Tamalin Is a Scaffold Protein That Interacts with Multiple Neuronal Proteins in Distinct Modes of Protein-Protein Association*

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Tamalin is a scaffold protein that comprises multiple protein-interacting domains, including a 95-kDa postsynaptic density protein (PSD-95)/discs-large/ZO-1 (PDZ) domain, a leucine-zipper region, and a carboxyl-terminal PDZ binding motif. Tamalin forms a complex with metabotropic glutamate receptors and guanine nucleotide exchange factor cytohesins and promotes intracellular trafficking and cell surface expression of group 1 metabotropic glutamate receptors. In the present study, using several different approaches we have shown that tamalin interacts with multiple neuronal proteins through its distinct protein-binding domains. The PDZ domain of tamalin binds to the PDZ binding motifs of SAP90/PSD-95-associated protein and tamalin itself, whereas the PDZ binding motif of tamalin is capable of interacting with the PDZ domain of S-SCAM. In addition, tamalin forms a complex with PSD-95 and Mint2/X11p/X11L by mechanisms different from the PDZ-mediated interaction. Tamalin has the ability to assemble with these proteins in vivo; their protein complex with tamalin was verified by coimmunoprecipitation of rat brain lysates. Interestingly, the distinct protein-interacting domains of tamalin are evolutionarily conserved, and mRNA expression is developmentally up-regulated at the postnatal period. The results indicate that tamalin exists as a key element that forms a protein complex with multiple postsynaptic and protein-trafficking scaffold proteins.

Multimolecular protein assembly through protein-protein interaction is important as a general mechanism for diverse cellular functions in neuronal and other cells (reviewed in Refs. 1–5). Molecular assembly of protein complexes is built around one or more central scaffold proteins that contain multiple domains for protein-protein interaction. The 95-kDa postsynaptic density protein (PSD-95)/discs-large/ZO-1 (PDZ) domain is a key protein-binding domain comprised of ~90 amino acid residues and interacts with a PDZ binding motif with the consensus sequences STxxV/I/L (X is any amino acid) (6, 7). In neurons, postsynaptic PDZ domain-containing scaffold proteins, PSD-95 and S-SCAM, interact with a number of membrane and cytoplasmic proteins, including NMDA receptors (7–9) and SAP90/PSD-95-associated proteins (SAPAPs) (also called guanylate kinase-associated proteins/human Discs Large-associated proteins) (10–12). PSD-95 and S-SCAM are localized at the PSD of excitatory synapses and play an important role in functional assembly of a postsynaptic macromolecular complex. The PDZ domain-containing scaffold proteins are also important in subcellular trafficking of their partner proteins (reviewed in Refs. 1, 4, and 13). LIN-2, LIN-7, and LIN-10 of Caenorhabditis elegans are all PDZ domain-containing proteins and are important for the proper localization of the LET-23 receptor tyrosine kinase (13). LIN-2 is the homolog of mammalian scaffold protein CASK, which was simultaneously discovered as a protein interacting with the cell surface protein neurexin (14, 15). The mammalian homolog of LIN-7 (16) was cloned and named as Veli (17), mLIN-7 (18), or MALS (19), whereas LIN-10 was found to be a homolog of mammalian scaffold protein, Mint/X11 (20–22). CASK, Veli, and Mint form a tripartite complex in the mammalian brain and are considered to be involved in protein targeting in mammalian polarized cells (1, 4, 17, 23). Thus, the PDZ domain-containing scaffold proteins participate in diverse mechanisms underlying neuronal activity and function.

Tamalin (also termed GRP1-associated scaffold protein) is a scaffold protein that comprises multiple protein-interacting domains (24, 25). It possesses a PDZ domain, a leucine-zipper region, a proline-rich region, and a carboxyl-terminal PDZ binding motif (24, 25). The PDZ domain of tamalin interacts with the carboxyl termini of group 1 and group 2 metabotropic glutamate receptors (mGlRs) and GABAA receptor (24), whereas the leucine-zipper region binds to the coiled coil region of guanine nucleotide exchange factor cytohesins (24, 25). Tamalin promotes intracellular trafficking and cell surface expression of group 1 mGlRs in COS-7 cells and cultured hippocampal neurons through the interaction with cytohesins (24). Because tamalin has multiple, distinct protein-interacting domains and is enriched in the PSD fraction, we sought in this investigation to examine a protein complex formation of tamalin with other neuronal proteins, using several different approaches. Here we report that tamalin exists as a key element that forms a protein complex with several postsynaptic and protein-trafficking scaffold proteins through its distinct protein-binding domains.

