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N-linked Glycosylation on the N-terminus of the dopamine D_2 and D_3 receptors determines receptor association with specific microdomains in the plasma membrane

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ABSTRACT

Numerous G protein-coupled receptors (GPCRs) are glycosylated at extracellular regions. The regulatory roles of glycosylation on receptor function vary across receptor types. In this study, we used the dopamine D_2 and D_3 receptors as an experimental model to understand the underlying principles governing the functional roles of glycosylation. We used the pharmacological inhibitor, tunicamycin, to inhibit glycosylation, generated chimeric D_2 and D_3 receptors by swapping their respective N-termini, and produced the glycosylation site mutant D_2 and D_3 receptors to study the roles of glycosylation on receptor functions, including cell surface expression, signaling, and internalization through specific microdomains. Our results demonstrate that glycosylation on the N-terminus of the D_3 receptor is involved in the development of desensitization and proper cell surface expression. In addition, glycosylation on the N-terminus mediates the internalization of D_2 and D_3 receptors within the caveolae and clathrin-coated pit microdomains of the plasma membrane, respectively, by regulating receptor interactions with caveolin-1 and clathrin. In conclusion, this study shows for the first time that glycosylation on the N-terminus of GPCRs is involved in endocytic pathway selection through specific microdomains. These data suggest that changes in the cellular environment that influence posttranslational modification could be an important determinant of intracellular GPCR trafficking.

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1. Introduction

Extracellular, cytoplasmic, and transmembrane domains are commonly found in G protein-coupled receptors (GPCRs). Domain roles in ligand binding, intracellular trafficking, and signal transduction vary depending on the receptor families of the protein. The GPCR extracellular domain is composed of N-terminus and extracellular loops. GPCRs are grouped into several classes based on the organization of their N-terminus [1]. Because the N-terminus is highly variable in size and sequence, functional roles of the extracellular domain are less clear than for other domains [2].

N-linked glycosylation is a highly conserved post-translational modification that occurs on the Asn-X-Ser/Thr motif on the extracellular domains of GPCRs [3–5]. The extent of glycosylation and its influence on receptor function varies according to receptor type and cellular environment. Therefore, carefully controlled experiments are necessary to understand the broader functional roles of glycosylation and the underlying regulatory mechanisms.

Dopamine D_2 and D_3 receptors, the primary targets of antipsychotic drugs, possess high amino acid sequence homology, similar pharmacological properties, and act similarly in signaling pathways. Despite pharmacological and functional similarities, D_2 and D_3 receptor exhibit distinct intracellular trafficking and desensitization properties [6]. Thus, these two receptors can serve as an excellent experimental model to assess the broad roles of glycosylation in receptor function, specifically cell surface expression, internalization, and signaling.

The human D_2 receptor contains three potential *N*-linked glycosylation sites, all in the N-terminus: Asn-5, Asn-17, and Asn-23. The D_3 receptor has four potential glycosylation sites: Asn-12 and Asn-19 in the N-terminus, Asn-97 in the first extracellular loop, and Asn-173 in the second extracellular loop. Glycosylation of these receptors was reported in the porcine pituitary D_2 receptor [7] and cloned D_2 and D_3

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receptors [8,9]. In the D_2 receptor, glycosylation is essential for proper cell surface expression and internalization [10,11]. The functional roles of glycosylation for the D_3 receptor, however, have not been reported.

The objective of this study was to determine whether a generalized principle exists to predict the roles of *N*-linked glycosylation in receptor function. We hypothesized that distinct N-terminal functions, specifically characteristic glycosylation patterns, could determine the differential regulatory properties of these receptors. Parallel and combined studies of the D_2 and D_3 receptors demonstrate that *N*-linked glycosylation of the N-terminus regulates association of the receptor with specific microdomains in the plasma membrane. Our results reveal a novel principle governing the control of intracellular GPCR trafficking of through regulation of *N*-linked glycosylation.

2. Materials and methods

2.1. Materials

Human embryonic kidney HEK-293 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Tissue culture media and fetal bovine serum were obtained from Life Technologies (Grand Island, NY, USA). Dopamine (DA), quinpirole, haloperidol, phorbol 12-myristate 13-acetate (PMA), forskolin, methyl-β-cyclodextrins (MβCD), and antibodies to actin, FLAG, and green fluorescent protein (GFP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Endoglycosidase H (Endo H) and peptide N-glycosidase F (PNGase F) were purchased from New England Biolabs (Ipswich, MA, USA). [^{3}H]-Sulpiride (84 Ci/mmol) and [^{3}H]-spiperone (85.5 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Tunicamycin, antibodies to GRK2 and clathrin heavy chain, and HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caveolin-1 antibody was purchased from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Antibody to β-arrestins was provided by Dr. Lefkowitz (Duke University, Durham, NC, USA).

2.2. Plasmid constructs

Wild-type human D_2 receptor (short form) and D_3 receptor were reported previously [12]. The D_2 and D_3 receptors were tagged with the M2-FLAG epitope (DYKDDDDA) at the N-terminus, inserted after Met[1]. Chimeric receptors with exchanged N-termini (D_2R-D_3NT and D_3R-D_2NT), WT-D_2 and D_3R-D_2NT receptor glycosylation mutants (N5Q/N17Q/N23Q), a series of N-terminus deletion mutants (from Δ2 to Δ20), D_3R glycosylation mutants (N12Q, N19Q, N11Q/N19Q, N97Q/N173Q, N12Q/N19Q/N97Q/N173Q), and a D_2R-D_3NT (N12Q/N19Q) glycosylation mutant were produced by site-directed mutagenesis and subcloned into pCMV5. Some constructs were FLAG-tagged at the N-terminus or GFP-tagged at the C-terminus. Small hairpin RNAs for clathrin heavy chain, caveolin-1, GRK2, and β-arrestins were used as described previously [13–16]. To study the effects of tunicamycin treatment on ligand binding, signaling, and internalization, we adjusted the amount of plasmids for transfection (2–3 times more plasmids were used in the tunicamycin-treated groups compared to the vehicle-treated groups).

2.3. Determination of ligand-binding properties

HEK-293 cells expressing D_2 or D_3 receptors were incubated with 2.2 or 7.2 nM [^{3}H]-sulpiride and with increasing concentrations of DA (10^{-9}–10^{-4} M for D_2 receptor, 10^{-13}–10^{-8} M for D_3 receptor) for 150 min at 4 °C [17]. Cells were washed three times with ice-cold serum-free Minimum Essential Medium containing 10 mM HEPES, pH 7.4, lysed with 1% sodium dodecyl sulfate (SDS), and counted using a liquid scintillation counter.

2.4. Receptor internalization assay

D_2 and D_3 receptor internalization was assessed based on the hydrophilic properties of [^{3}H]-sulpiride [17]. HEK-293 cells expressing the D_2 or D_3 receptor were seeded 1 day post-transfection at a density of 1.5×10^5 cells/well in 24-well plates. On the following day, cells were rinsed once and pre-incubated with 0.5 ml of pre-warmed, serum-free media containing 10 mM HEPES, pH 7.4, for 15 min at 37 °C. Subsequently, cells were stimulated with 10 μM DA for 60 min or 100 nM PMA for 30 min. Cells were incubated with 250 μl [^{3}H]-sulpiride (final concentration of 2.2 nM for D_2 receptor and 7.2 nM for D_3 receptor) at 4 °C for 150 min in the presence and absence of 10 μM haloperidol. Cells were washed and then lysed with 1% SDS. Remaining radioactivity was counted using a liquid scintillation counter.

2.5. Immunoprecipitation

Cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, 10% glycerol) on a rotation wheel for 1 h at 4 °C. Supernatant was combined with 20 μl of a 50% slurry of anti-FLAG agarose beads for 2–3 h on a rotation wheel. Beads were washed with washing buffer (50 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% NP-40) three times for 10 min each. The resulting immunoprecipitates were analyzed by immunoblotting.

