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Short Communication

Transcriptome comparison of murine wild-type and synaptophysin-deficient retina reveals complete identity

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ARTICLE INFO

Article history:

Accepted 22 January 2006

Keywords:

Synapse
Synaptic vesicle
Knockout
Photoreceptor
High-density microarray

Abbreviations:

SPH^{-/-}, synaptophysin knockout,
i.e. mice carrying two mutated
synaptophysin alleles
wt, wild-type

ABSTRACT

Loss of synaptophysin, one of the major synaptic vesicle membrane proteins, is surprisingly well tolerated in knockout mice. To test whether compensatory gene transcription accounts for the apparent lack of functional deficiencies, comparative transcriptome analyses were carried out. The retina was selected as the most suitable tissue since morphological alterations were observed in mutant photoreceptors, most notably a reduction of synaptic vesicles and concomitant increase in clathrin-coated vesicles. Labeled cRNA was prepared in triplicate from retinæ of age- and sex-matched wild-type and mutant litter mates and hybridized to high-density microarray chips. Only three differentially expressed RNAs were identified in this way, one of which was synaptophysin. Further validation by quantitative RT-PCR could only corroborate the results for the latter. We therefore conclude that, despite the distinct morphological phenotype, no significant changes in gene expression are detectable in synaptophysin-deficient animals and that therefore compensatory mechanisms are either pre-existent and/or act at the posttranscriptional level.

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One of the first and most abundant synaptic vesicle polypeptides to be characterized at the molecular level is synaptophysin, an integral tetraspan membrane protein (Sudhof et al., 1987; Jahn et al., 1985; Leube et al., 1987; Wiedenmann and Franke, 1985). Synthesis of the phosphorylated glycoprotein is strictly restricted to neurons and neuroendocrine cells, and its detection has therefore become an established tool in tumor and histodiagnosis (Hubner et al., 2002). Multiple functions have been assigned to synaptophysin concerning all aspects of the synaptic vesicle cycle. Its ability to segregate into a special vesicle type in non-neuroendocrine cells (Leimer et al., 1996; Leube et al., 1989, 1994) and its interaction with cholesterol (Thiele et al., 2000) suggested a function in vesicle biogenesis. It was further postulated that synaptophysin is involved in

vesicle exocytosis. Although the original proposition of its participation in fusion pore formation could not stand the test of time, a regulatory role in neurotransmitter secretion is very likely (Hubner et al., 2002). Notably, it was shown that synaptophysin and the vSNARE synaptobrevin form a developmentally and functionally regulated complex presumably limiting the amount of synaptobrevin available for SNARE complex formation (Washbourne et al., 1995; Becher et al., 1999a,b; Hinz et al., 2001; Edelmann et al., 1995; Calakos and Scheller, 1994). And, finally, evidence has accumulated supporting a contribution of synaptophysin to vesicle endocytosis (Hubner et al., 2002). In support, a calcium-dependent interaction of synaptophysin with the endocytotic factor dynamin has been characterized (Daly et al., 2000; Daly and Ziff, 2002).

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Given all these potential functions, it came as a surprise that depletion of synaptophysin in mice did not result in strong phenotypic deficiencies (McMahon et al., 1996; Eshkind and Leube, 1995). A likely explanation is that compensatory mechanisms are at work which could be provided either by polypeptides of similar design and/or by very different molecules that are involved in alternative pathways. In support of the first possibility, it was noted that double knockout mice lacking not only synaptophysin but also synaptogyrin, another synaptic vesicle phosphoprotein with the same multipass transmembrane topology, exhibit altered synaptic plasticity (Janz et al., 1999). Furthermore, in synaptophysin knockout ($SPH^{-/-}$) mice, significant synaptic vesicle alterations were seen in photoreceptors which are also devoid of the neuronal synaptophysin isoform synaptoporin (Spiwox-Becker et al., 2001). The second alternative has gained considerable attention in the recent past as different synaptic vesicle recycling pathways have been recognized (Rizzoli and Betz, 2005; Sudhof, 2004). Since clathrin-coated vesicles are significantly increased in photoreceptor terminals of $SPH^{-/-}$ mice, it was therefore proposed that synaptophysin may be involved in a clathrin-independent pathway (Spiwox-Becker et al., 2001).

