Differential Expression of Genes Encoding Subthreshold-Operating Voltage-Gated K^+ Channels in Brain

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The members of the three subfamilies (eag, erg, and elk) of the ether-a-go-go (EAG) family of potassium channel pore-forming subunits express currents that, like the M-current (I_M), could have considerable influence on the subthreshold properties of neuronal membranes, and hence the control of excitability. A nonradioactive *in situ* hybridization (NR-ISH) study of the distribution of the transcripts encoding the eight known EAG family subunits in rat brain was performed to identify neuronal populations in which the physiological roles of EAG channels could be studied. These distributions were compared with those of the mRNAs encoding the components of the classical M-current (Kcnq2 and Kcnq3). NR-ISH was combined with immunohistochemistry to specific neuronal markers to help identify expressing neurons. The results show that each EAG subunit has a specific pattern of expression in rat brain. EAG and Kcnq transcripts are prominent in several types of excita-

tory neurons in the cortex and hippocampus; however, only one of these channel components (erg1) was consistently expressed in inhibitory interneurons in these areas. Some neuronal populations express more than one product of the same subfamily, suggesting that the subunits may form heteromeric channels in these neurons. Many neurons expressed multiple EAG family and Kcnq transcripts, such as CA1 pyramidal neurons, which contained Kcnq2, Kcnq3, eag1, erg1, erg3, elk2, and elk3. This indicates that the subthreshold current in many neurons may be complex, containing different components mediated by a number of channels with distinct properties and neuromodulatory responses.

Key words: EAG; ERG; ELK; Kcnq; potassium channels; M-currents; mRNA; nonradioactive *in situ* hybridization; immunohistochemistry; GABAergic interneurons; parvalbumin; brain

Potassium channels that are open at membrane potentials close to the threshold for action potential generation have a major influence on neuronal excitability governing the responsiveness of neurons to incoming inputs. The classical example is the M-current (I_M), first described in sympathetic neurons (Brown and Adams, 1980) and later found in central neurons (Brown, 1988; Yamada et al., 1989). I_M becomes significant above -60 mV and thus may influence the resting potential and the input resistance of the cell. The current opposes depolarizing signals and influences the responsiveness of the cell to synaptic inputs. Moreover, the channels mediating this current (M-channels or M-type K^+ channels) do not inactivate, contributing K^+ current during long depolarizations and producing adaptation in repetitive firing neurons. The inhibition of I_M by neurotransmitters and neuropeptides generates slow depolarizing synaptic potentials and mediates increases in excitability. Another example is subthreshold-activating A-type K^+ channels. These inactivating channels have been shown to regulate the delay between membrane depolarization and action potential generation (delay to first spike) and to modulate firing frequency during repetitive activity (Connor and Stevens, 1971; Rudy, 1988; Baxter and Byrne, 1991; Hille, 1992).

They are prominently expressed in dendrites, where they regulate the back-propagation of action potentials from the soma into the dendritic tree and allow cells to filter fast synaptic inputs (Hoffman et al., 1997; Johnston et al., 1999; Schoppa and Westbrook, 1999).

It was shown recently that the classic M-channel in sympathetic neurons is a heteromeric protein containing Kcnq2 and Kcnq3 subunits (Wang et al., 1998). All of the eight known members of the EAG family of K^+ channel pore-forming subunits express homomeric subthreshold- or near threshold-operating K^+ channels, when expressed in heterologous expression systems (see Table 3). Moreover, similar to I_M , some EAG currents do not inactivate and can contribute an M-like steady outward current during long depolarized potentials. Even for EAG family channels that display inactivation, a large component of non-inactivating current is present.

The EAG family is subdivided into three subfamilies [eag, erg (eag-related genes), and elk (eag-like K^+ channels)] on the basis of sequence similarities (Warmke and Ganetzky, 1994; Ganetzky et al., 1999). [Because the term EAG is the name of the entire family as well as the name of one of the subfamilies, we have used capital letters (EAG) when referring to the family and lowercase when referring to the subfamily or individual subfamily members.] In the better-studied Kv family of K^+ channel pore-forming subunits, members of the same subfamily (or closely related subfamilies), but not of different subfamilies, can interact to form heteromultimeric channels, often with novel functional properties, resulting in a large increase in the diversity of voltage-gated K^+ channels (McCormack et al., 1990; for review, see Coetzee et al., 1999). Preliminary evidence suggests that members of the same EAG subfamily can also express heteromeric channels in

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Table 1. NR-ISH probe information

Probe	Accession number	Primers	Position/size	% Identity/gaps ^a
eag1	Z34264	<i>Eco</i> RI restriction fragment from partial eag1 clone from phage screening <i>Eco</i> RI @ pos 2175 and polylinker <i>Eco</i> RI from pBluescript	2175 3087 912 bp	w/Eag2 57/6
eag2	AF185637	Forward: CGGAAGGTTTT(CT)(AG)A(N)GA(AG)CA(CT)C Reverse: CTGCTCGGG(TGA)AT(TGCA)GG(GA)TA(GA)AA	1849 2937 1089 bp	w/Eag1 64/5
erg1	Z96106	Forward: GACCTGCA(CT)AAGAT(CT)CA(GT)CGAG [ERGfam] Reverse: GGGAAACCTGAGAAAGCGAGT	2491 3349 858 bp	w/Erg2 47/7 w/Erg3 42/7
erg1b	Z96106	Forward: GAGCTGCTTCCTGTGTTTGG Reverse: CTATGATTTCCCGTCACTG	309 1084 776 bp	w/Erg2 36/18 w/Erg3 52/12
erg2	AF016192	Forward: [ERGfam] Reverse: CCTGTAAGCTACCTCTGAGCA	2074 3137 1063 bp	w/Erg1 40/7 w/Erg3 38/6
erg3	AF016191	Forward: [ERGfam] Reverse: GAGACCCAAGATCCCTACAGT	2676 3731 1055 bp	w/Erg1 45/6 w/Erg2 38/6
elk1	AF061957	Forward: GATCGT(GAC)GATGG(AC)ATTGA(AG)GA [ELKfam] Reverse: CAGTATAGAGGTGGCTCTGC	2490 3521 1031 bp	w/Elk2 36/9 w/Elk3 36/8
elk2	AJ007627	Forward: [ELKfam] Reverse: GACAGAGGACAGTGGAGATG	2500 3523 1023 bp	w/Elk1 35/9 w/Elk3 44/7
elk3	AJ007628	Forward: [ELKfam] Reverse: GAATGCTTTGAGCTGCTGGC	2572 3514 942 bp	w/Elk1 36/8 w/Elk2 45/7
Kcnq2	AF087453	Forward: TCGATGACAGCCCAAGCAAG Reverse: CAACCCACACTACTCTATGC	2916 4221 1305 bp	w/Kcnq3 46/8 w/Kcnq4 38/36 w/Kcnq5 44/4
Kcnq2b	AF087453	Forward: CGCAAGCTGCAGAATTTTCCT Reverse: GTAGGTGTGCAAGTGGTCAT	1774 2344 570 bp	w/Kcnq3 64/5 w/Kcnq4 73/0 w/Kcnq5 68/0
Kcnq3	AF091247	Forward: GATGCCATAGAAGAAAGCCC Reverse: CACATGAGTCCAGAAGAGTC	1326 2247 921 bp	w/Kcnq2 45/9 w/Kcnq4 42/14 w/Kcnq5 42/12

^aIdentity to closest relatives. Full-length genes were first aligned using Clustal W. Matching residues and residues aligning with gaps within the probe region were then counted and divided by probe length for percentage.

heterologous expression systems (Wimmers et al., 2001). Many neurons in the CNS coexpress multiple Kv subunits, and heteromeric Kv channels have been shown to exist in native cells (Sheng et al., 1993; Wang et al., 1993; Chow et al., 1999; Hernández-Pineda et al., 1999). Similarly, heteromeric EAG K⁺ channels may exist in neurons coexpressing more than one member of the same subfamily.

Moreover, in neurons containing EAG family subunits in addition to Kcnq2–Kcnq3 proteins, the M-like current might be a complex combination of several components mediated by different channels. To begin to understand the modulation of the excitability of different neuronal populations and to facilitate manipulation of these properties, it is necessary to know the distribution of different EAG and Kcnq2 products in CNS neurons. This knowledge is also necessary to select neuronal populations in which the properties and functional roles of native EAG channels might be studied. In this paper, we report a high-resolution mapping of the patterns of expression of mRNA transcripts for the eight known members of the EAG family in the CNS and compare these distributions with those of Kcnq2 and Kcnq3 transcripts.

