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Expression and Coexpression of CO_2-sensitive Kir Channels in Brainstem Neurons of Rats

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Abstract. Several inward rectifier K^+ (Kir) channels are inhibited by hypercapnic acidosis and may be involved in CO_2 central chemoreception. Among them are Kir1.1, Kir2.3, and Kir4.1. The Kir4.1 is expressed predominantly in the brainstem. Although its CO_2 sensitivity is low, coexpression of Kir4.1 with Kir5.1 in *Xenopus* oocytes greatly enhances the CO_2/pH sensitivities of the heteromeric channels. If these Kir channels play a part in the central CO_2 chemosensitivity, they should be expressed in neurons of brainstem cardio-respiratory nuclei. To test this hypothesis, we performed in-situ hybridization experiments in which the expression of Kir1.1, Kir2.3, Kir4.1 and Kir5.1, and coexpression of Kir4.1 and Kir5.1 were studied in brainstem neurons using non-radioactive riboprobes. We found that mRNAs of these Kir channels were present in several brainstem nuclei, especially those involved in cardio-respiratory controls. Strong labeling was observed in the locus coeruleus, ventralateral medulla, parabrachial-Kölliker-Fuse nuclei, solitary tract nucleus, and area postrema. Strong expression was also seen in several cranial motor nuclei, including the nucleus of ambiguus, hypoglossal nucleus, facial nucleus and dorsal vagus motor nucleus. In general, the expression of Kir5.1 and Kir4.1 was much more prominent than that of Kir1.1 and Kir2.3 in all the nuclei. Evidence for the coexpression of Kir4.1 and Kir5.1 was found in a good number of neurons in these nuclei. The expression and coexpression of these CO_2/pH-sensitive Kir channels suggest that they are likely to contribute to CO_2 chemosensitivity of the brainstem neurons.

Key words: CO_2 central chemoreceptor — Cardio-respiratory control — Ion channel — Gene expression — In-situ hybridization

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Introduction

Inward rectifier K^+ channels (Kir) play an important role in the maintenance of membrane potential and cellular excitability (Hille, 1992). Activity of these channels is regulated by G proteins, nucleotides and hydrogen ions (Nichols & Lopatin, 1997). The modulation of these Kir channels by protons is significant, as changes in intra- and extracellular pH are seen in a wide variety of physiological and pathophysiological conditions, in which the Kir modulation may enable cells to sense and respond to their ambient PCO_2 and pH. Indeed, Kir channels have been shown to be involved in cellular responses to hypercapnia and acidosis in various tissues and cells (Ito, Vereecke & Carmeliet, 1992; Vivaudou & Forestier, 1995; Faraci & Sobey, 1996; Wang, Hebert & Giebisch, 1997; Ballanyi, Onimaru & Homma, I., 1999). One of the most important regulations takes place in the brainstem. Certain unidentified Kir channels have been shown to play a part in respiratory rhythmic activity (Smith et al., 1995; Johnson, Smith & Feldman, 1996) and CO_2 chemosensitivity of brainstem neurons (Pineda & Aghajanian, 1997). Hypercapnia produces depolarization and increase in firing activity of neurons in the locus coeruleus, a brainstem CO_2 chemosensitive area. This effect is mediated by certain pH-sensitive Kir channels that are inhibited during hypercapnia (Pineda & Aghajanian, 1997). Clearly, further understanding of these CO_2-sensitive Kir channels depends on the demonstration of specific channels and their expression in the brainstem, which may lead to a molecular identity of the CO_2 chemosensitive cells in the cardio-respiratory nuclei.

The precise species of Kir channels sensitive to CO_2 and pH have been studied over the past 5 years. Among them are the heteromeric Kir4.1-Kir5.1 and homomeric Kir1.1 and Kir2.3. These Kir channels are strongly inhibited by hypercapnia and intracellular acidosis with pKa values at near physiological pH levels (Coulter et al., 1995; Tsai et al., 1995;

Doi et al., 1996; Choe et al., 1997; Shuck et al., 1997; McNicholas et al., 1998; Zhu et al., 1999, 2000; Pearson et al., 1999; Tucker et al., 2000; Tanemoto et al., 2000; Xu et al., 2000). Other members in the Kir family are only slightly inhibited by hypercapnia and acidic pH or not at all (Shuck et al., 1997; Baukrowitz et al., 1999; Pearson et al., 1999; Yang & Jiang, 1999; Zhu et al., 2000). The heteromeric Kir4.1-Kir5.1 channels are particularly interesting in this regard. These channels have a pKa 7.45 (Xu et al., 2000; Yang et al. 2000), which allows them to detect modest hypercapnia and hypocapnia (Cui et al., 2001). The Kir4.1 is expressed predominantly in the brainstem in the central nervous system (Bredt et al., 1995), whereas the Kir5.1 does not express functional channels by its own homomers (Bond et al., 1994). Its sole biological significance known as yet is to form heteromeric channels, mainly with the Kir4.1 (Pessia et al., 1996; Tucker et al., 2000; Tanemoto et al., 2000; Xu et al., 2000; Yang et al., 2000; Casamassima et al., 2003). Thus, the Kir5.1 may be coexpressed with the Kir4.1 in neurons in brainstem cardio-respiratory nuclei. To test this hypothesis, we performed in-situ hybridization cytochemical experiments. We wanted to determine whether these Kir channels were expressed and co-expressed in the neurons in the medulla and pons where the cardio-respiratory neurons are located. In addition, we studied the distribution of Kir1.1 and Kir2.3 mRNAs in the brainstem, as these two Kir channels are also highly sensitive to CO_2 and pH (Coulter et al., 1995; Tsai et al., 1995; Doi et al., 1996; Zhu et al., 2000). Our results show that all these Kir channels are expressed in the brainstem, although their expression density and patterns appear different.

Materials and Methods

Reagent

DIG-11-UTP, T7 RNA polymerase, proteinase K, anti-DIG antibodies conjugated with alkaline phosphatase (AP), 4-nitrobluetetrazolium-chloride (NBT), and 5-bromo-4-chloro-3-indol-phosphate (BCIP) were purchased from Roche Diagnostics (Indianapolis, ID). The rest of chemicals including bovine serum albumin (BSA), dextran sulfate (MW 500,000), polyvinylpyrrolidone (PVP), Ficoll 400, yeast tRNA, salmon sperm DNA, deionzed formamide and levamisol were purchased from Sigma (St Louis, MI). All proteins and other macromolecules were purchased as molecular biology grade and RNase free.

Animals

Adult Sprague-Dawley rats (250–300g, either sex) were used in these studies. All procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Georgia State University Institutional Animal Care and Use Committee. The rats were deeply anesthetized by inhalation of saturated ether vapor, and then perfused transcardially with 100 ml phosphate buffer saline (PBS, containing in mM: 140 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4), followed by 500 ml 4% paraformaldehyde in the PBS buffer (0.1 M, pH 7.4). The brainstem was removed, post-fixed in the same fixative solution for 2 h at 4°C, and then dehydrated in 30% sucrose at 4°C overnight.

