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

Project title: Generation and characterization of synthetic single domain VHH antibodies against nicotinic acetylcholine receptor subtypes

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. Murine models have also shown that functional antagonists of the α3β4*-nAChRs decrease the self-administration of not only nicotine but other addictive drugs such as cocaine and morphine. Therefore, understanding the role α3β4*-nAChRs play in addiction is an important aspect to developing efficient therapeutics. The goal of the project is to develop VHHs against the α3β4-nAChR, which can be used as tools and also as potential therapeutics which may help alleviate the drug dependence of smoking and other drug addictions, thereby reducing the economic burden that cancer and other addiction-related diseases have 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 verification/characterization of robustly expressing α3β4-nAChR cell-lines will be the first major stage (Work Plan 1 [**WP1**]). **WP2**: these cell-lines will be used to isolate unique VHH nanobodies from an already generated randomized library. The isolated VHH nanobodies will be produced and finally, in **WP3**, characterized for their binding, via immunofluorescence studies; function, via electrophysiological studies; and general properties, such as their thermo-stability.

1.3. Work plan

WP1: Creation and verification of stable cell line expressing $(\alpha3)_3(\beta4)_2$- and $(\alpha3)_2(\beta4)_3$-nAChR (~9 months): Unique expression of the stoichiometric assemblies will be created from ratiometric transfection of HEK293 cells. All the stable cell-lines will be verified via immunofluorescence using cytoplasmic eGFP or mCherry conjugation. Additionally, functional evaluation through patch-clamp electrophysiology may distinguish whether the 2:3 or 3:2 variant is dominantly expressed.

WP2: Selection and production of VHH nanobodies begins with a ribosome display panning, of an already existing library, done on each of the cell-lines generated in **WP1**. Subsequent phage-display panning will be used through rounds of depletion and positive selection to isolate a series of VHH nanobodies specific to the cell-line being used. After three rounds of panning the isolated cDNAs from the selected colonies will be sequenced and analyzed (2a: ~3 months). They will be subsequently expressed in a bacterial expression system (2b: ~4 months), isolated from the periplasmic space through osmotic shock, and purified via an incorporated tag on the VHH to allow for subsequent characterization.

Characterization (**WP3**) will begin with identifying VHH nanobodies which strongly bind to the receptors through immunofluorescence experiments, 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 transiently transfected HEK293 cells will validate the selectivity of strong binders (3a: ~9 months). Afterwards, a thorough functional characterization, via two-electrode voltage clamp electrophysiology, will be performed (3b: ~18 months).

In addition to the functional and binding properties of the VHH against α3β4-nAChRs, the intrinsic properties, such as thermo-stability, isoelectric point, and ability to cross the blood-brain barrier will be evaluated (3b), with data analysis occurring at the end (3c: ~6 months).
A final six months is allocated in 2027 to write up articles and prepare the dissertation.

Timeline:

	2023				2024				2025				2026			
	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
WP1																
1																
WP2																
2a.																
2b.																
WP3																
3a.																
3b.																
3d.																

1.4. Literature *(max. 10 listed, as a suggestion for a PhD candidate)*

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

Picciotto, M. R.; Kenny, P. J. "Mechanisms of Nicotine Addiction." *Cold Spring Harb Perspect Med* (2021), *11* (5), a039610. DOI:10.1101/cshperspect.a039610.

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.

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

Zimmermann, I.; Egloff, P.; Hutter, C. A.; Arnold, F. M.; Stohler, P.; Bocquet, N.; Hug, M. N.; Huber, S.; Siegrist, M.; Hetemann, L.; Gera, J.; Gmür, S.; Spies, P.; Gygax, D.; Geertsma, E. R.; Dawson, R. J.; Seeger, M. A. "Synthetic Single Domain Antibodies for the Conformational Trapping of Membrane Proteins." *eLife* (2018), *7*, e34317. DOI:10.7554/eLife.34317.

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.

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.

Gransagne, M.; Aymé, G.; Brier, S.; Chauveau-Le Friec, G.; Meriaux, V.; Nowakowski, M.; Dejardin, F.; Levallois, S.; Dias de Melo, G.; Donati, F.; Prot, M.; Brûlé, S.; Raynal, B.; Bellalou, J.; Goncalves, P.; Montagutelli, X.; Di Santo, J. P.; Lazarini, F.; England, P.; Petres, S.; Escriou, N.; Lafaye, P. "Development of a Highly Specific and Sensitive VHH-Based Sandwich Immunoassay for the Detection of the SARS-CoV-2 Nucleoprotein." *Journal of Biological Chemistry* (2022), *298* (1), 101290. DOI:10.1016/j.jbc.2021.101290.

Hultberg, A.; Temperton, N. J.; Rosseels, V.; Koenders, M.; Gonzalez-Pajuelo, M.; Schepens, B.; Ibañez, L. I.; Vanlandschoot, P.; Schillemans, J.; Saunders, M.; Weiss, R. A.; Saelens, X.; Melero, J. A.; Verrips, C. T.; Gucht, S. V.; Haard, H. J. de. Llama-Derived Single Domain Antibodies to Build Multivalent, Superpotent and

Broadened Neutralizing Anti-Viral Molecules. *PLOS ONE* **2011**, *6* (4), e17665. DOI:10.1371/journal.pone.0017665.

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.
- Potential for travel to international conferences.
- Successful completion of the Ph.D. may open the door to potential post-doctoral at these international locations.
- Monthly stipend: 5 000 zł brutto/brutto for 36 months