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Expression of Kv1.3 potassium channels regulates density of cortical interneurons

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Abstract

The Kv1.3 protein is a member of the large family of voltage-dependent K⁺ subunits (Kv channels), which assemble to form tetrameric membrane-spanning channels that provide a selective pore for the conductance of K⁺ across the cell membrane. Kv1.3 differs from most other Kv channels in that deletion of *Kv1.3* gene produces very striking changes in development and structure of the olfactory bulb, where Kv1.3 is expressed at high levels, resulting in a lower threshold for detection of odors, an increased number of synaptic glomeruli and alterations in the levels of a variety of neuronal signaling molecules. Because Kv1.3 is also expressed in the cerebral cortex, we have now examined the effects of deletion of the *Kv1.3* gene on the expression of interneuron populations of the cerebral cortex. Using unbiased stereology we found an increase in the number of parvalbumin (PV) cells in whole cerebral cortex of *Kv1.3*^{-/-} mice relative to that in wild type mice, and a decrease in the number of calbindin (CB), calretinin (CR), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP) and somatostatin (SOM) interneurons. These changes are accompanied by a decrease in the cortical volume such that the cell density of PV interneurons is significantly increased and that of SOM neurons is decreased in *Kv1.3*^{-/-} animals. Our studies suggest that, as in the olfactory bulb, Kv1.3 plays a unique role in neuronal differentiation and/or survival of interneuron populations and that expression of Kv1.3 is required for normal cortical function.

Keywords

Potassium channels; Kv1.3; interneurons; cerebral cortex; CDP

INTRODUCTION

K⁺ channels comprise one of the largest and most diverse groups of membrane proteins and as such subserve a host of physiological functions. The Kv1.3 is one of seven members of the Kv1 subfamily, which in turn is one of the twelve subfamilies of the Kv family of voltage-dependent potassium channels (Stühmer et al., 1989; Swanson et al., 1990). Typically, delayed rectifiers and A-current channels, members of the Kv1 subfamily are important in shaping the action potential waveform and controlling the rate of neuronal firing, both key aspects of the regulation of neuronal communication and information processing (Levitan and Kaczmarek, 2002). Structurally, Kv1.3 resembles other Kv family

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members in having six transmembrane segments with one pore loop between the fifth and sixth segments. The biophysical behavior of Kv1.3 is, however, unusual in that it gives rise to delayed-rectifier currents that undergo long-lasting cumulative inactivation with repeated depolarizations, a feature that distinguishes it from other Kv1 family member channels (Marom and Levitan, 1994).

A variety of recent work has demonstrated that, in addition to regulating excitability, many ion channels can influence cell behavior in ways that may not require ion flux through the channel (Kaczmarek, 2006). Such interactions have been termed “non-conducting” functions of ion channels and may contribute to the role of potassium channels during development in the processes of cell proliferation, migration and survival (Hendriks et al., 1999; Pardo, 2004). In the case of Kv1.3, work with non-neuronal cells has suggested that gating of this channel may influence cytoplasmic and nuclear signaling pathways, perhaps through the formation of tight physical links with α -integrins (Artym and Petty, 2002; Levite et al., 2000). In addition to regulating integrin signaling, Kv1.3 may influence cell proliferation and the killing of neurons by microglia (Pardo, 2004; Fordyce et al., 2005). Such non-conducting effects may contribute to the extraordinary effects of deletion of the *Kv1.3* gene on the structure and function of the olfactory bulb, where Kv1.3 is normally expressed at high levels in mitral cells (Fadool et al., 2004; Pardo, 2004). Kv1.3^{-/-} mice have a 1,000–10,000-fold lower threshold for detection of odors and a twofold increase in the number of synaptic glomeruli in the olfactory bulb. Deletion of Kv1.3 also affects the neuronal levels of TrkB, Src, nShc, PSD-95 and other signaling molecules (Fadool et al., 2004). Genetic deletion of other Kv family channels, such as the Kv1.1 and the Kv3.1 channel, are known to influence excitability but are not known to have such striking developmental effects (Ho et al., 1997; Smart et al., 1998).

In addition to the mouse olfactory bulb, Kv1.3 is expressed in several other brain areas including the mouse auditory brainstem (Gazula et al., 2010) and the rat cerebral cortex (Beckh and Pongs, 1990; Kues and Wunder, 1992; Veh et al., 1995). In the present work, we have found that Kv1.3 is expressed in both pyramidal cells and interneurons of the mouse cerebral cortex. Deletion of the Kv1.3 gene does not affect the total number of cortical cells *per se*, nor the lamina structure of the cortex. Loss of Kv1.3, however, suppresses the expression of the transcription factor CDP, a marker of neurons in cortical layers II/III/IV that are not parvalbumin-positive interneurons (Nieto et al., 2004). This is accompanied by an increase in numbers and density of parvalbumin-containing interneurons and a decrease in somatostatin-expressing interneurons. Interneurons are vital for the preservation of a balanced inhibitory tone within the cortex (Woo and Lu, 2006; Li et al., 2008), are readily identifiable by immunohistochemistry, and comprise about 20–30% of all cortical cells (DeFelipe and Farinas, 1992; Corbin and Butt, 2011). Thus a change in the number, density and/or distribution of interneurons may have a profound effect on cortical function.

