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30. We thank A. J. Waker for helpful comments and suggestions. Supported by the Medical Research Council of Canada.

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Dual Signaling Regulated by Calcyon, a D1 Dopamine Receptor Interacting Protein

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The synergistic response of cells to the stimulation of multiple receptors has been ascribed to receptor cross talk; however, the specific molecules that mediate the resultant signal amplification have not been defined. Here a 24-kilodalton single transmembrane protein, designated calcyon, we functionally characterize that interacts with the D1 dopamine receptor. Calcyon localizes to dendritic spines of D1 receptor–expressing pyramidal cells in prefrontal cortex. These studies delineate a mechanism of G_q- and G_s-coupled heterotrimeric GTP–binding protein–coupled receptor cross talk by which D1 receptors can shift effector coupling to stimulate robust intracellular calcium (Ca^{2+}_i) release as a result of interaction with calcyon. The role of calcyon in potentiating Ca^{2+}-dependent signaling should provide insight into the D1 receptor–modulated cognitive functions of prefrontal cortex.

Dopamine (DA), acting through D1 receptors, modulates synaptic transmission in neural circuits, which mediate learning and memory (*1*, *2*). In heterologous expression systems, D1 DA receptors stimulate formation of adenosine 3′,5′-monophosphate (cAMP) by coupling to G_s heterotrimeric GTP-binding (G) proteins (*3*). However, in brain and kidney, D1 receptor agonists also produce increases in inositol 1,4,5-trisphosphate (IP_3) turnover and intracellular calcium (Ca^{2+}_i) (*4*). It is not yet known whether production of these second messengers involves linkage of D1 receptors to multiple G proteins or activation of alternative D1-like receptor subtypes.

We conducted a yeast two-hybrid screen (*5*) with the COOH-terminal 81 residues (residues 365 to 446) of the human D1 (hD1) receptor used as bait (*6*). One interacting clone, designated calcyon (for "calcium on"), contained a cDNA encoding a 217-residue protein (*7*). Calcyon is a putative type II membrane protein with a predicted single transmembrane segment extending from residues 88 to 103 (*8*). BLAST searches of GenBank databases indicated that calcyon displays a high degree of sequence similarity with two previously identified proteins of unknown function, P19 and P21 (*9*). The three proteins exhibit extensive sequence similarity (*7*). Calcyon appears to be the most highly diverged member of the P19/21/calcyon family of proteins.

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Antibodies to a 20-residue segment of calcyon bound to a strong band of about 34 kD and a weaker band of about 28 kD on immunoblots of microsomal protein fractions purified from rhesus monkey brain and spleen (*7*, *10*). The bands were present in samples prepared from prefrontal cortex and caudate putamen but not spleen. Preincubation of antibodies with immunizing peptide prevented detection of the bands. The predicted calcyon protein includes a potential N-linked glycosylation site at residue 73 (*7*). After digestion of prefrontal cortex microsomal proteins with N-glycosidase F, calcyon antibodies reacted with an ~24-kD protein, suggesting that the 28- and 34-kD bands corresponded to calcyon protein modified by N-linked oligosaccharides (*7*). Chaotropic salts failed to solubilize the immunoreactive protein, consistent with the notion that calcyon is an integral membrane protein (*10*). Together, these results suggest an NH_2-terminal extracellular, COOH-terminal intracellular transmembrane orientation of calcyon.

Calcyon antibodies labeled cell bodies and processes of neurons throughout the cerebral cortex and hippocampus (*11*) (Fig. 1, B and F). The labeling was characterized by high density in the vicinity of the plasma membrane and variably in neuronal processes. D1 receptors exhibit a similar distribution in these brain regions (Fig. 1, A and E). Calcyon appears to be preferentially expressed in pyramidal neurons, but expression in interneurons cannot be discounted until a more detailed anatomic investigation is conducted. Extensive labeling was also observed in many other subcortical structures (Fig. 1D). Omission of calcyon antibodies, or preincubation of antibodies with the immunizing peptide, prevented neuronal labeling. Immunogold electron microscopy of prefrontal cortex further revealed calcyon protein in small and medium-sized dendrites and den-

dritic spines receiving asymmetric inputs (Fig. 2, A and B). Similar to D1 receptors (*12*), calcyon was localized at the periphery of postsynaptic densities in dendritic spines (Fig. 2A).

Brain sections were double-labeled with calcyon and D1 receptor antibodies (*11, 13*), followed by a cocktail of species-specific Cy3-conjugated and fluorescein isothiocyanate (FITC)–conjugated immunoglobulin G (IgG). Overlays of the FITC and Cy3 fluorescent staining of prefrontal cortex indicated coexpression of D1 receptors and calcyon in the same population of pyramidal neurons (Fig. 2C). In the caudate, calcyon localized to a subpopulation of D1 receptor expressing medium spiny neurons (Fig. 2D), in which the D1 antibody labeled the neuropil so densely that cell body labeling was obscured (Fig. 1C).

