Beta-Endorphin Cell Therapy for Cancer Prevention
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Abstract

β-Endorphin (BEP)-producing neuron in the hypothalamus plays a key role in bringing the stress axis to a state of homeostasis and maintaining body immune defense system. Long-term delivery of BEP to obtain beneficial effect on chemoprevention is challenging, as the peptides rapidly develop tolerance. Using rats as animal models, we show here that transplantation of BEP neurons into the hypothalamus suppressed carcinogens- and hormone-induced cancers in various tissues and prevented growth and metastasis of established tumors via activation of innate immune functions. In addition, we show that intracerebroventricular administration of nanosphere-attached dibutyryl cyclic adenosine monophosphate (dbcAMP) increased the number of BEP neurons in the hypothalamus, reduced the stress response, enhanced the innate immune function, and prevented tumor cell growth, progression, and metastasis. BEP neuronal supplementation did not produce any deleterious effects on general health but was beneficial in suppressing age-induced alterations in physical activity, metabolic, and immune functions. We conclude that the neuroimmune system has significant control over cancer growth and progression, and that activation of the neuroimmune system via BEP neuronal supplementation/induction may have therapeutic value for cancer prevention and improvement of general health. Cancer Prev Res; 1–12. ©2014 AACR.

Introduction

The stress-controlling mechanisms of the body that are disrupted and implicated in tumor progression are causally connected to an enhanced immune defense system of the body. Specifically, increased cancer incidence, malignancy, and growth rate have been shown to be prevalent in patients with chronic stress, whereas managing stress of these patients promoted immune function that fights against their cancer (1–4). The increasing understanding of the mechanism and control of the neuroimmune system in the regulation of cancer chemoprevention offers promise for the design of therapeutic strategies. Recently, the use of BEP neuron transplantation to activate endogenous opioid system for regulation of stress and neuroimmune functions showed the most promising results. It has been shown that BEP not only inhibits the stress response of the hypothalamic–pituitary–adrenal (HPA) axis through interaction with corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus but also inhibits the sympathetic nervous system (SNS) and activates the parasympathetic nervous system through innervations in the paraventricular nucleus where these BEP molecules bind to δ- and β-opioid receptors to modulate the neurotransmission in neurons of the autonomic nervous system (ANS; ref. 5). Abnormalities in BEP neuronal function are correlated with a higher incidence of cancers (6). Limited study has demonstrated the ability of BEP neuron transplantation in suppression of carcinogen-induced prostate and breast cancer incidence in rats (5, 7). It is not yet known whether BEP neuronal transplants have preventive effects on the growth and metastasis of other cancers, and whether the anti-cancer property of the transplants involves specific immune cell population. In addition, translation of this cell therapy into clinical settings remains difficult because of the need of isogenic cells population for transplantation. A method to in situ differentiate BEP neurons to prevent cancer growth and metastasis should be a major advancement to the preclinical development of anticancer therapy. These problems dealing with the BEP neuron activation to increase body’s immune defense system for cancer reduction were addressed in this study.

Materials and Methods

Animals

Adult Sprague-Dawley (SD), Fischer 344 (F344) rats were purchased from Charles River and maintained in a controlled environment with a 12-hour light/dark cycle at Rutgers Laboratory Animal Services Facility. Female athymic nude mutant rat (nude rats) were also purchased from Charles River and housed under pathogen-free conditions. Animal care was performed in accordance with institutional guidelines and complied with NIH policy.

Preparation of BEP cells from neural stem cells and effect on cancer

We isolated neural stem cells (NSC) from 17-day-old fetal rat brains of SD rats and then differentiated these cells into BEP neurons in culture as we have previously described (7).
previous studies in rats suggested that NSC-derived BEP cells are not rejected in host animals of various strains possibly due to the lack of MHC-I protein in NSCs (5, 7). To control for transplanation, we used cortical cells prepared from 17-day-old fetal rat brains of the host strain. The justification for the use of animals with transplants of cortical neurons as controls is previously described (7). Animals were anesthetized and injected with cortical neurons or BEP neurons (20,000 viable cells/μL) in both sides of the paraventricular nucleus of the hypothalamus using stereotactic procedures (the coordinates were set 1.8 mm behind bregma, 0.5 mm lateral, and 7.5 mm below the cortex). We verified the functionality of the transplanted cells by doing a physiologic test in the transplanted animals and then by confirming the presence of BEP neurons at the site of transplantation after the termination of the experiments as previously described (8). All the non-control animals included in the cancer study showed transplanted BEP cells in the paraventricular nucleus.

Tumor induction and characterization

Hepatocellular carcinoma.

