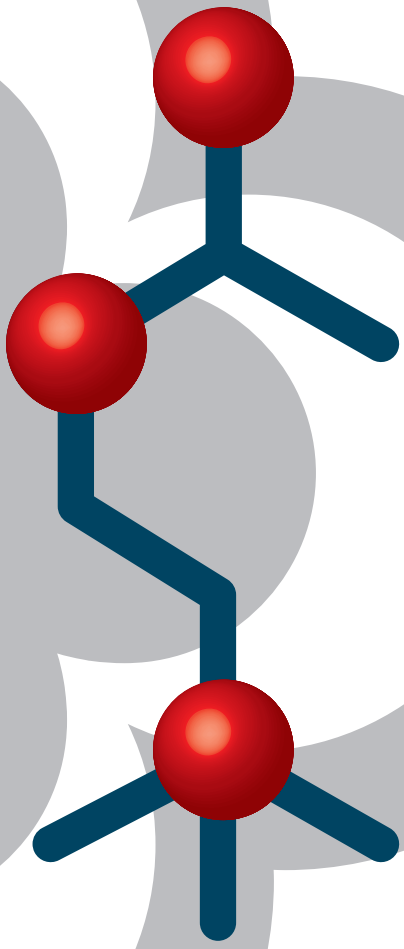
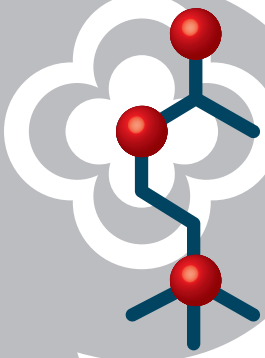
17th International Symposium on
Cholinergic Mechanisms



ISCM2022

8 - 12 May 2022
Dubrovnik • Croatia

17th International Symposium on
Cholinergic Mechanisms



ISCM2022

8 - 12 May 2022 · **Dubrovnik · Croatia**

Programme and Abstracts

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The 17th International Symposium on Cholinergic Mechanisms, **ISCM**2022, 8 - 12 May 2022, Dubrovnik

Publisher
Institute for Medical Research and Occupational Health

Editors
Zrinka Kovarik and Ines Primožič

Edition
112 pages, 120 copies

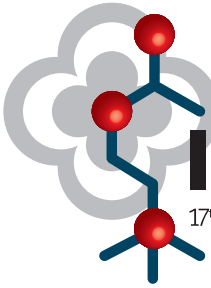
Cover page and logo design
Indira Filipović, Grafokor d.o.o., Zagreb

Photographs
Sheraton Dubrovnik Riviera Hotel
Igor Brautović

Produced by
Grafokor d.o.o., Zagreb

A CIP catalogue record for this book is available in the
Online Catalogue of the National and University Library in Zagreb as **001134853**

ISBN 978-953-96817-8-2



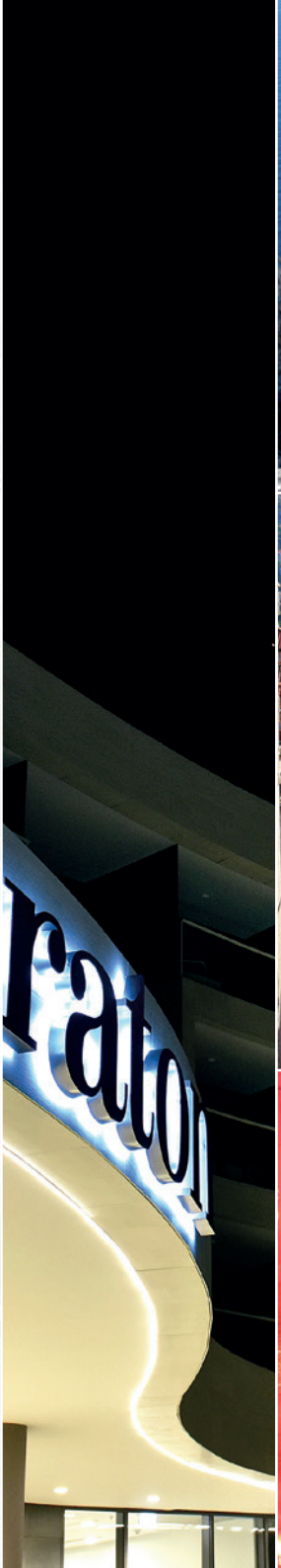
ISCM 2022

17th International Symposium on Cholinergic Mechanisms

8 - 12 May 2022 – Dubrovnik – Croatia

Programme and Abstracts

2022 • Dubrovnik • Croatia • EU



Welcome to the ISCM2022!

We have the pleasure and honour to welcome you to the 17th International Symposium on Cholinergic Mechanisms (ISCM2022). With this welcome address we also thank you all for your participation and for helping us make this Symposium possible.

The first International Symposia on Cholinergic Mechanisms was organized by Edith Heilbronn and took place in Skokloster, Sweden, in 1970. The tradition was continued over the years on a very regular basis and attracted significant numbers of participants from all over the world. The past ISCM took place in different countries and on different continents: Skokloster, Sweden (1970), Boldern, Switzerland (1974), La Jolla, USA (1977), Florence, Italy (1980), Oglebay Park, USA (1983), Buxton, UK (1986), Lidingo, Sweden (1989), Sainte-Adèle, Canada (1992), Mainz, Germany (1995), Arcachon, France (1998), St. Moritz, Switzerland (2002), Alicante, Spain (2005), Foz do Iguacu, Brazil (2008), Hangzhou, China (2013), Marseille, France (2016), and Rehovot, Israel (2019).

The principal objective of these ISCMs has been to highlight the progress in the molecular, cellular, pharmacological, toxicological, behavioural, and clinical aspects of the cholinergic system, to openly and critically discuss, consolidate collaborative initiatives, and, of course, establish and sustain long-term friendships. Over more than half of the past century, ISCM participants have created and established “a family” that is continuously incorporating new members, thereby broadening and expanding worldwide contacts and collaborations. We very much hope that this trend will persist in the future.

The 17th cholinergic conference will continue this great tradition. Along with a great scientific program, the participants will be able to enjoy Dubrovnik, a magnificent historic city, founded in the 7th century as a free city-state, which developed flourishing seafaring and international trade from the 14th to the 19th century which ensured its economic and cultural prosperity for centuries. "Those who seek paradise on Earth must come to Dubrovnik”, wrote George Bernard Shaw, charmed by the beauty of the city's 1940 m long defensive walls. The Old City, virtually unchanged since the 13th century, and a mild Mediterranean climate provide the fundaments for combining business with pleasure. Moreover, Dubrovnik is one of the Mediterranean's top tourist destinations, as well as a popular filming location.

Enjoy a successful and stimulating ISCM2022 and let’s all participants in person have a pleasant stay in Dubrovnik and Croatia!

Zrinka Kovarik
Chair of ISCM2022

Hermona Soreq
Chair of the International Advisory Board on Cholinergic Mechanisms

Organising Committee

Zrinka Kovarik (Chair of the ISCM2022), Zagreb, Croatia
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Croatian Society of Biochemistry and Molecular Biology

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The Young Investigator Travel Awardees

Tena Čadež
Institute for Medical Research and Occupational Health, Croatia

Serafima Dubnov
The Hebrew University of Jerusalem, Israel

Federico Fabris
Department of Biomedical Sciences, University of Padua, Italy

Muslum Gok
Mugla Sitki Kocman University, Turkey

Tibor Hodbod
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School of Medicine, University of Zagreb, Zagreb, Croatia

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Institute for Medical Research and Occupational Health, Zagreb

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Venue

Sheraton Hotel
Šetalište Dr. F. Tuđmana 17,
20207 Srebreno, Dubrovnik, Croatia

Technical Organizer

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Programme

SUNDAY, 8 MAY 2022

12:00-17:00	Registration
17:00-17:30	**Welcome Addresses and Introductory Remarks**
	Zrinka Kovarik (Zagreb, Croatia) Chair of the Organising Committee
	Hermona Soreq (Jerusalem, Israel) Chair of the ISCM International Advisory Board
17:30-18:15	**HDBMB Plenary Lecture**
17:30-17:35	**Jean-Pierre Changeux** (Paris, France) Introduction
17:35-18:15	**Palmer Taylor** (La Jolla, USA) Molecular Recognition in the Cholinergic Nervous System
18:15-19:00	**Keynote EMBO Lecture**
18:15-18:20	**Zrinka Kovarik** (Zagreb, Croatia) Introduction
18:20-19:00	**Hermona Soreq** (Jerusalem, Israel) Small RNA Regulators of Cholinergic Pathway Genes Exert Sex and Disease-Related Neuroinflammation and Mental Changes
19:00-21:00	Welcome Reception

MONDAY, 9 MAY 2022

8:30-10:10	**Session 1: Cholinergic Cross Talk between the Neural and Immune Systems** **Chairs: A. Meisel and J. Sussman**
8:30-8:50	**Andreas Meisel** (Berlin, Germany) Role of Cholinergic vs. Sympathetic Pathways in the Pathobiology of Stroke
8:55-9:15	**J. Michael McIntosh** (Salt Lake City, USA) Alpha 10-Containing Nicotinic Receptors – Implications for Neuropathic Pain, Cancer and Inflammation
9:20-9:40	**Jon Sussman** (Manchester, UK) Phenotype and Genotype Variation in Myasthenia Gravis
9:45-10:05	**Sergej Pirkmajer** (Ljubljana, Slovenia) Agrin Signalling in Human Skeletal Muscle Cells
10:10-10:30	Coffee Break
10:30-12:30	**Session 2: Neuromuscular Junction and Neurotransmission** **Chairs: E. Krejci and L. Mei**
10:30-10:50	**Kinji Ohno** (Nagoya, Japan) Three Newly Identified Molecules That Facilitate the Formation of Neuromuscular Junction *(virtual)*
10:55-11:15	**Claire Legay** (Paris, France) ColQ from Structure to Molecular Functions and Pathology
11:20-11:40	**Lin Mei** (Cleveland, USA) Phase-Separated Rapsyn Condensates as a Signaling Platform for Neuromuscular Junction Formation
11:45-12:05	**Eric Krejci** (Paris, France) Acetylcholine Toxicities: Surprising Lessons From Mouse Genetics
12:10-12:20	**Marco Stazi** (Padua, Italy) Functional Recovery of the Neuromuscular Junction From Neurotoxic Snake Envenoming Elicited by CXCR4 Agonist
12:20-12:30	**Wen-Cheng Xiong** (Cleveland, USA) Muscular Swedish Mutant APP-to-Brain Axis in the Development of Alzheimer's Disease
12:30-13:00	**Poster Highlights 1, Chair: F. Ekström** Lucia Markusková, Slovakia – Patrik Meglić, Croatia – Serafina Dubnov, Israel – Daniel Wiktelius, Sweden – Josip Madunić, Croatia – Tena Čadež, Croatia
13:00-14:30	Lunch

14:30-17:05	**Session 3: Cholinergic Modulators and Neurotoxins** **Chairs: C. Montecucco and M. Aschner**
14:30-14:50	**Carrie K. Jones** (Nashville, USA) Development of M5 NAM and Orthosteric Inhibitors for Opioid Use Disorder
14:55-15:15	**Cesare Montecucco** (Padua, Italy) Extremely Potent Human Monoclonal Antibodies are Effective for Prophylaxis and a Novel Therapy of Tetanus
15:20-15:40	**Michael Aschner** (New York, USA) Combined Exposure to Methylmercury and Manganese during L1 Larval Stage Causes Motor Dysfunction, Cholinergic and Monoaminergic Up-Regulation and Oxidative Stress in L4 *Caenorhabditis elegans*
15:45-15:55	**Federico Fabris** (Padua, Italy) Unravelling the Inhibitory Activity of Botulinum Toxins on the Enteric Nervous System
15:55-16:15	Coffee Break
16:15-16:35	**Edna F. Pereira** (Baltimore, USA) Organophosphorus Insecticides and the Developing Brain: Detrimental Effects of Early-Life Exposures *(virtual)*
16:40-17:00	**John A Dani** (Philadelphia, USA) Adolescent Nicotine Enhances Adult Alcohol Self-Administration (*virtual*)
17:05-17:35	**Poster Highlights 2, Chair: I. Primožič** Lenka Pulkrabkova, Czech Republic – Tibor Hodbod, Slovakia – Tomaž Trobec, Slovenia – Ana Matošević, Croatia – Petra Šoštarić, Croatia – Borna Puljko, Croatia
17:35-18:30	**Poster Session 1**

TUESDAY, 10 MAY 2022

8:30-9:15	Plenary Lecture
8:30-8:35	**Palmer Taylor** (La Jolla, USA) Introduction
8:35-9:15	**Thomas Balle** (Sydney, Australia) A Dynamic View on α4β2 nAChR Agonist and Modulator Binding
9:15-10:40	**Session 4: Nicotinic Cholinergic Pathway in Consciousness, Pain and COVID Chairs: T. Balle and B. M. Olivera**
9:15-9:35	**Jean-Pierre Changeux** (Paris, France) The Nicotinic Receptor Modulation of Higher Brain Functions: From Molecules to Consciousness
9:40-10:00	**Konstantinos Poulas** (Patras, Greece) COVID-19 and the Nicotinic Cholinergic Pathway
10:05-10:25	**Baldomero M Olivera** (Salt Lake City, USA) Towards Defining the Function of Nicotinic Receptor Subtypes in Sensory Physiology and Pathology
10:30-10:40	**Anna Hrabovska** (Bratislava, Slovakia) Cardiac Nicotinic Receptors Show β-Subunit-Dependent Compensatory Changes
10:40-11:00	Coffee Break
11:00-12:50	**Session 5: Drug Design and Potential Treatments in Cognitive Decline Chairs: M. L. Bolognesi and A. Bosak**
11:00-11:20	**Ezio Giacobini** (Geneva, Switzerland) Rethinking Cholinergic Therapy for Alzheimer's Disease *(virtual)*
11:25-11:45	**Maria Laura Bolognesi** (Bologna, Italy) Sustainable Drug Discovery of Multi-Target Acetylcholinesterase Inhibitors for Alzheimer's Disease
11:50-12:10	**Anita Bosak** (Zagreb, Croatia) Evaluation of 4-Aminoquinolines as Potential Anticholin-esterase Agents in the Treatment of Alzheimer's Disease
12:15-12:35	**Abraham Fisher** (Rehovot, Israel) Muscarinic Stimulation of Post-Synaptic Receptors for the Potential Treatment and prevention of Cognitive Decline
12:40-12:50	**Selena Maxwell** (Halifax, Canada) The Cholinergic System as a Potential Mechanism of Cognitive Preservation in Octogenarians and Older

12:50-14:00	Lunch
14:00-15:50	**Session 6: Young Investigators in Cholinergic Mechanisms Chairs: N. Maček Hrvat and T. Zorbaz**
14:00-14:20	**Niko Amend** (Munich, Germany) Biomedical Treatment of Organophosphate Poisoning
14:25-14:45	**Nikolina Maček Hrvat** (Zagreb, Croatia) Neuroprotective Effect of AChE Reactivator RS194B on Neuronal and Glial Cells of Mice Exposed to Nerve Agent
14:50-15:10	**Tamara Zorbaz** (Jerusalem, Israel) Cholinergic Links to mRNA Decay Processes in Schizophrenia and Bipolar Disorder
15:15-15:35	**Sebastian Lobentanzer** (Heidelberg, Germany) Integrating Multi-Omics with Prior Knowledge to Enable Causal Molecular Reasoning in Cholinergic Systems
15:40-15:50	**Muslum Gok** (Mugla, Turkey) A Novel Look on the Catalytic Activity of Butyrylcholinesterase Using Unusual Substrates: Saturated and Unsaturated 18C Fatty Acid
16:30-21:30	Excursion by Karaka to Dubrovnik

WEDNESDAY, 11 MAY 2022

8:30-11:30	**Session 7: Structural Insights in Cholinergic Mechanisms** **Chairs: Z. Radić and T. L. Rosenberry**
8:30-8:50	**Zoran Radić** (La Jolla, USA) Insights Into Functionally Relevant Conformational Flexibility of Protein Backbones in Cholinesterase Structures
8:55-9:15	**Tzviya Zeev-Ben-Mordehai** (Utrecht, The Netherlands) Cryo-Electron Microscopy of Cholinesterases
9:20-9:40	**Fredrik Ekström** (Umeå, Sweden) Discovery of the *in situ* Assembly Drug Modality of Choline-O-Acetyltransferase
9:45-10:05	**Terrone L. Rosenberry** (Jacksonville, USA) Out-of-Register Parallel β-Sheets and Antiparallel β-Sheets Coexist in 150-kDa Oligomers Formed by Amyloid-β (1–42)
10:10-10:30	**Pascale Marchot** (Marseille, France) Molecular Bases for Partnership of Two *C. elegans* Synaptic Organizers: The Cholinesterase-Like Cell-Adhesion Molecule Neuroligin-1 and the ADAMTS-Like Glycoprotein Punctin/MADD-4
10:35-10:55	Coffee Break
10:55-11:15	**Ryan Hibbs** (Dallas, USA) Structural Pharmacology of the Muscle-Type Nicotinic Acetylcholine Receptor
11:20-11:30	**Ali Kusay** (Sydney, Australia) Molecular Dynamics Insight into Modulator Binding at the α4α4 Binding Site of the α4β2 Nicotinic Acetylcholine Receptors
11:30-12:45	**Session 8: Synapses, Cognition and Neurodegeneration** **Chars: S. Kalanj Bognar and D. Montag**
11:30-11:50	**Svjetlana Kalanj Bognar** (Zagreb, Croatia) Cell Adhesion Glycoprotein Neuroplastin in Human Cognition and Neurodegeneration
11:55-12:15	**Dirk Montag** (Magdeburg, Germany) From Acetylcholinesterase Inhibitors Ameliorating Alzheimer's Disease to Retrograde Amnesia
12:20-12:40	**R. Jane Rylett** (London, Canada) Impact of Expression of 82-kDa ChAT on Alzheimer Pathology in a Mouse Model *(virtual)*
12:45-14:00	Lunch

14:30-15:45	Plenary Lectures
14:30-14:35	**Hermona Soreq** (Jerusalem, Israel) Introduction
14:35-15:15	**Marco A. M. Prado** (London, Canada) Regulation of Cholinergic Tone, Cognition and Alzheimer's Pathology by the Vesicular Acetylcholine Transporter (VAChT)
15:15-15:45	**Kevin J. Tracey** (Manhasset, USA) Famotidine Activates the Vagus Nerve Inflammatory Reflex to Attenuate Cytokine Storm *(virtual)*
15:45-16:30	**Poster Session 2 with Coffee and Tea**
16:30-17:45	**Session 9: Cholinergic Mechanisms in Dementias** **Chairs: M. A. M. Prado and T. Schmitz**
16:30-16:50	**Melissa Murray** (Jacksonville, USA) Paradoxical Aging in Alzheimer's Disease: A Clinicopathologic Perspective of Nucleus Basalis of Meynert *(virtual)*
16:55-17:15	**Taylor Schmitz** (London, Canada) Age-Dependent Effects of the p75 Modulator LM11A-31 on Alzheimer's Disease Biomarkers in a Six-Month Safety and Exploratory Endpoint Trial
17:20-17:40	**Frank Longo** (Stanford, USA) LM11A-31 Small Molecule p75 Receptor Modulator – Analysis of Safety and Exploratory Endpoints in a Phase 2A Trial in Alzheimer's Disease *(virtual)*
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THURSDAY, 12 MAY 2022

