The KCNQ2 potassium channel: splice variants, functional and developmental expression. Brain localization and comparison with KCNQ3

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Abstract Benign familial neonatal convulsions, an autosomal dominant epilepsy of newborns, are linked to mutations affecting two six-transmembrane potassium channels, KCNQ2 and KCNQ3. We isolated four splice variants of KCNQ2 in human brain. Two forms generate, after transient expression in COS cells, a potassium-selective current similar to the KCNQ1 current. L-735,821, a benzodiazepine molecule which inhibits the KCNQ1 channel activity (EC_{50} = 0.08 μM), also blocks KCNQ2 currents (EC_{50} = 1.5 μM). Using in situ hybridization, KCNQ2 and KCNQ3 have been localized within the central nervous system, in which they are expressed in the same areas, mainly in the hippocampus, the neocortex and the cerebellar cortex. During brain development, KCNQ3 is expressed later than KCNQ2.

Key words: Potassium channel; Benign familial neonatal convulsion; Splice variant; In situ hybridization

1. Introduction

KCNQ1 (KvLQT1) [1] was the first characterized member of a new group of six-transmembrane domain potassium channels, the KCNQ family. Recently two other channels belonging to this family were cloned, KCNQ2 [2] and KCNQ3 [3]. All three KCNQ members are associated with inherited diseases.

KCNQ1, which, in association with a regulatory protein, KCNE1 (IsK), produces the cardiac slow delayed rectifier (I_{Ks}) current, [4,5] is responsible for inherited cardiac long QT disorders [1,6]. These syndromes are the result of mutations in the KCNQ1 gene which alter the functionality of this channel [7–9]. KCNQ2 and KCNQ3 are both linked to benign familial neonatal convulsions (BFNC). BFNC is an autosomal dominant idiopathic epilepsy. Typically, seizures start within the first week of life and disappear spontaneously after several weeks or months. Subsequent child development is normal. However, 10–15% of patients develop seizures later in life. Investigations of pedigrees of families affected by BFNC have revealed that single mutations, deletions or insertions in KCNQ2 or KCNQ3 genes are tightly associated with this disease [2,3,10]. When expressed in *Xenopus* oocytes KCNQ2 produces a K^+ current similar to the KCNQ1 currents [10,11].

In this study, we describe the cloning of four splice variants of KCNQ2 from human brain and we analyze the functional expression and the pharmacological profile of this channel type. In situ hybridization shows that both KCNQ2 and KCNQ3 are expressed in the same brain areas.

2. Materials and methods

2.1. Amplification

Human brain cDNAs were used to amplify KCNQx with the Advantage GC cDNA polymerase kit (Clontech). Amplifications were performed with the following profile: 94°C, 1 min, 65°C, 1 min, and 68°C, 4 min, 35 cycles. The primers used for KCNQ2 were designed from the clone published by Biervert et al. [10] (GenBank database accession number Y15065): K2M 5′-CCTCCGAATTCCCACCATGGTGCAGAAGTC-3′, K2T 5′-GTCCACTGGTCTAGAGCCGCCTCACTTCCT-3′.

For KCNQ3, primers K3.1 and K3.2 were designed from GenBank clones, accession numbers R36327 and R49258 respectively: K3.1 5′-CGAAGACATGATCCCCACCCTGAA-3′, K3.2 5′-TGGGGCCACCACGCACACGCATGC-3′. Insert K3.1-K3.2 encodes the stop codon. PCR products were subcloned into pGEMT-easy vector (Promega) and sequenced. Clones retained for expression were subcloned into the pSI mammalian expression vector (Promega). The integrity of the different pSI-KCNQx was checked by sequencing.

2.2. Electrophysiological measurements in transfected COS cells

COS-7 cells (American Type Culture Collection, Rockville, MD, USA) were seeded at a density of 20 000 cells per 35 mm diameter Petri dish 24 h prior to transfection, and transfected using the classical DEAE-dextran precipitate method with 0.5 μg or 1 μg (in the coexpression experiments) of pSI-KCNQx per dish. Cells were tested in electrophysiology 48 h after transfection.

