Calcyon, a Novel Partner of Clathrin Light Chain, Stimulates Clathrin-mediated Endocytosis

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In the central nervous system, clathrin-mediated endocytosis (CME) is crucial for efficient synaptic transmission. Clathrin-coated vesicle assembly and disassembly is regulated by some 30 adaptor and accessory proteins, most of which interact with clathrin heavy chain. Using the calcyon cytosolic domain as bait, we isolated clathrin light chain in a yeast two-hybrid screen. The interaction domain was mapped to the heavy chain binding domain and C-terminal regions of light chain. Further, the addition of the calcyon C terminus stimulated clathrin self-assembly in a dose-dependent fashion. Calcyon, which is a single transmembrane protein predominantly expressed in brain, localized to vesicular compartments within pre- and postsynaptic structures. There was a high degree of overlap in the distribution of LC and clathrin in neuronal dendrites, spines, and cell bodies. Co-immunoprecipitation studies further suggested an association of calcyon with the clathrin-mediated endocytic machinery. Compared with controls, HEK293 cells overexpressing an association of calcyon with the clathrin-mediated endocytic machinery was mapped to the heavy chain binding domain and C-terminal regions of light chain. Further, the addition of the calcyon C terminus stimulated clathrin self-assembly in a dose-dependent fashion. Calcyon, which is a single transmembrane protein predominantly expressed in brain, localized to vesicular compartments within pre- and postsynaptic structures. There was a high degree of overlap in the distribution of LC and clathrin in neuronal dendrites, spines, and cell bodies. Co-immunoprecipitation studies further suggested an association of calcyon with the clathrin-mediated endocytic machinery. Compared with controls, HEK293 cells overexpressing an association of calcyon with the clathrin-mediated endocytic machinery was mapped to the heavy chain binding domain and C-terminal regions of light chain. Further, the addition of the calcyon C terminus stimulated clathrin self-assembly in a dose-dependent fashion.

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**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—The predicted cytoplasmic domain of human calcyon (residues 123–217) was inserted in the yeast two-hybrid bait plasmid, pGBK, in-frame with the GAL4 binding domain, and used to screen a human brain cDNA library (~3.9 × 10^6 independent clones) made in the prey vector, pACT2. Bait and prey plasmids were transformed into the yeast strain AH109, and transformants were screened on quadruple dropout medium lacking adenine, histidine, tryptophan, and leucine and containing X-a-gal (Clontech). GAL4 binding domain fusions to α2-laminin and caveolin-1 were used as unrelated baits to screen for false positives.

**DNA Constructs**—Plasmids used in this study include pEGFP-C2 (Clontech), pCMV-Tag2C, or Tag3C (Strategene) vectors expressing, respectively, N-terminal EGFP, FLAG, or Myc epitope-tagged cDNA of human calcyon and light chain a (GenBank™ accession number AF225903 and NM_007096, respectively). For pulldown assays, segments of the calcyon or clathrin light chain a (LCa) open reading frame were PCR-amplified using primers containing restriction enzyme sites for subcloning into the multiple cloning site of PET30a (Novagen) and pGEX4T-3 (Amersham Biosciences), respectively. The DNA sequence of each construct was determined by an Applied Biosystems automated DNA sequencer (model 377XL) by the Molecular Core facility at the Medical College of Georgia.

**Clathrin Purification and Assembly Assays**—CCVs were purified from 30 rat brains exactly as described in Ref. 15. The clathrin coats were subsequently stripped from the vesicles, and purified clathrins

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2 The abbreviations used are: CME, clathrin-mediated endocytosis; Tfn, transferrin; TfnR, transferrin receptor; EGFP, enhanced green fluorescent protein; CCV, clathrin-coated vesicle; HC, heavy chain; LC, light chain; MES, 4-morpholineethanesulfonic acid; GST, glutathione S-transferase; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; TMB, tetramethylbenzidine; LCa, light chain a; TGN, trans-Golgi network; EM, electron microscopy; ANOVA, analysis of variance.

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were isolated by centrifugation in a 5–20% sucrose gradient exactly as described in Ref. 12. Fractions of 1 ml were collected and analyzed by Coomassie Blue staining and immunoblotting with clathrin HC (1:2000; BD Biosciences) or LC (1:200; Covance) antibodies. Purified clathrin was dialyzed overnight in clathrin assembly buffer (10 mM Tris-HCl, pH 8.5) for self-assembly assays. Assembly assays were performed with purified clathrin and different concentrations of S-calcyon-C and S-calcyon-N fusion proteins as determined by the S-Tag rapid assay kit (Novagen) in a final volume of 90 µl of clathrin assembly buffer. Assembly was initiated at 4 °C by the addition of 10 ml of 1 M MES, pH 6.7. The mixture was incubated on ice for 45 min. and centrifuged at 400,000 × g for 6 min. Supernatants or pellets were separated on SDS-polyacrylamide gels, and HC was detected by staining gels with Coomassie Blue.

