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Contrasting expression of Kv4.3, an A-type K^+ channel, in migrating Purkinje cells and other post-migratory cerebellar neurons

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Abstract

Kv4.3, an A-type K^+ channel, is the only channel molecule showing anterior–posterior (A–P) compartmentalization in the granular layer of mammalian cerebellum known so far. Kv4.3 mRNA has been detected from the posterior but not anterior granular layer in adult rat cerebellum. To characterize this A-P compartmentalization further, we examined Kv4.3 protein expression in rat cerebellum by immunohistochemistry at the embryonic, early postnatal and adult stages. Specificity of the Kv4.3 antibody was confirmed by both Western blot and immunoprecipitation analysis. In adulthood, Kv4.3 was detected from the somatodendritic domain of posterior granule cells, with a restriction boundary in the vermal lobule VI extending laterally to the hemispheric crus 1 ansiform lobules. At the early postnatal stage, this A–P pattern first appeared on postnatal day 8, when significant numbers of granule cells had migrated into the posterior granular layer and started to express Kv4.3. Similar Kv4.3 expression in the somatodendritic domain of post-migratory neurons in the cerebellum was also observed in basket cells, stellate cells, a subset of GABAergic deep neurons, Lugaro cells and, probably, deep Lugaro cells. However, none of them showed A–P compartmentalization. Strikingly, we found Kv4.3 in several clusters of migrating Purkinje cells with mediolateral compartmentalization. These Purkinje cells no longer expressed Kv4.3 after completing the migration. By contrasting the expression in migrating and post-migratory neurons, our results suggest that Kv4.3 may play an important role in the development of cerebellum, as well as in the mature cerebellum.

Introduction

Kv4.3, a voltage-gated K^+ (Kv) channel α subunit, can evoke A-type K^+ currents (I_As) in heterologous expression systems, which are transiently activated and rapidly inactivated (Serôdio *et al*., 1996; Ohya *et al*., 1997; Tsaur *et al*., 1997). When coexpressed with certain Ca^{2+} binding proteins KChIPs, Kv4.3 can produce I_As activated at membrane potentials below the threshold of action potentials, a property closely resembling native I_As recorded from mammalian central nervous system neurons (An *et al*., 2000). A-type K^+ channels are important in the control of electrical signal transduction in neuronal circuits. When A-type K^+ channels are localized in dendrites and somata, I_As dampen excitatory post-synaptic potentials to prevent the initiation of action potentials and thus control neuronal excitability (Hoffman *et al*., 1997). When A-type K^+ channels are localized in axons and nerve terminals, I_As reduce the frequency of action potentials and thus decrease the amount of neurotransmitters released (Debanne *et al*., 1997). Although the functions of A-type K^+ channels have been well studied in mature neurons, their roles in immature neurons remain unclear. Mapping the expression of A-type K^+ channels in the developing nervous system will be a useful first step.

Among the large number of ion channels, Kv4.3 is distinct because it is the only channel molecule showing anterior–posterior (A–P) compartmentalization in the granular layer of mammalian cerebellum. Kv4.3 mRNA has been found in the posterior but not anterior granular layer in adult rat cerebellum (Serôdio & Rudy, 1998). Similar A–P compartmentalization has been detected from some transcription factors crucial for the development of cerebellum (reviewed in Herrup & Kuemerle, 1997). In addition, Kv4.3 mRNA has also been detected from Purkinje cells, basket cells, stellate cells and deep neurons in adult cerebellum (Tsaur *et al*., 1997; Serôdio & Rudy, 1998). Whether these Kv4.3(+) cerebellar neurons also show compartmentalization remains unclear. To elucidate the physiological significance of the A–P compartmentalization, one needs to know where the A–P boundary is, which cell types are involved and when this pattern first appears. Because cerebellar neurons originate from regions different from their final locations after migration (Hallonet *et al*., 1990; Zhang & Goldman, 1996; Altman & Bayer, 1997), it will be helpful to trace these cerebellar neurons back to when they first express Kv4.3 during development.

In this study, at the protein level, we examined Kv4.3 expression in rat cerebellum at the embryonic and early postnatal stages, in addition to adulthood. The A–P compartmentalization was further characterized. The granule cell was the only cell type involved, and the A–P pattern first appeared when post-migratory granule cells started to express Kv4.3 in their somatodendritic domain. Most notably, we found Kv4.3 in several clusters of migrating Purkinje cells, which showed transient mediolateral (M-L) compartmentalization in the developing cerebellum.

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Materials and methods

Animal preparation

Sprague-Dawley rats aged 8–12 weeks (in adulthood), from postnatal day 2 (P2)–P14 and from embryonic day 15.5 (E15.5)–E19.5 were supplied by the Animal Facility of National Yang-Ming University (Taipei, Taiwan). The day of birth was defined as P1. Timed pregnancies were established by checking for vaginal smears in the morning following mating, and we designated noon on the day sperm was detected as E0.5. National guidelines of animal care were followed, and the experiments were approved by the local ethics committee of the National Yang-Ming University. In order to prevent blood clotting for the immunohistochemical analysis, rats in the adult and early postnatal stages were injected intraperitoneally with 2000 units of heparin/kg of body weight. After 10 min, animals were anaesthetized by another intraperitoneal injection of sodium pentobarbital (120 mg/kg), and perfused transcardially with normal saline briefly and 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. Pregnant rats were anaesthetized with sodium pentobarbital without heparin pretreatment, and embryos were quickly removed into cold PBS before perfusion directly with 4% paraformaldehyde in PBS. To stain GABA, 1% glutaldehyde was added to 4% paraformaldehyde in PBS. After perfusion, brains were removed and post-fixed in 4% paraformaldehyde in PBS at 4 °C for 10 min (E15.5–E19.5), 30 min (P2–P14) or 2 h (adults). After dehydration in 30% sucrose twice, brains were kept frozen before use.

Membrane protein preparation and Western blotting

Adult rats were anaesthetized with sodium pentobarbital and decapitated. The brains were removed, chopped into small pieces and homogenized in ice-cold sucrose buffer [0.32 M sucrose, 1 mM EDTA, 50 mM Tris (pH 7.4)] containing 1 μg/mL pepstatin, leupeptin and aprotinin, 0.2 mM 4-(2-aminoehtyl)-benzenesulphonyl fluoride, 0.1 mg/mL benzamidine, and 8 μg/mL calpain inhibitors I and II (Sigma, St Louis, MO, USA). After centrifugation at 2000 ***g*** for 10 min at 4 °C to remove debris, the supernatant was centrifuged at 100 000 ***g*** for 1 h at 4 °C. The pellet was suspended in sucrose buffer, and aliquots were stored at −72 °C. Protein concentrations were determined by the Bradford method (Harlow & Lane, 1999). Approximately 25 μg of membrane proteins was solubilized in sodium dodecyl sulphate (SDS) sample buffer containing 5% 2-mercaptoethanol, separated by 7.5% SDS/polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes. After a brief rinse in PBS, nitrocellulose membranes were blocked for non-specific binding with 5% non-fat milk in PBST (PBS containing 0.05% Tween 20) for 1 h, washed in PBST and incubated with rabbit anti-Kv4.3 primary antibody at 1 : 1000 (Alomone Laboratories, Jerusalem, Israel) in PBST containing 1% bovine serum albumin (BSA) for another hour, with or without pre-absorption with Kv4.3 immunogenic peptides at 10 μg/mL (Alomone Laboratories). After washing, goat-anti-rabbit secondary antibody conjugated with horseradish peroxidase at 1 : 10 000 (Amersham, Arlington Heights, IL, USA) was applied for 45 min. In the presence of chemiluminescent reagents (NEN, Boston, MA, USA), immunoreactive bands were visualized by exposing the nitrocellulose membranes to X-ray film. All steps were performed at room temperature.

In vitro *transcription/translation and immunoprecipitation analysis*

Plasmids containing rat Kv4.3 cDNA (Tsaur *et al*., 1997) or Kv4.2 cDNA (Baldwin *et al*., 1991) were linearized with NotI and ApaI, and transcribed *in vitro* with GpppG capping using T7 and T3 RNA polymerases, respectively (Ambion, Austin, TX, USA). In the presence of [^{35}S]methionine (Pharmacia, Uppsala, Sweden), 1 μg of transcript was translated *in vitro* using a rabbit reticulocyte lysate system (Promega, Madison, WI, USA). Aliquots of *in vitro*-synthesized protein products were diluted with protein buffer [100 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), 0.5% nonidet-P40], pre-absorbed with normal rabbit serum for 45 min and then incubated with rabbit anti-Kv4.2 (1 : 100; Alomone Laboratories) or rabbit anti-Kv4.3 (1 : 100) primary antibody for 1 h, followed by protein A–sepharose (Pharmacia) for 45 min. All steps above were performed at 4 °C with gentle mixing. After brief centrifugation, reaction mixtures were washed five times with the protein buffer, boiled in SDS sample buffer in the presence of 5% 2-mercaptoethanol, and seperated by SDS/polyacrylamide gel electrophoresis. Protein bands with labelled polypeptides were visualized by exposing dried gels to X-ray film. For competition analysis, anti-Kv4.2 or anti-Kv4.3 primary antibody was pre-incubated with Kv4.2 or Kv4.3 immunogenic peptides at 10 μg/mL (Alomone Laboratories) for 1 h before being added to the reaction mixtures.

