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Production of Glycosylated Soluble Amyloid Precursor Protein Alpha (sAPPalpha) in *Leishmania tarentolae*

Stephan Klatt,[†,‡] Michael Rohe,[§] Kathirvel Alagesan,[∥] Daniel Kolarich,[∥] Zoltán Konthur,*[,†] and Daniela Hartl*[,⊥]

[†]Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany

[‡]Faculty of Biology, Chemistry and Pharmacy, Free University Berlin, 14195 Berlin, Germany

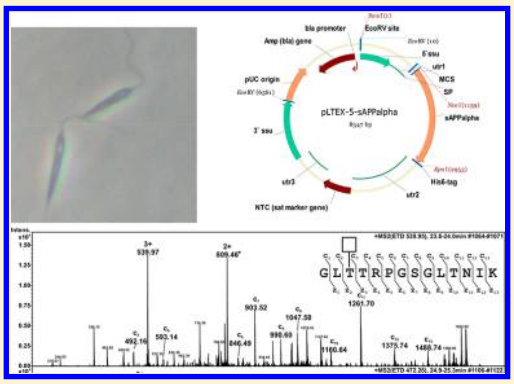
[§]Max-Delbrueck-Center for Molecular Medicine, 13092 Berlin, Germany

[∥]Max Planck Institute of Colloids and Interfaces, 14476 Potsdam, Germany

[⊥]Charité, Institute for Medical Genetics and Human Genetics, 13353 Berlin, Germany

Supporting Information

ABSTRACT: Soluble amyloid precursor protein alpha (sAPPalpha) is a cleavage product of the amyloid precursor protein (APP), the etiologic agent in Alzheimer's disease (AD). Reduced expression of sAPPalpha was previously found in the brains of AD patients, and it was suggested that sAPPalpha might counteract neurotoxic effects of Abeta, another APP cleavage product with enhanced abundance in Alzheimer's diseased brains. However, little is known about the biological functions of sAPPalpha. Thus, efficient production of this protein is a prerequisite for further studies. The unicellular eukaryotic parasite *Leishmania tarentolae* has recently emerged as a promising expression system for eukaryotic proteins due to its ability to posttranslationally modify proteins combined with easy cultivation and high protein yield. Interestingly, sAPPalpha produced in *L. tarentolae* was biologically active and glycosylated. In contrast to nonglycosylated sAPPalpha expressed in *Eschericha coli*, it also featured higher stability against enzymatic degradation. Detailed analysis of the glycosylation pattern of sAPPalpha produced in *L. tarentolae* by PGC−LC−ESI−MS/MS N-glycan analysis identified among eukaryotic species the highly conserved core pentasaccharide (Man3GlcNAc2) as being attached to Asn467 of sAPPalpha. Using oxonium ion scanning of CID−MS/MS spectra in combination with ETD fragmentation, we also identified two peptides (peptides 269−288 and 575−587) modified with *N*-acetyl hexosamine (HexNAc) residues. One of these O-glycosylation sites could be unambiguously assigned to Thr576 of sAPPalpha. This is the first time that O-glycosylation of a recombinant protein expressed in *L. tarentolae* has been demonstrated. Together, human sAPPalpha produced in *L. tarentolae* was N- and O-glycosylated on similar sites as described for mammalian-expressed sAPPalpha and showed similar biological activity. This demonstrates that *L. tarentolae* is a very suitable and simple to handle expression system for mammalian glycoproteins.



KEYWORDS: *amyloid precursor protein alpha, Leishmania tarentolae, Alzheimer's disease, PGC−LC−ESI−MS/MS N-glycan analysis*

INTRODUCTION

Expression of recombinant proteins in prokaryotic systems frequently entail drawbacks, such as improper protein folding, formation of inclusion bodies, or the lack of posttranslational protein modifications (PTMs).[1−3] In contrast, eukaryotic systems offer PTMs, and in general, more proteins are expressed in their soluble form. However, protein yield is usually lower in eukaryotic expression systems as compared to prokaryotic ones. Thus, a heterologous protein expression system should offer advantages of both systems, posttranslational protein modifications and high yield. The ability of the eukaryotic trypanosomatid parasite *Leishmania tarentolae* to express complex eukaryotic proteins with highly diverse PTMs in combination with its easy, bacteria-like handling makes the parasite a promising expression system for recombinant eukaryotic proteins.[4−7] Mammalian cytokines, such as interleukin-2 and γ-interferon have already been successfully expressed in trypanosomatids containing glycosylations.[8] In addition, human tumor protein 53 (p53) expressed in *Leishmania* was shown to be biological active and properly phosphorylated.[9]

Glycosylation is one of the most common PTMs to occur in eukaryotic protein synthesis, and *Leishmania* are specifically rich in glycoproteins. *L. tarentolae* has been reported to produce mammalian-type biantennary *N*-glycans, including galactose and fucose residues. Breitling et al. demonstrated that human erythropoietin (hEPO) produced in *Leishmania* was bio-

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logically active, natively processed at the N-terminus, and N-glycosylated.[10] The yield of cytoplasmic or secretory recombinant proteins purified from *L. tarentolae* cultures ranges between 0.1 and 30 mg/L.[10,11]

The aim of the current study was the production of human sAPPalpha in *L. tarentolae*. sAPPalpha is one cleavage product of the transmembrane glycoprotein amyloid precursor protein (APP), the etiologic agent in Alzheimer's disease (AD). APP can be cleaved in either an amyloidogenic or a non-amyloidogenic way. In the first case, subsequent cleavage of APP by β- and γ-secretases produces Abeta, soluble beta-APP (sAPPbeta), and a C-terminal fragment (AICD). Alternatively, non-amyloidogenic APP cleavage by α- and γ-secretases produces the longer fragment sAPPalpha as well as the short peptide P3. Accordingly, only one of both products, Abeta or sAPPalpha, can be obtained by cleavage from one APP molecule. The biological functions of individual APP cleavage products are still largely unknown.[12] Abeta is the best-analyzed APP cleavage product and is known to have neurotoxic properties, to cause oxidative stress, and to modulate synaptic activity.[13−15] The physiological functions of sAPPalpha are poorly analyzed. However, it recently has emerged to be a good predictor for AD and other neurodegenerative diseases.[16−19] Importantly, because of overproduction of Abeta, sAPPalpha levels are inevitably reduced in Alzheimer's diseased brains.[20] As sAPPalpha has been shown to be beneficial for memory function and to possess neurotrophic properties, it was suggested to counteract neurotoxic effects of Abeta. The observed decrease of sAPPalpha production in the brain of AD patients might therefore as well be one important disease-causing factor.[21−25] However, future studies are required to decipher the biological functions of this secreted APP cleavage product. The production of recombinant sAPPalpha in its properly modified form is one first step towards this aim.

