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GLYCOSYLATION AND DIMERIZATION OF THE HUMAN δ-OPIOID RECEPTOR POLYMORPHIC VARIANTS
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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium F202 of the Faculty of Medicine (Aapistie 5 B), on 14 December 2018, at 12 noon

UNIVERSITY OF OULU, OULU 2018
Cellular signaling by G protein-coupled receptors (GPCRs) governs a wide array of physiological functions throughout the body. The human δ-opioid receptor (hδOR) is a GPCR that modulates the sensation of pain and mood and has great potential for the treatment of pain and a variety of neurological disorders. A common single-nucleotide polymorphism (SNP) in the extracellular N-terminal tail of hδOR changes Phe to Cys at position 27. Using various biochemical and cell biological methods, the study demonstrates that several events during receptor biosynthesis and cell surface delivery are affected by the SNP. These events participate in the multifaceted regulation of the receptor and modulate receptor behavior at the cell surface.

Two distinct pathways were shown to scrutinize the quality of the synthesized hδOR in the endoplasmic reticulum (ER) and target some for degradation in N-glycan-dependent and -independent ways. The hδOR\textsubscript{Cys27} that matures inefficiently required N-glycan-mediated interactions with the lectin-chaperone calnexin to be expressed in a fully functional form at the cell surface, whereas the N-glycan-independent pathway was sufficient for hδOR\textsubscript{Phe27}. For both variants, the N-glycan-independent quality control, which is likely to operate as a back-up pathway, led to a more rapid export from the ER and receptors at the cell surface that were less stable.

Receptor dimerization emerged as an important regulatory step for receptor cell surface delivery. In co-transfected cells, interactions between the newly-synthesized variants led to the retention and subsequent ER-associated degradation of hδOR\textsubscript{Phe27}. This dominant-negative attenuation of hδOR\textsubscript{Phe27} cell surface expression by hδOR\textsubscript{Cys27} may have unpredictable consequences for opioid signaling in heterozygous individuals.

Finally, the study shows that N-acetylgalactosamine (GalNAc)-type O-glycosylation catalyzed in the Golgi modulates hδOR expression at the cell surface by enhancing receptor stability and inhibiting constitutive downregulation. The modification of Ser residues in the receptor N-terminus by GalNAc-transferase 2 was affected by the SNP, which presents another distinction in the cellular processing of the two variants.

The findings highlight the importance of the biosynthetic pathway in the regulation of GPCR behavior and pave way for strategies for treatments targeting GPCRs at this level.

**Keywords:** calnexin, cell surface receptors, delta opioid receptors, dimerization, endoplasmic reticulum, endoplasmic reticulum-associated degradation, G protein-coupled receptors, glycoproteins, glycosylation, membrane proteins, N-acetylgalactosaminyltransferases, opioid receptors, post-translational protein processing, protein biosynthesis, secretory pathway, signal transduction, single-nucleotide polymorphism
Tiivistelmä

Solujenvälillä viestinnällä on keskeinen tehtävä kehon kaikissa toiminnoissa. δ-opioidireseptori (δOR) on solusignalointiin erikoistuneen kalvoproteiiniperheen (G-proteiiniin kytketyt reseptorit) jäsen, joka ohjaa kivuntuntemusta ja mielialoja. Sitä pidetään mahdolliseena lääkekehyksen kohteena paitsi kivunlievityksen, myös useiden neurologisten häiriöiden hoidossa. δOR ilmenee kahtena polymorfisena muotona sen solunulkoisessa osassa tapahtuneen aminohappomutuksen vuoksi (Phe27Cys). Työssä tutkittiin reseptorin glykosylaatiota ja dimerisaatiota, jotka säätävät sen prosessointia, käyttäytymistä ja toimintaa. Käyttäen useita biokemiallisia ja solubiologisia menetelmiä työssä osoitettiin polymorfian vaikuttavan useisiin prosessointivaiheisiin ja muokkaavan siten reseptorin viestintää.


Tutkimus luo uutta tietoa biosynteesireseptoreiden merkityksestä G-proteiiniin kytkettyjen reseptoreiden säättelyssä sekä antaa pohjaa keinoille, joilla tätä voitaisiin hyödyntää farmakologisesti.

Asiakirjat:

To Mom and Dad
Acknowledgements

The work included in this thesis was carried out at the Cancer and Translational Medicine Research Unit (2010-2016) and the Research Unit of Biomedicine (2016-2018), at the University of Oulu. The work was done under the supervision of Dr. Ulla Petäjä-Repo in a research group that was part of the Glycoscience Graduate School and Medical Research Center Oulu during the time of the research. This study was financially supported by personal grants and salary from the Glycoscience Graduate School, Finnish Cultural Foundation, Magnus Ehrnrooth Foundation and the University of Oulu Graduate School and research group funds of the Academy of Finland, Medical Research Center Oulu and Sigrid Juselius Foundation.

I am grateful to my supervisor and the leader of the GPCR research team Dr. Ulla Petäjä-Repo for her guidance and supervision during the thesis project. I want to thank her for sharing her expertise and knowledge of GPCRs and for the countless opportunities to discuss our research. I am also grateful for the critical review of the many applications and texts that I had the chance to write.

I wish to thank the head of the Research Unit of Cancer and Translational Medicine, Juha Tuukkanen, and the head of the Research Unit of Biomedicine, Jukka Hakkola, for the opportunity to work in their respective departments and for their supportive attitude towards my research.

I am grateful to the past and present members of the GPCR research team, Orvokki Mattila, Hanna Tuhkanen, Olli-Pekka Tapio, Jussi Tuusa, Piia Markkanen, Tarja Leskelä, Anna Hakalahti, Hamayan Khan, Mervi Kreus and others with whom I had the chance to discuss and collaborate during my research. Especially, I wish to express my gratitude to my partners-in-crime Orvokki and Hanna, who made the struggle to finish the experiments infinitely more enjoyable. I also wish to thank the laboratory technicians Miia Vierimaa and Tuula Taskinen for their technical assistance and patience.

I wish to express my sincere gratitude to my co-authors Piia and Tarja who were instrumental in the studies of the thesis. I am also grateful to Christoffer Goth for his guidance and ideas during my research visit in the Copenhagen Center for Glycomics, and to Prof. Henrik Clausen for hosting me during this visit and for his suggestions for the research. Assistance provided by the staff in Denmark, especially Dr. Adnan Halim and Dr. Sergey Vakhrushev was also greatly appreciated. I am also grateful to Prof. Michel Bouvier for the opportunity to collaborate with his group during the thesis.
I wish to thank Prof. Jukka Finne and Dr. Tarja Kokkola for their critical review of my work and for the valuable comments and suggestions for the thesis, and Deborah Kaska, Ph.D., for the revision of the language in the thesis. I thank Jukka Käräjäoja for the revision of the Finnish abstract of the thesis.

I am grateful to my family and friends, Laura, Petri, Basti, Elisa, Stella, Kristiina, Sami, Kyösti and Leila for their support and friendship, for taking care of my dog and house when my research required my absence, and for giving me moments to think about other things besides science. My deepest gratitude goes to my parents Markus and Helli for all the support and care during my life.

Finally, I wish to thank my dog, Rapsu, for patiently waiting for my return from the lab after many long days.

19.10.2018

Jarkko Lackman
Abbreviations

αAR α-adrenergic receptor
βAR β-adrenergic receptor
dlOR δ-opioid receptor
κOR κ-opioid receptor
µOR µ-opioid receptor
AC adenylyl cyclase
AMP adenosine monophosphate
AR adrenergic receptor
ATP adenosine triphosphate
BiP binding immunoglobulin protein
BLAST basic local alignment search tool
BRET bioluminescence resonance energy transfer
BSA bovine serum albumin
C- carboxy-
cAMP cyclic adenosine monophosphate
cGMP cyclic guanosine monophosphate
CHO Chinese hamster ovary
CNS central nervous system
CNX calnexin
COP II coatamer protein II
CRT calreticulin
DDM n-dodecyl-β-D-maltopyranoside
DMEM Dulbecco’s modified Eagle medium
ECL extracellular loop
EDEM ER degradation-enhancing α-mannosidase-like protein
EDTA ethylenediaminetetraacetic acid
EndoH endo-β-N-acetylglicosaminidase H
ER endoplasmic reticulum
ERGIC endoplasmic reticulum-Golgi intermediate compartment
ERK1/2 extracellular signal-regulated kinase 1/2
ERManI ER-α1,2 mannosidase I
ERQC endoplasmic reticulum quality control
ERAD endoplasmic reticulum-associated degradation
ESCRT endosomal protein sorting complex
ESI-LIT-FT electrospray ionization-linear ion trap-Fourier transform
Idl  low density lipoprotein
LHR  luteinizing hormone receptor
MAF  minor allele frequency
MALDI matrix assisted laser desorption/ionization
MS mass spectrometry
MVB multi vesicular body
N- amino-
NC-IUPHAR International Union of Pharmacology, Committee on Receptor Nomenclature and Classification
NEM N-ethylmaleimide
NTX naltrexone
OST oligosaccharyltransferase
PBS phosphate-buffered saline
PDI protein disulfide isomerase
PDZ postsynaptic density protein of 95 kilodaltons, disc large, zona occludens-1
PE phycoerythrin
PI3K phosphoinositide 3 kinase
PKA protein kinase A
PKC protein kinase C
PLC β phospholipase C-β
PNGase F peptide:N-glycosidase F
PPI peptidyl-prolyl isomerase
PVDF polyvinylidene fluoride
RTP receptor transport protein
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM standard error of mean
SNP single-nucleotide polymorphism
TBS Tris-buffered saline
TGN trans-Golgi network
TM transmembrane domain
TOF time of flight
TR transferrin receptor
TR-FRET time-resolved Förster/fluorescence resonance energy transfer
TSHR thyroid stimulating hormone receptor
UDP uridine diphosphate
UGGT UDP-glucose:glycoprotein glucosyltransferase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>VVA</td>
<td><em>Vicia villosa</em> agglutinin</td>
</tr>
<tr>
<td>WB</td>
<td>western blotting</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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Original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:


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1 Introduction

G protein-coupled receptors (GPCRs) are a large family of signaling proteins mediating the signals of extracellular stimuli into cells in a wide variety of physiological events. Signals in the form of small molecules, hormones, ions, light, peptides, light, among others, activate the receptors via conformational changes in their seven transmembrane helical structures, and mediate the cellular response via G protein interactions and downstream signaling cascades. GPCRs are targeted by more than a third of the known pharmaceutical drugs, underlining the importance of the receptor family (Hauser, Attwood, Rask-Andersen, Schiöth, & Gloriam, 2017; Katritch, Cherezov, & Stevens, 2013).

The human δ-opioid receptor (hδOR) is a member of the opioid receptor subgroup of GPCRs that mediate the signals of endogenous opioid peptides and opiate alkaloids and participate in the modulation of pain and emotional responses. It is predominantly expressed in the central nervous system. (Darcq & Kieffer, 2018; Gendron, Cahill, von Zastrow, Schiller, & Pineyro, 2016). The opioid receptor family is relevant in the treatment of chronic pain, as well as many neurological disorders such as alcoholism, addiction and depression (Chu Sin Chung & Kieffer, 2013). A single-nucleotide polymorphism (SNP) in the extracellular N-terminus results in two distinct wild-type (WT) variants (Phe27 and Cys27) that are characterized by differences in the cellular processing with implications for various physiological conditions (Gelernter & Kranzler, 2000; Leskelä, Markkanen, Alahuhta, Tuusa, & Petäjä-Repo, 2009).

The number of functional GPCRs at the plasma membrane governs the output of their signaling and consequently, the subsequent response. The receptor levels are regulated at many levels, from the biosynthetic pathway to trafficking from the cell surface, and many of them involve receptor processing that modifies receptor structure and behavior (Dong, Filipeanu, Duvernay, & Wu, 2007; Petäjä-Repo & Bouvier, 2005). The receptor folds in the endoplasmic reticulum (ER) where the N-terminus is modified by N-glycans that govern the ER quality control (ERQC), a process that ensures the expression of functional glycoproteins at the cell surface and targets misfolded proteins for ER-associated degradation (ERAD) (Tannous, Pisoni, Hebert, & Molinari, 2015). The hδORs, like most other GPCRs, form functional oligomers, which not only alters receptor signaling but adds another aspect to receptor processing in the biosynthetic pathway (Cvejic & Devi, 1997; George et al., 2000; Hasbi et al., 2007). In the Golgi, the N-terminus is further modified by O-glycans that are likely to play a role in receptor processing and
behavior (Bennett et al., 2012; Markkanen & Petäjä-Repo, 2008; Petäjä-Repo, Hogue, Laperriere, Walker, & Bouvier, 2000). The present study attempts to shed light on these events and to understand their role in the processing of the receptor and in the regulation of functional receptors at the cell surface.
2 Review of the literature

2.1 G protein-coupled receptors

Signal transduction is a fundamental part of cellular communication within an organism. Signals of various hormones, neurotransmitters, ions, chemokines, odors and small organic molecules are converted to cellular responses by a vast and versatile superfamily of GPCRs. At the cell surface, they convey the extracellular messages into cells by interacting with intracellular heterotrimeric guanine-nucleotide-binding proteins (G proteins) and other effectors that facilitate the response (Katritch et al., 2013; Lagerström & Schiöth, 2008; Pierce, Premont, & Lefkowitz, 2002). According to databases (GPCR database [GPCRdb] and International Union of Basic and Clinical Pharmacology [IUPHAR]), about 800 genes in humans encode GPCRs that share a distinct structural similarity of seven cell membrane passing regions, an extracellular N-terminus and an intracellular C-terminus (Alexander et al., 2017; Fredriksson, Lagerström, Lundin, & Schiöth, 2003; Pandy-Szekerés et al., 2018) (Fig. 1). The ligand-induced activation of GPCRs governs a wide range of physiological processes, which explains why the GPCRs are the largest family of targets for pharmaceutical drugs and an active subject of scientific research (Hauser et al., 2017; Rask-Andersen, Masuram, & Schiöth, 2014).

2.1.1 Classification

The structure of GPCRs varies greatly. However, they share two defining characteristics. First, they all possess seven hydrophobic segments of 25 to 35 amino acid residues that make up the seven α-helical transmembrane domains (TMs) in the protein structure (Fig. 1). The second characteristic is their ability to interact with heterotrimeric G proteins. However, many GPCRs have not yet been demonstrated to interact with G proteins and for a few GPCRs, G protein-independent signaling is well established (Fredriksson et al., 2003).
Fig. 1. Schematic structure of GPCRs. GPCRs are polytopic membrane proteins that share a common structure of seven cell membrane passing α-helical domains that are connected by intracellular and extracellular loops. In the native conformation, the domains form a barrel-like bundle that is flanked by N-terminal and C-terminal domains on the extracellular and intracellular sides, respectively. A short alpha-helical segment (H8) is conserved in the membrane-proximal region of the C-terminal tail. The receptor structure contains conserved microdomains (red) and intramolecular bonds [green lines. The line thickness represents the number of interactions (Venkatakrishnan et al., 2013), that maintain the structure and govern the conformational changes during receptor activation and function. The orientation of the TM (1-7) as viewed from the intracellular side is depicted below the barrel-like bundle model. Modified from Venkatakrishnan et al., 2013.

GPCRs have been divided into subfamilies based on sequence homology, phylogeny and similarities in structure and ligands. A number of different systems for the division and nomenclature have emerged over the years. One of the early classifications of the protein family divided the identified GPCRs into six classes, A-F, based on sequence homology (Table 1) (Kolakowski, 1994). More recent phylogenetic analyses divide GPCRs by the GRAFS system (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, Secretin) denoted by the initial letters of the prototypical receptor members in the separate subgroups (Table 1) (Fredriksson et al., 2003). These two classifications are the most commonly used and overlap.
with each other. Recent revisions to these classifications place Taste2 receptors in their own group; these are thought to have diverged from Rhodopsin/Class A family receptors. Furthermore, some receptors have been placed in a group of Others as these receptors do not clearly belong to any of the designated groups (Nordström, Sallman Almen, Edstam, Fredriksson, & Schiöth, 2011). The IUPHAR Committee on Receptor Nomenclature and Classification (NC-IUPHAR) uses a revised A-F system with Adhesion and Frizzled receptors in their own groups (Alexander et al., 2017). The revised A-F classification used by NC-IUPHAR and the GRAFS classification are compiled in Table 1.

Table 1. Classification of GPCRs.

<table>
<thead>
<tr>
<th>Classification</th>
<th># Receptors*</th>
<th>Non-sensory</th>
<th>Sensory</th>
<th>Olfactory</th>
<th>Vision</th>
<th>Pheromone</th>
<th>Taste</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC-IUPHAR GRAFS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A Rhodopsin</td>
<td>682 / 81</td>
<td>197</td>
<td>390</td>
<td>9</td>
<td>5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>B Secretin</td>
<td>15 / 0</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C Glutamate</td>
<td>22 / 8</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Adhesion</td>
<td>33 / 33</td>
<td>33</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Frizzled</td>
<td>11 / 0</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>T Taste2</td>
<td>25 / 0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>O Other</td>
<td>6 / 6</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>D*</td>
<td>-</td>
<td>0</td>
<td>-</td>
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<td>E†</td>
<td>-</td>
<td>0</td>
<td>-</td>
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</tr>
</tbody>
</table>

* Number of human receptors. † Orphan, receptors without identified ligands. ‡ Vomeronasal 1. § Taste 1. ‡ Fungal mating pheromone receptors, none found in humans. ¶ cAMP receptors, none found in humans (Fredriksson et al., 2003, Nordström et al., 2011). Abbreviations: NC-IUPHAR, International Union of Basic and Clinical Pharmacology, Committee on Receptor Nomenclature and Classification; cAMP, cyclic adenosine monophosphate.

GPCRs are also divided based on their general functional properties to sensory and non-sensory GPCRs (Table 1). In humans, sensory GPCRs include roughly 450 receptors that mediate olfaction, pheromone, vision and taste signaling (Mombaerts, 2004). Non-sensory GPCRs make up about 350 receptors and they participate in cellular signaling and are targets for a majority of pharmaceutical drugs (Rask-Andersen et al., 2014). The families of GPCRs are often further subcategorized into groups based on endogenous ligands. The GPCRdb groups receptors into aminergic (e.g. adrenergic receptors), peptide (e.g. opioid receptors), protein (e.g. chemokine receptors), lipid (e.g. cannabinoid receptors), melatonin, nucleotide (e.g. adenosine receptors), steroid (e.g. GPCR estrogen receptors), alicarboxylic, sensory (e.g. rhodopsin), adhesion, ion, amino acid [e.g. gamma-
aminobutyric acid (GABA) receptors], orphan and other subgroups. Orphan GPCRs are receptors that still lack an identified endogenous ligand (Munk et al., 2016; Pandy-Szekeres et al., 2018). Some of these orphan receptors have putative ligands suggested while others have been suggested to interact with other GPCRs (Fredriksson et al., 2003).

The Rhodopsin receptor family (Class A) is the largest GPCR family with nearly 700 members in humans. The family is further divided into four phylogenetic subgroups of α, β, γ and δ, each containing closely related receptor clusters (Fredriksson et al., 2003). The family is characterized by receptors with a wide range of endogenous ligands (all subgroups except adhesion, ion and amino acid) that bind to the pocket within the bundle made by the TM domains. The opioid receptors belong to the γ-subgroup of the Rhodopsin/Class A family (Fredriksson et al., 2003). The Secretin (Class B) family is a small family of 15 peptide receptors that bind peptide hormones (Grauschopf et al., 2000; Lagerström & Schiöth, 2008). The Adhesion family contains 33 receptors and is sometimes called the Class B2 receptors due to the similarities with Secretin receptors (B1) (Fredriksson et al., 2003). However, they have distinct N-termini that bind extracellular matrix proteins (Lagerström & Schiöth, 2008). The Glutamate receptor family consists of 22 receptors that bind ions, amino acids or small molecules and often form dimeric receptors connected by disulfide bridges. The Frizzled receptor family consists of 10 frizzled receptors and the smoothened receptor. The Frizzled receptors bind Wnt glycoproteins, whereas the smoothened receptor operates in a signaling complex (Bhanot et al., 1996; Murone, Rosenthal, & de Sauvage, 1999). The GPCR receptor families with their characteristic structures are illustrated in Figure 2.

2.1.2 Structure

The primary structure of GPCRs is characterized by seven hydrophobic segments that form the distinctive seven TM domains (TM1-TM7) (Fig. 1). Early on, it was predicted that these segments form a bundle of TM helices that make up the core of the receptor structure (Baldwin, 1993) and the low-resolution structure of bovine rhodopsin supported this notion (Unger, Hargrave, Baldwin, & Schertler, 1997). The first high resolution structure of a GPCR, again the bovine rhodopsin, confirmed the predicted geometry and revealed some details in the structure. The TM domains are connected by extracellular (ECL1-3) and intracellular (ICL1-3) loops and are flanked by the extracellular N-terminus and the intracellular C-terminus (Palczewski et al., 2000). The general structure is depicted in Figure 1.
Fig. 2. GPCR families. GPCRs are divided phylogenetically into six different receptor families that have the most variability in the extracellular receptor N-terminus. Most GPCRs belong to the Rhodopsin/Class A family where the ligand binding pocket (red) lies within the TM core. The families with large N-terminal tails (Secretin, Adhesion, Glutamate and Frizzled) typically have a disulfide-bond stabilized N-terminal tail with functional domains (blue) that participate in the binding of their corresponding ligands (Fredriksson et al., 2003). The class C receptors exist in obligatory dimers (Jones et al., 1998). The Taste2 receptor group was recently separated into its own family (Lagerström & Schiöth, 2008; Nordström et al., 2011). The number of human receptors in each family is shown in parentheses. Modified from Fredriksson et al., 2003.

According to the GPCRdb, 252 GPCR crystal structures have been solved including 50 unique receptor structures (June 2018) (Pandy-Szekeres et al., 2018). Most structures have been crystallized in a ligand-bound inactive conformation. Opsin was the first structure solved in the active conformation (Scheerer et al., 2008). GPCR structures in complex with a G protein have also been revealed, as the beta-2 adrenergic receptor (β2AR) together with the G protein subunit Gαs, and
the mu-opioid receptor (µOR) with Ga, were successfully crystallized (Koehl et al., 2018; Rasmussen et al., 2011). Importantly, the crystallized “snap shots” of active and inactive receptor conformations only provide partial insights to the dynamic range of conformations that are subject to modulation by several factors and conditions.

A general division of the GPCR structure into three domains can be made, each with a role in the activation of the receptor and signal transduction. The extracellular part (N-terminus and ECLs) modulates the ligand access, the TM core mediates the signal by conformational changes upon ligand binding, and the intracellular domains (C-terminus and ICLs) convey the changes in the TM core by binding intracellular proteins that initiate the signaling cascades (Venkatakrishnan et al., 2013). The ligand binding domains in the TM core, or in the N-terminal tail in the case of Secretin, Glutamate and Frizzled families (Fig. 2), are often structured, whereas the C-terminal tail, ILC3 and most N-terminal tails are mostly disordered (Venkatakrishnan et al., 2014).

Extracellular domains

Extracellular domains show the most variety both in sequence and in structure between GPCR families and individual receptors (Lagerström & Schiöth, 2008; Unal & Karnik, 2012). This has also been seen in the crystallography of GPCR family members that reveal distinct secondary structures and disulfide bond patterns in the extracellular domains (Wheatley et al., 2012). Members of the Secretin, Adhesion, Glutamate and Frizzled receptor families have a characteristic large folded N-terminal domain (Fig. 2). Ligands of the Secretin family receptors bind to the receptor extracellular domains but also penetrate the ligand binding pocket within the TM bundle (Fig. 2) (Hemley, McCluskey, & Keller, 2007; Hollenstein et al., 2013). Receptors of the Adhesion family have an N-terminus that contains a number of different domains that likely contribute to cellular and cell-to-matrix interactions (Fig. 2) (Bjarnadottir et al., 2004; H. H. Lin et al., 2001), and a GPCR proteolytic domain that mediates the autoproteolytic cleavage of the N-terminus (Krasnoperov et al., 1997). The first structure of a receptor from the Glutamate family revealed a two-domain ligand binding site that is stabilized by disulfide bonds (Kunishima et al., 2000). The Frizzled family receptors have cysteine-rich domains in the N-terminus that bind Wnt ligands (Janda, Waghray, Levin, Thomas, & Garcia, 2012). The smoothened receptor ligand binding and
receptor activity involves both the cysteine rich domains and the TM bundle (Byrne et al., 2016; Sharpe, Wang, Hannoush, & de Sauvage, 2015).

Most Rhodopsin/Class A GPCRs have a small non-folded N-terminal tail with no distinct functional domains. The few exceptions include, e.g. protease-activated receptors that have a unique conserved proteolytic site that is cleaved by extracellular proteases to form a tethered N-terminal ligand for the receptor (Oikonomopoulou et al., 2006; Vu, Hung, Wheaton, & Coughlin, 1991; W. F. Xu et al., 1998). On the other hand, glycoprotein hormone receptors [luteinizing hormone receptor (LHR), follicle-stimulating hormone receptor (FSHR), and thyroid stimulating hormone receptor (TSHR)] have leucine-rich repeats that take part in hormone binding (Q. R. Fan & Hendrickson, 2005). These domains are also found in the N-termini of other leucine-rich repeat containing GPCRs. Some of these receptors also have low-density lipoprotein receptor Class A domains (Hsu et al., 2000).

The extracellular regions of Rhodopsin/Class A GPCRs may leave the binding pocket open and water accessible, form lid-like structures, or participate in the selectivity of ligands (Venkatakrishnan et al., 2013). The structure of the µOR revealed that the proximal N-terminus forms a lid-like structure and interacts with the bound agonist in the active crystal structure of the receptor (W. Huang et al., 2015). A study on β2AR demonstrated how ligands in the binding pocket also stabilize distinct conformations in the ECLs and proposed that drugs targeting the extracellular domains could modulate receptor activity via conformational coupling (Bokoch et al., 2010). The N-terminal tail has also been suggested to have an impact on downstream signaling. Mutations in the N-terminal tail of the serotonin receptor 5-HT2B were reported to have an effect on receptor activity (Belmer et al., 2014). Similarly, the activity of the melanocortin-4 receptor in the absence of a ligand was demonstrated to be maintained by the receptor N-terminal tail (Ersoy et al., 2012). The role of the N-terminal tails in cell surface expression has been suggested for dopamine D2 and α2c-adrenergic receptors (Angelotti, Daunt, Shcherbakova, Kobilka, & Hurt, 2010; Cho et al., 2012). Likewise, the N-terminal tail of the yeast GPCR Ste2p has an impact on cell surface expression as well as on receptor-receptor interactions (Uddin, Hauser, Naider, & Becker, 2016; Uddin, Kim, Deyo, Naider, & Becker, 2012).

Extracellular domains of membrane proteins, such as GPCRs, are typically subjected to co- and post-translational modifications that take place along the early secretory pathway. Protein glycosylation is a ubiquitous modification that adds an additional layer of ‘molecular information’ to structures and has crucial biological
and physiological roles (Moremen, Tiemeyer, & Nairn, 2012). N-glycan modifications in the extracellular domains mediate protein-protein interactions that guide folding during glycoprotein biosynthesis (see 2.2) (Tannous et al., 2015). During processing in the ER, the GPCR extracellular domains also attain disulfide bonds that stabilize the folded structure. Rhodopsin and Secretin receptor families have a conserved disulfide bond between ECL2 and TM3 that stabilizes the conformation of the TM bundle during receptor folding (Venkatakrishnan et al., 2013). A number of GPCRs have also been reported to be modified with O-glycans in the extracellular domains (see 2.2.5).

**Transmembrane core**

The TM core is the most conserved region in GPCR architecture. Despite distinct receptor sequences between different GPCRs, the overall structure is very similar within the protein family. Topologically equivalent residues within the structure form conserved noncovalent inter-TM contacts that provide the conformational scaffold for the structure (Fig. 1). For the Rhodopsin/Class A family, the TM bundle provides a pocket-like fold for ligand binding. The pocket typically has conserved topologically equivalent residues that mediate the interaction with ligands entering different depths into the pocket. Different GPCRs have varying amino acids in these sites, leading to selective ligand-receptor interactions (Venkatakrishnan et al., 2013; Venkatakrishnan et al., 2014). The TM core also has a number of structural motifs conserved in the family. These motifs have a role in receptor activation as functional micro-domains that modulate the transitions between receptor states. The conserved Asp/Glu-Arg-Tyr (“DRY”) motif in the cytoplasmic end of TM3 participates in conformational switches between inactive and active receptor states (Fig. 1). For some receptors it forms an “ionic lock” that is present in the basal and inactive conditions (Nygård, Frimer, Holst, Rosenkilde, & Schwartz, 2009; Venkatakrishnan et al., 2013). For opioid receptors, it forms a salt bridge between TM3 and ICL2 (Thompson et al., 2012; Venkatakrishnan et al., 2013). In the crystallized β2AR-Gaα complex, arginine in the motif directly interacts with the Gaα subunit (Rasmussen et al., 2011). The conformation of Arg in the motif is restrained by a hydrophobic cage facilitated by conserved residues in TM3 and TM6 (Prioleau, Visiers, Ebersole, Weinstein, & Sealfon, 2002). The Asn-Pro-x-x-Tyr-x-Phe (“NPxxY”) motif in TM7 is responsible for a conserved interaction with helix 8 (H8, see the following section) and TM2 (Fig. 1) (Angel, Chance, & Palczewski, 2009; Prioleau et al., 2002). The Cys-Trp-x-Pro (“CWxP”) motif in the TM6 is a
ligand binding-sensitive switch that regulates the ionic lock and controls the transitions between active and inactive states (Fig. 1) (Visiers, Ballesteros, & Weinstein, 2002).

**Intracellular domains**

The intracellular domains of GPCRs have a role in responding to conformational changes in the TM domain. The binding of a ligand initiates conformational changes in the binding pocket and the TM core that consequently elicit changes in the intracellular domains. These changes expose binding sites for G proteins and other signaling effectors that initiate intracellular downstream signaling (Katritch, Cherezov, & Stevens, 2012; Katritch et al., 2013). The crystallized receptor structures typically have unstructured ICLs or ICLs with \( \alpha \)-helical turns (Venkatakrishnan et al., 2013). The ICL2 of \( \beta_2 \)AR was shown to interact with the N-terminus of the \( \mathrm{G}_\alpha \) subunit in the crystallized complex (Rasmussen et al., 2011). Many solved structures for the Rhodopsin family have a short amphipathic helix (H8) at the C-terminal side of the TM bundle (Venkatakrishnan et al., 2013). Cysteines at the C-terminal end of the H8 are often modified by palmitoylation that anchors the C-terminal tail to the plasma membrane (Moench, Moreland, Stewart, & Dewey, 1994). Several GPCRs, including the \( \delta \)OR have been demonstrated to be palmitoylated (Chini & Parenti, 2009; Petäjä-Repo et al., 2006; Qanbar & Bouvier, 2003). The palmitoylation of \( \delta \)OR in the early compartments of the secretory pathway was demonstrated to be required for the efficient trafficking of the receptor to the cell surface, and at the cell surface it is regulated in an activation-dependent manner (Petäjä-Repo et al., 2006).

The C-terminus and ICL3 are typically intrinsically unstructured regions, giving them flexibility and exposure of amino acid segments that have likely functional importance (Gsponer & Babu, 2009; Jaakola, Prilusky, Sussman, & Goldman, 2005). The intracellular domains also contain a number of residues that are phosphorylated by different kinases. The phosphoserines in the C-terminal tail mediate the recruitment of \( \beta \)-arrestins that facilitate the endocytic trafficking and downregulation of the receptor (Gurevich & Gurevich, 2006). A number of GPCRs also have so-called PDZ (postsynaptic density protein of 95 kilodaltons, disc large, zona occludens-1)-interacting motifs in the C-terminal tail. PDZ-proteins interact with these motifs and modulate receptor trafficking and signaling (Dunn & Ferguson, 2015).
2.1.3 Signaling

The classical view of GPCR signaling involves ligand binding to the extracellular side that induces conformational changes in the receptor. The changes in the intracellular side enable coupling of heterotrimeric G proteins to the receptor, which dissociate upon activation and interact as separate subunits with downstream effectors that then trigger the cellular response (Fig. 3) (Pierce et al., 2002). The process is not a simple on/off event, rather, it is a dynamic process based on conformational plasticity of the receptor and the capacity of ligands to shift the conformational equilibrium to different receptor states that produce a biological effect (Manglik, Kobilka, & Steyaert, 2017; D. M. Rosenbaum, Rasmussen, & Kobilka, 2009). The activation of the receptor depends on the ligand binding pocket that is located differently in the various families of GPCRs (Fredriksson et al., 2003). A study on 27 Rhodopsin/Class A GPCRs concluded that activation of different receptors has highly conserved features involving similar structural rearrangement between TM3, TM6 and TM7 that exposed the G protein binding sites. This explains how diverse ligands can activate GPCRs that bind the same G proteins (Venkatakrishnan et al., 2016).

