Localization of the GoLoco motif carrier regulator of G-protein signalling 12 and 14 proteins in monkey and rat brain

Manuel F. López-Aranda,[1] Maria J. Acevedo,[1] Francisco J. Carballo,[1] Antonia Gutiérrez[2] and Zafar U. Khan[1]

[1]Departamento de Medicina y Centro de Investigaciones Medico Sanitarias, Facultad de Medicina, Universidad de Málaga, Campus Teatinos, 29071-Ma´laga, Spain

[2]Departamento de Biologı´a Celular, Facultad de Ciencias, Universidad de Málaga, Campus Teatinos, 29071-Ma´laga, Spain

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Abstract

Regulator of G-protein signalling (RGS)12 and -14 proteins possess the RGS domain, Ras-binding domains and the GoLoco motif. Emerging evidence suggests that these proteins are involved in several cellular functions in addition to stimulation of GTPase activity of G-protein a subunits. However, our understanding of the role of the two proteins in brain function remains marginal. Here, we have studied the expression pattern of RGS12 and RGS14 proteins in brain at regional, cellular and subcellular levels. Both proteins were expressed throughout the brain regions, including cortex, hippocampus, striatum, thalamus and substantia nigra. The most intense immunostaining for RGS12 was seen in cortex and that of RGS14 was found in striatum. In cortex, RGS12 and RGS14 proteins were associated with pyramidal and nonpyramidal cell types. Apical dendrites of pyramidal cells were also labelled. RGS12 was found in both nuclear and cytoplasmic compartments. In contrast to RGS12 protein, RGS14 was localized in astrocytes in addition to neurons. Pyramidal cells in the CA1 area showed labelling for both RGS proteins. The presence of RGS12 was predominantly nuclear in the striatum of rat brain; however, the labelling of this protein was non-nuclear in adult monkey brain. To our surprise, in 1-month-old monkey brain the immunostaining pattern of the same protein was changed to nuclear. Non-nuclear staining for RGS12 was also evident in thalamus of adult monkey brain; however, in 1-month-old monkey brain, it was seen into two different populations, one with nuclear and the other with cytoplasmic staining. Both RGS12 and RGS14 were exclusively localized at postsynaptic sites of excitatory synapses. Our results demonstrate a highly dynamic expression pattern of RGS12 and RGS14 proteins in the central nervous system, and support the view that these proteins may participate not only in G-protein receptor signalling pathways but also in other cellular activities.

Introduction

Regulator of G-protein signalling (RGS) proteins act as GTPaseactivating proteins (GAP) targeting the Ga subunit to terminatethe G-protein-mediated signal transmission in mammalian cells (Arshavsky & Pugh, 1998; Berman & Gilman, 1998; Hepler, 1999; Ross & Wilkie, 2000). To date, > 35 genes are known to encode the ~120 amino-acid RGS core domain which accounts for their GAP activity. This RGS domain consists of a nine-alpha-helix bundle which, by interacting with Ga switch regions, stabilizes the transition state of GTP hydrolysis (Berman et al., 1996; Tesmer et al., 1997). RGS proteins catalyse rapid GTP hydrolysis and attenuate agonist–G-protein-coupled receptor-stimulated cellular responses in vivo (Hollinger & Hepler, 2002; Neubig & Siderovski, 2002). Apart from the RGS domain, some RGS proteins have also been reported to carry distinctive accessory domains and emerging evidence indicates their importance in various cellular functions (Ishii & Kurachi, 2003). For example, the G-protein c-like domain targets the RGS protein to Gb5 subunits (Snow et al., 1998a), the Gαi/o-Loco interacting motif binds to GDP-bound Gia G-protein and inhibits nucleotide release (Kimple et al., 2002), the Ras-binding domains interact with the GTP-bound form of Rap1 and Rap2 (Traver et al., 2000; Hollinger et al., 2001), and the PSD95 / Dlg / ZO-1 (PDZ) domain binds to Ephrin-B (Lu et al., 2001) and to the Interleukin-8 receptor (Snow et al., 1998b).

RGS12 and RGS14 belong to a subfamily that, in addition to RGS domains, share tandem Ras-binding domains and a GoLoco motif (Siderovski et al., 1999; Kimple et al., 2001). The larger RGS12 isoform also has amino terminal PDZ and phosphotyrosine-binding (PTB) domains (Snow et al., 1998b). It has been shown that the PTB domain participates in phosphotyrosine-dependent recruitment of RGS12 to the N-type calcium channels during desensitization of $GABA_B$ receptor signalling in dorsal root ganglia (Schiff et al., 2000) where RGS12 interacts with the SNAP receptor (SNARE)-binding region of this calcium channel (Richman et al., 2005). Both RGS12 and RGS14 interact selectively with members of the Gia and Goa subfamily of G-proteins to regulate their guanine nucleotide binding and hydrolysis activity (Willard et al., 2004). In addition to GAP activity, RGS14 inhibits the guanine nucleotide exchange activity of Gia (Hollinger et al., 2001; Kimple et al., 2001; Mittal & Linder, 2004; Hepler et al., 2005). Phosphorylation of RGS14 at threonine 497 enhances its GDP dissociation inhibitor activity (GDI; Hollinger et al., 2003). In addition, the accessory domain of the RGS14 protein can also bind to the inactive form of Go and Gi to influence Gα nucleotide binding and / or hydrolysis by mechanisms distinct from the RGS domain (Hepler et al., 2005). Recently, it has been shown in HeLa cells that RGS14 is localized in centrosomes and promyelocytic leukaemia protein (PML) nuclear bodies and regulates gene transcription (Cho et al., 2005). RGS12 has also been found in nuclear compartments of COS7 cells where it represses transcription (Chatterjee & Fisher, 2000, 2002). The presence of RGS12 and RGS14 in brain has been demonstrated by mRNA Northern blot (Snow et al., 1997), quantitative PCR (Larminie et al., 2004), in situ hybridization (Grafstein-Dunn et al., 2001) and Western blot (Hollinger et al., 2001) assays, but their distribution pattern in the brain remains unknown. Here, we have used affinity-purified antibodies specific to

RGS12 and RGS14 to study the regional, cellular and subcellular localization of the two proteins in monkey and rat central nervous system. We found that RGS12 expression was more pronounced in cortical areas and RGS14 in stiratum. RGS14 was present in both neuronal and glial cells, whereas RGS12 was mainly in neuronal nuclear compartments. A broader rather than area-specific expression of both proteins was observed.

Materials and methods

All experiments were conducted according to European Union guidelines on the ethical use of experimental animals (86 / 609 / EEC), with particular care to minimize the number of animals used. This study was approved by the University of Malaga Animal Experimentation and Ethics Committee. Animals were deeply anaesthetized with pentobarbital or acepromazine prior to experimentation. Antibodies The carboxyl-terminal peptide of human RGS12 (GenBank accession no. AY987042), ARDPRLSKREES, and the N-terminal peptide of human RGS14 (GenBank accession no. AY987041), RKKPKLKPGKS, corresponding to residues 494–505 and 55–65, respectively, were synthesized. The amino acid sequence of both peptides is homologous to rat. Peptides were coupled to keyhole limpet haemocyanin (KLH) protein. Conjugation of peptides with KLH, immunization of rabbits and affinity purification of antisera were performed as described previously (Khan et al., 1993, 1994, 1998; Gutierrez et al., 1994; Khan & Gutierrez, 2004). Peptide and KLH coupling was performed with the use of the Pierce Imject Activated Immunogen Conjugation Kit (Pierce, Rockford, IL, USA). Peptide–KLH conjugates (150 μg) were emulsified in complete Freund's adjuvant and were injected into rabbits for antibody production. The development and titre of the antibodies was determined by ELISA assay. For affinity purification, peptide (5 mg) was coupled to 1 g activated thiopropyl–Sepharose 6B (Amersham Biosciences, Piscataway, NJ, USA). Antisera diluted in phosphate-buffered saline (PBS) at 1:5 (v/v) were circulated through the prepared columns. After washing with PBS, the antibodies were eluted with glycine-HCl, pH 2.3, and dialysed against PBS.

