Published in final edited form as:

Biochemistry. 2011 October 18; 50(41): 8780–8791. doi:10.1021/bi200637e.

Interaction between the D2 Dopamine Receptor and Neuronal Calcium Sensor-1 analyzed by fluorescence anisotropy

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Abstract

Neuronal calcium sensor-1 (NCS-1) is a small calcium binding protein that plays a key role in the internalization and desensitization of activated D2 dopamine receptors (D2Rs). Here, we have used fluorescence anisotropy (FA) and a panel of NCS-1 EF hand variants to interrogate the interaction between the D2R and NCS-1. Our data is consistent with the following conclusions: 1) FA titration experiments indicate that at low D2R peptide concentrations calcium-loaded NCS-1 binds to the D2R peptide in a monomeric form. At high D2R peptide concentrations, the FA titration data is best fit by a model in which the D2R peptide binds two NCS-1 monomers sequentially in a cooperative fashion. 2) Competition FA experiments in which unlabeled D2R peptide was used to compete with labeled peptide for binding to NCS-1 shifted titration curves to higher NCS-1 concentrations, suggesting that the binding of NCS-1 to the D2R is highly specific and that binding occurs in a cooperative fashion. 3) N-terminally myristoylated NCS-1 dimerizes in a calcium-dependent manner. 4) Co-immunoprecipitation experiments in HEK293 confirm that NCS-1 can oligomerize in cell lysates, and that oligomerization is dependent on calcium binding and requires functionally intact EF hand domains. 5) Ca²⁺/Mg²⁺ FA titration experiments revealed that NCS-1 EF-hands 2-4 contributed to binding with the D2R peptide. EF-2 appears to have the highest affinity for Ca²⁺ and occupancy of this site is sufficient to promote high-affinity binding of the NCS-1 monomer to the D2R peptide. Magnesium ions may serve as a physiological co-factor with calcium for NCS-1/D2R binding. Finally, we propose a structural model that predicts that the D2R peptide binds to the first 60 residues of NCS-1. Together, our results support the possibility of using FA to screen for small molecule drugs that can specifically block the interaction between the D2R and NCS-1.

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SUPPORTING INFORMATION Supplemental information includes methods, one table, 8 figures and 6 supplemental references. Supplemental materials may be accessed free of charge online at <http://pubs.acs.org>.

Keywords

Neuronal Calcium Sensor-1; dopamine-2 receptor; dopamine-3 receptor; fluorescence anisotropy; calcium-binding; magnesium-binding

Neuronal calcium sensor-1 (NCS-1) is the primordial member of the neuronal calcium sensor (NCS) family of EF-hand calcium binding proteins that includes recoverin, neurocalcin, hippocalcin, VILIP-1-3, guanylate cyclase-activating proteins (GCAPs), and Kv channel-interacting proteins (KChIPs) (1). NCS-1 is the mammalian ortholog of frequenin, a protein critical for synaptic transmission within the *Drosophila* nervous system (2). In mammalian cells, NCS-1 has been shown to promote exocytosis from dense core vesicles in both neurons and neuroendocrine cells (3), and plays a key role in G-protein coupled receptor desensitization via a direct interaction with the D2 dopamine receptor (D2R) (4). Characterization of the R102Q NCS-1 variant, identified in an individual with autism, resulted in the rapid cycling between cytosolic and membrane pools in mouse N2A neuroblastoma cells (5). Further, increased expression of NCS-1 has been observed in the prefrontal cortex of individuals with schizophrenia and bipolar disorder (6, 7), and is up-regulated in injured neurons and in epilepsy (8, 9) suggesting that the D2R/NCS-1 interaction may represent a novel target for the development of drugs that may be useful for treatment of these increasingly prevalent diseases.

NCS-1, in common with all NCS family members, contains four EF-hand domains. The EF-hand motif is a 12 residue loop flanked on either side with an α -helix (10). In NCS family members, typically three of these motifs bind calcium with low to mid-nM affinity and Hill coefficients of ~ 2 (for review see (11)). Most members contain N-terminal myristoylation consensus sequences (excluding KChIP2 and KChIP3) (11) that may be involved in membrane targeting. In NCS-1 specifically, the first EF-hand (EF1) does not bind Ca^{2+} due to the substitution of cysteine and proline residues at critical positions in the first helix (for review see (11)). Calcium is a known regulator of NCS-1 function. *In vivo* experiments have demonstrated that Ca^{2+} -binding modulates the functional interaction of NCS-1 with the D2R and other binding partners to affect cellular signaling (4, 12-14). However, the detailed mechanism(s) by which calcium modulates these effects is unclear.

Calcium ion-binding has been previously shown to promote a set of conformational changes in NCS-1 that stabilize the native, functional conformation required for binding to its cellular partners (15). In general, the oligomeric state of NCS-1 in its signaling complex(es) is unknown; however, several other NCS family members self-associate in response to changes in calcium ion concentrations (16, 17), or form covalent disulfide linkages when saturated with Ca^{2+} (18). The formation of NCS-1 oligomers could provide multiple binding regions for interaction with several different binding partners, or alternatively form a single binding domain contributed by multiple monomer subunits.

In this study, we have used fluorescence anisotropy (FA) and a panel of NCS-1 variants to assess the contribution of Ca^{2+} binding to the interaction between the D2R and NCS-1. Our experiments support the idea that the D2R and NCS-1 form a high affinity calcium-dependent complex that requires occupancy of all three calcium-binding EF-hands for

optimal stability. The results presented here provide new insight into the regulation and assembly of the D2R/NCS-1 complex, and highlight the possibility of using FA as a tool to screen for compounds capable of specifically blocking the association between the D2R and NCS-1.

EXPERIMENTAL PROCEDURES

NCS-1 Expression and Purification

All NCS-1 constructs contained either a C-terminal His₆-tag (19), or were engineered with a TEV protease cleavable N-terminal His₆-tag within the pET-28-TEV vector (gift of Stephen Burley, SGX Pharmaceuticals, San Diego). Site specific variants (E84Q (within EF2), E120Q (within EF3), E168Q (within EF4), and the NCS-1 EF (E84Q+E120Q+E168Q) variant (within EF2, 3 and 4) were generated using a Quikchange site-directed mutagenesis kit (Stratagene). All mutations were verified by direct DNA sequencing.

Each recombinant NCS-1 protein was overexpressed in *E. coli* BL21 (DE3) cells (Novagen) grown in ZYP-5052 auto-induction media (20), and purified by nickel-nitriloacetic acid (NiNTA) affinity chromatography as previously described (19). The cleavable N-terminal His₆-tag on the pET-28-TEV-expressed NCS-1 fusion protein was removed by an overnight digestion with His₆-tagged TEV protease (50:1 NCS-1:TEV mg/mg) followed by an additional round of NiNTA affinity chromatography. The resulting protein (NCS-1 TEV) contains a residual vector sequence of glycine-histidinemethionine present at the N-terminus of the endogenous NCS-1 sequence. All of the bacterially expressed proteins were further purified by gel filtration chromatography on a Superdex 75 10/300 GL gel filtration column (GE Healthcare Lifesciences) equilibrated in sample buffer (1 mM dithiothreitol (DTT), 20 mM HEPES, 100 mM NaCl; pH 7.5) where they eluted as monomers, as judged by multi-angle light scattering and refractive index detection (Wyatt Technology Corp, Santa Barbara CA).

