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LECTURES

Developmental neurotoxicity of the organophosphorus pesticide chloryprifos: from animal behavior to molecular mechanisms

Edson Albuquerque[a], Richard Burke[a], Rao Gullapalli[b], Jacek Mamczarz[a], Edna Pereira[a]

[a]*Division of Translational Toxicology, Department of Epidemiology and Public Health, University of Maryland School of Medicine, Baltimore, USA*
[b]*Department of Diagnostic Radiology, University of Maryland School of Medicine, Baltimore, USA*

Exposure of the developing brain to chlorpyrifos (CPF), an organophosphorus (OP) pesticide used extensively in agriculture worldwide, has been associated with cognitive deficits and disruption of the structural of the brain in children, particularly boys [Rauh et al. Proc Natl Acad Sci USA 109:7871–6, 2012]. The present study was designed to test the hypothesis that sex-dimorphic cognitive deficits and disruption of the structural brain integrity induced by prenatal exposure to sub-acute doses of CPF can be reproduced in a precocial small species. To this end, pregnant guinea pigs were injected s.c. with CPF (25 mg/kg/day) or vehicle (peanut oil) for 10 days starting on gestation day (GD) 53–55. Offspring were born around GD65, weaned on postnatal day (PND) 20. Offspring were then subjected to behavioral tests starting around PND 30 and either magnetic resonance imaging (MRI) or *in vitro* electrophysiological analysis of hippocampal synaptic transmission starting around PND60–70. All studies were followed by histological or immunohistochemical analysis of the structural brain integrity. On PND1, butyrylcholinesterase (BuChE), an OP bioscavenger used as a biomarker of OP exposures, and acetylcholinesterase (AChE), a major molecular target of OP compounds, were significantly inhibited in the blood of CPF-exposed offspring. In their brains, BuChE, but not AChE, was significantly inhibited. These findings strongly indicated that CPF crossed the placenta and the blood brain barrier of the fetuses. Prenatal CPF exposure had no significant effect on locomotor activity and on a form of non-associative memory assessed in open fields. However, it resulted in significant learning impairment, as assessed in the Morris water maze, more prominently among male than female guinea pigs [Mamczarz J et al. Neurotoxicology, 2016 Jun 10. doi: 10.1016/j.neuro.2016.06.008]. Increased GABAergic synaptic transmission in CA1 pyramidal neurons correlated with poor learning performance, which was also accompanied by a selective increase in microglia and an increase in TNF-α levels in the hippocampus of CPF-exposed guinea pigs. MRI measures also revealed reduced white matter integrity within the striatum and amygdala that correlated with spatial learning performance of the animals in the Morris water maze [Mullins et al., Neurotoxicology 48:9–20, 2015]. In conclusion, the results presented here support the test hypothesis and suggest that disruption of glia-neuron interactions is an important determinant of the developmental neurotoxicity of CPF.

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Cholinergic control of the frequency and power of the motor output during locomotor activity

Lili Anglister[a], Aharon Lev-Tov[a]

[a]*Hebrew University Medical School, Jerusalem, Israel*

Identification of neural networks and pathways involved in activation and modulation of spinal central pattern generators (CPGs) in the absence of the descending control from the brain is important for understanding of neural control of movement and for developing novel therapeutic approaches to improve the mobility of spinal cord injury patients. Previously we showed that the sacral and lumbar cholinergic system could potently modulate the locomotor CPGs in newborn rodents. Here we describe our recent studies of sacral relay neurons with lumbar projections to the locomotor CPGs and to lumbar flexor motoneurons and demonstrate that sacral and lumbar cholinergic components have the capacity to control the frequency of the locomotor CPGs and at the same time the motor output of the activated lumbar motoneurons during motor behavior. The physiological, pathophysiological and clinical implications of our findings will be discussed.

Human pluripotent stem cells for the study and treatment of neuromuscular diseases

Sandrine Baghdoyan[a], Julien Come[a], Stephane Nedelec[a], Vincent Mouilleau[a], Sylvain Roquevière[a], Lea Lesueur[a], Jacqueline Gide[a], Marc Peschanski[a], Cécile Martinat[a]

[a]*INSERM UMR U861, I-STEM, Corbeil-Essonnes, France*

Neuromuscular diseases correspond to a vast group of diseases that perturb the function of the skeletal muscles by affecting motoneurons, muscles and/or NMJs. To date, no efficient curative treatments have been identified for NMDs. Progress towards identification of new treatment has been hampered by the incomprehension of disease pathogenesis, particularly in early phases, as well as the availability of relevant screening tools. Disease-specific human pluripotent stem cells offer the unique opportunity to have access to a large spectrum of disease-specific cell models. Due to their ability of self-renewal and differentiation into various tissues affected in each pathological condition, the use of these human disease-specific pluripotent stem cells should provide new insights into pathological mechanisms.

Validating this concept, we previously demonstrated that human pluripotent stem cells and derivatives which express the causal mutation implicated in Myotonic Dystrophy type 1 (DM1), offer pertinent disease-cell models, applicable for a wide systemic analysis ranging from mechanistic studies to therapeutic screening. Thus, we identified, through a genome-wide analysis, two early developmental molecules involved both in myogenesis and in neurite formation and establishment of neuromuscular connections. These neuropathological mechanisms may bear clinical significance related to the functional alteration of neuromuscular connections associated with DM1. In parallel to these functional pathological studies, we also demonstrated the pertinence of this new disease-specific cell model to identify new therapeutic strategies. Thus, our results identified the possibility to repurpose metformin, the most

commonly prescribed drug for type 2 diabetes, for DM1, leading to a phase 2 clinical trial that is now ongoing.

We are now extending our approach to another incurable neuromuscular disease, spinal muscular atrophy (SMA). This disease, considered as the leading genetic cause of infant death, is due to mutations or deletions in the "Survival of Motor Neuron" gene, SMN1, which results in low levels of the expressed SMN protein. Despite this ubiquitous SMN expression, the pathology is characterized by degeneration of spinal motor neurons whereas other neuronal types are relatively preserved suggesting that spinal motor neuron's specific features control this differential sensitivity. Based on our recent development allowing the efficient and robust conversion of human pluripotent stem cells into affected spinal motor neuron's and non- affected cranial motor neurons, our objective is to identify the mechanisms involved in the specific degeneration of spinal motor neurons in SMA as well as the mis communication of these neurons with their muscular target.

Structural and functional analysis of acetylcholinesterase complexes with *in situ* click-chemistry inhibitors

Yves Bourne[a], Zoran Radić[b], K. Barry Sharpless[c], Palmer Taylor[b], Pascale Marchot[a]

[a]*Centre National de la Recherche Scientifique and Aix-Marseille Université, "Architecture et Fonction des Macromolécules Biologiques" Laboratory, Faculté des Sciences Campus Luminy, Marseille, France*
[b]*Dept of Pharmacology, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, CA, USA*
[c]*Dept of Chemistry, The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA, USA*

Ligand binding sites on acetylcholinesterase (AChE) comprise the active center, buried at the base of a deep and narrow gorge lined by aromatic residues, and the peripheral anionic site, located at the gorge entry. These features propelled AChE as an initial reaction vessel suited for *in situ* click-chemistry synthesis of high-affinity *syn*-TZ2PA6 and TZ2PA5 inhibitors, forming a central *syn*-triazole upon cycloaddition within the gorge from alkyne and azide reactants bound to the active site and peripheral site, respectively [1,2].

Subsequent crystallographic analyses of mouse AChE or its Tyr337Ala mutant in complexes with the *syn*- and *anti*-TZ2PA6 regioisomers demonstrated that association of the *syn* product is accompanied by distinctive side chain positions, freezing-in-frame a conformation distinct from an unbound state or an *anti* complex [3,4].

To correlate the inhibitor dimensions with reactivity and explore whether cycloaddition can occur in a crystalline template, we developed crystal-soaking procedures and solved structures of AChE complexes with the two TZ2PA5 regioisomers and their TZ2 and PA5 precursors [5]. These new structures, complementary to those of the preformed TZ2PA6 complexes, point to yet undescribed motions in the active site and at the gorge mouth' associated with TZ2 binding' and reveal coordinated side chain motions that guide the enzyme toward a state favoring *syn*-triazole formation.

We will present the rationale of the work, describe those molecular determinants that dictate, in a rate-limiting step, proper alignment of the azide and alkyne reactants to form the triazole product within the gorge, and compare our data with those from other *in situ* click chemistry studies.

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Pinnatoxins are potent antagonists of nicotinic receptors and a potential threat for global health: characterization of their neuro-developmental effects in embryos

Sophie Creuzet[a], Aurélie Couesnon[a], Vincent Dive[b], Armen Zakarian[c], Jordi Molgó[a,b], Denis Servent[b]

[a]*Institut des Neurosciences Paris-Saclay, UMR 9197, CNRS/ Université Paris-Sud, 91190, Gif sur Yvette, France*
[b]*CEA Saclay, Laboratoires de Chimie pour le vivant et de Toxinologie Moléculaire et Biotechnologies. Service d'Ingénierie Moléculaire des Protéines (DRF/IBITECS/SIMOPRO), Université Paris-Saclay, 91191, Gif sur Yvette, France*
[c]*Department of Chemistry and Biochemistry, University of California, Santa Barbara, California 93106, USA*

Pinnatoxins (PnTx) belong to an emerging class of potent fast-acting marine toxins of the cyclic imine group, and are known to exert selective potent antagonistic effects on nicotinic acetylcholine receptors (nAChRs). PnTx are potent and selective inhibitors of both muscle-type ($\alpha1_2\beta\gamma\delta$, $\alpha1_2\beta\delta e$) and neuronal ($\alpha7$, $\alpha3\beta2$ and $\alpha4\beta2$) nAChRs. The dual tropism of theses toxins towards the muscular and neuronal nAChRs leads to questions about the noxious downstream effects of single and chronic exposures on developing organisms.

The present study was designed in an attempt to determine whether PnTx had teratogenic effects on the embryo, or exerted other toxic actions when administered at early stages of embryonic development.

Due to the ease of access at any time during incubation, the chick embryo was used as a model for our functional assay. During embryonic development, we show that PnTX are prone to block the neuromuscular junction. Exposure to PnTx impinges on the embryonic motility. The detrimental effects on stereotypical flexion - longitudinal motor activity in embryos results in a drastic reduction in the spontaneous movements, and eventually leads to a global fetal ataxia.

Notably, motor alterations coincide with defects in the maturation of the musculoskeletal system. Cartilage of the appendicular skeleton appears reduced in size and density, as embryos are significantly smaller when compared to controls. *In* situ hybridization analyses of the expression of myogenic factors, required for the development and the maturation of the myogenic cells are misregulated, and confirm that PnTx-dependent blockade of the neuromuscular junction hampers the activity of these transcription factors. In addition, the transcriptomic alterations in the musculoskeletal system coincide with aberrant modifications in the

programmed cell death of the spinal motor neurons at the transverse level.

Our results reveal the detrimental effect of PnTx on the peripheral nervous system, hence preventing the maturation of the musculoskeletal system during pre-natal life. Our data shed new light on the potential risk of recurrent exposures to these environmental toxins during pregnancy for embryonic and fetal development.

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Pre-clinical SPECT imaging of butyrylcholinesterase for the diagnosis of Alzheimer's disease

Sultan Darvesh[a], Drew Debay[b], Andrew Reid[b], Ian Pottie[c], Chris Bowen[d], Earl Martin[c]

[a]*Dalhousie University, Mount Saint Vincent University and Biomedical Translation Imaging Centre, Halifax, Canada*
[b]*Dalhousie University, Halifax, Canada*
[c]*Mount Saint Vincent University, Halifax, Canada*
[d]*Biomedical Translation Imaging Centre, Halifax, Canada*

Background: Despite efforts toward molecular imaging of Alzheimer's Disease (AD) pathologies, early definitive diagnosis of AD during life remains elusive. Butyrylcholinesterase (BChE) is an enzyme with numerous functions in the normal brain, including cholinergic co-regulation. However, in AD, BChE also becomes incorporated into plaques and tangles, the neuropathological hallmarks of this disease. Imaging of BChE represents a potential diagnostic test for AD as elevated expression of this enzyme is associated with pathology. A radioligand that enters the brain and engages BChE as a target represents a potential means to identify AD pathology. In particular, analysis of radioligand retention in the cerebral cortex, which normally has low levels of BChE but high levels in AD, may provide the specificity and sensitivity for AD diagnosis during life.

Methods: Radioligand ^{123}I-1-methylpiperidin-4-yl 4-iodobenzoate was synthesized by rapidly replacing its tin precursor moiety with ^{123}Iodine radical. This agent was injected into Alzheimer mice (5XFAD) and age-matched wild-type (WT) controls for comparison. Dynamic Single Photon Emission Computed Tomography (SPECT) images were obtained in conjunction with computed tomography (CT) and magnetic resonance imaging (MRI) analysis. SPECT radioactivity source maps were generated and co-registered with CT/MRI, from which radioligand retention over time was assessed.

Results: ^{123}I-1-methylpiperidin-4-yl 4-iodobenzoate was produced in up to 70% radiochemical yield. SPECT imaging revealed initial brain uptake of the radiotracer indicating blood-brain barrier penetrance, followed by washout up to 20 min post-injection, at which point a relatively stable distribution volume was reached. Importantly, whole brain uptake demonstrated significantly higher cerebral retention in the 5XFAD brain compared to wild-type (WT) controls. Cortical retention index measurement, that expresses tracer retention in the cortex normalized to whole brain radiotracer uptake, was found to be greater (22–26%) in 5XFAD brains compared to WT. These results suggest target engagement of the tracer with BChE and increased cortical retention above uptake observed in cortical regions of WT mouse brains.

Conclusions: Preliminary findings indicate that the radioligand ^{123}I-1-methylpiperidin-4-yl 4-iodobenzoate can be rapidly synthesized in acceptable radiochemical yields and provides *in vivo* evidence of crossing the blood-brain barrier with BChE target engagement within the brain. Regional SPECT imaging and corroboration with paired BChE brain histopathology suggests positive predictive value of this radiotracer in accurately detecting BChE-associated AD pathology for AD diagnosis.

Allosteric mechanisms in ionotropic and metabotropic acetylcholine receptors

Stuart Edelstein[a]

[a]*Ecole Normale Supérieure, Paris, France*

Allosteric interactions of membrane receptors can be represented by transitions between pre-existing conformational states as formulated in the MWC model, which distinguishes between the fraction of molecules in the active conformation (the state function) versus the number of agonist molecules bound (the binding function). Since receptor activity reflects the state function, measurements of cooperativity cannot be interpreted as simply as for agonist binding using the Hill coefficient.[1]

The transitions between states entail a number of consequences for both ionotropic and metabotropic receptors. For oligomeric ionotropic receptors such as the nAChR, the transitions between open and closed states vary with the number of sites occupied, as quantitatively described by linear free-energy relations with a single critical transition state parameter.[2] The limits established by these relations suggest re-interpretation of some models implying a novel "flip" state.[3] The distinction between weak agonists and competitive antagonists can also require clarification, since their characterization can change depending on the value of L, the allosteric constant defining the stability between active and inactive conformations.[4]

For metabotropic receptors, biased agonism can occur, whereby different agonists of a specific GPCR favor responses either by G-proteins or by beta-arrestins. Biased agonism has recently been evaluated with a global allosteric model based on alternative pre-existing conformations that bind more strongly, but non-exclusively, either G-proteins or beta-arrestins.[5] Due to reciprocal effects among all interacting molecules, G-proteins and beta-arrestins are in steric competition, and the strength of their interactions potentiates the apparent affinity for the corresponding agonist, effectively equating these two transfer proteins to allosteric modulators. The balance between response alternatives can also be influenced by the physiological concentrations of either G-proteins or beta-arrestins, as well as by phosphorylation or interactions with positive or negative allosteric modulators.

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Rattlesnake PLA2 as a novel NAM of the prokaryotic pentameric proton-gated ion channel GLIC and PAM of CFTR

Grazyna Faure[a], Dorota Porowinska[a], Frederick Saul[a], Maciej Ostrowski[a], Aleksander Edelman[b], Pierre-Jean Corringer[a]

[a]*Institut Pasteur, Paris, France*
[b]*INSERM U1151, Paris, France*

The snake venom neurotoxic phospholipases A_2 ($sPLA_2$) are multifunctional proteins which interact with various protein targets, regulating their function essential to life processes including neuronal or neuromuscular chemoelectric signal transduction. These PLA_2 exhibit high pharmacological potential conferred by regions of their structure not involved in catalysis but directly implicated in protein-protein interactions with PLA_2-receptors or PLA_2-channels. The determination of PLA_2-receptor binding sites represents a challenging objective in receptor-channel biochemistry and pharmacology.

To investigate the mechanism of interaction of neurotoxic PLA_2 with their PLA_2-targets at the molecular level, we used as a model crotoxin, a heterodimeric $sPLA_2$ from rattlesnake venom. Crotoxin consists of the noncovalent association of a basic and weakly toxic $sPLA_2$ subunit (CB) with a small acidic, nonenzymatic, and nontoxic subunit (CA). As potential PLA_2-protein targets we investigated the proton gated ion channel GLIC, a bacterial homolog of the pentameric ligand-gated ion channel family and Cystic Fibrosis Transmembrane Regulator CFTR, a cyclic AMP-regulated chloride channel.

The SPR technology was critical to demonstrate that CB forms specific complexes with GLIC [1] and CFTR[2]. We investigated the functional effect of CB on proton-gated ion channel activity and showed that the CB interacting with full length purified and solubilized pentameric GLIC is a negative allosteric modulator NAM of GLIC, since it inhibits a proton-gated ion channel activity [1].

We also provide evidence that CB behaves as a positive allosteric modulator PAM of CFTR [2]. The correcting activities of CB on F508del-CFTR (the most frequent mutation responsible for cystic fibrosis) were studied at the molecular and cellular level and we showed that CB acts as a corrector by addressing F508del-CFTR to the plasma membrane. We characterized the binding interface between the two proteins. This study provides novel perspectives for the development of new drugs against cystic fibrosis.

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Cutaneous non-neuronal cholinergic system

Sergei Grando[a]

[a]*University of California, Irvine, USA*

Significant progress has been achieved in understanding structure and function of the cutaneous non-neuronal cholinergic system and its role in the pathogenesis and treatment of skin diseases. It is now well-established that acetylcholine (ACh) is an ubiquitous molecule that plays important roles in various aspects of skin cell biology and homeostasis. ACh has been acting as a signaling molecule in non-neuronal cells for about 3 billion years, whereas its neuronal function spans only ~1/2 billion years. The highest concentration of non-neuronal ACh is found in human skin. During the two decades after discovery of the cutaneous non-neuronal cholinergic network, substantial progress was made in defining the role for ACh in the physiological control of skin homeostasis, including epithelial cell proliferation, migration, adhesion, differentiation and apoptosis, pigmentation, dermal remodeling and inflammation The cutaneous non-neuronal ACh axis is an example of the more general neuroendocrine-like mechanisms that mediate peripheral responses to environmental factors and of evolutionary conservation of neuroendocrine systems in the periphery. The environmental, neural, endocrine and paracrine stimuli affect cutaneous ACh metabolism and signaling. On the other hand, skin cells can use ACh as a common cyto/neurotransmitter to send signals to the CNS. The cholinergic control can be mediated by synergistic, additive, and reciprocal effects triggered by two different ACh receptor classes expressed on skin cells. The ionic events are generated by ACh-opening of nicotinic receptor channels and the metabolic events—due to ACh-binding to G protein-coupled muscarinic receptors. Simultaneous stimulation of both receptor classes may be required to synchronize and balance ionic and metabolic events in a single cell, and a crosstalk between these receptors may provide for fine-tuning of the signals emanating from the CNS and endocrine glands, and environmental stimuli. The clinical and laboratory investigations produced first evidence that the cutaneous cholinergic system may be involved in the pathophysiology of allergic dermatoses, psoriasis, palmoplantar pustulosis, cutaneous lupus erythematosus, necrobiosis lipoidica diabeticorum, hair disorders (grayness and baldness), acne (vulgaris, inversa, rosacea), skin ulcers and immunobullous diseases (pemphigus, mucous membrane pemphigoid, dermatitis herpetiformis). There are also anecdotal reports about using cholinergic agents to treat Buerger's disease, Behçet's disease, recurrent aphthous stomatitis, pyoderma gangrenosum, erythema nodosum, Degos' disease, Kimura's disease, eosinophilic pustular folliculitis, oral lichen planus, pemphigus vulgaris, and psoriasis. Learning the cholinergic physiology and pathophysiology of cutaneous cells, given availability of ACh receptor-selective drugs, should offer novel specific and effective treatment regimens.

Molecular determinants of metabotropic G protein signaling by α7 nAChRs in neural cells

Nadine Kabbani[a]

[a]*George Mason University, Fairfax, USA*

Neuronal α7 nicotinic acetylcholine receptors (nAChRs) are expressed in synaptic terminals and contribute to neurotransmitter release, synaptic growth, and structural plasticity. A subset of α7 nAChRs is also expressed postsynatpically and regulates the formation of dendritic spines underlying learning and memory. Because α7 nAChRs exhibit a unique kinetic profile consisting of fast activation, rapid inactivation, and a transition into a long-lasting desensitized state, I will discuss the implications of our findings that show that α7 nAChRs can participate in metabotropic (non-conventional) channel signaling in neural cells. The hypothesis is presented that α7 nAChRs operate in two, non-exclusive, functional states that can be divided into ionotropic and metabotropic signaling. Based on proteomic, cell imaging, and mutagenesis experiments we show the existence and function of a new G protein binding cluster in the intracellular loop of α7 nAChR. G protein interactions mediate metabotropic calcium signaling by the α7 nAChR including calcium-induced calcium release (CICR) and PLC/IP_3 receptor caclium release from local ER stores. Metabotropic signaling via the a7

nAChR is found essential for rapid cytoskeletal regulation at sites of synaptic growth and may contribute to cholinergic mechanisms of neuronal plasticity and repair.

Peptide neurotoxins as tools for the study of acetylcholine receptors and as templates for new drugs

Igor Kasheverov[a], Denis Kudryavtsev[a], Igor Ivanov[a], Maxim Zhmak[a], Anton Chugunov[a], Roman Efremov[a], Yuri Utkin[a], Victor Tsetlin[a]

[a]*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russian Federation*

Among various ligands of nicotinic acetylcholine receptors (nAChR), (poly)peptide neurotoxins play not the least role. The best known are polypeptides with the three-finger fold (α-neurotoxins from the snake venoms and proteins of the Ly6 family) and shorter α-conotoxins from marine *Conus* molluscs. However, the list of peptides affecting nAChRs is constantly updated, i.e. with the recently discovered linear peptide azemiopsin from the Fea's viper venom. α-Conotoxins, due to their large number, small size, rigid structure and variety of their affinities for different nAChR subtypes, provide a convenient basis for the targeted design of highly selective ligands for studying subtle structural differences in the organization of orthosteric binding sites of distinct nAChRs. So, on the basis of the crystal structure of acetylcholine-binding protein (AChBP) bound to α-conotoxin GIC, we suggested which GIC amino acid residues might determine its high affinity for the α3β2 nAChR. The study of activity of a series of synthetic α-conotoxin GIC analogues on α3β2 nAChR confirmed these suggestions. Design of α-conotoxin PnIA analogues, aimed at creating potent and selective α7 nAChR ligands, was carried out using a new calculation PST (protein surface topography) method. Three peptides containing the [A9R]-mutation showed high affinity to α7 nAChR; a radioactive derivative of one of them bound to this receptor with Kd of 1.3 nM making it a prospective marker for the α7 subtype. Some other conotoxins (for example, αO-conotoxin GeXIV interacting with the α9α10 nAChR) or recombinant Slurp1 (one of the Ly6 family proteins, for which the interaction with the α7 nAChR allosteric site was recently shown), could be a useful research tool for the study of allosteric binding sites in different nAChR subtypes. Azemiopsin, the inhibitor of muscle-type nAChR, showed good prospects for practical application; it is now at the stage of preclinical trials as a local myorelaxant, and its shorter modified fragment has been successfully tested as an agent to reduce facial wrinkles.

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Sympathetic coinnervation of NMJs and its importance for synaptic homeostasis

Muzamil Khan[a], Danilo Lustrino[b], Willian Silveira[b], Wild Franziska[a], Benoit Evelyne[c], Jordi Molgó[c], Veit Witzemann[d], Hanns Lochmuller[e], Isis Kettelhut[b], Luiz Navegantes[b], Tullio Pozzan[f], Ruediger Rudolf[g]

[a]*Mannheim University of Applied Sciences, Mannheim, Germany*
[b]*University of Sao Paolo, Ribeirao Preto, Brazil*
[c]*CEA - iBiTec-S, Gif-sur-Yvette, France*
[d]*Max-Planck Institut fuer medizinische Forschung, Heidelberg, Germany*
[e]*University of Newcastle, Newcastle upon Tyne, UK*
[f]*University of Padua, Padua, Italy*
[g]*Interdisciplinary Center for Neurosciences, University of Heidelberg and Mannheim University of Applied Sciences, Mannheim, Germany*

The vertebrate neuromuscular junction (NMJ) has been considered as a purely cholinergic synapse. Sympathetic agonists like salbutamol or ephedrine have recently shown high efficiency in treating several forms of congenital myasthenic syndromes (for reviews see e.g. Cruz et al., 2014 and Engel et al., 2015), but the underlying mechanism has remained elusive. We have found (Khan et al., 2016) that sympathetic neurons regularly approach NMJs in different mouse skeletal muscles and often form a network of connections with blood vessels, motor neurons, muscle fibers and NMJs. Direct stimulation of sympathetic neurons in combination with simultaneous *in vivo*-imaging of muscles transfected with molecular biosensors revealed activation of postsynaptic beta2-adrenergic receptors and cAMP production. Furthermore, sympathetic neuron stimulation induced rapid nuclear import of the transcriptional coactivator PGC1alpha. Treatment with the sympathicomimetic clenbuterol corrected electrophysiological and morphological deficits of NMJs upon local chemical sympathectomy and in myasthenic mice. This study identifies the NMJ as a target of direct sympathetic innervation, which is crucial for synapse maintenance and function.

