Neuroscience 157 (2008) 720–732

I.C.V. ADMINISTRATION OF OREXIN-A INDUCES AN ANTIDEPRESSIVE-LIKE EFFECT THROUGH HIPPOCAMPAL CELL PROLIFERATION

N. ITO,[a] T. YABE,[a,b] Y. GAMO,[a,c] T. NAGAI,[a,b] T. OIKAWA,[a] H. YAMADA[a,b] AND T. HANAWA[a,c]*

[a]*Oriental Medicine Research Center, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan*

[b]*Kitasato Institute for Life Sciences and Graduate School of Infection Control Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan*

[c]*Department of Oriental Medicine, Doctoral Program of Medical Science, Graduate School of Medical Science, Kitasato University, 1-15-1 Kitasato, Sagamihara-City, Kanagawa 228-8555, Japan*

Abstract—A decrease in orexin-A (OX-A) levels has been reported to be associated with depression. It is also well known that stress and depression can disrupt neurogenesis in the dentate gyrus of the hippocampus; however, it is unclear how OX-A is involved in depression and/or neurogenesis. In the present study, we investigated the effect of i.c.v. administration of OX-A on the forced swimming test (FST), an accepted behavioral screen of antidepressant-like activity, and on the cell proliferation with bromodeoxyuridine (BrdU) in the dentate gyrus at 4 days after i.c.v. administration of OX-A. OX-A administration (140 pmol/mouse) led to a significant reduction in animal immobility in the FST, without affecting spontaneous locomotor activities or serum corticosterone levels. In addition, the number of BrdU-positive cells in the dentate gyrus was significantly increased in OX-A-treated mice *in vivo*; however, OX-A did not affect the percentage of doublecortin-positive cells in the dentate gyrus. The proliferation of neural progenitor cells derived from rat fetal brain was not affected by OX-A treatment *in vitro*, and the orexin receptor 1 (OXR1) protein was not expressed in these cells. Treatment with the OXR1 antagonist SB-334867 (30 mg/kg, i.p.) blocked both the OX-A-induced decrease in the immobility of FST and increase in BrdU-positive. Moreover, the OX-A-induced increase in neuropeptide Y (NPY)–positive cells in the hilus of the dentate gyrus was blocked by SB-334867. These results suggest that OX-A induces an antidepressive-like effect, at least in part, via the enhancement of cell proliferation in the dentate gyrus. These effects of OX-A also may be partly relevant to the regulation of the NPY system in the hilus of the dentate gyrus.

Key words: orexin-A, antidepressive-like effect, forced swimming test, cell proliferation, SB-334867, neuropeptide Y.

Orexin-A and -B (OX-A and OX-B, also known as hypocretin-1 and -2) were first identified as novel peptide ligands for two orphan G protein–coupled receptors. These neuropeptides are derived from a common precursor peptide (prepro-orexin) by posttranslational proteolytic cleavage (Sakurai et al., 1998). OX-A has a molecular weight of 3562 Da and is composed of 33 amino acid residues with two sets of intrachain disulfide bonds, whereas OX-B has a molecular weight of 2937 Da and comprises 28 amino acid residues. The amino acid sequence of OX-A is completely conserved between mouse, rat, and human, and that of OX-B is completely conserved between mouse and rat. Two subtypes of orexin receptor, termed orexin receptor-1 (OXR1) and orexin receptor 2 (OXR2), also have been identified in mammals. OXR1 has an affinity for OX-A that is almost 50 times greater than its affinity for OX-B, while OXR2 has comparable affinities for OX-A and OX-B.

Orexin-producing neurons are specifically localized in the lateral hypothalamic area (LHA) and in the posterior hypothalamus (Peyron et al., 1998; Nambu et al., 1999). Except for the cerebellum, these neurons project throughout most of the CNS, including the cerebral cortex, olfactory bulb, hippocampus, amygdala, septum, hypothalamus, midbrain, brain stem, and spinal cord (Peyron et al., 1998; Nambu et al., 1999). Through widespread distribution of orexinergic projections, OX-A efferently modulates the neural activity of several neurotransmitters, such as 5-HT (Brown et al., 2001; Liu et al., 2002), noradrenaline (Horvath et al., 1999), and histamine (Eriksson et al., 2001; Yamanaka et al., 2002). Conversely, orexin-producing neurons are afferently regulated by various neurotransmitters, such as 5-HT, noradrenaline, acetylcholine, GABA, corticotropin-releasing hormone, glutamate, ghrelin, and leptin (Li et al., 2002; Eggermann et al., 2003; Yamanaka et al., 2003a,b; Acuna-Goycolea et al., 2004; Muraki et al., 2004; Winsky-Sommerer et al., 2004). The following findings imply that orexin peptides may play an important role in various physiological functions, including the control of food intake (Sakurai et al., 1998), drinking behavior (Kunii et al., 1999), nociception (Bingham et al., 2001), sleep wakefulness (Piper et al., 2000; Thakkar et al., 2001), neuroendocrine homeostasis (Hagan et al., 1999; Jaszberenyi et al., 2000), and autonomic regulation (Peyron et al., 1998; Date et al., 1999). Moreover, OX-A improves the

*Correspondence to: T. Hanawa, Department of Clinical Research, Oriental Medicine Research Center, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan. Tel: +81-3-3444-6161; fax: +81-3-3448-1438.
E-mail address: hanawa-t@kitasato.or.jp (T. Hanawa).

Abbreviations: ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; BrdU, bromodeoxyuridine; DAB, diaminobenzidine; DCX, doublecortin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FST, forced swimming test; GCL, granule cell layer; HPA, hypothalamic–pituitary–adrenal; LHA, lateral hypothalamic area; NPCs, neural progenitor cells; NPY, neuropeptide Y; OFT, open field test; OX-A, orexin-A; OX-B, orexin-B; OXR1, orexin receptor 1; OXR2, orexin receptor 2; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline containing 0.3% Triton X-100; PVDF, polyvinylidene difluoride; PVN, paraventricular nucleus; RT, room temperature; SGZ, subgranular zone; SSC, saline sodium citrate; TBS, Tris-buffered saline.

0306-4522/08 © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2008.09.042

consolidation of the learning and retrieval processes in rats (Telegdy and Adamik, 2002), and the blockade of OXR1 impairs the acquisition and consolidation of spatial memory in rats (Akbari et al., 2006). In the hippocampus, OX-A and OX-B regulate synaptic plasticity (Selbach et al., 2004). From a neurological point of view, the loss of the orexinergic system is an important factor in narcolepsy and in Parkinson's and Huntington's diseases (Chemelli et al., 1999; Petersen et al., 2005; Thannickal et al., 2007). These findings indicate that the orexinergic system may be associated not only with the maintenance of homeostasis, but also with neuropsychological function and various neurodegenerative diseases.

A decrease in the number and size of OX-A-producing neurons in the LHA is observed in male Wistar-Kyoto rats, which exhibit a hyper-responsiveness of the hypothalamic–pituitary–adrenal (HPA) axis, a characteristic of depression (Allard et al., 2004). Suicidal patients with major depression exhibit significantly lower OX-A levels in the cerebrospinal fluid when compared with patients suffering from adjustment disorder and dysthymia (Brundin et al., 2007a). In addition, low levels of OX-A in the cerebrospinal fluid are related to pronounced symptoms of inertia and lassitude in suicide attempters (Brundin et al., 2007b). Several lines of evidence in these studies thus suggest that dysfunction of orexinergic neurons may be involved in the pathology of depression.

Stimulation of OX-A through OXR1 induces the proliferation of rat adrenocortical cells *in vitro* (Spinazzi et al., 2005). It has been reported that dietary restriction ameliorates depressive symptoms in humans (Demet et al., 1999), and that dietary restriction, during which the expression of OX-A in the LHA is upregulated (Sakurai et al., 1998), enhances neurotrophin expression and neurogenesis in the hippocampus (Lee et al., 2002; Kitamura et al., 2006). In turn, disruption of neurogenesis in the dentate gyrus of the hippocampus plays an important role in the mechanism by which stress facilitates depression (Tanapat et al., 1998; Duman et al., 1999). Chronic antidepressant treatment increases neurogenesis in the hippocampus (Malberg et al., 2000), and hippocampal neurogenesis requires the behavioral recovery effects of antidepressants (Santarelli et al., 2003). Although a plethora of studies have reported that regulation of neurogenesis in the dentate gyrus is associated with both the pathology of depression and the therapeutic action of antidepressants, the involvement of OX-A in depression and/or neurogenesis remains unclear.

To assess the involvement of OX-A in depression, in the present study we investigated the behavioral effect of i.c.v. administration of OX-A by the forced swimming test (FST), a well-accepted behavioral screen for antidepressant-like activity of specific compounds (Porsolt et al., 1977). To evaluate a potential role of OX-A in the neurogenesis process, we also performed *in vitro* and *in vivo* studies to examine the effect of OX-A on cell proliferation. Finally, we assessed the effects of a selective OXR1 antagonist on the OX-A-induced antidepressive-like effect and cell proliferation.

EXPERIMENTAL PROCEDURES

Animals

Adult (7-week-old) male ddY mice (Japan SLC, Hamamatsu, Japan) weighing 35–40 g were used in all experiments (n=231). Mice were housed individually under conditions of constant temperature (23±2 °C) and humidity (55±10%) with food and water available *ad libitum*, unless otherwise specified, and a 12-h light/dark cycle (light at 08:00 h and dark at 20:00 h). All animal experiments were performed according to the Guidelines for Care and Use of Laboratory Animals at the Kitasato Institute and Kitasato University, and conformed to the US National Institutes of Health guidelines on the ethical use of animals. Every effort was made to minimize the number of animals used and their suffering.

