Neurogenesis in the Hypothalamus of Adult Mice: Potential Role in Energy Balance

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Supporting Online Material
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Neurogenesis in the Hypothalamus of Adult Mice: Potential Role in Energy Balance

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Ciliary neurotrophic factor (CNTF) induces weight loss in obese rodents and humans, and for reasons that are not understood, its effects persist after the cessation of treatment. Here we demonstrate that centrally administered CNTF induces cell proliferation in feeding centers of the murine hypothalamus. Many of the newborn cells express neuronal markers and show functional phenotypes relevant for energy-balance control, including a capacity for leptin-induced phosphorylation of signal transducer and activator of transcription 3 (STAT3). Co-administration of the mitotic blocker cytosine-β-D-arabinofuranoside (Ara-C) eliminates the proliferation of neural cells and abrogates the long-term, but not the short-term, effect of CNTF on body weight. These findings link the sustained effect of CNTF on energy balance to hypothalamic neurogenesis and suggest that regulated hypothalamic neurogenesis in adult mice may play a previously unappreciated role in physiology and disease.

The obesity epidemic has prompted major efforts to develop safe and effective therapies (*1*, *2*). However, approved drugs for obesity have limited efficacy and act only acutely, with patients rapidly regaining weight after terminating treatment (*3*). Only the neurocytokine ciliary neurotrophic factor (CNTF) and Axokine, an analog of CNTF developed as a drug candidate for the treatment of obesity, appear to deviate from this paradigm. Rodents and patients treated with Axokine were reported to maintain lowered body weights weeks to months after the cessation of treatment (*4*, *5*). This feature of Axokine/CNTF action is unexplained and suggests that CNTF induces long-lasting changes in one or more elements of the energy-balance circuitry.

In rodents, CNTF is most potent when administered directly into the cerebrospinal fluid (*6*) and activates signaling cascades in hypothalamic nuclei involved in feeding control (*5*, *7*, *8*). For instance, CNTF activates phosphorylation of signal transducer and activator of transcription 3 (STAT3) in a population of hypothalamic neurons that substantially overlaps with those activated by leptin (*5*). However, in contrast to CNTF, leptin-treated animals do not maintain their lowered body weight after the cessation of treatment. We thus sought a CNTF-specific mechanism to explain this long-term effect.

CNTF supports the survival of neurons in vitro and in vivo (*9*) and has also been implicated in the maintenance of adult neural stem cells (*10*). Furthermore, other trophic factors, such as epidermal growth factor and fibroblast growth factor 2, are known to act as mitogens on adult neuronal progenitors (*11*, *12*), and they promote the functional regeneration of hippocampal pyramidal neurons (*13*). Neurogenesis in the adult brain is most clearly defined in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal formation (*14*). However, recent reports indicate that the neuroproliferative potency in the adult extends to other brain structures, including the hypothalamus (*15*–*17*). On the basis of these findings, we hypothesized that the long-term effect of CNTF on body-weight regulation might involve neurogenesis in the hypothalamus, which is the brain region most relevant for energy-balance regulation.

To assess the mitogenic potency of CNTF in the adult nervous system in vivo, we delivered the cell-proliferation marker bromodeoxyuridine (BrdU) alone (vehicle treatment) or together with CNTF directly into the cerebrospinal fluid of mouse brains (*18*). CNTF and BrdU were continuously infused for 7 days into the right lateral ventricle using osmotic minipumps. Mice were switched to a high-fat diet two months before surgery and were kept on this diet throughout the experiments. In accordance with previous results (*5*), CNTF-treated mice showed a marked reduction in body weights (Fig. 1A), which persisted after termination of CNTF delivery. Mice were killed 22 days after surgery, and brain sections were immunostained with an antibody against BrdU. Because BrdU incorporates into DNA of dividing cells, BrdU-positive ($BrdU^+$) cells are thought to represent newborn cells. Figure 1B shows coronal sections of vehicle- and CNTF-infused animals at the level of the arcuate, ventromedial, and dorsomedial nuclei, well-known hypothalamic centers for energy-balance regulation (*19*). In vehicle-infused animals, few $BrdU^+$ cells were detected in the parenchyma surrounding the third ventricle (Fig. 1B, left). Administration with CNTF led to a dramatic increase of BrdU+ cells (Fig. 1B, right). Note the higher density of $BrdU^+$ cells at the base of the third ventricle, which is part of the arcuate nucleus/median eminence.

