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Regional Expression and Cellular Localization of the α_1 and β Subunit of High Voltage-Activated Calcium Channels in Rat Brain

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The neuronal high voltage-activated calcium channels are a family of ion channels composed from up to five different α_1 and four different β subunits. The neuronal distribution and subunit composition of calcium channels were investigated using subunit-specific antibodies and riboprobes. The β subunit-specific antibodies identified the presence of β_{1a} in skeletal muscle; β_2 in heart; and β_2, β_3, and β_4 in brain. The β_3 protein was widely distributed in rat brain, with prominent labeling of olfactory bulb, cortex, hippocampus, and habenula. The β_4 protein was also widely expressed, most prominently in the cerebellum. β_2 protein was expressed at only low levels. *In situ* hybridization with β subunit-specific riboprobes confirmed the differential expression pattern of the individual subunits. Hybridization with riboprobes specific for the α_{1A}, α_{1B}, α_{1C}, and α_{1D} subunits showed a broad distribution of α_{1A} and α_{1B} transcripts, whereas the expression level of α_{1C} and α_{1D} mRNA was lower and more spatially restricted. The overall expression pattern and cellular localization suggested that β_4 may associate predominantly, but probably not exclusively, with the α_{1A} subunit, and β_3 with the α_{1B} subunit. In certain brain areas such as the habenula, the β_3 subunit may associate with other α_1 subunits too. Furthermore, the β_2 subunit may form complexes with different α_1 subunits in brain and cardiac muscle. These results demonstrate that a given β subunit may associate with different α_1 subunits in a cell type-dependent manner, contributing to the diversity of the neuronal calcium channels.

Key words: ion channel; calcium channel; brain; hippocampus; cerebellum; olfactory bulb; habenula

The high voltage-activated calcium channels are multimeric protein complexes containing the channel-forming α_1 subunit and the auxiliary β, α_2/δ, and γ subunits (for additional references, see Hofmann et al., 1994). The α_2/δ and γ subunits are encoded by single genes, whereas six and four genes have been identified for the α_1 and β subunits, respectively. The α_2/δ subunit, five of the six α_1 subunits, and all four β subunits are expressed in brain, suggesting that brain calcium channels contain at least one of the five α_1 subunits, one of the four β subunits, and an α_2/δ protein (Snutch and Reiner, 1992; Catterall, 1995; De Waard et al., 1996). Expression studies with the various cloned subunits have shown that each α_1 subunit reconstitutes with each β subunit to yield a functional channel. This apparent indiscriminatory channel complex formation was supported by the finding that a highly conserved region of the linker of the I–II loop of all α_1 subunits interacts with each β subunit (De Waard et al., 1994; Pragnell et al., 1994).

A careful comparison of the electrophysiological and pharmacological properties between expressed and native channels showed that the cloned channel subunits encode channels that have similar but not identical properties as the native channels (Zhang et al., 1993; Stea et al., 1994, Randall and Tsien, 1995; Reuter, 1996). An attractive explanation for the differences between expressed and native channels would be a subunit composition of the native channel that was not matched in the expression studies and the occurrence of diverse splice variants of the α_1 and β subunits. It seemed unlikely that the native calcium channels are the result of a random combination of a given α_1 subunit with any of the four β subunits. The analysis of the subunit composition of three calcium channels only partially confirmed this consideration. The purified skeletal muscle channel is a complex of the $\alpha_{1S}/\beta_{1a}/\alpha_2/\delta/\gamma$ subunit (for references, see Hofmann et al., 1994), whereas the neuronal $\alpha_{1B}/\alpha_2/\delta$ subunits of the N-type channel complex were immunoprecipitated together with the β_3 and β_4 subunits (Scott et al., 1996), and the neuronal $\alpha_{1A}/\alpha_2/\delta$ subunits of the ω-conotoxin MVIIC-sensitive calcium channel were associated with the β_{1b}, β_2, β_3, and β_4 subunits (Liu et al., 1996). The latter channel was immunoprecipitated and purified from microsomes derived from whole brains. Therefore, these results do not contradict the hypothesis that an α_1 subunit associates only with one type of β subunit in each neuron at a defined subcellular localization.

We have used site-directed anti-β antibodies and α_1 and β subunit-specific riboprobes to determine the regional expression and cellular colocalization of α_1 and β subunits in rat brain. This analysis did not include the α_{1E} subunit, because this subunit is rather ubiquitiously distributed in the brain and has been described previously (Soong et al., 1993; Yokoyama et al., 1995; Day et al., 1996). Our results indicate that the calcium channel subunits do not combine in a random fashion in the brain.

MATERIALS AND METHODS

Sprague Dawley rats and New Zealand rabbits were obtained from Charles River (Kisslegg, Germany). Keyhole limpet hemocyanin, antipain, pepstatin A, and benzamidine were obtained from Calbiochem (La Jolla, CA); *m*-maleimido benzoyl-*N*-hydroxysuccinimide and toluidine blue from Sigma (St. Louis, MO); epoxy-Sepharose 6B, CNBr-activated

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Sepharose 4B, and dextran from Pharmacia, Piscataway, NJ; the anti-FLAG antibody M2 from IBI; immobilon membrane from Millipore (Bedford, MA); the ECL detection kit and Hybond nitrocellulose from Amersham (Arlington Heights, IL); iodacetamide, 1,10-phenantroline, and leupeptin from Fluka, Neu-Ulm, Germany; goat anti-rabbit IgG–peroxidase conjugate from Jackson Laboratories (West Grove, PA); blotting paper from Schleicher & Schüll (Dassel, Germany); proteinase K and tRNA from Boehringer Mannheim (Mannheim, Germany); [^{35}S]UTP (1000–1500 Ci/mmol) from DuPont NEN (Wilmington, DE); T7 RNA polymerase from New England Biolabs (Beverly, MA); T3 RNA polymerase and pcDNA III from Invitrogen (San Diego, CA); and *Escherichia coli* BL21(DE3)pLysS from Novagen. The vector pAR(ΔRI) was a gift from Dr. M. A. Blanar, Hormone Research Institute, University of California, San Francisco (Blanar and Rutter, 1992).

Production of β subunit-specific antibodies. Peptides B30 CDRNWQRNRPWPKDSY (aa 463–477 of β_3; Hullin et al., 1992), B35 CYNRGSPGGCSHDSRHRL (aa 504–519 of β_4; Castellano et al., 1993), B36 CDSETQESRDSAYVEPKEDY (aa 502–520 of β_{2a}; Hullin et al., 1992), and B37 CSQRSSRHLEEDYADAYQDLY (aa 411–430 of β_3; Hullin et al., 1992) were synthesized by the solid-phase method. The N terminal C of B30, B36, and B37, as well as CY of B35, is not present in the native sequences and was added for coupling and detection purposes. The peptides were purified by reverse-phase HPLC and coupled to keyhole limpet hemocyanin with *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester (Green et al., 1982). The conjugates were emulsified with Freund's complete adjuvant and injected into New Zealand white rabbits. The animals were boostered three to four times with conjugate emulsified in Freund's incomplete adjuvant. For affinity purification of antibodies, the peptides B30, B35, B36, and B37 were coupled to epoxy-Sepharose 6B. Sera were diluted in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4), pH 7.4, and were cycled over the affinity matrix for 16 hr at 4°C. Bound antibodies were eluted with 4.5 M $MgCl_2$ and concentrated with Centricon-30 devices (Amicon, Beverly, MA).

An antibody against a sequence common to all β subunits was generated with a fusion protein produced in *E. coli*. A 567 bp fragment (nt 886–1452 of β_3) was PCR amplified from a cDNA plasmid carrying β_{3a} and cloned into pAR(ΔRI) (Blanar and Rutter, 1992) containing the N-terminal FLAG peptide DYKDDDDKL. The 21 kDa fusion protein was expressed in BL21(DE3)pLysS, detected on immunoblots with the anti-FLAG antibody M2 (IBI) and purified from inclusion bodies by preparative SDS-PAGE with the Model 491 Prep Cell (BioRad, Hercules, CA). Rabbits were immunized with the fusion protein, as described above. Antibodies were affinity-purified by incubation of serum with fusion protein blotted onto Immobilon, and bound antibodies were eluted with 4.5 M $MgCl_2$. To remove contaminating cross-reactive activities, the purified antibody was adsorbed on rat liver acetone powder coupled to CNBr-activated Sepharose 4B.

