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Region-specific expression of a K^+ channel gene in brain

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ABSTRACT **Northern blot analysis and *in situ* hybridization studies reveal the highly localized expression in rat brain of transcripts from a gene (KShIIIA) encoding components for voltage-gated K^+ channels. KShIIIA expression is particularly prominent throughout the dorsal thalamus. The expression of KShIIIA is compared to that of a closely related gene, here called NGK2-KV4. These two genes encode transcripts that induce currents in *Xenopus* oocytes that are as of yet indistinguishable, but they show very different patterns of expression in rat brain. NGK2-KV4 transcripts are particularly abundant in the cerebellar cortex, where KShIIIA expression is very weak. These results demonstrate the existence of cell-type-specific K^+ channel components and suggest that one reason for the unusually large diversity of K^+ channel proteins is the presence of subtypes that participate in specific brain functions.**

Studies in brain slices, acutely dissociated and primary cultured neurons from mammalian brain, and molluscan nerve cells have revealed that individual neuron types have distinct intrinsic electrophysiological properties (1–4). This diversity is fundamental to the functional specificity of neuronal circuits and is provided by various combinations of ion channels (1–4). Among these, potassium (K^+) channels constitute the group with the most variants, and a single cell may contain several subtypes (5–7). In addition, K^+ channels are one of the most frequent targets of the action of many second messengers, with subtypes differing in their response to these modulators. Changes in the function of specific K^+ channel types are frequently the mechanism by which neurotransmitters, neuropeptides, and other stimuli affect excitability (6–9). Thus, K^+ channel diversity is a key factor in determining the heterogeneity of intrinsic electrophysiological properties of neurons and their response to stimuli.

Recently a large number of related genes (the Shaker or Sh gene family; reviewed in refs. 10–12) encoding components of voltage-gated K^+ channels was discovered. These advances open up the possibility of carrying out studies to gain insights into how the intrinsic electrophysiological properties of neurons are regulated. To achieve this it is first necessary to discover the relationship between the cloned products and native voltage-gated K^+ channels. The functional role of the cloned products and the relationship between the diversity of molecular components and the functional diversity of K^+ channels also remain to be discovered.

We are interested in the functional role of KShIIIA, a Shaw-like or ShIII[||] gene encoding several alternatively spliced transcripts (14, 15).[¶] KShIIIA transcripts express in *Xenopus* oocytes voltage-dependent, delayed-rectifier-type, K^+ currents that activate somewhat slowly and at membrane potentials more positive than −20 mV. Such currents, if expressed *in vivo*, could play little role in subthreshold phenomena and may not be activated during single fast sodium spikes. However, they could play a role in the electrical behavior of neurons in which depolarization is maintained by slowly inactivating inward currents (such as Ca^{2+} currents) or in neurons exhibiting repetitive firing. For instance, these currents could be involved in the modulation of the oscillatory behavior of certain neurons during long-lasting depolarizations (1, 2).

To unravel the functional role of the proteins encoded by the KShIIIA gene it is important to know which cells express these proteins. As a first step toward this end, we have explored the expression of KShIIIA transcripts in rat brain by *in situ* hybridization. The expression of KShIIIA transcripts is compared to that of transcripts from RBK2, a Shaker-like or ShI gene (16), and to transcripts from another ShIII gene (called here NGK2-KV4), a gene encoding NGK2 (17) and a second alternatively spliced product, KV4 (18). The comparison of the distribution of KShIIIA and NGK2-KV4 transcripts is particularly interesting because they all induce very similar currents in *Xenopus* oocytes.

MATERIALS AND METHODS

Design and Preparation of Probes for Northern Blots and *in Situ* Hybridization. Hybridization probes were prepared by labeling 30 ng of DNA (with ^{32}P or ^{35}S for Northern blots and brain sections, respectively) from purified restriction fragments by the random hexamer primer method (19). To prepare gene-specific probes we chose fragments of the cDNAs showing minimal similarity to other Sh cDNAs. In addition, hybridization was done under high stringency conditions.