Preparation of Riboprobes

Kir4.1 and Kir5.1 cDNAs were kindly provided by Dr. John Adelman (Bond et al., 1994), Kir1.1 by Dr. Steven Hebert (Ho et al., 1993), and Kir2.3 by Dr. Carol Vandenberg (Perier et al., 1994). The Kir4.1 and Kir5.1 were cloned into pCDNA3.1(+) (Invitrogen, Carlsbad, CA) for antisense riboprobe synthesis and into pCDNA3.1(−) for the sense riboprobe. This was reversed in Kir1.1. The cDNAs were linearized at a restriction site (*Eco*RI for antisense Kir1.1, *Not*I for sense Kir1.1, *Xho*I for antisense Kir4.1 and Kir5.1, and *Kpn*I for sense Kir4.1 and Kir5.1) immediately before the 5′ untranslated sequence. The antisense and sense riboprobes were produced by in-vitro transcription using T7 polymerase for Kir4.1, Kir5.1 and Kir1.1. The original vector pBluescript was used for sense (using T3 polymerase and Hind III site for linearization) and antisense (T7, Xba I) synthesis in Kir2.3. The antisense and sense riboprobes were labeled with Digoxigenin using a riboprobe labeling kit (Roche Diagnostics). The cRNAs were treated with a buffer containing 60 mM Na_2CO_3 and 40 mM $NaHCO_3$, pH 10.5 at 60°C for 25 min for Kir4.1 and Kir5.1, and 28 min for Kir1.1 and Kir2.3. The length of the hydrolytic products was confirmed to be 400–500 bp with electrophoresis, in which only RNAs that migrated as sharp bands with correct sizes were used for further studies. To estimate riboprobe concentration, serial dilutions of labeled riboprobes and a standard labeled RNA were spotted on the nitrocellulose member (BIO-RAD, Hercules, CA), immunostained using 1:4000 diluted anti-DIG antibodies conjugated with AP, and visualized with NBT/BCIP as per the instructions of the kits (Roche Diagnostics). The riboprobes were diluted with 0.1% diethyl pyrocarbonate (DEPC)-treated water to 10 ng/μl and stored in aliquots at −80°C. Before hybridization, the riboprobes were diluted with hybridization buffer to 200 ng/ml, well mixed, and denatured at 80°C for 8 min.

In Situ Hybridization

The brainstem was trimmed into a tissue block, embedded with the TBS Tissue Freezing Medium (Ngle Biomedical Sciences, Durham, NC), and frozen to −20°C. Coronal sections of 10 μm were then obtained using a Cryostat (Leica, Allendale, NJ) at −24°C, and thaw-mounted onto 3-amino-propyltriethoxysilane-coated slides. The sections were dried overnight at 42°C, and then stored at −70°C. All pre-hybridization and hybridization buffers and solutions were sterile-filtered, treated with 0.1% DEPC, and autoclaved.

The sections were thawed at 24°C, and incubated in PBS for 5 min. The tissue-endogenous phosphatases were inactivated with a treatment of 0.2 M HCl for 8 min. Following two washes with PBS (5 min each), the tissues were permeabilized with 1 μg/ml RNase-free proteinase K in the TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 37°C for 30 min, and then post-fixed with 4% paraformaldehyde in PBS at 4°C for 5 min. For acetylation, the sections were placed in a container on a rocking platform and incubated with 0.1 M triethanolamine (TEA) buffer containing 0.25% (v/v) acetic anhydride (Sigma) at pH 8.0 for 15 min.

Subsequently, the sections were incubated with the pre-hybridization buffer containing 40% deionized formamide, 10% dextran sulfate, 0.02% BSA, 0.02% PVP, 0.02% Ficoll 400, 4 × SSC (20 × SSC containing 3 M NaCl, 30 mM Sodium citrate, pH 8.0), 10 mM dithiothreitol (DTT), 1 mg/ml yeast tRNA, 1 mg/ml denatured and sheared salmon sperm DNA at 50°C for 2 h. After draining the pre-hybridization buffer, sections were overlaid with 50 µl hybridization buffer containing 200 ng/ml cRNA probe that had been denatured at 80°C. Then the sections were incubated at 50°C in a humidified chamber for 16 h.

In a shaker water bath of 45°C, sections were washed twice with 2 × SSC (15min each) and then twice with 1 × SSC (15 min each). To digest unbound riboprobes, the sections were further incubated in TE buffer (100 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0) containing 20 µg/ml RNase A for 30 min at 37°C. Sections were washed with 2 × SSC plus 50% formamide for 30 min at 50°C, followed by one 30-min-wash with 0.1 × SSC and two with TN buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) on a shaking platform at 24°C (10 min each). The sections were covered with blocking buffer (100 mM Tri-HCl, 150 mM NaCl, 0.1% Triton X-100 and 2% normal sheep serum, pH 7.5) for 30 min. After draining the blocking buffer, sections were incubated with 1:400 sheep anti-DIG antibodies in the blocking buffer for 2 h. The sections were then washed twice with TN buffer (10 min each), and once with TM buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM Mg_2Cl, pH 9.5) for 15 min. In a humidified chamber, sections were covered with 200 µl detection buffer (0.18 mg/ml BCIP, 0.34 mg/ml NBT, and 480 µg/ml levamisol in the TM buffer). Following incubation in the dark at 37°C for 2 h (4 h for Kir1.1 and Kir2.3), color development was examined. Reactions were stopped at the optimal color development by dipping the sections briefly in distilled water. Sections were then covered with an aqueous mounting solution (Crystal/Dry Mounting medium (Biomeda, Foster City, CA).

Data Analysis

Positive stains were examined under bright-field microscope and digit-imaged using a CCD camera (UVP, Upland, CA). Four transectional levels were studied at the midbrain-pontine border, caudal pons, rostral medulla and caudal medulla. To assess relative expression levels of these mRNAs, the optical density of labeling was empirically estimated according to the relative optical intensity: i.e., ++++, highest levels of labeling; +++, high; ++, moderate; +, low levels; +/−, uncertain; −, below the threshold limit for detection. These estimates were averaged from 3–5 animals at each level for data presentation. All images were prepared using the Photoshop software (Adobe, San Jose, CA) and adjusted for optimal brightness and contrast.

Results

Overall Distribution of Kir4.1, Kir5.1, Kir1.1 and Kir2.3 mRNAs in the Brainstem

Under the low-power microscope, Kir4.1, Kir5.1, Kir1.1 and Kir2.3 mRNAs showed a heterogeneous distribution in the brainstem. The expression density of Kir5.1 was generally high throughout the medulla and pons, followed by Kir4.1. Although low, the expression of Kir1.1 and Kir2.3 was clearly detectable in several brainstem nuclei (Table 1).

The expression of Kir4.1 and Kir5.1 was studied in four brainstem levels, i.e., the midbrain-pontine border, caudal pons, rostral medulla and caudal medulla. Positive labeling of these Kir mRNAs was seen in several cardio-respiratory nuclei, including the locus coeruleus (LC), parabrachial-Kölliker-Fuse (PBKF) nuclei, ventralateral medullary (VLM) area, and solitary tract nucleus (NTS). Their signals were also detected in area postrema inferior olivary nucleus and spinal trigeminal nucleus. In addition, several cranial motor nuclei showed strong expression of Kir4.1 and Kir5.1, including the nucleus ambiguus, hypoglossal nucleus, dorsal vagus motor nucleus (DVM), facial nucleus and motor trigeminal nuclei (Table 1). In these areas the expression pattern of the two Kir channels showed a clear overlap, whereas low levels of expression of the Kir5.1 were also detected in choroid plexus of the fourth ventricle indicating non-neuronal expression of Kir5.1.

