Advanced fluorescent ligand-based techniques for the pharmacological and anatomical investigation of dopamine receptors

PhD thesis

Susanne Prokop

János Szentágothai Doctoral School of Neurosciences
Semmelweis University

Supervisors: István Katona, DSc

Official reviewers: Katalin Schlett, PhD
Zoltán Varga, PhD

Head of the Complex Examination Committee:
Alán Alpár, DSc

Members of the Complex Examination Committee:
Miklós Réthelyi DSc
Ádám Dénes, PhD

Budapest
2022

1. Introduction

G protein-coupled receptors (GPCRs) serve as the major targets of currently applied clinical drugs and continued efforts are underway to expand the current set of GPCR ligands for therapeutic purposes. Despite of the huge pharmacological interest, many aspects of ligand-GPCR interactions remain elusive. However, the precise understanding of drug binding is a key requirement for the successful establishment of novel pharmacological treatment strategies.

Labeled ligands have long been instrumental tools for the experimental investigation of drug-target interactions. Radioactive small molecules represent the traditional research tools to study GPCRs. Radioligand binding assays are gold standard methods to determine key parameters that describe the dynamics and kinetics of ligand-receptor interactions. Radioligands can also assist the anatomical visualization of drug targets in tissue samples via autoradiography. Overall, the contribution of radioligands to our current understanding of pharmacological principals cannot be overemphasized.

However, radioligand-based techniques face constraints, which limit their application for the investigation of some modern pharmacological paradigms.

Fluorescent ligands serve as more environmentally friendly alternatives to study drug binding events. Certainly, the synthesis of a fluorescent ligand that preserves the pharmacological characteristics of the unmodified compound can be challenging, since the attachment of a relatively large fluorescent moiety may substantially affect the molecular recognition of the ligand. However, due to the recent development of structural biology and computational tools, the number of available fluorescent ligands is rapidly rising. Generally, all fundamental pharmacological experiments that apply radioactive compounds can be conducted with the help of fluorescent ligands as well. Several different fluorescent detection methods have been applied to perform quantitative ligand binding measurements. However, there is a growing need for novel easily reachable proximity-based approaches, that can be readily applied to investigate unmodified receptors of interest.

Furthermore, the broad array of optical microscopic techniques offers the possibility to detect the target engagement of fluorescence ligands at multiple anatomical levels. Although previous studies have applied fluorescent small molecules in different modalities of optical microscopy, pharmacological probes are still not routinely considered for anatomical research. There is still a lack of experience with their application for histological studies and a rising demand for high-resolution microscopic approaches, which can detect individual drug-target interactions in a cell-type specific manner.

2. Objectives

We aimed to broaden the applicability of fluorescent pharmacoprobes and to develop refined fluorescent small molecule-based assays. Considering the leading role of dopamine receptors (DRs) in the pharmacological management of neuropsychiatric diseases, we decided to apply fluorescently labeled DR ligands to introduce our novel technical approaches and make an assessment about

our advancements by the investigation of prototypical D_1- and D_2-like dopamine receptors.

1. Our first specific aim was to apply a novel bioluminescence resonance energy transfer (BRET) assay for the quantitative measurement of D_1 DR ligand binding. In contrast to direct fluorescence measurements, BRET assays possess several advantages, because they do not require extrinsic excitation of the sample. Instead, energy is produced by an oxidative reaction of a luciferase enzyme. According to a recently developed BRET-based platform, if NanoLuc small luciferase is fused to the N-terminus of the receptor, only receptor-bound fluorescent ligands are excited and unbound molecules do not have to be separated from the reaction well, since energy transfer is highly dependent on the distance between the energy donor and acceptor. Therefore, ligand binding can be readily measured without additional washing steps and with a high signal-to-noise ratio. Our goal was to develop a novel BRET platform that preserves all the advantages of the previous assay but overcomes its major drawbacks. Firstly, we intended to avoid direct receptor tagging since covalent modifications can result in improper protein

folding or may directly alter ligand binding. Secondly, we aimed to make the assay more cost-efficient, which can facilitate its adaptation for high-throughput platforms.

