CALCYON FORMS A NOVEL TERNARY COMPLEX WITH DOPAMINE D1 RECEPTOR THROUGH PSD-95 AND PLAYS A ROLE IN DOPAMINE RECEPTOR INTERNALIZATION

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Running Title: Calcyon/PSD-95/D1DR complex in dopamine receptor trafficking

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Key words: Calcyon; G protein-coupled receptors; D1 dopamine receptor; post-synaptic density-95; Phosphorylation
Background: Calcyon has been associated with various dopamine D1 receptor signalings despite no direct interaction between them. Result: Calcyon forms a novel ternary complex with D1DR through PSD-95. Conclusion: Calcyon, by forming a ternary complex, regulates D1DR internalization in a phosphorylation dependent manner

Significance: Regulation of dopaminergic signaling by calcyon/PSD-95/D1DR complex may represent a novel target for related neuropsychiatric disorders

SUMMARY

Calcyon, once known for interacting directly with the dopamine D1 receptor (D1,DR), is implicated in various neuropsychiatric disorders including schizophrenia, bipolar disorder and ADHD. Although its direct interaction with D1DR has been shown to be misinterpreted, it still plays important roles in the D1,DR signaling. Here, we found that calcyon interacts with the PSD-95 and subsequently forms a ternary complex with D1,DR through PSD-95. Calcyon is phosphorylated on Ser169 by the PKC activator PMA or by the D1DR agonist SKF81297, and its phosphorylation increases its association with PSD-95 and recruitment to the cell surface. Interestingly, the internalization of D1,DR at the cell surface was enhanced by PMA and SKF81297 in the presence of calcyon, but not in the presence of its S169A phospho-deficient mutant, suggesting that the phosphorylation of calcyon and the internalization of the surface D1,DR are tightly correlated. Our results suggest that calcyon regulates D1,DR trafficking by forming a ternary complex with D1,DR through PSD-95 and thus, possibly linking a glutamatergic and dopaminergic receptor signalings. This also raises the possibility that a novel ternary complex could represent a potential therapeutic target for the modulation of related neuropsychiatric disorders.

As a brain specific protein, calcyon is mainly localized in the intracellular endosomal vesicles of dendritic spines in D1,DR expressing pyramidal cells in the prefrontal cortex and hippocampus and dorsal striatum region (1,2). Initially, calcyon had been reported as a D1,DR-interacting protein (DRIP) but later studies revealed there was no direct interaction between calcyon and D1,DR (3). Recent studies, however, have introduced calcyon as a candidate gene for D1,DR-related neuropathological disorders. Calcyon levels were elevated in schizophrenia patients (4,5) while calcyon transgenic mice showed reduced anxiety and an impaired working memory (6,7). Calcyon was up-regulated in the rodent model of attention deficit hyperactivity disorder (8) and its gene variations are known to be associated with cocaine dependence (9). In addition, calcyon is known to potentiate crosstalk between Gαs-linked D1,DR and heterologous Gq,11-coupled receptors. When primed with agonists to Gq,11-coupled receptors, calcyon induces D1,DR to stimulate intracellular Ca²⁺ release (2,10). Therefore, all of the above results suggest a possible functional interaction between calcyon and D1,DR despite no direct interaction between them. Indeed, a recent study suggested that calcyon-containing vesicles might transport D1,DR by associating calcyon with D1,DR through their assembly to clathrin (11). However, as a single transmembrane protein, it is not clear how calcyon can regulate the internalization of D1,DR from the plasma membrane to endocytic vesicles.

PSD-95 is prominently expressed in post synaptic densities (PSDs) and is a prototypical scaffolding protein with multiple protein interaction domains: NH2-terminus, Discs large/zona occludens-1 (PDZ), SH3 (src homology 3), and guanylate kinase-like (GK) domains (12). It forms the backbone of the postsynaptic protein complex that organizes receptors and signal transduction molecules, enabling the functional effects of the receptors at PSDs. PSD-95 is known to regulate D1,DR signaling and the formation of the D1,DR associated protein-protein complex (13,14). The NH2-terminal of PSD-95 interacts with the carboxyl-terminal tail of D1,DR and facilitates constitutive D1,DR internalization as well as internalization of the NMDA receptor, which complexes with D1,DR thus linking dopamine signaling and glutamatergic signaling (15).

Here, we show that calcyon interacts with PSD-95. This interaction formed a ternary protein complex containing PSD-95/calcyon and D1,DR in the dendritic spines of hippocampal neurons. Furthermore, we found that calcyon was phosphorylated on the Ser169 residue through a PKC dependent pathway. Phosphorylation of calcyon strongly enhanced its interaction with PSD-95, increased its surface localization, and consequently induced the internalization of the surface D1,DR.