Fractions containing purified NCS-1 proteins were incubated overnight in gel filtration buffer containing 50 mM EGTA to remove free and protein-bound calcium and magnesium. Calcium was subsequently removed, to a sub-nanomolar concentration, by buffer exchange against Chelex 100 (Bio-Rad)-treated buffer containing 20 mM HEPES, 100 mM NaCl; pH 7.5 using an Amicon Ultra centrifugal concentrator. The residual calcium and magnesium concentrations of the final purified NCS-1 protein were determined by atomic absorption (Huffman Laboratories Inc.) to be ~47 nM and ~3 nM, respectively, corresponding to ion:NCS-1 ratios of 1:100 and 1:1700, respectively.

Synthesis and Labeling of D2R-Peptide Tracer

A 17 amino acid residue peptide corresponding to the C-terminal tail of the D2R (residues 428-443, spanning the NCS-1 binding site) plus an additional lysine residue, was synthesized (New England Peptide) and used for binding experiments. For fluorescence experiments, a fluorescein-labeled peptide (D2R-FL) (TFNIEFRKAFLKILHCK-fluorescein) was synthesized to include a C-terminal lysine that provided a site for fluorophore attachment. MALDI-TOF MS analysis indicated a purity of >95% for the D2R-

FL peptide. An unlabeled D2R-peptide (uD2R) containing an acetylated C-terminus instead of fluorescein was also synthesized (Proteomics Core Facility, Penn State College of Medicine). The unlabeled peptide was purified by reverse phase-HPLC and analyzed by MALDI-TOF MS. Labeled and unlabeled peptides were lyophilized and resuspended in water containing 1 mM DTT and 0.001% Tween 20; pH 7.5. The concentration of the D2R-FL peptide was determined from its absorbance at 490 nm (Molecular Probes Handbook), and the concentration of the unlabeled D2R-peptide was determined as previously described (21).

Fluorescence Anisotropy

Fluorescence anisotropy (FA) was performed using an ISS PC1 fluorimeter (ISS Corporation) equipped with Glan-Thompson polarizers on emission and excitation channels. All measurements were made at 30° C (Peltier controlled) in a 10 mm special optical glass cuvette (Starna Cells, catalog # 3/SOG/10) with a reaction volume of 1.5 mL of a buffer containing 20 mM HEPES, 100 mM NaCl, 2 mM DTT; pH 7.5. Samples were excited at 490 nm and the emission was monitored at 530 nm with a cutoff filter (Schott OG-515) and a band pass filter (Ealing catalog # 35-3607) having a 10 nm bandwidth.

FA titration experiments were performed by serial dilution of saturating concentrations of NCS-1 in buffer containing 1 nM D2R-FL, 20 mM HEPES, 100 mM NaCl, 2 mM DTT, 2 mM CaCl₂; pH 7.5 with the same solution lacking NCS-1. This method results in NCS-1 concentrations that are equally spaced on a logarithmic scale. Due to differences in the affinity of NCS-1 variants for the D2R-FL peptide, the final protein concentration was adjusted for each NCS-1 variant tested (as indicated in figure legends). For competition FA titrations, samples contained 1 nM D2R-FL and 0 μM, 1 μM, or 5 μM of uD2R, at saturating concentrations of NCS-1 TEV (5 μM).

For calcium FA titrations, serial additions of CaCl₂ were made to samples containing 1 nM D2R-FL and either 1 μM, 5 μM, or 10 μM NCS-1 TEV. For calcium/magnesium FA titrations, serial additions of CaCl₂ were made to samples containing 1 nM D2R-FL, 5 μM NCS-1, and either 0 mM, 1 mM or 50 mM MgCl₂.

Data Analysis

The titration data for the binding of the D2R-FL peptide to NCS-1 TEV was fit to five models, where the change in D2R-FL anisotropy reflects:

Binding of a single NCS-1 monomer (MODEL 1) to form D2R·NCS-1 complex:

$$A_{calc} = \frac{A_f + A_m K_{A1} [NCS1]}{1 + K_{A1} [NCS1]} \quad \text{eq 1.}$$

Where A_{calc} is the expected anisotropy for the model, A_f and A_m are the anisotropies of free D2R-FL and bound to a single NCS-1 monomer, respectively, $[NCS1]$ is the total concentration of NCS-1 in the reaction and K_{A1} (μM^{-1}) is the equilibrium association constant for complex formation.

Cooperative binding of two NCS-1 monomers (MODEL 2) to form D2R·NCS-1₂ complex:

$$A_{calc} = \frac{A_f + A_d K_{dimer} [NCS1]^2}{1 + K_{dimer} [NCS1]^2} \quad \text{eq 2.}$$

Where K_{dimer} (μM^{-2}) is the equilibrium association constant for cooperative assembly of the 1:2 D2R-FL:(NCS-1)₂ complex from the free peptide and two NCS-1 monomers and A_d is the anisotropy of this complex. To facilitate comparison, the apparent equilibrium association constant, defined as the $\sqrt{K_{dimer}}$ in units of μM^{-1} , was used. This value corresponds to the concentration of NCS-1 monomer that results in half saturation of the 1:2 complexes.

Cooperative binding of three NCS-1 monomers (MODEL 3) to form D2R·NCS-1₃ complex:

$$A_{calc} = \frac{A_f + A_{tri} K_{trimer} [NCS1]^3}{1 + K_{trimer} [NCS1]^3} \quad \text{eq 3.}$$

Where K_{trimer} (μM^{-3} , $K_{ap,trimer} = \sqrt[3]{K_{trimer}}$) is the equilibrium association constant for the cooperative binding of three NCS-1 monomers, and A_{tri} is the anisotropy of the 1:3 D2R-FL:(NCS-1)₃ species.

Sequential binding of two NCS monomers (MODEL 4) to form D2R·NCS-1 and D2R·NCS-1₂ complexes:

$$A_{calc} = \frac{A_f + A_m K_{A1} [NCS1] + A_d K_{A1} K_{A2} [NCS1]^2}{1 + K_{A1} [NCS1] + K_{A1} K_{A2} [NCS1]^2} \quad \text{eq 4.}$$

Sequential binding of three NCS-1 monomers (MODEL 5) to form D2R·NCS-1, D2R·NCS-1₂ and D2R·NCS-1₃ complexes:

$$A_{calc} = \frac{A_f + A_m K_{A1} [NCS1] + A_d K_{A1} K_{A2} [NCS1]^2 + A_{tri} K_{A1} K_{A2} K_{A3} [NCS1]^3}{1 + K_{A1} [NCS1] + K_{A1} K_{A2} [NCS1]^2 + K_{A1} K_{A2} K_{A3} [NCS1]^3} \quad \text{eq 5.}$$

Where K_{A1} , K_{A2} , A_m , A_d are defined as above, and K_{A3} is the association constant for adding a third NCS-1 monomer to the complex and A_{tri} is the anisotropy of this species.