Adult F344 male rats received transplants of either cortical neurons or BEP neurons in the paraventricular nucleus and 4 weeks later were injected with 0.9% saline solution intraperitoneally (i.p.) and 0.5% carboxymethyl cellulose (CMC) intragastrically as a vehicle control or with 200 mg/kg body DEN (Sigma) in 0.9% saline i.p. Cancer was promoted by administering 2-acetylaminofluorene (2-AAF) intragastrically, suspended in 0.5% CMC, beginning 2 weeks after DEN was injected. The promoter was administered for 3 days per week for 13 weeks. At the end of 18-week period, all the animals were sacrificed by decapitation. A portion of the liver tissue was removed from the animals and fixed in 10% neutral-buffered formalin for 24 hours. Slides from each liver tissue were stained with hematoxylin and eosin (H&E) to evaluate tissue histology and tumor pathology. Slides were evaluated by a pathologist blindly.

Colon cancer.

Adult SD male rats implanted with either cortical or BEP neurons in the paraventricular nucleus were used. To induce colon cancer in these rats, the colon-specific procarcinogen 1,2-dimethylhydrazine dihydrochloride (DMH; Santa Cruz Biotechnology) at a dosage of 20 mg/kg body weight was administered subcutaneously to the animals once a week for 15 weeks. Before injection, the DMH was dissolved in 0.9% saline containing 1.5% EDTA and was adjusted to a pH of 6.5 with 1 mol/L sodium hydroxide. After sacrifice, the abdomen was opened and the entire large intestine (~7–8 cm long) was cut between the cecum and anal canal by a scissors. It was then opened longitudinally, washed with PBS, and the mucosal surface examined for gross pathology. After the gross examination, the distal, mid, and proximal colon tissues were separated and prepared for histopathologic examination according to the Swiss roll technique (9). Tissues from all groups were processed and embedded in paraffin blocks.

Pituitary tumors.

Adult F344 female rats were transplanted with BEP or cortical cell transplants. Four weeks following surgery, they were ovariectomized and implanted s.c. with a 1-cm-long silastic capsules containing 17β-estradiol (E2) or with an empty capsule. The E2 capsule maintained plasma levels of 17β-estradiol between 120 and 150 pg/mL. Sixty days following the ovariectomy, the animals were killed by decapitation and the anterior pituitary was removed and weighed to determine the tumor growth.

Mammary tumors.

To determine the effect of BEP neuronal transplants on the growth and survival of existing tumors were also tested, 50-day-old ovary-intact virgin SD rats were injected i.p. with a dose of NMU (50 mg/kg body weight). Beginning at 4 weeks postinjection, animals were weighed and palpated every week to check for tumor growth. Six to 10 weeks after the NMU injection, animals showing one or more 0.5-cm size tumors were anesthetized and injected with cortical neurons or BEP neurons in both sides of the paraventricular nucleus. Tumor length, width, and depth were measured weekly with calipers as previously described (5). Sixteen weeks after the NMU injection, animals were sacrificed by decapitation and processed for histology staining. One slide from each tissue was H & E stained to evaluate tissue histology and tumor pathology. Slides were evaluated by a pathologist blind to the treatment as describe previously (5).

Metastasis study.

Female nude rats at 2 months of age were anesthetized with sodium pentobarbital (50 mg/kg body weight, Sigma) and injected with 20,000 cortical neurons (control) or BEP neurons in both sides of the paraventricular nucleus of the hypothalamus using stereotactic procedures. Four weeks after transplantation, these rats were injected with MADB106 cells (100,000/0.2 μL) on the right flank as described previously (5). Tumors were monitored and their size measured with calipers every other day. After 2 weeks, the animals were sacrificed, and the whole body of each animal was inspected for the presence of visible tumors. Tumors 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 tumors.

Nanosphere-attached dbcAMP effects on cancer cAMP release test.

Nanospheres that deliver 70 and 350 nmol cAMP in 10 μL were purchased from Cupuscular Inc. To test the efficacy of nanospheres in releasing cAMP, 10 μL nanospheres were placed into 1 mL of artificial cerebrospinal fluid (ACSF) and incubated at 37°C. After 6 hours, nanospheres were spun down by centrifuging at 10,000 × g for 1 minute and the supernatant was collected for analysis of cAMP concentration. Nanospheres were resuspended in another 1 mL of ACSF and incubated for 18 hours. Similarly, supernatants were removed at 24, 48, and 72 hours and analyzed for cAMP concentration using ELISA kit (Enzo Life Sciences).

Nanosphere-attached dbcAMP administration.

To determine the effect of cAMP-delivering nanosphere on hormone levels in rats, we used adult male SD rats. Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, Sigma) and injected with 10 μL of plain nanospheres (control) or nanospheres that deliver 70 nmol cAMP or 350 nmol cAMP in the third ventricle using a 10-μL Hamilton syringe and a stereotactic instrument with the coordinates of 2.0 mm behind the bregma, midline, and 8.0 mm below the cortex using. Each injection was over 5-minute duration. After the injection, the cannula was left in place for 3 minutes to prevent nanospheres from backflowing during the removal of the cannula. The cannula was then slowly removed over a 3-minutes period. The skin was closed with...
wound clips. Animals then received i.p. injection of bromodeoxyuridine (BrdUrd, 50 mg/kg body weight; Sigma) every day for 7 days. One month after nanosphere injection, the animals were sacrificed and brains were collected for identification of BEP neurons. Another group of male SD rats was used for examining the effect of nanospheres injection. Animals were injected with nanospheres that deliver 70 nmol cAMP in 10 μL at 2 months, 1 month, and 2 weeks before the day of sacrifice. Control animals were kept intact until sacrifice. Hypothalamic tissues, pituitary, and blood plasma were collected for detection of hormones. The site of injection of nanosphere was verified histochemically by localizing fluorescently labeled nanosphere in the third ventricle.