EXPERIMENTAL PROCEDURES

 Constructs To construct expression vectors for calcyon, rat full-length calcyon cDNA (accession number: AF303658) by PCR from rat brain cDNA library and inserted into target vectors (EGFP-C1, pCDNA3-HA and C-terminal FLAG tagged pFLAG-CMV (Sigma, St. Louis, MO). Serine phosphorylation site of calcyon (S169A) was generated by site-directed point mutations using QuickChange® Site-directed Mutagenesis Kit (Stratagene. Austin, TX). Full-length rat PSD-95 (accession number: P31016) was amplified by PCR and subcloned into pCDNA3-HA and EGFP-N1 vectors (Invitrogen, Carlsbad, CA). The HA-D,DR was kindly provided by Dr. Wei-Dong Yao (Harvard University), and all DNA constructs were
verified by DNA sequencing. These expression vectors were transfected into HEK293T, SH-SY5Y cells and neuron for western blot and imaging analysis.

**Coinmunoprecipitation and immunoblotting** To verify the interaction of calcyon with PSD-95, adult rat brain was homogenized with a modified RIPA buffer (50 mM Tris–HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM leupeptin, 1.5 mM pepstatin, and 1 mM aprotenin) (16) and incubated with anti-calcyon antibody for immunoprecipitation (Santa Cruz Biotechnology, Santa Cruz, CA) and for immunoblotting (Abcam, Cambridge, MA). Mouse anti-PSD-95 monoclonal antibody and rabbit anti-PSD-95 polyclonal antibody were from NeuroMab (Davis, CA) and Synaptic Biosciences (Göttingen, Germany), respectively. HEK293T cells were cotransfected with FLAG-calcyon, PSD-95-EGFP and HA-D1DR and the cells were washed twice with cold PBS and extracted at 4°C with a modified RIPA buffer. The mixtures were then incubated with 30 µl of protein A-sepharose (50% slurry) for 1 h, pelleted by centrifugation, and analyzed by SDS–PAGE (8–15% gels). Proteins on gels were transferred to PVDF membranes (Pall Life Sciences, Ann Arbor, MI) and the membranes were incubated with anti-calcyon (1:800), anti-D1DR (1:1,000), or anti-PSD-95 (1:2,000) primary antibodies for 1 hour at room temperature or overnight at 4°C. Immunoblot was performed with anti-HA antibody (1:1000; Covance, Princeton, NJ), anti-FLAG (1:1,000; Sigma) or anti-GFP (1:3,000; GeneTex, Irvine, CA). The immunoreactions were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). The mixtures were then incubated with 30 µl of protein A-sepharose (50% slurry) for 1 h, pelleted by centrifugation, and analyzed by SDS–PAGE (8–15% gels). Proteins on gels were transferred to PVDF membranes (Pall Life Sciences, Ann Arbor, MI) and the membranes were incubated with anti-calcyon (1:800), anti-D1DR (1:1,000), or anti-PSD-95 (1:2,000) primary antibodies for 1 hour at room temperature or overnight at 4°C. Immunoblot was performed with anti-HA antibody (1:1000; Covance, Princeton, NJ), anti-FLAG (1:1,000; Sigma) or anti-GFP (1:3,000; GeneTex, Irvine, CA). The immunoreactions were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). After chemiluminescence detection using ImageQuant LAS 4000 (GE Healthcare Bio-Science, Uppsala, Sweden), images were analyzed using Image J software.

To identify the endogenous ternary complex of calcyon/PSD-95/D1DR, cultured hippocampal neuron lysates (DIV 16) was prepared after treatment of SKF81297 (10 µM) for 15 min and harvested with modified RIPA buffer. They were then clarified by centrifugation at 15,000 × g for 30 minutes. Protein concentrations were measured using a BCA assay (Pierce, Rockford, IL). The 800–1500 µg of lysates were incubated with 20 µl Protein A-Sepharose 4 Fast Flow (GE Healthcare Bio-Science) for 1 h at 4°C to remove nonspecific proteins and re-incubated with anti-calcyon, anti-PSD-95 (Synaptic Systems) or anti-D1DR (Santa Cruz biotechnology) antibodies overnight at 4°C. To test the roles of phosphorylation of calcyon, EGFP-calcyon transfected HEK293T cells and FLAG-calcyon/PSD-95-EGFP/HA-D1DR cotransfected HEK293T cells were incubated with 1 µM PMA (Sigma) and 10 µM SKF81297 (Santa Cruz biotechnology), respectively. Cell lysates were incubated with anti-GFP (GenTex) or anti-FLAG (Sigma) antibody, and immunoblotted with anti-phosphoserine antibody (Acris Antibodies GmbH, Herford, Germany).

