Orexin-A Suppresses Postischemic Glucose Intolerance and Neuronal Damage through Hypothalamic Brain-Derived Neurotrophic Factor

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ABSTRACT

Orexin-A (a glucose-sensing neuropeptide in the hypothalamus) and brain-derived neurotrophic factor (BDNF; a member of the neurotrophin family) play roles in many physiologic functions, including regulation of glucose metabolism. We previously showed that the development of postischemic glucose intolerance is one of the triggers of ischemic neuronal damage. The aim of this study was to determine whether there was an interaction between orexin-A and BDNF functions in the hypothalamus after cerebral ischemic stress. Male ddY mice were subjected to 2 hours of middle cerebral artery occlusion (MCAO). Neuronal damage was estimated by histologic and behavioral analyses. Expression of protein levels was analyzed by Western blot. Small interfering RNA directed BDNF, orexin-A, and SB334867 [N-(2-methyl-6-benzoxazolyl)-9-1,5-naphthyridin-4-yl urea; a specific orexin-1 receptor antagonist] were administered directly into the hypothalamus. The level of hypothalamic orexin-A, detected by immunohistochemistry, was decreased on day 1 after MCAO. Intrahypothalamic administration of orexin-A (1 or 5 pmol/mouse) significantly and dose-dependently suppressed the development of postischemic glucose intolerance on day 1 and development of neuronal damage on day 3. The MCAO-induced decrease in insulin receptor levels in the liver and skeletal muscle on day 1 was recovered to control levels by orexin-A, and this effect of orexin-A was reversed by the administration of SB334867 as well as by hypothalamic BDNF knockdown. These results suggest that suppression of postischemic glucose intolerance by orexin-A assists in the prevention of cerebral ischemic neuronal damage. In addition, hypothalamic BDNF may play an important role in this effect of orexin-A.

Introduction

It is well known that diabetes and/or hyperglycemia are one of the risk factors for strokes such as focal cerebral ischemia (Widmer et al., 1992; Dietrich et al., 1993). However, despite a patient’s history of diabetes, a hyperglycemic condition was reported to be induced after ischemic stroke in a clinical study (Matz et al., 2006). That is, postischemic hyperglycemia/glucose intolerance may be induced by cerebral ischemic stress, and normalization of glucose levels within 48 hours of hospitalization appears to confer greater survival outcomes in stroke patients (Matz et al., 2006). Furthermore, systematic reviews report that, in stroke patients, even a moderate elevation of blood glucose levels is associated with mortality and poor functional recovery compared with lower glucose levels (Capes et al., 2001). In contrast, the administration of intravenous insulin with the objective of maintaining serum glucose within a specific range in the first hours of acute ischemic stroke 1) does not provide benefit in terms of functional outcome, death, or improvement in final neurologic deficit and 2) significantly increased the number of hypoglycemic episodes (Bellolio et al., 2011), suggesting the need for ongoing discussion about the importance of postischemic glucose metabolism regulation. We recently reported that focal cerebral ischemia per se causes a hyperglycemic condition (i.e., postischemic glucose intolerance) and that this may worsen the ischemic neuronal damage in mice (Harada et al., 2009a, 2012b).

The orexin family of peptides (orexin-A and orexin-B) is an identified group of neuropeptides that are mainly expressed in the lateral hypothalamic area (LHA), perifornical area, and posterior hypothalamus (Sakurai et al., 1998). They play roles in many physiologic functions including arousal and energy metabolism such as glucose metabolism, feeding behavior, sleep, and wakefulness (de Lecea et al., 1998; Sakurai, 2007). The orexin-A and orexin-B are derived from a single precursor prepro-orexin and act via two types of G-protein-coupled receptors which have a seven-transmembrane topology as

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ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; EIA, enzyme immunoassay; FBG, fasting blood glucose; G6Pase, glucose-6-phosphatase; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; HRP, horseradish peroxidase; InsR, insulin receptor; LHA, lateral hypothalamic area; MCAO, middle cerebral artery occlusion; NDS, neurologic deficit score; NTS, nucleus of the solitary tract; OX1R, orexin-1 receptor; OX2R, orexin-2 receptor; p-InsR, tyrosine-phosphorylated insulin receptor; PEPCK, phosphoenolpyruvate carboxykinase; POMC, pro-opiomelanocortin; rCBF, relative cerebral blood flow; SB334867, N-(2-methyl-6-benzoxazolyl)-9-1,5-naphthyridin-4-yl urea; siRNA, small interfering RNA; TTC, 2,3,5-triphenyltetrazolium chloride; VMH, ventromedial hypothalamus.