2.6. Endo H and PNGase F cleavage

Crude membrane fractions were obtained from cells expressing FLAG-tagged D_2 or D_3 receptor according to protocols described previously [18]. Cell lysates were further solubilized with RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% NP-40, 0.5% deoxycholate, 0.1% SDS). Immunoprecipitates were dissolved in 30 μl denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) and heated at 65 °C for 20 min. Samples were then treated with 10 units/μl Endo H or PNGase F at 37 °C for 4 h. Samples were heated at 65 °C for 20 min and analyzed by immunoblotting.

2.7. Whole cAMP assay

Cellular cAMP was measured through an indirect method: a reporter plasmid containing the firefly luciferase gene under control of cAMP response elements and a pRL-TK control vector [19]. Transfected cells were seeded in 24-well plates, and each transfection set was organized into three identical groups. Cells were treated with 2 μM forskolin and quinpirole (10^{-13}–10^{-8} M) for 4 h and harvested. Relative luciferase expression was measured using a dual luciferase assay kit (Promega, Madison, WI, USA). Data were normalized by expressing the cAMP level as a percentage of forskolin-stimulated cAMP in each experiment. Dose–response curves were fitted with the GraphPad Prism software (GraphPad, San Diego, CA, USA).

2.8. Confocal microscopy

One day post-transfection, cells were seeded onto the 35-mm confocal dishes, which contain a 1-cm well in the center sealed by a glass coverslip on the bottom. Cells were allowed 1 day to recover, and then were examined with a TCS SP5/AOBS/Tandem laser scanning confocal microscope (Leica, Jena, Germany).

2.9. Primary brain cell culture

Culture of primary rat brain cortical neuronal cultures was described previously [20]. Cells were transfected using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). Cells were observed with an LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.10. Statistical analysis

All data are expressed as means ± SEM. Comparisons among groups were shown using two-way analysis of variance with Bonferroni post-tests. Student's *t*-test was also used to compare results between groups.

3. Results

3.1. Functional characterization of the N-terminus of dopamine D_3 receptors

The crystal structure of the D_3 receptor was previously reported [21]. However, details regarding the N-terminus attached at the 1st transmembrane domain (TM) have not been resolved due to its high flexibility in the crystal. The N-terminal regions of GPCRs are highly variable. Alignment of amino acid sequences of the N-terminal regions of D_2 and D_3 receptors show that they are disparate in composition, except for several amino acid residues near the 1st TM (Fig. 1A).

The functional roles of the N-terminus were studied by sequentially deleting the amino acid residues from the beginning of the D_3 receptor. Total amount of receptor was not affected by deletion up to 20 amino acid residues within the N-terminus (Fig. S1A) but surface expression was significantly inhibited by deletion of as few as 5 amino acid residues (Fig. S1B). The decrease in surface expression was not due to altered affinity of the receptor to the radioligand, $[^3H]$-sulpiride (Fig. S1C). Moreover, the last 4 amino acids in the N-terminus of the D_3 receptor were important for D_3 receptor desensitization (Fig. S1D and S1E).

Because this region contains two *N*-linked glycosylation sites (N12 and N19), we tested the involvement of glycosylation in surface expression. As shown in Fig. S1F, inhibition of D_3 receptor glycosylation by treatment with tunicamycin (1 μg/ml, 24 h) yielded similar outcomes as the glycosylation mutant (Δ(2–20)). These results suggest that glycosylation on the N-terminus of D_3 receptor could serve important roles in various receptor functions.

3.2. Both dopamine D_2 and D_3 receptors are glycosylated

Tunicamycin inhibits the synthesis of *N*-linked glycoproteins and has been used to study the functional roles of glycosylation. When cells were treated with tunicamycin, the molecular weights of D_2 and D_3 receptors, as observed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), decreased (Fig. 1B and 1C). With tunicamycin treatment (1 μg/ml, 24 h), a protein band at approximately 45 kDa shifted lower on the gel, suggesting that both the D_2 and D_3 receptors are glycosylated. These results were confirmed by treatment with enzymes that remove glycans. For example, Endo H selectively removes *N*-linked glycans with high mannose and/or hybrid types from glycoproteins, but does not remove the complex type [22]. PNGase F removes all *N*-linked glycans from glycoproteins [23]. Glycoproteins, which are correctly processed through the endoplasmic reticulum and Golgi apparatus, become resistant to Endo H. In agreement with this, the cell lysates from cell membranes containing D_2 or D_3 receptors were sensitive to PNGase F, and exhibited a protein band shift from 45 kDa to approximately 35 kDa (Fig. 1D and 1E).

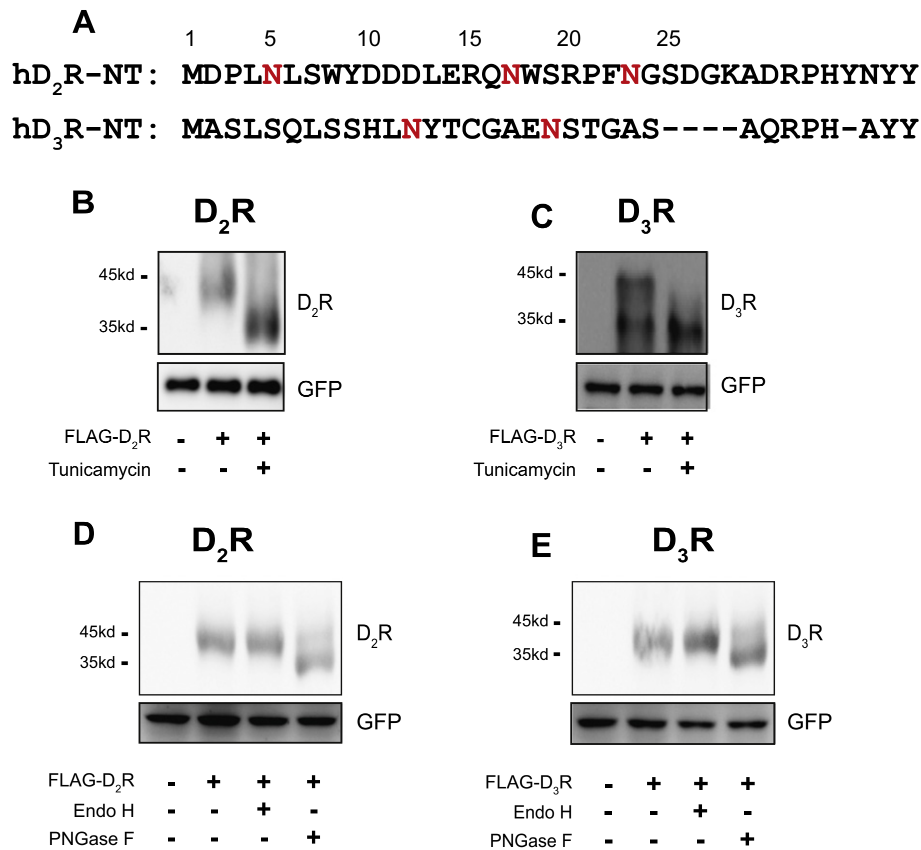

Fig. 1. Effect of tunicamycin treatment on dopamine D_2 and D_3 receptors migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Alignment of amino acid sequences in the N-terminal regions of the human D_2 and D_3 receptors. Potential glycosylation sites on the D_2 and D_3 receptors are shown in red. (B, C) Cells expressing green fluorescence protein (GFP) and FLAG-tagged D_2 receptor or D_3 receptor were treated either with vehicle or 1 μg/ml tunicamycin for 24 h. Cell lysates were immunoprecipitated with FLAG beads, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with antibodies to FLAG (immunoprecipitates) and GFP (lysates). Transfection efficiencies were normalized by including pEGFP vector. (D, E) Cells were transfected with FLAG-tagged D_2 or D_3 receptor, and cell lysates were immunoprecipitated with FLAG beads. Immunoprecipitates were treated with endoglycosidase H (Endo H) or peptide N-glycosidase F (PNGase F). Samples were analyzed by SDS-PAGE and blotted with FLAG antibody. Data (B–E) represent results from at least five independent experiments.

3.3. Role of glycosylation on agonist affinity and surface expression of D_2 and D_3 receptors

To test the effect of deglycosylation on the receptor affinities for agonist, cells were treated with tunicamycin and radioligand/agonist competition experiments were conducted. As shown in Fig. 2A and 2B, a change in D_2 and D_3 receptor affinity for DA was not evident when glycosylation was inhibited. This was expected because the ligand-binding pockets of the D_2 and D_3 receptors are buried within the transmembrane domains, away from the N-terminus [21,24].