We decided to produce human sAPPalpha in *L. tarentolae*. We demonstrate the successful purification of human sAPPalpha from culture supernatant of *L. tarentolae* and demonstrate N- and O-glycosylation, the latter of which has not been demonstrated before in a recombinant protein produced by *L. tarentolae*. The produced sAPPalpha was biologically active and exhibited enhanced stability compared to the nonglycosylated form produced in *Escherichia coli*.

MATERIALS AND METHODS

Cloning of sAPPalpha into the Leishmania Expression-Secretion Vector pLTEX-5

sAPPalpha (human full length APP695, neuron-specific isoform) was amplified by PCR from the full-length APP clone pcDNA 3.1/myc-B_APP using primers sAPPalpha-NcoI F (CATGCCATGGCTGAGGTGCCCACTGATGGTAATGC) and sAPPalpha-KpNI_R (CGGGGTACCTTTTTGATGATG-AACTTCATATCC). The forward primer introduces a silent mutation corresponding to amino acid Val_2 (GTA→ GTG) to eliminate a *Kpn*I restriction enzyme site and introduces a NcoI restriction enzyme site. The reverse primer introduces a *Kpn*I restriction enzyme site. For cloning into pLTEX-5, the amplicon as well as the destination vector were digested with NcoI and *Kpn*I, and the fragments were ligated. The ligated DNA was purified and transformed into electrocompetent DH10B cells. A positively evaluated single clone from this transformation was used in subsequent experiments to generate expression clones in *L. tarentolae* strain P10 as previously described.[26]

Production and Purification of sAPPalpha

Positive transfectants were used for sAPPalpha-production. A 200−500 mL amount of LEXSY BHI medium containing porcine Hemin, Pen/Strep, and Nourseothricin (NTC) was inoculated 1:40 with a densely grown culture of a clone and cultivated in agitated suspension culture (125 rpm) for 2−3 days at 26 °C until OD600 = 2.5−4.5 was reached. Cells were harvested for 20 min, at 3000 rpm and 4 °C, followed by a second centrifugation step for 5 min, at 4000 rpm and 4 °C, to remove pellet deposits. Supernatants were collected and pooled. Furthermore, 4 mL/L PI (Protease inhibitor cocktail, Roche Diagnostics), 8 mL/L PMSF (0.1 M; phenylmethanesulfonyl fluoride, Sigma-Aldrich), and 0.02 mL/L benzonase (Merck Chemicals) were added to inhibit protease activities and to remove nucleic acids. Next, for isolation of sAPPalpha, supernatant was concentrated using Amicon Ultra-15 columns (20 min, 4000 rpm, 4 °C) with a size cutoff of 50 kDa. Concentrates were mixed 1:5 with binding buffer (10 mM $NaH_2PO_4 \cdot H_2O$, pH 7) and loaded onto a HiTrap Heparin column (GE Healthcare) for fast protein liquid chromatography (FPLC)-based purification on an Äkta Explorer system. Washing and elution were carried out using a NaCl gradient (elution buffer: 10 mM $NaH_2PO_4 \cdot H_2O$, 1.8 M NaCl, pH 7) from 0.75 to 1.8 M. All FPLC fractions were analyzed for protein content at UV_{280nm}.

Isolation of sAPPalpha was verified by Western blot analysis using a specific monoclonal antibody (clone 2B3, IBL Hamburg), which recognizes the C-terminus of human sAPPalpha (DAEFRHDSGYEVHHQK). The purity of isolates was assayed by silver staining of SDS-PAGE gels.

Preparation and Treatment of Primary Cortical Mouse Neurons

Primary cortical neurons were prepared from newborn Balb/c mice at postnatal day 1 as described before.[27] Cortical cells were dissociated using papain (1 h at 37 °C) and cultured on poly-D-lysine/collagen-coated culture dishes. Neurons were cultured for 5 days in Neurobasal-A medium (Gibco) containing B27 supplement (Sigma-Aldrich) and GlutaMAX (Invitrogen). Neurons were then treated with 300 ng/mL sAPPalpha or medium only (control) for 1 h by replacing half of the culture medium with fresh medium. After treatment, neurons were still viable, and no differences in death rate were observed between treated and untreated cells. The cells were harvested in ice cold PBS, and cell pellets were frozen immediately in liquid nitrogen. Four individual samples of treated and control neurons were collected for Western Blot analysis using antibodies specific for ERK, phosphorylated ERK, and actin (n = 6; Cell Signaling Technology).

Stability Assay and Glycosidase Treatment

For analysis of sAPPalpha stability, we incubated equal amounts of sAPPalpha derived from *L. tarentolae* or *E. coli* (Sigma-Aldrich) with Endoproteinase GluC (Sigma-Aldrich) or buffer only (control) for 1 h at 37 °C. After incubation, samples were separated by SDS-PAGE, and protein signals were analyzed by Western blot as described above.

Equal amounts (2 μg) of sAPPalpha produced in *L. tarentolae* or *E. coli* (obtained from Sigma-Aldrich), respectively, were incubated with PNGase F and *O*-glycosidase (1 U and 2 mU, respectively, Roche Diagnostics) for 2 h at 37 °C. In parallel,

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control samples were treated with buffer only and incubated under the same conditions. Samples were separated by SDS-PAGE and analyzed by Western blotting as described above.

Glycoproteomic Sample Preparation and MS Analysis of sAPPalpha

Coommassie-stained bands identified as sAPPalpha were excised from the gel and digested with trypsin as described in detail previously.[28] Tryptic (glyco)peptides were analyzed by LC−ESI−MS/MS on an amaZon speed ETD Ion trap (Bruker Daltonics, Bremen, Germany) coupled to an Ultimate 3000 UHPLC system (Dionex, part of Thermo Fisher). The instrument was tuned to perform both CID and ETD fragmentation of selected precursors. An m/z range from 400 to 1500 Da was scanned for precursor scanning. The three most intense signals in every MS scan were selected for MS/MS experiments, and just signals with a charge state ≥2+ were selected for MS/MS. For MS/MS experiments, an m/z range from 100 to 2500 Da was scanned. Both MS precursor and MS/MS scans were recorded in the instruments "enhanced resolution mode". After digest, dried (glyco)peptides were taken up, loaded onto the precolumn (300 μm × 10 mm, ProteCol, SGE, Ringwood, Australia), desalted in 100% solvent A (0.1% formic acid), and separated prior MS detection by reversed phase chromatography (300 μm × 150 mm, ProteCol, SGE, Ringwood, Australia) using a linear gradient [0.5%/min increase of solvent B (acetonitrile with 0.1% formic acid)] up to 30%, followed by a steeper increase to 60% (2%/min) and a washing step (90% solvent B) before equilibration of the column in starting conditions (2% solvent B).