The pattern of CNTF receptor (CNTFR) mRNA expression is consistent with this observation. In situ hybridization using a riboprobe against CNTFR mRNA revealed strong staining in the walls of the basal third ventricle and surrounding arcuate nucleus parenchyma (Fig. 1C). Because this section originated from an animal treated with both CNTF and BrdU, we colabeled with antibodies to BrdU. Many $BrdU^+$ cells were positive for CNTFR expression, indicating that CNTF, at least in part, directly promotes cell division by binding to CNTFR on putative neural progenitor cells (Fig. 1D, inset). By counting all newly generated cells in the caudal hypothalamus, CNTF treatment led to a marked increase of $BrdU^+$ cells over vehicle-infused animals (Fig. 1E). The total number of $BrdU^+$ cells in CNTF-treated animals remained constant for at least 2 weeks after the infusion period. Subsequently, the numbers decreased but plateaued at a high level. Vehicle-infused animals showed a similar fractional decrease over time. Thus it appears that the majority of hypothalamic $BrdU^+$ cells do not die or migrate to distant areas as reported for newborn neurons of the SVZ, which follow the rostral migratory stream toward the olfactory bulb (*20*).

To investigate the origin of adult-born cells in the hypothalamus, we examined CNTF and vehicle-infused brains every 12 hours starting 48 hours after surgery, a time when the infused CNTF/BrdU should just reach the ventricular system (*18*). Hypothalamic BrdU incorporation was first detected 60 to 72 hours

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after surgery (fig. S1). Even at these early time points, BrdU$^+$ cells were found scattered within the parenchyma, suggesting that at least a fraction of the newly generated cells, endogenous and CNTF-induced, arise within hypothalamic parenchyma distant from the ependymal lining. Also at these early time points, BrdU$^+$ cells were often observed as close contacting pairs, suggestive of recently divided daughter cells (fig. S1B, insets).

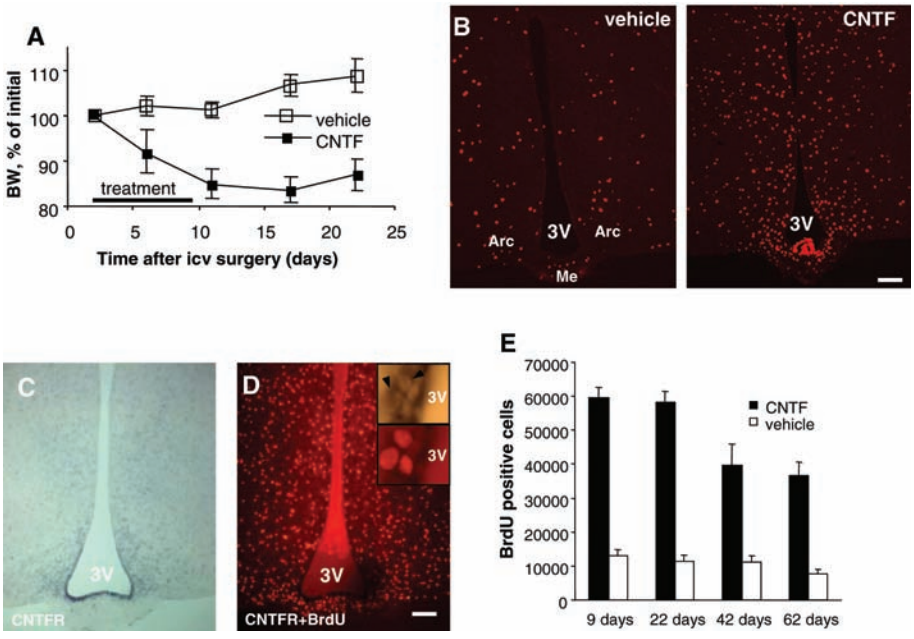

Fig. 1. CNTF reduces body weights long term and induces cell proliferation in the hypothalamus. (**A**) Mice were icv infused for 7 days with BrdU (12 μg/day) in artificial cerebrospinal fluid alone or together with CNTF (0.75 μg/day) at a flow rate of 12 μl/day. Body weight (BW) is shown as percentage difference from initial body weight. All data are mean ± SEM (n = 5 animals per group). (**B**) BrdU-labeled cells in coronal sections of the hypothalamus on the level of the arcuate nucleus. (**C**) In situ hybridization with a digoxygenin-labeled probe directed against CNTFR mRNA. Blue precipitate indicates staining. (**D**) Fluorescence image of the same section reveals BrdU$^+$ cells (red). (Insets) High-power magnification of BrdU$^+$ cells that express CNTFR (arrowheads). (**E**) Total number of BrdU$^+$ cells detected in the caudal hypothalamus of vehicle- and CNTF-infused animals. Brains were inspected at the indicated times after surgery. Error bars represent mean ± SEM (n = 3 animals per group). 3V, third ventricle; Arc, arcuate nucleus; Me, median eminence. Scale bars, 100 μm

