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Research report

Expression of PLA2G6 in human fetal development: Implications for infantile neuroaxonal dystrophy

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ABSTRACT

Mutations in PLA2G6, which encodes calcium-independent phospholipase A_2 group VIA (iPLA2-VIA), underlie the autosomal recessive disorder infantile neuroaxonal dystrophy (INAD). INAD typically presents in the first year of life, and leads to optic atrophy and psychomotor regression. We have examined PLA2G6 expression in early human embryonic development by *in situ* hybridization. At Carnegie Stage (CS) 19 (approximately 7 post-conception weeks [PCW]), strong expression is evident in the ventricular zone (VZ) of midbrain and forebrain suggestive of expression in neural stem and progenitor cells. At CS23 (8 PCW) expression is also detectable in the VZ of the hindbrain and the subventricular zone (SVZ) of the developing neocortex, ganglionic eminences and diencephalon. By 9 PCW strong expression in the post-mitotic cells of the cortical plate can be seen in the developing neocortex. In the eye, expression is seen in the lens and retina at all stages examined. PLA2G6 expression is also evident in the alar plate of the spinal cord, dorsal root ganglia, the retina and lens in the eye and several non-neuronal tissues, including developing bones, lung, kidney and gut. These findings suggest a role for PLA2G6 in neuronal proliferation throughout the developing brain and in maturing neurons in the cortical plate and hindbrain. Although widespread PLA2G6 expression is detected in neuronal tissues, the pattern shows dynamic changes with time and indicates that INAD pathogenesis may begin prior to birth.

1. Introduction

Infantile neuroaxonal dystrophy (INAD) is an autosomal recessive disease with early onset and rapid progression of hypotonia, hyperreflexia and tetraparesis. INAD belongs to a larger class of neuroaxonal dystrophies, which include pantothenate-kinase associated neurodegeneration (PKAN), idiopathic neurodegeneration with brain iron accumulation (NBIA), and Schindler disease. Within this group, INAD has the earliest onset, with motor and cognitive skill regression presenting at a median age of 15 months [14,18]. Optic atrophy is also commonly observed. Historically, INAD was diagnosed from histological evidence of neuroaxonal spheroids in peripheral nerves [24]. The detection of cerebellar atrophy and iron accumulation in the globus pallidus by MRI is also diagnostic of INAD, although iron accumulation often is not observed until later in the disease [14,18,23].

In 2006, the causative gene for INAD was identified as *PLA2G6*, which encodes a calcium-independent phospholipase (A_2 group IVA) [23]. *PLA2G6* mutations are found in the vast majority of INAD patients and have also been described in individuals previously diagnosed with idiopathic NBIA [14], as well as individuals with adult-onset dystonia-parkinsonism [29]. Phospholipases A_2 comprise a large family of enzymes that catalyze the hydrolysis of sn-2 ester bonds of glycerophospholipids, producing free fatty acids and lysophospholipids [12]. Arachidonic acid and other fatty acids released by $iPLA_2$-VIA can initiate apoptosis, inflammation, and cell growth [4,36]. The lysophospholipid remaining in the cell membrane can also trigger cellular processes, including chemotaxis and fusion of biological membranes [2,10].

The early presentation of INAD suggests that $iPLA_2$-VIA may have a developmental role. Malik et al. [20] showed that the *Pla2g6* knockout mouse [5] develops neurological impairments by 13 months and accumulates neuroaxonal spheroids similar to those in human INAD patients. In addition, 4-month-old *Pla2g6* knockout mice have decreased brain docosahexaenoic acid (DHA) metabolism and signaling [6]. In a second INAD mouse model, point mutations within *Pla2g6* result in motor dysfunction and neuroaxonal spheroids as early as 7 weeks of age [39]. These studies, however, did not examine *Pla2g6* expression during mouse development. *Pla2g6* expression has been shown in mouse sagittal

Abbreviations: INAD, infantile neuroaxonal dystrophy; NBIA, neurodegeneration with brain iron accumulation; PCW, post-conception weeks; PKAN, pantothenate-kinase associated neurodegeneration; SVZ, subventricular zone; VZ, ventricular zone.

