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·Original Article·

Differential distributions and trafficking properties of dopamine D1 and D5 receptors in nerve cells

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Abstract: Objective To explore the possible differential trafficking properties of the dopamine D1-like receptor subtypes, D1 receptor and D5 receptor. **Methods** To visualize distributions of dopamine D1-like receptor subtypes at subcellular level, the yellow and cyan variants of green fluorescent protein (GFP) were used to tag D1 and D5 receptors. After transfection with the tagged dopamine receptors, the neuroblastoma cells NG108-15 were treated with D1 agonist SKF38393 or acetylcholine (ACh). Then we observed the subcellular distributions of the tagged receptors under the confocal microscopy and tried to determine trafficking properties by comparing their distribution patterns before and after the drug treatment. **Results** In resting conditions, D1 receptors located in the plasma membrane of NG108-15 cells, while D5 receptors located in both plasma membrane and cytosol. With the pre-treatment of SKF38393, the subcellular distribution of D1 receptors was changed. The yellow particle-like fluorescence of tagged D1 receptors appeared in the cytosol, indicating that D1 receptors were internalized into cytosol from the cell surface. Same situation also occurred in ACh pre-treatment. In contrast, the subcellular distribution of D5 receptors was not changed after SKF38393 or ACh treatment, indicating that D5R was not translocated to cell surface. Interestingly, when D1 and D5 receptors were co-expressed in the same cell, both kept their distinct subcellular distribution patterns and the trafficking properties. **Conclusion** Our present study reveals that in NG108-15 nerve cells, dopamine D1 and D5 receptors exhibit differential subcellular distribution patterns, and only D1 receptor has a marked trafficking response to the drug stimulation. We further discuss the potential role of the differential trafficking properties of D1-like receptors in complex modulation of DA signaling.

Keywords: dopamine D1 receptor; dopamine D5 receptor; trafficking; internalization; green fluorescent protein; SKF38393; acetylcholine

1 Introduction

Dopamine (DA) receptors are divided into two categories: D1-like receptors (D1 and D5 receptors) and D2-like receptors (D2, D3 and D4 receptors). With the advant of the D1 selective ligands, D1-like receptors are distinguished from the D2-like receptors[1-3]. Then D1-like receptor subtypes, D1 receptor (D1R) and D5 receptor (D5R), are further identified in mammalian brain by cDNA cloning[4-7]. However, currently there is no selective ligand available to distinguish between D1R and D5R.

Recently, accumulating evidences have indicated that D1R and D5R play the distinct roles in brain: (1) D5R shows affinity to dopamine ten-fold higher than D1R[5,6]; (2) D1R knockdown in brain prevents the contralateral rotations of 6-hydroxdopamine-lesioned rats induced by D1 agonist, whereas D5R knockdown enhances such rotation behavior[8]. And D1R, but not D5R, knockout mouse exhibits higher level

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of locomotor activity[9]; (3) Genetic deletion of D1R impairs corticostriatal long-term potentiation (LTP), while blocking the remaining D5R receptors impairs corticostriatal long-term depression (LTD)[10].

All dopamine receptor subtypes belong to G-protein coupled receptors (GPCRs) family. The trafficking of GPCRs between cell surface and intracellular compartment indicates an important functional regulation[11]. Two situations are included in the trafficking process: one is the internalization of the receptor from the cell surface to the intracellular compartment; the other is the translocation to cell surface from the intracellular compartment. In both situations, the intensity of surface receptor is changed, which in turn, results in the changes of the intracellular signal strength. To explore the possible differences in trafficking properties of D1-like receptors, we constructed a series of green fluorescent protein (GFP) variants tagged D1R and D5R, and observed their subcellular distribution pattern change in neuroblastoma cell line NG108-15 before and after the drug treatments. The subcellular distributions of D1R and D5R expressed in HEK293 and HeLa cell lines were also observed.

2 Materials and methods

2.1 Cell culture and transfection NG108-15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented 10% fetal bovine serum containing amimopterin 0.176 μg/L, thymidine 4.125 μg/L and hypoxanthine 13.6 μg/L. HEK 293 cells and HeLa cells were cultured in DMEM supplemented 10% fetal bovine serum. After being cultured in the coverslips for 24h, cells were transfected using the LipofectAMINE technique (Invitrogen, Milano) with 1 μg plasmid per 35 mm dish at 70% confluence.

2.2 Plasmid construction For generation of C-terminal EYFP-tagged D1R, the coding sequence of D1R cDNA (generous gift from Prof. Kim Neve) without stop codon was amplified using *Pfu* DNA polymerase (forward primer: 5'-TTATGAATTCATGAGGACTCTGAACACCTCT-3'; reverse primer: 5'-ACTTGGATCCCCAGTTGGGTGCTGTCCGTT-3'). Products were digested in EcoRI and BamHI sites, and ligated into the corresponding sites in pEYFP-N1 vector (Invitrogen) in frame with EYFP. Then the D1R cDNA was sub-cloned into the corresponding sites in pECFP-N1 from the pEYFP-D1R. For generation of C-terminal tagged D5R, the coding sequence of D5R cDNA (generous gift from Prof. Robert Levenson) without stop codon was amplified (forward primer: 5'-TCGTGAATTCATGGACTACAAGGACGAC-3'; reverse primer: 5'-TAATGTCGACGTATGGAATCCATT-CGGGG-3'). Products were digested in EcoRI and SalI sites, and ligated into the corresponding sites of pEYFP-N1 vector (Invitrogen) in frame with EYFP. Then this D5R cDNA was sub-cloned into the corresponding sites of pECFP-N1 from the pEYFP-D5R. Before transfection, all the constructs were sequenced to confirm no induced mutation.