**GST pull-down assays** Full-length rat PSD-95 (1-724), N-terminus (1-64 amino acid), PDZ1-3 (65-393 amino acid), PDZ1 (65-151 amino acid), PDZ2 (160-246 amino acid), PDZ3 (313-393 amino acid), ASH3 (lacking amino acid 428-498) and SH3 (428-498 amino acid) of PSD-95, full-length rat calcyon (1-226 amino acid), Extracellular (1-88 amino acid), C-terminus (109-226 amino acid), C-End (176-226 amino acid) of calcyon were amplified by PCR and subcloned into pGEX4T-1 for the GST pull-down assays. PCR products of calcyon (175-200 and 201-226) were subcloned into pDESTc15 vector (Invitrogen) using the Gateway Cloning System (Invitrogen). The plasmids were transformed into BL-21, and the transformants were cultured in 2x-YT medium supplemented with ampicillin. After 5 hours induction with 0.5 mM isopropyl-1-thio-ß-D-galactopyranoside at 30 °C, the cultures were sonicated in lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 0.1 mM PMSF) and centrifuged at 15,000 × g for 15 min, and the supernatants were incubated with Glutathione-Agarose-4B beads (GE Healthcare Bio-Science) for 1 h at 4 °C. After washing three times with lysis buffer, the beads were incubated for 2 h at 4 °C or overnight with brain lysates in lysis buffer. The beads were then washed extensively with lysis buffer and analyzed by SDS-PAGE and immunoblotting.

**In vitro phosphorylation Assay** The 156-206 a.a domain of calcyon (P-WT) and S169A mutant (P-SA) were cloned into pDEST15 vector using the Gateway Cloning System and transformed to BL-21. The E. coli was sonicated with lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris, 150 Mm NaCl, 1 mM MgCl2, 1 mM EGTA, PH 7.2, 0.1 mM PMSF). The phosphorylation of wild type (P-WT) and phospho-mutant S169A (P-SA) were purified using Glutathione Sepharose 4B resin (GE Healthcare Bio-Science). For the *in vitro* phosphorylation assay, 25 µg of purified GST-P-WT and GST-P-SA were incubated with 20 ng of PKC-catalytic subunit (Millipore, Billerica, MA) in PKC assay dilution buffer II with 2 mM Mg2+-ATP containing Phosphatase inhibitor cocktail III (Sigma) at 30 °C for 40 min. The reaction was stopped by adding 10 mM EDTA and centrifuged 1000 × g for 1 min, and the supernatant was removed, added 2x SDS-Laemmli buffer without washing followed by SDS-PAGE. P-WT phosphorylation was detected by immunoblotting with phosphoserine antibody (Acris Antibodies GmbH). 

**cAMP enzyme immunoassay** HEK293T cells were transiently transfected with FLAG-calcyon, PSD-95-EGFP and HA-D1DR using
Lipofectamine 2000 (Invitrogen) and grown for 27 h in complete DMEM medium and then serum-starved for 16 h before SKF81297 treatment. The cells were stimulated by 10 μM SKF81297 for 30 min and cAMP accumulation was measured with a cAMP direct immunoassay kit (Abcam). In brief, DMEM was removed from the plate and cells were washed with warmed 1x DPBS briefly and incubated with 0.1 M HCl at room temperature for 20 min. Cells were dissociated by pipetting up and down and neutralizing buffer and acetylation reagent mixture were added to the cell lysates (≈1 μg/μl concentration) and reaction mixtures were incubated at room temperature for 10 min. Each acetylated samples and standards were transferred to a Protein G coated white 96-well plate. Rabbit anti-cAMP polyclonal antibody was added to standard and samples and reaction mixtures were incubated for 1 hr at room temperature, re-incubated with CAMP-HRP for 1 hr and washed, developed with the HRP developer for 1 hr. Stop the reaction by adding 1M HCl and read the plate at OD 450 nm.

**Cell culture, immunocytochemistry and image acquisition**
HEK293T and SH-SY5Y cells were transfected with various BiFC pairs vectors. After sequence verification, SH-SY5Y cells were transfected with various BiFC pairs with a polished half-bore Pasteur pipette. The transfection was carried out using Lipofectamine 2000 (Invitrogen), and cells were observed after 24-36 hours. The confocal images were acquired with an Olympus FV 1000 using a sequential scan tool mounted on an Olympus IX-81 microscope fitted with a 100X/1.4 NA objective lens driven by Olympus Fluoview software. For immunocytochemistry, cells were fixed in 4% formaldehyde with 4% sucrose in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (Hyclone, Logan, UT).

**Immunocytochemistry**
Cortical cultures were permeabilized with 0.25% Triton X-100, PBS and blocked for 30 minutes in 10% BSA, PBS at 37°C. The cells were incubated with primary antibodies in 3% BSA, PBS for 2 h at 37°C or overnight at 4°C, washed in PBS, and incubated with secondary antibodies in 3% BSA, PBS for 45 minutes at 37°C. For colocalization experiment of calcyon, PSD-95 and D1DR primary cultured neurons were permeabized with 0.25% Triton X-100 for 5 min at RT before primary antibodies. Analysis and quantification of data were performed with MetaMorph software (Molecular Device, Sunnyvale, CA) and SigmaPlot 8.0 (Systat Software, Point Richmond, CA), and data are presented as means ± SEM.