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sections at embryonic day 14.5 as part of the high-throughput Eurexpress project [38]. At this stage, the strongest expression seen in the brain is in the alar plate of the developing hindbrain with prominent expression also in an analogous region of the midbrain. *Pla2g6* also appears to be expressed weakly in the developing diencephalon and telencephalon of the forebrain. Expression was also detected in spinal cord and the bones of the developing skull, face, ribcage and limbs (EMAGE entry 18166; genex.hgu.mrc.ac.uk/).

To better understand the potential role for *PLA2G6* in neuronal development and pathogenic mechanisms underlying disease, we examined *PLA2G6* expression by *in situ* hybridization across several stages of human embryonic and early fetal development.

2. Materials and methods

2.1. Human tissue collection and processing

Human embryonic and fetal tissues were obtained from the MRC-Wellcome Trust Human Developmental Biology Resource (http://www.hdbr.org/), Institute of Human Genetics, Newcastle University. The samples were collected with appropriate maternal consents and ethical approval by the Newcastle and North Tyneside Research Ethics Committee. Tissue sections from seven samples were analyzed: Carnegie Stage (CS) 19 (~7 PCW, $n = 2$), CS23 (8 PCW, $n = 3$) and 9 PCW ($n = 2$). The stage of embryonic development (CS19 and CS23) was determined by assessment of external morphology as described [9,25]. For fetal samples (9 PCW) age was estimated from measurements of foot length and heel to knee length. These were compared with a standard growth chart [15].

2.2. In situ hybridization

Three fragments of the cDNA for human *PLA2G6*; 453 bp of *PLA2G6* exon 2–4 (probe 1), 517 bp of *PLA2G6* exon 11–15 (probe 2) and 557 bp of the *PLA2G6* 3'UTR (probe 3) were amplified from Homo sapiens *PLA2G6* mRNA (Accession: CU013143) and cloned into pCR-BluntII-TOPO (Invitrogen). The construct sequences were verified and prepared using HiSpeed Plasmid Midi kit (Qiagen). To create the *PLA2G6* antisense probe, the plasmid was linearized with SpeI and amplified from the T7 promoter. To create the *PLA2G6* sense control probe, the plasmid was linearized with Not1 and amplified from the Sp6 promoter. Probes were labeled with digoxygenin (DIG) using the DIG-RNA labeling kit (Roche Applied Science) according to manufacturer's instructions. All probes were tested and *PLA2G6* probe 3 was selected for the studies subsequently described.

In situ hybridizations were performed as previously described [22] with some modifications. Briefly, sections were dewaxed in xylene, gradually hydrated in decreasing ethanol concentrations before incubation in Proteinase K (20 μg/ml) at room temperature (RT), followed by fixation in 4% paraformaldehyde in PBS. Background was reduced by treating with 0.1 M Triethanolamine pH 8. Sections were

