Spatial diversity in gene expression for VDCCγ subunit family in developing and adult mouse brains

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Abstract

The γ subunit of voltage-dependent Ca^{2+} channels (VDCCs) is characterized by molecular diversity and regulation of AMPA-type glutamate receptors as well as VDCCs. In the present study, we examined expressions for the VDCCγ1-8 subunit mRNAs in developing and adult mouse brains by *in situ* hybridization. In adult brains, the γ2 and γ7 subunit mRNAs were widely expressed in various grey matter regions with the highest level in cerebellar Purkinje cells and granule cells. The γ3 and γ8 subunit mRNAs predominated in the telencephalon, with the latter being at striking levels in the hippocampus. The γ4 subunit mRNA was enriched in the olfactory bulb, striatum, thalamus, and hypothalamus. The γ5 subunit mRNA was abundant in the olfactory bulb, hippocampal CA2, thalamus, inferior colliculus, and Bergmann glia. Transcripts of these subunits were detected in embryonic brains: some showed well-preserved spatial patterns (γ2, γ5, γ7 and γ8), while others underwent developmental up- (γ3) or down-regulation (γ4). In contrast, the γ1 and γ6 subunit mRNAs were negative or very low throughout brain development. Therefore the present study has revealed spatial diversity in gene expression for individual VDCCγ subunits, presumably reflecting functional diversity of this protein family and their differential involvement in neural function.

Key words: VDCCγ subunit, Stargazin, TARPs, Development, mRNA, *In situ* hybridization, Brain.

Introduction

The γ subunit of voltage-dependent Ca^{2+} channels (VDCCs) has been originally identified as an auxiliary subunit of 1,4-dihydropyridine (DHP)-sensitive or L-type VDCCs in skeletal muscles (Bosse et al., 1990; Jay et al., 1990; Powers et al., 1993). The second subunit, γ2 subunit or stargazin, was identified as a gene responsible for the spontaneous mutant mouse, *stargazer*, which is characterized by absence epilepsy and ataxia (Letts et al., 1998). Subsequent studies have revealed six additional subunits and, hence, there are eight members to date in the VDCCγ family (Black and Lennon., 1999; Burgess et al., 1999, 2001; Klugbauer et al., 2000; Chu et al., 2001; Moss et al., 2002). Each γ subunit contains four putative transmembrane domains with intracellularly located N- and C-termini (Chu et al., 2001; Black, 2003). The γ1, γ2, γ3, γ4, γ5, and γ7 subunits have been shown to affect the function of L-type, T-type, and P/Q-type VDCCs, when expressed in various combinations (Wei et al., 1991; Eberst et al., 1997; Letts et al., 1998; Klugbauer et al., 2000; Freise et al., 2000; Rousset et al., 2001; Green et al., 2001; Moss et al., 2002, Held et al., 2002). On the other hand, the γ2 subunit and its structurally-related members, γ3, γ4, and γ8 subunits, are crucial for cell surface expression, synaptic targeting, recycling, channel activity, and gating of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and hence named as transmembrane AMPA receptor regulatory proteins (TARPs) (Chen et al., 2000; Tomita et al., 2003, 2004, 2005; Yamazaki et al., 2004; Priel et al., 2005). The γ subunit family is expressed in various tissues, including the brain, skeletal muscle, heart, lung, and testis (Chu et al., 2001). In the brain, all γ subunits, except the γ1 subunit, are expressed (Chu et al., 2001). Of these, four TARPs have been reported to display high and distinct expressions

in the developing and adult brains (Klugbauer et al., 2000; Chen et al., 2000; Tomita et al., 2003). However, comparative and systematic analysis on the expression of the VDCCγ family has not yet been performed. In the present study, we examined expressions for the VDCCγ1-8 subunit mRNAs in developing and adult mouse brains by *in situ* hybridization with [^{33}P]dATP-labeled antisense oligonucleotide probes, and have revealed their distinct regional and cellular expression in the brain.

Materials and Methods

Probes

To detect mRNAs for each VDCCγ subunit, specific antisense oligonucleotide probes were synthesized as follows: 5’-gtgctctggctcagcgtccatgcaggattcccaggggttctgagg-3’ for the γ1 subunit (GenBank accession No. AJ006306), 5’-gatgcgggtgatggcggaggcctggaggtagtcggtggcgcgggc-3’ for the γ2 subunit (accession No. AF077739), 5’-cggcaggcgcgcaaatgtagacttcttcaggagctctgaatggga-3’ for the γ3 subunit (accession No. AJ272044), 5’-ggcgtaaggagaggaggaagaggccttaaggaactcccgcttggt-3’ for the γ4 subunit (accession No. AJ272045), 5’-catctggtcatagtctgggcacttgagcaaagctgggtagttgct-3’ for the γ5 subunit (accession No. AF361347), 5’-ttgggccaccccacttggggcacagtgacctccagggccaggaag-3’ for the γ6 subunit (accession No. AF361348), 5’-gcgatagtgaaagtactgctcagagctgctgggcctgttcat-3’ for the γ7 subunit (accession No. AF361349), and 5’-acaccacaaacccctctcttcattccagcgtttcaatgactccag-3’ for the γ8 subunit (accession No.

