostly available for urinary metabolites used for biomonitoring, and the concentrations were maximally in the low nM range (Li et al., 2020; Li and Kannan, 2020; Zhang et al., 2019). Due to the lack of more direct data, we built physiology-based toxicokinetic (PBTK) models to predict plasma and brain concentrations (Table MS2_S11). An exposure to Imida (0.16 mg/kg; corresponding to a maximally expected level in the normal population; see above) was predicted to lead to plasma concentrations in the 0.8–1.6 μM range and to brain concentrations of 0.5–1.2 μM (Fig. MS2_S9). Reverse modelling showed that a brain concentration of 2 μM Imida (considered a point-of-departure from our in vitro studies) would be reached after an intake of 0.2 mg/kg body weight in the average population. The in vitro–in vivo

comparisons yield plausible data, in the sense that normal population exposure would normally yield sub- μM concentrations, and that such concentrations would not be sufficient to trigger nAChRs. Vice versa, an uptake exceeding the ARfD is predicted to lead to concentrations that may be sufficient to affect nAChR signaling.

3.2.5 Conclusion and outlook

In the present study, we investigated the functional expression and impact of nAChRs on $[\text{Ca}^{2+}]_i$ in two well-established model systems for dopaminergic human neurons. Based on this characterization, we asked whether the most widely used neonicotinoids (Bass et al., 2015; Jeschke et al., 2011) affected human nAChRs, in addition to their known action on insect receptors (Brown et al., 2006; Tan et al., 2007). We provided here compelling evidence for the triggering of cholinergic signal transduction by the neonicotinoid pesticides Aceta, Imida, Cloth and Thiac. Thus, our study is in line with earlier findings obtained with other mammalian cells, i.e., primary rat brain cultures (Kimura-Kuroda et al., 2012), and it provides clear evidence for a potential human hazard of such compounds.

Many subtypes of nAChRs are expressed in dopaminergic neurons, where they modulate the electrical activity and the release of dopamine (de Kloet et al., 2015; Grady et al., 1992; Mameli-Engvall et al., 2006; Quik and Kulak, 2002; Quik and Wonnacott, 2011; Rapier et al., 1988). As the nicotinic signaling thereby affects the functioning, plasticity and development of the dopaminergic nervous system, it is of crucial importance to learn about a potential effect of neonicotinoids in neurons and to determine the risk potential for the dopaminergic system or other important circuits of the brain (Lozada et al., 2012; Miwa et al., 2011; Romoli et al., 2019; Slotkin et al., 2006; Stevens et al., 2003; Welsby et al., 2006; Wheeler and Cooper, 2004; Ziviani et al., 2011). While our study provides unambiguous evidence for the agonist role of some neonicotinoids on human nAChRs, follow-up studies will be required to judge the full toxicological implication of our findings. We provide here some initial conclusions and highlight important gaps of knowledge to be addressed.

Concerning the toxicant–target interaction (also termed molecular initiating event, using the terminology of adverse outcome pathways), two findings are remarkable. First, it is clear from a broad range of data provided, that various subtypes of nAChR may be activated by neonicotinoids. We provide here evidence for a role of both $\alpha 7$ and non- $\alpha 7$ receptors. This is consistent with binding experiments and functional studies based on $^{86}\text{Rb}^+$ -flux, all of which demonstrated the interaction of, e.g., Imida or Aceta with various receptor types (Tomizawa and Casida, 2005, 1999). Notably, the available literature

data on the potencies of Imida and Aceta have a wide spread (0.7–320 μM , depending on the assessment method). Our data on a physiological signaling response (change of $[\text{Ca}^{2+}]_i$) provide important information on toxicologically important concentration ranges. Our data on direct stimulation of naive cells by neonicotinoids suggest that the lowest concentrations eliciting a neuronal response are in the 10 μM range. However, it needs to be considered that $[\text{Ca}^{2+}]_i$ signaling effects in our model systems may be masked by extremely rapid receptor desensitization. Prevention of tachyphylaxis, combined with allosteric enhancement by the modulator PNU, allowed Aceta, Imida, Cloth and Thiac to be detected in SH-SY5Y cells at concentrations of $\geq 1 \mu\text{M}$. This potency range is fully consistent with data from a desensitization assay in LUHMES cells, where the functional consequence of neonicotinoid exposure on subsequent cholinergic signaling was investigated. In addition, in this setup, compound activity started to be detected in the very low micromolar range for all four active compounds. Notably, the desensitizing aspect may be equally problematic for neurodevelopment as a potential overstimulation. To use such data for in vitro-to-in vivo extrapolations and as point-of-departure for risk assessment, the corresponding free toxicant concentrations need to be known: biokinetics calculations applied to our cell models showed that the free drug concentration was very close (mostly $< 1\%$ deviation) to the nominal concentration (Table MS2_S10).

The second important aspect of target interaction is our observation of two distinct subgroups of neonicotinoids with respect to neuronal signaling. While four test compounds showed relatively similar effects and effect potencies, two other agents (Thiam, Dino) were inactive within the test range (i.e., at least $100 \times$ less potent). The activity differences correlated well with favored docking poses of the ligands in a molecular model of the nAChR. While this will need further elucidation on the level of receptor binding and other molecular pharmacology approaches, this clear subgrouping suggests a high specificity of the LUHMES assay.

Whether the signaling disturbances measured here have lasting neurofunctional effects needs to be studied further. However, many studies on other nicotinic agonists (including nicotine) suggest that compounds triggering nAChR will affect the nervous system function not only acutely, but also affect its plasticity and development (Lozada et al., 2012; Romoli et al., 2019; Slotkin et al., 2006; Wheeler and Cooper, 2004; Ziviani et al., 2011). One study in rat neurons also shows that Aceta/Imida may alter the gene expression of neurons upon prolonged exposure (Kimura-Kuroda et al., 2016). Concerning human data, four studies on chronic effects of neonicotinoids have been reported (Cimino et al., 2017). In all of them, some significant general developmental/neurological effects were observed when the population was stratified

according to urine biomarkers, proximity to agricultural production or to use of neonicotinoid-containing anti-tick sprays.

A more difficult question is whether neonicotinoids have the potential to affect the development of the fetal brain (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013) and thus trigger developmental neurotoxicity (DNT). Many other compounds that trigger alterations in neurotransmitter signaling, but no overt structural defects have been shown to trigger DNT. They include for instance MDMA, heroine (Aschner et al., 2017) and nicotine itself (Dwyer et al., 2009; Slikker Jr et al., 2005; Slotkin et al., 2016). Moreover, compounds that hardly affect the adult brain (in typical exposure situations) have been shown to affect the developing brain with late life consequences of fetal exposure. Well-documented examples are methylmercury and lead (Grandjean and Landrigan, 2014). These examples from other compounds make it plausible that neonicotinoids pose a DNT hazard, but direct evidence is quite limited at present. As is good practice in toxicology, each individual compound needs to be evaluated for its proper effect/hazard. A transfer of knowledge and conclusions from one compound (e.g., nicotine) to others (neonicotinoids) always bears uncertainties (Rovida et al., 2020). More definite data on individual neonicotinoids are required in the future to confirm or disprove the DNT alert triggered by our study.

For a full risk assessment, some additional aspects need to be addressed. First, the molecular structure–activity relationship will require new technical approaches. Receptors with defined subunit stoichiometry may be expressed in *Xenopus laevis* oocytes to clearly identify the molecular targets of active neonicotinoids and to verify that the inactive ones do not interact with any of the receptors. Second, metabolism and metabolites will require additional attention. In general, neonicotinoids have relatively long half-lives, and we have provided data on the direct activity of parent compounds. However, there may be also some active metabolites. Several studies showed that the Imida metabolite desnitro-imidacloprid exhibits higher potency at inhibiting the binding of [³H]nicotine or [¹²⁵I]α-BGT to mammalian nAChRs compared to its precursor Imida, but similar to nicotine (Chao and Casida, 1997; D'Amour and Casida, 1999; Tomizawa et al., 2000; Tomizawa and Casida, 1999). The same finding has also been reported for chicken α4β2 nAChR expressed in M10 cells (Tomizawa and Casida, 2000). Thus, generation of toxicokinetics data on this metabolite together with bioactivity measurements on human neurons will be interesting. Third, PBTK models for all compounds will need to be applied to different exposure scenarios. A model validation with human data would be desirable to show reliability of the data and predictions from these models. Previous attempts into this direction have used microdosing in volunteers with deuterium-labeled compounds (Harada et al., 2016). Concerning the question of neurological consequences, controlled short-term experiments with volunteers have

been performed to study effects of nicotine (Grundey et al., 2018). Whether such studies would be ethically acceptable for pesticides is doubtful for most countries in Europe.

3.2.6 Acknowledgments

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3.2.7 Conflict of interest

The authors declare no conflict of interest.

3.2.8 Author contributions

DL: conceived or designed the study, performed the research, analyzed the data and wrote the paper. MH, JS, YJ, and IS: performed the research and analyzed the data. JB and MB: performed the research, analyzed the data and wrote the paper. TD, CM, IG, KG, and GE: contributed new methods or models and analyzed the data. SB, AF, ML, and UK: conceived or designed the study and wrote the paper.

3.2.9 Supplementary information

The online version contains supplementary material available at <https://doi.org/10.1007/s00204-021-03031-1>.

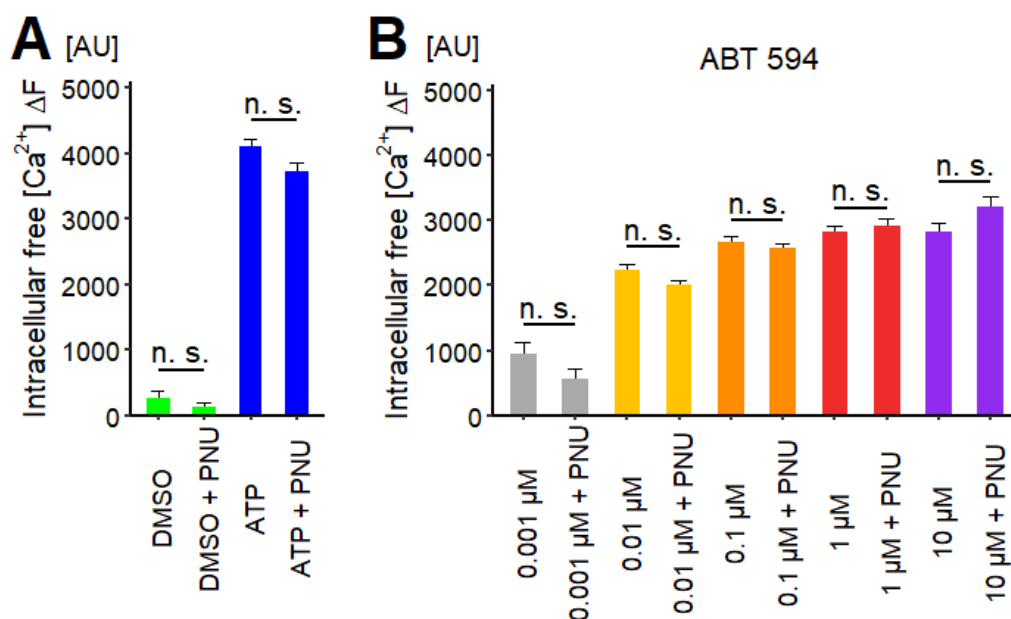


Fig. MS2_S2: Effects of PNU-120596 on LUHMES neurons.

(A-B) Ca^{2+} -imaging experiments to examine the effects of PNU-120596 (PNU), a selective positive allosteric modulator of $\alpha 7$ nAChR, on LUHMES neurons. The cells were pretreated with 10 μM PNU for 4.5 min. (A) Effect of the absence and presence of 10 μM PNU on the responses induced by the application of 0.1% DMSO (DMSO, $n \geq 5$), as vehicle control, and 1 μM α, β -meATP (ATP, $n = 6$), a selective P2X receptor agonist. (B) Influence of the absence and presence of 10 μM PNU on the responses evoked by the addition of ABT 594 ($n = 6$), a neuronal non- $\alpha 7$ nAChR agonist. Statistical significance was determined between recordings without and with PNU present (n. s., not significant).

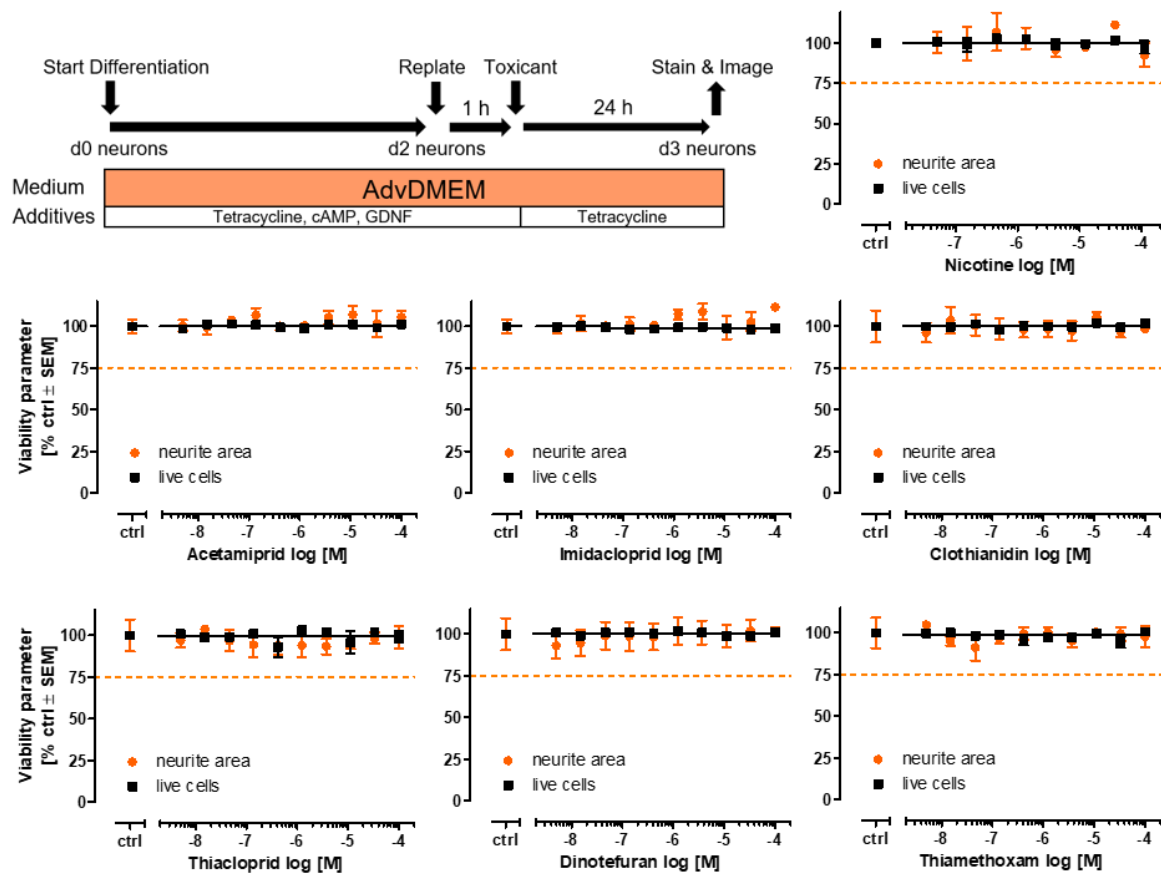


Fig. MS2_S3: Impact of neonicotinoids on neurite outgrowth of LUHMES neurons.

LUHMES cells were differentiated for two days. On day 2 of differentiation (d2 neurons) cells were replated and after 1 h of attachment, cells were treated for 24 h with nicotine and different neonicotinoids (5 nM - 100 μ M). High content imaging was performed and live cells (black) and neurite area (orange) were assessed in parallel. All data are means $\pm$ SEM from at least two biological replicates. Dashed line indicates 75% of control.

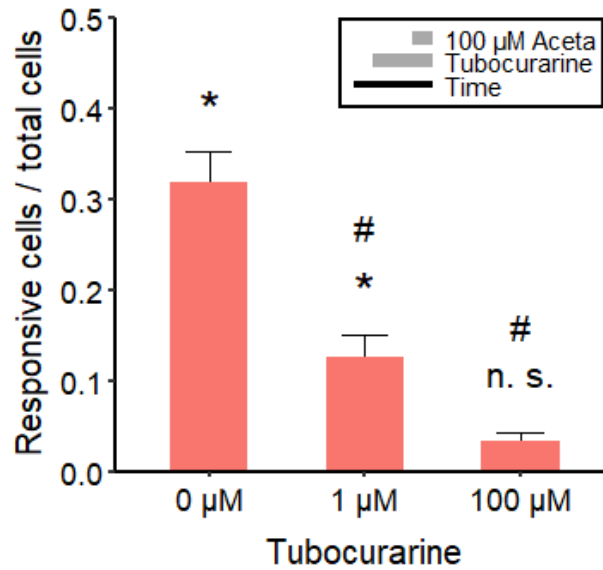


Fig. MS2_S4: Inhibition of acetaminophen (Aceta) triggered responses of LUHMES neurons by tubocurarine.

Percentage of cells that responded in single-cell Ca^{2+} -imaging to the application of 100 μM Aceta in the absence and presence of the nAChR antagonist tubocurarine. Statistical significance was determined against negative control recordings (*, significant; n. s., not significant) and against the control recordings with 100 μM Aceta in the absence of tubocurarine (0 μM) (#, significant). Note the treatment scheme (upper right corner), illustrating the experimental design and the enlarged y-axis. Detailed data on n numbers and percentages of responsive cells are found in table MS2_S7 & MS2_S8, respectively.

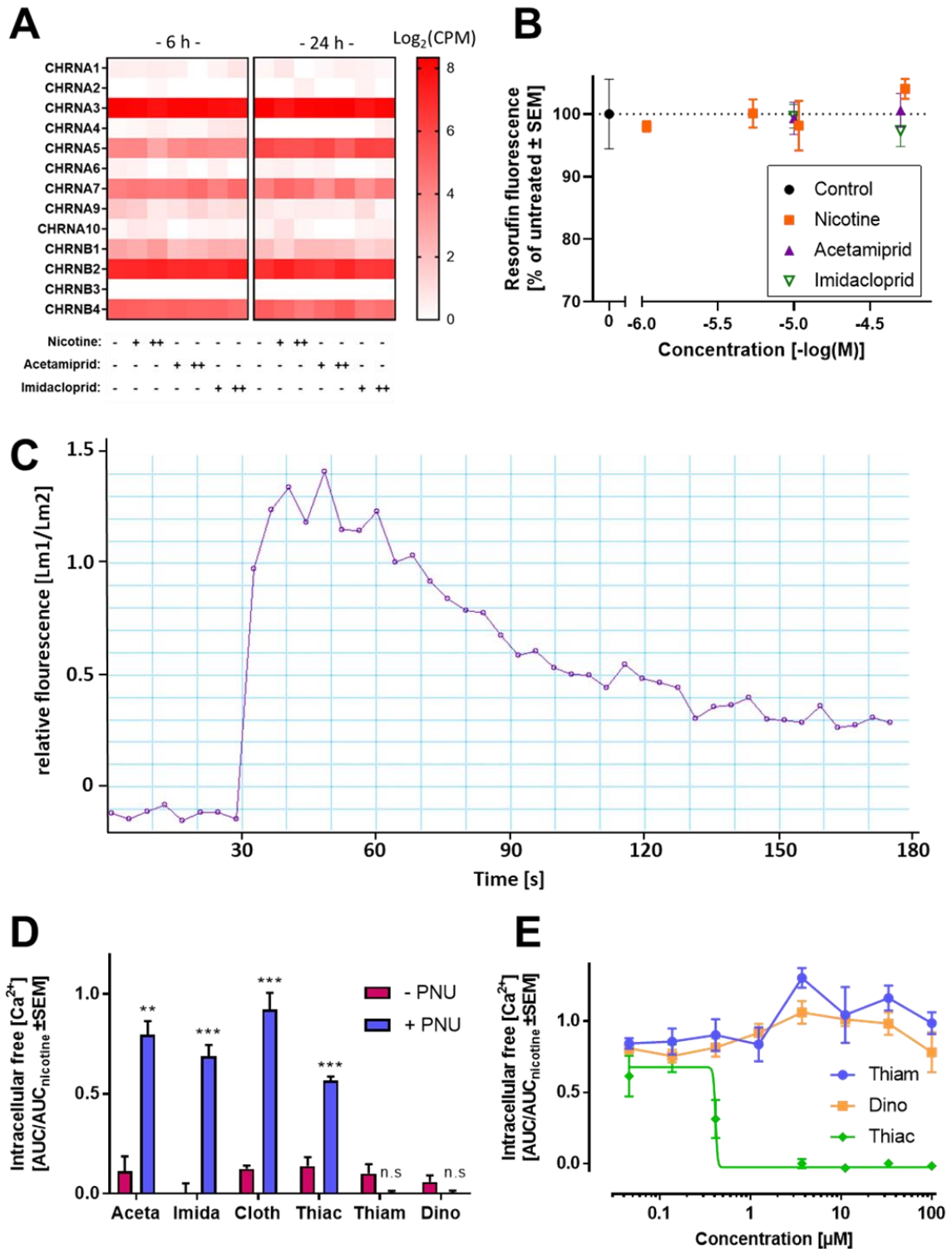


Fig. MS2_S5: Effects of neonicotinoids on SH-SY5Y cells.

(A) SH-SY5Y cells were treated with nicotine, acetamiprid, or imidacloprid on DoD3. After 6 and 24 h, samples for TempO-Seq analysis were taken. Counts were normalized to total counts per million and log2 transformed. - $\triangleq$ not present, Nicotine: + $\triangleq$ 1 μ M ++ $\triangleq$ 5 μ M, acetamiprid/imidacloprid: + $\triangleq$ 10 μ M ++ $\triangleq$ 50 μ M. Data represent mean of ≥ 4 replicates from 2 experimental occasions. No significant differences between treatment and control were observed when $p < 0.05$. (B) Cell viability of SH-SY5Y cells was determined by a resazurin assay. Cells were treated on DoD3 for 24 h. Resorufin fluorescence was normalized to untreated controls. (C)

Example curve of the increased Fura-2 fluorescence after $[Ca^{2+}]_i$ response of SH-SY5Y triggered by 30 mM KCl. Cells were treated on DoD3. (D) Effects of neonicotinoids (33.3 μ M) in absence and presence of 10 μ M PNU. The responses to Aceta, Imida, Cloth and Thiac were strongly increased. Significance was evaluated by multiple t-tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (E) The $[Ca^{2+}]_i$ responses triggered by the acute exposure to nicotine (11.1 μ M) was negatively modulated by pre-applied Thiac. The pre-exposure to Thiam and Dino did not modulate the nicotine-evoked response.

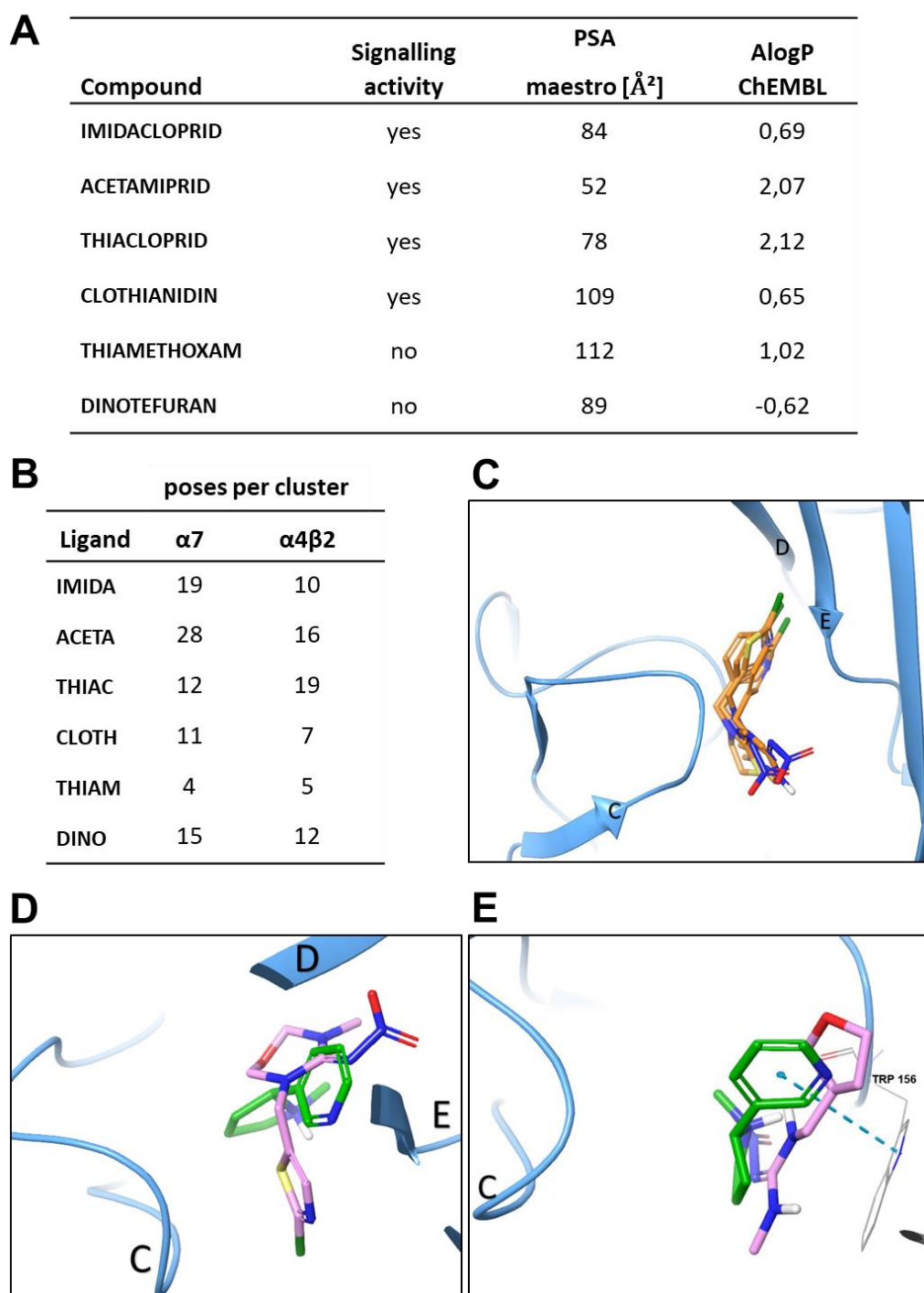


Fig. MS2_S6: Physicochemical properties and molecular docking studies.

(A) The octanol:water distribution coefficient (given as AlogP) and polar surface area (PSA) were calculated as mentioned in the methods section. In contrast to the other compounds, dinotefuran reported a negative AlogP value (i.e. higher hydrophilicity than the other compounds). The structurally similar compounds thiamethoxam and clothianidin had the highest values for PSA. (B) Up to 80 docked poses per ligand and per nAChR isoform were generated during the induced fit docking procedure. The poses were subsequently clustered according to the interaction-fingerprint of the docking-output. For each isoform, the highly populated clusters were analyzed further, and exemplary docking poses are displayed in C-E. (C) The neonicotinoids were docked to the $\alpha 7$ nAChR and to the $\alpha 4\beta 2$ receptor at the $\alpha 4\beta 2$ interface and at the $\alpha 4\alpha 4$ -interface. The representative poses of imidacloprid, thiachloprid, acetamiprid and clothianidin in the binding site

of the $\alpha 4\alpha 4$ -interface are superimposed. The poses looked essentially similar in the other modelled receptor binding sites; for better view only one ribbon from the docked complexes is shown in blue. For spatial orientation in Figures C-E: the loops (and associated beta-sheet regions) A-C belong to the principal (+) binding subunit (e.g. alpha-4), while structures labelled D-F belong to the complementary (-) subunit (e.g. beta-2). Ligands: Chlorine-atoms are depicted in green, nitrogen-atoms in blue, sulfur-atoms in yellow, carbon-atoms in orange and nitrogen-atoms in blue. Chloroheteroaryl-moieties of the ligands are aligning well and are pointing towards loop D and E from the complementary subunit while the electronegative nitro- or cyano-functionalities are pointing towards the tip of the loop C of the principal chain. This binding orientation is outlining the “common” binding mode, which is also reported in co-crystallized homologous ACh-binding protein (AChBP)-complexes. **(D)** Thiamethoxam (pink structure) exhibits an “inverted” binding mode with the nitroguanidine-group being placed reversed with respect to the heteroaryl substructure, which is pointing towards the tip of loop C in this binding conformation. Nicotine (green structure) has been docked as reference compound into the homo-pentameric $\alpha 7$ -structure and shows a “common” nicotinoid binding mode, with the amino-feature pointing away from loop C. **(E)** The $\alpha 4(+)$ - $\beta 2(-)$ -interface is shown: Dinotefuran (pink structure) accommodates a distinct binding mode compared to the other compounds. The reference compound nicotine (green structure) is shown as it was actually found in the co-crystallized complex. In this common binding mode it undergoes stabilizing pi-stacking-interactions with a Tryptophan (Trp156) of the B-loop of the binding site. Such an interaction was found here to be impossible for dinotefuran (lack of the heteroaryl moiety; altered orientation).

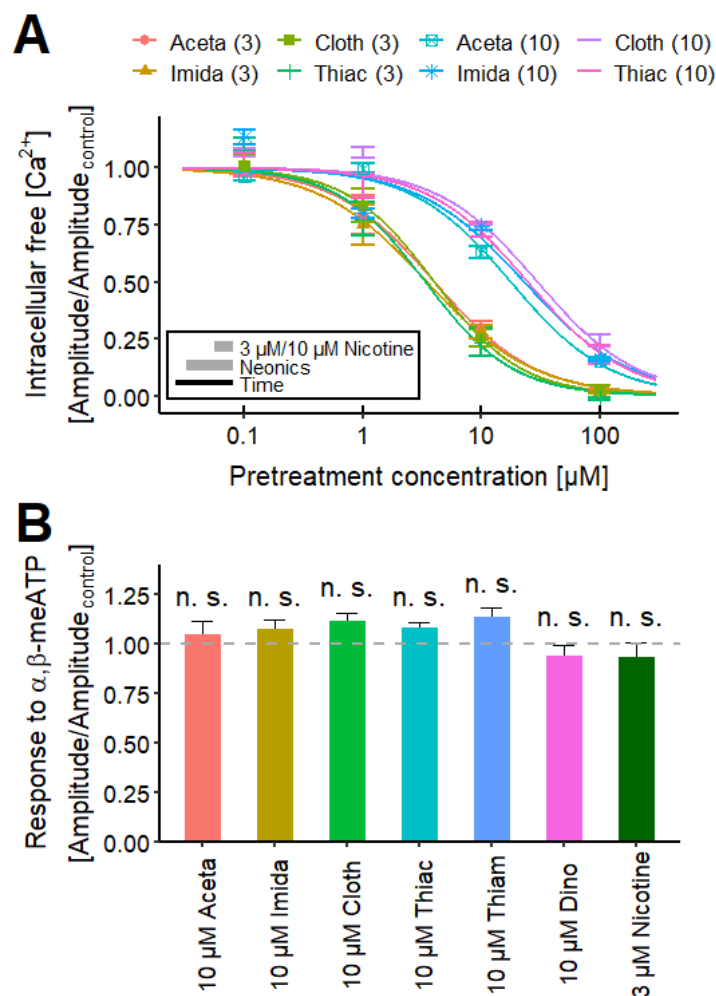


Fig. MS2_S7: Effects of neonicotinoids on responses of LUHMES to selective agonists.

