

Identification of pannexins in rat nasal mucosa

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ABSTRACT

Pannexins are a second family of gap-junction proteins in vertebrates, classified as pannexin-1, pannexin-2, and pannexin-3. Pannexin-1 is one of the candidates for channel-mediated ATP release into the extracellular space. In airway epithelia, ATP signaling modulates multiple cellular functions such as mucus/ion secretion and mucociliary clearance systems. However, the expression of pannexins in the upper airway has not been investigated. Nasal septal mucosae were collected from adult male Wistar rats aged 20–24 weeks. The expression of pannexin-1, pannexin-2, and pannexin-3 was examined by reverse transcription polymerase chain reaction (RT-PCR) and by whole-mount fluorescence immunohistochemistry. Transcripts for pannexin-1, pannexin-2, and pannexin-3 were detected in nasal septal mucosae of adult rats by RT-PCR. Distinct immunohistochemical fluorescence for pannexin-1 was observed in the epithelial layer, whereas there was no immunoreactivity for pannexin-2 or pannexin-3. This is the first article establishing the existence of pannexins (predominantly pannexin-1) in the upper airway, suggesting their possible participation in the physiological functions of ATP release and signaling in this tissue.

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Pannexins in vertebrates are a family of transmembrane channel proteins that are homologous to innexins, which are invertebrate gap-junction proteins.¹ The pannexin family contains three subtypes: pannexin-1, pannexin-2, and pannexin-3. These proteins have no significant sequence similarity to connexins, the prototypical vertebrate gap-junction proteins.² The expression of pannexins has been reported in the brain,^{1,3} muscle, bone, and skin. Pannexin-1 is the most thoroughly investigated member of the pannexin family; it forms an ATP-permeable, large-conductance (~500 pS), nonselective channel.^{4,5}

Current evidence suggests that release of ATP into the extracellular space is mediated by two different mechanisms, including vesicle exocytosis and release through a conductive channel such as pannexin-1.^{4,5} In the airway, extracellular ATP is important for the regulation of mucus/ion secretion and mucociliary clearance systems.^{6–9} Recent work shows that pannexin-1 is expressed on the apical surface of primary-cultured lung epithelial cells and participates in ATP release.¹⁰

However, there are no previous reports on the expression of pannexins in the upper airway.

In this study, we investigated the expression of pannexins in the nasal mucosa of rats. This report provides the first evidence for the presence of pannexins in the upper airway.

MATERIALS AND METHODS

Sampling of Rat Nasal Septal Mucosa

Eight adult male Wistar rats (Kyudo, Saga, Japan) aged 20–24 weeks were used. The animals were housed in standard plastic cages at 23–25°C in a 12-hour light/dark cycle. All experiments were performed in accordance with the guidelines of the Physiologic Society of Japan and approved by the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health. Anesthesia was administered to the rats by diethyl ether inhalation, and the animals were decapitated. The dorsum nasi was surgically cut open at the midline (Fig. 1 A), and the nasal septal mucosa was collected.

Reverse Transcription Polymerase Chain Reaction

The collected mucosal segments for each animal were pooled in a polymerase chain reaction (PCR) tube with carrier poly-A RNA (Qiagen, Valencia, CA). Total RNA was purified with an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. The purified total RNA was mixed with 5 μM of oligo-(dT) primers (Takara, Shiga, Japan) and 1 mM of dNTPs (Takara), heated at 65°C for 5 minutes, and cooled on ice. The sample was then mixed with 1× PrimeScript Buffer (Takara), 40 U of RNase inhibitor (Takara), and

