Differential distribution of classical inwardly rectifying potassium channel mRNAs in the brain: comparison of IRK2 with IRK1 and IRK3

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Abstract Distribution of IRK2 inwardly rectifying potassium channel mRNA in the mouse brain was studied using in situ hybridization histochemistry and compared with those of other classical inwardly rectifying potassium channel (IRK1 and IRK3) mRNAs. All these IRK channel mRNAs were detected in neurons, but not in glial cells. Their distribution patterns in the brain were, however, quite divergent: IRK2 mRNA was detected extremely high in granule cells of cerebellum, relatively high in motor trigeminal nucleus and moderate in olfactory bulb, piriform cortex, cerebral cortex, CA1 through CA3 regions of hippocampus, dentate gyrus and pontine nucleus. On the other hand, IRK1 mRNA was expressed throughout whole brain but in particular subsets of neurons, and IRK3 mRNA was in forebrain. Expression of these three IRK mRNAs overlapped in hippocampus, olfactory bulb, and cerebral cortex. This differential distribution of IRK mRNAs suggests that each of these channels has its specific function in regulation of the excitability of brain neurons.

Key words: Inwardly rectifying potassium channel; Hybridization (in situ); Brain; Neuron

1. Introduction

Classical inwardly rectifying potassium (K^+) channels play a pivotal role in maintaining resting potential and also in regulating the duration of action potential in various cells, including neuronal cells [1]. Theoretically, the neurons, which express enough quantity of classical inwardly rectifying K^+ channels, are expected to have a deep resting potential near to the K^+ equilibrium potential and may not excite automatically [2]. Being excited with stimulation, these cells may exhibit action potential with a long-lasting plateau when expression of voltage-gated outward K^+ channels is low. The electrical properties of neurons arisen from the inwardly rectifying K^+ channels, therefore, are quite important for regulation of neural excitability. However, few studies have been performed on the expression and functional roles of the classical inwardly rectifying K^+ channels in the brain.

We have cloned three kinds of classical inwardly rectifying K^+ channel cDNAs (IRK1, IRK2 and IRK3) from mouse brain cDNA library ([3–5]; see also [6] for rat IRK2). All of these K^+ channels have two putative membrane-spanning regions and one potential pore forming region [7,8]. Identities of amino acid sequences of IRK2 and IRK3 with that of IRK1 were 70% and 61%, respectively. The Northern blot analyses suggest differential distribution of the mRNAs of these clones in the brain [4,5,8]; i.e. IRK1 mRNA was expressed in both forebrain and cerebellum, while IRK2 and IRK3 mRNAs were dominantly in the cerebellum and the forebrain, respectively. Although the distributions of IRK1 and IRK3 mRNAs were further examined using in situ hybridization technique [3,9,10], that of IRK2 mRNA has not yet been studied. In addition, there were significant discrepancies in the distributions of IRK3 mRNA reported by Falk et al. (rIRK3: [9]) and by Bredt et al. (BIRK2: [10]). In the present study, therefore, to provide one experimental basis for elucidating the differential functional roles of classical inwardly rectifying K^+ channels in the brain, we compared the distributions of mRNAs of these three IRKs by performing in situ hybridization histochemistry under the same condition of hybridization.