MATERIALS AND METHODS

Animals

A total of 65 mice were used in these experiments. Of these, 15 consisted of 3 dams and 4 embryos per dam that were used for cortical cell cultures. Cultures were done 3 times and in each the cerebral cortices of 4 embryos were mixed. The rest of the embryos per dam are not accounted for. Otherwise, 50 adult mice were split into 2 groups: 25 controls and 25 Kv1.3^{-/-}. From these 25 brains per group 3 brains were used for NeuN, CDP, ER81, and FoxP2; 12 brains were processed for CB, CR and PV; and, 10 brains were used for NPY, VIP and SOM immunohistochemistry. Some of these were selected for stereological counts based on the quality of staining and integrity of the tissue. All experimental protocols involving animals were approved by the Yale University Animal Use and Care Committee,

and experiments were carried out on either 2-month-old C56BL/6J wild-type mice and/or Kv1.3^{-/-} mice in the same C56BL/6J background (Koni et al., 2003; Fadool et al., 2004). The genotype of all animals was confirmed using the polymerase chain reaction (PCR) on extracts of tail DNA. For wild-type mice, the primers used for PCR were KCNA-F (5'-GTACTTTGACCCACTCCG CAATGA-3') and KCNA-R (5'-GCAGAAGATGACAATGGA GATGAG-3'; 342-bp product). For Kv1.3^{-/-} mice, the primers used were Neo-F (AGGATCTCCTGTCATCTCACCTT) and Neo-R (CCTCAGAAGAACTCGTCAAGAAG; 499-bp product). The PCR conditions were: 94°C 30 minutes, 60°C 30 minutes, 72°C 40 minutes for 32 cycles

Perfusion and Immunohistochemistry

Adult mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused through the left ventricle with a PBS solution (100 mM Na₂HPO₄/NaH₂PO₄, pH 7.4, and 150 mM NaCl) containing 0.5% NaNO₂ and 1,000 U heparin, followed by a 0.1 M phosphate buffer (PB) solution (100 mM Na₂HPO₄/NaH₂PO₄, pH 7.2) containing 4% paraformaldehyde (PFA). Brains were removed, postfixed in 4% PFA for 2 hours at 4°C, rinsed twice with PB, and then either placed in 30% sucrose at 4°C for 24 hours or immediately sliced at 50 µm sagittally on a vibratome. Brains not sectioned were embedded in O.C.T. compound (Tissue-Tek, Torrance, CA), rapidly frozen in acetone containing dry ice, and stored at -80°C until cryostat sectioning. Slices were collected in multiwell plates containing PBS and were then postfixed in 4% PFA for 5 minutes, followed by two washes with PBS (for 5–10 minutes on orbital shaker). Slices were then permeabilized with a PBS solution containing 1% bovine serum albumin (BSA), 5% normal donkey serum, 0.2% glycine, 0.2% lysine, and 0.2% Triton X-100 (blocking solution) for 1 hour at room temperature or overnight at 4°C and were rinsed twice with PBS containing 1% BSA alone.

Cortical cell cultures

In brief, mouse embryos were removed at E14.4 from the uterine horn and placed in ice-cold Hank's balanced salt solution (HBSS). The skin and cartilage of the head were removed and the brain was extracted. The telencephalon was then dissected with a microknife. The olfactory bulb and basal ganglia were removed. Cortical cells were dissociated enzymatically with papain, neutral protease, and DNase I in HBSS twice for 15 min at 37°C with gentle triturating (10x) with fire-polished Pasteur pipettes after the first incubation and at the end of enzyme treatment. The cells were then left to settle for 5 min and the supernatant was transfer to a new tube. After spinning at 800 rpm for 5 min the supernatant was discarded and the cells were re-suspended in 10–50 ml of Neurobasal medium. Finally the cells were plated on glass coverslips in well plates. The next day (E15.5) the cells were fixed with 4% PFA for 10 min and stained for Kv1.3 using Rabbit anti Kv1.3-FITC (Immunogen: Peptide KDYPASTSQDSFEA(C), corresponding to amino acid residues 263–276 in the extracellular loop between domains S1 and S2 of human Kv1.3 (accession number P22001) from Alomone labs). For a complete description of the cortical cell culture protocol see Sestan et al., 1999.

Antibody and staining characterization

Rabbit anti-Kv1.3: (polyclonal, from Alomone Labs), control experiments with antigen-pre-absorbed antibody resulted in complete prevention of staining. We used this antibody for light and confocal microscopy experiments.

Rabbit anti-Kv1.3-FITC: (polyclonal, from Alomone Labs) We used this antibody for confocal microscopy experiments only.

Mouse anti-Kv1.3: (monoclonal, from NeuroMab), on Western blots of rat postganglionic sympathetic neurons, it detected a band close to 70 kDa, the predicted weight for Kv1.3 (Doczi et al., 2008).

Mouse anti-PV: (monoclonal, IgG1 isotype; from Sigma), derived from the PARV-19 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunized mouse. Purified muscle PV was used as the immunogen.

Rabbit anti-CR: (polyclonal, from Swant), specifically reacts with CR in tissue originating from human, monkey, rat and mouse. This antibody does not cross react with calbindin D-28K or other known calcium binding proteins.