Relative levels of clathrin assembly in wild type and Cal−/− brains were evaluated by differential centrifugation of forebrain homogenates as described (11, 16). Briefly, mid- and forebrain regions were dissected and Dounce-homogenized (15 strokes) in homogenization buffer D (10 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EGTA, 0.5 mM MgCl2, 0.02% NaN3, protease inhibitors (complete mini, EDTA-free; Roche Applied Science). Homogenates were passed twice through a 27.5-gauge needle and centrifuged at 1,000 × g for 1 min to remove unbroken cells. Supernatants were then centrifuged at 1,000 × g for 15 min to pellet nuclei and plasma membranes (P1). Supernatants were recentrifuged at 100,000 × g for 30 min at 4 °C in a Beckman tabletop TL100 ultracentrifuge to separate cytosolic components, including unassembled clathrin (supernatant) from a vesicular fraction enriched in Golgi, endosomes, and clathrin assembled in CCVs (P2). The P1 and P2 pellets recovered were resuspended in a volume of buffer D equal to that of the supernatant fraction. Equal volumes of P1, P2, and supernatant fractions were loaded on a 10–15% SDS-polyacrylamide gel and immunoblotted with clathrin HC (1:2000; BD Biosciences) or α-adaptin (1:1000; BD Biosciences) antibodies. Immunoblots were subjected to densitometric analysis using NIH Image J software (National Institutes of Health). The ratio of assembled to unassembled clathrin was compared between wild type and Cal−/− samples in a pairwise fashion using the P2 and supernatant (S) band intensities. “Assembly” levels (P2/S) detected in Cal−/− brain samples were normalized to those obtained for the wild type samples.

**Mammalian Cell and Primary Neuronal Cultures—**HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma), supplemented with 10% fetal calf serum (Sigma). The Myc-calcyon HEK293 stable cell line was established by selection in culture medium containing 900 µg/ml G418 (Sigma) following transfection with plasmid DNA (Novagen). The immunoblots were washed three times in PBS containing 0.1% Tween 20 prior to detection of bound antibodies by ECL (Amersham Biosciences).

Co-immunoprecipitation studies in Myc-calcyon HEK293 cells were conducted with cells solubilized in ice-cold lysis buffer. Cells were homogenized in lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 10% glycerol, 25 mM HEPES, pH 7.5) containing a mixture of protease inhibitors (complete mini, EDTA-free; Roche Applied Science). Homogenates were then centrifuged at 4 °C for 20 min at 14,000 rpm, and the protein concentration of the supernatants was determined. Rabbit anti-Myc or anti-β-galactosidase antibodies were added to the supernatants, followed by incubation with protein A/G slurry for 3 h at 4 °C. The slurry was washed four times with lysis buffer, and bound proteins were eluted with SDS loading buffer, resolved by SDS-PAGE, and immunoblotted with α-adaptin (1:1000; BD Transduction Laboratories), γ-adaptin (1:1000; BD Transduction Labs), δ-adaptin SA4 (1:500; NICHD, National Institutes of Health, and the University of Iowa Developmental Studies Hybridoma Bank), FLAG M2 (1:1000; Sigma) mouse monoclonal antibodies, or an HRP-conjugated Myc rabbit antibody (1:1000; Roche Applied Science).

**Immunocytochemistry—**Neurons were seeded on poly-l-lysine and laminin-coated glass coverslips. Cells were fixed in PBS containing 4% paraformaldehyde for 20 min at room temperature. After blocking in PBS containing 5% nonfat dry milk, 5% normal goat serum, and 0.1% Triton X-100 for 1 h at room temperature, cells were incubated with rabbit anti-Myc (1:2000; AbCam) and mouse EGF (1:200; Molecular Probes) for 1 h at room temperature. Bound primary antibodies were detected by incubation with the appropriate secondary antibodies: goat anti-mouse Alexa 488 (1:2000) and goat anti-rabbit Alexa 594 (Molecular Probes) (1:2000) for 30 min at room temperature. Cells were washed five times in PBS after fixation and each antibody incubation step. Coverslips were mounted in Prolong Antifade (Molecular Probes), and immunolabeling was observed under a laser-scanning confocal microscope (Zeiss Axiovert LSM 510, Carl Zeiss, Jena, Germany) using a Zeiss Plan-Apo 63 × 1.40 numerical aperture oil immersion objective lens, and documented using LSM510 software. Cells were excited using a 488-nm argon/krypton laser and detected with a 515–540-nm band pass filter. Alexa 594 was excited using a 543-nm helium/neon laser and detected with a 560-nm band pass filter. Images were collected at 1024 × 1024 resolution with a scan speed setting of 9 and pinhole adjustments of 0.8 and 0.9 µm, respectively, for the 488- and 594-nm channels.

For transferrin (Tfn) co-localization studies, HEK293 cells were plated on polylysine-coated coverslips and transfected with Myc-calcyon. 16–24 h later, the cells were incubated on ice for 45 min with 25 µg/ml Alexa-488-conjugated Tfn. Unbound Tfn was removed by washing cells with ice-cold wash medium. The cells were shifted to 37 °C for 2–15 min. Uptake was stopped by placing the cells on ice and washing twice with acid PBS (30 mM glycine, pH 2.5) for 2 min at 4 °C to remove any surface-bound Tfn. Myc-calcyon was detected with rabbit anti-Myc antibodies (1:2000), followed by Alexa-594-conjugated goat anti-rabbit secondary antibodies. Confocal images were taken using the Zeiss LSM-510 microscope.

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Tfn Endocytosis and Recycling Assays—For the HRP-Tfn uptake and recycling protocols (18, 19), Myc-calcyon HEK293 and control HEK293 cells were plated on polylysine-coated six-well plates at a density of 2 × 10^5/well. The next day, the cells were washed twice with warm Dulbecco’s modified Eagle’s medium and incubated for 30 min at 37 °C in prewarmed Transport Medium (Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin and buffered with 20 mM HEPES, pH 7.4). For HRP-Tfn endocytosis studies, the cells were shifted to 4 °C by placing on ice and washed once with ice-cold Dulbecco’s modified Eagle’s medium.

