Chapter 2

Characterization of D1 Dopamine Receptor Posttranscriptional Regulation

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Abstract

Posttranscriptional regulation (PTR) of gene expression describes regulatory mechanisms that control the expression of protein from its cognate mRNA. Studies that investigate changes in gene expression, for a variety of reasons, typically focus on measuring levels of mRNA or protein but not both. Even studies that measure both mRNA and protein levels of the gene of interest rarely assess the temporal discordance between the two. Given that PTR provides a mechanism for spatial and temporal regulation of gene expression, it likely plays a major role in physiological and pathophysiological conditions. In this chapter, we describe methods to assess PTR using the D1 dopamine receptor gene as an example. PTR mechanisms can be broadly classified into mechanisms that regulate mRNA turnover and those that control mRNA translation. The mouse catecholaminergic CAD cell line which expresses endogenous D1 dopamine receptor is a tractable model system for deciphering the molecular mechanism of D1 receptor PTR. We describe methods to measure D1 dopamine receptor mRNA stability using actinomycin D and methods using reporter constructs to assess microRNA (miRNA)-mediated regulation of D1 receptor protein translation. Using these methods we demonstrate that the D1 dopamine receptor exhibits PTR in which the expression of D1 receptor protein is regulated by miRNAs. The chapter provides detailed methods for studying potential D1 dopamine receptor PTR during development and in disease states.

Key words Dopamine receptor, Gene expression, Posttranscriptional regulation, mRNA stability, mRNA translation, MicroRNA, 3′ untranslated region, RT-PCR, Western blotting

1 Introduction

PTR of gene expression provides mechanisms by which expression of protein can be regulated spatially and temporally [1]. For example, in the nervous system, mRNA is synthesized in the nucleus of cell bodies and trafficked to axonal or dendritic terminals where local protein translation can be initiated by cues at a later time [2]. This process requires molecular mechanisms that can stabilize and protect the mRNA as well as suppress its translation. Ontogeny studies in rodents have shown that while the expression of D1 receptor mRNA commences around embryonic day 14 and reaches steady state expression level around postnatal day 5, the D1

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receptor protein levels increase postnatally and reach peak values between postnatal day 7 and 14 [3–5]. This lack of correlation in expression of D1 receptor mRNA and protein is also seen during human brain development [6]. While these studies suggest that D1 receptor expression is regulated at the posttranscriptional level, the molecular mechanisms that mediate the posttranscriptional regulation (PTR) of D1 receptor expression are not well understood. In this chapter, we focus on methods used to determine if the D1 dopamine receptor gene is posttranscriptionally regulated and describe approaches to study two mechanisms of PTR. We will describe the use of actinomycin D to study D1 dopamine receptor mRNA stability and methods used to study the role of miRNA in regulation of D1 dopamine receptor protein translation. To study PTR of any gene, three tools are necessary—(a) a tissue or cell line that endogenously expresses the gene and exhibits PTR of the gene under defined conditions, (b) method to quantitate the level of mRNA, and (c) good quality antibodies that can detect and quantitate the expression of the cognate protein.

In the case of D1 dopamine receptor, we have shown that the mouse CAD catecholaminergic cell line expresses endogenous D1 dopamine receptor mRNA and protein [7]. Furthermore, CAD cells undergo reversible differentiation in serum-free media (Fig. 1) during which there is an increase in D1 receptor mRNA but no concomitant increase in D1 receptor protein levels [7]. Thus the CAD cell line is a useful model to study PTR of D1 dopamine receptor. In this chapter, we will discuss the culture conditions for growing non-differentiated and differentiated CAD cells. The mouse D1 receptor gene has a promoter that is ~6.4 kb, one intron in the noncoding region, as well as 5′ and 3′ untranslated regions (5′UTR and 3′UTR) [7, 8]. We have detected and quantitated D1 receptor mRNA using methods such as RNase protection assay and quantitative real-time PCR [7, 8]. In this chapter we will describe the latter method for quantifying D1 receptor mRNA levels using the Taqman® PCR methods. Antibodies for detecting membrane proteins such as G-protein coupled receptors are notoriously poor, lacking selectivity and exhibiting inconsistency from lot to lot. Following years of testing various D1 receptor antibodies, we have validated a commercially available anti-D1 dopamine receptor rat monoclonal antibody that consistently detects the non-glycosylated and multiple glycosylated isoforms of mouse, rat, and human D1 dopamine receptor protein (Fig. 2).

