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# Expression of Kv2.1 delayed rectifier $K^+$ channel isoforms in the developing rat brain

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Mammalian central neurons regulate a diverse set of functions by changes in spatial and temporal expression patterns of a large family of $K^+$ channel gene products. Here the temporal patterns of expression of Kv2.1 (drk1) $K^+$ channel mRNAs and polypeptides have been studied in rat brain. Northern blots using Kv2.1-specific probes show that three size classes (4.4, 9.0, 11.5 kb) of Kv2.1 $K^+$ channel transcripts are present in rat brain. These mRNAs show differential patterns of expression over embryonic and postnatal developmental, with the 4.4 kb transcript being the predominant embryonic Kv2.1 mRNA, and the 11.5 kb transcript being the predominant adult mRNA. Immunoblots using Kv2.1-specific site-directed antibodies show the existence of multiple Kv2.1 polypeptides, which differ in their mobility on SDS polyacrylamide gels, in their immunoreactivity to a carboxyl terminal-directed anti-Kv2.1 antibody, and in their developmental expression. Thus Kv2.1 polypeptide isoforms exhibit discrete temporal patterning during neuronal development, implying distinct roles for these channel proteins in vivo.

$K^+$ channel, Rat brain; Developmental expression; Northern blotting; Immunoblotting; Polypeptide isoform

## INTRODUCTION

Voltage-sensitive $K^+$ channels are proteins intrinsic to the plasma membranes of excitable cells and are fundamental components in the control of neuronal excitability [1]. Within neurons, these channels underlie the determination of the resting potential, the shaping of the action potential, the modulation of transmitter release, and the regulation of rhythmic firing patterns; thus regulation of $K^+$ channel activity is of major physiological and pathological importance [2]. During development, $K^+$ currents are the first voltage-gated currents to appear in embryonic neurons [3]. Diversity of $K^+$ channels increases dramatically during development such that more than a dozen physiologically distinct $K^+$ currents have been observed in adult rat central neurons, with many neurons exhibiting more than one current type [2]. In addition, there may be specific changes in the properties of $K^+$ channels within a class during neuronal development [4]. The pattern of expression of specific classes of $K^+$ currents varies substantially among neurons isolated from either different regions of the brain, or from a given region at different stages of development [2]. The molecular bases for the physiological and pharmacological distinctions between these distinct neuronal $K^+$ conductance pathways is not yet understood.

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Studies of voltage-sensitive $Na^+$ and $Ca^{2+}$ channels have been greatly facilitated by the availability of specific agonists and antagonists. The lack of such naturally occurring high affinity probes for $K^+$ channel polypeptide components, combined with the diversity of $K^+$ channels, even within a given tissue, has prevented direct biochemical studies and determination of anatomical localization of most $K^+$ channel types, however, cDNAs encoding a number of $K^+$ channel polypeptides have been isolated over the past 3 years (reviewed in [5,6]). Using these cDNAs as probes, levels of $K^+$ channel transcripts have been found to change dramatically during development [7–9]. Duplication and divergence of $K^+$ channel genes, and alternative splicing of single gene products, have each been shown to play a role in generating mammalian $K^+$ channel diversity [10]. The extent to which post-translational processes may contribute to additional diversity is not yet known.

The recently isolated Kv2.1 cDNAs have been used to express fragments of the encoded channel polypeptides for use as immunogens [11]. Production of antibodies specific for $K^+$ channel subtypes defined in molecular cloning studies in many ways circumvents the problem of the lack of naturally occurring toxins and ligands, providing tools to address questions of the structure, abundance and distribution of $K^+$ channel polypeptides. Here cDNA and antibody probes specific for the Kv2.1 (drk1) $K^+$ channel have been used to characterize the temporal patterns of expression of Kv2.1 transcripts and polypeptides in the developing rat brain.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

$^{125}$I-labeled protein A was from ICN (Irvine, CA). [$^{32}$P]dCTP was from NEN-DuPont (Wilmington, DE). Nitrocellulose paper was from Bio-Rad (Mellville, NY). Other reagents, unless noted otherwise, were molecular biology grade from Sigma (St. Louis, MO) or Boehringer-Mannheim (Indianapolis, IN).