Taking the abovementioned considerations into account, we wanted to identify factors that compensate for the loss of synaptophysin. To do this, complete transcriptome analyses

were performed to pinpoint gene products that may participate in this process. The retina was selected for these experiments, reasoning that compensatory mechanisms should have reached their maximum since synaptic vesicle alterations are not prevented in this tissue.

Electron microscopy of photoreceptors of $SPH^{-/-}$ mice revealed significant alterations in comparison to their wt counterparts. Typically, terminals had a brighter cytoplasm than those of age- and sex-matched litter mate controls (Figs. 1A, B). Most notably, the mutant synapses presented a lower vesicle density with a significant increase of clathrin-coated vesicles (Figs. 1C, D). Given these significant alterations in addition to the more subtle differences described previously (Spiwox-Becker et al., 2001), we wanted to find out whether these changes were caused by altered gene transcription. To accomplish this, a genome-wide screen was performed using high-density microarray chips. Care was taken to match the isolates as much as possible, and experiments were performed in triplicate to avoid false-positive results. RNA was prepared in each experiment from wt and mutant male siblings of the same litter 6 weeks postpartum. In each instance, RNA was pooled from at least two male siblings. To minimize variability caused by illumination and/or endogenous cycling, animals were kept at a rigorous 12:12 h light-dark cycle and retinæ were dissected 4 h after switch to light

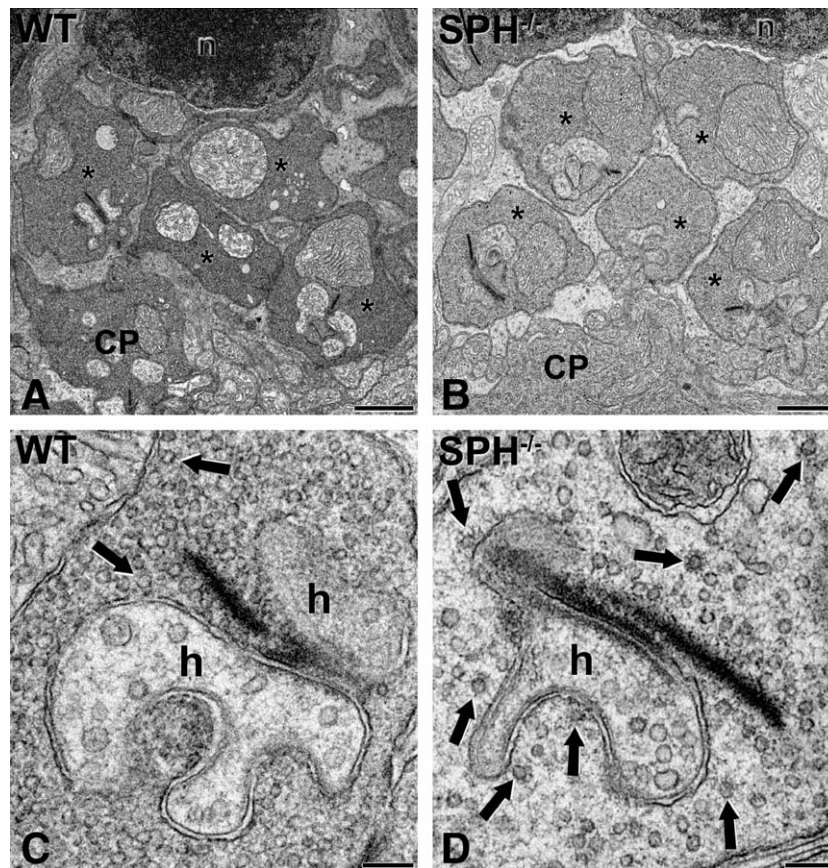


Fig. 1 – Electron microscopy of photoreceptors in $SPH^{-/-}$ mice and wt (WT) litter mate controls. Note the reduced staining of mutant photoreceptor terminals (*, rod; CP, cone pedicle) in panels A and B. The higher magnifications in panels C and D reveal a reduced vesicle density and an increase in clathrin-coated vesicles (examples labeled by arrows) in $SPH^{-/-}$ terminals. n, nucleus; h, horizontal cell process. Scale bars: 1 μ m in A, B; 0.2 μ m in C, D.

condition corresponding approximately to the time point at which the samples shown in Fig. 1 were also prepared. All samples were processed in parallel and hybridized individually to murine high-density microarray chips.

