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Genetic deletion of the GATA1-regulated protein α -synuclein reduces oxidative stress and nitric oxide synthase levels in mature erythrocytes

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Abstract

α -Synuclein is highly expressed in neural tissue and during erythropoiesis, where the key erythroid regulator GATA1 has been found to modulate its expression. While specific α -synuclein (*SNCA*) mutations are known to cause autosomal dominant familial Parkinson's disease, its wild-type function remains under debate. To investigate the role of α -synuclein in murine hematopoiesis and erythropoiesis, we utilized *Snca* knock-out mice and analyzed erythroid compartments for maturation defects, in vivo erythrocyte survival, and erythrocyte-based reactive oxygen species (ROS) and nitric oxide synthase (NOS) levels. Our findings show that while bone marrow and spleen erythropoiesis and peripheral blood erythrocyte survival in *Snca*^{-/-} mice was comparable to controls, the levels of ROS and of NOS-2 were significantly decreased in mature erythrocytes in these animals. These results indicate a role for α -synuclein in regulating oxidative stress in erythrocytes in vivo and could open new avenues for the investigation of its function in non-neural tissue

Introduction

Mutations and multiplications of the gene encoding for α -synuclein (*SNCA*) cause dominant Parkinson's disease (PD; OMIM#163890), where it is abundantly found in Lewy bodies, the histopathological hallmark of several neurodegenerative disorders [1,2]. While the biochemical properties of the mutated forms of α -synuclein have been extensively studied, the wild-type function of this small 14 kDa protein has eluded investigations for more than a

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decade. It is localized in the cellular cytoplasm and evidence exists for it to function as part of vesicle trafficking, neurotransmitter metabolism, neurogenesis, melanin synthesis, mitochondrial activities, and cell viability [3]. Some studies suggest that α -synuclein could be neuroprotective in its physiological state with disease-associated mutations abrogating this function by favoring aggregation [3–5]. In vitro data suggest the α -synuclein aggregation could be associated with the generation of oxidative stress [6,7]. *Snca*^{-/-} mice appear healthy, with mild electrophysiological changes in dopamine release upon Ca²⁺ stimulation [8]. In humans, it has been speculated that mitochondrial dysfunction, via increased reactive oxygen species (ROS) generation, could lead to the pathogenesis of PD [9–11].

Apart from CNS neurons, *SCNA/Snca* is highly expressed in human and murine erythrocytes, which opens avenues to studying α -synuclein in abundant, readily available tissue [7,12]. Furthermore, during erythropoiesis, *SNCA/Snca* expression is coordinately up-regulated with a wide array of heme metabolism genes, and is regulated by the master transcription factor GATA1 [7]. Despite these observations suggesting a possible functional role for α -synuclein in red blood cells (RBCs), no obvious deficits in peripheral erythrocyte cell counts have been reported in the *Snca* null mouse [12]. In patients affected by PD, abnormally low hemoglobin levels have been identified when compared to matched controls even before the formal clinical diagnosis of the neurodegenerative phenotype [13].

Whether a common cellular function is exerted by α -synuclein in neural and erythroid compartments is currently not clear, but intriguing similarities in neurogenesis and hematopoiesis have been known for several years [14,15]. For example, in mesencephalic A9 dopaminergic neurons, globin chains are expressed and the hemoglobin tetramer is found [14]. As both substantia nigra dopaminergic neurons and erythrocytes are exposed to high ROS levels under physiological conditions, we investigated the hypothesis that α -synuclein may be involved in oxidative stress during erythropoiesis [8,11,16]. We therefore examined erythropoietic differentiation, erythrocyte survival, and ex vivo ROS metabolism in *Snca*^{-/-} mice.

Methods

Animals

Previously described genetic null *SNCA*^{-/-} mice, heterozygous *SNCA*^{+/-} and wild-type littermates were kept under pathogen free conditions in approved facilities [8]. All animal care and study protocols were approved by the appropriate institutional review committees in accordance with NIH guidelines. Before any investigation involving *SNCA*^{-/-} mice, littermate *SNCA*^{+/-}, and wild-type controls, we verified the complete absence of α -synuclein in the erythroid tissue of the *SNCA*^{-/-} mice (Supporting Information Fig. 1a).

Hematological counts and peripheral blood iron (Prussian blue) stain

Counts were measured with an Advia (Siemens) machine, and cross-verified with a Hemavet 950 (Drew Scientific). Prussian blue staining to identify intra-corpuscular iron deposits/granules was performed as per routine clinical laboratory practices.

Peripheral blood, spleen, and bone marrow flow cytometry

Procedures were adapted from previously published methods [17]. Peripheral blood was collected into EDTA-coated tubes by tail venipuncture or direct cardiac puncture, and bone marrow was collected by femoral flushing. Single cell suspensions were generated from whole bone marrow and spleen as described previously [18]. Flow cytometry was performed after staining with fluorochrome bound antibodies against murine Ter119, CD71, CD44 (BD Pharmingen) and the viability marker 7-AAD (Invitrogen). Data were analyzed with the BD-Diva (BD Bioscience) and FlowJo (Treestar) software.

