Expression of Brain-Derived Neurotrophic Factor and Its Receptors in the Median Eminence Cells with Sensitivity to Stress

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The median eminence (ME) is considered as the final common pathway connecting the nervous and endocrine systems. In this neurohemal structure, dynamic interactions among nerve terminals, tanycytes, and astrocytes determine through plastic processes the neurohormones access to the portal blood. Because brain-derived neurotrophic factor (BDNF) is involved in plastic changes, we investigated its presence and that of its receptors (TrkB) in the different cellular types described in the ME. Using in situ hybridization and immunohistochemical techniques, we demonstrated that BDNF immunoreactivity was essentially located in the astrocytes and to a lesser extent in tanycytes. By contrast, BDNF was not detected in nerve terminals reaching the external layer of the ME. TrkB antibodies recognizing the extracellular receptor domain labeled all of these different cell types, suggesting an autocrine or paracrine action of BDNF at this level. More selective antibodies showed that TrkB.FL immunostaining was found in tanycytes and nerve endings, whereas TrkB.T1 immunostaining was localized in all cellular types. Immobilization stress increased BDNF mRNA and BDNF immunoreactivity patterns and induced biphasic BDNF release from the ME, as analyzed by push-pull perfusion. In addition, we observed that 60-min stress intensified BDNF immunoreactivity in the internal layer and also its colocalization with glial fibrillary acidic protein. Stress also accentuated BDNF immunostaining in the perivascular space in elements that were not labeled with antibodies recognizing fibroblast or endothelial cells. These data disclosed a novel location of BDNF and its receptors in the ME, which are presumably involved in dynamic processes such as hormone release. (Endocrinology 145: 4737–4747, 2004)

Brain-derived neurotrophic factor (BDNF) is a member of the nerve growth factor family, the so-called neurotrophins (1). These molecules are involved in a variety of functions in the central nervous system (CNS) including synaptic plasticity in adult rats (2, 3). Neurotrophins interact with high-affinity protein kinase receptors of the Trk family, the TrkB receptor being the specific entity for BDNF (4). At least three main TrkB isoforms have been identified in rodents (5, 6), the full-length catalytic receptor (TrkB.FL) and two truncated forms (TrkB.T1 and TrkB.T2) with different signaling capacities (7, 8).

Neuronal plasticity (9) is an important property allowing adaptive processes in several regions of the CNS, e.g. in the hippocampus, cerebral cortex, or hypothalamus. In the hypothalamus, this phenomenon has notably been studied in the supraoptic nucleus (10), paraventricular nucleus (11) and periventricular nucleus (12, 13). The hypothalamus is one of the brain regions containing the highest levels of BDNF mRNA (14) and BDNF peptide (15). In this region, we (16–18) and others (19, 20) recently reported a punctuated distribution of BDNF mRNA and protein as well as their physiological regulation in several hypothalamic nuclei. However, no special attention has been paid to the possible presence and regulation of BDNF and its receptors in the median eminence (ME). The ME belongs to the circumventricular organs and is considered to be the final common pathway connecting the nervous and endocrine systems. This organ is a conspicuous neurohemal structure that is devoid of neuronal cell bodies and contains only axons and two types of glial cells including astrocytes and tanyocytes (21, 22). Important morphological plasticity changes occur at the ME level to ensure appropriate physical contact between hypothalamic nerve endings and the vascular endothelium, which could facilitate neurohormone entry into the portal circulation. Thus, neurohormone release from nerve endings at the ME level results from dynamic plastic interactions of cell-to-cell communication processes involving astrocytes, tanyocytes, and collagen fibrils of the extracellular matrix (23) with endothelial cells of portal vessels (24).

Given the already recognized role of BDNF in plastic processes (3), we investigated whether BDNF and its receptors (TrkB.FL and TrkB.T1) were present in the different cell types found in the ME. Because stress represents a physiologic stimulus that triggers neuropeptide release processes at the ME level (25–27) and modulate cell plasticity (28), we also delivered immobilization stress to rats at different times to
determine whether BDNF mRNA, protein levels, and BDNF release from ME would be sensitive to this manipulation. As well, we monitored BDNF release from ME in anesthetized free-moving rats by using push-pull perfusion technique (25, 26).