Several genes were either up- or downregulated in each experiment exhibiting a signal log ratio of more than 1 or less than -1 (43 up and 24 down in experiment 1, 24 up and 96 down in experiment 2, 61 up and 199 down in experiment 3). In each instance, the gene with the highest degree of change was synaptophysin. After pooling the data and comparing all wt with all knockout samples, only three genes remained with a signal log ratio <-1 and none with a signal log ratio >1 (Table 1). Of these, synaptophysin showed the most significant, yet still comparatively moderate change with remaining, still significant signals in the knockout samples (590.4 ± 71.5 in knockout versus 5116.2 ± 133.4 in wt samples). To examine the possibility that transcripts were still produced in the mutant, RT-PCR was performed using primer pairs either bridging exons 6 and 7 or corresponding only to exon 7 (Fig. 2A). Fig. 2B shows that no product could be amplified from exons 6/7 but that lower, yet significant amounts could still be produced from exon 7. This demonstrates that partial transcripts are still generated in the mutant animals, thereby explaining the incomplete shut off of gene transcription detected in the chip experiment which employed probes taken from exon 7 (Fig. 2A). The decrease of transcript levels of the other two genes (BG067686, BG230349) was just above the recommended twofold cut-off level with signal log ratios of -1.29 and -1.05 , respectively (see Table 1), and the signals were very weak (198.4 ± 14.3 in wt versus 97.4 ± 17.3 in the knockout for BG067686 and 166.7 ± 4.0 in wt versus 96.4 ± 10.9 in the knockout for BG230349). Furthermore, both gene fragments do not contain long open reading frames and are not assigned to complete genes despite their presence in EST data bases. We therefore felt that it was imperative to validate the chip results by quantitative RT-PCR. Whereas the changes determined for synaptophysin could be reproduced quantitatively quite well in five PCR determinations of two different RNA pools (copy numbers $1.33 \times 10^8 \pm 0.01 \times 10^8$ in wt and $1.50 \times 10^7 \pm 0.19 \times 10^7$ in the knockout), this was not the case for either BG067686 which showed a slight upregulation instead of a downregulation (copy numbers $1.72 \times 10^6 \pm 0.67 \times 10^6$ in wt and $3.26 \times 10^6 \pm 1.29 \times 10^6$ in knockout samples) or BG230349 with hardly any detectable change (copy numbers $1.10 \times 10^7 \pm 0.22 \times 10^7$ in wt and $1.12 \times 10^7 \pm 0.15 \times 10^7$ in

