Brain-derived neurotrophic factor (BDNF) is generated by proteolytic cleavage of a prodomain from the proBDNF precursor either intracellularly by furin-like proteases or extracellularly by plasmin or matrix metalloproteinases. ProBDNF carries a single N-glycosylation sequon (Asn-127) that remains virtually unstudied despite being located in a highly conserved region proximal to the proteolytic site. To study the proBDNF structure and function, we expressed the protein and its nonglycosylated N127Q mutant in HEK293F cells. We found that mutation of the Asn-127 prevents intracellular maturation and secretion, an effect reproduced in WT proBDNF by tunicamycin-induced inhibition of N-glycosylation. Absence of the N-glycan did not affect the kinetics of proBDNF cleavage by furin in vitro, indicating that effects other than a direct furin–proBDNF interaction may regulate proBDNF maturation. Using an optimized LC-MS/MS workflow, we demonstrate that secreted proBDNF is fully glycosylated and carries rare N-glycans terminated by GalNAcβ1-4GlcNAcβ1-R (LacdiNac) extensively modified by terminal sulfation. We and others noted that this type of glycosylation is protein-specific, extends to proBDNF expressed in PC12 cells, and implies the presence of interacting partners that recognize this glycan epitope. The findings of our study reveal that proBDNF carries an unusual type of N-glycans important for its processing and secretion. Our results open new opportunities for functional studies of these protein glycoforms in different cells and tissues.

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This article contains Figs. S1–S6 and Tables S1.

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3 The abbreviations used are: BDNF, brain-derived neurotrophic factor; ACN, acetonitrile; CE, collision energy; ER, endoplasmic reticulum; HCD, higher-energy collisional dissociation; HexNAc, N-acetylgalactosamine; LacdiNac, N-acetylgalactosamine-β(1-4)-N-acetylglucosamine; LacNAc, galactose-β(1-4)-N-acetylgalactosamine; Neu5Ac, N-acetylneuraminic (sialic) acid; IDA, information dependent acquisition; PAPS, 3'-phosphoadenosine,5'-phosphosulfate; PEI, polyethyleneimine; PNGase F, N-glycanase F; proBDNF, prohormone brain-derived neurotrophic factor; PVDF, polyvinylidene fluoride; TAE, Tris acetate EDTA buffer; TBS-T, Tris-buffered saline, 0.1% Tween 20; XIC, extracted ion chromatogram; HEK, human embryonic kidney; NNTA, nickel-nitritoltriacetic acid; CMV, cytomegalovirus; HRP, horseradish peroxidase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

N-Glycosylation is required for secretion of the precursor to brain-derived neurotrophic factor (proBDNF) carrying sulfated LacdiNac structures

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compartments (12, 13). As the glycans mature, distinct structures of the evolving oligosaccharides carry out distinct functions at successive stages of the post-translational maturation process (13, 14). Immature N-glycans assist with protein folding in the ER, their trimmed versions assist with sorting of the (mis)folded glycoproteins for degradation or further development, and expansion of the structures by glycosyltransferases in the Golgi compartment conveys specific properties to the maturing glycoproteins. Thus glycosylation ultimately adjusts physicochemical properties of glycoproteins that may have profound impact on interactions with their partners. We therefore optimized expression of proBDNF and its N121Q mutant, which cannot be N-glycosylated, in the human embryonic kidney HEK293F cells and analyzed secretion and glycoforms of the neurotrophin by our newly optimized mass spectrometric methods. Our results show that proBDNF is fully occupied by N-glycans and that the N-glycans are required for maturation and secretion of the neurotrophin in the HEK293 cells. We further show that the N-glycans carry sulfated GalNAc1-4GlcNAc1-R (LacdiNAc) structures found to date on a limited number of glycoproteins. We further confirmed that LacdiNAc is present also on proBDNF originating from rat pheochromocytoma PC12 cells. Our results show that N-glycosylation regulates the maturation of proBDNF and suggests that

![Diagram](https://via.placeholder.com/150)

**Scheme 1.** A, schematic illustration of proBDNF with zoom into the region containing the N-glycosylation and proteolytic cleavage sites involved in the generation of mature BDNF; B, alignment of the sequences demonstrates a high degree of conservation among species (source www.uniprot.org) (please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site) (multiple alignments were performed with Align tool of the Clustal Omega 1.2.4 engine). **Letters in red** represent amino acids that differ from the human sequence. **Underlined text** highlights the NX(S/T) N-glycosylation sequon.

| Entry | Sequence | Organism |
|-------|----------|----------|
| P23560 | EEKNYLDAANMSRVRHSDPARRG 134 | Homo sapiens (Human) |
| 051578 | EEKNYLDAANMSRVRHSDPARRG 134 | Pan troglodytes (Chimpanzee) |
| H9M468 | EEKNYLDAANMSRVRHSDPARRG 132 | Elephas maximus (Indian elephant) |
| H9M466 | EEKNYLDAANMSRVRHSDPARRG 132 | Diceros bicornis (Black rhinoceros) |
| 006AB7 | EEKNYLDAANMSRVRHSDPARRG 134 | Equus caballus (Horse) |
| 095186 | EEKNYLDAANMSRVRHSDPARRG 137 | Bos taurus (Bovine) |
| P14882 | EEKNYLDAANMSRVRHSDPARRG 139 | Sus scrofa (Pig) |
| 018752 | EEKNYLDAANMSRVRHSDPARRG 134 | Ursus arctos (Brown bear) |
| 06LC15 | EEKNYLDAANMSRVRHSDPARRG 134 | Alluropoda melanoleuca (Giant panda) |
| 018755 | EEKNYLDAANMSRVRHSDPARRG 134 | Procyn Lotor (Raccoon) |
| 07YR84 | EEKNYLDAANMSRVRHSDPARRG 134 | Canis lupus familiaris (Dog) |
| 09T573 | EEKNYLDAANMSRVRHSDPARRG 134 | Felis catus (Cat) |
| 070183 | EEKNYLDAANMSRVRHSDPARRG 142 | Cavia porcellus (Guinea pig) |
| P23365 | EEKNYLDAANMSRVRHSDPARRG 136 | Rattus norvegicus (Rat) |
| P21237 | EEKNYLDAANMSRVRHSDPARRG 136 | Mus musculus (Mouse) |
| 01PTG4 | EEKNYLDAANMSRVRHSDPARRG 134 | Myotis luciflous (Little brown bat) |
| P25429 | EEKNYLDAANMSRVRHSDPARRG 133 | Gallus australis (Chicken) |
| 04N29 | EEKNYLDAANMSRVRHSDPARRG 133 | Meleagris aalloaavo (Wild turkey) |
| 04H7T7 | EEKNYLDAANMSRVRHSDPARRG 133 | Taeniopygia guttata (Zebra finch) |
| C5H908 | EEKNYLDAANMSRVRHSDPARRG 128 | Anolis carolinensis (Green anole) |
| 01X708 | EEKNYLDAANMSRVRHSDPARRG 128 | Boa constrictor (Boa) |
| 01W38 | EEKNYLDAANMSRVRHSDPARRG 119 | Xenopeltis unicolor (Sunbeam snake) |
| A3FP99 | EEKNYLDAANMSRVRHSDPARRG 134 | Xenopus tropicalis (Western clawed frog) |
| 06ZM5 | EEKNYLDAANMSRVRHSDPARRG 134 | Xenopus laevis (African clawed frog) |
| 09Z322 | EEKNYLDAANMSRVRHSDPARRG 157 | Cyrtinus carpio (Common carp) |
| 06NZ01 | EEKNYLDAANMSRVRHSDPARRG 157 | Danio rerio (Zebrafish) |