We next explored the phenotype of hypothalamic BrdU$^+$ cells by using immunofluorescence double staining combined with confocal microscopy on hypothalamic sections of CNTF-infused animals. Double labeling with an antibody against Hu, which is a marker for immature and mature neurons that labels nuclei and perikarya (*21*), indicates that a substantial number of hypothalamic BrdU$^+$ cells take on a neuronal fate (Fig. 2A). Three-dimensional (3D) reconstruction using multiple confocal images clearly demonstrates that BrdU$^+$ cells express Hu (Fig. 2B). Based on confocal analysis of brain sections from CNTF-infused animals 42 days after surgery, 42.7% (±8.8) of the BrdU$^+$ cells in the caudal hypothalamus expressed Hu. Vehicle-infused animals had 20.7% (±9.6) co-labeled cells. Colabeling with an antibody against β-tubulin type III (TuJ1) (*22*), another marker for immature and mature neurons, confirmed these results (fig. S2, A and B). Another population of newborn cells could be assigned to a glial phenotype of the oligodendrocyte lineage (Fig. 2, C and D). Confocal analysis of CNTF-infused brains 42 days after surgery revealed that 22.9% (±6.0) of hypothalamic BrdU$^+$ cells expressed the oligodendrocyte marker adenomatosis polyposis coli (APC) (*23*). The percentage of colabeled cells was not substantially different in vehicle-infused animals (31.7 ± 12.1%). In contrast, we detected few if any BrdU$^+$ cells expressing the astrocytic marker glial fibrillary acidic protein (GFAP) (fig. S2C).

We also tested hypothalamic BrdU$^+$ cells for the expression of doublecortin (Dcx), a transient marker for early postmitotic neurons (*24*). In contrast to Hu and TuJ1, Dcx is expressed in migrating and differentiating neurons but not in mature neurons. Because Dcx is a microtubule-associated protein present in

