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Research report

The Ca^{2+} binding protein, frequenin is a nervous system-specific protein in mouse preferentially localized in neurites

Petur Olafsson [a], Holly D. Soares [b], Karl-Heinz Herzog [b], Ti Wang [a], James I. Morgan [b], Bai Lu [a,*]

[a] *Laboratory of Developmental Neurobiology, NICHD, NIH, 49 Convent Dr., MSC 4480, Bethesda, MD 20892-4480, USA*
[b] *Department of Developmental Neurobiology, 332 North Landerdale, St. Jude Children's Research Hospital, Memphis, TN 38105, USA*

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Abstract

Frequenin is a Ca^{2+}-binding protein that has been implicated in the regulation of neurotransmitter release at the neuromuscular junction [15,16]. However, its cellular and subcellular localization in brain have not been determined. Therefore, we cloned mouse frequenin (Mfreq) and investigated its expression both in vivo and in vitro. The amino acid sequence of Mfreq is homologous to that of frequenins from other species. Northern and Western blot analyses indicated that the Mfreq mRNA is a single species of 4.2 kb, and that the protein has a mass of 24 kDa protein on SDS gel, respectively. Expression of Mfreq is nervous system specific. However, Mfreq mRNA and protein are widely distributed in the brain, spinal cord, and dorsal root ganglia. Mfreq is expressed in early embryonic brain and the levels of Mfreq remain high throughout development. In situ hybridization and immunocytochemistry demonstrated that Mfreq is expressed primarily in neurons and presumptive astrocytes. The Mfreq protein was preferentially localized in neurites (dendrites and axons). Double immunofluorescence microscopy established that Mfreq was co-localized with the dendritic marker, MAP-2 and the synapse marker, SV2 in cultured hippocampal neurons. The distribution and subcellular localization of Mfreq may help understand its cellular function.

Keywords: Frequenin; Ca^{2+}-binding protein; Hybridization, in situ; Immunocytochemistry; Synapse; Astroglia

1. Introduction

Ca^{2+}-binding proteins (CBPs) belong to a large family of cytosolic proteins involved in Ca^{2+}-dependent signaling mechanisms in diverse systems [4]. The recoverin-like proteins (also called neuronal calcium sensor, or NCS) are a subfamily of CBPs that share several common features including their characteristic EF-hand motifs and specific localization in the nervous system [14]. While many members of this family have been discovered, their biological function remains largely unknown. Recently, characterization of a *Drosophila* mutant led to the discovery of a new member in this family, frequenin, which may shed light on the function of CBPs. The mutant fly, V7 over-expresses frequenin and shows an enhanced, frequency-dependent facilitation of transmitter release at neuromuscular junctions. Thus, paired-pulse facilitation was significantly enhanced, and high-frequency stimulation gave rise to much larger postsynaptic responses in these flies [16,18]. Detailed analyses of the *Drosophila* mutant have also revealed several additional effects of the protein. Ca^{2+}-dependent modulation of type A K^+ currents in muscle cells is absent in flies overexpressing frequenin [17]. In addition, the number and length of motor terminal branches in the mutant are significantly reduced in the mutant [1]. Moreover, introduction of exogenous frequenin into *Xenopus* embryonic spinal neurons by early blastomere injection resulted in an enhancement of synaptic efficacy at the neuromuscular junction [15]. Taken together, these results suggest that frequenin may be involved in synaptic transmission and plasticity.

Despite this progress, it is currently difficult to envision the cellular and molecular mechanisms by which frequenin achieves its effects at the neuromuscular junction. While high levels of frequenin have also been detected in the central nervous system (CNS) of several species [5,14–16], little is known of its distribution in the mammalian nervous system. Indeed, it is not known whether it is present

* Corresponding author. Fax: +1 (301) 480-3526.

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in neurons or glia or both. Furthermore, knowledge of its cellular and subcellular localization could provide vital clues to its function. For example, localization of frequenin to synapses would suggest a role in regulation of synaptic efficacy. In the present study, we cloned mouse frequenin (Mfreq), and examined its tissue distribution and developmental expression. Moreover, we studied the cellular and subcellular localization of frequenin in neurons and glia by in situ hybridization and immunocytochemistry. Our results support the notion that frequenin is a protein preferentially localized in neuronal processes.

2. Materials and methods

2.1. Cloning of mouse frequenin

Degenerated primers MR-2 (5′-ATW TAY AAR CAR TTY TTY CC-3′) and MR-6 (5′-CCR TCW SWR TTY TTR TCC AT-3′), derived from identical sequences of *Drosophila* and *Xenopus* frequenin [15,16], were used for polymerase chain reaction (PCR) with DNA from a mouse brain cDNA library (Strategene). The resulting 335 bp fragment was cloned into M13mp18 vector and sequenced. This fragment has 82% identity at the DNA level to the *Xenopus* frequenin gene. The missing 5′ and 3′ sequences were obtained from mouse brain mRNA by using a protocol for the rapid amplification of cDNA ends (RACE) [8]. The primers used were: MF-7 (5′-ACC CTC TTC TCT GGT GTG-3′) and MF-8 (5′-GAT GTA GCC ATC GTT ATC-3′) for the 5′-end, and MF-9 (5′-AAC GTC TTC GAC GAG AAC-3′) and MF-10 (5′-ATG CTC GAC ATA GTG GAC-3′) for the 3′-end. The 3′-RACE fragment was 345 bp in length, the one from the 5′-end 567 bp. The overlapping sequences, a total of 924 bp, cover the entire coding region of Mfreq except the initiation ATG codon.