Deletion of the COOH-terminal 11 and NH_2-terminal 55 amino acids had no apparent effect on the ability of the D1 receptor bait to interact with calcyon (*14*), indicating that residues 421 to 435 of the D1 receptor comprise a core domain sufficient for interaction. We tested the ability of S-calcyon, a bacterial fusion protein containing the COOH-terminus of calcyon (residues 93 to 217), to associate with a GSTD1 (*15*), a glutathione S-transferase fusion protein containing the D1 receptor bait sequence or with full-length D1 receptors expressed in HEK293 cells (*16*). GSTD1 was bound by S-calcyon but not by the negative control.

Inclusion of a peptide containing D1 receptor residues 421 to 435 (pep421–435) in the pull-down reaction, but not an unrelated 17-residue peptide (pep2) (*17*), prevented detection of GSTD1. Full-length D1 receptor polypeptide migrates with a molecular mass of 48 to 50 kD on immunoblots of D1 HEK293 cell lysates. Bands of similar size were pulled down from D1 HEK293 cell lysates by S-calcyon, but not by S-β-gal. The rat D1 monoclonal antibody (*13*) coimmunoprecipitated calcyon from pCI-calcyon–transfected cells (*18*) (Fig. 4C).

We tested whether calcyon plays a role in D1 receptor signaling in D1 HEK293 cells after activation of endogenous G-protein–coupled receptors (GPCRs). The D1 agonist SKF81297 (10 μM), when applied after stimulating endogenous P2Y purinergic receptors (*19*) with adenosine triphosphate (ATP) (50 μM), triggered an immediate increase in $Ca^{2+}{}_i$ (Fig. 3A) (*20*) in cells transfected with either pEnhanced green fluorescent protein (EGFP)–calcyon or pCI-calcyon plasmids (*18*). SKF81297 produced a gradual but small increase in $Ca^{2+}{}_i$ in untransfected and vector-transfected cells (Fig. 3B). Without priming, the D1 agonist-stimulated responses were undetectable (*21*). Similar responses were observed in transfected cells bathed in Ca^{2+}-free medium containing EGTA, indicating release of Ca^{2+} from intracellular stores. As D1 receptors and the $G_{q/11}$ G protein physically associate (*22*), priming may increase the affinity of the D1 receptor for $G_{q/11}$. In calcyon-transfected cells, D1 receptor–stimulated $Ca^{2+}{}_i$ release was comparable in magnitude to that elicited by the conventional $G_{q/11}$-coupled P2Y receptor in the same cells (92.6% ± 8.3% of the ATP response; eight independent transfection experiments). However, D1-stimulated cAMP levels were unaltered by calcyon expression (Fig. 3C) (*23*). These findings could be explained by the ability of calcyon to further strengthen coupling of D1 receptors to $G_{q/11}$ or to regulate enzymes or substrates that influence $G_{q/11}$ signaling (*24*).

Functional interaction of Calcyon and D1 receptors was required for maximal SKF81297-stimulated $Ca^{2+}{}_i$ release as the response was blocked by the D1 receptor antagonist SCH23390 (50 μM) (Fig. 3D) and undetectable in HEK293 cells expressing calcyon only (Fig. 3E). D1 HEK293 cells were cotransfected with expression plasmids encoding EGFP-calcyon, and either enhanced yellow fluorescent protein (EYFP) or EYFP fused to the core calcyon-interaction domain (EYFP-$D1^{421-435}$) (*25*). The SKF81297-evoked response of cells cotransfected with 1:1 and 3:1 molar ratios of pEYFP-$D1^{421-435}$ to pEGFP-calcyon was reduced by about 60% and 75%, respectively ($P < 0.01$, independent t test) compared with cells cotransfected with similar ratios of pEYFP and pEGFP-calcyon (Fig. 3, F and G). Presumably, expression of $D1^{421-435}$ decreased the SKF81297-stimulated response in a DNA concentration-dependent manner by competitively