Immunohistochemistry

Rat brains were cut with a cryostat at 20–50 μm for free-floating sections, or at 12–25 μm for sections attached directly onto slides. After washing in Tris-buffered saline [TBS, 25 mM Tris (pH 7.5), 0.85% NaCl], sections were treated with 0.2% hydrogen peroxide until no bubbles appeared. Non-specific binding was blocked by 3% normal goat serum plus 2% BSA in TBS containing 0.3% Triton X-100 for 1.5 h. The primary antibodies applied to floating sections included rabbit anti-Dab1 (1 : 1000; Alomone Laboratories), rabbit anti-Kv4.3 (1 : 200 or 1 : 400), rabbit anti-mGluR5 (1: 100; Calbiochem, San Diego, CA, USA), rabbit anti-N-type Ca^{2+} channel (1 : 200; Alomone Laboratories), mouse anti-calbindin (1 : 1000; Sigma), mouse anti-calretinin (1 : 200; Chemicon, Temecula, CA, USA) and mouse anti-MAP2 (1 : 100; Sigma). The concentrations of these primary antibodies were doubled for sections attached directly onto slides. After overnight shaking at room temperature, sections were incubated for 1.5 h with the biotinylated secondary antibody (1 : 1000), and either goat-anti-rabbit IgG (Pierce, Rockford, IL, USA) or horse-anti-mouse IgG (Vector, Burlingame, CA, USA), followed by the avidin–biotin–horseradish peroxidase complex (Pierce) for another 1.5 h, with intervening washes with TBS containing 0.3% Triton X-100. Antigen was visualized by combining equal volumes of an ammonium nickel sulfate solution (30 mg/mL in 0.1 M sodium acetate, pH 6.0) and a diaminobenzidine solution (4 mg/mL in TBS) in the presence of 0.01% hydrogen peroxide. After washing and dehydration through a graded series of ethanol solutions, sections were coverslipped with mounting medium Permount (Merck, Darmstadt, Germany). Images were acquired with a Nikon DMX1200 connected digital camera to a Nikon Eclipse E800 light microscope (Nikon, Melville, NY, USA), and processed with Adobe Photoshop 6.0 software (Adobe, Mountain View, CA, USA).

Double immunofluorescent staining

Sections for immunofluorescent staining were processed as for immunohistochemistry, except that treatment with hydrogen peroxide was omitted. Sections were incubated simultaneously with two primary antibodies, rabbit anti-Kv4.3 (1 : 100) and one of the following antibodies: mouse anti-calbindin (1 : 500), mouse anti-calretinin (1 : 100), mouse anti-GABA (1 : 50; Sigma), mouse anti-MAP2 (1 : 50), mouse anti-parvalbumin (1 : 3000; Sigma), mouse anti-TUJ1 (1 : 500; Chemicon). Secondary antibodies, fluorescein-conjugated goat-anti-rabbit IgG (1 : 200; Jackson ImmunoResearch, West Grove, PA, USA) and

rhodamine-conjugated donkey-anti-mouse IgG (1 : 200; Chemicon) were also simultaneously applied. To augment the intensity of the fluorescein, the tertiary antibody goat-anti-fluorescein IgG (1 : 200; Molecular Probes, Eugene, OR, USA) and the quatenary antibody fluorescein-conjugated donkey-anti-goat IgG (1 : 200; Jackson ImmunoResearch) were sequentially applied. For double staining with rabbit anti-Kv4.3 and goat anti-somatostatin (1 : 50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), fluorescein-conjugated donkey-anti-rabbit IgG (1 : 200; Jackson ImmunoResearch) and rhodamine-conjugated donkey-anti-goat IgG (1 : 200) were used as secondary antibodies. Then, goat-anti-fluorescein IgG (1 : 200) was added to increase the signal of Kv4.3. Sections were mounted with Vectashield (Vector Laboratories). Images were collected by a Leica TCS-NT confocal laser scanning microscope with a PowerScan software (Leica, Buffalo, NY, USA). An average of four aquisitions with each channel were used for merging and images were processed by Adobe Photoshop 6.0 software.

Results

Specificity of the Kv4.3 antibody

Using rat brain membrane proteins for Western blot analysis, we found that the Kv4.3 antibody recognized a unique protein band with a molecular weight of ~69 kDa, which could be abolished when the Kv4.3 antibody was pre-absorbed with excess Kv4.3 immunogenic peptide (Fig. 1A). Kv4.2, a molecule highly homologous to Kv4.3, has a molecular weight (72 kDa) similar to that of Kv4.3 (69 kDa). To test whether the Kv4.3 antibody also recognizes Kv4.2, we synthesized both Kv4.2 and Kv4.3 protein products by *in vitro* transcription/translation and performed immunoprecipitation analysis. [^{35}S]methionine-labelled Kv4.3 protein products were immunoprecipitated by the Kv4.3 antibody, and it could be specifically blocked by the pre-incubation with excess Kv4.3 immunogenic peptides (Fig. 1B, lanes 1–3). By contrast, the Kv4.3 antibody did not immunoprecipitate Kv4.2 protein products (Fig. 1B, lanes 4–5). These results indicate that the Kv4.3 antibody is specific to Kv4.3 protein.

Expression of Kv4.3 in posterior granule cells at the post-migratory stage

To clarify the location of the boundary, we examined the distribution of Kv4.3 in adult rat cerebellum by immunohistochemistry. In the vermis, Kv4.3 immunoreactivity was found at very low levels in the anterior granular layer (from lobule I to the anterior face of lobule VIc), but at moderate levels in the posterior granular layer (from the dorsal face of lobule VIc to lobule X) (Fig. 2A and C). In the hemispheres, this A–P boundary was detected within the crus 1 ansiform lobule (Fig. 2B and D). These characteristic immunostaining patterns were completely abolished when the Kv4.3 antibody was pre-incubated with excess Kv4.3 immunogenic peptide (data not shown). Our data indicate that A–P compartmentalization of Kv4.3 appears only in the granular layer, and that the boundary is located in lobule VIc of the vermis extending laterally to the crus 1 ansiform lobules in the hemispheres.

To understand which cell types in the granular layer contribute to the A–P compartmentalization, we examined the posterior granular layer in adult rat cerebellum. Kv4.3 immunoreactivity was concentrated in the glomeruli and appeared lightly on somatic surfaces of granule cells (Fig. 3A). A glomerulus is formed by a bulbous terminal of mossy fibre wrapped around by Golgi cell axons and many granule cell dendrites. MAP2, a dendritic marker (Viereck *et al*., 1988), was co-localized with Kv4.3 in the glomeruli (see Fig. 7A). These results indicate that, in the glomeruli, Kv4.3 is expressed in the dendrites of posterior granule cells, instead of the axonal terminals of mossy fibres and Golgi cells. In addition, within the granular layer, Kv4.3 also appeared in deep Lugaro cells (described later), but no difference between the anterior and posterior granular layers was observed (data not shown). Thus, the granule cell is the only cell type in the granular layer responsible for this A–P compartmentalization.