2. Materials and methods

In situ hybridization was carried out essentially as described previously [11,12]. Brains from 8-week-old male ddY mice (Nippon Doubutsu, Osaka, Japan) were frozen on powdered dry ice. Coronal and parasagittal sections (20 μm) were cut on a cryostat and thaw mounted on 3-aminopropyltriethoxysilane-treated slides. All probes used in the experiments were located in the terminal region of coding sequence and 3′ non-coding region of each cDNA to exclude cross-hybridization. For IRK1 and IRK3, cDNA fragments of nucleotide positions 1499 to 1801 of IRK1 cDNA [3] and nucleotide positions 1135 to 1434 of IRK3 cDNA [5] were synthesized using PCR and inserted into pCRII plasmid (Invitrogen, San Diego, CA). T7 RNA polymerase and SP6 RNA polymerase were used for synthesis of ^{35}S-labeled antisense and sense riboprobes, respectively, in the presence of [α-^{35}S]UTP (New England Nuclear, Wilmington, MA). For IRK2, *Pst*I-*Stu*I fragment (nucleotide positions 1264–1591) of IRK2 cDNA [4] was subcloned into pBluescript (Stratagene, La Jolla, CA) and used as a template. ^{35}S-labeled riboprobes were synthesized using T7 RNA polymerase (for antisense probe) and T3 RNA polymerase (for sense probe). For IRK1, full-length cDNA (5.5 kb) in pBluescript was also used as a template. Sense and antisense riboprobes of full-length IRK1 were synthesized using T3 and T7 RNA polymerases, respectively, and partially hydrolyzed by treating the probes with 40 mM sodium bicarbonate and 60 mM sodium carbonate at 60°C for 90 min. These full-length probes of IRK1 gave the same results as those from IRK1 PCR fragment when used as a template. Hybridization (2×10^6 cpm/100 μl/slide) was carried out in 50% formamide, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.3 M NaCl, 10 mM sodium phosphate, pH 8.0, 1 × Denhaldt's solution, 10% dextran sulfate, 0.2% sarcosyl, 0.5 mg/ml yeast tRNA and 0.2 mg/ml salmon sperm DNA at 55°C for 12–16 h. After hybridization, sections were washed in 5 × SSC and 1% 2-mercaptoethanol at 55°C for 20 min, in a high stringency buffer (50% formamide, 2 × SSC and 10% 2-mercaptoethanol) at 65°C for 30 min and then 4 × in RNase buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 0.5 M NaCl) at 37°C for 10 min each. Sections were treated with RNase A (1 μg/ml) in RNase buffer at 37°C for 30 min, and then washed again with a high stringency buffer at 65°C for 30 min. After dehydration, sections were

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Abbreviations: IRK channel, inwardly rectifying potassium channel; SSC, saline-sodium citrate.

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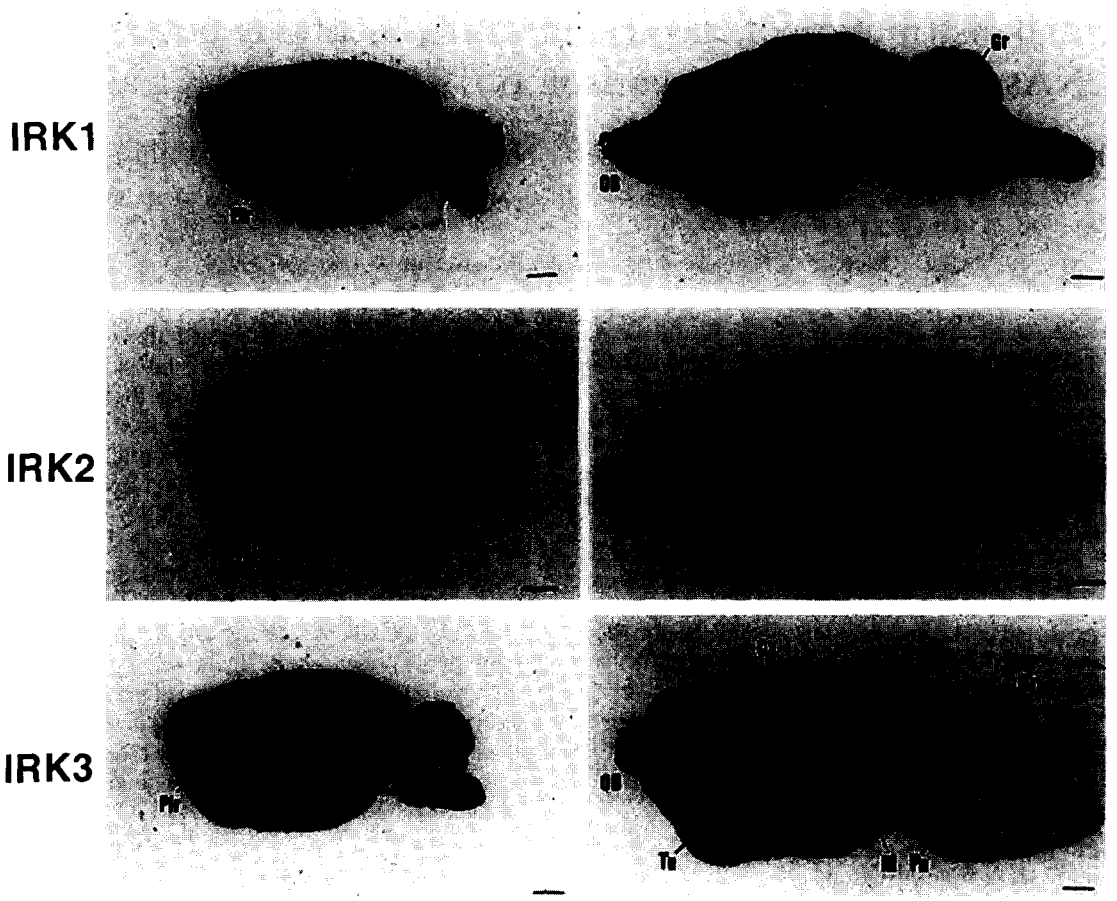