Next, we tested the effects of deglycosylation on cell surface expression of the D_2 and D_3 receptors. Treatment of cells with tunicamycin increased localization of receptors in the cytosol (Fig. 2C). The proportion of D_2 and D_3 receptors on the cell surface (cell surface/total) decreased by about 20% and 50%, respectively (Fig. 2C).

3.4. Identification of N-linked glycosylation sites of the D_3 receptor

Four possible *N*-linked glycosylation sites are located within the extracellular region of the D_3 receptor (N12, N19, N97, and N173; Fig. 3A) [6,25], these sites were mutated either individually or in combination by site-directed mutagenesis. As observed with tunicamycin treatment, the 45-kDa protein band disappeared and a new band about 35 kDa appeared with N12Q/N19Q- and N97Q/N173Q-D_3R (Fig. 3B). If the D_3 receptor was glycosylated on both the N-terminus and extracellular loop, we expected that the 35 kDa band would be observed with another receptor band of higher molecular weight. However, it was not clear whether the N12/19Q- and N97/N173Q-D_3R receptor bands were different from that of the WT-D_3 receptor treated with tunicamycin. We speculate that glycosylation on the N-terminus and extracellular loops might exert a mutual regulatory effect.

Individual mutation of the four potential *N*-linked glycosylation sites did not have any effect on cell surface expression of the D_3 receptor (Fig. 3C). The simultaneous mutation of N12 and N19 residues (N12Q/N19Q), but not N97 and N173 residues (N97Q/N173Q), resulted in decreased cell surface expression of D_3. The mutations of all four possible *N*-linked glycosylation sites (N12Q/N19Q/N97Q/N173Q) did not further decrease D_3 receptor surface expression compared to the N12Q/N19Q mutant. These results suggest that *N*-linked glycosylation on the N-terminus is involved in receptor cell surface expression. Mutation

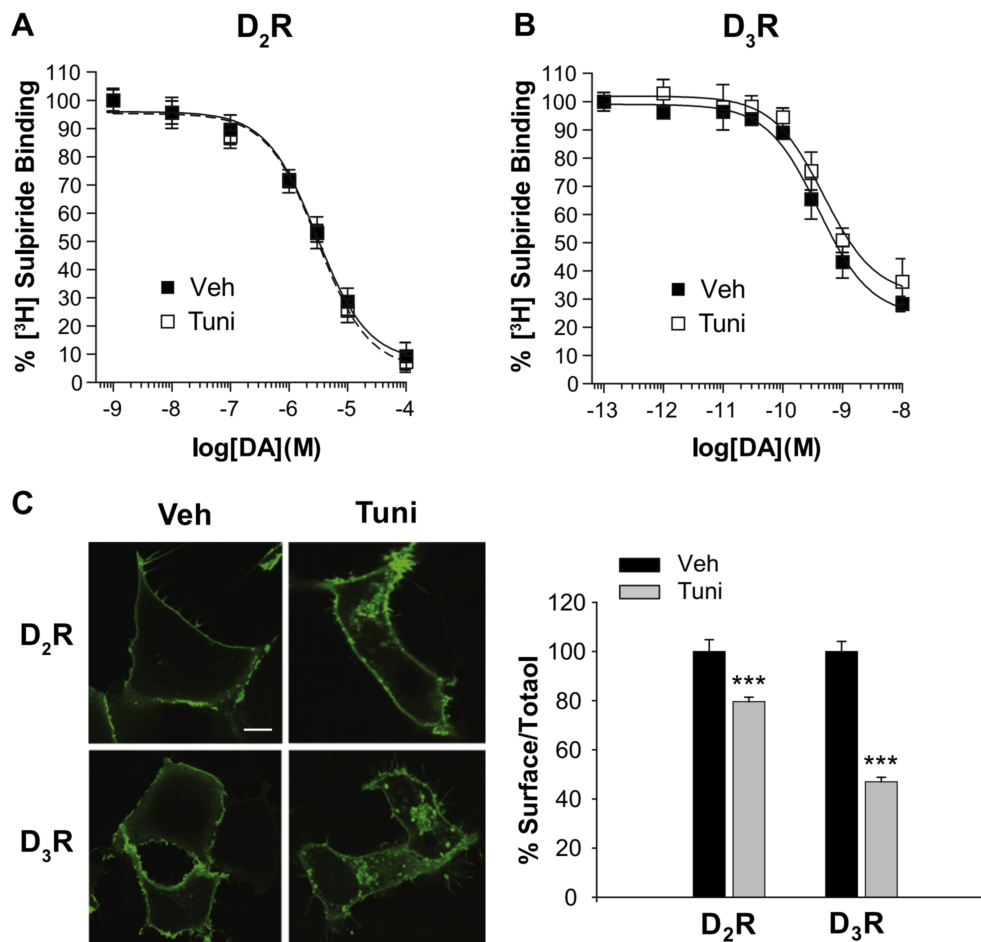

Fig. 2. Effect of tunicamycin treatment on agonist affinity for and cell surface expression of dopamine D_2 and D_3 receptors. (A, B) Cells expressing D_2 or D_3 receptor were treated either with vehicle or with 1 μg/ml tunicamycin for 24 h. Cells expressing D_2 or D_3 receptors were incubated with 2.2 or 7.2 nM [^{3}H]-sulpiride, respectively, and with increasing concentrations of dopamine (DA) for 1 h at room temperature in the presence or absence of 10 μM haloperidol. Cells were washed three times with ice-cold serum-free medium, dissolved in 1% SDS, and then were counted using a liquid scintillation counter. The Ki value of DA for D_2 receptor was 1.088–2.018 μM and 0.63–3.76 μM for vehicle- and tunicamycin-treated group at 95% confidence intervals, respectively. The K_i value of DA for D_3 receptor was 13.0–30.5 pM and 20.2–47.8 pM for vehicle- and tunicamycin-treated group at 95% confidence intervals, respectively. Each data point represents mean ± SEM (n = 3). Data represent results from two independent experiments with similar outcomes. (**C**) Effects of tunicamycin treatment on subcellular localization of D_2 and D_3 receptors. Cells were transfected with D_2 or D_3 receptor constructs. After 4 h, cells were treated with 1 μg/ml tunicamycin for 24 h. The horizontal bar represents 10 μM. Ratio of cell surface/total was determined with binding studies using [^{3}H]-sulpiride and [^{3}H]-spiperone, which bind cell surface receptors and total receptors, respectively. Each data point represents mean ± SEM (n = 3). Data represent results from two independent experiments with similar outcomes.