KShIIIA probes. Some of the experiments illustrated here utilized an *Eco*RI fragment containing the first 380 nucleotides of KShIIIA.1 (14, 15). This fragment is common to all KShIIIA cDNAs but shows less than 60% identity with other ShIII cDNAs. Identical labeling results (data not shown) were obtained with probes derived from a fragment between nucleotide 964 and nucleotide 1296 of KShIIIA (14). Transcript-specific probes were used to explore differences in the distribution of alternatively spliced transcripts (see Fig. 1). A *Bgl* II–*Xba* I fragment of KShIIIA.1 (nucleotides 1828–2481) was used to detect expression of KShIIIA.1 and KShIIIA.2 mRNA. A *Hin*dIII–*Eco*RI fragment of KShIIIA.3 (nucleotides 1798–2658) was used to detect KShIIIA.3 mRNA expression. The difference in the pattern of expression of KShIIIA.1 and KShIIIA.2 was not explored in this study.

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||Based on degrees of amino acid sequence similarity of the protein products, and hence probable evolutionary relatedness, Sh genes are organized into four subfamilies (10–13) designated here as ShI, ShII, ShIII, and ShIV.
¶The sequences reported in this paper have been deposited in the GenBank data base (KShIIIA.2, M84202; KShIIIA.3, M84203).

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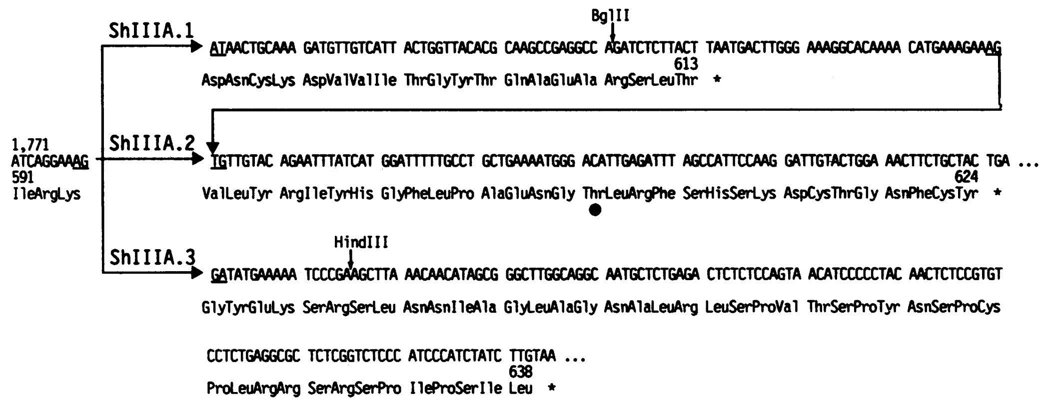

FIG. 1. Nucleotide and deduced amino acid sequences of the region of divergence of three KShIIIA cDNAs. After the underlined AG (position 1779–1780 in KShIIIA.1; ref. 15), the nucleotide and deduced amino acid sequences of KShIIIA products diverge. The nucleotide sequence of KShIIIA.2 immediately following this AG is found in KShIIIA.1 95 bases downstream from the point of divergence. After this new overlap the sequences of KShIIIA.1 and KShIIIA.2 are identical. However, a stop codon in the 95-nucleotide insert terminates the KShIIIA.1 protein. KShIIIA.3 diverges at the same point as the other two variants and the sequence that follows bears no relation to the other two. The restriction sites for the 5′ ends of the transcript-specific DNA probes are marked with arrows. A filled circle indicates a threonine residue in KShIIIA.2 within the consensus sequence for protein kinase C phosphorylation (25).

NGK2-KV4 probes. A 351-bp fragment encoding the last 117 residues of a rat NGK2 protein (20) was used for the experiments on the expression of NGK2-KV4 transcripts shown here. This fragment includes 328 bases common to NGK2 (17, 20) and KV4 (18). Identical results were obtained with a probe encoding the H1–H6 membrane spanning domains of NGK2 and KV4 (data not shown).

RBK2 probes. A *Pst* I fragment spanning the last 1295 bases of RBK2.1, of which 741 bases are 3′ untranslated sequence (16), was used to study RBK2 expression.

