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CHARACTERIZATION OF HUMAN AUDITORY BRAINSTEM CIRCUITS BY CALCIUM-BINDING PROTEIN IMMUNOHISTOCHEMISTRY

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Abstract—The cochlear nucleus (CN) and superior olivary complex are auditory brainstem centers with essential roles in encoding temporal features of vocalizations, localization of sound sources and descending modulation of the cochlea. Numerous neuronal populations, across a multitude of laboratory mammals, have been characterized within these brainstem centers based on cell body morphology, dendritic architecture, afferent/efferent connections and neurochemistry. However, scant details are available for these neuronal populations in humans. The objective of this study is to further characterize human auditory hindbrain nuclei and examine the axonal connections between these structures. To this end, we have used immunohistochemistry and morphometric techniques to characterize neuronal populations and axonal projections in the human brainstem. Herein, we provide evidence for calretinin immunoreactive neurons and synaptic boutons in the ventral CN, axons in the trapezoid body, peridendritic boutons in the medial superior olive and calyceal endings in the medial nucleus of the trapezoid body (MNTB). Further, we demonstrate that the majority of neurons in the human MNTB are calbindin and Kv3.1b immunoreactive and that perisomatic profiles in this nucleus are vesicular glutamate transporter and Rab3a positive, suggesting that such profiles are in fact synaptic terminals.

Key words: calyx of held, cochlear nucleus, superior olive, trapezoid body.

1. INTRODUCTION

Calcium-binding proteins (CBP) are expressed widely in neuronal cell bodies, axons and synaptic terminals throughout the vertebrate nervous system and play important roles in intracellular Ca^{++} signaling that impacts neuronal excitability and plasticity (reviewed in Christel et al., 2012). Calretinin (CR) and calbindin (CB) are both cytosolic EF-hand Ca^{++}-binding proteins which function as Ca^{++} buffers to regulate cell excitability and release of synaptic vesicles (Camp and Wijesinghe, 2009). Further, mice lacking CR demonstrate reduced long-term potentiation (Schurmans et al., 1997), altered action potential properties and ataxia (Schiffmann et al., 1999; Gall et al., 2003). Differential expression of CBP, especially CR and CB, has been used to characterize neuronal populations and circuits throughout the brains of many mammals (see below).

CBP are widely expressed in auditory brainstem nuclei and expression patterns of CR, CB and parvalbumin (PV) have been used to characterize auditory nuclei and axonal projections in numerous laboratory species (mouse – Bazwinsky et al., 2008; rat – Friauf, 1993, 1994; see also Fredrich et al., 2009; gerbil – Braun and Piepenstock, 1993; chinchilla – Kelley et al., 1992; guinea-pig – Caicedo et al., 1996; cat – Matsubara, 1990; horseshoe bat – Vater and Braun, 1994; rhesus – Bazwinsky et al., 2005) and human (Bazwinsky et al., 2003). Further, there are established developmental patterns of CBP expression in auditory brainstem nuclei (CB – Friauf, 1994; CR, PV – Lohmann and Friauf, 1996), CBP expression is altered as a result of sensorineuronal hearing loss (Caicedo et al., 1997; Förster and Illing, 2000; Alvarado et al., 2004; Fuentes-Santamaria et al., 2005) and aging (Idrizbegovic et al., 2003; Hong et al., 2009; Ouda and Syka, 2012; Ouda et al., 2012). Despite developmental changes and significant intraspecies variation, certain neuronal populations in the cochlear nucleus (CN) and superior olivary complex (SOC) can be characterized across mammalian species by their expression of CR and/or CB. In particular, globular bushy cells (GBC) in the CN are characteristically CR immunopositive (CR+; Caicedo et al., 1996; Bazwinsky et al., 2008) and principal neurons in the medial nucleus of the trapezoid body (MNTB) are characteristically CB immunopositive (CB+; Celio, 1990; Matsubara, 1990; Zettel et al., 1991; Kelley et al., 1992; Friauf, 1993; Caicedo et al., 1996; Bazwinsky et al., 2005). Additionally, the calyx of Held in the MNTB (arising from GBC axons) is characteristically CR+ (Arai et al., 1991; Résibois and Rogers, 1992; Lohmann and Friauf, 1996; Bazwinsky et al., 2005; although see mouse – Felmy and

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Abbreviations: AN, auditory nerve; CB, calbindin; CB+, calbindin immunopositive; CBP, calcium-binding proteins; CN, cochlear nucleus; CR, calretinin; CR+, calretinin immunopositive; GBC, globular bushy cell; LNTB, lateral nucleus of the trapezoid body; LSO, lateral superior olive; MNTB, medial nucleus of the trapezoid body; MSO, medial superior olive; NDS, normal donkey serum; PB, phosphate buffer; PV, parvalbumin; SBC, spherical bushy cell; SOC, superior olivary complex; SPON, superior paraolivary nucleus; tz, trapezoid body; VCN, ventral cochlear nucleus; VGLUT, vesicular glutamate transporter.