The expression of Kir1.1 and Kir2.3 was also examined in these levels of brainstem. Although their expression was rather low in all areas, positively labeled cells were seen in several brainstem nuclei, including the mesencephalic trigeminal nucleus, facial nucleus, VLM and hypoglossal nucleus. Even within these nuclei, only a small proportion of cells was labeled (Table 1). In the LC where the Kir4.1 and Kir5.1 showed strong expression, there was barely detectable signal of Kir1.1 and Kir2.3.

Several control experiments were performed using 1) the sense riboprobes, 2) unlabeled antisense probes, 3) staining without the probes and 4) RNase digestion. No labeling was found in any of these experiments, suggesting that the antisense riboprobes specifically label mRNAs in these cells.

Expression of Kir4.1 and Kir5.1 in Brainstem Neurons

Under the high-power bright-field microscope, the expression of the Kir channels was revealed in individual neurons. Positive labeling was found only in the cytoplasm, while the nucleus is completely devoid of stain.

Pons

Several pontine nuclei had strong expression of Kir4.1 and Kir5.1. A high level of labeling was seen in a large number of LC cells. The labeled cells had a bipolar or triangular shape with the soma measuring 10–20 µm (Fig. 1*A*,*B*). These cells were densely packed in the LC and did not show any detectable difference from those labeled with the Nissl stain (*not shown*), suggesting that most LC neurons, if not all, express these Kir channels. Strong signals of these Kir channels were also found in the adjacent mesencephalic trigeminal nucleus (Fig. 1*A*,*B*). Caudal to the LC, positively labeled cells were found in several

Table 1. Distribution of Kir mRNA in the brainstem

Brain Region	Kir5.1	Kir4.1	Kir1.1	Kir2.3
Pons				
Facial	+ + +	+ + +	+	+
Kölliker-Fuse nucleus	+ +	+	–	–
Lateral parabrachial nuclus	+ +	+	+/–	+/–
Locus coeruleus	+ + + +	+ + + +	–	–
Medial parabrachial nuclus	+	+	–	–
Mesencephalic trigeminal nucleus	+ + + +	+ + +	+	+
Raphe nuclei	+/–	–	–	–
Tegmental nuclei	+ +	+	+	+
Medulla				
Area postrema	+ +	+ +	–	–
Caudal Raphe nuclei	+	+/–	–	–
Cochlear nuclei	+	+	–	–
Dorsal vagus motor nucleus	+ +	+ +	+/–	+/–
Gracile/cuneate nuclei	+	+	–	–
Hypoglossal nucleus	+ + + +	+ + + +	+	+
Inferioe olivary nucleus	+ +	+ +	+/–	+/–
Lateral reticular nucleus	+ + +	+ + +	+	+
Nucleus of ambiguus	+ +	+ +	+/–	+/–
Nucleus of para-ambiguus	+ + +	+ + +	–	–
Nucleus of paragigantocelluris	+ + +	+ + +	+	+
Retrofacial nucleus	+	+	+	+
Retrotrapezoid nucleus	+	+	–	–
Solitary tract nucleus	+	+	–	–
Spinal trigeminal nuclei	+ +	+	–	–

In situ hybridization signals were estimated based on relative optical density. + + + +, highest levels of labeling; + + +, high; + +, moderate; +, low levels; +/–, uncertain; –, below the threshold limit for detection. Note the heterogeneity of labeling within a number of regions; *see* corresponding sections in Results for details.

nuclei of the parabrachial-Kölliker-Fuse complex. These labeled cells were rather small (8–12 μm) and densely distributed in the Kölliker-Fuse and lateral parabrachial nuclei. A small number of cells in the medial parabrachial nucleus also expressed the Kir4.1 and Kir5.1 mRNAs (Fig. 1*C–F*). In the more caudal and ventral area, the expression of these Kir channels was observed in the facial nucleus. Cells in this nucleus were triangular and multipolar with large cell bodies (20–30 μm), suggesting that they are the facial motor neurons (Fig. 1*G*,*H*).

VLM Areas

Figure 2 shows the expression of Kir4.1 and Kir5.1 in the VLM area. Heavily labeled cells were clustered in this area, which belong to several distinct brainstem nuclei (Fig, 2*A*,*B*). In the dorsal area of this cluster, cells expressing these Kir channels showed large cell bodies packed in a small area, which are presumably the ambiguus motor neurons (Fig. 2*C*,*D*). Ventral and ventralateral to these neurons were cells with intermediate size and triangular cell bodies. These cells were located in the para-ambiguus nucleus (Fig. 2*E*,*F*). Cells located within 100–150 μm to the ventral surface of the medulla also showed strong signal of these Kir channels (Fig. 2*G*,*H*). These cells in the lateral reticular nucleus and para-gigantocellular nucleus had bipolar and triangular cell bodies with various soma sizes (10–30 μm).

Dorsal Medulla

Several groups of neurons in the dorsal area of the medulla showed high levels of expression of Kir4.1 and Kir5.1 (Fig. 3*A*,*B*). Neurons in the hypoglossal nucleus were strongly labeled. These cells were large (20–30 μm) with multipolar soma (Fig. 3*C*,*D*). Neurons in the DVM were also labeled. The expression level of these Kir channels was slightly lower than that of hypoglossal neurons (Fig. 3*C*,*D*). Lateral to these two groups of neurons were some positively labeled cells in the NTS that were small (10–15 μm) and showed irregular shape of soma (Fig. 3*E*,*F*). In addition to neurons in these groups, the signal of these Kir channels was also detected in some small cells (8–10 μm) in the area postrema (Fig. 3*G*,*H*).

Caudal Medulla

Figure 4 illustrates the expression of Kir4.1 and Kir5.1 in the caudal medulla. Sections were obtained at the level of pyramidal decussation. Positive labeling of both Kir4.1 and Kir5.1 mRNAs was found in

