

Ciliary neurotrophic factor recruitment of glucagon-like peptide-1 mediates neurogenesis, allowing immortalization of adult murine hypothalamic neurons

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ABSTRACT The distinct lack of cell lines derived from the adult brain is evident. Ciliary neurotrophic factor (CNTF) triggers neurogenesis in primary culture from adult mouse hypothalamus, as detected by bromodeoxyuridine and Ki67 immunostaining. Using SV-40 T-antigen, we immortalized dividing neurons and generated clonal cell lines expressing neuropeptides and receptors involved in neuroendocrine function. We hypothesized that proglucagon-derived peptides may be the mechanistic downstream effectors of CNTF due to documented neuroprotective and proliferative effects. Indeed, proglucagon gene expression was induced by CNTF, and exposure of primary cells to glucagon-like peptide-1 receptor (GLP-1) agonist, exendin-4, induced cell proliferation. Intracerebroventricular injection of CNTF into adult mice caused increased expression of proglucagon peptide in the hypothalamus. Using a specific GLP-1-receptor antagonist, we found that neurogenesis was significantly attenuated and primary culture from GLP-1-receptor-knockout mice lacked CNTF-mediated neuronal proliferation, thus linking the induction of neurogenesis in the hypothalamus to GLP-1-receptor signaling.—Belsham, D. D., Fick, L. J., Dalvi, P. S., Centeno, M.-L., Chalmers, J. A., Lee, P. K. P., Wang, Y., Drucker, D. J., Koletar, M. M. Ciliary neurotrophic factor recruitment of glucagon-like peptide-1 mediates neurogenesis allowing immortalization of adult murine hypothalamic neurons. *FASEB J.* 23, 000–000 (2009). www.fasebj.org

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CILIARY NEUROTROPHIC FACTOR (CNTF) is a member of the neuropoietic cytokine family that enhances the survival and differentiation of several classes of neurons in the central and peripheral nervous system (1).

Clinical trials in human amyotrophic lateral sclerosis (ALS) patients demonstrated that CNTF caused unexpected and substantial weight loss (ALS CNTF Treatment Study Group, ref. 2). The CNTF-mediated sustained reduction in body weight and appetite was attributed to the regeneration of hypothalamic neurons presumably involved in resetting the energy balance set point (3), although their phenotypes are not yet determined. Structurally, the hypothalamus consists of an array of fully differentiated neurons regulating many vital functions that include energy homeostasis (4). The specific cell types and neuropeptides involved in these complex functions have been extensively investigated; however, little is known about the potential for hypothalamic neurogenesis. Until recently, it was thought that neurogenesis in the adult brain was limited to the hippocampal dentate gyrus region, subventricular zone lining the lateral ventricles, and olfactory bulb (5), but recent reports indicate that the hypothalamus also possesses neuroproliferative potency (3, 6–8). Kokoeva *et al.* (3, 8) demonstrated that the hypothalamus is capable of low levels of sustained neurogenesis that can be augmented by CNTF administration. The downstream effectors/factors responsible for CNTF-induced *de novo* generation of neurons are not yet identified.

Candidates responsible for the downstream neurogenic effects of CNTF present in the hypothalamus are the proglucagon-derived peptides (PGDPs) due to their reported ability to stimulate cell proliferation. Many reports have suggested that GLP-1 action in the brain is neuro-

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protective *via* activation of antiapoptotic signaling pathways in specific neurons (9, 10), and recently it has been shown that exendin-4, a stable GLP-1-receptor agonist, is able to promote adult neurogenesis *in vitro* and *in vivo* in the subventricular zone and hippocampus (11). These findings indicate that PGDPs have great neuroproliferative potential, particularly in the hippocampus. We speculated that the PGDPs may play a role in facilitating CNTF to turn quiescent neurons or progenitors into an active proliferative state. It is this neuroproliferative effect that we anticipated would be advantageous in the generation of immortalized adult hypothalamic neurons.

Mechanistic studies of neuropeptide gene regulation and signal transduction events occurring within hypothalamic neurons are difficult to perform in the whole brain. The lack of adult neuronal cell models is currently hindering these studies. Unlike embryonic cells, which actively proliferate, fully differentiated, nonproliferating adult hypothalamic neurons are nearly impossible to immortalize, as incorporation of SV40 T-antigen requires proliferating cells (12, 13). Therefore, on the basis of CNTF inducing neuronal proliferation *in vivo*, we hypothesized that CNTF treatment could induce neurogenesis in primary hypothalamic cultures from adult mice, thus permitting immortalization of the proliferating neurons by using SV40 T-antigen.

MATERIALS AND METHODS

Animals

Wild-type C57BL/6 male mice (Charles River Canada, Montreal, PQ, Canada) or GLP-1-receptor-knockout mice with age-matched wild-type controls (14) were housed under standard vivarium conditions in a 12-h light-dark cycle with food and water available *ad libitum* for the duration of each experiment. All procedures were conducted in accordance with the regulations of the Canadian Council on Animal Care and approved by the University of Toronto Animal Care Committee.

Adult mouse primary culture

Animals 8 to 12 wk old were euthanized by CO₂ and immediately decapitated. Brains were explanted, and the individual hypothalamus was dissected and stored on ice in Hank's balanced salt solution (HBSS; Gibco Invitrogen, Carlsbad, CA, USA). Tissues were transferred to cell culture dishes, washed in 0.1 M PBS (pH 7.4), incubated for 5 min in a 37°C water bath in 0.25% trypsin (Gibco Invitrogen), and washed again with PBS. Each hypothalamus was transferred into 1 ml of adult neuronal growth medium consisting of Neurobasal-A medium (Gibco Invitrogen), B-27 supplement (1:50; Gibco Invitrogen), 0.5 mM L-glutamine (Gibco Invitrogen), 10% FBS (Hyclone, Logan, UT, USA), 5% normal horse serum (heat inactivated; Gibco Invitrogen), and 1% penicillin-streptomycin (Gibco Invitrogen). Each tissue piece was gently triturated, starting with a 1000- μ l pipette followed by a 200- μ l pipette, until uniform cellular dissociation was achieved, then incubated on ice for 3 min, eliminating larger tissue debris, and the supernatant was further diluted 1:16 in adult neuronal growth medium. Aliquots were distributed into 8-chamber

cell culture slides (BD Biosciences, San Jose, CA, USA) coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA). Cell culture slides were divided into treatment or control groups, receiving either recombinant rat CNTF in PBS (10 ng/ml; Cedarlane Laboratories Ltd., Hornby, ON, Canada), exendin 4 or exendin 9–39 (100 nM each, Phoenix Pharmaceuticals, Phoenix, AZ, USA) or PBS alone, for 7 consecutive days. The final day of treatment included 10 μ M 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich). Cell culture slides were processed for immunocytochemical detection on the following day.

