Novel vertebrate- and brain-specific driver of neuronal outgrowth

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ABSTRACT

During the process of neuronal outgrowth, developing neurons produce new projections, neurites, that are essential for brain wiring. Here, we discover a relatively late-evolved protein that we denote Ac45-related protein (Ac45RP) and that, surprisingly, drives neuronal outgrowth. Ac45RP is a paralog of the Ac45 protein that is a component of the vacuolar proton ATPase (V-ATPase), the main pH regulator in eukaryotic cells. Ac45RP mRNA expression is brain specific and coincides with the peak of neurogenesis and the onset of synaptogenesis. Furthermore, Ac45RP physically interacts with the V-ATPase V0-sector and colocalizes with V0 in unconventional, but not synaptic, secretory vesicles of extending neurites. Excess Ac45RP enhances the expression of V0 subunits, causes a more elaborate Golgi, and increases the number of cytoplasmic vesicular structures, plasma membrane formation and outgrowth of actin-containing neurites devoid of synaptic markers. CRISPR-cas9-mediated Ac45RP knockdown reduces neurite outgrowth. We conclude that the novel vertebrate- and brain-specific Ac45RP is a V0-interacting constituent of unconventional vesicular structures that drives membrane expansion during neurite outgrowth and as such may furnish a tool for future neuroregenerative treatment strategies.

1. Introduction

Neurite outgrowth, one of the central steps in early brain development, is a dynamic process driven by actin- and microtubule rearrangements, resulting in axons and dendrites that interconnect and form neuronal networks (Miller and Suter, 2018). During initial elongation processes of the nascent axon, new membrane components are delivered via the secretory pathway and transported to the tip of the extending neurite (Ledesma and Dotti, 2003; Penfninger, 2009). Axonal outgrowth continues by the delivery of new membrane components to the growth cone through vesicles that originate from the Golgi (D’Alessandro and Meldolesi, 2019; Meldolesi, 2010). Later in development the new membrane is provided to dendrites mostly by vesicles derived from their ‘Golgi outposts’ (Penfninger, 2009). The membrane deliveries are accomplished by two types of unconventional secretory vesicles, namely plasmalemma precursor vesicles containing the tetanus toxin-insensitive vesicle membrane-associated membrane protein (TI-VAMP, also referred to as VAMP7) and the VAMP4-positive vesicles, the so-called enlargeosomes (Coco et al., 1999; Meldolesi, 2010) that function in the early stages of neuronal outgrowth (Meldolesi, 2010). TI-VAMP-positive vesicles have been identified in growth cones of neurites where they participate in the more mature outgrowth process.

Abbreviations: Ac45RP, Ac45-related protein; ATP6AP1, ATP6-associated protein-1; ATP6AP1l, ATP6AP1-like; AT20, mouse pituitary corticotrope tumor cells; BDNF, brain-derived neurotrophic factor; BRET, bioluminescence resonance energy transfer; CB, cerebellum; CRISPR, clusters of regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9; DG, dentate gyrus; dbcAMP, dibutyryl cyclic adenosine monophosphate; DIV, days in vitro; DSCAM, down syndrome cell adhesion molecule; E14.5, embryonic day 14.5; ER, endoplasmic reticulum; EST, expressed sequence tag; HA, hemagglutinin; N2a, neuroblastoma 2a; PFC, prefrontal cortex; OB, olfactory bulb; PTSD, posttraumatic stress disorder; SNARE, vesicular soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptor; SVZ, subventricular zone; SV2, synaptic vesicle protein 2; TI-VAMP, tetanus toxin-insensitive vesicle membrane-associated membrane protein; Tuj1, neuron-specific class III beta-tubulin; V-ATPase, vacuolar proton ATPase; V0, membrane sector of the V-ATPase; V0-0, cytoplasmic sector of the V-ATPase.

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2. Materials and methods

2.1. Animals

Female BALB/c surplus mice (12 months old) were obtained from the Central Animal Facility (Radboud University, Nijmegen, The Netherlands). Timed, pregnant mice with a C57Bl/6J background were euthanized quickly by means of cervical dislocation to prevent stress. The day of vaginal plug observation was defined as E0.5. E10.5 to P140 mouse brains were carefully dissected, deep frozen in liquid nitrogen and kept at -80 °C until RNA isolation. Rat E18.5 embryos were derived from timed-pregnant Wistar rats (Harland laboratories, B.V., Boxmeer, The Netherlands). All animal experiments were approved by the Animal Ethics Committee of Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands), and were conducted in accordance with Dutch legislation (Herziene Wet op Dierproeven, Art 10.a.2, 2014) and the European Communities Council Directive 86/609/EEC for animal welfare.

2.2. Bioinformatics analysis

Expressed sequence tag (EST), mRNA and genomic sequences were derived from NCBI using the TBLASTN algorithm (http://www.ncbi.nlm.nih.gov/), Ensembl Genome Browser (http://www.ensembl.org/index.html) and UCSC Genome Browser (http://genome.ucsc.edu/) using the BLAT algorithm. Nucleotide sequences were translated using the Expasy - Translate tool (http://www.expasy.ch/tools/dna.html). Alignments were made using Clustal Omega http://www.phylogeny.fr/version2/cgi/one_task.cgi?task_type=treedyln (Chevenet et al., 2006; Dereeper et al., 2010, 2008). An overview of search references is listed in Supplementary Table 1. For the prediction of protein domains and post-translational modifications, the public CBS Prediction Server (http://www.cbs.dtu.dk/services/) was used.

2.3. Molecular cloning

For molecular cloning of the full-length nucleotide sequence of mouse Ac45RP, cDNA synthesized from mouse olfactory bulb (OB) total RNA was used. For PCR amplification, High-Fidelity PCR Enzyme Mix (Thermo scientific) with primers based on the predicted mouse gene sequence EG435376 (mmAc45RP5' UTR forward primer: 5'-CAAATTACAGATGCGTTGGAATGGAATGGGTTAATGTAATAGTTTCGAGATCTG-3' and mmAc45RP3' UTR primer 5'-AGCTTTTGGAGATGGAATGGGTTAATGTAATAGTTTCGAGATCTG-3') was used. For cloning of the haemagglutinin (HA)-tagged mouse Ac45RP (mAc45RP-HA) expression construct, a PCR was performed using the full-length mouse Ac45RP cDNA clone as template and primers MmAc45RP 5'UTR-FW-2 5'-GGGGGAATTCACCAATTAACAGATATCGGTGGGAATGGGTTAATGTAATAGTTTCGAGATCTG-3' and MmAc45RP-HA-RV 5'-CCCCGGATCCATGCGTTGGAATGGAATGGGTTAATGTAATAGTTTCGAGATCTG-3' was used. For cloning of the V-ATPase V0 sector containing the rotation mechanism that translocates the protons over the membrane, while the second main sector of the V-ATPase, the cytoplasmic V1 sector, supplies the required energy by ATP hydrolysis (reviewed by Schoonwoert and Martens, 2001). For proton pumping, the fully assembled V-ATPase enzyme complex (V0 plus V1) is necessary. However, as mentioned and independent from V1, the V0 sector may also regulate exocytotic membrane fusion events (Bayer et al., 2003; Di Giovanni et al., 2010; Hiesinger et al., 2005; Liegeois et al., 2006; Morel and Poua-Guyon, 2015; Peters et al., 2001; Poua-Guyon et al., 2013; Zhang et al., 2008). For instance, recent evidence suggests that in hippocampal neurons V0 has a modulatory role in exocytosis prior to vesicle docking and priming (Bodzeta et al., 2017).