Preparation of RNA and Northern Blots. Total cellular RNA from adult (175–200 g) male Sprague–Dawley rat brain was prepared by a guanidinium thiocyanate method (21). The rats were quickly decapitated, brains were removed (up to the medullo-spinal junction), the desired regions were dissected, and the tissue was immediately homogenized in the denaturing solution at 4°C. The RNA (8 μg of total RNA per lane) was electrophoresed in denaturing formaldehyde gels and transferred to Hybond (Amersham) as described (22). The blots were hybridized with $\approx 1 \times 10^6$ cpm ($\approx$1.2 ng of DNA) of ^{32}P-labeled probes per ml as described (23).

***In Situ* Hybridization.** The regional distribution of Sh transcripts in rat brain was determined by *in situ* hybridization utilizing ^{35}S autoradiography as described in Stone *et al.* (24). Hybridization was carried out at 48°C in a solution containing 50% formamide, 2× SSC, 10% dextran sulfate, 3× Denhardt's solution, 50 mM dithiothreitol, and 0.5 mg of sonicated denatured salmon sperm DNA per ml. After hybridization the sections were washed at 48°C in SSC solutions of decreasing concentration (from 2× to 0.1×). After a final wash in phosphate buffer the sections were mounted on slides and dried. The slides were exposed to Kodak XAR-5 x-ray film for 1–3 days and then dipped in photographic emulsion (Kodak NTB-2) and stored at 4°C (in the dark) for 2–4 weeks. After developing the slides in Kodak D-19, they were counterstained with cresyl violet, dehydrated through graded alcohols, and coverslipped with Permount (Fisher Scientific).

RESULTS

Sequence of Three Alternatively Spliced KShIIIA Transcripts. We have isolated three different types of KShIIIA cDNAs from rat brain cDNA libraries (15). These cDNAs have identical nucleotide sequences up to a position close but prior to the in-frame stop codon and then diverge, thereby predicting proteins with different carboxyl ends. The nucleotide and deduced amino acid sequences of the region of divergence of the three KShIIIA cDNAs are shown in Fig. 1. The first sequence, KShIIIA.1, corresponds to RKShIIIA published by McCormack *et al.* (ref. 14; see also corrected sequence in ref. 15), and KShIIIA.3 is probably the same as KV3.2b of Luneau *et al.* (26). The point of divergence of the three sequences is the same and takes place after an AG, the dinucleotide characteristic of the exon boundary of 5′ splice junctions (27). This pattern is consistent with the hypothesis that the cDNAs correspond to alternatively spliced transcripts from the same gene. Furthermore, the expression studies described below show that these cDNAs correspond to transcripts normally expressed *in vivo*. Interestingly, the predicted amino acid sequences of the three KShIIIA carboxyl termini have distinct putative sites for protein kinase phosphorylation (Fig. 1). For example, KShIIIA.2 contains a putative site for protein kinase C-dependent phosphorylation absent in the other two variants. When injected into *Xenopus* oocytes, KShIIIA.2 and KShIIIA.3 induce currents (data not shown) with voltage dependence, kinetics, and pharmacology similar to those described for KShIIIA.1 (14). Luneau *et al.* (26) have isolated a partial cDNA for what might be a fourth transcript from the same gene.

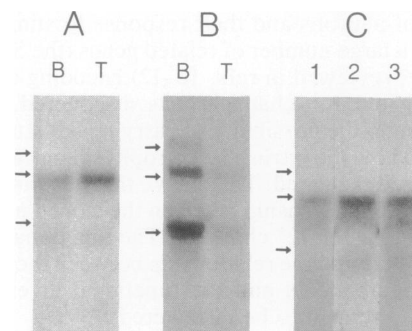

FIG. 2. Northern blot analysis of rat brain RNA hybridized with KShIIIA and NGK2-KV4 probes. (*A* and *B*) Northern blots of whole brain RNA (lanes B) and thalamus-enriched RNA (lanes T) were hybridized with the 380-bp *Eco*RI KShIIIA probe (*A*) or the 351-bp rat NGK2 probe (*B*). (*C*) Northern blots of whole brain RNA were hybridized with the 380-base *Eco*RI KShIIIA probe (lane 1), the *Bgl* II–*Xba* I fragment of KShIIIA.1 (lane 2), or the *Hin*dIII–*Eco*RI fragment of KShIIIA.3 (lane 3). The blots were exposed to x-ray film at −70°C for 8 hr in *A* and *B* and for 18 hr in *C* with two intensifying screens. Arrows indicate the positions of three RNA size markers (9.5, 7.5, and 4.4 kb).

