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Original Article

The Kv12 voltage-gated K^+ channels are expressed in the Phox2b-expressing neurons in the nucleus tractus solitarii in mice

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Abstract: Accumulating evidence demonstrates that the nucleus tractus solitarii (NTS) neurons serve as central respiratory chemoreceptors, but the underlying molecular mechanisms remain undefined. The present study investigated the expression of acid-sensitive ether-à-go-go-gene-like (Elk, Kv12) channels in the NTS of mice. Immunofluorescence staining was used to observe the distribution and cellular localization of the Kv12 channels in NTS neurons. Western blot and quantitative real-time PCR (qPCR) were used to evaluate protein and mRNA expression levels of Kv12 channels. The results showed that all of the three members (Kv12.1, Kv12.2, Kv12.3) of the Kv12 channel family were expressed in NTS neurons, and their expressions were co-localized with paired-like homeobox 2b gene (Phox2b) expression. The expression of Kv12.1 mRNA was the largest, whereas the expression of Kv12.3 was the least in the NTS. The results suggest Kv12 channels are expressed in Phox2b-expressing neurons in the NTS of mice, which provides molecular evidence for pH sensitivity in Phox2b-expressing NTS neurons.

Key words: nucleus tractus solitarii; Kv12; Phox2b

小鼠孤束核Phox2b神经元表达电压门控钾离子通道Kv12

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摘 要：大量研究表明孤束核神经元具有呼吸化学敏感特性，但是其分子机制仍有待揭示。本研究旨在观察电压门控钾离子通道Kv12在小鼠孤束核的表达。通过免疫荧光染色实验检测不同亚型Kv12 (Kv12.1，Kv12.2，Kv12.3)在孤束核的分布和细胞定位，用Western blot和定量PCR检测其不同亚型蛋白和mRNA在孤束核的表达差异。结果表明Kv12三种亚型在孤束核神经元都有表达，并且与Phox2b (paired-like homeobox 2b gene)存在共表达。三种亚型中Kv12.1 mRNA表达量最大，Kv12.3表达量最低。本研究结果表明小鼠孤束核Phox2b神经元表达Kv12，为孤束核神经元的pH敏感性提供了相关证据。

关键词：孤束核；Kv12；Phox2b
中图分类号：Q4

The nucleus tractus solitarii (NTS), located in the dorsomedial medulla, are the primary sites for integrating cardiorespiratory reflexes. Previous reports have shown that NTS neurons can sense CO_2 stimulation and induce hypercapnic ventilatory response (HCVR) [1]. Focal lesions of a portion of the NTS region attenuated HCVR [2]. In isolated brain slices, the discharge frequency of NTS neurons is altered when CO_2/H^+ concentration changes [3]. Blocking chemical and electrical synaptic transmissions does not abolish the chemosensitive response of NTS neurons [4]. Taken together, these findings indicate that NTS neurons are inherently

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pH-sensitive, supporting the hypothesis that the NTS is an important central respiratory chemosensitive region. However, the molecular signature of NTS neurons for their chemosensitivity remains undefined, and the mechanism of their pH sensitivity has not been entirely elucidated. Recently, our studies found that paired-like homeobox 2b gene (Phox2b)-expressing NTS neurons are CO_2/H^+-sensitive. Activation of these neurons augments breathing [5], and destruction of these neurons results in an impaired HCVR in mice [6].

The ether-à-go-go-gene-like (Elk, Kv12) channels are members of the ether-à-go-go (Eag) superfamily of voltage-gated K^+ (Kv) channels. These channels are activated at more hyperpolarized potentials than other outwardly-rectifying Kv channels and have a strong influence on resting membrane potential [7]. There are three members in the Kv12 family: Kv12.1, Kv12.2, and Kv12.3, encoded by the *Kcnh* genes. Previous studies indicate that all of the three members of the Kv12 K^+ family are inhibited by extracellular acidosis [8], indicating that Kv12 channels are pH-sensitive. Despite significant progress in understanding biophysical properties of these channels, there is still little information on the physiological functions and distribution of Kv12 channels in brain neurons.

We hypothesized that Phox2b-expressing NTS neurons express Kv12 channels and these channels may be the potential molecular pH base in Phox2b-expressing NTS neurons.