Materials and Methods

Animals
Adult male Sprague Dawley rats (Depéret, St. Douichard, France) weighing 250–260 g at the beginning of the experiments were housed for 1 wk under constant temperature (21 ± 1 C) and lighting (lights on from 0700 to 1900 h) regimens. Food pellets and water were freely available throughout the experiment. Procedures involving animals and their care were conducted in conformity with French laws on laboratory animals that are in compliance with international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 December, 1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals, NIH Publication 85-23, 1985). All protocols were approved by the Animal Welfare Committee at the University of Montpellier II. All experiments were performed in conscious rats (five to six in each group) between 0900 and 1400 h, i.e. during the diurnal trough of the circadian hypothalamic-pituitary-adrenal rhythm. On the day of the experiments, animals were immobilized according to a well-established protocol (16, 18, 29, 30). Briefly, rats were attached for different times (indicated in each experiment) to wooden boards in a prone position by taping their forelimbs and hindlimbs to metal mounts; head movements were restricted by means of two metal loops around the neck area. Controls and unstressed animals were handled daily, and on the day of the experiment, they were killed at the same time as the stressed ones.

Immunohistochemical procedures

Immunofluorescence labeling. On the day of the experiment, control and 60-min immobilized rats were deeply anesthetized with an injection of 0.2 ml of a mixture of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg) and rapidly perfused transcardially with 4% paraformaldehyde in 0.2 M phosphate buffer. Brains were removed and postfixed in the same fixative for 4 h at 4 C and placed in 15% sucrose (10 mg/kg) and rapidly perfused transcardially with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed in 0.2 ml of a mixture of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg) and rapidly perfused transcardially with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed in 0.2 ml of a mixture of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg) and rapidly perfused transcardially with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer.

Peroxidase labeling. For BDNF peroxidase immunohistochemistry determination, on the day of the experiment, the animals were immobilized for 30, 60, or 180 min. Control unstressed and experimental stressed animals were anesthetized and rapidly perfused as described for the immunofluorescence procedures. Brains were removed and postfixed in the same fixative for 72 h at 4 C, mounted on a vibrating blade microtome (Leica), and serially cut into 30-μm coronal sections. The protocol was conducted according to an immunohistochemistry-free-floating section method (31). Sections were carefully rinsed three times for 10 min each in PBS (pH 7.4) and then treated with 0.3% hydrogen peroxide for 30 min in PBS, rinsed three times in PBS, and blocked for 30 min in 5% GNS and 0.4% Triton X-100. Sections were incubated overnight at room temperature with the primary rabbit anti-BDNF antiserum (Santa Cruz Biotechnology) diluted at 1/100 in blocking solution. Then sections were rinsed three times in PBS and incubated for 2 h with a secondary biotinylated goat antirabbit antibody diluted at 1/1000 (Sigma-Aldrich Chemical) in PBS containing 1.5% GNS and 0.3% Triton X-100. After rinsing three times in PBS, sections were incubated for 1 h in avidin-biotin complex diluted at 1/100 (Vector Laboratories, Santa Cruz, CA, France). Sections were then carefully rinsed 3 times in PBS and the signal was detected with the diaminobenzidine kit (Vector Laboratories, AbCys, Paris, France) according to the manufacturer’s instructions. The immunostaining specificity was determined with the same protocol but by control incubation sections with the secondary antiserum alone.

Electron microscopy. After being deeply anesthetized with sodium pentobarbital (50 mg/kg), animals were perfused through the ascending aorta with PBS (pH 7.4), followed by 500 ml of fixative composed of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was then dissected and fixed by immersion in the fixative without glutaraldehyde for 12 h at 4 C. The hypothalamus was then cut coronally into 40- to 50-μm-thick sections with a vibratome. After careful rinsing in PBS, sections were successively incubated: 1) for 4 h at 4 C with the rabbit antibody against BDNF (diluted 1: 500), 2) for 12 h at 4 C with a peroxidase-labeled Fab fragment of goat IgG antirabbit IgG ( Biosys, Compiègne, France), diluted 1:1000, and 3) with 0.1% 3,3’-diaminobenzidine diluted in 0.05 M Tris-buffer (pH 7.3) and postfixed in 0.1% OsO4 in the presence of 0.2% H2O2. The primary and secondary antibodies were diluted in PBS containing 1% BSA, 1% normal goat serum, and 0.1% saponin. Immunostained sections were carefully rinsed in 0.1 M cacodylate buffer (pH 7.3) and postfixed in 1% OsO4 in the same buffer. They were then dehydrated in graded concentrations of ethanol and embedded in araldite. Punches of 1.5 mm in diameter were cut through the ME and mounted on araldite blocks. After being cut into ultrathin sections, they were observed in a Hitachi H 7110 electron microscope (CRIC, Montpellier, France) without counterstaining.