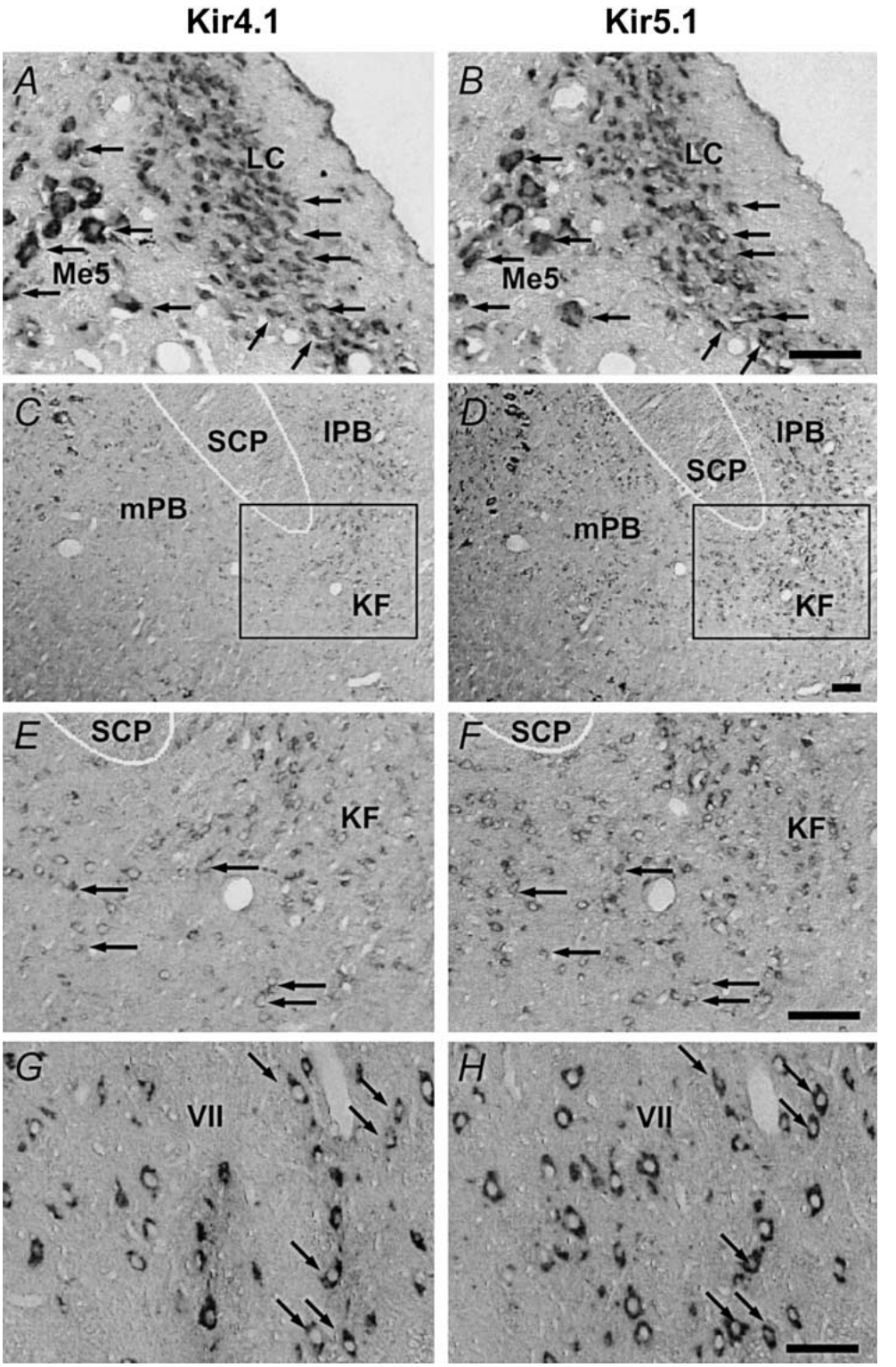

Fig. 1. Kir4.1 and Kir5.1 are expressed in several pontine nuclei. In-situ hybridization cytochemistry was performed in brainstem sections using non-radioactive riboprobes. Two consecutive sections (10 μm) were obtained at levels immediately caudal to the inferior colliculus (*A*, *B*), ~400 μm caudal to these sections (*C*, *D*), and more caudal areas (*G*, *H*). Note that *E* and *F* are enlarged displays of the boxed areas in *C* and *D*. Positive labeling is seen only in the cytoplasm of the soma. The nucleus and processes (dendrites and axons) are devoid of labeling. *A*, *C*, *E*, *G*. The Kir4.1 mRNA was found in the locus coeruleus (*LC*), mesencephalic trigeminal (*Me5*), medial parabrachial (*mPB*), lateral parabrachial (1*BP*), Kölliker-Fuse (*KF*) and facial (*VII*) nuclei. *B*, *D*, *F*, *H*: Sections taken immediately next to *A*, *C*, *E* and G. The Kir5.1 was also expressed in neurons in the same areas. Colocalizations of both Kir4.1 and Kir5.1 in single cells were examined in the two adjacent sections. A good number of cells show colabeling of Kir4.1 and Kir5.1, as indicated by arrows. *SCP*, superior cerebellar peduncle. Up, dorsal; down, ventral. Bar = 100 μm.

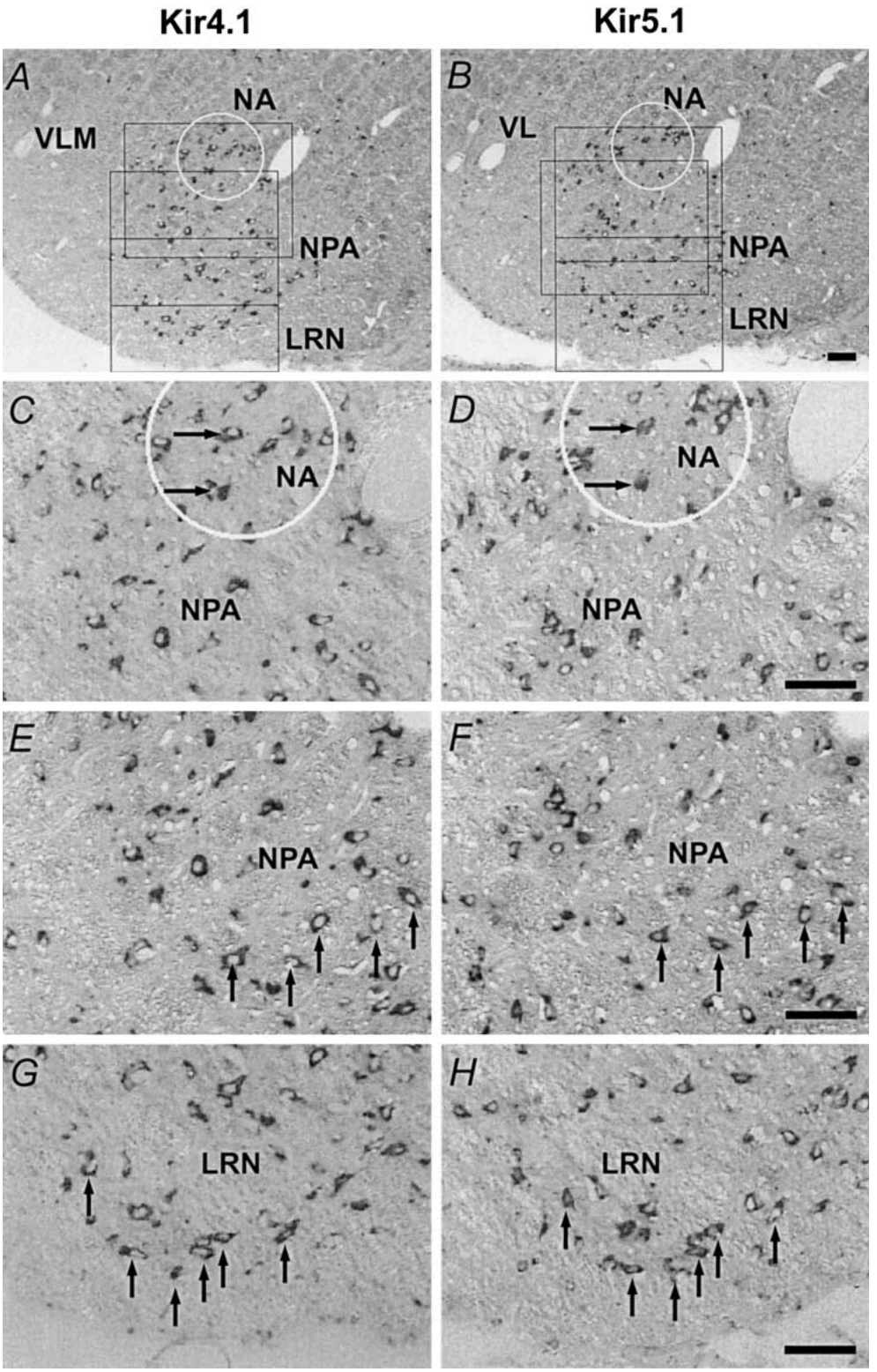

Fig. 2. High levels of expression of Kir4.1 and Kir5.1 are observed in the ventralateral medullary areas. *A*, *B*. Two successive sections (10 μm) taken from the medulla oblongata ~100 μm caudal to the obex. Cells expressing Kir4.1 (*A*) and Kir5.1 (*B*) are found to cluster in several nuclei of the ventralateral medullary (*VLM*) area. Neurons in the nucleus ambiguus (*NA*) and nucleus para-ambiguus (*NPA*) are positively labeled, and are better seen at higher magnification in *C*, *D* and *E*, *F*. Neurons in the nucleus of lateral reticular nucleus (*LRN*) are also strongly labeled (*G* and *H*). Some of the cells are found within 100 μm of the ventral surface of the medulla. Coexpression of both of the Kir channels are seen in a number of cells (*arrows*) within these nuclei. Bar = 100 μm.