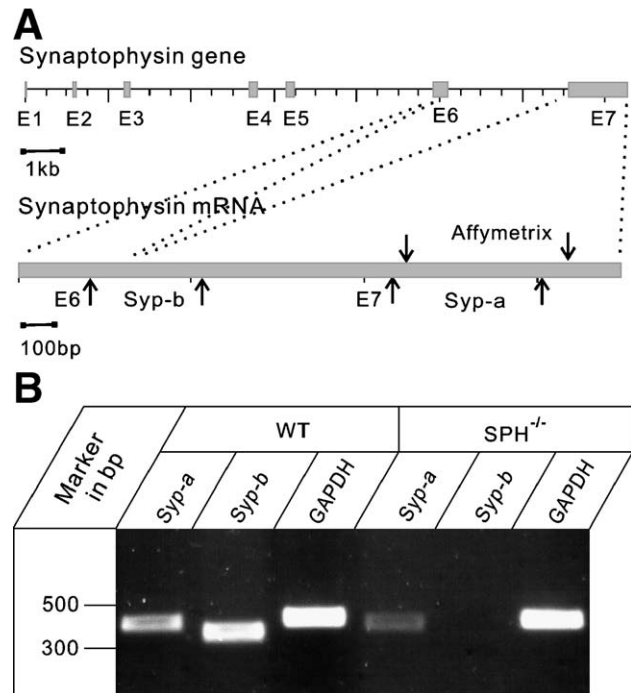


Fig. 2 – Analysis of gene transcription in wt (WT) and $SPH^{-/-}$ mice. (A) Scheme depicting the position of primer pairs used to amplify either fragment Syp-b corresponding to parts of exons 6 (E6) and 7 (E7) or fragment Syp-a corresponding to exon 7 only. Probes used for the high-density microarray are all localized within exon 7 as indicated (Affymetrix). (B) Photograph of agarose gel after electrophoretic separation of RT-PCR fragments obtained using either amplifiers for synaptophysin or for GAPDH as a control. The position of the co-electrophoresed 300 bp and 500 bp marker fragments is shown at the left. Note the reduced level of Syp-a and the complete absence of Syp-b in the knockout samples.

knockout samples). Comparisons of the fold changes are summarized in Table 1. Statistical analyses revealed no significant difference between the wt and knockout samples using either the Student's *t* test or the Wilcoxon–Mann–Whitney *U* test ($P = 0.421$ for BG067686 and $P = 0.929$ for BG230349), although highly significant differences were found for synaptophysin ($P \leq 0.001$) as was the case for all

Table 1 – Summary of results obtained for synaptophysin and two other genes in three independent microarray experiments (Exp. 1–3) and in two quantitative RT-PCR experiments (Exp. 3, 4)

Gene	Microarray SLR	Microarray SLR	Microarray SLR	Microarray average SLR	Microarray average fold change	RT-PCR average fold change	RT-PCR average fold change
	Exp. 1	Exp. 2	Exp. 3			Exp. 3	Exp. 4
Synaptophysin	-4.11	-3.54	-3.37	-3.67	-12.74	-11.07	-8.14
BG067686	-1.58	-1.30	-0.98	-1.29	-2.44	1.61	2.05
BG230349	-0.78	-1.13	-1.24	-1.05	-2.07	-1.13	1.42

The signal log ratios (SLR) are given for three independent microarray experiments and are averaged demonstrating a significant decrease for synaptophysin and only a minor, yet still significant decrease for the two other genes. The corresponding values for the average microarray fold change are also given and correlated to the changes observed in two RT-PCR experiments using either the same RNA samples as in microarray experiment 3 (Exp. 3) or RNAs prepared from yet another litter (Exp. 4). Note that only the results for synaptophysin could be replicated by this method.

comparisons in the microchip experiments. The inconsistency between the gene chip analyses and the RT-PCR experiments cannot be explained fully, although the low signal level in the gene chip hybridization may have resulted in false positives. We therefore conclude that, except for synaptophysin, no experimentally reproducible changes occur in gene transcription of SPH^{-/-} retinæ.

The last decade has witnessed an ever increasing number of knockout mice many of which do not present strong phenotypic defects. A common assumption is that compensatory mechanisms are responsible for the lack of apparent dysfunction. Presumably, such compensation involves not only posttranscriptional alterations but is also linked to changes in gene transcription. In fact, a widely held notion is that such alterations occur in all knockout mice whether they present overt phenotypes or not. Although it cannot be formally excluded that minor changes escaped the detection in our experiments, the results provide a prominent example demonstrating that loss of a major polypeptide, i.e. the synaptic vesicle membrane protein synaptophysin, can occur without the slightest repercussion on the transcriptome.