### 2.2. *RNA preparation and Northern blot analysis*

RNA was prepared and quantified by ultraviolet absorbance at 260 nm and by densitometry of photographs of gels after staining in ethidium bromide as described [12]. For Northern blots, total RNA (10 $\mu$g) was denatured at 65°C in loading buffer containing 50% formamide, and size fractionated on denaturing 0.8% agarose gels containing 6% formaldehyde. After transfer to nylon membranes (Hybond N, Amersham, Arlington Heights, IL) and UV crosslinking, immobilized RNA was hybridized overnight with a random primed $^{32}$P-labelled cDNA fragment derived from the Kv2.1 coding region (corresponding to nucleotides 387–900, encoding amino acids 129–300) or 3′-UT (corresponding to nucleotides 2,588–2,929, all in the 3′-UT) at 42°C in standard hybridization solution containing 50% formamide, 10% dextran sulfate and 0.5% SDS. Washes were performed at 68°C in 0.1 × SSC/0.1% SDS.

### 2.3. *Brain membrane preparations and immunoblotting*

A crude synaptosomal membrane fraction [11] was prepared from freshly dissected rat brains from ages E14, E18, P1, P3, P7, P12 and P90 (adult). A description of the generation and analysis of the anti-Kv2.1 antibodies, KC and pGEX-drk1, and immunoblotting procedures can be found in [11].

## 3. RESULTS

### 3.1. *Expression of Kv2.1 mRNAs changes during development*

Northern blot analyses on RNA samples isolated from the brains of different aged rats show the existence of multiple Kv2.1-related mRNAs. Three size classes of hybridizing mRNA, of 11.5, 9.0 and 4.4 kb, are detected with probes derived from either the 5′ portion of the coding region (Fig. 1) or from the 3′-UT (not shown). The 4.4 kb mRNA is the major Kv2.1-hybridizing transcript present in E14 rat brain (50% of total hybridization signal, Fig. 2, Table I). The levels of this transcript increase slightly but steadily through embryonic and postnatal development, such that adult levels are approximately 2.5 × higher than those found at E14 (Figs. 1 and 2; Table I). In contrast, the level of the 11.5 kb transcript changes more dramatically during development (Fig. 1), with a 6.5 × increase between E14 and P30 (Fig. 2; Table I). The 11.5 kb transcript is present at very low levels in embryonic brain (25% of total hybridization signal), rises dramatically during the first postnatal week, and more gradually thereafter, such that in adult (P30) brain it is the major Kv2.1-hybridizing mRNA (46% of the total Kv2.1 signal). A third transcript of 9.0 kb is also detected; levels of this transcript do not change significantly during development and never exceed 25% of the total hybridization signal. While the relative levels of the three mRNAs hybridizing with these Kv2.1 probes at high stringency change dramati-

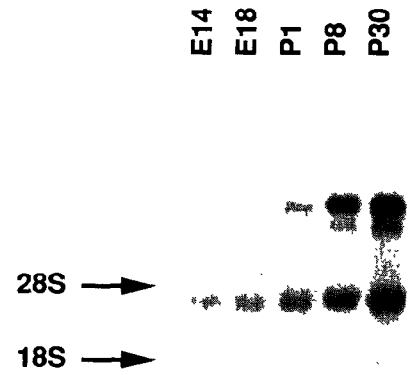


Fig. 1. Northern blot analysis of Kv2.1 transcripts in developing rat brain. Total RNA (10 $\mu$g) isolated from the brains of different aged rats was size fractionated on a 0.8% denaturing agarose gel, transferred to nitrocellulose and probed with a random primed Kv2.1-specific cDNA probe. Hybridization and washes were performed under high stringency conditions, using a probe from the coding region of Kv2.1. This probe yields a somewhat complex pattern of hybridization, with hybridizing transcripts at 11.5, 9.0 and 4.4 kb. The 4.4 kb mRNA is the major hybridizing transcript in embryonic brain, while the 11.5 kb transcript is the major hybridizing transcript in adult brain. 28 S and 18 S arrows show the position of ribosomal RNAs identified by ethidium bromide staining.

