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α-Synuclein A30P decreases neurodegeneration and increases synaptic vesicle release probability in CSPα-null mice

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ABSTRACT

α-Synuclein and Cysteine-string protein-α (CSPα) are presynaptic proteins that participate in the maintenance of synaptic function. Mutations or overexpression of the wild type form of α-synuclein have been related to Parkinson's disease, and CSPα mutations cause one type of neuronal ceroid lipofuscinosis. Both are adult-onset neurodegenerative diseases characterized by neuronal protein aggregations. Strikingly, while in mouse the lack of CSPα produces defective neurotransmission and neurodegeneration of motor terminals, blindness and early lethality, the moderate overexpression of wild-type α-synuclein fully rescues the CSPα-null phenotype. Contrarily, the overexpression of the mutated human α-synuclein A30P (α-synuclein^{hA30P}) has much less effect in CSPα KO mice. To explore how the A30P mutation affects the neuroprotective function of α-synuclein we investigated synaptic structure and neurotransmission in motor nerve terminals of wild-type and CSPα-null mice transgenic for α-synuclein^{hA30P}. We found that although α-synuclein^{hA30P} did not fully prevent neurodegeneration, it significantly improved synaptic organization and function in CSPα-null mice by enhancing quantal content, release probability, synaptic vesicle content, active zone number, postsynaptic area, and microtubule appearance. These results demonstrate that α-synuclein^{hA30P} is able to ameliorate synapse degeneration, despite its apparent lack of functionality and its long-term pathogenic effects in neurons. These findings may help to understand better the dual function of α-synuclein regarding neurodegeneration.

This article is part of the Special Issue entitled 'The Synaptic Basis of Neurodegenerative Disorders'.

1. Introduction

α-Synuclein is an abundant soluble presynaptic protein that interacts with membrane phospholipids (Burre et al., 2012; Iwai et al., 1995; Maroteaux et al., 1988; Maroteaux and Scheller, 1991), and associates with synaptic vesicles (Clayton and George, 1999; Irizarry et al., 1996; Iwai et al., 1995; Jensen et al., 1998). Although the physiological functions of α-synuclein are not fully understood, it seems to have an activity-dependent regulatory role in neurotransmission (Abeliovich et al., 2000; Liu et al., 2004; Nemani et al., 2010), to participate in synaptic vesicle mobilization, and to regulate the size of the recycling pool (RP) of synaptic vesicles (Cabin et al., 2002; Murphy et al., 2000). In addition, it has been proposed to contribute to the maintenance of presynaptic function (Chandra et al., 2004, 2005) as a SNARE-complex chaperone (Burre et al., 2010; Greten-Harrison et al., 2010) through its direct interaction with synaptobrevin-2/vesicle-associated membrane protein 2 (VAMP2) (Burre et al., 2010; Sharma et al., 2011). While these effects of α-synuclein are clearly beneficial, long-term overexpression produces its aggregation into Lewy body filaments, a landmark of Parkinson's disease and other synucleinopathies.

Cysteine String Protein alpha (CSPα) is a synaptic vesicle membrane protein that contains a DNA-J domain that interacts with the chaperone Hsc70 (heat-shock cognate 70; (Braun et al., 1996; Chamberlain and Burgoyne, 1997)) and with the cochaperone SGT (small glutamine-rich tetratricopeptide repeat protein) (Tobaben et al., 2001). The CSPα-Hsc70-SGT complex binds to SNAP-25 and, as wild-type α-synuclein, promotes SNARE complex formation, in this case by chaperoning SNAP-25 (Sharma et al., 2011). At the functional level, the lack of CSPα produces severe neurotransmission defects at the neuromuscular junction (NMJ) consisting of anomalous bursts of spontaneous release, decreases in quantal content, short-term plasticity alterations, and reduced