Electrophysiological recordings were carried out at 22 ± 2°C in the whole-cell configuration of the patch-clamp technique [12]. The intracellular pipette filling solution contained 150 mM KCl, 0.5 mM $MgCl_2$, 5 mM EGTA and 10 mM HEPES/KOH at pH 7.2 and the standard extracellular solution contained 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES/NaOH at pH 7.4. Drug solutions were freshly prepared from stock solutions.

2.3. Northern blots

Northern blots of human tissues were from Clontech. Membranes were probed in Xpress buffer as described by the manufacturer (Clontech). Blots were exposed on Kodak XAR film for 30–40 days. Probes used were a 402 bp insert corresponding to amino acids 167–301 of KCNQ2 which is common to all isoforms, and a 1092 bp fragment corresponding to the sequence upstream of the stop codon of KCNQ3. Poly(A)$^+$ RNAs, isolated from the brains of 3 day old, 1 week old, 1 month old and adult mice, were blotted as described previously [13] and probed with the human KCNQ2 and KCNQ3 DNA probes used for human Northern blots. For quantification, blots were exposed for 18 h with an imaging plate (Fuji) for a photo imager (Fuji BAS 1500).

2.4. In situ hybridization

Antisense and sense RNA probes were generated from linearized plasmids containing, respectively, a 706 bp fragment of KCNQ2 cDNA in the 5′-coding sequence (corresponding to amino acids 101–336) and the 1092 bp fragment of KCNQ3 cDNA. Tissue preparation and hybridization were performed as described in [14]. The hybridization temperature was 50°C. Specimens were exposed to Amersham β-max Hyperfilm for 10–15 days.

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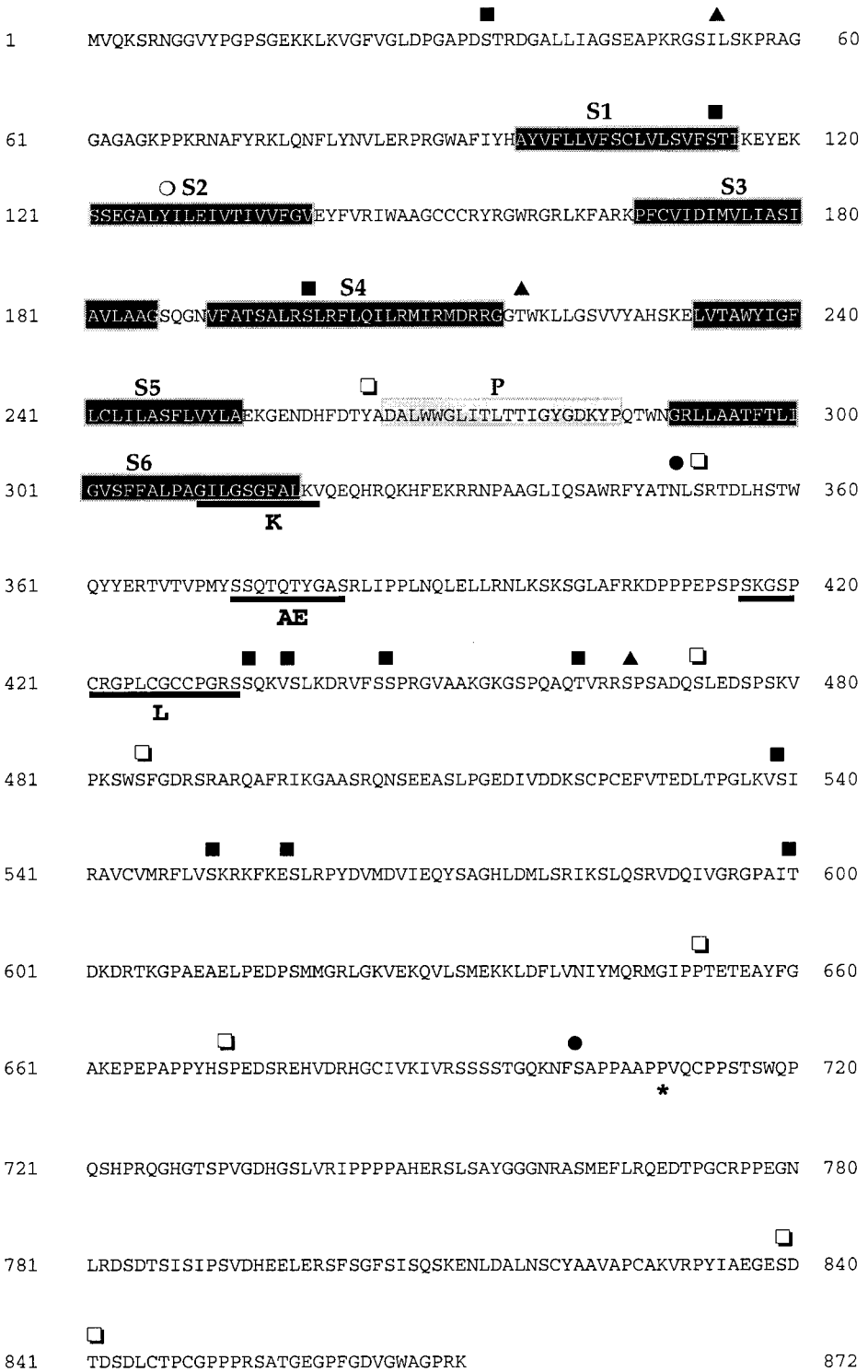