2.3 Confocal microscopy 48-72 h after transfection, the NG108-15 cells expressing GFP-tagged D1R or/and D5R were examined using laser confocal microscopy (Leica SP2 system, Leica Microsystems, Heidelberg, Germany). For cells treated with SKF38393 or ACh, they were first incubated with 1 μmol/L indicated agent in PBS for 40 min, and then immediately fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. After three brief washes with PBS, the fixed cells were incubated with TRITC–conjugated wheat germ agglutinin (TRITC-WGA) (Sigma, St. Louis, MO) at a concentration of 10 μg/mL in PBS for 30 min, and then transferred to 5 μg/mL Hoechst 33258 (Molecular Probes) in PBS to stain the nuclei for 10 min. Triple fluorescence imaging was performed using 458 nm, 514 nm or 543 nm plus UV to excite ECFP, EYFP and TRITC-WGA and Hoechst 33258, respectively, in sequential scanning.

2.4 Quantification of receptor subcellular distribution pattern To quantitatively describe the receptor subcellular distribution pattern, the "distribution index" (DI) is introduced. It is defined for individual cell, DI=Fcyto/Fmem, where Fcyto represents the average fluorescence intensity in the cytosol, and Fmem represents that in the plasma membrane. In Leica Systems LAS AF software, the cytosol area and the plasma membrane area of individual cell were manually selected. Briefly, region of interest 1 (ROI1) and region of interest 2 (ROI2) were selected to represent YFP fluorescence along the outside or inside edge of the plasma membrane, respectively; region of interest 3 (ROI3) indicated Hoechst 33258 fluorescence around the nucleus. To get YFP fluorescence in

the plasma membrane area, ROI2 is subtracted from ROI1; To get the cytosol area, ROI3 is subtracted from ROI2. The corresponding fluorescence intensities were measured by Leica Systems LAS AF software. Due to the background fluorescence in the cytosol and image sampling process, Fcyto is not usually zero. Student's *t*-test was performed to determine the DI difference between cells expressing D1R-YFP and cells expressing D5R-YFP in Microsolf Office Excel software. Data were expressed as mean ± SD.

After drug treatment, the YFP fluorescence in the plasma membrane was not as clear as that before treatment. For such cells, ROI1 was first selected along the most outside fluorescent signal, while ROI2 was selected empirically according to the previous average plasma membrane width from the resting condition. Differences in DI means between different groups were determined by one-way ANOVA analysis in OriginPro7.5 Software. Data were expressed as mean ± SD.

3 Results

3.1 Distinct subcellular distributions of D1R and D5R in NG108-15 cells To visualize the subcellular localizations of the D1R and D5R, a series of yellow or cyan variant GFPs tagged D1 or D5 receptor (termed D1R-YFP, D1R-CFP, D5R-YFP and D5R-CFP) were constructed, and the subcellular distributions of the tagged receptors were examined 48-72 h after the transfection. In NG108-15 cells expressing D1R-YFP, the yellow fluorescence of D1R-YFP was clearly confined to the plasma membrane of NG108-15 cells (n=46 cells, 4 independent transfection experiments, Fig.1A), whereas D5R-YFP fluorescence appeared not only in the plasma membrane but