Drugs

OX-A (Bachem, King of Prussia, PA, USA) was dissolved in saline. Bromodeoxyuridine (BrdU) (Roche Diagnostics, Indianapolis, IN, USA) was dissolved in saline with 0.007 N NaOH. SB-334867 (Tocris Bioscience, Ellisville, MO, USA), a selective OXR1 antagonist, was dissolved in saline with 10% (w/v) hydroxypropyl-β-cyclodextrin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) containing 1% (v/v) dimethyl sulfoxide (Sigma, St. Louis, MO, USA).

Administration of OX-A, SB-334867, and BrdU

I.c.v. administration of OX-A was performed using a stereotaxic apparatus (Narishige, Tokyo, Japan) under anesthesia with Nembutal (sodium pentobarbital, 40 mg/kg, i.p.) (Dainippon Sumitomo Pharma, Osaka, Japan) (Fig. 1). OX-A solution (14 or 140 pmol/2 μl/mouse, i.c.v.) was administered over a period of 1 min using a 10 μl syringe (Hamilton, Bonaduz, Switzerland) via a stainless steel needle placed into the right lateral ventricle (0.3 mm poste-

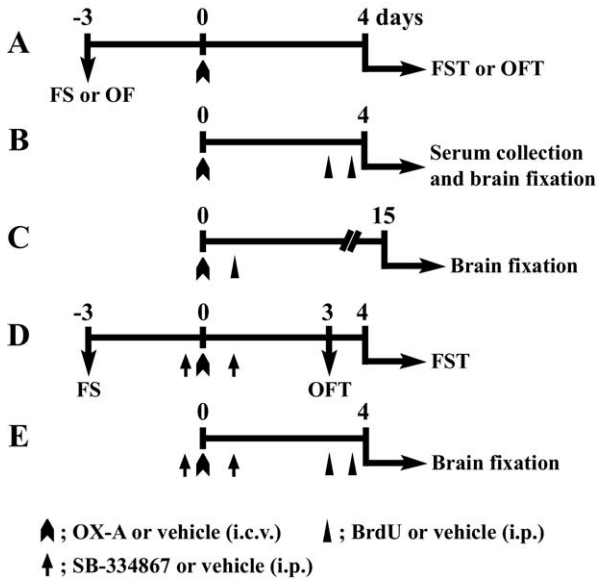

Fig. 1. Schematic representation of experimental schedules for mouse behavioral studies (A, D) and immunohistochemical analyses (B, C, E) after i.c.v. administration of OX-A. (A) Behavioral effects of OX-A on the FST or the OFT. (B) Effect of OX-A on serum corticosterone levels and cell proliferation in the dentate gyrus of the hippocampus. (C) Effect of OX-A on the differentiation of NPCs in the dentate gyrus. (D) Inhibitory effect of SB-334867 on OX-A-induced behavioral activities (FST and OFT). (E) Inhibitory effect of SB-334867 on OX-A-induced cell proliferation and NPY expression. Detailed explanations represent experimental procedures. FS, forced swimming; OF, open field.

rior to bregma, 0.9 mm lateral from midline, and 2.3 mm ventral to the cortical surface) (Suzuki et al., 2005). The choice of dosage of OX-A was based on previous studies addressing physiological responses to this molecule (Jaszberenyi et al., 2000; Thorpe et al., 2005). Control mice were injected with saline (2 μl). The needle was held in position for an additional 3 min after injection to ensure complete diffusion of the drug and prevent backflow. Correct placement of the injection needle was verified by blue ink injection.

Administration of SB-334867 (30 mg/kg, i.p.) was performed 1 h before and 24 h after i.c.v. administration of OX-A (Fig. 1D and E). The dose of SB-334867 was selected based on the previously observed behavioral effects of SB-334867 in rats (Rodgers et al., 2001; Ishii et al., 2004). SB-334867 reaches peak plasma and brain concentrations at 30 min after administration, at which point it significantly suppresses food intake and accelerates behavioral satiety. SB-334867 has a terminal elimination half-life of approximately 4 h; however, the effects disappeared 48 h after administration of SB-334867 (Ishii et al., 2005). Therefore, SB-334867 was administered twice to achieve complete blocking of the binding of OX-A to OXR1.

BrdU (100 mg/kg, i.p.), a thymidine analog that labels dividing cells in the S-phase of the cell cycle (Takahashi et al., 1992), was administered twice: 24 and 3 h prior to brain fixation (Fig. 1B and E). BrdU (200 mg/kg, i.p.) was also administered once after i.c.v. administration of OX-A (Fig. 1C).

FST

The FST was performed, with some modification, following previously described procedures (Porsolt et al., 1977) (Fig. 1A and D). Briefly, 3 days before i.c.v. administration of OX-A, mice were placed individually into 5 l beakers (height 27 cm, diameter 18 cm) filled with 4 l of water (23±1 °C) for 15 min. Animals were then removed from the water and allowed to dry before being returned to their home cages. Individual immobility times during the first 5 min of the first swim were used to assign animals to the test groups so that mean immobility times were similar in all groups. This procedure reduces the initial variability among groups. A mouse was considered to be immobile when it ceased struggling and remained floating motionless, making only those movements necessary to keep its head above water. After a 4-day recovery period post-i.c.v. administration of OX-A, the mice were again placed into the 5 l beakers containing 4 l of water, and the total duration of immobility during the 5 min FST was measured (Fig. 1A and D). This behavioral experiment was carried out between 13:00 h and 16:00 h.

Open field test (OFT)

The spontaneous locomotor activity of mice was measured using OFT. Briefly, 3 days before i.c.v. administration of OX-A, mice were placed individually in the opaque open field box (40×40×40 cm) and were allowed to move freely for 15 min. Individual total distance and duration of movement during the first 5 min using a video tracking system (EthoVision; Noldus, Wageningen, Netherlands) were used to assign animals to the test groups so that mean distance and duration of movement were similar in all groups. This procedure reduces the initial variability among groups. After a 4-day recovery period post-i.c.v. administration of OX-A, the mice were again placed in the open field box, and the total distance and duration of movement were measured during the 5 min of the test (Fig. 1A). For the study of the antagonizing effect of SB-334867, the total distance and duration of movement in the OFT were measured on the third day after i.c.v. administration of OX-A (Fig. 1D). This behavioral experiment was carried out between 13:00 h and 16:00 h.

Measurement of serum corticosterone

Blood samples were collected from the iliofemoral artery under light anesthesia, immediately before perfusion of animals with 4% paraformaldehyde solution (Fig. 1B). Samples were centrifuged at 4 °C and sera were stored at −80 °C, until corticosterone level assays were performed.

Serum corticosterone was measured using the non-radioactive immunoassay AssayMax Corticosterone ELISA Kit (Assaypro, St. Charles, MO, USA), according to the manufacturer's instructions. The sensitivity of the measurement was 26.99 pg/ml. The intra- and inter-assay coefficients of variations were 8% and 13%, respectively.

Brain fixation and tissue storage

Mice were anesthetized with ether and perfused transcardially with cold phosphate-buffered saline (PBS), followed by cold 4% paraformaldehyde solution. Brains were collected and postfixed in 4% paraformaldehyde solution at 4 °C overnight (Fig. 1B, C, and E). Serial coronal sections (50 μm thickness) were obtained throughout the hippocampus (bregma −1.2 mm to −2.5 mm) using a vibratome (Technical Products International Inc., St. Louis, MO, USA), and sections were stored in PBS/NaN_3 at 4 °C until needed for subsequent experiments.

Immunohistochemistry

For OXR1 immunohistochemistry, free-floating sections were incubated in antigen-activated solution (pH 9.0) (Nichirei, Tokyo, Japan) for 3 min at 100 °C, followed by incubation in 3% H_2O_2/80% methanol for 40 min at room temperature (RT) to remove the endogenous peroxidase. After washing in PBS, sections were blocked for 2 h with 1% bovine serum albumin (BSA) in phosphate-buffered saline containing 0.3% Triton X-100 (PBS-T), and then incubated for 10 days at 4 °C with goat anti-OXR1 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were subsequently rinsed in PBS-T, incubated for 2 h at RT with biotinylated donkey anti-goat IgG (1:200; Santa Cruz Biotechnology), and incubated for 2 h at RT with avidin–biotin complex using the ABC kit (Vector Laboratories, Burlingame, CA, USA). OXR1-positive cells were visualized by incubating sections with diaminobenzidine (DAB) solution (Vector DAB, Vector Laboratories) as the chromogen, which results in a brown reaction product. Sections were mounted on silane-coated slides and dried, and then counterstained with 0.05% Toluidine Blue (Sigma), dehydrated, and coverslipped using Permount (Fisher Scientific International Inc., Fair Lawn, NJ, USA).

For BrdU immunohistochemistry, free-floating sections were incubated in 50% formamide/2× saline sodium citrate (SSC) for 2 h at 65 °C, followed by a rinse in 2× SSC. Sections were then incubated in 2 N HCl for 30 min at 37 °C to denature double-stranded DNA, rinsed in 0.1 M borate buffer (pH 8.5). After blocking for 2 h with 1% BSA in PBS-T, sections were incubated overnight at 4 °C with mouse anti-BrdU IgG (1:1000; Chemicon, Temecula, CA, USA). After rinsing in PBS-T, sections were incubated for 2 h at RT with biotinylated horse anti-mouse IgG (1:200; Vector Laboratories), and incubated for 2 h at RT with the ABC kit. BrdU-positive cells were visualized by incubating sections with Vector DAB. As mentioned above, sections were mounted and coverslipped.