Data analysis was performed using ProteinScape 3 (Bruker Daltonics) and MASCOT 2.3 (MatrixScience, United Kingdom) using the following search parameters: Cysteine as carbamidomethyl was set as fixed modification, and deamidation (Asn/Gln) and oxidation (Met) were set as variable modifications. Up to two missed cleavages were allowed. Peptide tolerance (both MS and MS/MS) was set at ±0.2 Da. The data were searched against the SwissProt protein database (taxonomy restriction: *Homo sapiens*, SwissProt 2011_08; 531,473 sequences; 188,463,640 residues). Furthermore, tandem MS spectra identified to contain glycopeptide signatures were manually analyzed and interpreted using Data Analysis 4.0 (Bruker Daltonics, Germany). The criteria applied for acceptance of peptide assignments were as follows: minimum peptide length = 5, taxonomy = *H. sapiens* (human), and enzyme = trypsin.

N-Glycan analysis was performed from electrophoretically separated sAPPalpha electroblotted onto PVDF membranes as described by Jensen et al.[29] Characterization of *N*-glycans using porous graphitized carbon (PGC)−LC−ESI−MS/MS was essentially performed as described in detail by Jensen et al. using the same LC−ESI−MS/MS system as described for the glycoproteomic analysis above. The acquired data were screened manually to characterize sAPPalpha *N*-glycans.

RESULTS

Vector Design and Signal Peptide Cleavage Analysis

APP naturally carries an N-terminal secretory signal peptide (SP), which is cleaved off during maturation of the preprotein in the endoplasmic reticulum. To express sAPPalpha in *L. tarentolae*, the natural human SP was replaced by the SP of secreted acid phosphatase 1 (SAP1) of *Leishmania mexicana*. We analyzed the resulting expression construct for proper SP cleavage as imperfect SP cleavage can perturb protein folding and function and decrease protein expression or secretion.[30−32] First, we performed in silico cleavage site analysis for the SP of sAPPalpha in the pLTEX-5 vector. The vector pLTEX-5 was described previously.[26] We employed the software SignalP to test for proper SP cleavage of sAPPalpha produced in *L. tarentolae* as documented before.[33−35] In line with results from our previous study,[26] the SP implemented in the pLTEX-5 vector was demonstrated to be efficiently cleaved. Therefore, to express sAPPalpha in *L. tarentolae*, we inserted the coding sequence of human sAPPalpha (neuron-specific isoform of APP695) into pLTEX-5 vector. Proteins expressed with pLTEX-5 are provided with a C-terminal His6-tag and with an optimized SP (N-terminal) for protein secretion. The final expression construct was named pLTEX-5-sAPPalpha (Figure 1).

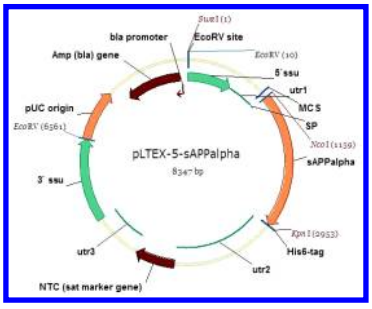

Figure 1. Vector map of pLTEX-5-sAPPalpha.

Sequence analysis of the N-terminal amino acid sequence of sAPPalpha by Edman degradation ultimately documented efficient SP cleavage as the first eight amino acids of sAPPalpha were identified as EVPTDGNA. This sequence completely lacks the SP. In combination with the C-terminally located His6-tag and with the additional amino acids glycine and threonine located between the sAPPalpha and the His6-tag (sAPPalpha_GT_HHHHHH), sAPPalpha has a theoretical size of 68.6 kDa. Together, pLTEX-5 vector was demonstrated to be an appropriate choice for expression and secretion of sAPPalpha in *L. tarentolae*.

Purification of sAPPalpha

sAPPalpha possesses several heparin binding domains and purification of this protein from conditioned cell culture medium using heparin-coated columns has previously been demonstrated.[36,37] We purified sAPPalpha using FPLC with immobilized heparin columns (HiTrap; Figure 2). Protein yield was typically about 150 μg/100 mL of initial culture medium.

After concentration and rough enrichment of sAPPalpha from the collected cell culture medium by size-exclusion centrifugation (50 kDa cutoff), the concentrate was mixed 1:5 with binding buffer. Heparin columns were washed with binding buffer, and culture media concentrates were then applied to heparin columns. Elution was carried out using a NaCl gradient ranging from 0.75 to 1.8 M, and pure sAPPalpha was eluted from columns at a NaCl concentration of ∼0.8 M (Figure 2A). Fractions containing purified sAPPalpha were applied to SDS-PAGE. Silver staining of gels determined the high purity of sAPPalpha, and Western blot analysis using an antibody specific for human sAPPalpha verified the presence of this protein in the eluted fractions as well as in cell culture media (Figure 2B,C). Moreover, sAPPalpha migrated at a relative mass of ∼100 kDa.

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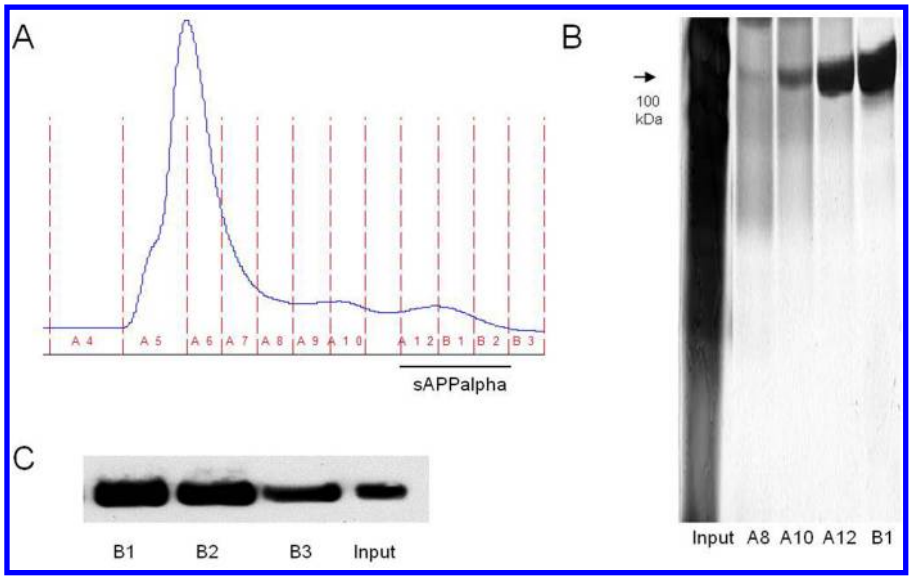

Figure 2. (A) Isolation of recombinant sAPPalpha produced in *L. tarentolae* by FPLC. The diagram shows protein concentrations as measured during FPLC corresponding to fractions A4 to B3. Pure sAPPalpha was present in fractions A9 to B3. (B) Silver-stained SDS-PAGE gel confirmed the high purity of sAPPalpha in FPLC fractions A8 to B1 as compared to concentrated cell culture supernatant (Input). (C) Western blot analysis confirmed the presence of sAPPalpha in fractions B1 to B3 as well as in the concentrated cell culture supernatant (Input).