8:30-10:35	**Session 10: Exposure to Organophosphorus Pesticides and Nerve Agents** **Chairs: F. Nachon and K. Musilek**
8:30-8:50	**Florian Nachon** (Brétigny sur Orge, France) Design and Evaluation of Novel Reactivators of Cholinesterases Inhibited by Nerve Agents
8:55-9:15	**Kamil Musilek** (Hradec Králové, Czech Republic) Modification of Oxime Nucleophiles for Enhanced Reactivation of Cholinesterases
9:20-9:40	**Ines Primožič** (Zagreb, Croatia) Modulating Cholinesterases Activity by Quinuclidine and Cinchona-Based Compounds
9:45-10:05	**Yvonne Rosenberg** (Washington, USA) Development of Pre- and Post-Countermeasures against OP Toxins in Macaques
10:10-10:30	**Zrinka Kovarik** (Zagreb, Croatia) Assessment of Four Organophosphorus Pesticides as Inhibitors of Human Cholinesterases
10:35-10:55	Coffee Break
10:55-12:10	**Session 11: Cholinergic System in Health and in the Alzheimer's Pathology** **Chairs: A. C. Cuello and I. Primožič**
10:55-11:15	**A. Claudio Cuello** (Montreal, Canada & Oxford, UK) The Impact of a Novel NGF Metabolic Pathway on the Forebrain Cholinergic System in Health and in the Alzheimer's Pathology
11:20-11:40	**Agneta Nordberg** (Stockholm, Sweden) The Role of Alpha 7 Nicotinic Acetylcholine Receptor in Alzheimer´s Disease Pathology and Other Proteinopathies
11:45-12:05	***In memory to Professor Giancarlo Pepeu*** **Maria Grazia Giovannini** (Florence, Italy) More than the Cholinergic System: The Evolving Role of Glia in Memory, Aging, and Neurodegeneration *(virtual)*
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12:20-12:35	Summary of the ISCM2022: Marco A. M. Prado
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P2 **Pralidoxime analogues as efficient reactivators of ChE inhibited by OP nerve agents and pesticides**
Tena Čadež, Zrinka Kovarik

P3 **Distinct distribution of cholinesterases in mouse heart**
Dominika Dingova, Eric Krejci, Anna Hrabovská

P4 **The macro problem of live human microglia**
Serafima Dubnov, Iddo Paldor, Hermona Soreq

P5 **The effect of high-fat diet on the cholinergic system**
Tibor Hodbod, Kristína Szmicseková, Zuzana Kiliánová, Dominika Dingová, Anna Hrabovská

P6 **Expression of cholinesterases and their anchoring proteins in rat heart**
Zuzana Kilianová, Natalia Ciznarova, Anna Hrabovská

P7 **Characterization of novel imidazolium oximes as selective reactivators of nerve agent-inhibited butyrylcholinesterase**
Dora Kolić, Ana-Marija Lulić, Zlatan Spahić, Ines Primožič, Maja Katalinić, Zrinka Kovarik

P8 **Amiridine derivatives as multitarget drugs related to Alzheimer's disease therapy**
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P9 **The effects of vitamin B3-based compounds on neuronal and muscle cells**
Josip Madunić, Antonio Zandona, Ana-Marija Lulić, Katarina Miš, Valentina Bušić, Dajana Gašo-Sokač, Sergej Pirkmajer, Maja Katalinić

P10 **Changes in the cholinergic system in patients with severe COVID-19**
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P11 **Synthesis and biological evaluation of heterocyclic meta-biscarbamates**
Ana Matošević, Anamarija Knežević, Antonio Zandona, Nikola Maraković, Maja Katalinić, Zrinka Kovarik, Anita Bosak

P12 **Low dose tetanus toxin injections into the rat motor cortex and striatum impair the narrow beam walking performance**
Patrik Meglić, Petra Šoštarić, Ivica Matak

P13 ***In vitro* evaluation of uncharged thienostilbene oximes as reactivatiors of organophosphate-inhibited cholinesterases**
Milena Mlakić, Tena Čadež, Danijela Barić, Zrinka Kovarik, Irena Škorić

P14 **Commercial Herbicides Screened for Toxicity *in silico* and Examined as Inhibitors of Acetylcholinesterase and Butyrylcholinesterase**
Vesna Pehar, Višnja Stepanić and Zrinka Kovarik

P15 **Cellular model expressing acetylcholinesterase for evaluation of neurotoxicity and neuroprotection**
Lenka Pulkrabkova, Lubica Muckova, Martina Hrabinova, Tereza Kobrlova, Lukas Gorecki, Ondrej Soukup

P16 **The effect of ganglioside composition on enzyme activity, protein expression and submembrane localization of Na+ /K+ -ATPase in mouse brain**
Borna Puljko, Mario Stojanović, Katarina Ilić, Nikolina Maček Hrvat , Marija Heffer, Kristina Mlinac Jerković, Svjetlana Kalanj Bognar

P17 **Synthesis, characterization and evaluation of 10,11-dihydrocinchonidine carbamates as potential cholinesterase inhibitors**
Alma Ramić, Ines Primožič, Ana Mikelić, Tomica Hrenar

P18 **Exploring structure-activity relationship of new *N*-alkyl quaternary quinuclidines through cholinesterase inhibition and impact on cell homeostasis**
Donna Vadlja, Antonio Zandona, Alma Ramić, Ines Primožič, Suzana Žunec, Maja Katalinić

P19 **7-phenoxytacrine is a AChE inhibitor and NMDA antagonist with neuroprotective efficacy *in vivo***
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P20 **Long-term motor central effects of botulinum toxin type a in rats**
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P22 **Development of an *in situ* reactive Coenzyme A trapper to enable efficient extraction and characterization of choline acetyltransferase from tissue**
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P23 **Investigation of antioxidant potential of pyridinium oximes with halogen moiety and its relevance to therapeutic application**
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P24 **Utilizing isothermal spectral shift detection to quantify challenging biomolecular interactions with Monolith X**
Andreas Langer, Tanja Bartoschik, Natalia Kubisa, Claire Hatty

Abstracts

Molecular Recognition in the Cholinergic Nervous System

Palmer Taylor, Kwok-Yiu Ho, Gisela Andrea Camacho, Xi Zhang, William Fenical, Zoran Radić, K Barry Sharpless

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If we consider proteins recognizing acetylcholine and its congeners, we find distinct responses spanning a time frame of microseconds to hours and variation of structural themes involving ligand-gated ion channels, G-protein coupled receptors and catalysis of the released transmitter, acetylcholine. Typically, these proteins are arranged as oligomers to achieve localization, rapid synaptic transmission and far slower desensitization. Specificity not only relies on an active center selectivity, but also conformational or state variations of the target molecule. Pharmaceutical development often starts with natural product characterization, dating back to Sir Henry Dale. Cholinesterase inhibition, muscarinic and nicotinic receptor activation all have been achieved by natural product leads for over a century. For acetylcholinesterase, reactivation of the irreversibly inactivated enzyme becomes a dominate goal; antidote efficacy requires agents that act on targets centrally as well as peripherally. Here we have developed novel nucleophiles that rapidly convert among multiple ionization states and enter a narrow, tortuous, active center gorge within each subunit. We rely on strong nucleophiles that cross the blood-brain barrier rapidly to access central and peripheral nervous system sites in order to minimize toxicity of acute exposure. Zwitterionic reactivators enable us to form oximate anions and ionizable base cations devoid of toxicity of surface-active agents. Nicotinic receptors depart from the cholinesterases by having a subunit interfaces contributing to recognition. With five subunits and corresponding faces, some contribute to recognition of the ligand and others create an asymmetry around a circular axis leading down a central channel. State changes, as originally formulated by Katz and Thesleff and described further for oligomeric proteins by Monod, Wyman and Changeux, depend on ligand occupation and differ for the sites that are proximal and distal to the initial site of occupation. Hence, with multi-subunit proteins possessing an axis of symmetry, both subunit face composition and maintenance of symmetry are deciding factors in receptor occupation and agonist responses. Antagonists, whether they act reversibly or irreversibly, serve their function by blocking agonist access and disrupting subunit symmetry. Both agonists and antagonists, upon prolonged exposure, more slowly create desensitized states resistant to activation.

Small RNA regulators of cholinergic pathway genes exert sex and disease-related neuroinflammation and mental changes

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Cholinergic pathway genes are targeted by small regulatory RNAs including MicroRNAs and transfer RNA fragments (CholinomiRs, CholinotRFs) via complementary, few nucleotides long motifs in the mRNAs 3' non-coding regions. Such targeting reduces translation and induces destruction of the cholinergic transcripts. Therefore, common single nucleotide polymorphisms (SNPs) in the motif-carrying domains of the AChE gene entail diverse reactions to both anti-cholinesterase poisoning and stressful events (e. g. via the acetylcholinesterase (AChE) targeting miR-132). Since regulatory RNAs can each block many different genes that carry complementary motifs, SNPs in the small RNAs-reacting non-coding 3'-regions of target genes, which do not change their protein products, may affect numerous other genes including the recognition motifs. Men to women differences in cortical CholinomiRs may therefore explain the distinct therapeutic responses in men and women with psychiatric and neurodegenerative diseases. Additionally, circular RNAs may operate as 'sponges' to small regulatory RNAs, disabling their suppressive capacities, for example in the brain of Parkinson's disease or mental disease patients. Moreover, inherited differences in genomic regions affected by small regulatory RNAs may affect the cholinergic anti-inflammatory response and block metabolic reactions. However, there are differences between the distinct regulatory RNAs: recruiting CholinomiRs requires transcription and transport to where they should function, whereas CholinotRFs produced by nuclease destruction of pre-existing tRNA molecules can rapidly yield oligonucleotides with miR-like features. Therefore, CholinotRFs may be advantageous compared to CholinomiRs under acute states. We addressed this issue in blood cells from ischemic stroke patients, where the need to block excess inflammation is accompanied by the requirement to avoid risky infections. Achieving this goal is fulfilled by a 'changing of the guards' process where blood cells CholinomiRs decline whereas CholinotRF levels increase. In conclusion, both mRNA and small RNA profiles of cholinergic regulator transcripts yield dynamic sex and age-related differences in health and disease.

A Dynamic View on α4β2 nAChR Agonist and Modulator Binding

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Neuronal nicotinic acetylcholine receptors (nAChRs) are cation-selective, ligand-gated ion channels that mediate fast neuronal transmission in the central and peripheral nervous systems. nAChRs are involved in a range of physiological and pathological functions and hence are important therapeutic targets.

Over the past decade we have made significant progress in understanding the structure and function of nAChRs. In particular, the recognition of the importance of different stoichiometric isoforms of heteromeric nAChRs and development of methods to control their assembly has enabled interpretation of pharmacological data in a structural context. More recently, the revolution in nAChR structure determination through X-ray crystallography and cryo-electron microscopy, has made computational studies and structure-based drug design more feasible than ever.

To further our understanding of nAChR ligand binding we have used extensive molecular dynamics simulations of agonist and modulators at both the extracellular and transmembrane binding sites in α7 and α4β2 nAChRs. For the transmembrane region, we explored the binding of modulators in the inter-subunit space that is occupied by PNU-120596 in a recent α7 nAChR structure. The simulations revealed stark similarities in binding modes for the ligands and lend themselves to development of pharmacophore models for allosteric modulators. Moreover, simulations of modulators at the transmembrane inter-subunit space provides a possible explanation for their impact on channel modulation and opening. For the extracellular region, simulations provided exciting insight into the dynamics of binding of modulators and agonists and again suggests new opportunities for development of novel selective drugs.

Regulation of cholinergic tone, cognition and Alzheimer's pathology by the vesicular acetylcholine transporter (VAChT)

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The vesicular acetylcholine transporter (VAChT) regulates that last cholinergic specific step for acetylcholine (ACh) secretion, the storage of neurotransmitter into synaptic vesicles. VAChT levels directly regulate the amount of ACh released by cholinergic nerve terminals providing fine control of cholinergic tone. *In vivo* recordings using fibre photometry and new ACh fluorescent sensors have started to provide unprecedented information of how brain ACh tone contributes to specific behaviors. Understanding the multiple roles of cholinergic signaling from specific brain regions to regulate behaviors in health and diseased states will be fundamental to improve cholinergic dysfunction in disease. Cholinergic deficiency is a hallmark of many neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease, both of which present decrease in VAChT levels in specific brain regions. In mice, brainsteam VAChT deficits affect mainly gait, but does not disturb high-level cognitive function such as attention or behavioral flexibility. Mice with selective VAChT deficiency in the striatum are prone to habitual behavior and present deficits in associative learning due to abnormal cholinergic-dopaminergic balance. In contrast, mice with decreased forebrain VAChT levels present attention, behavioral flexibility and spatial working memory deficits. Interestingly, combined PET/MRI analysis suggests that decreased VAChT levels in the course of AD is inversely associated with the degree of brain pathology in humans. To test for causal relationships between VAChT expression and AD pathology, we have generated humanized APP knock-in (KI) mice lacking VAChT in cholinergic forebrain neurons or mice overexpressing VAChT. Elimination of forebrain VAChT led to increased levels of insoluble Ab compared to mice expressing normal levels of VAChT. In contrast, mice with VAChT overexpression showed decreased Ab levels. Surprisingly, this causal relationship was not found in intact female mice, suggesting an important sex difference in cholinergic regulation of AD pathology. Collectively, the present work has started to reveal how VAChT regulates cholinergic tone associated with specific cognitive functions. Mechanistic understanding of how VAChT levels regulate pathological changes related to AD may provide new insights on cholinergic signalling involved in disease modification in dementia.

Role of Cholinergic vs. Sympathetic Pathways in the Pathobiology of Stroke

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The tight interconnection between the nervous and immune systems plays an essential role in the homeostasis of the organism. Acute damage of the central nervous system, such as an ischemic stroke, leads to a rapid but transient depression of the peripheral immune system. Both cholinergic and sympathetic signaling pathways play an essential role in this process. Simultaneous overactivation of these two autonomic systems leads to apoptosis or dysfunction of lymphocytes and myeloid cells via stimulation of the corresponding receptors (nicotinic acetylcholine receptor, ß2-receptor) in spleen, blood and lung, thereby impairing antibacterial defense. In addition, mucociliary clearance of the lung as well as motility and immune barrier of the intestine were severely disrupted by the autonomic pathways. These factors are causally involved in stroke-associated infections. Moreover, there are massive changes in gene expression in peripheral immune cells that involve cholinergic signaling pathways regulating immune function, suggesting functional relevance to infection and homeostasis after stroke. Pneumonia is the most common complication after stroke, which contributes significantly to the high burden of stroke by increasing the risk of recurrent vascular events, vascular cognitive impairment, and death. Changes in the peripheral immunity as well as infections are likely to be involved in both acute and chronic neuroinflammation associated with damaging and regenerative processes after acute CNS trauma. A better understanding of pathobiology is needed to develop immunomodulatory treatment approaches for protecting lung and brain.

Alpha 10-Containing Nicotinic Receptors – Implications for Neuropathic Pain, Cancer and Inflammation

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Nicotinic acetylcholine receptors containing α9 and α10 nAChR subunits have been implicated in several pathological states including neuropathic pain, breast and lung cancers, and inflammation. Selective antagonists of α9-containing nAChRs have been developed, are analgesic and appear to have disease-modifying properties. One conopeptide analog has entered human clinical trials. The role of the α10 subunit is less clear and has been thought to function only as a partner to α9. Contrary to long-held assumptions, heterologous expression studies indicate that human α10 subunits can readily assemble as homomeric receptors. These findings have implications for the possible nAChRs subtypes expressed in native tissues and their roles in human disease states.

Phenotype and Genotype Variation in Myasthenia Gravis

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Myasthenia Gravis is the prototypic organ-specific antibody-mediated autoimmune disease caused by immune dysregulation, in which muscular weakness directly reflects disruption of nicotinic cholinergic neurotransmission. What was considered a single disease is better thought of as a series of related conditions. Although all produce weakness in a range of muscle groups, the condition can be subdivided. Disease onset under the age of 40-50 is associated with thymic inflammation, however late onset disease which is increasing in prevalence is associated with an atrophic thymus, distinct genetic markers that may be related to the loss of immune tolerance, and a specific range of antibodies. Antibodies of several subtypes against a range of proteins at the neuromuscular junction disrupt neurotransmission by a number of mechanisms including complement -mediated endplate destruction and receptor modulation. The phenotypes and genotypes of myasthenia and their clinical and therapeutic significance will be described.

Agrin signalling in human skeletal muscle cells

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Degeneration of the neuromuscular junction (NMJ) and a decline in skeletal muscle regeneration contribute to the ageing-related sarcopenia. Neural agrin, a heparan-sulphate proteoglycan secreted by the α-motor neuron, stabilizes the NMJ, suggesting that agrin-based therapies might protect against sarcopenia. As well as stabilizing the NMJ, neural agrin increases or decreases proliferation of various non-muscle cells. We investigated whether neural agrin might affect skeletal muscle regeneration by modulating intracellular signalling and proliferation of primary human myoblasts. Effects of neural agrin were compared with those of muscle agrin, a non-neural isoform that lacks NMJ stabilizing properties. An acute treatment with exogenous neural or non-neural agrin modulated the activity of Abl and the ERK1/2 signalling pathways, while other pathways, such as STAT3 and focal-adhesion kinase, did not show a significant response. Despite similar signalling responses to the agrin treatment, two distinct groups of responses were observed with regard to the proliferation. As assessed by the BrdU incorporation, agrin did not alter the proliferation of myoblasts obtained from younger donors (<30 years), while it increased the proliferation of myoblasts from middle-aged/older donors (>58 years). Collectively, our data show that primary human myoblasts are responsive to neural as well as non-neural agrin, but functional consequences of the agrin treatment seem to be at least partially age-dependent. Grants: This study was supported by grants from the Slovenian research agency (P3-0043, J7-3153) and from University of Trieste (FRA-2013).

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Three newly identified molecules that facilitate the formation of neuromuscular junction

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Secreted molecules play essential roles in the clustering of muscle nicotinic acetylcholine receptors (AChR) and the formation of the neuromuscular junction (NMJ). The most characterized secreted molecule is a neural isoform of agrin. We identified that polypyrimidine binding protein 1 (PTBP1) is a key RNA-binding protein that enables splicing of a neuron-specific isoform of agrin. Extensive search for molecules expressed at the NMJ, as well as in cellulo and *in vivo* studies of specific molecules, revealed three novel secreted molecules that are essential for the NMJ formation. First, R-spondin 2 (Rspo2) binds to leucine-rich repeat-containing G-protein, Lgr5, on the motor endplate, and phosphorylates MuSK to induce AChR clustering in an agrin-independent manner. We also showed that spinal motor neuron-derived Rspo2 plays a major role in AChR clustering and postsynaptic NMJ formation, and muscle-derived Rspo2 also plays a substantial role in the juxtaposition of the active zones and synaptic folds. Second, multiple fibroblast growth factor (FGF) ligands and multiple FGF receptors are expressed at the NMJ, but little is known about their roles at the NMJ. Presynaptic Fgf receptor 2 (Fgfr2) is involved in the formation of the NMJ, but its ligand remains to be identified. We found that Fgf18-knockout mice showed an abnormal aggregation of multiple nerve terminals in the diaphragm. In particular, the nerve terminals made a gigantic pre-synapse, but had few synaptic vesicles in each nerve terminal. Thus, Fgf18 is likely to be a specific ligand that activates presynaptic Fgfr2. In addition to the effects of Fgf18 on the nerve terminal, we observed that Fgf18-knockout mice showed simplified motor endplates and reduced gene expression of NMJ-specific Chrne and Colq. Third, we found that the CT domain of connective tissue growth factor (Ctgf/Ccn2) directly binds to the third β-propeller domain of LRP4. Ctgf/Ccn2 enhances the binding of LRP4 to MuSK and facilitates the localization of LRP4 on the plasma membrane. Ctgf/Ccn2 enhances agrin-induced MuSK phosphorylation and AChR clustering in cultured myotubes. Ctgf-deficient mouse embryos have small AChR clusters and abnormal dispersion of synaptic vesicles along the motor axon. Ultrastructurally, the presynaptic nerve terminals have reduced numbers of active zones and mitochondria. Functionally, Ctgf-deficient embryos exhibit impaired NMJ signal transmission. Thus, Ctgf/Ccn2 interacts with LRP4 to facilitate clustering of AChRs at the motor endplate and the maturation of the nerve terminal.