AF361350). Oligonucleotide probes were labeled with [^{33}P]dATP using terminal deoxyribonucleotidyl transferase (Invitrogen, Carlsbad, CA).

In situ hybridization

Under deep pentobarbital anesthesia, the brains were freshly obtained from C57BL/6J mice at embryonic days 13 (E13), E18, postnatal days 1 (P1), P7, P14, P21, and adult (4 months). The day after overnight mating was designated as E0, and the day of birth as P1. Fresh frozen sections (20 μm thickness) were cut with a cryostat (CM1900, Leica, Nussloch, Germany) and mounted on glass slides precoated with 3-aminopropyltriethoxysilane. Probe labeling and hybridization were performed as described (Fukaya et al., 2005) with minor modifications. Sections were treated at room temperature with the following incubation steps: fixation with 4% paraformaldehyde-0.1M sodium phosphate buffer (pH 7.2) for 10 min, 2mg/ml glycine-phosphate-buffered saline (pH 7.2) for 10 min, acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min, and prehybridization for 1 hr in a buffer containing 50% formamide, 50 mM Tris-HCl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.6 M NaCl, 0.25% SDS, 200 μg/ml tRNA, 1 mM EDTA, and 10% dextran sulfate. Hybridization was performed at 42 °C for 12 hr in the prehybridization buffer supplemented with 10,000 cpm/μl of [^{33}P]dATP-labeled oligonucleotides. Slides were washed twice at 55 $^{\circ}$C for 40 min in 0.1 x SSC containing 0.1% sarcosyl. Sections were exposed either to BioMax (Kodak, Rochester, NY) or to Nuclear Track emulsion (NTB-2, Kodak) for 4 weeks. Emulsion-dipped sections were Nissl-stained with methyl green pyronine solution.

Results

In situ hybridization was employed to reveal the spatiotemporal expression patterns of each VDCCγ subunit mRNA in the mouse brain using radiolabeled antisense oligonucleotide probes. Overall distribution was examined by X-ray film macroautoradiography (Fig. 1), while detailed regional and cellular expression was determined by emulsion microautoradiography (Fig. 2, 3). The specificity of hybridizing signals was verified by the disappearance of signals when hybridization was carried out in the presence of a 100-fold excess amount of unlabeled oligonucleotides (Fig. 1A-H insets).

Distribution in adult mouse brain

Regional and cellular expressions in the cerebral cortex, hippocampus, and cerebellum are mainly described in the text, and expressions in other regions are summarized in Table 1.

γ1 subunit. No significant signals for the γ1 subunit mRNA were detected in any regions of the adult brain (Fig. 1A, 2A, 2I, 2Q).

γ2 subunit. The γ2 subunit mRNA was detected at high levels in various grey matter regions (Fig 1B). The highest expression was found in the cerebellar cortex, where the γ2 subunit mRNA was expressed strongly in Purkinje cells and granule cells, and moderately in the molecular layer neurons (basket and stellate cells) and deep cerebellar nuclei (Fig. 2R, 3M). In the cerebral cortex, the γ2 subunit mRNA was distributed in layers II-VI (Fig. 2B). In the hippocampus, it was detected strongly in pyramidal cell layer of the CA3 region and

granule cell layer of the dentate gyrus, and weakly in CA1 pyramidal cell layer (Fig. 1B, 2J, 3A, 3G). Intense signals were also detected in neurons of polymorphic layer of the dentate gyrus and interneurons dispersed in the CA1 region (Fig. 2J, 3A, 3G).

γ3 subunit. Hybridization signals for the γ3 subunit mRNA were predominantly detected in the telencephalon, including the olfactory bulb, cerebral cortex, hippocampus, striatum, septum, and amygdala (Fig. 1C). In the hippocampus, the γ3 subunit mRNA was intense in CA1 pyramidal cell layer, moderate in CA3 pyramidal cell layer and granule cell layer of the dentate gyrus, and very low in the CA2 region (Fig. 2K, 3B, 3H). Expression was low in lower brain regions, including the cerebellum (Fig. 1C). Emulsion microautoradiography revealed the presence of dispersed signal clusters in the cerebellum, which originated from large cells dispersed in the granular layer, i.e., Golgi cells, an interneuron in the granular layer (Fig. 2S, 3N).