2.2. Northern blotting

Total RNAs extracted from brain and a number of different tissues were separated by 1% agarose gel electrophoresis, and transferred to nitrocellulose filters. The 5′-end cDNA fragment (567 bp) was labeled with [α-^{32}P]dCTP by nick translation to a specific activity of around 10^9 cpm/μg. This probe was used to hybridize with the nitrocellulose filter at high stringency (65°C). The filter was then washed at 54°C in 0.1 × SSC and exposed to Kodak XAR-5 films.

2.3. Western blotting

Mouse tissues were quickly dissected, frozen in liquid nitrogen, and stored at −80°C before use. The tissues were homogenized in 1% SDS using a Dounce-homogenizer followed by sonication. Protein samples (40 μg for each sample) were mixed with loading buffer (0.125 M Tris–HCl, pH 6.8, 20% glycerol, 3% SDS, 3% β-mercaptoethanol, bromophenol blue), separated by SDS-polyacrylamide electrophoresis (0.025 M Tris, 0.2 M glycine, 0.1% SDS), and blotted onto nitrocellulose filters. The nitrocellulose filters were blocked by milk-TBS (2% powder milk, 20 mM Tris, pH 7.4, 100 mM NaCl, 0.1% Triton X-100). An antiserum (XF-3C) raised against recombinant *Xenopus* frequenin showed a significant cross reactivity with Mfreq, and therefore was used to react with the filter. Mfreq protein was detected by a horseradish peroxidase method coupled to the 4-chloronaphthol (Sigma)/H_2O_2 color reaction.

2.4. In situ hybridization

Adult mice were intracardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer/diethylpyrocarbonate (DEPC) water, pH 7.4. Tissue was extracted and post-fixed for an additional 4 h at 4°C then transferred to 25% sucrose in 0.1 M phosphate/DEPC-treated water, pH 7.4. In situ hybridization and autoradiography were performed as described [12]. Briefly, sections were treated for 10 min with 0.001% proteinase K in 0.1 M Tris-EDTA buffer followed by a rinse in distilled water. Sections were acetylated for 10 min with acetic anhydride in 0.1 M triethanolamine (TEA) pH 8.0 and rinsed in 0.1 M TEA. Sections were then rinsed in 2 × SSC and dehydrated. Subsequently, sections were hybridized at 55°C overnight with 100 μl of 5×10^6 cpm/ml ^{33}P-labeled riboprobes generated by in vitro transcription (T_3 for sense and T_7 polymerase for antisense probes). Sections were rinsed in 2 × SSC for 15 min, then incubated for 30 min at 37°C with 100 μg/ml of RNase A in 0.5 M NaCl, 10 mM Tris–HCl and 1 mM EDTA. After RNase treatment, sections were washed for 30 min at 50°C with the RNase buffer. Sections were then rinsed for one hour at 60°C with 2 × SSC and one hour in 0.2 × SSC then dehydrated in ethanol. Slices were dipped in NTB2 emulsion and developed 8 days later.

2.5. Immunocytochemistry

Rabbit antibody XF-3C was used in conjunction with mouse monoclonal antibodies against specific antigens for double immunostaining. Mixed cultures of hippocampal neurons and glia derived from E17 mice were plated on plastic coverslips and grown in low serum (1%) medium at a density of 0.1 million cells/well in a 12-well dish for 10–15 days, according to standard procedure [11]. The cultures were fixed with 4% paraformaldehyde in 0.1 M $NaPO_4$, pH 7.4 for 1 h at room temperature. Immunofluorescence staining was performed at room temperature as follows: (1) washing for 2 × 5 min in 0.1 M $NaPO_4$, pH 7.4, then 1 × 30 min with 0.1 M glycine/NaOH, pH 7.4. (2) Permeablizing with 0.3% Triton X-100 in 0.1 M $NaPO_4$ (washing buffer) for 30 min. (3) Incubation in

blocking solution (10% horse serum in washing buffer) for 1 h. (4) Incubation with two primary antibodies in blocking solution for 2 h. One was rabbit anti-frequenin polyclonal antibody XF3C, 1:200. The other was one of the following mouse monoclonal antibodies (all from Boehringer Mannheim except anti-SV2, which was kindly provided by Dr. Richard Scheller): anti-SV2 1:1000; anti-Map-2, 1:100; anti-GFAP, 1:200. (5) Washing 3×5 min with washing buffer. (6) Incubation with Cy3 conjugated goat anti rabbit IgG antibody (1:150) and FITC conjugated goat anti mouse IgG antibody (1:200) in blocking solution for 2 h. (7) Washing 3×5 min in washing buffer and 1×5 min in H_2O. (8) The coverslips were mounted onto glass slides with Vectorshield mounting solution (Vector), and examined and photographed with a Zeiss microscope.

3. Results

3.1. Cloning and expression of mouse frequenin

A pair of degenerate primers based upon the *Drosophila* and *Xenopus* frequenin sequences were used to PCR clone a 355 bp fragment of mouse frequenin from a mouse brain cDNA library. Upon sequencing, this was found to be 82% identical to the *Xenopus* coding sequence. Additional 5′ and 3′ RACE PCR resulted in the isolation of a 924 bp sequence that covers the entire frequenin coding region (Fig. 1). This Mfreq sequence has 83% identity to *Xenopus* frequenin at the nucleic acid level and 98% identity at the amino acid level. Like *Xenopus* frequenin, Mfreq has four putative Ca^{2+}-binding motifs and an N-terminal myristoylation site. Northern blot using a Mfreq cDNA probe detected a single mRNA of 4.2 kb (data not shown).

