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Differential Expression of *Shaw*-related K^+ Channels in the Rat Central Nervous System

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The family of mammalian genes related to the *Drosophila Shaker* gene, consisting of four subfamilies, is thought to encode subunits of tetrameric voltage-gated K^+ channels. There is compelling evidence that subunits of the same subfamily, but not of different subfamilies, form heteromultimeric channels *in vitro,* and thus, each gene subfamily is postulated to encode components of an independent channel system. In order to identify cells with native channels containing subunits of one of these subfamilies (*Shaw*-related or ShIII), the cellular distribution of ShIII transcripts was examined by Northern blot analysis and *in situ* hybridization. Three of four ShIII genes (KV3.1, KV3.2, and KV3.3) are expressed mainly in the CNS. KV3.4 transcripts are also present in the CNS but are more abundant in skeletal muscle. *In situ* hybridization studies in the CNS reveal discrete and specific neuronal populations that prominently express ShIII mRNAs, both in projecting and in local circuit neurons. In the cerebral cortex, hippocampus, and caudate-putamen, subsets of neurons can be distinguished by the expression of specific ShIII mRNAs.

Each ShIII gene exhibits a unique pattern of expression; however, many neuronal populations expressing KV3.1 transcripts also express KV3.3 mRNAs. Furthermore, KV3.4 transcripts are present, albeit at lower levels, in several of the neuronal populations that also express KV3.1 and/or KV3.3 mRNAs, revealing a high potential for heteromultimer formation between the products of three of the four genes. Expression of ShIII cRNAs in *Xenopus* oocytes was used to explore the functional consequences of heteromultimer formation between ShIII subunits. Small amounts of KV3.4 cRNA, which expresses small, fast-inactivating currents when injected alone, produced fast-inactivating currents that are severalfold larger when coinjected with an excess of KV3.1 or KV3.3 cRNA. This amplification is due to both an increase in single-channel conductance in the heteromultimeric channels and the observation that less than four, perhaps even a single KV3.4 subunit is sufficient to impart fast-inactivating properties to the channel. The oocyte experiments indicate that the apparently limited, low-level expression of KV3.4 in the CNS is potentially significant. The anatomical studies suggest that heteromultimer formation between ShIII proteins might be a common feature in the CNS. Moreover, the possibility that the subunit composition of heteromultimers varies in different neurons should be considered, since the ratios of overlapping signals change from one neuronal population to another. In order to proceed with functional analysis of native ShIII channels, it is important to know which subunit compositions might occur *in vivo.* The studies presented here provide important clues for the identification of native homo- and heteromultimeric ShIII channels in neurons.

[Key words: potassium channels,* Shaker, Shaw*-related, heteromultimer,* in situ *hybridization, GABA, cortical interneurons, hippocampal interneurons]

Electrophysiological and pharmacological studies in the nervous system have revealed the existence of a large variety of ligand- and voltage-gated ion channels, leading to the view that individualities in membrane electrical properties play key roles in determining the functional specificity of neural circuits (Llinas, 1988; Steriade and Llinas, 1988; Betz, 1990; McCormick, 1990; Seeburg et al., 1990; Steriade et al., 1990; Baxter and Byrne, 1991; Kandel et al., 1991; Lüddens and Wisden, 1991; Hille, 1992; Nakanishi et al., 1992). The presence of a similar array of neurotransmitter and neuropeptide receptors acting through second-messenger cascades to modulate the activity of ion channels is also now well established (Kaczmareck and Levitan, 1987; Hille, 1992). Among the ion channels, those that conduct K^+ ions selectively constitute a group exhibiting a particularly large functional diversity (Rudy, 1988; Latorre et al., 1989; Hille, 1992). K^+ channel types vary in their response to factors, such as intracellular Na^+ and Ca^{2+} concentrations and the membrane potential, which determine the opening and closing of the channel. K^+ channels also vary in kinetics, permeability properties, and in their response to toxins, drugs, and modulating factors. This diversity allows a fine tuning of action potential waveforms by affecting the rates of repolarization and a modulation of firing and neurotransmitter-secretion patterns by influencing both membrane potential and resistance (Klein et al., 1982; Kaczmareck and Levitan, 1987; Levitan, 1988; Llinas, 1988; Rudy, 1988; McCormick, 1990; Baxter and Byrne, 1991; Hille, 1992).

The application of cDNA cloning methods has allowed the

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identification of a large number of components of K^+ channels, revealing a molecular diversity that parallels the functional diversity. However, since this work has been done in the absence of prior biochemical analysis, the relationship between the molecular components identified by cloning and native K^+ channels is not known. The physiological significance of the molecular diversity also remains to be elucidated.

Two groups of genes encoding putative subunits of mammalian neuronal voltage-gated K^+ channels have been discovered. One group consists of homologs of the *Drosophila eag* (*ether 'a-go-go*) gene (Warmke et al., 1991; Warmke and Ganetzky, 1993), the other of the homologs of the *Drosophila Shaker* gene (called here the *Shaker* or *Sh* gene family; reviewed in Jan and Jan, 1990; Perney and Kaczmarek, 1991; Rudy et al., 1991a).

Close to 20 *Sh* genes have been identified in mammals. Based on sequence similarities and, hence, probable evolutionary relationships, *Sh* genes are organized into four subfamilies (reviewed in Jan and Jan, 1990; Perney and Kaczmarek, 1991; Rudy et al., 1991a; Salkoff et al., 1992) designated here as ShI, ShII, ShIII, and ShIV. A member of a subfamily in mammals is also more similar to one of four *Drosophila* genes identified by cross-hybridization with *Shaker* probes (*Shaker, Shab, Shaw,* and *Shal*; Butler et al., 1989; Wei et al., 1990) than to a mammalian member of a different subfamily. ShI mammalian genes are thought to be homologs of *Shaker*; ShII of *Shab,* ShIII of *Shaw,* and ShIV of *Shal.* Multiple genes of each subfamily are known in mammals: ShI, 7; ShII, 2; ShIII, 4; and ShIV, 3. In addition, two *Sh* cDNAs were recently isolated from rat libraries that appear to belong to new, yet undefined subfamilies (Drewe et al., 1992). Moreover, some *Sh* genes encode more than one product by the process of alternative splicing (Luneau et al., 1991a,b; Rudy et al., 1992; Vega-Saenz de Miera et al., 1992) leading to the existence of at least 25 known *Sh* transcripts.

Each *Sh* transcript induces the expression of voltage-gated K^+ currents with a characteristic voltage dependence, kinetics, and pharmacology when introduced into *Xenopus* oocytes and other cells (Jan and Jan, 1990; Perney and Kaczmarek, 1991; Rudy et al., 1991a; Salkoff et al., 1992). The underlying channels are homomultimers, probably tetramers of *Sh* proteins (MacKinnon, 1991), and may or may not be identical to the K^+ channels normally present in cells (Rudy et al., 1991a).

One major factor that could influence the subunit composition and diversity of native K^+ channels containing *Sh* subunits is heteromultimer formation. Indeed, there is compelling evidence, from *in vitro* expression studies, that subunits of the same subfamily, but not of different subfamilies, are capable of forming heteromultimeric channels (Christie et al., 1990; Isacoff et al., 1990; K. McCormack et al., 1990; Ruppersberg et al., 1990; Covarrubias et al., 1991). It is still unknown, however, how important heteromultimer formation is in determining channel composition *in vivo.* Other factors such as interaction with non-*Sh* subunits and posttranslational modifications may also contribute to the functional properties of native channels containing *Sh* proteins (Rehm and Lazdunsky, 1988; Rudy et al., 1988, 1991a; Rehm et al., 1989a,b). Furthermore, the influence of heteromultimer formation, interaction with non-*Sh* subunits and posttranslational modifications, could vary from cell to cell. Therefore, K^+ channel diversity arising from these factors may contribute to the individualities in electrophysiological properties of single neurons in the nervous system.

In order to be able to identify cells where one can explore the functional properties of channels containing ShIII subunits and begin to elucidate the functional role of these proteins, we have studied the tissue expression of the products of the four known ShIII or *Shaw*-related genes (KV3.1, KV3.2, KV3.3, and KV3.4) by Northern blot analysis and *in situ* hybridization. The potential significance of heteromultimer formation emphasizes the importance of studying these genes as a group. In addition, we explored the functional consequences of *in vitro* heteromultimer formation in this subfamily, by coexpression of transcripts from two ShIII genes in *Xenopus* oocytes.

This study comprises a detailed comparison of the expression patterns in the CNS of the ShIII mRNAs. Although the expression of the mRNA products of two of these genes in the CNS has been previously studied with some depth (KV3.2 or KShIIIA, Rudy et al., 1992; KV3.1, Perney et al., 1992; see also Rettig et al., 1992), a detailed study of the complete ShIII subfamily has not been carried out. Moreover, only in one of these reports (Perney et al., 1992) were the neuronal populations expressing the RNA within a given brain region identified. This is necessary to estimate whether ShIII mRNAs are coexpressed in the same cells. A different view from that obtained by comparisons of the expression of only KV3.1 and KV3.2 emerges when the expression patterns of the four genes are considered. The comparison of the distribution of the products of the four ShIII genes in the CNS begins to provide us with a picture of the functional diversity that this subfamily may be able to generate.

Materials and Methods

Nomenclature of Shaw*-related genes and their transcripts*

Four *Shaw*-related or ShIII genes have been identified in rodents and humans (Yokoyama et al., 1989; K. McCormack et al., 1990; T. McCormack et al., 1990; Luneau et al., 1991a,b; Rudy et al., 1991b; Schroter et al., 1991; Ghanshani et al., 1992; Rudy et al., 1992; Vega-Saenz de Miera et al., 1992). Each one of these genes generates several alternatively spliced transcripts with divergent 3′ ends. The predicted proteins differ only in their carboxyl ends. The various names given originally to these genes and their products are shown in Table 1, which also includes the corresponding names in a simplified nomenclature proposed by several investigators in the field (Chandy et al., 1991) and the name assigned to each gene by the Human Genome Nomenclature Committee. The simplified nomenclature of Chandy et al. (1991) will be implemented throughout the text; readers can refer to Table 1 for equivalent names.

ShIII protein products of different ShIII genes are very similar (≥70% amino acid identity), particularly in the putative membrane spanning domains (Vega-Saenz de Miera et al., 1992). They are all more similar to the product of the *Drosophila Shaw* gene (≥50% amino acid identity in a core region used to compare *Sh* proteins, which includes all the membrane portion of the polypeptide) than to the products of the three other K^+ channel genes in *Drosophila* (*Shaker, Shab,* and *Shal*) or the ShI, ShII, and ShIV genes in mammals (≈40% amino acid identity). A comparison of amino acid sequences and a description of the main structural features that characterize ShIII proteins can be found in Vega-Saenz de Miera et al. (1992, 1993).

For three of the four genes, KV3.1 (Perney et al., 1992), KV3.2 (Rudy et al., 1992), and KV3.3 (M. Weiser and B. Rudy, unpublished observations), *in situ* hybridization with probes specific for different alternatively spliced variants produces indistinguishable hybridization patterns. The experiments illustrated in this article utilized gene-specific probes that differentiate between the products of different genes but do not distinguish among alternatively spliced variants.

Design and preparation of probes for Northern blot analysis and in situ *hybridization.* Probes were prepared by labeling cDNA fragments by the random hexamer primer method (Feinberg and Vogelstein, 1983) with ^{32}P or ^{35}S (for Northern blots and *in situ* hybridization, respectively). Untranslated regions (UTRs) are often preferred to obtain specific probes. However, each ShIII gene generates more than one product by alternative splicing of the 3′ end (Luneau et al., 1991a,b; McCormack et al., 1991; Rudy et al., 1991b, 1992; Schroter et al., 1991; Vega-Saenz

Table 1. Nomenclature of *Shaw*-related K^+ channel transcripts in mammals

Gene (nomenclature of Chandy et al., 1991)	Gene names previously used in this laboratory	Transcripts (nomenclature of Chandy et al., 1991)	Other names[1]	Gene symbol[2]
KV3.1	KShIIIB; NGK2-KV4	KV3.1a	NGK2;[a] KShIIIB[b]	KCNC1
		KV3.1b	KV4[c]	
KV3.2	KShIIIA	KV3.2a	RKShIIIA;[d] KShIIIA.1[e]	KCNC2
		KV3.2b; KV3.2d	KV3.2b;[f] KShIIIA.3[e] KShIIIA.2[e]	
KV3.3	KShIIID	KV3.3a	KShIIID.1[g]	KCNC3
		KV3.3b	KShIIID.2[g]	
KV3.4	KShIIIC	KV3.4a	Raw3[h]	KCNC4
		KV3.4b	KShIIIC[i]	
		KV3.4c	KV3.4c[j]	

[1] Names given to the cDNAs in publications reporting their identification.
[2] Gene symbol by the Human Genome Nomenclature Committee.
[a] Yokoyama et al. (1989).
[b] Vega-Saenz de Miera et al. (1990).
[c] Luneau et al. (1991a).
[d] T. McCormack et al. (1990).
[e] Rudy et al. (1992).
[f] Luneau et al. (1991b).
[g] Vega-Saenz de Miera et al. (1992a).
[h] Schroter et al. (1991).
[i] Rudy et al. (1991b).
[j] Vega-Saenz de Miera et al. (1993).

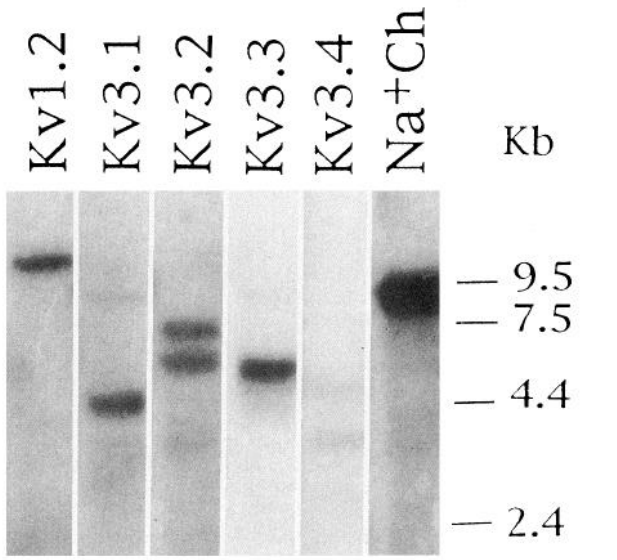


Figure 1. Northern blot analysis of rat brain polyA RNA (3 μg/lane) hybridized with probes specific to the KV1.2 (*first lane*), KV3.1 (*second lane*), KV3.2 (*third lane*), KV3.3 (*fourth lane*), KV3.4 (*fifth lane*) K^+ channels as well as with a probe specific for the α subunits of brain voltage-gated Na^+ channels (*Na^+ Ch; sixth lane*). The blots were exposed to Kodak XAR-5 x-ray film at −70°C for 3 d with two intensifying screens. The size of the RNAs as obtained with Bethesda Research Labs RNA size markers is shown on the *right*.

de Miera et al., 1992) and perhaps also of the 5′ UTRs (Kentros et al., 1992). Hence, to prepare probes that will recognize all the transcripts of each gene but distinguish between the products of the four ShIII genes it was necessary to use probes derived from the coding sequence. Short (220–454 bases) fragments from the constant region of a product of each ShIII gene, showing minimum similarity (~60%) with the products of other ShIII genes and lacking any long stretches of sequence identity, were utilized to generate gene-specific probes. The sequences were also checked in blocks of 100 bases against the sequences in the GenBank database, and none revealed sequence identities larger than 60%. In order to eliminate cross-reactivity, *in situ* hybridization was conducted under high-stringency conditions (equivalent to a hybridization temperature of ~80°; see below). These hybridization conditions will select for long stretches of sequence identity. As shown in Results (Fig. 1), each ShIII probe gave a specific banding pattern in Northern blots (even under the slightly lower stringency conditions used in these experiments), indicating that the probes do not cross-react between ShIII transcripts. Signal specificity was also assessed by the use of competition experiments in which radiolabeled probes were hybridized in the presence of a large excess (50-fold) of unlabeled probe. When the appropriate DNA fragment was used in these competition experiments, the autoradiograms were blank (data not shown). The characteristics of the probes used in each case, as well as additional controls for specificity, are described below:

KV3.1 probes (see Table 1 for nomenclature). A fragment between nucleotide 1303 and nucleotide 1536 of a rat KV3.1 cDNA (rat NGK2, Ried et al., 1993) was used for experiments on the expression of KV3.1 transcripts shown here. Identical results were obtained with a probe encoding the H1 to H6 membrane-spanning domains of KV3.1a and KV3.1b (Rudy et al., 1992). Transcript-specific antisense oligonucleotide probes also produce very similar expression patterns (Perney et al., 1992).