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orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R) (Sakurai et al., 1998). OX1R binds orexin-A with high affinity that is almost 50 times greater than that for orexin-B, while OX2R is less selective, binding both orexin-A and orexin-B with high affinities (Sakurai et al., 1998). It was recently reported that the autonomic nervous system, which plays an important role in interorgan metabolic communication such as conveying metabolic information between the hypothalamus and peripheral organs, is apparently essential for maintaining systemic homeostasis, particularly glucose and energy metabolism (Yamada and Katagiri, 2007; Imai et al., 2008). In addition, vagus nerve activity is the unique mechanism of insulin action on liver gluconeogenesis. Previous reports have shown that insulin, used as an antidiabetic drug, affects the arcuate nucleus of the hypothalamus and suppresses hepatic gluconeogenesis through the vagus nerve (Brüning et al., 2000; Pocai et al., 2005). Interestingly, injection of orexin-A into the ventromedial hypothalamus (VMH) of mice or rats directly activated the VMH and enhanced insulin-stimulated glucose uptake in skeletal muscle by activating the sympathetic nervous system (Shiuchi et al., 2009). Furthermore, elevated blood glucose levels in diabetic mice were reduced by intracerebroventricular administration of orexin-A without changing serum insulin levels (Tsuneki et al., 2002).

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has been reported to have a neuroprotective effect against brain injury (Han and Holtzman, 2000). Some reports have suggested that BDNF regulates glucose metabolism by improving insulin sensitivity and increasing pancreatic insulin expression (Nakagawa et al., 2000; Nonomura et al., 2001). In addition, it has been reported that BDNF enhances hepatic insulin signaling in streptozotocin-induced diabetic mice (Tsuchida et al., 2001). Furthermore, we previously reported that the development of posts ischemic glucose intolerance and neuronal damage are suppressed by intracerebroventricular or intrahypothalamic administration of orexin-A and BDNF (Harada et al., 2011, 2012a).

In this study, we determined the effects of intrahypothalamic administration of orexin-A on the development of posts ischemic glucose intolerance, including changes in insulin signaling and ischemic neuronal damage. In addition, we analyzed the interaction between orexin-A and BDNF functions in the hypothalamus after cerebral ischemic stress.

**Materials and Methods**

**Animals.** All experimental procedures were approved by the ethics committee for animals at Kobe Gakuin University (approval number: A 060601-10). The experiments were performed on male ddY mice (5 weeks old, 25–30 g) obtained from SLC (Shizuoka, Japan). The animals were housed at a temperature of 23–24°C with a 12-hour light-dark cycle (lights on 8:00 AM to 8:00 PM). Food and water were available ad libitum. The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Japanese Pharmacological Society.

**Animal Model of Focal Cerebral Ischemia.** The experimental transient focal ischemia mouse model was generated by performing middle cerebral artery occlusion (MCAO), as previously described (Harada et al., 2009b). Briefly, an 8-0 filament with a thin silicon coating (Provil Novo Medium; Heraeus Kulzer, Hanau, Germany) was inserted into the left internal carotid artery to occlude the left middle cerebral artery at its origin through the common carotid artery for 2 hours, followed by reperfusion, while the mice were under isoflurane anesthesia. Sham-operated mice underwent the same surgical procedure without suture insertion. Relative cerebral blood flow (rCBF) was measured by laser Doppler flowmetry (TFB-LN1; Unique Medical, Osaka, Japan) to assess the adequacy of the vascular occlusion and reperfusion, as previously described (Harada et al., 2009b). A laser Doppler probe inserted into the acrylic sheath was positioned over the left skull 2 mm posterior to the bregma and 6 mm to the left side of the midline. Baseline rCBF values measured before the occlusion were defined as 100%. The MCAO was documented by a decrease in rCBF to 40% of control values and rCBF was recovered to about 100% by reperfusion. In addition, physiologic parameters were measured before, during, and 30 minutes after MCAO using a sphygmomanometer (TK-370C; BrainScience Idea, Osaka, Japan) and an i-STAT Portable Clinical Analyzer (300F; FUSO Pharmaceutical Industries, Osaka, Japan) (Harada et al., 2009b). Physiologic parameters were within normal physiologic ranges in all animals at baseline, during MCAO, and during early reperfusion. We eliminated mice with pricking brain on the silicon-coated 8-0 nylon monofilament and unsuccessful infarction on day 1 or 3 after MCAO. The final number of mice is described in each figure legend. In addition, no mice in this study died by MCAO.