Immunoblots

Membranes from the frontal cortex of monkey brain tissues were prepared as shown earlier (Khan et al., 1993, 1998; Khan & Gutierrez, 2004). Briefly, brain tissues were homogenized in 50 mm Tris-HCl, pH 7.4, and centrifuged at 105,000 *g* for 1 h, and the resultant pellet was washed three times in Tris-HCl buffer. The supernatant and suspended membranes were stored at -80 ºC until used. Brain membranes were treated with sodium dodecyl sulphate (SDS) buffer and processed for Western blot analysis similarly to descriptions given elsewhere (Khan et al., 1993, 1994, 2000; Khan & Gutierrez, 2004). Membrane proteins (100 μg / lane) were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. These nitrocellulose strips were then incubated with antibodies to RGS12 and RGS14 (5 μg / mL), followed by incubation with antirabbit IgGHRP (1:2000; Amersham Biosciences). Reactive protein bands were visualized using the ECL kit (Amersham Biosciences). Light and electron microscopy immunohistochemistry Four adult monkeys and 12 rats were deeply anaesthetized and

perfused transcardially with a fixative containing 4% paraformaldehyde, 0.2% glutaraldehyde and 0.2% picric acid. Brains were then dissected out and postfixed in the same fixative for 3 h and cryoprotected with 30% sucrose. One-month-old monkey brain sections were obtained from Dr Nenad Sestan (Yale University). Sagittal and coronal sections of 30 µm thick were cut on a freezing microtome and processed for immunohistochemistry as described earlier (Gutierrez et al., 1994; Khan et al., 1994, 1998, 2000, 2001; Khan & Gutierrez, 2004). In brief, brain sections were incubated with antibodies to RGS12 (1:1000) and RGS14 (1:100) for 2 days at 4 ºC; this was followed by incubation with biotinylated goat antirabbit antibody (1:200; Jackson ImmunoResearch, West Grove, PA, USA) and then the ABC Elite kit (1:100; Vector Laboratories, Burlingame, CA, USA). The bound antibodies were visualized with either 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide or DAB–glucose oxidase reaction. For double-label immunofluorescence studies, sections were incubated with RGS12 or RGS14 antibodies and mouse monoclonal antibodies to calretinin (1:1000), parvalbumin (1:1000) or tyrosine hydroxylase (1:1000), followed by incubation with goat antirabbit IgG-Cy 3 (1:200; Jackson Immuno-Research) and goat antimouse IgG-FITC (1:100; Jackson Immuno-Research).

For electron microscopic-level analysis, postfixed brains were cut into 50-lm sections with a vibratome and processed as above for the detection of RGS12 and RGS14 antibodies by immunoperoxidase reaction using the ABC Elite kit (Vector Laboratories). Sections were then osmicated, dehydrated and flat-embedded in Durcupan ACM (Fluka Chemical Corp., Milwaukee, WI, USA). The resin-embedded sections were cut into ultrathin sections on an ultramicrotome (Reichert, Leica, Germany) and examined in a Philips transmission electron microscope.

Results

RGS14 is a membrane protein but RGS12 is both a cytosolic and a membrane protein

To study whether the RGS12 and RGS14 are membrane-bound or not, we performed immunoblot analysis of prepared membrane and cytosolic fractions from monkey brain. The results of the immunoblot are illustrated in Fig. 1. Affinity-purified antibodies to RGS12 recognized two polypeptide bands of 74 and 51 kDa in membrane fractions; however, in cytosolic fractions this antibody bound to only the larger polypeptide band. The size of 74 kDa is very similar to that expected (76 kDa) from the RGS12 gene obtained from human brain (GenBank accession no. AY987042) but is lower in size than the other reported larger variant of the human RGS12 gene, which is 149 kDa (GenBank accession no. NM_002926). Though the peptide selected for the preparation of antibodies is common to all RGS12 genes published to date, we were unable to see the larger size band. It is probable that the expression of other RGS12 species is very low or null in brain. Therefore, our results suggest that there are two isoforms of RGS12 proteins in monkey brain, one (51 kDa) bound to membrane and the other (76 kDa) existing in both membrane and cytosol. In contrast to RGS12, RGS14 showed immunoreactivity to a 44-kDa protein band in membrane and was absent in the cytosolic fraction. The observed size is similar to the estimated molecular weight of the RGS14 gene (45 kDa) from human

brain (GenBank accession no. AY987041) and very similar to another human RGS14 gene (GenBank accession no. NM_006480; 48 kDa).

Furthermore, when RGS12 and RGS14 antibodies were preabsorbed with their respective cognate peptide, the immunoreactive bands were abolished (Fig. 1). In addition, replacements of antibodies with their respective preimmune sera resulted in no reaction. These antibodies also showed reactivity to GST-fusion proteins of the expected size of RGS12 and RGS14, when tested on immunoblots (not shown). Taken together, these results suggest that the appearance of the polypeptide bands was due to the specific reaction with antibodies to RGS12 and RGS14. For data on characterization of both antibodies, please see on-line Supplementary material, Appendix S1.

RGS12 and 14 in cerebral cortex

Table 1 summarizes the expression pattern of both RGS proteins in brain where cortical region shows strong to very strong immunoreactivity. Both RGS12 and RGS14 were highly expressed in the cortex of rat brain (Fig. 2A and B). Homogenous cellular immunostaining for RGS12 was observed throughout all the layers (Fig. 2A); however, the RGS14 was more concentrated in layers II–V (Fig. 2B). The labelling of RGS12 was in both pyramidal and nonpyramidal neurons of rat and monkey brain (Figs 3A and C, and 4A). Double labelling immunofluorescence studies of RGS12 and calretinin, a marker for GABAergic neurons, further confirmed the localization of this protein in nonpyramidal neurons of rat cerebral cortex (Fig. 4A). Although RGS12 was present in the cytoplasm of all cells, the predominant staining was associated with nuclear compartments (Fig. 3A–C). In addition, pyramidal neurons showed immunoreactivity in apical dendrites. The labelling in the cortex was very similar in the two species except in layer 1 of monkey brain where we observed an intricate web of fine fibres (Fig. 3D). In contrast to RGS12, the immunolabelling for RGS14 was predominantly cytoplasmic in both rat and monkey brains (Fig. 6B and C). Similar to RGS12, dendritic staining in pyramidal neurons was also observed with antibodies to RGS14 (Fig. 6B). Furthermore we found that RGS14 was not only present in neurons but also in astrocytes of rat (Fig. 5A) and 1-monthold monkey brain tissues (Fig. 6D).

Ultrastructural analysis of monkey brain area 46 confirmed the nuclear as well as cytoplasmic localization of RGS12 protein (Fig. 7A and B). However, we found that there were two populations of neurons, one that showed labelling in both cytoplasm and nucleus (Fig. 7A) and the other that presented only cytoplasmic staining (Fig. 7B). In addition, RGS12 was localized only postsynaptically (Fig. 7C and D) and not presynaptically. The localization of RGS14 in subcellular compartments of astrocytes and neurons is presented in Fig. 8A and B, respectively. Although it was not noticeable at the light-microscopy level, a light staining for RGS14 protein was also observed in nuclei (Fig. 8B). Similar to RGS12, the RGS14 protein was expressed postsynaptically (Fig. 8D). In addition, RGS14 was found to be localized around the perivascular cells of capillary vessels (Fig. 8C).

RGS12 and 14 in hippocampus and striatum

Strong expression of RGS12 as well as RGS14 was observed in the hippocampus of both rat and monkey brain. Pyramidal cells of the CA1 field were intensely labelled with RGS12 antibodies; however, a moderate staining was seen in stratum radiatum dendritic fibres that originated from the pyramidal cells of CA1 (Figs 9A and E, and 4E). This protein was also localized in parvalbumin-positive GABergic neurons of the CA1 area (Fig. 4E). Nuclear expression was more prominent in rat brain than monkey brain. In contrast to RGS12, the RGS14 protein was present with equal intensity in CA1 cells and dendritic fibres of rat brain (Fig. 9B). In addition, an abundance of labelled astrocytes was also observed in both stratum oriens and stratum radiatum of the CA1 field. In monkey brain, cells with long dendrites were observed in the CA1 area (Fig. 9C) and in the subiculum (Fig. 9D). The expression of RGS14 was very low to null in the CA2 region (Fig. 9F); however, RGS12 expression was as strong as in the CA1 field (Fig. 4C). In striatum of rat brain, the labelling of both RGS12 and RGS14 was more intense than in the hippocampus. RGS12 showed typical dot-like nuclear staining (Figs 10A and 4D) and RGS14 showed cytoplasmic staining (Fig. 10D). The dot-like nuclear staining for RGS12 was also evident in double-immunofluorescence experiments where this protein was stained with tyrosine hydroxylase, a marker for dopaminergic cells and structures. However, in adult monkey brain, both proteins were cytoplasmic where nuclei were unlabeled (Fig. 10B and E). In contrast to the adult, in 1-month-old monkey brain RGS12 was predominantly in nuclei (Fig. 10C). The presence of both proteins was observed in medium-size spiny neurons; however, RGS12 was also found in large cholinergic neurons (Fig. 10B and C). In 1-month-old monkey brain, labelling for RGS14 was seen in neurons as well as in astrocytes (Fig. 10F).

RGS12 and 14 in thalamus, cerebellum, globus pallidus and midbrain

Besides the cortex and striatum, thalamus and reticular nucleus cells were the other area where strong immunolabelling of RGS12 protein was observed. In adult monkey, the staining was associated with the cytoplasm and not with the nucleus (Fig. 11A–C); however, in 1-month-old monkey, the RGS12 protein was localized in two populations of cells, one that showed nuclear staining and the other presenting cellular labelling (Fig. 11C and D). Immunolabelling of RGS14 in the thalamus and reticular nucleus was cytoplasmic and was similar in the two species (Figs 12A, C and D, and 5C). In monkey brain, the RGS14 protein was strongly expressed in the ventrolateral dorsal and lateral dorsal thalamic nuclei (Fig. 5C) and the lateral geniculate nucleus (Fig. 5D). The immunostaining was also observed in cells of the inferior colliculus in rat brain.