The specificity of the antibodies was tested by immunoblots using the purified calcium channel from rabbit skeletal muscle (Schneider et al., 1992) and membranes from HEK 293 cells that were transfected individually with the full-length cDNA of the β_{2a}, β_3, or β_4 subunit and the α_{1Ca} subunit (see Fig. 2*A*). The four affinity-purified anti-β subunit antibodies labeled no bands in immunoblots using membrane preparations from control cells transfected with the pcDNA3 vector alone (Invitrogen). The β_2 subunit-specific antibody stained specifically the β_{2a} subunit with an apparent molecular mass of 72 kDa, which is in close agreement to the calculated M_r of 68 kDa (Hullin et al., 1992). The anti-β_3 and anti-β_4 subunit antibodies labeled the expressed β_3 and β_4 subunits with apparent molecular masses of 60 and 58 kDa, which are close to the predicted M_r values of 54 and 58 kDa, respectively (Hullin et al., 1992; Castellano et al., 1993). The anti-β_{common} antibody labeled specifically the expressed full-length β_{2a}, β_3, and β_4 subunits as well as the skeletal muscle β_1 subunit (Ruth al., 1989). Immunostaining of the β subunits was abolished by preincubation of the antibodies with the corresponding peptides respective fusion protein.

Immunoblot. Microsomal membranes were prepared from rat liver, skeletal muscle, brain, uterus, and heart as well as from transfected HEK 293 cells at 4°C. Tissues were homogenized in buffer A containing (in mM): 20 MOPS, pH 7.4, 300 sucrose, 2 EDTA, 1 iodacetamide, 1 1,10-phenantroline, and 0.1 phenylmethanesulfonyl fluoride, and 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μM pepstatin A, and 1 mM benzamidine. The homogenate was centrifuged for 10 min at 5000 × *g*. The pellet was reextracted and centrifuged for 10 min at 5000 × *g*. The combined supernatants were brought to 0.6 M KCl and centrifuged for 35 min at 100,000 × *g*. The pellet was resuspended in buffer A and stored at −70°C. Protein concentrations were determined with the BCA method (Pierce, Rockford, IL).

Membrane proteins (50–100 μg) were separated using 7.5% SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were blocked with TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 0.1% Tween and 5% dry milk powder and probed with the primary anti-β subunit antibody and subsequently with a secondary goat anti-rabbit IgG–peroxidase-conjugated antibody. Bound antibodies were detected by the ECL chemiluminescence method(Amersham).

Histoblots. In situ detection of the β subunit proteins in rat brain slices was accomplished by a modification (Benke et al., 1995) of the original *in situ* blotting method (Taraboulos et al., 1992; Okabe et al., 1993). Slices of unfixed rat brain (16 μm thick) were cut on a cryostat and mounted onto nitrocellulose. The membrane was placed for 15 min on blotting paper (Schleicher and Schüll), soaked in transfer buffer (39 mM glycine, 48 mM Tris, 2% SDS, 20% methanol), and incubated subsequently in 0.1 M Tris, pH 7.0, 2% SDS, 0.1 M β-mercaptoethanol for 1 hr at 45°C and for 16 hr at room temperature (RT). The membrane was blocked in TBS containing 0.1% Tween and 5% dry milk powder and labeled with the anti-β subunit antibodies, followed by a secondary peroxidase-conjugated goat anti-rabbit IgG antibody. Bound antibodies were detected by ECL. Adjacent sections were processed according to standard histological techniques, stained with toluidine blue, and viewed with a stereomicroscope in comparison with overlaid histoblot film images. Neuronal structures were identified according to Paxinos and Watson (1986).

In situ *hybridization (ISH).* Adult Sprague Dawley rats (250–350 gm) were anesthetized with sodium pentobarbital and perfused with 50 ml ice-cold PBS, pH 7.4. The brain was removed and quickly frozen in isopentane cooled in a dry ice/ethanol bath. Sections (16 μm thick) were cut in a cryostat, thaw-mounted onto polyLlysine-coated slides, fixed with 4% paraformaldehyde in PBS, pH 7.4, and dehydrated. Before hybridization, slices were pretreated with 7 μg/ml proteinase K for 15 min at RT and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min. Dehydrated slices were prehybridized for 2 hr at 42°C in hybridization buffer (10 mM Tris, pH 8.0, 0.3 M NaCl, 1 mM EDTA, 1 × Denhardt's solution, 10% dextran, 50% deionized formamide, 50 mM DTT). The slices were then incubated with the radiolabeled probe (5×10^6 cpm/ml hybridization buffer) for 16 hr at 55°C. After hybridization, the slides were washed two times in 2 × SSC, 1 mM DTT, 1 mM EDTA, and incubated in RNase A (20 μg/ml) for 30 min at RT and washed twice in 2 × SSC, 1 mM DTT, 1 mM EDTA. Subsequently, sections were washed at high stringency in 0.1 × SSC, 1 mM DTT, 1 mM EDTA for 2 hr at 75°C. The slices were dehydrated, dried, and exposed to Kodak BioMax MR film for 6 d. The slides were then dipped in autoradiography emulsion Kodak NTB-2, exposed for 6–8 weeks, and developed in Kodak D-19 developer. Sections were lightly counterstained with toluidine blue and examined with bright- and dark-field illumination.

For construction of vectors for *in vitro* transcription, 157–278 bp fragments of β_{1b} (Pragnell et al., 1991), β_{2a} (Perez-Reyes et al., 1992), β_3 (Castellano and Perez-Reyes, 1994), β_4 (Castellano et al., 1993), α_{1A} (Starr et al., 1991), α_{1B} (Dubel et al., 1992), α_{1C} (Snutch et al., 1991), and α_{1D} (Hui et al., 1991) were PCR-amplified from rat brain first-strand cDNA with the following primer pairs:

AL9 (5′-GGTCCTTAATCCCCAGCTGTA-3′) (nt 2031–2051 of β_{1b}) and AL10 (5′-GGGTCTGGGGTTTGTGGAAGA-3′) (nt 2302–2282 of β_{1b})

AL5 (5′-GGACCACTGTTTCTTGCTTGT-3′) (nt 2555–2576 of β_{2a}) and AL4 (5′-CTGCTGACTTGGCATTAAGA-3′) (nt 2791–2772 of β_{2a})

AL1 (5′-CGCCACCTGGAGGAAGACTA-3′) (nt 1406–1425 of β_3) and AL2 (5′-CGCTGCCAGTTCCGGGTCATTG-3′) (nt 1563–1544 of β_3)

AL3 (5′-CAGCCATGACTCCCGACATA-3′) (nt 1737–1754 of β_4) and AL4 (5′-CCTCCTAGACTCAAGGGCATA-3′) (nt 1952–1932 of β_4)

AL19 (5′-CTCCCGAGAACAGCCTTATC-3′) (nt 3257–3294 of α_{1A}) and AL20 (5′-GGGGTCTGCCTCCTCTTCCT-3′) (nt 3483–3464 of α_{1A})

AL17 (5′-GGGGATAAGGAAACTCGAAAT-3′) (nt 3031–3051 of α_{1B}) and AL18 (5′-GGCCTTCCAGGTCCGTGTTA-3′) (nt 3304–3285 of α_{1B})

AL13 (5′-CTCCAGCCCAGTGAAAATGA-3′) (nt 2745–2764 of α_{1C}) and AL14 (5′-GCCAGGGAGATGCTACTGAG-3′) (nt 3022–3003 of α_{1C})