1 MATERIALS AND METHODS

1.1 *Animals*

All experiments were performed in male 8-week-old C57BL/6 mice (25–30 g). Mice were housed under controlled temperature [(21 ± 1) °C] and humidity [(50 ± 10)%] conditions, with a fixed 12-h light/12-h dark cycle. The mice had *ad libitum* access to both food and water. All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Hebei Medical University Animal Care and Ethics Committee.

1.2 *Immunofluorescence staining*

Previously-published immunohistological protocols were used [6]. Briefly, three mice were anesthetized using urethane (1.8 g/kg, i.p.) and transcardially perfused with 200 mL chilled saline, followed by 4% phosphate-buffered paraformaldehyde (0.1 mol/L, pH 7.4). The animals were then decapitated; the brainstems were removed and stored in perfusion fixative at 4 °C for 48 h, and then immersed in 30% sucrose in PBS at 4 °C for at least 2 days. The rostrocaudal position of each coronary section was determined (bregma: 7.20 to 7.76 mm, approximately corresponding to intermediate and caudal NTS) according to the mouse brain in stereotaxic coordinates [9]. A series of 25-μm sections were cut by a cryostat (CM1950; Leica Microsystems, Wetzlar, Germany). Tissue sections were washed 3 times with PBS, and immersed in blocking buffer (2% BSA in PBS) for 30 min at room temperature. For immunofluorescence staining, sections were incubated with primary antibodies at 4 °C overnight. Primary antibodies were omitted from the incubation solution in the negative control. After being rinsed with PBS, sections were incubated with secondary antibodies for 1 h at room temperature. Finally, sections were mounted and examined with a fluorescence microscope (DM6000B, Leica Microsystems, Wetzlar, Germany). Images were acquired and processed with the Leica Application Suite, version 4.6.

1.3 *Antibody characterization*

Phox2b was detected using a mouse monoclonal antibody (dilution 1:200, sc-376993; Santa Cruz, CA, USA), as in our earlier publication [5]. Kv12.1, Kv12.2, and KV12.3 were detected with rabbit polyclonal antibodies (#APC-113, 1:200, Alomone labs, Israel; #NBP2-14142, 1:500, Novus, USA; and #APC-116, 1:200, Alomone Labs, Israel), respectively. Specificity of Kv12 antibodies was examined by Western blot by confirming that each antibody detected a single protein band at the predicted molecular weight. Primary antibodies were detected by using a matched secondary antibody tagged with fluorescent reporters to reveal Phox2b (dilution 1:200, goat anti-mouse Cy3; Jackson Laboratories Inc., West Grove, PA, USA), Kv12.1, Kv12.2, and Kv12.3 (dilution 1:200, donkey anti-rabbit FITC; Jackson Laboratories Inc.) expression in the NTS region.

1.4 *Western blot analysis*

Mice were anesthetized with urethane (1.8 g/kg, i.p.). The brain stem was quickly removed, and coronary slices (300 μm) were cut using a vibratome (VT1200S; Leica Biosystems, Wetzlar, Germany) in ice-cold saline. According to the anatomical map [9], the NTS regions (n = 3) from 8 mice were removed using a microscope and homogenized in 100 μL of RIPA buffer

(150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton-X 100, 50 mmol/L Tris-HCl at pH 7.5) with protease inhibitor cocktail (Roche Diagnostics, Canada). The homogenate was centrifuged at 12 000 r/min at 4 °C for 15 min. The supernatant was retained, as it contained the protein fractions of interest. The concentration of this protein extract was determined using the colorimetric BCA protein assay (Pierce Corp., USA). After denaturation, an equal amount of protein (30 μg) was loaded into each lane, separated by electrophoresis on a 10% polyacrylamide gel, and then transferred to a PVDF membrane (Millipore Corporation, USA). The membranes were blocked with bovine serum albumin (BSA) and incubated at 4 °C overnight with primary antibodies: anti-Kv12.1 (#APC-113, 1:200, Alomone Labs, Israel), anti-Kv12.2 (#SAB2101210, 1:500, Sigma, USA), anti-Kv12.3 (#APC-116, 1:200, Alomone Labs, Israel), and anti-GAPDH (#T0022, 1:3 000, Affinity Biosciences, USA). The membranes were then incubated with corresponding secondary antibodies for 1 h at room temperature. The reaction was visualized using the enhanced chemiluminescence (ECL) method.

