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MDPI

# Identification of novel adenylyl cyclase 5 (AC5) signaling networks in $D_1$ and $D_2$ medium spiny neurons using bimolecular fluorescence complementation screening

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## SUPPLEMENTAL METHODS

*Development and culture of CAD VN-AC5/$D_{2L}$ stable cell line*

The Cath a. differentiated (CAD) cell line was constructed to express the long isoform of the human $D_2$ dopamine receptor ($D_{2L}$) and human AC5 linked by the N-terminus to the N-terminal BiFC fragment of the Venus fluorescent protein (VN155-AC5) through a short alanine rich, flexible linker CAD VN-AC5/$D_{2L}$ cells). Briefly, CAD VN-AC5/$D_{2L}$ stable cell clones expressing VN-AC5 were assessed by their response to acute forskolin stimulation. Clones providing a robust FSK response were then transfected with the human $D_{2L}$, and subsequently selected based upon the development of $D_2$-mediated heterologous sensitization. Final CAD VN-AC5/$D_{2L}$ clones were selected based upon functional results, as well as their ability to produce a strong BiFC response with complementary interacting partners. Stable cell clones were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Grand Island, NY), supplemented with 5% bovine calf serum (Hyclone, Logan, UT), 5% fetal bovine serum (Hyclone, Logan, UT), 1% Antibiotic-Antimycotic 100x solution (Life Technologies, Grand Island, NY). Stable cell clones were maintained in culture media containing 800µg/ml G418 (Invivogen, San Diego, CA) and 200µg/ml puromycin. All cell lines were maintained in a humidified incubator at 37°C and 5% $CO_2$.

*Adenylyl cyclase assay procedures*

Acute cAMP accumulation was measured by adding 10µl/well a mixture of FSK and 3-isobutyl-1-methylxanthine (IBMX) (3µM and 500µM final concentrations, respectively) to the wells, plates were incubated at room temperature for 1 hour before cell lysis and cAMP accumulation measurements. Assay plates using the Cisbio HTRF cAMP dynamic-2 assay kit was excited using 330nm wavelength and analyzed for fluorescent emissions at 620 and 665nm using a Synergy4 (BioTek, Winooski, VT). Ratiometric analyses were performed with GraphPad Prism (GraphPad Software, La Jolla, CA) by dividing the 665nm emission by the 620nm emission to interpolate cAMP concentrations from a cAMP standard curve.

Acute cAMP inhibition experiments were conducted by adding 5µl/well quinpirole (final concentration 3µM), and then the plates were briefly centrifuged to ensure all liquid was collected at the bottom of the well. Plates were incubated at room temperature for 15 minutes, then stimulated by adding 5µl/well forskolin in 3-isobutyl-1-methylxanthine (IBMX) (3µM and 500µM final concentrations, respectively) to the wells, plates were incubated at room temperature for 1 hour before cell lysis and cAMP accumulation measurements.

Sensitization cAMP accumulation was measured by adding 5µl/well of the $D_2R$ ligand quinpirole (3µM final concentration) and incubating the plate at 37°C and 5% $CO_2$ for 2 hours.

5µl/well forskolin (300nM final concentration) in 2mM 3-isobutyl-1-methylxanthine (IBMX) and 1µM spiperone was added to the wells, then plates were incubated at room temperature for 1 hour before cell lysis and cAMP accumulation measurements.

*siRNA reverse transfection*

Reverse transfection of siRNA for cAMP accumulation in 384-well plates.

20µM siRNA stocks were diluted to 0.5µM in OptiMEM, adding 2.4µl of dilute stock to each appropriate well. Lipofectamine RNAiMAX was diluted in OptiMEM (9µl/mL) and 8µl added to each well containing siRNA. The plate was centrifuged briefly and incubated at room temperature for 30 minutes. Cells in culture were re-suspended in OptiMEM containing 7.5% heat inactivated fetal bovine serum, and 20µl cell solution plated on top of siRNA/RNAiMAX. The plate was centrifuged briefly, then incubated in a humidified incubator at 37°C and 5% $CO_2$ for 72 hours. Acute and sensitization cAMP assays were conduced as described above, adjusting concentrations for an increased 40µl final well volume.

Cell viability following siRNA transfection was measured using the CellTiter-Fluor (Promega, Madison, WI) according to the manufacturer's directions. Assay plates using the cell titer-fluor assay kit were excited using 380nm wavelength and analyzed for fluorescent emissions at 508 using a Synergy4 (BioTek, Winooski, VT).