Mouse anti-CR: (monoclonal, from Swant), specifically reacts with CR and does not cross react with calbindin D-28k or other known calcium binding proteins.

Mouse anti-CB: (monoclonal, from Swant), specifically reacts with calbindin D-28k on immunoblots of extracts of tissue originating from human, monkey, guinea pig, rabbit, rat mouse and chicken. This antibody does not cross react with CR or other known calcium binding proteins. This antibody specifically stains the ⁴⁵Ca-binding spot of calbindin D-28k MW 28,000.

Rabbit anti NPY: (polyclonal, from Immunostar). In rat central nervous system this antibody has significant staining with a very low background. Cross reactivity experiments in which diluted NPY antiserum was absorbed with excess peptide YY, avian pancreatic polypeptide -endorphin, VIP, CCK or SOM showed no affect in blocking the intensity of staining.

Rabbit anti-VIP: (polyclonal, from Immunostar). VIP immunolabeling was completely abolished by pre-adsorption with VIP. Pre-adsorption with the following peptides resulted in no reduction of immunostaining: Secretin, gastric inhibitory polypeptide, somatostatin, glucagon, insulin, ACTH, gastrin 34, FMRF-amide, rat GHRF, human GHRF, peptide histidine isoleucine 27, rat pancreatic polypeptide, motilin, peptide YY, substance P, neuropeptide Y, and CGRP.

Rabbit anti-SOM: (polyclonal, from Immunostar). Immunolabeling was completely abolished by pre-adsorption with somatostatin, somatostatin 25, and somatostatin 28. Pre-adsorption with the following peptides resulted in no reduction of immunostaining: substance P, amylin, glucagon, insulin, NPY, and VIP.

Mouse anti-NeuN: (monoclonal, from Chemicon). Staining is primarily in the nucleus of the neurons with lighter staining in the cytoplasm.

Goat anti-Fox P2: (polyclonal, from Novus). Immunogen is a synthetic peptide conjugated to KLH derived from within residues 700 to the C-terminus of Human Fox P2.

Goat anti-CDP: (polyclonal, from Santa Cruz Biotechnology). This CDP (C-20; CDP for CCAAT displacement protein, Cux-1) is raised against a peptide mapping at the C-terminus of CDP of mouse origin; MW of 180kDa on western blot.

Goat anti ER81: (polyclonal, from Santa Cruz Biotechnology). Epitope corresponding to amino acids 121–190 mapping within an internal region of ER81 of human origin.

PI: Propidium Iodide (Sigma-Aldrich): nuclear fluorescent stain. Used as per manufacturer instructions.

Diaminobenzidine (DAB) staining

Free-floating sections (50 μm thick) were washed with PBS 3×10 minutes each, incubated with 1% H_2O_2 for 10 minutes, washed 3 times with PBS, and then incubated with Rb anti-Kv1.3 in blocking solution for 48 hours at 4°C under constant agitation. The tissue was washed twice with PBS containing 1% BSA, followed by incubation with biotinylated donkey anti-rabbit antibody at room temperature for 1 hour under constant agitation. The sections were again washed and then reacted with an avidin-biotin peroxidase complex (Vectastain; Vector Laboratories, Burlingame, CA), and the immunoreactivity was visualized using nickel-enhanced diaminobenzidine (DAB). In brief, 10 mg DAB (Sigma, St. Louis, MO) was dissolved in 18.4 ml PB, followed by the addition of 200 μl of 0.4% NH_4Cl , 200 μl of 20% -D-glucose (Sigma), and 800 μl of 1% $(\text{NH}_4)_2\text{Ni}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (Sigma). The solution was then filtered by a 0.45- μm syringe filter, followed by the addition of 20 μl glucose oxidase (Sigma). This solution was then added to the sections, and the reaction was allowed to proceed for 10 minutes. The reaction was terminated by adding fresh PB (0.1 M), and this was then followed by two rinses with PB. Stained sections were mounted on gelatin-coated slides, dehydrated through graded ethanol followed by xylene, and coverslipped with Permount (Fisher Scientific, St. Louis, MO).

Fluorescent staining

After postfixation, permeabilization, and blocking; free-floating slice sections were processed for either single labeling with fluorescein isothiocyanate (FITC)-conjugated anti-Kv1.3 or double labeling with rabbit anti-Kv1.3 (Alomone Labs) or with mouse anti Kv 1.3 (Neuromab) and mouse/rabbit anti-PV, CR, CB, VIP, SOM, and NPY. After 48 hours of incubation at 4°C in primary antibody, sections were washed three times for 10 minutes each with $1 \times$ PBS. The fluorescent secondary antibodies used in double-labeling experiments were donkey anti-rabbit Alexa 488 (Invitrogen, Carlsbad, CA) for Kv1.3 staining and donkey anti-mouse / donkey anti-rabbit Cy3 (Jackson ImmunoResearch, West Grove, PA) for CB, CR, SOM, VIP and NPY staining. Incubation was for 1 hour at room temperature. Sections were mounted on gelatin-coated slides, air dried, and coverslipped with 2.5% DL-2-amino-5-polyvinyl alcohol/1,4-diazabicyclo (2.2.2) octane (PVA-DABCO). Control sections were processed through similar immunohistological procedures, except that primary or secondary antibodies were omitted. In all cases, the omission of primary or secondary antibodies resulted in the lack of specific labeling, confirming the specificity of immunocytochemical analysis (Santi et al., 2006; Chen et al., 2009). In addition to DAB or fluorescent staining, selected sections were stained for nissl using cresyl violet via standard protocols. In the case of cultures, Kv1.3 was visualized with FITC and nuclei with PI.