2. Since nanoscale biochemical processes can determine the physiological or pathophysiological effects of receptor-ligands interactions in a cell-type specific manner, our second objective was to develop a novel methodology that enables the analysis of drug binding in precise anatomical context. More specifically, our intention was to design a framework in which we can visualize the receptor engagement of a dopamine receptor-targeting drug even at the nanoscale level by its fluorescently labeled analogue. We chose a novel, D_3R-preferring third generation antipsychotic drug, cariprazine, as the basis of a novel microscopic pharmacoprobe. We hypothesized that a fluorescently labeled analogue of cariprazine would be an advantageous research tool for multiple purposes. First, due to its high D_3R affinity, it would represent a useful labeling tool for this subtype. Second, the identification of nanoscale cariprazine binding sites in brain tissue could reveal important details about its hitherto enigmatic mechanism of action. We decided to

optimize a procedure for the multi-scale imaging of fluorescent cariprazine binding in cell-culture and tissue samples. Additionally, we aimed to test the feasibility of cell-type specific super-resolution imaging of drug binding sites.

3. Methods

In BRET measurements, luminescence was detected in HEK 293T cells using a Varioskan Flash multimode plate reader.

Fluorescent images were taken with 3DHISTECH Panoramic MIDI II slide scanner, a Nikon A1R confocal laser-scanning system, and correlated confocal and STORM iaging was performed on a Ti-E inverted microscope equipped with an N-STORM system, a Nikon C2 confocal scan head, and an Andor iXon Ultra 897 EMCCD camera.

All animal experiments were approved by the Hungarian Committee of the Scientific Ethics of Animal Research and were carried out according to the Hungarian Act of

Animal Care and Experimentation in accordance with the European Communities Council Directive.

Microscopic cell culture experiments were performed on transiently transfected HEK 293 cells. Histological samples were prepared from D_3R wild-type and knock-out mice on C57BL/6 genetic background. *In vivo* cariprazine was administered intraperitoneally to live mice. Pharmacological labeling was performed on acute brain slices, immunolabeling was performed on thin, free-floating sections. Custom-written Python scripts were used to assist the quantitative analysis of images.

4. Results

Our first set of experiments aimed to measure ligand binding of D_1R with the help of a novel BRET-assay that exploits a fluorescent receptor ligand. Instead of directly tagging D_1R with the energy donor, we measured BRET between receptor-bound fluorescent ligands and a plasma membrane-anchored Gaussia luciferase enzyme (GLuc-PM). Moreover, the GLuc-PM biosensor uses coelenterazine as a substrate for the luciferase reaction,

making our measurements exceptionally cost-effective compared to the previous NanoLuc-based BRET assays. Treatment with BODIPY-FL-SKF-83566, a commercially available fluorescent D_1R drug, led to an increase of the BRET signal in cells co-expressing D_1R and the GLuc-PM. The binding of a non-labeled antagonist (SCH-23390) could be readily measured in a competitive manner. Co-treatment with an unlabeled D_1R ligand (SCH-23390) decreased the BRET signal in a concentration-dependent manner, and the half maximal inhibitory concentration (IC_{50}) value of SCH-23390 was successfully assessed from the displacement assay (1.07 nM).

In our second set of experiments, we have first successfully designed and synthesized a novel sulfo-Cy5-tagged fluorescent probe (Fluo-CAR) that preserved the main pharmacological characteristics of the original antipsychotic drug. It acted as a weak D_3R partial agonist in a BRET-based G_{i1} protein activation assay and displayed highest affinity towards the D_3 dopamine receptor (D_3R) subtype. We tested its utility for the

visualization of D_3R in cell cultures by confocal and STORM super-resolution microscopy. Fluo-CAR selectively visualized HA-tagged D_3Rs in the plasma membrane of HEK 293 cells, which were simultaneously labeled with anti-HA immunolabeling as well. The receptor binding sites of Fluo-CAR molecules could be localized with excellent lateral localization precision by STORM microscopy. To demonstrate the quantitativity of our approach, we implemented a PharmacoSTORM-based competitive- and saturation ligand binding assay. Notably, due to the single molecule detection sensitivity of STORM, the receptor binding of individual Fluo-CAR molecules could be specifically visualized even at low (subnanomolar) ligand concentration.

