English Summary
There is MORe than meets the eye in MOR signaling. The µ-opioid receptor (MOR) is amongst the most extensively studied G-protein coupled receptors (GPCRs), yet there has been an inflow of new emerging concepts that leave us bewildered about the different roles it can take up. In this thesis, we have studied MOR from two different angles, giving rise to Part 1 and Part 2 of this thesis. Part 1 mainly focusses on the role of GPCR heterodimerization in modulating the functional outcome, with a primary focus on MOR. In this part, we have shown how the interaction of MOR with another GPCR may modulate its signaling. In Part 2 we have developed and applied MOR reporter systems for the evaluation of novel synthetic opioids (NSO), which is one of the rapidly growing classes of new psychoactive substances (NPS). We did this by monitoring the capacity of these NSO to cause recruitment of distinct cytosolic proteins to MOR.

Chapter 1 provides a broad introduction about GPCRs, along with a detailed introduction about MOR and the dopamine D_2 receptor (D_2R), as the physical and functional interaction of these two receptors has been assessed further on in this thesis. Along with this, also the interplay between opioid and dopamine systems is discussed, together with a brief section dedicated to GPCR-GPCR interactions.

Chapter 2 provides a brief outline on the aim of the thesis.

Part 1, Chapter 3 of this thesis reviews protein complementation assays (PCA) based on functional complementation of the split luminescent or fluorescent proteins, as these have been widely used to study GPCR dimerization/oligomerization. Starting from the evolution of these bio-assays, this review moves on to their current applicability in in vivo settings.

There exists an interplay between opioid and dopamine systems. Using Human Embryonic kidney 293T (HEK293T) and HeLa cells transfected with MOR and D_2R, we have demonstrated that these receptors heterodimerize, using an array of biochemical-biophysical techniques such as co-immunoprecipitation (co-IP), Bioluminescence Resonance Energy Transfer 1 (BRET\(^1\)), Förster Resonance Energy Transfer (FRET) and functional complementation of a split luciferase. Moreover, live-cell imaging also revealed that D_2L R, when co-expressed with MOR, slowed down the internalization of MOR, following activation with the MOR agonist [D-Ala\(^2\), N-MePhe\(^4\), Gly-ol] enkephalin (DAMGO) (Chapter 4).
Part 2 of this thesis focuses on another aspect of MOR signaling: its ligand binding and how this translates to signaling. In this part, we focus on NSO as a relevant class of NPS, which are synthetic derivatives of existing psychoactive drugs with a slightly tweaked structure to circumvent detection and legislation. NSO are one of the deadliest classes of NPS emerging on the illegal drug market. These opioids exert their effect by binding to and activating MOR, which then leads to the recruitment of cytosolic transducers, such as G protein and/or β-arrestin. The recruitment of these cytosolic transducers has been studied by various techniques by many researchers all around the globe and the literature about NSO and signaling has been summarize under three major topics in Chapter 5. First, modification of the core structure of the highly potent analgesic fentanyl has been found to have a profound influence on the activity of the resultant moieties. Second, the activity of bivalent ligands built using fentanyl as one of the ligands is briefly discussed. Third, functional selectivity has been extensively studied for MOR in general, but only to a limited extent for NSO. Some studies have tested a signaling bias demonstrated by fentanyl both in vitro and in vivo, which has been discussed here. Additionally, we review some biased ligands built on the core structure of piperidine that have also been tested both in vitro and in vivo. This, together with our own results, provides a holistic knowledge about the recent developments in this field.

Opioids are undoubtedly the best painkillers on the market, but unfortunately, they carry the risk of causing unwanted side effects. Enormous efforts are channelized to develop drugs that retain their analgesic potential but are devoid of side effects, which is where the concept of biased agonism comes into the picture. On the signaling level downstream from MOR, analgesia has been reported to be mainly mediated through the G protein pathway, while the undesirable effects of opioids have been linked to the β-arrestin pathway. As mentioned in Chapter 5, the NSOs have newly emerged on the illegal market and little is known about their potential ‘bias’ (i.e. the preferential activation of one pathway over the other). With this in mind, we have developed and applied a novel, stable, robust bio-assay platform to study the activity of twenty-one synthetic opioids through both the G protein pathway, via coupling of mini-Gi (GTPase domain of Gαi subunit), and the β-arrestin pathway, via recruitment of β-arrestin 2, to activated MOR. The utilized technology (NanoBiT®) relies on functional complementation of two split fragments of nanoluciferase {LargeBiT (LgBiT) and SmallBiT (SmBiT)}, either fused to the receptor or to a cytosolic protein (mini-Gi/ β-arrestin 2 in this
case), that is recruited to the receptor upon activation. This ultimately leads to a restoration of the luciferase activity, which generates a bioluminescent signal in the presence of a suitable substrate. The bio-assays developed and used in this study allowed us to gain insight into the structure-activity relationships of NSO. Although none of the tested NSO showed a significant bias for any of the two pathways, remarks about some tendencies for some NSO were made cautiously, which could be useful to gain some preliminary insights and may be a tool for future compound design (Chapter 6).

Recently, nanobodies derived from camelids have received significant attention owing to their applicability in stabilizing the structure of activated MOR, via their specific recognition of and binding to the active receptor conformation. In Chapter 7, we have developed and applied a novel bio-assay, utilizing intracellular expression of one such nanobody, Nb39. This bio-assay also utilizes the NanoBiT® technology, as explained above. Here, the cytosolic protein Nb39 was linked to one part of a split luciferase and, upon MOR activation, this fusion protein was recruited to the receptor (fused to the other part of a split luciferase). All the evaluated synthetic opioids (n = 5) demonstrated a concentration-dependent response in this MOR-Nb39 bio-assay. Moreover, when comparing the obtained results with those obtained using the MOR-mini-Gi bio-assay (as described in Chapter 6), very similar potencies (expressed as pEC$_{50}$) were obtained. Interestingly, the raw activation profiles of the novel MOR-Nb bio-assay revealed that the activation pattern was sustained over time, as opposed to the profile obtained from the mini-Gi coupling bio-assay, where the activation profile drops relatively quickly, although both are a reflection of activated MOR. This could possibly stem from signaling from internal membranes such as the Golgi apparatus or endosome, in addition to the canonical activation from the plasma membrane, although this postulation needs to be supported by further studies.

Just like a coin that has two sides, and both sides have equal importance, we have presented in this thesis two sides of our coin, “MOR”, providing evidence that there is more to MOR than meets the eye. Indeed, the more is known about MOR signaling, the more we realize a lot is not known, from the impact of its interaction with other receptors to the differential signaling by (synthetic) opioids: we’re just at the start of a new journey. This thesis aimed at contributing some pieces to the massive puzzle of this ever MORE intriguing receptor.