The V0-sector of the mammalian V-ATPase interacts with the ubiquitously expressed Ac45 protein (ATP6AP1) that is localized to regulated secretory vesicles (Supek et al., 1994) and controls the proton pumping activity of the V-ATPase (Jansen et al., 2010a, 2008; Jansen et al., 2012). Ac45-deficient patients suffer from ATP6AP1-congenital disorder of glycosylation (CDG) with a diversity of symptoms such as immunodeficiency with pathophysiology, cognitive impairment or cutis laxa and pancreatic insufficiency (Dimitrov et al., 2018; Jansen et al., 2016), pointing to a critical and cell-specific role of this protein in the regulation of V-ATPase functioning. Ac45 has been previously considered to be an accessory subunit of the V-ATPase (Jansen and Martens, 2012; Supek et al., 1994). However, recent cryo-electron microscopy and mass spectrometry analyses have shown that the Ac45 protein is an integral subunit of the V-ATPase V0-sector (Abbás et al., 2020; Wang et al., 2020a, b). Furthermore, Ac45 appears to regulate V0-assembly in the ER, and V1-/V0-assembly and -disassembly in the later stages of the secretary pathway (Abbás et al., 2020; Guida et al., 2018; Jansen et al., 2016; Roh et al., 2018). In the present study, we identified and characterized a novel vertebrate- and brain-specific paralog of the Ac45 protein which is encoded by ATP6APIL and termed it Ac45-related protein (Ac45RP). Like Ac45, Ac45RP interacts with the V0-sector, but is localized to TI-VAMP/VAMP4-positive unconventional, and not synaptic, secretory vesicles and drives neuronal membrane delivery during neurite outgrowth. As such, our study links for the first time the V-ATPase V0-sector to the process of neurite outgrowth.
RV-3 5′-GGCAGCACTTTGTTAATTTGTTA-3′ for mouse Ac45RP. For mouse actin: Mm B-actin-FW 5′-CTGACCTGAATGCCACCTT-3′ and Mm B-actin-RV 5′-AGAGCCATACAGGGAGCAGCA-3′. The annealing temperature was 58 °C and 40 cycles were run. For quantitative PCR analysis (qPCR), a 1:16 dilution of the total cDNA pool was used in a 10-μl reaction using the SensiFAST SYBR/noROX qPCR kit (Bioline #BIO 98020) and a Rotor-Gene™ 6000 real-time analyzer (Corbett Research, Sydney, Australia).

For mouse Ac45RP amplification, forward primer 5′-AGA-
TACTGGACAGGAAGAATGAGG-3′ and reverse primer 5′-TCTGACTCTGTTCCACCTCCAC-3′, and for mouse Ac45, forward primer 5′-TCTGATGGGCCAGGACCAT-3′ and reverse primer 5′-AGCAGATGGGATTAGGACGC-3′, Tyrösine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta peptide (Y-Whaz, forward primer 5′-AAATTATCAGGAAATTGGTCCAGTC-3′ and reverse primer 5′-TGATCCACCCAGGGTTTGT-3′) or β-actin (forward primer Mm B-actin-FW 5′-CTGACCTGAATGCCACCTT-3′ and MmB-actin-RV 5′-AGAGCCATACAGGGAGCAGCA-3′) were used. qPCR data were analyzed by setting the lowest Ct value to 1 and calculating the relative Q-values of the genes of interest. The normali-


cation factor for the reference genes was determined using the GenORM program (medgen.ugent.be/genorm) and used to normalize the Q-values (Valles et al., 2011). Individual experiments were performed in triplicate. For the analysis of V0-subunit expression in N2a cells, 25-μl reactions using the SensiFAST SYBR/noROX qPCR kit (Bioline #BIO 98020) in a Biocart CFX96 Real-Time System were performed. The following primers were used for V0c: MmATP6V0bFW1 5′-ATCTTCTTGAGAGCGGTGGC-3′ and MmATP6V0bRV1 5′-GCCCAAGATCACCTT-3′, MmATP6V0cFW1 5′-CATCACCCTCTAGCAGGGT-3′ and MmATP6V0cRV1 5′-ATCATGCCACGCAAGTCTG-3′, V0α1: MmAtp6V0a1FW1 5′-AGAGAAGAAGCATTTGGGAACT-3′, and MmAtp6V0a1RV1 5′-AAACAATCTTGCCAAGTACGCTT-3′, ATP6P2: MmAt-
p6ap2FW1 5′-CTATGGTGGGAACGCAGTGG and MmAtp6ap2RV1 5′-AGAGACCTTTGGTCTTCTCTTG-3′ Ac45RP: MmAc45RPFW1 5′-CATCACCCTCTAGCAGGGT-3′ and MmAc45RP-RV1: 5′-GAGTACGGTTAGGAGAAAGCAGT-3′. For specifically measuring wild-type Ac45RP levels the following primers, generating an amplicon from the CRISPR-cas9 targeted region, were used: MmAc45RPcrisp-FW1 5′-GAGTACGGTTAGGAGAAAGCAGT-3′ and MmAc45RPcrisp-RV1 5′-CATCACCCTCTAGCAGGGT-3′. Individual experiments were performed in triplicate. qPCR data were analyzed by setting the lowest Ct value to 1 and using the delta-delta-Ct method.

2.5. Cell culture

Mouse neoblastoma N2a cells were cultured in complete MEM medium (Gibco, #41090) containing 10 % fetal bovine serum (Gibco #A1608). Cells were differentiated into dopaminergic neuron-like cells in MEM medium containing 0.1 % BSA (Evangelopoulos et al., 2005) with 0.5 mM or 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP, Sigma-Aldrich #D6027) for 24 h (Tremblay et al., 2010). To analyze N-glycosylation of Ac45RP, cells were cultured in the presence or absence of 1 μM tunicamycin for 24 h. Mouse anterior pituitary AtT-20 cells were cultured in complete DMEM (Gibco #41966) containing 7% horse serum and 7% fetal bovine serum. All cells were maintained at 37 °C and 5.5 % CO2 atmosphere.

2.6. Transient and stable cell transfections

One day prior to transfection, cells were seeded on 12-wells plates at a confluence of ~70 %. Cells were transfected using Lipofectamine LTX (Invitrogen #15381800) according to the manufactures’ guidelines. For the generation of stably transected N2a cell lines, cells were seeded on a 10-cm culture dish at a confluency of 70 % and transfected using Lipofectamine 2000 according to the manufactures’ guidelines. One day following transfection, cells were split (1:10) and cultured in MEM/10 % FCS. Two days following transfection, culture medium was replaced by selection medium (culture medium containing 600 μg/mL neomycin). After 14 days, individual clones were isolated and trans-

ferred into 48-wells plates. Finally, semi-confluent cultures were transferred to T25 culture flasks and subjected to further analysis.