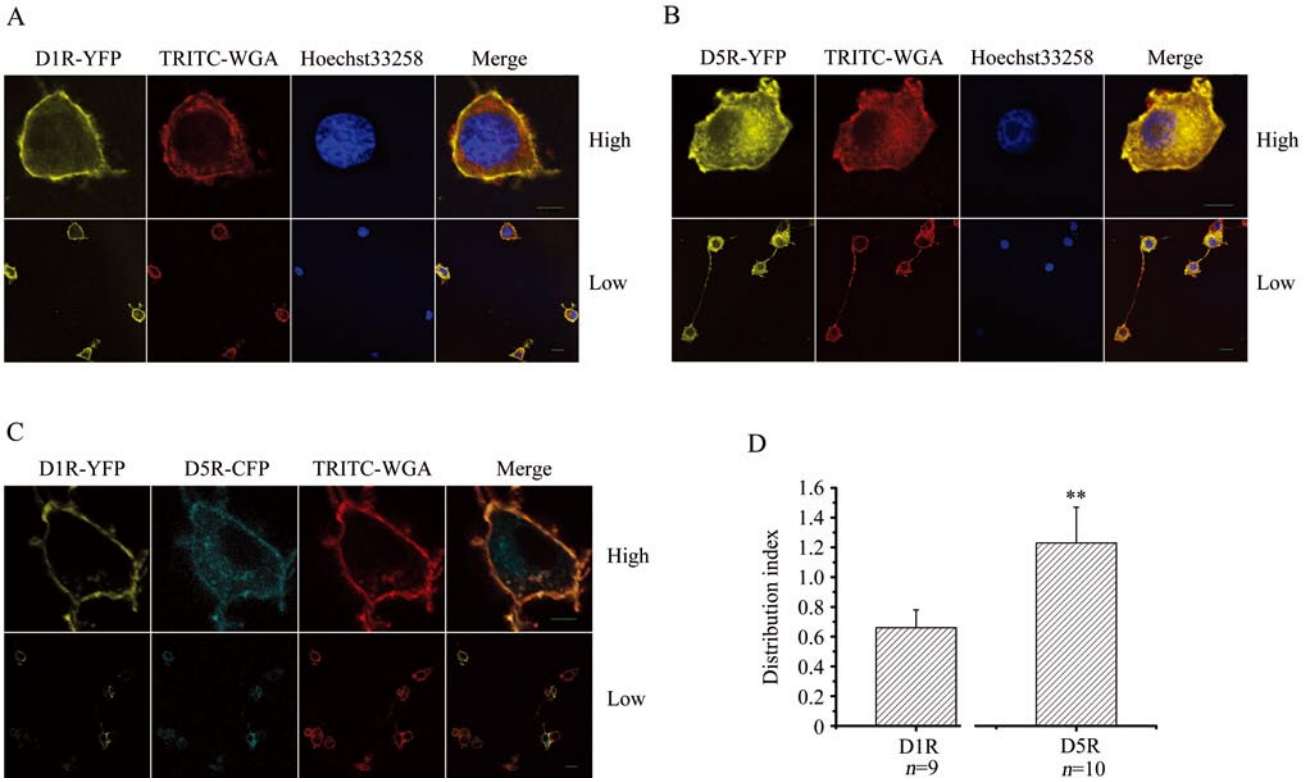

Fig. 1 The distinct subcellular distributions of D1R and D5R in NG108-15 cells. TRITC-WGA was used to label plasma membrane structure; Hoechst33258 was used to label the nucleus. A, The representative NG108-15 cells expressing D1R-YFP in high or low magnification. B, The representative NG108-15 cells expressing D5R-YFP in high or low magnification. C, The representative NG108-15 cells co-expressing D1R-YFP and D5R-CFP in high or low magnification. D1R-YFP and D5R-CFP kept their distinct distribution patterns. High: high magnification. Low: low magnification. Scale bar, 8 μm for the high magnification images; 20 μm for the low magnification images. D, The significant difference in distribution index values between cells expressing D1R-YFP and cells expressing D5R-YFP. This difference indicated their differential subdistribution patterns, **P<0.01 *vs* D1R-YFP group.

also in cytosol as particles in all other 4 transfection experiments with 54 detected cells (Fig. 1B). To better identify the relative subcellular location, Hoechst 33258 was used to label the nucleus; and TRITC-WGA was used to generally label the plasma membrane, as WGA specifically binds to carbohydrate residues of membrane glycosylated proteins. However, TRITC-WGA also labeled some intracellular compartments of NG108-15 cells some time, especially in cells expressing D5R-YFP (Fig. 1B). The D5R-YFP yellow fluorescence signals were co-localized with the TRITC-WGA labeled intracellular compartments, and the specific binding of TRITC-WGA indicated that these organelles were rich in glycosylated proteins. To quantitatively describe their distribution patterns, the "distribution index" (DI) values were measured for 9 NG108-15 cells expressing D1R-YFP and 10 NG108-15 cells expressing D5R-YFP. The result showed a significant difference in DI values between cells expressing D1R-YFP and that expressing D5R-YFP (Fig. 1D), which was consistent with the observations of their differential distributions.

To further evaluate their distinct distribution patterns, D1R-YFP and D5R-CFP were co-expressed in NG108-15 cells. The results showed that D1R-YFP was still confined to the plasma membrane, while D5R-CFP exhibited both plasma membrane and intracellular locations (Fig.1C) in 2 independent transfection experiments with 19 cells. In the merged image, the co-localization of D1R-YFP and D5R-CFP in TRITC-WGA labeled plasma membrane resulted in a golden ring along the plasma membrane, and the cyan fluorescence of D5R-CFP was also observed in the cytosol (Fig. 1C). It indicated that D1R and D5R kept their distinct subcellular distributions even when they were co-expressed in NG108-15 cells. We wondered whether these distinct subcellular distributions of D1R and D5R implied the potential functional difference. Next, their trafficking responses to drug stimulation were examined.

3.2 D1R was internalized following SKF38393 or ACh treatment It has been reported that D1R can be internalized by it's agonists[12,13]. In our experiments, the internalization of D1R-YFP induced by D1 agonist SKF38393 was also observed. After the pre-treatment with SKF38393 (1 μmol/L, 40 min), the yellow fluorescent particles appeared in the cytosol of NG108-15 cells in 3 independent transfection experiments with 26 cells, and these particles were co-localized with TRITC-WGA labeled vesicle structures (Fig. 2B). These vesicles appeared in the cells treated with SKF38393 (Fig. 2B), but not in the untreated cells (Fig. 2A), indicating that the double-labeled vesicles were internalized. However, under the same experimental conditions, *l*-stepholidine (100 nmol/L, 40 min), which possesses D1 agoinst/D2 antagonist dual actions[14], failed to induce the redistribution of D1R-YFP (data not shown).

In addition to the D1 agonists, the effect of ACh on internalization of D1 receptors was investigated in NG108-15 cell, a neuroblastoma x glioma hybrid cell line with endogenous muscarinic acetylcholine receptors (mAChR)[15]. Surprisingly, the pre-treatment with ACh (1 μmol/L, 40 min) also induced the internalization of D1R-YFP (Fig. 2C). The vesicular D1R-YFP co-localized with TRITC-WGA clearly appeared in the cytosol in 3 independent transfection experiments with 18 cells. The results indicated the heterogenous internalization of D1R induced by ACh.