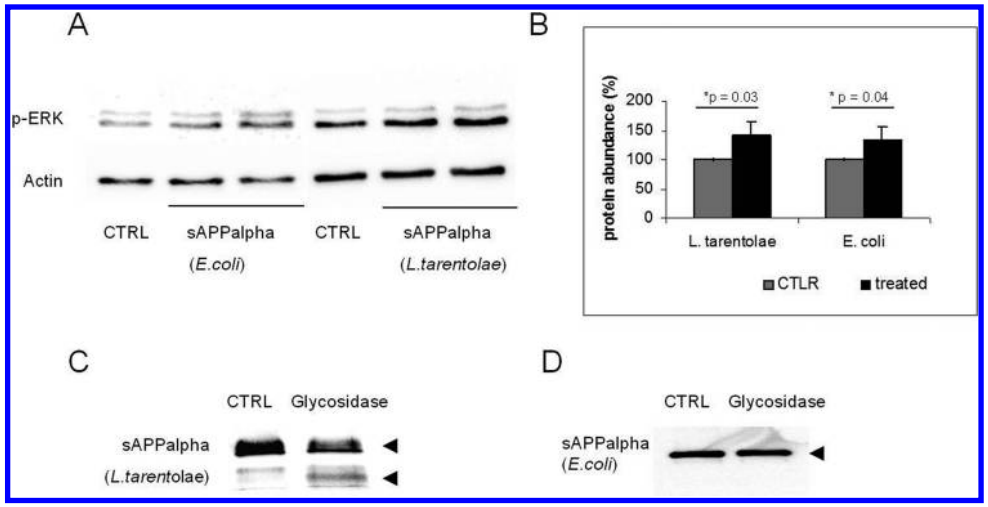

Figure 3. (A) Phosphorylated ERK (p-ERK) was up-regulated in neurons treated with sAPPalpha as compared to control neurons treated with medium only (CTRL). Neurons were treated with sAPPalpha produced in *L. tarentolae* or *E. coli*, respectively. Actin served as a loading control. (B) Upregulation of p-ERK was statistically significant in neurons treated with sAPPalpha produced either in *L. tarentolae* or in *E. coli* (untreated signals set to 100%, Mann−Whitney U test, $n = 6$). (C) After treatment of sAPPalpha produced in *L. tarentolae* with glycosidase, two signals (arrowheads) appeared on the Western blot representing glycosylated (higher molecular weight) and deglycosylated (lower molecular weight) forms of the protein. (D) When sAPPalpha produced in *E. coli* was treated with glycosidase, only the original signal (arrowhead) was observed on the Western blot after treatment.

Biological Activity of sAPPalpha

To test whether recombinant sAPPalpha produced in *L. tarentolae* was biologically active, we analyzed the activation of downstream signaling cascades in mouse primary cortical neurons. sAPPalpha has previously been shown to specifically activate mitogen-activated protein kinases (MAPK, ERK).[38] We thus tested whether ERK was increasingly phosphorylated in neurons after treatment with sAPPalpha.

We applied 300 ng/mL sAPPalpha dissolved in medium (or medium only as control) to primary cortical neurons for 1 h ($n = 6$). Neurons were treated with sAPPalpha produced in *L. tarentolae* or *E. coli*, respectively. Western blot analysis and subsequent quantification of signals revealed significantly enhanced ERK phosphorylation in treated as compared to control neurons (Mann−Whitney U test, $p \leq 0.05$). This was true for both versions of sAPPalpha, sAPPalpha produced in *L. tarentolae* and *E. coli* (Figure 3A,B). In contrast, total ERK levels were not altered documenting specific activation of this signaling cascade (data not shown). Together, our results demonstrate that with respect to ERK-phosphorylation, recombinant sAPPalpha produced in *L. tarentolae* as well as sAPPalpha produced in *E. coli* are biologically active.

Glycoproteomic Analysis of sAPPalpha

One significant advantage of using *L. tarentolae* for recombinant protein expression is its principle capacity to perform eukaryotic protein glycosylation.[10] To study this in more detail,

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Table 1. Peptides and Corresponding Modifications of Peptides Identified by Mass Spectrometry Analysis of sAPPalpha Produced in *L. tarentolae*[a]

m/z meas.	Δ*m/z* (ppm)	*z*	score	range	sequence	modification
440.2	−1.8	3	60.1	041−051	R.LNMHMNVQNGK.W	oxidation: 3, 5; deamidated: 9
496.7	−39.5	2	24.5	052−060	K.WDSDPSGTK.T	
422.9	8.9	3	35.6	107−116	K.THPHFVIPYR.C	
422.9	8.9	3	34.0	107−116	K.THPHFVIPYR.C	
415.2	−46.7	2	21.9	135−140	K.FLHQER.M	
1064.0	−34.6	2	77.1	269−288	R.TTSIATTTTTTTESVEEVVR.E	
845.1	23.8	3	24.7	269−288	R.TTSIATTTTTTTESVEEVVR.E	2 × HexNAc
709.7	9.0	3	48.7	269−288	R.TTSIATTTTTTTESVEEVVR.E	
479.5	60.7	4	28.2	302−319	K.YLETPGDENEHAHFQK.A	
571.6	28.4	3	53.0	331−343	R.MSQVMREWEEAER.Q	oxidation: 1, 5
510.8	122.8	4	47.0	331−346	R.MSQVMREWEEAERQAK.N	oxidation: 1, 5
474.7	−13.7	2	41.4	337−343	R.EWEEAER.Q	
550.3	3.0	2	39.8	355−363	K.AVIQHFQEK.V	
819.1	26.2	3	48.1	355−375	K.AVIQHFQEKVESLEQEAANER.Q	
687.8	−41.3	2	92.0	364−375	K.VESLEQEAANER.Q	
410.2	−20.2	3	40.3	376−385	R.QQLVETHMAR.V	oxidation: 8
474.3	138.4	2	17.1	386−393	R.VEAMLNDR.R	
482.2	−66.0	2	63.6	386−393	R.VEAMLNDR.R	oxidation: 4
545.3	−33.2	4	68.8	395−413	R.RLALENYITALQAVPPRPR.H	
452.7	−86.4	2	27.8	414−420	R.HVFNMLK.K	oxidation: 5
494.3	89.7	2	53.8	453−460	R.SQVMTHLR.V	oxidation: 4
998.9	−49.5	2	85.1	494−510	K.EQNYSDDVLANMISEPR.I	oxidation: 12
878.4	−30.9	2	91.4	511−526	R.ISYGNDALMPSLTETK.T	oxidation: 9
539.9	−120.5	3	31.8	574−587	R.GLTTRPGSGLTNIK.T	HexNAc: 3
472.3	59.4	3	33.8	574−587	R.GLTTRPGSGLTNIK.T	
583.3	−33.4	4	43.5	574−595	R.GLTTRPGSGLTNIKTEEISEVK.M	
467.7	−85.6	2	57.6	588−595	K.TEEISEVK.M	
567.3	59.4	3	50.7	588−601	K.TEEISEVKMDAEFR.H	oxidation: 9