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The Abbreviations Used Are: PSD-95, 95-kDa postsynaptic density protein; PDZ, PSD-95/discs-large/ZO-1; SAPAP, SAP90/PSD-95-associated protein; Mint, munc18-interacting protein; GST, glutathione S-transferase; SH3, Src-homology 3; GK, guanylate kinase; MJD, munc18-interacting domain; PTB, phosphotyrosine-binding domain; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; mGlRs, metabotropic glutamate receptors; C-tam, carboxyl-terminal portion of tamalin lacking the PDZ domain; N-tam, amino terminal portion of tamalin with the PDZ domain; NMDA, N-methyl-D-aspartate.
Experimental Procedures

Sequence Analysis—The amino acid sequence of rat tamalin (GenBankTM AF374272) was used as a probe in a BLAST data base search for the expressed sequence tag (EST) and nr data base at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST) with the TBLASTN algorithm. The Xenopus ESTs (GenBankTM BU906716, BU901589, BJ033586, BF163520, BJ029954, AI886945, BJ038540, BG472606, BJ048855, BJ043787, BJ053111, and BQ984122), the zebrafish EST (GenBankTM AW422012), and the zebrafish genomic DNA sequence (GenBankTM LA09757.9) were assembled to create electronic DNA sequences from which the amino acid sequences of Xenopus and zebrafish tamalin were predicted. Human IMAGE clone cDNAs 4422992 and 615168 (GenBankTM BC035500 and BU165906), Drosophila melanogaster RH12258 full-insert cDNA (GenBankTM AA909845.1), and C. elegans cDNA encoding the PDZ domain-containing protein (27.8 kDa) (GenBankTM NM 057583.1) were also identified as the cDNAs encoding proteins homologous to rat tamalin. Protein sequences were analyzed by the pfam program on the ISREC profile scan server (hits.itsb-sib.ch/cgi-bin/PFSCAN). Sequence alignment was performed with the Clustal W software (26) and DNA-SIS software (Hitachi). The phylogenetic tree was generated with Clustal W software using the Neighbor-Joining method and displayed with NJPLOT (27).

DNA Constructs—The carboxyl-terminal portion of SAPAP3 (amino acid residues 853–977, GenBankTM U67139), the first and second PDZ domains of PSD-95 (PSD-95 PDZ1 + 2) (residues 48–274, GenBankTM M96853), the third PDZ domain of PSD-95 (PSD-95 PDZ2) (residues 266–411, GenBankTM M96853), the Src homology 3 (SH3) domain of PSD-95 SH3 (residues 504–533, GenBankTM M96853), the guanylate kinase (GK) domain of PSD-95 (PSD-95 GK) (residues 504–724, GenBankTM M96853), the fifth PDZ domain of S-SCAM (S-SCAM PDZ5) (residues 1104–1277, GenBankTM AF04863), the PDZ domain of CASK (CASK PDZ) (residues 469–598, GenBankTM U47110), two PDZ domains of Mint1 (Mint1 PDZ1 + 2) (residues 640–839, GenBankTM AF291065), and the Munc18-interacting domain (MID) (residues 1–356, GenBankTM AF029107), the phosphotyrosine-binding domain (PTB) (residues 349–601), and two PDZ domains (residues 550–705) of Mint2 were cloned by reverse transcriptase-mediated polymerase chain reaction (RT-PCR) from rat brain RNA. The PDZ domain of Veli2 (residues 57–207, GenBankTM AF173082) was amplified by PCR, using mouse brain Marathon-Ready cDNA (Clontech) as a template. Isolated domains were subcloned in frame into pACT2 (Clontech), pGEX-4T-1/2 (Amersham Biosciences), or pET32a (Novagen). The deletion mutants of tamalin and SAPAP3 lacking the last 3 amino acid residues were constructed by PCR. To prepare the full-length cDNAs for rat Mint2 and SAPAP3, we first cloned several partial cDNAs by RT-PCR using total RNA as template and constructed full-length cDNAs by connecting the partial cDNAs (28). The isolated rat Mint2 cDNA was inserted in frame into pCMV-Tag3B (Stratagene). Proper in frame insertions and the absence of any sequence errors of all PCR products were confirmed by DNA sequencing. Mammalian expression vectors for myc-tagged full-length S-SCAM and myc-tagged full-length PSD-95 were kindly provided by Dr. Yutaka Hata (27). Proper in frame insertions and the absence of any sequence errors of all PCR products were confirmed by DNA sequencing. Mammalian expression vectors for myc-tagged full-length S-SCAM and myc-tagged full-length PSD-95 were kindly provided by Dr. Yutaka Hata (27).