DIs were measured in 7 cells expressing D1R-YFP treated with SKF38393 and 5 cells expressing D1R-YFP treated with ACh. The DI values of the drug-pretreated groups were significantly different from DI values of control group (Fig. 2D), which also indicated the D1R-YFP internalization caused by drug treatments.

3.3 D5R did not translocate to cell surface from intracellular compartments after the treatment It has been reported that the GPCRs can not only be internalized from the cell surface but also display the regulated translocation to the cell surface from intracellular compartments[11]. As D5R locates both in the plasma membrane and in the intracellular compartments in resting conditions, there raises the question: whether D5R would be translocated by the drug treatment? However, results revealed no change of the subcellular distribution pattern of D5R-YFP before and after SKF38393 treatment (1 μmol/L, 40 min) (Fig. 3B) in 3 independent transfection experiments with 22 cells versus the untreated cells (Fig. 3A), which indicated that D5R-YFP was not translocated to cell surface from intracellular compartments after drug treatment.

Besides, D5R-YFP exhibited the similar distribution

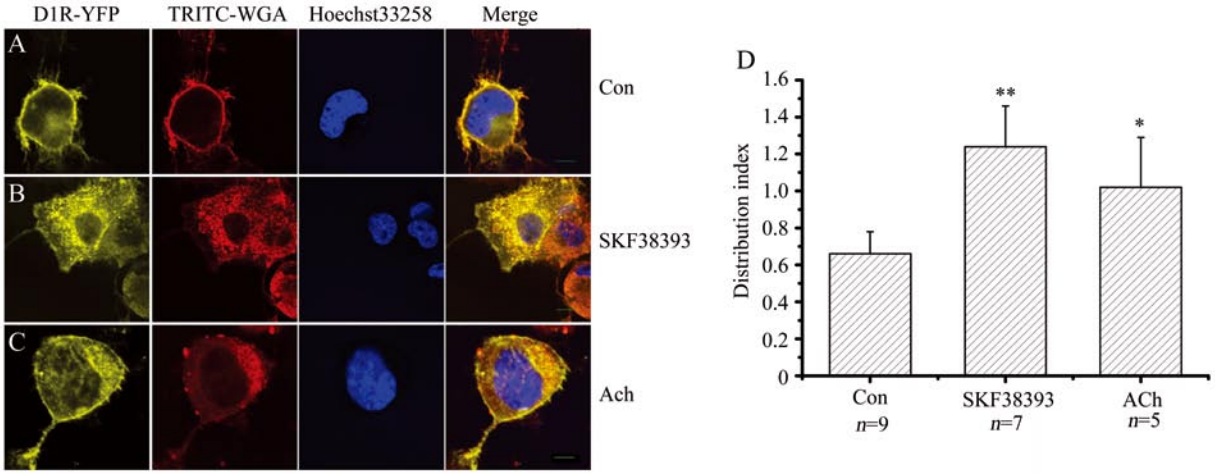

Fig. 2 D1R-YFP was internalized by SKF38393 or ACh pre-treatment. TRITC-WGA was used to label membrane structure; Hoechst33258 was used to label the nuclues. A, D1R-YFP was confined to the plasma membrane in resting conditions (Con) in NG108-15 cell. B, After 40 min pre-treatment with 1 μmol/L SKF38393, D1R-YFP appeared in the cytosol. The co-localization of D1R-YFP and TRITC-WGA indicated the internalization of D1R-YFP. C, After 40 min pre-treatment with 1 μmol/L ACh, D1R-YFP appeared in the cytosol. Scale bar, 8 μm. D, The distribution index values of the drug-pretreated groups are significantly different from that of control group, *P<0.05, **P<0.01 *vs* control group.