In the cerebellum, RGS12 protein was mainly localized in the nucleus of Purkinje cells and granule cells (Figs 11F and 4F). The staining was also observed in a few cells of the molecular layer and in dendrites of Purkinje cells (Fig. 11F). However, RGS14 was only localized in dendritic fibres of the molecular layer in the cerebellum (Fig. 12B).

A strong staining for RGS14 was seen in cells of the globus pallidus area of rat brain (Fig. 13A). However, in 1-month-old monkey, this labelling was not only associated with cells but was also localized in astrocytes (Fig. 13B).

In rat brain, RGS14 was present in tyrosine hydroxylase-negative cells of the pars reticulata of the substantia nigra but not in the pars compacta (Fig. 5B) whereas, in monkey brain, this protein was localized in pars compacta cells (Fig. 13C and D). In contrast to RGS14, the RGS12 protein immunostaining was observed in cells of the substantia nigra pars compacta that were positive for tyrosine hydroxylase (Fig. 4B) and in cells of pars reticulata.

Discussion

Here we have presented the distribution of RGS12 and RGS14 proteins in brain at regional, cellular and subcellular levels. A dynamic expression profile of both proteins suggests their importance in brain function. A shift in the expression pattern of RGS14 from glia cells in 1-month-old monkey brain to neurons in adult monkey brain suggests a change in the role of this protein from the growth period to the adult. This dynamic change in the expression pattern of RGS12 was also observed where the difference was associated not only with age but also with species. Though the expression of RGS12 in cytoplasmic compartments was evident at both cellular and subcellular levels, this protein was present predominantly in nuclear compartments. Notably, the expression of this protein in striatal and thalamic neurons of adult monkey brain was associated with cytoplasmic compartments.

However, in striatum of 1-month-old monkey brain it was mostly expressed in nuclei. In contrast to striatum, thalamic neurons of 1-month-old monkey showed localization in both cytoplasmic and nuclear compartments. Together these results indicate that RGS12 and RGS14 proteins are much more versatile and may participate in different cellular functions regulated by compartmentalized expression. Additionally, the presence of RGS12 in membrane as well as in the cytosolic supernatant fraction further adds to the versatility of this protein. In agreement with our observation, RGS12 and RGS14 are known to have Ras-binding domains and the Go-Loco motif in addition to a conserved RGS domain and there is mounting evidence that suggests the multifunctional role of these proteins (Siderovski et al., 1999; Kimple et al., 2001; Martin-McCaffrey et al., 2004, 2005; Cho et al., 2005; Hepler et al., 2005; Richman et al., 2005). In addition, PDZ and PTB domains have also been reported in RGS12 (Snow et al., 1998b).

At the subcellular level, both proteins were observed in dendrites and in postsynaptic sites where spines and axons were making asymmetric synaptic contacts. Localization of RGS12 and RGS14 in spines at synaptic contacts where neurotransmission flow is expected to be very high confirm the participation of these proteins in signal transmission and further extend our understanding that they act only postsynaptically and at excitatory synapses. These proteins may not take part in presynaptic activities. At the regional level, the expression level of the two proteins in cerebral cortex was similar whereas in hippocampus RGS14 was stronger, suggesting a greater role of RGS14 in hippocampal signalling mechanisms. In substantia nigra, RGS12 was found in the tyrosine hydroxylase-positive dopaminergic cells where RGS14 was absent; instead it was localized in nondopaminergic cells. Dopaminergic neurons of this area innervate the striatum, cerebral cortex, nucleus accumbens and amygdala and modulate motor

functions, working memory, sensorimotor learning and reward-related learning and memory (Lewis & Sesack, 1997; Nicola et al., 2000). In addition, degeneration of these cells is known to produce Parkinson's disease. Though the participation of these proteins in dopaminergic pathways remains to be investigated, our results suggest that RGS12 may take part in the signalling process of these dopaminergic cells. However, exclusive postsynaptic localization of RGS12 protein at synaptic boutons excludes the possibility of involvement of this protein in presynaptic activities. Therefore, it is suggested that RGS12 in dopaminergic cells may participate in other cellular functions in addition to postsynaptic signalling. Furthermore, high expression of RGS12 and RGS14 in areas such as cerebral cortex, hippocampus, striatum and globus pallidus, which are known to participate in the formation of dopaminergic pathways, suggests their possible role in this system. In support of this argument, it has been shown that peptides of the Ga-specific GoLoco motif from RGS12 and RGS14 selectively decouple dopamine D2 receptor-mediated activation of potassium channels (Webb et al., 2005).

Localization of both RGS12 and RGS14 protein was observed in nuclear compartments. However, the expression of RGS14 was much less, to the extent that it was not seen at light microscopy level. This protein was observed only at the ultrastrucural level with electron microscopy. The findings of RGS12 protein in brain tissues not only confirm the reports of Chatterjee & Fisher (2000; 2002), who have shown nuclear localization in COS7 cells, but also demonstrate the presence of this protein in cytoplasmic compartments. The interaction of RGS12 with the SNARE-binding region of the CaV2.2 calcium channel during GABAB-mediated inhibition of calcium currents (Richman et al., 2005) further supports the cytoplasmic localization of this protein. Structural analysis of a variant of RGS protein (RGS12TS-S) has revealed that this protein plays an important role in transcriptional repression and cell cycle regulation, both nucleus-based activities (Chatterjee & Fisher, 2002). In line with our finding of nuclear RGS14 protein in brain, it has recently been shown that RGS14 localizes in centrosomes and PML nuclear bodies of HeLa cells (Cho et al., 2005). It has also been shown that RGS14 shuttles between the cytoplasm and nucleus and mild heat stress translocates this protein to PML nuclear bodies. RGS14 is also a mitotic spindle protein and is critical for the first cell division of the fertilized zygote (Martin-McCaffrey et al., 2004).

Results of the present study provide fundamental information concerning the localization of RGS12 and RGS14 in brain. A dynamic change in expression pattern of these proteins, such as from one cell type to other and even within cellular compartments, seen in 1-monthold and adult monkeys, suggests that they might have different functional roles during these periods. In addition, variation in the compartmental localization of RGS12 protein in the striatum of adult rat and monkey brain further indicate that this protein may participate in two different cellular functions. The dynamic changes in RGS12 expression were frequently associated with striatum and thalamus. The thalamus is considered the gateway to the cortex, transferring peripheral information to the cortex through first-order relay nuclei. The function of the thalamus is to integrate sensory and motor activities. It also plays roles in consciousness, affective behaviour and memory. We also found that the lateral geniculate nucleus, which is known to be a relay thalamic nucleus of the visual pathway, was strongly labelled with RGS14. This nucleus receives

fibres from the optic tract conveying visual information from both retinae and also from the primary visual cortex (area 17). In addition to the lateral geniculate nucleus, RGS14 was also prominent in the ventrolateral dorsal and lateral dorsal nuclei. These thalamic nuclei have been shown to participate in the limbic pathway. However, the striatum takes part in motor function including the preparation for and execution of cortically initiated movements. In addition to its role in motor function, it also participates in cognitive functions. Thus, the localization of both RGS12 and RGS14 in these areas suggests their possible role in some of the thalamic and striatal functions.

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Abbreviations

GAP, GTPase-activating protein; GDI, GDP dissociation inhibitor activity; KLH, keyhole limpet haemocyanin; PBS, phosphate-buffered saline; PDZ, PSD-95 / Dlg / ZO-1; PML, promyelocytic leukaemia protein; PTB, phosphotyrosine binding; RGS, regulator of G-protein signalling; SDS, sodium dodecyl sulphate; SDS, sodium dodecyl sulphate; SNARE, SNAP receptor.