Multiple KShIIIA and NGK2-KV4 Transcripts in Brain RNA. Northern blots of adult rat brain RNA hybridized under high stringency conditions with KShIIIA or NGK2-KV4 probes are shown in Fig. 2 *A* and *B*, respectively. These probes do not differentiate between alternatively spliced variants. Hybridization signals with KShIIIA probes are stronger in thalamus-enriched RNA than in whole brain RNA (Fig. 2*A*). The opposite is observed in Northern blots hybridized with NGK2-KV4 probes (Fig. 2*B*). Multiple hybridization bands are seen in Northern blots probed with the NGK2-KV4-specific sequence (Fig. 2*B*). Luneau *et al.* (18) showed that the more abundant 4.5-kilobase (kb) band corresponds to KV4, whereas the 8-kb band corresponds to NGK2 transcripts. The faint large molecular size band (≈10 kb) is seen with probes specific to either type of transcript and its nature is not clearly understood (18).

Several bands are also observed in Northern blots of whole brain RNA hybridized with the KShIIIA probe that does not differentiate between alternatively spliced transcripts (Fig. 2*A*, lane B, and 2*C*, lane 1). In addition to two major bands (≈7.5 and 6.5 kb) there is a diffuse band around 4 kb. Interestingly, the 6.5-kb band is not seen with thalamus RNA (Fig. 2*A*, lane T). These multiple bands are not explained by the existence of the alternatively spliced transcripts that have been described thus far. The probe specific to KShIIIA.1 and KShIIIA.2 (Fig. 2*C*, lane 2) produces multiple bands of sizes similar to those seen with the nonspecific probe (Fig. 2*C*, lane 1). A KShIIIA.3-specific probe also hybridizes to multiple bands of similar sizes (Fig. 2*C*, lane 3). This observation of multiple hybridization bands with presumably transcript-specific probes is similar to results reported for a different ShIII gene (23) and indicates the existence of differentially processed species or of subtypes of each of the transcripts identified thus far. As is the case for most Sh transcripts, the NGK2-KV4 and KShIIIA RNA species are significantly larger than the cDNAs (2–3 kb). The presence of as of yet unidentified unusually long 5′ untranslated sequences may account for the greater part of the total length of the transcripts (12).

KShIIIA Transcripts Are Highly Localized in Rat Brain. The results of Northern blot analysis suggest that KShIIIA transcripts are not evenly distributed throughout the brain. Consistent with this conclusion, we find by *in situ* hybridization that the expression of KShIIIA transcripts is highly localized (Fig. 3). KShIIIA expression is particularly prominent in the thalamus, with most nuclei of the dorsal thalamus showing high levels of hybridization. Strong hybridization signals are also seen in the optic layer of the superior colliculus. Additional regions of the brain show distinct but less prominent hybridization signals. These areas include deep layers (4–6) of all cortical areas of the neocortex, the piriform cortex, the red nucleus, and the CA3 region of the