**Immunohistochemical and Histologic (H&E Staining) Analyses.** Mice were decapitated on day 1 after MCAO, and brains were dissected immediately. The brains were cut into 2-mm-thick coronal slices. The brain slices were incubated in ice-cold phosphate-buffered 4% paraformaldehyde (pH 7.4) at 4°C. The entire brain was postfix in the same fixative overnight at 4°C. Then, paraffin embedding was performed on the fixed brain slices, and sections were cut using a sliding microtome (thickness, 6 µm). Cut brain sections were dehydrated with xylene and ethanol. Immunohistochemical staining for anti-mouse orexin-A antibody (1:50; R&D Systems, Inc., Minneapolis, MN) or anti-rabbit c-Fos antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) was undertaken with a VECTASTAIN avidin–biotin peroxidase complex using goat anti-rabbit biotinylated secondary antibodies and avidin–biotin peroxidase according to the manufacturer's instructions (VECTASTAIN Elite ABC Kit; Vector Laboratories, Peterborough, UK). H&E staining (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) was performed on the same area previously described.

**Hypothalamic Orexin-A Levels.** Hypothalamic orexin-A levels were assessed using an enzyme immunoassay (EIA). Tissue samples of hypothalamus were collected into Vacutainer tubes containing EDTA and aprotinin (50 µg/ml) and gently rocked several times to inhibit proteinase activity and homogenization. Homogenized tissue was centrifuged at 15,000g for 5 minutes at 4°C and supernatants were collected. Tissue samples were stored at –80°C before EIA, and then warmed to 4°C before analysis. Levels of orexin-A were measured using a commercially available EIA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA). Briefly, standard brain tissue samples or positive control were added at 50 µg/well in a secondary antibody-coated plate. Then, primary antibody and biotinylated peptide were added at 25 µg/well, and incubated at room temperature for 2 hours. Afterward, plates were washed four times with 300 µl/well of 1× assay buffer, streptavidin–horseradish peroxidase (HRP) solution was added 100 µl/well in plate, and incubated at room temperature for 1 hour. After each plate was washed four times with 300 µl/well of 1× assay buffer, tetramethylbenzidine substrate solution was added 100 µl/well in plate, and incubated at room temperature for 1 hour. Finally, to stop the reaction, 2 N HCl was added 100 µl/well in plate. The color in plate was read using Micro Plate Reader (absorbance O.D. at 450 nm; Bio-Rad Laboratories, Hercules, CA).

**Western Blot Analysis.** Western blotting was performed as previously described (Harada et al., 2009b, 2011, 2012a) but with some modifications. Briefly, the hypothalamus, liver, and skeletal muscle were homogenized in homogenization buffer and protein samples (30 µg) were electrophoresed in 7.5% SDS-PAGE acrylamide gels and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Insulin receptor (InsR) and tyrosine-phosphorylated...
InsR (p-InsR) were detected using primary antibodies from Abcam (Tokyo, Japan; 1:1000). OX1R, gluconeogenic regulatory enzymes (phosphoenolpyruvate carboxykinase [PEPCK] and glucose-6-phosphatase [G6Pase]), and BDNF were detected using antibodies from Santa Cruz Biotechnology (1:1000 or 1:500). Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a loading control and was detected using primary antibodies from Chemicon (Temecula, CA; 1:20,000). Blots for InsR and p-InsR were incubated overnight.

Fig. 1. Changes in hypothalamic orexin-A and orexin-1 receptor on day 1 after cerebral ischemic stress. (A–H) Immunohistochemical and histologic analysis. (A–D) Analysis of orexin-A immunostaining on day 1 after MCAO. (E–H) Analysis of neuronal damage using H&E stain on day 1 after MCAO. Scale bars: for panels A, B, E, F = 100 μm; for panels C, D, G, H = 300 μm. (I) the hypothalamic levels of orexin-A were analyzed for EIA (n = 13). (J) Representative Western blots of OX1R and GAPDH levels in hypothalamus and ipsilateral cortex are shown. (K) Relative levels were analyzed by determining the ratio of OX1R/GAPDH on day 1 after MCAO; n = 6. (I, K) Results are presented as mean ± S.E.M. **P < 0.01, *P < 0.05; (I) unpaired Student’s t test, (K) Scheffe’s test.