Fig. 1. Amino acid sequence of the K2KL form of KCNQ2. Transmembrane domains are boxed in black. The pore domain, P, is boxed in gray. Particular exons, K, L and AE, are underlined; AE, additional exon described in [10]. The K2ΔLMP amino acid sequence change due to the point mutation is indicated by an asterisk. Using the Prosite server with the ppsearch software based on the Mac Pattern program we identified *N*-glycosylation sites (●), and phosphorylation sites of protein kinase A (▲), protein kinase C (■), casein kinase II (□) and tyrosine kinase (○).

3. Results

3.1. KCNQ2 splice variants

From the known sequence of KCNQ2 [10] we chose two oligonucleotides, K2M including the start codon and K2T including the stop codon. Using these oligonucleotides on human brain cDNAs we amplified four different species of cDNA encoding KCNQ2 variants. The first one, herein called K2ΔL, corresponded to the KCNQ2 sequence published by Biervert et al. [10]. It contains the sequence that these authors

called the additional exon, corresponding to amino acids 373–382 (Fig. 1). Actually this additional exon was present in all our clones. Two other KCNQ2 forms, K2KL and K2ΔKΔL, corresponded to splice forms of K2ΔL. The K2KL form is characterized by the presence of exon L, corresponding to amino acids 417–434 (Fig. 1). The K2ΔKΔL form is characterized by the absence of exon K corresponding to amino acids 310–319 (Fig. 1). The fourth form, K2ΔLMP, corresponds to a single base deletion at position 2110 and to a replacement of a G by an A at position 2120 (positions are given from the cDNA of the K2KL form where the A of the ATG codon is at position 1). We verified that this last form was not due to a mistake of the reverse transcriptase and/or Taq DNA polymerases by preparing new cDNAs with another reverse transcriptase enzyme (AMV RTase instead of MMuLV RTase) and by amplifying with other Taq DNA polymerases (from Gibco and Boehringer). We were able in all cases to detect the K2ΔLMP form. The point mutation leads to a change in the open reading frame which lengthens the C-terminal end of the K2ΔLMP protein, which comprises 930 amino acids instead of 872 for the K2KL protein.

