Research report

Co-localization of NMDA receptors and AMPA receptors in neurons of the vestibular nuclei of rats

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Abstract

We are interested in studying the co-localization of NMDA glutamate receptor subunits (NR1, NR2A/B) and AMPA glutamate receptor subunits (GluR1, GluR2, GluR2/3 and GluR4) in individual neurons of the rat vestibular nuclei. Immunoreactivity for NR1, NR2A/B, GluR1, GluR2, GluR2/3 and GluR4 was found in the somata and dendrites of neurons in the four major subdivisions (superior, medial, lateral, and spinal vestibular nuclei) and in two minor groups (groups x and y) of the vestibular nuclei. Double immunofluorescence showed that all the NR1-containing neurons exhibited NR2A/B immunoreactivity, indicating that native NMDA receptors are composed of NR1 and NR2A/B in a hetero-oligomeric configuration. Co-expression of NMDA receptor subunits and AMPA receptor subunits was demonstrated by double labeling of NR1/GluR1, NR1/GluR2/3, NR1/GluR4 and NR2A/B/GluR2 in individual vestibular nuclear neurons. All NR1-containing neurons expressed GluR2/3 immunoreactivity, and all NR2A/B-containing neurons expressed GluR2 immunoreactivity. However, only about 52% of NR1-immunoreactive neurons exhibited GluR1 immunoreactivity and 46% of NR1-containing neurons showed GluR4 immunoreactivity. The present data reveal that NMDA receptors are co-localized with variants of AMPA receptors in a large proportion of vestibular nuclear neurons. These results suggest that cross-modulation between NMDA receptors and AMPA receptors may occur in individual neurons of the vestibular nuclei during glutamate-mediated excitatory neurotransmission and may in turn contribute to synaptic plasticity within the vestibular nuclei.

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1. Introduction

Glutamate, a major excitatory neurotransmitter in the central nervous system, has been proposed as the principal neurotransmitter at the excitatory synapses in the vestibular nucleus [33,34,41,45]. This neurotransmitter is released from the vestibular afferent nerve terminals in mammals [15,23,45], and drives the excitatory synaptic neurotransmission mainly via glutamate-gated ion channels [26]. Various ligand-gated ion channels (i.e. ionotropic glutamate receptors) mediate the glutamate neurotransmission in the central nervous system [26]. On the basis of sequence homologies and agonist-affinities, ionotropic glutamate receptors are classified into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), and kainate (KA) receptors. Structurally, these receptors are composed of subunits: glutamate receptor 1–4 (GluR1–4) subunits in the case of AMPA receptors, GluR5–7 and KA1–2 subunits in the case of KA receptors, and NMDAR1 (NR1) and NR2A-D subunits in the case of NMDA receptors [16,20,25,26].

Physiological and pharmacological data indicate that NMDA and AMPA receptors play important roles in the mediation of glutamate neurotransmission and synaptic plasticity in the vestibular nuclei. Vestibular nuclear neurons are activated by the primary vestibular afferent synaptic inputs via NMDA or AMPA receptors in vivo and
in vitro [19,21,23,40,43]. Electrophysiological recording shows that the monosynaptic excitatory postsynaptic potential of second-order vestibular nuclear neurons in frog is mediated in part by NMDA receptors and in part by AMPA receptors [40]. In addition, electrophysiological studies ([2,7,15,23]; see review: [13]) have demonstrated that glutamatergic input from the ipsilateral vestibular nerve is mediated predominantly by AMPA receptors. A recent study [31] has suggested that AMPA receptors are also involved in non-primary vestibular excitatory transmission. The AMPA receptors are therefore involved in both the primary and non-primary vestibular transmission. Furthermore, NMDA receptors, with the characteristics of high Ca\(^{2+}\) permeability, play more roles in the long-term modulation (or long-term potentiation, LTP) of synaptic neurotransmission [3,6,11,32,42]. Recent evidence indicates that functional AMPA receptors are important for the maintenance of neuronal dendritic spines where most central excitatory synapses reside [1]. It is of interest that progressive co-expression of functional NMDA receptors and AMPA receptors was observed in hippocampal neurons during postnatal development and LTP [24,28]. We hypothesize that the co-localization of NMDA receptors and AMPA receptors in individual vestibular nuclear neurons provides a substrate for cross-talk between the receptors during excitatory synaptic events of the vestibular nuclear neurons. The AMPA receptors (GluR1, GluR2/3, and GluR4 subunits) were previously reported in the chinchilla vestibular nuclei [31]. Several NMDA receptor proteins and their mRNAs were also found in vestibular nuclear neurons [29,33,44]. However, it is not yet known whether or not NMDA and AMPA receptors are co-expressed in individual vestibular nuclear neurons. The co-localization of NMDA and AMPA receptor subunits, viz. NR1, NR2A/B, GluR1, GluR2, GluR2/3 and GluR4, was therefore investigated in individual vestibular nuclear neurons of the rat with the use of double immunofluorescence. The present study provides the basis for elucidating the interaction between NMDA receptors and AMPA receptors in excitatory synaptic neurotransmission and synaptic plasticity within the vestibular nucleus.