KV3.2 probes. The experiments illustrated here utilized a DNA fragment containing the first 380 nucleotides of RKShIIIA (McCormack et al., 1990a). Identical labeling results (Rudy et al., 1992) were obtained with probes derived from a fragment between nucleotide 964 and nucleotide 1296 of RKShIIIA and with probes derived from the 3′ UTRs of two alternatively spliced variants (Rudy et al., 1992).

KV3.3 probes. A fragment (nucleotides −222 to +232 of KShIIID.1, Vega-Saenz de Miera et al., 1992) corresponding to sequence prior to the divergence of KV3.3a and KV3.3b was used to study KV3.3 expression. Identical results were obtained with a KV3.3a-specific probe (nucleotides 2041–2807 of the KShIIID.1 cDNA).

KV3.4 probes. This probe was a fragment from a rat KV3.4b cDNA corresponding to nucleotides −34 to +193 of HKShIIIC (Rudy et al., 1991b) and is identical in sequence to nucleotides 365–592 of KV3.4a (Schroter et al., 1991).

The levels of ShIII transcripts were compared to those of KV1.2 (also known as RBK2 and RCK5), a K^+ channel transcript of the ShI subfamily, as well as to the levels of transcripts encoding α subunits of Na^+ channels by Northern blot analysis. The KV1.2 probe was a fragment spanning the last 1295 bases of RBK2.1 (McKinnon, 1989), of which 741 bases are 3′ untranslated sequence, and was a kind gift of Dr. D. McKinnon. The Na^+ channel α subunit probe was the 5′-specific probe ($pEAF_1$) of Goldin et al. (1986) and was a kind gift of Drs. A. Goldin and N. Davidson. This probe does not distinguish among the various isoforms of the Na^+ channel α subunit found in rat brain (Noda et al., 1986; Auld et al., 1988).

Preparation of RNA and Northern blot analysis. Total cellular RNA from adult (175–200 gm) Sprague-Dawley rat brain was prepared by a guanidinium-thyocianate method (Chomczynski and Sacchi, 1987). The rats were quickly decapitated, an extensive craniotomy performed, the brains removed (up to the medullospinal junction), and the tissue immediately homogenized in the denaturing solution at 4°C. PolyA RNA was prepared in oligo-dT cellulose columns as previously described (Rudy et al., 1988). The RNA was electrophoresed in denaturing formaldehyde gels and transferred to Hybond (Amersham, Arlington Heights, IL) as previously described (Rudy et al., 1988). The blots were hybridized with 1×10^6 cpm (≈1.2 ng of DNA/ml) of ^{32}P-labeled probes per ml at 42°C in a solution containing 50% formamide, 5× Denhardt's, 5× SSPE (0.15 M NaCl, 1 mM EDTA, 10 mM NaH_2PO_4, pH 7.4), 0.3% SDS, and 200 μg/ml denatured salmon sperm DNA. After hybridization, the blots were washed at 68°C in 0.2× SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7) with 0.1% SDS.

In situ *hybridization.* Adult male rat brain sections were hybridized with ^{35}S-labeled probes utilizing methods described in Stone et al. (1990). Briefly, the rats were anesthetized with sodium pentobarbital (Nembutal; 90 mg/kg, i.p.) and perfused transcardially with normal saline containing 0.5% $NaNO_2$ and 10 U/ml of heparin followed by 400 ml of cold 0.1 M phosphate buffer pH 7.2, containing 4% formaldehyde. The brains were removed, cut in blocks, and postfixed for 2 hr in the 4% formaldehyde solution. After several washes in 0.1 M phosphate buffer prepared in diethyl procarbonate (DEPC)-treated water, the brain blocks were placed in 30% sucrose overnight. Coronal sections (30–40 μm) were cut on a freezing sliding microtome and collected in scintillation vials containing cold 2× SSC made in DEPC water. The sections were prehybridized for 1–2 hr at 48°C in a solution containing 50% formamide, 2× SSC, 10% dextran sulfate, 4× Denhardt's, 50 mM dithiothreitol, and 0.5 mg/ml sonicated denatured salmon sperm DNA. After prehybridization, denatured labeled probe was added and the sections hybridized overnight at 48°C. After hybridization, the sections were washed at 48°C in SSC solutions of decreasing concentration (from 2× to 0.1×). Following a final wash in 0.1 M phosphate buffer, the sections were mounted on slides and air dried. The slides were exposed to Du Pont Cronex 4 x-ray film for 7 d and then dipped in photographic emulsion (Kodak NTB-2) and stored at 4°C (in the dark) for 2–4 weeks. After developing in Kodak D-19, the slides were fixed, counterstained with cresyl violet, and coverslipped with Permount (Fisher Scientific, St. Louis, MO). The data were analyzed by dark-field and bright-field microscopy with an Olympus BH2 photomicroscope. The atlases and books by Paxinos and Watson (1986), Swanson (1992), Paxinos (1985), and Jones (1985) were used as aids to identify CNS neuronal populations. For purposes of comparison, probes of similar specific activity (per mole of labeled nucleotide) were used and the autoradiograms exposed for similar times.

Functional expression in Xenopus *oocytes.* The following cDNA clones were used to prepare *in vitro* transcribed RNA (cRNA) for injection into *Xenopus* oocytes: RKShIIIA (KV3.2a) in pBluescript (T. McCormack et al., 1990), NGKV (KV3.1a) (Ried et al., 1993), KV4 (KV3.1b) in pGEM-A (Luneau et al., 1991a), HKShIIIC (KV3.4b) in pBluescript (Rudy et al., 1991b), and KShIIID.1 (KV3.3a) in pBluescript (Vega-Saenz de Miera et al., 1992). The recombinant plasmids were linearized by digestion with the appropriate restriction enzyme and full-length capped RNA transcripts (cRNA) synthesized with the appropriate RNA polymerase as described for each cDNA clone in the publications referred to above. The cRNAs were stored in distilled water at 200 ng/μl at −70°C. Stage V and VI *Xenopus laevis* oocytes were prepared and injected with 2–5 ng of cRNA/oocyte as in Iverson and Rudy (1990), and incubated for 2–3 d, before electrophysiological recording, at 20°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Macroscopic currents were obtained with a standard two microelectrode voltage clamp (Iverson and Rudy, 1990) at 21–22°C, under continuous perfusion with ND96. The records were low-pass filtered at 3 kHz with an 8-pole Bessel filter before digitization. Single-channel recordings were obtained by the cell-attached configuration of the patch-clamp technique as described in Iverson and Rudy (1990). The data were digitized and analyzed using the PCLAMP system (Axon Instruments, Foster City, CA).

Results

Relative abundance of ShIII transcripts in rat brain

A Northern blot of rat brain polyA RNA comparing the banding patterns of the transcripts of the four ShIII genes is shown in Figure 1. This blot was hybridized under high-stringency conditions with ShIII probes that do not differentiate between alternatively spliced variants, as described in Materials and Methods. The same probes were used for the *in situ* hybridization studies. The amounts of ShIII transcripts are compared to the levels of a ShI transcript (KV1.2, Chandy et al., 1991; also known as RBK2, McKinnon, 1989; and RCK5, Stühmer et al., 1989) and to the levels of mRNAs encoding the α subunits of voltage-gated Na^+ channels (Noda et al., 1986; Auld et al., 1988). In the rat brain, KV3.1 and KV3.3 transcripts are the most abundant ShIII products, followed by KV3.2 and, lastly, by KV3.4, which are the least abundant. Multiple hybridization bands are seen on lanes hybridized with ShIII probes, indicating the existence of several transcripts for each ShIII gene. In some cases these represent alternatively spliced variants. For example, two main bands of approximately 8 and 4.5 kilo bases (kb) are seen with the KV3.1 probe (see Fig. 1). Luneau et al. (1991a) showed that the more abundant 4.5 kb band corresponds to KV3.1b transcripts, while the 8 kb band to KV3.1a transcripts. A faint 10 kb size band is seen with probes specific to either type of transcript and its nature is not clearly understood (Luneau et al., 1991a). However, in the case of KV3.2 (Rudy et al., 1992) and KV3.3 (Vega-Saenz de Miera et al., 1992), similar banding patterns are seen whether one uses gene-specific or transcript-specific probes, suggesting that the multiple bands represent processing variants.

The autoradiogram shown here was exposed for sufficient time so that less abundant transcripts will be clearly visible, although signals by the most abundant transcripts had probably reached saturation earlier. An autoradiogram of the same blot exposed for 8 hr was used for scanning and quantification of the bands. The results show similar amounts of KV1.2 mRNAs in the rat brain as the most abundant KV3.1 (KV3.1b) and KV3.3 transcripts. These mRNAs are approximately three times more abundant than KV3.2 and approximately six times more abundant than KV3.4 transcripts. There are about six times more α subunit Na^+ channel mRNAs than the most abundant K^+ channel transcripts tested.

Tissue distribution

The presence of ShIII transcripts in various tissues was explored by Northern blot analysis (Fig. 2). KV3.1, KV3.2, and KV3.3 are expressed mainly in the brain, although low but detectable levels of KV3.1 (mainly KV3.1a) transcripts are also seen in skeletal muscle. KV3.1 and KV3.3 products have also been identified and isolated from PC12 pheochromocytoma cells (Vega-Saenz de Miera et al., 1991; Ried et al., 1993). In contrast, KV3.4 transcripts are more abundant in skeletal muscle than in brain.

Two ShIII expression patterns in the CNS

A panoramic comparison of the expression patterns of the four ShIII genes in the CNS obtained from x-ray film autoradiograms of brain sections hybridized with each probe is shown in Figures 3 and 4. Two important features of the localization of ShIII mRNAs emerge from the analysis at this level of resolution.

(1) As is the case with neurotransmitters, receptors, and other molecular markers, ShIII mRNAs are distributed in a nonhomogeneous fashion throughout the CNS. In fact, some neuronal types (e.g., most neurons in the cerebral cortex, the caudate-putamen, the amygdala and the hypothalamus) exhibit undetectable labeling, suggesting that they do not express significant amounts of any ShIII mRNAs (Fig. 3).

(2) The expression patterns of KV3.1 and KV3.3 are very similar in x-ray film autoradiography (Figs. 3, 4). Moreover, KV3.4 mRNAs that, in accordance with the results from Northern blot analysis, are the least abundant in the CNS, are seen mainly in areas that also contain KV3.1 and/or KV3.3 transcripts, usually at higher levels (Figs. 3, 4). Thus, there appears to be a high degree of overlap between the expression patterns of these three genes. On the other hand, there are many areas where KV3.2 transcripts do not overlap with the products of the other ShIII genes. KV3.2 transcripts are particularly abundant throughout the dorsal thalamus (Figs. 3, 4) but they are

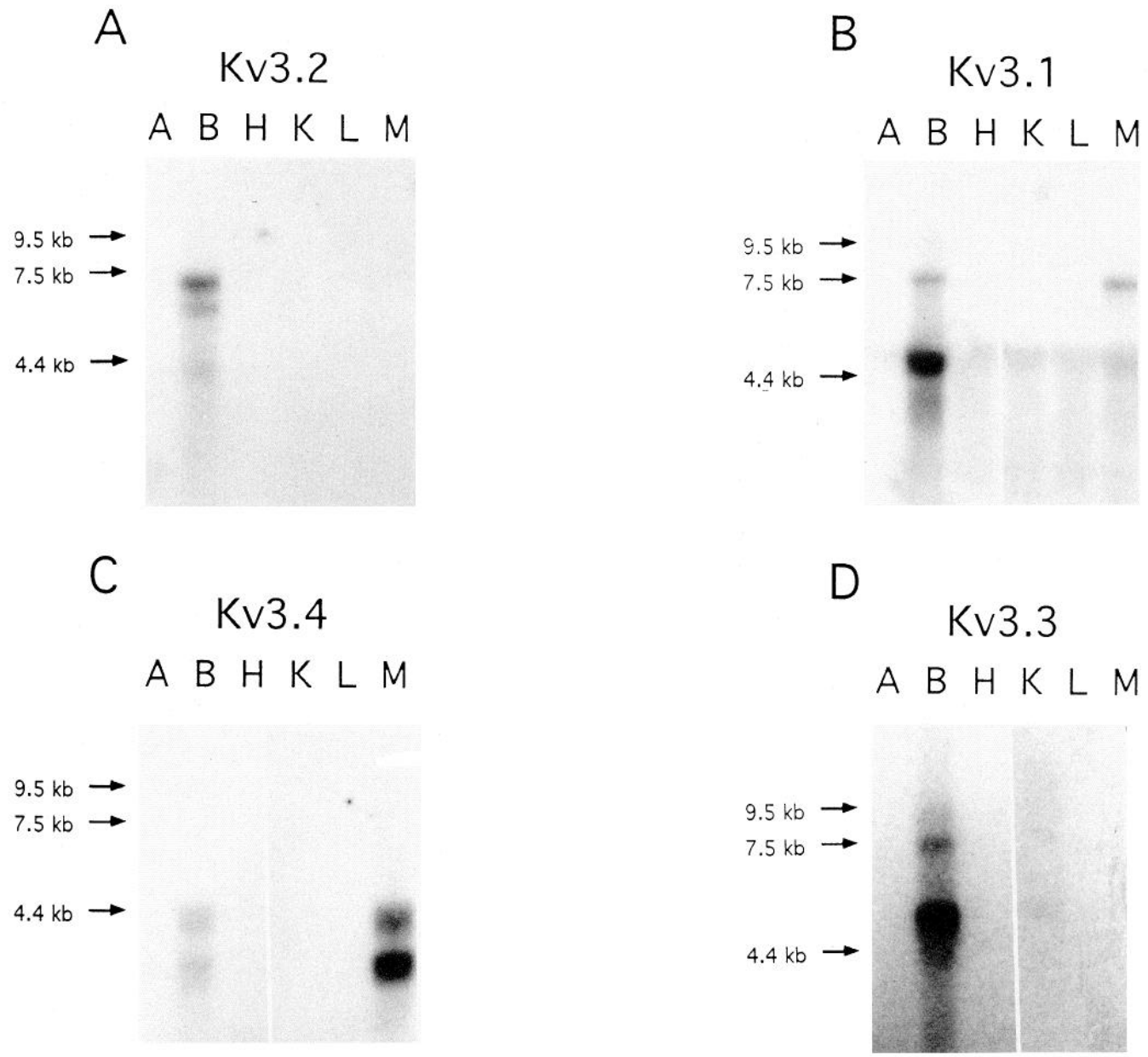


Figure 2. Tissue expression of four ShIII genes. Northern blot analysis of total rat RNA (20 μg/lane) from adrenal gland (*first lane*), brain (*second lane*), heart (*third lane*), kidney (*fourth lane*), liver (*fifth lane*), and skeletal abdominal muscle (*sixth lane*) hybridized with KV3.2 (*panel A*), KV3.1 (*panel B*), KV3.4 (*panel C*), or KV3.3 (*panel D*) probes. The blots were exposed to Kodak XAR-5 x-ray film at −70°C with two intensifying screens for 3 d for KV3.1, KV3.2, and KV3.3 probes and 7 d for KV3.4 probes. *Arrows* indicate the positions of three RNA size markers (9.5, 7.5, and 4.4 kb).

rare in the cerebellar cortex, most of the hindbrain, and the spinal cord (Fig. 4). KV3.1 and KV3.3 mRNAs are present at highest levels in the cerebellar cortex, the inferior colliculus, several structures in the hindbrain, and the spinal cord (Fig. 4).

A higher-resolution study obtained by microscopic analysis of emulsion-dipped sections was necessary to assess the overlap of the expression patterns of the various transcripts. Hybridization appears to be only on neurons and not glia, based both upon cell size and morphology. In all cases, hybridization is in the larger-diameter cells having characteristics of neuronal somas. In some areas such as the cortex and the hippocampus having multiple, distinct, complex neuronal populations, ShIII mRNAs are present in a small subset of neurons, making it difficult to ascertain whether the same neurons express more than one of these mRNAs, even if the overall pattern of expression appears to be the same. On the other hand, labeling was observed with more than one ShIII probe in other areas where neuronal subpopulations are more easily identifiable. When most or all of the neurons of such neuronal subpopulations are labeled with one of these probes, then labeling of the same subpopulation with another probe strongly suggests that the mRNAs are coexpressed in the same neurons. Definitive proof of coexpression will require colocalization experiments, for example, by using distinct labeling for two probes in the same preparation.