Tumor induction and characterization.

To determine the effect of cAMP-delivering nanosphere injection on mammary tumor growth and progression, 50-day-old ovary-intact virgin SD rats were injected i.p. with a dose of NMU (50 mg/kg body weight, Sigma). Six weeks after the NMU injection, animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.; Sigma) and injected with plain nanospheres (control) or 70 nmol cAMP-delivering nanospheres in the third ventricle using stereotaxis procedures as previously described. No tumors were detected at this time. Beginning 1 week after surgery, when the animals had recovered from the brain surgery, animals were weighed and palpated every week to check for tumor growth. Tumor length and width were measured with calipers, and tumor volumes were calculated as \( \frac{1}{2} \times (\text{length} \times \text{width}) \). 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 to evaluate tumor pathology (5).

Metastasis study.

Female F344 rats were injected with control or cAMP-delivering nanospheres at 60 days of age. After 1 month, these nanosphere-injected rats were inoculated with MADB106 tumors cells (100,000 cells/0.2 mL) into the jugular vein under isoflurane anesthesia and used for metastasis study as previously described.

Plasma analysis of hormones.

Plasma was analyzed for corticosterone levels by a competitive ELISA (Immunodiagnostic Systems) according to the manufacturer’s direction. All samples were run on one 96-well plate. Prolactin hormone was determined in plasma by ELISA (Alpco Diagnostics) for 15 minutes at 4°C. The cells were washed and incubated with rabbit anti-TH (1:500) antibody for 2 hours or rabbit anti-CRH antibody (1:2,000) overnight. For double staining, sections were incubated with rabbit anti-β-EP (1:2,000; Invitrogen) anti-body and mouse anti-BrdUrd antibody (1:2,000) overnight. The sections were then washed twice and incubated with Alexa anti-mouse IgG (1:500) and FITC anti-rabbit IgG (1:500) for 45 minutes. Stained slides were mounted with DAPI, and pictures were taken by a confocal microscope with a 20x objective lens (Nikon EZ-C1. 3.60 build 770, Gold version). Cell numbers on each section were manually counted, and the sum of the total cell number from all sections of one brain was calculated.

Immune system characterization

Immune reaction after tumor cell inoculation.

We checked immune reaction to tumor cell inoculation in animals after they received injection of control or cAMP-delivering nanospheres for 4 weeks. Animals were anesthetized with isoflurane and inoculated with MADB106 cells (100,000/0.2 mL) into the jugular vein. After 24 hours, animals were sacrificed, and blood and spleens were collected. Peripheral blood mononuclear cells (PBMC) were collected from the blood, and spleenocytes were collected from the spleens. PBMCs and splenocytes were used for natural killer (NK) cell cytolytic assay and macrophage migration assay (Cell BioLab) and also stained by fluorescein isothiocyanate (FITC)-conjugated antibodies for CD161a and CD11b/c for flow cytometry (FACS).

NK cell depletion.

To deplete the NK cells, rats were injected i.p. with rabbit anti-mouse asialo-GM-1 (AsGM-1; 150 μL per rat; Wako Chemicals USA, Inc.) antibodies. Control animals were injected with rabbit γ-globulin (150 μL per rat; ARGG). The antibody was administered i.p. for 4 days prior and a day after the inoculation of MADB cells. After 24 hours of cancer cell inoculation, the effects of the NK cell depletion were confirmed in blood by cytotoxic assay and flow cytometric analysis. Four weeks after the inoculation of cancer cells, the animals were sacrificed and used for lung metastasis study.

Isolation of NK cells from blood.

The tail vein blood was collected and put on histopaque column (3 mL) and centrifuged at 1,400 rpm for 30 minutes at 4°C to isolate WBC. The buffy coat was collected in 15-mL tube and cells were counted. The cells were centrifuged again at 3,000 rpm for 5 minutes and pelleted was resuspended in MACS running buffer (Miltenyi Biotec). The cells were stained with FITC-anti-granulocyte (H1S48) and FITC-anti-MHC-II antibody for 30 minutes. The cells were washed 2 times with running buffer and then incubated with anti-PerC, anti-45RA, and anti-FITC microbeads (BD Biosciences) for 15 minutes at 4°C. The cells were washed and then separated with MACS (Miltenyi Biotec) on Posell-S program and negative port cells (containing NK cells) were selected and used for cytotoxicity assay.

FACS measurements of T cell and NK cell numbers.