2. Materials and methods

Eight adult male Sprague–Dawley rats weighing 230–260 g were used in the present study. All procedures in each preparation conformed to the Principles of Laboratory Animal Care and were approved by the University of Hong Kong Committee on the Use of Live Animals in Research. Briefly, the animals were anesthetized with sodium pentobarbital (60–80 mg/kg, i.p.), and then perfused transcardially with 100 ml of saline, followed by 500 ml of 0.1 M phosphate buffer (PB; pH 7.4) containing 4% paraformaldehyde. The brain stems were removed immediately and placed in 0.1 M PB containing 30% sucrose overnight at 4°C. The brain stems were then serially cut into frontal sections (40 μm thickness) with a freezing microtome and then processed for the following immunocytochemical reactions.

Double immunofluorescence was employed to demonstrate the co-localization of different receptor subunits in individual vestibular nuclear neurons in sections of eight rats. Alternate sections of the brain stem that contained the vestibular nuclei were processed to double immunofluorescence. A mixture of one mouse antibody and one rabbit antibody in 0.01 M phosphate-buffered saline (PBS) containing 3% normal goat serum and 0.1% Triton X-100 was used. The following primary antibodies that were obtained from commercial source (Chemicon International, Temecula, CA) were employed in the present study: mouse-anti-NR1 monoclonal antibody (1:1000), rabbit-anti-NR2A/B polyclonal antibody (1:500), rabbit-anti-GluR1 polyclonal antibody (1:500), mouse-anti-GluR2 monoclonal antibody (1:1000), rabbit anti-GluR2/3 polyclonal antibody (1:500), and rabbit anti-GluR4 polyclonal antibody (1:500). The following double immunofluorescence was then performed: NR1 and NR2A/B (written as NR1/NR2A/B), NR1/GluR1, NR1/GluR2/3, NR1/GluR4 and NR2A/B/GluR2. The sections were incubated in the mixtures of primary antibodies for 24–48 h at 4°C. Subsequently, the sections were washed in PBS (3×PBS) and incubated for 2–4 h at room temperature in a mixture of secondary antibodies, i.e., either donkey anti-mouse IgG conjugated with tetramethyl rhodamine isothiocyanate (TRITC, 1:100–200; Chemicon) and donkey anti-rabbit IgG conjugated with dichlorotriazinyl aminofluorescein (DTAF; 1:100–200; Chemicon), or donkey anti-mouse IgG conjugated with aminomethylcoumarin (AMCA, 1:100–200; Chemicon) and donkey anti-rabbit IgG conjugated with TRITC. After several washes in PBS, the sections were mounted in flurosave medium (Calbiochem). The sections were examined both with fluorescence microscope (Axioplan, Zeiss) and a laser scan confocal microscope (LSM 510, Zeiss) for DTAF-, AMCA- and TRITC-labeled neurons. In addition, other sections of four rats were processed with immunoperoxidase procedures using 3,3′-diaminobenzidine (DAB) as the chromogen (ABC kit, Vector Labs.) to visualize the cellular localization of NR1, NR2A/B, GluR1, GluR2, GluR2/3 and GluR4 in the rat vestibular nuclei. The sections were reacted in a single immunoperoxidase reaction for comparison of the pattern of distribution and number of immunopositive neurons in the regions of the vestibular nuclei.