MATERIALS AND METHODS

RNA probe design and labeling. Antisense RNA probes were prepared for eag1, eag2, erg1, erg2, erg3, elk1, elk2, elk3, Kcnq2, and Kcnq3 potassium channel subunits. The cloning of cDNAs encoding eag2, erg2, erg3, elk2, elk3, Kcnq2, and Kcnq3 subunit fragments was obtained by PCR from single-stranded rat cortex cDNA. The cloning of erg1 was obtained by PCR from rat cerebellum cDNA. Several attempts to amplify elk1 from

cortical, cerebellar, or total brain cDNA were unsuccessful. Instead, the elk1 probe was obtained by PCR using the full-length elk1 cDNA clone as the template (gift from D. McKinnon and J. Dixon, State University of New York, Stony Brook). The primers used in all PCRs are listed in Table 2. The thermocycler protocol for all PCRs was as follows: 94°C, 1 min; 55°C, 1 min; 72°C, 1 min; for 35 cycles. Single-stranded cDNA was prepared from random-primed total RNA using Maloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) as described previously (Saganich et al., 1999). The eag1 probe was made from a partial eag1 clone obtained from screening a rat brain cDNA library. The details of each probe are listed in Table 1.

Each PCR amplification product was cloned into vectors containing the T7 and/or Sp6 promoters for RNA polymerase, linearized with the appropriate restriction enzyme, and template for *in vitro* transcription prepared by treatment with Proteinase K (10 µg/ml), followed by two phenol/chloroform extractions and ethanol precipitation. Antisense digoxigenin (DIG)-labeled RNA probes (or control sense probes) were made following the manufacturer's protocol by *in vitro* transcription in the presence of DIG-labeled UTP (Roche, Hertfordshire, UK) using ~1 µg of template and the appropriate RNA polymerase. The concentration and integrity of each RNA probe was analyzed by gel electrophoresis, and the level of DIG-UTP incorporation was tested by dot blot by comparison to a known DIG-labeled RNA standard (Roche). For each probe, the transcription reaction resulted in ~10 µg of DIG-labeled RNA, which was diluted with RNase-free H₂O (Sigma, St. Louis, MO) to a concentration of 25 ng/µl, aliquoted, and stored at –80°C. All probes were used at a concentration of 50 ng/ml of hybridization buffer in the *in situ* hybridization (ISH) reaction.

To avoid possible cross-reactivity, each probe was designed to include regions of low nucleotide identity with other related family members or other sequences located in the National Center for Biotechnical Information nucleotide database. The highest level of identity found for any probe was calculated to be 73% (Table 1). To further ensure that probes

Figure 1. Differential expression of EAG and Kcnq K⁺ channels in brain. NR-ISH for EAG family and Kcnq K⁺ channel transcripts in rat brain using DIG-labeled RNA antisense probes is shown. DIG-labeled probes were detected using the alkaline phosphatase substrate NBT/BCIP for 14 hr. *AccN*, Accumbens nucleus; *CPu*, caudate/putamen; *Cx*, cerebral cortex; *Cer*, cerebellum; *Hipp*, hippocampus; *IC*, inferior colliculus; *IO*, inferior olive; *ob*, olfactory bulb; *Pn*, pontine nucleus; *Rt*, reticular thalamic nucleus; *Th*, thalamus; *Tu*, olfactory tubercle.

had no cross-reactivity with their closely related subfamily members, we also performed a dot-blot hybridization for each probe against the cDNAs of each EAG family member. As predicted from similarity calculations, each probe proved to be highly specific for its intended EAG subunit when hybridized at the same stringency conditions used in the ISH protocol.

Combined *in situ* hybridization–immunohistochemistry. The nonradioactive (NR)-ISH protocol used was based on a modified radioactive ISH method developed by Dr. Harriet Baker (Burke Medical Research Institute) (Weiser et al., 1994; Saganich et al., 1999). Briefly, 6- to 8-week-old male rats were perfused intracardially with 100 ml of cold saline solution (0.9% NaCl with 0.5% NaNO₂ and 1000 U heparin), followed by 300 ml of cold 4% paraformaldehyde solution in 0.1 M phosphate buffer, pH 7.4. The brains were removed carefully, cut in blocks, and post-fixed for 1 hr. After post-fixing, the brains were washed several times in cold, 0.1 M phosphate buffer, pH 7.4, and placed in 30% sucrose overnight. Slices were obtained on a freezing-microtome at 40 μ m thickness, and floating sections were prehybridized at 60°C in a solution containing 60% formamide, 3.5 $\times$ SSC, 5% dextran sulfate, 3.5 $\times$ Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA, 0.2 mg/ml t-RNA, and 0.25 mg/ml SDS. After 1 hr of prehybridization, 50 ng/ml of DIG-labeled RNA probe was added, and the hybridization reaction was allowed to proceed for 17 hr.

After hybridization, the sections were washed in decreasing concentrations of SSC (2 $\times$ to 0.1 $\times$) buffer at 65°C followed by a single wash in buffer B1 (150 mM NaCl, 100 mM Tris, pH 7.4) at room temperature. Sections were then treated for 1 hr at room temperature in buffer B1 + 10% normal sheep serum followed by overnight incubation at 4°C with anti-DIG Fab fragments conjugated with alkaline phosphatase (AP) in buffer B1 + 1% normal sheep serum. When co-labeling for neuronal nuclear protein (NeuN), parvalbumin (PV), glutamate decarboxylase (GAD67), and/or calbindin (Cb) was desired, the antibodies were added with the anti-DIG antibodies. Antibodies were used at the following

concentrations: anti-DIG Fab, 1:3000 (Roche); NeuN, 1:500 (MAB377; Chemicon, Temecula, CA); PV, 1:500 (Sigma); GAD67, 1:2500 (AB108; Chemicon); Cb, 1:500 (Sigma). Overnight incubation with antibodies was followed by three 15 min washes in buffer B1 followed by 2 hr incubation at room temperature with secondary antibodies (anti-rabbit Cy2 and/or anti-mouse Cy3; Molecular Probes, Eugene, OR) in buffer B1 + 1% normal goat serum + 0.1% BSA + 0.02% cold water fish gelatin. After treatment with secondary antibodies, sections were washed three times for 15 min at room temperature in buffer B1 followed by a single wash in DIG detection buffer (100 mM NaCl, 100 mM Tris, 50 mM MgCl₂, pH 9.5). DIG detection was performed using the AP substrate nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche) for 8–24 hr in DIG detection buffer. The reaction was stopped by rinsing sections four times for 15 min in ddH₂O; then these were mounted in 0.1 $\times$ SSC, partially dried, and coverslipped in 50% glycerol on glass slides. Images were acquired using an Olympus Provis microscope equipped with a MagniFire digital camera. Fluorescent images were acquired using filter sets for Cy2 and Cy3.

RESULTS

***In situ* hybridization** is an extremely useful technique for determining the expression pattern of genes of interest in the brain. The development of the NR-ISH method has provided a higher level of resolution as compared with traditional techniques that use radiolabeled probes and photographic emulsion. NR-ISH facilitates identification of individual expressing cells by producing an opaque precipitate within the cell body, rather than silver grains that reside in a layer of emulsion above the cell. Because most of the mRNA in neurons is located within the cytoplasm of

Table 2. Distribution of mRNA for EAG and Kcnq K⁺ channel subunits in rat brain

	eag1	eag2	erg1	erg2	erg3	elk2	elk3	Kcnq2	Kcnq3
Forebrain									
Olfactory bulb									
Granular cell layer	++	+++	++	—	++	++	—	++	—
Mitral cell layer	+++	+++	++	++	++	++	—	+++	+
Periglomerular layer	—	—	++	++	++	—	—	+	+
Piriform cortex	+++	+++	+	—	++	++	++	+++	+++
Anterior olfactory nucleus	+++	++	—	—	+	+	—	+++	++
Olfactory tubercle	+++	+	—	—	++	+	++	+++	+++
Cerebral cortex									
Layer II	+++	+	+	—	+++	++	++	+++	++
Layer III	+++	++	+	—	+++	++	++	+++	+++
Layer IV	+++	+++	+	—	+	+/-	+	+/-	+++
Layer V	++	+f	+	—	++	+	+	+++	++
Layer VI	++	—	+/-	—	+	+	+	++	+
Inhibitory interneurons	—	—	++ ^a	—	—	—	—	+f	—
Hippocampus									
Pyramidal cells									
CA1	++	—	++	—	+++	++	+	+++	+++
CA2	+++	—	—	—	—	+/-	—	+++	+
CA3	+++	+/-	—	—	—	+/-	—	+++	+++
DG	++	+/-	—	—	—	++	+	+++	+++
Inhibitory interneurons	—	—	++	—	—	—	—	—	—
Septum	+	+/-	++	—	+/-	—	—	++	+
Basal ganglia									
Caudate/putamen	++	—	Int	—	+/-	+	++	++	+++
Accumbens nucleus	++	—	—	—	—	—	++	++	++
Globus pallidus	—	—	+	—	—	—	—	+/-	+/-
Thalamus									
Medial geniculate nucleus	—	++	++	—	++ ^b	—	—	++	+++
Ventral posterior thalamic complex	—	++	++	—	+/-	—	—	++	+++
DLG	—	++	++	—	+/-	—	—	++	+++
Reticular thalamic nucleus	—	—	+++	—	++	—	—	++	+++
Habenula	+	++	++	—	+	—	—	++	—
Mammillary nucleus	+++	—	++	—	—	—	—	+	+
Amygdala	++	++ ^c	+	—	+	++	—	++	++
Hypothalamus	++	+	++	—	+/-	—	—	++	++
Midbrain									
Superior colliculus									
SuG	—	+	+	—	++	+	—	+	++
InG	—	++	+	—	++	+	—	+	++
Inferior colliculus	+	+++	+++	—	+	—	—	++	++
Substantia nigra									
Pars reticulata	—	—	+f	—	—	—	—	+f	++
Pars compacta	—	—	++	—	—	—	—	++	+
Nucleus lateral lemniscus	—	+++	+++	—	+++	—	—	+	+
Ventral tegmental nucleus	—	—	+++	—	—	—	—	+++	+
Raphe nucleus	—	+	+++	—	++	—	—	+	++
Central gray	+	+	+	—	+	—	—	+	+
Red nucleus	++	—	+++	—	—	—	—	++	+++
Oculomotor nucleus (3)	++	—	+++	—	—	—	—	++	+
Hindbrain									
Cerebellum									
Granule layer	+++	—	++	—	—	++	—	+++	—
Molecular layer	—	—	—	—	—	—	—	—	—
Purkinje cell layer	—	—	+++	—	++	—	—	+++	—
Deep nuclei	+	—	+++	—	+	—	—	++	+/-