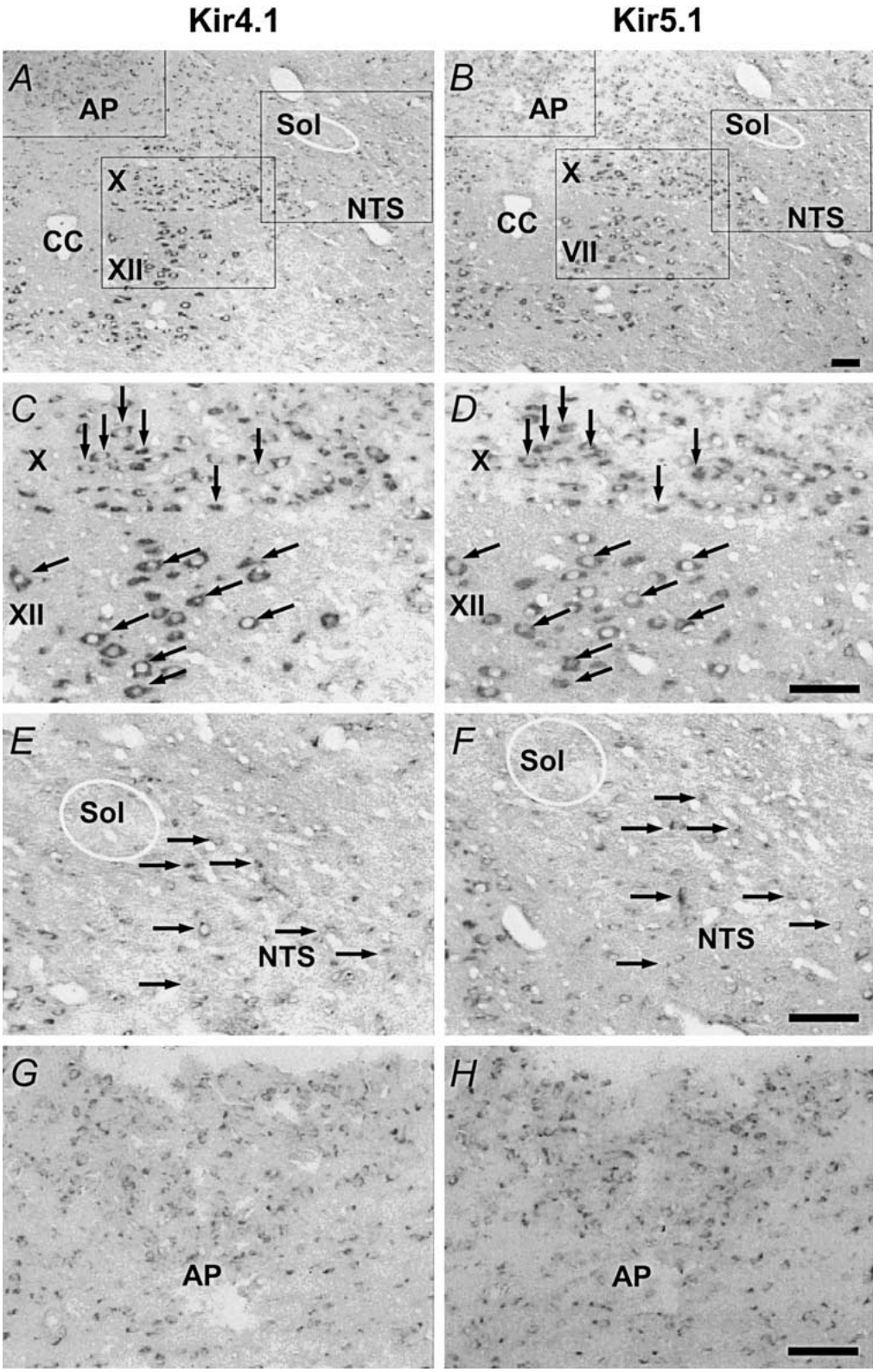

Fig. 3. Several groups of neurons in the dorsal area of the medulla express Kir4.1 and Kir5.1. (*A*, *B*) Two adjacent sections were taken from the medulla about 200 μm caudal to the obex. Positively labeled cells are seen in the hypoglossal nucleus (*XII*), dorsal vagus motor nucleus (*X*), solitary tract nucleus (*NTS*), and area postrema (*PA*). Note that *C*, *D*, *E*, *F*, *G* and *H* are obtained from *A* and *B* with higher magnification. *Sol*, the solitary tract; *CC*, central canal. A good number of cells coexpressed both Kir4.1 and Kir5.1, as indicated by arrows. Up, dorsal; down, ventral. Bar = 100 μm.

