Journal of Chemical Neuroanatomy 35 (2008) 334–345

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Morphometric analysis of the AMPA-type neurons in the Deiters vestibular complex of the chick brain

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ARTICLE INFO

Article history:
Received 11 September 2007
Received in revised form 18 February 2008
Accepted 18 February 2008
Available online 2 March 2008

Keywords:
Vestibular system
Postural control
Glutamate receptors
Avian brain
Comparative neuroanatomy

ABSTRACT

Chicken (*Gallus gallus*) brains were used to investigate the typology and the immunolabel pattern for the subunits composing the AMPA-type glutamate receptors (GluR) of hindbrain neurons of the dorsal (dND) and ventral nuclei (vND) of the Deiter's vestibular complex (CD), which is the avian correspondent of the lateral vestibular nucleus (LVN) of mammals. Our results revealed that neurons of both divisions were poor in GluR1. The vND, the GluR2/3+ and GluR4+ label presented no area or neuronal size preference, although most neurons were around 75%. The dND neurons expressing GluR2/3 are primarily around 85%, medium to large-sized 85%, and predominantly 60% located in the medial portion of the rostral pole and in the lateral portion of the caudal pole. The majority of dND neurons containing GluR4 are also around 75%, larger (70% are large and giant), exhibiting a distribution that seems to be complementary to that of GluR2/3+ neurons. This distinct arrangement indicates functional differences into and between the DC nuclei, also signaling that such variation could be attributed to the diverse nature of the subunit composition of the GluRs. Discussion addresses the morphological and functional correlation of the avian DC with the LVN of mammals in addition to the high morphological correspondence, To include these data into the modern comparative approach we propose to adopt a similar nomenclature for the avian divisions dND and vND that could be referred as dLVN and vLVN.

1. Introduction

Postural control in vertebrates typically depends on a continuous management provided by polysensoryl inputs, and, especially to bipeds, those offered by the vestibular apparatus receive greater importance (Peusner and Giaume, 1997; Gdowski and McCrea, 1999). Vestibular information is conveyed by several brainstem nuclei and their functioning are critical to integrate head movements with body response to maintain the animal positioning (static response) or to correct ongoing motor activities (dynamic response). In birds, experimental studies involving the vestibular nuclear complex (VNC) have shown different degrees of differentiation between species, mostly regarding their specific abilities to fly (Brandis, 1894; Wold, 1976) and to sing (Craigie, 1928; Wold, 1976), which requires singular and proper adjustments. VNC topography, cytoarchitecture, and nomenclature are all similar, but not equal between many species (Wold, 1976), and although the general description of VNC used for avian species is comparable to those found for mammals and amphibians some nuclei and groups of small cells had received different names (Wold, 1975, 1976; Reichenberger et al., 1997; Vibert et al., 2000; Fanardjian et al., 2001) adding unnecessary difficulties (Reiner, 2005).

Actual terminology include embryological criteria following recent revisions about the VNC segmental organization in chickens (Diaz and Glover, 2002; Diaz and Puelles, 2002; Diaz et al., 2003) as well some earlier studies about its cytoarchitecture and anatomy (Wold, 1975, 1976, 1978a, 1978b, 1979a, 1979b, 1981). It is assumed so far that the avian VNC is composed of six major nuclei: the superior vestibular nucleus, the medial vestibular nucleus, the descending vestibular nucleus, the tangential nucleus, the small

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Abbreviations: A, cell group A; Ang, angular nucleus; B, cell group B; Cbl, lateral cerebellar nucleus; CCN, central cervical nucleus; DC, Deiters complex; dND, dorsal nucleus of Deiters; DVN, descendent vestibular nucleus; La, laminar nucleus; LVN, lateral vestibular nucleus; Mcc, magnocellular cochlear nucleus; MVN, medial vestibular nucleus; nV, trigeminal nucleus; NVIII, vestibular nerve; rb, restiform body; SVN, superior vestibular nucleus; Ta, tangential nucleus; VCR, vestibular-cervical reflex; VNC, vestibular nuclear complex; vND, ventral nucleus of Deiters; VOR, vestibular-ocular reflex; VSR, vestibular-spinal reflex.

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doi:10.1016/j.jchemneu.2008.02.003

cells groups (A and B), and the dorsal and ventral nuclei of Deiters, collectively designated as the Deiters complex (DC).