Table 2 continues.

Table 2. Continued

	eag1	eag2	erg1	erg2	erg3	elk2	elk3	Kcnq2	Kcnq3
Pontine nucleus	+	++	+++	–	–	–	–	++	+
Superior olive	++	++	++	–	+	–	–	+	–
Vestibular nucleus	+	+/-	+++	–	–	–	–	+	+
Dorsal cochlear nucleus	–	++	+++	–	–	–	–	++	++
Ventral cochlear nucleus	–	++	+++	–	–	–	–	+/-	+
Facial nucleus (7)	++	+	+++	–	–	–	–	++	++
Spinal trigeminal nucleus (5)	–	+	+++	–	+	–	–	++	+
Gracile nucleus	–	–	+++	–	++	–	–	+	+
Cuneate nucleus	+	+	+++	–	++	–	–	++	+
Dorsal motor nucleus, vagus (10)	–	–	+	–	++	–	–	–	–
Gigantocellular reticular nucleus	+/-	+/-	+++	–	+/-	–	–	+	+
Hypoglossal nucleus (12)	+	–	++	–	–	–	–	++	+
Lateral reticular nucleus	–	+	+++	–	++	–	–	++	+
Inferior olive	–	–	+++	–	++	–	–	++	++

The data for this table (except for Kcnq2) were generated from NR-ISH of serial coronal sections from the same rat brain in which all genes were processed in parallel in the same experiment under identical conditions. Relative levels of expression were first determined for each individual gene and then normalized between the different genes. +++, Very high; ++, moderate to high; +, low; +/-, just detectable; –, not detectable; f, few neurons; Int, in PV+ interneurons.

^aMainly in cingulate cortex.

^bIn amygdala projection nucleus of medial geniculate only.

^cEag2 was particularly strong in the intercalated nucleus.

the cell body, the signal produced by NR-ISH usually has a ring or “donut” shape encircling the nucleus. Sometimes the shape of the cell body can be determined from the outline, as observed frequently with relatively abundant transcripts in large layer V pyramidal cells in the cortex, but often the shape of the cell is difficult to obtain.

The regional distribution of EAG and Kcnq K⁺ channel transcripts was determined using NR-ISH using DIG-labeled RNA probes. To achieve a higher level of understanding of the distribution of these channel transcripts within heterogeneous populations of neurons (such as what is found in the cerebral cortex), this technique was combined with immunohistochemistry using antibodies against NeuN, a marker for all neurons (except, according to the manufacturer, Purkinje, mitral, and photoreceptor cells), and markers for inhibitory neurons: GAD, PV, and Cb (Kawaguchi and Kubota, 1997).

Differential expression of EAG and Kcnq K⁺ channel transcripts in rat brain

Each EAG family transcript shows a distinct pattern of expression in rat brain, although some genes have a wider expression pattern than others, as illustrated in representative sagittal images in Figure 1.

The overall pattern of eag1 and eag2 transcripts was similar to previously reported results using lower-resolution methods (Saganich et al., 1999; Ludwig et al., 2000). Eag1 expression was most prominent in the cerebral cortex, hippocampus, cerebellum, and olfactory bulb (Fig. 1, Table 2). Several nuclei of the amygdala and the caudate/putamen also showed prominent expression. Eag1 transcripts were not detected in the thalamus, and only low levels were detected in the brainstem. Unlike eag1, overall expression of eag2 was much more restricted (Fig. 1, Table 2). Eag2 was very abundant in the cerebral cortex and olfactory bulb. Lower levels were also detected in the thalamus, inferior colliculus, intercalated nucleus of the amygdala, and a few brainstem nuclei. Unlike eag1, little or no expression was detected in the hippocampus and cerebellum.

Erg1 is mostly known for its role in the heart, where erg1

subunits combine with accessory mink proteins to form the channels responsible for the IKr current (Trudeau et al., 1995; Sanguinetti et al., 1995; Abbott et al., 1999). Mutations of this gene in humans cause a form of arrhythmia known as long-QT syndrome (Sanguinetti et al., 1995). Erg2 and erg3 were cloned by homology to erg1 from cDNA derived from superior cervical ganglia. Highly sensitive RNase protection assays suggested that erg1 and erg3, but not erg2, were also expressed in brain (Shi et al., 1997).

In our NR-ISH studies, the three members of the Erg subfamily showed different expression levels and patterns in rat brain (Fig. 1). Overall, erg1 levels were low, except for select brain regions. Low levels of erg1 expression seemed to be found in almost every region of the brain; however, in the reticular thalamus, olfactory bulb, and several brain stem nuclei, erg1 expression was more abundant. The widespread low-level expression of erg1 was also found using a separate, non-overlapping probe (erg1b) (Table 1) from a different region of the gene and is therefore believed not to be the result of nonspecific background. Erg3 expression was more abundant but also showed a highly specific pattern of expression, being very prominent in the cerebral cortex, olfactory bulb, and hippocampus. Similar to erg1, erg3 was also found in a few brainstem structures (Table 2). Among EAG family members, erg1 and erg3 were the most prominently expressed transcripts in the brainstem. Interestingly, although RNase protection assays suggested that erg2 is not found in brain (Shi et al., 1997), significant levels of erg2 mRNA were detected in the olfactory bulb. It is possible that the olfactory bulb was not included in the tissue isolated to prepare brain mRNA for the RNase protection assays. Low levels of erg2 (undetectable above normal background using NR-ISH), however, may also be found in the neocortex because the probe was derived from an RT-PCR using total RNA from rat brain cortex (see Materials and Methods). Erg2 had by far the most restricted pattern of expression observed in this study.

The mRNAs of the Elk subfamily also had specific patterns of expression (Fig. 1). Of the three members, elk2 was the most abundant with an overall pattern similar to eag1, with expression

Figure 2. *Eag1* and *eag2* transcripts have overlapping expression in the cerebral cortex, olfactory bulb, and amygdala. *A–D*, ISH with DIG-labeled *eag1* antisense probe. *A*, *Eag1* expression in coronal section at the level of the hippocampus showing strong labeling in the cerebral cortex (Cx), hippocampus (Hipp), and lateral nuclei of the amygdala (La). *B*, High magnification of *A* showing *eag1* expression in the cerebral cortex with specific lamina identified. Note labeling in layers II–VI, with particular high expression levels in layer IV. *C*, *Eag1* expression in the hippocampus showing strongest signals in the CA2 and CA3 fields and in the dentate gyrus (DG). *D*, *Eag1* staining of the granule cell layer (Gr) of the cerebellum. *E–H*, ISH using *eag2* DIG-labeled antisense probe. *E*, *Eag2* expression in serial section of the same brain as *A*. Note strong expression in cerebral cortex (Cx) and much weaker signals in the thalamus (Th) and lateral amygdala (La). *F*, High magnification of the cortex shows *eag2* expression in layer IV. Unlike *eag1*, little or no *eag2* expression was found in the hippocampus (*G*) or the cerebellar cortex (*H*). *I–J*, Overlapping expression of *eag1* (*I*) and *eag2* (*J*) transcripts was also found in the internal granule layer (IGr) and the mitral cell layer (Mi) of the olfactory bulb. Scale bar (shown in *J*): *A*, *E*, 1500 μ m; *B*, *F*, 150 μ m; *I*, *J*, 500 μ m; *C*, *D*, *G*, *H*, 600 μ m. ML, Molecular layer of the cerebellum; GI, periglomerular layer of the olfactory bulb.

in the cerebellum, cerebral cortex, olfactory bulb, and hippocampus. *Elk3* expression was relatively weak but very restricted. Like *elk2*, *elk3* was also located in the cerebral cortex; however, it was most prominent in the caudate/putamen and the accumbens nuclei. In this study, the only other transcripts that had similar staining patterns in the caudate were *eag1*, *Kcnq2*, and *Kcnq3*. Our regional results for *elk2* and *elk3* were in good agreement with Northern blot data of the human *Elk* homologs *bec1* and *bec2* (Miyake et al., 1999). Finally, no *elk1* expression was found in rat brain. Furthermore, several attempts to amplify *elk1* by RT-PCR, using the same RNA that yielded all other members of the EAG and *Kcnq* families, was unsuccessful (see Materials and Methods), although the primers and PCR conditions tested allowed robust amplification of *elk1* when using *elk1* cDNA as template. The finding of undetectable levels of *elk1* by our ISH methods in rat brain is in agreement with previously reported RNase protection assays that ranked *elk1* expression as “just detectable” (Shi et al., 1998). However, a partial *elk1* sequence was isolated from rat cortex cDNA by RT-PCR (Engeland et al., 1998). Therefore, the overall expression of *elk1* in brain must be very low and requires highly sensitive methods, such as PCR, for detection.

