Click Chemistry-mediated Biotinylation Reveals a Function for the Protease BACE1 in Modulating the Neuronal Surface Glycoproteome*

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The cell surface proteome is dynamic and has fundamental roles in cell signaling. Many surface membrane proteins are proteolytically released into a cell’s secretome, where they can have additional functions in cell-cell-communication. Yet, it remains challenging to determine the surface proteome and to compare it to the cell secretome, under serum-containing cell culture conditions. Here, we set up and evaluated the ‘surface-spanning protein enrichment with click sugars’ (SUSPECS) method for cell surface membrane glycoprotein biotinylation, enrichment and label-free quantitative mass spectrometry. SUSPECS is based on click chemistry-mediated labeling of glycoproteins, is compatible with labeling of living cells and can be combined with secretome analyses in the same experiment. Immunofluorescence-based confocal microscopy demonstrated that SUSPECS selectively labeled cell surface proteins. Nearly 700 transmembrane glycoproteins were consistently identified at the surface of primary neurons. To demonstrate the utility of SUSPECS, we applied it to the protease BACE1, which is a key drug target in Alzheimer’s disease. Pharmacological BACE1-inhibition selectively remodelled the neuronal surface glycoproteome, resulting in up to 7-fold increased abundance of the BACE1 substrates APP, APLP1, SEZ6, SEZ6L, CNTN2, and CHL1, whereas other substrates were not or only mildly affected. Interestingly, protein changes at the cell surface only partly correlated with changes in the secretome. Several altered proteins were validated by immunoblotting in neurons and mouse brains. Apparent nonsubstrates, such as TSPAN6, were also increased, indicating that BACE1-inhibition may lead to unexpected secondary effects. In summary, SUSPECS is broadly useful for determination of the surface glycoproteome and its correlation with the secretome. Molecular & Cellular Proteomics 17: 1487–1501, 2018. DOI: 10.1074/mcp.RA118.000608.

Cell surface membrane proteins are essential for the communication between cells and their environment. They have fundamental roles in physiology and pathophysiology and represent major protein targets for existing drugs and drug development (1). The cell surface proteome is dynamic, which allows cells to regulate the levels and functions of its surface membrane proteins, for example in cell adhesion and signal transduction. Removal of membrane proteins from the surface may occur through endocytosis or proteolysis, where proteases in the membrane or the extracellular space, such as a disintegrin and metalloprotease 10 and 17 (ADAM10, ADAM17) and β-site APP cleaving enzyme 1 (BACE1), cleave off a membrane protein’s ectodomain (2–4). This proteolytic process is referred to as ectodomain shedding and does not only affect the surface proteome, but also a cell’s secretome, which comprises both soluble, secreted proteins and shed membrane protein ectodomains. The latter may be active biomolecules, such as growth factors (e.g. TGFβ) and cytokines (e.g. TNFα) but may also act as decoy receptors for their membrane-bound counterparts (5). Therefore, it is important to investigate how changes in the surface proteome are linked to alterations in the secretome during (patho)physiological conditions. But despite the tight coupling of a cell’s surface proteome and secretome, it remains challenging to directly analyze both in the presence of serum proteins within the same experiment.
Determination of the cell surface membrane proteome by mass spectrometry-based proteomics critically depends on the purity of surface membrane protein preparations. Besides subcellular fractionation and surface protein shaving, the biotinylation of cell surface proteins has become popular (6). However, biotinylation of lysine side chains in surface proteins has the drawback to block subsequent tryptic digestion during sample preparation. More recent approaches, such as cell surface capturing (CSC) and periodate oxidation and aniline-catalyzed oxime ligation (PAL), circumvented this issue by exploiting the fact that most surface membrane proteins are glycosylated or predicted to be glycosylated (7, 8). CSC and PAL use a two-step chemical protocol, which involves oxidation of protein glycans to aldehydes and subsequent labeling with a biotin-containing tag for further glycoprotein enrichment. The sugar oxidation is performed on living cells, which facilitates the selective enrichment of cell surface, but not intracellular glycoproteins. Despite their suitability for surface protein analysis, CSC and PAL are not well suited for proteomic analysis of the corresponding cell secretome as cells are mostly cultured in the presence of serum or serum-like supplements, which contain high concentrations of glycoproteins, in particular immunoglobulins. Those serum-derived glycoproteins would also be labeled, but—because of their high abundance—prevent efficient detection of the low-abundant cellular secretome proteins. Thus, alternative methods for secretome analysis such as SPECS (secretome protein enrichment with click sugars) have been developed (9). SPECS metabolically labels only newly synthesized cellular glycoproteins with click chemistry-suitable sugars (9–12). Subsequently, click chemistry allows selective enrichment of the glycoproteins from the serum protein-containing conditioned medium. A similar approach was developed where proteins are labeled with nonnatural amino acids amenable to click chemistry-mediated labeling (13). SPECS has previously been used to enrich cellular glycoproteins (11) but was not able to distinguish between surface and intracellular proteins. Here, we set up a method for specific labeling and relative quantification of cell surface membrane glycoproteins, which only requires a single chemical reaction and is complementary to secretome analyses using the SPECS method in the same experiment. The new method is named SURface-Spanning Protein Enrichment with Click Sugars (SUSPECS).

Our new approach identified nearly 700 transmembrane glycoproteins at the surface of primary murine neurons using label-free quantitative proteomics. To demonstrate the utility of SUSPECS, we applied it to study the protease BACE1, which is a key drug target in Alzheimer’s disease, as it cleaves the membrane protein APP to generate the pathogenic amyloid β peptide (14). Yet, BACE1 also cleaves numerous other membrane protein substrates (9, 15–19), whose functions may be affected when BACE1 is therapeutically blocked. In fact, BACE1-deficient mice show several defects and phenotypes (20). Therefore, we investigated the neuronal surface proteome after pharmacological inhibition of BACE1 and compared the results to changes in the secretome.