For each model, the values of the anisotropy of D2R-FL-containing species and their association constant(s) were estimated by non-linear least squares analysis in the program Origin 8 (OriginLab, Northampton MA) or using MATLAB (Mathworks Inc, Natick MA) routines developed in-house. For these analyses, the total concentration of NCS-1 used in equations 1-5 was used as an estimate of its free concentration, since $[D2R-FL] \ll K_{D,app}$, the apparent dissociation constant for the reaction and the effect of ligand depletion can be ignored. The values and associated errors for the fitted parameters reflect the mean and standard deviations obtained from at least three independent experiments, unless noted otherwise. The reported value for the reduced χ^2 is defined in (22)

The binding constant for an unlabeled D2R peptide (uD2R) was determined in a competition experiment where D2R-FL was titrated with NCS-1 at fixed concentrations of the unlabeled peptide. The calculated anisotropy of the D2R-FL peptide in the presence of fixed concentrations of uD2R and varying concentrations of NCS-1 is given by equation 6

COMPETITION MODEL:

$$A_{calc} = \frac{A_f + A_m K_{A1} [NCS1]_f + A_d K_{A1} K_{A2} [NCS1]_f^2}{1 + K_{A1} [NCS1]_f + K_{A1} K_{A2} [NCS1]_f^2} \quad \text{eq 6.}$$

Where the free concentration of NCS-1, as well as those of D2R-FL and uD2R, are the simultaneous roots of mass balance equations for each species:

$$[NCS1]_T = [NCS1]_f \left(1 + K_{A1} [D2RFL]_f + K'_{A1} [uD2R]_f \right) + [NCS1]_f^2 (K_{A1} K_{A2} [D2RFL]_f + K'_{A1} K'_{A2} [uD2R]_f) \quad \text{eq 7a.}$$

$$[D2RFL]_T = [D2RFL]_f + K_{A1} [NCS1]_f [D2RFL]_f + K_{A1} K_{A2} [NCS1]_f^2 [D2RFL]_f \quad \text{eq 7b.}$$

$$[uD2R]_T = [uD2R]_f + K'_{A1} [NCS1]_f [uD2R]_f + K'_{A1} K'_{A2} [NCS1]_f^2 [D2RFL]_f \quad \text{eq 7c.}$$

In these experiments, the values of K_{A1} , K_{A2} , A_f , A_m , and A_d , determined in equivalent titrations in the absence of the unlabeled peptide, were used. The values of the association constants, K'_{A1} and K'_{A2} for unlabeled peptide, were estimated from equations 3b and 4a-c by minimizing the reduced χ^2 value between the experimental and calculated anisotropy values using MATLAB routines developed in house.

Analysis of titration curves for the effects of calcium ion concentration on the binding of the D2R-FL peptide to NCS-1 is described elsewhere (Supporting Information).

DNA Transfection and Coimmunoprecipitation

HEK-293 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum as previously described (4) and transfected with NCS-1 constructs containing either a C-terminal mycHis epitope tag (NCS-1-mycHis and NCS-1 EF-mycHis), or a C-terminal hemagglutinin (HA) epitope tag (NCS-1-HA), respectively. Transient transfections were performed using the Effectine transfection reagent (Qiagen) under conditions described by the manufacturer.

Coimmunoprecipitations (coIPs) were carried out on lysates prepared from HEK-293 cells expressing various NCS-1 constructs. Lysates contained 100 μ M CaCl_2 and 10 mM DTT. NCS-1-HA was immunoprecipitated with a rabbit anti-HA antibody (Covance). Western blot analysis of immunoprecipitated proteins was performed using a chicken anti-frequencein (anti-NCS-1) polyclonal antibody (1:14000 dilution, Rockland Immunochemicals). Immunoreactivity was detected with a horseradish peroxidase-conjugated secondary

antibody (1:14000 dilution, Jackson Immuno-Research), and visualized using enhanced chemiluminescence (ECL plus, GE Healthcare).

Size Exclusion Chromatography Multi-angle Laser Light Scattering (SEC-MALS)

Analytical SEC-MALS measurements were made using an Agilent 1200 series HPLC system (Agilent Technologies) equipped with an isocratic pump and auto-sampler. Chromatographic separations were accomplished using either a Superdex 75 10/300 GL or a 200 10/300 size exclusion column (GE Healthcare). The equilibration buffer and flow rate are given in the figure legends. On-line measurements of statically scattered light were determined using a Wyatt Technologies DAWN HELEOS MALS detector with concurrent change in refractive index measured by a Wyatt Technologies Optilab rEX instrument. The assumed dn/dc (change in refractive index with concentration) value for these experiments was 0.185 mL/g. Data analysis, including molecular weight determination and sample polydispersity, was performed using the ASTRA V software package (Wyatt Technology). In order to minimize the MALS contribution of dust particles, solutions were pre- and in-line filtered with an Anodisc 0.1 μ m filter membrane (Whatman).

Micro-batch Multi-angle Laser Light Scattering (MB-MALS)

NCS-1 constructs were dialyzed into a buffer composed of 150 mM NaCl, 25 mM HEPES, 1 mM TCEP, and either 2 mM $CaCl_2$ or 1 mM EGTA at pH 7.5 and then filtered through an Anotop 0.02 μ m syringe filter (Whatman). Samples of known concentration were introduced into the flow cell of the Wyatt Technologies DAWN HELEOS detector using a syringe pump (Razel Scientific) at a constant flow rate of 0.15 mL/min. Samples were in-line filtered with an Anotop 0.02 μ m syringe filter (Whatman). Light scattering data analysis and molecular weight measurements were determined as described above after subtracting a buffer baseline established at the beginning and end of each experiment.

Plots of molecular weight vs. concentration and equilibrium dissociation constants for self-association were determined using Origin (OriginLab) curve fitting software. The monomer-dimer self-association model was fit to the observed molecular weight using the following relationship:

$$MW_{Obs} = MW_{mon} \frac{C_{mon}}{C_{tot}} + 2 * MW_{mon} \frac{C_{dim}}{C_{tot}} \quad \text{eq. 8}$$

Where MW_{Obs} is the observed molecular weight, MW_{mon} is the molecular weight of the monomer, C_{mon} is the concentration of the monomer species, C_{dim} is the concentration of the dimer species, and C_{tot} is the total protein concentration. For any given total protein concentration expressed as a function of monomer (M_t) concentration, the concentration of the dimer species dependent on equilibrium dissociation constant (K_d) can be given as:

$$C_{dimer} = \frac{4M_t + K_d - \sqrt{-(4M_t + K_d)^2 + 16M_t^2}}{8} \quad \text{eq. 9}$$

Values for K_d adjusted by non-linear least squares analysis were then used to determine dimer concentrations in Equation 8.

Analytical Equilibrium Ultracentrifugation

Sedimentation equilibrium experiments were performed using a Beckman XL-A ultracentrifuge. NCS-1 samples were dialyzed into a buffer composed of 100 mM NaCl, 25 mM HEPES, 2 mM DTT and 2 mM CaCl_2 or 1 mM EGTA at pH 7.5. Centrifugation experiments with initial protein concentrations between 0.1 and 0.8 mg/mL were performed at speeds ranging from 15,000 to 30,000 RPM at 20° C using an An-60 Ti rotor. UV absorbance scans (278 nm) were collected in radial step mode with 0.001 cm steps averaged over 5 replicates. The models used for curve fitting the experimental data included a model corresponding to an ideal monomer and a self-associating ideal monomer-dimer system, which have been derived and discussed extensively elsewhere (23, 24). During data analysis, the solvent density was assumed to be 1.0, and the partial specific volume of NCS-1 was taken to be $0.730 \text{ cm}^3\text{g}^{-1}$, which was estimated from its amino acid sequence (25).