(A) The concentration-response curves present the effects of the neonicotinoids Aceta, Imida, Cloth and Thiac, which were preincubated for 4.5 min, on the response of LUHMES neurons to the addition of 3 μM (3) and 10 μM (10) nicotine. The resulting pIC_{50} values are ~ 5.4 for the effects of Aceta, Imida, Thiac and Cloth on the response evoked by 3 μM nicotine, as described in fig. MS2_8C. The pIC_{50} s for the experiment with 10 μM nicotine are ~ 4.6 for Aceta, Imida, Cloth and Thiac. Detailed data on pIC_{50} values and n numbers are found in table MS2_S5 & MS2_S6, respectively. The impact of the four neonicotinoids was significantly different between the recordings with 3 μM and 10 μM nicotine. Note the treatment scheme (lower left corner), illustrating the experimental design. (B) Investigation of unspecific effects of neonicotinoids and nicotine on the responses evoked by 0.1 μM α, β -meATP, a P2X receptor agonist, using Ca^{2+} -imaging. The neonicotinoids Aceta (n = 5), Imida (n = 5), Cloth (n = 5), Thiac (n = 5), Thiam (n = 6) and Dino (n = 6) and nicotine (n = 7) were preincubated for 4.5 min prior to the application of α, β -meATP. Statistical significance was determined against control recordings (n = 6; n. s., not significant).

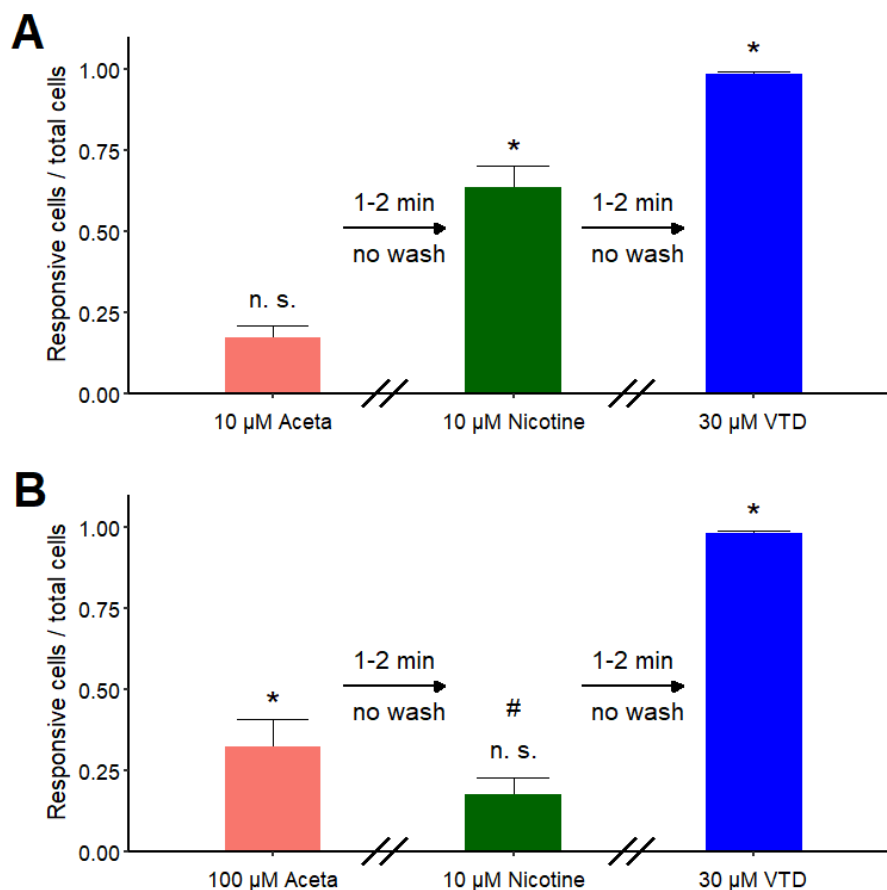


Fig. MS2_S8: Effects of acetamiprid (Aceta) on Ca^{2+} -signaling of individual LUHMES neurons.

Percentage of responsive cells during the serial application of (A) 10 μ M or (B) 100 μ M Aceta, followed by the addition of 10 μ M nicotine and 30 μ M veratridine (VTD) in single-cell Ca^{2+} -imaging. Compounds were incubated for 1-2 min, before the next application. The Na_v channel modulator VTD was used as a positive control. Statistical significance was determined against negative control recordings (*, significant; n. s., not significant) and between the values of 10 μ M nicotine (#, significant). Detailed data on n numbers and percentages of responsive cells are found in table MS2_S7 & MS2_S8, respectively.

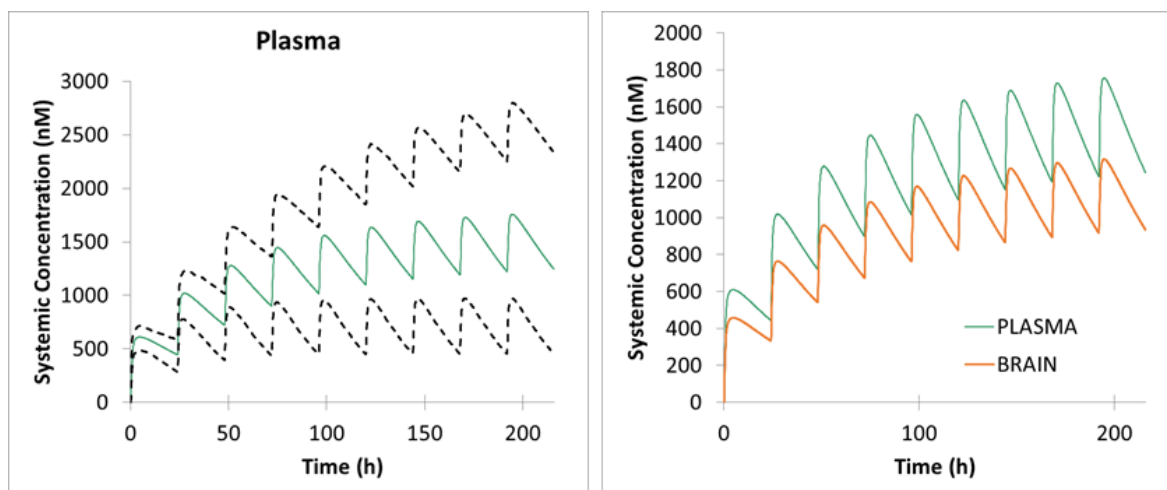


Fig. MS2_S9: Physiologically-based toxicokinetic modelling of imidacloprid concentrations in the human population.

A physiology-based toxicokinetic model (PBTk model) was established in the Simcyp Simulator V19 (Certara, Sheffield, UK), using a previously published approach (Albrecht et al., 2019). The input parameters for imidacloprid are given in table MS2_S11. The systemic exposure following an oral dose of 0.16 mg/kg, given every 24 h intervals to 100 individuals (50% female) with randomly assigned phenotypic and genotypic properties typical for a Caucasian Northern European population are shown. In the panel to the left, the predicted mean plasma concentrations (green line) are shown with the 5th and 95th percentiles of the population represented as dashed lines. In the panel to the right, mean plasma and brain concentrations are compared. A description of the full model building and model application for all compounds will be reported elsewhere, as this would exceed the scope of the current study.

Table MS2_S1. Compound list.

Compound	Supplier	Order number	Function	Reference
α,β -Methyleneadenosine 5'-triphosphate trisodium salt (α,β -meATP)	Tocris	3209	P2 receptor agonist	Bianchi et al. 1999
ABT 594	Sigma	Q53646	neuronal non- $\alpha 7$ nACh receptor agonist	Donnelly-Roberts et al. 1998; Michelmore et al. 2002
Acetamiprid	Sigma Aldrich	33674	Neonicotinoid	
Acetylcholine chloride (ACh)	Sigma Aldrich	A6625	Endogenous neurotransmitter	
Advanced DMEM/F12	Gibco	12634010		
AR-R 17779	Sigma Aldrich	SML2049	$\alpha 7$ nACh receptor agonist	Mullen et al. 2000; Michelmore et al. 2002; Papke et al. 2004
Boric acid	Sigma Aldrich	B7660		
Cal-520 AM	Biomol	ABD-21130		
Clothianidin	Sigma Aldrich	33589	Neonicotinoid	
(-)-Cytisine	Sigma Aldrich	C2899	non-selective nACh receptor agonist	Capelli et al. 2011
Dibutyl cyclic-AMP sodium salt (cAMP)	Sigma Aldrich	D0627		
Dinotefuran	Sigma Aldrich	32499	Neonicotinoid	
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D8418		
Recombinant human FGF basic (FGF-2)	R&D Systems	4114-TC		
Fibronectin	Sigma Aldrich	F1141		
Recombinant human GDNF protein (GDNF)	R&D Systems	212-GD		
L-glutamine solution	Sigma Aldrich	G7513		
Imidacloprid	Sigma Aldrich	37894	Neonicotinoid	
Mecamylamine	Sigma Aldrich	M9020	nACh receptor antagonist	Papke et al. 2008; Capelli et al. 2011
Methyllycaconitine	Sigma Aldrich	M168	nACh receptor antagonist	Palma et al. 1996; Capelli et al. 2011
MG 624	Sigma Aldrich	M3184	nACh receptor antagonist	Gotti et al. 2000; Capelli et al. 2011
N2-supplement	Gibco	17502048		
(-)-Nicotine	Sigma Aldrich	N3876	non-selective nACh receptor agonist	Capelli et al. 2011
PNU-120596	Sigma Aldrich	P0043	Positive allosteric nACh receptor modulator	Hurst et al. 2005; Grønlien et al. 2007; Williams et al. 2011
Polyethyleneimine solution (PEI)	Sigma Aldrich	P3143		
Poly-L-ornithine (PLO)	Sigma Aldrich	P3655		
Sodium tetraborate	Sigma Aldrich	221732		
Thiacloprid	Sigma Aldrich	37905	Neonicotinoid	
Thiamethoxam	Sigma Aldrich	37924	Neonicotinoid	
Tetracycline hydrochloride	Sigma Aldrich	T7660		
Trypsin-EDTA (0.05 %)	Gibco	25300062		
(+)-Tubocurarine chloride pentahydrate	Sigma	93750	non-selective nACh receptor antagonist	Chavez-Noriega et al. 2000; Jonsson et al. 2006
Varenicline tartrate	Sigma	PZ0004	nACh receptor agonist	Coe et al. 2005; Mihalak et al. 2006; Capelli et al. 2011
Veratridine	Tocris	2918	Na _v channel modifier	Loser et al. 2021

Table MS2_S2. Overview of pEC₅₀ values for agonist experiments.

Compound	pEC ₅₀ ± SEM	Pharmacology	Figure
ACh	5.98 ± 0.03	AChR agonist (neurotransmitter)	1B
Nicotine	5.93 ± 0.05	nAChR agonist	1B
Cytisine	5.95 ± 0.05	nAChR agonist	1B
Varenicline	6.08 ± 0.04	nAChR agonist	1B
ABT 594	8.36 ± 0.05	non-α7 nAChR agonist	3C
AR-R 17779	n. d.	α7 nAChR agonist	-
AR-R 17779 (+10 μM PNU-120596)	6.20 ± 0.05	α7 nAChR agonist + α7 nAChR positive allosteric modulator	3C

Table MS2_S3. Overview of pIC₅₀ values for antagonist experiments.

Compound	pIC ₅₀ ± SEM	Pharmacology	Figure
Effect on response to 3 μM ACh		AChR agonist (neurotransmitter)	
Tubocurarine	6.13 ± 0.04	nAChR antagonist	2C
Effect on response to 3 μM nicotine		nAChR agonist	
Tubocurarine	6.08 ± 0.04	nAChR antagonist	2C
MLA	6.33 ± 0.04	nAChR antagonist	2F
Mecamylamine	6.17 ± 0.05	nAChR antagonist	2F
MG 624	6.80 ± 0.07	nAChR antagonist	2F
Effect on response to 3 μM varenicline		nAChR agonist	
Tubocurarine	6.13 ± 0.09	nAChR antagonist	2C
Effect on response to 0.03 μM ABT 594		non-α7 nAChR agonist	
Tubocurarine	5.70 ± 0.05	nAChR antagonist	3F
Mecamylamine	6.08 ± 0.03	nAChR antagonist	3F

Table MS2_S4. Overview of concentrations and technical replicates for agonist and antagonist experiments.

Compound	Concentration (n-values)	Figure
ACh	0.3 μ M (7), 1 μ M (10), 3 μ M (10), 10 μ M (10), 30 μ M (10), 100 μ M (10)	1B
Nicotine	0.001 μ M (8), 0.01 μ M (9), 0.1 μ M (10), 1 μ M (9), 10 μ M (10), 100 μ M (10)	1B
Cytisine	0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 100 μ M (6)	1B
Varenicline	0.001 μ M (6), 0.003 μ M (5), 0.01 μ M (6), 0.03 μ M (6), 0.1 μ M (5), 0.3 μ M (6), 1 μ M (6), 3 μ M (6), 10 μ M (6), 30 μ M (5), 100 μ M (6)	1B
ABT 594	0.0001 μ M (3), 0.001 μ M (5), 0.003 μ M (5), 0.01 μ M (5), 0.03 μ M (5), 0.1 μ M (5), 1 μ M (5), 10 μ M (5)	3C
AR-R 17779	0.1 μ M (15), 1 μ M (18), 10 μ M (13), 100 μ M (15)	-
AR-R 17779 (+10 μ M PNU-120596)	0.03 μ M (12), 0.1 μ M (12), 0.3 μ M (12), 1 μ M (10), 3 μ M (12), 10 μ M (11), 30 μ M (11)	3C
Effect on response to 3 μM ACh		
	Control for tubocurarine (6)	
Tubocurarine	0.0001 μ M (5), 0.001 μ M (6), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 100 μ M (6)	2C
Effect on response to 3 μM nicotine		
	Control for tubocurarine (6), MLA (5), mecamylamine (5) and MG 624 (6)	
Tubocurarine	0.0001 μ M (4), 0.001 μ M (5), 0.01 μ M (4), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 100 μ M (6)	2C
MLA	0.01 μ M (6), 0.03 μ M (6), 0.1 μ M (5), 0.3 μ M (4), 1 μ M (5), 3 μ M (6), 10 μ M (5)	2F
Mecamylamine	0.001 μ M (6), 0.03 μ M (5), 0.1 μ M (6), 0.3 μ M (5), 1 μ M (5), 3 μ M (4), 10 μ M (6)	2F
MG 624	0.003 μ M (5), 0.03 μ M (6), 0.1 μ M (6), 0.3 μ M (6), 1 μ M (6), 10 μ M (6)	2F
Effect on response to 3 μM varenicline		
	Control for tubocurarine (6)	
Tubocurarine	0.0001 μ M (5), 0.001 μ M (4), 0.01 μ M (6), 0.1 μ M (6), 1 μ M (6), 10 μ M (6), 100 μ M (5)	2C
Effect on response to 3 μM ABT 594		
	Control for tubocurarine (11) and mecamylamine (17)	
Tubocurarine	0.01 μ M (11), 0.1 μ M (8), 0.3 μ M (11), 1 μ M (11), 3 μ M (12), 10 μ M (12), 100 μ M (12)	3F
Mecamylamine	0.03 μ M (12), 0.1 μ M (13), 0.3 μ M (13), 1 μ M (15), 3 μ M (14), 10 μ M (12)	3F

Table MS2_S5. Overview of pIC₅₀ values for experiments with neonicotinoids.

Compound	pIC ₅₀ ± SEM	Pharmacology	Figure
Effect on response to 100 µM Aceta (+10 µM PNU-120596)			
Tubocurarine	5.28 ± 0.10	nAChR antagonist	6F
Effect on response to 100 µM Imida (+10 µM PNU-120596)			
Tubocurarine	5.18 ± 0.09	nAChR antagonist	6F
Effect on response to 100 µM Cloth (+10 µM PNU-120596)			
Tubocurarine	5.45 ± 0.08	nAChR antagonist	6F
Effect on response to 100 µM Thiac (+10 µM PNU-120596)			
Tubocurarine	5.64 ± 0.08	nAChR antagonist	6F
Effect on response to 100 µM AR-R (+10 µM PNU-120596)			
Tubocurarine	5.22 ± 0.09	nAChR antagonist	6F
Effect on response to 3 µM nicotine		nAChR agonist	
Aceta	5.40 ± 0.08		8C
Imida	5.47 ± 0.10		8C
Cloth	5.41 ± 0.07		8C
Thiac	5.48 ± 0.08		8C
Thiam	n. d.		8C
Dino	n. d.		8C
Effect on response to 3 µM ACh		AChR agonist (neurotransmitter)	
Aceta	5.53 ± 0.09		8F
Imida	5.43 ± 0.08		8F
Cloth	5.46 ± 0.07		8F
Thiac	5.64 ± 0.04		8F
Thiam	n. d.		8F
Dino	n. d.		8F

(Table continued on next page.)

Table MS2_S5. Overview of pIC₅₀ values for experiments with neonicotinoids. (continued)

Effect on response to 0.03 µM ABT 594		non-α7 nAChR agonist
Aceta	5.07 ± 0.03	9C
Imida	5.24 ± 0.03	9C
Cloth	5.05 ± 0.05	9C
Thiac	5.08 ± 0.06	9C
Thiam	n. d.	9C
Dino	n. d.	9C
Effect on response to 10 µM nicotine		nAChR agonist
Aceta	4.75 ± 0.04	S7A
Imida	4.64 ± 0.08	S7A
Cloth	4.53 ± 0.06	S7A
Thiac	4.61 ± 0.07	S7A
Thiam	n. d.	-
Dino	n. d.	-

Table MS2_S6. Overview of concentrations and technical replicates for experiments with neonicotinoids.

Compound	Concentration (n-values)	Figure
Aceta	0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (7)	4C
Imida	0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (7)	4C
Cloth	0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (6)	4C
Thiac	0.1 µM (6), 1 µM (7), 10 µM (7), 100 µM (7)	4C
Thiam	0.1 µM (6), 1 µM (7), 10 µM (7), 100 µM (7)	4C
Dino	0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (6)	4C
Nicotine	0.1 µM (7), 1 µM (7), 10 µM (5), 100 µM (5)	4C
Effect on response to 0.1 % DMSO	100 µM (5), 100 µM +10 µM PNU120596 (5)	-
Effect on response to 100 µM Aceta	100 µM (7), 100 µM +10 µM PNU120596 (7)	6C
Effect on response to 100 µM Imida	100 µM (6), 100 µM +10 µM PNU120596 (5)	6C
Effect on response to 100 µM Cloth	100 µM (6), 100 µM +10 µM PNU120596 (5)	6C
Effect on response to 100 µM Thiac	100 µM (6), 100 µM +10 µM PNU120596 (5)	6C
Effect on response to 100 µM Thiam	100 µM (6), 100 µM +10 µM PNU120596 (5)	6C
Effect on response to 100 µM Dino	100 µM (5), 100 µM +10 µM PNU120596 (5)	6C
Effect on response to 100 µM Aceta (+10 µM PNU-120596)	Control for tubocurarine (6)	
Tubocurarine	0.1 µM (5), 1 µM (5), 10 µM (6), 100 µM (5)	6F
Effect on response to 100 µM Imida (+10 µM PNU-120596)	Control for tubocurarine (5)	
Tubocurarine	0.1 µM (6), 1 µM (6), 10 µM (5), 30 µM (7), 100 µM (4)	6F

(Table continued on next page.)

Table MS2_S6. Overview of concentrations and technical replicates for experiments with neonicotinoids. (continued)

Effect on response to 100 µM Cloth (+10 µM PNU-120596)		
	Control for tubocurarine (4)	
Tubocurarine	0.1 µM (5), 1 µM (5), 10 µM (6), 30 µM (7), 100 µM (7)	6F
Effect on response to 100 µM Thiac (+10 µM PNU-120596)		
	Control for tubocurarine (5)	
Tubocurarine	0.1 µM (4), 1 µM (6), 10 µM (3), 30 µM (4), 100 µM (3)	6F
Effect on response to 100 µM AR-R 17779 (+10 µM PNU-120596)		
	Control for tubocurarine (5)	
Tubocurarine	0.1 µM (5), 1 µM (5), 10 µM (5), 30 µM (5), 100 µM (5)	6F
Effect on response to 3 µM nicotine		
	Control for Aceta (9), Imida (9), Cloth (21), Thiac (21), Thiam (15), Dino (6)	8C
Aceta	0.01 µM (7), 0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (7)	8C
Imida	0.01 µM (5), 0.1 µM (5), 1 µM (7), 10 µM (7), 100 µM (7)	8C
Cloth	0.01 µM (13), 0.1 µM (14), 1 µM (14), 10 µM (14), 100 µM (14)	8C
Thiac	0.01 µM (12), 0.1 µM (12), 1 µM (12), 10 µM (13), 100 µM (12)	8C
Thiam	0.01 µM (13), 0.1 µM (13), 1 µM (12), 10 µM (13), 100 µM (13)	8C
Dino	0.01 µM (6), 0.1 µM (6), 1 µM (6), 10 µM (6), 100 µM (6)	8C
Effect on response to 3 µM ACh		
	Control for Aceta (14), Imida (14), Cloth (14), Thiac (14), Thiam (26), Dino (14)	
Aceta	0.01 µM (6), 0.1 µM (7), 1 µM (5), 10 µM (7), 100 µM (6)	8F
Imida	0.01 µM (7), 0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (7)	8F
Cloth	0.01 µM (7), 0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (7)	8F
Thiac	0.01 µM (7), 0.1 µM (16), 1 µM (19), 3 µM (12), 10 µM (20), 100 µM (21)	8F
Thiam	0.01 µM (7), 0.1 µM (6), 1 µM (7), 10 µM (6), 100 µM (6)	8F

(Table continued on next page.)

Table MS2_S6. Overview of concentrations and technical replicates for experiments with neonicotinoids. (continued)

Dino	0.01 µM (7), 0.1 µM (7), 1 µM (7), 10 µM (6), 100 µM (7)	8F
Effect on response to 3 µM ABT 594	Control for Aceta (12), Imida (6), Cloth (13), Thiac (6), Thiam (6), Dino (6)	
Aceta	0.3 µM (9), 1 µM (9), 3 µM (9), 10 µM (12), 30 µM (10), 100 µM (9)	9C
Imida	0.3 µM (4), 1 µM (4), 3 µM (6), 10 µM (6), 30 µM (6), 100 µM (6)	9C
Cloth	0.3 µM (9), 1 µM (9), 3 µM (9), 10 µM (11), 30 µM (12), 100 µM (11)	9C
Thiac	0.3 µM (3), 1 µM (4), 3 µM (5), 10 µM (5), 30 µM (6), 100 µM (6)	9C
Thiam	1 µM (4), 10 µM (4), 100 µM (6)	9C
Dino	1 µM (3), 10 µM (3), 100 µM (4)	9C
Effect on response to 10 µM nicotine	Control for each compound (13)	
Aceta	0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (7)	S7A
Imida	0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (7)	S7A
Cloth	0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (6)	S7A
Thiac	0.1 µM (7), 1 µM (6), 10 µM (7), 100 µM (7)	S7A
Thiam	0.1 µM (6), 1 µM (7), 10 µM (7), 100 µM (7)	-
Dino	0.1 µM (7), 1 µM (7), 10 µM (7), 100 µM (6)	-

Table MS2_S7. Overview of concentrations and technical replicates for single-cell Ca²⁺-imaging.

Compound	Concentration (n-values)		Figure
Nicotine	0.001 µM (5), 0.01 µM (5), 0.1 µM (7), 1 µM (4), 10 µM (7), 100 µM (8)		5C
Aceta	1 µM (5), 10 µM (3), 100 µM (6)		5C
Imida	1 µM (4), 10 µM (3), 100 µM (5)		5C
Cloth	100 µM (9)		5D
Thiac	100 µM (9)		5D
Thiam	100 µM (8)		5D
Dino	100 µM (3)		5D
Effect on response to 100 µM Aceta	Control for tubocurarine (9)		S4
Tubocurarine	1 µM (6), 100 µM (6)		S4
Serial application			
Aceta	10 µM (8)	100 µM (4)	S8
Nicotine	10 µM (8)	10 µM (4)	S8
VTD	30 µM (8)	30 µM (4)	S8

Table MS2_S8. Overview of percentages of responsive cells of single-cell Ca²⁺-imaging.

Compound	Concentration	Responsive cells $\pm$ SEM	Figure
Aceta	1 μ M	0.025 $\pm$ 0.010	5C
Aceta	10 μ M	0.135 $\pm$ 0.024	5C
Aceta	100 μ M	0.275 $\pm$ 0.051	5C
Imida	1 μ M	0.031 $\pm$ 0.010	5C
Imida	10 μ M	0.092 $\pm$ 0.010	5C
Imida	100 μ M	0.275 $\pm$ 0.071	5C
Nicotine	0.001 μ M	0.008 $\pm$ 0.004	5C
Nicotine	0.01 μ M	0.022 $\pm$ 0.007	5C
Nicotine	0.1 μ M	0.393 $\pm$ 0.083	5C
Nicotine	1 μ M	0.810 $\pm$ 0.033	5C
Nicotine	10 μ M	0.745 $\pm$ 0.042	5C
Nicotine	100 μ M	0.846 $\pm$ 0.019	5C
Cloth	100 μ M	0.277 $\pm$ 0.046	5D
Thiac	100 μ M	0.125 $\pm$ 0.012	5D
Thaim	100 μ M	0.034 $\pm$ 0.006	5D
Dino	100 μ M	0.011 $\pm$ 0.008	5D
100 μ M Aceta +			
Tubocurarine	0 μ M	0.319 $\pm$ 0.033	S4
Tubocurarine	1 μ M	0.126 $\pm$ 0.025	S4
Tubocurarine	100 μ M	0.033 $\pm$ 0.010	S4
Aceta	10 μ M	0.175 $\pm$ 0.036	S8A
Nicotine	10 μ M	0.636 $\pm$ 0.069	S8A
VTD	30 μ M	0.988 $\pm$ 0.005	S8A
Aceta	100 μ M	0.323 $\pm$ 0.085	S8B
Nicotine	10 μ M	0.177 $\pm$ 0.050	S8B
VTD	30 μ M	0.981 $\pm$ 0.008	S8B

Table MS2_S9. Benchmark responses (BMR) for the neonicotinoids desensitization of nicotine and acetylcholine signaling.

Overview of the BMR10 and BMR25 concentrations (in log[M]) of the Ca²⁺ response induced by nicotine and acetylcholine after an initial stimulation of LUHMES cells with neonicotinoids. For each BMR, the benchmark concentrations (BMC) and their confidence interval (BMCL and BMCU) are shown; n.a.: BMR could not be determined due to low/no effect within the tested concentration range.

Compounds		Intracellular free [Ca ²⁺]					
Treatment	Pre-treatment	BMR10			BMR25		
		BMCL	BMC	BMCU	BMCL	BMC	BMCU
nicotine	Acetamiprid	-6.6	-6.3	-6.1	-6.1	-5.9	-5.7
	Imidacloprid	-7.3	-6.4	-5.6	-6.6	-5.9	-5.3
	Clothianidin	-6.4	-6.2	-6.0	-5.9	-5.8	-5.7
	Thiacloprid	-7.1	-6.3	-5.4	-6.5	-5.9	-5.3
	Thiamethoxam	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Dinotefuran	-4.8	-4.0	-3.2	n.a.	n.a.	n.a.
acetylcholine	Acetamiprid	-6.3	-6.1	-5.8	-6.0	-5.8	-5.6
	Imidacloprid	-6.7	-6.0	-5.3	-6.2	-5.7	-5.2
	Clothianidin	-6.8	-6.0	-5.1	-6.4	-5.7	-5.1
	Thiacloprid	-6.3	-6.2	-6.1	-6.0	-5.9	-5.9
	Thiamethoxam	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Dinotefuran	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table MS2_S10. Prediction of free drug concentrations in recording buffer of LUHMES.

The free drug concentrations [μM] were calculated for the exemplary nominal concentrations of 1, 10 and 50 μM , based on the known recording buffer content of lipids and proteins and the number of cells used in each well. Calculations were performed as detailed in Kisitu et al. (2020) and Fisher et al. (2019).

Nominal concentration [μM]	1	10	50
Acetamiprid	0.96	9.55	47.73
Imidacloprid	1.00	10.00	49.99
Clothianidin	1.00	10.00	49.98
Thiacloprid	1.00	9.99	49.94
Thiamethoxam	1.00	10.00	50.00
Dinotefuran	1.00	10.00	50.00

Table MS2_S11. Input parameters used in the imidacloprid PBTK model.

Parameter	Value	Method/Reference
Molecular weight (g/mol)	255.7	Pubchem
log P	0.4	Predicted <i>in silico</i> ^b .
Compound type	Neutral	
blood/plasma ratio [B/P]	0.954	measured
free fraction [fu]	0.724	measured
Main plasma binding protein	Human serum albumin	assumed
Absorption parameters:^b		
fa	0.95	

(Table continued on next page.)

Table MS2_S11. Input parameters used in the Imidacloprid PBTK model. (continued)

ka (1/h)	1.16	
fu _{gut}	1	assumed
Q _{gut} (L/h)	18.6	
P _{eff,man} (10 ⁻⁴ cm/s)	2.82	
P _{trans,0} (10 ⁻⁶ cm/s)	35.4	
Distribution Model	Minimal PBTK Model	
V _{ss} (L/kg)	0.85	Predicted ^c
Brain: Plasma ratio	0.75 ^d	
Kp Scalar	2	Optimized
Enzyme	CYP3A4	
CL _{int} (μL/min/pmol)	0.003 ^e	
CL _R (L/h)	0.17 ^f	

^a: Predicted according to a mechanistic permeability model (Sugano, 2009)

^b: Average of 5 estimates from different source models

^c: Method 2 (Rodgers and Rowland, 2007)

^d: Assumed same as mouse (Ford and Casida, 2006)

^e: Retrograde calculation based on predicted V_{ss} and half-life of renal elimination reported for deuterated imidacloprid in humans (Harada et al., 2016)

^f: Estimated from total CL and renal excretion of deuterated imidacloprid in human urine as reported by Harada et al. (2016).