Receptor states

GPCRs exist in different receptor states that are in equilibrium in physiological conditions. A crude division can be made into inactive and active receptor states, mirroring their capacity to activate G proteins (Fig. 3). Crystallographic data suggest intermediate conformations, such as low-affinity agonist-bound inactive states, and active states with varying degrees of G protein interaction (Katritch et al., 2013). In the basal state, the receptor may exist in different states, some of which may be susceptible to binding to G proteins in the absence of a ligand. This so-called constitutive activity has been observed for several GPCRs (Kobilka & Deupi, 2007; Seifert & Wenzel-Seifert, 2002), including the δOR (Befort, Zilliox, Filliol, Yue, & Kieffer, 1999). A commonly used pharmacological model for GPCR activation is the extended ternary complex (ETC) model that describes the equilibrium of receptor conformations and complexes with a ligand and/or a G-protein (Samama, Cotecchia, Costa, & Lefkowitz, 1993) (Fig. 3B). In the model, equilibrium exists between active (Rₐ) and inactive receptor states (Rᵢ), both of which can be in complex with a ligand (LRₐ and LRᵢ). However, only the active receptor state leads to activation. The active receptor in complex with a ligand (or
not) may form a complex with a G protein (LRaG and RaG). A complex cubic ternary model further allows the interaction of an inactive receptor with a G protein, with or without a ligand (LRiG and RiG; Fig. 3C) (Weiss, Morgan, Lutz, & Kenakin, 1996). The model is supported by the discovery of G protein-dependent binding of ligands to inactive forms of the receptor (Brown & Pasternak, 1998). The landscape of different receptor states is further complicated by receptor states in complex with effectors such as β-arrestins and GPCR kinases (GRKs) and with allosteric modulators (Katritch et al., 2013).

**Fig. 3. Ligand-induced activation of GPCRs.** The signaling of GPCRs is mediated by receptor-specific ligands (shown in red) and distinct receptor states that can activate specific intracellular signaling pathways. **A**, Agonists bind to the receptor and promote conformational changes that shift the equilibrium to active states (LRa) that have affinity for heterotrimeric G proteins (shown in green) and other signaling effectors. Binding to G proteins (LRaG) results in the dissociation of G protein subunits that can initiate the typical signaling cascades. **B-C**, The classical model of receptor activation has been extended to the more complex models of the extended ternary complex model (B) and cubic ternary complex model (C). Modified from Katritch et al., 2013, Rasmussen et al., 2011 and Weiss et al., 1996.
**Ligands and allosteric modulators**

Various small molecules, such as hormones, neurotransmitters, amino acids, peptides and other extracellular signals can act as ligands for GPCRs by interacting with the GPCR binding pocket. Ligands are classified by their affinity and capacity to stabilize different receptor conformations (Fig. 4), leading to different outcomes in the cellular response. The different responses arise from the capacity of the ligands to alter the equilibrium of the receptor states. Stabilization of active receptor states by the agonist, for example, leads to a higher number of active receptors that can bind the signaling effectors (Fig. 4) (Katritch et al., 2013).

The primary site for the interaction of ligands with GPCRs is the orthosteric site. Ligands bound to this site stabilize ligand-specific active or inactive conformations of the receptor. Further complexity is added to the activation of GPCRs by allosteric modulators that bind to non-competitive allosteric sites in different parts of the receptor. Allosteric modulators can fine-tune the pharmacological response by modulating the receptor ligand-bound conformation. The impact can be on the efficacy or affinity of the orthosteric ligand, or the modulator can shift the equilibrium towards an active conformation in a process known as allosteric agonism (May, Avlani, Sexton, & Christopoulos, 2004). Positive and negative allosteric modulators can enhance or antagonize the activation, respectively, whereas silent modulators have no net effect. Bitopic ligands bind both to the orthosteric and allosteric sites (Keov, Sexton, & Christopoulos, 2011). Ions that are bound to the ligand binding pocket are typical allosteric modulators. Structures of opioid receptors have revealed sodium ions that elicit allosteric effects on ligand binding for these receptors. A similar mechanism occurs for many other receptors of the Rhodopsin family (Fenalti et al., 2014; Katritch et al., 2014; W. Liu et al., 2012). Recently, a β2AR structure with a small molecule allosteric modulator revealed an allosteric binding site at the intracellular side of the receptor (X. Liu et al., 2017).

**G proteins and downstream signaling**

Heterotrimeric G proteins comprise three subunits, α, β and γ (Fig. 3A). The Ga subunit is a guanosine triphosphate (GTP) hydrolase that in the inactive guanosine diphosphate (GDP)-bound conformation is coupled to the other subunits and interacts with the receptor. The interaction activates the Ga subunit, causing the exchange of GDP to GTP and dissociation from the Gβγ dimer. Ga-GTP and Gβγ
can activate downstream effectors that carry out the cellular signaling via modulation of the amount of second messengers in various pathways (Oldham & Hamm, 2008; Syrovatkina, Alegre, Dey, & Huang, 2016).

Fig. 4. Classification of GPCR ligands. Ligands are characterized by their capacity to activate or inhibit GPCR signaling. In the absence of a ligand, active and inactive receptor states are in an equilibrium that, results in basal activity. Full and partial agonists bind to and stabilize the active receptor states causing a shift of different degrees in the equilibrium towards the active state followed by G protein coupling and GPCR signaling. Antagonists bind both the active and inactive receptor states causing no change in the equilibrium. Thus, they produce no response and can inhibit signaling of competing agonists for the same binding sites. Inverse agonists have a higher affinity for inactive states, leading to a shift towards the inactive state and inhibition of basal activity (Katritch et al., 2013). Modified from Rosenbaum et al., 2009.

The Gα subunits have been divided into four families, whereas Gβ and Gγ subunits are each included in one family (Table 2). The most common families of Gαs and Gαi are named based on the capacity of their members to activate (Gαs, s = stimulatory) or inhibit (Gαi, i = inhibitory) adenylyl cyclases (AC), respectively (Landry, Niederhoffer, Sick, & Gies, 2006; Syrovatkina et al., 2016; Watson, Katz, & Simon, 1994; Wettschureck & Offermanns, 2005; Wilkie et al., 1992). The Gβγ dimer dissociated from Gα can also regulate effectors, such as phospholipase C-β and certain AC isoforms (Landry et al., 2006; Oldham & Hamm, 2008). A more detailed list of G protein subunit families and the effectors that their members activate and inhibit is in Table 2. The GPCR-G protein interface has multiple residues that can be employed, providing determinants for receptor selectivity. Different receptors may bind to the same G proteins, using slightly different
residues. Similarly, receptors may bind to different types of G proteins. The G protein-coupling of GPCRs is complicated by the fact that the receptors are subject to functional selectivity (see 2.1.3, functional selectivity) by different ligands, oligomerization and allosteric modulators that may alter the active receptor conformation to favor specific G proteins (Flock et al., 2017).

The effectors stimulated or inhibited by G proteins modulate the amount of second messengers that convey the signal further into the cellular response. Typical second messengers include cyclic adenosine monophosphate (cAMP), diacylglycerol, inositol triphosphate, cyclic guanosine monophosphate (cGMP) and Ca\(^{2+}\). Usually, the second messenger-mediated pathways lead to phosphorylation and/or protein activation (Landry et al., 2006; Syrovatkinsa et al., 2016). The most important GPCR second messenger pathway is the cAMP pathway. The cAMP-dependent signaling pathways can activate protein kinases and regulate the function of ion channels. The activation of protein kinase A (PKA) by cAMP leads to further steps in the signaling cascade as it phosphorylates various proteins, such as transcription factors that regulate gene expression, e.g. enzymes that regulate glucose metabolism, promote muscle contraction or increase heart rate (Landry et al., 2006; Oldham & Hamm, 2008). A well-characterized pathway involves the activation of cAMP response element-binding protein (CREB) that stimulates the transcription of CRE-genes responsible for various cellular responses (De Cesare & Sassone-Corsi, 2000; Shaywitz & Greenberg, 1999).

Typically, several parallel signaling pathways are initiated upon GPCR activation. For example, opioid receptor activation leads to G\(\alpha\)-mediated inhibition of AC and activation of the G protein-regulated inwardly rectifying potassium channel (Kir3), and G\(\beta\gamma\)-mediated inhibition of Ca\(^{2+}\) channels (Al-Hasani & Bruchas, 2011).

Active conformations of certain GPCRs may have affinity for G proteins in the absence of ligands, leading to constitutive activity. Similarly, certain GPCRs in their active conformations may possess affinity for downstream effectors and scaffold proteins, in their G protein-free state. The G protein-independent signaling of GPCRs is a relatively recently discovered phenomenon, in which certain receptors interact with intracellular effectors independently of G protein interaction, and thus modulate their activity and the associated signaling pathways (Tilley, 2011). G protein-independent activation of signaling effectors has been recently discovered, for example, for opioid, melanocortin, adrenergic and chemokine receptors (Ghamari-Langroudi et al., 2015; Ilegorova, Fisyunov, & Krishtal, 2010; Shenoy et al., 2006; Steen, Larsen, Thiele, & Rosenkilde, 2014). GPCRs also activate signaling pathways via interactions with \(\beta\)-arrestins, that are
typically required for receptor endocytic trafficking (Hilger, Masureel, & Kobilka, 2018; Peterson & Luttrell, 2017).

Table 2. G protein subunits and effectors.

<table>
<thead>
<tr>
<th>Family</th>
<th>Subtypes</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_s$</td>
<td>$\alpha_s$, $\alpha_{olf}$</td>
<td>Adenylyl cyclase↑, Src tyrosine kinase</td>
</tr>
<tr>
<td>$\alpha_i$</td>
<td>$\alpha_{i1-3}$, $\alpha_{i4}$, $\alpha_{o}$, $\alpha_{t1}$, $\alpha_{t2}$</td>
<td>Adenylyl cyclase↓, Src tyrosine kinase, cGMP-PDE↑</td>
</tr>
<tr>
<td>$\alpha_{q/11}$</td>
<td>$\alpha_q$, $\alpha_{11}$, $\alpha_{14}$, $\alpha_{15/16}$</td>
<td>Phospholipase C-β↑, p63RhoGEF, G protein-coupled receptor kinase 2, Phosphoinositide 3 kinases</td>
</tr>
<tr>
<td>$\alpha_{12}$</td>
<td>$\alpha_{12}$, $\alpha_{13}$</td>
<td>p115RhoGEF↑, LARG, PDZ-RhoGEF</td>
</tr>
<tr>
<td>$\beta$</td>
<td>$\beta_{1-5}$</td>
<td>Adenylyl cyclase↑, Phospholipase C-β↑, Phosphoinositide 3 kinases↑, G protein-coupled receptor kinases↑, K+ and Ca2+ channels↑</td>
</tr>
</tbody>
</table>

* signaling by G$\beta$γ dimer; ↑, stimulation; ↓, inhibition. Abbreviations: cGMP-PDE, cyclic guanosine monophosphate phosphodiesterase; LARG, leukemia-associated RhoGEF; RhoGEF, Rho-Guanine nucleotide exchange factor (Landry et al., 2006; Syrovatkina et al., 2016; Watson et al., 1994; Wettschureck & Offermanns, 2005; Wilkie et al., 1992).

Functional selectivity

The conformational plasticity of GPCRs results in further complexity in the activation and signaling of these receptors. Minute changes in the active conformations may alter the activation of G proteins and downstream effectors and may cause differential signaling. The concept of functional selectivity or biased signaling has been coined to describe the distinct signaling pathways via activation of the same receptor (Urban et al., 2007). Different ligands for the same receptor may stabilize these different conformations (Nygaard et al., 2013) that lead to distinct pathways or even G protein switching. Furthermore, these biased ligands may alter the events following receptor activation, including subcellular trafficking, binding kinetics with the effectors, and spatially differential signaling (Costa-Neto, Parreiras, & Bouvier, 2016). Certain signaling pathways have been attributed to distinct membrane micro-domains and subcellular compartments (Eichel, Jullie, & von Zastrow, 2016; Irannejad et al., 2013), indicating distinct dimensions in GPCR signaling due to functional selectivity.
2.1.4 Functional regulation

Physiological functions mediated by GPCR activation are sensitive to the number of receptors at the cell surface. In order to respond to extracellular stimuli, and to maintain the signaling capability in the complex and dynamic environment, the cells control the amount of receptors by regulating the ER export and delivery of GPCRs to the cell surface, and by desensitization and endocytic trafficking of receptors from the cell surface (Duvernay, Filipeanu, & Wu, 2005). Increase in the number of δOR at the cell surface, for example, leads to enhanced δOR-mediated signaling and function (C. M. Cahill et al., 2001; Walwyn, John, Maga, Evans, & Hales, 2009). Conversely, receptor trafficking from the cell surface decreases the δOR-mediated responses (A. A. Pradhan et al., 2009; Scherrer et al., 2006).

ER export and cell surface delivery

The cell surface delivery of GPCRs is governed by processes in the ER and along the secretory pathway, and for GPCRs localized predominantly in the intracellular compartments, such as the δOR, these steps have been suggested to regulate the signaling of the receptor (C. M. Cahill, Holdridge, & Morinville, 2007; X. Zhang, Bao, & Ma, 2010). Nascent GPCRs in the ER are immediately scrutinized by the ERQC machinery that regulates the quality of exported receptors from the compartment. Receptors unable to pass the check points are targeted for degradation in a process known as ERAD (Braakman & Hebert, 2013; Tannous et al., 2015). The ERQC and ERAD are important steps in GPCR biosynthesis that are discussed in more detail later (see 2.2). Protein degradation by ERAD acts also as a quantity control process for a subset of membrane protein substrates, including GPCRs. The ER quantity control responds to environmental cues by regulating the degradation and thus levels of exported receptors (Printsev, Curiel, & Carraway, 2016). The events in the ER during synthesis of δOR have been demonstrated to be rate-limiting in the receptor maturation (Petäjä-Repo et al., 2000), suggesting important regulatory steps during its cell surface delivery. In the ER, a portion of the δOR is retained by the ERQC and degraded by the ERAD mechanisms (Markkanen & Petäjä-Repo, 2008; Petäjä-Repo et al., 2001).

A number of ER-localized proteins are involved in the folding and quality control of GPCRs (see 2.2), but certain chaperones are also directly involved in the regulation of the export itself. For example, the cyclophilin homolog NinaA (Colley, Baker, Stamnes, & Zuker, 1991), the membrane-associated protein ODR-
4 (Dwyer, Troemel, Sengupta, & Bargmann, 1998) and the dynein light chain Tctex-1 (Tai, Chuang, Bode, Wolfrum, & Sung, 1999) have been found to regulate the export of their corresponding GPCR substrates. Likewise, the human cornichon homolog protein 4 (CNIH4) was recently demonstrated to regulate the export of β2AR in a manner that is very sensitive to changes in the amount of CNIH4 and its substrate (Sauvageau et al., 2014), and ribophorin I (RPNI), an ER resident chaperone, was shown to modulate the export of µOR and δOR (X. Ge, Loh, & Law, 2009). Receptor activity-modifying proteins (RAMPs) that modify the functional activity of certain GPCRs have also been suggested to participate in the cell surface delivery of GPCRs (Hay, Poyner, & Sexton, 2006). Still another important determinant of receptor export is the formation of oligomers between receptors. The oligomerization of GPCRs is required for certain GPCRs to exit the ER and may have a more general role in GPCR cell surface delivery (see 2.1.5).

The export of ER-synthesized proteins to the Golgi is mediated by the Coatomer protein II (COPII)-coated vesicle trafficking (Barlowe, 2003). GPCRs are packed into COPII-coated vesicles that are transported to the Golgi via ERGIC (Dong et al., 2007; Duvernay et al., 2005). Introduction of the cargo to the COPII machinery typically involves interactions with specific motifs in the protein structure (Aridor, Weissman, Bannykh, Nuoffer, & Balch, 1998; B. L. Tang, Wang, Ong, & Hong, 2005). Proteins involved in the export of GPCRs have been shown to recruit COPII proteins to facilitate their export (Sauvageau et al., 2014). For GPCRs, the membrane-proximal region is likely to be the most important molecular determinant that governs the ER export. Several motifs within the region have been identified in GPCRs. These typically include dileucine residues (LL) coupled with Phe (F) or Glu (E) residues. The export of vasopressin receptor 2 and 3, dopamine receptor D1, α2AR and angiotensin II receptor type 1 have been found to be impaired when their corresponding motifs have been mutated (Bermak, Li, Bullock, & Zhou, 2001; Duvernay, Zhou, & Wu, 2004; Robert, Clauser, Petit, & Ventura, 2005; Schülein et al., 1998). However, the proximity of the motifs to the H8 domain that is important for GPCR conformation, may explain the impaired export in some of these studies. Interestingly, a conserved motif governing the ER export of α2AR was located in the extracellular N-terminal tail (Angelotti et al., 2010), suggesting that the GPCR N-terminal tail is important for GPCR anterograde trafficking and that GPCR ER retention may be regulated also from the luminal side of the ER. The ER to Golgi anterograde trafficking after export from the ER is a step that is also regulated. Recently, a small GTPase, Rab43, was reported to interact and control the ER-Golgi trafficking of nascent GPCRs. The
interaction was shown to be Rab43 activation-dependent and involve a shift of Rab43 from the generic trafficking to GPCR-dependent trafficking (C. Li et al., 2017).

**Desensitization and endocytic trafficking**

Another fundamental cellular process that regulates GPCR availability and function is endocytic trafficking (Fig. 5). The process serves to allow cells to rapidly adapt to changes in extracellular signals and to control cell responsiveness by modulating receptor sensitivity to stimuli. This is achieved by receptor desensitization and internalization from the cell surface via endocytosis. The endocytosis is followed by critical sorting steps in endosomes that lead to either receptor resensitization by recycling back to the cell surface or downregulation by lysosomal degradation (Hanyaloglu & von Zastrow, 2008). The endocytosis has been demonstrated to occur after agonist-mediated receptor activation, but also constitutively in the absence of ligands (von Zastrow & Kobilka, 1992, 1994). There is a great diversity in the endocytic behavior and trafficking properties even between similar GPCR homologs (von Zastrow, Link, Daunt, Barsh, & Kobilka, 1993).

The desensitization of GPCR signaling involves phosphorylation of the intracellular serine and threonine residues by protein kinases and subsequent recruitment of GPCR regulating proteins (Fig. 5, see B). The phosphorylation promotes β-arrestin binding that attenuates G-protein coupling, thus desensitizing the receptor. The β-arrestin also mediates the interaction with the endocytic machinery, leading to receptor internalization (Hanyaloglu & von Zastrow, 2008). The desensitization can be homologous or heterologous depending on the nature of the process. Homologous desensitization requires receptor activation and phosphorylation by GRKs that recruit the β-arrestins (Gurevich & Benovic, 1997; Kovoor, Celver, Wu, & Chavkin, 1998). This is an adaptive measure to control the sensitivity of specific GPCRs to their cognate agonists. However, GRKs are also important initiators of G protein-independent signaling pathways mediated by arrestins (T. J. Cahill, 3rd et al., 2017; Lefkowitz & Shenoy, 2005; Premont & Gainetdinov, 2007; Webel, Menon, O'Tousa, & Colley, 2000). Heterologous desensitization is a more general effect governed by second messenger kinases that can phosphorylate multiple GPCRs even in the absence of their respective agonists (Kelly, Bailey, & Henderson, 2008).
Fig. 5. Endocytic trafficking of GPCRs. After receptor activation and initiation of downstream signaling (lightning icon) via effectors, such as ACs (A), GPCRs are typically subjected to desensitization and internalization. Receptor phosphorylation (red dots) by GRKs prevents further coupling of G proteins and recruits β-arrestin (B) that promotes receptor internalization via endocytosis but may also act as an initiator of downstream signaling. The GPCRs accumulate in clathrin-coated pits (C) that detach from the plasma membrane to form endosomes (D). GPCRs are sorted in endosomes (E) for different fates by either recycling them to the cell surface (F), effectively resensitizing the receptor, or by sorting them to late endosomes (G). Certain GPCRs, such as hδOR, end up in intraluminal vesicles when the late endosomes transform into multivesicular bodies that merge with lysosomes, leading to degradation and downregulation of the receptors (H). GRK, GPCR kinase. Modified from Sposini & Hanyaloglu, 2017.
Internalization of desensitized GPCRs occurs typically via clathrin-mediated endocytosis (Fig. 5, see C), but clathrin-independent pathways have also been described (Hanyaloglu & von Zastrow, 2008; McMahon & Boucrot, 2011). Cargo-specific adaptor proteins, such as the adaptor protein 2 (AP2) complex mediate cargo selection during the clathrin-mediated vesicle formation. The AP2 interacts with the cytoplasmic motifs of the cargo and recruits the coat-forming clathrin molecules to the site. The vesicle scission facilitated by dynamin completes the formation of endosomes carrying the receptor cargo (Fig. 5, see C-D) (B. T. Kelly et al., 2008; McMahon & Boucrot, 2011). The GPCR internalization is followed by endosomal sorting that dictates the fate of the receptor and its capacity for further signaling (Fig 5, see E). The receptor may be dephosphorylated and recycled back to the cell surface with various rates (Fig 5, see F) or downregulated altogether by degradation in the lysosomes (Fig5, see G-H) (Hanyaloglu & von Zastrow, 2008). The endocytic sorting is usually attributed to specific molecular determinants in the C-terminal tail of GPCRs that bind endocytic adaptor proteins. These proteins facilitate the distinct trafficking outcomes for individual GPCRs. Short sequence signal motifs, such as dileucine, tyrosine, and PDZ-binding motifs are recognized by adaptor proteins that mediate the endocytic sorting (Marchese, Paing, Temple, & Trejo, 2008).

The opioid receptors are subjected to clathrin-mediated internalization, but they are sorted to different fates afterwards. The δOR is targeted for lysosomal degradation for receptor downregulation, whereas μOR is recycled back to the cell surface (A. A. Pradhan et al., 2009; Tsao & von Zastrow, 2000; Whistler et al., 2002; Williams et al., 2013; von Zastrow, 2010). The δOR has also been found to be internalized constitutively, in the absence of a ligand (Trapaidze, Gomes, Bansinath, & Devi, 2000). It has been suggested that the post-endocytic trafficking of the δOR can vary depending on the engagement with different ligands and distinct sorting machineries. The δOR has also been reported to recycle back to the plasma membrane in a process that may be subjected to functional selectivity and ligand bias (Audet et al., 2012; Pineyro & Archer-Lahlou, 2007; Trapaidze et al., 2000). Furthermore, the δOR that is targeted for degradation was recently demonstrated to be subjected to retrograde transport to the trans-Golgi Network (TGN) from late endosomes and recycled back to the plasma membrane (Charfi, Abdallah, Gendron, & Pineyro, 2017). This implies additional regulatory steps in the late endosomes capable of retargeting the receptors from a degradation pathway to delivery to the cell surface.
The recycling of proteins may occur via a bulk-recycling pathway or via the sequence specific recycling pathway. Many GPCRs are recycled via specific sequences in their structure. The recycling of several GPCRs, such as β2AR and κOR, have been attributed to the PDZ-ligand motifs (Cao, Deacon, Reczek, Bretsch, & von Zastrow, 1999; J. G. Li, Chen, & Liu-Chen, 2002), and the motifs induce receptor recycling even when transplanted to non-recycling receptors, such as δOR (Gage, Matveeva, Whiteheart, & von Zastrow, 2005). Certain unique sequence motifs, such as the Leu-Glu-Asn-Leu-Glu-Ala-Glu (LENLEAE)-motif of μOR, have been identified to sort the receptor for recycling (Tanowitz & von Zastrow, 2003). The μOR C-terminal tail fails to target the receptor for recycling if the sorting sequence is mutated. Furthermore, the chimeric δOR with the μOR C-terminal tail is recycled back to the cell surface (Tanowitz & von Zastrow, 2003).

The lysosomal sorting requires segregation of receptors from the bulk recycling pathway by forming multivesicular bodies (MVBs) (Fig. 5, see G). The receptors are packed into intraluminal vesicles (ILVs) within the MVBs. This involves the involution of vesicles away from the cytoplasm inside the endosomes and is governed by the endosomal protein sorting complex (ESCRT) family members (Henne, Stenmark, & Emr, 2013). Ubiquitination has been shown to be the main sorting signal for the lysosomal pathway (Katzmann, Babst, & Emr, 2001), also for the GPCRs (Shenoy, McDonald, Kohout, & Lefkowitz, 2001), although certain GPCRs, such as δOR can also be targeted for lysosomal degradation in a ubiquitin-independent manner (Tanowitz & Von Zastrow, 2002). Ubiquitination is a versatile sorting determinant for GPCR regulation with different outcomes. The protease-activated receptor-1 undergoes constitutive ubiquitination and is deubiquitinated upon activation (Wolfe, Marchese, & Trejo, 2007). Ubiquitination also stimulates δOR assembly into ILVs during formation of MVBs (Henry, White, Marsh, von Zastrow, & Hislop, 2011). Nonactivated GPCRs may also be subjected to transubiquitination by other activated GPCRs, or co-ubiquitination and co-degradation by GPCRs in receptor oligomers (Dores & Trejo, 2012).

Downstream signaling steps have been also shown to control the endocytic sorting of GPCRs, implying that downstream events during signaling can guide the fate of the desensitized receptor. The PKA kinase that is activated downstream of β2AR signaling has been found to regulate β2AR recycling (Vistein & Puthenveedu, 2013). Similarly, μOR phosphorylation by downstream signaling kinases was revealed to regulate its recycling, as it was shown to decrease upon direct cAMP activation by forskolin (Roman-Vendrell, Yu, & Yudowski, 2012).
While the typical division between recycling and downregulation has been the prevalent cross road for regulation of GPCR signaling, it is becoming evident that in certain cases GPCRs display sustained signaling that continues after internalization (Sposini & Hanyaloglu, 2017). The β2AR was found to activate Ga in the early endosomes, leading to activation of distinct transcriptional profiles (Irannejad et al., 2013; Tsvetanova & von Zastrow, 2014). The thyroid-stimulating hormone receptor was demonstrated to activate Ga after internalization and trafficking to the TGN (Godbole, Lyga, Lohse, & Calebiro, 2017). Recently, the μOR and δOR were suggested to remain active after receptor internalization, and mediate opioid signaling from endosomes and from the Golgi (Stoeber et al. 2018). The activation of GPCRs from the intracellular compartments has also been reported to occur independently from internalization. In this manner, the β1AR was found to be activated by an intracellular pool of Ga-proteins in the Golgi (Irannejad et al., 2017).

### 2.1.5 Oligomerization

GPCR signaling is often depicted as a unit comprising a single receptor. In reality, evidence has accumulated suggesting that GPCRs can form functional receptor dimers and oligomers comprising more than a single receptor protomer. The oligomerization complicates all aspects of GPCR assembly and signaling via receptor cross-talk and allosteric interactions. The GPCR receptor-receptor interaction has been established using several methods, including co-immunoprecipitation, Förster (or fluorescence) resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) (Ferre et al., 2014; Gomes et al., 2016). The nomenclature for oligomerization makes a distinction based on its requirement for receptor function. The function of heteromeric receptors and homomeric receptors requires oligomerization of different or identical receptor monomeric subunits, respectively. In contrast, receptor heteromers and receptor homomers comprise different or identical functional receptor monomers, respectively, that have different biochemical properties compared to the formed receptor oligomer (Ferre et al., 2009). The GABA<sub>B</sub> receptor is a typical heteromeric receptor in that its signaling was found to require the interaction of two different monomers (Jones et al., 1998). A similar functional requirement has been observed for metabotrophic glutamate receptors (El Moustaine et al., 2012), and with other members of the Glutamate receptor family GPCRs (see Fig. 2) (Rondard, Goudet, Kniazeff, Pin, & Prezeau, 2011).
Many Rhodopsin/Class A GPCRs, including opioid receptors, have been reported to form receptor homo- and heteromers. Opioid receptor homomers and heteromers have been widely studied and demonstrated for all subtypes (Cvejic & Devi, 1997; George et al., 2000; Jordan & Devi, 1999; Massotte, 2015). Oligomers have been suggested to be dynamic in nature, transiently forming oligomers that can utilize multiple interfaces, in a receptor density-dependent manner (Dijkstra et al., 2018; Hern et al., 2010; Kasai, Ito, Awane, Fujiwara, & Kusumi, 2017). Rhodopsin/Class A GPCR homomers and heteromers have also been detected in living cells and native tissues. The β2AR was the first Rhodopsin family receptor to be demonstrated to form oligomers in living cells, using BRET (Angers et al., 2000). Using selective fluorescent ligands and FRET, oxytocin receptor oligomers were detected in mammary glands (Albizu et al., 2010), and µOR-δOR heteromers were found in the murine spinal cord, using a heteromer-selective opioid agonist (Waldhoer et al., 2005).

Despite the number of examples of Rhodopsin/Class A heteromers, the stoichiometry of the functional unit is unclear. Rhodopsin, β2AR and µOR have been shown to activate G proteins in their monomeric form (Bayburt, Leitz, Xie, Oprian, & Sligar, 2007; Kuszak et al., 2009; Whorton et al., 2007), but this may not reflect the preferred assembly under physiological conditions in native membranes. Rhodopsin was revealed to be expressed in dense rows of dimers in native disk membranes (Fotiadis et al., 2003). Similarly, β2ARs on the surface of cardiomyocytes appear to consist of clusters of signalosome complexes (Ianoul et al., 2005). Studies on rhodopsin behavior in photoreceptor membranes and mathematical simulations suggest that the G protein may bind to a complex with more than a single receptor (Dell'Orco & Schmidt, 2008; Govardovskii, Korenyak, Shukolyukov, & Zueva, 2009). The findings imply signaling from higher ordered oligomers and argue that this organization would provide a kinetic advantage in responding to a stimulus in a rapidly varying environment (Dell'Orco & Schmidt, 2008). Single-molecule imaging has also revealed functional aspects in the oligomerization of GPCRs. The dopamine D2 receptor was shown to form transient receptor dimers that were stabilized by agonists (Kasai et al., 2017). Using similar methods, the chemokine receptor CXCR4 decoupling from G proteins by pertussis toxin led to a decrease in the number of receptor dimers and higher oligomers (B. Ge et al., 2017). The oligomeric formation is also supported by crystallography, as the solved structures typically depict an assembly of receptor dimers, albeit sometimes in anti-parallel orientation. Crystals with receptors in parallel orientation, such as µOR and κOR display dimeric contact regions in TM1, TM2
and H8 (Manglik et al., 2012; H. Wu et al., 2012). Interestingly, some of these structures suggest multiple dimeric interphases (Manglik et al., 2012), tempting the speculation for higher ordered oligomers.

**Oligomerization and receptor functional cross-talk**

The oligomerization can lead to functional cross-talk between receptor protomers and to heteromer-specific functional output. The δOR-µOR heteromers have dissimilar pharmacological properties to those of the subtype protomers (George et al., 2000; Jordan & Devi, 1999), and it was shown that the activation of a single monomer in the δOR-µOR heteromer by a ligand was able to initiate G protein mediated signaling of its heteromeric partner (Snook, Milligan, Kieffer, & Massotte, 2006). The cross-talk between heteromerizing receptors can be mainly attributed to the allosteric effect by the receptor-receptor interaction. Like allosteric modulators, the receptor-receptor interaction can fine-tune active conformations, leading to distinct pharmacological properties. This often entails alterations in ligand binding, or even G protein activation and coupling (Rozenfeld & Devi, 2011). The heteromerization of δOR and µOR in live cells, demonstrated by BRET, altered µOR signaling and analgesic output, and the introduction of a δOR antagonist led to an enhancement of µOR signaling activity (Gomes et al., 2004). The δOR-µOR heteromerization has also been reported to cause a G protein-coupling switch from Gαi to Gαz (Hasbi et al., 2007). Heteromer interactions may also introduce conformations with an affinity for heteromer-selective ligands and induce heteromer-biased signaling pathways (Gomes et al., 2016; Rozenfeld & Devi, 2011). The stimulation of the δOR-µOR heteromer with µOR and δOR ligands that activate the typical G protein-coupled signaling has been found to activate heteromer-biased β-arrestin-dependent signaling (Rozenfeld & Devi, 2007). The δOR has also been shown to form heteromers with other Rhodopsin/Class A GPCRs. Heteromerization with cannabinoid receptors led to a change in δOR activity during modulation of neuropathic pain (Bushlin, Gupta, Stockton, Miller, & Devi, 2012). The co-expression of δOR and the chemokine receptor CXCR4 in immune cells caused a formation of heterodimers and a suppression in signaling for both receptors (Pello et al., 2008). Recently, a dynamic association and dissociation of Frizzled receptor dimers was suggested to modulate the constitutive signaling to ERK1/2 (extracellular signal-regulated kinase 1/2) pathway (Petersen et al., 2017). The receptor heteromerization may elicit functional cross-talk even beyond GPCR families. The serotonin receptor 5-HT2A of the
Rhodopsin/Class A family was reported to form functional oligomers with metabotropic glutamate receptors of the Glutamate/Class C receptor family in the brain cortex (Gonzalez-Maeso et al., 2008). Oligomerization may also present ligand-independent functions for orphan receptors that do not have identified ligands by altering the function of their heteromeric non-orphan receptor partners (Levoye, Dam, Ayoub, Guillaume, & Jockers, 2006).

The receptor cross-talk can also lead to co-trafficking of the receptor protomers after activation. For example, opioid receptor heteromerization has been reported to result in distinct trafficking pathways (van Rijn, Whistler, & Waldhoer, 2010). The co-expression of κOR with δOR inhibited its agonist-induced internalization (Chu, Murray, Lissin, & von Zastrow, 1997). In contrast, the heteromerization of δOR and β2AR in co-expressed cells led to co-internalization of receptors when either of the receptors was activated (Jordan, Trapaidze, Gomes, Nivarthi, & Devi, 2001).