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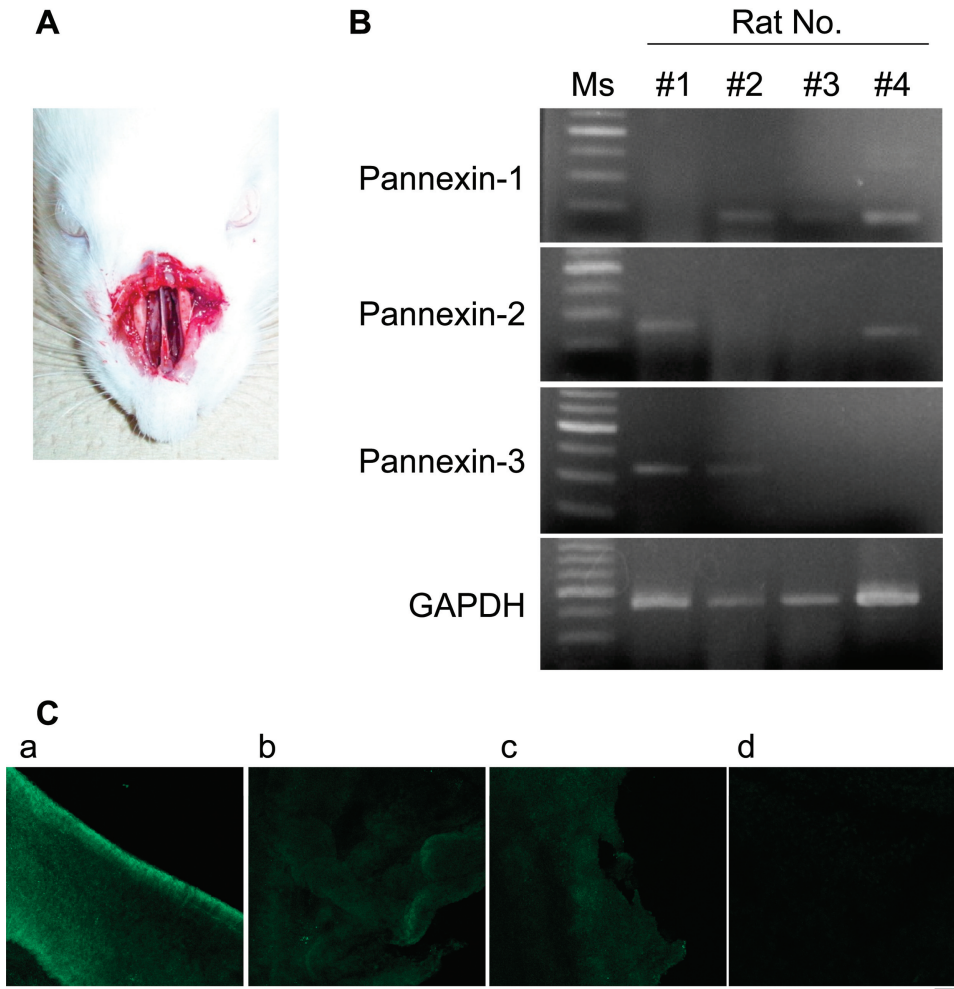


Figure 1. Pannexin expression and localization in rat nasal mucosa. (A) Photograph of the dissection of the rat nasal cavity. The dorsum nasi is surgically cut open at the midline, and the nasal cavity is exposed. (B) Expression of pannexin mRNA transcripts in rat nasal mucosa. Product sizes for pannexin-1, pannexin-2, pannexin-3, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) are 185, 258, 336, and 429 bp, respectively. The 100-bp marker ladder is denoted as Ms; the bright bands indicate 500-bp. (C) Photomicrographs of whole-mount immunohistochemical fluorescence staining of rat nasal mucosa. A, Pannexin-1; b, pannexin-2; c, pannexin-3; d, negative control. Distinct fluorescence for pannexin-1 is observed in the epithelial layer; there is no immunoreactivity for pannexin-2 or pannexin-3 (scale bar indicates, 60 μ m).

200 U of PrimeScript RTase (Takara), in a total volume of 40 μ L. Reverse transcription (RT) was performed at 42°C for 60 minutes. After the reaction was stopped at 70°C for 15 minutes, the products were stored at -20°C until further use.

PCR amplification of pannexin-1, pannexin-2, pannexin-3, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, positive control) mRNAs was performed in a 50- μ L reaction mixture containing 3.3 μ L of cDNA template, 1 \times Ex Taq Buffer, 0.2 mM of dNTPs, 0.12 μ M each of the forward and reverse primers, and 0.05 U of Ex Taq (Takara). The following sets of primers, which were consistent with previous reports,^{11,12} were used: 5'-TTC TTC CCC TAC ATC CTG CT-3' (forward) and 5'-GGT CCA TCT CTC AGG TCC AA-3' (reverse) for pannexin-1 (GenBank accession no., NM_199397.1; product size, 185 bp); 5' -TGG ACA TCG TAT TGC TCT GC-3' (forward) and 5'-CCA CGT TGT CGT ACA TGA GG-3' (reverse) for pannexin-2 (GenBank accession no., NM_199409.2; product size, 258 bp); 5'-GAC CCC CTG AAA CAC TTT GA-3' (forward) and 5'-TCT AAC CCA CTG GCC TTC AC-3' (reverse) for pannexin-3 (GenBank accession no.,