We illustrated the advantages of multi-scale imaging by combining epifluorescence, confocal and STORM microscopy for the mapping of Fluo-CAR binding in the mouse brain. Living acute brain slices were treated with the fluorescent pharmacoprobe before chemical fixation and further processed to conform various microscopic settings.

Low-magnification images revealed the highest intensity Fluo-CAR labeling in the vicinity of dense cell masses in the basal forebrain, representing the so-called Islands of Calleja (IoC). The islands are formed by the aggregations of small granule cells, and the nearby Fluo-CAR rich areas were identified as “hilar” subregions of the IoC. We performed a precise three-dimensional reconstruction of the Fluo-CAR rich areas from consecutive coronal brain samples which corroborated that the IoC represents a continuous structure throughout a surprisingly large portion of the ventral striatum. In accordance with its *in vitro* receptor affinity profile, Fluo-CAR labeling was fully eliminated from the IoC of D_3R KO animals. Next, we demonstrated the feasibility of fluorescent ligand-based super-resolution imaging of pharmacological interactions in tissue. We performed STORM imaging with Fluo-CAR in the IoC of D_3R WT and KO mice. The number of Fluo-CAR localization points (LPs) was significantly reduced in the absence of D_3Rs, which confirmed the high specificity of the PharmacoSTORM signal. As opposed to diffraction limited techniques, PharmacoSTORM had the capacity to

discern individual fluorescent drug molecules from background with nanoscale precision.

We further challenged the efficiency of our approach and performed nanoscale imaging in striatal regions where Fluo-CAR labeling was substantially weaker than in the IoC. Indeed, the PharmacoSTORM LP density was significantly lower in the adjacent ventral and dorsal striatum, where the specificity of the low-density Fluo-CAR signal was further validated by competitive binding experiments with unlabeled cariprazine. In contrast to the IoC, specific Fluo-CAR binding was detected in the dorsal and ventral striatum even in the absence of D_3Rs. These results agree with the high expression level of the other lower affinity target of cariprazine, the D_2 dopamine receptors.

Considering the D_3R preference of Fluo-CAR in the IoC, we visualized the distribution of drug binding sites together with immunolabeling of dopaminergic signaling proteins. Firstly, we selected dopamine- and cAMP-regulated neuronal phosphoprotein of molecular weight 32,000 (DARPP-32) as a concomitant target, which represents a key intracellular modulator of

dopaminergic signaling in striatal medium spiny neurons. DARPP-32 was below the detection sensitivity in the IoC on large scale confocal microscopic images. We next performed anti-tyrosine hydroxylase (TH) immunolabeling, which visualizes the key neurotransmitter synthesizing enzyme in dopaminergic afferents. Contrarily to DARPP-32, TH-immunoreactive afferents constitute an extensive meshwork in the IoC. However, in contrast to our low magnification images, combined confocal and PharmacoSTORM imaging of TH immunostaining and Fluo-CAR binding sites revealed that Fluo-CAR LPs rather avoided TH-immunolabeled dopaminergic terminals at the nanoscale level.

To further search for the cellular structures that underly the prominent Fluo-CAR binding in the IoC, granule cells were filled with biocytin by electrophysiological methods within acute brain slices that were subsequently treated with the fluorescent probe. Systematic correlated three-dimensional analysis of granule cell morphology with Fluo-CAR distribution revealed that a thin, gracile process, which shows morphological features of an axon, consistently enters the Fluo-CAR rich hilus. At the

nanoscale level, correlated confocal and PharmacoSTORM imaging of granule cell axons and drug binding sites demonstrated that Fluo-CAR molecules are preferentially associated to the axons of granule cells.