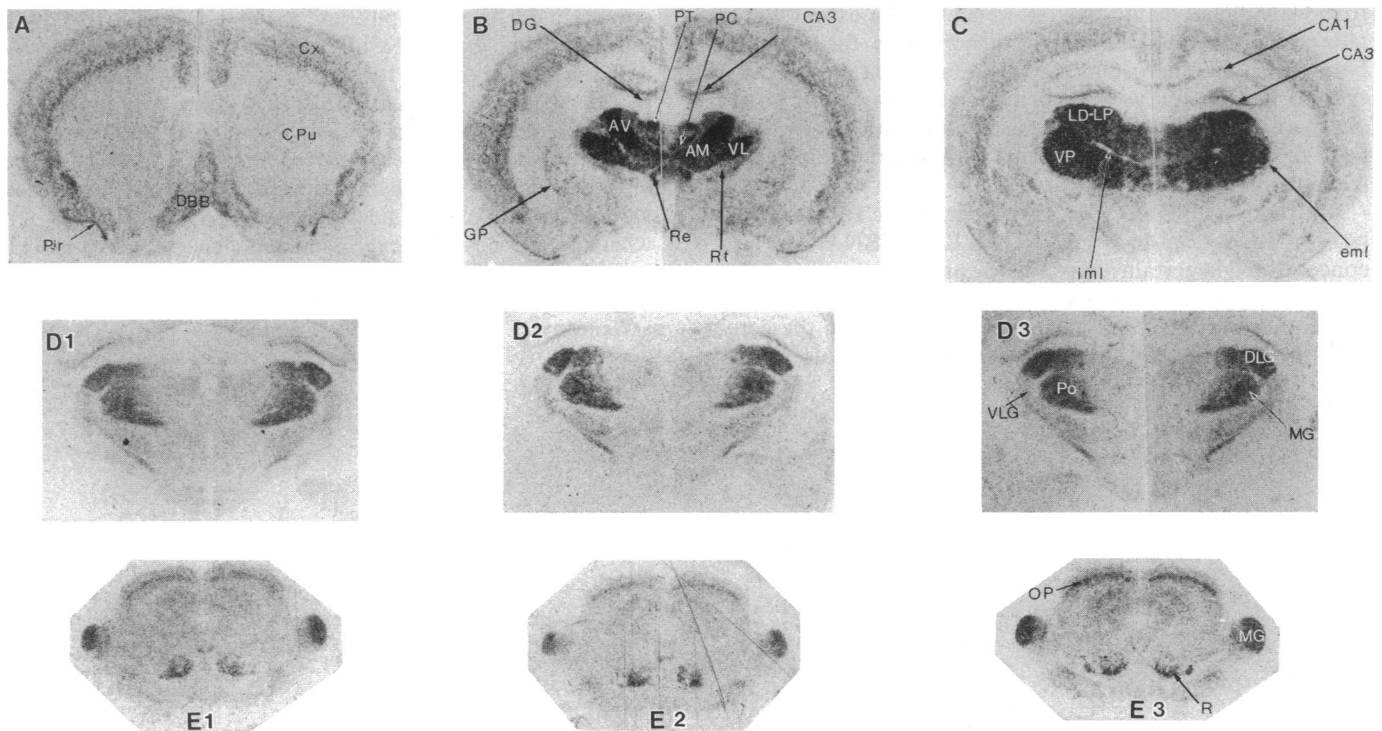

FIG. 3. Localization of KShIIIA mRNAs by *in situ* hybridization. Autoradiography of coronal sections (40 μm) at various (rostral to caudal) levels of an adult rat brain were hybridized with the following ^{35}S-labeled KShIIIA probes: the *Hin*dIII–*Eco*RI fragment of KShIIIA.3 (*A–C*, *D3*, and *E3*), the 380-base *Eco*RI KShIIIA common probe (*D1* and *E1*), the *Bgl* II–*Xba* I fragment of KShIIIA.1 (*D2* and *E2*). The sections were exposed to x-ray film for 1 day at room temperature. (*A*) Rostral section at the level of the diagonal band of Broca showing hybridization in deep layers of the cortex and the piriform cortex. (*B*) Section illustrating hybridization in the anterior thalamic complex. (*C*) Section illustrating the center of the thalamus. (*D*) Sections at the level of the diencephalon caudal to the section shown in *C* illustrating the lateral geniculate complex (LG) and the rostral part of the medial geniculate complex (MG). (*E*) Sections at the level of the rostral part of the mesencephalon showing hybridization in the MG complex, the superior colliculi, and the red nucleus. The following symbols were used in this and the next two figures: (3), oculomotor nucleus; AD, AM, AV, anterodorsal, anteromedial, and anteroventral thalamic nuclei; CA1–CA3, fields CA1–CA3 of Ammon's horn; Cx, cerebral cortex; CPu, caudate-putamen; DBB, diagonal band of Broca; DG, dentate gyrus; DLG, dorsal lateral geniculate complex; eml, external medullary lamina; Gi, gigantocellular reticular nucleus; GP, globus pallidus; Gr, granular cell layer cerebellar cortex; iml, internal medullary lamina; LD, LP, MD, laterodorsal, lateroposterior, and mediodorsal thalamic nuclei; Mi, mitral cell layer; Mo, molecular cell layer cerebellar cortex; OP, optic layer superior colliculus; P, Purkinje cell layer cerebellar cortex; Pir, piriform cortex; PC, paracentral thalamic nuclei; Po, posterior thalamic nuclear group; PT, paratenial thalamic nucleus; R, red nucleus; Re, Rt, reuniens and reticular thalamic nuclei; SC, superior colliculus; SNR, substantia nigra reticulata; Ve, vestibular nuclei; VL, ventrolateral thalamic nucleus; VLG, ventral lateral geniculate complex; VP, ventral posterior thalamic complex; ZI, zona incerta. (×4.2.)