Immortalization of adult hypothalamic primary cell culture and characterization

Primary cultures from 2-mo-old mouse hypothalamii were prepared as described above. The cultures were treated with 10 ng/ml recombinant rat CNTF in PBS for 5 to 7 consecutive days. Control plates without treatment were also prepared in order to determine the efficiency of antibiotic treatment for selection. The cell cultures were then immortalized, as described previously (15). The primary cells were incubated for 1 h, twice successively, with fresh virus-containing medium harvested from confluent culture of ψ 2 cells (psitex cells) harboring the intact cDNA sequence for simian virus (SV40) large T antigen and neomycin resistance gene. The producer psitex cells harboring the oncogene were cocultured with NIH3T3 cells at a 1:4 ratio. The viral supernatant was prepared at a titer between 10⁵ and 10⁶ CFU/ml. The supernatant was stored at –80 C until primary cell culture infection. Retrovirus-infected cells (after 48 h in culture medium with retrovirus) were incubated with medium containing geneticin (G418) in selective concentrations (400–600 μ g/ml for initial selection, 250 μ g/ml for cell maintenance).

Resistant colonies, appearing after 2–3 wk, were picked using cloning cylinders and further expanded. The growth curves of cloned cell lines, still representing a mixed population of hypothalamic cells, displayed a doubling time of ~24–48 h. The new clones were selected if they demonstrated predominantly neuronal lineage morphology, as small, rounded or ovoid perikarya and long neuritic processes. Generally, the cloned lines form monolayers, grow rapidly, and retain growth contact inhibition. Further expansion was performed only after evidence for expression of SV40 T antigen, neuron-specific enolase (NSE), and lack of glial fibrillary acidic protein (GFAP). Mixed populations of hypothalamic cells were further subcloned through successive dilutions of the trypsinized cells into 96-well tissue culture plates coated with poly-L-lysine. The optimal dilution allowed only 1 or 2 cells/well. The cells were incubated in conditioned medium (*i.e.*, medium was taken from the mixed cultures) at a 1:1 ratio with culture medium, DMEM with 15% FBS (Life Technologies/Gibco). Cell colonies were allowed to grow and then were successively split into 24-well plates, and finally 60-mm plates, for RNA analysis and cryopreservation. Immortalized cell lines were grown in DMEM supplemented with 10% FBS, 20 mM glucose and penicillin/streptomycin, and maintained at 37°C with 5% CO₂.

Each cell line was analyzed for the expression of specific markers by reverse transcriptase PCR (RT-PCR). First-strand cDNA was synthesized from 10 μ g of DNaseI-treated RNA, using SuperScript II RT (Life Technologies/Gibco). The RT reaction was primed with random primers. cDNA synthesis was followed by RNase H (180 U/ml) digestion of RNA in a total volume of 20 μ l. Control reactions were performed in which amplification was carried out on samples in which the RT was omitted (RT⁻). Whenever possible, primer sequences flanked an intron, as an extra control for DNA contamination. The primer sequences used have been documented previously (15). All products were sequenced to confirm identity.

Immunocytochemistry of primary cell culture

The primary culture was plated in an 8-well chamber pre-coated glass slides (BD Laboratories, Franklin Lakes, NJ, USA), as described previously. The cultured cells were rinsed briefly in PBS and fixed with 4% paraformaldehyde for 25 min at room temperature. Following fixation, cells were washed with PBS with 1% Triton X-100 and then bathed in ice-cold 1 N HCl for 10 min, then 2 N HCl for 10 min at room temperature, followed by 20 min at 37°C. Following the acid treatment to break open the DNA structure, the slides were neutralized with 0.1 M borate solution for 12 min at room temperature. The slides were then washed with PBS/1% Triton X-100. The cells were then blocked with PBS/1% Triton X-100/1 M glycine/5% BSA for 1 h. Cells were then washed in PBS and incubated at 4°C overnight in PBS/1% BSA/0.5% Triton X-100 with antibodies specific to nestin (1:200; Abcam, Cambridge, MA, USA), doublecortin (DCX; 1:200; Cell Signaling Technology, Inc., Beverly, MA, USA), neurofilament (NF; 1:200; Abcam), NSE (1:200; Abcam), Ki67 (1:200; Abcam), and BrdU (1:200; Neo Markers, Fremont, CA, USA). Cells were washed with PBS/1% Triton X-100 and incubated with FITC-conjugated or biotinylated secondary antibodies followed with Texas Red streptavidin to rabbit, mouse, and chicken (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:200; Vector Laboratories, Burlingame, CA, USA) for 60 min. Cells were poststained with DAPI present within the mounting medium (Molecular Probes, Invitrogen). BrdU-positive nuclei were counted using an Olympus fluorescent microscope (Olympus, Tokyo, Japan) in a blind study to prevent bias in the results.