Ligand Docking

The HADDOCK webserver (26) was used for flexible docking simulations of the peptide into the receptor binding site of NCS-1. The coordinates from Chain A of PDB ID:1G8I were used as the rigid docking model for NCS-1. For the flexible receptor, residues 385-400 of PDB ID:3PBL were extracted, visualized in Chimera and serine 399 changed to histidine in its most commonly observed rotamer to match the sequence of the D2 peptide used in our assays. The structures of other NCS family members in complex with peptides were superimposed onto the structure of NCS-1. The peptides bound to similar parts of their respective proteins. The D2R peptide was superimposed onto the peptide helix of PDB ID: 2I94.pdb. This placed residues D37 and L89 of the NCS-1 protein close to R391 and I397 of the D2R peptide. Therefore, these residues were set as active and the surrounding residues defined as passive in the analysis.

RESULTS AND DISCUSSION

Fluorescence Anisotropy Binding Isotherm for NCS-1 TEV

To determine whether the isolated D2R peptide (residues 428-443) binds NCS-1 TEV, a fluorescein labeled version of this peptide, D2R-FL, was synthesized. Titration of 1 nM D2R-FL, in the presence of 2mM CaCl_2 , with increasing concentrations of non-myristoylated NCS-1 TEV resulted in increases in the anisotropy of fluorescence polarization from ~0.04, for the free peptide, to a maximum value of ~0.23, at high NCS-1 concentrations (Figure 1). By contrast, in the absence of CaCl_2 , no significant change in anisotropy was observed at 1 μM NCS-1 TEV and the increase was only 0.01 units at 10 μM NCS-1 TEV (data not shown). The changes in anisotropy were not the result of alterations in the environment of the fluorophore in the presence of NCS-1, since the fluorescence intensity was unaffected throughout the titration (27) (See Supplemental Figure SF1). These data are consistent with D2R-FL binding to NCS-1 TEV in a specific and calcium-dependent manner.

The limiting anisotropy of the D2R-FL:NCS-1 TEV complex observed in the titration experiment is larger than expected for a 1:1 complex of an ~4 kDa peptide and 22 kDa NCS-1 monomer and is more consistent with a complex of 1:2 D2R-FL:NCS-1. Therefore, to determine the stoichiometry and affinity of NCS-1 binding, the FA titration data was fit to each of five models: D2R-FL binds either a one (MODEL 1), two (MODEL 2) or three (MODEL 3) NCS-1 TEV monomers with perfect cooperativity (i.e. no significant intermediate species are present) or sequential assembly of two (MODEL 4) or three (MODEL 5) NCS-1 TEV monomers. Comparison of the χ^2 values for the fits of each model to the data indicate that only sequential binding MODEL 4 accurately describes the data ($\chi^2 = 0.7$ compared with $\chi^2 > 1.5$ for MODELS 1-3 and 5, for a summary see Supplemental Table 1, and Supplemental Figure SF2). Moreover, a model in which D2R-FL and NCS-1 form 2:2 complexes had a χ^2 value > 100 indicating that the complex contains only a single D2R-FL at these peptide concentrations (data not shown). In MODEL 4, the D2R-FL peptide binds two monomers sequentially with a disassociation constant for the first and second monomers of K_{D1} , of $0.27 \pm 0.03 \mu\text{M}$ and K_{D2} , of $0.53 \pm 0.06 \mu\text{M}$, respectively. Under these conditions, NCS-1 with or without 2 mM CaCl_2 was monomeric by SEC-MALS ($20.2 \pm 0.3\%$ and $22.1 \pm 0.1\%$ kDa, for apoNCS-1 and calcium loaded Supplemental Figure SF3). In addition, the concentration normalized scattering curves of EGTA-treated (3 mg/mL) and calcium-loaded (6 mg/mL) NCS-1 were essentially identical by small angle X-ray scattering. Their radius of gyration (20.4 Å EGTA, 20.8 Å calcium-loaded), and longest vector (60 Å for either) were also similar, indicating that they had the same shape in solution (Supplemental Figure SF4). In addition, the intensities extrapolated to zero angle and normalized for protein concentration ($I(0)/c$) are essentially the same and the molecular weights calculated from the scattering intensity by the Porod method (28) were each $22 \pm 10\%$ kDa, in agreement with the calculated molecular weight of a monomer. Together, this suggests that the non-myristoylated NCS-1 dimer formation observed in the FA binding experiments involves an allosteric transition resulting from D2R-FL peptide binding.

To determine the affinity of NCS-1 TEV for the unlabeled peptide, we performed a competition assay in which 1nM D2R-FL was titrated in the presence of 0, 1 and 5 μM uD2R with increasing NCS-1 TEV concentrations (Figure 1). As expected, inclusion of the unlabeled peptide shifted the titration curves to higher NCS-1 TEV concentrations, with higher concentrations of uD2R having a greater effect, indicating that the unlabeled peptide competes with D2R-FL for NCS-1 binding. Fitting of this data to the COMPETITION MODEL, in which uD2R sequentially binds two NCS-1 monomers, accurately described the data ($\chi^2 \sim 1$ for all three uD2R peptide concentrations compared with values > 5 if the unlabeled peptide forms only 1:1 or 1:2 complexes with NCS-1). Under these conditions, the unlabeled peptide has a ~1.50-fold lower affinity ($K'_{D1} = 0.39 \pm 0.08 \mu\text{M}$ and $K'_{D2} = 0.81 \pm 0.09 \mu\text{M}$) for NCS-1 compared with D2R-FL. The weaker affinity may reflect either the effects of the carboxyl-terminal lysine residue and the fluorescein moiety in the D2R-FL peptide, and/or, aggregation of the unlabeled peptide at the concentrations used in these experiments. This latter possibility is supported by the observation that, in the presence of 10 μM uD2R, the measured anisotropy of the D2R-FL peptide alone was significantly higher (0.06, data not shown) than in its absence. The limited solubility of the D2R peptide used in this study precluded detailed structural analysis. In any case, the uD2R peptide binds

NCS-1 with the same stoichiometry and similar affinity to D2R-FL, under our measurement conditions.

NCS-1 Can Oligomerize in Cell Lysates

The FA experiments imply that purified monomeric non-myristoylated NCS-1 TEV can dimerize in the presence of the D2R derived peptides. To assess whether NCS-1 can associate in cell lysates, we tested whether an anti-HA epitope-tag antibody co-immunoprecipitated NCS-1-HA from lysates of HEK-293 cells expressing either NCS-1-HA alone or co-transfected with either NCS-1-mycHis or NCS-1 EF-mycHis. The resultant blot was probed with an anti-NCS-1 antibody in order to detect the presence of either the HA-tagged or mycHis-tagged NCS-1 in the coIP (Figure 2). In untransfected cells, the anti-NCS-1 antibody failed to immunoreact with any cellular proteins (Figure 2, lanes 1 and 2). An immunoreactive band of ~22 kDa, the predicted size of HA-tagged NCS-1, was detected in lysates prepared from cells transfected with NCS-1 HA alone (Figure 2, lane 3). A band migrating with a similar molecular mass was detected in the immunoprecipitated (IP) lane (Figure 2, lane 4), indicating that the anti-HA antibody specifically immunoprecipitated NCS-1-HA from transfected cells. An immunoreactive band of ~25 kDa, the predicted size of NCS-1 mycHis, was detected in lysates prepared from cells transfected with NCS-1-mycHis alone (Figure 2, lane 5). However, no band was detected in the IP lane (Figure 2, lane 6), indicating that the NCS-1-mycHis protein did not immunoprecipitate with the anti-HA antibody. Similarly, an immunoreactive band of ~25 kDa was detected in lysates prepared from cells transfected with the mycHis-tagged NCS-1 EF variant (Figure 2, lane 7), whereas no band was detected in the NCS-1 EF-mycHis IP lane (Figure 2, lane 8). Two bands, ~22 and ~25 kDa in size, were detected in lysates from cells cotransfected with NCS-1-HA and NCS-1-mycHis (Figure 2, lane 9). Two bands of similar molecular mass were also detected in the IP lane (Figure 2, lane 10), indicating that NCS-1-HA and NCS-1-mycHis, were coIP'd with anti-HA antibodies. Likewise, two bands of ~22 and ~25 kDa were detected in lysates prepared from cells cotransfected with NCS-1-HA and mycHis-tagged NCS-1 EF (Figure 2, lane 11). However, only the 22 kDa NCS-1-HA protein was detected in the IP lane (Figure 2, lane 12). Therefore, the ~25 kDa mycHis-tagged NCS-1 EF protein was unable to coIP with NCS-1-HA. Taken together, these coIP experiments indicate that NCS-1 can form oligomers in cell lysates. It is important to note that the ability of NCS-1 to form oligomers was dependent on the addition of calcium (100 μ M CaCl_2) to the lysates, suggesting that NCS-1 oligomerization is mediated by physiologically relevant concentrations of localized calcium (29, 30). Our observation that the NCS-1 EF-mycHis variant did not interact with NCS-1-HA, even in the presence of 100 μ M calcium, further argues that NCS-1 oligomerization is a calcium-dependent process, since this construct does not bind calcium, but maintains proper secondary structure (19). We therefore conclude NCS-1 oligomerization was dependent on Ca^{2+} -binding and requires functionally intact EF-hand domains, although we cannot determine whether oligomerization requires the presence of additional binding partners present in the lysate or is the result of myristoylation of NCS-1 in HEK293 cells.