Double immunohistochemistry of doublecortin (DCX), a marker of immature neurons, and BrdU, were performed using a two-step staining processes (Rao and Shetty, 2004). Sections were first processed for DCX immunohistochemistry using the Vector SG (Vector Laboratories) as the chromogen, which yields a gray reaction product, before performing BrdU immunohistochemistry using Vector DAB. Briefly, free-floating sections were incubated in 3% H_2O_2/80% methanol for 40 min at RT. After

washing in PBS, sections were blocked for 2 h with 1% BSA in PBS-T and incubated overnight at 4 °C with goat anti-DCX antibody (1:500; Santa Cruz Biotechnology). The sections were then rinsed in PBS-T, incubated for 2 h at RT with biotinylated donkey anti-goat IgG (1:200; Santa Cruz Biotechnology), and incubated for 2 h at RT with the ABC kit. DCX-positive cells were visualized by incubating sections with Vector SG solution. Sections were then incubated in 3% H_2O_2/80% methanol for 40 min at RT to eliminate the peroxidase activity present after the first DCX immunohistochemistry step. Next, BrdU immunohistochemistry was performed according to the method described above. As mentioned above, sections were mounted and coverslipped.

For neuropeptide Y (NPY) immunohistochemistry, free-floating sections were incubated in 3% H_2O_2/80% methanol for 40 min at RT. After washing in PBS, sections were blocked for 2 h with 1% BSA in PBS-T and incubated for 24 h at 4 °C with rabbit anti-NPY antibody (1:4000; GeneTex, San Antonio, TX, USA). Sections were then rinsed in PBS-T, incubated for 2 h at RT with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories), and incubated for 2 h at RT with the ABC kit. NPY-positive cells were visualized by incubating sections with Vector DAB solution. As mentioned above, sections were mounted and coverslipped.

All counts of BrdU-labeled cells, BrdU-labeled cells co-expressing DCX (BrdU/DCX double-positive cells) or NPY-positive cells were performed on every third section throughout the hippocampus (bregma −1.2 mm to −2.5 mm) at 400× and 1000× magnification under a light microscope (Olympus BX-41, Tokyo, Japan) to avoid over-counting the same cells. The number of BrdU-labeled cells in the dentate gyrus, or NPY-positive cells in the hilus or the subgranular zone (SGZ) of the dentate gyrus of each mouse was defined as total cell counts in six or four sections, respectively. The percentage of BrdU/DCX double-positive cells in the dentate gyrus of each mouse was calculated from total cell counts in six sections. All immunohistochemical procedures included negative controls lacking primary antibodies. No staining was detected in the controls.

Preparation of neural progenitor cells (NPCs) and cell proliferation assay

NPCs were isolated from fetal rat brain and prepared using the neurosphere method. Briefly, Wistar rat embryos (Japan SLC) were collected on embryonic day 14 and tissues of the striatum region were dissected from the embryonic brain under a stereoscopic microscope. The tissues were dissociated and suspended in papain dissociation system (Worthington Biochemical Corporation, Lakewood, NJ, USA), according to the manufacturer's instructions. After filtration of the cell suspension through a Cell Strainer (40 μm) (BD Biosciences, Bedford, MA, USA) to remove tissue debris, cells ($\sim 2\times10^5$ cells/ml) were incubated for 7 days under humidified 5% CO_2 conditions at 37 °C in a non-coated 10 cm dish (Iwaki, Tokyo, Japan) with Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 1% (v/v) N-2 Supplement (Invitrogen, Carlsbad, CA, USA), recombinant murine epidermal growth factor (EGF) (20 ng/ml) (PeproTech, London, UK), recombinant human fibroblast growth factor-basic (20 ng/ml) (PeproTech), heparin (2 μg/ml) (IBL, Minneapolis, MN, USA), and 1% (v/v) penicillin/streptomycin (Invitrogen). After 7 days of incubation, neurospheres were collected into a 50 ml tube and resuspended by thorough pipetting. A single cell suspension was pelleted by gentle centrifugation (120×g) for 5 min at 4 °C and resuspended. The NPCs were harvested and transferred into 0.01% poly-L-lysine (Sigma)-coated 96-well plates (1.0×10^4/well). OX-A was added to the cells at a final concentration of 0.1, 1.0, 20, or 100 ng/ml (equivalent to 0.28, 2.8, 5.6, or 28 nM, respectively). EGF (10 ng/ml) was added as a positive control. After incubation for 3 days under humidified 5% CO_2 conditions at 37 °C, supernatant was removed. Cells were then incubated for 2 h under humidified 5% CO_2 conditions at 37 °C in DMEM containing 10% (v/v) Alamar Blue (Biosource, Carnarillo, CA, USA). Cell proliferation was subsequently measured by fluoroscopy (fluoroscan, Labsystems, Chicago, IL, USA) (excitation, 544 nm; emission, 590 nm).

Western blot analysis

NPCs (3.0×10^7 cells) prepared as described above were homogenized in lysis buffer (CelLytic™ MT, Sigma) containing 1% (v/v) protein inhibitor cocktail (Sigma) according to the manufacturer's instructions. The protein content in the lysate was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Total protein lysate (20 μg), and adult mouse brain extract (Santa Cruz Biotechnology) as a positive control were separated by 10% SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membranes using the iBlot™ Gel Transfer System (Invitrogen). The membranes were incubated in blocking buffer containing Tris-buffered saline (TBS) (20 mM Tris–HCl, pH 7.5; 100 mM NaCl), 1% BSA, and 0.1% Tween 20 (Sigma) for 2 h at RT, and incubated with goat anti-OXR1 antibody (1:1000) or mouse anti-β-actin antibody (1:20,000; Sigma) for 1–2 h at RT. After washing with TBS containing 0.1% Tween 20, the membranes were incubated with peroxidase-conjugated anti-goat IgG (1:20,000; Sigma) or peroxidase-conjugated anti-mouse IgG (1:10,000; Cell Signaling Technology, Tokyo, Japan) for 1 h at RT. Immunoreactive bands were visualized by chemiluminescence using the enhanced chemiluminescence detection system (GE Healthcare, Tokyo, Japan).

Statistical analysis

Results are presented as mean±standard error of mean (S.E.M.). For calculations including two groups per experiment, statistical analysis was performed by unpaired t-test. For calculations using three or more groups per experiment, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's test, using the StatView 5.0 software (SAS Institute, Cary, NC, USA). Differences were considered significant at $P<0.05$.

RESULTS

I.c.v. administration of OX-A reduced the duration of immobility in the FST without affecting spontaneous locomotor activities and serum corticosterone levels

The duration of immobility in the FST and the spontaneous locomotor activity of mice in the OFT, which were performed in separate experiments, were measured 4 days after i.c.v. administration of OX-A. In the FST paradigm, OX-A (140 pmol) significantly reduced the immobility (115.5±13.9 s) when compared with saline-injected control mice (175.1±15.5 s) (Fig. 2A; one-way ANOVA, $F=3.721$, $P=0.0356$; post hoc test, $P<0.05$). Furthermore, OX-A (14 and 140 pmol) reduced the duration of immobility in a dose-dependent manner (127.8±18.2 and 115.5±13.9 s, respectively). In contrast, OX-A (14 and 140 pmol) did not affect the total distance (2667.7±148.4 and 2580.4±129.5 cm, respectively) and the duration of movement (261.1±6.8 and 265.2±8.0 s, respectively) in the OFT paradigm, when compared with saline-injected control mice (2475.7±158.7 cm and 255.2±5.9 s, Fig. 2B and C). Serum corticosterone levels were not affected in animals injected with OX-A (14 and 140 pmol resulted in corticosterone levels of 49.7±11.6 and 53.4±8.2 ng/ml, respectively), when compared with saline-injected control mice (42.9±6.9 ng/ml, Fig. 2D).