To measure D1 receptor mRNA stability in non-differentiated and differentiated CAD cells, we quantitate the levels of mature D1 receptor mRNA at different time points after the synthesis of new mRNA is blocked using actinomycin D. To assess regulation of D1 receptor protein expression by miRNA, we use plasmid constructs in which the β-galactosidase reporter gene is fused to D1 receptor 3′UTR with and without mutations in putative miRNA

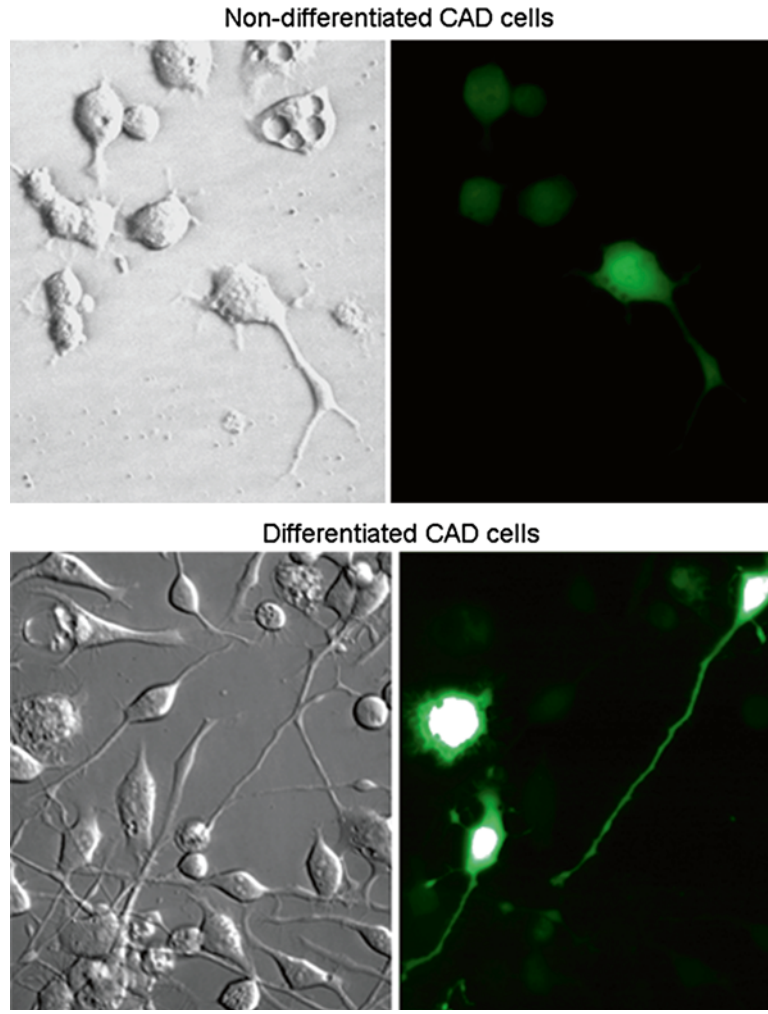

Fig. 1 Representative bright field and fluorescent images of non-differentiated and differentiated CAD cells that were transfected with a plasmid expressing enhanced green fluorescent protein using Lipofectamine 2000 as described in Sect. 3.3. The differentiated cells were cultured in serum-free media for 48 h

binding sites. We describe the spectrophotometric assay used to measure β-galactosidase activity using widely available reagents and equipment.

2 Materials

2.1 Cell Culture Materials

1. Dulbecco's modified Eagle's medium (DMEM)/F12 media (catalog# 12-719Q, Lonza-BioWhittaker, Walkersville, MD, USA).
2. Fetal calf serum (catalog# 26140-079, Invitrogen, Carlsbad, CA, USA).

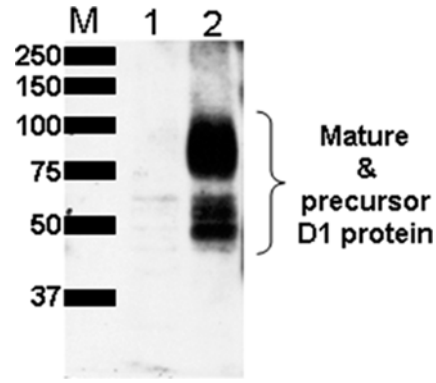

Fig. 2 Representative Western blot using the anti-D1 receptor rat monoclonal antibody shows the level of mouse D1 receptor protein in AtT-20 neuroendocrine cells (which does not express endogenous D1 receptor) transiently transfected with empty plasmid vector (*lane 1*) or a constitutive expression plasmid encoding the mouse D1 dopamine receptor (*lane 2*). Each lane was loaded with 30 μg of total cell protein and the D1 receptor protein detected using the anti-D1 receptor rat monoclonal antibody. Multiple glycosylated forms of mature D1 receptor are detected with this antibody

3. Penicillin/streptomycin (catalog# 15140-148, Invitrogen).
4. 10× Phosphate-buffered saline (catalog# 70011-044, Invitrogen).
5. Sodium selenite (catalog# S9133, Sigma-Aldrich, St. Louis, MO, USA).
6. Transferrin (catalog# T8158, Sigma-Aldrich).
7. Actinomycin D, Streptomyces sp. (catalog# 114666, EMD-Millipore, Billerica, MA, USA).
8. 6- or 12-well Costar tissue culture plates (catalog# 07-200-80 or 07-200-81, Fisher Scientific, Pittsburgh, PA, USA).
9. 100-mm tissue culture plates (catalog# 83. 1802.003) (Sarstedt Inc., Newton, NC, USA).
10. T-25 tissue culture flasks (catalog# 83.1810.001) (Sarstedt Inc., Newton, NC, USA).
11. T-75 tissue culture flasks (catalog# 83. 1813) (Sarstedt Inc., Newton, NC, USA).
12. Cellulose acetate 0.22 μm bottle top filters (catalog# 09-761-50, Corning, Tewksbury, MA, USA).