**Oligomerization and receptor cell surface delivery**

GPCR oligomerization appears to occur already early in the biosynthetic pathway, and in a constitutive manner. The chemokine receptor CCR5 homomer interaction was demonstrated with BRET in intact cells and in the ER and plasma membrane subfractions (Issafras et al., 2002). Using co-immunoprecipitation and BRET, similar results were obtained for oxytocin and vasopressin V1a and V2 receptors (Terrillon et al., 2003). Opioid receptors appear to behave similarly, as the δOR-μOR heteromers form before reaching the cell surface (Hasbi et al., 2007). The GPCR oligomerization in the secretory pathway is also supported by the fact that LHR oligomers co-localize with the ER marker calnexin (CNX) (R. Guan et al., 2009). The localization in the ER suggests a possible role for oligomerization in GPCR synthesis and export. The β2AR that oligomerizes in the ER inhibited trafficking to the cell surface when its oligomerization was inhibited. Furthermore, β2AR with a mutated ER export motif forms oligomers with the co-expressed WT β2AR, leading to retention of the WT receptor in the ER (Salahpour et al., 2004). A similar dominant negative effect of mutant receptors in the cell surface delivery has been suggested for other GPCRs as well (Wilson, Wilkinson, & Milligan, 2005; Zhou, Filipeanu, Duvernay, & Wu, 2006).

Further evidence for the role of oligomerization in GPCR cell surface delivery has been obtained with cells expressing δOR and μOR. Chronic morphine (μOR agonist) treatment was reported to enhance δOR expression at the cell surface in
dorsal root ganglia, but not in cells lacking µOR, indicating a role for µOR-δOR heteromerization in the cell surface expression of δOR (C. M. Cahill et al., 2001; Morinville et al., 2003). Morphine treatment also resulted in increased abundance of δOR-µOR heteromers in the central nervous system (CNS), visualized by heteromer-selective antibodies (Gupta et al., 2010). Morphine is able to rescue the cell surface delivery of an ER-retained mutant µOR (Chaipatikul, Erickson-Herbrandson, Loh, & Law, 2003), likely by acting as a pharmacological chaperone (see 2.2.3). This offers a likely mechanism for how morphine targets δOR-µOR heteromers in the ER and promotes their export. Interestingly, another study reported that increased δOR expression can lead to attenuation of µOR maturation, in co-expressed cells, and this effect was alleviated by a Golgi-localized chaperone, receptor transport protein 4 (RTP4) (Decaillot, Rozenfeld, Gupta, & Devi, 2008). This indicates that not only does heteromerization participate in GPCR cell surface delivery, but it is also regulated in the secretory pathway.

### 2.2 Biosynthesis of GPCRs

The biosynthesis of secreted and membrane-bound proteins takes place along the secretory pathway, starting in the ER (Fig. 6). These proteins are directed to the ER by signal segments in the primary structure (Ellgaard, McCaul, Chatsisvili, & Braakman, 2016). The folding of multi-spanning polytopic membrane proteins, such as GPCRs, occurs through co-translational insertion and assembly of the TM domains into the ER membrane that follows the basic principles of protein folding and the Gibbs free energy (Anfinsen, 1973; Ellgaard et al., 2016). Co-translational modifications of GPCRs, such as N-glycosylation and disulfide bonds formation aid to stabilize the correct fold and add elements that modulate the interaction with molecular chaperones that optimize the folding and monitor the quality of the protein (Hartl, Bracher, & Hayer-Hartl, 2011; Lamriben, Graham, Adams, & Hebert, 2016). Poorly folded receptors are targeted for degradation, while receptors that attain the native structure are exported from the ER (Fig. 6) (Christianson & Ye, 2014; Kroeger, Chiang, & Lin, 2012). Receptors mature further in the Golgi before reaching the plasma membrane as functional proteins (Fig. 6) (Dong et al., 2007).
Fig. 6. GPCR folding, maturation and cell surface delivery. Internal or cleavable signal peptides target GPCRs to the ER membrane where they are synthesized. GPCRs begin to fold co-translationally and co-translocationally after being inserted into the membrane (A). They continue to fold with the help of folding chaperones and attempt to attain their native conformation while scrutinized by the ERQC machinery (B). The ERQC targets the non-native receptors for ERAD via the ubiquitin-proteasome pathway that involves retrotranslocation to the cytosol (C). The receptors with a native conformation are targeted for ER export (D). During ER processing, receptors are modified by N-glycans that modulate the interactions with ER-resident proteins. The receptors exported from the ER traverse to the Golgi where they are further modified by O-glycans (E), before delivery to the cell surface (mature N- and O-glycans are depicted in the inset, see more details in Fig. 7). Modified from Petäjä-Repo & Lackman, 2014.
2.2.1 Membrane insertion and ER folding machinery

The translation of GPCRs is directed to the ER membrane via two distinct mechanisms, by a signal sequence located at the N-terminus as a cleavable signal peptide, or by a segment of the protein, usually the first TM domain. Most GPCRs lack the cleavable N-terminal peptide and anchor to the ER membrane via the TM segment instead (Schülein, Westendorf, Krause, & Rosenthal, 2012; Wallin & von Heijne, 1995). Many members of the Secretin and Glutamate receptor families, and other GPCRs with large N-termini have N-terminal signal peptides. This ensures that the N-terminus attains a stable fold that is typically necessary for these receptors (Kochl et al., 2002; Schülein et al., 2012). The cleavable signal peptides interact with the signal recognition particle (Janda et al., 2010), and the ER translocon facilitates the insertion into the membrane (Cymer, von Heijne, & White, 2015). The insertion of GPCRs without a signal peptide may occur via a process in which distinct TMs act as signal-anchors or stop-transfer regions, to facilitate the lateral insertion of the TM domains into the ER membrane. For example, the timing of opsin translocation was shown to be determined by the properties of the TM segment (Ismail, Crawshaw, Cross, Haagsma, & High, 2008).

The insertion of multi-spanning polytopic proteins into the ER membrane is a complex and not fully characterized process that likely requires the coordination of chaperones in all three topological domains. The intracellular parts remain on the cytosolic side, extracellular loops must enter the ER lumen and the TM segments integrate laterally into the ER membrane from the translocon (Cymer et al., 2015; Ellgaard et al., 2016). The TM segments of multi-spanning polytopic proteins have been suggested to be inserted into the ER membrane in a loop by loop manner, and the loop exposure to the cytosol or ER lumen is controlled by structural rearrangements in the ribosome, translocon and associated proteins (P. J. Lin, Jongsma, Liao, & Johnson, 2011). The folding of polytopic proteins follows a pattern in which the α-helical segments are first formed followed by attempts to reach a stable native conformation as they interact with the lipid bilayer and with the other helices (Popot & Engelman, 2000).

At the ER membrane, the receptors begin to fold already during translocation and membrane insertion. The nascent receptor polypeptide segments entering the ER lumen are met with the ER folding machinery consisting of numerous molecular chaperones that interact with the receptor. They assist the receptor to reach optimal free-energy states and block unwanted interactions that would lead to protein aggregation. The primary chaperones in the ER include BiP (Binding...
immunoglobulin protein) of the 70-kDa heat shock protein family (Hsp70), Grp94 of the Hsp90 family and the lectin chaperones CNX and calreticulin (CRT) (Christianson & Ye, 2014; Hartl et al., 2011). BiP assists the folding of proteins and participates in the retention of non-native proteins in the ER (Hammond & Helenius, 1994; Simons, Ferro-Novick, Rose, & Helenius, 1995). BiP has been found to interact with GPCRs, such as rhodopsin, angiotensin II receptor type 1 and LHR (Anukanth & Khorana, 1994; Lanctot, Leclerc, Escher, Leduc, & Guillemette, 1999; Mizrachi & Segaloff, 2004). Gpr94 that typically has a more selective range of substrates, has also been reported to interact with the ER-localized rhodopsin and LHR (Anukanth & Khorana, 1994; Mizrachi & Segaloff, 2004). The lectin chaperones CNX and CRT are protein homologs primarily assisting the folding of membrane-bound and soluble substrates, respectively. Their interaction with newly-synthesized substrates is mediated by N-glycans that are added co-translationally to most proteins in the ER (see 2.2.2 and 2.2.4). Many GPCRs have been shown to interact with CNX, including rhodopsin (E. E. Rosenbaum, Hardie, & Colley, 2006), δOR and µOR (X. Ge et al., 2009; Markkanen & Petäjä-Repo, 2008), V2 and V3 vasopressin receptors (Morello et al., 2001; Robert, Auzan, Ventura, & Clauser, 2005) and dopamine D1 and D2 receptors (Free et al., 2007). GPCRs, such as δOR and LHR have also been demonstrated to interact with the sarcoendoplasmic reticulum Ca\textsuperscript{2+} ATPase 2b, an ER protein involved in calcium homeostasis and protein synthesis (Brostrom & Brostrom, 2003). The complex with hδOR also involved CNX and promoted hδOR expression at the cell surface (Tuusa, Leskelä, & Petäjä-Repo, 2010; Tuusa, Markkanen, Apaja, Hakalahti, & Petäjä-Repo, 2007).

The ER folding machinery also contains a number of folding enzymes that co-translationally modify the nascent proteins entering the ER. The modifications participate in the construction of the stable receptor conformation and provide molecular determinants for further processing and maturation in the ER (Ellgaard et al., 2016). The formation of disulfide bonds is a critical rate-limiting step in the maturation of most ER-synthesized proteins due to the stabilizing nature of the covalent bond formed. Protein disulfide isomerases (PDIs), which belong to a protein family of more than 20 members, interact with nascent membrane proteins and control the formation of disulfide bonds by breaking non-native bonds and rearranging cysteine linkages to match the native conformation (Ellgaard & Ruddock, 2005; Galligan & Petersen, 2012). A conserved disulfide bond between TM3 and ECL2 is critical to the stability of the overall GPCR structure, and is present in most GPCRs (Venkatakrishnan et al., 2013). Another group of important
folding enzymes are peptidyl-prolyl isomerases (PPI) that catalyze the \textit{cis-trans} isomerization of peptidyl-prolyl bonds, which is also a rate-limiting step during protein folding (Schmid & Baldwin, 1978; Schmidpeter & Schmid, 2015). Importantly, the lectin chaperone CNX is known to recruit members of the folding machinery to the translocon. It also interacts with ER-resident protein 57 (Erp57) of the PDI family, and with cyclophilin B of the PPI family (Frickel et al., 2002; Jessop, Tavender, Watkins, Chambers, & Bulleid, 2009; Kozlov et al., 2010).

\subsection{2.2.2 ER quality control}

Protein folding is a complex and time-consuming process that is prone to errors and aggregation. The protein may attain several intermediate folding stages before obtaining the native fold, or even fail to do so, assuming a non-native state incapable of functioning properly. Cells have elaborated ways to monitor the quality of the product during protein folding, and segregate non-native products in order to preserve proteostasis and cellular health. The ERQC prohibits premature export of folding intermediates and targets proteins failing to attain the native conformation for degradation (Christianson & Ye, 2014). The mechanism is imperative for ensuring that only functional GPCRs are delivered to the cell surface (Petäjä-Repo & Bouvier, 2005).

The ERQC consists of molecular chaperones, specialized folding factors, folding sensors, retention factors and escort proteins that work to ensure the functional expression of the ER-synthesized proteins (Hartl et al., 2011). It is unclear how the ERQC sorts native and non-native proteins from one another. It is likely that proteins have molecular determinants in their structure that act as signals that promote the retention or export. Nonnative conformations may expose motifs that are targeted by the passive ER retention machinery, whereas in native conformations these motifs are buried, leading to protein export from the ER. Similarly, ER export signals, such as in many GPCRs, may be accessible and functional only in native conformations (Kincaid & Cooper, 2007; G. Wu, 2013). The exposure of hydrophobic regions typically indicates a non-native fold and lead to interaction with folding factors and proteins that promote retention (Hartl et al., 2011). The sorting of proteins from the ER likely results from multiple competing interactions involved in ER export and ER retention (Gomez-Navarro & Miller, 2016).

The stability of a protein is a function of free energy present in the folded structure, and for GPCRs this is achieved by conserved inter-TM interactions that
guide the overall receptor fold (see Fig. 1). An intricate balance of stability and flexibility is required of the native structure of GPCRs, as receptor conformation changes upon activation and interaction with ligands, G proteins and effectors (Katritch et al., 2012). GPCRs are able to exhibit a number of native conformations depending on the state of the receptor activation and interaction with proteins and modulators (D. M. Rosenbaum et al., 2009). The flexibility makes them only marginally thermodynamically stable in their physical environment. This fact, and the polytopic membrane protein nature of GPCRs, which involves folding in distinct cellular environments (ER lumen, cytosol and membrane), makes the folding inefficient and prone to errors (Guerrier et al., 2017; Hartl et al., 2011).

Many diseases have been attributed to the ER retention of misfolded GPCRs. Misfolding and subsequent retention of rhodopsin has been implicated in some forms of retinitis pigmentosa (Noorwez et al., 2004), whereas the retention of the vasopressin V2 receptor leads to nephrogenic diabetes insipidus (Bichet, 2006; Birnbaumer, Gilbert, & Rosenthal, 1994). Similarly, retention causing mutations in the endothelin B receptor and melanocortin-4 receptor have been revealed to play a role in Hirschsprung’s disease and morbid obesity, respectively (Fuchs et al., 2001; Rene et al., 2010). The α-helical TM domains are naturally the most important for the integrity of the GPCR structure, as is evidenced by many mutations in this domain that lead to non-functional receptors (Stoy & Gurevich, 2015). For most GPCRs, especially in the Rhodopsin/Class A family, the N- and C-terminal tails are unstructured and thus less likely to be important for the stability of the structure (Jaakola et al., 2005; Katritch et al., 2012).

The most extensively-characterized ERQC machinery is the N-glycan dependent ERQC (Fig. 7). It utilizes the co-translational N-glycosylation of proteins and several lectin chaperones that interact with proteins via different N-glycoforms (Hammond, Braakman, & Helenius, 1994; Tannous et al., 2015; Yamamoto, 2014). Newly-synthesized membrane-bound and secreted proteins are typically modified with high-mannose N-glycans (glucose₃-mannose₉-N-acetylglucosamine₂) at Asn residues in glycosylation sequons (Asn-X-Ser/Thr). Oligosaccharyltransferase (OST) catalyzes the transfer as the polypeptide emerges from the translocon, initiating chaperone interactions already during protein folding (Mohorko, Glockshuber, & Aebl, 2011). The N-glycan is trimmed during ER processing to create transient structures that have affinity for chaperones in different steps of protein folding, quality control and trafficking (Ellgaard et al., 2016; Tannous et al., 2015). The initial high-mannose N-glycan precursor added is trimmed by glucosidases I and II that remove the terminal glucose from the
glucose–mannose–N-acetylglucosamine. The mono-glucosylated form interacts with CNX/CRT chaperones, initiating the quality control in a cyclic process known as the CNX/CRT cycle. Folding trials are followed by the removal of the final glucose from the N-glycan precursor by glucosidase II, disrupting the CNX/CRT interaction and subsequently promoting interaction with the folding sensor UDP-glucose:glycoprotein glucosyltransferase (UGGT). If the native conformation is not yet attained, glucose is added by UGGT, and the cycle continues as the CNX/CRT interaction is re-established. If the native conformation is reached, further processing of the glycan by mannosidases leads to glycoprotein export from the ER. In a process that works presumably in a time-sensitive fashion, several attempts are given to the glycoprotein to reach the native fold, before re-glycosylation is prevented by demannosylation, effectively resulting in failure to pass the quality control (Ellgaard et al., 2016; Lamriben et al., 2016). Failing to reach the native fold may be due to mutations or due to inherent inefficiency in reaching the native structure, as discussed above. A few native GPCRs, such as the δOR and LHR have been shown to fold inefficiently, leading to a substantial number of ER retained receptors (Leskela et al., 2009; Petajä-Repo et al., 2001; Petajä-Repo et al., 2000; Pietilä et al., 2005).

2.2.3 ER-associated degradation

Native proteins that have passed the ERQC are exported from the ER and continue maturation along the secretory pathway. In contrast, proteins failing to reach the native state in time must be eliminated in order to avoid aggregation and cellular stress (Christianson & Ye, 2014). This is achieved by the ERAD machinery that links the ERQC to the cytosolic degradation. Non-native proteins failing the quality control are recognized, transported to the cytosol and degraded by the cytosolic proteasome (Fig. 6 and 7). The transport of ERAD substrates to the cytosol involves ubiquitin conjugation and a process called retrotranslocation that utilizes a translocon to deliver the proteins from the ER lumen to the cytosolic side. In the case of TM proteins this also involves dislocation of the TM segments from the membrane (Christianson & Ye, 2014).
Fig. 7. N-glycan processing in the ER and Golgi. Glycoprotein processing in the ER is largely governed by N-glycan-mediated interactions. Processing of the N-glycans leads to the recruitment of different ER-lectins during ERQC, ERAD and ER export, effectively regulating the maturation and fate of the glycoproteins during early biosynthesis. A variety of glycoforms emerge due to the activity of glucosidases and mannosidases that trim the N-glycans of the glycoproteins. The most important aspect during glycoprotein ERQC is the CNX/CRT cycle that adds and removes glucoses from the N-glycan, in a process that monitors the quality of the fold before targeting the protein either for ER export (green arrow), or degradation (red arrow). In the Golgi, the N-glycans are further processed by mannosidases and transferases to generate mature N-glycans that decorate the proteins at the cell surface (typical structure of complex N-glycan is shown). Monosaccharide residues in the glycans are explained in the inset. Cnx, Calnexin; Gls, Glucosidase; ERm, ER mannosidase; EDEM, ER mannosidase-like; Golm, Golgi mannosidase; GnT, GlcNAc transferase; GT, Galactosyl transferase; Mal, Malectin; ST, Sialyl transferase; UGGT, UDP-glucose:glycoprotein glucosyltransferase. Modified from Yamamoto, 2014.
The specific steps leading to degradation, especially for polytopic membrane proteins like GPCRs, are not fully understood. The degradation of the cystic fibrosis transmembrane conductance regulator (CFTR), a large polytopic membrane protein, was the first multi-spanning membrane protein reported to be degraded by the ubiquitin-proteasome system (Jensen et al., 1995; Ward, Omura, & Kopito, 1995). Since then, several luminal ERAD substrates, such as the mutant yeast vacuolar carboxypeptidase, CPY* have been studied as models to understand the ERAD machinery (Hiller, Finger, Schweiger, & Wolf, 1996). The pathways for luminal (ERAD-L), membrane-associated (ERAD-M) and cytosolic (ERAD-C) substrates are somewhat different and include different components (Christianson & Ye, 2014).

The first step of ERAD involves the recognition of proteins that fail to attain the native conformation. These proteins are directed away from the folding machinery to proteins facilitating their delivery to the retrotranslocation complex (Christianson & Ye, 2014). Details of the recognition mechanisms and timing are substrate dependent. Some proteins are selected for degradation soon after synthesis while others remain in intermediate folding states for long periods of time (Pisoni & Molinari, 2016). Folding chaperones, such as BiP, have been suggested to participate in the recognition of ERAD substrates as it recognizes hydrophobic interfaces of folding intermediates and misfolded proteins (Hendershot et al., 1995; Plemper, Bohmler, Bordallo, Sommer, & Wolf, 1997). The most extensively characterized mechanism selecting substrates for ERAD involves N-glycan trimming and is deeply integrated with the N-glycan mediated quality control (Fig. 7). It is believed that N-glycan trimming acts as a timer for glycoprotein folding attempts (Pisoni & Molinari, 2016). The removal of mannose residues from the core high-mannose N-glycans exposes glycan structures for ERAD lectins that introduce the non-native glycoproteins for the ERAD pathway (Fig. 7). Concomitantly, the trimming of terminal mannose residue from the left branch of the high-mannose N-glycan precursor prevents reglucosylation by UGGT and thus re-entry to the CNX/CRT cycle and further folding attempts (Lamriben et al., 2016). Demannosylations by the ER-α1,2 mannosidase I (ERManI) and ER degradation-enhancing α-mannosidase-like proteins (EDEMs) 1-3 are responsible for creating trimmed mannose5,7 N-glycans that are recognized by ERAD lectins (Molinari, 2007; Ninagawa et al., 2014). EDEM1 has been found to interact with mutant rhodopsin and promote its degradation (Kosmaoglou, Kanuga, Aguila, Garriga, & Cheetham, 2009). In yeast, Yos9 (human orthologues are OS9 and XTP-3B) is one of the lectins believed to link the quality control and degradation
machineries in the ER. It recognizes trimmed mannoses and interacts with the ERAD machinery to direct the substrate for degradation (Christianson, Shaler, Tyler, & Kopito, 2008; Clerc et al., 2009; Gauss, Jarosch, Sommer, & Hirsch, 2006; Hosokawa, Kamiya, Kamiya, Kato, & Nagata, 2009; Hosokawa et al., 2008).

It is unclear how non-glycosylated proteins are targeted to ERAD. For certain substrates, like the non-glycosylated sonic hedgehog, the lectin chaperones were found to be involved, as OS9 and EDEM2 were required for its degradation (H. Y. Tang, Huang, Zhuang, Christianson, & Chen, 2014). Likewise, the degradation of two non-glycosylated substrates of BiP were demonstrated to be dependent on the interaction with OS9, XTP3-B and EDEM1 (Shenkman et al., 2013). The N-glycan independent ERAD pathway has also been found to operate as a backup system under ER stress (Ushioda, Hoseki, & Nagata, 2013).

The next step of ERAD after recognition introduces the substrates to polyubiquitination and subsequent retrotranslocation to the cytosol. This key ERAD complex contains the E3 ubiquitin ligase Hrd1 and the membrane adaptor protein Sel1L (Christianson et al., 2008; Christianson & Ye, 2014; Hosokawa et al., 2008; Sun et al., 2014). The retrotranslocated ERAD substrates are polyubiquitinated by the ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin-ligase E3 that operate in conjunction to modify the cytosolic lysine residues with ubiquitin molecules (Christianson & Ye, 2014; Pickart, 2001). Another complex containing the membrane protein Derlin-1 was shown to facilitate retrotranslocation of membrane proteins with misfolded luminal domains (Lilley & Ploegh, 2004; Ye, Shibata, Yun, Ron, & Rapoport, 2004). The retrotranslocation itself is powered by a conserved ATPase P97/VCP. It provides the energy for the retrotranslocation/dislocation of the substrate across the translocon to the cytosolic side (Jarosch et al., 2002; Ye, Meyer, & Rapoport, 2001).

The retrotranslocation/dislocation of polytopic substrates, such as GPCRs is poorly understood and may involve several processes. It has been suggested that the TM substrate may be retrotranslocated in a similar fashion as luminal substrates after being dislocated to the luminal side in a process that may involve intramembrane cleavages by proteases. The process may also involve translocation of luminal domains while the TM domains move laterally to the translocon before dislocation to the cytosol. The hydrophobicity of the TM segment is an energetic barrier and the biggest obstacle during translocation of integral membrane substrates (Avci & Lemberg, 2015; Guerriero et al., 2017). The ERAD of hδOR has been shown to involve polyubiquitination and the subsequent retrotranslocation...
of the non-native receptor species to the cytosol via the Sec61 translocon (Petäjä-Repo et al., 2001).

In the final step of ERAD, the retrotranslocated protein is delivered to the 26S proteasome for degradation. The 26S proteasome is a large cytosolic protease complex that is responsible for degradation of cytosolic polyubiquitinated proteins (Finley, 2009). A number of proteins are involved in the recognition and delivery of substrates to the proteasome (Finley, 2009; Misaghi, Pacold, Blom, Ploegh, & Korbel, 2004). Before degradation, the δOR was shown to be deglycosylated before delivery to the 26S proteasome (Petäjä-Repo et al., 2001). The inhibition of the proteasome by lactacystin resulted in an accumulation δOR in the cytosol, confirming the fate of the retrotranslocated receptor (Petäjä-Repo et al., 2001). Interestingly, the proteasomal inhibition led to an accumulation of LHRs only in the ER membrane, but not in the cytosol (Pietilä et al., 2005), implying that at least for some GPCRs, the retrotranslocation is more tightly coupled to proteasomal degradation.

Pharmacological chaperones

The export of GPCRs from the ER is promoted by molecular chaperones (Ellgaard et al., 2016; Ulloa-Aguirre, Janovick, Miranda, & Conn, 2006) but it can also be promoted by exogenous small membrane-permeable molecules with pharmacological affinity for the receptor (Morello, Petäjä-Repo, Bichet, & Bouvier, 2000). The pharmacological chaperone can be a substrate, ligand or allosteric modulator that binds to the target protein at a physiologically relevant site during biosynthesis (Lester, Miwa, & Srinivasan, 2012). Typically, membrane-permeable receptor specific ligands have been used to rescue the cell surface expression of misfolded, potentially disease causing GPCRs that are retained in the ER. This strategy has been employed to rescue mutant V2 vasopressin receptors that cause nephrogenic diabetes insipidus (Morello, Salahpour, et al., 2000). Similarly, the cell surface delivery of mutants of gonadotropin-releasing hormone receptor causing hypogonadism and mutants of LHR causing Leydig cell hypoplasia were both rescued by pharmacological chaperones (Janovick et al., 2009; Newton et al., 2011). This strategy was also used to improve the folding efficiency and ER export of mutant forms of rhodopsin that cause retinitis pigmentosa (Noorwez et al., 2003; Noorwez et al., 2004) and rescue melanocortin-4 receptors mutants implicated in obesity (Z. C. Fan & Tao, 2009). Pharmacological chaperones have also been successfully used in rescuing receptor oligomerization.
Namely, ER-retained mutants of β1AR and the α1bAR that are incapable of forming receptor homo-oligomers were rescued by pharmacological chaperones (Canals, Lopez-Gimenez, & Milligan, 2009; Kobayashi, Ogawa, Yao, Lichtarge, & Bouvier, 2009). Pharmacological chaperones have also been used to enhance the folding and maturation of WT GPCRs and natural variants of GPCRs that are inefficient in passing the ERQC and display relatively low levels of expression at the cell surface. Several opioid ligands have been successfully used as pharmacological chaperones (Petäjä-Repo & Lackman, 2014), and they have been found to enhance the cell surface expression of δOR, κOR and μOR (Fortin et al., 2010; Leskelä, Markkanen, Pietilä, Tuusa, & Petäjä-Repo, 2007; Petäjä-Repo et al., 2002).

The details of the enhancing effect of pharmacological chaperones on receptor ER exit are unclear. Like ligands promoting active conformations during receptor activation (Katritch et al., 2013), membrane-permeable ligands entering the ER may bind to and promote native conformations of folding intermediates, assisting them to pass the quality control. Upon binding to nascent receptors, they may also disrupt and inhibit interactions with folding sensors and the retrotranslocation machinery that might promote retention of the protein (Leidenheimer & Ryder, 2014; Tao & Conn, 2014). The number of hδOR precursors in the ER were found to increase when the cells were treated with opioid ligands, and when the anterograde trafficking was blocked with brefeldin A. The removal of the inhibition led to an increase in the number of receptors at the cell surface, indicating an enhanced export from the ER. Treatment with opioid ligands led to stabilization of receptor precursors in a heat-inactivation assay, and enhanced dissociation from CNX (Leskelä et al., 2007), implying an improved passing of the ERQC.

2.2.4 N-linked glycosylation

Most newly-synthesized membrane-bound and secreted proteins are cotranslationally modified with N-linked glycans. The OST at the translocon catalyzes the addition of a preassembled N-glycan from a dolichol pyrophosphate donor to Asn residues of certain glycosylation sequons (Asn-X-Ser/Thr) of substrate proteins. N-glycans have an important role during the folding and assembly of membrane proteins. They enhance intrinsically glycoprotein solubility, stability and the energetics of folding by glycan-protein interactions. The favorable folding is partially promoted by the restriction of conformational entropy by the glycan, but the effect is not elicited in a context-independent manner. Rather, the important glycan-protein interactions have emerged through evolution and are
generally site-specific (Hanson et al., 2009; Hebert, Lamriben, Powers, & Kelly, 2014; Price et al., 2010). The N-glycans also introduce molecular determinants that upon processing operate as signals for various steps in protein folding and quality control by the ER-resident lectin chaperones (see 2.2.2) (Hammond et al., 1994; Lamriben et al., 2016).

According to the GPCR database, a majority of GPCRs contain the Asn-X-Ser/Thr-sequon(s) in the N-terminal tail or in the ECLs (Pandy-Szekeres et al., 2018). The N-glycans are typically absent in the crystallized structures of GPCRs due to the removal of the N-terminal tail and/or enzymatic deglycosylation of the protein to improve crystallization (D. M. Rosenbaum et al., 2009). However, the crystal structure of rhodopsin includes N-glycans in the extracellular tail of the receptor (Palczewski et al., 2000). Most of the studied and biochemically characterized GPCRs, including rhodopsin, μOR, α₁AR, β₁AR, angiotensin II type 1 receptor, LHR and FSHR, are N-glycosylated in the extracellular domains (Davis, Liu, & Segaloff, 1995; He, Xu, Castleberry, Lau, & Hall, 2002; P. Huang, Chen, Mague, Blendy, & Liu-Chen, 2012; Kaushal, Ridge, & Khorana, 1994; Keinänen, 1988; Lanctot et al., 1999; Sawutz, Lanier, Warren, & Graham, 1987; Webel et al., 2000). Typically, the studies have employed site-directed mutagenesis of the N-glycosylation sequons, enzymatic deglycosylation or the use of an inhibitor of N-glycosylation, tunicamycin. The state of protein N-glycosylation is also often used to identify immature receptor precursors and mature receptor forms on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Receptor precursors residing in the ER carry high-mannose-type N-glycans and are sensitive to Endo-β-N-acetylglucosaminidase H (Endo H) digestion, whereas N-glycan processing in the Golgi results in more heterogeneous receptor forms that are Endo H insensitive (Petäjä-Repo et al., 2001; Petäjä-Repo et al., 2000).

During processes in the ER, the high-mannose N-glycans are demannosylated before the glycoproteins are exported to the Golgi or targeted for degradation (Aebi, Bernasconi, Clerc, & Molinari, 2010; Lamriben et al., 2016). In the Golgi, the processing of N-glycans continues by trimming and elongation of the structure to hybrid and complex mature N-glycans by Golgi glycosidases and glycosyltransferases (Fig. 7) (Stanley, Taniguchi, & Aebi, 2015). The processing of N-glycans occurs sequentially and relies on subcompartmentalization of the Golgi (Dunphy, Brands, & Rothman, 1985; Dunphy, Fries, Urbani, & Rothman, 1981; Farquhar & Palade, 1981; Roth & Berger, 1982). The elongation of the glycan structure is dependent on nucleotide-sugar transporters that bring the appropriate uridine diphosphate (UDP) sugars from the cytosol for the reaction. The high-
mannose N-glycans are demannosylated to the Man$_3$-GlcNAc$_2$ structure by Golgi α-mannosidases 1A and 2A in the cis-Golgi. In the medial-Golgi, the structure is elongated to GlcNAc-Man$_5$-GlcNAc$_2$ by N-acetylglucosaminyl-transferase 1, before further demannosylation by α-mannosidase 2A and 2B into GlcNac-Man$_3$-GlcNAc$_2$. Another elongation by N-acetylglucosaminyltransferase 2 creates the GlcNac$_2$-Man$_3$-GlcNAc$_2$ structure that is the precursor of all biantennary complex N-glycans. Multiple branches, or antennae, may be glycosylated by additional modifications by GlcNAcs. The GlcNac$_2$-Man$_3$-GlcNAc$_2$ structure is also often modified in vertebrates by a fucose residue in the Asn-linked GlcNAc. Hybrid N-glycans result from an incomplete demannosylation or the absence of the second GlcNAc elongation (Stanley et al., 2015). The maturation of N-glycans occurs in the trans-Golgi and the trans-Golgi network (TGN) where the N-glycan branches are modified by galactose and typically terminated by sialic acids (Stanley et al., 2015).

The mature N-glycans can have a number of different structures at each glycosylation site, leading to great heterogeneity in the mature GPCR structure. Any given glycosylation site may be occupied by differently processed and elongated mature N-glycans or may lack the N-glycans altogether (Stanley et al., 2015). The hδOR heterologously expressed in cells is typically expressed in two heterogeneous species distinguished by the number of N-glycosylation sites occupied. The heterogeneous bands on western blot analysis imply a variety of different N-glycoforms in the mature form, while the additional small molecular mass species indicate a population of mature receptors with only a single N-glycosylation site occupied (Markkanen & Petäjä-Repo, 2008; Petäjä-Repo et al., 2001; Petäjä-Repo et al., 2000).