**EXPERIMENTAL PROCEDURES**

**MATERIALS**

The following antibodies were used: polyclonal DNER (R&D Systems, AF2254), monoclonal SEZ6 clone 14E5 (19) and SEZ6L (R&D Systems, MN; AF4804), SEZ6L2 (R&D Systems, AF4916), monoclonal BACE1 clone 3D5 (kindly provided by Robert Vassar), polyclonal TSPAN6 (Abgent, CA; AP9224b), monoclonal MMP17 clone EP1270Y (Abcam, Cambridge, UK; ab51075), monoclonal Golgin-97 (Invitrogen, CA; A21270), monoclonal NEO1 clone 21A8 (11), polyclonal RGMA (R&D Systems, AF2458), monoclonal GluR2 (MAB397, Millipore, Merck, Darmstadt, Germany), monoclonal Calnexin (Enzo, Stressgen, Farmingdale, NY, ADI-SPA-860), monoclonal β-actin clone AC-74 (Sigma, Merck; A5316), HRP coupled anti-mouse (Promega, Mannheim, Germany; W4020) and anti-rabbit secondary (Promega, W401B), HRP-coupled anti-goat (Santa Cruz, sc-2020), anti-rat (Santa Cruz, TX; sc-2006) and anti-sheep (Santa Cruz, sc-2473). Monoclonal APPL1 clone 8G6 (mouse IgG2a/k) was generated by peptide immunization (AA75-EPDPQRSRRCLLDPQR-AA90 within APPL1 copper binding domain) of C57BL/6 mice using standard procedures (21).

The following reagents and media were used: DMEM, Neurobasal medium, HBSS, and B27 (Thermo Fisher Scientific, Darmstadt, Germany), protease inhibitor (Sigma, P8340), C3 (β-secretase inhibitor IV; Calbiochem, 565788, final concentration 2 μM), tetra-acetylated N-azidoacetylmannosamine (Ac4-ManNAz), Dichlorotriazinylaminino fluorescein DTAF-conjugated Streptavidin (Dianova, Hamburg, Germany), Sulfo-dibenzylcyclooctyne-biotin (Sulfo-DBCO-Biotin, Jena Biosciences, Jena, Germany).

**Immunofluorescence**—COS-7 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and 1% Penicillin-Streptomycin (Pen/Strep). For immunofluorescent labeling of cell surface proteins, COS-7 cells were seeded onto poly-d-lysine (PDL)-coated glass coverslips in culture medium supplemented with 50 μM tetra-acetylated N-azidomannnosamine (Ac4-ManNAz) and cultured for 48h. Unfixed cells were washed twice with 1x phosphate buffered saline (PBS)
and metabolically labeled glycoproteins were biotinylated with 100 μM sulfo-DBCO-Biotin for 2 h at 4 °C. Subsequently, cells were fixed with 4% paraformaldehyde (PFA) in 1x PBS for 10 min at room temperature (RT). Biotinylated proteins were labeled with 2 μg/ml streptavidin-dichlorotriazinylamino fluorescein (DTAF) for 1 h at room temperature and nuclei were stained with 4 μg/ml Hoechst 33342 for 30 min at RT. Coverslips were mounted in FluorSave (Calbiochem, Merck) onto glass slides and imaged with confocal microscopy using a Leica TCS SP5 Confocal Laser Scanning Microscope (LSM) or Zeiss LSM 510 Axio Observer. Pictures were processed with ImageJ (Fiji), Photoshop (Adobe) or ZEN2 (Zeiss).

Primary Cell Culture—Primary cortical neurons were extracted from wildtype C57BL/6 mouse embryos on embryonic day 16.5 like described before (22). Briefly, embryonic cortices were dissected and cleaned from meninges. Cortices were pooled and digested with 200 μP papain (in DMEM-high glucose supplemented with L-cysteine, pH 7.4) at 37 °C for 15 min. Using a pipette, tissue was mechanically dissociated to obtain a single-cell-suspension. Cells were plated in PDL-coated T-175 cm flasks (20 × 10³ cells for proteomic experiments) or on 6 cm dishes (4 × 10³ cells for immunoblots (IB)) in plating medium (DMEM-high glucose, 10% FCS) and medium was changed after 3–4 h to culture medium (Neurobasal, 500 μM l-glutamine, 1% Penicillin/Streptomycin, 1x B27 supplement).

Treatment of Cells and Labeling of Surface Proteins—For metabolic labeling of newly synthesized glycoproteins, primary cortical neurons at 5 days in vitro (DIV) were cultured for 48 h in neuronal culture medium supplemented with 50 μM Ac₄-ManNAz and the BACE inhibitor C3 or dimethyl sulfoxide (DMSO) as control. 40 μM Biotin and incubating 2 h at 4 °C. To remove sulfo-DBCO-Biotin in excess, cells were washed twice with 1x PBS and lysed in STET-lysing buffer (150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA, 1% Triton X-100) with protease inhibitor. Lysates were cleared by centrifugation for 5 min at maximum speed and filtered through a 0.45 μM syringe filter (Millipore).

For immunoblot analyses, cells were lysed in STET-lysing buffer with protease inhibitor or SDS buffer (50 mM Tris (pH 8), 150 mM NaCl, 2 mM EDTA, 1% SDS) with protease inhibitor. Lysates were cleared by centrifugation for 5 min at maximum speed. Cleared SDS-lysates were diluted 1:1 with RIPA buffer (10 mM Tris pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% Sodium deoxycholate) and sonicated.

