Accumulation of unfolded proteins in the endoplasmic reticulum initiates intracellular signaling termed the unfolded protein response (UPR). Although Xbp1 serves as a pivotal transcription factor for the UPR, the physiological role of UPR and Xbp1 in the central nervous system remains to be elucidated. Here, we show that Xbp1 mRNA was highly expressed during neurodevelopment and activated Xbp1 protein was distributed throughout developing neurons, including neurites. The isolated neurite culture system and time-lapse imaging demonstrated that Xbp1 was activated in neurites in response to brain-derived neurotrophic factor (BDNF), followed by subsequent translocation of the active Xbp1 into the nucleus. BDNF-dependent neurite outgrowth was significantly attenuated in Xbp1−/− neurons. These findings suggest that BDNF initiates UPR signaling in neurites and that Xbp1, which is activated as part of the UPR, conveys the local information from neurites to the nucleus, contributing the neurite outgrowth.

Endoplasmic reticulum (ER) is a site of synthesis, folding, and modification of secretory and cell surface proteins, and this organelle is widely distributed throughout neurons, including axons, dendrites, and growth cones (1). Various biological phenomena, such as increased protein synthesis, nutrient deprivation, and alteration in Ca2+ homeostasis, hamper protein folding in the ER, causing unfolded proteins to accumulate in the ER lumen. This condition is designated as ER stress, which triggers an adaptive reaction known as the unfolded protein response (UPR) (2, 3). The protective signaling of the UPR acts transiently to maintain homeostasis within the ER, but sustained ER stress ultimately leads to apoptosis. Most of the previous studies on ER stress focused on its pathological aspect, as the pathogenesis of ischemic and neurodegenerative disorders is characterized by the accumulation of protein aggregates. Nevertheless, recent studies suggested that the UPR is required for normal development for certain cell lineages and that Xbp1, a pivotal transcription factor of the UPR, is essential for liver development (4) and plasma cell differentiation (5).

Xbp1 is a basic leucine zipper type transcription factor; it is activated by spliceosome-independent mRNA splicing initiated by Ire1α on the cytosolic surface of the ER membrane (6). The endoribonuclease Ire1α cleaves a 26-nt fragment from an unspliced form of Xbp1 mRNA, inducing a frameshift of the open reading frame (ORF) of the message. Xbp1 protein translated from the unspliced mRNA (Xbp1u protein) has no transcriptional activity, whereas Xbp1 protein from the spliced mRNA (Xbp1s protein) is a potent transcription factor inducing expression of UPR-related genes. This type of transcription factor activation (the unconventional splicing of its mRNA in the cytoplasm not in the nucleus) is unique to Xbp1 in animals, and the mechanistic basis of Xbp1 splicing and its function has been studied intensively in non-neuronal cells (5–9). Although the mRNA of Xbp1 is expressed in the brains of adult rodents (10), little is known about the detailed expression and function of Xbp1 in the mammalian central nervous system (CNS).

In this study, by utilizing an isolated neurite culture system and time-lapse imaging, we examined the spatiotemporal dynamics of Xbp1 during mouse CNS development and demonstrated that brain-derived neurotrophic factor (BDNF) induced the splicing of Xbp1 mRNA in the neurites, contributing to neurite outgrowth.

**EXPERIMENTAL PROCEDURES**

**Animals**—The Xbp1 knock-out mice were kindly provided by Dr. L. H. Glimcher (Harvard School of Public Health, Cambridge, MA). The Animal Experiment Committee of RIKEN approved all experimental procedures.

**Antibodies and Reagents**—Rabbit polyclonal antibody (pAb) to Xbp1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit pAb to eIF-2α, phospho-eIF-2α (Stressgen, Victoria, BC, Canada), mouse monoclonal antibody (mAb) to phospho-neurofilaments (Sternberger Monoclonal, Lutherville, MD), mouse mAb to MAP2 (NeoMarkers, Fremont, CA), mouse mAb to PSD-95 (Upstate, Lake Placid, NY), mAb to actin (Calbiochem, La Jolla, CA), and Alexa 488- or 568-conjugated secondary antibodies (Invitrogen, Eugene, OR) were used. Mouse mAb to syn-
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aptophysin (11) was kindly provided by Dr. K. Obata (Brain Science Institute, RIKEN, Japan). Rhodamine-labeled phalloidin was purchased from Invitrogen, BDNF was from Sigma-Aldrich, and rapamycin was from Calbiochem.

