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ALPHA-SYNUCLEIN DEFICIENCY AFFECTS BRAIN *FOXP1* EXPRESSION AND ULTRASONIC VOCALIZATION

A. KURZ,[a1] M. WÖHR,[b1]* M. WALTER,[c] M. BONIN,[c] G. AUBURGER,[a] S. GISPERT[a]* AND R. K. W. SCHWARTING[b]

[a]*Section Molecular Neurogenetics, Department Neurology, Goethe University Medical School, Frankfurt am Main, Germany*

[b]*Experimental and Physiological Psychology, Philipps-University, Marburg, Germany*

[c]*Department of Medical Genetics, University of Tübingen, Tübingen, Germany*

Abstract—Alpha-synuclein is an abundant protein implicated in synaptic function and plasticity, but the molecular mechanism of its action is not understood. Missense mutations and gene duplication/triplication events result in Parkinson's disease, a neurodegenerative disorder of old age with impaired movement and emotion control. Here, we systematically investigated the striatal as well as the cerebellar transcriptome profile of alpha-synuclein-deficient mice via a genome-wide microarray survey in order to gain hypothesis-free molecular insights into the physiological function of alpha-synuclein. A genotype-dependent, specific and strong downregulation of *forkhead box P1* (*Foxp1*) transcript levels was observed in all brain regions from postnatal age until old age and could be validated by qPCR. In view of the co-localization and heterodimer formation of FOXP1 with FOXP2, a transcription factor with a well established role for vocalization, and the reported regulation of both alpha-synuclein and FOXP2 expression during avian song learning, we performed a detailed assessment of mouse movements and vocalizations in the postnatal period. While there was no difference in isolation-induced behavioral activity in these animals, the alpha-synuclein-deficient mice exhibited an increased production of isolation-induced ultrasonic vocalizations (USVs). This phenotype might also reflect the reduced expression of the anxiety-related GABA-A receptor subunit gamma 2 (*Gabrg2*) we observed. Taken together, we identified an early behavioral consequence of alpha-synuclein deficiency and accompanying molecular changes, which supports the notion that the neural connectivity of sound or emotion control systems is affected. **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: alpha-synuclein deficiency, transcriptome analysis, ultrasonic vocalizations, FOXP1, GABRG2.

Alpha-synuclein was initially described when its avian ortholog synelfin was identified to be dynamically altered in the neuroanatomical song circuit of juvenile male zebra finches during the critical period of song learning. It is presynaptically enriched and abundantly expressed in the central nervous system, particularly in motor regions such as striatum, cortex and cerebellum (Clayton and George, 1998; Maroteaux et al., 1988). The expression of the alpha-synuclein gene (SNCA) starts in late embryonic development and increases in the juvenile and adult mouse brain (Hsu et al., 1998), while it is reduced at old age (Adamczyk et al., 2005). Functional data confirm a role of alpha-synuclein for learning, synaptic plasticity and maintenance. Furthermore, alpha-synuclein was reported to regulate dopamine synthesis and to act as a chaperone (Surguchov, 2008). A hint for a role of alpha-synuclein in sound control such as with isolation-induced ultrasonic vocalizations (USVs) came from the observation of an enhanced level of USVs in a C57BL/6JOla mouse strain with several genetic anomalies, among them a spontaneous deletion of the SNCA gene (Wöhr et al., 2008).

The genetic interaction of alpha-synuclein with the SNARE apparatus of vesicle exocytosis (Chandra et al., 2005) together with electrophysiological data on altered neurotransmitter release probability in alpha-synuclein-deficient (knockout; KO) mice (Senior et al., 2008) led to the assumption that alpha-synuclein plays a role in the rapid cycling of synaptic vesicles. Mutations of alpha-synuclein have been thoroughly investigated, because they result in impaired movement and depression/anxiety, manifesting in humans as a progressive neurodegeneration known as Parkinson's disease (PD) (Gasser, 2009). In spite of the wealth of publications on alpha-synuclein, its effect on synaptic function is not yet understood at the molecular level.