Recent studies have identified a subgroup of CBPs called neural calcium sensors (NCS) in *C. elegans*, chicken and rat [13,14]. Sequence comparison indicated that these proteins are highly homologous to frequenin from *Drosophila*, *Xenopus* and mouse and, therefore, should be considered as frequenin in these species. The nucleotide and amino acid sequences of frequenin are evolutionarily highly conserved. At the amino acid levels, chick, mouse and rat frequenin sequences are 100% identical, and *C. elegans* frequenin differed by only 25% (Fig. 2A). To determine the relationship of frequenin to the other CBPs, we constructed a phylogenetic tree using the Geneworks program (Fig. 2B). The frequenins from different species clearly represent a subgroup of CBPs. An unexpected finding was that *C. elegans* frequenin was closer to *Xenopus* frequenin than *Drosophila*. Overall, the frequenin subgroup had a closer relationship with the neurocalcin/hippocalcin subgroup than with the recovering subgroup.

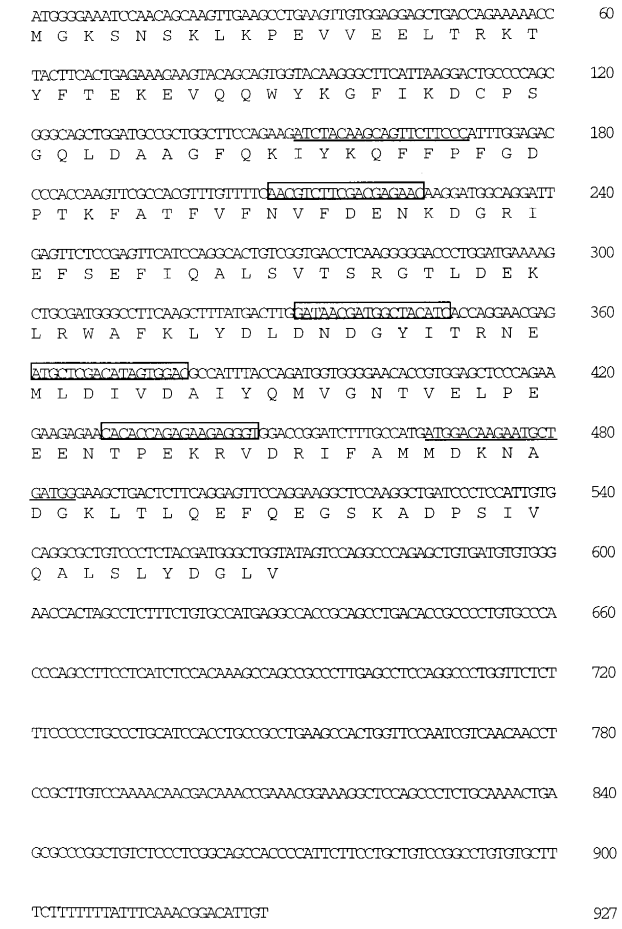


Fig. 1. Complete coding sequence for mouse frequenin. The positions for the degenerate primers used for PCR cloning are underlined (MR-2 and MR-6). Primer used for 5′ (MF-7 and MF-8) and 3′ (MF-9 and MF-10) RACE-PCR are boxed.

3.2. Mfreq: a nervous system specific protein

An antibody raised against *Xenopus* frequenin (XF-3C) cross-reacted very well with the Mfreq protein. This was expected since Mfreq is 98% homologous to *Xenopus* frequenin at amino acid levels. Immunoblotting of proteins from mouse brain with this antibody identified a single band of approximately 24 kDa, which is close to the size of Mfreq (Fig. 3). The tissue distribution of Mfreq was determined by immunoblotting using the XF-3C antibody (Fig. 3A). Although hippocampus showed the highest levels of expression, Mfreq protein was present at roughly equivalent amount in most brain regions. In contrast, no Mfreq was detected in any of the non-neural tissues examined. Within the nervous system, Mfreq protein was observed as early as embryonic day 12 (E12), and gradually increased during embryonic development (Fig. 3B). Interestingly, during postnatal development an additional, slightly lower molecular weight band was identified (Fig. 3B). Whether this band represents a cross-reacting protein, alternative splicers, proteolic cleavage or other post-translational modification, remains to be investigated.