Given the multiple alternative interaction partners of synaptophysin, its integration into the complex functional network of the synaptic vesicle cycle, and the permanent morphological alterations in photoreceptors of SPH^{-/-} mice (Hubner et al., 2002), we would have rather expected significant changes in the transcription of multiple genes. Most notably, none of the RNAs coding for the similarly built tetraspan vesicle membrane proteins or for any known synaptophysin interaction partner was significantly altered including also synaptobrevin II whose protein levels were previously reported to be slightly reduced in SPH^{-/-} mice (McMahon et al., 1996; see, however, Eshkind and Leube, 1995). The same was true for RNAs encoding molecules that are involved in the apparently upregulated clathrin-dependent endocytotic pathway. We cannot exclude at present, however, that changes in gene expression escaped detection given the still incomplete characterization of the murine transcriptome and potential problems in transcript detection with the probes present on the gene chips that were used. The observed lack of compensatory changes in the transcriptome can be explained in two ways: either all compensation occurs at the posttranscriptional level or endogenous redundancy ensures that synaptophysin is – for the most part – dispensable. Posttranslational modification and/or altered protein stability could account for the first alternative. The slight decrease in synaptobrevin II that was noted in SPH^{-/-} mice, however, is to date the only documented example of altered synthesis of a proven synaptophysin interaction partner (McMahon et al., 1996). We therefore favor the second alternative, especially in light of the accumulating evidence for alternate routes of synaptic vesicle recycling (Rizzoli and Betz, 2005; Sudhof, 2004). While either one of the different pathways may be sufficient to maintain the basic machinery of vesicle-mediated neurotransmission, its loss may still limit higher order cerebral functions requiring synaptic plasticity. Future experiments will show whether the increase of clathrin-coated vesicles in photoreceptors of SPH^{-/-} mice is an indication of a partial compensation for the loss of a synaptophysin-dependent recycling pathway to maintain proper neurotransmis-

sion and whether these animals suffer from more subtle defects in complex neuronal tasking.

Animals. Mice with an inactivating mutation in the X chromosomal synaptophysin gene were described previously (Eshkind and Leube, 1995). These animals do not synthesize detectable amounts of synaptophysin or synaptophysin fragments. They were inbred for several generations prior to outcrossing with C57BL/6 (Harlan Winkelmann, Borchon, Germany). For the experiments, SPH^{-/-} females were first mated with wild-type (wt) C57BL/6 males and the heterozygous SPH^{+/-} female offspring was subsequently crossed with SPH⁺⁰ C57BL/6 males. In this way, male litter mates were generated carrying either a wt or a mutant synaptophysin allele. The genotype of these males was determined by duplex PCR using primers 03-51 ACTTCCATCCCTATTTCCACACC, 03-52 TTCCACCCACCAGTTCAGTAGGA, and 03-53 TCGCCTTCTTGACGAGTTCCTTCTG, yielding a 236 bp fragment from the wt allele and a ~500 bp fragment from the mutant allele. Animals were kept at a constant light cycle (lights on at 6 a.m. and off at 6 p.m.) at 21°C and 60% humidity. Mice were killed by cervical dislocation after anesthesia with ether before tissue removal.

Electron microscopy. Retinæ were rapidly removed postmortem and fixed in freshly prepared 2% paraformaldehyde, 2.5% glutaraldehyde in PBS for 15 h. After a rinse in PBS containing 6.8% (w/v) sucrose, tissues were fixed in osmium tetroxide (2% w/v in PBS) for 90 min, washed three times in PBS, and dehydrated in a graded acetone series. Flat embedding was in Epon (Serva, Heidelberg, Germany). 50–60 nm sections were prepared and mounted onto Formvar-coated copper grids (Serva). Staining with 8% (w/v) uranyl acetate for 10 min and contrasting with lead citrate for 5 min ensued. Sections were viewed with a Leo 907 transmission electron microscope (Zeiss, Jena, Germany), and images were recorded digitally.

cRNA preparation and microarray hybridization. Retinæ were prepared from mice during the light cycle, i.e. at 10 a.m., and total RNA was immediately purified from up to 8 retinæ using 1 ml Trizol reagent (Invitrogen, San Diego, CA, USA) and the RNeasy kit (Qiagen, Hilden, Germany) following the hybrid protocol provided by Affymetrix (www.affymetrix.com). RNA concentration and quality were determined with the help of an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Fifteen micrograms total RNA was used to make labeled probes for microarray hybridization according to protocols recommended by Affymetrix. Briefly, reverse transcription was done with the help of the Poly-A-Control kit using an oligo (dT)₂₄-T7 primer (Invitrogen), and double-stranded cDNA was synthesized with the Superscript II double-stranded cDNA synthesis kit (Invitrogen). The double-stranded cDNA was then purified using the GeneChip Sample Cleanup Module (Affymetrix, High Wycombe, UK) and served as a template in the subsequent in vitro transcription reaction (IVT Labeling Kit, Affymetrix) using T7 RNA-polymerase and biotinylated nucleotides (Invitrogen). Fifteen micrograms of labeled and fragmented cRNA was then hybridized for 16 h at 45°C with Mouse Genome 430 2.0 GeneChip arrays (Affymetrix) containing 45,000 probe sets that represent more than 39,000 transcripts and variants from over 34,000 mouse genes and EST clusters plus controls. After hybridization, staining and washing with R-phycoerythrin-streptavidin (Merck, Darmstadt,