Glutathione-S-transferase (GST) Pull-down Assay, Immunoprecipitation, and Yeast Two-hybrid Screening—Protein purification and pull-down assay were performed as described previously (24, 30). For pull-down assay, 10 μg of GST fusion proteins were immobilized on glutathione-Sepharose 4B beads (25 μl) and incubated with either supernatants (100 μg) containing recombinant tamalin transiently expressed in COS-7 cells or the purified recombinant thioredoxin fusion proteins (10 μg). Yeast two-hybrid screening and immunoprecipitation were performed as described previously (24).

Antibodies—Anti-tamalin antisera were raised in rabbits against GST-N-tamalin (24) emulsified with TiterMax Gold Adjuvant (Panhakoish) and used for immunoprecipitation. For immunoprecipitation from brain lysates, the affinity-purified anti-tamalin peptide antibody was used as described previously (24). Polyclonal rabbit anti-S-SCAM and anti-Veli1/2/3 antibodies were gifts from Dr. Yutaka Hata (9, 31). Other primary antibodies were obtained from the following sources: mouse monoclonal anti-Mint1, anti-Mint2, anti-CASK, and anti-PSD-95 antibodies from BD Transduction Laboratories; mouse monoclonal anti-SAPAP1 antibody from StressGen; mouse monoclonal anti-hexahistidine tag and anti-myc tag antibodies from Clontech. The secondary antibodies were described previously (24).

Northern Blotting—Mouse Brain Aging Blot (20 μg of total RNA/ lane, Seegene) was probed with 32P-labeled cDNA fragments under high stringency conditions. The cDNA fragment used corresponded to amino acid residues 10–200 of mouse tamalin. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe was purchased from Clontech and used as a control.

Results

Evolutionary Conservation of the Domain Structures of Tamalin—Since we had previously reported the sequences of rat and mouse tamalin (24), the DNA data base of C. elegans, Drosophila, and human became available. We first addressed, by BLAST data base search of the above species as well as Xenopus and zebrafish with the rat tamalin sequence as a probe, whether the characteristic structure of tamalin has been conserved during evolution. This analysis showed the existence of homologs of tamalin in all species analyzed (Fig. 1). The deduced amino acid sequences of tamalin of rat, mouse, human, Xenopus, zebrafish, Drosophila, and C. elegans are composed of 394, 392, 395, 379, 706, and 245 amino acid residues, respectively (Fig. 1A). They all possess a PDZ domain, a leucine- zipper region, and a carboxyl-terminal PDZ binding motif in common (Fig. 1A). Although the amino-terminal position of the PDZ domain is deleted in the Drosophila tamalin homolog and the carboxyl-terminal portion between a leucine- zipper region and a carboxyl-terminal PDZ binding motif diverges between vertebrates and invertebrates, the amino acid sequences of tamalin are highly conserved over the PDZ domain, the leucine-zipper region, and the PDZ binding motif (Fig. 1, B–D). The result indicated that the multiple protein-interacting domains of tamalin are conserved during evolution and may serve as scaffold for other proteins in cell function.