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FIGURES AND TABLES

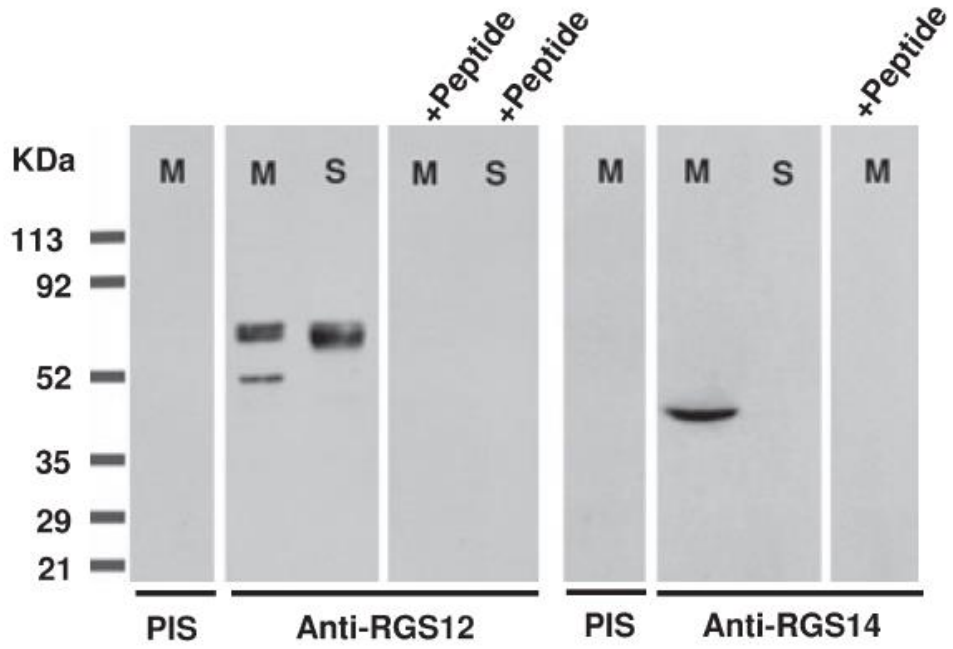

Fig. 1. Immunoblots of antibodies to RGS12 and RGS14 in monkey brain.
Incubation with an antibody to RGS12 reacted with two polypeptide bands of 74 and 51 kDa in the membrane (M) fraction; however, this antibody recognized only the larger band (74 kDa) in the cytosolic supernatant (S) fraction. Anti-RGS14 reacted with a single band of 44 kDa in the membrane fraction only. Incubation with preimmune sera (PIS), obtained prior to the immunization, showed no reactivity. In addition, preabsorption of both antibodies with their respective immunogen peptide (+ Peptide) abolished the observed bands.

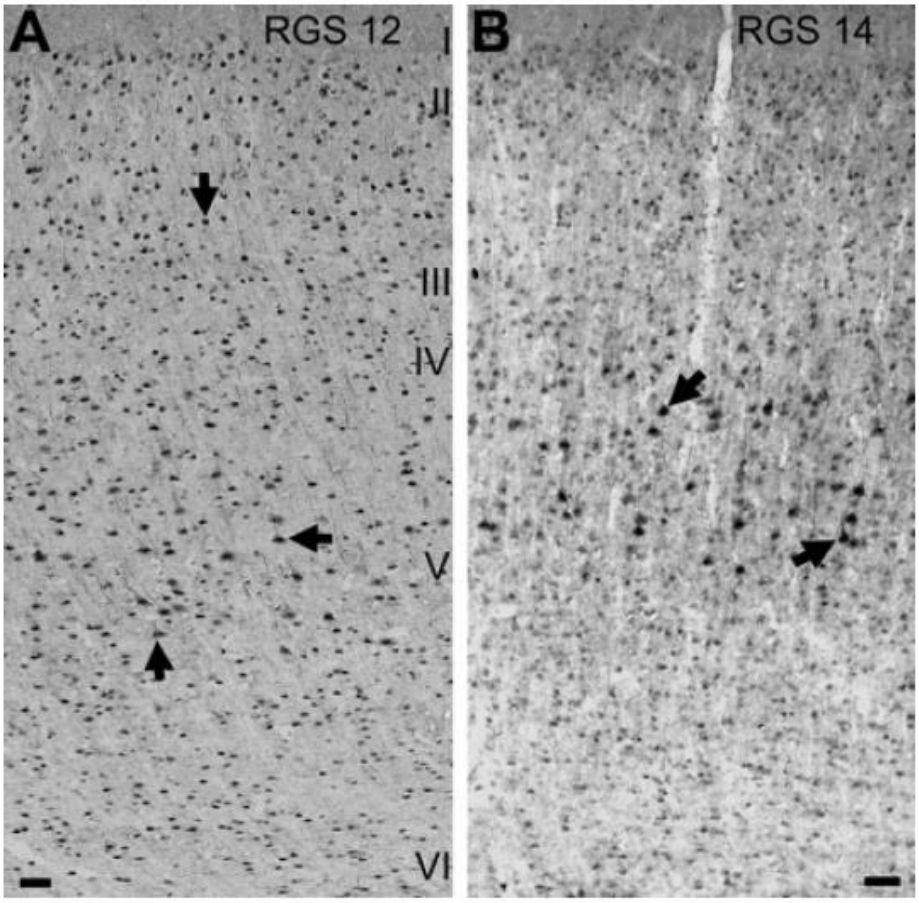

Fig. 2. Localization of RGS12 (A) and RGS14 (B) proteins in frontal cortical layers of rat brain. A homogenous expression of RGS12 was observed throughout the cortical layers. However, RGS14 was more abundant in layers II–V. Arrows indicate the typical labelling of neurons. I–VI show corresponding cortical layers. Scale bars, 100 μm.

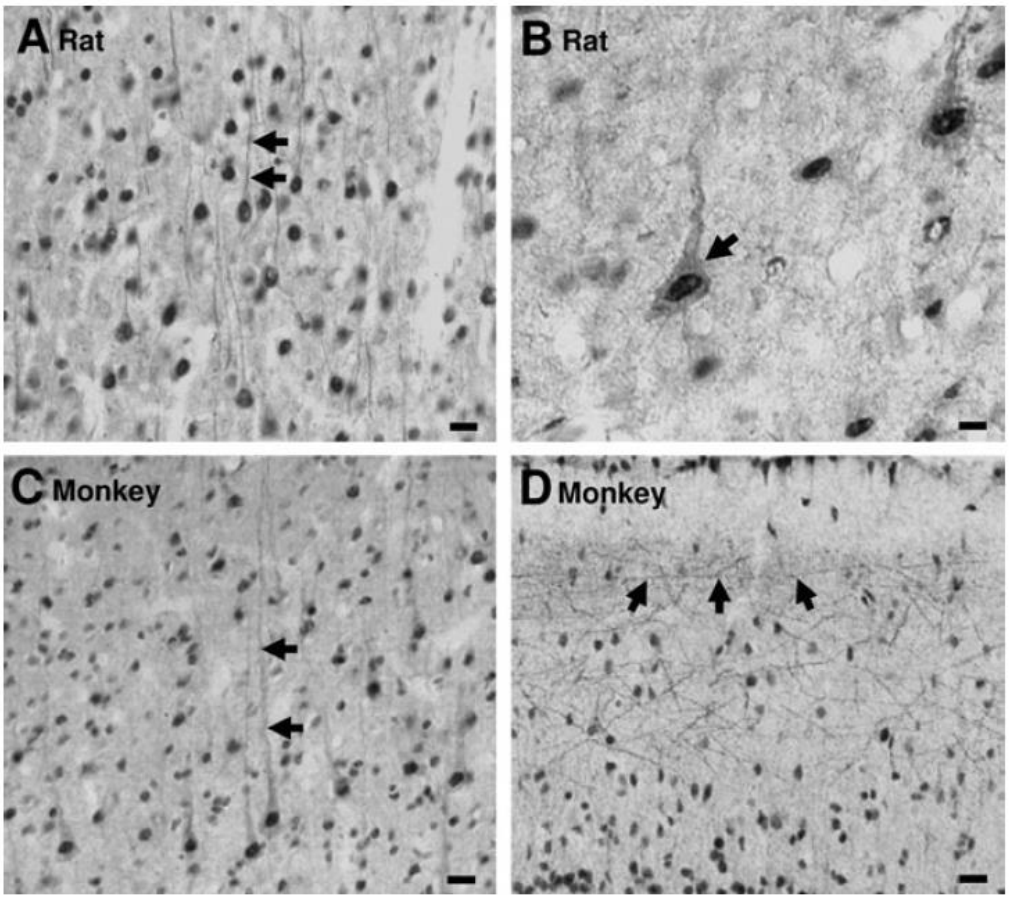

Fig. 3. Immunolabelling of RGS12 in cerebral cortex.
Cells of layers III–V showed labelling in proximal dendrites of pyramidal neurons of both (A) rat and (C) adult monkey as indicated by arrows. (B) Labelling in the cytoplasm and nucleus of a pyramidal neuron (arrow) was clearly evident. (D) In layers I and II, the immunostaining in fibre-like structures (arrows) was also observed. Scale bars, 50 μm (A, C and D), 25 μm (B).

