Hypothalamic neurogenesis is not required for the improved insulin sensitivity following exercise training

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Abstract

Neurons within the hypothalamic arcuate nucleus (ARC) are important regulators of energy balance. Recent studies suggest that neurogenesis in the ARC is an important regulator of body mass in response to pharmacological stressors. Regular exercise training improves insulin action and is a primary treatment modality for obesity and type 2 diabetes. We examined whether exercise training causes hypothalamic neurogenesis and whether this contributes to exercise-induced improvements in insulin action. Acute exercise in adult mice induced a pro-neurogenic transcriptional program involving growth factors, cell proliferation and neurogenic regulators in the hypothalamus. Daily exercise training for 7 days increased hypothalamic cell proliferation 3.5-fold above sedentary mice, and exercise-induced cell proliferation was maintained in diet-induced obese mice. Colocalization studies indicated negligible neurogenesis in the ARC of sedentary or exercise-trained mice. Blocking cell proliferation via administration of the mitotic blocker cytosine-1-β-D-arabinofuranoside (AraC) did not affect food intake or body mass in obese mice. While four weeks of exercise training improved whole-body insulin sensitivity compared with sedentary mice, insulin action was not affected by AraC administration. These data suggest that regular exercise training induces significant non-neuronal cell proliferation in the hypothalamus of obese mice, but this proliferation is not required for enhanced insulin action.
Introduction

Obesity dramatically increases the likelihood of developing metabolic complications including dyslipidemia, fatty liver, glucose intolerance and cardiovascular disease, which increase morbidity and mortality (1; 2). Insulin resistance is a prominent metabolic defect in many obese individuals and contributes to the development of type 2 diabetes. “Lifestyle” interventions involving reduced caloric intake and increased physical activity remain the cornerstone for the treatment of obesity-related diabetes. Laboratory-based studies demonstrate improved insulin sensitivity in the muscle and liver after a period of exercise training (3; 4) and exercise reduces the progression from glucose intolerance to type 2 diabetes in at risk individuals (5-7). The exercise-induced improvements in insulin action are mediated by a host of molecular adaptations that improve metabolic, vascular and endocrine functions in the major peripheral glucoregulatory tissues, which include skeletal muscle, liver and adipose tissue (for review see (8)).

The regulation of plasma glucose was traditionally viewed as a process controlled by the coordinated actions of peripheral tissues. The pancreas responds to post-prandial rises in glucose by secreting insulin, which in turn increases glucose disposal into skeletal muscle and other insulin-sensitive tissues, and reduces glucose output by the liver to restore euglycemia. However, studies in the last decade have shown that neuronal populations within the hypothalamus and hindbrain influence autonomic systems controlling hepatic glucose and lipid fluxes, pancreatic insulin secretion, and peripheral glucose uptake. For example, central injections of neuropeptide Y (NPY) inhibits hepatic glucose production (9) and stimulates hepatic VLDL-triglyceride secretion (10) via the sympathetic nervous system. Moreover, insulin signaling in the hypothalamus suppresses hepatic glucose production (11) via phosphoinositide 3-kinase dependent activation of ATP-dependent potassium (K\textsubscript{ATP}) channels in agouti-related peptide (AgRP) expressing neurons within the arcuate nucleus (ARC), which in turn regulate efferent vagal innervation of the liver (12). Insulin action in the CNS increases muscle glucose uptake, independent of insulin signalling in
muscle (13; 14) and this involves the activation of $K_{ATP}$ channels. While somewhat controversial (11; 15), it is suggested that the insulin-dependent CNS pathway may account for more than half of the insulin-mediated glucose uptake in lean mice (14), making this an important regulatory point for systemic glucose homeostasis. The deletion of insulin receptors in the CNS cause insulin resistance (16) and insulin resistance of the CNS occurs in obese rodents (17-19) and humans (20). These results suggest that the management of impaired insulin action in obesity may require restoration of CNS insulin sensitivity. Indeed, successful treatment of uncontrolled diabetes depends on intact brain insulin action (21) and selective modification of the CNS in several animal models of insulin resistance and type 2 diabetes improves peripheral insulin action and reverses hyperglycemia (22).