BDNF cRNA probe synthesis and preparation

As we previously reported (30), BDNF cDNA was cloned within the pGem-T vector and linearized with Sall (Promega, Charbonniere, France). Briefly, radioactive antisense cRNA copies were synthesized using the TransProbe SP Pharamacika kit (Amersham-Pharmacia, Orsay, France) by incubation of 250 ng of linearized DNA template in 5 μl transprobe buffer containing ATP, GTP, and CTP in 1 μl RNAguard (ribonuclease inhibitor), 0.5 μl of dUTP (200 μM); NEN Life Science Products, Paris, France), 0.5 μl RNase-free diethyl pyrocarbonate water, and 1.5 μl of T7 polymerase for 90 min at 37 °C. To reduce background, unincorporated nucleotides were removed using a Microspin column (Amersham-Pharmaica) 15 min after the addition of 1 μl of DNaseI (37 °C). Radioactive sense cRNA probe used as negative control was also synthesized to verify the specificity of the probe. Hybridization with this probe did not reveal any positive signal.
Semi-quantitative in situ hybridization

For in situ hybridization determination, on the day of the experiment, the animals were immobilized for 15 or 180 min. Control unstressed and stressed animals were anesthetized, perfused, postfixed, and frozen as described for the immunofluorescence procedure. Frozen brains were serially cut on a cryostat (Leica) into 10-μm coronal sections. The hypothalamic sections were mounted on Superfrost Plus glass slides (Labonord, Templemars, France) and kept at −80°C until use. Hybridization histochemical localization of BDNF transcripts was carried out using [35S]-labeled-cRNA probe, as already described (18, 30). Briefly, after prehybridization treatment, hybridization of BDNF transcripts was carried out using [35S]cRNA probe at a concentration of 8 × 10⁶ cpm/slide. After the posthybridization treatments, the sections were dehydrated and coated with liquid photographic emulsion (NTB-2, diluted 1:1 with water, Kodak, Rochester, NY). Slides were exposed for 4 wk, developed in Dektol developer (Eastman Kodak) for 2 min, and fixed in rapid fixer (Eastman Kodak) for 4 min. Analysis of BDNF mRNA signal was performed by computer image analysis using a Sony CCD XC-77 video camera with high resolution [570(H) to 485(V) TV lines] coupled to a Macintosh computer (Power PC G3) and NIH-Image software (version 1.65 non-FPU, W. Rasband, NIH, Bethesda, MD) (18, 30). For each animal, an OD from four to six sections of the ME was determined to calculate a mean per animal. The OD was corrected for the average background signal determined by sampling cells immediately outside the cell groups of interest. The results were expressed as means ± SEM calculated from four to five animals per experimental group. The experiments were performed twice independently, but all sections were hybridized at the same time to avoid intrinsic variations among different in situ hybridizations.

Push-pull perfusion

After a 7-d acclimation period in the laboratory, rats were anesthetized with sodium pentobarbital (40 mg/kg), and a stainless steel cannula (0.5 mm outer diameter) fitted with a stylette was stereotaxically implanted into the hypothalamic external ME according to Paxinos and Watson coordinates (AP, 2.8 mm; L, 0 mm; and DV, 10 mm) (32). Immediately after surgery, rats received an im injection of 60,000 IU penicillin (Bristopen, Paris, France) and were caged separately. They were handled every day for 1 wk and had recovered their preoperative body weight when they were subjected to push-pull perfusion. A control group (n = 4), not subjected to immobilization stress, was perfused during 3 h according to the experimental procedures already described (29, 33). Briefly, push-pull perfusion was performed on unanesthetized animals with artificial cerebrospinal fluid at a regular flow rate of 18 μl/min, as described (29, 33). Samples (270 μl/min) were collected every 15 min for 3 h and then acidified (acetic acid, 1 N final concentration) and evaporated in a Speed-Vac concentrator (Savant Instruments, Hicksville, NY). They were immediately stored at −20°C until BDNF assay. In the experimental group (n = 7), after 60 min of perfusion in basal conditions, animals underwent immobilization stress for 30 min, followed by another 75 min nonstimulated period. At the end of the experiments, all animals were killed and their brains removed and fixed for histological examination (see Fig. 9A). Rats with unsuitable cannula locations, i.e., outside the ME, were excluded from the study.