Image acquisition and analysis

Confocal microscopy—Sections were examined with a Zeiss laser scanning microscope (LSM 150 Meta) coupled to a computer with Zeiss image acquisition and analysis software. Images were acquired in multitrack mode using a C-Apochromat $\times 40/1.2$ W corr. or $\times 63/1.2$ W corr. objective (W corr., water correction). The optical thickness of the slices was constant for both tracks. Alexa Fluor 488 has excitation/emission of 496/519, and Cy3 has excitation/emission of 550/570. Double-immunofluorescence images were also displayed as dual-color merged images.

Light microscopy—We used a Zeiss Axiophot microscope with AxioCam HRc and AxioVision software. Adobe Photoshop (Adobe Systems, San Jose, CA) was used to sharpen images and/or adjust brightness and contrast level when necessary.

Stereology

Section outlines and cell plotting was carried out using a Zeiss Axioskop microscope fully motorized and interfacing to a Dell computer running StereoInvestigator® and NeuroLucida software (MicroBrightField Inc., Williston, VT). Outlines and fiduciary marks were drawn at 5x. Cell counts were done in bright field using a Zeiss Plan-Neofluar 20x objective lens with a 0.5 optical aperture. Regions of interest consisted of the whole cerebral cortex in the parasagittal plane from the rostral tip including the frontal association cortex and the ventrally located lateral orbital cortex to the caudal tip ventrally including the retrosplenial agranular cortex. The dorsal limit was pia and the ventral border that of the corpus callosum. Limits for areas of interest were drawn following a mouse brain Atlas (Paxinos and Franklin, 2001). Stereological analysis was performed by systematic random sampling (SRS) using an optical fractionator probe (West et al., 1991; Gundersen et al., 1999). All cell counts were made by an investigator blind to genotype. Optimal sampling parameters were obtained experimentally. Once a positive labeled cell was encountered, its position was plotted using one of the symbols provided by the program. Morphometric data (perimeters, areas, volumes, etc.) were obtained with NeuroLucida Explorer. Three parasagittal sections were done per brain and a mean was obtained. Because our interest was in detecting changes in whole cerebral cortex distinctions were not made between lamina or between different functional areas. See table 1 for stereological parameters.

RESULTS

Mouse cortical pyramidal and non pyramidal cells express Kv1.3 channels throughout development

We first examined whether Kv1.3 potassium channels are expressed in cortical neurons both before birth as well as in adult mice. We immunostained non-permeabilized cells in primary cortical cultures at E15.5 using an antibody against an extracellular epitope in Kv1.3. This antibody has no immunoreactivity against the nervous system of Kv1.3^{-/-} mice (Gazula et al., 2010 and data are not shown). The cells were counterstained with propidium iodide (PI), a nuclear stain. At this early developmental stage nearly all cortical cells express Kv1.3 and the peripheral pattern of immunolocalization is consistent with expression in the plasma membrane (Fig. 1A). To confirm that Kv1.3 is expressed in multiple types of neurons later in life, we immunohistochemically stained brain slices from adult animals for Kv1.3 using DAB as the chromogen. Under microscopic observation both putative pyramidal and non-pyramidal cells appear to express Kv1.3, as judged by the characteristic sizes and shapes of the stained cell types (Fig. 1B).

Deletion of Kv1.3 does not affect the number of cortical neurons nor alter the laminar organization of the cortex

We confirmed the previous observation that Kv1.3^{-/-} mice are smaller by weight than age-matched controls (Xu et al., 2003). We also observed that the brains of Kv1.3^{-/-} mice appeared slightly smaller than those of matched controls and our preliminary results indicated a slight decrease in the cortical volume of the Kv1.3^{-/-} mice, although no gross anatomical changes were observed. To investigate further the general cytoarchitecture of the cortex, sagittal brain slices from Kv1.3^{-/-} and control mice were stained for Nissl and for the neuronal marker NeuN (Fig. 1C–D). The numbers of NeuN⁺ cells were estimated stereologically (n=3 animals; 3 sections per animal) from selected sections matched at medio-lateral levels. Our microscopic observations indicated that the laminar structure of the cortex is preserved in Kv1.3^{-/-} mice and the stereological counts indicated that there is no difference in cortical NeuN⁺ cell numbers between the two groups (Fig. 1E).