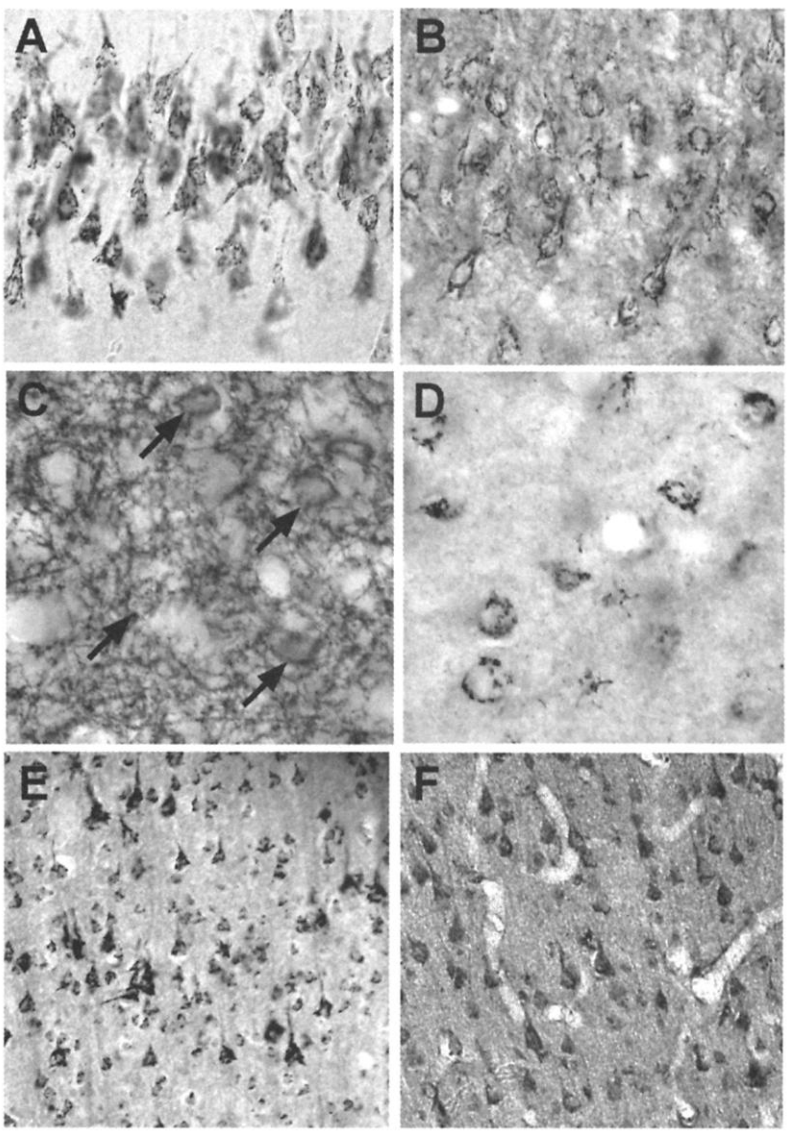

Fig. 1. Immunolocalization of calcyon and D1 DA receptors. Selected areas in the macaque brain labeled with subtype-specific antibodies to the D1 receptor (**A**, **C**, and **E**) and calcyon (**B**, **D**, and **F**). (A and B) Neurons in the CA3 region of the hippocampus; (C and D) caudate nucleus; (E and F) layer III neurons in area 46. Contrast and brightness of the images have been adjusted. Arrows in (C) point to labeled cell bodies.

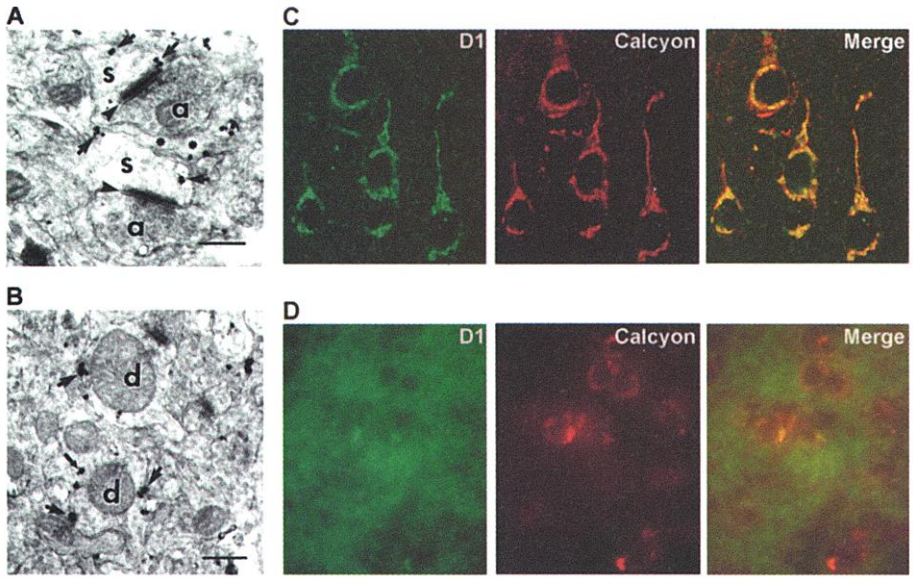

Fig. 2. Ultrastructural analysis of calcyon protein and colocalization of D1 DA receptors and calcyon in primate brain. (**A**) Immunogold localization of calcyon in two cortical dendritic spines (s), which receive asymmetric input (arrowheads) from unlabeled axons (a). Note extra- and perisynaptic membrane localization of immunogold particles (arrows). Immunopositive astrocytic process protruding between two spines is labeled with asterisks. Bars = 0.2 μm. (**B**) Calcyon protein localization in the small dendrites (d) of the cerebral cortex. Immunogold particles are associated with the plasma membranes (arrows) and cytoplasm (small arrow). Confocal (**C**) and epifluorescent (**D**) detection of D1 receptor and calcyon antibody double labeling of prefrontal cortex (C) and caudate nucleus (D).

