Conclusion and summary

Recent years have witnessed an increasing need for sensitive *in vitro* techniques for the characterization of the interaction of ligands with respective G protein-coupled receptors (GPCRs) to keep up with pharmacological insights. Phenomena of particular interest include the determination of structure-activity relationships (SAR) in diverging signaling pathways, the potentially biased agonism of ligands between these pathways, and the possible occurrence of inverse agonism. Furthermore, *in vitro* bioassays have been described to be applicable for the activity-based detection of new psychoactive substances (NPS) in biological samples, based on the particular activity of a substance rather than on its specific structure.

Part A of this thesis focuses on the development of *in vitro* assays for the activity-based detection and functional characterization of (psychedelic) agonists of the serotonin 2A receptor (5-HT_{2A}R). The 5-HT_{2A}R is the main pharmacological target of serotonergic psychedelics, a substance group of which a substantially expanding number of compounds appeared on the drug market as NPS throughout the past few years. Main issues encountered are the difficult detection and the poor characterization of these substances.

As a number of *in vitro* assays have been described, **Chapter A.1** provides an overview of those assays that have been specifically applied for the characterization of (psychedelic) agonists at the 5-HT_{2A}R. This overview includes the methodologies on which these assays are based, a discussion of their advantages and limitations, and the factors that should be considered when comparing outcomes from different studies. In this thesis, the NanoBiT® functional complementation technique was selected, a sensitive technique based on a real-time luminescent readout in live cells.

In **Chapter A.2**, this technique was applied to monitor the recruitment of the cytosolic protein β-arrestin 2 (βarr2) to the activated receptor in the presence of a psychedelic agonist, to explore whether an activity-based screening assay can be developed to detect the use of such substances in biological fluids. However, blank samples also resulted in an activation signal in the assay, and upon the exploration of different options, the endogenous agonist serotonin was uncovered as the responsible factor, precluding the use of activity-based screening assays for psychedelic substances.

A second issue with psychedelic substances, is the fact that their functional properties are only scarcely characterized at a molecular level. Therefore, in **Chapter A.3**, a cell line was developed that stably expresses the assay components from Chapter A.2. This cell line was

subsequently applied for SAR elucidation in a set of 30 (psychedelic) 5-HT_{2A}R agonists, for which the EC_{50} values were correlated with estimated common user doses.

An outstanding question about the molecular mechanisms of (psychedelic) 5-HT_{2A}R agonists, is their potentially biased agonism, which is addressed in **Chapters A.4, A.7,** and **A.8**. To this end, a second bioassay was developed, monitoring the recruitment of mini$G\alpha_q$ to the 5-HT_{2A}R in the NanoBiT® system. Hence, the described approach uses two highly analogous assay setups, detecting two signaling events upstream of the signaling cascade. Statistically significantly biased agonism has been detected in each of the chapters, with diverging interpretations depending on the method employed to analyze the data. **Chapter A.7** additionally evaluates the impact of a number of variable factors on the obtained outcomes, including the reference agonist used and the time point of data analysis. **Chapter A.8** identified strongly biased agonists, which almost exclusively induce the recruitment of βarr2, based on the 5-HT_{2A}R selective agonist 25CN-NBOH.

Furthermore, the described bioassays have been employed to determine which molecular features are indispensable for receptor activation in a set of 25H-NBOMe (**Chapter A.5**) and 25H-NBF (**Chapter A.6**) positional isomers. This aspect has been largely unexplored in these substance groups that have only recently emerged.

Part B describes *in vitro* assays for the characterization of pharmacological phenomena at certain purinergic receptors, including the A_3 adenosine receptor (A_3AR) and the $P2Y_2$ receptor ($P2Y_2$R). A brief introduction on signaling through purinergic GPCRs, its receptors, functionalities, and available ligands is provided in **Chapter B.1**.

Chapter B.2 describes the development of a cell line stably expressing the components of a NanoBiT® recruitment assay to assess mini$G\alpha_i$ recruitment to the A_3AR, complementing a highly analogous A_3AR-βarr2 recruitment assay. This allowed for the estimation of biased agonism in a set of A_3AR ligands. Even though the performance of the bioassay was appropriately demonstrated, none of the tested ligands displayed a signaling pattern divergent from that of the reference agonist.

Aiming to uncover biased agonism in a set of methanocarba nucleoside A_3AR ligands, in **Chapter B.3** inverse agonism was uncovered. So far, this phenomenon has only been scarcely explored in A_3AR ligands, with only one non-nucleoside ligand having been identified, and no

nucleoside derivatives showing this behavior. The further exploration of a set of structurally related substances unveiled remarkable patterns, in which a minor change of substituent in one position converted inverse agonism to partial agonism.

While for the A_3AR an extensive number of ligands has been described and the molecular mechanisms have been explored to a substantial extent (although the understanding is far from complete), the $P2Y_2R$ has been less intensively researched. One example of an elusive mechanism, is the recruitment of βarr1 and βarr2 to the receptor. **Chapter B.4** describes the search for $P2Y_2R$ structural determinants indispensable for the recruitment of either βarr in response to receptor activation by either endogenous ligand, ATP or UTP. While all generated receptor constructs could still effectively recruit both, the C-terminal tail apparently modulated this interaction.

Overall, the bioassays generated and applied in this thesis resulted in ‘An enlightening trip through psychedelic and purinergic signaling’, and prove(d) to be useful to address a variety of pharmacological questions.