Introduction

Breast cancer is the most frequent malignant disease among women. The National Cancer Institute estimated that there would be 40,170 deaths due to breast cancer (1), while the American Cancer Society predicted 192,370 new cases of invasive breast cancer among American women in the year 2009 (2). Stress has been shown to be a tumor-promoting factor (3–7). Emerging evidence suggests that chronic neurobehavioral stress can promote various tumor growth and progression secondary to sustained activation of sympathetic nervous system and inhibition of parasympathetic nervous system (8–10). Stress can significantly affect many aspects of the body’s immune systems. For example, higher levels of stress were shown to be associated with decrease in natural killer (NK) cell lysis activity, macrophage migration activity, decrease of T cell population, decreased lymphocyte proliferating following infection, and decrease in interferon-γ (IFN-γ) levels (reviewed in ref. 9). These factors are reported to be important components of immunity against cancer (10, 11). Therefore, manipulations to control the body’s stress response may be beneficial to increase immunity and fight against cancer. β-Endorphin (BEP), an endogenous opioid polypeptide primarily produced by the hypothalamus and pituitary gland, is known to have the ability to inhibit stress hormone production, produce analgesia, and a feeling of well-being (12, 13). BEP is a cleavage product of proopiomelanocortin (POMC), which is also the precursor hormone for adrenocorticotrophic hormone and α-melanocyte–stimulating hormone (α-MSH). BEP neuronal cell bodies are primarily localized in the arcuate nuclei of the hypothalamus, and its terminals are distributed throughout the CNS, including the paraventricular nucleus (PVN) of the hypothalamus (8). In the PVN these neurons innervate corticotropin releasing hormone (CRH) neurons and inhibits CRH release (14), while a μ-opioid receptor antagonist increases it (15). During stress, secretion of CRH and catecholamine stimulate secretion of hypothalamic BEP and other POMC-derived peptides, which in turn inhibit the activity of the stress system (15). BEP is known to bind to δ- and μ-opioid receptors and modulate the neurotransmission in sympathetic neurons via neuronal circuitry within the PVN to alter NK cell cytolytic functions in the spleen (16, 17). Abnormalities in BEP neuronal function are correlated with a higher incidence of cancers and infections in patients with schizophrenia, depression, and fetal alcohol syndrome and in obese patients (18–24).
We have recently shown that the neural stem cell–derived BEP neurons, when transplanted into the PVN, remained at the site of transplantation, decreased lipopolysaccharide (LPS)-induced levels of hypothalamic CRH and plasma corticosterone, increased NK cell cytolytic function and antiinflammatory cytokine productions in response to immune challenge, and suppressed carcinogen-induced prostate cancer development in rats (17, 25). It is not known whether BEP transplants prevent mammary tumor growth. Also, the effects of BEP neural activation on cancer progression and its metastasis to distant tissues are not evaluated. In this study, we examined the effect of transplantation of in vitro differentiated BEP neurons from fetal neuronal stem cells into the hypothalamus on tumor incidence, growth, malignancy rate, and metastasis using a rat model of breast cancer. In addition, we determined immunologic and neurochemical changes pertinent to BEP action on tumor.

Materials and Methods

Animals

Adult Sprague-Dawley and Fischer 344 male and female rats were purchased from Charles River and maintained in a controlled environment with a 12-hour light/dark cycle at Bartlett Hall Animal Research Facility of our institute. Male and female rats of each strain were bred and their fetuses or offspring were used in this study. All the animals were housed individually, allowed free access to regular rat chow, water, and maintained their normal physical activities throughout the study. Animal care was done in accordance with institutional guidelines and complied with NIH policy.