2.2 Enzymes and Reagents for Cloning D1 3′ UTR

1. Bacterial artificial clone (BAC) containing the entire mouse D1 dopamine receptor gene (catalog# RPCI23.C, clone ID: 47M2, Invitrogen).
2. Primers for amplifying and cloning the 1,277 bp mouse D1 receptor 3′UTR-forward Not I primer 5′-AAGGAAAAA

AGCGGCCGCATATTGGGTTCTCATCTCTGAAGCT ATGAGTTCC-3′, reverse Hind III primer 5′-G GTT TAA AC AAGCTT CACGTG C TTA AGC TCA TTA GCT AGT TTA CCA CTA ACA TTA AAG AGC-3′. The forward primers has a Not I restriction site and the reverse primer has restriction sites for Hind III, Afl II, and Pml I. To generate the mutation in the miR142-3p binding site in the D1 3′UTR, we used a reverse Ssp I/ Kpn I primer 5′-CTTTCCAAAATAT TTTTAGGGGCAGAGCATTGGGGTACCTAGTCACTT CTTACC-3′.

3. Restriction enzymes were from New England Biolabs, Ipswich, MA, USA.
4. Advantage® 2 Polymerase Mix (catalog# 639201, Clontech Labs, Mountain View, CA, USA).
5. Deoxynucleotides (dNTP) (catalog# 10297-018, Invitrogen).
6. Enzyme diluent (catalog#1773, BioFire Diagnostics, Salt Lake City, UT, USA).
7. QIAquick Gel Extraction Kit (catalog# 28704, Qiagen, Valencia, CA, USA).
8. T4 DNA ligase (catalog# 15224-017, Invitrogen).
9. MAX Efficiency® DH5α™-T1R competent *E. coli* cells (catalog# 12297-016, Invitrogen).
10. QIAprep Spin Miniprep Kit (catalog# 27104, Qiagen).

2.3 Transfection Reagents

1. Lipofectamine 2000 (catalog# 11668-019, Invitrogen).
2. Opti-MEM (catalog# 31985-070, Invitrogen).
3. p3XFLAG-CMV™-7-BAP transfection efficiency control plasmid (catalog# C7472, Sigma).
4. Carrier plasmid pUC19 (catalog#. 15364-011, Invitrogen).

2.4 RNA Isolation and RT-PCR Reagents

1. RNeasy mini kit (catalog# 74104, Qiagen).
2. TURBO DNA-free™ Kit (catalog# AM1907, Invitrogen).
3. Random primers (catalog#48190-011, Invitrogen).
4. SuperScript® III First-Strand Synthesis System (catalog# 18080-051, Invitrogen).
5. Real-time PCR was performed using the Roche Light Cycler carousel-based system (Indianapolis, IN, USA).
6. Glass capillaries (catalog# 04929292001, Roche).
7. Bovine serum albumin (BSA) (2.5 mg/mL) (catalog#1777, BioFire Diagnostics).
8. TaqMan® gene expression assay for D1 dopamine receptor (Assay ID: Mm01353211_m1, Invitrogen).

9. The TaqMan® gene expression assay for internal control GAPDH (Assay ID: Mm99999915_g1, Invitrogen).
10. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (catalog# 4324018, Invitrogen).

