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TRANSIENT EXPRESSION OF XPN, AN XLMR PROTEIN RELATED TO NEURITE EXTENSION, DURING BRAIN DEVELOPMENT AND PARTICIPATION IN NEURITE OUTGROWTH

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Abstract—*KIAA2022* has been implicated as a gene responsible for expressing X-linked mental retardation (XLMR) proteins in humans. However, the functional role of *KIAA2022* in the human brain remains unclear. Here, we revealed that depletion of *Kiaa2022* inhibits neurite outgrowth of PC12 cells, indicating that the gene participates in neurite extension. Thus, we termed *Kiaa2022* as an XLMR protein related to neurite extension (Xpn). Using the mouse brain as a model and ontogenetic analysis of *Xpn* by real-time PCR, we clearly demonstrated that *Xpn* is expressed transiently during the late embryonic and perinatal stages. *In situ* hybridization histochemistry further revealed that *Xpn*-expressing neurons could be categorized ontogenetically into three types. The first type showed transient expression of *Xpn* during development. The second type maximally expressed *Xpn* during the late embryonic or perinatal stage. Thereafter, *Xpn* expression in this type of neuron decreased gradually throughout development. Nevertheless, a significant level of *Xpn* expression was detected even into adulthood. The third type of neurons initiated expression of *Xpn* during the embryonic stage, and continued to express the gene throughout the remaining developmental stages. Subsequent immunohistochemical analysis revealed that Xpn was localized to the nucleus and cytoplasm throughout brain development. Our findings indicate that Xpn may participate in neural circuit formation during developmental stages via nuclear and cytoplasmic Xpn. Moreover, disturbances of this neuronal circuit formation may play a role in the pathogenesis of mental retardation.

Key words: X-linked mental retardation, KIAA2022, neurite outgrowth, neuronal circuit formation, XLMR protein related to neurite extension.

INTRODUCTION

More than 20 genes have been identified as members of the X-linked mental retardation (XLMR) family (Ropers and Hamel, 2005). Cantagrel et al. (2004) proposed *KIAA2022* as one such gene. Analysis of the breakpoints of pericentric inversion in families with two male members affected by severe mental retardation indicated that *KIAA2022* was disrupted by the breakpoint in the Xp13.2 chromosome (Cantagrel et al., 2004). Thus, it was suggested that the absence of *KIAA2022* was related to the pathogenesis of XLMR. However, screening of 20 probands from XLMR families failed to reveal any mutations in the *KIAA2022* gene (Cantagrel et al., 2004).

KIAA2022 encodes a large protein, containing 1516 amino acids. Previous reports have shown that *KIAA2022* has no functional motif or significant homology with other known proteins (Cantagrel et al., 2004, 2009). Cantagrel et al. (2009) demonstrated that *KIAA2022* mRNA was highly expressed in the brain, suggesting a role for *KIAA2022* in brain function. Elucidation of this role will facilitate a better understanding of the mechanism by which disruption of *KIAA2022* causes XLMR in humans.

In the present study, we demonstrated that *Kiaa2022* mRNA is expressed transiently in the mouse brain during the late embryonic and perinatal stages of development. *In situ* hybridization histochemistry revealed that *Kiaa2022*-expressing neurons could be categorized ontogenetically into three types. We further observed that *Kiaa2022* participates in neurite outgrowth of neurons. Using both *in situ* hybridization histochemistry and immunohistochemistry, we examined the distribution of *Kiaa2022* mRNA and Kiaa2022 protein in the mouse brain. We demonstrated that both *Kiaa2022* and Kiaa2022 expressions were closely related to the function

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Abbreviations: ATRX, α-thalassemia mental retardation X-linked; BSA, bovine serum albumin; CA, cornu ammonis; DAPI, phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; E, embryonic day; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; HS, horse serum; NGF, nerve growth factor; P, perinatal day; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; VPM, ventral posteromedial thalamic nucleus; XLMR, X-linked mental retardation; Xpn, XLMR protein related to neurite extension.

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of specific neuronal groups. Our results indicate that immature neuronal circuit formation is involved in the development of XLMR. Thus, we designated Kiaa2022 as an XLMR protein related to neurite extension (Xpn).

EXPERIMENTAL PROCEDURES

Ethics statement

All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of the Osaka University. The principles for animal care followed the United States National Institutes of Health Guide for the care and use of Laboratory Animals.

Cell culture

PC12 cells were maintained in tissue culture dishes (Thermo Fisher Scientific, Rochester, NY, USA). The cells were cultured on Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum and 5% horse serum (HS). The cultures were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2. The PC12 cells were transfected using Lipofectamine 2000 (Life Technologies Inc.), according to the manufacturer's instructions. For the neurite outgrowth studies, PC12 cells (5×10^5 cells) were plated onto 60-mm collagen-coated plastic tissue culture dishes, and cultured in DMEM containing 1% HS. When cells attained a length greater than diameter, they were scored for the production of outgrowths. The number of neurite-bearing cells was expressed as a percentage of the total cell number (> 25 μm; three independent experiments, 300 cells per experiment). The cells were observed under a light microscope (Model IX71; Olympus Corp., Tokyo, Japan).

