Immunohistochemical and biomolecular identification of 5-HT\textsubscript{7} receptor in rat vestibular nuclei

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Abstract. The association between migraine and balance disorder morbidities has been a topic of interest for many years, and serotonin (5-HT) receptor is known to be closely related with migraine and also to be associated with vestibular symptoms. However, the mechanism underlying the pathogenesis of migrainous vertigo and its association with 5-HT has not been elucidated. Of the many 5-HT receptors, 5-HT\textsubscript{7} receptor has recently attracted attention in the context of migraine treatment. The purpose of this study was to investigate the localization and expression of 5-HT\textsubscript{7} receptor in the rat vestibular nuclei by immunohistochemical staining and reverse transcriptase-polymerase chain reaction (RT-PCR). The present study might provide additional insight into the role of 5-HT\textsubscript{7} receptor in the pathogenesis of migraine-related vestibular symptoms.

Keywords: Migraine, vertigo, 5-HT\textsubscript{7} receptor, vestibular nuclei

1. Introduction

The association between migraine and balance disorders has been a subject of interest for many years [11]. Vestibular symptoms frequently occur in association with migraine, but the pathogenesis of migraine-related vestibular symptoms has not been determined. The activation or blockade of serotonin (5-hydroxytryptamine, 5-HT) receptors is known to be closely related with migraine and its associated vestibular symptoms [5]. However, the nature of this involvement has not been fully elucidated yet.

The pathophysiology of migrainous vertigo is unclear; however, there are several links between central vestibular pathways and proposed mechanisms involved in migraine. Such interactions may involve the vestibular nuclei, the trigeminal system and thalamocortical pathways. Reciprocal connections between the inferior, medial and lateral vestibular nuclei and the trigeminal nucleus caudalis may provide a mechanism whereby vestibular signals can influence trigeminovascular pathways and that produces a tight linkage between vestibular and trigeminal information processing during migraine [5].

Several neurotransmitters that are involved in the pathogenesis of migraine (serotonin, calcitonin-gene related peptide, noradrenaline, dopamine) are known to modulate the activity of vestibular neurons. Several lines of evidence in the literature have suggested that 5-HT\textsubscript{7} receptors may be involved in the pathogen-
esis of migraine [16]. The 5-HT7 receptor, coupled to Gs proteins and stimulating cAMP production [7], is the most recently identified member of the family of 5-HT receptors. Recently, a series of 5-HT7 receptor antagonists were developed, and studies on the selective 5-HT7 receptor antagonists SB-269970-A and SB-656104-A suggest that this receptor plays a role in various disorders, such as depression and migraine [1,20]. The 5-HT7 receptor and its mRNA are found distributed in meningeal tissues including middle meningeal arteries [18], trigeminal ganglion [17], and spinal trigeminal nucleus [21], which indicates that 5-HT7 receptors are located in the pathway of trigeminovascular system. The 5-HT7 receptors and their effect possibly include pain, hyperalgesia, and the facilitation of neurogenic inflammation [16]. However, no attempt has been made to investigate the localization and expression of 5-HT7 receptor in vestibular nuclei. Therefore, the aim of this study was to investigate the localization and expression of 5-HT7 receptor in rat vestibular nuclei by immunohistochemical staining and reverse transcriptase-polymerase chain reaction (RT-PCR).

2. Methods

A total of 16 male Sprague-Dawley rats (300–400 g; Central Laboratory Animal Inc., Seoul) were used in this study. All experimental protocols used complied with the Guidelines of the National Institute of Health and the Declaration of Helsinki, and were approved by the Committee on the Use and Care of Animals at Gyeongsang National University.

2.1. Immunohistochemical staining of 5-HT7 receptor in the four major vestibular nuclei

The ten rats for the immunohistochemistry study were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with phosphate-buffered saline (PBS, 0.9% NaCl in 50 mM phosphate buffer, pH 7.3) followed by paraformaldehyde-lysine-periodate (PLP) fixative [10]. Heads were removed, skinned, and brains were carefully removed and placed in 30% sucrose-PBS at 4°C for 48–72 h. Sections (35 μ) were cut on a freezing-slide microtome, and sets of every sixth section were placed in PBS containing 30% sucrose and 30% ethylene glycol and stored at −20°C until required. After washing with distilled water followed by PBS, free-floating sections were treated for 1 h with blocking buffer (PBS containing 2% bovine serum albumin (BSA)) and incubated with 5-HT7 antibody (1:250, raised in rabbits, ImmunoStar, Hudson, WI, USA) for 48 h at room temperature. The sections were then washed in PBS and incubated with a secondary antibody (biotinylated goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. They were then processed using immunoperoxidase procedures using 3,3’-diaminobenzidine tetrahydrochloride (DAB) as a chromogen (Vectastain ABC Elite Kit, Vector Laboratories) to visualize the cellular localizations of 5-HT7 receptor in the vestibular nuclei. The sections were rinsed with distilled water and mounted on gelatin/chrome alum-subbed slides. After air-drying overnight, the sections were dehydrated through a graded ethanol series, cleared with xylene, and coverslipped with DPX Mountant (Fluka, Milwaukee, WI, USA). The staining was visualized with a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan). The images obtained were adjusted for brightness and contrast, and then cropped using Adobe Photoshop 7.0 (Adobe system, Inc., San Jose, CA, USA). Specificity for 5-HT7 receptor was confirmed by omitting the primary antibody, and processed sections using the immunoperoxidase procedure described above.