ColQ, from structure to molecular functions and pathology

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ColQ is a non-fibrillar collagen expressed at the neuromuscular junction. It anchors the enzyme Acetylcholinesterase (AChE) in the synaptic cleft through interactions with perlecan, a proteoglycan and the transmembrane MuSK-LRP4 complex. Mutations or deletions causing myasthenic syndromes have been identified in human all along COLQ gene and in most cases can be attributed to the loss of molecular interactions or defects in ColQ structure leading to the absence or low levels of AChE. The consequences of the genetic defects are coherent with what we know about the functional domains. However, as for the C-terminus of ColQ, the link between mutations in this domain and the pathology is not completely clear. Analysis of mutations in this domain and ColQ molecular interactions provide new insights into its role that will be discussed.

Phase-Separated Rapsyn Condensates as a Signaling Platform for Neuromuscular Junction Formation

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Neuromuscular junction is a synapse between motoneurons and skeletal muscles, where nerve terminals are precisely aligned with postsynaptic members with high concentration of acetylcholine receptors (AChRs), to control muscle contraction. Although much has been learned about this peripheral synapse and related disorders, exactly how the NMJ is assembled is not well understood. Rapsyn is a membrane-attached, cytoplasmic protein, critical for NMJ formation. Earlier evidence suggests that it serves a scaffold protein to link AChRs to the cytoskeleton. Recent studies indicate that rapsyn may serve as a signaling protein. First, it possesses an E3 ligase activity that promotes AChR stability by neddylation. Mutation of C366 to alanine ablates the E3 ligase activity *in vitro*; mice carrying this mutation fail to form the NMJ. Second, rapsyn could be phosphorylated by MuSK and the phosphorylation enhances self-association and E3 ligase activity. Third and more recently, rapsyn was shown to undergo liquid-liquid phase separation (LLPS) and condensates into liquid-like assemblies. Such assemblies could recruit AChRs, cytoskeletal proteins, and signaling proteins for postsynaptic differentiation. Rapsyn LLPS requires multivalent binding of tetratricopeptide repeats and is increased by MuSK signaling. The ability of rapsyn to self-associate and phase-separate is diminished by mutations of congenital myasthenic syndrome (CMS). These results reveal novel mechanisms of NMJ formation and insight into neuromuscular disorders. I will provide an update of our studies of rapsyn in NMJ formation and pathology.

Acetylcholine toxicities: Surprising lessons from mouse genetics

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The pleiotropic effects of acetylcholinesterase (AChE) inhibitors are understood as cumulative effects of excess ACh in synaptic transmission, gene regulations that modify cholinergic mechanisms, non-enzymatic actions of AChE or its associated proteins, and non-specific effects of cholinesterase inhibitors (including highly reactive organophosphates and carbamates). Pleiotropic effects appear both during acute intoxications (e.g. chemical weapons or suicides with pesticides), as well as during chronic intoxications which last long after a normalization of AChE levels (e.g. Gulf syndrome).

We have developed genetic approaches in mice to evaluate specifically the consequences of excess ACh. We introduced a point mutation in the gene encoding AChE (WACHE mice) and a point mutation in the gene encoding BChE (SBChE mice). These mutations do not alter the normal AChE or BChE protein folding nor their adequate localization. However, they make them inactive (no ACh hydrolysis). Like BChE KO mice, SBChE mice develop normally; like AChE KO mice, WAChE mice show severe growth retardation, severe bloating and die before weaning. The growth of PRIMA KO mice is normal despite a severe deficit of AChE in cholinergic neurons in brain and peripheral nervous system. In contrast, the growth retardation and weakness is apparent in ColQ deficient mouse (point mutation in COLQ, essential for anchoring AChE in neuromuscular junctions). An AChE deficit in skeletal muscle is not sufficient to explain the growth retardation because a mouse without AChE in skeletal muscle (AChE1iRR) and two copies of active BChE has normal growth. We were surprised to find that an AChE1iRR mouse without active BChE dies in the perinatal period, while an AChE1iRR mouse with a single copy of active BChE shows severe growth retardation. Furthermore, we established that α7 nicotinic receptor activation in JNMs depresses ACh release when AChE is absent. Growth of an AChE1iRR mouse without α7 receptor is also delayed.

In contrast to the brain's ability to adapt to excess ACh, these observations show that excess ACh in the periphery is highly toxic if not hydrolyzed by AChE and BChE. We propose that this toxicity occurs when muscle ACh reaches numerous targets of non-neuronal cholinergic systems, rather than by a local alteration of cholinergic synapses.

Development of M_5 NAMs and Orthosteric Antagonists for the Treatment of Opioid Use Disorder

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Opioid use disorder (OUD) is a debilitating neuropsychiatric disorder associated with symptoms of compulsive opioid use, dependence, and repeated relapse following periods of abstinence. Accumulating evidence indicates that selective inhibition of the M_5 muscarinic acetylcholine receptor (M_5 mAChR) may provide an alternative therapeutic approach for the prevention and/or treatment of OUD. This presentation will review the recent identification and characterization of the novel, selective M_5 negative allosteric modulators (NAMs) ML375 and VU6008667 and the M_5 orthosteric antagonist VU6019650 in several preclinical models of opioid drug seeking behaviors. Using RNAScope *in situ* hybridization techniques, we confirmed the expression of the M_5 mAChR subtype within the mesolimbic dopaminergic reward circuitry, primarily on dopamine neurons in the ventral tegmental area (VTA). We also demonstrated that the M_5 antagonist VU6019650 blocked the nonselective muscarinic agonist oxotremorine-M-induced increases in VTA neuronal firing rates in acute brain slice electrophysiology studies. In addition, we determined that the short acting M_5 NAM VU6008667 produced dose-dependent attenuation of the μ-opioid agonist oxycodone self-administration and cue-induced reinstatement after extinction in rats. When administered daily to opioid-naïve rats, VU6008667 also prevented the acquisition of oxycodone self-administration behaviors. Importantly, selective inhibition of M_5 have no effect on opioid-induced antinociception using the hot-plate and tail-flick assays in rats. Collectively, these findings suggest that selective functional antagonism of the M_5 mAChR represents a novel, non-opioid-based treatment for opioid misuse and relapse behaviors.

Extremely potent human monoclonal antibodies are effective for prophylaxis and a novel therapy of tetanus

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Human monoclonal antibodies were identified and used to study the mechanism of neuron intoxication by tetanus neurotoxin (TeNT) and as a safe preventive and therapeutic substitute of hyperimmune sera for tetanus. Two monoclonal antibodies displayed specific exceptionally high neutralizing activities for TeNT and were extensively characterized both structurally and functionally. We found that these antibodies interfere with the binding and translocation of the neurotoxin into neurons by interacting with two epitopes, whose definition pinpoints defined events in the cellular pathogenesis of tetanus. Some mechanistic aspects of tetanus neurotoxin intoxication were revealed, explaining at the same time, the unprecedented neutralization ability of these antibodies. These antibodies were found to be exceptionally efficient in preventing experimental tetanus when injected in mice long before the neurotoxin. Moreover, their Fab derivatives neutralized tetanus neurotoxin in post-exposure experiments, suggesting their potential therapeutic use upon intrathecal injection. Preliminary experiments support this possibility. As such, these human monoclonal antibodies, as well as their Fab derivatives, meet all requirements for being considered for prophylaxis and therapy of human tetanus and are ready for clinical trials.

Combined exposure to methylmercury and manganese during L1 larval stage causes motor dysfunction, cholinergic and monoaminergic up-regulation and oxidative stress in L4 *Caenorhabditis elegans*

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Humans are exposed simultaneously to a variety of neurotoxic agents, including manganese (Mn) and methylmercury (MeHg). Therefore, the study of combined exposures to toxicants is timely. This work aimed to study changes in cholinergic system focusing on acetylcholinesterase (ace-2), monoaminergic system focusing on vesicular monoamine transporter (VMAT, cat-1) expression, to address changes in antioxidant enzymatic systems, namely, the expression of superoxide dismutase (sod-3 and sod-4) and catalase (ctl-3), as well as worm reproduction and locomotion. *C. elegans* in the L1 larval stage were exposed to Mn, MeHg or both. All analyses were done 24 h after the end of exposure, except for behavior and reproduction tests that were assessed in L4 larval stage worms. The values obtained for lethal dose 50% (LD_{50}) were 17.78 mM for Mn and 30.63 µM for MeHg. It was observed that body bends, pharyngeal pumping and brood size decreased in worms exposed to metals when undergoing combined exposures. Relative mRNA content of ace-2, cat-1, sod-3, sod-4 and ctl-3 was increased at the highest concentration of the interaction (50 mM Mn + 50 µM MeHg). Cholinergic degeneration was observed in all groups co-exposed to both metals. Notably, combined exposure to metals was more toxic to the worms than when exposed to a single metal.

Organophosphorus Insecticides and the Developing Brain: Detrimental Effects of Early-Life Exposures

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Gestational exposures to high doses of organophosphorus (OP) insecticides, including chlorpyrifos (CPF), induce a toxidrome that can be fatal and results from irreversible inhibition of acetylcholinesterase (AChE). Although OP-intoxicated pregnant women are generally treated with high doses of atropine to non-selectively inhibit overactive muscarinic receptors (mAChR) and/or oximes such as 2-PAM to reactive AChE, health outcomes are not always favorable. Recently, we examined the effectiveness of different treatments to counter the immediate and long-term effects of an acute gestational CPF exposure. In this study, pregnant guinea pigs were exposed to CPF (360 mg/kg, p.o.) and posttreated intramuscularly with 2-PAM (25 mg/kg) alone or with atropine (1 mg/kg) or THP (0.1 mg/kg), a more selective inhibitor of M1/M3 than M2 mAChRs. Treatment with 2-PAM-plus-THP was the most effective to ensure survival of the dams and their offspring and preservation of their functional and structural brain integrity, assessed in behavioral assays, imaging, and electroencephalography. The effectiveness of THP may be due to its ability to: (i) spare presynaptic M2 receptors, and, thereby, safeguard a negative feedback mechanism in which ACh limits its own release, and (ii) inhibit neuronal nicotinic ACh receptors, whose overactivation can contribute to poor health outcomes following OP poisoning. Our studies using the guinea pig model have also supported the notion that CPF is a developmental neurotoxicant. Specifically, guinea pigs prenatally exposed to low CPF doses presented learning deficits that correlated with increased hippocampal GABAergic tone. Heightened GABAergic activity was also detected in primary hippocampal cultures exposed to non-AChE inhibiting CPF concentrations and was likely due to (direct or indirect) inhibition of cannabinoid 1 receptors by CPF. Understanding the mechanisms that underly the sensitivity of the developing nervous system to different levels of OP insecticides is a necessary step to guide the assessment of health risks posed by these chemicals. [Support: NIH grant R01ES027822]

Adolescent Nicotine Enhances Adult Alcohol Self-administration

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Adolescent smoking is associated with pathological drinking later in life, but the biological basis for this vulnerability is unknown. To examine how adolescent nicotine influences subsequent ethanol intake, nicotine was administered during adolescence or adulthood and responses to alcohol were measured one month later. We found that adolescent, but not adult, nicotine exposure altered long-term GABA signaling within the ventral tegmental area (VTA) and led to a long-lasting enhancement of alcohol self-administration. We detected depolarizing shifts in $GABA_A$ reversal potentials arising from impaired chloride extrusion in VTA GABA neurons. This GABAergic dysregulation was reversed by applying the 5-$HT2_AR$ agonist TCB-2 ex vivo via functional enhancement of the potassium-chloride cotransporter KCC2. Importantly, enhancing chloride extrusion in midbrain GABAergic neurons in adolescent-nicotine treated animals restored VTA GABA signaling and alcohol self-administration to control levels. Taken together, this work suggests that adolescent nicotine increases the risk profile for increased alcohol drinking in adulthood.

The nicotinic receptor modulation of higher brain functions: from molecules to consciousness

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The evolutionary analysis together with the developmental data for the human newborn has suggested that"consciousness"is not an irreducible quality, but a bona fide brain function evolving stepwise through several nested levels of organization (Changeux 2006, 2017, 2021). Accordingly, the molecular level imposes mandatory bottom-up constraints to all these levels of functional organization, up to consciousness. For instance, the cholinergic system (ACh) coordinate the response of brain networks to internal and external inputs and shape global neuronal activity from multiple brain territories. Technical advances that combine molecular approaches, *in vivo* recordings in awake behaving animals, human brain imaging, and genetics have strengthened our understanding of the roles of nicotinic acetylcholine receptors (nAChRs). Four aspects are considered in this presentation.

1. The differential contribution of nAChR subunits to the reward system engaged into nicotine addiction and the relevant loss of control (Changeux 2010, 2018)

2. The role of nAChRs in ultraslow fluctuations in EEG thought to monitor conscious processing in humans (Koukouli et al 2016).

3. The alteration of nAChR modulation of layer II/III hierarchical inhibitory circuits as responsible of prefrontal deficits of schizophrenia (Koukouli et al 2016, 2017, 2020). The identification of the diverse nAChR components of the circuit engaged in cognition and its dysfunctions, both in animal models and in humans opening multiple novel strategies for the diagnosis, prevention, and therapies of neuropsychiatric diseases.

4. The contribution of our present knowledge of the allosteric transitions of the nAChR at the atomic level on drug design (Cecchini & Changeux 2022).

COVID-19 and the Nicotinic Cholinergic Pathway

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Immune dysregulation and cytokine storm appear to play a major role in the pathophysiology of severe COVID-19. The"cholinergic anti-inflammatory pathway"is an important regulator of the inflammatory response. Its effects are mediated mainly by the vagus nerve and by α7 nicotinic acetylcholine receptors (nAChRs) on macrophages and other immune cells. α7 nAChRs are also expressed in human bronchial epithelial and endothelial cells, which are the major targets of SARS-CoV-2. We have described a direct interaction between SARS-CoV-2 Spike Glycoprotein and the Nicotinic Cholinergic System. Such interactions are not only explaining part of the pathophysiology of the severe disease, but could have potential therapeutic implications considering that there are pharmacological agents, such as nicotine and other cholinergic agonists, which could protect these receptors through competitive binding to the receptors.

Towards Defining the Function of Nicotinic Receptor Subtypes in Sensory Physiology and Pathology

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Because nicotinic acetylcholine receptors (nAChRs) are pentameric, a vast number of potential molecular isoforms are possible. Which of these actually exist in native neurons, and how they influence physiological functions are challenging to address. We have developed a calcium imaging-based pharmacological approach,"Integrated Constellation Pharmacology", to investigate the expression of nAChRs in the sensory neurons of the dorsal root ganglion (DRG). We monitored the expression of different nAChR subtypes in 19 different neuronal cell types in the mouse DRG. The α7-nAChR subtype is widely expressed in most DRG neurons, compared to α6α3β4 nAChR subtype. A small subset of neurons express α3β4 receptor. Although transcripts for α6β2 nAChRs are found in a subset of DRG neurons by single-cell transcriptomic analysis, the functional expression and roles of these receptors have remained elusive.

The nAChRs are recognized as crucial modulators of pain signaling. One nAChR subtype, the α9α10 expressed by immune cells, appears to be pro-inflammatory; inhibitors of this nAChR subtype prevents chronic pain after nerve injury. We assessed how the distribution of nAChR subtypes change under pathological conditions and whether inhibitors of α9α10 alter the course of disease progression. In an animal model of chronic pain, the chronic constriction injury of mouse sciatic nerve, the pathology is triggered by nerve injury and we found cell-specific downregulation of nAChR subtypes in DRG neurons with disease progression. The molecular changes in specific sensory neuronal subtypes are altered when chronic pain is prevented by the application of inhibitors of the α9α10 nicotinic receptor.

Rethinking cholinergic therapy for Alzheimer disease

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Progressive loss of memory, particularly of episodic memory, starts early in AD together with a selective neurodegeneration of cholinergic neurons of the basal forebrain. A close relationship between progressive cholinergic degeneration and cognitive impairment is supported by the clinical efficacy of pharmacotherapy specifically targeting the cholinergic system. The correlation of severity of dementia with disruption of cholinergic markers, including reduced choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities, reduction of choline uptake, and of acetylcholine (ACh) synthesis and decrease of nicotinic receptors (nAChR) subtypes suggests a close link between loss of cholinergic function and cognitive decline in AD.

Presently, enhancement of cholinergic neurotransmission via cholinesterase inhibitors (ChEI) represents the main available approach to treat cognitive and behavioral symptoms of early as well as late stages of Alzheimer's disease. Restoring the cholinergic system has been a primary means of improving cognition in Alzheimer's disease, as four of the six approved therapies are ChEI. Recently, it has been demonstrated that ChEIs clinical effect can be as long-lasting as 3-5 years if the treatment is started early in the disease and is prolonged in time with adequate dosage.

New alternatives to cholinergic therapy should be developed to amplify their clinical effects:

1. A combination of a ChEI with anti-a-beta or anti-tau therapy to complement and strengthen the cognitive effect.
2. A combination of a ChEI with an anti-inflammatory agent to reduce microglia reaction. An increase of the immune neurotransmitter function via ChE inhibition may improve the immune response to brain inflammation in AD.
3. A significant body of preclinical evidence suggests that activation of M1 mAChR by selective M1 muscarinic agonists, in addition to beneficial effects on cognition functions, can alleviate major hallmarks of AD such as pathological amyloid, tau, α-synuclein, and neuroinflammation.
4. An early, pharmacologically based, NGF-targeted therapy directed to preserve cholinergic neurons and function delaying cognitive impairment.

Reference: Giacobini E, Cuello C. and Fisher A. Reimagining cholinergic therapy for Alzheimer Disease. Brain, In press 2022

Sustainable Drug Discovery of Multi-Target Acetylcholinesterase Inhibitors for Alzheimer's Disease

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Alzheimer's disease (AD) is among the most frequent type of dementia, with the number of AD patients increasing from current 35 million to an astonishing 135 million by 2050. Already 60% of them live in low- and middle-income countries, and this number will rise to 71%, concomitantly with global increases in population size and life expectancy. All in all, AD has become a global health problem.

The multifactorial nature of AD has been called into question as one of the factors contributing to the lack of effective drugs, although single-target cholinesterase inhibitors remain the predominant class of the currently available therapies. Cholinergic mechanisms are indeed known to have a key role in AD and to interplay with other AD hallmarks (neuroinflammation, amyloid and tau).[1] For this reason, cholinesterase inhibition has been so far a driving force in the development of "Multi-Target-Directed Ligands"(MTDLs),[2] i.e., single molecules that simultaneously modulate multiple targets involved in the neurodegenerative cascade.

As a further step, as cases increase in low- and middle-income countries, there is a need of new drugs that are not only effective, but also accessible and affordable to those in need. By using biowaste as starting material, we are motivated to develop MTDLs toward a sustainable drug discovery.[3] Clearly, this is a long-term aspirational goal, which, however, deserves attention by the medicinal chemistry community for the potential benefits to the global patient population and the environment.