The blood was collected and put on put on histopaque column (3 mL) and spin at 1,400 rpm for 30 minutes at 4°C to isolate WBC. The buffy coat was collected in 15-mL tubes and cells were counted and 0.5 million cells were used for staining NK and T cells. The cells were centrifuged at 3,000 rpm for 5 minutes, and pellet was washed with 1× PBS and then cells were blocked with 2.5% horse serum for 30 minutes at room temperature. The cells were centrifuged and resuspended and incubated with antibodies (all from BD Biosciences) for NK cells FITC-CD161a (1:250) and...
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FITC-CD4 (1:250) for T cells for 1 hour at room temperature (RT). The cells were washed 3 times with 1 x PBS and resuspended in PBS. The cells were sorted and analyzed on BD Accuri C6 flow cytometer. Unstained samples were analyzed first to determine optimal criteria for sorting test samples. Specifically, cells without antibody treatment were used to set the light scatter gate. A total of 10,000 events were counted for FACS analysis.

Measurement of cytotoxicity.

The cytotoxicity of PBMCs and splenocytes at each time point was determined by calcein AM assays using YAC-1 murine lymphoma cells (ATCC) as target cells. The YAC-1 cells were grown and maintained in RPMI-1640 medium containing 1% antibiotic (Sigma-Aldrich) and 10% FBS (Sigma-Aldrich). YAC-1 cells were washed and incubated with 5 mmol/L calcein AM (Sigma-Aldrich in serum-free RPMI-1640 medium for 40 minutes at 37°C. Labeled YAC-1 cells were washed and plated into U-bottom 96-well plates (BD Falcon) at 5 x 10^4 cells per well. PBMCs or splenocytes incubated with IL2 (100 ng/mL, R&D Systems) for 12 hours at 37°C were then added at various E/T ratios in triplicate. YAC-1 cells in RPMI alone were used to determine spontaneous calcein release, whereas maximal release was achieved by lysing target cells with 0.1% Triton X-100. Cells were incubated at 37°C for 4 hours, and fluorescence in the supernatant was measured using a fluorescence plate reader (Tecan). The percent cytotoxicity for each sample was calculated as follows: % cytotoxicity = [experimental well – spontaneous well]/(max lysed well – spontaneous well)] x 100. The percentage at each E/T ratio was converted to lytic units per 10^7 effector cells and based on 20% specific cell lysis (Supplementary Fig. S3).

Physiological function tests

Reproductive function test.

A group of SD male and female rats with BEP cell transplants or control transplants for a period of 3 months were group housed as unisex. Female rats were mated from the vaginal orifice daily and inspected microscopically for determination of estrous cycle phase for a period of 3 weeks. All animals showed 4 to 5 days estrous cycles. Male rats were bred from tails and used for measurement of testosterone concentrations using ELISA assay (Immuno-Biological Laboratories, Inc.).

Physical activity measurements by running wheel test.

Physical activity was measured by placing animals to individual running wheel cages that were built with a running wheel (diameter, 34 cm and width, 11 cm, Mini Mitter) and had ad libitum access to food and water throughout the experiment. Each revolution of the running wheel activated a microswitch that was monitored and recorded in a computer 24 h/d for 24 days. Data for running activity was analyzed using the ClockLab software package (Actimetrics) and were presented as the number of revolutions per hour and day.

Measurement of cardiovascular parameters.

Cardiovascular parameters were measured using a noninvasive tail-cuff volume pressure recording system (CODA, Kent Scientific). The animals were acclimated to the procedure by daily training for 1 week and 5 minutes before the recording day. The recordings are made for 4 consecutive days, and measurements for each day were determined by averaging the values for at least 3 successful recording. The measurements for mean blood pressure (BP), diastolic BP, and systolic BP was expressed as mm Hg and heart rate as BPM.

The oral glucose tolerance test.

The oral glucose tolerance test (OGTT) was conducted in animals after overnight fasting for 12 hours, the BEP and control neurons implanted animals were administered a 50% glucose solution (2 g/kg of body weight) dissolved in water by gavage for OGTT. Blood samples were taken by tail-tip withdrawal and blood glucose concentrations were determined with an Accu-check glucometer (Roche Diagnostics) at 0, 30, 60, 90, 120, and 180 minutes.

Measurement of body and organs weight.

At 14 months of age, a total of 12 to 15 per group SD male rats that had BEP cell transplant or control transplants were used for measurement of body weight followed by after sacrificed used for measurements of the weight of pituitary, liver, spleen, and various lobes of the prostate gland.

Statistical analysis.

Differences in average tumor incidence, tumor number, and tumor volume were assessed using 2-way ANOVA with a Bonferroni posttest at the level of α = 0.05. To evaluate tumor type, a χ² test was performed. Differences in tumor incidence and immune responses after various drugs were assessed using one-way ANOVA with a Newman–Keuls post hoc analysis at the level of α = 0.05.

Results

BEP neuron transplantation prevents the tumor growth, progression, and metastasis via activation of the innate immune system.