Interaction between Tamalin and SAPAP3—To identify tamalin-interacting proteins, we extended yeast two-hybrid screening of a rat brain cDNA library, using rat tamalin as bait. In addition to cytohesin-2 reported previously (24), 65 positive clones were isolated, in which 19 different cDNA species were included, 7 of them possessed a typical PDZ binding motif at their carboxyl termini (Table I). Four independent cDNA clones isolated encoded SAPAP3, which represents a postsynaptic scaffold protein containing the conserved carboxyl-terminal PDZ binding motif (11). The interaction between tamalin and SAPAP3 was analyzed in more detail with yeast two-hybrid assay by constructing a prey plasmid containing the intact carboxyl-terminal portion of SAPAP3 (SAPAP3-ct) or the truncated mutant of SAPAP3-ct lacking the last 3 amino acids (SAPAP3-ct del3). This analysis showed that tamalin bound to SAPAP3-ct, but not to SAPAP3-ct del3 (Fig. 2A). The recognition of the PDZ binding motif of SAPAP3 occurred through the PDZ domain of tamalin, because in the yeast two-hybrid assay SAPAP3-ct bound to the amino-terminal portion containing the PDZ domain of tamalin (N-tam), but not to the carboxyl-terminal portion lacking the PDZ domain (C-tam) (Fig. 2B). The interaction of tamalin with SAPAP3 was further confirmed by immunoprecipitation assay using COS-7 cells cotransfected with the full-length tamalin and myc-tagged full-length SAPAP3. Cell lysates were immunoprecipitated with anti-tamalin antibody, followed by immunoblotting with anti-myc antibody. Myc-SAPAP3 communoprecipitated from cotransfected cells with anti-tamalin antibody (Fig. 2C, lane 6) but not with nonimmunized serum (Fig. 2C, lane 8). In control, immunoprecipitation of myc-SAPAP3 was not seen in cells transfected with either myc-SAPAP3 or tamalin alone (Fig. 2C, lanes 5 and 7). The result indicated that tamalin and SAPAP3 form a protein complex in mammalian cells.

Tamalin Forms a Complex with Several Neuronal Scaffold Proteins in the Brain—We next addressed whether tamalin forms a protein complex with scaffold proteins involved in postsynaptic protein assembly or subcellular protein transport.
in the brain. Solubilized rat brain membrane fractions were immunoprecipitated with anti-tamalin antibody and immunoblotted with antibodies against candidate scaffold proteins (Fig. 3, A and B). S-SCAM, PSD-95, SAPAP1 were coimmunoprecipitated from rat brain membrane fractions with anti-tamalin antibody (Fig. 3A). In addition, Mint2 and CASK were immunoprecipitated, but neither Mint1 nor Veli1/2/3 was precipitated with anti-tamalin antibody (Fig. 3B). Because the last 13 amino acids covering the carboxyl-terminal PDZ binding motif are identical between SAPAP1 and SAPAP3 (11), it is likely that SAPAP1, like SAPAP3 (Fig. 2), directly binds to the tamalin PDZ domain. That there was no coimmunoprecipitation of either Veli1/2/3 or Mint1 was rather unexpected, because CASK is known to form a ternary complex with Veli1/2/3.
Multiple Binding Partners of Tamalin

TABLE I
Clones isolated by yeast two-hybrid screening with tamalin as bait

| Clones isolated by yeast two-hybrid screening | GenBank™ accession no. | Number of independent clones | PDZ binding motif |
|---------------------------------------------|------------------------|------------------------------|------------------|
| SAPAP3                                      | U67139                 | 4                            | QTRL             |
| Cytosin-2                                   | U53896                 | 4                            | GTSL             |
| Homology to human CG003                     | U50534                 | 4                            |                 |
| KARP-1-binding protein 2                    | AB022658               | 3                            |                 |
| Homology to human 80 K-H                    | J03075                 | 2                            |                 |
| Homology to human Zha1                      | AF106862               | 2                            |                 |
| Homology to human BCR                       | Y00661                 | 2                            |                 |
| Homology to mouse FMR2                      | NM 008032              | 2                            |                 |
| Homology to human KIAA0769                  | AB018312               | 2                            |                 |
| Homology to human KIAA0354                  | AB002352               | 2                            |                 |
| Homology to human NY-REN-58                 | AF155115               | 2                            |                 |

Fig. 2. Interaction of the PDZ domain of tamalin with the carboxyl-terminal PDZ binding motif of SAPAP3. A, under a schematic structure of rat SAPAP3, truncated and deletional constructs used for yeast two-hybrid assays are indicated; the above four truncated clones were isolated in the initial yeast two-hybrid screening. Positive and negative interactions as determined by filter β-galactosidase assay are indicated as + and −, respectively. B, schematic structures of rat tamalin and truncated N-tam and C-tam mutants are shown. Results of filter β-galactosidase assay are shown as in part A; COS-7 cells were transfected with myc-SAPAP3, tamalin, or both. C, lysates were immunoprecipitated with either anti-tamalin antibody (lanes 5–7) or nonimmunized control serum (lane 8) and immunoblotted with anti-myc antibody. Inputs (lanes 1–4) show 1/20 of cell lysates used for immunoprecipitation.