γ4 subunit. Hybridization signals for the γ4 subunit mRNA were found in various brain regions with higher levels in the olfactory bulb, striatum, thalamus, and hypothalamus (Fig. 1D). In these regions, signals were detected in major cellular populations. For example, most cells were labeled in thalamic nuclei. In other regions, the γ4 subunit mRNA was expressed in limited cellular populations, which were poorly labeled with Nissl staining and distributed irrespective of the grey and white matters (Fig. 2D, 2L, 2T, 3C, 3I). Moreover, laminar patterns of signal distribution were obscure in the cerebral cortex and hippocampus, suggesting its expression in non-neuronal cells. In the cerebellum, high levels of the γ4 subunit mRNA were detected in the Purkinje cell layer (Fig. 2T), where it was detected in Bergmann glia, but not in Purkinje cells (Fig. 3O).

γ5 subunit. The γ5 subunit mRNA was detected highly in the olfactory bulb, CA2 region of the hippocampus, thalamus, and cerebellum, and moderately in the septum and inferior colliculus (Fig. 1E). In the hippocampal CA2 region, signals for the γ5 subunit mRNA were detected over pyramidal cells, (Fig. 2M, 3D). In the cerebellum, the γ5 subunit mRNA was selectively expressed in Bergmann glia (Fig. 2U, 3P).

γ6 subunit. Hybridization signals for the γ6 subunit mRNA were very low in the adult brain, showing faint labeling in some telencephalic structures, such as the olfactory bulb and hippocampus (Fig. 1F, 2F, 2N, 2V).

γ7 subunit. The γ7 subunit mRNA was prominently expressed in various grey matter regions (Fig. 1G). In the cerebral cortex, moderate levels were observed in the layers II-VI (Fig. 2G). In the hippocampus, levels were higher in the CA2 region and dentate gyrus than in the CA1 and CA3 regions (Fig. 2O, 3E, 3K). In the cerebellum, the γ7 subunit mRNA was high in Purkinje cells, and also detected in other neurons, including granule cells, molecular layer interneurons, and neurons in the deep cerebellar nuclei (Fig. 2W, 3Q).

γ8 subunit. The γ8 subunit mRNA was highly enriched in the telencephalon with the highest levels in all hippocampal regions (Fig. 1H, 2P, 3F, 3L). In the cerebral cortex, levels were higher in the layers II/III and V than the layers IV and VI (Fig. 2H).

Developmental changes

Developmental changes were examined from E13 to P21 (Fig. 4). VDCCγ subunits that were expresssed highly in the adult brain (i.e., γ2-5, γ7, γ8) also exhibited high expression in embryonic brains. Of these, several subunits showed generally preserved patterns of spatial

expression throughout development. Higher expression of the γ2 subunit mRNA in the cerebellum (Fig. 4B), wide expression of the γ7 subunit mRNA in the brain (Fig. 4G), and higher expression of the γ8 subunit mRNA in the telencephalon (Fig. 4H) were all noted from embryonic and neonatal stages, and continued to the adult stage. In addition, higher expression of the γ5 subunit mRNA in the olfactory bulb, thalamus, inferior colliculus, and cerebellar cortex was observed from perinatal stages (Fig. 4E). On the other hand, the γ4 subunit mRNA was very strong throughout brains until P7, and the adult type of expression was established by thereafter downregulation in the grey matter, except for olfactory bulb, striatum, and thalamus (Fig. 4D). On the contrary, higher expression of the γ3 subunit mRNA in the telencephalon became apparent at P7 and thereafter (Fig. 4C).

Faint signals for the γ1 subunit mRNA was transiently detected in the telencephalon at E18 and P1 (Fig. 4A). At P7, low signals for γ6 subunit mRNA first appeared in the olfactory bulb, cerebral cortex, and hippocampus, which was maintained until the adult stage (Fig. 4F). In contrast to generally low expression in the brain, the γ1 and γ6 subunit mRNAs were clearly detected in the skeletal muscle (arrows in Fig. 4A, F).

Discussion

In the present study, we examined expression patterns for the VDCCγ subunit family in developing and adult mouse brains by *in situ* hybridization, and have disclosed their distinct regional and cellular expression as summarized in Table 1.