Sample Preparation for Mass Spectrometry—Cleared cell culture lysates of 6 control (DMSO) and 6 inhibitor treated (C3) cell samples were loaded on a polyprop chromatography column (Bio-Rad, Munich, Germany) containing 300 μl of high capacity streptavidin agarose beads (Thermofisher) and washed twice with 10 ml 2% SDS in PBS to remove nonspecifically bound proteins. The flow-through was reloaded to increase the yield of bound biotinylated proteins. Each column was washed twice with 10 ml 2% SDS in PBS. Streptavidin-beads were transferred into an Eppendorf tube in 2% SDS in PBS and liquid was removed completely with a Hamilton syringe. Beads were boiled 5 min at 95 °C in 150 μl Lämmli buffer supplemented with 8 M urea and 3 mM biotin to elute the proteins. Samples were separated with 10% SDS-polyacrylamide gel electrophoresis (PAGE) and stained with 0.025% (w/v) Coomassie Brilliant Blue in 10% acetic acid for 15 min with horizontal shaking. The gel was destained in 10% acetic acid (2 × 30 min, 1x overnight) with horizontal shaking.

In-gel Digestion and Peptide Purification—Each lane was cut into 14 horizontal slices (=14 fractions) at equal height and subjected to tryptic in-gel digestion (23). Briefly, proteins residing in the gel were denatured with 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate (ABC) and reduced with 55 mM iodoacetamide (IAA) in 100 mM ABC. Proteolytic digestion was performed at 37 °C overnight using 150 ng trypsin per fraction. Peptides were extracted with 40% acetonitrile (ACN) supplemented with 0.1% formic acid, dried by vacuum centrifugation, and reconstituted in 0.1% formic acid for proteomic analysis.

LC-MS/MS Analysis—Each gel fraction was analyzed on an Easy nLC-1000 (Thermo Proxeon), which was coupled online via a nano electrospray source (Thermo Proxeon) to a Velos Pro Orbitrap Mass Spectrometer (Thermo). Peptides were separated on a self-packed C18 column (300 mm × 75 μm, ReproSil-Pur 120 C18-AQ, 1.9 μm, Dr. Maisch) with a binary gradient of water (A) and acetonitrile (B) containing 0.1% formic acid (0 min, 2% B; 3:30 min 5% B; 48:30 min, 25% B; 59:30, 35% B; 64:30, 60% B). Full MS spectra were acquired at a resolution of 70,000. The ten most intense peptide ions per spectrum were chosen for collision-induced dissociation (CID) within the ion trap. A dynamic exclusion of 40 s was applied for CID acquisition.

LC-MS/MS Data Analysis and Statistical Evaluation—Database search and label free quantification was performed with the software MaxQuant (version 1.5.5.1, maxquant.org). Trypsin was defined as protease (cleavage specificity: C-terminal of K and R). Carbamidomethylation of cysteines was defined as fixed modification. Oxidation of methionines and acetylation of protein N termi were defined as variable modifications. Two missed cleavages were allowed for peptide identification. The first search option was enabled with a precursor mass tolerance and a fragment mass tolerance of 20 ppm. The mass tolerances for the main search were set to 4.5 and 20 ppm for precursor and fragment ion masses, respectively. The false discovery rate (FDR) for both peptides and proteins was adjusted to less than 1% using a target and decoy approach (concatenated forward/reverse database) including the reference mouse proteome (UniProt, download: June 8, 2018).
Experimental Design and Statistical Rationale—For MS experiments, we analyzed 6 biological replicates in 6 independent experiments. Each biological replicate originated a separate neuronal preparation (6 C57BL/6 mice). Cortices of all embryos (~6–9 per mouse) were pooled during one extraction (so that all neurons in one experiment have the same ‘background’). \(8 \times 10^6\) neurons were used for one biological replicate: half was treated with the BACE1 inhibitor C3 and half was vehicle-treated with DMSO. C3 and DMSO samples from one experiment were separated on a SDS-PAGE and analyzed by LC-MS/MS and quantified with a label-free approach. For quantification, we only considered proteins present in both groups (C3 and DMSO) in at least 3 out of 6 experiments. Statistical testing was performed with a one-sample \(t\) test and corrected for multiple hypothesis testing according to Benjamini-Hochberg.

Immunoblots of neurons were performed with at least 3 different neuronal preparations (divided in up to 3 experiments per preparation), which yielded 6–11 independent experiments per protein with each one C3 and one DMSO-treated group. Each protein was checked by immunoblotting in neurons originating 3 different mice. Immunoblots of mouse cortices were performed with 6 biological replicates. One biological replicate equals one WT and one B1/–/– animal. Immunoblot experiments were statistically tested by applying the Mann-Whitney-\(U\) test.

RESULTS

Selective Labeling of Plasma Membrane Proteins—Previously, we developed the “secretome protein enrichment with click sugars” (SPECS) method (9), which uses click chemistry to label and identify the cellular secretome—comprising both soluble proteins and proteolytically released membrane protein ectodomains—by mass spectrometry. To be able to compare secretome changes to alterations in the surface proteome using material from the same cellular experiment, we first set up a workflow referred to as ‘Surface-Spanning Protein Enrichment with Click Sugars’ (SUSPECS), where SPECS-like labeling is used to assess the surface proteome of glycosylated membrane proteins (Fig. 1). SUSPECS exploits the fact that according to the UniProt-database most membrane proteins are glycosylated (supplemental Fig. S1). For example, more than 90% of single-pass type I transmembrane proteins, to which most substrates of BACE1 and other surface proteases belong (9, 16, 17), are glycosylated (supplemental Fig. S1).