2.2. RT-PCR for 5-HT7 receptor mRNA expression

Another 6 animals were sacrificed for RT-PCR by decapitation under deep anesthesia. Brains were rapidly isolated and mounted on a rat brain matrix in ice-cold PBS. Coronal sections were cut to isolate the brain stem at 2 mm and 4 mm rostrally from caudal margin of the 4th ventricle. The location of the medial vestibular nucleus was the most easily recognized and therefore was selected for RT-PCR. Tissue containing the medial vestibular nuclei was selectively isolated from brain stem slices using a tissue punch (1.8 mm in ID; Fine Science Tools, USA). The landmark used for the medial vestibular nuclei was the lateral border of the 4th ventricle and the brown-colored borderline between the prepositus hypoglossal nucleus and the dorsal paragigantocellular nucleus. One tissue sample (1.8 mm in diameter) of each medial vestibular nucleus was isolated in the caudo-rostral direction [4]. The trigeminal ganglion was also harvested for comparison with the vestibular nuclei. Total RNA was extracted from dissected tissues using TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA). Briefly, each tissue sample was homogenized in 0.3 ml of TRI-
Chloroform was then added and the mixture was centrifuged at 12000 g for 15 min at 4°C. The RNA phase was precipitated with isopropanol, centrifuged at 12000 g for 8 min at 4°C, and pellets were washed with 75% ethanol, centrifuged at 12000 g for 5 min at 4°C, air-dried for 10 min, and dissolved in RNase-free water at 55–60°C for 10 min. RNA concentrations were determined using a UV-visible spectrophotometer (Optizen 3220 UV, Mecasys, Daejeon, Korea). Reverse transcription was carried out in a reaction volume of 20 A using iScript cDNA Synthesis Kits (Bio-Rad Laboratories, Hercules, CA, USA). The extracted RNA was reverse transcribed into cDNA using oligo-dT, and the synthesized cDNA derived from total RNA was then amplified with specific primers for 5-HT<sub>7</sub> receptor and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Two primers were designed from the cloned rat 5-HT<sub>7</sub> receptor sequence (accession no. L22558) as follows; 5'-CTGACGTCCAGCGAACCTGCTCCTG-3' (sense) and 5'-TGCCTGCAGACAGGACGCTTC-CGGT-3' (antisense) (964 base pairs). Two other primers (5'-TGCCTGCAGACAGGACGCTTC-CGGT-3' and 5'-TTGCTGGTGCAGGACGCTTC-CGGT-3') (515 base pairs) of the cloned GAPDH sequence (accession no. M17701) were used as a positive control. All primers used in this study were obtained from Bioneer Co. (Chungwon, Korea). After cDNA synthesis, a PCR mixture was prepared containing: 2 A of cDNA, 5 A of 10x PCR buffer, 4 A of 10 mM dNTP mix, 1.25 units of Taq polymerase (Takara Bio Inc, Otsu, Japan), 30 pmole of each 5'- and 3'- primer for 5-HT<sub>7</sub> receptor and GAPDH. PCR was performed using a thermocycler (Bio-Rad Laboratories, Hercules, CA) using; pre-denaturation at 94°C for 3 min followed by 30 amplification cycles (denaturation for 30 sec at 94°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C). The PCR products obtained were separated by electrophoresis in a 1.2% agarose gel and visualized using an UV illuminator in the presence of ethidium bromide.

3. Results
3.1. Immunoreactivity of 5-HT<sub>7</sub> receptor in the four major vestibular nuclei

The presence of DAB immunopositive products in vestibular nuclei sections was taken to indicate immunoreactivity for 5-HT<sub>7</sub> receptor in the four major vestibular nuclei. The nomenclature and boundaries defined in the rat brain atlas of Paxinos and Watson [13] were utilized throughout. 5-HT<sub>7</sub> receptor immunopositive neurons were found to be distributed throughout the four major vestibular nuclei. There appear to be two distinct staining patterns in the vestibular nuclear complex, one pattern in the superior (Figs 1a–b) and medial vestibular nuclei (Figs 1c–g) and the other in the lateral (Figs 1c–d) and spinal vestibular nuclei (Figs 1e–f). In the former, there is a significant reticulum of neuropil staining, whereas the immunolabeling in the lateral and spinal vestibular nuclei appears to be limited to cell bodies and their proximal dendrites. The other observation is that there are somata in each of the vestibular nuclei that are clearly unstained. Some nuclear labeling are also observed in the lateral, spinal, and medial vestibular nuclei. In control sections, which were not treated with primary antibody, no neuronal staining was observed (data not shown).