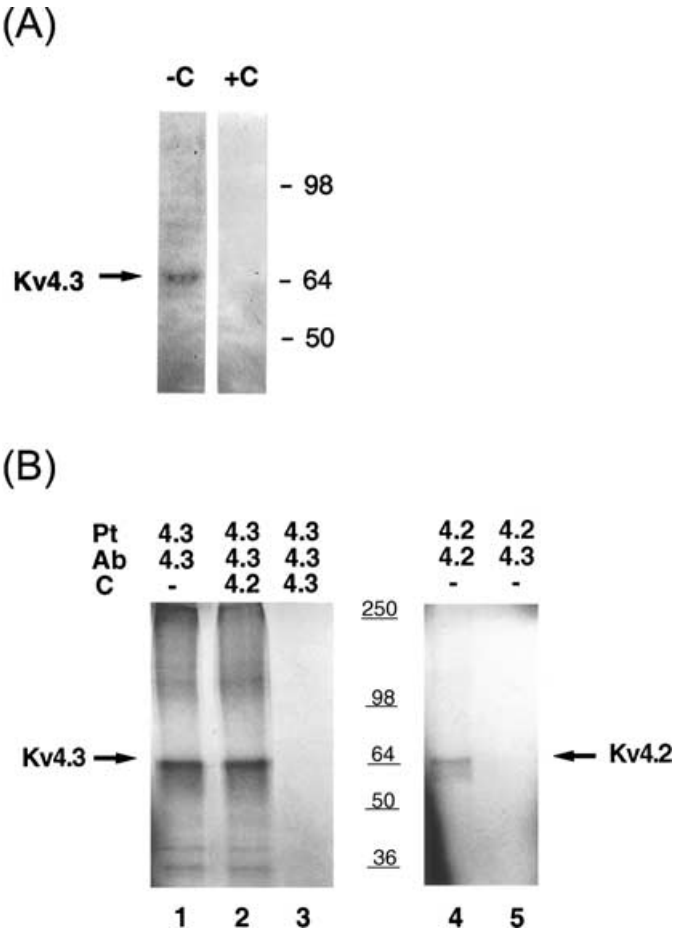

FIG. 1. Specificity of the Kv4.3 antibody. (A) Western blot analysis. Nitrocellulose membrane with rat brain membrane proteins was immunoblotted with Kv4.3 antibody in the absence (−C) or presence (+C) of excess Kv4.3 immunogenic peptides. Kv4.3 antibody recognizes a unique band of 69 kDa (arrow), which is consistent with the molecular weight of Kv4.3 protein. (B) Immunoprecipitation analysis. Identical amounts of [^{35}S]methionine-labelled Kv4.2 or Kv4.3 proteins (Pt) were immunoprecipitated by Kv4.2 or Kv4.3 antibody (Ab) in the absence or presence of excess Kv4.2 or Kv4.3 immunogenic peptides (C). Lanes 1 and 5, Kv4.3 antibody immunoprecipitates Kv4.3 protein but not Kv4.2 protein. Kv4.2 antibody immunoprecipitates Kv4.2 protein (lane 4). Lanes 2 and 3, Kv4.3 (but not Kv4.2) immunogenic peptides can prevent Kv4.3 antibody from immunoprecipitating Kv4.3 proteins. Positions of the molecular mass standards (in kDa) are indicated. The minor bands of 30–40 kDa (in lanes 1 and 2) are probably Kv4.3 degradation products.

To detect when A–P compartmentalization first appears, we examined Kv4.3 expression in immature granule cells and their precursors. At the embryonic stage, Kv4.3 was absent from both the external germinal layer (EGL, where granule cells are born) and the germinal trigone (where EGL neuron precursors originate) (see Fig. 6A and I). At the early postnatal stage, Kv4.3 remained undetectable from the EGL (Fig. 4A and C–F). Nevertheless, weak Kv4.3 immunostaining was found in granule cells located in the posterior granular layer, and thus A–P compartmentalization became visible on P8 (Fig. 4B and D). Owing to fewer granule cells on P8 than in adulthood, the intensity of Kv4.3 immunoreactivity in the posterior granular layer is weaker on P8

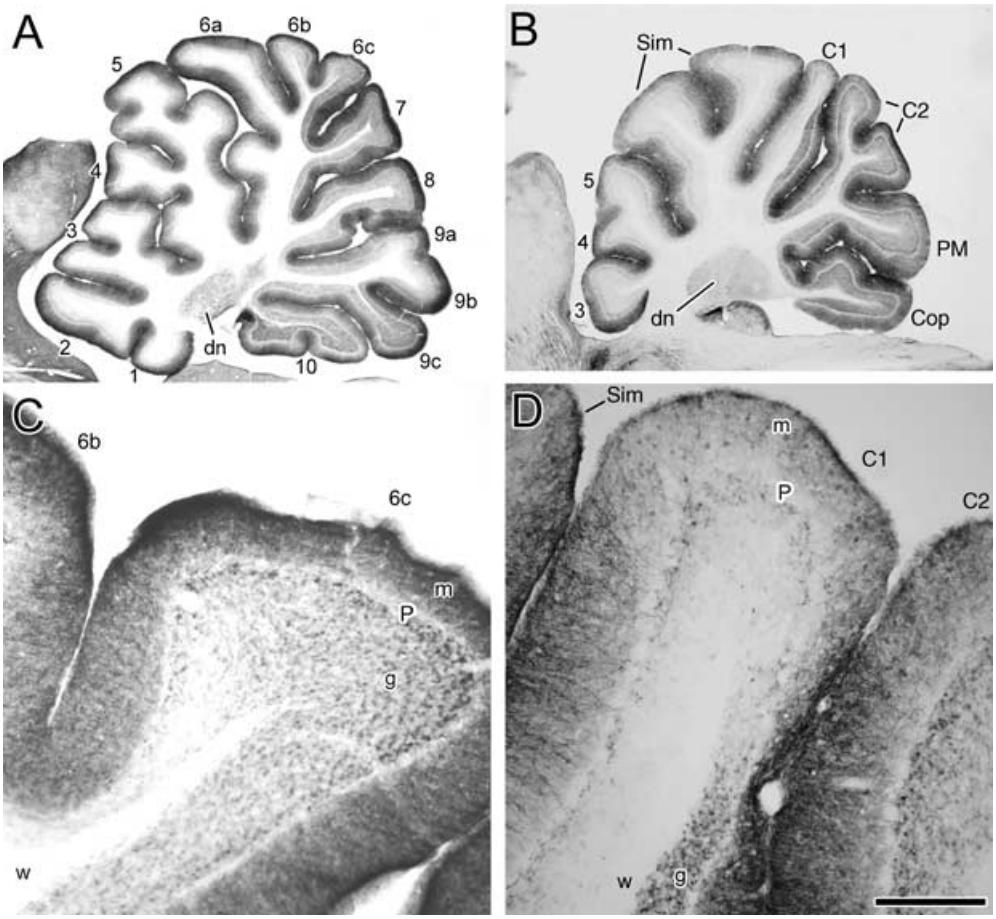

FIG. 2. Expression of Kv4.3 protein in adult rat cerebellum shows an A–P compartmentalization in the granular layer. Sagittal sections were used and immunostained with Kv4.3 antibody characterized in Fig. 1. Lobules of the vermis are numbered in Arabic numerals from 1 to 10 from rostral to caudal, and lobules of the hemisphere are indicated by their Latin names. (A and B) Patterns through the vermis and hemisphere, respectively. (C and D) Partial magnifications of the vermis lobule 6c in A and the hemispheric lobule crus 1 in B, respectively. In the granular layer, the intensity of the Kv4.3 immunoreactivity in the posterior cerebellum is stronger than it is in the anterior cerebellum, and the boundary is localized in the vermis lobule 6c extending laterally to the hemispheric crus 1 ansiform lobules. The outer part of the molecular layer in some regions had over-reacted. Scale bars, 1.6 mm (A and B); 187 μm (C and D).

than it is in adulthood (Figs 3A and 4D). These data indicate that A–P compartmentalization first appears on P8, after a significant number of post-migratory granule cells have settled in the posterior granular layer and begun to express Kv4.3.

Transient expression of Kv4.3 in several clusters of migrating Purkinje cells

In the embryonic cerebellum, Kv4.3 first appeared in cells located in the upper part of differentiating zone 3 (dz3) on E15.5 (Fig. 5A). The dz3 is a cell cluster formed by Purkinje cells after they are born from the neuroepithelium during E14–E16 (Altman & Bayer, 1997). During E16.5–E17.5, when dz3 has been divided into many Purkinje cell clusters and each cluster moves in unison along its unique migratory route, Kv4.3 was found in several migratory Purkinje cell clusters (Fig. 5B and C). TUJ1 has been used as a marker for newborn neurons (Menezes & Luskin, 1994). Co-expression of Kv4.3 and TUJ1 confirmed that these Kv4.3(+) cell clusters comprised newborn neurons (see Fig. 7B). Calbindin and Disabled-1 (Dab1) have been found in migrating Purkinje cells (Wassef *et al*., 1985; Rice *et al*., 1998), whereas an N-type Ca^{2+} channel has been detected in migrating granule cells (Komuro & Rakic, 1992). Interestingly, Kv4.3 was co-localized with Dab1 and an N-type Ca^{2+} channel in some Purkinje cell clusters (Fig. 5; Table 1), but not with calbindin (Fig. 6A–D; Table 1). On E19.5, Kv4.3(+) Purkinje cell clusters appeared discontinuous along the primitive Purkinje cell layer and remained non-overlapping with calbindin(+) Purkinje cell clusters (Figs 6E and F, and 7C). M-L compartmentalization of Kv4.3 thus appeared.