[a]The ions score cutoff/confidence level was a minimum ion score of 15; significance threshold = 0.05.

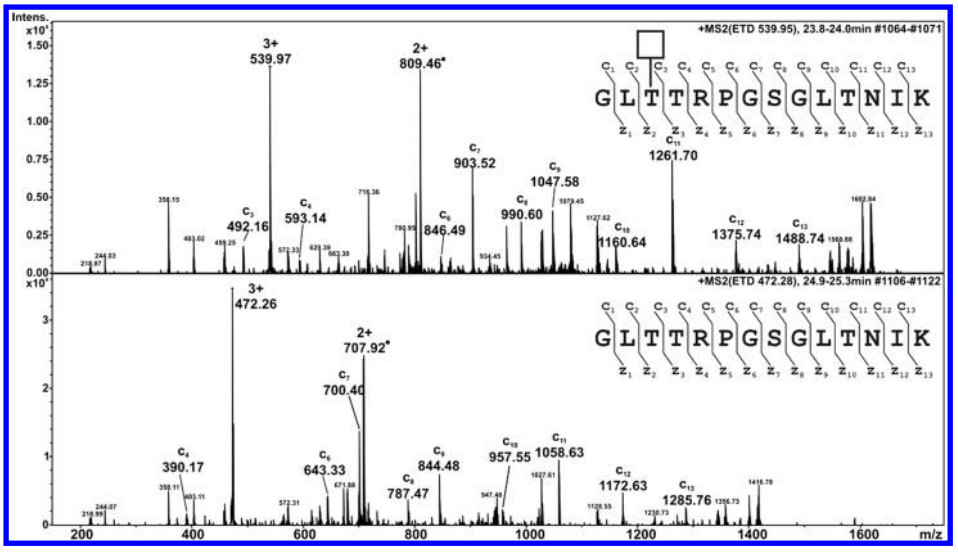

Figure 4. ETD−MS/MS spectra of the triply charged precursors of peptide 575−587 with (top) and without (bottom) a single HexNAc attached. Top: The almost complete c-ion series beginning with c_3 proves glycosylation of Thr576 with a single HexNAc residue. Bottom: peptide 575−587 was also identified in a nonglycosylated form.

we applied glycoproteomic analysis to identify glycosylations of sAPPalpha produced in *L. tarentolae*.

We first of all treated purified sAPPalpha with glycosidase (PNGase F and *O*-glycosidase). After treatment, a strong signal appeared at a lower molecular weight, indicating that sAPPalpha produced in *L. tarentolae* was glycosylated (Western blot, Figure 3C). In contrast, this was not observed when sAPPalpha derived from *E. coli* was treated in the same way with glycosidase (Figure 3D).

PGC−LC−ESI−MS/MS *N*-glycan analysis of electrophoretically separated sAPPalpha produced in *L. tarentolae* identified the core pentasaccharide (Man3GlcNAc2) as the only N-glycan

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released and isolated from sAPPalpha (Supporting Information). sAPPalpha contains two potential N-glycosylation sites, Asn467 and Asn496. Previous studies found N-glycosylation site Asn496 to be not modified by an *N*-glycan when expressed in Chinese hamster ovary (CHO) cells.[39] Our results show a similar pattern for *L. tarentolae* expressed sAPPalpha since peptide 495−510 was exclusively detected in its nonglycosylated form (Table 1).

The second expected N-glycopeptide (containing Asn467) could not be identified, neither glycosylated nor unglycosylated. Nevertheless, the fact that we were able to isolate and identify *N*-glycans from sAPPalpha and Asn496 is not glycosylated provides a strong indirect proof that Asn467 is posttranslationally modified with an *N*-glycan as it has been described for different mammalian expression systems.

O-Glycosylation of several serine and threonine residues of native sAPPalpha has been described previously.[40] Using oxonium ion scanning of CID−MS/MS spectra in combination with ETD fragmentation, it was possible to successfully identify two peptides modified with *N*-acetyl hexosamine (HexNAc) residues. A single HexNAc was found attached to tryptic peptide 575−587; using ETD fragmentation, it was possible to assign this HexNAc attached to Thr576 (Figure 4). Interestingly, the very same threonine residue has also been described to be O-glycosylated when sAPPalpha was expressed in a mammalian expression system.[41] We were also able to identify a second peptide (269−288) to be modified with two HexNAc residues; however, because of the particular peptide sequence containing in total 11 Ser and Thr residues, we were unable to unambiguously identify the particular glycosylated amino acids using ETD fragmentation (Table 1). However, no indications for more complex types of O-glycosylation or any signs of sialic acid attachment were found. Furthermore, substantial amounts of the corresponding peptides were also identified in their unmodified form (Figure 4). Despite these above-described findings, it cannot be completely ruled out that *L. tarentolae* is potentially capable of performing more complex types of O-glycosylation, and further studies with different types of O-glycosylated proteins would be required to investigate the degree of suitability for using *L. tarentolae* as an expression system for O-glycosylated proteins. However, to the best of our knowledge, this is the first report ever describing *L. tarentolae* as capable of posttranslationally modifying recombinant proteins with O-glycosylations.

Stability Assay

Having demonstrated glycosylation of sAPPalpha produced in our expression system, we next asked for the functional consequences of these modifications. In general, glycosylation has been shown to influence a variety of biological processes at both the cellular (e.g., intracellular targeting and protein folding) and the protein level (e.g., protein−protein binding, protein stability).[42] However, glycosylation also becomes more and more important for pharmaceutical applications as glycosylation has been found to protect pharmaceutically applied proteins against rapid proteolytic degradation and influence serum half-life.[43] Thus, protein glycosylation tuning can dramatically increase the effectiveness of applied protein therapeutics.