Table I

Changes in rat brain Kv2.1 mRNA during development

| Age | 4.4 kb mRNA | 9.0 kb mRNA | 11.5 kb mRNA | Total mRNA |
|---|---|---|---|---|
| E14 | 41 ± 10[a] (50%)[b] 1 ×[c] | 21 ± 13 (25%) 1 × | 21 ± 7 (25%) 1 × | 83 1 × |
| E18 | 49 ± 16 (52%) 1.2 × | 21 ± 12 (22%) 1.0 × | 24 ± 10 (26%) 1.1 × | 94 1.1 × |
| P1 | 60 ± 16 (47%) 1.5 × | 28 ± 15 (22%) 1.3 × | 39 ± 14 (31%) 1.8 × | 127 1.5 × |
| P8 | 81 ± 18 (35%) 2.0 × | 36 ± 9 (16%) 1.7 × | 112 ± 9 (49%) 5.3 × | 229 2.7 × |
| P30 | 100 (35%) 2.5 × | 55 ± 2 (19%) 2.6 × | 132 ± 12 (46%) 6.3 × | 287 3.5 × |

[a]Mean of three independent experiments, ± S.E.M.
[b]Percent of total Kv2.1 hybridization signal in that age.
[c]Fold increase in hybridization signal over E14 level.

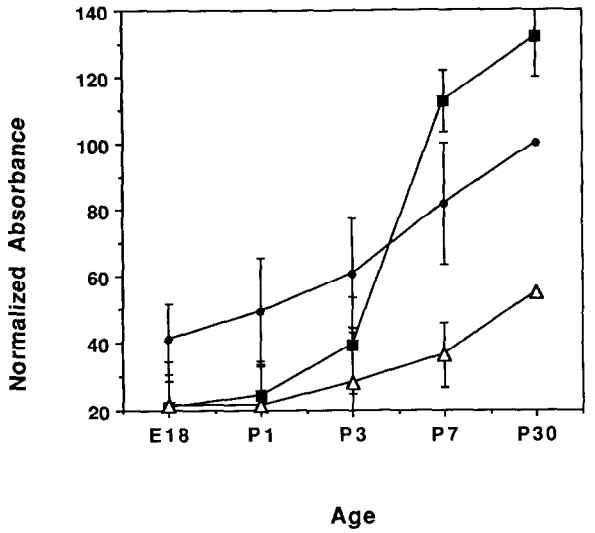


Fig. 2. Time course of developmental changes in the three size classes of Kv2.1 mRNAs. RNA was isolated from the brains of rats of different ages, and analyzed by Northern blots as in Fig. 1. The hybridization signal was quantified by densitometry. Means and standard deviations from three independent experiments are shown. ■, 11.5 kb Kv2.1 mRNA; ●, 4.4 kb Kv2.1 mRNA, △, 9.0 kb Kv2.1 mRNA.

cally during development, no obvious differences are seen in the pattern of Kv2.1 transcripts when RNAs isolated from different regions of adult brain are analyzed in a similar manner (data not shown).

### 3.2. *Developmental accumulation of Kv2.1 polypeptide isoforms*

Crude rat brain synaptosomal membranes were prepared in parallel from siblings of those used to prepare RNA for the assays described above. Equal amounts of membrane protein were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with two different, site-specific anti-Kv2.1 antibodies [11]. These antibodies were made to two different sites on the large putative cytoplasmic tail of the Kv2.1 polypeptide, one site just downstream of the last membrane-spanning segment ('pGEX-drk1', amino acids 506–533) and one corresponding to the C-terminal 17 amino acids of the predicted Kv2.1 polypeptide ('KC', amino acids 837–853). These affinity-purified anti-Kv2.1 antibodies show a specific reaction to the Kv2.1 polypeptide on these immunoblots of adult brain membranes; no specific staining was seen with preimmune serum from either rabbit or in the presence of competing recombinant fusion proteins containing the appropriate Kv2.1 sequences [11].

Strong immunoreactivity with the pGEX-drk1 antibody is present on immunoblots in samples from all embryonic and postnatal ages tested, from E14 to adult (Fig. 3A). In E14 brain, the major species of Kv2.1 polypeptide identified with the pGEX-drk1 antibody is an $M_r$ = 110 kDa moiety; a minor band of $M_r$ = 120 kDa is also present. With continuing prenatal development, the amount of the $M_r$ = 110 kDa Kv2.1 polypeptide stained with pGEX-drk1 antibody decreases coincident with an increase in the amount of the $M_r$ = 120 kDa form of the polypeptide. At postnatal ages, the $M_r$ = 120 kDa Kv2.1 polypeptide is less prominent, while an isoform of $M_r$ = 130 kDa becomes the major pGEX-drk1 immunoreactive species. This $M_r$ = 130 kDa Kv2.1 polypeptide is the major species reacting with the pGEX-drk1 antibody in adult rat brain [11].

