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Regulation of dense core vesicle release from PC12 cells by interaction between the D2 dopamine receptor and calcium-dependent activator protein for secretion (CAPS)

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Abstract

We identified CAPS1 (calcium-dependent activator protein for secretion) as a D2 dopamine receptor interacting protein (DRIP) in a yeast two-hybrid screen of a human brain library using the second intracellular domain of the human D2 receptor (D2IC2). CAPS1 is an evolutionarily conserved calcium binding protein essential for late-stage exocytosis of neurotransmitters from synaptic terminals. CAPS1 interaction was confirmed for both the long and short isoforms of the D2 receptor, but not with any other dopamine receptor subtype. Interaction between CAPS1 and the D2 receptor was validated using both pulldown and coimmunoprecipitation assays. Deletion mapping localized the D2 receptor binding site to a segment located within the C-terminal region of CAPS1 as well CAPS2. In PC12 cells, CAPS1 and D2 receptors were found to colocalize within both cytosolic and plasma membrane compartments. Overexpression of a truncated D2 receptor fragment caused a significant decrease in K^+-evoked dopamine release from PC12 cells, whereas no effect on norepinephrine or BDNF release was observed. These results suggest that D2 dopamine receptors may modulate vesicle release from neuroendocrine cells via direct interaction with components of the exocytotic machinery

Keywords: CAPS; Dopamine; Dopamine receptor; Exocytosis; G-protein coupled receptor; Neurotransmission

1. Introduction

The mechanisms that modulate dopamine neurotransmission in the human brain play an important role in regulating cognitive and behavioral functions and also contribute to various neurologic diseases and drug addiction [1,2]. The effects of dopamine are mediated through two classes of dopamine receptors, the D1-like (D1 and D5) and D2-like (D2–D4) receptors. All five dopamine receptor subtypes localize to postsynaptic sites and regulate important aspects of synaptic transmission. In addition to their postsynaptic localization, D2 and D3 dopamine receptors are also localized to presynaptic nerve terminals where they function as autoreceptors to negatively regulate dopamine release during synaptic transmission [3,4].

Neurotransmitters are packaged into two primary classes of secretory vesicles, small clear synaptic vesicles and large dense core vesicles (LDCVs) that contain either the fast-acting neurotransmitters (glutamate) or the slower acting peptides and biogenic amines (norepinephrine and dopamine), respectively [5]. Regardless of vesicle type, the mechanism of neurotransmitter release essentially involves the filling, docking, priming, and fusion of vesicles with the plasma membrane. These processes are tightly regulated and involve many evolutionarily conserved vesicle- and membrane-associated proteins [6]. In addition, neurotransmitter release is highly dependent on intracellular calcium levels, specifically during the late stages of

Abbreviations: BDNF, brain-derived neurotrophic factor; CAPS, calcium-dependent activator protein for secretion; D2L, dopamine receptor D2 long isoform; D2S, dopamine receptor D2 short isoform; LDCV, large dense core vesicle; DRIP, dopamine receptor interacting protein; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; GFP, green fluorescent protein; GST, glutathione-S-transferase; HEK, human embryonic kidney; IC2, second intracellular domain; KCl, potassium chloride; MHD, munc homology domain; NE, norepinephrine; PKA, protein kinase A; PKC, protein kinase C; RT-PCR, reverse transcriptase-polymerase chain reaction

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vesicular fusion and release [7]. Several proteins have been identified as essential for calcium-triggered exocytosis including the SNAREs (soluble *N*-ethylmaleimide-sensitive factor attached protein (SNAP) receptors), SNAP-25, vesicle-associated membrane protein (VAMP)/synaptobrevin, syntaxin, and the syntaxin binding protein, munc18 [6].

Rat pheochromocytoma PC12 cells are a useful model for studying the mechanisms underlying exocytosis in both neuronal and endocrine cell types. PC12 cells express a large number of neurosecretory markers present in neurons, and they synthesize a number of growth factors and neurotransmitters including dopamine and norepinephrine (NE) that are packaged in LDCVs [8,9]. Several studies demonstrate that dopamine is released from PC12 cells via Ca^{2+}-dependent exocytosis in a mechanism similar to neurons and this process is regulated via the activity of endogenous D2-like dopamine receptors [8].

We have been studying the regulation of dopaminergic neurotransmission by identifying and characterizing a group of proteins called dopamine receptor interacting proteins (DRIPs) that mediate the signaling properties of individual dopamine receptor subtypes. Functional studies indicate that DRIPs regulate most aspects of receptor activity from biosynthesis to desensitization [10]. For example, γ-COP (gamma coat protein) and DRiP78 (dopamine receptor interacting protein 78) are DRIPs that regulate post-translational processing and export of D1 receptors from the endoplasmic reticulum [11]. DRIPs linked to the cytoskeleton, including neurofilament M [12], filamin A [13] and protein 4.1N [14], regulate D1 and D2 receptor cell surface expression. Association of D2 receptors with G-protein receptor kinase-2 and signaling proteins such as neuronal calcium sensor-1 play an important role in regulating receptor desensitization and internalization [15]. The identification of DRIPs and additional components of dopamine signaling complexes now provides a molecular framework for elucidating the diverse signaling mechanisms that contribute to dopamine-mediated neurotransmission.

Many of the known DRIPs have been identified using the yeast two-hybrid system in which various intracellular segments of a dopamine receptor are used as bait to screen for interacting proteins in a brain cDNA library. In the present study, we have conducted a yeast two-hybrid screen using the second intracellular domain of the D2 dopamine receptor (D2IC2) as bait to identify novel DRIPs. From this screen we identified calcium-dependent activator protein for secretion (CAPS1, CAPS, CADPS) as a D2 receptor-specific DRIP. CAPS1 is an evolutionarily conserved, presynaptic calcium binding protein that functions in late-stage exocytosis [16]. In permeabilized PC12 cells, CAPS1 has been shown to function at the Ca^{2+}-dependent triggering step of LDCV exocytosis [17]. Current evidence indicates that CAPS1 functions selectively in LDCV exocytosis by interacting with LDCVs, the plasma membrane, and protein components of the fusion machinery [18]. Our studies indicate that CAPS1 interacts with the D2 dopamine receptor in mammalian cells. We show that disruption of this interaction causes a selective decrease in dopamine release from PC12 cells. These results suggest that CAPS/D2 interaction may serve to modulate the release of catecholamine-containing LDCVs from neurons and provide evidence that dopamine receptors may also interact with components of the exocytotic machinery.