Of especial interest as outputting vestibular signals the DC corresponds to the lateral nucleus in mammalian brains (Gstoettner and Burian, 1987; Newman et al., 1992; Dickman, 1996; Dickman and Fang, 1996; Suarez et al., 1997; Tellegen et al., 2001; Barmack, 2003) whereas the DC terminology is sometimes used in mammals as well (Brodal, 1984; Diaz et al., 1993; Fanardjian et al., 2001). The DC/vestibular lateral nucleus plays an important role coordinating the fine-tuning of postural control and ocular activities according to the movements of the head and body (Peusner, 2001; Chan et al., 2002). DC seems to be a relay station and many projections from the cerebellum areas as cortex, uvula, nodulus, and the flocculus terminate in the ipsilateral DC as well as efferent projections from the medial and intercalate nuclei (Wold, 1981). Despite its apparently small size compared to other vestibular brainstem nuclei, DC neurons give rise to many prominent descending outputs to low axial motoneurons (Wold, 1978a) and some ascending projections to mesencephalic oculomotor centers (Wold, 1978b).

In relation to cellular form and size, the dorsal nucleus of Deiters (dND) presents a homogeneous cytoarchitecture when compared to the ventral nucleus of Deiters (vND). The dND does not receive primary sensory afferents (Wold, 1975, 1976), but it receives numerous projections from the cerebellum (Wold, 1981) and commissural connections from the superior vestibular nucleus, the descending vestibular nucleus, the group A, and also the bilateral projections from the medial vestibular nucleus (Wold, 1979b). The dND sends efferent projections to ocular motor nuclei (Wold, 1978a), bilaterally to the reticular formation (Wold, 1979b), and to the spinal cord (Wold, 1978a).

The ventral nucleus of Deiters consists of a mixture of morphologically different neurons (Wold, 1976). It receives primary afferents probably from ampullar receptors (Wold, 1975), commissural connections from the descending vestibular nucleus, cell group A and also a bilateral projection from the medial vestibular nucleus (Wold, 1979b). The vND sends efferent projections to all segments of the spinal cord (Wold, 1978a) and scanty fibers to ocular motor nuclei (Wold, 1978b).

Both commissural connections and primary afferents from vestibular receptors use glutamate as their major neurotransmitter suggesting that this neurotransmitter has a key role in excitatory processes within vestibular nuclei (Li et al., 1996; Straka et al., 1996; Vidal et al., 1996; Popper et al., 1997; Reichenberger et al., 1997; Büttner-Ennever, 2000; Vibert et al., 2000). Glutamatergic influence is exerted through ionotropic and metabotropic receptor classes, but those that usually guarantee fast responses (Watkins et al., 1990; Gerber et al., 1991) controlling neural reflexes, are typically performed by ion channel receptor-type (Currie and Stein, 1992; Smith and Darlington, 1996; Priesol et al., 2000). Based on their pharmacologic properties, glutamate ionotropic family receptors include NMDA (*N*-methyl-D-aspartate) and two non-NMDA subtypes: α-amino-3-hidroxy-5-methyl-4-isoxalone propionate (AMPA) type and *kainate* receptor-type (Foster and Fagg, 1984; Ozawa et al., 1998; Chen et al., 2000; Chan et al., 2003). Presence of the AMPA-type receptor has been described for neural connections between both type I and type II hair cells and the primary vestibular afferents (Matsubara et al., 1999; Smith, 2000; Elezgarai et al., 2003), whereas type II hair cells target neurons also present NMDA receptors (Fujita et al., 1994; Chen et al., 2000).

AMPA receptors are formed by tetrameric combinations (Mosbacher et al., 1994; Ozawa et al., 1998) between four different subunits, namely, $GluR_1$, $GluR_2$, $GluR_3$, and $GluR_4$ (Popper et al., 1997; Chen et al., 2000, Chan et al., 2003). These four subunits can form homomeric (Ozawa et al., 1998) and heteromeric receptors (Mosbacher et al., 1994; Sans et al., 2000), and their composition determines the ion permeability and the kinetic properties of the receptor channel (Popper et al., 1997; Ozawa et al., 1998; Chen et al., 2000; Chan et al., 2003). This heterogeneity could lead to functional differentiation in neuronal subpopulations, and such a distribution may provide important clues about their physiological properties. Occurrence of glutamate acting via AMPA-type receptors in internal circuits of VNC has been described [Cochran et al., 1987 (frog); Popper et al., 1997 (chinchilla); Chen et al., 2000 (rat)]. Here, we focused on the typology and characterization of the DC neuronal population in chick brains. Also, based on such scrutiny, we present the description of the AMPA-type subunits distribution on those DC neurons. As one of the brainstem sources of motor input to the avian spinal cord, identifying its morphological compounds is an initial step to comprehend its functioning as a motor relay station to biped vertebrates.

