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Developmental molecular and functional cerebellar alterations induced by PCP4/PEP19 overexpression: Implications for Down syndrome

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ABSTRACT

PCP4/PEP19 is a modulator of Ca^{2+}-CaM signaling. In the brain, it is expressed in a very specific pattern in postmitotic neurons. In particular, Pcp4 is highly expressed in the Purkinje cell, the sole output neuron of the cerebellum. *PCP4*, located on human chromosome 21, is present in three copies in individuals with Down syndrome (DS). In a previous study using a transgenic mouse model (TgPCP4) to evaluate the consequences of 3 copies of this gene, we found that PCP4 overexpression induces precocious neuronal differentiation during mouse embryogenesis. Here, we report combined analyses of the cerebellum at postnatal stages (P14 and adult) in which we identified age-related molecular, electrophysiological, and behavioral alterations in the TgPCP4 mouse. While Pcp4 overexpression at P14 induces an earlier neuronal maturation, at adult stage it induces increase in cerebellar CaMK2alpha and in cerebellar LTD, as well as learning impairments. We therefore propose that PCP4 contributes significantly to the development of Down syndrome phenotypes through molecular and functional changes.

Introduction

PCP4 (Purkinje cell protein 4), a regulator of calcium signaling, is expressed from human chromosome 21 (HSA21); its murine ortholog, *Pcp4*, is located on mouse chromosome 16 (MMU16) (Cabin et al., 1996). The encoded amino-acid sequence is highly conserved between humans and mice (Mouton-Liger et al., 2011), with 100% identity between critical functional residues (Putkey et al., 2008). PCP4, also known as PEP19, modulates calmodulin activity by activating the interaction of calcium with calmodulin *via* an acidic-IQ motif (Putkey et al., 2003, 2008). It thereby regulates the activation of Ca^{2+}-CaM targets, in particular CaMKII (Johanson et al., 2000; Slemmon et al., 1996). CaMKIIs are multifunctional protein kinases encoded by four genes and are involved in neuronal differentiation and plasticity (Hansel et al., 2006; Hudmon and Schulman, 2002). Further, Erhardt et al. (2000) suggest that PCP4 protects cells against induced apoptosis. The biological importance of this small regulator of CaM signaling is linked to the high number of targets of Ca^{2+}-CaM which belong to various protein classes: namely, enzymes, nuclear proteins, cytoskeleton, exo-endocytosis, transmitter release and cell death (Berggård et al., 2006; Shen et al., 2005) and may depend on calcium and CaM levels in the cell compartments (Chin and Means, 2000; Putkey et al., 2003, 2008; Wang et al., 2013).

PCP4 exhibits a specific and conserved expression pattern in neurons (Arlotta et al., 2005; Ichikawa and Sugimoto, 2005; Ichikawa et al., 1999; Lein et al., 2005; Molyneaux et al., 2007; Mouton-Liger et al., 2011; Mugnaini et al., 1987; Murray et al., 2007; Thomas et al., 2005; Utal et al., 1998; Watakabe et al., 2012; Ziai et al., 1986). Expression of *Pcp4* initiates early in development in the ectoderm and neuroectoderm, including in cells derived from the neural crest, and is strongly associated with the postmitotic state *in vivo* (Bulfone et al., 2004; Mouton-Liger et al., 2011; Reymond et al., 2002; Thomas et al., 2003) as well as *in vitro* (Przyborski et al., 2003) suggesting it

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might be critical at development stages. However, the transcriptional regulators driving the expression pattern of *PCP4* remain unknown.

Dysregulation of Pcp4 has been observed in different brain disorders: Huntington disease and its related mouse model (Luthi-Carter et al., 2002; Utal et al., 1998), Alzheimer disease (Slemmon et al., 1994) and major depressive disorder (Teyssier et al., 2011), suggesting that precise regulation of its expression levels is associated to normal brain function. Moreover, altered cerebellar synaptic plasticity is observed in the absence of *Pcp4* (Wei et al., 2011) confirming that PCP4 is important at least for normal cerebellum function. Overexpression resulting from the presence of three copies of *Pcp4* has been clearly demonstrated in different parts of the brain of two mouse models of Down syndrome, called Ts1Cje and Ts65Dn, at RNA level during postnatal development (Amano et al., 2004; Kahlem et al., 2004; Laffaire et al., 2009; Lyle et al., 2004), as well as at RNA and protein levels during embryogenesis (Mouton-Liger et al., 2011). These findings therefore implicate PCP4 as a good candidate for a role in Down syndrome brain phenotypes. Currently, one mouse model has been developed for *PCP4* overexpression (without overexpression of other DS-related genes) to study the contribution of this gene to DS phenotypes. Importantly, this model, the TgPCP4 mouse (transgenic for 1 copy of the human gene), recapitulates the precocious neuronal differentiation that occurs during embryogenesis in the DS model Ts1Cje, which is also trisomic for 80 other chromosome 21 orthologs (Mouton-Liger et al., 2011). But it cannot be excluded that, in other mechanisms in DS, PCP4 contribution may be modified through its interactions with other genes in three copies.