Germany) were performed according to the provided protocols (Affymetrix). The arrays were scanned with an Affymetrix GCS 3000 scanner, and the images were quantified using Microarray Suite 5.0 (Affymetrix). The resulting data were normalized using global normalization, and all parameters of GeneChip quality control were within accepted ranges as described by the manufacturer. The signal log ratios of the expression levels in SPH^{-/-} and wt control samples were calculated using the GeneChip robust multi-array analysis method with the help of the Array Assist Software 3.0 (Stratagene, La Jolla, CA, USA).

Reverse transcription. Double-stranded cDNA was synthesized from 1 µg total RNA using the 4 U Omniscript reverse transcriptase (Qiagen) in a total volume of 20 µl containing 2.0 µl 10× buffer RT, 0.5 mM dNTPs, 10 U ribonuclease inhibitor (Ambion, Huntingdon, UK), and 1 µl oligo(dT) primer (MWG Biotech, Ebersberg, Germany) at 37°C for 60 min. A control sample was prepared in the absence of RNA. The reaction was terminated by heating the samples to 95°C for 5 min. The synthesized cDNA was diluted 1:10 in RNase-free water and stored at -20°C until use.

Quantitative real time PCR. Real time PCR was carried out in a total volume of 25 µl containing 12.5 µl absolute QPCR SYBR Green Fluorescein Mix (Abgene, Hamburg, Germany), 0.75 µl of each primer (10 mM stock solution), 5 µl cDNA, and 6 µl distilled water. Primers were designed using the Primer Designer 7 software version 7.0 (Scientific and Educational Software, Cary, USA). Primers GCAGACAGGCAGGTGAAGAG and GAGGGA-GAGGGCAGAGAAAG were employed to generate the 359 bp synaptophysin fragment Syp-a, primers TACGGGCTCAGGGT-GGTTATC and TGGGTGAAGGTAGGGCTCAGAC to produce the 326 bp synaptophysin fragment Syp-b, primers CATGCAACAG-CACCGTAAGAC and TGATGCCTGCTGGCTGTTAGAG to amplify a 487 bp fragment from gene BG067686, primers CAGAAG-AGGGCATCAGAC and GAGACACTCCCTGTTTCC for production of a 321 bp fragment from BG230349, and primers ATGAGCC-TTCCACAATG and TCCACTCAGGCAAATTC to detect a 374 bp GAPDH gene fragment (BC095932). PCR amplification was performed in an i-Cycler (BioRad, Munich, Germany) as follows: activation of the enzyme was at 95°C for 15 min followed by 40 cycles consisting of 30 s denaturation at 95°C, 30 s annealing at 61°C, and 20 s extension at 72°C. All amplifications were carried out in duplicate. The purity of the amplification products was checked by recording the melting curve and electrophoretic separation of the reaction products on 1% agarose gels followed by ethidium bromide staining for visualization. The amount of RNA was calculated from the measured threshold cycles. Data were normalized by determination of the amount of GAPDH present in the same cDNA preparations. The mean ± standard error of the mean (SEM) was calculated for the determined transcript copy numbers. Student's *t* test and the Wilcoxon-Mann-Whitney *U* test were used (SigmaStat 2.03; Jandel Scientific Corporation) for statistical analyses. A value of *P* equal to or smaller than 0.05 was considered to be significant.

Acknowledgments

The expert technical assistance of Ilse von Graevenitz is gratefully acknowledged. The work was supported by the

Stiftung Rheinland Pfalz für Innovation. The microchip array experiments were performed in the high-density microarray unit of the Johannes Gutenberg University.

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