The expression pattern of Kv4.3 in Purkinje cells changed dramatically after birth. On P2, Kv4.3 remained detectable from some Purkinje cells in the hemispheres but not in the vermis, where they had become calbindin(+) (Fig. 6G and H). On P5, when Purkinje cells had aligned into a monolayer, calbindin appeared in all Purkinje cells, whereas Kv4.3 had completely disappeared from Purkinje cells (Fig. 6I and J). Although Kv4.3 had been detected from some spindle-shaped cells located just beneath Purkinje cells starting from P5 (Fig. 4A), double immunostaining indicated that they were Lugaro cells (Fig. 7D) (described later). These data indicate that, after Purkinje cells have completed the migration, previous calbindin(+) Purkinje cells continue expressing calbindin, whereas previous Kv4.3(+) Purkinje cells stop expressing Kv4.3 and switch to express calbindin.

Kv4.3 mRNA has been detected from Purkinje cell somata in adulthood (Tsaur *et al*., 1997; Serôdio & Rudy, 1998). However, Kv4.3 was absent from the somata of Purkinje cells soon after the migration is completed (Fig. 7D). We wondered whether Kv4.3 protein was localized in another subcellular domain of Purkinje cells, such as the proximal and distal dendrites extending throughout the molecular

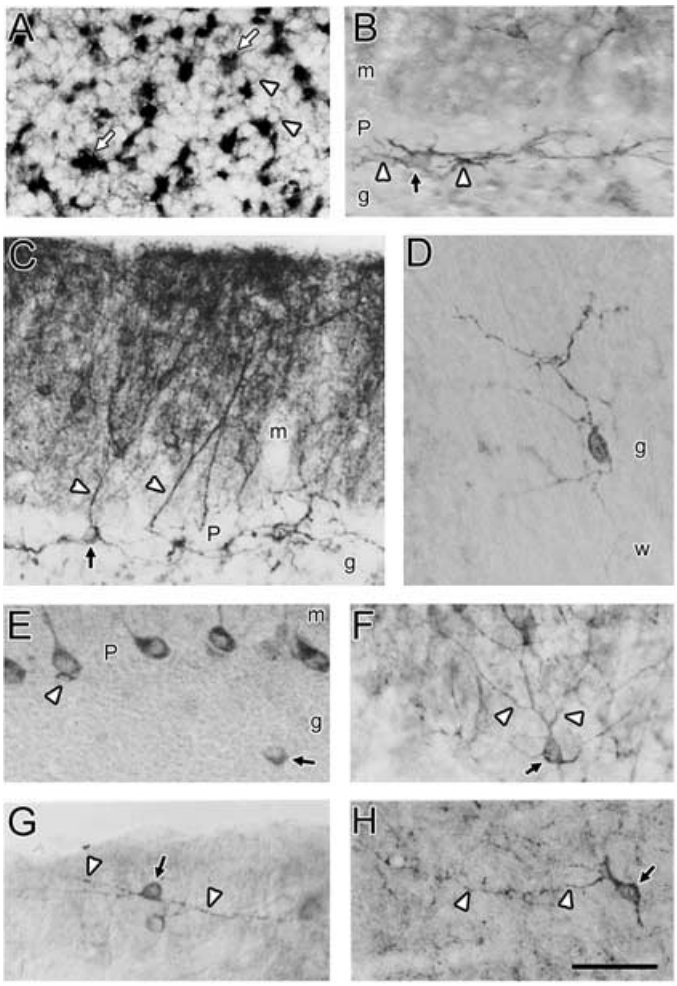

FIG. 3. Expression of Kv4.3 in adult rat cerebellar neurons. Horizontal (A–C) and sagittal (D–H) sections were used and immunostained with Kv4.3 antibody, except E for mGluR5 antibody. (A) In the posterior granular layer (at 12-μm thickness), Kv4.3 proteins appear in granule cells, mainly on the dendrites aggregated in the glomeruli (arrows) and to a lesser extent on the somatic surfaces (arrowheads). (B) In the anterior cerebellum, at the junction of the granular layer and the Purkinje cell layer, Kv4.3 could be clearly detected from the horizontally orientated dendrites (arrowheads) of Lugaro cells, but not their cell bodies (arrow). (C) At the same location as B, but on less frequent occasions, Kv4.3 could be found in the somata of Lugaro cells (arrow) and their vertical dendrites (arrowheads) extending into the molecular layer. (D) At various depths of the anterior granular layer, a few large Kv4.3(+) cells could be found, which are presumably deep Lugaro cells. (E) mGluR5 appears in both Lugaro cells (arrowhead) and deep Lugaro cells (arrow), a pattern similar to that seen for Kv4.3. This mGluR5 antibody also cross-reacts with mGluR1, which is highly expressed in Purkinje cells. (F) In the lower one-third of the molecular layer, Kv4.3 is present on the somata of basket cells (arrow) and their dendrites (arrowheads). (G) In the upper one-third of the molecular layer, Kv4.3 appears in the somata of stellate cells (arrow) and their major dendrites extending from the opposite sites of the cell body. (H) In the deep nuclei, Kv4.3 was found mainly in the dendrites of deep neurons (arrowheads), and less frequently in their somata (arrow). Scale bars, 38 μm (A, B and F); 54 μm (C and E); 32 μm (D); 27 μm (G and H).

layer, or the axons projecting to the deep nuclei. Calbindin is present in all the subcellular domain of Purkinje cells starting from P5 and continues throughout adulthood (Baimbridge & Miller, 1982; Garin & Escher, 2001), and therefore it could be used as a marker for Purkinje cells. Double immunofluorescent staining showed that Kv4.3 did not overlap with calbindin in any subcellular domain of Purkinje cells in adulthood or on P14 (Fig. 7E and F). In summary, Kv4.3 protein is present in a subset of Purkinje cells only at the migratory stage, and is absent from all Purkinje cells starting from the early postnatal stage and thereafter.

Expression of Kv4.3 in post-migratory Lugaro cells and deep Lugaro cells

In the adult cerebellum, along the junction of the Purkinje cell layer and the granular layer, we found Kv4.3 immunoreactivity in the horizontally elongated processes (Fig. 3B). Without the interference of immunostaining from the posterior granular cells, the processes could clearly be seen in the anterior cerebellar cortex (Fig. 3B). The processes originated from both poles of the spindle-shaped cell bodies located just below the Purkinje cell layer, and these cell bodies showed higher Kv4.3 immunoreactivity at the early postnatal stage than in adulthood (Figs 3B and 4F). Less frequently, Kv4.3 could be also found in their vertically orientated processes extending within the molecular layer (Fig. 3C). Co-localization of Kv4.3 and calretinin, a marker for Lugaro cells (Dieudonné & Dumoulin, 2000), supported the finding that Kv4.3 was expressed in Lugaro cells (Fig. 7G). Similar to the dendritic arborization described previously (Lainé & Axelrad, 1996), the horizontal and vertically orientated processes are presumably the dendrites of Lugaro cells. Post-migratory Lugaro cells can be morphologically recognized as early as P4.5 at this junction (Lainé *et al*., 1992). Consistently, we detected a few Kv4.3(+) Lugaro cells at this junction on P5 (Fig. 4A), and the cell number increased as the cerebellum gradually enlarged (Fig. 4C and E).

In addition to Lugaro cells, Kv4.3 also appeared in a very few large ovoid cells scattered at variable depths within the granular layer (Fig. 3D). They were not Golgi cells as indicated by the absence of somatostatin (data not shown), a marker for Golgi cells (Vincent *et al*., 1985; Geurts *et al*., 2001). Similar to Lugaro cells and unipolar brush cells, they also expressed calretinin (Geurts *et al*., 2001). Based on their morphology (Fig. 3D), fewer cell numbers than with Lugaro cells, their higher density in lobules I, II and VII–X of the vermis (data not shown) and that they do not overlap with calretinin(+) unipolar brush cells (Fig. 7H), we reason that these Kv4.3(+) cells are deep Luagro cells (Lainé & Axelrad, 2002). The expression pattern of Kv4.3 in the anterior granular layer was similar to that of mGluR5 (Fig. 3E), which has been found in both Lugaro cells and deep Lugaro cells (Neki *et al*., 1996). Taken together, these results support Kv4.3 being expressed in Lugaro cells and deep Lugaro cells.

Expression of Kv4.3 in post-migratory basket cells and stellate cells

In the adult cerebellum, Kv4.3 immunoreactivity was also present in cells scattered in the molecular layer (Fig. 3F and G). Basket cells are located in the lower one-third and stellate cells in the upper two-thirds of the molecular layer (Palay & Chan-Palay, 1974). Co-localization of Kv4.3 and parvalbumin (Fig. 7I), a marker for basket cells and stellate cells (Fortin *et al*., 1998), indicates that Kv4.3 is present in both types of interneurons. Because Kv4.3 was absent from the bases of Purkinje cell somata and their initial axon segments where basket cell axon terminal plexuses are concentrated, it is conceivable that the Kv4.3(+) processes arising from basket cell somata toward the pia surface are their dendrites (Fig. 3F).