Surgery and i.c.v. microinjections

At 8 wk of age, mice received a preanesthetic isoflurane, were shaved on the dorsal skull surface and cleaned with 70% isopropyl alcohol followed by 10% betadine iodine solution, then were transferred to a stereotactic apparatus (Stoelting Company, Wood Dale, IL, USA) and maintained on 0.5 to 1.0% isoflurane inhalant anesthetic. A 26-gauge stainless-steel guide cannula (Plastics One, Roanoke, VA, USA) was directed toward the midhypothalamus in the third ventricle using flat-skull coordinates from bregma (AP 0 mm, ML 0 mm, DV -5.1 mm). The guide cannula was secured with cyanoacrylate gel (Plastics One) and acrylic dental cement (Jet Denture Repair, Lang Dental Manufacturing Co., Wheeling, IL, USA); the incision was closed with 4-0 silk suture (Syneture; Tyco Healthcare Group, Mansfield, MA, USA), and the individual was allowed to recover for 7 d prior to manipulations. Mice ($n=4$ /treatment group) were divided into 3 treatment groups receiving 1 μ l of 100 ng/ml recombinant rat CNTF in saline (Cedarlane Laboratories), or saline alone, slowly infused through a 30-gauge internal cannula (Plastics One) with a 2- μ l Hamilton syringe (Fisher Scientific; Nepean, ON, Canada). Microinjections were administered over 7 consecutive days, 2 h prior to lights off each day in order to maximize the temporal effect (8). Additional BrdU (100 mg/ml i.c.v.; Sigma-Aldrich) was included on the last 4 d of injections. Daily food consumption (grams of food eaten) and body weight were recorded for each subject at time of treatment.

Tissue perfusion and sectioning

Brains were perfused through the ventricular aorta with 0.1 M PBS, followed by cold (4°C) 4% paraformaldehyde. The brains were postfixed in 4% paraformaldehyde for 4 h and cryoprotected in 15 and 30% sucrose for 48–60 h at 4°C with continuous gentle agitation before they were snap-frozen in a

isopentane bath cooled to the temperature of dry ice-ethanol and stored at -80°C. Frozen brain stems were sectioned on a cryostat in a rostral to caudal direction from the olfactory bulb to the caudal linear nucleus at the level of the substantia nigra. Sections were 25 μ m in thickness, and serial sections were collected in a cryoprotecting buffer (30% ethylene glycol, 20% glycerol in 0.05 M PBS). Every other section through the arcuate nucleus of the hypothalamus (at 25- μ m intervals) was selected for this study, and care was taken to ensure that there was similar representation of the areas from all animals. All sections were stored at -20°C until they were immunostained by specific antibodies against proglucagon and BrdU.

Proglucagon immunofluorescence in mouse brain

Immunofluorescence was performed to determine whether CNTF treatment increases the proliferation of proglucagon-expressing neurons in the hypothalamus. Free-floating sections were washed in 0.02 M KPBS. Nonspecific tissue antibody binding sites were blocked by incubating the tissue in 2% normal donkey serum (NDS) in 0.4% Triton X-100 for 1 h at room temperature. Sections were then incubated with the rabbit polyclonal anti-GLP-1 antibody (generated by D.J.D.) at a dilution of 1:1000 in 2% NDS for 48 h at 4°C. After KPBS washes, sections were incubated with FITC donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at a dilution of 1:200 in 0.4% Triton X-100 for 1 h at room temperature and darkness. Following KPBS washes, sections were mounted on slides and coverslipped with Pro-Long Gold antifade reagent (Molecular Probes). Control sections were processed by omitting the primary antibody. Tissue sections from all animals were immunostained together to ensure that the immunocytochemistry was performed identically for all of the groups. Sections were examined using a Zeiss LSM510 confocal microscope equipped with fluorescence filters (Carl Zeiss, Oberkochen, Germany). The same exposure time was used for all of the groups. The cytoarchitectonic identification of brain stem regions was based on the stereotactic atlas of the mouse brain.

Quantitative real-time RT-PCR

Cells were harvested at the indicated time points following treatment with CNTF, and total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform extraction method, as described previously (15), and reverse transcription was performed with 2.0 μ g of total RNA using SuperScript II and random primer, as described in the Superscript II cDNA Synthesis Kit (Invitrogen). Approximately 200 ng of template was used for the quantitative real-time RT-PCR that was performed with SYBR green PCR master mix (Applied Biosystems, Streetsville, ON, Canada), according to the manufacturer's instructions and run on the Applied Biosystems Prism 7000 real-time PCR machine. The SYBR primers were designed using Primer Express software (Applied Biosystems). The sequences of the primers for the γ -actin gene are as follows: actin-SYBR-F: 5'-CTTCCCCACGCCATCTTG-3' (sense) and actin-SYBR-R: 5'-CCC GTT CAG TCAG GAT CTC AT-3' (antisense). The sequences of the primers for the proglucagon gene are as follows: proglucagon-SYBR-F: 5'-GAGGAGAACCCAGATCATTCC-3' (sense) and proglucagon-SYBR-R: 5'-GTGGCGTTTGTCTTCATTCATC-3' (antisense). The proglucagon primer sequences flanked an intron, as an extra control for DNA contamination. Data were represented as C_t values, defined as the threshold cycle of PCR at which amplified product was first detected and analyzed using ABI Prism 7000 SDS software package (Ap-

plied Biosystems). Copy number of amplified Proglucagon gene was standardized to γ -actin using the standard-curve method (ABI Prism 7700 Users Bulletin). The final fold differences in proglucagon mRNA expression were relative to the corresponding time-matched control.

SDS-PAGE and Western blot analysis

Protein from confluent mHypoA-2/10 cells was prepared as described previously (16). Protein concentration was determined by the BCA protein assay kit (Thermo Scientific, Vernon Hills, IL, USA). Total protein (40 μ g) was resolved on SDS-PAGE gels and blotted onto Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA, USA). The resulting blot was blocked with 5% BSA in TBS containing 0.2% Tween 20 and incubated with primary antibody (Ab) overnight at 4°C. Phospho-STAT3 (Tyr 705, 1:1000), and phosphor-JAK2 (Tyr 1007/1008, 1:1000) antibodies were purchased from Cell Signaling Technology, and G protein β -subunit (1:1000) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoreactive bands were visualized with horseradish peroxidase-labeled secondary anti-rabbit IgG at a 1:10,000 dilution and enhanced chemiluminescence (ECL kit; GE Healthcare, New York, NY, USA), as described by the manufacturer. Protein was visualized on a Kodak Image Station 2000R using Kodak 1D Image Analysis Software 3.6 (Eastman Kodak Company, Rochester, NY, USA), allowing for the bands to be resolved within the linear range due to continuous digital imaging.