Myristoylated NCS-1 can form a stable calcium-dependent homo-dimer

To determine whether amino terminal myristoylation affects NCS-1 oligomerization, we expressed and purified myristoylated NCS-1 with a carboxyl terminal His₆-tag (MyrNCS-1 His₆) (19). Chromatography on a Superdex 75 column and in-line MALS analysis of EGTA treated MyrNCS-1 His₆ revealed a single peak with the monomer molecular mass (21.5 ± 0.3 kDa, Figure 3A). By contrast, replacement of EGTA with 2 mM CaCl₂ in the sample and elution buffer resulted in the appearance of an additional peak corresponding to that of a stable dimeric species (46.7 ± 0.1 kDa, Figure 3B), as well as a small amount of higher molecular weight species. The higher molecular weight species were not observed with non-myristoylated NCS-1 His₆ under any condition tested (data not shown). In the presence of CaCl₂, the peak corresponding to the MyrNCS-1 His₆ monomer was broader and the leading edge had a molecular mass slightly larger than that of the monomer (~ 2 -4 kDa). This leading edge effect was also observed in the absence of Ca²⁺ (Figure 3A). This lead us to conclude that dimerization of MyrNCS-1 His₆ involves two homodimeric species, one in rapid equilibrium (leading edge of main peak) that was weakly dependent on Ca²⁺ and the other, a stable homodimer (dimeric peak) that required Ca²⁺ bound to each of the three functional EF-hand domains. MyrNCS-1 His₆ dimerization was further characterized by MB-MALS (Supplemental Figure SF5) and analytical ultracentrifugation (Supplemental Figure SF6). In both cases, MyrNCS-1 His₆ formed a reversible dimer in the presence of 2 mM CaCl₂, but not its absence. The dissociation constant for dimerization was found to be in the low micromolar range (10 ± 1 μ M and 2 ± 0.2 μ M by MB-MALS and analytical ultracentrifugation respectively). These results confirm that myristoylated NCS-1 can form a calcium dependent, reversible dimer in solution which likely accounts for the dimerization observed in pulldown experiments from HEK293 lysates. The Ca²⁺ and myristoylation-dependent dimerization of NCS-1 observed here is similar to that reported for the NCS family member VILIP-1(31). MyrNCS-1 His₆ was also capable of binding to the D2R-FL peptide, albeit with reduced affinity. Non-myristoylated NCS-1 His₆ bound the D2R-FL peptide with a similar affinity to the NCS-1 TEV protein ($K_{D1} = 0.48$ μ M, $K_{D2} = 1.3$ μ M and $K_{D1} = 0.27$ μ M, $K_{D2} = 0.53$ μ M for His₆ and TEV protein respectively). The slight decrease in the affinity of NCS-1 His₆ may reflect the effects of the tag on local ion concentrations around the protein. The MyrNCS-1-His₆ bound the D2R-FL peptide with lower affinity compared with NCS-1 His₆ for both monomeric and dimeric complexes (K_{D1} of 3.4 ± 0.2 and K_{D2} of 5.9 ± 0.7 μ M, Table 1, Supplemental Figure SF6). The reduced affinity the MyrNCS-1-His₆ construct could reflect competition between the myristoyl moiety, which is thought to bind to EF-1, and the peptide for the same binding site in our solution-based assay, or the result of a conformational change that reduces affinity.

Disruption of EF-hands 2-4 affects D2R peptide binding

Our previous work suggested a role for EF-hand 1 in binding to the D2R receptor (4). Here, we demonstrate that binding of the isolated, fluorescently labeled D2R peptide (residues 428-443 of D2R) to NCS-1 also is calcium-dependent and therefore EF-hands 2-4 must play a role in peptide binding. To define the relative contributions of these EF-hands, we constructed and characterized, using a FA assay, variants of NCS-1 that prevented Ca²⁺ binding at specific sites (E84Q disrupts EF-2, E120Q disrupts EF-3, E168Q disrupts EF-4, and EF contains three point mutations to disrupt all functional EF-hands). These were

chosen because they disrupt Ca^{2+} binding without significantly affecting NCS-1 structure (19, 32, 33).

All of the single site variants of NCS-1 were assayed in the NCS-1 His₆ background, since this construct expressed to higher levels, which was beneficial for some of the poorly expressing variants. To account for the potential of altered binding affinities to D2R-FL caused by the affinity tag, NCS-1-His₆ was used for comparison. Titration of the three singly substituted non-myristoylated NCS-1-His₆ variants revealed that each bound the D2R-FL, albeit with differing affinities. By contrast, the NCS-1 EF construct, which disrupts all three calcium binding sites, displayed no observable binding even at 10 μM concentration. Table 1 summarizes the parameters obtained (K_{D1} , K_{D2} , A_f , A_m , A_d and reduced χ^2) for FA titrations of each variant fitted to MODEL 4. The reported reduced χ^2 values with this model are all near unity, demonstrating the quality of the fits. None of the other binding models tested accurately described the titration curves, based upon the χ^2 criteria. Moreover, with MODEL 4, there is good agreement between the fitted anisotropy values for each peptide-bound species for all of the NCS-1 variants, with the exception of A_d for NCS-1 E84Q, which is likely due to the presence of light scattering. Further, the limited solubility of this latter protein resulted in titration curves that only reached the K_d value, further reducing the quality of the fit. Taken together this data support a binding model in which the isolated D2R peptide and NCS-1 form stable 1:1 and 1:2 complexes under the experimental conditions.

Analysis of the affinities of NCS-1 E84Q, E120Q and E168Q for the D2R-FL peptide reveals that disruption of the Ca^{2+} binding site in EF-2 (E84Q) results in the largest decrease in binding affinity for this peptide. The affinity of the first NCS-1 monomer is reduced by ~15-fold and the second by >200-fold compared with NCS-1-His₆. The effect of the equivalent residue in EF-3 (E120Q) was more modest (~2 and 5-fold reduction in K_{D1} and K_{D2} respectively), and disruption of Ca^{2+} binding by EF-4 (E168Q) only significantly affected the value of K_{D2} (~5-fold). These data suggest that all three Ca^{2+} binding sites contribute, in some manner, to the stability of D2R-FL:NCS-1 complexes.