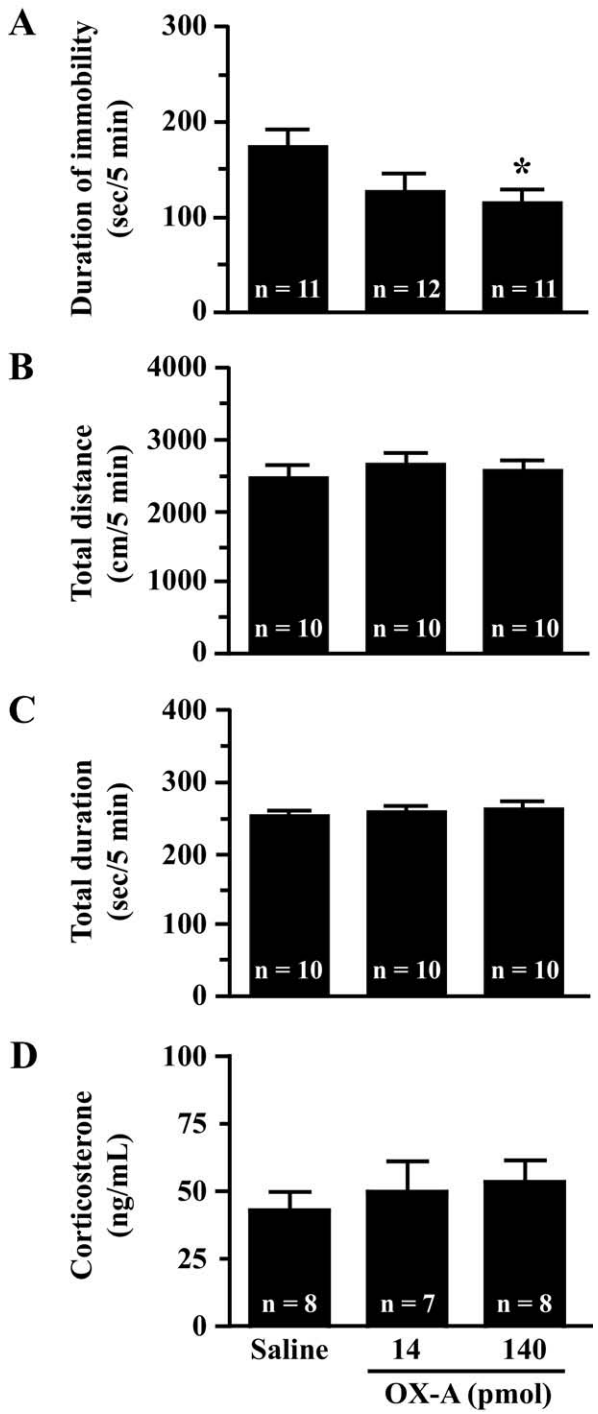

Fig. 2. I.c.v. administration of OX-A reduced the duration of immobility in the FST. (A) The duration of immobility was measured during a 5 min FST conducted 4 days after i.c.v. administration of OX-A (14 and 140 pmol). (B, C) The total distance and duration of movement were measured during a 5 min OFT conducted 4 days after i.c.v. administration of OX-A. (D) Serum corticosterone levels were measured using a corticosterone ELISA kit. Each column represents the mean±S.E.M. The number in each column represents the number of mice per group. * $P<0.05$ vs. saline-injected control mice with Dunnett's test.

OXR1 protein distribution in the hippocampus

Using immunohistochemistry, we then examined the distribution of OXR1 expression in selected regions of the mouse brain, such as the granule cell layer (GCL), SGZ, the hilus, and the CA1-3 area of the hippocampus which are associated with neurogenesis. OXR1 immunoreactivity was found on the surface of neurons in the GCL and CA1-3 areas, as well as in interneuron-like cells in the hilus (Fig. 3A–D). In contrast, OXR1 immunoreactivity was not found in cells of the SGZ (Fig. 3A).

I.c.v. administration of OX-A increased the number of BrdU-positive cells in the dentate gyrus

Using brain slices prepared from mice killed 4 days after i.c.v. administration of OX-A, we assessed the number of BrdU-labeled cells using immunohistochemistry. OX-A (140 pmol) significantly increased the number of BrdU-positive cells (460±21), compared with saline-injected control mice (318±16) (Fig. 4A; one-way ANOVA, $F=7.388$, $P=0.0035$; post hoc test, $P<0.05$). In addition, OX-A (14 and 140 pmol) tended to increase the number of BrdU-positive cells in the dentate gyrus in a dose-dependent manner (380±39 and 460±21, respectively).

I.c.v. administration of OX-A did not affect the differentiation of newborn cells

We subsequently investigated whether OX-A controlled the differentiation of newborn cells in the hippocampus. We used double immunohistochemistry of BrdU and DCX to quantify the number of DCX-positive cells among the BrdU-positive cells in the SGZ of the dentate gyrus. BrdU expression was visualized as a brown color in the nucleus and DCX expression was detected as a blue color in the soma and dendrites (Fig. 4B). Among the newborn cells of the dentate gyrus of the saline- or OX-A-injected mice, 61.8 or 62.1%, respectively, differentiated into immature neurons 14 days after BrdU injection.

OX-A treatment did not affect the cell proliferation *in vitro*

To investigate whether OX-A is capable of regulating cell proliferation *in vitro*, we treated NPCs derived from fetal rat brain with EGF (10 ng/ml), used as a positive control or with OX-A (0.1, 1.0, 20, or 100 ng/ml). The former significantly increased cell proliferation (one-way ANOVA, $F=28.890$, $P<0.0001$; post hoc test, $P<0.05$), whereas OX-A treatment did not affect this parameter, when compared with the control (Fig. 4C).

OXR1 is not expressed in proliferating NPCs

We next examined whether OXR1 protein was expressed in the NPCs, using Western blot analysis. OXR1 protein was detected in the adult mouse brain extract, used as a positive control, but not in the NPCs derived from fetal rat brain (Fig. 4D).

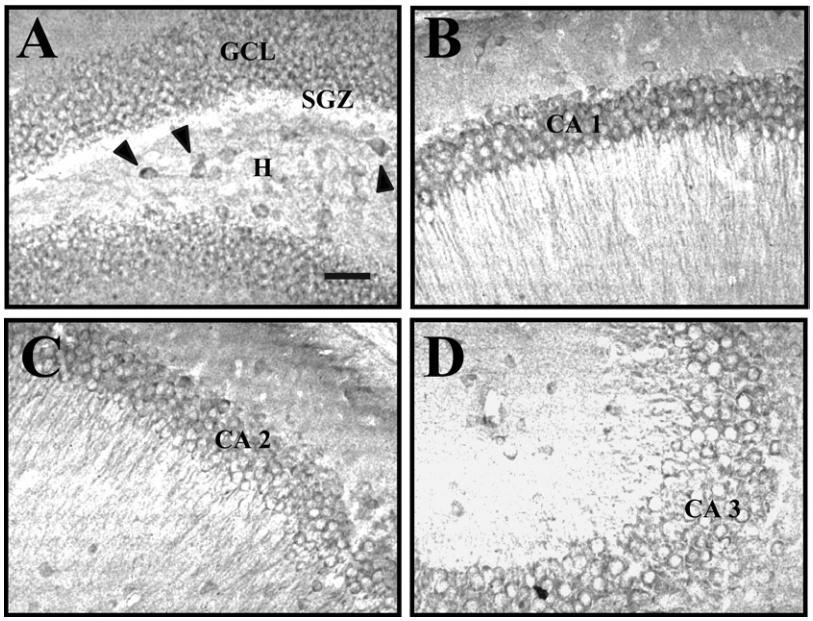

Fig. 3. Distribution of OXR1 protein expression in the mouse hippocampus. Photomicrographs represent OXR1-positive cells in the dentate gyrus (A) and the CA1-3 regions (B–D). Note that OXR1-positive signal was seen on the surface of neurons in the GCL, CA1-3 regions, and of interneurons (arrowhead) in the H, but not in the SGZ. H, hilus. Scale bar=50 μm (in all four photomicrographs).

Treatment with a selective OXR1 antagonist inhibited the OX-A-induced antidepressive-like effect

To establish the role of OXR1 in the OX-A-induced antidepressive-like effect described above, we treated mice with a selective OXR1 antagonist, SB-334867. The antagonist alone did not affect the duration of immobility in the FST (199±12.1 s), when compared with saline-injected control mice (212.5±12.8 s). As mentioned above, OX-A (140 pmol) significantly reduced the duration of immobility in the FST (121.4±13.3 s), when compared with saline-treated control mice (Fig. 5A; one-way ANOVA, $F=8.092$, $P<0.0001$; post hoc test, $P<0.01$), but treatment with SB-334867 potently inhibited the OX-A-induced decrease in immobility (193.8±18.4 s) (one-way ANOVA, $F=8.092$, $P<0.0001$; post hoc test, $P<0.01$). In the OFT paradigm, the total distance (2433.6±192.1 cm) and the duration of movement (264.7±5.0 s) were not affected in animals treated with SB-334867 alone, when compared with saline-pretreated control mice (2016.7±147.5 cm and 231.6±13.8 s, respectively, Fig. 5B and C). In addition, treatment of SB-334867 in conjunction with OX-A did not affect the total distance (2212.1±99.9 cm) or the duration of movement (247.2±6.2 s), when compared with OX-A-injected mice (2485.3±167.1 cm and 256.7±8.2 s, respectively).

Treatment with SB-334867 prevented the OX-A-induced increase in BrdU-positive cells in the dentate gyrus

We next investigated whether the OX-A-induced increase in BrdU-positive cells was prevented by treatment with SB-334867. SB-334867 alone did not affect the number of BrdU-positive cells (302±16), when compared with saline-pretreated control mice (289±26, Fig. 6). In contrast, treatment with SB-334867 significantly inhibited the OX-A-induced increase in BrdU-positive cells (362±17), when compared with animals treated with OX-A alone (470±33) (one-way ANOVA, $F=11.929$, $P<0.0001$; post hoc test, $P<0.05$).

Treatment with SB-334867 prevented the OX-A-induced increase in NPY-positive cells in the dentate gyrus

To determine the regulatory effect of OX-A on the expression of NPY in the dentate gyrus, we measured the number of NPY-positive cells in the dentate gyrus. Most NPY-positive cells were found in the hilus and SGZ of the dentate gyrus (Fig. 7A). In the hilus of the dentate gyrus, OX-A (140 pmol) significantly increased the number of NPY-positive cells (156±12), when compared with saline-injected control mice (103±6) (Fig. 7A and B; one-way ANOVA, $F=8.722$, $P=0.0008$; post hoc test, $P<0.01$). Furthermore, treatment with SB-334867 significantly inhibited the OX-A-induced increase in NPY-positive cells (106±3) (one-way ANOVA, $F=8.722$, $P=0.0008$; post hoc test, $P<0.01$), whereas injection of SB-334867 alone did not affect the number of NPY-positive cells (104±12), when compared with saline-injected control mice. In contrast, in the SGZ region neither OX-A nor SB-334867 treatment affected the number of NPY-positive cells (143±10 and 129±12, respectively), when compared with saline-injected control mice (110±5, Fig. 7C). In the paraventricular nucleus (PVN) of the hypothalamus, the distribution of axons of NPY-positive neurons was not affected in either OX-A or SB-334867-treated mice, when compared with saline-injected control mice (Fig. 7D).

