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Original Paper

Chorein Sensitive Dopamine Release from Pheochromocytoma (PC12) Cells

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Key Words
Dopamine • Exocytosis • Degranulation • Vesicles • VAMP8

Abstract
Background: Chorein, a protein supporting activation of phosphoinositide 3 kinase (PI3K), participates in the regulation of actin polymerization and cell survival. A loss of function mutation of the chorein encoding gene *VPS13A* (vacuolar protein sorting-associated protein 13A) leads to chorea-acanthocytosis (ChAc), a neurodegenerative disorder with simultaneous erythrocyte akanthocytosis. In blood platelets chorein deficiency has been shown to compromise expression of vesicle-associated membrane protein 8 (VAMP8) and thus degranulation. The present study explored whether chorein is similarly involved in VAMP8 expression and dopamine release of pheochromocytoma (PC12) cells. ***Methods:*** Chorein was down-regulated by silencing in PC12 cells. Transmission electron microscopy was employed to quantify the number of vesicles, RT-PCR to determine transcript levels, Western blotting to quantify protein expression and ELISA to determine dopamine release. ***Results:*** Chorein silencing significantly reduced the number of vesicles, VAMP8 transcript levels and VAMP8 protein abundance. Increase of extracellular K^+ from 5 mM to 40 mM resulted in marked stimulation of dopamine release, an effect significantly blunted by chorein silencing. ***Conclusions:*** Chorein deficiency down-regulates VAMP8 expression, vesicle numbers and dopamine release in pheochromocytoma cells.

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Introduction

Chorein interacts with the phosphoinositide-3-kinase (PI3K)-p85-subunit thus contributing to PI3K activation with subsequent activation of the small G protein ras-related C3 botulinum toxin substrate 1 (Rac1) and the p21 protein-activated kinase 1 (PAK), a signaling cascade eventually leading to actin polymerization and fostering cell survival [1-4]. Loss-of-function mutations of the chorein encoding gene VPS13A (vacuolar protein sorting-associated protein 13A) underlies chorea-acanthocytosis (CA), an autosomal recessive disease [5-10] leading to progressive hyperkinetic movement disorder, cognitive dysfunction, behavioral abnormalities, chronic hyperkalemia and erythrocyte acanthocytosis [6, 11]. Knockout of chorein in mice yields erythrocyte shape changes [12], neuronal apoptosis [13] and altered behavior [13].

Chorein is expressed in a variety of further tissues [14-16] and additional chorein sensitive functions include platelet activation [16], endothelial cell stiffness [15] and tumor cell survival [4]. Chorein sensitive disarrangement of several cytoskeletal structures was reported in human fibroblasts isolated from ChAc-patients [17]. In platelets, chorein deficiency decreases the number of intracellular granules and compromises platelet degranulation [16]. The defect is paralleled by decreased expression of vesicle-associated membrane protein 8 (VAMP8), a critical regulator of platelet degranulation [16]. In pheochromocytoma (PC12) cells chorein was observed in the termini of extended neurites and localized in dopamine containing dense-core vesicles [18]. Overexpression of the carboxyterminal fragment of chorein increased K^+-induced dopamine release [18].

The present study explored whether chorein deficiency modifies vesicle abundance, dopamine release and VAMP8 expression in PC12 cells.

Materials and Methods

Cells

Pheochromocytoma (PC12) cells [19] were grown in RPMI-1640 (Gibco) supplemented with 10% horse serum, 5% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Silencing of chorein

PC12 cells were seeded in 6 well plates on a density of 2 x 10^5 and cultured under standard culture conditions (37°C, 5% CO_2) for 24 h. The cells were subsequently transfected for 72 h with validated siRNA for VPS13A (chorein) (ID#s235043, Ambion, Darmstadt, Germany) or with a negative control siRNA (ID#s4390843, Ambion) using siPORT amine transfection agent (Ambion) according to the manufacturer's protocol. The efficiency of silencing was checked by RT-PCR.