* **********...**
ProBDNF carries specific N-glycosylation

![Image](https://via.placeholder.com/150)

**Figure 1. ProBDNF stably expressed in HEK293F cells is glycosylated.** N-Glycosylation of proBDNF and the prodomain is documented by mass shift following deglycosylation with PNGase F. A, detection of proBDNF and BDNF by an antibody recognizing the BDNF region; B, detection of proBDNF and the prodomain by an antibody recognizing the prodomain region. The gel images were spliced as indicated by space to exclude samples not related to the study.

its impact in different cells, tissues, or pathophysiological conditions should be further examined.

**Results**

**Recombinant proBDNF and N121Q mutant expressed in HEK293F cells**

BDNF and proBDNF are present in biological samples at sub-nanomolar concentrations that limits detectability and, especially, analysis of their glycoforms. We therefore expressed proBDNF, its prodomain, and the N121Q mutant (which eliminated the only Nxs/T sequon) in HEK293F cells adapted to serum-free conditions. We inserted a N-terminal His$_6$ tag between the signal peptide sequence and the proBDNF polypeptide chain to facilitate its purification by Ni$_2^+$ affinity and we generated stable transfectants expressing proBDNF under the control of a CMV promoter. Full-length proBDNF along with mature BDNF and the prodomain were constitutively co-secreted by the HEK293F cells (Fig. 1). The yields of the proteins were 2–3 μg of purified protein/ml of cell suspension, which allowed us to isolate sufficient amounts for detailed mass spectrometric characterization of the glycoforms as described below. This expression system provides good yields of the proBDNF and enables further exploration of its properties and glycoforms. Both secreted proBDNF and its prodomain are N-glycosylated as demonstrated by mass shift following deglycosylation with PNGase F, in line with previous reports (3, 4, 8, 10, 11). As expected, mature BDNF is not glycosylated and a mass shift following PNGase F is not observed (Fig. 1). Glycosylation site occupancy was determined after deglycosylation of secreted proBDNF with PNGase F in [18O]water. Under these conditions asparagine that is glycosylated undergoes deamidation to aspartic acid (mass difference 1 Da); reaction in [18O]water results in an additional 2 Da difference. By comparison of precursor XICs of unoccupied N-peptide NYLDAA$^{2+}$NMSMR and its deglycosylated D-form (NYLDAA$^{2+}$DMSMR) we were only able to detect the deglycosylated form indicating site-occupancy higher than 99.9% on the secreted proBDNF (Fig. 2).

Mutation of the only N-glycosylation sequon (N121Q) did not affect the expression of proBDNF mRNA (Fig. 3C) or protein (Fig. 3D) in the HEK293 cells but abolished the secretion of proBDNF or BDNF into the medium (Fig. 3A). Cells expressing WT proBDNF contain both full-length proBDNF and cleaved mature BDNF but only unprocessed full-length proBDNF (nonglycosylated) was observed in the N121Q mutant (Fig. 3B). Incubation of cells expressing N121Q mutant with proteasomal and lysosomal inhibitors, MG-132 and chloroquine, did not lead to the accumulation of proBDNF in the cells (Fig. 3D), as we expected, indicating that degradation of potentially misfolded proBDNF does not play a significant role in the observed effect of the mutation. Chemical chaperone, 4-phenylbutyric acid, partially restored release of proBDNF but mature BDNF was not detected (Fig. 3E).

**ProBDNF cleavage by furin in vitro**

ProBDNF is processed by furin-like convertases to mature BDNF (4) but the N121Q mutant seems resistant to cleavage (BDNF in cells is not observed, Fig. 3B). We therefore analyzed the impact of glycosylation on the proteolytic cleavage of proBDNF by furin *in vitro* (Fig. 4). The two forms of proBDNF were pre-mixed equimolarly prior to the addition of furin and the cleavage kinetic was analyzed as a loss of proBDNF over the incubation time in parallel with the corresponding increase in mature BDNF generated by the cleavage. The reaction conditions were optimized to obtain measurable products throughout the 60-min reaction time. The mass difference between glycosylated and nonglycosylated proBDNF enabled quantitation of each of the proteoforms individually. On the contrary, because mature BDNF is not glycosylated, its band combines intensities of BDNF originating from both proBDNF forms. We did not observe a difference in the furin-cleavage kinetics between the two proBDNF forms under these conditions and we did not find evidence that the N-glycan directly modifies the cleavage reaction. We therefore expect that some as yet undefined interaction regulates the maturation of proBDNF in the cells and *in vivo*.

**LC-MS analysis of proBDNF glycosylation**

*N-Glycosylation Profile—Occupancy and microheterogeneity of the N-glycosylation site was determined by data-dependent LC-MS/MS-HR analysis of the tryptic digest of secreted proBDNF purified by Ni$_2^+$-NTA affinity from conditioned media of the HEK293F cells. Precursor profile of the tryptic glyc peptide NYLDAANMSMR of proBDNF (oxidized at both Met residues in peptide backbone) is shown in Fig. 5A. Most of the glyc an structures are fucosylated and are terminated by the GalNAcβ1–4GlcNAcβ1-R (LacdiNAc), which is frequently sulfated (>60% of the LacdiNAc structures identified). This is unusual because the majority of human N-glycans are extended by the common Galβ1–4GlcNAcβ1-R (LacdiNAc) motif, which typically carries terminal sialylation. To confirm the presence of the LacdiNAc structures, we used fragmentation under limited collision energy (CE) optimized in our previous studies (15, 16). At low CE (10% of normalized CE), the major fragmentation event is the loss of the outer arm LacdiNAc structure (Hex-NAc-HexNAc). Accordingly, we observed dominant LacdiNAc oxonium ions HexNAc-HexNAc (m/z 407,17) and its fucosylated form (m/z 553.22) together with the complementary y-ions (m/z 1373, 1381, 1454, and 1555). In case of the mixed structures that carry LacNAc on one arm and LacdiNAc on the other, the limited HCD fragmentation yields two outer...
arm fragments of similar intensity (HexNAc-HexNAc \( m/z \) 407.17 and HexNAc-Hex \( m/z \) 366.14, data not shown). The sulfation of LacdiNAc was confirmed by the presence of a low abundant GalNAc-SO4 fragment ion (accurate mass \( m/z \) 284.0426) in positive MS/MS spectra (Fig. S1) with mass accuracy of 3 ppm (theoretical mass \( m/z \) 284.0435).