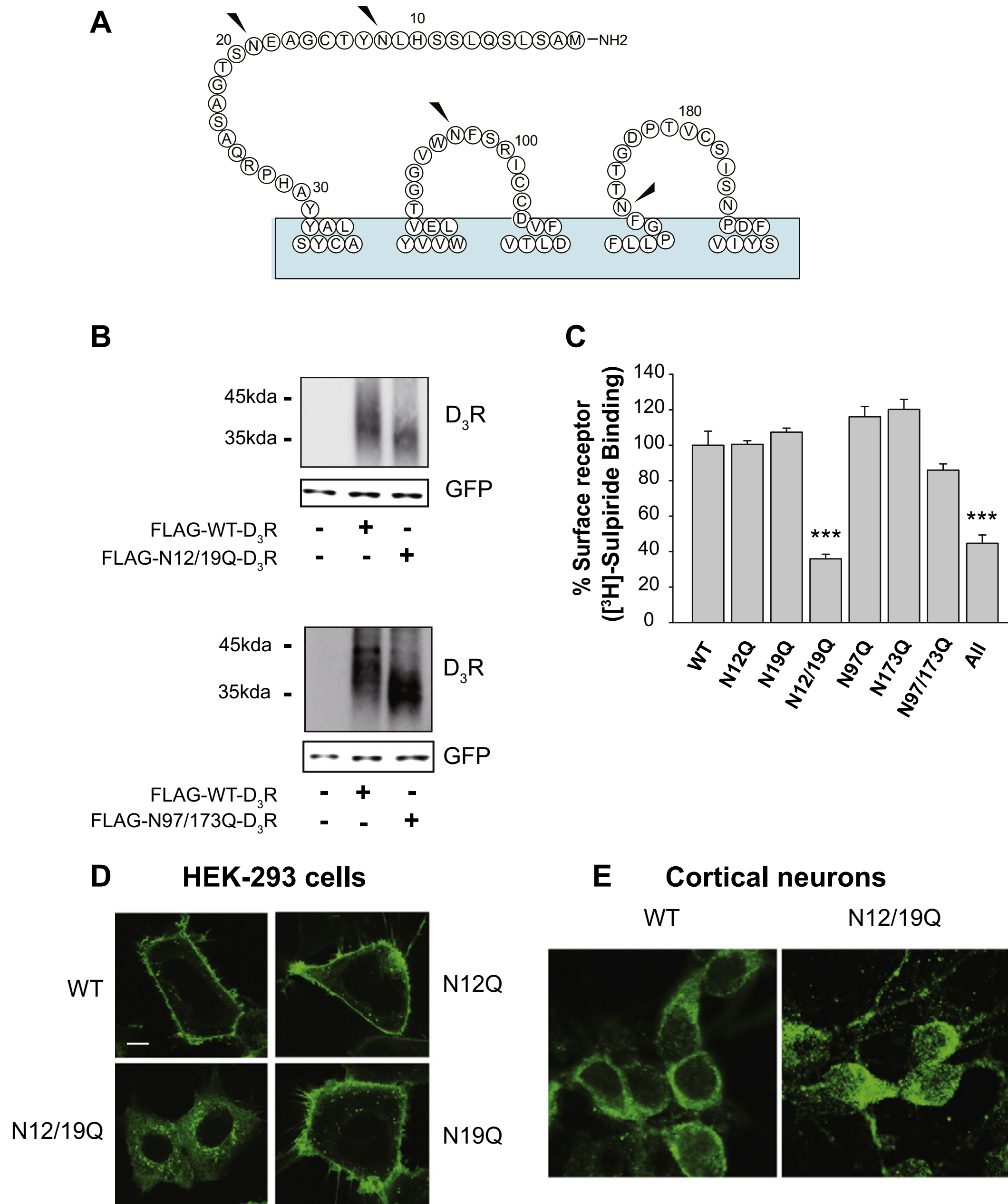

Fig. 3. Role of *N*-linked glycosylation on the N-terminus or extracellular loops of the D_3 receptor on cell surface expression. (A) Alignment of amino acid sequences within the N-terminal regions and extracellular loops of the D_3 receptor. Potential *N*-linked glycosylation sites are shown (arrow heads). Four potential *N*-linked glycosylation sites on the N-terminus (N^{12} and N^{19}) and extracellular loops (N^{97} and N^{173}) were mutated to glutamine (Q) residues individually or in combination. (B) (Upper panel) Cells were transfected with FLAG-tagged WT- or N12/19Q-D_3R together with GFP vector. (Lower panel) Cells were transfected with FLAG-tagged WT- or N97/173Q-D_3R together with GFP vector. Cell lysates were immunoprecipitated with FLAG beads, analyzed by SDS-PAGE, and immunoblotted with FLAG antibody. Cell lysates were immunoblotted with GFP antibody. Data represent results from four independent experiments. (C) Effects of mutations in the *N*-linked glycosylation sites on cell surface expression of the D_3 receptor. HEK-293 cells were transfected with 2 μg of each glycosylation mutant in pCMV5 per 100-mm culture dish. Cells were treated with 7.2 nM [^{3}H]-sulpiride dissolved in serum-free medium for 1 h at room temperature in the presence and absence of 10 μM haloperidol. ***$p < 0.001$ compared to WT-D_3 receptor. Each data point represents mean ± SEM ($n = 3$). Data represent results from three independent experiments. (D, E) Microscopic images of D_3 receptor glycosylation mutants in HEK-293 cells (D) and primary cortical neurons (E). Cells were transfected with WT-, N12Q-, N19Q-, and N12/19Q-D_3R in pEGFP-N1, and images were obtained 36 h later with a laser scanning confocal microscope. The horizontal bar represents 10 μM. Images were captured with TCS SP5/AOBS/Tandem laser scanning confocal microscope (D), and Carl Zeiss LSM710 (E). Data represent results from two independent experiments.

of either N12 or N19 alone did not affect the cell surface expression, suggesting that N12 and N19 may compensate for each other when one is blocked. These results were confirmed by observing the subcellular distribution of GFP-tagged D_3 receptor in HEK-293 cells. The WT-D_3 receptor and the individually mutated receptors were found primarily on the plasma membrane, while the doubly mutated receptors were found in the perinuclear regions and in the cytosol (Fig. 3D). Similar results were observed in the primary cultured cortical neurons (Fig. 3E).

3.5. Different functional roles of glycosylation on the D_3 receptor N-terminus and extracellular loops

Because glycosylation occurs on both the N-terminus and extracellular loops of the D_3 receptor, we predicted that glycosylation may have different functions for different domains. Mutation of the glycosylation sites on the N-terminus (N12, N19) inhibited both D_3 receptor signaling and desensitization (Fig. 4A). In contrast, the mutation of the N97 and

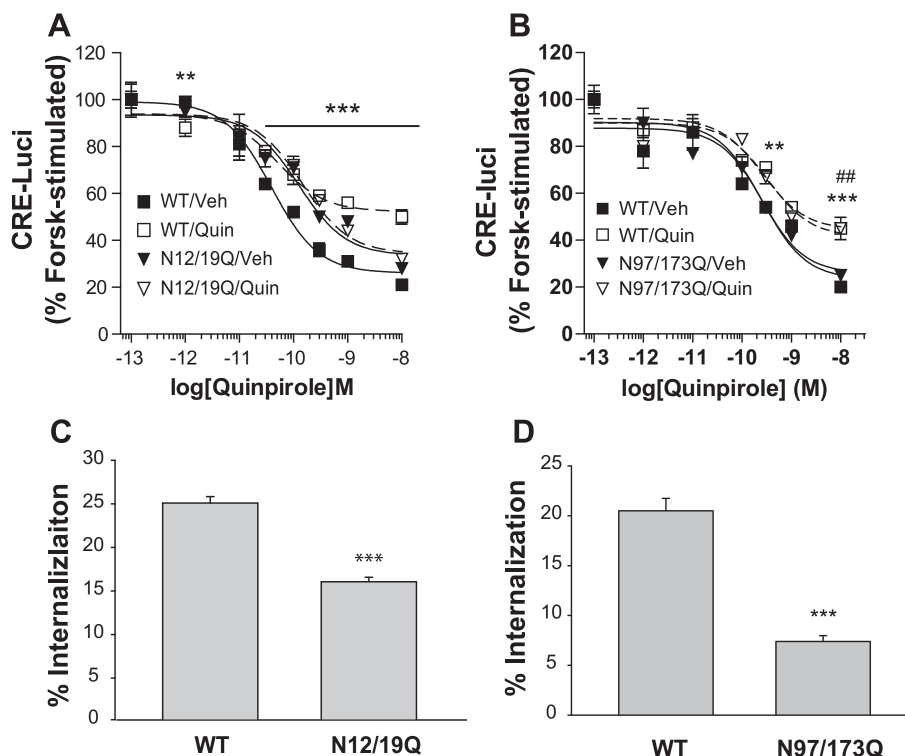

Fig. 4. Role of *N*-linked glycosylation on the N-terminus or extracellular loops on signaling, desensitization, and internalization of the D_3 receptor. (A, B) Effects of deglycosylation on desensitization properties of the D_3 receptor. (A) Cells were transfected with WT- and N12/19Q-D_3R at a ratio of 1:10. After 36 h, cells expressing WT- or N12/19Q-D_3R were treated with 100 nM quinpirole for 5 min to induce desensitization. $^{**}p < 0.01$, $^{***}p < 0.001$ when WT/Veh group was compared to WT/Quin group. When WT/Veh group was compared to N12/N19/Veh group, $p < 0.01$ at 3×10^{-10} M; $p < 0.001$ between 10^{-10} and 10^{-9} M of quinpirole. (B) Cells expressing WT- or N97/173Q-D_3R were treated with 100 nM quinpirole for 5 min to induce desensitization. $^{**}p < 0.01$, $^{***}p < 0.001$ when WT/Veh group was compared to WT/Quin group. $^{\#\#}p < 0.01$ when N97/173Q/Veh group was compared to N97/173Q/Quin group. Each data point represents mean ± SEM (n = 3). Data represent results from two independent experiments. (C, D) Cells were transfected with WT- or N12/19Q-D_3R at a ratio of 1:10 (C) or with WT- or N97/173Q-D_3R (D). After 36 h of transfection, cells were treated with 100 nM PMA for 30 min. $^{***}p < 0.001$ compared to the WT group. Each data point represents mean ± SEM (n = 3). Data represent results from two independent experiments.