N-glycosylation has a critical role in the synthesis of secreted and membrane-bound proteins, but this is poorly studied with GPCRs. The research is often limited to the characterization of steady state receptor expression levels at the surface, or to receptor subcellular localization. The lack of N-glycosylation often results in decreased cell surface expression. For example, the prostaglandin E$_2$ receptor (Boer, Neuschafer-Rube, Moller, & Puschel, 2000), dopamine D5 receptor (Karpa, Lidow, Pickering, Levenson, & Bergson, 1999) and gastrin-releasing peptide receptor (Benya et al., 2000) were shown to accumulate intracellularly and had decreased expression at the cell surface. The N-glycosylation was revealed to promote the cell surface expression of glucagon-like peptide-1 and glucose-dependent insulinoitropic polypeptide receptors via enhanced export from the ER, and decreased degradation in the ER. The lack of N-glycans had a more drastic
effect on the glucose-dependent insulinotropic polypeptide receptor, suggesting that the role of N-glycans are largely receptor-specific (Whitaker, Lynn, McIntosh, & Accili, 2012). Similarly, the mutation of N-glycosylation sites of the angiotensin II type 1 receptor and V₁a vasopressin receptor resulted in decreased cell surface expression that was attributed to the intracellular retention of the non-N-glycosylated receptor (Hawtin, Davies, Matthews, & Wheatley, 2001; Lanctot et al., 1999). The glycan processing in the ER was shown to be important for the cell surface delivery, even though the mutant receptor interacted with CNX, in the absence of N-glycans (Lanctot, Leclerc, Escher, Guillemette, & Leduc, 2006). Furthermore, the position of N-glycans appeared vital for receptor function, as changing the position of the N-glycan was found to lead to misfolding and inefficient quality control of the receptor (Lanctot et al., 2005). N-glycans have also been confirmed in the N-terminal tail of δOR, as well as the N-termini of other opioid receptors (P. Huang et al., 2012; J. G. Li, Chen, & Liu-Chen, 2007; Markkanen & Petäjä-Repo, 2008; Petäjä-Repo et al., 2000).

2.2.5 O-GalNAc glycosylation

In the Golgi, many proteins traversing through the compartment are modified by GalNAc (N-acetylgalactosamine) -type O-glycans, or O-GalNAc glycans. The O-glycosylation is a common term for glycosylation of the hydroxyl group of amino acid chains in substrate proteins. Several O-glycans, such as O-mannose, O-fucose, O-glucose, O-galactose, O-xylose, O-GlcNAc (N-acetylglucosamine) and O-GalNAc have been characterized and named after the initial monosaccharide added to the substrate (Bennett et al., 2012). The most abundant types of O-glycan modifications include the O-GlcNAc glycans found mainly in many cytosolic and nuclear proteins and the O-GalNAc glycans found in various secreted and membrane proteins. The O-GalNAc glycosylation is also known as mucin-type O-glycosylation due to the abundance of these glycans in mucins and mucin-like domains (Bennett et al., 2012; Tian & Ten Hagen, 2009). However, O-GalNAc glycosylation has also been reported in many other substrates, such as GPCRs.

The O-GalNAc glycosylation is catalyzed by members of the peptidyl-N-acetylgalactosaminyltransferase (GalNAc-T) family that add a GalNAc monosaccharide to a specific serine, threonine, and in some cases, tyrosine residues. Unlike N-glycosylation, O-GalNAc glycosylation lacks an identifiable consensus glycosylation sequence. Instead, members of the GalNAc-T family have different preferences for amino acids in the proximity of the glycosylation site.
The twenty isoforms of GalNAc-Ts, which have been identified in humans, form orthologous groups and pairs that display high conservation across species in sequence and in function (Tian & Ten Hagen, 2009). Close orthologues also share preferences in substrates and glycosylation sites of their substrates, implying a degree of overlapping activity and biological redundancy (Steenoft et al., 2013; Ten Hagen, Tran, Gerken, Stein, & Zhang, 2003). Different preferences in glycosylation sites also suggest distinct functional roles that have been conserved in many species (Tian & Ten Hagen, 2009). Deficiencies in GalNAc-Ts produce distinct, and often subtle outcomes in phenotype, suggesting unique functions of individual isoforms. In humans, familial tumoral calcinosis has been attributed to mutations in GalNAc-T3 (Kato et al., 2006), whereas mutations in GalNAc-T2 results in lower levels of high-density lipoprotein (Khetarpal et al., 2016). Unique roles for different isoforms are also suggested by their distinct expression patterns in mature and developing tissues (P. D. Kingsley, Hagen, Maltby, Zara, & Tabak, 2000; Ten Hagen et al., 2003).

After the initiation of O-GalNAc glycosylation by the GalNAc residue, the structure is further modified and elongated by several glycosyltransferases in the Golgi. Much like the processing of N-glycans, the elongation of O-glycans occurs through Golgi compartments after GalNAc has been added in the cis-Golgi. The Ser/Thr-GalNAc (known as the Tn antigen) is modified by the core-1 synthase (C1Gal-T1) or core 3 synthase (β3GnT6), creating the core 1 O-GalNAc glycan (GalNAc-Gal) or core 3 O-GalNAc glycan (GalNAc-GlcNAc), respectively. These core structures are further elongated to core 2 (from core 1) and core 4 (from core 3), creating four of the most abundant O-GalNAc core structures. A total of 8 core structures have been identified that all are elongated further to create a large variety of glycan structures, typically ending in terminal sialic acid residues. Any given glycosylation site may contain a variety of distinct O-GalNAc glycan structures leading to a great degree of heterogeneity in the O-glycoprotein (Brockhausen & Stanley, 2015).

The modification of a protein backbone with an O-glycan adds a stabilizing molecular determinant to the structure (Chaffey et al., 2017). Depending on the substrate and the glycosylated region, this can have varying effects on protein behavior. O-GalNAc glycan modifications have been attributed to have many roles. They have a functional role in cellular sorting and trafficking (Alfalah et al., 1999; Altschuler et al., 2000; Yeaman et al., 1997), inhibition and regulation of proteolytic processing (Goth et al., 2015; Shirakabe et al., 2017; van der Post et al., 2013), modulation of protein activity and interactions (T. C. Lin et al., 2017; Niang
et al., 2016; Sens et al., 2017; Tokhtaeva et al., 2016) and protein stability (Y. Xu, Pang, Lu, Shan, & Zhang, 2016).

A few GPCRs have been shown to be modified by GalNAc-type O-glycans. Unlike mucins and mucin-like domains, GPCRs with identified O-glycosylation typically only contain a handful of sites in their extracellular domains. Several acceptor sites were confirmed to be O-glycosylated in the extracellular N-terminal tail of the vasopressin V2 receptor and CC chemokine receptor 5 (Bannert et al., 2001; Sadeghi & Birnbaumer, 1999). The terminal sialylation in the CC chemokine receptor 5 O-glycans were demonstrated to promote interaction with chemokines. Site-specific O-GalNAc glycans in the N-terminus of β1AR were demonstrated to co-regulate the proteolytic processing of the receptor N-terminus (Goth et al., 2017; Park, Reddy, Wallukat, Xiang, & Steinberg, 2017). Within the opioid receptor family, δOR and κOR have been shown to contain O-GalNAc glycans (J. G. Li et al., 2007; Petäjä-Repo et al., 2000), but no clear functional role has been established.

2.3 The opioid system

The opioid system is a central modulator of pain management, emotional responses and hedonic homeostasis. It modulates pain behavior and antinociception, regulates mood and responses to stress and anxiety, and is critical for hedonic processing in reward-related motivation, learning and memory. It also regulates a variety of peripheral physiological processes, such as respiration, gastrointestinal motility, cardiovascular regulation, and has a role in hypoxia, motor control, as well as in endocrine and immune systems. The opioid system consists of endogenous opioid peptides and GPCRs of the opioid receptor family (Al-Hasani & Bruchas, 2011; Chu Sin Chung & Kieffer, 2013).

2.3.1 Modulation of pain, reward and mood

The most clinically important function of the opioid system is its role in reducing pain. Opioid receptors take part in the modulation of pain supraspinally in the locus ceruleus, nucleus raphe magnus, periaqueductal gray, medial thalamus, midbrain, cortex and the limbic system. Spinal opioid receptors are located presynaptically in neurons of dorsal root ganglia and the descending pathway entering the dorsal horn. Peripheral opioid analgesia is modulated by presynaptic receptors located in neurons extending from dorsal root ganglia. The activation of opioid receptors in
the supraspinal regions inhibit the neurons, resulting in inhibition of spinal pain transmission (Al-Hasani & Bruchas, 2011; Pasternak, 2014). A selective functional control of pain has been suggested for different opioid receptor subtypes. The δOR was shown to control mechanical pain, whereas μOR controls heat pain (Scherrer et al., 2009). Opioid receptors in peripheral primary afferent sensory neurons can modulate peripheral analgesia. Peripheral opioid analgesia appears to be important in inflammatory conditions and has a role in processes such as cardioprotection (Rachinger-Adam, Conzen, & Azad, 2011).

Opioid receptors couple primarily to inhibitory Ga_i/Ga_o-proteins and have an inhibitory effect on neuronal circuitry. The inhibition of pain transmission is achieved by receptor activation-mediated modulation of downstream effectors. After receptor activation, the dissociated Ga and Gβγ subunits interact with downstream effectors that initiate inhibitory signaling. Perhaps the most important pathway in opioid analgesia is the G protein-mediated modulation of Ca^{2+} and K^+ channels that cause hyperpolarization of the neuron and inhibition of the release of neurotransmitters. The dissociated Gβγ subunit stimulates Ca^{2+}-gated ion-channels and K_i, promoting hyperpolarization and inhibiting tonic neural activity (Madariaga-Mazon et al., 2017; Pasternak, 2014).

Positive emotions such as pleasure, hedonism and reward have evolutional significance for survival. When associated with the ability to learn, they have a vital impact on behavior, called positive reinforcement. Opioid receptors and opioid ligands are broadly expressed in reinforcement networks. Generally, δOR and μOR agonists induce positive reinforcement, such as pleasure and euphoria, while κOR agonists induce dysphoria, aversion and malaise (Chu Sin Chung & Kieffer, 2013; Le Merrer, Becker, Befort, & Kieffer, 2009). The role of opioid system in the positive reinforcement works in conjunction with dopaminergic and GABAergic pathways. For example, the activation of μOR inhibits GABAergic neurons that are inhibitory in nature. The inhibition of GABAergic neurons allows the release of more dopamine, which is the primary neurotransmitter responsible for inducing euphoria and pleasure (Mistry, Bawor, Desai, Marsh, & Samaan, 2014). Opioid receptors are also involved in the energy-driven and hedonic food reinforcement and multiple aspects of sexual reinforcement, and drug reinforcement (Le Merrer et al., 2009). While the μOR appears to be the main modulator of morphine-induced analgesia and euphoria (Matthes et al., 1996), the δOR has been shown to be involved in the control of cognitive and emotional aspects of pain, as well as in the alleviation of stress and anxiety. Knock-out mice for δOR show increased levels of anxiety and depressive-like behavior and increased ethanol self-administration to
reduce the increased levels of anxiety, corresponding to the role of the opioid system for drug reinforcement (Filliol et al., 2000; Roberts et al., 2001). The activation of δOR by introduction of δOR selective agonists has been suggested to decrease levels of anxiety and act as antidepressants (A. A. Pradhan, Befort, Nozaki, Gaveriaux-Ruff, & Kieffer, 2011). Due to the significant role of δOR in modulating mood, the receptor is implicated in many psychiatric and neurological disorders (Chu Sin Chung & Kieffer, 2013).

The functional output of the opioid system has been suggested to be influenced by the opioid receptor trafficking (C. M. Cahill et al., 2007; X. Zhang et al., 2010). The δOR, in particular, has been suggested to be largely localized to intracellular and vesicular compartments (Chen, Mestek, Liu, Hurley, & Yu, 1993; Elde et al., 1995; Gendron et al., 2006; X. Zhang, Bao, Arvidsson, Elde, & Hokfelt, 1998), and delivered to the cell surface under specific conditions. In striatal cholinergic interneurons, the δOR was found to traffic to the cell surface upon Pavlovian conditioning, as measured by food reward (Bertran-Gonzalez, Laurent, Chieng, Christie, & Balleine, 2013). Chronic pain and chronic alcohol exposure have also been reported to increase agonist binding to δOR, suggesting receptor upregulation in these conditions (A. Pradhan, Smith, McGuire, Evans, & Walwyn, 2013; van Rijn, Brissett, & Whistler, 2012).

### 2.3.2 Opioid ligands

The physiological processes in the opioid system are governed by activation of opioid receptors by the endogenous opioid peptides. The opioid receptors are subject to functional selectivity, which can lead to differences in cellular responses depending on the acting ligand (A. A. Pradhan, Smith, Kieffer, & Evans, 2012). The peptide ligands derive from different precursors and bind selectively to different opioid receptor subtypes, as specified in Table 3 (Gendron et al., 2016; Waldhoer, Bartlett, & Whistler, 2004). The opioid receptors are also targeted by various exogenous opioids, peptides and natural opioid alkaloids (opiates), and several synthetic and semisynthetic small molecule agonists, antagonists and inverse agonists have been developed. Most clinically used opioids have the highest affinity to μOR (Feng et al., 2012).
Table 3. Opioid peptides.

<table>
<thead>
<tr>
<th>Endogenous opioid peptides</th>
<th>Sequence</th>
<th>Precursor</th>
<th>Receptor selectivity</th>
</tr>
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<tbody>
<tr>
<td>Enkephalins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Met]-enkephalin</td>
<td>YGGFM</td>
<td>Proenkephalin</td>
<td>δOR</td>
</tr>
<tr>
<td>[Leu]-enkephalin</td>
<td>YGGFL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynorphins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynorphin A</td>
<td>YGGFL-X_{12}</td>
<td>Prodynorphin</td>
<td>κOR</td>
</tr>
<tr>
<td>Dynorphin B</td>
<td>YGGFL-X_{24}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α/β-neo-endorphin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endorphins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-endorphins</td>
<td>YGGFM-X_{11}</td>
<td></td>
<td>µOR</td>
</tr>
<tr>
<td>β-endorphins</td>
<td>YGGFM-X_{25}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-endorphins</td>
<td>YGGFM-X_{12}</td>
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<td></td>
</tr>
<tr>
<td>Endomorphines</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Endomorphine-1</td>
<td>YPWF</td>
<td></td>
<td>µOR</td>
</tr>
<tr>
<td>Endomorphine-2</td>
<td>YPFF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nociceptin/orphanin FQ</td>
<td>FGGFT-X_{12}</td>
<td>Pronociceptin/orphanin FQ</td>
<td>NOP</td>
</tr>
</tbody>
</table>

Abbreviations: δOR, delta-opioid receptor; κOR, kappa-opioid receptor; µOR, mu-opioid receptor; NOP, Nociceptin/orphanin FQ.

2.3.3 Opioid receptors

Already in the 1970s, it was evident that opioids with different characteristics can bind to different types of opioid receptors in multiple tissues. One of the tissues with opioid binding in mouse was the vas deferens, from which the δOR (δ for deferens) was first discovered (Lord, Waterfield, Hughes, & Kosterlitz, 1977; Pert & Snyder, 1973). The δOR was the first opioid receptor subtype to be cloned, first from mouse NG108-15 glioma hybridoma cells (Evans, Keith, Morrison, Magendzo, & Edwards, 1992; Kieffer, Befort, Gaveriaux-Ruff, & Hirth, 1992), then from rat (Fukuda, Kato, Mori, Nishi, & Takeshima, 1993) and finally from a human striatal cDNA library (Knapp et al., 1994). Cloning of the other receptor subtypes from multiple species followed: µOR (µ for morphine) (Chen et al., 1993; Wang et al., 1994), κOR (κ for ketocyclazocine) (Nishi, Takeshima, Fukuda, Kato, & Mori, 1993; Simonin et al., 1995) and nociception/orphanin FQ receptor (first called opioid receptor like-1) (Meunier et al., 1995; Mollereau et al., 1994), each named after their identified ligand (Lord et al., 1977). A study on the evolution of opioid receptors concluded that the subtypes arose from quadruplication during early vertebrate evolution. Thus, most vertebrate species have four opioid receptor subtypes (Dreborg, Sundstrom, Larsson, & Larhammar, 2008). The opioid
receptors are ~60% homologous with the other subtypes, with the most conserved regions in TM regions that bind the opioid ligands (Waldhoer et al., 2004).

The opioid receptors are peptide-binding receptors that have somewhat overlapping but distinct expression patterns that cover many areas in the CNS, especially in the cortex, limbic system and brain stem. In the CNS, δOR, µOR and κOR are expressed in many regions important for processing of reward, pain and mood. All three subtypes are highly expressed in the nucleus accumbens and caudate nucleus. The µOR shows also high expression in the cerebellum, κOR in the putamen, and δOR in the cerebral cortex, putamen, temporal lobe, olfactory bulb and hippocampus. All subtypes are also expressed in the spinal cord and dorsal root ganglia. The different patterns correlate with the different roles of the receptors in the opioid system (Le Merrer et al., 2009; Peng, Sarkar, & Chang, 2012). The opioid receptors are also expressed in many peripheral tissues, including the adrenal gland (δOR, µOR, κOR), pancreas and small intestine (δOR, µOR, κOR), lung, kidney and heart (δOR, κOR), as well as spleen (κOR) and thymus (δOR, κOR) (Peng et al., 2012).

The crystal structures of all opioid receptor subtypes have been solved (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; H. Wu et al., 2012). They have a typical Rhodopsin/Class A GPCR structure with a short extracellular N-terminal tail and a bundle of TMs containing the ligand binding pocket (Fig. 2). All opioid receptors possess the DRY motif, the CWxP motif and the NPxxY motifs described earlier (see 2.1.2) and a stabilizing disulfide bridge between the conserved cysteines in TM3 and ECL2 (Waldhoer et al., 2004). The extracellular loops of opioid receptors partially cover the open ligand binding pocket, providing ligand selectivity for the receptor. For δOR, ECL3 is the most important determinant for ligand selectivity (Fenalti et al., 2015; Granier et al., 2012; Waldhoer et al., 2004; Varga et al., 1996). The backbone structure of δOR is highly conserved with the other opioid receptors, even in regions with low sequence conservation. The ECL2 of opioid receptors has a β-hairpin structure that is commonly seen with receptors of the Rhodopsin/Class A family. The opioid receptor crystal structures also revealed an open ligand binding pocket with two distinct regions. The upper portion of the pocket confers subtype selectivity, whereas the bottom of the pocket is conserved among the opioid receptors. This is in concordance with the “message-address” model, in which the efficacy (message) and the selectivity (address) are contained within the opioid ligand that interacts with the binding pocket (Chavkin & Goldstein, 1981; Granier et al., 2012). The N-terminal tail is often omitted from receptor constructs used for crystallization but
interestingly, the structure of μOR in the active conformation revealed an interaction between the truncated N-terminal tail and the ligand (W. Huang et al., 2015), suggesting a possible role for the region as a lid-like structure for opioid receptors. The high-resolution crystal structure of the human δOR revealed the presence of water molecules in the orthosteric binding site and a sodium ion in the allosteric site (Fenalti et al., 2014). Furthermore, the “closed” conformation of ICL3 in all inactive opioid receptors suggested the presence of a stabilizing determinant compensating for the absence of the “ionic lock” present in many of the other Rhodopsin/Class A GPCRs (Fenalti et al., 2014). The μOR and κOR structures have been solved in the active receptor conformation (Che et al., 2018; W. Huang et al., 2015; Sounier et al., 2015), and the μOR in complex with the Go protein (Koehl et al., 2018). The structure of μOR reveals large changes in ICL1 and H8, suggesting their role in G protein coupling, possibly for all opioid receptors (W. Huang et al., 2015; Sounier et al., 2015). Both active conformations showed a general contraction of the extracellular side (ECL and TMs) and a reduction in the volume of the binding pocket. These conserved changes likely facilitate the changes in the intracellular side that result in binding of G proteins and other effectors (Che et al., 2018; W. Huang et al., 2015). The clinical potential of simultaneous blockade of δOR and stimulation of μOR to avoid development of morphine tolerance inspired the solution of the δOR structure with a bifunctional δOR antagonist/μOR agonist (Abdelhamid, Sultana, Portoghese, & Takemori, 1991; Fenalti et al., 2015). To date, this is the only opioid structure solved with a peptide ligand.

**Delta-opioid receptor and the OPRD1 gene**

The δOR shares high homology across species and with the other opioid receptor subtypes, μOR, κOR and NOR, but has unique features that make it an attractive target for pharmaceuticals (Waldhoer et al., 2004). It is a potential target for treating various neurological disorders and alleviating adverse effects in μOR-mediated analgesia (Dripps & Jutkiewicz, 2017; Gendron et al., 2016; A. A. Pradhan et al., 2011). The amino acid sequence and the arrangement of TM domains revealed from the crystal structure are depicted in Figure 8.
Fig. 8. Snake plot diagram of hδOR. The amino acid sequence with structural characteristics assigned to the residues according to the structure (4RWD, modeled with JSmol at RCSB Protein Data Bank) (Fenalti et al., 2015). The most conserved residues of each TM and the H8 domain [per Ballesteros-Weinstein (Ballesteros & Weinstein, 1995)], and the residues involved in ligand interaction in the purified crystal structure are indicated (Fenalti et al., 2015). The Phe27Cys SNP, phosphorylation sites (Guo et al., 2000), the putative palmitoylation site (Petäjä-Repo et al., 2006) and the two N-glycosylation sites (Markkanen & Petäjä-Repo, 2008) are indicated.
The h\(\delta\)OR gene, \textit{OPRD1}, has been localized to the short arm of chromosome 1 (Befort, Mattei, Roeckel, & Kieffer, 1994). The gene contains two introns between TM domains 1 and 4 (Simonin et al., 1994), and no alternative splicing has been reported. The gene for h\(\delta\)OR is highly regulated and sensitive to extracellular stimuli and epigenetic regulation. The promoter region lacks the classical TATA and any consensus initiator. The gene is preceded by a GC rich region that binds members of the specificity protein 1 transcription factor family (Crist & Clarke, 2016; Wei & Loh, 2011).

The 1000 genomes project concluded in 2015 identified 2240 SNPs and somatic natural variants (SNV) in the \textit{OPRD1} gene (Auton et al., 2015). Of these, 67 missense mutations, 50 silent mutations and 169 upstream gene variants were identified, but only a handful of these had a global minor allele frequency (MAF) higher than 1%. Two nonsynonymous SNPs (Ala309Thr and Ile52Val) and one synonymous silent mutation (Ala269Ala) with low MAF (~2%) have been reported in specific ethnic groups but have not been studied. Only Ile52Val is more abundant in the population in which it is the most prevalent (African populations, MAF 8%). One synonymous (Gly307Gly, MAF 42%) and one nonsynonymous (Phe27Cys, MAF 6%) SNP are widely present in many populations. The Phe27Cys polymorphism is more abundant in American and European populations (MAF 8% and 13%, respectively), and absent in East Asian populations (Auton et al., 2015). One SNP is also located 2 kb upstream of the transcription initiation site at the promoter region (A/G, MAF 6%). This SNP has been shown to enhance transcription factor binding that can lead to an increase in h\(\delta\)OR expression (H. Zhang et al., 2010). Interestingly, the SNP is in high linkage disequilibrium with the Phe27Cys polymorphism, suggesting association of the alleles, and possibly phenotypes (Mistry et al., 2014; H. Zhang, Kranzler, Yang, Luo, & Gelernter, 2008). The only common nonsynonymous mutation in the h\(\delta\)OR is the T80G change in exon 1. This results in an amino acid change Phe27Cys in the extracellular N-terminus of the receptor (Gelernter & Kranzler, 2000). The h\(\delta\)OR was first cloned from a neuroblastoma cell line carrying the G80 allele (Cys27), making this SNP the reference sequence. However, the T80 allele (Phe27) is the ancestral and more common allele (Gelernter & Kranzler, 2000; Simonin et al., 1994). The Phe27Cys polymorphism has been studied both at the genetic and protein level. The Cys27 variant has been reported to be associated with increased opioid dependence risk (H. Zhang et al., 2008), altered amyloid precursor protein processing (Sarajärvi et al., 2011) and compromised calcium signaling (Tuusa & Petäjä-Repo, 2011). Studies using heterologous expression of h\(\delta\)OR\textsuperscript{Phe27} and
h\textsuperscript{δ}OR\textsuperscript{Cys\textsubscript{27}} have concluded similar pharmacological and signaling properties but have shown distinct phenotypes in post-translational processing (Leskelä et al., 2009). This is in line with the position of the amino acid substitution in the N-terminus that is not part of the ligand binding pocket but may affect trafficking and processing of the protein in the secretory pathway. The less common h\textsuperscript{δ}OR\textsuperscript{Cys\textsubscript{27}} variant displays less efficient maturation leading to a larger portion of receptors in intracellular compartments. Dissimilarities can also be seen in trafficking from the cell surface as the turnover of h\textsuperscript{δ}OR\textsuperscript{Cys\textsubscript{27}} is more rapid in constitutive and ligand treated conditions than that of h\textsuperscript{δ}OR\textsuperscript{Phe\textsubscript{27}} (Leskelä et al., 2009).
3 Aims of the thesis

Opioid receptors are among the most clinically relevant drug targets and are subjects of highly active scientific research. These receptors hold tremendous potential in the adverse effect-free treatment of chronic pain, as well as in the treatment of various neurological disorders. While the signaling and pharmacological properties of GPCRs, including opioid receptors, are extensively studied, the biosynthesis and processing during receptor maturation in the secretory pathway are poorly understood. The thesis examines the molecular determinants and mechanisms that guide the δOR synthesis during these steps and thus modulate their behavior and functional output at the cell surface. Furthermore, the polymorphic variants of this important receptor, which are associated with severe medical conditions, are investigated in this context to possibly explain the observed phenotypic differences.

The specific aims of the thesis are:

1. To investigate the heteromerization of δOR variants and its possible role in receptor ER processing and cell surface delivery.
2. To study the functional role of δOR variant N-glycosylation in receptor folding and quality control in the ER and at the cell surface.
3. To identify O-glycosylation sites and enzymes O-glycosylating the δOR variants, and to study the role of O-glycans in the behavior and function of the receptor.
4 Materials and methods

The hδOR processing and cellular behavior were studied utilizing heterologous expression of the receptor in various mammalian cell models. WT and engineered cell models were transfected with the receptor cDNA constructs to produce transient or stable overexpression for the experiments. In a similar way, DNA constructs of other proteins of interest were transfected and expressed. N- and C-terminal epitope tags were utilized in the receptor constructs to allow efficient purification and specific detection by tag-specific antibodies. The hδOR was studied in experiments using live cells, fixed cells or cell extracts of different subcellular fractions. The experiments cover a wide range of complementary biochemical and cell biological methods aiming to answer the specific questions. The aims and selected approaches are listed in Table 4, and the outline of methods is depicted in Figure 9.
Table 4. Aims and approaches.

<table>
<thead>
<tr>
<th>Study</th>
<th>Aim</th>
<th>Cell models</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Characterization of hδOR variant processing and oligomerization in co-transfected cells</td>
<td>HEK293, SH-SY5Y</td>
<td>Transient and stable co-expression of hδOR variants</td>
</tr>
<tr>
<td></td>
<td>Receptor processing studied with metabolic labeling, western blotting, flow cytometry and confocal microscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Receptor oligomerization studied with co-immunoprecipitation and BRET</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Assessing the role of N-glycans in hδOR processing in the ER</td>
<td>HEK293</td>
<td>Site-directed mutagenesis of receptor N-glycosylation sites and stable transfection</td>
</tr>
<tr>
<td></td>
<td>Receptor maturation and turnover studied with metabolic labeling, western blotting and cell surface biotinylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Receptor processing in the ER studied by selective inhibitors of proteasome and glycan processing, as well as co-immunoprecipitation with calnexin and ubiquitin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Characterization and functional investigation of hδOR O-glycosylation</td>
<td>HEK293, HepG2, CHO K1, CHO K1 Id1D, HEK293 SimpleCells (HEK293 SC), GaINAc-T2 gene (GALNT2) knockout and/or knockout/knock-in in HEK293 (HEK293 ΔT2) and HepG2 (HepG2 ΔT2, HepG2 ΔT2+/ΔT2)</td>
<td>Characterization of receptor O-glycosylation by in vitro peptide glycosylation assay and mass spectrometry</td>
</tr>
<tr>
<td></td>
<td>Site-directed mutagenesis of receptor O-glycosylation sites, expression in cell models and lectin-mediated purification</td>
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<tr>
<td></td>
<td>Assessment of receptor expression and trafficking in engineered cell lines by flow cytometry and cell surface biotinylation.</td>
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<td></td>
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<tr>
<td></td>
<td>Functional analysis by cAMP accumulation assay</td>
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</tbody>
</table>

Abbreviations: BRET, Bioluminescence resonance energy transfer; HEK293, human embryonic kidney cell line; hδOR, human delta-opioid receptor; HepG2, human hepatocellular carcinoma cell line; SH-SY5Y, human bone marrow neuroblastoma cell line (see Table 7 for details).
Fig. 9. Outline of methods. General outline of experiments used in the thesis, starting from protein expression and purification and ending in detection and analysis. N- (Myc or HA) and C-terminal (FLAG, Venus or Rluc) epitope tags are illustrated in the inset on the right. *Detection of samples from metabolic labeling. BRET; Bioluminescence Resonance Energy Transfer; VVA, Vicia villosa Agglutinin.
4.1 Heterologous expression

The hδOR was studied by heterologous expression in various cell models. This entailed the design and preparation of receptor cDNA constructs, transfections of the constructs into various cell models, and transient or stable expression of the receptor. Certain approaches involved biochemical labeling of the receptor in order to follow and assess hδOR maturation and processing during receptor expression in the cellular models (see Fig. 9). In these cases, live cells were assayed with cell surface biotinylation (II, III) (see 4.1.6) to follow the cell surface receptor, or with metabolic labeling (I, II) (see 4.1.7) to follow the de novo synthesis and processing of hδOR in the cells.

4.1.1 DNA constructs

The receptor cDNA constructs included epitope-tags in the receptor N-terminus and/or C-terminus. The N-terminal tags myc (EQKLISEEDL) or human influenza hemagglutinin (HA) (YPYDVPDYA), and the C-terminal FLAG (DYKDDDDK) were used for efficient purification and detection of the receptor by tag-specific antibodies. The myc tag is a peptide tag derived from the c-myc gene product and HA is from the hemagglutinin corresponding to amino acids 98-106. The C-terminal Venus and Renilla luciferase (Rluc) tags were used for BRET analysis. Venus is a modified version of the yellow fluorescent protein and Rluc is a bioluminescent luciferase that acts as a BRET donor for Venus (Lohse, Nuber, & Hoffmann, 2012; Nagai et al., 2002). HA-hδORCys27-Rluc and myc-GABA2b-Venus constructs have been described elsewhere (Breit, Gagnidze, Devi, Lagace, & Bouvier, 2006; Villemure et al., 2005). HA-hδORCys27-Venus was prepared by subcloning HA-hδORCys27 into a modified pcDNA3.1/Zeo(+) vector using NheI and AvrII restriction enzymes. HA-hδORPhe27-Rluc and HA-hδORPhe27-Venus constructs were prepared from the corresponding hδORCys27 constructs by site-directed mutagenesis. All receptor cDNA constructs included a cleavable signal peptide from hemagglutinin: KTIALSYIFCLVFA. The signal peptide improves greatly the targeting of translation to the ER and improves receptor expression (X. M. Guan, Kobalika, & Kobalika, 1992). Several cDNA constructs were altered by site-directed mutagenesis to include nucleotide changes, such as disruptions of glycosylation sites. Most receptor cDNA constructs consisted of hδOR subcloned into a modified pcDNA5/FTR/TO vector (Invitrogen) called pft-SMMF (Pietilä et al., 2005). The pft-SMMF constructs contained the appropriate tags, cleavable
signal peptide and the receptor cDNA, separated by short N- and C-terminal linkers. Table 5 lists the constructs used, indicating the epitope-tags, mutations in the cDNA, expression vectors and the publications in which they appear.

Table 5. DNA constructs and expression vectors.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Mutations</th>
<th>Vector</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA- hδOR&lt;sup&gt;Thr&lt;sup&gt;27&lt;/sup&gt;</td>
<td>pcDNA3.1/Zeo&lt;sup&gt;+&lt;/sup&gt;</td>
<td>I, III</td>
<td></td>
</tr>
<tr>
<td>HA- hδOR&lt;sup&gt;Cys&lt;sup&gt;27&lt;/sup&gt;</td>
<td>pcDNA3.1/Zeo&lt;sup&gt;+&lt;/sup&gt;</td>
<td>I, III</td>
<td></td>
</tr>
<tr>
<td>HA- hδOR&lt;sup&gt;Thr&lt;sup&gt;27&lt;/sup&gt;-Venus</td>
<td>pcDNA3.1/Zeo&lt;sup&gt;+&lt;/sup&gt;</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>HA- hδOR&lt;sup&gt;Cys&lt;sup&gt;27&lt;/sup&gt;-Venus</td>
<td>pcDNA3.1/Zeo&lt;sup&gt;+&lt;/sup&gt;</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>hδOR&lt;sup&gt;Thr&lt;sup&gt;27&lt;/sup&gt;-RLuc</td>
<td>pcDNA3.1/Zeo&lt;sup&gt;+&lt;/sup&gt;</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>hδOR&lt;sup&gt;Cys&lt;sup&gt;27&lt;/sup&gt;-RLuc</td>
<td>pcDNA3.1/Zeo&lt;sup&gt;+&lt;/sup&gt;</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>myc- rLHR -FLAG</td>
<td>pft-SMMF</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>myc- GABA&lt;sub&gt;β&lt;/sub&gt;R2 -Venus</td>
<td>pcDNA3.1/Zeo&lt;sup&gt;+&lt;/sup&gt;</td>
<td>I</td>
<td></td>
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<tr>
<td>HA- Ubiquitin</td>
<td>pcDNA3</td>
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<td>pft-SMMF</td>
<td>I, II</td>
<td></td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Cys&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>pft-SMMF</td>
<td>I, II</td>
<td></td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Thr&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>N18;33E</td>
<td>pft-SMMF</td>
<td>II, III</td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Cys&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>N18;33E</td>
<td>pft-SMMF</td>
<td>II, III</td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Thr&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>N18;33E, S25;29A</td>
<td>pft-SMMF</td>
<td>III</td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Cys&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>N18;33E, S25;29A</td>
<td>pft-SMMF</td>
<td>III</td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Thr&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>N18;33E, S6;25;29A</td>
<td>pft-SMMF</td>
<td>III</td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Cys&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>N18;33E, S6;25;29A</td>
<td>pft-SMMF</td>
<td>III</td>
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<tr>
<td>myc- hδOR&lt;sup&gt;Thr&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>S25;29A</td>
<td>pft-SMMF</td>
<td>III</td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Cys&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>S25;29A</td>
<td>pft-SMMF</td>
<td>III</td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Thr&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>S6;25;29A</td>
<td>pft-SMMF</td>
<td>III</td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Cys&lt;sup&gt;27&lt;/sup&gt; -FLAG</td>
<td>S6;25;29A</td>
<td>pft-SMMF</td>
<td>III</td>
</tr>
</tbody>
</table>

*A semicolon (;) is used when the same amino acid is mutated in more than one position (i.e. N18,33E = N18E and N33E). * indicates modified.