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Evaluation of 4-aminoquinolines as potential anticholinesterase agents in the treatment of Alzheimer's disease

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Over the past years, although great efforts have been devoted to the development of drugs to treat Alzheimer's disease (AD), five of the six currently approved AD drugs act by temporally improving the cognitive abilities of patients by increasing the amount of the neurotransmitter acetylcholine or by blocking NMDA receptors in the brain. The problem in the development of AD drugs that act to slow or stop the progression of the disease lies in the fact that the aetiology of the disease is highly complex with clinical hallmarks such as a decline in acetylcholine levels, amyloid-β (Aβ) peptide deposits, oxidative stress, dyshomeostasis of bio-metals, tau protein hyperphosphorylation and accumulation, all concomitant with the fact that expression and level of appearance of AD hallmarks are highly individual. Recently, the new approach was suggested, that future research directed to slowing down or stopping the progression of AD and preserving brain function should move from single targeted drugs to "multi-target directed ligands". In line with that, one of the most promising directions combines the inhibition of ACh hydrolysis and additional AD features that target the promotion or progression of the disease, like oxidative stress and/or reduction of amyloid plaques aggregation. Design of new cholinesterase inhibitors, particularly acetylcholinesterase inhibitors, preferably includes "dual-binding site" inhibitors able to simultaneously interact with the catalytic and peripheral anionic sites of the enzyme. Such ligands have the potential to restore the cholinergic deficit by blocking acetylcholinesterase catalytic activity and at the same time interfering with Aβ deposition and aggregation by interaction with acetylcholinesterase's peripheral anionic site. Such desired characteristics could be met by 4-aminoquinolines. Their simple structure, high inhibitory potential toward cholinesterases and potential to cross the blood-brain barrier make 4-aminoquinoline derivatives a promising candidate for the design of novel AD drugs. Acknowledgements: The authors would like to thank the Croatian Science Foundation (Grant IP-2020-02-9343) and the MSTD (Grants No. 451-03-68/2022-14/200026 and 451-03-68/2022-14/200168).

Muscarinic stimulation of post-synaptic receptors for the potential treatment and prevention of cognitive decline

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The M1 muscarinic receptor (M1 mAChR) is a validated therapeutic target for the treatment of cognitive dysfunction in schizophrenia and Alzheimer's disease (AD) as it has a pivotal role in modulating cognitive deficits and the pathology of the disease. The rationale for M1 muscarinic agonists as a treatment strategy in AD is based on the preserved status of M1 mAChR in AD, the linkage of cognition, cholinergic presynaptic degeneration, Aβ and tau neuropathological hallmarks of AD and supported by the clinical efficacy of AChEIs treatments in AD. However, several agonists have been discontinued due to: i) adverse effects (AE) (e.g. the M1/M4 agonist Xanomeline, which improved cognition and reduced psychotic episodes in AD patients); ii) lack of beneficial effects in AD [the M1-positive allosteric modulator (M1 PAM), MK-7622; the M1/M3 agonist, Talsaclidine]; iii) or repurposed for treatment of Sjogren's syndrome despite some beneficial effects in AD patients [AF102B (Evoxac, Cevimeline)]. To mitigate the peripheral AE of Xanomeline, Trospium, a peripheral pan-muscarinic receptor antagonist was used in the combination of Xanomeline-Trospium (KarXT) resulting in significant improvements in schizophrenic patients and good tolerability. Activation of M1 mAChR can be achieved through M1 allosteric muscarinic agonists including bitopic (bind to both the orthosteric and allosteric site), M1 PAMs and direct-acting M1 muscarinic orthosteric agonists. While the efficacy of M1 PAMs depends upon the presence of acetylcholine, which declines as post-synaptic neurons loose cholinergic input from the basal forebrain in AD, the activity of M1 muscarinic orthosteric agonists is independent of the functional or anatomical integrity of pre-synaptic cholinergic terminals and would likely retain efficacy as the disease progresses even after pre-synaptic degeneration of cholinergic fibers. The preferred M1 agonist should fulfill stringent acceptance criteria such as: M1 subtype selectivity and specificity; a high therapeutic index; no central and peripheral AE; no tolerance when taken chronically; a brain/plasma ratio greater than 1; no drug-drug and detrimental pharmacodynamic interactions, respectively, with approved approved drugs including AChEIs, Memantine and Aducanumab; the potential to improve cognition and behavior (treatment); the potential for prevention, delaying or halting disease progression (disease modification, DM). In this context, some rigid analogs of acetylcholine from the *AF series* are relatively selective M1 orthosteric partial agonists including AF102B, AF150(S), AF267B, and AF292. Notably, AF102B (Evoxac) is the only M1 muscarinic agonist, approved by the FDA and Japanese health authorities for the treatment of dry mouth in Sjogren's disease. AF267B, which fulfills the criteria mentioned above, improved cognitive deficits in many animal models and chronic treatment decreased both Aβ and tau pathologies in the hippocampus and cortex, and reversed cognitive deficits in 3xTg-AD mice. Chronic treatment with AF267B and AF102B also decreased alpha-synuclein aggregates in human alpha-synuclein transgenic mice. Positive effects on some of the neuropathological hallmarks of AD were also reported in preclinical *in vivo* studies with EUK1001, VU0364572, a bitopic M1 agonist, VU0486846, an M1 PAM and AF710B, a mixed M1 allosteric/sigma-1 agonist. Chronic treatment with AF102B and Talsaclidine, decreased significantly CSF Aβ in AD patients whereas AChEIs had no effects on Aβ. In summary, preclinical evidence suggests that activation of M1 mAChR by selective M1 muscarinic agonists, in addition to beneficial effects on cognition functions, can alleviate major hallmarks of AD such as pathological amyloid, tau, and alpha-synuclein. Translating these findings to AD patients may evolve and extend the classical cholinergic hypothesis initially limited to symptomatic treatment. The compound with a high safety profile and selectivity for M1 mAChR, *in vivo*, should be selected as a therapeutic strategy designed to improve the cognitive deficits and to exhibit DM properties. Based on available data very few reported selective M1 muscarinic agonists (preferable M1 partial orthosteric) may fulfill such rigorous criteria as the next generation of therapies in AD, schizophrenia and related diseases such as Parkinson's disease dementia, dementia of Lewy body, prion diseases, and Sjogren's syndrome. *Ref: E. Giacobini, AC Cuello and A. Fisher. REIMAGINING CHOLINERGIC THERAPY FOR ALZHEIMER DISEASE, Brain, in press.*

Biomedical treatment of organophosphate poisoning

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Every year, more than 100.000 people die from organophosphorus (OP) pesticide poisoning. Furthermore, the deliberate release of OP nerve agents in military conflicts, e.g. Syria, or their use for assassination attempts, e.g. in the UK in 2018 underline the threat of OP compounds. The current treatment of OP poisoning comprises a muscarinic antagonist (e.g. atropine), an oxime (e.g. obidoxime or pralidoxime) and an anticonvulsant (e.g. diazepam or midazolam). These three pillars have been virtually unchanged for decades. The administration of an oxime is currently the only option to reactivate inhibited acetylcholinesterase (AChE). However, the currently licensed oximes lack effectiveness in mega-dose OP poisoning and in poisoning with certain nerve agents and pesticides, e.g. soman, tabun and profenofos. A promising approach to overcome the limitations of oxime treatment might be the use of (bio)scavengers. These scavengers seem to be a suitable treatment option for OP poisoning, primarily with agents having a long *in vivo* persistence. The concept comprises the use of large amounts of exogenous scavenger molecules that react irreversibly with the OP. One of the most advanced stoichiometric bioscavengers is butyrylcholinesterase (BChE). However, production costs and plasma stability limited a broad use. Another future treatment option might be the use of catalytic scavengers of bacterial origin that detoxify OP by formation of non-toxic hydroxyl metabolites. Chemical engineering currently optimizes different catalytic scavengers to create a treatment approach for OP poisoning. Depending on the used OP and route of exposure bioscavengers may be most effective as pre-treatment and post-exposure pre-symptomatic treatment. In spite of these promising approaches, further research and compound optimization is necessary to provide additional treatment options for OP poisoning.

Neuroprotective effect of AChE reactivator RS194B on neuronal and glial cells of mice exposed to nerve agent

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Acutely toxic, small lipophilic organophosphate (OP) molecules readily cross the blood-brain barrier and inhibit the physiological function of acetylcholinesterase (AChE) - hydrolysis of the neurotransmitter acetylcholine. Consequently, hypercholinergic activity will induce seizures which lead to brain damage and trigger neuroinflammation. Also, overstimulation of nicotinic and muscarinic membrane receptors will induce symptoms of cholinergic crises, which in severe cases can lead to hypoxia, vasodepression, and respiratory arrest, followed by death. Therapy against OP poisoning includes oxime compounds that can restore AChE activity preventing the cholinergic crisis and an anti-muscarinic drug, such as atropine. Nevertheless, oximes currently approved for therapy do not cross the blood-brain barrier readily, due to the permanent positive charge, and cannot restore the activity of synaptic AChE, leaving the brain vulnerable to long-term damage. We anticipate that the treatment with uncharged, but ionizable oximes that cross the blood-brain barrier and reactivate OP-inhibited synaptic AChE will act protectively on the brain of mice exposed to a nerve agent. For that purpose, we have investigated the effect of centrally acting oxime RS194B in the brain of mice exposed to a nerve agent and compared it to poisoned mice by monitoring effects on neuronal and glial cells. A microglial response, detected with IBA-1 protein, glial cells, detected with glial fibrillary acidic protein (GFAP), and neuronal cell viability, detected by the neuronal nuclei antigen NeuN were all monitored to track the level of GFAP and IBA-1 proteins, and the preservation of NeuN expression in order to indicate the survival of neurons and neuroprotection by uncharged, ionizable oxime in mice exposed to a nerve agent. This research was supported by the HDTRA-19-1-006-UCSD-113020, and Croatian Science Foundation (IP-2018-01-7683, and IP-2016-06-8636).

Cholinergic links to mRNA decay processes in schizophrenia and bipolar disorder

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The cholinergic system controls numerous immune functions, but its mental disease impact is less well- understood. Here, we report cholinergic involvement in schizophrenia (SCZ) and bipolar disorder (BD), complex psychiatric disorders affecting ca. 3% of the population. Genome and transcriptome studies identified SCZ and BD-related mis-regulation of dopaminergic, glutamatergic and GABAergic systems, but cholinergic contributions to these disorders were less intensively studied. Also, while SCZ and BD show similar prevalence in men and women, their onset and progression patterns differ sex-dependently. Therefore, we sought both disease- and sex-specific changes in molecular processes involving the cholinergic-neuroimmune network.

Bioinformatics analyses of the NIH's CommonMind RNA-sequencing dataset identified disease- and sex-specific changes in the dorsolateral prefrontal cortex from individuals with SCZ and BD compared to controls. Intriguingly, tristetraproline (TTP), which contributes to mRNA decay of cholinergic-regulated pro-inflammatory cytokines, was consistently modulated. Supporting cholinergic relevance, cholinergic-differentiated cultured neurons of human male and female origin revealed distinctly upregulated TTP and its brain-specific target NOVA1, which regulates alternative splicing and nonsense-mediated mRNA decay (NMD).

Interestingly, cholinergic-differentiated neurons also revealed changes in targets of the brain-specific miR-128, which regulates SCZ and/or BD-related transcripts (ARPP21, BDNF, RELN) and contributes to neuronal differentiation by controlling the levels of the NMD-related UPF1 transcript. Moreover, circSLC8A1 that acts as a 'sponge' for miR-128 and incapacitates its actions was modulated in middle temporal gyrus brain tissues of Netherland Brain Bank individuals with BD. Specifically, upregulated TTP and downregulated circSLC8A1 in women with BD validated the sex- and brain area-specific changes in mRNA decay. Finally, reflecting brain-to-body communication, we identified TTP upregulation in leukocytes from women with BD who show altered cholinergic immune functions. Taken together, our findings support functional relevance of the cholinergic system pathways to sex-related differences in SCZ/BD, which may open new venues for diagnosis and therapeutics.

Integrating multi-omics with prior knowledge to enable causal molecular reasoning in cholinergic systems

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Recently, biomedical science has seen a paradigm shift in the generation of experimental data. Meanwhile, our way of causal reasoning has not changed since the age of enlightenment. To use our high-dimensional"omics"data to their full extent, we must bridge the gap between human-level reasoning and computational analysis of biomedical big data. For instance, cholinergic systems are defined by the actions of the small molecule acetylcholine and facilitated by proteins (e.g. ChAT, AChE), whose expression in turn is controlled by microRNAs on the transcriptional level. In addition, the transmitter nature of acetylcholine implies involvement of multiple cell types, resulting in a multi-timescale, multi-omics problem. To face this computational challenge, we address it at multiple levels: 1) generalised machine language and object recognition, 2) abstraction of biological mechanisms, and 3) prior-knowledge-based mechanistic reasoning. We propose a unified language of biomedical property graph databases,"BioCypher", to tackle the first; and taylored algorithmic reasoning on the basis of aggregated biomedical prior knowledge to address problems two and three, using logic models (CellNOpt), integer linear programming (CARNIVAL, COSMOS), and artificial neural networks (LEMBAS).

Insights into functionally relevant conformational flexibility of protein backbone in cholinesterase structures

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Ligand binding to human, mouse and Torpedo californica acetylcholinesterases (EC 3.1.1.7; AChEs) can cause mild to moderate changes in their backbone conformations, revealed from pairwise comparisons with X-ray structures of unliganded AChEs. This holds for complexes with reversible ligands (substrates and inhibitors) and for covalent AChE conjugates of tetrahedral and trigonal-planar geometries. While backbone conformations in most organophosphate (OP)-conjugated human acetylcholinesterases (hAChEs) have been shown as similar to those in native hAChE, formation of the diethylphosphoryl-hAChE conjugate, with large ethoxy group inserted into the acyl pocket (AP) of hAChE, can trigger notable loop distortions and consequential dissociation of C-terminally associated hAChE homodimer. Nevertheless, recent X-ray structures of hAChE conjugated with large Novichok OPs, despite snug OP-conjugate fit, reveal only minor backbone changes. That was likely due to a 1.2–2.4 Å shift of the OP-conjugate, away from the AP, towards choline binding site. The small AP of AChEs can thus accommodate substituents of the size of ethoxy or butyryl groups, without AP loop distortion, provided that conjugated OP is "pulled" away from the AP. Therefore, conformationally adaptive AP loop may not necessarily exclusively serve as a static structural element for steric control of AChE specificity. And aromatic choline binding site has potential to attract and pull entities already covalently tethered to the active Ser. The pull can promote catalytic reactions or relieve steric pressure within the impacted space of the AChE active center gorge. Furthermore, conformational changes of this loop often coincide with shifts in C-terminal α-helical positions, revealing potential for allosteric interaction propagated within the AChE backbone. Those dynamic properties of the AChE backbone, inferred from the analysis of static X-ray structures thus contribute towards understanding of AChE as a template in the structure-based design of therapeutically active molecules, including AChE inhibitors, and reactivators of conjugated AChE.

Cryo-electron microscopy of cholinesterases

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Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) exist in a variety of oligomeric forms, each with defined cellular and subcellular distributions. Although crystal structures of AChE and BChE have been available for many years, structures of the physiologically relevant ChE tetramer were only recently solved by cryo- electron microscopy (cryo-EM) single-particle analysis. During my presentation I will briefly discuss how these structures contribute to our understanding of cholinesterase oligomerization, highlighting the advantages of using cryo-EM to resolve structures of protein assemblies that cannot be expressed recombinantly. I will further give an outlook on how cryo- electron tomography can be used to image membrane-anchored ChE oligomers directly in their native environment—the cell.

Discovery of the *in situ* assembly drug modality of choline-*O*-acetyltransferase

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Choline-*O*-acetyltransferase (ChAT) catalyses the production of acetylcholine in cholinergic neurons, T-cells, and B-cells. Although ChAT is a potential drug target, the progress towards therapeutically useful compounds has been slow, mainly due to an insufficient understanding of the biochemical and structural basis of inhibition. Herein, we investigate the inhibition of ChAT by the prototypical arylvinylpyridinium (AVP) class of inhibitors. Using X-ray crystallography and complementary methods, we show that AVPs are substrates of ChAT and that the actual inhibitors are assembled in the active site through a hydrothiolation reaction with endogenous coenzyme A. We show that the formed AVP-CoA adducts have a long target residence time and provide a remarkable stabilisation of the enzyme. Our study underscores the importance of interactions between the inhibitors and a hydrophobic pocket near the choline binding site, which may result in a gain in entropy upon binding and govern inhibitor potency. Our results solve the mechanistic conundrum of AVPs and establish an inhibitor modality that exploits a target-catalysed reaction between an exogenous substrate and an endogenous precursor. The study provides new directions for the development of ChAT inhibitors with improved bioactivity.

Out-of-Register Parallel β-Sheets and Antiparallel β-Sheets Coexist in 150-kDa Oligomers Formed by Amyloid-β(1–42)

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We have largely determined the structure of a 150-kDa oligomeric aggregate formed by the amyloid-β peptide (Aβ(1–42)) in dilute anionic micelles. We previously reported solid-state NMR measurements that showed a β-strand, spanned by residues 30–42, which arranges into an antiparallel β-sheet.

New results presented here indicate that there is a second β-strand formed by residues 11–24. Contrary to expectations, NMR data indicate that this second β-strand is organized into a parallel β-sheet despite the co-existence of an antiparallel β-sheet in the same structure. In addition, the in-register parallel β-sheet commonly observed for amyloid fibril structure does not apply to residues 11–24 in the 150-kDa oligomer. Rather, we present evidence for an inter-strand registry shift of three residues that likely alternate in direction between adjacent molecules along the β-sheet.

We corroborated this unexpected scheme for β-strand organization using multiple two-dimensional NMR and 13C–13C dipolar recoupling experiments and built a model of the structure based on cryo-electron microscopy. Our findings indicate a previously unknown assembly pathway composed of a planar array with a four-fold axis of symmetry.

Molecular bases for partnership of two *C. elegans* synaptic organizers: the cholinesterase-like cell-adhesion molecule neuroligin-1 and the ADAMTS-like glycoprotein punctin/MADD-4

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The cholinesterase-like, post-synaptic cell-adhesion molecules neuroligins (NLGs) organize mammalian synapses by interacting with the pre-synaptic neurexins (NRXs), among other functional partners. Mutations in the human *NLG* and/or *NRX* genes have been associated with autism or schizophrenia.

In the nematode *C. elegans*, the only identified NLG, Ce-NLG1, has been proposed to share functional redundancy with its mammalian relatives. It has also been shown to interact with punctin/MADD-4B, a secreted ADAMTS-like multidomain protein also identified as a synaptic organizer, to cluster GABA receptors at inhibitory/GABAergic but not excitatory/cholinergic synapses, thereby dictating the identity of postsynaptic domains. Whether the human relatives of punctin/MADD-4, ADAMTSL1/3, also bind human NLGs is unclear. However, the *ADAMTSL3* gene, expressed in the CNS, was identified as a susceptibility gene for schizophrenia.

To identify the molecular bases of their partnership, we generated recombinant forms of Ce-NLG1 and punctin/MADD-4B and carried out a comprehensive biochemical/biophysical study of their interaction, complemented by an *in vivo* localization study (Platsaki et al, 2020). These studies identified the Ig-like domain of punctin/MADD-4B as the primary determinant for interaction with Ce-NLG-1 *in vitro*, and as an essential component for efficient recruitment of GABA receptors at GABAergic synapses in *C. elegans*.

