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Expression and localization of the Parkin Co-Regulated Gene in mouse CNS suggests a role in ependymal cilia function

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ABSTRACT

Parkin Co-Regulated Gene (*PACRG*) is a gene that shares a bi-directional promoter with the Parkinson's disease associated gene *parkin*. The functional role of PACRG is not well understood, although the gene has been associated with parkinsonian syndromes and more recently with eukaryotic cilia and flagella. We investigated the expression of *Pacrg* in the mouse brain by *in situ hybridization* and observed robust expression of *Pacrg* in the cells associated with the lateral, third and fourth ventricle, in addition to the aqueduct of Sylvius and choroid plexus. For all regions of *Pacrg* expression identified, strong expression was observed in the newborn period and this was maintained into adulthood. Immunohistochemical analysis showed that Pacrg was a component of the ependymal cells and cilia lining the ventricles. Based on our results and the previous association of PACRG homologues with cilia and flagella, we propose that Pacrg is a component of the ependymal cilia and may play an important role in motile cilia development and/or function in the CNS.

The Parkin Co-Regulated Gene (*PACRG*) encodes a predicted protein of 257 amino acids that displays considerable evolutionary conservation among metazoan and protozoan species. *PACRG* is physically and functionally linked to the Parkinson's disease (PD) associated gene *parkin*. In addition to being oriented in a head-to-head arrangement on opposite DNA strands, the two genes share a common bi-directional promoter [21]. Pacrg and parkin have been shown to display a similar cellular distribution and the two proteins form a complex *in vivo*. These observations have suggested a role for PACRG in parkinsonian disorders and indeed there is evidence that PACRG is associated with PD and is a component of Lewy bodies [1,7,19,20].

However, the physiological function of PACRG remains to be fully established. Recent studies have suggested a role for PACRG orthologues in the structure and/or function of the flagella in eukaryotic and prokaryotic organisms. In the mouse, loss of Pacrg results in defects in the axonemal microtubule array of the flagellum and impaired spermiogenesis [11]. Similarly, PACRG homologues are necessary for the correct development and function of the flagellar axoneme in diverse organisms including the green alga and ciliated protozoa [6,9].

There is evidence that PACRG may play a role in microtubule dynamics and this could explain its potential role in flagella function. PACRG has been shown to interact with tubulins *in vitro* [5] and may function *in vivo* to stabilize axonemal outer microtubule doublets [3]. Similarly, PACRG homologues have been identified as a conserved constituent of the centriole/basal body [8]. The centriole is a component of the centrosome and functions to nucleate and organize microtubules within the cell, in addition to nucleating the mitotic spindle and cilium (reviewed in [2]).

To further investigate the potential neurological role of PACRG, we analysed the spatial and temporal distribution of *Pacrg* in mouse brain and demonstrated that Pacrg was a component of the ependymal cells and cilia of the ventricular system.

Wildtype c57/Bl6J mice were housed in a specific-pathogen-free environment under a 12 h light/dark cycle with free access to food and water. The study and all procedures were conducted in accordance with National Research Council Guidelines and ethical approval was obtained from the Murdoch Childrens Research Institute Animal Ethics Committee.

Mice aged 1, 3 and 12 weeks ($n=2$ male and $n=2$ female for each age group) were culled, brains were removed and fixed in 4% (w/v) paraformaldehyde in PBS overnight at 4 °C. After overnight cryoprotection in 20% (w/v) sucrose at 4 °C, brains were snap frozen in iso-pentane over dry-ice. Serial sections of 10 μm

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thickness were cut and collected on superfrost plus slides (Menzel-Glaser).

The cDNA clone encoding the entire open reading frame of mouse *Pacrg* (AK005771) was amplified by PCR and cloned into PCRII TOPO (Invitrogen, K4600-01) according to the manufacturer's instructions. The integrity of the insert was confirmed by sequence analysis and sense and antisense riboprobes were generated utilizing the DIG RNA labelling kit (Roche, 1175025). The riboprobes were purified using ChromaSpin 100 DEPC-H_2O columns (BD biosciences, 636090) and the integrity and abundance was determined by agarose gel electrophoresis.

Sense and antisense riboprobes were diluted 1:10 in hybridization buffer [1× salt solution (2 M NaCl, 100 mM Tris, 50 mM $NaH_2PO_4{\cdot}2H_2O$, 0.5 mM Na_2HPO_4, 50 mM EDTA), 50% formamide, 50× Denhardts, 10% dextran sulphate, 1 mg/ml yeast tRNA] and hybridized to sections overnight at 65 °C in a sealed chamber. Slides were washed at 65 °C in 50% 1× SSC, 50% formamide, followed by room temperature washes in Maleic acid buffer (MABT) pH7.5 (100 mM maleic acid, 150 mM NaCl, 0.1% Tween-20). Sections were blocked for 2 h in blocking buffer [MABT containing 20% heat inactivated sheep serum and 2% Blocking Reagent (Roche, 1096176)]. Riboprobe binding was detected by incubation with an alkaline phosphatase conjugated anti-DIG antibody (Roche, 1:2000, 1093274). Sections were sequentially washed in MABT and alkaline phosphatase staining buffer (100 mM NaCl, 50 mM MgCl, 100 mM Tris pH 9.5, 0.1% Tween-20). Staining was visualized after an overnight incubation in NBT/BCIP prepared according to manufacturer's instructions (Roche, 1383213/1383221). Slides were washed in PBS, mounted with Aquamount (Merck) and viewed with a LeicaDM IRB microscope. Images were captured with a Leica DFC 480 camera and examined using Adobe Photoshop.