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calcium sensitivity of exocytosis (Dawson-Scully et al., 2000, 2007; Ruiz et al., 2008). Unexpectedly, transgenic overexpression of human or mouse wild-type α-synuclein in the CSPα knock-out (CSPα KO) mouse eliminates its lethal phenotype, while the knock-out of endogenous α-synuclein in the CSPα KO mouse speeds up its neurodegenerative process (Chandra et al., 2005). On the other hand, α-synuclein^{hA30P}, which hosts a missense mutation at the first alpha-helix of the protein and causes familial Parkinson's disease (OMIM 601508; Kruger et al., 1998), has only a transient and limited beneficial effect in CSPα KO mice (Chandra et al., 2005). Because the overexpression of human α-synuclein in the CSPα KO mouse restores normal motor performance, abolishes neurodegeneration and restores survival and body weight (Chandra et al., 2005), it is reasonable to presume that neurotransmission is also well re-established at motor nerve terminals. However, the very limited effect on motor behavior in the CSPα KO synuclein^{hA30P} mouse raises the question of how this mutation affects the physiological function of α-synuclein at the level of the NMJ and whether the overexpression of the mutated α-synuclein is deleterious in wild-type mice. We addressed these issues by recording synaptic activity and studying synaptic structures in wild-type and CSPα KO mice overexpressing α-synuclein^{hA30P}. This study has the potential advantage of identifying the NMJ-autonomous specific changes induced by the overexpression of the mutated protein, without being influenced by the general phenotypic improvement found in mice overexpressing wild-type α-synuclein. Our results demonstrate that α-synuclein^{hA30P} in CSPα KO mice improves neurotransmission by specifically restoring vesicle release probability in a calcium-dependent manner. In addition, we found that α-synuclein^{hA30P} decreases neurodegeneration and enhances motor terminal growth as evidenced by its positive effect on active zone (AZs) number, synaptic vesicle readily releasable pool (RRP) size, postsynaptic terminal area, and axonal and intraterminal microtubules structure. On the other hand, we found that the overexpression of the A30P mutation on wild-type background had no effect on synaptic function, synaptic organization, or motor performance. These findings may contribute to a better understanding of α-synuclein^{hA30P} function, particularly its less known role in ameliorating neurodegeneration.

2. Material and methods

2.1. Animal models

Mouse lines overexpressing the synuclein^{hA30P} transgene under control of the murine Thy-1 promoter were kindly provided by Dr Südhof (Stanford University, CA). Mice were bred and maintained on C57BL/6 background. Identification of wild-type, CSPα KO, and transgenic mice was done as previously described (Chandra et al., 2005). Four genotypes were used for experiments after crossing CSPα +/−; α-synuclein^{hA30P} heterozygous pairs: CSPα wild-type (with and without α-synuclein^{hA30P}) and CSPα KO (with and without α-synuclein^{hA30P}). All comparisons were made between littermates to limit potentially confounding background effects. All experiments were performed according to the guidelines of the European Council Directive for the Care of Laboratory Animals.

2.2. Muscle preparation

Mice were killed by means of CO_2 and exsanguinated. The *levator auris longus* (LAL) muscle was dissected with its nerve branches intact and pinned to the bottom of a 2 ml chamber, over a bed of cured silicone rubber. Preparations were continuously perfused with a solution of the following composition (in mM): 125 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$ and 30 glucose. The bath Ca^{2+} concentration varied from 0.1 to 2 mM as specified in some experiments. The solution was continuously gassed with 95% O_2 and 5% CO_2, pH 7.35.

2.3. Neurological analyses

Neurological evaluation was performed as previously described (Fernandez-Chacon et al., 2004).