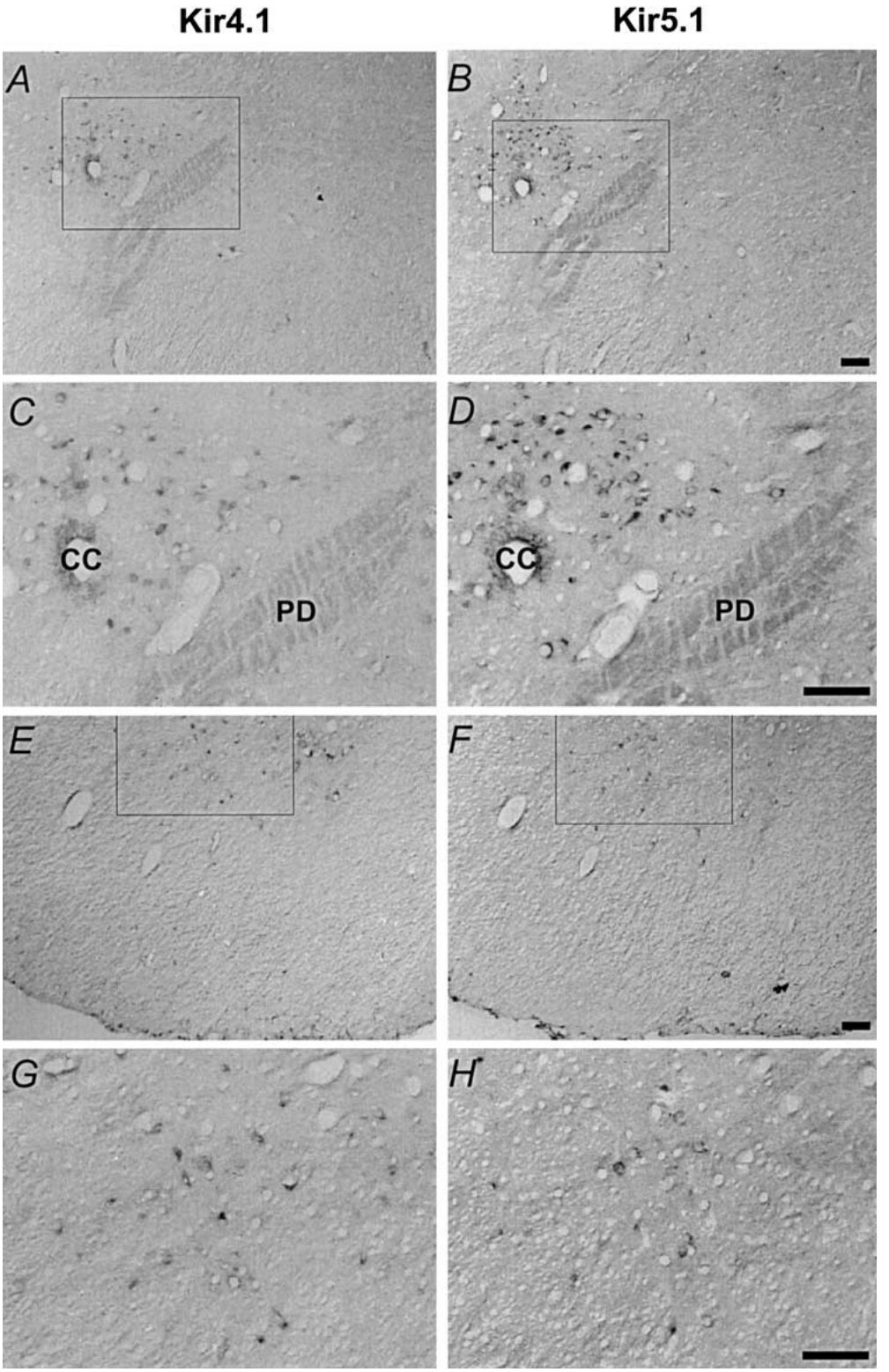

Fig. 4. Low levels of expression of Kir4.1 and Kir5.1 in the caudal area of the medulla. (*A*, *B*). Sections were taken in the caudal medulla at the level of the upper border of the pyramidal decussation. Positively labeled cells are seen around the central canal and the ventralateral field, but are generally absent in other areas. (*C*, *D*) Boxed areas in *A*, *B* at higher magnification (bar = 100 μm). *E–H*: Kir4.1 and Kir5.1 expression is also seen in areas around the retro-ambiguus nucleus. *CC*, central canal; *PD*, pyramidal decussation. Bar = 100 μm.

a small number of cells around the central canal (Fig. 4*A–D*). The expression of the two Kir channels was also seen in areas around the retro-ambiguus nucleus (Fig. 4*E–H*). In most areas, the Kir4.1 and Kir5.1 mRNAs were undetectable. The low levels of expression of these Kir channels make a clear contrast to those seen in more rostral areas of the medulla.

Coexpression of Kir4.1 and Kir5.1 in Single Neurons

The expression patterns of Kir4.1 and Kir5.1 were rather similar in most of the brainstem areas. This, plus the fact that these two Kir channels can express functional channels in their heteromers, suggests that they are coexpressed in these brainstem neurons. To test this hypothesis, we studied the colocalization of both Kir4.1 and Kir5.1 mRNAs in single neurons. Labeled cells displayed on two successive sections (10 μm) were compared. Positive labeling was examined based on tissue landmarks such as blood vessels, axonal pathways and the distribution pattern of the labeled cells. We found that neurons in most of the nuclei described above showed coexpression of Kir4.1 and Kir5.1 (Figs. 1, 2 and 3). To further identify the degree of coexpression, we examined three consecutive sections labeled alternatively with Kir4.1 and Kir5.1 riboprobes. Because of the soma size, most neurons in hypoglossal and facial nuclei can be traced in three sections. Lack of coexpression was shown when the labeling of a cell was missing. We found that very small numbers of cells showed only one of the Kir channels, whereas the vast majority of other cells expressed both channels (Fig. 5).

Expression of Kir1.1 and Kir2.3 in Brainstem Neurons

The expression of Kir1.1 and Kir2.3, though weak, was observed in several brainstem nuclei. In the pons, positive labeling of both of the Kir channels was seen in the mesencephalic trigeminal nucleus and the facial nucleus (Fig. 6). In contrast to the strong labeling of Kir4.1 and Kir5.1, the LC neurons had very little expression of the Kir1.1 and Kir2.3 (Fig. 6*A–D*). In the facial nucleus, only some of the cells showed weak stain (Fig. 6*E–H*). In the medulla, signals of Kir1.1 and Kir2.3 were detected in the VLM and the hypoglossal nuclei, in which the expression density and the number of positively labeled cells were both lower than for Kir4.1 and Kir5.1 (Fig. 7). The expression of Kir2.3 was particularly low throughout the medulla and pons. The coexpression of Kir1.1 and Kir2.3 was examined in two adjacent sections similarly as for Kir4.1 and Kir5.1. Colabeling was sparsely seen in all areas (Figs. 6, 7).

Discussion

In our current studies we have shown that Kir4.1, Kir5.1, Kir1.1 and Kir2.3 are expressed in several cardio-respiratory and cranial motor nuclei in the brainstem, although their relative expression density and patterns are different. The expression of these CO_2/pH-sensitive Kir channels supports their potential involvement in neuronal responses to changes in PCO_2 and pH in the microenvironment to these cells.

The Kir4.1 has previously been shown to be expressed predominantly in the brainstem (Bredt et al., 1995). With the non-radioactive riboprobes, we have detailed its expression in several brainstem nuclei. The Kir4.1 is highly expressed in several cardio-respiratory nuclei including the LC, VLM, PBKF and NTS. Its expression pattern shows a clear overlap with Kir5.1 but not Kir1.1 and Kir2.3. The highly similar expression pattern is not a result of cross-labeling of its mRNA by the Kir5.1 riboprobe, as the nucleotide sequence of Kir4.1 has much higher homology with Kir1.1 than Kir5.1 (Ho et al., 1993; Bond et al., 1994). The similar expression pattern suggests that Kir4.1 and Kir5.1 are coexpressed in some of the neurons. Indeed, their coexpression in single brainstem neurons has been demonstrated in the present studies. Our results have shown that a good number of cells in these brainstem areas are colabeled with the Kir4.1 and Kir5.1 riboprobes, and thus provide the first evidence for the coexpression of the Kir channels in central neurons at the mRNA level. We believe that this is an important finding, as the Kir5.1 does not express functional channels in its own homomers. The highly similar expression pattern of the two Kir channels and their coexpression in single neurons suggest that they are likely to express heteromeric Kir4.1-Kir5.1 channels in these neurons.