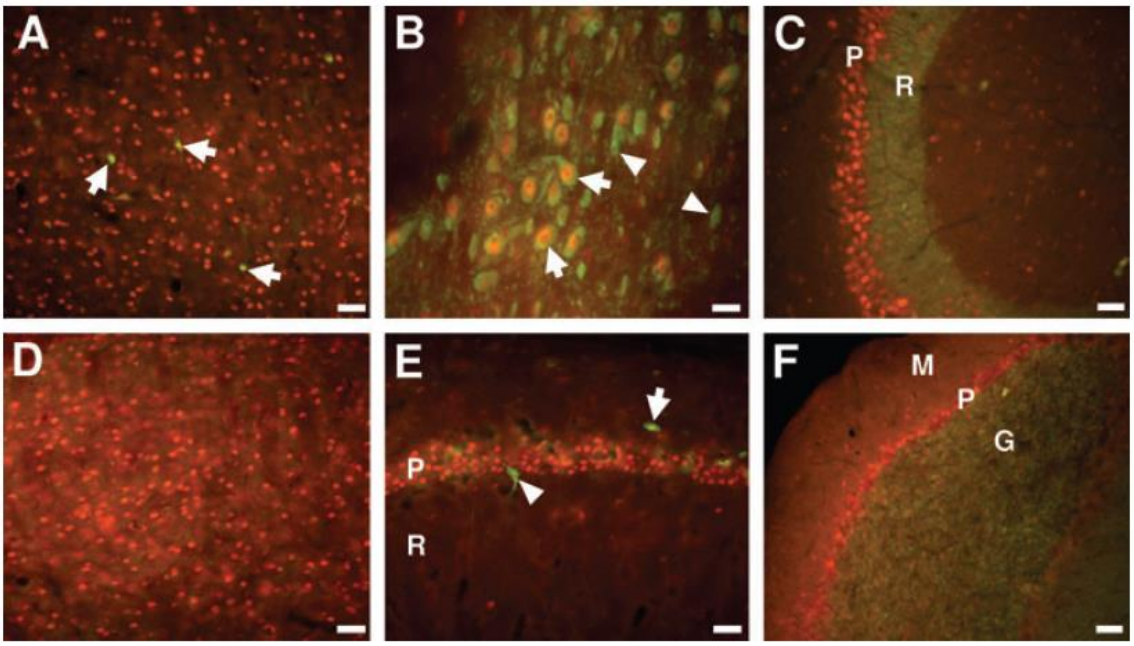

Fig. 4. Co-localization of RGS12 with calretinin, parvalbumin and tyrosine hydroxylase in rat brain.

Co-labelling of RGS12 (in red) with calretinin (in green) is shown in (A) cortex, (C) the CA2 region of hippocampus and (F) cerebellum. Co-labelling with tyrosine hydroxylase (green) is presented in (B) substantia nigra pars compacta and (D) striatum, and colabelling with parvalbumin (green) in CA1 area is shown in E. Co-labelled cells are indicated with arrows. Arrowheads show cells without RGS12. P, Purkinje cell layer; R, stratum radiatum; M, molecular cell layer; G, granule cell layer. Scale bars, 50 μm (A and C–F), 25 μm (B).

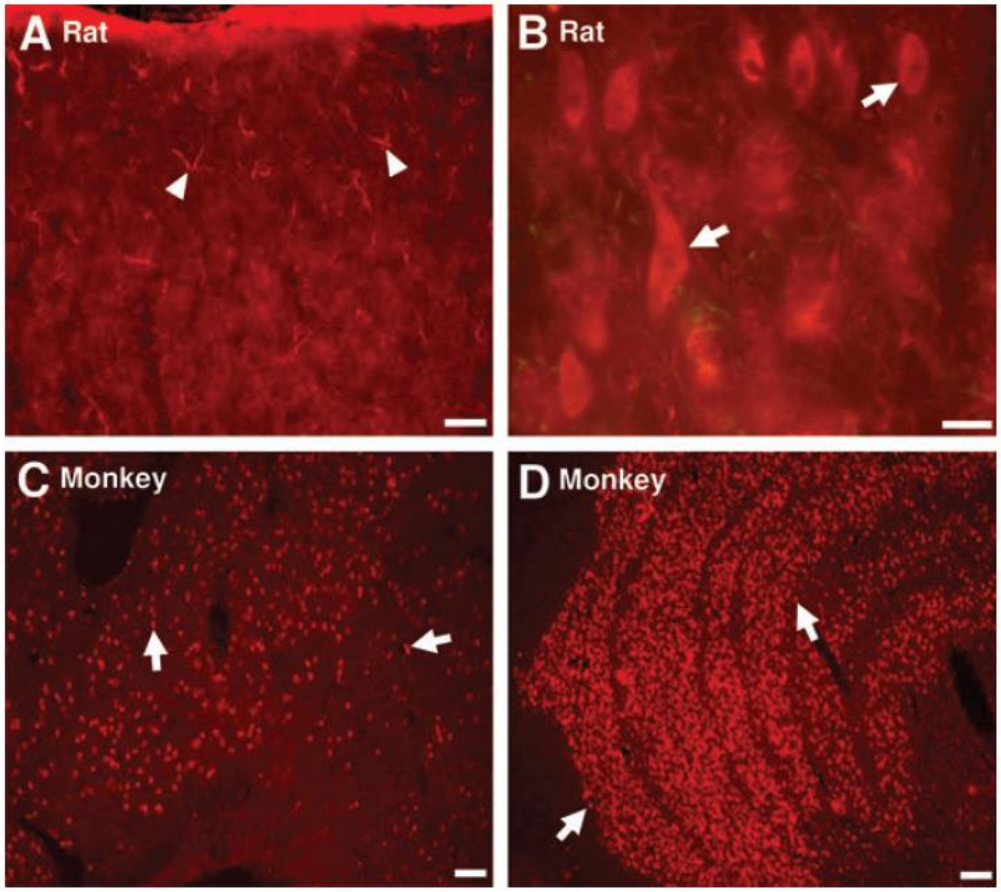

Fig. 5. Immunofluorescent labelling of RGS14 in rat and monkey brains.
(A) In rat cerebral cortex, labelling in astrocytes (arrowheads) was observed. (B) Localization of RGS14 (red) with tyrosine hydroxylase (green) in substantia nigra pars reticulata shows the presence of this protein in nondopaminergic cells(arrows). In monkey brain, intense staining for RGS14 was observed in (C) the ventrolateral dorsal and lateral dorsal nuclei of the thalamus and (D) layers oflateral geniculate nucleus. Arrows indicate the labelled neurons. Scale bars, 50 µm (A, C and D), 25 µm (B).

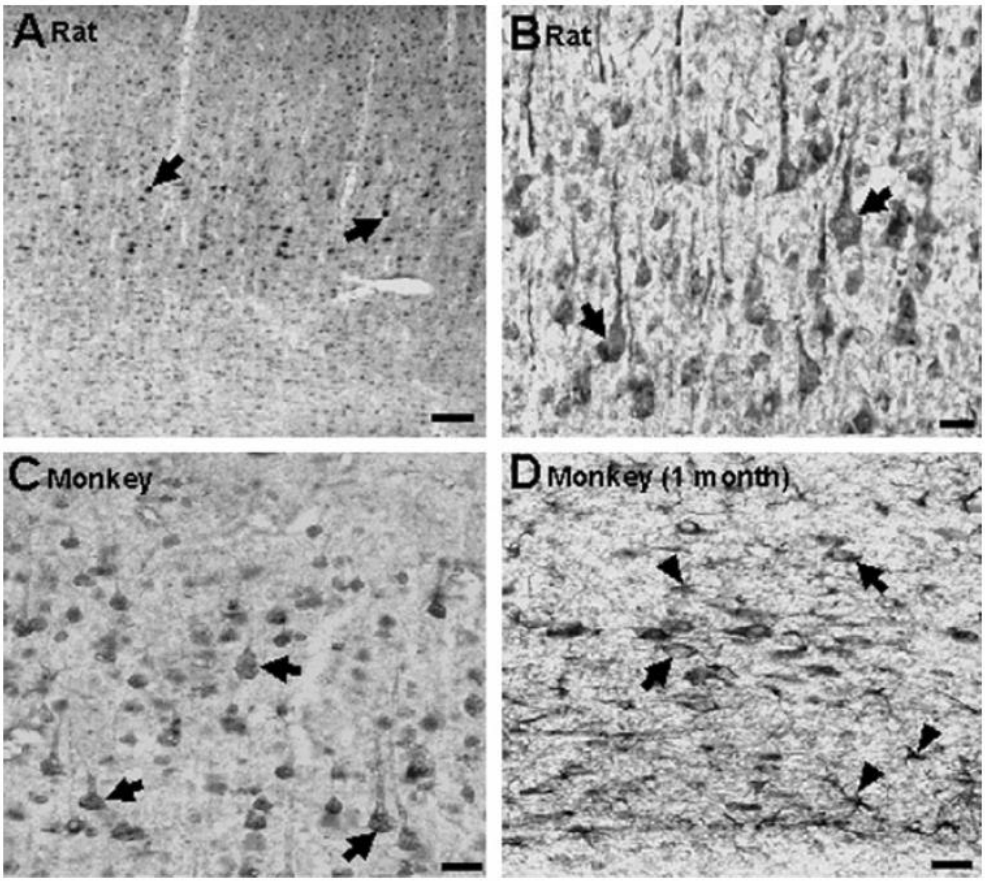

Fig. 6. RGS14 in rat and monkey cerebral cortex.
(A) Labelling of RGS14 in cerebral cortex at low magnification. Soma as well as proximal dendrites of pyramidal cells of (B) rat and (C) adult monkey were immunolabelled. (D) In the cortex of 1-month-old monkey brain, labelling was much more prominent in glial cells (arrowheads) than it was in adult monkey. Arrows indicate the immunolabelled neurons. Scale bars, 280 μm (A), 50 μm (B–D).