Preparation of BEP cells from neural stem cells

We isolated neural stem cells from 17 days old fetal rat brains of Sprague-Dawley rats and then differentiated these cells into BEP neurons in culture to use in this study. We used cAMP and pituitary adenylate cyclase-activating polypeptide (PACAP) to differentiate BEP neurons from rat fetal neural stem cells, as we have recently described (25). To control for transplantation, we used cortical cells prepared from 17-day-old fetal rat brains. The justification for the use of cortical neurons as control is previously described (25). Prior to transplantation, differentiated BEP cells were dissociated and resuspended at a concentration of 20,000 viable cells/μL in HEPES-buffered Dulbecco’s modified Eagle’s medium (DMEM)-containing serum supplement (30 nmol/L selenium, 20 nmol/L progesterone, 1 μmol/L iron-free human transferrin, 5 μmol/L insulin, 100 μmol/L putrescine, and antibiotics), cAMP (10 μmol/L), and PACAP (10 μmol/L) for the transplantation. Cells were placed on ice throughout the grafting session. Cell viability, assessed by the trypan blue exclusion assay, was routinely more than 90%. The composition of the differentiated cultures, with respect to the absence of undifferentiated neural stem cells and the presence of mature BEP-producing cells, was verified before grafting by staining for the immature neural marker nestin and/or vimentin, and for BEP using immunocytochemistry as described previously (25).

Determining the functionality of BEP cells

We verified the functionality of the transplanted cells by doing a physiologic test in the transplanted animals followed by confirming the presence of BEP neurons at the site of transplantation after the termination of the experiments. We have previously shown that animals transplanted with BEP neurons have increased expression of POMC mRNA and decreased expression of CRH mRNA in the PVN and reduced response of plasma corticosterone following a LPS challenge (17). In this study, we verified the function of transplanted BEP neurons by determining the changes in corticosterone response to LPS. We used a 100 μg/kg dose of LPS for a period of 2 hours (which was found to be an effective dose; 26) to determine the changes in the plasma corticosterone responses. After termination of the experiment, the brain was collected and processed for histochemical verification of the presence of transplanted BEP cells in the PVN of the hypothalamus using immunohistochemical methods (25). PN nuclei do not contain BEP neuronal cell bodies in situ (Ref. 14; control-transplanted PVN did not have any BEP staining; data not shown), hence the immunocytochemically detected BEP cells in this area were considered transplanted cells. All the animals included in cancer study showed transplanted BEP cells in the PVN.

Tumor induction and characterization

To determine the effect of BEP neuronal transplants on mammary tumor growth and progression, 50 days old intact virgin Sprague-Dawley rats were injected intraperitoneally (i.p.) with a dose of NMU (50 mg/kg body weight). Six weeks after the NMU injection, animals were anesthetized and injected with cortical neurons (control) or BEP neurons in both sides of PVN of the hypothalamus using stereotactic procedures described by us previously (25). No tumors were detected at this time. Beginning 1 week, when the animals recovered from the brain surgery, animals were weighed and palpated every week to check for tumor growth. Tumor length, width, and depth were measured with a calibrator as previously described (24). Sixteen weeks after the NMU injection, animals were sacrificed, tumors were collected, and slices of tumors were immersed in formalin and processed for histology staining. Fixed tissue was dehydrated, cleared, and paraffin infiltrated overnight using a tissue processor. The tissues were paraffin embedded, sectioned into 5-μm thick slices and placed on slides. One slide from each tissue was stained with hematoxylin and eosin (H&E) to evaluate tissue histology and tumor pathology. Slides were evaluated by a pathologist blinded to treatment. Ductal/cystic hyperplasia was defined by increased proliferation of benign glandular structures, with predominantly regular cells and nuclei. Adenomas were defined by a more solid phase glandular structure with regular cells and nuclei predominating. Adenocarcinomas presented primarily as solid-phase lesions containing many atypical and anaplastic cells, a high mitotic rate (including numerous atypical mitoses) and observable zones of tumor necrosis (apoptotic) and some show significant invasiveness.
Immunohistochemical localization of various proteins

Thin paraffin sections (5 μm) of mammary tumors were stained using the ABC Elite Vectastain Kit (Vector Labs) according to manufacturer’s instructions using various primary antibodies. Primary antibodies for immunohistochemistry were used as follows: polyclonal rabbit antibodies against TNF-α (1:250), NF-kB (1:100), E-cadherin (1:250), and N-cadherin (1:250; all from Abcam), as well as Snail (1:200), Slug (1:200), and Twist (all from Santa Cruz Biotechnology). After the primary antibody incubation and PBS wash, sections were incubated with peroxidase-coupled anti-rabbit Ig ImmPRESS reagent (Vector Laboratories, Inc.). Antigen localization was achieved by using the 3,3′-diaminobenzidine-peroxidase reaction and sections were dehydrated, and coverslipped. To evaluate the immunohistochemical staining, sections were photographed using Nikon-TE 2000 inverted microscope. Intensity of staining was categorized as negative (−) and strongly positive (+++). Western blotting