The TARP family, which consists of γ2, γ3, γ4, and γ8 subunits, was originally reported to participate in surface expression, synaptic targeting and recycling of AMPA receptors (Chen et al., 2000; Tomita et al., 2003, 2004). Recently, we and others have found that TARPs not only modulate AMPA receptor trafficking but also regulate their channel activities working as auxiliary subunits (Yamazaki et al., 2004; Priel et al., 2005; Tomita et al., 2005). Therefore, the TARP family, presumably other VDCCγ subunits as well, play diverse roles as ion channel modulators. Distinct regional expressions of the TARP-VDCCγ subunits in the present study are well consistent with previous reports (Chen et al., 2000; Klugbauer at al., 2000; Tomita et al., 2003). In general, major subunits in given brain regions are γ2, γ3, and γ8 subunits in the telencephalon, γ2 and γ4 subunits in the diencephalon, and γ2 subunit in other regions, including the cerebellum. Within the cerebellum, we have disclosed here distinct cellular expression: γ2 subunit in Purkinje cells, granule cells and molecular layer interneurons, γ3 subunit in Golgi cells, and γ4 subunit in Bergmann glia. The distinct cellular expression agrees with reported phenotypes in the *stargazer* mouse, a spontaneous mutant defective in the γ2 subunit (Letts et al., 1998). In the *stargazer* cerebellum, AMPA receptor-mediated fast responses are almost missing and AMPA receptor subunits are greatly reduced at mossy fiber-granule cell synapses (Hashimoto et al., 1999; Chen et al., 1999, 2000). On the other hand, AMPA receptors at hippocampal pyramidal cell synapses are intact in the *stargazer* mouse (Hashimoto et al., 1999). Based on our present results, this different phenotype can be interpreted that the γ2 subunit is the sole subunit of the TARPs expressed in cerebellar granule cells, whereas it is one of the subunits expressed in hippocampal pyramidal cells. In particular, the γ8 subunit is expressed most prominently in the hippocampus, and thus may compensate

the loss of γ2 subunit function in the *stargazer* (Fig. 1H and 2P in the present study; Tomita et al., 2003). If this is also true for other neurons, AMPA receptor function and localization might be greatly affected at synapses in cerebellar Purkinje cells and brainstem motoneurons of the *stargazer*, in which the γ2 subunit is predominantly expressed, like cerebellar granule cells. Since Ca^{2+}-permeable AMPA receptors expressed in Bergmann glia have been shown to play an important role in glial enwrapping of Purkinje cell synapses (Iino et al., 2001), the γ4 subunit may also control transmission at Purkinje cell synapses through regulating the glial AMPA receptors.

Regional and cellular distributions of mRNAs for non-TARP-VDCCγ subunits (γ1, γ5, γ6, and γ7 subunits) are also differential in the brain. Of these, the γ1, γ5 and γ7 subunits are known to regulate function and localization of VDCC complex (Wei et al., 1991; Eberst et al., 1997; Klugbauer et al., 2000; Moss et al., 2002). Although the γ1 and γ6 subunit mRNA are expressed in skeletal muscles (Fig. 4A, 4F; Powers et al., 1993), they are negative or showed low restricted expression in the adult brain, suggesting little or limited contribution to brain function. Transient expression of the γ1 subunit mRNA in newborn telencephalon implies its transient involvement in brain development. In contrast, the γ7 subunit mRNA is expressed highly and widely in developing and adult brains, suggesting that it is a major regulator for VDCCs in the brain. From the expression in particular neural regions, such as hippocampal CA2 region and Bergmann glia, the γ5 subunit may play some additional role in these regions. Functional assignment of the γ6 subunit remains unknown, and its expression in the brain is low throughout development.

The VDCCγ subunit family is thus diverse not only in molecular forms and function,

but also in their spatial expression in the brain. The present mapping study on the VDCCγ subunits will provide the molecular-anatomical basis for future studies.

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Legends

Figure 1. Distribution of VDCCγ1 (A), γ2 (B), γ3 (C), γ4 (D), γ5 (E), γ6 (F), γ7 (G), and γ8 (H) subunit mRNAs in the adult mouse brain. Images were made from parasagittal brain sections exposed to an X-ray film. Insets show negative hybridizing signals by adding unlabeled probes. Cb, cerebellum; CP, caudate-putamen; Cx, cerebral cortex; Hi, hippocampus; Hy, hypothalamus; Mb, midbrain; MO, medulla oblongata; OB, olfactory bulb; Th, thalamus. Scale bars, 1mm.