3.2. Expression of KCNQ2 splice variants in transfected COS cells

Among the four splice variants only two, K2KL and K2ΔL, were able to generate a selective K^+ current after transfection into COS cells (Fig. 2). Although K2KL and K2ΔL differ in their primary amino acid sequence (Fig. 1), they share exactly the same electrophysiological properties (Fig. 2). These two forms produce an outward K^+ current which is very similar to the KCNQ1 current [7]. The main biophysical differences are a slight shift of the activation curve toward negative voltage values ($V_{0.5} = -37.5 \pm 1.2$ mV, slope factor $k = 14.3 \pm 0.8$ mV, $n = 11$ for K2KL and $V_{0.5} = -23.6 \pm 1.6$ mV, slope factor $k = 11.1 \pm 0.9$ mV, $n = 8$ for KCNQ1) and a large increase in the deactivation kinetics (time constant at −40 mV of 141.3 ± 6.8 ms, $n = 8$ for K2KL and 621.4 ± 27.0 ms, $n = 10$ for KCNQ1 [7]) (Fig. 2). The non-functional variants, K2ΔKΔL and K2ΔLMP, were assayed for their ability to interfere with the KCNQ2 channel activity. We also tested the effect of another clone, called HNSPC, which had been isolated from neuroblastoma cells and which is also a splice variant of KCNQ2 [15]. None of the non-active forms was able to modify the current generated by either K2KL or K2ΔL channels. The single transmembrane domain regulatory subunit KCNE1 (IsK) [4,5] had no effect on the expression of both active forms of KCNQ2 channel expressed in COS cells.

As the KCNQ2 amino acid sequence contains 12 potential phosphorylation sites for protein kinase A (PKA) and three potential phosphorylation sites for protein kinase C (PKC) (Fig. 1), we examined the possibility of a regulation through these two phosphorylation pathways. Application of either 8-Cl-AMPc (500 μM), an activator of PKA, or of the phorbol 12-myristate 13-acetate (PMA), an activator of PKC, did not