To explore both the ability of Ce-NLG1 to bind the only NRX identified in *C. elegans*, Ce-NRX1, and how it may interact with the punctin/MADD-4 Ig-like domain, we carried out structural and modeling studies (Platsaki et al, in preparation). The crystal structure of Ce-NLG revealed several molecular features that differentiate it from vertebrate NLGs and may account for functional divergences. Modeling a Ce-NLG/NRX1 complex by homology with a crystalline mammalian NLG-NRX complex suggested that significant conformational rearrangement of either or both of the presumed partners would be required for complex formation. Modeling a Ce-NLG/Ig-like complex using molecular docking pointed to possible binding interfaces either overlapping, or not, the putative Ce-NLG1/NRX1 interface; however, whether Ce-NLG1 can simultaneously bind punctin/MADD-4B and Ce-NRX to form a large, trans-synaptic tripartite complex is unknown.

These data point to still another partner for the cholinesterase-like cell-adhesion molecules, NLGs, and raise questions about functional partnership redundancy across species.

Structural pharmacology of the muscle-type nicotinic acetylcholine receptor

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Our lab focuses on using structural and functional approaches to understand signaling by ligand-gated ion channels in the nervous system. A major emphasis is on understanding nicotinic receptor pharmacology from a structural perspective. Here I will present some of our recent work using the muscle-type nicotinic receptor from the *Torpedo* ray as a structurally tractable reference for the human receptor at the neuromuscular junction. I will focus on how toxins from animals and plants act on the receptor, and how we can use this information to understand state transitions that underlie ion channel gating. These studies have revealed a new class of allosteric site at the junction of the extracellular and transmembrane domains that small molecules can leverage to modulate channel desensitization. Our findings suggest that stabilizing a desensitized-like non-conducting state, through this allosteric site, may be an important alternative mechanism for antagonizing the receptor.

Cell-adhesion glycoprotein neuroplastin in human cognition and neurodegeneration

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Neuroplastin (Np) is a glycosylated transmembrane cell-adhesion glycoprotein, whose brain-specific isoform Np65 is abundantly expressed in synaptic membranes. Experimental finding that neuroplastin is involved in long-term potentiation, a cellular mechanism underlying learning and memory, initiated more extensive research on neuroplastin roles in mammalian brain. A substantial evidence, mostly from studies on mice and rats, shows that neuroplastin participates in different cellular processes such as neurite outgrowth, synaptic plasticity, maintenance of excitatory/inhibitory balance. Additionally, studying neuroplastin-deficient mice revealed that Np65 is necessary for associative memory formation. Novel data indicate that Np65 is involved in regulation of membrane excitability, most probably due to its cross-talk with plasma membrane calcium ATP-ases. In humans, genetic studies showed association of several Np gene variations with cortical thickness and intelligence in adolescents, and susceptibility to schizophrenia. However, very scarce data are available on neuroplastin expression and function during human brain development, aging and neurodegeneration. The results of our group show that specific pattern of neuroplastin immunoreactivity in human hippocampus delineates a major glutamatergic trisynaptic pathway. We found as well altered neuroplastin expression in hippocampi affected by sporadic Alzheimer's disease neuropathology, suggested to be an early tissue compensatory response to neurodegeneration. Here we present an overview of up-to-date knowledge about neuroplastin expression in human tissues, discuss on its genetic association with several neuropsychiatric disorders and its presumed role in molecular events underlying cognition and neurodegeneration, and provide data on high spatio-temporal neuroplastin gene expression in the human brain. We suggest that neuroplastin may be viewed as a housekeeper of neuroplasticity and propose several avenues for future investigations which should clarify neuroplastin functions in human brain in more details and support its role as a cognition-related molecule.

From acetylcholinesterase inhibitors ameliorating Alzheimer's disease to retrograde amnesia

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Because cholinergic augmentation improves cognition, the acetylcholinesterase inhibitors (AChEI) donepezil, galantamine, and rivastigmine serve as symptomatic treatment in mild to moderate Alzheimer's dementia. Galantamine binds reversibly to AChE and is unique because it acts also as an allosterically potentiating ligand of nicotinic acetylcholine receptors (nAChR). Chronic low-level stimulation by galantamine can up-regulate nAChR expression, slow neurodegeneration, and confers protection against β amyloid toxicity. Modulating microglial nAChRs, galantamine exerts neuroprotective effects enhancing Aβ phagocytosis and clearance in the brain.

We showed that galantamine delays behavioral decline, plaque deposition, and gliosis in 5XFAD mice. The derivative memogain can be administered nasally avoiding gastrointestinal side effects of AChEIs. Enzymatic cleavage of the pro-drug memogain liberates galantamine. We showed in 5XFAD mice that chronic nasal memogain application ameliorated affected behaviors even before cognitive decline. In addition, it efficiently lowered the plaque load at lower doses compared to galantamine. Furthermore, distribution and activity of nAChRs are influenced by plasma membrane Ca2+ ATPases (PMCA) which extrude Ca2+ to the extracellular space. We discovered that neuroplastin supports expression of PMCAs by forming functional complexes with PMCAs. These neuroplastin-PMCA complexes may engage in further interactions sequestering and tethering these complexes to places where Ca2+ extrusion to the extracellular space is required. In the absence of neuroplastin, PMCA levels are reduced resulting in elevated intracellular Ca2+ levels and prolonged decay time to reach resting Ca2+ levels after stimulation which interferes with signal transmission and may change network activities. We showed that neuroplastin is essential for associative learning and loss of neuroplastin after learning results in retrograde amnesia for associative memories. The assembly of nAChRs with PMCA and neuroplastin appears as a new pathway of significant functional importance.

Impact of expression of 82-kDa ChAT on Alzheimer pathology in a mouse model

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Hallmarks of Alzheimer disease (AD) pathology are increased amyloidogenic processing of amyloid precursor protein (APP) to β-amyloid (Aβ), decreased choline acetyltransferase (ChAT) activity, cholinergic neuron loss and cognitive dysfunction. ChAT protein is produced in cholinergic neurons in human brain by the M-transcript that encodes both 69- and 82-kDa ChAT proteins, with 82-kDa ChAT localized predominantly to neuronal nuclei. This suggests alternative functional roles for 82-kDa ChAT; importantly the presence of 82-kDa ChAT in nuclei of cholinergic neurons decreases with increasing age and in early AD. Using gene microarray and chromatin immunoprecipitation (ChIP)-sequencing analysis, 82-kDa ChAT-expressing neural cells have altered expression of genes for several proteins that regulate APP processing resulting in decreased Aβ production in 82-kD ChAT-containing cells. BACE1 levels and activity are significantly decreased with reduction in endogenous $A\beta_{1\text{-}42}$ release from neurons cultured from brains of APP/PS1 mice. We developed transgenic mice expressing human 82-kDa ChAT in forebrain under control of Nkx2.1-Cre gene, with expression confirmed in cholinergic neurons by immunofluorescence staining and RNAscope. Critical *in vivo* experiments showed that changes in subcellular localization of 82-kDa ChAT in cholinergic neurons in necropsy human brain during aging are recapitulated in mouse brain. These 82-kDa ChAT-expressing mice were crossed with AD model mice with mutant human APP knocked-in and we found that expression of 82-kDa ChAT reduces AD-like amyloid plaques deposition in cortex and hippocampus and lowers $A\beta_{1\text{-}42}$ production measured by ELISA. Iba1 staining revealed reduced microgliosis in AD model mice expressing 82-kDa ChAT compared to mice not expressing 82-kDa ChAT. Microglial cell morphology also differed in the 82-ChAT expressing AD model mice, suggesting a less activated phenotype. Thus, 82-kDa ChAT expression may have implications for forestalling AD pathology associated with amyloidogenic processing of APP and play a role in maintenance of cholinergic neurons.

Paradoxical Aging in Alzheimer's disease: A Clinicopathologic Perspective of Nucleus Basalis of Meynert

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The strongest risk factor for Alzheimer's disease (AD) dementia is increasing age, yet sporadic young-onset AD sufferers are often observed to have a more severe disease course. Moreover, neuroimaging and neuropathologic studies demonstrate skewed cortical involvement that may underlay a higher frequency of observed focal cortical syndromes in those less than age 65 at presentation. To provide clinicopathologic evidence of paradoxical aging in AD, the FLorida Autopsied Multi-Ethnic (FLAME) cohort will be utilized. This autopsied series is derived from memory disorder clinic referral services throughout the state of Florida, USA. The FLAME-AD cohort provides an invaluable opportunity to investigate the intersection of age (53-102 years at death) and sex (n=875/1625 [54%] females) in AD. While all young-onset AD patients would become amyloid positive, utilization of hippocampal involvement or speed of decline as a biomarker readout may interfere with interpretation relative to late onset AD sufferers. Throughout the presentation, parallels to what has been reported in neuropathologic subtypes of AD will be discussed. Neurofibrillary tangle patterns that diverge from the well-established Braak staging method (hippocampus > cortex) have identified extreme phenotypes of hippocampal sparing AD (hippocampus <<< cortex) and limbic predominant AD (hippocampus >>> cortex). Hippocampal sparing AD is enriched in young-onset AD patients, whereas limbic predominant AD is enriched in late onset AD patients. To examine the intersection between the concept of paradoxical aging and AD subtypes, neurofibrillary tangle accumulation in the cholinergic hub (i.e., nucleus basalis of Meynert) will be discussed.

Age-dependent effects of the p75 modulator LM11A-31 on Alzheimer's disease biomarkers in a six-month safety and exploratory endpoint trial

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Aims: Basal forebrain cholinergic neurons (BFCNs) degenerate during normal aging and early stages of Alzheimer's disease (AD). The p75 neurotrophin receptor (p75NTR) is expressed by BFCNs and modulates pro- and anti-apoptotic pathways as well as neurite and neuritic spine integrity. Pharmacological modulation of this receptor with LM11A-31 reverses basal forebrain axonal pathology in mouse models of AD. Older, late-onset AD subjects tend to have greater proportions of mixed etiology dementia while younger AD subjects are considered to have a greater proportion of pure AD pathology. This prompted the examination of age-dependent effects of LM11A-31 in human AD with longitudinal structural magnetic resonance imaging (sMRI) and cerebrospinal fluid (CSF) biomarkers.
Methods: Participants with mild to moderate AD (MMSE score=18-26; age 55-85) were enrolled in a six-month placebo-controlled phase 2a safety and exploratory endpoint trial of LM11A-31. For placebo and drug, age groups were defined using a median-split: younger (<72 years) and older (>=72 years).
Results: Older subjects exhibited smaller BF volumes at baseline than younger subjects in both placebo and drug groups, and exhibited no differences in AD stage according to MMSE score or CSF p-tau/Aβ42 ratio at baseline. Results outlining age-group specific effects of LM11A-31 on longitudinal CSF and neuroimaging biomarkers will be presented.
Conclusions: This investigation points to the possibility that certain biomarkers can demonstrate age-dependency in response to therapeutic interventions in the context of mild-moderate AD. Our results may inform biomarker selection and design of future studies of LM11A-31 in human AD.

LM11A-31 Small Molecule p75 Receptor Modulator –Analysis of Safety and Exploratory Endpoints in a Phase 2a Trial in Alzheimer's Disease

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Background: The p75 neurotrophin receptor regulates multiple degenerative signaling pathways related to synaptic degeneration and glial activation and known to be affected Alzheimer's disease (AD).
Aims: Assess the primary outcome of safety; along with exploratory biomarker and clinical assessment in a phase 2a trial in mild-moderate Alzheimer's disease (AD) for LM11A-31, a first-in-class small molecule modulating p75 neurotrophin receptor signaling.
Methods: Three study arms were included: placebo, low-dose (200mg) and high-dose (400mg) administered by oral capsules twice daily for 26 weeks. Inclusion criteria included CSF amyloid beta analysis. Safety assessments included clinical laboratory studies, MRI and EKGs. Exploratory outcome assessments included: AD core and other CSF biomarkers; structural MRI; mini mental status exam, ADAS-Cog-13, a Neuropsychological Testing Battery and other cognitive tests; and statistical region of interest and voxel-based FDG-PET.
Results: A total of 241 subjects were randomized and initiated on therapy with similar populations distributed across the three study arms. 221(91%) subjects completed the trial with 211 (87%) per protocol. Safety analysis demonstrated that 20 subjects discontinued the study; 16 with adverse events, 4 of these serious, and 4 (2 placebo and 2 drug) with withdrawal of consent. One death occurred in the placebo group. Exploratory outcome analyses suggest drug-associated effects consistent with slowing disease progression on multiple CSF and imaging biomarkers.
Conclusions: These findings indicate an overall satisfactory safety profile. Exploratory outcome analysis suggests that LM11A-31 engages mechanisms relevant to p75 receptor signaling and AD progression. Overall, this study suggests that LM11A-31 is suitable for subsequent larger-scale trials.
Funding: National Institute of Aging
Conflict of Interest: FL is a board member, has equity interest and has a consulting relationship with PharmatrophiX. FL and SM have intellectual property interest in LM11A-31.

Design and evaluation of novel reactivators of cholinesterases inhibited by nerve agents

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Organophosphorus nerve agents (OPNA) inhibit acetylcholinesterase (AChE) by phosphylating the catalytic serine. AChE inhibition disrupts the cholinergic transmission and leads to death if untreated. Current pyridinium-oxime-based reactivators like 2-PAM or HI-6 can restore the enzyme activity by displacing the phosphyl moiety from the catalytic serine. However, the reactivation efficiency depends on the chemical structure of the OPNA. None of the currently available reactivators display activity for a broad range of OPNA. Another drawback of these charged pyridinium-oxime reactivators is their poor ability to cross the blood-brain barrier to access the inhibited enzymes in the central nervous system. Strategies have been evaluated over the years to overcome these weaknesses. Our consortium focuses on the design and evaluation of bifunctional reactivators with activity for a broader spectrum of OPNA and enhanced blood-brain barrier permeability. Their general design is based on the coupling of a peripheral site ligand of AChE to a reactive 3-hydroxy-2-pyridinaldoxime moiety. Original families of molecules synthesized over the last decade will be presented. We will report their ability to reactivate OPNA-inhibited cholinesterases *in vitro*, related structural studies, and for the most promising candidates, the ability to cross efficiently the blood-brain-barrier *in vitro* and to protect against lethal doses of OPNA in a murine model.

Modification of oxime nucleophiles for enhanced reactivation of cholinesterases

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The cholinesterase reactivators (so called"oximes") are used as causal antidotes in case of organophosphorus intoxications. The effectiveness of the reactivator is strongly dependent on the formation of active nucleophile, the oximate anion [1]. Its formation can be supported by optimizing the physical-chemical properties (i.e. pKa) of the oxime and the decreased pKa should lead to enhanced reactivation of phosphylated cholinesterases.

For this reason, the charged chlorinated oximes were introduced and proved to highly effective *in vitro* for reactivation of acetylcholinesterase [2] and butyrylcholinesterase [3] inhibited by multiple organophosphorus agents. The chlorinated oximes were also proved to be not cytotoxic and effective after *in vivo* administration. More recently, the charged fluorinated oximes were prepared and thoroughly evaluated with promising reactivation results against multiple organophosphorus agents, although their particular lower stability was found [5]. Finally, further modified halogenated oximes with optimized oximate formation were designed and evaluated on cholinesterases inhibited by nerve agent surrogates. Some modified reactivators were proved to be *in vitro* effective for reactivation of NEMP, NIMP or NEDPA-inhibited AChE and surprisingly one oxime resulted as excellent reactivator of NEMP and NIMP-inhibited BChE. These promising results make prospects for further detailed investigation of modified oximes nucleophiles. This work was supported by *Czech Science Foundation* (no. 21-03000S).

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Modulating cholinesterases activity by quinuclidine and cinchona-based compounds

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Antidotes for organophosphorus poisoning are oximes, which antidotal properties are related to their ability to reactivate phosphorylated acetylcholinesterase (AChE, EC 3.1.1.7), and butyrylcholinesterase (BChE, EC 3.1.1.8) when used as scavenger [1]. In addition, it is well known that the protection of cholinesterases (ChEs) from phosphorylation can be achieved by the previous inhibition with carbamates. Given that there is no single compound applicable as antidote for poisoning with various organophosphorus agents, pursue for compounds with better chemical, physical and biochemical properties as well as better understanding of their interactions with both enzymes are still important. Therefore, over the years, a series of novel quinuclidine and cinchona-based oximes and carbamates were synthesized to be evaluated as inhibitors and/or reactivators of phosphorylated human ChEs [2,3]. Compounds were modified to gain divers structures and the hybrid molecules with known aromatic pyridine and imidazole oximes designed. Activity and selectivity of cholinesterases were described, and to clarify differences in the inhibition and oxime reactivation potency, conformational analysis of compounds as well as detailed docking studies were conducted. Orientations of studied compounds in the active site of ChEs have been proposed by QM/QM studies. Analyses of the obtained complexes pointed out significant hydrogen bonds and close contacts between functional groups of compounds and the residues of the active site. To facilitate the prediction and design of new and more potent compounds, the most optimal regression models for the prediction of bioactivity were established and validated by extensive machine learning protocols. (Supported by the *Croatian Science Foundation*, Project No. IP-2016-06-3775 ADESIRE)

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Development of pre- and post-countermeasures against OP toxins in macaques

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Deliberate sarin releases in Syria with large numbers of fatalities emphasize the need for OP countermeasures for both military and civilian populations. Therapeutic countermeasures involve several strategies: (i) preventing OP poisoning through administering pre-exposure_treatments that scavenge OPs before they inhibit their physiological AChE targets in the brain and in the periphery (ii) post-exposure oxime that can rapidly reactivate OP-inhibited AChE or (iii) a combination of both. In terms of a pretreatment, our recent studies have demonstrated that administration of an aerosolized (aer)-rHuBChE employing an improved, modified user friendly Aerogen (Gallway) Solo vibrating mesh nebulizer, forms a protective pulmonary bioshield in the lungs of macaques which to date remains intact for at least 5 days. Thus 4-5 mg/kg of aer-rHuBChE deposited in the lung can prevent symptoms and inhibition of RBC-AChE and plasma BChE following a high (55 μg/kg) inhaled dose of aer-paraoxon (Px) 4 days later; an amount known to inhibit circulating ChEs by >95% and cause tremors. In terms of oxime efficacy, macaque studies have demonstrated that a single IM post-exposure injection of the zwitterionic, centrally acting oxime RS194B (62-80 μg/kg) plus low-dose atropine rapidly reactivates OP-inhibited RBC-AChE and circulating BChE and dramatically reverse both early and advanced clinical OP symptoms following lethal inhalation exposure to both sarin vapor (49.6 μg/kg) and lethal aerosolized paraoxon (100 μg/kg). In addition post-exposure RS194B has been shown to protect macaques against lethal inhaled phosphorothioate insecticides e.g. parathion and chlorpyrifos.

The increased efficacy of nebulizers in humans and the known synergy between aer-rHuBChE pretreatment with IM RS194B post exposure bodes well for a prophylactic single or combination treatment which can protect against potent inhaled OP agents for >6 days without multiple injections.

Assessment of four organophosphorus pesticides as inhibitors of human cholinesterases

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Toxicity of organophosphorus compounds remains a major public health concern due to their widespread use as pesticides and the existence of nerve agents. Their common mechanism of action involves inhibition of enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) which are crucial for neurotransmission. Both chronic and acute poisoning by organophosphates can leave long-lasting health effects even when the patients are treated with standard medical therapy. Therefore, an increasing urgency exists to find more effective oxime reactivators for compounds which are resistant to reactivation, especially phosphoramidates. Here, we investigated *in silico* and *in vitro* interactions and kinetics of inhibition for human cholinesterases with four organophosphate pesticides - ethoprophos, fenamiphos, methamidophos and phosalone. Overall, ethoprophos and fenamiphos displayed higher potency as inhibitors for tested cholinesterases. Our results show that methamidophos-inhibited AChE was more susceptible to reactivation than AChE inhibited by fenamiphos by selected oximes. Molecular modelling enabled an evaluation of interactions important for specificity and selectivity of both inhibition and reactivation of cholinesterases. Two newly developed reactivators – bispyridinium triazole oxime 14A and zwitterionic oxime RS194B possess remarkable potential for further development of antidotes directed against pesticides and related phosphoramidate exposures, such as nerve agents tabun or Novichoks. Acknowledgments: This work was supported by the Croatian Science Foundation (IP-2018-01-7683).