To test whether sAPPalpha produced in our expression system was more resistant against degradation, we incubated it with endoproteinase GluC. In addition, we compared the characteristics of sAPPalpha, being either glycosylated (produced in *L. tarentolae*) or nonglycosylated (produced in *E. coli*). Incubation of both protein versions with endoproteinase GluC for 1 h resulted in degradation of both proteins, but in contrast to the version produced in *L. tarentolae*, sAPPalpa produced in *E. coli* was degraded more rapidly (97 ± 8% as compared to 75 ± 1%, p = 0.03, Mann−Whitney U test, n = 4) (Figure 5).

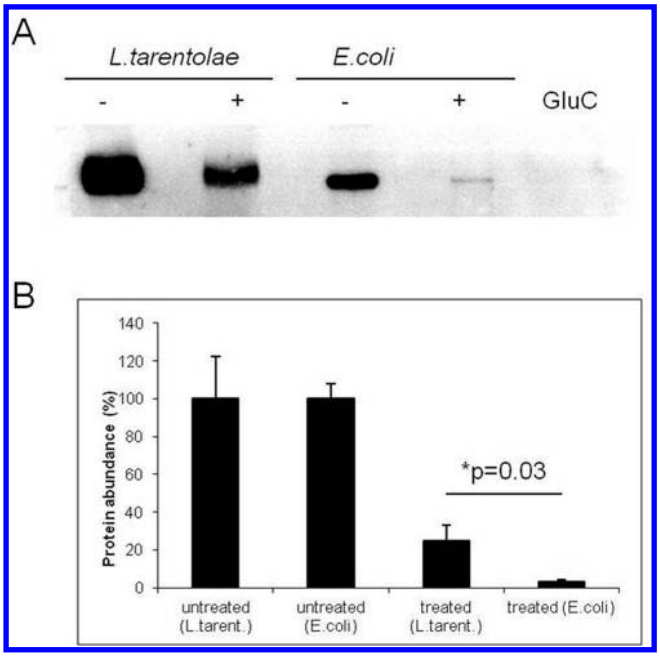

Figure 5. (A) sAPPalpha produced in *L. tarentolae* was more stable when treated with Endopeptidase GluC (+) as compared to sAPPalpha produced in *E. coli*. (B) Quantification of signals revealed that about 97% of sAPPalpha (produced in *E. coli*) was degraded when treated with GluC (treated). Only about 75% of sAPPalpha produced in *L. tarentolae* was degraded when treated with GluC (untreated signals set to 100%, Mann−Whitney U test, n = 4).

Together, proteinase treatment of nonglycosylated and glycosylated forms of sAPPalpha confirmed a protective effect against protein degradation for glycosylated sAPPalpha produced in *L. tarentolae*.

DISCUSSION

Prokaryotic cells are most commonly used for expression of recombinant proteins. However, in spite of clear advantages such as easy cultivation in high cell numbers, prokaryotic expression systems do not provide recombinant proteins with eukaryote-like PTMs. We expressed human sAPPalpha in the trypanosomatid parasite *L. tarentolae*. This relatively new expression system combines important characteristics of easy cultivation and eukaryote-like PTMs of proteins. Moreover, the organism is especially suited for the expression of glycoproteins.

Processing of full-length APP by α-secretase activity at the cell surface releases sAPPalpha into the extracellular space. Exogenous application of sAPPalpha to neurons was previously demonstrated to induce neurite outgrowth via induced ERK phosphorylation.[38,44] Accordingly, when applied to primary cortical neurons of mice, sAPPalpha produced in *L. tarentolae* induced ERK phosphorylation, demonstrating biological activity of the recombinant protein (Figure 3A,B).

Despite the fact that APP is well-known to carry both *N*- and *O*-glycan modifications, there is just a handful of studies addressing this issue in more detail.[39,41,45] To date, only five of

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nine potential glycosylation sites located within the sAPPalpha domain of APP have been described.[41,45,46] Interestingly, when analyzing glycosylation of sAPPalpha produced by *L. tarentolae*, we identified similar patterns of *N*-glycan site occupancy as described for sAPPalpha expressed in mammalian systems. However, in contrast to previous studies, we were unable to identify the presence of complex-type *N*-glycans since the only *N*-glycan structure to be present on *L. tarentolae* expressed sAPPalpha appeared to be the core *N*-glycan pentasaccharide.[10] This difference could possibly be explained by previous studies reporting *Leishmania* and *Trypanosoma* N-glycosylation to be highly variable-dependent on the particular life cycle stage.[47] Nevertheless, the observed N-glycosylation pattern could also be protein- or growth condition-dependent. The missing N-glycosylation on Asn496, as also reported for mammalian system expressed sAPPalpha, could be due to the 3′-position of Asp 499. Mellquist et al. showed that site occupancy rates are considerably reduced by the presence of an Asp in position 3′ after the N-glycosylation sequon.[48]

Importantly, our work describes the first report on the principle capacity of *L. tarentolae* to perform initial O-glycosylation steps. Single HexNAc were found to be specifically attached to threonine and/or serine residues in a similar manner as previously described for mammalian expression systems. The exact nature of the HexNAc residues requires further determination. Despite these findings, we were unable to identify any larger *O*-glycan structures commonly found in mammalian systems. The fact that considerable amounts of the respective O-glycosylated peptides were also found in their unglycosylated state could indicate slightly different substrate specificities of the respective *L. tarentolae* glycosyltransferases as compared to their mammalian counterparts. Optimization of the expression conditions could result in higher O-glycosylation occupancy rates, and the single HexNAc residues could potentially be further modified in vitro using specific glycosyltransferases if particular structural features are required to be present on *N*- or *O*-glycans of the expressed proteins.

Together, our study demonstrates that *L. tarentolae* is a well-suited system for the expression of mammalian glycoproteins. Recombinant sAPPalpha produced in *L. tarentolae* will provide a useful tool for future analyses of the biological functions of this important APP cleavage product.

■ ASSOCIATED CONTENT

Supporting Information

ETD-MS/MS spectra, CID-MS/MS spectra, EIC, table, and MS and CID-MS/MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +49 3084131586. Fax: +49 3084131388. E-mail: konthur@molgen.mpg.de (Z.K.). Tel: +49 30450566258. Fax: +49 30450566904. E-mail: Daniela.hartl@charite.de (D.H.).

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Sorensen, H. P.; Mortensen, K. K. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microb. Cell Fact.* **2005**, *4* (1), 1.