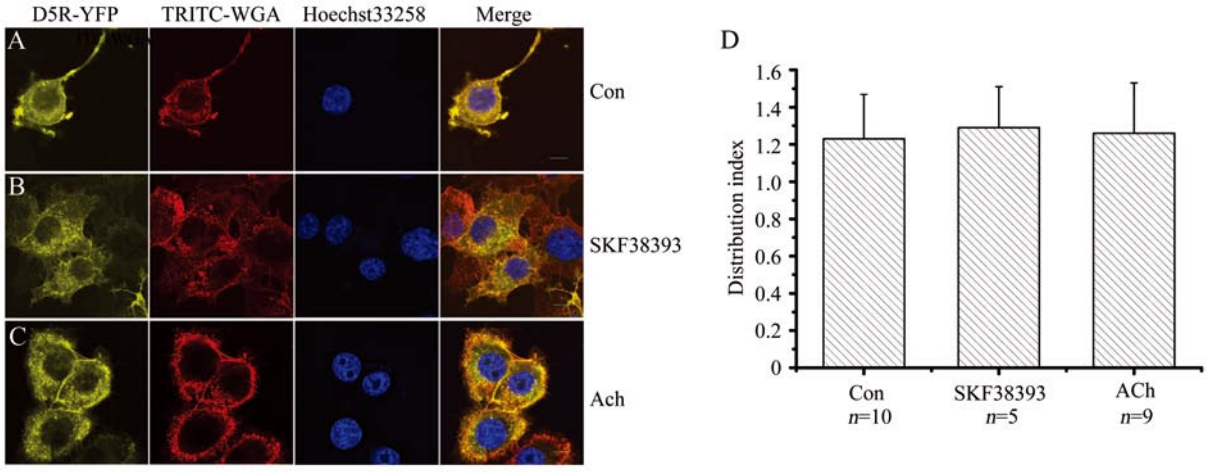

Fig. 3 D5R-YFP did not translocate to cell surface from the cytosol after SKF38393 or ACh pre-treatment. TRITC-WGA was used to label membrane structure; Hoechst33258 was used to label the nuclues. A, D5R-YFP located in both the plasma membrane and the cytosol in resting conditions (Con) in NG108-15 cell. B, After 40 min pre-treated with 1 μmol/L SKF38393, D5R-YFP remained in the cytosol. C, After 40 min pre-treated with 1 μmol/L ACh, D5R-YFP remained in the cytosol. Scale bar, 8 μm. D, The distribution index values of the drug-pretreated groups were similar with that of control group.

pattern after ACh treatment (1μmol/L, 40 min) in 3 independent transfection experiments with 18 cells, and it did not translocate to cell surface (Fig. 3C). However, the yellow fluorescence of D5R-YFP around the plasma membrane disappeared in some treated cells, which suggested the possibility of D5R-YFP internalization after the ACh pre-treatment.

DIs were also measured for 5 cells expressing D5R-YFP treated with SKF38393 and 9 cells expressing D5R-YFP treated with ACh. The DI values of the drug-pretreated groups were both similar with that of control group (Fig. 3D), which indicated no translocation of D5R-YFP by the drug pretreatment.

3.4 D1R and D5R co-expressed in the same cell kept their

distinct trafficking properties Dopamine D1 receptors can not only form the homodimer[16,17], but also form the heterodimer with other GPCRs[17,18]. The heterodimerized GPCRs can result in the mutual modulation of their function and/or the redistribution of their subcellular locations[17-20]. To find out whether there were similar hetero-regulations between D1R and D5R, trafficking properties of D1R-YFP and D5R-CFP co-expressed in NG108-15 cells were detected (2 independent transfection experiments, 9 cells).

When co-expressed with D5R-CFP in the same cell, D1R-YFP was still internalized after the SKF38393 pre-treatment (1 μmol/L, 40 min). Meanwhile, D5R-CFP still localized both in the plasma membrane and in the cytosol (Fig. 4B), just like that in resting conditions (Fig. 4A). The above results demonstrated that, D1R was internalized by SKF38393 treatment, while D5R did not translocate to cell surface, regardless of whether they were expressed separately or co-expressed with each other.

It was assumed that if D1R and D5R formed heterodimer, they should display the same distribution patterns, as one 'pulls down' or 'pulls up' another one. However, this was not observed between D1R-YFP and D5R-CFP in our experiments. It's shown clearly that in the cytosol, D5R-CFP was unlocalized with D1R-YFP (Fig. 4B, indicated by arrowhead), which implied that no hetero-dimerization existed between D1R and D5R in our expression system.

The trafficking of co-expressed D1R-YFP and D5R-CFP was also examined after the ACh pre-treatment (1μmol/L, 40 min) (2 independent transfection experiments, 12 cells). D1R-YFP was still internalized by ACh pre-treatment, whereas D5R-CFP did not translocate to cell surface when they were co-expressed in the same cell (Fig. 4C). However, the ACh treatment tended to induce more vesicular-like signals within cytoplasm for D1R-YFP and D5R-CFP. And some D5R-CFP co-localized with D1R-YFP (indicated by arrows in Fig. 4C). These implied a different mechanism between ACh-induced and D1 agonist-induced D1R internalization.