Results are substantially different when immunoblots are performed on samples from embryonic and newborn rat brain with the KC antibody (Fig. 3B). The $M_r$ = 110 kDa species, which exhibited prominent immunoreactivity with the pGEX-drk1 antibody in embryonic brain preparations, is not recognized by the KC antibody. The $M_r$ = 120 kDa and $M_r$ = 130 kDa polypeptides seen in pGEX-drk1 immunoblots are detected with the KC antibody. Because of the lack of reactivity to the major embryonic species of $M_r$ = 110 kDa, only low levels of KC immunoreactivity are present in the E14, E18, P1 and P3 samples; it increases in the P7 sample and rises dramatically in the second postnatal week to approach adult values by P12. The pGEX-drk1 and KC antibodies exhibit similar, if not identical patterns of immunoreactivity to the adult brain samples, as shown previously [11]. These immunoblot results indicate that multiple Kv2.1 polypeptide isoforms are present in developing and adult rat brain. These isoforms share common sequences in the region of pGEX-drk1 antibody binding, but differ at their carboxyl terminus, the site recognized by the KC antibody. The developmental expression of these isoforms is differentially regulated, the embryonic and early postnatal forms being pGEX-drk1 positive, KC negative, while the adult forms exhibit positive immunoreactivity with both site-specific antibodies.

## 4. DISCUSSION

These studies are aimed at relating Kv2.1 $K^+$ channel subtypes defined by molecular cloning studies with the wide variety of physiologically important $K^+$ conductance pathways in the CNS by analyzing the temporal and spatial distribution of specific subtypes in the adult and developing central nervous system. In this study, cDNA and antibody probes derived from the Kv2.1 cDNA have been used to investigate the corresponding transcripts and polypeptide isoforms in rat brain.

Kv2.1 transcripts can be detected in rat brain as early as embryonic day 14 (E14). Relatively high levels of Kv2.1 transcripts are present in brain at E14, a period when neuronal precursors in a number of brain regions are beginning to withdraw from mitosis and differentiate into neurons [13]. Among the $K^+$ channel transcripts that have been so characterized, Kv2.1 and Kv1.4 [7]