Fig. 1. X-ray film autoradiographs illustrating distributions of IRK1, IRK2 and IRK3 mRNA in parasagittal serial sections of the mouse brain. The sections were hybridized with antisense probes. CA1–CA3, fields CA1–3 of Ammon's horn; Cx, cerebral cortex; CPu, caudate putamen; DG, dentate gyrus; Gr, granular layer; M, mammillary nuclei; Mo5, Motor trigeminal nucleus; OB, olfactory bulb; Pir, piriform cortex; Pn, pontine nucleus; Tu, olfactory tubercule. Scale bars: 400 μm.

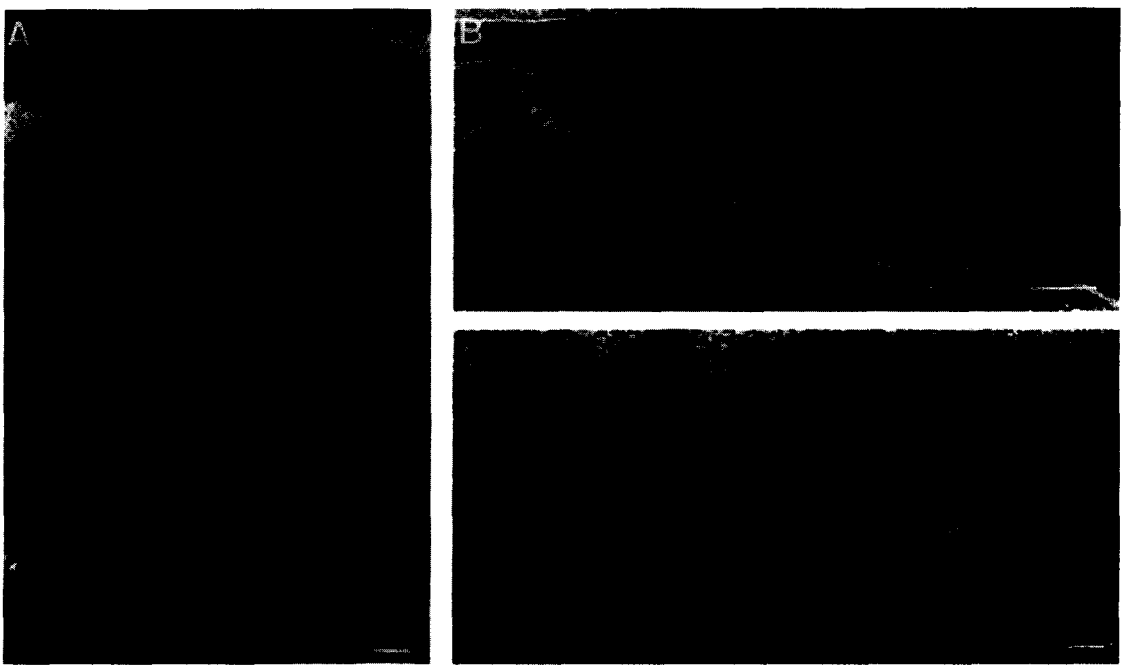

Fig. 2. Dark-field photomicrographs of IRK1 mRNA distribution on emulsion-dipped sections. A, caudate putamen, cerebral cortex and fields CA2–3 express IRK1 mRNA. B and C, facial nucleus and granular layer of cerebellum are labeled. CA2–CA3, fields CA2–3 of Ammon's horn; CPu, caudate putamen; Cx, cerebral cortex; Gr, granular layer; Mo, molecular layer; 7, facial nucleus. Scale bars: A,B, 400 μm; C, 100 μm.

exposed to Fuji RX film (Fuji Photo Film, Tokyo, Japan) for 4 days. For emulsion autoradiography, slides were dipped in an emulsion (NTB-2, Kodak, Rochester, NY) and then exposed at 4°C for 2 weeks. The signals obtained by these techniques were specific, because sense probes of IRK1, IRK2 and IRK3 gave no signals (data not shown).

3. Results

Distribution patterns of IRK1, IRK2, and IRK3 mRNAs were compared in parasagittal serial sections (Fig. 1). IRK1 mRNA was widely expressed in the adult mouse brain as described previously [3]. Olfactory bulb, piriform cortex, cerebral cortex, fields CA1, CA2 and CA3 of Ammon's horn in hippocampus, dentate gyrus, and granular layer of cerebellum contained IRK1 mRNA (Fig. 1–IRK1). The level of expression in dentate gyrus was higher than that of CA1 through CA3 regions. Because the signal of IRK1 mRNA was weaker than those of IRK2 and IRK3 mRNAs, we also used full-length IRK1 riboprobe. The same results were obtained in this condition. The IRK2 mRNA was expressed in an extremely high level in cerebellar granular layer (Fig. 1–IRK2). Moderate expression of IRK2 mRNA was observed in olfactory bulb, piriform cortex, cerebral cortex, CA1 through CA3 regions of hippocampus, dentate gyrus and pontine nucleus. The level of expression of IRK2 mRNA in dentate gyrus was almost