AL15 (5′-GCCAACAGTGACAACAAGGT-3′) (nt 2912–2931 of

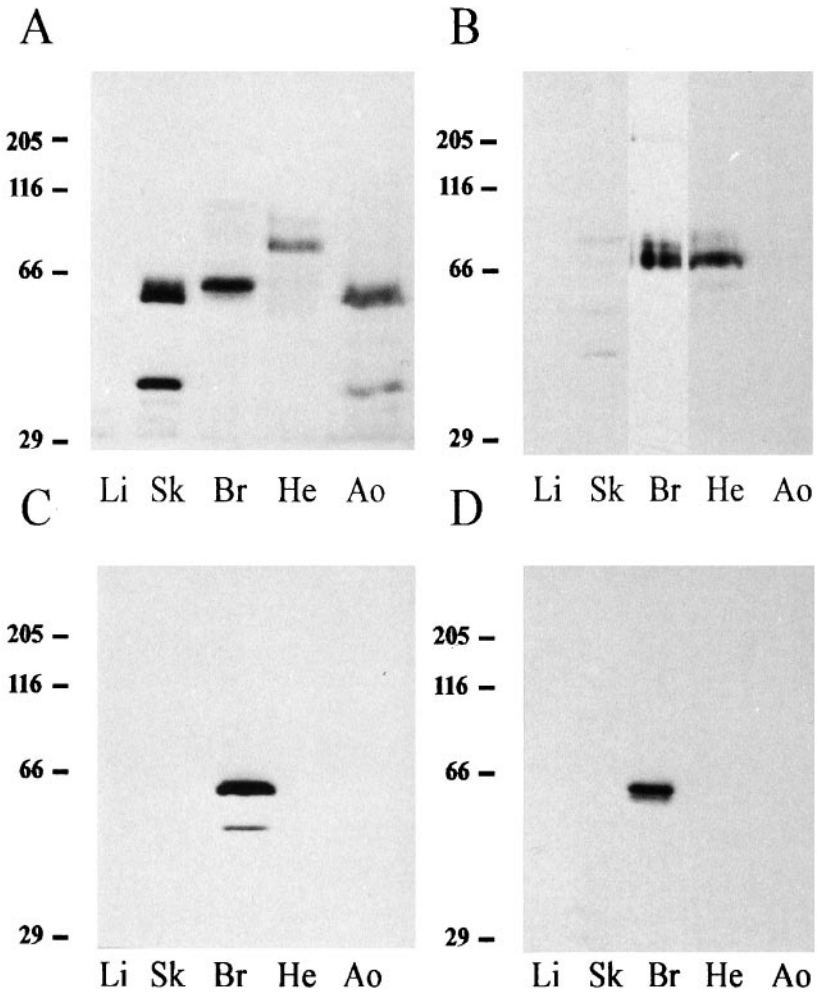

Figure 1. Expression of β subunit proteins in rat tissues. Microsomal membranes (50 μg per lane) of rat liver (*Li*), skeletal muscle (*Sk*), brain (*Br*), heart (*He*), and aorta (*Ao*) were separated using 7.5% SDS-PAGE and transferred to nitrocellulose. The blots were probed with the anti-β_{common} antibody (*A*), the anti-β_2 specific antibody (*B*), the anti-β_3 specific antibody (*C*), and the anti-β_4 specific antibody (*D*). In (*B*), the lane containing the separated brain proteins (*Br*) was from a separate immunoblot and was exposed four times longer than the other lanes. Molecular mass standards ($\times 10^{-3}$) are indicated on the *left* of each blot.

α_{1D}) and AL16 (5′-ACACGGATCGGGTTGGTCTT-3′) (nt 3153–3134 of α_{1D}).

A *Bam*HI and Asp718 site was added to the 5′end of sense and antisense primers, respectively. The PCR profile was as follows: initial denaturation for 1.5 min at 94°C; 40 cycles of 94°C 1 min, 52°C 1 min, 72°C 1 min, and a final extension at 72°C for 5 min. The reaction products were analyzed by restriction mapping and cloned into a pUC19-derived vector containing opposing T3 and T7 RNA polymerase promoters flanked by *Bam*HI respective Asp718 sites. All inserts were sequenced on both strands. Antisense and sense probes were *in vitro* transcribed with [^{35}S]UTP and T7 or T3 RNA polymerase, respectively.

RESULTS

Tissue-specific expression of the β subunits

In initial immunoblot experiments, the anti-β_{common} and the β_2, β_3, and β_4 subunit-specific antibodies (for details, see Materials and Methods) were used to determine the tissue distribution of the β subunits (Fig. 1). The anti-β_{common} antibody (Fig. 1*A*) recognized in skeletal muscle the β_1 subunit (55 kDa) and a 35 kDa band. The staining of the 35 kDa band varied in intensity among different membrane preparations and most likely represented a proteolytic fragment of the β_1 subunit. In brain membranes, the anti-β_{common} antibody stained a protein of 60 kDa; at weaker intensity, a band at 58 kDa; and after longer exposure times, a faint band at 72 kDa. The antibody labeled a 72 kDa protein in heart and a major protein species of 56 kDa and a minor species of 36 kDa in aorta. Labeling of immunoblots with the β subunit-specific antibodies confirmed the β subunit expression profile detected by the β_{common} antibody. The anti-β_2 subunit antibody recognized specifically the 72 kDa protein in heart and at weaker intensity in brain (Fig. 1*B*). The anti-β_3 subunit antibody strongly stained the 60 kDa protein in brain (Fig. 1*C*), whereas the anti-β_4 subunit antibody recognized a 58 kDa protein in this tissue (Fig. 1*D*). The monoclonal antibody 7C3 that was generated against the β_{1a} subunit from rabbit skeletal muscle (Nastainczyk et al., 1990) stained specifically the 55 kDa protein in rat skeletal muscle, but showed no signal with rat brain membranes (data not shown). None of the antibodies stained a band in rat liver membranes, providing additional evidence for the specificity of the antibodies used (Fig. 1). These immunoblots supported the following tissue distribution: the predominant β subunit in skeletal muscle is β_{1a} and that of the heart is the β_2 subunit. Brain contains at least the β_2, β_3, and β_4 subunits and, as shown previously, the β_{1b} subunit (Pragnell et al., 1991). The anti-β_{common} antibody recognized a 56 kDa protein species in aorta, which was not labeled by the β_3 subunit-specific antibody, suggesting that rat smooth muscle contains a β subunit that has a different C terminus than the rabbit β_{3a} subunit (Hullin et al., 1992). To identify a C-terminal truncated splice variant of the β_{3a} subunit, designated β_{3b} (Murakami et al., 1994), an antibody was generated against the C terminus of the β_{3b} subunit (aa 411–430). This sequence is identical for the β_{3a} and β_{3b} subunits. The new antibody labeled specifically the β_3 subunit expressed in HEK 293 cells and the β_3 subunit present in rat brain. However, this antibody was unable to

Table 1. Distribution of β subunit protein and mRNA and α_1 subunit mRNA in the rat brain

	β_{common} Protein	β_2 Protein	β_3 Protein	β_4 Protein	β_1 mRNA	β_2 mRNA	β_3 mRNA	β_4 mRNA	α_{1C} mRNA	α_{1D} mRNA	α_{1B} mRNA	α_{1A} mRNA	α_{1E} mRNA
Olfactory bulb	+++	−	+++	++	++	−	+++	++	++	++	+	++	+++
Cortex	+++	−	++	++	++	−	++	++	−	+	++	++	++
Hippocampus													
Ammon's horn	++	+	++	+	++	+	++	+	++	+	++	+++	+++
Dentate gyrus	+++	+	++	++	++	+	++	+	++	++	++	+++	+++
Caudate putamen	++	−	++	++	++	+	++	+	−	−	+	+	++
Amygdala	++	−	+	+	++	−	++	+	+	+	++	+	++
Hypothalamus	+	−	+	+	++	−	++	+	−	+	++	++	++
Thalamus	+	+	+	+	−	+	+	++	+	−	++	++	+
Habenula	+++	−	+++	−	−	−	+++	−	−	+	−	−	+++
Nucleus interpeduncularis	++	−	++	−	−	−	−	−	−	−	−	−	n.d.
Superior colliculus	++	−	++	+	+	−	+	+	−	++	++	+	n.d.
Inferior colliculus	++	−	−	++	++	−	+	++	−	−	++	+++	n.d.
Cerebellum	+++	+	++	+++	−	+	++	+++	++	+	++	+++	+++
Brainstem	+	−	−	+	+	−	−	+	−	−	++	++	++

In situ hybridization signals on film images and emulsion-dipped sections and chemiluminescence signals obtained with specific antibodies on histoblots were rated as +++, strong; ++, moderate; +, low; −, no signal above background; n.d., not determined. *Data from Soong et al., 1993.

immunolabel a band in membrane or cytosol preparations from rat aorta (data not shown), indicating that the β subunit of rat aorta differs considerably from that expressed in rat brain.

