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Oligophrenin-1 (*Ophn1*) is expressed in mouse retinal vessels ☆

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ABSTRACT

The Rho GTPase activating protein (RhoGAP) Oligophrenin 1 (Ophn1) regulates numerous members of the Rho family that are involved in neuronal morphogenesis of the central and peripheral nervous system. In the present study we investigated the spatial and temporal expression of *Ophn1* in the mouse eye.

The expression of *Ophn1* was analysed on both mRNA and protein level. To identify the *Ophn1* transcripts, adult retina and cerebrum (positive control) of postnatal day (P) 158 was subjected to reverse transcription polymerase chain reaction (RT-PCR) and sequencing of the amplified cDNA. The Ophn1 protein was analyzed in adult retina by Western blotting and in developing eyes at embryonic day (E) 12, E14, E16, E18, P0, P3, P7, P14 and P158 by immunohistochemistry.

Ophn1 transcripts were detected in adult retina by RT-PCR and confirmed by sequencing. Western blot analysis revealed the expression of Ophn1 protein in the adult retina. Immunohistochemical examination of developing eyes localized the protein to retinal vasculature with an onset of Ophn1 expression from P14 onwards.

The specific expression pattern suggests that Ophn1 could have a physiological role in the retinal vasculatures. At P14, the vessel development in the retina is widely completed, implying that Ophn1 has either a function during adulthood or for the generation of the intermediate plexus during the late vessel development of the retina.

During development of vertebrates, the retina outgrowths from the evolving brain to form a multi-layered structure composed of five neuronal cell types (ganglion cells, amacrine cells, horizontal cells, bipolar cells, cone and rod photoreceptors), Müller glia cells and retinal pigment epithelial cells (Byerly and Blackshaw, 2009). After birth, the retinal vasculature develops as it interpenetrates in three plexuses the inner portion of the retinal cell layers to support the retinal cells with oxygen and nutrition (Gariano, 2003).

Ophn1 expression and its function were extensively studied in brain. However, the relevance of Ophn1 in retina has not been examined yet. The *Ophn1* gene encodes a 92 kDa protein involved in the pathogenesis of X-linked mental retardation (MRX), a genetic disorder characterized by cognitive impairments. Over the past decade much interest has been focused on the molecular mechanisms responsible for MRX. In a long series of X-chromosome linked genes, *Ophn1* was the first that was found to be mutated in investigated MRX families (Annunziata et al., 2003; Billuart et al., 2000; Chabrol et al., 2005; Chelly and Mandel, 2001; Kohn et al., 2004; Menten et al., 2007; Tentler et al., 1999). The RhoGAP domain of Ophn1 is involved in the organization of the actin cytoskeleton by inhibition of Rho pathways through control of RhoGTPases. Thereby *Ophn1* regulates dendritic spine morphology associated with cognitive mechanisms such as neuronal plasticity, learning and memory (Fauchereau et al., 2003; Govek et al., 2004; Khelfaoui et al., 2007). Thus, impaired function of *Ophn1* has been regarded as a cause for the phenotype of individuals suffering from MRX (Billuart et al., 1998b). The MRX phenotype in humans is consistent with *Ophn1-null* mice. These mice exhibit dendritic spine immaturity leading to MRX-related defects in memory and social behaviour (Khelfaoui et al., 2009).

Extensive studies in brain tissue have determined *Ophn1* expression in different species including human, mouse, rat, chicken and guinea pigs (Billuart et al., 1998a; Fauchereau et al., 2003;

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Govek et al., 2004; Khelfaoui et al., 2007; Kohn et al., 2007, 2004; Ljubimova et al., 2001; Pinheiro et al., 2001; Wang et al., 2000; Xiao et al., 2003, 2004). Moreover, expression of *Ophn1* has also been reported in peripheral neurons such as the sciatic nerve and the enteric nervous system (Wang et al., 2000). Many other tissues were also found to be positive for *Ophn1* expression, i.e. murine testis, heart and liver (Khelfaoui et al., 2007; Kohn et al., 2007, 2004); human placenta, pancreas and kidney (Billuart et al., 1998a) and rat derived blood vessels, heart, lung, liver, skeletal muscle, kidney and testis (Govek et al., 2004). Expression has also been detected in colorectal cancer tissue (Pinheiro et al., 2001; Wang et al., 2000) and glioblastomas (Ljubimova et al., 2001). These observations raise the hypothesis that the function of Ophn1 is not restricted to a specific region and eventually not only to neuronal cell types.