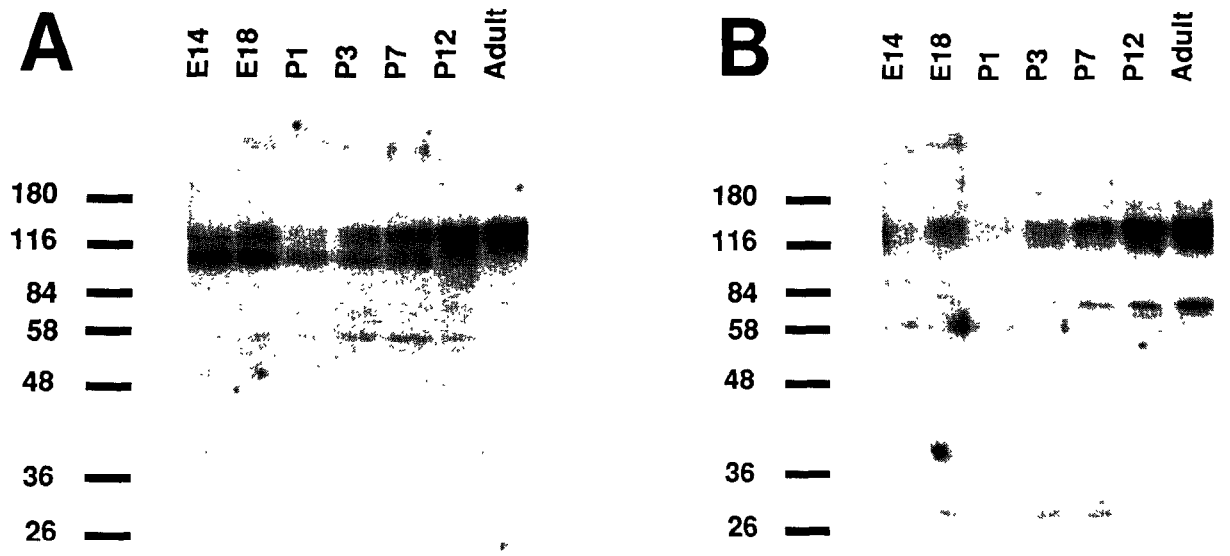


Fig. 3. Western blots of brain membranes isolated from different aged animals. 75 $\mu$g of crude rat brain membranes were fractionated on 10% SDS gels, transferred to nitrocellulose and probed with pGEX-drk1 (A) or KC (B) antisera. Note additional population of $M_r$ = 110 kDa Kv2.1 polypeptide detected by pGEX-drk1, but not KC, antibodies. Reactive bands on pGEX-drk1 blot at $\approx$ 60 kDa, and on KC blot at $\approx$ 30 and 80 kDa are not blocked by competing peptide, and thus are not related to the Kv2.1 polypeptide Numbers denote relative molecular mass of standard proteins.

are unique in their expression at these early time periods in the CNS. Kv2.1 transcripts differ from Kv1.4 in that they remain elevated throughout the adult brain, such that Kv2.1 remains a predominant $K^+$ channel transcript in this adult tissue, while Kv1.4 mRNA levels were found to decline postnatally in hindbrain [7].

Recently, cDNAs encoding a second member of the mammalian *Shab* subfamily, termed Kv2.2 ('CDRK' in the original publication) have been isolated [14]. The mRNA and polypeptide encoded at the Kv2.2 gene closely resemble Kv2.1, (66% nucleotide identity, 70% amino acid identity). Is it possible that some of the multiple mRNAs and polypeptides observed in our analyses of Kv2.1 could be due to the interfering presence of Kv2.2 gene products? A number of lines of evidence argue against but do not totally exclude this possibility. First, while multiple size classes of Kv2.2 mRNAs also exist in adult rat brain [14], the sizes of these mRNAs (13.5 and 6.2 kb) do not correspond to the sizes of the Kv2.1 mRNAs observed in our analyses (11.5, 9.0 and 4.4 kb). In addition, the Kv2.1 and Kv2.2 mRNAs in adult brain differ in their spatial distribution [14]. Southern blot analysis shows that cross-hybridization of Kv2.1 and Kv2.2 probes occurs at low stringency, but totally non-overlapping patterns of hybridization of Kv2.1 and Kv2.2 are seen at the high stringency conditions used for both their [14] and our (Fig. 1) Northern blot analyses. Based on these results, it is unlikely that the mRNA bands on the high stringency Northern blots presented here (Fig. 1) represent hybridization to Kv2.2 transcripts. These may represent alternative Kv2.1 transcripts, or products of mRNAs arising from another *Shab* subfamily gene (i.e. Kv2.3) more similar to Kv2.1 than is Kv2.2. Isolation of the cDNAs corresponding to each of the three size classes of developmentally regulated Kv2.1-related mRNAs will ultimately clarify this issue.

The Kv2.2 cDNAs predict an encoded polypeptide of 90.6 kDa (vs. 95.3 kDa for Kv2.1). Could the smaller polypeptides seen on our immunoblots with the pGEX-drk1 antibody be due to the presence of the smaller Kv2.2 polypeptide in embryonic brain? A comparison of the amino acid sequences of the Kv2.1 and Kv2.2 polypeptides in the region of the pGEX-drk1 immunogen reveal that there exists 50% identity between the two sequences. Thus it is possible that this antibody, but not the KC antibody (directed to a sequence not present in Kv2.2) would cross-react with Kv2.2 polypeptide. While the developmental patterns of expression of Kv2.2 are not known, in adult brain the most prominent in situ hybridization signals are obtained in the cerebellum, the major development of which proceeds postnatally in the rat. The fact that the smaller pGEX-drk1$^+$, KC polypeptide is seen predominantly in embryonic brain and is not detectable in adult brain argues against, but does not eliminate, this possibility. If the lower $M_r$