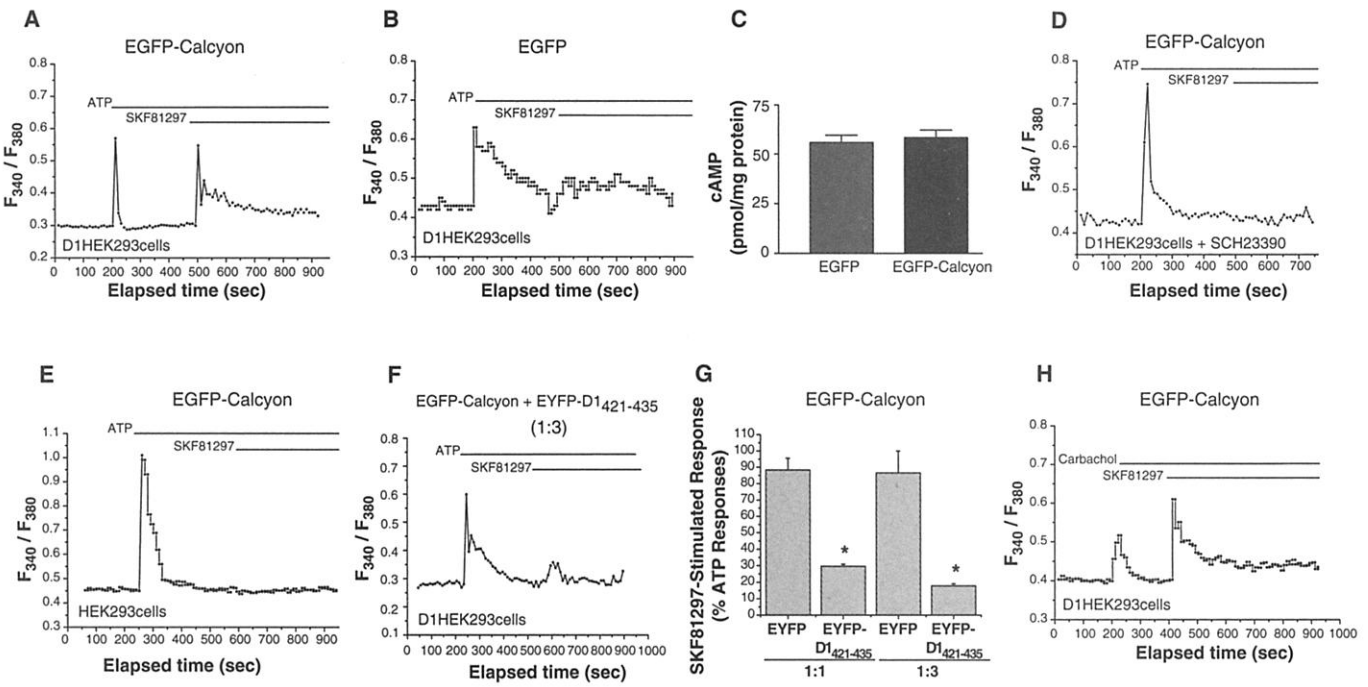

Fig. 3. Calcyon potentiates D1 receptor-stimulated of $Ca^{2+}{}_i$ release in HEK293 cells. (**A**, **B**, **D** to **F**, and **H**) Ligand-induced $Ca^{2+}{}_i$ release in fura-2–loaded D1 HEK293 or HEK293 cells expressing EGFP-calcyon, EGFP, or both EGFP-calcyon and EYFP-$D1^{421-435}$. Substances were applied for the times indicated by horizontal bars. $Ca^{2+}{}_i$ signals are reported as the mean of six to eight transfected cells. Similar results were obtained from four to eight additional independent transfection experiments. (**C**) cAMP accumulation in transfected D1 HEK293 cells treated with 50 μM ATP followed by 10 μM SKF81297 10 min later (t = 10 min). Reactions were stopped at t = 25 min. Results are reported as the average of three independent transfection experiments assayed in triplicate. (**G**) Grouped data from experiments similar to that shown in (F). The magnitude of the SKF81297 stimulated response in D1 HEK293 cells transfected with pEGFP-calcyon (0.02 pmol) and either pEYFP or pEYFP-$D1^{421-435}$ in a 1:1 or 1:3 molar ratio expressed relative to the size of the response elicited by ATP in the same cells. Bars show mean ± SE of (+EYFP) 1:1, n = 6; 1:3, n = 4 or (+EYFP-$D1^{421-435}$) 1:1, n = 3; 1:3, n = 3 independent transfection experiments. *$P < 0.01$; independent t test.

inhibiting formation of the calcyon-D1 receptor protein complex.