The impact of a novel NGF metabolic pathway on the forebrain cholinergic system in health and in the Alzheimer's pathology

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Endogenous NGF sustains the cholinergic phenotype of basal forebrain (BF) neurons. A system which becomes progressively atrophic in Alzheimer's disease (AD). An atrophy of NGF-dependent cholinergic neurons occurring, paradoxically, with normal NGF synthesis and abundance of the NGF precursor molecule.

This metabolic pathway implies the activity-dependent release of proNGF. The released NGF precursor molecule (proNGF) is converted to mature NGF (mNGF) and ultimately degraded in the extracellular space. This is achieved by a chain of zymogens, convertases, proteases and endogenous inhibitors. This pathway has been pharmacologically validated as controlling the cholinergic phenotype of BF neurons.

The NGF metabolic pathway has been found deregulated in the AD pathology explaining the BF cholinergic atrophy. In AD pathology there is a defective conversion of proNGF to mature NGF and an increased degradation of mNGF, thus creating a disconnect of the trophic support of the NGF-dependent BF cholinergic neurons and synapses, leading their progressive atrophy. The increased proNGF levels in AD brains reflects a build-up of"unconverted"NGF precursor molecules.

The AD-pathology induced NGF metabolic deregulation has been found proportional to the amyloid burden. It is also present in Down Syndrome (DS), even at its AD preclinical stages. In DS body fluids, proNGF levels are increasingly elevated in their transition from DS-AD asymptomatic to DS with clinical AD with the rise in proNGF plasma levels predicting a subsequent cognitive decline.

An NGF metabolic deregulation has been found in postmortem brain samples from non-cognitively impaired (NCI) individuals when presenting significant Aβ-amyloid pathology. The NGF deregulation in NCI brains correlated with lower cognitive scores and loss of cortical VAChT-immunoreactivity. The altered levels of key molecules of NGF metabolism in body fluids should assist the diagnoses of preclinical AD stages as reviewed in Giacobini, Cuello and Fisher, Reimagining cholinergic therapy for Alzheimer Disease. Brain, In press 2022

The role of brain alpha 7 nicotinic acetylcholine receptors in Alzheimer´s disease pathology and other proteinopathies

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The alpha 7 nicotinic receptors (α7-nAChR) are expressed in several region of the human brain. High expression α7-nAChR is found in the hippocampus and cortical brain regions which are important for cognition, memory and also show pathological vulnerability in neurodegenerative disorders as Alzheimer´s disease (AD) and Parkinson´s disease and in psychiatric disorders. The homo-pentameric α7-nACh show very high calcium permeability as well as metabotropic properties and is known to participate in neurotransmitter release, neural excitation, signal transduction, synaptic plasticity as well as neurogenesis. The α7-nAChRs are expressed in different types of neurons but also in non-neuronal cells such as glia cells as astrocytes and microglia. A significant increased number of astrocytic α7-nAChR has been measured in cortical brain tissue from both sporadic and autosomal dominant AD while a reduction in neuronal α7-nAChRs compared to healthy controls. The α7-nAChR show high binding affinity to amyloid-β and can mediate amyloid -ß release of glutamate in astrocytes in a calcium -dependent manner. It has also suggested that the α7-nAChR can medicate uptake of amyloid-ß to synapses. Due to the multiple roles of α7-nAChRs in normal brain as well as in different disease they may play a crucial role both in the develop of new diagnostic disease markers and well as new drug targets.

It is challenging to design a PET tracer for *in vivo* imaging of α7-nAChR in brain. Several radioligands for PET imaging have been explored and ^{18}F-ASEM has recently shown selective binding *in vitro* as well as high brain binding in human PET studies. Since the α7-nAChR may form also heteromeric receptors together with other subtypes we therefore decided to develop new α7-nAChR PET tracer candidates using ASEM (α7-nAChR antagonist) as lead compound. *In silico* and machine learning techniques were used to predict binding energy and other properties of ASEM analogues and to interpret these properties in term of atomic structures using ^{18}F-ASEM as lead structure and six selected candidates were labelled with ^{11}C and ^{3}H and to evaluate the binding properties of the compound *in vitro* and *in vivo* with the labelled candidates. All compounds showed high affinity binding in GH3-hα7 cells with IC_{50} in picomolar range. KIn83 demonstrated two binding sites (super-high, high) in human brain. Regional distribution was observed for ^{3}H-KIn83 using large hemisphere autoradiography and with high binding in AD temporal cortex, insula, hippocampus. *In vivo* PET with ^{11}C-Kin83 in non-human primate showed favourable properties for selective imaging of α7-nAChRs.Microdosing studies in rodents showed no toxic effect. The first *in vivo* PET studies with ^{11}C-Kin83 in cognitive healthy controls and patients with different stages of AD are planned during 2022.These studies are performed in parallel with *in vivo* PET imaging of astrogliosis with ^{11}C-deprenyl and amyloid plaque load with ^{11}C-PIB in the same individuals providing a deeper insight into to complex α7-nAChR interactive mechanisms in pathology of AD brain.

More than the cholinergic system: the evolving role of glia in memory, aging, and neurodegeneration

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The aims of this presentation are: 1) which cholinergic neurons are affected in brain neurodegenerative diseases; 2) the possible causes of the degeneration of the cholinergic neurons, 3) how glia may be involved in such mechanisms. In Alzheimer's disease an extensive loss of forebrain cholinergic neurons is accompanied by reduction of the cholinergic fiber network of the cortex and hippocampus. The cholinergic hypothesis of geriatric memory dysfunction, proposed 40 years ago, was followed by extensive investigations, but a definite answer to the underlying mechanisms has not yet been given.

The formation and accumulation of b-amyloid oligomers and plaques play a crucial role in AD by direct neuronal toxicity or through extensive inflammatory reaction. Glia in the last decades moved from a mere role in scaffolding or in damaging/neurodegenerative mechanisms to that of a major player in the physiology and pathology of the CNS. I grew up for years considering the brain formed by a network of neurons talking each other in a sort of vacuum until in the '90s suddenly astrocytes, microglia and oligodendrocytes shared the scene with neurons of which they became real partners. Glia is now accepted to play critical roles in CNS development, synaptogenesis, synaptic maintenance and maturation, significantly contributing to memory processes. The dogma that astrogliosis is a single uniform process, in any pathological situations, is rapidly evolving. Proper functioning of the neuron-astrocyte-microglia triad is fundamental for the functional organization of the brain and dysregulation of this interplay may be at the basis of neurodegenerative processes. The dynamics of neuronal degeneration in CA1 and CA3 are paralleled by differences in glia behavior in the two hippocampal areas. Comparing the similarities and differences of neuron and glia interplay will better define whether future therapeutic interventions should attempt to enhance or impair the actions of glia.

Functional recovery of the neuromuscular junction from neurotoxic snake envenoming elicited by CXCR4 agonist

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Snake envenoming is a major, but neglected, human tropical disease. Among venomous snakes, kraits (snakes of the *Bungarus* genus) are medically important venomous species that cause a peripheral neuroparalysis. Hospitalization and use of antivenoms derived from animal immunized with *Bungarus* venoms are the primary therapies that prevent death from early onset respiratory paralysis. There is a general consensus that additional and non-expensive treatments, that can be delivered even long after the snake bite, are needed. Traumatic or toxic degenerations of peripheral motor neurons, with ensuing neuroparalysis, are characterized by the activation of a pro-regenerative intercellular signaling programs. A major player is the intercellular signaling axis consisting of the chemokine CXCL12α, produced by perisynaptic Schwann cells and acting on the CXCR4 receptor expressed on the damaged neuronal axons. The CXCR4 agonist NUCC-390 was recently found to promote axonal growth. We have tested its efficacy on the neuroparalysis induced by the venoms of three major krait species, i.e. *Bungarus caeruleus*, *Bungarus multicinctus* and *Bungarus candidus* that are prevalent in Asia. These venoms cause a complete degeneration of motor axon terminals. Functional recovery of the neuromuscular junction was assessed by electrophysiological recordings and by imaging. We report that NUCC-390 administration to venom injected mice greatly accelerates the recovery from paralysis. These data candidate NUCC-390 to be tested as a novel therapeutic to reduce death by respiratory deficits and to improve the recovery of normal neuromuscular physiology, thus reducing the human and hospital costs of envenoming.

Muscular Swedish mutant APP-to-Brain axis in the development of Alzheimer's disease

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Alzheimer's disease (AD) is the most common form of dementia. Interestingly, patients with AD often suffer from severe sarcopenia. However, their direct link and relationship remain poorly understood. Here, we generated a mouse line, TgAPPsweHSA, by crossing LSL (LoxP-STOP-LoxP)-APPswe with HSA-Cre mice, which express APPswe (Swedish mutant APP) selectively in skeletal muscles. Interestingly, examining phenotypes in TgAPPsweHSA mice showed not only sarcopenia-like muscle deficits, but also AD-relevant hippocampal inflammation, impairments in adult hippocampal neurogenesis and blood brain barrier (BBB), and depression-like behaviors. Further studies suggest that APPswe expression in skeletal muscles induces senescence and expressions of senescence associated secretory phenotypes (SASPs)(e.g., inflammatory cytokines and chemokines); but decreases growth factors, such as PDGF-BB and BDNF. These changes likely contribute to the systemic and hippocampal inflammation, deficits in neurogenesis and BBB, and depression-like behaviors. Thereby uncovering an axis of muscular APPswe to brain in AD development.

Unravelling the Inhibitory Activity of Botulinum Toxins on the Enteric Nervous System

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Botulism is a rare, neuroparalytic syndrome caused by the ingestion of foods contaminated with Botulinum neurotoxin (BoNT), the most poisonus biological substance known. The syndrome is characterized by the inhibition of neurotransmission, in particular that of cholinergic neurons, causing descending flaccid paralysis and, in worst cases, death by respiratory failure. The mechanism of action of BoNTs on somatic motor neurons is well known. On the other hand, despite in natural botulism BoNTs adsorption is in the intestine, with constipation as one of the first symptoms, little is known about the possible action of BoNTs on the Enteric Nervous System (ENS). The ENS is a complex subdivision of the autonomic nervous system with a central role in the control of enteric motility, secretion, blood flow and response to infections, characterized by an extremely wide cholinergic innervation. Therefore, we are investigating the action of BoNTs on the great number of cholinergic neurons present in the ENS. After the treatment of mice by oral gavage with BoNT serotypes A or B, we tested the staining properties of two antibodies that specifically stain SNAP-25 truncated by BoNT/A and VAMP truncated by BoNT/B. For the first time, we could observe the proteolytic activity of BoNTs inside enteric cholinergic and non-cholinergic neurons by immunofluorescence. Moreover, we identified a dose of BoNTs that leads to a significant slowdown on peristalsis with instead no systemic signs of botulism, indicating cholinergic nerve terminals of the ENS as the possible first target of these toxins. Therefore, BoNTs may be established as a novel tool to study gut physiology, and, in particular, the interactions of enteric cholinergic neurons with the many different cell types present in the intestinal wall, including interstitial Cajal cells, muscle cells and the various cell types involved in the innate immune response.

Cardiac nicotinic receptors show β-subunit-dependent compensatory changes

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Recently, a hypothesis about the plasticity of β-subunits of nicotinic receptors (NR) was proposed, suggesting that the β4-subunit may substitute *in vivo* for a lacking β2-subunit and vice versa. This hypothesis, however, was never tested. Inspired by that, we assessed the consequences of missing β-subunits in the mouse heart. Using two mutant mice strains lacking β2 or β4 NR subunits, we examined the relative expression of NR subunits and other key cholinergic molecules. Additionally, we investigated the physiology of isolated hearts perfused by Langendorff's method at basal conditions and after cholinergic and/or adrenergic stimulation. We observed that an absence of β4-subunit was compensated by increased mRNA expression of β2-subunit but not vice versa. This alteration was accompanied with specific adaptation changes in the cholinergic system, namely, in acetylcholine synthesis and muscarinic receptors expression patterns. These adaptations were, however, insufficient for the preservation of cardiac phenotype. Thus, in support of the proposed plasticity of cardiac NR, our results confirmed subunit-dependent compensatory changes to missing cardiac NR subunits with consequences on isolated heart physiology. This project was supported by VEGA 1/0283/22, 1/0815/21, and SK-FR-19-0005.

The Cholinergic System as a Potential Mechanism of Cognitive Preservation in Octogenarians and Older

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Introduction: Cognitive impairment due to neurodegenerative disease or aging is highly prevalent in octogenarians and older. A subset of this population, cognitively normal octogenarians and older (CNOO), maintain cognitive function allowing for a higher quality of life and present an example of ideal brain aging. Cholinergic dysfunction is a hallmark of Alzheimer's disease (AD), and to a lesser extent, brain aging. The aim of this study was to characterize neuropathological and cholinergic changes in the rostral prefrontal cortex (rPFC) and hippocampal formation, regions responsible for attention and memory, respectively, to evaluate the cholinergic system as a potential mechanism of cognitive preservation in the CNOO.

Experimental: Sex- and age-matched formalin-fixed AD and CNOO rPFC and hippocampal tissues were stained immunohistochemically to visualize neuropathological aggregates associated with β-amyloid (Aβ), tau, phosphorylated TAR DNA-binding protein 43 (pTDP-43), and α-synuclein (α-syn). Histochemical stains were used to visualize acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) at pH 6.8. Neuropathological structures were scored semi-quantitatively using a modified Consortium to Establish a Registry for AD (CERAD) criteria in nine regions of interest: rPFC, dentate gyrus, CA1-3, subiculum, presubiculum, parasubiculum, and entorhinal cortex.

Results: BChE-positive pathology was significantly higher in AD than in CNOO cases in most regions of interest, followed by AChE. Aβ burden was comparable in all regions except the parasubiculum. Tau deposition was comparable in five of nine regions. α-syn burden was low in both the CNOO and AD, while pTDP-43 pathological burden was common and comparable in both groups. Abnormal protein aggregation was common in the CNOO in the form of intraneuronal Aβ, and thorny astrocytes consistent with age-related tau astrogliopathy.

Conclusion: The CNOO have significant deposition of Aβ, tau and pTDP-43 pathologies including unusual sequestering of both Aβ and tau. Cholinesterase-positive neuropathological burden, specifically BChE, remains an effective means of differentiating CNOO from AD brains.

A novel look on the catalytic activity of butyrylcholinesterase using unusual substrates: saturated and unsaturated 18C fatty acids

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Cholinergic neurotransmission is terminated by the two enzymes acetylcholinesterase and butyrylcholinesterase. Butyrylcholinesterase (BChE) is a multifunctional enzyme linked to lipid metabolism by an unknown mechanism. Based on lipid-related studies and the similarity of the catalytic domain of lipases with BChE, we recently found that BChE is able to hydrolyze 4-methylumbelliferyl (4-mu) palmitate. In this study, the lipolytic activity of BChE with various saturated and unsaturated 18C fatty acids was further investigated by molecular modeling experiments and enzyme kinetic studies and compared with pancreatic lipase. Analysis of the inhibition kinetics of BChE with monosaturated and polyunsaturated 18C fatty acids using butyrylthiocholine as substrate showed that oleic acid, linoleic acid, and α-linolenic acid exhibited noncompetitive inhibition when they interacted with BChE. Data were analyzed and kinetic parameters were calculated using GraphPad Prism. Ki and Km values for oleic acid, linoleic acid and linolenic acid were 321, 1400, 667 μM and 265, 145, 185 μM, respectively. Molecular docking was used to estimate the binding affinity and interactions of these fatty acids with human BChE and pancreatic lipase. The docking results showed that all lipids localized to and interacted with the substrate binding site of pancreatic lipase, whereas the saturated and unsaturated fatty acids, with the exception of palmitate, did not interact with the active site of BChE. These results suggest that free, differentially saturated 18C fatty acids interact with BChE to varying degrees and cause changes in enzyme activity. This research, which explores the possibility of using different lipids as substrates for BChE, will shed new light on the link between BChE and lipid metabolism. The possibility that BChE hydrolyzes other types of lipids in addition to 4-mu palmitate will lead to a new approach for the treatment of lipid-related diseases associated with increased BChE activity/expression.

Molecular dynamics insight into modulator binding at the α4α4 binding site of the α4β2 nicotinic acetylcholine receptors

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Neuronal nicotinic acetylcholine receptors (nAChRs) have an established role in devastating neurodegenerative diseases, yet few drugs have been successfully developed to target them. nAChRs assembled in a 3α:2β stoichiometry - (α4)3(β2)2, contain a unique agonist binding site at the α4α4 interface in addition to two binding sites at the α4β2 interface. Most documented compounds bind to both binding sites, CMPI and NS9283 however, exclusively target the α4α4 binding site. This selective binding, results in a modulator-like effect akin to benzodiazepines in GABAA receptors which prompts research into better understanding ligand binding to the α4α4 binding site. This presents a challenge given the flexibility of loops C and F as well as the presence of His-116 which exists in two neutral tautomeric forms. To address this, we conducted molecular dynamics simulations of the (α4)3(β2)2 nAChR in a large membrane system. The receptor was tested in the ligand bound and APO state and with both neutral tautomers of His-116. This systematic approach totalling to 24 microseconds of simulation time provided several key observations for ligand binding at the α4α4 binding site. In APO state simulations, loop C collapsed and altered the hydrogen bonding pattern of the protein. The binding stability of small molecules such as nicotine and acetylcholine were strongly correlated with a water mediated hydrogen bond to the backwall of the binding site. Furthermore, clustering analysis provided several uniform and overlapping binding modes between the compounds. Nicotine, CMPI and NS9283 displayed an overlapping binding mode extending under loop C to a hitherto unknown pocket. The validity of the CMPI binding mode is supported by its strong agreement to site directed mutagenesis data. The simulations presented here comprise one of the largest molecular dynamics studies on nAChRs to date and will help in drug discovery efforts targeting the α4α4 binding site.

Hydrazone-based compounds evaluated as inhibitors of human cholinesterases

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Hydrazones are a class of organic compounds with an RR'C=NNH_2 structure. This class of compounds has been reported to possess antimicrobial, anticonvulsant, analgesic, anti-inflammatory, antiplatelet, antitubercular, antitumoral properties. Recently, hydrazone-based compounds were also investigated as a class of compounds with the potential to be used in treating neurodegenerative diseases such as Alzheimer's (AD). Some hydrazone-based compounds have been reported to successfully cross the blood-brain barrier and inhibit the activity of acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and amyloid fibril formation in the brain.