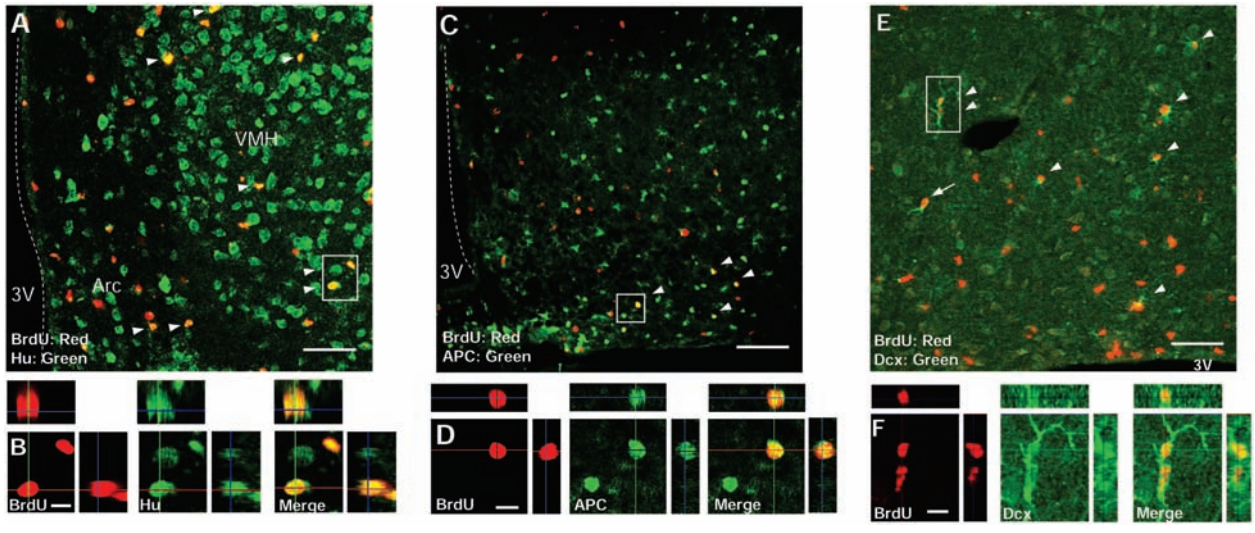

Fig. 2. Newborn hypothalamic cells exhibit neuronal and glial phenotypes. Brains were perfused 42 days (A to D) or 9 days (E and F) after icv surgery and immunolabeled sections of the caudal hypothalamus were inspected by laser-scanning confocal microscopy. (**A**) Numerous BrdU$^+$ cells (red) express the neuronal marker Hu (green, arrowheads). (**B**) Confocal 3D reconstruction of area boxed in (A). Top, x-z plane; right, y-z plane. (**C**) BrdU$^+$ cells expressing APC (arrowheads). (**D**) 3D reconstruction of area boxed in (C). (**E**) BrdU$^+$ (red) cells expressing the Dcx (green, arrowheads). (**F**) 3D reconstruction of the area boxed in (E). VMH, ventromedial hypothalamus. Scale bars in (A), (C), and (E), 50 μm; in (B), (D), and (F), 10 μm.

projections, antibodies to Dcx allow the visualization of dendritic arborization in immature neurons. Immunohistochemical inspection of hypothalamic brain sections from CNTF-treated animals revealed a large number of BrdU$^+$ cells expressing Dcx 9 days after surgery (Fig. 2E, arrowheads), estimated to match the number of BrdU$^+$/Hu$^+$ cells. Some BrdU$^+$/Dcx$^+$ cells exhibited fusiform shapes with a single process extending from their somata (Fig. 2E, arrow). Others displayed more complex morphologies with many often-arborized projections (Fig. 2F), which is a possible indication that these cells functionally integrate into the hypothalamic circuitry.

To determine whether newborn hypothalamic cells exhibit a critical functional phenotype relevant to energy-balance regulation, we used an antibody to phosphorylated (p) STAT3, a component of the leptin-activated signaling cascade in leptin receptor–containing cells of the hypothalamus (*25*). This cascade is a key signaling circuit for energy-balance regulation in hypothalamic feeding centers (*26*). Injection of leptin intraperitoneally (ip) induces STAT3 phosphorylation specifically in the arcuate, ventromedial, and dorsomedial nuclei of the hypothalamus (*27*, *28*). To induce STAT3 phosphorylation, we injected mice ip with leptin after overnight fasting, and 45 min later, we perfused them for immunohistochemical analysis. Labeling with antibody to pSTAT3 revealed strong nuclear staining throughout the arcuate, ventromedial, and dorsomedial nuclei in leptin-treated animals (fig. S3A). In contrast, the signal was virtually absent in saline-treated mice, confirming the specificity of the antibody to pSTAT3 (fig. S3B). We next applied this treatment to CNTF-infused mice 42 days after surgery. In these animals, many of the hypothalamic BrdU$^+$ cells were pSTAT3$^+$ after leptin treatment, indicating that these newborn cells acquired responsiveness to leptin (Fig. 3, A and B). 26.3% (±6.4) of all newly born cells confined to the arcuate, ventromedial, and dorsomedial nuclei were pSTAT3$^+$. A similar fraction of colabeled cells, 21.5% (±7.1), could be detected in vehicle-infused animals, indicating that CNTF-treatment substantially increases the absolute number of leptin-sensitive pSTAT3$^+$ cells.

If leptin-responsive STAT3 phosphorylation within newborn hypothalamic neurons is critical for the sustained weight-loss effect of CNTF, then mice lacking leptin signaling should show an altered CNTF response. To test this hypothesis, we intracerebroventricularly (icv) infused ob/ob mice, which lack endogenous leptin (*29*), with CNTF or leptin (Fig. 3C). CNTF enhanced cell proliferation in the hypothalami of ob/ob mice (fig. S4) similarly to mice with diet-induced obesity (DIO). However, in contrast to DIO mice, ob/ob mice did not maintain lowered body weights resulting from CNTF treatment. Instead, they regained weight shortly after drug cessation, similar to leptin-treated animals (Fig. 3C). For comparison, wild-type mice with DIO treated with an equal dose of CNTF displayed weight loss that was sustained well beyond treatment cessation (Fig. 3C). Consistent with our data, db/db mice lacking leptin receptors rapidly regained body weight after termination of peripheral CNTF treatment (*30*).