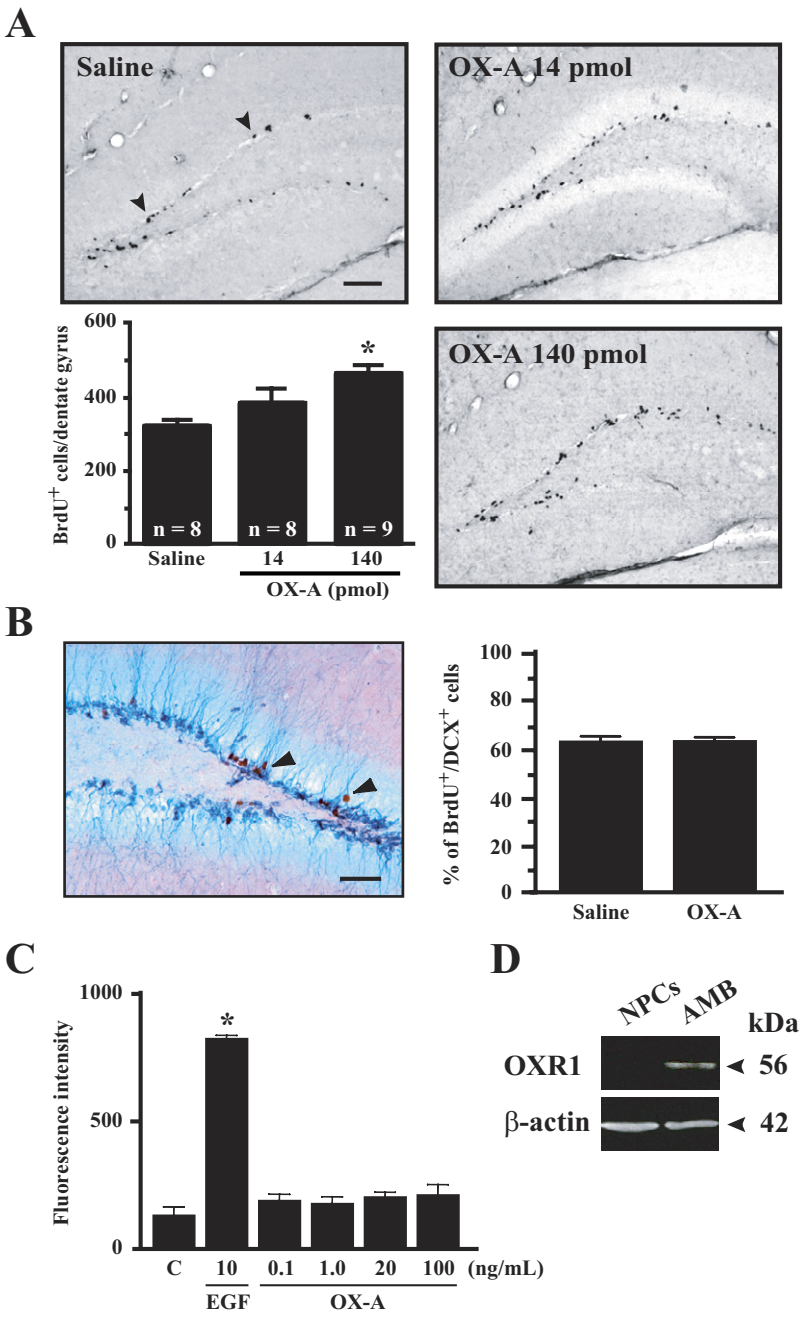

Fig. 4. I.c.v. administration of OX-A increased the number of BrdU-positive cells in the dentate gyrus, but did not affect the maturation of NPCs. OX-A treatment did not affect the proliferation of NPCs *in vitro*. (A) BrdU immunohistochemistry was performed on brain slices prepared from animals killed at 4 days after i.c.v. administration of OX-A (14 and 140 pmol). Photomicrographs of dentate gyrus with representative BrdU-positive cells indicated by arrowheads. The number in each column represents the number of mice per group. (B) BrdU/DCX double immunohistochemistry was performed at 15 days after i.c.v. administration of OX-A. Photomicrograph represents the BrdU- (brown) and/or DCX- (blue) positive cells in the dentate gyrus. Arrowheads represent BrdU/DCX double-positive cells. Each column represents the percentage of BrdU/DCX-positive cells in the dentate gyrus. (C) The proliferation of fetal rat brain-derived NPCs (1×10^5/ml) induced by treatment with OX-A (0.1–100 ng/ml) or EGF (10 ng/ml) was assayed *in vitro* by chemifluorescence method. C, Control. (D) Western blot analysis for OXR1. NPCs or adult mouse brain (AMB) extract used as a positive control was separated by SDS-PAGE. Proteins were transferred onto a PVDF membrane and immunoreacted with antibodies against OXR1 or β-actin. Each column represents the mean±S.E.M. * $P<0.05$ vs. saline-injected or control with Dunnett's test. Scale bar=100 μm (A); 50 μm (B).

DISCUSSION

The goal of the present study was to clarify the association of OX-A with depression and neurogenesis. Our results demonstrate that i.c.v. administration of OX-A induces an antidepressive-like activity in a behavioral screen/test of antidepressant-like activity of a compound and leads to an increase in cell proliferation in the dentate gyrus, mediated by OXR1. Furthermore, the OX-A-induced antidepressive-

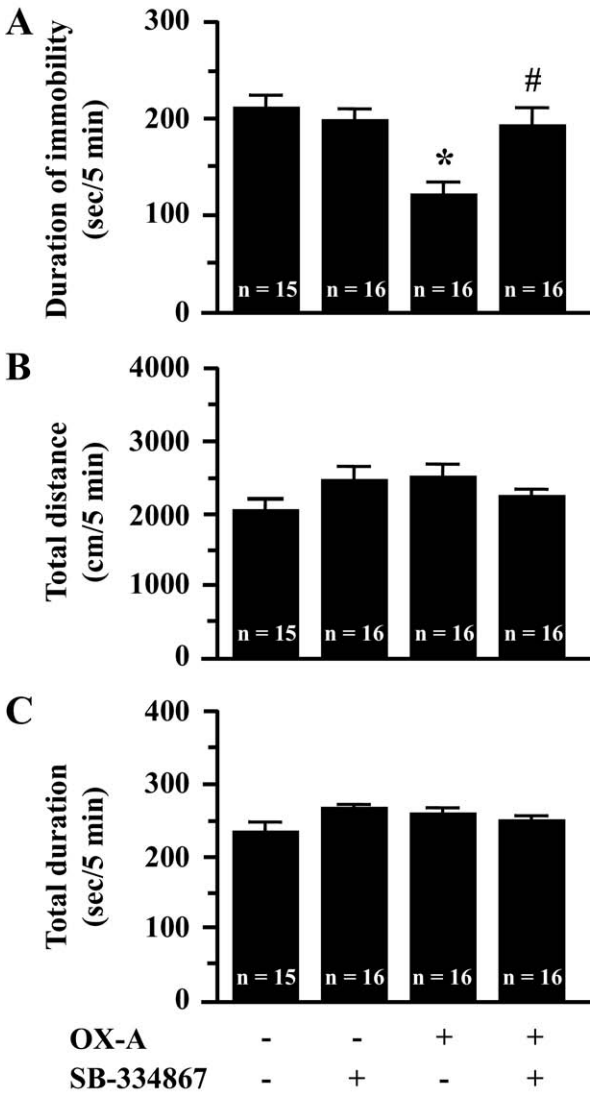

Fig. 5. Treatment with SB-334867 blocked OX-A-induced reduction of immobility in the FST. SB-334867 (30 mg/kg, i.p.) was administered at 1 h before and 24 h after i.c.v. administration of OX-A (140 pmol). (A) The duration of immobility was measured during a 5 min FST at 4 days after i.c.v. administration of OX-A. (B, C) The total distance and duration of movement were measured during a 5 min OFT at 4 days after i.c.v. administration of OX-A. Each column represents the mean±S.E.M. The number in each column represents the number of mice per group. * $P<0.01$ and # $P<0.01$ vs. saline-injected controls and OX-A-injected mice, respectively, with Tukey's test.

like activity and the increase in cell proliferation in the dentate gyrus observed here may be mediated by regulation of the expression of NPY.

In the present study, i.c.v. administration of OX-A reduced the duration of immobility in the FST without affecting spontaneous locomotor activities, resulting in an antidepressive-like activity at 4 days after i.c.v. administration of OX-A. Moreover, the antidepressive-like activity was abolished by treatment with SB-334867, an OXR1 antagonist. Therefore, these results indicate that OX-A may, at least in part, contribute to the amelioration of depression-like behaviors in mice, via OXR1.