In order to gain hypothesis-free insights into the physiological function of alpha-synuclein, we performed a genome-wide transcriptome survey via microarray analyses in brain tissue of KO mice. According to this transcriptional profile, only twelve genes showed altered expression levels. Here, we focused our investigations on *forkhead box P1* (*Foxp1*), a transcription factor implicated in neural connectivity and sound control, which exhibited consistently decreased transcript levels in the KO mice. For functional

[1] Joint first authorship.

*Corresponding author. Tel: +49-69-6301-7417; fax: +49-69-6301-7142 (S. Gispert) or Tel: +49-6421-28-23612; fax: +49-6421-28-23610 (M. Wöhr).
E-mail addresses: gispert-sanchez@em.uni-frankfurt.de (S. Gispert) or markus.woehr@staff.uni-marburg.de (M. Wöhr).

Abbreviations: A, adenine; ANOVA, analysis of variants; C, cytosine; cDNA, complementary DNA; cm, centimetre; cRNA, complementary RNA; db, decibel; DNase, desoxyribonuclease; Foxp1/2/4, forkhead box P1/2/4; G, guanine; Gabrg2, GABA-A receptor subunit gamma 2; Ggcx, glutamyl carboxylase; HET, heterozygote; IVC, individually ventilated cages; KO, knockout; LSD, least significant difference; mRNA, messenger RNA; PD, Parkinson's disease; pd(N)6, random-hexamer primer; pnd, postnatal day; qPCR, quantitative real-time RT–PCR; Rmnd5a, required for meiotic nuclear division 5 homolog A; SNARE, soluble *N*-ethylmaleimide receptor sensitive factor attachment; Snca, alpha-synuclein; T, thymine; Tbp, TATA binding protein; USV, ultrasonic vocalization; WT, wild-type.

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validation, we performed a detailed behavioral characterization of infant KO mice, including movement coordination and USVs, which are a widely used tool for the assessment of neurobehavioral development (Branchi et al., 2001; Scattoni et al., 2009). Infant mice emit USVs when isolated from dam and littermates (Costantini and D'Amato, 2006; Zippelius and Schleidt, 1956). USVs play an important role in pup survival, since they can elicit maternal behavior, like retrieval (Allin and Banks, 1972; Ehret and Haack, 1982; Sewell, 1970; Wöhr et al., 2008; Wöhr et al., 2009). The importance of genetic factors was indicated by early studies where inbred strain differences in USVs were observed within the species *Mus musculus* (Cohen-Salmon et al., 1985; Hennessy et al., 1980; Sales and Smith, 1978). Subsequent genetic studies have shown that the rate of USV emission and probably all acoustic call characteristics have a multiple genetic background (Hahn et al., 1987; Roubertoux et al., 1996; Thornton et al., 2005).

EXPERIMENTAL PROCEDURES

Animals and housing

Alpha-synuclein-deficient (knockout, KO) mice with a targeted replacement of exons 4 and 5 of the SNCA gene (Cabin et al., 2002) were compared to wild-type (WT) animals with the corresponding 129S6/SvEvTac background. Mice were obtained from a mutant line originally generated by Cabin et al. (2002) as follows: A mouse genomic bacterial artificial chromosome library constructed from the 129S6/SvEvTac strain was screened for *Snca* using a sequence of mouse alpha-synuclein cDNA from the 3′ end. The structure of the alpha-synuclein gene was determined and a targeting vector was generated to replace exons 4 and 5 with the aminoglycoside phosphotransferase gene (Neo) conferring neomycin resistance, transcribed in the opposite direction to *Snca*. For identification of correctly targeted cell clones, colonies of embryonic stem cells resistant to the aminoglycoside G418 were screened by Southern blotting. One correctly targeted clone was used for blastocyst injections to establish the mutant line on an inbred 129S6/SvEvTac background (for details of the procedure see: Cabin et al., 2002).

Mice were housed in Type II L cages ($365\times207\times140$ mm^3, floor area 530 cm^2; IVC-based) in the same animal room (where the environmental temperature was maintained between 20 and 24 °C, humidity: 55 ± 10%) under 12/12 h light/dark cycle with food and water *ad libitum*.

Heterozygous littermates were used as founders to breed over six generations and establish homozygous KO and WT cohorts to be aged in parallel within the same facility, in an effort to minimize the necessary animal cage space to generate the number of male animals and tissues required for transcriptome analyses.