N173 sites on the extracellular loops did not affect D_3 receptor signaling and desensitization (Fig. 4B). In agreement with these results, deletion of the N-terminus region of the D_3 receptor containing N-linked glycosylation sites abolished desensitization (Fig. S1E). In case of D_2 receptor, mutation of consensus *N*-linked glycosylation sites (Fig. S2A) or treatment with tunicamycin (Fig. S2B) did not affect signaling. These results again suggest that functional roles of *N*-linked glycosylation are highly dependent of molecular environments surrounding glycosylation sites, such as sequence homology and location of N-linked glycosylation site within the receptor.

Next, the role of glycosylation on D_3 receptor internalization was tested for using PMA-induced internalization since it was reported that PKC-mediated internalization is the major endocytic pathway of D_3 receptor [26]. Mutation of the glycosylation sites on both the N-terminus (Fig. 4C) and the extracellular loops (Fig. 4D) inhibited D_3 receptor internalization.

3.6. Glycosylation on the N-terminus regulates cell surface expression of D_2 and D_3 receptor independent of the respective N-terminus

Additional studies were conducted to test whether the roles of glycosylation on receptor function are determined by specific characteristics of the N-terminus. To answer this question, we generated chimeric D_2 and D_3 receptors, in which the respective N-termini were exchanged (Fig. 5A). Glycosylation of the chimeric receptors was inhibited either by treating with tunicamycin or by mutating the consensus glycosylation sites, and the resulting receptors were tested for surface expression and internalization.

Interestingly, the newly introduced N-termini influenced surface expression of the chimeric receptors. As shown in Fig. 5B, surface expression of the D_2 receptor decreased with its substitution by the D_3 receptor N-terminus, and surface expression of the D_3 receptor increased with its substitution by the D_2 receptor N-terminus. When the *N*-linked glycosylation sites on the N-termini were mutated, surface expression of each chimeric receptor was inhibited. In agreement with these results, inhibition of glycosylation with tunicamycin reduced surface expression of each chimeric receptor (Fig. 5C). These results suggest that *N*-linked glycosylation is critical in determining proper cell surface receptor expression, independent of the respective N-terminus.

3.7. Glycosylation on D_2 and D_3 N-termini exerts opposite effects on receptor internalization

Next, we tested the effects of N-terminus glycosylation on receptor internalization. DA- and PMA-induced internalization was employed for D_2 and D_3 receptors, respectively, because they are the primary internalization pathways for the receptors [26]. Receptor internalization increased when glycosylation of the WT-D_2 receptor was inhibited by tunicamycin treatment (Fig. 6A) or with mutation of consensus N-terminus glycosylation sites (Fig. 6B, WT vs. GlyX). Similarly, receptor internalization increased with mutation of glycosylation sites in the chimeric D_2 receptor in which the N-terminus was replaced with that of the D_3 receptor (D_2R-D_3NT) (Fig. 6B, D_3NT vs. D_3NT-GlyX).

PMA-induced internalization decreased when glycosylation of the WT-D_3 receptor was inhibited by tunicamycin (Fig. 6C) or when N-terminus glycosylation sites were mutated (Fig. 6D, WT vs. GlyX). Similarly, mutation of glycosylation sites of the chimeric receptor,

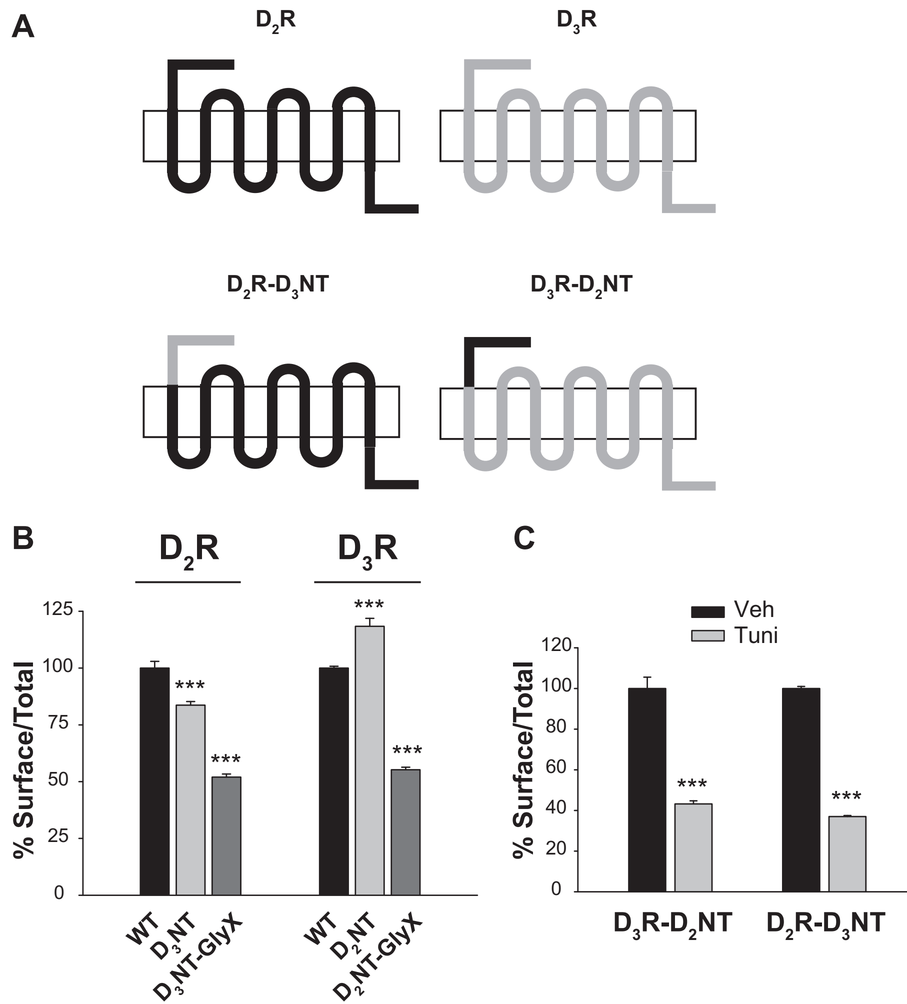

Fig. 5. Role of N-terminus glycosylation on surface expression of the D_2 and D_3 receptors. (A) Scheme for chimeric D_2 and D_3 receptors, in which N-termini of D_2 and D_3 were switched. (B) Effects of switching N-termini between the D_2 and D_3 receptors and deglycosylation of N-termini on the surface expression of the D_2 and D_3 receptors. Receptor expression was determined using [^{3}H]-spiperone (3 nM) for total receptors and [^{3}H]-sulpiride (2.2 and 7.2 nM for the D_2 and D_3 receptor, respectively) for the receptors expressed on the cell surface. ***$p < 0.001$, compared to the corresponding WT group. (C) Effects of tunicamycin on cell surface expression of the chimeric receptors, in which the N-termini of the D_2 and D_3 receptors were swapped. Cells expressing D_3R-D_2NT or D_2R-D_3NT were treated with either vehicle or 1 μg/ml tunicamycin for 24 h. ***$p < 0.001$ compared to the vehicle-treated group. For Fig. 5B and 5C, each data point represents mean ± SEM (n = 3). Data represents results from two independent experiments.