the same as those of CA1 through CA3 regions. The IRK3 mRNA was expressed dominantly in the forebrain (Fig. 1–IRK3): i.e. olfactory bulb, piriform cortex, olfactory tubercle, cerebral cortex, caudate putamen, CA1 through CA3 regions, dentate gyrus showed high to moderate expression of IRK3 mRNA. The level of expression of IRK3 mRNA in dentate gyrus was higher than those of CA1–3. Mammillary nuclei and pontine nucleus also expressed moderate amount of IRK3 mRNA. The granular layer of cerebellum expressed weak level of IRK3 mRNA. The distribution of IRK3 mRNA shown in this study is consistent with the preliminary report by Bredt et al. [10], but different from the study by Falk et al. [9].

Expression of these three IRK mRNAs was further examined using dark-field photomicrographs of emulsion-dipped brain sections (Figs. 2–4). To compare quantitatively expressions of these three IRK channel mRNAs each other, same amounts of radiolabeled probes of almost equal specific activities were used and also same exposure time to an emulsion was introduced. The distribution of IRK1 mRNA was expressed moderately in caudate putamen and facial nucleus (Fig. 2A,B). Cerebellar granule cells and some cells in the molecular layer also expressed IRK1 mRNA (Fig. 2C). Cerebellar Purkinje cells did not show detectable expression of IRK1 mRNA with the present protocol (2 weeks), but did so with a longer exposure (~3–4 weeks) of brain slices to an emulsion as described previously [3] (data not shown). Figs. 3 and 4 show dark-field and bright-field photomicrographs of emulsion dipped sections for IRK2 and IRK3 mRNAs, respectively. The expression of IRK2 mRNA was extremely high in cerebellar granule cells (Fig. 3A,D), relatively high in motor trigeminal nucleus (Fig. 3C,F) and periglomerular cells of olfactory bulb (Fig. 3E), moderately high in cerebral cortex, medial habenular nucleus, and paraventricular thalamic nucleus (Fig. 3B), and low in thalamus (Fig. 3B). In Fig. 4A,D, the abundant expression of the IRK3 mRNA was clearly depicted in the olfactory system, including olfactory bulb, anterior olfactory nucleus, olfactory tubercle and piriform cortex. Moderate expression of IRK3 mRNA was detected in cerebral cortex, caudate putamen, me-