**Plasmid Construction and Transfection**—Full-length cDNAs for mouse Xbp1u (unspliced form) and Xbp1s (spliced form) were obtained by RT-PCR from the total RNA of NIH3T3 cells. Generation of Xbp1ns (never spliced by Ire1α) by site-directed mutagenesis was performed as described previously (7). The resulting products were subcloned into pcDNA4/myc-His (Invitrogen) to create pcDNA/Xbp1s-His or pcDNA/Xbp1ns-His. A full-length Xbp1u cDNA was also inserted into the BamHI site of Venus/pcS2 vector (kindly provided by Dr. A. Miyawaki, Brain Science Institute, RIKEN) to create pCS2/Xbp1-Venus, in which the Venus cDNA was located at the downstream region of Xbp1u cDNA; Xbp1s-Venus fusion protein was translated only when Ire1α-dependent splicing occurred. Cultured cells were transfected with expression plasmids by the use of LipofectAMINE2000 (Invitrogen). In standard analyses, neurons were transfected after 1 day in vitro (DIV) and maintained for 1–4 days after transfection.

**In Situ Hybridization (ISH)**—Mouse brains were prepared by perfusion fixation, embedded in paraffin, and cut into 8-μm thick sections. The procedures were as previously described (12). Briefly, sections were hybridized with digoxigenin-labeled antisense (or sense, as a negative control probe) RNA complementary to the coding region of the mouse full-length Xbp1 cDNA using a DIG RNA labeling kit (Roche, Mannheim Germany) with T7 or T3 RNA polymerase. After hybridization and washes, sections were incubated in a color-developing buffer containing NBT and BCIP for 8–12 h.

**Cell Cultures and Isolated Neurites**—Mouse embryonic fibroblasts and primary telencephalic neuronal cultures with Xbp1+/+ or Xbp1−/− genotype were generated from embryos at embryonic day 12.5 (E12.5) according to the methods described previously (13). Hippocampal neurons were isolated from mouse embryos at gestational day 17–18 as described previously (14).

The cells were plated on laminin- and poly-d-lysine-coated cover glasses or plastic culture dishes at a density of 2 × 10⁶ cells/cm² for high density culture or 1.3 × 10⁴ cells/cm² for low density culture. The hippocampal neurons were maintained in a serum-free medium (Neurobasal medium (Invitrogen)) supplemented with 0.5 mM glutamine and B27 supplement (Invitrogen). Isolated neurites were prepared according to the two surface culture techniques (15), with slight modifications. Hippocampal neurons were plated at a density of 2 × 10⁵ cells/cm² into a culture insert containing a polycarbonate filter membrane with 3-μm-diameter pore (Chemotaxis 3μ; Kurabo, Osaka, Japan) that had been coated with poly-d-lysine. After 5 days in culture in the serum-free medium, the upper membrane surface was scraped with a cotton swab (a thoroughly flattened tip) to remove cell bodies. Scraping was repeated three times with a fresh swab. The scraped membrane was analyzed by TO-PRO3 nuclear staining (Invitrogen) to ensure complete removal of cell bodies and non-neuronal cells. Only preparations containing no cell bodies were used for experiments. At 20 h after the removal of cell bodies, the isolated neurites remaining on the lower surface were stimulated with BDNF (100 ng/ml) or BDNF plus rapamycin (20 ng/ml).

**Quantitative RT-PCR**—Total RNA was prepared from mouse tissue or cultured cells using TRIzol reagent (Invitrogen). The SuperScript II first-strand synthesis system (Invitrogen) was used to synthesize cDNA according to the manufacturer’s instructions. For isolated neurites, total RNA was extracted using an RNeasy Micro kit (Qiagen, Hilden, Germany) and was processed through the antisense RNA amplification (16). Briefly, total RNA (100 ng) was first reverse-transcribed using a T7-oligo (dT) promoter primer (Affymetrix Japan, Tokyo, Japan) in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA was purified using a cRNA in vitro transcription (IVT) cleanup kit (Affymetrix) and served as a template in the subsequent IVT reaction. The IVT reaction was carried out in the presence of T7 RNA polymerase (Ambion Inc., Austin, TX), and then an additional procedure of double-stranded cDNA (ds-cDNA) synthesis was performed to obtain a sufficient amount of cDNA for analysis. cDNAs were subjected to a TaqMan RT-PCR assay (Applied Biosystems, Foster City, CA). The primer sequences were as follows (nucleotide difference between Xbp1s and Xbp1u underlined): (sense) 5’-CTGAGTGGCCAGCATCGGT-3’ (Xbp1s) 5’-CTGAGTGGCCAGCATCTACA-3’ (Xbp1u); (antisense) 5’-TGTCAGGTCATGGGAAGA-3’ (Xbp1s) 5’-TCAAGTCATGGCAATGGAA-3’ (Xbp1u) (FAM-labeled probe): 5’-GGGCCAGTGTACCTCCCTCCCC’-3’ (Xbp1s) and 5’-CTATGTGCACCTCTGC-3’ (Xbp1u).