Analysis of the values of the dissociation constants in light of the structure of NCS-1 (34) provides insight into the possible individual roles of each EF-hand in D2R-FL-binding. The NCS-1 E84Q mutation results in the largest increase in K_D s for both the monomer (K_{D1} ~9 μM) and dimer (K_{D2} ~71 μM) when compared to the equivalent wild type protein. The D2R interaction motif lies in the first 71 amino acids of NCS-1 (4), suggesting that EF-2 affects the direct binding of NCS-1 to the D2R peptide. In addition, the significant destabilization of the 1:2 D2R-FL:(NCS-1) complex suggests that Ca^{2+} binding at EF-2 also contributes to NCS-1 dimer stability in the complex.

EF-hands within a protein typically occur in pairs that form discrete domains (35) allowing communication and positive cooperativity between them. Such coupling is seen in NCS-1 where EF-1/EF2 and EF-3/EF-4 form pairs (34). NCS-1 E120Q (EF 3) and NCS-1 E168Q (EF 4) both have minimal effects upon binding of the peptide to monomeric NCS-His₆ (K_{D1} of 1.01 μM and 0.64 μM , respectively) compared to K_{D1} of NCS-1-His₆ (0.48 μM). These minimal decreases may reflect either a slight alteration on calcium binding affinity of

EF-2 (32) or that there are contributions to binding of the peptide from regions outside of EF-1. Both substitutions (E120Q and E168Q) result in a modest decrease in the stability of the dimeric NCS-1 peptide complex (K_{D2} of 7 μM and 8 μM , respectively) consistent with a supporting role in NCS-1 dimer formation in the complex. This similarity could be attributed to the structured pairing of these EF-hands, in that non-functionality of one affects the function of the other in dimer formation.

Effect of Calcium/Magnesium ion binding on the D2R-FL/NCS-1 Interaction

All three calcium ion binding EF-hands in NCS-1 contribute to D2R-FL peptide binding at saturating CaCl_2 concentrations. However, intracellular calcium concentrations are likely to be much lower than 2 mM. To investigate the calcium ion dependence of D2R peptide-binding, we titrated 1 nM D2R-FL in the presence of 1 or 5 μM NCS-1 TEV with increasing concentrations of CaCl_2 (Figure 4). In the absence of calcium, a weak association between NCS-1 TEV and the D2R-FL peptide was observed as a small increase in anisotropy at high NCS-1 concentrations. This increase may be due to weak calcium ion-independent or non-specific binding. Titration with CaCl_2 resulted in a biphasic increase in anisotropy that reached its maximum value at Ca^{2+} :NCS-1 concentrations of $\sim 5:1$. The limiting anisotropy value of each curve was dependent upon the concentration of NCS-1 TEV in the solution, suggesting that this concentration is limiting. This is in agreement with the peptide-NCS-1 titrations using 2 mM CaCl_2 described above. The biphasic nature of the anisotropy curve is consistent with the formation of both 1:1 and 1:2 D2R:NCS-1 complexes with differing dependence on added Ca^{2+} .

A previous study has shown that non-myristoylated NCS-1 binds Ca^{2+} independently in EF2, EF3 and EF4 (36). In our studies, we observed a differential effect of Ca^{2+} -binding at these sites on binding of the D2R-FL peptide. To better characterize the Ca^{2+} -dependence of complex formation, the CaCl_2 titration curves were fit to several binding models. Due to the complexity of the reaction, several assumptions were made, specifically: (1) In the absence of Ca^{2+} , NCS-1 binds non-specifically to the D2R-FL peptide; (2) The anisotropy of the 1:1 and 1:2 D2R-FL:NCS-1 complexes are independent of the number of bound Ca^{2+} ; (3) Formation of the 1:2 D2R-FL:NCS-1 complex requires at least 5 total bound Ca^{2+} . The first assumption appears valid since the dependence of the D2R-FL peptide anisotropy on NCS-1 concentration in the absence of CaCl_2 is low. Assumptions 2 and 3 were necessary to restrict the number of parameters to allow convergence of the fitting routines, and thus represent clear limitations of the current model. **Figure 4A** is a schematic that describes the linkage between Ca^{2+} and D2R-FL binding to NCS-1. It fits the CaCl_2 titration data reasonably well (χ^2 of 1.1 and 1.3 for the titrations with 1 and 5 μM NCS-1 TEV respectively, see Supplemental Methods and Supplemental Figure SF8 for details of this model). The key features of this model are: 1) EF-2 and EF-3 bind randomly to Ca^{2+} while the last site is only filled after the first two; 2) D2R-FL only binds to one of the mono Ca^{2+} bound forms of NCS-1; and 3) the 1:2 D2R-FL:NCS-1 dimer primarily contains 6 bound Ca^{2+} . In the current study, we propose that EF-2 has the highest affinity for Ca^{2+} in the presence of the D2R-FL peptide and that occupancy of this site is sufficient to promote high affinity binding of the peptide to the NCS-1 monomer. This is consistent with the significant reduction in binding affinity of the E84Q NCS-1 variant. The second sharp rise in the anisotropy, at $\sim 5:1$

Ca²⁺:NCS-1, likely corresponds to the formation of stable Ca²⁺ saturated dimers bound to the D2R peptide. Since this occurs at higher concentrations of CaCl₂ and, disruption of EF-4 significantly affects only the 1:2 complexes (E168Q), we propose that binding of Ca²⁺ to EF-4 accounts for the observed changes in anisotropy. A clear strength of this model is that it implies a role(s) for all three EF-hands in complex formation, and is consistent with the NCS-1 TEV titration data obtained at saturating concentrations of CaCl₂. However, because of the simplifications necessary to obtain stable fits to the data, and since Ca²⁺ binding is measured indirectly, the association constants for Ca²⁺ binding from these fits should be viewed with some caution.