1.5 *Quantitative real-time PCR (qPCR)*

NTS tissues were rapidly harvested as described above and frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen, USA) method. RNA integrity was assessed using a Nano Drop 2000 spectrophotometer (Thermo, USA). Total RNA was reverse-transcribed using the Invitrogen 2-step RT kit and Superscript II first strand synthesis kit (Invitrogen, USA) according to the manufacturer's instructions. The cDNA was diluted 1:20 and used for qPCR. Platinum SYBR Green qPCR Super Mix-UDG with ROX (Invitrogen, USA) was used to measure gene expression using specific primers of *Kcnh8*, *Kcnh3*, *Kcnh4*, and *Gapdh*. These primers were designed in our lab, and their sequences are provided in Table 1. Dissociation curve analysis was performed following all qPCRs to ensure uniformity in the amplified product. All reactions were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems, USA). Gene expression was normalized to *Gapdh*, and the data were analyzed using the comparative $2^{-\triangle\triangle Ct}$ method.

Table 1. PCR primers used to analyze Kv12 gene expression

Target gene	Primer sequences
Kcnh8	5'-ACCTAACCATGGTAGAAAGAAAGA-3'
	5'-GTTTCCTCATTGCTGTTACCATCT-3'
Kcnh3	5'-AGTCCATTTGCCTCCACTACGTCA-3'
	5'-AAGGTGAGCACAACCGCGCTGTAC-3'
Kcnh4	5'-TGACATCGCTGTGGAAATGCTCTT-3'
	5'-TCAAAGGGCAAAGCAGCAATGAGG-3'
Gapdh	5'-GCAAATTCAACGGCACAGTCAAGG-3'
	5'-TCTCGTGGTTCACACCCATCACAA-3'

1.6 *Statistical analysis*

Statistical analysis was performed using Prism version 7 software (GraphPad Software Inc., La Jolla, CA, USA). Values are presented as mean ± SEM. Data were compared using one-way ANOVA followed by Tukey's tests. Results were considered significant when $P < 0.05$.

2 RESULTS

2.1 Co-expression of Kv12 and Phox2b in NTS neurons

There was no immunoreactivity for Kv12 in the negative control (Fig. 1*A*). In contrast, Kv12 immunoreactivity was observed in the NTS and adjacent areas when Kv12 antibodies were present (Fig. 1*B–D*).

To assess whether Kv12 and Phox2b were co-expressed in NTS neurons, double-immunofluorescence staining was performed. As shown in Fig. 2, all of the three Kv12 subtypes (Kv12.1, Kv12.2, and Kv12.3) were present in NTS neurons. Kv12 was mainly expressed in the cell membrane. We also observed Kv12 expression in neurons of adjacent areas such as the dorsal motor nucleus of vagus (DMNV) and the area postrema (AP). All three Kv12 subtypes were found to be co-expressed with Phox2b (Fig. 2).

2.2 Kv12 expression in the NTS region

Western blot analysis showed that all of the Kv12 subtypes (Kv12.1, Kv12.2, and Kv12.3) were expressed in the NTS region of mice (Fig. 3*A*). qPCR showed that among the three genes, the mRNA expression of Kv12.3 (*Kcnh4*) was the lowest, and the mRNA expression of Kv12.1 (*Kcnh8*) and Kv12.2 (*Kcnh3*) was 170% and 140% higher than Kv12.3 (*Kcnh4*) respectively (Fig. 3*B*).

3 DISCUSSION

In this study, we used morphological and molecular biology methods to investigate the expression of Kv12 channels in the NTS region. We found that all of three members of the Kv12 channel family were expressed