Neuropeptide Y (NPY) and pro-opiomelanocortin (POMC)–expressing neurons in the arcuate nucleus play crucial antagonistic roles in the regulation of energy balance (*19*). To assess whether BrdU$^+$ cells in the arcuate nucleus express either of these markers, we combined anti-BrdU immunolabeling and in situ hybridization using digoxigenin-labeled riboprobes against NPY or POMC mRNAs. In CNTF-infused animals 42 days after surgery, we identified several BrdU$^+$ cells per brain section expressing NPY or POMC (Fig. 4). Although the role of these specific neurons in the sustained CNTF effect is unknown, it is clear that CNTF can induce neurogenesis within neurocircuitry that is critical to energy balance in the adult mouse hypothalamus.

To investigate whether the stimulatory effect of CNTF on neurogenesis/cell proliferation underlies its ability to induce long-term weight loss, we used the antimitotic drug Ara-C (cytosine-β-D-arabinofuranoside), which prevents neural progenitor cells of the adult SVZ from dividing when centrally administered (*31*). Mice kept for 2 months on a high-fat diet were infused with Ara-C and/or CNTF into the lateral ventricle for 7 days. Inspection of brains 9 days after surgery revealed that, as before, CNTF treatment markedly increased the number of newborn cells (Fig. 5A). In contrast, we detected few if any BrdU$^+$ cells throughout the brain parenchyma of animals exposed to Ara-C (Fig. 5A). Thus, Ara-C efficiently blocks cell proliferation in the adult mouse brain, including the hypothalamus.

We next explored energy-balance regulation in the Ara-C–treated animals. As before, mice receiving CNTF alone had reduced body weights compared with animals infused with vehicle only or Ara-C only, and this was

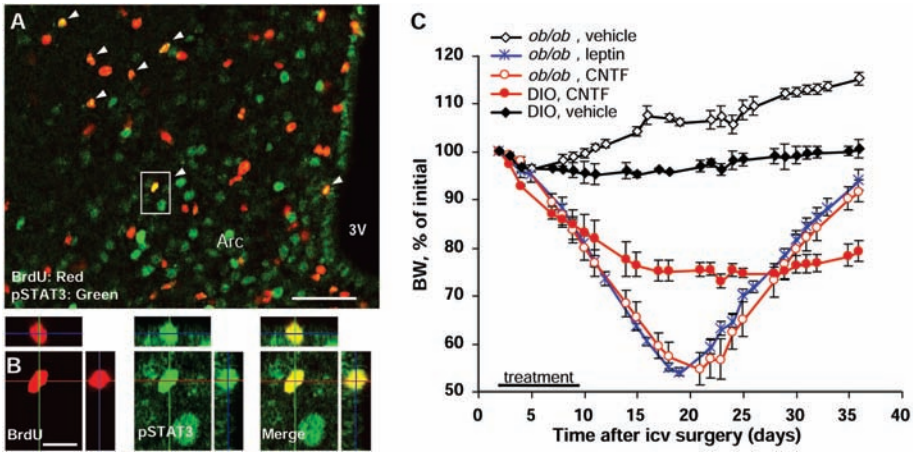

Fig. 3. (**A**) Newborn hypothalamic cells respond to leptin. Many BrdU$^+$ (red) cells of CNTF-treated mice were also positive for pSTAT3 (green) after ip leptin injection. (**B**) 3D confocal reconstruction of area boxed in (A). (**C**) Groups of DIO or ob/ob mice (n = 5) were icv infused for 7 days with CNTF (0.75 μg/day) or leptin (0.60 μg/day). For all animals, BrdU (12 μg/day) was coadministered. To induce DIO, mice were placed on a high-fat diet for 5 months. Body weight is shown as percentage difference from initial body weight. All data are mean ± SEM. Scale bars in (A), 50 μm; in (B), 10 μm.