Figure 2. Distribution of VDCCγ1 (A, I, Q), γ2 (B, J, R), γ3 (C, K, S), γ4 (D, L, T), γ5 (E, M, U), γ6 (F, N, V), γ7 (G, O, W), and γ8 (H, P, X) subunit mRNAs in the adult cerebral cortex (A-H), hippocampus (I-P), and cerebellum (Q-X). Images were made from emulsion-dipped sections. CA1-3, CA1-3 regions of the hippocampus; CC, corpus callosum; CP, caudate-putamen; DCN, deep cerebellar nuclei; DG, dentate gyrus; GL, granular rayer; I-VI, layer I-VI of the cerebral cortex; ML, molecular layer; PL, Purkinje cell layer; Su, subiculum. Scale bars, 500 μm in A and I; 1 mm in Q.

Figure 3. Bright-field miscrographs showing VDCCγ2 (A, G, M), γ3 (B, H, N), γ4 (C, I, O), γ5 (D, J, P), γ7 (E, K, Q), and γ8 (F, L, R) subunit mRNAs in the adult hippocampal CA1 or CA2 region (A-F), dentate gyrus (G-L), and cerebellar cortex (M-R). No significant signals for the γ1 and γ6 subunit mRNAs were detected in emulsion-dipped sections. White arrowheads in C and I indicate γ4 mRNA-positive glial cells. Black and white arrows in M-R indicate the cell body of Purkinje cells and Bergmann glia, respectively. Double arrows in N

indicate a Golgi cell. CA1 and CA2, CA1 and CA2 regions of the hippocampus; Cb, cerebellum; DG, dentate gyrus; Gr, granular layer; Mo, molecular layer; Or, stratum oriens; Po, polymorphic layer; Py, pyramidal cell layer; Ra, stratum radiatum. Scale bars, 20 μm.

Figure 4. Developmental changes in expressions for VDCCγ1 (A), γ2 (B), γ3 (C), γ4 (D), γ5 (E), γ6 (F), γ7 (G), and γ8 (H) mRNAs in mouse brains from E13 to P21. Each set of hybridized sections was exposed to a single X-ray film. Arrows indicate expression of γ1 and γ2 mRNAs in the skeletal muscles. Scale bar, 1 mm.