TUNEL assay

Cell death during the experiments was assessed using the TMR Red *in situ* cell death detection kit (Roche Diagnostics Corp., Indianapolis, IN, USA). Cells were observed by fluorescent microscopy. Different populations in the total cell population (based on counting 400 nuclei) were visualized by overlaying phenylindole dihydrochloride (DAPI) images (displaying all nuclei) with TUNEL staining images. Thereafter, TUNEL-positive cells were expressed as a percentage of total DAPI-positive cells. For each experiment, at least four fields were examined by an independent observer, who was unaware of the experimental protocol.

Knockdown experiment using siRNA

Stealth siRNA against *Xpn* (5′-UAUCAAAGAAGGCAGAUCGAGAGUC-3′) and negative control duplexes (scrambled siRNA for *Xpn*, 5′-UAUGAACGAAGAAACGUAGAGCGUC-3′) were provided by Life Technologies Inc. The PC12 cells were transfected with 200 pM of each siRNA and a scrambled siRNA using Lipofectamine RNAiMAX (Life Technologies Inc.), according to the manufacturer's instructions. The knockdown of *Xpn* was confirmed by real-time reverse transcriptase reaction and real-time polymerase chain reaction (PCR).

Plasmid construction

Plasmids containing hemagglutinin (HA)-fused mouse Xpn were derived from the eukaryotic expression vector pcDNA3.1 (Life Technologies Inc.). Mouse Xpn was amplified from a mouse brain cDNA library by using PCR. Xpn was amplified using rTaq DNA polymerase (Takara Bio Inc., Kyoto, Japan) with the following primer set: 5′-GCGGCCGCGCCACCATGGATAACCAACAAGATAAAGTT-3′ (forward) and 5′-GGTACCAATGTCTTTCTGGAAAATGTG-3′ (reverse). To comprise the negative control, HA was amplified using rTaq DNA polymerase (Takara Bio Inc.) with the following primer set: 5′-GGTACCTACCCTTATGATGTGCCGGAT-3′ (forward) and 5′-AAGCTTTTAGGCATAATCCGGCACATCATA-3′ (reverse). The amplified fragments were TA-cloned into the pGEM-T vector (Promega Corp., Madison, WI, USA). The *Xpn* siRNA-insensitive mouse Xpn was amplified from pGEM-Xpn by using the following primer sets: 5′-GAtTCTCGtTCTGCtTTCTTcGATAAAAAG-3′ (forward) (the mutated sequences are indicated in lowercase letters; these mutations do not change the amino acid sequence of mouse Xpn) and 5′-CTCACTGGAACCAGGCATATCTGC-3′ (reverse).

RT-PCR

Total RNA was prepared from the mouse brain and PC12 cells using ISOGEN (NipponGene, Toyama, Japan) according to the manufacturer's instructions. The total RNA extract was reverse transcribed using oligo(dT) 12–18-mer primers and SuperScript III RNaseH reverse transcriptase (Life Technologies Inc.), according to the manufacturer's instructions. Real-time PCR was performed using an ABI PRISM 7900HT Sequence Detection System with the Sybr Green PCR Master Mix (Life Technologies Inc.). To quantify the expression levels of *Xpn* (*Kiaa2022*), the following primers were used: mouse *Xpn* forward primer, 5′-AAAAGAGTCCCCGATGTTTCCC-3′ (complement of bases 162–183); mouse *Xpn* reverse primer, 5′-TGATCGGGTGCTTCAATCAGG-3′ (reverse complement of bases 246–266); rat forward primer, 5′-CGGGAGCTACTAAGAATCACAGG-3′ (complement of bases 4160–4182); rat *Xpn* reverse primer, 5′-CAAAGAAGGCAGATCGAGAGTCC-3′ (reverse complement of bases 4263–4285). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5′-GTGTTCCTACCCCCAATGTG-3′ and GAPDH reverse primer 5′-AGGAGACAACCTGGTCCTCA-3′ were used as the initial controls. Sybr Green I fluorescence from the double-stranded PCR products was measured according to the manufacturer's instructions (Life Technologies Inc.).

In situ hybridization

The partial cDNAs for the coding region of mouse *Xpn* (nucleotides 2543–3182) were amplified by RT-PCR using the following primers: *Xpn* forward primer, 5′-AAGCATTTGTACCTCTCCAGTCTG-3′ (complement of bases 2543–2566); partial *Xpn* reverse primer, and 5′-TTGTCAGGAGTGAAGTTGGAGATG-3′ (reverse complement of bases 3159–3182). The amplified fragments were TA-cloned into the pGEM-T vector (Promega Corp.). Serial sagittal sections, 12-μm thick, were prepared and thaw-mounted on Matsunami adhesive silane (MAS)-coated glass slides (Matsunami Glass Ind., Osaka, Japan). The sections were processed for *in situ* hybridization as described previously (Koyama et al., 2008; Miyata et al., 2011).

Western blot analysis

We used the anti-TrkA rabbit polyclonal antibody (1:500) (Millipore, Billerica, MA, USA), anti-phospho-TrkA rabbit polyclonal antibody (1:500) (Cell Signaling, MA, USA), and anti-β tubulin rabbit polyclonal antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Treated cells were washed twice in phosphate-buffered saline (PBS), harvested, and lysed in TNE buffer (10 mM Tris–HCl, pH 7.8, 1 mM EDTA, and 150 mM NaCl) containing 1% (v/v) NP-40 and protease inhibitor cocktail (Roche Diagnostics Corp.). Equal amounts of protein were subjected to 7% (v/v) SDS–PAGE for phospho-TrkA, total TrkA and GAPDH and transferred to a PVDF membrane (Millipore). The membrane