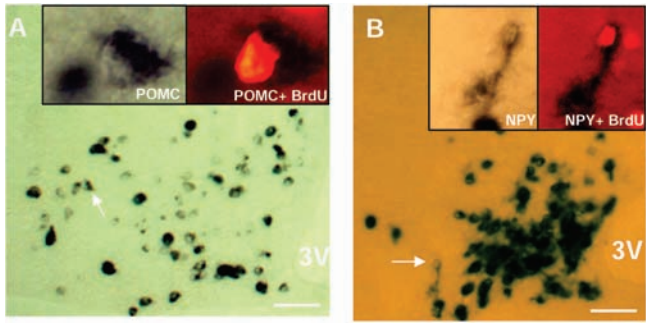

Fig. 4. In situ hybridization combined with anti-BrdU immunohistochemistry reveals newborn cells expressing NPY and POMC. Coronal sections at the level of the arcuate nucleus of a CNTF-treated mouse 42 days after surgery. (**A**) Brain section after hybridization to a digoxigenin-labeled probe for POMC. Inset left: High-power magnification of a POMC expressing cell (arrow). Inset right: Fluorescence image of the same cell demonstrating colocalization with BrdU. (**B**) Brain section hybridized to a NPY probe. The NPY-expressing cell marked by an arrow is also positive for BrdU (Insets). Scale bars: 100 μm.

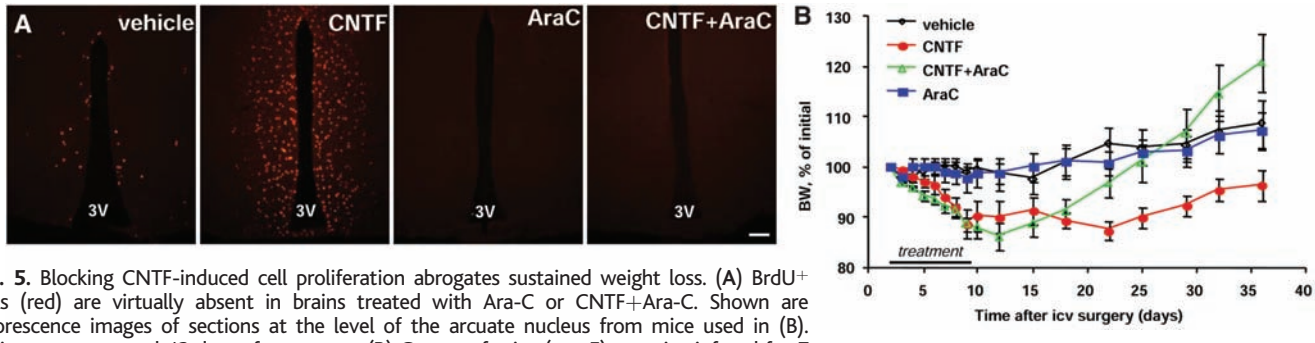

Fig. 5. Blocking CNTF-induced cell proliferation abrogates sustained weight loss. (**A**) BrdU+ cells (red) are virtually absent in brains treated with Ara-C or CNTF+Ara-C. Shown are fluorescence images of sections at the level of the arcuate nucleus from mice used in (**B**). Brains were removed 42 days after surgery. (**B**) Groups of mice (n = 5) were icv infused for 7 days with CNTF (0.75 μg/day) and/or Ara-C (40 μg/day). For all animals, BrdU (12 μg/day) was coadministered. Body weight is shown as percentage difference from initial body weight. All data are mean ± SEM. Scale bar, 100 μm.

maintained beyond cessation of the drug (Fig. 5B). In contrast, the time course of body-weight changes of mice treated with both CNTF and Ara-C (CNTF+Ara-C) showed a distinct pattern. First, the acute CNTF-induced weight loss during the infusion period (days 0 to 9) was unaffected by Ara-C. However, after cessation of treatment, CNTF+Ara-C–infused animals rapidly regained weight, reaching body weights of vehicle-treated animals at about 20 days after treatment. In accordance with previous results (*5*, *30*), CNTF-induced weight loss was associated with reduced food intake, whereas Ara-C–dependent body-weight rebound after treatment cessation was paralleled by increased food intake (fig. S5).

Although Ara-C specifically inhibits mitosis, it has been reported that this mitotic blocker can also act as a cytotoxin, triggering apoptotic degradation of postmitotic neurons (*32*). To address this potential concern, we inspected Ara-C–treated brains using the sensitive cell-death marker Fluoro-Jade (*33*). We observed no signs of cell degeneration throughout the brain parenchyma after treatment (fig. S6A). As positive controls, we used mice treated with gold thioglucose, which induces cell death in the ventromedial hypothalamus (fig. S6B).

To determine whether Ara-C interferes with acute CNTF signaling and action, we examined the CNTF-dependent induction of hypothalamic STAT3 phosphorylation (*5*). STAT3 phosphorylation, which is acutely triggered during CNTF exposure, was unaffected by Ara-C (fig. S7A). Also, the CNTF-induced activation of astrocytes (*34*) appears unperturbed by Ara-C. The up-regulation of GFAP expression associated with glial activation is equally evident in mice treated with CNTF or CNTF+Ara-C, but not in vehicle-infused animals (fig. S7B). Thus, at the dose used in our experiments, Ara-C blocked the cell proliferation/neurogenesis effect of CNTF without detectable toxicity, or inhibition of its acute actions.