Statistical analysis

Data were analyzed using 1-way ANOVA by GraphPad Prism (GraphPad Software, San Diego, CA, USA), and statistical significance was determined using Bonferroni's multiple comparisons test or Student's *t* test with $P < 0.05$.

RESULTS

CNTF induces proliferation in adult hypothalamic primary culture

To test the feasibility of whether we could immortalize adult neurons, we first determined whether CNTF could induce neurogenesis in a dispersed cell culture environment. Following culture for 1 wk, adult murine hypothalamic primary cultures were treated with 10 ng/ml CNTF or vehicle for 7 d. At the end of the week, the BrdU labeling index was assessed in the cultures. CNTF treatment increased proliferation, as assessed by either Ki67 or BrdU (Fig. 1A). A representative Ki67 staining is presented, while quantitative analysis of BrdU staining indicated an approximate 2-fold increase in BrdU incorporation within the adult mouse primary cell cultures (Fig. 1A). In addition, we characterized the nature of the BrdU-positive cells using colabeling for neuronal differentiation markers, such as nestin, a marker of pluripotent neuronal progenitors; DCX, a marker of immature neuronal cells; and NF and NSE, markers of mature, fully developed neurons. We found that there was evidence of proliferation in all the colabeled populations, indicating that the proliferation was not limited to a certain population of cells (Fig.

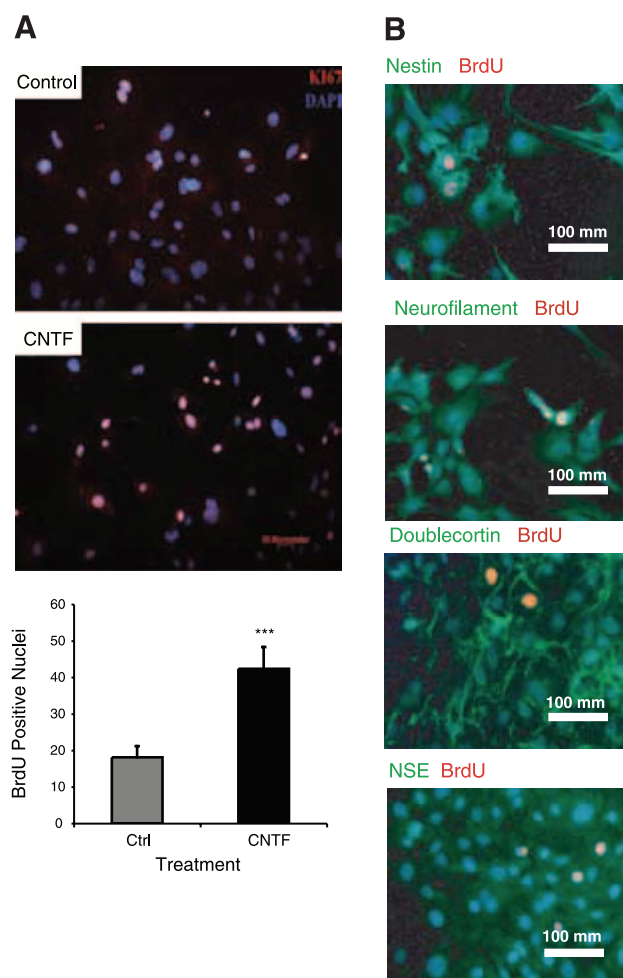


Figure 1. CNTF induces proliferation of neurons at different developmental stages. A) Adult hypothalamic primary culture was treated with either 10 ng/ml CNTF or vehicle and assessed for proliferation by either Ki67 or BrdU. Top panels: representative Ki67 immunostain, with or without CNTF treatment. Bottom panel: histogram of immunocytochemical analysis using BrdU-positive nuclei. Results are expressed as means \pm SE; $n = 4$. *** $P < 0.001$; Student's *t* test. B) CNTF and BrdU-treated adult hypothalamic primary culture were probed with antibodies against markers for neuronal differentiation, including nestin, DCX, NF, and NSE, as well as BrdU; $n = 4$. Nuclei are stained with DAPI (blue).

1B). Further analysis of the cell marker expression in primary cultures overall indicated that $\sim 10\%$ of neurons stained for NF; 20–30% for DCX; 50% for nestin; while 80–90% of neurons were immunopositive for NSE. The representative panels shown in Fig. 1B are taken from fields with high amounts of immunostaining for each marker and may not be representative of the more global immunoreactivity patterns in the primary cultures.

CNTF induces proliferation in and permits immortalization of adult hypothalamic neurons

We exploited the ability of CNTF to stimulate proliferation in primary cultures of adult mouse hypothalamus

to create adult immortalized murine hypothalamic cell lines. When we used CNTF at 10 ng/ml, primary adult hypothalamic cultures were immortalized with retroviral transfer of the SV40 T-Ag. Control primary hypothalamic cultures, not exposed to CNTF, did not exhibit any signs of proliferation, could not be immortalized by T-Ag, and resulted in complete cell death after 3 wk in culture. Following immortalization, incorporation of the SV40 T-Ag into the neuronal genome and expression was confirmed with RT-PCR. In addition, the mixed culture was screened using RT-PCR for specific markers of neuronal and glial cells, including NSE and GFAP, respectively (Fig. 2A). Both NSE and GFAP were found to be expressed in these mixed adult cultures, indicating the presence of both neural and glial cells. This heterogeneous mixture of cell types was then serially diluted to obtain individual clonal cell lines (Fig. 2B) that were then characterized *via* semiquantitative RT-PCR, revealing unique phenotypic profiles (Fig. 2C). Each of the 23 cell lines created expresses the CNTF receptor yet differentially expresses a variety of other neuropeptides, hormones, and receptors (Supplemental Table 1). We performed immunocytochemistry with the neuronal differentiation markers on one of the 23 clones, the mHypoA-2/10 neurons, to verify that they express characteristics of mature neurons. The mHypoA-2/10 neurons demonstrate robust ex-

pression of NSE and a reduced expression of nestin and DCX (Fig. 3A), indicating that the clonal, immortalized cell lines represent fully differentiated neurons.