Fig. 2. Effects of orexin-A treatment on ischemic neuronal damage. Mice were intrahypothalamically administered with orexin-A (1 and 5 pmol/mouse) immediately after MCAO. (A) Intrahypothalamic administration site (arrow). (B) Representative photographs of TTC staining on day 3 after MCAO. (C) Quantitative analysis of the infarct volume. Results are presented as mean ± S.E.M. **P < 0.01, *P < 0.05. Scheffe’s test: SAL, n = 12; OXA 1 pmol, n = 4; OXA 5 pmol, n = 10; OXA 5 pmol + SB334867, n = 7. (D–E) Results of the NDS and the step-through–type passive avoidance learning test on day 3 after MCAO. **P < 0.01; #P < 0.01; ##P < 0.001; #P < 0.05; tP < 0.05. Steel-Dwass test, sham-treated group: SAL, n = 10; OXA 1 pmol, n = 3; OXA 5 pmol, n = 6. MCAO-treated group: SAL, n = 12; OXA 1 pmol, n = 4; OXA 5 pmol, n = 10; OXA 5 pmol + SB334867, n = 7. SAL, saline; OXA, orexin-A.
with the primary antibody at 4°C in Tris-buffered saline containing 0.1% Tween 20 and 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), whereas those for OXIR, PEPCK, and G6Pase were incubated overnight in phosphate-buffered saline containing 0.1% Tween 20 and blocking agent (GE Healthcare, Tokyo, Japan). After washing, the blots were incubated with HRP-conjugated anti-rabbit IgG (1:1,000; KPL, Guildford, UK) for InsR, p-InsR and PEPCK, HRP-conjugated anti-mouse IgG (1:10,000; KPL) for GAPDH, or HRP-conjugated anti-goat IgG (1:1,000; KPL) for OXIR and G6Pase, for 1 hour at room temperature. Immunoreactive bands were visualized using a Light-Capture instrument (AE-6981; ATTO, Tokyo, Japan) with the ECL Western Blotting Analysis System (GE Healthcare). The signal intensity of immunoreactive bands was analyzed using ImageJ software (Version 3.0; ATTO).

Measurement of Infarct Volume. On day 3 after MCAO, mice were killed and the brains were immediately dissected to assess infarct outcome. The brains were cut into 2-mm-thick coronal slices. Brain slices were incubated in saline containing 2% 3,3′,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) for 10 minutes at 37°C. The stained slices were then fixed with 4% paraformaldehyde (Sigma-Aldrich) and the infarct volumes were measured using image analysis software (NIH ImageJ; National Institutes of Health, Bethesda, MD and Adobe Photoshop Elements 5.0, Adobe Systems Incorporated, Tokyo, Japan), as previously described (Harada et al., 2009b; Shichii et al., 2011). The infarct volume was calculated based on infarct area and intensity (intensity = intensity of left hemisphere – intensity of right hemisphere).

Neurologic Examination. Neurologic examinations were performed on day 3 after reperfusion using the neurologic deficit score (NDS), which consists of consciousness (0, normal; 1, restless; 2, lethargic; 3, stuporous; 4, seizures; and 5, death), walking (0, normal; 1, paw; 2, unbalanced walking; 3, circling; 4, unable to stand; and 5, no movement), limb tone (0, normal; 1, spastic; and 2, flaccid), and the pain reflex (Harada et al., 2009b). The pain reflex was assessed using the tail flick test (pain reflex = latency on day 3 after MCAO – latency before MCAO). Difference latency means NDS points. A cutoff time of 10 seconds was used to prevent any injury to the tail.

Learning and Memory Tests. A one-trial step-through–type passive avoidance learning test was performed as previously described (Harada et al., 2009a). Briefly, in the training trial (on day 2 after MCAO), the mice were placed in the illuminated compartment facing away from the dark compartment. When the mice entered the dark compartment, an electric shock (50 V, 3 seconds in duration) was delivered. The mice were then returned to the home cage. In the test trial (on day 3 after MCAO), conducted 24 hours after the training trial, the mice were placed in the illuminated compartment, and the latency time to entering the dark compartment (maximum of 600 seconds) was measured.