Priming or cross talk with $G_{q/11}$-coupled receptors apparently is necessary to activate the calcyon-D1 receptor complex as D1 agonists triggered a large increase in $Ca^{2+}{}_i$ in calcyon expressing cells if applied after stimulating endogenous M1 muscarinic receptors (*19*) (Fig. 3H), but not after stimulation of endogenous β-adrenergic receptors (*19*). Pretreatment with two different protein kinase C (PKC) inhibitors significantly attenuated D1-stimulated $Ca^{2+}{}_i$ release in calcyon-transfected cells (Fig. 4, A and B). Whereas the calcyon-D1 receptor complex forms in the absence of agonist (+HBS), interaction between calcyon and the receptor appears to be strengthened in the presence of agonists and reduced, but not abolished, by treatment with the PKC inhibitor bisindolylmaleimide (Fig. 4C) (*18*). The cytoplasmic domain of calcyon, which contains consensus PKC phosphorylation sites at Ser^{154} and Ser^{196}, can be phosphorylated by purified PKC isoforms (Fig. 4D) (*26*). Preliminary data indicate that this region of calcyon can bind the acidic phospholipid phosphoinositol-4,5-bisphosphate (PIP_2) (*27*).

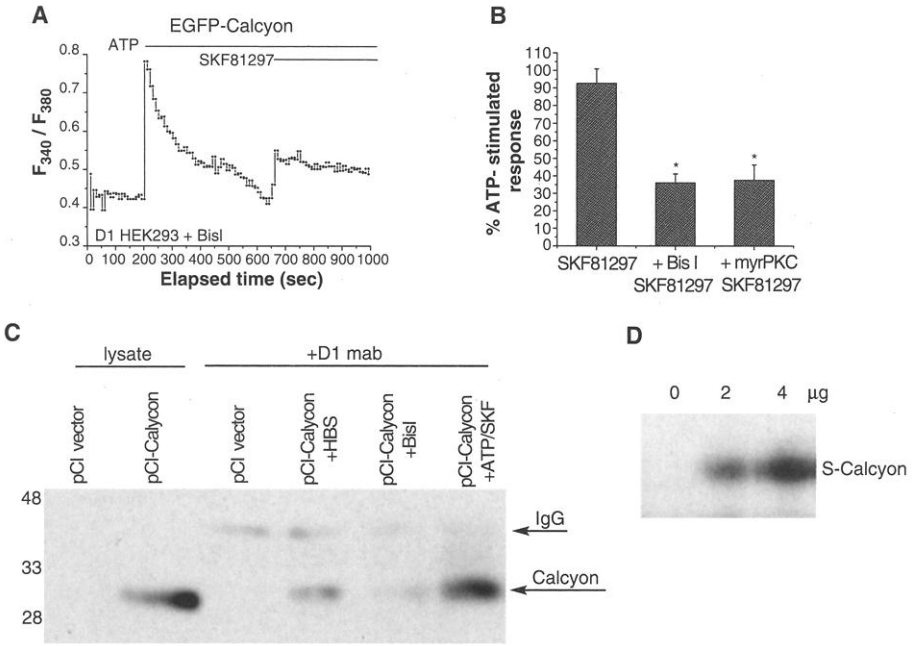

Fig. 4. PKC dependence of calcyon function. (**A**) Ligand-induced $Ca^{2+}{}_i$ release in fura-2–loaded D1 HEK293 cells expressing EGFP-calcyon. Substances were applied for the times indicated by horizontal bar. $Ca^{2+}{}_i$ signals are reported as the mean of six to eight cells. Cells were treated with the PKC inhibitor (2 μM bisindolylmaleimide) (+BisI) or 10 μM myristoylated PKC (19–27) (+myrPKC) for 10 or 60 min, respectively, before application of ATP. (**B**) Grouped data from five to eight experiments similar to that shown in (A). The magnitude of the SKF81297 response is expressed relative to that elicited by ATP in the same cells. The response elicited by SKF81297 in the absence of PKC inhibitors is shown for comparison. Bars show mean ± SE. *$P < 0.01$, independent t test; untreated, $n = 8$; +BisI, $n = 5$; +myrPKC, $n = 5$. (**C**) Calcyon antibody reaction with immunoblots containing solubilized proteins (20 μg) from pCI or pCI-calcyon–transfected D1 HEK293 cells (lysate) and proteins immunoprecipitated by the rat D1 receptor monoclonal antibody (+D1 mab) (*17*). Transfected cells were untreated (+HBS), treated with bisindolylmaleimide (2 μM) (+BisI), or treated with 50 μM ATP followed by 10 μM SKF81297 5 min later ($t = 5$ min) (+ATP/SKF) and before solubilization at $t = 10$ min. Calcyon was detected with rabbit calcyon antibodies and HRP-conjugated antirabbit (Fc fragment specific). Position of rat monoclonal antibody heavy chain is indicated by IgG, and positions of the molecular mass markers in kilodaltons are indicated on the left. (**D**) Autoradiographic detection of S-calcyon (0, 2, and 4 μg) after incubation with purified PKC and [γ-^{32}P]ATP. Proteins were separated on an SDS–12% polyacrylamide gel.

Calcyon appears to represent a prototype for a GPCR cross talk–specific accessory protein. Calcyon also may interact with other DA receptor subtypes and/or other GPCRs. Indeed, preliminary functional data suggest that calcyon can interact with the D5 DA receptor, which contains a region similar in sequence to the D1 receptor's core calcyon binding domain. The D1 receptor is of particular interest because it is the most prominent DA receptor in the cerebral cortex (*28*), and it modulates excitatory transmission in prefrontal neurons during working memory performance (*1*). The mechanism by which calcyon alters D1 receptor signaling after $G_{q/11}$-coupled receptor priming in HEK293 cells provides a molecular framework for understanding D1 receptor–mediated neuromodulation. Other neurotransmitters may prime the D1 receptor–stimulated $Ca^{2+}{}_i$ release, as D1 receptors and calcyon localize to pyramidal cell dendritic spines, which are the site of excitatory amino acid input. Several electrophysiological as well as molecular models of synaptic plasticity require both D1 receptor activation and *N*-methyl-D-aspartate receptor-mediated glutamate transmission (*29*). Furthermore, M1 muscarinic receptors also localize to spines of pyramidal neurons (*30*). The cross talk between muscarinic/dopaminergic signaling shown here thus may be relevant in vivo (*31*).