CNTF up-regulates proglucagon expression within hypothalamic neurons

With the generation and characterization of these adult mouse hypothalamic neurons, our next goal was to determine the underlying mechanism by which CNTF induces neurogenesis, thereby permitting the immortalization of these cells. Therefore, we analyzed the PDGPs because of their proliferative effects within the brain and gut (17–19). We first conducted experiments with the mHypoA2/10 neurons, one of the newly immortalized proglucagon-expressing cell lines. Utilizing these neurons, we defined CNTF responsiveness by analyzing known signaling responses on activation of the CNTF receptor. Using Western blot analysis and phospho-specific antibodies, we found that both Janus kinase 2 and signal transducers and activators of transcription 3 (JAK2/STAT3) were activated on CNTF stimulation (Fig. 3B). We then characterized the regulation of proglucagon gene expression by CNTF in the mHypoE-2/10 immortalized neurons. The mHypoA2/10 neurons were treated with 10 ng/ml CNTF; total RNA

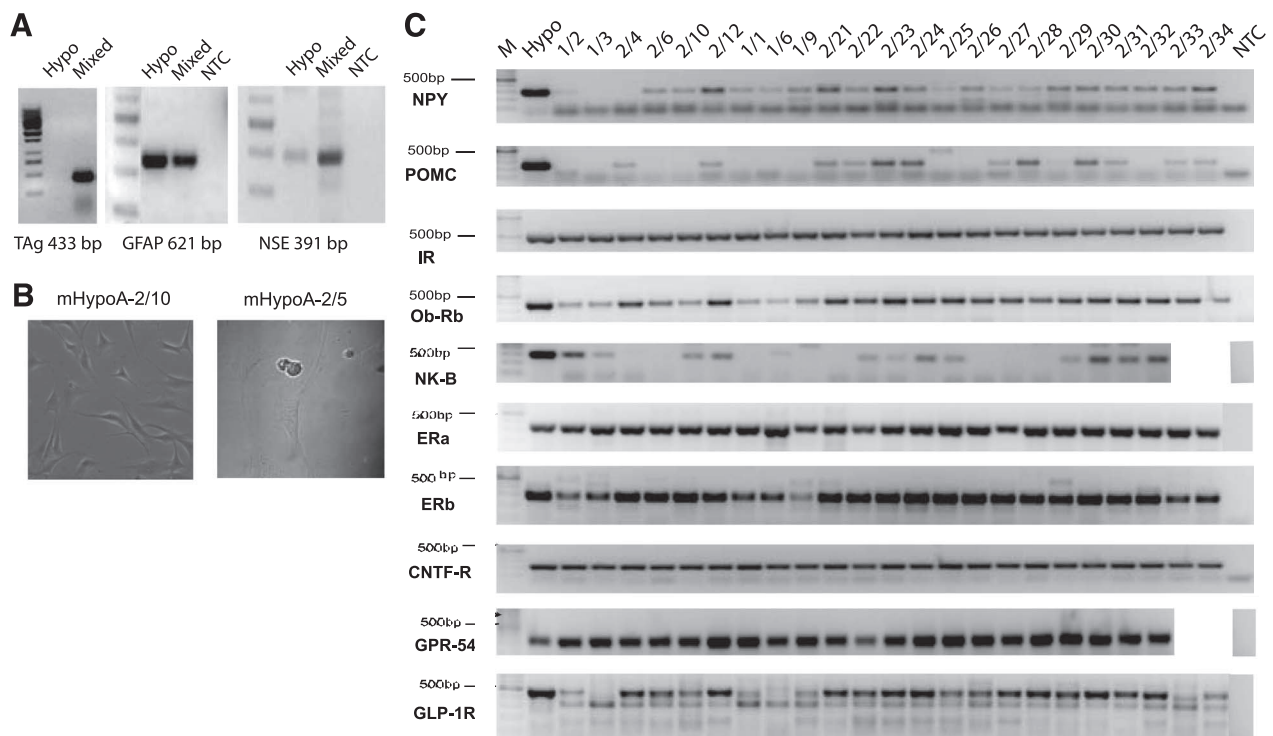


Figure 2. Generation and characterization of adult, immortalized hypothalamic cell lines. *A*) Mixed immortalized cell lines derived from adult hypothalamic primary cultures were screened by RT-PCR for the presence of the immortalization factor T-Antigen (T-Ag), the glial marker GFAP, or a neuron-specific marker, NSE. *B*) Phase-contrast image of live mHypoA-2/10 neurons and a magnified differential interference contrast confocal image of live mHypoA-2/5 neurons. *C*) The array of adult hypothalamic neurons was screened for 28 markers by RT-PCR, 10 of which are shown here. NPY, neuropeptide Y; POMC, proopiomelanocortin; IR, insulin receptor; ObRb, leptin receptor, long form b; NK-B, neurokinin B; ER, estrogen receptor; CNTF-R, ciliary neurotropic factor receptor; GPR-54, G-protein-coupled receptor 54 or Kisspeptin receptor; GLP-1 R, glucagon-like peptide-1 receptor; Hypo, hypothalamus tissue; NTC, no template control; M, molecular weight marker.