Deletion of Kv1.3 affects the expression of the transcription factor CDP

Although there were no detectable lamina differences observed in the Nissl and NeuN stains, we further characterized the integrity of cortical laminae by immunostaining the cortex for transcription factors that are selectively expressed in the different laminae. In the deeper cortical layers, identified by immunohistochemistry with ER81 (layer V) and FoxP2 (layer VI) no differences were observed between cortices from wild type and Kv1.3^{-/-} animals (Fig 2). A very clear and striking difference was, however, detected in the expression of CDP (CCAAT displacement protein, Cux-1) (n=6 animals). This transcription factor is a marker for most post-mitotic neurons in layers II/III/IV but it does not label parvalbumin-positive interneurons (Nieto et al., 2004). Immunostaining for this marker was present in all three layers in cortices of wild type mice, but was greatly reduced in layers III/IV of Kv1.3^{-/-} mice (Fig. 2).

Cortical interneurons express Kv1.3 channels

Different classes of interneurons in the cerebral cortex can be specifically identified by their immunoreactivity to markers such as calbindin (CB), calretinin (CR), parvalbumin (PV), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP) and somatostatin (SOM); some of which constitute partially overlapping populations (Cauli et al., 1997; Markram et al., 2004). To determine whether any of these classes of neurons express Kv1.3 channels in adult animals, we carried out double immunofluorescence staining using antibodies against each of these markers with the antibody against the extracellular epitope in Kv1.3. In each case the interneurons were labeled using secondary antibodies conjugated to Cy3 (red) and the Kv1.3 channels were labeled using secondary antibodies conjugated to Alexa488 (green). Overlay of the corresponding images indicates that all of these classes of interneurons express Kv1.3 channels (Fig. 3). As expected for a plasma membrane ion channel, immunoreactivity for Kv1.3 was localized primarily but not exclusively at the periphery of the cells.

Deletion of Kv1.3 affects cortical interneuron numbers and densities

To determine how loss of the Kv1.3 channel influences interneuron populations we carried out unbiased stereological measurements of the numbers and density of each of the labeled interneuron types in wild-type and Kv1.3^{-/-} mice (see Methods) in serial sagittal brain sections at 50 µm intervals (Fig. 4A). These were then immunohistochemically processed for CB, CR, PV, NPY, VIP or SOM. Stereological cell counts were carried out for the cerebral cortex using SRS with an optical fractionator probe. In brief, the program randomly places a grid on top of the area of interest (Fig. 4B,C) and displays a counting box on the lower right corner of each grid box. Stained cells inside the counting box or touching the inclusion border are counted; those outside the counting box or touching the exclusion border are not (Fig. 4D). Adequate sampling parameters (grid size, counting box size, etc.) were obtained empirically for each type of staining (Table 1).

We found that deletion of Kv1.3 increases the number of parvalbumin-expressing neurons in the cerebral cortex. Figure 5 shows immunostaining for PV in representative sections from wild type and Kv1.3^{-/-} animals. Also shown are diagrams of the locations of the images along the cortex and of the positions of stained cells within the counting boxes. The increased number of parvalbumin neurons in the Kv1.3^{-/-} sections is evident by visual inspection. The mean data for 12 wild type and 12 Kv1.3^{-/-} animals each show that number of PV positive neurons is increased by over 40% (Fig. 7A, Table 2).

In contrast to parvalbumin, the numbers of CB, CR, NPY and SOM expressing neurons was reduced in the cerebral cortices of Kv1.3^{-/-} mice, although no statistically significant change was found for VIP-immunoreactive cells (Fig. 7A, Table 2). The decrease in number

was particularly striking for somatostatin-positive cells, for which numbers were reduced by 70% (Fig. 6, Fig. 7, Table 2).

As described above, the cerebral cortices of *Kv1.3*^{-/-} animals appeared reduced in thickness when compared to those of wild type animals, although the rostrocaudal extent of the cortex did not seem to be affected (see diagrams in Figs 5,6). When quantified, this difference was found to be highly statistically significant (Fig. 7C, Table 2). We therefore normalized the cell counts for the different classes of interneurons to cortical volume in each animal to estimate cell density rather than cell number. The mean density of CB, CR, NPY and VIP expressing cells was found not to differ between the cortices of wild type and *Kv1.3*^{-/-} animals. The inverse relation between the expression of parvalbumin and somatostatin neurons was, however, also highly significant for the density of these two cell types, with a greater than 68% increase in density of PV neurons and a 65% decrease in density of SOM neurons in the cortices of *Kv1.3*^{-/-} animals.

DISCUSSION

We have found that, in the mouse cerebral cortex, *Kv1.3* potassium channels are present in both pyramidal and non-pyramidal cells. Expression can be detected as early as embryonic day 15.5, and continues into adult life. Deletion of the *Kv1.3* gene does not change the total number of cortical neurons nor the overall laminar organization of the cortex. It does, however, alter the expression of the CDP transcription factor, which is normally present in layer II/III/IV cortical neurons other than parvalbumin-containing interneurons. This finding is consistent with the finding that the number of parvalbumin (PV) cells in the cerebral cortex of *Kv1.3*^{-/-} mice is increased relative to that in wild type mice, and that there is a decrease in the number of somatostatin (SOM) interneurons. PV, SOM, CB, CR, NPY and VIP expressing cells, are likely to represent the majority of, if not all, cortical interneurons (Xu et al., 2010). The nature of the staining at the periphery of the cells is consistent with the presence of *Kv1.3* in the plasma membrane, suggesting that the channels contribute to the electrical excitability of these cells, although this was not tested explicitly. Our studies suggest that, as in the olfactory bulb, *Kv1.3* plays a unique role in neuronal differentiation and/or survival of interneuron populations and that expression of *Kv1.3* is required for normal cortical function.