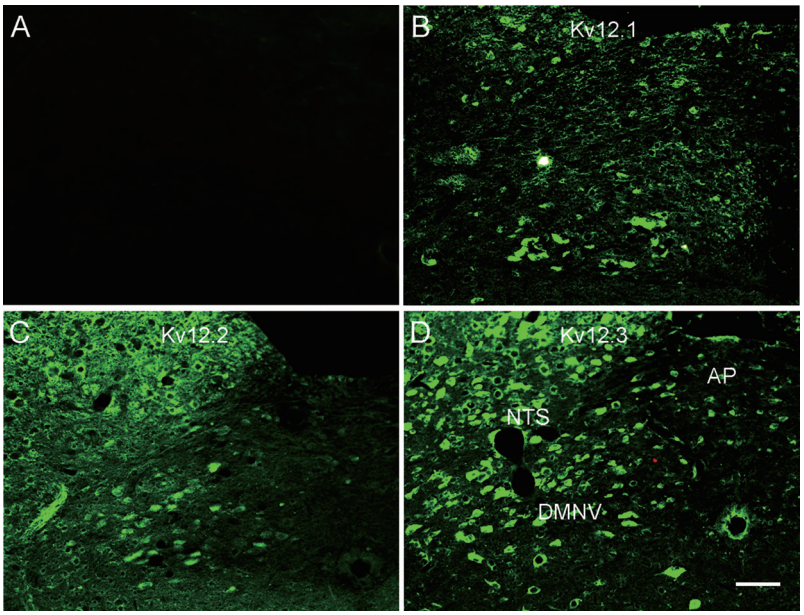

Fig. 1. Expression of Kv12 in nucleus tractus solitarii (NTS) neurons of mice detected by immunofluorescence staining. *A*: Negative control for immunofluorescence experiments. There was no immunoreactivity in the NTS or adjacent areas when Kv12 antibody was not present. Kv12 immunoreactivity was observed when Kv12.1 (*B*), Kv12.2 (*C*) or Kv12.3 (*D*) antibody was included respectively. Scale bar, 100 μm. AP, area postrema; DMNV, dorsal motor nucleus of vagus.

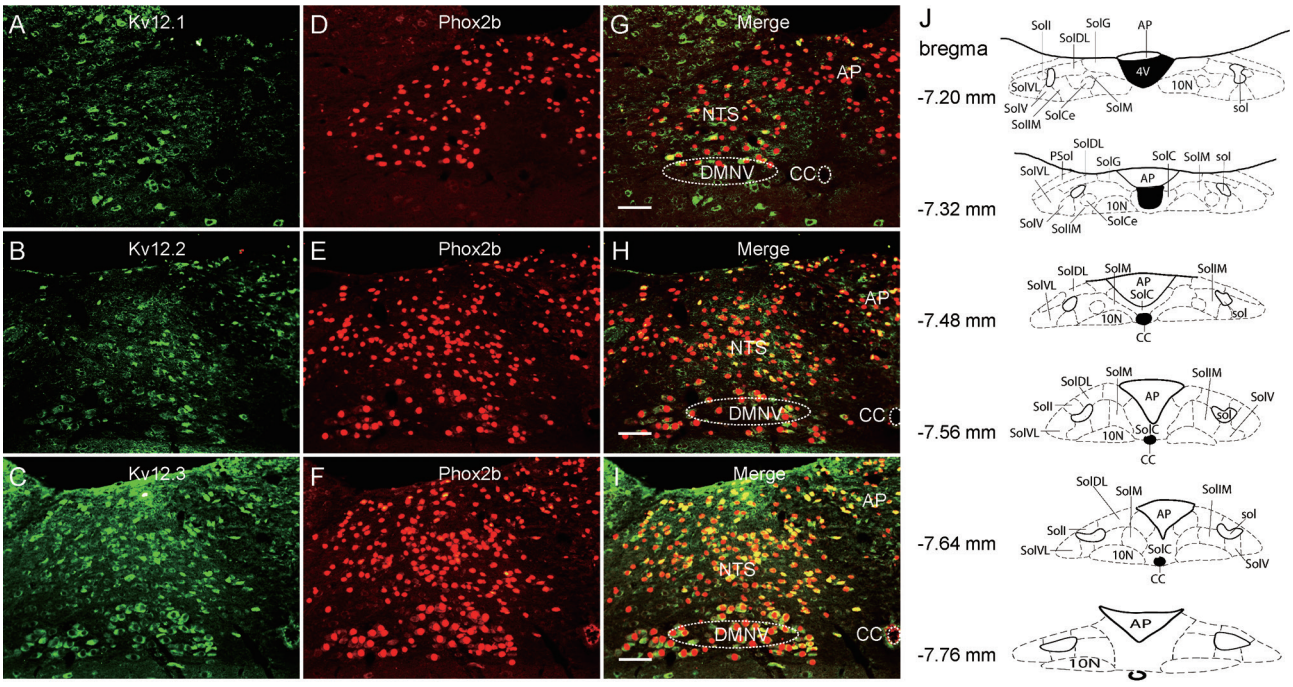

Fig. 2. Photomicrographs of immunofluorescence staining for Kv12 and Phox2b in nucleus tractus solitarii (NTS) neurons of mice. All three members of the Kv12 family exhibited immunoreactivity which were labeled green in the membrane (*A*, *B*, *C*) and Phox2b immunoreactivity which were labeled red in the nucleus (*D*, *E*, *F*). The merged photomicrograph shows that most Kv12-expressing cells were also Phox2b-positive (*G*, *H*, *I*). Scale bar, 100 μm. *J*: The schematic drawing of the slice based on the atlas of the mouse brain in sterotaxic coordinates. AP, area postrema; DMNV, dorsal motor nucleus of vagus; CC, central canal; SolDL, dorsolateral part of the NTS; SolIM, intermediate part of NTS; SolM, medial part of the NTS; sol, solitary tract; SolV, ventral part of NTS; PSol, parasolitary nucleus.