Figure 1 also shows the results of NR-ISH for *Kcnq2* and *Kcnq3* (see also Table 2). The localization of *Kcnq2* in rat brain was extensive, being found to some degree or another in most brain areas (Schroeder et al., 1998; Tinel et al., 1998). The widespread distribution of *kcnq2* was also confirmed using a second probe (*kcnq2b*) from a different region of the gene (Table 1). The highest levels of expression were detected in the hippocampus, cerebral cortex, olfactory bulb, caudate, and cerebellum. Interestingly, *Kcnq3* expression was more restricted than *Kcnq2* (see implications in Discussion). The highest levels of this transcript were found in the cerebral cortex, thalamus, hippocampus, and caudate/putamen. Several nuclei of the amygdala and the hypothalamus also demonstrated *Kcnq3* expression. In the brainstem, expression of both *Kcnq2* and *Kcnq3* was moderate to weak,

but the patterns of both were highly overlapping (see Fig. 12, Table 2).

Analysis at higher magnification, as well as dual staining with antibodies to the neuronal marker NeuN and to markers of GABAergic neurons, allowed the scoring of the expression levels of EAG and *Kcnq* transcripts in many neuronal populations throughout the brain (Table 2). This analysis and the data shown in Figure 1 show clearly that there is overlap between members of the same subfamily in several neuronal populations. Furthermore, many neurons in the brain express multiple EAG and *Kcnq* transcripts. Some examples of the data used to generate Table 2 are shown below. We illustrate examples that emphasize the absence or presence of overlap.

Overlapping expression of EAG and *Kcnq* transcripts

The eag subfamily

Eag1 and *eag2* transcripts have overlapping expression in the cerebral cortex and the olfactory bulb (Fig. 2). Serial coronal sections hybridized with *eag1* or *eag2* antisense probes were used to characterize the expression of both transcripts in the cortex (Fig. 2*A,E*). *Eag1* expression in the cortex was strongest in layers IV and VI (Fig. 2*A,B*). *Eag2* expression, however, was much more restricted to the lower layer III and layer IV (Fig. 2*E,F*). Products of both genes were seen in the majority of neurons in layer IV (Fig. 3). Co-staining with antibodies to GAD showed that neither *eag1* nor *eag2* is expressed in inhibitory neurons within layer IV or any other layer in the cortex (Fig. 3). Together the data suggest that *eag1* and *eag2* are most likely found within the same excitatory neurons in layer IV.

Strong expression of *eag1* was also found in the hippocampus and the cerebellum (Fig. 2*A,C,D*). Higher magnification of the hippocampus showed *eag1* to be strongest within the pyramidal layers of the CA2 and CA3 subfields and the granule cell layer of the dentate gyrus (Fig. 2*C*). In contrast, little or no expression of *eag2* was found within the hippocampus (Fig. 2*G*). In the cerebellum, *eag1* was very strong and restricted to the granule cell

Figure 3. *Eag1* and *eag2* transcripts are not found in inhibitory cells of the cortex. *A–D*, Dual detection of *eag1* and GAD in cortical layer IV. *A*, Low-magnification bright-field image of *eag1* expression in cortical layer IV. *B*, High-magnification bright-field image of *A* showing *eag1* expression in many small non-pyramidal neurons. *C*, Immunofluorescent detection of GAD immunoreactive interneurons. *D*, Overlay of *B* and *C* with *eag1* expression pseudocolored green. Note GAD⁺ neurons are not labeled for *eag1* (arrows). *E–H*, Same as *A–D*, but for *eag2*. Note *eag2* transcripts do not colocalize with GAD⁺ immunoreactive neurons (arrows). Scale bar (shown in *H*): *B–D*, *F–H*, 50 μ m; *A*, *E*, 200 μ m.

layer (Fig. 2*D*). In contrast, little *eag2* expression was found within the cerebellum (Fig. 2*H*).

Expression of *eag1* and *eag2* was also found to colocalize within the olfactory bulb (Fig. 2*I,J*). In the case of both genes, expression was found in the mitral cell layer and the granule cell layers of the olfactory bulb. In contrast, neither gene was found within cells of the periglomerular region of the bulb.

One of the most striking features of *eag2* expression in brain is its specific laminar expression in the neocortex (Figs. 1, 2) (Saganich et al., 1999). Using radioactive probes, we were unable to ascertain which neurons were expressing the gene, both because of the lack of delineation of neuronal morphology with radioactive ISH on Nissl counter-stained sections, as well as the fact that given the cell density in layer IV it was very difficult to assign emulsion grains to underlying cells. NR-ISH showed that the laminar distribution of *eag2* transcripts varied with cortical region, being most abundant in the somatosensory cortex as compared with other cortical areas (Fig. 4*A*). In tangential sections through the rat somatosensory barrel cortex, a barrel pattern with hollow centers is observed after NR-ISH for *eag2* (Fig. 4*B*), indicating that *eag2* transcripts are concentrated in spiny stellate cells (Egger and Sackmann, 2001). In coronal sections, it is also clear that the strongest hybridization signals are seen in the barrel sides and barrel margins with layer III and layer V (Fig. 4*C–E*). Many of the neurons prominently expressing *eag2*, in the barrel margin with layer III, have clear pyramidal morphology (Fig. 4*I–K*), whereas those inside layer IV are small, non-pyramidal or have a star-shaped appearance (Fig. 4*F–H*).

The *Erg* subfamily

As mentioned above, the three members of the *Erg* family have very different distributions. Overlap of two or more of the *Erg* genes does occur, however, in several areas (Figs. 5–7). For example, expression of both *erg1* and *erg3* was seen in the reticular thalamus (Fig. 5*A,B*). Given that the reticular thalamus is composed mainly of a single population of GABAergic neurons (Jones 1985), and that most neurons in this nucleus express *erg1* and *erg3* (data not shown), each reticular thalamus neuron most likely expresses both transcripts. This was confirmed after co-labeling with antibodies to GABA and PV (data not shown). The reticular thalamus is in fact one of the areas in which *erg1* is most abundant. *Erg3* expression is much weaker than *erg1* in the

reticular thalamus as well as in dorsal thalamic nuclei (Fig. 5*B*). *Erg1* is expressed throughout the dorsal thalamus, but at lower levels than in the reticular thalamus (Fig. 5*A*, Table 2).

Both *erg1* and *erg3* transcripts were also expressed in the cerebral cortex (Figs. 5*A–D*, 7). *Erg1* expression in the cortex was much weaker than *erg3* and was found throughout all layers of the cortex (Fig. 5*C*). In contrast, *erg3* expression in the cortex was very strong and produced a bilaminar pattern easily observed at low magnification (Fig. 5*B*). This pattern was the result of strong staining of neurons within cortical layer II/III and layer V (Fig. 5*D*). Weaker staining was also apparent in layers IV and VI. High-magnification images of the *erg3 in situ* experiments and co-labeling with NeuN antibodies (as performed for *eag2* above) confirmed that *erg3* transcripts in layers III and V were found mostly within neurons with pyramidal morphology (data not shown). Unfortunately, the weak expression of *erg1* transcripts in the cortex made more detailed characterization of this gene product difficult (with the exception of the cingulate and retrosplenial cortices; see below). However, it was clear, on the basis of co-labeling with NeuN antibodies, that *erg1*, like *erg3* transcripts, was located within most large layer V pyramidal neurons (Fig. 5*C*).