2.7. Mouse and rat primary cortical neuron cultures

Cortices of E14.5 mouse or E18.5 rat embryonic brains were dissectioned and collected in ice-cold HBSS without Mg2+ and Ca2+ (Gibco #14175). Rat tissue was trypsinized using 0.5 % trypsin in HBSS con-

taining Glutamax (Invitrogen) and pen/strep at 37 °C for 15 min and following washing steps with HBSS, transferred into seeding medium (Neurobasal (Gibco # 21103049) containing 10 % FCS and Glutamax). Mouse tissue was directly transferred to seeding medium. The tissue was subsequently triturated using a polished Pasteur’s pipet to dissociate the cells, transferred to a syringe and filtered through a nylon filter (pore size 80 μm). The number of isolated cells was counted and cells were seeded onto poly-D-lysine-coated (for rat cells) or poly-L-ornithine-coated (for mouse cells) coverslips in 24-wells-plates (~2 x 10^5 cells/well) in seeding medium. After 4 h, the seeding medium was replaced by culture medium (Neurobasal containing B27 supplement, Glutamax and 10 μM citosine-arabinofuransoside, CAR (Sigma C1768)). Cells were cultured at 37 °C under 5.0 % CO2 for the time periods indicated.

2.8. Antibodies

A 15-aminio acid synthetic peptide representing a conserved region in the cytoplasmic tail of mouse Ac45RP (GSQGAEYELRNNQ) was used as an antigen for the generation of a rabbit anti-Ac45RP polyclonal antibody (#2086) that was subsequently affinity purified against the peptide (Open Biosystems, Thermo Scientific). The anti-mouse Ac45 antibody (directed towards Ala271-Thr283 and Leu443-Ile457 of mouse Ac45) was obtained from Dr. J. Creemers (Catholic University Leuven, Belgium). Anti-SV2 mouse monoclonal antibody was obtained from Developmental Studies Hybridoma Bank, anti-VAMP2 (Syb2, clone 69.1) monoclonal antibody was from Dynatech Systems (Germany) and the anti-TI-VAMP (SYBL1) monoclonal antibody 158.2 was from Abcam. Anti-Giall Fibrillary Acidic protein (GFAP) was from Sigma (G8393) and anti-NeuN antibodies were from Millipore (ABN91). Anti-VAMP4 monoclonal antibodies (Cocucci et al., 2008) were kindly provided by Drs. J. Meldolesi and G. Racchetti (Vita-Salute San Raffaele University, Milano, Italy), and the anti-rat 16K-N antibody (Nezu et al., 1992) by dr. S. Ohkuma (Kanazawa University, Ishikawa, Japan). Rabbit-anti-HA (HA-probe (Y-11) was from Santa Cruz Biotechnology.

2.9. Protein isolation and Western blot analysis

5 x 10^5 cells were grown for 24 h in a 6-wells plate (Greiner). To isolate membranes, cells were collected in 1 mL PBS. Following centri-
fugation (5 min, 500 g, 4 °C), cells were resuspended in homogenization buffer (0.32 M sucrose, 10 mM HEPEs pH 7.4, 2 mM EDTA and protease inhibitors (Roche)) using a pellet pestle and 10 freeze-thaw cycles. To remove the nuclear fraction, samples were centrifuged 15 min at 1000 g and the supernatant (S1) was subsequently centrifuged for 30 min at 200,000 g in a Sorvall Discovery Micro-Ultracentrifuge (M150SE) using a fixed angle rotor (S120AT6). The pellet was washed in homogenization buffer, centrifuged again for 30 min at 200,000 g and dissolved in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5 % SDS, 50 mM Tris pH8.0, 2 mM EDTA) to obtain the membrane fraction. For Western blotting, proteins were separated using 10 % SDS-PAGE and subsequently transferred to PVDF membrane (Amersham...
Biosciences) and the membrane was incubated with the purified anti-Ac45RP antibody (1:1000) or the anti-Ac45 antisemur (1:5000) and secondary peroxidase-conjugated Goat-anti-rabbit antibodies (Thermo-Fisher, 1:5000) followed by chemiluminescence (LuminLight Plus, Roche). Signals were detected using a Bioimaging system with Labworks 4.0 software (UVP Bioimaging Systems, Cambridge, UK) or ImageQuant LAS4000 (GE Healthcare).

2.10. Immunofluorescence assay

Cells cultured on coverslips were washed twice with PBS and fixed with 4% paraformaldehyde/PBS for 1 h at room temperature (RT). Next, cells were washed with PBS/50 mM NH₄Cl and permeabilized with PBS/0.1% TritonX100 (PBS-T). Subsequently, cells were incubated with the first antibodies rabbit-anti-Ac45RP (1: 500), mouse-anti-VAMP4 (1:500), mouse-anti-TI-VAMP (1:200), anti-VAMP2 (1:1000), mouse-anti-SV2 monoclonal antibodies (1:200), mouse anti-Neuron-specific class III beta-tubulin (Tuj-1, 1:1000), rabbit-anti rat-V-ATPase proteolipid 16k-N (1:200) or rabbit-anti-HA (1:500) in blocking buffer (PBS-T/1% BSA) for 1 h at 4°C. Following PBS washing steps, cells were incubated for 1 h at RT with secondary antibodies (Goat-anti-rabbit-Alexa488 (Molecular Probes, 1: 200) or Goat-anti-mouse-Alexa 568 (Molecular Probes, 1: 200) in blocking buffer. Finally, cells were washed with PBS, MilliQ water, dehydrated with isopropanol, air dried and embedded in Mowiol containing 2.5 μM DAPI to visualize the nuclei. Microscopic imaging was performed using an Olympus FV1000 laser scanning microscope. Co-localization analysis was performed and Manders’ coefficients (M₁ and M₂) were calculated (n = 10) using the JACoP toolbox under Fiji (Bolte and Cordelieres, 2006). Quantitative photon counting was performed using the HyD detector of the Leica SP8x laser scanning microscope using the photon counting mode and LAS-X software. Density measurements were performed using Fiji.

2.11. Immunohistochemistry on mouse brain cryosections

P22 mouse brains were isolated, fixed in 4% PFA/PBS, cryo-protected in 30% sucrose/PBS, deep frozen and sectioned (15 μm). Immunohistochemistry was performed as described previously (Kolk et al., 2009) using the affinity-purified rabbit anti-Ac45RP polyclonal antibody (1:200), anti-NeuN (1:1000), anti-GFAP (1:200). Microscopic analysis was performed using a fluorescence microscope (Leica DM RA fluorescence microscope with DFC340FX camera) and the Fiji free software package. Confocal laser scanning microscopy was done using the Leica SP8x AOB S WLL microscopy system using the Las X software package.

2.12. Analysis of membrane expansion and neurite outgrowth

N2a cells were plated on a black/clear 96-wells tissue culture plate (BD Falcon) in densities of 4000, 3000, 2000 or 1000 cells per well and differentiated during 24 h using 1 mM dbcAMP. Then, cells were washed in 1 x PBS and fixed using 4% paraformaldehyde/PBS overnight at 4°C. Subsequently, cells were washed with PBS / 50 mM NH₄Cl and permeabilized with PBS / 0.1% Triton X-100 (Sigma) for 15 min at room temperature, washed three times 5 min with 1 x PBS and stained with 50 μM HCS Cell Mask Red (Invitrogen)/DAPI/PBS for 30 min at RT. Cells were then washed three times with 1 x PBS for 5 min and stored in PBS at 4°C.