To check whether BEP neuronal protective effects on tumor clearance are tissue- and/or carcinogen-specific, we determined the efficacy of in vitro differentiated BEP neuronal transplants on diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC; ref. 11), 1,2-dimethylhydrazine (DMH)-induced colon cancer (12), and estradiol (E2)-induced pituitary prolactin-secreting tumors (13). In the liver cancer induction study in male Fischer 344 (F344) rats, we observed that the rats injected with DEN and implanted with control transplants before the carcinogen treatment developed liver tumors with a 60% tumor incidence. However, animals with BEP neuron transplants developed no tumors (Fig. 1A and B). Histopathologic evaluation revealed that rats with control transplants had well-differentiated HCC, whereas rats with BEP transplants showed no abnormalities in the liver (Supplementary Fig. S1). In the colon cancer study, SD male rats with control transplants and given weekly DMH injections for 16 weeks developed tumors at a 100% incidence. However, DMH treatment in the BEP transplant group produced tumors at a much-reduced incidence (30%; Fig. 1C and D). In addition, the mean tumor volume in the colon of BEP-treated rats was about one third of those in control rats (Fig. 1E). In the pituitary tumor study, E2 supplementation in control female F344 rats increased pituitary weight about 5-fold and plasma prolactin level 6-fold (Fig. 1F and G). The pituitary weight and plasma prolactin levels of BEP-treated animals were about 40% lower than those in control group, indicating that BEP neuronal transplants suppress the growth of E2-induced pituitary tumors. Together, these data
suggest that BEP neuron transplants have protective effects against tumorigenesis in general, as they suppressed the actions of various carcinogens on tumor development and growth in various tissues.

The BEP neuronal transplant effects on the growth and survival of existing tumors were also tested in SD female rats injected with NMU to induce mammary tumors. Animals with mammary tumors about 0.5 cm in diameter received transplants of either

![Liver cancer incidence](image)

**Figure 1.** Protective effects of BEP neuronal transplants in the hypothalamus on tumor incidence, growth, survival, and metastasis. A and B, incidences of hepatocellular carcinoma in control and BEP neuron-transplanted SD animals after DEN treatment. Representative images of livers of animals with control transplants (control, left) or BEP neuron transplants (right). Multiple HCCs were shown by arrows. HCC incidence is shown in the histograms figure on the right. C–E, BEP transplantation effects on DMH induced colon cancer incidence in F344 male rats. The macroscopic section shows tumor nodule in control transplanted (left; inlet higher magnification) and small tumor in BEP neuron-transplanted animals (right; inlet higher magnification) within the distal colon. The histograms show the incidence of colonic adenocarcinoma and the tumor volume in cortical neurons and BEP neuron-transplanted animals. F and G, BEP transplants effects on the E2 implant-induced prolactinoma development in female F344 rats as determined by pituitary weights and plasma PRL levels. H–K, effects on the survival of NMU-induced mammary tumors and their growth in SD female rats as determined by tumor volume, tumor numbers, and malignancy. L and M, BEP effects on tumor metastasis following intravenous injection of MADB106 cells in female F344 rats. Representative pictures of lungs sampled from an animal transplanted with cortical neurons (control, left) and from a BEP neuron-transplanted animal (right). Data shown in B, D, E, H, I, J, and M were analyzed using the Student t test; F and G with one-way ANOVA followed by the Newman–Keuls post hoc test; K with the χ² test. *, P < 0.05; **, P < 0.01; ***, P < 0.001, BEP versus control; a, P < 0.001, versus E2 + Cont and E2 + BEP. N = 6–18.
BEP or control neurons. In the group treated with BEP neurons, 3 of 8 animals had their tumors completely disappear. In the control group, none of the 15 animals had their tumors disappear (Fig. 1H). The volume of the remaining tumors in the BEP-treated group was less than a third of the tumor volume in control rats, and the average tumor number in the BEP-treated group was less than half of the number in the control group (Fig. 1I and J). Furthermore, control animals had primarily adenocarcinoma, whereas BEP-treated animals had mostly benign adenoma (Fig. 1K). These data suggest that BEP neuron transplants reduce the growth and survival of existing tumors.

The BEP neuronal transplant effect on tumor metastasis was determined by studying the lung retention of MADB106 mammary cancer cells following intravenous inoculation in F344 rats. Four weeks after inoculation of MADB106 cells, all rats with control transplants showed visible multiple tumor foci in lungs (Fig. 1L). A small number of animals also had a single visible tumor at the site where the tumor cells were inoculated (Supplementary Fig. S2). However, 9 of 10 BEP-transplanted animals showed no visible tumors either in the lung or any other body sites (Fig. 1M), suggesting an antimitastatic effect of BEP neurons transplantation in animals.