Measurement of Fasting Blood Glucose Levels. The mice were fasted for 15 hours for measurement of fasting blood glucose (FBG) levels, and blood samples (~1.5 μl) were obtained from the tail veins. Plasma FBG was measured using the Glucose Pilot (Aventin Biotech, Carlsbad, CA). The increment in FBG was calculated using the following formula: FBG increment = FBG after MCAO – FBG before MCAO (pre-MCAO FBG), as previously described (Harada et al., 2009a). The pre-MCAO FBG were measured at 48–96 hours before MCAO.

Administration in the Hypothalamus (Orexin-A, SB334867, BDNF Small Interfering RNA). Mice were intrahypothalamically administered 1 or 5 pmol/mouse orexin-A (Wako, Osaka, Japan); was dissolved in saline immediately after MCAO. SB334867 [N-(2-methyl-6-benzoxazolyl)-N'-1,5-naphthyridin-4-yl urea], a selective nonpeptide orexin-1 receptor antagonist inhibitor (Tocris Bioscience, St. Louis, MO) was dissolved in 1% dimethyl sulfoxide and intrahypothalamically administered (200 pmol/mouse) 30 minutes before saline or orexin-A administration. All intrahypothalamic administrations were performed as previously reported (Tsao et al., 2008; Harada et al., 2012a). The intrahypothalamic administration volume was 0.2 μl/mouse (orexin-A and SB334867) and 2 μl/mouse (BDNF small interfering RNA [siRNA] and control siRNA). Briefly, mice were anesthetized with pentobarbital (40 mg/kg) and immobilized on a stereotaxic surgery instrument (ST-5M; Narishige Co. Ltd., Tokyo, Japan). A microsyringe with a 30-gauge stainless-steel needle was used for all experiments. The needle was inserted unilaterally into the hypothalamus (1.3 mm posterior to the bregma, 0.5 mm lateral from the midline, and 5.7 mm deep), as previously reported (Tsao et al., 2008; Harada et al., 2012a). Orexin-A (0.2 μl), SB334867 (0.2 μl), and BDNF siRNA (2 μl) were injected into the hypothalamus incrementally over the course of 1 minute. The needle was kept at this position for 1 minute after injection and then raised 1 mm. After another 30 seconds, the needle was slowly removed over a period of 1 minute. The injection site of hypothalamus was confirmed with 0.5% trypan blue in saline (0.2 μl/mouse).

Induction of Hypothalamic-Specific BDNF Knockdown. BDNF ON-TARGETplus SMART pool (BDNF siRNA; Thermo Fisher Scientific, Kanagawa, Japan) and ON-TARGETplus nontargeting Pool (control siRNA; Thermo Fisher Scientific) siRNAs and in vivo jetPEI–BDNF ON-TARGET plus SMART pool (BDNF siRNA; Thermo Fisher Scientific, Kanagawa, Japan) and ON-TARGETplus nontargeting Pool (control siRNA; Thermo Fisher Scientific) siRNAs and in vivo jetPEI were used for all experiments. The needle was inserted unilaterally into the hypothalamus on day 3 after reperfusion using the neurologic deficit score (NDS), which consists of consciousness (0, normal; 1, restless; 2, lethargic; 3, stuporous; 4, seizures; and 5, death), walking (0, normal; 1, paw; 2, unbalanced walking; 3, circling; 4, unable to stand; and 5, no movement), limb tone (0, normal; 1, spastic; and 2, flaccid), and the pain reflex (Harada et al., 2009b). The pain reflex was assessed using the tail flick test (pain reflex = latency on day 3 after MCAO – latency before MCAO). Difference latency means NDS points. A cutoff time of 10 seconds was used to prevent any injury to the tail.

Statistical Analysis. The infarct volume, FBG levels, and results of Western blots were analyzed using one-way analysis of variance followed by Scheffe’s post hoc test. Results of EIA were analyzed using the F test followed by unpaired Student’s t tests. The data are presented as means ± S.E.M. NDS and one-trial step-through–type passive avoidance data were analyzed using the Steel-Dwass test with post hoc nonparametric multiple comparison tests, and the data are presented as medians (25th–75th percentile). Before statistical post hoc test, we have checked normality and equal variance test for all data. P < 0.05 was regarded as significant.

Results

Changes in Hypothalamic Orexin-A on Day 1 after Cerebral Ischemic Stress. Orexin-A–positive cells were observed in the hypothalamus of the sham group (Fig. 1, A and C), but were not detected in the MCAO group (Fig. 1, B and D). Furthermore, by H&E staining, some ischemic neuronal damage was observed in the hypothalamus on day 1 after MCAO (Fig. 1, E–H). In addition, the level of
hypothalamic orexin-A was significantly decreased on day 1 after MCAO by EIA analysis (Fig. 1I). On day 1 after MCAO, the expression levels of OX1R were not affected in hypothalamus, but were significantly increased in cortex (Fig. 1, J and K).