The higher-resolution analysis reveals that the expression patterns of KV3.1 and KV3.3 are similar but not identical, contrary to what might have been concluded from the x-ray film autoradiography. In some neuronal populations, the signals produced by KV3.1 probes are more intense than those produced

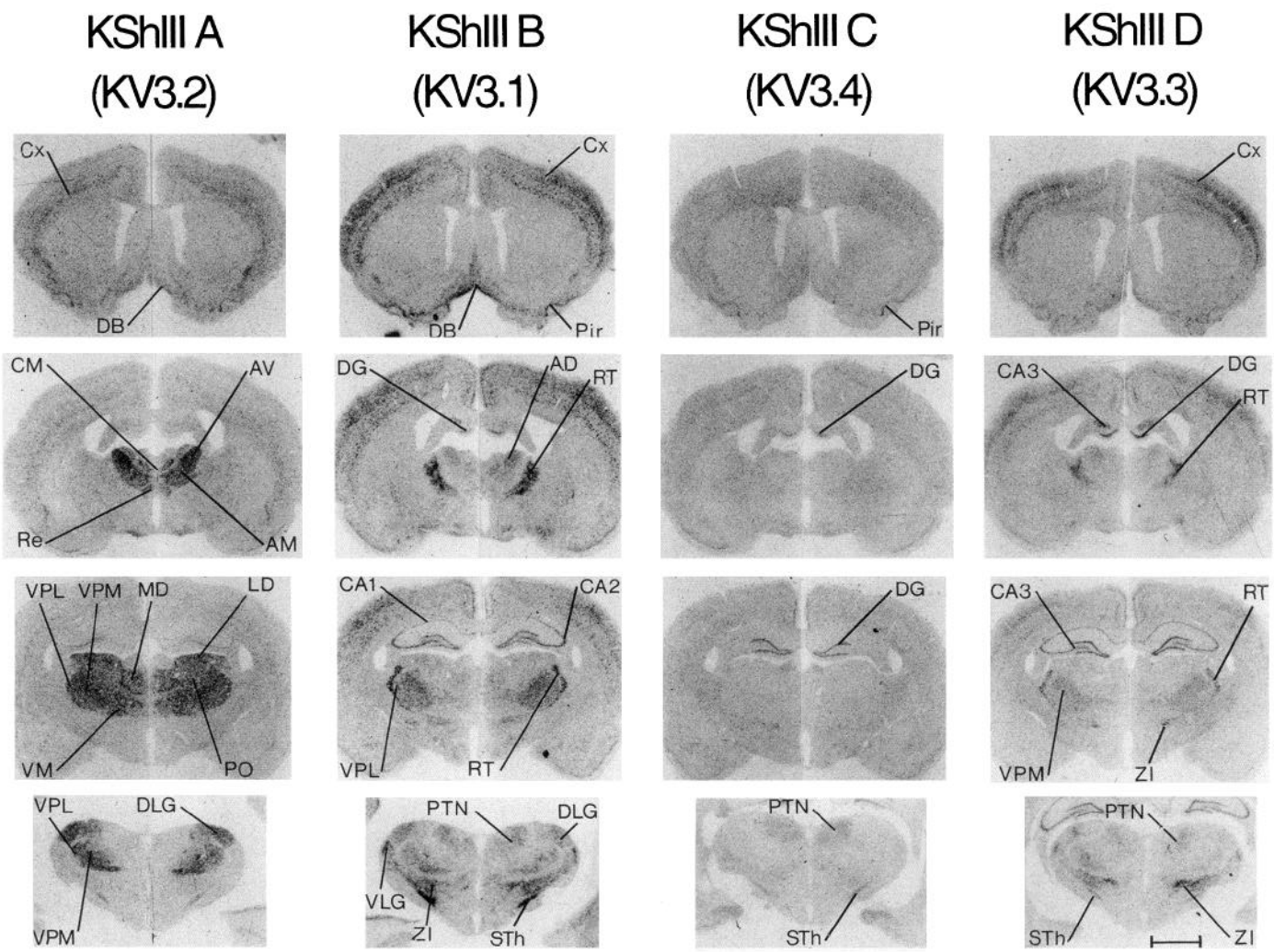


Figure 3. Panoramic view of the localization of ShIII transcripts in the rostral rat brain illustrated with x-ray film autoradiograms of 40 μm coronal sections hybridized with probes to four ShIII K^+ channel mRNAs. Contiguous sections were used for each of the four probes. Shown for each probe are sections through four levels of the brain. *First row,* sections at the level of the diagonal band of Broca; *second row,* sections at the level of the anterior thalamus; *third row,* sections at the level of the posterior thalamus; *fourth row,* sections at the level of the dorsal lateral geniculate (without most of the overlying cortex). Note the unique and specific regional distribution of the four K^+ channel mRNAs, and the lack of significant signal in the caudate-putamen (*first and second rows*) and the hypothalamus (*second and third rows*). *AD,* anterodorsal thalamic nucleus; *AM,* anteromedial thalamic nucleus; *AV,* anteroventral thalamic nucleus; *CA1,* CA1 region of hippocampus; *CA2,* CA2 region of hippocampus; *CA3,* CA3 region of hippocampus; *CM,* centeromedial thalamic nucleus; *Cx,* cortex; *DB,* nucleus of the diagonal band of Broca; *DLG,* dorsal lateral geniculate; *LD,* lateral dorsal thalamic nucleus; *Pir,* piriform cortex; *PO,* posterior thalamic nucleus; *PTN,* pretectal nucleus; *Re,* reuiniens nucleus; *RT,* reticular thalamic nucleus; *STh,* subthalamic nucleus; *VLG,* ventral lateral geniculate; *VM,* ventromedial thalamic nucleus; *VPL,* ventral posterior lateral thalamic nucleus; *VPM,* ventral posterior medial thalamic nucleus; *ZI,* zona incerta. Scale bar, 2.5 mm.

by KV3.3 probes, but the reverse is true in other areas. An extreme case is seen in regions such as the cerebellar cortex where both KV3.1 and KV3.3 mRNAs are abundantly present but basically in distinct neuronal populations.

A summary of the levels of expression of the four ShIII genes in the CNS based on the high-resolution analysis obtained from emulsion autoradiograms is shown in Table 2. Interestingly, in several brain areas, illustrated here in the cerebral cortex, the hippocampus, and the caudate-putamen, individual ShIII probes label specifically, sometimes intensely, a small number of neurons indistinguishable morphologically from other cells in the area. Thus, it appears that subsets of neurons in these regions can be distinguished by their expression of ShIII mRNAs. Details of the localizations of ShIII transcripts in specific brain areas are described below.

Olfactory bulb. KV3.1 and KV3.3 mRNAs are found in the olfactory bulb in similar neuronal populations. In this structure, the strongest signals are seen over mitral cells (Mi in Fig. 5) but some tufted cells also show clear but weaker signals (indicated by arrows in Fig. 5). Weak, diffuse periglomerular labeling is also seen (Gl in Fig. 5). No significant hybridization is seen in the olfactory bulb with KV3.2 and KV3.4 probes (data not shown).

Neocortex. All four ShIII genes are expressed in the cerebral cortex. Two different patterns of hybridization are evident in x-ray film autoradiograms (Fig. 3). Probes specific for KV3.2 mRNAs produce a band localized toward deep layers of the cortex (Fig. 3, first, second, and third rows). KV3.1, KV3.3, and KV3.4 probes produce a different labeling pattern characterized by a double band, one in superficial layers, which is more in-

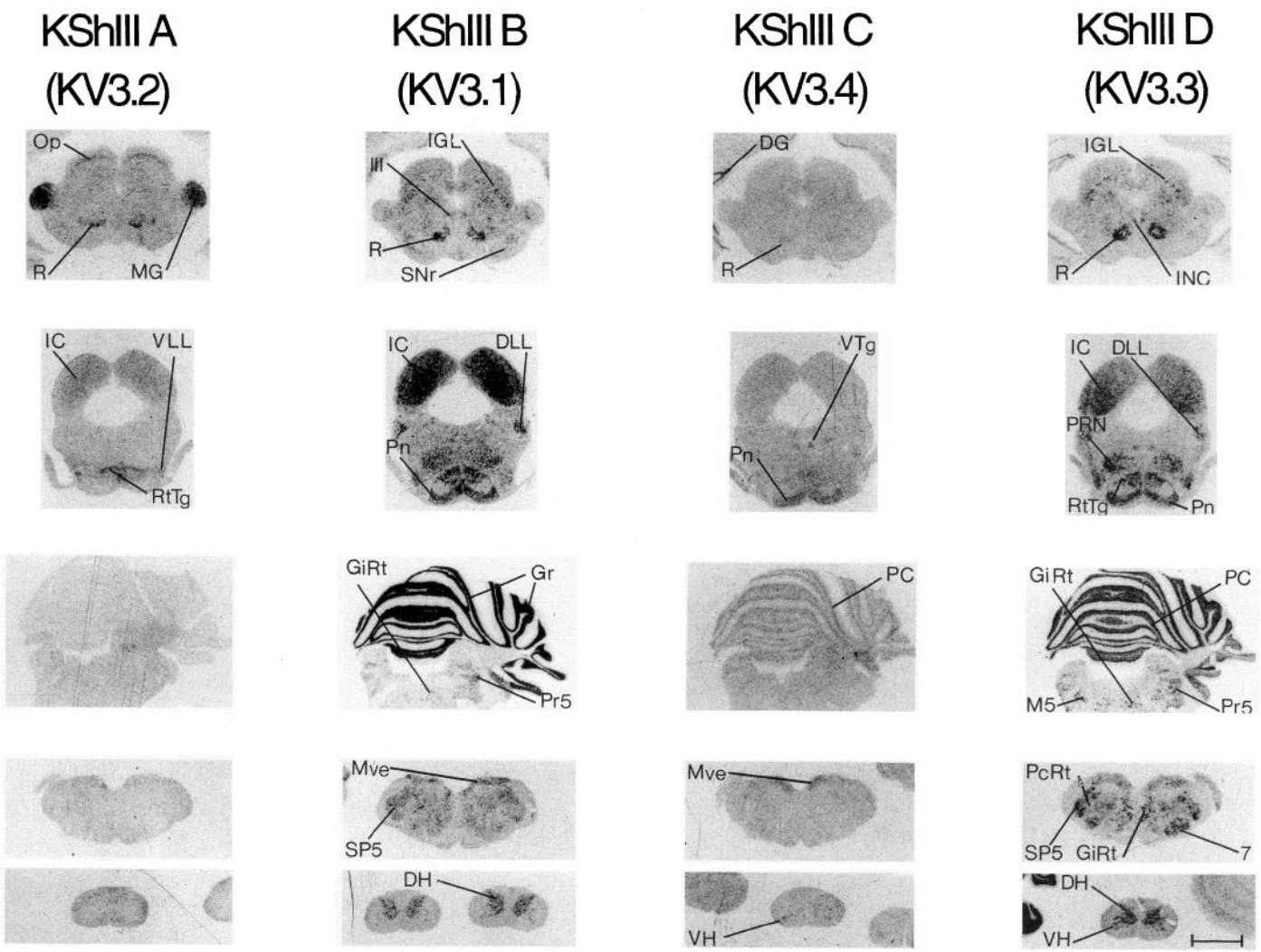


Figure 4. Panoramic view of the localization of ShIII transcripts in the caudal rat brain and cervical spinal cord illustrated with x-ray film autoradiograms of 40 μm coronal sections hybridized with probes to four ShIII K^+ channel mRNAs. Contiguous sections were used for each of the four probes. Shown for each probe are sections through four levels of the brain and one section through the cervical spinal cord. *First row,* sections at the level of superior colliculus (without most of the overlying cortex); *second row,* sections at the level of inferior colliculus (without the overlying cortex); *third row,* sections at the level of the cerebellum; *fourth row,* sections of the brainstem at the level of the facial nucleus; *fifth row,* sections of the cervical spinal cord. Note the unique and specific regional distribution of the four K^+ channel mRNAs. *7,* facial nucleus; *DG,* dentate gyrus; *DH,* dorsal horn; *DLL,* dorsal lateral lemiscus; *GiRt,* gigantocellular reticular nucleus; *Gr,* granule cell layer; *IC,* inferior colliculus; *IGL,* intermediary gray layer; *III,* oculomotor nucleus; *INC,* interstitial nucleus of Cajal; *M5,* trigeminal motor nucleus; *MG,* medial geniculate; *Mve,* medial vestibular nucleus; *Op,* optic layer of interior colliculus; *PC,* Purkinje cell layer; *PcRt,* parvocellular reticular nucleus; *Pn,* pontine nucleus; *Pr5,* trigeminal primary sensory nucleus; *PRN,* pontine reticular nucleus; *R,* red nucleus; *RtTg,* reticulotegmental nucleus; *SNr,* substantia nigra pars reticulata; *SP5,* spinal trigeminal nucleus; *VH,* ventral horn; *VLL,* ventral lateral lemiscus; *VTg,* ventral tegmental nucleus. Scale bar, 2.5 mm.

tense, and one in deeper layers (Fig. 3, first, second, and third rows). In all cases, hybridization in the cortex appears weak in x-ray film autoradiography.

However, analysis of emulsion-dipped sections reveals an uneven signal characterized by strong but infrequent hybridization patches (shown for KV3.2 and KV3.1 in Fig. 6, *A* and *C,* respectively). These patches are due to multiple grains present over single cells or sometimes two or three cells close to each other (see Fig. 6*E–G*). The distribution of these cells throughout the layers of the cortex is characteristic for KV3.2 versus KV3.1 mRNAs and explains the signals seen in the x-ray film autoradiograms. KV3.2 mRNAs are present in cells located in layers V–VI (Fig. 6*A,B*). KV3.1 mRNAs are present in cells located throughout layers II–VI, being more numerous in layers II, the superficial part of III, and layer IV (Fig. 6*C,D*). Consistent with the x-ray autoradiography (Fig. 3, first, second, and third rows), the hybridization patches seen with KV3.3 and KV3.4 probes show a distribution pattern similar to that of KV3.1 but they are of lower intensity (KV3.1 > KV3.3 ≫ KV3.4; data not shown).

The hybridization patterns in the cortex suggest that ShIII mRNAs are present in nonprojecting cortical interneurons (see Discussion). Consistent with this conclusion, hybridization grains are accumulated over small- and medium-sized neurons rather than the large pyramidal cells (Fig. 6*E–G*).