2. Materials and methods

2.1. DNA constructs and protein interaction assays

All constructs were generated by subcloning PCR amplification or restriction enzyme fragments into appropriate expression vectors. Each construct was verified by DNA sequence analysis. For the yeast two-hybrid screen, the second intracellular loop of the human D2 receptor (D2IC2; residues 131–151) was subcloned in the yeast GAL4 DNA-binding domain vector pAS2-1 (BD Biosciences Clontech) and used as bait to screen a human fetal brain cDNA library expressed in the yeast GAL4-activation domain vector pACT2 (BD Biosciences Clontech). Bait and prey constructs were simultaneously cotransformed into yeast strain MaV103, and a total of 1×10^6 independent clones was screened by selective growth on $Leu^-/Trp^-/His^-/Ade^-$ plates as previously described [19].

Sites within the human dopamine receptors and human CAPS that contribute to CAPS/dopamine receptor interaction were mapped using a directed two-hybrid approach. CAPS1 (residues 617–959, 791–1129, 960–1301) and CAPS2 (residues 755–1029) truncation fragments were inserted into pACT2. Human CAPS2 cDNA was generously provided by Dr. Peter Ray (The Hospital for Sick Children, Toronto, Canada). IC2 domains of D1 (residues 120–138), D2 (residues 131–151), D3 (residues 127–149), D4 (residues 132–151) and D5 (residues 137–158) dopamine receptors were subcloned in pAS2-1. Bait and prey constructs were cotransformed into MaV103, and positive clones were identified by growth on Leu^-/Trp^- selection plates followed by expression of β-galactosidase activity [19]. For each protein–protein interaction screen, β-galactosidase assays were repeated five times. For all β-galactosidase assays performed in this study, we detected either a robust signal indicating positive interactions or an absence of signal indicating no interaction; therefore, results are reported as "+" or "−" to designate the presence or absence of β-galactosidase activity, respectively.

2.2. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). PC12

cells were plated in tissue culture dishes precoated with 10 μg/cm^2 Collagen IV (Sigma). Cells were maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% horse serum, 5% FBS. PC12 cells were transfected with the LipofectAMINE 2000 transfection reagent (Invitrogen Life Technologies), whereas HEK 293 cells were transfected using Effectene (Qiagen) under conditions recommended by the manufacturers.

2.3. Pulldowns and coimmunoprecipitation

Glutathione-S-transferase (GST)–D2L dopamine receptor fusion protein (GST–D2L) and GST–D2IC2 fusion protein were constructed in the bacterial expression vector pGEX-4T-1 (Amersham Pharmacia). GST–D2L and GST–D2IC2 fusions were induced in *E. coli* strain BL21, then purified by adsorption to glutathione-sepharose beads (Amersham Pharmacia) according to the manufacturer's instructions. Full-length CAPS1 containing an N-terminal myc tag (CAPS1-myc; generous gift from Dr. Thomas Martin, University of Wisconsin, Madison, WI) was constructed in pcDNA3.1, transiently expressed in HEK 293 cells, and total cell lysates prepared 24 h after transfection. GST pulldown assays were performed as described previously [13]. Proteins were eluted from the glutathione-sepharose beads, separated by SDS-PAGE, transferred to nitrocellulose, and probed with a rabbit polyclonal anti-myc antibody (1:250 dilution; Santa Cruz Biotechnology). Proteins were visualized with a horseradish peroxidase-conjugated goat anti-rabbit (1:2000 dilution) secondary antibody (Jackson ImmunoResearch). Immunoreactivity was detected by enhanced chemiluminescence with an ECL Plus kit (Amersham Pharmacia).

For coimmunoprecipitation, HEK 293 cells were cotransfected with cDNAs encoding CAPS1-myc and a FLAG-tagged dopamine receptor (either D1, D2S, D2L, D3, D4, or D5, constructed in the mammalian expression vector pCB6). Immunoprecipitations were performed from crude membranes as previously described [20]. FLAG-tagged dopamine receptors were immunoprecipitated using the anti-FLAG monoclonal antibody M2 (Sigma). Immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose, and probed using a rabbit polyclonal anti-myc antibody (1:250 dilution, Santa Cruz Biotechnology). Proteins were visualized using a peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000 dilution, Jackson ImmunoResearch). Immunoreactivity was detected by enhanced chemiluminescence.

2.4. Immunocytochemistry

PC12 cells were plated on poly-lysine and laminin coated coverslips and transiently cotransfected with GFP-tagged CAPS1 (CAPS1–GFP) and D2–FLAG cDNAs. Cells were grown on coverslips for 24 h, then fixed in acetone:methanol (1:1 v/v) and permeabilized with 0.03% Triton-X-100. To detect D2–FLAG, cells were stained with an anti-FLAG M5 monoclonal antibody (1:1000 dilution, Sigma), and staining was visualized with a rhodamine red-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch). GFP epifluorescence was visualized directly. Confocal imaging was performed using a DMIRE2 confocal microscope powered by Leica Confocal Software (Leica).

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was prepared from PC12 cells using Trizol reagent (Invitrogen Life Technologies) according to the manufacturer's specifications. cDNA was prepared using the SuperScript First-strand Synthesis System (Invitrogen Life Technologies), and PCR was performed following the suggested protocol for REDTaq DNA polymerase (Sigma) at an annealing temperature of 55 °C. All primers were designed to encompass regions that include introns, thereby providing a means to distinguish between genomic DNA amplification and RT-PCR products. The expected size for the CAPS1 RT-PCR product is 613 nucleotides (nts), while genomic DNA is predicted to yield fragments >65,000 nts. The predicted CAPS2 RT-PCR product is 849 nts compared to 63,000 nts from CAPS2 genomic DNA. For D2 (D2S and D2L) amplification, primers were designed to span the third intracellular domain. The expected size of the D2S product is 609 nts, whereas the expected size of the D2L product is 696 nts. In contrast, amplification of D2 receptor genomic DNA sequences would produce fragments >4807 nts in size. For each primer pair, plasmid cDNA was used as a positive control, and negative controls included a "no RT" reaction. In addition, the CAPS1 primers were tested using the CAPS2 plasmid, and vice versa, to ensure template specificity.