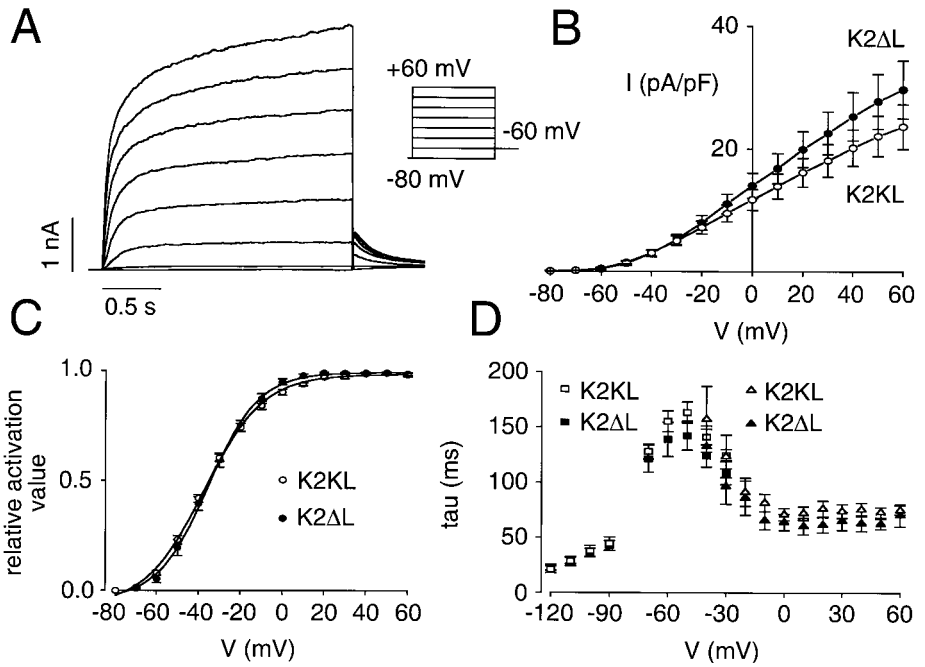

Fig. 2. Electrophysiological characterization of K2KL and K2ΔL currents expressed in transfected COS cells. A: Typical traces of K2KL currents elicited by depolarizing voltage steps. Currents are activated by 2 s pulses applied in 20 mV from −80 mV to +60 mV. Tail currents elicited upon repolarization to −60 mV (voltage protocol inset) increased progressively with voltage and saturated at +20 mV. B: K2KL and K2ΔL currents measured at the end of the test pulses normalized to cell capacitance (current density) and plotted against test potentials. Data points are mean ± S.E.M., $n = 11$ cells for K2KL and $n = 7$ cells for K2ΔL. C: Voltage dependence of activation determined from peak tail currents from experiments as in A. Activation curves were determined by fitting peak values of tail currents (I_{tail}) versus test potential (V_t) to a Boltzmann function: $I_{tail} = I_{tail-max}/(1+\exp[(V_{0.5} - V_t)/k]$ where $I_{tail-max}$ is the maximum tail current. The voltage at which the current was half activated ($V_{0.5}$) and the slope factor (k) calculated from these data were: -37.5 ± 1.2 mV and 14.3 ± 0.8 mV for K2KL ($n = 11$ cells) and -35.8 ± 1.8 mV and 12.0 ± 0.9 mV for K2ΔL ($n = 7$ cells). D: Kinetics of K2KL and K2ΔL channel activation and deactivation. Activation time constant (triangle) was assessed by the same voltage protocol as in A. For deactivation (square), the voltage potential was stepped to +40 mV and then stepped back to voltages ranging from −120 mV to −30 mV in 10 mV increments, where tail current deactivation time constants were measured. Activation and deactivation were fitted with a single exponential. Data points are mean ± S.E.M., $n = 7$ cells for activation of both channels, $n = 8$ and 9 cells for deactivation of K2KL and K2ΔL, respectively.

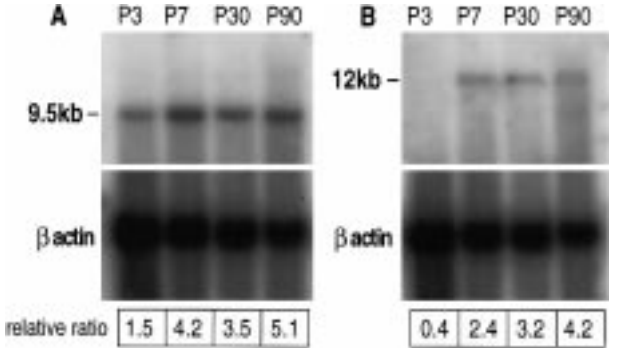

Fig. 3. Expression in mouse brain of KCNQ2 (A) and of KCNQ3 (B) mRNAs. Each lane contains 4 μg of poly(A)$^+$ mRNAs isolated from brain of 3 day old (P3), 1 week old (P7), 1 month old (P30) and adult mice (P90). Autoradiograms were exposed for 8 days at −70°C. Blots were hybridized with a β-actin probe for relative quantification. Relative ratios KCNQx signal/β-actin signal are calculated for each lane using the TINA 2.09 program on BAS reader 1500 apparatus (Fuji).

produce variations of the KCNQ2 channel activity (data not shown).

The pharmacological properties of the KCNQ2 channel were investigated using two different strategies. First we tested molecules which are known to have an epileptogenic action such as the MCD peptide (a bee venom toxin) and DTXI (a mamba venom toxin) [16,17]. Both MCD peptide 1 μM and DTXI 0.1 μM had no effect on the KCNQ2 channel activity (data not shown). Next we tested compounds which are known to be potent activators or inhibitors of KCNQ1 channel activity such as the benzodiazepine derivative L-364,373 (activator) [18], or chromanol 293B [19] and another benzodiazepine derivative, L-735,821 (inhibitors). The KCNQ1 inhibitor chromanol 293B had no effect on KCNQ2 activity. The benzodiazepine L-364,373, which activates KCNQ1 even when expressed alone either in *Xenopus* oocytes [18] or in COS cells (not shown), is unable to activate the KCNQ2 channel. Interestingly, the inhibitor L-735,821 reversibly blocks both KCNQ1 and KCNQ2 channel activities with EC_{50}s of 0.08 μM for KCNQ1 and 1.5 μM for KCNQ2.

3.3. Northern blot analysis

The KCNQ2 distribution was analyzed by Northern blots on different human tissues and compared to the KCNQ3 distribution. KCNQ2 and KCNQ3 are almost exclusively expressed in the brain where they hybridize three different species of RNA poly(A)$^+$ corresponding to 9.5 kb, 4.0 kb and 1.8 kb for KCNQ2 and 12.0 kb, 7.0 kb and 4.0 kb for KCNQ3. KCNQ2 is also expressed as a band at 1.4 kb in testis and KCNQ3 as a band at 12.0 kb in spleen (data not shown).