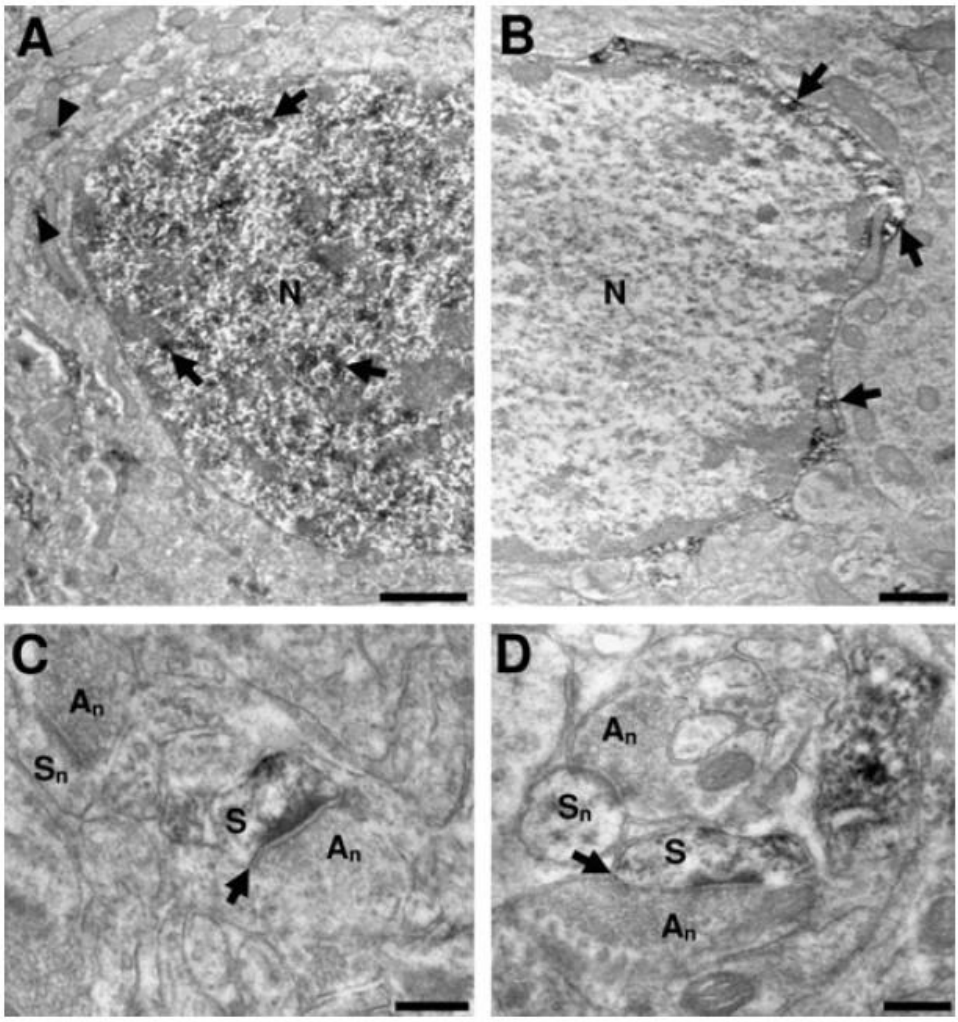

Fig. 7. Electron microscopic-level immunostaining for RGS12 in area 46 of monkey brain.

(A) Labelling in the nucleus (arrows) of a cell where cytoplasmic staining (arrowheads) was also observed. (B) A cell where only the cytoplasmic compartment (arrows) is labelled. (C and D) Postsynaptic localization of this protein. Arrows in C and D indicate the synapses of immunoreactive spines. N, nucleus; An, unlabelled axon; Sn, unlabelled spine; S, labelled synapse. Scale bars, 1 μm (A), 500 nm (B and C), 200 nm (D).

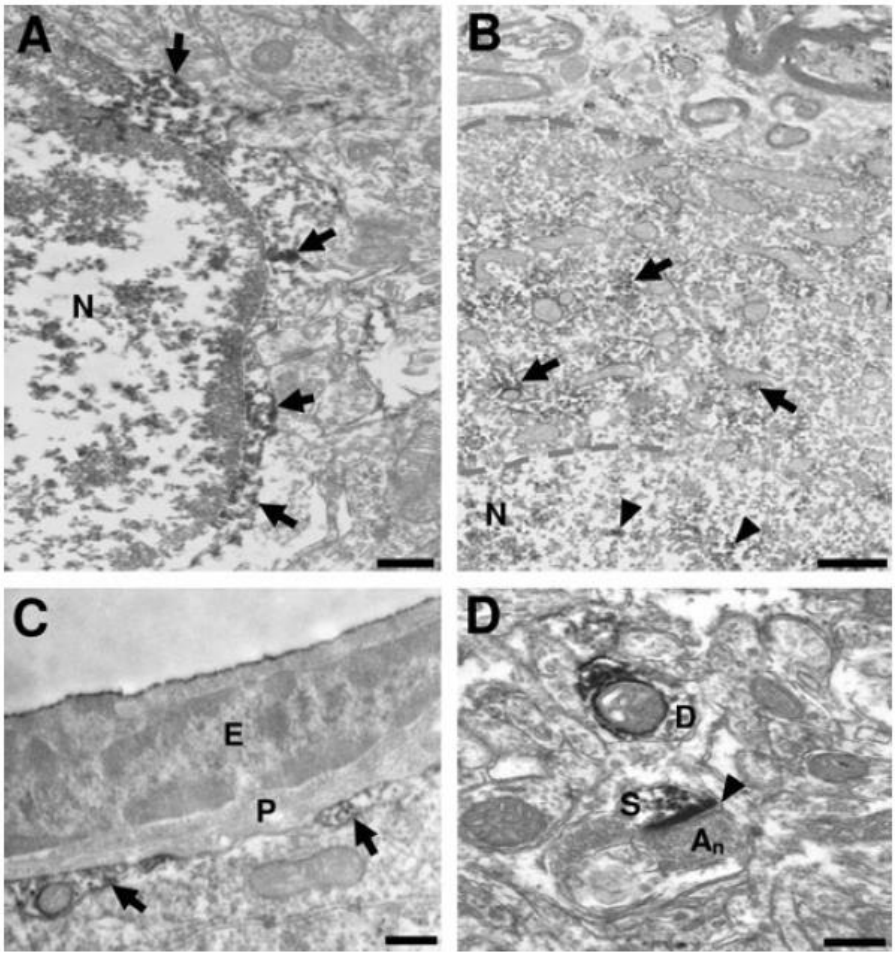

Fig. 8. Immunolabelling of RGS14 in area 46 of monkey brain at the electron microscopic level.

(A) An astrocyte cell where arrows indicate the typical astrocytic labelling. (B) Representative immunolabelled neuron showing labelling in cytoplasm (arrows) as well as in the nucleus (arrowheads). (C) Dendritic structures surrounding blood vessels (arrows) were also stained. (D) Postsynaptic localization of RGS14 in spines and in dendrites. N, nucleus; E, epithelial cells; P, pericytic profile; An, unlabelled axon; D, labelled dendrite; S, labelled synapse. Scale bars, 250 nm (A), 1 µm (B), 200 nm (C and D).