Basket cells and stellate cells originate from the neuroepithelium and further divide in the white matter during migration before arriving in the molecular layer (Hallonet *et al*., 1990; Zhang & Goldman, 1996). On P8, soon after the peak production of basket cells (P6–P7), many Kv4.3(+) cells were found in the lower one-third of the

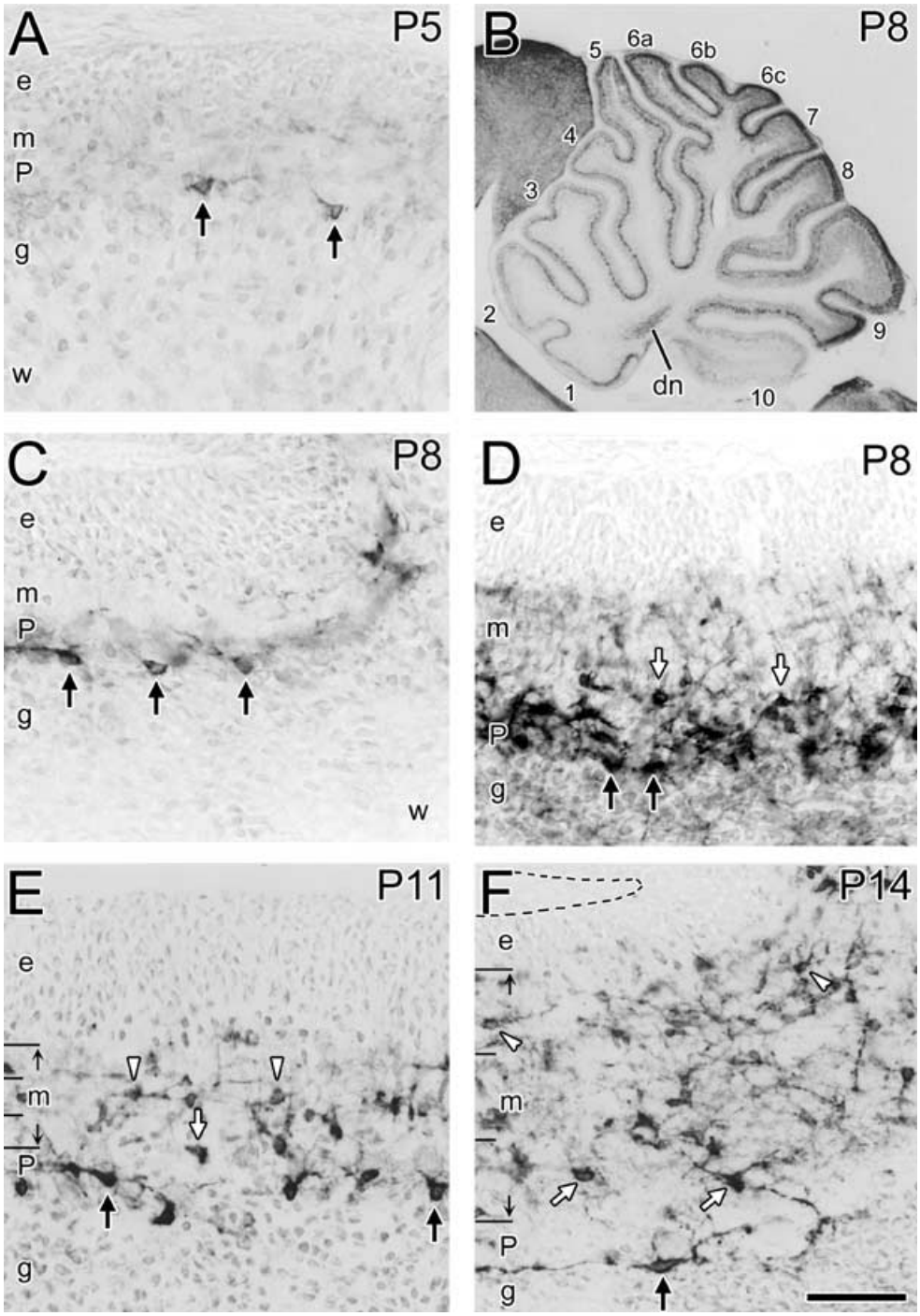

FIG. 4. Expression of Kv4.3 in P5–P14 rat cerebellum. Sagittal sections were used for immunohistochemistry. Lugaro cells (black arrows), basket cells (white arrows) and stellate cells (white arrowheads) are indicated. (A) In P5 anterior cerebellum, Kv4.3 first appears in a few post-migratory Lugaro cells located at the junction of the granular layer and the Purkinje cell layer. Kv4.3 is absent from the EGL. (B) On P8, when sufficient granule cells have migrated into the posterior granular layer and started to express Kv4.3, the A–P compartmentalization becomes visible (compare with the granular layer in C and D). (C) Higher magnification of anterior cerebellum shown in B. More Kv4.3(+) Lugaro cells are observed than on P5. (D) Higher magnification of posterior cerebellum shown in B. Basket cells begin to express Kv4.3 after they have migrated into the lower one-third of the molecular layer. (E) In P11 anterior cerebellum, there are only a few Kv4.3(+) cells in the middle third of the molecular layer, which are presumably post-migratory stellate cells. The molecular layer is roughly divided into three parts, as shown also in F. (F) In P14 anterior cerebellum, more Kv4.3(+) stellate cells appear in the upper two thirds of the molecular layer. The dashed line marks the outer margin of the EGL. Note that Kv4.3 is absent from the entire EGL throughout the early postnatal stage. Scale bars, all 38 μm, except 920 μm in B.

molecular layer (Fig. 4D). Similarly, on P11, on the last day for the peak production of stellate cells (P8–P11), there were a few Kv4.3(+) cells in the middle third of the molecular layer (Fig. 4E). Several days later, on P14, some Kv4.3(+) cells could also be observed in the upper one-third of the molecular layer (Fig. 4F). Kv4.3 was not found in the migrating basket cells or stellate cells (data not shown). These results indicate that basket cells and stellate cells express Kv4.3 only at their post-migratory stages.

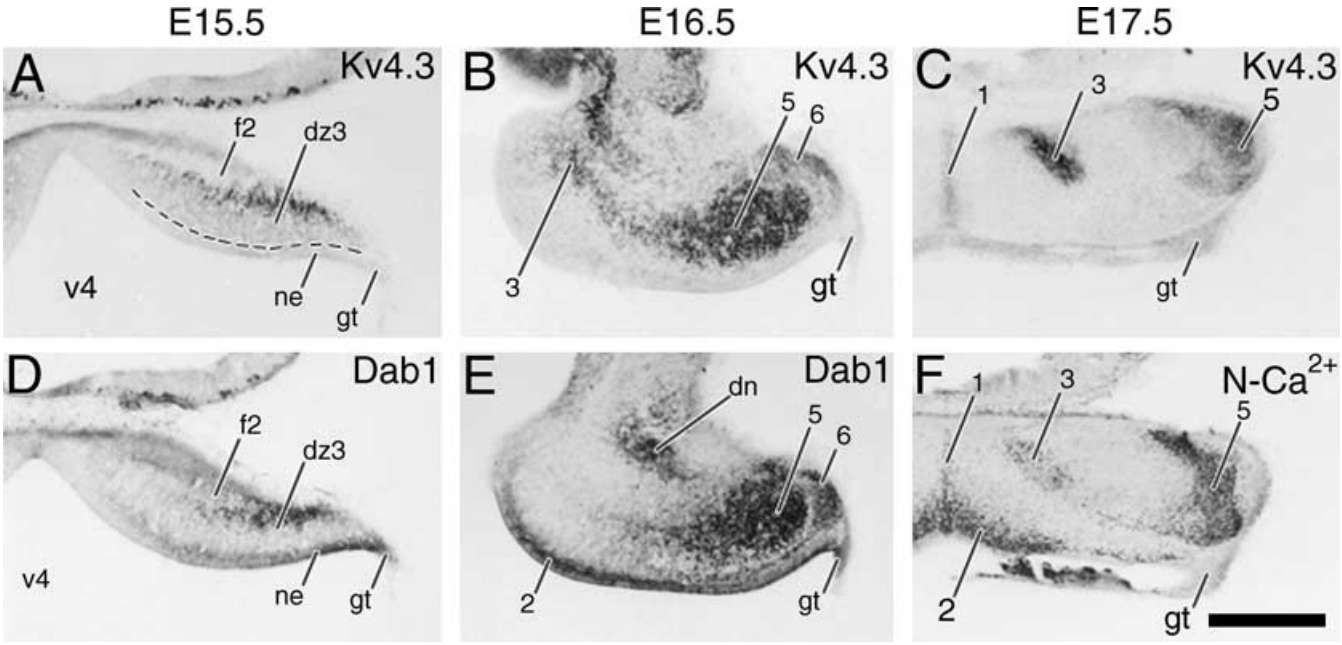

FIG. 5. Expression of Kv4.3 in E15.5–E17.5 rat cerebellum. Coronal sections were used for immunohistochemistry. Purkinje cell clusters are designated with Arabic numerals from medial to lateral. (A and D) On E15.5, both Kv4.3 and Dab1 were detected from the upper margin of dz3, which represents the first group of migrating Purkinje cells. The remainder of dz3 contains premigratory Purkinje cells. Dab1 is also present in the neuroepithelium. (B and E) On E16.5, when Purkinje cells are migrating in clusters, Kv4.3 is co-localized with Dab1 in some clusters (nos. 5 and 6). Dab1(+) migrating deep neurons do not express Kv4.3. (C and F) On E17.5, Kv4.3 is co-expressed with N-type Ca^{2+} channel in certain Purkinje cell clusters (nos. 3 and 5). Scale bar, 375 μm (A, B, D and E); 530 μm (C and F).