In the present study we used experimental approaches to identify the expression of *Ophn1* on both mRNA and protein levels. The immunohistochemical analysis revealed an exclusive localization of the protein in the retinal vasculature with an onset of expression at P14. In contrast to our initial assumptions deduced from other studies that described expression in central and enteric nervous system, immunoreactivity was absent from neuronal cell types in the retina.

1. Materials and methods

1.1. Tissue preparation

Experiments were performed with C57Bl/6J mice in accordance with the ARVO statement for the use of animals in ophthalmic and vision research. Eyes and cerebrum were explanted from sacrificed animals. For retina isolation, adult eyes were enucleated, hemisected at the pars plan, the lens removed and the retina peeled away from the retinal pigment epithelium (RPE). For RNA extraction retina and cerebrum were submerged with the RNAlater RNA stabilization reagent (Qiagen, Hilden, and Germany) and stored at −20 °C. For protein extraction retinal and cerebral tissues were snap-frozen in liquid nitrogen and kept at −80 °C until use. For immunohistochemistry, the entire eyes were perfused with 4% formaldehyde fixation buffer (A + E. Fischer, Wiesbaden Germany) and stored in this fixative.

1.2. RT-PCR and sequencing

Total RNA from retina and cerebrum was extracted with RNeasy Micro kit (Qiagen). Genomic DNA was digested with 1 U DNaseI per μg total RNA (Fermentas, St. Leon-Rot, Germany) at 37 °C for 30 min. Subsequently, the reaction was inactivated in 2.5 mM EDTA (Fermetas) for 10 min at 65 °C. The first strand cDNA was synthesized at 65 °C for 5 min with 10 nmol of each dNTP, 50 pmol oligo$(dT)_{20}$-primer (both from Invitrogen, Karlsruhe, Germany) and 1 μg total RNA in a total volume of 13 μl and chilled on ice for 1 min. The first strand buffer (1 μmol Tris–HCl (pH 8.3), 1.5 μmol KCl, 60 nmol $MgCl_2$, 0.1 μmol DTT, 200 U of the SuperScript III RT (all reagents from Invitogen) and 40 U RiboLock RNase Inhibitor (Fermentas) were given to the mixture and incubated for 60 min at 50 °C in a total volume of 20 μl. The reaction was stopped by heating 15 min at 70 °C. In negative controls, the addition of the enzyme SuperScript III RT was omitted. For PCR the primer pair of Ophn1 (Gene Bank ID: NM 052976; forward: 5′-ACT CGT CTC TGC TGC CAA AT-3′ (from exon 17 (ENSMUSE00000207337) and exon 18 (ENSMUSE00000207348)), reverse: 5′-CAG CCA CAG TGT CCT CTT GA-3′ (from exon 19 (ENSMUSE00000292444)) generated a 221 bp fragment of three adjacent exons. The PCR was carried out in 25 μl reaction using *Taq* polymerase within *Taq* buffer (Invitrogen) in 45 cycles at 94 °C for 45 s, 53 °C for 45 s, 72 °C for 45 s and a final extension at 72 °C for 5 min. The β-actin (actin) mRNA was amplified as a positive control (Gene Bank ID: NM 007393; forward: 5′-CCC TGA AGT ACC CCA TTG AA-3′, exon 3 (ENSMUSE00000510722) reverse:5′-GGG GTG TTG AAG GTC TCA AA-3′, exon 4 (ENSMUSE00000517504)). In negative controls, cDNA was replaced by ddH_20 (double distilled water). Results from RT-PCR were verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany) with the dideoxy chain-termination sequencing technique (Sanger et al., 1977) and the internet-based BLASTn program for sequence correspondence.