3.4. Developmental expression of KCNQ2 in the brain and comparison with KCNQ3

KCNQ2 is already present 3 days after birth (P3) in the mouse and its expression is increased by a factor of 2.5 at 1 week after birth. Then the KCNQ2 expression level remains stable until the adult stage (P90) (Fig. 3A). KCNQ3 is present in very low amounts at P3 but its expression increases continuously until the adult stage. This expression is about two-fold higher at P30 than at P7 (Fig. 3B). At earlier stages KCNQ2 expression is higher than that of KCNQ3. The ratio is maximal at P3 because KCNQ3 expression is very low then.

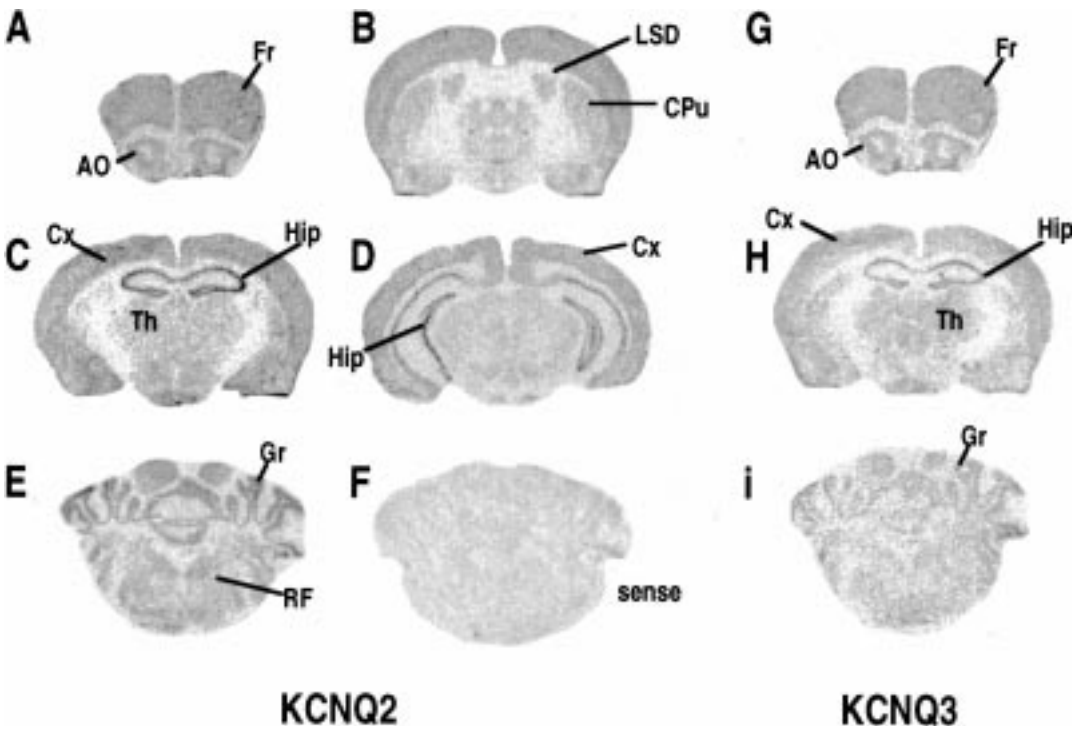

Fig. 4. Localization of KCNQ2 (A–F) and KCNQ3 (G–I) mRNAs in the mouse brain. Photomicrographs of the autoradiograms of mouse coronal sections after in situ hybridization histochemistry with an antisense riboprobe of a 706 bp fragment of KCNQ2 (A–E), the corresponding sense riboprobe (F) or an antisense riboprobe of a 1092 bp fragment of KCNQ3 (G–I). Abbreviations: AO, anterior olfactory nuclei; CPu, caudate putamen; Cx, neocortex; Fr, frontal olfactory cortex; Gr, cerebellar granular layer; Hip, hippocampus; LSD, lateral septal nucleus (dorsal part); RF, reticular formation; Th, thalamus. Adjacent sections hybridized with corresponding sense probes revealed no signal.