The cells were then incubated on ice with cold Transport Medium containing HRP-conjugated human Tfn (1 μg/ml) for 90 min to allow binding of Tfn to the cell surface. The samples were washed three times for 5 min each in ice-cold PBS to remove unbound ligand. Subsequently, endocytosis but not recycling was allowed to proceed by shifting the cells to 16 °C. Time points were taken at 0, 5, 15, 30, and 60 min by returning the plates to ice and washing in PBS containing 30 mM glycine, pH 2.5, followed by two washes in PBS prior to lysing the cells in PBS, 1% Triton X-100.

For the HRP-Tfn recycling studies, the cells were preloaded with HRP-Tfn by incubating cells at 37 °C for 60 min in warm Transport Medium containing 1 μg/ml HRP-Tfn. The cells were then placed on ice and washed twice in PBS containing 30 mM glycine, pH 2.5, to remove surface-bound HRP-Tfn, followed by two additional washes in PBS for 5 min each. Recycling was stimulated by incubating the cells at 37 °C in prewarmed transport medium containing 100 mM unlabeled Tfn and 100 μM desferoxamine to block reuptake of recycled Tfn. Time points were taken at 0, 5, 15, 30, 60, and 120 min by shifting samples to ice and washing three times in PBS containing 30 mM glycine, pH 2.5, followed by two washes in PBS before lysing the cells.

For quantitation of Tfn endocytosis in wild type and Cal^−/−^ neurons, neocortical neurons were prepared from embryonic day 16 mice as described above, and plated on polylysine- and laminin-coated 35-mm dishes at a density of 2 × 10^6 cells/dish. At 4 days in vitro, the neurons were washed and incubated for 30 min at 37 °C in prewarmed Transport Medium (basal medium Eaging containing 0.1% bovine serum albumin and buffered with 20 mM HEPES, pH 7.4). Neurons were then incubated on ice for 90 min with 5 μg/ml mouse HRP-Tfn in transport medium and shifted to 16 °C, a temperature permissive for endocytosis but not recycling. Time points were taken at 0, 5, 15, 30, 45, and 60 min by placing dishes on ice and washing six times with cold PBS.

For determining rates of HRP-Tfn recycling, neurons at 4 days in vitro were washed and incubated for 30 min at 37 °C in prewarmed Transport Medium and then preloaded with HRP-Tfn by incubating cells at 37 °C for 60 min with 5 μg/ml mouse HRP-Tfn in Transport Medium. After loading, cells were placed on ice and washed twice with ice-cold acid PBS, pH 2.5, followed by two washes with ice-cold PBS. Recycling was measured in warm transport medium containing 100 mM desferoxamine at 37 °C. Time points were taken at 0, 5, 15, 30, 60, and 120 min by placing the cells on ice and washing in PBS containing 30 mM glycine, pH 2.5, and PBS before lysing cells in PBS, 1% Triton X-100.

In all of the endocytosis and recycling assays described above, HRP-Tfn values were determined for using the HRP tetramethylbenzidine (TMB) substrate (Sigma). OD values obtained with the TMB substrate at 450 nm after the addition of 2 m H_2SO_4, were normalized such that the intracellular HRP-Tfn/mg protein values were determined for each sample with standard curves both for protein and HRP-Tfn.

Calcyon Gene Targeting—A c57Bl/6 ES cell line was electroporated with a 12.8-kb calcyon gene-targeting construct containing loxp sites inserted in introns 2 and 6, and a GK-neo cassette flanked by flp sites in intron 6. ES cell clones bearing the “floxed” calcyon allele (Cal^fl/fl^) were selected by PCR and Southern blotting, injected into Balb/c blastocysts, and implanted into ICR (white) females. Male chimeras were back-crossed to C57Bl/6 females, and black progeny were tested for germ line transmission by PCR of tail DNA. Mice homozygous for the targeted calcyon allele (Calcyon^+/−^) are viable and were crossed with CMV^CRE^ mice (Jackson Laboratories). The CMV^CRE^ transgene deleted both floxed calcyon alleles in Cal^fl/fl^ mice, generating a whole animal deletion of the calcyon gene (Cal^−/−^).

Primers a (GGC CTA TGG CCC TTC AAG), b (AGG CCG TGG GTG CAT TTT CTC), c (GAC CCA GCC CTT CTC CAC TTC), d (CAA TTT ACA GAA GGC CAA CCA C), and e (CTG TCC ATC TGC ACG AGA CT) were used for screening the targeted ES cell clones and for genotyping mice. Forward and reverse primers designated MusH164-185 (GAGACCAGGAT-GGGGCTGAC) and muscal 3’ 949–928 (ACAGAGTCAGAGT-CACAGG), which anneal to the second and sixth exons, respectively, were used to detect expression of calcyon transcripts in mice brain samples by reverse transcription-PCR (20). All wild type and transgenic mice were housed and bred in the animal facility at the Medical College of Georgia with protocols approved by the animal care and use committee and in accordance with the Public Health Service guidelines for animal husbandry and welfare.