All of the other assays were carried out using the Assay-on-Demand service (Applied Biosystems). We calculated the relative values by measuring ΔCt = Ct (each gene) − Ct (Gapdh or Actb) for each sample in quadruplicate. For the assessment of a ratio of Xbp1s to Xbp1u mRNA (Xbp1s/u ratio), an external control standard curve was determined by a PCR with the serial dilution of pcDNA/Xbp1s or pcDNA/Xbp1u plasmid as template. These standard curves displayed a linear relationship between Ct values and the logarithm of the input plasmid amounts (supplemental Fig. S2). To validate the Xbp1 isoform specificity of each probe, we performed quantitative PCR with the Xbp1s plasmid template and Xbp1u-specific probe or with the Xbp1u plasmid template and Xbp1s-specific probe. Although PCR efficiency of the undesirable cross-reaction was less than 2% relative to the specific reaction, the contribution of the cross-reaction was subtracted from the absolute value estimated by the specific reaction. Each value was compared statistically with the control by a Kruskal-Wallis test followed by a Games-Howell multiple comparison test.

**Western Blot Analysis**—Cells were lysed in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 1% β-mercaptoethanol, 10% glycerol, and 10 μg/ml bromphenol blue). The lysates were subjected to SDS-PAGE and immunoblot analysis using standard procedures.

**Immunofluorescence Analysis**—Neurons or isolated neurites were fixed for 20–30 min at room temperature in PBS containing 4% paraformaldehyde plus 0.1% glutaraldehyde, and then incubated for 60 min at room temperature in a blocking solution (PBS containing 5% goat serum and 0.1% Triton X-100).
They were then incubated for 2 h at room temperature with primary antibodies diluted in the blocking solution. After washing with PBS, the cells were incubated for 1 h at room temperature with corresponding Alexa 488- or 568-conjugated secondary antibodies diluted in the blocking solution, washed again with PBS, and mounted. For quantitative fluorescence analysis, low density cultured hippocampal neurons were fixed and stained with pAb to phospho-eIF2α and MAP2. Images were acquired with a ×40 objective lens (N.A. = 1.00) of a confocal microscope (FV1000, Olympus, Tokyo, Japan) in 35 randomly selected fields from each independent culture preparation. The integration of fluorescence intensity was measured using Fluoview imaging software (Olympus). The background intensity (based on a neighboring region that did not contain neurons) was subtracted from each value obtained. Because MAP2 is widely distributed throughout the early developing neuron, a ratio of phospho-eIF2α to MAP2 fluorescence signals was regarded as the phospho-eIF2α level in the neurons.

**Time-lapse Imaging**—Neurons were kept in a humidified incubator (MI-IBC-IF, Olympus) at 37 °C and 5% CO2, and they were imaged every 5 min for 12 h with a ×60 objective lens (N.A. = 1.40) of a confocal microscope (FV1000, Olympus) with an argon laser (3% intensity) adjusted to a wavelength of 515 nm. For the fluorescence recovery after photobleaching (FRAP) assay, a region of interest (ROI) in the nucleus was scanned with the 515-nm laser (100% intensity) for 10 s to photobleach the fluorescence of Venus, and postbleach images were captured as well.

**Quantitative Morphological Analysis**—Low density cultured neurons derived from E12.5 telencephalon of Xbp1+/+ or Xbp1−/− mice at 4 DIV were fixed and stained with Ab to pNF, an axonal marker, and rhodamine phalloidin. The length of axons, which were at least twice as long as the cell body, was measured in 150 randomly selected neurons with large pyramidal morphologies. The length was calculated with Scion image software (Frederick, MD). Three independent analyses were carried out while blinded to Xbp1 genotype. Nonparametric statistical tests (Mann-Whitney U test and a two-sample Kolmogorov-Smirnov test) were used to compare the distributions of values in two data sets.

**RESULTS**

**Xbp1 Is Highly Expressed in the Developing CNS**—It has been reported that Xbp1 mRNA is expressed ubiquitously in the mouse embryo, and especially strongly in the exocrine glands, liver, and bone precursors (17). Although Xbp1 is also expressed in the brains of adult rodents (10), the regional, and developmental variability of Xbp1 expression in the CNS has not been well characterized. We examined the expression profile of Xbp1 mRNA during mouse CNS development. Mice ranging in age from E18 to postnatal day 300 (P300) were subjected to ISH with a riboprobe that hybridized to both Xbp1s and Xbp1u mRNAs. At E18, Xbp1 was expressed throughout the brain. After birth, it was preferentially present in the regions with abundant neuronal cell bodies, namely the cerebral cortex, olfactory bulb, olfactory tubercle, thalamic nuclei, striatum, and cerebellum in postnatal development (Fig. 1A). Hippocampus was most strongly labeled throughout the various developmental stages (Fig. 1B). The pyramidal neurons of the frontal cortex and hippocampal cornu ammonis area 3 were intensely labeled with the Xbp1 probe at the early postnatal period (P7), but the signal seemed to be weaker at the adult stage (P300; Fig. 1C). In particular, apical dendrites of the pyramidal neurons were labeled with the Xbp1 probe during the early postnatal period. Xbp1 sense control probe gave no specific hybridization signal (supplemental Fig. S1A). To characterize the subcellular localization of Xbp1 mRNA, cultured hippocampal neurons at 4 and 10 DIV were subjected to ISH. Xbp1 mRNA was abundant in the cell body, and it was also present in neurites and growth cones (Fig. 1D). No hybridization signal was observed in either soma or neurites with Xbp1 sense control probe (supplemental Fig. S1B).