2.6. Catecholamine and BDNF release assays

Catecholamine release assays were performed essentially as described [21]. PC12 cells were transfected with EGFP-tagged D2IC2 (D2IC2–EGFP) or with an empty pEGFP-C2 (EGFP) vector and cultured for 24 h. On average, transfection efficiency was approximately 60% as determined by EGFP epifluorescence. Transfected cells were plated at a density of 5×10^5 cells/well in 24-well tissue culture dishes coated with collagen IV. Cells were grown for an additional 24 h, washed in PBS, then incubated in HEPES-buffered saline containing 0.5 μCi/mmol [^{3}H]-dopamine (59.7 Ci/mmol, Perkin Elmer Life Sciences) or 0.5 μCi/mmol [^{3}H]-NE (37.0 Ci/mmol, Amersham Biosciences) for 1 h at 37 °C. Cells were washed twice, then incubated in HEPES-buffered saline for 1 h at 37 °C. Cells were transferred into growth medium (RPMI 1640 supplemented with 10% horse serum, 5% FBS), and after a 5 min

incubation period, stimulated by the addition of 56 mM KCl. To measure basal catecholamine release, cells were incubated in growth medium lacking KCl. After 10 min, media was collected and cells were washed once with PBS, lysed by the addition of 2% Triton-X-100, and the levels of [^{3}H]-catecholamine in media and cell lysates measured by scintillation counting. Each release assay was performed three times, in triplicate, and the data expressed as the mean ± standard error of the mean (S.E.M.). The levels of [^{3}H]-catecholamine released were normalized to total levels of [^{3}H] in each well. For each experiment, the level of [^{3}H]-catecholamine released following KCl stimulation is expressed as the percent above basal release. Data were analyzed using a one-way ANOVA, followed by a Tukey's HSD multiple comparison analysis. Dopamine release from mock and EGFP transfected cells was not statistically different. Dopamine release from D2IC2–EGFP cells differed significantly from both mock ($p < 0.001$) and EGFP ($p = 0.002$) transfected cells. For NE release assays, no statistical differences were detected between mock, EGFP and D2IC2–EGFP transfected cells.

BDNF release assays were performed essentially as described [22]. Briefly, PC12 cells were cotransfected with BDNF/pCMVsport6 (American Type Culture Collection) and D2IC2–EGFP. Cells were grown for 24 h, then plated into 6-well dishes coated with collagen IV and grown for an additional 24 h. Cells were transferred into fresh media (RPMI 1640, 10% FBS and 0.2% BSA), cultured for 30 min at 37 °C, transferred into fresh media and stimulated by exposure to 56 mM KCl for 5 min. To measure basal release, cells were incubated in fresh media for 5 min in the absence of KCl. Media was then collected and levels of BDNF were determined using the BDNF Emax ELISA kit (Promega) according to the manufacturer's recommendations. Assays were performed in triplicate and data is expressed as the mean ± standard error of the mean (S.E.M.). For each experiment, the level of BDNF released following KCl stimulation is expressed as the percent above basal. Data was analyzed using a one-way ANOVA, followed by a Tukey HSD multiple comparison analysis. No significant differences in BDNF levels were found for mock, EGFP and D2IC2–EGFP transfected cells.

3. Results

3.1. Interaction of CAPS1 with D2 dopamine receptors

We have undertaken a systematic approach using the yeast two-hybrid system to identify novel DRIPs. The presence of seven membrane-spanning regions in all dopamine receptor subtypes requires truncation of the receptors for successful two-hybrid screening [19]. We have previously identified DRIPs using the third intracellular and C-terminal domains of the D2 dopamine receptor as bait [13–15]. In the present study, we conducted a yeast two-hybrid screen using the second intracellular domain of the D2 receptor (D2IC2) as bait to screen a human brain cDNA library. The 22 amino acid D2IC2 domain is of particular interest due to its high degree of amino acid similarity not only to other dopamine receptor subtypes but also to the majority of GPCRs [23]. In addition, mutagenesis studies of various IC2 domains suggest an involvement of the IC2 domain in both adenylate cyclase activity and G-protein coupling [23,24].

In control experiments, neither the D2IC2 construct nor any other bait was found to autoactivate β-galactosidase expression. Using the D2IC2 construct as bait, one clone was identified that contained an ~2.1 kb cDNA insert. Analysis of the predicted amino acid sequence revealed that the cDNA encoded a 684 amino acid-long fragment corresponding to the C-terminal-half (residues 617–1301) of CAPS1. CAPS1 is a cytosolic calcium binding protein essential for the final stages of dense core vesicle exocytosis and the regulated secretion of the biogenic amine class of neurotransmitters [16].

We used the yeast two-hybrid system to examine the specificity of the CAPS1/D2 interaction by testing for CAPS1 interaction with additional dopamine receptor family members. To this end, bait constructs encoding the IC2 domains of the D1–D5 dopamine receptors were tested for interaction with CAPS1 cDNA in a directed two-hybrid screen. CAPS1 was found to interact specifically with the D2 receptor, but not with any of the other dopamine receptor subtypes (Fig. 1A). An alignment of the five dopamine receptor IC2 domains is depicted in Fig. 1C. All five dopamine receptor subtypes contain a conserved nine amino acid-long segment located within the N-terminal portion of the IC2 domain. The D2 and D3 receptors show identity at eight of nine positions within this segment, whereas the D2 and D4 receptors show identity at six of the nine positions. The D1 and D5 receptors differ at four of the nine positions compared to the D2 receptor. The fact that CAPS1/D2 interaction is limited to the D2 receptor subtype strongly suggests that the region of interaction most likely resides within the C-terminal non-conserved segment (residues 141–151) of the D2IC2 domain. We also did not detect interaction of CAPS1 with other intracellular regions of the D2 receptor including the first and third intracellular domains and the C-terminal tail (data not shown).

