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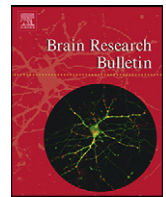

Research report

The presenilin-1 familial Alzheimer's disease mutation *P117L* decreases neuronal differentiation of embryonic murine neural progenitor cells

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ABSTRACT

The *presenilin-1* gene is mutated in early-onset familial Alzheimer's disease. The mutation *Pro117Leu* is implicated in a very severe form of the disease, with an onset of less than 30 years. The consequences of this mutation on neurogenesis in the hippocampus of adult transgenic mice have already been studied *in situ*. The survival of neural progenitor cells was impaired resulting in decreased neurogenesis in the dentate gyrus. Our intention was to verify if similar alterations could occur *in vitro* in progenitor cells from the murine ganglionic eminences isolated from embryos of this same transgenic mouse model. These cells were grown in culture as neurospheres and after differentiation the percentage of neurons generated as well as their morphology were analysed. The mutation results in a significant decrease in neurogenesis compared to the wild type mice and the neurons grow longer and more ramified neurites. A shift of differentiation towards gliogenesis was observed that could explain decreased neurogenesis despite increased proliferation of neural precursors in transgenic neurospheres. A diminished survival of the newly generated mutant neurons is also proposed. Our data raise the possibility that these alterations in embryonic development might contribute to increase the severity of the Alzheimer's disease phenotype later in adulthood.

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1. Introduction

Mutations in the *presenilin-1* gene are the most commonly recognized cause of early-onset familial Alzheimer's disease (FAD). Presenilin-1 (PS-1) is an approximately 48 kDa transmembrane protein that is activated when cleaved into two sets of heterogeneous fragments, one corresponding to the N-terminal (about 30 kDa) and the other to the C-terminal part (about 18 kDa) [24]. PS-1 participates in a protein complex [23], the secretasome, which works as a γ-secretase, a protease cleaving the precursor protein of the β-amyloid peptide or APP. It leads, subsequently to the action of a β-secretase, to the release of β-amyloid peptides. The 42–43 amino acid form of the β-amyloid peptide is generated preferentially resulting in oligomeric and fibrillary compounds that are particularly toxic and proposed to be key players in the etiopathogenesis of Alzheimer's disease according to the β-amyloid cascade hypothesis [11]. Presenilin-1 mutations also result in an increased sensitivity of neurons to various damages [10].

Some mutations in the *PS-1* gene such as the *Pro117Leu* (*P117L*) missense mutation cause very-early-onset Alzheimer's disease, i.e. usually before the age of 30 years [14,27], suggesting that they might contribute to deleterious effects already during development. This hypothesis is strengthened by the fact that PS-1 is expressed widely during development including in neural progenitor cells of the primitive subventricular zone [18] and that targeted *PS-1* null mutant mice die shortly after birth [21]. Furthermore, PS-1 is physically and functionally associated with several intercellular signaling pathways involving, among others, Notch, GSK-3β and cadherins, all proteins known for their implications in the development of the nervous system [1,3,8]. However, *PS-1* mutations might differ in their consequences. Indeed, *PS-1* FAD mutations can rescue the embryonic lethality of *PS-1* knock out mice suggesting that their

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effects on development might be rather subtle [7]. The effect of the FAD *PS-1 P117L* mutation has been studied in neuron-specific enolase (NSE) driven *PS-1* transgenic mice. Levels of the 42 amino acid form of the β-amyloid peptide were significantly increased in the *PS-1 P117L* transgenic compared to the wild type mice [25]. Moreover, in these studies the survival of neural progenitor cells was impaired in the dentate gyrus of adult mice by the FAD mutation leading to a decrease in neurogenesis, whereas overexpression of wild type PS-1 had no effect or was found to promote neurogenesis in the same region [25,26]. Interestingly PS-1 was also suggested to have survival promoting properties in neurodegenerative disorders [9].

Thus, given the widely distributed expression and the importance of PS-1 in neurogenesis, we wondered whether the *P117L* mutation might also affect or not prenatal development of the forebrain and not just the dentate gyrus. As a first approach, we decided to study the differentiation of neural progenitor cells (NPCs) isolated from the E14 murine striata. Here we show that the FAD *PS-1 P117L* mutation impairs neurogenesis of NPCs *in vitro* but promotes neuritic outgrowth in newly generated neurons. Thus, this study supports a broader role for the *PS-1 P117L* mutation beyond its effects limited to the metabolism of the APP in adulthood and might contribute to explaining the severity of the disease phenotype.