We show that CNTF robustly induces cell proliferation in the hypothalamus with many of the newborn cells taking on a neuronal fate. In the past, research on adult neurogenesis has mainly focused on the following two brain regions: the SVZ, which gives rise to neuronal precursors that migrate to the olfactory bulb, and the SGZ, which fuels the granular layer of the dentate gyrus with new neurons (*14*). In contrast, adult neurogenesis in the hypothalamus has received little attention (*17*, *35*). This may be attributable to the strong proliferative potency of the SVZ or the SGZ and the relative insensitivity of the methods used to reveal newborn cells in other brain regions (*36*). In our study, instead of injecting BrdU ip, the route commonly used to mark newborn cells, we administered BrdU centrally. This approach allowed the detection of newborn hypothalamic cells in response to CNTF and might be generally suitable to detect neurogenesis in brain regions with a proliferative potency lower than that in the SVZ or SGZ. The origin of newborn hypothalamic cells is presently unclear. It appears that rather than being exclusively restricted to the ependymal lining of the third ventricle, neural progenitors also reside within the hypothalamic parenchyma.

Many of the newborn hypothalamic cells induced by CNTF exhibit phenotypes important for energy-balance regulation, including neuropeptide expression and capacity for leptin-induced activation of STAT3. Mitotic blockade of CNTF-stimulated cell proliferation does not alter the acute CNTF-dependent weight loss but abrogates the long-term effect on body-weight regulation. These observations support a model in which the short-term effects of CNTF result from acute signaling in existing neurons, whereas the long-term effects on body weights of CNTF-treated animals require functional neurogenesis in hypothalamic structures that subserve energy homeostasis. Because CNTF stimulates hypothalamic cell proliferation yet does not cause a sustained weight loss in ob/ob mice, it is plausible that a leptin-sensitive component of the newborn cell population is central to the sustained antiobesity effect of CNTF, possibly by enhancing the satiety response of the leptin signaling circuitry. The precise identity and function of the responsive cells remain to be determined.

Because CNTF also induces cell proliferation/neurogenesis in the SVZ of the lateral ventricles (*37*) and because Ara-C blocks cell proliferation throughout the ventricular system, these studies cannot exclude a role for extra-hypothalamic neurogenesis in the long-term effect of CNTF on energy balance. This possibility seems unlikely, however, because CNTFR expression is exceptionally strong in the parenchyma surrounding the third ventricle at the level of the arcuate, ventromedial, and dorsomedial nuclei, all of which are key structures involved in energy homeostasis (Fig. 1C). We also observe a particularly dense population of newborn cells at the bottom of the third ventricle after CNTF administration (Fig. 1B). Furthermore, there are no structures adjacent to the walls of the lateral ventricles known to be involved in the control of energy balance. Thus, cell proliferation within the hypothalamus is likely to be responsible for the CNTF-induced sustained effects on energy balance.

Hypothalamic plasticity has recently been proposed to play a role in energy-balance regulation (*38*). It was shown that leptin can influence the number and types of synaptic inputs to POMC and NPY neurons in the adult arcuate nucleus (*39*) and that perinatal leptin adminstration to leptin-deficient mice increases the density of certain projections emanating from the arcuate nucleus (*40*). Our observation that CNTF-induced neurogenesis occurs within hypothalamic feeding centers represents another type of plastic change, with the capacity to reset the energy-balance set point.

Axokine appears capable of lowering body weights in obese humans, but the development of neutralizing antibodies has limited the development of this drug (*4*). Given that CNTF induces hypothalamic neurogenesis, which contributes to the sustained weight-loss effect of this neurocytokine, further investigation into the potential role of hypothalamic neurogenesis in the pathophysiology and treatment of obesity is warranted.