Fig. 3. Association of tamalin with several scaffold proteins in the brain. Rat brain membrane fractions were immunoprecipitated with either anti-tamalin antibody or nonimmunized control IgG, followed by immunoblotting with anti-S-SCAM, anti-PSD-95, anti-SAPAP1 (A), anti-Mint1, anti-Mint2, anti-CASK, and anti-Veli1/2/3 (B) antibodies. Inputs show equivalent amounts of extracts used for immunoprecipitation.

cDNA encoding either S-SCAM, Mint2, or PSD-95 was transiently co-transfected with the tamalin cDNA in COS-7 cells. Cell lysates were immunoprecipitated with anti-tamalin antibody and immunoblotted with the respective antibody. Anti-tamalin antibody immunoprecipitated all these scaffold proteins, whereas no such immunoprecipitate was detected in cells untransfected with the tamalin cDNA (Fig. 4, A–C). The result indicated that tamalin interacts with S-SCAM, Mint2, and PSD-95 in mammalian cells. CASK was found to be endogenously highly expressed in COS-7 cells (Fig. 4D). Only tamalin was exogenously transfected into COS-7 cells, and anti-tamalin immunoprecipitates were immunoblotted with anti-CASK antibody. No immunoblotting band of CASK was detected in anti-tamalin immunoprecipitates (Fig. 4D), suggesting that no direct interaction occurs between tamalin and CASK.

Identification of Tamalin-binding Domains of S-SCAM, PSD-95, and Mint2—We performed in vitro pull-down assay to identify tamalin-binding domains of S-SCAM, PSD-95, and Mint2. GST was fused to the PDZ domains of the scaffold proteins analyzed in this investigation. The resultant GST fusion proteins were expressed in Escherichia coli and purified. The GST fusion proteins were immobilized on glutathione-Sepharose 4B beads and incubated with the recombinant tamalin expressed in COS-7 cells. Bound proteins were eluted and immunoblotted with anti-tamalin antibody. This analysis showed GST-S-SCAM PDZ5 bound to tamalin (Fig. 5A). Inter-
lysates used for immunoprecipitation. Further pull-down assay showed that His-tagged N-tam was retained on glutathione-Sepharose 4B beads attached with GST-C-tam, but not on glutathione-Sepharose 4B beads attached with either GST-N-tam or GST-C-tam del3 (Fig. 5C), indicating that tamalin forms a homomeric complex through its PDZ domain and PDZ binding motif.

To identify the tamalin-binding domain of PSD-95, we divided the carboxyl-terminal portion following the PDZ domains of PSD-95 into the SH3 domain and the GK domain. These two domains were fused to GST and their ability to bind to tamalin was determined by GST pull-down assay (Fig. 5C). This analysis showed that tamalin bound to GST-GK but not to other protein domains of PSD-95 (Fig. 5C), indicating that the GK domain of PSD-95 is responsible for the specific interaction with tamalin.

We also examined the tamalin-binding domain of Mint2 by GST pull-down assay. Mint2 was dissected into the amino-terminal MID, the middle PTB, and the carboxyl-terminal PDZ domain, each of which was fused to GST. GST pull-down assay showed that tamalin bound to GST-MID and GST-PTB, but not to GST-PDZ1 + 2 (Fig. 5D). The result indicated that Mint2 interacts with tamalin through its MID and PTB domains rather than the PDZ domain. Collectively, these results demonstrated that tamalin distinctly interacts with many scaffold proteins through its multiple protein-binding domains.