The lack of significant changes in the numbers of NeuN⁺ cells between controls and *Kv1.3*^{-/-} mice suggest that *Kv1.3* plays a role in neuronal differentiation rather than regulating cell death or proliferation *per se*. Nevertheless selective block of *Kv1.3* and *Kv3.1* potassium channels has been found to increase neuronal progenitor cell proliferation and both channels have been previously found to be present in almost all neural progenitor cells (Liebau et al., 2006). Deletion of the *Kv1.3* gene is also accompanied by a decrease in overall cortical volume, although this is perhaps not surprising given that *Kv1.3* channels may regulate energy homeostasis, cell volume and apoptosis, and that *Kv1.3*^{-/-} mice weigh significantly less than control littermates (Xu et al., 2003).

Although the mammalian cortex contains a variety of inhibitory interneurons, each with distinct morphological, immunochemical, and/or physiological properties; in general, there are important differences in the expression, co-expression and distribution of cortical interneuron markers between different species and even in the same species between different regions and lamina (Rogers, 1992; Kawaguchi and Kubota, 1993, 1996; Kubota et al., 1994; Cauli et al., 1997; Gonchar and Burkhalter, 1997; Somogyi et al., 1998; Kawaguchi and Kondo, 2002; Markram et al., 2004; Wang et al., 2004; Halabisky et al., 2006; Xu et al., 2006, 2010; Gonchar et al., 2007; Ascoli et al., 2008; Zaitsev et al., 2009). PV interneurons are typically basket or chandelier cells while about half of the SOM cells

are typically Martinotti cells and the other half have different morphologies (Kawaguchi and Kubota, 1996; DeFelipe, 2002; Kawaguchi and Kondo, 2002; Xu et al., 2006; McGarry et al., 2010).

Both PV and SOM interneurons share similar developmental origins (Pleasure et al., 2000; Ang et al., 2003; Miyoshi et al., 2010; Corbin and Butt, 2011). Subclasses of GABA-ergic interneurons originate from distinct parts of the ganglionic eminence. PV, SOM and NPY interneurons develop and migrate from the median ganglionic eminence. In contrast, CR and VIP cells, which we found to be unchanged by deletion of the Kv1.3 gene, develop and migrate from the caudal ganglionic eminence. Interestingly the normal density of cells for both PV and SOM populations is very close (See Figure 4 and Table 2) and the deletion of Kv1.3 produces a change that is similar in magnitude, but opposite in direction, for the two classes of cells. This raises the possibility that a developmental switch dependent on Kv1.3 expression determines the fate of cells to become either PV or SOM interneurons.

A prime candidate for the developmental pathway that is regulated by Kv1.3 is the bone morphogenetic protein 4 (BMP4) signaling pathway. Overexpression of bone morphogenetic protein 4 (BMP4) in mice *in vivo* increases the number of interneurons expressing PV and reduces the number of those expressing SOM, an effect that exactly matches that of deletion of the Kv1.3 gene (Mukhopadhyay et al., 2009). The effects of BMP4 on the choice of neuropeptide and calcium binding protein are mediated by BMP type I receptors (BMPRI). This receptor also regulates the specification of calbindin-positive interneurons in the dorsomedial cortex, as well as the suppressive effect of BMP signaling on oligodendrocyte lineage commitment (Samanta et al., 2007).

Elimination of Kv1.3 could influence developmental regulation through the BMP4 or other signaling pathways through several distinct mechanisms. In many species, Kv1.3 plays a crucial role in T lymphocytes, where inhibition of the channel prevents immune responses (DeCoursey et al., 1984; Wulff et al., 2003; Nicolaou et al., 2007), although experiments in mice found normal immunological activity in Kv1.3^{-/-} animals (Koni et al. 2003). In addition this channel influences the fate of platelets and megakaryocytes (McCloskey et al., 2010). In each of these cases Kv1.3 is thought primarily to act through its effects on the resting membrane potential. Increases in potassium current render the membrane potential more negative, increasing the driving force for calcium entry, enhancing intracellular calcium-dependent responses that lead to proliferation. As stated in the introduction, however, Kv1.3 also interacts directly with a variety of cytoplasmic signaling pathways including α -integrins (Artym and Petty, 2002; Levite et al., 2000). Loss of these interactions could influence cell proliferation (Pardo, 2004), killing of cells by microglia (Fordyce et al., 2005) and structure and function (Biju et al., 2008; Fadool et al., 2004) in ways that go beyond the simple influence of channels on membrane potential. Moreover, Kv1.3 is potently regulated by a variety of signaling pathways including the insulin receptor and other growth factors linked to tyrosine phosphorylation (Bowlby et al., 1997; Fadool and Levitan, 1998; Fadool et al., 2000; Colley et al., 2004, 2007, 2009; Tucker et al., 2010). Loss of a key final effector of these signaling pathways could strongly influence developmental choices.