(2) Villaverde, A.; Carrio, M. M. Protein aggregation in recombinant bacteria: Biological role of inclusion bodies. *Biotechnol. Lett.* **2003**, *25* (17), 1385−1395.

(3) Konthur, Z.; Hust, M.; Dubel, S. Perspectives for systematic in vitro antibody generation. *Gene* **2005**, *364*, 19−29.

(4) Kushnir, S.; Gase, K.; Breitling, R.; Alexandrov, K. Development of an inducible protein expression system based on the protozoan host *Leishmania tarentolae*. *Protein Expression Purif.* **2005**, *42* (1), 37−46.

(5) Mureev, S.; Kushnir, S.; Kolesnikov, A. A.; Breitling, R.; Alexandrov, K. Construction and analysis of *Leishmania tarentolae* transgenic strains free of selection markers. *Mol. Biochem. Parasitol.* **2007**, *155* (2), 71−83.

(6) Fritsche, C.; Sitz, M.; Weiland, N.; Breitling, R.; Pohl, H. D. Characterization of the growth behavior of *Leishmania tarentolae*: A new expression system for recombinant proteins. *J. Basic Microbiol.* **2007**, *47* (5), 384−393.

(7) Fritsche, C.; Sitz, M.; Wolf, M.; Pohl, H. D. Development of a defined medium for heterologous expression in *Leishmania tarentolae*. *J. Basic Microbiol.* **2008**, *48* (6), 488−495.

(8) La Flamme, A. C.; Buckner, F. S.; Swindle, J.; Ajioka, J.; Van Voorhis, W. C. Expression of mammalian cytokines by *Trypanosoma cruzi* indicates unique signal sequence requirements and processing. *Mol. Biochem. Parasitol.* **1995**, *75* (1), 25−31.

(9) Zhang, W. W.; Charest, H.; Matlashewski, G. The expression of biologically active human p53 in Leishmania cells: A novel eukaryotic system to produce recombinant proteins. *Nucleic Acids Res.* **1995**, *23* (20), 4073−4080.

(10) Breitling, R.; Klingner, S.; Callewaert, N.; Pietrucha, R.; Geyer, A.; Ehrlich, G.; Hartung, R.; Muller, A.; Contreras, R.; Beverley, S. M.; Alexandrov, K. Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. *Protein Expression Purif.* **2002**, *25* (2), 209−218.

(11) Basile, G.; Peticca, M. Recombinant protein expression in *Leishmania tarentolae*. *Mol. Biotechnol.* **2009**, *43* (3), 273−278.

(12) Lazarov, O.; Demars, M. P. All in the Family: How the APPs Regulate Neurogenesis. *Front. Neurosci.* **2012**, *6*, 81.

(13) Hartl, D.; Rohe, M.; Mao, L.; Staufenbiel, M.; Zabel, C.; Klose, J. Impairment of adolescent hippocampal plasticity in a mouse model for Alzheimer's disease precedes disease phenotype. *PLoS One* **2008**, *3* (7), e2759.

(14) Hartl, D.; Schuldt, V.; Forler, S.; Zabel, C.; Klose, J.; Rohe, M. Presymptomatic Alterations in Energy Metabolism and Oxidative Stress in the APP23 Mouse Model of Alzheimer Disease. *J. Proteome Res.* **2012**, *11*, 3295−3304.

(15) Huang, Y.; Mucke, L. Alzheimer mechanisms and therapeutic strategies. *Cell* **2012**, *148* (6), 1204−1222.

(16) Steinacker, P.; Fang, L.; Kuhle, J.; Petzold, A.; Tumani, H.; Ludolph, A. C.; Otto, M.; Brettschneider, J. Soluble beta-amyloid precursor protein is related to disease progression in amyotrophic lateral sclerosis. *PLoS One* **2011**, *6* (8), e23600.

(17) Ray, B.; Long, J. M.; Sokol, D. K.; Lahiri, D. K. Increased secreted amyloid precursor protein-alpha (sAPPalpha) in severe autism: Proposal of a specific, anabolic pathway and putative biomarker. *PLoS One* **2011**, *6* (6), e20405.

(18) Perneczky, R.; Tsolakidou, A.; Arnold, A.; Diehl-Schmid, J.; Grimmer, T.; Forstl, H.; Kurz, A.; Alexopoulos, P. CSF soluble