The Wistar-Kyoto rat model, which is representative of depression (Dugovic et al., 2000), has been reported to display a decrease in the number and size of OX-A-positive cells in the LHA (Allard et al., 2004). Accordingly, deprivation of rapid eye-movement sleep, which ameliorates depressive symptoms in human (Demet et al., 1999), results in an increase in the number and/or size of OX-A-positive cells in Wistar-Kyoto rats (Allard et al., 2007). Stress-induced depression-like model mice (Ito et al., 2006) also exhibited a decrease in the number of OX-A-positive cells in the LHA (data not shown). Clinical studies showed that a decrease in OX-A levels in the cerebrospinal fluid in humans correlates with depression and its symptoms (Brundin et al., 2007a); however, the possibility that the reduced levels of OX-A may be a consequence of depression has not been fully addressed. Among studies supporting a relationship between OX-A and depression, our results in the present study seem to provide evidence that OX-A may be a neuropeptide that induces antidepressive-like effects in mice. Further studies on the influence of endogenous OX-A regulation on this antidepressive-like activity are needed to further clarify the efficacy of the antidepressive effect of OX-A.

We found OXR1 protein to be expressed in the GCL, hilus, and CA1-3 regions of the hippocampus, which is consistent with a previous report by Hervieu et al. (2001). It is well known that the hippocampus is a major neurogenesis-associated region of the brain. Hence, we investigated the possible involvement of OX-A in neurogenesis. When mice were administered OX-A by i.c.v. injection, the number of BrdU-positive cells was increased in the dentate gyrus of the hippocampus, and this increase was significantly blocked by treatment with SB-334867. These results suggest that OX-A induces an increase in cell proliferation through its interaction with OXR1. Interestingly, it is noteworthy that the increase in cell proliferation was concom-

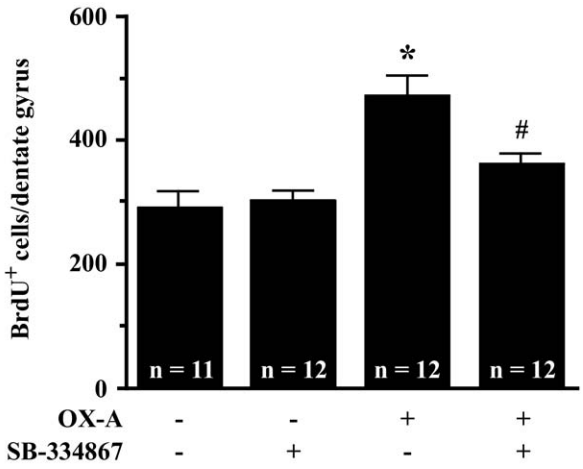

Fig. 6. Treatment with SB-334867 blocked OX-A-induced increase in BrdU-positive cells in the dentate gyrus. BrdU immunohistochemistry was performed at 4 days after i.c.v. administration of OX-A (140 pmol). Each column represents the mean±S.E.M. The number in each column represents the number of mice per group. * $P<0.01$ and # $P<0.05$ vs. saline-injected control mice and OX-A-injected mice, respectively, with Tukey's test.

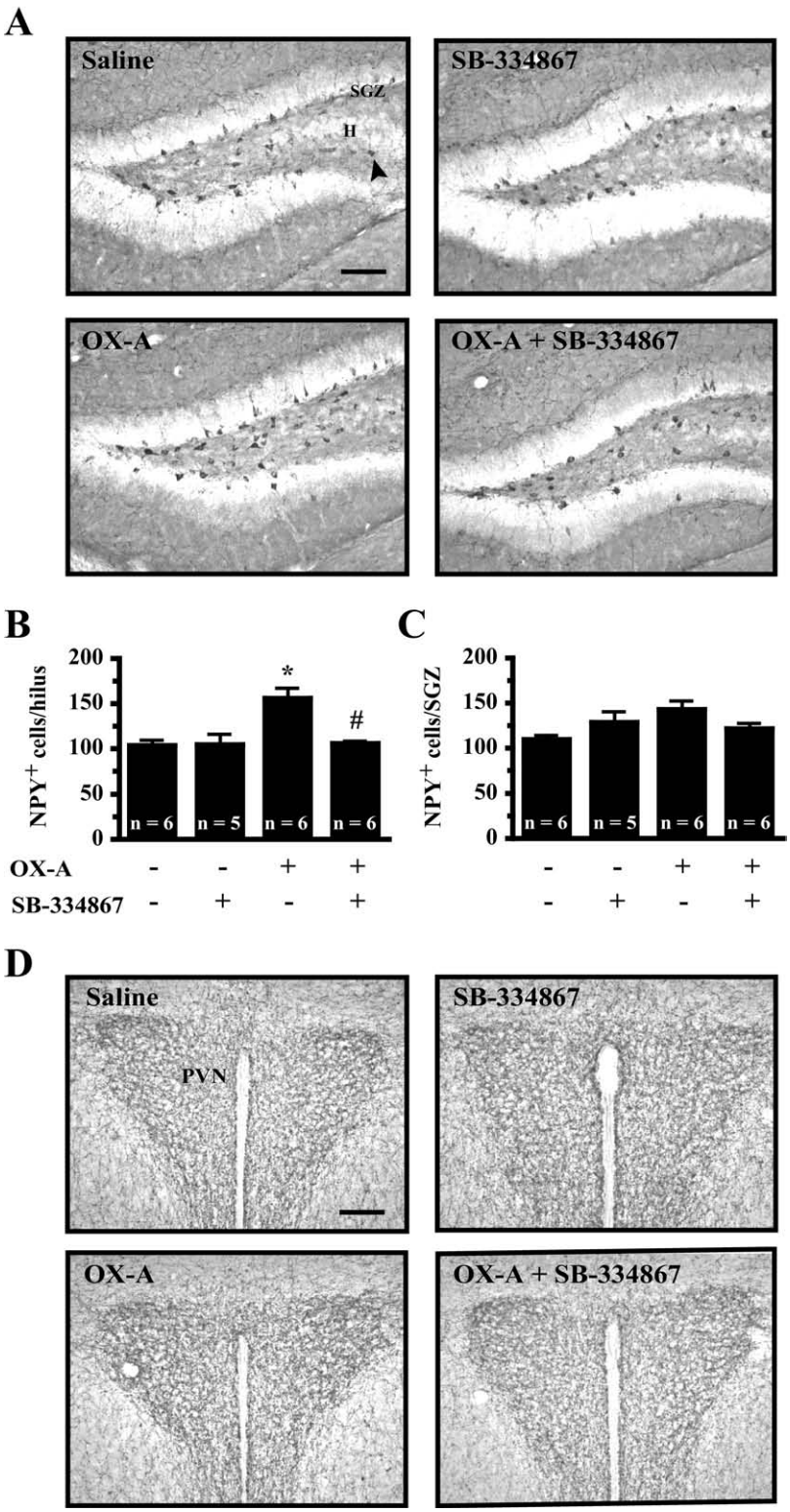

Fig. 7. I.c.v. administration of OX-A increased the number of NPY-positive cells in the H of the dentate gyrus, and treatment with SB-334867 blocked this OX-A-induced increase in NPY-positive cells. (A) NPY immunohistochemistry was performed on brain slices prepared from animals killed at 4 days after i.c.v. administration of OX-A (140 pmol). Photomicrographs represent the NPY-positive cells (arrowhead) in the dentate gyrus. The number of NPY-positive cells in the H (B) and SGZ (C) of the dentate gyrus was counted. (D) Photomicrographs represent the axons of NPY-positive cells in the PVN. Each column represents the mean±S.E.M. The number in each column represents the number of mice per group. * $P<0.01$ and # $P<0.01$ vs. saline-injected control mice and OX-A-injected mice, respectively, with Tukey's test. Scale bar=100 μm (in all eight photomicrographs). H, hilus.

itant with OX-A-induced antidepressive-like activity in behavioral studies, and that both effects were abolished by blockage of OXR1. The relationship between the antidepressive-like activity and the cell proliferation associated with OX-A may be further supported by a previous report demonstrating that the duration of immobility in the FST and the number of BrdU-positive cells in mice were negatively correlated (Llorens-Martin et al., 2007). Therefore, the OX-A-induced antidepressive-like activity may be associated with the regulation of cell proliferation in the hippocampus.

To our knowledge, there are no reports describing the induction of depressive-like behavior after i.c.v. administration of OX-A; however, Suzuki et al. (2005) reported that i.c.v. administration of OX-A was possibly involved in the onset of anxiety-like behavior. In this report, anxiety-like behavior was evaluated at 15 min after i.c.v. administration of OX-A in mice. Levels of corticosterone and adrenocorticotropic hormone (ACTH) in sera are increased immediately after i.c.v. administration of OX-A and are maintained for a few hours (Kuru et al., 2000; Jaszberenyi et al., 2001; Russell et al., 2001; Brunton and Russell, 2003). This suggests that OX-A temporarily activates the HPA axis. Taylor and Samson (2003) also reported that increase in corticosterone and ACTH levels by i.c.v. administration of OX-A may represent a temporary stress response. Therefore, these findings imply that the OX-A-induced anxiety-like behavior and concomitant increase in corticosterone and ACTH levels might be caused by an acute compensatory biological response to stress. In the present study, we performed behavioral evaluations at 4 days after i.c.v. administration of OX-A in mice. The rationale for this approach was to rule out possible effects of the temporal compensatory response against surgical stresses and OX-A. Several studies have underscored the necessity for an extended recovery period (Shirayama et al., 2002; Taylor and Samson, 2003). In the present study, the reduction of immobile behavior in the FST at 4 days after i.c.v. administration of OX-A implies that OX-A has an antidepressive-like effect that is independent of the stress inherent to the technical procedure.