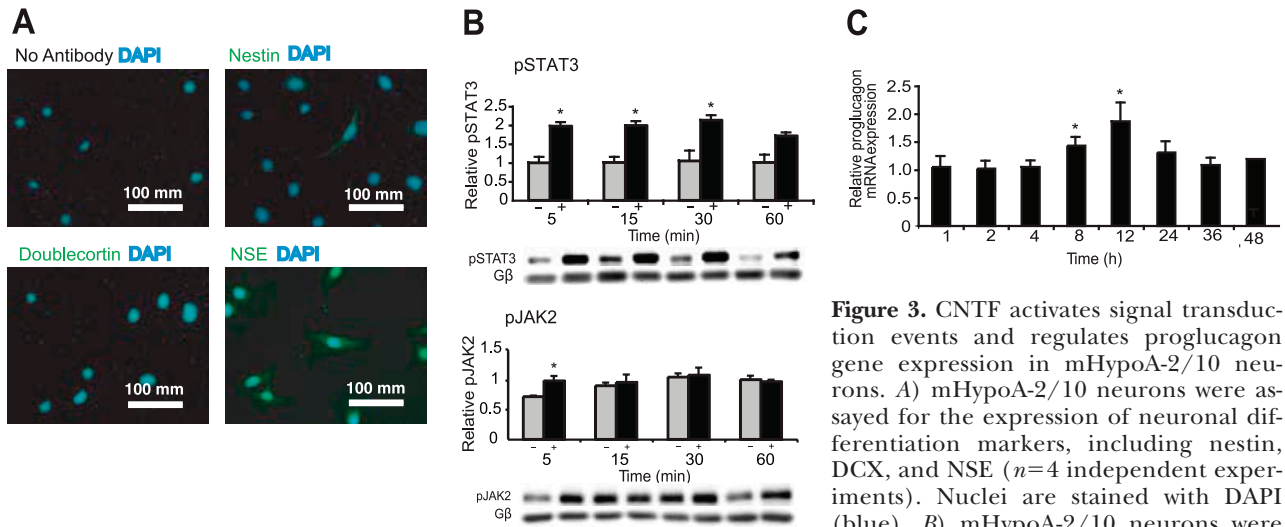


Figure 3. CNTF activates signal transduction events and regulates proglucagon gene expression in mHypoA-2/10 neurons. *A*) mHypoA-2/10 neurons were assayed for the expression of neuronal differentiation markers, including nestin, DCX, and NSE ($n=4$ independent experiments). Nuclei are stained with DAPI (blue). *B*) mHypoA-2/10 neurons were serum starved for 12–16 h before treatment

with 10 ng/ml (0.45 nM) CNTF (+) or vehicle alone (–) over a 60-min time course. Western blot analysis of cell lysates was performed using phospho-specific antibodies directed against STAT3 (Tyr 705) and JAK2 (Tyr 1007/1008). All results are normalized to G- protein β subunit and are expressed as means \pm SE; $n = 4$ independent experiments. Representative Western blots are shown. *C*) mHypoA-2/10 neurons were exposed to 10 ng/ml (0.45 nM) CNTF, and real-time RT-PCR was performed over a 48-h time course. Real-time RT-PCR with primers specifically designed to amplify proglucagon mRNA was performed. Proglucagon mRNA levels were quantified using the CT method and normalized to the internal control (γ -actin). All results are relative to corresponding control mRNA levels (set to 1.0) at each time point and are expressed as means \pm SE; $n = 4$ independent experiments. * $P < 0.05$.

was harvested over a 48-h period, and then quantitative real-time RT-PCR was performed. We found that CNTF up-regulated proglucagon gene expression between 8 and 12 h posttreatment ($P < 0.05$; Fig. 3C).

To demonstrate that CNTF could up-regulate the expression of proglucagon, and thus PGDPs, *in vivo* in the intact hypothalamus, we treated i.c.v. cannulated mice with 100 ng/ml CNTF over 7 d. The mice had comparable food intake, but those treated with 100 ng/ml CNTF had an average weight loss of ~ 3 g over 7 d. Through immunohistochemistry, we found that CNTF induced the expression of GLP-1 immunoreactivity in the hypothalamus, particularly the periventricular and dorsomedial nuclei of the hypothalamus (Fig. 4). The antibody used was generated toward a GLP-1 antigen; however, it will also detect the unprocessed proglucagon peptide if present in these cells.

Blockade of GLP-1-receptor signaling abrogates both basal neuronal proliferation and CNTF-induced proliferation

Once we established the CNTF-mediated up-regulation of proglucagon gene expression within our immortalized cell line and *in vivo* hypothalamus, we assayed the effects of the proglucagon peptides, specifically GLP-1, in primary hypothalamic neurons. Although it is likely that both GLP-1 and GLP-2 are processed by prohormone convertase 1/3 and released from the same neurons, we postulated that it was more likely that GLP-1 was inducing proliferation due to the high levels of GLP-1 receptors in the hypothalamus compared to the scarce expression of GLP-2 receptors in this same

region. To verify that GLP-1 is involved in the CNTF-induced neurogenesis in the hypothalamus, we treated primary adult hypothalamic culture with exendin-4, a GLP-1-receptor agonist, for 1 wk following a 1-wk acclimatization period and then performed immunocytochemistry for BrdU or Ki67 staining. Exendin-4 (100 nM) led to a 2-fold increase in the number of BrdU-positive cells (Fig. 5A), indicating that GLP-1-receptor signaling is involved in neurogenesis. To confirm this, we treated acclimated primary adult hypothalamic tissue with exendin 9–39 amide, a GLP-1-receptor antagonist, to determine whether GLP-1-receptor signaling was required for basal neurogenesis. We found that pretreatment of the primary culture with exendin 9-39 amide, and then throughout the 7-d treatment period, decreased Ki67 staining by 80% as compared to vehicle controls (Fig. 5B). Therefore, it is likely that CNTF acts through proglucagon expression to promote neurogenesis within the adult hypothalamus.

To link GLP-1-receptor signaling directly to the CNTF-mediated induction of neuronal proliferation, we used primary cell culture derived from either GLP-1-receptor-knockout mice (14) or their age-matched controls. Primary cultures were established as described above and then treated for 7 consecutive days with 10 ng/ml CNTF. The neurons were then exposed to BrdU and proliferation was quantified *in vitro*. The CNTF-treated cultures from the control mice displayed a significant increase in BrdU-positive neurons per well, whereas the cultures derived from GLP-1R KO mice did not exhibit an increase in BrdU incorporation (Fig. 5C).