The Kir1.1 and Kir2.3 are also highly sensitive to CO_2 and pH (Coulter et al., 1995; Tsai et al., 1995; Doi et al., 1996; Zhu et al., 1999, 2000; Schultz et al., 2003). Our current studies have shown that both of these Kir channels are also expressed in several brainstem nuclei, although their relative expression density and patterns were different from the Kir4.1 and Kir5.1. In comparison to Kir4.1 and Kir5.1, the expression density of Kir1.1 and Kir2.3 appeared low in all brainstem areas; their mRNAs are detected in only a few nuclei, including the VLM and some cranial motor nuclei. Even within these nuclei, only a small proportion of cells is labeled. Consistent with the low level of expression of Kir2.3 is a previous study using radioactive oligonucleotide probes (Karschin & Karschin, 1997). Because of the low expression level of Kir2.3 and the resolution of the probes, this previous study failed to detect the Kir2.3 mRNA in the brainstem (Karschin & Karschin, 1997).

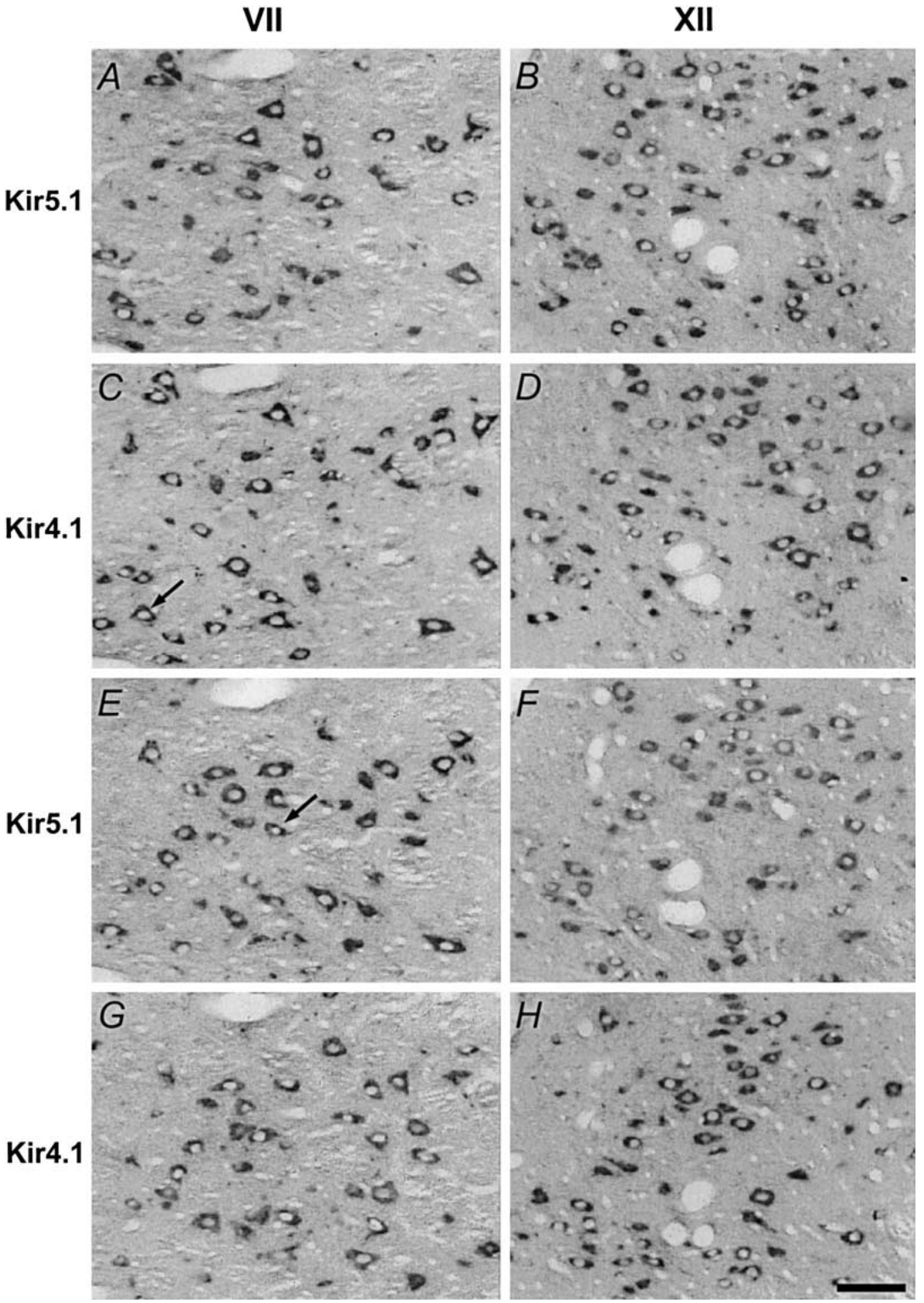

Fig. 5. Identification of cells that lack coexpression of Kir4.1 and Kir5.1. *A*, *C*, *E* and *G*. Consecutive sections (10 μm) were obtained from the facial nucleus. *A*, *E* were labeled with the Kir5.1 riboprobe, and *C*, *G*, with the Kir4.1 riboprobe. The lack of labeling was examined in three adjacent sections. Because of the soma size, the facial neurons can be traced in three or even four sections. Negative coexpression was shown when labeling of the cell was missing in the next section (*arrows*). (*B*, *D*, *F* and *H*). Similar experiments were performed in the hypoglossal neurons. These Kir channels are coexpressed in all the hypoglossal neurons displayed in the sections. Bar = 100 μm for all sections.