BDNF release

BDNF release was measured with a conventional two-site enzyme-immunoassay system (ELISA) assay as previously reported (18, 30, 34). The BDNF Emax immunoassay (Promega) was performed according to the manufacturer’s protocol. Briefly, each evaporated sample was reconstituted with 100 μl assay buffer and used to determine BDNF release. The assay sensitivity was 15 pg/ml and the cross-reactivity with other related neurotrophic factors was less than 3%. The BDNF concentration was expressed as picograms per fraction. The intra- and interassay coefficients of variations were 3 and 8%, respectively.

Corticosterone RIA

Corticosterone was analyzed to control the efficacy of the stress paradigm. As previously reported (30, 35, 36), blood samples were collected after killing the rats after different times of immobilization stress (15, 30, 60, and 180 min). Blood was collected on 1 mg/ml EDTA (Sigma-Aldrich), centrifuged at 4°C, and plasma stored at −20°C until assayed for corticosterone. Plasma corticosterone was assayed in 20-μl samples after ethanol extraction using the RIA kit kindly provided by Dr. P. Siaud (36). The intra- and interassay coefficients of variation were 6 and 8%, respectively. The assay sensitivity was 0.5 ng/ml.

Statistical analysis

Quantitative data are presented as mean ± SEM. Mean and SEM were calculated from five to six animals per group for determination of plasma corticosterone, BDNF immunoreactivity, and BDNF mRNA levels. Comparisons between control and stressed groups were performed by an ANOVA (Statview 4.5) followed by Fisher’s protected least significant difference test (18, 30, 35), and P < 0.05 was considered significant. To evaluate the effect of stress on BDNF release at the ME level, each 15-min sampled fraction obtained after the beginning of stress was compared with mean basal values obtained before the stress session and a variance analysis with repeated measures (Statview 4.5), followed by a nonparametric Wilcoxon signed rank test for individual comparisons, and P < 0.05 was considered significant.

Results

Distribution of BDNF, TrkB, TrkB.FL, and TrkB.T1 immunoreactivities in the ME

Fluorescent immunohistochemical data, obtained on 10-μm coronal cryostat sections, revealed the presence of BDNF and TrkB receptors in the ME (Fig. 1). BDNF immunoreactivity was essentially restricted to the internal layer of the ME, although the perivascular space, beyond the external layer, was also weakly labeled (Fig. 1A). The antibody recognizing the TrkB extracellular domain strongly labeled the internal layer of the ME and in a less extent the external layer.
TrkB.FL immunoreactivity was essentially localized in the external layer of the ME (Fig. 1C), whereas immunostaining for TrkB.T1 was found in both external and internal layers (Fig. 1D). TrkB.T2 could not be identified because antibodies recognizing this isoform are not available yet. Negative controls did not exhibit any detectable immunostaining in ME regions that had positive signals with specific BDNF and TrkB antibodies (data not shown).

**BDNF mRNA expression in the ME**

To determine whether BDNF was synthesized in situ, we looked for its mRNA expression in the ME. *In situ* hybridization labeling of BDNF mRNA was identified in the internal and external layers of the ME, whereas no hybridization signal was observed in the intermediary part (see Fig. 5A, control). A hybridization signal was also detected in the medial and lateral part of the arcuate nucleus, an anatomical region shown in the adjacent scheme. This hybridization signal served as positive control in this experiment because BDNF signal in the arcuate nucleus has been previously reported by others (19). Hybridized sections with sense probe (negative control) did not exhibit any detectable reaction in the ME (data not shown).

**Identification of cell populations expressing BDNF, TrkB, TrkB.FL, and TrkB.T1 in the ME**

Cell populations expressing BDNF and the different isoforms of TrkB receptor were identified on 10-μm coronal cryostat sections (Fig. 2) double immunostained for: 1) BDNF (first column), the extracellular TrkB domain (second column), TrkB.FL (third column), and TrkB.T1 (fourth column) and 2) specific markers for the different cellular components of the ME including VIM for tanycytes (37, 38) (top panels), GFAP for astrocytes (middle panels), or SYN for nerve terminals (bottom panels). As previously reported, VIM immunostaining was found to be associated with tanycytes, which are cells surrounding the lumen of the third ventricle (37). Tanycyte cell bodies were present in the internal part of the ME, exhibited long processes projecting into the periventricular parenchyma and perpendicularly to the main axis of the organ toward the external limit of the external layer (Fig. 2, top panels). GFAP immunostaining was associated with cells showing typical astrocytes morphology, i.e. stellate cells, with several processes radiating from the cell body localized in the internal layer of the ME (Fig. 2, middle panels). SYN immunostaining was exclusively found in the external layer of the ME in the vicinity of portal vessels (Fig. 2, bottom panels). This label should correspond to nerve terminals that deliver hypothalamic hormones in portal blood.