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Cholinergic activation during lithium-pilocarpine-induced status epilepticus

Jochen Klein[a], Imran Imran[a], Markus Hillert[a], Anna Thinnes[a]

[a]*Goethe University, Frankfurt, Germany*

Status epilepticus (SE) is a life-threatening condition which requires intensive therapeutic management. The lithium-pilocarpine model is an epilepsy model in rats which reliably induces SE with a low mortality rate. In our studies [1,2], we measured acetylcholine (ACh) levels in the extracellular fluid by microdialysis in rat hippocampus before, during and after SE, in the absence of anesthesia. In addition, we measured glucose, lactate and pyruvate to monitor energy metabolism and 8-OH-isoprostanes to evaluate oxidative stress. After 90 min of SE, seizures were terminated, and in some cases, blood was withdrawn and/or rats were decapitated for

the isolation of mitochondria from brain. We found that administration of pilocarpine (30 mg/kg s.c.) to rats which were pretreated with lithium chloride (127 mg/kg) caused a massive, six-fold increase of hippocampal ACh release concomitant with the development of tonic seizures. When seizures were terminated with diazepam (10 mg/kg) or ketamine (75 mg/kg), ACh levels returned to normal. The same observation was made when valproic acid, pregabalin, or levetiracetam were used as anticonvulsants. Local infusions of tetrodotoxin (TTX, 1 μM) or hemicholinium (HC-3, 10 μM) strongly reduced ACh release. Increases of ACh were also seen when striatal areas were sampled, or when kainate was used to induce SE. Among the metabolites, glucose showed only small changes during SE, whereas lactate increased up to 4–6 fold. 8-OH-isoprostanes increased more than ten-fold, and glycerol, an indicator of membrane damage, increased up to eightfold, during 90 min of status. Surprisingly, mitochondrial respiration was found to be unchanged after 90 min of SE. Taken together, our results demonstrate that seizure development in the SE model is accompanied by massive increases of extracellular ACh, lactate and glycerol, whereas glucose levels and mitochondrial integrity remain largely unaltered. Blockade of cholinergic receptors may be a therapeutic option in therapy-resistant seizures.

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Efficient detoxification of soman, tabun, and VX by oxime assisted reactivation of acetylcholinesterase mutants

Zrinka Kovarik[a], Nikolina Macek Hrvat[a], Suzana Zunec[a], Maja Katalinic[a], Palmer Taylor[b], Zoran Radić[b]

[a]*Institute for Medical Research and Occupational Health, Zagreb, Croatia*
[b]*Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, USA*

Newly considered strategies in medical protection against nerve agents focus on the use of exogenously administered butyrylcholinesterase (BChE). The overall idea is to supplement endogenous BChE in combination with a specific oxime, to scavenge an organophosphate (OP) before it can reach and inhibit native acetylcholinesterase (AChE) in target tissues, thus helping organism detoxification from the excess OP. However, oxime antidotes commonly used to reactivate OP inhibited AChE are ineffective against soman and tabun, while the efficacy of the recommended nerve agent bioscavenger BChE is limited by strictly stoichiometric scavenging. Our previous research showed that AChE mutagenesis can enable aldoximes to substantially accelerate the reactivation of OP-enzyme conjugates, while dramatically slowing down rates of OP-conjugate dealkylation (aging). Herein we demonstrate through a combination of *in silico*, *in vitro*, *ex vivo*, and *in vivo* results, a feasible approach to the development of oxime assisted catalytic bioscavengers of soman, tabun and VX based on human AChE mutants modified at the choline binding site (Y337A) and an aging resistant (Y337A/F338A mutant) in combination with its efficient reactivator. Ultimately, the oxime assisted catalytic scavenging of the nerve agents in mice improved therapeutic outcomes preventing lethality, and resulted in delayed onset of toxicity symptoms.

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Human secreted Ly-6/uPAR related proteins: insights into specificity of interaction with acetylcholine receptors. Part 1: SLURP-1 exclusively binds to human α7 nicotinic acetylcholine receptors on the outside of the orthosteric binding site

Dmitrii Kulbatskii[a], Mikhail Shulepko[a], Zakhar Shenkarev[b], Maxim Bychkov[b], Denis Kudryavtsev[b], Igor Kasheverov[b], Morten Thomsen[c], Viktor Tsetlin[b], Mikhail Kirpichnikov[a], Dmitry Dolgikh[a], Ekaterina Lyukmanova[a]

[a]*Lomonosov Moscow State University, Moscow, Russian Federation*
[b]*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow, Russian Federation*
[c]*Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen, Denmark*

SLURP-1 is a secreted toxin-like Ly-6/uPAR protein found in epithelium, sensory neurons and immune cells. Point mutations in the slurp-1 gene cause the autosomal inflammation skin disease Mal de Meleda. SLURP-1 is considered an autocrine/paracrine hormone that regulates growth and differentiation of keratinocytes and controls inflammation and malignant cell transformation. The majority of previous studies of SLURP-1 have been made using fusion constructs containing, in addition to the native protein, extra polypeptide sequences.

Here we describe the activity and pharmacological profile of a recombinant analogue of human SLURP-1 (rSLURP-1) differing from the native protein only by one additional *N*-terminal Met residue. rSLURP-1 significantly inhibited proliferation (up to ~ 40%, EC_{50} ~ 4 nM) of human oral keratinocytes (Het-1A cells). Application of mecamylamine and atropine, - non-selective inhibitors of nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors, respectively, and anti-α7-nAChRs antibodies revealed α7 type nAChRs as an rSLURP-1 target in keratinocytes. Using affinity purification from human cortical extracts, we confirmed that rSLURP-1 binds selectively to the α7-nAChRs. Exposure of *Xenopus* oocytes expressing α7-nAChRs to rSLURP-1 caused a significant non-competitive inhibition of the response to acetylcholine (up to ~ 70%, IC_{50} ~ 1 μM). It was shown that rSLURP-1 binds to α7-nAChRs overexpressed in GH_4C_1 cells, but does not compete with ^{125}I-α-bungarotoxin for binding to the receptor. These findings imply an allosteric antagonist-like mode of SLURP-1 interaction with α7-nAChRs outside the classical ligand-binding site. Contrary to rSLURP-1, other inhibitors of α7-nAChRs (mecamylamine, α-bungarotoxin and Lynx1) did not suppress the proliferation of keratinocytes. Moreover, the coapplication of α-bungarotoxin with rSLURP-1 did not influence antiproliferative activity of the latter. This supports the hypothesis that the antiproliferative activity of SLURP-1 is related to the 'metabotropic' signaling pathway through α7-nAChR, which activates intracellular signaling cascades without opening the receptor channel.

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High fidelity: cholinergic mechanisms and prefrontal attention circuits

Evelyn Lambe[a], Eliane Proulx[a], Michael Tian[a], Derya Sargin[a]

[a]*University of Toronto, Toronto, Canada*

The cholinergic modulation of the prefrontal cortex is essential for working memory and attention. In particular, acetylcholine exerts robust excitation of deep prefrontal pyramidal neurons to achieve optimal performance on challenging cognitive tasks. From an integrated approach of electrophysiology, multiphoton imaging, and optogenetics, we demonstrate that nicotinic and muscarinic acetylcholine receptors work together to sculpt the excitation of these prefrontal output neurons in a calcium-dependent manner. We extend this examination to characterize and explore disruptions of this signalling by other neurotransmitters and its vulnerability in models of brain disorders. Taken together, our results point to a trade-off between excitation and spiking fidelity in pyramidal cells of the major corticothalamic layer in prefrontal cortex. This compromise has important ramifications for the regulation of excitability in prefrontal executive circuits.

The heteromeric nicotinic receptors of the Renshaw cell are likely (3 alpha)(2 beta) LS receptors

Boris Lamotte d'Incamps[a], Philippe Ascher[b]

[a]*CNRS UMR 8119, Université Paris Descartes, Paris, France*
[b]*CNRS UMR 8118, Université Paris Descartes, Paris, France*

At the motoneuron-Renshaw cell synapse in young mice (P5-P10), the component of the synaptic current mediated by heteromeric nicotinic receptors (nAChRs) has a bi-exponential decay with a large fast component (time constant around 10 ms) and a small slow component (time constant around 100 ms). Because the Renshaw cell nAChRs are known to contain multiple α and ß subunits, we had initially considered the possibility that the two components of the decay may involve different subunits, but found that the decay of the EPSCs remained bi-exponential in a series of knock-outs in which a given subunit had been eliminated (Lamotte d'Incamps and Ascher, 2013). We now explored the possibility that the two time constants of the decay may arise from the dual affinity of heteromeric "low sensitivity" (LS) nAChRs with a 3α2ß stoichiometry. The concentration response curve of ACh on LS nAChRs has a small foot (high affinity) and a main part (low affinity) (Harpsoe et al. 2011). Our hypothesis was that the fast decay of the EPSC may result from the fast dissociation of ACh from the low affinity sites, while the slow decay would be due to slow dissociation from the high affinity sites.

To test this hypothesis, we used NS9283, a compound known to act selectively on the 3α-2ß stoichiometric variants of α*ß* receptors. NS9283 has been shown to potentiate selectively LS nAChRs receptors (Timmermann et al. 2012) by slowing deactivation (Grupe et al. 2013) and transforming the concentration-response curve to ACh from a two-component curve into a single high affinity curve (Wang et al., 2015). NS9283 (10 μM) induced a marked prolongation of the Renshaw cell EPSCs, reducing the fast component and prolonging the slow one. NS9283 also prolonged the mEPSCs. This effect is similar to the effect of low doses of neostigmine (0.1 μM, Lamotte d'Incamps et al. 2012) and led us to test the inhibitory effect of NS9283 on acetylcholinesterase. The enzymatic activity of purified acetylcholinesterase was tested *in vitro* in presence of NS9283 (Krejci, Dingova and Zorbaz, unpublished observations). NS9283 proved to be a non-competitive antagonist of acetylcholinesterase with a K_i of 7.2 μM.

In conclusion, NS9283 cannot support nor negate our hypothesis on the stoichiometry of Renshaw cell nAChRs. More generally, the anticholinesterase activity of NS9283 severely weakens the possible use of this compound in characterizing LS nAChRs in physiological conditions.

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Acetylcholinesterase regulates skeletogenesis both dependently and independently of its substrate

Paul Layer[a], Janine Spieker[b], Astrid Vogel-Höpker[a]

[a]*Technische Universität Darmstadt, Darmstadt, Germany*
[b]*Universitätsklinikum Heidelberg, Heidelberg, Germany*

Acetylcholinesterase (AChE) can function conventionally at synapses, but also as a component of *non-neuronal cholinergic systems*. However, it remains open when and how this coopting protein acts as an ACh-degrading enzyme, or otherwise, e.g. via some structural mechanism. Focusing on the developing vertebrate limb as an excellent study model, we found profound changes of endochondral ossification in prenatal homozygous AChE-/BChE-double knockout mice, affecting skeletogenic genes, e.g. *Col-II*, *Col-X*, *Ihh*, *Mmp-13 and Alp*. In chicken limb buds, cholinesterases and choline acetyltransferase (ChAT) are both expressed early, but do not overlap completely. By *in vitro* analyses, we examined cholinergic effects in micromass cultures of chick limb mesenchyme. In this system, in which neurons and muscles are absent and ACh availability was largely limited, cartilage-like nodules treated with the AChE inhibitor BW284c51, or with nicotine similarly decreased proteoglycan content and accelerated mineralization. Using a more natural *in vivo* approach, we implanted beads unilaterally into chicken limb buds *in ovo*. Here, both ACh- and ChAT-soaked beads accelerated bone formation, while inhibition of AChE by BW284c51 or MAB304 decelerated mineralization. Notably, BW284c51 binds bi-functionally to the active and a peripheral site of AChE, while the monoclonal antibody MAB304 binds only to a peripheral site. We conclude that skeletogenesis in mice and birds is strongly supported by an ACh-dependent cholinergic system (as shown *in vitro*), but also is affected by a non-enzymatic aspect of the AChE protein (as shown *in ovo*). These findings could have far-reaching medical and environmental implications.

Effects of tobacco smoke constituents on nicotine reinforcement and dependence

Frances Leslie[a], James Belluzzi[a], Candice Gellner[a], Daisy Reynaga[a]

[a]*Department of Pharmacology, University of California, Irvine, USA*

Tobacco dependence is extremely difficult to treat and the majority of those who try to quit will relapse within a year. Many smoking cessation therapies also fail at the clinical phase of drug development. One explanation for this may be that the majority of preclinical tests use nicotine alone, ignoring ~8,000 constituents also found in tobacco smoke. Furthermore, although the majority of smokers initiate during adolescence, most preclinical studies use

adult rodents. To investigate the role of non-nicotine constituents in tobacco dependence, we have studied the effects of individual constituents and of combined constituents in aqueous cigarette smoke extract (CSE) on nicotine self-administration and withdrawal in male rats. We have found that tobacco smoke constituents influence the behavioral effects of nicotine in an age-dependent manner. In adults, tobacco smoke constituents in CSE enhance self-administration, drug- and stress-induced reinstatement and somatic withdrawal signs as compared to nicotine alone. Whereas nAChRs appear to mediate these effects of CSE, pharmacological analysis of AT-1001, a functional antagonist of $\alpha3\beta4$ nAChRs, suggests a differential role for this receptor type in the behavioral effects of nicotine as compared to CSE. In prior studies (Belluzzi et al., Neuropsychopharmacology, 30, 705–12, 2005), we have shown the tobacco smoke constituent, acetaldehyde, to have a synergistic interaction with nicotine on reinforcement in adolescent males. In contrast, our current findings suggest that the non-nicotine constituents in CSE do not influence adolescent responding for drug, extinction or reinstatement of drug-seeking behavior. These differences in the effects of CSE in adolescents and adults suggest that both age and smoke constituents should be considered when developing preclinical models of tobacco dependence.

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Hupresin, a superior affinity gel for purifying human butyrylcholinesterase

Oksana Lockridge[a], Lawrence Schopfer[b], Patrick Masson[a], Emilie David[c], Steven Hinrichs[b]

[a]*University of Nebraska Medical Center, Omaha, USA*
[b]*University of Nebraska Medical Center, Omaha, NE, USA*
[c]*CHEMFORASE, Mont-Saint-Aignan, France*

Human butyrylcholinesterase (BChE) is a bioscavenger of nerve agents. Animals pretreated with BChE are protected from lethal doses of nerve agents. Tetrameric BChE purified from human plasma is stable at 4?C for years and is the gold standard for protection studies in terms of long residence time in the circulation and ability to scavenge a variety of organophosphorus toxicants. Plasma-derived BChE is purified by anion exchange and affinity chromatography. The procainamide affinity gel introduced by Lockridge and La Du in 1978 has been used successfully for lab and industrial scale purification for nearly 40 years. Brazzolotto et al. (2012) introduced a new affinity gel, hupresin, for purification of recombinant human BChE. We have purified gram quantities of plasma-derived human BChE extracted from 80 kg of Cohn fraction IV-4 by passing the filtered extract through a 30 Liter Q-ceramic anion exchange column at pH 4.5 followed by affinity chromatography on hupresin at pH 8. The optimal loading of BChE was 0.3 to 0.6 mg BChE per mL hupresin, using 1.5 to 3 L hupresin for 1000 mg BChE. At this loading, contaminating proteins washed off hupresin with 0.3 M NaCl pH 8 without loss of BChE. Recycled hupresin stripped with 2 M NaCl or with 0.1 M NaOH has been reused 13 times with no loss of performance. Hupresin is available from the CHEMFORASE company by contacting Emilie David. Hupresin separates AChE from BChE because AChE binds to hupresin, but AChE does not elute with procainamide. In contrast BChE elutes from hupresin with 0.1 M procainamide and is pure by SDS gel electrophoresis. AChE can be eluted with 3% Tween-20, but AChE activity is unstable in Tween-20. In conclusion, hupresin works better than the procainamide affinity gel for purification of human BChE.

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Slow-binding inhibition of acetylcholinesterase by an alkylammonium derivative of 6-methyluracil: molecular modeling, X-ray crystallography and kinetic study of the inhibition mechanism

Sofya Lushchekina[a], Ekaterina Kots[a], Florian Nachon[b], Konstantin Petrov[c], Alexandra Kharlamova[c], Evgeny Nikolsky[d], Patrick Masson[d]

[a]*Emanuel Institute of Biochemical Physics of Russian Academy of Sciences, Moscow, Russian Federation*
[b]*Institut de Recherche Biomédicale des Armées, Brétigny-sur-Orge, France*
[c]*Arbuzov Institute of Organic and Physical Chemistry of Russian Academy of Sciences, Kazan, Russian Federation*
[d]*Kazan Federal University, Kazan, Russian Federation*

Slow-binding inhibitors present considerable advantages over classical reversible inhibitors in pharmacology [1]. Inhibition of human AChE and BChE by an alkylammonium derivative of 6-methyluracil, C-547, a potential drug for the treatment of *myasthenia gravis*' was studied. Progressive AChE inhibition was observed. Molecular docking, steered molecular dynamics (SMD) and free energy profile calculations of binding/dissociation processes of C-547 showed that the inhibitor rapidly binds to the AChE PAS. This is followed by a slow step for crossing the AChE bottleneck. The slow step is reflected in high force peaks for SMD simulations and a 4 kcal/mol energy barrier in the free energy profile. Then, tight complex below the AChE gorge bottleneck is established. This complex was observed by X-ray crystallography (3.13 Å resolution) [2]. For the dissociation process, passing through the bottleneck was more hindered than for binding. This mechanism corresponds to slow-binding inhibition of type B, i.e. after formation of the initial enzyme-inhibitor complex (K_i = 140 pM), an induced-fit step allowed establishment of the final complex (K_i* = 22 pM). A slow k_{off} = 0.05 min^{-1} determines a long residence time on target, τ = 20 min, much longer than for other reversible inhibitors used in the treatment of *myasthenia gravis*. This makes C-547 a promising drug for the treatment of this disease.

On the other hand, due to the absence of a bottleneck in pre BChE gorge, SMD simulations of C-547 binding and dissociation processes did not display a slow step. Kinetic studies showed that inhibition of human BChE is a reversible fast-binding process of mixed-type (K_i = 1.77 μM; K_i = 3.17 μM). The non-charged analog of C-547, C-35, was not a slow-binding inhibitor of AChE. It did not cross the bottleneck because it is not sensitive to the electrostatic driving force that pulls charged ligands to the bottom of the gorge. These results demonstrated that slow-binding inhibition of AChE by C-547 is determined both by the existence of a bottleneck in the enzyme active gorge and by the cationic nature of this ligand.

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Bioscavenger is effective as a delayed therapeutic intervention following percutaneous VX poisoning in the guinea pig

Thomas M. Mann[a], Matthew E. Price[a], John E. H. Tattersall[a], A. Christopher Green[a], Helen Rice[a]

[a]*Toxicology, Trauma & Medicine Group, Defence Science and Technology Laboratory, Porton Down, Salisbury, UK*

The treatment of percutaneous nerve agent is challenging due to the duration of poisoning outlasting the effectiveness of first aid medical countermeasures (MedCMs). Previous animal studies have shown that administration of bioscavengers in conjunction with MedCMs on signs of poisoning is protective by reducing the toxic load of nerve agent. This study investigated the effectiveness of delayed bioscavenger administration.

Cannulated guinea pigs were dosed with 2.45 mg/kg VX percutaneously (4 × 24-h LD_{50}). On unequivocal signs of nerve agent poisoning all animals were administered a bolus of MedCM compounds (atropine: 17.4 mg/kg, avizafone: 3.14 mg/kg, HI-6: 27.9 mg/kg) intramuscularly followed by a second 2 h later intravenously (i.v.). Immediately following this, the bioscavenger group (n = 6) was dosed i.v. with rHuBChE (100 mg/kg) and the control group (n = 6) received an equivalent volume of saline.

All but one of the animals in the bioscavenger group survived to the end of the study (6 days) with all the control animals dying before 12 h. Data will be presented comparing survival, clinical signs, body temperature and post-mortem findings.

These data suggest that intravenous bioscavengers may have utility for treating nerve agent poisoning if their administration is delayed and they are given as part of the clinical management of poisoning. The current guinea-pig model should be used to explore further questions relevant to the integration and optimisation of treatment strategies into the nerve agent casualty chain.

Diversity in the binding interactions of marine phycotoxins to AChBP, the soluble nAChR surrogate

Pascale Marchot[a], Gerlind Sulzenbacher[a], Zoran Radić[b], Romulo Araoz[c], Morgane Reynaud[d], Evelyne Benoit[c], Armen Zakarian[e], Denis Servent[d], Jordi Molgó[c], Palmer Taylor[b], Yves Bourne[a]

[a]*Architecture et Fonction des Macromolécules Biologiques, Centre National de la Recherche Scientifique/Aix-Marseille Université, Marseille, France*

[b]*Department of Pharmacology, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla CA, USA*

[c]*Institut des Neurosciences Paris-Saclay, CNRS, CEA, iBiTecS, Service d'Ingénierie Moléculaire des Protéines, Gif sur Yvette, France*

[d]*CEA, iBiTecS, Service d'Ingénierie Moléculaire des Protéines, Gif sur Yvette, France*

[e]*Department of Chemistry and Biochemistry, University of California Santa Barbara, Santa Barbara CA, USA*

The pentameric acetylcholine-binding protein (AChBP) from marine and freshwater snails is a soluble surrogate of the extracellular, ligand-binding domain of the nicotinic acetylcholine receptor. Nicotinic agonists and competitive antagonists bind primarily within a nest of aromatic side chains contributed by loops C and F on opposing faces of each subunit interface. The macrocyclic imine phycotoxins belong to an emerging class of chemical agents associated with marine algal blooms and shellfish toxicity. Binding and voltage-clamp recordings on muscle-type and neuronal nAChRs revealed subnanomolar affinities governed by slow dissociation, potent antagonism, and varying levels of subtype selectivities [1]. Common AChBP determinants imbedded within the aromatic nest are involved in conferring high affinity binding to the various phycotoxins. In contrast, distinctive determinants brought about by loop F and located within the nest, or extending outside the nest towards apical, radial or "membrane" subsites of the interface, dictate either broad or narrow selectivity of the toxins for muscle-type or neuronal nAChR subtypes [2,3]. A comprehensive overview of binding, functional, and structural data will be presented. These data offer unique means for detecting spiroimine toxins in shellfish and identify distinctive ligands, functional determinants and binding regions for the design of new drugs able to target nAChR subtypes with high affinity.

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Learning performances and vulnerability to amyloid toxicity in acetylcholinesterase and butyrylcholinesterase knockout mice

Tangui Maurice[a], Manon Strehaiano[a], Julie Espallergues[b], Christelle Bertrand[c], Arnaud Chatonnet[c]

[a]Inserm U1198, University of Montpellier, Montpellier, France
[b]Inserm U710, University of Montpellier, Montpellier, France
[c]INRA UMR 866, University of Montpellier, Montpellier, France

Cholinergic neurons in the basal forebrain play a crucial role in plasticity, memory and vulnerability to neurodegenerative pathologies, such as Alzheimer's disease (AD). Like acetylcholinesterase (AChE), butyrylcholinesterase (BChE) hydrolyses the neurotransmitter acetylcholine ACh, contributing to choline generation and recycling. We here characterized the behavioral phenotypes of heterozygous AChE knockout (hetAChE KO) mice and homozygous BChE KO mice, focusing on memory functions and vulnerability to amyloid toxicity. First, AChE activity was significantly decreased in the hippocampus and cortex of male and female hetAChE KO mice, but BChE activity was preserved. hetAChE KO mice failed to show any difference in terms of locomotion, exploration and anxiety parameters in the open-field test. Animals were then tested for place learning in the water-maze using a 'sustained acquisition' protocol (three swim trials per day) or a 'mild acquisition' protocol (two swim trials per day) to locate an invisible platform in fixed position (reference memory procedure). Then, during 3 days, they were trained to locate the platform in a variable position (working memory procedure). Learning profiles and probe test performances were similar for hetAChE KO and wildtype mice. When mice were administered intracerebroventricularly (ICV) an oligomeric amyloid β_{25-35} peptide, generating AD-like toxicity, they failed to show learning deficits. The peptide also failed to generate oxidative stress in forebrain structures. Second, male and female BChE KO mice tested for place learning in the water-maze showed increased acquisition slopes and presence in the training quadrant during the probe test. An increased passive avoidance response was also observed for males. BChE KO mice therefore showed enhanced learning ability in spatial and non-spatial memory tests. In BChE KO mice, the $A\beta_{25-35}$-induced deficit in place learning was attenuated in males and blocked in females. No changes in lipid peroxidation or ACh levels were observed after $A\beta_{25-35}$ treatment in BChE KO mice. We conclude that, on the one hand, the increase in cholinergic tonus observed in hetAChE KO mice did not result in increased memory functions but allowed a significant prevention of the deleterious effects of amyloid toxicity. On the other hand, the genetic invalidation elimination of BChE in mice increased learning capacities and lowered the vulnerability to Aβ toxicity.