Both *erg1* and *erg3* are found within the cerebellum as well (Fig. 5*E,F*). *Erg1* is located within the granule cell layer and in Purkinje cells. Because of the small amount of cytoplasm found within cerebellar granule neurons, and background from fibers, it is easier to appreciate the granule layer staining at lower magnifications (Fig. 5*E*, top panel). Higher magnifications reveal clear labeling of the large Purkinje cells at the border of the granule cell layer (Fig. 5*E*, bottom panel). Unlike *erg1*, *erg3* expression was extremely weak in the granule cell layer (Fig. 5*F*, top panel). However, as observed for *erg1*, this transcript was expressed in Purkinje neurons (Fig. 5*F*, bottom panel).

All three *Erg* transcripts were expressed within the olfactory bulb (Fig. 5*G*). *Erg1* and *erg3* had similar patterns, being located within the majority of neurons of the mitral cell and granule cell layers and in scattered cells within the periglomerular area (Fig. 5*G*, top and bottom panels). *Erg2* expression, which was not found anywhere else in the brain, had an even more restricted pattern in the bulb as compared with its relatives *erg1* and *erg3*. *Erg2* was found only in the periglomerular and mitral cell layers, with no

Figure 4. Characterization of *eag2* expression in the cerebral cortex. *A*, Changes in *eag2* expression with cortical region. *Eag2*-expressing neurons are more abundant in somatosensory cortex (SS) as compared with the striate (Str) and frontal (Fr) cortical regions. *B*, ISH for *eag2* in tangential sections through rat somatosensory barrel cortex reveals a whisker barrel pattern with hollow centers. *C–E*, Combined ISH for *eag2* and immunofluorescent detection of NeuN in a coronal section through rat somatosensory barrel cortex. *C*, Fluorescent detection of all neurons using NeuN antibodies. *D*, Same section in bright field showing labeling for *eag2* by ISH. Note that *eag2* staining demarcated cortical layer IV with strong labeling of neurons lining the barrel sides (arrows), as well as neurons on the margins between layer IV and neighboring layers. *E*, Overlay of *C* and *D* with *eag2* pseudocolored green, and NeuN red. *F–K*, High-magnification images of *C* identifying *eag2*-positive neurons along barrel sides in cortical layers IV (*F–H*) and in deep cortical layer III (*I–K*). Note that *eag2*-positive neurons in layer IV are small and non-pyramidal and have a star-shaped appearance (*F–H*, arrows). In contrast, *eag2*-positive cells in deep layer III are clearly pyramidal in shape with identifiable apical dendrites that are orientated toward the pia surface (*I–K*, arrow). Scale bar (shown in *K*): *A*, 400 μ m; *B*, 575 μ m; *C–E*, 500 μ m; *F–K*, 50 μ m.

labeling in the granule cell layer (Fig. 5*G*, middle). The staining pattern of *erg2* was similar to *erg1* and *erg3* in the periglomerular region but was different in the mitral cell layer, being found within slightly larger and more scattered cells. Interestingly, high-power images of *erg1* within the periglomerular layer revealed that this transcript was found in a small number of neurons, with larger soma size, that did not co-label with PV or Cb (Fig. 8*E–H*). This suggests that *erg1* transcripts within the glomerular region of the olfactory bulb are probably expressed in the external tufted cells and not the PV and Cb containing periglomerular or superficial short axon cells (Crespo et al., 1997).

Erg1 and *erg3* expression patterns were quite different in the hippocampus but did show some areas of overlap (Fig. 6). *Erg1* expression was relatively weak and appeared to be concentrated in the pyramidal cells of the CA1 field and in scattered cells located throughout the hippocampus (Fig. 6*A–G*). Many of these scattered cells located outside or near the pyramidal cell layers were PV positive (Fig. 6*B–G*) and most likely correspond to the inhibitory basket cells (Freund and Buzsaki, 1996). *Erg3* was also found within CA1 pyramidal cells, but at much higher levels (Fig.

6*H–N*). Unlike *erg1*, *erg3* expression was restricted to the pyramidal cell layer and was not found in surrounding PV-positive cells (Fig. 6*I–N*).

The expression of *erg1* in PV-containing inhibitory neurons in the brain was not restricted to the hippocampus. As mentioned above, *erg1*, as well as *erg3*, was located within the reticular thalamus (Figs. 1, 5), in which all neurons are inhibitory PV-expressing neurons (Jones, 1985). In the cerebral cortex, *erg1* was also found to be expressed in a population of PV-containing interneurons located within the cingulate and retrosplenial cortices (Fig. 7*A*). Within these areas, the majority, and the strongest *erg1*-labeled neurons, were PV positive (Fig. 7*B–D*). Interestingly, outside these two cortical areas, it was increasingly difficult to find strongly labeled cells and to show coexpression with PV in the cortex (data not shown). In contrast, *erg3* did not co-label with PV-containing interneurons in the cortex (Fig. 7*E–H*). Finally, *erg1* was also found to be located within PV-positive cells in the caudate (Fig. 8*A–D*). These neurons, which correspond to locally projecting aspiny interneurons (Kita et al., 1990; Hontanilla et al., 1998), were labeled strongly for *erg1*, scattered, and few in num-

Figure 5. Overlapping expression of Erg mRNA transcripts occurs in the reticular thalamus, cerebellum, hippocampus, and olfactory bulb. *A*, ISH with *erg1* antisense probe. Note that *erg1* expression was relatively weak with the exception of the reticular thalamic nucleus (*Rt*). Weaker expression was found in the cerebral cortex (*Cx*), hippocampus (*Hipp*), thalamus (*Th*), and ventral medial hypothalamic nuclei (*VMH*). *B*, ISH with *erg3* antisense probe. *Erg3* expression was strong in the cerebral cortex (*Cx*) and the CA1 subfield of the hippocampus (*CA1*). Weaker *erg3* expression was also found in the *Rt* and the *VMH*. *C–D*, Expression of *erg1* and *erg3* mRNA in cerebral cortex. *C*, High magnification of the cortex in *A* showing weak *erg1* expression throughout cortical layers II–V. *D*, High magnification of *B* showing strong *erg3*-positive neurons in layers II/III and V. *E*, *F*, Expression of *erg1* and *erg3* transcripts, respectively, in the cerebellar cortex. Note that both transcripts were found in the Purkinje

cell layer (*PL*). Comparison at low power revealed that only *erg1* was found in the granule cell layer (*E*, *F*, top panels). *G*, Colocalization of *erg1* (top), *erg2* (middle), and *erg3* (bottom) in the olfactory bulb. *Erg1* and *erg3* transcripts were located in neurons of the internal granule layer (*IGr*), mitral cell layer (*Mi*), and periglomerular layer (*GL*). *Erg2* transcripts, however, were located only within the mitral cell layer and the periglomerular cell layer. Scale bar (shown in *G*): *A*, *B*, 2000 μ m; *C*, *D*, 200 μ m; *E*, *F* (top), 720 μ m; *E*, *F* (bottom), 360 μ m; *G*, 500 μ m.

Figure 6. *Erg1*, but not *erg3*, is located in PV-containing interneurons throughout the hippocampus, but both are co-expressed in CA1 pyramidal cells. *A–G*, ISH using *erg1* antisense probe. *A*, *Erg1* expression within the CA1 pyramidal cell layer and scattered cells throughout the hippocampus. *B–D*, Dual labeling for *erg1* and PV in the CA1 subfield. *B*, Bright-field image of *erg1*-positive neurons. *C*, Immunofluorescent detection of PV-reactive interneurons found on the margin of the CA1 pyramidal cell layer. *D*, Overlay of *C* and *D* with *erg1* pseudocolored green. Note that many of the strongly labeled neurons in *B* are also PV positive (*B–D*, arrows). *E–G*, Dual labeling for *erg1* and PV in the CA3 hippocampal subfield. *E*, *Erg1* expression is found in the pyramidal cell layer and stratum radiatum. *F*, PV immunoreactive interneurons in the CA3 (arrows). *G*, Overlay of *E* and *F* with *erg1* pseudocolored green. Note less *erg1* expression in the CA3 pyramidal cell layer as compared with the CA1 (compare green cells in *D* and *G*). Similar to the CA1, several PV-positive neurons also expressed *erg1* (*E–G*, arrows). However, not all *erg1*-positive neurons located outside the pyramidal cell layer were PV positive (*E*, *G*, arrowheads). *H–N*, ISH using *erg3* antisense probe. *H*, *Erg3* expression was very strong and concentrated on the CA1 hippocampal subfield. *I–N*, Dual labeling of *erg3* transcripts and PV-immunoreactive interneurons in the CA1. *I*, *Erg3* expression was located within the pyramidal cell layer of the CA1, but not in scattered cells located outside as observed using *erg1* probe (compare *B* and *I*). *J*, Immunolocalization of PV+ interneurons located along the CA1 pyramidal cell layer. *K*, Overlay of *I* and *J* with *erg3* pseudocolored green. *L–M*, High magnification of *I–K*, respectively, showing that *erg3* labeling does not colocalize with PV (arrows). Scale bar (shown in *N*): *A*, 1000 μ m; *B–G*, *I–K*, 100 μ m; *H*, 810 μ m; *L–N*, 50 μ m.

ber (Fig. 8*A,B*). In contrast, no *erg3* signal was detected in the caudate (Fig. 1, Table 2).