All identified glycans on the secreted proBDNF were core-fucosylated with varying degrees of outer-arm fucosylation. Fucosylation was confirmed under higher collision energy (25% of normalized CE) where a pair of fragment peaks at \( m/z \) 1520.6 and 1666.6 represent GlcNAc-peptide and fucosylated GlcNAc-peptide fragments, respectively (Fig. S2C). Intensity of the fucosylated GlcNAc-peptide fragment is low compared with the GlcNAc-peptide fragment, because of the energy required to break the core GlcNAc-GlcNAc bond, necessary to determine core fucosylation, also leads to cleavage of core-linked fucose. However, because the fragment originates from a single glycoform, its presence serves as a proof of core-fucosylation. The NYLDAANMSMR glycopeptide contains two Met residues; ions obtained at higher CE were used to determine oxidation status of the peptide backbone to correctly assign the glycan structures. Fig. S2, A and B show characteristic peptide-HexNAc and peptide-HexNAc-fucose fragments with one or two Met oxidized, respectively, which confirms our structural assignments.

More than 90% of the identified N-glycan structures of proBDNF carry LacdiNAc; 70% of those exclusively (on all arms). The LacNAc motif is present in less than 10% of all identified structures of the proBDNF. All the identified glycans are biantennary, we did not observe glycans with higher branching.

Figure 2. Extracted ion chromatograms (XIC) of (A) nonglycosylated, NYLDAANMSMR and (B) deglycosylated, NYLDAADMSMR glycopeptide of proBDNF secreted by HEK293F cells confirm site occupancy higher than 99%. C, tandem mass spectrum verifies deglycosylation of the proBDNF peptide under \([^{18}O]\)water.
The two major structures that represent >50% of all the glycoforms are the biantennary sulfo-LacdiNAc with core-fucose (precursor m/z 1083) or two fucose residues (precursor m/z 1132). The identified proBDNF glycoforms and their relative proportions are summarized in Table 1.

N-Glycosylation of the Prodomain—The prodomain was prepared as the proBDNF truncated immediately after the furin-cleavage site by insertion of a stop codon (see “Experimental procedures”). This is important because it was suggested that the prodomain may itself carry out important functions. Expressed prodomain was constitutively secreted into the conditioned medium as confirmed by Western blotting and Coomassie Blue staining (not shown) and purified by Ni-NTA affinity as described for the proBDNF. The yields of the prodomain were lower compared with proBDNF possibly due to instability of the prodomain characterized as intrinsically disorganized but we obtained sufficient quantities for our optimized MS workflow. High resolution LC-MS/MS analysis confirmed that LacdiNAc remained the dominant structure (Fig. S3) present on 90% of the identified glycoforms (Table 1). However, compared with the full-length proBDNF, we observed a lower proportion of structures carrying LacdiNAc on both arms (30% versus 70% on proBDNF) and appearance of major Man6/LacdiNAc hybrid glycoform (m/z 1119) (Fig. S3). Moreover, the sulfation level was substantially lower (<5% as opposed to 60% on proBDNF) and sialylation became the dominant terminal modification present on 50% of all the glycoforms (Table 1). This is an important observation because synthesis of LacdiNAc is associated with defined sequence/structure motives (17–20) and the results show that the LacdiNAc determinant is present in the prodomain region. Drop in the level of sulfation is surprising as the peptide sequence/structures motive-dependence was not described for the 4-O-sulfotransferases that carry out LacdiNAc sulfation.

N-Glycosylation of ProBDNF Expressed in PC12 Cells—To verify that (sulfo) LacdiNAc synthesis is not restricted to HEK293F cells, we expressed proBDNF in rat pheochromocytoma PC12 cells as described under “Experimental procedures.” The mRNA expression of the enzymes related to the formation of sulfated LacdiNAc: (1,4)-N-acetylgalactosaminyltransferases 3 (B4GALNT3) and 4 (B4GALNT4), carbohydrate sulfotransferases 8 (CHST8) and 9 (CHST9); and (1,4)-galactosyltransferase 1 (B4GALT1) in these cells was confirmed by RT-PCR (Fig. S4). HEK293F cells expressed all four LacdiNAc-related enzymes, PC-12 cells expressed both 4-O-sulfotransferases but the B4GALNT4 was the dominant N-acetylgalactosaminyltransferase (Fig. S4). To facilitate proBDNF isolation, we cultured the cells in the presence of a proprotein
ProBDNF carries specific N-glycosylation

Effect of glycosylation inhibitors

Effect of glycosylation inhibitors on maturation of the proBDNF was analyzed in HEK293F cells cultured in the absence of the proprotein convertase inhibitor to analyze the proportion of mature BDNF and proBDNF produced and secreted by the cells. In the presence of 50 mM sodium chlorate, an inhibitor of cellular sulfation, we observed a 10-fold decrease in the sulfation of proBDNF from 60 to 6% (Fig. S5A). Treatment with 1 μg/ml of kifunensine, an inhibitor of α-mannosidase I, enriched immature oligomannose glycans Man9, Man8, and Man7 glycans >70% (Fig. S5B) as expected and verified by LC-MS/MS analysis.

Effect of the inhibitors of glycosylation on the ratios of BDNF to proBDNF or prodomain to proBDNF is shown in Fig. 7 for the secreted and intracellular protein pools. For both secreted and intracellular forms, BDNF/proBDNF and prodomain/proBDNF ratios were diminished by 5 μg/ml of tunicamycin due to barely detectable amounts of mature BDNF and prodomain. On the other hand, kifunensine (Fig. 7) did not have any effect on these ratios in either pool. Inhibition of cellular sulfation by sodium chlorate largely reduced intracellular content of mature BDNF (Fig. 7A), whereas secreted BDNF was not affected (Fig. 7B). Chlorate treatment reduced intracellular and secreted prodomain content to a similar degree (Fig. 7, C and D) but the effect on intracellular prodomain was less pronounced than that for mature BDNF.