Cyclic imine toxins exhibiting high affinity for muscle- and neuronal-subtypes of nicotinic acetylcholine receptors

Jordi Molgó[a], Romulo Aráoz[a], Evelyne Benoit[a], Bogdan I. Iorga[b], Armen Zakarian[c], Denis Servent[d]

[a]Institut des Neurosciences Paris-Saclay, UMR 9197 CNRS/ Université Paris-Sud and CEA Saclay, Laboratoire de Toxinologie Moléculaire et Biotechnologies. Service d'Ingénierie Moléculaire des Protéines (DRF/iBiTEC-S/SIMOPRO) 91191, Gif sur Yvette, France
[b]CNRS, Institut de Chimie des Substances Naturelles, UPR 2301, Labex LERMIT 91198, Gif sur Yvette, France
[c]Department of Chemistry and Biochemistry, University of California, Santa Barbara, California 93106, Santa Barbara, USA
[d]CEA Saclay Laboratoire de Toxinologie Moléculaire et Biotechnologies. Service d'Ingénierie Moléculaire des Protéines (DRF/iBiTEC-S/SIMOPRO) 91191, Gif sur Yvette, France

A large number of compounds found in nature are known to interact with nicotinic acetylcholine receptors (nAChRs), including a number of alkaloids and peptides of various origins. In recent years several heterogeneous groups of macrocyclic marine compounds, globally distributed, known as cyclic imine toxins including: gymnodimines, spirolides, pinnatoxins, pteriatoxins, portimine, spiro-prorocentrimine and prorocentrolides, have been associated with marine harmful algal blooms and shellfish toxicity. Their chemical structure is represented by a macrocycle, with the ring size between 14 and 27, and two conserved features that include the cyclic imine group (which in most cases is found as a spiroimine) and the spiroketal ring system. The producers of gymnodimines, spirolides, and pinnatoxins known to date have been identified as being the dinoflagellates *Karenia selliformis, Alexandrium ostenfeldii/Alexandrium peruvianum*, and *Vulcanodinium rugosum,* respectively. Successful strategies for the synthesis of pinnatoxins have been developed by the Zakarian group. The cyclic imine toxins have been reported to be "fast acting" toxins because they induced rapid onset of neurological symptoms in mouse bioassays followed by death within a few minutes. The neurotoxic effects reported for the different phycotoxins are mainly due to their specific interaction with muscle and neuronal-types of nicotinic acetylcholine receptors (nAChRs) which are the principal molecular targets. In this presentation we summarize our current understanding of the functional effects of gymnodimines, spirolides and pinnatoxins on muscle and neuronal nAChRs expressed at the neuromuscular junction or in *Xenopus* oocytes using conventional electrophysiological techniques. In addition, we compare their selectivity profile using binding studies on clonal cell lines expressing various nAChRs subtypes. The nAChR subtype selectivity of the cyclic imine toxins is discussed on the basis of crystal structures determined with the AChBPs (Bourne et al., 2010, 2015), and molecular docking models.

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Splicing regulation of the human acetylcholinesterase gene

Kinji Ohno[a], Mohammad Nazim[a], Akio Masuda[a]

[a]*Neurogenetics, Nagoya University Graduate School of Medicine, Nagoya, Japan*

RNA processing, including alternative splicing (AS) and alternative polyadenylation (APA) of pre-mRNA, is a highly specialized mechanism that enables enhanced transcriptome and proteome diversity. AS causes skipping/inclusion of exons or retention of introns through differential selection of splice sites (ss's) in pre-mRNA, and APA results in distinct 3′ ends through regulated processing of the 3′ end including transcription termination, cleavage and polyadenylation. Specific *cis*-elements on pre-mRNA and the cognate *trans*-acting factors facilitate coordinated regulation of alternative RNA processing in tissue-specific and developmental stage-specific manners.

ACHE encoding acetylcholinesterase (AChE) has 5 exons, including 3 invariant exons (exons 2, 3, and 4) that encode the core catalytic domain, and variable 5′ and 3′ exons. Exon 5 has two alternative 3′ splice sites (ss's): one at the boundary of intron 4 and exon 5a (proximal 3′ ss) and the other at the boundary of exon 5a and exon 5b (distal 3′ ss). Splicing from exon 4 to exon 5a makes the $AChE_H$ isoform, whereas splicing from exon 4 to exon 5b makes the $AChE_T$ isoform. The readthrough $AChE_R$ isoform is generated when the transcript is unspliced after exon 4. In addition to alternative splicing, a cryptic polyadenylation site (PAS) is present in exon 5a.

We found that hnRNP H binds to two specific G-runs in exon 5a of human *ACHE* and activates the distal 3′ ss to generate $AChE_T$. Specific effect of hnRNP H was corroborated by siRNA-mediated knockdown and artificial tethering of hnRNP H. Furthermore, hnRNP H competes for binding of CstF64 to the overlapping binding sites in exon 5a, and suppresses the selection of the cryptic polyadenylation site (PAS), which additionally ensures transcription of the distal 3′ ss required for the generation of $AChE_T$. Expression levels of hnRNP H were positively correlated with the proportions of the $AChE_T$ isoform in three different cell lines. HnRNP H thus critically generates $AChE_T$ by enhancing the distal 3′ ss and by suppressing the cryptic PAS. Global analysis of CLIP-seq and RNA-seq also revealed that hnRNP H competitively regulates alternative 3′ ss and alternative PAS in other genes. We propose that hnRNP H is an essential factor that competitively regulates alternative splicing and alternative polyadenylation.

Identification of positions crucial for the pentameric assembly of the intracellular domain of pentameric ligand-gated ion channels

Akash Pandhare[a], Antonia Stuebler[a], Elham Pirayesh[a], Min Ahmadi[a], Pablo Artigas[a], Michaela Jansen[a]

[a]*Department of Cell Physiology and Molecular Biophysics, Center for Membrane Protein Research, TTUHSC, Lubbock, USA*

Receptors of the nicotinic acetylcholine family belong to a large superfamily of ligand-gated ion channels in eukaryotes that is also known as "Cys-loop receptors" based on a conserved disulfide-linked loop. Members of the superfamily include cation (e.g. 5-HT_{3A}, nAChR) and anion-conducting channels (e.g. $GABA_A$, Gly). A decade ago, more than a dozen prokaryotic homologues were identified in an elegant computational study (Tasneem et al., 2005). Since those lack the eponymous disulfide-linked loop, the more commonly used nomenclature now is pentameric ligand-gated ion channels (pLGICs). All pLGICs contain an extracellular and a transmembrane domain, whereas only the eukaryotic members contain an intracellular domain (ICD). Interestingly, the intracellular domain is by far the most diverse domain, exemplified by lengths of 50–280 amino acids. None of the so-far published structures of prokaryotic or more recently also eukaryotic pLGICs provide complete structural insights into the ICD, because the former entirely lack this domain and the latter were engineered to not contain an ICD or it was proteolyzed for structural studies. The consensus assumption in the field has been for decades that the ICD is largely disordered. We have recently shown that chimeras between maltose binding protein (MBP) and diverse ICDs assemble into homopentamers in solution (Pandhare et al., 2016). While contributions of the two other domains to oligomerization have been studied in the past, the observed pentameric oligomerization of the ICD adds a novel function to its repertoire. Here we identify single-point mutations that disrupt the oligomerization and instead lead to lower assembly intermediates, mostly monomer with some dimer. Further studies are aimed at identifying why these positions are pivotal for pentameric assembly, as well as deducing the mechanisms by which the structural role corroborates different aspects of the ICD to ion-channel function. Chimeric proteins for the present study are heterologously expressed in *E. coli*. Assembly behaviors are assessed with size-exclusion chromatography followed by multi-angle light scattering, as well as isothermal titration calorimetry. Mutants identified to be structurally-important are additionally generated in the wildtype ion channel background and studied with patch-clamp electrophysiology.

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Paradoxical interactions of alpha7 nAChR silent agonists and allosteric modulators; equilibration between desensitized states and persistent currents

Roger Papke[a], Khan Manther[a], Ganesh Thakur[b], Clare Stokes[c]

[a]*University of Florida Department of Pharmacology, Gainesville, Florida, USA*

[b]*Department of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115, USA*

[c]*Department of Pharmaceutical Sciences, Northeastern University, Gainesville, Florida, USA*

Investigation of alpha7 nAChR as a therapeutic target has led to the discovery of promising new drug types, including ago-PAMs and silent agonists. Ago-PAMs are positive allosteric modulators (PAMs) that also produce direct allosteric activation (DAA). While DAA by the prototypical ago-PAM GAT107 is transient, a single GAT107 application followed by a drug washout results in a

prolonged period of primed potentiation of acetylcholine responses lasting an hour or longer. Silent agonists have little or no efficacy for activating the alpha7 ion channel but induce desensitization states. Some of these desensitized states can be converted into channel-active states by PAMs. NS6740 is a silent agonist that induces prolonged nonconducting states that are insensitive to activation by acetylcholine but activatible by a PAM. A single application of 30 μM NS6740 leaves receptors in a PAM-sensitive state for over an hour. GAT107 and NS6740 therefore appear to stably induce different peri-activatable states, which can work in concert to produce large ion-channel responses, and with sequential applications these two drugs induce varying levels of activation that persist following washout. With 30 μM GAT107 application following 30 μM NS6740, there was a biphasic activation with an initial peak 60 ± 20-fold greater than the amplitude of a response to 60 μM ACh alone. Within 60 s, current decreased to a level about 20-fold higher than ACh controls. This late-phase current decayed with a time constant of about 10 min and was still detectable as a mecamylamine-sensitive baseline current after 1 h. Drugs that are intermediate in their induction of desensitization or allosteric potentiation are also intermediate in their ability to induce persistent currents. Despite their seemingly opposite effects on channel activation, both GAT107 and NS6740 have been shown to be very effective analgetic agents in the same *in vivo* pain models. This suggests that the different peri-activational states induced by GAT107 and NS6740 have common signal transduction activity, which we hypothesize is due to similar effects on the receptor's intracellular interactome that is independent of their opposing effects on channel activation.

Cholinergic co-transmission in the striatum: roles in cognition and drug abuse

Vania Prado[a]

[a]*Robarts Research Institute, Anatomy & Cell Biology, Physiology and Pharmacology, Neuroscience Graduate Program, University of Western Ontario, N6A5B7, London, Canada*

Striatum, the major input gateway to the basal ganglia, plays important roles in controlling motor functions, goal-directed and reward-related behaviours. The striatum is the brain region mostly affected in motor diseases, such as Parkinson's disease (PD), Huntington's disease, and dystonia. Cholinergic interneurons (CINs) in the striatum are thought to play major regulatory functions in motor behaviours, cognition and reward. These neurons express two vesicular transporters that can load either acetylcholine (ACh) or glutamate (Glu) into synaptic vesicles. Consequently, CINs can release both neurotransmitters, making it difficult to discern their individual contributions for the regulation of striatal functions. We have been dissecting the specific roles of ACh or Glu release on striatal-dependent behaviours in mice by selective elimination of the vesicular acetylcholine transporter (VAChT) from CINs or global deletion of the vesicular glutamate transporter 3 (VGLUT3). Our data suggest that ACh and Glu from CINs exert opposite effects on striatal dopamine release. Analysis of several behavioural parameters indicates that while elimination of VAChT does not affect spontaneous locomotion, cocaine-induced hyperactivity, or its reward properties; these behaviors are heavily affected by elimination of VGLUT3. On the other hand, VAChT elimination affects a number of cognitive functions that are not changed when VGLUT3 is eliminated. These observations indicate that one population of neurons can use two distinct neurotransmitters to differentially regulate a given circuitry. Furthermore, these data indicate that, by specifically targeting VAChT or VGLUT3 from CINs, one can differentially modulate dopaminergic function as well as various striatal-regulated behaviors. A deeper understanding of the contributions of VAChT and VGLUT3 from CINs to striatal circuitry could pave the way for developing new therapeutic strategies for drug abuse, Parkinson's disease (PD) and other striatal-related diseases.

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Role for the nicotinic cholinergic system in Parkinson's disease and other movement disorders; mechanisms and therapeutic implications

Maryka Quik[a], **Tanuja Bordia**[a], **Xiomara Perez**[a], **Danhui Zhang**[a]

[a]*SRI, Menlo Park, USA*

Accumulating evidence suggests that the nicotinic cholinergic system represents a therapeutic target for Parkinson's disease and other movement disorders. Numerous pre-clinical reports using parkinsonian animals indicate that nicotine and nicotinic receptor agonists protect against nigrostriatal damage as occurs in Parkinson's disease. In addition, studies show that nicotinic receptor agonists alleviate L-dopa-induced dyskinesias, debilitating abnormal involuntary movements that arise with prolonged L-dopa treatment for Parkinson's disease. Our data also show that nicotinic receptor agonists reduce other drug-induced abnormal movements such as tardive dyskinesia that arise as a side effect of antipsychotic use. Since nicotinic receptor-mediated function is strongly influenced by cholinergic activity, we initiated studies to understand how these neurons regulate abnormal movement as such knowledge may lead to improved therapies. To approach this we used optogenetics with a focus on the striatum because of its central role in motor control. Parkinsonian choline acetyltransferase (ChAT)-Cre mice expressing channelrhodopsin2 (ChR2) in striatal cholinergic interneurons were treated with L-dopa until dyskinetic. Continuous single pulse optical stimulation (20 ms to 1 s) reduced dyskinesias ~60% via a nicotinic receptor-mediated mechanism. We also optically stimulated striatal cholinergic interneurons in antipsychotic-treated ChAT-Cre mice expressing ChR2-eYFP. A ~50% decrease was observed in abnormal movements that occurred via a nicotinic receptor-mediated mechanism. These combined studies indicate that striatal cholinergic interneurons play an important role in motor control and suggest that nicotinic receptor drugs may be useful for reducing abnormal movements. This latter idea is consistent with our studies in animal models, which show that nicotinic receptor drugs reduce L-dopa- and antipsychotic-induced dyskinesias up to 60%. In fact, several nicotinic agonists that showed safety and tolerability in human clinical trials for Alzheimer's disease and schizophrenia very effectively reduced L-dopa-induced dyskinesias in parkinsonian nonhuman primates. This included the β2 agonists ABT-089 and ABT-894, as well as the α7 agonists ABT-107 and ABT-126, at doses that compared well to those used in the clinical trials.

Altogether these data indicate a prominent role of striatal nicotinic cholinergic transmission in motor function. Moreover, they suggest that nicotinic receptor agonists may be useful for Parkinson's disease therapy and for the treatment of movement disorders associated with L-dopa and antipsychotic drugs.

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Receptor - membrane interactions as a potential basis of functional selectivity of xanomeline for M1 and M4 muscarinic receptors

Alena Randáková[a], Pavel Zimčík[a], Eva Dolejší[a], Vladimír Doležal[a], Jan Jakubík[a]

[a]*Institute of Physiology CAS, Prague, Czech Republic*

Xanomeline is one of the first described muscarinic agonists displaying subtype selectivity for $M_{1,4}$ subtypes of muscarinic receptors at the level of functional response. The molecular mechanism underlying functional selectivity of xanomeline is not known and its elucidation may contribute to development of new selective muscarinic agonists suitable for therapeutic use. We compared amino acid sequences of $M_{1,4}$ muscarinic receptors with other subtypes to identify amino acids with potential role in functional selectivity of xanomeline. In position 6.46 (according to Ballesteros-Weinstein numbering) there is leucine in M_1 and M_4 receptors and isoleucine in the rest of the subtypes. This amino acid is oriented to the cell membrane and can mediate interaction of receptor with membrane components. We mutated leucine 6.46 to isoleucine in M_1 and M_4 receptors (L376I in sequence of M_1 receptor, L411I in sequence of M_4 receptor) and analyzed the effect of this mutation on functional response of xanomeline (xanomeline-stimulated accumulation of inositolphosphates) and on persistent activation of the $M_{1,4}$ receptors by wash-resistantly bound xanomeline. Mutation of L376I in M_1 receptor as well as L411I in M_4 receptor caused decrease in xanomeline efficacy and completely abolished persistent activation by wash-resistantly bound xanomeline. The mutations had no effect on xanomeline potency. Our results point to the crucial role of leucine 6.46 in persistent activation of $M_{1,4}$ receptors by wash-resistantly bound xanomeline, and suggest that interaction of $M_{1,4}$ receptors with the membrane via leucine 6.46 may by related with functional selectivity of xanomeline for these two subtypes.

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Superior efficacy of HI-6 dimethanesulfonate over pralidoxime methylsulfate against Russian VX poisoning in cynomolgus monkeys (Macaca fascicularis)

Chloé Reymond[a], Nina Jaffré[a], Franck Dhote[a], Frédéric Dorandeu[a]

[a]*Institut de Recherche Biomédicale des Armées, Brétigny-sur-Orge, France*

Organophosphorus agents, either chemical warfare agents (a.k.a. nerve agents) or pesticides, still represent a serious risk to human health. More efficient medical countermeasures are needed, especially more effective cholinesterase (ChE) reactivators. In the French armed forces, the current emergency treatment is a fully licensed wet-dry dual-chambered autoinjector (Ineurope ®). It contains pralidoxime methylsulfate (Contrathion®), to reactivate inhibited ChE, atropine sulfate (AS), an anticholinergic drug, and avizafone chlorhydrate (AVZ), a prodrug of the anticonvulsivant diazepam. While this treatment is effective against several of the identified nerve agents on the threat list, it shows little efficacy against the Russian VX (VR), one of the most toxic compounds. Among the oximes currently being investigated, HI-6 dimethanesulfonate (HI-6 DMS) shows interesting ability to reactivate VR-inhibited ChE *in vitro*. To assess the *in vivo* efficacy of HI-6 DMS prior to licensing, we compared the two 3-drug-combinations (HI-6 vs Pralidoxime, 33 and 18 mg/kg respectively, equimolar doses; AS/AVZ 0.25/0.175 mg/kg respectively) in VR-poisoned cynomolgus macaques. Drugs were injected intramuscularly (i.m.) in separate locations. This model was required by the French drug regulatory agency. A better efficacy of the HI-6 DMS combination was clearly observed: up to 5 LD_{50} of VR (i.m.), a single administration of the HI-6 DMS combination, shortly after the onset of clinical signs, prevented death of the four intoxicated animals. As expected with V agents, reinhibition of blood ChE was observed but without any apparent impact on the recovery of the animals. Conversely, pralidoxime only prevented death in one out of three subjects.

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Degeneration and regeneration of the neuromuscular junction: novel modulators and possible therapeutic agents

Michela Rigoni[a], Elisa Duregotti[a], Samuele Negro[a], Bryan C. Dickinson[b], Christopher J. Chang[b], Cesare Montecucco[a]

[a]*Department of Biomedical Sciences, University of Padova, Padova, Italy*

[b]*Department of Chemistry and Molecular and Cell Biology, University of California, Berkeley, USA*

The neuromuscular junction is one of the few human tissues capable of complete regeneration after major damages (1). We have set up a reliable model of acute degeneration of the motor axon terminals followed by complete recovery of function (2). We have found that alarmins (Hydrogen Peroxide, mt-DNA and cyt c) are released by mitochondria of the degenerating nerve terminal (2). In addition, motor axon terminals right after damage release ATP which activates perisynaptic Schwann cells (3). Stimulated by mitochondrial alarmins by ATP, these cells are activated, become phagocytes and release signals that act retrogradely on the nerve terminal, inducing its regeneration. As an example we will show the effect of a chemokine which was identified via a specific transcriptomics analysis. This chemokine strongly stimulates axonal growth and recovery of function after nerve terminal degeneration. Other retrograde signals are currently being investigated by imaging and transcriptomics methods.

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ATP released by toxin or immunocomplexes-injured neurons activates Schwann cells

Michela Rigoni[a], Samuele Negro[a], Umberto Rodella[a], Elisa Duregotti[a], Elisanna Bergamin[a], Michele Scorzeto[a], Kees Jalink[b], Nobuhiro Yuki[c], Cesare Montecucco[a]

[a]*Department of Biomedical Sciences, University of Padua, Padua, Italy*
[b]*Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, Netherlands*
[c]*Department of Neurology, Mishima Hospital, Niigata, Japan*

Injured nerve terminals of neuromuscular junctions can regenerate. This remarkable and complex response is governed by molecular signals that are exchanged among the cellular components of this synapse: motor axon nerve terminal 3 (MATs), perisynaptic Schwann cells (PSCs), and muscle fibre 3. The nature of signals that govern MAT regeneration is unknown.

In the present study the spider toxin α-Latrotoxin or an anti-polysialoganglioside *plus* complement complex (the *pathogen* responsible for a group of autoimmune peripheral neuropathies) have been used as tools to investigate the mechanisms underlying peripheral neuroregeneration. Both agents induce acute, specific, localized and reversible damage of the MAT, and their action mimics the cascade of events that leads to nerve terminal degeneration in injured patients and in other neurodegenerative conditions.

Here we provide evidence that degenerating neurons release ATP as *alarm* messenger, and that ATP activates multiple intracellular signaling pathways within SCs that are crucial for nerve regeneration: Ca^{2+}, cyclic AMP, MAP kinases. These results contribute to define the cross-talk taking place among degenerating nerve terminals and PSCs involved in the functional recovery of the NMJ.

- Duregotti E et al. (2015) Mitochondrial alarmins released by degenerating motor axon terminals activate perisynaptic Schwann cells. Proc Natl Acad Sci USA 112, E497–505.

- Negro S et al. (2016) ATP released by injured neurons activates Schwann cells. Front Cell Neurosci. 10,134.

Cholinergic control of fear behaviors and fear memory engrams in cortical-amygdala circuits

Lorna Role[a], David Talmage[a]

[a]*Stony Brook University, Stony Brook, USA*

We examined the contribution of endogenous cholinergic signaling to the acquisition and extinction of fear-related memory by optogenetic labelling and photo-stimulation of cholinergic input from the nucleus basalis/substantia innominata (NB/SI) to the basal lateral amygdala (BLA). Stimulation of cholinergic terminal fields within the BLA in awake-behaving mice during training in a cued fear-conditioning paradigm slowed the extinction learning. Inhibition of cholinergic activity during training reduced the acquisition of learned fear behaviors. Circuit mechanisms underlying the behavioral effects of cholinergic signaling in the BLA were assessed by *in vivo* and *ex vivo* electrophysiological recording and engram mapping. Fear learning induces a region-specific increase in engram enrollment in both the BLA and in a subset of cholinergic neurons in the NBM. Photo-stimulation of endogenous cholinergic input: (1) enhances BLA principal neuron firing through activation of acetylcholine receptors (AChRs); (2) enhances glutamatergic synaptic transmission to BLA principal neurons and (3) induces LTP of cortical-amygdala circuits. These studies support an essential role of cholinergic modulation of BLA circuits in the inscription and retention of fear memories.

Hopeahainol A is a reversible inhibitor at the acetylcholinesterase (AChE) peripheral site that inhibits enzyme activity with a novel higher order concentration dependence

Terrone Rosenberry[a], A. Jeremy Nix[a], Jonah Cheung[b], Scott Wildman[c], Scott Snyder[d], Ren Xiang Tan[e]

[a]*Mayo Clinic, Jacksonville, FL, USA*
[b]*New York Structural Biology Center, New York, NY, USA*
[c]*University of Wisconsin, Madison, WI, USA*
[d]*University of Chicago, Chicago, IL, USA*
[e]*Nanjing University, Nanjing, China*

Natural product inhibitors of AChE are of interest both because they offer promise as inexpensive drugs for symptomatic relief in Alzheimer's disease and because they may provide insights into the structural features of the AChE catalytic site. Hopeahainol A (HopA) is an uncharged polyphenol AChE inhibitor from the stem bark of *H. hainanensis* with a constrained, partially dearomatized bicyclic core. Molecular modeling indicates that HopA is too bulky to be accommodated in the long but narrow AChE active site gorge without severe distortion of the gorge as depicted in AChE crystal structures. The observed pattern of inhibition by HopA was completely novel for AChE inhibitors, with the extent of inhibition showing a striking dependence on the HopA concentration. Plots analogous to classic Dixon plots showed a dependence on HopA concentrations to the third- or fourth order. This dependence suggested the binding of multiple HopA molecules to the AChE active site. A series of AChE inhibitors with binding sites known from crystal structures was employed to better define where these multiple interactions occur. Previous experiments indicated that HopA initially bound to the AChE peripheral site and that edrophonium binding to the acylation site at the base of the active site gorge blocked higher order HopA inhibition. Here we particularly focused on hopeahainol A effects on organophosphorylation by the fluorogenic organophosphate 7-[(methylethoxyphosphonyl)oxy]-4-methylcoumarin (EMPC). We hypothesize that HopA binding slowly distorts the AChE active site, a phenomenon that would be unprecedented for a reversible inhibitor that apparently forms no covalent bonds with the enzyme.

Acetylcholinesterase folding, assembly and trafficking to the synapse

Richard Rotundo[a]

[a]*Department of Cell Biology, University of Miami Miller School of Medicine, Miami, USA*

Acetylcholinesterase (AChE) is an essential component of cholinergic synapses in the central and peripheral nervous systems. AChE has one of the highest turnover numbers of any enzyme, making it easy to detect and measure. It is also unique in that thousands of reversible and irreversible inhibitors exist that allow specific subsets of AChE molecules, both spatial and temporal, to be analyzed. For example, newly synthesized AChE can be analyzed by inhibiting expressing cells with a membrane permeant inhibitor, DFP, followed by washout and recovery of active enzyme through

de-novo protein synthesis. The newly synthesized enzyme molecules can then be followed over several hours as they traffic through the cell and become externalized on the cell surface or secreted. Using these approaches, we have found that most of the newly synthesized AChE, approximately 80% in muscle, is catalytically inactive and rapidly degraded through the ERAD. During synthesis the AChE polypeptide chain interacts with several identified chaperons and oxidoreductases in the RER including BiP, PDI, Erp72 and calnexin' which in some cases may be rate limiting. The newly-synthesized AChE molecules that become catalytically active remain in a highly labile state for at least 1–2 h, they are rapidly inactivated at 45°C, are inactivated by 1 mM DTT and are trypsin-sensitive, whereas the mature AChE molecules are resistant to these treatments. Oligomerization is at least in part necessary for the enhanced stability of the enzyme. Once assembled into stable oligomers including the PRiMA-linked tetramer, the secreted AChE tetramer and the collagen-tailed asymmetric AChE, the enzyme is transported to the cell surface via the constitutive secretory pathway. In every case, the assembled AChE form is targeted to the apical domain corresponding to the villi surface in polarized epithelial cells and the axon in neurons. Finally, all these events of protein folding and assembly can be regulated both *in vitro* and *in vivo* by altering the interactions of catalytic subunits with the noncatalytic ones, or fragments thereof, or even through the interaction with specific inhibitors that bind to residues in the catalytic site. These interactions can be used to advantage to increase expression of AChE *in vivo* at peripheral synapses in the CNS, or can be a disadvantage when the desired effect is to decrease AChE activity, as in the treatment of Alzheimer's disease patients.