Sequence analysis of the CAPS1 binding site on the D2 receptor revealed that Ser147 represents a putative phosphorylation site. To determine if this residue contributes to the CAPS1/D2 interaction, Ser147 was converted to alanine (S147A) and the mutated D2IC2 cDNA tested for interaction with CAPS1 in a directed two-hybrid screen. Cotransformation of CAPS1 and the S147A mutant D2IC2 construct failed to activate β-galactosidase expression (Fig. 1B), suggesting that Ser147 is likely to be one of the amino acids that contributes to the CAPS1/D2 interaction. To determine if phosphorylation of Ser147 contributes to the CAPS1/D2

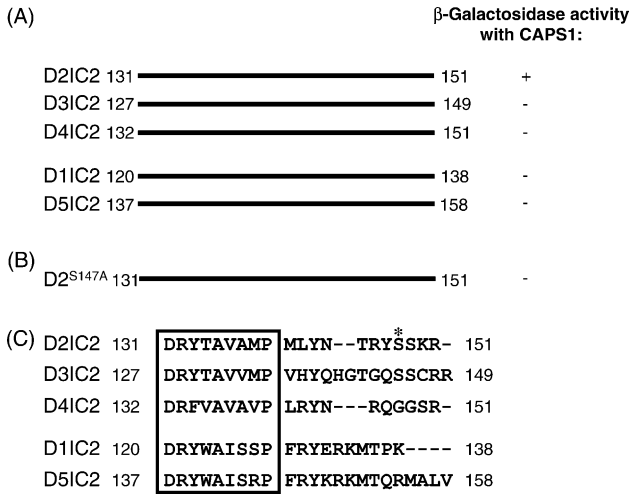

Fig. 1. CAPS1 interacts with the IC2 domain of the D2 receptor. (A) Schematic representation of the IC2 domains of the five dopamine receptor subtypes. Each dopamine receptor IC2 construct was separately cotransformed into yeast with CAPS1 (amino acids 619–1301) and interaction indicated by the presence (+) or absence (−) of β-galactosidase activity. (B) Conversion of Ser147 to alanine (S147A) in D2IC2 prevents interaction with CAPS1. (C) Amino acid sequence alignment of the dopamine receptor IC2 domains. The conserved N-terminal region is boxed. Serine147 is designated by the asterisk.

interaction, we performed preliminary coimmunoprecipitation experiments in the presence of the specific PKA inhibitor, H-89, and the specific PKC inhibitor, bisindolylmalemide I. Blocking either PKC- or PKA-mediated phosphorylation did not affect CAPS1/D2 interaction (data not shown), suggesting that phosphorylation is not involved in regulation of the CAPS1/D2 interaction.

The CAPS1/D2 interaction was initially verified using pulldown techniques. A lysate prepared from HEK 293 cells expressing a myc-tagged CAPS1 construct (CAPS1-myc) was tested for the ability to associate with a GST fusion protein containing the D2IC2 domain (D2IC2–GST). As shown in Fig. 2A, a Western blot containing lysate prepared from HEK 293 cells produced immunoreactive bands of ∼145 and 135 kDa when probed with anti-myc antibodies (lane 1). These bands correspond to the expected size of CAPS1 as reported previously [16,25]. The same bands were detected in pulldown assays after the cell lysate was incubated with the D2IC2–GST fusion protein (lane 3), but not when the lysate was adsorbed onto GST alone (lane 2). Similar results were obtained in GST-pulldown assays conducted with a full-length D2L–GST fusion protein (Fig. 2B). Total cell lysate from transfected HEK 293 cells was also tested for the ability to associate with D2L–GST fusion protein in the presence (10 μM $CaCl_2$) or absence (1 mM EGTA) of calcium. No apparent differences were observed in the level of CAPS1-myc associated with D2L–GST when calcium was present or chelated in the pulldown assay (data not shown). These results support the validity of the CAPS1/D2 interaction and suggest that the interaction between these proteins occurs in a calcium-independent fashion.

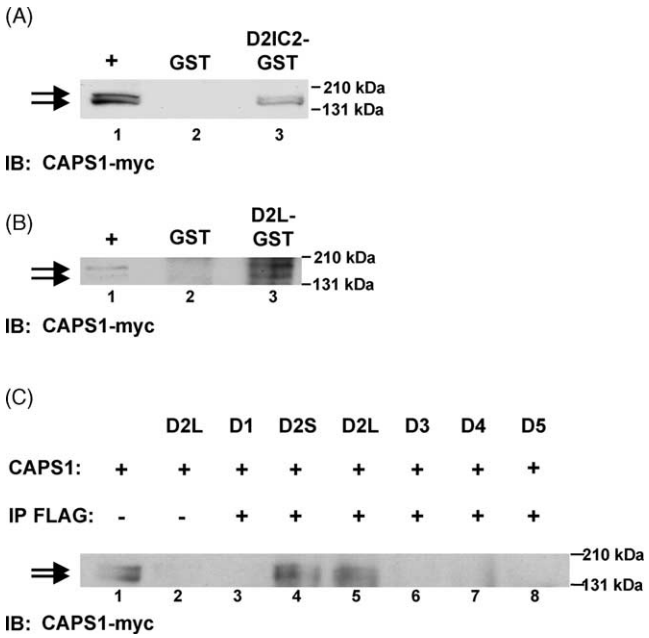

Fig. 2. Pulldown and coimmunoprecipitation of CAPS1/D2 receptor complexes. A D2IC2–GST fusion protein (A) and a D2L–GST fusion (B) were used to pull down myc-tagged CAPS1 (CAPS1-myc) from HEK 293 cell lysates. In lysates prepared from HEK 293 cells transfected with CAPS1-myc, CAPS1-myc is seen as a doublet running at the expected size of 135 and 145 kDa (A and B, lanes 1). In pulldown assays, CAPS1-myc was pulled down in the presence of D2L–GST (A and B, lanes 3) but not with GST alone (A and B, lanes 2). (C) The anti-FLAG M2 monoclonal antibody was used to coimmunoprecipitate FLAG-tagged dopamine receptors and CAPS1-myc from HEK 293 cells. CAPS1-myc was detected in cell lysates prepared from HEK 293 cells transfected with CAPS1-myc (lane1) and immunoprecipitates prepared from cells expressing D2S and D2L (lanes 4 and 5), but not from cells transfected with D1, D3, D4, or D5 receptors (lanes 3, 6–8, respectively), or CAPS1-myc alone (lane 2). Arrows in (A) and (B) indicate positions of the 135 and 145 kDa CAPS1 bands. IP, immunoprecipitation; IB, immunoblot. For all experiments, $n = 4$.

Interaction between the dopamine receptor and CAPS1 was also validated using coimmunoprecipitation methods. To demonstrate interaction, we tested the ability of an anti-FLAG monoclonal antibody to coimmunoprecipitate CAPS1 and dopamine receptors from crude membranes prepared from HEK 293 cells transiently expressing CAPS1-myc and FLAG-tagged dopamine receptor cDNAs. As shown in Fig. 2B, anti-FLAG antibodies were capable of coimmunoprecipitating CAPS1 and D2L or D2S dopamine receptors. We were unable to coimmunoprecipitate CAPS1 from cells coexpressing either FLAG-tagged D1, D3, D4, or D5 receptors, or when the anti-FLAG antibody was omitted from the immunoprecipitation reaction. Taken together, these results strongly suggest a specific interaction between CAPS1 and D2L and D2S dopamine receptor splice variants.