Discussion

As for many other bioactive neurotropins or proteins, concentrations of proBDNF/BDNF in biological samples are in low nanograms per gram of tissue, which limits detectability of proBDNF and structural resolution of its glycoforms. To overcome this limitation, we optimized expression of proBDNF in a mammalian system using suspension human embryonic kidney HEK293F cells adapted to serum-free conditions. We generated stable transfectants expressing proBDNF under control of a CMV promoter and took advantage of the serum-free conditions to improve purification of the expressed proteins. This expression system will serve as a valuable resource for studies of the glycosylated proBDNF. Previous experiments showed that adherent HEK293 cells co-secrete glycosylated proBDNF with mature BDNF (8) and our experiments confirm that the HEK293F cells are a suitable model to study glycosylation of this neurotrophin. The cells stably expressing WT proBDNF secreted the expected mixture of proBDNF and mature BDNF but the N121Q mutation almost completely abolished secretion suggesting a crucial role of N-glycosylation in the BDNF maturation process. Intracellular mRNA expression of the WT and the N121Q mutant were comparable indicating that differences occur at the post-translational levels. Intracellular protein content of proBDNF was also similar to that of the secreted form in line with the range/charge of precursors of purified proBDNF produced in HEK293F cells.

4 J. Benicky, M. Sanda, Z. Brnakova Kennedy, and R. Goldman, unpublished observation.
between WT and N121Q mutant; however, the majority of proBDNF/BDNF is constitutively secreted by the HEK293F cells and the overall proBDNF production in the N121Q mutant is therefore lower. In addition, we only observed unprocessed proBDNF in the N121Q mutant (Fig. 3) and the effect of N-glycosylation sequon’s mutation is reproduced by pharmacological inhibition of N-glycosylation by tunicamycin (Fig. 7) indicating that the processing of nonglycosylated proBDNF into the mature secreted form is compromised. This could be due to direct impact of the N-glycan on interaction with the convertases or due to altered interactions of the glycosylated proBDNF with other proteins; such interactions could affect its maturation or its ability to reach the relevant intracellular compartments.
Table 1
Relative abundance of the glycoforms of HEK293F-produced proBDNF and prodomain expressed as % of all identified glycoforms.

| Structure          | Symbolic representation | m/z     | ProBDNF (%) | Prodomain (%) |
|--------------------|-------------------------|---------|-------------|---------------|
| 6Hex-4HexNAc-2Fuc  |                         | 1121.444| -           | 25            |
| 5Hex-4HexNAc-Fuc   |                         | 1029.403| 7           | -             |
| 5Hex-4HexNAc-3Fuc-Neu5Ac |                  | 1213.144| -           | 8             |
| 4Hex-5HexNAc-Fuc   |                         | 1043.079| 7           | -             |
| 4Hex-5HexNAc-Fuc-Sulf |                      | 1069.731| 10          | -             |
| 4Hex-5HexNAc-2Fuc-Neu5Ac |                 | 1188.797| 4           | 19            |
| 4Hex-5HexNAc-3Fuc  |                         | 1129.787| -           | 7             |
| 4Hex-5HexNAc-3Fuc-Sulf |                     | 1156.440| -           | 1             |
| 4Hex-5HexNAc-3Fuc-Neu5Ac |                  | 1226.319| -           | 11            |
| 3Hex-4HexNAc-2Fuc-Sulf |                       | 1083.407| 17          | -             |
| 3Hex-4HexNAc-2Fuc  |                         | 1105.440| 3           | -             |
| 3Hex-4HexNAc-2Fuc-Neu5Ac |                 | 1132.093| 35          | -             |
| 3Hex-4HexNAc-2Fuc-Neu5Ac |                 | 1202.472| 10          | 13            |
| 3Hex-4HexNAc-3Fuc  |                         | 1154.126| 8           | 16            |
| 3Hex-4HexNAc-3Fuc-2Sulf |                   | 1196.768| -           | 2             |

ProBDNF can be intracellularly cleaved by furin-like subtilisin-kexin proprotein convertases, including furin, PC1 (4), or PC7 (22). HEK293F cells only possess constitutive secretory pathway, implication of PC1 mostly localized to acidic secretory granules (23) is therefore not likely in these cells. Proprotein convertase inhibitor used in our studies strongly inhibits furin (Kᵢ = 16 ps) but not the PC7 convertase. The inhibition efficiently prevents cleavage of proBDNF by HEK293F cells (Fig. S6) indicating that furin is the main intracellular proBDNF convertase in this cell type. To evaluate whether N-glycosylation has direct influence on the proBDNF-furin interaction, we compared the cleavage kinetics of nonglycosylated human proBDNF produced in Escherichia coli and N-glycosylated proBDNF produced in human HEK293F cells in an in vitro reaction with recombinant human furin. To minimize the effect of assay to assay variability and possible influence of contaminants in the proBDNF preparations, we pre-mixed the two forms of proBDNF equimolarly prior to the addition of furin. The mass difference between glycosylated and nonglycosylated proBDNF enabled us to analyze each form separately in the mixed reaction. Cleavage of both proteoforms by furin proceeded at the same rate indicating that proximity of the N-glycan to the proteolytic site (Scheme 1A) is not essential for the direct interaction with furin and the glycan does not directly affect the cleavage reaction. This simple in vitro model, however, does not take into account the complex intracellular environment where other carbohydrate-interacting proteins (e.g. intracellular lectins) could substantially change the equilibrium. In addition, the secretion of proBDNF was compromised in both N121Q mutant and tunicamycin-treated cells indicating that glycosylation regulates traffic of the protein through the secretory pathway. This is further supported by the observation that the N-glycosylation site of secreted proBDNF is fully occupied (Fig. 2) indicating that only N-glycosylated proBDNF is secreted.

We used LC-MS/MS analysis with energy-optimized fragmentation conditions to determine the structural motives of the N-glycans associated with proBDNF. This selective fragmentation approach identifies structure-specific ions not detectable under standard collision-induced dissociation conditions (Fig. 5B, Fig. S2). Our results determine N-glycan structures associated with proBDNF and the cleaved prodomain, which significantly extends previous reports of the N-glycan presence based on gel shifts (3, 4, 8, 10, 11). Analysis of the fragmentation spectra confirmed that proBDNF carries preferentially N-glycans terminated by the GalNacβ1–4GlcNacβ1–R (LacdiNac) structures, which are rare compared with the common Galβ1–4GlcNacβ1–R (LacNac). The LacdiNac-carrying glycans are further modified by fucosylation (both core and outer arm) and sulfation of the terminal GalNAc residue. Glycans terminated by sulfated LacdiNac were so far observed on a limited number of glycoproteins, including pituitary glycoprotein hormones luteinizing hormone and thyrotropin, proopiomelanocortin, carbonic anhydrase VI, sialoadhesin, or tenascin-R as reviewed in Ref. 24; their dominant contribution to the proBDNF glycoforms was therefore a surprising observation. In general, unusual oligosaccharide sequences or specific carbohydrate modifications often mediate more specific or vital biological functions of glycoproteins (14). This suggests, together with the high evolutionary conservation of the proBDNF sequence around the N-glycosylation site (9) (Scheme 1B), an important biological function of the N-glycans on this neurotrophin.