Careful examination of double immunostained sections for VIM, GFAP, or SYN and BDNF indicated that BDNF labeling was colocalized in the internal layer of the ME with GFAP immunostaining but scarcely and weakly with VIM immunostained elements. By contrast, BDNF was not found in the SYN-positive terminal ends (Fig. 2, first column). Im-

![Fig. 2. Colocalization of BDNF, TrkB, TrkB.FL, and TrkB.T1 with VIM, GFAP, or SYN in ME sections. Specific antibodies against BDNF, TrkB, TrkB.FL, and TrkB.T1 were revealed with Alexa Fluor 488-labeled secondary antibodies (green immunolabeling) and specific antibodies against VIM, GFAP, and SYN with Cy3-labeled secondary antibodies (red immunolabeling). Colocalizations appear in yellow (arrows). 3v, Third ventricle. Scale bar, 60 μm.](https://academic.oup.com/endo/article-abstract/145/10/4737/2500103/ ADDITIONAL IMAGES 3B, 3C, 3D, 3E, 3F, AND 3G FROM FIG. 2)
munostaining obtained with the antibody addressed against the extracellular domain of TrkB (which labels the three isoforms) was colocalized with VIM immunostained tanycytes, GFAP-immunostained astrocytes, and weakly with SYN-labeled nerve endings (Fig. 2, second column). Double-immunostained sections for VIM, GFAP, or SYN and TrkB.FL showed that TrkB.FL was colocalized in the external layer of the ME with VIM immunostained tanycytes and SYN-labeled nerve endings (Fig. 2, third column). TrkB.T1 immunostaining showed that this truncated receptor was essentially colocalized with VIM-immunostained tanycytes throughout the different ME layers but also with GFAP immunostaining and SYN-labeled nerve endings in the external layer of the ME (Fig. 2, fourth column).

Ultrastructural localization of BDNF in the ME
Throughout the different regions of the ME, immunostaining was clearly recognized as electron-dense precipitates associated with different cellular structures. In all the sections examined, BDNF immunostaining was essentially associated with glial structures, including astrocytic processes located in the internal layer of the ME (identified by their contents of dense bundles of intermediate filaments; Fig. 3A) and tanycytic processes that project throughout the ME layers (that were identified by their lipid droplet contents; Fig. 3B). Strong to moderate BDNF immunostaining was associated with fibroblast-like cells located in the perivascular space (Fig. 3C).

Levels of plasma corticosterone during immobilization stress
Figure 4 shows that plasma corticosterone levels rose significantly after 15 min of immobilization stress (359 ± 31 ng/ml in basal conditions at t0, P < 0.01) and reached maximal concentrations after 30 min of immobilization stress (471 ± 21 ng/ml). After 180 min of stress application, corticosterone levels were still highly elevated (374 ± 21 vs. 10 ± 4 ng/ml at t0, P < 0.01). These concentrations are similar to those previously reported with a similar paradigm (18, 30, 34).

Effects of immobilization stress on BDNF mRNA in the ME
Figure 5 shows the distribution of mRNA label in control unstressed male rats and after immobilization stress in the ME. In response to 15 or 180 min of stress exposure, we observed an increase in signal intensities in the internal but notably in the external layer of the ME (Fig. 5A). The statistical analysis (Fig. 5B) showed that the OD was increased in the two layers. Thus, in the internal layer, the OD values expressed as arbitrary units were 114 ± 20 (15 min) and 140 ± 10 (180 min) vs. 51 ± 2 in control rats; P < 0.05 and P < 0.01, respectively. In the external layer, values were 96 ± 17 (15 min) and 150 ± 4 (180 min) vs. 53 ± 5 OD in control rats; P > 0.05 and P < 0.01, respectively. These data show an increase in BDNF mRNA levels in the external layer of the ME. Interestingly, signals in the arcuate and ventromedial nuclei seem to be also increased after stress.

