



Glial A30P alpha-synuclein pathology segregates neurogenesis from anxiety-related behavior in conditional transgenic mice



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ARTICLE INFO

Article history:

Received 23 March 2013

Revised 23 June 2013

Accepted 1 July 2013

Available online 16 July 2013

Keywords:

Gliosis

Parkinson's disease

S100B

Transgenic

A30P alpha-synuclein

Conditional

Propagation

ABSTRACT

In Parkinson's disease (PD) patients, alpha-synuclein (α -syn) pathology advances in form of Lewy bodies and Lewy neurites throughout the brain. Clinically, PD is defined by motor symptoms that are predominantly attributed to the dopaminergic cell loss in the substantia nigra. However, motor deficits are frequently preceded by smell deficiency or neuropsychological symptoms, including increased anxiety and cognitive dysfunction. Accumulating evidence indicates that aggregation of α -syn impairs synaptic function and neurogenic capacity that may be associated with deficits in memory, learning and mood. Whether and how α -syn accumulation contributes to neuropathological events defining these earliest signs of PD is presently poorly understood.

We used a tetracycline-suppressive (tet-off) transgenic mouse model that restricts overexpression of human A30P α -syn to neurons owing to usage of the neuron-specific CaMKII α promoter. Abnormal accumulation of A30P correlated with a decreased survival of newly generated neurons in the hippocampus and olfactory bulb. Furthermore, when A30P α -syn expression was suppressed, we observed reduction of the human protein in neuronal soma. However, residual dox resistant A30P α -syn was detected in glial cells within the hippocampal neurogenic niche, concomitant with the failure to fully restore hippocampal neurogenesis. This finding is indicative to a potential spread of pathology from neuron to glia. In addition, mice expressing A30P α -syn show increased anxiety-related behavior that was reversed after dox treatment. This implies that glial A30P α -synucleinopathy within the dentate gyrus is part of a process leading to impaired hippocampal neuroplasticity, which is, however, not a sole critical event for circuits implicated in anxiety-related behavior.

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Introduction

In Parkinson's disease (PD), the natively unfolded protein alpha-synuclein (α -syn) aggregates into Lewy bodies (LB) and Lewy neurites (LN) through the transition from monomers into stable fibrils via intermediate oligomers (Conway et al., 2000b). Differences in fibrillization rate have been observed in relation to distinct mutations within the α -syn gene. In particular the A30P mutation appears to promote its conversion into oligomers (Conway et al., 1998; Li et al., 2002; Narhi et al., 1999) whilst exhibiting a slow propensity to fibrillize (Choi et al., 2004; Conway et al., 2000a; Li et al., 2001). Additionally, increased levels

derived from impaired clearance of the protein by lysosome and/or the proteasome (Cuervo et al., 2004; Nonaka and Hasegawa, 2009) may incite both release (Jang et al., 2010; Lee et al., 2005) and seeding effects of neurotoxic α -syn species on its soluble counterpart; thus starting a vicious cycle of further α -syn conversion, overload of protein degradation systems and exocytosis that may hasten cell death. These neurodegenerative processes are typically accompanied by reactive gliosis. Contributing glial cells overexpress GFAP to form stable astrocytic processes and S100B, a neurotrophic factor that is known to stabilize calcium homeostasis and to balance oxidative stress (Rothermundt et al., 2003). The topographical pattern of the neurotoxic progression suggests a spread of α -synucleinopathy within regions of the central nervous system, probably through cell-to-cell transfer between neurons (Desplats et al., 2009) or neuron to glia (Lee et al., 2010a). Despite the detection of this pattern the underlying mechanisms of disease progression that contribute to earliest PD symptoms as smell deficit, anxiety,

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Available online on ScienceDirect (www.sciencedirect.com).

and depression prior to motor effects remains unclear. Changes in anxiety and/or depression related behavior however, were directly attributed to alpha-synucleinopathy in transgenic animal models (George et al., 2008; Nuber et al., 2011; Oaks et al., 2013). Lifelong adult neurogenesis is implicated in memory formation and mood disorders (Eisch and Petrik, 2012). *In vitro*, α -syn mutations were observed to influence the fate and differentiation of neuronal progenitors (Schneider et al., 2007) and may therefore influence the capability of adult neurogenesis (Song et al., 2002). A detrimental impact of human α -syn on adult neurogenesis has been reported in mouse models expressing wild-type (WT) and mutant (A53T, A30P) α -syn (Winner et al., 2004, 2008, 2012). Importantly, in conditional mouse models, the suppression of either WT or A30P expression is able to restore the negative influence of α -syn on neurogenesis related to the olfactory bulb (Marxreiter et al., 2009; May et al., 2012; Song et al., 2002). The functional role of hippocampal neurogenesis is matter of intensive debate, e.g. recent studies showed that depletion of adult hippocampal neurogenesis led to a prominent anxiety- and/or depression-related behavior when stress was invoked (Revest et al., 2009; Snyder et al., 2011), whereas other studies did either not observe any change or an decrease in anxiety (recently reviewed by (Petrik et al., 2012).