**Effects of Orexin-A on the Development of Ischemic Neuronal Damage on Day 3 after Cerebral Ischemic Stress.** The injection site of orexin-A in the hypothalamus was identified using trypan blue (Fig. 2A). Orexin-A (1 or 5 pmol/mouse) significantly and dose-dependently suppressed the development of infarction, neurologic abnormalities, and memory disturbances on day 3 after MCAO, compared with saline treatment (Fig. 2, B–E). These effects of orexin-A were significantly inhibited by SB334867 (200 pmol/mouse).

**Effects of Orexin-A on the Elevation of FBG on Day 1 after Cerebral Ischemic Stress.** Orexin-A (1 or 5 pmol/mouse) significantly and dose-dependently suppressed the elevation of FBG on day 1 after MCAO, compared with saline treatment. However, the FBG level in orexin-A–treated MCAO mice remained significantly higher than that in the sham group (Fig. 3). These effects of orexin-A were significantly inhibited by SB334867 (200 pmol/mouse).

**Effects of Orexin-A on Hepatic and/or Skeletal Muscle InsR, p-InsR PEPCK, and G6Pase Expression on Day 1 after Cerebral Ischemic Stress.** Hepatic InsR and p-InsR protein expression levels were significantly lower in the MCAO group than in the sham group on day 1 after MCAO.
In the sham group, orexin-A significantly increased hepatic InsR and p-InsR levels compared with saline treatment (Fig. 4, A and B). Orexin-A (5 pmol/mouse) significantly suppressed the decrease in InsR and p-InsR levels in the liver on day 1 after MCAO (Fig. 4).

Hepatic PEPCK and G6Pase levels were significantly higher in the MCAO group than in the sham group (Fig. 4, C and D). In the sham group, orexin-A significantly decreased hepatic PEPCK and G6Pase protein levels compared with saline treatment (Fig. 4, C and D). In addition, orexin-A (5 pmol/mouse) significantly suppressed the increases in hepatic PEPCK and G6Pase protein levels in the MCAO group (Fig. 4).

On the other hand, in skeletal muscle, the InsR and p-InsR levels were significantly lower in the MCAO group than in the sham group (Fig. 4, E and F). In the sham group, orexin-A significantly increased skeletal muscular InsR and p-InsR levels compared with saline treatment (Fig. 4, E and F). Orexin-A (5 pmol/mouse) significantly suppressed the decrease in InsR and p-InsR levels in skeletal muscle on day 1 after MCAO (Fig. 4).

These effects of orexin-A were significantly inhibited by SB334867, an OXR1 inhibitor (200 pmol/mouse) (Fig. 4).

**Intrahypothalamic Administration of Orexin-A Induces Nucleus of the Solitary Tract c-fos Expression/Neuronal Activation in the Medulla Oblongata.** c-Fos-positive cells were clearly observed at 90 min after intrahypothalamic administration of orexin-A in the nucleus of the solitary tract (NST) of the medulla oblongata, but were not seen in the sham group (Fig. 5).

**Effect of Intrahypothalamic BDNF siRNA Administration on BDNF and FBG Levels in Naive Mice.** The injection site of BDNF siRNA in the hypothalamus was
identified using trypan blue (Fig. 2A). BDNF levels in the hypothalamus were significantly decreased in a time-dependent manner, reaching a minimum at 48 hours after intrahypothalamic BDNF siRNA administration, while no effect was observed in the cortex (Fig. 6, A and B). In addition, FBG was not significantly affected by intrahypothalamic BDNF siRNA administration (Fig. 6C).

**Effect of Hypothalamic BDNF Knockdown on BDNF Levels in the Hypothalamus and on the Increase in FBG Level on Day 1 after Cerebral Ischemic Stress.** The expression of hypothalamic BDNF was significantly lower in the MCAO group than in the sham group (Fig. 7A). In the sham group, orexin-A significantly increased the expression of hypothalamic BDNF compared with saline treatment (Fig. 7A). Orexin-A (5 pmol/mouse) significantly suppressed the decrease in BDNF levels in the hypothalamus on day 1 after MCAO (Fig. 7A). The effect of orexin-A was significantly inhibited by hypothalamic BDNF knockdown in the sham and MCAO groups (Fig. 7A). On the other hand, the suppressive effect of intrahypothalamic orexin-A administration on increase of FBG tended to be inhibited by hypothalamic BDNF knockdown (Fig. 7B).

