



Poster abstracts

Nicotinic Acetylcholine Receptors as Therapeutic Targets: Emerging Frontiers in Basic Research and Clinical Science October 14–16, 2009, Chicago, IL

Section 1. Basic sciences

1.1

Functional consequences of adaptive evolution of the mammalian $\alpha 9\alpha 10$ nicotinic receptor

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The $\alpha 9\alpha 10$ nicotinic acetylcholine receptor (nAChR) mediates efferent inhibition of cochlear hair cells in mammals and birds. This inhibition results from activation of calcium-dependent potassium current thought to depend on calcium entry through the activated nAChR. Sequence analysis of the *CHRNA10* genes (but not of *CHRNA9*) of different species revealed signs of adaptive evolution in the mammalian lineage [1]. Therefore, one could propose that the mammalian $\alpha 9\alpha 10$ receptor (i.e., from the rat, *Rattus norvegicus*) would have functional properties different from those of the avian receptor (i.e., from the chicken, *Gallus gallus*) as a result of specific, adaptive non-synonymous substitutions acquired by the *CHRNA10* gene in the mammalian lineage. To begin to test this hypothesis, we analyzed the properties of the recombinant chicken $\alpha 9\alpha 10$ receptor, using the two-electrode voltage-clamp technique in *Xenopus laevis* oocytes expressing these subunits. The sensitivity to acetylcholine of the *G. gallus* receptor was lower than that of the *R. norvegicus* receptor ($EC_{50} = 21.7 \pm 1.2 \mu M$ and $13.8 \pm 1.7 \mu M$, respectively). In addition, the *G. gallus* $\alpha 9\alpha 10$ receptor did not desensitize significantly, in a manner similar to that of the homomeric $\alpha 9$ from rat, and different from the strong desensitization of the heteromeric $\alpha 9\alpha 10$ *R. norvegicus* receptor. Perhaps most notably, the oocyte's endogenous calcium dependent chloride current stimulated by rat $\alpha 9\alpha 10$ was not activated by the *G. gallus* $\alpha 9\alpha 10$ receptors, suggesting that calcium permeability of the avian receptor is substantially lower than that of the mammalian receptor. These results indicate that the process of adaptive evolution in the mammalian $\alpha 9\alpha 10$ receptor resulted

in new functional properties which are different from those of the homologous receptor in non-mammalian vertebrate species.

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Reference

- [1] Franchini LF, Elgoyhen AB. Adaptive evolution in mammalian proteins involved in cochlear outer hair cell electromotility. *Mol Phylogenet Evol* 2006;41(3):622–35.

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1.2

The nicotinic acetylcholine receptors of *Ascaris suum*

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Parasitic nematodes are important aetiological agents of disease in humans and domesticated animals. Currently, and for the foreseeable future, control of these infections is largely via treatment with chemical anthelmintics. Nicotinic acetylcholine receptors (nAChRs) are significant drug targets for human and animal parasitic nematodes, with pyrantel and levamisole being notable examples, and a new class of drugs, the amino-acetonitrile derivatives, currently in development. However, increasing anthelmintic resistance presents a problem for sustainable helminth control. Despite their importance as drug targets in parasites, most molecular studies of nematode nAChRs have previously been carried out using *C. elegans*. Using a bioinformatics approach, we found that the parasites *Brugia malayi* and *Trichinella spiralis* have fewer nAChR genes than *C. elegans*, but some subunits of the levamisole-sensitive neuromuscular nAChR (*unc-38*, *unc-29* and *unc-63*) are well conserved. We have cloned cDNAs encoding UNC-29 and UNC-38 from *Ascaris suum* (*A. suum*); antibody labelling shows that *Ascaris* UNC-

38 and UNC-29 co-localise on the muscle cell membrane. We have co-expressed *Ascaris* UNC-38 and UNC-29 in *Xenopus* oocytes to form functional ACh-, nicotine and levamisole-gated ion channels, the first successful heterologous expression of a parasite nAChR. Changing the subunit stoichiometry produces receptor populations with different pharmacological properties: injecting different RNA ratios to drive expression towards a 3:2 UNC-38:UNC-29 stoichiometry produces a receptor population more sensitive to nicotine and oxantel, whereas driving expression to favour a 2:3 UNC-38:UNC-29 stoichiometry produces receptors more sensitive to levamisole and pyrantel. The pharmacology of these receptors resembles the L- and N-subtypes previously observed in native *A. suum* muscle cell membranes. In addition, we describe a novel nAChR subunit gene, *acr-26*, that is conserved in several evolutionary distinct parasitic species but, to date, not in any free-living or plant parasitic species. Immunofluorescence using an antibody against *A. suum* ACR-26 designed and tested in-house demonstrated expression in the head region of the nematode. Sequence data similarities with other nAChR subunits, such as the nematode ACR-16 and the vertebrate $\alpha 7$ and $\alpha 9$ subunits, combined with a computer modelling approach predicted that ACR-26 could form a homomeric receptor. We injected cRNA encoding ACR-26 into *Xenopus* oocytes and observed that a novel homomeric receptor was expressed in these cells, forming cation channels sensitive to both acetylcholine and nicotine.