Using a conditional model with doxycycline (dox) controllable neuronal expression of human mutant (A30P) α -syn we demonstrate here, that A30P overexpression in the hippocampus correlates with its posttranslational modification, astrogliosis, and a reduced survival of newborn neurons. We further observed an age-dependent and site-specific increase in co-localization of A30P α -syn and S100B within astrocytes that persisted after dox treatment, although the overall transgenic protein levels were reduced. Importantly, presence of glial A30P α -syn was concomitant with the pronounced impact and inability to restore hippocampal neurogenesis while these changes were not observed in the subventricular zone/olfactory bulb (SVZ/OB) circuit. Functional analyzes showed an increased anxiety in transgenic mice that was reversed by dox treatment. Thus our findings suggest that both neuronal and glial A30P α -syn pathology lead to the severely impaired neuronal plasticity of the hippocampus, which however may not be the sole site for anxiety related networks. These data also shed some light into the progression of synucleinopathies being transmitted between distinct cell-types and may help to explain in part the spatial progression of neuropathology.

Materials and methods

Transgenic mice

Construction of tet regulated mutant A30P α -syn expressing mice has been described in detail before (Marxreiter et al., 2009). The same cohort of mice was analyzed for the present study. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the local governmental commission for animal health. Animals for the analysis of adult neurogenesis were 2 months of age at the beginning of the experiment. Animals for the behavioral testing were 2 months of age and received. To suppress transgenic expression, animals received 2 mg/ml doxycycline (dox) (Sigma) in a 5% sucrose solution in the drinking water for 8 weeks. Sucrose was weekly reduced by 0.1% until a final concentration of 0.2% was reached as previously published (Nuber et al., 2011).

Sequential protein extraction

For sequential extraction of cytosolic, membrane-bound and detergent-stable α -syn, hippocampi and olfactory bulb were subdivided from dissected brains on a chilled stage. Sequential extraction of α -syn was performed as previously described (Tofaris et al., 2006). Shortly, tissues were homogenized in 100 μ l of TBS + [50 mM Tris-HCl, pH 7.4,

175 mM NaCl; 5 mM EDTA, protease inhibitor cocktails (Calbiochem, CA)] and spun for 30 min at 120,000 $\times$ g. Afterwards, the pellet was extracted in TBS + containing 1% of Triton X-100 (TX), and TBS +, 1 M sucrose, and RIPA buffer (TBS +, 1% NP-40, and 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate), each extraction step followed by centrifugation for 20 min at 120,000 $\times$ g. The detergent-stable pellet was finally solubilized in 8 M urea/5% SDS.

Western blot analyses

To analyze biochemical pattern of human α -syn, S100B, GFAP and influence of dox on protein expression, we used dissected brains of $n = 3$ untreated and dox-treated 4 months old adult mice. For western blot analyses 25 μ g of TBS, TX of mouse brain protein extracts, and 0.2 μ g of mono-ubiquitinated recombinant α -syn (Hejjaoui et al., 2011) were run on 4–12% Bis-Tris gels (Invitrogen, Life Technologies, Carlsbad, CA) and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After washing in phosphate-buffered saline (PBS) each membrane was blocked for 30 min in PBST (phosphate-buffered saline with 0.2% Tween-20) containing 5% of bovine serum albumine (BSA) at room temperature and subsequently incubated with either human-specific antibody syn211 (1:600; Sigma, Saint Louis, MO; named hSYN), human and rodent specific antibody syn1 (1:1000; clone 42, BD Bioscience, San Diego, CA; named h + mSYN), or an antibody that recognizes mouse α -syn (1:1000; #4179, Cell Signaling Technology, Danvers, MA; named mSYN), or antibodies against astroglial markers S100B (1:500; Sigma) or GFAP (1:1000; Abcam, Cambridge, MA) in PBST containing 5% of BSA over night. After washing with PBST, membranes were probed with corresponding secondary antibodies (1:5000, American Qualex, CA), visualized with enhanced chemiluminescence (ECL, PerkinElmer, Boston, MA), and analyzed with the VersaDoc gel imaging system (BioRad). Proteins were normalized to β -actin (1:3000), used as a loading control. Quantification of signal intensities was performed as described previously (Nuber et al., 2008).

Analysis of neurogenesis

Single transgenic mice CaMKII α -tTA served as control (named ctl, $n = 4$) for A30P α -syn transgenic mice (named A30P, $n = 5$). In an additional group, α -syn expression was suppressed starting at the age of 2 months for 8 weeks via dox application in the drinking water (named A30P + dox, $n = 5$). In order to label newly generated cells, all groups received intraperitoneal injections of bromodeoxyuridine (BrdU, 50 mg/kg) for 5 consecutive days at the age of 2–3 months and were killed 32 days after the first BrdU injection (see also Fig. 1A). All mice were kept in normal light/dark cycle (12 h light/12 h dark) and had free access to food. The animals water intake and weight was monitored daily. Animal perfusion and histological processing were performed as previously described (Marxreiter et al., 2009). Brains were cut sagittally into 25 μ m sections using a sliding microtome (Leica, Bensheim, Germany) on dry ice. Sections were then stored in cryoprotectant (ethylene glycol, glycerol, PB, pH 7.4, 1:1:2 by volume) at -20°C until further processing.

Antibodies, immunohistochemistry and confocal microscopy

For evaluation of neurogenesis, the following primary antibodies and final dilutions were used: mouse monoclonal anti-proliferating cell nuclear antigen (PCNA), 1:500 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rat monoclonal anti-BrdU, (1:500, Oxford Biotechnology, Oxford, UK), goat polyclonal anti-doublecortin C18 (DCX), (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse monoclonal anti-neuronal nuclei (NeuN) (1:500, Chemicon, Temecula CA, USA), rat monoclonal anti-human α -syn syn211, (1:1000, Sigma), rabbit anti-S100B (1:500, Sigma), mouse anti-glial fibrillary acidic protein (GFAP; 1:500, Abcam), rabbit anti-Calbindin 1:500 (Sigma). BrdU

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