Distribution of the β subunit proteins in rat brain

The β subunit-specific antibodies were used to map the regional expression of β_2, β_3, and β_4 subunits in cryostat sections of rat brain. No acceptable signal could be obtained using conventional immunohistochemical techniques. This failure was probably attributable to the limited accessibility of the β subunit epitopes in the native channel complex. However, the β subunit distribution was mapped *in situ* using the histoblot technique (Okabe et al., 1993; Benke et al., 1995). This procedure allows direct protein mapping from a single cell layer of an SDS-denatured tissue section mounted onto nitrocellulose. Histoblotting was used successfully to map among others the distribution of prion protein PrPSc (Taraboulos et al., 1992) and NMDA–receptor NR1 subunit (Benke et al., 1995) in brain. A series of horizontal, sagittal, and coronal sections spaced at 1 mm intervals was labeled with the anti-β subunit antibodies (see Fig. 3; Table 1). No signal was seen in adjacent sections processed without primary antibody or with primary antibody blocked with an excess of peptide (Fig. 2*B*). The anti-β_{common} antibody (Fig. 3*A*) labeled most intensely the olfactory bulb, cortex, hippocampus, habenula, and cerebellum. Moderate labeling was seen in the basal ganglia, amygdala, nucleus interpeduncularis, and superior and inferior colliculus (Table 1).

The overall staining with the anti-β_2 subunit antibody was much lower than that observed with the anti-β_3 and anti-β_4 subunit antibodies (Fig. 3*B*). The anti-β_2 subunit antibody weakly labeled the hippocampus, thalamus, and cerebellum (Table 1). The staining observed after application of the anti-β_3 subunit antibody (Figs. 3*C*, 5*A*) was strongest in the olfactory bulb and habenula. Moderate signals were detected in the cortex, hippocampus, basal ganglia, nucleus interpeduncularis, superior colliculus, and cerebellum (Table 1). Immunoreactivity in the hippocampus was found throughout the histological layers, with slightly more prominent signals in a formation that represents the stratum radiatum of Ammon's horn. In contrast to the distribution of the β_3 subunit, the expression of the β_4 subunit was highest in the cerebellum (Fig. 3*D*). Most prominent staining was detected over the molecular cell layer, whereas the granular cell layer was only weakly stained. Moderate β_4 subunit immunoreactivity was found in the olfactory bulb, cortex, hippocampus, basal ganglia, and inferior colliculus (Table 1). In the hippocampal formation, the β_4 subunit immunoreactivity was quite prominently localized at the molecular layer of the dentate gyrus, whereas the other hippocampal layers were stained moderately. The anti-β_4 subunit antibody did not stain the habenula (see Fig. 5*C*).

Regional expression of β subunit mRNAs in rat brain

To confirm the immunocytochemical distribution of the β subunit proteins, ISH with riboprobes specific for the β_1, β_2, β_3, and β_4 subunits was performed (Fig. 4). The different riboprobes had similar lengths of 157–278 bp and contained between 60 and 70 [^{35}S]UTP molecules. Sections of one anatomical plane were processed together. These conditions were chosen to obtain information on the relative concentration of the different mRNA species. Control ISH experiments with the sense riboprobes on adjacent sections showed no signals (Fig. 2*C*). Hybridization with the β_1 subunit riboprobe showed moderate labeling in the olfactory bulb, cortex, hippocampus, caudate putamen, amygdala nuclei, hypothalamus, and inferior colliculus (Fig. 4*A*; Table 1). As already observed for the β_2 protein, the β_2 subunit mRNA was expressed at a low level (Fig. 4*B*) in the hippocampus, caudate putamen, thalamus, and cerebellum (Table 1).

In contrast to the β_2 subunit mRNA, the β_3 subunit transcripts were highly expressed (Figs. 4*C*, 5*B*). Strong hybridization signals were detected in the olfactory bulb and habenula. Moderate signals were detected in the cortex, hippocampus, caudate putamen, amygdala nuclei, hypothalamus, and cerebellum (Table 1). The expression pattern of the β_4 subunit transcripts (Figs. 4*D*, 5*D*) showed remarkable differences to that of the other β subunits. The strongest hybridization signals were found in the cerebellum. Moderate labeling was observed in the olfactory bulb, cortex, thalamus, and inferior colliculus. In general, the intensity of the ISH signals corresponded with that of the histoblots (compare Fig. 5*A* with Fig. 5*B* and see Table 1), suggesting that the mRNA levels correlated well with the protein concentration.

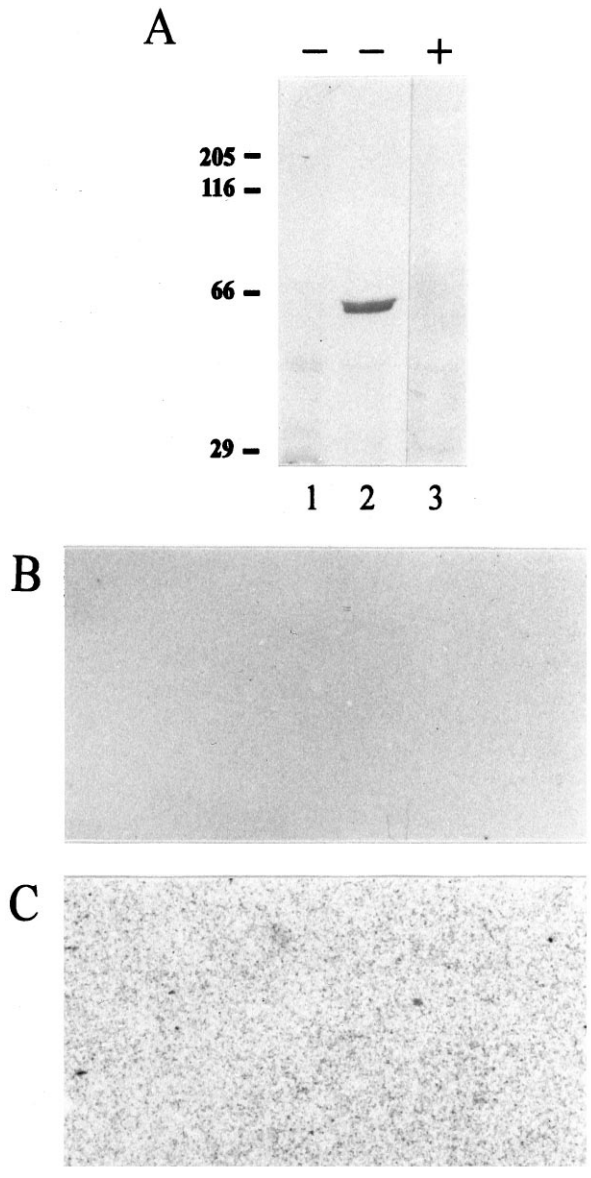

Figure 2. Controls for antibody specificity, histoblots, and ISH sections. *A*, Microsomal membranes from HEK 293 cells transfected with control vector (*lane 1*) or full-length β_3 cDNA expression vector (*lanes 2*, *3*) were electrophoresed on a 7.5% SDS gel and blotted onto nitrocellulose. *Lanes 1* and *2* were probed with the anti-β_3 antibody preincubated in the absence (−) and *lane 3* with the anti-β_3 antibody preincubated in the presence (+) of 10 μM peptide B30. *B*, A sagittal histoblot section adjacent to the section of Figure 3*B* was probed with the anti-β_2-specific antibody blocked with 10 μM peptide B36. *C*, A sagittal ISH section adjacent to the section of Figure 4*B* was labeled with the β_2 sense riboprobe.