Effects of immobilization stress on BDNF immunoreactivity in the ME
Figure 6 shows the localization of BDNF immunoreactivity in the ME of adult male rats after 30 (Fig. 6, C and D), 60 (Fig. 6, A), 90 (Fig. 6, B), and 120 (Fig. 6, E) min of immobilization stress. Values for control animals (t0) and after 15, 30, 60, and 180 min of immobilization stress (n = 5–6 per experimental group) are shown. The results are expressed as mean ± sem. **, P < 0.01 vs. basal concentrations at t0.
E and F), and 180 min (Fig. 6, G and H) of immobilization stress. These results obtained on 30-μm sections showed stronger immunolabeling in the internal layer of the ME after 30 and 60 min of immobilization stress, with no major modification at 180 min. This was followed by stronger immunostaining in the external layer of the ME after 60 and 180 min of immobilization stress. Because the most important variations were detected after 60 min of stress, this time was chosen for double immunohisto-
chemistry analysis (Fig. 7). Fluorescent microscopy analysis of 10-μm sections double immunostained for BDNF and VIM, GFAP, or SYN basically indicated that stress seems to increase the colocalization of BDNF and GFAP immunoreactivities in the internal layer of the ME. The double-immunostaining analysis of TrkB (antibody detecting the extracellular binding domain) and these cellular markers showed a larger colocalization of TrkB with VIM and GFAP after stress stimulus than in control animals. The colocalization of TrkB with SYN immunoreactivity was not apparently modified. Then sections were double immunostained with antibodies detecting the different TrkB receptor isoforms and VIM, GFAP, or SYN. Stress stimulus seems to increase the expression of TrkB.FL and TrkB.T1 in glial cells and to a lesser extent that of TrkB.T1 in the tanyocytes feet (Fig. 7) because the signal intensity looks more pronounced.

Attempts to identify the BDNF-containing cellular elements of the perivascular space

Although these elements certainly do not belong to the ME, we tried to identify the cell population(s) of the perivascular space expressing BDNF immunoreactivity after stress stimulus. For this purpose two specific markers were used: Fibro for fibroblasts (39) and EBA recognizing brain mature endothelial cells (40). Thus, 10-μm coronal cryostat sections from control (Fig. 8, A, B, E, and F) or 60 min-stressed rats (Fig. 8, C, D, G, and H) were double immunostained with antibodies against BDNF and EBA (Fig. 8, A–D) and for BDNF and Fibro (Fig. 8, E–H). Analysis of the double-immunostained sections indicated that even if some BDNF label was localized in fibroblasts, the huge amounts of immunoreactive BDNF observed after stress was not localized in the cellular types studied.

Effect of 30-min immobilization stress on BDNF release in the ME

Immobilization stress induced biphasic stimulation of BDNF release from the ME (Fig. 9), promptly at the onset of the stress stimulus, and a belated second peak, at the onset of the recovery period. BDNF release first rose from basal levels (16.7 ± 0.3 pg per 15 min) to stimulated levels (23.7 ± 4.1 pg per 15 min; n = 7; P < 0.05) 15 min after the beginning of stress and progressively returned to basal values 15 min after the end of the stress stimulus (17.0 ± 0.7 pg per 15 min). During the recovery period, BDNF release was again increased 30 min after the end of the stress application and rose from 16.7 ± 0.3 pg per 15 min to 27.8 ± 6.5 pg per 15 min (n = 7, P < 0.05). Then the BDNF values were not significantly different from basal values observed before immobilization stress. An illustrative individual specimen is shown in Fig. 9A and a statistical time-course analysis is shown in Fig. 9B.

Control animals perfused during 3 h exhibited a minimal release of BDNF near of the limit of the assay detection, in the same range of basal values measured in the experimental group. Therefore, stress-induced BDNF release could not be a natural peak of BDNF secretion. Control and experimental animals were perfused at the same time of day (Fig. 9A).

Discussion

The major finding of this study was the localization of BDNF in the ME, a hypothalamic structure mainly devoted...

![Fig. 7. Double-immunostained sections of the ME in control and stressed adult rats. After 60 min of immobilization stress, colocalization of BDNF-immunoreactivity (IR), TrkB-IR, TrkB.FL-IR, or TrkB.T1-IR with VIM, GFAP, and SYN were analyzed in 10-μm ME sections. Fluorescence was visualized with Alexa Fluor 488-labeled specific antibodies against BDNF, TrkB, TrkB.FL, or TrkB.T1 (green immunolabeling) and Cy3-labeled antibodies against VIM, GFAP, and SYN (red immunolabeling). Colocalization appears in yellow. 3v, Third ventricle. Scale bar, 60 μm.]
to neurohemal function and belonging to circumventricular organs. Actually, in situ hybridization and immunocytochemical experiments allowed us to disclose BDNF mRNA and protein localization in the internal and external layers of the ME as well as secondarily in the perivascular space. Double-immunofluorescence staining revealed the presence of BDNF in two types of glial cells, i.e., astrocytes whose classical role seems to be the regulation of cellular metabolism and activity (41) and tanycytes, which are assumed to be important in hormonal or transport (42).