Presence of the LacdiNac was confirmed in low CE spectra by a specific outer-arm fragment (HexNAc-HexNAc, m/z 407.17 and its fucosylated form, m/z 553.22) together with complementary y-ions representing fragments with glycan arm loss (Fig. 5B). It is important to note that at higher CE, the HexNAc-HexNAc fragment is also present in standard LacNac N-glycopeptide fragmentation where it originates from the fragmentation of the glycan mannose core (GlcNAC-GlcNAC fragment). Under the optimized low CE conditions, this fragment is specific for the LacdiNac containing structures because the core-derived GlcNAC-GlcNAC fragment is not produced as we demonstrated previously (25). However, fragmentation spectra at higher CE (producing peptide-GlcNAc fragment) were used to determine peptide backbone oxidation (Fig. S2) because tryptic glycopeptide [113]NYLDAANMSMR[125] (glycosylation site underlined) of proBDNF contains two Met residues that can be oxidized producing a mass difference corresponding to the difference between deoxyhexose (fucose) and hexose (mannose, galactose). Knowledge of oxidation status is therefore vital to correctly assign fucose residues. In addition, we used retention behavior prediction (21) to resolve overlapping glycopeptide isotopic patterns such as di-fucosylated and sialylated glycopeptides. This is to our knowledge the first study that documents feasibility of the MS/MS assignment of these...
ProBDNF carries specific N-glycosylation

Sulfated species were confirmed by the presence of a sulfogalactosyl fragment (exact mass 284.0426, theoretical mass 284.0435) in the HCD fragmentation spectra (Fig. S1). In addition, the presence of sulfate was indirectly confirmed by selective changes after inhibition of sulfation by the sodium chlorate treatment (Fig. S5) where we observed a drop in the peak intensity at m/z 1132 (3Hex-6HexNAc-2Fuc-Sulfo) together with an increase in peak intensity at m/z 1106 (3Hex-6HexNAc-2Fuc).

glycopeptide structures and will enable further studies of the glycosylated neurotrophins.

Figure 6. Extracted ion chromatograms of four of the most abundant glycopeptides of proBDNF expressed in PC12 cells. A, C, and D, LacdiNAc containing structures; B, LacNAc containing structure. The arrow points to the peaks of glycopeptides aligned with slight retention time shifts as expected based on the hydrophilicity of the attached glycan. Numbers represent intensity of the extracted peaks (×10^3).

Figure 7. Impact of modulators of glycosylation on the BDNF/proBDNF ratio in HEK293F cells. Western blotting using antibodies to prodomain and mature BDNF regions was analyzed by densitometry for the following: A, intracellular BDNF to proBDNF ratio; B, secreted BDNF to proBDNF ratio; C, intracellular prodomain to proBDNF ratio; and D, secreted prodomain to proBDNF ratio. The representative Western blotting is labeled as the graphs: CTRL, control; CHLOR, 50 mM sodium chlorate; KIF, 1 μg/ml kifunensine; TNMC, 5 μg/ml of tunicamycin. Results are expressed as scatter plot (mean ± S.D., n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with CTRL, one-way analysis of variance with Bonferroni adjustment.
Core fucose was confirmed by the presence of the peptide-HexNAc-Fuc fragment at high CE (see fragmentation spectrum in Fig. S2C, m/z 1666); the outer arm fucosylation produced the HexNAc-HexNAc-Fuc fragment (Fig. S5B, m/z 553.2) at low CE when the glycans core is not fragmented.

LacdiNAc was also dominant on the prodomain where it represents 90% of all identified glycoforms. Compared with the full-length proBDNF, we observed a smaller proportion of glycans that carried LacdiNAc on both arms and sialylation was the main terminal modification of the LacdiNAc (instead of sulfation observed on the full-length proBDNF) (Table 1).

Addition of LacdiNAc is carried out by specific B1,4-N-acetyl-galactosaminyltransferases (GalNAc transferases B4GALT3 or B4GALT4) (26, 27) expressed in a limited number of tissues including pituitary, brain, and kidney (18). The HEK293 cells express both enzymes (17, 28), which we confirmed in our HEK293F cells (Fig. S4). As GalNAc transferases compete for the same oligosaccharide substrate with the ubiquitously expressed β1,4-galactosyltransferases, addition of GalNAc is protein-specific driven by sequence/structure-based determinants. Such determinants have been described for the pituitary hormones and carbonic anhydrase as stretches of basic amino acids on α-helical structures (18–20) and for the trefoil factor family domains as patches of hydrophobic amino acids on cysteine loops forming a hydrophobic cleft, also proposed for some neuropeptide hormones (17). In support of protein-specific GalNAc transfer, the presence of the recognition determinant can increase catalytic efficiency for GalNAc transfer 500-fold (19, 20). This is further supported by the observation that Cys/ Gly mutations in trefoil factor family domains lead to reversal from almost exclusive LacdiNAc to common LacNAc glycans (17). This strongly suggests that a specific motif or structure is required for the in vivo LacdiNAc synthesis. Such a recognition motif for proBDNF is yet to be determined. Regions surrounding the N-glycosylation site lack similarities with the published determinants whose sequences are themselves heterogeneous. However, our study shows that the expressed BDNF prodomain truncated immediately after the furin-cleavage site (Arg-128) carries LacdiNAc structures, albeit with somewhat different motive distribution than proBDNF (Table 1), suggesting that the prodomain itself is sufficient to drive the LacdiNAc synthesis. The structure of the prodomain, as opposed to BDNF (29), is not known and the prodomain is characterized as intrinsically disordered (30); follow-up studies will be required to determine the exact recognition motif within the prodomain sequence.

LacdiNAc present on the proBDNF is sulfated to a large extent. Addition of sulfate to proBDNF has been observed previously using pulse-chase study with radioisotope 35S in cultures of mouse pituitary AtT20 cells (3, 4). Our study explains the long-standing puzzle of what structural epitope carries the sulfate. As a charge carrying modification, sulfation can substantially modify the properties at the site of attachment and may thus affect interactions. LacdiNAc sulfation is carried out by specific GalNAc-4-sulfotransferases (CHST8 or CHST9) highly expressed in the pituitary and brain (31) and we confirmed the expression of both enzymes in HEK293F cells (Fig. S4). The induction of 4-O-GalNAc sulfotransferase transcription and activity by DMSO has been reported previously in AtT20 and αT3 cells (31) but we did not observe additional increases in proBDNF sulfation in HEK293F cells exposed to DMSO (analyzed by LC-MS/MS, not shown) indicating that this may not be universally applicable to all cell types or that the sulfation enzymes in this cell line are already highly expressed. On the other hand, sodium chlorate, an inhibitor of the formation of PAPS, which serves as a sulfate donor in the sulfotransferase reaction, reduced proBDNF sulfation 10-fold (Fig. S5A).