2.4. Morphological studies

Immunofluorescence staining was performed on brainstem cryosections with mouse monoclonal anti-human α-synuclein S5566 antibodies whose epitope is localized to amino acids 121–125 (Sigma). For immunohistochemical study of NMJs, LAL muscles from mice at P30 were dissected in a solution containing (in mM): 145 NaCl, 2.7 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes and 15 glucose (pH 7.35). Preparations were washed and incubated in clean solution with 2 mM $CaCl_2$ for 30 min, and 10 min in solution without calcium, and fixed and processed for immunolabeling as previously described (Torres-Benito et al., 2011) using the following primary antibodies: mouse monoclonal anti-human α-synuclein S5566 (1:100; Sigma), mouse monoclonal anti-bassoon (1:600, Stressgen), rabbit polyclonal anti-vesicular acetylcholine transporter (vAChT, 1:500, SYSY), or mouse monoclonal anti-acetylated tubulin (1:1000, Sigma). Preparations were incubated for one hour with secondary antibodies: 1:500 goat anti mouse Alexa647 for Bassoon, 1:500 donkey anti-rabbit CF488 for vAChT, or donkey anti-mouse CF488 for tubulin. Postsynaptic acetylcholine receptors (AChRs) were labeled with bungarotoxin-rhodamine (0.5 μg/ml; Sigma). Muscle preparations were mounted on glass slides using the antifade reagent Slow Fade Gold (Invitrogen). The procedure was used for all four genotypes. Images of LAL muscle rostral band were acquired with an upright Olympus FV1000 confocal laser scanning microscope equipped with three excitation laser lines. An alternating sequence of laser pulses was used during the acquisition of images for sequential activation of the different fluorescent probes. Images were taken using a 63× oil-immersion objective (n.a. 1.42). Images from wild type and mutant littermate preparations were taken with similar conditions (laser intensity and photomultiplier voltage) and, usually, during the same day. The analysis of images was blind and performed independently by two persons. ImageJ was used for analysis of the labeled structures (W. Rasband, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/). Postsynaptic terminals and synaptic vesicles were delineated with outline masks based on brightness thresholding from maximal projected confocal images. Bassoon puncta were counted using the noise tolerance application. All statistics given are mean ± standard error of the mean (SEM) unless stated otherwise. Differences between two groups were tested using the *t*-test (2 tailed).

2.5. Electrophysiology

Synaptic transmission recordings were performed *ex vivo* in acute neuromuscular preparations of the LAL muscle at room temperature. The nerve was stimulated by means of a suction electrode. The stimulation consisted of square-wave pulses of 0.2 ms duration and 2–40 V amplitude, at variable frequencies (0.5–100 Hz). A glass microelectrode (10–20 MΩ) filled with 3 M KCl was connected to an intracellular recording amplifier (TEC-05X; npi electronic) and used to impale single muscle fibers of the rostral band near the motor nerve endings. Evoked endplate potentials (EPPs) and miniature EPPs (mEPPs) were recorded as described previously (Ruiz et al., 2008). Muscle contractions were prevented by including in the bath 3–4 μM μ-conotoxin GIIIB (Alomone Laboratories). For two-electrode voltage clamp experiments, muscle fibers were visualized with an x60WI objective (Olympus) in order to indentify nerve terminals and position the electrodes as close as possible to the terminal endings. Holding potential was −40 mV.

The mean amplitudes of the EPP and mEPPs recorded at each NMJ were linearly normalized to −70 mV resting membrane potential and EPPs corrected for nonlinear summation (McLachlan and Martin, 1981). The estimation of the readily releasable pool (RRP) size was performed as previously reported (Ruiz et al., 2011). The variance and the mean of quantum content (*m*) were calculated only during stable EPP and mEPP recordings. All data are given as groups mean values ± SEM. All experiments reported include the results of at least three animals per genotype. In all experiments, significance between the two groups was estimated by the use of a directional Student's *t*-test for uncorrelated samples (unpaired two-tailed). Three levels of statistical significance were distinguished: $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$. Results were considered statistically different when the *P* value was ≤0.05. Data in parentheses (*n, N*): *n*, the number of muscles fibers per group; *N*, number of mice per group.

3. Results

3.1. α-Synuclein^{hA30P} rescues evoked neurotransmission in CSPα KO motor nerve terminals

Because neurotransmitter release in CSPα-null mice at motor nerve terminal is greatly impaired (Fernandez-Chacon et al., 2004; Rozas et al., 2012; Ruiz et al., 2008) we first explored the effect of transgenic overexpression of α-synuclein^{hA30P} on synaptic function of CSPα KO, focusing in particular on measures of quantum content (*m*), release probability (*p*), and size of the RRP of synaptic vesicles.

We first compared at low frequency of stimulation (0.5 Hz) the size of the end-plate potentials (EPPs) in wild-type controls, wild-