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Wolfram syndrome 1 (*Wfs1*) mRNA expression in the normal mouse brain during postnatal development

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ABSTRACT

Wolfram syndrome is a rare genetic disorder accompanying diabetes insipidus, sensorineural hearing loss, neurological complications, and psychiatric illness. This syndrome has been attributed to mutations in the *WFS1* gene. In this study, we made a detailed histochemical analysis of the distribution of *Wfs1* mRNA in the brain of developing mice. There were three patterns of change in the strength of *Wfs1* mRNA signals from birth to early adulthood. In type 1, the signals were weak or absent in neonates but strong or moderate in young adults. This pattern was observed in the CA1 field, parasubiculum, and entorhinal cortex. In type 2, the signals were of a relatively constant strength during development. This pattern was seen in limbic structures (e.g. subiculum and central amygdaloid nucleus) and brainstem nuclei (e.g. facial and chochlear nuclei). In type 3, the signals peaked in the second week of age. This pattern was observed in the thalamic reticular nucleus. Thus, *Wfs1* mRNA was widely distributed in the normal mouse brain during postnatal development. This evidence may provide clues as to the physiological role of the *Wfs1* gene in the central nervous system, and help to explain endocrinological, otological, neurological, and psychiatric symptoms in Wolfram syndrome patients.

1. Introduction

Wolfram syndrome (OMIM 222300) is an autosomal recessive neurodegenerative disorder defined by young-onset non-autoimmune insulin-dependent diabetes mellitus and progressive optic atrophy (Wolfram and Wagener, 1938; Minton et al., 2003). The nuclear gene responsible for Wolfram syndrome has been identified as *WFS1* (Wolfram syndrome 1; Inoue et al., 1998; Strom et al., 1998), and is located at 4p16.1 (Polymeropoulos et al., 1994; Collier et al., 1996). The *WFS1* gene is also responsible for autosomal dominant low frequency sensorineural hearing loss (Bespalova et al., 2001; Young et al., 2001), and is a candidate to contribute low risk for type 2 diabetes mellitus (Minton et al., 2002; Sparsø et al., 2008; Wasson and Permutt, 2008). The WFS1 protein, also called wolframin, localizes primarily to the endoplasmic reticulum (ER) membrane, and contains nine transmembrane segments with the amino-terminus in the cytosol and the carboxy-terminus in the ER lumen (Takeda et al., 2001; Hofmann et al., 2003). Subsequent functional studies showed that the WFS1 protein is important in the regulation of intracellular Ca^{2+} homeostasis (Osman et al., 2003; Takei et al., 2006), contributes to cell cycle progression (Yamada et al., 2006), and is produced under conditions of troubled homeostasis, including ER stress (Yamaguchi et al., 2004; Fonseca et al., 2005; Ueda et al., 2005). In addition, screening for mutations in Wolfram syndrome patients demonstrated more than 50 distinct mutations of the *WFS1* gene, including stop, frameshift, deletion and missense mutations (Inoue et al., 1998; Strom et al., 1998; Hardy et al., 1999; Gómez-Zaera et al., 2001; Khanim et al., 2001; Tessa et al., 2001; Cano et al., 2007). Thus loss-of-function mutations in the *WFS1* gene have been linked to Wolfram syndrome, however, molecular functions of the WFS1 protein and the mechanism by which mutations of the *WFS1* gene cause Wolfram syndrome remain unclear.

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Although the defining characteristics of Wolfram syndrome are diabetes mellitus (100%)[1] and optic atrophy (100%), other symptoms include cranial diabetes insipidus (73%), sensorineural deafness (62%), neurological complications (cerebellar ataxia and myoclonus; 62%), and psychiatric illness (60%) (Swift et al., 1990; Barrett et al., 1995). Accordingly, the term DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness) is used to describe Wolfram syndrome with more widespread complications (Barrett et al., 1995). The prevalence of Wolfram syndrome is one per 770,000 in the UK population, and the median age at death (commonly central respiratory failure with brainstem atrophy) is 30 years (range 25–49 years) (Barrett et al., 1995). Neuroradiological (Rando et al., 1992; Scolding et al., 1996; Ito et al., 2007) and neuropathological (Genís et al., 1997; Shannon et al., 1999) studies have reported severe atrophy in the brainstem, cerebellum, and optic nerve of Wolfram syndrome patients. Mild atrophy was also observed in the cerebral cortex and hypothalamus. Thus, clinical and pathological facts concerning brain-related (ophthalmological, endocrinological, otological, neurological, and psychiatric) symptoms in Wolfram syndrome have been accumulated. However, the site of pathology for these symptoms remains unclear. To obtain insights into the site of pathology for the symptoms, it is necessary to examine *WFS1* expression in the brain not only at the adult stage, but also at the developmental stages, since there is a possibility that lack of *WFS1* expression during development contributes to the progression of the brain-related symptoms of Wolfram syndrome caused by loss-of-function mutations in the *WFS1* gene. Insights into the site of pathology may provide hypotheses about the pathophysiology of the brain-related symptoms of Wolfram syndrome.

In the rodent brain, expression of the *Wfs1* gene has previously been described in the cerebral cortex, the basal ganglia, the hypothalamus, the brainstem motor and sensory nuclei, the reticular formation, and in the cerebellar cortex, as well as in the CA1 field of the hippocampus and in the amygdala (Takeda et al., 2001; Ishihara et al., 2004; Kato et al., 2008; Kawano et al., 2008; Luuk et al., 2008). To obtain neuroanatomical evidence for understanding the endocrinological, otological, neurological, and psychiatric symptoms of Wolfram syndrome, and to establish a basis for functional studies of the WFS1 protein in the brain, we performed a detailed histochemical analysis of the distribution of *Wfs1* mRNA signals in the brain of normal mice during postnatal development.