1.3. Western blot analysis

Retinal and cerebral tissues were grinded (Ultra Turrax T25 basic, IKA Werke, Staufen, Germany) in 200 μl RIPA lysis buffer (Cao et al., 2005) with 218 mM PMSF and protease inhibitor cocktail (Complete, Roche, Grenzach, Germany). Of each tissue protein extract, 40 μg were resolved in 10% SDS gel. Proteins and molecular weight marker (PageRuler™ Prestained Protein Ladder, Fermentas, Germany) were transferred to nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany) and blocked in 5% filtered non-fat dry milk (Roth, Karlsruhe, Germany) in PBST (137 mM NaCl, 1.47 mM KH_2PO_4, 7.81 mM Na_2HPO_4, 2.68 mM KCl, 0.1% Tween 20) at 4 °C for 40 min under agitation. The blot was incubated with 0.5 μg/ml rabbit anti-mouse Ophn1 antibody (1:400; 11076-1-AP, Lot #1, PTG, Manchester, UK) in 0.25% filtered non-fat dry milk in PBST overnight and the membrane was incubated with 0.4 μg/ml HRP-conjugated secondary goat anti-rabbit IgG antibody (sc-2301, Lot #E1909, Santa Cruz Biotechnology, Santa Cruz, USA) in PBST for 1 h at 4 °C. Immunoreactivity was detected using detection reagent (TRIS/HCl 100 mM pH 8.5, 2.5 mM luminal, 0.4 mM p-coumaric acid, 2.5 mM hydrogenperoxide) in a Fuji LAS 3000 imaging system (Raytest, Germany).

1.4. Immunohistochemical analysis

The fixative was removed from the tissues in running tap water overnight and samples were dehydrated in graded 2-propanol solutions (1 h each: 30%, 50%, 60%, and 70%, 2x Abs.). Subsequently, the tissues were cleared in a preheated 1:1 solution of 2-propanol/paraffin at 65 °C overnight (Memmert, Schwabach, Germany). Afterwards, the tissue was infiltrated with three changes of paraffin for 90 min intervals at 65 °C and embedded in paraffin. Sections were cut at 2–5 μm (pfm-2002, Cologne, Germany), deparaffinized in 2 × 10 min changes of Roti-Histol (Roth, Karlsruhe, Gemany) and rehydrated in a graded series of ethanol (5 min each: Abs., 96%, 70%, 50%, ddH_2O). Heat-induced epitope recovery was carried out in citrate buffer (pH 6, 1.8 mM citric acid, 8.2 mM sodium citrate) in a microwave at 600 W. Endogenous peroxidase activity was blocked in 3% H_2O_2 for 20 min. Endogenous avidin/biotin activity was blocked according to the instruction of the manufacturer from Avidin/Biotin blocking kit (Vector laboratories, Biozol, Eching, Germany). Then, unspecific antibody binding was blocked for 30 min in TBS (pH 7.4, 10 mM Tris, 136 mM NaCl) supplemented with 5% non immune serum (Vectastain ABC elite kit, Biozol, Eching, Germany). Primary anti-Ophn1 antibody (11076-1-AP, Lot #1, PTG, Manchester, UK), diluted 1:200 in TBS, was given to the slides and at 4 °C overnight in a humid chamber. Sections were incubated at room temperature with the biotinylated secondary antibody (Vectastain ABC elite kit) diluted 1:200 in TBST and 2% non immune serum (Vectastain ABC elite kit) for 30 min. Immunoreactivity was visualized with the DAB chromogen (DAB kit, Vector laboratories) according to the manufacturer's recommendations. Sections were counterstained with Mayer's hemalaum solution (Roth). Tissue slides were mounted with an aqueous mounting