The capacity of BEP neuron transplants to protect against cancer cell growth and metastasis via immunomodulation is tested. The BEP transplant effect on MADB106 cells growth and metastasis was first determined in the athymic nude rats, which have a complete loss of thymus and therefore lack T cells but non-thymus-dependent immune activities are intact (14). Within several days, the subcutaneously injected MADB106 cells in the back developed into a palpable clump at the site of cell injection. Measurement of the growth of the original tumor showed that BEP transplantation decreased the tumor growth compared with control animals (Fig. 2A). In about half of the control animals, another tumor appeared at the distant side on the neck from the original tumor (Fig. 2B), indicating a metastasis ability of the MADB106 tumor cells. However, this kind of secondary tumor did not occur in any of the BEP neuron–transplanted rats. After sacrifice of these animals, we found metastasis of tumor granules in the lungs of most of the control animals (9 of 10), but in the lungs of only 2 of the 10 BEP-transplanted animals (Fig. 2C and D). Further analysis of the immunomodulatory role of BEP transplants was carried out by determining changes in the function of the innate immune system following MADB106 tumor cell inoculation in F344 rats. We found that BEP-transplanted rats had no change in the number of T cells (Fig. 2E) but had higher number of NK cells and its cytolytic activity (Fig. 2F and G) in peripheral blood than those in controls. Treatment with anti-asialo GMI antiserum, which ablated circulating NK cells, increased the number and volume of tumor foci in the lung following tumor cell inoculation in rats with control transplants, and eliminated the therapeutic benefit of BEP neuron transplantation on lung tumor clearance (Fig. 2E–J). These data suggest that BEP neuronal ability to prevent tumor growth and metastasis involves innate immune system, primarily NK cells.

A novel method to increase BEP neuronal differentiation in the hypothalamus

The data presented above and reported previously (5, 7) demonstrated a cancer-preventive effect of BEP neuron transplantation irrespective of cancer cell types. However, this rat treatment is difficult to translate into human treatment conditions because of the need for isogenic BEP neurons for transplantation in humans and because of the rapid degradation of BEP peptide and the tolerance to the opioid peptide. Hence, we evaluated whether in situ differentiation and growth of BEP neurons can be chemically induced. Previous studies have demonstrated that hypothalamic NSCs differentiate to BEP neurons following treatment with cAMP-activating agents (e.g., dbcAMP) in vitro (7). NSCs are multipotent cells that have the property of self-renewal (15). Because neuronal differentiation from NSC persists in the adult and most of neurons in the hypothalamus are derived from the proliferative neuroepithelium of the third ventricle (16), we tested whether it is possible to induce NSCs to differentiate into BEP neurons in vivo by administering dbcAMP into the third ventricle. To immobilize and ensure a slow sustainable release of dbcAMP molecules to the surrounding epithelium and the NSCs with them, we used commercially available carrier nanospheres, spherical particles about 20 nm in diameter with multiple binding sites where a chemical can be covalently attached (17). A single administration of 70 or 350 nmol of nanosphere-attached dbcAMP (N-CAMP), which maintains CAMP levels at a concentration of 2 to 10 or 10 to 50 nmol in cerebrospinal fluid in vivo (Supplementary Fig. S3) increased BEP neuron differentiation as identified by the enhanced number of BrdUrd-incorporated BEP neurons (Supplementary Fig. S4; Fig. 3A and B). Both doses of N-CAMP increased the total number of BEP neurons by about 30% to 40% in the arcuate nucleus of the hypothalamus (Fig. 3C). The N-CAMP treatment primarily enhanced the BEP neuronal differentiation as the number of another CAMP-responsive neuron, tyrosine hydroxylase (TH)-containing neurons (18), did not change in the hypothalamus (Supplementary Fig. S5A and S5B).

The N-CAMP–differentiated BEP neurons were functional for a long period of time, as levels of the BEP protein and the precursor gene POMC mRNA were maintained at about 2-fold higher and the levels of hormones of the HPA axis (CRH, ACTH, and corticosterone), which BEP inhibits, were at about 2-fold lower in N-CAMP–treated rats than those in control rats for 2 months studied (Fig. 3D–H). Plasma levels of BEP did not change in N-CAMP–treated group (control, 1.47 ± 0.19 ng/mL; 70 nmol cAMP, 1.39 ± 0.09 ng/mL; N = 7, P > 0.05), suggesting that N-CAMP treatment affected primarily the hypothalamic, but not pituitary, production of the peptide. Besides the HPA axis, the activity of the SNS was also suppressed by injection of N-CAMP, as shown by decreased plasma levels of epinephrine (control, 6.925 ± 0.89 ng/mL; 70 nmol cAMP, 3.91 ± 0.29 ng/mL; N = 7, P < 0.001) and norepinephrine (control, 14.37 ± 1.81 ng/mL; 70 nmol cAMP, 8.34 ± 0.98 ng/mL; N = 7, P < 0.001). The effectiveness of in vitro N-CAMP–differentiated BEP neurons in the hypothalamus in altering the endogenous HPA axis hormones is comparable to that achieved after transplantation of BEP neurons in the hypothalamus (5, 7).