We performed a real-time RT-PCR assay to compare quantitatively Xbp1 mRNA expression in brain samples from different stages. To distinguish between Xbp1s and Xbp1u mRNAs,
we designed Xbp1s- and Xbp1u-specific TaqMan probe sets (supplemental Fig. S2). Consistent with the findings of ISH with a riboprobe that hybridized to both Xbp1s and Xbp1u mRNAs (Fig. 1), total Xbp1 (Xbp1t) was highly expressed during early postnatal developing stages in comparison with the developed period P360 (Fig. 2D). While Xbp1u mRNA has a similar expression profile to that of Xbp1t (Fig. 2B), Xbp1s was abundant through both the prenatal and early postnatal period (Fig. 2A). We quantified the splicing efficiency of Xbp1 mRNA, the ratio of Xbp1s mRNA to Xbp1u (Xbp1s/u ratio; see also supplemental Fig. S2). Xbp1s/u ratio was larger in the developing period E12 compared with the developed period P360 (Fig. 2C). Grp78, an ER chaperone up-regulated during the UPR, was also highly expressed in developing stages (Fig. 2A). A relatively high level of 54-kDa Xbp1s protein was seen at the developing stage (18 DIV), especially in the nucleus (Fig. 3A). To investigate the Xbp1 protein expression profile, total cell lysates prepared from cultured hippocampal neurons of several developmental stages were subjected to immunoblot analysis. A relatively high level of 54-kDa Xbp1s protein was seen at the developing stage (4–10 DIV) compared with the developed stage (18–26 DIV; Fig. 3B). In contrast, we failed to detect 33-kDa Xbp1u protein, possibly because of the shorter half-life of Xbp1u protein than that of Xbp1s protein (18). Indeed, we could see both Xbp1s and
Xbp1u clearly in the presence of MG132, a proteasome inhibitor that blocks Xbp1 degradation (supplemental Fig. S3).

We next examined the detailed subcellular localization of Xbp1 protein by co-staining with neuronal markers at various stages. At 6 DIV, Xbp1-IR was present at relatively high levels in the soma, and it was detected along axons stained with mAb to phosphorylated neurofilaments (pNF), an axonal marker (open arrows). Growth cones, as well as the soma, exhibited high Xbp1-IR (Fig. 3C, arrowheads). Xbp1 was also detected in the dendrites stained with MAP2 (solid arrows). These results suggest that Xbp1 protein localizes not only to the soma but also to axons, dendrites, and growth cones of developing hippocampal neurons.

To distinguish clearly the distribution of Xbp1s and Xbp1u proteins in the neurons, we generated two expression constructs. In the first construct, the 26-nt sequence was already spliced out of Xbp1 cDNA; thus, it produced only Xbp1s protein. The second one contained mutant Xbp1 cDNA, which produced mRNA that was resistant to splicing by Ire1α (7) and produced only Xbp1u protein. Each cDNA was fused with a His tag sequence and named His-Xbp1s or His-Xbp1ns (never spliced), respectively. Expression of the His tag proteins was tested by immunoblot analysis to ensure that each expression construct produced the correct molecular weight, 54-kDa or 33-kDa, respectively (Fig. 3D). Cultured hippocampal neurons were transfected with His-Xbp1s or His-Xbp1ns construct, and the cells were immunostained with mAb to His and MAP2 (Fig. 3D). His-Xbp1u protein expressed from the His-Xbp1ns construct was distributed in the cytoplasm, including dendrites, except for the nucleus. On the other hand, His-Xbp1s protein was concentrated in the nucleus, suggesting differential subcellular localizations of Xbp1s and Xbp1u proteins. The ratio of fluorescence intensity between the nucleus and cytoplasm in the cultured hippocampal neurons was analyzed to confirm the differential localization of Xbp1s and Xbp1u. Xbp1u exhibited a nuclear/cytoplasmic fluorescence ratio of 3.59, whereas Xbp1ns had a ratio of 0.39, the difference of which was statistically significant (Fig. 3D). The same staining pattern of Xbp1s and Xbp1u proteins was also observed in NIH3T3 cells (supplemental Fig. S4) and HeLa cells (43). The differential subcellular localizations of Xbp1s and Xbp1u proteins might be generally shown in various regional and developmental cell lines.