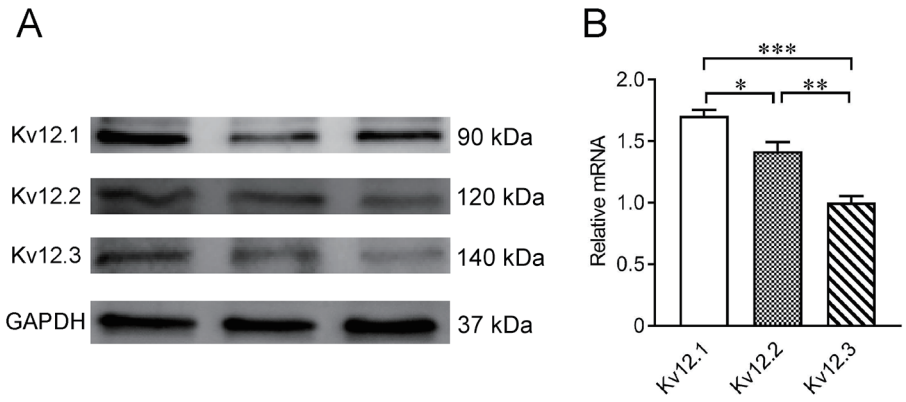

Fig. 3. Kv12 protein and mRNA expression in the nucleus tractus solitarii (NTS) region of mice. Western blot (*A*) and qPCR (*B*) analysis of all three members of the Kv12 channel family in the NTS. Mean ± SEM, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with Tukey's *post hoc* test.

in NTS neurons, with the abundance of Kv12.1 the highest and that of Kv12.3 the least. Furthermore, all three members of Kv12 were highly expressed in Phox2b-positive NTS neurons.

Both *in vivo* and *in vitro* evidence suggests that NTS neurons are respiratory chemosensitive [1, 4]. Phox2b is a disease-defining gene for congenital central hypoventilation syndrome (CCHS), and a mutation in this gene in animal models results in hypoventilation [10]. Our recent studies show that 69% of NTS neurons express Phox2b in mice, and 43% of these Phox2b-expressing NTS neurons exhibit an intrinsic sensitivity to CO_2/H^+ [6]. However, the membrane molecules responsible for their chemosensitivity remain unclear. The Elk Kv channels have a low activation threshold, suggesting that they are well-adapted to control the intrinsic excitability of neurons. In addition, the Kv12 channels have been reported to be inhibited by extracellular acidosis [8], but little information is available regarding whether the acidosis-induced inhibition is related to Kv12 channels. In this study, we found that all of the three members of the Kv12 channel family are expressed in some Phox2b-expressing NTS neurons, which might account for their intrinsic sensitivity to CO_2/H^+. Further studies are needed to confirm this postulation.

As central respiratory chemoreceptors, NTS neurons integrate peripheral and visceral sensory information, and relay it to the parasympathetic preganglionic neurons in the DMNV, from which the axons project and innervate many visceral organs via GABAergic, glutamatergic, and catecholaminergic terminals [11]. The AP located on the wall of the fourth ventricle is a sensory circumventricular organ. AP neurons sense and integrate blood-borne baroreceptor information from the carotid sinus and aorta, osmoreceptor information from the liver, and mechanical information from stretch receptors in the stomach [12]. In this study, we found that all of the three members of Kv12 are expressed in DMNV and AP neurons in addition to the NTS. Based on the neurochemical evidence, we speculate that Kv12 may participate in the control of the excitability of DMNV and AP neurons; however, further investigation is required to confirm this speculation.

In summary, our study demonstrates that all of the three members of the Elk subfamily of Kv channels (Kv12.1, Kv12.2, and Kv12.3) are expressed in Phox2b-expressing NTS neurons. The results provide molecular evidence for pH sensitivity in Phox2b-expressing NTS neurons.

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