Electron Microscopy—Animals used for immunohistochemistry were housed and treated according to federal and institutional guidelines. Two adult rhesus monkeys (Macaca mulatta) were transcardially perfused under deep Nembutal anesthesia with a fixative containing 0.1 or 0.2% glutaraldehyde, respectively, 4% paraformaldehyde, and 0.2% picric acid in sodium phosphate buffer (PB); 0.1 m, pH 7.35). The brains were then postfixed for an additional 2 h with the same fixative. Coronal blocks from the PFC were cut on a Vibratome at 60 μm. Sections were washed in PB and placed in an ascending series of sucrose solutions, frozen, and then stored at −70 °C. After washing in PBS and blocking (3% normal goat serum, 1% bovine serum albumin, 0.1% glycine, and 0.1% lysine in PBS) for 40 min, sections of the PFC were incubated with a polyclonal rabbit anti-calcyon (1:5000) antibody (21) for 2 days at 4 °C followed by an incubation in a biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA) secondary antibody. Sections were then treated according to the avidin–biotin protocol using Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, for 1 h) and tetramethylbenzidine as a chromogen. Briefly, sections, washed in PBS following the ABC step, were rinsed in phosphate buffer (pH 6.0) (PB6) and incubated in a solution containing 0.005% TMB (Sigma), 0.05% ammonium paratungstate, 0.004% NH_4Cl in PB6. The reaction was developed according to the glucose oxidase method (22). Rinsing the sections in PB6 stopped the development of the TMB reaction. The reaction product was stabilized using 0.05% 3’3’-diaminobenzidine (Sigma), 0.004% NH_4Cl, 0.02% CoCl_2 in PB6 with the aid of the glucose oxidase technique. Sections were then osmicated, dehydrated in ethanol and propylene oxide, and then flat embedded in Durecupan ACM (Fluka Chemical Corp., Milwaukee, WI). Ultrathin sections cut from the PFC were poststained with lead citrate and examined on a JEOL 1010 transmission electron microscope.

The percentage of labeled axons, spines, and dendrites was defined in each of the three different categories on electron micrographs taken from the neuropil of the PFC. Altogether, our analysis included 637 profiles. On each micrograph, TMB-labeled profiles were identified and classified by...
ultrastructural criteria (23). Profiles were identified as spines based on size (0.3–1.5 μm in diameter), presence of spine apparatus, absence of mitochondria or microtubules, and, in some cases, the presence of asymmetric synaptic contacts. Dendrites were identified by their larger size (0.5 μm or greater in diameter) and the presence of microtubules, mitochondria, and, in some cases, synaptic contacts. Axon terminals were characterized by the presence of numerous vesicles, mitochondria, and occasionally a presynaptic specialization. Preterminal, unmyelinated axons were identified by their small size (0.1–0.3 μm in diameter), regular round shape, and occasional presence of synaptic vesicles or neurofilaments.

![Figure 1](image)

**Figure 1.** Both the HC binding domain and C terminus of LCa are necessary for interaction with calcyon. A, schematic diagram of the overall domain structure of LCa, indicating regions included in the GST-LCa fusion proteins used in pulldown assays. B–D, subscript numbers refer to the LCa residues contained in the respective constructs. E, immunoblots of material pulled down by the GST-LC fusion proteins shown in B–D. Equivalent amounts of GST only or GST-LCa fusion protein were prebound to glutathione resin, blocked with 1% bovine serum albumin, and then incubated with purified S-calcyon-C. Resins were washed five times with binding buffer, and bound proteins were eluted with SDS-PAGE loading buffer. Gels were loaded with half of the eluate for each sample and one-quarter of the input S-calcyon-C for comparison. The S-calcyon-C fusion protein was detected with the HRP-conjugated S protein antibody. F, results of densitometric analysis using NIH Image J software of two independent replicates of the pulldown experiments shown in E. Bars and error bars, show the means ± S.E. of the percentage of total input S-calcyon-C pulled down by the GST-LCa fusion proteins indicated. Levels of S-calcyon-C associated with GST-LCa1-248, GST-LCa102-248, and GST-LCa102-248Δ163-192 were found not to significantly differ from each other when analyzed by one-way ANOVA followed by Tukey–Kramer multiple comparison posttest but were found to be significantly greater than for the other GST fusion proteins (*, p < 0.001). In contrast, the ability of all other GST-LCa fusions to pull down calcyon did not significantly differ from that of GST only.
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**RESULTS**

**Calcyon Interacts with the HC Binding Domain and C-terminal Segment of LC—**A yeast two-hybrid screen of an adult human brain cDNA library using human calcyon C-terminal residues 123–217 as bait yielded an interacting clone encoding the neuronal isoform of LCa (24, 25). Growth of colonies containing the calcyon bait and the LCa activation domain clone could be detected within 2 days of plating on quadruple dropout selective medium. In contrast, colonies containing the LCa construct and either empty bait vector or an unrelated bait construct were not detectable 5 days after plating, suggesting that the interaction between calcyon and LCa is specific and sufficient for growth on quadruple dropout medium.

Clathrin LCa are encoded by two ubiquitously expressed genes, LCa and LCb, that share ~60% sequence identity at the amino acid level but differ slightly in length (10). The neural isoform of LCa is composed of a series of domains, including the “conserved sequence,” Hsc70-binding site, Ca2+-binding domain, HC binding site, neuron-specific insert, and a C-terminal segment that includes the calmodulin binding site (Fig. 1A) (25). We localized the calcyon interaction domain with a panel of GST-LCa fusion proteins (Fig. 1, B–D). For example, immobilized GST fusion protein including full-length LCa (GST-LCa1–248) quantitatively precipitated the S-calcyon-C, an S-tagged bacterial fusion protein, including the C terminus (residues 114–217) of human calcyon. In contrast, similar assays with equivalent amounts of GST alone yielded no recovery of the S-tagged protein (Fig. 1, B and E).