2.5 Protein Isolation and Western Blotting Reagents

1. CelLytic™ M reagent (catalog# C2978, Sigma).
2. Phenylmethylsulfonylfluoride (PMSF; catalog# P7626, Sigma).
3. Protease inhibitor cocktail (catalog# P8340, Sigma).
4. BCA Protein Assay Reagent (catalog# 23227, Thermo-Fisher Scientific).
5. Sample loading buffer (62.5 mM Tris–Cl [pH 6.8], 2%SDS, 10 % glycerol, 10 mM EDTA, 50 mM TCEP bond-breaker (catalog# 77720, Thermo-Fisher), 0.01 % bromophenol blue).
6. SDS-PAGE was performed using the Mini-PROTEAN® Tetra Cell system using homemade gels or commercially available precast gels (Bio-Rad, Hercules, CA, USA).
7. Nitrocellulose membranes (catalog# 88013, Thermo-Fisher Scientific).
8. Nonfat milk dry milk powder (Carnation).
9. Tris-buffered saline (pH 7.4) [20 mM Tris base (catalog# T6066, Sigma), 137 mM sodium chloride (catalog#S9888, Sigma), adjust to pH 7.4 with HCl].
10. Tween®-20 (catalog# P9416, Sigma).
11. Monoclonal anti-D1 dopamine receptor antibody produced in rat (catalog# D2944, Sigma).
12. Monoclonal anti-GAPDH antibody produced in rabbit (catalog# 2118, Cell Signaling Technology, Danvers, MA, USA).
13. Monoclonal ANTI-FLAG® M2 antibody produced in mouse (catalog# F3165, Sigma).
14. Goat anti-rat secondary antibody conjugated to horse radish peroxidase (HRP) (catalog# 31470, Thermo-Fisher Scientific).
15. Goat anti-rabbit secondary antibody conjugated to HRP (catalog# A6154, Sigma).
16. Sheep anti-mouse secondary antibody conjugated to HRP (catalog# A5906, Sigma).
17. Blot stripping buffer—Western ReProbe™ (catalog# 786-119, G-Biosciences, St. Louis, MO, USA).
18. SuperSignal West Dura chemiluminescent substrate (catalog# 34075, Thermo-Fisher Scientific).
19. HyBlot CL™ autoradiography film (catalog# E3018, Denville Scientific, Metuchen, NJ, USA).

2.6 Reagents for β-Galactosidase Assay

1. β-gal lysis buffer (10 mM KCl, 1 mM $MgSO_4$, 2.5 mM EDTA, 0.25 % NP-40 detergent, 50 mM β-mercaptoethanol, and 100 mM sodium phosphate buffer [pH 7.2]).
2. 100 mM sodium phosphate buffer (3.4 mL 1 M Na_2HPO_4 + 1.6 mL 1 M NaH_2PO_4 + 45 mL sterile deionized water).
3. Chlorophenol red-β-D-galactopyranoside (CPRG, catalog# 10884308001, Roche Applied Science, Indianapolis, IN, USA).
4. Coomassie (Bradford) Protein Assay Kit (catalog# 23200, Thermo-Fisher Scientific, Rockford, IL, USA).

3 Methods

3.1 Culture and Actinomycin D Treatment of CAD Cells

Maintain CAD cells in DMEM/F12 media, 8 % fetal calf serum, and 100 U/mL penicillin/streptomycin in T-25 or T-75 tissue culture flasks. Plate and grow the CAD cells used in the experiments in either 6- or 12-well Costar tissue culture plates or 100 mm tissue culture plates. To prepare CAD cells for differentiation, grow CAD cells in serum-containing media for 24–48 h. Subsequently remove the serum-containing media, wash the cells once with phosphate-buffered saline, and treat with serum-free media for 48 h to induce differentiation. Differentiation is induced by treating cells with serum-free medium consisting of DMEM/F12, 20 μg/mL transferrin, 50 ng/mL sodium selenite, and 100 U/mL penicillin/streptomycin for 48 h (*See* **Note 1**).

To determine the stability of D1 receptor mRNA in the non-differentiated and differentiated cells, treat the cells with 1 μg/mL actinomycin D or vehicle (DMSO) control dissolved in either serum-containing (non-differentiated CAD cells) or serum-free (differentiated CAD cells) media (Fig. 3). Harvest cells immediately or after 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h. For harvesting, the cells wash with ice-cold 1× PBS twice, scrape, transfer to a prechilled centrifuge tube, and spin in a refrigerated centrifuge at 1,000 × *g* for 5 min. Remove the supernatant and lyse cell pellet in the buffer provided with RNeasy® mini kit. The lysate can be stored at −80 °C or processed immediately and total RNA isolated as described in Sect. 3.4.

3.2 Cloning and Mutagenesis of D1 Receptor 3′ UTR

Amplify the mouse D1R 3′UTR region (the 1,277 bp fragment) using specific primers and a BAC construct containing the entire mouse D1R gene. The primers include Not I and HindIII/AflII/PmlI restriction sites which facilitates the cloning of the amplified D1R 3′UTR into the pcDNAβ-gal reporter plasmid (Fig. 4a). To amplify the 1,277 bp D1 3′UTR from the BAC construct, set up a PCR reaction containing 200 ng of BAC template, 400 nM forward and reverse primers, 1× Advantage2 PCR buffer, 250 μg/mL