3.2. Colocalization of CAPS1 and D2 receptors in PC12 cells

The intracellular distribution of CAPS1 and D2 was examined in PC12 cells by confocal laser microscopy.

PC12 cells were cotransfected with GFP-tagged CAPS1 (CAPS1–GFP) and D2–FLAG constructs and cultured for 24 h. Cells were stimulated with 56 mM KCl for ten minutes, then fixed and stained with an anti-FLAG antibody. As shown in Fig. 3, D2–FLAG and CAPS-GFP colocalize in perinuclear and plasma membrane compartments. This distribution pattern is similar in both untreated (top panels) and K^+-stimulated (bottom panels) cells. These results support the notion that CAPS1 and D2 colocalize within PC12 cells under basal as well as stimulated neurotransmitter release conditions.

3.3. Mapping the D2 dopamine receptor binding site on CAPS1

Deletion mapping studies were performed to localize sites within CAPS1 that contribute to CAPS1/D2 interaction. Truncated fragments of the C-terminal domain of CAPS1 (amino acids 617–1301) were tested for interaction with the D2IC2 domain in a directed yeast two-hybrid screen. Construct B (residues 791–1129) tested positive in the β-galactosidase assay, whereas constructs A (residues 617–959) and C (residues 960–1301) failed to interact, indicating that the D2 receptor binding site resides within the middle of the CAPS1 C-terminal domain (Fig. 4). We also tested subfragments of construct B, including construct D (residues 903–1016) which spans the munc homology domain (MHD) of CAPS1. This construct failed to interact with D2IC2 in directed yeast two-hybrid assays, suggesting that either the MHD alone is not sufficient for D2 interaction or that the truncation lacks the necessary secondary structure needed for D2 interaction. Overall, these mapping studies indicate that the D2 receptor binding site on CAPS1 is contained within a region spanned by residues 791–1129 that includes the MHD, and that either the overall structure or multiple segments within this region may contribute to the CAPS1/D2 interaction.

3.4. Interaction of D2 dopamine receptors with CAPS2

Recently CAPS2, a CAPS1 paralog, has been cloned and characterized [25,26]. An alignment of CAPS1 and CAPS2 reveals that the two proteins share 80% overall amino acid sequence identity. Within the region encompassing the D2 receptor binding domain (CAPS1 residues 791–1129), the two proteins show 76% identity (Fig. 5). To examine interaction between the CAPS2 isoform and the D2 receptor, we used a directed yeast two-hybrid screen and tested interaction of D2IC2 with a CAPS2 truncation fragment (residues 755–1029) analogous to the D2 receptor binding domain present in CAPS1. As shown in Fig. 5B, the CAPS2 truncation fragment displayed a positive interaction with the D2 receptor IC2 domain and produced similar levels of β-galactosidase activity as the CAPS1/D2 interaction. Taken together, these results provide support for the idea that CAPS1 and CAPS2 are capable of interaction with the D2 dopamine receptor.

3.5. Selective modulation of dopamine release from PC12 cells by CAPS/D2 receptor interaction

To determine the physiological significance of the CAPS/D2 interaction, we overexpressed a D2 dopamine receptor truncation fragment and examined its effect on dopamine release from PC12 cells. The D2 truncation fragment spans the IC2 domain (residues 131–151) of the D2 receptor. When overexpressed, the D2 truncation fragment should act in a dominant-negative fashion to compete with the wild-type D2 receptors endogenously expressed in PC12 cells for binding to CAPS (both CAPS1 and CAPS2). RT-PCR analysis demonstrates that the PC12

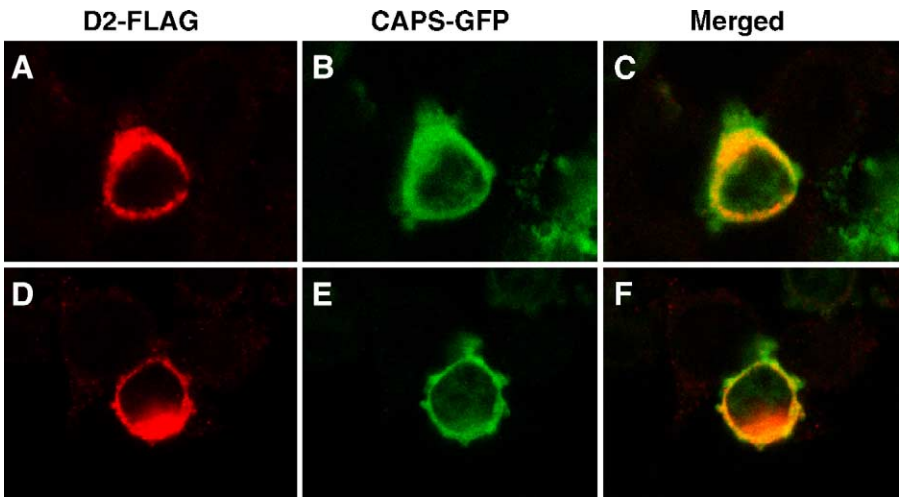

Fig. 3. CAPS1 and D2 receptors colocalize in PC12 cells. PC12 cells were cotransfected with GFP-tagged CAPS1 (CAPS1–GFP) and FLAG-tagged D2S (D2–FLAG) receptors. Cells were either untreated (top panels) or stimulated with 56 mM KCl for 10 min (bottom panels). Localization of D2–FLAG (A and D) and CAPS1–GFP (B and E) was determined by confocal laser microscopy. Merged images show that in unstimulated (C) and K^+-treated (F) cells, D2–FLAG and CAPS1–GFP colocalize in the cytoplasm as well as the plasma membrane.