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The three-finger toxin fold: a multipotent structural fold to modulate cholinergic function

Denis Servent[a], Guillaume Blanchet[a], Nicolas Gilles[a], Gilles Mourier[a]

[a]*CEA/DRF/ibitecS/SIMOPRO, Gif sur Yvette, France*

Despite their extraordinary diversity, animal toxins belong to a limited number of structural superfamilies of which the three-finger fold is probably the most common in snake venoms.

Three-finger toxins (3FTx) have a molecular mass within the range of 6000–8000 Da, and they contain four or five disulphide bridges, of which four are conserved in all members and located in the small globular core from where three β-strands emerge. Members of the 3FTx family show a wide array of pharmacological effects by targeting different receptors, enzymes and ion channels with often high specificity and affinity. In particular, they interfere with cholinergic transmission by targeting the nAChRs, the AChE or the mAChRs.

We have focused our studies on 3FTx from mambas that display the unique property to interact with various GPCRs. Indeed, in addition to the well-known muscarinic toxins, we have recently identified several toxins active on α-adrenoceptors as well as on the dopamine D3 subtype, highlighting the multipotent interacting property of 3FTx for aminergic GPCRs. These toxins may display either absolute selectivity for one receptor subtype or a polypharmacological property for various aminergic receptors. Moreover, to study the mode of action of some of these toxins (MT7 and ρ-Da1a) on their respective targets (muscarinic M1 and $\alpha 1_A$-adrenoceptor'), equilibrium and kinetic binding experiments as well as functional assays were performed using wild type and modified toxins as well as receptor mutants. Our results highlight that both toxins interact in a completes different way with GPCRs, MT7 being an allosteric modulator of the M1 muscarinic receptor while ρ-Da1a acts as a competitive ligand on the $\alpha 1_A$-adrenoceptor. Based on these results, toxin engineering using a loop permutation strategy was used in order to design new three-finger toxins with original pharmacological profiles.

Finally, phylogenic analyses of these 3FTx show that muscarinic, adrenergic and dopaminergic toxins may be pooled in one family called aminergic toxins, this family coming probably from a specific radiation of ligands present in mamba venoms. We recently applies the ancestral protein resurrection strategy to aminergic toxins in order to pinpoint important functional mutations which probably occurred during their evolution, and analyze them to modulate their binding properties on various GPCRs and in particular on muscarinic receptors.

Alpha-1 antitrypsin inhibits ATP-induced release of monocytic IL-1β via activation of CD36 and nicotinic receptor subunits α7, α9, α10

Robin Siebers[a], Katrin Richter[a], Zakrzewicz Anna[a], Bijan Fink[a], Sigrid Wilker[a], Mira Küllmar[a], Andreas Hecker[a], Nupur Aggarval[b], Winfried Padberg[a], Michael J. McIntosh[c], Sentot Santoso[d], Sabina Janciauskiene[b], Veronika Grau[a]

[a]*Justus-Liebig-University, Department of General and Thoracic Surgery, Laboratory of Experimental Surgery, Giessen, Germany*
[b]*Hannover Medical School, Department of Pulmonology, Hannover, Germany*
[c]*University of Utah, Department of Biology, Salt Lake City, USA*
[d]*Justus-Liebig-University, Institute for Clinical Immunology and Transfusion Medicine, Giessen, Germany*

Introduction: Interleukin-1β (IL-1β), a potent cytokine produced by monocytes/macrophages, is essential for defense against infections. High systemic IL-1β concentrations, however, cause life-threatening systemic inflammation. Danger- or pathogen-associated molecular patterns induce the expression of pro-IL-1β. Extracellular ATP originating from damaged cells typically activates receptor P2X7, induces inflammasome activation, cleavage of pro-IL-1β and release of mature IL-1β. We recently described that activation of nicotinic receptors (nAChR) inhibits the P2X7 receptor and IL-1β release by human monocytes (Hecker, Küllmar et al. J Immunol 2015). The anti-protease α-1 antitrypsin (AAT) forms complexes with lipids, predominantly linoleic and oleic acids, and exerts anti-inflammatory functions via poorly defined pathways. Here, we test the hypothesis that AAT also inhibits ATP-induced IL-1β release and describe a novel cholinergic signaling pathway.

Materials and Methods: Primary human monocytes or LPS-primed human monocytic U937 cells were stimulated with ATP, and IL-1β was measured in cell culture supernatants by ELISA. CD36, calcium-independent PLA2 (iPLA2) and nAChR gene expression were silenced by siRNA. Lipid-containing and lipid-free AAT were applied together with ATP either alone or in combination with antibodies to CD36, inhibitors of phospholipase A2 (PLA2) or nicotinic antagonists. ATP-induced ion currents were monitored using the patch-clamp technique. Conditioned medium from AAT-treated U937 cells underwent ultrafiltration at a cut-off of 10 kDa.

Results: ATP-induced release of IL-1β by human monocytic cells was dose-dependently inhibited by lipid-containing AAT, whereas corresponding free fatty acid concentrations and lipid-free AAT were ineffective. Inflammasome activation by the pore-forming toxin nigericin, however, was unimpaired. In contrast, ATP-induced current responses in U937 cells were abrogated by AAT. The effect of AAT was blunted after silencing of CD36, iPLA2 or nAChR α9 expression and upon double knock-down of subunits α7 and α10. Furthermore, the effect was sensitive to antibodies against CD36, inhibitors of iPLA2 and specific antagonists of nAChR. A low molecular weight nicotinic agonist was detected in conditioned medium of AAT-treated U937 cells that prevented IL-1β release like AAT itself.
Conclusions: We suggest that AAT is a potent inhibitor of ATP-induced release of IL-1β by human monocytic cells that triggers a novel triple membrane-passing signaling cascade. Lipids bound to AAT seem to signal via CD36 and activate iPLA2. A soluble factor X, most probably a choline-containing cleavage product of phospholipids, is released. Finally, factor X induces metabotropic functions at nAChR composed of subunits α9 and either α7 or α10, which efficiently inhibit P2X7 receptor activation and IL-1β release.

Agonist efficacy in the nicotinic superfamily

Lucia Sivilotti[a]

[a]*University College London, London, UK*

Efficacy is the ability of an agonist to stabilise the active form of the receptor.

Channels in the nicotinic superfamily have been very useful models to test our hypothesis about efficacy, because members of this superfamily such as the muscle nicotinic acetylcholine receptor and the glycine receptor are very suitable for single molecule recording techniques.

The traditional view of agonist efficacy in nicotinic channels held for a long time that the channel goes from resting to open in a single step, which is favoured by the bound agonist. Progress in the analysis techniques of single channel patch clamp data has allowed us to show that there are at least two steps in the activation of Cys-loop channels (Burzomato, Beato, Groot-Kormelink, Colquhoun & Sivilotti, *J. Neurosci.*, 24, 10924, 2004). There is a first step to an intermediate state of the channel, which is still closed (but already has increased affinity for the agonist) and this is followed by the channel opening.

It is the first step that differs for agonists with different efficacy (Lape, Colquhoun & Sivilotti, *Nature*, 454, 722, 2008, Mukhtasimova, da Costa & Sine, *J. Gen. Physiol.*, 148,43, 2016). The second (opening) transition is similar across different agonists, or at least not measurably different with our techniques. This pattern has now been observed not only in the muscle nicotinic receptor and the glycine receptor, but also in the 5-HT_3 receptor (Corradi & Bouzat, *J. Neurosci.*, 34, 16865, 2014).

The key role of α7 nicotinic acetylcholine receptors in neuroinflammation and memory impairment

Maryna Skok[a], **Olena Lykhmus**[a], **Lyudmyla Koval**[a], **Kateryna Uspenska**[a]

[a]*Palladin Institute of Biochemistry, Kyiv, Ukraine*

Nicotinic acetylcholine receptors of the α7 subtype (α7 nAChRs) expressed in the brain regulate cognitive functions, cell survival and inflammatory reactions. The α7 nAChRs are found on both plasma membranes and mitochondria of the brain cells and interact with amyloid-beta (Aß) peptides involved in pathogenesis of Alzheimer disease. We have found that either regular injections of bacterial lipopolysaccharide (LPS) or immunizations with recombinant extracellular domain of α7 nAChR subunit, resulting in generation of α7 (1-208)-specific antibodies, decreased the level of α7 nAChRs in the brain and brain mitochondria, stimulated astrogliosis and accumulation of Aß(1–42), resulting in significant memory impairment in mice. Even a short-term LPS challenge decreased the levels of α7 nAChR RNA and protein, as well as acetylcholinesterase expression and activity, in distinct mouse brain regions, modified brain microRNA profiles in favor of anti-inflammatory and pro-apoptotic ones, and sensitized brain mitochondria to the apoptogenic effect of Ca^{2+}. The α7(1–208)-specific antibodies, which readily penetrated the brain parenchyma of LPS-treated mice upon intravenous injection, prevented elevation of both the anti-inflammatory and pro-apoptotic miRNAs and supported the resistance of brain mitochondria to Ca^{2+}. The α7-specific antibodies stimulated IL-6 production by glioblastoma U373 cells by activating the Src/p38-dependent signaling pathway. However, *in vivo*, IL-6 was elevated in the brain only upon immunization with mannosylated α7(1–208) produced in yeast but not with the deglycosylated one. Nevertheless, antibodies against either glycosylated or deglycosylated α7(1–208) decreased α7 nAChR, stimulated accumulation of Aß(1–42) in the brain and produced memory impairment. Oligomeric Aß(1–40) induced mitochondrial pore opening and cytochrome *c* release in mitochondria of α7-/- but not wild-type mice. These data indicate that LPS-induced inflammation results in α7 nAChR down-regulation, which makes ineffective the cholinergic anti-inflammatory pathway in the brain and decreases mitochondria, protection against apoptogenic stimuli and Aß toxicity. The α7-specific antibodies also decrease the number of functional α7 nAChRs and aggrevate neuroinflammation by dampening anti-inflammatory miRNAs. We conclude that the decrease of α7 nAChR density in the brain, caused by either LPS or α7(1–208)-specific antibody, is a key mechanism for the development of Alzheimer-like symptoms like neuroinflammation, Aß(1–42) accumulation and episodic memory impairment.

Visualization of acetylcholine distribution in intestinal tissue sections by tandem imaging mass spectrometry and its function in mice

Toshio Takahashi[a]

[a]*Suntory Foundation for Life Sciences, Kyoto, Japan*

Acetylcholine (ACh) has been considered as a neurotransmitter residing in central, parasympathetic, and neuromuscular synapses of mammals. On the other hand, non-neuronal ACh is predicted to function as a local cell signaling molecule. However, the physiological significance of the synthesis of non-neuronal ACh in the intestine remains unclear. Here, experiments using cultured crypt-villus organoids that lack nerve and immune cells led us to suggest

that endogenous ACh is synthesized in the intestinal epithelium to evoke growth and differentiation of the organoids through activation of muscarinic ACh receptors (mAChRs). Extracts of cultured organoids exhibited a noticeable capacity for ACh synthesis that was sensitive to a potent inhibitor of choline acetyltransferase (ChAT). Although ACh is one of the first neurotransmitters to be characterized, it has yet to be specifically localized. Imaging mass spectrometry (IMS) is gaining popularity as a means of visualizing the distribution of molecular ions in tissue sections. IMS is therefore expected to allow the imaging of ACh not only in the nervous system but also in other tissues, such as epithelium. Tandem IMS revealed distribution of endogenous ACh that is localized in the epithelial layer in mouse small intestinal epithelium *in vivo*, suggesting non-neuronal sources of ACh. Treatment of organoids with carbachol down-regulated growth of organoids and expression of marker genes for each epithelial cell type, but not the marker gene (*Lgr5*) for intestinal stem cells. On the other hand, mAChR antagonists enhanced growth and differentiation of organoids, indicating involvement of mAChRs in regulating proliferation and differentiation of Lgr5-positive stem cells. Collectively, our data provide evidence that endogenous ACh released from mouse intestinal epithelium maintains the homeostasis of intestinal epithelial cell growth and differentiation via mAChRs.

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Molecular determinants governing recognition properties of nicotinic acetylcholine receptors and acetylcholinesterase: a tale of two active sites with distinctive functions

Palmer Taylor[a], Gisela Camacho Hernandez[a], K. Barry Sharpless[a], Zoran Radić[a], Kasia Kaczanowska[b]

[a]*University of California, San Diego, La Jolla, CA, USA*
[b]*University of California, San Diego, La Jolla, USA*

Apart from obvious disparities of recognition capacities of AChE inhibitors or reactivators being directed to the transition state for catalysis and the nicotinic acetylcholine receptor (nAChR) recognizing predominant conformations of the native neurotransmitter or a congeneric pharmacological agent, distinguishing differences have emerged from the crystallographic structures and other physical studies of these two families of molecules. For AChE, inhibition is achieved with reversible ligands, by using slowly turning over substrates (carbamoylating agents) or by irreversible covalent conjugation with organophosphates (OP's). Initially shown by Harel, Sussman and Silman in *Torpedo*, and then by other groups, this is achieved by occupying the active center at the base of a deep gorge, either as a reversible complex or a covalent conjugate. High affinity reversible complexes are achieved by stabilization by cation-pi interactions or by a complement of partnering residues between inhibitor, as seen with donepezil, and the active center gorge base and walls. A peripheral anionic site has also been defined through interactions with propidium and other, more bulky ligands. Bisquaternary ligands can achieve extremely high affinities through occupation of both sites. Also under consideration are the reactivating agents, since, similar to the deacylation step of acetylcholine catalysis involving a water molecule, oxime reactivations of OD, show similar properties as the deacylation of the trigonal acetyl-enzyme in terms of substrate activation and inhibition, and its pH dependence. These phenomena will be analyzed by comparing enzymatic and general base catalysis. For the acetylcholine receptor (nAChR), we require a subunit interface for demonstrating cooperative binding and channel activation by agonists and contributions from both subunits at the subunit interface. Studies using the soluble acetylcholine binding protein as a crystallographic model and the homologous α7-AChR reveal three distinct modes of association; (1) the cation-pi model for quaternary amines at the subunit interface, also seen with AChE for its subunit internal gorge; (2) stabilization of secondary, tertiary amines and imines involving hydrogen bonding from a protonated basic nitrogen to a backbone carbonyl of a conserved tryptophan on the principal subunit interface, and (3) stabilization of less basic amines involving 4,6 di-substituted 2-aminopyrimidines. In each case, stabilization of the complexes and selectivity of binding depend on molecular determinants on both subunits. Crystallography forms the basis of structure-guided design, but for AChE and AChR complexes, solution-based, mechanistic studies with spectroscopy and mass spectrometry are invaluable complements to analyses and future structure-based antidote and drug design, yielding useful therapeutic outcomes.

Acetylcholinesterase knock-out mice develop osteoporosis: a role of the globular form of the enzyme in osteoblastic differentiation

Karl W. K. Tsim[a], Cathy W. C. Bi[a], Miranda L. Xu[a], Etta Y.L. Liu[a], Tina T. X. Dong[a]

[a]*Division of Life Science and Shenzhen Research Institute, The Hong Kong University of Science and Technology, Hong Kong, China*

Acetylcholinesterase (AChE) is a hydrolytic enzyme in cholinergic transmission; however, emerging evidences indicate that AChE exerts other functions in different systems. Here, we demonstrated that PRiMA-linked AChE, a tetrameric globular form, has a possible role in the differentiation process of bone cells. In *ACHE-/-* mice, a robust decrease of bone density and growth, as well as the levels of bone differentiation markers, e.g. ALP, ostonectin, osteocalcin, Runx2 and osterix, were markedly decreased in *ACHE-/-* mice. Bone tissues expressed mRNAs encoding AChE and PRiMA. In cultured rat osteoblasts and osteosarcoma MG-63 cells, PRiMA-linked AChE was the major form of enzyme detected. The expressions of AChE and PRiMA mRNAs, as well as PRiMA-linked AChE, were stimulated during the osteogenic differentiation. Over-expression of PRiMA-linked AChE in osteoblasts induced cell differentiation and expression of osteoblastic protein markers; however, this osteogenic effect of AChE did not require the enzymatic activity. In parallel, the differentiation process was markedly reduced in cultured AChE-depleted osteoblasts: the reduction was a result of defective signaling mediated by bone morphogenetic protein (BMP) and Wnt/β-catenin. These results showed the existence of PRiMA-linked AChE in cultured osteoblasts and bone tissue, which may participate in the osteogenic differentiation process regardless of its enzymatic activity.

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Novel modes of targeting allosteric sites at muscarinic receptors: Implications for drug discovery

Celine Valant[a]

[a]*Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia*

The muscarinic acetylcholine receptors (mAChRs) are prototypical members of the G protein-coupled receptor (GPCR) superfamily that regulate numerous fundamental processes in both the central and peripheral nervous system (1). Unfortunately, this family of receptors remains suboptimally targeted due to the very high degree of conservation of the orthosteric, acetylcholine-binding site across all 5 receptor subtypes. However, it is now well acknowledged that these (and other) GPCRs possess spatially distinct allosteric sites that provide greater selectivity in modulating receptor function (2). This novel type of subtype selectivity begs several questions: i) where does the selectivity come from? ii) where do allosteric modulators bind? Do they both share a common binding motif, or are they using a distinct set of residues within each receptor subtype? And finally, since virtually all GPCRs appear to display allosteric sites, iii) is it possible that each of these receptors possess endogenous allosteric ligands present physiologically, or perhaps pathologically (3)? To begin to answer these questions, we have recently solved the structures of the multiple mAChRs, including the activated M_2 mAChR bound to both an agonist and a positive allosteric modulator (4, 5), to understand the structural basis of orthosteric and allosteric ligand interaction with this important class of GPCRs. In addition, we have developed computational and chemical biology approaches to enriching allosteric ligand structure-activity studies (6–9). These recent findings will facilitate the development of new mAChR subtype-selective ligands that could prove useful for the treatment of numerous pathophysiological conditions.

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Royal jelly of honeybees and pluripotent stem cells of mammals: two examples for non-neuronal acetylcholine (ACh) and the non-neuronal cholinergic system

Ignaz Wessler[a]

[a]*Institute of Pathology, University Medical Center, Johannes Gutenbergy-University, D-55101 Mainz, Germany*

During the past two decades our understanding about the biological roles of ACh outside the nervous system has been markedly extended. The terms non-neuronal ACh and non-neuronal cholinergic system were established to describe the expression in almost all taxa (Wessler et al., 1998; Wessler and Kirkpatrick, 2008). Via auto- and paracrine modes of action non-neuronal ACh promotes cell proliferation, differentiation, regulation of cell-cell contact, locomotion and transport of ions and water. The present experiments describe the expression of non-neuronal ACh in honeybees and pluripotent stem cells. Royal jelly contains unusually high amounts (mM) of ACh (Colhoun and Smith, 1960). We confirmed these concentrations by HPLC-measurement and identified the hypopharyngeal gland of nursing honeybees as the source, where membrane-bound choline acetyltransferase [ChAT; 2.2 nmol/mg/h] is responsible for ACh synthesis. Also the brood food for working bee larvae contains mM ACh concentrations. ACh was removed from brood food by raising its pH and adding butyrylcholinesterase for testing in artificial larval breeding experiments. At day 5 the larval survival rate was higher with ACh-supplemented than with non-supplemented food. Chronic exposure of honeybee colonies to the neonicotinoids clothinanidin (1, 10 and 100 ppb) and thiacloprid (200 and 8800 ppb) reduced the ACh content in larval food and impaired ACh-synthesis, demonstrating a so far unknown adverse effect of these neonicotinoid pesticides. Murine embryonic stem cells express the complete non-neuronal cholinergic system (Kaltwasser et al., 2015). In the present experiments we also found ACh-like activity in the pellet and supernatant of 2 human iPS cell lines (ATCC-DYP0250; Gibco A18945). Moreover, the ATCC-DYP0250 cells expressed M2, M3 and M4 as well as multiple nicotinic receptor subunits (α3, α4, α9, ß1, ß3, ß4). In conclusion, larval food of honeybees, as well as pluripotent embryonic stem cells, represent examples for non-neuronal ACh and the non-neuronal cholinergic system. In honeybees, neonicotinoids can impair this system.

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POSTERS

Novel benzimidazole derivatives with multi functions on cholinesterase enzymes and neuroprotection

Beyza Ayazgok[a], Tuba Tuylu Kucukkilinc[a], Keriman Ozadali-Sari[b], Oya Unsal-Tan[b], Ayla Balkan[b]

[a]*Hacettepe University, Faculty of Pharmacy,Department of Biochemistry, Ankara, Turkey*
[b]*Hacettepe University, Faculty of Pharmacy,Department of Department of Pharmaceutical Chemistry, Ankara, Turkey*

Alzheimer's disease (AD) is a multifactorial neurodegenerative brain disorder with many contributers such as cholinesterase enzymes, β-amyloid (Aβ), tau protein and oxidative stress [1]. In this study we report design, synthesis and activity studies of novel benzimidazole derivatives as multifunctional anti-Alzheimer candidates.

The target compounds were synthesized via the route outlined in Scheme [1]. Chemical structures of the compounds were elucidated by FT-IR, ^{1}H-NMR, mass spectra and elemental analysis.

Scheme1: Synthetic route of the target compounds

Novel derivatives were tested for their potential to inhibit human AChE and equine BChE by Ellman's assay [2]. For cell culture studies SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1% antibiotics at 37°C in 5% CO_2. SHSY5Y cells (5000 cells/well) were treated with novel derivatives (10 mM) for 3 h prior to amyloid β 1–40 treatment (10 mM), and incubated for 24 h. The MTT reduction assay was performed to evaluate cell viability.

Results show cholinesterase inhibitory effects of novel candidates with IC_{50} values of 5–95 mM. Compounds presented a significant potential for neuroprotection against amyloid-induced cytotoxicity compared to the reference drug donepezil (100% and 60% respectively). The results indicated that the compounds are protective against β-amyloid induced cytotoxicity and good AChE/BChE inhibitors.

Keywords: Acetylcholinesterase, butyrylcholinesterase, Alzheimer's disease, neuroprotection

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miR-211 is a neuronal regulator of cholinergic-induced seizures

Uriya Bekenstein[a], Nibha Mishra[a], Dan Milikovsky[b], Amit Berson[a], Geula Hanin[a], David Greenberg[a], Alon Friedman[b], Hermona Soreq[c]

[a]*Department of Biological Chemistry, Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel*
[b]*Department of Physiology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel*
[c]*Department of Biological Chemistry and The Edmond and Lily Safra Center for Brain Sciences, Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel*

Epilepsy, one of the most common brain disorders, poses a world-wide health and social problem. Recent reports indicate that both cholinergic and microRNA (miRNA) mis-regulation are involved in generating the neuronal hyper-excitability and hyper-synchronization hallmarks of convulsive and non-convulsive epileptic seizures; however, how the healthy brain avoids such seizures remains unclear. Here, we report that miRNA-211 suppresses non-convulsive seizures via the cholinergic and the TGFβR-II pathways. Our working hypothesis predicted that neuronal miRNAs that regulate synaptic vesicle functioning could control neuronal hyper-excitability and seizure-related synchronized firing. By intersecting publicly available miRNA datasets we found miRNA-211 to be a putative regulator of synaptic vesicle processes, regulated by cholinergic-activation. Notably, neuronal miRNA-211 is located in the 15q13.3 chromosomal locus (OMIM #612001), in which heterozygote microdeletions result in widely differing degrees of mental retardation and recurrent epileptic seizures, and homozygous deletions entail epileptic encephalopathy. Engineered double-transgenic mice (dTg-211), over-expressing doxycycline-suppressible forebrain miRNA-211, showed impaired spatial learning and memory, but otherwise normal behavior. RNA-sequencing demonstrated that doxycycline-induced suppression of miR-211 excess induced a wide transcriptional change in the frontal cortex, particularly in genes involved in synaptic activity, Ca^{+2} transmembrane transport, TGFβR-II signaling and higher brain functions. Concordantly, electrocorticography (ECoG) documented spontaneous, non-convulsive seizures following miR-211 suppression. These events were accompanied by downregulation of the cholinergic muscarinic receptors. mAChR4 and mAChR2, known to negatively affect cholinergic synaptic transmission, and elevation in mAChR5, and the nicotinic nAChR5 and nAChR7, which have a reciprocal excitatory influence on synaptic transmission. Alongside the emergence of spontaneous seizures we noted a stepwise elevation of the convulsive-seizure related miR-134, which does not change following mild epileptic preconditioning, and the inhibition of which prevents prolonged seizure-suppressive effects. Taken together, our findings indicate that miR-211 downregulation may have a strong excitatory influence on CNS cholinergic transmission, and support cholinergic-mediated miRNA-211 control over neuronal hyper-excitability and synchronized firing, which limits the consecutive emergence of non-convulsive and convulsive seizures.