In vivo peripheral blood erythrocyte survival analysis

Mice were tail vein injected with 100 mg/kg of Sulfo-NHS-Biotin (Sigma-Aldrich) in DMSO (Thermo Scientific). One hour after injection, in vivo erythrocyte biotinylation was verified by flow cytometry using Streptavidin-Fluorescein isothiocyanate (FITC) (BD Pharmingen) (Supporting Information Fig. 1b). Analysis was repeated after 14 and 21 days.

ROS analysis

Erythrocytes were stained with 10 μ M live-cell ROS indicator CM-H₂-DCFDA (Molecular Probes/Life Technologies) and quantified by flow cytometry.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, and Oxyblot

Erythrocytes were lysed in 1% NP-40 substitute/PBS containing protease inhibitors (Roche), protein normalized, and assayed by SDS-PAGE/Western blot (NuPAGE 4–12% Bis Tris gel/MES running buffer, Invitrogen; PVDF membranes, Millipore) according to manufacturer's instructions, using anti-murine iNOS (Cell signaling #2982) antibody and anti-murine β -spectrin antibody (courtesy of Dr B. Paw, BWH, Boston). Oxyblot™ (Millipore) was performed according to manufacturer's instructions.

Results

First, to investigate the role of α -synuclein during erythroid differentiation and maturation, we analyzed the erythroid compartments of the bone marrow, spleen, and peripheral blood of adult *Snca*^{-/-} mice compared to littermate *Snca*^{+/-} and wild-type controls. Erythrocyte, leukocyte, and platelet morphology, as well as erythrocyte and reticulo-locyte counts and indices were normal when compared to littermate *Snca*^{+/-} and wild-type controls at two time points (6 weeks and 10 months of life, Supporting Information Fig. 2). In addition, Prussian blue stained peripheral blood smears showed no abnormal intra-corpuscular iron deposition in *Snca*^{-/-} mice (data not shown).

Second, we analyzed the bone marrow and spleen by immunophenotyping. To this end, we stained isolated cells with the differentiation markers Ter119, CD71, CD44, and the viability marker 7AAD and performed multicolor flow cytometry. The differentiation and maturation of spleen and bone marrow erythroid progenitors of *Snca*^{-/-} mice were identical to age-matched, littermate controls at two time points (6 weeks and 10 months of life, Fig. 1a,b). These findings indicate globally undisturbed erythropoiesis in *Snca*^{-/-} mice.

Third, to examine a potential role for α -synuclein in mature erythrocyte survival, we determined the RBC life span in age-matched wild-type and *Snca*^{-/-} mice by in vivo biotin labeling (Fig. 1). Biotin was injected intravenously, and blood samples were taken weekly and analyzed for the rate of decrease of FITC-streptavidin-biotin signal by flow cytometry. Overall, we observed a modest trend toward slower RBC turnover in *Snca*^{-/-} mice, which did not reach statistical significance (Fig. 1c), but may indicate a subtle tendency toward increased stability of peripheral erythrocytes in the *Snca*^{-/-} mice.

Fourth, to investigate erythrocyte ROS formation in the absence of α -synuclein, we analyzed freshly isolated RBCs using the ROS sensor CM-H2DCFDA and flow cytometry, to detect peroxides and mixed ROS. Unexpectedly, we found a significant decrease of ROS in erythrocytes in the *Snca*^{-/-} mice (Fig. 2a). We employed a second independent method (Oxyblot™) to confirm this finding and measure its impact on protein carbonylation and found that protein carbonylation was accordingly reduced in the erythrocytes of the *Snca*^{-/-} mice (Fig. 2b,c). Mutated species of α -synuclein have also been shown to contribute to nitrosative stress and lead to neuronal cell death [19]. We concordantly found the levels of iNOS (NOS2), an enzyme crucial for ROS/nitric oxide synthase (NOS) homeostasis, to be decreased in *Snca*^{-/-} erythrocyte (Fig. 2d).

Discussion

Mutations in α -synuclein cause PD and are associated to intra-neuronal aggregates. Accessing α -synuclein in neuronal tissue of healthy and PD-affected patients remains a challenge for biomedical investigation. Understanding the physiological function of α -synuclein could possibly enhance the study of its role in the pathogenesis of PD. The aim of our work was to explore whether a cryptic phenotype in the erythroid compartment of *Snca*^{-/-} mice had eluded prior studies. The expression of α -synuclein is regulated by the erythroid transcription factors GATA1/2, and coincides with the induction of heme metabolism genes, such as transferrin receptor [7]. In addition, murine *Snca* mRNA is abundant in terminally differentiated erythrocytes, its expression being approximately 30-fold higher than in neural tissue and peripheral blood leukocytes, suggesting a functional role in these cells [20].

