1. PHD PROJECT DESCRIPTION (4000 characters max., including the aims and work plan)

Project title: Creating and characterizing biological tools to study the α3β4*-nicotinic acetylcholine receptors

1.1. Project goals: Smoking addiction has long been linked to the cholinergic system, specifically nicotinic acetylcholine receptors (nAChRs), through the principal addictive component in tobacco: nicotine. The nAChRs are implicated in many neurological disorders such as schizophrenia, Alzheimer's disease, in addition to addiction. Recent genomic studies have identified the α3-β4-α5 gene cluster as having a strong role in nicotine addiction. Understanding the role α3β4*-nAChRs play in addiction is an important aspect to developing efficient therapeutics. The goal of the project is to develop biological tools that will isolate specific stoichiometries of the α3β4*-nAChR to help understand their role in addiction. Alleviating the drug dependence of smoking would reduce the economic burden that lung cancer has on the healthcare system.

1.2. Outline: The proposed project can be broken into three separate stages which are mostly in a sequential order. The creation of and characterization of chimeric acetylcholine binding proteins (AChBPs) that mimic various regions of the α3- and β4-nAChR subunits. These characterized proteins will be used to generate VHH nanobodies either from alpaca immunization or from an already generated synthetic randomized library. The isolated VHH nanobodies will be produced and finally characterized for their binding against specific stoichiometries of the α3β4-nAChRs primarily through immunofluorescence studies and functional electrophysiological studies.

1.3. Work plan: WP1: Creation and verification of C-terminally tagged apical-α3-nAChR/*A.c.*-AChBP and apical-β4-nAChR/*A.c.*-AChBP, as well as N-terminally tagged α3/α3-AChBP and β4/β4-AChBP chimeras (~12months). Overlap-extension PCR will be used to create various version of the constructs in a vector which incorporates a C-terminally linked GFP and then these constructs will be transiently transfected into HEK-GnT1$^-$ cell lines. Evaluation of proper expression and specifically pentameric oligomerization will be performed using fluorescence size-exclusion chromatography.

WP2: The chimeric proteins from WP1 will be used to screen an already existing synthetic VHH nanobody library, and potentially injected into alpacas to generate natural VHH nanobodies against the specific domains incorporated into the chimeras. The screening of the alpaca serum and/or synthetic library will be done via phage-display panning through multiple rounds of depletion and positive selection to isolate a series of VHH nanobodies specific to the chimeric proteins being used. After three rounds of panning the isolated cDNAs from the selected colonies will be sequenced and analyzed (2a:~3months). They will be subsequently expressed in a bacterial expression system (2b: ~3months), isolated from the periplasmic space through osmotic shock, and purified via an incorporated tag on the VHH to allow for subsequent characterization.

WP3: Characterization will begin with identifying VHH nanobodies which strongly bind to the receptors through immunofluorescence experiments on already created stable cells lines expressing them, using either direct fluorophore-conjugated VHH nanobodies or fluorophore-conjugated antibodies that recognize the VHH nanobody's purification tag. Screening through

a library of the predominant neuronal nAChR subtypes through the use of a mixture of transiently transfected HEK293 cells and cells lines stably expressing these receptors will validate the selectivity of strong binders (3a: ~9months). Afterwards, a thorough functional characterization, via two-electrode voltage clamp electrophysiology, will be performed to validate specific stoichiometric binding to the $(\alpha3)_3(\beta4)_2$-nAChRs and $(\alpha3)_2(\beta4)_3$-nAChRs (3b: ~18months).

A final six months is allocated in 2029 to write up articles and prepare the dissertation.

Timeline

	2025				2026				2027				2028			
	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
WP1																
1																
WP2																
2a.																
2b.																
WP3																
3a.																
3b.																

1.4. **Literature** *(max. 7 listed, as a suggestion for a PhD candidate preliminary study):*

1. Nemecz D.§, Nowak W., ***Nemecz Á.§*** "VHH nanobody versatility against pentameric ligand-gated ion channels." *J Med Chem* (2024), Jun 13;67(11):8502-8518.DOI: 10.1021/acs.jmedchem.4c00231
2. Nemecz Á.*, Prevost, M.S.*, Menny A.*, Corringer P-J. "Emerging Molecular Mechanisms of Signal Transduction in Pentameric Ligand-Gated Ion Channels." *Neuron* (2016), May 4, 90(3):452-70. Review. DOI: 10.1016/j.neuron.2016.03.032
3. Picciotto, M. R.; Kenny, P. J. "Mechanisms of Nicotine Addiction." *Cold Spring Harb Perspect Med* (2021), *11* (5), a039610. DOI:10.1101/cshperspect.a039610.
4. Bertrand, D.; Wallace, T. L. A Review of the Cholinergic System and Therapeutic Approaches to Treat Brain Disorders. *Curr Top Behav Neurosci* (2020), *45*, 1–28. DOI:10.1007/7854_2020_141.
5. Gharpure, A., Teng, J., Zhuang, Y., Noviello, C. M., Walsh, R. M., Cabuco, R., Howard, R. J., Zaveri, N. T., Lindahl, E., & Hibbs, R. E. "Agonist Selectivity and Ion Permeation in the α3β4 Ganglionic Nicotinic Receptor." *Neuron*, (2019), Nov. 6, 104; 1-11. DOI: 10.1016/j.neuron.2019.07.030
6. Li Q., Nemecz Á., Aymé G., Baachaoui R., Prevost M.S., Pons S., Dejean de la Bâtie G., Barilone N., Maskos U., Lafaye P., Corringer P-J. "Generation of nanobodies acting as silent and positive allosteric modulators of the α7 nicotinic acetylcholine receptor." *Cellular and Molecular Life Sciences* (2023), Apr.
7. Uchański, T.; Pardon, E.; Steyaert, J. "Nanobodies to Study Protein Conformational States." *Current Opinion in Structural Biology* (2020), *60*, 117–123. DOI:10.1016/j.sbi.2020.01.003.

1.5. Required initial knowledge and skills of the PhD candidate

Master of Science in biochemistry, pharmacology, biology, or related field, completed before beginning of doctoral dissertation.

- Experience in a biochemical laboratory
- Good communication skills
- Proficiency in written and spoken English

1.6. Expected development of the PhD candidate's knowledge and skills

- Scientific development through the preparation of a doctoral dissertation,
- Experience with ligand-gated ion channels in a leading laboratory with international connections.
- Travel to international conferences.
- Successful completion of the Ph.D. may open the door to potential post-doctoral at these international locations.