Cellular localization of the β subunit mRNAs

The cellular distribution of the four transcripts was studied in the cerebellum, olfactory bulb, and frontal cortex (Fig. 6), because these regions were heavily labeled by the β_3 and β_4 subunit probes. Pronounced differences in the cellular expression profile were found. In slices of the cerebellum, the β_4 subunit riboprobe labeled intensely granule cells and the Purkinje neurons (Fig. 6*D*). In addition, scattered cells were labeled in the molecular layer representing basket or stellate neurons.

In comparison with the β_4 subunit signal, the cerebellar signal of the β_3 subunit riboprobe was less intense (Fig. 6*C*). The β_3 subunit was present in granule cells and in some cells of the molecular layer. The Purkinje cells expressed the β_3 subunit mRNA only at very low levels or not at all. Hybridization with the β_2 subunit antisense probe showed a thin line beneath the molecular layer on autoradiographic film images (Fig. 4*B*). On emulsion-dipped slides, this signal was identified as labeling of Purkinje neurons (Fig. 6*B*). Hybridization with the β_1 subunit riboprobe showed no specific labeling of cerebellar cells, suggesting that the β_1 subunit is not expressed in the cerebellum (Fig. 6*A*).

In the olfactory bulb, virtually no signal was observed with the β_1 and β_2 subunit-specific riboprobes (data not shown). The β_3 subunit-specific probe gave strong to moderate signals, which were confined to the granule cell layer, and weak labeling of the glomerular layer (Fig. 6*E*). In contrast, the β_4 subunit-specific riboprobe yielded prominent signals over mitral cells and scattered tufted cells. The granule cell layer was only weakly labeled (Fig. 6*F*).

No obvious difference was observed in the type of expression of β_1, β_3 (Fig. 6*G*), and β_4 (Fig. 6*H*) subunit transcripts in the cerebral cortex. In cortical layers II–VI, numerous cortical neurons were strongly labeled. All four β subunits were expressed in the neurons of the CA1, CA2, and CA3 fields of the hippocampus and in the granule cells of the dentate gyrus. Highest expression levels were observed for the β_3 transcripts followed by the β_1 mRNA. The β_4 and β_2 subunit-specific probes showed considerably weaker signals, but both subunits were clearly expressed in the neurons of the hippocampal formation. One prominent feature of the expression of the β_3 subunit that was not observed with the other β subunit riboprobes was labeling of the habenula. The medial habenulae were very strongly labeled by the β_3-specific riboprobe, both on autoradiographic film images (Fig. 5*B*) and on emulsion-dipped slides (Fig. 6*I*) and by the anti-β_3 antibody on histoblots (Fig. 5*A*).

Expression profile of the α_1 subunit mRNAs in rat brain

In the next series of experiments, we investigated the colocalizations of the β subunits with the α_1 subunits of voltage-gated calcium channels. The expression of the α_{1E} subunit was not investigated in detail, because previous studies showed that this subunit is rather ubiquitously expressed in the brain (Soong et al., 1993; Yokoyama et al., 1995). ISH was performed with probes specific for α_{1A}, α_{1B}, α_{1C}, and α_{1D} subunit mRNA. The probes were chosen to recognize all described splice variants of these α_1 subunits and followed the same criteria as described for the β subunit riboprobes. All four riboprobes were directed against the loop between segments IIS6 and IIIS1, which is highly variable and characteristic for each type of α_1 subunit. An abundant and broad distribution of the α_{1A} and α_{1B} transcripts was observed (Fig. 7*C*,*D*; Table 1) contrasting with a lower and spatially more restricted expression of the α_{1C} and α_{1D} mRNA (Fig. 7*A*,*B*;, Table 1). On the autoradiographic film images, moderate hybridization signals with the α_{1C} specific riboprobe (Fig. 7*A*) were detected in the olfactory bulb, hippocampus, and cerebellum (Table 1). In the hippocampus, α_{1C} transcripts were moderately expressed in the dentate gyrus and the CA2 and CA3 fields of Ammon's horn, whereas the CA1 field was only weakly labeled. The overall expression level of α_{1D} transcripts (Fig. 7*B*) in rat brain was similar to that of the α_{1C} subunit. Moderate signals were observed in the olfactory bulb, the dentate gyrus of the hippocampal formation, and the superior colliculus (Table 1). In contrast to the expression profile of both L-type α_1 calcium channel subunits,

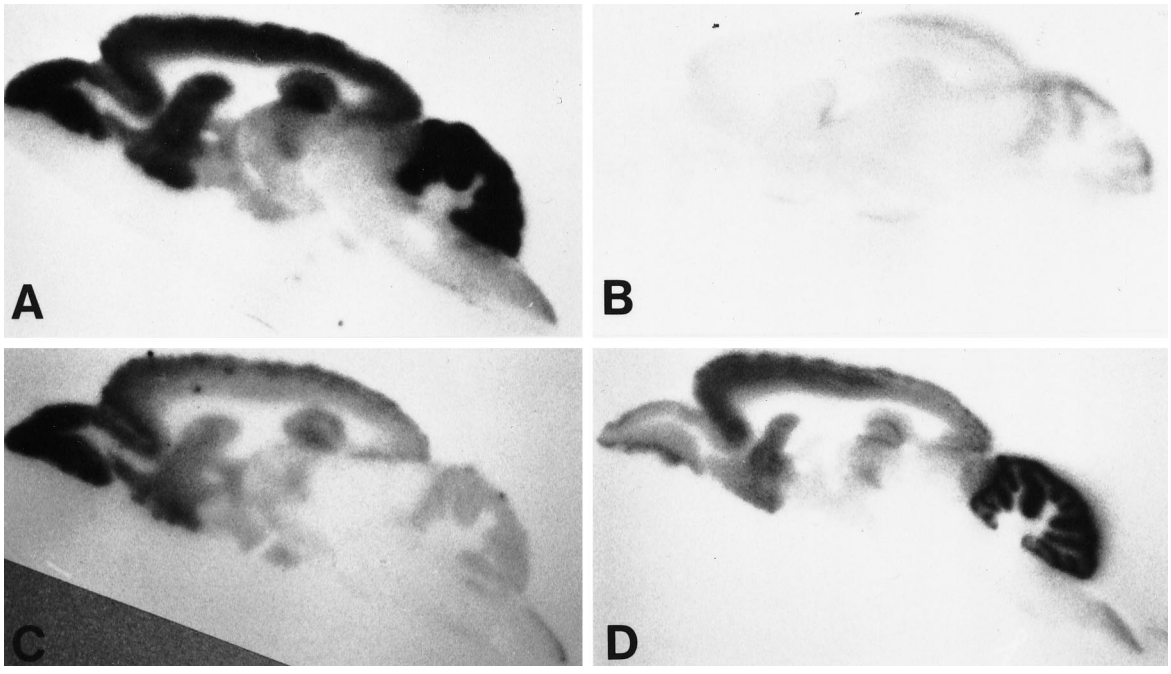

Figure 3. Histoblots showing the neuronal distribution of β subunit proteins. Sagittal sections of rat brain were transferred to nitrocellulose and labeled with the anti-β_{common} antibody (*A*), the anti-β_2 antibody (*B*), the anti-β_3 antibody (*C*), and the anti-β_4 antibody (*D*).