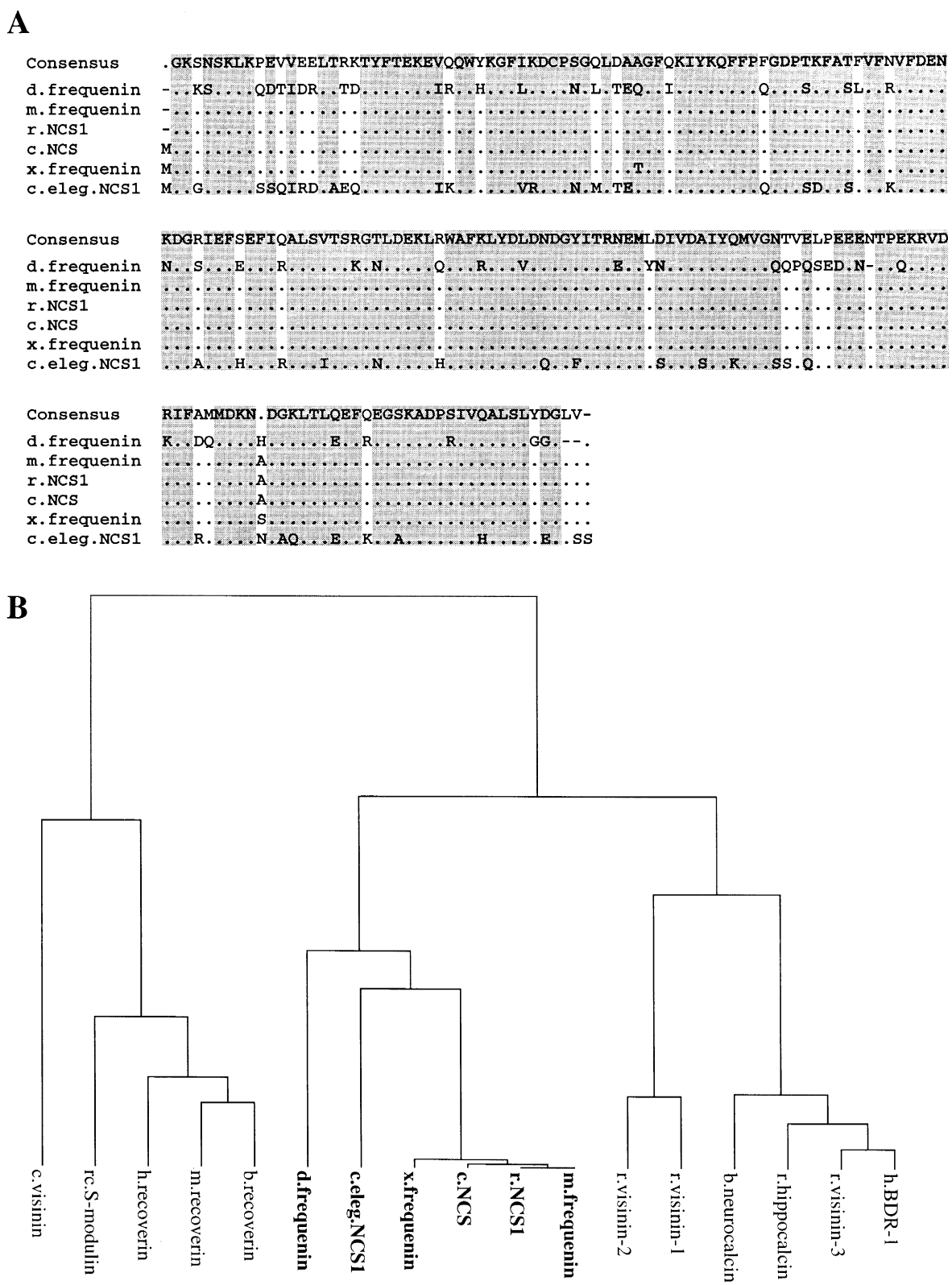


Fig. 2. (A) Amino acid sequence comparison of frequenin (also called neural calcium sensor, or NCS) in mouse and other species. The first letter denotes the species: m, mouse; r, rat; c, chicken; x, *Xenopus*, d, *Drosophila*, c.eleg., *C. elegans*. Dots indicate identical amino acids. Conserved sequences are shown as shaded areas. (B) Phylogenetic tree showing the relationship of the frequenin family of proteins with other calcium binding proteins. The evolutionary relationships of these sequences are calculated by GeneWorks program. The length of horizontal lines is proportional to the estimated genetic distance between the sequences.

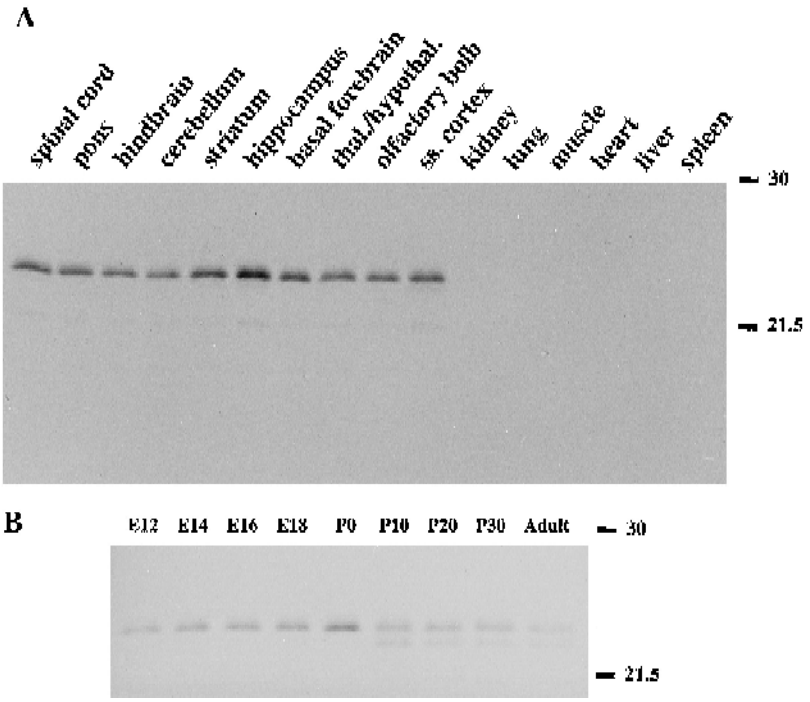


Fig. 3. (A) Tissue distribution of Mfreq protein detected by Western blots. Equal amounts of proteins (40 μg/lane) from adult tissues as indicated were separated by SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The filter was reacted with a polyclonal antibody against frequenin, followed by a detection method based on horseradish peroxidase reaction. Note that only neural tissues exhibit a band at about 24 kDa. (B) Expression of Mfreq protein during development. The same method as above was used to detect Mfreq from whole brain of embryonic and neonatal mice at the indicated stages. Forty μg of whole brain proteins were loaded in each lane. Note that a second, slightly lower molecular weight protein is detected in postnatal brain.