In situ differentiated BEP neurons prevented cancer growth and metastasis

Because injection of N-CAMP into the third ventricle increased BEP neuronal activity, we tested the effectiveness of injection of these nanospheres on NMU-induced breast cancer development in SD female rats (5). Rats were injected with N-CAMP 4 weeks after NMU injection. The N-CAMP treatment significantly reduced NMU-induced mammary tumor incidence (Fig. 4A), tumor number (Fig. 4B), and tumor volume (Fig. 4C). Histopathologic
analysis of tumors revealed that control animals had more adenocarcinoma, whereas N-cAMP-injected animals had mostly benign adenoma (Fig. 4D and E).

To determine the effect of N-cAMP on metastasis, we used the MADB106 cancer cell inoculation in F344 female rats and found that about 80% of the control nanosphere–injected animals showed visible tumor foci in lungs compared with only 20% of the N-cAMP–injected rats had tumor foci in lungs (Fig. 4F and G). Furthermore, this beneficial effect of N-cAMP on preventing tumor metastasis was reversed by injection of naloxone, a competitive antagonist of the μ-opioid receptor. These observations indicated that the beneficial effect of nanospheres was acting through μ-opioid receptors, which are the primary binding sites of BEP peptide. Analysis of plasma samples drawn from these rats at the time of tumor inoculation revealed that N-cAMP decreased the corticosterone level, an indicator of stress level, whereas naloxone-treated animals had corticosterone levels similar to that in controls (Fig. 4H). These data suggest that injection of N-cAMP into the third ventricle significantly inhibited retention of mammary cancer cells in the lung, and this effect was mediated by the increased of BEP neuronal function in the animals.
Knowing that N-cAMP increases BEP peptide production in the hypothalamus and that BEP increases innate immune function (7, 19), which is critical for inhibition of cancer development, we compared the number and activity of innate immune cells in N-cAMP- and vehicle-treated rats. Animals injected with N-cAMP had increased NK cell numbers in PBMC (Fig. 4I) and in the spleen (Fig. 4J) and higher NK cytolytic activities in these cell populations (Fig. 4K and L). The N-cAMP treatment also increased percentage population and migration activities of macrophages in PBMC (Fig. 4M and O) and splenocytes (Fig. 4N and P). Together, these data suggest that injection of N-cAMP into the third ventricle significantly inhibited metastasis of mammary cancer cells into the lung, and this effect was possibly mediated through an increase in BEP, decrease of stress, and increase of innate immune function in the animals.

Increasing BEP neuronal number in the brain is beneficial to general health

We have also determined whether the added BEP neurons in the hypothalamus produce any detrimental effects on general health by determining various body parameters at various ages following BEP or control neuronal transplantation at 2 month of age in SD rats. Reproductive functions of rats with BEP transplants were normal at 4 months of age, and like control

Figure 3.
Nanosphere-delivered dbcAMP administration increased differentiation of neural stem cells into BEP neurons in the hypothalamus. A, representative fluorescent microscope photographs of BEP\(^{+}\)BrdUrd double-stained neurons (red, BrdUrd; green, BEP) in the arcuate nucleus of the animals treated with various doses of cAMP-attached nanospheres (N-cAMP). B and C, changes in the numbers of neurons double-stained with BEP and BrdUrd and the total number of BEP neurons in the arcuate nucleus of animals treated with various doses of N-cAMP. D and E, changes in BEP peptide levels and POMC levels in the hypothalamus at various time periods after 70 nmol of N-cAMP. F–H, changes in the HPA axis function as determined by hypothalamic CRH levels, pituitary ACTH levels (G), and plasma corticosterone levels (H) at various time periods after 70 nmol of N-cAMP. Data were analyzed using one-way ANOVA followed by the Newman–Keuls post hoc test. \(^*\), \(P < 0.05\), versus control; \(^{**}\), \(P < 0.01\), versus control; \(^{***}\), \(P < 0.001\), versus control. \(n = 8\).
animals, BEP neuron–transplanted female had regular estrous cycles (4–5 days) and BEP-treated male rats showed normal levels of blood testosterone (control, 3.04 ± 0.43 ng/mL, n = 8; BEP, 2.86 ± 0.61 ng/mL, n = 7). Body weights of BEP neuron–transplanted and control cell–transplanted adult male and female rats were similar until 8 months of age (male control, 643.0 ± 16.2 g, n = 9; male BEP, 657.0 ± 29.5 g, n = 9; female control, 333.8 ± 18.6 g, n = 7; female BEP, 341.8 ± 9.5 g, n = 8). At older age (14 months), rats with BEP neuron transplants when exposed to running wheels performed better during the dark period than the rats with control cell transplants (Fig. 5A). In addition, BEP neurons transplanted rats continued to improve the daily total activity with time, which was not evident in control animals (Fig. 5B). At old age, male rats with BEP transplants were leaner than those of controls (Fig. 5C). The basal blood glucose levels were similar between control and BEP neuron–transplanted rats, although animals with BEP transplants had lower glucose loads during glucose tolerance.
Figure 5.
Long-term effects of BEP neuronal transplants on various body functions. A group of male rats treated with control cell transplants or BEP neuron transplants were maintained in the animal facility for 14 months and then analyzed to determine their physical, metabolic, cardiovascular, and immune functions by using the following measures: (A and B) hourly running activity and total daily running activity; (C) body weight; (D) oral glucose tolerance; (E) mean heart rate; (F) systolic, diastolic and mean blood pressure; (G) pituitary weight; (H) liver weight; (I) spleen weight; (J) weights of prostate lobes. Data shown in C, G, H, I were analyzed with the Student t test, and data shown in A, B, D, E, F, and J were analyzed using 2-way ANOVA. Data in A: *** P < 0.001, between treatment (F = 40.13, DF = 23); data in B: *** P < 0.001, between treatment (F = 438.2, DF = 19); data in D: *** P < 0.001, between treatment (F = 95.7, DF = 6), n = 8/group.
tests (Fig. 5D). Heart rates, systolic, diastolic, and mean blood pressures of BEP and control male rats were similar (5E and F). At sacrifice, 38% of control male rats showed tumors in neck, but no visible tumors were identified in BEP neuron–transplanted animals. The weights of the pituitary, liver, prostate, and spleen were identical in control and BEP neuron–transplanted rats (Fig. 5G–J). These data suggest that increasing the number of BEP neurons in the hypothalamus in rats did not produce any deleterious effects on their general health but produced some beneficial effects on their aging-altered physical, cardiovascular, metabolic, and immune health.