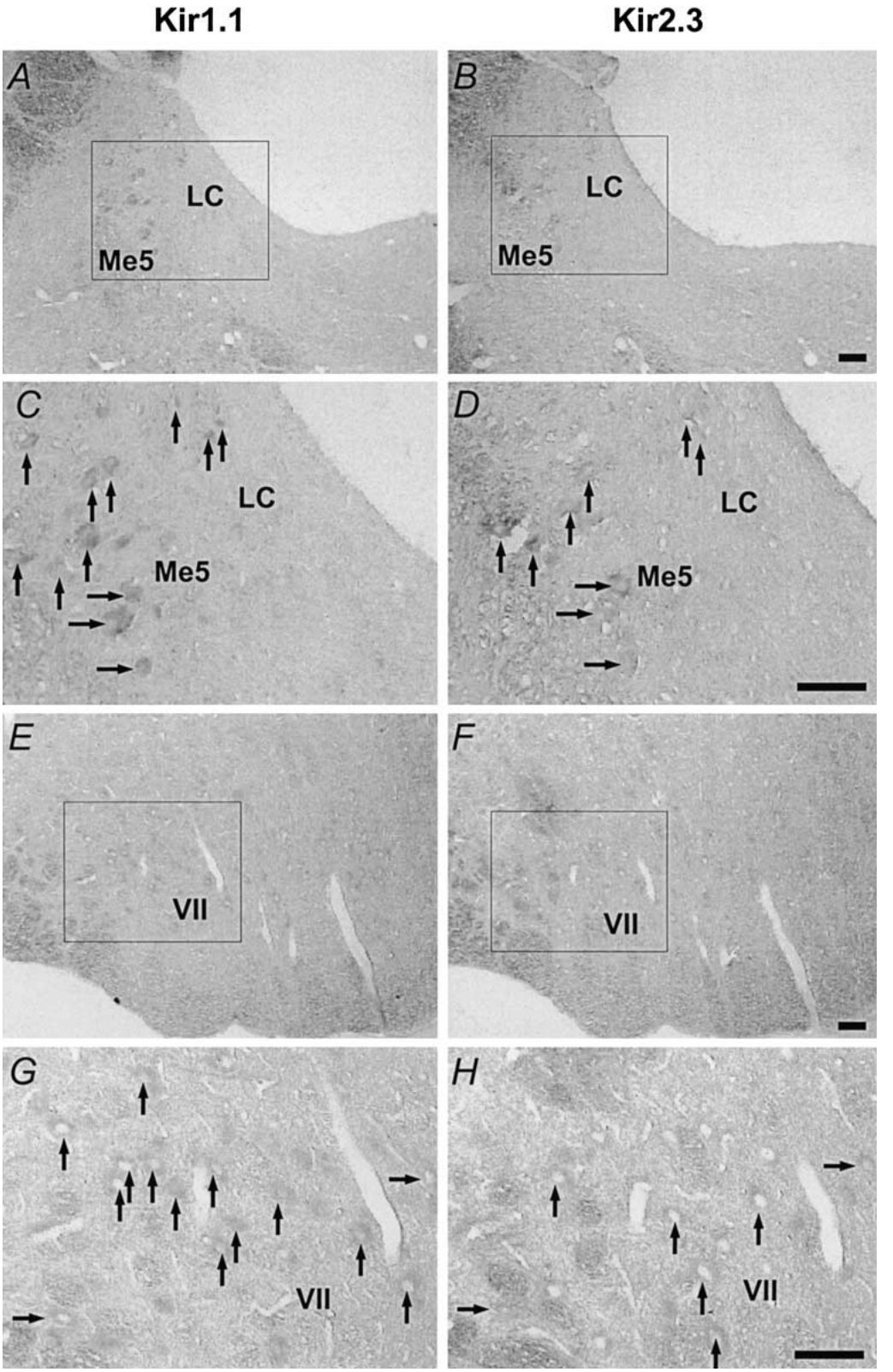

Fig. 6. Expression of Kir1.1 and Kir2.3 in pontine nuclei. (*A*, *B*). Two successive sections were taken from the rostral pons. Positive signals are seen in the mesencephalic trigeminal nucleus (Me5), whereas neurons in the lacus coeruleus (*LC*) show generally no labeling. These are better viewed in a higher magnification (*C* and *D*). Positive labeling is indicated by vertical arrows, and coexpression by horizontal arrows. (*E*, *F*). In the caudal pons, signals of Kir1.1 and Kir2.3 were also detected in the facial nucleus with very low density. (*G*, *H*) Magnified areas from *E*, *F*. Bar = 100 μm.

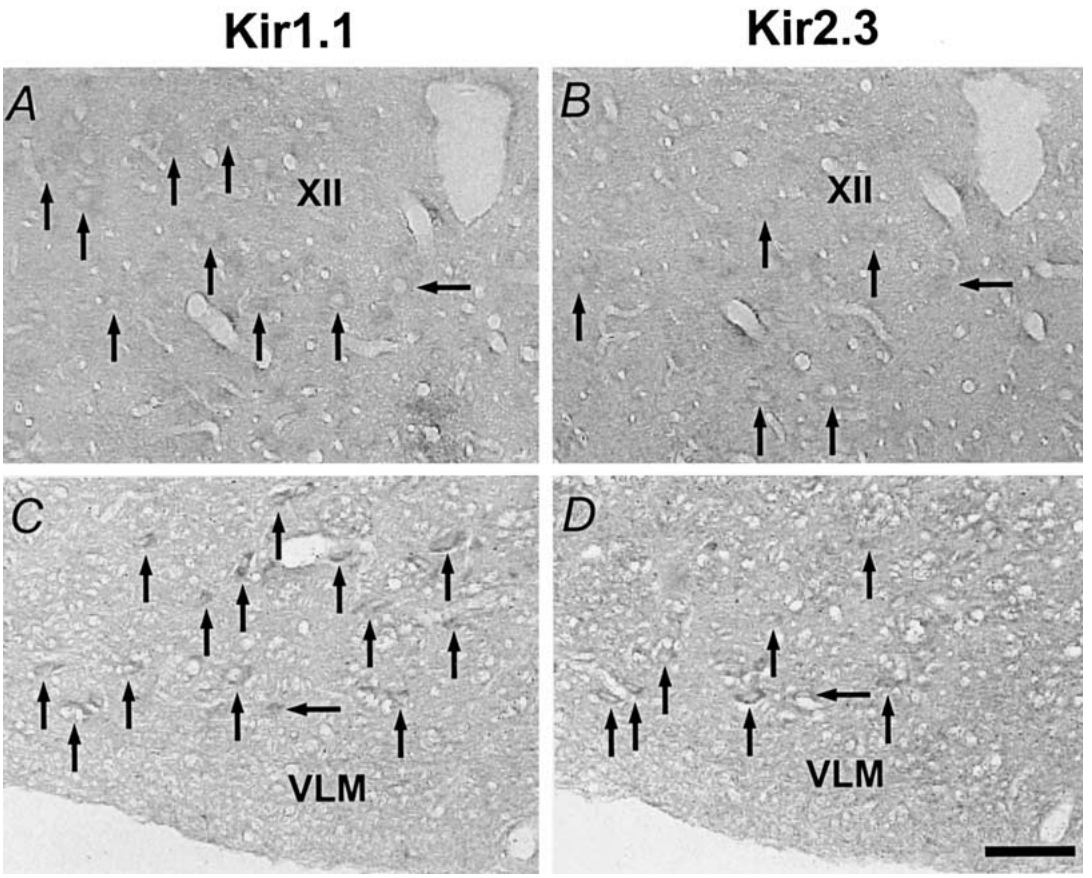

Fig. 7. Expression of Kir1.1 and Kir2.3 in the hypoglossal nucleus (*A*, *B*) and ventralateral medullary area (*C*, *D*). Positive labeling is indicated by vertical arrows, and coexpression by horizontal arrows. Bar = 100 μm.

The Kir channels are important players in the maintenance of plasma membrane excitability. Inhibition of these K^+ channels by hypercapnia and acidosis produces depolarization and an increase in membrane excitability. Certain Kir channels in the LC neurons have been shown to be inhibited by intracellular protons leading to depolarization and increase in firing activity during hypercapnia (Pineda & Aghajanian, 1997). Our previous studies have indicated that the heteromeric Kir4.1 and Kir5.1 channels are highly sensitive to CO_2 (Xu et al., 2000; Yang et al., 2000). This together with our current finding that they are coexpressed in the LC neurons strongly suggests that Kir4.1 and Kir5.1 appear to be some of the unidentified Kir channels and contribute to the CO_2/pH sensing of the LC cells. Our results also show strong expression of all the CO_2/pH-sensitive Kir channels in the VLM consisting of the nucleus ambiguus, para-ambiguus nucleus, lateral reticular nucleus and para-gigantocellular nucleus. Since neurons in these areas are known to be also involved in CO_2 central chemoreception, the expression of these CO_2/pH sensitive Kir channels including Kir1.1 and Kir2.3 may allow the cells to sense hypercapnia and acidosis. Our current studies have also shown strong expression of these Kir channels in several cranial motor nuclei. Since most of the cranial motor neurons are recruited during hypercapnic acidosis, it is possible that these Kir channels are also involved in the excitation of these cranial motor neurons during respiratory stress. Thus, expression of the Kir channels in the brainstem neurons that have direct or indirect connections with cardio-respiratory neuronal networks may enable these cells as well as the cardio-respiratory system to respond to high PCO_2 in the cerebral spinal fluid and blood circulation.

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