D_3R-D_2NT, exerted the same influences in the receptor internalization (Fig. 6D, D_2NT vs. D_2NT-GlyX). These results suggest that *N*-linked glycosylation on the N-terminus represses and facilitates internalization of the D_2 and D_3 receptors, respectively, independent of the respective N-terminus.

3.8. Glycosylation on the N-terminus determines endocytic pathway selection of D_2 and D_3 receptor

Because glycosylation of the N-terminus plays important roles in the internalizations of D_2 and D_3 receptors, molecular mechanisms underlying their regulation was further examined. To examine whether glycosylation on the N-terminus determines the endocytic routes through clathrin-coated pits or caveolae, clathrin heavy chains (CHC) were knocked down by stable transfection of shRNA or cells were treated with MβCD (methyl-β-cyclodextrin) which disrupts caveolae [27].

Internalization of the D_2 receptor and D_2R-D_3NT increased approximately twofold with N-terminus deglycosylation. With cellular CHC knockdown, internalization of both receptors was significantly inhibited regardless of glycosylation status (Fig. 7A). In contrast, knockdown of caveolin-1 inhibited internalization of the D_2 receptor and D_2R-D_3NT by approximately 50%, but did not affect internalization of deglycosylated mutants (Fig. 7B). Essentially the same results were obtained from the cells treated with 3 mM MβCD (Fig. S3A), suggesting that the glycosylation of D_2 receptor is required for caveolae-dependent internalization of D_2 receptor, independent of the respective N-terminus.

Internalization of both the WT-D_3 receptor and D_3R-D_2NT were inhibited by mutation of consensus *N*-lined glycosylation sites on the N-terminus (Fig. 7C, Con-KD). Knockdown of CHC selectively inhibited internalization of receptors with intact glycosylation sites by approximately 50% (Fig. 7C, compare between Con-KD and CHC-KD). In contrast, knockdown of caveolin-1 did not affect internalization of either the WT-D_3 receptor or D_3R-D_2NT, regardless of glycosylation status of the N-terminus (Fig. 7D). Virtually the same results were obtained from the cells treated with 3 mM MβCD (Fig. S3B), suggesting that glycosylation of the D_3 receptor on the N-terminus is required for clathrin-dependent internalization, independent of the respective N-terminus.

Thus, our results overall suggest that the *N*-linked glycosylation on the N-terminus is involved in the regulation of the endocytosis of D_2 and D_3 receptor. In case of D_2 receptor, only glycosylated ones undergo caveolar endocytosis; both glycosylated and deglycosylated D_2 receptors undergo endocytosis through clathrin-mediated pathway. On the other hand, D_3 receptors undergo glycosylation-dependent clathrin-mediated endocytosis.

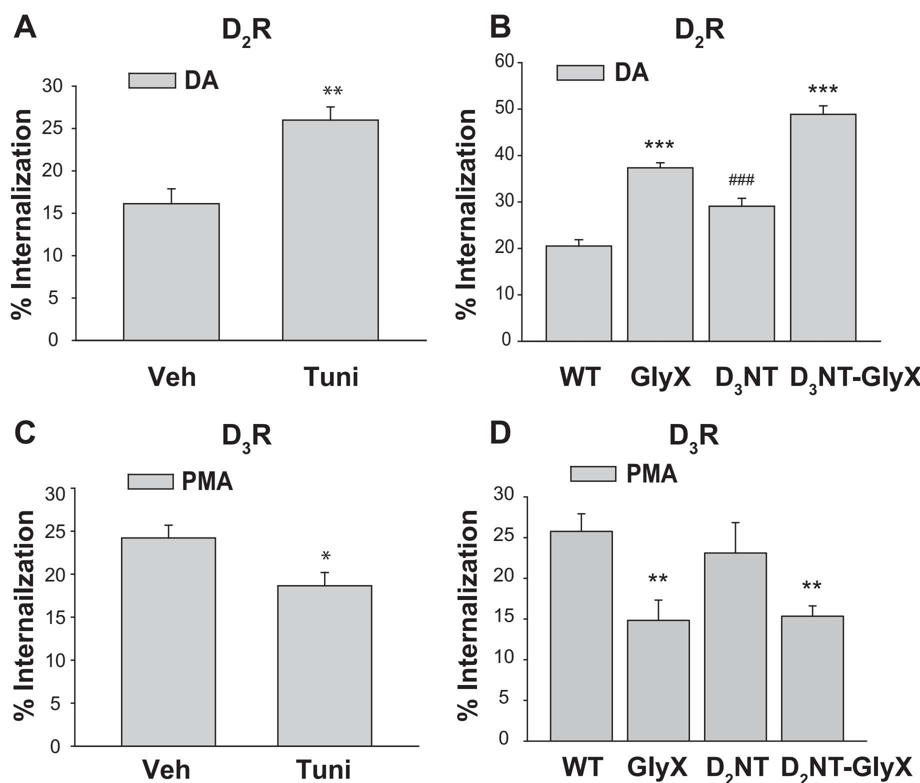

Fig. 6. Role of glycosyation in the internalization of the D_2 and D_3 receptors. (A) Cells expressing the D_2 receptor were treated with either vehicle or 1 μg/ml tunicamycin for 24 h, followed by treatment with 10 μM DA for 1 h. $^{**}p < 0.01$ compared to the vehicle-treated group. (B) Cells were transfected with WT-D_2 receptor (WT), glycosylation mutant of D_2 receptor (GlyX), D_2R-D_3NT (D_3NT), or glycosylation mutant of D_2R-D_3NT (D_3NT-GlyX) in pCMV5. Cells were treated with 10 μM DA for 1 h. $^{***}p < 0.001$ compared to corresponding glycosylation WT. $^{\#\#\#}p < 0.001$ compared to WT-D_2 receptor. (C) Cells expressing D_3 receptors were treated with either vehicle or 1 μg/ml tunicamycin for 24 h, followed by treatment with 100 nM PMA for 30 min. $^{*}p < 0.05$ compared to the vehicle group. (D) Cells were transfected with WT-D_3 receptor (WT), glycosylation mutant of WT-D_3 receptor (GlyX), D_3R-D_2NT (D_2NT), or glycosylation mutant of D_3R-D_2NT (D_2NT-GlyX) in pCMV5. Cells were treated with 100 nM PMA for 30 min. $^{**}p < 0.01$ compared to corresponding glycosylation WT. All experiments (A–D) were repeated three times. Each data point represents mean ± SEM (n = 3).

3.9. Glycosylation on the N-terminus determines microdomain-specific internalization of the D_2 and D_3 receptors by regulating interaction with caveolin-1 and clathrin

To understand the molecular basis for the *N*-linked glycosylation on microdomain-specific receptor internalization, we tested the interactions between receptor and cellular components responsible for receptor endocytosis, including GRK, β-arrestin, PKC, clathrin, and caveolin-1. Knockdown of GRK2 or β-arrestins did not inhibit the effects of tunicamycin on DA-induced internalization of D_2 receptor (Fig. S4A and S4B). However, tunicamycin treatment did inhibit the interaction between D_2R and caveolin-1, but did not affect interaction with clathrin (Fig. 8A). These results are consistent with the results in Fig. S3A, in which the treatment with MβCD failed to inhibit the internalization of D_2R-GlyX. Thus, glycosylation on the N-terminus of the D_2 receptor is required for interaction with caveolin-1; further, N-terminus glycosylation plays important roles in intracellular trafficking by selectively directing receptors toward the caveolae pathway.

In the case of the D_3 receptor, PMA-induced internalization was inhibited by deglycosylation independent of the respective N-terminus (Fig. 6D), but in a clathrin-dependent manner (Fig. 7C). As expected, the interaction with clathrin was inhibited by tunicamycin treatment (Fig. 8B). Interaction between the D_3 receptor and PKCβ was not affected (Fig. S4C). These results suggest that *N*-linked glycosylation on the N-terminus regulates receptor internalization by controlling the association with different microdomains on the plasma membrane.