Piriform cortex. The four ShIII genes are expressed in the piriform cortex at low to moderate levels (Fig. 3, first row). At high resolution, we find, however, that KV3.2 mRNAs are present in different layers than KV3.1, KV3.3, and KV3.4 mRNAs. KV3.2 mRNAs are present mainly in cells located in the bound-

Table 2. Distribution of transcripts from four ShIII genes in the CNS

	KShIIIA (KV3.2)	KShIIIB (KV3.1)	KShIIIC (KV3.4)	KShIIID (KV3.3)
Olfactory bulb				
Periglomerular cells	−	+	−	+
Tufted cells	−	+	−	+
Mitral cells	−	++	−	++
Neocortex				
Interneurons	Layers V–VI	Layers II–IV > V–VI	Layers II–IV > V–VI	Layers II–IV > V–VI
Pyramidal cells	−	−	−	−
Pyriform cortex	+	+	±	+
Hippocampus				
CA1 pyramidal cells	+	+	−	+
Str. radiatum[a]	−	++	−	+
Str. oriens[a]	+++	++	−	+
CA3 pyramidal cells	++	+++	−	+
Str. radiatum[a]	++	+	−	+
Str. oriens[a]	++	+	−	+
DG granule cells	−	+++	++	++
Borders with str. granulosum	−	+	−	±
Hilus	+++	±	−	−
Basal nuclei				
Caudate-putamen	++ scattered	−	−	−
Globus pallidus	++	+	±	±
Subthalamic nucleus	++	+++	+	++
Septum				
Diagonal band of Broca	++	+	±	±
Medial septum	++	+	±	±
Lateral septum	−	−	−	−
Epithalamus				
Medial habenula	−	−	−	−
Lateral habenula	−	±	+	±
Paraventricular nuclei	−	−	−	−
Pretectum	−	++	+	±
Thalamus (dorsal)				
Anterodorsal nu.	++++	+	−	±
Anteroventral nu.	++++	±	−	−
Anteromedial nu.	++++	±	−	−
Laterodorsal nu.	+++	+	−	±
Parataenial nu.	++++	−	−	−
Reuniens nu.	+++	±	−	−
Mediodorsal nu.	++++	±	−	−
Intermediodorsal nu.	++	−	−	−
Lateral posterior nu.	+++	+	−	−
Ventral posterolateral nu.	++++	++	−	+
Ventrolateral nu.	++++	±	−	−
Ventral posteriomedial nu.	++++	++	−	+
Ventro medial nu.	++++	±	−	−
Central medial nu.	+++	±	−	−
Posterior nu.	++++	±	−	−
Dorsal lateral geniculate	++++	++	−	+
Medial geniculate	++++	−	−	−
Thalamus (Ventral)				
Reticular thalamic nu.	±	++++	±	++
Ventral lateral geniculate nu.	−	+	−	+
Brain stem				
Substantia nigra pars compacta	−	−	−	−
Substantia nigra pars reticulata	+	++	±	+
Zona incerta	+	+++	±	+++
Superior colliculi, optic layer	+++	−	−	−

Table 2. Continued

	KShIIIA (KV3.2)	KShIIIB (KV3.1)	KShIIIC (KV3.4)	KShIIID (KV3.3)
Superior colliculi, IGL[b]	+ scattered	+++ scattered	+ scattered	++ scattered
Darkshevich nu.	−	++	±	+
Interstitial nu.	+	−	−	++
Oculomotor nu.	±	±	±	+++
Red nu.	++	+++	±	+++
Inferior colliculi	+	++++	±	+++
Lateral lemniscus nuclei	++ (ventral)	+++	±	+++
Reticulo tegmental nu. pons	+	+++	+	+++
Pontine nuclei	−	+++	++	+++
Pontine reticular nu.	−	+++	+	+++
Cochlear nu. dorsal	++	± scattered	−	+ scattered
Cochlear nu. ventral	−	++	±	+++
Spinal trigeminal nu.	±	+	+	+++
Principal sensory trigeminal nu.	−	++	±	+++
Motor trigeminal nu.	−	++	++	+++
Locus coeruleus	++	−	−	−
Superior olivary complex	++ scattered	±	±	++
Nu. trapezoid body	−	++	−	++
Vestibular nu. lateral	+	++	±	+++
Vestibular nu. medial	−	+	+	++
Parvocellular reticular nu.	+	+	+	++
Gigantocellular reticular nu.	+	++	+	+++
Intermediate reticular nu.	+	++	+	+++
Abducens nu.	−	−	−	++
Facial nu.	−	−	±	+++
Prepositus hypoglossal nu.	++	−	−	±
Inferior olive	−	−	−	−
Hypoglosal nu.	−	±	±	+++
Dorsal column nu.	−	−	−	++
Cerebellum				
Molecular cell layer	−	±	+	+
Purkinje cells	−	+	+	++++
Granular cell layer	−	++++	±	++
Deep cerebellar nu.	++	+++	−	+++
Spinal cord				
Dorsal horn	−	+++	−	+++
Ventral horn	−	+	++	+++

For the interneurons in the cerebral cortex and the hippocampus, symbols indicate numbers of cells labeled; otherwise, the symbols indicate signal intensity as follows: − (signals undistinguishable from background); ± (very weak signals but clearly above surrounding background); + (weak); ++ (moderate); +++ (high); ++++ (very high).

[a] Refers to labeled neurons in the indicated stratum or on their interface with the stratum pyramidalis.

[b] IGL, intermediary gray layer.

ary between the plexiform and the pyramidal cell layer and the most superficial part of the pyramidal cell layer. In contrast, the other mRNAs are localized throughout the pyramidal layer (data not shown).

Few scattered hybridizing cells are detected in the amygdala. Weakly labeled cells are seen with KV3.4 in the medial amygdala and with KV3.1 in the basolateral amygdaloid nuclei.

Hippocampus. All ShIII mRNAs are expressed in the hippocampus, but each with a characteristic distribution (Fig. 6*H–K*).

Diffuse hybridization signals are seen in the pyramidal cell layers of the CA1–CA3 fields with KV3.2, KV3.1, and KV3.3 probes (Fig. 6*H,I,K*). In the three cases there is a gradient of expression beginning in CA1 and continuing toward CA3. Little signal is seen in these areas with KV3.4 probes. Particularly in the CA3 region, where cell density is lower, and both cell size and grain density higher, it is clear upon examination at high magnification that grains are over pyramidal cells and that most cells are labeled with KV3.1 and KV3.2 probes (data not shown). In the stratum granulosum of the dentate gyrus, KV3.1, KV3.3, and KV3.4 mRNAs are present at higher levels than KV3.2 transcripts.

In addition to the diffuse labeling seen throughout the pyramidal cell layers of the CA fields and the granular cell layer of the dentate gyrus, sections hybridized with KV3.1 and KV3.2 and to a lesser degree with KV3.3 probes show a small number of densely labeled cells scattered throughout the hippocampus, having the localization described for interneurons in this struc-

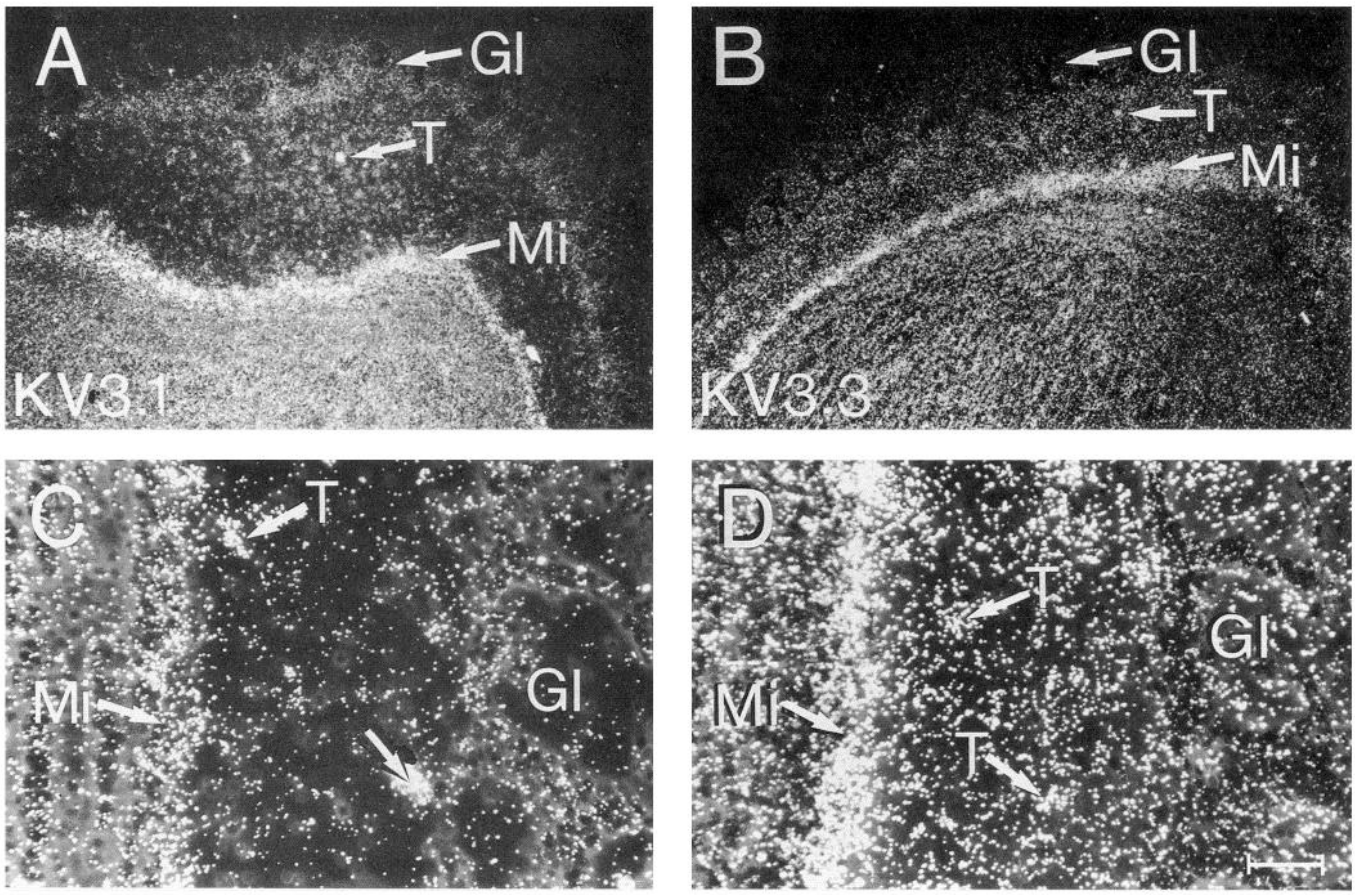


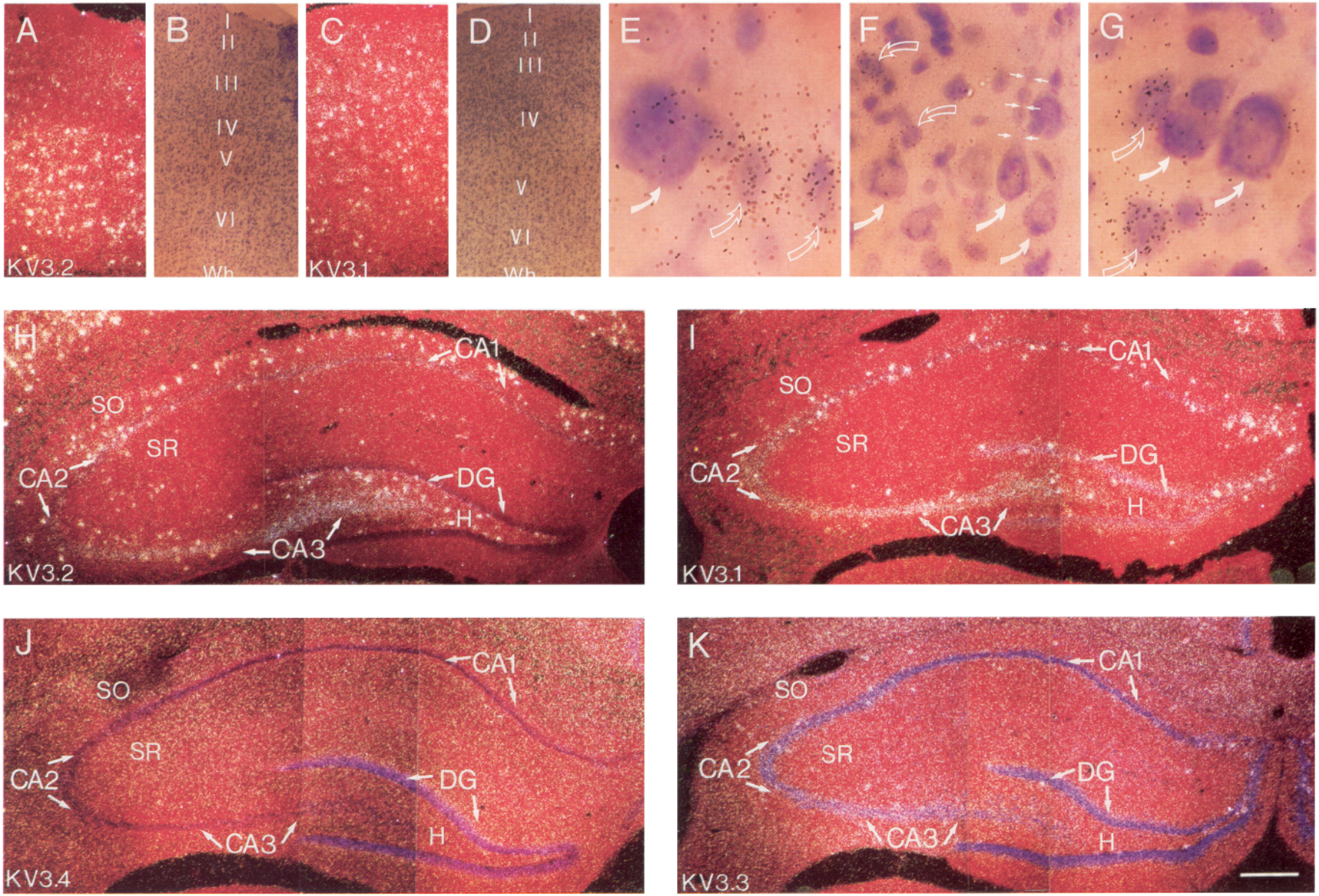
Figure 5. Expression of KV3.1 and KV3.3 mRNAs in the olfactory bulb. *A* and *B*, Low-power dark-field photomicrographs of emulsion autoradiograms following *in situ* hybridization with probes to KV3.1 (*A*) and KV3.3 (*B*) mRNAs. *C* and *D*, High-power dark-field photomicrographs of emulsion autoradiograms following *in situ* hybridization with probes to KV3.1 (*C*) and KV3.3 (*D*) mRNAs. *Gl*, glomerular cell layer; *Mi*, mitral cell layer; *T*, tufted cell. Scale bar; 125 μm for *A* and *B*, 60 μm for *C* and *D*.

ture (see Discussion). As in the neocortex, the pattern is different for KV3.2 versus KV3.1 and KV3.3 mRNAs. The differences are most notable in the CA1 region and in the area dentata. In the CA1 region, cells labeled with KV3.2 probes are numerous in the stratum oriens and occasionally along the border between the stratum oriens and the stratum pyramidalis, but are rarely seen in the stratum radiatum or along the stratum radiutum–pyramidalis border. In contrast, cells hybridizing with KV3.1 probes (Fig. 6*I*) and also a few weakly labeled cells with KV3.3 probes (Fig. 6*K*) are seen both along the stratum radiatum–pyramidalis border and in the stratum oriens–pyramidalis border. The number of densely labeled cells in the stratum radiatum and the stratum–radiatum–pyramidalis border increases in the CA3 region with the KV3.2 probes (Fig. 6*H*). In the area dentata, KV3.2-labeled cells are numerous in the hilus (Fig. 6*H*), while KV3.1-and KV3.3-labeled cells (Fig. 6, *I* and *K*, respectively) are more numerous in the interfaces of the granular cell layer than in the hilus proper.