TABLE 1. Expression of Kv4.3, Disabled-1 (Dab1), N-type Ca^{2+} channel (N-Ca^{2+}) and calbindin (CaB) in different clusters of migrating Purkinje cell clusters in E16.5–E17.5 rat cerebellum

Cluster	Kv4.3	Dab1	N-Ca^{2+}	CaB
1	+	–	+	–
2	–	+	+	+
3	+	–	+	–
4	–	–	–	+
5	+	+	+	–
6	+	+	–	–
7	–	–	ND	+
8	–	+	ND	+

+, significant expression; –, no expression; ND, not determined.

Expression of Kv4.3 in a subset of post-migratory GABAergic deep neurons

In the embryonic cerebellum, Kv4.3 first appeared in deep neurons on E19.5 (Fig. 6E), when they had just completed migration (Altman & Bayer, 1997). Kv4.3 immunoreactivity was absent from the neuroepithelium where deep neurons originate (data not shown), as well as the migrating deep neurons (compare Fig. 5B and E). After birth and thereafter, in addition to the somata of some deep neurons, Kv4.3 also appeared in their processes spreading within the deep nuclei (Fig. 3H). Double immunostaining of Kv4.3 and GABA showed that Kv4.3 was expressed in some but not all GABAergic deep neurons (Fig. 7J). These results indicate that only a subset of GABAergic deep neurons express Kv4.3 in their post-migratory stage.

Discussion

We have examined the spatiotemporal expression of Kv4.3 protein in rat cerebellar neurons. The relative intensities of Kv4.3 immunostaining in the neuronal cell types at various developmental stages are summarized in Fig. 8. At the subcellular level, except for Purkinje cells, Kv4.3 appeared on the somata soon after these neurons had finished migration, and gradually concentrated in their dendrites. Most interestingly, Kv4.3 showed M-L compartmentalization in migrating Purkinje cells, in addition to the A–P compartmentalization in post-migratory granule cells. The possible functions of Kv4.3 in Purkinje cells, granule cells and Lugaro cells are discussed below.

Possible function of Kv4.3 with M-L compartmentalization in Purkinje cells

Kv4.3 was transiently expressed in several migrating Purkinje cell clusters during E16.5–P2 (Figs 5 and 6), and a pattern of M-L compartmentalization could be clearly detected on E19.5 (Figs 6E and 7C). Similar M-L compartmentalization at the embryonic stage has also been found in many other molecules, such as N-type Ca^{2+} channel, Dab1 (Fig. 5; this report), calbindin, a cyclic GMP-dependent protein kinase, a Purkinje cell-specific glycoprotein (Wassef & Sotelo, 1984, Wassef *et al*., 1985), En-1, En-2, Wnt-7b, Pax-2, PEP19, L7 (reviewed in Herrup & Kuemerle, 1997), cadherins (Arndt *et al*., 1998), Eph receptors and ephrins (Lin & Cepko, 1998; Karam *et al*., 2000). It has been suggested that molecules showing compartmentalization during development are involved in the formation of the cerebellar map. Cerebellar map formation includes foliation and lobulation, as well as A–P and M-L patterning. The map divides the cerebellum into many subregions, which differ in anatomy and function (Oberdick *et al*., 1998). The heterogeneity of Purkinje cells and precerebellar afferents may underlie the formation of the cerebellar map. For example, the inferior olive (one of the origins of precerebellar afferents) can be divided into many subregions, and each subdivision projects to a particular longitudinal strip of the cerebellar cortex (reviewed in Voogd, 1995). Altman & Bayer (1997) suggested that, during the formation of the cerebellar map, different Purkinje cell

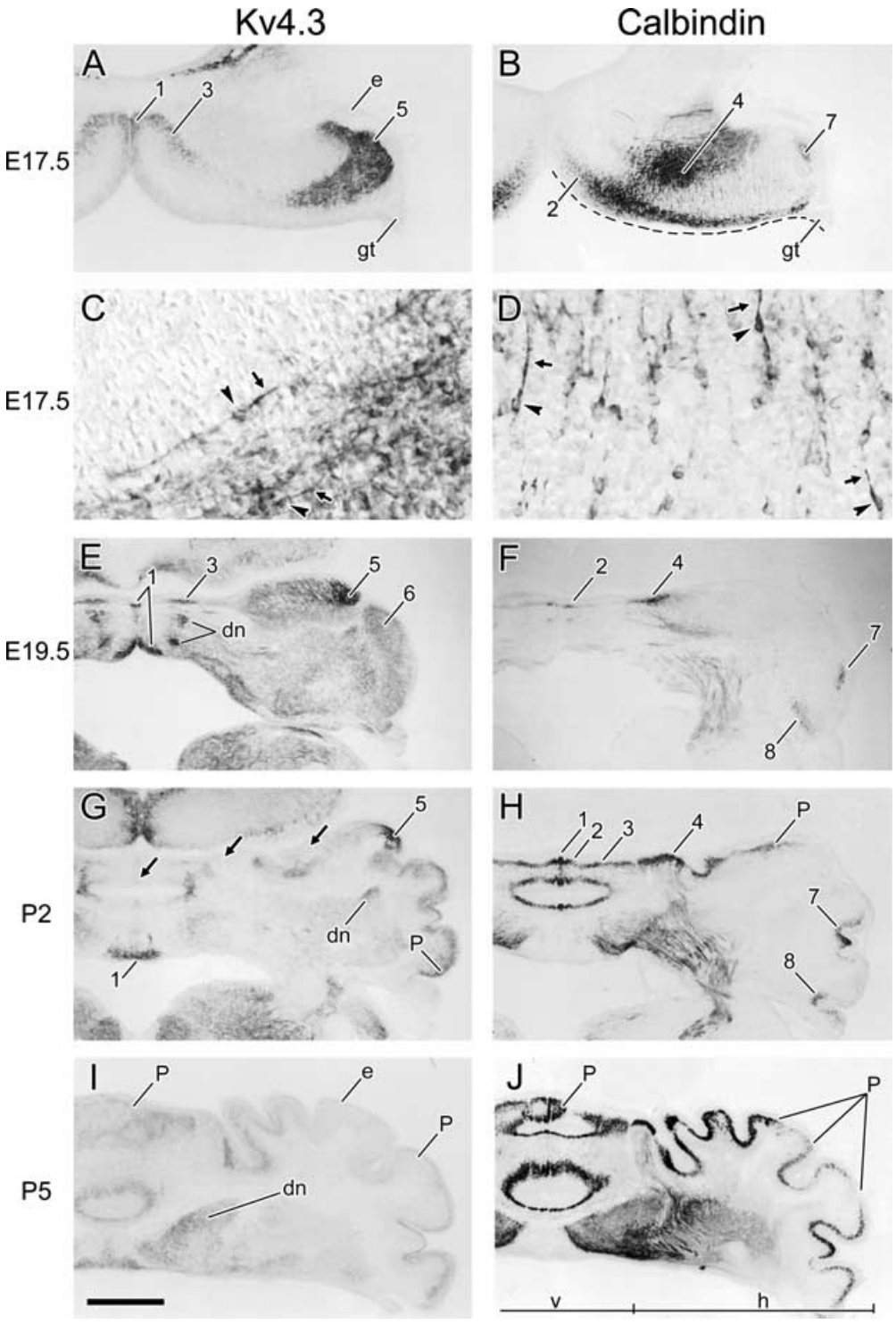

FIG. 6. Expression of Kv4.3 and calbindin in E17.5–P5 rat cerebellum. Coronal sections were used for immunohistochemistry. (A and B) On E17.5, Kv4.3 and calbindin are localized in different clusters of migrating Purkinje cells. (C and D) Higher magnifications of cluster no. 5 in A and cluster no. 4 in B, showing migrating Purkinje cells with spindle-shaped cell bodies (arrowheads) and long leading processes extending in the direction of migration (arrows). (E and F) On E19.5, Kv4.3(+) (nos. 1, 3 and 5) and calbindin(+) (nos. 2 and 4) Purkinje cell clusters appear complementary along the primitive Purkinje cell layer. (G) On P2, Kv4.3 has disappeared from most Purkinje cells (arrows), except some in clusters 1 and 5. (H) On P2, Purkinje cells that were calbindin(+) on E19.5 (in clusters 2, 4, 7 and 8) continue expressing calbindin, whereas those that were Kv4.3(+) on E19.5 (in clusters 1 and 3) have switched to express calbindin. (I and J) On P5, Kv4.3 is no longer detectable from Purkinje cells, whereas calbindin is highly expressed in all Purkinje cells except some in the hemispheres. Note that Kv4.3 begins to appear in deep neurons on E19.5, but it has never been found in the germinal trigone or the external germinal layer. Scale bar, 375 μm (A and B); 38 μm (C); 32 μm (D); 530 μm (E–J).