Emerging evidence shows that new neurons are generated within the adult hypothalamus (2; 23-25). In sedentary mice, the rate of neuronal turnover is relatively slow, with new neurons accounting for ~6% of the total neuronal population (26). Markers of hypothalamic subtypes such as pro-opiomelanocortin (POMC), AgRP and NPY are expressed in the newly formed neurons, suggesting involvement in metabolic regulation (2; 23; 25). Pioneering studies (2) have demonstrated pronounced neurogenesis in the energy balance circuitry of the ARC in response to central ciliary neurotropic factor (CNTF) administration. That neurogenesis was required to elicit CNTFs sustained and powerful anorexigenic effects further indicate that postnatal neurogenesis may be important in regulating hypothalamic functions. Consistent with this notion are the findings that obesity is associated with impaired neurogenesis in the ARC (24; 26), which leads to a relative ageing of hypothalamic neuronal populations. Further, reducing neurogenesis by expressing constitutively active IKKβ in hypothalamic neural stem cells causes overeating, weight gain, glucose intolerance and hyperinsulinemia (26). Together, these data suggest that neurogenesis within the hypothalamic circuitry and/or turnover of energy-balance neurons is important for the maintenance of normal energy balance and glucose metabolism in mice. On the basis of these
observations, strategies aimed at enhancing hypothalamic neurogenesis may be a viable strategy to combat obesity and diabetes.

Regular exercise training is commonly used to treat obesity and diabetes and is also known to induce neurogenesis in several brain regions (27), although exercise-induced neurogenesis in the hypothalamus is poorly described. In the present study, we tested the hypothesis that neurogenesis is an adaptive response to exercise that is important for the weight loss and insulin sensitizing effects of regular exercise-training in obesity.

**Materials and Methods**

*Animal care and husbandry*

Experimental procedures were approved by the School of Biomedical Sciences Animal Ethics Committee (Monash University). Male C57BL/6J mice were purchased from Monash Animal Services (MAS). Mice were fed a standard low-fat laboratory diet (LFD, 10% of total energy from fat) or a micronutrient matched high-fat diet (HFD, 59% of total energy from fat) (Specialty Feeds, Glen Forrest, WA, Australia).

*Study Design and analytical methods*

Experiment 1: Transcriptional responses to acute exercise.

C57BL/6J mice were familiarized to the treadmill (Columbus Instruments, OH) for 3 days before the experiment, where mice ran for 30 min at 15 m/min on a 5% grade. Sedentary ‘control’ mice were placed on the stationary treadmill for the same amount of time. Mice were culled 6 h later and the hypothalamus was dissected (defined caudally by the mamillary bodies, rostrally by the optic chiasm, laterally by the optic tract, and dorsally by the apex of the hypothalamic third ventricle).
RT² Profiler PCR Array

RNA from the hypothalamus was extracted in Qiazol extraction reagent and by isolated using an RNeasy Tissue Kit (Qiagen). RNA quality was determined (NanoDrop p2000 Spectrometer, Biolab) and reverse transcribed (Invitrogen). Gene products were determined by real-time quantitative RT-PCR (ep realplex Mastercycler, Eppendorf) using a RT² profiler PCR Array (SA Biosciences) with targeted expression of genes related to neurogenesis and neural stem cell activation. Hspcb was used as a reference gene and did not vary between groups. The mRNA levels were determined by a comparative Cₜ method.

Experiment 2: Exercise training and cell proliferation.

An intracerebroventricular (ICV) cannula and osmotic mini-pump were implanted in 20 week old C57BL/6J mice (12 weeks on their respective diet). Mice commenced training 1 day later, which consisted of 30 min treadmill running daily for 7 days at ~12 m/min and a 5% slope (protocol in Table S1). Mice were perfused transcardially with 4% paraformaldehyde 24 h or 28 days after the final exercise bout and brains were removed for immunohistochemical analysis (Figure 1A).

Experiment 3: Prolonged exercise training and insulin action.

Mice were fed a HFD for four weeks then randomized into a vehicle or AraC treatment group. Mice were implanted with an ICV cannula attached to an osmotic mini-pump (see below) and recovered for two days. Thereafter, mice remained sedentary or commenced exercise training, which consisted of daily treadmill running, five times a week for four weeks (Table S2). Body weight was monitored throughout the training period and exercise capacity was assessed after the exercising training period. Insulin sensitivity was assessed 72 hours after the final exercise bout (Figure 2A).
**Intracerebroventricular (ICV) cannulation**

Mice were anesthetized under isoflurane inhalation and stereotactically implanted with steel guide cannulae (Plastics One, Roanoke, VA America) targeted to the right lateral ventricles (-0.3 mm anteroposterior, +1.0 mm laterally to bregma, -2.5 mm below the skull).