2. Materials and methods

2.1. Culture and differentiation of neural stem cells

B6D2 wild type mice and *PS-1 P117L* transgenic mice [26] were used to isolate striatal neural progenitor cells (NPCs) from embryos (E14–15). Animal manipulations were carried out in accordance with Federal Swiss Veterinary regulations and with institutional approval. The NPCs were cultured following the protocol of Reynolds and Weiss [19,20]. After animal decapitation, striata, subventricular zone included, were dissected out under sterile conditions. Cells were mechanically dissociated and then placed in basic stem cell medium: 1 to 1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient (Sigma, no. D8062, Buchs, Switzerland) including 0.6% glucose, 2 mM glutamine, 3 mM sodium bicarbonate, 25 μg/ml insulin (Sigma), 100 μg/ml apotransferrin (Sigma), 20 nM progesterone (Sigma), 60 μM putrescine (Sigma) and 30 nM sodium selenite (Sigma). To this medium, Epidermal Growth Factor (EGF; Sigma), basic Fibroblast Growth Factor (bFGF; Invitrogen, Carlsbad, USA) and heparin (Sigma) were added at 20 ng/ml, 10 ng/ml and 2.5 μg/ml final concentrations, respectively. After 4–6 days in culture, floating clusters of cells or neurospheres formed. The neurospheres were either used to study differentiation or NPCs proliferation. In the last case neurospheres were mechanically dissociated and cells were grown at clonal dilution [20] in 4 ml growth medium; after 7 days of proliferation the number of newly generated neurospheres and their average diameter were analysed in 100 μl neurosphere suspensions.

For cell differentiation, the procedure applied was derived from Wu et al. [29] and consisted in a first step of "preconditioning" in which dissociated cells were allowed to proliferate for 6 days as adherent cells on poly-L-ornithine (15 μg/ml) and laminin (2 μg/ml; Sigma) treated glass coverslips in basic stem cell medium containing bFGF (20 ng/ml) and laminin (1 μg/ml), followed by a differentiation step in basic stem cell medium containing no growth factors but B27 supplement (2% final concentration; Invitrogen) for 8 days. According to Wu et al. [29], this procedure should yield a higher proportion of newly generated neurons. Differentiated cells were immunocytochemically analysed.

2.2. Genotyping, sequencing and RT-PCR

Tail-extracted blood samples or NPCs from B6D2 wild type and *PS-1 P117L* hemizygous transgenic mice were genotyped by polymerase chain reaction (PCR) according to the protocol of Wen et al. [26]. The presence of mutation *PS-1 P117L* was verified by sequencing (Automated DNA sequencing, Applied Biosystems EBI 3100 DNA sequencer, Rotkreuz, Switzerland) a DNA fragment amplified with primers corresponding to the neuron-specific enolase (NSE) intron 1 (5′-GCTCCACCCTTCTAAGCCTC-3′; [26]) and to a PS-1 DNA sequence 5′-GGCATGGATGACCTTATAGCACCT-3′.

Transcription of the NSE driven transgene was monitored by amplifying synthesized cDNAs (First-strand cDNA synthesis; Amersham/GE Healthcare, Otelfingen, Switzerland) from DNAse I treated RNA extracted from 10^6 proliferating NPCs (TRIzol reagent, Invitrogen) using human *PS-1* primers 5′-ATGACAGAGTTACCTGCACCG-3′ and 5′-ACAGAACCACCAGGAGGATA-3′ (L76517).

2.3. Immunoprecipitation

Five millions dissociated progenitor cells were collected from cultured wild type or *PS-1 P117L* neurospheres by centrifugation (5 min, 1000 rpm, 4 °C), lysed for 30 min on ice in 500 μl NP40 buffer (20 mM Tris, 150 mM NaCl, 0.5% Na deoxycholate and 0.5% NP40, pH 8.0) in the presence of 1 μl of a protease inhibitor cocktail (10 μl/ml, Sigma). They were sonicated (1 × 10 s; Bandelin Sonopuls HD200, Bandelin, Germany) and centrifuged 10 min at 10,000 rpm to remove unsolubilized material. Ten microliters of rabbit polyclonal anti-PS-1 IgG (Santa Cruz, H70; Santa Cruz, USA) that recognizes human as well as murine PS-1 was added to each lysate and incubated overnight at 4 °C under mild agitation. Twenty microliters protein A beads suspension (magnetic Bio-Adembeads protein A, Ademtech, Pessac, France), washed three times in NP40 buffer and separated each time from the buffer by magnetic separation, was added to the lysate of each sample and incubated 1 h at room temperature under mild mixing. After magnetic separation, the pellets were washed four times with 500 μl NP40 buffer using magnetic separation. Finally, beads from each sample were resuspended in 20 μl of SDS polyacrylamide gel electrophoresis (PAGE) buffer 1× (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2.5% β-mercaptoethanol, 2% SDS, 0.01% bromophenol blue) and boiled 1 min. The supernatants were isolated after a last magnetic separation and 15 μl per sample was loaded onto the SDS-PAGE gel (10% acrylamide; Miniprotean II dual slab cell, Bio-Rad, Reinach, Switzerland). After electrophoresis proteins were electroblotted (overnight, 35 V; Mini Trans-Blot electrophoretic transfer cell, Bio-Rad) onto a nitrocellulose membrane (Schleicher and Schuell/GE Healthcare).