Xbp1 mRNA Is Spliced in Neurites in Response to BDNF—The highly polarized neurons have a characteristic protein synthesis mechanism, local protein synthesis, which is characterized by regulated mRNA localization and local translation in the neurites. This is the well-documented mechanism that provides the neurons with a means of rapidly altering protein composition in a spatially restricted manner, and it is known to play central roles in the regulation of neurite outgrowth and synaptic plasticity (19). BDNF strongly enhances protein synthesis by promoting translation initiation in neurons (20). Because ER stress is often accompanied by increased protein synthesis (21), we investigated whether BDNF triggers the UPR and induces Xbp1 splicing in mouse hippocampal neurons. RT-PCR revealed that bath application of BDNF (100 ng/ml) strikingly increased the splicing of Xbp1 mRNA within 8 h of stimulation (Fig. 4A). The Xbp1s/u ratio was dramatically increased in response to BDNF. Rapamycin, which suppresses BDNF-dependent protein synthesis (22), significantly blocked the Xbp1 mRNA splicing.

As Xbp1 mRNA (Xbp1u and Xbp1s) was detected throughout developing neurons (Fig. 1, C and D), we next asked where the splicing event occurs in neurites. For this purpose, we used a two surface culture technique (15), by which we isolated neurites remaining on the lower surface were stimulated with BDNF (100 ng/ml) or BDNF plus rapamycin (20 ng/ml) for quantitative RT-PCR. Bars represent means ± S.E. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001, Kruskal-Wallis test followed by Games-Howell multiple comparison test. Xbp1s, spliced form of Xbp1; Xbp1u, unspliced form of Xbp1; Xbp1t, total Xbp1 mRNA.

FIGURE 4. The effect of BDNF on Xbp1 splicing in cultured hippocampal neurons. A, RT-PCR analysis of Xbp1s mRNA levels in response to BDNF. Hippocampal neurons at 4 DIV were treated with or without BDNF (100 ng/ml) for indicated times, and rapamycin (20 ng/ml) or vehicle was added to the culture 20 min prior to BDNF application. Total RNA was extracted from the culture samples, and mRNA levels were measured by quantitative RT-PCR. Bars represent means ± S.E. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001, Kruskal-Wallis test followed by Games-Howell multiple comparison test. Xbp1s, spliced form of Xbp1; Xbp1u, unspliced form of Xbp1; Xbp1t, total Xbp1 mRNA.
ng/ml) for quantitative RT-PCR. In isolated neurites, BDNF dramatically induced splicing of Xbp1 mRNA within 8 h of stimulation, and rapamycin significantly blocked this event (Fig. 4B). This assay provided good evidence that Xbp1 splicing occurred in neurites in response to BDNF.

**BDNF Also Activates eIF-2α Signaling Pathway in Cultured Neuron**—Phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α) is a well-documented mechanism of down-regulating protein synthesis under a variety of stress conditions. During ER stress, ER stress-mediated eIF-2α phosphorylation is carried out by PERK (21), an ER-resident kinase functioning as an ER stress sensor protein, which is a parallel UPR pathway yet distinct to Ire1α-Xbp1 signaling. We tried to ascertain whether BDNF also activates eIF-2α signaling. Cultured hippocampal neurons were treated with BDNF or vehicle, followed by immunoblot or immunofluorescence analysis. Immunoblot analysis with pAb to phospho-eIF-2α revealed enhanced phosphorylation of eIF-2α in response to BDNF (Fig. 5A). Phosphorylation of eIF-2α was also detected in its mobility shift in immunoblot with pAb to eIF-2α. This was supported statistically by quantitative immunofluorescence analysis (Fig. 5C). Phospho-eIF-2α was distributed not only in soma but also in neurites, suggesting that UPR signaling could indeed be triggered in neurites (Fig. 5B).

**Spliced Form of Xbp1 Is Translated in the Neurites and Is Transported into the Nucleus**—To visualize the splicing of Xbp1u into Xbp1s mRNA, translation of Xbp1s mRNA, and localization of Xbp1s protein in cultured neurons spatiotemporally, we generated a construct consisting of the full-length mouse Xbp1u cDNA fused with Venus (a variant of yellow fluorescent protein with fast maturation) (25) in the frame of Xbp1s (Fig. 6A). Under an unstressed condition, the mRNA transcribed from the construct was not spliced, and only Xbp1u protein was produced. In contrast, during ER stress, the 26-nt fragment was spliced out by endogenous Ire1α, leading to a frameshift to induce translation of Xbp1s-Venus fusion protein (Fig. 6B, arrowhead). The hippocampal neurons were transfected with the Xbp1-Venus construct and bath-applied with BDNF.