Erg1 was also expressed in several brainstem nuclei (Fig. 1). A more detailed analysis of the pattern of expression in this brain area is presented later to compare the expression of *erg1* transcripts with the products of *Kcnq* genes (see Fig. 12).

The Elk subfamily

Among the two Elk transcripts found in brain, *elk2* and *elk3*, there was little overlap in expression. *Elk2* had very strong expression in the granule cell layer of the cerebellum (Fig. 9*A,B*). No expression of *elk2* was found in the Purkinje cells, which could

Figure 7. Erg1 is located in a population of parvalbumin-containing interneurons in the cingulate cortex, whereas *erg3* is found only in excitatory neurons in the cerebral cortex. *A*, Relatively strong *erg1* expression was found within neurons of the cingulate cortex. *B–D*, Identification of a population of *erg1*-positive interneurons in the cingulate cortex by dual labeling with PV. *B*, High-power bright-field image of *erg1*-positive neurons from *A*. Note that *erg1*-positive neurons are scattered and strongly labeled. *C*, Immunofluorescent detection of PV-containing interneurons. *D*, Overlay of *B* and *C* with *erg1* signals pseudocolored green. Note that nearly all PV-positive neurons are also expressing *erg1* transcripts. Characteristic labeling of interneurons by ISH, revealing a bipolar shape, is evident in bright field (arrows). *E–H*, Unlike *erg1*, *erg3* expression is not found in inhibitory neurons of the cortex. *E*, Low-power bright-field image showing *erg3* expression in cortical layers II/III. *F*, High-magnification bright-field image of *erg3*-positive cells in cortical layer III. *G*, Immunodetection of GAD-immunoreactive interneurons. *H*, Overlay of *F* and *G* with *erg3* signals pseudocolored green. Note that GAD-positive cells do not express *erg3* (*F*, arrows point to unlabeled GAD+ cells). Scale bar (shown in *H*): *A*, *E*, 500 μ m; *B–D*, *E–H*, 50 μ m.

Figure 8. Erg1 transcripts are located in a few scattered PV-positive cells in the caudate/putamen but do not colocalize with PV or Cb in the olfactory bulb. *A–D*, Erg1 transcripts are located in PV-positive interneurons of the caudate/putamen (CPu). *A*, Low-magnification bright-field image of *erg1*-expressing cells in the CPu. Note that cells are strongly labeled but scattered and few in number. *B*, Higher magnification image of *erg1*-positive neurons in the CPu. Fiber tracts appear as light gray (arrowhead). *C*, Immunofluorescent detection of PV-containing neurons. *D*, Overlay of *B* and *C* with *erg1* signals pseudocolored green. Note that most PV-positive neurons also express *erg1* (*B–D*, arrows). *E–H*, Erg1-expressing neurons in the periglomerular layer of the olfactory bulb are not immunoreactive for PV or Cb. *E*, Low-magnification bright-field image of *erg1* in the olfactory bulb. *F*, High magnification of *E* (arrow) showing three glomeruli (GI). Note that *erg1* expression is strong and located in large neurons within the periglomerular layer (arrows). *G*, Immunofluorescent detection of PV- and Cb-positive periglomerular neurons (PV and Cb monoclonal antibodies were mixed and detected with the same secondary antibody). *H*, Overlay of *F* and *G* with *erg1* labeling pseudocolored green. Note that none of the large *erg1*-positive neurons are PV or Cb positive. Scale bar (shown in *H*): *A*, 250 μ m; *E*, 500 μ m; *B–D*, *F–H*, 100 μ m.

be identified by labeling with PV (Fig. 9*B*). In contrast, *elk3* was not expressed significantly anywhere in the cerebellum (Fig. 9*C,D*).

Elk2 expression was also strong within the olfactory bulb (Fig. 9*E,F*). At high magnification, it was clear that *elk2* was found within both the mitral and granule cell layers (Fig. 9*F*). *Elk2* was not found within the cells of the periglomerular layer (Fig. 9*F*). *Elk3* expression was not detected in the olfactory bulb (Fig. 1).

In contrast, *elk3* expression was relatively strong within the caudate (Fig. 9*G,H*). Higher magnification revealed that most neurons within the caudate were *elk3* positive (Fig. 9*H*), suggesting that this transcript is expressed in the principal neurons of this nucleus, the medium spiny neurons (Heimer et al., 1995). This was similar to the pattern observed for *eag1* (data not shown) and *Kcnq2* and *Kcnq3* (Fig. 10) (see below) but unlike the interneuron staining found in the caudate using the *erg1* probe (Fig. 8).

Expression of both *elk2* and *elk3* was observed in the hippocampus (Fig. 9*I–J*). *Elk2* signals were much stronger than *elk3* in the hippocampus, but they overlapped. Signals for both transcripts were located in the pyramidal cell layer of the CA1 field and the granule cells of the dentate gyrus.

Both *elk2* and *elk3* were also located in the cerebral cortex (Fig. 9*K–N*). Each gene was found throughout all cortical layers, but both were most abundant within the neurons of the upper lamina (layers II–III).

Kcnq2 and *Kcnq3*

The overlapping expression of *Kcnq2* and *Kcnq3* transcripts is of particular interest because it is believed that heteromultimers of these two channel subunits are responsible for the native M-currents that have been recorded within several brain areas, including the cerebral cortex and the hippocampus (Halliwell, 1986; Brown, 1988; McCormick and Williamson, 1989; McCormick, 1992; Wang et al., 1998; Cooper et al., 2000). The lack of overlapping expression of the products of these two genes is also interesting because *Kcnq3* homomultimers have been reported to conduct little current in heterologous expression systems (Wang et al., 1998).

The hippocampus was a brain area in which both *Kcnq2* and *Kcnq3* transcripts were prominent and colocalized in the same neuronal populations (Fig. 10*A,B*). *Kcnq2* was located in the pyramidal cell layer of the CA1–CA3 subfields and the granule cells of the dentate gyrus (Fig. 10*A*). *Kcnq3* expression in the

Figure 9. Elk2 and elk3 expression in rat brain have overlapping expression in the cerebral cortex and hippocampus. *A–B*, Dual labeling of elk2 and PV in rat cerebellar cortex. *A*, Bright-field image showing elk2 expression localized to the granule cell layer (*Gr*) of the cerebellum. *B*, Immunofluorescent detection of PV-labeled Purkinje neurons within the Purkinje layer (*PL*) and inhibitory cells within the molecular layer (*ML*).

hippocampus was slightly more restricted, being most prominent within the CA1, CA3, and dentate gyrus, but lower in the CA2 subfield (Fig. 10*B*).

Both Kcnq2 and Kcnq3 were expressed in the majority of cells in the caudate (Fig. 10*C,G*). Neither Kcnq3 (Fig. 10*D–F*) nor Kcnq2 (data not shown) colocalized with the interneuron marker PV. As for elk3, on the basis of the number and size of the Kcnq2 and 3-labeled neurons, we hypothesize that they correspond to the medium spiny neurons (Heimer et al., 1995). On the other hand, one area in which Kcnq2 and Kcnq3 expression did not overlap was the cerebellar cortex. Kcnq2, but not Kcnq3, was expressed in the cerebellar cortex (Fig. 10*H*). Kcnq2 was prominently expressed in granule and Purkinje cells but not in the interneurons of the molecular layer (Fig. 10*H–K*).

Both Kcnq2 and Kcnq3 were expressed in the cerebral cortex. Kcnq3 was most prominent in layer IV and was present in a large majority of the small abundant cells of this layer, probably colocalizing with *eag1* and *eag2*. In contrast, Kcnq2 signals were negligible in this highly populated cortical lamina (Figs. 1, 11*A,H*). Nevertheless both Kcnq2 and Kcnq3 were strongly expressed in most pyramidal cells in layers II–III and V, where they are likely to be coexpressed in the same neurons (Fig. 11*A,B,E–H,I,L*). Dual labeling for PV (Fig. 11*I–N*) and GAD (data not shown) showed that Kcnq3 is not expressed in GABAergic interneurons. Interestingly, in some experiments Kcnq2 was seen to colocalize in a few PV-containing cells in layers II–III (Fig. 11*B–D*). Co-labeling of Kcnq2 and PV was less common in layer V (Fig. 11*E–G*).

