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Research Report

Differences in parvalbumin and calbindin chemospecificity in the centers of the turtle ascending auditory pathway revealed by double immunofluorescence labeling

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ABSTRACT

Using double immunofluorescence labeling, quantitative ratio between parvalbumin- and calbindin-containing neurons, neurons that co-localize both peptides, as well as the intensity of their immunoreactivities were studied in the brainstem, midbrain and forebrain auditory centers of two chelonian species, *Testudo horsfieldi* and *Emys orbicularis*. In the spiral ganglion and first-order cochlear nuclei, highly immunoreactive parvalbumin-containing neurons predominated, and almost all neurons in these nuclei also exhibited weak immunoreactivity to calbindin. The number of strongly calbindin-immunoreactive (-ir) cells increased in the second-order brainstem auditory centers (the laminar cochlear nucleus, superior olivary complex, lateral lemniscal nucleus), and co-localization with parvalbumin in some of them was observed. In the midbrain, a complementary distribution of parvalbumin and calbindin immunoreactivity was found: the central (core) region of the torus semicircularis showed strong parvalbumin immunoreactivity, while the laminar (belt) nucleus was strongly calbindin-ir. In the thalamic nucleus reuniens, almost complete topographic overlapping of the parvalbumin-ir and calbindin-ir neurons was shown in its dorsomedial region (core), with the intensity of immunoreactivity to calbindin being much higher than that to parvalbumin. The predominance of calbindin immunoreactivity in neurons of the dorsomedial region of the nucleus reuniens is correlated with

Abbreviations: ADVRvm, ventromedial area of the anterior dorsal ventricular ridge; CB, calbindin; Ce, nucleus centralis of the torus semicircularis; CoA, nucleus cochlearis angularis; CoL, nucleus cochlearis laminaris; CoM, nucleus cochlearis magnocellularis; CR, calretinin; GS, ganglion spiralis; ir, immunoreactive; L, nucleus laminaris of the torus semicircularis; MGB, medial geniculate body; MLd, nucleus mesencephalicus lateralis, pars dorsalis; nLl, nucleus lemnisci lateralis; OD, optical density; OS, oliva superior; OSd, nucleus dorsalis of the oliva superior; OSv, nucleus ventralis of the oliva superior; PV, parvalbumin; Re, nucleus reuniens; TS, torus semicircularis

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the existence of the dense calbindin-ir terminal field in its projection area in the telencephalon. We conclude that the turtle auditory pathway is chemically heterogeneous with respect to calcium-binding proteins, the predominance of parvalbumin in the brainstem and midbrain centers giving way to that of calbindin in the forebrain centers; the portion of neurons co-localizing both peptides nonlinearly decreases from lower to higher order centers.

1. Introduction

A variety of electrophysiological and neurochemical studies (DiFiglia et al., 1989; Celio, 1990; Heizmann and Braun, 1990; Andressen et al., 1993; Chard et al., 1993; Schwaller et al., 2002; Camp and Wijesinghe, 2009; Schwaller, 2009) has shown that several neuronal populations, each with a particular, distinct, pattern of activity, may also be characterized by the different calcium-binding proteins that they incorporate. Differences in the biophysical properties of these proteins, and hence in their functional roles, may thus render them selective markers of functionally distinct neuronal populations.

Calcium-binding proteins (parvalbumin, PV; calbindin, CB) have been widely used in investigations of sensory systems, particularly for defining different neuronal subpopulations (Jones and Hendry, 1989; Wong-Riley, 1989; Celio, 1990; Wild et al., 1993; Puelles et al., 1994; Hevner et al., 1995; de Venecia et al., 1995, 1998; Partata et al., 1999; Jones, 2003; Ashwell and Paxinos, 2005; Anderson et al., 2007). Selective PV and CB labeling of different morphofunctional types of neurons in mammalian thalamic nuclei led Jones (1998, 2003) to postulate a core-matrix principle of the organization of mammalian thalamus. According to the hypothesis, projection neurons of central (core) divisions of sensory lemniscal thalamic nuclei, being highly active, contain PV. At the same time, metabolically less active neurons of their peripheral non-lemniscal (belt or shell) divisions of relay sensory nuclei which belong to the diffuse matrix system are CB-ir. However, alternative distribution of PV and CB in the core and belt subdivisions correspondingly appears to be more evident in primates than in other mammalian species. Non-primate mammals markedly differ in a strong diversity in the distribution of these proteins in the projection neurons of the lemniscal, core, subdivisions of sensory thalamic nuclei, thus containing either PV or CB or both proteins (Celio, 1990; Zettel et al., 1991; Braun and Piepenstock, 1993; Vater and Braun, 1994; Ashwell and Paxinos, 2005). It has therefore been suggested that, in the relay sensory nuclei, PV regulation of neuronal calcium balance is a more recent feature, in contrast to the phylogenetically ancient CB regulation (Jones, 1998, 2007; Parvizi and Damasio, 2003). The Jones' core-matrix model of the thalamic organization is, however, much less applicable to thalamic organization in non-mammalian vertebrates including reptiles (Belekhova et al., 2003, 2010).

More specifically, calcium-binding proteins serve as effective markers of different neuronal populations in the vertebrate auditory system. While data on the distribution of calcium-binding proteins in the auditory centers of mammals (Celio, 1990; Zettel et al., 1991; Braun and Piepenstock, 1993; Vater and Braun, 1994) and birds (Braun et al., 1985; Takahashi et al., 1987; Braun, 1990; Rogers et al., 1990; Braun et al., 1991; Kubke et al., 1999) are considerable, comparable information in reptilian species is limited (lizards: Dávila et al., 2000; Yan et al., 2010; turtles: Belekhova et al., 2004, 2008, 2010). Even so, the results from different species of reptiles are not completely congruent. At the same time, our knowledge of morphofunctional and neurochemical properties of the organization of the central auditory system of reptiles, and particularly of turtles, provides a great opportunity to clarify both the basic mechanisms of the transmission of auditory information and the phylogenesis of this system in amniotes.

In our recent investigations, we showed that all centers of the turtle auditory system, including the sensory (spiral) ganglion, contained both PV-ir and CB-ir neurons (Belekhova et al., 2004, 2008, 2010) and that high metabolic activity is a typical feature of the lemniscal pathway. We also came to the conclusion that in turtles, the distinction between the core and the belt of the various auditory centers progressively diminishes as one ascends the neuraxis. Though we have found both proteins in projection neurons of turtles' auditory centers, the data obtained were not sufficient to assume which type of calcium-binding proteins predominates in each center of the turtle auditory pathway. As in many other studies, we also noted that the intensity of immunolabeling either for PV or CB in neurons of these centers strongly varied (Belekhova et al., 2004, 2008, 2010). Usually the difference in the intensity of labeling is explained by different concentration of corresponding protein in neurons, and therefore, the predominance of any protein in each auditory center may be estimated not only by the number of immunoreactive neurons but also by the intensity of their immunoreactivity.

To complement our previous findings, in the present study we attempt to estimate (i) the degree of the intensity of immunoreactivity both to PV and CB in neurons of each center of the turtle auditory system; (ii) the quantitative ratio between "weakly"- and "strongly"-labeled either PV- or CB-immunoreactive neurons; (iii) the number of PV- and CB-ir neurons; and (iiii) the number of neurons that co-localize both proteins. With this aim, we used double immunofluorescence techniques followed by counting the number of mono- and double-immunolabeled neurons as well as by measuring the optical density of neuronal immunofluorostaining.

2. Results

The terminology we use in describing the turtle auditory nuclei is based on the accepted nomenclature of the