While the effect of loss of Kv1.3 on the function of the olfactory system has been established (Fadool et al., 2004), it is not yet clear what specific effect(s) the change in the relative ratio of PV and SOM interneurons may have on the function of the cerebral cortex. The relative ratios of different types of neurons maintain the excitatory-inhibitory balance and alterations in this balance are likely to alter cortical network function (Haider et al., 2006). Electrophysiologically, PV and SOM are very different, PV cells are fast spiking (FS) and SOM cells are regular spiking (RS). Hence, their contributions to cortical dynamics

and in particular to the inherent oscillatory behavior of cortical networks are also very different (Buzsaki and Draguhn, 2004). PV cells contribute to cortical gamma oscillations (30–80 Hz) while SOM cells contribute to beta oscillations (15–30 Hz) (Marin, 2012). Although both PV and SOM cells fire during UP cortical network states only SOM cells fire persistently during DOWN-states (Fanselow and Connors, 2010). Furthermore, within the subpopulations of PV or SOM interneurons, electrophysiologically distinct categories may exist (Halabisky et al., 2006; Fanselow et al., 2008). Nevertheless, based on our findings, we may speculate that an increase in basket and/or chandelier PV GABAergic cell numbers would increase the perisomatic and axo-axonic inhibition of excitatory pyramidal cells while a decrease on Martinotti SOM GABAergic cell numbers would decrease the inhibition of dendritic shafts and spine heads receiving excitatory inputs in upper layers of the cortex. This would be likely to produce an increase in gamma and a decrease in beta oscillations. Such changes may perhaps provide a mechanism for the increased locomotion and metabolism in Kv1.3^{-/-} animals (Tucker et al., 2008).

A wide variety of experiments have demonstrated that alterations in interneuron populations are correlated to a myriad of disorders of the nervous system (Corbin and Butt, 2011, Marin 2012). Moreover reduced excitability and/or reduced numbers of PV cells have been a prominent marker in Schizophrenia and related conditions (Marin, 2012). Nevertheless, substantial differences in neuronal densities may exist without functional changes. For example, the ratio of different neurons in visual areas of the macaque can vary by more than a factor of 2 without a clearly altered phenotype (van Essen et al 1984, 1986, Williams and Rakic 1986, Williams and Herrup 1988). Resolution of these issues will require further combined electrophysiological/behavioral investigations.

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Figure 1.

A. Kv1.3 stains the extracellular membrane of all cultured primary cortical cells (not permeabilized) that were visualized using the confocal microscope. Cell cultures were obtained at E14.5 and staining was done at E15.5. Kv1.3 is visualized with an antibody conjugated to FITC (green) and propidium iodide (PI - red) nuclear stain was used as counterstaining. Scale bar applies to lower magnification but at 20 μm . **B.** Kv1.3 is expressed throughout the cortex in both pyramidal and non-pyramidal cells. Here a sagittal view of the frontal cortex stained for Kv1.3 developed with DAB. Black arrow heads point to putative pyramidal cells while white arrowheads point to putative non-pyramidal cells. Pyramidal vs. non-pyramidal classification is based on cellular size and shape. **C–D.** Nissl and NeuN stainings indicate that the laminar structure of the Kv1.3^{-/-} mouse cortex is

preserved. **E.** No difference in NeuN⁺ cell numbers between controls and Kv1.3^{-/-} mice was detected by stereological counts.

Figure 2.

Expression of the transcription factor CDP (CCAAT displacement protein), a marker for layers II/III/IV, appears mostly absent in layers III/IV of *Kv1.3*^{-/-} mice while expression of ER81 (layer 5 marker) and FoxP2 (layer 6 marker) appear unchanged in wild type and *Kv1.3*^{-/-} mice. Top four panels show staining for CDP in wild type and *Kv1.3*^{-/-} mice using DAB as well as fluorescent staining. Lower panels show fluorescent staining for ER81 and FoxP2.

Figure 3.

Different classes of cortical interneurons express Kv1.3 channels. Double immunofluorescence staining was used to determine the presence of Kv1.3 channels in the different types of interneurons tested. In every case interneurons are labeled with secondary antibodies conjugated to Cy3 (red) and Kv1.3 channels with secondary antibodies conjugated to Alexa488 (green). Overlay of corresponding images indicates that all interneurons labeled expressed Kv1.3 channels in the soma membrane. Images were obtained using confocal microscopy with a Zeiss LSM 150 Meta laser scanning microscope coupled to a computer running Zeiss image acquisition and analysis software.

Methodology

Figure 4.

Methodology. **A.** Brains fixed with 4% PFA from transcardially perfused mice were obtained and serially cut in the sagittal plane at 50 μm thick. **B.** Sections were immunohistochemically processed to reveal the presence of CB, CR, PV, NPY, VIP or SOM. Section outlines and cell plotting was done using a Zeiss Axioskop microscope fully motorized and interfacing to a Dell computer running StereoInvestigator® (MicroBrightField Inc., Williston, VT). **C.** Stereological cell counts were done in the cerebral cortex using systematic random sampling (SRS) with an optical fractionator probe. In short, the program randomly places a grid on top of the area of interest and displays a counting box (**D**) on the lower right corner of each grid box. Stained cells inside the counting box or touching the inclusion border are counted; those outside the counting box or touching the exclusion border are not. Adequate sampling parameters (grid size, counting box size, etc.) are empirically obtained for each type of staining. See table 1 for stereological parameters and materials and methods for further details. Diagrams, including the grid and boxes, are not to scale.