RT-PCR

For quantitative real-time PCR (RT-PCR) total RNA was isolated from cells using Trifast Reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. Subsequently approximately 2.5 µg of total RNA was reverse transcribed to cDNA using oligo(dT)$_{12-18}$ primers and SuperScript II reverse transcriptase. To determine transcript levels of chorein and Vamp8, RT-PCR was performed with the BioRadiCycleriQ™ Real-Time PCR Detection System (Bio-Rad Laboratories) and GoTaqSybr Green Master Mix (Promega). The reaction was applied in a final volume of 20 µl containing 2 µl of cDNA under following conditions: an initial incubation at 95°C for 5 min, 40 cycles at 95°C for 15 s, 59°C for 20 s and 72°C for 30s. Specificity of the PCR products was verified by melting curve analysis. The subsequent primers were used (5'→3' orientation):

Vps13a fw: TCATCCGGAATCTTCTTCCCTAC
Vps13a rev: TGCCACAACTGTCTGTCCAGTA
Vamp8 fw: ATGACCGAGTCAGGAACCTGC

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Vamp8 rev: TCTTGAAGTGTTCAGACGTGGC
Gapdh fw: TGTGAAGGTCGGTGTGAACG
Gapdh rev: ACATACTCAGCACCAGCATCAC

The mRNA levels of the respective genes were normalized to the expression levels of GAPDH in the same cDNA sample. Relative quantification was calculated according to the $2^{-\Delta\Delta Ct}$method [20].

Western Blotting

To quantify protein abundance, cells were washed twice with ice cold PBS and suspended in 100 µl ice-cold RIPA-lysis buffer (Thermo Fisher Scientific) containing protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration was determined using the Bradford assay (BioRad, München, Germany). Forty µg of protein were solubilized in sample buffer at 95ºC for 5 min and resolved by 12% SDS-PAGE. For immunoblotting proteins were electro-transferred onto PVDF membranes and blocked with 5% BSA in TBS-0.10% Tween 20 at room temperature for 1 h. Then, the membranes were incubated with either anti-Vamp8 antibody (1:4000, Abcam) or anti-GAPDH antibody (1:2000, Cell Signaling) at 4°C overnight. After washing (TBST) the blots were incubated with HRP-conjugated anti-rabbit (1:2000, Cell Signaling) antibody for 1 h at room temperature. After additional washes (TBST) protein abundance was detected with the ECL detection reagent (Amersham, Freiburg, Germany) and quantified with Quantity One Software (BioRad, Munich, Germany).

Dopamine release

PC12 cells were silenced for 72 hours and treated with 40 mM KCl at 37°C for 3 minutes to induce dopamine release in the cell culture medium. For determination of dopamine in the cell culture medium a dopamine ELISA kit (IBL International GmbH) was used according to the manufacturer's protocol.

Transmission electron microscopy

After washing with warmed PBS, PC12 cells were fixed with warmed Karnovsky's fixative for 1 h at RT and stored at 4°C. For electron microscopic analyses, the cell pellets were embedded in 3.5% agarose at 37°C, coagulated at room temperature, and fixed again in Karnovsky's solution. Post-fixation was based on 1.0% osmium tetroxide containing 1.5% K-ferrocyanide in aqua bidest for 2 h. Using standard methods, blocks were embedded in glycide ether and cut using an ultra microtome (Ultracut, Reichert, Vienna, Austria). Ultra-thin sections (30 nm) were mounted on copper grids and analyzed using a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) operating at 80 kV.

Statistics

Data are expressed as arithmetic means ± SEM. Statistical analysis was made by unpaired t-test or Mann-Whitney test, as appropriate. $p < 0.05$ was considered statistically significant.

Results

The present study explored whether degranulation of and dopamine release from pheochromocytoma (PC12) cells is sensitive to the presence of chorein. In a first approach, the morphology of the cells was visualized by electron microscopy. As illustrated in Fig. 1, silencing of chorein was followed by a significant decrease of the number of vesicles bound to the cell membrane.

In order to test whether chorein silencing influences degranulation of PC12 cells, dopamine was determined in the supernatant following an increase of extracellular K^+ from 5 mM to 40 mM. As illustrated in Fig. 2, the amount of dopamine released upon depolarization of the cell membrane was significantly decreased following silencing of chorein.