Basal ganglia: caudate-putamen, globus pallidus, and subthalamic nucleus. One notable feature in these brain nuclei is the lack of significant expression of any ShIII K+ channel genes in the caudate-putamen (Fig. 3, first and second rows). Nevertheless, we find, in emulsion-dipped sections, that a very small number of neurons, scattered throughout the caudate-putamen,

→

Figure 6. Expression of ShIII K+ channel transcripts in the cerebral cortex and the hippocampus. *A*, Low-power dark-field photomicrograph of an emulsion autoradiogram of a coronal section through the cerebral cortex following *in situ* hybridization with probes to KV3.2 mRNAs. *B*, Bright-field photomicrograph of the section in *A*. Note that the hybridization patches in *A* are more frequent in deep rather than superficial layers of the cortex. *C*, Low-power dark-field photomicrograph following hybridization with probes to KV3.1 mRNAs. *D*, Bright-field photomicrograph of the section in *C*. Note that for KV3.1 mRNAs, hybridization patches are present throughout the layers of the cortex but are more frequent in the superficial layers. *E*, High-power photomicrograph of *B* demonstrating that silver grains are located mainly over small cortical neurons, putatively interneurons (see *open curved arrows*) rather than over the large pyramidal neurons (see *solid curved arrows*). *F* and *G*, High-power bright-field photomicrograph of the section in *D* (KV3.1). Note that silver grains are also located over small (*open curved arrows*) but not large (*solid curved arrows*) cortical neurons. Also note in *F* the ascending dendrite of the large pyramidal cell (*small arrows*). *H–K*, Dark-field photomicrographs of coronal section through the hippocampus with probes to KV3.2 (*H*), KV3.1 (*I*), KV3.4 (*J*) and KV3.3 (*K*). Note that the four potassium channel probes label different hippocampal neuronal populations. *CA1–CA3*, fields CA1–CA3 of Ammon's horn; *DG*, dentate gyrus; *H*, hilus; *SO*, stratum oriens; *SR*, stratum radiatum; *Wh*, white matter; *I–VI*, layers I–VI of the cerebral cortex. Scale bar; 375 μm for *A–D*, 25 μm for *E* and *G*, 50 μm for *F*, and 375 μm for *H–K*.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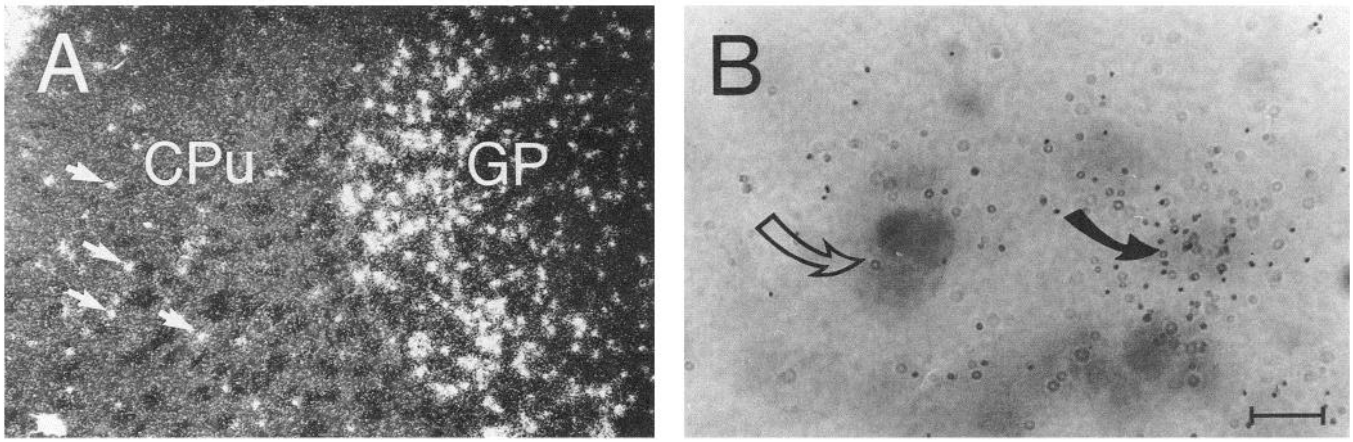

Figure 7. Expression of KV3.2 transcripts in the caudate-putamen and globus pallidus. *A,* Low-power dark-field photomicrograph of an emulsion autoradiogram following *in situ* hybridization with probes specific for KV3.2 mRNAs. Note the scattered appearance of dense signal over a subpopulation of cells in the caudate nucleus (some indicated with *arrows*). *B,* High-power bright-field photomicrograph of cells in *A*. Note that the large cell (*open arrow*) has much fewer grains than the small cell (*solid arrow*). *Cpu,* caudate putamen; *GP,* globus pallidus. Scale bar; 225 μm for *A,* 30 μm for *B*.

contain low to moderate levels of KV3.2 mRNAs (Fig. 7*A*). Grains are seen over small- to medium-sized cells rather than the very large cells occasionally seen in this structure (Fig. 7*B*).

In the globus pallidus, KV3.2 mRNAs are present at moderate levels (Fig. 7*A*). Grains are seen over most of the large- or medium-sized oval or polygonal cells, the principal neurons of the globus pallidus. The patchy appearance seen in Figure 7*A* is due to the relatively low density of neurons in this brain area. KV3.1, KV3.3, and KV3.4 mRNAs are also present in the globus pallidus on most neurons, but in lower amounts (Fig. 3, second row).

All ShIII genes are expressed in the subthalamic nucleus but at different levels (Fig. 8). KV3.1 and KV3.2 mRNAs are more abundant than KV3.4 and KV3.3 transcripts. Most medium to large cells, similar in appearance to the Golgi type I cells that comprise 94% of the neurons in this nucleus (Hammond and Yelnik, 1983; Kitai et al., 1983), are labeled (data not shown). Since the majority of neurons are labeled, it is very likely that the same neuronal populations in this brain nucleus are expressing more than one, if not all, ShIII mRNAs.

Thalamus. The dorsal thalamus is the brain region where KV3.2 mRNAs are most abundant (Fig. 3, second, third, and fourth rows; Fig. 4, first row). Hybridization signals are seen throughout the dorsal thalamus. Most of the nuclei in the anterior, medial, lateral, ventral, intralaminar, and posterior nuclear groups of the dorsal thalamus display high to very high levels of expression. There appears to be a general tendency for hybridization signals to be somewhat lower in some of the nuclei that are more medial and posterior. An extreme case is the parafascicular nucleus, which is weakly labeled with KV3.2 probes (data not shown).

KV3.1 and KV3.3 mRNAs are also present in some of the nuclei of the dorsal thalamus, particularly in the more lateral nuclei of the ventral nuclear group (ventral posterolateral and ventral posteromedium nuclei; Fig. 3, third and fourth rows), in the dorsal nuclei of the anterior nuclear group (anterodorsal and laterodorsal nuclei; Fig. 3, second row), and in the dorsal lateral geniculate (Fig. 3, fourth row). Hybridization signals in these nuclei are stronger with KV3.1 than with KV3.3 probes, but the overall distribution is the same. No signal over background is seen with KV3.4 probes in the dorsal thalamus.

In all of the nuclei of the dorsal thalamus expressing KV3.1 and KV3.3 mRNAs, the levels of KV3.2 transcripts appear to be much higher (Fig. 3, second, third, and fourth rows; Fig. 9). Apparently, almost every large neuron in these structures (probably thalamic relay neurons, which constitute 75–95% of the neurons in these nuclei; Yen et el., 1985; Steriade et al., 1990) expresses high levels of KV3.2 mRNAs (e.g., in the ventral posterolateral nucleus in Fig. 9*A,E*). KV3.1 and KV3.3 mRNAs are also in the large neurons (Fig. 9*G,I*).

Another notable feature in this part of the brain is the difference in ShIII mRNA expression between dorsal and ventral thalamus. These two parts of the thalamus have distinct embryological origins (Rose, 1942) and are functionally distinct, since only the nuclei in the dorsal thalamus project to the cerebral cortex (Jones, 1985; Steriade et al., 1990). KV3.2 mRNAs are present at very low levels in the ventral thalamus. For example, background KV3.2 hybridization signals are seen on the ventral-lateral geniculate (Fig. 3, fourth row) and very weak signals in the reticular thalamic nucleus (Fig. 3, second and third rows; Fig. 9*A*). In contrast, the reticular thalamic nucleus is one of the sites showing strongest KV3.1 signals (Fig. 3, second and third rows; Fig. 9*B*). There is also moderate labeling of the reticular thalamic nucleus with KV3.3 probes (Fig. 3, second and third rows; Fig. 9*C*). These mRNAs are also present in the ventral lateral geniculate (Fig. 3, fourth row).

In the reticular thalamic nucleus the levels of KV3.1 mRNAs are higher than those of KV3.3 transcripts (Fig. 9*B* and *C,* respectively). However, in both cases, grains are seen over most of the neurons of this nucleus (Fig. 9*F,H*). The reticular thalamic nucleus appears to contain only one type of neuron showing oval or fusiform somas such as those seen here (Steriade et al., 1990). These neurons are one of the cell types where it is very likely that the same neuron expresses KV3.1 and KV3.3 mRNAs.

In contrast to the prominent expression of ShIII genes in the thalamus, the rest of the diencephalon does not show significant labeling (Fig. 3, second, third, and fourth rows; see also Table 2). In the epithalamus, moderate to low levels of KV3.1, KV3.3, and KV3.4 mRNAs are present in pretectal nuclei (Fig. 3, fourth row) and very low levels in the lateral habenula (data not shown) but KV3.2 mRNAs are not detected (Fig. 3, fourth row). On the other hand, no hybridization signals above background are seen in the medial habenula and the paraventricular nucleus

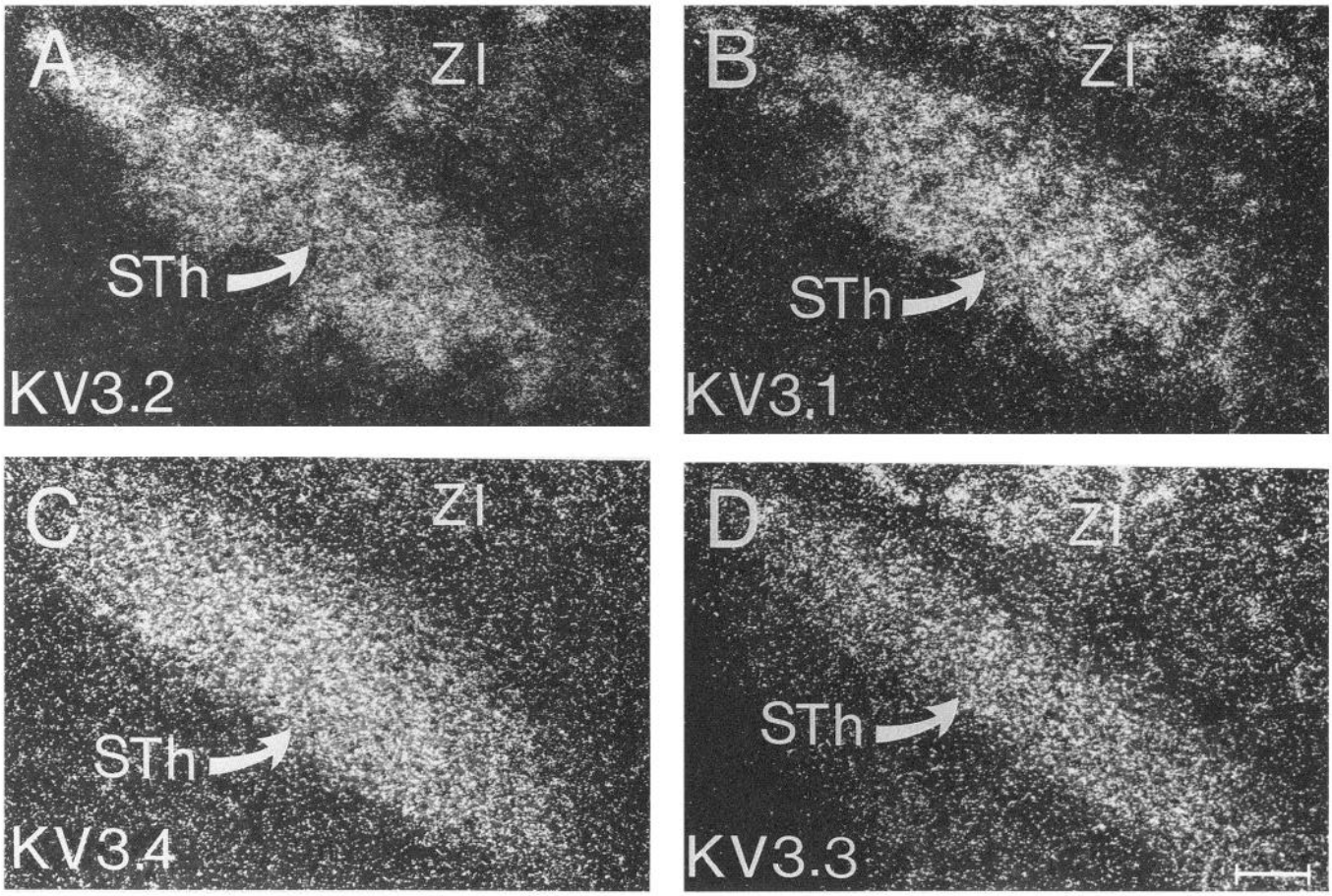

Figure 8. Expression of ShIII K^+ channel transcripts in the subthalamic nucleus; low-power dark-field photomicrographs of emulsion autoradiograms following *in situ* hybridization with probes to KV3.2 mRNAs (*A*), KV3.1 mRNAs (*B*), KV3.4 mRNAs (*C*), and KV3.3 mRNAs (*D*). Note that all four K^+ channel transcripts appear to be expressed in the subthalamic nucleus, albeit at different levels. Hybridization in the neighboring zona incerta is also seen. *STh,* subthalamic nucleus; *ZI,* zona incerta. Scale bar, 125 μm.

with any of the four ShIII probes (Fig. 3, third row). No signal is seen in the hypothalamus on x-ray film autoradiograms (Fig. 3, second and third rows), but a few scattered hybridizing cells are detected by microscopic observation of emulsion-dipped sections. Weakly labeled cells are seen with KV3.4 probes in the anterior hypothalamic area, with KV3.2 probes in the preoptic nuclei and with KV3.1 probes in the preoptic nuclei and the anterior hypothalamic area. Diffuse weak labeling of the suprachiasmatic nucleus is also seen with KV3.2 probes (data not shown).

Midbrain. Several structures in the midbrain express one or more ShIII mRNAs (Fig. 3, fourth row; Fig. 4; first and second rows). As in other brain regions, the pattern of expression of KV3.2 differs substantially from the patterns of expression of the other three genes. In the superior colliculus, for example, KV3.2 transcripts are present mainly in the optic layer (Fig. 4, first row), while KV3.1, KV3.3, and KV3.4 probes produce patchy hybridization signals located mainly in the intermediary and deep gray layers (Fig. 4, first row).

In contrast, the inferior colliculus is one of the regions of the CNS most strongly labeled with KV3.1 and KV3.3 probes (Fig. 4, second row). High amounts of both types of mRNA appear to be present on most large- and medium-size neurons in this structure, except for its most dorsal portions. Hybridization signals with KV3.2-specific probes are much weaker and diffuse (Fig. 4, second row).

Other midbrain structures show hybridization signals of varying intensity with one or more ShIII probes (Fig. 3, fourth row; Fig. 4, first and second rows; see Table 2). All probes, but mainly KV3.1 and KV3.3, label the substantia nigra pars reticulata, but not the pars compacta (Fig. 4, first row). The pontine nuclei, the reticulotegmental nucleus of the pons, and the pontine reticular nuclei coexpress, most likely in the same neuronal populations, KV3.1, KV3.3, and KV3.4 mRNAs (Fig. 4, second row). On the other hand, it is highly probable that transcripts of all four ShIII genes are present in the same cells in the gigantocellular part of the red nucleus (Fig. 4, first row). Every one of the large cells of this structure shows hybridization signals with the four ShIII probes, although of different intensities (see Table 2). KV3.3 and KV3.1 are present in the dorsal and ventral parts of the lateral lemniscus, but KV3.2 signals are seen mainly in the ventral part (Fig. 4, second row).

Cerebellum. The cerebellar cortex is the region showing strongest signals with KV3.1 and KV3.3 probes in x-ray film autoradiograms. Some signal is also seen with KV3.4 probes but almost none with KV3.2 (Fig. 4, third row; Figs. 10, 11).

