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DIFFERENTIAL G PROTEIN ACTIVATION BY ISOFORMS OF THE DOPAMINE D_2 RECEPTOR

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Abstract:
Background and Purpose
The dopamine D_2 receptor is expressed as a short (D2S) and a long (D2L) isoform with 29 additional amino acids in the third intracellular loop. The D2S isoform shows higher presynaptic expression than the D2L isoform, and decreased D2S expression has recently been linked to an increased risk for schizophrenia. Here, we present the first investigation, at receptor isoform level, of kinetic differences in the G protein activation profiles of the D2S, compared with the D2L isoform.
Experimental Approach
We employed a Nano BRET-based approach to G protein dissociation to interrogate the time-resolved coupling profile of 3×HA-tagged D2L and D2S to $G\alpha_{i/o/z}$ proteins in vitro.
Key Results
Using dopamine as a D_2 receptor agonist, we observed a more pronounced activation of $G\alpha_o$ and $G\alpha_z$ than $G\alpha_i$ proteins by D2L compared with D2S. This differentiation was not observed for D2S, which activated $G\alpha_o$ and $G\alpha_z$ with lower efficacy than D2L. These signalling differences were preserved on second messenger level and were not due to differences in receptor expression. Expanding to a set of seven full and partial D_2 receptor agonists showed these effects were not restricted to dopamine but rather a mutual, receptor-associated property. Contrasting this trend, we found that D2S activated G proteins faster than D2L upon full receptor activation.
Conclusion and Implications
The findings highlight that both D2L and D2S are mechanistically able to activate all non-visual $G\alpha_{i/o}$ proteins. Thereby, they add to previous reports about isoform-specificity to certain $G\alpha_{i/o}$ proteins observed in specific cell types.

Keywords : iso-form , receptors , cell types

Introduction :

Negative feedback of neurotransmitter release serves to avoid excessive postsynaptic action and is largely guided by presynaptic autoreceptors. For a considerable number of central neurotransmitters, the autoreceptor is a GPCR. In the case of the dopaminergic system, this feedback regulation is governed by the dopamine D_2 receptor (Centonze et al., 2003, 2004; Usiello et al., 2000).
The D_2 receptor is an established drug target for numerous CNS disorders including Parkinson's disease, restless-legs syndrome, and schizophreniform disorders (Beaulieu et al., 2015, 2023;

Ferraiolo & Hermans, 2023; Uchida et al., 2011). However, such drugs target two different receptor isoforms. The *DRD2* gene generates two principal transcripts that are highly expressed in the brain (Dal Toso et al., 1989; Grandy et al., 1989; Montmayeur et al., 1991; Usiello et al., 2000). They differ by 29 amino acids (29aa) in the intracellular loop 3 (ICL3), which are retained in case of a long (D2L) isoform and removed by alternative splicing of exon 6, leading to a short (D2S) isoform of the D_2 receptor (Figure S1a) (Dal Toso et al., 1989; Grandy et al., 1989). The isoforms have similar pharmacology in terms of drug binding to the orthosteric site (Dal Toso et al., 1989; Itokawa et al., 1996; Martres et al., 1992) but differ in their cellular and subcellular expression patterns (Figure S1b) (Blagotinsek Cokan et al., 2020; Itokawa et al., 1996; Kubale et al., 2016; Montmayeur et al., 1991). While D2L seems to govern postsynaptic functions together with D2S (Khan et al., 1998; Lindgren et al., 2003), only D2S is considered to be the presynaptic autoreceptor for the brain dopaminergic system (Centonze et al., 2003; Khan et al., 1998; Usiello et al., 2000).

Material and method :

HEK293T cells (RRID:CVCL_0063) were cultured on T75 or T175 tissue culture flasks in culture medium (DMEM supplemented with 10% [v/v] FBS and penicillin–streptomycin [$100\ U\cdot ml^{-1}$, each]) in a humidified atmosphere (37°C, 5% CO_2). Cells were split routinely every 2–3 days, briefly, by washing with DPBS, detaching with Trypsin-EDTA solution, which was stopped by adding approx. a volume of 2:1 cell culture medium and followed by 5-min centrifugation at 170 x *g*. The cell lines were regularly checked for the absence of mycoplasma contamination by quantitative PCR (qPCR) diagnosis (Eurofins Genomics, Ebersberg, Germany).

For G protein activation assays, 1×10^6 cells were plated on six-well tissue culture dishes and transfected with 250 µl of a pcDNA sample of 7 µg in OptiMEM, which was mixed with PEI in OptiMEM (4 g PEI per ng pcDNA) and incubated for 30 min prior to addition to cells. Such pcDNA samples consisted of pcDNA3.1(+) encoding $Venus_{156–239}$-Gβ1, $Venus_{1–155}$-Gγ2, masGRK3ct-Nluc (500 ng each), the Gα protein of interest (750 ng), and either DRD2-414aa or DRD2-443aa (1125 ng), filled up to 7 µg with empty pcDNA3.1(+)-vector plasmids and were prepared in 150-µl OptiMEM. Validation samples during assay implementation were prepared by substituting receptor- (R mock) or G protein- (G mock) encoding pcDNA for empty pcDNA3.1(+) as negative controls.

For CAMYEL (cAMP accumulation by cAMP-sensor using YFP-EPAC-Rluc) assays, 8×10^5 cells were plated on 6-cm tissue culture dishes in culture medium and incubated at 37°C in a humidified atmosphere (see above); 24 h later, 1-µg pcDNA3.1-D2S or pcDNA3.1-D2L, 1-µg CAMYEL sensor, and 1-µg empty pcDNA3.1(+) in 200-µl OptiMEM were mixed with 6-µl Lipofectamine 2000 in 200-µl OptiMEM (prepared 5 min prior to adding to pcDNA samples), incubated for 30 min and added to the cells.