To facilitate the enrichment of glycoproteins, glycans were labeled with tetra-acetylated N-azidomannosamine (Ac4-ManNAz), a click chemistry-suitable azido-modified mannoside derivative. When taken up by cells, Ac4-ManNAz is modified to N-azido-sialic acid and metabolically incorporated into the terminal glycan structures of newly synthesized glycosylated proteins (25, 26). After 2 days of continuous metabolic labeling, the conditioned medium was removed, and living cells
were washed with PBS and subsequently biotinylated with sulfo-dibenzocyclooctyne (DBCO)-biotin on ice for two hours. In a reaction referred to as copper-free click reaction, the DBCO moiety reacts with the azido moiety of the glycoprotein, thereby leading to covalent biotinylation of the glycoprotein (Fig. 1, inset). Afterwards, cells were lysed, and the biotinylated proteins were enriched with streptavidin agarose and separated on 1D polyacrylamide gels (a representative Coomassie stained gel is shown in supplemental Fig. S2). The gel was cut into 14 fractions, subjected to in-gel tryptic digestion and analyzed by label free quantitative mass spectrometry. To specifically label cell surface proteins, but not intracellular proteins, the sulfo-variant of DBCO-biotin was used, which carries a negative charge and should not cross the plasma membrane. To evaluate whether this is indeed true, COS7 cells were subjected to SUSPECS-mediated biotinylation. Instead of lysing cells and precipitating biotinylated proteins with streptavidin, cells were fixed and permeabilized for confocal microscopy. Biotinylated proteins were visualized with fluorescent streptavidin (streptavidin-dichlorotriazinylamino fluorescein (DTAF)) and nuclei were stained with Hoechst dye. SUSPECS-labeled cells showed a cell surface staining, with no apparent signal in the cytoplasm or intracellular organelles (Fig. 2A and 2B). As a control, cells were cultured without Ac₄-ManNAz (Fig. 2A, lower panel), which, as expected, did not yield a positive streptavidin-DTAF signal. This demonstrates the specificity of the fluorescent signal for biotinylated proteins as well as the selectivity of the click reaction. As a further control experiment we determined biochemically whether the SUSPECS workflow indeed leads to an enrichment of cell surface proteins compared with the whole cell lysate. To this aim, cell surface proteins of primary murine neurons were biotinylated according to the SUSPECS workflow, precipitated with streptavidin, separated by gel electrophoresis and analyzed by immunoblot. Four known cell surface proteins, NEO1 (27), GluR2 (28), RGMa (29), and MT4MMP (MMP17) (30), were enriched in the surface-labeled samples (Fig. 2C and quantification in 2D). Conversely, the Golgi marker Golgin-97 (31), the ER marker calnexin (32) and the cytoskeletal protein beta actin, which do not reside at the plasma membrane, were largely depleted from the SUSPECS workflow. Fig. 1. Workflow of SUSPECS method. Cell culture medium is supplemented with tetra-acetylated N-azidoacetylmannosamine (Ac₄-ManNAz) for metabolic labeling and cells are treated with inhibitor (C3) or control (DMSO). Ac₄-ManNAz is metabolized and incorporated as modified terminal N-azido-sialic acid into glycan structures of newly synthesized proteins. After 48h cells are subjected to click mediated biotinylation for 2 h at 4 °C, during which the alkyne group of the sulfo-dibenzocyclooctyne-biotin conjugate (DBCO) covalently reacts with the azide group of the azido-sialic acids of glycoproteins in the cell membrane (inset box). Biotinylated glycoproteins are enriched and purified with streptavidin-beads and separated via 1D-SDS-PAGE. Fractionated, in-gel digested proteins are analyzed by mass spectrometry using label-free quantification.
Surface Glycoproteomics of Neurons

Figure A: Immunofluorescence images showing the localization of Ac4-ManNAz in +Ac4-ManNAz and -Ac4-ManNAz conditions. Merge images demonstrate the combined fluorescence of Hoechst and Streptavidin-DTAF. Scale bars are 10µm.

Figure B: Confocal microscopy images at different planes (bottom, mid, top) with scale bars of 5µm. The images depict the localization of various proteins.

Figure C: Western blot analysis of total lysates and surface lysates at 25x magnification. Proteins include NEO1, GluR2, MT4MMP, RGMa, Goiln-97, Calnexin, and Actin.

Figure D: Relative enrichment (%) of proteins in different compartments (Surface, Golgi, ER, Cytoskeleton) with logarithmic scale.
Surface-labeling samples, demonstrating that SUSPECS enriches cell surface proteins (Fig. 2C).

**SUSPECS Identifies Transmembrane Proteins at Neuronal Surface**—Next, we applied SUSPECS to determine the surface glycoproteome of primary murine neurons treated with or without the BACE1 inhibitor C3. First, we generally determined the proteins at the cell surface and subsequently how their levels were changed upon BACE1 inhibition.

Proteins were considered as identified, if they were detected by at least 2 unique peptides in at least 3 out of the 12 samples (six samples each with or without C3). This yielded 3957 identified proteins (see supplemental Data S1). Although SUSPECS metabolically labels glycoproteins, the total number of proteins may also include nonglycoproteins or peripherally membrane-associated proteins, which either are bound unspecifically to the beads or are co-purified during the streptavidin pulldown as they are binding partners of glycoproteins. Thus, we focused specifically on glycosylated transmembrane and GPI-anchored proteins, which we consider as the cell surface glycoproteome in our study. Therefore, we filtered the proteins according to reviewed Uniprot annotations using the keyword “glycoprotein” as well as the subcellular location terms: single-span membrane proteins of type I, II, III or IV, as well as GPI-anchored and multipass membrane proteins. Our criteria yielded 691 transmembrane and GPI-anchored glycoproteins at the neuronal surface, with the majority being single-span type I membrane proteins (40%) or multipass membrane proteins (45%). 5 proteins had double annotations and are therefore separated into new categories (TM1/GPI, TM1/TM2, and TM1/Multi pass) (Fig. 3A, see supplemental Data S1 for a complete list of identified proteins). The identified unique tryptic peptides were matched to the sequence of the 691 membrane proteins using the QARIP webserver (33). As expected, the peptides were derived from the extra- and intracellular protein domains (for selected examples, see supplemental Fig. S3), but generally not from the transmembrane domains, which contain long stretches of mostly hydrophilic amino acids, but rarely a tryptic cleavage site. Only for 20 multipass proteins some peptides also contained amino acids originating from a possible transmembrane region. However, for those proteins the Uniprot transmembrane annotations were incomplete. Interestingly, for three known BACE1 substrates (CHL1, L1CAM, APLP1) we also identified peptides spanning the previously identified BACE1 cleavage sites (9, 15, 34), which are located close to the transmembrane domain (supplemental Fig. S3).