Further experiments revealed that truncating LCa from the C terminus negatively impacted the yield of S-calcyon-C pulled down. The reduced effectiveness correlated with the extent of deletion, such that the combination deletion of the C-terminal domain, neuron-specific sequences, and HC binding site abolished the ability of GST-LCa1–101 to pull down S-calcyon-C. In contrast, an N-terminal deletion construct, GST-LCa102–248, resulting from removal of the conserved sequences and the HSC70 and Ca2+ binding sites, was as effective as full-length LCa in pulling down S-calcyon-C (Fig. 1, C and E). We next tested a set of deletions of the HC binding, C-terminal, and neuron-specific domains alone or together in varying combinations (Fig. 1, A–E). Whereas GST fusion proteins including only the neural specific insert (GST-LCa163–193), HC binding domain (GST-LCa192–248), or C-terminal domain (GST-LCa191–248) were ineffective (Fig. 1, B, C, and E), one containing the HC binding and C-terminal domains in combination was as effective as full-length LCa in pulling down S-calcyon-C (Fig. 1, D–F). Altogether, these results indicate that the heavy chain and C-terminal sequences are necessary and sufficient for LCa interaction with calcyon.

**Calcyon Associates with the Clathrin-mediated Endocytic Machinery—**The above biochemical data are consistent with a direct interaction of calcyon and LCa in vitro. CCV transport proteins localize in a variety of different membrane compartments, including the plasma membrane, endosomes, and the trans-Golgi network (TGN) (26, 27). Therefore, we next sought to learn where the association of LC and calcyon might occur in vivo, using affinity-purified polyclonal antibodies to human calcyon (21). Electron microscopic (EM) examination revealed calcyon antibody labeling both in perikarya (Fig. 2A) and in distal neuronal processes of the neuropil in the macaque prefrontal cortex (Fig. 2, B–E). The subcellular localization of the calcyon immunoreactivity was highly restricted to membranous, vesicular structures. In neuronal somata, calcyon antibodies specifically labeled the TGN (Fig. 2A), whereas vesicular intracellular organelles indicative of the endocytic and/or recycling machinery (28, 29) were labeled in extrasynaptic and intracellular regions of spines (Fig. 2B), dendrites (Fig. 2D), and axon terminals (Fig. 2C). The association of calcyon immunoreactivity to the intracellular side of the plasma membrane (Fig. 2, B–D) and the absence of labeling of the multivesicular bodies (MVB; Fig. 2, B and D) and of the synaptic membrane specialization of axon terminals and dendrites (Fig. 2, B–D) further support the observation that calcyon specifically localizes to intracellular endocytic pathways within neurons of the primate prefrontal cortex. Collectively, the above EM data highlight the potential for calcyon to regulate clathrin-mediated vesicle trafficking in neurons at several points along the endocytic and late secretory pathways (30).

We also examined the relative subcellular distributions of calcyon with respect to LC. Primary cultures of mouse neocortical neurons were co-transfected with an N-terminal Myc-tagged calcyon (Myc-calcyon) and EGFP-LCa plasmids, since the polyclonal antibodies raised against human calcyon do not recognize rat or mouse calcyon. Both Myc-calcyon and EGFP-LCa exhibited a vesicular-like distribution in neuronal cell bodies and processes as reflected by the punctate nature of the labeling patterns (Fig. 3). Further, there was a high degree of overlap in the LC and calcyon puncta in neuronal dendrites, spines, and cell bodies (Fig. 3, A–C). Quantitation of the dendritic distribution revealed that the vast majority (~80%) of calcyon-labeled puncta are also labeled by LC or abut LC-labeled puncta (Fig. 3D). Similar evidence for co-localization was detected in HEK293 cells expressing Myc-calcyon and N-terminal FLAG-tagged LCa (data not shown). In addition, co-immunoprecipitation studies were carried out to examine the association of calcyon with CCVs in mammalian cells. FLAG-LCa was immunoprecipitated from lysates of transfected Myc-calcyon stable HEK293 cells by anti-Myc but not by anti-β-galactosidase antibodies, as shown in Fig. 3E. Endogenous α-adaptin, a subunit of the AP-2 adaptor complex, was also co-immunoprecipitated by anti-Myc but not anti-β-galactosidase antibodies. AP-2 is involved in recruiting clathrin to the plasma membrane for CCV formation. By directly interacting with HC, AP-2 forms a link between the outer clathrin coat and the inner membrane-bound cargo (31). However, neither LCa nor α-adaptin could be immunoprecipitated from HEK293 cells with the Myc antibody (Fig. 3F). Since EM studies reveal calcyon antibody labeling of the TGN, lysosomes, and endosomes (Fig. 2), we asked whether calcyon might associate with CCVs containing either the AP-1 or AP-3 adaptor proteins (32, 33). As shown in Fig. 3E, γ-adaptin and δ-adaptin, subunits of the AP-1 and AP-3 adaptor protein complexes, respectively, can be detected in the Myc antibody immunoprecipitates of the Myc-calcyon but not HEK293 cell lysates. However, comparison of the adaptor protein band intensity in the immunoprecipitates with that of the lysates shows a larger fraction of α-adaptin co-immunoprecipitated with Myc-calcyon than was observed for γ- or δ-adaptin (Fig. 3F). The relatively stronger co-precipitation of the α-adaptin subunit of the AP-2 adaptor protein complex presumably reflects a more prominent association of calcyon with the clathrin-based endocytic machinery.