**Effect of Hypothalamic BDNF Knockdown on the Suppressive Effects of Orexin-A on Hepatic and/or Skeletal Muscle InsR, p-InsR PEPCK, and G6Pase Expression on Day 1 after Cerebral Ischemic Stress.** The orexin-A–treated sham group showed significantly increased hepatic and skeletal muscular InsR and p-InsR levels and decreased hepatic PEPCK and G6Pase levels compared with the saline-treated sham group; this effect of orexin-A was significantly reversed by hypothalamic BDNF knockdown (Fig. 8). On the other hand, the MCAO-induced decrease in hepatic and skeletal muscular InsR and p-InsR levels and the MCAO-induced increase in hepatic PEPCK and G6Pase levels on day 1 was recovered to control levels by orexin-A, and this effect of orexin-A tended to be reversed by hypothalamic BDNF knock down (Fig. 8).

**Discussion**

Recently, there has been increased demand not only for chemically synthesized compounds, but also for genetically engineered endogenous peptides for the development of therapeutic agents. Orexin-A, a neuropeptide produced in hypothalamic neurons, has been reported to be an important regulator of glucose metabolism in peripheral organs via the activation of the autonomic nervous system (Shiuchi et al., 2009). Orexin-A is generated from a single precursor prepro-orexin, and its effects are mediated by two types of G-protein–coupled receptors, OX1R and OX2R (Sakurai et al., 1998). It was reported that injection of orexin-A into the VMH enhances insulin-stimulated glucose uptake and glycogen synthesis in skeletal muscle by activating the sympathetic nervous system (Shiuchi et al., 2009). With regard to cerebral ischemia, gene and protein expression of OX1R were reported to be increased in the ischemic hemisphere after focal ischemia in rats (Irving et al., 2002). In addition, the level of orexin-A in cerebrospinal fluid was found to be reduced in subsets of patients with vascular diseases (Ripley et al., 2001). The mechanisms of the neuroprotective effects of orexin-A have been proven to be related with some intracellular signal transduction pathway, such as hypoxia-inducible factor-1α (Yuan et al., 2011). Therefore, it was hypothesized that hypothalamic orexin-A might be involved in the development of postischemic glucose intolerance and neuronal damage. In the present study, we found that hypothalamic orexin-A, the level of which is decreased by cerebral ischemic stress, is involved in the development of postischemic glucose intolerance. Although the complete mechanism underlying this role is still unknown, it is possible that the decrease of hypothalamic orexin-A induced by the neuronal damage in the hypothalamus might be involved in transcription factor changes for orexin-A expression such as the forkhead box transcription factor Foxa2, which binds to the orexin-A promoter and stimulates orexin-A expression (Silva et al., 2009). Indeed, expression of Foxa2 is suppressed by oxidative stress (Ohtsubo et al., 2011), suggesting that the decrease in the level of hypothalamic orexin-A may be due to inhibition of Foxa2 expression by cerebral ischemia. Furthermore, intrahypothalamic administration of orexin-A significantly suppressed the development of neuronal damage on day 3 and postischemic glucose intolerance on day 1 after cerebral ischemic stress. Interestingly, in liver and skeletal muscle,
the ischemic stress-induced decreases in the level of InsR and the ischemic stress-induced increase in the level of gluconeogenic enzymes were returned to the control levels by orexin-A. Furthermore, the effects of orexin-A were reversed by SB334867, a specific OX1R antagonist. These results suggest that orexin-A treatment may lead to an improvement in peripheral insulin sensitivity and to the suppression of postischemic glucose intolerance through OX1Rs.

In the hypothalamus, orexin receptors are highly expressed in the LHA, the periventricular hypothalamic nucleus, the