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1.3

Pharmacological chaperoning of nicotinic receptors begins in the endoplasmic reticulum: Compartments and stoichiometries

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Pentameric $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptors (nAChRs) assemble in two possible stoichiometries, $(\alpha 4)_2(\beta 2)_3$ or $(\alpha 4)_3(\beta 2)_2$. The proportion of the total receptor population represented by each stoichiometry in a cell is influenced by the local environment. Selective pharmacological chaperoning of nicotinic acetylcholine receptor (nAChR) number and stoichiometry (SePhaChARNS) is an important aspect of nicotine addiction and can explain the inadvertent therapeutic effects of smoking in Parkinson's disease. We employed fluorescent protein (FP)-tagged nicotinic acetylcholine receptor (nAChR) subunits to study the effects of nicotine and cytosine on: (1) intracellular receptor stoichiometry using pixel-by-pixel Förster Resonance Energy Transfer (FRET) and (2) trafficking of assembled nAChRs to the plasma membrane (PM) by total internal reflection fluorescence microscopy (TIRFM). Neuroblastoma (N2a) cells were transiently co-transfected with $\alpha 4$ mCherry and $\beta 2$ GFP nAChR subunits. Nicotine (1 μ M for 4 h) incubation increased the assembly of the $(\alpha 4\text{mCherry})_2(\beta 2\text{GFP})_3$ nAChR stoichiometry. Subcellular stoichiometry analysis revealed that nicotine induced preferential $(\alpha 4\text{mCherry})_2(\beta 2\text{GFP})_3$ receptor assembly in the endoplasmic reticulum (ER). TIRFM showed that nicotine exposure restricted localization of the newly assembled $(\alpha 4\text{GFP})_2(\beta 2)_3$ receptors to the ER. Conversely, cytosine treatment (1 μ M for 4 h) of $\alpha 4$ mCherry and $\beta 2$ GFP transfected N2a cells resulted in preferential assembly of the $(\alpha 4\text{mCherry})_3(\beta 2\text{GFP})_2$ stoichiometry in the ER and an

increase in surface trafficking of assembled nAChRs relative to non-treated controls. To study the influence of $\beta 2$ on $\alpha 4\beta 2$ nAChR trafficking, N2a cells were transiently co-transfected with either $\alpha 4\text{GFP}\beta 2$ or $\alpha 4\text{GFP}\beta 4$ and imaged 48 h post-transfection by TIRFM. mCherry with a lyn kinase membrane localization signal was included in transfections to visualize the PM. The $\alpha 4\text{GFP}\beta 2$ receptors trafficked to the PM in ~10 % of the cells while ~90% of cells displayed $\alpha 4\text{GFP}\beta 4$ at the PM. Together, these data reveal a rate-limiting role for $\beta 2$ subunits in ligand-induced $\alpha 4\beta 2$ nAChR trafficking and stoichiometry-based differences in subcellular receptor localization.

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1.4

Pharmacological chaperoning of nicotinic receptors begins in the endoplasmic reticulum: High-resolution imaging

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Nicotine addiction is the world's leading preventable cause of mortality. Smokers also have a much lower incidence of Parkinson's disease. Previous experiments show that ligand interactions with $\alpha 4$ - and $\beta 2$ -nicotinic receptor subunits are necessary and sufficient for nicotine addiction. A plausible cellular/molecular mechanism for some responses to nicotine exposure is selective pharmacological chaperoning of acetylcholine receptor number and stoichiometry (SePhaChARNS). To investigate SePhaChARNS in a neuronlike environment, we used single-molecule resolution fluorescence microscopy to monitor localization and trafficking of $\alpha 4\text{GFP}\beta 2$ and $\alpha 4\text{GFP}\beta 4$ receptors expressed in mouse neuroblastoma (N2a) cells. As in previous investigations on native neurons and heterologous expression systems, we find large pools of endoplasmic reticulum (ER) localized $\alpha 4\text{GFP}\beta 2$ receptors. Strikingly, cells expressing $\alpha 4\text{GFP}\beta 4$ display plasma-membrane (PM) localized receptors. Pharmacological chaperoning was investigated by incubating N2a cells expressing $\alpha 4\text{GFP}\beta 2$ receptors in nicotine or the partial agonist cytosine. Furthermore, we simultaneously imaged $\alpha 4\text{GFP}\beta 2$ receptors and a mCherry-tagged ER exit site (ERES) marker to monitor the ER exit of $\alpha 4\text{GFP}\beta 2$ receptors and the associated changes in ERES. Nicotine induced an increase of ER localized $\alpha 4\text{GFP}\beta 2$ receptors, and ERES activity did not change markedly. In contrast, cytosine treatment increased the number of ERES fusion events, which may be an ERES adaptive response to increased cargo load. Consistent with an increase in cargo load we observed increased $\alpha 4\text{GFP}\beta 2$ receptor PM localization. Data from cytosine and nicotine treatments directly indicate that pharmacological chaperoning is initiated in the ER. Nicotine and cytosine induce assembly of differential $\alpha 4\beta 2$ nicotinic receptor stoichiometries (see accompanying abstract by Srinivasan et al.), which leads to differential receptor localization, trafficking and ERES response. Thus, high-resolution imaging of SePhaChARNS is providing data required to understand, and manipulate nicotinic receptors with drugs.