Additional experiments explored whether the decrease of dopamine release is paralleled by altered expression of vesicle-associated membrane protein 8 (VAMP8). To this end, VAMP8 transcript levels were quantified by RT-PCR. As shown in Fig. 3, the VAMP8 transcript levels were significantly decreased by chorein silencing.

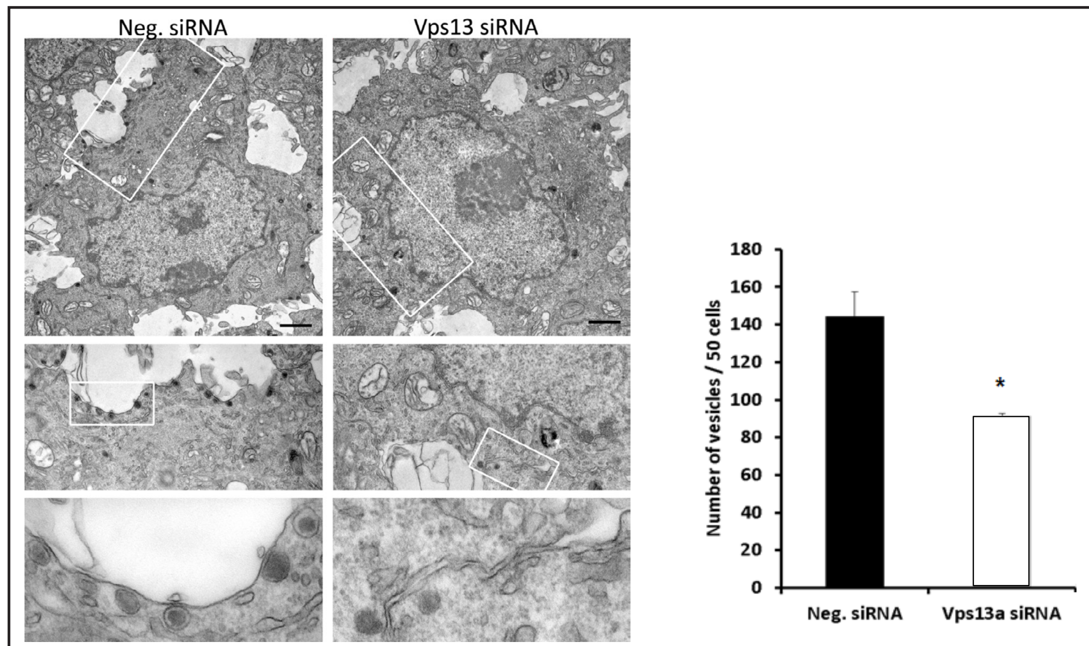

Fig. 1. Chorein sensitive number of vesicles bound to the membranes of PC12 cells. A. Original electron micrograph of pheochromocytoma (PC12) cells following treatment with siRNA without (left panel, Neg. siRNA) and with (right, Vps13 siRNA) specific silencing of chorein. B. Arithmetic means ± SEM (n = 5 preparations with 50 cells each) of the number of vesicles bound to the cell membrane in PC12 cells treated with unspecific (Neg. siRNA, black bar) and chorein specific (Vps13 siRNA, white bar) silencing RNA. * significant difference ($p<0.05$; t-test).