3.3 Manuscript 3: Potent neuromodulatory activity of the imidacloprid metabolite desnitro-imidacloprid via human nAChR agonism

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Keywords Live-cell calcium imaging, Pesticide metabolism, Nicotine, Developmental neurotoxicity, Molecular docking, Oocyte recording

3.3.1 Abstract

Several neonicotinoids have recently been shown to activate the nicotinic acetylcholine receptor (nAChR) on human neurons. Moreover, imidacloprid (IMI) and other members of this pesticide family form a set of diverse metabolites within crops. Amongst these, desnitro-imidacloprid (DN-IMI) is of special toxicological interest, as there is evidence (i) for human dietary exposure to this metabolite, (ii) and that DN-IMI is a strong trigger of mammalian nicotinic responses. We set out here to quantify responses of human nAChRs to DN-IMI and an alternative metabolite, IMI-olefin. To evaluate toxicological

hazards, these data were then compared to those of IMI and nicotine. Ca^{2+} -imaging experiments on human neurons showed that DN-IMI exhibits an agonistic effect on nAChRs at sub-micromolar concentrations (equipotent with nicotine) while IMI-olefin activated the receptor less potently (in a similar range as IMI). Molecular docking studies, using receptor models for the $\alpha 7$ and $\alpha 4\beta 2$ nAChR subtypes supported an activity of DN-IMI similar to that of nicotine. Direct experimental data on the interaction with defined receptor subtypes were then obtained by heterologous expression of various human nAChR subtypes in *Xenopus laevis* oocytes and measurement of the transmembrane currents evoked by exposure to putative ligands. DN-IMI acted on human $\alpha 7$, $\alpha 3\beta 4$, and the high-sensitivity variant of $\alpha 4\beta 2$ nAChRs with similar potency (nM range) as nicotine. IMI and IMI-olefin were confirmed as nAChR agonists, although with 2-3 orders of magnitude lower potency. In summary, these data suggest that DN-IMI functionally affects human neurons similar to the well-established neurotoxicant nicotine by triggering $\alpha 7$ and several non- $\alpha 7$ nAChRs.

3.3.2 Introduction

The toxicological assessment of many pesticides is complicated by the fact that there is not only exposure to the original substances, but also to their many metabolites formed in the environment. This also applies to the neonicotinoids, a class of insecticides with long persistence within crops (Craddock et al., 2019; Simon-Delso et al., 2015; Thompson et al., 2020). They comprise, e.g., imidacloprid (IMI), acetamiprid, clothianidin, and thiacloprid. With a global market turnover of > 1 billion € (Jeschke et al., 2011; Sparks and Nauen, 2015), this group of compounds has dominated many pesticide application domains and thus has led to widespread human exposure (Craddock et al., 2019; Klarich et al., 2017; Thompson et al., 2020). The insecticidal mode of action is based on the over-activation of the nicotinic acetylcholine receptor (nAChR) of the target species. This activity has been assumed to be relatively specific for the insect nervous system (Brown et al., 2006; Tan et al., 2007), as neonicotinoids have been developed to exhibit a higher affinity for insect nAChRs compared to vertebrate paralogs (Casida, 2018; Tomizawa et al., 2000; Tomizawa and Casida, 2005). However, some studies suggest adverse effects of neonicotinoids on mammals (Abou-Donia et al., 2008; Berheim et al., 2019; Burke et al., 2018; Duzguner and Erdogan, 2012). A broad toxicological debate has been triggered by the observation that acetamiprid and IMI activated the nAChRs on neonatal rat neurons in the low μM range (Kimura-Kuroda et al., 2012). The relevance of this finding for human toxicology is

further supported by a recent study using cultured human neurons. Clear nAChR signaling and also pronounced receptor desensitization were demonstrated for several neonicotinoids at concentrations that may be reached by dietary or accidental exposure (Loser et al., 2021a).

Food products intended for human consumption have high detection rates for IMI (Chen et al., 2014; Craddock et al., 2019; Thompson et al., 2020). In addition, several metabolites are found. The transformation of the parent compounds can arise via abiotic (photolysis, hydrolysis, and chlorination) or biological (microbial, fungal, and plant) processes (Simon-Delso et al., 2015; Thompson et al., 2020). One important metabolic step is the reduction of the nitro group of IMI to form aminoguanidine derivatives or derivatives that entirely lack the nitro group (e.g., DN-IMI). Besides cytochrome P450 enzymes, especially aldehyde oxidase seems to play an important role in this biotransformation (Dick et al., 2005; Ford and Casida, 2006; Schulz-Jander et al., 2002; Schulz-Jander and Casida, 2002; Shi et al., 2009; Simon-Delso et al., 2015; Swenson and Casida, 2013; Thompson et al., 2020).

Imidacloprid-olefin (IMI-olefin) has been detected in honey (Codling et al., 2016; Thompson et al., 2020), and DN-IMI is a major IMI degradation product in the environment (Anon, 2006; Koshlukova, 2006). The latter metabolite is produced abiotically by photodegradation (17% of all IMI), but also biotically as the dominant bacterial metabolite, and as a major metabolite in many plants (Anon, 2006; Koshlukova, 2006). It has, e.g., been found in drinking water (Klarich Wong et al., 2019; Wan et al., 2020) and it has been reported to be formed in diverse foods such as apples, tomatoes, eggplants, and potatoes, where it accounted for around 10-30% of IMI degradation products. It reached concentrations in the 10-30 µg/kg range in apples and potatoes and up to 300 µg/kg in fodder corn (Anon, 2006). Although the use of IMI has been banned in Europe due to unacceptable toxicity to bees, exposure via the diet still occurs via imported food commodities. The current dietary risk assessment on IMI in Europe covers the exposure to the parent compound IMI and its metabolites. However, there is no specific residue definition for DN-IMI (European Food Safety Authority (EFSA) et al., 2019). This means that it is assumed that the toxicological potency is similar to the parent compound.

DN-IMI has also been detected in mice exposed to IMI. This suggests that it can also be produced within mammals by endogenous metabolism (Ford and Casida, 2006; Swenson and Casida, 2013). This is consistent with findings of DN-IMI and IMI-olefin in human urine samples analyzed in a recent biomonitoring study (Wang et al., 2020).

The previous knowledge of IMI metabolism shows that a shift in the bioactivity spectrum can occur. For instance, some metabolites have a strongly reduced potency on insect nAChRs, but they instead increase their affinity for mammalian nAChRs (Chao and

Casida, 1997; D'Amour and Casida, 1999; Tomizawa et al., 2000; Tomizawa and Casida, 1999). For instance, binding assays using mammalian nAChRs have shown that DN-IMI has an affinity similar to the high-affinity ligand nicotine (D'Amour and Casida, 1999; Tomizawa et al., 2000; Tomizawa and Casida, 1999). Moreover, studies in mice suggested a higher toxicity of DN-IMI, compared to its parent compound IMI (Chao and Casida, 1997; Tomizawa et al., 2000). Nicotine is a well-known neurotoxicant and developmental neurotoxicant for vertebrates, including man (Dwyer et al., 2009; Grandjean and Landrigan, 2006; LeSage et al., 2006; Levin et al., 1993; Slikker Jr et al., 2005; Slotkin et al., 2016; Zahedi et al., 2019). Therefore, IMI metabolites mimicking the activity profile of nicotine on human receptors are of high toxicological concern.

The activation of ionotropic receptors like nAChRs on neurons leads to a depolarization of the cell membrane and, thereby, activates voltage-dependent Ca^{2+} channels. The transient influx of Ca^{2+} into the cell increases the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which can be measured by Ca^{2+} -imaging in neuronal cell cultures (Grunwald et al., 2019; Leist and Nicotera, 1998; Loser et al., 2021b; Sirenko et al., 2019). This method is based on the quantifications of fluorescence signals of calcium-sensitive dyes introduced into the cells, and it is amenable to high throughput formats (Brüll et al., 2020; Karreman et al., 2020; Loser et al., 2021b; Sirenko et al., 2019). Alternatively, xenobiotic effects on individual nAChR subtypes may be measured directly, by the recording of the transmembrane currents in *Xenopus laevis* oocytes that heterologously express human receptors of interest. The basis of this method is the injection of mRNA coding for human neurotransmitter receptor subunits into the cells. It is well-known that this experimental system has a high efficiency for protein translation and functional insertion of the respective receptors in the cell membrane. The large size of the oocytes allows the current flow through the cell membrane (triggered by agonists) to be measured by two sharp microelectrodes placed inside the cell. The test method obtains its specificity from the strong heterologous expression of the respective receptor. (Benallegue et al., 2013; Bermudez and Moroni, 2006; Carbone et al., 2009; Harpsøe et al., 2011; Jonsson et al., 2006; P. Li et al., 2011; Mazzaferro et al., 2011; Mineur et al., 2009; Moroni et al., 2006).

The human neuronal precursor cell line LUHMES and the neuroblastoma cell line SH-SY5Y can be differentiated into post-mitotic neurons (Lopes et al., 2010; Scholz et al., 2011), and they are often used as a model system to investigate adverse effects on human neurons (Attoff et al., 2020, 2016; Brüll et al., 2020; Delp et al., 2019, 2018b, 2018a; Gustafsson et al., 2010; Harris et al., 2017; Krug et al., 2014, 2013; Lohren et al., 2015; Ring et al., 2015; Smirnova et al., 2016; Tomizawa and Casida, 1999; Tong et al., 2017; Witt et al., 2017; Zhang et al., 2014). The utility of these cell models for

functional neurotoxicity testing has been demonstrated for agents that affect voltage-dependent sodium channels or ionotropic receptors (Loser et al., 2021b, 2021a). Both cell types express functional nAChRs and have been used in Ca^{2+} -imaging assays to study the effects of several neonicotinoids (Loser et al., 2021a).

In this study, we explored whether DN-IMI poses a potential neurotoxicity or developmental neurotoxicity hazard, by acting on nAChR of human neurons. The IMI metabolite was chosen for this study, as it may be directly ingested by food. However, it is also relevant as it may be generated in individuals exposed to IMI. We compared the signaling effects of DN-IMI on LUHMES neurons and SH-SY5Y to that of IMI and nicotine. To determine differences in nAChR subtype selectivity of the compounds, we further investigated the agonist activity of these compounds on human $\alpha 4\beta 2$, $\alpha 7$, and $\alpha 3\beta 4$ nAChR subtypes, expressed in *Xenopus laevis* oocytes, and we developed a molecular docking approach explaining these findings. To gather background information on the persistence and distribution of DN-IMI in man, a toxicokinetic model was implemented and parameterized by metabolism data from human hepatocytes. The broad data set of this study was used for a preliminary risk assessment of DN-IMI.

3.3.3 Materials and methods

Materials and chemicals

An overview of experimental tool compounds and toxicants is given in table MS3_S1. Consumables are indicated in the specific methods paragraphs. Chemical structures of imidacloprid (IMI)

(<https://pubchem.ncbi.nlm.nih.gov/compound/86287518#section=2D-Structure>),

desnitro-imidacloprid (DN-IMI)

(<https://pubchem.ncbi.nlm.nih.gov/compound/10130527#section=2D-Structure>) and

imidacloprid-olefin (IMI-olefin)

(<https://pubchem.ncbi.nlm.nih.gov/compound/14626249#section=2D-Structure>) were obtained from PubChem and visualized in ChemDraw JS (version 19.0.0-CDJS-19.0.x.9+da9bec968, PerkinElmer).

LUHMES cell culture

The cultivation of the LUHMES cells was performed as described earlier (Krug et al., 2013; Schildknecht et al., 2013; Scholz et al., 2011). In brief, LUHMES cells were cultured in standard cell culture flasks (Sarstedt) that were pre-coated with 50 $\mu\text{g}/\text{ml}$ poly-L-ornithine (PLO) and 1 $\mu\text{g}/\text{ml}$ fibronectin (Sigma-Aldrich) in H_2O overnight at 37

°C. The cells were maintained in proliferation medium containing advanced DMEM/F12 (Gibco) with 2 mM L-glutamine (Sigma Aldrich), 1x N2-supplement (Gibco), and 40 ng/ml recombinant human basic fibroblast growth factor (FGF-2, R&D Systems). The cells were kept at 37 °C and 5% CO₂ and passaged three times a week when the culture reached a confluency of 75-90%. Cells were used until passage 18. For differentiation, cells were cultured in differentiation medium consisting of advanced DMEM/F12 (Gibco) supplemented with 2 mM L-glutamine (Sigma Aldrich), 1x N2-supplement (Gibco), 1 mM N6,2'-O-dibutyryl 3',5'-cyclic adenosine monophosphate (cAMP) (Sigma Aldrich), 1 µg/ml tetracycline (Sigma Aldrich) and 2 ng/ml recombinant human glial cell-derived neurotrophic factor (GDNF, R&D Systems).

For Ca²⁺-imaging the cells were pre-differentiated for 48 h in cell culture flasks, detached and plated at a density of 20,000 cells and 30,000 cells per well on 0.1% PEI-coated 384-well and 96-well plates (Greiner Bio-One), respectively, for the Ca²⁺-imaging. The cells were further differentiated for another 7 days. 50% of the medium was exchanged every 2-3 days.

Cell culture of SH-SY5Y cells

SH-SY5Y cells were cultured as previously described (Attoff et al., 2016). Briefly, they were cultured in MEM supplemented with 10% fetal bovine serum (Gibco, 31330095), 1% non-essential amino acid solution (Gibco, 11140035), 2 mM L-glutamine (Gibco, 25030024), 100 µg/ml streptomycin, and 100 U/ml penicillin (Gibco, 15140122). For maintenance culture, SH-SY5Y cells were seeded at 27,000 cells/cm² in 75 cm² cell culture flasks (Corning). The cells were passaged once a week using TrypLE Express Enzyme (Gibco). SH-SY5Y cells were differentiated into a neuronal-like phenotype by exchanging the maintenance medium with differentiation medium consisting of DMEM/F12 (Gibco, 31330095) supplemented with 1 mM L-glutamine (Gibco, 25030024), 100 µg streptomycin/mL, 100 U penicillin/mL, 1x N2 supplement (Gibco, 17502048) and 1 µM all-trans retinoic acid (RA, Sigma, R2625) 24 h after seeding. The cells were incubated in 100% humidity at 37°C in air with 5% CO₂.

LUHMES Ca²⁺-imaging

Ca²⁺-imaging was performed using HT Functional Drug Screening System FDSS/µCELL (Hamamatsu Photonics) at nominal 37 °C. The FDSS/µCell system enables the indirect recording of changes of intracellular Ca²⁺ [Ca²⁺]_i via a Ca²⁺-sensitive fluorescent dye. The fluorescence signal of a complete 384-well plate is acquired at once with a high-speed and high-sensitivity digital ImagEM X2 EM-CCD camera (Electron Multiplying Charge-Coupled Device, Hamamatsu Photonics), but with limited spatial resolution. Therefore, the software only determines the mean fluorescence signal of each well

rather than of individual cells. For compound application, the integrated dispenser head with 384 pipette tips was used, which can add the test compound to all wells simultaneously. Cells were preincubated with Cal-520 AM (AAT Bioquest) at a concentration of 1 μ M for 1 h at 37 °C. For recording, the medium was exchanged by a buffer solution containing [mM]: 135 NaCl, 5 KCl, 0.2 MgCl₂, 2.5 CaCl₂, 10 HEPES, and 10 D-glucose, pH 7.4. Test compound application was executed after obtaining a 1.5 min baseline recording. Where applicable, a second application was executed 4.5 min after the first application. The total recording never exceeded 8 min.

For Ca²⁺-imaging experiments with a higher resolution on the single-cell level, the Cell Observer (Carl Zeiss Microscopy) was used. The Ca²⁺-sensitive dye, the cell handling before the experiment, and the buffer were the same as described above for the experiments with the high-throughput FDSS/ μ CELL system. The recordings were performed with 2x2 binning and a 42 ms exposure time. The compounds were applied after a baseline recording of at least 10 s.

Ca²⁺ measurements in SH-SY5Y

To measure acute changes in the average [Ca²⁺]_i of a population, SH-SY5Y cells were examined in the 96-well plate fluorescence reader FlexStation II (Molecular Devices) by using the fluorophore Fura-2AM. SH-SY5Y (35,000 cells/well; 109,375 cells/cm²) were seeded in maintenance culture medium in black 96-well plates with clear bottom (Corning, #3603). 24 h after seeding, maintenance medium was replaced with differentiation medium. After 72 h of differentiation, Fura-2AM dissolved in DMSO and diluted in KRH buffer (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.0 mM CaCl₂, 6.0 mM D-glucose, and 25 mM HEPES (free acid), pH adjusted to 7.4 by 1.0 M NaOH) were added to the medium to a final concentration of 4 μ M (Gustafsson et al., 2010). The plates were incubated for 30 minutes at 37 °C before cells were washed once with 200 μ l KRH buffer. 90 μ l of KRH buffer without or with 10 μ M PNU-120596 (PNU) and/or 125 μ M mecamylamine (Mec) and/or test chemicals in different concentrations for antagonist experiments, were added to the Fura-2AM loaded cells. The plate was again incubated for 20 min to allow full hydrolysis of the AM group before the experiment. The fluorescence was assessed at 37 °C in the fluorescence plate reader (FlexStation II; Molecular Devices) at two different excitation wavelengths, 340 nm for Ca²⁺-bound Fura-2 and 380 nm for free Fura-2, and at 510 nm emission, every 3.1 seconds using bottom read settings. After 26-29 seconds of initial baseline recording of the fluorescence intensity, 10 μ l of the compound dilution (10 times higher than the final concentration to the cells) were transferred automatically by the FlexStation II ("Flex mode") to the cell plate wells (five wells per concentration) and the fluorescence intensity was monitored for another 150 seconds. The ratio of fluorescence intensity at

340/380 nm was determined and the mean values from the baseline recording before the addition of test compounds was set to zero. The acute change in the Ca^{2+} influx after the addition of the compounds was quantified as the area under the curve (AUC) using the SoftMax Pro 4.8 software (Molecular Devices). All test compounds were dissolved in DMSO. Compounds were diluted in KRH buffer in 1:3 series, with 100 μM as the highest concentration. As a negative control, 0.1 % DMSO in KRH buffer was used. Nicotine (11 μM) and KCl (30 mM) in KRH were used as positive controls. The Ca^{2+} influx induced by DN-IMI was normalized to the response triggered by nicotine (11 μM) or KCl (30 mM).

Structure-based approach – docking studies and binding free energy calculations

In order to gather more information about the binding mode of nicotinoids (DN-IMI) and neonicotinoids (IMI) on human nAChRs, with the focus on the $\alpha 7$ - and $\alpha 4\beta 2$ -isoforms, we also searched the protein data bank (PDB, rcsb.org) and relevant literature for accessible structures.

IMI and DN-IMI are available in complex of the soluble acetylcholine binding protein (AChBP) (PDB-ID: 2zju (Ihara et al., 2008) and 3wtn (Ihara et al., 2014)), which is homologous to the extracellular domain of nAChRs.

For the structure-based approach, we conducted induced-fit docking (IFD) experiments and binding energy calculations by using respective tools from the software suite of Schrödinger's Maestro20-2 (Schrödinger Release 2020-2, 2020). A detailed description of the IFD can be found in the methods section of our previous publication about neonicotinoid effects on human neurons (Loser et al., 2021a). In brief, before the docking studies, the proteins and ligands of interest need to be prepared to enrich the quality of the docking outcome (Madhavi Sastry et al., 2013). Both have been prepared at $\text{pH } 7.0 \pm 0.5$ using LigPrep and Protein Preparation Wizard, respectively, and additionally have been checked manually (Schrödinger Release 2020-2, 2020). Nicotine has been structurally elucidated in complex of the human $\alpha 4\beta 2$ -isoform at distinct stoichiometries, so that PDB-ID 6cnj suited the purpose for the $\alpha 4(+)\beta 2(-)$ - and 6cnk for the $\alpha 4(+)\alpha 4(-)$ -binding pocket (Walsh et al., 2018). The co-crystallized ligand nicotine between chains A and B, [A:402] in 6cnj and [A:405] in 6cnk (Walsh et al., 2018), has been used as a centroid for the docking grid, respectively. Before the structure of the human nAChR $\alpha 7$ -isoform has recently been experimentally resolved (Noviello et al., 2021), published homology models of the ligand-binding domain were usually used for docking studies on this subtype (Ng et al., 2018). The centroid of previously defined key ligand-binding residues (Ng et al., 2018; Sakkiah et al., 2020) has been used as the center of the grid box on this isoform.

The docking poses are scored by the docking algorithm which implements the OPLS3e force field (Harder et al., 2016; Roos et al., 2019), according to their estimated binding energy of the generated protein-ligand complex (Schrödinger Release 2020-2, 2020): Scoring parameters from log-file: (1) IFDScore [kcal/mol] = + 1.0* Prime_Energy + 9.057 Glide_gscore + 1.428 Glide_ecoul; (2) docking Score [kcal/mol] = GlideScore + Epik State Penalty (from LigPrep). The Prime technology is not only applied during the IFD but is also used for calculations of the ligand binding energy with the molecular mechanics generalized Born surface area (MM-GBSA)-tool within the Schrödinger package (Schrödinger Release 2020-2: Prime, Schrödinger, LLC, New York, NY, 2020 (Schrödinger Release 2020-2, 2020)). This method utilizes molecular mechanics energies in combination with a generalized Born model to assess residue-dependent effects and electrostatic solvation energy (Fig. MS3_S3A). Within the VSGB solvation model used in this study (J. Li et al., 2011), water is set as a solvent that is suitable for the ligand-binding domain of nAChRs since it is located extracellularly and is, therefore, water exposed (Velisetty et al., 2014).

Also, surface areas have been incorporated in this binding energy assessment tool (Genheden and Ryde, 2015; Schrödinger Release 2020-2, 2020). The force field for refinement, OPLS3e (Harder et al., 2016) is the same as in the IFD protocol (Schrödinger Release 2020-2, 2020). In this study, minimization of all residues' atoms has been chosen as a sampling method to assess protein flexibility within a defined distance of 12 Å around the ligand. As an initial step for calculating the MM-GBSA binding energies of protein complexes, originating either from co-crystallized ligands or from IFD-output structures, the ligand has to be extracted as a separate entry for the use of this tool.

In general, MM-GBSA is an established method for rationalization of experimental findings (binding energies from co-crystallized ligands) and for improving the results of docking (Genheden and Ryde, 2015). We used the following two parameters: (1) MM-GBSA dG Bind: The binding energy of the receptor and ligand as calculated by the Prime Energy, a Molecular Mechanics + Implicit Solvent Energy Function [kcal/mol] = PrimeEnergy (Optimized Complex) – PrimeEnergy (Optimized Free Ligand) – PrimeEnergy (Optimized Free Receptor); (2) MM-GBSA dG Bind (NS): A version of dG Bind that does not include contributions from receptor or ligand strain. [kcal/mol] = PrimeEnergy (Optimized Complex) – PrimeEnergy (Ligand Geometry From Optimized Complex) – PrimeEnergy (Receptor Geometry From Optimized Complex).

Oocyte recordings

The human $\alpha 3$ (GenBank: U62432.1), $\alpha 4$ (GenBank: L35901.1, silent base exchanges to reduce GC content), $\beta 2$ (GenBank: X53179.1), and $\beta 4$ (GenBank: U48861.1) nAChR

subunits were synthesized using FragmentGene service by Genewiz company and subsequently cloned in the pNKS2 vector (Gloor et al., 1995) using Gibson Assembly. The human $\alpha 7$ nAChR subunit was cloned in the pCDNA3.1 vector.

The recordings of human $\alpha 7$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, and $\alpha 4\beta 4$ nAChRs expressed in *Xenopus laevis* oocytes (EcoCyte Bioscience) were performed in two-electrode voltage-clamp mode using the Roboocyte2 system and the corresponding software (version 1.4.1; Multi Channel Systems MCS). Prior to the recordings, the oocytes were maintained at 19 °C in modified Barth's solution containing [mM]: 88 NaCl, 1 KCl, 0.33 $\text{Ca}(\text{NO}_3)_2$, 0.82 MgSO_4 , 2.4 NaHCO_3 , 0.41 CaCl_2 , 5 Tris, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, pH 7.4.

For the generation of the mRNA for injection, the plasmid DNAs of $\alpha 3$, $\alpha 4$, $\beta 2$, and $\beta 4$ were linearized with the NotI restriction endonuclease (New England Biolabs) and the plasmid DNA of $\alpha 7$ was linearized with the XbaI restriction endonuclease (New England Biolabs). The mRNAs of $\alpha 3$, $\alpha 4$, $\beta 2$, and $\beta 4$ were generated by in vitro transcription using the mMESSAGE mMACHINE SP6 Transcription Kit (Invitrogen). For the generation of $\alpha 7$ mRNA, the mMESSAGE mMACHINE T7 Transcription Kit (Invitrogen) was used. For the separation of the DNA and mRNA, a phenol-chloroform extraction (Chomczynski and Sacchi, 2006) was performed. The mRNA was then obtained by ethanol precipitation from the aqueous phase; for quantification, the BioPhotometer (Eppendorf) was used. To express human $\alpha 7$ nAChR, we injected 50 nl of mRNA solution (30 ng mRNA) per oocyte, using the Roboinject and the corresponding software (version 1.2.1; Multi Channel Systems MCS). The subunits of the heteromeric human $\alpha 3\beta 4$ nAChRs and high-sensitivity (HS) $(\alpha 4)_2(\beta 2)_3$ combination were injected in a ratio of 1:10 (α : β subunit) with a mRNA amount of 0.33 ng of $\alpha 3$ and 3.33 ng of $\beta 4$ for $\alpha 3\beta 4$, and 3 ng of $\alpha 4$ and 30 ng of $\beta 2$ for $\alpha 4\beta 2$. The mRNA for the subunits of the low-sensitivity (LS) $(\alpha 4)_3(\beta 2)_2$ stoichiometry was injected in a ratio of 10:1 (α : β subunit) with 10 ng of $\alpha 4$ and 1 ng of $\beta 2$. The subunits of the $\alpha 4\beta 4$ nAChR subtype were injected in a ratio of 1:1 with 3.33 ng of $\alpha 4$ and 3.33 ng of $\beta 4$. After mRNA injection, the oocytes were maintained for 3-6 days before recordings were performed. The experiments were executed in a ND96 buffer solution containing [mM]: 96 NaCl, 2 KCl, 1 MgCl_2 , 1.8 CaCl_2 , 5 HEPES, pH 7.4. The oocyte membrane potential was kept at -50 mV in all recordings. In experiments with $\alpha 7$, the compounds were applied for 5 s followed by a 60 s wash period. At the end of each recording, a reference application of 1 mM nicotine was performed. In experiments with heteromeric nAChRs, the compound was applied for 3 s, followed by a washout of 10 s, and an application of acetylcholine (ACh) for 1 s, which was followed by a washout of 60 s. The recordings for $\alpha 3\beta 4$, $\alpha 4\beta 2$ (HS), $\alpha 4\beta 2$ (LS) and $\alpha 4\beta 4$ were

performed with 200 μ M, 3 μ M, 100 μ M and 100 μ M ACh, respectively. ACh was applied as an additional reference for run-down detection and positive control. Therefore, ACh was applied four times before the addition of the first compound concentration and after the application of each compound concentration. After the measurement of all compound concentrations, the last application was a reference exposure to nicotine with 1 mM for $\alpha 3\beta 4$, 10 μ M for $\alpha 4\beta 2$ (HS), 100 μ M for $\alpha 4\beta 2$ (LS) and 100 μ M for $\alpha 4\beta 4$ and used for normalization.

For the antagonist recordings, DN-IMI was applied for 3 s after a 5 s baseline period. The application of DN-IMI was followed by a wash period of 70 s. Initially, four control recordings were performed, followed by three recordings in the presence of each of the three antagonist concentrations. Finally, three recordings were executed during washout. DN-IMI was applied at 1 μ M in recordings with $\alpha 4\beta 2$ (HS) and at 30 μ M in recordings with $\alpha 3\beta 4$ and $\alpha 7$.

Physiologically-based toxicokinetic modeling

A physiology-based toxicokinetic (PBTK) model for DN-IMI was established in the Simcyp Simulator V20 (Certara) using a previously published approach (Albrecht et al., 2019). Due to the lack of published human metabolism and exposure data for DN-IMI, an analog approach was used to inform the PBTK model using a compound, in our case atenolol, with known human pharmacokinetics and similar physicochemical properties. The input parameters for DN-IMI and atenolol are given in table MS3_S8 and further details are found in fig. MS3_S9.

Data analysis

For the high-throughput Ca^{2+} -imaging data obtained in LUHMES cells, an offset correction using the FDSS software (version 3.2) was performed. Afterward, the data was exported and further analyzed with scripts written in R (version 3.6.3) (R Core Team, 2020). The concentration-response curves were fitted using a log-logistic model described by Ritz et al. (2015), utilizing the R package *drc* with its function *drm()* and *LL2.2()* with the following equation: $f(x) = d / [1 + \exp(b(\log(x) - \tilde{e}))]$ (Ritz et al., 2015). The logarithm of the half-maximal effective concentration ($\log\text{EC}_{50}$) between 0 and the upper limit (d), which was set to 1, is represented by $\tilde{e}$, x denotes the concentration, and b stands for the slope parameter (Ritz et al., 2015). In cases with normalizations to responses induced by other compounds, the function *LL2.3()* was used with a variable upper limit (d ; Ritz et al., 2015). The same equation was used to determine the half-maximal inhibitory concentration ($\log\text{IC}_{50}$). Then the $\log\text{EC}_{50}$ and $\log\text{IC}_{50}$ values were converted into the pIC_{50} and pEC_{50} values, which are the negative logarithms to base 10.

Concentration-effect responses in the SH-SY5Y $[Ca^{2+}]_i$, were analyzed by the GraphPad Prism8.0 software using the four-parameter sigmoidal curve fit settings and the concentrations giving 50% increase in $[Ca^{2+}]_i$ in relation to the nicotine response were estimated.

The single-cell Ca^{2+} -imaging recordings were exported and analyzed in Fiji ImageJ (version 1.52i) to get the average fluorescence signal of each cell. These signals were further analyzed in R, where a threshold detection was performed to detect responding cells. For this, the offset was corrected by subtracting the mean of 20% to 65% of the fluorescence signal of the pre-application period from the recording, to be robust against spontaneous activity. The threshold was defined as mean + 3 * SD of the negative control recordings, during the detection phase of 6.5 s during the application.

The baseline correction of voltage-clamp oocyte recordings were performed with the Roboocyte2+ software (version 1.4.3; Multi Channel Systems MCS, Germany). The maximal current influx and further analysis were executed in scripts written in R. In the antagonist experiments with oocytes, the maximal inward current was determined for the last response of each period (control, three antagonist concentrations, and washout).

The following R packages were utilized for data handling: cowplot (Wilke, 2019), dplyr (Wickham et al., 2020), drc (Ritz et al., 2015), ephys2 (Danker, 2018), ggplot2 (Wickham, 2016), htmlwidgets (Vaidyanathan et al., 2019), lemon (Edwards, 2019), magick (Ooms, 2020), magrittr (Bache and Wickham, 2014), matrixStats (Bengtsson, 2020), miniUI (Cheng, 2018), modelr (Wickham, 2020), multcomp (Hothorn et al., 2008), plotrix (Lemon, 2006), proto (Grothendieck et al., 2016), shiny (Chang et al., 2020), shinyjs (Attali, 2020), shinyTree (Trestle Technology, LLC, 2017), tidyverse (Wickham et al., 2019).