Analysis of membrane staining was performed using the BD Pathway 855 spinning disc confocal fluorescent microscope. A 3 × 4 montage data set was collected using a 20 x objective for all wells. Image data were analyzed using Fiji software. A threshold was set for the cell staining such that only the membrane became visible. In addition, using the watershed option, close-lying nuclei were separated. To obtain per cell a quantitative measure of membrane expansion following differentiation, the ratio of the amount of membrane and the number of nuclei was determined. An automated analysis tool was designed by Dr. Louis Wolf (Microscopic Imaging Centre, RIMLS) and is available as ImageJ plugin IntDenPerCell.

For neurite outgrowth measurements, the Incucyte Live-Cell analysis system and software were used. Cells were plated on 96-wells plates (12 000 cells/well, six wells/cell line) and imaging started one hour following plating. Per well, each 45 min nine regions of interest (ROI) were imaged in the phase mode. Image segmentation of neurites and analysis of cell body clusters and neurite development was performed using the Incucyte NeuroTrack analysis software module.

2.13. CRISPR-cas9-mediated downregulation

CRISPR-cas9 nickase-mediated dual guide-RNA (gRNA) targeting was used for genome editing of N2a cells using pcCas9D10A-GFP (Addgene plasmid #44720) and two gRNAs (Ac45RP guide A and Ac45RP guide B) that were cloned into the gRNA_Cloning vector (Addgene plasmid #18424). gRNA sequences were designed for the N2a Ac45RP exon4 genomic sequence using the Zhang lab guide-RNA design tool (CRISPR.mit.edu). Primer sequences for gRNA cloning were: Ac45RP-GuideA-forward: 5′- TTTCTTGCTTTATATATCTTGGAAGGACGAAACACCGTGATGGCTGAACGTACTCC-3′, Ac45RP-GuideB reverse: 5′- TTTCTTGCGTTATATATCTTTGAGAAAGGACGAAACCGTGATGGCTGCTTACAC-3′. Forward and reverse oligos were annealed and extended using Phusion polymerase (New England Biolabs), then the extended oligo duplexes were recombined into AffiII-linearized gRNA cloning vector using the Gibson Assembly Mastermix (New England Biolabs) and incubated overnight (12 h). The two N2a cell lines (1F3 and 1G11) were cultured from the sorted fluorescent cells. These were trypsinized and GFP-fluorescent cells were selected using Fluo-3 (Invitrogen) and M200 (Thermo Scientific) at 1:10 dilution to discriminate GFP in fluorescence. For each cell line, 10 000 cells were imaged in the phase mode. Image segmentation of neurites and analysis of cell body clusters and neurite development was performed using the Incucyte NeuroTrack analysis software module.

2.14. Bioluminescence Energy Transfer (BRET) analysis

To perform protein–protein interaction studies on recombinant Ac45RP and Ac45 proteins, and a number of recombinant V-ATPase V₀ subunits ex vivo, we performed a BRET analysis. Cloning of the expression construct pcDNA-Ac45RP-eYFP for BRET analysis is described in section 2.3 Molecular Cloning, and expression constructs pcDNA-Rluc-V-ATPase-a3, pcDNA-Rluc-V-ATPase-d, pcDNA-Rluc-V-ATPase-c, pcDNA-Rluc-V-ATPase-c” and pcDNA-Ac45-eYFP have been described previously (Feng et al., 2008). BRET assays were performed as previously described (Feng et al., 2008; Kroeger et al., 2001).
2.15. Statistics

Data are presented as means ± SEM. Statistical evaluation was performed using a Student’s t-test.

3. Results

3.1. Identification and phylogenetic analysis of a novel paralog of the Ac45 protein

To identify protein sequences related to the Ac45 (ATP6AP1) amino acid sequence, we searched public mouse genomic and EST databases with the carboxy-terminal part of the mouse Ac45 protein sequence (the most conserved portion of the protein) as bait, and found two novel sequences structurally related to Ac45. One sequence represents a highly N-glycosylated, lung- and kidney-specific Ac45 isoform (termed Ac45-like protein, Ac45LP) that we previously characterized and is present exclusively in non-mammalian tetrapod species (Fig. 1C; Jansen et al., 2010b). In the present study, we characterize the second Ac45-related gene of unknown function (ATP6AP1L) that is localized on mouse chromosomal region 13C3 and human chromosome 5 (5q14.2). Cloning of a full-length mouse ATP6AP1L cDNA revealed a protein of 336 amino acids (apparent molecular weight of ~37 kDa) containing a signal of a full-length mouse gene of unknown function (Jansen et al., 1998) (Fig. S1).

To allow protein expression studies, we generated an affinity-purified anti-Ac45RP antibody that we used for Western blot analysis of N2a cell lysates treated with the protein N-glycosylation inhibitor tunicamycin. The analysis showed the specificity of the antibody and revealed that, like the Ac45 protein (Holhuis et al., 1999), Ac45RP is a highly N-glycosylated protein (Fig. 1B).

Using the newly identified mouse Ac45RP amino-acid sequence as bait, we found Ac45RP orthologues in vertebrate but not invertebrate species. To construct a phylogenetic tree, we used the ancestral sequence present in the Ac45-luminal domain ((RPSRVAR, Louagie et al., 2008) and a longer carboxy-terminal tail that lacks the autonomous Ac45-routing sequence (Jansen et al., 1998) (Fig. S1). The Ac45RP protein is present only in vertebrate species such as human (Homo sapiens, Hs), chimpanzee (Pan troglodytes, Pt), gorilla (Gorilla gorilla gorilla, Gg), armadillo (Dasypus novemcinctus, Dn), orca (Orcinus Orca, Oc), dog (Canis familiaris, Cf), rat (Rattus norvegicus, Rn), mouse (Mus musculus, Mm), oppossum (Monodelphis domestica, Md), chicken (Gallus gallus, Gg), zebra finch (Taeniopygia guttata, Tg), duck (Anas platyrhynchos, Ap), lizard (Anolis carolinensis, Ac), frog (Xenopus tropicalis, Xe), Xenopus laevis, (Xi), platypus (Ornithorhynchus anatinus, Oa), turtle (Chrysemys picta bellii, Cpb), zebra fish (Danio rerio, Dr), stickleback (Gasterosteus aculeatus) and tilapia (Oreochromis niloticus, On), and not in non-vertebrates such as lancelet (Branchiostoma floridae, Bf), mosquito (Aedes aegypti, Aa), fruit fly (Drosophila melanogaster, Dm), and worm (Caenorhabditis elegans, Ce).