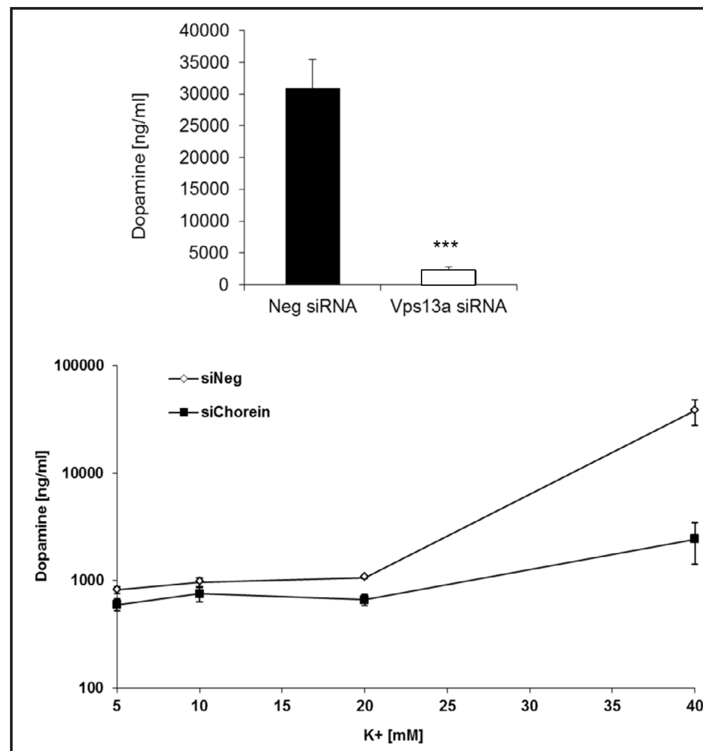

Fig. 2. Chorein sensitive dopamine release from PC12 cells. Arithmetic means ± SEM (n=5) of dopamine released following increase of extracellular K^+ from 5 mM to 40 mM from pheochromocytoma (PC12) cells treated with unspecific (Neg. siRNA, black bar) and chorein specific (Vps13 siRNA, white bar) silencing RNA. ***significant difference ($p<0.001$; unpaired t-test).

Western blotting was employed to test, whether the decrease of VAMP8 transcript levels following chorein silencing was paralleled by a similar decrease of protein abundance.

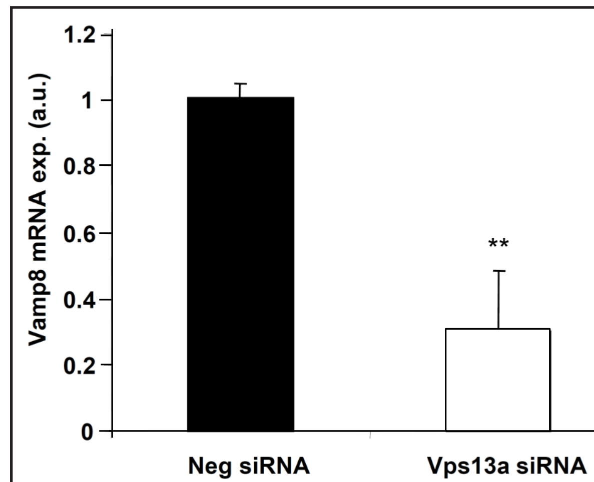

Fig. 3. Chorein sensitive VAMP8 transcript levels in PC12 cells. Arithmetic means ± SEM (n=5) of transcript levels encoding vesicle-associated membrane protein 8 (VAMP8) in pheochromocytoma (PC12) cells treated with unspecific (Neg. siRNA, black bar) and chorein specific (Vps13 siRNA, white bar) silencing RNA. **significant difference (p<0.01; unpaired t-test).