For the control experiments of immunofluorescence and immunocytochemistry, the primary antibody was substituted with normal mouse serum (for NR1 and GluR2) or normal rabbit serum (for NR2A/B, GluR1, GluR2/3 and GluR4) respectively. The sections were then processed with the same double immunofluorescence or immunoperoxidase reaction sequence as described above.

In the present study, the perikaryal size of immuno-
positive cells was measured under the light microscope with the use of a morphometric micrometer. For semi-quantification of immunopositive cells in representative sections, double-labeled neurons in eight randomly selected fields (40× objective) of each of the following nuclei, namely the superior vestibular nucleus (SuVe), medial vestibular vestibular nucleus (MV e), lateral vestibular nucleus (LV e), spinal vestibular nucleus (SpVe), group x and group y were counted.

3. Results

3.1. Control experiments for immunocytochemistry

In those sections that were incubated in normal mouse or rabbit serum in place of the primary antibodies, no immunoreactivity was detected (data not shown). For double labeling procedures, only single immunoreactivity was detected when one of the two primary antibodies was substituted by normal serum during the reaction sequence. When two primary antibodies were substituted, no immunoreactivity was detected (data not shown).

3.2. Immunoreactivity for NMDA and AMPA receptor subunits in different regions of the vestibular nuclei

Immunoreactivity for NR1, NR2A/B, GluR1, GluR2, GluR2/3 and GluR4 was identified by the presence of DAB immunoreaction products in the sections of the rat brain stem. The nomenclature and boundaries defined in the rat brain atlas of Paxinos and Watson [27] were utilized in this study. In the vestibular nuclei, immunoreactivity for NR1 (Fig. 1), NR2A/B (Fig. 2), GluR1, GluR2, GluR2/3 or GluR4 (Figs. 3 and 4) was predominantly observed in neuronal somata and in their proximal dendrites. The immunopositive neurons were primarily distributed in the superior vestibular nucleus (SuVe), medial vestibular nucleus (MV e), lateral vestibular nucleus (LV e), spinal vestibular nucleus (SpVe) and group x, y subnuclei (Figs. 1–4). NR1 immunoreactivity was highly expressed in the LV e (Fig. 1C–D), SpVe (Figs. 1E–F) and MV e (Figs. 1G–H). In SuVe, however, only low NR1-immunoreactivity was observed (data not shown). Moderate expression of NR1 immunoreactivity was found in the groups x and y subnuclei (see Fig. 1A–B for y subnucleus). Within the MV e, a majority of NR1-immunoreactive (IR) neurons was spherical, oval or fusiform in shape (Fig. 1H). The size of these neurons ranged from small to medium with a diameter between 10 μm to 20 μm. The LV e was characterized with the presence of giant NR1-IR neurons, which were multi-polar with diameters of 30 μm to 50 μm (Fig. 1D). A number of small and medium-sized NR1-IR neurons were also found in the LV e (Fig. 1D). The NR1-IR neurons in the SuVe were similar to those of MV e in their shape and size but most of them were weakly labeled. The NR1-IR neurons in the SpVe were triangle, oval, or multi-polar in shape, and their diameters ranged from 10 μm to 30 μm (Fig. 1F). The NR1-IR neurons in groups x and y subnuclei were mainly oval in shape and their diameters ranged from 10 μm to 20 μm (Fig. 1B).

Immunoreactivity for NR2A/B (Fig. 2A–D), GluR2 (Figs. 3C–D, 4C–D) and GluR2/3 (Figs. 3E–F, 4E–F) subunits was also found to be abundant in the vestibular nuclei. The immunoreactive neurons were similar to the NR1-IR neurons in terms of cell number and distribution pattern in the SuVe, MV e, LV e, SpVe and group x, y subnuclei. Immunoreactivity for GluR1 (Figs. 3A–B, 4A–B) or GluR4 (Figs. 3G–H, 4G–H), however, was less abundant than that for NR1. The GluR1-IR or GluR4-IR neurons were also fewer in number than NR1-IR neurons in different regions of the vestibular nuclei.