*PCR Conditions and primers for amplification of cDNA insert*

Thermocycler Program:

| | | |
|---|---|---|
| Initial Denaturation | 95°C | 30sec |
| 40 Cycles | 95°C | 30sec |
| | 60°C | 60sec |
| | 72°C | 5min |
| Final Extension | 72°C | 10min |
| Hold | 4°C | |

Primers:

PCR primers were purchased from Integrated DNA Technologies (Coralville, IA)

Forward - 1st PCR

5′-ACACTCTTTCCCTACACgACgCTCTTCCgATCTggAgACCCAAgCTggCTAgCg-3′

Reverse - 1st PCR

5′-gTgACTggAgTTCAgACgTgTgCTCTTCCgATCTgTCggATCCACCTgATCCgCC-3′

Forward Vehicle -2nd PCR Forward Vehicle – FACS#1

5′-AATGATACGGCGACCACCGAGATCTACACTGAACCTTACACTCTTTCCCTACACGAC-3′

Reverse Vehicle -2nd PCR Reverse Vehicle – FACS#1

5′-CAAGCAGAAGACGGCATACGAGATATCACGACGTGACTGGAGTTCAGACGTG-3′

Forward Quinpirole- 2nd PCR Forward Treatment – FACS#1

5′-AATGATACGGCGACCACCGAGATCTACACTGCTAAGTACACTCTTTCCCTACACGAC-3′

Reverse Treatment - 2nd PCR Reverse Treatment – FACS#1

5′-CAAGCAGAAGACGGCATACGAGATACAGTGGTGTGACTGGAGTTCAGACGTG-3′

Forward Vehicle – 2nd PCR Forward Vehicle – FACS#2

5′-AATGATACGGCGACCACCGAGATCTACACTGTTCTCTACACTCTTTCCCTACACGAC-3′

Reverse Vehicle - 2nd PCR Reverse Vehicle – FACS#2

5′-CAAGCAGAAGACGGCATACGAGATCAGATCCAGTGACTGGAGTTCAGACGTG-3′

Forward Quinpirole - 2nd PCR Forward Treatment – FACS#2

5′-AATGATACGGCGACCACCGAGATCTACACTAAGACACACACTCTTTCCCTACACGAC-3′

Reverse Treatment - 2nd PCR Reverse Treatment – FACS#2

5′-CAAGCAGAAGACGGCATACGAGATACAAACGGGTGACTGGAGTTCAGACGTG-3

Forward Vehicle – 2nd PCR Forward Vehicle – FACS#3

5′-AATGATACGGCGACCACCGAGATCTACACCTAATCGAACACTCTTTCCCTACACGAC-3′

Reverse Vehicle - 2nd PCR Reverse Vehicle – FACS#3

5′-CAAGCAGAAGACGGCATACGAGATACCCAGCAGTGACTGGAGTTCAGACGTG-3′

Forward Quinpirole - 2nd PCR Forward Treatment – FACS#3

5′-AATGATACGGCGACCACCGAGATCTACACCTAGAACAACACTCTTTCCCTACACGAC-3′

Reverse Treatment - 2nd PCR Reverse Treatment – FACS#3

5′-CAAGCAGAAGACGGCATACGAGATAACCCCTCGTGACTGGAGTTCAGACGTG-3′

*qPCR Primers*

GAPDH

Forward: 5′-GTCGGAGTCAACGGATTTG-3′
Reverse: 5′-GACGGTGCCATGGAATTT-3′

GNAS

Forward: 5′-TGAACGTGCCTGACTTTG-3′
Reverse: 5′-TCGATCTTGTCCAGGAAGTA-3′

AC5

Forward: 5′-AGATGAACCGCCAGAGAA-3′
Reverse: 5′-CTCAGACCGAAGCCTATCA-3′

PPP2CB

Forward: 5′-GTGGAGACTGTGACTCTTCTTG-3′
Reverse: 5′-CTTGGGTAATTTGTCGGCTTTC-3′

NAPA

Forward primer: 5′-CTGTTTGATGCGAGCAATCG-3′
Reverse primer: 5′-GTCCACCAACTCTGTCTCATAG-3′