Unless mentioned differently, values are presented as means $\pm$ SEM. Experiments were usually performed with at least three technical replicates per condition. Detailed data on pEC₅₀, pIC₅₀, and numbers of experimental repetitions are given in supplementary tables. Unless mentioned differently, statistical significance was defined as P<0.05 and was determined by one-way ANOVA with Dunnett's post hoc test as indicated. To determine benchmark concentrations (BMC), and their upper and lower 95% confidence intervals (BMCL, BMCU), the BMC online software of UKN was used (Krebs et al., 2020).

3.3.4 Results and discussion

Effect of imidacloprid metabolites desnitro-imidacloprid (DN-IMI) and imidacloprid-olefin on LUHMES neurons.

LUHMES neurons express functional $\alpha 7$ and non- $\alpha 7$ nAChRs, and they have proven useful for the characterization of different neonicotinoids like IMI by high-throughput Ca^{2+} -imaging (Loser et al., 2021a). We used this system here for a functional characterization of the two IMI metabolites DN-IMI and IMI-olefin (Fig. MS3_1A). Both metabolites produced clear signals (Fig. MS3_1B, C). A quantification of $[\text{Ca}^{2+}]_i$ responses yielded a pEC_{50} of 6.6 for DN-IMI (Fig. MS3_1D). DN-IMI appeared at least as potent as ACh and nicotine (pEC_{50} values of ~ 6.0 in LUHMES neurons) (Loser et al., 2021a). These signaling data are in line with published binding data that suggest a similar affinity of DN-IMI and nicotine for mammalian nAChRs (D'Amour and Casida, 1999; Tomizawa et al., 2000; Tomizawa and Casida, 1999).

For IMI-olefin the pEC_{50} was not reached within the measurement range ($\leq 100 \mu\text{M}$), but a significant response (around 15% of the maximal response to DN-IMI) was found at 30-100 μM . Thus, the potency of IMI-olefin was similar to that of its parent compound IMI (Loser et al., 2021a). Our observations on signaling potency are consistent with literature data for differences in binding affinity to mammalian nAChRs (Chao and Casida, 1997; D'Amour and Casida, 1999; Tomizawa et al., 2000; Tomizawa and Casida, 1999).

The data on DN-IMI were confirmed by a different analytical method. Instead of the whole-culture-based high-throughput $[\text{Ca}^{2+}]_i$ assay, we used traditional time-lapse fluorescence microscopy to quantify responses of individual cells (Fig. MS3_1E). The percentage of responsive cells ($\sim 80\%$) was similar to that measured in a previous study for nicotine (Loser et al., 2021a). The quantification of single-cell responses confirmed the sub-micromolar potency of DN-IMI and suggested that the majority of all cells responded functionally to the IMI metabolite (Fig. MS3_1F).

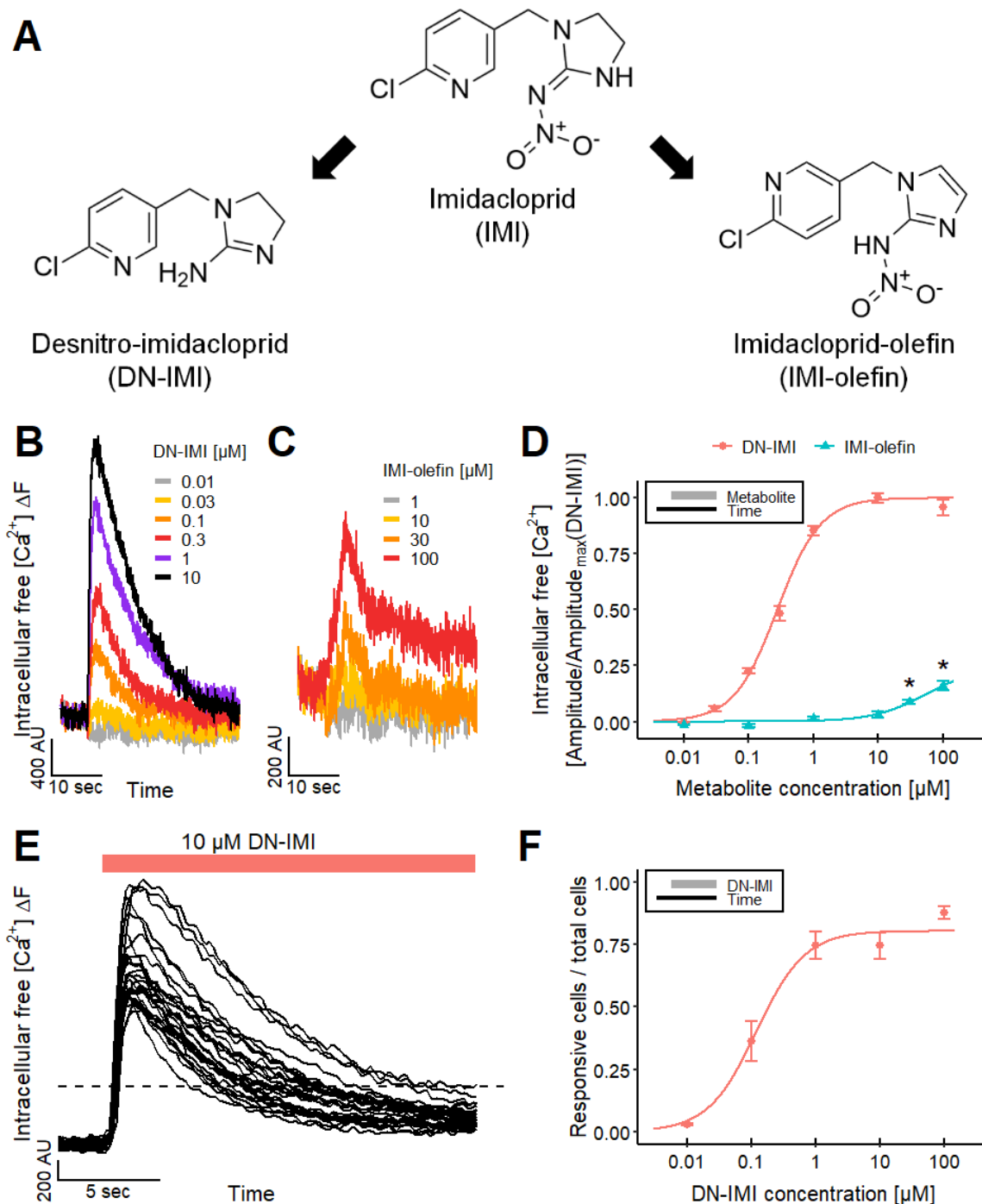


Fig. MS3_1: Effect of desnitro-imidacloprid (DN-IMI) and imidacloprid-olefin (IMI-olefin) on LUHMES neurons.

(A) Chemical structures of imidacloprid (IMI) and its two metabolites desnitro-imidacloprid (DN-IMI) and imidacloprid-olefin (IMI-olefin). (B-F) LUHMES neurons were differentiated for 9 days, before they were loaded with a $[Ca^{2+}]_i$ indicator dye and used for Ca^{2+} -imaging. (B, C) The cells cultivated in 384-well plates were exposed to various concentrations of DN-IMI and IMI-olefin, and exemplary recordings of the fluorescence signal from a whole well are shown. (D) The fluorescence data (peak amplitude) of multiple experiments were quantified and normalized to the maximal response stimulated by DN-IMI (means $\pm$ SEM are displayed). The significance of the responses triggered by IMI-olefin was determined between control recordings and the responses evoked by IMI-olefin (*: $p < 0.05$). For DN-IMI, a sigmoid curve was fitted to the data, and a pEC_{50} value of 6.6 ± 0.03 was obtained as a potency estimate. Note the treatment scheme

(upper left corner), illustrating the experimental design. (E, F) LUHMES cultures in 96-well plates were used to image the $[Ca^{2+}]_i$ responses of single cells with a fluorescent microscope. Regions of interest were assigned for all individual cell bodies in the image section. (E) Ca^{2+} -imaging traces of the responses of individual cell bodies are shown after exposure to DN-IMI (10 μ M). (F) The percentage of cells that responded with a clear fluorescence increase (= rise in $[Ca^{2+}]_i$) to different concentrations of DN-IMI was determined. Note the treatment scheme (upper left corner), illustrating the experimental design. Detailed data on n numbers are listed in table MS3_S6.

Activation of human $\alpha 7$ nAChR by DN-IMI

There is a large variety of nAChR subtypes with distinct functions in the nervous system. To get initial information, we examined whether the human $\alpha 7$ nAChR is affected by DN-IMI. This Ca^{2+} permeable receptor is widely distributed in the central nervous system and involved in the modulation of neurotransmitter release (Alkondon et al., 1999; Gotti et al., 2006; Gray et al., 1996; McGehee et al., 1995; Zoli et al., 2015). We utilized PNU-120596 (PNU), a selective positive allosteric modulator of the $\alpha 7$ nAChR, to slow down the $\alpha 7$ nAChR inactivation and enable thereby the detection of the $\alpha 7$ nAChR-mediated response in Ca^{2+} -imaging (Chatzidaki et al., 2015; Dickinson et al., 2007; Grønlien et al., 2007; Hurst et al., 2005; Larsen et al., 2019; Ng et al., 2007; Papke et al., 2009; Williams et al., 2011). The response of LUHMES neurons to DN-IMI was strongly enhanced and prolonged in the presence of PNU (Fig. MS3_2A). A quantification at multiple DN-IMI concentrations showed that this effect is less pronounced at sub-maximal receptor stimulation (Fig. MS3_2B). The maximal amplitude triggered by DN-IMI was increased by PNU by around 40%. This strongly suggests the activation of $\alpha 7$ nAChRs. These data are fully in line with findings showing the enhancement of neonicotinoid effects by PNU in LUHMES neurons (Loser et al., 2021a). The activation of non- $\alpha 7$ nAChRs at low concentrations of DN-IMI (0.03-0.3 μ M) is most likely the reason for an absence of PNU enhancement in the low concentration range.

To further support these findings, we examined the effect of DN-IMI on a second human cell system. SH-SY5Y neuroblastoma cells predominantly express the $\alpha 7$ nAChR subtype, together with $\alpha 3$ -containing receptors (Loser et al., 2021a). Therefore, they show little response to neonicotinoids or nicotine in the absence of PNU, and also DN-IMI only triggered small responses reaching about 44% of the maximal response obtained in the presence of PNU (Fig. MS3_2C). In this experimental setup (presence of PNU), DN-IMI led to a strong, concentration-dependent $[Ca^{2+}]_i$ response, with half-maximal responses at about 0.3 μ M, a peak at ~ 3 μ M, and declining responses at even higher concentrations (Fig. MS3_2C). The maximal response triggered by DN-IMI was

roughly similar to the one evoked by nicotine. The strong signal increase in the presence of PNU is in line with our results for LUHMES.

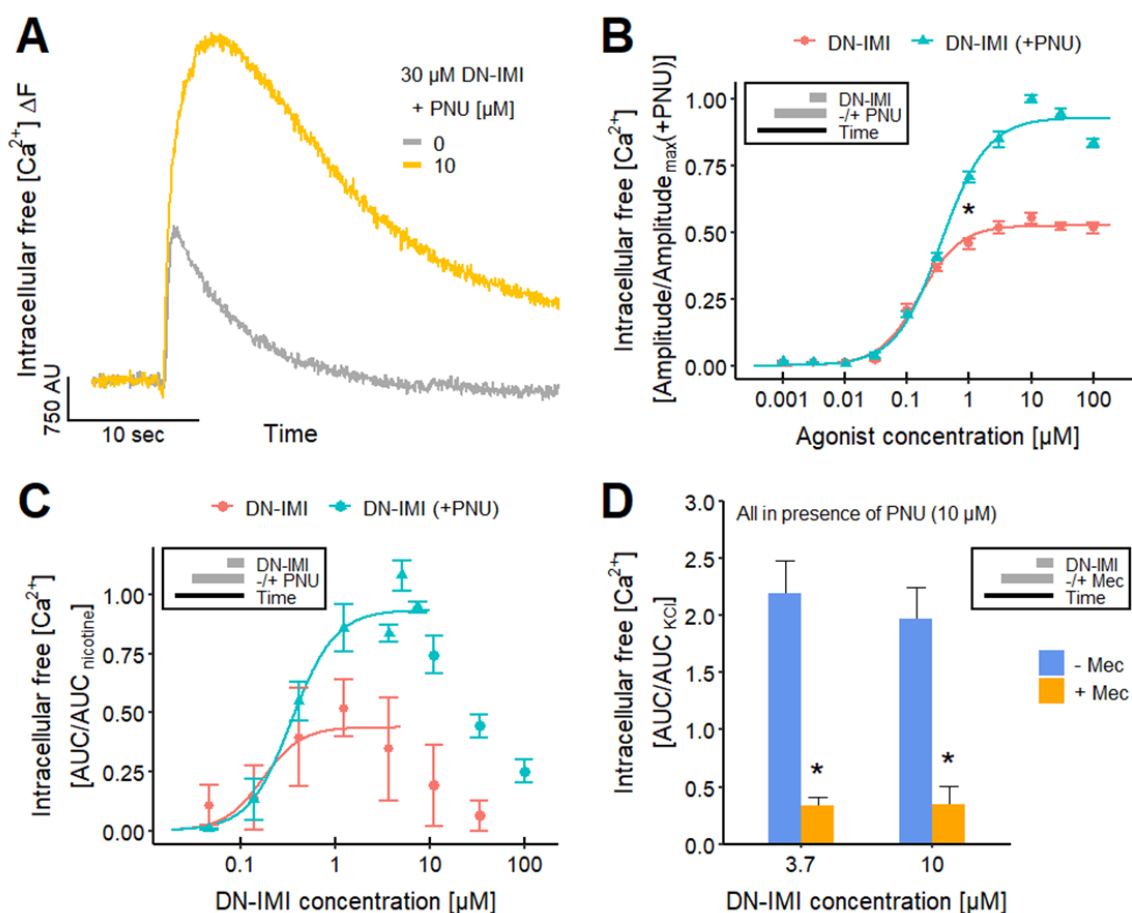


Fig. MS3_2: Activation of human $\alpha 7$ nAChRs on LUHMES and SH-SY5Y cells by DN-IMI.

(A, B) LUHMES neurons differentiated in 384-well plates were exposed to various concentrations of DN-IMI in the absence and presence of PNU-120596 (PNU, 10 μM), a selective positive allosteric modulator of $\alpha 7$ nAChRs. (A) Representative recordings of the Ca^{2+} -imaging fluorescence signal from a whole well are shown. (B) The fluorescence data (peak amplitude) of multiple experiments were quantified and normalized to the maximal response triggered by DN-IMI in the presence of PNU (means $\pm$ SEM are displayed). After sigmoidal curve fitting, the relative half maximum (turning point) was determined: they were on a $-\log(M)$ scale: 6.8 ± 0.04 in the absence of PNU and 6.5 ± 0.03 in the presence of PNU. The upper asymptote was at 53% of the maximal response (found in all experiments at all conditions) in the absence of PNU and at 93% in the presence of PNU. The significance of the difference between the effects of DN-IMI (1 μM) in the absence and presence of PNU was evaluated (*: $p < 0.05$). Detailed data on n numbers are found in table MS3_S6. (C, D) SH-SY5Y cells were used for automated $[Ca^{2+}]_i$ monitoring, with the area under the curve (AUC) of the fluorescence intensity as assay endpoint. Data were normalized to a reference signal (10 μM nicotine in C, 30 mM KCl in D). All data are from multiple experiments and are displayed as means $\pm$ SEM. (C). Data were obtained for multiple concentrations of DN-IMI in the absence and presence of PNU, and the ascending arms of the curves were fitted for concentrations $< 10 \mu M$. The sigmoidal curve fit yielded relative pEC_{50} s of 6.8 ± 0.36 in the absence of PNU (estimated maximum at ~ 0.44 , $n = 5$) and 6.5 ± 0.07 (0.3 μM , estimated maximum at ~ 0.93 , $n = 4$) in the presence of PNU. (D) The $[Ca^{2+}]_i$ response of SH-SY5Y cells triggered by DN-IMI (in the presence of PNU (10 μM)) was measured in the absence and presence

of the nAChR antagonist mecamylamine (Mec, 125 μ M) ($n = 5$); *: $p < 0.05$. Note the treatment schemes, illustrating the experimental design.

We also examined the effect of IMI-olefin in the absence and presence of PNU on $[Ca^{2+}]_i$ of LUHMES neurons (Fig. MS3_S1A), and we observed a strong enhancement of the signal. This allowed for the determination of a pEC_{50} (5.5 in the presence of PNU) (Fig. MS3_S1B). The significant increase of the responses indicates the activation of human $\alpha 7$ nAChRs by IMI-olefin, but with a significantly lower potency compared to DN-IMI.

Block of responses to DN-IMI by selective nAChR antagonists.

We used a pharmacological approach to verify that the signaling ($[Ca^{2+}]_i$) effect of DN-IMI is mediated exclusively by nAChRs. For this purpose, LUHMES cells were pretreated with several well-known nAChR antagonists. Tubocurarine (Tubo) (Jonsson et al., 2006) antagonized the responses evoked by DN-IMI with a pIC_{50} of 5.9 (Fig. MS3_3A, C). Tubo completely blocked the response at 100 μ M, indicating that the entire DN-IMI-evoked Ca^{2+} -signaling was mediated by nAChRs. The obtained pIC_{50} value is comparable to the values determined for several nAChR agonists in experiments with LUHMES (Loser et al., 2021a).

To further substantiate this finding, we utilized the non-competitive nAChR antagonist mecamylamine (Mec) (Capelli et al., 2011; Papke et al., 2008). It blocked the DN-IMI-induced response in LUHMES neurons with a pIC_{50} of 6.8 (Fig. MS3_3B, C), which is in line with literature data of 6.6 for human $\alpha 3\beta 2$ (Chavez-Noriega et al., 2000). We also used Mec in the SH-SY5Y cultures, and the $[Ca^{2+}]_i$ responses induced by DN-IMI were strongly blocked (Fig. MS3_2D) This confirmed that also in this cell model, DN-IMI signaling was strictly dependent on nAChRs.

To further investigate the agonism of DN-IMI on nAChRs on LUHMES, we researched the effect of the antagonist methyllycaconitine (MLA), which is highly potent (low nM range) on $\alpha 7$ nAChRs compared to other nAChR subtypes (Buisson et al., 1996; Capelli et al., 2011; Gopalakrishnan et al., 1995; Palma et al., 1996; Puchacz et al., 1994). MLA inhibited the response to DN-IMI with a pIC_{50} of 6.8 (Fig. MS3_3D, F), which is comparable to the value obtained for nicotine with LUHMES (Loser et al., 2021a). The pIC_{50} is similar to literature data for human $\alpha 4\beta 2$ and $\alpha 6$ -containing ($\alpha 6/3\beta 2\beta 3$) nAChRs (Capelli et al., 2011). This (relatively low) potency of MLA in LUHMES indicates the involvement of non- $\alpha 7$ nAChRs in the $[Ca^{2+}]_i$ response evoked by DN-IMI.

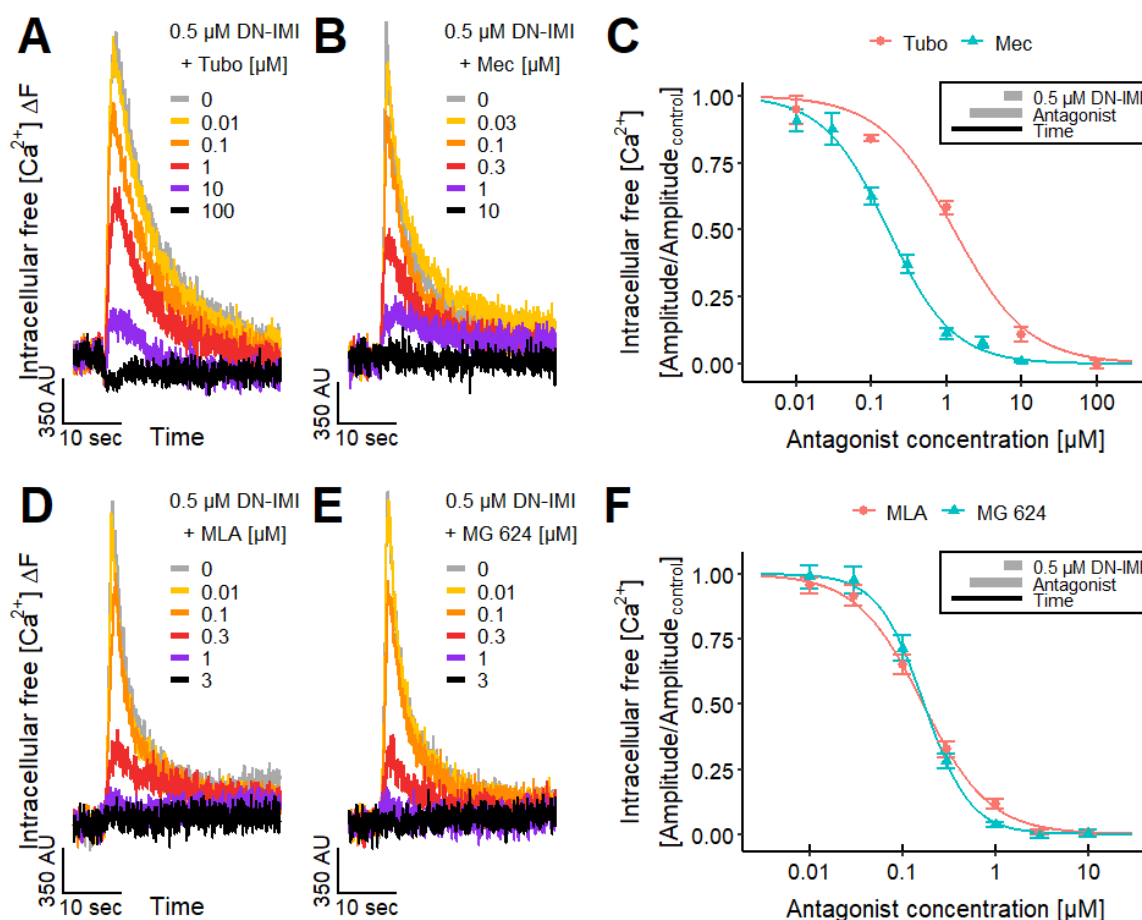


Fig. MS3_3: Block of DN-IMI signaling by nAChR antagonists.

LUHMES neurons differentiated in 384-well plates were pretreated with various concentrations of nAChR antagonists before DN-IMI (0.5 μM) was applied in Ca^{2+} -imaging experiments. (A, B) Exemplary recordings of the fluorescence signal from a whole well are shown for the effects of (A) tubocurarine (Tubo) and (B) mecamylamine (Mec) on the responses evoked by DN-IMI. (C) The fluorescence data (peak amplitude) of multiple experiments were quantified and normalized to control recordings (means $\pm$ SEM are displayed). After curve fitting, pIC_{50} values of 5.9 ± 0.05 for Tubo and 6.8 ± 0.03 for Mec were determined for their inhibitory effects on DN-IMI-induced $[\text{Ca}^{2+}]_i$ responses. Note the treatment scheme (upper right corner), illustrating the experimental design. (D, E) Exemplary recordings of the fluorescence signal from a whole well are shown for the effects of (D) MLA and (E) MG 624 on the $[\text{Ca}^{2+}]_i$ responses evoked by DN-IMI. (F) The fluorescence data (peak amplitude) of multiple experiments were quantified and normalized to control recordings (means $\pm$ SEM are displayed). After curve fitting, pIC_{50} s of 6.8 ± 0.03 for MLA and 6.8 ± 0.03 for MG 624 were determined. Note the treatment scheme (upper right corner), illustrating the experimental design. Detailed data on n numbers are found in table MS3_S6.

Finally, we applied the nAChR antagonist MG 624 (Capelli et al., 2011; Gotti et al., 2000) on LUHMES neurons. The resulting pIC_{50} of 6.8 (Fig. MS3_3E, F) is comparable to the pIC_{50} of nicotine obtained with LUHMES neurons (Loser et al., 2021a) and previously reported data for $\alpha 4\beta 2$, $\alpha 3\beta 4$, $\alpha 7$, and $\alpha 1\beta 1\delta \epsilon$ nAChRs (Capelli et al., 2011).

In summary, the antagonist data demonstrate the activation of nAChRs by DN-IMI. They indicate the involvement of different nAChR subtypes.

Modulation of cholinergic responses by DN-IMI and IMI-olefin.

An important feature of nAChRs is desensitization. This is the inactivation of the receptor during agonist exposure or upon closely-timed repeated agonist applications. Thus, even in the presence of an agonist, the receptor can stop the signaling and may not be activated again within a certain period after an initial stimulation (Arias et al., 2015; Campling et al., 2013; Capelli et al., 2011; Eaton et al., 2014; Fenster et al., 1997; Lester, 2004; Marks et al., 2010; Papke et al., 2011; Paradiso and Steinbach, 2003; Quick and Lester, 2002; Rollema et al., 2010; Rollema and Hurst, 2018). The desensitization of a receptor is typically caused by an agonist concentration that activates the receptor, but it can also occur at low concentrations that are not sufficient to activate it (Fenster et al. 1997; Paradiso and Steinbach 2003; Lester 2004; Rollema et al. 2010; Capelli et al. 2011; Arias et al. 2015; Rollema and Hurst 2018). As our previous results indicate an agonistic effect of both IMI metabolites, we investigated whether they would also desensitize the nAChRs on LUHMES neurons. In these experiments, the metabolites were pre-applied at various concentrations and then the response of LUHMES neurons was triggered by the exposure to nicotine and measured by Ca^{2+} -imaging. The pretreatment led to a pronounced reduction of the nicotinic signaling (Fig. MS3_4A, B). The corresponding concentration-response curves yielded pIC_{50} values of 6.9 for DN-IMI and 4.9 for IMI-olefin (Fig. MS3_4C). The pIC_{50} of IMI-olefin is comparable to the effects of its parent compound IMI and other neonicotinoids (Loser et al., 2021a). The pIC_{50} of DN-IMI is comparable to pIC_{50} values reported for the desensitizing effect of nicotine on human $\alpha 4\beta 2$, $\alpha 4\beta 4$, and $\alpha 3\beta 4$ nAChRs (Capelli et al., 2011; Fenster et al., 1997; Lester, 2004). Thus, DN-IMI was more potent than several neonicotinoids (pIC_{50} s of ~ 5.4) (Loser et al., 2021a) and IMI-olefin at attenuating the response evoked by nicotine. For confirmation of the desensitization in a different cell model, we used SH-SY5Y cells. Also here, pretreatment with DN-IMI reduced/abolished the response to nicotine in the submicromolar range. This effect was clearly more potent than the desensitization observed by IMI and another neonicotinoid pesticide, acetamiprid (Fig. MS3_S2). Thus, desensitization by neonicotinoids was confirmed in a second cell model, and the particularly high potency of DN-IMI was reproduced.

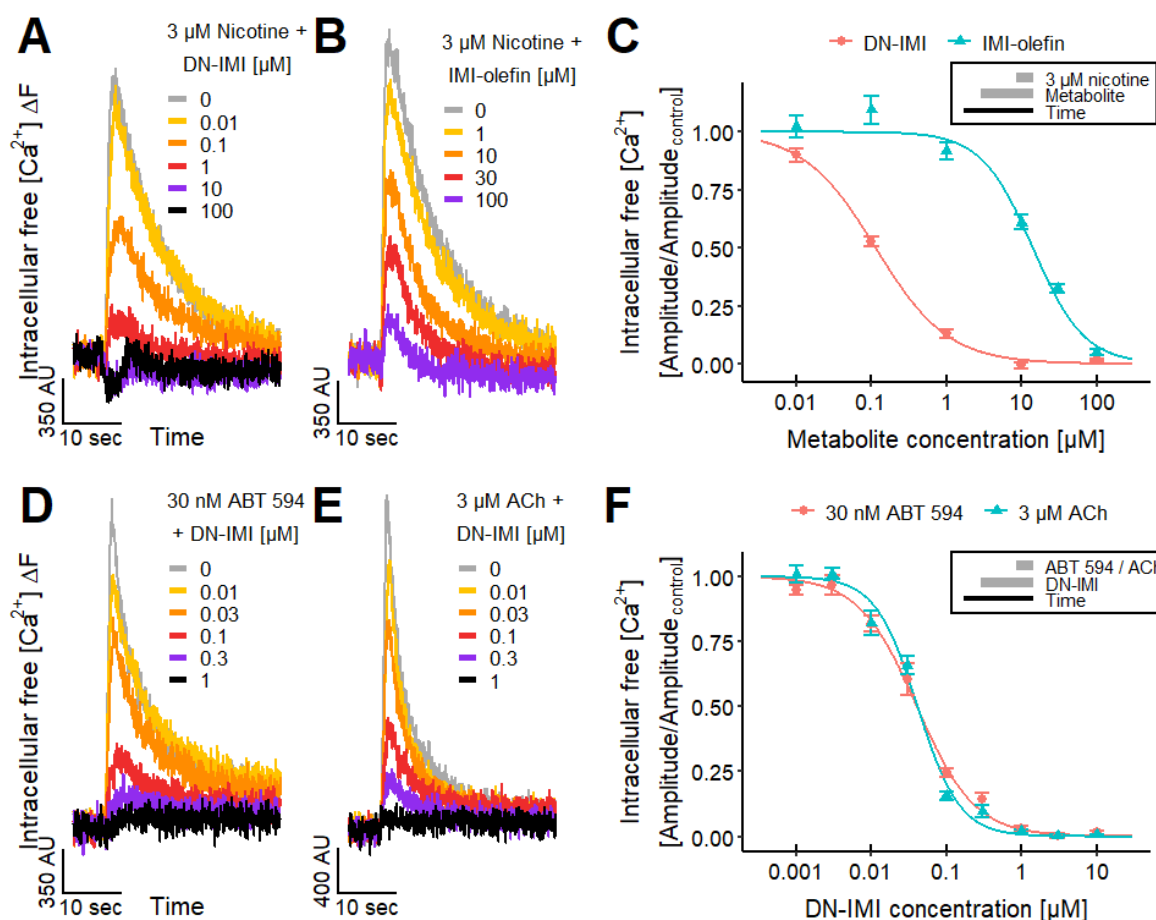


Fig. MS3_4: Desensitizing effects of DN-IMI and IMI-olefin on nAChRs

LUHMES neurons differentiated in 384-well plates were pretreated with various concentrations of DN-IMI or IMI-olefin for 4.5 min, before different nAChR agonists were applied and $[\text{Ca}^{2+}]_i$ signals were recorded. (A, B) Exemplary traces of the fluorescence signal from a whole well are shown for the desensitizing effects of different concentrations of (A) DN-IMI and (B) IMI-olefin on the $[\text{Ca}^{2+}]_i$ responses evoked by nicotine. (C) The fluorescence data (peak amplitude of 3 μM nicotine) of multiple experiments were quantified and normalized to control recordings (means $\pm$ SEM are displayed). After curve fitting, pIC_{50} values of 6.9 ± 0.03 for DN-IMI and 4.9 ± 0.03 for IMI-olefin were determined. Note the treatment scheme (upper right corner), illustrating the experimental design. (D, E) Exemplary traces of the fluorescence signal are shown for the effects of DN-IMI on the responses evoked by (D) 30 nM ABT 594 and (E) 3 μM ACh. (F) The fluorescence data (peak amplitude of the agonist stimulus) of multiple experiments were quantified and normalized to control recordings (means $\pm$ SEM are displayed). After curve fitting, pIC_{50} values of 7.4 ± 0.03 (ABT 594) and 7.4 ± 0.03 (ACh) were determined. Note the treatment scheme (upper right corner), illustrating the experimental design. Detailed data on n numbers are found in table MS3_S6.