**RESULTS AND DISCUSSION**
A previous study showed that calcyon localizes to the dendritic spines of D1 receptor-expressing pyramidal cells in the prefrontal cortex (2). PSD-95 is a prominent scaffolding protein with multiple protein interaction domains in the dendritic spine (19,20). Since the subcellular fractionation data in this study showed that calcyon and PSD-95 protein existed in the postsynaptic density (PSD) and had similar expression patterns during the development of rat hippocampal neurons...
(Supplemental Fig.1), we wondered whether calcyon interacts with PSD-95.

To test this possibility, an immunoprecipitation (IP) analysis was performed with adult rat whole brain lysates. Fig. 1 shows that calcyon and PSD-95 co-precipitated, suggesting they interact with each other endogenously (Fig. 1A). When HEK293T cells were cotransfected with FLAG-calcyon, EGFP-C empty vector, clathrin light chain (Lca)-EGFP, and PSD-95-EGFP, calcyon coprecipitated with Lca (lane 2) and PSD-95 (lane 3) but not with the EGFP empty vector (Fig. 1B). This interaction was further confirmed by bimolecular fluorescence complementation (BiFC) assay using the improved yellow fluorescence protein, Venus, which allows for direct visualization of protein interactions at the subcellular sites of their interactions in living cells (21-23). Fluorescence complementation was only observed in a group of cells that coexpressed fragments of Venus (VN173 or VC155) conjugated to the C-terminus of calcyon and to the N-terminus of PSD-95 (Fig. 1C). No complementation was detected when fragments of Venus (VN173 or VC155) was fused to the N-terminus of calcyon or C-terminus of PSD-95 (Supplemental Fig. 2A).

We further investigated which domain of PSD-95 binds to calcyon. A series of GST pull-down assays were done with various domain mutants of PSD-95 and calcyon (Fig. 2A, B). Calcyon was found to interact with the full-length PSD-95, the PDZ1-3 domain, and the PDZ1 domain, but not with the N-terminal region, PDZ2, PDZ3, or SH3 domain (Fig. 2C). In addition, the full-length and PDZ1 domain of PSD-95 interacted with the C-terminus (a.a. 109-226) and C-terminal end (a.a. 175-226) of calcyon, respectively and performed GST-pull down assay. We found that a.a. 201-226 of calcyon strongly bound to PSD-95 whereas a.a.175-200 did not. This was further supported by the BiFC results in which a group of cells that coexpressed fragments of PSD-95-VN173 or VN173-PSD-95 with the calcyon-VC155 (a.a. 201-226) only showed fluorescent complementation (Fig. 2E and Supplemental Fig. 2B). Because both C-terminal fragments contain AXXV motif, these results indicate that AXXV motif doesn’t seem to be involved in PSD-95 binding. To test whether last four amino acid sequence (QSPK) of calcyon is the PSD-95 binding motif, we have introduced S224A point mutation in the Ser residue of the sequence since Ser/Thr at -2 position is the most conserved residue among various PDZ binding motifs (21). We found that S224A mutant failed to bind PSD-95 while the wildtype strongly binds to PSD-95 (Fig. 2F).

Interaction of calcyon with PSD-95 was further confirmed by immunocytochemistry. Neurons were doubly stained with calcyon and PSD-95 antibodies. Although calcyon was found to be present throughout the cytosol, it partially colocalized with PSD-95 in the dendritic spines (Supplemental Fig. 3A). When exogenously expressed, calcyon also was found in the punctate structures, which were partially colocalized with the endosomal marker, EEA1 in the dendritic spines (Supplemental Fig. 3B). Consistent with endogenous staining, exogenously expressed calcyon also exhibited partial colocalization with PSD-95 (Supplemental Fig. 3E).