**Calcyon Enhances CCV Self-assembly in Vitro—**The role played by LCa in assembly and disassembly is still not fully understood (34). However, proteins that interact with LCa are known to regulate the dynamics of CCV formation and breakdown. For example, Hip1 promotes assembly of CCVs (13), whereas Hsc70 brings about disassembly (25, 35). Therefore, we examined whether calcyon also regulates lattice self-assembly using clathrin purified from rat brain (Fig. 4, A and B) (12). Varying amounts of either S-calcyon-C or S-calcyon-N were included in the self-assembly assays to achieve molar ratios of input fusion protein to purified clathrins ranging from 0.25 to 2.0. Following self-assembly in vitro, assembled unassembled clathrin were separated by centrifugation, such that clathrin lattices are enriched in the pelleted material (12, 15). As shown in Fig. 4, C and E, the levels of HC pelleted increased proportionally with the “dose” of purified S-calcyon-C included in the reactions, such that the addition of
FIGURE 2. Ultrastructural localization of calcyon in the macaque prefrontal cortex. A, calcyon immunoreactivity is associated with the Golgi apparatus (G) in the perinuclear region (pN), whereas the cytoplasm enriched in the cisterns of the rough endoplasmic reticulum (rER) is devoid of labeling. Note that the nucleus cannot be seen in this section. Note also the strong labeling between the Golgi apparatus and the lysosome (L). Labeling occurs toward the trans side of the Golgi apparatus (opposite to the perinuclear, cis side). Inset, the strongest labeling appears in the trans-Golgi network (TGN), whereas the cis-Golgi (opposite, down side) is unlabeled. The Golgi-cisterns appear to be only weakly labeled, but this signal might reflect diffusion of the chromogen TMB. N, nucleus. B, tangential section through a calcyon-positive spine (sp) receiving putative excitatory synaptic contacts (arrowheads) as defined by the asymmetric membrane specialization of the synapses and by the round synaptic vesicles in the axon terminal (at). Calcyon immunoreactivity shown by the circumflex is restricted to a vesicular region of the spine well removed from the postsynaptic density. This is also shown in another example in the inset. Note the remote localization of the multivesicular body (MVB) relative to the synaptic contacts on the tangential section of the spine. The open arrows point to the immunonegative region. C, calcyon-positive axon terminal (at) forming asymmetric synaptic contact (arrowhead) with an immunonegative spine (sp). Immunolabeling is associated with the intracellular side of the plasma membrane away from the synapse around amorphous vesicular structures (open arrow) different from the synaptic vesicles occupying the central part of the terminal. Nonsynaptic vesicular structures can also be seen in the right side of the bouton. D, calcyon immunoreactivity in distal dendritic (dd) processes of the neuropil. The labeling is concentrated at the plasma membrane (open arrows) in the vicinity of the lysosome (L), which is apparently immunonegative. Note also the absence of calcyon immunoreactivity of the multivesicular body (MVB) despite its proximity to the immunoreactive sites shown by the open arrowheads. E, percentage labeling of axons (including the terminal and preterminal, myelin-free segment), spines, and dendrites is shown in the three ultrastructural categories. The data table includes calcyon immunopositive (Positive) as well as immunonegative (Negative) counts for a total of 637 profiles. Scale bars represent 0.5 μm in A and 200 nm in B–D and insets.
equimolar or greater amounts of the C-terminal fusion protein significantly stimulated clathrin assembly. Additionally, there was a concomitant “dose-dependent” decrease in the levels of HC detected in the supernatants that was significant with the addition of equimolar or greater amounts of S-calcyon-C (Fig. 4, C and D). In contrast, the intensity of the HC band detected in either the supernatants or pellets was unaltered by the addition of the
S-calcyon N-terminal fusion protein (Fig. 4, C–E). Taken together, these data indicated that interaction of the calcyon C terminus with LC promotes the assembly of clathrin lattices.

**Overexpression of Calcyon Accelerates Tfn Endocytosis**—Tfn is a useful marker for monitoring the dynamics of CME as well as for examining the role of CCVs in endocytic recycling (27). Further, calcyon is not endogenously expressed in HEK293 cells. Therefore, HEK293 cells represent a good null cell line in which to examine the effects of calcyon expression on TfnR trafficking. Consistent with a potential involvement in CME, we found that calcyon partially co-localized with internalized Alexa-488 conjugated Tfn in transfected HEK293 cells (Fig. 5A). Further, functional studies performed in Myc-calcyon and untransfected HEK293 cells revealed a marked effect of calcyon expression on TfnR internalization. Specifically, upwards of a 2-fold enhancement in the levels of internalized HRP-Tfn was detected in the Myc-calcyon compared with control HEK293 cells within 15 min of the onset of TfnR endocytosis. By 60 min, 92% of the prebound Tfn was internalized in the Myc-calcyon cells, whereas only 60% was taken up by the HEK293 cells within the same time frame (Fig. 5B). That calcyon expression significantly enhanced the rate of TfnR endocytosis was evidenced by the difference in the time constants (t) for Tfn uptake in the Myc-calcyon cells versus HEK293 cells (t = 32.7 and 67.5 min, respectively, based on curve fitting with a single exponential equation). The difference in amounts of Tfn internalized could not be attributed to a competing recycling process, since the assays were performed at 16 °C, a temperature permissive for endocytosis but not recycling or degradation (36). Nor could they be accounted for by a difference in the number of surface TfnRs, since the levels of HRP-Tfn initially bound to both cell types did not significantly differ (p = 0.18). In contrast to endocytosis, expression of calcyon exerted no detectable impact on TfnR recycling (i.e. the levels
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and apparent rates of depleting internalized Tfn in the Myc-calcyon and control HEK293 cells were indistinguishable (Fig. 5C). Altogether, these results point to a specific role for calcyon in regulating CME.