To confirm that the desensitizing effect was not specific for nicotine stimulation, we used the endogenous nAChR agonist ACh and the selective non- $\alpha 7$ nAChR agonist ABT 594 for stimulation (Donnelly-Roberts et al., 1998; Michelmore et al., 2002) (Fig. MS3_4D, E). Here, we observed pIC_{50} values of ~ 7.4 for the desensitization (Fig. MS3_4F). This high potency is in agreement with other observations that desensitization of nAChR can occur at lower concentrations than required for stimulation (Fenster et al. 1997;

Paradiso and Steinbach 2003; Lester 2004; Rollema et al. 2010; Capelli et al. 2011; Arias et al. 2015; Rollema and Hurst 2018). The large difference in potency of DN-IMI and its parent compound IMI and other neonicotinoids is consistent with literature data for potency differences in binding assays with mammalian nAChRs (Chao and Casida, 1997; D'Amour and Casida, 1999; Tomizawa et al., 2000; Tomizawa and Casida, 1999). In summary, DN-IMI desensitized nAChRs in the nM range, and this may be of toxicological significance, as nAChR-signaling plays an important role in the central nervous system (Alkondon et al., 1999; Champtiaux et al., 2003; Gotti et al., 2006; Levin et al., 2006; Zoli et al., 2015).

Molecular docking studies of nicotine, IMI, and IMI metabolites on nAChR models

We were interested in how the particularly high potency of DN-IMI (as compared to other neonicotinoids) may be explained on the level of receptor binding. For this reason, we used the extensive data on nAChR structures to build models for molecular docking. Ligand binding sites of nAChRs are positioned at the interface of an alpha subunit (principal or + face) with the complementary or - face of another, adjacent subunit. Thus, a typical binding site is found at the interface of an $\alpha 4$ subunit and a $\beta 2$ subunit (Fig. MS3_5A), or between two $\alpha 7$ subunits. A more detailed characterization of the ligand-binding site showed that it is formed by six polypeptide loops (designated A-F) (Karlin, 2002). The amino acid side chains from these loops are forming a tightly packed "aromatic box" surrounding the agonist (Fig. MS3_5B) (Morales-Perez et al., 2016). A conspicuous structural element is loop C, which is stabilized by a disulfide bond, and winds around the ligand (Fig. MS3_5C, D) (Karlin, 2002; Morales-Perez et al., 2016). It undergoes ligand-induced conformational changes and contributes to steric constraints (S.-X. Li et al., 2011). Data on co-crystallized complexes (nAChR with ligands) suggest that several neonicotinoids occupy the upper (extracellular-oriented) part of the ligand-binding pocket in an essentially similar fashion as nicotine. This means that the heteroaryl moiety overlaps with the position of nicotine's pyridine ring. However, the nitroguanidine substructure occupies space that would normally be covered by loop C. This feature was very well reproduced by our dynamic docking model (Fig. MS3_5B). In this binding mode, the electronegative nitro- or cyano-groups are pointing towards the tip of loop C of the principal subunit, while the aryl moieties are pointing towards loop E from the complementary subunit. DN-IMI is lacking the electronegative and space-requiring nitro-functionality. Instead, it features a positively chargeable guanidine-functionality, embedded in the imidazolidine ring (Fig. MS3_5B). Our docking experiments at human nAChR structures suggest that this feature results in a flip of the imidazolidine-ring (relative to IMI), and therefore a much better alignment with the most likely poses of nicotine (Fig. MS3_5C).

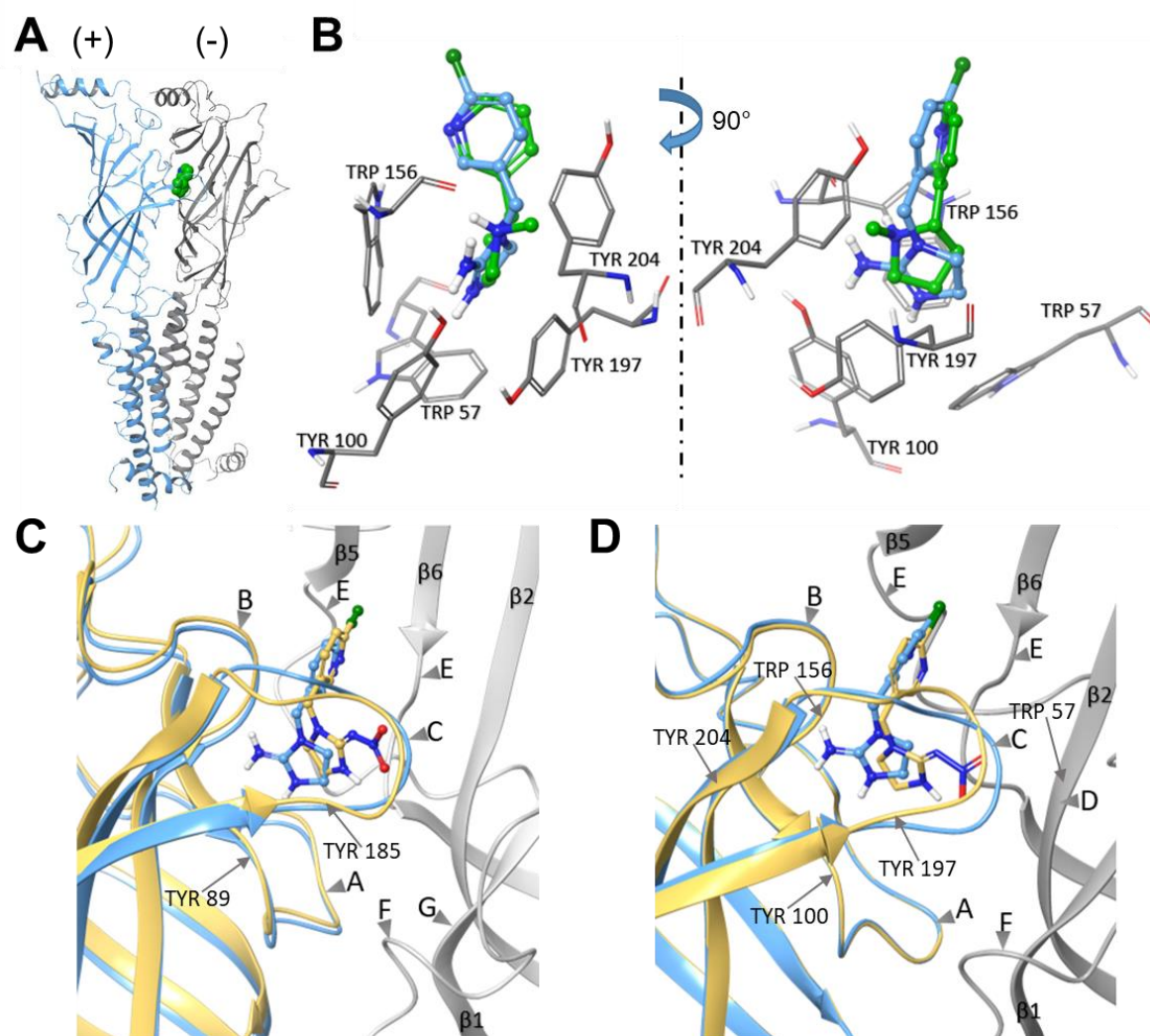


Fig. MS3_5: Molecular docking studies on nAChR structures.

(A) A 3D model of the human pentameric $\alpha 4 \beta 2$ nAChR in its ligand-bound state was established. A side view (top: extracellular part; bottom: intracellular part) of two of the five receptor subunits is shown. Nicotine (green) is shown at its binding site (based on legacy data of co-crystallization studies) between an α subunit (+; blue ribbon) and a β subunit (-; grey ribbon). (B) Details are shown of the nicotine binding site, with particular focus on the amino acids of the "aromatic box". Nicotine (green carbon atoms, ball-stick model) and DN-IMI (blue carbon atoms) are docked to the same site. Two view angles (turned by 90 degrees) are provided to show the close similarities in positioning and space requirements of the ligands. The amino features of both ligands are framed by residues from the aromatic box (a conserved protein structure amongst nAChRs). The canonical nomenclature of nAChR peptide backbone loops (shown in C, D) is used here for the localization of particular amino acids. Displayed amino acids from the principal binding site (α subunit) are Tyr100 (on loop A), Trp156 (on loop B), Tyr197, and Tyr204 (on loop C). From the complementary binding site loop D (β subunit), Trp57 is shown. Note that DN-IMI is adopting a binding mode that resembles the one of nicotine, concerning both, the orientation of the aryl moiety as well as the amino feature. (C) IMI (yellow carbon atoms; blue nitrogen- and red oxygen atoms) and DN-IMI (blue carbon atoms) in complex with the human nAChR $\alpha 4 \beta 2$ -structure. The structures were aligned based on the C_{α} -atoms. Note that the nitro-imidazolidine ring of IMI, with its nitro group, is facing towards the loop C (different from DN-IMI), while the pyridine rings of DN-IMI and IMI align well in their representative poses. IMI possibly interacts with Glu198 from loop C, and this interaction may reduce the affinity by steric hindrance and electrostatic repulsion.

(D) Data on AChBP have been used to model $\alpha 7$ nAChRs (as is common in the field). Data on IMI (yellow carbon atoms) and DN-IMI (blue carbon atoms), co-crystallized with AChBP were extracted from a structure database. The ligands were superimposed here, based on the C α -atoms. The protein ribbons of the principal side are shown in blue and yellow for the two structures used. The ribbon of one complementary face of the binding site is shown in light gray for a better view. Note that a very similar difference in binding poses can be noted for the $\alpha 7$ model (AChBP-based) as for the $\alpha 4\beta 2$ nAChR model (in C).

To obtain information on binding pockets from other nAChR subtypes, we used structural data on ACh binding protein (AChBP) from *Lymnaea stagnalis*. Good X-ray structures for this protein, also with several co-crystallized ligands are available and it is commonly accepted that this protein is a good model for mammalian $\alpha 7$ receptors (Ihara et al., 2008). Also here, neonicotinoids with an N-heteroaromatic ring (e.g., IMI) assume a binding mode that positions this structure in a very similar way as the pyridine ring of nicotine, while the nitroguanidine group faces loop C. Similar to the binding pocket of the $\alpha 4\beta 2$ receptor, the docking suggested a much more favorable position for DN-IMI, with the aminoguanidine group facing away from loop C (Fig. MS3_5D).

Molecular docking studies of such a complex target as the nAChR are not yet able to predict absolute binding affinities. However, there are several approaches to compare potential ligands. One such strategy is to calculate the ensemble of all likely docking poses for a ligand and to compare them with an optimal pose or with the exact positioning of a known ligand. For instance, it has been suggested that neonicotinoids may bind at mammalian receptors in the “common” mode or in a so-called “inverted” mode (pyridine moiety facing away from the central pore). In this inverted binding mode, the electronegative functionalities are located at the place of the N-heteroaromatic moieties in the common mode (Fig. MS3_S3B, C), and this appears energetically less favored (Tomizawa et al., 2008). Docking experiments on various receptor models suggested that the least potent neonicotinoids have the highest propensity to bind in the inverted mode (Loser et al., 2021a). Our docking experiments suggested that DN-IMI prefers the common binding mode compared to the inverted position. This preferential behavior is also seen for nicotine (Fig. MS3_5B). Docking scores were also obtained for IMI-olefin. The results suggest a similar behavior as that of IMI, i.e., there was a good fit with the binding site (in line with clear signaling data in LUHMES cells), but a predicted interference with loop C, showing less favorable contacts to amino acids from the aromatic box, which is also reflected in the worsened docking score and MM-GBSA binding energy approximate (Table MS3_S7) (in line with a lower potency than the one of DN-IMI and nicotine). The docking studies showed that the imidazolidine ring of IMI behaves similar to the imidazole ring (double bond between carbon atoms C4 and C5) of IMI-olefin. (Fig. MS3_S3D).

Besides the scoring of docking poses, some semi-quantitative data may be obtained by the calculation of proxies for binding energies. We evaluated via docking experiments and subsequent binding energy (MM-GBSA) calculations whether the inverted binding mode (more likely for IMI) shows differences in calculated binding energy compared to the common binding mode (more likely for DN-IMI and nicotine) (Fig. MS3_S3B, C). Indeed, the common binding mode ranked better (according to the “MM-GBSA dG bind” parameter). In addition, our approach showed for the $\alpha 4\beta 2$ model that IMI scored worse in the binding pose ranking than IMI-olefin. Moreover, the highest binding energy was predicted for DN-IMI (Table MS3_S7). However, some inconsistencies concerning nicotine still need to be explained. But this would require applying methods such as linear free energy calculations, which are much more time and computational cost-intensive than state-of-the-art docking approaches. As an alternative experimental approach to obtain data from individual specified receptors, we performed recordings in the *Xenopus laevis* oocyte expression system.

Activation of human $\alpha 4\beta 2$, $\alpha 7$, and $\alpha 3\beta 4$ nAChRs by DN-IMI.

To verify an agonistic effect of DN-IMI on the important neuronal nAChR subtypes $\alpha 4\beta 2$, $\alpha 7$, and $\alpha 3\beta 4$, we expressed each of them in *Xenopus laevis* oocytes and performed two-electrode voltage-clamp recordings (Fig. MS3_6A).

First, we focused our experiments on the $\alpha 4\beta 2$ receptor, which can assemble in two different stoichiometries. The high-sensitivity (HS) variant (two $\alpha 4$ subunits and three $\beta 2$ subunits) has been reported to have a $pEC_{50}(\text{ACh})$ of ~ 5.7 in the *Xenopus laevis* oocyte expression system, while the low sensitivity variant (three $\alpha 4$ subunits and two $\beta 2$ subunits) had a $pEC_{50}(\text{ACh})$ of ~ 4.1 (Benallegue et al., 2013; Bermudez and Moroni, 2006; Carbone et al., 2009; Harpsøe et al., 2011; Jonsson et al., 2006; P. Li et al., 2011; Mazzaferro et al., 2011; Mineur et al., 2009; Moroni et al., 2006). In our system, we found for the $\alpha 4\beta 2$ (HS) receptor a $pEC_{50}(\text{ACh})$ of ~ 5.7 (Fig. MS3_S4A, B). For nicotine, we found a pEC_{50} of 6.3, in line with literature data (Moroni et al., 2006). DN-IMI yielded a relative pEC_{50} of 6.3 (Fig. MS3_6B, C). The data show a high potency for this nAChR subtype; our data suggest that DN-IMI has a similar potency but slightly lower efficacy (64% of full stimulation) than nicotine (Fig. MS3_S4C, D). Its parent compound IMI did not trigger a concentration-dependent activation of the receptor in the tested concentration range ($\leq 100 \mu\text{M}$). For control purposes, we applied DN-IMI (30 μM) to *Xenopus laevis* oocytes without additional receptor expression (injection of water without mRNA). In this experimental setup, we did not detect any current responses ($n = 5$, data not shown). These findings show that DN-IMI only triggered inward currents via the activation of the heterologously expressed human nAChRs. This was further

confirmed by antagonist experiments, where the response of the human $\alpha 4\beta 2$ (HS) receptor to DN-IMI was concentration-dependently and reversibly blocked by the non-competitive nAChR antagonist Mec (Fig. MS3_S5).

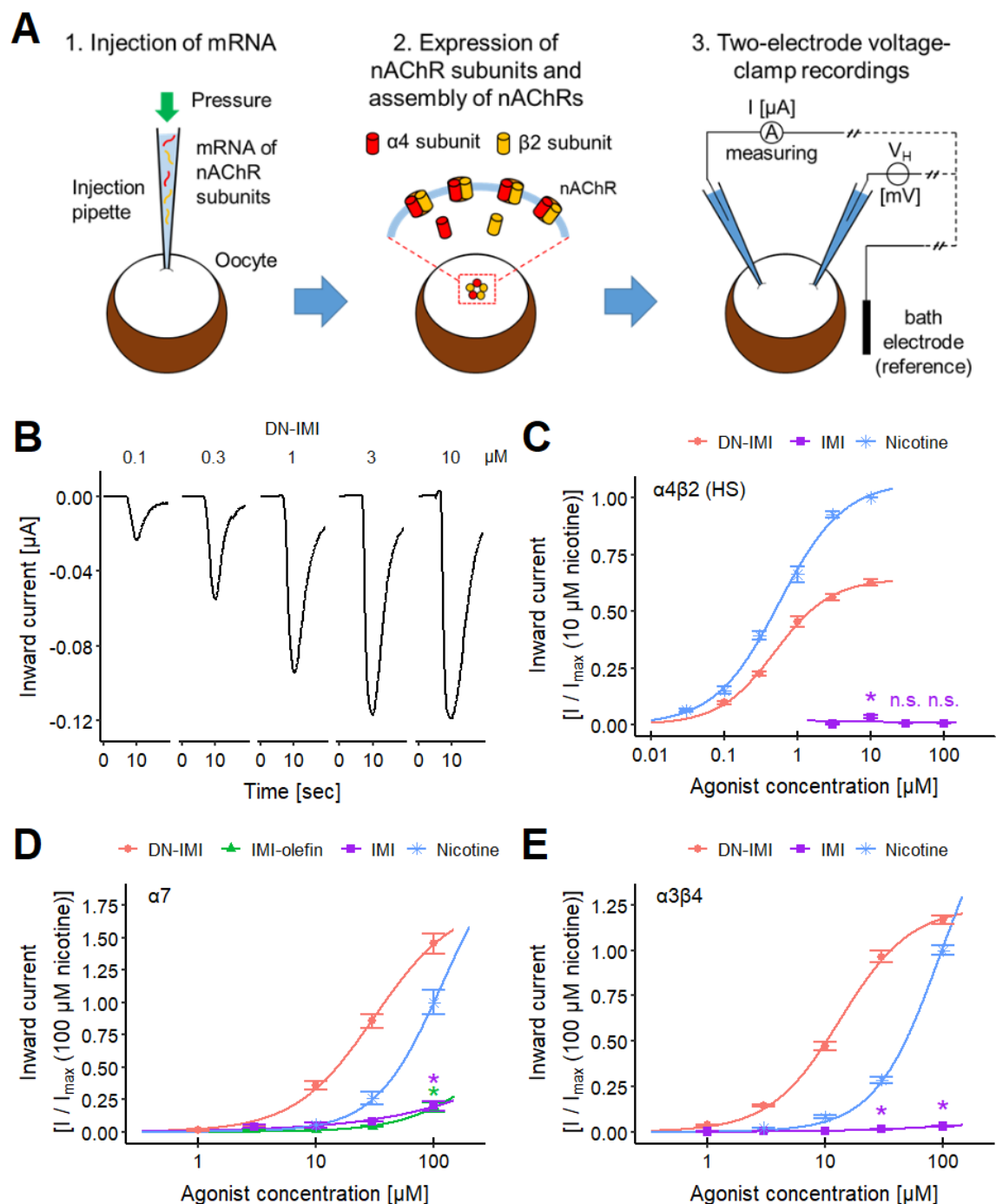


Fig. MS3_6: Effects of DN-IMI on human nAChR subtypes expressed by *Xenopus laevis* oocytes.

(A) The basic principle of the experiments with human nAChRs expressed by *Xenopus laevis* oocytes is presented. 1.) The genetic information (mRNA) of the respective nAChR subunits, in this example $\alpha 4$ (red) and $\beta 2$ (orange), is injected at the desired ratio (here: 1 ($\alpha 4$):10 ($\beta 2$)) into the oocytes. 2.) The oocytes are incubated for a few days to allow protein expression and

membrane integration as functional nAChRs 3.) The experiments were performed in two-electrode voltage-clamp recording mode. The agonist evoked inward current through the nAChRs was measured by the current electrode, while the membrane potential of the oocyte was kept constant ($V_H = -50$ mV) by a regulated voltage electrode and its reference electrode in the bath solution. (B) Increasing concentrations of DN-IMI were added to the bath solution, with washout phases between the recordings. Exemplary inward currents through human $\alpha 4\beta 2$ (HS) nAChRs are shown. Note that an excess of $\beta 2$ subunits was used here to generate pentameric receptors with two α subunits (designated here as high sensitivity (HS) variant, compared to receptors with > 2 α subunits). (C) The inward current data (amplitude) of human $\alpha 4\beta 2$ (HS) nAChRs expressed by *Xenopus laevis* oocytes of multiple experiments were quantified (means $\pm$ SEM are displayed). After curve fitting, relative pEC_{50} values (curve inflection point) of 6.3 ± 0.04 (estimated maximum amplitude at 64% of nicotine's) for DN-IMI and 6.3 ± 0.04 for nicotine were determined. The significance of the responses triggered by IMI was evaluated between the lowest concentration (3 μ M) and the other concentrations (*: $p < 0.05$; n.s., not significant). The inward current amplitudes were normalized to the response induced by nicotine (10 μ M). Exemplary current traces of DN-IMI and nicotine are shown in fig. MS3_S4C and MS3_S4D, respectively. (D) The inward current data (amplitude) of human $\alpha 7$ nAChRs expressed by *Xenopus laevis* oocytes of multiple experiments were quantified (means $\pm$ SEM are displayed), and after curve fitting relative pEC_{50} values of 4.5 ± 0.09 (estimated maximum of 83%) for DN-IMI and 3.9 ± 0.04 for nicotine were obtained. The significance of the responses triggered by IMI-olefin and IMI was evaluated between the lowest concentration (3 μ M) and the other concentrations (*: $p < 0.05$). The current amplitudes were normalized to the response induced by nicotine (100 μ M). Exemplary current traces of DN-IMI, IMI-olefin, and nicotine and the complete concentration-response curve for nicotine are shown in fig. MS3_S6. (E) The inward current data (amplitude) of human $\alpha 3\beta 4$ nAChRs expressed by *Xenopus laevis* oocytes of multiple experiments were quantified (means $\pm$ SEM are displayed), and after curve fitting relative pEC_{50} values of 4.9 ± 0.03 for DN-IMI and 4.0 ± 0.01 for nicotine were determined. The significance of the responses triggered by IMI was determined between the lowest concentration (1 μ M) and the other concentrations (*: $p < 0.05$). The current amplitudes were normalized to the response induced by nicotine (100 μ M). Exemplary current traces of DN-IMI, IMI, and the complete concentration-response curve for nicotine are shown in fig. MS3_S7C-F. Detailed data on n numbers are found in table MS3_S6.

To verify an agonistic effect of DN-IMI and IMI-olefin on human $\alpha 7$ nAChRs, we expressed this nAChR subtype in *Xenopus laevis* oocytes and performed two-electrode voltage-clamp recordings (Fig. MS3_6D & MS3_S6). DN-IMI had a relative pEC_{50} of 4.5 with a lower efficacy than nicotine (Fig. MS3_6D & MS3_S6A, D). Compared to nicotine, DN-IMI thus showed a slightly higher potency and a partial agonistic effect (81% of the maximal response to nicotine) on human $\alpha 7$ nAChRs, well in line with our Ca^{2+} -imaging data (Fig. MS3_2B, C). IMI-olefin and its parent compound IMI also stimulated significant inward currents but with a lower potency and efficacy than DN-IMI (Fig. MS3_6D & MS3_S6B, D). The results for IMI match our previous findings with LUHMES and SH-SY5Y neurons (Loser et al., 2021a). The application of nicotine yielded a pEC_{50} of 3.9 (Fig. MS3_6D & MS3_S6C, D), which is comparable to literature data (Briggs et al., 1995). As an internal consistency check, we performed antagonist experiments, where the DN-IMI-triggered response of the human $\alpha 7$ nAChR was concentration-dependently and reversibly blocked by the selective $\alpha 7$ receptor antagonist MLA (Fig. MS3_S5).

As a third approach, we investigated the effects of DN-IMI on human $\alpha 3\beta 4$ nAChRs expressed by *Xenopus laevis* oocytes. The application of nicotine and ACh resulted in pEC₅₀s of 4.0 and 3.8 (Fig MS3_6E & MS3_S7A, B, D), respectively, which are both comparable to literature data (Jonsson et al., 2006; Nelson et al., 2001; Wang et al., 1996). The addition of DN-IMI to oocytes expressing human $\alpha 3\beta 4$ nAChR yielded a relative pEC₅₀ of 4.9 (Fig. MS3_6E & MS3_S7C, D). IMI evoked small but significant inward currents in a concentration-dependent manner (Fig. MS3_6E & MS3_S7D-F). Responses of the human $\alpha 3\beta 4$ nAChR triggered by DN-IMI were concentration-dependently and reversibly blocked by the nAChR antagonist Tubo (Fig. MS3_S5). For the further characterization of DN-IMI on individual receptors, we investigated the effects of DN-IMI and IMI on the low-sensitivity variant of $\alpha 4\beta 2$ (LS) and on $\alpha 4\beta 4$ nAChRs. DN-IMI yielded a pEC₅₀ of 5.3 for $\alpha 4\beta 2$ (LS) and 5.5 for $\alpha 4\beta 4$ (Fig. MS3_S8). IMI did not trigger a concentration-dependent activation of these two nAChR subtypes in the tested concentration range (≤ 100 μ M).

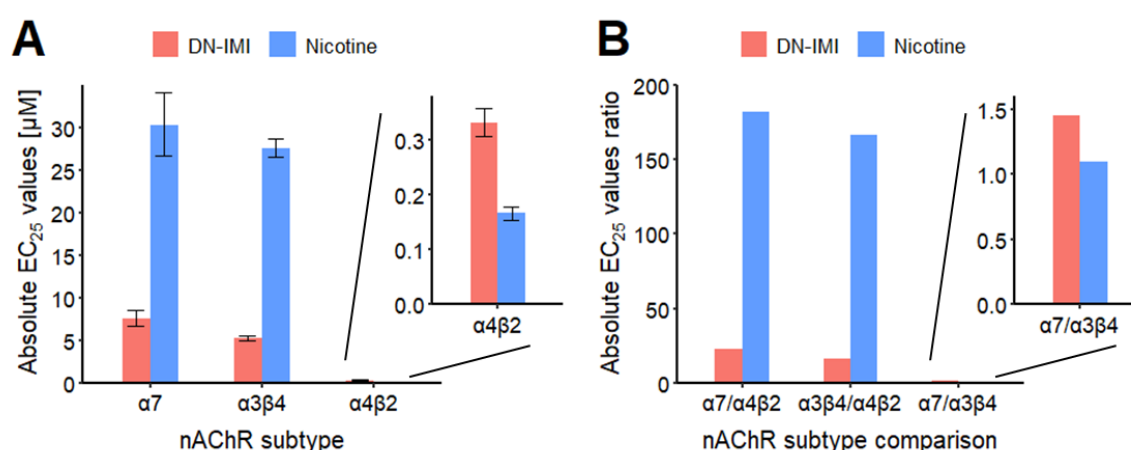


Fig. MS3_7: Comparative display of agonist potencies at nAChRs.

Oocyte recordings were performed, and data for nicotine and DN-IMI stimulations were normalized as in Fig. MS3_6. From the curve-fitted concentration-response data, EC₂₅ values were determined. (A) The absolute EC₂₅ values are shown for the effects on $\alpha 7$ (7.6 μ M by DN-IMI and 30.2 μ M by nicotine), $\alpha 3\beta 4$ (5.2 μ M by DN-IMI and 27.5 μ M by nicotine), and $\alpha 4\beta 2$ (HS) (0.33 μ M for DN-IMI and 0.17 μ M for nicotine) nAChRs. Note that the latter data set is shown as insert, because of the altered y-axis. (B) The ratios of the absolute EC₂₅ values between the nAChR subtypes $\alpha 7$, $\alpha 3\beta 4$, and $\alpha 4\beta 2$ (HS) are displayed for the effects of DN-IMI and nicotine. HS = high sensitivity variant of the receptor (two $\alpha 4$ subunits per receptor); note the different y-axis of the insert.

Having obtained data on nicotine and DN-IMI for nAChR subtypes, we used them for a comparison of their potencies. For this purpose, we determined the absolute EC₂₅ values (Fig. MS3_7A). These data suggest that DN-IMI and nicotine were about equipotent on

the $\alpha 4\beta 2$ (HS) nAChR (less than half a log step difference). On the other receptors, DN-IMI appeared slightly more potent than nicotine (about 0.6 log steps). To understand differences between experimental systems or possibly to predict toxicological consequences for brain areas with different receptor expression patterns, it was interesting to compare apparent (functional) receptor affinities: this showed that both ligands were more potent on the $\alpha 4\beta 2$ (HS) nAChR than on other subtypes (> 1 log-step for DN-IMI; > 2 log-steps for nicotine), while there was no difference between, e.g., $\alpha 7$ and $\alpha 3\beta 4$ (Fig. MS3_7B). This might explain mixed responses, e.g., on LUHMES cultures that express all these receptor types, and it provides an explanation for differences between, e.g., SH-SY5Y cells and LUHMES (the former cells predominantly express $\alpha 7$ receptors but also $\alpha 3$ -containing receptors (Loser et al., 2021a)).

In summary, the metabolite DN-IMI exhibits significantly higher potency and efficacy on the human nAChR subtypes than its parent compound IMI.

Exposure considerations and in vitro-to-in vivo comparisons.

While the above approaches inform on potential hazards by DN-IMI, the interpretation of the data and their use for risk assessment requires some understanding of concentrations to be reached in human tissues/body fluids. Due to the lack of more direct human data, we built a physiology-based toxicokinetic (PBTK) model to predict the plasma concentrations of DN-IMI. Because of the limited availability of human metabolism and exposure data for DN-IMI, the model construction was based on data from atenolol, a compound with similar physicochemical properties, and with well-known human pharmacokinetics. As DN-IMI-specific parametrization of the model, we used metabolic turnover data from human primary hepatocytes and physicochemical properties of DN-IMI as predictors for passive membrane permeability and protein binding (Table MS3_S8). As input (oral exposure), we used 0.016 mg DN-IMI/kg body weight. This amount corresponds to 10% of the value used earlier for IMI PBTK modeling (Loser et al., 2021a). Our rationale was that DN-IMI can reach about 10% of the IMI content in fruits, vegetables, and cereals (see the introduction for reference).

Under these conditions, the model predicted average plasma concentrations of around 50 nM and peak concentrations in a subfraction of the human population of at least 100 nM (Fig. MS3_S9A). The plasma concentrations predicted for atenolol from our PBTK model were in good agreement with measured data found in the literature (Fig. MS3_S9B). We see this as an indication of a good predictive capacity of our model. As the central nervous system is a main target tissue of DN-IMI, we also predicted brain concentrations. They were even slightly higher than the plasma concentrations (Fig. MS3_S9C). It is likely that the free diffusion of the compound through the blood-brain barrier also predicts a free distribution into the fetus. It is, therefore, reasonable to

assume that also fetal brains would be exposed to DN-IMI at concentrations up to the three-digit nM range.