2. Materials and methods

Fifteen young adult (15–20 days old) chicks (*Gallus gallus*) weighing 130–140 g, with food and water always available *ad libitum*, were anesthetized with i.m. ketamine (Vetalar, PARKE-DAVIS, 0.5 mg/100 g), *xylazine* (Rompun, MILES Lab., 0.1 mg/100 g) and perfused transcardially with a saline solution (pH 7.4) followed by perfusion with 4% paraformaldehyde in phosphate buffer (PB). Brains were removed, post-fixed in the same solution for 12 h and cryoprotected in 30% sucrose in 0.1 M PB for at least 24 h. The brainstem was transversely cut at 40 μm on a freezing microtome. All animal procedures were made in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, Society for Neuroscience Guidelines, and the experimental protocol was approved by the Institutional Ethics Committee of the City University of São Paulo.

2.1. Cytoarchitecture and morphometric analysis

To perform the morphological studies, brainstem of four chicks were cut and each section was sequentially placed in individual compartments, mounted into subbed slides, and stained with *Giemsa*. That procedure allowed us to reconstruct the brainstem. Identification of the limits of the dorsal and ventral portions of the DC was based on the previous descriptions by Wold (1976) and Kuenzel and Masson (1988). Morphology of neurons had also been based on Wold (1976). Size of the cells was determined by measuring the longest axis of the neuronal body with the aid of a computational system (NIH Image 1.57) through representative sections throughout the DC nuclei. According to its size, neurons were categorized as small ≤20 μm, medium >20 μm to ≤35 μm, large >35 μm to ≤50 μm, and giant >50 μm following a scale proposed by Suarez et al. (1993). Numbers were counted using the Abercrombie correction factor (Abercrombie, 1946) and to avoid duplicity only neurons with a visible nucleus were counted. Also to specifically validate the data for giant neurons (over 50 μm) the neuronal nucleus instead of the whole cell was used as reference to the counting procedures as well as to apply Abercrombie's factor (Abercrombie, 1946). The neuronal shape was also investigated and neurons were classified as triangular, round, or fusiform. A criterion to differentiate round from fusiform was an apparent presence of a largest axis, and a neuron was defined as triangular when the typical three edges were clearly visible. To investigate uniformity in and among dorsal and ventral nuclei of CD, each nucleus was analyzed along its rostrocaudal extension. In the dorsal nucleus of Deiters we investigated the rostral pole and the caudal pole, but in the ventral nucleus of Deiters an intermediate region could also be identified between the rostral and the caudal poles comprising three subdivisions. The intermediate region is the larger portion and it is traversed by fibers supposedly arriving at the medial vestibular nuclei (Wold, 1976).

2.2. Immunohistochemical studies of the AMPA receptor subunits

Immunohistochemistry was used to analyze the distribution of the AMPA-type glutamate receptor subunits in neurons at the dorsal and ventral nuclei of Deiters of chick brainstems. The sections were rinsed 3 × 10 min in PB, at room temperature and incubated with the primary antibody for 48 h in a cold room (4 °C). In the present study we employed commercial antibodies (Chemicon International, Temecula, CA) against GluR1 and GluR4 subunits, and an antibody which recognizes a common epitope to $GluR_2$ and $GluR_3$ subunits (GluR2/3). Specificity of the antibodies was already certified in previous works (Ravindranathan et al., 1996; Reng et al., 1999; Cornil et al., 2000) and co-substantiated by our *preabsorbin* trials with specific peptides. The concentration varied from 1:500 to 1:2000 in a solution containing Triton-X-100 0.3% in phosphate buffer 0.1 M, plus 5% normal serum. Next, the sections were rinsed once more 3 × 10 min in PB, at room temperature and incubated with a biotinylated secondary antibody (Jackson ImmunoResearch Labs Inc., West Grove, PA) for 1 h at a 1:250 dilution. The sections were rinsed