Fig. 1. Ac45RP is an N-glycosylated transmembrane protein and vertebrate-specific Ac45 family member. (A) The structural organisation of the Ac45RP protein resembles that of the Ac45 protein. The level of amino-acid sequence identity (%) identity between Ac45RP and Ac45 is highest in the portion comprising the transmembrane region and the adjacent region containing the pair of cysteines. Note that Ac45RP has a shorter N-terminal region lacking the cleavage site (CS) of the endoprotease furin. SP: signal peptide sequence; C: cysteine; TM: transmembrane region. (B) Western blot analysis of endogenous Ac45RP in mouse neuroblastoma (N2a) cells. Cells were cultured in the absence (-) or presence (+) of tunicamycin and lysed, and the protein lysates were analysed using the anti-Ac45RP antibody. (C) Phylogenetic analysis of Ac45, Ac45-like protein (Ac45LP) and Ac45RP. The Ac45RP protein is present only in vertebrate species such as human (Homo sapiens, Hs), chimpanzee (Pan troglodytes, Pt), gorilla (Gorilla gorilla gorilla, Gg), armadillo (Dasypus novemcinctus, Dn), orca (Orcinus Orca, Oc), dog (Canis familiaris, Cf), rat (Rattus norvegicus, Rn), mouse (Mus musculus, Mm), oppossum (Monodelphis domestica, Md), chicken (Gallus gallus, Gg), zebra finch (Taeniopygia guttata, Tg), duck (Anas platyrhynchos, Ap), lizard (Anolis carolinensis, Ac), frog (Xenopus tropicalis, Xe), Xenopus laevis, (Xi), platypus (Ornithorhynchus anatinus, Oa), turtle (Chrysemys picta bellii, Cpb), zebra fish (Danio rerio, Dr), stickleback (Gasterosteus aculeatus) and tilapia (Oreochromis niloticus, On), and not in non-vertebrates such as lancelet (Branchiostoma floridae, Bf), mosquito (Aedes aegypti, Aa), fruit fly (Drosophila melanogaster, Dm), and worm (Caenorhabditis elegans, Ce).
distances of Ac45RP to the ancestral Ac45 genes in Drosophila and C. elegans are larger than those of Ac45 and Ac45LP, we conclude that the third, vertebrate-specific branch harbouring Ac45RP evolved later than the two other branches. The Ac45RP protein is evolutionarily highly conserved, with the highest degree of sequence identity found in the region containing the transmembrane domain (Fig. S1).

3.2. Expression of Ac45RP mRNA and protein in developing and adult mouse brain

Quantitative PCR analysis revealed that the onset of Ac45RP mRNA expression in the developing mouse brain was at E 13.5 (i.e., the peak of neurogenesis; Gotz and Huttner, 2005) and its expression slightly increased postnatally. Interestingly, Ac45RP mRNA expression greatly increased from postnatal day (P) 7 onwards and peaked at P14 with relatively high expression levels thereafter (Fig. 2A), in particular in the prefrontal cortex (PFC), cerebellum (CB) and especially the OB (Fig. 2B).

Semi-quantitative PCR analysis showed that, except for a relatively low level of expression in ovary, in adult mice Ac45RP mRNA is expressed exclusively in the brain, with the highest level of expression in the OB (Fig. 2B, C). In contrast, Ac45 mRNA is ubiquitously expressed, and enriched in neuronal and neuroendocrine tissues (Jansen et al., 2016; Louagie et al., 2008) (Fig. 2C). The fact that Ac45RP mRNA is expressed in mouse neuroblastoma 2A (N2a) cells, albeit at low levels, but not in mouse neuroendocrine anterior pituitary AtT-20 cells (Fig. 2D) indicates that it is expressed specifically in neuronal and not neuroendocrine cells.

To study Ac45RP protein expression in mouse brain, we performed Western blot and immunocytochemical analyses. In line with the Western blot analysis of N2a cell lysates, we observed the ~37-kDa Ac45RP protein and its glycosylated form (~80 kDa), but not its ~55-kDa form, in OB and hippocampal tissue lysates of adult mice (Fig. 2E).

Immunostainings of P22 mouse brain sections confirmed the relatively high plexiform layer, mitral cell layer, external plexiform layer and granular cell layer (Fig. 2H). Furthermore, we detected Ac45RP protein and its glycosylated form (~80 kDa), but not its ~55-kDa form, in OB and hippocampal tissue lysates of adult mice (Fig. 2E). The Ac45RP protein is expressed in the subventricular zone (SVZ) of the OB (Fig. S1). Quantitative colocalization analysis showed that in developing network formation in eight- and 22-DIV cultures of mouse cortical neurons Ac45RP significantly colocalized with VAMP4 and TIA-VAMP, but not with SV2; Manders’ coefficients for VAMP4 colocalization: M1 = 0.82 ± 0.02, M2 = 0.73 ± 0.03 (eight-DIV; Fig. 4B, D) and M1 = 0.72 ± 0.02, M2 = 0.71 ± 0.02 (22-DIV; Fig. S3B, C, E); Manders’ coefficients for TIA-VAMP colocalization: M1 = 0.69 ± 0.02, M2 = 0.67 ± 0.04 (eight-DIV; Fig. 4C, D) and M1 = 0.69 ± 0.04, M2 = 0.67 ± 0.03 (22-DIV; Fig. S3D, E); Manders’ coefficients for SV2 colocalization: M1 = 0.21 ± 0.03, M2 = 0.36 ± 0.05 (eight-DIV; Fig. 4A, D) and M1 = 0.34 ± 0.04, M2 = 0.50 ± 0.03 (22-DIV; Fig. S3A, E). In eight-DIV neuronal cultures, Ac45RP was expressed only in TuJ1-positive and thus neuronal cells (Fig. 4B). We conclude that in differentiating mouse and rat primary cortical neurons Ac45RP localizes to pools of VAMP4/-TIA-VAMP-positive non-secretory vesicles, clearly distinct from SV2-positive (immature) synaptic vesicles.

3.4. Physical interaction between Ac45RP and V0-subunit c” but not V0-subunits c, a3 and d

The V-ATPase assembly factor Act45 interacts with the V0-sector of the proton pump (Supek et al., 1994), more specifically with subunits V0c, V0c” and V0a3, but not V0d (Feng et al., 2008). Immunolocalization studies on rat primary neurons revealed that V0c-subunits V0c and V0d colocalized with the Ac45RP protein in varicosities and at the tips of the extending neurites (Fig. 5A, B). We therefore decided to explore whether Ac45RP physically interacts with the V-ATPase V0-sector. For this purpose, we performed a BRET assay in COS-1 cells with recombinant Ac45-eyFP and Ac45RP-eyFP proteins and recombinant Rluc-V0d, Rluc-V0-c, Rluc-V0-c” and Rluc-V0-a3 proteins. This analysis confirmed the interaction between the Ac45-eyFP protein and the V0c/c”-subunits (Feng et al., 2008), and revealed a physical interaction between Ac45RP-eyFP and Rluc-V0-c”, albeit less strong than between Ac45RP-eyFP and Rluc-V0-c” (Fig. 5C). Ac45RP-eyFP did not interact with the Rluc-V0-c, Rluc-V0-a3 or Rluc-V0d subunits (Fig. 5C). Thus, both Ac45 and Ac45RP interact with the V0-sector of the V-ATPase.