The N-terminus of PSD-95 is known to interact with the C-terminus of D1DR (13). Although a direct interaction between calcyon and D1DR turned out to be misinterpreted since our results indicated that calcyon and PSD-95 associates with each other, there is a possibility that calcyon and D1DR interact indirectly through PSD-95 as a mediator. To test this possibility, we first incubated purified GST-calcyon or GST-only with HEK293T cell lysates that expressed HA-D1DR and PSD-95-EGFP and immunoprecipitated with GST antibody followed by immunoblotting with HA-antibody. We found that HA-D1DR was coimmunoprecipitated with GST-calcyon (Fig. 3A). Second, HEK293T cells were transfected with various combinations of HA-D, DR, PSD-95-GFP, and FLAG-calcyon, and coimmunoprecipitation followed by immunoblotting with specific antibodies was carried out. PSD-95-GFP was coimmunoprecipitated with either FLAG-calcyon or HA-D, DR when doubly transfected. Interestingly, HA-D, DR and FLAG-calcyon only coprecipitated if PSD-95-GFP was triply co-transfected, strongly suggesting that PSD-95 acts as a linker between calcyon and D1DR (Fig. 3B). Finally, we confirmed that a calcyon/PSD-95/D1DR ternary complex exists endogenously in the brain by immunoprecipitation assay in cultured neurons after treatment with a D1DR specific agonist, SKF81297 for 15 min. Immunoblot analysis with antibodies against each protein showed that calcyon, PSD-95 and D1DR coprecipitated together (Fig. 3C). Immunocytochemistry also showed that the three proteins are colocalized in the dendritic spines, especially the spine heads (Fig. 3D and Supplemental Fig. 5).
The D1-like receptors, activate cyclic AMP production, bidirectionally modulates protein kinase A (PKA) and DARPP-32 through G_{olf} (24). Intriguingly, D1DR-stimulated Ca^{2+} release was significantly attenuated by treatment with a PKC inhibitor in calcyon expressing cells, and calcyon was found to be phosphorylated by purified PKC although no detailed analysis has been done (2). We also found that calcyon was phosphorylated on the serine residue by the PKC activator PMA, and this phosphorylation was blocked by the PKC inhibitors GF109203X and Rottlerin but not by the PKA inhibitor H-89 (Fig. 4A). We further investigated the specific phosphorylation sites of calcyon by PKC. Human calcyon is phosphorylated on Ser154 and Ser196 by PKA (2), whereas the rat calcyon has a putative PKC phosphorylation site on Ser169 in the cytoplasmic domain (NetPhos K in ExPASy Bioinformatics Resource Portal). HEK293T cells were transfected with an EGFP-tagged wild-type calcyon or S169A phospho-deficient mutant and incubated with PMA. Fig. 4B shows that the wild type, but not the S169A mutant, was rapidly phosphorylated on the serine residues by PMA, suggesting that Ser169 is the major phosphorylation site of calcyon by PKC. This was further confirmed by an in vitro phosphorylation assay (Fig. 4C). We purified a.a 156-187 from wild type (P-WT) and the phospho-deficient mutant S169A (P-SA) of calcyon and incubated them with the purified PKC catalytic subunit in the presence of ATP. Phosphorylation on the serine residues was observed in the P-WT but not in the P-SA, suggesting that calcyon is directly phosphorylated by PKC on Ser 169 (Fig. 4C).

We next tested whether the phosphorylation of calcyon modulates its interaction with PSD-95. HEK293T cells were transfected with FLAG-calcyon and incubated with 1 μM of PMA for 10 min. Then, the lysates were precipitated with purified GST-PSD-95 beads and immunoblotted with FLAG-antibodies. Fig. 4D shows that the interaction between calcyon and PSD-95 was dramatically increased by treatment with PMA. Interestingly, calcyon was also phosphorylated on the serine residues after SKF81297 treatment, whereas the S169A mutant was not (Fig. 4E). Direct activation of PKC by PMA resulted in a maximum increase of calcyon phosphorylation only 5 min after treatment (Fig. 4B), whereas SKF81297 treatment induced maximal calcyon phosphorylation 15 min after treatment (Fig. 4E), suggesting that D1,DR activation by SKF81297 indirectly causes PKC activation during D1,DR signal transduction. Consistently, SKF 81297 treatment significantly increased the interaction of PSD-95 with calcyon but not with the S169A mutant (Fig. 4F).

PSD-95 interacts with D1,DR, promoting the internalization of the surface D1,DR (13). Thus, we next investigated whether calcyon could regulate the internalization of the surface D1,DR through the ternary complex with PSD-95. HEK293T cells were triply co-transfected with HA-D1,DR, PSD-95-EGFP, and the FLAG-calcyon or FLAG-S169A mutant. The surface biotinylation assay showed that the surface level of D1,DR rapidly decreased after treatment with SKF 81297 in the presence of calcyon. Compared to D1,DR only or D1,DR with PSD-95 group, when calcyon was cotransfected with D1,DR and PSD-95, the slope of the decrease was much steeper (Fig. 5A). The phospho-deficient S169A mutant of calcyon failed to induce D1,DR internalization after treatment with SKF81297 (Fig. 5A).