Although both KV3.1 and KV3.3 mRNAs are abundant in the cerebellar cortex, the distribution of these RNAs in the neuronal components of this brain area is quite different. The almost crystalline cytoarchitecture of the cerebellar cortex allows for the relatively unambiguous assignment of particular transcripts to neuronal subpopulations. KV3.1 mRNAs are

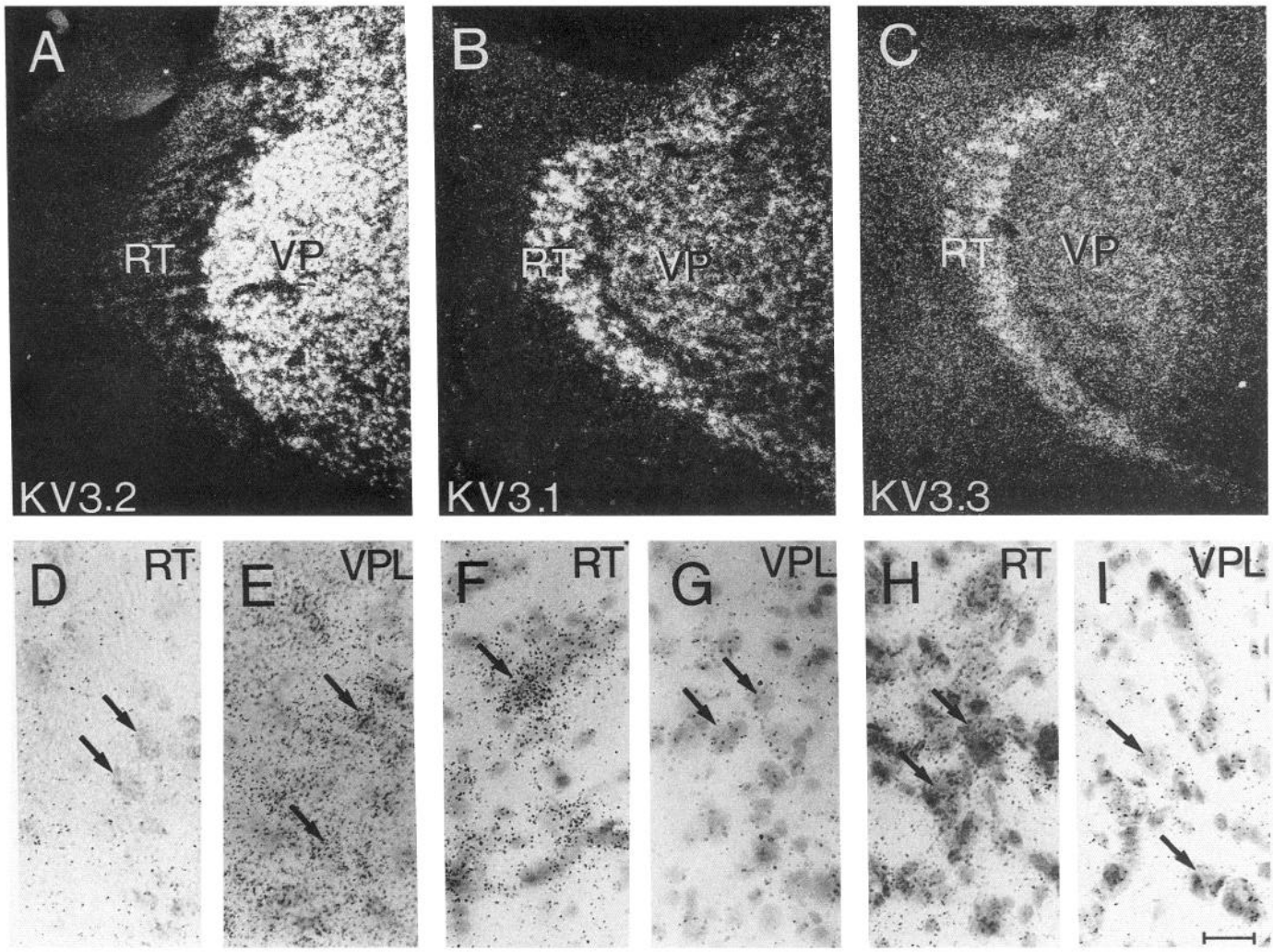

Figure 9. Expression of ShIII K+ channel transcripts in the reticular thalamic nucleus and in the ventral posterior complex of the dorsal thalamus. *A–C,* Low-power dark-field photomicrographs of emulsion autoradiograms following *in situ* hybridization with probes to KV3.2 mRNAs (*A*), KV3.1 mRNAs (*B*), and KV3.3 mRNAs (*C*). *D–I,* High-power bright-field photomicrographs of the reticular thalamic nucleus (*RT*) (*D, F, H*) and ventral posterior lateral thalamic nucleus (*VPL*) (*E, G, I*) areas in *A–C*. *D* and *E* were hybridized with KV3.2 probes; *F* and *G,* with KV3.1 probes; and *H* and *I,* with KV3.3 probes. Note that for KV3.2 there are few grains over cells in the *RT* but many grains over cells in the *VPL* (see *arrows* in *D* and *E*). In contrast, for KV3.1 and KV3.3 there are more grains over cells in the *RT* than in the *VPL* (see *arrows* in *F–I*). *VP,* ventral posterior thalamic complex. Scale bar; 200 μm for *A–C,* 40 μm for *D–I*.

present predominantly on granule cells in the granular cell layer (Fig. 10*A,D*), although some Purkinje cells, particularly in the paraflocculus and flocculus, also express these transcripts (Fig. 11*B*). In contrast, KV3.3 mRNAs are concentrated on Purkinje cells (Figs. 10*C,F*; 11*D*). KV3.4 is expressed at low levels in the Purkinje cell layer, as well as somewhat in the granular and molecular cell layers (Figs. 10*B,E*; 11*C*).

Interestingly, while signals produced by KV3.3 probes are

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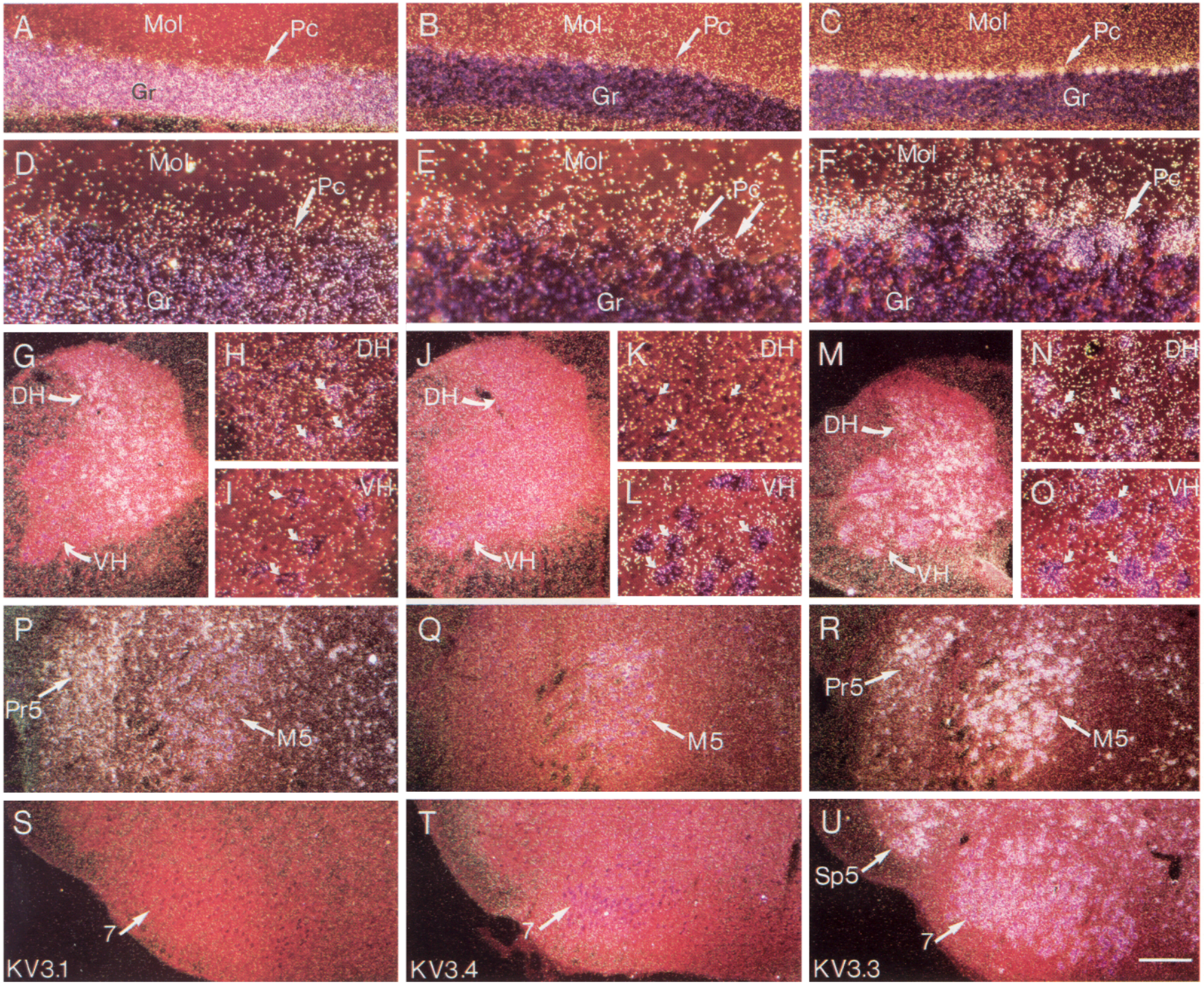
Figure 10. Expression of ShIII K+ channel transcripts in the cerebellum, spinal cord, and cranial nerve nuclei V and VII. *A–C,* Low-power dark-field photomicrograph of an emulsion autoradiogram of a coronal section through the cerebellum following *in situ* hybridization with probes to KV3.1, KV3.4, and KV3.3 mRNAs, respectively. Note in *A* that the granule cell layer has dense hybridization signal while weak signal is seen over the Purkinje cell layer (*PC*). In contrast, note in *C* the strong hybridization signal over the Purkinje cell layer (*PC*) but not the granule cell layer (*Gr*). *D–F,* High-magnification photomicrographs of the sections in *A–C*. *G, J,* and *M,* Low-power dark-field photomicrographs of the spinal cord following hybridization with probes to KV3.1, KV3.4, and KV3.3 mRNAs, respectively. *H* and *I,* High-power photomicrographs of the section in *G*. *K* and *L,* High-power photomicrographs of the section in *J*. *N* and *O,* High-power photomicrographs of the section in *M*. Note that KV3.3 has strong hybridization signal over neurons in both the dorsal horn (*N*) and ventral horn (*O*). In contrast, KV3.1 mRNA is expressed mostly in neurons in the dorsal horn, while KV3.4 mostly in neurons in the ventral horn. *P–R,* Low-power dark-field photomicrograph of the trigeminal nucleus following hybridization with probes to KV3.1, KV3.4, and KV3.3 mRNAs, respectively. Note that KV3.3 has stronger hybridization signals in the motor nucleus of the trigeminal nerve but cells are also labeled with KV3.1 or KV3.4 probes. The neighboring principal sensory nucleus of the trigeminal nerve is also seen in these sections. *S–U,* Low-power dark-field photomicrographs of the facial nucleus hybridized with probes to KV3.1, KV3.4, and KV3.3 mRNA, respectively. Note that only KV3.3 has a significant hybridization signal. *DH,* dorsal horn; *Gr,* granule cell layer; *M5,* motor trigeminal nucleus; *Mol,* molecular cell layer; *Pc,* Purkinje cell layer; *Pr5,* principal sensory trigeminal nucleus; *Sp5,* spinal trigeminal nucleus; *VH,* ventral horn; *7,* facial nucleus. Scale bar; 300 μm for *A–C,* 85 μm for *D–F*; 400 μm for *G, J,* and *M*; 85 μm for *H, I, K, L, N,* and *O*; 300 μm for *P–U*.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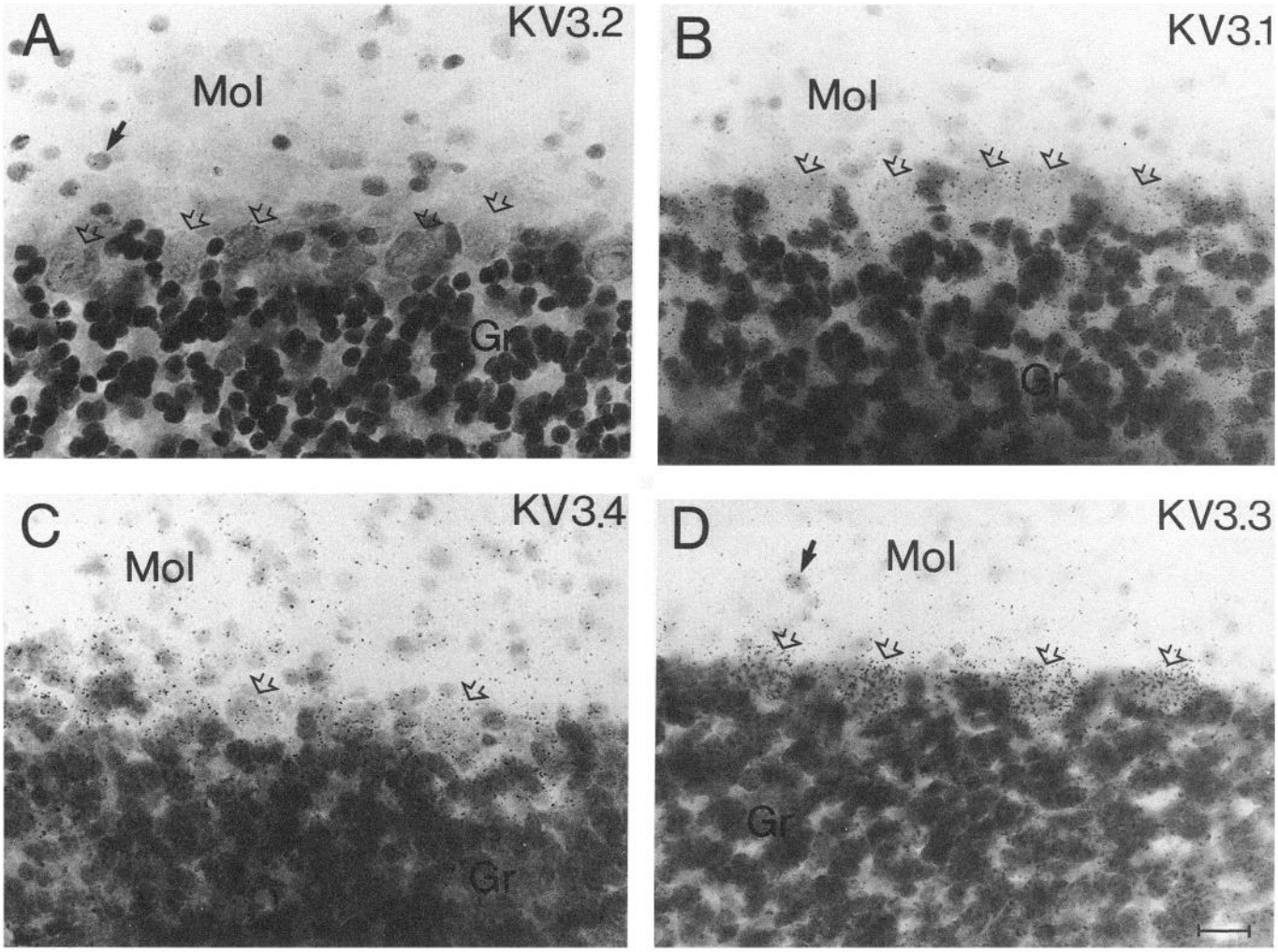

Figure 11. Expression of ShIII K^+ channel transcripts in the flocculus of the cerebellum. *A*, High-power bright-field photomicrographs of emulsion autoradiograms following *in situ* hybridization with probes to KV3.2 mRNAs (*A*), KV3.1 mRNAs (*B*), KV3.4 mRNAs (*C*), and KV3.3 mRNAs (*D*). In *D* (KV3.3) note that there are many more silver grains over Purkinje cells (*open arrows*) than for the other transcripts, especially compared to *A* (KV3.2) where there appears to be very little hybridization signal. In *B*, note that the hybridization signal over the granule cell layer is strong but lower than in Figure 10*A*, and there is also some labeling of Purkinje cells. Also note that some smaller cells, perhaps basket or stellate cells, in the molecular layer in *A* and *D* appear to have silver grains (see *solid arrows*). *Mol*, molecular cell layer; *Gr*, granule cell layer. Scale bar, 30 μm.

uniform throughout the various lobules of the cerebellum, we see some regional differences with KV3.1 probes. With KV3.1 probes, signals are strong in the granular cell layer and very weak in Purkinje cells in most lobules (Fig. 10*B*). However, in the paraflocculus and flocculus, signals in the granular cell layer are weaker and those in Purkinje cells stronger than in other lobules (Fig. 11*B*).

In most images of the Purkinje cell layer, in sections hybridized with KV3.3 probes, there are strong and dense hybridization signals on the molecular cell layer immediately above the Purkinje cells (Fig. 10*C,F*). These signals are most likely not the result of scattering by grains that are over the soma of the cell since they are clearly asymmetric; no such intense labeling is seen on the side of the Purkinje cells facing the granular cell layer. We speculate that this represents hybridization in the large primary dendrites of these neurons.

All four ShIII mRNAs are present in the deep cerebellar nuclei. Although the signals with KV3.1 and KV3.3 probes are more intense, in all cases the grains are seen over the large cells of these nuclei (data not shown).

Hindbrain. Many nuclei in the hindbrain also show labeling by one or more ShIII probe (Fig. 4, third and fourth rows; see Table 2). Several of these nuclei have neuronal populations that are easy to recognize in Nissl stains, thus facilitating conclusions regarding coexpression of the ShIII mRNAs.