**Experiment 2:** Cannulae were connected to subcutaneously implanted osmotic minipumps (model 1007D, flow rate 0.5 µL/h, 7 days, Alzet, Cupertino, CA) via 65 mm long vinyl tubing filled with artificial cerebrospinal fluid (aCSF). Pumps were filled with vehicle solution, vehicle solution containing the known neurogenic agent Axokine (100 ng/µL, 1.2 µg/day, modified CNTF from Regeneron Pharmaceuticals), AraC (3.3 µg/µL, 40 µg/day, Sigma, St Louis, MO; selectively inhibits DNA synthesis), or Axokine plus AraC. The vehicle solution was aCSF containing 1 µg/µL 5-bromoeoxyuridine (BrdU) (12 µg/day, Sigma). BrdU incorporates into DNA of dividing cells and is commonly used for the birth dating and monitoring of cell proliferation (2; 24; 26).

**Experiment 3:** Cannulae were connected to subcutaneously implanted osmotic minipumps (model 1004, flow rate 0.11 µL/h, 28 days, Alzet) via 33 mm long vinyl tubing. Pumps and their tubing extension were filled with vehicle solution or vehicle solution containing AraC (15.2 µg/µL, 40 µg/day Sigma). The vehicle solution was aCSF containing 4.5 µg/µL BrdU (12 µg/day). The different concentrations of compounds between experiment 2 and 3 ensured that mice received the same total amount of chemical per day, irrespective of the flow rate. Mice were housed singly and monitored daily for body weight and food intake for all experiments.

**Tissue processing and immunohistochemistry**

Mice were anesthetized under isoflurane inhalation and perfused transcardially with 0.9% NaCl with 10 mg/L heparin followed by 4% paraformaldehyde (PFA) (Sigma, St Louis, MO). Brains were removed, post-fixed by standard methods and sectioned on a cryostat in the coronal plane.
Sections (30µm thick) were collected in four series. For BrdU immunostaining, after mounting (Superfrost Ultra Plus Slides, Thermo Scientific) and drying overnight, sections were fixed with 4% PFA for 10 min at room temperature (RT), rinsed in PBS, then rinsed with 100% methanol for 20 min, rinsed in PBS and incubated in 2N HCl for 30 min at 37°C. Sections were rinsed in PBS, blocked for 1 hour with 5% normal horse serum in PBS/0.02% Triton X-100 and incubated with sheep anti-BrdU antibodies (1:400, Abcam) overnight at 4°C. Sections were rinsed and incubated with the appropriate secondary antibody for 1 hour, rinsed in PBS and coverslipped. For co-labeling analyses, sections first underwent BrdU immunohistochemistry and were then incubated with primary antibodies (neuronal nuclei, NeuN 1:1000; Abcam, or glial fibrillary acidic protein (GFAP 1:1000 Dako) in blocking solution. Sections were rinsed and incubated with the appropriate secondary antibody for 1 hour, washed in PBS and coverslipped.

BrdU+ cells in the caudal hypothalamus were counted using stereological principles of quantifying BrdU+ cells from systematically collected serial sections with the initial section chosen at random. Per animal, every 4th coronal section (30 µm thickness) throughout the caudal hypothalamus (-1.22mm to -2.70mm from bregma) was analyzed by standard fluorescence microscopy. BrdU+ cells within the hypothalamic parenchyma were counted for each section analysed, excluding cells of the uppermost focal plane to avoid oversampling. The average number of BrdU+ cells for any given section of the caudal hypothalamus was calculated as the sum of the counted BrdU+ cells divided by the total number of sections counted.

Exercise capacity test

Mice ran on the treadmill at 10 m/min for 2 minutes (5% grade) and the speed was increased by 2 m/min every 2 min until the mouse reached exhaustion.
Insulin Tolerance Test (ITT)

An ITT was conducted 72 h after the last exercise bout. Mice were fasted for 4 h and venous blood glucose was assessed (Accu-Check glucometer, Roche) before and after intraperitoneal insulin administration (1 U/kg body weight, Actrapid, Novo Nordisk, Bagsvaerd, Denmark).

Assessment of tissue specific insulin sensitivity

After a 3 h fast (0800 to 1100 h) mice were injected via a tail vein with 0.5 U/kg of insulin, 10 μCi of 2-[1-3H]deoxyglucose (3H-DOG) and 2 μCi of [U-14C]glucose. Blood samples were obtained from a cut in the tail at 2, 5, 10, 15 and 20 min. Mice were killed by decapitation and tissues collected. 2-DOG clearance from the blood and into tissues was performed in mice as described (28; 29).

Immunoblot analysis

pAKT (Ser473) and AKT 1:1000 (Cell Signalling, #9271 and #4685) were assessed in hypothalamic lysates by standard methods (30).