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Schneggenburger, 2004). However, quantitative data on CBP expression and distribution in the human CN and SOC are scarce. Specifically, there are no detailed reports on the localization of CBP in the human CN and only a single, limited qualitative report on CBP in the human SOC which, interestingly, denies the existence of the MNTB (Bazwinsky et al., 2003).

The pathway from the auditory nerve (AN) to GBC and then to the MNTB is perhaps one of the best characterized in the auditory brainstem, playing an essential role in the detection of interaural intensity differences. Within this circuit, AN axons give rise to modified endbulbs which contact GBCs (Harrison and Warr, 1962; Ryugo and Rouiller, 1988) and GBCs axons form elaborate synaptic endings (calyces of Held; Harrison and Warr, 1962) which are characteristically CR+ (Arai et al., 1991) and surround MNTB principal neurons (Morest, 1968a,b). Although this circuit has been characterized across a number of mammalian species, its existence in the human brainstem is in doubt (Harrison and Warr, 1962; Moore, 2000; but see Grothe et al., 2010 – their appendix A). The objective of this study is to further characterize neuronal populations in the human CN and SOC and investigate the existence of a GBC/MNTB circuit in the human brainstem. To this end, we have applied immunohistochemical and morphometric techniques to human brainstem tissue to localize CR and CB and additional markers to further characterize human MNTB neurons.

2. EXPERIMENTAL PROCEDURES

2.1. Materials and fixation

This investigation is based on the examination of 11 human brainstems from individuals ranging in age from 59 to 96 years of age (average 79.72 ± 3.11; 3 male and 8 female). Table 1 shows age, cause of death and post-mortem interval for specimens used in this study. Specimens used in this study were donated to the medical school through the Pennsylvania Humanity Gifts Registry and the LECOM Institutional Review Board granted exempt status for all procedures. Brainstems were only included in this study if: (1) the cause of death was not neurological, (2) there were no signs of degenerative disease affecting the brain on gross inspection or sectioning, (3) there were no signs of pathology affecting the brainstem or posterior cranial fossa and (4) brainstems could be preserved within 24 h of death. Specimens 2007.01 through 2009.45 were perfused through the right common carotid artery with an embalming solution (33.3% glycerin, 33.3% methanol, 27.8% phenol and 5.6% formaldehyde, diluted 1:3 in water; King Chemical, Inc., St. Louis, Missouri) and dissected from the skull after a short post-embalming interval. Specimens 2011.62 through 2013.05 were dissected from the skull immediately upon arrival to our morgue. After dissection and trimming, specimens were post-fixed for at least 2 weeks in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2.

2.2. Sectioning

Brainstem blocks were cryoprotected in a solution of 30% sucrose and 4% paraformaldehyde in 0.1 M PB until they were saturated (approximately 2 weeks). Tissue blocks were sectioned in the transverse plane on a freezing microtome at a thickness of 40 μm and collected free-floating. Every tissue section was collected beginning from the pontobulbar body (caudal to the CN) and extending rostrally to the exit of the trigeminal nerve. Alternating series of sections were designated for Nïssl staining (Giemsa; Iñiguez et al., 1985; see Kulesza, 2007 and Wagoner and Kulesza, 2009) and myelin staining (modified Mahon's method; Jebb and Woolsey, 1977; see Wagoner and Kulesza, 2009; Schmidt et al., 2010).

2.3. Immunohistochemistry

Free-floating sections were rinsed in 0.1 M PB pH 7.2, endogenous peroxidase activity was quenched by a wash in 1.5% hydrogen peroxide in PB and sections were permeabilized in 0.5% Triton X. Tissue sections were blocked for 1 h in 1% normal donkey serum (NDS) and then incubated for at least 20 h at room temperature with primary antisera diluted in 1% NDS in PB. The antisera and dilutions used in this investigation are summarized in Table 2.

Following incubation in primary antisera, tissue sections were rinsed and incubated for 1–2 h in biotinylated secondary antisera (Vector Laboratories, Burlingame, California; 1:100 in PB). Sections were then

Table 1. Summary of specimen age, cause of death and post-mortem interval (PMI)

Specimen	Age	Sex	Cause of death	PMI (h)
2007.01	75	F	Cardiac	< 24
2007.03	85	F	Metastatic breast cancer	< 24
2007.04	76	M	Respiratory failure	< 24
2008.02	81	F	Failure to thrive	< 4
2009.45	72	F	Cardiac arrhythmia	< 24
2011.62	85	F	Atrial fibrillations	< 18
2011.64	77	F	Diabetes/chronic renal disease	< 8
2011.65	95	F	COPD	< 5
2012.16	96	F	Melanoma	< 24
2012.22	59	M	Lung cancer	< 5
2013.05	79	M	COPD	< 7