Unlike other members of the NCS-1 family, two of the EF-hands (-2, -3) can bind Mg²⁺ with micromolar affinity (32). Due to the high cellular Mg²⁺ levels relative to Ca²⁺, binding of magnesium ions could conceivably contribute to NCS-1 function (32). To determine whether Mg²⁺ can substitute for Ca²⁺ in D2R peptide binding, we analyzed the effects of replacing Ca²⁺ with Mg²⁺. In the absence of CaCl₂, all of the anisotropy values of the D2R-FL peptide in the presence of 5 μM NCS-1 TEV were identical and independent of MgCl₂ concentration (0 – 50 mM), demonstrating that NCS-1 TEV requires Ca²⁺ to interact with the D2R-FL. Since Mg²⁺ and Ca²⁺ share two binding sites on NCS-1, it is possible that the presence of Mg²⁺ could affect Ca²⁺-dependent D2R-FL/NCS-1 complex formation. To test this idea, 1 nM D2R-FL and 5 μM NCS-1 TEV were titrated with CaCl₂ either in the absence or presence of MgCl₂ concentrations ranging from 0 to 50 mM (Figure 5). Addition of 10 mM MgCl₂ (Figure 5A) altered the biphasic shape of the calcium FA titration curve compared to titrations without Mg²⁺. At MgCl₂ concentrations up to 10mM, the measured anisotropy was elevated in the intermediate CaCl₂ range corresponding to ratios of Ca²⁺:NCS-1 from ~1:1 and 2.5:1. By contrast, at 50 mM MgCl₂ the measured anisotropy was lower than the corresponding values in its absence over the entire CaCl₂ concentration measured (at 15 μM CaCl₂ the anisotropy decreases from 0.20 to 0.17 in the presence of 50 mM MgCl₂). This observation suggests that, at near physiological Mg²⁺ levels (<10 mM), it can partially substitute for Ca²⁺ in stabilizing D2R:NCS-1 complexes; however, at higher levels, Mg²⁺ competes with Ca²⁺. The enhanced stability of D2R-FL:NCS-1 complexes observed with 10 mM MgCl₂ may reflect occupancy of EF-3 with Mg²⁺, the relatively higher affinity site for this ion (K_D ~ 17 μM). Since binding of Ca²⁺ by EF-2 and EF-3 appears to be sequential in the presence of the D2R-FL peptide and the affinity of EF-2 for Mg²⁺ is much lower than for Ca²⁺, the presence of 10 mM MgCl₂ could allow occupancy of EF-3 with Mg²⁺ resulting in a higher proportion of NCS-1 TEV molecules having both EF-2 and -3 occupied with divalent ions in the intermediate range of CaCl₂. In this case, with EF-2 occupied with Ca²⁺ and EF-3 occupied with Mg²⁺ NCS-1 TEV would be able to support binding of the D2R-FL peptide. The loss of binding observed at higher MgCl₂ concentration could reflect binding of Mg²⁺ to EF-2 preventing complex formation. This interpretation is consistent with the results of divalent ion binding studies with apo NCS-1 (32) and the observation that the Ca²⁺ binding site in EF-2 plays a critical in binding of D2R-FL.

Qualitatively, these results are consistent with a model in which Mg²⁺ can functionally replace Ca²⁺ at one of its low affinity binding sites (EF-3), but is a competitive inhibitor of binding to EF-2. To further explore the possibility that physiological levels of Mg²⁺ and

Ca^{2+} might play a role in supporting D2R:NCS-1 complex formation, we titrated the D2R-FL peptide with NCS-1 TEV in the presence of 200 μM CaCl_2 with and without 5mM MgCl_2 (Figure 5B). In the presence of physiologically-relevant Ca^{2+} and Mg^{2+} concentrations, the titration curve shifted to lower NCS-1 concentrations ($K_{D1\text{app}} = 0.20 \pm .04$ and $K_{D2\text{app}} = 0.47 \pm .02 \mu\text{M}$, $\chi^2 = 0.6$, compared with $0.44 \pm .05$ and $0.62 \pm .02 \mu\text{M}$, $\chi^2 = 0.7$ for the titration with CaCl_2 alone) suggesting that Mg^{2+} could act as a physiological co-factor with Ca^{2+} for binding.

Modeling of the of the D2R-FL peptide binding site in NCS-1

In order to interpret the biochemical data in a structural context, the peptide was docked into the crystal structure of NCS-1 (PDB ID:1G8I). The crystal structure of the D3 receptor (D3R) was recently determined (37), which also binds NCS-1 (4). It contains a sequence that is virtually identical to the D2R peptide used in our studies. In the context of the D3R structure, the D2R peptide appears to form an α -helix. To model the interaction between NCS-1 and the D2R peptide, the coordinates of this region in the D3 structure were extracted and the sequence changed to agree with D2R by replacing the serine with the most commonly observed rotamer of histidine. Three structures of NCS family members in complexes with peptides are currently available (PDB ID:1S6C, 2I94, 2JU0). Although the details of binding are different in each case, they share a common binding site for their substrates (PDB ID:1S6C, residues 217-225 of the potassium voltage gated subfamily D member 2; PDB ID:2I94, residues 1-16 of rhodopsin kinase; PDB ID:2JU0, residues 145-169 of phosphatidylinositol 4-kinase, respectively) that binds to EF1 and helix 5 (within EF2). The D2R peptide was superimposed onto the bound peptide helix of PDB ID:2I94 and its new position was used as starting coordinates for docking of the D2R peptide onto the surface of NCS-1. The docking algorithm was run multiple times with different constraints. The models that gave the best composite scores bound the peptide in a similar orientation; a representative model is shown (Figure 6). In order to visualize the dimer, the coordinates of neurocalcin were used (code PDB ID:1BJF) (38) as a template, and the coordinates of NCS-1 superimposed on each monomer. This allowed us to estimate the distance between the two D2R putative binding sites on NCS-1, which were approximately 45 Å apart. An interesting feature of this peptide-binding site is that it overlaps with the myristoyl-binding pocket identified in the NCS homolog GCAP-1 (39). Like NCS-1 (40), the myristoyl moiety is not involved in a calcium-myristoyl switch, and thus interacts with the protein in the Ca^{2+} free state. This would provide an explanation for the reduced affinity of Myr-NSC-1 for the D2R peptide, since it would have to compete with the myristoyl-moiety for binding. However, in the presence of a membrane bilayer, the myristoyl-moiety is likely to interact with the bilayer and not with NCS-1 relieving inhibition.

CONCLUSIONS

At physiological concentrations of Ca^{2+} and Mg^{2+} , the three functional EF-hands in NCS-1 support D2R peptide binding. Currently, there are no high resolution structures for the complex between NCS-1 and the D2R or D3R receptors, respectively, and thus the binding site on NCS-1 for the peptide cannot be identified unambiguously. However, deletion of EF-1 (1-60) abrogates D2R binding (4) and disruption of Ca^{2+} binding in EF-2, which pairs

with EF-1, severely affects D2R peptide binding. In contrast, disruption of Ca^{2+} binding in either EF-3, or EF-4 primarily affects dimerization. This suggests that the D2R peptide binds to the first 60 residues of NCS-1. In our structural model, the peptide binds within EF-1 with its N-terminus on the same face as the membrane anchored myristoylated N-terminus of NCS-1 (Figure 6), as is observed in other NCS family members (38, 41). This placement is consistent with the observed reduction in affinity for the D2R peptide for myristoylated NCS-1. In the absence of a membrane, the myristoyl moiety is thought to interact with EF-1 and thus in our solution experiments would directly compete with the fluorescently labeled D2R peptide. The NCS-1 monomer has a hydrophobic groove that runs the length of the protein. Although we cannot rule out binding of the D2R peptide within this groove, it seems unlikely, as it would place the N-terminus of the D2R peptide further from the plasma membrane.