Next, we functionally categorized the transmembrane proteins according to Gene Ontology classifications using PANTHER (35). The identified transmembrane glycoproteins fell into five main functional categories (Fig. 3B), namely transporters (18.20%), receptors (17.57%), hydrolases (16.11%), transferases (8.79%), and signaling molecules (6.07%). Other categories (33.26%) included cell adhesion molecules, extracellular matrix proteins and defense/immunity proteins (Fig. 3B). Many of the identified proteins are known to be located at the neuronal cell surface according to Uniprot, which validates SUSPECS as a method for investigating the cell surface proteome. Taken together, these results demonstrate that SUSPECS identified nearly 700 neuronal surface transmembrane glycoproteins with a broad range of physiological functions, making the method applicable to various studies in the life sciences investigating cell surface proteins.

**BACE Activity Modulates the Protein Composition of Neuronal Surface Membranes**—Inhibition of BACE1 increases neuronal surface levels of some of its substrates, including SEZ6, SEZ6L, and CNTN-2, which led to the hypothesis that BACE1 cleavage is a general mechanism to control the levels of its substrates at the cell surface (19, 36). However, this hypothesis has not yet been tested for the numerous additional BACE1 substrates. Moreover, BACE1, which has an acidic pH optimum, does not cleave its substrates at the cell surface, but in the endosomes and the trans-Golgi network (14, 37), thus questioning whether BACE1 can generally control surface levels of all its substrates.

To resolve this open issue, we used SUSPECS and determined how pharmacological BACE1 inhibition with the established inhibitor C3 (38) affects the neuronal surface proteome. Although C3 also blocks the BACE1-homolog BACE2, we
BACE1 inhibition. Benjamini-Hochberg correction was applied to correct for multiple hypothesis testing using a 5% false discovery rate (FDR) which resulted in q-values below the pie charts.

![Fig. 3. Topology and protein classification of identified transmembrane glycoproteins. A, Glycosylated transmembrane proteins (691) identified with minimum 2 unique peptides in at least 3 measurements were classified using Uniprot 'subcellular locations': shown are single-pass type I membrane proteins (TM1), single-pass type II membrane proteins (TM2), single-pass type III membrane proteins (TM3), single-pass type IV membrane proteins (TM4), GPI-anchored (GPI) and multipass membrane proteins (Multi pass). B, Classification of identified proteins was done using the PANTHER (protein annotation through evolutionary relationship) classification system. Total number of proteins in each class is given and their percentage relative to all classified proteins that were functionally annotated in PANTHER. Note that individual proteins may fall into multiple functional classes in PANTHER. Sub-categorization of the 5 main protein classes as well as an overview of the remaining, other categories is shown as pie charts under each class. Relative percentages of functional classes in each category are given below the pie charts.

The significantly enriched proteins can be grouped into three different categories: mildly increased (<1.5-fold), moderately increased (1.5 < fold-change < 2), and strongly increased (>2-fold). Nine proteins showed a mild increase (<1.5-fold), three multispans membrane proteins a moderate increase (>1.5 fold, but < 2-fold) and seven proteins had a strong increase (>2-fold, up to 7-fold) (Fig. 4). Six out of the seven proteins with the strongest increase (>2-fold) are single-span and GPI-anchored proteins and are known BACE1 substrates: APLP1, CNTN2, CHL1, SEZ6, SEZ6L1 and APP (14, 15, 18, 19, 41). SEZ6, SEZ6L, CNTN-2, APP, APLP1, and CHL1 were previously known to be enriched at the neuronal surface upon BACE1 inhibition (15, 19, 36, 42, 43). The seventh protein in this group is PLD3, a type II membrane protein, which is studied in AD research (44–46). In the group of proteins with a mild increase, one more known BACE1 substrate (L1CAM) and a BACE1 substrate candidate (TMEM132a) were detected (9, 15, 16). For L1CAM an accumulation on the membrane has not been shown so far (see supplemental Data S1 for a complete list of 471 proteins). The other single-span membrane proteins in the mild enrichment category, such as PODXL2 and CADM2, may be additional BACE1 substrates or be enriched as a secondary consequence of BACE1 inhibition. Taken together, the identification of known BACE1 substrates among the enriched proteins demonstrates that BACE1 can control surface levels of its substrates beyond the previously investigated proteins. Yet, the list of 471 quantified transmembrane proteins also contains several other BACE1 substrates or substrate candidates, which did not show FDR-significant changes of their surface levels. This includes DNER, SEZL2, GLG1, LRRN1, and NTM demonstrating that BACE1 does not lead to a sig-
significant alteration of the surface levels of all its known substrates.

**Validation of Regulated Cell Surface Proteins in Primary Neurons and BACE1-deficient Mouse Cortices**—To validate the proteomic data by employing an orthogonal method, we treated primary neurons as above in an independent experiment and used immunoblot analysis for protein detection after immunoprecipitation and pull-down (Fig. 5). We have chosen selected BACE1 substrates that either had shown a significant increase (APLP1, SEZ6, SEZ6L), a mild, but not significant, increase (DNER) or no increase at all (SEZ6L2) in the proteomic data set. Additionally, we have chosen a presumed nonsubstrate with an increase (TSPAN6) and a known nonsubstrate MT4MMP (MMP17) (11). The increase for APLP1, SEZ6, SEZ6L and TSPAN6 observed by mass spectrometry (MS) was comparable to the significant increases seen in immunoblots (IB) (APLP1: 7.5× (MS) versus 7× (IB), SEZ6: 2.9× (MS) versus 3.6× (IB), SEZ6L: 2.6× (MS) versus 1.8× (IB), TSPAN6: 1.8× (MS) versus 3.5× (IB)) (Fig. 4 and 5A, 5B). Likewise, the unaltered abundance of SEZ6L2 and MT4MMP was validated by immunoblot analysis. Also, for DNER the mild increase (<1.5×) in the proteomic experiment was confirmed by immunoblot, demonstrating the reliability of the mass spectrometry-based quantification of SUSPECS labeling. Interestingly, although the mild increase of DNER levels was not significant after FDR correction in the proteomic experiment, it did reach significance in the immunoblot quantification (Fig. 5A, 5B).