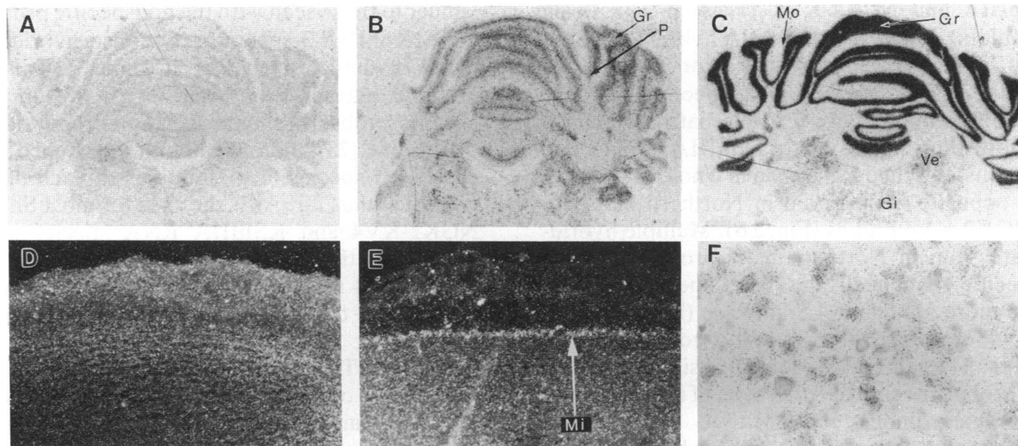

FIG. 4. (*A–C*) Autoradiography of coronal sections at the level of the cerebellum hybridized with the 380-base *Eco*RI KShIIIA probe (*A*), RBK2 probe (*B*), or NGK2-KV4 probe (*C*). Note that several deep cerebellar nuclei (not individually labeled) show hybridization to the RBK2 probe. For purposes of comparison we show autoradiograms with exposure times similar to those used for the sections shown in Fig. 3. (*D* and *E*) Dark-field images of emulsion-dipped lateral sections through the olfactory bulb hybridized with the KShIIIA.3 probe (*D*) or NGK2-KV4 probe (*E*). (*F*) Bright-field image at higher magnification of the ventral posterior nucleus of the thalamus in the section shown in Fig. 3*C* illustrating preferential labeling over the soma of large neurons. (*A–C*, ×4.0; *D* and *E*, ×25; *F*, ×100.)

hippocampus. Very weak signals are seen in the cerebellar cortex (Fig. 4*A*) but we find discrete labeling of the dorsal cochlear nucleus. At the present level of analysis we have not found significant qualitative differences in the expression of alternatively spliced KShIIIA transcripts in the adult rat brain (Fig. 3). There may be quantitative differences that require further study. It is also possible that the different alternatively spliced transcripts are developmentally regulated or that they encode proteins with different subcellular localizations.