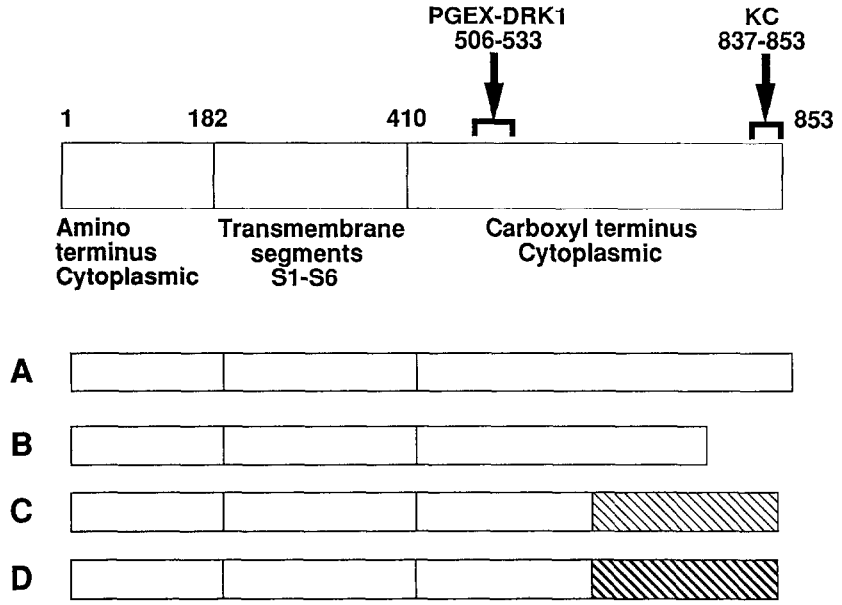


Fig. 4. Model of primary structures of putative Kv2.1 isoforms in rat brain. Cartoons showing a linear representation of possible Kv2.1 polypeptide isoforms. (A) The location of the pGEX-drk1 and KC antibody binding sites on the adult rat brain Kv2.1 polypeptide. (B–D) Alternative models for the major embryonic brain Kv2.1 polypeptides. In B, the pGEX-drk1$^+$, KC Kv2.1 polypeptide isoform is depicted as a truncated form of the adult Kv2.1 polypeptide. In C, this embryonic form exists as an alternatively spliced product that contains a different carboxyl terminus. In D, the isoforms are encoded by different but highly related genes that share highly conserved sequences in the region of the pGEX-drk1 antibody, but diverge in the region of the KC antibody.

pGEX-drk1 immunoreactive polypeptides present in embryonic rat brain do, in fact, represent Kv2.2, it means that these $K^+$ channel polypeptides have an early appearance in development that may quantitatively overshadow their adult expression, as reported [14]. A detailed analysis of the developmental patterns of expression of the Kv2.2 polypeptide will lead to further insights as to the temporal patterning of different members of the mammalian *Shab* subfamily in the developing rat brain.

Kv2.1 transcripts in brain are found in three size classes, two of which (the 11.5 and 4.4 kb mRNAs) are major compared to the third (9.0 kb). The differences in nucleotide sequence between these multiple size classes of Kv2.1 mRNAs is not yet known, and relies upon the future isolation of cDNAs corresponding to each of the alternative Kv2.1 mRNA isoforms. The fact that identical hybridization patterns were obtained on Northern blots probed at high stringency with both coding and 3′-UT cDNA probes suggests, but does not prove, that the multiple size classes were transcribed from the same gene. Alternative start or polyadenylation sites, or alternative splicing of a single nascent Kv2.1 mRNA, could all occur in a developmentally regulated manner and contribute to the size differences observed in the Kv2.1 mRNAs. Alternative splicing of a single nascent $K^+$ channel transcript has been shown to give rise to the Kv3.1$\alpha$ (Kv4) and Kv3.1$\beta$ (NGK2) mRNAs [10]. These alternatively spliced mRNAs exhibit different developmental patterns of expression in rat brain [15]. Isolation and analysis of cDNAs corresponding to each of the Kv2.1 mRNAs will allow for further insights into the neuronal mechanisms important in the developmental regulation of $K^+$ channel expression and diversity.