From the same experiments, we also blotted the total cell lysates, which comprise the cell surface proteins, but also the intracellular proteins, including those in intracellular organelles. Protein changes in the total lysate were like the changes at the surface (Fig. 5A, 5B). SEZ6 and SEZ6L in the cell lysate displayed two protein bands in immunoblots, one carrying the immature N-glycosylation typical for a protein in the endoplasmic reticulum and Golgi (immature SEZ6 and SEZ6L) and
Surface Glycoproteomics of Neurons

TABLE I
Significantly changed single-pass membrane proteins and GPI-anchored proteins. False discovery rate-corrected significant proteins are sorted by their effect size (C3/DMSO). Proteins are shown with their Uniprot accession number (Uniprot AC), their topology, change in surface levels detected upon BACE1 inhibition over control (C3/DMSO), and references in case that they were described before as BACE1 substrates.

| Uniprot AC | Gene name | Protein name | Topology | C3/DMSO | p value | References |
|------------|-----------|--------------|----------|---------|---------|------------|
| Q03157     | Aplp1     | Amyloid precursor-like protein 1 | Type 1   | 7.45    | 9.72E-06 | (9, 15–19) |
| Q61330     | Cntn2     | Contactin-2  | Type 1   | 4.08    | 3.88E-05 | (9, 15, 18, 19) |
| P70232     | Ch1       | Close homolog of neural cell adhesion molecule L1 | Type 1   | 3.43    | 1.29E-06 | (9, 15, 18, 19) |
| Q7TSK2     | Sez6      | Seizure protein 6 | Type 1   | 2.88    | 1.38E-03 | (9, 18, 19) |
| Q6P1D5     | Sez6l     | Seizure 6-like protein | Type 1   | 2.60    | 4.06E-04 | (9, 17–19) |
| O3S405     | Pld3      | Phospholipase D3 | Type 2   | 2.30    | 1.45E-03 | |
| P12023     | App       | Amyloid beta A4 protein (APP) | Type 1   | 2.07    | 1.76E-03 | (9, 15–18) |
| Q922P8     | Tmem132a  | Transmembrane protein 132A | Type 1   | 1.49    | 2.11E-03 | (9, 16) |
| Q6JMB8     | Cntn6     | Contactin-6  | Type 1   | 1.35    | 1.46E-03 | (9, 15, 16) |
| P11627     | L1cam     | Neural cell adhesion molecule L1 | Type 1   | 1.34    | 2.13E-04 | |
| Q8BKG3     | Ptk7      | Inactive tyrosine-protein kinase 7 | Type 1   | 1.33    | 2.85E-04 | |
| Q8BLQ9     | Cadm2     | Cell adhesion molecule 2 | Type 1   | 1.31    | 4.50E-04 | |
| Q6IE6E     | Tmem132e  | Transmembrane protein 132E | Type 1   | 1.30    | 3.2E-05 | |
| Q8CAE9     | Pdx12     | Podocalyxin-like protein 2 | Type 2   | 1.29    | 1.57E-03 | |
| P10852     | Stc3a2    | 4F2 cell-surface antigen heavy chain | Type 1   | 1.20    | 4.33E-04 | (16) |
| O89026     | Robo1     | Roundabout homolog 1 | Type 1   | 1.20    | 4.33E-04 | |

one with a larger molecular weight carrying complex glycans (mature SEZ6 and SEZ6L) (19). At the cell surface only, the mature forms were detected (Fig. 5A). Interestingly, in the lysate, where both forms were detected, only the mature form but not the immature form was increased, which is consistent with BACE1 being active in post-Golgi compartments, such as trans-Golgi network and endosomes (37, 47, 48).

Given that increased protein levels were not only visible at the neuronal cell surface, but also in total neuronal lysates, we further validated the SUSPECS data in vivo using postnatal day 7 wild-type and BACE1-deficient mouse brain cortices. We have chosen proteins that are well detected in mouse brain by immunoblots. As cell surface labeling is not possible in brain extracts, membrane fractions containing membrane proteins and membrane-associated proteins were generated and blotted for several BACE1 substrates (APLP1, SEZ6, SEZ6L, and DNER) and for apparent nonsubstrates (TSPAN6) (Fig. 5C, 5D). Like the observations in primary neurons, levels of APLP1, SEZ6 and SEZ6L were increased in BACE1−/− brains, although the effect size was not as strong. In contrast to neurons, no significant change in protein level in brain cortices was observed for DNER and TSPAN6 (Fig. 5C, 5D). Unlike BACE1, which is most highly expressed in neurons and CNTN2 and TSPAN6 are also expressed in other brain cell types (49), where their levels may be controlled by proteases other than BACE1. This would explain increased full-length levels of both proteins specifically in neurons, but not in brain tissue upon BACE1 inactivation. SEZ6L2, which was not changed in neurons, was also not changed in the BACE1-deficient brains. Taken together, these results demonstrate that the BACE1-dependent accumulation of selected membrane proteins seen by SUSPECS was validated by immunoblots in vitro in primary neurons and in vivo in mouse brains.