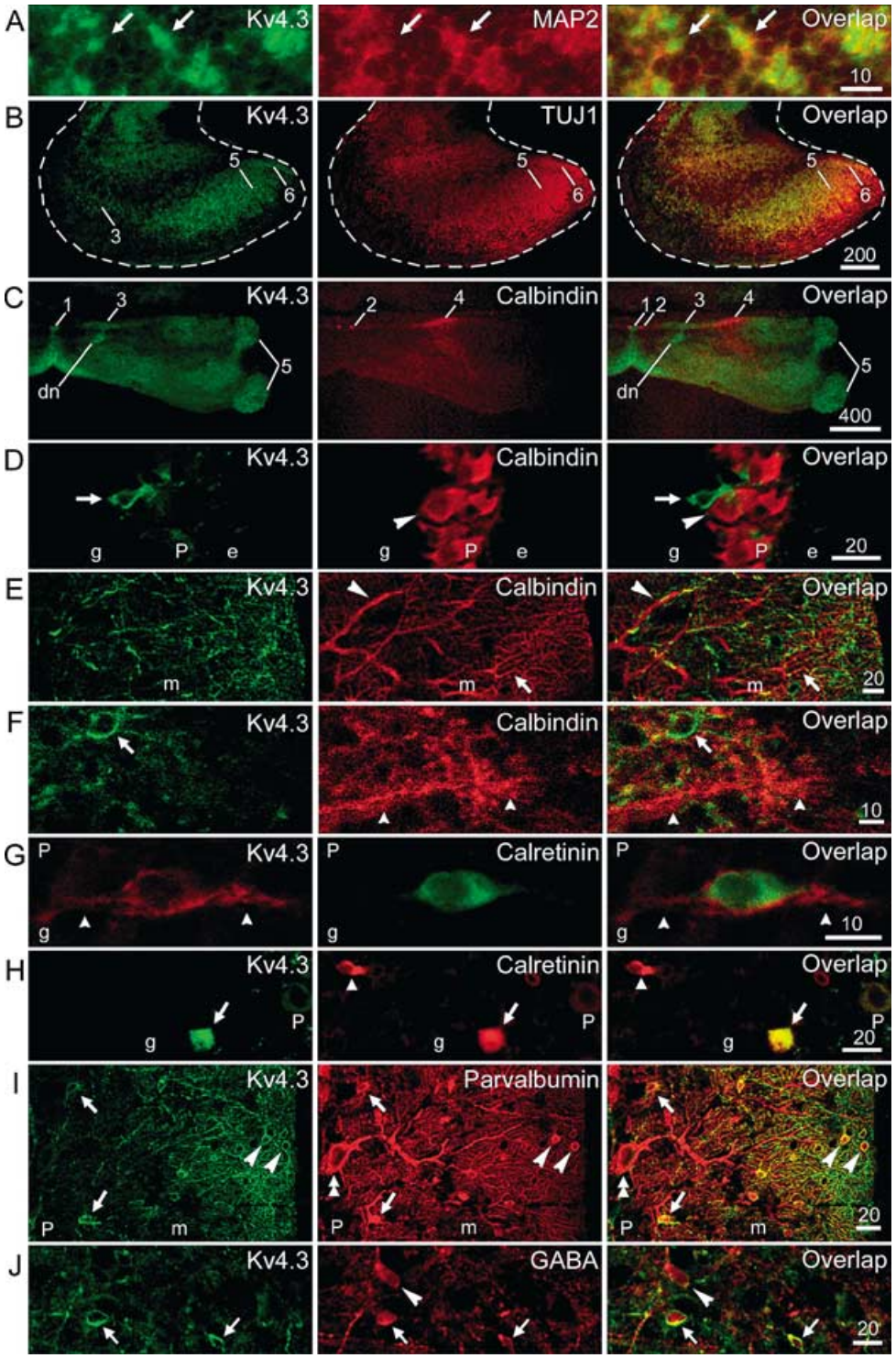

FIG. 7. Confocal microscopic images of Kv4.3, various markers in rat cerebellum at various developmental stages. Sagittal (except B and C in coronal) sections were used for double immunofluorescent staining. (A) In adult granular layer (12-μm thickness), co-localization of Kv4.3 (green) and MAP2 (red) in the dendrites of posterior granule cells, which are aggregated in the glomeruli (arrows). (B) On E16.5, coexistence of Kv4.3 (green) and TUJ1 (red) in newborn neurons (clusters 5 and 6, as shown in Fig. 5B). Each Arabic numeral indicates a cluster of migrating Purkinje cells. (C) On E19.5, Kv4.3(+) (green; nos. 1, 3 and 5) and calbindin(+) (red; nos. 2 and 4) Purkinje cell clusters did not overlap along the primitive Purkinje cell layer. (D) On P5, calbindin (red) was present in all Purkinje cells (arrowhead). A Kv4.3(+) cell (green, arrow) appeared just below the Purkinje cell layer and it is likely to be a Lugaro cell. (E) In adult molecular layer, calbindin (red) was present in the proximal dendrites (arrowhead) and distal dendrites (arrow) of Purkinje cells. Non-overlapping of Kv4.3 (green) and calbindin (red) indicates that Kv4.3 is absent from the dendrites of Purkinje cells. (F) In P14 deep nuclei, Kv4.3 (green) appeared on the somatic surfaces of some deep neurons (arrow) but is absent from the calbindin-positive (red) Purkinje cell axons (arrowheads). (G) On P14, at the position just below the Purkinje cell layer, Kv4.3 (red) was present on the somatic surfaces and the horizontally orientated dendrites (arrowheads) of a Lugaro cell, whereas calretinin (green) was localized in its cytosol. (H) In P14 granular layer, co-expression of Kv4.3 (green) and calretinin (red) in deep Lugaro cells (arrow). Arrowhead indicates a calretinin(+) unipolar brush cell. (I) In adult molecular layer, Kv4.3 (green) is co-localized with pavalbumin (red) in basket cells (arrows) and stellate cells (arrowheads), but not Purkinje cells (double arrowhead). (J) On P6, Kv4.3 (green) was expressed in a subset of GABA(+) (red) deep neurons (arrows). Arrowhead indicates a Kv4.3(–) GABAergic deep neuron. Scale bars (in μm) are indicated in the right-hand panels.

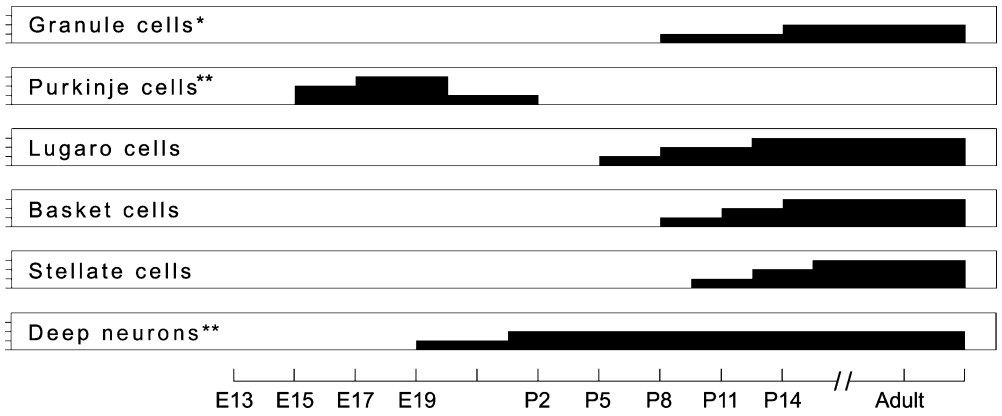

FIG. 8. Temporal expression of Kv4.3 in cerebellar neurons. Expression of Kv4.3 in each type of cerebellar neuron at different developmental stages is rated on an arbitrary scale according to the intensity of Kv4.3 immunoreactivity. A single asterisk indicates granule cells located in the posterior granular layer; a double asterisk indicates only a subpopulation of Purkinje cells or deep neurons.

clusters make selective somato-axonic contact with specific precerebellar afferents and drag them toward the EGL. After Purkinje cells have completed migration, settled Purkinje cells and precerebellar afferents they have dragged segregate into different structural and functional compartments, such that the cerebellar map forms. Therefore, it is likely that expression of Kv4.3 in migrating Purkinje cells is involved in the formation of the cerebellar map. In Kv4.3(+) migrating Purkinje cells, neuronal excitability modulated by Kv4.3 may be required to maintain the somato-axonic contact to drag the precerebellar afferents. Alternatively, the appearance of I_A evoked by Kv4.3 may induce the expression of certain molecules necessary for dragging the afferents.