Acetylcholine controls the somatotropic axis during mouse development

Sylvie Berrard[a], Nélina Ramanantsoa[a], Marie-José Lecomte[b], Françoise Saurini[a], Chloé Bertolus[c], Jacques Callebert[d], Corinne Collet[d], Thomas Bourgeois[a], Maud Ringot[a], Jacques Mallet[e], Guilan Vodjdani[a], Jorge Gallego[a], Jean-Marie Launay[d]

[a]*PROTECT, UMR1141-INSERM/Université Paris-Diderot/ Sorbonne Paris Cité, Paris, France*
[b]*Endocells, Paris, France*
[c]*AP-HP, Paris, France*
[d]*AP-HP, InsermU942, Paris, France*
[e]*ICM, UMR S1127 UPMC/CNRS /Inserm, Paris, France*

Growth hormone (GH) is produced by somatotroph cells within the anterior pituitary gland and, through its release into the blood flow, exerts widespread actions involving multiple organs and physiological processes. Most of the effects of GH are mediated by hepatic Insulin-like Growth Factor (IGF-1), which is secreted in response to GH stimulation and circulates to target organs to produce a number of cellular responses. The activity of the GH/IGF-1 axis is regulated by a complex neuroendocrine system which includes two hypothalamic neuropeptides, GH-releasing hormone (GHRH) and somatostatin, and a gastrointestinal hormone, ghrelin. Several neurotransmitters, including acetylcholine (ACh), are also involved in tuning GH secretion. The pharmacological manipulation of the cholinergic tone, mainly performed at adulthood in humans and animals, revealed an overall stimulatory action of ACh on GH secretion. In the present study, we investigated the role of the cholinergic system in the regulation of the somatotropic axis during development.

We generated choline acetyltransferase knockout mice and showed that heterozygous animals display a transitory deficit in ACh levels during the developmental period. Detailed exploration of the pup's phenotype revealed that this developmental deficiency has no major impact on their weight development and cardiorespiratory status. In contrast, we found that endogenous ACh levels determine the concentrations of circulating GH and IGF-1 at late gestational and postnatal stages. Moreover, we showed that the cholinergic control of pituitary GH secretion is mediated, at least largely, by the regulation of GHRH, somatostatin and ghrelin production.

These findings demonstrate that ACh plays a crucial role in the regulation of somatotropic function during development and consequently in the control of physiological functions governed by GH and IGF-I.

Flavonoids induce the expression of acetylcholinesterase in cultured osteoblasts

Cathy W. C. Bi[a], Miranda L. Xu[a], Etta Y. L. Liu[a], Tina T. X. Dong[a], Karl W. K. Tsim[a]

[a]*Division of Life Science and Shenzhen Research Institute, The Hong Kong University of Science and Technology, Hong Kong, China*

Flavonoids, a group of natural compounds found in a variety of vegetables and herbal medicines, are well known to possess diverse biological effects. They are structurally very similar to estrogen and many of them display strong estrogen-like activities, which have been used as alternatives of estrogen. Recent researches have intensively reported on their ability to affect bone metabolism. In order to search for potential therapeutic agents against osteoporosis, different sub-classes of flavonoids were analyzed to determine their osteogenic activities. Here, we aimed to test if flavonoids could induce a cholinergic enzyme, acetylcholinesterase (AChE), as well as bone differentiation. In cultured rat osteoblasts, twenty flavonoids, deriving from Chinese herbs and having known induction of alkaline phosphatase (ALP) expression, were tested for induction of AChE expression. Eleven flavonoids showed induction, and five of them displayed robust activation of AChE expression, including baicalin, calycosin, genistin, hyperin and pratensein: the induction of AChE included the levels of mRNA, protein and enzymatic activity. Moreover, the flavonoid-induced AChE expression in cultured osteoblasts was in the proline-rich membrane anchor (PRiMA)-linked tetrameric globular form (G_4) only. In parallel, the expression of PRiMA was also induced by the application of flavonoids. The flavonoid-induced AChE in the cultures was not affected by the estrogen receptor blocker, ICI 182,780. Taken together, the induction of PRiMA-linked AChE in osteoblasts should be independent of the classical estrogen signaling pathway.

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In vivo efficacy assessment of a novel uncharged reactivator of a NOP-inhibited acetylcholinesterase based on a tetrahydroacridine pyridine-aldoxime hybrid in mouse compared to pralidoxime

André-Guilhem Calas[a], Aurélie Nervo[a], José Dias[a], Rachid Baati[b], Florian Nachon[a]

[a]*Institut de Recherche Biomédicale des Armées, Brétigny-sur-Orge, France*
[b]*UMR CNRS 7515 ICPEES, Strasbourg, France*

Organophosphorus nerve agents are highly toxic due to their strong inhibition potency against acetylcholinesterase. Indeed, inhibited acetylcholinesterase cannot hydrolyze acetylcholine, and the subsequent accumulation of this neurotransmitter into the synaptic cleft or neuromuscular junction leads to the over-stimulation of cholinergic receptors. Neurotransmission is seriously impaired, causing seizures in the central nervous system (CNS), muscle fasciculation and generally death by respiratory arrest. One of these most toxic nerve agents is VX, but a pesticide such as paraoxon shows similar toxic properties at the appropriate dose. Acetylcholinesterase inhibited by NOP (Neurotoxic OrganoPhosphorus) can be reactivated using powerful nucleophilic molecules, most commonly oximes, which are one major component of the emergency treatment in cases of nerve agent intoxication. But currently marketed oximes do not readily cross the blood-brain barrier and have, therefore, poor activity in the CNS.

We present here a comparative *in vivo* study on C57BL/6 and Swiss mice of pralidoxime and a reactivator based on 3-hydroxy-2-pyridinaldoxime. This new non-quaternary oxime has shown efficient reactivation properties *in vitro*. The lack of a permanent cationic charge should facilitate its blood-brain barrier crossing thus allowing reactivation of cholinesterases in the CNS. Blood clearance of both drugs was established by a new and simple pharmacokinetic method. The results obtained will be compared with survival tests on

VX-intoxicated and Paraoxon-intoxicated mice using the up-and-down method. The new oxime presents reactivation of NOP-inhibited AChE but also inhibition at higher doses. The potential toxic effects of such a dual behavior of this compound will be assessed, particularly on breathing.

A new structural landscape for ligand binding to the α7 nicotinic acetylcholine receptor

Gisela Camacho Hernandez[a], Katarzyna Kaczanowska[b], Michal Harel[b], MG Finn[c], Larissa Kohs[b], Larissa Bendicks[b], Christina Beck[b], Palmer Taylor[b]

[a]*Universidad Autonoma de Baja California-University of California San Diego, Tijuana, BC, Mexico*
[b]*University of California San Diego, La Jolla, California, USA*
[c]*Georgia Tech University, Atlanta, GA, USA*

In our previous studies we identified a series of 4,6-disubstituted 2-aminopyrimidines interacting with Acetylcholine Binding Proteins (*Lymnaea* AChBP) in a cooperative fashion [Kaczanowska K *et al.*, *Proc. Natl. Acad. Sci. USA* 111, 10749 (2014)]. To examine receptor interactions of this family of compounds on nicotinic acetylcholine receptors (nAChRs) and related pentameric ligand-gated ion channels, we employed HEK cells transfected with cDNA's encoding one of three requisite receptor subtypes: α7-nAChR, α4β2-nAChR and $5HT_{3A}R$, along with a fluorescent reporter. Initial screening of a series of over 50 2-aminopyrimidines showed only two of the compounds interacting with AChBP to be agonists on the α7-nAChR below 13.3 μM concentration. Based on X-ray crystal structures of AChBP in complex with the cooperative ligands and the results of receptor interaction, we designed a new subset of molecules that target α7-nAChR. Seventeen compounds were synthesized with a o,*m*-picolyl or dibenzyl moiety at the 4-position and a variety of heterocycles at the symmetric 6-position. These compounds were then characterized in cell-based functional assays containing one of the three receptors and a fluorescent reporter of Ca^{++} permeation. The most potent ligands, AC-171A2 and AC-171H, have α7-nAChR EC_{50}'s between 60-70 nM, reflecting selectivity for α7-nAChRs. Some members of the large family are α4β2partial agonists or antagonists at higher concentrations, while others show a high degree of selectivity as agonists for the a7-nAChR subtype. These ligands were also examined for binding to *Lymnaea stagnalis* and *Aplysia californica* AChBPs and show competition with epibatidine with K_d values in the low nM range for *Lymnaea* and typically somewhat higher K_d's for *Aplysia*. Selected *Ls*-AChBP ligands (AC-171A, AC-171A2, AC-171C, AC-171D) were then crystallized with AChBP, revealing a unique binding pose that differs from classical nicotinic agonists and antagonists and from the previously analyzed set of substituted 2-aminopyrimidines that displayed distinct cooperative interactions with AChBP. Orientations of the protein aromatic side chains in the subunit interfacial binding site, harbored behind the C loop, are distinctive for these complexes, suggesting new modes of binding at the agonist-antagonist site and perhaps an allosteric mode of action for heteromeric nAChRs, occurring at the subunit interface in the extracellular domain.

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TDP-43 regulates AChE expression and activity: possible implication in ALS

Maria-Letizia Campanari[a], Claire Legay[b], Edor Kabashi[c]

[a]*Institut du Cerveau et de la Moelle Épinière (ICM), Paris, France*
[b]*Centre de Neurophysique, Physiologie et Pathologie, Centre CNRS, UMR 8119, Université Paris Descartes, Sorbonne Paris Cité, Paris, France*
[c]*Sorbonne Université, Université Pierre et Marie Curie (UPMC), Université de Paris 06, Unité Mixte 75, Institut National de la Santé et de la Recherche Médicale (INSERM) Unité 1127, Centre National de la Recherche Scientifique (CNRS) Unité Mixte de Recherc, Paris, France*

TAR DNA-binding protein 43 (TDP-43) is a multifunctional RNA-binding protein, ubiquitously expressed, mainly localized in the nucleus, where it is implicated in several steps of RNA metabolism. Since TDP-43 is associated with a spectrum of neurodegenerative diseases, it is crucial to identify RNA and protein targets that could unravel disease onset [1]. Here, we show that TDP-43 interacts with acetylcholinesterase (AChE), one of the most multi-faceted proteins with a wide range of vital functions, also crucially linked with Amyotrophic Lateral Sclerosis (ALS) and Alzheimer's disease (AD) [2,3]. We modulated TDP-43 expression through knockdown by siRNA or overexpression of WT or mutant TDP-43 in SH-SY5Y cells. Both up and downregulation induce a significant decrease of AChE activity and protein content. We demonstrate that this decrease is linked to AChE mRNA destabilization. Interestingly, the decrease of TDP-43 expression causes a switch in AChE splicing, enriching the monomeric form, AChE-R. We also describe that three out of five different point mutations in TDP-43 identified in ALS patients induce changes in AChE messenger/protein level and activity. Furthermore, both TDP-43 partial depletion and mutants overexpression cause a down-regulation of the mRNA encoding for the Proline-Rich Membrane Anchor (PRiMA) that targets AChE to the plasma membrane. Although the impact of TDP-43/AChE interaction in disease pathology is still being determined, the results presented here can explain, at least in part, the AChE changes seen in AD brain [4] and the neuromuscular junction deficiency in ALS [5].

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Selective and sensitive near-infrared fluorescent probe for acetylcholinesterase imaging

Sovy Chao[a], Ludovic Jean[a], Eric Krejci[b], Pierre-Yves Renard[a]

[a]*Normandie Univ, COBRA, UMR 6014 & FR 3038, UNIV Rouen-Normandie, INSA Rouen, CNRS, IRCOF, 1 Rue Tesnières, Rouen, France*

[b]*Université Paris Descartes CNRS SSA COGNAC G UMR 8257 45 rue des Saints Pères, Paris, France*

Acetylcholinesterase is a well-known enzyme, important in neurosciences and toxicology. Detection of AChE illustrates the complexity of quantifying proteins, even enzymes that have remarkable properties and substrates. AChE remains a model to propose novel strategies to detect proteins by light. Here we derived fluorescent probes from huprine, one of the most evolved AChE inhibitors. Two Near Infra-Red (NIR) fluorescent probes HupNIR1 and HupNIR2 based on the huprine scaffold and cyanine 5.0 dyes have been synthesized and evaluated *in situ* for the detection of acetylcholinesterases in different tissues. As anticipated by the initial properties of huprine, both probes displayed a high affinity and selectivity for AChE vs BChE, with IC_{50} values in the nanomolar range and no non-specific binding in the tissues. HupNIR2 appears the best probe for AChE with a great selectivity and sensitivity for AChE even in a low AChE region like striatum. Moreover, the binding of HupNIR2 is affected when AChE is inhibited with toxic molecules such as organophosphates. This work provided a new tool to visualize AChE activity in biological tissues.

ESTHER database: update on the alpha/beta hydrolase fold superfamily of proteins

Arnaud Chatonnet[a], Pascale Marchot[b], Yves Bourne[b], Nicolas Lenfant[a], Thierry Hotelier[c]

[a]*Dynamique Musculaire et Métabolisme lab, INRA, Place Viala, Montpellier, France*

[b]*Architecture et Fonction des Macromolécules Biologiques lab, CNRS/AMU, Campus Luminy, Marseille, France*

[c]*Unité Informatique INRA-AgroM, Place Viala, Montpellier, France*

The database ESTHER (ESTerase and alpha/beta Hydrolase Enzymes and Relatives, (http://bioweb.supagro.inra.fr/esther), now cross-referenced in UniProt (http://www.uniprot.org), is growing at a fast pace: today it contains ca. 48.000 Gene-proteins grouped in 182 families [1,2,3]. As many as 1650 crystal structures covering 429 distinct members of the superfamily are available in the RCSB PDB; for more than 109 families at least one structure is known. For the human genome, 120 genes that encode proteins belonging to 53 families are referenced in the database. For only 36 of these proteins, a structure is available. For 25 of these genes, mutations have been associated with a disease. The database also includes 856 natural or artificial substitutions reported in 93 cholinesterases, carboxylesterases or non-catalytic cholinesterase-like proteins. These substitutions are spread along 235 positions of the 575 amino-acid sequence of Torpedo acetylcholinesterase, used as a reference. 203 distinct substitutions in acetylcholinesterase or carboxylesterase found in insecticide-resistant populations of 29 arthropod species are listed [4]. 618 inhibitors, 275 substrates, and 44 reactivators are included and indexed by InChL, PubChem, Canonical Smiles. An interface "LiKid" is being built for analysis and interpretation of enzyme kinetic data.

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Toward an innovative treatment of Alzheimer's disease: Synthesis and evaluation of multi-target directed ligands (MTDLs) targeting acetylcholinesterase (AChE) and alpha7 nicotinic acetylchloline receptors (alpha7 nAChRs)

Monika Cieslikiewicz-Bouet[a], Sovy Chao[b], Ludovic Jean[a], Romulo Araoz[c], Denis Servent[d], Nicolas Coquelle[e], Jacques-Philippe Colletier[e], Pierre-Yves Renard[a]

[a]*Normandie Univ, UMR 6014 CNRS COBRA, Univ Rouen, INSA Rouen, CNRS, Rouen, France*

[b]*Normandie Univ, COBRA, UMR 6014 & FR 3038, UNIV Rouen-Normandie, INSA Rouen, CNRS, IRCOF, 1 Rue Tesnières, Rouen, France*

[c]*CNRS, Insitut de Neurosciences Paris-Saclay, and CEA, iBiTecS, Service d'Ingenierie Moleculaire des Proteines, Toxines Recepteur et Canaux, Gif sur Yvette, France*

[d]*CNRS, Insitut de Neurosciences Paris-Saclay, and CEA, iBiTecS, Service d'Ingenierie Moleculaire des Proteines, Toxines Recepteur et Canaux, Gis sur yvette, France*

[e]*CNRS, Institut de Biologie Structurale, Grenoble, France*

Alzheimer's disease (AD) is a complex and progressive neurodegenerative disorder. The available therapy is limited to symptomatic treatment and its efficacy remains unsatisfactory. In view of the prevalence and expected increase in the incidence of AD, the development of an effective therapy is crucial for public health. Due to the multifactorial etiology of this disease, the multi-target-directed ligand (MTDL) approach is a promising method to search for new drugs for AD. Aiming at developing new MTDLs, this project consists in the development of new multifunctional agents, which will act simultaneously on the different players in AD pathology. This disease is incurable for the moment, only palliative and long-term treatment is available having acetylcholinesterase and alpha7 nicotinic acetylcholine receptor as molecular targets. In our synthetic work, we took advantage of ''click-chemistry'' to couple modified acetylcholinesterase inhibitors (Cl- or NH2-tacrine and Cl-NH2- and acyl-huprine) to azide-alkyne derivatized agonists of alpha7 nicotinic acetylcholine receptor in order to obtain a series of multi-target directed ligands which could ideally simultaneously interact with acetylcholinesterase and with the alpha7 nicotinic acetylcholine receptor.

Acetylcholinesterase inhibition by the synthesized two-target ligands ranged between 3 and 15 nM. The agonistic behaviour of the two-target compounds was evaluated by manual and automated two-electrode voltage clamp on Xenopus oocytes expressing human alpha7 nicotinic acetylcholine receptors. Among the double functionalized organic molecules, one compound showed an *in vitro* inhibitory activity towards human acetylcholinesterase on a

nanomolar scale and an agonistic activity in the micromolar range. Moreover, this compound showed a good ability to cross the blood-brain barrier.

Selective beta3-adrenoceptor agonists inhibit cholinergic activity in the urinary bladder: direct evidence in isolated porcine detrusor by 3H-acetylcholine release experiments

Gianluigi D'Agostino[a], Anna-Maria Condino[a], Stefano Palea[b]

[a]*Department of Drugs Science, University of Pavia, Pavia, Italy*
[b]*Palea Pharma & Biotech Consulting, Toulouse, France*

Adrenergic receptors of the beta$_3$-subtype (b_3-ADRs) represent a target for superior therapeutics for overactive bladder (OAB). This study aimed to set up a reliable experimental model for the screening of selective b_3-ADRs agonists in the urinary bladder.

Neuronal stores of porcine detrusor strips were labeled with tritiated acetylcholine (ACh) according to a validated protocol [1]. Electrical field stimulation (EFS) at 20 Hz produced [^{3}H]-ACh release into perfusion fluid and smooth muscle contraction, measured respectively by liquid scintillation spectrometry and by PowerLab apparatus in different experimental protocols (A and B).

Protocol A: two intermittent EFS caused [^{3}H]-ACh release (S_1 or S_2) and peak tensions (C_1 or C_2). The neural and muscular effects of drugs were expressed as the respective ratio (S_2/S_1; C_2/C_1), in comparison with the equivalent ratio in the absence of the drug. Concentration-response curves (CRCs) using a non-cumulative protocol were constructed for isoprenaline and mirabegron in the absence and presence of the selective b_3-ADRs antagonist L-748,337. Drug potencies were calculated as -log EC_{50} and pK_b values.

Protocol B: continuous EFS was applied for 75 min and, when reproducible evoked-responses were observed, two periods of [^{3}H]-ACh release (P_1 and P_2) and contraction (T_1 and T2) were calculated. The % change in the respective ratios (P_2/P_1 and T_2/T_1) in the presence of drug compared to controls was taken as a measure of neural and muscular effects caused by subtype-preferring activators or inhibitors for K^+ channels (BK_{Ca} and SK) [2].

Results: Intermittent EFS-evoked [^{3}H]-ACh and contractile response mostly reflect the release of neural TTX-sensitive ACh from cholinergic terminals. Isoprenaline and mirabegron produced inhibitory CRCs for the two EFS-evoked effects, both competitively antagonized by L-748,337, with potencies similar to those shown in human detrusor [1].

Continuous EFS-evoked [^{3}H]-ACh release and contractile responses were affected to a different extent by activators (NS1619 and SKA-31) or inhibitors (paxilline and apamine) for BK_{Ca} and SK channels, respectively. The most significant variations were observed for apamine and SKA-31, which respectively increase and decrease both effects by about 40%.

Our results are consistent with the presence of b_3-ADRs at pre- and postjunctional sites and demonstrate that the pig represents a reliable animal species for the screening of selective b_3-ADRs agonists for OAB therapy. Moreover, we suggest that SK channels are involved in modulating ACh release.

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Hupresin, a new affinity resin to purify butyrylcholinesterase

Emilie David[a], Cyril Ronco[b], Ludovic Jean[b], Xavier Brazzolotto[c], Florian Nachon[c], Pierre-Yves Renard[b]

[a]*CHEMFORASE, Mont-Saint-Aignan, France*
[b]*COBRA, Université de Rouen, Mont-Saint-Aignan, France*
[c]*IRBA, Brétigny-sur-Orge, France*

Poisoning by organophosphorus nerve agents (OPNAs) and pesticides is a serious public and military health issue. Butyrylcholinesterase has been shown to inhibit OPNAs and is currently developed as a prophylactic countermeasure to prevent the effects of OPNA poisoning.

A new class of huprine derivatives functionalized at position 9 has been developed as potent cholinesterase inhibitors [1-3]. One of the huprine derivatives presented a high affinity towards butyrylcholinesterase and acetylcholinesterase and was coupled to a sepharose matrix to generate a new affinity resin named Hupresin. Hupresin is an innovative technology whose characteristics are adapted to the very efficient purification of endogenous or recombinant butyrylcholinesterase [4,5]. It presents obvious advantages in terms of yield, purity, ease of use and time saving, compared to the procaïnamide sepharose resin which is classically used to purify butyrylcholinesterase. This new chromatographic support will help to reduce the costs associated with the production of butyrylcholinesterase-based nerve agents bioscavengers.

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Design, synthesis and *in vitro* evaluation of a promising new class of bifunctional uncharged hybrid reactivators for nerve agent-inhibited human acetylcholinesterase

José Dias[a], Julien De Sousa[b], Anne-Julie Gastellier[a], Catherine Rousseau[a], Charlotte Courageux[a], Christopher Timperley[c], Richard Brown[d], Gianluca Santoni[e], Martin Weik[e], Rachid Baati[f], Florian Nachon[a]

[a]*Institut de Recherche Biomédicale des Armées, Brétigny-sur-Orge, France*
[b]*Université de Strasbourg, ICPEES, UMR CNRS 7515, University of Southampton, Strasbourg, France*
[c]*Dstl Porton Down, Salisbury, UK*
[d]*University of Southampton, Southampton, UK*
[e]*Institut de Biologie Structurale, Grenoble, France*
[f]*Université de Strasbourg, ICPEES, UMR CNRS 7515, Strasbourg, France*

Acetylcholinesterase (AChE) is a key enzyme of the Central Nervous System (CNS) hydrolyzing the neurotransmitter acetylcholine. By targeting AChE, OPNAs (OrganoPhosphorus Nerve Agent) and organophosphorus pesticides irreversibly inhibit cholinergic transmission leading to certain death if untreated. The current treatment available in the French army consists of an auto-injector containing a methanesulfonate salt of 2-PAM for AChE reactivation, an anticholinergic drug, atropine and avizafone, a prodrug of diazepam for limiting convulsions. However, this treatment displays

major drawbacks in terms of CNS bioavailability, restricted spectrum of action and effectiveness.

The aim of this project is to develop a new class of more efficient human nerve agent-inhibited acetylcholinesterase. We designed, synthesized and evaluated a new class of bifunctional uncharged hybrid reactivators composed of a 3-hydroxypyridinaldoxime linked to a tacrine derivative. The *in vitro* efficacy of these reactivators has been assessed. We show that this new class of reactivators outperform HI-6 in restoring human AChE activity inhibited by VX, sarin, tabun and paraoxon. By X-ray crystallography, we have been able to observe some of these new hybrids inside the catalytic site of *Tc*AChE.

Dopaminergic/mu opioid modulation of tobacco smoking release of cortisol and prolactin

Edward Domino[a], Mika Hirasawa-Fujita[a]

[a]*University of Michigan, Ann Arbor, USA*

Purpose: The mass dose effects of carfentanil, a mu opioid agonist, and raclopride, a dopamine D2/3 antagonist, were determined on venous plasma cortisol and prolactin levels before and after tobacco smoking in 24 overnight abstinent healthy male smokers.

Methods: The total [^{11}C] labeled plus unlabeled doses of each radioligand were used to assess the association between doses of the ligands and hormone levels by linear regression analyses.

Results: Peak cortisol levels after average nicotine (avnic) tobacco smoking were decreased with greater carfentanil doses ($p = 0.03$). The change in plasma cortisol levels after avnic smoking compared to before avnic smoking was also decreased with greater carfentanil doses ($p = 0.003$). Unexpectedly, the changes in cortisol levels after denicitinized (denic) tobacco smoking compared to before denic smoking were increased with greater carfentanil doses ($p = 0.04$). No dose effects of raclopride were found on cortisol levels. Peak prolactin levels after avnic smoking were increased with greater raclopride doses ($p = 0.003$). The changes in prolactin levels after avnic smoking compared to before were also increased with greater doses of raclopride ($p < 0.001$). There was no effect of carfentanil on plasma prolactin.

Conclusion: As expected, no correlations between the doses of the radioligands and the basal hormone levels were found. However, in the presence of nicotine tobacco smoking the PET ligands had significant subtle clinical pharmacological effects on both cortisol and prolactin.