KShIIIA and NGK2-KV4 Transcripts Have Different Patterns of Expression in Brain. NGK2-KV4 mRNAs are also concentrated in certain areas, but the pattern of expression is quite different from that exhibited by KShIIIA transcripts. NGK2-KV4 mRNAs are particularly abundant in the cerebellar cortex, where KShIIIA expression is minimal (Fig. 4 *C* and *A*, respectively). NGK2-KV4, but not KShIIIA, transcripts are also seen in mitral cells of the olfactory bulb (Fig. 4 *D* and *E*). The distribution of NGK2-KV4 transcripts in the cortex, in the hippocampus, and in the thalamus is also quite different from that of KShIIIA mRNAs (compare Fig. 5*A* with Fig. 3). In contrast to the results seen with KShIIIA and NGK2-KV4, we find that RBK2, a ShI transcript, is distributed more diffusely (Figs. 4*B* and 5*B*), as reported by McKinnon (16). The three genes explored here are expressed in the hippocampus but with distinct patterns. KShIIIA is expressed particularly in the CA3 field of Ammons horn (Fig. 3 *B–D*), whereas NGK2-KV4 is expressed in the dentate gyrus as well as in CA3 (Fig. 5*A1*), and RBK2 is expressed throughout the hippocampal formation (Fig. 5*B1*; see also ref. 16).

Neuronal Expression of Sh Transcripts. The patterns of distribution of the transcripts explored here suggest that they

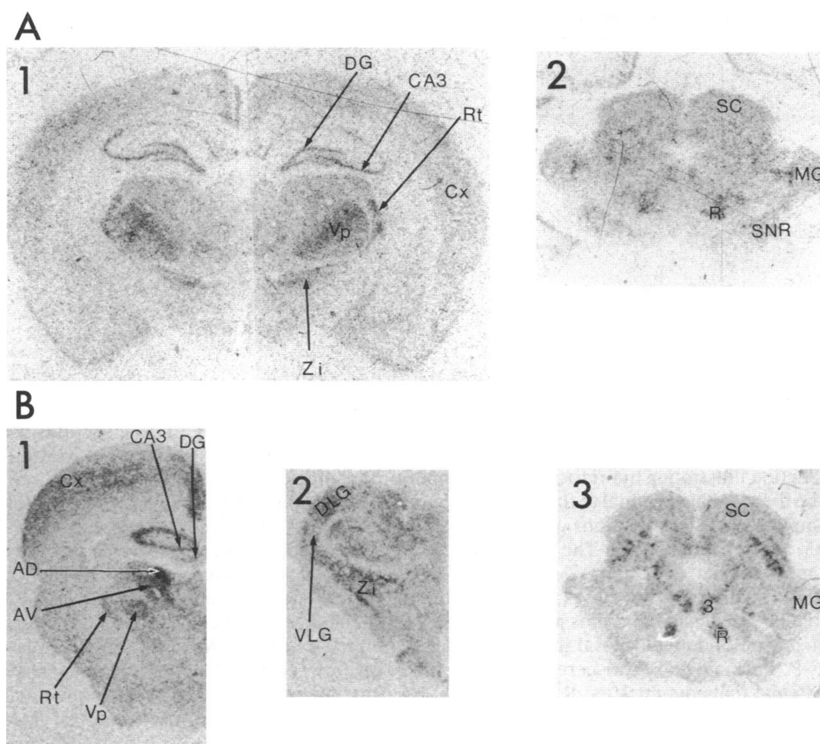

FIG. 5. Autoradiography of coronal sections of an adult rat brain hybridized with NGK2-KV4 (*A*) and RBK2 (*B*) probes. For purposes of comparison we show autoradiograms with exposure times similar to those used for the sections shown in Fig. 3. (*A1*) Section at a level comparable to that in Fig. 3*D*. (*A2*) Section at a level comparable to that in Fig. 3*E*. (*B1*) Left half of a section at a level in between that in Fig. 3*B* and that in Fig. 3*C*. (*B2*) Left half of a section at a level comparable to that in Fig. 3*D*. (*B3*) Section at a level comparable to that in Fig. 3*E*. (×5.2.)

are expressed at highest levels in neurons. Bright-field images of KShIIIA hybridization in thalamic nuclei (Fig. 4*F*) confirm this view. Hybridization grains are concentrated preferentially over the soma of large neuronal cells (probably thalamic relay neurons). Their levels in glia appear to be similar to background.