It is well known that cell proliferation in the dentate gyrus is suppressed by corticosterone (Cameron and Gould, 1994; Tanapat et al., 1998). This suggested that i.c.v. administration of OX-A would lead to the suppression of cell proliferation in the dentate gyrus accompanied by the elevation of corticosterone levels; however, in our study the levels of corticosterone in the serum were not affected, and the cell proliferation was not suppressed but rather enhanced by OX-A treatment. A possible explanation for this observation may be that the time point at investigation of the effect of OX-A on cell proliferation was at 4 days after i.c.v. administration of OX-A. It is also possible that OX-A can induce other neuropeptides, affecting cell proliferation in opposite ways than corticosterone, but it remains unclear whether OX-A induces the neuropeptides that affect cell proliferation. Further studies will be needed to address the issue.

OX-A treatment did not promote the proliferation of NPCs *in vitro*, suggesting that OX-A has no direct effect on the proliferation of NPCs. In our immunohistochemical studies, the OXR1 protein was not apparently expressed in the SGZ of the dentate gyrus, in which newborn cells such as NPCs are generated. Moreover, OXR1 was not expressed in fetal brain-derived NPCs, even though the protein was expressed in the adult brain. These results raise the possibility that the absence of OXR1 expression in NPCs may lead to a failure of OX-A to directly induce the proliferation of NPCs; however, the fact that expression of OXR1 is found in certain neurogenesis-associated brain regions, such as the hippocampus, may implicate OXR1 signaling in the regulation of neurogenesis. Further studies are needed to clarify the interaction between OXR1 signaling and neurogenesis.

With respect to the maturation of NPCs in the dentate gyrus, the differentiation of NPCs into immature neurons in OX-A-injected mice described here was induced by about 60%, which was similar that seen in saline-injected mice at 14 days after BrdU injection. Under physiological conditions, NPCs differentiate into immature neurons at a rate of about 60% after 14 days (Brown et al., 2003), which is consistent with the results presented here. Consequently, these results suggest that OX-A may induce cell proliferation without affecting the differentiation of NPCs in the dentate gyrus.

NPY as well as OX-A has been implicated in the stimulation of feeding behavior (Clark et al., 1985). NPY-expressing neurons are distributed not only in the arcuate nucleus, but also in the hippocampus (in interneurons of the hilus and CA1-3 regions) (Kharlamov et al., 2007). Several studies have also shown that NPY plays a role in the mode of action of antidepressants (Heilig et al., 1988) and exhibits an antidepressive-like activity itself (Redrobe et al., 2002). More interestingly, NPY stimulates the proliferation of neuronal precursor cells in the dentate gyrus (Howell et al., 2005, 2007). In the present study, the OX-A-induced increase in NPY-positive cells in the hilus was blocked by SB-334867 treatment, but not in the SGZ. These results imply that OX-A may be associated with the regulation of the NPY system in the hilus through OXR1 signaling. This may be supported by the finding that NPY neurons are directly activated by OX-A via OXR1 (Muroya et al., 2004). However, it is still unclear whether the regulation of the NPY system is one possible mechanism underlying the antidepressive-like effect and the regulation of cell proliferation induced by OX-A. Further studies using an antagonist of NPY receptors might help to elucidate whether the mediation of the NPY system plays a critical role in the effects of OX-A. NPY-positive neurons also project from the arcuate nucleus to the PVN, and this projection seems to play a role in the activation of the HPA axis (Suda et al., 1993). In the present study, OX-A injection did not affect the distribution of axons of NPY-expressing neurons in the PVN. These findings and the analysis of corticosterone levels in sera imply that the OX-A-induced increase in NPY-positive cells in the hilus may not be

related to the activation of the HPA axis. In addition, the difference in distribution of NPY between the dentate gyrus and the PVN may suggest region-specific actions of NPY.

CONCLUSION

In conclusion, this study provides the first evidence that OX-A is a neuropeptide that exhibits antidepressive-like activity through the regulation of cell proliferation in the dentate gyrus. However, it remains unclear whether an extensive action of OX-A throughout the brain is associated with the antidepressive-like effect. Further studies on the effects of repeated administration of OX-A or overexpression of OX-A using transgenic approaches may clarify the association of the orexinergic system with depression. Finally, additional studies will be needed to identify the molecular and cellular adaptations underlying the indirect action of OX-A to further elucidate the mechanism of OX-A-induced antidepressive-like activity.

Acknowledgments—We would like to thank Ms. Y. Kobayashi and Ms. I. Harada for their technical assistance. This work was supported by a Grant-in-Aid for Young Scientists (B) of KAKENHI (19790463).

REFERENCES

Acuna-Goycolea C, Li Y, Van Den Pol AN (2004) Group III metabotropic glutamate receptors maintain tonic inhibition of excitatory synaptic input to hypocretin/orexin neurons. J Neurosci 24: 3013–3022.

Akbari E, Naghdi N, Motamedi F (2006) Functional inactivation of orexin 1 receptors in CA1 region impairs acquisition, consolidation and retrieval in Morris water maze task. Behav Brain Res 173: 47–52.

Allard JS, Tizabi Y, Shaffery JP, Manaye K (2007) Effects of rapid eye movement sleep deprivation on hypocretin neurons in the hypothalamus of a rat model of depression. Neuropeptides 41: 329–337.

Allard JS, Tizabi Y, Shaffery JP, Trouth CO, Manaye K (2004) Stereological analysis of the hypothalamic hypocretin/orexin neurons in an animal model of depression. Neuropeptides 38:311–315.

Bingham S, Davey PT, Babbs AJ, Irving EA, Sammons MJ, Wyles M, Jeffrey P, Cutler L, Riba I, Johns A, Porter RA, Upton N, Hunter AJ, Parsons AA (2001) Orexin-A, an hypothalamic peptide with analgesic properties. Pain 92:81–90.

Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG (2003) Transient expression of doublecortin during adult neurogenesis. J Comp Neurol 467:1–10.

Brown RE, Sergeeva O, Eriksson KS, Haas HL (2001) Orexin A excites serotonergic neurons in the dorsal raphe nucleus of the rat. Neuropharmacology 40:457–459.

Brundin L, Bjorkqvist M, Petersen A, Traskman-Bendz L (2007a) Reduced orexin levels in the cerebrospinal fluid of suicidal patients with major depressive disorder. Eur Neuropsychopharmacol 17:573–579.

Brundin L, Petersen A, Bjorkqvist M, Traskman-Bendz L (2007b) Orexin and psychiatric symptoms in suicide attempters. J Affect Disord 100:259–263.

Brunton PJ, Russell JA (2003) Hypothalamic-pituitary-adrenal responses to centrally administered orexin-A are suppressed in pregnant rats. J Neuroendocrinol 15:633–637.

Cameron HA, Gould E (1994) Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. Neuroscience 61:203–209.

Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98:437–451.

Clark JT, Kalra PS, Kalra SP (1985) Neuropeptide Y stimulates feeding but inhibits sexual behavior in rats. Endocrinology 117:2435–2442.

Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M, Nakazato M (1999) Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc Natl Acad Sci U S A 96:748–753.

Demet EM, Chicz-Demet A, Fallon JH, Sokolski KN (1999) Sleep deprivation therapy in depressive illness and Parkinson's disease. Prog Neuropsychopharmacol Biol Psychiatry 23:753–784.

Dugovic C, Solberg LC, Redei E, Van Reeth O, Turek FW (2000) Sleep in the Wistar-Kyoto rat, a putative genetic animal model for depression. Neuroreport 11:627–631.

Duman RS, Malberg J, Thome J (1999) Neural plasticity to stress and antidepressant treatment. Biol Psychiatry 46:1181–1191.

Eggermann E, Bayer L, Serafin M, Saint-Mleux B, Bernheim L, Machard D, Jones BE, Muhlethaler M (2003) The wake-promoting hypocretin-orexin neurons are in an intrinsic state of membrane depolarization. J Neurosci 23:1557–1562.

Eriksson KS, Sergeeva O, Brown RE, Haas HL (2001) Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus. J Neurosci 21:9273–9279.

Hagan JJ, Leslie RA, Patel S, Evans ML, Wattam TA, Holmes S, Benham CD, Taylor SG, Routledge C, Hemmati P, Munton RP, Ashmeade TE, Shah AS, Hatcher JP, Hatcher PD, Jones DN, Smith MI, Piper DC, Hunter AJ, Porter RA, Upton N (1999) Orexin A activates locus coeruleus cell firing and increases arousal in the rat. Proc Natl Acad Sci U S A 96:10911–10916.

Heilig M, Wahlestedt C, Ekman R, Widerlov E (1988) Antidepressant drugs increase the concentration of neuropeptide Y (NPY)-like immunoreactivity in the rat brain. Eur J Pharmacol 147:465–467.

Hervieu GJ, Cluderay JE, Harrison DC, Roberts JC, Leslie RA (2001) Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. Neuroscience 103:777–797.

Horvath TL, Peyron C, Diano S, Ivanov A, Aston-Jones G, Kilduff TS, van Den Pol AN (1999) Hypocretin (orexin) activation and synaptic innervation of the locus coeruleus noradrenergic system. J Comp Neurol 415:145–159.

Howell OW, Doyle K, Goodman JH, Scharfman HE, Herzog H, Pringle A, Beck-Sickinger AG, Gray WP (2005) Neuropeptide Y stimulates neuronal precursor proliferation in the post-natal and adult dentate gyrus. J Neurochem 93:560–570.

Howell OW, Silva S, Scharfman HE, Sosunov AA, Zaben M, Shatya A, McKhann G 2nd, Herzog H, Laskowski A, Gray WP (2007) Neuropeptide Y is important for basal and seizure-induced precursor cell proliferation in the hippocampus. Neurobiol Dis 26:174–188.