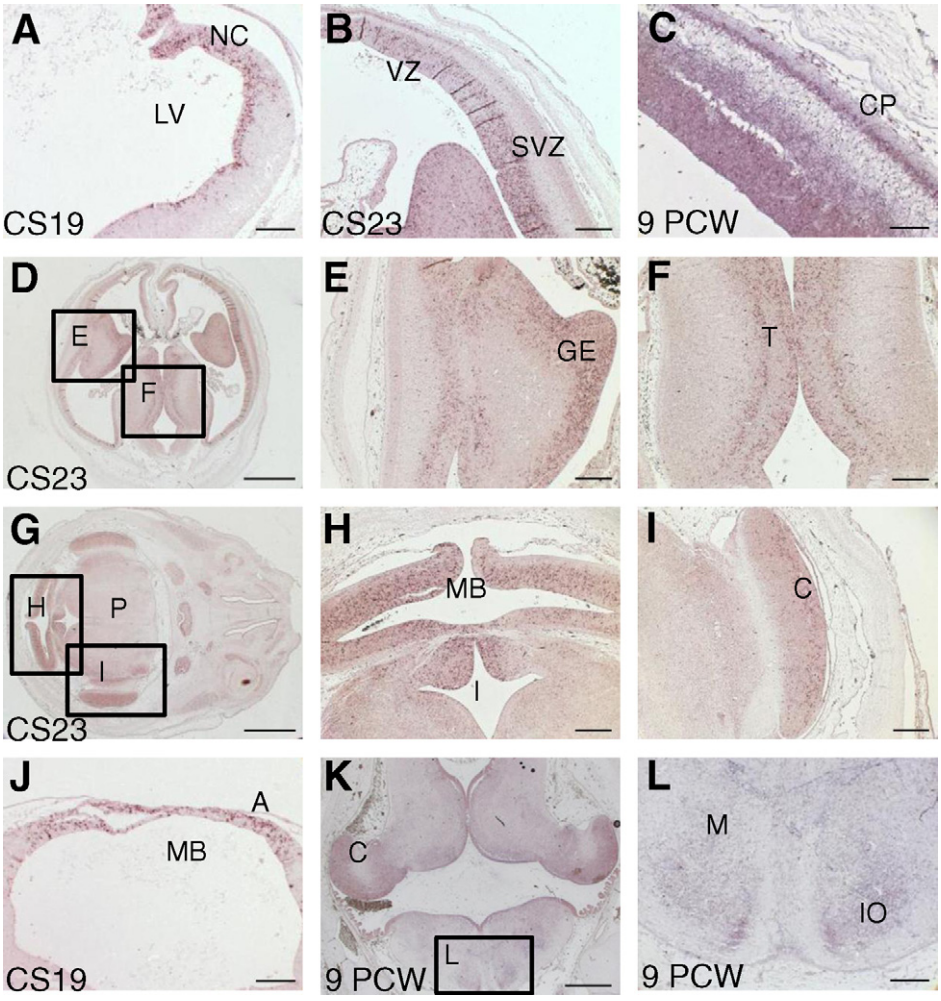

Fig. 1. *PLA2G6* expression in human brain at CS19, CS23 and 9 PCW. (A)–(L) show images of *in situ* hybridization with antisense probes for *PLA2G6* to sections at CS19 (A and J), CS23 (B and D–I) and 9 PCW (C, K and L). Signal is detected as a purple stain. (A)–(C) *PLA2G6* staining in developing neocortex. At CS19 (A), expression can be seen close to the lateral ventricle (VZ), by CS23 (B) expression is in the VZ and SVZ and by 9 PCW (C) expression is also seen in the CP. (D) Low magnification image showing developing neocortex, ganglionic eminences and thalamus. The boxed areas are shown at higher magnification in (E) and (F). (G) Low magnification image showing developing midbrain, isthmus, cerebellum and pons. The boxed areas are shown at higher magnification in (H) and (I). (J) *PLA2G6* expression is seen in the alar plate of the developing midbrain. (K) *PLA2G6* expression in the hindbrain is seen in the developing cerebellum, the VZ of medulla and neurons in developing nuclei (IO). Boxed area is seen at higher magnification in (L). No signal was detected using sense control probes (Supplemental Fig. 1). C—cerebellum, CP—cortical plate; GE—ganglionic eminences, IO—inferior olive, I—isthmus, LV—lateral ventricle, M—medulla, MB—midbrain, NC—neocortex, P—pons, SVZ—subventricular zone, T—thalamus, VZ—ventricular zone. Scale bars are: 100 μm in (A), (C), (J) and (L); 200 μm in (B), (E), (F), (H) and (I); 1000 μm in (D), (G) and (K). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)