The presence and intensity of *Pacrg* staining in specific regions of the brain was determined with reference to anatomical landmarks [12]. Semi-quantitative expression analysis was conducted, with the signal intensity in the specific region of interest being designated as no signal (−), weak (+), moderate (++) or strong (+++).

Postnatal c57/Bl6J mice aged 0, 2, 4, 7 and 21 days ($n = 3$ for each age group) were culled by cervical dislocation and brains were immediately collected and snap frozen in iso-pentane over dry-ice. 10 μm cryostat sections were cut in the coronal plane, collected onto superfrost plus slides and fixed in 4% PFA/PBS for 5 min. After washing in PBS, sections were processed for immunohistochemical analysis utilizing the Vector MOM Immunodetection Kit according to manufacturer's instructions (Vector Laboratories, BMK 2202). Sections were blocked for 1 h in Mouse Ig Blocking reagent and incubated with primary antibodies for 30 min at room temperature. After washing in PBS, binding of the primary antibodies was visualized utilizing goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 (1:1000, Molecular Probes, Invitrogen, A11034 and A11032). Sections were washed in PBS and mounted with Vectorshield medium containing DAPI (Vector Laboratories). Sections were viewed with a Zeiss Axio Imager M1 microscope and captured using Axiovision Release 4.7 software. Primary antibodies used were polyclonal anti-$PACRG_{66-257}$ antibody (1:100, MC1291, epitope and production previously described [10]), monoclonal anti-acetylated tubulin (1:500, Sigma, T6793), and monoclonal anti-s100β (1:100, Sigma, S2532). The specificity of MC1291 was validated by an antigen absorption test. One microgram of affinity purified antibody was diluted 1:300 and pre-incubated with 20 μg of the immunizing protein (approximately 100× excess) for 2 h at room temperature prior to application to the tissue sections as above.

To investigate the functional role of PACRG in the mammalian brain, we determined the expression pattern of *Pacrg* in the mouse CNS utilizing *in situ hybridization* (ISH). For all sections analysed, the specificity of the *Pacrg* riboprobe staining was confirmed by comparison to serial sections stained with the sense control probe. No gender differences were observed; therefore images generated from 1 week male are presented. Low to moderate expression of *Pacrg* was evident throughout the cerebral cortex, hippocampus and the cerebellum. In addition, expression was also observed in distinct CNS nuclei (Table 1, Supplementary figures). To assess the developmental expression of *Pacrg*, we performed ISH on mice aged 1, 3 and 12 weeks old. The regional distribution of *Pacrg* expression was maintained at all ages examined and was generally consistent with our previous investigation of the distribution of the protein in mouse brain [1].

Table 1
Regional and temporal analysis of *Pacrg* expression in the mouse brain.

Region	1 week	3 week	3 months
Hippocampus	+++	+	+
Oculomotor nucleus (3N)	+++++	+++	++
Motor trigeminal nucleus	++++	++++	+++
Cerebellum	++	+	+
Doral motor nucleus of vagus (10N)	++++	++	++
Hypoglossal nucleus (12N)	++++	++	+++
Facial nucleus (7N)	++++	+++	+++
Medial habenular nucleus (Mhb)	++	+++	++
Cerebral cortex	++	+	+
Substantia nigra	–	–	–
Striatum	–	–	–
Ventricular system			
Lateral ventricle	++++	+++	+++
Third ventricle	+++	+++	++
Dorsal third ventricle	+++	+++	++
Aqueduct	+++++	++++	+
Fourth ventricle	+++++	+++	+++
Choroid plexus	+++	++++	++++

Symbols represent semi-quantitative evaluation of the intensity of labelling in the indicated regions and correspond to no signal (−), weak (+), moderate (++), strong (+++) and very strong *Pacrg* expression (++++).

In addition to the regions described above, we also observed expression of *Pacrg* in association with the ventricular system. Robust staining was identified in the cells lining the lateral ventricle, third and fourth ventricles and the aqueduct of Sylvius. Furthermore, *Pacrg* expression was observed in the choroid plexus within the lateral and fourth ventricles (Fig. 1A–D). As noted for other regions, this staining pattern was maintained at all ages examined (Table 1).

This pattern of transcript expression appeared to be associated with the cell body of the multiciliated ependymal cells that line the cerebral ventricles. Transcript expression was not evident within the ependymal cilia. Protein components of the cilia are generally translated in the ependymal cell body and undergo intraflagellar transport (IFT) during cilial assembly. To test the possibility that Pacrg may be a component of ependymal cilia, we performed co-localization studies with antibodies directed against the cilial axoneme. Analysis of the ventricular system in 1 week old mice revealed intense Pacrg staining associated with cells lining the lateral ventricle, third and fourth ventricles and the aqueduct of Sylvius (Fig. 2A–D). Co-labelling with acetylated α-tubulin, a major structural component of the ciliary axoneme [13], suggested that the Pacrg immunoreactivity was primarily associated with the tufted structures corresponding to cilia of the ependymal cells (Fig. 2A–L). A similar localization also was observed using an independent polyclonal anti-Pacrg antibody ([MC1290, [10]], data not shown). This pattern of Pacrg immunoreactivity was absent when the sections were stained with anti-Pacrg antibody pre-absorbed with an excess of the immunizing protein or pre-immune sera (Supplementary figure 3).

To investigate a possible role for Pacrg in ependymal ciliogenesis we determined Pacrg localization in the lateral ventricle during cilia development (P0–P7). Few ciliary axonemes were detected with