To further test whether the decrease in the surface level of D1,DR depends on the phosphorylation of calcyon, triply co-transfected HEK293T cells were incubated with 10 μM of SKF 81297, 1 μM of PMA, or 5 μM of GF109203X and the surface levels of D1,DR were measured. Fig. 5B shows that both PMA and SKF81297 treatments induced significant decreases in the surface level of D1,DR while GF109203X treatment did not. Evidently, the S169A mutant did not induce any significant changes in the surface levels of D1,DR after treatment with PMA, SKF81297, or GF109203X (Fig. 5B). To test whether the formation of the ternary complex has effect on D1,DR-mediated signaling, HEK293T cells were transfected with various combinations of constructs, and after incubation with 10 μM of SKF 81297 for 30 min, the levels of cAMP, a downstream effector of D1,DR-mediated signaling pathway, were measured (Fig. 5C). We found that in the presence of calcyon, PSD-95 and D1,DR, SKF81297-stimulated cAMP production was significantly reduced compared to D1,DR only or PSD-95 with D1,DR. The phospho-deficient mutant of calcyon (S169A) failed to affect cAMP production. Since the interaction between calcyon and PSD-95 increased by the phosphorylation of calcyon, our results suggest that D1,DR activation by SKF81297 induces the PKC-mediated phosphorylation of calcyon, which promotes the formation of a ternary complex between calcyon and D1,DR through PSD-95 and consequently, induces the internalization of D1,DR, thus inhibiting D1,DR signaling.

Previously, calcyon and D1,DR were thought to interact with each other, but later on, it was discovered there was no direct interaction between them (8). Growing evidence, however, indicates that calcyon has important roles in D1,DR-mediated signaling pathways as well as in various D1,DR-related neuropsychiatric disorders (4,5,25-27). Therefore, it was widely assumed that calcyon could interact with D1,DR either functionally or indirectly. D1,DR is regulated by the glutamatergic scaffolding protein PSD-95 through direct interaction, which facilitates the constitutive internalization of D1,DR as well as the internalization of the NMDA receptor that complexes with D1,DR (15).
In addition, D1DR interacts with NMDA receptor and balances NMDA receptor-mediated responses through both a PKA-dependent pathway and a Ca2+-dependent mechanism (28). Furthermore, recent studies revealed that calcyon is required for the NMDA activity-dependent internalization of AMPAR, which leads to long-term depression (29). All of these results imply there could be many possible interactions involving D1DR, calcyon, and D1DR-specific agonist SKF81297 also increased evidence has not been provided (2). We found phosphorylated by PKC although direct signaling pathways downstream to these proteins may compete with other proteins for binding to PSD-95, which subsequently affects the spines would indirectly inhibit D1DR-mediated signaling by “physically” reducing the surface levels of D1DR and thus may provide a novel target for D1DR related neuropsychiatric diseases such as schizophrenia and ADHD. Whether the phosphorylation dependent formation of a ternary complex among D1DR/PSD-95/calcyon directly affects D1DR-mediated signaling and plays a role in linking dopaminergic and glutamatergic signaling in the brain would be of great interest but certainly requires further investigation.

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FIGURE LEGENDS

**Figure. 1** Calcyon associates with PSD-95. (A) Adult rat brain extracts were immunoprecipitated with mouse monoclonal anti-PSD-95 antibody (left panel) or rabbit polyclonal anti-calcyon antibody (right panel) followed by immunoblot analysis with rabbit polyclonal anti-calcyon (left panel) or mouse monoclonal anti-PSD-95 antibody (right panel). Arrows indicate the size of calcyon (left panel) and PSD-95 (right panel) NS; nonspecific band. HC; heavy chain. Sup; Immunoprecipitated supernatants. (B) HEK293T cells were cotransfected with FLAG-calcyon and EGFP-C1, PSD-95-EGFP and Lca (clathrin light chain)-EGFP. Cell lysates were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti-GFP antibody. Lower panel: the expression of each protein was detected by anti-GFP or anti-FLAG antibody. (C) Schematic figures showing the successful combination of BiFC between calcyon and PSD-95. Lower panels: SH-SY5Y cells were co-transfected with various combinations of BiFC pairs. Note that except for the calcyon-VC155/VN173-PSD-95 pair and calcyon-VN173/VC155-PSD-95 pair, all other combinations failed to exhibit a positive fluorescence complementation. 5-HT6R/VN: 5-HT6 receptor conjugated to VN173. Cal/VC (a.a. 175-200): calcyon c-terminus region (a.a.175-200) conjugated to VC155. Scale bar = 20 μm; 10 μm in inset.