BDNF increased expression of both exogenous Xbp1s-Venus fusion protein and endogenous Xbp1 protein, corresponding to 80- or 54-kDa, respectively, which was determined by immunoblot analysis with antibodies against both Xbp1 and Venus (Fig. 6C). Neurons transfected with Xbp1-Venus construct were also imaged by confocal microscopy. The time-lapse imaging revealed that the fluorescence of Xbp1s-Venus emerged in the neurites, as well as in the cell soma, and then translocated to the nucleus (Fig. 6D; see also supplemental Video S1). The fluorescence repeatedly appeared at the tips of developing neurites that were rapidly moving and shrinking (Fig. 6D, red circles). Because this observation implied the nuclear transportation of Xbp1s protein, we performed a FRAP assay to verify this finding. Before photobleaching, hippocampal neurons were treated and were imaged in the same way as the experiment mentioned above (Fig. 6E, prebleach). An ROI (shown as a red polygon in each image) was photobleached after the Venus fluorescence was concentrated in the nucleus, and subsequent fluorescence recovery due to translocation of unbleached Venus was recorded (Fig. 6E, postbleach). We observed that the Venus fluorescence in the nucleus was gradually recovered. The quantitation of the fluorescence intensity in the ROI showed that the rate of increase in the nuclear fluorescence was similar before and after the bleaching (Fig. 6E, right graph), suggesting continuous nuclear transport of Xbp1-Venus during the entire period.
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**FIGURE 6. Visualization of the molecular dynamics of spliced formed Xbp1 protein.** A, schema of the Xbp1-Venus construct that consists of full-length mouse Xbp1 cDNA fused with Venus cDNA. B, exogenous expression of Xbp1 protein derived from the Xbp1-Venus construct. Lysates of NIH3T3 cells transfected with mock vectors or expression vectors for Xbp1-Venus and treated with thapsigargin (thap.), a potent inducer of ER stress, were subjected to immunoblot analysis with pAb to Xbp1 or Venus. Xbp1s-Venus protein (arrowhead) was produced when Xbp1 mRNA was spliced upon ER stress. C, hippocampal neurons were transfected with Xbp1-Venus construct and treated with vehicle, BDNF, or thapsigargin. The expression of exogenous Xbp1s-Venus (closed arrowhead), endogenous Xbp1s (open arrowhead), and exogenous and endogenous Xbp1u protein (arrow) was determined. D, spatiotemporal translation and localization of Xbp1s protein in neurons. Cultured hippocampal neurons at 4 DIV were transfected with the Xbp1-Venus expression vectors and treated with 100 ng/ml BDNF. After 8 h, a neuron was imaged by time-lapse confocal microscopy with a 515-nm laser (3% intensity) captured at 5-min intervals (see also supplemental Video S1). DIC, differential interference contrast. Scale bars, 20 μm. E, FRAP assay showing the nuclear transport of Xbp1-Venus in cultured neurons. Hippocampal neurons at 4 DIV were treated as noted in C. After the Venus fluorescence was concentrated in the nucleus, a ROI (shown as a red polygon in each image) was photobleached for 10 s with the 515-nm laser (100% intensity). The fluorescence recovery was monitored (postbleach); fluorescence intensity within ROI was measured throughout the entire duration and plotted (right graph). Shaded area indicates the bleached period.

**DISCUSSION**

In this study, we demonstrated that the distribution of Xbp1 and its unique molecular dynamics in developing neurons. Although the expression of BDNF and its cognate receptor TrkB is low during the prenatal period (28), we confirmed all major components of BDNF signaling, including TrkB, PI3K, and Akt, were present to a similar extent in Xbp1+/+ and Xbp1−/− neurons by GeneChip analysis.3 We also observed no differential gene expression of immediately early genes, which are reportedly induced upon BDNF treatment, between Xbp1+/+ and Xbp1−/− neurons, suggesting that BDNF signaling was equally triggered in the neurons with both genotypes (supplemental Fig. 6A). Under our culture condition, BDNF markedly increased neurite outgrowth in Xbp1+/+ neurons and to a lesser degree in Xbp1−/− neurons (Fig. 7A). For quantification, cultured neurons with large pyramidal morphologies were subjected to morphological analyses, which were carried out while blinded to Xbp1 genotype. No significant difference in axon length was observed between each genotype under a basal condition, whereas Xbp1−/− neurons exhibited a significantly shorter axonal length after BDNF application (p = 0.002, Mann-Whitney U test, Fig. 7B; p = 0.001, Kolmogorov-Smirnov two-sample tests, Fig. 7C). Furthermore, there was also a significant reduction in the number of axonal branches in Xbp1−/− neurons both under basal and BDNF-treated condition (p = 0.006 and 0.0001, respectively, Mann-Whitney U test, Fig. 7D; p = 0.001 and 0.006, respectively, Kolmogorov-Smirnov two-sample tests, Fig. 7E).