Ishii Y, Blundell JE, Halford JC, Upton N, Porter R, Johns A, Rodgers RJ (2004) Differential effects of the selective orexin-1 receptor antagonist SB-334867 and lithium chloride on the behavioural satiety sequence in rats. Physiol Behav 81:129–140.

Ishii Y, Blundell JE, Halford JC, Upton N, Porter R, Johns A, Rodgers RJ (2005) Satiety enhancement by selective orexin-1 receptor antagonist SB-334867: influence of test context and profile comparison with CCK-8S. Behav Brain Res 160:11–24.

Ito N, Nagai T, Yabe T, Nunome S, Hanawa T, Yamada H (2006) Antidepressant-like activity of a Kampo (Japanese herbal) medicine, Koso-san (Xiang-Su-San), and its mode of action via the hypothalamic-pituitary-adrenal axis. Phytomedicine 13:658–667.

Jaszberenyi M, Bujdoso E, Pataki I, Telegdy G (2000) Effects of orexins on the hypothalamic-pituitary-adrenal system. J Neuroendocrinol 12:1174–1178.

Jaszberenyi M, Bujdoso E, Telegdy G (2001) The role of neuropeptide Y in orexin-induced hypothalamic-pituitary-adrenal activation. J Neuroendocrinol 13:438–441.

Kharlamov EA, Kharlamov A, Kelly KM (2007) Changes in neuropeptide Y protein expression following photothrombotic brain infarction and epileptogenesis. Brain Res 1127:151–162.

Kitamura T, Mishina M, Sugiyama H (2006) Dietary restriction increases hippocampal neurogenesis by molecular mechanisms independent of NMDA receptors. Neurosci Lett 393:94–96.

Kunii K, Yamanaka A, Nambu T, Matsuzaki I, Goto K, Sakurai T (1999) Orexins/hypocretins regulate drinking behaviour. Brain Res 842:256–261.

Kuru M, Ueta Y, Serino R, Nakazato M, Yamamoto Y, Shibuya I, Yamashita H (2000) Centrally administered orexin/hypocretin activates HPA axis in rats. Neuroreport 11:1977–1980.

Lee J, Seroogy KB, Mattson MP (2002) Dietary restriction enhances neurotrophin expression and neurogenesis in the hippocampus of adult mice. J Neurochem 80:539–547.

Li Y, Gao XB, Sakurai T, van den Pol AN (2002) Hypocretin/orexin excites hypocretin neurons via a local glutamate neuron: A potential mechanism for orchestrating the hypothalamic arousal system. Neuron 36:1169–1181.

Liu RJ, van den Pol AN, Aghajanian GK (2002) Hypocretins (orexins) regulate serotonin neurons in the dorsal raphe nucleus by excitatory direct and inhibitory indirect actions. J Neurosci 22: 9453–9464.

Llorens-Martin MV, Rueda N, Martinez-Cue C, Torres-Aleman I, Florez J, Trejo JL (2007) Both increases in immature dentate neuron number and decreases of immobility time in the forced swim test occurred in parallel after environmental enrichment of mice. Neuroscience 147:631–638.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104–9110.

Muraki Y, Yamanaka A, Tsujino N, Kilduff TS, Goto K, Sakurai T (2004) Serotonergic regulation of the orexin/hypocretin neurons through the 5-HT1A receptor. J Neurosci 24:7159–7166.

Muroya S, Funahashi H, Yamanaka A, Kohno D, Uramura K, Nambu T, Shibahara M, Kuramochi M, Takigawa M, Yanagisawa M, Sakurai T, Shioda S, Yada T (2004) Orexins (hypocretins) directly interact with neuropeptide Y, POMC and glucose-responsive neurons to regulate Ca^{2+} signaling in a reciprocal manner to leptin: orexigenic neuronal pathways in the mediobasal hypothalamus. Eur J Neurosci 19:1524–1534.

Nambu T, Sakurai T, Mizukami K, Hosoya Y, Yanagisawa M, Goto K (1999) Distribution of orexin neurons in the adult rat brain. Brain Res 827:243–260.

Petersen A, Gil J, Maat-Schieman ML, Bjorkqvist M, Tanila H, Araujo IM, Smith R, Popovic N, Wierup N, Norlen P, Li JY, Roos RA, Sundler F, Mulder H, Brundin P (2005) Orexin loss in Huntington's disease. Hum Mol Genet 14:39–47.

Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18:9996–10015.

Piper DC, Upton N, Smith MI, Hunter AJ (2000) The novel brain neuropeptide, orexin-A, modulates the sleep-wake cycle of rats. Eur J Neurosci 12:726–730.

Porsolt RD, Bertin A, Jalfre M (1977) Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther 229:327–336.

Rao MS, Shetty AK (2004) Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. Eur J Neurosci 19:234–246.

Redrobe JP, Dumont Y, Fournier A, Quirion R (2002) The neuropeptide Y (NPY) Y1 receptor subtype mediates NPY-induced antidepressant-like activity in the mouse forced swimming test. Neuropsychopharmacology 26:615–624.

Rodgers RJ, Halford JC, Nunes de Souza RL, Canto de Souza AL, Piper DC, Arch JR, Upton N, Porter RA, Johns A, Blundell JE (2001) SB-334867, a selective orexin-1 receptor antagonist, enhances behavioural satiety and blocks the hyperphagic effect of orexin-A in rats. Eur J Neurosci 13:1444–1452.

Russell SH, Small CJ, Dakin CL, Abbott CR, Morgan DG, Ghatei MA, Bloom SR (2001) The central effects of orexin-A in the hypothalamic-pituitary-adrenal axis in vivo and in vitro in male rats. J Neuroendocrinol 13:561–566.

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92:573–585.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805–809.

Selbach O, Doreulee N, Bohla C, Eriksson KS, Sergeeva OA, Poelchen W, Brown RE, Haas HL (2004) Orexins/hypocretins cause sharp wave- and theta-related synaptic plasticity in the hippocampus via glutamatergic, gabaergic, noradrenergic, and cholinergic signaling. Neuroscience 127:519–528.

Shirayama Y, Chen AC, Nakagawa S, Russell DS, Duman RS (2002) Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. J Neurosci 22:3251–3261.

Spinazzi R, Rucinski M, Neri G, Malendowicz LK, Nussdorfer GG (2005) Preproorexin and orexin receptors are expressed in cortisol-secreting adrenocortical adenomas, and orexins stimulate in vitro cortisol secretion and growth of tumor cells. J Clin Endocrinol Metab 90:3544–3549.

Suda T, Tozawa F, Iwai I, Sato Y, Sumitomo T, Nakano Y, Yamada M, Demura H (1993) Neuropeptide Y increases the corticotropin-releasing factor messenger ribonucleic acid level in the rat hypothalamus. Brain Res Mol Brain Res 18:311–315.

Suzuki M, Beuckmann CT, Shikata K, Ogura H, Sawai T (2005) Orexin-A (hypocretin-1) is possibly involved in generation of anxiety-like behavior. Brain Res 1044:116–121.

Takahashi T, Nowakowski RS, Caviness VS Jr (1992) BUdR as an S-phase marker for quantitative studies of cytokinetic behaviour in the murine cerebral ventricular zone. J Neurocytol 21:185–197.

Tanapat P, Galea LA, Gould E (1998) Stress inhibits the proliferation of granule cell precursors in the developing dentate gyrus. Int J Dev Neurosci 16:235–239.

Taylor MM, Samson WK (2003) The other side of the orexins: endocrine and metabolic actions. Am J Physiol Endocrinol Metab 284:E13–E17.

Telegdy G, Adamik A (2002) The action of orexin A on passive avoidance learning. Involvement of transmitters. Regul Pept 104: 105–110.

Thakkar MM, Ramesh V, Strecker RE, McCarley RW (2001) Microdialysis perfusion of orexin-A in the basal forebrain increases wakefulness in freely behaving rats. Arch Ital Biol 139:313–328.

Thannickal TC, Lai YY, Siegel JM (2007) Hypocretin (orexin) cell loss in Parkinson's disease. Brain 130:1586–1595.

Thorpe AJ, Cleary JP, Levine AS, Kotz CM (2005) Centrally administered orexin A increases motivation for sweet pellets in rats. Psychopharmacology (Berl) 182:75–83.

Winsky-Sommerer R, Yamanaka A, Diano S, Borok E, Roberts AJ, Sakurai T, Kilduff TS, Horvath TL, de Lecea L (2004) Interaction between the corticotropin-releasing factor system and hypocretins (orexins): a novel circuit mediating stress response. J Neurosci 24:11439–11448.

Yamanaka A, Beuckmann CT, Willie JT, Hara J, Tsujino N, Mieda M, Tominaga M, Yagami K, Sugiyama F, Goto K, Yanagisawa M, Sakurai T (2003a) Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron 38:701–713.

Yamanaka A, Muraki Y, Tsujino N, Goto K, Sakurai T (2003b) Regulation of orexin neurons by the monoaminergic and cholinergic systems. Biochem Biophys Res Commun 303:120–129.

Yamanaka A, Tsujino N, Funahashi H, Honda K, Guan JL, Wang QP, Tominaga M, Goto K, Shioda S, Sakurai T (2002) Orexins activate histaminergic neurons via the orexin 2 receptor. Biochem Biophys Res Commun 290:1237–1245.

(Accepted 23 September 2008)
(Available online 1 October 2008)