**Fig. 8.** Effect of intrahypothalamic BDNF siRNA administration on the suppressive effect of postischemic glucose intolerance by hypothalamic orexin-A. BDNF siRNA was intrahypothalamically administered 48 hours before MCAO. Effect of intrahypothalamic BDNF siRNA administration on the changes in hepatic InsR (A), p-InsR (B), and hepatic PEPCK (C), G6Pase (D), and skeletal muscular InsR (E), p-InsR (F) expression on day 1 after MCAO by hypothalamic orexin-A. Representative Western blots of InsR, p-InsR, PEPCK, G6Pase, and GAPDH levels are shown. Relative levels were analyzed by determining the ratio of InsR/GAPDH, p-InsR/GAPDH, PEPCK/GAPDH, and G6Pase/GAPDH on day 1 after MCAO. Results are presented as mean ± S.E.M. **P < 0.01; #P < 0.05; *P < 0.05; †P < 0.05; ‡P < 0.05. Scheffe’s test, n = 8. SAL, saline; OXA, orexin-A.
VMH, and the arcuate nucleus (Trivedi et al., 1998; Marcus et al., 2001). These areas are involved in the origins of sympathetic or parasympathetic (vagus nerve) signals to liver or skeletal muscle (Trivedi et al., 1998; Marcus et al., 2001). That is, the hypothalamus, which is where orexin-A cooperates to produce autonomic outflow to the peripheral tissues, is the most important site for the central regulation of glucose metabolism (Prodi and Obici, 2006; Karnani and Burdakov, 2011; Marino et al., 2011). Orexin-A not only senses peripheral metabolic signals but also controls glucose production and utilization in peripheral tissues through the autonomic nervous system (Karnani and Burdakov, 2011). In the present study, c-Fos levels, a neuron activation maker in the NST, were increased by intrahypothalamic administration of orexin-A in the NST, suggesting that some neuron was activated by orexin-A in hypothalamus. In other reports, orexin-positive fibers have been described as being distributed throughout the brainstem, including neurons in the dorsal motor nucleus of the vagus, the major source of parasympathetic innervation to peripheral tissues including liver, and neurons in the NST (Peyron et al., 1998; Harrison et al., 1999). In addition, orexin-A directly depolarizes neurons in the dorsal motor nucleus of the vagus (Hwang et al., 2001). These results suggest that the effect of orexin-A may be mediated by the activation of parasympathetic nerves (for example, the vagus nerve) through the activation of the nucleus of the solitary tract in the medulla oblongata. It has been reported that the activation of LHA and VMH neurons could regulate insulin receptor substrate activity and glucose production in the liver and skeletal muscle via parasympathetic and sympathetic neurons, respectively (Shimazu and Ogasawara, 1975). The intrahypothalamic administration of orexin-A may stimulate these pathways. These findings also provide significant insight into the therapeutic effectiveness of this endogenous neuropeptide.

BDNF has potent neuroprotective effects against brain injuries such as cerebral ischemic neuronal damage (Han and Holtzman, 2000). In our previous reports, we showed that regulation of postischemic glucose intolerance by intrahypothalamic administration of BDNF suppressed ischemic neuronal damage (Harada et al., 2012a). We have suggested that there are important functional relationships between orexin-A and BDNF in the hypothalamus. In the present study, the suppressive effect of orexin-A was completely inhibited in the sham group, but tended to be inhibited in the MCAO group. It is possible that BDNF knockdown mice retain some expression of BDNF; therefore, the effect of BDNF may not be entirely blocked by BDNF knockdown. However, these results suggest that the effect of orexin-A is mediated at least in part by a BDNF action in hypothalamus. Interestingly, pro-opiomelanocortin (POMC)–positive neurons in the arcuate nucleus of the hypothalamus were previously shown to release α-melanocyte-stimulating hormone (Xu et al., 2003), which binds melanocortin 4 receptors and induces BDNF expression (Xu et al., 2003; Toriya et al., 2010). That is, orexin-A might activate POMC-positive neurons in the arcuate nucleus of the hypothalamus as a first step, and then POMC-positive neurons might induce the expression of BDNF as a second step. In addition, interactions between orexin-positive neurons and BDNF–positive neurons cause parasympathetic nerve activation (Blouet and Schwartz, 2010; Berglund et al., 2012; Liao et al., 2012).

In conclusion, regulation of postischemic glucose intolerance by intrahypothalamic administration of orexin-A significantly suppressed cerebral ischemic neuronal damage. In addition, hypothalamic BDNF may play an important role in the induction of this action of orexin-A. These findings provide some insight into the therapeutic effectiveness of the use of this endogenous neuropeptide.

Authorship Contributions

Participated in research design: Harada, Tokuyama.

Conducted experiments: Harada, Yamazaki, Tokuyama.

Contributed new reagents or analytic tools: Harada, Tokuyama.

Performed data analysis: Harada, Yamazaki, Tokuyama.

Wrote or contributed to the writing of the manuscript: Harada, Tokuyama.

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