4.1.2 Site-directed mutagenesis

Site-directed mutagenesis was used to introduce point mutations into the cDNA constructs (II, III). Codons of specific amino acids were changed to disrupt confirmed and putative glycosylation sites in the receptor N-terminal tail. The methods offered a substrate-specific tool to complement the global glycosylation deficiencies in engineered cell lines. This is important as the global glycosylation deficiencies may lead to indirect effects on hδOR processing. Oligos used in mutagenesis are listed in Table 6.
Protocol

1. The mutagenesis reaction was prepared with the cDNA template (50 ng) and mutagenesis oligos (125 ng), according to the protocol of the QuikChange Lightning site-directed mutagenesis kit manufacturer (Agilent).

2. Mutagenesis was performed with the polymerase chain reaction (PCR) using the following cycles:

   x 95°C, 2 min
   18 x (95°C, 20 s; 60-68°C, 10 s; 68°C, 30 s/kb of plasmid length)
   1 x 68°C, 5 min

3. Parental cDNA was digested with the \textit{Dpn I} enzyme.

4. Mutated cDNA was transformed into competent \textit{E. coli} cells and new constructs were isolated and verified by sequencing.

Table 6. Oligos for site-directed mutagenesis.

<table>
<thead>
<tr>
<th>cDNA construct</th>
<th>Mutation</th>
<th>Mutagenesis oligo</th>
</tr>
</thead>
<tbody>
<tr>
<td>myc- hδOR\textsuperscript{N18;33E} -FLAG</td>
<td>N18;33E</td>
<td>CCTACCCTAGCCCTTCCCCAGCGCTGGGC\textsuperscript{C}</td>
</tr>
<tr>
<td>myc- hδOR\textsuperscript{S25;29A} -FLAG</td>
<td>S25;29A</td>
<td>GGACGCCTACCCTGCGCCCTCCCCGCCGCCCTGGGC\textsuperscript{C}</td>
</tr>
<tr>
<td>myc- hδOR\textsuperscript{S25;29A} -FLAG</td>
<td>S25;29A</td>
<td>GGACGCCTACCCTGCGCCCTCCCCGCCGCCCTGGGC\textsuperscript{C}</td>
</tr>
<tr>
<td>myc- hδOR\textsuperscript{S25;29A} -FLAG \textsuperscript{N18;33E}</td>
<td>S25;29A</td>
<td>GGAGGCCTACCCTGCGCCCTCCCCGCCGCCCTGGGC\textsuperscript{C}</td>
</tr>
<tr>
<td>myc- hδOR\textsuperscript{S25;29A} -FLAG \textsuperscript{N18;33E}</td>
<td>S25;29A</td>
<td>GGAGGCCTACCCTGCGCCCTCCCCGCCGCCCTGGGC\textsuperscript{C}</td>
</tr>
</tbody>
</table>
| myc- hδOR\textsuperscript{S25;29A} -FLAG \textsuperscript{N18;33E} | S6A       | CGGGCCCCGCGGGGCCGGG |}

The mutated codons are underlined. A semicolon (;) is used when more than one amino acid is mutated (i.e. N18;33E = N18E and N33E).

4.1.3 \textit{Cell culture}

The scope of the thesis entails processes that are conserved at a cellular level in mammalian cells, making expressions in selected cell models suitable tools for the study. Cell models of neuronal origin were included to validate the findings in cells that express the hδOR endogenously. Site-directed mutagenesis of receptor
constructs was complemented with the use of engineered cell lines using WT DNA constructs. These cell lines had specific alterations in the genome to facilitate knock-out and/or knock-in of relevant targets in receptor processing.

Human embryonic kidney cells (HEK293i, 293S, Flp-In-293, HEK-SC, HEKΔT2), Chinese hamster ovary cells (CHO-K1, CHO-ldID) and neuroblastoma cells (SH-SY5Y) were cultured at 37 C in a humidified atmosphere with 5% CO2. The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) or in F-12 medium with Kaighn’s modification supplemented with fetal bovine serum (FBS) as indicated in Table 7. The CHO-ldID cell line has a nonfunctional epimerase-4 that is required to catalyze UDP-Gal and UDP-GalNAc synthesis from UDP-Glc (UDP-Glucose) and UDP-GlcNAc (UDP-N-acetylglucosylamine), respectively (D. M. Kingsley, Kozarsky, Hobbie, & Krieger, 1986; Krieger et al., 1989). UDP-GalNAc is required for the initiation of O-glycosylation by GalNAc-transferases, and UDP-Gal is used for the elongation of O-glycans and N-glycans. These modifications only take place if the culture medium is supplemented with GalNAc and/or Gal, as they have salvage pathways that utilize extracellular monosaccharides for protein glycosylation (Krieger et al., 1989).

The Flp-In system (Invitrogen) was employed in pre-existing and newly-established cell lines with isogenic receptor expression from the same genetic locus (Hillen & Berens, 1994; Sauer, 1994). Flp-In-CHO-ldID cells were generated by introducing a Flp Recombination target (FRT) site to the CHO-ldID cells by transfection with the FRT-lacZeo plasmid (Invitrogen) (III). Positive clones were selected with 100 µg/ml Zeocin and tested with the β-galactosidase assay and Southern blotting. The Flp-In-CHO-ldID cells were subsequently co-transfected with receptor constructs (see Table 5) and the pOG44 plasmid (Invitrogen), which resulted in isogenic stable integration of the receptor to the FRT site. Positive clones were selected with 400 µg/ml hygromycin. The HEK293i cells used were generated using the same approach with an additional transfection of the tetracycline repressor (pcDNA6/TR, Invitrogen) to establish stable lines with tetracycline-inducible receptor expression (Table 7) (I, II). In these cells the expression was induced by introduction of tetracycline in the culture medium. Stable HEK-SCs expressing hδOR were established by transfecting the constructs in pcDNA3.1 into the cells and selecting for positive clones under 100 µg/ml Geneticin selection (III). Cell culture media of all cells were supplemented with 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich), and if appropriate with 100 µg/ml Zeocin (Invitrogen), 400 µg/ml Hygromycin (Invitrogen), 4 µg/ml Blasticidin (Invitrogen)
and 100 µg/ml Geneticin (Thermo Fisher Scientific), as indicated in Table 7. PBS used in the study does not contain calcium or magnesium.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Phenotype</th>
<th>Source</th>
<th>Antibiotics</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>293S</td>
<td></td>
<td>Prof. M. Bouvier</td>
<td>PS</td>
<td>III</td>
</tr>
<tr>
<td>Flip-In-293</td>
<td></td>
<td>Invitrogen</td>
<td>PS, Zeoc.</td>
<td>I</td>
</tr>
<tr>
<td>HEK293,</td>
<td>FRT-site</td>
<td>Made in Lab</td>
<td>PS, Hyg., Blast.</td>
<td>II</td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Phe27&lt;/sup&gt;-FLAG (i)</td>
<td>Made in Lab</td>
<td>PS, Hyg., Blast.</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Cys27&lt;/sup&gt;-FLAG (i)</td>
<td>Made in Lab</td>
<td>PS, Hyg., Blast.</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Phe27&lt;/sup&gt;-N18;33E-FLAG (i)</td>
<td>Made in Lab</td>
<td>PS, Hyg., Blast.</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Cys27&lt;/sup&gt;-N18;33E-FLAG (i)</td>
<td>Made in Lab</td>
<td>PS, Hyg., Blast.</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>HA- hδOR&lt;sup&gt;Phe27&lt;/sup&gt; (i)</td>
<td>Made in Lab</td>
<td>PS, Hyg., Blast.</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>HA- hδOR&lt;sup&gt;Cys27&lt;/sup&gt; (i)</td>
<td>Made in Lab</td>
<td>PS, Hyg., Blast.</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>HEK-SC</td>
<td>COSMC knockout</td>
<td>Prof. H. Clausen</td>
<td>PS</td>
<td>III</td>
</tr>
<tr>
<td>HEK T2</td>
<td>GALNT2 knockout</td>
<td>Prof. Clausen</td>
<td>PS</td>
<td>III</td>
</tr>
<tr>
<td>HepG2</td>
<td></td>
<td>Prof. Clausen</td>
<td>PS, NEAA</td>
<td>III</td>
</tr>
<tr>
<td>HepG2 T2</td>
<td>GALNT2 knockout</td>
<td>Prof. Clausen</td>
<td>PS, NEAA</td>
<td>III</td>
</tr>
<tr>
<td>HepG2 T2/T2</td>
<td>GALNT2 knockout/knock-in</td>
<td>Prof. Clausen</td>
<td>PS, NEAA</td>
<td>III</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>endogenous hδOR&lt;sup&gt;Cys27&lt;/sup&gt;</td>
<td>Prof. M. Hiltunen</td>
<td>PS</td>
<td>I, III</td>
</tr>
<tr>
<td>CHO-K1</td>
<td></td>
<td>ATCC</td>
<td>PS</td>
<td>III</td>
</tr>
<tr>
<td>CHO-ldlD</td>
<td>epimerase-4 deficiency</td>
<td>ATCC</td>
<td>PS</td>
<td>III</td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Phe27&lt;/sup&gt;-N18;33E-FLAG (i)</td>
<td>Made in Lab</td>
<td>PS, Hyg.</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>myc- hδOR&lt;sup&gt;Cys27&lt;/sup&gt;-N18;33E-FLAG (i)</td>
<td>Made in Lab</td>
<td>PS, Hyg.</td>
<td>III</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ATCC, American Type Culture Collection; Blast., Blasticidin; CHO, Chinese hamster ovary; COSMC, Cosmc gene; δOR, delta-opioid receptor; FRT, flippase recognition target; GALNT2, N-acetylgalactosaminyltransferase 2 gene; Genet., Geneticin; HEK, human embryonic kidney; Hyg., Hygromycin; i, inducible; NEAA, non-essential amino acids; PS, penicillin/streptomycin; s, stable; SC, SimpleCells.

### 4.1.4 Transfections

The heterologous expression of hδOR entails the introduction of the receptor DNA constructs into the cell models via transfection. In a similar way, other proteins of interest in the study were transiently expressed. The primary method of transfection was the use of liposomes (lipofection), in which the DNA is complexed with a reagent to form cationic liposomes. The complexes comprise a positively charged outer surface surrounding the negatively charged DNA. The composition and charge of the liposome allows the complex to permeate the negatively charged cell
membrane and transfer the DNA into the cell. An alternative strategy of transfection that was utilized used a stable cationic polymer, polyethylene imine (PEI), that condenses DNA into positively charged particles that are endocytosed and released into the cells (Boussif et al., 1995; Sonawane, Szoka, & Verkman, 2003).

Transient transfections of the plasmid cDNA constructs only yield short-term protein expression (up to 4 days) since the DNA typically does not integrate into the replicating genome. Stable transfections can be established by using antibiotics to select for transfected cells, in which the DNA integration has made the expression permanent. Co-transfections were done by either forming the complexes in the presence of two different cDNA constructs, or in some cases by separate formation of complexes that were introduced to the same cells. The cDNA constructs were transfected into the cells using Lipofectamine 2000 (I, II, III) and 3000 (III) transfection reagents (Invitrogen), or linear 25-kDa PEI (III) (Polysciences).

Protocol
1. The cell culture medium was changed 3 h prior to transfection to Opti-MEM (Invitrogen) with 4% FBS.
2. The DNA and the reagent were diluted separately into Opti-MEM and incubated for 5 min before combining the dilutions and incubating for 15-20 min. With the Lipofectamine 3000 reagent, the DNA dilution contained an additional reagent (P3000).
3. The reagent:DNA ratios used were 2-3:1 (Lipofectamine 2000) or 2:1 (Lipofectamine 3000, PEI)
4. The combined dilutions with the reagent and the DNA were added to the cells.
5. The medium was changed back to the normal culture medium after 4 h, and the cells were cultured for 20 h.

4.1.5 Ligands and treatments
Several hypotheses in the study were related to interacting proteins and cellular processes involved in hδOR processing. To test these, reagents such as inhibitors and ligands were used during hδOR synthesis and expression. Inhibitors of glycoprotein processing were utilized to investigate the role of glycans and glycan processing during early steps of hδOR biosynthesis (II). Castanospermine (CST) is a glucosidase inhibitor, which prevents the trimming of the three glucose from
high-mannose N-glycans of nascent polypeptides (Saul, Ghidoni, Molyneux, & Elbein, 1985). The role of this step is central during glycoprotein quality control, as described earlier (see 2.2.2). The timing of the CST treatment leads to different outcomes in glycan processing. The co-translational treatment inhibits glucose trimming and the consequent introduction of the glycoprotein to the CNX/CRT cycle, whereas the treatment later on can inhibit merely the removal of the last glucose, leading to stabilization of the CNX/CRT-glycoprotein interaction (Lamriben et al., 2016). Tunicamycin can be used to prevent N-glycosylation altogether, due to its inhibitory effect in the synthesis of the N-glycan precursor (Mahoney & Duksin, 1980). This also induces ER stress due to the global absence of the modification (Banerjee et al., 2011). Tunicamycin was used to study the receptor in these conditions (I). The co-translational addition of opioid ligands was used to assess the capacity of ligands to act as pharmacological chaperones and rescue hδOR from the ERAD, and to identify the hδOR species and conditions that lead to this pathway (I, II). The degradation of hδOR was also assessed by treating cells with lactacystin (I, II). This common inhibitor of proteasome activity leads to accumulation of proteins that are targeted for degradation (Fenteany et al., 1995). The treatments used are listed in Table 8.

Table 8. Ligands and treatments.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration / Manufacturer</th>
<th>Target / Assay</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castanospermine</td>
<td>150 µg/ml Enzo Life Sciences</td>
<td>ER glucosidase inhibitor / N-glycan processing during ERAD and ERQC</td>
<td>II</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>10 µM Enzo Life Sciences</td>
<td>Inhibition of N-glycan synthesis / induction of ER Stress</td>
<td>I</td>
</tr>
<tr>
<td>Lactacystin</td>
<td>10 µM Enzo Life Sciences</td>
<td>Proteasomal inhibitor / Identification of substrates of ERAD</td>
<td>I, II</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>10 µM Tocris</td>
<td>Opioid antagonist / hδOR degradation</td>
<td>I, II</td>
</tr>
<tr>
<td>Nalttriben</td>
<td>10 µM Tocris</td>
<td>Opioid antagonist / hδOR degradation</td>
<td>I, II</td>
</tr>
</tbody>
</table>

ER, Endoplasmic reticulum; ERAD, ER-associated degradation; ERQC, ER quality control.

4.1.6 Cell surface biotinylation

The cell surface biotinylation is a method to label proteins exclusively at the cell surface, as the sulfo-N-hydroxysuccinimide (NHS)-biotin reagent used does not pass through the plasma membrane (Daniels & Amara, 1998). The NHS-activated
biotin reagents react with amine residues (such as in lysine) in cell surface proteins, and the biotinylated proteins can be purified or detected with streptavidin conjugates that bind to the biotin-group. After quenching the reaction, the turnover of biotinylated proteins can be assessed in a chase assay. Cell surface biotinylation offers a convenient tool to detect and analyze the hδOR at the cell surface. It was used to identify receptor species reaching the cell surface (II, III), and to quantitate receptor turnover due to constitutive downregulation (II, III).

Protocol

1. Before biotinylation of cell surface receptors, cells were washed twice with phosphate-buffered saline (PBS) supplemented with 10 mM MgCl₂ and 100 µM CaCl₂ (D-PBS).
2. D-PBS was added, and the plates were cooled on a metal plate on ice for 10 min before the addition of EZ-linked sulfo-NHS-biotin (Pierce) /PBS to a final concentration of 0.5 mg/ml.
3. Cells were biotinylated on ice for 30 min. The reaction was stopped by adding 1 M Tris-HCl, pH 7.4 to a final concentration of 50 mM, and incubating for 10 min on ice. Finally, the solution was aspirated, cells were washed with PBS and harvested. Alternatively, the stopping solution was aspirated and replaced with fresh culture medium and cells were chased at 37°C in the cell incubator before harvesting.

4.1.7 Metabolic pulse-chase labeling

The de novo synthesis and maturation of proteins can be assessed by metabolic labeling during translation. Using [³⁵S]-labeled methionine/cysteine in the cell culture medium after depletion of these amino acids results in [³⁵S]-labeled proteins that can be detected by autoradiography and fluorography (see 4.3.2). The strategy was used to assess the maturation and processing of the newly-synthesized hδOR. After a short pulse with the radiolabel, the maturation of radiolabeled receptor can be followed by harvesting the cells after different time points. The analyses of the maturation efficiency and kinetics was done after receptor purification and detection.

Protocol
1. Cells transiently transfected for 24 h, or stable cell lines induced to express the hδOR for 1 h were depleted of methionine and cysteine for 1 h in methionine/cysteine-free DMEM at 37°C in the cell incubator.

2. Newly-synthesized proteins were labeled with 50-150 µCi [35S]-methionine/cysteine (Easytag Express [35S]-protein labeling mix, PerkinElmer) for 30-40 min at 37°C.

3. Cells were washed three times with DMEM supplemented with 5 mM methionine and chased for 0.5-6 h at 37°C. After the chase, the medium was removed, and the cells were harvested in PBS.

4.2 Protein purification

After expression in cell models, the hδOR and the other proteins of interest were assayed and analyzed with different methods relevant for the specific questions (see Fig. 9). This often required the biochemical characterization or quantification at the protein level and purification of the required protein. Harvested cells were used to obtain whole cell extracts or different cellular fractions by utilizing detergent-containing buffers and differential centrifugation. Breaking down the cells and membranes was achieved using detergents that disrupt the membrane structures into small micelles. The detergent used was n-dodecyl β-D-maltoside (DDM) that is an alkyl maltoside detergent typically used in the study of GPCRs (Grisshammer, 2009) (I, II, III). An alternative, gentler detergent, digitonin, was used in a few experiments to assess protein-protein interactions (II). The integrity of the released proteins was maintained by buffers that mimicked the physiological conditions and contained protease inhibitors that prevent proteolysis. N-ethylmaleimide (NEM) was included in the first buffers used to alkylate the cysteine residues in order to prevent the formation of inappropriate in vitro disulfide bonds and aggregations. Differential centrifugation was used for the removal of unwanted cellular material from the samples and for the separation of different cellular fractions. After the desired samples were obtained, the protein concentration was measured using a detergent-compatible DC™ protein assay (Bio-Rad) that is a colorimetric assay based on the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The luminescence at 750 nm was measured with a spectrophotometer or the Varioskan® LUX (Thermo Fisher Scientific) microplate reader, using bovine serum albumin (BSA) as a standard (Bio-Rad). For the following steps of purification by immunoprecipitation or VVA lectin, or for the sample separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), an equal amount of protein in
each sample was used. Finally, the purified receptors and other proteins of interest were detected with antibodies or other detection methods.

4.2.1 Preparation of whole cell extracts

In order to extract total cellular proteins for the assays without the need to obtain a more specific cellular fraction, cells were directly lysed with a detergent-containing buffer, followed by separation of the nonsolubilized material by centrifugation (I, II, III). The obtained samples included all the detergent-soluble membrane proteins, as well as cytosolic proteins.

Protocol

1. After washing the cell culture plates with PBS, the cells were harvested in cold PBS by scraping. Cell pellets were collected by centrifuging at 1,500 g at 4°C for 5 min. Pellets were washed once with PBS, frozen in liquid nitrogen and stored at -70°C.

2. Harvested cell pellets were suspended in cell lysis buffer: 25 mM Tris-HCl, pH 7.4, 0.5% DDM (Enzo Lifesciences/Thermo Fisher Scientific), 140 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 μg/ml benzamidine, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM 1,10-phenanthroline and 20 mM NEM (Sigma-Aldrich). Alternatively, the cell lysis buffer was supplemented with 0.5% digitonin (Sigma-Aldrich) (instead of DDM) and 10 mM CaCl₂.

3. Cells were lysed at 4°C in a mixer for 30 min, and the insoluble material was separated by centrifugation at 16,000 g at 4°C for 30 min.

4.2.2 Cellular fractionation and solubilization of cellular membranes

GPCRs are membrane proteins attached to cellular membranes after initial insertion in the ER. They remain bound to the membranes, unless cellular pathways subject them to dislocation from the membrane. This can occur in the ER where targeting of hO1Rs for ERAD leads to their retrotranslocation to the cytosol (Petäjä-Repo et al., 2001). The distinct receptor localization and trafficking can be assessed by obtaining specific cellular fractions. The cellular fractionation was accomplished by homogenization of the cells in a buffer that did not contain detergent, followed by removal of unbroken cells and nuclei with low-speed centrifugation, and finally
by separation of cellular membranes from the cytosolic fraction with high-speed differential centrifugation (I, II, III). The membrane-bound proteins remained in the high-speed pellet, whereas the cytosolic proteins ended up in the supernatant. The cytosolic fraction was used to obtain the retrotranslocated receptors (II).

The extraction of cellular membranes was used not only to analyze proteins in the membrane fraction, but also to obtain a cleaner sample of δOR for analyses without cytosolic proteins. Membrane-bound proteins were solubilized from the membrane fraction by introducing detergent to the buffer, and after mixing the suspension, the detergent insoluble material was removed by ultracentrifugation.

Protocol

1. Cell pellets were homogenized in lysis buffer without DDM and NaCl using the Polytron Ultra-Turrax T-25 homogenizer.

   A) For the cytosolic fraction containing retrotranslocated receptors, cells were broken down with a Dounce glass homogenizer in the lysis buffer without DDM.

2. The homogenates were centrifuged at 1,000 g for 5 min, and the unbroken cells were rehomogenized using the same buffer. Centrifugation was repeated, and the supernatants were pooled.

3. The crude membrane fraction and the cytosolic fraction were obtained by centrifuging at 45,000 g for 20 min (or 10,000g for 30 min), and collecting the pellet (membrane fraction) and the supernatant (cytosolic fraction).

4. The pellet containing the membrane fraction was washed twice using the same buffer and finally solubilized with the solubilization buffer (Lysis buffer without NEM) using a 26-G needle and a syringe and mixing the suspension on a magnetic stirrer at 4°C for 1 h. The insoluble material was separated by centrifugation at 100,000 g for 1 h.

5. The supernatant (from 3.) was centrifuged at 100,000 g for 1 h to obtain a supernatant containing soluble cytosolic proteins. The centrifugation was repeated to clear the fraction and DDM was added to a final concentration of 0.5%.
4.2.3 Immunoprecipitation

Immunoprecipitation was utilized to purify the receptor and other proteins of interest from cell extracts and solubilized membrane and cytosolic fractions (I, II, III). This resulted in samples with higher concentrations of the desired protein and lower amounts of other nonspecific proteins that would lower the signal to noise ratio in the subsequent detection assays. Epitope-tag antibodies immobilized to agarose were utilized to recover the proteins of interest and antibodies against non-tag epitopes were coupled to Protein G Sepharose to purify CNX and CNX-bound receptors (II). After thorough washing, the purified proteins were eluted with an excess of competing epitope tag peptides or with sodium dodecyl sulfate (SDS) that denatured the antibody and released the proteins from the resin. Immunoprecipitations were done in a one-step or a two-step manner. In the two-step method, the eluate was purified a second time with the same or a different antibody.

Protocol

1. The agarose or Protein G Sepharose (GE Healthcare) beads were equilibrated with the lysis buffer (see 4.2.1) containing 0.1% BSA at 4°C for 1 h in a mixer. In the case of nonimmobilized antibodies, the samples were first precleared with Protein G Sepharose, after which the antibodies were bound to Protein G Sepharose by mixing for 1 h at 4°C.

2. Samples were added to the resins in the presence of 0.1% BSA and incubated at 4°C for 2-16 h.

3. The unbound proteins were removed by washing 5 times with the lysis buffer (4 times with a buffer containing 0.5 % DDM and once with buffer containing 0.1 % DDM), and each wash was followed by centrifugation at 1,500 g for 1 min.

4. Bound proteins were eluted with 25 mM Tris, pH 7.4, 1% SDS, with 50 mM sodium phosphate, 1% SDS or with SDS-PAGE sample buffer (Bio-Rad). The agarose and protein G Sepharose beads were first incubated at 22°C for 15 min, then at 95°C for 5 min. Eluates were recovered by centrifugation at 1,500 g for 1 min. Alternatively, the FLAG-tagged receptors were eluted with FLAG peptide (0.2 mg/ml) in lysis buffer containing 0.1% DDM (I, II). The volume of the eluates was reduced using Microcon-30 concentrators (Merck Millipore).
5. In two-step immunoprecipitation, the first elution was performed using 25 mM Tris, pH 7.4, 1% SDS, and the agarose beads were washed with a 10-fold volume of lysis buffer. The wash buffer was combined with the elute to dilute the SDS amount, and the mixture (including 0.1% BSA) was subjected to a second immunoprecipitation step at 4°C for 1 h.

4.2.4 VVA lectin purification

Lectins that bind to distinct glycan structures can be utilized to purify and detect glycoproteins. Proteins containing \( O\)-GalNAc glycans have been successfully enriched from samples by lectin affinity chromatography using immobilized \textit{Vicia villosa} agglutinin (VVA) (Levery et al., 2015; Steentoft et al., 2013). This strategy has been coupled with protein expression in HEK-SC that have been engineered to terminate O-glycosylation after the initial addition of GalNAc. This has been accomplished by the zinc-finger nuclease mediated mutation of the \textit{COSMC} gene (Steentoft et al., 2013). Cosmc is a necessary coenzyme for the core1-synthase that initiates the elongation of \( O\)-GalNAc residues (Ju & Cummings, 2002). Thus, the truncated \( O\)-GalNAc residues of glycoproteins can be targeted by VVA purification after expression in these cells. Using this strategy, the h\( \delta \)OR was expressed and purified with agarose-immobilized VVA from cellular extracts or solubilized membranes. The bound proteins were eluted with an excess of GalNAc that competed with the bound \( O\)-GalNAc-modified proteins. This method was used to assess h\( \delta \)OR O-glycosylation and to identify the glycosylation sites in the receptor N-terminus (III).

Protocol

1. VVA resin (Vector) was equilibrated by washing with 25 mM Tris, pH 7.4 and incubating in the same buffer with 0.1% BSA for 1 h at 4°C.
2. Solubilized membrane fractions from HEK-SCs expressing h\( \delta \)OR were added to the equilibrated resin and incubated overnight in the presence of 0.1% BSA at 4°C in a mixer.
3. Receptors were eluted with the elution buffer (Vector) that contains 200 mM GalNAc. The buffer was supplemented with 0.1% DDM. The elution was repeated 2-3 times.
4. The combined eluates were concentrated using Amicon centrifugal 30-kDa filters (Merck) twice, replacing the buffer with 25 mM Tris, pH 7.4 in between to a final volume of 60 µl.

5. For deglycosylation, a two-step purification was performed using immobilized FLAG M2 antibody for the first step, and immobilized VVA for the second step. The final elution was done using 50 mM sodium phosphate, 1% SDS.

4.3 Protein detection

The detection of receptors and other proteins of interests was accomplished with antibodies against the epitope-tags or against specific antigens in the proteins. Receptors were generally detected using antibodies against the epitope tags due to the lack of commercial antibodies with sufficient specificity and sensitivity. The primary and secondary antibodies used are listed in Table 9. Alternatively, fluorescent epitope tags were sufficient for the detection without the use of antibodies, such as in the case of bioluminescent resonance energy transfer assays.

4.3.1 SDS-PAGE and western blotting

SDS-PAGE and subsequent western blotting offered a versatile method for the biochemical characterization and quantification of h6OR expression, and the expression of other proteins of interest. Proteins in immunoprecipitated samples or direct cell extracts and solubilized membranes were first separated by SDS-PAGE in denaturing and reducing conditions. SDS coats the proteins with a negative charge allowing separation with electric current in the gel. The conditions break disulfide bonds and oligomers that are sensitive to SDS and the reducing agent dithiothreitol, allowing separation by molecular mass. The separation was followed by western blotting, in which proteins are transferred from the gel to a polyvinylidene fluoride (PVDF) membrane, and immunoblotted with specific antibodies to detect the protein of interest (Table 9).

Protocol

1. Samples were diluted with SDS-PAGE sample buffer (Bio-Rad) and reduced by heating at 95°C for 2 min in the presence of 50 mM dithiothreitol.
2. Proteins were separated by SDS-PAGE using the Mini-PROTEAN 3 electrophoresis cell (Bio-Rad) using Tris-Glycine 10% SDS-PAGE gels.
prepared beforehand. The separation was done using 150V for 70-90 min, in Tris-Glycine-SDS running buffer.

3. The Immobilon P (Merck) PVDF membranes were rinsed in 100% ethanol and Tris-Glycine transfer buffer, and the gels were equilibrated in the transfer buffer.

4. A Mini Trans-Blot cell (Bio-Rad) was used at 35-100V for 1-16 h at 4°C for electroblotting.

5. Membranes were rinsed with water and 100% ethanol and dried. Thereafter, they were rinsed again with 100% ethanol and molecular mass markers were stained with the Ponceau S dye solution (Sigma-Aldrich).

6. Nonspecific binding to membranes was blocked with 2.5% skim milk in Tris-buffered saline (TBS) for 1 h at 22°C before probing with appropriate antibodies.

7. Antibody incubations were done at 22°C with gentle agitation for 1 h, washing several times with 0.1% Tween/TBS in between.

8. Proteins were detected with enhanced chemiluminescence and exposed to films (Kodak), or imaged with the LiCOR Odyssey Fc imager.

4.3.2 Autoradiography and fluorography

The \(^{35}\text{S}\)-isotope-labeled proteins obtained from metabolic labeling experiments can be detected with autoradiography and fluorography. After purification and SDS-PAGE, the gels were treated with liquid autoradiography enhancer that produces photons when the radioactive particles from the sample interact with the reagent (Skinner & Griswold, 1983). Detection of these photons by fluorography can be done by drying the gels and exposing them to an X-ray film. Thus, the nascent proteins containing \(^{35}\text{S}\)-cysteines and \(^{35}\text{S}\)-methionines could be quantified efficiently using this method (I, II).

Protocol

1. SDS-PAGE gels containing radiolabeled proteins were stained with Coomassie Brilliant Blue (Bio-Rad) at 22°C for 1 h.
2. Excess dye was removed by incubation in 40% methanol/10% acetic acid at 22°C for 30 min.
3. The gel was incubated in an EN3HANCE (PerkinElmer) solution on a mixer at 22°C for 1 h.
4. Gels were washed and dried between cellophane membranes and exposed to MR autoradiography films (Kodak) at -70°C.
5. The films were developed with the AGFA Curix 60 tabletop film processor (AGFA Healthcare) and scanned with the Umax PowerLook 1120 Color scanner (Amersham Biosciences) for image analysis and quantification.
Table 9. Antibodies.

<table>
<thead>
<tr>
<th>Primary Ab</th>
<th>Type/Clonality</th>
<th>Conjugation/Marker</th>
<th>Manufacturer</th>
<th>Method</th>
<th>Study</th>
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<tr>
<td>FLAG</td>
<td>M2, Mouse mc</td>
<td>HRP-conj.</td>
<td>Sigma-Aldrich</td>
<td>WB</td>
<td>I, II</td>
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<td>I, II, III</td>
</tr>
<tr>
<td>FLAG</td>
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<td></td>
<td>Sigma-Aldrich</td>
<td>WB</td>
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</tr>
<tr>
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<td>WB, IF</td>
<td>I, II</td>
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<tr>
<td>cMyc</td>
<td>9E10, Mouse mc</td>
<td></td>
<td>Santa Cruz</td>
<td>FC</td>
<td>I, II</td>
</tr>
<tr>
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<td>9E10, Mouse mc</td>
<td></td>
<td>Covance</td>
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<tr>
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<td>Biolegend</td>
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<td>III</td>
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<tr>
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<td>I, II</td>
</tr>
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<td>Sigma-Aldrich</td>
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</tr>
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<td>TR</td>
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<td></td>
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<td>WB, FC</td>
<td>I</td>
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<td>PerkinElmer</td>
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<td>Study</td>
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<td>Invitrogen</td>
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<td></td>
<td></td>
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<tr>
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<td>Anti-mouse</td>
<td>HRP-conj. F(ab)2</td>
<td>Bio-Rad</td>
<td>WB</td>
<td>I, II</td>
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<tr>
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<td>HRP-conj. F(ab)2</td>
<td>Bio-Rad</td>
<td>WB</td>
<td>I</td>
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<td>WB</td>
<td>I</td>
</tr>
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<td>Anti-mouse</td>
<td>PE-conj.</td>
<td>BD Biosciences</td>
<td>FC</td>
<td>I, II, III</td>
</tr>
</tbody>
</table>

The secondary antibodies, and some of the primary antibodies, were conjugated with enzymes and molecules to suit the desired endpoint assay. HRP-conjugates were utilized for western blotting, fluorescent Alexa-conjugates for confocal microscopy and phycoerythrin conjugates for flow cytometry. Abbreviations: Ab, Antibody; As, Assay; FC, flow cytometry; HRP, Horse radish peroxidase; IF, Immunofluorescence (confocal microscopy); IP, immunoprecipitation; mc, monoclonal; pc, polyclonal; PE, phycoerythrin; Thermo Fisher Sci, Thermo Fisher Scientific; TR, Transferrin receptor; WB, western blotting.
4.3.3 Confocal microscopy

The expression and subcellular localization of hδOR was investigated using fluorescent antibodies (Table 9) and confocal microscopy (I). Fixing the cells preserved the structure and morphology of the cells and enabled the visual analysis of hδOR trafficking along the secretory and endocytic pathways. By permeabilizing the cell membranes and utilizing antibodies against subcellular markers, the hδOR could be localized in the ER, ERGIC, Golgi, plasma membrane, endosomes and lysosomes.