To model the NCS-1 dimer, we used neurocalcin, a dimeric member of the NCS family. In neurocalcin, the hydrophobic groove is shorter, due to a different packing of the C-terminal helix (38). The residues in the interface are largely conserved in NCS-1, raising the possibility that receptor binding to EF-1 in Ca^{2+} loaded NCS-1 could trigger a conformational change in the C-terminal helix that promotes dimerization in a similar way. The enhanced dimerization of myristoylated NCS-1 may reflect a general effect of occupancy of EF-1. This model would also explain why NCS-1 is monomeric in its complex with $\text{PI4K}\beta$ (42), because both the C-terminal helix and $\text{PI4K}\beta$ utilize the same binding surface. In our model, the contribution of D2R peptide and divalent cation binding are similar to that of divalent cation binding to the myristoylated form of VILIP-1 (31). In any case, the question still remains: Why does NCS-1 dimerize at all? One possible explanation is that the NCS-1 dimer stabilizes its interaction with the D2 and D3 receptors, which are themselves dimeric (43). In this highly speculative model, the two putative D2R peptide binding sites are about 45 Å apart. Based on the crystal structure of the D3R-lysozyme chimera, the two NCS-1 binding helices (named helix VII) sit approximately parallel to the plane of the membrane, and perpendicular to the transmembrane helices. If each helix VII changed orientation upon NCS-1 binding to become perpendicular to the surface of the membrane, the 2 helix VIIs would be approximately 45 Å apart and a 2:2 D2R-FL:NCS-1 could form. Although we did not observe a 2:2 D2R-FL:NCS-1 complex, the biochemical experiments were performed at low concentrations of peptide and high concentrations of NCS-1. These conditions favor 1:2 complexes. Alternatively, the 1:2 D2R:NCS-1 complex that we observed may allow recruitment of other factors to the signalsome modulating the activity of the receptor complex (44). The answer to these critical questions will require a high resolution structure of the D2R-NCS-1 complex. The results presented here do highlight the possibility of using FA as a high throughput tool to screen for compounds capable of specifically blocking the association between the D2R and NCS-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We would like to thank Drs. Jorg Roesgen and Ira Ropson for a careful reading of the manuscript. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081). We thank Drs Marc Allaire and Lin Yang from X9 at the National Synchrotron Light Source, Brookhaven National Laboratory for their support and discussions.

Funding Sources: The authors acknowledge financial support from NIDA (DA025995) (RL) and a grant from the Pennsylvania Department of Health using Tobacco Settlement Funds. Use of the National Synchrotron Light Source, Brookhaven National Laboratory was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-98CH10886.

ABBREVIATIONS

Ca²⁺	calcium
D2R	D2 dopamine receptor
NCS-1	neuronal calcium sensor-1
FA	fluorescence anisotropy
D2R-FL	fluorescein-labeled D2 dopamine receptor peptide
uD2R	unlabeled D2 dopamine receptor peptide

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Figure 1. FA Titration of NCS-1 TEV

Anisotropy of 1nM D2R-FL (▪) is plotted as a function of increasing NCS-1 concentration at fixed $[Ca^{2+}]$. The error bars are small enough to be completely covered by the individual sample points. The solid black line shows the NLLS fit of MODEL 4 to the data. This experiment was performed in triplicate. The D2F-FL peptide was also titrated with NCS-1 in the presence of 1 μ M uD2R (●) or 5 μ M (▲). The results of the fit of these data to the competition model are shown in as the green solid line for the titration in the presence of 1 μ M or red solid line 5 μ M uD2R.

Figure 2. CoIP of the NCS-1/NCS-1 dimers

Anti-HA antibody was used to immunoprecipitate HA tagged NCS-1 from crude membrane fractions of HEK-293 cells containing either no NCS-1-HA (HEK-293, lanes 1 and 2), NCS-1-HA (HA, lanes 3 and 4), NCS-1-mycHis (His, lanes 5 and 6), NCS-1 Δ EF-mycHis (Δ EF, lanes 7 and 8), NCS-1-HA + NCS-1-mycHis (HA + His, lanes 9 and 10), or NCS-1-HA + NCS-1 Δ EF-mycHis (HA + Δ EF, lanes 11 and 12). CoIPs were performed in the presence of 100 μ M CaCl_2 and 10 mM DTT. The blot was probed using an anti-NCS-1 antibody. (L) indicates lysate only samples, and (IP) indicates samples immunoprecipitated with anti-HA antibody.

Figure 3. SEC-MALS analysis of NCS-1 oligomeric state

Profiles of myristoylated 4 mg/mL NCS-1-His₆ in the (A) absence and (B) presence of calcium. Change in relative refractive index (right y axis -solid line) and MW (left y axis - open circles) are given as a function of runtime. The elution buffer for these experiments was 25 mM Hepes pH 7.5, 150 mM NaCl, 1mM TCEP and either 1 mM EGTA or 2 mM CaCl₂. Separations were conducted at a flow of 0.5 mL/min.

Figure 4. FA calcium titration assay

Anisotropy is plotted as a function of total Ca^{2+} molecules per NCS-1 molecules. Sample titrations contained 1 nM D2R-FL, and either 1 μM NCS-1 TEV (Q_{α}^1) or 5 μM NCS-1 TEV (Q_{α}^1). The solid and dashed black lines represent the fitted anisotropy as described in Supplemental 1.

A.

B.

Figure 5. $\text{Ca}^{2+}/\text{Mg}^{2+}$ NCS-1 titrations

(A): Anisotropy is plotted as a function of total Ca^{2+} molecules per NCS-1 molecule. Error bars are small enough to be completely covered by individual sample points. Sample titrations contained 1 nM D2R-FL, 5 μM NCS-1 TEV, and either a) 0 mM Mg^{2+} ($\circ$), b) 10 mM Mg^{2+} (∇) or c) 50 mM Mg^{2+} ($\bullet$).

(B): Titration of 1nM D2R-FL peptide with increasing NCS-1 and with 5 mM MgCl_2 , and 200 μM CaCl_2 ($\circ$, and solid line is best fit to the data) and 200 μM CaCl_2 ($\bullet$, and dashed line is best fit to the data)

Figure 6. Model of Putative NCS-1 dimer with D2R peptide

The molecular surface of the NCS-1 dimer is colored according to EF hand: EF-1 (residues 2-59) green, EF-2 (residues 60-95) red, EF-3 and EF-4 (residues 96-190) gray. The amino terminal residue is colored blue. The D2R peptide is shown as a yellow helix. This figure was produced using Chimera (45).

Table 1
Binding isotherms of all NCS-1 proteins

Abbreviations: Equilibrium dissociation constant for D2R-FL/NCS-1 monomer interaction (K_{D1}), equilibrium dissociation constant for D2R-FL/NCS-1 dimer interaction (K_{D2}), anisotropy of the unbound free D2R-FL (A_f), anisotropy of the NCS-1 monomer bound D2R-FL (A_m), anisotropy of the dimer bound D2R-FL (A_d), chi square value of the fluorescence anisotropy curve fit (χ^2). NB denotes no measurable binding.

Construct	K_{D1} (μ M)	K_{D2} (μ M)	A_f	A_m	A_d	χ^2
NCS-1TEV	0.27 $\pm$ 0.08	0.53 $\pm$ 0.09	0.045 $\pm$ 0.002	0.098 $\pm$ 0.007	0.236 $\pm$ 0.001	0.7
NCS-1-His ₆	0.48 $\pm$ 0.05	1.3 $\pm$ 0.1	0.042 $\pm$ 0.007	0.092 $\pm$ 0.009	0.234 $\pm$ 0.003	0.8
E84Q	9 $\pm$ 1	71 $\pm$ 5	0.047 $\pm$ 0.003	0.10 $\pm$ 0.01	0.28 $\pm$ 0.05	1.3
E120Q	1.0 $\pm$ 1	7 $\pm$ 1	0.046 $\pm$ 0.003	0.102 $\pm$ 0.007	0.232 $\pm$ 0.005	0.9
E168Q	0.64 $\pm$ 0.07	8 $\pm$ 1	0.051 $\pm$ 0.005	0.10 $\pm$ 0.01	0.222 $\pm$ 0.003	1.1
NCS-1 EF	NB	NB	NB	NB	NB	-
myrNCS-1	3.4 $\pm$ 0.2	5.9 $\pm$ 0.7	0.044 $\pm$ 0.002	0.096 $\pm$ 0.009	0.238 $\pm$ 0.005	0.7