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Research article

Developmental study of vitamin C distribution in children's brainstems by immunohistochemistry

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ABSTRACT

Vitamin C (Vit C) is an important antioxidant, exerts powerful neuroprotective brain effects and plays a role in neuronal development and maturation. Vit C is present in brain tissue at higher concentrations than in other organs, but its detailed distribution in brain is unknown. Immunohistochemical detection of this vitamin has been performed by using a highly specific antibody against Vit C. The aim of the present work was to analyze the distribution of Vit C in children's brainstems during postnatal development, comparing two groups of ages: younger and older than one year of life. In general, the same areas showing neurons with Vit C in young cases are also immunostained at older ages. The distribution of neurons containing Vit C was broader in the brainstems of older children, suggesting that brainstem neurons maintain or even increase their ability to retain Vit C along the life span. Immunohistochemical labeling revealed only cell bodies containing this vitamin, and no immunoreactive fibers were observed. The distribution pattern of Vit C in children's brainstems suggests a possible role of Vit C in brain homeostatic regulation. In addition, the constant presence of Vit C in neurons of *locus coeruleus* supports the important role of Vit C in noradrenaline synthesis, which seemed to be maintained along postnatal development.

Abbreviations: 1, dorsal longitudinal fasciculus; 2, caudal part of the dorsal motor nucleus of the vagus; 3, solitary nucleus and tract; AbdNu, abducens nucleus; ALS, anterolateral system; AMV, anterior medullary velum; ArNu, arcuate nucleus; CA, cerebral aqueduct; Cbl, cerebellum; CC, crus cerebri; CeGy, central gray; CoBul, corticobulbar fibers; CoCNr, cochlear nerve; CortNig, corticonigral fibers; CSNu, chief sensory nucleus (caudal end); CSp, corticospinal fibers; CTT, central tegmental tract; DAO, dorsal accessory olivary nucleus; DCNu, dorsal cochlear nucleus; DLF, dorsal longitudinal fasciculus; DMNu, dorsal motor nucleus of the vagus; DSCT, dorsal spinocerebellar tract; DTTr, dorsal trigeminothalamic tract; EAF, external arcuate fibers; FacNu, facial nucleus; FCu, cuneate fasciculus; FGr, gracile fasciculus; FPon, frontopontine fibers; GIF, glossopharyngeal fibers (intramedullary); GlNr, glossopharyngeal nerve; HyF, fascicles of hypoglossal nerve; HyNu, hypoglossal nucleus; IAF, internal arcuate fibers; IC,Br, inferior colliculus, brachium; IPN, interpeduncular nucleus; ISNu, inferior salivatory nucleus; LCNu, lateral cuneate nucleus; LL, lateral lemniscus; LL, Nulateral lemniscus, nucleus; LoCer, locus coeruleus; LRNu, lateral reticular nucleus; MAO, medial accessory olivary nucleus; MCP, middle cerebellar peduncle; MesNu, mesencephalic nucleus; MesTr, mesencephalic tract; MGB, medial geniculate body; ML, medial lemniscus; MLF, medial longitudinal fasciculus; MVN, medial vestibular nucleus; NiSt, nigrostriatal fibers; NuAm, nucleus ambiguus; NuCu, cuneate nucleus; NuGr, gracile nucleus; NuPp, nucleus prepositus; OCbF, olivocerebellar fibers; OcNr, oculomotor nerve; OcNu, oculomotor nucleus; OPon, occipitopontine fibers; PalNig, pallidonigral fibers; PBNu, pontobulbar nucleus NA; PCbF, pontocerebellar fibers; PO, principal (inferior) olivary nucleus; PonNu, pontine nuclei; PPon, parietopontine fibers; Py, pyramid; RB, restiform body; RetF, reticular formation; RetSp, reticulospinal tract; RNu, red nucleus; RuSp, rubrospinal tract; SC, superior colliculus; SCP, superior cerebellar peduncle; SM, stria medullaris of fourth ventricle; SN, substantia nigra; SO, superior olive; SolNu, solitary nucleus; SolTr, solitary tract; SpTec, spinotectal tract; SpTh, spinothalamic tract; SpTNu, spinal trigeminal nucleus; SpTT, spinal trigeminal tract; SpVN, spinal (inferior) vestibular nucleus; TecSp, tectospinal tract; TegDec, tegmental decussation; TPon, temporopontine fibers; TriMoNu, trigeminal motor nucleus (caudal part); TroNr, exit, trochlear nerve exit; TroNu, trochlear nucleus; VCNu, ventral cochlear nucleus; VesSp, vestibulospinal tract; VSCT, ventral spinocerebellar tract; VTegDec, ventral tegmental decussation; VTTr, ventral trigeminothalamic tract.