Protocol

1. The cells expressing hδOR were seeded onto poly-L-lysine coated glass coverslips in 6 and 12-well plates and cultured for 1-3 days.
2. Cells were washed with PBS and fixed with 2 % paraformaldehyde in PBS for 20 min and permeabilized with 0.1 % Triton X-100 in PBS for 45 min.
3. The coverslips were blocked with 0.5% BSA in PBS for 45 min and washed with PBS.
4. The coverslips were stained sequentially with appropriate primary and secondary antibodies (Table 9) for 30 min.
5. Cell nuclei were stained with TO-PRO-3 iodine (Invitrogen) for 10 min, followed by washing with PBS.
6. The coverslips were then mounted on glass slides with Immu-mount (ThermoShandon) and dried in the dark.
7. The confocal microscopic analysis was performed with the Zeiss LSM510 confocal microscope.

4.3.4 Flow cytometry

The analysis of receptor expression levels at the surface of live cells was possible by flow cytometry and fluorescence-assisted cell sorting (FACS). Cells transiently or stably expressing the receptor were incubated with cMyc or HA antibodies directed against the receptor N-terminal epitope tag. Fluorescent secondary antibodies were then used to allow the detection of receptors at the cell surface (I, II, III). Alternatively, a membrane-impermeable opioid ligand conjugated to a fluorescent molecule was used to quantify the ligand binding competent receptors at the cell surface (II, III). Broken and dead cells were omitted from the analysis by specific staining (TO-PRO3). Flow cytometry provided quantitative data on hδOR.
expression at the cell surface and complemented other methods used for similar purposes.

Protocol
1. Cells on culture plates were washed with PBS to remove detached cells. Live cells were then detached in cold PBS, centrifuged at 200 g for 3 min and suspended in 1% FBS/PBS.
2. Cells were stained with the appropriate antibody (Table 9) or the fluorescein isothiocyanate (FITC)-conjugated opioid antagonist naltrexone (NTX) (FITC-NTX, Invitrogen).
3. After antibody staining, cells were washed twice with 1% FBS/PBS and stained with phycoerythrin (PE)-conjugated anti-rabbit secondary antibody (Table 9).
4. Cells were washed again as above and dead cells were stained with 7-amino-actinomycin D (BD Biosciences).
5. Fluorescence was quantified from triplicate samples by measuring 10,000 cells using the FACSCalibur flow cytometer and CellQuest Pro 6.0 software (BD Biosciences).

4.3.5 Bioluminescence resonance energy transfer

The bioluminescence energy transfer (BRET) is a phenomenon that occurs when a bioluminescent donor molecule excites the fluorescent acceptor molecule, leading to light emission from the acceptor (Lohse et al., 2012). This method was utilized by engineering hδOR constructs with donor- and acceptor-tags (I). A close proximity of the tagged receptors led to a detectable BRET signal, making it a convenient tool to assess receptor oligomerization in live cells.

Protocol
1. The BRET donor Renilla luciferase (Rluc) and the BRET acceptor Venus were subcloned into the C-terminal tail of hδOR constructs and appropriate control proteins.
2. Cells transiently transfected with Rluc- and Venus-tagged constructs were washed and detached with PBS containing 5 mM EDTA and resuspended in PBS. The total protein amount was quantified, and cells corresponding to 2 µg protein were distributed on gray 96-well plates (PerkinElmer).
3. The total fluorescence was measured using the FlexStation IV (Molecular Devices) with 485-nm excitation and 538-nm emission.
4. The cells were transferred onto white 96-well plates (Corning) and 5 µM of the Rluc substrate coelenterazine H (Invitrogen) was added.
5. BRET signal was generated upon the close proximity of the acceptor and donor and was defined as the ratio of emission at 510-550 nm (Venus) over 460-500 nm (Rluc) using a Mithras LB 940 microplate reader (Berthold Technologies).
6. The luminescence of the Rluc-tagged construct transfected alone was measured for background signal. The BRET values were plotted as a function of the ratio of total fluorescence over luminescence.

4.3.6 Radioligand binding

The saturation ligand binding assay is a tool to quantify total receptor expression levels, as well as to assess the ligand binding properties of the receptor (I, II, III). The assay was performed on cellular membrane fractions that contain the total membrane-bound receptor pool. Aliquots of the membranes were incubated with increasing amounts of a [3H]-labeled opioid ligand. High concentrations eventually saturate the ligand binding receptors in the sample, making it possible to analyze their amount (pmol/mg of protein). The analysis also provided the affinity of the ligand for the receptor (Kₐ). The radioactivity was measured with a liquid scintillator after harvesting the samples onto filtermats.

Protocol
1. Cellular membranes were isolated as in 4.2.2, using a buffer containing 5 mM MgCl₂.
2. Membrane fractions (2-15 µg) were incubated with increasing concentrations of [15,16-3H]-diprenorphine (PerkinElmer) in the presence of 0.1% BSA.
3. The nonlabeled antagonist NTX (Tocris) was used to determine the non-specific binding. The total binding was determined in the absence of NTX.
4. Triplicates with or without NTX were incubated in deep 96-well plates (PerkinElmer) at 22°C for 1 h.
5. Samples were harvested with 25 mM Tris-HCl, pH 7.4 onto glass fiber Filtermat filters (PerkinElmer) using the Brandel MWR-96T harvester.
6. Filters were dried and the Meltilex B/HS scintillator (PerkinElmer) was melted onto the filters before measuring the radioactivity with the liquid scintillator Wallac MicroBeta TriLux (PerkinElmer).

4.4 Other methods

4.4.1 Enzymatic deglycosylation

Enzymatic deglycosylation was employed to characterize hδOR glycosylation (II, III) and to identify receptor species with distinct states of maturity (I, II, III). Treatments with specific O-glycan and N-glycan glycosidases were coupled with SDS-PAGE to determine a possible shift in mobility. A shift in molecular mass indicates the presence of glycosidase specific glycans in the receptor. High-mannose type N-glycans present in proteins that have not been processed through the Golgi are sensitive to Endo H treatment (Mulloy, Dell, Stanley, & J, 2015). This has been exploited to identify ER-resident hδOR precursors (Petäjä-Repo et al., 2000). Peptide N-glycosidase F (PNGase F) treatment leads to deglycosylation of N-glycans, both the high-mannose and the complex-type glycans. The digestion of O-GalNAc glycans require the removal of terminal sialic acids by neuraminidase, before the remaining O-glycan can be removed by the endo-α-N-acetylgalactosaminidase (O-glycosidase) (Mulloy et al., 2015).

Protocol

1. Cellular membranes or whole cell extracts were purified by immunoprecipitation or VVA purification and eluted in 50 mM sodium phosphate, pH 5.5, 1% SDS.

2. The deglycosylation reaction was set up with the appropriate enzyme, sample and the deglycosylation buffer:

   - Deglycosylation buffer: 50 mM Tris-HCl, pH 5.5 (or pH 7.5 for PNGase F), 140 mM NaCl, 0.5% DDM, 2 mM EDTA, 10 µg/ml benzamidine, 5 µg/ml leupeptin, 5 µg/ml soybean trypsin inhibitor, 2 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM 1,10-phenanthroline.

   - Neuraminidase and/or O-glycosidase, both 50 mU/ml; Roche Applied Science), Endo H (100 mU/ml, Roche Applied Science) or PNGase F (50 mU/ml, Roche Applied Science).
The sample volumes corresponded to one fifth of the reaction volume to dilute the inhibiting SDS used for elution.

3. Deglycosylation was done for 5 or 16 h at 30°C and stopped by cooling and adding SDS-PAGE sample buffer containing 1% SDS.

4. Samples were analyzed for mobility shift by SDS-PAGE and western blotting.

4.4.2 Cyclic AMP accumulation

The hδOR is a GPCR coupled to the G\textsubscript{\alpha}i-mediated inhibition of AC. The hδOR functional output was assessed by a cAMP accumulation assay, in which the forskolin-induced activation of cAMP accumulation was inhibited by a hδOR selective opioid agonist (III). The assay utilized homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) between a europium-labeled cAMP tracer complex and an Alexa Fluor 647-conjugated cAMP antibody. The accumulating cAMP competed with the cAMP tracer complex leading to a decrease in the TR-FRET signal. The assay allowed quantitative determination of hδOR-mediated signaling in live transfected cells.

Protocol

1. Cells transfected with hδOR constructs were detached from culture plates 24 h after transfection and resuspended in PBS/0.1% BSA/1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich).
2. Triplicate aliquots with 1,500–6,000 cells/well were transferred to white OptiPlate-384 plates (PerkinElmer) with the Alexa Fluor 647-labeled cAMP antibody (PerkinElmer), 1:100.
3. The cells were incubated for 60 min at 20°C in the presence or absence of 5 μM forskolin (Sigma-Aldrich) and a saturating concentration of the δOR peptide \([D-Ala\textsubscript{2}, D-Leu\textsubscript{5}]-enkephalin (DADLE, Sigma-Aldrich; 10 \mu M). The total reaction volume in each well was 10μl.
4. The reaction was terminated with the kit’s detection buffer (10 μl; 0.35% Triton X-100, 10 mM CaCl\textsubscript{2}, 50 mM HEPES, pH 7.4), containing LANCE Eu-W8044 labeled streptavidin and biotin cAMP.
5. The signal was measured after 60 min using the Victor3 plate reader (PerkinElmer Life and Analytical Sciences) and the build-in LANCE protocol.
6. The cyclic AMP generated was interpolated from a cyclic AMP standard curve generated in parallel for each experiment.
4.4.3 Southern blotting

Southern blotting was used for the generation of the Flp-In-CHO-lldID cell line (see 4.1.3). After the introduction of a Flp Recombination target (FRT) site to the CHO-lldID cells, the method was used to confirm that only a single integration event had occurred. This was a perquisite for establishing stable hδOR cell lines with a single isogenic locus. The method involved isolation of genomic DNA from the selected cell clones (see 4.1.3), digestion of the DNA samples and subsequent FRT fragment detection by radioactive probe hybridization.

Protocol

1. Genomic DNA was isolated in buffer (1 M Tris-HCl, pH 8.0, 250 mM EDTA, pH 8.0, 4 M NaCl, 20% SDS) containing Proteinase K (20 mg/ml) in a mixer at 55°C for 16 h. The DNA was precipitated with isopropanol/ethanol precipitation at -22°C, dried and resuspended in water.
2. Samples were digested with HindIII at 37°C for 3 h and analyzed on agarose gel.
3. The DNA in the gel was depurinated with 250 mM HCl for 15 min, denatured with 500 mM NaOH/1.5 M NaCl for 20 min, and neutralized with 1 M Tris, pH 7.4/1.5 M NaCl for 20 min, washing the gel in between the incubations.
4. The gel was equilibrated in saline-sodium citrate buffer and the DNA was transferred to a nylon filter (Immobilon-ny+, Millipore) for 16 h using the same buffer for the transfer.
5. The gel was air dried for 30 min and UV-crosslinked to the filter.
6. The probe was prepared using the Nick translation system (Invitrogen) according to the manufacturer’s instructions and purified with nickel column.
7. The nylon filter was placed in a hybridization tube and prehybridized in the Church-hybridization buffer (0.8 M NaPi, pH 7.2; 20% SDS; 0.5 mM EDTA; pH 8.0; 0.5 % BSA; 0.1 mg/ml salmon sperm DNA) at 60°C for 30 min.
8. The Church-buffer containing the radioactive probe was boiled in 100°C water bath for 5 min before hybridization of the nylon filter at 65°C for 18 h.
9. The gel was washed thoroughly with the Church-buffer (without EDTA, BSA or salmon sperm DNA) and sealed in plastic. Finally, the gel was exposed into X-ray film for detection of a single FRT-positive band.
4.5 Glycopeptides and mass spectrometry

The O-glycosylation of hδOR was studied using an *in vitro* glycopeptide assay, followed by mass spectrometric analyses of the products (III). Synthetic peptides corresponding to the N-terminal tail of the hδOR were designed and assayed with GalNAc-Ts to screen for GalNAc-Ts that glycosylate the receptor, to analyze the kinetics of glycosylation, and to identify the glycosylation sites. Mass spectrometry (MS) is a powerful tool used in glycoproteomics to analyze glycosylation of peptides and proteins. The addition of glycans results in a specific change in mass that can be detected and analyzed. While not necessarily depicting perfectly the *in vivo* glycosylation of hδOR, this *in vitro* approach gave valuable information on the responsible GalNAc-T isoforms involved, and an accurate prediction of possible glycosylation sites. The synthetic peptides used are listed in Table 10.

**Table 10. Receptor N-terminal tail and model peptides.**

<table>
<thead>
<tr>
<th>Region / peptide</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hδOR N-terminal tail</td>
<td>MEPAPSAGAELQPLFANASDAYPSAXSAGANA$GPPGAR</td>
</tr>
<tr>
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</tr>
<tr>
<td>C27</td>
<td>ANASDAYPSACPSAGANA$GPPGAR</td>
</tr>
</tbody>
</table>

X= F/C (polymorphic site), Predicted O-glycosylation sites (bold font), confirmed N-glycosylation sequon (underlined).

4.5.1 GalNAc-T screening

The synthetic peptides were used for screening of the potential GalNAc-T isoforms responsible for hδOR O-glycosylation. Recombinant GalNAc-Ts were produced as soluble secreted truncated proteins in insect cells (Wandall et al., 1997). The peptides were analyzed using time-course product development assays by setting up reactions with the UDP-GalNAc substrate and a panel of recombinant GalNAc-T isoforms. The glycosylation of peptides was monitored at different time points by matrix assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectrometry. The assay allowed the semi-quantitative evaluation of the rate of reactions as well as the number of GalNAc residues incorporated.
Protocol

1. Peptides (10 µg) were incubated with 1.5 mM UDP-GalNAc and a panel of recombinant polypeptide GalNAc-Ts (0.5 – 5 µg) in 25 mM cacodylic acid, pH 7.4, MnCl₂ at 37°C for 5 min – 16 h.
2. Aliquots from the reactions were diluted into 0.1% trifluoroacetic acid/H₂O (1:10), and 1 µl of the dilutions were mixed with 1 µl 10 mg/ml 2,5-dihydrobenzoic acid dissolved in acetonitrile/H₂O (7:3).
3. The mixtures were spotted onto MS plates and analyzed using MALDI-TOF MS for peptide mass change corresponding to GalNAc addition.
4. The MS spectra were obtained using a Voyager-DE™ (Applied Biosystems) MALDI-TOF device operating at an accelerating voltage of 20 kV in the linear mode and with the Autoflex (Bruker) using the Flex Control 3.4 software (Bruker). The spectra were recorded in the positive ion mode and the raw spectra were processed using the Voyager Data Explorer 5.1 and FlexAnalysis 3.4 software (Bruker).
5. For further analysis (see 4.5.2), the glycopeptides were produced in reactions with a 100–200-µg scale and purified by HPLC using a Kinetex™ 2.6-µm C18 100-Å LC column (Phenomenex).

4.5.2 Characterization of O-glycosylation sites

The hδOR glycopeptides produced in the in vitro assay were characterized for glycosylation sites. Tandem mass spectrometry (MS/MS) and peptide fragmentation were employed to identify the serines glycosylated in the presence of each GalNAc-T. This was done by the electrospray ionization-linear ion trap-Fourier transform mass spectrometry (ESI-LIT-FT-MS) in an LTQ-Orbitrap XL hybrid spectrometer (Thermo Fisher Scientific). The peptide sequence analysis was carried out by MS/MS with equipped electron transfer dissociation and retention of glycan site-specific fragments. The tandem MS analysis of the glycopeptides was carried out by Dr. Sergey Vakhrushev and Dr. Adnan Halim at the Copenhagen Center for Glycomics.

Protocol

1. Samples were dissolved in methanol/water (1:1) containing 1% formic acid and introduced by direct infusion via a TriVersa NanoMate ESI-Chip interface (Advion BioSystems) at a flow rate of 100 nl/min and 1.4-kV spray voltage.
2. Mass spectra were acquired in the positive ion FT mode using parameters similar to a previous study (Steentoft et al., 2011), except at a nominal resolving power of 30,000 or 60,000.

3. Electron transfer dissociation-MS2 spectra were analyzed by comparison with theoretical c and z• fragment m/z values calculated for all positional combinations of one HexNAc residue distributed at all potential glycosylation sites in the sequence. Calculations were performed using the web-based Protein Prospector MS-Product software routine.

4. Samples were analyzed on a setup composed of an EASY-nanoLC 1000 (Thermo Fisher Scientific) connected via a nanoSpray Flex ion source to an LTQ-OrbitrapVelos Pro hybrid mass spectrometer.

5. Glycopeptides were dissolved in 0.1% formic acid and separated on an in-house packed reverse phase column (1.9 µm Reprosil-Pure-AQ C18 particles). The MS1 precursor ion scan was performed in the orbitrap using a nominal resolution of 30,000 followed by two MS2 scan events (15,000 resolving power at m/z 400) utilizing higher-energy collisional dissociation (HCD) and electron-transfer dissociation (ETD) fragmentation modes.

6. Glycopeptide identification and glycosite assignments were accomplished by the Proteome Discoverer 1.4 with final validation through manual inspection of the assigned peaks.

4.6 **In silico analyses and bioinformatics**

The hδOR amino acid sequence was analyzed with online prediction algorithms and alignment tools to assess the conservation and presence of putative O-glycosylation sites (III). The probability of O-glycan modifications in the hδOR extracellular N-terminus of the hδOR was predicted using the online algorithm of the NetOGlyc 4.0 server that produces neural network predictions of mucin-type GalNAc O-glycosylation sites in mammalian proteins (Steentoft et al., 2013). The hδOR sequence was submitted to the server and N-terminal positive predictions were collected. The N-terminal sequence homology analysis of mammalian δORs was done by submitting the hδOR sequence to the Basic Local Alignment Search Tool (BLAST) (III). Several of the closest matches were aligned in the analysis.
4.7 Quantification and data analysis

Fluorograms and western blots were analyzed by densitometric scanning with the Umax PowerLook 1120 color scanner using the Magic Scan software (I, II, III). Alternatively, the chemiluminescence of western blots was subjected to digital imaging by the LiCOR Odyssey Fc imager (III). Relative intensities of the receptor bands were quantified using the Image J software (I, II, III). Uncalibrated optical densities were quantified, and local background was subtracted. Data were analyzed with the GraphPad Prism software v.4.0-v.7.02 (I, II, III). The ligand binding data were analyzed using the one-site binding (hyperbola) model. Receptor maturation data and the receptor precursor turnover data (II) were subjected to the non-linear regression analysis using sigmoidal dose response and one-phase decay models, respectively. Statistical analyses were performed using paired, unpaired or one-sample t-tests for comparisons between two groups or the one- or two-way analysis of variance followed by Tukey’s or Bonferroni’s post hoc analysis for multiple comparisons. The limit of significance was set to $p < 0.05$. The data are presented as mean with standard error of mean (SEM).
5 Results

The processing steps during hδOR synthesis and maturation that govern the expression level, trafficking and the behavior of functional receptors at the cell surface were studied in a variety of mammalian cell models utilizing an array of cell biological and biochemical methods. The focus of the thesis is centered around hypotheses, in which receptor glycosylation and oligomerization play an important role in regulating the delivery and expression of functional receptors at the cell surface. The hδOR was chosen as a model for the apparent importance of its N-terminal tail in receptor processing, and for the clinical importance and potential of the receptor itself. The N-terminal tail has been shown to be glycosylated and it contains a SNP that alters receptor behavior at the cellular level. The study builds upon earlier characterization of hδOR processing and accumulates new evidence for molecular determinants and processes that govern receptor function and behavior.

5.1 Expression and processing of hδOR variants in mammalian cells (I)

The first studies of hδOR cellular processing in the past were done using HEK293 cells constitutively expressing the less common hδOR\(^{\text{Cys27}}\) variant. Petäjä-Repo et al. (Petäjä-Repo et al., 2001; Petäjä-Repo et al., 2000) concluded that the rate limiting step in the processing of hδOR\(^{\text{Cys27}}\) occurs in the ER where it is inefficiently processed, resulting in the retention of a substantial portion of immature receptors, and their subsequent degradation by ERAD. The processing steps in the ER were shown to lead to the poor maturation efficiency of hδOR\(^{\text{Cys27}}\) that is in concordance with the observations that place the receptors primarily in intracellular compartments. Further studies have included the common hδOR\(^{\text{Phe27}}\) variant and have demonstrated that it is processed more efficiently in the ER than the less common hδOR\(^{\text{Cys27}}\), leading to a relatively higher portion of receptors expressed at the plasma membrane (Leskelä et al., 2009). Thus, the minute amino acid change in the N-terminal tail of the receptor produces two distinct phenotypes of hδOR, warranting further investigation of how the N-terminal tail of hδOR is processed in the early secretory pathway and what is its significance for receptor expression. In Caucasians, about 25% of the population carry the hδOR\(^{\text{Cys27}}\) allele, leading to a large number of heterozygous individuals (Auton et al., 2015; Sarajärvi et al., 2015).
5.1.1 Dominant-negative effect of hδOR\textsuperscript{Cys27} in co-expressed cells

The distinct processing of the hδOR variants in the ER and the specific characteristics of the hδOR\textsuperscript{Cys27} variant raise a question of the possible outcome in cells co-expressing the variants. Oligomerization within the GPCR protein family has been demonstrated to take place already in the early biosynthetic pathway (Hasbi et al., 2007; Issafras et al., 2002; Terrillon et al., 2003), and may present an unforeseen regulatory role in the expression of receptor heteromers. In order to conveniently study receptor co-expression, the tetracycline-inducible expression system was utilized. Cell lines generated with the Flp-In T-Rex system express a tetracycline repressor that prevents the expression of the gene of interest unless tetracycline is introduced to the culture medium (Hillen & Berens, 1994; Sauer, 1994). A previously established HEK293 cell line expressing the hδOR\textsuperscript{Cys27} in a tetracycline-inducible manner (Leskelä et al., 2009) was further stably transfected with hδOR\textsuperscript{Phe27}. The hδOR\textsuperscript{Cys27} contained an N-terminal myc-tag and a C-terminal FLAG-tag, whereas the hδOR\textsuperscript{Phe27} contained an N-terminal HA-tag. The strategy of distinct epitope tags allowed specific purification and detection of the variants expressed in the same cells.

The analysis of the steady-state variant expression pattern by western blotting revealed that the induction of hδOR\textsuperscript{Cys27} expression led to a decrease in the expression of the mature hδOR\textsuperscript{Phe27} at the cell surface (I, Fig. 1A-D). The major 55-kDa species seen in western blots is the mature receptor form containing two fully processed N-glycans that were insensitive to Endo H and sensitive to PNGase F (I, Fig. 1E). Concomitantly, the Endo H-sensitive receptor precursor form (43 kDa) showed accumulation upon hδOR\textsuperscript{Cys27} induction (I, Fig. 1A-E). Thus, hδOR\textsuperscript{Cys27} that has a poor maturation efficiency elicits a dominant negative effect on hδOR\textsuperscript{Phe27}, impairing its maturation and cell surface delivery. The effect was confirmed by flow cytometric analysis of cell surface receptors in co-transfected live cells (I, Fig. 1F). In this assay, only cell surface receptors are labeled with an antibody. Similar to the western blot, the induction of hδOR\textsuperscript{Cys27} expression led to a decreased labeling of hδOR\textsuperscript{Phe27} at the cell surface. Importantly, the dominant negative effect of hδOR\textsuperscript{Cys27} was dose-dependent (I, Fig. 1A-D, F) and receptor specific, as no effect was elicited with the co-transfected TR or with another GPCR, the LHR (I, Fig. 2C, E, and 4C-D). The effect could also be seen using transiently co-transfected receptor variants, using different ratios of hδOR\textsuperscript{Cys27}:hδOR\textsuperscript{Phe27} DNA for transfection. The dominant negative attenuation of hδOR\textsuperscript{Phe27} maturation in transiently co-transfected cells was confirmed by western blotting and flow
cytometric analysis (Fig. 10 and 1, Fig. 4A-B and S1). The same effect was observed in HEK293 cells and SH-SY5Y neuroblastoma cells, indicating that the effect is not specific to the cell model either.

The observed dominant negative effect of the $\delta$OR$^{\text{Cys27}}$ variant on the $\delta$OR$^{\text{Phe27}}$ variant was studied in more detail with metabolic pulse-chase labeling that followed the *de novo* synthesis of $\delta$OR$^{\text{Phe27}}$ in the co-transfected cells (1, Fig. 2A-B and D). The $\delta$OR$^{\text{Phe27}}$ expressed alone displayed rapid and efficient maturation, leading to a fully mature receptor form within four hours. The induction of $\delta$OR$^{\text{Cys27}}$ co-expression led to a dose dependent attenuation of the $\delta$OR$^{\text{Phe27}}$ maturation efficiency, indicated by a decreased mature/precursor receptor ratio (1, Fig. 2B). The concurrent accumulation of receptor precursors suggested a more robust ER retention and subsequent targeting to ERAD. This was confirmed by inhibiting proteasomal degradation with lactacystin treatment (1, Fig. 3). The inhibition led to a further accumulation of receptor precursors and accumulation of polyubiquitinated receptor forms, indicating that the retained $\delta$OR$^{\text{Phe27}}$ precursor is targeted for degradation via the proteasome-ubiquitin pathway of ERAD. Furthermore, supplementation of the chase medium with NTX increased the maturation efficiency of $\delta$OR$^{\text{Phe27}}$ during $\delta$OR$^{\text{Cys27}}$ induction, indicating that the prematurely degraded $\delta$OR$^{\text{Phe27}}$ can be rescued using pharmacological chaperones.

The subcellular localization of $\delta$OR variants in co-transfected cells was examined by confocal microscopy. Subcellular markers were used to determine $\delta$OR variant localization. The $\delta$OR$^{\text{Phe27}}$ was found predominantly at the plasma membrane when expressed alone. The co-transfection led to the intracellular accumulation of the receptor variants in compartments with positive staining for ER and ERGIC markers (Fig. 10C and 1, Fig. 5 and S1-2). This is in concordance with previous results and suggests that the co-expression leads to an impairment of $\delta$OR$^{\text{Phe27}}$ cell surface delivery. In summary, $\delta$OR$^{\text{Cys27}}$ attenuates the cell surface delivery of the $\delta$OR$^{\text{Phe27}}$ variant and targets it for degradation by ERAD in cells co-expressing the variants.
Fig. 10. Dominant-negative effect of hδOR\textsuperscript{Cys27}. SH-SY5Y cells were co-transfected with different ratios of HA-hδOR\textsuperscript{Phe27} and myc-hδOR\textsuperscript{Cys27}-FLAG (1:2 in C). After 24 h, whole cell extracts were prepared and subjected to immunoprecipitation (IP), SDS-PAGE and western blotting (WB) (A). Alternatively, the cells were analyzed by flow cytometry (B) or confocal microscopy (C). In panel B, the GeoMean values obtained using HA or cMyc antibodies followed by PE-conjugated secondary antibody were normalized to those obtained with the same antibodies from single-transfected cells. The data were analyzed by Bonferroni’s Multiple Comparison’s test after repeated measures one-way analysis of variance before normalization. *, p < 0.05; **, p < 0.001; ***, p < 0.001. C, Fixed and permeabilized cells were stained with the indicated antibodies followed by Alexa Fluor 488/568 secondary antibodies. The Nuclei were stained with TOP-RO3 iodide. RGB profile blot analysis is performed from the red line. Modified from I (panel A and B: Fig. 4A-B, panel C: Fig. 5).
5.1.2 Heteromerization of hδOR variants

The hδOR has been demonstrated to form functional homomers and heteromers with other GPCRs (Bushlin et al., 2012; Cvejic & Devi, 1997; George et al., 2000; Jordan & Devi, 1999; Pello et al., 2008). The heteromerization of the receptor variants in the biosynthetic pathway is a likely explanation for the observed dominant-negative effect in co-expressed cells. To test this hypothesis, metabolic pulse-chase labeling experiments were performed using the HEK293 cells inducible for co-expression. A two-step co-immunoprecipitation assay using epitope tag-specific antibodies was used to assess how the receptors interact with each other during synthesis and maturation (I, Fig. 6A-B). From samples collected after a 40-min labeling pulse, both variant precursors were successfully purified using the two-step HA-FLAG and FLAG-HA purification schemes, indicating that the receptor precursors interact already in the ER. Six hours after the pulse, both variants had matured to the fully processed 55-kDa form. This species, too, was successfully co-immunoprecipitated, as above, suggesting that the receptors exist as oligomers also at the cell surface. To complement the findings of the co-immunoprecipitation assay, a BRET assay was utilized. The hδOR constructs were tagged with Rluc or Venus that can operate as donors and acceptors, respectively, to create a specific BRET signal if they come into close proximity. The co-transfection of Rluc and Venus tagged h δOR variants yielded specific BRET signal, indicating formation of heteromers in live cells. The BRET signal was obtained using either variant, as a BRET donor/acceptor. The signal was a specific for hδOR variants as co-transfection of the Venus-tagged GABA(A)R2 did not yield a positive signal with hδorcys27-Rluc.

The results imply that the hδOR variants interact at an early step during biosynthesis via receptor heteromerization. This predisposes a portion of hδORphe27 to the fate of hδorcys27, for retention in the ER and targeting to ERAD, in a dominant-negative manner. Furthermore, the receptor variants exported from the ER and delivered to the cell surface form oligomers also at the plasma membrane.

5.2 N-glycosylation of hδOR variants (II)

The hδOR processing in the ER is the rate-limiting step during maturation of the receptor (Petäjä-Repo et al., 2000). As this step in biosynthesis leads to distinct phenotypes and behavior of the hδOR variants distinguished by a single amino acid change in the N-terminal tail, the processing of this receptor domain during early
receptor biogenesis is apparently important. One of the first steps in the processing is the modification of specific asparagine residues with N-glycans. The N-glycosylation guides the folding and quality control of glycoproteins, dictating the outcome of functional protein expression (Lamriben et al., 2016).

The hδOR\textsuperscript{Cys27} N-terminal tail was previously found to carry two N-glycans (Petäjä-Repo 2000, Markkanen & Petäjä-Repo 2009) that have an important role in the expression of functional hδOR\textsuperscript{Cys27} (Markkanen & Petäjä-Repo 2009). Interestingly, the comparison of the hδOR\textsuperscript{Phe27} and hδOR\textsuperscript{Cys27} variants revealed that the N-glycosylation of hδOR\textsuperscript{Cys27} is inefficient, as it is often seen as a species with only a single N-glycan (Leskelä et al., 2009). This suggests that the SNP may have an impact on receptor N-glycosylation and N-glycan mediated processes. The role of N-glycosylation in the ER processing of hδOR variants was studied using stable HEK293i cell lines that express epitope-tagged hδOR variants in a tetracycline-inducible manner, and a HEK293 cell line that expresses hδOR\textsuperscript{Cys27} constitutively (II).

5.2.1 Compromised cell surface expression of hδOR lacking N-glycans

To study hδOR N-glycosylation, the two sites in the N-terminus (Asn18 and Asn33) were disrupted by site-directed mutagenesis. The initial characterization of the WT and N18;33E mutant receptors in HEK293 cells demonstrated that the non-N-glycosylated receptors migrate as two closely migrating bands of 42-45 kDa on SDS-PAGE (II, Fig. 1B). Cell surface biotinylation and enzymatic deglycosylation confirmed that the two species are the receptor precursor and the mature O-glycosylated hδOR that reaches the cell surface (II, Fig. 1C and E). Thus, N-glycans are not required for the anterograde trafficking of the receptor. Flow cytometric analysis of cells expressing the hδOR-N18;33E mutant confirmed this finding and revealed that the expression levels of the non-N-glycosylated receptor variants were significantly lower compared to the levels of the corresponding WT variants. Furthermore, both cell surface biotinylation and flow cytometric analyses showed a significantly higher expression for hδOR\textsuperscript{Phe27}, in the presence and absence of N-glycans (II, Fig. 1D, F). This implies that the differences between the variants are intrinsic to the amino acid change, rather than N-glycosylation.