3.5 The subcellular distributions of D1R and D5R in other

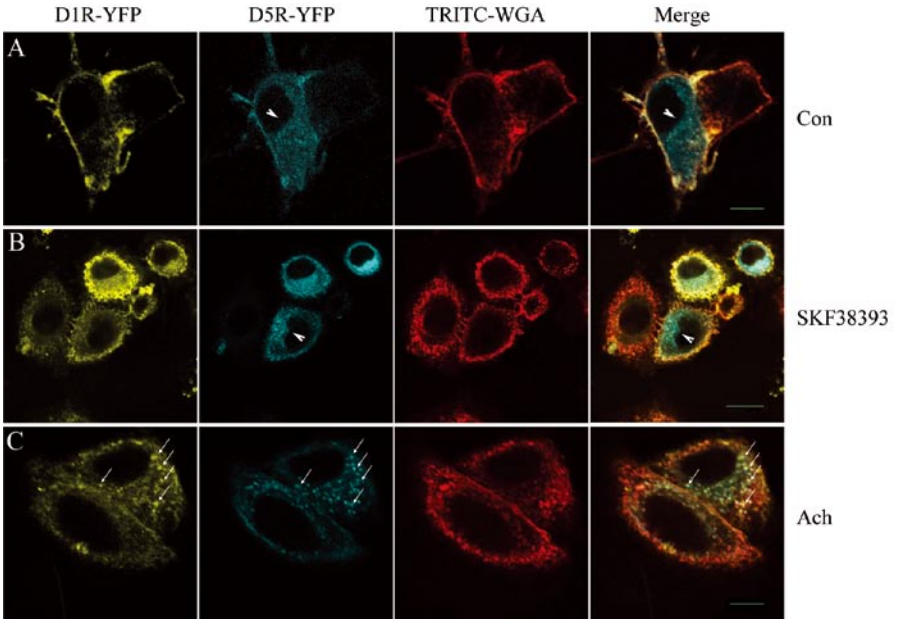

Fig. 4 The trafficking properties of D1R and D5R induced by SKF38393 or ACh pre-treatment when they were co-expressed in NG108-15 cells. TRITC-WGA was used to label membrane structure. A, D1R-YFP and D5R-CFP co-expressed in NG108-15 cell in resting conditions. They exhibited the distinct subcellular distribution patterns. D1R-YFP was confined to the plasma membrane, whereas D5R-CFP localized in both the cytosol (arrowhead) and the plasma membrane. B, After 40 min pretreatment with 1 μmol/L SKF38393, D1R-YFP was internalized in the cytosol (the leftmost panel), while the D5R-CFP remained in the intracellular compartment (arrowhead, the second left panel). C, After 40 min pretreatment with 1 μmol/L ACh, D1R-YFP was internalized in the cytosol (the leftmost panel), while the D5R-CFP remained in the intracellular compartment (the second left panel). Some vesicles double-labeled by D1R-YFP and D5R-CFP appeared (arrows). Scale bar, 8 μm.

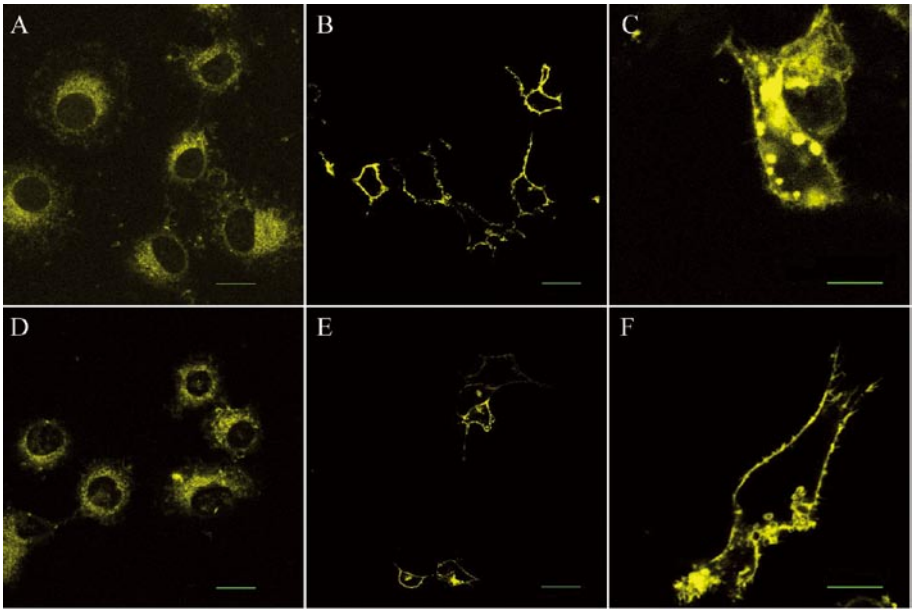

Fig. 5 Subcellular distributions of D1R-YFP and D5R-YFP in HeLa and HEK293 cells. In HeLa cells, both D1R-YFP (A) and D5R-YFP (D) located in the intracellular compartments. In HEK293 cells, both D1R-YFP (B) and D5R-YFP (E) were confined to the plasma membrane. The pre-treatment with 1 μmol/L SKF38393 for 40 min induced the internalization of D1R-YFP in the cytosol (C), but there was no such internalization for D5R-YFP (F). Scale bar, 20 μm for A, B, D and E, 8 μm for C and F.

cell lines In other non-neuronal cell lines, the subcellular distributions of D1R-YFP and D5R-YFP were also investigated. Interestingly, in the tumor cell line Hela, both D1R-YFP and D5R-YFP were shown to locate in the intracellular compartments (Fig. 5A, D) in 2 independent transfection experiments for all observed cells.

In epithelial cell line HEK293, both D1R-YFP and D5R-YFP were confined to the plasma membrane in 3 independent transfection experiments for D1R-YFP (Fig. 5B), and in 2 independent transfection experiments for D5R-YFP (Fig. 5E). After the pre-treatment of SKF38393 (1 μmol/L, 40 min), the D1R-YFP was internalized into the cytosol (Fig. 5C) in 3 independent transfection experiments with 16 cells. In contrast, no internalized fluorescent signal of D5R-YFP in the cytosol was detected after SKF38393 pre-treatment (Fig. 5F) in 2 independent transfection experiments with 12 cells. These results indicated that in HEK293 cells, the trafficking of D5R-YFP was less active than D1R-YFP, which was consistent with the previous results in NG108-15 cell line.

Taken together, above results implied that the distinct distributions of D1R and D5R might depend on some components in NG108-15, but not in HEK293 or HeLa cells. On the other hand, the distinct distributions and trafficking properties of D1R and D5R in NG108-15 cells are not due to GFP variants fused to C-terminus of the receptor, since the tagged D1R and D5R showed the same subcellular distributions in other cell lines.