Protein sequence/structure recognition motif was not described for the 4-O-sulfotransferases CHST8 and -9 and β1,4-linked terminal GalNAc (added by protein-specific GalNAc transferases) seems to be a sufficient determinant for the 4-O-sulfotransferase activity (24). It was therefore surprising to see a substantial reduction of sulfation (below 5%) on the expressed prodomain despite the presence of the LacdiNAc on most glycoforms, which were rather modified by terminal sialylation (Table 1). If protein structure/sequence is not limiting interactions with sulfotransferases, this may indicate different accessibility to enzymes possibly due to different spatial distribution of proBDNF and the prodomain. In support of this, inhibition of sulfation did not affect secretion of BDNF and proBDNF (or their ratio) but almost completely depleted the intracellular pool of mature BDNF, whereas intracellular prodomain remained detectable (Fig. 7). The mechanism behind this effect needs further study but may indicate decoupling of proBDNF/BDNF and cleaved off prodomain under these circumstances, which could explain differences in glycan modifications between proBDNF and the prodomain.

Although LacdiNAc formation seems to be protein-specific, we wanted to confirm in cells of different types and origin that it is not a peculiar feature of suspension in HEK293F cells. Because we do not have sufficient sensitivity of the methods to observe native proBDNF in tissues or neurons, we expressed proBDNF in rat pheochromocytoma PC12 cells and showed that LacdiNAc is present on major identified glycoforms (Fig. 6). This observation, together with previous reports of proBDNF sulfation in mouse pituitary AtT20 cells (3, 4) further supports protein-specific addition of this rare glycan structure to proBDNF. Further studies will be needed to determine the glycoforms of proBDNF in neuronal cells and their impact on the proBDNF maturation process. We are aware that HEK293 cells possess only a constitutive secretory pathway and their applicability as a model for neuronal control of proBDNF is therefore limited. However, the secretion of proBDNF/BDNF utilizes both regulated (activity-dependent) and constitutive pathways (32–34) and our model may be useful to distinguish their regulatory elements. Although activity-dependent (pro)BDNF secretion is prevalent in neurons (35), BDNF or proBDNF have been found in a number of nonneuronal tissues or cell types where constitutive secretion prevails, including megakaryocytes and platelets (36, 37), activated monocytes and T- and B-cells (38), cells of the cardiovascular system (39) or hepaticstellate cells (40), and BDNF signaling is up-regulated in many types of cancer (41, 42). Although the role of this neurotrophin in nonneuronal tissues and cells is not as well-elucidated as for the brain, nonneuronal regulation of proBDNF may have broader implications.
ProBDNF carries specific N-glycosylation

To our best knowledge, this is the first report demonstrating a specific type of N-glycosylation of proBDNF by detailed mass spectrometric analysis. Our study suggests that glycosylation impacts processing and secretion of proBDNF. Biological impact of specific glycoforms of proBDNF and the prodomain are yet to be elucidated but high conservation of the N-glycosylation site and unique properties of proteins conferred by the presence of LacdiNAc support biological significance. Lacdi-sylation site and unique properties of proteins conferred by the impact of specific glycoforms of proBDNF and the prodomain impacts processing and secretion of proBDNF. Biological spectrometric analysis. Our study suggests that glycosylation

TAC CTA GAT GCT GCA CAG ATG TCC ATG AGG GTC
TGC AGC ATC TAG GTA ATT 3

using primer pair 5′-CCG GAC CCT CAT GGA CAT CTG
ACT ACC ACC ATC ACG CCC TC-3″ (sense) and 5′-TTT CAT GGG GGC GTG ATG GTG GTG GTG AGC CTT CAT GCA ACC AAA GTA TG-3″. Nonglycosylated His-proBDNF was generated by N121Q substitution as above

GGT CCG GCG CTA GTC TGA CCC TGC CC-3″
ACT AGC GCC GGA CCC TC-3″

at 0.3–3 × 10<sup>5</sup> cells/ml and subcultured every 5 days. Rat phe-ochromocytoma PC12 (ATCC, Manassas, VA, number CRL-1721) were grown in RPMI 1640 supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 50 μg/ml of gentamycin at 37 °C under 5% CO<sub>2</sub>. The binding was performed directly with undiluted conditioned medium at 1 ml/min followed by washing with 10 column volumes of PBS, 300 mM NaCl, and 25 mM imidazole. Elution of bound proBDNF/prodomain was achieved with PBS, 300 mM NaCl, 125 mM imidazole. The fraction corresponding to the proBDNF/prodomain peak was concentrated and buffer exchanged to PBS, 0.01% Tween 20 using Amicon Ultra centrifugal filters with 10-kDa cut-off for proBDNF and 3-kDa cut-off for prodomain (both Millipore Sigma). Purity of proBDNF (>90%) was assessed by SDS-PAGE and identity of the protein in the band was confirmed by MS. The concentration of proBDNF in individual fractions was routinely quantified by commercial sandwich proBDNF Duoset ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions and confirmed by Coomassie Blue staining compared with proBDNF standards (Alomone Labs, Jerusalem, Israel) of known concentrations. Due to trace quantities of proBDNF in conditioned media of PC12 cells, we concentrated serum-free conditioned media using Amicon Ultra centrifugal filters with 10-kDa cut-off (Millipore Sigma) and separated the concentrate by SDS-PAGE followed by Coomassie Blue staining. The bands around the position of glycosylated proBDNF produced in HEK293F (± 5 kDa) were excised and used for LC-MS analysis as described below.

Treatment of cells with inhibitors of glycosylation and proteolysis

Enrichment of nonsulfated proBDNF was achieved by culturing the HEK293F cells in the presence of 50 μM sodium chloride (Sigma), an inhibitor of cellular sulfation that prevents formation of PAPS, a donor of sulfate for the sulfotransferase reaction. Kifunensine (1 μg/ml, Cayman Chemical, Ann Arbor, MI), an α-mannosidase I inhibitor, was used to prevent pro-

Purification of proBDNF or its prodomain

Conditioned media were harvested every 5 days and used for proBDNF or prodomain purification by His-affinity chroma-
tography performed on an AKTA Start FPLC system (GE Healthcare) and Ni-NTA resin (Thermo Scientific, Rockford, IL) packed in a Tricorn 5/100 column (GE Healthcare). The expression as described below.