NM_199398.1; product size, 336 bp); and 5'-CAT GCC GCC TGG AGA AAC CTG CCA-3' (forward) and 5'-GGG CTC CCC AGG CCC CTC CTG T-3' (reverse) for GAPDH (GenBank accession no., NM_017008.3; product size, 429 bp). Amplification was performed by denaturing at 94°C for 30 seconds, 40 cycles of 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The amplified products were analyzed by electrophoresis in a 2% agarose gel, stained with ethidium bromide, and visualized under UV irradiation. Gel images were captured with an AE-6905 Hours Image Saver HR (ATTO, Tokyo, Japan).

Whole-Mount Fluorescence Immunohistochemistry

The nasal mucosa was fixed with 4% paraformaldehyde in 0.1 M of phosphate-buffered saline (PBS) at pH 7.4 for 4 hours at room temperature and washed with PBS. After 1 hour of incubation with 1% fetal bovine serum and 0.3% Triton X-100 in PBS, the mucosa was washed and incubated at 4°C for 2 hours with rabbit anti-rat pannexin-1 polyclonal antibody (1:500), rabbit

anti-rat pannexin-2 polyclonal antibody (1:500), or rabbit anti-rat pannexin-3 polyclonal antibody (1:500; all from Abcam, Cambridge, U.K.) in PBS. After washing, the samples were incubated with the fluorescently labeled secondary antibody, Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA), in PBS for 1 hour. Samples were mounted with coverslips, and images were acquired using a confocal laser scanning microscope (LSM 5 PASCAL; Carl Zeiss Co., Heidelberg, Germany). For controls, primary antibodies were omitted from the protocol.

RESULTS AND DISCUSSION

The results of RT-PCR are presented in Fig. 1 B. Four rats were used for this experiment. Positive bands were observed in three animals (rats 2, 3, and 4) for pannexin-1, in two animals (rats 1 and 4) for pannexin-2, and in two animals (rats 1 and 2) for pannexin-3. The GAPDH band was positive in all four animals. These results indicate that pannexin transcripts are present in rat nasal mucosa.

Representative photomicrographs of the fluorescence immunohistochemistry results are presented in Fig. 1 C. Four rats were used for this experiment. Distinct fluorescence for pannexin-1 was observed in the epithelial layer of two of the animals. By contrast, no immunoreactivity was detected for pannexin-2 or pannexin-3 in any of the animals. These results provide further evidence for the existence of pannexin-1 in rat nasal mucosa.

Pannexins form single membrane-localized channels; their role as gap-junction proteins has not been recognized *in vivo*.^{4,5,10,13} Pannexin-1 is one of the leading candidates for channel-mediated ATP release into the extracellular space.^{4,5} ATP signaling modulates multiple cellular functions such as mucus/ion secretion and mucociliary clearance.^{6–9,14} The expression of pannexins in the upper airway has not been investigated previously, despite their potential role in regulating airway epithelial functions. Using RT-PCR, the present study provides the first evidence that transcripts for pannexin-1, pannexin-2, and pannexin-3 are expressed in rat nasal mucosa. Immunohistochemical analyses indicate that pannexin-1 is the predominant pannexin subtype, which is localized in the epithelial layer. These results suggest that pannexin-1 in the nasal mucosa is likely to play an important role in the release of ATP into the extracellular space.

The present study reveals differences in the distributions of transcripts (RT-PCR) and proteins (immuno-

fluorescence staining) of pannexin-1, pannexin-2, and pannexin-3 in each animal. At the current time, definitive evidence to explain these variations is lacking. Additional biochemical evidence is needed.

In conclusion, we investigated the expression of pannexins in rat nasal mucosae. This is the first report showing the existence of pannexins, predominantly pannexin-1, in the upper airway. These data may provide new insight into respiratory physiology. The physiological and pathological significance of pannexins in nasal mucosa remains to be investigated further in animal and human studies.

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