Levels of receptors at the plasma membrane are modulated by ER retention/export and downregulation from the cell surface. The decrease in hδOR expression levels at the plasma membrane in the absence of N-glycans may occur
due to either one of these scenarios. The cell surface biotinylation of cells expressing hδOR-N18;33E mutants showed that both variants are constitutively downregulated (II, Fig. 2B). Interestingly, hδOR\(^{Cys27}\) downregulated more rapidly. The quantification of ligand-binding competent native hδOR levels at the plasma membrane was performed to assess the conformational integrity of the rapidly downregulating receptor variants. The FITC-conjugated opioid ligand NTX was utilized and the fluorescence signal obtained by FACS was compared to that obtained using the cMyc antibody. The normalized FITC-NTX labeling was similar for the WT hδOR\(^{Phe27}\), for the corresponding non-N-glycosylated mutant, and for the WT hδOR\(^{Cys27}\), suggesting that all these receptors at the cell surface are in a native conformation. In contrast, for hδOR\(^{Cys27-N18;33E}\) the normalized FITC-NTX labeling was decreased compared to that obtained using the cMyc antibody, suggesting that some of the receptors are in a conformation that is incapable of ligand binding in the absence of receptor N-glycosylation (Fig. 11B and II, Fig. 2A). Importantly, no differences in ligand binding affinity were seen between the WT and non-N-glycosylated receptor variants in a saturation binding assay with radiolabeled diprenorphine. The results imply that a population of receptors is in a conformation incapable of ligand binding rather than having an overall decrease in ligand binding affinity.

These results suggest that N-glycans stabilize the receptor at the cell surface, decreasing the rate of constitutive downregulation that leads to lower levels of expression in the absence of N-glycans. The N-glycans also appear to play a role during processing of the hδOR in the ER, as is evidenced by the appearance of ligand-binding incompetent receptors at the cell surface.

### 5.2.2 N-glycan mediated ER processing of hδOR

The emergence of receptors incapable of ligand binding and receptors with decreased stability at the cell surface in the absence of N-glycans suggest that the quality control in the ER is compromised, leading to export of non-native hδORs. Quality control mechanisms that monitor the folding status of glycoproteins during protein synthesis are imperative to the expression of functionally active proteins (Lamriben et al., 2016; Tannous et al., 2015). Already during folding, the immature hδORs are scrutinized by the ERQC machinery. The process is suggested to function in a cyclic manner that permits several folding attempts before targeting the protein for export or degradation. N-glycans are a central part of the quality control for glycoproteins, whereas the molecular determinants for the quality
control of non-glycosylated proteins are unclear (Lamriben et al., 2016; Pisoni & Molinari, 2016).

In order to assess the role of N-glycans in the maturation and ER processing of hδOR, the turnover of newly-synthesized WT and mutant N18;33E variants were analyzed by metabolic pulse-chase labeling (II, Fig. 3A-F). The WT hδOR matured relatively slowly, only reaching completion after two hours. The difference in maturation efficiency between the variants was apparent as hδOR\textsuperscript{Cys27} displayed substantial retention of the precursor. This is in concordance with previous observations (Leskelä et al., 2009; Petäjä-Repo et al., 2001). Protein-protein interactions in the rate-limiting compartment are likely to delay the ER export and target receptor folding intermediates for degradation after prolonged futile interactions with the ERQC machinery. The hδOR\textsuperscript{Cys27} thus appears to be more prone to failure during folding attempts and is subject to more robust retention by the ERQC. Co-immunoprecipitation experiments revealed that both hδOR variant precursors interact with the molecular chaperone CNX (II, Fig. 4A). As expected, the interaction with the lectin-chaperone is mediated by N-glycans as the non-N-glycosylated receptor variants did not co-precipitate. Further investigation by metabolic pulse-chase labeling demonstrated that CNX interaction occurs during receptor translocation or immediately thereafter, as CNX co-precipitated with the receptor precursor already after the short labeling pulse (II, Fig. 4B-E). During the chase, the CNX-bound receptor precursors disappeared as the mature receptor species appeared, indicating release from CNX and subsequent export from the ER. However, there was a striking difference between the variants. The half-life of the CNX-bound hδOR\textsuperscript{Cys27} was significantly longer than that of hδOR\textsuperscript{Phe27} (II, Fig. 4B-E), implying slower dissociation from CNX. This suggests that CNX-mediated interactions have a role in the retention of hδOR\textsuperscript{Cys27}.

The CNX interaction during the N-glycan mediated ER processing of hδOR was further assessed by modulating the interaction with a glucosidase inhibitor CST. CST inhibits N-glycan trimming, and depending on the time when cells are treated, it will either inhibit or stabilize the CNX interaction. The introduction of CST to the chase medium stabilizes the interaction of substrate proteins with CNX by inhibiting the removal of the final glucose from the substrate N-glycans (see 2.2.2) (Hammond et al., 1994; Hebert, Foellmer, & Helenius, 1995). Indeed, this slowed down the turnover of CNX-bound precursors (II, Fig. 5A-B, E-F), supporting the conclusion of the CNX-mediated retention of hδOR\textsuperscript{Cys27}. Thus, the increased retention was likely to lead to decreased levels of receptors being exported out of the ER. Interestingly, the co-translational addition of CST that
inhibits the required trimming of the first glucoses of substrate glycoproteins and the subsequent CNX interaction had a similar effect in reducing the maturation efficiency (II, Fig. 5A-D). This suggests that hδOR\textsuperscript{Cys27} is targeted for degradation when it fails to interact with CNX, or alternatively, that other lectins bind and retain the di- and tri-glucosylated hδOR. The fate of the retained hδOR\textsuperscript{Cys27} during the inhibition of CNX interaction was revealed by inhibiting the proteasome with lactacystin (II, Fig. 5J). The accumulation of receptor precursors indicate that the receptor is targeted for degradation by ERAD. These results demonstrate that CNX is a central factor in mediating the N-glycan mediated ERQC of hδOR.

5.2.3 N-glycan independent quality control of hδOR

It is poorly understood how proteins with no N-glycans fold and are processed in the ER. Metabolic pulse-chase labeling experiments with stable HEK293 cells expressing the hδOR-N18;33E mutants were performed to further assess the role of N-glycans in hδOR retention and export, and to investigate possible distinct outcomes between the variants. Despite the fact that CNX was demonstrated to be responsible for WT hδOR\textsuperscript{Cys27} retention, in the absence of N-glycans and CNX interaction, the non-N-glycosylated hδOR variants showed patterns of maturation efficiency similar to those of the WT counterparts (Fig. 11A and II, Fig. 3D-F). The hδOR\textsuperscript{Phe27-N18;33E} matured efficiently, whereas a portion of the hδOR\textsuperscript{Cys27} variant did not reach maturity. This suggests the presence of a machinery that is able to process and retain hδOR\textsuperscript{Cys27} also independently of N-glycans. The pulse-chase labeling experiments also showed enhanced kinetics of receptor maturation for both mutants compared with the corresponding WT receptors. The maximal levels of mature hδOR-N18;33E were achieved after one hour (Fig. 11A and II, Fig 3D-F), indicating more rapid processing in the ER. Evidently, the delay caused by lectin-mediated interactions was averted, leading to a more rapid export from the compartment. Yet, some of the hδOR\textsuperscript{Cys27-N18;33E} mutants were retained in the ER by an unknown mechanism. The exported receptors reached the cell surface and displayed increased turnover in a similar manner as was observed in the biotinylation chase assay (II, Fig. 2B). It is not clear whether this is due to an intrinsic effect of the N-glycans at the cell surface or to the lack of N-glycan-mediated processing in the ER. The fast kinetics of ER export of the non-N glycosylated receptors suggest less stringent quality control, which explains the less stable and partially nonnative hδOR\textsuperscript{Cys27-N18;33E} at the cell surface. Instead
of the time-consuming lectin cycle, there appears to be a back-up quality control system that utilizes different ER proteins and allows fewer folding attempts.

Fig. 11. N-glycan-independent quality control of hδOR. The myc-hδOR-FLAG WT and N18;33E mutant variants were expressed in stably transfected HEK293, cells that were subjected to metabolic pulse-chase labeling (A) or flow cytometry (B). A, After pulse-labeling with [35S]methionine/cysteine and subsequent chase for the indicated periods of time, receptors in whole cell extracts were purified by two-step immunoprecipitation with FLAG antibody and analyzed by SDS-PAGE and fluorography. Fluorograms were quantified and the values were normalized to the corresponding precursors at the end of the pulse. B, Cells were labeled with cMyc antibody and PE-conjugated secondary antibody to quantify the total receptor pool (black bars), or with FITC-conjugated NTX to detect the ligand-binding capable receptor pool (light gray bars) at the cell surface. The GeoMean values are normalized to those obtained for the hδOR\(^{Cys27}\) WT. Two-way analysis of variance followed by Bonferroni’s post hoc tests were used for statistical comparisons. *, \( p < 0.05 \); **, \( p < 0.001 \); ***, \( p < 0.001 \). Modified from II (panel A: Fig. 3D-F, panel B: Fig. 2A).

The fate of the hδOR\(^{Cys27}\)-N18;33E mutant that was retained independently of N-glycans was investigated by metabolic pulse-chase labeling. The newly-synthesized hδOR accumulated in the presence of lactacystin, suggesting that some
receptors are targeted for degradation (II, Fig. 6A). Experiments with additional transfection of HA-tagged ubiquitin constructs were performed to confirm the degradation via the ubiquitin-proteasome pathway. A two-step immunoprecipitation with immobilized HA- and FLAG-tag antibodies was utilized to identify high molecular mass receptor forms representing polyubiquitinated receptors (Petäjä-Repo et al., 2001). These species accumulated in lactacystin treated cells (II, Fig. 6B), indicating that the degradation of the non-N-glycosylated hδOR involves utilization of the ubiquitin-proteasome pathway. Cellular fractionation by differential centrifugation was used to follow and identify the receptor species that were targeted for this pathway (II, Fig. 7A-D). The retrotranslocated cytosolic and soluble hδOR was identified for both WT and N18;33E receptor constructs. Altogether, these results imply that while the non-N-glycosylated receptor is retained by a distinct mechanism and different molecular determinants, it is similarly retrotranslocated and degraded via the ubiquitin-proteasome pathway.

5.2.4 Pharmacological chaperones assist hδOR to pass ERQC

The inefficient maturation and complex cellular trafficking of δOR presents a challenge in the clinical potential of hδOR as a drug target. The use of opioid ligands as pharmacological chaperones presents an attractive approach to enhance functional opioid receptor levels at the cell surface, and to alleviate problems in cell surface delivery related to mutations, problematic SNPs and physiological conditions. Opioid ligands that permeate cellular membranes reach the immature folding intermediates in the ER and stabilize the native conformation, thus aiding them to pass the ERQC pathways (Leskelä et al., 2007; Petäjä-Repo et al., 2002). Opioid antagonists NTX and NTB were introduced to the cells in an attempt to see whether pharmacological chaperones could be used to assist hδOR to pass N-glycan dependent and -independent QC pathways. NTX was able to enhance the export of hδORCys27, evidenced by the increased mature receptor/precursor ratio in metabolic pulse-chase labeling experiments, whether the receptor-CNX interaction was inhibited with CST or not (II, Fig. 5K). The export was also enhanced when the interaction was stabilized by adding CST to the culture medium post-translationally during the chase. This suggests that the pharmacological chaperone action of NTX is able to rescue hδOR independently of CNX interaction and rescue hδOR trapped in the CNX cycle. Importantly, the results also suggest that a substantial portion of hδORs retained in the ER are not terminally misfolded.
Rather, receptors with intermediate folding states failing to attain the native fold are able to leave the ER upon binding NTX. In a similar fashion, NTB enhanced the maturation of hδORCys27 in N-glycosylated and non-N-glycosylated conditions (II, Fig. 7A-D), by inhibiting the retrotranslocation and subsequent degradation by ERAD. This is in line with the hypothesis and observation that the rescued hδORs reach the native state and are exported from the ER, instead of being targeted for degradation. Thus, pharmacological chaperones present a valid strategy for enhancing receptor expression at the plasma membrane in conditions that utilize either N-glycan dependent or independent QC pathways.

5.3 O-GalNAc glycosylation of hδOR variants (III)

After leaving the ER, hδORs proceed to the Golgi, where the N-glycans are processed to their mature form, and the receptor N-terminus is further modified by O-linked glycans. The O-glycosylation of hδOR was first suggested by the finding that two receptor species were expressed in cells treated with tunicamycin (Petäjä-Repo et al., 2000), which is used to prevent protein N-glycosylation (Mahoney & Duksin, 1980). A small difference in the molecular mass of the non-glycosylated receptor precursors and mature receptors suggested further modification of the mature receptor. Enzymatic deglycosylation of the hδOR confirmed the presence of O-GalNAc glycans in the receptor expressed in HEK293 cells (Petäjä-Repo et al., 2000). Further investigation has been hindered by methodological challenges related to this modification and have left the functional role of receptor O-glycosylation unclear. O-glycans appear in many conserved sites in glycoproteins and may have a role in receptor trafficking and behavior at the plasma membrane and may even contribute to the distinct behavior of the variants at the cell surface. The Phe27Cys SNP itself may alter receptor O-glycosylation, as the modification lacks a well-defined consensus sequence, and the sites modified appear largely to be modulated by amino acid preferences in the vicinity of the glycosylation site. Certain aspects of hδOR behavior and processing can be attributed to N-glycans but the presence of several serines in the receptor N-terminus suggests a role for O-glycans as well. The role of O-glycosylation in hδOR processing and behavior was studied with very recently developed O-glycoproteomic tools and O-glycosylation deficient cell lines, which have made the study of this modification possible (III).
The O-GalNAc glycosylation involves typically serine and threonine residues in extracellular protein domains (Bennett et al., 2012; Brockhausen & Stanley, 2015). Intracellular domains and portions within the membrane are inaccessible to glycosyltransferases. Online algorithms producing neural network predictions for O-GalNAc glycosylation in proteins have been established for initial screening for putative O-glycosylation sites. The Uniprot amino acid sequence of hδOR (P41143) and the amino acid sequence of hδORPh27 (rs1042114) were submitted to the NetOGlyc 4.0 server that has a high general success rate in predicting (over 84%) known glycosylation sites (Steentoft et al., 2013). The algorithm provides numeric values from 0 to 1 for the probability of O-glycosylation. A threshold of 0.5 is given as a limit of positive prediction. Five putative sites were identified by the algorithm in the N-terminus of the two hδOR sequences (III, Fig. 1A). Ser6 (0.9) and Ser29 (0.9-0.93) obtained the highest values of prediction. This is in line with the observations that proline residues are commonly seen in close proximity of O-glycosylation sites (Gerken et al., 2011). Ser25 was also above the threshold (0.55-0.65). Ser20 (0.52-0.53) and Ser35 (0.84-0.85) were predicted to be O-glycosylation sites, but these sites are within the N-glycosylation sequons and possibly inaccessible in cells, in which Asn18 and Asn33 are glycosylated. However, a large degree of heterogeneity exists in N-glycosylation, leading not only to distinct glycoforms in glycoproteins but also sites that are only partially used. A small portion of hδORs is often seen as a species with only one N-glycan modified, at Asn18. This is more prevalent for hδORCys27 (Markkanen & Petäjä-Repo, 2008), which may be due to the fact that by the time Asn33 is to be glycosylated during receptor translocation, cysteine at position 27 has emerged from the translocon. The absence of an N-glycan at either Asn18 or Asn33 leaves the putative O-glycosylation sites at Ser20 and Ser35 available for modification. This may not only affect the usage of the sites but may lead to differences in the overall stoichiometry of O-glycosylation. The amino acid at position 27 causes minor differences in the prediction of the sites. The sequence corresponding to hδORPh27 had slightly higher prediction values, especially at -2 and +2 sites (Ser25 and Ser29). It has been established that glycosyltransferases have clear preferences for certain amino acids close to the potential glycosylation site (especially -2 to +2) (Gerken et al., 2011; Gerken, Raman, Fritz, & Jamison, 2006).
Many N-glycosylation sites in glycoproteins are conserved between species, underlining their potential importance during folding and processing in the ER. Conservation in general is an indication of an important part of a protein preserved by evolution. The conservation of the N-terminus and the putative O-glycosylation sites of δORs between closely related mammalian species were investigated using BLAST (III, Fig. 1A). Although the N-terminal tail is generally the least conserved portion in the GPCR structure, the BLAST analysis showed a conservation both of N-glycosylation sites and the putative O-glycosylation sites. Thus, the putative O-glycosylation sites may be modified in most species and may have an important role in the processing or function of δORs.

5.3.2 Characterization of O-GalNAc glycosylation of hδOR variants

The presence of O-glycans in hδOR\textsuperscript{Phe27} was confirmed by purifying the receptor from whole cell extracts by immunoprecipitation and subjecting it to enzymatic deglycosylation with neuraminidase and O-glycosidase. The samples were then analyzed by SDS-PAGE to confirm a minor but clear downward shift in molecular mass (III, Fig. 1B), indicating removal of O-glycans. The O-GalNAc glycosylation is catalyzed by enzymes expressed differentially in cells and tissues, likely leading to specific glycosylation in different milieus (P. D. Kingsley et al., 2000; Ten Hagen et al., 2003). The hδOR O-glycosylation in HEK293 cells may thus not be a ubiquitous modification found in other cells. Thus, receptor O-glycosylation was confirmed in SH-SY5Y neuroblastoma cells that endogenously express the receptor. The mutant hδOR-N18;33E variants were chosen for the analysis, in order to see a clearer shift in mass in the absence of N-glycans. This was seen after treatment with the enzymes, indicating O-glycosylation of hδOR in its native environment (III, Fig. 1C).

The O-glycan analysis with enzymatic digestion relies on the detection of minor molecular mass shifts on glycoproteins and is specific only to the most common type of O-glycosylation. To complement the results obtained from deglycosylation experiments, the presence of O-glycans was assessed using O-glycoproteomic tools, employing engineered cell lines producing truncated O-glycans, and specific lectins to purify the glycoproteins. The HEK293-SC cell line that has been engineered to halt O-glycosylation after GalNAc addition [see 4.2.4 and Steentoft et al. (2011)] were stably transfected to express HA-tagged hδOR variants, and the receptors were purified from solubilized membranes with immobilized HA antibody or VVA lectin (III, Fig. 1D). Purification with HA
antibody yielded receptor species similar to the ones purified from normal HEK293; cells expressing the same receptor constructs. The purification with VVA-lectin that binds proteins modified with GalNAc-residues yielded the mature hδOR species from HEK293-SCs, indicating the presence of hδOR variants modified by truncated O-glycans. As expected, the VVA purification of hδORs was unsuccessful from the normal HEK293, cells that produce elongated O-glycans. The results demonstrate that the hδOR variants are O-glycosylated in HEK293 and SH-SY5Y cells and are modified by GalNAc-type O-glycans.

5.3.3 In vitro screening for hδOR modifying GalNAc-transferases

The O-glycosylation is initiated by a family of GalNAc-Ts that have distinct preferences for glycosylation sites (Bennett et al., 2012; Gerken et al., 2011). The hδOR O-glycosylation was investigated in more detail by screening for the GalNAc-Ts responsible for receptor glycosylation. Synthetic peptides corresponding to the variant N-terminal sequence covering four of the five putatively O-glycosylated serine residues and the polymorphic site were subjected to glycosylation assays with a UDP-GalNAc substrate and a panel of recombinant GalNAc-Ts ranging through the entire family (III, Fig 2A-B). GalNAc-T1, T2, T3, T4, T5, T7, T12, T13, T14 and T16 were assayed for various periods of time and the products were analyzed by MALDI-TOF MS. The products were analyzed for changes in m/w corresponding to the addition of GalNAc to the peptide (+203). Only GalNAc-T2, and to some extent GalNAc-T16, were able to glycosylate the hδOR peptides (III, Fig. 2A-B). The initial peptide analysis excluded the fifth putative site near the N-terminal end due to interest in the region in the proximity of the polymorphic site.

A more detailed analysis with multiple time points was then performed to assess the efficiency and kinetics of glycosylation of the hδOR peptides. The assay demonstrated that GalNAc-T2 is able to glycosylate both variant peptides already within 5 min, suggesting a primary role of this transferase (III, Fig. 2D). GalNAc-T2 was able to glycosylate multiple sites, whereas GalNAc-T16 only glycosylated a single site. Furthermore, GalNAc-T2 glycosylated more sites in the peptide corresponding to the hδORPhe27 variant, which is in concordance with the variant being the more likely substrate in the in silico analysis. All four sites (corresponding to Ser20, Ser25, Ser29 and Ser35) were glycosylated to some extent in the peptide corresponding to the hδORPhe27 variant, while the peptide corresponding to the hδORCys27 variant had mainly two sites glycosylated (III, Fig.
This indicates that the hδOR with phenylalanine at position 27 is a better substrate for GalNAc-T2. As discussed previously, the in vitro assay lacks N-glycosylation that may mask two of the sites in vivo, and these sites may be available only in certain conditions.

The purification of the produced glycopeptides by HPLC and the subsequent analysis by ESI-LIT-FT MS/MS revealed also differences in the occupation of the sites between the variants. The GalNAc-T16 glycosylated Ser25 of both peptides, whereas differences were found for GalNAc-T2. The peptide corresponding to the hδORPhe27 variant appeared to be glycosylated at the site corresponding to Ser25, whereas the peptide corresponding to the hδORCys27 variant appeared to be glycosylated at a site corresponding to Ser29 (II, Fig. 2C). Apparently, the SNP not only alters the efficiency of glycosylation, but also the modified glycosylation site. The differences in the number of glycans added and the sites occupied in the peptides likely reflect in vivo conditions and intact proteins, at least at the stoichiometric level.

5.3.4 GalNAc-T2 mediated glycosylation of hδOR variants in cells

To validate the in vitro results and to assess hδOR glycosylation by GalNAc-T2 in cells, the variant constructs were transiently transfected to GALNT2 knockout HEK293 cells (HEK293-ΔT2). The immunoprecipitated receptors from whole cell extracts were analyzed by SDS-PAGE and western blotting to see shifts in mobility. An incomplete heterogeneous downward shift in the migration of the receptor could be seen with the knockout cells (III, Fig. 3A) indicating impaired glycosylation related to the lack of GalNAc-T2. The hδORPhe27-N18;33E was similarly analyzed after expression in HEK293-WT, HEK293-SC and HEK293-ΔT2 cells to assess the shift more clearly in the absence of heterogeneous N-glycans. A clear downward stepwise shift was seen from WT to SCs and finally to ΔT2 cells (III, Fig. 3B), indicating truncation and absence of O-glycans, respectively. HEK293 cells also express GalNAc-T16, which may to some extent compensate for the lack of GalNAc-T2. Thus, the receptor expression pattern was also assessed in HepG2 cells that do not express GalNAc-T16. The hδOR-N18;33E variant constructs showed a similar mobility shift in HepG2-ΔT2 cells compared to HepG2 WT cells as in the corresponding HEK293 cells (III, Fig. 3C), indicating a loss of GalNAc-T2 mediated O-glycosylation. The O-glycosylation was rescued in a GALNT2 knockout/knock-in HepG2 cell line, which showed a similar pattern
of expressed receptors to the HepG2 WT cells (III, Fig. 3C). These results confirm that the primary isoform responsible for hδOR O-glycosylation is GalNAc-T2.

According to the O-glycosylation prediction algorithm and the in vitro assay, Ser25 and Ser29 of hδOR are the most likely sites to be O-glycosylated in cells. The modification of serines in the receptor N-terminal tail was assessed by mutating Ser25 and Ser29 to alanine. The hδOR WT and the mutants were transfected into HEK293-SC cells and purified with VVA lectin to test for the presence of GalNAc O-glycans (III, Fig. 4A-B). The western blot analysis showed a successful purification of hδOR WT constructs with the lectin, as expected. Interestingly, the VVA-purified receptor showed a relative increase in the amount of Endo H resistant mature receptors with a single N-glycan (III, Fig. 4A-C). Likely, the hδOR with only one N-glycan has O-glycans more accessible for VVA binding, resulting in a higher yield of this species. The mutagenesis of Ser25 and Ser29 (S25;29A) resulted in a drastic reduction in VVA binding, implying disruption of O-glycosylation sites. An additional mutation of the final putative site at Ser6 (S6;25;29A) resulted in almost complete absence of VVA binding. Finally, the same O-glycosylation sites were mutated in the N18;33E receptor constructs to analyze the effect of N-glycans on the use of the O-glycosites. The hδOR-N18;33E variants bound to VVA similarly to the WT constructs (III, Fig. 4A-B). Again, the S25;29A mutation resulted in a drastic reduction in VVA binding, which was non-existent after an additional mutation at Ser6. Importantly, the Ser20 and Ser35 were available for modification in these constructs, but according to the VVA binding results, they were not used. These results confirm that the hδOR variants are O-glycosylated at Ser6, Ser25 and Ser29.

5.3.5 Stabilization of hδOR expression at the cell surface by O-glycosylation

The functional significance of the hδOR O-glycosylation was studied in an engineered cell line, CHO-lldD. In these cells O-glycosylation can be controlled by the supplementation of the cell culture medium with GalNAc and Gal [see 4.1.3 for more details (D. M. Kingsley et al., 1986; Krieger et al., 1989)]. The hδOR variants expressed transiently in these cells showed a low molecular mass receptor form migrating slightly above the receptor precursor (III, Fig. 5A). This form is likely modified by N-glycans that have been only partially processed in the ER and Golgi, but the elongation is stopped in the absence of UDP-Gal. The addition of Gal rescued N-glycosylation, leading to a faint band co-migrating closely with the fully
processed form in WT CHO-K1 cells. The processing of N-glycosylation apparently contributes the most to the apparent increase in the molecular mass of the mature receptor, which is in line with the results of the enzymatic deglycosylation (see III, Fig. 1B). The addition of GalNAc resulted in a minor increase in the size of the receptor, indicating initiation of O-glycosylation. Importantly, the truncated GalNAc-O-glycan is not further elongated by sialic acids, as the sialic acid transferase responsible, ST6GalNAc, is not expressed in CHO cells (Yang et al., 2014). The addition of both monosaccharides allows fully elongated O- and N-glycosylation and rescued the near-normal phenotype. However, the N-glycan processing appeared to be somewhat incomplete, leading to a heterogeneous migration of the mature receptor form. To avert the problems in N-glycan processing, the hδOR<sup>Δexo27</sup>-N18;33E mutant was transfected and analyzed as above. In the absence of the N-glycans, the initiation and full elongation of the O-glycans was clearly visible. The addition of GalNAc initiated O-glycosylation, increasing receptor size slightly. The addition of both sugars led to a further increase in size as the O-glycans are elongated further (III, Fig. 5A). To avoid the impact of heterogeneous N-glycan processing on the clarity of results, the hδOR-N18;33E mutants were chosen for further studies in this cell model.

For studying the role of O-glycosylation in hδOR maturation and expression, the hδOR-N18;33E variants were stably transfected into CHO-ldlD cells. The cell lines were established with the Flp-In system, resulting in isogenic incorporation of the receptor variants into the genome. The receptor cell surface expression was studied in various conditions of glycosylation by supplementing the cell culture medium and analyzing live cells by flow cytometry. In O-glycosylation prohibiting conditions (no supplementations), receptor expression was detected by labeling with a tag-specific antibody, but at significantly lower levels compared to the O-glycosylation permitting conditions (Gal and GalNAc supplementation) (III, Fig 5B). This suggests that while O-glycosylation is not required for the cell surface delivery of hδOR, the receptor levels at the plasma membrane are modulated by O-glycosylation. Interestingly, in conditions, in which O-glycosylation sites only carried GalNAc (GalNAc supplementation), the receptor levels were higher and at similar levels to the levels of O-glycosylation permitting conditions.

A further analysis of receptor cell surface expression was performed by the cell surface biotinylation chase assay. The method was used to detect and follow the receptor at the cell surface and to assess the rate of receptor turnover. Again, in O-glycan permitting conditions (GalNAc or both Gal and GalNAc supplementation), higher levels of immunoprecipitated receptor variants were detected on western
blots with the streptavidin-HRP detection (Fig. 12A and III, Fig. 6A-C). Furthermore, the turnover of receptors during the 4-h chase was significantly slower in these conditions (Fig. 12A and III, Fig. 6A and D). The hδOR\textsubscript{Cys27} variant showed faster turnover in both conditions, implying that the decreased stability of this variant is intrinsic to the amino acid substitution. Altogether, these results suggest that the O-glycans modulate hδOR levels by decreasing receptor turnover at the cell surface. O-glycans may elicit a stabilizing effect on the receptor that slows down the constitutive downregulation.

5.3.6 The impact of O-glycosylation on hδOR ligand binding and functional activation

In order to assess the impact of receptor O-glycosylation on the functional output of hδORs, the ligand-binding capable cell surface hδOR-WT and hδOR-S6;25;29 mutant were quantified. The DNA constructs were transiently transfected into HEK293 WT and ΔT2 cells, and the cells were labeled with FITC-conjugated NTX (III, Fig. 7A) or cMyc antibody (Fig. 12B and III, Fig. 7B) to quantify the ligand-binding capable and total pool of receptors at the cell surface, respectively. Flow cytometric analysis of live cells using both labeling methods showed a decrease in cell surface receptor levels in HEK293 WT cells when hδOR-S6;25;29A variants were compared to hδOR-WT variants, confirming the effect of the absence of O-glycans. Similarly, both methods showed a decrease when comparing hδOR-WT variants expressed in HEK293 WT cells to HEK293-ΔT2 cells, suggesting that the effect is GalNAc-T2 dependent. In contrast, no decrease was seen in the staining of hδOR-S6;25;29A in these cells, as expected. The FITC-NTX signal obtained paralleled the cMyc signal, confirming that hδOR at the cell surface is ligand-binding competent with or without O-glycans and suggests that the effects might directly translate to altered signaling.
Fig. 12. Impact of O-glycosylation on hδOR downregulation and signaling. **A**, Stable CHO-ldlD cells expressing myc-hδOR-FLAG N18;33E mutant variants were cultured with the indicated supplementations and subjected to cell surface biotinylation. Cells were harvested after quenching the residual biotin reagent or after 4-h chase in a medium containing the indicated supplements. Receptors were purified from whole cell extracts by immunoprecipitation (IP) and analyzed by western blotting (WB) with streptavidin-HRP. Densitometric scans were analyzed to quantify the decrease in cell surface receptor levels after the chase (right graph). **B-C**, HEK293 WT and ΔT2 cells were transfected with myc-hδOR-FLAG WT and S6;25;29A mutant variant constructs. After 24h, cells were analyzed by flow cytometry (B) or subjected to cAMP accumulation assay (C). *B*, The GeoMean values obtained using cMyc antibody and PE-conjugated secondary antibody were normalized to those obtained for hδORPhe27. *C*, Cells were incubated in the presence of 5 µM forskolin and in the absence or presence of 10 µM δOR-selective agonist, DADLE, for 60 min, and cAMP was measured. Data are presented as percentage of forskolin-induced cAMP accumulation. Two-way analysis of variance followed by Bonferroni’s (A-B) or Tukey's (C) post hoc tests were used for statistical comparisons. *, p < 0.05; **, p <0.001; ***, p < 0.001. Modified from III (panel A: Fig. 6A and D, panel B: Fig. 7B, panel C: Fig. 7C).
The signaling output of cells transfected with hδOR-WT and hδOR-S6;25;29A was further studied by quantifying their capacity to inhibit AC-mediated accumulation of cAMP (Fig. 12C and III, Fig. 7C). HEK293 cells transfected with hδOR-WT and hδOR-S6;25;29A were subjected to the LANCE cAMP accumulation assay to measure the agonist-induced inhibition of forskolin stimulated cAMP accumulation. The accumulation was inhibited with a δOR-selective opioid agonist, DADLE. As expected, the inhibition was significantly reduced for hδOR-S6;25;29 compared to the WT receptor. This is in concordance with the decreased levels of functional hδORs at the cell surface in the absence of O-glycans. In summary, these results suggest that the site-specific O-glycosylation of Ser6, Ser25, and Ser29 by GalNAc-T2 modulates cell surface expression and consequently, agonist mediated activation of hδOR.
6 Discussion

This study investigated the processing of hδOR during receptor maturation along the secretory pathway. Critical steps in processing in the ER and in the Golgi were studied in order to understand how different molecular elements govern the levels of functional receptors at the plasma membrane. A summary of the findings is illustrated in Figure 13. The hδOR variants with a Phe27Cys substitution in the N-terminal tail were used in the study to gain a wider perspective on receptor processing that results in distinct phenotypes, and to accumulate evidence on the significance of this polymorphism. The variants were expressed in HEK293, HepG2, CHO and SH-SY5Y cells and studied with a variety of biochemical and cell biological methods.