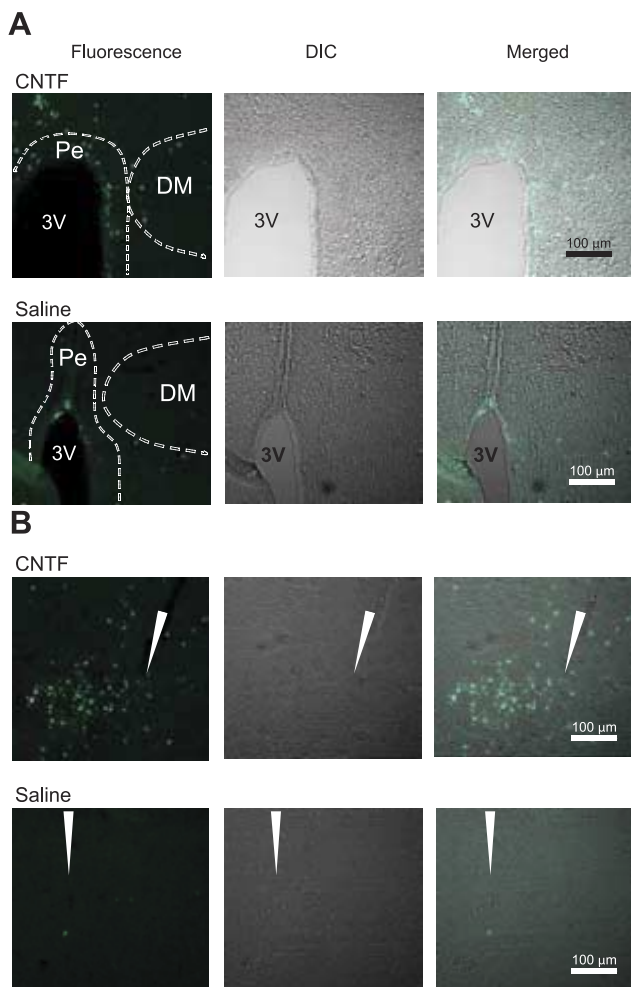


Figure 4. CNTF induces expression of proglucagon peptide in the adult mouse hypothalamus. Representative confocal immunofluorescence and differential interference contrast (DIC) images of coronal brain sections at the level of the hypothalamic periventricular (Pe) and dorsomedial (DM) nuclei (A) or the cannula injection site stained with an antibody against proglucagon (B). Mice received i.c.v. micro-injections of either CNTF (100 ng/ml) or saline ($n=4$ each). CNTF-treated animals exhibited increased proglucagon-immunoreactive cells at the area of the nuclei or at the injection site compared with saline-treated animals. White arrow indicates the cannula track.

DISCUSSION

CNTF is expressed in glial cells within the central and peripheral nervous systems and is thought to convey its cytoprotective effects after release from adult glial cells by some mechanism induced by injury. CNTF stimulates gene expression, cell survival, or differentiation in a variety of neuronal cell types, such as sensory, sympathetic, ciliary, and motor neurons. Owing to this effect on motor neurons (20), it was investigated as a treatment for ALS, in which it was found to induce weight loss, later found to be due to its potential to induce neurogenesis (3). Exploiting the neurogenic effects of CNTF to generate the adult clonal hypothalamic cell lines represents an innovation in the field of neuronal

cell line creation. Until now, no one has succeeded in immortalizing adult neurons. The immortalization method used, retroviral delivery of SV40 T antigen, requires the dissolution of the nuclear envelope that occurs during mitosis for integration into the genome. Treatment of primary culture with CNTF allows the integration event to occur, triggering cell proliferation or neurogenesis (3, 8). During neurogenic proliferation, the dividing cells express specific differentiation factors, which represent different stages in neuronal development. These factors include nestin, an intermediate filament that is expressed within neuronal/glia pluripotent progenitor cells (21); DCX, which is a microtubule associated protein that is expressed in differentiating, but not fully differentiated neurons (22, 23); and NSE and NF, which represent factors expressed in mature, fully-differentiated neurons (24, 25). Immunocytochemistry indicated that all of these factors are expressed in cells that had previous evidence of division or were actively dividing. One of these factors, nestin, demonstrated extensive expression within both the vehicle and CNTF-treated primary neuronal culture shortly after plating. This expression diminished markedly after a week of acclimatization. It appears that the stress of harvest, trituration, and plating of the primary culture induced nestin expression. A similar increase in nestin expression has been described in the rat brain after ischemia (26).

Previous research with CNTF indicates that the neurons generated as a result of CNTF exposure function as mature adult neurons (3, 8). CNTF-treated mice demonstrate a persistent anorectic effect well after the cessation of CNTF treatment, and this effect was purported to be due to the increased number of anorexigenic neurons within the hypothalamus (3). Neurogenesis is not restricted to a specific neuronal phenotype or nuclei but occurs throughout the hypothalamus. When treated with CNTF, the hypothalamus exhibits broad-spectrum cell division (8). Accordingly, it is reasonable to assume that the immortalization permitted by CNTF administration allowed for the generation of many different neuronal phenotypes. The preliminary screening experiments corroborated this hypothesis. The cell lines express a wide variety of receptors, protein, and neuropeptides; however, every cell line exhibited expression of the CNTF receptor—a fact that indicates that each cell line is responsive to CNTF, even while being phenotypically distinct and representative of the heterogeneous nature of the intact hypothalamus.

CNTF treatment up-regulates the neuronal expression of the proglucagon gene. PGDPs play a powerful role in the activation of neurogenesis within the hypothalamus. PGDPs have been detected in the mammalian brain, and indeed, the proglucagon gene has been shown to be expressed in the hypothalamus (27–29). Proglucagon is a larger precursor molecule that undergoes tissue-specific processing to generate PGDPs, including GLP-1, GLP-2, glicentin, oxyntomodulin, and