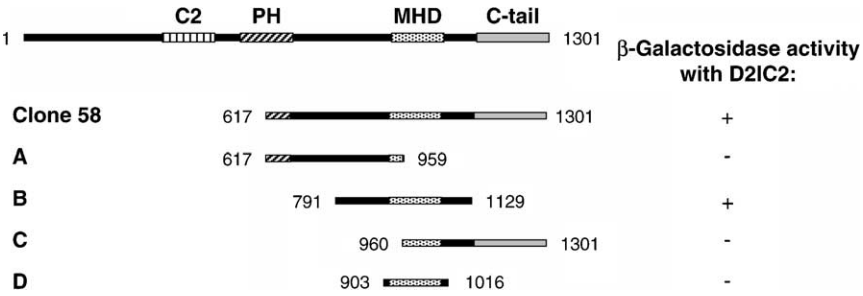

Fig. 4. Localization of the D2 binding site on CAPS1. Schematic representation of constructs encoding truncations of CAPS1. Full-length CAPS1 protein is shown above with shaded boxes depicting the C2, plextrin homology (PH), munc homology (MHD) and C-terminal tail (C-tail) regions. CAPS1 truncation fragments A–D were tested for interaction with D2IC2 (residues 131–151) in the two-hybrid assay. Interaction is indicated by the presence (+) or absence (−) of β-galactosidase activity. Clone 58 was the CAPS1 cDNA identified in the original yeast two-hybrid library screen.

cells used in these studies express D2, CAPS1 and CAPS2 (Fig. 6A). Probing Western blots with available antibodies revealed expression of CAPS1 and D2 receptors in PC12 cell lysates (Fig. 6B). Previous studies have shown that the PC12 cells used in this study also secrete dopamine [27].

In order to monitor expression and transfection efficiency of the D2IC2 truncation fragment, we generated a D2IC2 and EGFP (D2IC2–EGFP) fusion protein. The D2IC2–EGFP construct was cotransfected with CAPS1-myc into cells, and coimmunoprecipitation experiments were performed to verify interaction between D2IC2–GFP and CAPS1. As shown in Fig. 7A, cell lysates cotransfected with CAPS1-myc and either EGFP (lane 2) or D2IC2–EGFP (lane 1) contain a band at ∼30 kDa, the expected size of both EGFP (283 residues) and D2IC2–EGFP (303 residues). An anti-myc antibody was capable of coimmunoprecipitating the ∼30 kDa band that represents the D2IC2–EGFP fusion protein (lane 3), but not EGFP alone (lane 4). These experiments indicate that D2IC2–EGFP binds to CAPS1 in mammalian cells.

For the functional assays, we transfected the D2IC2–EGFP construct into PC12 cells and examined the effect of

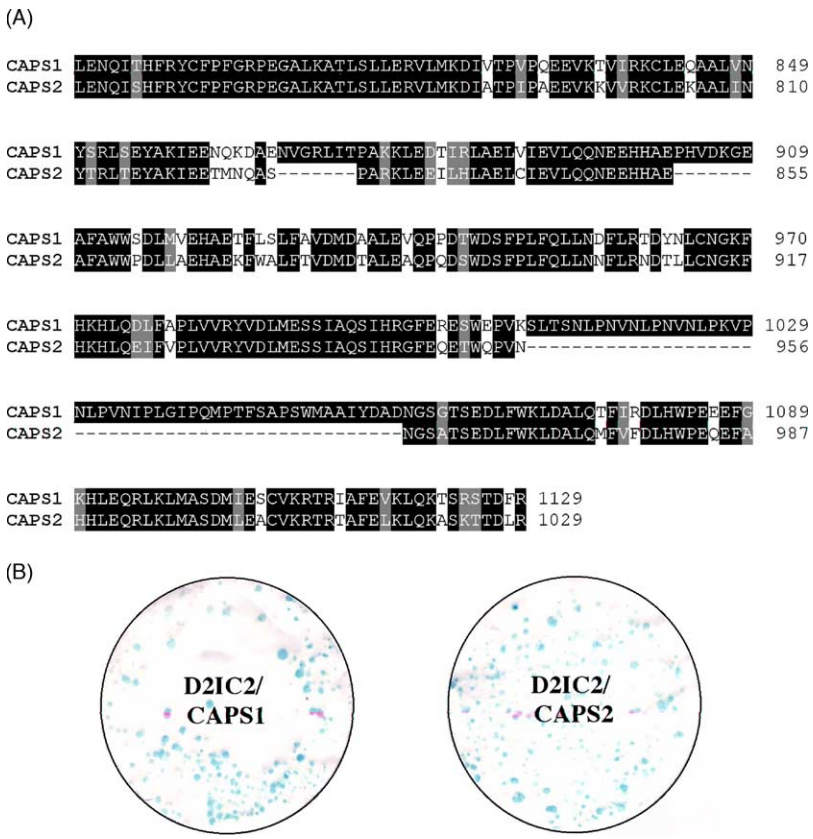

Fig. 5. D2 receptor interacts with CAPS2. (A) Amino acid sequence alignment of CAPS1 (residues 791–1120) and CAPS2 (residues 755–1024) in the region spanning the D2IC2 binding site. Amino acids are numbered to the right of each line. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in gray. (B) Representative β-galactosidase assays comparing the interaction of D2IC2 with CAPS1 and CAPS2. CAPS1 and CAPS2 produced approximately the same levels of β-galactosidase activity.