In this pilot-study, we tested the inhibitory potential of seven new hydrazone derivatives of pyridoxal and pyridine-4-carbaldehyde against human AChE and BChE. Our results showed that all of the tested compounds reversibly inhibited AChE and BChE with dissociation constants of the enzyme-hydrazone complex (K_i) in 9.9 – 301 μM range. The most potent AChE inhibitor was compound **2**, a pyridoxal derivative with two nitro groups on benzene ring with K_i = 16 μM, while the lowest inhibitory potential was that of 4-pyridinecarboxaldehyde based hydrazone (**9**) with non-substituted benzene. Generally, pyridoxal-based derivatives were more potent inhibitors of AChE than 4-pyridinecarboxaldehyde based. The most potent BChE inhibitor was compound **1**, a pyridoxal-based derivative with non-substituted benzene, while the lowest inhibition potency was detected for compound **11**, a 4-pyridinecarboxaldehyde based hydrazone with fluorine on benzene ring. It was noticed that pyridoxal-based derivatives were generally low selective to AChE, while 4-pyridinecarboxaldehyde based were BChE selective. The exceptions were compound **1** that was BChE selective, and **11** that was slightly AChE selective. As target enzymes are located in neurons, we evaluated hydrazones' potential for crossing the blood-brain barrier by passive transport based on their calculated physicochemical properties and comparison to the recommended physicochemical properties of central nervous system drugs. Six hydrazones were determined to possess the ability to penetrate the blood-brain barrier by passive transport, while compound **2** has one violation of Lipinski's rule of five and should be able to passively penetrate the blood-brain barrier. In conclusion, a pyridoxal-based hydrazone **1** can be pointed out as compound that has potential for further evaluation and structural refinement as molecule with possible applicability in treatment of Alzheimer's disease symptoms. ACKNOWLEDGEMENTS: The authors would like to thank the CSF (Grant IP-2020-02-9343)

Pralidoxime analogues as efficient reactivators of ChE inhibited by OP nerve agents and pesticides

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The covalent inhibition of cholinesterases (ChE) with organophosphates (OPs) requires immediate treatment mostly based on oximes as reactivators of acetylcholinesterase (AChE). A downfall of this well-known treatment is its inapplicability for the reactivation of butyrylcholesterase (BChE) or targeted AChE in brain due to the poor blood brain barrier (BBB) penetration of the oximes. Thus new oximes were synthesized, referring to standard oxime pralidoxime (2-PAM), with permanently charged pyridinium rings, but with longer aliphatic linkers which improved their ability to penetrate the BBB regarding *in silico* results. We singled out three analogs of 2-PAM for which both enzymes had moderate affinity, with a 2- to 14-fold higher binding proficiency of AChE than BChE. In a previous study, certain pyridinium oximes were identified with a significantly improved *in vitro* reactivation potential for tabun inhibited-AChE. So in this research we wanted to test the reactivation ability, not only for ChE inhibited by nerve agents but also by organophosphorus pesticides that are analogs of tabun, metamidophos and fenamiphos. From several combinations of an oxime and the OP-ChE conjugate, 2-PAM analogs have shown the highest reactivation efficiency for cyclosarin, with a 300-fold higher overall kinetic reactivation rate for BChE than standard oxime. Moreover, the reactivation potency of cyclosarin-inhibited AChE was up to 90% for one oxime. With regard to ChE inhibited with pesticides, the acquired results were slightly better than for 2-PAM, with the highest reactivation of 60% recorded for fenamifos-inhibited BChE. Taking into account the reactivation efficacy for cyclosarin exposure, oximes were also evaluated in ex vivo conditions together with exogenous BChE. The obtained results demonstrated up to 80% of restored phosphonylated cholinesterase activity within a short time. Considering our results, we identified three efficient reactivators of phosphylated BChE that, due to a cumulative capacity to reactivate both AChE and BChE, possess the potential for further evaluation in OP bioscavenging. Acknowledgments: This work was supported by the Croatian Science Foundation (IP-2018-01-7683).

Distinct distribution of cholinesterases in mouse heart

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Cholinergic control of the heart, exerted by neuronal acetylcholine (ACh) secreted from nervus vagus and by non-neuronal ACh released by cardiomyocytes, has a protective role during many cardiac pathologies, including heart failure and atrial and ventricular fibrillation. ACh level is controlled by two enzymes: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), but information about them in cardiac tissue is sparse. The aim of this work was therefore to provide a detailed picture of the cholinesterases (ChE) in mouse heart, by characterizing their activity, molecular forms and precise localization. For this, we used mutant mouse strains lacking ChE or their anchoring proteins – ColQ and PRiMA. The molecular forms in heart compartments were separated on sucrose gradients and activities were determined by modified Ellman's assay. ChE were visualized in whole-mounted, gelatin-filled hearts or in cryosections by Tsuji's staining method or immunohistochemistry. We found that BChE activity exceeds AChE activity in mouse heart. Distribution of ChE was compartment-specific, with the highest AChE activity in the atria and highest BChE activity in the ventricles. Activities of both ChE were higher in the base of the heart than in the apex. Predominantly anchored forms of AChE were present, visualized mainly in the atria and in the heart base, and colocalizing with neuronal marker TUJ1. No anchored forms of BChE were detected by biochemical analysis. Soluble BChE was visualized predominantly in the heart ventricles. Our findings suggest distinct distribution of ChE throughout the heart. Colocalization of AChE with a neuronal marker suggests its participation in the hydrolysis of neuronal ACh. BChE is predominantly in the ventricles and its participation in the hydrolysis of non-neuronal ACh can be proposed. Acknowledgement: This project was supported by VEGA 1/0283/22, 1/0815/21, APVV SK-FR-19-0005

The macro problem of live human microglia

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The cholinergic anti-inflammatory pathway is associated with a range of neurological disorders, such as poststroke immune blockade and Alzheimer's disease. There is evidence that acetylcholine might reduce inflammation levels in the brain by deactivating microglia – the main immune cells of the CNS. Although it is known that microglia respond to cholinergic signaling via the α7 nicotinic acetylcholine receptor, the full pattern of cholinergic regulation in microglia is yet to be discovered. Furthermore, it appears that live human brain samples feature higher biological diversity of microglial cells, as compared to post-mortem brain tissue. We are combining powerful computational techniques and experimental pipelines to explore the cholinergic transcriptome of live human microglia and its role in brain immune response. Applying a novel data analysis method based on the Information Bottleneck algorithm to single cell RNA-Seq data obtained from the microglia of live human brain samples allows us to refine the current knowledge of microglial transcriptomic diversity. Furthermore, we are characterizing the cholinergic activation in live human microglia as a function of sex, age, and brain region. Moreover, we are constructing a unique short RNA-seq dataset from live human microglia to further address the question of cholinergic regulation by short non-coding RNA, such as microRNAs and tRNA fragments.

Revealing the cholinergic identity of microglia might deepen the current scientific knowhow of the cholinergic modulation in various neurological conditions.

The effect of high-fat diet on the cholinergic system

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Introduction: Recent research in human and animals suggests that butyrylcholinesterase (BChE) plays an important role in the mechanism of obesity development. Individuals with reduced BChE activity are more prone to become obese. In connection with this finding, we decided to look closer at changes in BChE expression and activity in obese rats induced by increased dietary fat intake. The aim of our study was therefore to investigate the effect of a high-fat diet (HFD) on expression and activity of cholinesterases and in the lipid profile in Wistar rats. Methods: In the experiment, we used adult male Wistar rats fed with a standard diet (SD) or HFD, with or without parallel treatment with a BChE inhibitor, isoOMPA, dissolved in drinking water for 13 weeks. During the experiment, we monitored the food and water intake, change in the body weight and calculated BMI. Selected tissues were collected post mortem for further anatomical, morphological and biochemical analyses. Cholinesterase activity was measured using Ellman's method, relative expression of cholinesterases was assessed by RT-qPCR, and the lipid profile of plasma was assessed in a certified laboratory. Results: As expected, in rats fed with HFD, we observed higher body weight gain, adipose tissue weight, BMI and serum triacylglycerol levels when compared to rats on SD. Moreover, higher BChE activities were recorded in serum. Although BChE relative expression was increased in liver, its activity level did not change, suggesting secretion of the de novo synthesized BChE into the bloodstream. Surprisingly, systemic inhibition of BChE activity in rats on SD caused a weight gain comparable to that observed in rats on HFD. Conclusion: Our results suggest reciprocal relationship between serum BChE activity and obesity, as obesity caused by HFD led to an increase in serum BChE activity and vice-versa inhibition of BChE activity induced weight gain in Wistar rats. This project was supported by VEGA 1/0283/22, 1/0815/21, and SK-FR-19-0005.

Expression of cholinesterases and their anchoring proteins in rat heart

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Acetylcholine (ACh)-mediated vagal transmission as well as nonneuronal ACh release are considered cardioprotective in pathological situations with increased sympathetic drive such as ischemia–reperfusion and cardiac remodeling. Although cardioprotective effects of ACh were studied in detail, the information about ACh degradation enzymes, acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) in cardiac tissue is missing. Here we therefore assessed the expression of specific cholinesterase (ChE) molecular forms in different compartments of rat heart using RT-qPCR. We observed that both ChE were expressed in all heart compartments, while BChE was expressed at higher levels than AChE (average BChE CT value 26.88 versus average AChE CT value 29.41). Additionally, ChE expression differed in specific heart compartments. While BChE was found in all heart compartments, AChE was predominantly expressed in rat atria. ChE expression was accompanied by expression of both ChE anchoring proteins PRiMA and ColQ. Interestingly, we detected two PRiMA amplification products. The size of the first amplification product corresponded to the predicted rat PRiMA sequence, while the size of the second PRiMA amplification product corresponded to a predicted PRiMA transcript previously described in human and mouse brain. Altogether, here we report differential expression of AChE and BChE in rat heart. In the future experiments, the role of cardiac AChE and BChE should be examined in regard to degradation of ACh from the neuronal and nonneuronal sources. This project was supported by VEGA 1/0283/22, 1/0815/21, and SK-FR 2019.

Characterization of novel imidazolium oximes as selective reactivators of nerve agent-inhibited butyrylcholinesterase

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Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are enzymes crucial for neurotransmitter acetylcholine metabolism in both central and peripheral nervous system. The progressive inhibition of these enzymes by nerve agents (NA) during poisoning disrupts acetylcholine breakdown, leading to excessive impulse transmission and cholinergic crisis. Since BChE circulates freely in the bloodstream, it has been considered as a possible exogenous bioscavenging enzyme which would act as an oxime-assisted catalytic scavenger of NAs, neutralising them before they reach target tissues. Oximes are used in standard clinical therapy to recover activity of inhibited cholinesterases, but reactivating BChE with standard pyridinium oximes proved to be ineffective as they are mainly AChE reactivators. To approach this issue, based on previous research we synthesized eight novel *N*-benzyl supstituted imidazolium oximes to be considered as reactivators of nerve agent sarin-, cyclosarin- and tabun-inhibited human AChE and BChE. Two oximes emerged as effective reactivators of all three NA-BChE conjugates, possessing superior overall reactivation rates (kr) than standard oxime HI-6 which was up to 3000-times higher for cyclosarin conjugate and 210-times for sarin conjugate, or 100-times higher than oxime TMB-4 in case of tabun conjugate. Binding affinities of phosphylated BChE for novel oximes in terms of $1/K_{OX}$ increased up to 1900-fold compared to standard oximes and they achieved their reactivation maximum in a shorter time frame. However, standard pyridinium oximes remain better reactivators of phosphylated AChE which indicates that imidazolium oximes with 1,3-aromatic substituents do not position optimally into the AChE active site gorge. Novel oximes' low toxicity on hepatic (HepG2) and neuronal (SH-SY5Y) cell lines and high capacity for reactivation of BChE in the studied concentration range establishes them as promising candidates for future treatment development. This research was supported by the Croatian Science Foundation (HrZZ-IP-2018-01-7683 and HrZZ-IP-2016-06-3775).

Amiridine derivatives as multitarget drugs related to Alzheimer's disease therapy

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Amiridine is anticholinesterase drug used in Russia for treatment of AD and a number of other neurological disorders. Amiridine was developed in the USSR in the mid-80s, and is currently used as a stimulator of learning and memory for the treatment of Alzheimer's disease (AD) and other forms of senile dementia. From a structural perspective, it shares high degree of similarity to tacrine, a well-established FDA-approved drugs. Both compounds act as cholinesterase inhibitors. Within our contribution, we will report on the development of novel amiridine derivatives. Specifically, we will focus on their chemical synthesis, evaluate their acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition properties and discuss structural-activity relationship. As some of amiridine derivatives were developed as AChE dual-bining site inhibitors, we will also provide insight into their AChE-induced β-amyloid aggregation. Besides, results from other experiments, like antioxidiant capacity, will be reported. The study was supported by the Czech Science Foundation #20-29633J.

The effects of vitamin B3-based compounds on neuronal and muscle cells

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Compounds based on vitamin B3 active form nicotinamide, have been associated with many targets due to their antitumoral, antibacterial, anti-inflammatory, and various other biological activities. Our previous work has characterized these compounds as inhibitors of human cholinesterases which makes them possible drugs for cholinergic neurotransmission-linked pathologies. Our aim was to assess in more detail the safety of these compounds on target cells.

In the present study we evaluated the effects of these nicotinamides on neuronal (SH-SY5Y) and muscle cells, as types of cells involved in neuromuscular junction. Possible cytotoxic effect in both cell types was evaluated by MTS assay and the involvement of several nicotinamide-associated signalling pathways in the mechanism behind observed cellular effects was further analysed using flow cytometry.

Results showed that out of nine tested compounds, four have displayed time-dependent cytotoxicity in treated cells. Cytotoxicity profiling revealed that compounds in doses of 100 μM could induce events possibly leading to cell death. These responses likely arise from modulation of nicotinamide-linked pathways such as MAPK-, AMPK-, Akt- and mTOR signalling that have an important role in multiple cellular functions. Nevertheless, the concentration affecting the cells, especially for the potent ChE-inhibitor 1-(4'-phenylphenacyl)-3-carbamoylpyridinium bromide, was far greater than predicted to be used in therapy according to the determined inhibition potency or in the general aspect of drug administration. However, the observed effects on cells should not be neglected in any future detailed studies of nicotinamide derivatives as new drug scaffolds. This research was supported by the Croatian Science Foundation grant UIP-2017-05-7260, Croatian-Slovenian Bilateral project BI-HR/20-21 and Slovenian Research Agency grant P3-0043 in J7-8276.

Changes in the cholinergic system in patients with severe COVID-19

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The COVID-19 disease caused by SARS-CoV-2 viral infection represents an excessive burden on health care systems in many countries of the world. Despite the implementation of public health measures and the accessibility of vaccination, their effectiveness in Slovakia is low and with the outreach of new variants of SARS-CoV-2 virus, there is still an unmet need for early and effective identification of people who develop severe to critical disease. Although several biochemical parameters associated with severe COVID-19 disease progression have been identified to date, the presence of a suitable prognostic marker of disease progression remains absent. Butyrylcholinesterase (BChE), an enzyme regulating the activity of the cholinergic system, has been investigated mainly in the past from a toxicological point of view in organophosphate poisoning and from a pharmacological point of view in the context of the metabolism of drugs such as succinylcholine or mivacurium. Its physiological function is still not well understood despite its high activity in serum. In recent years, BChE has been investigated in metabolic disease development such as obesity, hyperlipidemia, type 2 diabetes mellitus and inflammatory processes, factors identified as risk factors for the development of a more severe course of COVID-19. In our monocentric clinical study, we have focused on the relationships between changes in serum BChE activity, demographic data and biochemical parameters in relation to disease severity, present comorbidities, pharmacotherapy and clinical outcomes. Further understanding of these relationships provides the promise for the use of new prognostic markers useful for practice. Acknowledgement: This project was supported by VEGA 1/0283/22, 1/0815/21 and APVV SK-FR-19-0005.

Synthesis and biological evaluation of heterocyclic meta-biscarbamates

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Carbamates are a structural part of many drugs for the treatment of various diseases, including neurodegenerative disorders like Alzheimer's disease (AD). AD is one of the most common causes of mental deterioration in elderly people caused by a loss of cholinergic innervation in the cerebral cortex and characterized by decreased levels of the neurotransmitter acetylcholine in neurons, which has led to the development of AD drugs that inhibit the activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The mechanism of action of carbamates and cholinesterases is similar to that of the physiological substrate of AChE, acetylcholine; the only difference lies in the turnover rate of the enzyme's activity where the decarbamylation rate is much slower than the deacetylation.

We synthesized 17 new heterocyclic biscarbamates with aliphatic carbamate groups in meta position on the benzene ring and different substituents in the amino part of the molecule. All biscarbamates were potent inhibitors of both cholinesterases with inhibition rate constants within 103-106 M^{-1} min^{-1}. The most potent BChE inhibitors were ethyl-methyl carbamates with piperidine or adamantylamine in the amino part of the molecule, while the most potent AChE inhibitor was diethyl carbamate with tert-pentylamine. The inhibition potential and selectivity were analysed by molecular docking studies. Nine biscarbamates were determined to possess the ability to penetrate the blood-brain barrier by passive transport, while seven biscarbamates exhibited one deviation from the recommended values for CNS active drugs. Twelve biscarbamates didn't exhibited hepatotoxicity, nephrotoxicity not neurotoxicity, while five biscarbamates were toxic to at least one of the cell lines at concentrations in which they showed inhibition activity. The ethyl-methyl biscarbamate with piperidine in the amine moiety could be pointed out as the most promising compound for further evaluation and structural modifications for development as a potential AD drug. Supported by CFS grants IP-2018-01-7683, IP-2020-02-9343 and UIP-2017-05-7260.

Low Dose Tetanus Toxin Injections into the Rat Motor Cortex and Striatum Impair the Narrow Beam Walking Performance

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Basal ganglia and motor cortex integrate the sensory proprioceptive input arriving from periphery and the planning and execution of movement. Abnormalities in the motor cortex and basal ganglia excitability play a central role in the onset of movement disorders such as dystonias and parkinsonism. Tetanus neurotoxin (TeNT) prevents the inhibitory neurotransmission in central synapses. The aim of present study was to examine the behavioral effect of neuronal disinhibition in mentioned brain regions induced by low, non-convulsive doses of TeNT.

The rats were injected unilaterally into caudate putamen or motor cortex with 0.5 ng TeNT. After 2 weeks, the injections were repeated into the aforementioned contralateral motor regions, and the effect of TeNT was assessed for another 2 weeks. Various behavioral tests were repeatedly performed to assess the effect of TeNT-induced disinhibition on normal motor performance in rats, as well as to exclude possible epileptogenic toxin action. First unilateral toxin injection exerted a short-lasting impairment in the ability to transverse a narrow beam, and with no visible effects on rota-rod, swimming, gait analysis (catwalk) and EMG. Upon the second toxin injection, the animals developed a lasting impairment in the beam walking performance while the performance in other motor tests remained unimpaired. The plantar misplacement of the hind-limb during the narrow beam traversing were more evident on the hind-paw contralateral to the injected brain region. Open field test, pre-pulse inhibition and attempted audiogenic seizure tests did not indicate the possible epileptogenic actions of TeNT.

The motor cortex or striatal disinhibition with TeNT induces subtle motor impairment in the relatively complex motor task of crossing the elevated narrow beam, requiring correct prediction of paw placement. After unilateral toxin applications, the animals appear to be able to compensate the proprioceptive motor deficit, which is then aggravated and becomes long lasting after contralateral regional disinhibition. Funding: Croatian Science Foundation (project ID: UIP-2019-04-8277)

In vitro Evaluation of Uncharged Thienostilbene Oximes as Reactivatiors of Organophosphate-inhibited Cholinesterases

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The inhibition of AChE and BChE by organophosphates (OPs) as nerve agents and pesticides compromises normal cholinergic nerve signal transduction in the peripheral and central nervous systems (CNS) leading to cholinergic crisis. The treatment comprises an antimuscarinic drug and an oxime reactivator of the inhibited enzyme. Oximes in use have quaternary nitrogens, and therefore poorly cross the brain–blood barrier. In this work, we synthesized novel uncharged thienostilbene oximes by sequence of three reactions in very high yields. The expected targeted products were pure *cis*- and *trans*-isomers of *syn*- and *anti*-oximes containing different substituents bound in the *para*-position of the benzene ring.[1]

Eight *trans*,*anti*- and *trans*,*syn*-isomers of oximes were tested as reactivators of nerve-agent-inhibited AChE and BChE. Four derivatives reactivated cyclosarin-inhibited BChE up to 70% in two hours of reactivation, and docking studies confirmed their productive interactions with the active site of cyclosarin-inhibited BChE. Based on the moderate binding affinity of both AChE and BChE for all selected oximes, these compounds present a new class of oximes with the potential for further development of CNS-active therapeutics in OP poisoning. This is the first study to show the potential of thienostilbene oximes as therapeutics in OP poisoning, and it seems that further design of the compounds e.g., with amide, OH, mono- and dimethylamino groups, or triazole ring, could provide a new platform for further antidote and scavenger development for exposure to organophosphates.