**Figure. 2** Calcyon interacts with PSD-95. (A) Schematic figures showing PSD-95 domain structures and each GST fused domain of PSD-95. (B) Schematics of calcyon domain and full-length calcyon (Cal) and extracellular region (Extra), C-terminus (C-term) and the 175-226 amino acid part of the C-terminus (End) of calcyon. (C) Brain lysates were pulled down with GST fusions of PSD-95-full length; NT, N-
Brain lysates were pulled down with GST-wild type (QSPK) or GST-S224A point mutant (QAPK). Note fusion proteins were identified using Coomassie blue staining (lower panel). Arrowhead indicates GST-calcyon and asterisks indicate GST-fusion proteins. (E) GST-End of calcyon was divided into two domains (a.a. 175-200 and a.a.201-226) in which each domain contains one putative PDZ binding motif (See Supplemental Fig. 2C). Brain lysates were pulled down with each GST-fusion protein and immunoblotted with anti-PDZ-95 antibody. Note that strong immunoreactivity of PSD-95 was identified in the 201-226 lane only. Purified GST-fusion proteins were identified using Coomassie blue staining (right panel). (F) Brain lysates were pulled down with GST-wild type (QSPK) or GST-S224A point mutant (QAPK). Note that S224A mutant failed to bind PSD-95 while the wild-type strongly binds to PSD-95. Purified GST-fusion proteins were identified using Coomassie blue staining (right panel).

Figure. 3 Calcyon interacts indirectly with D1DR via PSD-95. (A) HEK293T cells were cotransfected with EGFP tagged PSD-95 and HA-tagged D1,DR, lysed, immunoprecipitated with GST or GST-calcyon, and immunoblotted with anti-HA antibody. Arrowhead indicates HA-tagged D1,DR (left panel) and purified GST-calcyon wild type (Right panel). Purified GST-calcyon was identified using Coomassie blue staining (right panel). (B) HEK293T cells were cotransfected with EGFP tagged PSD-95, HA-tagged D1,DR and FLAG tagged calcyon. Cells were lysed, immunoprecipitated, and immunoblotted with various combinations of indicated antibodies. Note that only when three proteins were co-transfected, calcyon was co-immunoprecipitated with D1,DR. (Left panel). The model depicting a putative molecular interaction of calcyon/PSD-95/ D1,DR (Right panel). (C) Endogenous complex of calcyon/PSD-95/ D1,DR in the cultured neurons. The cultured primary hippocampal neurons (DIV 21) were incubated with 10 μM SKF81297 for 15 min, lysed in RIPA buffer, immunoprecipitated, and immunoblotted with rabbit polyclonal anti-D1,DR, calcyon, or –PSD-95 antibody. 10% neuron lysates (70 μg) was loaded as the positive control. (D) D1,DR, PSD-95, and calcyon were colocalized in the dendritic spines and dendritic shaft. Since the antibody species matter, GPP-tagged calcyon was transfected while PSD-95 and D1,DR were detected endogenously with mouse anti-PSD-95 antibody (secondary antibody Alexa405 conjugated anti-mouse antibody) and rabbit anti-D1,DR antibody (secondary antibody Alexa594 conjugated anti-rabbit antibody). Arrows indicate the spines where the D1,DR/PSD-95/calcyon complex was colocalized. Scale bar = 50 μm; scale bar = 10 μm in inset.

Figure. 4 Phosphorylation of calcyon by PKC enhances its interaction with PSD-95. (A) The phosphorylation of calcyon by PKC. HEK293T cells were transfected with EGFP-calcyon and preincubated with DMSO and 20 μM H-89 (PKA inhibitor) for 20 min or two different PKC inhibitors, 5 μM GFX (GF109203X) for 45min and 30 μM Rot (Rottlerin) for 30 min. After preincubation, cells were treated with 1 μM PMA, a PKC activator, for 10 min, harvested and coimmunoprecipitated with anti-EGFP antibody followed by immunoblotting with anti-phospho-serine antibody. Lower bar graphs: Quantification from 3 independent experiments. Data are the mean ± S.E. Asterisks indicate a significant change compared to the group(s) indicated (one-way ANOVA; * p<0.05, ** p<0.01). (B) HEK293T cells expressing EGFP-calcyon or phospho-deficient mutant EGFP-calcyon (S169A) were incubated with PMA for the indicated time, coimmunoprecipitated with anti-GFP antibody followed by immunoblotting with phospho-serine antibody. (C) Purified GST tagged wild type (P-WT) and phospho-deficient mutant S169A (P-SA) of calcyon were incubated with the PKC catalytic subunit in the presence of ATP. The phosphorylation was analyzed by SDS-PAGE and immunoblotting with phospho-serine antibody (left panel). Arrowheads indicate the PKC catalytic subunit and phosphorylated serine. Purified GST-fusion proteins were identified using Coomassie blue staining (right panel). (D) HEK293T cells expressing FLAG-calcyon were incubated with 1 μM PMA for 10 min and lysates were coprecipitated with GST-PSD-95 beads and immunoblotted with anti-FLAG antibody. The amount of FLAG-calcyon pulled down with GST-PSD-95 increased more than 3-fold after treatment with PMA for 10 min. Statistical analysis was performed using Student’s t-test. ** p<0.01. (E) HA-D1,DR/PSD-95-EGFP and FLAG-calcyon or FLAG-calcyon (S169A) were cotransfected into HEK293T cells. After treatment with SKF81297 for the indicated times, cells were lysed, immunoprecipitated with anti-FLAG antibody followed by immunoblotting with phospho-serine antibody. Note that in the absence of HA-D1,DR expression, SKF81297 treatment failed to induce the phosphorylation of calcyon. Lower graph: Quantification from 3 independent experiments. Asterisks indicate significant changes in the second group compared to the first and the third group at matching time points. ANOVA and Tukey’s HSD post hoc test; ** p<0.01. (F) HA-D1,DR/PSD-95-EGFP and FLAG-calcyon or FLAG-calcyon (S169A) were cotransfected into HEK293T cells. After treatment of SKF81297 for the indicated times, cells were lysed and immunoprecipitated with anti-GFP antibody followed by immunoblotting with anti-FLAG antibody.
Phosphorylation of calcyon by SKF81297 increased its interaction with PSD-95. Asterisk indicates significant changes in the second group compared to the first group at matching time points. ANOVA and Tukey's HSD post hoc test; * p < 0.05.