BACE1 Differentially Regulates Surface and Secretome Levels of its Substrates—Using the SPECS method for secretome analysis we previously reported that different substrates are cleaved by BACE1 to different extents (9). Some substrates were only cleaved by BACE1, such that BACE1 inhibition nearly completely blocked substrate cleavage and ectodomain release into the secretome, e.g. for SEZ6, APLP1, L1CAM, and CHL1. However, other substrates were also cleaved by additional proteases, such as ADAM10 in the case of APP and DNER, so that BACE1 inhibition only led to a mild reduction of total ectodomain cleavage. To test whether a strong reduction in the secretome is typically coupled to a strong increase at the surface, we carried out a substrate meta-analysis comparing the effect size of surface increases obtained by SUSPECS to the secretome changes obtained previously by SPECS (Figure 6) under the same conditions, where primary neurons were treated with the BACE1 inhibitor C3 (9).

Four out of the six substrates, which showed the strongest (2–7-fold) surface increase by SUSPECS (APLP1, SEZ6, SEZ6L, CHL1), also had the most pronounced reductions in the secretome, ranging from 4–35% of cleavage remaining (Fig. 6). Conversely, DNER, SEZ6L2 and TMEM132a had a mild (<1.5-fold) or no increase of surface levels and only a mild reduction (20–40%) of their secretome levels. Thus, there is a reasonable correlation between changes at the surface and in the secretome for several substrates. Yet, a lack of correlation was observed for other substrates. APP and CNTN2 belonged to the group of substrates with strongest surface increases, but only showed a mild reduction of less than 40% in the secretome. The opposite was seen for additional substrates. For example, L1CAM, which showed a mild (1.35-fold) increase in cell surface levels, is one of the proteins with the strongest reduction in the secretome (21%
**FIG. 5. Validation of identified surface protein changes by immunoblot.** A, Primary neurons were treated with DMSO as control or the BACE inhibitor C3 and subjected to SUSPECS. streptavidin-enriched glycoproteins from the surface and proteins in the total lysates were analyzed by immunoblotting. Actin was used as loading control to evaluate the purity of the enriched surface protein fraction. No actin signal was detected in the surface fraction. Note: The vertical dashed line in the APLP1 blot indicates that the samples were run on the same gel and next to each other, but were loaded in reverse order (first total lysates then surface). For illustrative purposes, order was changed. B, Signals were normalized to actin levels (total lysates) or MT4MMP (surface) and ratios were quantified relative to the control condition (DMSO). APLP1, SEZ6, SEZ6L, DNER, and TSPAN6 significantly increased in total lysates (upper panel) and on the cell surface (lower panel). Statistical testing was performed with n = 6–11 replicates, using the Mann-Whitney-U test with the significance criteria of p < 0.05. C, Membrane fractions of postnatal day 7 (P7) BACE1-knockout (B1-/−) and wildtype (WT) mouse cortices were investigated by immunoblotting for protein accumulation in vivo. Actin- or calnexin-normalized signals were compared and quantified. Ratios are shown relative to the control (WT). APLP1, SEZ6, and SEZ6L showed significant accumulation in the membrane fraction of B1-/− cortices. DNER, TSPAN6, and SEZL2 levels did not increase significantly in B1-/− cortex membranes. Statistical testing was performed with n = 6 replicates, using the Mann-Whitney-U test with the significance criteria of p < 0.05. (APLP1: arrowhead = specific bands, asterisk = unspecific signals; SEZ6: m = mature, im = immature).
remaining). Likewise, GLG1, LRRN1, and NTM had their cleaved ectodomain levels reduced by more than 65%, but did not reveal a significant increase at the cell surface.

Taken together, the comparison of the SPECS and SUSPECS results reveals important insights into the molecular consequences of BACE1 inhibition in neurons. We conclude that loss of BACE1 substrate cleavage is partly coupled to corresponding substrate level increases at the neuronal surface.

**DISCUSSION**

Cell surface membrane proteins have a fundamental role for cell signaling in health and disease and may be proteolytically released into a cell’s secretome, where they can have additional functions in cell-cell communication. Here, we demonstrate that SUSPECS is a new method for enrichment, identification, and quantification of the cell surface proteome. SUSPECS identified nearly 700 transmembrane glycoproteins at the surface of primary murine neurons and demonstrated a selective role for the protease BACE1 in remodeling the neuronal surface proteome.

SUSPECS compares well to two previously developed methods for surface protein biotinylation, cell surface capturing (CSC) and periodate oxidation and aniline-catalyzed oxime ligation (PAL). As described in more detail in the introduction, CSC and PAL use a two-step chemical labeling, where surface glycoproteins are first chemically oxidized and then labeled with a biotin-containing reagent (7, 8, 50). In contrast, SUSPECS uses a single chemical reaction based on copper-free click chemistry, which makes the method easy and fast to carry out.

In PAL and CSC sample complexity is strongly reduced by focusing on the analysis of N-glycopeptides. To this aim, PNGase F digestion is performed after tryptic digest to release the specific peptides containing the N-glycosylation site. CSC typically identified a few hundred surface glycoproteins per cell type (7), which is like the nearly 700 proteins identified here by SUSPECS. CSC was recently also used to generate a cell surface protein atlas including 1296 mouse and 1492 human cell surface glycoproteins from over 70 different cell types (51). PNGase F digestion may also be carried out after SUSPECS protein enrichment and tryptic on bead-digestion to focus on N-glycopeptides. Specific identification of the glycopeptides or biotinylated peptides has the advantage of reduced sample complexity but comes with the disadvantage that only few peptides are identified per protein and can be used for quantitative proteomics. Therefore, we have chosen to analyze all tryptic peptides in SUSPECS to achieve reliable relative label free protein quantification, which may also be feasible with CSC and PAL.