Acknowledgment**:** This research was partially funded by the Croatian Science Foundation (project IP-2018-01-7683).

[1]M. Mlakić et. al. *Pharmaceuticals* **2021**, *14*, 1147.

Commercial Herbicides Screened for Toxicity *in silico* and Examined as Inhibitors of Acetylcholinesterase and Butyrylcholinesterase

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The widespread and frequent use of herbicides has led to the emergence of two major global issues: 1) increasing weed resistance to herbicides and 2) detrimental impact on health. A set of 509 herbicides collected from the literature and online databases was classified based on their modes of action and screened for toxic effects on human health based on predicted toxicities for *in vitro* and animal models and impact on environment. The analyses enabled us to estimate the toxicity profiles for herbicides of different herbicidal modes of action, including also their physicochemical properties (MW, TPSA, log P, log D, HBA, HBD, log BB, BBB penetration, etc.). We selected 11 compounds with potential neurotoxic effect for biological tests focusing on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), enzymes involved in neurotoxicity and detoxification mechanisms, respectively. Out of organophosphate herbicides (anilophos, piperophos, bensulide and butamiphos) and carbamates (flufenacet, desmedipham and phenmedipham), all of which exhibited an inhibition of both cholinesterases, anilophos was the most potent inhibitor of AChE (IC_{50}=26 μM) and BChE (8 μM). Moreover, the inhibition of all of the listed herbicides was with a slight preference to bind BChE, which is known as a natural endogenous bioscavenger of various xenobiotics. The selected glyphosate, oxadiazon, tembotrione and terbuthylazine were poor inhibitors with an estimated IC_{50} above 1 mM. Our results given by *in silico* and *in vitro* analyses could give insight into the toxic effects of herbicides in use and may be the origin of new herbicides with less hazardous effects to humans and the environment. This research was supported by the Croatian Science Foundation (HrZZ-IP-2018-01-7683).

Cellular model expressing acetylcholinesterase for evaluation of neurotoxicity and neuroprotection

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Neurodegenerative disorders and organophosphate poisoning is commonly associated with neurotoxicity. Neuronal death leads primarily to acute and life-threatening symptoms and to secondary disorders that may persist for a long time. We have focused on the development and validation of an *in vitro* cellular model based suitable for studying the neurotoxicity and its potential countermeasures based on the differentiation of the SH-SY5Y cell line into mature human neurons. The mature human neuron model was obtained by differentiating the neuroblastoma cell line SH-SY5Y. The protocol involved stimulation of the cell line with retinoic acid and brain-derived neurotrophic factor for 9-12 days. Observation of morphological signs of neurons (characteristic synaptic connections), using lighting microscopy and a detection of specific neuronal markers (tau protein, microtubulle-associated protein (MAP), synaptophysin (SYN), post-synaptic density protein (PSD-95)) using fluorescence microscopy was used for validating this model. Furthermore, the quantification of acetylcholinesterase in differentiated and undifferentiated cells was assessed. The result supported our hypothesis that the level of enzyme in differentiated cells is substantially higher than the original undifferentiated SH-SY5Y. Based on the obtained results, the model is suitable for further *in vitro* testing and will be used in the study of neurotoxic effects of organophosphates and in the screening of potential prophylactics or drugs for neurodegenerative diseases treatment.

This work was supported by the Ministry of Education, Youth and Sports of Czech Republic (project ERDF no. CZ.02.1.01/0.0/0.0/18_069/0010054) and student project *SV/FVZ202010.*

The effect of ganglioside composition on enzyme activity, protein expression, and submembrane localization of Na+ /K+ -ATPase in mouse brain

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The Na+ /K+ -ATPase (NKA) is an enzyme that asymmetrically distributes Na+ and K+ ions across the plasma membrane to generate and maintain the membrane potential, also having a role in signaling processes. NKA dysfunction has been implicated in several neurodegenerative disorders. Its positioning as well as different functions of active and inactive pools depend on interactions with neighboring membrane lipids. Ganglioside enriched lipid rafts (LR) are housing active NKA pool, while the bulk membrane contains the inactive pool. Gangliosides are known to modulate the structure, function and localization of membrane proteins thus having an impact on ion homeostasis. The aim of this study was to investigate the effect of altered ganglioside composition on activity, protein expression and submembrane localization of NKA, using St8sia1 null mice with impaired synthesis of gangliosides. Adult wild type (WT) and null mice littermates were sacrificed, brains neuroanatomically dissected and cortical and cerebellar homogenates prepared. NKA activity was measured spectrophotometrically. Protein expression of NKA in homogenates was analyzed by Western blotting. LR and non-raft (nLR) fractions from cortices and cerebella were isolated by ultracentrifugation in discontinuous sucrose gradients, and submembrane localization of NKA analyzed by Western blotting. Data revealed statistically lower NKA activity in cortices of null mice compared to the WT mice, whilst there was no disparity in the cerebella. Total protein amount of NKA was statistically lower in null mice cortices compared to their WTs, whilst it was unvaried in the cerebella. Analysis of submembrane localization has shown higher amount of NKA to be positioned within LR of cortices than those derived from the cerebella. These results demonstrate that altered ganglioside composition may contribute to lower NKA expression influencing NKA activity and cellular ion homeostasis.

Synthesis, characterization and evaluation of 10,11-dihydro cinchonidine carbamates as potential cholinesterase inhibitors

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Cinchonidine belongs to class of alkaloids found in the bark of Cinchona tree, together with cinchonine, quinine and quinidine. These alkaloids have various uses in chemistry, for example they are used as organocatalysts in asymmetric synthesis or as chiral stationary phases and chiral solvating agents in chromatographic separations. Also, they posses a wide range of biological activity such as antimalarial, antibiotic and antiparasitic. By transfer hydrogenation of cinchonidine using formic acid/ammonium formate as hydrogen donor and palladium on carbon as a catalyst 10,11 dihydrocinchonidine was prepared and used as starting material for synthesis of his carbamate derivatives. We have prepared a series of mono- and disubstituted aliphatic (methyl, ethyl) and aromatic (phenyl) 10,11 dihydrocinchonidine carbamates. Carbamate derivatives have been prepared by reaction of 10,11 dihydrocinchonidine with appropriately substituted isocyanates or carbamoyl chlorides. Synthesized compounds were characterized by FT-IR, 1D and 2D 1H and 13C NMR spectroscopy. Extensive docking studies and quantum chemical calculations were used to define intermediates and transition states in the course of the carbamylation reaction as well as other possible interactions of prepared compounds within the active sites of enzymes. All prepared carbamate derivatives will be screened for their ability to inhibit human acetylcholinesterase and butyrylcholinesterase activity. Supported by the Croatian Science Foundation, Project No. IP-2016-06-3775 ADESIRE.

Exploring structure-activity relationship of new *N*-alkyl quaternary quinuclidines through cholinesterase inhibition and impact on cell homeostasis

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Quinuclidine based derivatives are a class of compounds that has been attracting increased attention in modern drug discovery based on their anticholinergic, antimicrobial, antioxidative and antitumor activity. Guided by an interest to develop drugs with polypotent chemical structures which result in interaction with various molecular targets or receptors, we have synthesized and characterized 14 quaternary quinuclidine compounds with the variation in *N*-alkyl chain length and incorporation of alcohol or oxime headgroup at the position 3 of the quinuclidine ring. We analysed reversible inhibition of human acetylcholinesterase (AChE) and human butyrylcholinesterase (BChE) by these compounds and screened their cytotoxic effect on three selected cell types: neuronal cells (SH-SY5Y), hepatocytes (Hep G2) and kidney cells (HEK293). All of the tested compounds reversibly inhibited both AChE and BChE activity in the micromolar range. The most potent inhibitors were bisquaternary 3-hydroxy and 3-hydroxyimino compounds with C10 alkyl chains (K_i = 0.2 – 1.6 μM). Analysis of the impact of functional group revealed oximes with C8 to C12 alkyl chains and alcohols with C14 and C16 alkyl chains as more potent reversible inhibitors than their analogues. AChE showed high affinity toward compounds with a long alkyl side chain (C14 and C16). BChE affinity was not affected much by the length of the alkyl chain, although an opposite preference to AChE was observed. MTS assay showed toxicity (IC_{50} = 3 – 204 μM) of compounds with a long side alkyl chain (C12, C14 and C16) from both groups, alcohols and oximes. Neuronal cells were the least sensitive to the toxicity of the tested compounds. According to inhibition potency for both AChE and BChE and non-cytotoxic profile, it seems that quinuclidine oximes with short chain length (< C12) can be considered as candidates for further investigations with possible therapeutic potential in central nervous system.

Acknowledgment: This work was supported by the Croatian Science Foundation grant UIP-2017-05-7260.

7-Phenoxytacrine is a AChE inhibitor and NMDA antagonist with neuroprotective efficacy *in vivo*

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NMDARs are a valid pharmacological target for the treatment of neurodegenerative disorders; however, novel drugs targeting NMDARs are often associated with specific psychotic side effects and abuse potential. Motivated by currently available treatment against neurodegenerative diseases involving the inhibitors of acetylcholinesterase (AChE) and NMDARs, administered also in combination, we developed a dually-acting compound 7-phenoxytacrine (7-PhO-THA) and evaluated its neuropsychopharmacological and drug-like properties for potential therapeutic use. Indeed, we have confirmed the dual potency of 7-PhO-THA, i.e. potent and balanced inhibition of both AChE and NMDARs. We discovered that it selectively inhibits the GluN1/GluN2B subtype of NMDARs via an ifenprodil-binding site, in addition to its voltage-dependent inhibitory effect at both GluN1/GluN2A and GluN1/GluN2B subtypes of NMDARs. Furthermore, whereas NMDA-induced lesion of the dorsal hippocampus confirmed potent anti-excitotoxic and neuroprotective efficacy, behavioral observations showed also a cholinergic component manifesting mainly in decreased hyperlocomotion. From the point of view of behavioral side effects, 7-PhO-THA manages to avoid these, notably those analogous to symptoms of schizophrenia. Thus, CNS availability and the overall behavioral profile are promising for subsequent investigation of therapeutic use. The work has been supported by Czech Health Research Council (No. NU20-08-00296)

Long-term Motor Central Effects of Botulinum Toxin Type A in Rats

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Botulinum toxin type A (BoNT-A) is a potent presynaptic neurotoxin and a standard therapy in hyperkinetic movement disorders, presumably due to its local muscular anticholinergic effect. However, recent experimental data point to possible central effects in the CNS. The present aim was to examine the contribution of the transcytosis-dependent central toxin action on the long term muscular function recovery in rats, as well as TeNT-evoked spastic paralysis. Rats were bilaterally injected with BoNT-A into the gastrocnemius muscle (2 U/kg) or sciatic nerve (5 U/kg). The following day, the animals were injected intrathecally (i.t.) with BoNT-A-neutralizing antitoxin. On day 62, animals were injected i.m. with tetanus neurotoxin (TeNT). In different motor tests (gait ability score, digit abduction score, rota-rod, beam walking and swimming performance), i.t. antitoxin significantly accelerated the flaccid paralysis and motor performance recovery. BoNT-A reduced the lower hind-limb diameter and muscle size without significant recovery during the entire experiment, which resulted in reduction of CMAP and H reflex. The TeNT-evoked increase in muscle tone was reduced by BoNT-A dependently on its central effect. However, the H-reflex, when corrected for reduced muscle size or reduced CMAP, was not affected by the toxin treatment, suggestive of the lack of the toxin's direct effect on monosynaptic reflex. The enzymatic activity of the toxin, examined by cleaved synaptosomal-associated protein 25 (cSNAP-25) immunohistochemistry, was still present in neuromuscular junctions and spinal cord. The central occurrence of the cSNAP-25, present in second order spinal cord cholinergic neurons, depended on the toxin's central transcytosis. Conclusion: Long term motor effects of BoNT-A both on normal motor performance (day 1-62), as well as the spastic paralysis (days 62-78), are influenced by the toxin's ongoing central action mediated by retrograde transport and transcytosis. These data suggest that clinically relevant beneficial effect of BoNT-A result from toxin's combined peripheral and central effects. Funding: Croatian Science Foundation (project ID: UIP-2019-04-8277)

Effects of organoruthenium(II) complexes on the activity of mammalian cholinesterases and on neuromuscular cholinergic system

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Metal complexes are used in the therapy of various diseases and also for diagnostic purposes. Ruthenium complexes possess a wide range of biological effects, such as anticancer, antibacterial, antiviral, antiparasitic, and imunosupressive activities. These complexes selectively inhibit several medically important enzymes involved in pathophysiological conditions, such as cholinesterases (ChEs), glutathione S-transferases, protein kinase, aldo-keto reductases, thioredoxin reductase, cathepsin B, topoisomerase II, and HIV-1 reverse transcriptase.

It is known that some ChE inhibitors have a dual effect; they can inhibit not only ChEs but also different subtypes of nicotinic acetylcholine receptors. Therefore, we have been investigating the effects of organoruthenium(II) complexes with various ligands on the activity of mammalian ChEs and on cholinergic system using a combined biochemical and electrophysiological approach. Our kinetic experiments showed that some newly synthesised organoruthenium(II) complexes inhibit both acetylcholinesterase and butyrylcholinesterase, or selectively inhibit butyrylcholinesterases of human and animal origin in the low micromolar range. We have performed electrophysiological experiments with the most potent ChE inhibitors on neuromuscular preparations of the mouse hemidiaphragm to study the effects of these complexes on the cholinergic system in the mammalian neuromuscular junction. The results show that some complexes do not affect muscle twitch, resting membrane potential, endplate potential, and miniature endplate potential, strongly suggesting that they do not affect neuromuscular transmission. However, we have also synthetized a organoruthenium nitrophen (5-nitro-1,10-phenanthroline) complex, [(η6-p-cymene)Ru(nitrophen)--Cl]Cl, that has a dual effect. Enzyme inhibition assays showed that this complex acts as a competitive inhibitor of ChEs, but in neuromuscular preparations the complex competitively and reversibly blocks (IC_{50} = 19.44 μM) muscle contraction. It decreases endplate potentials (IC_{50} = 7.6 μM), strongly suggesting that it reversibly blocks the muscle type of nicotinic acetylcholine receptors. Overall, we have found interesting complexes for further preclinical studies and potential use in veterinary and human medicine.

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Development of an *in situ* reactive Coenzyme A trapper to enable efficient extraction and characterization of choline acetyltransferase from tissue

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Choline acetyltranferase (ChAT) catalyzes synthesis of acetylcholine, and is responsible for charging cholinergic neurons with the neurotransmitter. Exploiting our discovery that ChAT is a hydrothiolation catalyst,[1] we have developed a chemical probe for the isolation of native ChAT from tissue homogenates. A biotinyl-linked arylvinylpyridine (B-AVP) derivative was prepared and found to bind to the active site tunnel of ChAT. A subsequent *in situ* chemical reaction between the B-AVP and coenzyme-A yielded an inhibitor with a notable long residence time. Streptavidin coated magnetic beads were subsequently used to extract the enzyme from brain homogenate, allowing efficient removal of matrix components by simple washing. Unspecific binding of ChAT proved to be substantial, and current work is focused on improving bead loading and extraction yields. We anticipate that, in its final form, this method can be applied to isolate ChAT from various tissues and cellular compartments to study the distribution of isoforms, posttranslational modifications and regulatory mechanisms.

[1] Wiktelius D, Allgardsson A, Bergström T, Hoster N, Akfur C, Forsgren N, Lejon C, Hedenström M, Linusson A, Ekström F. *In situ* assembly of choline acetyltransferase ligands by a hydrothiolation reaction reveals key determinants for inhibitor design. *Angew Chem Int Ed* **2021**, *60*, 813-819.

Investigation of antioxidant potential of pyridinium oximes with halogen moiety and its relevance to therapeutic application

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Pyridinium oximes are an essential part of antidotal therapy used in organophosphorous (OP) compounds poisoning. Through reactivation of phosphorylated acetylcholinesterase (AChE) oximes ameliorate symptoms arising from acute OP poisoning. However, survivors of OP exposures often have delayed neurologic symptoms of memory loss and cognitive dysfunction. It was shown that oxidative stress mediates secondary OP injury so long-term neurological consequences may be amendable to antioxidant therapy. Pyridiunium oximes have incorporated iminium and oxime moieties that are electron-affinic which enables them various physiological properties and might contribute to their overall therapeutic efficacy. The antioxidant activity of novel pyridinium oximes with the addition of chlorine or fluorine atoms was evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) assays and compared to their non-halogenated analogues, standard pyridinium oximes and standard antioxidants. According to calculated EC_{50} values (the concentration of a compound necessary to decrease the initial concentration of DPPH radicals by 50 %), di-fluorinated oximes exhibited the best radical scavenging activity, with the strongest effect being observed for oxime 7b (EC_{50} 250 μM). In case of chlorinated oximes, mono-chlorinated oximes showed better DPPH radical scavenging activity than di-chlorinated. Standard oximes and nonhalogenated analogues showed weak scavenging capacity (EC_{50} 1.05 – 2.99 mM). In addition, FRAP assay highlighted di-fluorinated analogue 7b as the oxime with the highest antioxidant activity in terms of its reducing capacity (FRAP value 1.6 mM). Generally, a trend of higher reduction capacity of fluorinated oximes compared to chlorinated and non-halogenated analogues was observed. Both assays clearly indicated that 7b possesses an antioxidant potential probably due to the radical stabilizing effect of fluorine substituents associated with the resonance delocalization of the unpaired electron. Therefore, a multi-purpose (reactivation and antioxidant) activity of studied oximes could contribute to the entire therapeutic outcome in OP poisoning.

Acknowledgement: Supported by the Croatian Science Foundation (HrZZ IP-2018-01-7683).

Utilizing isothermal spectral shift detection to quantify challenging biomolecular interactions with Monolith X

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Monolith X is the latest addition to the Monolith product line, combining **isothermal spectral shift detection with MST (MicroScale Thermophoresis) technology** to characterize biomolecular interactions in solution. When a target is labelled with a fluorophore it generates a particular emission spectrum, and if a ligand binds to this labelled target, the fluorophore's chemical environment is changed, causing a shift in fluorescence spectra. Monolith X exploits this phenomenon by performing ratiometric measurements at two emission wavelengths of a labelled target in the presence of various concentrations of an unlabeled ligand to derive the affinity constant (Kd) for the interaction.

Isothermal spectral shift detection enables characterization of in solution interactions for a wide range of biomolecules, even for challenging samples such as membrane proteins, intrinsically disordered proteins, and cell lysates. Since the binding partners are in solution, there is no lost activity due to immobilization, and evaluation is size independent. Measurements can be performed in any buffer, including detergents, using low sample volumes and concentrations. The spectral shift analysis also facilitates the evaluation of competition assays and ternary binding events. Monolith X provides a valuable orthogonal method to validate your results from other biophysical methods and to characterize your most challenging interactions.

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International Society
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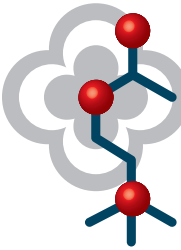

ISCM 2022

17th INTERNATIONAL
Symposium on Cholinergic Mechanisms
8 - 12 May 2022 – Dubrovnik – Croatia

ISBN 978-953-96817-8-2