The cellular localization of Mfreq mRNA was determined by in situ hybridization. ^{33}P-labeled sense and antisense riboprobes were generated by in vitro transcription. Sense probe did not detect any specific signals (data not shown), whereas antisense probe detected neural specific expression of Mfreq mRNA (Figs. 4 and 5). No Mfreq signal was detected in non-neural tissues such as liver and muscle (Fig. 4A,B). The Mfreq mRNA was widely distributed within the peripheral and central nervous systems. Positive signals were found in dorsal root ganglion (DRG), and in the ventral horn of spinal cord (Fig. 4C and Fig. 5A). Within the forebrain, the expression of Mfreq mRNA was high in cortex but low in caudate putamen (Fig. 5B). High levels of Mfreq message were also found in cerebellar cortex (Fig. 5C). In the hippocampus, the expression of Mfreq was evident in CA3 and CA1 pyramidal neurons, dentate granule neurons, and presumptive astrocytes (Fig. 5D).

3.3. Neuronal and glial expression of Mfreq

In the peripheral nervous system, Mfreq appeared to be localized only to neurons but not glia (Schwann cells). In the DRG, for example, Mfreq was expressed in sensory neurons (Fig. 4C) but not in Schwann cells (Fig. 4C). Within the spinal cord, Mfreq signals were concentrated in gray matter (Fig. 4C and Fig. 5A), suggesting that Mfreq mRNA was localized to neurons and/or astroglia but not to oligodendrocytes, which are primarily in the white matter. In the cerebellum, Mfreq signals were primarily associated with the granular and molecular layers, which consist of Purkinje and basket neurons, and Bergman glia (a type of glia) (Fig. 5C). These results suggest that frequenin contributes to both neuronal and astroglial function within the nervous system.

To confirm neuronal as well as astroglial expression of Mfreq protein, immunocytochemistry experiments were performed on hippocampal cultures. Intense Mfreq positive staining was found in neurons, which were identified by co-staining with the neuronal marker neuron specific enolase (data not shown). However, low but readily detectable levels of Mfreq immunoreactivity were also observed in glial fibrillary acidic protein (GFAP)-positive astroglia (Fig. 6E).

3.4. Subcellular localization of Mfreq

In adult hippocampus, the immunocytochemical staining of Mfreq was primarily localized in the stratum radiatum and stratum moleculare but not the stratum pyramidale. This result suggests that the protein is preferentially localized on neuritic processes and not in the cell body (data not shown). Double immunofluorescence of hippocampal cultures were used to determine the subcellular