BMC modeling of our $[Ca^{2+}]_i$ signaling and single nAChR data showed 20% response (in different systems) at about 100-300 nM of DN-IMI (Table MS3_S9 & MS3_S10). Such concentrations are close to the ones reachable in some subjects by dietary exposure. While such concentrations may not be reached for the average of the population, the gap between realistic internal exposure levels and the minimal effect concentration is less than 10-fold. This marginal safety buffer is eliminated, if receptor desensitization is considered as an effect parameter: the BMC for this endpoint was at ~17 nM (Table MS3_S11, for a 20% effect). Such concentrations may be reached by the consumption of food derived from crops treated with IMI. Notably, the desensitizing effect may be equally problematic for normal brain function and neuronal development, as the direct activation of the nAChRs.

To conclude these preliminary risk assessment considerations, it is important to consider that exposure to DN-IMI may also occur through the metabolism of IMI AFTER it has been ingested. From rodent experiments, it is clear that DN-IMI is generated after exposure to IMI, and that the endogenous metabolite DN-IMI distributes to the brain (Chao and Casida, 1997; Ford and Casida, 2006). Also, goat data suggest that IMI is converted to DN-IMI (about 25% of the IMI dose recovered in the liver) (Anon, 2006). In rabbits, DN-IMI was excreted in the urine after exposure to IMI (Vardavas et al., 2018), and this agrees well with human biomonitoring data that identified high (several-fold higher than IMI) levels of DN-IMI in urine (Wang et al., 2020).

If one assumes that 10% of ingested IMI is converted to DN-IMI, then the endogenously formed metabolite may reach levels of a similar magnitude as those generated from direct ingestion of the metabolite (assuming that the intake of IMI is 10 times higher than that of DN-IMI (input parameter of our PBTK model, based on food consumption data)).

Even though such considerations of potential internal exposure are consistent with literature data, they need to be considered as very preliminary. There is still considerable uncertainty on the human metabolism. It is not known which percentage of IMI is metabolized to DN-IMI within the liver and whether other tissues also contribute to the metabolism. The situation is complex, as several competing enzymes may oxidize or reduce IMI. Besides cytochrome P450 enzymes, there is evidence for the contribution of cytosolic aldehyde oxidases (Dick et al., 2005; Swenson and Casida, 2013). These enzymes show high species variation in their expression and activity (Dick et al., 2005; Pryde et al., 2010). Considering that humans express relatively high levels of aldehyde oxidase, data from animals cannot be easily translated to humans, and experiments are ongoing to better quantify IMI metabolism by different cell compartments.

3.3.5 Conclusion and outlook

The present study shows that the IMI metabolite DN-IMI potently (at sub-micromolar concentrations) affects human nAChRs. This was found both in neuronal cultures and in defined individual receptor subtypes expressed in *Xenopus laevis* oocytes. The evidence from all systems clearly indicates a much higher potency of DN-IMI relative to its parent compound IMI. The comparative data show that the desnitro metabolite is equipotent to nicotine, while another IMI metabolite, IMI-olefin, rather was equipotent to IMI. The study on DN-IMI showcases the role of metabolism for human neurotoxicology, as it demonstrates that a particular metabolite can be several orders of magnitude more potent as a neuronal signaling disrupter (desensitization) than its parent compound. This may have consequences for the risk assessment of the parent compound and for the need of additional data on metabolite generation in the environment and in man. Our preliminary modeling suggests that bioactive, potentially toxic DN-IMI concentrations may be reached by nutritional exposure in the normal (not professionally exposed) population.

Median lethal dose (LD₅₀) studies with mice showed that DN-IMI (LD₅₀: 6-24 mg/kg) is more toxic than its parent compound IMI (LD₅₀: 35-50 mg/kg) (Chao and Casida, 1997; Tomizawa et al., 2001, 2000) and IMI-olefin (no lethality at the highest tested dose of 50 mg/kg) (Chao and Casida, 1997). Little information is available on more subtle forms of neurotoxicity, and to our knowledge, no data are available on the potential developmental neurotoxicity of DN-IMI. The latter is important, considering that nicotine is an established developmental neurotoxicant (Aschner et al., 2017; LeSage et al., 2006; Levin et al., 1993). The former is relevant, as different nAChR subtypes are present on dopaminergic neurons and play an important role in the modulation of the electrical activity and neurotransmitter release (de Kloet et al., 2015; Grady et al., 1992; Mameli-Engvall et al., 2006; Quik and Kulak, 2002; Quik and Wonnacott, 2011; Rapier et al., 1988). Thus, substance-induced disturbances of nicotinic signaling can have an impact on the functioning, plasticity, and development of the dopaminergic system (Lozada et al., 2012; Miwa et al., 2011; Romoli et al., 2019; Slotkin et al., 2006; Stevens et al., 2003; Welsby et al., 2006; Wheeler and Cooper, 2004; Ziviani et al., 2011).

Several studies reported that the binding affinity of DN-IMI to mammalian or chicken nAChRs was similar to the affinity of nicotine and clearly higher than the one of its parent compound IMI (Chao and Casida, 1997; D'Amour and Casida, 1999; Tomizawa et al., 2000; Tomizawa and Casida, 2000, 1999). Our functional data, using a physiological signaling response in human neurons are in line with these observations. DN-IMI triggered [Ca²⁺]_i responses at concentrations ≥100 nM, i.e. it was at least two

orders of magnitude more potent than its parent compound (Loser et al., 2021a). These findings were further supported by oocyte recordings, which showed an agonistic effect of DN-IMI on human $\alpha 7$ and several non- $\alpha 7$ nAChRs. DN-IMI activated $\alpha 4\beta 2$ (HS) receptors at 20-fold lower concentrations than $\alpha 7$ and $\alpha 3\beta 4$ nAChRs. This potency difference on $\alpha 7$ and $\alpha 3\beta 4$ vs $\alpha 4\beta 2$ (HS) is also seen for nicotine. Such relative receptor preferences may be responsible for a selective toxicity on certain brain regions or neuronal functions, and future studies should also include assays on non-neuronal nAChR.

In desensitization experiments with LUHMES, the pretreatment with DN-IMI inhibited the subsequent activation of the nAChRs at concentrations ≥ 10 nM (BMR10, Table MS3_S11), which is ~ 70 times more potent compared to the effects of IMI (Loser et al., 2021a). The more potent desensitization effect of DN-IMI in comparison with IMI was confirmed in SH-SY5Y neurons (Fig. MS3_S2). After prolonged agonist exposure, nicotinic receptors desensitize by adopting a high-affinity and agonist-bound, non-conducting conformation (Morales-Perez et al., 2016; Nemecz et al., 2016). This may adversely affect normal neuronal function and neurodevelopment.

For adverse outcome pathways (AOP), it is important to understand the molecular initiating events both for parent compounds and also for the relevant metabolites formed (Leist et al., 2017). Until now, few such cases have been fully resolved, as the focus in neurotoxicology has either been on toxicants acting independent of metabolism, e.g., rotenone or vinca alkaloids (Delp et al., 2021, 2018b), or on compounds that act by a single toxic metabolite, without any effect of the parent, such as methylphenylpyridinium (Schildknecht et al., 2015; Terron et al., 2018) or methylmercury (Aschner et al., 2017). In many other cases, the target is little defined (e.g., for solvents or acrylamide). In this context, mechanistic studies on neonicotinoids and their metabolites should eventually provide an explanation for different potencies and activity spectra of all metabolites on various nAChRs. We progressed here some steps towards this vision, by including molecular docking studies, and by using them to provide a rationale for the experimental findings. The availability of several X-ray structures with co-crystallized neonicotinoids has facilitated the establishment of a robust docking model (Ihara et al., 2014, 2008; Loser et al., 2021a). In the present work, we focussed on the overlap of the pesticide N-heteroaromatic ring with the pyridine ring of nicotine. The comparison with published studies showed good accuracy of our model. Structural alignments of these complexes and docking studies at human nAChRs demonstrate that the electronegative moiety in IMI can contribute to a flip of the imidazolidine ring in the binding pocket. We demonstrate here, that this is less likely to happen with DN-IMI. This feature may explain its higher affinity/potency. Our binding hypothesis is supported by ranking via different docking scores, binding free energy approximates, and

comparisons of nicotinoids and neonicotinoids bound to homologous proteins. This gives a rationale for the functional differences of neonicotinoids and nicotine that were reported for cell experiments with LUHMES and SH-SY5Y cells (Loser et al., 2021a). These studies are still mainly qualitative, and their applicability domain is most likely narrow (applying only to the compounds of this study). However, our approach forms the basis for the development of a more powerful and refined model in the future. Eventually, this might then be able to quantitatively predict molecular initiating events (MIE) for the dozens of neonicotinoid metabolites found in food. Such a model might distinguish, e.g., high- vs. low-affinity ligands or discriminate between agonists and antagonists.

Further research is also needed to elucidate whether the signaling disturbances revealed here have lasting effects on neuronal function. It has been reported that other nAChR agonists (including nicotine) may affect nervous system plasticity and development (Levin et al., 1993; Lozada et al., 2012; Romoli et al., 2019; Slotkin et al., 2006; Welsby et al., 2006; Wheeler and Cooper, 2004; Ziviani et al., 2011). Epidemiological studies are quite scarce, but some general developmental/neurological effects have been reported for neonicotinoids used in agriculture or anti-tick sprays (Cimino et al., 2017). Some other compounds that evoke disturbances of neuronal network activity without causing structural changes have been reported to induce developmental neurotoxicity (DNT). Examples are 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), heroin, or nicotine (Aschner et al., 2017; Dwyer et al., 2009; LeSage et al., 2006; Levin et al., 1993; Slikker Jr et al., 2005; Slotkin et al., 2016). Moreover, compounds like for example methylmercury and lead can have severe effects on the developing brain, although they have a low neurotoxicity for adults (Grandjean and Landrigan, 2014). These examples make it conceivable that neonicotinoids and their metabolites such as DN-IMI may exhibit a DNT risk. However, a transfer of knowledge from one compound (e.g., nicotine) to others (e.g., DN-IMI) holds the risk of uncertainties (Rovida et al., 2020). Therefore, further mechanistic studies are needed to address the difficult question of a DNT hazard of DN-IMI and other neonicotinoid metabolites like a descyano metabolite of thiacloprid, which has also been reported to exhibit a higher affinity for mammalian and chicken nAChRs than its parent compound (Tomizawa et al., 2000; Tomizawa and Casida, 2000).

3.3.6 Acknowledgments

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3.3.7 Conflict of interest

The authors declare no conflict of interest.

3.3.8 Author contributions

DL: Conceived of or designed the study, performed research, analyzed data, and wrote the paper. KG, JB: Performed research, analyzed data, and wrote the paper. MH, YJ, CB, PW: Performed research and analyzed data. YH, GE, AN, TD, CM, IG: Contributed new methods or models. AF, SB, UK, ML: Conceived of or designed the study and wrote the paper.

3.3.9 Supplementary information

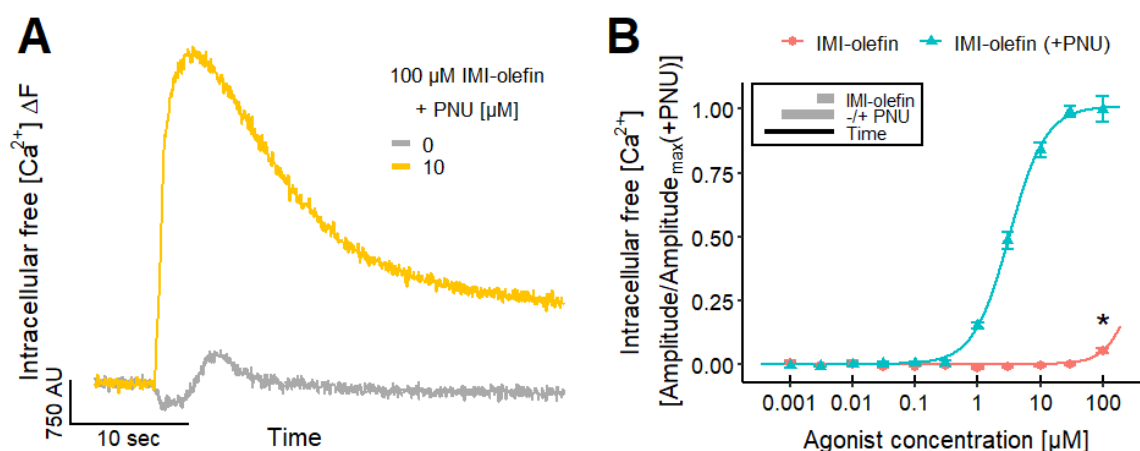


Fig. MS3_S1: Activation of human $\alpha 7$ nAChRs on LUHMES neurons by IMI-olefin.

LUHMES neurons were cultivated in 384-well plates and used for Ca^{2+} -imaging. **(A)** The cells were exposed to various concentrations of IMI-olefin in the absence and presence of PNU-120596 (PNU, 10 μM), and exemplary recordings of the fluorescence signal from a whole well are shown. **(B)** The fluorescence data (peak amplitude) of multiple experiments were quantified and normalized to the maximal response triggered by IMI-olefin in the presence of PNU (means $\pm$ SEM are displayed), and after curve fitting a pEC_{50} value of 5.5 ± 0.03 was determined for IMI-olefin in the presence of PNU. The significance of the responses triggered by IMI-olefin was evaluated between the responses evoked by IMI-olefin in the absence and presence of PNU (*: $p < 0.05$). Note the treatment scheme (upper left corner), illustrating the experimental design. Detailed data on n numbers are found in table MS3_S6.

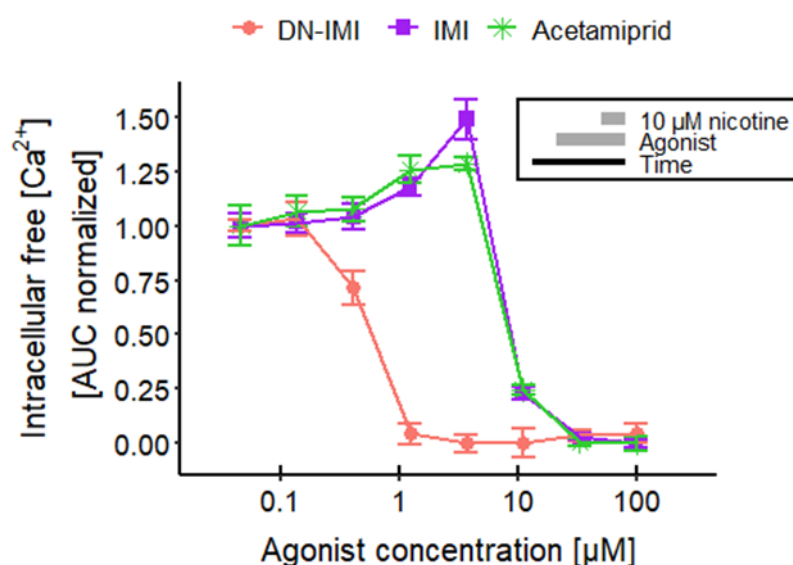


Fig. MS3_S2: Modulation of cholinergic responses of SH-SY5Y cells by DN-IMI, IMI, and acetamiprid.

SH-SY5Y cells were pretreated with various concentrations of DN-IMI, IMI, and acetamiprid for 20 min, before nicotine (10 μM) was applied in the presence of PNU-120596 (PNU, 10 μM) and the $[\text{Ca}^{2+}]_i$ response was recorded. The area under the curve (AUC) was determined for the fluorescence intensity of the responses triggered by nicotine. The data were normalized to the $[\text{Ca}^{2+}]_i$ responses stimulated by nicotine in the presence of the lowest agonist concentration. No curve fitting was performed. Note the treatment scheme (upper right corner), illustrating the experimental design.

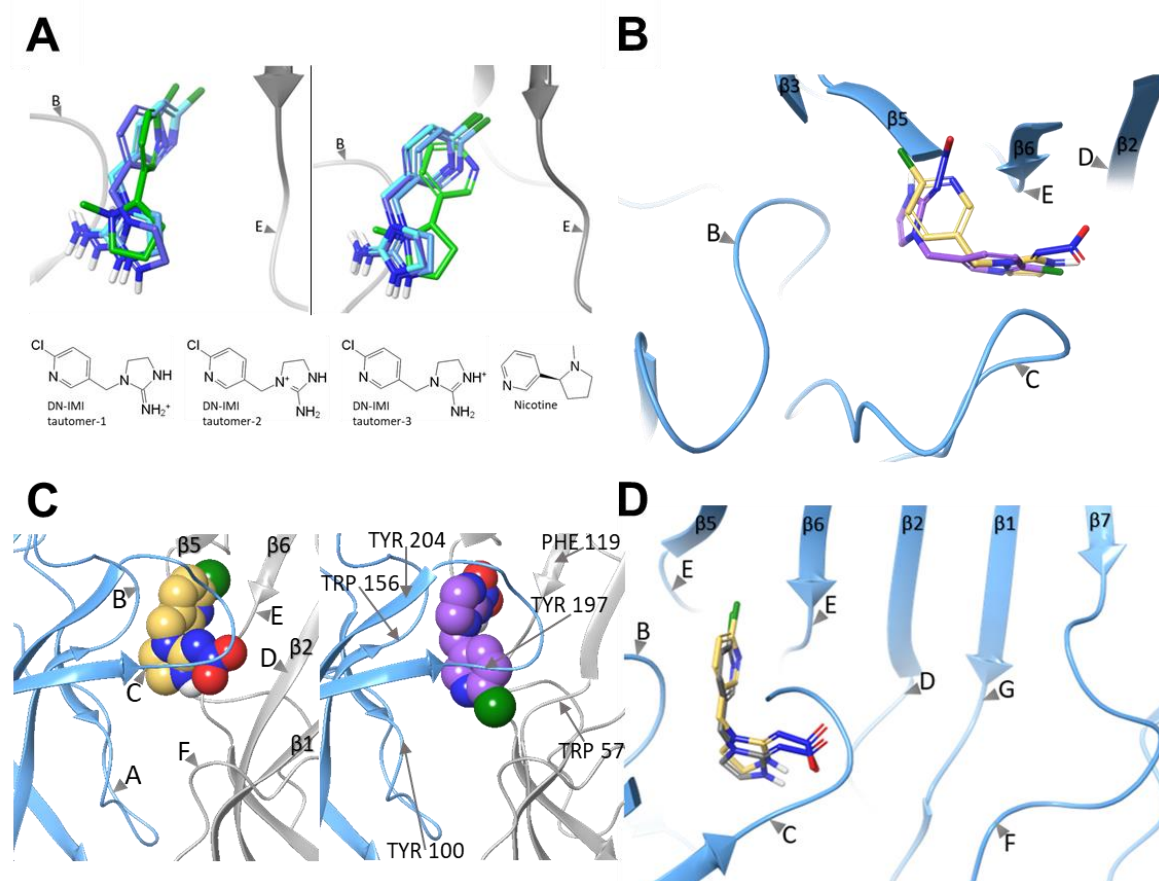


Fig. MS3_S3: Molecular docking studies.

(A) The IFD poses of DN-IMI in human $\alpha 4\beta 2$ nAChR (grey ribbon) before (top left) and after (top right) the MMG-BSA incorporated minimization step are displayed, together with the chemical structures of the presented DN-IMI tautomers (at the bottom from left to right: tautomer 1 (turquoise), tautomer 2 (medium blue), tautomer 3 (dark blue)) and nicotine (bottom right, green). The different tautomer states of the guanidine moiety embedded in the imidazolidine ring and their effect on the docking score, MMG-BSA energy, and binding orientation in $\alpha 4(+)\beta 2(-)$ -binding site were investigated (Table MS3_S7). After the MMG-BSA applications, some torsion angles were optimized for a better alignment of the DN-IMI poses. **(B)** IMI-olefin is shown in the common (yellow) and inverted (purple) binding mode in the binding pocket of the human $\alpha 7$ nAChR (blue ribbon). **(C)** IMI is displayed in the common (yellow) and inverted (purple) binding mode in the binding pocket of the human $\alpha 4\beta 2$ nAChR. The ribbon of one complementary face of the binding site is shown in light gray for a better view. **(D)** IMI (yellow) and IMI-olefin (gray) bind in the common binding mode to the structure of the human $\alpha 4\beta 2$ nAChR (blue ribbon). MMG-BSA binding energy calculations predicted the inverted binding mode of IMI to be less favorable for the overall thermodynamic state of the protein-ligand complex compared to the common binding mode in the $\alpha 4\beta 2$ - and $\alpha 7$ -binding site (Table MS3_S7).

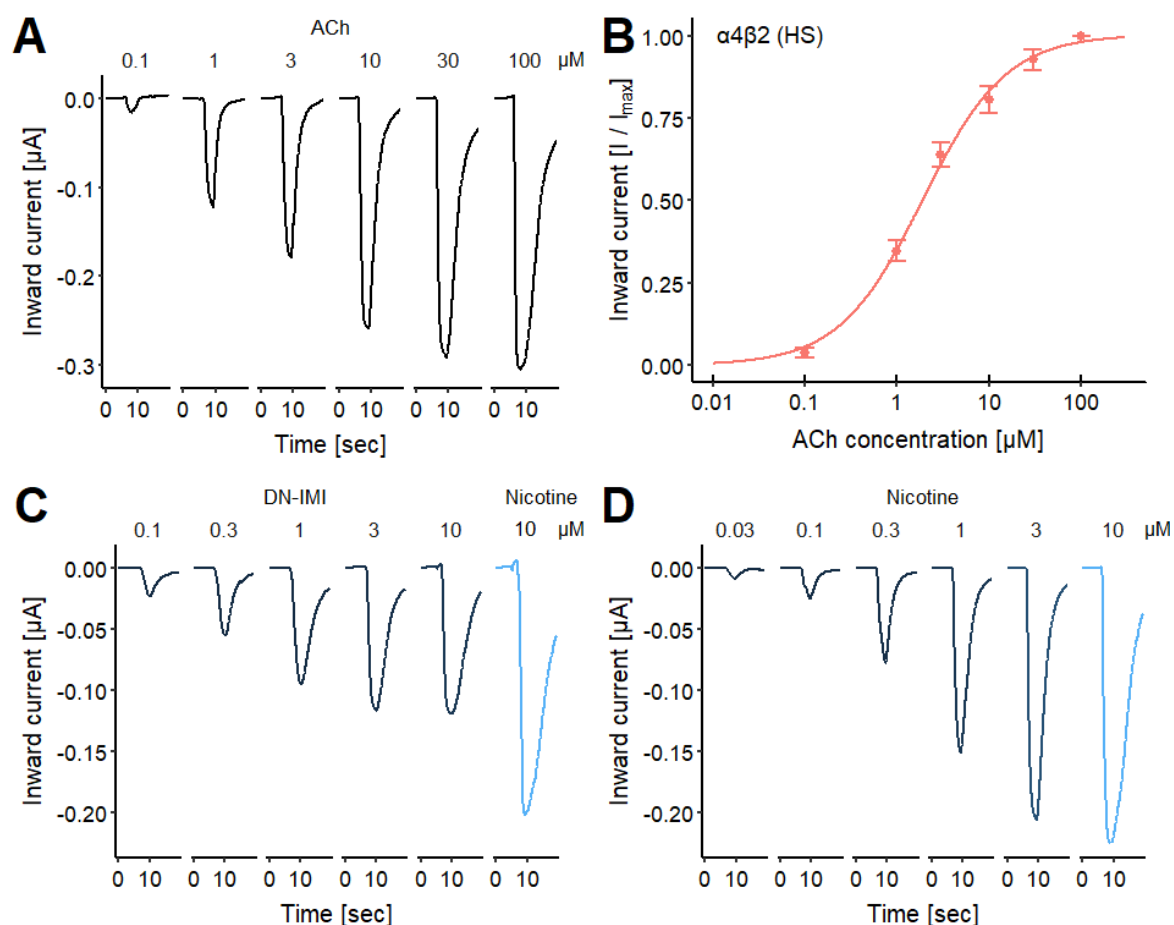


Fig. MS3_S4: Effects of ACh, DN-IMI, and nicotine on human $\alpha 4 \beta 2$ (HS) nAChRs expressed by *Xenopus laevis* oocytes.

(A) Exemplary inward currents through human $\alpha 4 \beta 2$ (HS) nAChRs expressed by *Xenopus laevis* oocytes stimulated by various ACh concentrations are shown. **(B)** The inward current data (amplitude) of human $\alpha 4 \beta 2$ (HS) nAChRs expressed by *Xenopus laevis* oocytes of multiple experiments were quantified (means $\pm$ SEM are displayed), and after curve fitting a pEC₅₀ value of 5.7 ± 0.06 for ACh was determined. **(C, D)** Exemplary inward currents through human $\alpha 4 \beta 2$ (HS) nAChRs expressed by *Xenopus laevis* oocytes triggered by various concentrations of **(C)** DN-IMI and **(D)** nicotine are shown together with the response to a final reference application of nicotine (10 μ M, reference response (blue)).

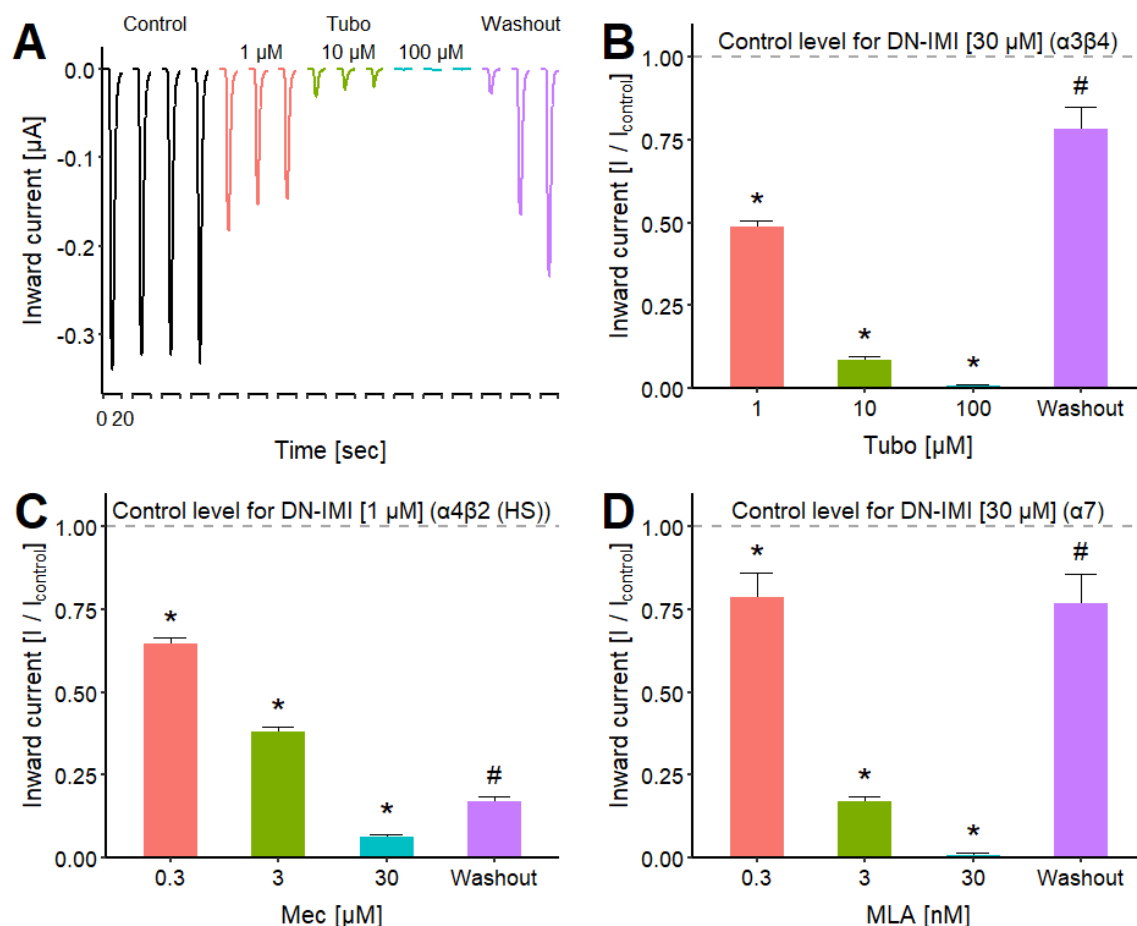


Fig. MS3_S5: Inhibition of DN-IMI-induced current responses of human nAChRs expressed by *Xenopus laevis* oocytes.

(A) Exemplary traces of inward currents of the human $\alpha 3\beta 4$ nAChR triggered by DN-IMI (30 μM) during control, in the presence of different concentrations of the nAChR antagonist Tubo, and during washout are shown. **(B)** The inhibitory effect of Tubo on the inward current amplitude of the human $\alpha 3\beta 4$ nAChR evoked by DN-IMI (30 μM) was evaluated. **(C)** The inhibitory effect of the non-competitive nAChR antagonist Mec on the inward current amplitude of the human $\alpha 4\beta 2$ (HS) nAChR triggered by DN-IMI (1 μM) was determined. **(D)** The antagonistic effect of the potent $\alpha 7$ nAChR blocker MLA on the inward current amplitude of the human $\alpha 7$ nAChR stimulated by DN-IMI (30 μM) was evaluated. The significance of the inward current block was determined between control recordings and recordings in the presence of the antagonist (*: $p < 0.05$). The significance of the inward current increase after washout of the antagonist was evaluated between recordings of the highest antagonist concentration and recordings during washout (#: $p < 0.05$). Detailed data on n numbers are found in table MS3_S6.

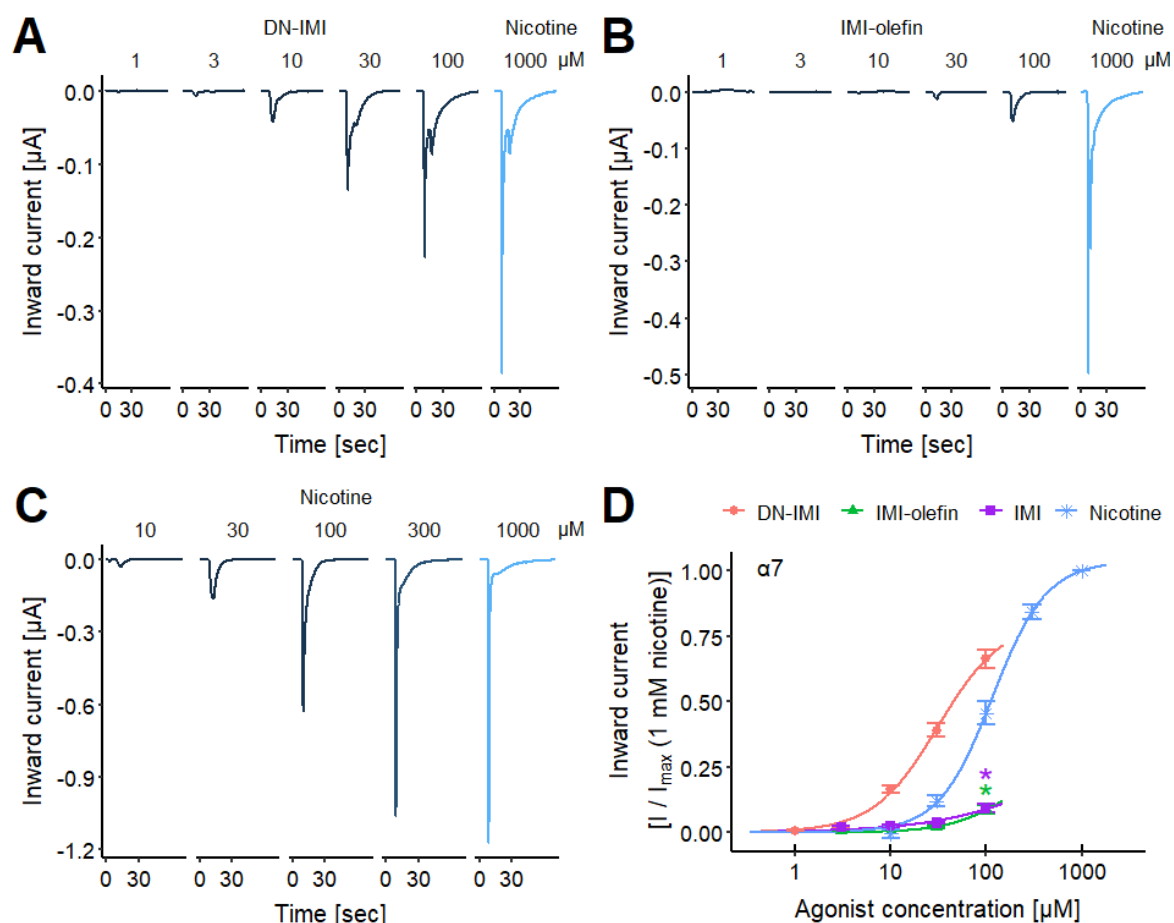


Fig. MS3_S6: Effects of DN-IMI, IMI-olefin, IMI, and nicotine on human $\alpha 7$ nAChRs expressed by *Xenopus laevis* oocytes.