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Supporting Online Material

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Material and Methods
Figs. S1 to S7
References

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NPY/AgRP Neurons Are Essential for Feeding in Adult Mice but Can Be Ablated in Neonates

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Hypothalamic neurons that express neuropeptide Y (NPY) and agouti-related protein (AgRP) are thought to be critical regulators of feeding behavior and body weight. To determine whether NPY/AgRP neurons are essential in mice, we targeted the human diphtheria toxin receptor to the *Agrp* locus, which allows temporally controlled ablation of NPY/AgRP neurons to occur after an injection of diphtheria toxin. Neonatal ablation of NPY/AgRP neurons had minimal effects on feeding, whereas their ablation in adults caused rapid starvation. These results suggest that network-based compensatory mechanisms can develop after the ablation of NPY/AgRP neurons in neonates but do not readily occur when these neurons become essential in adults.

The arcuate nucleus (ARC) of the hypothalamus is a site of convergence of central and peripheral signals of energy stores, and it contains at least two distinct populations of neurons that are critically involved in the regulation of body weight (*1*–*3*). Orexigenic neuropeptide Y/agouti-related protein (NPY/AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons respond to circulating satiety and hunger signals, including glucose, leptin, insulin, ghrelin, and peptide YY (*4*, *5*). Both populations exert an inhibitory tone onto each other, and they also send dense projections to other hypothalamic areas, including the paraventricular nucleus (PVN), zona incerta, perifornical area, and lateral hypothalamic area (*6*, *7*). POMC neurons reduce food intake and increase energy expenditure by releasing α-melanocyte-stimulating hormone (αMSH), a product of POMC processing, which activates melanocortin-4 receptors (MC4R). NPY/AgRP neurons have the opposite effects, inhibiting POMC neurons and antagonizing the action of αMSH on MC4R-bearing cells via the release of AgRP (a natural antagonist of αMSH) (*8*). Despite the fact that intracranial injection of either NPY or AgRP stimulates robust feeding in rodents (*1*–*3*), mutations that prevent the expression of AgRP, NPY, or various receptors for NPY have little impact on feeding behavior (*3*, *9*–*11*). In contrast, mutations that prevent production of leptin, leptin receptor, POMC, or MC4R lead to obesity in mice and other species (*12*–*17*). These observations raise the question of whether signaling by NPY, AgRP, or any other transmitter made by these cells is important for the regulation of body weight.

To assess whether NPY/AgRP neurons are essential for feeding, we adopted a "toxin receptor–mediated cell knockout" strategy (*18*) to specifically ablate these neurons in a temporally controlled manner (*19*). Because *Agrp* gene expression is restricted to NPY/AgRP neurons in the brain (*20*, *21*), we targeted the expression of the human diphtheria toxin receptor cDNA (*DTR*) to the *Agrp* locus in embryonic stem cells and generated $Agrp^{DTR/+}$ mice that express the human *DTR* in NPY/AgRP neurons (fig. S1). In situ hybridization revealed that human *DTR* mRNA was expressed in the ARC of $Agrp^{DTR/+}$ mice but not in controls (fig. S2).

Neonatal ablation of NPY/AgRP neurons was performed by injecting 1-day-old $Agrp^{DTR/+}$ and control $Agrp^{+/+}$ pups (genotype unknown at time of injection) with diphtheria toxin (DT) at 50 μg of DT per kg mouse (μg/kg) (subcutaneous), a dose tolerated by controls (*18*, *21*). After 9 weeks, all mice were fasted for 2 days to increase NPY and AgRP expression before they were killed (*22*). Brains were fixed, sectioned, and analyzed for NPY expression by immunohistochemistry. DT injection reduced the number of NPY-positive cells in the ARC by ~85% ($Agrp^{DTR/+}$ mice had 9.7 ± 0.9 neurons per section, $n = 5$ mice; controls had 78 ± 2 neurons per section, $n = 3$, $P < 0.001$) (Fig. 1, A to D). There was a concomitant reduction of NPY fibers in the PVN (Fig. 1, E and F), but NPY-expressing cells outside the ARC were spared (fig. S3). AgRP staining in the ARC and PVN was also reduced after DT treatment in $Agrp^{DTR/+}$ mice (fig. S3). The integrity of POMC neurons was demonstrated by using antibodies to adrenocorticotropic hormone (ACTH), another peptide product of POMC (Fig. 1, G and H). The loss of NPY/AgRP cells and the retention of POMC cells in the ARC was also documented by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) of *Agrp* and *Pomc* mRNA (Fig. 1I).

If NPY/AgRP neurons are critical regulators of energy balance, then their ablation should negatively affect food intake and body weight. However, when newborn pups generated from a cross of $Agrp^{DTR/+}$ and $Agrp^{+/+}$ mice were injected with DT and their body weights recorded starting at weaning, there

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