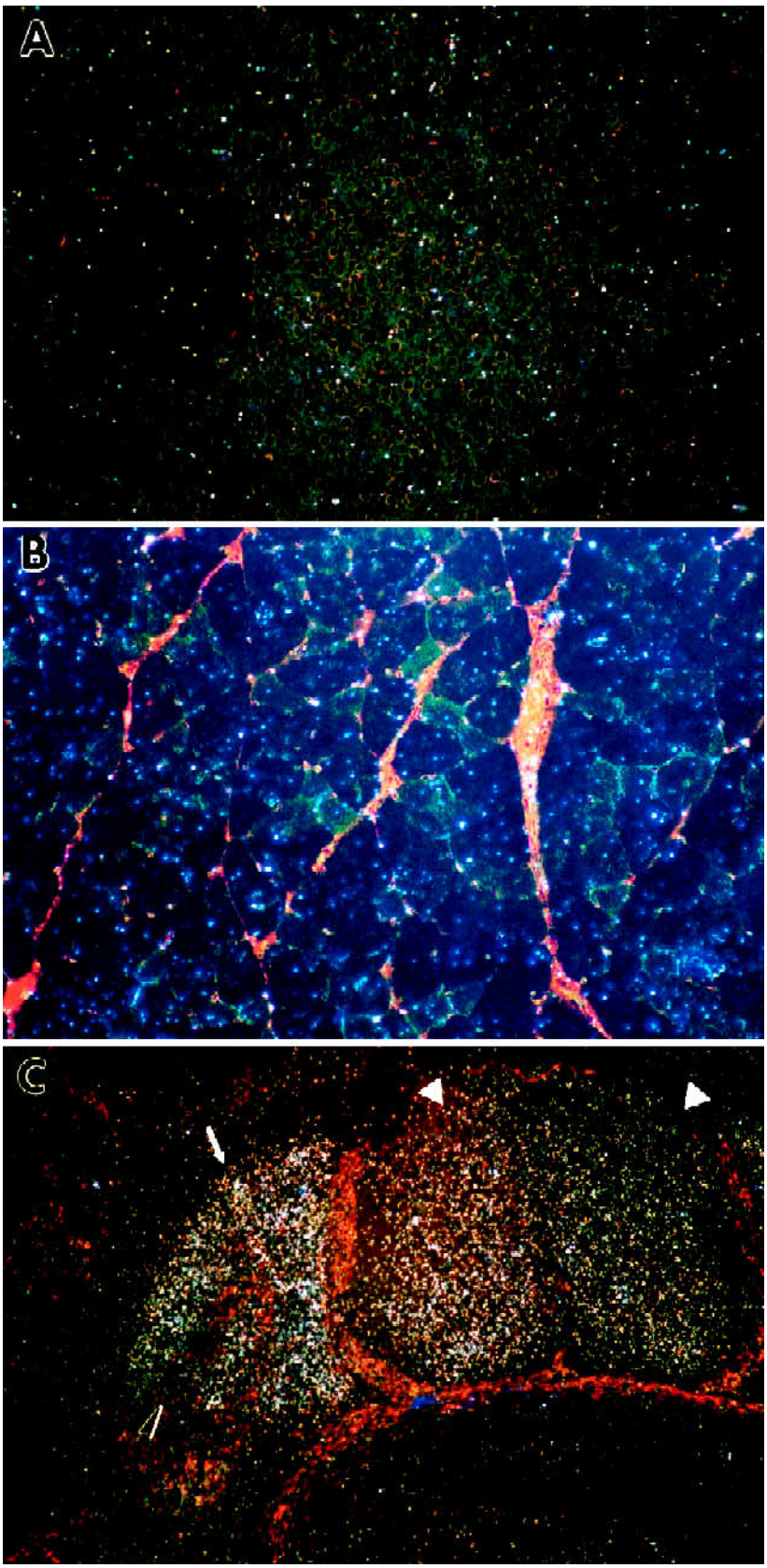


Fig. 4. Tissue distribution of Mfreq mRNA determined by in situ hybridization. An antisense riboprobe for Mfreq was used to label sections from liver (A), skeletal muscle (B) and spinal cord (C). Neural tissues, such as spinal cord exhibit high levels of expression while no expression was detected in liver and skeletal muscle. In dorsal root ganglion (DRG), Mfreq is expressed in sensory neurons (white arrows) but not in Schwann cells (black arrowhead). Within the spinal cord, Mfreq signal was concentrated in gray matter (white arrowheads).