Developmental Change of Tamalin Expression—Tamalin mRNA is expressed mainly in the telencephalic region of the mouse adult brain (24). We examined developmental changes of tamalin mRNA expression in the mouse brain by Northern blotting analysis. Expression levels of tamalin mRNA were low before and at birth, continuously increased during the postnatal period, and reached the highest level after postnatal 2 weeks (Fig. 6, upper panel). In control, G3PDH mRNA levels remained unchanged during the time course analyzed (Fig. 6, lower panel). The result suggested that tamalin may play some roles in developing and mature brain.

**DISCUSSION**

Tamalin is a scaffold protein possessing evolutionarily conserved protein-protein-interacting domains including a PDZ domain, a leucine-zipper region, and a carboxyl-terminal PDZ binding motif. The multiple protein domains of tamalin are now revealed to interact with many neuronal proteins in different modes (Fig. 7). The PDZ domain of tamalin binds to the PDZ binding motifs of not only mGluR1/mGluR5 and GABAB2 receptor (24) but also SAPAP3. Anti-SAPAP3 antibody was presently not available, and an interaction between tamalin and SAPAP3 in the brain remained elusive, but the identical carboxyl-terminal sequences between SAPAP1 and SAPAP3 (11) most likely drive a protein assembly between tamalin and both SAPAP1 and SAPAP3 in vivo. The carboxyl-terminal PDZ binding motif of tamalin directs a specific binding with the PDZ domains of S-SCAM and tamalin. To our knowledge, the latter is the first example indicating that the PDZ domain has the ability to associate with its own PDZ binding motif. Mint2 and PSD-95 also bind to tamalin by mechanisms different from a PDZ-mediated interaction. In addition, CASK forms a complex with tamalin indirectly via unknown scaffold proteins. Thus, tamalin has the ability to associate with many key proteins involved in neuronal function.

The PDZ is a highly organized signal-processing machinery composed of supramolecular protein complexes. Many proteins identified as a composite of the PDZ have PDZ domains and are involved in organizing the complex protein lattice at the PDZ domain of PSD-95.
For example, PSD-95 and S-SCAM are specifically localized at the postsynaptic site and serve to link NMDA receptors and the SAPAP family proteins to the PSD (7–12). Tamalin is enriched in the PSD fraction and promotes cell surface expression of mGluR1 in heterologously expressed COS-7 cells (24). Interestingly, mGluR1 is present in the NMDA receptor-associated macromolecular assembly (32), but it is located at the perisynaptic site and thus differs from NMDA receptors with respect to the synaptic localization (33). Tamalin interacts with typical postsynaptic scaffold proteins, PSD-95, S-SCAM, and SAPAP1/3, and the tamalin-mediated different assembly of postsynaptic scaffold proteins may have an distinct and important role in organizing a postsynaptic signal-processing machinery specific for group 1 mGluRs.

Tamalin interacts with cytohesins, and its transfection with the tamalin-containing adenovirus promotes a neurite distribution of endogenous mGluR5 in cultured hippocampal cells (24). Cytohesins are guanine nucleotide exchange factors specific for the ADP-ribosylation factor family of small GTP binding proteins and control intracellular protein transport (34, 35). Tamalin also associates with Mint2 and CASK. Mint2 binds to munc-18 (20), a protein required for exocytosis and essential for protein transport from Golgi apparatus to cell surface in epithelial cells (36). CASK is also involved in synaptic targeting of N-type calcium channels in cultured hippocampal neurons (37).
Furthermore, the orthologs of the Mint family protein (LIN-10) and CASK (LIN-2) in C. elegans are required for the normal basolateral localization of LET-23 (1, 4, 13). Moreover, LIN-10 is essential for postsynaptic localization of the glutamate receptor GLR-1 in nematode neurons (1, 4, 38). It is thus possible that the transport of group 1 mGluRs could be regulated by tamalin-associated trafficking complexes, including not only cytohesins but also Mint2 and CASK.

Several postsynaptic proteins show significant changes in expression levels during development. The expression of tamalin mRNA is regulated developmentally and is well correlated with other scaffold proteins, may contribute to developmentally regulated distribution of mGluR1 and other signaling proteins in neurons. In summary, our study shows that tamalin interacts with many important scaffold proteins involved in postsynaptic organization and protein trafficking in neurons. This indicates that tamalin may participate in receptor clustering, trafficking, and intracellular signaling. Further study will help to understand regulatory mechanisms of receptor-mediated neuronal function.

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