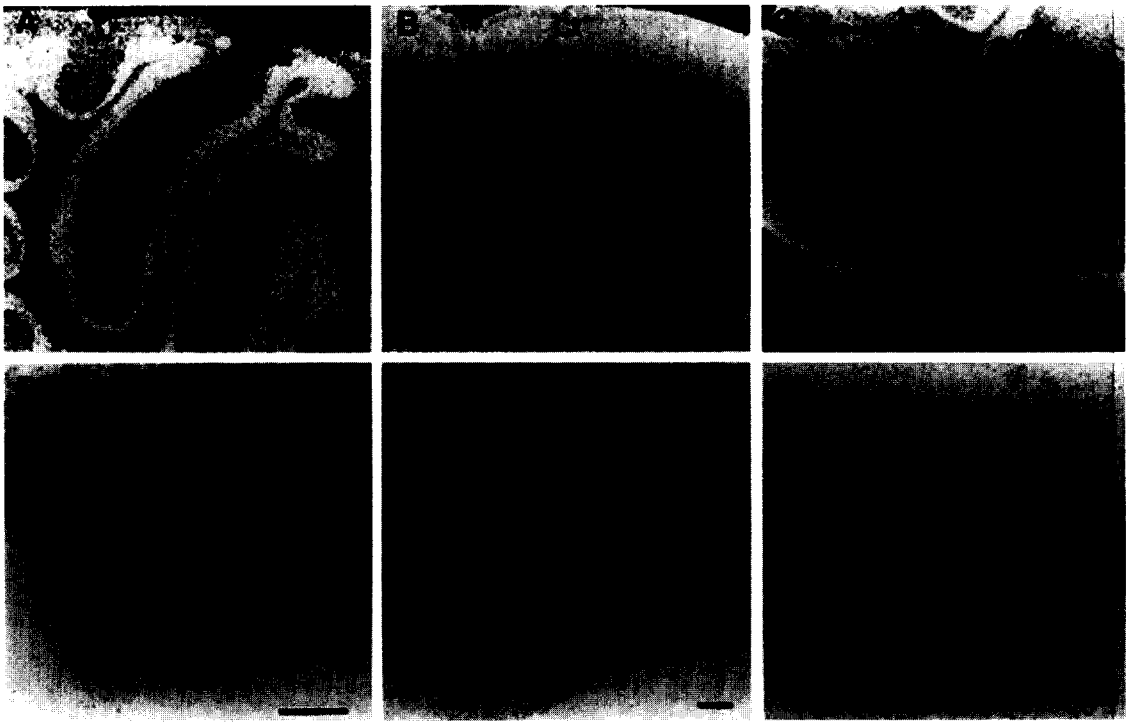

Fig. 3. Dark-field (A–C) and bright-field (D–F) photomicrographs of IRK2 mRNA distribution on emulsion-dipped sections. A,D, granule cells of cerebellum express IRK2 mRNA. B, cerebral cortex, fields CA1–3, medial habenular nucleus and paraventricular nucleus are stained. C,F, motor trigeminal nucleus; E, periglomerular cells of olfactory bulb. CA1–CA3, fields CA1–3 of Ammon's horn; Cx, cerebral cortex; DG, dentate gyrus; Gr, granular layer; Int, interposed cerebellar nucleus; MHb, medial habenular nucleus; Mo, molecular layer; Mo5, Motor trigeminal nucleus; pg, perigromerular cell; PVP, paraventricular thalamic nucleus posterior; Ve, vestibular nucleus; Scale bars: A–C, 400 μm; D–F, 40 μm.