Kcnq2 and *Kcnq3*, along with *erg1*, were the genes most strongly expressed within the midbrain and hindbrain regions (Table 2, Fig. 12). The expression levels of Kcnq2 and the Kcnq3 in the brainstem were moderate, with many areas of overlap. Many of the brainstem nuclei expressing Kcnq2 and Kcnq3, such as medial vestibular nucleus, inferior olive, dorsal cochlear nucleus, pontine nucleus, inferior colliculus, substantia nigra, and red nucleus, also prominently expressed *erg1* (Fig. 12). A few of the *erg1*-containing nuclei were also positive for *erg3* (Table 2). Many of these nuclei contain heterogeneous populations of neurons. Therefore, to establish whether there is colocalization of EAG and Kcnq transcripts in brainstem neurons still requires an anal-

Comparison of *A* and *B* shows that elk2 is not expressed within the Purkinje cell layer as demarcated by PV staining. *C–D*, Low- and high-magnification bright-field images, respectively, showing no elk3 expression within the cerebellar cortex. *E–F*, Low- and high-magnification bright-field images, respectively, showing strong elk2 expression in the olfactory bulb. Elk2 was abundant in the internal granule (*IGr*) and mitral cell (*Mi*) layers but not within the periglomerular area (*GI*). *G–H*, Elk3 expression in the caudate/putamen (*CPu*). *G*, Low-magnification image showing elk3-positive neurons in the *CPu*. *H*, Higher magnification of *G* showing that most neurons in the *CPu* were elk3 positive (as compared with *erg1*) (Fig. 8*A,B*). *I–J*, Localization of elk2 and elk3 transcripts, respectively, in the hippocampus. Note that both genes were found in the CA1 subfield and the DG, with elk3 expression being weaker than that of elk2. *K–L*, Elk2 expression in the cerebral cortex. *K*, Low-power bright-field image showing weak elk2 expression throughout all the cortical lamina with higher levels in upper layers II/III. *L*, High-power image of *K* showing cortical layer II. Note large number of weakly stained neurons. *M–N*, Elk3 expression in cerebral cortex. *M*, Similar to elk2, elk3 expression was found throughout the cortex with higher levels within upper lamina. *N*, High-power image of *M* showing elk3 in a large proportion of layer II neurons. Scale bar (shown in *N*): *A, B, D, F, H, L, N*, 200 μ m; *I, J*, 550 μ m; *G*, 2000 μ m; *C, E, K, M*, 500 μ m.

Figure 10. Kcnq2 and Kcnq3 mRNA transcripts overlap in the hippocampus and caudate/putamen, but not the cerebellum. *A–B*, Comparison of Kcnq2 and Kcnq3 transcripts in the hippocampus. *A*, Abundant Kcnq2 expression was detected in CA1–CA3 pyramidal cells and granule cells of the DG. *B*, Strong Kcnq3 expression was found in similar neurons but was weaker than Kcnq2 in CA2. *C–G*, Kcnq3 and Kcnq2 expression in the caudate/putamen (CPu). *C*, Low-magnification bright-field image showing strong Kcnq3 expression in the large majority of CPu neurons. *D–F*, Kcnq3 is not expressed in PV-immunoreactive neurons in the CPu. *D*, High-magnification bright-field image of Kcnq3-positive neurons in the CPu. *E*, Immunofluorescent detection of PV-containing neurons in same section as *D*. *F*, Overlay of *D* and *E* with Kcnq3 pseudocolored green. Note that no PV neurons were Kcnq3 positive (arrows). *G*, Kcnq2 is also located in the caudate/putamen. *H–K*, Kcnq2, but not Kcnq3, is expressed in the cerebellar cortex. *H*, Low-magnification image of Kcnq2 (left) and Kcnq3 (right) expression in the cerebellar cortex. *I–K*, Kcnq2 is located in the granule and Purkinje cell layer of the cerebellum. *I*, High-power bright-field image showing Kcnq2 expression in the cells of the Purkinje layer (PL) and granule layer (Gr) but not the molecular layer (ML). *J*, Same image as *I*, with immunodetection of PV-reactive Purkinje cells and interneurons of the molecular layer. *K*, Overlay of *I* and *J*, with Kcnq2 pseudocolored green. Scale bar (shown in *K*): *A*, *B*, 275 μ m; *C*, 250 μ m; *D–F*, 100 μ m; *G*, 300 μ m; *H*, 475 μ m; *I–K*, 50 μ m.

ysis of individual neuronal populations, but is likely to be extensive given the regional patterns observed in this study.

DISCUSSION

The M-current is a slow, non-inactivating K⁺ current, believed to be one of the most important modulators of the subthreshold excitability of neurons and their responsiveness to synaptic inputs (Brown, 1988). The K⁺ channels expressed in heterologous expression systems by subunits of the EAG family also have interesting properties (Table 3) and could have functional consequences similar to those of M-currents (Shi et al., 1997, 1998; Stansfeld et al., 1997; Meves et al., 1999; Saganich et al., 1999; Selyanko et al., 1999). Most EAG channels activate significantly at voltages close to physiological resting potentials and hence near or below the threshold for action potential generation (Table 3). Moreover, they have little or only incomplete inactivation. These low-threshold-activating channels could thus resemble M-channels in their ability to carry steady outward currents that can suppress the overall excitability of neurons and oppose action potential generation. Furthermore, the diversity in voltage-dependent and kinetic behavior, and perhaps distinct responses to neuromodulators, of the various channels of this group could provide neurons with divergent integrative properties and modulatory responses.

The presence of time-dependent relaxations of K⁺ currents after hyperpolarization from depolarized (more than −40 mV) holding potentials has been interpreted typically as indicating the presence of M-channels. However, the closing of different types of EAG channels can produce similar relaxations, albeit with different kinetics. Moreover, muscarinic agonists also inhibit eag

and erg channels (Stansfeld et al., 1996; Selyanko et al., 1999; Ludwig et al., 2000), demanding more detailed kinetic and pharmacological experiments to identify the channels mediating M-like currents in specific neurons. The distributions reported in this study will be an important aid in this process.

Roles for EAG potassium channels in the mammalian CNS remain to be found. However, the importance of these channels in the control of neuronal excitability in *Drosophila* (Wu et al., 1983; Ganetzky et al., 1999) and in human cardiac function (Curran et al., 1995; Sanguinetti, 1999) has been well established. The expression patterns shown here provide a framework to identify neurons in rodent brain where the roles of EAG family channels can be investigated.

Subunit composition of M-channels

It has been suggested that the channels mediating I_M in sympathetic neurons are heteromultimers of two members of the Kcnq family, Kcnq2 and Kcnq3 (Wang et al., 1998). When expressed alone, Kcnq2 channels produce small currents, whereas Kcnq3 proteins express negligible currents. It has been assumed that M-channels in the CNS have a subunit composition similar to that in sympathetic neurons, and therefore it was expected that cells expressing Kcnq3 subunits would also contain Kcnq2 proteins. Our results show that Kcnq2 and Kcnq3 transcripts indeed overlap in many neuronal populations, including neurons in which M-currents have been recorded [hippocampal pyramidal neurons (Madison and Nicoll, 1984); cortical layer V pyramidal cells (McCormick and Prince, 1986; Brown, 1988; Brown et al., 1990)]. However, to our surprise we also found that there are several neuronal populations that express one but not the other. For

Figure 11. Differential distributions of Kcnq2 and Kcnq3 transcripts in rat cerebral cortex. *A*, Cross section of rat cerebral cortex showing strong Kcnq2-positive neurons in layers II/III and V, and little or no signal in layer IV. *B*, High-power bright-field image showing layer II/III Kcnq2-positive neurons. *C*, Same image as *B*, with immunodetection of PV-reactive interneurons. *D*, Overlay of *B–D* with Kcnq2 pseudocolored green. Note that some Kcnq2 transcripts colocalize with PV-positive interneurons (*B–D*, arrows), and some do not (*B–D*, arrowheads). *E*, High-power bright-field image showing strong Kcnq2 signal in a large layer V pyramidal neuron. *F*, Same image as *E*, with immunodetection of PV-reactive interneurons. *G*, Overlay of *E* and *F* with Kcnq2 pseudocolored green. Note that most Kcnq2 transcripts did not colocalize with PV-positive interneurons (*E*, arrows). *H*, ISH for Kcnq3 showing labeling of neurons in cortical layers II–VI, with highest levels found in layer IV. *I–N*, Kcnq3 expression is not found in PV-positive cortical interneurons in cortical layer IV (*I–K*) or V (*L–N*). *I*, High-power image of Kcnq3-labeled neurons in cortical layer IV. *J*, Immunofluorescent detection of PV in *I*. *K*, Overlay of *I* and *J* with Kcnq3 pseudocolored green. *L*, High-power image of Kcnq3-labeled neurons in cortical layer V. *M*, Immunofluorescent detection of PV in *L*. *N*, Overlay of *L* and *M* with Kcnq3 pseudocolored green. Arrows in *I* and *L* point to Kcnq3-negative cells that were immunoreactive for PV. Scale bar (shown in *N*): *A*, *H*, 200 μm; *B–G*, *I–N*, 50 μm.