3.5. Transient overexpression of Ac45RP provokes extensive outgrowth of neurites

Next, we transiently overexpressed HA-tagged Ac45RP in N2a cells and in embryonic mouse primary cortical neurons. Immunostainings showed that in the transfected N2a cells the recombinant Ac45RP-HA protein was localized to vesicular structures in the cytosol, and in varicosities and growth cones of the neurites (Fig. 6A), comparable with the localization of the endogenous Ac45RP protein in rodent primary neurons (Fig. 3). In non-transfected N2a cells and in cells expressing moderate Ac45RP-HA levels, Ac45RP, SV2 and VAMP2 were expressed in neurites (data not shown). Intriguingly, N2a cells expressing a high level of the Ac45RP-HA protein were characterized by VAMP4-positive neurites containing a high number of filopodia and a high degree of branching (Fig. 6C, asterisks), while most of the neurites did not contain SV2 or VAMP2 (Fig. 6A, B, asterisks). Thus, Ac45RP-HA-overexpressing cells apparently led to the presence of VAMP4, but not SV2- or VAMP2-containing, membrane structures. Ac45RP-HA-positive vesicles were observed in the tips of extending neurites and filopodia that were positively stained for actin (Fig. 6D, inset). Immunostainings showed that three-DIV mouse primary cortical neurons transfected with the Ac45RP-HA expression construct had developed HA-positive, SV2-
lacking neurites (Fig. 6E). Interestingly, Ac45-RP-HA-transfected fiveDIV mouse primary cortical neurones had developed an Ac45-HA-positive neurite network largely lacking SV2 (Fig. S4). We conclude that overexpression of Ac45RP in N2a cells and in mouse primary cortical neurones provokes the generation of new, immature neurites which do not contain neurotransmitter vesicles.

3.6. Stable overexpression of Ac45RP increases V-ATPase V0-subunit expression and enhances neuronal network formation

We then generated two stable N2a cell lines expressing either Ac45RP-HA (line #2.6; ~2.6 and ~1.5 times increased Ac45RP mRNA and protein expression, respectively) or non-tagged Ac45RP (line #30; ~7 and ~3 times Ac45RP mRNA and protein overexpression, respectively) as well as an empty-vector-transfected stable control cell line (pcDNA3 line #4; unaltered Ac45RP mRNA and protein expression) (Fig. 7B). Since Ac45 is an integral part of the V-ATPase V0-sector and considering its role in V0-assembly (Abbas et al., 2020; Guida et al., 2018), we wondered whether overexpression of Ac45RP affects the expression levels of endogenous V0-subunits. Because of their recently established interactions with the Ac45 protein (Abbas et al., 2020; Wang et al., 2020a, b), we chose to study the mRNA levels of V0c, V0c, and V0a1 and of the V-ATPase assembly factor ATP6AP2 by qPCR analysis. First, we confirmed the increased expression of Ac45RP mRNA in lines #2.6 (~2.6-fold) and #30 (~7-fold) (Fig. 7A). While the mRNA expression levels of V0c, V0c, V0a1, ATP6AP2 and Ac45 were, except for ATP6AP2 (~1.6-fold), not significantly affected by moderate Ac45RP overexpression in line #2.6 cells, stronger Ac45RP overexpression in line #30 cells resulted in significantly increased expression of V0c, V0c, V0a1, ATP6AP2 and Ac45 (~1.7, ~1.6, ~1.7, ~3, and ~1.6-fold, respectively) (Fig. 7A). We conclude that the expression of V0-subunits, and most dramatically of the assembly factor ATP6AP2, is affected by increased Ac45RP expression levels.

Undifferentiated #4 empty-vector-transfected control N2a cells were mostly roundly shaped, little neurite outgrowth was observed, and Ac45RP and SV2 were localized to the Golgi area (Fig. SSA), whereas VAMP4-positive vesicles were distributed over the cytoplasm (Fig. S6A). Intriguingly, undifferentiated #2.6 Ac45RP-overexpressing cells were more flattened than the control cells and displayed short extensions that contained Ac45RP, SV2 and VAMP4 (Figs. S7A and S8A). Four hours following dbcAMP-induced differentiation of the #4 control cells, the SV2 protein was still mostly found in the Golgi (Fig. S5B), whereas in the #2.6 Ac45RP-transfected cells this synaptic vesicle marker was absent from the Golgi and exclusively found in the varicosities and the extremes of the newly formed neurites (Fig. S7B) that were also positive for Ac45RP and VAMP4 (Fig. S8B). Twenty-four hours following differentiation the #2.6 cells had formed an SV2-positive network that was clearly more extensive than that in differentiated #4 control cells (compare Figs. S7C and S5C). The phenotype of the Ac45RP-overexpressing clone #30 cells was similar to that of clone #2.6 cells (Fig. S7D).

Using high-throughput microscopy in combination with whole-cell staining, we quantified the levels of membrane expansion in the N2a Ac45RP-overexpressing cell lines ~2.6 and ~30, and mask-transfected N2a cell line #4 following dbcAMP-induced differentiation by calculating per nucleus the total cell circumference (indicative for the amount of plasma membrane). The total amount of plasma membrane was independent of cell density and positively correlated with the level of Ac45RP overexpression in that the membranes of clones #2.6 and #30 (~1.5 and ~3 times Ac45RP protein overexpression, respectively) were ~1.5 and ~4 times larger than the control line #4 membrane, respectively (Fig. 7C). Thus, overexpression of Ac45RP in N2a cells induced an increase in neuronal membrane formation.

Finally, since excess Ac45RP caused an increase in membrane expansion and neurite outgrowth in N2a cells, we used electron microscopy to study the morphologies of Ac45RP-overexpressing ~30 and control ~4 N2a cells at the subcellular level. No morphological alterations or changes in the number of organelles such as mitochondria, lysosomes and the endoplasmatic reticulum were observed in the two cell lines. However, Golgi-related structures, including lumen vesicular structures, were more abundant in the ~30 Ac45RP-overexpressing than the ~4 control cells (Fig. 7D, E), indicating elevated membrane biogenesis in the overexpressing cells.

3.7. CRISPR-cas9n-mediated Ac45RP knockdown in N2a cells results in decreased neurite outgrowth capacity

We next used CRISPR-cas9n double-guide RNA treatment (CRISPR-Cas9n, Ran et al., 2013) to accomplish Ac45RP downregulation in order to generate genomic deletions in exon 4 of Ac45RP in N2a cells. N2A cells display aneuploidy (Mehrabian et al., 2014) and to increase the chances to hit all Ac45RP alleles two rounds of CRISPR-cas9treatment were performed. Genomic sequence analysis of two clonal N2a cell lines (1F3_3 and 1G11_3) showed deletions ranging from 10 to 102 base pairs (see Methods section). qPCR analysis using primers specifically recognizing the CRISPR target sequence revealed the complete absence of wild-type Ac45RP mRNA in both CRISPR-cas9n-treated cell lines, showing the success of the CRISPR-cas9n approach (Fig. 8A). In the CRISPR-cas9treated line 3 F3_3, the level of total (mutant) Ac45RP mRNA was reduced (~0.7-fold), whereas in line 1G11_3 cells an ~1.2-fold increase of total (mutant) Ac45RP mRNA was observed (Fig. 8B). Ac45 mRNA was still expressed in these Ac45RP knockdown cell lines (Fig. 8C). Quantitative fluorescence imaging and Western blot analysis using the anti-Ac45RP antibody revealed that the deletions also resulted in significantly reduced Ac45RP protein expression levels (~0.6-fold; Fig. 8D, E).