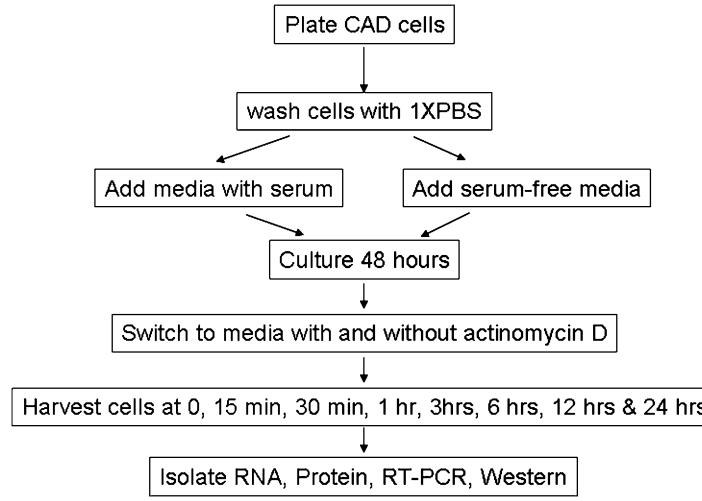

Fig. 3 The experimental design used to measure the stability of D1 receptor mRNA in non-differentiated and differentiated CAD cells. Non-differentiated and differentiated CAD cells are treated with 1 μg/mL actinomycin D and the D1 receptor mRNA and protein levels measured at the indicated time points

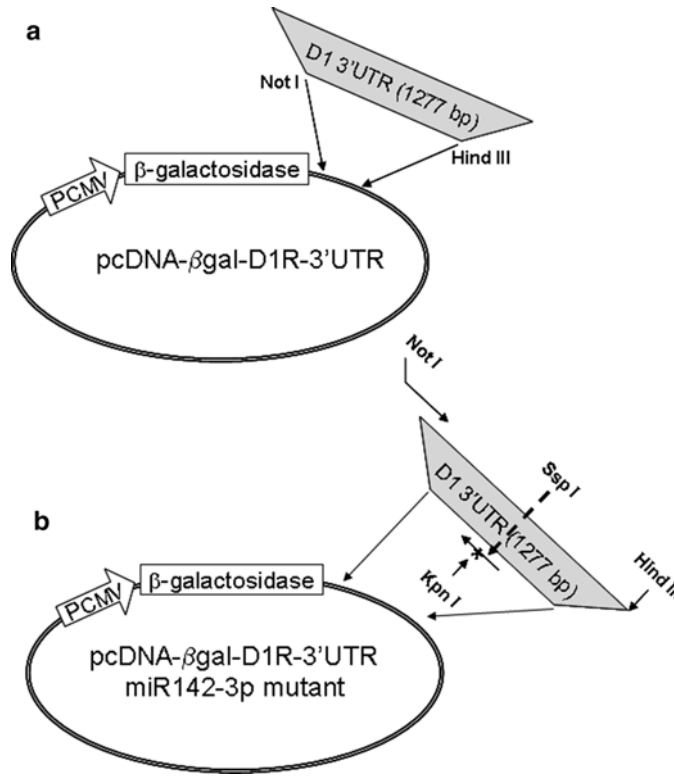

Fig. 4 Schematic representation of the β-galactosidase reporter plasmid under the control of the constitutively active Cytomegalovirus promoter (pCMV) and the wild-type (**a**) or miR142-3p binding site mutated (**b**) D1 receptor 3′UTR. The restriction enzymes used for cloning and the mutagenic primer (*asterisk*, Kpn I) are indicated

BSA, 200 μM dNTP, 0.8 μL enzyme diluent, and 1× Advantage 2 polymerase mix in a final volume of 10 μL. Perform the PCR using a Idaho Technology Light cycler in glass capillary tubes with a 5 min pre-incubation at 94 °C followed by 40 cycles of denaturation (94 °C for 3 s), annealing (57 °C for 3 s), and extension (68 °C for 60 s). At the end of 40 cycles a polishing step (72 °C for 10 min) is included to ensure that all products are full length. Run 16 reactions at one time and pool at the end of the PCR. Run the pooled reactions on a 1 % TBE (Tris-borate-EDTA)-agarose gel and excise the 1,277 bp PCR product from the gel and extract using the QIAquick gel extraction columns. Determine the concentration of PCR product and use 2 μg for restriction digests with Not I and Hind III. In parallel, digest the pcDNA-βgal plasmid vector with Not I and Hind III. Perform the restriction digestions for 2 h at 37 °C and run on a 1 % TBE-agarose gel. Excise the digested products from the gel and extract the fragment with the QIAquick gel extraction columns. Run a portion (1/5th) of the eluted products on a diagnostic 1 %TBE-agarose gel and determine the size and concentration by comparing to molecular weight ladder (*See* **Note 2**). Mix the digested plasmid vector and D1 3′UTR PCR product in a 1:5 ratio with T4 DNA ligase and ligate at 16 °C for 15 h. Use a portion (1/6th) of the ligation reaction to transform the competent *E. coli* cells. Plate the transformed competent cells on Luria broth (LB) plates with 100 μg/mL ampicillin. Next day pick individual colonies, culture in liquid LB media with 50 μg/mL ampicillin, and isolate the plasmid DNA in individual clones using the QIAprep Spin Miniprep Kit. Sequence plasmid DNA isolated from several individual bacterial colonies at commercial sequencing labs using the forward and reverse primers that were used in the PCR amplification step.