*Immunoprecipitation*

Unless otherwise specified, reagents were purchased from Sigma Aldrich (St. Louis, MO). Immunoprecipitation of adenylyl cyclase activity was conducted as previously described. Briefly, non-transfected HEK AC5/$D_{2L}$ cells were grown to 90% confluency in 10-cm dishes. Cells were washed with 3-5mL ice-cold PBS, removing PBS by aspiration, and lysis buffer added to plate, and left on ice for 5 min (300µL; 50mM HEPES, pH 7.5, 1mM EDTA, 1mM $MgCl_2$, 150mM NaCl, 0.5% $C_{12}E_{10}$, plus protease inhibitor cocktail). Cells were scraped and collected in an Eppendorf tube, a 23-gauge needle and 1mL syringe were used to homogenize cells. Cell lysate was centrifuged at 13,000xg for 10 min at 4°C to remove cellular debris. Supernatant was collected in new Eppendorf tube, and protein concentration determined by BCA assay. Samples were diluted to 500µg/mL, and 500µL aliquoted in Eppendorf for each condition. 1-2µg of antibody was added to appropriate vial, and rotated overnight at 4°C. 30µl of washed anti-protein A agarose beads were added to each vial, then rotated for 1hr at 4°C. After incubation, samples were centrifuged, and supernatant removed. The beads were washed three times with 300µl wash buffer (lysis buffer containing 0.05% $C_{12}E_{10}$). Samples for western blotting were resuspended in 40µl 1x SDS sample buffer and run as western blots described previously. Samples for adenylyl cyclase activity assays were resuspended in 50µl membrane buffer (50mM HEPES, pH 7.5, 1mM EDTA, 1mM $MgCl_2$, 0.05% $C_{12}E_{10}$) and 10µl/well plated in white, flat bottom, tissue culture-treated 384-well plate (PerkinElmer, Shelton, CT). 5µl membrane buffer without $C_{12}E_{10}$ was added to all wells. 5µl 4x stimulation buffer (33mM HEPES, 0.05% $C_{12}E_{10}$, 10mM $MgCl_2$, 1mM ATP, 4µM GTPγS, 2mM IBMX, 200µM forskolin, 200nM purified, $G\alpha_s$-GTPγS) was added to appropriate wells. 5µl stimulation buffer lacking forskolin and purified $G\alpha_s$ were added to basal wells. Plates were incubated at room temperature for 1 hour before cell lysis and cAMP accumulation measurements as described previously for Cisbio HTRF cAMP assays.

*Western Blotting*

Unless otherwise listed, reagents were purchased from Sigma Aldrich (St. Louis, MO) Anti $G\alpha_{s/olf}$ antibody was purchased from Santa Cruz biotechnology (Dallas, TX). Anti-NAPA, anti-vinculin, and anti-alpha tubulin antibodies were purchased from Novus biological (Littleton, CO). Anti-PPP2CB antibodies were purchased from Abcam (Cambridge, MA).

Cells were briefly with phosphate buffered saline, before being dissociated from the plate with non-enzymatic cell dissociation buffer and centrifuged at 800xg for 5 minutes. The supernatant was aspirated, and the cell pellet re-suspended by pipetting in RIPA buffer (final concentrations in water, 150mM NaCl, 5mM EDTA, 50mM Tris, 1.0% TritonX, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate) containing phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktail before being placed on ice for 30 minutes. The cell samples were centrifuged for 15 minutes at 18,000xg at 4°C, with the soluble fraction being preserved. A BCA protein assay (Biorad, Hercules, CA) was used according to the manufactures directions to determine the protein concentration of each sample. 15ug of each sample were combined with Laemmli buffer (final concentration, 60mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 5 minutes. Denatured proteins were separated via SDS-PAGE on a 4-18% polyacrylamide gel (BioRad, Hercules, CA) and transferred to a PVDF membrane, pore size 0.45µm (Millipore, Billerica, MA). Membranes were blocked in 5% non-fat milk for 1 hour at room temperature, the membrane was probed for the protein of interest with primary antibodies diluted in PBS + 0.5% Tween20 (PBST) with 1% milk, by rocking overnight at 4°C. The membrane was washed with PBST, then incubated with a secondary IRDye 680RD anti-mouse or IRDye 800CW anti-rabbit (LICOR Biotechnology, Lincoln, NE) at 1:10,000 for 1 hour at room temperature. Detection of immunostaining was carried out using LICOR Odessey CLx imager. Bands were quantified using ImageJ Software (NIH, Bethesda, MD).