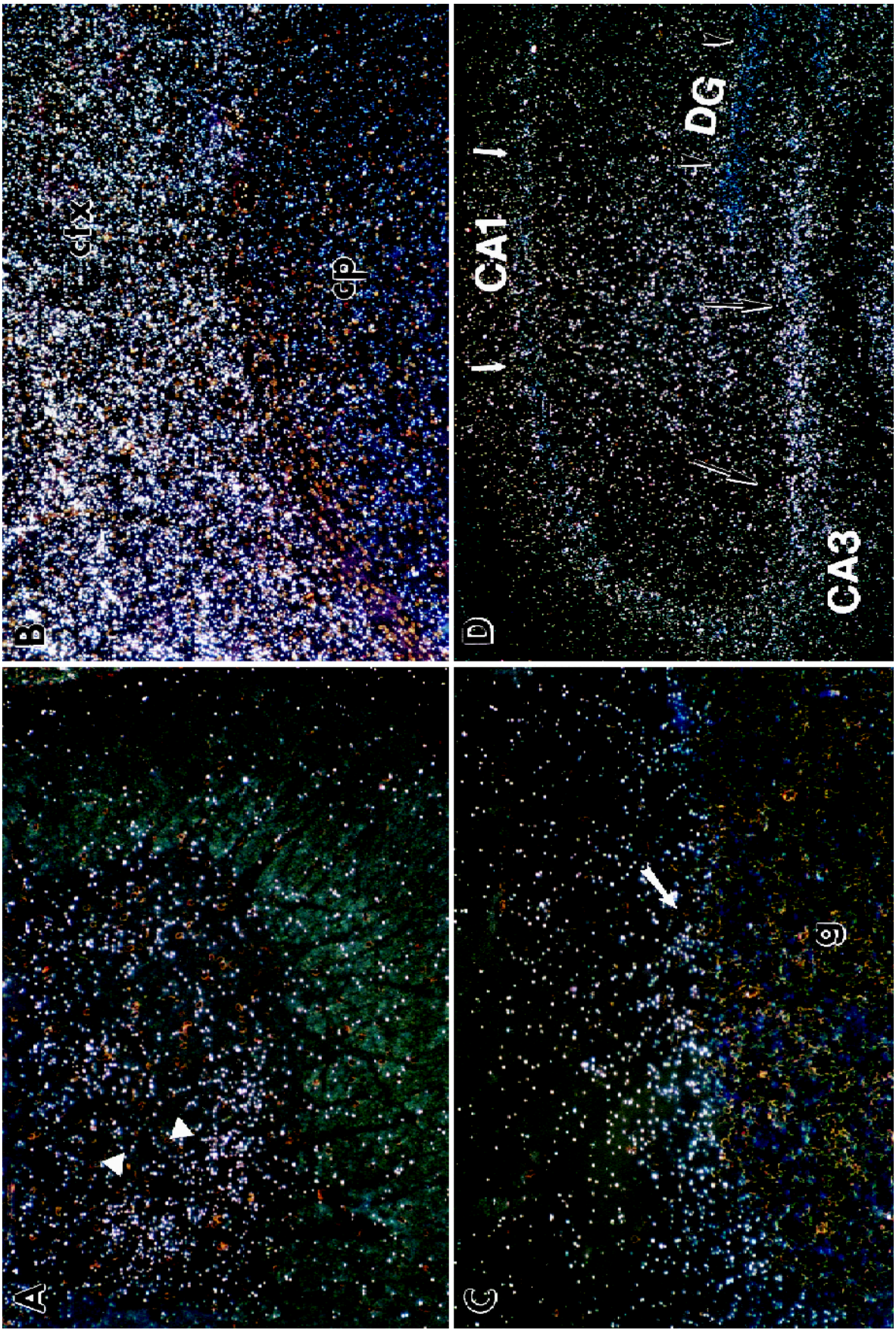
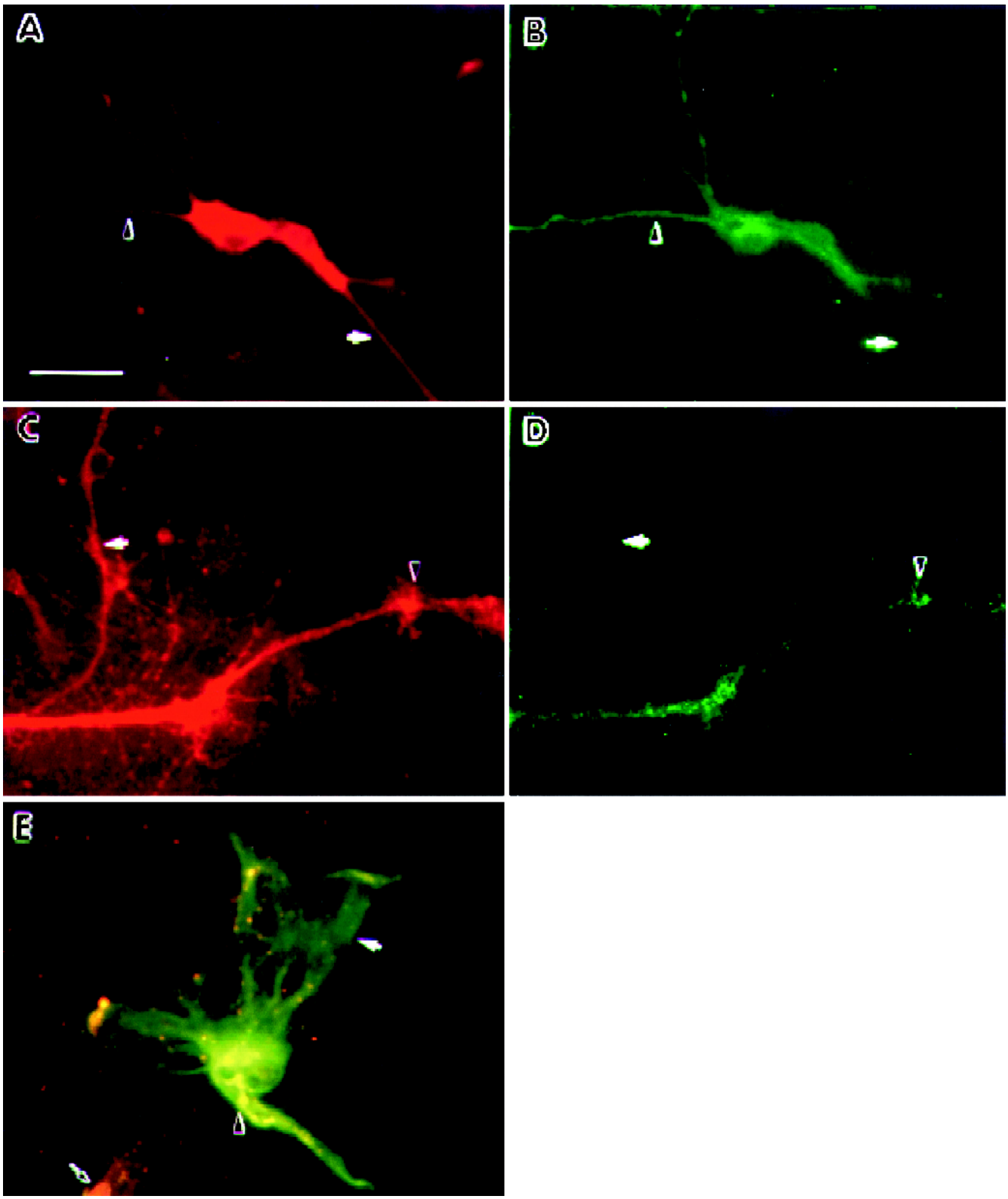


Fig. 5. In situ hybridization for Mfreq mRNA in adult mouse brain. (A) Mfreq mRNA in the ventral horn of spinal cord. Signal is diffuse throughout spinal cord gray matter with localization of labeling over spinal motor neurons (white arrowheads). (B) Mfreq mRNA in the forebrain. Cortex (ctx) exhibits high levels of signal while labeling within the caudate putamen (cp) is low. (C) Mfreq mRNA in the cerebellum. Most of label in the cerebellum is localized to the Purkinje cell layer where Bergman glia and Purkinje soma reside. (D) Mfreq mRNA in the hippocampus. Hybridization signal is present in CA3 (black arrows), CA2 and CA1 (white arrows) neurons. Expression is detectable in granule cells of the dentate gyrus (DG, arrowheads).

localization of Mfreq protein. Map-2 has been frequently used as a marker for neuronal dendrites [7]. Virtually all Map-2 positive dendrites were stained by anti-frequenin antibody (Fig. 6A,B, black arrowhead). However, Map-2 negative processes, which are most likely axons, also expressed a high level of Mfreq (Fig. 6A, white arrow).