Figure 12. Erg1, Kcnq2, and Kcnq3 mRNA expression was often overlapping in the rat midbrain and hindbrain. 3, Oculomotor nucleus; 7, facial nucleus; 12, hypoglossal nucleus; Cu, cuneate nucleus; DC, dorsal cochlear nucleus; IC, inferior colliculus; IO, inferior olive; LDT, laterodorsal tegmental nucleus; LRt, lateral reticular nucleus; MG, medial geniculate nucleus; MVe, medial vestibular nucleus; Pn, pontine nucleus; RdN, red nucleus; SN, substantia nigra; Sp5, spinal trigeminal nucleus; VLL, ventral nucleus lateral lemniscus; VTg, ventral tegmental nucleus.

example, Kcnq2 (but not Kcnq3) is prominently expressed in cerebellar granule and Purkinje cells (Figs. 1, 10, Table 2). On the other hand, Kcnq3 is strongly expressed in layer IV in the cortex, where Kcnq2 signals are weak (Figs. 1, 11, Table 2). These

examples were particularly puzzling at the time when we completed these experiments, because the other two known members of the family, Kcnq1 and Kcnq4, either are not found in brain (Kcnq1) or are restricted to the brainstem auditory pathway

Table 3. Functional properties of EAG K⁺ channels in heterologous expression systems

	Activation							Inactivation		Pharmacology (IC ₅₀)		
	<i>V</i> _{on} (mV)	<i>V</i> _{1/2} (mV)	<i>k</i> (mV)	<i>τ</i> _{fast} (msec)	<i>τ</i> _{slow} (msec)	Cole– Moore shift	Deactivation <i>τ</i> _{deact} (msec)	<i>τ</i> _{inact} (msec)	<i>τ</i> _{recov} (msec)	TEA	4-AP	E-4031
Eag1	−60 to −50	−11.8 to −4.1	23.5	12 @ 40 mV ^a	210 @ 40 mV ^a	y	1–6	No inactivation		28	NB by 20 mM	NB
Eag2	−100	−35.5	29	14.6 @ 40 mV ^a	202 @ 40 mV ^a	y	Fast	No inactivation		19 @ 40 mV	NB by 20 mM	NB by 1 μM
Erg1	−60 to −50	−21	6 to 8	200 @ 0 mV		n	Seconds @ −70 mV	4.3 @ 30 mV	NA	Slows channel inactiva- tion	NA	<i>K</i> _D 99 nM
Erg2	−40	−3.5	8.3	111 @ 0 mV	500 @ 0 mV	n	Seconds @ −70 mV	4.7 @ 30 mV	NA	NA	NA	<i>K</i> _D 116 nM
Erg3	−80 to −70	−44	7.2	25 @ 0 mV		n	~100 @ −70 mV	8 @ 30 mV	NA	NA	NA	<i>K</i> _D 193 nM
Elk1	−40	9.3	13.1	676 @ 0 mV		n	111 @ −50 mV	No inactivation		NB by 10 mM	NB by 10 mM	NB by 10 μM
Elk2	−80 to −70	−6.4 to −24.4	20.1 to 28.3	7.7 @ 30 mV	70.0 @ 30 mV	n	<i>τ</i> _{fast} = 93.7 <i>τ</i> _{slow} = 622 @ −50 mV	10.2 @ 30 mV	6.7 @ −50 mV	Slows channel inactiva- tion	NA	NB by 10 μM
Elk3	−90	−59.1	10.8	38 @ 0 mV	360 @ 0 mV	n	201 @ −80 mV	No inactivation		NB by 100 mM	NB by 10 mM	NB by 10 μM

Data were obtained from Ludwig et al. (1994), Robertson et al. (1996), Terlau et al. (1996), Shi et al. (1997, 1998), Bijlenga et al. (1998), Engeland et al. (1998), Frings et al. (1998), Saganich et al. (1999), Schonherr et al. (1999), and Trudeau et al. (1999).

y, Yes; n, no; NA, not available; NB, not blocked; V_{on}, “activation” voltage; time constants at room temperature.

^a Holding potential = –90 mV.

(Kcnq4) (Coetzee et al., 1999; Kubisch et al., 1999; Kharkovets et al., 2000). While our experiments were in progress, a new Kcnq subunit, Kcnq5, was identified (Lerche et al., 2000; Schroeder et al., 2000). This subunit has also been shown to coassemble with Kcnq3 proteins and produce M-channels *in vitro*, suggesting that it contributes to the formation of M-channels in brain. Moreover, Kcnq5 mRNAs are strongly expressed in the neocortex (Schroeder et al., 2000). It is thus possible that Kcnq5 forms heteromultimeric M-channels with Kcnq3 in cortical layer IV neurons. More puzzling at present is the situation in the cerebellar cortex, where Kcnq5 is expressed very weakly (Schroeder et al., 2000).

Heteromultimeric EAG-family channels

Recent evidence shows that different members of one of the EAG subfamilies (erg) can heteromultimerize to form channels with novel electrophysiological properties when coexpressed in Chinese hamster ovary cells (Wimmers et al., 2001). Furthermore, erg subunits do not seem to coassemble with eag or elk proteins (Wimmers et al., 2001). Further investigation of the ability of EAG family members to heteromultimerize within and between different subfamilies is still necessary to uncover the “rules” that govern EAG channel assembly; however, it is quite possible that a situation similar to that observed in the Kv family of K⁺ channels will emerge in the EAG family. In the Kv family, members of the same subfamily, but not of different subfamilies, can form heteromeric channels in heterologous expression systems, and heteromeric complexes have been shown to exist in brain tissue (Coetzee et al., 1999). Our data show overlap of multiple members of the same EAG subfamily in the same neuronal population in which they may form heteromeric channels. Moreover, different combinations of subunits of a given EAG subfamily are found in different neuron types. For example, both erg1 and erg3 transcripts are expressed in cerebellar Purkinje cells and the neurons of the reticular thalamus, but erg1 is found alone in the basket cells of the hippocampus (Figs. 1, 5, 6, Table 2). Therefore, as in the case of Kv channels, the subunit compo-

sition (and perhaps the functional properties) of EAG channels containing a particular subunit, could vary between different neurons (Weiser et al., 1994; Coetzee et al., 1999).

Multiple channels may contribute to the subthreshold K⁺ current in many CNS neurons

This study showed that many neurons contain transcripts for multiple subthreshold EAG and/or Kcnq channels. For example, on the basis of the observation that most layer V pyramidal neurons contained Kcnq2, Kcnq3, eag1, erg1, and erg3, it is very likely that all of these transcripts are coexpressed in many of these neurons. Pyramidal cells in the hippocampus also coexpressed multiple EAG and Kcnq mRNAs, as did neurons in several other brain areas (see Table 2, and the appropriate sections in Results). In contrast, other neurons, such as inhibitory interneurons in the hippocampus, had only a single member (erg1), as judged by colocalization with inhibitory cell markers.

Although it still remains to be shown that the protein products are expressed and localized in somatodendritic membrane, this overlap of multiple EAG and Kcnq transcripts suggests the possibility that the voltage-dependent subthreshold K⁺ current of many neurons may include the contribution of different components, produced by channels with different properties, including distinct responses to neuromodulators. The situation in many CNS neurons may resemble that recently described for the native M-like currents in neuroblastoma NG108–15 cells. The I_M of these cells was shown to include a Kcnq2–Kcnq3-mediated component resembling the M-current in sympathetic neurons, and a slower component probably mediated by channels containing erg proteins (Meves et al., 1999; Selyanko et al., 1999).

Of additional interest was the observation that cortical inhibitory interneurons seem to express few members of the EAG or Kcnq family in comparison to local and projecting excitatory neurons. The only clear exception was erg1, which seemed to be prominent in populations of inhibitory interneurons in several brain areas, including neocortex and hippocampus. Differences in

the expression and properties of M-channels and other subthreshold currents in inhibitory interneurons compared with excitatory neurons could result in markedly different susceptibilities to subthreshold modulation of excitability.

The complexity of subthreshold K⁺ currents in neurons is further increased by currents from inward rectifiers (particularly those displaying weak rectification) and “leak” K⁺ channels composed of proteins of the recently discovered tandem or two-pore K⁺ channel family (Goldstein et al., 1998). The window current of subthreshold-activating A-type K⁺ currents and the contributions from Kv1 channels showing sufficient activation in the subthreshold voltage range (such as those mediating the slowly and incompletely inactivating D current), and under some conditions calcium-activating K⁺ currents, can also contribute to this complexity. How these different channels impact neuronal excitability remains to be explored. This diversity may be partially associated with differential subcellular localization of the channels (Cooper et al., 2000). This is an issue of great importance and will require the development of specific antibodies to determine the localization of protein products. For example, localization of multiple subthreshold operating channels with diverse kinetics and voltage responses in compartments receiving synaptic inputs provides a substrate for tuning cells to differentially filter synaptic inputs. An analogous role has already been described for A-currents on AMPA versus NMDA responses (Schoppa et al., 1999). Distinct subthreshold-operating channels may also respond differently to neuromodulators, allowing specific temporal and spatial control of the membrane impedance and the resting potential.

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