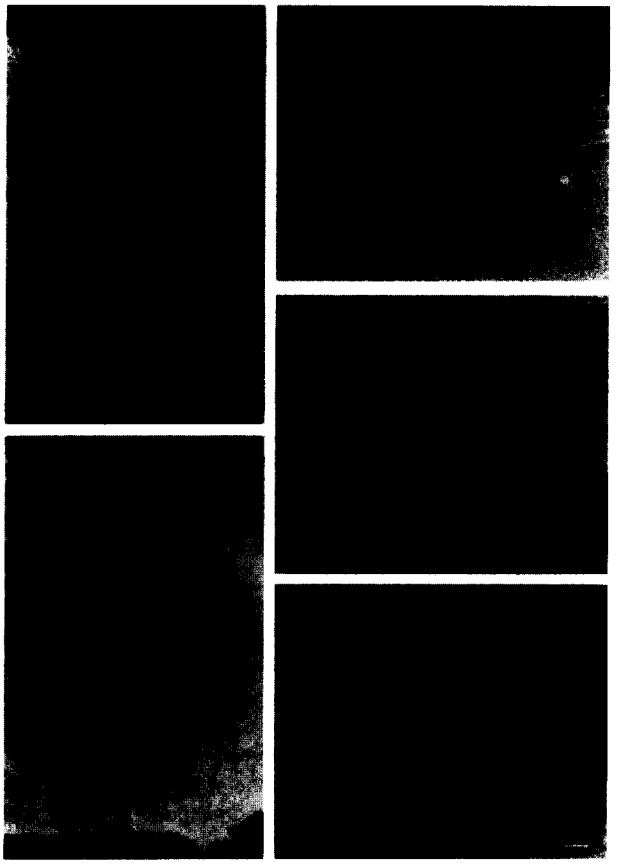

Fig. 4. Dark-field photomicrographs of IRK3 mRNA distribution on emulsion-dipped sections. A,D, olfactory system expresses IRK3 mRNA. B,C, dentate gyrus, fields CA1–3 are heavily labeled and cerebral cortex, medial habenular nucleus, reticular nucleus and caudate putamen are moderately labeled. E, very weak signal is shown in granular layer of cerebellum. AOL, anterior olfactory nucleus; CA1–CA3, fields CA1–3 of Ammon's horn; CPu, caudate putamen; Cx, cerebral cortex; DG, dentate gyrus; MHb, medial habenular nucleus; OB, olfactory bulb; Pir, piriform cortex; Rt, reticular nucleus; Tu, olfactory tubercule. Scale bars: 400 μm.

dial habenular nucleus and reticular nucleus of thalamus (Fig. 4B,C). Motor trigeminal and facial nuclei contained moderate to low level of IRK3 mRNA (data not shown). Very low level of expression was detected in granule cells of cerebellum (Fig. 4E).

In the cerebral neocortex, layer II neurons expressed the mRNAs of all these three channels (Fig. 5). Although layers III–VI showed expressions of three IRK channel mRNAs in the longer exposure (3–4 weeks) of brain slices to an emulsion (data not shown), relatively moderate to high expressions of IRK2 and IRK3 mRNAs were detected on layers III–VI (Fig. 5). The density of IRK2 mRNA on layer II was higher than those of layers III–VI. On the other hand, IRK3 mRNA was detected almost equally in layers II–VI (Fig. 5).

4. Discussion

Using in situ hybridization histochemistry, expression of mRNAs of the classical inwardly rectifying K^+ channels, i.e. IRK1, IRK2 and IRK3, was examined in the mouse brain. The present study showed that the classical inward-rectifier IRK mRNAs seemed to be expressed abundantly in neurons, but not in glial cells. In glial cells, expression of another inwardly rectifying K^+ channel, K_{AB}-2, was recently identified [10]. Although K_{AB}-2 channel exhibited classical inwardly rectifying properties similar to those of IRKs, its amino acid sequence had only ~40% homology to those of IRKs and was characterized with a Walker type-A putative ATP-binding domain. Thus, the glial K_{AB}-2 may belong to a different subfamily from that of IRKs. This suggests that neurons and glial cells differentially regulate transcription of different subfamilies of inwardly rectifying K^+ channels.