Production of proBDNF and its variants

Expression vector pCMV6-Entry bearing human proBDNF cDNA (NM_001709, Origene, Rockville, MD) with C-terminal Myc-DDK tags was modified to introduce His<sub>N</sub> affinity tag between signal peptide sequence and proBDNF polypeptide chain using QuikChange Lightning Multi-Site-directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions and mutagenic primers: 5′-CAT ACT TTG GTT GCA TGA AGG CTC ACC ATC ACC ACC ATC ACG CCC CCA TGA AA-3″ (sense) and 5′-TTT CAT GGG GGC GTG ATG GTG GTG GTG AGC CTT CAT GCA ACC AAA GTA TG-3″. Nonglycosylated His-proBDNF was generated by N121Q substitution as above using primer pair 5′-CCG GAC CCT CAT GGA CAT CTG TGC AGC ATC TAG ATA TAG 3″ (antisense) and 5′-AAT TAC CTA GAT GCT GCA CAG ATG TCC ATG AGG GTC CGG-3″ (sense). To express proBDNF prodomain truncated after the furin-cleavage site, 123RVRR ↓ H129, we used site-di-
directed mutagenesis to convert the 129<sup>H</sup> codon CAC into a stop codon TAG using mutagenic primers 5′-GGG CAG GGT CAG ACT AGC GCC GCA CCC TC-3″ (antisense) and 5′-GAG GGT CCG GCG CTA GTC TGA CCC TGC CC-3″ (sense). ProBDNF not carrying C-terminal Myc-FLAG tags was generated by insertion of TAA stop codon between the proBDNF sequence and the linker using primer pair 5′-CCG CGG CGT TTA AGC CGT TCT TCC CCT TT-3″ (antisense) and 5′-AAA GGG GAA GAA CGC GTT AAA CGC GGC CG-3″ (sense). Before transfection, the constructs were linearized with unique cutter DraIII (New England Biolabs, Ipswitch, MA) disrupting the f1 origin region and transfected into HEK293F cells using linear 25-kDa polyethylenimine (Polysciences, Warrington, PA) at nitrogen/phosphate ratio 20. Stable transfectants were generated by antibiotic resistance selection using 500 μg/ml of G418. For production of full-length proBDNF, the cells were cultured in the presence of 1 μg/ml of proprotein convertase inhibitor (Millipore Sigma, Burlington, MA, number 537076) to prevent intracellular cleavage of proBDNF into BDNF.

To produce proBDNF from rat PC12, we transfected cells with the above proBDNF construct using Avalanche-Omni Transfection Reagent (Vita Scientific, College Park, MD) according to the manufacturer’s instructions. Twenty four hours after transfection, the cells were in complete medium and subjected to selection by antibiotic resistance selection as above. For experiments, the culture medium was exchanged to serum-free Opti-MEM medium (Invitrogen) in the presence of 1 μg/ml of proprotein convertase inhibitor.

Experimental procedures

Cell cultures

Suspension HEK293F cells (Invitrogen) were grown in serum-free 293 Freestyle medium (Invitrogen) at 37 °C and 5% CO<sub>2</sub> on an orbital shaking platform at 150 rpm. Cells were kept at 0.3–3 × 10<sup>5</sup> cells/ml and subcultured every 5 days. Rat pheochromocytoma PC12 (ATCC, Manassas, VA, number CRL-1721) were grown in RPMI 1640 supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 50 μg/ml of gentamycin at 37 °C under 5% CO<sub>2</sub>.
cessing of high mannose into complex glycans (47). Overall N-glycosylation was blocked by tunicamycin (5 µg/ml, Sigma). For all treatments, the cells were uniformly plated to 6-well plates (3 ml of cell suspension at 0.3 × 10⁶ cells/ml per well) and pre-conditioned with inhibitors overnight to ensure that the pathways are inhibited before initiating the experiment. Inhibitors were replenished at half-concentration 48 h after initiation of the experiments. Proprotein convertase inhibitor was omitted to not interfere with the process of proBDNF maturation. The cells were harvested 5 days after initiation of the experiment. The cells were pelleted at 300 g/10 min, the conditioned media were saved, and cell pellets were lysed in M-PER buffer (Thermo Scientific) supplemented with protease inhibitor mixture (Roche Diagnostics). Lysates were cleared by centrifugation at 16,000 × g for 15 min and stored at −20 °C. The protein concentration in lysates was measured by Micro BCA assay (Thermo Scientific) and total protein content per well was used for normalization of samples prior to the detection of proBDNF, BDNF, and prodomain by Western blotting as described below. HEK293F cells expressing the N121Q proBDNF mutant were treated with 10 µM MG-132 (Millipore Sigma), a proteasome inhibitor, 10 µM chloroquine (Sigma), an inhibitor of lysosomal acidification, and activation of lysosomal proteases, or 8 mM 4-phenylbutyric acid (Sigma), a chemical chaperone. The cells and conditioned media were harvested after 48 h of treatment and processed as above. ProBDNF deglycosylation in vitro was achieved by incubation with PNGase F (New England Biolabs, number P0704) at a ratio of 5 units/µg of proBDNF for 60 min at 37 °C.

**LC-MS analysis of proBDNF**

**Sample preparation**—ProBDNF enriched by Ni-NTA affinity as above was buffer exchanged to 50 mM ammonium bicarbonate buffer, reduced/alkylated, and digested with Trypsin Gold (Promega, Madison, WI) using Barocycler NEP 2320 (Pressure BioScience, Easton, MA). The volume of the digest was reduced in a refrigerated CentriVap concentrator (Labconco, Kansas City, MO) and subjected to LC-tandem MS (LC-MS/MS) analysis without further processing as described below.

**Identification of ProBDNF Glycoforms on Orbitrap Fusion Lumos**—Digested proteins were separated using a 90 min acetonitrile (ACN) gradient on a 150 mm × 75-µm C18 pempmap column at a flow rate of 0.3 µl/min. In brief, peptide and glycopeptide separation was achieved by a 5-min trapping/washing step using 99% solvent A (2% ACN, 0.1% formic acid) at 10 µl/min followed by a 90-min ACN gradient at a flow rate of 0.3 µl/min: 0–3 min 2% B (0.1% formic acid in ACN), 3–5 min 2–10% B; 5–60 min 10–45% B; 60–65 min 45–98% B; 65–70 min 98% B; 70–90 min equilibration by 2% B. Glycopeptides were analyzed using Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) with the electrospray ionization voltage set at 3 kV and the capillary temperature at 275 °C. MS1 scans were performed over m/z 400–1800 with the wide quadrupole isolation at a resolution of 120,000 (m/z 200). RF Lens at 40%, intensity threshold for MS2 set to 2.0e4, selected precursors for MS2 with charge state 2–7, and Dynamic exclusion 30 s. Data-dependent HCD tandem mass spectra were collected with a resolution of 15,000 in the Orbitrap with fixed first mass 110 and normalized collision energy 5–30% (see below). LC-MS datasets were processed by Protein Discoverer 2.2. (Thermo Fisher Scientific) with Byonic node (Protein Metrics, Cupertino, CA) followed by manual confirmation of the glycopeptides.

To facilitate structure-specific analysis, the standard glycoproteomics workflow was modified by modulation of the CE. Optimization was achieved using glycosylated proBDNF produced in the HEK293F cells and purified as described above. Changes in structure-specific oxonium ions and complementary y-ions were monitored across the CE range (5–30% of normalized CE). At low CE, outer arm-specific signals (LacdiNAc, fucosylated LacdiNAc, and sulfated LacdiNAc) were observed in the low mass range. Fragmentation at higher CE values provided glycan core-specific fragments (core fucose, etc.) and peptide backbone modification status (e.g. Met oxidation) necessary for correct assignment of the attached glycan structures (Met oxidation is isobaric change to the difference between hexose and fucose).