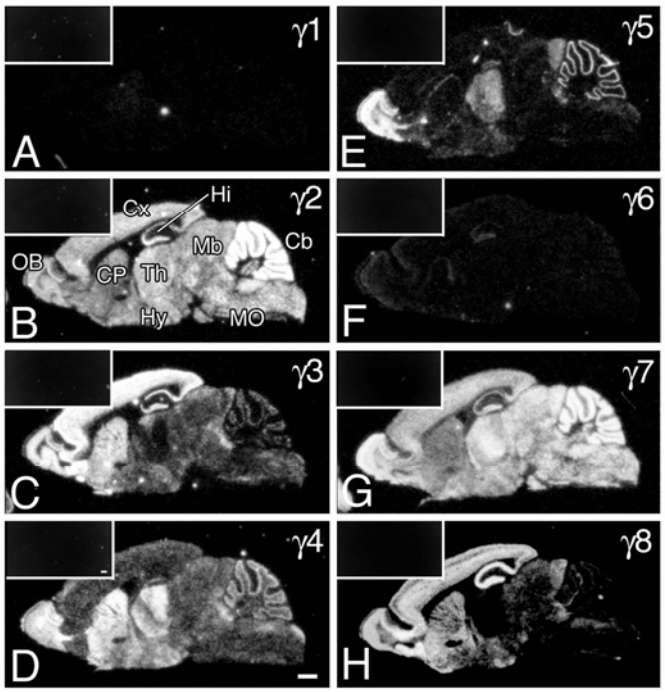

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Figure 1

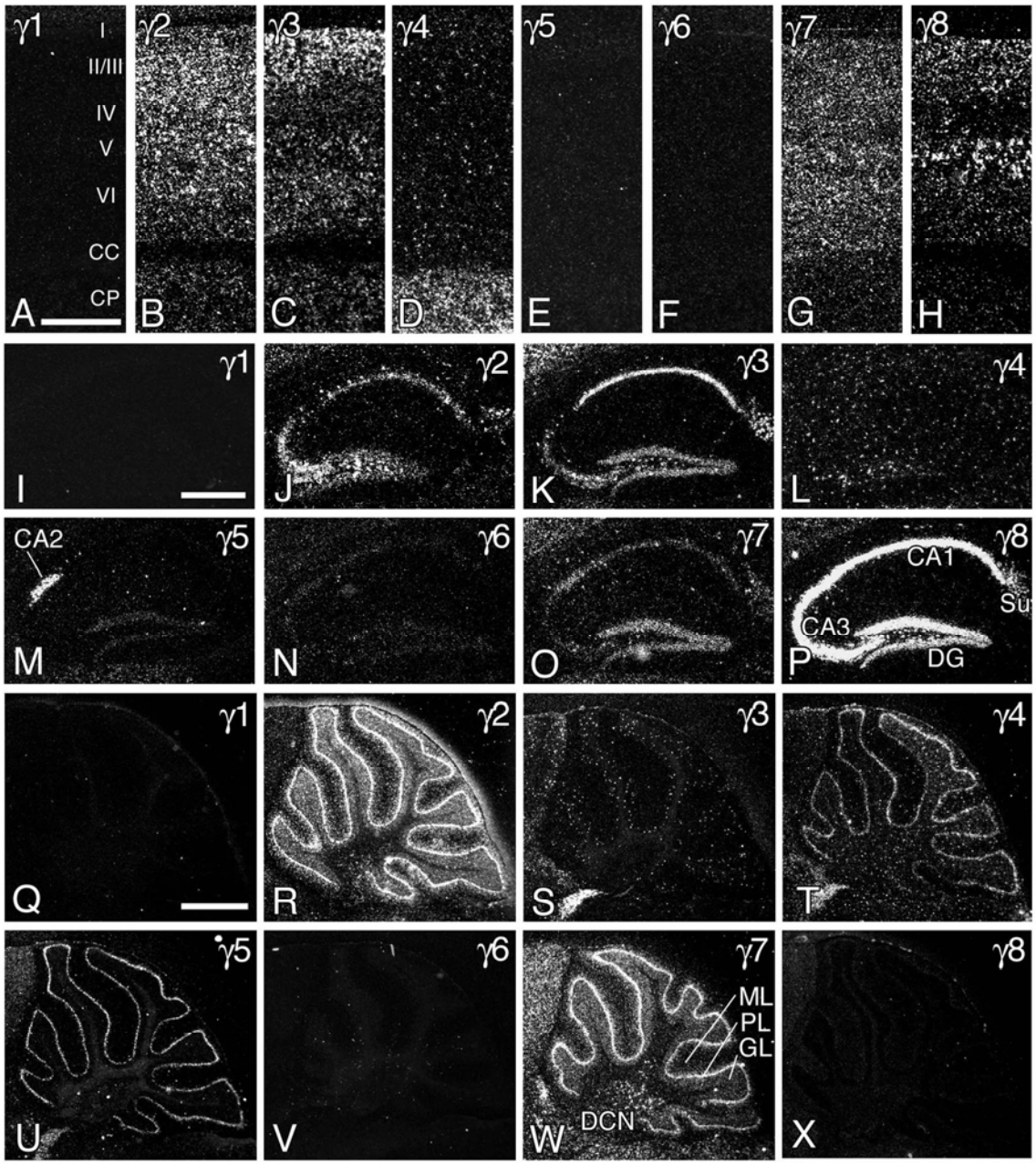

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Figure 2

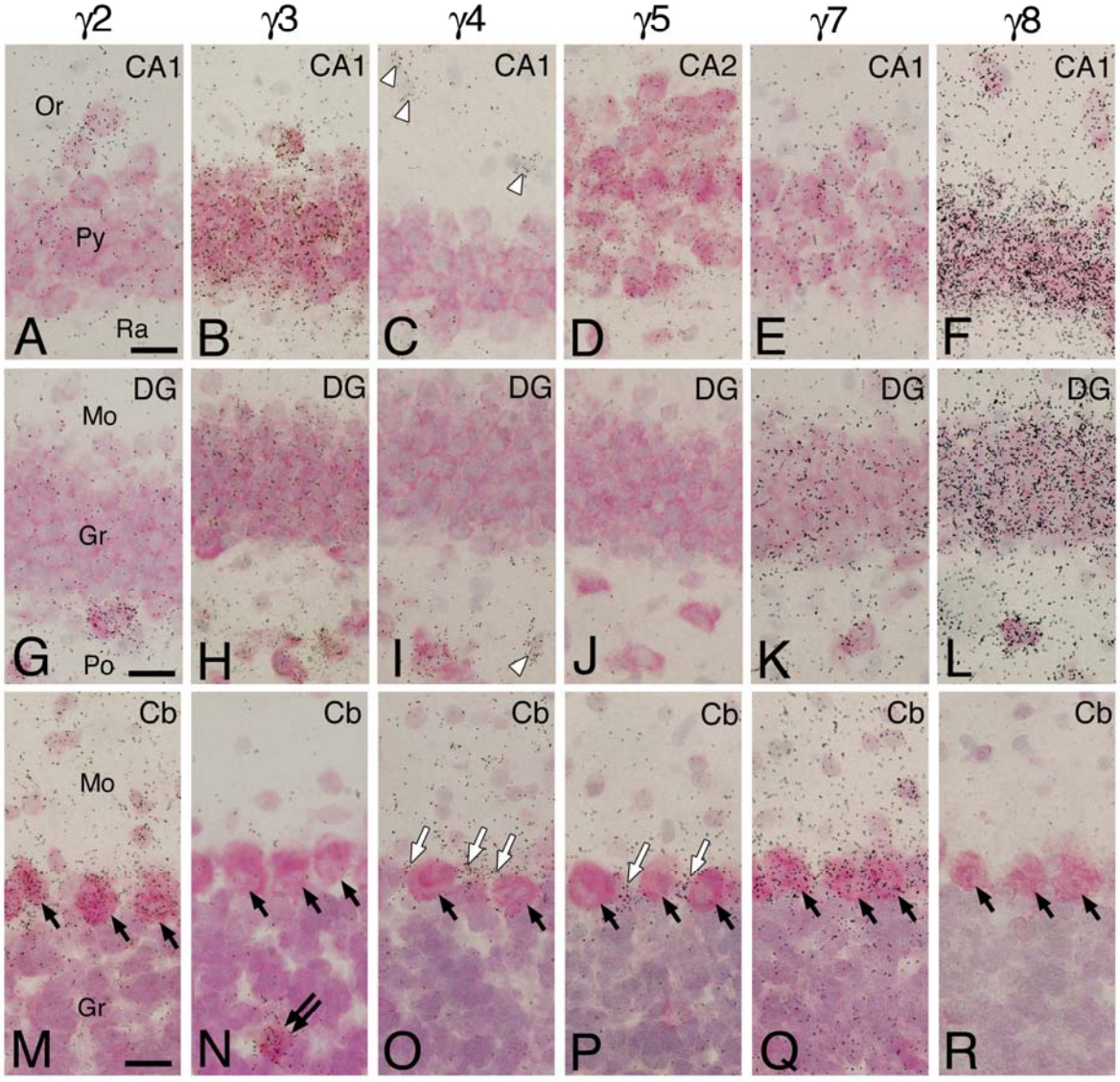

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Figure 3

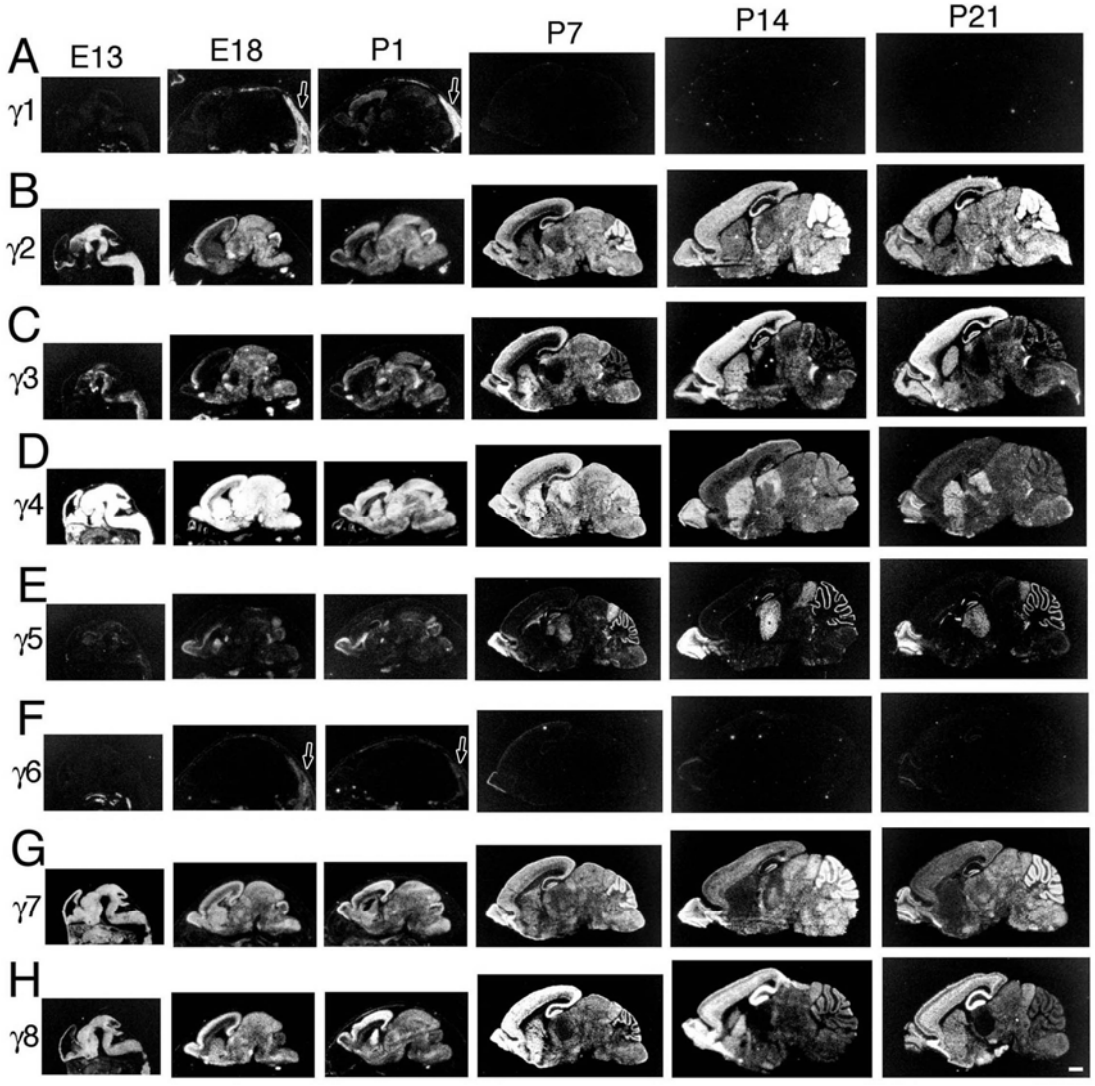

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Figure 4

Table 1. Relative abundance*[1] of VDCCγ subunit mRNAs in adult mouse brain

	γ1	γ2	γ3	γ4	γ5	γ6	γ7	γ8
Olfactory bulb								
Mitral cell layer	-	1	1	1	4	2	4	1
Granule cell layer	-	3	4	4	4	2	4	4
Glomerular layer	-	3	1	4	4	2	4	3
Cerebral cortex								
Layer I	-	1	-	2	-	-	-	-