The primary structure of all the Kv2.1 polypeptides encoded by the three different size classes of Kv2.1 mRNAs is also not known; however, the observed differential immunoreactivity of the isoforms to the site-specific pGEX-drk1 and KC antibodies, and the primary structure derived from the original cloning of the Kv2.1 cDNA from adult brain [15] allow us to make some predictions about the major embryonic and adult isoforms. Fig. 4 shows one possible schematic model of the primary structure of the adult Kv2.1 polypeptide isoform (A), derived from the original Kv2.1 sequence [15] and consistent with our immunoreactivity data. Two possible alternative structures (B and C) for the major embryonic rat brain Kv2.1 polypeptide isoforms are also presented. These models are consistent with the observed differences in immunoreactivity between the embryonic and adult isoforms. All of the Kv2.1 polypeptide isoforms are recognized by the pGEX-drk1 antibody, thus the models are shown to be identical in this region. The embryonic isoforms (B and C) are not recognized by the KC antibody, indicating that they differ from the adult isoform at the carboxyl terminus. This difference could be due to the presence of a trun-

cated carboxyl terminus (form B) or an alternative carboxyl terminus (form C). The exact nature of the differences between the adult and embryonic Kv2.1 isoforms will have to be resolved through the isolation of cDNAs corresponding to the alternative embryonic isoforms.

What then is the correlation between the developmentally regulated Kv2.1 polypeptide isoforms seen on immunoblots and the developmentally regulated mRNAs seen on Northern blots? The relative proportion of the two Kv2.1 polypeptide isoforms changes dramatically during development, such that the $M_r$ = 110 kDa population is prevalent in embryonic brain, while the $M_r$ = 130 kDa polypeptide is the major form in adult brain. Likewise, the smaller Kv2.1 mRNA (4.4 kb) is the major embryonic isoform, while the largest (11.5 kb) is the major adult isoform. The simplest relationship between these results is that the smaller (4.4 kb) Kv2.1 transcript encodes the smaller ($M_r$ = 110 kDa) Kv2.1 polypeptide, as both are the predominant Kv2.1 species expressed in embryonic brain. Conversely, the expression of the larger transcript (11.5 kb) and polypeptide ($M_r$ = 130 kDa) species parallel, both being low in embryonic brain and rising during postnatal development to become the major Kv2.1 species in adult brain. Based on these data it seems likely that the 11.5 kb transcript encodes for the polypeptide containing the KC epitope that was defined in the original Kv2.1 cloning studies [15], depicted as form A in Fig. 4. One piece of data that is inconsistent with this correlation is the fact that the Kv2.1 cDNA was originally isolated from an adult rat brain library prepared from mRNA that had been size-selected for 3.3–4.2 kb transcripts [15]. It is possible, however, that some of the 11.5 kb Kv2.1 mRNA contaminated the lower molecular weight size fractions during isolation. The eventual cloning, sequencing and expression of cDNAs representing each of the three Kv2.1 transcript classes will allow for a direct determination of the specific differences between the respective encoded polypeptides, and the channel complexes of which they are components.

### 4.1. *Correlation with neuronal* $I_K$

Kv2.1 is thought to be a component of a delayed rectifier-type $K^+$ channel, based on the properties of currents expressed from homotetrameric Kv2.1 channels expressed in *Xenopus* oocytes [15]. Its expression early in development corresponds temporally to the appearance of $I_K$ as the first voltage-dependent ionic current to appear in developing neurons [3]. It may be that Kv2.1 is a component of a similar, early-occurring $K^+$ conductance pathway. Slowly inactivating, TEA-sensitive, outward currents have been observed in cultured embryonic neurons from hippocampus [16–19], cerebellum [4], cortex [20], and diencephalon [21]. In each of these cases, these $I_K$-like currents are the first current present in these cultured embryonic neurons; expression is maintained with time in culture. The expression of Kv2.1 in distinct subpopulations of neurons during development may be important in determining their electrical properties. Spitzer [3] has proposed that $I_K$ plays a determining role in the maturation of the neuronal action potential, which in turn may influence the cells synaptic connectivity and morphology. It is interesting to speculate that changes in Kv2.1 expression may play a role in the emergence of these adult patterns. Future studies utilizing such neuronal cultures may help to determine if the emergence of Kv2.1 isoform-specific immunostaining correlates with the appearance of a distinct $I_K$-like current in these developing neurons. Antibodies such as KC and pGEX-drk1 may also prove to be useful in identifying Kv2.1-derived currents via antibody-mediated channel blockade.

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