Since our results demonstrated that the *N*-linked glycosylation determines the endocytosis D_2 and D_3 receptor within specific microdomains, we were curious whether glycosylation of each receptor normally occurs within specific microdomains. As shown in the upper panel of Fig. 8C, the D_3 receptor was normally glycosylated in clathrin-KD cells where the receptor endocytosis was inhibited in a glycosylation-dependent manner. D_2 receptor was also normally glycosylated in Cav1-KD cells (lower panel).

4. Discussion

Glycosylation differentially affects GPCR function, depending upon the specific receptor and the cell type. Thus, it is difficult to identify general principles governing glycosylation-mediated regulation of receptor function. Here, we employed the dopamine D_2 and D_3 receptors as an experimental model. Because the receptors are highly homologous at the amino acid level and similar with respect to pharmacology and signaling, we expected our experiments to reveal the principles dictating the functional roles of glycosylation.

On SDS-PAGE, the molecular weights of D_2 and D_3 receptors decreased by 7–10 kDa when deglycosylated with Endo H or PNGase F (Fig. 1). Considering that the N-termini of the D_2 and D_3 receptors contain only 37 amino acid (4.5 kDa) and 32 amino acid (3.4 kDa) residues, respectively, glycosylation imposes a significant amount of weight and volume on the N-terminus of each receptor. The attached glycans may provide a larger and potentially more flexible binding surfaces and add negative charge to the N-terminus of the GPCRs [15].

One specific aim in this study was to understand the functional roles of glycosylation on the N-terminus of GPCRs in terms of specificity of the N-terminus. As illustrated in Fig. 9, our study showed that *N*-linked glycosylation on the N-terminus plays important roles in determining the various receptor properties and functions. This was required for

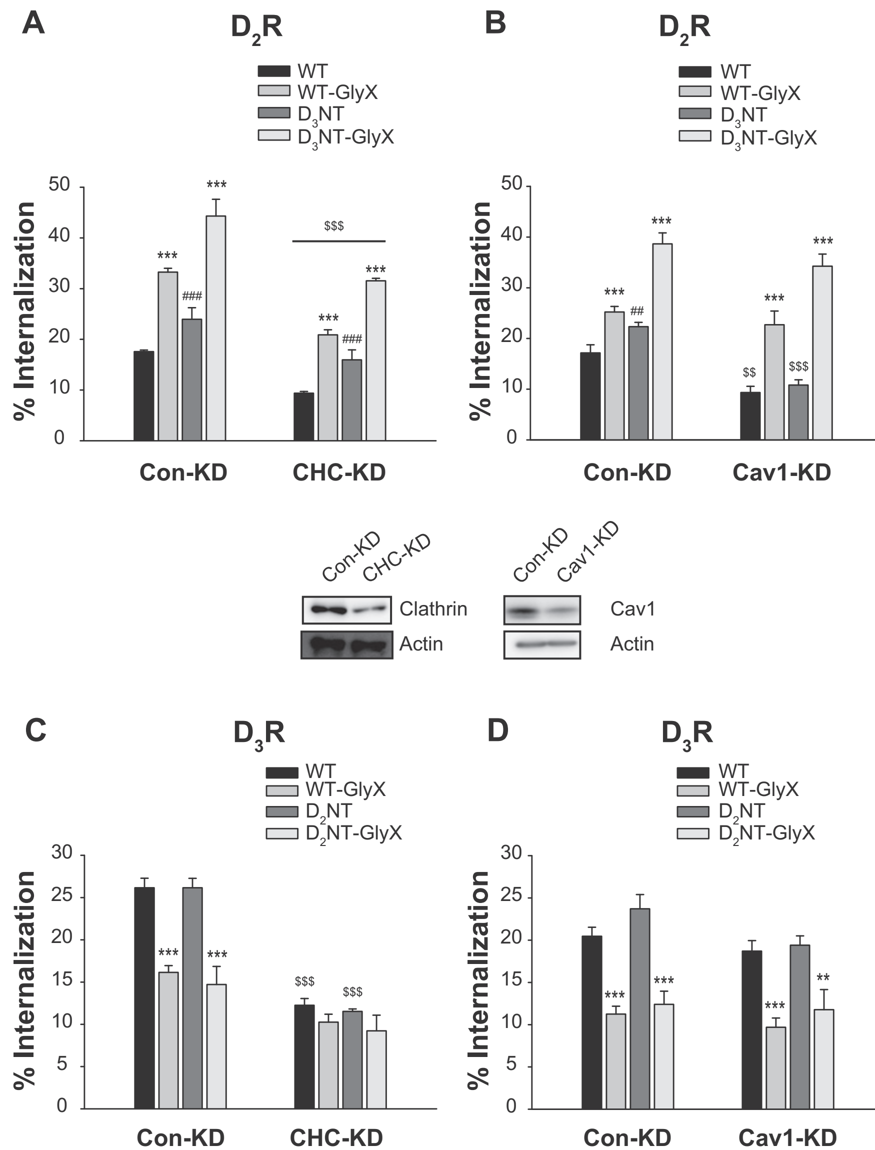

Fig. 7. Role of glycosylation in the internalization of the D_2 and D_3 receptors via specific plasma membrane microdomains. (A) Cells stably expressing either control shRNA (Con-KD) or clathrin heavy chain shRNA (CHC-KD) were transfected with WT-D_2 receptor (WT), glycosylation mutant of WT-D_2 receptor (WT-GlyX), D_2R-D_3NT (D_3NT), or glycosylation mutant of D_2R-D_3NT (D_3NT-GlyX) in pCMV5. Cells were treated with 10 μM DA for 1 h. $^{***}p < 0.001$ compared to the corresponding glycosylation WT; $^{\#\#\#}p < 0.001$ compared to the WT-D_2 receptor in each cellular knockdown group; $^{\$\$\$}p < 0.001$ compared to the corresponding Con-KD group. (B) Cells stably expressing either control shRNA (Con-KD) or caveolin-1 shRNA (Cav1-KD) were transfected with WT-D_2 receptor (WT), glycosylation mutant of WT-D_2 receptor (WT-GlyX), D_2R-D_3NT (D_3NT), or glycosylation mutant of D_2R-D_3NT (D_3NT-GlyX) in pCMV5. Cells were treated with 10 μM DA for 1 h. $^{***}p < 0.001$ compared to corresponding glycosylation WT; $^{\#\#}p < 0.01$ compared to the WT-D_2 receptor; $^{\$\$}p < 0.01$ compared to WT/Con-KD group. $^{\$\$\$}p < 0.001$ compared to D_3NT/Con-KD group. (C) Cells stably expressing control shRNA (Con-KD) or clathrin heavy chain shRNA (CHC-KD) were transfected with WT-D_3 receptor (WT), glycosylation mutant of WT-D_3 receptor (WT-GlyX), D_3R-D_2NT (D_2NT), or glycosylation mutant of D_3R-D_2NT (D_2NT-GlyX) in pCMV5. Cells were treated with 100 nM PMA for 30 min. $^{***}p < 0.001$ compared to corresponding glycosylation WT; $^{\$\$\$}p < 0.001$ compared to corresponding experimental group Con-KD cells. (D) Cells stably expressing control shRNA (Con-KD) or caveolin-1 shRNA (Cav1-KD) were transfected with WT-D_3 receptor (WT), glycosylation mutant of WT-D_3 receptor (WT-GlyX), D_3R-D_2NT (D_2NT), or glycosylation mutant of D_3R-D_2NT (D_2NT-GlyX) in pCMV5. Cells were treated with 100 nM PMA for 30 min. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to corresponding glycosylation WT group. Cell lysates from Con-KD, CHC-KD, and Cav1-KD cells were blotted with antibodies to clathrin, caveolin-1, and actin (middle panel). All experiments (A–D) were repeated three times. Each data point represents mean ± SEM (n = 3).

both correct cell surface expression and internalization through a specific microdomain. Both activities occurred independent of which N-terminus, D_2 or D_3, was attached. For signaling, glycosylation on the N-terminus of the D_3 receptor was required for basal signaling and desensitization.