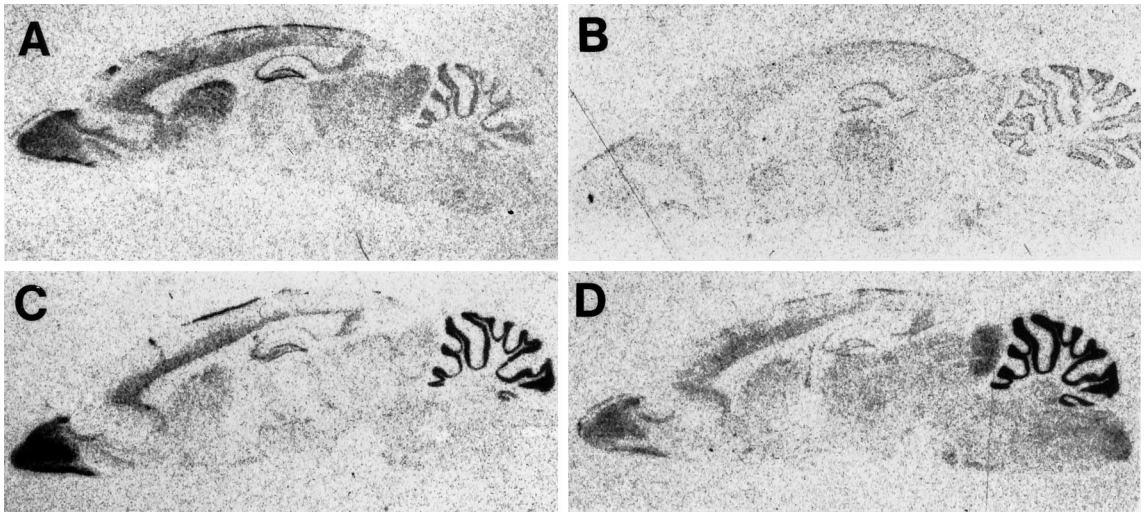

Figure 4. Neuronal distribution of β subunit mRNAs. Autoradiographic film images of sagittal sections hybridized with antisense riboprobes specific for β_1 subunit mRNA (*A*), β_2 subunit mRNA (*B*), β_3 subunit mRNA (*C*), and β_4 subunit mRNA (*D*).

the α_{1B} transcripts (Fig. 7*C*) were expressed almost ubiquitously, with moderate labeling of most brain regions (Table 1). Expression of the α_{1A} subunit (Fig. 7*D*) was most prominent in the cerebellum. Strong signals were also observed in the hippocampus and inferior colliculus. Nearly all other brain regions were labeled moderately on autoradiographic film images (Table 1).

Cellular localization of α_1 subunit mRNAs

The cellular localization of the α_1 subunit transcripts in the cerebellum is shown in Figure 7. The α_{1c} subunit was expressed moderately in neurons of the granular layer, whereas no labeling of Purkinje cells was observed (Fig. 8*A*). In contrast, the α_{1D} transcript was moderately expressed both in granule cells and in Purkinje neurons (Fig. 8*B*). The α_{1B}-specific riboprobe gave moderate signals over neurons in the granule cell layer. The Purkinje cells were not labeled significantly (Fig. 8*C*). Hybridization with the α_{1A}-specific riboprobe showed heavy labeling of Purkinje neurons and granule cells as well as neurons in the molecular layer (Fig. 8*D*). These results are in accordance with Northern blot experiments using cerebella from mutant mice with different types of cerebellar degeneration (Mori et al., 1991).

In the olfactory bulb, the α_{1D} (Fig. 8*E*) and α_{1C}, as well as α_{1B} (data not shown) mRNA was expressed moderately in granule cells. Mitral cells, tufted cells, and periglomerular cells were lightly labeled too. Hybridization with the α_{1A}-specific probe (Fig. 8*F*) showed intense labeling of mitral cells and some scattered tufted cells. Neurons in the granular layer and periglomerular

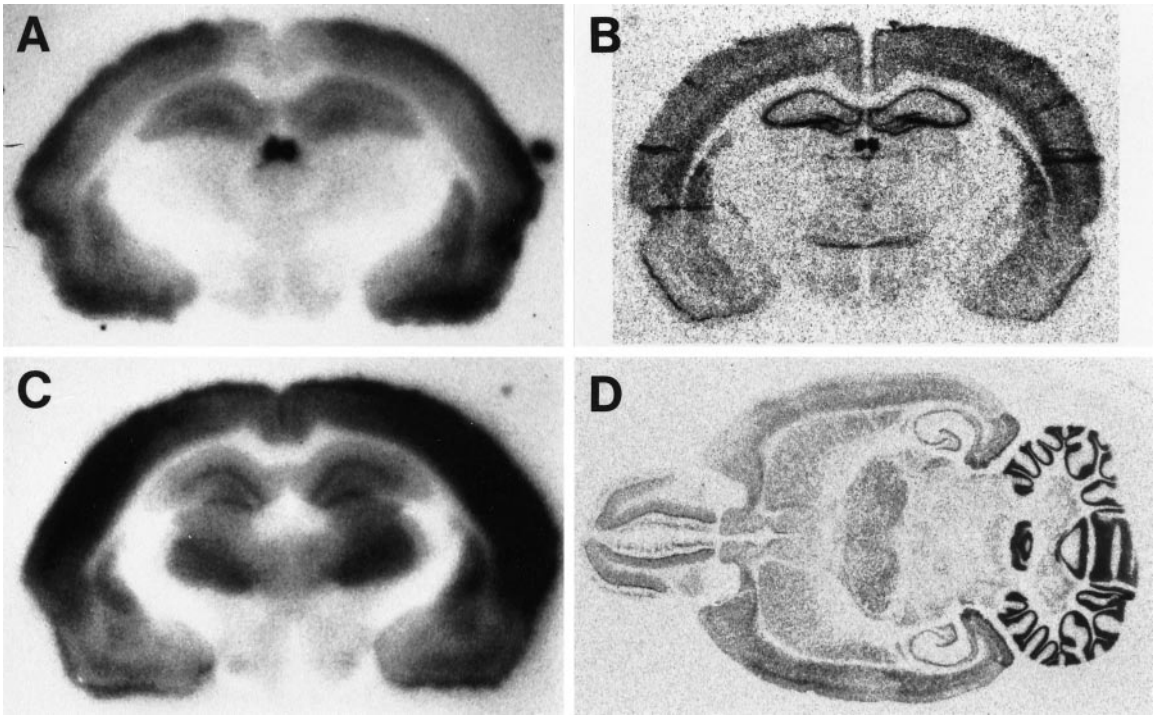

Figure 5. Distribution of β subunits in rat brain. *A*, Histoblot of a coronal section labeled with the β_3-specific antibody. *B*, ISH of an adjacent coronal section with the β_3-specific riboprobe. *C*, Histoblot of an adjacent coronal section labeled with the β_4-specific antibody. *D*, ISH of a horizontal section with the riboprobe directed against β_4 mRNA.

cells gave moderate signals. Expression of the α_{1C} subunit was not detected in the cerebral cortex. The α_{1D} probe labeled moderately and the α_{1A} and α_{1B} riboprobes labeled intensely most neurons throughout cortical layers II–VI (data not shown).

DISCUSSION

This study analyzed the regional expression and localization of various α_1 and β subunits of voltage-activated calcium channels at the mRNA and protein levels. The specificity of the anti-β subunit antibodies was demonstrated (see Fig. 2*A*) by (1) immunoblots of microsomal membranes from HEK 293 cells transfected with expression vectors coding for the various full length β subunits, (2) block of the immunostaining by preincubation of the antibodies with the specific peptides, (3) no signals with liver microsomes, and (4) the use of antibodies directed against different regions of the β subunit (C terminal vs common region). In agreement with previous Northern blot results (Hullin et al., 1992; Perez-Reyes et al., 1992; Castellano et al., 1993), these antibodies confirmed that the β_{1a} subunit is predominantly expressed in skeletal muscle; the β_2 subunit in heart and brain; and the β_{1b}, β_2, β_3, and β_4 subunits in brain. Labeling of histoblots with the anti-β_{common} antibody gave signals in all brain regions where immunoreactivity with the β subunit-specific antibodies was detected. Furthermore, the close correspondence between the results from immunohistoblot and mRNA ISH demonstrated further the specificity of the antibodies and riboprobes as well as the usability of the histoblot method to determine the distribution of brain proteins. Taken together, these results demonstrate that the immunostaining pattern observed in rat brain slices was caused specifically by the expressed β subunits and not by other potentially cross-reactive proteins. The staining pattern summarized in Table 1 indicates that the expression of α_1 and β subunits varies considerably among different brain regions. This highly differentiated regional and neuronal expression of the calcium channel genes is undoubtedly important for neuronal functions.