Nicotine upregulation of alpha7 (α7) nAChR IN Xenopus oocytes

Joseph Farley[a], Mohammad Islam[a], Patrick Schwartz[a], Kristi Deboeuf[a], Jayharsh Panchal[a], Thangaraju Murugesan[b], Jed Rose[b]

[a]*Indiana University Bloomington, Bloomington, USA*
[b]*Duke University Medical Center, Durham, USA*

The alpha7 (α7) subtype of nAChR appears to play critical roles in learning, cognition, and various neuropathologies including nicotine addiction. Nicotine-upregulation of α7 Rs may play a significant role in the latter phenomenon. But whether nicotine-upregulation of α7 Rs reliably occurs, and its underlying mechanism (s), are largely unknown. Previous studies of α7 nAChRs heterologously expressed in *Xenopus* oocytes failed to observe nicotine-upregulation. These failures might have been due to intracellular accumulation of nicotine and its subsequent slow release from oocytes, resulting in desensitization of α7 Rs during functional assays. In our experiments, 12-14 h exposure to nicotine (100 μM) 4-5 days post cRNA-injection (PI), followed by extensive washout, yielded statistically-significant ~2-fold increases in peak α7 currents and net charge as determined by TEVC and α7-protein (by Western blot). Less-extensive washout, as well as 100 nM nicotine incubation, failed to produce upregulation. Instead, inactivated/desensitized currents were observed. GC/MS measurement of nicotine in the washout fluid confirmed that nicotine was continually released from oocytes. The concentration was ~20 nM at 3-4 h following washout onset, well in excess of the ~ 3 nM IC_{50} value. Exposure of α7-expressing oocytes to cumulative washout fluid suppressed/desensitized α7 currents (~ 8% of controls). Similar to nicotine, methyllycaconitine (MLA), a cell-permeable competitive antagonist of α7 Rs, and carbachol, a stable membrane-impermeable agonist, also produced ~ 2×-upregulation. However, ACh incubation (which can be hydrolyzed by oocytes) failed to produce upregulation. We also found that chelation of intracellular Ca^{2+} by BAPTA-AM completely blocked nicotine upregulation. However, elimination of extracellular Ca^{2+} had no effect. These results suggest that persistent ligand-binding to α7 Rs (but not necessarily channel gating), leading to increases in $[Ca^{2+}]_i$, was critical for α7-upregulation. Several Ca^{2+}-dependent signaling pathways were implicated in nicotine-upregulation of α7, including calcineurin/PP2B and PKC. In contrast, PTK-signaling pathways, while potent regulators of upregulation of α7 Rs in their own right, do not appear to participate in nicotine-upregulation (in oocytes). For example, genistein (100 μM), a potent broad-spectrum PTK inhibitor (and PAM) at α7, produced ~ 5×-upregulation, after 24 h exposure followed by washout. The combination of genistein and nicotine produced upregulation that appeared to be *supra*-additive; genistein failed to occlude nicotine's upregulation. The preceding major conclusions concerning functional upregulation were also confirmed for numerical upregulation of α7, as determined by Western blot of plasmamembrane protein and an α7 antibody (Chemicon AB15322).

Reactivation potential of non-quaternary cholinesterase reactivators

Martina Hrabinova[a], Vendula Sepsova[a], Ondrej Soukup[b], Jan Misik[c], Jitka Turanova[a], Daniel Jun[d]

[a]*University of Defence, Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy, Hradec Kralove, Czech Republic*
[b]*Biomedical Research Center, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic*
[c]*University of Defence, Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy, Hrade kralove, Czech Republic*
[d]*University of Defence, Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy, Hradec kralov, Czech Republic*

Cholinesterase reactivators are able to restore acetylcholinesterase (AChE) activity at cholinergic synapses during organophosphorus poisoning (OP). An example of such compounds, are HI-6, obidoxime and pralidoxime commonly used for the treatment of OP poisoning. Nevertheless, reactivation potency is

usually limited due to poor penetration across the blood-brain barrier (BBB). Thus, non-quaternary (uncharged) reactivators are tested, to see if they reach a higher therapeutic concentration in the brain in comparison with standard therapy using quaternary (charged) reactivators such as HI-6.

In this study, three novel uncharged oximes have been evaluated *in vitro*. Their ability to reactivate sarin, tabun, and paraoxon-inhibited human AChE and human butyrylcholinesterase (BChE) was measured by Ellman's spectrophotometric method. Two concentrations of reactivators were chosen for testing (10^{-4}; 10^{-5} M). The lower oxime concentration (10^{-5} M) is attainable after administration via autoinjectors. The activity of non-quaternary reactivators was compared to the reference oxime HI-6. Regarding the reactivation of sarin-inhibited AChE and BChE, all new compounds showed lower reactivation potency compared with HI-6. On the other hand, all three compounds presented potential to reactivate tabun-inhibited AChE more than HI-6 and two of them were able to reactivate tabun-inhibited BChE at 10^{-4} M. In addition, two compounds showed a better reactivation potency for paraoxon-inhibited AChE and all tested antidotes displayed a better reactivation ability of paraoxon-inhibited BChE.

All benefits (lipophilicity, better reactivation potency of tabun-inhibited AChE) and negatives (e.g. low solubility in hydrophilic media) of quaternary versus non-quaternary reactivators should be considered to decide whether there is some potential in this new strategy of antidotal therapy.

Binding of N-methylscopolamine to the allosteric site of M2 muscarinic acetylcholine receptor

Jan Jakubík[a], Alena Randáková[a], Vladimír Doležal[a]

[a]*Institute of Physiology CAS, Prague, Czech Republic*

Interaction of orthosteric ligands with extracellular domains was described at several aminergic G protein-coupled receptors, e.g. β-adrenergic, adenosine, including muscarinic acetylcholine receptors. The competitive antagonists quinuclidinyl benzilate (QNB) and N-methylscopolamine (NMS) bind to the binding pocket of the muscarinic acetylcholine receptor formed by transmembrane α-helices. We show that high concentrations of either QNB or NMS slow down dissociation of their radiolabeled species from all five subtypes of muscarinic acetylcholine receptors, suggesting allosteric binding. The affinity of NMS at the allosteric site is in the micromolar range for all receptor subtypes. Using site directed mutagenesis and molecular modelling of the M_2 receptor we demonstrate that E172 and E175 in the second extracellular loop and N419 in the third extracellular loop are involved in allosteric binding of NMS. The allosteric binding site of NMS overlaps with the binding site of some allosteric, ectopic and bitopic ligands. Understanding of interactions of NMS in the allosteric binding site is essential for correct analysis of binding and action of these ligands.

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Cytotoxicity study of novel centrally acting non-quaternary acetylcholinesterase reactivators

Daniel Jun[a], Lubica Muckova[a], Martina Hrabinova[a], Vendula Sepsova[a], Petr Jost[a]

[a]*Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic*

Acetylcholinesterase (AChE; EC 3.1.1.7) reactivators based on pyridinium aldoximes are used as causal antidotes in case of nerve agent or pesticide poisoning. Due to the presence of one or two quaternary nitrogens, they have low blood-brain barrier (BBB) permeation and thus they are not capable to fully reactivate phosphylated AChE in the central nervous system, where nerve agents or pesticides can be responsible for the acute cholinergic crisis or chronic neural disorders.

Development of novel centrally acting reactivators able to cross more efficiently the BBB is one of the most promising strategies. However, several drawbacks of physico-chemical, pharmacological and toxicological origin are expected.

In our work, the cytotoxicity of novel non-quaternary AChE reactivators was compared with the most potent AChE reactivators deployed in the NATO armies, like asoxime and methoxime. The testing was performed on the human neuroblastoma cell line SH-SY5Y and hepatoma cell line HepG2 using a standard MTT assay. The IC_{50} values were calculated for the comparison of cytotoxicity. After 4 h of incubation, fluorogenic 2,7-dichlorofluorescein diacetate dye was utilized to measure the generation of reactive oxygen species.

The tested reactivators showed different cytotoxicity effects. The novel centrally acting reactivators have IC_{50} values in the micromolar range where as the methoxime and asoxime were toxic in the millimolar range.

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Relationship of AChE inhibition and hippocampal ACh levels in mice

Jochen Klein[a], Franziska Mohr[a], Eric Krejci[b], Sebastian Lobentanzer[a]

[a]*Goethe University, Frankfurt, Germany*
[b]*Université Paris Descartes, Paris, France*

In a series of investigations in mice, we have characterized extracellular concentrations of acetylcholine (ACh) and its relationship to acetylcholinesterase (AChE) activity by microdialysis. In normal mice, ACh levels in hippocampal dialysates were 1.03 ± 0.15 nM (N = 24). This value was approximately doubled to 1.86 ± 0.20 nM in mice that were heterozygous for AChE and had 40% less AChE activity in brain homogenates [1]. Very high ACh levels (175.6 ± 64.2 nM) were measured in mice that did not express any AChE (knockout mice) [2]. Similarly high levels were obtained in mice which did not express the proline-rich membrane anchor (PRiMA), a transmembrane protein that organizes AChE into its tetrameric, functional, synaptic form; these mice had less than 10% of AChE activity when compared to wild-type levels [3,4]. These findings, together with data obtained in mice treated with AChE inhibitors, show that brain extracellular ACh levels are inversely correlated to AChE activity (ACh levels ≈ 1/AChE activity). In Alzheimer's disease, AChE inhibitors are given as

therapeutic agents and, according to PET studies, reduce AChE activity by 20-30% in a therapeutic setting. Judging from murine data, this extent of AChE inhibition would not be sufficient to increase extracellular ACh levels by more than 50%. Adverse effects due to systemic inhibition of AChE, especially in the parasympathomimetic system, preclude a higher and possibly more effective dosing regimen for AChE inhibitors.

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Development of multi-target drugs for Alzheimer's disease based on acetylcholinesterasse inhibitors

Jan Korabecny[a], Ondrej Soukup[a], Jan Ricny[a], Katarina Spilovska[a], Eugenie Nepovimova[b], Kamil Kuca[b]

[a]National Institute of Mental Health, Klecany, Czech Republic
[b]University Hospital Hradec Kralove, Hradec Kralove, Czech Republic

Alzheimer's disease (AD) is a multifactorial disorder and apparently involves several different etiopathogenetic mechanisms. To date, there are no curative treatments or effective disease modifying therapies for AD. On the other hand many aspects of AD are currently debated or even unknown. Current efforts in the development of novel drugs aimed against AD are represented by the so-called Multi-Target- Directed Ligands (MTDLs), the therapeutic strategy followed not only in AD research but also for other diseases. MTDLs combine drugs action at different levels of the neurotoxic cascade. MTDLs represent a challenging approach giving people suffering from AD a new hope to slow down or even cure this insidious disease. Within our contribution, novel trends in design and development of MTDLs based on a tacrine scaffold as potential anti-AD drugs will be presented.

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siRNA-mediated downregulation of aromatase decreases acetylcholinesterase specific activity and mRNA levels

Tuba Tuylu Kucukkilinc[a]

[a]Hacettepe University, Faculty of Pharmacy, Department of Biochemistry, Ankara, Turkey

Estrogen is the primary female sex hormone, which also contributes to many states in brain such as cognition, mood, pain and neuroprotection [1]. By converting C19 steroids to estrogen, Aromatase (CYP19) is the major source of postmenopausal estrogen levels [2]. Although aromatase is suggested to have neuroprotective effects, the underlying mechanism is still not clear.

Estrogen influences on cholinergic neurons are reported within cerebral cortex and hippocampus. Previous studies have shown estrogen induces choline acetyl transferase (ChAT) and acetylcholine release [3]. In the present study I report that short-interfering RNA-mediated aromatase silencing reduces acetylcholinesterase (AChE) mRNA level and spesific activity in SH-SY5Y cells.

SH-SY5Y cells were seeded in 6-well plates at a density of 3×10^5 cells per well, and cultured in DMEM at 37°C with 5% CO_2. Cells were transfected with siRNA targeting aromatase. Downregulation of aromatase mRNA was analyzed by Real Time PCR. To justify the downregulation, decreases of estradiol (E2) levels were verified using a commercial elisa kit. AChE activity of SH-SY5Y cells was determined using the Ellman method [4].

My findings indicated that silencing aromatase in SH-SY5Y cells showed a significant decrease in AChE mRNA levels (98%, $p < 0.0001$) and specific activity (64%, $p < 0.005$). Taken altogether, these results prove a possible interaction between aromatase and acetylcholinesterase providing new insights to enlighten our understanding of the mechanism of Alzheimer's disease.

Keywords: Aromatase, acetylcholinesterase, Alzheimer's disease.

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Design of bacterial expression systems of poorly studied three-finger human proteins Lypd6 and Lypd6b

Dmitrii Kulbatskii[a], Evgeny Loktyushov[b], Mikhail Shulepko[a], Zakhar Shenkarev[b], Alexander Paramonov[b], Dmitry Dolgikh[a], Ekaterina Lyukmanova[a]

[a]Lomonosov Moscow State University, Moscow, Russian Federation
[b]Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow, Russian Federation

Human Lypd6 and Lypd6b are members of the Ly6/uPAR protein family. Ly6/uPAR proteins share characteristic a "three-finger" fold which is stabilized by four disulfide bonds. Lypd6 plays important roles in embryogenesis by modulating the activity of the Wnt/β-cathenin pathway. It was shown that Lypd6 interacts with both Lrp6 and Frizzled8 proteins, the members of the receptor complex on the cellular membrane. Lypd6b has a high sequence homology with Lypd6 (~ 60%). It is proposed that both proteins are targeting nicotinic acetylcholine receptors. At present, the biological role of Lypd6b is unknown. Lypd6 and Lypd6b are tethered to the membrane with a GPI anchor. Because the production of GPI-linked Lypd6 and Lypd6b as individual proteins represents an unfeasible task, and functional and structural studies of proteins in such form is difficult, it is important to develop recombinant expression systems for production of Lypd6 and Lypd6b in a soluble form.

The main goal of the present work was the design of efficient expression systems for both Lypd6 and Lypd6b. These three-finger proteins contain twelve cysteine residues which form six disulfide bonds. Thus the recombinant production of these proteins in correct structural form is not a simple task. We developed bacterial expression systems for Lypd6 and Lypd6b combining protein production in the form of inclusion bodies with subsequent refolding. These systems allowed us to obtain milligram quantities of both Lypd6 and Lypd6b and their isotope-labeled ^{15}N-^{13}C and ^{15}N analogues. The structural properties of Lypd6 and Lypd6b have been explored using high-resolution heteronuclear NMR spectroscopy and resulting spectra were typical for proteins with well-defined spatial structure. Engineering of systems for recombinant production of Lypd6 and Lypd6b opens new perspectives for the future study of their role in organism functioning.

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Amyloid beta peptides act as allosteric modulators of cholinesterases

Rajnish Kumar[a], Taher Darreh-Shori[a]

[a]*Dept of Neurobiology, Care Sciences and Society, Center for Alzheimer Research, Division of Translational Alzheimer Research, Karolinska Institutet, Stockholm, Sweden*

Amyloid beta peptides are universally produced and released in an action potential synchronized manner into the interstitial fluids in the brain. Their native function is unknown. However, the amyloid hypothesis of neurodegenerative disorders such as Alzheimer Disease (AD) ascribes them as toxic for the neurons [1]. The central cholinergic signaling is also affected selectively and early in the AD brain without clear reasons. Various reports suggest that the Aβ interacts with BuChE and apoE [2]. We show that Aβ peptides interact readily in an apoE protein-facilitated manner with BuChE, forming highly stable and soluble BuChE-Aβ-ApoE-complexes (BAβACs), which can be separated in their native states by the sucrose density gradient technique. The enzymological analyses revealed that Aβ concentration-dependently increased the ACh-hydrolyzing capacity of cholinesterases. This was further supported with *in silico* molecular modeling studies. The docking studies deciphered the active site amino acid residues responsible for the Aβ-ChE molecular interaction in formation of BAβACs. In the case of BuChE, the results indicated that Aβ interacts with a putative activation site at the mouth of its catalytic tunnel, most likely leading to increased ACh influx into the catalytic site, and thereby increasing the intrinsic catalytic rate of BuChE. The current study proposes that one of the native functions of amyloid beta peptides is to allosterically modulate the cholinesterases through formation of soluble, ultra-reactive ACh-hydrolyzing complexes termed BAβACs, which may alter both synaptic and extracellular ACh-signaling.

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Evolution of animal cholinesterases

Nicolas Lenfant[a], Murray E. Selkirk[b], Thierry Hotelier[c], Yves Bourne[a], Pascale Marchot[a], Arnaud Chatonnet[d]

[a]*Architecture et Fonction des Macromolécules Biologiques lab, CNRS/AMU, Campus Luminy, Marseille, France*
[b]*Department of Life Sciences, Imperial College, London SW7 2AZ, UK*
[c]*Unité Informatique INRA-AgroM, Place Viala, Montpellier, France, Montpellier, France*
[d]*Dynamique Musculaire et Métabolisme UMR866 INRA University of Montpellier, Montpellier, France*

Cholinesterases emerged from a family of enzymes and proteins with adhesion properties. This family is absent in plants and expanded in multicellular animals [1,2]. True cholinesterases appeared in triploblastic animals together with the cholinergic system. Lineage specific duplications resulted in two acetylcholinesterases in most hexapods and in up to four genes in nematodes and arachnids. In the vertebrates, the duplication leading to acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) is now considered to be an ancient event which occurred before the split of the Ostheichthyes [3]. The product of one or the other of the paralogues is responsible for the physiological hydrolysis of acetylcholine, depending on the species lineage and tissue considered. The BChE gene seems to have been lost in some fish lineages [4]. The complete genome of amphioxus (*Branchiostoma floridae*: cephalochordate) or Saccoglossus kowalevskii (Acorn worm) contains a large number of duplicated genes or pseudogenes of cholinesterases [5]. Parasitic nematodes or ticks also show amplification of cholinesterase like genes. The mode of attachment through alternative C-terminal exons seems to have evolved independently from the catalytic part of the gene.

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Erythropoietin (Epo)-induced signaling is interfered with by acetylcholinesterase (AChE) in erythroblasts and neurons

Etta Y. L. Liu[a], Wilson K. W. Luk[a], Cathy W. C. Bi[a], Miranda L. Xu[a], Tina T. X. Dong[a], Karl W. K. Tsim[a]

[a]*Division of Life Science and Shenzhen Research Institute, The Hong Kong University of Science and Technology, Hong Kong, China*

Acetylcholinesterase (AChE) is a key enzyme in terminating cholinergic neurotransmission in the nervous system. AChE is encoded by a single gene and occurs in different isoforms by alternative splicing, $AChE_R$ as monomer, $AChE_H$ as dimer and $AChE_T$ as oligomer via association with proline-rich membrane anchor (PRiMA) or collagen Q (ColQ). Apart from this cholinergic function, emerging evidence suggests additional non-enzymatic functions of AChE in different tissues. Our preliminary findings demonstrate ACHE knock-out mice suffer from anemia, which is a result of a defect of erythropoietin (Epo) signaling during

erythropoiesis. The Epo-induced gene expression in erythroblasts is interfered with by AChE, and this interference is triggered by a physical association of Epo receptor (EpoR) with AChE protein on the cell surface. In addition, this Epo signaling interference is revealed in cultured neurons. Here, we hypothesize that AChE, localized on the cell surface, interferes with Epo signaling by binding to EpoR in erythroblasts and neurons. To test this hypothesis the binding, direct or indirect, of AChE to EpoR, and in parallel the signaling triggered by Epo is revealed in AChE knock-out or over-expressed erythroblasts and/or neurons. The dimeric lipid raft (G2) AChE is expressed in erythroblasts, while neurons, express mainly PRiMA-linked tetramer (G4): The binding of EpoR to two forms of AChE could be different in two specific cell types. Establishing this novel function of AChE in Epo-induced signaling will not only provide new insight into the non-enzymatic function of AChE, but will also contribute to the understanding of cell differentiation in general both is erythroblasts and neurons. Specifically, the elucidation of molecular mechanism(s) by which AChE induces blood/neuron differentiation will forge a new direction in developing therapeutic modalities targeting anemia and neurodegenerative diseases.

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Deletion of α2* nicotinic acetylcholine receptors ablates learning and memory in maternal nicotine treated adolescent offspring

Shahrdad Lotfipour[a]

[a]*UC Irvine, Irvine, USA*

Maternal cigarette smoking is linked with deficits in learning and memory in adolescent offspring. The neurocircuitry regulating the mechanisms of learning and memory coincide with the expression patterns of α2* nicotinic acetylcholine receptors (nAChRs) (Ishii et al., 2005). Targeted deletion of α2* nAChRs (*Chrna2*^{KO}) influences emotional memory processing and context-dependent nicotine withdrawal in adult mice (Lotfipour et al., 2013). Adolescent hypersensitive α2$^{L9'S/L9'S}$ nAChRs (*Chrna2*$^{L9'S}$) mice exhibit enhanced nicotinic facilitation of long-term potentiation in the hippocampal CA1 and baseline deficits in learning and memory that are rescued by acute nicotine exposure (Lotfipour et al., *under revision*). The findings demonstrate that α2* nAChRs can influence adolescent hippocampal synaptic plasticity and learning and memory. Maternal nicotine exposure is hypothesized to influence the learning and memory consequences of maternal nicotine exposure via α2* nAChRs (Nakauchi et al., 2015, Chen et al., 2016). Our current studies test this hypothesis in maternal nicotine treated adolescent wild type and *Chrna2*^{KO} mice using a pre-exposure dependent contextual fear conditioning paradigm. The findings demonstrate that the deletion of α2* nAChRs ablates learning and memory in adolescent maternal nicotine treated offspring.

Lynx1 competes with Aβ1-42 for the binding to nicotinic acetylcholine receptors

Ekaterina Lyukmanova[a], **Maria Arvaniti**[b], **Majbrit Jensen**[b], **Mikhail Shulepko**[c], **Dmitry Dolgikh**[c], **Lars Pinborg**[d], **Wolfgang Haertig**[e], **Jens Mikkelsen**[d], **Mikhail Kirpichnikov**[c], **Morten Thomsen**[b]

[a]*Lomonosov State University, Moscow, Russian Federation*
[b]*University Hospital Copenhagen, Copenhagen, Denmark*
[c]*Lomonosov Moscow State University, Moscow, Russian Federation*
[d]*University of Copenhagen, Copenhagen, Denmark*
[e]*Paul Flechsig Institute for Brain Research, University of Leipzig, Leipzig, Germany*

Lynx1 regulates synaptic plasticity in the brain by regulating nicotinic acetylcholine receptors (nAChRs). It is not known to what extent Lynx1 can bind to endogenous nAChR subunits in the brain or how this interaction is affected by Alzheimer's disease pathology.

We apply affinity purification to demonstrate that a water-soluble variant of human Lynx1 (ws-Lynx1) isolates α3, α4, α5, α6, α7, β2, and β4 nAChR subunits from human and rat cortical extracts, and rat midbrain and olfactory bulb extracts, suggesting that Lynx1 forms complexes with multiple nAChR subtypes in the human and rodent brain. Incubation with ws-Lynx1 decreases nicotine-mediated ERK phosphorylation in PC12 cells, indicating that binding of ws-Lynx1 is sufficient to inhibit signaling downstream of nAChRs. The effect of nicotine in this assay is independent of α7 or α4β2 nAChRs, suggesting that Lynx1 can affect the function of native non-α7, non-α4β2 nAChR subtypes. Finally, oligomeric Aβ1-42 inhibits the interaction between ws-Lynx1 and α3, α4, α5, and α7 nAChR subunits. We further show that ws-Lynx1 prevents Aβ1-42-induced cytotoxicity in cortical neurons, and cortical Lynx1 levels are decreased in a transgenic mouse model with concomitant Aβ and tau pathology.

Our data suggest that Lynx1 binds to multiple nAChR subtypes in the brain and that this interaction may have functional and pathophysiological implications.

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Human secreted Ly-6/uPAR related proteins: insights into specificity of interaction with acetylcholine receptors. Part 2: SLURP-2 regulates keratinocytes proliferation by interaction with different types of acetylcholine receptors

Ekaterina Lyukmanova[a], **Mikhail Shulepko**[a], **Zakhar Shenkarev**[b], **Dmitrii Kulbatskii**[c], **Maxim Bychkov**[b], **Alexander Paramonov**[b], **Anton Chugunov**[b], **Maria Arvaniti**[d], **Eva Dolejsi**[e], **T Schaer**[f], **Morten Thomsen**[g], **Vladimir Doležal**[e], **Daniel Bertrand**[f], **Mikhail Kirpichnikov**[c], **Dmitry Dolgikh**[c]

[a]*Lomonosov State University, Moscow, Russian Federation*
[b]*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow, Russian Federation*
[c]*Lomonosov Moscow State University, Moscow, Russian Federation*
[d]*University Hospital Copenhagen, Copenhagen, Denmark*
[e]*Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic*
[f]*HiQScreen Sàrl, Geneva, Switzerland*
[g]*Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen, Denmark*

Secreted Ly-6/uPAR protein SLURP-2 represents an alternative splicing isoform of the membrane-tethered neuromodulator Lynx1.

SLURP-2 regulates growth and differentiation of epithelial cells. Here we describe the structure and pharmacology of a recombinant analogue of human SLURP-2 (rSLURP-2) having one additional *N*-terminal Met residue. NMR spectroscopy revealed structural homology of recombinant human SLURP-2 to three-finger snake neurotoxins having a conserved β-structural core and three protruding loops. Affinity purification from cortical extracts revealed that SLURP-2 interacts with the α3, α4, α5, α7, β2, and β4 subunits of nicotinic acetylcholine receptors (nAChRs). SLURP-2 causes a modest inhibition of acetylcholine-evoked currents in α4β2 and α3β2-nAChRs expressed in *Xenopus* oocytes (IC_{50} ~ 0.17 and >3 μM, respectively). SLURP-2 at concentrations <1 μM significantly enhances acetylcholine-evoked currents at α7-nAChRs, but causes inhibition at higher concentrations. SLURP-2 allosterically interacts with human M1 and M3 muscarinic acetylcholine receptors (mAChRs) increasing the binding of the orthosteric antagonist N-methylscopolamine. Using inhibitors of nAChRs and mAChRs it was shown that SLURP-2 promotes the proliferation of human oral keratinocytes via interaction with α3β2-nAChRs and M3-mAChRs, while SLURP-2 interaction with α7-nAChRs inhibits cell growth. Computer modeling of complexes with α7 and α3β2-nAChRs revealed possible SLURP-2 binding to the 'classical' orthosteric agonist/antagonist binding site.