Astrocytes are classic glial cells present in the ME whose processes, unlike those of tanycytes, stretch parallel to the ventricular surface (38). Astrocytes appear as an important source of BDNF because, besides expressing GFAP, a specific marker of these cells, they were also positive for BDNF. Tanycytes represent the most abundant cell type of the ME. They are specialized glial cells with an elongated shape and one cytoplasmic end that contact the surface of the third ventricle and the other end contacting neurosecretory terminals or the perivascular space limiting the external layer of the ME. This represents a major finding because so far, in vivo BDNF localization has been reported to be restricted to neurons in the CNS (43).

Staining in the internal ME layer might correspond to BDNF brought by anterograde transport from hypothalamic magnocellular neurons in which abundant BDNF mRNA expression has been described (17, 18). Indeed, preterminal axons have been detected in the internal ME directed to the posterior pituitary, predominantly derived from the magnocellular nuclei, i.e., supraoptic and paraventricular nucleus nuclei (44). Nevertheless, negative results were obtained with electron microscopy and synaptophysin, a specific marker of axon endings, thus discarding this possible BDNF origin. Therefore, it is probable that BDNF visualized in the internal layer is originated in tanycyte bodies present at this level, especially because colocalization of BDNF/VIM was observed here.

Electron microscopy observations indicated that BDNF immunostaining was present, although with weaker expression, in the external ME, a region in which feet tanycytes are located. Interestingly, in the ME, regenerative axon sprouting always occurs in close association with tanycytes (37, 45), and when tanycytes are transplanted in the spinal cord, they...

**Fig. 8.** ME sections double immunostained with BDNF and EBA or Fibro in control and stressed adult rats. The 10-μm ME sections from control or 60-min stressed animals were analyzed. Fluorescence was visualized with Alexa Fluor 488-labeled specific antibody against BDNF (green immunolabeling) and Cy3-labeled antibodies against EBA and Fibro (red immunolabeling). A, C, E, and G show lower magnification of BDNF-immunoreactivity (IR) with EBA-IR (A and C) or Fibro-IR (E and G). B, D, F, and H show higher magnification of BDNF-IR with EBA-IR (B and D) or Fibro-IR (F and H) in the external layer of the ME. Colocalization appears in yellow. 3v, Third ventricle. Scale bar in lower magnification, 60 μm (A, C, E, and G); scale bar in higher magnification (B, D, F, and H), 30 μm.
BDNF in this region overlying the brain. Failure of staining this region with specific cell markers such as Fibro and EBA allowed us to exclude fibroblasts and endothelial cells as a source of BDNF, respectively. Therefore, it is conceivable that cellular elements in the perivascular space other than those analyzed here, but intimately juxtaposed to it, can synthesize this neurotrophin. Good candidates are the meninges because they have been reported to contain a soluble factor possessing important neurotrophic properties (47).

We observed a wide BDNF receptor distribution in the internal layer of the ME, in VIM- and GFAP-labeled regions, close to tanycyte cell bodies and processes. TrkB labeling was also coexpressed with synaptophysin staining in the external layer. This peculiar TrkB receptor distribution, within or in the close vicinity of cells containing BDNF, led us to envisage a paracrine or autocrine mode of action for BDNF in the ME. This possibility is in keeping with data showing that most neurotrophin actions are exerted according to a paracrine or autocrine mode (48, 49).

Receptor detection was first performed with an antibody recognizing all of the TrkB isoforms because it was addressed against the extracellular domain. Then the characterization of BDNF receptor isoforms was undertaken using specific TrkB receptor antibodies against the full-length (TrkB.FL) and truncated (TrkB.T1) receptors. TrkB.FL receptor localization was rather restrictive to the external ME layer in which it colocalizes with VIM and SYN labeling but not with GFAP. TrkB.T1 receptors were visualized throughout the ME, presenting a similar distribution to the tanycytes trajectory. Location of truncated receptors in the ME tanycytes is not surprising because these isoforms have been reported to be predominant in other circumventricular regions already analyzed (5). TrkB.T1 staining also colocalizes with GFAP labeling, thus showing a preferential nonneuronal localization in keeping with reported data (50, 51). Indeed, it has been reported that TrkB.T1 is the major isoform expressed in non-neuronal cells of adult brain (52). Although to a lesser degree, TrkB.T1 receptor also colocalizes with SYN marker that is also coherent with its already reported coexpression with the catalytic isoform TrkB.FL in neurons (53). Because it is established now that truncated receptors are also endowed with biological activity (7, 8), their presence in the ME, in addition to the full-length form, further strengthens that BDNF localized in the ME is physiologically relevant. Furthermore, the different colocalizations described above are also well visualized after stress application. Interestingly, the TrkB staining pattern seemed to be widespread in tanycytes after stress. BDNF could be released from astrocytes to act on themselves or on tanycytes and even on ME nerve endings. Other growth factors, TGFα and IGF-I, are known to be released from astrocytes for controlling GnRH release, likely through tanycyte mediation as reported in vitro (54, 55).