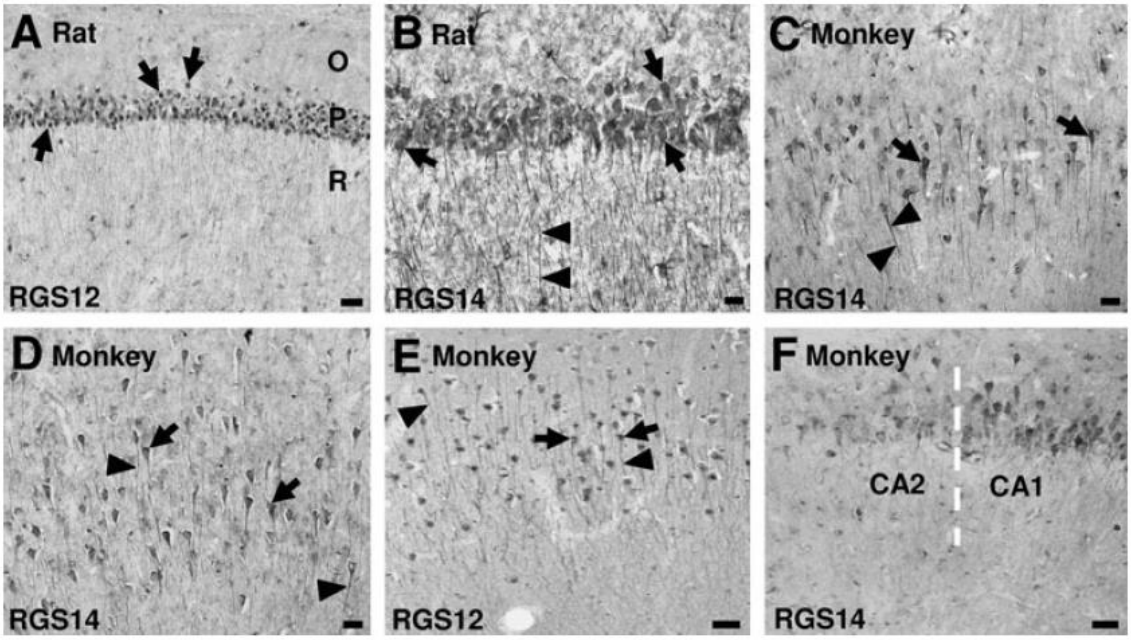

Fig. 9. Labeling of RGS12 and RGS14 in hippocampus.
Pyramidal cells (arrows) and proximal dendrites (arrowheads) of the CA1 field showed immunoreactivity with (A and E) RGS12 antibodies and (B and C) RGS14 antibodies. (D) RGS14 labelling in neurons and dendrites of the subiculum. (F) Pyramidal cells of the CA2 region expressed much lower RGS14 protein than did those of CA1. Scale bars, 100 μm (A and E), 50 μm (B–D and F).

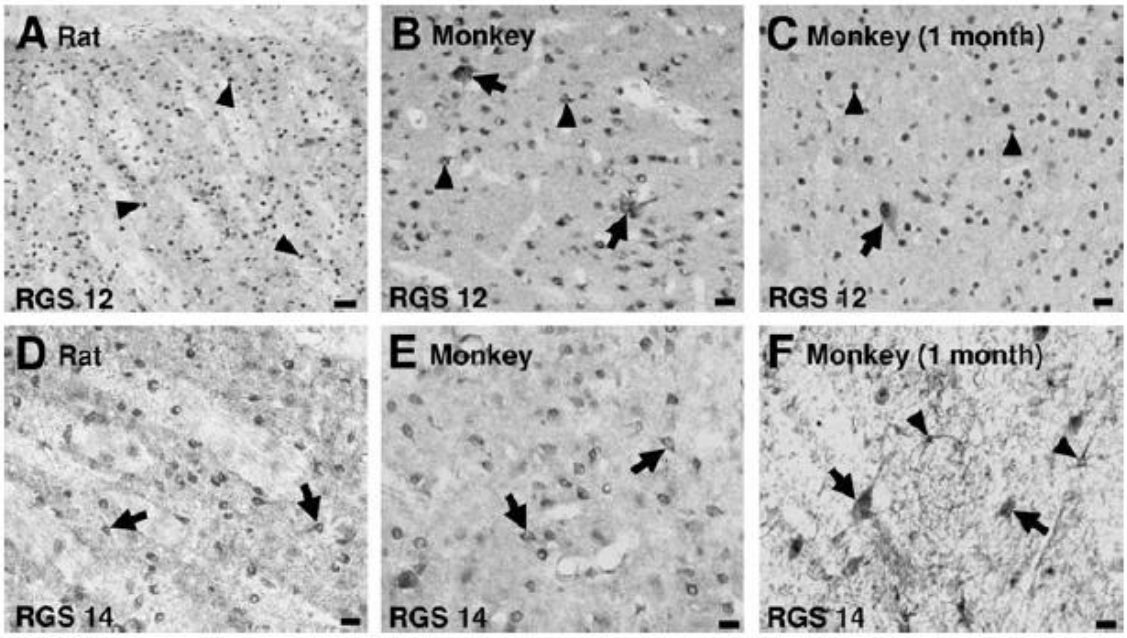

Fig. 10. RGS12 and RGS14 in striatum.
(A) A prominent nuclear labelling of RGS12 in striatal neurons (arrowheads) of rat brain was observed. (B) However, in monkey brain the immunoreactivity was associated with cytoplasm and was absent in the nucleus. (C) In contrast to adult monkey but similar to reactivity seen in rat, in 1-month-old monkey the labelling was present in nucleus. In B and C, arrows indicate large cholinergic neurons and arrowheads show small-size cells. (D and E) RGS14 immunostaining was in spiny neurons; (F) however, in 1-month-old monkey, this protein was also found in astrocytes. In D, E and F, arrows indicate the labelled cells and arrowheads show immunostained astrocytes. Scale bars, 100 μm (A), 50 μm (B–F).

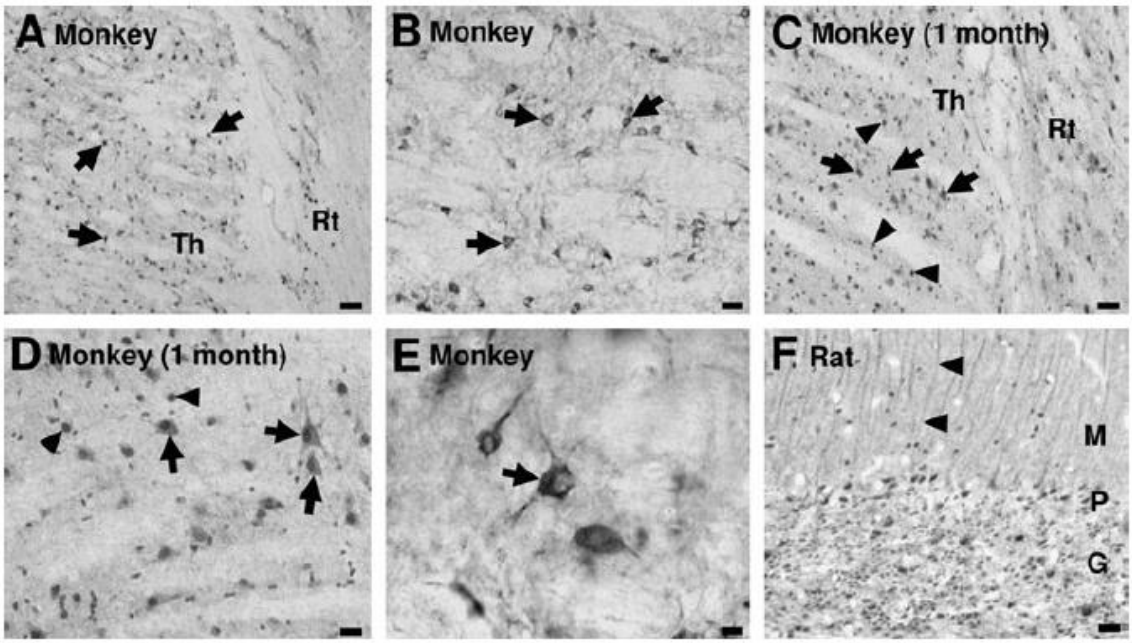

Fig. 11. RGS12 in thalamus and cerebellum.
(A) Immunostaining in the reticular as well as in the thalamic nucleus was observed. (B and E) High magnification of thalamic neurons showing no nuclear localization. (C and D) However, in 1-month-old monkey, RGS12 was present in two populations, one in which the nucleus was labelled (arrowheads) and other in which cytoplasm was stained (arrows); C is at low and D at high magnification. (F) The expression of RGS12 protein in cerebellum where Purkinje cells, granular layer cells and a few cells in the molecular layer were immunostained. Labeling in dendrites of Purkinje cells (arrows) was also observed. Th, thalamus; Rt, reticular nucleus; M, molecular layer; P, Purkinje layer; G, granule cell layer. Scale bars, 280 µm (A), 50 µm (B, D and F), 100 µm
(C), 25 µm (E).