For Western blotting, tumor tissue extracts equivalent to 50 μg total protein were separated by 4% to 20% SDS-PAGE and transferred overnight to immobilon-P polyvinylidene difluoride membranes. Membranes were incubated with primary antibody for 18 hours at 4°C in blocking buffer. Membranes were then washed and incubated with peroxidase-conjugated secondary antibody (1: 5,000) for 1 hour. Afterwards membranes were washed and then incubated with enhanced chemiluminescence Western blot chemiluminescence reagent (Pierce). Membranes were exposed to X-ray films and developed. Membranes were washed and then incubated with peroxidase-conjugated antibody incubation and PBS wash, sections were exposed to X-ray films and developed. Membranes were washed and then incubated with peroxidase-coupled anti-rabbit Ig ImmPRESS reagent (Vector Laboratories, Inc.). Antigen localization was achieved by using the 3,3′-diaminobenzidine-peroxidase reaction and sections were dehydrated, and coverslipped. To evaluate the immunohistochemical staining, sections were photographed using Nikon-TE 2000 inverted microscope. Intensity of staining was categorized as negative (−) and strongly positive (+++).

Metastasis study

We used the MADB106 cells, a Fischer 344 rat mammary adenocarcinoma cell line maintained in DMEM containing 10% fetal calf serum. Female Fischer-344 rats were transplanted with BEP cell transplants or control cell transplants at 50 days of age. After about 2 weeks, these PVM cells–transplanted rats were inoculated with MADB106 tumour cells (100,000 cells/0.2 mL/rat) into jugular vein under sodium Nembutal solution (50 mg/kg) anesthesia. Wounds were closed with a surgical clip, and rats were left on heating pad till recover. Rats were sacrificed at 4 weeks, after the tumor cells inoculation, whole body of each animal was inspected for the presence of visible tumors. Tumors were only located in lungs in most animals, and at the inoculation site in a few animals. Lungs were collected, fixed in formalin, embedded with paraffin, and sectioned for H&E staining. Stained slides were examined under the microscope for the verification of the presence of tumor. Brain tissues were processed for verification of the site and viability of BEP cell transplantation.

Determination of pharmacologic modification of autonomic influence on immune system on lung metastasis

We hypothesize that the beneficial effect of BEP neuron transplants on immune function is regulated through autonomic nervous system, and that central BEP modulate peripheral immune function by inhibiting the sympathetic activity and activating the parasympathetic activity. Metaproterenol (MET) is an agonist for β-receptors, which are a class of G protein-coupled receptors that are targets of the catecholamines, especially norepinephrine and epinephrine (27, 28). Methylylcacnitine (MLA) is a selective antagonist of α7 nicotinic acetylcholine receptor (α7 nAChR; ref. 29). By injecting MET and MLA i.p., we mimicked the stimulation of sympathetic nerves and inhibition of parasympathetic nerves. We also used naloxone, an opiate receptor antagonist to test whether BEP neuronal effects is acted via opiodergic receptor (16). Forty-eight BEP neuron–transplanted Fischer rats and 48 control rats were divided into 8 groups and treated with saline, naloxone (10 mg/kg body weight), MET (0.8 mg/kg body weight), or MLA (2.5 mg/kg body weight) for 8 days (the doses used for these drugs are recognized as effective biological doses). A day after the first injection of these agents, animals were inoculated with MADB106 cells (100,000/0.2 mL/rat). Twenty-four hours after tumor cell inoculation, blood was collected by orbital puncture. Plasma was collected, and peripheral blood mononuclear cells (PBMC) were separated for migration assay and NK cytolytic assay. These animals continued receiving the blocker treatments for 7 days. After 4 weeks they were sacrificed, their lungs were fixed in formalin and processed to H&E staining for determination of cancer pathology. Brain tissues were processed for verification of the site and viability of BEP cell transplantation.