Inspection of the ISH images indicates that the α_{1A} subunit forms a complex with the β_4 subunit in most neurons. In the cerebellum, the unique localization of the α_{1A} transcripts matches excellently that of the β_4 subunit mRNA. Both α_{1A} and β_4 mRNA was strongly expressed in cerebellar Purkinje cells and granule cells. In the olfactory bulb, the α_{1A} and β_4 mRNA was coexpressed in mitral cells. Both α_{1A} and β_4 subunits were expressed at high levels in thalamic neurons. The cellular localization of the β_4 protein in the molecular layer of the cerebellum corresponded well with the distribution of α_{1a} immunoreactivity (Volsen et al., 1995; Westenbroek et al., 1995), which was found most prominently along dendrites of Purkinje cells and also at cell bodies and parallel fiber dendrites of cerebellar granule cells. Taken together, these results demonstrate that the α_{1A} and β_4 subunits are expressed together in the same cells at similar or identical locations and that therefore the α_{1A} and β_4 subunits are part of a P/Q-type calcium channel complex in most cells. However, the α_{1A} subunit could combine also with the β_2 and/or β_3 subunits in specific neurons, because some neuronal cell types expressed the mRNA for both α_1 and β_2 or β_3 subunits. For example, the β_3 subunit mRNA was expressed in cerebellar granule cells, suggesting that the α_{1A} subunit could also couple with the β_3 subunit. The expression of the β_2 subunit in Purkinje cells is in agreement with electrophysiological results (Stea et al., 1994), which showed that the β_2 subunit slowed inactivation of the α_{1A} channel current, and with the recent finding that the purified α_{1A} subunit is associated among other subunits with the β_2 protein (Liu et al., 1996).

The very broad and uniform distribution of the α_{1B} transcripts corresponds well with the overall distribution of the β_3 mRNA. This is also true at the cellular level, e.g., both transcripts are

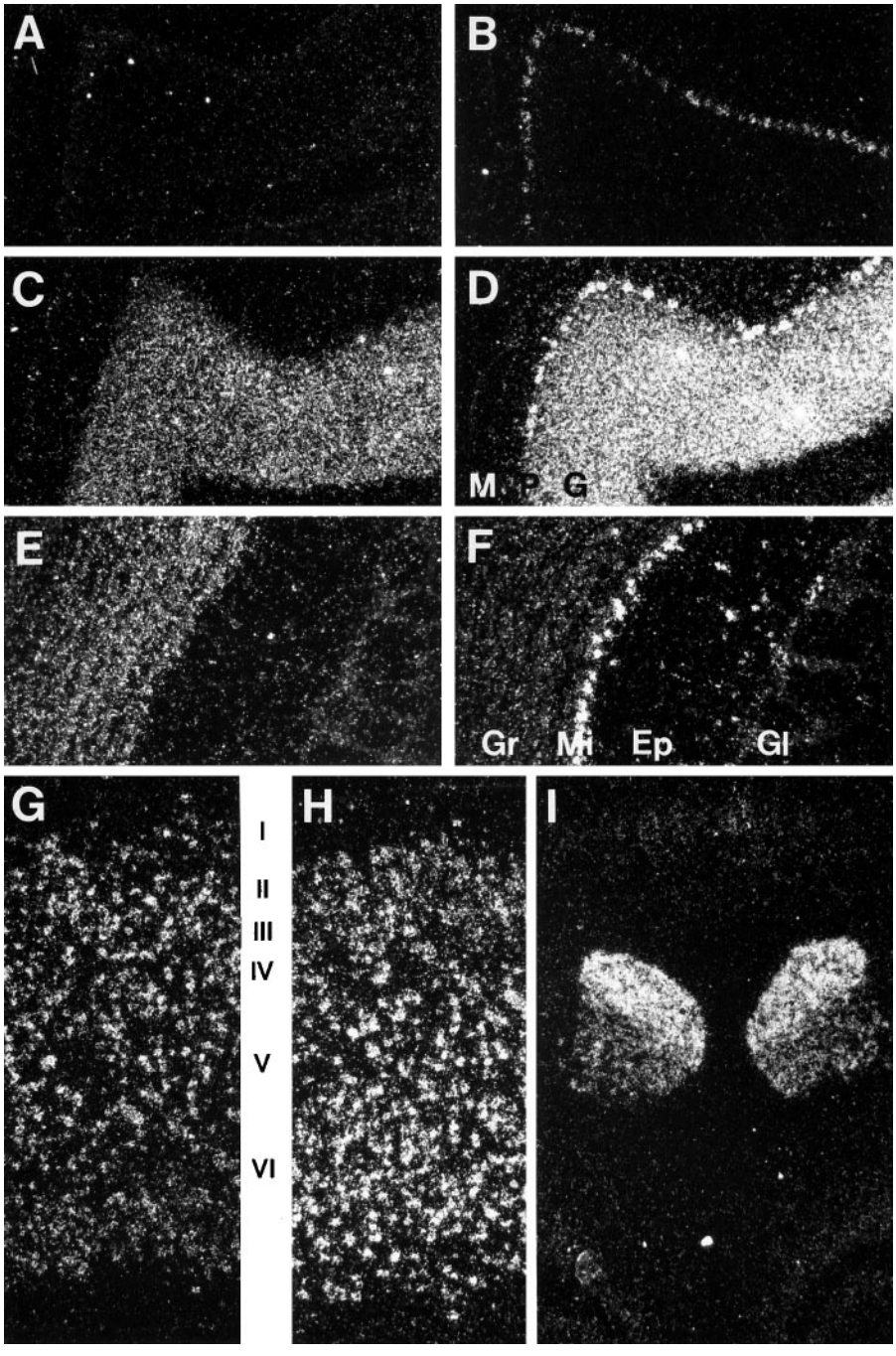

Figure 6. Cellular localization of β subunit mRNA in the cerebellum, olfactory bulb, and cortex. Dark-field microscopy views showing emulsion-dipped sagittal sections through the cerebellum labeled with antisense riboprobes specific for β_1 subunit mRNA (*A*), β_2 subunit mRNA (*B*), β_3 subunit mRNA (*C*), and β_4 subunit mRNA (*D*). *M*, Molecular cell layer; *P*, Purkinje cell layer; *G*, granular cell layer. *E*, *F*, Coronal sections through the olfactory bulb hybridized with the riboprobes directed against the β_3 subunit mRNA (*E*) and β_4 subunit mRNA (*F*). *Gr*, Granular layer; *Mi*, mitral cell layer; *Ep*, external plexiform layer; *Gl*, glomerular layer. *G*, *H*, Coronal section through the frontal cortex showing the expression of β_3 subunit mRNA (*G*) and β_4 subunit mRNA. *H*, *I–VI*, Cortical layers I–VI. *I*, Coronal section through the habenular complex labeled with the β_3-specific riboprobe. Magnification, 100×.

expressed in cerebellar granule cells, suggesting that α_{1B} associates with the β_3 subunit.

However, the strong expression of β_4 subunit mRNAs in granule cells opens the possibility that α_{1B} may associate also with the β_4 subunit. This expression pattern is in accordance with the subunit composition of the N-type calcium channel immunoprecipitated from brain (Scott et al., 1996), which contained the β_3 and β_4 subunits in association with the α_{1B} subunit. The β_3 subunit associates also with other α_1 subunits, as exemplified by neurons of the habenular complex. The anti-β_3 and anti-β_{common} antibodies and the β_3 antisense riboprobes gave very intense signals in the medial habenula. The nucleus interpeduncularis, which is a projection area of habenular neurons, was also labeled by the anti-β_{common} and anti-β_3 antibodies, whereas no expression of β_3 mRNA was detected in this region. This suggests that the β_3 protein is synthesized in habenular neurons and transferred via axonal transport to the nucleus interpeduncularis. None of the other β subunit-specific antibodies or riboprobes gave any signal in the habenula. Remarkably, no expression of α_{1B} transcripts could be detected in the habenular complex. Only the α_{1D} riboprobe gave a faint signal. This pattern indicates that the β_3 subunit is not associated exclusively with the α_{1B} subunit, but forms complexes with other α_1 subunits, most likely with the α_{1E} subunit. It was shown previously that transcripts of the α_{1E} subunit are heavily expressed in the medial habenula (Soong et al., 1993). These results strongly support the hypothesis that the β_3 subunit binds to and modulates the current through the α_{1B} and α_{1E} subunits.