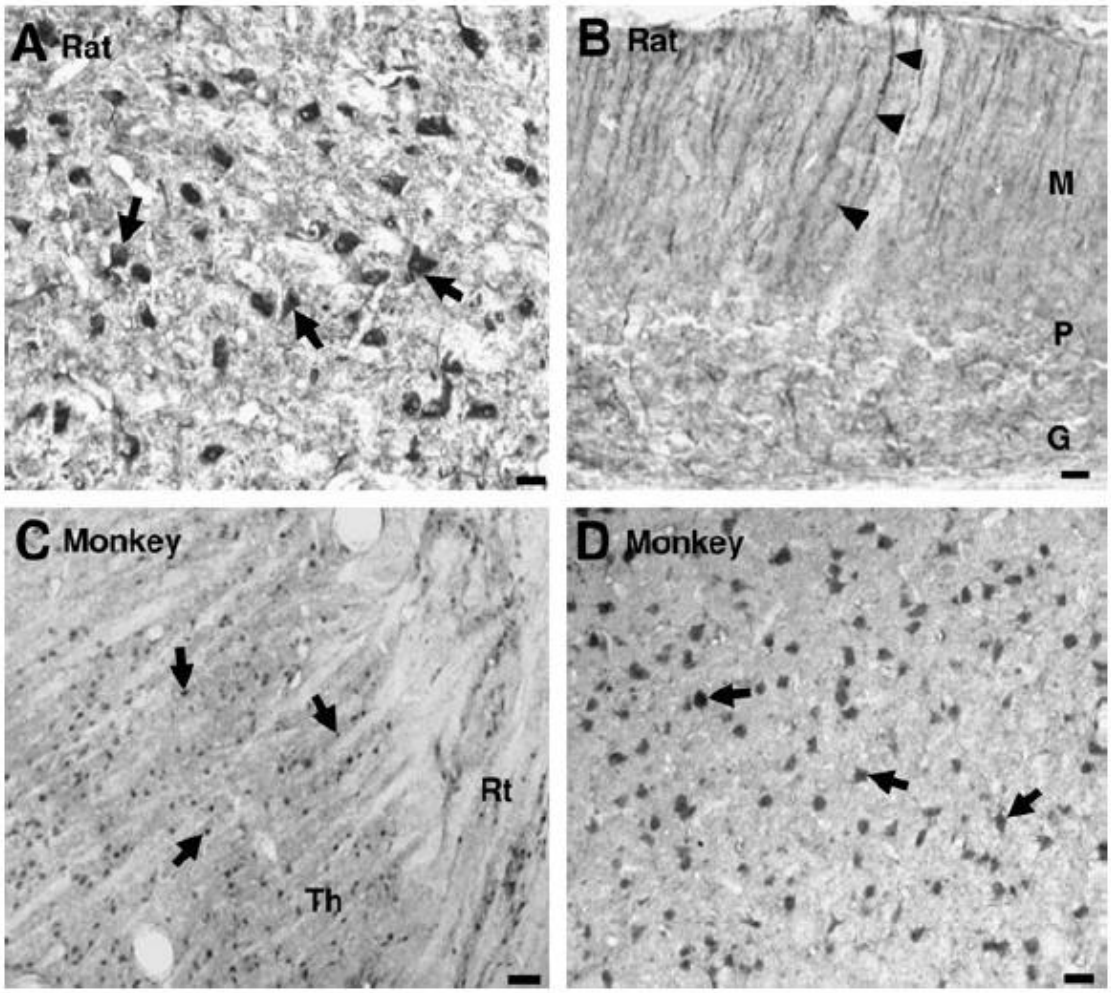

Fig. 12. RGS14 in thalamus and cerebellum.
The labelling of RGS14 was observed in both reticular nuclei and thalamus. (A) Labelling in thalamic neurons (arrows). (B) In cerebellum, staining was seen in fibre-like structures of the molecular cell layer. Cellular labelling was absent in all three cell layers. (C) Low magnification of thalamic neurons; (D) labelling in neurons at high magnification. M, molecular layer; P, Purkinje layer; G, granule cell layer; Th, thalamus; Rt, reticular nucleus. Scale bars, 50 μm (A and B), 280 μm (C), 100 μm (D)

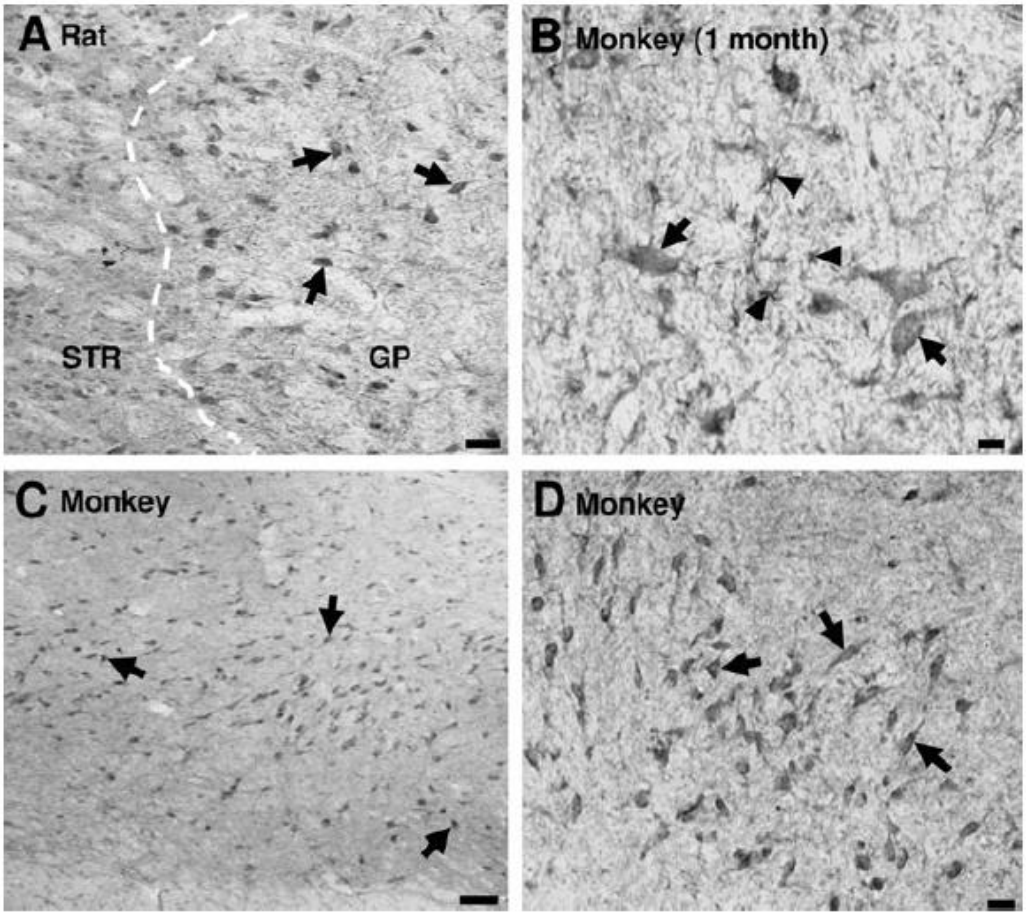

Fig. 13. RGS14 in substantia nigra and globus pallidus.
(A) In rat, immunolabelling in globus pallidus was observed only in large cells; (B) however, in1-month-old monkey, staining was found in neurons (arrows) as well as in astrocytes (arrowheads). (C) Low magnification and (D) high magnification of cells labelled in substantia nigra (arrows). STR, striatum; GP, globus pallidus. Scale bars, 100 µm (A), 50 µm (B and D), 280 µm (C).

TABLE 1. Expression pattern of RGS12 and RGS14 proteins in brain

Localization sites	RGS12 labelling	RGS14 labelling
Cerebral cortex	Very strong intensity	Strong intensity
Pyramidal and nonpyramidal neurons	Yes	Yes
Apical dendrites	Yes	Yes
Astrocytes	No	Yes
Nucleus	Yes (predominantly)	Yes (only at EM level)
Cytoplasm	Yes	Yes (predominantly)
Layer I fibres	Yes	No
Presynaptic	No	No
Postsynaptic	Yes	Yes
Striatum	Strong intensity	Very strong intensity
Neurons of rat brain	Nucleus	Cytoplasm
Neurons of monkey	Cytoplasm	Cytoplasm
Neurons of 1-month-old monkey	Nucleus	Cytoplasm
Medium size spiny neurons	Yes	Yes
Large cholinergic neurons	Yes	No
Astrocytes	No	Yes
Hippocampus	High intensity	Strong intensity
Neurons in rat	Yes (mostly in nucleus)	Yes (cytoplasm)
Neurons in monkey	Yes (cytoplasm)	Yes (cytoplasm)
Ca1	Yes	Yes
Ca2	Yes	No
Stratum radiatum	Yes (low)	Yes
Astrocytes	No	Yes
Midbrain	Medium intensity	Medium intensity
Neurons	Nucleus	Cytoplasm
SN-pars reticulata	Yes	Yes
SN-pars compacta	Yes (in TH-positive cells)	Yes in Monkey (and no in rat)
Thalamus	Strong intensity	Medium intensity
Neurons in rat	Nucleus	Cytoplasm
Neurons in monkey	Cytoplasm	Cytoplasm
Neurons in 1-month-old monkey	Nucleus and cytoplasm	Cytoplasm
Reticular nucleus	Yes	Yes
Lateral geniculate nucleus	No	Yes (very strong)
Lateral dorsal nucleus	Yes (low)	Yes (strong)
Cerebellum	Medium intensity	Medium intensity
Purkinje cells	Yes (nucleus)	No
Dendrites of Purkinje cells	Yes	Yes
Granule cells	Yes	No
Globus pallidus	Medium intensity	Strong intensity
Inferior colliculus	Medium intensity	Medium intensity

The labelling intensity is described as low, medium, high, strong and very strong. TH, tyrosine hydroxylase.