Immune reaction after tumor cell inoculation

We checked immune reaction to tumor cell inoculation in animals after they received BEP neuron transplants or cortical neuron transplants for 4 weeks. Animals were anesthetized with Nembutal, 1 mL of blood was drawn from jugular vein, and MADB106 cells (100,000/0.2 mL/rat) were inoculated into the jugular vein. After 24 hours, animals were anesthetized again. One milliliter of blood was drawn from the jugular vein, and then animals were sacrificed by decapitation. Vein blood before and after tumor inoculation was used for flow cytometry to determine cell populations in PBMC as described previously (27). Trunk blood PBMCs and splenocytes were used for NK cell cytolytic assay (25) and macrophage migration assay (CytoSelect 96-well cell migration assay, 5 μm, Cell BioLab). Trunk plasma was used for multicytokine assay. After termination of experiment, spleen were obtained and splenocytes were prepared and used for measuring mRNA levels of various cytokines and cytotoxic factors using real-time RT-PCR methods as described by us previously (27).

Plasma analysis of hormones and cytokines

Plasma was analyzed for corticosterone levels by a competitive ELISA (Immunodiagnostic Systems) according to manufacturer’s recommendations. All samples were run on one 96-well plate for each variable. Plasma cytokine levels were measured by AssayGate, Inc. using their multiplex platform which measures multiple cytokines in a single plasma sample. Each sample was measured in triplicate. Immune-related cytokines data are presented in figures.
Figure 1. BEP neuronal transplant in the hypothalamus suppresses mammary tumor growth and progression. Animals were administered with a single i.p. injection of NMU at 50 mg/kg at 50 days of age. After 6 weeks of NMU, rats were administered with 20,000 cells/μL of BEP cells or cortical cells (control) into the PVN bilaterally. Rats were palpated for tumors once a week following injections. A, a representative photomicrograph showing BEP cells in primary cultures after staining for the BEP peptide by immunofluorescence technique. B, a representative immunofluorescence pictures of BEP cells in the PVN area of the hypothalamus after 6 months of transplants. C, corticosterone response to LPS. D, body weight changes. E, graph represents percent (tumor incidence) of rats presenting with tumors each week postinjection. F, graph represents average number of tumors per animal in each group. G, average volume of tumor per animal in each group. H, tumors malignancy rate as determined by histologic evaluation. Percentage of each histologic tumor type that developed per treatment group. Data of C were analyzed using 1-way ANOVA followed by Newman–Keuls post-hoc analysis. Data in panels D–G were analyzed using a 2-way ANOVA and only the data shown in E–G were found to have significant treatment and time interactions at \( P < 0.001 \) (8–12/group), and F was analyzed using a \( \chi^2 \) test with a \( P < 0.0001 \). I–L, representative images of different histologic tumor types developed in our model.

Statistics

Differences in average tumor incidence, tumor number, and tumor volume were assessed using 2-way ANOVA with a Bonferroni posttest at the level of \( \alpha = 0.05 \). To evaluate tumor type, a \( \chi^2 \) test was done. Differences in tumor incidence and immune after various drugs were assessed using 1-way ANOVA with a Newman–Keuls post-hoc analysis at the level of \( \alpha = 0.05 \). \( t \) tests were used to evaluate the differences in various protein and cytokine levels.

Results

BEP neuronal transplantation into the hypothalamus suppresses mammary tumor growth and progression

We used the \( \text{in vitro} \) produced BEP neuron in this study (Fig. 1A). Before testing BEP neuronal effects on mammary cancer growth, we evaluated the functionality of BEP-neuron transplantation into the hypothalamus following long-term transplants. For this, female rats at age of 50 days were implanted with BEP neuronal cells or control cells in both sides of PVN. After 2 to 3 months of cell transplants, these rats were inspected for changes in reproductive cyclicity and body growth. Like the untreated rats, both control and BEP cell–transplanted rats showed regular 4 to 5 days estrous cycle. Body weights of untreated control, control cell–transplanted rats and BEP cell–transplanted rats were similar (Untreated, 243 ± 6; control cell transplanted, 251 ± 5; BEP cell transplanted, 253 ± 6; \( n = 8–10 \)). After 6 months, they were used to determine their plasma corticosterone response to LPS, as a functional test of BEP neuronal activity (17), and their brains were employed for immunohistochemical verification of transplanted neurons in the PVN. Transplanted BEP cells remained at the site of transplantation, as they were detected in the PVN by immunostaining (Fig. 1A and B). As reported by us previously (17), BEP-transplanted animals showed lower plasma corticosterone response to LPS (Fig. 1C).
We used N-methyl-N-nitrosourea (NMU) to induce mammary cancer in rats (24). Six weeks after the administration of NMU, animals were anesthetized and injected with in vitro-differentiated BEP neurons or in situ cortical neurons, which served as the controls, in both sides of the PVN of the hypothalamus (25). Weekly body weight gain were similar between rats with control transplant or BEP transplants (Fig. 1D). Weekly measurement of tumor number, length and width for a period of 16 weeks revealed that BEP neurons implanted animals had lower tumor incidence, tumor number, and tumor volume (Fig. 1E–G). At the termination of the experiment, whole body inspection revealed that tumors were localized only in mammary glands. Tumors from the study were classified by histologic analysis (Fig. 1H–L). Histopathologic evaluation of tumors showed that, unlike control animals, which had mostly adenocarcinoma (both invasive and noninvasive), most of the BEP-treated animals had benign adenoma with glandular hypertrophy.