Then, in order to avoid litter effects and to make behavioral comparisons between precisely age-matched littermates of different genotypes possible, homozygous KO and WT individuals were mated and the offspring used in heterozygous breeding. A total of 10 such litters from heterozygous crossings were used, comprising 66 pups as subjects (13 KO; 37 HET; 16 WT–38 females; 28 males).

All experiments employing mice were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Transcriptome analyses

Three age groups were employed: mouse pups at the age of postnatal day 7 (pnd 7), young adult mice at the age of 6 months and old mice of at least 19 months of age. Following cervical dislocation of young adult and old mice, the brain was dissected into brainstem, midbrain, cerebellum and striatum (dorsal and ventral striatum as a tissue block). In case of the mouse pups, the brain was dissected into both hemispheres. Immediately after dissection, the tissues were frozen in liquid nitrogen and stored at −80 °C until further analyses.

Transcriptome survey via oligonucleotide microarrays

From the brains of adult (6 months) versus old (21 months) male mice, striatal ($n=3$ vs. $n=3$) and cerebellar ($n=3$ vs. $n=4$) tissue was dissected, both for KO mice and for WT controls. Double-stranded cDNA synthesized from total RNA was linearly amplified and biotinylated. Labeled and fragmented cRNA was hybridized to MOE430 2.0 Gene Chip® arrays (Affymetrix, Santa Clara, CA, USA). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO series accession number GSE19534. For details of microarray performance and biomathematical analyses, see supplementary notes.

Transcript level assays via qPCR

Total RNA was extracted from brain hemispheres derived from mouse pups and from brainstem and midbrain derived from adult male mice with TRIZOL (Invitrogen, Karlsruhe, Germany) and digested with DNase (amplification grade I; Invitrogen) following the manufacturer's instructions. In case of the striata derived from 19 months old male mice, RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) was used for RNA extraction. Two micrograms of DNase treated RNA were reverse transcribed in a 36 μl reaction, using pd(N)$_6$ and *Not*I-d(T)$_{18}$ primers (First Strand cDNA Synthesis Kit; Amersham Bioscience, Freiburg, Germany).

Expression levels of the transcript for TATA binding protein (*Tbp*) were used for the normalization of *Foxp1*, *Foxp2* and *Gabrg2* transcript levels. Expression changes were analyzed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The following TaqMan® assays and primers were employed: Mm00474845_m1 (*Foxp1*), Mm00475030_m1 (*Foxp2*), Mm00446973_m1 (*Tbp*), *Gabrg2*-forward (5'- GCT CAC AAC CTT CTC CCT CA -3'), *Gabrg2*-reverse (5′- AAG GTA AAA CAT TAG CAA GCA CA -3′), *Tbp*-forward (5′- CAC TTC GTG CAA GAA TGC TGA AT -3′) and *Tbp*-reverse (5′- CGT GGC TCT CTT ATT CTC ATG ATG A -3′). For details of qPCR performance, see supplementary notes.

Isolation-induced behavioral activity and USVs

To induce behavioral activity and USVs, pups were isolated from the mother and nest on pnd 7 for 5 min under room temperature (23.17 ± 0.08 °C; range: 21.80–24.20 °C). The isolation occurred between 9 and 16 h during the light phase of the 12/12 h light/dark cycle. Prior to each test, behavioral equipment was cleaned using a 0.1% acetic acid solution followed by drying. Pups were removed individually from the nest at random and gently placed into an isolation box ($23\times28\times18$ cm^3) surrounded by a sound attenuating cubicle (for details see: Wöhr and Schwarting, 2008). An UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics, Berlin, Germany) placed in the roof of the isolation box, 12 cm above the floor, was connected via an Avisoft UltraSoundGate 116 USB Audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 300,000 Hz in 16 bit format by Avisoft RECORDER (version 2.7; Avisoft Bioacoustics). The microphone that was used for recording was sensitive to frequencies of 15–180 kHz with a flat frequency response (±6 dB) between 25 and 140 kHz. After isolation, animals were weighed, marked and tissue samples for genotyping were taken (tail-snipping).

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