After a brief rinse in distilled H_2O the membrane was washed (3× 10 min) in TB buffer (Tris–HCl 0.02 M, pH 8.0, 130 mM NaCl, 0.05% Tween 20), non-specific binding sites were saturated by 1 h incubation in a 2% blocking solution (ECL advance kit, Amersham/GE Healthcare) in TB. The membrane was then incubated overnight at 4 °C with the mouse monoclonal anti-human PS-1 primary antibody NT.1 (diluted 1:3000 in blocking solution, Paul Mathews, New York University School of Medicine, Nathan Kline Institute, Orangeburg, NY).

The membrane was then washed as before and incubated 1 h with a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (GE Healthcare) diluted 1:100,000 in blocking solution. After washing (3× 10 min in TB), the membrane was briefly rinsed in distilled H_2O and bands detected by chemiluminescence (ECL advance Western blotting detection, Amersham/GE Healthcare) on film (Hyperfilm ECL, Amersham/GE Healthcare).

After washing (3× 10 min in TB) the same blot was reprobed with HRP-labelled anti-rabbit IgG (1:100,000; GE Healthcare) to detect the immunoglobulin used in the immunoprecipitation. After washing, the membrane was stripped in 0.2 M glycine–HCl, pH 2.5 with 0.1% Tween 20 for 2× 2 h at 60 °C, neutralized in TB for 2× 15 min and incubated 1 h in 2% blocking solution, then 2 h at room temperature with rabbit polyclonal anti-PS-1 antibody (Santa Cruz, H70; dilution 1:1000); after washing (3× 10 min in TB) the membrane was incubated 1 h with the HRP-labelled anti-rabbit antibody to detect the total amount of transgenic (human) and murine PS-1 immunoprecipitated.

For the densitometric analysis, films were scanned with a GS-700 imaging densitometer (Bio-Rad) and the band intensities were measured as optical density unit × area (mm^2) using the molecular analyst software program (Bio-Rad) and compared as percentages.

2.4. Immunocytochemistry

Indirect immunocytochemistry was carried out on preconditioned and differentiated NPCs. Cells were fixed for 30 min at room temperature in 4% paraformaldehyde (PAF) in PBS. After washing in PBS (3× 5 min), cells were permeabilized for 5 min in PBS containing 0.25% Triton X-100. Following a second washing step in PBS (10 min), coverslips were incubated for 30 min in PBS containing 10% horse serum (PBS/HS) and then overnight at 4 °C with primary antibodies diluted in PBS/HS. After washing (3× 10 min) in PBS, coverslips were incubated with appropriate secondary antibodies diluted in PBS/HS for 1 h at room temperature and finally washed (3× 10 min) in PBS. At the end of the procedure, coverslips were incubated in 0.2 μg/ml of bisbenzimide (Hoechst 33258; Calbiochem/Merck, Darmstadt, Germany) for 15 min and then washed three times 5 min in PBS. Hoechst reagent stains cell nuclei and allows counting the number of dead and living cells attached on coverslips. Coverslips were finally rinsed three times 10 min in PBS, then in distilled water, mounted on glass slides coverslipped with Fluorosave (Calbiochem).

The mouse monoclonal anti-βIII-tubulin antibody (Sigma), diluted 1/1000, was used as a marker for newly generated neurons and the rabbit polyclonal anti-glial fibrillary acidic protein antibody (GFAP, Sigma), diluted 1:500, as a marker for astrocytes.

Secondary antibodies, diluted 1:1000, were either anti-mouse IgG coupled to cyanide 3 (Jackson ImmunoResearch Laboratories, West Grove, USA) or coupled to AlexaFluor 488 (Molecular probes/Invitrogen), anti-rabbit IgG coupled to cyanide 3 (Chemicon) or to AlexaFluor 488 (Molecular probes/Invitrogen). Pictures of immunostained cells were taken using a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Feldbach, Switzerland) equipped with epifluorescence and were digitalized with an Axiocam camera.

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