The role of tanycytes in the ME has long been associated with either transport mechanisms (41) between the cerebrospinal fluid and external layer of the ME (56) or control-releasing events occurring in this region (57, 58). This latter action seems to be exerted through a retraction or enlargement process, a property of tanycyte foot, which are able to interpose their terminal podia between neurohormonal nerve endings and portal blood vessels (59). Moreover, the existence of neuron-parenchymal transmission pathways and the expression of synaptic proteins, like Syn and VIM, in tanycytes (60) and the fact that astrocytes express the TrkB receptor isoforms (53) strongly suggest that BDNF synthesized in tanycytes could be responsible or contribute to axon regeneration.
glial synaptoid contacts has been reported for tanyocytes in the ME (60, 61). Because neurohormones are secreted from nerve endings, tanyocytes seem to play an important indirect role in neuroendocrine control (21, 24, 55) by influencing the degree of accessibility of neurohormones to the bloodstream (58). A BDNF action on tanyocytes could underlie the sprouting and retracting capacities of these cells, given that similar actions are recognized for BDNF in neuronal sprouting phenomena at different levels in the CNS (20, 62). Moreover, because adrenalectomy induces morphological changes on tanyocytes (63), it is possible that plasma glucocorticoid levels increased by stress could affect tanyocytes through BDNF changes. In fact, glucocorticoids regulate BDNF expression in some regions of the CNS (64, 65).

In the present study, significant increases in BDNF mRNA levels were noted as soon as after 15 min of stress in the internal ME layer. This was followed by the accumulation of BDNF peptide at 30 and 60 min of stress, as revealed by immunohistochemistry. These rapid increases were not surprising because we recently reported that BDNF mRNA levels and protein content in the hypothalamus (17, 18) and pituitary (30) are extremely and rapidly sensitive to stress stimuli.

A more accurate study using specific cellular markers revealed that 60 min of stress strongly increased BDNF immunoreactivity within GFAP-labeled cells, indicating that BDNF content increased in astrocytes. In addition, stressed animals exhibited a peak of BDNF release 30–45 min after the onset of stress, measured by push-pull perfusion of the ME. Caution is necessary in interpreting data obtained with push-pull perfusion because this technique cannot accurately discriminate the ME locus in which BDNF is released, i.e., the external or internal layer. Some degree of diffusion in the perfusion system could be responsible for the inaccuracy in determining the exact locus of secretion (66). Whatever the locus intra-ME from which BDNF is released, its action direct or indirectly might influence neurohormone release because its specific receptor, TrkB, is widely located in tanyocytes but also in nerve endings. The profile of BDNF secretion was biphasic, and the second peak could correspond to some additional stress provoked when animals were released. Another possibility, although more speculative, could be that the first peak of release induces the second one because it has been shown that at least in vitro, BDNF is able to induce BDNF release (67). In this eventuality, the second peak of release might serve to reinforce the physiological response. The present results represent the first in vivo evidence of stress-induced neurotrophin release. So far, BDNF release has been measured only in vitro (68), in which it seems to play a role in synaptic plasticity processes (69, 70), a property that in the ME might be a crucial event in morphological changes displayed by the tanyocytes.

In summary, we have demonstrated that both BDNF mRNA and protein are localized in the ME. BDNF was essentially found in astrocytes and weakly in tanyocytes. In addition, we observed a wide TrkB receptor distribution on tanyocytes but also in astrocytes and on nerve endings. Stress affected BDNF accumulation and release from ME and seemed to modify the TrkB distribution in tanyocytes. Taken as a whole, these results suggest a novel role of BDNF in the hypothalamus, likely associated with adaptive changes facilitating neurohormonal release during the stress response. These data provide the first evidence of a BDNF location in the ME, suggesting a novel role of this neurotrophin in neuroendocrine function.

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