Our findings reveal that, although dispensable in erythropoiesis, α -synuclein could be important for ROS production in erythrocytes. This observation correlates with the notion that upregulation and aggregation of α -synuclein in PD are associated with ROS-mediated cellular toxicity, and with data showing that RNAi-mediated knockdown of α -synuclein in neural cell lines reduces ROS formation and increases cell survival [16,21–23]. In fact, despite the robust and redundant mechanisms for ROS protection in erythrocytes, the presence of high concentrations of molecular oxygen and iron in erythrocytes can lead to oxidative damage to proteins and membrane lipids. Such damage directly and adversely impacts red cell function and survival, and could provide a potential mechanism for the modest increase in red cell lifespan in *Snca*^{-/-} mice [24,25]. Interestingly, prior studies have shown NO donors to negatively affect membrane fluidity and also the deformability of RBCs [26,27]. In PD, NOS-related neuronal insults contribute to neurodegeneration, and

nitrosative activation may be a consequence of α -synuclein dysfunction [19,28]. Our data hint at a link between α -synuclein and NO and ROS homeostasis in the erythroid setting.

Other proteins involved in the pathogenesis of neurodegenerative disorders by intraneuronal aggregate formation seem to cause alterations in the oxidative metabolism of mature erythrocytes. For example, in familial amyotrophic lateral sclerosis (FALS), mutations in the gene encoding the homodimeric Cu/Zn-superoxide dismutase (*SOD1*) cause the aggregation of this protein leading to neuronal death. Murine *Sod1* knock-out mice develop a microcytic anemia associated with increased erythroid oxidative stress [29–31]. The erythrocyte phenotype in the *Sod1* knockout mice can be reversed by the human SOD1 protein suggesting a biological relevance of murine models for the study of the human biology of this group of proteins [32].

There are several limitations to our findings. First, similar to every study involving murine model systems, the fundamental question of the relevance of the observed erythroid phenotype to human biology remains to be proven [33]. Second, only mechanistic understanding of the findings in the erythrocytes will definitively reveal whether our findings can truly be employed to interpret and further study the physiologic function and pathologic features of α -synuclein in the human. Third, the role and presence of NOS in the erythrocyte has been the center of an array of contradictory data and some controversy [34]. Thus, it will be important for further work to determine whether the radicals reduced in the *Sncd*^{-/-} erythrocyte are truly derived from NO, as suggested by the decrease iNOS levels in our study.

In summary, our findings constitute a novel in vivo observation of the impact of the absence of α -synuclein on cellular ROS and NOS levels in a non-neural context, but where the protein is expressed at supra-neural levels. Taken altogether, our data suggest a potential novel erythrocyte pathway linking α -synuclein to ROS generation, possibly via NOS levels. This study opens new avenues for functional studies of α -synuclein in an abundant, high-oxygen exposed, non-neural setting, but also highlight the need for the investigation of α -synuclein as potential regulator of erythrocyte NO and ROS homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Characterization of erythropoiesis in wild-type (WT) and *Snca*^{-/-} (KO) mice. A: Flow cytometry of whole bone marrow with CD71 (transferrin), and Ter119 (glycophorin) immunostaining. Maturation starts with the progressive acquisition of first CD71 and second Ter119. More mature RBC loose expression of CD71. No significant differences are identified at all stages between WT and KO mice ($n = 9$ per genotype). Timepoint 10 months is shown. Student-*T* testing applied to measure significance (n.s. = non significant, $P > 0.05$). B: Detailed sub-analysis of the differentiation stages of erythroblasts to mature erythrocytes by staining with CD44, as described by Liu and colleagues [18]. The arrow indicates this staining to differentiate the cells losing CD71 to become mature erythrocytes. No significant differences are identified during any stage between WT and KO mice ($n = 9$ per genotype). Pro = proerythroblast, Baso = basophilic erythroblast, Poly = polychromatophilic erythroblast, Ortho = Orthochromatic erythroblasts, Reticulo = Reticulocyte, Erythro = erythrocyte. Student-*T* testing was applied to measure significance (n.s. = non significant, $p > 0.05$). C: RBC survival by in vivo biotinylation. Turnover of erythrocytes ($n = 9$ per genotype) was measured by flow cytometry as shown in Supporting Information Fig. 1). A linear regression model was applied and no significant difference identified ($P = 0.5$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 2.

ROS and NOS analysis of wild-type (WT) and *Snca*^{-/-} (KO) erythrocytes. A: ROS sensor CMH2DCF-DA flow cytometry ($n = 9$ per genotype), Student-*T* testing was applied to measure significance. B: OxyBlotTM analysis detecting protein carbonylation indicative of ROS toxicity. Membranes were stripped/reprobed for β -spectrin as a loading control. C: Numerical quantification of global protein carbonylation based on OxyBlotTM analysis. Student-*T* testing applied to measure significance ($n = 3$ per genotype). D: Western immunoblot analysis of peripheral RBCs for iNOS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]