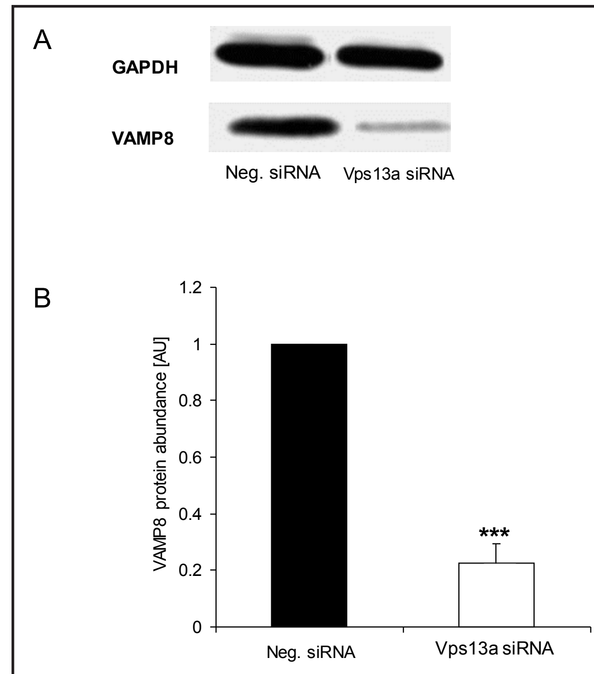

Fig. 4. Chorein sensitive VAMP8 protein expression in PC12 cells. A. Original Western blot showing protein abundance of GAPDH (upper lanes) and vesicle-associated membrane protein 8 (VAMP8, lower lanes) in pheochromocytoma (PC12) cells treated with unspecific (left, control) and chorein specific (right, siChorein) silencing RNA. B. Arithmetic means ± SEM (n=5) of vesicle-associated membrane protein 8 (VAMP8) in pheochromocytoma (PC12) cells treated with unspecific (Neg. siRNA, black bar) and chorein specific (Vps13 siRNA, white bar) silencing RNA. ***significant difference (p<0.001; unpaired t-test).

As illustrated in Fig. 4, the VAMP8 protein abundance was significantly lower in chorein silenced PC12 cells than in cells transfected with negative siRNA.

Discussion

The present study confirms the impact of chorein on dopamine release from pheochromocytoma (PC12) cells [18]. The present observations further reveal that chorein participates in the regulation of vesicle formation. The effect of chorein in PC12 cells is thus similar to the effect in blood platelets. As shown previously [16], in platelets drawn from patients with chorea-acanthocytosis activation-induced platelet secretion from dense granules and alpha granules was significantly less than in platelets drawn from healthy individuals.

The present study further sheds some light on possible mechanisms involved. Similar to what has been observed in megakaryocytes [16], chorein silencing decreases the expression of vesicle-associated membrane protein 8 (VAMP8). The protein is required for granule

secretion [21, 22]. VAMP8 forms core SNARE complexes with different partners in different tissues or cell lines [23]. In PC12 cells and NRK cells VAMP8 interacts with syntaxin 7 and 8, as well as Vtilb [24, 25]. In platelets, VAMP8 targets syntaxin 4 [21]. VAMP8 deficiency compromizes thrombin-induced secretion of blood platelets [26]. Conversely, VAMP8 overexpression results in hyperreactive platelets [27]. In pancreatic acinar cell granule membranes, VAMP8 interacts with syntaxins 2 and 3 as well as SNAP-23 [23]. VAMP8 participates in the formation and is required for fusion of pancreatic acinar cell granules [23, 28], which are significantly smaller in VAMP8 knockout mice than in wild type mice [23].

In cytotoxic T cells VAMP8 interacts with Vtib [29], in kidney with syntaxins 3 and 4 [30], in mast cells with SNAP23 and syntaxin 4 [31]. Given the function of VAMP8, the protein is most likely involved in the effects of chorein on degranulation. However, at this stage, we cannot predict to which extent the down-regulation of VAMP8 contributes to the impaired formation of vesicles and/or dopamine release.

The present study did not address the signaling involved in altered VAMP8 expression, vesicle formation and dopamine release following chorein silencing. Chorein has previously been shown to interact with PI3K [1, 2], which is known to participate in the regulation of degranulation in a wide variety of cells [32-40]. Possibly, chorein similarly modifies degranulation of those cells.

PI3K signaling is further known to confer survival of a wide variety of cells including cancer cells [41-55] and neurons [56-59]. Chorein is particularly important for function and viability of neurons and skeletal muscle cells [6, 11]. Moreover, chorein polymerizes cortical actin filaments [3, 17], which is expected to affect a variety of cellular functions including exocytosis [3, 60-69]. Along those lines several cytoskeletal structures including actin microfilaments, microtubules and desmin-, as well as cytokeratin-intermediate filaments are disorganized in human fibroblasts from chorea-acanthocytosis-patients [17]. Interestingly, actin redistribution has been previously described to regulate catecholamine secretion in PC12 cells [70, 71]. However, whether chorein-associated actin cytoskeleton polymerization contributes to the observed chorein sensitive dopamine release in PC12 remains to be elucidated.

In conclusion, the present observations reveal that chorein participates in the regulation of vesicle formation and dopamine release, effects which may contribute to the pathophysiology of chorea-acanthocytosis.

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Disclosure Statement

The authors declare that they have nothing to disclose.

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