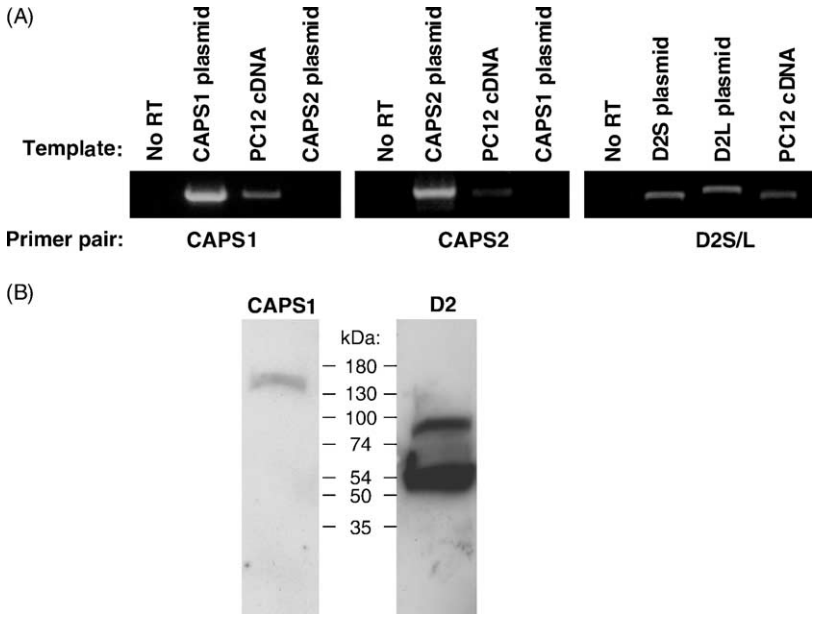

Fig. 6. PC12 cells express CAPS and D2. (A) RT-PCR analysis shows expression of CAPS1, CAPS2 and D2S in PC12 cells. Plasmids containing CAPS1, CAPS2 and D2 were used as positive controls for each primer pair and each primer pair was also tested in a "no RT" negative control reaction. (B) Western blot analysis demonstrates expression of CAPS1 and D2 at the protein level. Whole cell lysates were prepared from PC12 cells and lysates were analyzed using antibodies against CAPS1 (left panel; 1:500 dilution of monoclonal CAPS antibody, BD Pharmingen, San Diego, CA) and D2 (right panel; 1:1000 dilution rabbit polyclonal D2 antibody, Chemicon, Temecula, CA). The two bands detected by the D2 antibody represent the monomeric and dimeric forms of the protein. Shown is a representative blot, $n = 3$.

the truncation peptide on K^+-evoked dopamine release. As shown in Fig. 7B, overexpression of the D2IC2–EGFP fusion peptide in stimulated cells produced only an ~10% (10.91 ± 6.67%) increase in dopamine release compared to PC12 cells transfected with EGFP alone (42.41 ± 2.09%) or in mock transfected cells (49.46 ± 8.36%). We also examined the effect of D2IC2–EGFP on the release of norepinephrine from PC12 cells. As shown in Fig. 7C, expression of the D2IC2 fragment had virtually no effect on NE release (mock, 51.89 ± 1.04%; EGFP, 53.18 ± 2.16%; D2IC2–EGFP, 55.35 ± 7.71%). Disrupting the CAPS/D2 interaction thus appeared to cause a specific reduction in

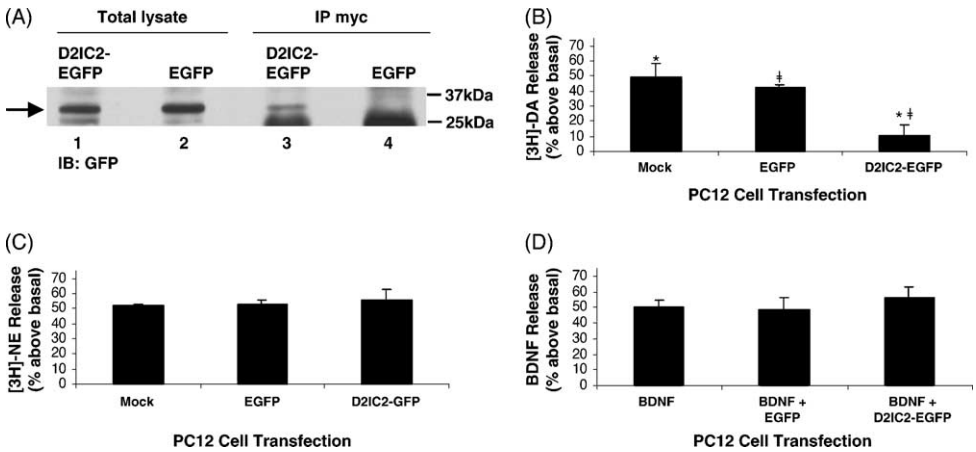

Fig. 7. CAPS/D2 interaction specifically modulates dopamine release from PC12 cells. (A) The D2IC2 truncation fragment interacts with CAPS1. An anti-myc antibody was used to coimmunoprecipitate (IP) CAPS1-myc and D2IC2 from cells transfected with CAPS1-myc and either empty EGFP vector (EGFP) or D2IC2–EGFP. Cell lysates (lanes 1 and 2) or immunocomplexes (lanes 3 and 4) were immunoblotted (IB) with an anti-GFP antibody. An ~30 kDa band (arrow) was detected in cells expressing D2IC2–EGFP but not in the negative (EGFP only) control. The 25 kDa bands in lanes 3 and 4 are the expected size of the IgG light chain. (B) D2IC2 truncation fragment blocks dopamine release. PC12 cells were mock transfected or transfected with either EGFP or D2IC2–EGFP, loaded with [^{3}H]-dopamine, then stimulated with 56 mM KCl. [^{3}H]-dopamine release was measured as described in Section 2. Basal levels were 5.59 ± 0.34% (mock), 6.22 ± 0.13% (EGFP) and 6.32 ± 0.40% (D2IC2–EGFP) of total cellular content. DA, dopamine; $^*p < 0.001$, $p = 0.002$. (C) D2IC2 truncation fragment does not block norepinephrine release. PC12 cells were transfected and treated as described in (B). [^{3}H]-NE release was measured as described in Section 2. Basal levels were 6.06 ± 0.35% (mock), 5.79 ± 0.73% (EGFP) and 5.50 ± 1.19% (D2IC2–EGFP) of total cellular content. (D) CAPS/D2 interaction does not regulate BDNF release. PC12 cells were transfected with BDNF alone or cotransfected with BDNF and either EGFP or D2IC2–EGFP. BDNF release assays were performed as described in Section 2. Basal levels were 9.37 ± 0.56 pg/ml (BDNF), 9.82 ± 0.43 pg/ml (BDNF + EGFP) and 8.63 ± 0.67 pg/ml (BDNF + D2IC2−EGFP).

dopamine release from K^+-stimulated PC12 cells, suggesting that the CAPS/D2 interaction may modulate release of dopamine-containing LDCVs from PC12 cells.

Recent studies have reported that CAPS2 plays an important role in K^+-stimulated neurotrophin release [22]. To determine whether the CAPS/D2 interaction contributes to neurotrophin secretion, we cotransfected the D2IC2–EGFP construct and BDNF into PC12 cells and examined the effect of the truncation peptide on K^+-stimulated BDNF release. The results are shown in Fig. 7D. Expression of the D2IC2–EGFP peptide had no apparent effect on K^+-evoked BDNF release ($56.26 \pm 6.41\%$) compared to cells cotransfected with BDNF and EGFP ($48.55 \pm 8.08\%$) or cells transfected with BDNF alone ($50.12 \pm 4.62\%$). These results suggest that the CAPS/D2 interaction does not significantly contribute to BDNF secretion from PC12 cells.