amyloid precursor proteins in the diagnosis of incipient Alzheimer disease. *Neurology* **2011**, *77* (1), 35−38.
(19) Wu, G.; Sankaranarayanan, S.; Hsieh, S. H.; Simon, A. J.; Savage, M. J. Decrease in brain soluble amyloid precursor protein beta (sAPPbeta) in Alzheimer's disease cortex. *J. Neurosci. Res.* **2011**, *89* (6), 822−832.
(20) Sennvik, K.; Fastbom, J.; Blomberg, M.; Wahlund, L. O.; Winblad, B.; Benedikz, E. Levels of alpha- and beta-secretase cleaved amyloid precursor protein in the cerebrospinal fluid of Alzheimer's disease patients. *Neurosci. Lett.* **2000**, *278* (3), 169−172.
(21) Mattson, M. P.; Cheng, B.; Culwell, A. R.; Esch, F. S.; Lieberburg, I.; Rydel, R. E. Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of the beta-amyloid precursor protein. *Neuron* **1993**, *10* (2), 243−254.
(22) Meziane, H.; Dodart, J. C.; Mathis, C.; Little, S.; Clemens, J.; Paul, S. M.; Ungerer, A. Memory-enhancing effects of secreted forms of the beta-amyloid precursor protein in normal and amnestic mice. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95* (21), 12683−12688.
(23) Postina, R.; Schroeder, A.; Dewachter, I.; Bohl, J.; Schmitt, U.; Kojro, E.; Prinzen, C.; Endres, K.; Hiemke, C.; Blessing, M.; Flamez, P.; Dequenne, A.; Godaux, E.; van Leuven, F.; Fahrenholz, F. A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alzheimer disease mouse model. *J. Clin. Invest.* **2004**, *113* (10), 1456−1464.
(24) Stein, T. D.; Anders, N. J.; DeCarli, C.; Chan, S. L.; Mattson, M. P.; Johnson, J. A. Neutralization of transthyretin reverses the neuroprotective effects of secreted amyloid precursor protein (APP) in APPSW mice resulting in tau phosphorylation and loss of hippocampal neurons: support for the amyloid hypothesis. *J. Neurosci.* **2004**, *24* (35), 7707−7717.
(25) Bell, K. F.; Zheng, L.; Fahrenholz, F.; Cuello, A. C. ADAM-10 over-expression increases cortical synaptogenesis. *Neurobiol. Aging* **2008**, *29* (4), 554−565.
(26) Klatt, S.; Konthur, Z. Secretory signal peptide modification for optimized antibody-fragment expression-secretion in *Leishmania tarentolae*. *Microbial Cell Factories* 2012, *11* (97), DOI: 10.1186/1475-2859-11-97.
(27) Rohe, M.; Nebrich, G.; Klein, O.; Mao, L.; Zabel, C.; Klose, J.; Hartl, D. Kainate promotes alterations in neuronal RNA splicing machinery. *J. Proteome Res.* **2011**, *10* (4), 1459−1467.
(28) Kolarich, D.; Jensen, P. H.; Altmann, F.; Packer, N. H. Determination of site-specific glycan heterogeneity on glycoproteins. *Nat. Protoc.* **2012**, *7* (7), 1285−1298.
(29) Jensen, P. H.; Karlsson, N. G.; Kolarich, D.; Packer, N. H. Structural analysis of N- and O-glycans released from glycoproteins. *Nat. Protoc.* **2012**, *7* (7), 1299−1310.
(30) Borrero, J.; Jimenez, J. J.; Gutiez, L.; Herranz, C.; Cintas, L. M.; Hernandez, P. E. Protein expression vector and secretion signal peptide optimization to drive the production, secretion, and functional expression of the bacteriocin enterocin A in lactic acid bacteria. *J. Biotechnol.* **2011**, *156* (1), 76−86.
(31) Futatsumori-Sugai, M.; Tsumoto, K. Signal peptide design for improving recombinant protein secretion in the baculovirus expression vector system. *Biochem. Biophys. Res. Commun.* **2010**, *391* (1), 931−935.
(32) Palomares, L. A.; Estrada-Mondaca, S.; Ramirez, O. T. Production of recombinant proteins: Challenges and solutions. *Methods Mol. Biol.* **2004**, *267*, 15−52.
(33) Emanuelsson, O.; Brunak, S.; von Heijne, G.; Nielsen, H. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protoc.* **2007**, *2* (4), 953−971.
(34) Choo, K. H.; Tan, T. W.; Ranganathan, S. A comprehensive assessment of N-terminal signal peptides prediction methods. *BMC Bioinf.* **2009**, *10* (Suppl. 15), S2.
(35) Petersen, T. N.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 4.0: Discriminating signal peptides from transmembrane regions. *Nat. Methods* **2011**, *8* (10), 785−786.
(36) Cappai, R.; Mok, S. S.; Galatis, D.; Tucker, D. F.; Henry, A.; Beyreuther, K.; Small, D. H.; Masters, C. L. Recombinant human amyloid precursor-like protein 2 (APLP2) expressed in the yeast Pichia pastoris can stimulate neurite outgrowth. *FEBS Lett.* **1999**, *442* (1), 95−98.
(37) Turner, P. R.; Bourne, K.; Garama, D.; Carne, A.; Abraham, W. C.; Tate, W. P. Production, purification and functional validation of human secreted amyloid precursor proteins for use as neuropharmacological reagents. *J. Neurosci. Methods* **2007**, *164* (1), 68−74.
(38) Rohe, M.; Carlo, A. S.; Breyhan, H.; Sporbert, A.; Militz, D.; Schmidt, V.; Wozny, C.; Harmeier, A.; Erdmann, B.; Bales, K. R.; Wolf, S.; Kempermann, G.; Paul, S. M.; Schmitz, D.; Bayer, T. A.; Willnow, T. E.; Andersen, O. M. Sortilin-related receptor with A-type repeats (SORLA) affects the amyloid precursor protein-dependent stimulation of ERK signaling and adult neurogenesis. *J. Biol. Chem.* **2008**, *283* (21), 14826−14834.
(39) Pahlsson, P.; Shakin-Eshleman, S. H.; Spitalnik, S. L. N-linked glycosylation of beta-amyloid precursor protein. *Biochem. Biophys. Res. Commun.* **1992**, *189* (3), 1667−1673.
(40) Tomita, S.; Kirino, Y.; Suzuki, T. Cleavage of Alzheimer's amyloid precursor protein (APP) by secretases occurs after O-glycosylation of APP in the protein secretory pathway. Identification of intracellular compartments in which APP cleavage occurs without using toxic agents that interfere with protein metabolism. *J. Biol. Chem.* **1998**, *273* (11), 6277−6284.
(41) Perdivara, I.; Petrovich, R.; Allinquant, B.; Deterding, L. J.; Tomer, K. B.; Przybylski, M. Elucidation of O-glycosylation structures of the beta-amyloid precursor protein by liquid chromatography-mass spectrometry using electron transfer dissociation and collision induced dissociation. *J. Proteome Res.* **2009**, *8* (2), 631−642.
(42) Georgopoulou, N.; McLaughlin, M.; McFarlane, I.; Breen, K. C. The role of post-translational modification in beta-amyloid precursor protein processing. *Biochem. Soc. Symp.* **2001**, *67*, 23−36.
(43) Sola, R. J.; Griebenow, K. Effects of glycosylation on the stability of protein pharmaceuticals. *J. Pharm. Sci.* **2009**, *98* (4), 1223−1245.
(44) Gakhar-Koppole, N.; Hundeshagen, P.; Mandl, C.; Weyer, S. W.; Allinquant, B.; Muller, U.; Ciccolini, F. Activity requires soluble amyloid precursor protein alpha to promote neurite outgrowth in neural stem cell-derived neurons via activation of the MAPK pathway. *Eur. J. Neurosci.* **2008**, *28* (5), 871−882.
(45) Halim, A.; Brinkmalm, G.; Ruetschi, U.; Westman-Brinkmalm, A.; Portelius, E.; Zetterberg, H.; Blennow, K.; Larson, G.; Nilsson, J. Site-specific characterization of threonine, serine, and tyrosine glycosylations of amyloid precursor protein/amyloid beta-peptides in human cerebrospinal fluid. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108* (29), 11848−11853.
(46) Liu, T.; Qian, W. J.; Gritsenko, M. A.; Camp, D. G., 2nd; Monroe, M. E.; Moore, R. J.; Smith, R. D. Human plasma N-glycoproteome analysis by immunoaffinity subtraction, hydrazide chemistry, and mass spectrometry. *J. Proteome Res.* **2005**, *4* (6), 2070−2080.
(47) Parodi, A. J. Biosynthesis of protein-linked oligosaccharides in trypanosomatid flagellates. *Parasitol. Today* **1993**, *9* (10), 373−377.
(48) Mellquist, J. L.; Kasturi, L.; Spitalnik, S. L.; Shakin-Eshleman, S. H. The amino acid following an asn-X-Ser/Thr sequon is an important determinant of N-linked core glycosylation efficiency. *Biochemistry* **1998**, *37* (19), 6833−6837.