Calcyon Knock-out Mice Exhibit Deficits in Endocytosis but Not Recycling—Calcyon is predominantly expressed in the brain and can be detected in embryonic stages as well as in the adult (data not shown). We generated calcyon knock-out (Cal^-/-) mice by homologous recombination of a floxed allele of calcyon (Calfl) into the calcyon locus, followed by breeding with CMV-CRE transgenic mice. Initially, in an effort to create a forebrain-restricted deletion of calcyon, we tested whether the CRE transgene driven by the Emx1 promoter could delete the Cal^beta allele (37). However, Emx1-CRE-mediated deletion of the Cal^beta allele was only partially effective (~50% based on PCR band intensity; data not shown) either due to the long distance between loxP sites (5.8 kb) in the targeted allele or because calcyon and Emx1 are not expressed in the same population of neurons. In contrast, the CRE recombine encoded by the strong and ubiquitously expressed cytomegalovirus promoter effectively disrupted the targeted locus, generating a null allele (Cal^-) (Fig. 6, A and B). Cal^-/- mice are viable and fertile, but compared with wild type and heterozygous animals, they do not express detectable levels of calcyon mRNA in the brain (Fig. 6C). Whereas calcyon is endogenously expressed in cultures made from wild type animals, transcripts could not be detected in the cultures prepared from the Cal^-/- mice (data not shown).

We examined the effect of deleting the calcyon gene on clathrin assembly in brain using a differential centrifugation strategy. In this approach, the pellets (P2) obtained at 100,000 x g are relatively enriched in CCVs, endosomes, and Golgi, whereas the supernatants (S) contain soluble cytosolic constituents, including unassembled clathrin (11, 16). Fractions from wild type and Cal^-/- mid- and forebrain were immunoblotted with anti-clathrin HC, alpha-adaptin (AP-2), and actin antibodies. Pairwise comparisons revealed a significant reduction in the steady state levels of assembled clathrin (P2/S ratio) in Cal^-/- compared with wild type brain (Fig. 7A). Similar results (individual and grouped) were obtained whether samples were immunoblotted with alpha-adaptin (p < 0.01, paired t test) or HC (p < 0.05, paired t test) antibodies (Fig. 7B). In contrast, the P2/S ratio for actin was unaffected by genotype. Altogether, these findings suggest that the calcyon/LC interaction might be an important determinant of clathrin assembly in brain.

Since overexpression of calcyon in HEK293 cells stimulates Tfn uptake, we used the calcyon knock-out mice to ask whether deletion of endogenous calcyon might alter CME in neurons. Tfn uptake assays were performed in the wild type and Cal^-/- neocortical neurons using a protocol developed for the neuron-like PC12 cells (16). In this protocol, we incubated the primary neuronal cultures with HRP-Tfn at 4 °C and then shifted the samples to 16 °C (without prior washing) to stimulate endocytosis. Thus, Tfn uptake in the neurons is reported as the total "cell-associated" HRP-Tfn (surface and intracellular) detected after washing samples taken at a given time point (16). Tfn recycling in the neocortical cultures was measured using the protocol described above for HEK293 cells. However, the recycling results are also reported as cell-associated Tfn, because, as reported for PC12 cells (16), acid washing of neurons was not effective in completely removing cell surface-bound Tfn. As shown in Fig. 7C, no significant differences in Tfn recycling could be detected in cultures made from the Cal^-/- as compared with the wild type mice. However, compared with levels measured in wild type neurons, significantly reduced levels of Tfn uptake were observed in the Cal^-/- neurons within 45 min of shifting the cells to 16 °C (p < 0.05, by two-way ANOVA and Bonferroni posttest for multiple comparisons (time x genotype))(Fig. 7D). By 60 min, Tfn levels in the wild type neurons exceeded that detected in the knock-out neurons by ~2-fold (238 ± 15% versus 124 ± 8%; p < 0.05), indicating that genetic deletion of the calcyon gene produces significant deficits in CME.
DISCUSSION

The data presented above indicate that calcyon stimulates CME by directly interacting with clathrin LC. Specifically, a fusion protein containing the C terminus but not the N terminus of calcyon significantly increases clathrin assembly in vitro, and full-length calcyon co-precipitates LC and the clathrin adaptor AP-2 in vivo. Importantly, neurons from calcyon knock-out mice exhibit significant deficits in TfR uptake. Conversely, HEK293 cells overexpressing calcyon display a marked enhancement of TfR endocytosis. As such, we propose that calcyon is a novel component of the elaborate molecular machinery involved in clathrin coat assembly in the brain. Consistent with such a role for calcyon, EM analysis localized calcyon to vesicles in dendritic spines and axon terminals, two sites in the central nervous system where CME is crucial for efficient synaptic transmission (1, 4). Calcyon is also found in endosomal vesicles in dendrites as well as in the TGN, which suggests a potential involvement in the transport of cargo from the TGN or from endosomes. However, at least with respect to the TfR receptor, our work indicates that calcyon functions primarily to enhance CME. Future studies will be required to unravel the role of calcyon in clathrin-mediated sorting from the TGN and/or endosomes, as would be consistent with the ability of calcyon to co-immunoprecipitate AP-1 and AP-3 subunits.