Generate the D1R 3′UTR constructs with mutations in the microRNA binding sites using a mutagenic primer with a KpnI restriction site replacing the microRNA seed recognition sequence. To mutate the miR142-3p site in D1 receptor 3′UTR, set up a PCR as described above using the D1 3′UTR as a template and the forward Not I and mutagenic reverse SspI/Kpn I primers (Fig. 4b). The PCR will generate the 5′ end of the D1 3′UTR from the Not I site to the native SspI site. To obtain the 3′end of the D1 3′UTR from the SspI to Hind III site, cut the parent plasmid with the 1,277 bp D1 3′UTR with SspI and Hind III. Ligate the 5′- and 3′- ends of the D1 3′UTR to the parent plasmid cut with Not I and Hind III to generate the D1 3′UTR with the mutated miR142-3p binding site. The ligation, transformation, and screening of plasmid from bacterial clones are performed as above. Sequence all recombinant plasmids and compare to the wild-type D1R 3′UTR sequence, confirming that the sequence matches the sequence in the NCBI database. Isolate all plasmids using the alkaline-lysis plasmid DNA isolation method and purify two sequential CsCl density gradients prior to sequencing and transfection.

3.3 Transfection of CAD Cells

Plate CAD cells and culture for 24 h or more in serum-containing media to about 60 % confluence before transfection. Perform transfections on cells plated in either 6- or 12-well tissue culture plates. For transfection of 6-well plates, for each well, dilute 6 μL of Lipofectamine 2000™ transfection reagent in 250 μL OPT-MEM and mix with 2.0 μg test plasmid, 0.4 μg of BAP-Flag™ transfection control plasmid, and 2.4 μg of pUC19 carrier plasmid in 250 μL OPTI-MEM media and incubate the combined mixture at 25 °C for 30 min. For transfection of 12-well plates, for each well, dilute 2 μL of Lipofectamine 2000™ transfection reagent in 100 μL of OPT-MEM and mix with 1.2 μg of test plasmid, 0.2 μg of BAP-Flag™ transfection control plasmid, and 0.2 μg of pUC19 carrier plasmid in 100 μL of OPTI-MEM media and incubate the combined mixture at 25 °C for 30 min. After a 30-min incubation, overlay the Lipofectamine, DNA, and OPTI-MEM mixture on non-differentiated CAD cells in antibiotic-free serum containing CAD cell culture media for 6 h. After 6 h, replace the media with fresh serum-containing or serum-free media and harvest the cells 48 h later (Fig. 5).

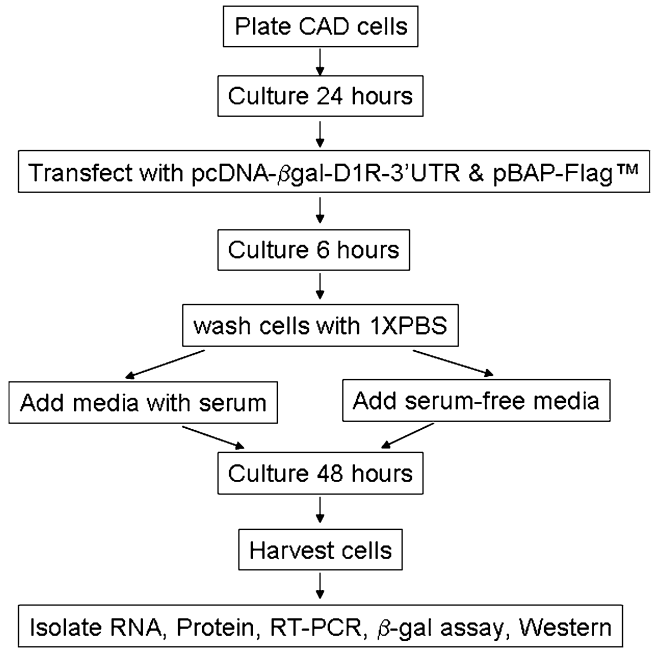

Fig. 5 The experimental design used for transfecting the β-galactosidase reporter plasmid with the wild-type and mutated D1 receptor 3′UTR into non-differentiated and differentiated CAD cells. Non-differentiated and differentiated CAD cells are harvested ~54 h after transfection and the levels of β-gal mRNA, β-gal activity, and BAP-Flag™ transfection control mRNA and protein measured