Pcp4 expression level is high in the GABAergic Purkinje cell, the sole output neuron of the cerebellum (Slemmon et al., 1996), suggesting that PCP4 participates to specific functional properties in calcium-dependent signaling (Fierro and Llano, 1996; Simons et al., 2009; Wei et al., 2011). In mice, expression in Purkinje cells is detectable as early as embryonic day (E) 14.5 in the cerebellum primordium, showing that *Pcp4* is a very early gene in the specification of these neurons (Mouton-Liger et al., 2011; Thomas et al., 2003). From its initial detection at E14.5, the level of cerebellar *Pcp4* expression gradually increases up to the adult stage (Sangameswaran et al., 1989; Ziai et al., 1986). We hypothesised that, given these expression data, PCP4 overexpression has important consequences for the molecular and functional development of Purkinje cells and may contribute to motor skill impairment as observed in Down syndrome.

Here, we performed a detailed analysis of the cerebellum of TgPCP4 mice at two postnatal stages: P14, a stage at which the Purkinje cell layer is formed and most molecular interneurons have already migrated; and adult stage, at which outcomes of neuronal differentiation, maturation, and synaptic connection formation can be identified at the molecular and functional (electrophysiological and behavioral) levels. We provide evidence that *Pcp4* overexpression contributes to molecular and functional alterations that may play a significant role in Down syndrome phenotypes.

Material and methods

Animals

Mice were housed in standard cages with access to food and water *ad libitum*, under a controlled environment (temperature = 20 ± 1 °C; humidity = 60%), and with a light/dark cycle of 12 h. The transgenic TgPCP4 (Mouton-Liger et al., 2011) was maintained on a C57Bl/6J background. All experiments reported were performed after the N9 generation of backcross on this genetic background. Animals (transgenic and their control littermates) were handled and experimental procedures carried out at the Institut Jacques Monod—Université Paris Diderot-Paris7 animal facility, according to the guidelines of the French Ministry of Agriculture for experiments with laboratory animals (law 87848) and EU directive 86/609. Official authorization from the French Ministry of Agriculture was granted to carry out research and experiments on animals (accreditation 75-367) and this study was approved by the local ethical committee (University Paris-Diderot). All manipulations of animals were conducted carefully in order to reduce stress at the minimum. For behavior testing on adults, two cohorts of males were tested. The first cohort was analysed at a phenotypic company (KeyObs, Orleans, France) using the SHIRPA protocol (13 Wt and 15 TgPCP4 from same litters, 4–5 months of age) to evaluate general health, activity and anxiety (supplementary methods). The tests were conducted according to established protocols approved by the committee on animal ethics and regulations (CNREEA-27). The second cohort (11 wild-type and 12 adult TgPCP4 male mice from same litters, 6–8 months of age) was used for behavioral testing specially focused on motor learning and memory at the CRG (Center for Genomic Regulation). All experimental protocols were performed under authorization (CEEA-PRBB; MDS-08-1060P1 and JMC-07-1001P1-MDS), and met the guidelines of the local (law 32/2007) and European regulations (EU directive 86/609, EU decree 2001-486) and Statement of Compliance with Standards for Use of Laboratory Animals by Foreign Institutions nº A5388-01, National Institutes of Health (USA). All investigators involved in animal experimentations have the appropriate training (Department de Medi Ambient i Habitatge, 214/1997). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14). All the behavioral testing was conducted in an isolated room by the same experimenter at the same time of the day (between 8:30 a.m. and 3:30 p.m.). The experimenter was blinded as to the genetic status of the animals.

Molecular analysis

Tissue collections

Adult mice were euthanized using isoflurane followed by perfusion with PFA for staining procedures. For molecular studies, P14 and adult mice were euthanized by decapitation followed by brain dissection (cerebellum and cerebrum) and freezing on liquid nitrogen. Both procedures were approved by the local Ethical Committees (CEEA PRBB; University Paris Diderot), and met the guidelines of the local (Catalan law 5/1995 and Decrees 214/97, 32/2007; French Ministry of Agriculture, law 87848) and the European regulations (EU directives 86/609 and 2001-486). For immunohistochemical analyses, adult and P14 brains were placed further into paraformaldehyde 4% in PBS at +4 °C (48 h) then transferred to 30% sucrose-PBS for cryopreservation.

DNA and RNA preparation

Transmission of the *PCP4* transgene in the progeny was followed by amplification with previously described 5P10 and INTP16 primers, which are highly specific for the human gene (Mouton-Liger et al., 2011). DNA was prepared from lysate obtained by overnight incubation of tail tissue with 25 mM Tris (pH 8.0), 75 mM NaCl, 25 mM EDTA (pH 8.0) and 1% sodium dodecyl sulfate (SDS) supplemented with 650 ng/μL proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) at 42 °C. Gene quantification was carried out with a 1/10 dilution of the DNA solution.

Total RNA from P14 cerebellum was prepared with the NucleoSpin RNAII kit (Macherey-Nagel Hauptsitz, Germany), which includes DNaseI treatment. Quantity and purity of RNA were assessed by measuring optical absorbance at 260 and 280 nm. RT-PCR was performed with PrimeScript™ Reverse Transcriptase (Takara Bio, Shiga, Japan), using 2 μg of total RNA and random primer 9 (New England Biolabs, Ipswich, MA, USA). cDNA for mouse and human transcripts were amplified with species-specific primers (mouse, SPM-S and -R and human, SPH-S and -R) to validate the expression of the transgene in TgPCP4 while quantification was performed with the set of primers MH3PCP4 which amplified both cDNAs (Mouton-Liger et al., 2011). PCR was performed in a Hybaid thermocycler (30 cycles) with 0.01 U/μL Dynazyme II (Finnzyme, Thermo Scientific, France). Primer sequences are listed in Table 1.