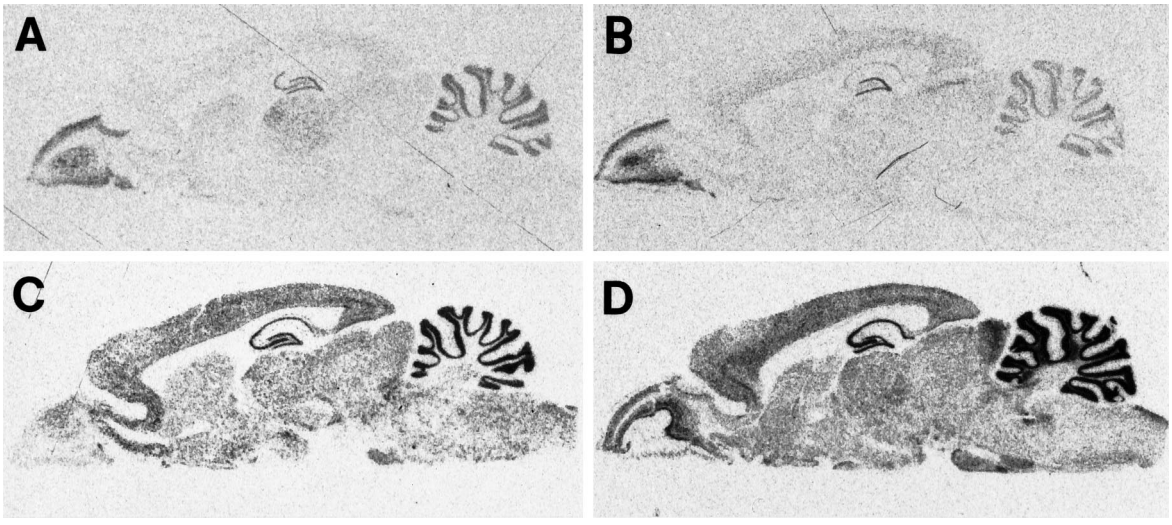

Figure 7. Neuronal distribution of α_1 subunit mRNAs. Autoradiographic film images of sagittal sections hybridized with antisense riboprobes specific for α_{1C} subunit mRNA (*A*), α_{1D} subunit mRNA (*B*), α_{1B} subunit mRNA (*C*), and α_{1A} subunit mRNA (*D*).

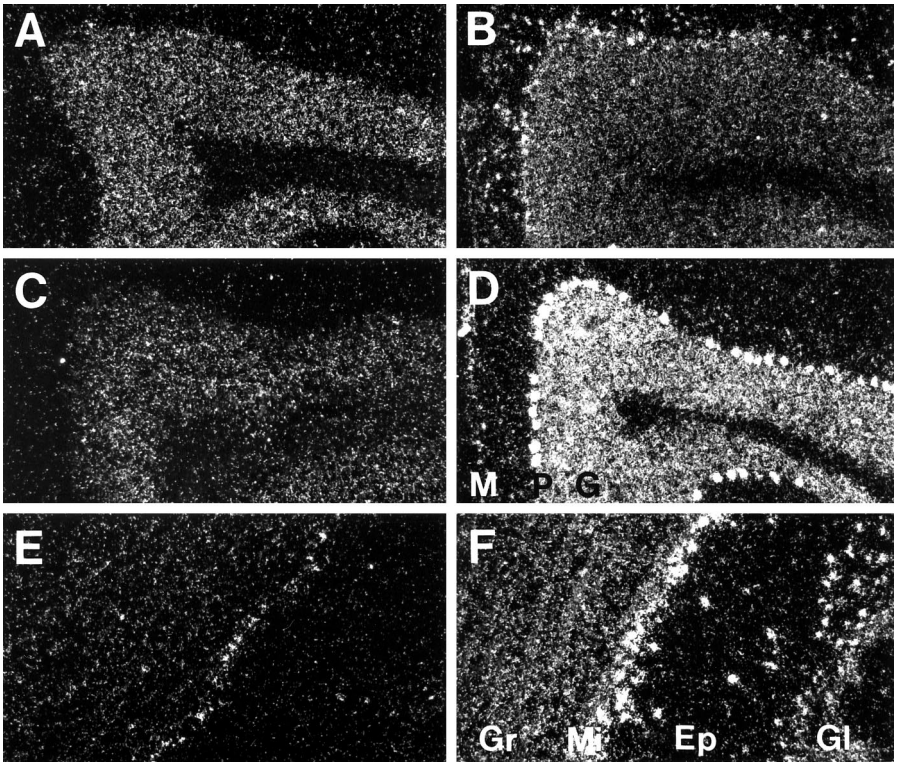

Figure 8. Cellular localization of the α_1 subunit mRNAs in the cerebellum and olfactory bulb. *A–D*, Sagittal sections through the cerebellum hybridized with riboprobes directed against the α_{1C} subunit mRNA (*A*), α_{1D} subunit mRNA (*B*), α_{1B} subunit mRNA (*C*) and α_{1A} subunit mRNA (*D*). *E*, *F*, Sections through the olfactory bulb labeled with the riboprobes specific for α_{1D} subunit mRNA (*E*) and α_{1A} subunit mRNA (*F*). Abbreviations are as in the legend to Figure 6. Magnification, 100×.

In contrast to β_1, β_3, and β_4 subunits, the β_2 subunit was expressed at low levels in rat brain, as shown by Western blots, histoblots, and mRNA ISH. Expression of the β_2 subunit was confined to pyramidal and granular cells of the hippocampus, thalamic neurons, and cerebellar Purkinje cells. The α_{1C} and β_2 subunits are coexpressed in rat cardiac myocytes (A. Ludwig and F. Hofmann, unpublished results). The distribution pattern determined for the two L-type calcium channel α_{1C} and α_{1D} subunits is in accordance with results from earlier studies (Ahlijanian et al., 1990; Chin et al., 1992). Direct comparison of the slices labeled with each α_1 riboprobe demonstrated that α_{1C} and α_{1D} transcripts were expressed at low levels in brain. Their expression profile showed distinct differences to that of the β_2 subunit. In the cerebellum, α_{1D} and α_{1A} mRNA, but not α_{1C} mRNA, was detected in Purkinje cells. Both L-type α_1 subunit transcripts were prominently expressed in the cerebellar granular layer, which labels heavily for the β_3 and β_4 subunits but not for the β_2 subunit. Moderate expression of the α_{1C} subunit was detected in the dentate gyrus and hippocampal CA2 and CA3 fields but was observed at only low levels in the CA1. In contrast, the β_2 subunit was expressed evenly in all three hippocampal fields. Taken together, these results suggest that the β_2 subunit is associated not only with the α_{1C} subunit, as in cardiac muscle, but also with α_{1A} in cerebellar Purkinje cells and probably also with other α_1 subunits. Conversely, the α_{1C} subunit may bind not exclusively β_2 but also other β subunits. In cerebellar granule cells and olfactory

neurons, where α_{1C} transcripts were prominently found, β_3 and β_4 transcripts but not β_2 mRNA were detected. This distribution pattern suggests that the β_2 and α_{1C} subunits associate with different channel subunits in brain and heart muscle.

This study provides evidence that a given β subunit associates with different types of α_1 subunits depending on the type of neuron. This interpretation is in accordance with the finding that β subunits bind to α_1 subunits through a conserved motif located at the intracellular loop between repeat I and II of each α_1 subunit (De Waard et al., 1994; Pragnell et al., 1994). Coexpression studies of the cloned cDNAs demonstrated that all four β subunits are able to interact with a given α_1 subunit. They modulated calcium channel activity similarly but differed in their relative effectiveness (Singer et al., 1991; Welling et al., 1993; Castellano and Perez-Reyes, 1994; Olcese et al., 1994; Stea et al., 1994; Lacinova et al., 1995). The subunit composition of the calcium channel is obviously important for the identified electrophysiological properties of the channel. Beyond this functional aspects, the combination of a given α_1 subunit with different β subunits may affect the subcellular localization of the channel and interaction with G-protein subunits (Campbell et al., 1995; Herlitze et al., 1996; Ikeda, 1996). It is evident from this work that the association of an α_1 subunit with different types of β subunits contributes to the diversity of calcium channel activity in the brain.

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