Numerous studies have shown that the inflammatory tumor microenvironment potentiates not only tumor development...
BEP neuron transplants have prevented the development of tumors and morphologic data of tumors suggest the possibility that transplanted animals (Fig. 2A–G). In summary, the cellular E-cadherin in mammary gland as compared with control-decreased in the level of N-cadherin, and increased the level of NF-kB, as well as Snail, Slug, and Twist with concomitant with BEP cell transplants showed reduced levels of TNF-α (N-cadherin) and epithelial adhesion factor (E-cadherin). Immunohistochemical and Western blot determination of invasiveness and more advanced tumor stage for many cancers including breast cancer (33). Therefore, we determined the expression of proinflammatory cytokine TNF-α and proinflammatory NfκB, transcription factors linked to the morphogenetic processes causing EMT (Snail, Slug, and Twist) expression, and expression of mesenchymal adhesion factor (N-cadherin) and epithelial adhesion factor (E-cadherin). Immunohistochemical and Western blot determination of various cytokines in tumor tissues obtained from rats with BEP cell transplants showed reduced levels of TNF-α and NF-κB, as well as Snail, Slug, and Twist with concomitant decreased in the level of N-cadherin, and increased the level of E-cadherin in mammary gland as compared with control-transplanted animals (Fig. 2A–G). In summary, the cellular and morphologic data of tumors suggest the possibility that BEP neuron transplants have prevented the development of advanced stage carcinoma possibly by suppressing the inflammatory response and repressing EMT factors in tumor tissues.

**BEP neuron transplantation into the hypothalamus prevents mammary tumor metastasis to lung**

To determine the effects of BEP neuron transplants on tumor metastasis, we used nonimmunogenic syngeneic MADB106 mammary cancer cells, which are widely used for lung metastasis studies in rats (34). Four weeks after inoculation of MADB106 cells through jugular vein, 70% to 80% of inoculated control cell–transplanted animals showed visible multiple tumor foci in lungs (Fig. 3A). Some of these animals also had a single visible tumor at the site where the tumor cells were inoculated (data not shown). None of the BEP-transplanted animals showed visible tumors either in the lung or any other body sites. Histologic examinations of lung tissues identified focal and invasive tumors in control-transplanted rats while no tumors in BEP-transplanted rats (Fig. 3C). These data suggest that the BEP transplantation completely eliminated retention of MADB106 tumor cells in the lungs.

**Activation of hypothalamic BEP neurons increases innate immune functions**

Because BEP transplantation increases immune activity in normal animals and since the innate immune system is critical for tumor cell clearance (reviewed in refs. 35, 36), we hypothesize that the elimination of tumor cells from lungs of BEP-transplanted animals may have been caused by increased innate immune function. Using the MADB106 cancer model, we checked immune response (37) 24 hours following tumor cell inoculation. Several studies have shown that MADB 106 tumor cells metastasize only to the lungs and subjected to NK cell cytotoxic activity when injected intravenously (34). In this study, we have not quantified the cytokine profile for longer observation, because it may be possible that there may be some changes in the cytokines profile by adaptive antitumor immune response only in control cell–transplanted rats that may not reflect the innate immune responses as like the early stage of tumor development.