At P30 and later no significant difference exists between the respective expressions of the two mRNAs species.

3.5. Localization of KCNQ2 in mouse brain by in situ hybridization and comparison with KCNQ3

KCNQ2 is highly expressed in the hippocampus and dentate gyrus, neocortex, and in the granular layer of the cerebellum. It is also present in the olfactory bulb, caudate putamen, and several septal, thalamic and brainstem nuclei (Fig. 4). KCNQ2 and KCNQ3 hybridization signals are largely overlapping (Fig. 4), although KCNQ2 signals are stronger.

4. Discussion

We have isolated four variants of KCNQ2 from human adult brain. Two of them, K2KL and K2ΔL, are capable of generating a K^+ current when they are expressed in COS cells. This K^+ current has the same major characteristics as the KCNQ1 current an essential component of the I_{Ks} channel in the heart [4,5]. The function of exon L is unknown. The cytoplasmic segment it encodes at the C-terminal end of the channel bears no putative phosphorylation site; however, it could play a role in the association with a putative regulatory protein.

The two other KCNQ2 forms, K2ΔKΔL and K2ΔLMP, are unable to produce a K^+ current. This is not surprising for K2ΔKΔL since the ΔK deletion occurs at the end of the sixth transmembrane segment. This deletion will probably dramatically change the integration of the channel protein in the lipid bilayer. Results obtained with the K2ΔLMP form have to be associated with those obtained with HNSPC, another KCNQ2 splice variant [15]. HNSPC, which corresponds to a protein with a very short C-terminal tail, is inactive (our results and [20]). K2ΔLMP differs from K2KL at amino acid 709. These data indicate that the cytoplasmic C-terminal part (approximately the last 150 amino acids) of the KCNQ2 protein is very important for channel activity. It is therefore not surprising that recently described mouse brain KCNQ2 isoforms, which do not match in their C-terminal part with K2KL, are inactive [20]. The cytoplasmic C-terminal part of the KCNQ1 channel is also important for its activity. Mutations associated with long QT syndromes are found in this portion of the structure and lead to inactive mutants which in addition act as dominant negatives [7–9]. We analyzed the possibility that K2ΔKΔL, K2ΔLMP and HNSPC could act as negative regulators of the K2KL current, and found that these variants are without effect on K2KL expression in COS cells.

BFNC is an idiopathic epilepsy with seizures occurring in the first days of life and is due to mutations in both KCNQ2 and KCNQ3 [2,3,10]. A recent report indicates that KCNQ2 and KCNQ3 interact to give a current ∼15-fold larger than the individual currents obtained with either KCNQ2 or KCNQ3 alone, suggesting a probable interaction between the two subunits [11]. Such an interaction would explain why mutations on either KCNQ2 or KCNQ3 genes lead to the same symptoms. In situ hybridization shows that KCNQ2 is mainly present in the cerebellar cortex, the neocortex and the hippocampal formation including the dentate gyrus. This is interesting since these three structures present distinct epileptic seizure susceptibility [21]. KCNQ3 is localized in the same areas in mouse brain. However, KCNQ2 expression appears earlier than that of KCNQ3 and rapidly increases during the first week of life. At birth, KCNQ3 is expressed in a very low amount whereas KCNQ2 is already expressed at a significant level. This indicates that different profiles of association of KCNQ2 and KCNQ3 will probably occur during development. It will be important to know at the protein level, using specific antibodies, how and where KCNQ2 and KCNQ3 appear in human brain during development, and how differences in expression might be related to the age-dependent seizure remission seen in BFNC. This seizure remission is probably explained by a compensation mechanism for defects in KCNQ2/KCNQ3 channels by other types of K^+ channels which appear a few days after birth [13,22,23].

Although KCNQ2 and KCNQ1 belong to the same protein family, their pharmacological profiles are different except for their sensitivity to benzodiazepine L-735,821 which blocks both channels, but is a more potent blocker of the KCNQ1 channel.

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