Figure 5.

Deletion of Kv1.3 increases the number of PV interneurons in the cerebral cortex. The upper diagrams show actual samplings. The punctate marks inside of the cortex represent positively stained cells plotted inside counting boxes ($200\ \mu\text{m} \times 200\ \mu\text{m}$). The thickness of the cortex appears reduced in Kv1.3^{-/-} animals but the rostrocaudal extent does not appear to be affected. Panels A and C show staining for parvalbumin for the entire extent of the cortex while panels B and D show magnified images of the boxes shown in A and C.

Figure 6.

Deletion of Kv1.3 decreases the number of SOM interneurons in the cerebral cortex. The upper diagrams show the actual samplings. The punctate marks inside of the cortex represent positively stained cells plotted inside counting boxes, which were the same size ($200\ \mu\text{m} \times 200\ \mu\text{m}$) for both SOM and PV (see Fig. 5). Notice that the difference in density of plotted cells is easily seen between control and Kv1.3^{-/-} cases even though the grid (sampling frequency) is different for PV and SOM (see table 1). Panels A and C show staining for somatostatin at a higher magnification while panels B and D show lower power views.

Figure 7.

Deletion of Kv1.3 affects cortical interneuron numbers, densities and cortical volume. **A.** The mean number of cortical interneurons is always higher in controls than in Kv1.3^{-/-} except for PV in which the trend is reversed. Also, the difference in mean cortical interneuron numbers between the two groups is always statistically significant except for the case of VIP. However, because cortical volume is reduced in all Kv1.3^{-/-} cases as compared to controls (**C**), the crucial changes are those in interneuron densities (**B**). In this case, the changes in density are statistically significant only in the case of two interneuron types, PV and SOM. Also, the changes are opposite so that there is a much higher density of PV cells in the Kv1.3^{-/-} cases than in controls and much lower density of SOM cells in in Kv1.3^{-/-} than in controls.. **C**, the reduction in cortical volume was observed for all experiments, independent of interneuron staining type. Therefore, in each case when the mean cortical volume for a particular interneuron staining is compared to its control counterpart, the difference in cortical volume is always statistically significant.

Table 1

Animals and stereological parameters

		n (# of animals)	Counting frame	Grid size	Probe height
CB	CON	12	200 × 200	1000 × 1000	12
	Kv1.3^{-/-}	12			
CR	CON	6	250 × 250	500 × 500	8
	Kv1.3^{-/-}	6			
PV	CON	12	200 × 200	1000 × 1000	12
	Kv1.3^{-/-}	12			
NPY	CON	9	250 × 250	500 × 500	8
	Kv1.3^{-/-}	10			
VIP	CON	8	250 × 250	500 × 500	8
	Kv1.3^{-/-}	9			
SOM	CON	6	200 × 200	500 × 500	8
	Kv1.3^{-/-}	6			

Table 2

Stereological results

Cell type			# of cells	Cortical Volume (mm ³)	Density (#cells/mm ³)
CB	CON	Mean	166590.6	13.6	12277.5
		SD	64295.2	1.2	4782.8
		SEM	19385.7	0.4	1442.1
	KO-	Mean	114270.5	11.5	9982.4
		SD	53242.2	0.6	4731.9
		SEM	15369.7	0.2	1366.0
CR	p-value		0.04490	0.00003	0.26066
	CON	Mean	35466.7	14.6	2438.2
		SD	8714.7	1.1	587.2
		SEM	3557.8	0.5	239.7
	KO-	Mean	24833.3	12.5	2000.4
		SD	7784.5	0.4	649.1
PV	p-value		0.04993	0.00178	0.24865
	CON	mean	77985.7	13.2	5906.9
		SD	19270.8	0.9	1377.0
		SEM	5563.0	0.3	397.5
	KO-	mean	111041.4	11.0	9971.3
		SD	38124.6	0.7	3119.0
NPY	p-value		0.01366	0.00000	0.00044
	CON	mean	51966.7	14.5	3642.6
		SD	13822.9	1.9	1203.2
		SEM	4607.6	0.6	401.1
	KO-	mean	34750.0	11.6	2991.5
		SD	11077.8	1.4	967.0
		SEM	3503.1	0.4	305.8

Cell type			# of cells	Cortical Volume (mm ³)	Density (#cells/mm ³)
	p-value		0.00787	0.00148	0.20878
VIP	CON	Mean	36471.4	13.5	2790.8
		SD	17677.9	1.1	1507.6
		SEM	6681.6	0.4	569.8
	KO ⁻	Mean	31955.6	11.4	2876.2
		SD	8966.1	1.0	1033.8
		SEM	2988.7	0.3	344.6
SOM	p-value		0.51490	0.00139	0.89479
	CON	Mean	86015.2	13.6	6436.1
		SD	15590.4	1.2	1658.9
		SEM	6364.8	0.5	677.3
	KO ⁻	Mean	26692.3	12.0	2244.2
		SD	9143.7	0.8	813.8
	p-value		3732.9	0.3	332.2
			0.00001	0.01929	0.00024