(A-C) Exemplary inward currents through human $\alpha 7$ nAChRs expressed by *Xenopus laevis* oocytes stimulated by various concentrations of (A) DN-IMI, (B) IMI-olefin, and (C) nicotine are shown together with the response to a final reference application of nicotine (1 mM, reference response (blue)). (D) The inward current data (amplitude) of human $\alpha 7$ nAChRs expressed by *Xenopus laevis* oocytes of multiple experiments were quantified (means $\pm$ SEM are displayed), and after curve fitting relative pEC_{50} values of 4.5 ± 0.09 (estimated maximum ~ 0.81) for DN-IMI and 3.9 ± 0.04 (estimated maximum ~ 1) for nicotine were determined. The significance of the responses triggered by IMI-olefin and IMI was evaluated between the lowest concentration (3 μ M) and the other concentrations (*: $p < 0.05$). The current amplitudes were normalized to the response induced by nicotine (1 mM) for each oocyte. Detailed data on n numbers are found in table MS3_S6.

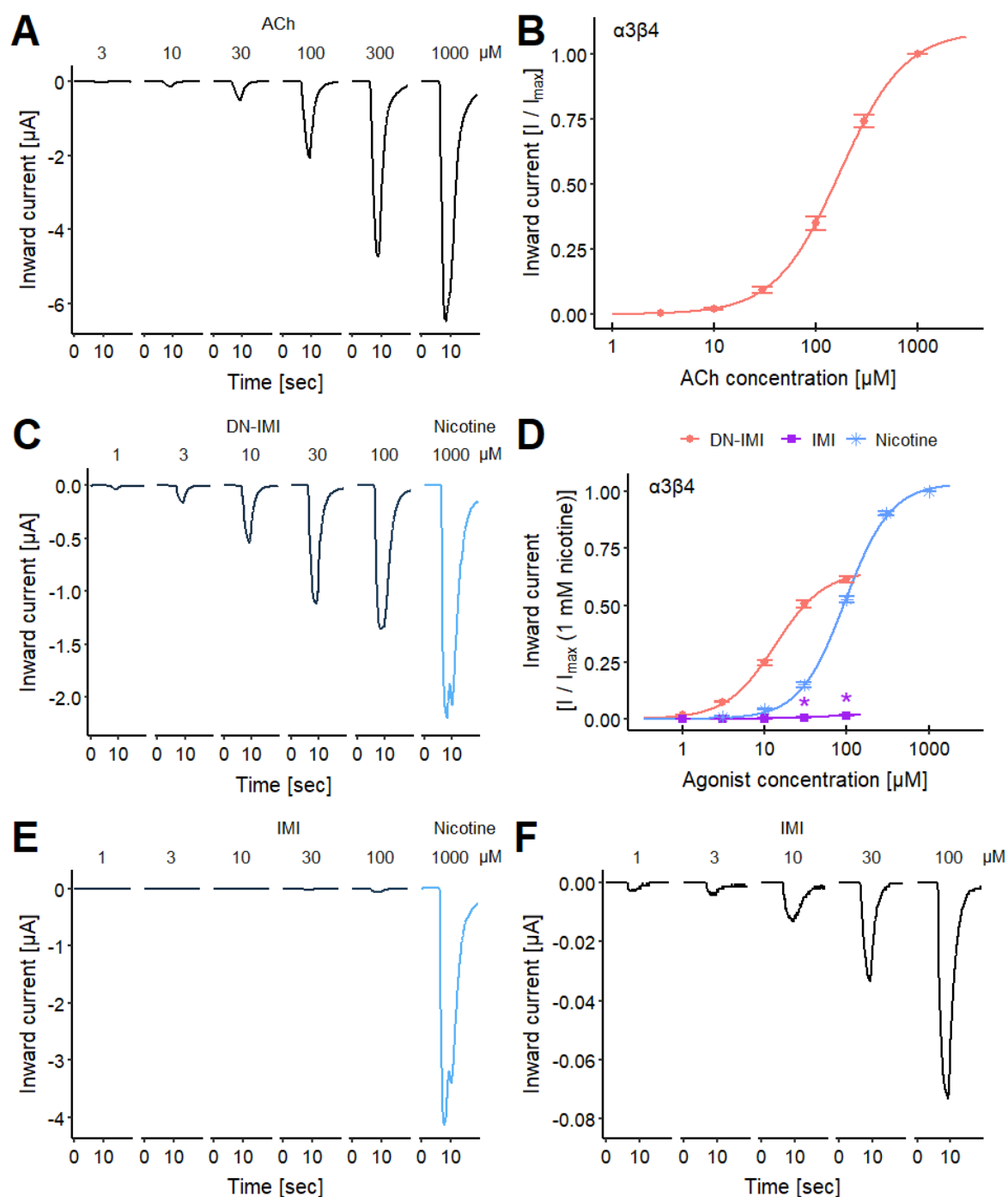


Fig. MS3_S7: Effects of ACh, DN-IMI, IMI, and nicotine on human $\alpha 3\beta 4$ nAChRs expressed by *Xenopus laevis* oocytes.

(A) Exemplary inward currents through human $\alpha 3\beta 4$ nAChRs expressed by *Xenopus laevis* oocytes stimulated by various ACh concentrations are presented. (B) The inward current data (amplitude) of human $\alpha 3\beta 4$ nAChRs expressed by *Xenopus laevis* oocytes of multiple experiments were quantified (means $\pm$ SEM are displayed), and after curve fitting a relative pEC_{50} value of 3.8 ± 0.03 for ACh was determined. (C) Exemplary inward currents through human $\alpha 3\beta 4$ nAChRs expressed by *Xenopus laevis* oocytes triggered by various concentrations of DN-IMI are shown together with the response to a final reference application of nicotine (1 mM, reference response (blue)). (D) The inward current data (amplitude) of human $\alpha 3\beta 4$ nAChRs expressed by *Xenopus laevis* oocytes of multiple experiments were quantified (means $\pm$ SEM are displayed), and after

curve fitting relative pEC₅₀ values of 4.9 ± 0.03 (estimated maximum ~ 0.65) for DN-IMI and 4.0 ± 0.01 (estimated maximum ~ 1) for nicotine were determined. The significance of the responses triggered by IMI was evaluated between the lowest concentration (1 μ M) and the other concentrations (*: $p < 0.05$). The current amplitudes were normalized to the response induced by nicotine (1 mM) for each oocyte. **(E, F)** Exemplary inward currents through human $\alpha 3\beta 4$ nAChRs expressed by *Xenopus laevis* oocytes triggered by various concentrations of IMI are shown with a y-axis adjusted to **(E)** the amplitude of the response to a final reference application of nicotine (1 mM, reference response (blue)) and **(F)** the highest current amplitude stimulated by IMI (100 μ M). Detailed data on n numbers are found in table MS3_S6.

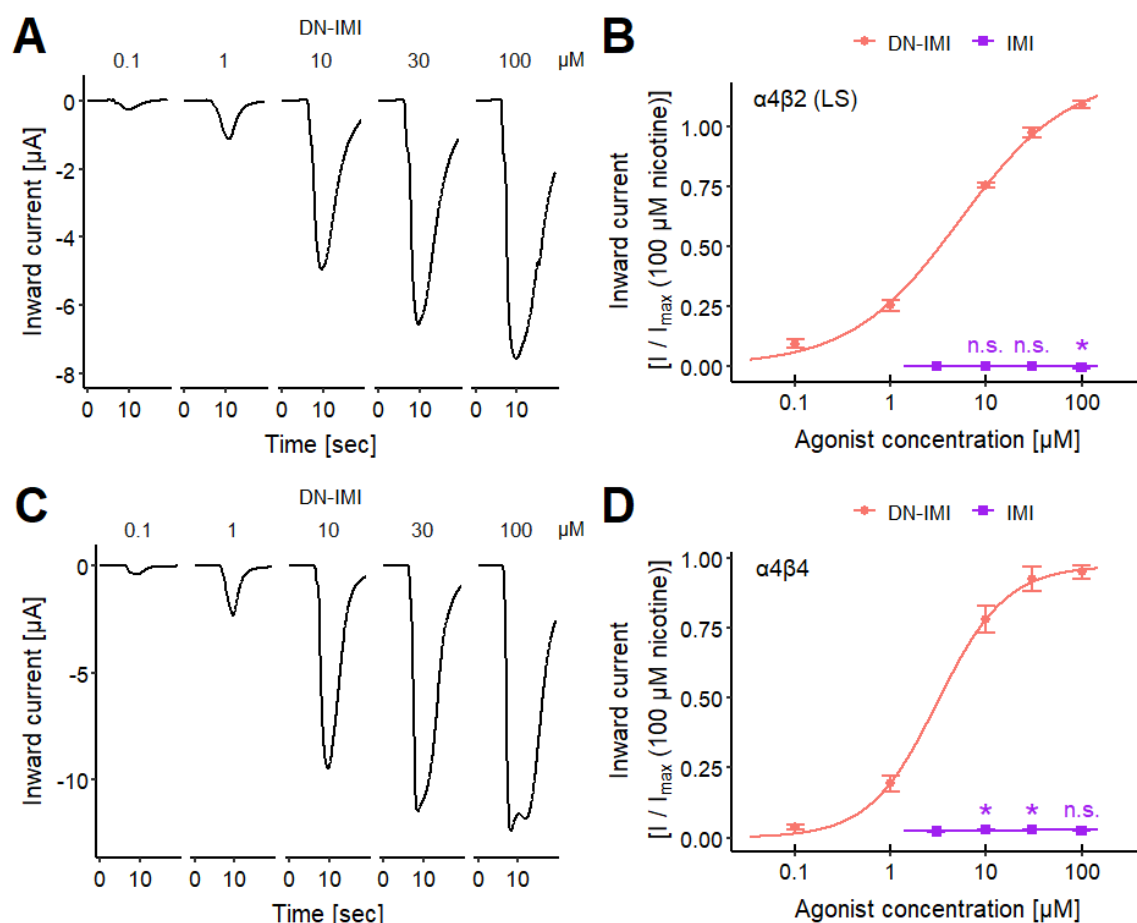


Fig. MS3_S8: Effects of DN-IMI and IMI on human $\alpha 4 \beta 2$ (LS) and $\alpha 4 \beta 4$ nAChRs expressed by *Xenopus laevis* oocytes.

(A) Exemplary inward currents through human $\alpha 4 \beta 2$ (LS) nAChRs expressed by *Xenopus laevis* oocytes stimulated by various DN-IMI concentrations are shown. **(B)** The inward current data (amplitude) of human $\alpha 4 \beta 2$ (LS) nAChRs expressed by *Xenopus laevis* oocytes of multiple experiments were quantified (means $\pm$ SEM are displayed), and after curve fitting a relative pEC_{50} value of 5.3 ± 0.06 (estimated maximum ~ 1.22) for DN-IMI was determined. The significance of the responses triggered by IMI was evaluated between the lowest concentration (3 μM) and the other concentrations (*: $p < 0.05$; n.s., not significant). The current amplitudes were normalized to the response induced by nicotine (100 μM) for each oocyte. **(C)** Exemplary inward currents through human $\alpha 4 \beta 4$ nAChRs expressed by *Xenopus laevis* oocytes stimulated by various DN-IMI concentrations are shown. **(D)** The inward current data (amplitude) of human $\alpha 4 \beta 4$ nAChRs expressed by *Xenopus laevis* oocytes of multiple experiments were quantified (means $\pm$ SEM are displayed), and after curve fitting a relative pEC_{50} value of 5.5 ± 0.07 (estimated maximum ~ 0.97) for DN-IMI was determined. The significance of the responses triggered by IMI was evaluated between the lowest concentration (3 μM) and the other concentrations (*: $p < 0.05$; n.s., not significant). The current amplitudes were normalized to the response induced by nicotine (100 μM) for each oocyte. Detailed data on n numbers are found in table MS3_S6.

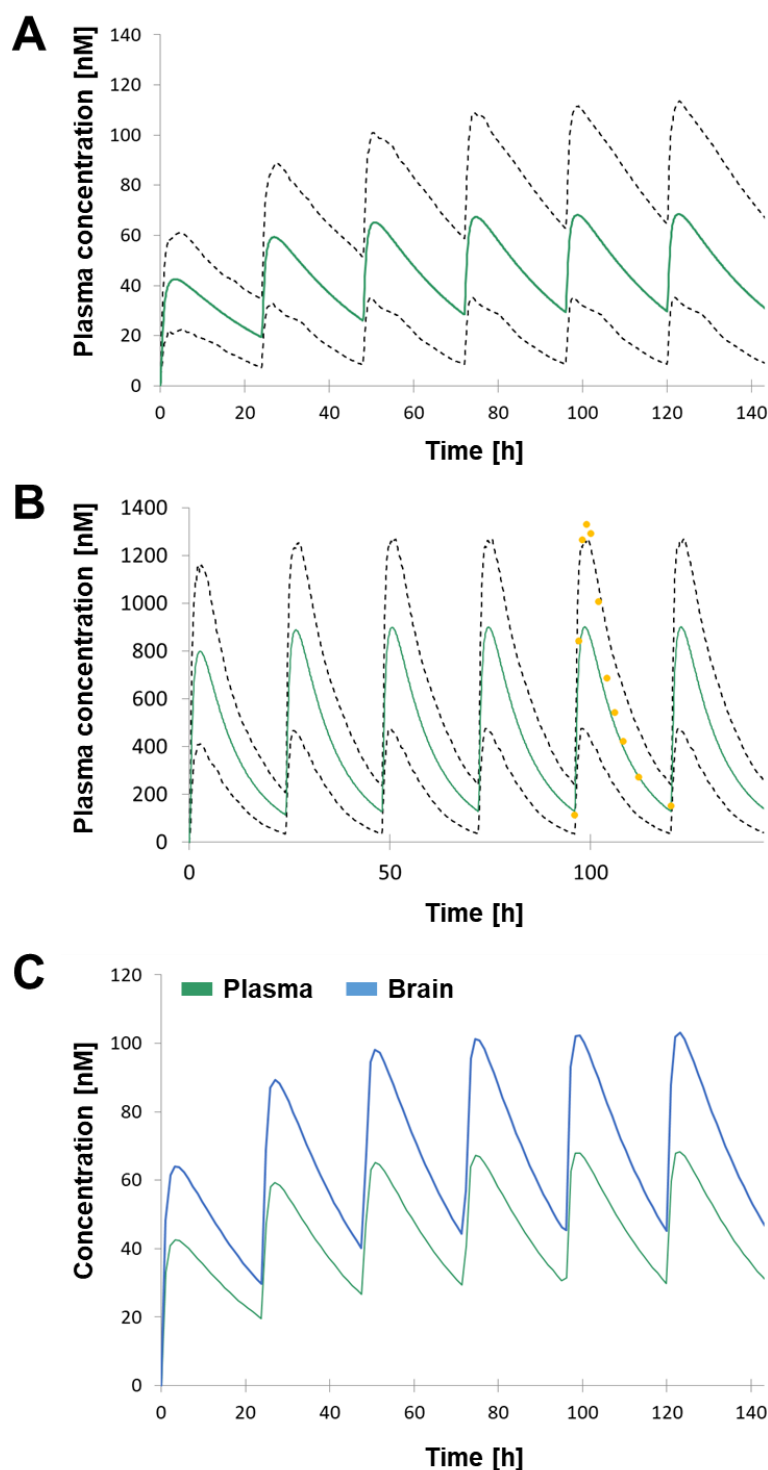


Fig. MS3_S9: Physiologically-based toxicokinetic (PBTk) modeling of DN-IMI and atenolol in the human population.

(A) The systemic exposure following an oral dose of 0.016 mg DN-IMI/kg body weight, given every 24 h intervals to 100 individuals (aged 20-50; 50% female) with randomly assigned phenotypic and genotypic properties typical for a caucasian Northern European population is shown. The predicted mean plasma concentrations of DN-IMI (green line) are shown with the 5th and 95th percentiles of the population (dashed lines). **(B)** The mean plasma concentrations of atenolol following multiple oral exposures of 50 mg every day for 6 days (green line) are shown with the 5th and 95th percentiles of the population (dashed lines). The population for the atenolol simulation matched the demographics of the subjects used in a clinical study (10 trials of 16

subjects aged 36-65; 31% female, (Andrawis et al., 2000)). The reported data are shown as yellow circles. The simulated mean atenolol C_{max} and AUC were 245 ng/mL and 2867 ng*h/mL, respectively, and are in reasonable agreement with the reported mean values (409.3 ng/mL for C_{max} and 3534 ng*h/mL for AUC). The simulated C_{max} and AUC were within 1.7 and 1.2 fold, respectively, of the observed values from this clinical study and were considered to be adequate for the purpose of this exercise. **(C)** The mean plasma (green) and brain (blue) concentrations of DN-IMI following multiple oral exposures of 0.016 mg DN-IMI/kg body weight, given every 24 h intervals to 100 individuals (aged 20-50; 50% female) with randomly assigned phenotypic and genotypic properties typical for a caucasian Northern European population are shown. The input parameters for DN-IMI and atenolol are given in table MS3_S8.

In clearance studies conducted with DN-IMI in a hepatocyte co-culture system, the measured in vitro intrinsic clearance was <0.143 ml/min/ 10^6 cells (i.e. below the limit of quantitation of the assay; in the same experiment the positive control compounds ketoprofen and prednisolone had measured intrinsic clearance values of 4.39 and 0.379, ml/min/ 10^6 cells, respectively (data not shown). As the rate of metabolism of DN-IMI was below the limit of quantitation of the clearance assay, no metabolic clearance was included in this DN-IMI PBTK model. This is a conservative assumption and metabolism components can be included in the model if data becomes available. Renal clearance for DN-IMI was assumed to be governed only by passive processes, with both filtration and passive reabsorption being accounted for. Passive permeability was scaled from the predicted permeability in the intestine (P_{eff} $0.37 \cdot 10^{-4}$ cm/s) accounting for the surface area of the proximal tubules in the kidney. The renal CL for atenolol also includes a component of active secretion and was entered into the model using reported transporter kinetic data (Yin et al., 2015). In the absence of equivalent data for DN-IMI, no active secretion component was included for this compound. If active secretion does occur for DN-IMI this would result in a lower exposure of DN-IMI in the body.

Table MS3_S1: Compound list.

Compound	Supplier	Order number	Function	Reference
ABT 594	Sigma	Q53646	neuronal non- $\alpha 7$ nACh receptor agonist	Donnelly-Roberts et al.1998; Michelmore et al. 2002
Acetylcholine chloride (ACh)	Sigma Aldrich	A6625	Endogenous neurotransmitter	
Advanced DMEM/F12	Gibco	12634010		
Boric acid	Sigma Aldrich	B7660		
Cal-520 AM	Biomol	ABD-21130		
Desnitro-Imidacloprid	Sigma Aldrich	37052	Metabolite of Imidacloprid	
Dibutyl cyclic-AMP sodium salt (cAMP)	Sigma Aldrich	D0627		
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D8418		
Recombinant human FGF basic (FGF-2)	R&D Systems	4114-TC		
Fibronectin	Sigma Aldrich	F1141		
Recombinant human GDNF protein (GDNF)	R&D Systems	212-GD		
L-glutamine solution	Sigma Aldrich	G7513		
Imidacloprid	Sigma Aldrich	37894	Neonicotinoid	
Imidacloprid-olefin	Sigma Aldrich	34534	Metabolite of Imidacloprid	
Mecamylamine	Sigma Aldrich	M9020	nACh receptor antagonist	Papke et al. 2008; Capelli et al. 2011
Methyllycaconitine	Sigma Aldrich	M168	nACh receptor antagonist	Palma et al. 1996; Capelli et al. 2011
MG 624	Sigma Aldrich	M3184	nACh receptor antagonist	Gotti et al. 2000; Capelli et al. 2011
N2-supplement	Gibco	17502048		
(-)-Nicotine	Sigma Aldrich	N3876	non-selective nACh receptor agonist	Capelli et al. 2011
PNU-120596	Sigma Aldrich	P0043	Positive allosteric nACh receptor modulator	Hurst et al. 2005; Grønlien et al. 2007; Williams et al. 2011
Polyethyleneimine solution (PEI)	Sigma Aldrich	P3143		
Poly-L-ornithine (PLO)	Sigma Aldrich	P3655		
Sodium tetraborate	Sigma Aldrich	221732		
Tetracycline hydrochloride	Sigma Aldrich	T7660		
Trypsin-EDTA (0.05 %)	Gibco	25300062		
(+)-Tubocurarine chloride pentahydrate	Sigma	93750	non-selective nACh receptor antagonist	Chavez-Noriega et al. 2000; Jonsson et al. 2006

Table MS3_S2: Overview of pEC₅₀ values for agonist experiments.

Compound	pEC ₅₀ ± SEM	Maximum ± SEM	Figure
DN-IMI	6.56 ± 0.03	1.00 ± 0.02	1D
IMI-olefin	n.d.	n.d.	1D
DN-IMI	6.80 ± 0.04	0.53 ± 0.01	2B
DN-IMI + PNU	6.45 ± 0.03	0.93 ± 0.01	2B
IMI-olefin	n.d.	n.d.	S1B
IMI-olefin + PNU	5.49 ± 0.03	1.01 ± 0.01	S1B
For experiments with SH-SY5Y cells			
DN-IMI	6.77 ± 0.36	0.44 ± 0.11	2C
DN-IMI + PNU	6.46 ± 0.07	0.93 ± 0.05	2C

Table MS3_S3: Overview of percentages of responsive cells of single-cell Ca²⁺-imaging.

Compound	Concentration	Responsive cells ± SEM	Figure
DN-IMI	0.01 µM	0.031 ± 0.004	1F
DN-IMI	0.1 µM	0.364 ± 0.080	1F
DN-IMI	1 µM	0.745 ± 0.054	1F
DN-IMI	10 µM	0.744 ± 0.054	1F
DN-IMI	100 µM	0.876 ± 0.026	1F

Table MS3_S4: Overview of pIC₅₀ values.

Compound	pIC ₅₀ ± SEM	Pharmacology	Figure
Effect on response to 0.5 µM DN-IMI			
Tubocurarine	5.90 ± 0.05	nAChR antagonist	3C
Mecamylamine	6.78 ± 0.03	nAChR antagonist	3C
MLA	6.78 ± 0.03	nAChR antagonist	3F
MG 624	6.76 ± 0.03	nAChR antagonist	3F
Effect on response to 3 µM nicotine		nAChR agonist	
DN-IMI	6.94 ± 0.03		4C
IMI-olefin	4.85 ± 0.03		4C
Effect on response to 30 nM ABT 594		nAChR agonist	
DN-IMI	7.40 ± 0.03		4F
Effect on response to 3 µM ACh		nAChR agonist	
DN-IMI	7.39 ± 0.03		4F

Table MS3_S5: Overview of pEC₅₀ and maximum values for *Xenopus laevis* oocyte experiments.

Compound	pEC ₅₀ ± SEM	Maximum ± SEM	Figure
human α4β2 (HS) nAChR			
Nicotine	6.26 ± 0.04	1.07 ± 0.03	6C
DN-IMI	6.31 ± 0.04	0.64 ± 0.02	6C
ACh	5.72 ± 0.06	1.00 ± 0.03	S4B
human α7 nAChR			
Nicotine	3.93 ± 0.04	1.04 ± 0.04	6D/S6D
DN-IMI	4.50 ± 0.09	0.81 ± 0.08	6D/S6D
human α3β4 nAChR			
Nicotine	4.02 ± 0.01	1.04 ± 0.01	6E/S7D
DN-IMI	4.87 ± 0.03	0.65 ± 0.02	6E/S7D
ACh	3.76 ± 0.03	1.09 ± 0.03	S7B
human α4β2 (LS) nAChR			
DN-IMI	5.28 ± 0.06	1.22 ± 0.04	S8B
human α4β4 nAChR			
DN-IMI	5.51 ± 0.07	0.97 ± 0.03	S8D

Table MS3_S6: Overview of concentrations and technical replicates.

Compound	Concentration (n-values)	Figure
DN-IMI	0.01 μ M (20), 0.03 μ M (7), 0.1 μ M (20), 0.3 μ M (7), 1 μ M (21), 10 μ M (21), 100 μ M (7)	1D
IMI-olefin	0.01 μ M (3), 0.1 μ M (5), 1 μ M (19), 10 μ M (20), 30 μ M (14), 100 μ M (16)	1D
DN-IMI	0.001 μ M (6), 0.003 μ M (5), 0.01 μ M (7), 0.03 μ M (7), 0.1 μ M (7), 0.3 μ M (7), 1 μ M (7), 3 μ M (5), 10 μ M (7), 30 μ M (7), 100 μ M (7)	2B
DN-IMI (+PNU)	0.001 μ M (7), 0.003 μ M (7), 0.01 μ M (7), 0.03 μ M (6), 0.1 μ M (6), 0.3 μ M (6), 1 μ M (5), 3 μ M (7), 10 μ M (6), 30 μ M (7), 100 μ M (6)	2B
IMI-olefin	0.001 μ M (7), 0.003 μ M (7), 0.01 μ M (4), 0.03 μ M (6), 0.1 μ M (5), 0.3 μ M (5), 1 μ M (5), 3 μ M (7), 10 μ M (7), 30 μ M (7), 100 μ M (7)	S1B
IMI-olefin (+PNU)	0.001 μ M (6), 0.003 μ M (6), 0.01 μ M (7), 0.03 μ M (4), 0.1 μ M (7), 0.3 μ M (7), 1 μ M (7), 3 μ M (7), 10 μ M (7), 30 μ M (7), 100 μ M (7)	S1B
Effect on response to 0.5 μM DN-IMI	Control for Tubocurarine (7), Mecamylamine (42), MLA (72), MG 624 (87)	
Tubocurarine	0.01 μ M (5), 0.1 μ M (5), 1 μ M (7), 10 μ M (7), 100 μ M (7)	3C
Mecamylamine	0.01 μ M (14), 0.03 μ M (6), 0.1 μ M (19), 0.3 μ M (20), 1 μ M (21), 3 μ M (11), 10 μ M (20)	3C
MLA	0.01 μ M (29), 0.03 (9), 0.1 μ M (30), 0.3 μ M (31), 1 μ M (30), 3 μ M (31), 10 μ M (14)	3F
MG 624	0.01 μ M (28), 0.03 (12), 0.1 μ M (27), 0.3 μ M (34), 1 μ M (28), 3 μ M (29), 10 μ M (12)	3F
Effect on response to 3 μM nicotine	Control for DN-IMI (34) and IMI-olefin (34)	
DN-IMI	0.01 μ M (13), 0.1 μ M (13), 1 μ M (14), 10 μ M (14), 100 μ M (20)	4C
IMI-olefin	0.01 μ M (4), 0.1 μ M (6), 1 μ M (13), 10 μ M (14), 30 μ M (7), 100 μ M (27)	4C

(Table continued on next page.)

Table MS3_S6: Overview of concentrations and technical replicates. (continued)

Effect on response to 3 μM ACh		Control for DN-IMI (25)	
DN-IMI	0.001 (13), 0.003 μ M (14), 0.01 μ M (12), 0.03 μ M (17), 0.1 μ M (13), 0.3 μ M (7), 1 μ M (7), 3 μ M (6), 10 μ M (6)	4F	
Effect on response to 0.03 μM ABT 594		Control for DN-IMI (35)	
DN-IMI	0.001 μ M (6), 0.003 μ M (11), 0.01 μ M (19), 0.03 μ M (11), 0.1 μ M (20), 0.3 μ M (7), 1 μ M (14), 3 μ M (7), 10 μ M (14)	4F	
Cellobserver		Control for DN-IMI (7)	
DN-IMI	0.01 μ M (6), 0.1 μ M (5), 1 μ M (5), 10 μ M (10), 100 μ M (7)	1F	
Oocyte recordings human $\alpha 4\beta 2$ (HS) nAChR			
Nicotine	0.03 μ M (5), 0.1 μ M (4), 0.3 μ M (5), 1 μ M (5), 3 μ M (5), 10 μ M (5)	6C	
DN-IMI	0.1 μ M (9), 0.3 μ M (8), 1 μ M (9), 3 μ M (9), 10 μ M (9)	6C	
IMI	3 μ M (5), 10 μ M (5), 30 μ M (5), 100 μ M (5)	6C	
ACh	0.1 μ M (4), 1 μ M (4), 3 μ M (4), 10 μ M (4), 30 μ M (4), 100 μ M (4)	S4B	
human $\alpha 7$ nAChR			
Nicotine	10 μ M (3), 30 μ M (7), 100 μ M (7), 300 μ M (7), 1000 μ M (7)	6D/S6D	
DN-IMI	1 μ M (5), 3 μ M (7), 10 μ M (7), 30 μ M (7), 100 μ M (7)	6D/S6D	
IMI-olefin	3 μ M (4), 10 μ M (4), 30 μ M (4), 100 μ M (4)	6D/S6D	
IMI	3 μ M (4), 10 μ M (4), 30 μ M (4), 100 μ M (4)	6D/S6D	
human $\alpha 3\beta 4$ nAChR			
Nicotine	3 μ M (5), 10 μ M (5), 30 μ M (5), 100 μ M (5), 300 μ M (5), 1000 μ M (5)	6E/S7D	
DN-IMI	1 μ M (8), 3 μ M (8), 10 μ M (8), 30 μ M (8), 100 μ M (8)	6E/S7D	
IMI	1 μ M (4), 3 μ M (4), 10 μ M (4), 30 μ M (4), 100 μ M (4)	6E/S7D	
ACh	3 μ M (5), 10 μ M (5), 30 μ M (5), 100 μ M (5), 300 μ M (5), 1000 μ M (5)	S7B	

(Table continued on next page.)