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6. Five hundred micrograms of pACT2 human brain cDNA library, amplified from 8×10^6 recombinant clones, was transformed into Y190 containing pGBTD1$^{365–446}$, containing hD1 residues 365 to 446 inserted into pGBT9 (*5*). Nitrocellulose lifts, β-galactosidase assays, and prototrophic assays on Leu$^-$ Trp$^-$ His$^-$ plates containing 30 mM 3-amino-1,2,4-triazole were done as described [T. Durfee *et al.*, *Genes Dev.* **7**, 555 (1993)]. pACT-calcyon failed to interact with GAL4 binding domain fused to lamin, RNA1, actin, p53, cdk2, or snf1. The nucleotide sequence of both strands of pACT-calcyon insert DNA was determined with an ABI automated DNA sequencer. Protein sequences were aligned with CLUSTALW and BOXSHADE 3.3.1 software.
7. GenBank accession number for calcyon is AF225903. Supplemental data are available at www.sciencemag.org/feature/data/1044333.shl
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10. Three female New Zealand White rabbits were immunized with keyhole limpet hemocyanin conjugated to a 20-residue peptide (QLSSPDQQNFPDLEGQRLNC) (*32*) as described (*12*). Calcyon antibodies were affinity-purified with bovine serum albumin (BSA)–conjugated peptide coupled to Affigel-15. Membrane and soluble proteins were prepared from monkey tissues as described [L. Mrzljak *et al.*, *Nature* **381**, 245 (1996)]. For blocking experiments, diluted calcyon antibodies were preincubated with 50 μg of BSA or BSA conjugated to the immunizing peptide for 30 min. *N*-glycosidase F digestion of membrane protein fractions was carried out with 1.0 unit of recombinant enzyme. Calcyon was present only in sedimented fractions after resuspension of prefrontal cortex microsomal proteins in 100 mM Na_2CO_3 (pH 11) or 10 mM Hepes (pH 7.4) and 5 mM EDTA buffer containing 500 mM NaCl, 6 M urea, or 100 mM NaI. For immunoblotting, proteins in loading buffer [U. K. Laemmli, *Nature* **227**, 680 (1970)] were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride sheets [H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)]. Molecular mass was determined relative to mobility of Perfect protein markers or prestained broad range markers. Immunoblots were incubated with rabbit antibodies to calcyon (1:100) followed by horseradish peroxidase (HRP)–conjugated antibodies to rabbit (antirabbit antibodies) (1:25,000).
11. Perfusion and preparation of brain tissue from adult macaque monkeys (*Macaca mulatta*) were carried out as described [L. Mrzljak, A. I. Levey, S. Belcher, P. S. Goldman-Rakic, *J. Comp. Neurol.* **390**, 112 (1998)]. For double-labeling experiments, 40-μm tissue sections were incubated with a cocktail of rat D1 monoclonal antibody (*13*) and rabbit calcyon antibodies for 48 hours at 4°C, washed, and incubated with Cy3-conjugated antirabbit or FITC-conjugated secondary antirat antibodies (*12*). Sections were viewed with a Molecular Dynamics confocal argon/krypton laser and Nikon Diaphot 200 microscope, and data were collected with Molecular Dynamics Image Space software and analyzed as a Ray model projection of 30 1-μm sections. For electron microscope analysis, sections were processed by the preembedding silver intensified immunogold method with goat antirabbit IgG coupled to 1.4-nm gold particles.
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14. NH_2- and COOH-terminal truncations of the D1 receptor bait pGBTD1$^{365–446}$ were generated by polymerase chain reaction (PCR), subcloned into pGBT9 (*5*), verified by DNA sequencing with an ABI automated DNA sequencer, and then tested for interaction with pACT-calcyon in Y190 (*6*). Positive two-hybrid interactions were visibly blue within 2 to 6 hours at 30°C.
15. A 550-base-pair Nco I–Bgl II fragment containing calcyon residues 93 to 217 was placed in-frame with the S-peptide [F. M. Richards and H. W. Wycoff, in *The Enzymes*, P. D. Boyer, Ed. (Academic Press, New York, 1971), p. 647] in pET30a. S-β-gal contained full-length β-galactosidase subcloned into pET30. Fusion proteins were induced in BL21(DE3) *Escherichia*