Recently, a method for enrichment and quantification of biotinylated peptides called “direct detection of biotin-containing tags” (DiDBiT) was developed, which reduces binding of nonspecific proteins to beads (52). A modified version of our protocol for cell surface labeling protocol may also be combined with DiDBiT. Because glycans and not amino acids are labeled in our SUSPECS protocol, the metabolic labeling of amino acids would have to be performed using azidohomocysteine (AHA) (13), an amino acid analog of methionine which contains an azido moiety, instead of Ac4-ManNAz. After biotin labeling using sulfo-DBCO-biotin, DiDBiT could be employed to quantify only biotinylated peptides (52). Yet, AHA is cyt-
Upon BACE1 inhibition may result from different molecular mechanisms. Yet, this limitation appears acceptable for proteomic discovery experiments, given that a large fraction of membrane proteins is indeed glycosylated (supplemental Fig. S1). Moreover, compared with direct biotinylation of lysine side chains in surface proteins, e.g. by sulfo-NHS-biotin, the three methods SUSPECS, CSC and PAL allow tryptic digest of the biotinylated proteins which is well suited for subsequent mass spectrometric analysis.

An advantage of SUSPECS over other cell surface proteomics methods is the possibility to combine it with SPECS for secretome analysis in the presence of serum or other protein containing cell culture medium supplements, as only newly synthesized cellular proteins are metabolically labeled with the click sugar. Serum and supplements are often required for primary cells and to keep cells under physiological conditions (13). Because of the metabolic labeling step, SUSPECS is particularly useful for cells or tissues in culture, whereas CSC and PAL may also be used with freshly prepared cells or tissues that are not taken into culture as no metabolic labeling step is required.

A comparison of surface proteome and secretome can yield new and unexpected insights into biology. This is shown in our study with the example of BACE1 inhibition, which selectively remodeled a small fraction (4%) of the quantified surface proteome (21 out of 471 proteins), including eight BACE1 substrates. This was unexpected, because under the same conditions BACE1 inhibition reduced shedding of more than 30 substrates and substrate candidates in the secretome (9). Potentially, BACE1 controls surface levels of more than the eight substrates identified here, as seen with the protein DNER. Although DNER did not show a significant increase at the cell surface in the proteomic analysis when applying strict multiple hypothesis testing corrections, we validated the mild increase of DNER surface levels by immunoblot analysis.

The selective surface accumulation of only some substrates upon BACE1 inhibition may result from different molecular mechanisms. Several of the strongly (> 2-fold) accumulating BACE1 substrates (APLP1, SEZ6, SEZ6L, CHL1) are nearly exclusively cleaved by BACE1 in neurons and not by other proteases (15, 19, 41), demonstrating that in the absence of BACE1 activity no other protease compensates and consequently the full-length membrane proteins accumulate within cells and at the cell surface. Conversely, another substrate, SEZ6L2, which is only partially shed by BACE1 and mostly by other proteases (9), was not significantly increased at the cell surface. However, a correlation between the extent of BACE1 cleavage and surface accumulation was not occurring for all substrates, including CNTN-2 and APP. Both proteins are also shed by other proteases, but still accumulated at the neuronal surface upon BACE1 inhibition. The opposite was seen for L1CAM, where shedding was nearly completely inhibited, but surface levels were only mildly increased. The poor correlation seen for some substrates may result from different molecular mechanisms. For example, if only a small fraction of the substrate is shed, BACE1 inhibition may completely block shedding but only mildly increase surface levels of the substrate, as it was seen for L1CAM. Another mechanism explaining the selective surface substrate enrichment might be the cellular compartment where BACE1 cleaves its substrates. BACE1 has an acidic pH and cleaves its substrates in acidic cellular compartments, in endosomes and the trans-Golgi network (14, 37). If BACE1 cleavage is blocked, some uncleaved substrates may be transported to the cell surface and accumulate. Yet other substrates, if uncleaved, may not be transported further, but instead reside within endosomes/TGN or be degraded, and thus, not accumulate at the cell surface. As an additional mechanism it is conceivable that BACE1 substrates with a long half-life could possibly show a stronger accumulation upon BACE1 inhibition than substrates with a short half-life, as an excess of the latter ones may be rapidly degraded by other cellular mechanisms. Yet, proteins with both a long and a short half-life are well detected by SUSPECS because of the continuous labeling with the click sugar. This is for example seen with the efficient detection of APP, a protein with a known half-life of less than one hour (53, 54).

Even though only few proteins were increased at the neuronal surface upon BACE1 inhibition, this may have consequences for the function of the affected proteins, for example for CNTN-2 and APLP1. Increased surface levels of the neural adhesion molecule CNTN-2 were suggested to alter cell adhesion, neurite outgrowth and axon guidance (36), and may, thus, contribute to some of the phenotypes reported for BACE1-deficient mice (55). APLP1 acts as a transcellular adhesion molecule at the synapse (56). Altered APLP1 expression affects synapse formation in vitro and in vivo (57) such that the increased APLP1 surface levels in BACE1-inhibited and BACE1-deficient conditions may interfere with synapse formation and maintenance, which are known to be altered in BACE1-deficient mice (58–60). Additionally, also loss of APLP1 results in reduced spine density in aged mice (57). Not only substrates, but also apparent nonsubstrates of BACE1, such as TSPAN6, accumulated at the neuronal cell surface, suggesting that BACE1 inhibition may have more potential mechanism-based consequences than expected.

In summary, SUSPECS is a new, easy, and rapid method for determination of the cell surface glycoproteome. A major strength of the method is that—beyond surface proteome analysis—it can be combined with SPECS-based secretome analyses in the presence of serum or other media supplements within the same experiment. Although we have demonstrated the power of the approach using the example of the Alzheimer protease BACE1, other applications of combining SUSPECS with secretome studies may range from cell-secre-
Surface Glycoproteomics of Neurons

tome changes under inflammatory conditions to allow different cell types and the study of other cell surface proteases, many of which have essential functions in (patho) physiological conditions.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (24) partner repository with the data set identifier PXD008619. Project webpage: http://www.ebi.ac.uk/pride/archive/projects/PXD008619.

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