4. Discussion

Using a yeast two-hybrid based approach, we identified CAPS1 as a DRIP that interacts specifically with the D2 dopamine receptor subtype. The interaction was verified by the ability of a GST–D2L fusion construct to pull down myc-tagged CAPS1 from whole cell lysates. Coimmunoprecipitation studies further verified the CAPS1/D2 interaction in intact cells. The presence of a complex containing D2 receptors and CAPS suggests that CAPS may link D2 dopamine receptors to the cellular secretory machinery. Indeed, disruption of the CAPS/D2 interaction by expression of a D2 truncation fragment was found to inhibit K^+-evoked release of dopamine-containing LDCVs from PC12 cells. Together, these results suggest that the CAPS/D2 interaction may serve to modulate dopamine secretion from neuroendocrine cells.

CAPS1 was first discovered as an essential neuron/endocrine-specific cytosolic protein that functions in Ca^{2+}-triggered neurotransmitter release from PC12 cells. CAPS1 acts at a step following vesicle docking and ATP-dependent vesicle priming [16] but prior to Ca^{2+}-triggered fusion [28]. CAPS1 has been shown to play a selective role in LDCV exocytosis, as neutralizing CAPS1 antibodies disrupt LDCV, but not synaptic vesicle, exocytosis [29]. Recent work has shown that CAPS1 is a PIP_2 (phosphatidylinositol 4,5-bisphosphate) binding protein that facilitates LCDV exocytosis [28]. However, the molecular basis of CAPS1 function remains to be clarified.

Our results indicate that CAPS1 interacts directly with both the D2S and D2L dopamine receptor isoforms. Since CAPS1 is localized in presynaptic nerve terminals, we view this to suggest that CAPS interacts with presynaptic D2 receptors which are predicted to be mainly of the D2S type [30]. Presynaptic dopamine receptors function as inhibitory autoreceptors [3], and are known to regulate neurotransmitter release via the G-protein modulation of synaptic calcium and potassium channels [31,32]. Recent evidence, however, indicates that presynaptic receptors may also regulate neurotransmission by directly interacting with secretory vesicles and/or molecular components of the exocytotic pathway. For example, muscarinic acetylcholine receptors have been shown to directly interact with syntaxin, synaptotagmin and SNAP-25 [33]. The interaction of D2 receptors with CAPS may thus provide a functional mechanism for linking presynaptic dopamine receptors with components of the exocytotic pathway.

The interaction of D2 receptors with CAPS could provide a mechanism for physically linking dopamine receptor function to LDCV release. In this view, the CAPS/D2 interaction would be expected to negatively regulate dopamine secretion, since presynaptic D2 receptors are known to function as inhibitory autoreceptors [30]. However, the dopamine release experiments described here demonstrate that disruption of the CAPS/D2 interaction via expression of a D2 truncation fragment caused specific inhibition of dopamine release from PC12 cells. These results suggest instead that, in PC12 cells, the CAPS/D2 complex may function in a positive fashion to enhance dopamine-containing LDCV release from these cells. It will clearly be of interest to determine whether the CAPS/D2 interaction has a similar effect on dopamine secretion in neurons. A further issue that should be raised in this context is that D3 receptors have also been localized to presynaptic terminals where they are also believed to function as inhibitory autoreceptors [34,35]. Our protein interaction screens and coimmunoprecipitation experiments failed to uncover an interaction between CAPS and D3 receptors. It is thus tempting to speculate that D2 and D3 autoreceptors may modulate presynaptic neurotransmitter release via distinct molecular mechanisms.

CAPS and its invertebrate orthologs form a family of proteins with limited sequence homology to members of the Munc13 family of vesicle priming proteins that is known to function in synaptic vesicle fusion [36]. Genetic analysis of CAPS in *C. elegans* (UNC-31) and *Drosophila* (dCAPS) suggests a specific role for CAPS in LDCV release [37], while studies in vertebrate cell lines implicate CAPS in the regulation of biogenic amine secretion [17,38]. Our data suggests a specific role for CAPS in modulating dopamine secretion in PC12 cells. This conclusion is based principally on the observation that disrupting the CAPS/D2 interaction specifically blocked dopamine, but not NE, release following K^+ stimulation of PC12 cells. To our knowledge, this represents the first report indicating potential differences in the mechanism underlying the release of these two neurotransmitters from PC12 cells. This finding presents that intriguing possibility that the release of LDCVs containing biochemically similar neurotransmitters may be under differential regulatory control.

In directed two-hybrid screens, we detected interaction between D2 receptors and CAPS2, a recently discovered CAPS isoform [25,26] with high sequence similarity to CAPS1. The two CAPS isoforms exhibit distinct differences in expression patterns within the brain [25], and CAPS2 has been shown to selectively mediate release of the neurotrophins, BDNF and neurotrophin-3, and cell survival in PC12 cells [22]. However, the functional significance for multiple CAPS isoforms has not yet been clearly explained. It is possible, for example, that CAPS1 functions as a calcium sensor in regulated exocytosis, whereas CAPS2 functions in constitutive vesicle trafficking and secretion [26]. Alternatively, CAPS1 and CAPS2 may for the most part be functionally redundant [25]. CAPS2 was recently shown to regulate neurotrophin secretion from PC12 cells. However, we found that disrupting the CAPS/D2 interaction (by expression of the D2IC2 domain peptide) had virtually no effect on BDNF release from PC12 cells. These results suggest that CAPS2-regulated BDNF secretion occurs by a mechanism that is unrelated to the CAPS/D2 receptor interaction.

The recent identification of a cohort of DRIPs has led to an increased understanding of the mechanisms that regulate the activity of dopamine receptors in the central nervous system [10]. Elucidating the complete array of DRIPs and other unknown interacting partners of dopamine signaling complexes is important for understanding of the mechanisms of dopamine neurotransmission in the brain. The discovery that two DRIPs, calcyon and neuronal calcium sensor-1, are upregulated in schizophrenia points to the possibility that alterations in DRIPs and defects in Ca^{2+} homeostasis may contribute to abnormalities in the brain dopamine system in neuropsychiatric diseases [39,40]. Because CAPS appears to play a pivotal role the regulation of presynaptic dopamine receptor function, it is possible that alterations in CAPS structure and/or function could contribute to aberrancies in dopamine release as seen in neuropathologies such as schizophrenia and in conditions of drug addiction.

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