Neuroscience Letters 563 (2014) 96–100

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Transgenic mice expressing S129 phosphorylation mutations in α-synuclein

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HIGHLIGHTS

- We developed transgenic mice expressing only S129D or S129A human α-synuclein.
- Transgene expression and localization were normal and at endogenous levels.
- No gross abnormalities in behavior or motor defects indicating toxicity were seen.
- Mice had normal gut motility and no neurodegeneration or aggregation in the brain.
- Presynaptic vesicle localization was normal in phosphomutant mice.

ARTICLE INFO

Article history:
Received 6 October 2013
Received in revised form 10 January 2014
Accepted 18 January 2014

Keywords:
α-synuclein
Parkinson's disease
Phosphorylation
Lewy bodies
Transgenic

ABSTRACT

Aggregated α-synuclein is a predominant constituent of Lewy bodies, the intracellular protein aggregates seen in Parkinson's disease. While most α-synuclein in the nervous system is unphosphorylated, the majority of α-synuclein in Lewy bodies is phosphorylated at serine 129 (S129). We developed transgenic mice expressing human *SNCA* with either a phosphomimic (S129D) or a non-phosphorylatable (S129A) mutation, on a mouse *Snca* knockout background. Transgenic lines with each mutation expressing the human α-synuclein protein at levels ranging from 0.3 to 1.9 fold of endogenous mouse protein were chosen to avoid toxic overexpression effects. We previously demonstrated an altered distribution of presynaptic vesicles in *Snca* knockout mice, as well as enhanced interaction between presynaptic cytoskeletal proteins and α-synuclein when phosphorylated at S129 or carrying an S129D mutation. We therefore examined α-synuclein's synaptic localization and the distribution of presynaptic vesicles in these mutants. In addition, we evaluated the transgenic lines for reduced colonic motility, an early marker of α-synuclein pathology, and α-synuclein aggregates. No abnormalities were detected in mice expressing either phosphorylation mutant protein as their only α-synuclein protein. These results suggest the S129A and S129D mutations have no obvious effect on α-synuclein function.

1. Introduction

Several of lines of evidence implicate α-synuclein (αSyn) in the pathogenesis of Parkinson's disease (PD) including familial human mutations and genome wide association studies [15]. The pathognomonic Lewy bodies (LBs) found in PD brains consist predominantly of highly aggregated αSyn [23] that is phosphorylated at S129 [2,9]. The physiological function of αSyn phosphorylation and its role in the disease process remain unknown.

Attempts to study the effect of αSyn phosphorylation in animal models by overexpression, particularly with viral vectors, have produced conflicting results [3,6,8,12]. We studied the *in vivo* effect of S129 phosphorylation by analyzing transgenic mouse lines that express αSyn at approximately endogenous levels with mutations that either mimic (S129D) or prevent (S129A) phosphorylation at serine 129, on an endogenous murine *Snca* knockout background.

First we looked at the expression of the αSyn phosphomutant proteins using immunohistochemistry to detect aggregated LB-like αSyn deposits. Since αSyn phosphorylation has been shown

Abbreviations: PD, Parkinson's disease; αSyn, α-synuclein; LB, Lewy bodies; PAC„ P1 artificial chromosome.

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http://dx.doi.org/10.1016/j.neulet.2014.01.033

to influence binding to synaptic proteins *in vitro* [16], we examined synaptic localization of αSyn and synapse formation using immunocytochemistry of neuronal cultures. Based on studies suggesting an effect of αSyn on presynaptic vesicle pools [5,18] we also analyzed vesicles using electron microscopy of neuronal cultures. Finally, we measured colonic motility in the transgenic mice, as enteric nervous system dysfunction is one of the earliest and most robust manifestations of αSyn pathology, preceding movement and cognitive dysfunction in both humans and mouse models of PD [4,13].

2. Material and methods

2.1. Transgenic mice

The 145 kb P1 artificial chromosome (PAC) RP1-27M07 (BAC/PAC Resource Center, Children's Hospital, Oakland, CA) containing wildtype human *SNCA* was described previously [24]. The S129A and S129D mutations were introduced by recombineering in the *E. coli* strain DY380 [14] by introducing GCA (S129A) or GAT (S129D).

We generated transgenic mouse lines carrying either the $SNCA^{S129A}$ PAC or $SNCA^{S129D}$ PAC in a FVB/N background using previously described methods [11]. Two independent lines for each construct were selected. Mice homozygous for each of the four S129 PAC transgenes in a $Snca^{+/+}$ background grew, developed normally and bred, indicating the PAC transgenes did not cause insertion site mutations. Each of the PAC transgenic lines was crossed with a $Snca^{-/-}$ knock-out (129S6//SvEvTac) [5] to generate homozygous PAC transgenics on a $Snca^{-/-}$, mixed FVB/N × 129S6 background. PCR and interphase FISH were used for genotyping and to determine transgene zygosity as reported previously [13]. The two S129A and two S129D homozygous transgenic lines on $Snca^{-/-}$ were designated A1, A2, D1 and D2 respectively. Transgenic mice expressing wildtype human αSyn [13] and non-transgenic littermates with $Snca^{+/+}$ or $Snca^{-/-}$ genotypes were selected as controls with a similar mixed background. All animals were handled in accordance with the requirements of NIH, the UCSF Animal Care and Use Committee, and EC Directive 86/609/EEC.