Layer II/III	-	3	4	2	-	1	3	4
Layer IV	-	3	3	2	-	-	3	2
Layer V	-	3	4	2	-	-	3	4
Layer VI	-	3	4	2	-	-	3	3
Hippocampus								
CA1 (pyramidal cells)	-	2	4	2	-	1	3	5
CA2 (pyramidal cells)	-	1	1	2	5	1	3	5
CA3 (pyramidal cells)	-	4	3	2	-	1	3	5
Dentate gyrus (granule cells)	-	4	3	2	2	2	3	5
Interneurons	-	3	3	-	-	-	3	4
Subiculum	-	3	3	2	-	1	3	5
Striatum								
Caudate-putamen	-	3	4	4	-	-	2	4
Globus pallidus	-	3	4	4	-	-	2	4
Septum	-	3	3	3	4	-	2	4
Amygdala	-	3	3	3	3	-	3	4
Corpus callosum	-	-	-	2	-	-	-	-
Thalamus	-	4	2	4	4	-	4	1
Hypothalamus	-	4	3	4	2	-	4	3
Superior colliculus	-	4	2	2	1	-	4	2
Inferior colliculus	-	4	2	2	3	-	4	4
Substantia nigra	-	3	2	2	-	-	3	2
Precerebellar nuclei								
Pontine nuclei	-	3	1	2	-	-	4	1
Reticulotegmental nucleus	-	3	1	2	-	-	4	1
Lateral reticular nucleus	-	3	1	1	-	-	4	1
Inferior olive	-	2	2	1	-	-	4	1
Motor nuclei*[2]		-	4	1	1	1	-	4
1								
Trigeminal nucleus (sensory)	-	2	1	1	-	-	4	1
Cochlear nucleus (dorsal)	-	3	1	1	1	-	4	1
Cerebellum								
Purkinje cells	-	5	-	-	-	-	5	-
Granule cells	-	5	1	1	-	-	4	-
Stellate/Basket	-	3	4	3	-	-	3	-
Bergmann glia	-	-	-	4	4	-	-	-
Deep cerebellar nuclei	-	3	1	1	-	-	4	-

*[1] Relative expression levels were estimated by visual comparison using both X-ray film autoradiograms and emulsion-dipped sections. -, not detected; 1, very low; 2, low; 3, moderate; 4, high; 5, very high. *[2] Trigeminal motor nucleus, facial nucleus, and hypoglossal nucleus.