Table MS3_S6: Overview of concentrations and technical replicates. (continued)

human $\alpha 4\beta 2$ (LS) nAChR		
DN-IMI	0.1 μ M (6), 1 μ M (6), 10 μ M (6), 30 μ M (6), 100 μ M (6)	S8B
IMI	3 μ M (6), 10 μ M (6), 30 μ M (6), 100 μ M (6)	S8B
human $\alpha 4\beta 4$ nAChR		
DN-IMI	0.1 μ M (6), 1 μ M (6), 10 μ M (6), 30 μ M (6), 100 μ M (6)	S8D
IMI	3 μ M (5), 10 μ M (5), 30 μ M (5), 100 μ M (5)	S8D
Effect on response of human $\alpha 3\beta 4$ nAChR to 30 μM DN-IMI		
Tubocurarine	Control for tubocurarine (5) 0.3 μ M (5), 3 μ M (5), 30 μ M (5), washout (5)	S5B
Effect on response of human $\alpha 4\beta 2$ (HS) nAChR to 1 μM DN-IMI		
Mecamylamine	Control for mecamylamine (6) 0.3 μ M (6), 3 μ M (6), 30 μ M (6), washout (6)	S5C
Effect on response of human $\alpha 7$ nAChR to 30 μM DN-IMI		
MLA	Control for MLA (6) 0.3 μ M (5), 3 μ M (6), 30 μ M (6), washout (6)	S5D

Table MS3_S7: IFD and MMG-BSA-energy calculation parameters.

Ligand in human nAChR $\alpha 4\beta 2$	IFDScore	Docking score	MMG-BSA dG bind (NS)	Prime energy
DN-IMI (tautomer 1)	-18018.7	-10.723	-75.34	-18431.8
DN-IMI (tautomer 2)	-17978.0	-11.229	-78.02	-18435.7
DN-IMI (tautomer 3)	-18006.7	-10.293	-78.31	-18341.8
IMI (common)	-17939.9	-6.648	-60.85	-18372.0
IMI (inverted)	-17998.3	-6.699	-61.94	-18338.3
IMI-olefin (common)	-17997.5	-6.510	-63.26	-18314.1
IMI-olefin (inverted)	-17962.1	-7.000	-47.02	-18481.6
Nicotine-cocryst-6cnj	n.d.	n.d.	-59.01	-18236.9

Ligand in human nAChR $\alpha 7$	IFDScore	Docking score	MMG-BSA dG bind(NS)	Prime energy
DN-IMI (tautomer 1)	-12373.3	-7.280	-73.86	-12973.8
DN-IMI (tautomer 2)	-12384.6	-10.209	-71.79	-12854.0
DN-IMI (tautomer 3)	-12319.6	-7.191	-78.37	-12988.0
IMI (common)	-12267.1	-8.138	-59.15	-12876.2
IMI (inverted)	-12261.5	-6.737	-53.59	-12942.0
IMI-olefin (common)	-12247.6	-7.454	-65.15	-12876.8
IMI-olefin (inverted)	-12245.5	-7.008	-65.32	-12906.6
(S)-Nicotine	-12255.8	-9.159	-57.06	-12766.0

Table MS3_S8: Input parameters used in the DN-IMI and atenolol PBTK model.

Parameter	DN-IMI	Atenolol	Source
Molecular weight (g/mol)	210.7	Pubchem	Pubchem
log P	0.23	0.25	DN-IMI average of 6 estimates from different sources.
Compound type	Monoprotic base	Monoprotic base	
pKa	9.6	9.6	DN-IMI average of values in ChEMBL and ACD. Atenolol value taken from Dahlgren et al. (2016).
Blood/plasma ratio [B/P]	0.759	1.07	DN-IMI was measured. Atenolol was taken from Taylor and Turner (1981).
Free fraction [fu]	0.841	0.97	DN-IMI was measured. Atenolol data was taken from Barber et al. (1978).
Main plasma binding protein	Human serum albumin	Human Serum albumin	Assumed.
Absorption parameters			
Jejunal permeability (10^{-4} cm/s)	0.63	0.45	Predicted – mechanistic permeability model (Sugano, 2009). For atenolol the $P_{trans,0}$ value was calibrated so that the jejunal permeability corresponded to the observed value in humans reported by Dahlgren et al. (2016). The same calibration to $P_{trans,0}$ was applied to DN-IMI.
$P_{trans,0}$ (10^{-6} cm/s)	120	120	
f_{ugut}	1	1	
Distribution Model		Full PBTK Model	
V_{ss} (L/kg)	0.85	0.88	Predicted - Method 2 (Rodgers and Rowland, 2007). Atenolol value was adjusted to be in line with the value reported by Dahlgren et al. (2016).
Metabolic Clint CYP3A4 (ml/min/pmol enzyme)	0	0.00073	Atenolol is 95% excreted into urine.
CL_R (L/h)	3.2	9.64	Predicted using Mech Kim model (Burt et al., 2016). Atenolol CL_R was adjusted to recover the mean CL_R reported after IV dosing by Kirch et al. (1981), Mason et al. (1979), and Wan et al. (1979).

Table MS3_S9: Benchmark responses (BMR) for the $[Ca^{2+}]_i$ responses of LUHMES neurons triggered by DN-IMI and IMI-olefin.

Overview of the BMR10 and BMR20 concentrations (in log[M]) of the $[Ca^{2+}]_i$ response of LUHMES cells induced by DN-IMI and IMI-olefin. For each BMR, the benchmark concentrations (BMC) and their confidence interval (BMCL and BMCU) are shown; n.a.: BMR could not be determined due to low/no effect within the tested concentration range.

Compound	Intracellular free $[Ca^{2+}]$	
	BMR10	BMR20
	BMC [95% CI]	BMC [95% CI]
DN-IMI	7.3 [7.5; 7.1]	7.0 [7.1; 6.9]
IMI-olefin	4.4 [4.5; 4.2]	n.a.

Table MS3_S10: Benchmark responses (BMR) for the agonistic effects of nicotine and DN-IMI on several human nAChR subtypes expressed in *Xenopus laevis* oocytes.

Overview of the BMR10 and BMR20 concentrations (in log[M]) of the inward current response induced by nicotine or DN-IMI on several human nAChR subtypes expressed in *Xenopus laevis* oocytes. For each BMR, the benchmark concentrations (BMC) and their confidence interval (BMCL and BMCU) are shown.

nAChR subtype	Compound	Inward current (norm. to 100 μ M nicotine)	
		BMR10	BMR20
		BMC [95% CI]	BMC [95% CI]
$\alpha 7$	Nicotine	4.6 [6.6; 2.6]	4.5 [5.1; 3.9]
	DN-IMI	5.3 [6.4; 4.2]	5.1 [6.0; 4.3]
$\alpha 3\beta 4$	Nicotine	4.7 [5.1; 4.2]	4.6 [4.8; 4.4]
	DN-IMI	5.5 [6.3; 4.7]	5.3 [5.9; 4.7]
$\alpha 4\beta 2$ (LS)	DN-IMI	6.4 [5.6; 7.3]	6.1 [5.5; 6.8]
$\alpha 4\beta 4$	DN-IMI	6.3 [6.1; 6.5]	6.0 [5.8; 6.2]
		Inward current (norm. to 10 μ M nicotine)	
$\alpha 4\beta 2$ (HS)	Nicotine	7.2 [7.4; 7.0]	6.9 [7.0; 6.7]
	DN-IMI	6.9 [7.1; 6.7]	6.6 [6.8; 6.4]

Table MS3_S11: Benchmark responses (BMR) for the desensitizing effect of DN-IMI and IMI-olefin on nAChR signaling.

Overview of the BMR10 and BMR20 concentrations (in log[M]) of the $[Ca^{2+}]_i$ response induced by nicotine, ACh, or ABT 594 after an initial stimulation of LUHMES cells with DN-IMI or IMI-olefin. For each BMR, the benchmark concentrations (BMC) and their confidence interval (BMCL and BMCU) are shown.

Compound		Intracellular free $[Ca^{2+}]$	
Treatment	Pre-treatment	BMR10	BMR20
		BMC [95% CI]	BMC [95% CI]
Nicotine	DN-IMI	8.0 [8.2; 7.8]	7.6 [7.8; 7.5]
	IMI-olefin	5.6 [6.2; 5.1]	5.3 [5.7; 5.0]
ACh	DN-IMI	8.1 [8.3; 7.8]	7.8 [8.0; 7.6]
ABT 594	DN-IMI	8.3 [8.4; 8.1]	7.9 [8.0; 7.8]

4 Overarching discussion and outlook

LUHMES are a well-established model for neurotoxicity assays with morphological and biochemical endpoints (Brüll et al., 2020; Delp et al., 2021, 2019, 2018b, 2018a; Harris et al., 2017; Krug et al., 2014, 2013; Lohren et al., 2015; Smirnova et al., 2016; Tong et al., 2017; Witt et al., 2017; Zhang et al., 2014). To the best of our knowledge, we provided here for the first time a comprehensive overview of compound-induced neurophysiological changes in LUHMES neurons and demonstrated the capability and applicability of these cells for the assessment of functional neurotoxicity. We exemplified this for several meaningful neurotoxicant targets like P2X receptors as an example for ionotropic receptors, Nav channels as an example for voltage-gated ion channels, and the DAT as an example for electrogenic neurotransmitter transporters (Table 1).

Table 1: Toxicity types measurable with our high-throughput Ca^{2+} -imaging assays based on LUHMES neurons.

Toxicity type measurable	Example assay	Potential applications
Ion channel modification	Na_v channel toxicity	Marine neurotoxins, insecticides, local anesthetics (lidocaine), anticonvulsants (phenytoin) and antiarrhythmics
Receptor modification	P2X receptor function	Antipsychotics, antiepileptic drugs anti-inflammatory drugs, central neuropathic pain and antimigraine drugs
	nAChR function	Pesticides, marine neurotoxins and plant neurotoxins
Membrane transporter modulation	DAT function and DAT- Ca_v channel coupling	Psychostimulants (ritalin) and antidepressants (bupropion)

Our results demonstrate the presence of different P2X receptor subtypes, amongst others the P2X3 receptor subtype. We highlighted the high reproducibility of the differentiation of LUHMES neurons by the low variation of pEC_{50} values obtained for α, β -meATP over several differentiations, which is a very important characteristic of this cell line, as it is crucial for reliable and reproducible functional neurotoxicity assays. The usefulness of our test system to assess substance-induced effects on P2X receptors was shown by the determination of the known inhibitory side-effect of suramin on these receptors, together with effects of other agonists and antagonists that were in line with literature data. Future applications could be the assessment of adverse effects of antiepileptic, anti-inflammatory drugs, or analgesics targeting P2X receptors, as they

are widely expressed in the nervous system (Burnstock and Verkhratsky, 2012; Di Virgilio and Vuerich, 2015; Riquelme et al., 2020).

We also investigated the overall action potential firing behavior of LUHMES neurons using manual patch clamp to assess the maturation state of the differentiated neurons. 96% of cells were able to generate action potentials on days 9-10 of the differentiation, which highlights a homogenous maturation state of the differentiated LUHMES neurons. This finding is very important for a cell model that builds the basis of functional neurotoxicity assays.

Next, we demonstrated the suitability of high-throughput Ca^{2+} -imaging in combination with LUHMES neurons to quantify adverse effects on Nav channels and assessed modulatory and inhibitory effects of several biotoxins (e.g., pCTX and TTX). In the future, this assay could be also utilized for the detection of other marine neurotoxins, e.g., cyanobacterial toxins like kalkitoxin (LePage et al., 2005). For a more detailed investigation of the present Nav channels, we used LUHMES neurons in automated planar patch clamp experiments and examined the biophysical properties of these channels. Based on the results, we implemented pulse protocols for the assessment of acute and use-dependent inhibitory effects on Nav channels, which could be useful in the future for the detection of side-effects of local anesthetics or pyrethroids on Nav channels of the central nervous system (Cao et al., 2011; Casida and Durkin, 2013; Groban, 2003; Mather et al., 2005; Neal et al., 2010) and the examination of anticonvulsants, like phenytoin (Brodie, 2017; Goldenberg, 2010). It has been reported that LUHMES have TTX-sensitive Nav channels (Scholz et al., 2011). We expanded this work and demonstrated a major contribution of Nav1.2 to the response of functional Nav channels on LUHMES neurons, which is important in terms of subtype-specific potency differences of toxicants.

Furthermore, we showed the presence of functional DAT in LUHMES neurons and the suitability of this test system to investigate substance-induced alterations of DAT activity using high-throughput Ca^{2+} -imaging. Another finding was the presence of $[\text{Ca}^{2+}]_i$ oscillations that were synchronized over long distances, which suggests a functional coupling of LUHMES neurons. The oscillations were stimulated by DA and AMP and could be blocked by different antagonists that block the DAT, Ca_v channels, or Nav channels. Our results show a strong coupling of DAT and T-type Ca_v channels in LUHMES neurons. However, an in-depth examination of the underlying mechanism of $[\text{Ca}^{2+}]_i$ oscillations was out of the scope of this study. In the future, this assay could be applied for the investigation of compounds that modify neuronal network function, like seizurogenic substances and antiepileptic drugs that do not solely affect the GABA-glutamate system. The applicability of LUHMES neurons for the investigation of ionotropic receptors was exemplified for P2X receptors. In the next step, we extended this work and researched

the presence of human nAChRs on LUHMES neurons and utilized several well-known nAChR agonists and antagonists in Ca^{2+} -imaging experiments. The corresponding results show the presence of functional $\alpha 7$ and neuronal non- $\alpha 7$ nAChRs on LUHMES and highlight the suitability of our test system for the investigation of adverse effects on nAChR-mediated signaling. On this basis, we examined the effects of several neonicotinoid pesticides on human nAChRs and found strong evidence for an impact of Aceta, Imida, Cloth, and Thiac on nAChR signaling. These four neonicotinoids triggered an increase of $[\text{Ca}^{2+}]_i$ in LUHMES neurons, which is in good agreement with an earlier study of Aceta and Imida with primary rat neuronal cultures (Kimura-Kuroda et al., 2012).

Our findings on the direct stimulation of LUHMES cells by the four active neonicotinoids provide clear evidence that low concentrations ($\sim 10 \mu\text{M}$) can trigger $[\text{Ca}^{2+}]_i$ changes. In these experiments, the effect of $\alpha 7$ nAChRs might be masked by their extremely rapid desensitization, which we overcame by using the positive allosteric modulator of the $\alpha 7$ nAChR PNU. The strong potentiation of the responses triggered by the four active neonicotinoids strongly suggests an activation of the $\alpha 7$ nAChR subtype. Furthermore, our data from desensitization experiments with LUHMES neurons show that very low micromolar concentrations of the four active neonicotinoids significantly desensitized nAChR signaling. At high concentrations ($100 \mu\text{M}$), the responses evoked by the subsequent exposure to other nAChR agonists were blocked entirely. Notably, the desensitizing effect may be equally problematic for normal neuronal function and neurodevelopment as a potential overstimulation. Our results provide evidence that $\alpha 7$ and non- $\alpha 7$ nAChRs are affected by these four active neonicotinoids. This finding is in line with earlier reported data on binding and $^{86}\text{Rb}^+$ -flux experiments, which showed the interaction of several neonicotinoids with various nAChR subtypes (Tomizawa and Casida, 2005, 1999).

The results for the neonicotinoids suggest a potential risk for humans, as nAChRs are widely distributed in the nervous system, among others on dopaminergic neurons, where they modulate the electrical activity and the release of dopamine (de Kloet et al., 2015; Grady et al., 1992; Mameli-Engvall et al., 2006; Quik and Kulak, 2002; Quik and Wonnacott, 2011; Rapier et al., 1988). The signaling via nAChRs is involved in several processes, e.g., the development and the functioning of the nervous system. It is, therefore, crucial to investigate potential adverse effects of neonicotinoids on human neurons and to assess its hazard potential for the dopaminergic system or other critical neuronal circuits in the brain (Lozada et al., 2012; Miwa et al., 2011; Romoli et al., 2019; Slotkin et al., 2006; Stevens et al., 2003; Welsby et al., 2006; Wheeler and Cooper, 2004; Ziviani et al., 2011).

The exposure to neonicotinoids needs to be distinguished into at least three scenarios (Thompson et al., 2020). One scenario is the intentional exposure for suicidal attempts with the ingestion of large amounts of neonicotinoids (Mohamed et al., 2009). Another scenario is the occupational (non-intentional) exposure, which usually is low due to mostly controlled use in Europe (Marfo et al., 2015). A third scenario is the normal dietary exposure, which is the case for the general population. The acute reference doses (ARfD) were set by EFSA to, e.g., 25 µg/kg/day for Aceta and 80 µg/kg/day for Imida. Monitoring studies (e.g., in 2018 in Europe (European Food Safety Authority (EFSA) et al., 2020)) revealed a maximal acute exposure of 57 µg/kg/day for Aceta, which is more than twice the ARfD. Data on internal exposure are relatively limited. The treatment of mice with NOAELs of Imida and Aceta (10 mg/kg) led to brain levels of around 3-6 ppm and similar plasma levels (Ford and Casida, 2006), resulting in a broad concentration range of approximately 15-30 µM. Biomonitoring data for humans is primarily available for urine samples, where maximal concentrations in the low nM range were reported (Li et al., 2020; Li and Kannan, 2020; Zhang et al., 2019).

Due to the limited availability of direct data, a PBTK model was implemented to predict plasma and brain concentrations (Loser et al., 2021a). We assumed that an exceedance of the ARfD by a factor of two is realistic. Thus, a maximal exposure on a single day could be 160 µg/kg for Imida (see ARfD above), which resulted in predicted plasma concentrations in the range of 0.8-1.6 µM and brain concentrations of 0.5-1.2 µM (Loser et al., 2021a). The reverse modeling data demonstrate that a brain concentration of 2 µM Imida (considered a point of departure from our in vitro studies) would be obtained after ingestion of 200 µg/kg body weight.

The comparison of in vitro and in vivo data results in a plausible overall picture, as the average population is usually exposed to sub-micromolar concentrations, which would not be sufficient to activate or desensitize nAChRs. However, concentrations higher than the ARfD might affect nAChR signaling.

Another key aspect of our findings is that the tested neonicotinoids can be distinguished in two subgroups concerning nAChR signaling. Unlike the four active compounds with relatively similar effects, two other neonicotinoids (Thiam and Dino) were inactive in the tested concentration range (≤ 100 µM). This difference might be explained by the preference of other docking poses of these two agents as suggested by a molecular docking model of nAChRs (Loser et al., 2021a). Though, this has to be further investigated in detail in the future. The clear subgrouping of the neonicotinoids in our assays shows a high specificity of our LUHMES-based test system.

While our findings provide strong evidence of an agonistic effect of a subgroup of neonicotinoids on human nAChRs, further research is needed to assess the full toxicological implication of our results. In future studies, new technical approaches are

required to identify the nAChR subtypes affected by these neonicotinoids. For this purpose, *Xenopus laevis* oocytes could be used to investigate the effects on nAChRs with a defined subunit stoichiometry. A verification that the inactive agents do not interact with any of the receptors would also be necessary. Furthermore, metabolites of the neonicotinoids will require additional attention, as several binding studies reported a higher affinity of, e.g., the Imida (IMI) metabolite DN-IMI to mammalian nAChRs compared to its parent compound IMI and similar to the affinity of nicotine (Chao and Casida, 1997; D'Amour and Casida, 1999; Tomizawa et al., 2000; Tomizawa and Casida, 1999). Therefore, we investigated the effects of DN-IMI on human nAChR signaling. Our findings for DN-IMI on a functional level using a physiological signaling response (changes of $[Ca^{2+}]_i$) were in line with these previous observations. Our data demonstrate a higher potency of DN-IMI compared to its parent compound IMI and similar to the potency of nicotine for human nAChRs present on LUHMES neurons. We demonstrated that DN-IMI is an agonist, which triggered $[Ca^{2+}]_i$ responses of LUHMES neurons at concentrations of ≥ 100 nM. This is a potency increase of around two orders of magnitude in comparison to its parent compound. Moreover, our results show that human $\alpha 7$ and neuronal non- $\alpha 7$ nAChRs on LUHMES were activated by DN-IMI. These findings were further supported by oocyte recordings, which showed an agonistic effect of DN-IMI on human $\alpha 7$ and several non- $\alpha 7$ nAChRs. DN-IMI exhibited significantly high effectiveness than its parent compound IMI, and similar potencies for human $\alpha 4\beta 2$ (HS), $\alpha 7$, and $\alpha 3\beta 4$ subtypes compared to nicotine.

These results were in good agreement with our LUHMES data, highlighting the suitability and applicability of our LUHMES-based assays to detect adverse effects on human nAChR signaling using Ca^{2+} -imaging. The findings in LUHMES of a human $\alpha 7$ nAChR activation by IMI could be supported by *Xenopus laevis* oocyte recordings, whereas only small but significant effects on $\alpha 3\beta 4$ and no effects on the other tested nAChR subtypes were detected. This observation may be explained by the presence of other nAChR subtypes on LUHMES neurons, like $\alpha 6$ - or $\alpha 5$ -containing receptors. In desensitization experiments with LUHMES, DN-IMI inhibited the subsequent activation of nAChRs at concentrations ≥ 10 nM (BMR10 (manuscript 3)), which is ~ 70 times more potent compared to the effects of IMI. As mentioned above, a desensitizing effect may be equally problematic for normal neuronal function and neurodevelopment as a potential overstimulation, because both processes lead to a long-lasting inactivation of the receptor in the presence of the neonicotinoid parent compound or metabolite (Fig. 3).

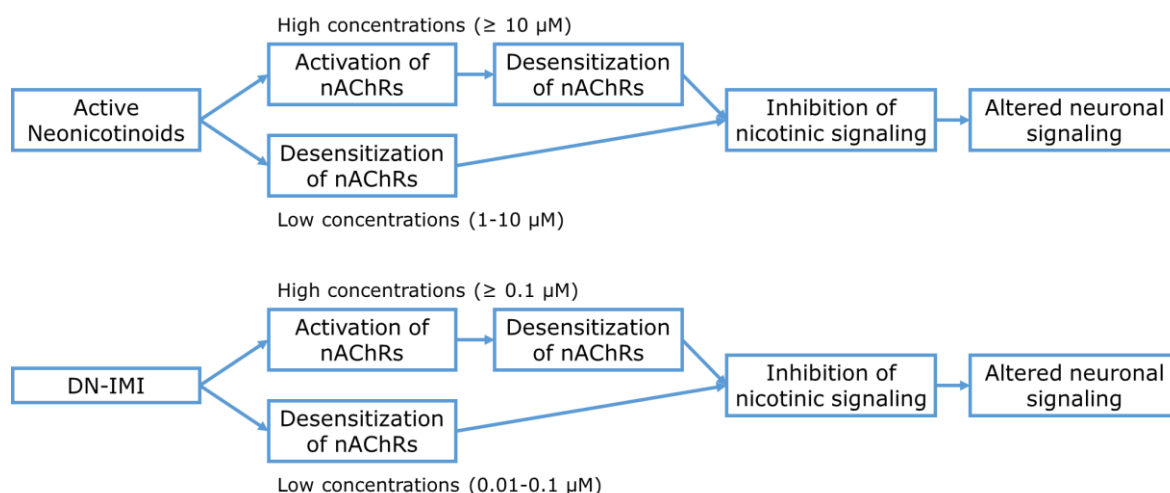


Fig. 3: Effects of active neonicotinoids and DN-IMI on nAChR signaling.

Our results demonstrate an agonistic effect of the active neonicotinoids (Aceta, Imida/IMI, Cloth, and Thiac) and DN-IMI on the nAChR signaling of LUHMES neurons with a higher potency of DN-IMI compared to the other active neonicotinoids, and for the desensitization compared to the activation of the nAChRs. These effects lead to an alteration of the neuronal signaling via inhibition of the nAChRs.

Another IMI metabolite IMI-olefin also exhibited an agonistic effect on LUHMES neurons but with a lower potency and efficacy than DN-IMI, comparable to IMI. It negatively modulated the responses of LUHMES neurons evoked by nicotine with a potency similar to its parent compound. These findings show that not all metabolites exhibit a gain of potency for human receptors but can still be equally active compared to their parent compound. IMI-olefin shows an increased insecticidal effect (Codling et al., 2016; Dai et al., 2006; Thompson et al., 2020) and lower toxicity in mice than IMI (Chao and Casida, 1997).

Toxicity studies with mice showed that the median lethal dose (LD₅₀) of DN-IMI (6-24 mg/kg) is lower compared to its parent compound IMI (35-50 mg/kg) (Chao and Casida, 1997; Tomizawa et al., 2000). IMI-olefin was not lethal and only induced tremors at the highest tested dose of 50 mg/kg (Chao and Casida, 1997). Furthermore, IMI and DN-IMI were found in the brain and the liver of mice treated with 10 mg/kg, whereas IMI-olefin was detected in the liver (Ford and Casida, 2006). Urine samples of these mice contained 22% of the administered dose of IMI together with different metabolites in the first 24 h (Ford and Casida, 2006). In human urine samples, mostly parent compound concentrations of neonicotinoids are assessed (Li et al., 2020; Li and Kannan, 2020; Zhang et al., 2019). In a recent study, the concentration of IMI and its metabolites DN-IMI and IMI-olefin were determined in human urine samples (Wang et al., 2020). They detected low nM concentrations of the three compounds and showed that these two metabolites account for 92% of the detected summated amount of the

three compounds, indicating a high metabolization rate of IMI in humans and/or a substantial dietary uptake of the IMI metabolites.

Due to the lack of more direct human data and for a better understanding of the DN-IMI concentrations that may be reached in humans, a PBTK model was built to predict the plasma concentrations of this metabolite. As input (oral exposure), 0.016 mg DN-IMI/kg body weight was used, which corresponds to 10% of the value used earlier for IMI PBTK modeling (Loser et al., 2021a). The rationale was that DN-IMI can reach about 10% of the IMI content in food products like vegetables and fruits (Anon, 2006). The model predicted average plasma concentrations of around 50 nM and peak concentrations in a subfraction of the human population of at least 100 nM (manuscript 3). The predicted brain concentrations were even slightly higher than the plasma concentrations. The free diffusion of DN-IMI through the blood-brain barrier may also predict a free distribution into the fetus. Thus, an exposure of the fetal brains to DN-IMI concentrations in a similar range is reasonable to be assumed. Our results showed that a 20% response via the activation of nAChRs can be triggered at about 100-300 nM of DN-IMI. This concentration range is close to the peak concentrations reachable in some subjects by dietary exposure. Such concentrations may not be reached by the average population but the gap between realistic internal exposure levels and the minimal effect concentration is less than 10-fold. This marginal safety buffer is eliminated, if receptor desensitization is considered as a 20% effect occurred at ~17 nM of DN-IMI. These concentrations may be reached by the consumption of food derived from crops treated with IMI. As mentioned before, the desensitization of nAChRs may be equally problematic for normal brain function and neuronal development, as the direct activation of these receptors. These observations, the increased toxicity of DN-IMI for mammals, and our findings of an increased potency of DN-IMI similar to nicotine for human nAChRs demonstrate the importance of a proper risk assessment for neonicotinoid metabolites.

For a full risk assessment, a thorough investigation of the species-specific toxicological implications of neonicotinoids and their many metabolites is necessary in future studies. Because other neonicotinoid metabolites, besides DN-IMI, might also exhibit higher affinities for mammalian nAChRs than their parent compounds. One example is a descyano metabolite of thiacloprid (Tomizawa et al., 2000). Moreover, the effects of DN-IMI and other neonicotinoid metabolites should be evaluated not only for the most common nAChR subtypes (e.g., $\alpha 4\beta 2$ and $\alpha 7$) but also for other neuronal (e.g., $\alpha 5$ - or $\alpha 6$ -containing) nAChR subtypes and muscle nAChRs.

In future studies, it should be investigated whether the signaling disturbances detected here have lasting effects on neuronal function, as other nAChR agonists (including nicotine) may not only have an acute effect on nervous system function but also a long-

lasting effect by influencing plasticity and development (Lozada et al., 2012; Romoli et al., 2019; Slotkin et al., 2006; Welsby et al., 2006; Wheeler and Cooper, 2004; Ziviani et al., 2011), or alter gene expression of neurons upon prolonged exposure (Kimura-Kuroda et al., 2016). Some studies on chronic effects of neonicotinoids on humans reported general developmental/neurological effects (Cimino et al., 2017).

Some other compounds have been reported to induce DNT by disturbing the neuronal network activity without causing structural changes, e.g., MDMA, heroin, or nicotine (Aschner et al., 2017; Dwyer et al., 2009; Slikker Jr et al., 2005; Slotkin et al., 2016). Besides, compounds that only cause small changes in the adult brain (in typical exposure situations) can have severe effects on the developing brain with consequences for later life, as reported, e.g., for methylmercury and lead (Grandjean and Landrigan, 2014). Therefore, a DNT risk of neonicotinoids or their metabolites seems conceivable. However, the availability of data is still limited and a knowledge transfer from one compound (e.g., nicotine) to another (e.g., DN-IMI) comprises the risk of uncertainties (Rovida et al., 2020). Thus, further studies are needed to properly evaluate the risk potential and to address the difficult question of a DNT hazard of neonicotinoids and their metabolites.

In summary, we demonstrated in this study the capability of a LUHMES-based approach for high-throughput functional neurotoxicity testing using Ca^{2+} -imaging. The main results were supported by patch clamp and oocyte recordings. We showed the presence of several important neuronal toxicant targets and exemplified our assay's suitability for the assessment of adverse effects on these targets. Furthermore, we revealed the effects of several neonicotinoids and two metabolites on human nAChR signaling, which may bear a potential DNT risk for humans. One of these metabolites (DN-IMI) exhibits a considerably higher potency and effectiveness on human nAChRs than its parent compound, but similar to the well-known developmental neurotoxicant nicotine. The findings on neonicotinoids and two metabolites expand the common knowledge of the effects of these pesticides and two of their metabolites, which was mainly based on a small amount of data from binding affinity and animal toxicity studies, on a functional level using human nAChRs.

5 Author contribution

In the following, my contributions to the three manuscripts are presented.

I conceived or designed, performed, and analyzed all high-throughput and single-cell Ca^{2+} -imaging experiments with LUHMES neurons. I also conceived or designed and analyzed all manual patch clamp experiments, and performed some of them and supervised the rest. Moreover, I conceived or designed some and performed and analyzed all automated planar patch clamp and oocyte experiments. Furthermore, I visualized the data of the above-mentioned experiments for the three manuscripts and wrote substantial parts, concerning the above-mentioned experiments, of the three manuscripts together with my supervisors.

The other data included in the three manuscripts of RNA analysis, radioactively-labeled DA uptake, neurite outgrowth, experiments with SH-SY5Y cells, modeling (molecular docking, BMC, PBTK models), production of plasmid DNA for $\alpha 3$, $\alpha 4$, $\beta 2$, and $\beta 4$ nAChR subunits, and so forth were contributed by my colleagues.

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