3.2. Expression of 5-HT<sub>7</sub> receptor mRNA in the medial vestibular nuclei

The presence of 5-HT<sub>7</sub> receptor mRNA was investigated by RT-PCR in the rat medial vestibular nuclei and trigeminal ganglia. The cDNA of the constitutively expressed housekeeping gene GAPDH was also amplified for control purposes. PCR products of the expected size (964 base pairs) for 5-HT<sub>7</sub> receptor were consistently amplified in all samples (Fig. 2). Sequence analysis of the selected PCR product confirmed its identity with the published sequence of the corresponding rat 5-HT<sub>7</sub> receptor gene.

4. Discussion

It is being increasingly recognized that the symptoms of migraine and balance system disorders are interrelated [5,19,20]. Recent studies have shown that dizziness occurs in 28–30% [3] and motion sickness in ∼50% of migraine patients [8]. Furthermore, migrainous vertigo has been reported in 9% of migraine sufferers [12]. However, the pathophysiology of migraine-associated vestibular disorders is not well understood.

5-HT receptors have been reported to be important neuromediators of migraine development [9,14], and recently, the link between 5-HT and migrainous vertigo has attracted renewed interest. Vestibular nucleus complex neurons express a number of different serotonergic receptor subtypes, including 5-HT<sub>1A</sub>, 5-HT<sub>1F</sub> and 5-HT<sub>2A</sub> receptors [2,15]. Some studies have concluded
Fig. 1. Photomicrographs of 5-HT7 receptor immunoreactive neurons in the rat vestibular nuclei. The photomicrographs in the right panel are high magnification views of those in the left panel (a+b, c+d, e+f and e+g). 5-HT7 receptor immunoreactive neurons were observed in the superior vestibular nucleus (SuVe), the lateral vestibular nucleus (LVe), the spinal vestibular nucleus (SpVe), and the medial vestibular nucleus (MVe). scp, superior cerebellar peduncle; icp, inferior cerebellar peduncle; g7, genu of facial nucleus; Pr, prepositus nuclei; DC, dorsal cochlear nucleus. Scale bars: 200 μm (in e for a and c); 25 μm (in g for b, d and f).
that 5-HT makes important contributions to the development of migraine-related vestibular symptoms [2,5]. It has been reported that the vestibular nuclei receive major serotonergic projections from the dorsal raphe nucleus and minor serotonergic and nonserotonergic projections from the nuclei raphe pallidus and obscurus [6]. Thus, the presence of serotonin and its receptors in the vestibular pathway suggests a specific neural basis for the occurrence of migrainous vertigo.

The present study demonstrates that 5-HT$_7$ receptor mRNA is expressed in the rat medial vestibular nucleus and trigeminal ganglion, and the 5-HT$_7$ receptor is expressed in each of the four vestibular nuclei. The reciprocal link between vestibular nuclei and the trigeminal nucleus caudalis implies that trigeminal and vestibular signals are likely to be simultaneously activated during the development of migraine or migrainous vertigo. Given that 5-HT$_7$ receptor is localized and expressed in rat vestibular nuclei, presumably a serotonergic mechanism in vestibular pathways operates in parallel to the trigeminal pathways involved in migraine. Accordingly, a better understanding of the role of 5-HT$_7$ receptor is likely to provide additional insight into the pathogenesis of migrainous vertigo.

It has been suggested that migraine may result from sudden increases in the amounts of 5-HT released from perivascular serotonergic and 5-HT-containing noradrenergic fibers due to the activations of the dorsal raphe nucleus and the locus coeruleus, respectively, and that these sudden releases lead to the stimulation of 5-HT$_7$ receptor in large conduit vessels and to vasodilatation, which would lead to the activations of trigeminal sensory nerves [16]. In fact, the majority of migraine prophylactic 5-HT antagonists display relatively high affinity for 5-HT$_7$ receptor, and it has been reported that vasodilatation of the middle meningeal artery can be blocked in vivo by SB-269970 (a selective 5-HT$_7$ receptor antagonist) in the rat [18]. Furthermore, the therapeutic efficacies of 5-HT$_7$ receptor antagonists in migrainous vertigo might be explained by the blockade of 5-HT$_7$ receptor, and thus, by its mediation of craniovascular vasodilatation.

In conclusion, the present study demonstrates, for the first time, that 5-HT$_7$ receptor is localized and expressed in rat vestibular nuclei. This study may provide new insights into the role of 5-HT$_7$ receptor in the vestibular signal processing. Further studies are needed for clarifying the role of 5-HT$_7$ receptor in serotonergic neurotransmissions in normal as well as pathological states.

References