The present study further showed that, even among the members of IRK subfamily (IRK1–3), the distribution of their mRNAs was quite divergent in the brain: i.e. IRK1 mRNA distributed in the whole brain but in particular subsets of neurons, IRK2 mRNA in granule cells of the cerebellum and IRK3 mRNA predominantly in neurons of forebrain. This result suggests that each IRK channel has its specific function in regulation of neuronal excitability in the brain. To elucidate the mechanism underlying the area-specific expression of IRK mRNAs in the brain and their specific functional roles, further studies, such as analysis of transcriptional regulation and knocking-out of each gene, are needed.

The distribution of IRK3 mRNA in this study is consistent with the preliminary report of Bredt et al. [10], but different from that of Falk et al. [9]. Falk et al. detected moderate expression of IRK3 mRNA in Purkinje cells of cerebellum where we could not observe specific expression. On the other hand, different from their report we found moderate expression in frontal and parietal cortex as well as occipital and temporal cortex. We also detected moderate expression of IRK3 mRNA in caudate putamen and reticular nucleus of thalamus, but few or weak expression was reported by Falk et al. We and Bredt et al. used ^{35}S-labeled probes, while Falk et al. used fluorescein-labeled cRNA and alkaline phosphatase coupled anti-fluorescein antiserum. Therefore, the discrepancies might be caused by the different techniques used in these studies.

The neuronal inwardly rectifying K^+ channels might be regulated by various cell signalling systems and thus convert chemical signals to the cellular electrical signal in the brain. For example, substance P inhibits a classical inwardly rectifying K^+

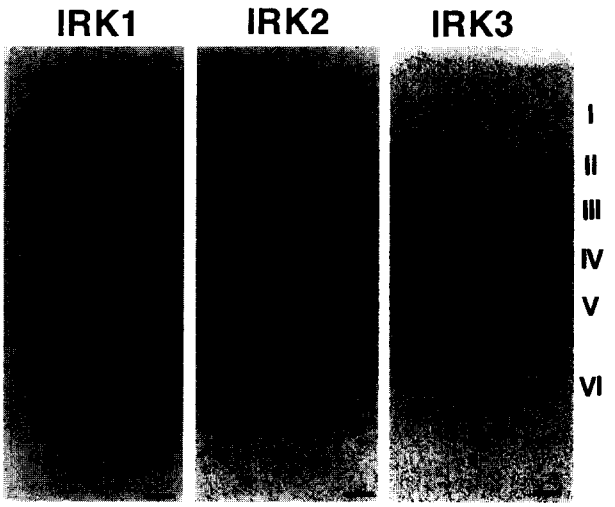

Fig. 5. Distribution of IRK mRNAs in the cerebral cortex. Layers I–VI were based on Nissl stained sections. Scale bars: 40 μm.

current in cholinergic neurons of basal nucleus, which raises excitability of the neurons [13–15]. Because both intracellular ATP and phosphorylation were reported to be essential to maintain IRK1 channel activity [16], IRKs can be targets of protein kinases and phosphatases. Actually localization of calmodulin-dependent adenylyl cyclase in the brain are quite similar to that of IRK2 mRNA [17]. The distributions of mRNAs of nitric oxide synthase [18] and IRK1 are also very similar in the brain. These suggest that IRKs can be regulated by various second messengers and may play important roles in regulation of neural excitability.

It was noted that expression of IRK mRNAs overlapped in some neurons in hippocampus, olfactory bulb and cerebral cortex: e.g. pyramidal cells of CA1–3 and granule cells of dentate gyrus were likely to express more than two IRK channels. Because the cardiac G protein-gated inwardly rectifying muscarinic K^+ channel was found to be a functional heteromultimer of inwardly rectifying K^+ channel subunits (GIRK1 and CIR) [19], IRK channels might also compose a heteromultimer in some cells. Co-expression experiments of IRK channels in *Xenopus* oocytes will clarify whether IRK channels can be functional as a heteromultimer or not.

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