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1. Introduction

Vitamins are widely distributed in nature and are essential for several reactions. In this sense, several studies have demonstrated the involvement of vitamins in very different metabolic processes (see Mangas et al., 2009b). Among vitamins, vitamin C (Vit C) is a water-soluble vitamin acting as an antioxidant and thus preventing and ameliorating brain damage. Vit C is probably the most important antioxidant in plasma and contributes as an electron donor in several important biological reactions in the body (Frei et al., 1989; Drew et al., 2002).

Physiologically, Vit C is present as the ascorbate anion. Humans, non-human primates (including macaque monkeys), and guinea pigs cannot synthesize ascorbate from glucose, unlike other animals that have the enzyme required for the last step in ascorbate biosynthesis. The uptake and distribution of Vit C in the body is under homeostatic control and is regulated by tissue-specific sodium-dependent Vit C co-transporters (SVCT) 1 and 2, which actively transport Vit C (Tsukaguchi et al., 1999; Fischer et al., 2004; Corti et al., 2010; Lindblad et al., 2013). In the body, Vit C displays complex non-linear pharmacokinetics, and in brain tissue the Vit C is present at higher concentrations than in other organs (Kaufman, 1966; Spector, 1977; Schreiber and Trojan, 1991; Lindblad et al., 2013). Additionally, the brain is able to preferentially retain Vit C at the expense of other tissues during chronic states of severe deficiency, and to uphold concentrations 100-fold higher than other organs, which are readily depleted (Hughes et al., 1971; Lykkesfeldt et al., 2007). The brain depends on the SVCT2 receptor to drive active Vit C transport across the choroid plexus to the brain extracellular fluid and farther onto neuronal cells, where several lines of evidence indicate that ascorbate anion is preferentially localized (Rice, 2000).

Thus, ascorbate is absorbed from the diet and enters the central nervous system via the choroid plexus (Rice, 2000) and the SVCT2 transporter. Ascorbate levels are modulated by a glutamate-ascorbate heteroexchange across cell membranes, mainly in neurons (Grunewald, 1993). This mechanism might provide neuroprotection by minimizing the excitotoxic consequences of glutamate release. Furthermore, Vit C has also been found to induce the expression of brain-derived-neurotrophic-factor – a component of several neuronal survival pathways – and may thereby contribute to the defense mechanisms of the brain (Grant et al., 2005). Ascorbic acid has been also implicated in neuronal development and functional maturation (Qiu et al., 2007), as well as in glial and neuronal differentiation. In fact, mice lacking the Vit C transporter SVCT2 die at birth with respiratory failure, probably due to brainstem haemorrhaging (Harrison et al., 2010, 2014).