For ELISA (enzyme-linked immunosorbent assays), 4×10^4 cells were plated on 96-well TC-assay plates in 100-µl culture medium per well. Per well, 23-ng receptor-encoding pcDNA was mixed with PEI (4 ng PEI per ng pcDNA) in 10-µl OptiMEM and incubated for 30 min before addition to cells.

All transfection samples were incubated at 37°C, 5% CO_2 in a humidified atmosphere until further usage as indicated below.

Results

We used an established nBRET-based G protein dissociation approach to test for differences in the activation of G protein subtypes by D2L and D2S (Hollins et al., 2009; Masuho, Martemyanov, & Lambert, 2015; Masuho, Ostrovskaya, et al., 2015; Moo et al., 2021). Briefly, HEK293T cell lines transiently transfected with a Venus-Gβ-Gγ construct and mas-GRK3ct-Nluc construct will show increased BRET when receptor activation and subsequent G protein activation occurs. This is due to the G protein activation leading to dissociation of the Gα subunit from the Venus-Gβ-Gγ sensor, which then can physically interact with the C-terminal recognition motif in GRK3, leading to

proximity of Venus and Nluc (Hollins et al., 2009). Informed by previous studies (Avet et al., 2022; Hauser et al., 2022;Masuho et al., 2023; Masuho, Ostrovskaya, et al., 2015; Moo et al., 2021), we decided to overexpress either of the $G\alpha_{i1-3}$, $G\alpha_{oA}$, $G\alpha_{oB}$, and $G\alpha_z$ subtypes of the Gα subunit of the heterotrimeric G proteins, together with either of the 3×HA-tagged D2 isoforms (Figure 1a). We confirmed an absence of measurable G protein activation in absence of Gα proteins and in presence of $G\alpha_s$ and $G\alpha_q$ proteins (Figure S1e, f), in line with the previously observed G protein-coupling pattern (Avet et al., 2022; Hauser et al., 2022; Masuho et al., 2023; Masuho, Ostrovskaya, et al., 2015; Moo et al., 2021).

We monitored full concentration–response curves . Here, we have observed that D2L differed from D2S in the maximal activation (E_{max}-ΔBRET) for $G\alpha_{oA}$, $G\alpha_{oB}$, and $G\alpha_{i1-3}$ subtypes and showed a similar trend for $G\alpha_z$; however, it was not statistically significant . Furthermore, we determined the temporally resolved activation of the distinct G protein subtypes (yielding the activation rate constant $1/\tau$ and the amplitude-ΔBRET). We discovered a higher activation amplitude (amplitude-ΔBRET) by D2L, compared with D2S among all studied G proteins, and this was significant for $G\alpha_{oA}$ and $G\alpha_z$. This suggests a considerable effect of the 29aa difference in ICL3 on the overall efficacy of D2L. While the isoforms distinguished between towards the distinct G proteins in terms of efficacy, the corresponding potencies and activation kinetics were unaltered.

Discussion

Recent G protein activation studies among up to 148 GPCRs have created a comprehensive overview of receptor-G protein coupling specificity but have not covered distinct physiologically relevant receptor isoforms (Avet et al., 2022; Hauser et al., 2022; Inoue et al., 2019; Pándy-Szekeres et al., 2023). Therefore, we have studied the G protein coupling behaviour of the D_2 receptor, which exists in a long and short isoform with a 29aa deletion within ICL3. To the best of our knowledge, this is the most extensive assessment of D_2 receptor isoforms covering all six human non-olfactory and non-retinal $G\alpha_{i/o}$ subtypes, in parallel. The present study warrants detailed analysis of other physiologically relevant isoforms of GPCRs.

Going beyond classical concentration–response analysis, we intended to investigate the kinetic differences in G protein activation, using an nBRET-based assay platform that has not been studied for D2S in comparison with D2L, to date. The applied nBRET platform has the advantage of not requiring labelling of either receptor or Gα subunits and thereby manipulating their properties. Still, constraints to the translatability of the results could arise because we only used the Gβ1γ2 out of 59 other possible combinations (Masuho et al., 2021). The effective BRET values are often compared among different G protein isoforms given that the released Gβ1γ2-Venus protein will have a similar BRET efficiency with the masGRK3ct-nLuc construct (Masuho, Ostrovskaya, et al., 2015). However, potential limitations for this comparison can be differences in expression of the respective Gα subunit. Also, kinetic differences between the activation of the Gα subtypes can be dominated by intrinsic activation properties of the respective G protein as an intrinsically low activation rate of $G\alpha_z$ and $G\alpha_i$ over $G\alpha_o$ proteins (M. Jiang & Bajpayee, 2009). Lastly, the kinetics are influenced by differences in their deactivation mechanisms (Masuho et al., 2020). Special care must be taken in comparing the results obtained for D2L and D2S with the different Gα proteins, because similar expression levels between them cannot be guaranteed. Nevertheless, the observed rank order of G protein subtype activation is in accordance with previous results derived for D2L (Masuho et al., 2023; Masuho, Ostrovskaya, et al., 2015; Moo et al., 2021). Moreover, those results align with the previous notion of $G\alpha_o$ being the main effector for D_2 receptors in the brain (M. Jiang et al., 2001). We determined a higher difference in the efficacy (kinetically or at equilibrium) for all $G\alpha_{i/o}$ subtypes for D2L over D2S, while dopamine did not show differences in kinetics or activation speed via either isoform.

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