2. Materials and methods

2.1. Animals and tissue preparation

Male mice (n = 10; C57BL/6NCrlCrlj; Charles River Laboratories Japan, Inc., Yokohama, Kanagawa, Japan) were used in this study. The delivery day was designated as postnatal day 0 (P0). Three mice at 8 weeks old (P8W, early adulthood), two mice at P28, and five neonates at early postnatal ages, P0, P4, P7, P14, and P21, were used. Prior to the experiments, they were housed in an animal care facility with a 12-h light (lights on 8:00–20:00), 12-h dark photoperiod and free access to tap water and rodent chow. The mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and perfused transcardially with 4% paraformaldehyde dissolved in 0.1 M sodium phosphate buffer (PB; pH 7.4) at 4 °C. Brains were removed from the skull, stored in the same fixative for 48 h, and then immersed in 30% saccharose in 0.1 M PB at 4 °C until they sank. The brains were frozen in powdered dry ice and coronally cut at a thickness of 40 μm. The sections were collected as a 1-in-5 series in a cryoprotectant medium (33.3% saccharose, 1% polyvinylpyrrolidone (K-30), and 33.3% ethylene glycol in 0.067 M sodium phosphate buffer (pH 7.4) containing 0.067% sodium azide; Warr et al., 1981) and stored at −30 °C prior to use. In each experimental case at ages P0 and P4, heads including the brain were processed as described above without decalcification.

All experimental protocols for this study were approved by the committee on the Ethics of Animal Experimentation at Yamaguchi University School of Medicine, and were conducted according to the guidelines for Animal Research of Yamaguchi University School of Medicine and The Law (No. 105) and Notification (No. 6) of the Japanese Government.

2.2. Preparation of cRNA probes

To synthesize a cRNA probe for *in situ* hybridization, a 1548-base fragment of the mouse *Wfs1* cDNA was amplified by RT-PCR, and subcloned into the vector pCR-Blunt (Invitrogen, Carlsbad, CA). The primers used were MOUSE-U2, 5′-T CCG TAC TCT CAC CGA CCT G-3′, and MOUSE L3, 5′-C TCA GGC GGC AGA CAG GAA T-3′. The fragment encoded the 3′-end of the protein-coding region including the stop codon, and occupied 85% of exon 8 where many mutations have been reported in the *WFS1* gene of Wolfram syndrome patients (Inoue et al., 1998; Strom et al., 1998; Hardy et al., 1999; Gómez-Zaera et al., 2001; Khanim et al., 2001; Cano et al., 2007). Two independent clones containing the insert with a different orientation (pCR-clone 19 for sense, pCR-clone 1 for anti-sense) were used. A sense or an anti-sense cRNA probe was obtained by in vitro transcription with a DIG RNA labeling kit (SP6/T7; Roche Diagnostics GmbH, Penzberg, Germany).

2.3. In situ hybridization histochemistry

In situ hybridization histochemistry was carried out as described previously (Kawano et al., 2008). Free-floating sections washed for 5 min in diethylpyrocarbonate-treated phosphate-buffered saline (DEPC-PBS) were pretreated with 0.2 N HCl for 20 min, washed twice for 5 min in DEPC-PBS, and then acetylated in 0.1 M triethanolamine–HCl (pH 8.0) containing 0.25% acetic anhydride for 10 min. Before the hybridization step, sections were washed again twice for 5 min with DEPC-PBS. All pretreatments were performed at 4 °C. Following the pretreatment, sections were preincubated in hybridization buffer (50% deionized-formamide; 10 mM Tris–HCl, pH 7.5; 1 mM EDTA, pH 8.0; 600 mM NaCl; 1× Denhardt's solution; 10% dextran sulfate; 0.25% sodium dodecyl sulfate; and 200 μg/ml yeast tRNA) at 55 °C for 1 h and then hybridized with DIG-labeled anti-sense cRNA probes (0.5 μg/ml; denatured at 95 °C for 5 min and cooled at 4 °C for 5 min shortly before use) in the same buffer at 55 °C for 16 h. After hybridization, the sections were washed with 2× SSC (300 mM NaCl, and 30 mM sodium citrate, pH 7.0) containing 50% formamide at 55 °C for 1 h, rinsed in wash buffer (500 mM NaCl, 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA, pH 8.0) for 10 min and then incubated with RNase A (20 μg/ml; Sigma–Aldrich, St. Louis, MO) in wash buffer at 37 °C for 30 min. After being rinsed in wash buffer again for 10 min, they were soaked in 2× SSC containing 50% formamide and 0.2× SSC containing 50% formamide at 55 °C for 30 min each. To perform the immunoreaction, the sections were blocked in buffer 2 (buffer 1 (150 mM NaCl, and 100 mM Tris–HCl, pH 7.5) containing 2% blocking reagent) at 20 °C for 1 h and then incubated in buffer 2 containing alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Diagnostics) diluted 1:3000 at 20 °C for 16 h. After two washes in buffer 1 for 10 min, they were rinsed in buffer 3 (100 mM NaCl, 50 mM $MgCl_2$, and 100 mM Tris–HCl, pH 9.5) for 5 min and incubated with NBT/BCIP substrate (1:50; Roche Diagnostics) in buffer 3 at 37 °C for 2–4 h to visualize the immunocomplex. The coloring reaction was stopped with buffer 4 (1 mM EDTA, and 10 mM Tris–HCl, pH 8.0), and the sections were washed in

[1] Percentage in parentheses shows frequency of the feature in Wolfram syndrome patients.