Also of interest here is an apparent reversal of the pattern seen in the forebrain and to some degree also in the midbrain, where overall hybridization signals with KV3.1 probes are stronger than with KV3.3 probes. In the hindbrain (as well as in the spinal cord), the reverse tends to be true (Table 2). To illustrate these points further, Figure 10 (*P–U*) shows the expression of KV3.1, KV3.3, and KV3.4 in two of the cranial nerve nuclei, the trigeminal motor nucleus, and the facial nerve nucleus. These nuclei are characterized by the presence of a population of very large and distinct cells, so it is possible to predict whether it is likely that the same neuron expresses more than one of these mRNAs. The motoneurons of the trigeminal motor nucleus coexpress the three mRNAs, although KV3.3 transcripts appear to be more abundant (Fig. 10*P–R*). Neurons in the sensory trigeminal nucleus (Pr5) express mainly KV3.1

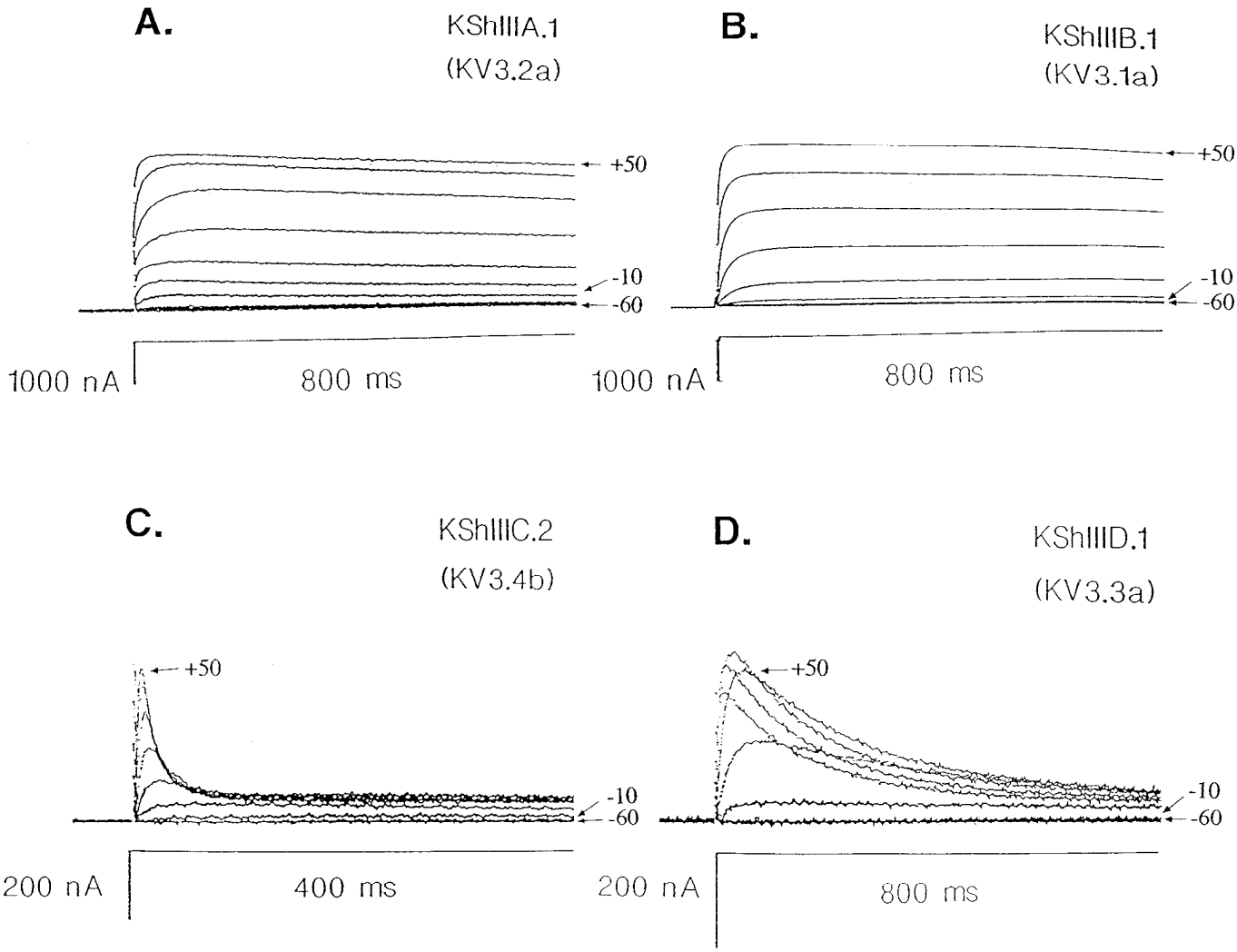

Figure 12. Currents expressed by one alternatively spliced variant of each of four ShIII genes in *Xenopus* oocytes: KV3.2a (*A*), KV3.1a (*B*), KV3.4b (*C*), and KV3.3a (*D*). Shown are the currents recorded under voltage clamp during depolarizing pulses from −60 to +50 mV from a holding potential of −100 mV. The oocytes were bathed in ND96 solution and the electrodes filled with 3 M KCl. Note that the current increment decreases at high voltages. In the case of KV3.3a, the currents at +50 mV are even smaller than the currents at +40 mV. Other alternatively spliced variants of each gene, tested thus far, express currents indistinguishable from those expressed by the subtypes shown here.

and KV3.3 transcripts (Fig. 10*P,R*). On the other hand, in the facial nucleus, only KV3.3 is prominently expressed (Fig. 10*S–U*). Dense labeling by KV3.3 probes appears to be a property of most motoneurons in cranial nerve nuclei (Table 2) as well as in the spinal cord (see below). KV3.2 mRNAs are not prominent anywhere in the hindbrain (Fig. 4, third and fourth rows; see also Table 2).

Given the variations in the ratios of various ShIII mRNAs in different neurons, if heteromultimer formation depends on the availability of subunit proteins, and if the intensity of *in situ* hybridization signals reflects the concentration of subunits, then the composition of heteromultimeric channels may change in different neuronal populations.

Spinal cord. High levels of KV3.1 and KV3.3 mRNAs are present in the dorsal horn of the cervical spinal cord (Fig. 4, fifth row; Fig. 10, *G,H* and *M,N*, respectively). Labeling is strong in many large- and medium-sized neurons in the central part of the dorsal horn but not in cells of the most dorsal laminae. However, the pattern is different in the ventral horn. Large cells in the ventral horn, probably motoneurons, express high levels of KV3.3 (Fig. 10*M,O*) but little KV3.1 mRNAs (Fig. 10*G,I*). On the other hand, KV3.4 mRNAs are seen mainly in the putative motoneurons of the ventral horn (Fig. 4, fifth row; Fig. 10*J–L*).

Functional consequences of heteromultimer formation in the ShIII subfamily

Xenopus oocytes injected with ShIII *in vitro* transcripts express voltage-dependent potassium currents absent in uninjected oocytes. The currents expressed by one of the alternatively spliced transcripts from each of the four ShIII genes are shown in Figure 12. All ShIII currents have a similar voltage dependence; currents are first seen when the membrane is depolarized beyond −10 mV (Fig. 12). They also show very similar pharmacological properties [they are very sensitive to 4-aminopyridine (4-AP) and tetraethylammonium (TEA); data not shown]. However, the kinetics of the currents expressed by products of distinct ShIII genes are different. KV3.2 and KV3.1 transcripts express delayed-rectifier type currents (Fig. 12*A,B*) while KV3.4 and KV3.3 express transient or A-type currents (Fig. 12*C,D*). KV3.4

channels inactivate much faster than KV3.3 channels, but we have yet to find any significant differences between KV3.1 and KV3.2 currents. Alternatively spliced variants express currents with similar electrophysiological properties (Luneau et al., 1991a,b; Rudy et al., 1992).

Since all ShIII channels have similar voltage dependencies we expect that this parameter will not be affected in heteromultimers. However, we do expect changes in kinetics when subunits expressing channels that inactivate at different rates are combined. The *in situ* hybridization data suggest that the most interesting heteromultimers would be those formed between inactivating KV3.3 or KV3.4 subunits and noninactivating KV3.1 proteins, as well as those between fast-inactivating KV3.4 and slow-inactivating KV3.3 subunits. A full account of our studies on the effects of coinjection of different ShIII RNAs is beyond the scope of this paper. However, given the results from *in situ* hybridization, it is important to illustrate what the consequences of heteromultimer formation are for this subfamily.

Demonstration of heteromultimer formation through electrophysiological analysis requires proving that when two different subunits are coexpressed, currents are obtained that are not the algebraic sum of the two channels acting independently. In the experiment shown in Figure 13*C*, a small amount of a KV3.4 cRNA was coinjected with an approximately four- to fivefold excess of a KV3.1 cRNA. This is a potentially relevant combination since several neuronal populations in the CNS coexpress these two mRNAs, possibly in similar ratios. When an amount of KV3.4 mRNA similar to that injected in the experiment shown in Figure 13*C* is injected alone, small transient currents such as those illustrated in Figure 13*A* are obtained. The magnitude of the peak current at +40 mV for a number of experiments like the one in Figure 13*A* is shown in the histogram with solid bars in Figure 13*D*. When the two RNAs are coinjected, the currents consist of a transient and a noninactivating component (Fig. 13*C*). The noninactivating component is presumably due to homomultimers of KV3.1 channels. Indeed, the currents seen in these oocytes, after prepulses eliminating the inactivating component, are indistinguishable from those seen in oocytes injected with KV3.1 alone at similar concentrations (Fig. 13*B*).

The transient component in Figure 13*C* is much larger than that seen in oocytes injected with the same amount of KV3.4 alone. This is contrary to what might be expected from the algebraic sum of two independent currents and is consistent with the formation of heteromultimeric channels containing mainly KV3.1 subunits and one or more KV3.4 subunits. The magnitude of the peak current at +40 mV of the inactivating component in oocytes coinjected with KV3.1 and KV3.4 cRNAs, for a large number of experiments, is shown in the histogram with hatched bars in Figure 13*D*. The rates of inactivation of KV3.4 homomultimers are compared to those of the putative heteromultimers in Figure 13*E*. These channels inactivate approximately three times slower than KV3.4 homomultimers (see Fig. 13*E*). These results also support the hypothesis that the transient component seen in coinjected oocytes is due to heteromultimers since the algebraic sum of KV3.1 currents and KV3.4 currents should result in a current with time constants of inactivation not very different from those of homomultimeric KV3.4 channels.

On the average, we see 6.0-fold more transient current in the coinjected oocytes. These currents are probably the result of heteromultimeric channels containing few (perhaps only one) KV3.4 subunit and several KV3.1 subunits, which are present in an excess. We utilized a large excess of KV3.1 mRNA in this experiment so that most heteromultimers have only one KV3.4 subunit. Calculations of the relative numbers of inactivating channels in oocytes injected with KV3.4 alone versus oocytes injected with KV3.4 and KV3.1 suggest that these heteromultimeric channels indeed contain, on the average, only one inactivating subunit.[1] The inactivation due to fast inactivating KV3.4 subunits also dominates the kinetics of the currents in oocytes coinjected with small amounts of KV3.4 cRNA and an excess of KV3.3 cRNA. An amplification of the fast-inactivating current similar to that illustrated here is seen in these oocytes (data not shown).

Heteromultimer formation thus amplifies the amounts of inactivating currents that can be expressed by KV3.4 subunits. The *in situ* hybridization data suggest that the situation simulated in the oocyte might be encountered in several neuronal populations in the CNS.

Discussion

These studies provide necessary insight into the potential for *in vivo* heteromultimer formation in the ShIII subfamily. In addition, they present the first analysis of the expression of a complete *Sh* subfamily at the cellular level.

Expression of ShIII mRNAs in the CNS

The *in situ* hybridization studies provide a number of interesting features of the expression of ShIII or *Shaw*-related K^+ channel transcripts, primarily in the CNS. These results will be particularly useful in attempts to identify native channels containing ShIII proteins utilizing electrophysiological methods.

The following are the most notable features of the expression of ShIII mRNAs in the CNS. (1) Seen as a group of proteins that may be components of related channels, a trait expected from the functional similarities seen in the channels expressed in heterologous expression systems, it is interesting to note that several neuronal populations appear to be devoid of any ShIII mRNAs. Most neurons in the cerebral cortex, the caudate-putamen, the epithalamus, and the hypothalamus, the amygdala, the substantia nigra compacta, and a few structures in the brainstem such as the inferior olive showed undetectable labeling with ShIII probes. Others, such as the pyramidal neurons in the hippocampus, particularly in the CA1 subfield of Ammons horn, were only weakly labeled. ShIII gene expression is prominent in the thalamus, cerebellum, brainstem, and spinal cord.

Interestingly, the main neuronal population of the caudate-putamen appears to express few *Sh* transcripts of the other subfamilies as well. Of those tested, only KV1.4, of the ShI subfamily (Sheng et al., 1992) and KV4.2, of the ShIV subfamily

[1] The number of inactivating channels in oocytes injected with KV3.4b and KV3.1b (n_{hr}) over the number of inactivating channels in oocytes injected with KV3.4b alone (n_{ho}) is given by $n_{hr}/n_{ho} = (Ip_{hr}/Ip_{ho})\,(i_{ho}\cdot p_{ho}/i_{hr}\cdot p_{hr})$, where Ip is the peak inactivating current at a given voltage, i is the single channel current at that voltage, and p the probability that the channels available to open at that voltage are opened at the peak of the macroscopic current; hr denotes heteromultimers, and ho, homomultimers.

Single-channel measurements show that $i_{hr}/i_{ho} \cong 1.35$. The probabilities that the channels available to open at a given voltage are opened at the peak of the macroscopic current were obtained from the increases in current produced by removing inactivation with H_2O_2 (Vega-Saenz de Miera and Rudy, 1992) and found to be similar for oocytes expressing homomultimeric or heteromultimeric channels. Hence, $p_{ho}/p_{hr} \cong 1$. Therefore, $n_{hr}/n_{ho} \cong 6.0 \cdot 1/1.35 \cong 4.4$, close to the value (4) expected if the heteromultimeric channels contain one inactivating (KV3.4) and three noninactivating (KV3.1) subunits.

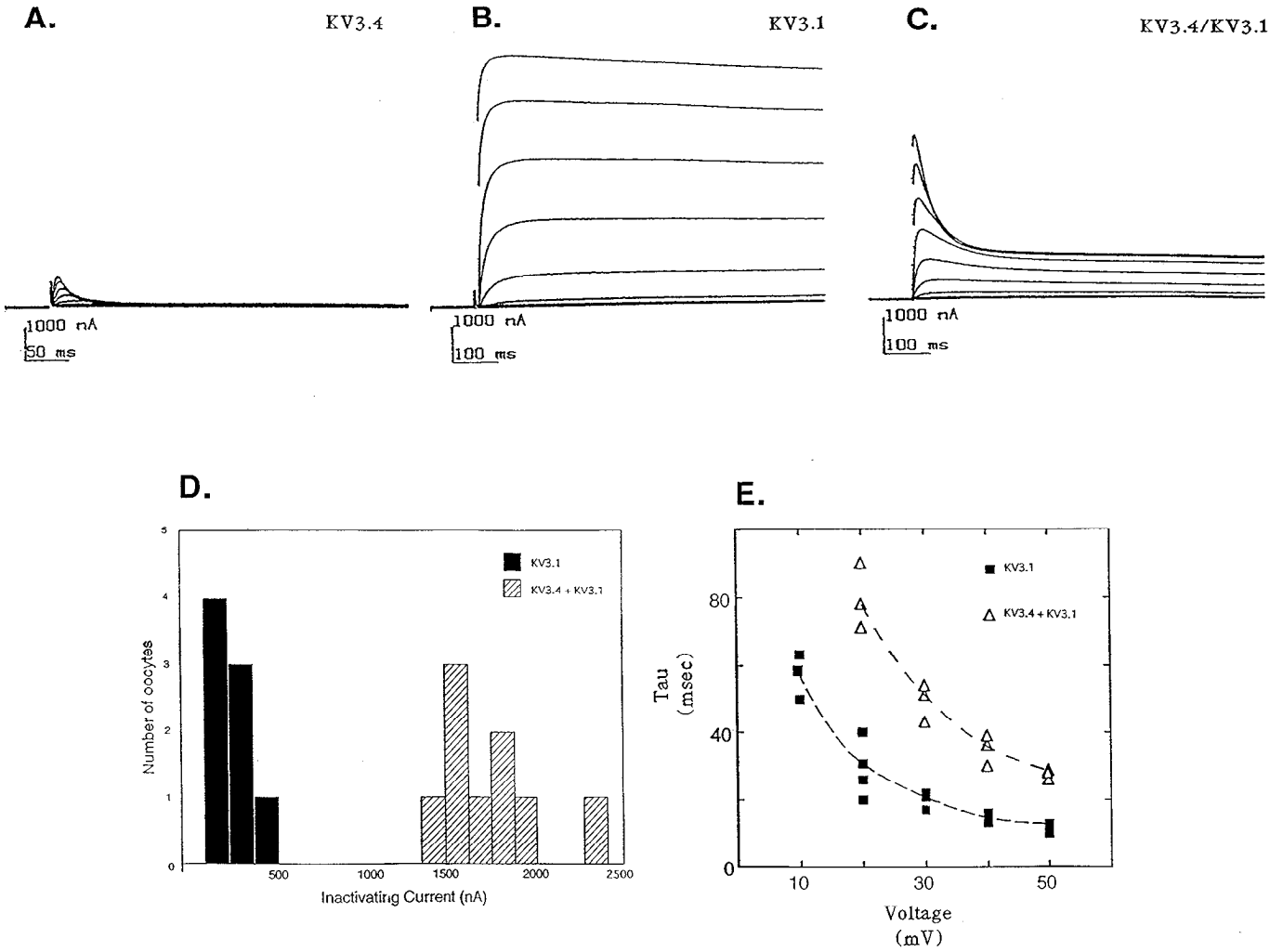

Figure 13. KV3.1 and KV3.4 heteromultimeric channels expressed in *Xenopus* oocytes. *A,* Currents recorded in an oocyte injected with KV3.4b cRNA alone. *B,* Currents recorded in an oocyte injected with KV3.1b cRNA alone. *C,* Currents recorded in an oocyte injected with the same amount of KV3.4b cRNA as the oocyte in *A* plus the same amount of KV3.1b cRNA as the oocyte in *B.* In all cases, the recordings were obtained as in Figure 12. *D,* Histograms depicting the number of oocytes giving the indicated amount of transient current at +40 mV in different experiments such as that shown in *A* (*solid bars*) or *C* (*hatched bars*). *E,* Voltage dependence of the average time constants of inactivation of the transient currents recorded in eight oocytes such as the one shown in *A* (*solid squares*) or nine oocytes such as the one shown in *C* (*open triangles*). The decline of the currents to a steady-state value, at the indicated potential, was fitted to a single exponential. These fits followed the decay of the currents quite closely.