In addition, astrocyte-like immunoreactive cells are prominent in NMDA2A/B antibody-incubated sections. Weakly stained astrocytes were also seen in GluR1 and GluR2/3-stained sections. GluR4-like immunoreactive astrocytes were hardly detected in our experiment (data not show). These results were consistent with previous report [30].

3.3. Co-localization of NMDA and AMPA receptor subunits

Co-expression of immunoreactivity for NMDA receptor subunits, NR1 and NR2A/B, was observed in individual vestibular nuclear neurons (Fig. 5). Double labeled neurons were found throughout all subdivisions of the vestibular nucleus including the SuVe, MV e, LV e, SpVe and group x, y subnuclei. Virtually all the NR1-IR neurons were found to exhibit NR2A/B immunoreactivity (Table 1). Fig. 5A, A’ illustrates individual SpVe neurons displaying co-localization of NR1 and NR2A/B immunoreactivity in the somata and dendritic processes. Similarly, co-expression of immunoreactivity for NR2A/B and GluR2, NR1 and GluR1, NR1 and GluR2/3, or NR1 and GluR4, was also found in individual vestibular nuclear neurons. Double labeled neurons were found in the SuVe, MV e, LV e, SpVe and group x, y subnuclei. In addition, all the vestibular neurons that expressed GluR1 (Fig. 5) or GluR4 (Fig. 5) immunoreactivity were found to express NR1 immunoreactivity. However, the NR1/GluR1 and NR1/GluR4 double-labeled neurons were fewer in number. This was particularly evident in the SuVe, MV e and group x, y subnuclei. Representative NR1/GluR1 and NR2A/B/GluR2 double labeled neurons in the SpVe are shown in Fig. 5B, B’, C, C’.

3.4. Semi-quantification

Results of the semi-quantification analyses of the immunopositive cell numbers in the vestibular nuclei are shown in Table 1. All of the NR1-IR neurons were found...
Fig. 1. Photomicrographs showing the immunoreactivity for NMDA receptor subunit NR1 in subdivisions of the 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 G+H). NR1-immunoreactive neurons are shown in regions including the group y subnucleus (Y), lateral vestibular nucleus (LVe), medial vestibular nucleus (MVe), spinal vestibular nucleus (SpVe). Abbreviations: DC, dorsal cochlear nucleus; icp, inferior cerebellar peduncle; Int, interpositus nucleus of the cerebellum. Scale bars: 300 μm (in G for A, C and E); 80 μm (in H for B, D and F).
to display NR2A/B and GluR2/3 immunoreactivity. Similarly, all of the NR2A/B-IR neurons displayed GluR2 immunoreactivity. Only a proportion of NR1-IR neurons, however, exhibited GluR1 or GluR4 immunoreactivity. About 52% of the NR1-IR neurons displayed GluR1 immunoreactivity, while 46% of NR1-IR neurons displayed GluR4 immunoreactivity.

4. Discussion

Our present data reveal that neurons of the rat vestibular nucleus are abundantly endowed with NMDA and AMPA receptors. The immunostaining patterns of individual NMDA receptor subunits (NR1, NR2A/B) and individual AMPA receptor subunits (GluR1, GluR2/3, GluR4) in the vestibular nuclei are in line with previous observations [29–31]. The present study has, however, provided novel evidence that: (a) GluR2-IR neurons are substantially distributed in the vestibular nuclei and their distribution pattern is similar to that of NR1-IR neurons, (b) the NR1 receptor subunit is co-localized with NR2A/B receptor subunits in individual vestibular nuclear neurons, and (c) NMDA receptors and AMPA receptors are co-localized in a large proportion of vestibular nuclear neurons.