We tested the effect of Ac45RP knockdown on neurite outgrowth by live time-lapse imaging of the cells before and after induction of

Fig. 2. Temporal and spatial mRNA and protein expression of Ac45RP in developing and adult mice. (A) Total brain Ac45RP mRNA expression starts at E13.5 and peaks postnatally. (B) Ac45RP mRNA expression in olfactory bulb (OB), prefrontal cortex (PFC) and cerebellum (CB) peaks at ~P21. Expression levels were determined by qPCR-analysis relative to mouse Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta peptide (Y-Whaz) and mouse ß-actin as reference transcripts. (C) Semi-quantitative RT-PCR analysis of Ac45 and Ac45RP mRNA expression in mouse tissues. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control for RNA integrity. Br, brain; OB, olfactory bulb; Oc, oocytes; Ov, ovary; St, small intestine; Sr, skin; Sp, spleen; Tg, testis; St, stomach; R, - absence of reverse transcriptase (negative control). (D) Quantitative analysis of Ac45 and Ac45RP mRNA expression in mouse N2a and ART-20 cells. Peptidyl prolyl isomerase (mPPIa) and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta peptide (Y-Whaz) served as reference transcripts. (E) Western blot analysis of Ac45RP protein expression in OB and hippocampus (HI). Total OB and HI tissue lysates were analysed with an affinity-purified anti-Ac45RP antibody. (F, G,) Staining of P22 mouse brain sections with the anti-Ac45RP antibody (green) confirms relatively high expression levels of the Ac45RP protein in OB, PFC and CB; in the CB, Ac45RP was found in the Purkinje cells. (H) In the OB, expression of Ac45RP was restricted to the inner plexiform layer (IPL), mitral cell layer (MCL), external plexiform layer (EPL) and glomerular layer (GL), and no Ac45RP expression was found in the granular cell layer (GCL). Sections were counterstained with Nissl (blue). (J) Immunohistochemistry combined with confocal laser scanning microscopy on sagittal P-65 mouse brain sections using Ac45-RP (green) and NeuN (red) antibodies. Ac45RP is located in the membrane of neuronal NeuN-positive cells in the neocortex. I-II-III-IV refer to cortical layers. (J) Immunohistochemistry on sagittal P-65 mouse brain sections using Ac45-RP (green) and GFAP (red) antibodies. White-matter astrocytes in the corpus callosum were devoid of Ac45RP. NC, neocortex, CC, corpus callosum, CP, caudate putamen. Scale bar: 50 μm.
neuronal cell differentiation by serum deprivation and in the presence of dbcAMP. Ac45RP-overexpressing N2a cell line #30 was used as a positive control. In line with our membrane expansion analysis and compared to wild-type cells, differentiated line #30 cells showed a significant increase in neurite outgrowth (calculated as the total neurite length / cell or cell cluster (Fig. 8F)) compared to wild type cells. The 1F3_3 and 1G11_3 Ac45RP-knockdown N2a cells displayed significantly reduced neurite outgrowth during the differentiation process (Fig. 8G). These results show that reduced levels of endogenous Ac45RP negatively affect the ability of the N2a cells to effectively extend their neurites.

Fig. 3. Subcellular distributions of Ac45RP, VAMP4, TI-VAMP and SV2 during neurite outgrowth of rat primary cortical neurons. (A-D) E19 rat cortical neuronal cells were cultured for six hours, fixed and stained with the anti-Ac45RP antibody and monoclonal antibodies against (A) the neural β-tubulin marker TuJ1, the unconventional secretory vesicular markers (B) VAMP4 and (C) TI-VAMP or (D) synaptic vesicle marker SV2. (A) Ac45RP-positive vesicles were localized in the cytoplasm, in the tips of extending neurites and in close proximity to neural β-tubulin. (B) Both Ac45RP- and VAMP4-positive vesicular structures were found in the cytosol and in the newly-formed neurites. (C) Both Ac45RP and TI-VAMP were found in the cytosol and in the tip of the extending neurite. (D) Ac45RP-positive vesicles were present in the cytosol and in the extreme tips of the extending neurites. SV2-positive vesicles were mostly observed in the Golgi region and to some extent in the neurites, but clearly not in the tips of these neurites. Bar, 10 μm.
4. Discussion

In this study, we functionally characterized a novel brain-specific paralog of the V-ATPase assembly factor Ac45 that we named Ac45RP. Ac45RP evolved only in vertebrates and thus later than its relatives Ac45 and the lung- and kidney-specific Ac45LP. The similar structural characteristics of Ac45, Ac45LP and Ac45RP (all are N-glycosylated type I transmembrane glycoproteins with a relatively long amino-terminal luminal domain, a pair of cysteine residues adjacent to the transmembrane domain and a short cytoplasmic tail) indicate that they belong to a small family of V-ATPase regulators. A structural difference among the three family members concerns the absence of a consensus cleavage site for the endoprotease furin in the Ac45RP sequence. This difference is of note because cleavage of Ac45 at the furin site is required for its efficient transport through the secretory pathway (Jansen et al., 2008) and cleaved Ac45 has been found to be an integral part of the V₀-sector of mammalian V-ATPase (Abbas et al., 2020; Wang et al., 2020a, b). Interestingly, cleaved Ac45 and intact Ac45RP are of similar length, implying that an endoproteolytic cleavage event may not be necessary to allow secretory pathway transport of Ac45RP. Therefore, Ac45RP may well represent a constitutively active Ac45 family member.

Our mRNA and protein expression studies in developing mouse brain point to a role for Ac45RP during neural development. For example, the onset of Ac45RP mRNA expression in mouse E13.5 embryos coincided with the peak of neurogenesis. Also, the strong increase in Ac45RP
expression from P0 onwards occurred concomitant with extensive network formation and is in line with the onset of synaptogenesis. The observed spatiotemporal changes in Ac45RP expression may reflect the maturation states of the outgrowth processes in the various brain regions over time. Furthermore, a remarkable finding was the relatively high level of Ac45RP mRNA and protein expression in the mouse OB, a brain region constantly receiving newborn neurons (neuroblasts) (Alvaréz-Buylla and Garcia-Verdugo, 2002; Curtis et al., 2009; Lledo et al., 2006; Whitman and Greer, 2009). Within the OB, Ac45RP expression was found only in the subregions that contain developing interneurons and not in the OB granular layer nor in the subventricular zone, suggesting a role for Ac45RP in the outgrowth of OB interneurons, but not in neuroblast migration. Interestingly, Ac45RP is downregulated in patients suffering from Posttraumatic Stress Disorder (PTSD) together with genes involved in neuron projection development and plasticity, namely Down syndrome cell adhesion molecule (DSCAM) and brain-derived neurotrophic factor (BDNF) (Logue et al., 2015), also hinting to a role for Ac45RP in brain development and plasticity.