Neurons containing excess amount of frequenin exhibit enhanced synaptic transmission [15,16]. Double immunofluorescence staining indicated that frequenin was distributed along the processes containing SV2 positive varicosities (Fig. 6C,D, arrowhead). Thus, the protein is indeed localized in the presynaptic compartments as well.



4. Discussion

In the present study, we cloned the mouse CBP, frequenin and demonstrated that it is a member of the recoverin-like protein family that are specifically expressed in the nervous system. The recoverin-like proteins can be divided into two subgroups: those that restrict their expression in the pineal gland and retina (such as recovering [6], visinin [20], S-modulin [9]); and those that are widely distributed in the brain (such as neurocalcin [19] and VILIP [10]). It is clear that frequenin belongs to the latter. In *Drosophila*, frequenin is highly concentrated in the central and peripheral nervous system, particularly in neuropil and synaptic regions [16]. Although frequenin has also been shown to be a nervous system specific protein in *Xenopus* [15], and chicken [14], detailed analysis of its regional distribution has not been performed. Our study indicated that the expression of frequenin within the nervous system is quite ubiquitous. However, subtle differences in cellular distribution can be detected by in situ hybridization. For example, frequenin was expressed in the Purkinje cell layer of the cerebellum but not in the granule cell layer. Within the spinal cord, frequenin was present in gray matter but not in fiber tracts.

Although frequenin has been shown to be a nervous system specific protein, the cellular localization of the protein has not been resolved. The present study suggests that frequenin is localized to the nervous system where it is expressed in both neurons and astrocytes. It appears to be absent in Schwann cells and oligodendrocytes as well as some neuronal populations such as cerebellar granule cells. Thus, frequenin may play a role in both neuronal and glial biology.

Physiological experiments using *Drosophila* and *Xenopus* neuromuscular junctions have indicated a presynaptic function of frequenin [15,16,18]. In the present study, we used immunocytochemistry to determine the subcellular localization of the protein in neurons. Synaptic localization has also been confirmed by co-localization of the protein with the synaptic vesicle protein, SV2. However, functional roles of frequenin in presynaptic terminals are not well established. In *Drosophila*, over-expression of frequenin in mature motoneurons leads to facilitation of synaptic responses to high frequency stimulation (>5 Hz), while basal synaptic transmission (<1 Hz) is not affected [16,18]. In *Xenopus*, however, loading of exogenous frequenin into developing spinal neurons results in enhancement of basal synaptic transmission [15]. It is also unclear whether frequenin modifies the secretion process itself, or regulate the electric signals associated with transmission, such as Ca^{2+} or K^{+} channels. A recent study indicated that in crayfish, a frequenin-like molecule is selectively expressed in phasic but not tonic motoneurons [3]. The nerve terminals of phasic neurons have high initial quantal output, but exhibit less facilitation and more depression than those of tonic neurons [2]. It is possible that the ability of frequenin to enhances transmitter release may depend upon the intrinsic properties of the neurons. High levels of frequenin in phasic neurons may ensure high quantal output. In contrast, high frequency activity may be necessary for frequenin to function at low level in tonic neurons. Consistent with this idea is our present finding that different levels of frequenin are expressed in different population of neurons in the brain. The relationship between the level of frequenin, and synaptic properties in different types of neurons represents an important area for future research.

Finally, our double staining experiments indicated that frequenin is not restricted to the presynaptic terminals and axons, since it is also present in the postsynaptic dendrites. Whether frequenin is localized in synaptic vesicles or postsynaptic densities requires further investigation using electron microscopy. Nevertheless, our results raised an interesting and testable hypothesis, namely, frequenin plays a role not only in presynaptic transmitter release but also in the regulation of postsynaptic properties. Physiological experiments that introduce frequenin into neurons will test this hypothesis.

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Fig. 6. Double immunofluorescence staining of hippocampal cultures. The cultures were stained with a rabbit anti-frequenin antibody (A, C and E) and a mouse monoclonal antibody against MAP-2 (B), SV2 (D), or GFAP (E). The Mfreq was detected by rhodamine-conjugated secondary antibody (red fluorescence) and other markers were detected by fluorescence-conjugated secondary antibody (green fluorescence). Bar: 20 μm. (A and B) Staining with anti-Mfreq and anti-Map-2. A Map-2 and Mfreq positive dendrite was indicated by black arrowhead. White arrow indicates a Mfreq positive, Map-2 negative process, presumably axon. (C and D) Staining with anti-Mfreq and anti-SV2. A SV2 and Mfreq positive axon was indicated by black arrowhead. White arrow indicates a Mfreq positive, SV2 negative process, presumably dendrite. (E) Double staining with anti-Mfreq and anti-GFAP, indicating astroglial expression of Mfreq. Black arrowhead indicates double staining (yellow). White arrow indicates parts of the astroglia that is not stained by Mfreq antibody (green). Small black arrow indicates a Mfreq positive GFAP negative, unidentified cell type that occasionally observed in glial cultures (orange).

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