Whether Kv4.3 is expressed in mature Purkinje cells in adulthood remains controversial. Our data showed that Kv4.3 protein was absent from all the subcellular domains of mature Purkinje cells, which is inconsistent with previous findings with Kv4.3 mRNA from *in situ* hybridization (Tsaur *et al*., 1997; Serôdio & Rudy, 1998). This discrepancy suggests that Kv4.3 mRNAs could not be translated into Kv4.3 proteins in mature Purkinje cells. Alternatively, the probes used for *in situ* hybridization might recognize another molecule, which is highly expressed in mature Purkinje cells.

Kv4.3 with A–P compartmentalization in granule cells: possible function and mechanism of formation

Serôdio & Rudy (1998) have shown an A–P compartmentalization of Kv4.3 mRNA in the granular layer. Here, at the protein level, our results indicate that the granule cell is the only cell type in the granular layer involved in A–P compartmentalization, and Kv4.3 protein is selectively expressed in the somatodendritic domain of posterior granule cells (this report). By contrast, another Kv4 member, Kv4.2, is evenly expressed in all granule cells within the same domain (Sheng *et al*., 1992). It has been found that I_A is the dominant K^+ current in all granule cells (Bardoni & Belluzzi, 1993), and Kv4 channels account for this current (Shibata *et al*., 2000). No report has thus far indicated that the property of I_A in anterior granule cells differs from that in posterior granule cells. It is likely that Kv4.2 is the major component of I_A detected in all granule cells, whereas Kv4.3 provides a fine tuning for it only in the posterior granule cells, via biochemical (such as phosphorylation) or electrical modification (such as forming a complex with KChIP auxiliary proteins).

Using a transgenic animal model, Hawkes *et al*. (1999) observed an A–P compartmentalization in granule cells, which has a boundary similar to that of Kv4.3 (Fig. 2). Their data suggest that the formation of A–P compartmentalization is due to endogenously different pools of granule cell precursors in the germinal trigone, which is one of the models proposed by Ozol & Hawkes (1997). Among the genes (< 10) showing A–P compartmentalization in granular cells (reviewed in Herrup & Kuemerle, 1997; McAndrew *et al*., 1998), the expression pattern of Kv4.3 is most similar to that of *Otx2*, a homeobox gene encoding a transcription factor (Simeone *et al*., 1992). A strong *Otx2* mRNA signal has been detected from the posterior EGL during E18–P3 in the mouse (McAndrew *et al*., 1998), with a time course earlier than that of Kv4.3. Whether *Otx2* contributes to the selective expression of Kv4.3 in posterior granule cells will be an interesting question for future investigation.

Possible function of Kv4.3 in Lugaro cells

We observed Kv4.3 expression in Lugaro cells, including those located just beneath the Purkinje cell layer and those randomly distributed within the granular layer (also known as deep Luagro cells). Lugaro cells are selectively excited by serotonin, and the serotonergic input to the cerebellum is crucial for several types of ataxia, in addition to the glutaminergic input (Trouillas & Fuxe, 1993). Both signalling pathways are illustrated in Fig. 9 (reviewed in Voogd, 1995; Altman & Bayer, 1997; Lainé & Axelrad, 1998). The functioning of Lugaro cells allows part of the cerebellar cortex to switch from a glutaminergic to serotonergic influence, when fine control of ongoing movement is required. An I_A activated around the resting potential has been detected from the somatodendritic domain of Lugaro cells, and inhibition of I_A is necessary for the depolarization of Lugaro cells after binding of serotonin (Dieudonné & Dumoulin, 2000). Abundant expression of Kv4.3 in the dendrites of Lugaro cells in mature cerebellum suggests that Kv4.3 could be the molecular determinant underlying this I_A.

Kv4.3M is the major splicing form of Kv4.3 in the cerebellum

Three Kv4.3 splicing variants have been isolated from rat brain: Kv4.3L (Ohya *et al*., 1997), Kv4.3M (Serôdio *et al*., 1996) and Kv4.3S (Tsaur *et al*., 1997). Although we have demonstrated the specificity of Kv4.3 antibody by both Western blot and immunoprecipitation analysis, this antibody cannot distinguish the three variants because the immunogenic peptide sequence is present in all of them. Therefore, the immunohistochemical staining represents a combined pattern of these variants. These variants have similar electrophysiological properties, but they may have differing modulatory mechanisms, such as phosphorylation. By quantitative RT-PCR, Ohya *et al*. (2001) detected Kv4.3M and Kv4.3L from adult rat cerebellum in a ratio of 86 : 14, indicating that Kv4.3M is the major

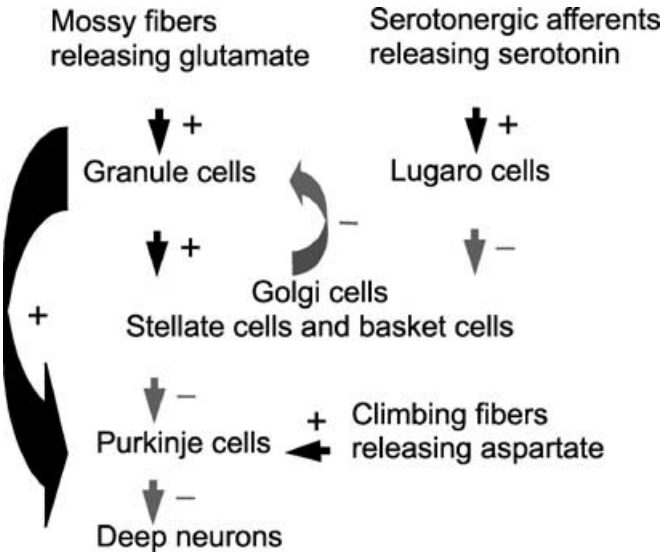

FIG. 9. Diagram of the inputs and intrinsic connections within the rat cerebellum. Two major inputs (mossy fibres and climbing fibres) and one minor input (serotonergic afferents) are shown. Climbing fibres directly contact with Purkinje cells, while mossy fibres transmit input signals via granule cells to Purkinje cells. The functions of Purkinje cells and granule cells are modulated by interneurons scattered within the cerebellum, such as basket cells, stellate cells and Golgi cells. These interneurons are further inhibited by Lugaro cells, which are activated by serotonergic afferents. After receiving the integrated information from Purkinje cells, deep neurons send the output signals to various brain regions that are under cerebellar influence. '+', excitatory effect; '–', inhibitory effect.

form of Kv4.3 in the cerebellum. To determine which Kv4.3 form is expressed in certain types of cerebellar neurons, single-cell RT-PCR will be a useful approach.

Functions of A-type K^+ channels in immature neurons

By contrasting the expression of Kv4.3 in post-migratory and migratory neurons, it is clear that A-type K^+ channels play important roles not only in mature neurons but also in immature neurons. In addition to Kv4.3 in migrating Purkinje cells, other A-type K^+ channel α subunits have also been detected from immature neurons. For example, Kv1.4 was found in migrating Cajal–Retzius cells (Mienville *et al*., 1999), a type of neuron that appears at the onset of corticogenesis and disappears at the end of neuronal migration. Recently, Kv3.4- and Kv4.2-like proteins were detected from the growth cones of the developing *Xenopus* retinal ganglion cells (Pollock *et al*., 2002). These data suggest that A-type K^+ channel α subunits are required for the development of central and peripheral nervous systems. Taken together, these findings will be useful in elucidating the possible functions of A-type K^+ channels in immature neurons, and subsequently promote a better understanding of the developing nervous system.

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Abbreviations

(+), immunoreactive to a specific molecule as indicated; A–P, anterior–posterior; C1, crus 1 ansiform lobule; C2, crus 2 ansiform lobule; cp, choroid plexus; Cop, copula pyramis lobule; dn, deep neurons; dz3, differentiating zone 3; E, embryonic day; e (in figures) and EGL (in text), external germinal layer; f2, fibrous zone 2; g, granular layer; gt, germinal trigone; h, hemisphere; I_As, A-type K^+ currents; m, molecular layer; ne, neuroepithelium; P, Purkinje cell layer (in figures) and postnatal day (in text); PM, paramedian lobule; Sim, simple lobule; v, vermis; v4, fourth ventricle; w, white matter.

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