**Xbp1−/− Neurons Show Morphological Alternation in Axonal Growth in Vitro**—We investigated the morphology of primary neurons obtained from Xbp1−/− mice. Xbp1−/− embryos do not survive beyond E14.5 because of severe liver hypoplasia (4). Thus, we took advantage of a low density culture technique of dissociated cells derived from E12.5 telencephalons of Xbp1+/+ or Xbp1−/− littermates. Considering that BDNF plays a key role in regulating neurite extension and branching (26, 27) and strongly elevated Xbp1 splicing (Fig. 4), we examined the effect of BDNF on the low-density cultured neurons of each genotype. Although the expression of BDNF and its cognate receptor TrkB is low during the prenatal period (28), we confirmed all major components of BDNF signaling, including TrkB, PI3K, and Akt, were present to a similar extent in Xbp1+/+ and Xbp1−/− neurons by GeneChip analysis.3 We also observed no differential gene expression of immediately early genes, which are reportedly induced upon BDNF treatment, between Xbp1+/+ and Xbp1−/− neurons, suggesting that BDNF signaling was equally triggered in the neurons with both genotypes (supplemental Fig. 6A). Under our culture condition, BDNF markedly increased neurite outgrowth in Xbp1+/+ neurons and to a lesser degree in Xbp1−/− neurons (Fig. 7A). For quantification, cultured neurons with large pyramidal morphologies were subjected to morphological analyses, which were carried out while blinded to Xbp1 genotype. No significant difference in axon length was observed between each genotype under a basal condition, whereas Xbp1−/− neurons exhibited a significantly shorter axonal length after BDNF application (p = 0.002, Mann-Whitney U test, Fig. 7B; p = 0.001, Kolmogorov-Smirnov two-sample tests, Fig. 7C). Furthermore, there was also a significant reduction in the number of axonal branches in Xbp1−/− neurons both under basal and BDNF-treated condition (p = 0.006 and 0.0001, respectively, Mann-Whitney U test, Fig. 7D; p = 0.001 and 0.006, respectively, Kolmogorov-Smirnov two-sample tests, Fig. 7E).

**DISCUSSION**

In this study, we demonstrated

3 A. Hayashi and T. Kato, unpublished data.
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ISH analyses of brain slices (Fig. 1C) and cultured hippocampal neurons (Fig. 1D) demonstrated that Xbp1 mRNA was found in the developing neurites in hippocampus and cerebral cortex. Recent studies indicated that a population of localized mRNA is strictly regulated by development and by activity (29). Our isolated neurite culture showed the existence of both Xbp1s and Xbp1u mRNAs in the neurites, and it clearly revealed the local splicing of Xbp1 in the neurites in response to BDNF (Fig. 4B). Spliceosome-dependent splicing capability of live neuronal dendrites was reported in a previous study (30), which showed the conventional mRNA splicing in dendrites. In contrast, this is the first evidence of the unconventional splicing of Xbp1 mRNA in the neurites, which is independent of spliceosome and specifically cleaved by endoribonuclease Ire1α. Based on this observation, we hypothesized that Xbp1 proteins in the neurites (Fig. 3, A and C) might be locally translated from Xbp1 mRNA in the neurites rather than being transported from the soma after translation there.

To further test the local translation of Xbp1 mRNA in neurites, we constructed the Xbp1-Venus expression vector. A similar Xbp1-Venus construct based on Ire1α-dependent splicing was developed as an ER stress reporter system (31), and our Xbp1-Venus construct was modeled after that construct. In that construct, the Venus cDNA was fused just after the spliced site of Xbp1, and the C-terminally truncated Xbp1-fused Venus protein was translated from the construct. In contrast, our construct contained the full-length cDNA of Xbp1 followed by Venus cDNA. Because of the property of the C-terminal regions that dominates the subcellular localizations of Xbp1 protein (Fig. 3D), our Xbp1-Venus construct allowed us to visualize not only real-time translation but also a molecular dynamics of Xbp1s protein. Time-lapse imaging and FRAP assay revealed that the fluorescence of Xbp1-Venus protein emerged in the neurite, continuously concentrating into the nucleus (Fig. 6). These observations suggested local translation of Xbp1s, the potent transcription factor, from Xbp1s mRNA and its subsequent nuclear transport.