The roles of *N*-linked glycosylation on the N-terminus in correct cell surface expression are consistent with a previous study, in which the inhibition of glycosylation was shown to decrease surface expression of the D_2 receptor by interfering with the calnexin interaction [10]. Inhibition of glycosylation interferes with cell surface expressions of other GPCRs, including the thyrotropin receptor [28], β_1 adrenergic receptor [29], and protease-activated receptor type 2 [30]. Thus, it is postulated that one important role of glycosylation is to regulate the surface expressions of GPCRs.

It is clear that *N*-linked glycosylation of the D_3 receptor on the N-terminus is responsible for surface expression, desensitization, and internalization. It should be noted, however, that *N*-linked glycosylation within the extracellular loops is exclusively responsible for internalization. Similar results were reported previously. For example, glycosylation on the N-terminus and within the 2nd extracellular loop of protease-activated receptor type 1 (PAR1) are important for transport to the cell surface and internalization, respectively [31]. Similar were

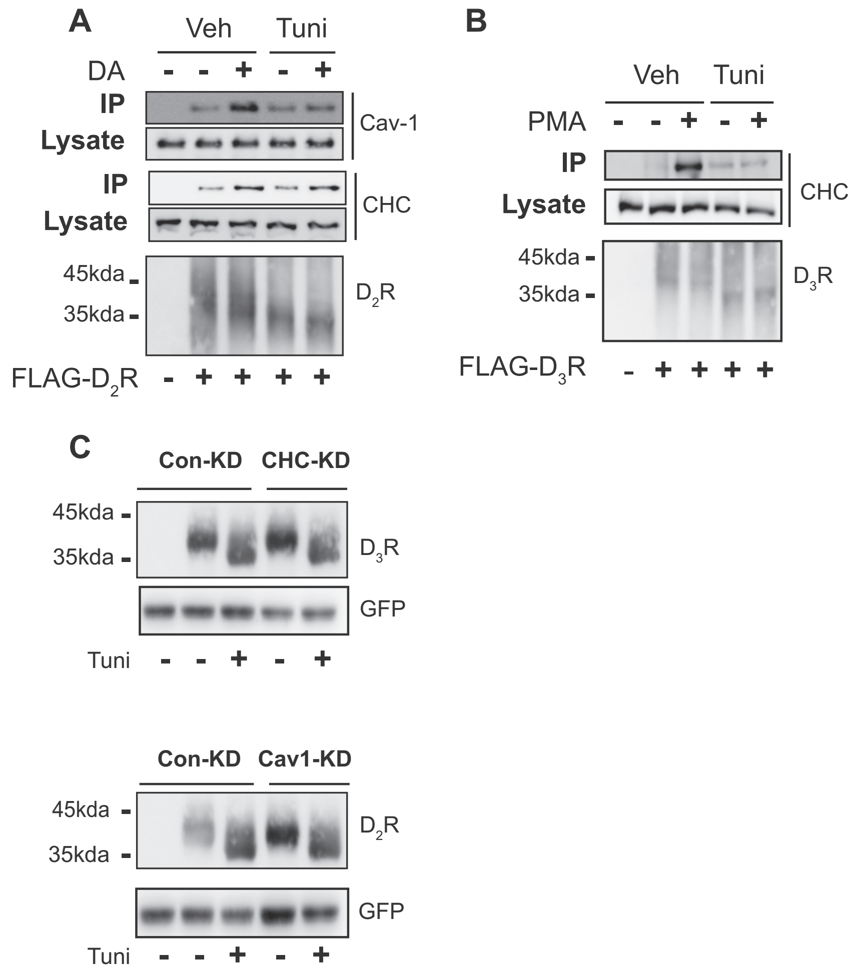

Fig. 8. Molecular mechanisms involved in microdomain-selective internalization of the D_2 and D_3 receptors. (A) Cells expressing FLAG-tagged D_2 receptors were treated with either vehicle or 1 μg/ml tunicamycin for 24 h, followed by treatment with 10 μM DA for 2 min. Cell lysates were immunoprecipitated with FLAG beads and blotted with antibodies to clathrin heavy chain, caveolin-1, and FLAG. (B) Cells expressing FLAG tagged D_3 receptors were treated with either vehicle or 1 μg/ml tunicamycin for 24 h, followed by treatment with 100 nM PMA for 2 min. Cell lysates were immunoprecipitated with FLAG beads and blotted with antibodies to clathrin heavy chain and FLAG. (C) Con-KD, CHC-KD, or Cav1-KD cells were transfected with green fluorescence protein (GFP), FLAG-tagged D_3 receptor (upper panel), and FLAG-tagged D_2 receptor (lower panel). Cells were treated either with vehicle or 1 μg/ml tunicamycin for 24 h. Cell lysates were immunoprecipitated with FLAG beads, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with antibodies to FLAG and GFP. Transfection efficiencies were normalized by including pEGFP vector. Data (A–C) represent results from three independent experiments.

reported for the prostacyclin receptor [32]. More experimental results are needed to identify a common principle dictating the functional roles of glycosylation of the extracellular loop.

It is notable that glycosylation states determined internalization of D_2 and D_3 receptors within different microdomains. The posttranslational modifications seem to play important roles in association with

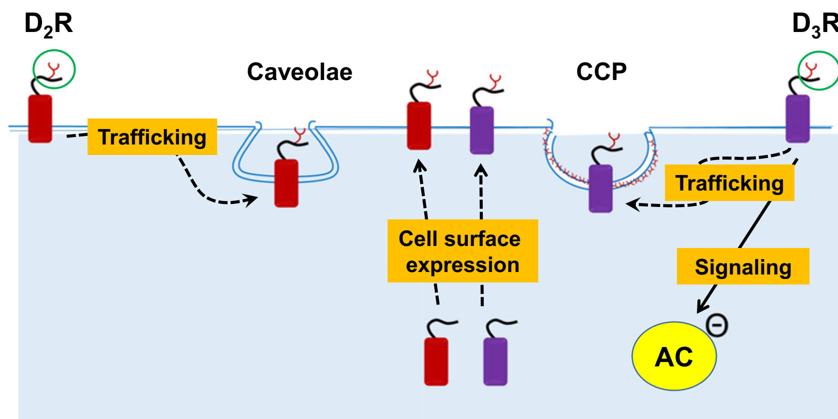

Fig. 9. Roles of glycosylation on the N-terminus in cell surface expression, endocytosis, and signaling of the dopamine D_2 and D_3 receptors. Independent of the respective N-terminus, glycosylation on the N-terminus is required for expression of the dopamine D_2 and D_3 receptors on the plasma membrane, and for endocytosis of the D_2 and D_3 receptors via caveolae and clathrin-coated pits, respectively. Glycosylation of the D_3 receptor N-terminus is involved in basal signaling and desensitization.

specific plasma membrane microdomains of integral proteins. For example, glycosylation of Edg-1, a receptor for sphingosine-1-phosphate, is required for agonist-induced internalization and association with caveolae [33]. Introduction of *N*-linked glycosylation sites to the basolateral Na^+, K^+-ATPase β1 subunit results in its clustering in caveolae [34]. However, the understanding the detailed mechanisms involved in the glycosylation-mediated associations between the receptor and the constituents of plasma membrane microdomains requires further study.

The main purpose of this study was to identify general principles governing the functional roles of glycosylation on GPCR functions. Our results show that the functional roles of glycosylation are too diverse to be generalized based on the structural and functional homology between the dopamine D_2 and D_3 receptors. However, our results indicate that N-linked glycosylation on the N-terminus of both the D_2 and D_3 receptors are involved in the selection of endocytic pathways through specific microdomains. Additional studies in more controlled experimental systems could provide a valuable answer to the fundamental question—what determines endocytic pathways selection for specific GPCRs?

Because glycosylation status strongly influences the various functional properties of D_3 receptor, it would be interesting to examine the glycosylation patterns of D_3 receptor in different regions of the brain and in pathological conditions involving D_3 receptors. A similar case was reported with dopamine transporters, which are co-expressed with the D_3 receptor in dopaminergic neurons that are differentially glycosylated depending where they are expressed in the brain regions [35]. Additionally, another study reported that dopamine transporter glycosylation is correlated with vulnerability of midbrain dopaminergic cells in Parkinson's disease [36].

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Appendix A. Supplemental data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.09.024.

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