Finally, we established a competitive ligand binding experiment to confirm that PharmacoSTORM LPs represent real *in vivo* binding sites of the original drug cariprazine. Fluo-CAR molecules were effectively displaced by live cariprazine treatment of the animals, which indicates that PharmacoSTORM has the capacity to visualize the binding of the clinically applied drug in its native tissue environment.

5. Conclusions

The current thesis introduces two novel techniques that demonstrate the pertinence of fluorescent ligands in quantitative pharmacological as well as in anatomical studies. The Gaussia luciferase-based BRET assay is an excellent tool for the characterization of ligand binding of unmodified D_1 dopamine receptors with the help of its commercially available fluorescent ligand and holds the

potential for cost-efficient high-throughput screening of unlabeled small molecules in a competitive manner.

The PharmacoSTORM method provides a new fluorescent-ligand based framework that brings quantitative pharmacology and precise anatomical studies together. The successful rational design of a fluorescent cariprazine analogue (Fluo-CAR) demonstrates that the generation of specific pharmacological probes can be greatly facilitated by structure-based rational design and advanced chemical techniques. The new compound is a suitable labeling probe for D_3R in both overexpression and native systems for conventional and super-resolution fluorescence microscopy as well, and pharmacological receptor labeling can be readily combined with the visualization of immunological markers.

Fluo-CAR binds D_3Rs at an outstanding density in the Islands of Calleja, which represents a large, continuous assembly of granule cells in the basal forebrain. We have verified that Fluo-CAR binding sites in the IoC represent real *in vivo* targets of the unlabeled antipsychotic, cariprazine. Our multi-scale analysis revealed a prominent

density of cariprazine binding sites on DARPP-32 negative granule cell axons in the Islands of Calleja, which were surrounded by tyrosine hydroxylase-positive dopaminergic nerve terminals.

6. Bibliography of the candidate's publications

Publications related to the thesis:

1. Tóth AD, Garger D, **Prokop S**, Soltész-Katona E, Várnai P, Balla A, Turu G, Hunyady L. (2021) A general method for quantifying ligand binding to unmodified receptors using *Gaussia* luciferase. *J Biol Chem*, 296:100366. I.F.: 5.157
2. **Prokop S***, Ábrányi-Balogh P*, Barti B, Vámosi M, Zöldi M, Barna L, Urbán GM, Tóth AD, Dudok B, Egyed A, Deng H, Leggio GM, Hunyady L, van der Stelt M, Keserű GM*, Katona I. (2021) PharmacoSTORM nanoscale pharmacology reveals cariprazine binding on Islands of Calleja granule cells. *Nat Commun*, 212(1):6505.
   I.F.: 14.919

Publications unrelated to the thesis:

1. Szalai B, Hoffmann P, **Prokop S**, Erdélyi L, Várnai P, Hunyady L. (2014) Improved methodical approach for quantitative BRET analysis of G Protein Coupled Receptor dimerization. *PLoS One*, 9(10):e109503
   I.F.: 3.234
2. Elek Z, Dénes R, **Prokop S**, Somogyi A, Yowanto H, Luo J, Souquet M, Guttman A, Rónai Z. (2016) Multicapillary gel electrophoresis based analysis of genetic variants in the WFS1 gene. *Electrophoresis*, 37(17-18):2313-21
   I.F.: 2.744
3. **Prokop S**, Perry NA, Vishnivetskiy SA, Toth AD, Inoue A, Milligan G, Iverson TM, Hunyady L, Gurevich VV. (2017) Differential manipulation of arrestin-3 binding to basal and agonist-activated G protein-coupled receptors. *Cell Signal*, 36:98-107.
   I.F.: 3.487

4. Tóth AD*, **Prokop S***, Gyombolai P, Várnai P, Balla A, Gurevich VV, Hunyady L, Turu G. (2018) Heterologous phosphorylation-induced formation of a stability lock permits regulation of inactive receptors by β-arrestins. *J Biol Chem*, 293(3):876-892.
I.F.: 4.106