(Tsaur et al., 1992) label the caudate-putamen strongly. Only weak labeling is seen with ShII probes (Drewe et al., 1992; Hwang et al., 1992).

(2) Each one of the four ShIII genes explored here has a unique expression pattern in the CNS. However, transcripts of three of these genes, KV3.1, KV3.3, and KV3.4, exhibit localizations more similar to each other than those of KV3.2 transcripts, thus defining two trends in the expression of ShIII genes in the CNS.

Many neurons expressing KV3.2 mRNAs express these transcripts predominantly (relative to the other ShIII transcripts) if not exclusively (e.g., in thalamic relay neurons of many nuclei of the dorsal thalamus, and in neurons of the optic layer of the superior colliculus and the locus coeruleus). At the same time, many regions that express KV3.1, KV3.3, or KV3.4 mRNAs prominently, such as the cerebellar cortex, the spinal cord, the reticular thalamic nucleus, the inferior colliculus, and many nuclei in the brainstem, appear to express little or no KV3.2 transcripts.

Although KV3.1 and KV3.3 transcripts are expressed in the same regions of the CNS, leading to very similar expression patterns in x-ray film autoradiograms, the higher-resolution analysis reveals that their patterns of expression are overlapping but different. Some neuronal populations (e.g., local circuit neurons in the cerebral cortex and the hippocampus, pyramidal cells in the hippocampus, granule cells in the dentate gyrus, principal neurons in the reticular thalamic nucleus, granule cells in the cerebellum) express predominantly KV3.1 transcripts, but others, such as Purkinje cells in the cerebellum, neurons of the facial nucleus, and motoneurons in the spinal cord, express mainly KV3.3 mRNAs.

ShIII mRNA expression distinguishes among subsets of interneurons in the cortex, hippocampus, and caudate-putamen

In the cortex and the hippocampus some ShIII mRNAs appear to be expressed in distinct sets of interneurons. Subsets of cor-

tical and hippocampal interneurons have been described based on the expression of various neurotransmitters, neuropeptides, and receptors (Somogyi et al., 1984; Lin et al., 1986; Penny et al., 1986; Sloviter and Nilaver, 1987; Demeulemeester et al., 1988; Woodson et al., 1989; Douglas and Martin, 1990; Zilles et al., 1990) or morphology (DeFelipe and Jones, 1988; Zilles, 1990).

In the rat cortex, interneurons represent a small percentage of the total neuronal population (Lin et al., 1986; DeFelipe and Jones, 1988; Douglas and Martin, 1990; Zilles, 1990; Zilles et al., 1990). The localization of KV3.2-labeled cells is most similar to the distribution of D_1 dopamine receptor–containing cells (Boyson et al., 1986; Dawson et al., 1986; Weiner et al., 1991) and somatostatin-binding sites (McCarty and Plunkett, 1987). In addition, Ramon y Cajal and other authors described a group of neurons called Martinotti cells that are localized in layers V–VI but preferentially in layer VI (reviewed in DeFelipe and Jones, 1988), as seen here for cells expressing KV3.2 mRNAs. However, it has proven difficult to differentiate these cells from other nonpyramidal cells by other criteria, and their existence as a distinguishable group of cells is controversial (DeFelipe and Jones, 1988). Some investigators even give a totally different laminar distribution to Martinotti cells (Zilles, 1990). Further studies will be required to ascertain whether the cells containing KV3.2 mRNAs correspond to any of these subsets of cortical neurons.

The distribution of cells expressing KV3.1 (or KV3.3 and KV3.4) mRNAs seen here is qualitatively similar to that obtained from immunohistochemical observations using antibodies against GABA or the GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD) (Somogyi et al., 1984; Gabbott and Somogyi, 1986; Demeulemeester et al., 1988; Zilles et al., 1990; Pernev et al., 1992).

In the hippocampus, the pattern of labeling seen here with KV3.2 probes is similar to that reported for somatostatin immunoreactivity (Somogyi et al., 1984; Sloviter and Nilaver, 1987) while the labeling pattern with KV3.1 and KV3.3 probes is more like that seen for GABA or cholecystokinin immunoreactivity, although the number of labeled cells is less in our case (Perney et al., 1992; Somogyi et al., 1984; Sloviter and Nilaver, 1987; Woodson et al., 1989).

In the caudate-putamen, KV3.2 probes label a very small number of neurons. These are a subset of small- to medium-sized cells distinct from the large cholinergic local circuit neurons (Fig. 7*B*) that represent 2–5% of the total neuronal population of the caudate-putamen (Woolf and Butcher, 1981; Paxinos and Butcher, 1985). Thus, the KV3.2 mRNA–containing neurons may reflect a subpopulation of the principal GABAergic neurons which comprise over 90% of the cells in the caudate-putamen or perhaps another subpopulation of interneurons (Carpenter, 1984).

Coexpression of ShIII transcripts in the same neuronal populations

Because ShIII subunits can form heteromultimeric channels *in vitro*, we have put emphasis on determining whether the same neuronal populations are labeled with two or more ShIII probes. Coexpression of transcripts in the same cell is, of course, not evidence that heteromultimers are formed *in vivo*. If the proteins are targeted to different parts of the cell, heteromultimers will not be formed. Other factors regulating the synthesis and assembly of channel proteins may also intervene. However, determining whether the mRNAs are coexpressed in the same cells gives a good indication of the potential significance of heteromultimer formation. Moreover, it gives clues as to which subunits and in which cells further experimentation should be directed to explore heteromultimer formation directly. The question of heteromultimer formation between ShIII subunits would have little physiological significance if RNA expression studies showed that the RNAs are rarely coexpressed in the same cells.

Our analysis revealed that neurons in several CNS areas are likely to coexpress transcripts of more than one ShIII gene. This is clearest in nuclei having easily identifiable neuronal subpopulations where most, if not all, neurons are labeled with multiple probes. Based on such criteria, there is little doubt that KV3.1, KV3.3, and KV3.4 mRNAs or at least two of these transcripts are often coexpressed in the same cells. The mitral cells of the olfactory bulb, the principal neurons of the reticular thalamic nucleus, the lateral leminiscus, the reticulotegmental nucleus of the pons, the pontine nuclei, the motor trigeminal nucleus, and some cerebellar Purkinje cells are some examples. Other neuronal populations appear to express all ShIII mRNAs, albeit at different levels, such as the principal neurons of the globus pallidus, the subthalamic nucleus, the diagonal band of Broca, the substantia nigra pars reticulata, the zona incerta, and the red nucleus. Thalamic relay neurons of some nuclei of the dorsal thalamus are likely to coexpress KV3.2, KV3.1, and KV3.3 mRNAs. Based on this analysis we conclude that heteromultimer formation is potentially a significant feature of the channels formed by subunits of the *Shaw* subfamily.

Many neurons in the inferior colliculi are heavily labeled with KV3.1 and KV3.3 probes. Although in many fields most cells contain grains, this brain area has a number of different neuronal populations that are not easily distinguishable with Nissl stains. Given this and the high density of cells in this region, the certainty that the mRNAs are present in identical neuronal population is less than that for the areas described above. Similarly, in various subdivisions of the reticular nuclei of the hindbrain there is strong labeling, but since these nuclei are composed of scattered cells it is more difficult to assess whether cells are colabeled.

Since KV3.4 transcripts appear to be much less abundant throughout the CNS than the products of the three other ShIII genes, and are mainly expressed in neurons also expressing KV3.1 or KV3.3 mRNAs, KV3.4 subunits may act in CNS neurons as modulators of the inactivation properties of channels composed mainly of KV3.1 and KV3.3 proteins. The electrophysiological studies described in this article indicate that small amounts of KV3.4 transcripts might be sufficient to impart fast inactivating properties to channels composed mainly of the other ShIII subunits. Similar studies with ShI subunits have also shown that the presence of a single inactivating subunit is sufficient to impart inactivating properties in the resultant channels (e.g., Isacoff et al., 1990; K. McCormack et al., 1990; Ruppersberg et al., 1990; MacKinnon et al., 1991). This can be understood in terms of the amino "ball and chain" model of inactivation (Hoshi et al., 1990; Zagotta et al., 1990). The "ball and chain" of a single inactivating subunit is sufficient to inactivate the channel, although the rate of inactivation increases with the number of

inactivating subunits (MacKinnon et al., 1991), as seen here also for ShIII heteromultimers.

Functional role of ShIII proteins in CNS neurons

In vitro ShIII channels produce slow, high-voltage-activating, delayed-rectifier or A-type currents (depending on whether they have KV3.3 and/or KV3.4 subunits that provide inactivating properties) that are blocked by low concentrations of TEA (1 mM).

Some ShIII transcripts have been identified both in rodents and humans (e.g., KV3.1a in mouse, Yokoyama et al., 1989; and in rat and human, Ried et al., 1993; KV3.3a in rat, Vega-Saenz de Miera et al., 1992; and in human, J. Lee and J. E. Rose, unpublished observations; and KV3.4b in human, Rudy et al., 1991b; and in rat, E. Vega-Saenz de Miera and B. Rudy, unpublished observations). In all these cases the predicted proteins are highly conserved. For example, there is only a single amino acid difference in the 511 residues of KV3.1a in rat and human (Ried et al., 1993). This argues that these proteins play important and specific roles. Voltage-gated K^+ channels could modulate neuronal excitability in a number of ways: they can affect action potential waveforms by their influence on the rates of repolarization and firing patterns through their effects on the membrane potential and membrane resistance in between spikes. Voltage-gated K^+ channels can affect the threshold for action potential generation if they are opened at resting membrane potentials and they have been shown to influence the delay to first spike in a train of action potentials. These channels could also influence the rates of neurotransmitter release in presynaptic terminals or the generation and spread of postsynaptic responses (Hille, 1992). A given voltage-gated K^+ channel could influence one or more of these parameters, depending on its voltage dependence and its subcellular localization. In addition, voltage-gated K^+ channel function is affected by phosphorylation by a number of different kinase systems, and therefore their role as one of the mediators of the modulatory actions of neurotransmitters and neuropeptides is of considerable physiological significance (Kaczmarek and Levitan, 1987; Levitan, 1988).

Homomultimeric and heteromultimeric ShIII channels expressed in heterologous expression systems begin to activate when the membrane is depolarized beyond -10 mV, and they do so relatively slowly (Fig. 12). We would expect these channels to be activated significantly mainly during action potentials of sufficient duration, and to play little role in subthreshold phenomena. However, Ruppersberg et al. (1991) found that inactivated KV3.4a channels reopen during repolarization. The channels appear to be unable to enter the resting closed state when the membrane is hyperpolarized until the inactivation is first removed. A long-lasting transient current is carried by the channels at negative potentials while inactivation is being removed and channels have not completely closed. Hence, after a depolarization activating and then inactivating these channels, for example during an action potential, they could affect the probability of generating the next spike. KV3.3 channels that inactivate more slowly may or may not behave in a similar fashion.

Given the difficulties in isolating K^+ current components, electrophysiological experiments specifically designed to search for native channels with properties similar to those of ShIII channels *in vitro* will be required to ascertain the properties of these channels *in vivo*. For example, it is possible that channels composed of KV3.3 subunits, which are abundant in cerebellar Purkinje cells, account for some of the inactivating component of the maintained outward current seen in Purkinje cells in organotypic cultures (Bossu et al., 1988; Gähwiler and Llano, 1989). Nevertheless, currents similar to those seen in oocytes injected with ShIII transcripts are not among the most typical K^+ currents seen in somatic recordings of neurons. Take, for example, thalamic relay neurons where KV3.2 mRNAs appear to be most abundant. Two delayed-rectifier currents I_{K1} and I_{K2} and a low-voltage-activating transient current have been described in acutely dissociated thalamic relay neurons (Huguenard and Prince, 1991). None of these currents show properties identical to those of KV3.2 channels (or those of any KV3.2 heteromultimeric combination) in *Xenopus* oocytes. I_{K1}, which accounts for only 2% of the total outward current, is similar to KV3.2 channels in voltage dependence and sensitivity to TEA, but is proposed to be Ca-activated and is more sensitive to 4-AP. I_{K2} activates at more negative voltages and is less sensitive to TEA.

The lack of many clear cut examples of *in vivo* currents similar to those of *in vitro* ShIII channels could be explained in at least three ways. First, most voltage-clamp studies from neurons utilize dissociated embryonic or early postnatal tissue that may not express ShIII subunits; second, native channels containing ShIII proteins might, at least in some cell populations, have electrophysiological properties different than *in vitro* ShIII channels. Alternatively, these channels might be more prominent in neuronal processes or terminals not accessible to the electrophysiological methods used. Determination of the significance of these factors requires the localization of ShIII proteins.

We were unable to find a common characteristic of the neurons expressing any or all ShIII transcripts. Had we found such a correlation, this would have provided strong hints as to the functional role of ShIII subunits. However, the lack of correlation is not necessarily surprising. There are usually multiple receptors for most neurotransmitters and neuropeptides, each acting on different second-messenger cascades. In addition to this divergence, there is a convergence of different neurotransmitter and neuropeptide receptors eliciting the same second-messenger response. Moreover, a given voltage-gated K^+ channel could play different roles in different neurons or even in different places in the same neuron. In some cells it may mediate the response of one neurotransmitter; it could mediate the response to another neurotransmitter acting on the same second-messenger system in another cell. Moreover, the same channel could be affected by more than one kinase and, hence, mediate the effect of a second-messenger system in a third group of cells or play a role as a voltage-gated channel independent of second-messenger modulation in another group of cells.

In this context, the fact that many, but certainly not all, of the neurons expressing ShIII transcripts, in particular, KV3.1, KV3.3, and KV3.4 mRNAs (e.g., interneurons in the cerebral cortex and the hippocampus; the neurons of the reticular thalamic nucleus, globus pallidus, and substantia nigra pars reticulata; and cerebellar Purkinje cells), are GABAergic, a feature first noted by Perney et al. (1992) for KV3.1 mRNAs, is quite intriguing. Perhaps in these neurons the channels containing these subunits mediate the effects of putative $GABA_B$ autoreceptors. If this is the case, some important GABAergic systems, notably the main neuronal population of the caudate-putamen

and the olfactory bulb, do not use the same modulating mechanism since they do not express ShIII mRNAs.

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