Discussion

BEP neurons are involved in maintaining a variety of functions including metabolic control, a feeling of well-being, stress regulation, and immune functions (8). Abnormalities in BEP neuronal function are correlated with various pathologies associated with increased incidence of cancer (23–27). Furthermore, the endogenous function of BEP neurons is reduced in patients with cancer (6). We demonstrated that increasing BEP neuronal numbers in the hypothalamus via cell transplantation or via in situ differentiation enhances the body’s ability to control stress, increase innate immunity, and promote tumor cell clearance and tumor growth control without producing detectable side effects. One advantage of BEP neuronal transplantation is that the number of BEP cells transplanted can be precisely determined on the basis of what is needed to replenish the body’s immune defense. The drawbacks that make this technique hard to be adapted to clinical applications are the restrictions regarding use of stem cells from fetal origin and the transplant rejection caused by host versus graft response, although our study in rats suggests that NSC-derived BEP cells are not rejected in various strains of host animals, which may be due to the lack of MHC-I protein in NSCs (28). One possible solution to the cell transplantation problem is to differentiate BEP neurons from NSCs in situ in the host. The combination use of dbcAMP and a nanosphere delivery system is effective in increasing BEP neuronal population in the hypothalamus. Thus, nanosphere is a useful delivery system of dbcAMP effective in increasing BEP neuronal population in the hypothalamus via cell transplantation or via in situ differentiation enhances the body’s ability to control stress, increase innate immunity, and promote tumor cell clearance and tumor growth control without producing detectable side effects. One advantage of BEP neuronal transplantation is that the number of BEP cells transplanted can be precisely determined on the basis of what is needed to replenish the body’s immune defense. The drawbacks that make this technique hard to be adapted to clinical applications are the restrictions regarding use of stem cells from fetal origin and the transplant rejection caused by host versus graft response, although our study in rats suggests that NSC-derived BEP cells are not rejected in various strains of host animals, which may be due to the lack of MHC-I protein in NSCs (28). One possible solution to the cell transplantation problem is to differentiate BEP neurons from NSCs in situ in the host. The combination use of dbcAMP and a nanosphere delivery system is effective in increasing BEP neuronal population in the hypothalamus. Thus, nanosphere is a useful delivery system of dbcAMP for the differentiation of NSCs at a specific region in the brain and is useful in generating BEP neurons in the hypothalamus. Both transplanted BEP neurons and in situ differentiated BEP neurons persisted functioning within the hypothalamus for a long period of time. BEP neurons produce 2 major peptides, BEP and α-melanocyte-stimulating hormone (α-MSH) in the hypothalamus. It is most likely that the opioidergic system is involved in increasing the immune function because the tumor-preventive effect of transplanted or dbcAMP-differentiated BEP cells can be blocked by an opiate antagonist. The cancer-preventive role of BEP is believed to be caused by the suppression of SNS function and activation of the parasympathetic neuronal function (5), primarily resulting from an increased peripheral NK cell activity. The weight control and glucose response improvement effects during aging in BEP neuron–treated/activated rats may have been related to the influence of the melanocortin system and glucose-sensing mechanism of these neurons (20, 22). The improvement of physical activity on running wheel may be a reflection of overall better health in BEP-treated animals. Thus, the BEP cell enhancement procedure to treat cancer could have a potential therapeutic use in humans because it uses and optimizes the body’s own defense system to continuously recognize and control abnormal cell proliferation without producing any unwanted side effects. However, the aspect of pharmacologic induction of BEP in the brain by local injections may not be attractive to patients and translatable to the clinic. Hence, better delivery methods need to be developed for induction of BEP in the brain via peripheral administration of N-cAMP to achieve a more tolerable and acceptable treatment therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.K. Sarkar
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