4 Discussion

In the present study, we reported the differential subcellular distributions and trafficking properties of D1-like receptor subtypes, D1R and D5R in NG108-15 cells. Firstly, D1R localized in the plasma membrane, whereas D5R localized in both the plasma membrane and the cytosol. Secondly, D1R showed more active intracellular trafficking than D5R, following the D1R agonist stimulation. D1R exhibited regulated internalization not only by D1 agonist SKF38393, but also by ACh. In contrast, D5R failed to show the SKF38393-induced translocation in the same conditions. In addition, D1R was internalized in HEK293 cells by D1 agonist stimulation, while D5R did not show a marked internalization. The potential molecular determinants for the differential trafficking properties

of D1R and D5R, and the possible physiological implications of such difference will be discussed in the following.

4.1 The potential molecular determinant for the differential trafficking properties of D1R and D5R DA receptors are regulated through their intracellular segments by a group of proteins called dopamine receptor-interacting proteins (DRIPs)[21]. D1-like receptors have a short third intracellular loop (IL3) and a long intracellular carboxyl terminus (C-terminus). In most cases, D1 receptors interact with the DRIPs through C-terminus rather than IL3[22-25]. It has been reported that the substituted mutation of a hydrophobic motif, or the truncated mutation of C-terminus would disturb the cell surface targeting of D1R[22,23]. The two D1-like receptor subtypes, D1R and D5R, exhibit more diversity in their C-termini amino acid sequence than that in transmembrane segments[4-7]. Correspondingly, D1R and D5R can bind to different DRIPs through their C-termini. D1R interacts with NMDA receptor subunits NR1 and NR2A and inhibits the NMDA-mediated current through its C-terminal tails[24]; While D5R interacts with GABAa receptor γ2 (short) subunit and enables mutually functional inhibition through it's C-terminus[25]. In a systematic binding study of the C-termini of all dopamine receptor subtypes, D5R but not D1R C-terminus selectively and strongly bound to a membrane-associated protein sorting nexin 1 (SNX1)[26]. In addition, the C-termini of D1-like receptors also governed their distinct binding affinity to agonists [27,28]. Swapping the C-termini of D1R and D5R resulted in the full switch of the affinities. The chimeric D1R harboring D5R C-terminus displays an increased affinity to the agonist which is indistinguishable from the wild-type D5R. Taken together, these data suggested that the different C-termini of D1R and D5R played as the intrinsic molecular determinant, and interacted with some components expressed in NG108-15 cells, resulting in the differential subcellular distributions and trafficking properties between D1R and D5R.

4.2 The physiological implication of differential trafficking properties of D1-like receptors DA acts as a complex modulator in brain through the five metabotropic receptors. The activation of D1-like receptor family alone, just D1R and D5R two subtypes in mammalian brain, is capable of inducing bidirectional modulation effects in a single neuron[29] or modulating the working memory performance of primates with the characteristic DA "inverted U" dose-effect relationship [30,31]. It's intriguing that limited receptor subtypes implement complex modulation functions.

Potential mechanisms have been attributed to the different receptor subtypes, their differential affinities to agonist and their differential cellular or subcellular distributions[1,5,6,31-34]. In present study, we observed the differential trafficking properties of D1R and D5R, adding a new factor into consideration of how the two D1-like receptor subtypes implement complex modulation of DA.

D1-like receptor family displays the sub-micromolar EC_{50} values for SKF38393-stimulated cAMP accumulation in *in vitro* assay. The EC_{50} value ranges from 0.2 to 0.8 μmol/L in D1R-mediated cAMP accumulation assay, and stays at about 0.3 μmol/L in D5R-mediated assay[28,35]. In present study, 1 μmol/L SKF38393 was adopted, because this concentration is higher than EC_{50} values, and is capable of efficiently triggering intracellular signaling in most cAMP accumulation assays *in vitro*. D5R also displays about 5-fold higher affinity to SKF38393 than D1R[28], so intracellular DA signaling activated by SKF38393 should be preferentially through D5R rather than D1R at low SKF38393 concentration. When the concentration increased above the EC_{50} values, our results revealed that D1R was internalized by 1 μmol/L SKF38393, which would decrease the surface receptors. It is implied that the D1R-mediated signaling would be weakened due to the internalization at 1 μmol/L SKF38393 stimulation. In contrast, D5R-mediated signaling would not be weakened, as D5R was not trafficked from cell surface to the cytosol (Fig. 6A, B, C). In other words, D5R can mediate the intracellular signaling not only at the low agonist level but also at the high level, whereas D1R may primarily mediate the signaling at the intermediate agonist level due to its low affinity and the sensitive agonist-induced internalization. As D5R also exhibits higher affinity to endogenous neurotransmitter DA than D1R[5,7,28] and these differential pharmacological characteristics are their inherent properties, it is proposed that D5R can mediate endogenous intracellular DA signaling in wide DA dynamic range, and D1R in narrow DA dynamic range. Future approaches of simultaneously recording or imaging the recep-