3.4 Isolation of RNA and RT-PCR

Isolate RNA using the RNeasy® Mini Kit (Qiagen) according to the manufacturers' instructions. Use TURBO DNA-free™ Kit (Invitrogen) to remove DNA contamination from the total RNA sample. Mix 10 μg total RNA, 1× TURBO DNase buffer, and 1 μL TURBO DNase and incubate at 37 °C for 45 min. Inactivate the DNase using the inactivation reagent supplied with the kit (*See* **Note 3**). Confirm the quality and integrity of the DNase-treated RNA by running 2 μg of total RNA on a 1.2 % TBE agarose gel. To set up the reverse transcriptase reaction, use 2 μg of DNase-treated RNA and 300 ng random primers. Incubate this mix at 65 °C for 5 min followed by quick chilling on ice. After the addition of 1× SuperScript III RT buffer (Invitrogen), 0.5 mM deoxynucleotide triphosphates, 10 mM dithiothreitol, 1 U RNaseOut (Ambion), and 200 U SuperScript III RT, incubate the entire mix at 25 °C for 10 min followed by 45 °C for 2 h. Heat-inactivate the reaction at 70 °C for 15 min and then immediately chill on ice (*See* **Note 4**). Perform the real-time PCR using the Roche Light Cycler (Indianapolis, IN, USA) with gene-specific TaqMan® gene expression assays (Applied Biosystems). To measure D1 dopamine receptor cDNA levels use the TaqMan® gene expression assay, Mm0135211. To detect the internal control GAPDH cDNA, use the mM99999915 TaqMan® gene expression assay. For the Light Cycler, use the following amplification parameters: Initial denaturation 95 °C for 5 min, 20 °C/s transition rate, followed by 40 cycles with a denaturation step of 95 °C for 5 s, 2 °C/s transition rate, and a combined annealing and extension step of 60 °C for 60 s, 2 °C/s transition rate with a fluorescence acquisition at the end of the 60 s extension step (*See* **Note 4**). Incorporate two negative and one positive control in each RT-PCR run. Negative control includes a PCR reaction in which water is substituted for the template. For a second negative control, PCR is run with products from a RT reaction in which the SuperScript III RT enzyme is omitted. Use mouse brain cDNA as a positive control.

3.5 Isolation of Protein and Western Blotting

Harvest cells and lyse using the CelLytic™ M reagent supplemented with 1 mM PMSF and 1 % protease inhibitor cocktail (*See* **Note 5** for protein lysate that is to be used for the β-gal assay.). Determine protein amounts in the lysates using the BCA assay. Based on the concentration, mix 20 μg to 50 μg of total cell proteins with sample loading buffer, heat at 37 °C for 10 min, load on to 10 % gels, and separate using SDS-PAGE using the Mini-PROTEAN® Tetra Cell system (*See* **Note 6**). Transfer the proteins on the gel onto nitrocellulose membranes with a Mini-PROTEAN® tank transfer system. Block the nitrocellulose membranes for 2 h at 25 °C in blocking solution (10 % nonfat milk in Tris-buffered saline (pH 7.4) with 0.1 % Tween®-20 (TBS-T)). Wash the

membranes three times, 1 × 10 min and 2 × 5 min, with TBS-T between blocking and antibody incubations. First detect the D1 receptor using a rat monoclonal anti-D1R antibody (1:2,000 dilution in blocking solution in blocking solution) and goat anti-rat secondary antibody conjugated to horseradish peroxidase (1:20,000 dilution). Following the detection of D1R protein, strip the membrane with Western ReProbe™ for 30 min at 25 °C, wash three times (5 min each) with 0.1 % TBS-T, and block with 5 % nonfat milk in 0.05 % TBS-T for 1 h at 25 °C. After washing the blots with 0.05 % TBS-T, use a rabbit monoclonal antibody (1:5,000 dilution in 5 % blocking solution with 0.05 % TBS-T) to detect GAPDH. Incubate the membranes with GAPDH antibody for 1 h at 25 °C and wash three times (5 min each) with 0.05 % TBS-T. To detect the GAPDH antibody, incubate the membranes for 1 h at 25 °C with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:20,000 dilution in 5 % blocking solution with 0.05 % TBS-T). To detect the Flag™-tagged bacterial alkaline phosphatase protein, which is used as a transfection control, the conditions are identical to those used for the GAPDH antibody except use the M2 anti-Flag™ mouse monoclonal antibody (1:2,000 dilution) and the sheep anti-mouse HRP conjugated secondary antibody (1:20,000 dilution). Detect the horseradish peroxidase conjugated secondary antibodies with the SuperSignal® West Dura extended duration substrate chemiluminescence detection kit using an imaging system or autoradiography film.