**Figure 5.** The phosphorylation of calcyon and the internalization of the surface D₁DR are tightly correlated. (A) HEK293T cells were transfected with various combinations of constructs as indicated. Cells were then exposed to 10 μM SKF 81297 for the indicated times and surface biotinylation experiments were performed as described in the Methods. The surface levels of D₁DR were gradually decreased after SKF 81297 treatments in cells co-expressing D₁DR and PSD-95 or D₁DR, PSD-95 and calcyon although when the three proteins were co-expressed, the rate of decrease was much faster. The phospho-deficient mutant of calcyon (S169A) failed to induce D₁DR internalization after treatment with SKF81297. Lower graphs: Quantification from 5 independent experiments (left graph). The surface intensity values of D₁DR at the indicated times were normalized to the that at time zero to emphasize the rate of internalization of surface D₁DR after SKF81297 treatment (right graph). Asterisks indicate significant changes in each group compared to the HA- D₁DR group at matching time points. ANOVA and Tukey's HSD post hoc test; * p<0.05.** p<0.01. (B) HEK293T cells expressing the FLAG-calcyon or –calcyon (S169A) with HA-D₁DR/PSD-95-EGFP were incubated with 10 μM SKF 81297 or 1 μM PMA for 30 min in the presence or absence of 5 μM GFX (GF109203X). Surface biotinylation experiments were performed, and surface as well as total levels of D₁DRs were measured by immunoblotting with anti-HA antibody. The surface biotinylated proteins intensity was normalized to the total protein intensity. Quantification from 3 independent experiments (lower graph). Asterisks indicate significant changes in PMA or SKF 81297 treated group compared to the DMSO treated group or GFX treated group. Data represent the mean ± SE. ANOVA and Tukey's HSD post hoc test; * p<0.05.** p<0.01. (C) HEK293T cells were transfected with same constructs as A. Cells were then exposed to 10 μM SKF 81297 for 30 min and cAMP direct immunoassay were performed as described in the Methods. The SKF81297 induced-cAMP accumulation levels were significantly diminished in cells co-expressing D₁DR, PSD-95 and calcyon compared to D₁DR or D₁DR with PSD-95. The data are means ± SEM from at least three independent experiments. Asterisks indicate significant changes between linked groups.* p<0.05, ANOVA and Tukey's HSD post hoc test.
Figure 1.

A

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<tr>
<th>Brain lysates</th>
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<tr>
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IB: FLAG

IB: GFP

IB: GFP

IP: PSD-95

IP: Cal

IP: Lc-EGFP

IP: EGFP

IB: GFP

IB: Cal

IB: FLAG

B

IB: GFP

IB: FLAG

IB: GFP

IP: PSD-95

IP: Cal

IP: Lc-EGFP

IP: EGFP

IB: GFP

IB: Cal

IB: FLAG

C

Cal/VC + VN/PSD-95

Cal/VC + VN/PSD-95

Cal/VC + 5-HT1R/VN

Cal/VC + 5-HT1R/VN

Cal/VC(175-200) + PSD-95/VN

Cal/VC(175-200) + PSD-95/VN
Figure 3.

A

GST-GST-Cal

HA-D, DR+ PSD-95-EGFP

IP: GST GST-Cal

IB: KDa

Staining

B

HA D, DR:
PSD95-GFP:
FLAG-Cal:

IP: HA
IB: FLAG
IP: GFP
IB: HA

IB: KDa

Cultured Neuron

C

Input (10%) D, DR Cal PSD-95:

IB

D

EGFP-Cal

PSD-95

D1DR

Merge

Staining

Scale bars
Figure 4.

A.  

B.  

C.  

D.  

E.  

F.
Figure 5.

A

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B

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<td>-</td>
</tr>
<tr>
<td>SKF</td>
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C

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