Although many authors have highlighted the key role of Vit C in the neuroprotective mechanisms of the brain, there have been few studies addressing the specific distribution of Vit C within the mammalian brain. The presence and distribution of Vit C have been described in some mammal brains (Mefford et al., 1981; Oke et al., 1987; Mangas et al., 2009b; Harrison et al., 2010), but the specific distribution of Vit C in adult and developing human brains has not yet been described. It is worth mentioning that earlier works studying the presence and distribution of Vit C used different techniques. In the past few decades, chromatographic techniques have been used to detect Vit C in human and animals models (Mefford et al., 1981; Harrison et al., 2010), as well as other chemical methods (Oke et al., 1987). However, the works of Mangas et al. (2009b) and Coveñas et al. (2011a, 2011b), using a highly specific antiserum directed against Vit C, are the only ones in which immunohistochemical approaches have been implemented to assess the presence and the specific distribution of fibers and/or cell bodies containing Vit C in the brain of a non-human primate (*Macaca fascicularis*). It is important to examine the distribution of Vit C in the central nervous system by immunohistochemistry because this technique provides information about which cells display Vit C, and as to where the molecule is located inside the cells. Moreover, immunohistochemistry allows the immunoreactive structures containing Vit C to be located anatomically not only across a widespread region of the human brain, but also in specific central nervous central nuclei of our species. Thus, using an immunohistochemical technique the main aim of this work was to determine for the first time the presence (in fibers and/or cell bodies) and the distribution of Vit C in human brainstems during postnatal development. The results obtained here are compared with recently published data concerning the distribution of MAP-2 and HIF-1α in the same cases (Coveñas et al., 2014).

In sum, knowledge of the distribution of immunoreactive structures containing Vit C in the human central nervous system is necessary in order to gain insight into the role played by this vitamin in the regions of the human brain in which its presence is found. Thus, the roles of Vit C may vary depending on the brainstem nuclei in which it is located and the data reported here suggest that it may play hitherto unsuspected roles.

2. Materials and methods

2.1. Tissue processing

For this study, seven human infant brains obtained from the Andalusian Public Health System Biobank (ISCIII-Red de Biobancos RD09/0076/00085) were used. These brains were collected from routine autopsies, and their utilization for medical research was authorized by the informed consent of the next of kin. Pursuant to Spanish laws and the Helsinki Declaration for Medical Research in humans, approval was obtained from the Ethical Committee of Clinical Research of the University Hospital in Albacete (Spain). The privacy rights of human subjects were always observed, samples being non-identifiable. According to the neuropathological examination performed by an experienced neuropathologist, no evidence of neurological damage was found, then all cases were considered as controls. Information concerning the age, sex, cause of death, *post-mortem* time and other demographic aspects of the infants is given in Table 1.

All brains were subjected to the same experimental protocol. As previously described (Cebada-Sánchez et al., 2014; Coveñas et al., 2014), brains were immediately immersion-fixed in 10% formalin after dissection and then transferred to 4% paraformaldehyde in 0.1 M phosphate buffer-saline (PBS), pH 7.4, following the standardized protocol carried out in our Laboratory (Insausti et al., 2010). Brainstems were dissected out and cryoprotected in increasing sucrose solutions (15–30% in PBS) until they sank. Then, the brainstems were cut on a cryostat (Micron, Heidelberg, Germany) in 50-μm thick coronal sections. In all cases, one-in-five sections were mounted onto gelatin-coated slides for Nissl staining.

In this work, the free-floating immunohistochemical procedure was performed following a previously described protocol (Cebada-Sánchez et al., 2014; Coveñas et al., 2014; Mangas et al., 2009b; Marcos et al., 2011, 2013) specially adapted to improve immunolabeling sensitivity and minimize background in human tissue (Ramos-Vara, 2005). The protocol applied here has been previously validated for the detection of calcium-binding proteins (Graterón et al., 2003), neuropeptides such as somatostatin and neuropeptide Y (Cebada-Sánchez et al., 2014), and for the mapping of HIF-1α and MAP-2 (Coveñas et al., 2014) in the brains of the same cases studied here. The highly specific polyclonal primary antibody against Vit C (GEMAC S. A., Saint Jean d'Illac, France; reference: AP-131) was developed in rats and was diluted 1/500, and incubations with