We found that the BEP-transplanted rats had greater NK cell cytolytic activity in PBMCs and in splenocytes as well as greater macrophage migration and cell proliferation activity than those in control cell–transplanted rats (Fig. 4A). Consistent with these data, we found that splenocytes of BEP-transplanted rats had higher mRNA and protein levels of NK cell cytolytic activity regulatory factors (e.g., granzyme B; 38), NK cell activator receptor (e.g., NKG2; 39), cytokines that is produced after NK activation (e.g., IFN-γ; but lower TNF-α; 40), a chemokine that recruits and activates macrophages (MCP1; 41), and a protein that is produced in an increased amount from activated macrophages (IL-1β; 42; Fig. 4B). Furthermore, measurement of cytokines and protein levels in PBMCs of tumor-inoculated rats showed that the levels of macrophage-regulatory/derived factors (GM-CSF, MIP-1α, IL-18; 43–45) and an NK cell–regulatory/derived factor (IFN-γ; 40) are higher in BEP-transplanted rats than those in control-transplanted rats (Fig. 4C). Also, lower levels of several inflammatory cytokines (IL-1α, IL-12, TNF-α; 46) were observed in plasma of BEP-transplanted rats.
Figure 4. Evaluation of effects of BEP-cell transplantation on cancer cell retention and immune functions. Rats were transplanted with 20,000 viable cells/μL of BEP cells or cortical cells (control) into 2 PVN, and then after 4 weeks they were inoculated with 100,000/0.2 mL/rat of MADB106 cells via jugular vein. A, NK cell cytolytic activity and macrophage migration activity in splenocytes or in PBMC at 24 hours after tumor inoculation. B, splenocytes mRNA levels of cytotoxic factors and cytokines genes known to regulate NK cell and macrophage functions at 24 hours after tumor inoculation. C, plasma levels of various cytokines at 24 hours after tumor inoculation. D, NK cell populations in PBMC before and after tumor inoculation, and in splenocytes 24 hours after tumor inoculation. E, macrophage cell populations in PBMC before and after tumor inoculation, and in splenocytes 24 hours after tumor inoculation. n = 8–12 rats. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus control.
as compared with PBMCs of control-transplanted rats. In addition, BEP transplants increased NK and macrophage cell numbers in PBMCs, but decreased their numbers in spleno-ocytes after tumor cell inoculations (Fig. 4D and E), suggesting that the transplants also promoted migration of these immune cells out of the spleen to the blood and/or inhibited emigration from the blood into the spleen to promote defense against tumor cells. These data suggest that the BEP neuronal supplement promotes innate immunity and produces an anti-inflammatory environment in recipient animals.

Antagonists of opiate receptor and α7 nicotine acetylcholine receptors and agonists of β-adrenergic receptors prevent BEP neuronal ability to enhance immunity and suppress tumor metastasis

BEP neurons produce opioid peptides and other products of POMC (47). To address the question whether BEP alone and/or other peptide products from the transplanted neurons are responsible for the observed actions on the immune system and cancer, we tested the ability of a general opiate antagonist naloxone to block the effects of BEP neuron transplants on immune activation and metastasis prevention. In addition, we tested the effects of norepinephrine agonist MET and a α7 nicotine acetylcholine receptor (α7 nAChR), antagonist MLA. We found that 24 hours after tumor inoculation, PBMC NK cell activity (Fig. 5A) and macrophage migration activity (Fig. 5B) were higher in saline-treated BEP-transplanted animals than in saline-treated cortical cells–transplanted controls. Naloxone, MET, and MLA all had moderate or strong inhibitory effect on basal and BEP-stimulated NK cell activity and macrophage migration activity. Consistent with these findings, we observed that naloxone, MET, and MLA prevented, at various degrees, the beneficial effect of BEP in eliminating tumor cell lung retention (Fig. 5C). These data suggest that BEP neurons activate innate immunity for cancer cell clearance via altering the function of the autonomic nervous system.

Discussion

In situ, BEP neurons originating from the arcuate nucleus terminate in the PVN and are known to regulate both stress axis and immune functions. The data presented here show that the neural stem cell–derived BEP neurons, when transplanted in the PVN, remained viable and increased endogenous opioid inhibitory tone to the HPA axis so that plasma corticosterone levels responded lower during stressful conditions like immune challenge. BEP cell transplantation does not seem to affect general body growth or reproductive hormone profiles as the body weight and reproductive cyclicity of these rats were similar to those in controls. Within the context of immune-related function, in situ BEP neurons in the
hypothalamic arcuate hypothalamic nucleus make synaptic contacts on to nucleus preopticus medianus neurons projecting to the paraventricular hypothalamic nucleus containing projection fibers from the


cell. Cancer Res 2011;71:19 October 1, 2011


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