coli with isopropyl β-D-thiogalactopyranoside and coupled to S-agarose resin. Interaction assays were done with GSTD1 purified as described (*12*) or with lysates of D1 HEK293 cells (*16*) solubilized in 25 mM Hepes (pH 7.4), 50 mM NaCl, 10% glycerol, 1% BSA containing 0.5% Nonidet P-40. Resin-bound S-calcyon (50 μg) or S-β-gal (50 μg) and protein targets (GSTD1, 50 μg; D1 HEK293 lysates, 500 μg) or peptides (200 μg) were nutated for 2 hours at room temperature, washed twice in 10 vol of 25 mM Hepes (pH 7.4) containing 50 mM NaCl, and then resuspended in gel loading buffer.

16. D1 HEK293, a human embryonic kidney 293 (HEK293) cell line expressing hD1 receptors, was established by calcium phosphate transfection of pLXSN plasmid containing hD1 receptor cDNA. Stable transfected cells were maintained in Dulbecco's modified Eagle's medium containing 2 mM glutamine, 10% fetal calf serum, and 250 μg of G418 per milliliter. Cells contained a D1 receptor density of 3.1 pmol per microgram of protein as determined by [^{3}H]SCH23390 radioligand binding.
17. pep 421–435 (SVILDYDTDVSLEKI) and pep2 (NEDQKIGIEIIKRALKI) were synthesized with an ABI model 430A peptide synthesizer using the FastMoc procedures and reagents supplied by Perkin-Elmer, Applied Biosystems division, and resuspended in 100 mM Hepes (pH 7.4) at 1 mg/ml.
18. A 0.9-kb Eco RI–Xho I restriction fragment encoding full-length calcyon was subcloned into pCI and pEGFP-C3 expression plasmids. Twenty-four hours after transfection, about 10^6 pCI or pCI-calcyon-transfected D1 HEK293 cells were solubilized in mild lysis solution containing protease inhibitor cocktail. Protein concentrations were determined and adjusted by dilution so that equal amounts of total protein were incubated with rat anti-D1 monoclonal antibody or normal rat serum (diluted 1:100) for 12 hours at 4°C. Immunoprecipitated proteins were obtained by addition of protein A/G agarose slurry. The resin was washed three times with mild lysis solution, and adsorbed proteins were eluted in SDS-PAGE loading buffer.
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20. Cells (2.5×10^5) were seeded on cover slips and transfected with 0.4 μg of plasmid DNA per well using Effectene. Cells were rinsed with HBS [150 mM NaCl, 10 mM sodium Hepes, 10 mM glucose, 2.5 mM KCl, 4 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 7.4)] 16 to 20 hours after transfection and loaded with 5 μM fura-2 AM [G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **260**, 3440 (1985)] in HBS at room temperature. After 20 min, cells were washed three times with HBS. Assays were done at room temperature in 1.5 ml of HBS. Drugs were prepared in HBS and manually applied. For EGTA experiments, Ca^{2+}-free HBS was prepared containing 0.25 mM EGTA. Samples were viewed with an Axiovert 135 microscope (×63 objective). Cells transfected with pEGFP- and pEYFP-containing plasmids were identified at 490 nm and selected as areas for analysis. Images were collected with a charge-coupled device camera connected to a silicon graphics workstation and analyzed with Inovision-Ratiotool 4.3.5. Cells were sequentially illuminated for less than 100 ms, first at 340 nm and then at 380 nm. Fluorescence emission at 510 nm was monitored for each excitation wavelength at 10-s intervals. Average pixel intensities within six to eight selected areas (each area corresponded to a single transfected cell) for both wavelengths at each time point were digitized and stored.
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23. For cAMP assays, transfected D1 HEK293 cells were washed once with HBS and then exposed to agonists DA-HCl, SKF81297-HBr, ATP, or isoproterenol hydrochloride at 37°C in HBS. After timed incubation, cells were placed on ice, washed once in cold phosphate-buffered saline, and then lysed by addition of HCl. cAMP levels in the supernatants were determined with a direct cAMP enzyme immunoassay kit. Protein pellets were resuspended in boiling 10% SDS; protein concentrations were determined after dilution of SDS to 0.9% by addition of 10 mM tris (pH 7.4).