The binding of calcyon to LC requires the combined presence of the HC binding domain and C-terminal region, suggesting that the interaction depends on the formation of a binding interface in LC. Recent cryo-EM analysis of assembled clathrin lattices indicates that the HC binding domain of LC lies on the exterior of the lattice along the edges of the facets formed by the HC proximal segment (34). By being externally localized, LC is expected to interact with cytosolic elements and is predicted to play a much different role in regulating clathrin polymerization than adaptor proteins that link membrane-bound cargo to the interior of the clathrin coat (34). In cells, calcyon would also probably be associated with the CCV exterior, since calcyon is a single transmembrane protein that interacts with LC via C-terminal cytosolic sequences. Given the position of LC along the lattice surface, it seems reasonable to speculate that the mechanism by which calcyon stimulates CCV assembly might involve altering important regulatory contacts between LC and the HC proximal segment. At this point, our data do not allow us to distinguish whether calcyon becomes stably associated with the fully assembled CCV or simply regulates the assembly process in a transient fashion. Along these lines, calcyon was not identified as a clathrin-associated protein in a recent proteomic analysis of CCVs from the brain (8). As for a number of well-characterized clathrin accessory proteins like amphiphysin I and II, dynamin, and synaptojanin that were also not detected, the inability to discover calcyon by this strategy might be consistent with a transient association of calcyon with LC or perhaps reflect the relatively low abundance of calcyon.

FIGURE 6. Generation of calcyon knock-out (Cal−/−) mice. A, diagram of the calcyon locus and targeted (Cal+) and knock-out (Cal−) alleles. The numbers above exons and below introns indicate the size of the corresponding segments in base pairs (19). The targeted allele (Cal+) includes a GKneo cassette (shown in orange), flanked by flp sites (purple bars) inserted in the sixth intron. Additionally, homologous recombination of the 12.8-kb targeting vector into the calcyon locus resulted in the insertion of LoxP sites (green arrowheads) into intron 2 as well as intron 6, downstream of the GK-neo cassette. Outside probe indicates the genomic region used to detect ES cell clones bearing the Cal+ allele by Southern blotting. The arrows labeled a, b, c, d, and e refer to PCR primer annealing sites for genotyping. R1 and H indicate EcoRI and HindIII restriction sites. B, ethidium bromide-stained gel showing PCR products derived from the wild type, floxed, and knock-out alleles using the primer pairs indicated. C, reverse transcription-PCR confirmation of the lack of calcyon expression in brain samples from Cal−− mice by from hind brain and cortex. Parallel samples processed from wild type (Cal+/+) and heterozygous (Cal+/−) littermates are included for comparison.
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Interaction with LC presumably occurs with vesicular calcyon. It is also possible that the minor fraction of calcyon found inserted in the plasma membrane plays a role in clathrin assembly in cells (38). However, since the bulk of calcyon is vesicular, it seems more likely that calcyon might bind to light chain on internalized vesicles and promote trafficking to intracellular membranes. Alternatively, a potential function of cell surface-localized calcyon might be to demarcate nucleation sites for clathrin assembly or coat stabilization prior to cargo capture mediated by the adaptors (39).

Although LC is present in brain CCVs in a 1:1 stoichiometry with HC (8), LC is thought to negatively regulate the spontaneous assembly of clathrin lattices (25, 40, 41). Thus, the observed stimulatory effect of calcyon on clathrin assembly (8), LC is thought to negatively regulate the spontaneous assembly of clathrin. It is also possible that calcyon is vesicular, it seems more likely that calcyon might bind to light chain on internalized vesicles and promote trafficking to intracellular membranes. Alternatively, a potential function of cell surface-localized calcyon might be to demarcate nucleation sites for clathrin assembly or coat stabilization prior to cargo capture mediated by the adaptors (39).

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Calcyon probably regulates vesicular trafficking via a mechanism distinct from that of NEEP21, since it interacts with LC via C-tail sequences that are not well conserved in NEEP21. Consistent with this idea, NEEP21 influences receptor recycling by a mechanism that involves a clathrin-independent, phosphatidylinositol 3-kinase-dependent pathway (26, 45). Further studies are necessary to clarify similarities and differences in the mechanisms by which calcyon and NEEP21 regulate the movement of proteins between vesicular compartments as well as to elucidate the specific function of P19 in neuronal endocytic trafficking.

Extensive evidence suggests that CME is a crucial step in the stimulus-dependent removal of AMPA GluR2 subunits from synapses in dendritic spines during the synaptic weakening phenomenon of long term depression (1). Underscoring the potential for calcyon to regulate CME dynamics at excitatory synapses, double label immunofluorescence studies show that calcyon co-localizes with LC in dendritic spines of neocortical neurons in culture. Further, EM analysis of prefrontal cortex localized calcyon along the lateral walls of the spine head, well removed from the postsynaptic density, where other components of the clathrin coat, including dynamin and AP-2, are found (29, 46). Since genetic
deletion of calcyon produced a significant deficit in Tfn uptake consistent with a role for calcyon in CME, further investigations with the calcyon knock-out mice might provide additional mechanistic insights regarding CME in dendritic spines, especially during paradigms of synaptic plasticity.

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