A few studies have reported transcription factors whose mRNA is present and translated in neurite (32, 33). There are several mechanisms by which transcription factors are activated in the neurites, namely post-translational modification of proteins that are already present (e.g. phosphorylation of CREB (34) and NF-kB (35) or protein cleavage of neuregulin-1 (36)). Once being translated and/or activated into a mature protein, the transcription factor can undergo neurite-to-nucleus translocation within neurons, if it serves as a signal transducer from neurites to the nucleus. Because neurites are highly polarized cells with morphologically and functionally distinct subcellular compartments, the signaling from neurites to the nucleus plays a crucial role in CNS development and function. Our findings suggest that Xbp1 is a member of the neurite-to-nucleus signaling system with a novel mechanism: local splicing of its mRNA in the neurites as the result of the UPR. To our knowledge, this is the first report of a physiological role of Xbp1 splicing within neurons.

Mammalian cells have three ER stress sensors, ATF6, Ire1α, and PERK, all of which exist in the ER of dendrites in to the nucleus, thus mediating neurodevelopment. Indeed, BDNF-induced neurite outgrowth was attenuated in Xbp1−/− neurons (Fig. 7).

FIGURE 7. Altered axonal outgrowth of cultured neurons derived from Xbp1+/+ mice. A, the representative neurons derived from E12.5 telencephalon of Xbp1+/+ or Xbp1−/− cultured in the absence or presence of BDNF (100 ng/ml). Neurons at 4 DIV were stained with a mAb to pNF and rhodamine phalloidin. Xbp1−/− neurons tended to show less morphological complexity than Xbp1+/+ neurons. The captured images were subjected to quantitative morphological analysis in a blind manner (B–D). The arrowheads indicate branching points of axons. Scale bars, 50 μm. B, mean ± S.E. (n = 150 per group) of axon lengths with or without BDNF. C, frequency histogram analysis of axon lengths of each group. Mean ± S.E. (n = 150 per group) of axonal branches per neuron with or without BDNF. D, frequency histogram analysis of axonal branches per neurons of each group. n.s., not significant; **, p < 0.01; ***, p < 0.001, Mann-Whitney U test for B and D, two-sample Kolmogorov-Smirnov test for C and E.
primary mouse neurons (37). Therefore, to characterize the UPR properly, the detection of only Ire1α-Xbp1 signaling might not be sufficient. Indeed, the transgenic mouse model based on ATF6-Grp78 signaling to monitor ER stress in vivo (38) showed inconsistent results with another mouse model based on Ire1α-Xbp1 signaling (31). Therefore, we next examined eIF-2α, which is regulated by PERK signaling. BDNF induced the phosphorylation of eIF-2α, and the phosphorylated eIF-2α was found in neurites as well as cell bodies (Fig. 5). This finding also indicated that BDNF initiates UPR signaling locally in neurites. A detailed mechanism by which BDNF induces the splicing of Xbp1 has yet to be determined, but our results showed that BDNF does induce the splicing, at least partially, in a rapamycin-dependent fashion (Fig. 4). Rapamycin inhibits the mammalian target of rapamycin (mTOR), a signaling component closely related to translation initiation (22). Considering that BDNF strongly enhances protein synthesis by promoting translation initiation in neurons, it is possible that BDNF-induced increased protein synthesis might elicit UPR signaling. By utilizing GeneChip analysis, we observed the up-regulated gene expression of glycosylation-related enzymes and components of the ubiquitin-proteasome system in response to BDNF (supplemental Table S1). Glycosylation is the process of addition of saccharides to proteins, and the majority of proteins synthesized in the ER undergo glycosylation inside the ER and Golgi apparatus. The ubiquitin-proteasome system has an essential role in protein degradation, and this system is used during ER-associated protein degradation, which eliminates misfolded proteins inside the ER (39). These data might imply that BDNF increased protein turnover, leading to the initiation of the UPR. A molecular target downstream of Xbp1 signaling for neurite outgrowth still remains to be elucidated. One possible explanation is the role of Xbp1 in lipid biosynthesis. In developing neurons, growth cones show extreme motility, continuously extend and retract, and are characterized by a large amount of smooth ER. The ER membrane is involved in the recycling of plasma membrane, when growth cones change their shape (40). Interestingly, Xbp1 is reported to promote ER biogenesis by enhancing lipid biosynthesis in NIH3T3 cells (41) and secretory organs (42). The impairment of BDNF-induced neurite outgrowth in Xbp1−/− neurons (Fig. 7) could be accounted for by this mechanism. BDNF is crucial for the formation of the neural network, and it is locally secreted in an activity-dependent manner. This local secretion of BDNF might initiate the UPR in developing neurites in vivo. Indeed, TaqMan-based quantitative PCR indicated that the expression and splicing of Xbp1 mRNA was significantly greater during the development of the CNS in vivo (E12-P21) in comparison with the developed CNS (P360; Fig. 2). Together with the fact that the majority of glial proliferation occurs either during late embryogenesis or in the early postnatal period after the majority of neurons are formed, the activation of Xbp1 might be required for the differentiation of neurons. Further research on the role of UPR signaling mediated by Xbp1 during CNS development in vivo is warranted.

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