2.2. Quantitative RT-PCR

Brains were harvested from 6-week-old mice and stored in RNAlater (Qiagen). The mice expressed either endogenous mouse *Snca* (wildtype), a wildtype human *SNCA* transgene, or a phosphomutant human *SNCA* transgene (A1, A2, D1 and D2). The transgenic mice were homozygous for the transgenes on a $Snca^{-/-}$ background. cDNA preparation and real-time PCR on 16–20 replicates were performed as previously described [13]. The difference (ΔCt) between the average cycle number at which threshold was reached (Ct) for each line and the average Ct for endogenous *Elavl4* or endogenous *Syp* was recorded. The difference of differences (ΔΔCt) and relative amount $2^{(\Delta\Delta Ct)}$ between transgenic wildtype or phosphomutant human *SNCA* and endogenous mouse *Snca*, (both normalized to *Elavl4* or *Syp*), was calculated.

2.3. Western blotting

Brains from 8-week-old female mice from each transgenic line (A1, A2, D1 and D2), a $Snca^{-/-}$ littermate, and two wildtype littermates were dissected. Western blotting with the brain protein lysates was performed as previously described [5,13] using anti-αSyn antibody (BD Transduction Laboratories) that recognizes both human and mouse αSyn. αSyn signals were normalized for total protein per lane using mouse anti-α-tubulin antibody (Calbiochem).

2.4. Immunohistochemistry

Immunohistochemistry was performed on the brains of one male 9-month-old A2, and one 12-month-old D2 mouse using anti-αSyn antibody Syn505 as described previously [13]. Anti-αSyn antibodies Syn505 and pSer129/81A were used on the brain of a 12-month-old mouse expressing A53T human αSyn (line M83) as a positive control [10]. Tissue sections were counterstained with hematoxylin.

2.5. Immunocytochemistry of cultured neurons

Hippocampal and cortical neurons from E15.5 mouse embryos were isolated and plated in Neurobasal medium (Invitrogen) with B-27 supplement (Invitrogen) following manufacturer's protocol. At 17 days post-plating, neurons were fixed and stained with mouse anti-αSyn antibody (BD Transduction Laboratories) and rabbit anti-synapsin antibody (Stressgen). They were then stained with Alexa Fluor 488 conjugated goat anti-mouse and Alexa Fluor 594 conjugated goat anti-rabbit secondary antibodies (Invitrogen) and mounted on slides using Vectashield containing DAPI nuclear stain (Vector Laboratories). Images were taken using a SPOT microscopy camera and software (SPOT imaging). Final images were corrected for brightness and contrast at a similar level for each genotype using the Photoshop Elements program (Adobe).

2.6. Electron microscopy

Two eight-week-old mice from each genotype (A2, D2, wildtype and knockout) were intracardially perfused with 4% paraformaldehyde/2% gluteraldehyde fixative. The brains were post-fixed in fresh fixative. Coronal brain sections of 70 nm were cut at the level of the caudate/putamen using a vibratome and fixed overnight. Vesicles were counted in fifteen synapses from each mouse in the center of the CA1 stratum radiatum as previously described [5].

2.7. Colonic motility

Colonic motility was measured in 6-month-old male and female A2 and D2 mice by the bead expulsion test as previously described [13]. Six-month-old mice expressing the wildtype human *SNCA* PAC on a mouse $Snca^{-/-}$ background [13] were included as a control.

2.8. Statistical analysis

Standard deviations of ΔCts and ΔΔCts were calculated from the standard deviations (SD) as $\sqrt{[(SD_1)^2 + (SD_2)^2]}$. Student's *t* tests were used for pair-wise comparison between wildtype and mutant lines in Fig. 3. The Mann–Whitney *U* test was used in Fig. 4. The significance level was $p < 0.05$ for both tests. All data were analyzed by GraphPad Prism 4.0 statistical software (GraphPad Software).

3. Results

3.1. Expression of mutant human αSyn in transgenic mice

Human *SNCA* or mouse *Snca* mRNA expression was measured in brains from each of the two S129D and S129A transgenic lines and wildtype mice using qRT-PCR. The transgenic lines expressed human *SNCA* mRNA at 0.7-fold–39-fold higher levels in brain relative to expression of mouse *Snca* in wildtype mice, in all cases