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25. pEYFP-D1$^{421–435}$ was constructed by subcloning a PCR product containing D1 receptor residues 421 to 435 into pEYFP-C1 and verified by DNA sequencing.
26. Phosphorylation assays were done in triplicate with purified PKC and S-calcyon or S-β-gal in reaction buffer containing 23 mM tris-HCl (pH 7.4), 0.44 mM $MgCl_2$, 250 mM $CaCl_2$, 1 μM ATP, and 1.5 to 2.0 μCi of [γ-^{32}P]ATP supplemented with 1 μg of diacylglycerol and 6 μg of phosphatidylserine. The reactions were stopped after 5 min at room temperature by addition of SDS-PAGE loading buffer and incubation at 80°C for 5 min. Proteins were resolved by SDS-PAGE on 12% acrylamide gels. Gels were dried before autoradiography.
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32. Amino acid residues are abbreviated as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.
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Epitopes Involved in Antibody-Mediated Protection from Ebola Virus

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To determine the ability of antibodies to provide protection from Ebola viruses, monoclonal antibodies (mAbs) to the Ebola glycoprotein were generated and evaluated for efficacy. We identified several protective mAbs directed toward five unique epitopes on Ebola glycoprotein. One of the epitopes is conserved among all Ebola viruses that are known to be pathogenic for humans. Some protective mAbs were also effective therapeutically when administered to mice 2 days after exposure to lethal Ebola virus. The identification of protective mAbs has important implications for developing vaccines and therapies for Ebola virus.

Ebola viruses cause acute, lethal hemorrhagic fevers for which no vaccines or treatments currently exist. Knowledge about the immune mechanisms mediating protection is limited. The membrane-anchored glycoprotein (GP) is the only viral protein known to be on the surfaces of virions and infected cells and is presumed to be responsible for receptor binding and fusion of the virus with host cells. As a result, Ebola GP may be an important target of protective antibodies. However, the contribution of antibodies to Ebola GP in disease resistance is unclear. Negligible serum titers of neutralizing antibodies in convalescent patients, together with inconsistent results in achieving protection through experimental transfers of immune sera to animals (*1*, *2*), have led to suggestions that antibodies to Ebola GP cannot confer protection to Ebola virus (*3*).

The role of antibodies to GP in protection is further confounded by the observation that Ebola GP occurs in several forms. Transcriptional editing of the GP mRNA is required for production of the virion-associated GP (*4*, *5*). Proteolytic processing of GP results in two products, GP_1 and membrane-bound GP_2, that covalently associate to form a monomer of the GP spike found on the surfaces of virions (*6*). GP_1 is also released from infected cells in a soluble form (*7*). The unedited GP mRNA encodes a secreted glycoprotein (sGP) that is synthesized in abundance early in infection (*4*, *5*, *8*). sGP and GP_1 are identical in their first 295 NH_2-terminal amino acids, whereas the remaining COOH-terminal 69 amino acids of sGP and 206 amino acids of GP_1 are encoded by different reading frames. It has been suggested that secreted GP_1 or sGP may effectively bind antibodies that might otherwise be protective (*5*, *7*).

This study identified protective GP-specific mAbs (*9–11*) that were classified into five groups on the basis of competitive binding assays (*12–14*). Individual mAbs in these five groups were protective against Ebola challenge when administered prophylactically or thera-

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