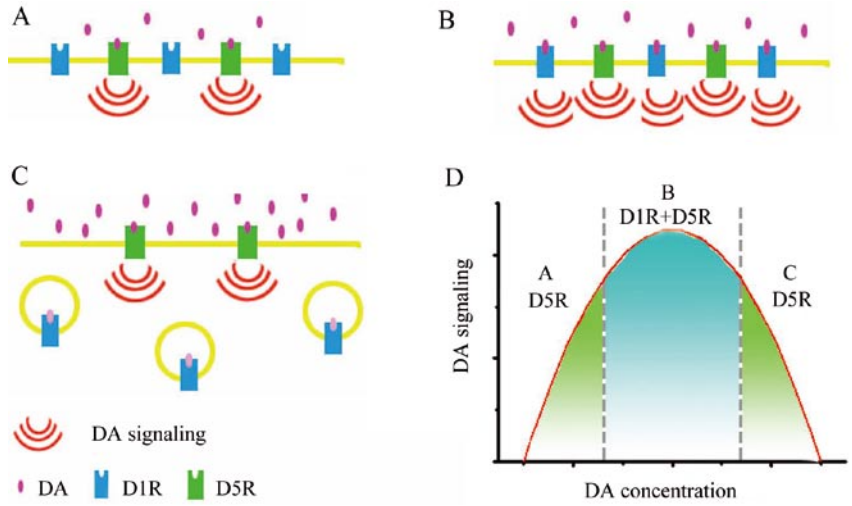

Fig. 6 D1-like receptors (D5R and D1R) could preferentially mediate the DA signaling in different dynamic range. Different concentrations of DA (or D1 agonist) are indicated by the numbers of pink dot. A, D5R with high affinity to DA mediates the main DA signaling at low DA concentration. B, Both D1R and D5R mediate the DA signaling at intermediate DA concentration, and the signaling strength increases. C, D5R mediates the main DA signaling at high DA concentration, as internalization of D1R will decrease D1R-mediated signaling strength in this condition. D, The synergism of D1R- and D5R-mediated DA signaling over the full dynamic DA range in co-localization sites will result in a non-linear dose-effect relationship, such as an "inverted-U".

tor trafficking and intracellular cAMP signaling in real-time manner will be beneficial for the test of this hypothesis.

Recent ultrastruturally morphological evidence proved that D1R and D5R co-localized in the same spine of pyramidal neurons in primate prefrontal cortex[36]. In such co-localization sites, the synergism between D1R- and D5R-mediated DA signaling over the full dynamic range will result in non-linear dose-effect relationship (Fig. 6D). Although such cellular mechanism can not be directly utilized to interpret the animal behavior *in vivo*, such as the DA "invert-U" dose-effect in working memory performance[30,31], it enriches the understanding of the potential mechanisms for the complex modulation of DA.

4.3 ACh induced the hetero-regulation of D1 receptors The present study also showed that ACh induced the internalization of D1R that expressed alone or co-expressed with D5R in NG-108 cells.

The heterodimerization of different GPCRs can result in the modulation of their function and/or the redistribution of their subcellular locations[17-20]. It was documented that β2-adrenergic agonist isoproterenol could induce the internalization of opioid δ receptors through the hetero-dimer of β2-adrenergic receptor and δ opioid receptor[20]. Several lines of evidence raise the possibility of the hetero-dimerization between D1R and mAChR: (1) In NG108-15 cells, the endogenous mAChR was reported to be internalized by ACh stimulation[37], and the mAChR could form heterodimer with other GPCRs[38]. (2) D1R could also form the heterodimer with other GPCRs and mutually regulate each other, just like the heterodimer between D1R and adenosine A1 receptor[18]. (3) D1R and mAChR were found to co-localize in striatonigral neurons both *in vivo*[39,40] and in our expression system. So the possible hetero-interaction between mAChR and D1R and the resulting hetero-regulation of D1R internalization are worthy of investigating in future.

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神经细胞中多巴胺 D1 和 D5 受体不同亚细胞分布和胞内转运的特性

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摘要：目的 探索多巴胺D1样受体家族的D1、D5亚型，可能有的不同的胞内转运(trafficking)特性。**方法** 为了直接观察D1样受体的亚细胞分布，用绿色荧光蛋白(GFP)变体分别标记D1和D5受体，转染进神经瘤细胞系NG108-15中，并对它们进行定位，然后比较在D1受体激动剂SKF38393或乙酰胆碱(ACh)处理前后D1、D5受体亚细胞分布的异同，以研究它们胞内的转运特性。**结果** 在静息条件下，D1受体特异分布在NG108-15的细胞膜上，而D5受体分布于细胞膜和胞浆内。以D1受体激动剂SKF38393孵育处理后，D1受体亚细胞分布发生改变，胞浆中出现代表D1受体的黄色荧光信号，提示D1受体被内吞； ACh的孵育处理也能引起D1受体异源内吞。与D1受体不同，经SKF38393或ACh处理后，D5受体亚细胞分布与静息条件下比较，未显示明显变化。这提示药物处理不引起D5受体由胞浆向细胞膜的转位(translocation)。当D1和D5受体共同表达在同一个NG108-15细胞中时，它们仍然保持各自的亚细胞分布模式。而且，SKF38393和ACh引起的D1或D5受体的细胞内定位状态，也并未因另一种亚型的存在而改变。**结论** 当分别表达在神经瘤细胞系NG108-15上时，D1、D5两个受体亚型显示出不同的亚细胞定位和药物引起的不同胞内转运特性。当两者共同表达时，D1和D5又互不干扰。SKF38393和ACh都可以引起D1受体的内吞。我们对D1样受体家族这种不同的胞内转运特性在DA信号复杂调控中的可能作用，进行了讨论。

关键词： 多巴胺D1受体；多巴胺D5受体；胞内转运；内吞；绿色荧光蛋白；SKF38393；乙酰胆碱