**Glycopeptide Analysis by nano-LC-MS/MS on 6600 Triple-TOF**—Glycopeptide separation was achieved on a NanoAcuity LC (Waters, Milford, MA) using capillary trap, 180 µm × 0.5 mm, and analytical 75 × 150-µm Atlantis DB C18, 3 µm, 300-Å columns (Waters) interfaced with a 6600 TripleTOF (Sciex, Framingham, MA). A 1-min trapping step using 2% ACN, 0.1% formic acid at 15 µl/min was followed by chromatographic separation at 0.4 µl/min as follows: starting conditions 5% ACN, 0.1% formic acid; 1–35 min, 5–50% ACN, 0.1% formic acid; 35–37 min, 50–95% ACN, 0.1% formic acid; 37–40 min, 95% ACN, 0.1% formic acid followed by equilibration to starting conditions for an additional 20 min. Tryptic digest (0.1 µg of protein) was injected directly on the column. We have used an information dependent acquisition (IDA) workflow with one MS1 full scan (400–1800 m/z) and 50 MS/MS fragmentations, with rolling collision energy (different equation for CE calculation for low and high CE mode). MS/MS mass spectra were recorded in the 100–1800 m/z range with resolution 30,000 and mass accuracy less than 15 ppm using the following experimental parameters: declustering potential 80 V, curtain gas 30, ion spray voltage 2,300 V, ion source gas 11, interface heater 150 °C, entrance potential 10 V, collision exit potential 11 V.

**Determination of N-Glycosylation Site Occupancy**—Occupancy of the N-glycosylation site of proBDNF was quantified by comparison of precursor XICs of unoccupied N-(asparagine) peptide (NYLDA[12]NMSMR) and deglycosylated D-(aspartic acid) peptide (NYLDA[12]DMSMR) following PNGase F deglycosylation under [18O]water as described previously (48). Briefly, proBDNF (2 pmol) was digested after reduction and alkylation by trypsin as described above. The enzymatic digest was heated for 15 min at 90 °C to deactivate trypsin and evaporated to dryness using a SpeedVac. Separately, 2 µl of Glycobuffer 2 (New England Biolabs) and 0.5 µl of PNGase F (New England Biolabs) were evaporated and diluted in 20 µl of [18O]water (Cambridge Isotope Laboratories, Andover, MA). This solution was used to dissolve the dried peptides for de-glycosylation (1.5 h at 37 °C) using Barocycler NEP2320 (Pressure BioSciences). De-glycosylated [18O]-labeled peptides (2 pmol) were analyzed directly after the PNGase F enzymatic treatment.
ProBDNF carries specific N-glycosylation

on a TripleTOF 6600 mass analyzer using an IDA workflow as described above.

ProBDNF cleavage by furin

Glycosylated human proBDNF without C-terminal Myc-FLAG tags produced in HEK293F cells and nonglycosylated human proBDNF produced in E. coli (Alomone Labs, number B-257) were used to study the kinetic of cleavage by furin (R&D Systems, number 1503-SE-010). Nontagged proBDNF was used to avoid possible competition of the C-terminal KRGR sequence of proBDNF that represents the potential convertase-cleavage site (23). An equinmolar mix of the two forms of proBDNF was incubated with recombinant human furin. Reaction buffer consisted of 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM CaCl₂, and 0.5% Brij-35 in a volume of 100 μL. Ten pmol of proBDNF (300 ng) was incubated in the presence of 12.5 ng of furin (specific activity 125 pmol of pERTKR-AMC/min/μg) at 37 °C at 300 rpm using Thermomixer R (Eppendorf, Haup- paue, NY). The aliquots corresponding to 10% of the reaction volume were collected at 0, 5, 10, 30, and 60 min and the reaction was immediately stopped by addition of 4× LDS sample buffer (Invitrogen). Aliquots were analyzed by SDS-PAGE and proBDNF and BDNF were detected by antibody recognizing the BDNF region as described below.

SDS-PAGE and Western blotting

ProBDNF and BDNF/prodomain were separated by SDS-PAGE using NuPAGE 12% BisTris gels (Invitrogen) and transferred to PVDF membranes by dry transfer using an iBlot2 instrument (Invitrogen). Samples for prodomain detection were immediately cross-linked to PVDF membrane by 30 min exposure to 2.5% glutaraldehyde in PBS to prevent its dissociation from the membrane as reported previously (10). Membranes were blocked with 5% BSA in TBS-T for 60 min at room temperature and exposed overnight at 4 °C to the respective antibodies as follows: rabbit monoclonal anti-human BDNF antibody (1:10,000, Abcam, Cambridge, MA, number ab108319) (49) was used to detect the proBDNF and BDNF. Mouse monoclonal anti-human proBDNF antibody (1:10,000, GeneCopeia, Rockville, MD, number H10001G-MA) recognizing the prodomain region, was used to detect the proBDNF and prodomain (10). Membranes were washed in TBS-T and exposed to secondary horsedarsh peroxidase (HRP)-conjugated antibodies: goat anti-rabbit IgG-HRP (1:50,000, Abcam, number ab6721) or rabbit anti-mouse IgG-HRP (1:100,000, Sigma, number A9044) for 2 h at room temperature. Membranes were developed using Clarity Western ECL reagent (Bio-Rad). For semi-quantitative analysis, the images were acquired by Amersham Biosciences Imager 600 (GE Healthcare) with automated high dynamic range operation and control of image intensity saturation and the intensities of individual bands were determined by densitometry using Gel Analyzer in ImageJ software (NIH, Bethesda, MD). Some images not used for quantitative analysis were acquired by exposure to autoradiography film. Experiments for semi-quantitative analysis were repeated three times and acquired on the same membrane to avoid assay to assay variability. Results shown in other blots show representative results of more than three repeats with the exception of the experiment shown in Fig. 3, D and E, which was performed once.

RT-PCR

Total RNA from cell lysates was isolated using RNeasy Mini Kit with on-column DNA digestion (Qiagen, Germantown, MD) and reverse transcribed using SuperScript IV Reverse Transcriptase and random hexamer primers (both Invitrogen) according to the manufacturer’s instructions. RT-PCR was carried out using Platinum Green Hot Start PCR 2× Master Mix (Invitrogen) and 200 nm primers using cycling conditions as follows: initial denaturation 2 min at 94 °C followed by 25–35 cycles of 94 °C/30 s, 60 °C/30 s, 72 °C/60 s, and a final extension step for 10 min at 72 °C. The products of the PCR were separated on 2% Ultrapure Agarose (Invitrogen) in 1× TAE at 100 V and visualized using GelGreen Nucleic Acid Stain (Biotium, Fremont, CA) on a SmartBlue Transilluminator (Accuris Instruments, Edison, NJ). The primers are listed in Table S1.

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