Our study indicates that the expression of Ac45RP is restricted to neurons and does not occur in other cell types such as neuroendocrine cells and astrocytes, although at present we cannot exclude that Ac45RP is expressed by other brain cell types. Since Ac45RP-overexpressing N2a cells displayed a more elaborate Golgi, an increased number of vesicular structures in the cytoplasm and more extended plasma membrane

Fig. 5. Localization of Ac45RP and the V-ATPase V₀ subunits c and d during neurite outgrowth of rat primary cortical neurons, and determination of in vitro Ac45RP-V₀ subunit interactions. (A) Two-DIV E18.5 rat primary cortical neurons were stained with the anti-Ac45RP antibody (green) and an anti-V-ATPase V₀d subunit monoclonal antibody (red). Both proteins were found in vesicular structures throughout the cytoplasm, and in varicosities and in the tips of the neurites. (B) E18.5 rat neuroblasts were cultured for twelve hours and stained with a V₀c polyclonal antibody (green) and the V₀d subunit monoclonal antibody (red). Both V₀c and V₀d were present in membranous structures throughout the cell and accumulated in the tip of the extending neurite. Nuclei were stained by DAPI (blue). (C) In vitro measurement of the physical interaction strength between Ac4S or Ac45RP and V₀ subunits V₀a3, V₀c and V₀c" in transfected COS-1 cells using a BRET assay.
formation, Ac45RP may be involved in the supply of membrane during vesicular biogenesis. Furthermore, in these cells extensive neurite outgrowth was observed, indicating a role for Ac45RP in membrane delivery during the process of neurite outgrowth. The appearance of Ac45RP-positive vesicular structures preceding the neural tubules and situated in close proximity of the actin filaments in the extending neurite supports a role for Ac45RP in neurite outgrowth. Neurite outgrowth depends on unconventional exocytotic membrane fusion involving vesicles containing the vesicular soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptor (SNARE) proteins VAMP4 or TI-VAMP (Coco et al., 1999; Cocucci et al., 2008; D’Alessandro and Meldolesi, 2019; Martínez-Arca et al., 2000; Meldolesi, 2010), but devoid of classical marker proteins of synaptic neurotransmitter or neuropeptide vesicles, such as SV2 and VAMP2 (Buckley and Kelly, 1985; Coco et al., 1999; Meldolesi, 2010). Using these vesicular markers, our colocalization studies in N2a cells and rodent primary cortical neurons revealed that Ac45RP is indeed present in unconventional vesicles, distinct from the classical neurotransmitter-/neuropeptide-containing vesicles.

In line with our results obtained through Ac45RP overexpression in N2a cells, our CRISPR-cas9n approach to knockdown endogenous Ac45RP resulted in a clear reduction in the capability of the N2a cells to...
Fig. 7. Analysis of V₀ subunit expression and membrane biogenesis in N2a cells stably overexpressing Ac45RP. (A) qPCR analysis revealed an Ac45RP-dose-dependent increase of V-ATPase-V₀ subunit expression in N2a cells. Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein zeta peptide (Y-Whaz) and peptidyl prolyl isomerase (mPPiα) were used as reference genes. (B) Membrane fractions of N2a cells stably transfected with an empty expression vector (line #4), or an expression construct for Ac45RP-HA (line #2.6) or Ac45RP (line #30) were subjected to Western blot analysis using the anti-Ac45RP antibody and an anti-Ac45 antibody. (C) Quantification of the amount of membrane generated following 24 h of differentiation of the stably transfected N2a cell lines #4, #2.6 and #30 using high-throughput microscopy. The amount of membrane generated per cell was found to be independent of cell density. (D,E) Transmission Electron Microscopy on undifferentiated (D) line #4 (mock-transfected control) and (E) line #30 (Ac45RP-overexpressing) N2a cells. No morphological alterations or changes in the amounts of organelles such as mitochondria, lysosomes and the endoplasmatic reticulum (ER) were observed. Golgi-related structures, including lucent vesicular structures (arrows), were more abundant in the #30 Ac45RP-overexpressing than the #4 control cells. G, Golgi; N, nucleus; L, lysosome. Bar equals 200 nm.
provoke outgrowth of neurites, indicating that Ac45RP is a critical component of the membrane-expanding machinery to achieve neurite outgrowth.

Interestingly, PC12 cells expressing a TI-VAMP mutant protein lacking its N-terminal domain that acts as an intramolecular inhibitor of the SNARE-binding motif show unusually long neurites with an increased number of filopodia (Martinez-Arca et al., 2000), similar to those found in our Ac45RP-transfected N2a cells. The severity of the transfected PC12 cell phenotypes was positively linked to the capacity of the mutant protein to form stable SNARE complexes, which then results in increased unconventional exocytosis and neurite outgrowth. Ac45RP overexpression enhances V₀-subunit and in particular ATP6AP2 expression. This is of great interest since ATP6AP2 and Ac45 have recently been found to physically interact (Wang et al., 2020a), and both proteins act together to allow V₁/V₀-association and -dissociation (Guida et al., 2018). Future studies may disclose whether the Ac45-structurally related Ac45RP protein also interacts with ATP6AP2 and as such fulfills a comparable role in vesicle-specific...
and spatio-temporal V0-assembly.

Since Ac45RP physically interacts with the V-ATPase V0-sector and V0 on its turn interacts with v-SNAREs (Di Giovanni et al., 2010; Galli et al., 1996), we hypothesize that a neuronal-cell-specific and TI-VAMP/VAMP4-containing Ac45RP/V0-SNARE complex drives unconventional exocytotic membrane fusion in the growth cone of the extending neurite. Reasoning along this line and because excess Ac45 induced enhanced biogenesis of immature secretory vesicles, large membrane expansions and an increase in the secretory capacity of neuroendocrine cells (Jansen et al., 2008, 2012), this Ac45 family member may be associated with the biogenesis and fusion of regulated secretory granules, and a neuronal-/neuroendocrine-enriched and SV2/VAMP2-containing Ac45/V0-SNARE complex may be involved in regulated exocytosis. Thus, the two Ac45 family members may determine whether the V-ATPase V0-sector becomes involved in either unconventional or secretory vesicle fusion events. As such, Ac45RP and Ac45 would be crucial components of the molecular machinery driving membrane expansion during neuronal outgrowth and regulated exocytosis, respectively. The fact that Ac45RP evolved relatively late and only during vertebrate evolution indicates that the ancestral role of the V-ATPase concerns its function as subcellular pH regulator, whereas subsequent evolution led to the role for the V0-sector in membrane fusion events. Finally, recent evidence suggests that Ac45, and therefore perhaps also Ac45RP, is a V0-assembly sector and an integral rather than a transiently interacting V-ATPase-subunit (Abbas et al., 2020; Wang et al., 2020a, b), indicating that these Ac45 family members are not true accessory subunits.

In conclusion, our findings link for the first time the V-ATPase to the, at first sight, unrelated process of neuronal outgrowth. The discovery of the role of Ac45RP and the V-ATPase V0-sector in membrane fusion and expansion during the outgrowth of neuronal cells greatly advances our mechanistic understanding of this crucial neurodevelopmental process. Together with the fact that the Ac45RP gene emerged only after the vertebrate-invertebrate lineage separation and is expressed brain specifically, the disclosure of this neurite outgrowth-promoting role further raises the intriguing possibility that Ac45RP has been part of the machinery that has enabled the evolution of the vertebrate brain. Finally, our discovery of the role of the neuron-specific Ac45RP protein provides new and exciting opportunities for the development of neuroregenerative therapies and thus for the treatment of a number of neurological diseases.

Author contributions

E.J.R. designed and performed experiments, interpreted the results and co-wrote the manuscript. N.H.M.v.B., B.B., N.F.M.O.L., Th.G.M.H., and S.M.C. performed experiments. J.X. supplied materials. S.M.K. gave advice on the developmental experiments and supplied embryonic tissues, and G.J.M.M. supervised the project and co-wrote the manuscript. All co-authors critically read and approved the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. The Peer Review Overview and Supplementary data

The Peer Review Overview and Supplementary data associated with this article can be found in the online version: https://doi.org/10.1016/j.pneurobio.2021.102069.

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