Fig. 13. Summary. The events during hδOR processing that were studied in the thesis were receptor oligomerization, N-glycosylation and O-glycosylation. Their functional roles in the secretory pathway and at the plasma membrane, as suggested by the results, are listed.
6.1 Oligomerization and processing of hδOR variants in co-expressed cells

Receptor oligomerization has been demonstrated for numerous GPCRs, but the subcellular location of the phenomenon has remained controversial. The oligomerization of receptors in the early biosynthetic compartments could present an important step in their processing and maturation. The hδOR variants showed distinct behaviors during ER export and receptor maturation, as the hδOR$_{\text{Cys27}}$ variant was found to be inherently inefficient in folding and ER export (Leskelä et al., 2009). This study assessed the possible role of heteromerization in governing the maturation of the variants. Using transient co-transfections and stable cell lines induced to co-express the variants, the hδOR$_{\text{Cys27}}$ variant was found to elicit a dominant negative effect on hδOR$_{\text{Phe27}}$. This was shown by western blotting, metabolic labeling, flow cytometry and confocal microscopy. In a dose-dependent manner, the co-expression of the hδOR$_{\text{Cys27}}$ variant led to a decrease in the cell surface delivery of hδOR$_{\text{Phe27}}$. Simultaneous accumulation of the hδOR$_{\text{Phe27}}$ precursor confirmed the retention in the ER and suggested that it is rerouted for proteasomal degradation via ERAD. This was confirmed by using the proteasomal inhibitor lactacystin, and by rescuing the retained receptor precursors with pharmacological chaperones. Importantly, the dominant negative effect was specific for hδOR, as the LHR and TR did not affect hδOR receptor levels in co-transfected cells.

The attenuation of hδOR ER export in co-expressed cells implies receptor-receptor interaction in the ER where the defective or inefficient protomer dominantly interferes with the processing of the other and suppresses its export. This has been observed for other class A GPCRs, such as rhodopsin and α$_2$AR whose delivery to the cell surface is inhibited by their corresponding mutants (Rajan & Kopito, 2005; Zhou et al., 2006). The effect is not limited to misfolding mutations, as the CXC chemokine receptor 1 with an inserted ER retention motif was also found to retain its WT counterpart (Wilson et al., 2005). Here, the underlying mechanism was directly demonstrated to be receptor-receptor interaction in the ER. Direct evidence for receptor heteromerization was obtained by metabolic labeling of newly-synthesized receptors in the co-transfected cells, followed by sequential co-immunoprecipitation of the variants with different variant-specific epitope tags. Both the mature and the precursor receptor forms were successfully co-precipitated, indicating receptor heteromerization already in the ER. The receptor heteromerization was confirmed in live cells by co-
transfection of Venus and Rluc-tagged hσOR variants, and performing a BRET analysis. Confocal microscopic analysis of co-transfected HEK293 and SH-SY5Y neuroblastoma cells indicated receptor accumulation in the early secretory compartments. These results are in agreement with earlier studies that localize GPCR heteromerization to the ER (Salahpour et al., 2004; Terrillon et al., 2003). Markers for ER, and to some extent ERGIC, co-localized with the hσOR variants in co-transfected SH-SY5Y cells. The results suggests that the retention may occur from as far as ERGIC, which has been shown to be involved in cellular quality control pathways and where certain ER quality control chaperones also localize (Appenzeller-Herzog & Hauri, 2006; Hammond & Helenius, 1994; Zuber et al., 2001).

The interaction of receptor variants in the ER and the interference of hσOR^{Phe27} processing mediated by hσOR^{Cys27} were further suggested by the observation that there were changes in the variant N-glycoforms expressed in co-expressed cells. The hσOR^{Phe27} carries normally two N-glycans but hσOR^{Cys27} is expressed in two forms with either two or one N-glycans, the latter with only a single N-glycosylation site occupied at Asn18 (Markkanen & Petäjä-Repo, 2008). In the co-transfected cells, the hσOR^{Phe27} showed an increased amount of a glycoform with only a single N-glycan. This suggests that the heteromerization may occur already during folding, as the N-glycosylation is a co-translational event. The inefficient glycosylation of Asn33 in hσOR^{Phe27} may occur due to a suboptimal conformation of the emerging polypeptide, or due to interfering interactions with other ER proteins. In cells expressing both variants, the proximity of receptor N-termini during early biosynthesis may also hinder N-glycosylation of hσOR^{Phe27} in similar ways. Small qualitative changes could also be seen in the hσOR^{Phe27} glycoforms, even in the heterogeneous mature receptor species containing two N-glycans. Heteromerization in the Golgi could similarly alter the availability of the N-terminal sites for post-translational glycan modifications.

Receptor-receptor interactions may elicit allosteric modulations on receptor protomer conformations, similar to other receptor-interacting proteins, such as G proteins and arrestins (Katritch et al., 2013), leading to altered receptor behavior. This is well established in pharmacological studies that show novel heteromer-dependent signaling (Hasbi et al., 2007; Kabli et al., 2010; Rozenfeld & Devi, 2007). Thus, interactions in the ER may alter protomer conformations and directly impact their folding and processing, also in homomeric receptors. The dynamics of the receptor monomer-oligomer balance are largely unclear, but the tendency to form oligomers in the ER would likely have an impact on the folding and
processing of the receptor. There is also an intriguing possibility of a heteromer-specific quality control that scrutinizes the oligomers formed during or immediately after folding. Due to the apparent timing of the early formation of receptor oligomers, it would be plausible that the quality of the entire oligomer is monitored. Therefore, the hδOR<sup>Cys27</sup>-hδOR<sup>Phes27</sup> oligomer as such may attain suboptimal conformation that is more prone to retention.

In addition to the observed impact on ER processing and cell surface delivery, the heteromerization is likely to alter hδOR behavior at the cell surface. The heteromerization was observed at the cell surface with co-immunoprecipitation and BRET, suggesting that the variant oligomerization is not limited to the early secretory pathway. In earlier studies, the variants were revealed to differ in behavior at the plasma membrane. The hδOR<sup>Cys27</sup> was found to be more prone to constitutive internalization (Leskelä et al., 2009), suggesting that it is less stable. Here, we observed a faster turnover for the metabolically labeled hδOR<sup>Cys27</sup>, indicating faster constitutive downregulation for this variant. In cells co-expressing the variants, the less stable variant heteromers prone to retention in the ER might show a similar tendency for more rapid internalization at the plasma membrane. The oligomerization of opioid receptors has been shown to lead to co-internalization after receptor activation (Kabli et al., 2010). Furthermore, single-molecule imaging has revealed that an activation-mediated internalization of CXCR4 is more robust with the receptors in oligomeric forms (B. Ge et al., 2017). Thus, the destabilizing nature of the hδOR<sup>Cys27</sup> variant in the hδOR heteromer likely has further implications at the cell surface and in the endocytic pathway. This possibility has already emerged in a study, in which heterozygote individuals were overrepresented among patients suffering from Alzheimer’s disease, and the endocytosis of the hδOR<sup>Cys27</sup> variant was associated with altered amyloid precursor protein processing in a heterologous expression system (Sarajärvi et al., 2011). Altogether, the hδOR variant heteromerization presents an important step in receptor processing that has multifaceted effects on receptor folding, quality control and cell surface delivery and may translate to altered opioid receptor signaling. The mechanism likely affects the cell surface expression of the rest of the GPCR family and thus modulates the signaling of many physiological functions.

6.2 N-glycosylation of hδOR variants

N-glycans and N-glycan-mediated interactions play a crucial role in the folding and quality control of glycoproteins (Lamriben et al., 2016). Many GPCRs with
disrupted N-glycosylation show lower levels of receptors at the cell surface (Hawtin et al., 2001; Lanctot et al., 1999; Markkanen & Petäjä-Repo, 2008), implying an important role of N-glycosylation for the synthesis and cell surface delivery. The N-glycosylation and N-glycan-mediated ER processing of the hδOR variants were studied to investigate the importance of the modification for the receptors and to assess whether the amino acid substitution further affects N-glycan-mediated ER processing that translates to phenotypic differences at the cell surface.

The disruption of hδOR N-glycosylation by site-directed mutagenesis and analysis of expressed mutant receptors by SDS-PAGE revealed similar low molecular mass species for two variants in various mammalian cells. This indicates that both variants carry N-glycans on the predicted N-glycosylation sites. Flow cytometric analysis and cell surface biotinylation confirmed the presence of hδOR-N18;33E at the cell surface, suggesting that the N-glycans are not required for the cell surface delivery. However, the receptor levels at the cell surface were decreased compared to WT receptors. The impact of N-glycosylation is generally substrate specific but several GPCRs have been found to be expressed at the cell surface at lower levels when the N-glycosylation sites have been disrupted (Compton, Sandhu, Wijesuriya, & Hollenberg, 2002; X. Ge et al., 2009; Hawtin et al., 2001). For µOR, this has been attributed to attenuated interaction with a putative export chaperone (RPNI) (X. Ge et al., 2009), and for κOR to increased retention of the mutant receptor (J. G. Li et al., 2007). Disruption of any of the five N-glycosylation sites of µOR results in a decrease in cell surface receptor levels, and the total absence of N-glycans completely abolishes the expression (X. Ge et al., 2009).

The metabolic pulse-chase labeling of the mutant hδOR-N18;33E variants showed increased turnover for the mature receptor, suggesting that decreased stability of the mature receptor contributes to lower receptor levels at the cell surface, similar to hκOR (J. G. Li et al., 2007). It is not clear whether the N-glycans play a part in receptor trafficking from the cell surface or whether the extrinsic and/or intrinsic effects of glycans contribute to receptor properties that make it less stable and prone to downregulation. Glycans play a part in polypeptide stability, as they stabilize the conformation by reducing dynamic fluctuations and reduce the formation of other secondary structures and unfolding (Jayaprakash & Surolia, 2017). The absence of N-glycans may thus shift the conformational equilibrium towards receptor states more prone to downregulation. The N-glycans also have an indirect effect on protein stability and conformation via glycan-mediated interactions that guide the receptor folding in the ER (Hebert et al., 2014). The cell
surface biotinylation chase assay of the hδOR-N18;33E variants confirmed the rapid downregulation of the variants in the absence of N-glycans, and showed that hδOR\textsuperscript{Cys27}, which is less efficiently processed in the ER, is more rapidly downregulated. Furthermore, the flow cytometric quantitation of ligand binding competent hδOR variants at the cell surface revealed a population of non-native hδOR\textsuperscript{Cys27}, implying more severe N-glycan dependent conformational defects. The hδOR\textsuperscript{Phe27} variant, however, was expressed as a fully native, binding competent form. Importantly, the non-N-glycosylated hδOR mutants did not show altered ligand binding properties in the saturation ligand binding assays. These results support the idea that the decreased stability of the mature non-N-glycosylated hδOR at the cell surface can be attributed to events taking place during receptor processing in the ER that generate more complications for the hδOR\textsuperscript{Cys27} variant.

The metabolic labeling experiments also showed that the rate of hδOR receptor maturation for both mutant variants was drastically enhanced compared to the corresponding WT receptors. This implies more rapid export of the non-N-glycosylated receptors from the ER. Similar observations have been obtained for hκOR (J. G. Li et al., 2007). Interestingly, the N-glycans have been demonstrated to accelerate folding of a glycoprotein (Hanson et al., 2009). Thus, the accelerated receptor maturation in the absence of N-glycans is likely to occur due to more rapid quality control and ER export than to faster folding. In the ER, interactions with the folding machinery, and the repeated interactions with CNX can delay the export until the native conformation has been reached (Lamriben et al., 2016). The CNX co-precipitated with the hδOR precursor in an N-glycan-dependent manner, and this interaction was seen to take place co-translationally. Importantly, the half-life of CNX-bound hδOR\textsuperscript{Cys27} was longer than that of hδOR\textsuperscript{Phe27}. This is in line with the observations that the processing of hδOR\textsuperscript{Cys27} in the ER is more inefficient, and implies that CNX is responsible for the retention hδOR\textsuperscript{Cys27}. This was supported by the fact that long-term expression of hδOR\textsuperscript{Cys27}, which has been shown to lead to increased accumulation of the receptor precursor in the ER (Leskelä et al., 2009), further slowed down the dissociation from CNX. The interaction of CNX is mediated by glucose trimming of the high-mannose N-glycans in glycoprotein substrates. The enzymatic removal of the first two and the last of the three glucoses in the N-glycan controls the association and dissociation of the CNX, respectively (Lamriben et al., 2016). The post-translational inhibition of the removal of the third glucose by CST resulted in a slower turnover of the receptor precursor and diminished efficiency of maturation. The post-translational timing of the inhibition prolongs the interaction with CNX. Thus, the results support the notion of CNX
being responsible for the ER retention of the receptor. The absence of the interactions apparently bypasses this delay and leads to enhanced kinetics of receptor export from the ER.

Interestingly, a portion of the metabolically labeled hδOR_{Cys27-N18;33E} was found to be retained in the ER, even in the absence of N-glycans, suggesting that an N-glycan-independent mechanism not involving CNX is able to scrutinize the receptor precursors and prevent their export from the ER. The hδOR_{Cys27-N18;33E} did not co-precipitate with CNX, confirming that CNX does not interact with hδOR in an N-glycan independent manner, unlike certain other GPCRs, such as the angiotensin II type 1 receptor (Lanctot et al., 2006), and D1 and D2 dopamine receptors (Free et al., 2007). The CNX-independent retention of N-glycosylated receptors was also observed when the CNX interaction with the receptor was inhibited with CST. Not only was the retention observed, but also the retained receptor precursor appeared to accumulate. This is likely due to incomplete inhibition of the removal of the first two glucose of nascent N-glycans, and subsequent accumulation of the monoglucosylated forms that promote CNX binding. The retained receptor precursor during CST treatment accumulated when the proteasome was inhibited by lactacystin, suggesting that the precursor is subjected to degradation via ERAD. This was also seen when the proteasomal degradation of hδOR_{Cys27-N18;33E} was inhibited. The fate of the hδOR-N18;33E in the ERAD pathways was further confirmed by the evidence of increased polyubiquitination of the receptors during proteasomal inhibition. Furthermore, subcellular fractionation revealed a soluble receptor species that was evidently retrotranslocated to the cytosol in the absence of the N-glycans.

The two cellular pathways that can scrutinize hδORs in the presence or absence of receptor N-glycosylation may work as distinct pathways operating in different conditions. The quality control mediated by CNX is slower but at the same time it is more efficient in preventing the export of nonnative receptors, whereas the N-glycan-independent quality control appears to be more prone to errors. The quality control of newly-synthesized proteins has been found to vary depending on the conditions existing in the ER. During the unfolded protein response, the requirement for N-glycosylation of EDEM1 substrates that are targeted for ERAD is altered. In normal conditions, EDEM1 targets nonnative proteins for degradation in a process that requires N-glycan trimming (Ron et al., 2011) although non-glycosylated substrates have been reported as well (Shenkman et al., 2013). Similarly, the null Hong Kong variant of α-antitrypsin, a common ERAD substrate model, was show to be subjected to CNX and EDEM1 mediated targeting to
ERAD, but shifted to the N-glycan independent pathway involving BIP, in the absence of N-glycans, and during ER stress (Ushioda et al., 2013). Thus, it is likely that cells have adaptive back-up pathways for quality control of proteins to maintain cellular homeostasis, and these pathways are also implemented for GPCRs in the absence of N-glycans.

Multiple ER proteins interact with the nascent polypeptide that emerges from the translocon during early biosynthesis. The OST that N-glycosylates proteins, the glucosidases, CNX and the other quality control proteins are accompanied by members of the PDI and PPI families (Hartl et al., 2011). The interplay of glycosylation and disulfide bond formation is well established with the association of CNX and ERp57 (Oliver, van der Wal, Bulleid, & High, 1997). The ERp57 catalyzes disulfide bridge formation in nascent polypeptides, and via interaction with CNX it can selectively target glycoproteins that have not yet attained the native conformation (Jessop et al., 2007). Interestingly, the hδOR Cys27 residue is an unpaired cysteine in the receptor extracellular domain that may be prone to form mixed disulfide bonds with PDIs during receptor synthesis. These interactions may contribute to the inefficient processing, and the subsequent retention of the receptor in the ER. If this is the case, this would likely not be limited to interactions with ERp57, as the retention occurs also in the absence of CNX interaction.

In previous studies, opioid antagonists and agonists have been used as pharmacological chaperones that release the receptors from the CNX cycle and assist their export from the ER (Leskelä et al., 2007; Petäjä-Repo et al., 2002). In this study, they were used to rescue both the WT and the non-N-glycosylated hδOR from retrotranslocation and subsequent degradation. This suggests that the rescue is not limited to quality control mediated by N-glycans and CNX interaction. Rather, pharmacological chaperones may also assist incompletely folded receptors that are scrutinized by the more rapid, N-glycan-independent quality control. The results imply a direct role of the pharmacological chaperones in aiding receptors to attain the native conformation or inhibiting retrotranslocation, rather than blocking interactions with retaining ER chaperones, and offer a potential treatment in conditions where the receptor glycosylation and/or quality control is impaired.

Altogether, the study demonstrates the importance of N-glycosylation in the biosynthesis of hδOR, and presents a general role for the N-glycans in the modulation of GPCR cell surface delivery and functionality. It also presents ways in which the cells attempt to monitor the quality of their signaling receptors and paves the way for developing strategies to treat individuals carrying mutations and SNPs that cause retention of GPCRs.
6.3 O-GalNAc glycosylation of hδOR variants

Protein O-GalNAc glycosylation is a protein modification that is poorly understood apart from the archetypical heavily glycosylated mucin-type proteins. A number of GPCRs have been shown to contain O-glycans in their extracellular domains, without further characterization of their role in receptor function or processing. In previous studies, the hδOR was found to carry O-glycans (Markkanen & Petäjä-Repo, 2008; Petäjä-Repo et al., 2000), and in this study, the O-GalNAc glycosylation was characterized in more detail by identifying the GalNAc-Ts responsible and the sites glycosylated and by assessing the functional role of the modification for the receptor.

The research on protein O-glycosylation has been challenging due to the lack of identifiable glycosylation sites and the large number of catalyzing enzymes. New online prediction algorithms were used to predict the sites used for the O-glycosylation of hδOR variant N-termini. Five serines (Ser6, Ser20, Ser25, Ser29 and Ser35) were found to be above the prediction threshold of the NetOGlyc 4.0 algorithm that relies on recent O-glycoproteomic studies (Steentoft et al., 2013). The predictions were in line with established amino acid preferences in close proximity to serines. Especially prolines at -1 (Ser6, Ser25, Ser29) and +3 (Ser25, Ser35) positions and glycines at +2 (Ser6, Ser29) have been discovered to be favorable amino acids for the most common GalNAc-T isoforms, T1, T2 and T3 (Gerken et al., 2011). The polymorphic site at position 27 is between two of the putative O-glycosylation sites and the enzymes may have a preference for one or the other polymorphic amino acid. Interestingly, glycosylation predicted at Ser25 (0.66 vs 0.55) and Ser29 (0.93 vs 0.90) in the hδOR_{Cys27} variant were less likely, suggesting that the polymorphism may indeed alter receptor O-glycosylation. Importantly, the prediction does not account for the N-glycosylation sites that overlap with two of the putative O-glycosylation sites (Asn18-x-Ser20 and Asn33-x-Ser35). Furthermore, the sequence alignment analysis suggested a high degree of conservation of serines in the N-termini with other mammalian δORs, which implies a possible functional role for these residues.

The O-glycosylation of hδOR_{Cys27} has been demonstrated previously by enzymatic deglycosylation, followed by assessment of the mobility shift in SDS-PAGE (Markkanen & Petäjä-Repo, 2008; Petäjä-Repo et al., 2000). A similar strategy was used in this study to confirm the O-glycosylation of hδOR_{Phe27} in HEK293 cells, and to confirm hδOR O-GalNAc glycans in neuronal SH-SY5Y cells that endogenously express the receptor. The enzymatic cleavage by O-
glycosidase not only confirmed the presence of O-GalNAc-glycans in the protein but suggested that the glycans are core-1 type structures containing Gal elongations. The method is limited by the specificity of the enzyme and by challenges in interpreting the migration shifts on SDS-PAGE. As an alternative, a more direct method utilizing novel O-glycoproteomic tools was used to assess the presence of O-glycans in hδOR variants. The VVA lectin binds to terminal GalNAc residues that emerge during the initiation of O-GalNAc glycosylation. The VVA purification has been successfully used to enrich O-glycoproteins from HEK293 SCs that are engineered to terminate the O-glycosylation after the initial GalNAc addition (Steenoft et al., 2013; Steentoft et al., 2011). This strategy was used to assess O-glycosylation of hδOR by expressing the receptor in these cells and purifying the receptor from whole cell extracts by VVA. Both variants were successfully purified, confirming the presence of O-GalNAc glycans.

A further characterization of hδOR variant O-glycosylation was done by designing peptides corresponding to the receptor N-termini and subjecting them to in vitro glycosylation assays. The in vitro glycosylation assay has been demonstrated to accurately predict glycosylation in cells (Goth et al., 2015; Kong et al., 2015). A panel of GalNAc-Ts covering a large portion of the protein family was included in the screening. Only GalNAc-T2, and to some extent GalNAc-T16, produced glycopeptides in the assay. GalNAc-T2 is an enzyme widely expressed throughout the body, including tissues where hδOR is expressed endogenously, and GalNAc-T16 is a close homologue that shares many of its substrates with GalNAc-T2 (Homa, Hollander, Lehman, Thomsen, & Elhammer, 1993; Kong et al., 2015). Both peptides were substrates for the enzymes, albeit with different efficiencies. While GalNAc-T16 glycosylated a single site in both peptides, GalNAc-T2 showed a tendency to glycosylate more sites in the peptide corresponding to the hδORPhe27 variant during longer incubations. The kinetic analysis showed that GalNAc-T2 glycosylated the peptides already after a 5-min incubation, suggesting that GalNAc-T2 has a high affinity for the region, and that it is likely the primary enzyme responsible for hδOR glycosylation. Interestingly, the identification of the glycosylation sites with tandem MS revealed that the peptides are glycosylated at different serines. The hδORPhe27 peptide was glycosylated at Ser25 by both GalNAc-T2 and T16. In contrast, the hδORCys27 peptide was glycosylated at Ser25 by GalNAc-T16 but at Ser29 by GalNAc-T2. It appears that cysteine at +2 may be unfavorable for GalNAc-T2, leading to altered glycosylation at these sites. Only after a 16-h incubation, a hδORCys27 peptide carrying a second glycan at Ser25 began to emerge. The glycosylation at Ser25 may also promote further
glycosylations in the peptide. This would explain why the hδOR\textsuperscript{Phe27} peptide was more efficiently glycosylated at multiple sites. The second glycosylation had begun during the first 4 h of incubation, and after 16 h, most of the peptides had three or four sites occupied. This hypothesis is supported by a study that determined that O-GalNAc-Ser at position -4 is favorable for glycosylation by GalNAc-T2 (e.g. glycosylation of Ser29 after Ser25 in hδOR\textsuperscript{Phe27}), and unfavorable at position +4 (e.g. glycosylation of Ser25 after Ser29 in hδOR\textsuperscript{Cys27}) (Perrine et al., 2009).

Importantly, the \textit{in vitro} results are unlikely to fully reflect hδOR glycosylation that takes place \textit{in vivo}. Some GalNAc-T isoforms have been shown to prefer substrates with different degrees of glycosylation already present in the protein (Pratt et al., 2004). Thus, the use of single isoforms in the \textit{in vitro} assay may not reveal enzymes that glycosylate only sites that have O-GalNAc residues already in the close proximity. Furthermore, the presence of N-glycans in cells are likely to at least inhibit glycosylation at the overlapping sites at Ser20 and Ser35 of hδOR. Interestingly, differential N-glycosylation of the variants, and the presence of receptors with only a single N-glycan may lead to further differences between the variants due to differences in the steric effect of the N-glycans.

The \textit{in vitro} tools provide easily interpretable results and accurate predictions of O-glycosylation of proteins. To complement these findings, both the GalNAc-T isoforms involved and the glycosylation sites of hδOR variants were confirmed in cells. To this end, site-directed mutagenesis of glycosylation sites followed by mutant receptor expression in HEK293 SC cells and subsequent purification with VVA was performed. The mutagenesis of Ser25 and Ser29 to alanine resulted in drastic reduction in VVA binding, indicating a loss of O-GalNAc glycans in one or both of these sites. Only the hδOR-S25:29A mutant species with a single N-glycan still bound VVA, which may be due to the fact that the species was the most abundant one, as the absence of an N-glycan may remove some of the steric hindrance and increase the affinity for VVA. The remaining VVA binding was almost completely abolished with an additional mutation at Ser6. The lack of VVA binding after these mutations confirms that Ser20 and Ser35 overlapping with N-glycosylation sites are unlikely to be occupied by O-glycans. Importantly, the ability of the hδOR-N18;33E variants to bind to VVA revealed that the same sites were O-glycosylated in the mutants as well as the WT receptors, and no additional sites were used, despite the absence of N-glycans.

The hδOR was also expressed in \textit{GALNT2} knockout HEK293 and HepG2 cells to confirm the role of GalNAc-T2 in hδOR O-glycosylation. The absence of GalNAc-T2-mediated O-glycosylation in these cells resulted in a shift in mobility.
in SDS-PAGE. The effect was clearly visible when the non-N-glycosylated hδOR-N18;33E constructs were expressed in HepG2 cells that do not express GalNAc-T16. The GalNAc-T16 isoform produced a small amount of glycopeptide in the *in vitro* assay and may, to some extent, complement for the loss of GalNAc-T2 in HEK293 cells.

Further investigation of the manner by which the O-glycans affect hδOR processing and trafficking was performed using CHO-ldlD cells in O-glycosylation permitting and omitting conditions. O-glycosylation in these cells was apparent after medium supplementation with GalNAc and Gal that rescue O- and N-glycosylation (Krieger et al., 1989). The hδOR-N18;33E mutant variants lacking N-glycans were further studied to exclude any possibility of N-glycan processing and to simplify the interpretation of the results. The hδOR-N18;33E variants were observed at the cell surface by FACS, indicating that the O-glycans are not required for trafficking to the plasma membrane. However, the expression levels were drastically reduced, suggesting that O-glycans are required to maintain normal receptor levels at the cell surface. The effect was already observed after GalNAc supplementation that merely initiates receptor O-glycosylation. The CHO cells lack the sialyltransferase enzyme that catalyzes the sialylation of the O-GalNAc residue in certain other cells (Yang et al., 2014). This implies that these effects are unlikely to be explained by receptor O-glycan-mediated interactions with other proteins as the terminal sialic acids that have been demonstrated to be involved in these interactions (Remacle et al., 2006; Rutledge & Enns, 1996) were missing. Instead, glycans more likely elicit a stabilizing effect on the polypeptide, an effect that has been demonstrated for other proteins (Y. Xu et al., 2016). Indeed, the cell surface receptor levels of the biotinylated hδOR-N18;33E were significantly higher in O-glycosylation permitting conditions, and the turnover was significantly slower. Again, this effect was seen by mere GalNAc supplementation. Thus, O-glycans and N-glycans alike appear to have a stabilizing role in maintaining adequate receptor levels at the cell surface. The role of O-glycans, however, is directly related to the stability of the receptor at the cell surface, whereas N-glycans affect the receptor conformation already during biosynthesis.

Levels of cell surface receptor expression defines the cellular capacity to transmit extracellular signals into the cells (C. M. Cahill et al., 2007; X. Zhang et al., 2010). This is modulated via molecular determinants and cellular trafficking. In particular, the levels of constitutively active receptors, such as hδOR directly modulate its signaling capability. The S6;25;29A mutation with disrupted receptor O-glycosylation resulted in lower levels of ligand binding-capable receptors at the
cell surface as measured by FACS with a FITC-conjugated opioid antagonist. Lower levels were also obtained for the WT hδOR expressed in GALNT2 knockout HEK293 cells, confirming that the effect is specific to GalNAc-T2 mediated O-glycosylation. The lack of O-glycosylation on hδOR-S6;25;29A resulted in diminished signaling output as the hδOR agonist DADLE-mediated inhibition of cAMP accumulation was attenuated in HEK293 cells. Altogether, these results support the notion that O-glycosylation directly modulates the levels of functional hδORs at the cell surface via receptor stabilization, and as a result maintains the adequate cellular signaling capacity of the receptor.

The GalNAc-type O-glycosylation of dozens of tandem repeats in mucins and mucin-like domains produces heterogeneous glycoproteins, in which the glycans make up a large portion of the molecular mass of the glycoproteins and dictate the physical properties of the protein (Perez-Vilar & Hill, 1999; Round et al., 2002). In contrast, the functional role of isolated O-GalNAc glycans in proteins with only a few glycans is poorly understood. Site-specific, isolated O-GalNAc glycans added by specific GalNAc-T isoforms have only recently emerged as regulators of protein function. Site-specific glycosylation of the fibroblast growth factor 23 at Thr178 was found to inhibit the proteolytic cleavage of the adjacent proprotein convertase-processing site (Kato et al., 2006). Interestingly, the glycosylation was catalyzed specifically by a single isoform, GalNAc-T3 (Kato et al., 2006), the deficiency of which has been attributed to cause familial tumoral calcinosis (Topaz et al., 2004). Likewise, GalNAc-T13 that is upregulated during neuronal development was shown to specifically glycosylate certain sites in podoplanin. The knockout of GalNAc-T13 and knockdown of podoplanin both suppressed neuronal differentiation, suggesting that O-glycosylation of podoplanin by GalNAc-T13 has an important role during these events. This was supported by an observation that the O-glycosylation improved the stability of podoplanin (Y. Xu et al., 2016). The O-glycosylation of Ser25 and Ser29 of hδOR were similarly catalyzed primarily by a single isoform, resulting in a distinct behavior and altered signaling output of the receptor at the cell surface. Unlike with the heavily O-glycosylated mucins, the few glycans in the receptor N-terminus are unlikely to dominate the physical characteristics of the protein. Rather, the site-specific protein O-glycosylation is more likely to present a fine-tuning element in the processing and modulation of proteins, such as GPCRs. A recent example of this is the site-specific O-glycosylation of β1AR by GalNAc-T2 that was demonstrated to co-regulate the proteolytic processing of the receptor (Goth et al., 2017).
The peptides corresponding to the hδOR variants were differentially O-glycosylated in the in vitro assays. However, no significant differences in O-glycosylation were observed with the variants expressed in cells. Mutation of Ser6, Ser25 and Ser29 resulted in a similar reduction in VVA binding, with no apparent O-glycosylation involving Ser20 and Ser35. Furthermore, a similar capacity of the O-glycans in promoting hδOR stability and cell surface signaling was observed for the variants. It is yet possible that stoichiometric differences exist in the glycosylation of Ser25 and Ser29, as suggested by the in vitro results, or that the differences are more apparent in other cells or in vivo in natural tissues. More detailed investigation of this possibility would require individual mutagenesis of these sites, or preferably a MS analysis of purified hδOR N-termini. The indistinguishable O-glycosylation-characteristics of the variants are in agreement with the notion that polymorphic differences in the constitutive downregulation arise from specific behavior and processing of the hδORCys27 variant in the ER, rather than processing in the Golgi or at the cell surface. However, the site-specific O-glycosylation by GalNAc-T2 can potentially accentuate the phenotypic differences in specific cells and conditions. The hδORCys27 that is more prone to constitutive internalization may display even more rapid downregulation, leading to diminished signaling capacity, compared to the hδORPhe27. The differences may also alter the development of opioid tolerance, which has been suggested to involve receptor internalization (Koch & Hollt, 2008). Furthermore, in light of the recent evidence suggesting sustained δOR-mediated signaling from endocytic compartments (Stoeber et al., 2018), the receptor O-glycosylation may directly influence the spatiotemporal opioid signaling.

The study sheds light on the enigmatic role of this glycan modification of GPCRs occurring during its synthesis. The O-glycans present an important molecular determinant in the modulation of GPCR signaling at the cell surface. Site-specific modification of receptor N-termini by the GalNAc-T family reveals an entirely new aspect in the regulation of the GPCR family.
7 Conclusions

The cellular signaling by GPCRs is a highly regulated process. The capacity to respond to extracellular signals and the magnitude of cellular response are largely dictated by the number of functional receptors at the cell surface. The on-demand nature of responses in a highly complex and sensitive environment requires intricate control of functional receptor levels at the cell surface. This is achieved by regulation of the export and delivery of GPCRs to the cell surface, and by trafficking away from the cell surface. The hδOR is a GPCR that is subjected to time consuming and error-prone processing during its synthesis. Two common variants of hδOR have identical pharmacological properties but differ in cellular processing. The SNP is associated with serious neurological conditions, such as Alzheimer’s disease and opioid addiction, underlining the importance of these processes in the function of the receptor.

Here, several key findings on hδOR processing in the secretory pathway were obtained to further the knowledge of GPCR regulation during its maturation, and to elucidate the differences between the variants at the cellular level.

1. Two pathways scrutinize the maturing receptors in the ER for the quality of their conformation. An N-glycan-dependent pathway employing CNX is required for the fully functional expression of hδOR(Cys27), whereas the more rapid N-glycan independent back-up pathway is sufficient for hδOR(Phe27).

2. Receptor oligomerization in the ER modulates the cell surface delivery of hδOR. In cells expressing both variants, this leads to an attenuation of hδOR(Phe27) cell surface targeting by hδOR(Cys27), which retains and targets the hδOR(Phe27) for degradation in a dominant-negative manner.


The multiple pathways that mediate intracellular receptor retention in different conditions, and the impact of glycosylation and oligomerization in modulating the levels of functional receptors at the cell surface underline the importance of these events for opioid signaling. They modulate directly the signaling capacity of the receptor in the opioid system and may alter the sensitivity to various opioid treatments. The pathways and molecular determinants examined here likely affect
other GPCRs as well, albeit in a receptor-specific manner. Thus, the findings provide insights into the regulation of the entire protein family, and have implications for emerging promising strategies, such as for targeting δOR-μOR heteromers in the treatment of chronic pain, or for treating conditions caused by GPCR-retaining mutations and SNPs.
References


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