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7-methoxytacrine-4-pyridinealdoxime hybrid as a novel prophylactic agent with reactivation properties in organophosphate intoxications

Eugenie Nepovimova[a], Jan Korabecny[b], Rafael Dolezal[b], Daniel Jun[a], Ondrej Soukup[b], Petr Jost[b], Kamil Musilek[b], Kamil Kuca[b]

[a]*Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic*
[b]*Biomedical Research Center, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic*

Chemical warfare agents constitute an increasing threat to both military and civilian populations. Therefore, an effective prophylactic approach is urgently needed. Herein, we present a novel prophylactic agent combining 7-methoxytacrine (7-MEOTA) with a 4-pyridinealdoxime moiety. The presumed mechanism of action of such hybrid drugs would primarily consist in reversible inhibition of acetylcholinesterase (AChE, EC 3.1.1.7), due to occupation of the catalytic active site of AChE by the 7-MEOTA moiety, protecting the enzyme from OP inhibition. Secondly, in case intoxication occurs it would serve as immediate causal antidote to restore the function of phosphylated AChE via its 4-pyridiniumaldoxime fragment. Various *in vitro* assays of 7-MEOTA-4-pyridinealdoxime heterodimer exhibited promising results making this molecule worthy of further investigation.

Nepovimova E., Korabecny J., Dolezal R., Nguyen T.D., Jun D., Soukup O., Pasdiorova M., Jost P., Muckova L., Malinak D., Gorecki L., Musilek K., Kuca K. 7-Methoxytacrine-4-pyridinealdoxime hybrid as novel prophylactic agent with reactivation properties in organophosphate intoxications. *Toxicology Research*, **2016**, 5:1012-1016.

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Acetylcholinesterase and respiration: where is this essential enzyme required?

Aurélie Nervo[a], André-Guilhem Calas[a], Chloé Reymond[a], Florian Nachon[b], Eric Krejci[c]

[a]*Institut de recherche biomédicale des armées and COGnition and ACtion Group, Brétigny sur Orge, France*
[b]*Institut de recherche biomédicale des armées, Brétigny sur Orge, France*
[c]*COGnition and ACtion Group, Paris, France*

Respiration is a vital function controlled by acetylcholine (ACh) that plays a critical role at the neuromuscular junction (NMJ). ACh is absolutely required to breathe. Indeed when curare blocks muscle nicotinic receptors, synaptic transmission stops, muscle contraction weakens and inspiration fails. Inhibition of acetylcholinesterase (AChE), the enzyme that terminates synaptic transmission at the NMJ, also blocks respiration. The study of muscle contractions and synaptic transmission *ex-vivo*, suggests that muscles cannot sustain repetitive synaptic transmission when ACh is not hydrolized and accumulates. Unexpectedly, AChE1irr mutant mice that do not produce AChE in skeletal muscles are able to breathe. Moreover AChE1irr mice are more sensitive to neostigmine, a carbamate inhibitor of AChE. AChE1irr mice have a severe muscle weakness and adapt to the excess of ACh by the fragmentation of the NMJ and reduction of muscle nAChR. This sensitivity to neostigmine is not related to a change of permeability of the blood brain barrier. Indeed, PRiMA KO mice, which have severe AChE deficit in brain including in the respiratory centers, breathe normally. In addition, PRiMA KO mice are as sensitive as control mice to AChE inhibitors. Similar results were obtained with paraoxon, an archetypal organophosphate pesticide. This suggests that excess of ACh not only perturbs brain or skeletal muscles but also additional non classical cholinergic systems. To explore the perturbation of those cholinergic systems involved in the respiratory function, we have recorded the body movements and air exchanges of mice in a double-chambers plethysmography device (DCP). We can monitor the change of the inspiration and expiration parameters, the pause between them, the discrepancy between the flux of air through the airways and the movement of the body that reveals constriction of airways. We have also compared ventilation of mice with partial deficit in AChE (lack of expression of AChE in muscle or lack of anchoring). For each genotype, the nasal flows and body movements were recorded before and after intoxication with different inhibitors of cholinesterases such as paraoxon, physostigmine (carbamate that crosses the blood brain barrier) or neostigmine. We will present how the DCP and the genetic approach help to identify different alterations of ventilation. All the inhibitors affect dramatically the ventilation in mice despite not altering ACh concentration (brain microdialysis), synaptic transmission or skeletal muscle contraction. We will discuss how these results challenge the canonical view that AChE acts essentially to terminate synaptic transmission at cholinergic synapses.

G protein-coupled receptor (GPCR) signaling underlies the nicotine-induced upregulation of alpha7 (α7) nicotinic acetylcholine receptors (nAChRs) expressed in Xenopus oocytes.

Jayharsh Panchal[a], Kristi Deboeuf[a], Mohammad Islam[a], Joseph Farley[a]

[a]*Indiana University, Bloomington, USA*

Alpha7 nAChRs are widely distributed throughout the nervous system, playing important roles in learning, memory, a variety of diseases and neurodegenerative processes including schizophrenia, Alzheimer's and Parkinson's diseases, inflammation, pain, cancer, and nicotine addiction (Changeux et al 2012). A variety of compounds (agonists, antagonists, PAMs) produce functional and/or numerical upregulation of alpha7 Rs in different cells, implicating multiple signaling pathways and mechanisms. Prolonged nicotine exposure can also upregulate alpha7 nAChRs, which may contribute to nicotine addiction (Govind et al 2012, Brunzell et al 2014). Earlier, we found ~ 2-fold functional and numerical upregulation of murine alpha7 nAChRs in *Xenopus* oocytes following 12 h of 100 μM nicotine and extensive washout. Nicotine-upregulation was dependent upon intracellular Ca^{2+}, being abolished by BAPTA-AM, and involved several Ca^{2+}-dependent enzymes (e.g., PP2B, PKC). However, upregulation was independent of Ca^{2+} influx, being unaffected by removal of extracellular Ca^{2+}. Similar to another pentameric, Cys-loop LGIC, glycine receptor 1 (GlyR1), the alpha7 nAChR contains a conserved G protein-binding cluster (GPBC) in the M3-M4 loop. Coupling of G-protein signaling with alpha7 Rs has been shown in neurons and PC12 cells (Kabbani et al 2013). Here, we show that GPCR signaling mediates nicotine-upregulation of alpha7 nAChR. We first observed that a Substance P-analogue peptide (a putative specific inhibitor of G-alpha-q/11 binding to cognate GPCRs) prevented nicotine-induced upregulation of alpha7 Rs. However, the Substance-P analogue also significantly reduced control alpha7 R currents, raising the possibility that its effects were nonspecific. This led us to study mutation of the alpha7 nAChR GPBC (RMKR to AAAA; denoted as alpha7 344-347A) which we expected would block interaction of G-alpha-q with the GPBC. Receptor expression levels, peak current amplitude, and kinetics were equivalent for mutant and wild type (wt) alpha7 Rs, but nicotine-upregulation of alpha7 344-347A R was completely inhibited. In contrast, exposure to the cell-permeable, competitive antagonist Methyllycaconitine (MLA), produced ~ 2-fold upregulation of both wt and mutant alpha7 Rs; upregulation was unaffected by BAPTA-AM. MLA upregulation of mutant and wt alpha7 Rs appears to be due to a chaperone-like mechanism (Nashmi and Lester 2007, Lester et al 2009). Our results reinforce the idea that alpha7 nAChRs may function as both ionotropic and metabotropic receptors. They further indicate that GPCR-signaling of the alpha7 R is critical for its upregulation by nicotine (through several Ca^{2+}-signaling pathways), but not for its upregulation by MLA.

Hsp90, thioredoxin and thioredoxin reductase form a chaperone-redox machinery enabling the catalytic activity of clostridial neurotoxins inside nerve terminals

Marco Pirazzini[a], Domenico Azarnia Tehran[a], Giulia Zanetti[a], Oneda Leka[a], Andrea Mattarei[b], Thomas Binz[c], Romano Lista[d], Ornella Rossetto[a], Cesare Montecucco[a]

[a]*University of Padova - Department of Biomedical Sciences, Padova, Italy*

[b]*University of Padova - Department of Chemical Sciences, Padova, Italy*

[c]*Medizinische Hochschule Hannover, Institut für Physiologische Chemie, Hannover, Germany*

[d]*Army Medical and Veterinary Research Center - Histology and Molecular Biology Section, Roma, Italy*

Botulinum (BoNTs) and tetanus (TeNT) neurotoxins are the most toxic substances known and form the growing family of clostridial neurotoxins (CNTs), the etiologic agents of botulism and tetanus. CNTs are composed of a metalloprotease light chain (L), linked via a disulfide bond to a heavy chain (H). H mediates the binding to nerve terminals and the membrane translocation of L into the cytosol, where its substrates, the three SNARE proteins, are localized. L translocation is accompanied by unfolding and, once delivered on the cytosolic side of the endosome membrane, it has to be reduced and reacquire the native fold to exert its protease activity. Starting from the previous observation that the Thioredoxin reductase-Thioredoxin system (TrxR-Trx) is capable of reducing the interchain disulphide bond of TeNT and BoNT/A, we performed a pharmacological inhibition of Trx or of its reductase on living cells and mice, and found that this treatment strongly prevents the neurotoxicity of all CNTs, suggesting that this redox system mediate the reduction of the interchain disulphide *in vivo*. Importantly, we also found that the TrxR-Trx are loosely bound to the cytosolic side of synaptic vesicles (SV), the organelles wherefrom L translocates in the cytosol. Moreover, by means of immunoprecipitation, we also found that, on SV, TrxR physically interact with the cytosolic chaperone Hsp90. Considering the well-known capability of Hsp90 in recognizing protein unfolded intermediates and its role in the cell entry of many bacterial exotoxins, we envisaged that the TrxR-Trx and the chaperone Hsp90 may orchestrate on SV a machinery exploited by CNTs to enable L catalytic activity inside nerve terminals. In fact, we report that CNTs toxicity is potently hampered also by Geldanamycin, a well-known inhibitor of Hsp90. Importantly, we also found that this molecule strongly synergises with PX-12, an inhibitor of thioredoxin, suggesting that the processes of L chain refolding and interchain disulphide reduction are strictly coupled. Together, our data indicate that CNTs have evolved to exploit a chaperone-redox machinery composed of TrxR, Trx and Hsp90 for the delivery and the activation of their catalytic part in the cytosol, offering a rational target for the development of new antitoxins.

Phosphocholine, a novel unconventional agonist of nicotinic receptors containing subunit α9

Katrin Richter[a], Verena Mathes[b], Martin Fronius[c], Mike Althaus[a], Andreas Hecker[a], Gabriela Krasteva-Christ[d], Winfried Padberg[a], Arik J. Hone[e], Michael J. McIntosh[e], Anna Zakrzewicz[a], Veronika Grau[a]

[a]*Justus-Liebig-University of Giessen, Gießen, Germany*
[b]*Justus-Liebig-University of Giessen, 35392, Germany*
[c]*University of Otago, Dunedin, New Zealand*
[d]*Julius-Maximilians-University of Wuerzburg, Würzburg, Germany*
[e]*University of Utah, Salt Lake City, USA*

Question: The proinflammatory cytokine interleukin-1β (IL-1β) plays an important role in innate immunity. Since excessive levels of IL-1β lead to life-threatening systemic inflammation, release of IL-1β is tightly controlled. Recently, we demonstrated that stimulation of α9-containing nicotinic acetylcholine receptors (nAChR) in human monocytes efficiently inhibits ATP-mediated IL-1β release (Hecker et al. 2015, J. Immunol. 195, 2325-2334). Moreover, phosphocholine (PC) seemed to function as a nicotinic agonist in this context. The purpose of this study was to test the hypothesis that PC is a novel ligand of non-canonical α9-containing nAChR.
Methods: In lipopolysaccharide-primed human monocytic U937 cells and freshly isolated mononuclear leukocytes from α9 as well as α10 gene-deficient mice, the ATP-sensitive P2X7 receptor was stimulated with the known agonist BzATP. IL-1β release was measured in cell culture supernatants via ELISA. In electrophysiological whole-cell patch-clamp measurements on U937 cells the BzATP-induced ion current responses were monitored. PC, choline (Cho; a known α9 agonist), the α-conotoxin RgIA4 (selective human α9 antagonist), and mecamylamine (general nAChR antagonist) were added together with BzATP. Furthermore, human homomeric α9 or heteromeric α9/α10 nAChR were heterologously expressed in *Xenopus laevis* oocytes and investigated in two-electrode voltage-clamp measurements.
Results: In U937 cells BzATP-induced IL-1β release was inhibited by PC and Cho. RgIA4 antagonized the inhibitory effects of PC and Cho, indicating an involvement of α9-containing nAChR. These results were confirmed in U937 cells treated with siRNA targeting α9 and in mononuclear leukocytes from α9 gene-deficient mice. Experiments on α10 gene-deficient mice indicated that this subunit is also mandatory for the control of BzATP-induced release of IL-1β by mononuclear leukocytes. In patch-clamp experiments on U937 cells, application of PC and Cho alone did not evoke ion currents. Furthermore, BzATP-induced current responses were completely inhibited by PC and Cho. Mecamylamine and RgIA4 antagonized the effects of PC and Cho. In α9- and α9/α10-expressing oocytes, PC had no effect on ion currents, while Cho induced a current response. Interestingly, the Cho-induced current responses were blunted by PC, suggesting silent desensitization.
Conclusion: We identified PC as agonist of monocytic α9-containing nAChR that inhibits signaling via the P2X7 receptor and hence prevents ATP-mediated release of IL-1β. PC does not trigger ionotropic effects at heterologously expressed human homomeric (α9) or heteromeric (α9/ α10) nAChR, whereas Cho does. In conclusion, PC exerts metabotropic functions at monocytic nAChR that inhibit ATP receptor P2X7, without activating conventional ionotropic functions that are typical for the nervous system.

Strategy for recovering neuromuscular transmission following organophosphate exposure

Susana Rossi[a], Richard Rotundo[a]

[a]*Department Cell Biology, University of Miami Miller School of Medicine, Miami, USA*

Acetylcholinesterase (AChE) is localized at the neuromuscular junction (NMJ) where it terminates synaptic transmission. Exposure to organophosphate nerve agents or pesticides can inactivate this enzyme leading to death. Studies from Massoulie's lab have shown that the noncatalytic subunit PRAD region can induce oligomerization of the catalytic subunits. We then designed peptides (PRAD-KDEL) that can be endocytosed by skeletal muscle cells and transported back to the endoplasmic reticulum where they stabilize newly synthesized AChE molecules, thereby increasing expression and secretion of the enzyme (Ruiz, C.A., Rossi, S.G. and Rotundo, R.L. JBC 2015). Intravenous (i.v.) injection of biotinylated PRAD-KDEL in mice showed that the peptide accumulates at the NMJ within 5 min after injection. Initial pharmacokinetic studies using a PRAD-KDEL ELISA developed in our lab indicated that serum peptide levels peak about 10 min after i.m. injection. Several problems could arise from the administration of the peptides including decrease in muscle strength, motor coordination and behavioral responses. To test this possibility we injected mice with either PRAD-KDEL or saline alone followed by a battery of physical and behavioral tests from 1 h to 7 days later. Food, water intake and body weight measurements were unchanged between the groups. No statistically significant differences were observed in either total distance traveled or horizontal activity between the groups in an automated open field test. In addition, mouse pole tests and grip strength tests showed no differences between groups. Furthermore, we demonstrated that i.m. injected PRAD-KDEL peptides can both protect and rescue mice and guinea pigs from 1xLD75 exposure to the organophosphate DFP. Addition of 0.4 mg/kg body weight atropine to the PRAD peptide diluent is essential for rescuing the animals. Intramuscular injection of PRAD-KDEL plus atropine 15 min after 1× LD75 DFP increases survival from 25% in DFP-treated animals to 96% in peptide-treated. In conclusion, we found that doses of PRAD-KDEL peptide that increases AChE at the NMJ and rescue mice and guinea pigs from lethal organophosphate exposure did not produce signs of adverse reactions for at least 7 days following injection and did not reduce muscle strength or function.

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Lipid raft localization of the M1 muscarinic receptor in plasma membranes of CHO cells

Vladimir Rudajev[a], Eva Dolejší[a], Alena Randáková[a], Jan Jakubík[a], Esam El-Fakahany[b], Vladimir Doležal[a]

[a]*Institute of Physiology CAS, Prague, Czech Republic*
[b]*College of Pharmacy UMN, Minneapolis, USA*

Lipid rafts are specialised regions of plasma membrane characterised by high cholesterol content, where many signalling molecules including trimeric G proteins and GPCRs are concentrated. Whereas alpha subunits of Gq/11, Gi, and Gs proteins are known to participate in lipid rafts to various extents, less information is available about raft localization of individual subtypes of muscarinic acetylcholine receptors (mAChR). To assess the size of raft-associated pools of the M1 and M2 subtypes of mAChR as well

as corresponding Gq/11 and Gi proteins, we compared detergent and non-detergent separation methods. Cell membranes from CHO cells transfected to stably express the M1 or M2 subtype of mAChR were exposed to a low concentration of Triton X-100 (detergent method) or sonicated in Na_2CO_3/$NaHCO_3$ buffer (pH 11.0; non-detergent method). As cholesterol is the main marker of lipid rafts, we compared its distribution after detergent and non-detergent treatment. Separation of raft from non-raft membrane fractions was achieved by equilibrium centrifugation in discontinuous sucrose gradients (SW41 rotor, 36000 rpm, 20 h). Our data show that both Triton X-100 and alkaline treatment of membranes lead to separation of protein poor and cholesterol rich membrane fractions which contain nearly all cholesterol, from the protein rich and cholesterol poor membrane phase. Non-detergent (alkaline) extraction is known to represent a somewhat milder procedure for extraction of lipid rafts, and isolated rafts are assumed to represent more natural structures than those after detergent treatment. We show that the non-detergent extraction method yields a different distribution of the M1 and M2 receptor subtypes and their preferred G-protein α-subunits. Under such conditions, the majority of trimeric G proteins, caveolin, and M1-AChR receptor, as well as nearly all M2-AChR receptor are present in a fraction corresponding to lipid rafts. These results demonstrate that significant pools of the M1 and M2 subtypes of mAChR are present in lipid rafts isolated from transfected CHO cells and that a non-detergent method compared to detergent treatment seems more suitable for isolation of membrane rafts.

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An oxidative mechanism for cholinergic dysfunction in neurons exposed to Alzheimer's-linked Aβ-oligomers

Luis Eduardo Santos[a], Cicero Figueiredo-Freitas[a], Sergio Teixeira Ferreira[b], Fernando Garcia De Mello[c]

[a]*Instituto de Bioquímica Médica Leopoldo de Meis - UFRJ, Rio de Janeiro, Brazil*
[b]*Instituto de Bioquímica Médica Leopoldo de Meis and Instituto de Biofísica Carlos Chagas Filho - UFRJ, Rio de Janeiro, Brazil*
[c]*Instituto de Biofísica Carlos Chagas Filho - UFRJ, Rio de Janeiro, Brazil*

Acetylcholine is a major neurotransmitter in the central nervous system (CNS), synthesized, usually near presynaptic terminals, by transfer of an acetyl group from acetyl-coenzyme A to choline. This reaction is carried out by choline acetyltransferase (ChAT), a phenotypic marker of cholinergic neurons. Disturbed cholinergic transmission may underlie the onset and development of several CNS pathologies. Amongst a number of well-established cholinergic hypotheses of disease is that of Alzheimer's disease (AD), an alarmingly prevalent form of dementia. Early work by our group, using the avian retina as a CNS model, showed that ChAT activity in cultured or *ex vivo* neurons is markedly and specifically down-regulated by excitotoxic stimuli, before any changes in cell viability or enzyme levels occur. This effect was shown to require calcium influx and nitric oxide (NO) production [J Neurochem 2001; 77:1136-1144]. More recently, we observed similar results in a more specific pathological context, using amyloid-β peptide oligomers (AβOs). These are diffusible toxins that accumulate in the brains of patients and animal models of AD, and are currently regarded as possible culprits of the disease. Exposing cultured cholinergic neurons to AβOs inhibited ChAT activity in the absence of neuronal death or changes in enzyme expression. We further showed that the effect of AβOs on ChAT activity is linked to excitotoxicity and to increased production of reactive oxygen species (ROS); inhibition is caused by oxidative damage to the enzyme [JBC 2012; 287:19377-19385]. In the current work, we expand those observations to a mammalian model, using cultured neurons of the rat septal region, and attempt to identify oxidative modifications involved ChAT inhibition. Using S-nitrosothiol resin-assisted capture and labeling of reduced thiols, we show that cysteine modification is not central to the mechanism of inhibition. Tyrosine nitration, on the other hand, was found to be induced in cultures exposed to glutamate, AβOs and NO donors, and correlated well with loss of ChAT activity. Results suggest a novel mechanism of cholinergic dysfunction preceeding neuronal death, which may be relevant in early-stage AD pathology.

The effect of acetylcholinesterase reactivators on the cholinergic system

Vendula Sepsova[a], Ondrej Soukup[a], Lubica Muckova[a], Petr Jost[a], Martina Hrabinova[a], Daniel Jun[a]

[a]*University of Defence, Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy, Hradec Kralove, Czech Republic*

One of the therapeutic approaches to organophosphate poisoning, resulting in overstimulation and desensitization of cholinergic receptor by acetylcholine, is to reactivate acetylcholinesterase (AChE) with oximes. However, reactivators probably also play a role in the synthesis of acetylcholine or via the interaction with pre- and post-synaptic cholinergic receptors. The exact pharmacological effects of oximes are still an open question.

The aim of the study was to evaluate ten standard and newly synthesized reactivators at two concentrations (100 and 10 μM), with respect to their abilities to inhibit M_1 muscarinic receptor (mAChR). A CHO cell line stably expressing the M_1 subtype mAChR was used for the assessment of such interaction. Other tests included affinity to AChE, their ability to reactivate sarin and tabun-inhibited AChE, and cytotoxicity on SHSY-5Y cell line was measured. Statistical analysis of results was performed in GraphPad Prism 6.

In conclusion, affinity to mAChR together with AChE reactivation may prolong the time of survival after organophosphate intoxication. Trimedoxime showed the best antimuscarinic results with good reactivation ability. On the other hand, prophylactic reactivator HI-6 inhibited AChE and had no effect on tabun-inhibited AChE. Thus, trimedoxime seems to be a better compound. However, due to a relatively weak affinity *in vitro*, *in vivo* validation seems to be essential to confirm this hypothesis.

"Betel nut" the orphaned addiction of 300 million people and its relationship to brain nicotine receptors

Clare Stokes[a], Roger Papke[b], Nicole Horenstein[c]

[a]*University of Florida, Department of Pharmacology, Gainesville, Florida, USA*
[b]*University of Florida Department of Pharmacology, Gainesville, Florida, USA*
[c]*University of Florida Department of Chemistry, Gainesville, Florida, USA*

Habitual chewing of "betel nut" is the fourth most common human self-administration of a psychoactive and potentially addictive substance after alcohol, caffeine, and nicotine. Areca nut is the main ingredient of a typical betel quid, along with spices, possibly sweeteners, and about 50% of the time, tobacco, wrapped in a betel vine leaf with slaked lime, which alkalizes the mixture and allows active agents to pass into the brain. Betel nut effects are variously characterized as stimulant or euphoric, but most obvious is the production of copious amounts of bright red saliva staining the lips, teeth, and gums. Long-term use of betel preparations is a known cause of oral cancer and other diseases of the mouth. A primary active ingredient of areca nut is arecoline, which is a relatively non-selective muscarinic agonist, accounting for many of the overt peripheral and central nervous system effects. This muscarinic activity, however, is not likely to account for the addictive properties of the betel. We found that arecoline is a nicotinic partial agonist with about 6-10% efficacy for alpha4 and alpha6-containing nicotinic acetylcholine receptors (nAChR) that are related to nicotine addiction, and a silent agonist of alpha7 nAChR. Our data also indicate that there are additional agents in areca with nicotinic activity beyond that accounted for by arecoline. Application of areca nut infusion to alpha4beta2 and alpha6beta3-containing nAChRs resulted in weak partial agonist responses and a subsequent refractory period when the receptors became insensitive to ACh. These observations provide an impetus to discover the active components responsible for these activities, with the hypothesis that such compounds will serve as the basis for development of new, more selective, smoking cessation agents compared to varenicline and cytisine. The activities of cytisine and varenicline were compared to arecoline and isoarecolone on key nAChRs. Both arecoline and isoarecolone lack agonist activity at alpha7, ganglionic, and muscle-type receptors while maintaining the partial agonist activity at the alpha4beta2 and alpha4beta2alpha6beta2beta3 receptors, which are the targets for smoking cessation therapies. Further, comparing isoarecolone, arecoline, and ACh as muscarinic agonists at 10 μM, isoarecolone was noteworthy in its weak partial agonism, nearly 6-fold less than arecoline on cells expressing multiple muscarinic receptor subtypes and essentially zero on M1 receptors. These observations suggest that there is a useful chemical space for compounds to be developed with enhanced selectivity for alpha4beta2 and alpha4beta2alpha6beta2beta3 nAChR and diminished activity at mAChRs.

Muscarinic suppression of ATP-sensitive K+ channels mediated by the M3/phospholipase C pathway contributes to smooth muscle contractions in mouse small intestine

Yasuyuki Tanahashi[a], Ban Wang[a], Yuri Murakami[a], Hayato Matsuyama[b], Seiichi Komori[b], Toshihiro Unno[b]

[a]*Department of Animal Medical Sciences, Kyoto Sangyo University, Kyoto, Japan*
[b]*Laboratory of Pharmacology, Department of Veterinary Medicine, Gifu University, Gifu, Japan*

It has been suggested that ATP-sensitive K^+ (K_{ATP}) channels are expressed in gastrointestinal smooth muscles and their activities are regulated by muscarinic receptor stimulation (1,2). However, the physiological significance and mechanisms of the muscarinic regulation of this channel are not fully understood. Changes in electrical and mechanical activities in mouse single ileal myocytes and ileal segment preparations, respectively, in response to the K_{ATP} channel-opener cromakalim and the K_{ATP} channel-blocker glibenclamide, were recorded. Cromakalim (10 μM) induced membrane hyperpolarization in single myocytes and relaxation in segment preparations, whereas glibenclamide (10 μM) induced membrane depolarization and contraction, respectively. To investigate the muscarinic regulation of K_{ATP} channel activity and its underlying mechanisms, the effects of carbachol (CCh) on cromakalim-induced K_{ATP} channel currents (IK_{ATP}) were studied in myocytes of wild-type (WT) mice and M_2 or M_3 receptor-knockout (KO) mice. CCh (100 μM) induced a sustained, comparable suppression of IK_{ATP} in cells from WT and M_2KO mice. However, CCh had a minimal effect on IK_{ATP} in M_3KO cells. The intracellular application of BAPTA (20 mM), a Ca^{2+} chelator, slightly reduced the CCh-induced I_{KATP} suppression in the WT cells. The phospholipase C (PLC) inhibitor U73122 (1 μM), but not its inactive analogue U73343 (1 μM), caused a marked reduction of the CCh-induced suppression of IK_{ATP}. These results indicate that K_{ATP} channels are constitutively active and contribute to the setting of resting membrane potentials in mouse ileal smooth muscles. M_3 receptors can inhibit the activity of these channels, thereby contributing to the membrane depolarization in smooth muscles, leading to their contraction. The major pathway of the M_3-induced K_{ATP} channel suppression is brought about via a PLC pathway, which is independent of intracellular Ca^{2+}.