3.6 Measurement of β-Galactosidase Reporter Activity

Following transfection of CAD cells (Fig. 5), for the β-galactosidase assay, lyse the CAD cell pellet for 10 min on ice in the β-gal lysis buffer by gently pipetting the mixture five times through a 200 μL micropipette tip. Spin the lysate at 14,000 × *g* for 10 min and determine the total protein concentration in the supernatant using the Bradford protein assay per instructions provided with the kit. Detect the β-galactosidase reporter enzyme activity in the lysate using CPRG as a substrate (*See* **Note 7**). For the β-gal assay, mix 5–10 μg of total protein with 20 μL of CPRG (4 mg/mL) and bring the volume of the assay reaction to 200 μL with the β-gal lysis buffer. Incubate the mixture at 37 °C till there is a visible difference between the blank sample and the test samples. Transfer 100 μL of each sample into a 96 well plate and read the absorbance using a spectrophotometer. The β-galactosidase enzyme cleaves the CPRG substrate to yield a colored product which has maximal absorbance at 575 nm which is detected using a visible wavelength spectrophotometer. Normalize the β-galactosidase reporter enzyme activity to the total protein amount present in the lysate and further normalize for transfection efficiency.

4 Notes

1. *CAD cell culture*: Media is prepared by adding DMEM/F12 with and without fetal calf serum and penicillin/streptomycin and filtering into a sterile glass bottle via 0.22 μm bottle top filter. The prepared media is stored in the dark at 4 °C. Be extremely careful when handling sodium selenite as it is highly toxic. It is also light-sensitive. Dissolve in sterile distilled/deionized water and aliquot 1 mL of the solution (10 μg/mL) into 1.5 mL microcentrifuge tubes and store at −20 °C. Transferrin is dissolved in sterile distilled/deionized water and 1 mL aliquots of the solution (4 mg/mL) are stored at −80 °C. Actinomycin D is dissolved in dimethyl sulfoxide (DMSO) at 1 mg/mL concentration and stored at −20 °C in the dark.

   CAD cells do not adhere to the substrate very well; therefore, to prevent dislodging cells, add solutions to the walls of the culture vessels rather than directly on the cells. Non-differentiated CAD cells divide rapidly; therefore, it is necessary to sub-culture the cells every 2–3 days. Differentiated CAD cells do not divide; therefore, they can be cultured for weeks provided half the media is replaced with fresh serum-free media every 3–4 days.

2. *Cloning*: To detect the nucleic acid bands on the TBE-agarose gels, we use ethidium bromide (10 mg/mL). We typically add 2 μL ethidium bromide to 60 mL of melted gel solution for both RNA and DNA gels. In addition, for DNA gels we add 6 μL ethidium bromide to 200 mL 1× TBE running buffer. Ethidium bromide is a suspected carcinogen and should be handled with care. Used gels and buffers containing ethidium bromide should be disposed by following local regulations. Alternate nontoxic dyes can also be substituted for ethidium where available.

3. *RNA isolation*: Use RNase- and DNase-free water, tubes, and micropipette tips with filters to minimize degradation and contamination. Use RNase-Zap to decontaminate micropipettes and centrifuge rotors and chamber. Always wear clean gloves and change them frequently. RNA pellets are difficult to dissolve when they are overdried. To dissolve RNA pellets, add RNase-free water and heat at 60 °C with intermittent vortexing. Do not pipette up and down to dissolve the pellet as the sticky pellet might get lost in the pipette tip. Store RNA at −80 °C.

4. *RT-PCR*: The cDNA generated in the RT reaction can be stored at −20 °C. We perform the PCR using the Roche Light Cycler and glass capillaries; given the fast transition times with this PCR machine, it will be necessary to alter the amplification parameters for other PCR machines, in particular, the Peltier-based PCR machines. Fortunately, the TaqMan® gene expression assay

probes are optimized for Peltier-based machines and the optimal amplification parameters can be obtained from Invitrogen.

5. *Protein isolation*: Keep all tubes on ice and ensure that they are prechilled. All centrifugation should be at 4 °C. For D1 receptor, BAP-Flag™, and GAPDH the protein isolation can be carried out using the Celytic M™ reagent; however, for the βgal assay it is important to note that the cell lysis has to be performed in the βgal lysis buffer. The cell pellet is resuspended in the lysis buffer and processed as in Sect. 3.6.
6. *Protein sample preparation for WB*: To detect membrane proteins such as the D1 dopamine receptor, the protein lysate should NOT be boiled before loading on the gel as this will aggregate glycosylated membrane proteins. We recommend adding TCEP bond breaker to the sample buffer and protein lysate and heating at 37 °C for 10 min before loading the gel. If necessary, samples can be heated to 50 °C for 5 min.
7. *β-galactosidase assay*: CPRG is dissolved in water and stored as aliquots at −20 °C in the dark. When setting up the assay, add the CPRG substrate at the very end to the reaction tube that already contains the protein lysate and β-gal lysis buffer. The reaction that leads to formation of the colored substrate cannot be terminated; therefore it is important to monitor the reaction and take the absorbance readings as soon as the test sample is different from the blank sample. If the color changes very rapidly, reduce the protein amount added to the lysate and/or perform the incubation at 25 °C rather than at 37 °C.

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