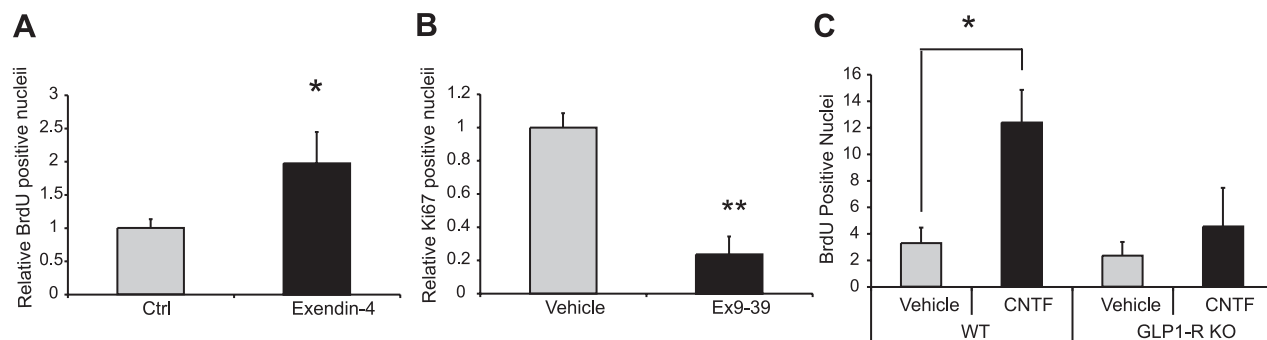


Figure 5. Effects of GLP-1 signaling and blockade on hypothalamic neurogenesis. *A*) Adult hypothalamic primary culture was treated with 100 nM exendin-4, a GLP-1 analog, for 7 d. During immunocytochemical analysis, BrdU-positive nuclei were counted. Results are expressed as means \pm SE; $n = 4$. *B*) Adult hypothalamic primary culture was treated with exendin 9-39, a GLP-1-receptor antagonist, for 7 d. During immunocytochemical analysis, Ki67-positive nuclei were counted. Results are expressed as means \pm SE; $n = 4$. *C*) Adult hypothalamic primary culture was generated from GLP-1-receptor-knockout (GLP-1-R KO) mice and age-matched control hypothalamii. Cultures were treated with 10 ng/ml (0.45 nM) CNTF for 7 consecutive days. During immunocytochemical analysis, BrdU-positive nuclei were counted. Results are expressed as means \pm SE; $n = 3$, with 8–24 wells/experiment counted. * $P < 0.05$; ** $P < 0.01$.

several others in the α -cells of the pancreatic islets, the intestinal L cells, and the brain (30, 31). Although the best-characterized actions of GLP-1 and GLP-2 are the regulation of glucose homeostasis and intestinal function, respectively, other physiological roles have also been described. Other actions of GLP-1 include islet β -cell proliferation, differentiation, and inhibition of apoptosis (32), whereas GLP-2 inhibits enterocyte and crypt-cell apoptosis (17). Outside the GI tract, GLP-2 stimulates cell proliferation in rat astrocyte cell cultures (18). Both GLP-1 and GLP-2 have been implicated in the regulation of feeding behavior in the hypothalamus through unidentified mechanisms (33). In fact, the GLP-1 receptor is highly expressed in the hypothalamus (34). Within the pancreas and intestine, the expression of GLP-1 leads to cell protection and proliferation, and it is now confirmed that some PGDPs have a similar effect within the central nervous system (35). Remarkably, it is still debated whether proglucagon is endogenously expressed in the mouse brain anywhere other than in the brain stem under basal conditions, although elegant studies from the late 1980s have demonstrated expression in the hypothalamus (27–29). We speculate that on an immune insult or injury, CNTF is released from glial cells, thereby increasing proglucagon levels in the hypothalamus. Interestingly, obesity increases cytokine levels, including leptin and other proinflammatory cytokines (36) and may trigger CNTF increases within the brain. Neurogenesis may, therefore, be a physiological response to the pathological condition of obesity, increasing the number of neurons that can regulate feeding behavior and energy homeostasis. Within the rodent brain, exendin-4 triggers neurogenesis within the adult subventricular zone (10, 11). Our results indicate that signaling through the GLP-1 receptor is important for the downstream effects of CNTF-mediated neurogenesis in primary culture. We have shown that the proliferative effects of the PGDPs may be activated by CNTF admin-

istration and were able to utilize this action to immortalize adult neurons.

The mammalian central nervous system is the most complex organ system with an estimated 10^{12} different neurons that are considered biochemically and phenotypically unique (37). The hypothalamus is a critical part of the brain that is considered by many to be the life control system in the body. Knowledge of the control mechanisms of unique peptidergic neurons from these brain regions is critical before we can understand how the brain achieves its diverse central control of basic physiology. The lack of appropriate cell models is currently hindering these studies, as analysis of brain slices or whole animal experimentation yields limited, and often conflicting, mechanistic data. Non-transformed primary hypothalamic cultures are difficult to maintain, have a short life span, and represent a heterogeneous neuronal and glial cell population, usually with a minimal number of healthy peptide-secreting neurons. Further, the cost of maintaining primary cultures, through either animal colonies or direct purchase, is often limiting.

Historically, it has proven to be difficult to establish immortalized hypothalamic cell lines, because of the lack of naturally occurring CNS tumors and the inherent difficulty of transforming or immortalizing highly differentiated neurons from primary culture (13). Cell lines from the peripheral nervous system have been established from neuroblastomas, such as the Neuro2A cell line, and pheochromocytomas, such as the PC12 cell line; however these models are not truly representative of differentiated CNS neurons. Previous infection of primary cultures of hypothalamic tissue from embryonic day 14 with SV40 large T-antigen in the early 1970s produced cell lines that were not considered fully differentiated (12). On the other hand, targeted tumorigenesis in transgenic mice has been used successfully to establish cell lines in specific tissues, such as the anterior pituitary and pancreas (38–43), and directed

tumorigenesis technique was used to develop a murine immortal cell line of gonadotropin-releasing hormone-secreting hypothalamic neurons (44). However, cell models from the hypothalamus, and from the entire brain for that matter, consist of a few isolated cell types, and represent an infinitesimal percentage of the neuronal phenotypes represented within the brain.

Therefore, our laboratory generated an array of immortalized cell models from mouse embryonic hypothalamus using retroviral transfer of SV40 T-antigen (15). These cell models express many specific neuropeptides and receptors involved in neuroendocrine function and have been used extensively as models for cellular and molecular analysis of hypothalamic function (45–49). Although these embryonic hypothalamic models are extremely useful in understanding the cellular biology of specific neuroendocrine cells, it is still not completely known whether these immortalized embryonic neurons differ from adult neurons in the basic control mechanism involved in neuronal function. It is quite important to understand that the adult, or fully differentiated neuron, may function differently than a fetal neuron. Further, adult neurons may respond differently to drugs and hormones than embryonic neurons. These new cell lines will allow further analysis of hypothalamic function based on models representative of the adult hypothalamus. **[F]**

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