4.1. Glutamate and neurotransmission between the vestibular afferents and vestibular nuclear neurons

Glutamate-containing axonal terminals have been shown to make synaptic contacts with the somata and the dendrites of vestibular nuclear neurons [35]. Pharmacological and electrophysiological findings showed that NMDA and AMPA receptors play a key role in mediating excitatory neurotransmission between the vestibular afferents and neurons in the vestibular nucleus [15,19,21,33,36,40,43]. An in vivo microdialysis study also demonstrated that electrical stimulation of the vestibular nerve induced the release of glutamate release in the MVe [43]. After sectioning of the vestibular nerve, a significant decrease in high affinity glutamate uptake was observed in the LVe and the rostral part of the SpVe on the ipsilateral side [33]. Neurons in the LVe and SpVe have also been shown to respond to natural stimulation of the otolith organs [9,22].
Fig. 3. Photomicrographs showing the immunoreactivity for GluR1 (A+B), GluR2 (C+D), GluR2/3 (E+F) and GluR4 (G+H) in the spinal vestibular nucleus (SpVe) at low and high magnifications. Abbreviations as in Fig. 1. Scale bars: 300 μm (in G for A, C, and E); 80 μm (in H for B, D and F).
Fig. 4. Photomicrographs showing the immunoreactivity for GluR1 (A, B), GluR2 (C, D), GluR2/3 (E, F) and GluR4 (G, H) in the vestibular nuclei at high magnification. The GluR1-(A), GluR2-(C), GluR2/3-(E) or GluR4-immunoreactive neurons (G) are demonstrated in LVe. In addition, the GluR1-(B), GluR2-(D), GluR2/3-(F) or GluR4-immunoreactive neurons (H) are demonstrated in MVe. Abbreviations as in Fig. 1. Scale bar: 80 μm (in H for all micrographs).
Fig. 5. Double immunofluorescence showing the double labeled neurons with distinct glutamate receptor subunits, viz. NMDA and AMPA receptor subunits in the vestibular nuclei. The confocal laser scanning images (A, A') show that individual vestibular nuclear neurons labeled with NR1 (A; Rhodamine-labeling) display immunoreactivity for NR2A/B (A'; DTAF-labeling) in spinal vestibular nucleus (SpVe). Photomicrographs B-B' show co-expression of NR1 (B; AMCA-labeling) and GluR1 (B'; Rhodamine-labeling) immunoreactivity in SpVe and the co-localization of NR2A/B (C; Rhodamine-labeling) and GluR2 (C'; DTAF-labeling) immunoreactivity in SpVe as well (Photomicrographs C-C'). Arrows indicate the doubly labeled neurons. Asterisks depict the blood vessels. Scale bars: 30 μm in panels A, A' and 50 μm in panels B, B', C, C'.
The present results show that all individual neurons in the vestibular nuclei co-express NR1 and NR2A/B subunit immunoreactivity, indicating that native NMDA receptors in vestibular nuclear neurons are most likely assembled from these subunits in a hetero-oligomeric configuration. On the other hand, the distributions of GluR1 and GluR4 subunits are uneven among central vestibular neurons. The LVe and SpVe showed an abundant expression of these subunits. There was less expression in the SuVe, MVe, and groups x, y subnuclei. In addition, another interesting finding is the co-expression of NMDA receptors and the AMPA receptor subunit GluR2 in individual vestibular neurons. The stability and maintenance of these spines depend both on AMPA receptors and spontaneous glutamate release [1].

4.4. Functional implications in the vestibular system

The present data do provide the possible cellular expression of NMDA and AMPA subunits in the central vestibular neurons. The present data however, do not directly provide functional data of the ionotropic glutamate receptors in these neurons. Previous physiological studies report that NMDA receptors contribute to the regulation of the resting discharge [14,37] and long-term modulation of their synaptic transmission [6] in vestibular neurons. NMDA receptors also play a crucial role in the synaptic plasticity associated with vestibular compensation [8,10,12,39]. However, the role of the AMPA receptors in regulating the functions of central vestibular neurons has received less attention though they are known to involve in non-primary vestibular excitatory transmission [31]. In addition, electrophysiological data obtained from frogs indicated that afferent-evoked responses in vestibular nuclear neurons involved NMDA and AMPA receptor-
mediated components [40], suggesting that functional interaction between NMDA and AMPA receptors occurs in vestibular nuclear neurons. The intracellular signal cascade and physiological consequence that follow from the cross-modulation between NMDA receptor and AMPA receptor in vestibular nuclear neurons deserve further investigation.

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