DISCUSSION

Differential Expression and Functional Properties of KShIIIA and NGK2-KV4 Proteins. The expression studies reported here demonstrate region-specific expression of K^+ channel transcripts in the mammalian central nervous system (see also ref. 28). These results shed new light on a theme that has emerged from electrophysiological recordings of mammalian and molluscan neurons: the existence of a diversity of channels to tailor the electroresponsiveness of the membrane of neurons of different regions (or even within the same region) to a specific function in a network (1–4).

We found here that transcripts from the KShIIIA and NGK2-KV4 genes, which express currents in *Xenopus* oocytes that are very similar in kinetics, pharmacology, and voltage dependence, nevertheless have markedly different patterns of distribution in rat brain. It is possible that transcripts of both genes also express similar channels *in vivo* and that the existence of two genes is a redundancy necessitated by divergent developmental pathways. However, the protein products of these genes in rat are nearly identical to their counterparts in human (unpublished data). This degree of conservation suggests very strong selection for the differences in structure and function of the protein products of the two genes. It is thus important to consider the possibility that there may be significant functional differences in the channels formed by KShIIIA and NGK2-KV4 proteins that remain to be discovered. KShIIIA proteins show about 70% amino acid identity with NGK2-KV4 proteins. Dissimilar regions (20, 23) may be involved in targeting the proteins to specific parts of neurons, in interacting with other proteins, or in determining responsiveness to different stimuli. For example, KShIIIA proteins, but not NGK2 or KV4, have a consensus sequence for cAMP-dependent phosphorylation (residues 561–564 in ref. 15). Whatever their function might be, the specific localization of KShIIIA and NGK2-KV4 transcripts found here, taken together with the evolutionary pressure that has resulted in the high degree of conservation of these genes throughout evolution, is a strong indication that the conductances resulting from these proteins play important and specific roles in the brain nuclei in which they are expressed. It has been suggested that mammalian-like ShIII genes might be a more recent evolutionary acquisition (12). It is thus possible that mammalian-like ShIII proteins are components of K^+ channels having a more specialized function. The highly localized distribution of transcripts from two ShIII genes in rat brain seen here may be an expression of this evolutionary specialization.

The generalized and prominent expression of KShIIIA transcripts throughout, and limited to, the nuclei of the dorsal thalamus is particularly intriguing. Hybridization signals in the epithalamus (e.g., the habenula and the anterior paraventricular thalamic nucleus) and the nuclei in the ventral thalamus (e.g. ventral lateral geniculate complex and reticular thalamic nuclei) are much weaker and in some cases within background levels. The area that gives rise to the dorsal thalamus is embryologically distinct from the areas that give rise to the epithalamus and the ventral thalamic nuclei (29). The dorsal thalamus is also distinct physiologically as it corresponds to the parts of the thalamus that project to the cerebral cortex (29, 30). Interestingly, this differential labeling between dorsal and ventral thalamus is not seen with the probes for NGK2-KV4 and RBK2 (compare Fig. 3 with Fig. 5 *A1*, *B1*, and *B2*).

Functional Role of KShIIIA and NGK2-KV4 Proteins. The results of *in situ* hybridization provide important clues for the identification of native KShIIIA and NGK2-KV4 channels utilizing electrophysiological methods. Since Sh proteins of the same subfamily appear to be capable of forming heteromultimeric channels (11, 12), KShIIIA and NGK2-KV4 proteins could be part of a number of different channel types. These include high-voltage activating, delayed-rectifier-type channels when homomultimeric or when forming heteromultimers with each other, but they could also be components of inactivating channels when forming heteromultimers with KShIIIC or KShIIID products since these proteins express inactivating channels (20, 23). Other factors may also affect the properties of native channels containing these components (12). To further explore the role of KShIIIA and NGK2-KV4 proteins it is important also to determine their localization in different regions of neurons (i.e., dendrites vs. soma or synapses).

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