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Treatment with a cholinergic precursor in an animal model of cerebrovascular disease: insights into mechanisms of neuroprotective activity

Seyed Khosrow Tayebati[a], Daniele Tomassoni[b], Ilenia Martinelli[a], Michele Moruzzi[a], Francesco Amenta[a]

[a]*University of Camerino, School of Pharmacy, Camerino, Italy*
[b]*University of Camerino, School of Biosciences and Veterinary Medicine, Camerino, Italy*

Cholinergic hypofunction is a common trait of adult-onset dementia including Alzheimer's disease (AD) and vascular dementia (VaD). Cholinergic replacing approaches represent an obvious strategy for treating cognitive dysfunctions due to impaired cholinergic neurotransmission. Cholinergic precursors increasing choline availability and acetylcholine synthesis/release were among the first approaches tried for countering cognitive impairment typical of AD or VaD. Alpha-glyceryl-phosphoryl-choline (GPC) is among cholinergic precursors the most effective in enhancing acetylcholine biosynthesis and release in animal models.

Arterial hypertension is the main risk factor for stroke and plays a role in the development of VaD. An association between hypertension and reduced cerebral blood flow is documented and arterial hypertension in midlife is associated with a higher probability of cognitive impairment. Spontaneously hypertensive rats (SHR) are a rat strain investigated for assessing hypertensive brain damage and treatment of it. They are characterized by time-dependent rise of arterial blood pressure, brain atrophy, glial reaction and cholinergic hypofunction. These phenomena are shared to some extent with hypertensive brain damage and VaD.

Treatment of SHR for 4 weeks treatment with 150 mg/kg/day of GPC countered the nerve cell loss in zones II, III and IV of frontal cortex and in the CA1 subfield of hippocampus and dentate gyrus. The compound reduced also parenchymal astrocytes hyperplasia and hypertrophy in the hippocampus of SHR. No significant changes in the size of perivascular astrocytes were observed in SHR, whereas the expression of the blood-brain barrier (BBB) marker aquaporin-4 increased in SHR. This phenomenon was countered by GPC treatment.

An increased expression of choline and of vesicular acetylcholine transporters (VAChT) was observed in different brain areas of SHR. This increase probably represents an up-regulation to counter cholinergic deficit of SHR. Treatment with GPC further increased choline transporter and to a greater extent VAChT expression.

Besides activity on cholinergic markers, GPC increased neuronal surveillance, prevented astrogliosis, reversed BBB changes and improved micro-vessels inflammatory pattern of SHR.

GPC's protective effect is probably due to its interference with membrane phospholipids and/or to an enhancement of cholinergic neurotransmission mechanisms. These findings may explain data of clinical trials reporting an improvement by GPC of cognitive function in subjects suffering from cerebrovascular disorders.

Activation of M1 and M4 muscarinic acetylcholine receptors biases excitatory synaptic transmission in hippocampus CA1

Catherine Thorn[a], Eda Stark[a], Dmitri Volfson[a], Jeremy Edgerton[a]

[a]*Pfizer Neuroscience and Pain Research Unit, Cambridge, USA*

Hippocampal networks are particularly susceptible to dysfunction in many neurodegenerative diseases and disorders including Alzheimer's disease, Lewy body dementia, and schizophrenia. The main output region of the hippocampus, CA1, receives glutamatergic input from both hippocampus CA3 and entorhinal cortex, via the Schaffer collateral (SC) and temporoammonic (TA) pathways, respectively. SC and TA inputs to CA1 are thought to be differentially involved in the retrieval of previously stored memories versus the encoding of novel information, and switching between these two crucial hippocampal functions is thought to critically depend on acetylcholine (ACh) acting at muscarinic receptors. In this study, we aimed to determine the roles of specific subtypes of muscarinic receptors in mediating the neuromodulatory effects of ACh on glutamatergic synaptic transmission in the SC and TA pathways of CA1.

Using selective pharmacological manipulation of M1 and M4 receptors and extracellular and intracellular electrophysiology recordings in hippocampus CA1 slices from adult rats, we have demonstrated that activation of these two specific receptor subtypes is sufficient to reproduce several of the major effects previously reported for ACh. Specifically, activation of M1 receptors increases intrinsic excitability, decreases spike frequency accommodation, and increases spontaneous firing rates of CA1 pyramidal neurons, without exerting major effects on evoked synaptic transmission in either the SC or TA pathways. By contrast, activation of M4 receptors dramatically suppresses evoked synaptic responses in the SC pathway by decreasing the presynaptic release of glutamate, while leaving the TA pathway relatively uninhibited.

Our results suggest specific mechanisms by which the activation of M1 and M4 receptors may normalize CA1 circuit activity in the wake of the disruption of cholinergic signaling that accompanies neurodegenerative dementias or neuropsychiatric disorders. These findings are of particular interest in light of the prior clinical findings that xanomeline, an M1/M4 preferring agonist, was able to improve cognitive and behavioral symptoms in patients with Alzheimer's disease or schizophrenia.

Brain cholinergic dysfunction in obese Zucker LEPR(fa/fa) rats

Daniele Tomassoni[a], Seyed Khosrow Tayebati[b], Michele Moruzzi[b], Ilenia Martinelli[b], Francesco Amenta[b]

[a]*University of Camerino, School of Biosciences and Veterinary Medicine, Camerino, Italy*
[b]*University of Camerino, School of Medicinal and Health Products Sciences, Camerino, Italy*

Increased food intake, reduced physical activity and altered metabolic processes are variables affecting energy balance inducing obesity. Obesity is now considered an increasing medical challenge associated with the development of chronic diseases. Metabolic syndrome (MetS), another disorder related to obesity and characterized by hypertension, hyperglycemia, hyper-triglyceridemia, reduced high-density lipoprotein cholesterol, is accompanied by abnormal adipose deposition and function. Obesity and Mets are recognized risk factors for adult-onset dementia disorders such as Alzheimer's disease (AD) and vascular dementia (VaD). They have been also associated with poorer cognitive performance. Moreover, combination of obesity and arterial hypertension could impair performance across various cognitive domains.

The cholinergic system is altered in age-related neurodegenerative diseases, such as AD and VaD. A decline in the integrity of the cholinergic system characterized by a decrease of acetylcholine

(ACh) biosynthetic enzyme choline acetyltransferase (ChAT) was also reported in patients with VaD. The present study has investigated the brain cholinergic pattern in Obese Zucker rats (OZRs) compared with lean control Zucker rats (LZRs) at different ages. This to clarify the possible relationships between Mets, cerebral injury and cholinergic system impairment.

Male OZRs and the littermate LZRs of 12, 16 and 20 weeks of age were used. The OZRs, with a mutation in the leptin receptor (fa/fa), represent a model of obesity related to type 2 diabetes mellitus, exhibiting dyslipidemia, and a moderate degree of arterial hypertension. Body weight, blood pressure and blood parameters were checked. The brain was processed for immunochemical and immunohistochemical analysis of vesicular acetylcholine transporter (VAChT), ChAT, acetylcholinesterase (AChE), nicotinic (nAChRalpha7) and muscarinic (mAChR) receptor subtypes. Behavioural tests were performed to identify cognitive changes.

Initiation of structural studies of human pancreatic lipase as bioscavenger candidate against nerve agent intoxication

Cédric Touvrey[a], Julie-Anne Chemelle[a], Isabelle Famery[a], Raphael Terreux[a], Florian Nachon[b], Xavier Brazzolotto[b]

[a]*Institut de Biologie et Chimie des Protéines, Lyon, France*
[b]*Institut de Recherche Biomédicale des Armées, Brétigny sur Orge, France*

The efficiency of human butyrylcholinesterase (BChE) as a stoichiometric bioscavenger against nerve agent intoxication has been proven. However, its wide use is currently limited by production and purification costs. A possible alternative exists in the development of a catalytic bioscavenger, i.e. a protein able to rapidly hydrolyze nerve agents, theoretically efficient at lower amounts.

Looking for a potential catalytic bioscavenger scaffold candidate we initiated a virtual screening of the Protein Data Bank for functional similarity using the SuMo software and a search model based on the BChE active site geometry. Besides the expected acetylcholinesterase and butyrylcholinesterase, we identified a set of bile salt activated lipases structures, among which is the human pancreatic lipase (hPanLip). This enzyme shares only 35% identity with BChE but further docking analyses with different nerve agents validated hPanLip as a potential candidate. We produced the recombinant protein in mammalian cells and purified it, allowing enzymatic characterization and determination of IC_{50} after 5 min inhibition by paraoxon and surrogates of VX, sarin and tabun. Following this biochemical study, we initiated a structural study by solving the X-ray structure of apo hPanLip and complexes with VX, sarin and paraoxon at resolutions in the 2-Å range. Parallel *in silico* work based on QM/MM techniques continues to determine possible modifications of the protein that would yield better nerve agents specificity and hydrolyzing properties.

Cholinergic contraction mediated via the M2 muscarinic receptor is predominant in mouse colonic circular muscles

Toshihiro Unno[a], Kasumi Suzuki[a], Hiroshi Nagano[a], Hayato Matsuyama[a], Yasuyuki Tanahashi[b], Seiichi Komori[a]

[a]*Gifu University, Gifu, Japan*
[b]*Kyouto Sangyo University, Kyoto, Japan*

In visceral smooth muscles including those of the gastrointestinal tract, M_2 and M_3 muscarinic receptor subtypes are coexpressed with a predominance of the former subtype. These receptors mediate the action of the parasympathetic neurotransmitter acetylcholine or muscarinic agonists to produce excitation and contraction. To characterize the functional roles of muscarinic receptor subtypes in the cholinergic contractions in colonic circular muscles, isometric contractile responses to electrical field stimulation (EFS) or exogenously applied carbachol (CCh) were studied in distal or proximal parts of colonic circular muscle strips isolated from wild-type (WT), M_2-subtype knockout (KO), M_3-KO or M_2/M_3-double KO mice. Under conditions where adrenergic, nitrergic and purinergic components were suppressed using guanethidine, L-NAME and MRS-2500, respectively, EFS (pulse width: 0.5 ms; strength: 50 V) at 1-50 Hz for 20 s evoked a transient contraction in a frequency-dependent manner in WT preparations. The transient contractile responses were abolished after treatment with atropine or tetrodotoxin. In M_2-KO preparations, EFS also elicited a transient contraction, but the amplitude of cholinergic contraction was markedly smaller than that of WT preparations. In M_3-KO preparations, the cholinergic contraction was also reduced as compared with WT preparations, but the reduction was much smaller than that in M_2-KO preparations. In M_2/M_3-double KO preparations, EFS did not evoke any cholinergic contractions. Similar results were obtained when we studied CCh-induced contractile responses in each KO preparation. In WT preparations pretreated with pertussis toxin, which is known to uncouple M_2 receptors from $G_{i/o}$ type G proteins, cholinergic contractions were significantly suppressed. In conclusion, although both M_2 and M_3 muscarinic receptors are involved in cholinergic contractions, the M_2-mediated component is predominant in mouse colonic circular muscles, in contrast to other tissues including ileal longitudinal (1), urinary bladder (2) or uterus (3) smooth muscles where the M_3-mediated component dominates.

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Action of the human Lynx1 on nicotinic acetylcholine receptors in the brain

Natalia Vasilyeva[a], Mikhail Shulepko[b], Dmitry Kulbatskii[b], Zakhar Shenkarev[b], Morten Thomsen[c], Dmitry Dolgikh[b], Mikhail Kirpichnikov[b], Ekaterina Lyukmanova[b]

[a]*Lomonosov Moscow State University, Institute for Higher Nervous Activity and Neurophysiology RAS, Shemyakin-Ovchinnikov Institute of Bioorganic chemistry, RAS, Moscow, Russian Federation*
[b]*Shemyakin-Ovchinnikov Institute of Bioorganic chemistry, RAS, Lomonosov Moscow State University, Moscow, Russian Federation*
[c]*University of Copenhagen, Copenhagen, Denmark*

Influence on the processes of cognition and their improvement is one of the most important problems of modern medicine. The nicotinic acetylcholine receptors (nAChRs) could be considered as potential targets for drugs that improve the cognitive functions of the brain. It has been shown that activation of α7-AChR has a positive effect on cognitive processes such as thinking, concentration, attention, and increases resistance to stress factors. Allosteric modulators of this receptor are regarded as the prototype for biomedical drugs affecting cognition. The human protein Lynx-1 belonging to the Ly6/uPAR family could be considered as such a drug prototype. This endogenous membrane-tethered neuromodulator is expressed in different areas of the brain and regulates the work of nAChRs. Previously was shown the involvement of Lynx1 in cognitive processes (memory, learning). Presently Lynx1 is considered as one of the key factors regulating neuronal plasticity. The molecular mechanisms underlying Lynx1 modulatory activity and its effects on cognition remain insufficiently studied.

Here we describe the results of an electrophysiology study of a water-soluble recombinant analogue of human Lynx1 (ws-Lynx1) in rat brain slices. We used a whole-cell patch-clamp configuration and fast drug-application system. It was revealed that ws-Lynx1 at 1 μM concentration didn't affect the ACh-evoked current in brain slices, while application of 10 μM ws-Lynx1 resulted in the ~ 30% enhancement of the current amplitude. Using the specific inhibitors MLA and DhbE, we showed that ws-Lynx1 substantially enhances response at the α7 nAChR.

The affinity purification of different nAChR subunits from human cortex extracts using ws-Lynx1 coupled to magnetic beads was assayed in the presence of different nAChR agonists and antagonists (nicotine, MLA, DhbE, MII, PIA, AUIB). It was shown that MLA and DhbE compete with ws-Lynx1 for binding to α7 and β4 nAChR subunits, respectively. This indicates that ws-Lynx1 binds to the receptors near the 'classical' agonist/antagonist binding site. Obtained results revealed new facets of Lynx1 action.

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Attenuated staining of cholinergic markers in the basal forebrain in murine model of Alzheimer's type tauopathy but not of frontotemporal lobar degeneration tauopathy

Adrianna Wysocka[a], Maciej Zadrozny[b], Marta Steczkowska[a], Grazyna Niewiadomska[a]

[a]*Nencki Institute of Experimental Biology, Warsaw, Poland*
[b]*Cardinal Wyszynski University, Warsaw, Poland*

During aging and the progression of Alzheimer's disease (AD) there is atrophy of cholinergic neurons accompanied by a reduction in ChAT, the synthetising enzyme for the neurotransmitter acetylcholine (ACh), in the vesicular acetylcholine transporter, in the cholinergic muscarinic and nicotinic acetylcholine receptors, as well as the requirement of cholinergic neurons to receive neurotrophic support by NGF-mediated high- (TrkA) and low-affinity ($p75^{NTR}$) receptors. However, the mechanisms underlying cholinergic neuron degeneration remain uncertain. Such loss may be due to the toxic interaction of tau with muscarinic receptors for ACh. Therefore, the aim of the study was to validate this hypothesis and to characterize morphology of the basal forebrain cholinergic neurons in animal models of human dementia with tauopathy. The research was conducted on two transgenic mouse models: line 1 (L1), with mild Alzheimer's disease-like tauopathy (AD), and Line 66 (L66) with severe frontotemporal lobar degeneration-like tauopathy (FTLD). The control animals were NMRI wild type mice. Experiments were carried out on 3 and 9 months old animals (n = 3 for each line and age group). Immunohistochemistry for cholinergic neuron markers, ChAT and $p75^{NTR}$ was performed. The microscopic analysis showed attenuated staining of ChAT and $p75^{NTR}$ in cholinergic neurons in basal forebrain in L1 mice, but not in L66 mice, as compared to NMRI wild type mice, where no differences were visible. The impairments in L1 were observed in various structures such as interneurons in striatum, as well as in projection neurons in medial septum, vertical and horizontal limbs of the diagonal band nucleus of Broca and the magnocellular basal nucleus of both age groups. These may suggest a loss of cholinergic phenotype or even neuronal death in L1 mice. Interestingly, the aforementioned changes in L1 appeared before fully developed tauopathy occurred. In summary, results obtained may indicate a possible divergence in the roles of tau in the two pathological conditions examined.

[1] Melis V. *et al*. (2015) Different pathways of molecular pathophysiology underlie cognitive and motor tauopathy phenotypes in transgenic models for Alzheimer's disease and frontotemporal lobar degeneration. *Cell Mol Life Sci*, 72, 2199-2222.

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2, 3, 7, 8-tetrachlorodibenzo-p-dioxin-induced alteration of acetylcholinesterase at the neuromuscular junction

Li Xu[a], Yingjie Xia[a], Tuan Xu[a], Yangsheng Chen[a], Hualing Fu[a], Heidi Q. Xie[a], Bin Zhao[a]

[a]*State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center of Eco-Environment Sciences, Chinese Academy of Sciences, Beijing, China*

Dioxin belongs to a group of compounds, which are highly toxic and both environmentally and biologically persistent. Dioxin or other dioxin-related compounds cause multiple toxic effects to humans, including cancer induction and developmental alteration of the nervous, immune and reproductive systems. Among them, the issue of investigating the side effects of dioxin in the nervous system

is emerging in the last decade. Evidence suggested a possible impact of dioxin on the neuromuscular system. A current understanding suggests that the PNS (neuromuscular system) may be one of the possible target sites of dioxin, based on the evidence of paralytic symptoms upon dioxin exposure.

Acetylcholinesterase (AChE) exerts important functions at neuromuscular junctions, the expression level of which was upregulated during myogenesis. In this work, we investigated the effect of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) on cultured neuronal cells and muscle cells. We found that low-dose TCDD treatment caused suppression of AChE activity by down-regulation of the AChE mRNA level in cultured neuronal cells [1]. Meanwhile, low-dose TCDD treatment caused dose-dependent-suppression of AChE mRNA levels during myogenic differentiation of cultured C2C12 cells, which implied that the differentiation profile of AChE expression was changed by TCDD treatment. The underlying mechanism needs further investigations to explain the toxicity of TCDD on neuromuscular junctions.

[1] H.Q.H. Xie, H.M. Xu, H.L. Fu, Q. Hu, W.J. Tian, X.H. Pei, B. Zhao, AhR-Mediated Effects of Dioxin on Neuronal Acetylcholinesterase Expression *in Vitro*, Environmental Health Perspectives, 121 (2013) 613-618.

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The enhancement of acetylcholinesterase by Wnt3a in osteoblasts: a signaling mediated by Runx2

Miranda L. Xu[a], Cathy Bi[a], Etta Liu[a], Tina T. X. Dong[a], Sibao Chen[b], Karl W. K. Tsim[a]

[a]*Division of Life Science and Shenzhen Research Institute, The Hong Kong University of Science and Technology, Hong Kong, China*
[b]*State Key Laboratory of Chinese Medicine and Molecular Pharmacology (Incubation), Shenzhen Research Institute of The Hong Kong Polytechnic University, Shen Zhen, China*

Acetylcholinesterase (AChE) plays a hydrolytic role in terminating cholinergic transmission. Apart from this notion, emerging evidence indicates that AChE exerts functions in bone formation. AChE mainly exists as a PRiMA-linked form in bone tissue and osteoblasts, which is proposed to play a role in osteoblast differentiation. Increase in bone cell-specific markers, e.g. ALP (alkaline phosphatase), COL1A1 (collagen type I), osteonectin and osteocalcin, and calcium mineralization, were used to characterize osteoblastic differentiation. Increase in AChE expression was observed during osteoblast differentiation. The Wnt/β-catenin signaling pathway plays a pivotal role in bone formation and osteoblast differentiation. A defect in Wnt signaling transduction was observed in osteoblasts from *ACHE-/-* mice when compared to those of *ACHE+/+* mice, which suggested the participation of AChE in the Wnt/β-catenin signaling pathway. Further, the expression of the AChE could be enhanced by application of Wnt3a, the agonist of the Wnt/β-catenin signaling pathway, in osteoblast cultures. This observation indicated the regulatory effect the Wnt/β-catenin signaling pathway in AChE expression. The binding site of Runx2 (Runt-related transcription factor 2), a downstream transcription factor of the Wnt/β-catenin signaling pathway, was found on the *ACHE* promoter. Here, the Runx2 occupancy of the *ACHE* promoter was detected by the chromosome immunoprecipitation (ChIP) assay. Further, the role of Runx2 in regulating AChE expression was tested by application of human AChE promoter with downstream tagged luciferase (pAChE-Luc) and $pAChE_{\Delta Runx2}$-Luc, which possesses a mutation of the Runx2 binding site on the *ACHE* promoter. This study, for the first time, suggested that enhancement of AChE by Wnt3a in osteoblasts may be attributed to the activation of Runx2.

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The effects of organophosphorus compounds and novel antidotes on human neuronal cells

Tamara Zorbaz[a], Maja Katalinic[a], Ivan Pavicic[a], Anissa Braïki[b], Julien Renou[b], Pierre-Yves Renard[b], Ludovic Jean[b], Zrinka Kovarik[a]

[a]*Institute for Medical Research and Occupational Health, Zagreb, Croatia*
[b]*Normandie Univ., COBRA, UMR 6014 & FR 3038, Univ. Rouen, INSA Rouen, CNRS, Rouen, France*

Lethal organophosphorus nerve agents (OPNA; e.g. sarin, cyclosarin, tabun, soman, VX) still present a major treatment challenge. Medical practice today utilises the antimuscarinic atropine and the oxime reactivator of OPNA-inhibited acetylcholinesterase (AChE, EC 3.1.1.7). The approved pyridinium oximes (2-PAM, HI-6, obidoxime) are not efficient for every OPNA, and they cannot cross the blood-brain barrier as they are charged. Our research was focused on several uncharged lipophilic oximes (JR595, JR585, GM508, CG193) designed to be passively transported across the blood-brain barrier and act in the central nervous system. Our primary testing *in vitro* showed that they can efficiently reactivate AChE inhibited by a range of OPNAs. However, little is still known about their *in vivo* beneficial effects that are not linked to reactivation and adverse effects that may be related to their structural characteristics. Preliminary testing showed higher chemosensitivity of human neuronal cells (SH-SY5Y) to this kind of compound. To get an insight into uncharged oximes, we thoroughly investigated their adverse effects on SH-SY5Y cells as the primary targets of these antidotes in humans. We studied the basic cytotoxicity of these oximes in terms of cell viability and cellular reactive oxygen species status. These two outcomes were also studied with OPNA compounds, which were applied at nanomolar concentrations in order to confirm oxidative stress, which is one of the proposed mechanisms of neuronal damage in OP intoxication.

Our results suggest that there is a thin line between beneficial and adverse effects of uncharged oximes on the cellular level. Therefore, our future studies will try to elucidate the mechanism behind the observed effects *in vitro* and to evaluate the toxicity of uncharged oximes *in vivo*.

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Optogenetic and pharmacological modulations of striatal cholinergic interneurons regulate motor, cognitive and emotional symptoms in Parkinson's disease

Samira Ztaou[a], Martine Liberge[a], Nicolas Maurice[b], Corinne Beurrier[b], Lydia Kerkerian-Le Goff[b], Marianne Amalric[a]

[a]Aix-Marseille Université/CNRS, Laboratoire de Neurosciences Cognitives (UMR7291), Marseille, France
[b]Aix-Marseille Université/CNRS, Institut de Biologie du Développement de Marseille (UMR7288), Marseille, France

In Parkinson's disease (PD), anticholinergic (ACh) compounds were the first widely accepted drugs before L-DOPA and recently regained interest for the treatment of neurodegenerative diseases. Here, we examined the involvement of striatal cholinergic interneurons (CINs) in the expression of motor, emotional and cognitive function. In transgenic mice specifically expressing halorhodopsin (eNpHR) in cholinergic neurons, photo-inhibition of striatal CINs reduced the asymmetric motor symptoms (postural asymmetry and turning bias) produced by unilateral 6-OHDA nigrostriatal lesions. To further investigate the muscarinic cholinergic subtypes involved, systemic and intrastriatal injections of telenzepine and tropicamide (M1 and M4 selective receptor antagonists, respectively) were tested in the same PD model. The beneficial effects on motor symptoms were reproduced by blocking either M1 or M4 mACh receptors in the dorsal striatum. As cognitive and neuropsychiatric symptoms are increasingly recognized in PD, we also examined the role of striatal CINs in these deficits in a mouse model of early PD. Decrease of striatal CINs activity by eNpHR photoillumination or M1 and M4 receptor pharmacological blockade was found to reduce anxiety in the elevated plus maze, and improve spatial and social recognition in partially lesioned mice. To decipher the mechanisms of cholinergic action on striatal post-synaptic M4 receptors, additional experiments were performed with mutant mice that lack M4 receptors only in D1 dopamine receptor-expressing cells. The present results indicate that cholinergic modulation of the dorsal striatal circuit, particularly M1 or M4 mACh receptor subtypes, plays a pivotal role in the regulation of motor, cognitive and emotional symptoms in PD.

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