Statistics

Results are expressed as the means ± SEM. Statistical analysis was performed by employing the appropriate student’s t test, one-way, two-way or repeated measures two-way ANOVA test with Bonferroni post hoc tests. Significance was established a priori at P ≤ 0.05.

Results

Acute exercise upregulates genes involved in hypothalamic neurogenesis and cell proliferation.

Acute exercise increased the expression of genes involved in cell proliferation including Egf, Fgf2, Il3 and Vegfa (Table 1). Pro-neurogenic growth factors were induced by exercise including Artn, Bdnf, Bmp15, Bmp2 and Ndp. Regulators of the cell cycle and known transcriptional regulators of
neurogenesis including *Arnt2*, *Hey1*, *Mef2c*, *Neurod1*, *Notch2*, *Pax3* and *Pax6* were also increased after exercise (Table 1). No gene was downregulated by exercise. Thus, acute exercise promotes a pro-neurogenic transcriptional program in the hypothalamus of adult mice.

*Regular exercise induces hypothalamic cell proliferation in lean mice.*

To determine whether regular exercise training induces hypothalamic cell proliferation, mice ran at a moderate intensity on a treadmill for 30 min/day for one week. Mice received a continuous ICV infusion of BrdU to label dividing cells and were killed one day after the last exercise bout. Hypothalamic cell proliferation in sedentary mice averaged 31 ± 5 BrdU+ cells per 30 µm section and this number increased 3.5-fold in exercise-trained mice (Figure 1B). This was substantially less than mice treated with the neurogenic factor CNTF, which was implemented as a positive control (Figure 1C). The number of BrdU+ labelled cells was reduced by 45% in exercise-trained mice at 33 vs. 8 days (Figure 1B), indicating that approximately half of the newborn cells survived. One caveat to this interpretation is that cells newly labelled with BrdU cannot be distinguished from cells that have incorporated BrdU and then divided. Thus, actual ‘survival’ rates are semi-quantitative. Together, these findings show that exercise training increases cell proliferation in the hypothalamus of mice.

Examination of coronal sections of sedentary and exercise-trained mice show that BrdU+ cells were diffusely distributed throughout the hypothalamus but were mostly concentrated in regions ~200-300 µm from the third ventricle including the ARC and the ventromedial and dorsomedial hypothalamic nuclei (Figure 1B). Very few BrdU+ cells were present in the ependymal lining of the third ventricle. Pairs of newly divided BrdU cells were distributed extensively within the hypothalamic parenchyma (Figure 1E), consistent with the notion that cell proliferation in the hypothalamus is not restricted to a specific zone or nuclear region.
Exercise-induced hypothalamic cell proliferation is not impaired in obesity.

Cell proliferation was not impaired in obese sedentary mice compared with lean sedentary mice, with an average of $43.4 \pm 6.6$ and $31.5 \pm 4.5$ BrdU$^+$ cells per section, respectively (Figure 1D). Exercise increased the number of BrdU$^+$ cells by 3.3-fold above that observed in obese sedentary mice and there was no significant reduction in BrdU$^+$ cells after 28 days in the obese exercise trained mice (Figure 1D), suggesting that the majority of the proliferating cells survived. Thus, the exercise-induced proliferation and survival rates in obesity were not impaired when compared with lean mice.

Given that ageing has been associated with reduced neuroregenerative capacity, comparison was made between groups of aged (~52 weeks) and relatively young (~18 weeks) mice that were exercise trained for one week. These experiments showed that hypothalamic cell proliferation is reduced by 2.2-fold in aged mice compared with young mice and that exercise increases cell proliferation by 1.5-fold in aged mice, even after one year of high-fat feeding (Figure 1F). The level of cell proliferation in exercise-aged mice was similar to sedentary young mice.

Limited neurogenesis and generation of astrocytes in the ARC after exercise training.

We next determined the neural contribution to cell proliferation in the ARC by assessing co-localization of BrdU with neuron (NeuN) and astrocyte (GFAP) markers. Only ~0.5% of the BrdU$^+$ cells were co-labelled with NeuN in exercise trained mice (33 days) and there was no BrdU/NeuN co-labelling in sedentary mice (Figure 1G). We applied this same approach to the subgranular zone of the hippocampal dentate gyrus, an area where neurogenesis is well established. The majority of BrdU labelled cells were colocalized with NeuN in exercise-trained mice, confirming marked neurogenesis. BrdU/NeuN co-labelling was less apparent in sedentary mice (Figure 1H). There was also limited colocalisation of BrdU and GFAP in sedentary and exercise trained mice, indicating restricted production of new astrocytes (Figure 1I). Because negligible neurogenesis was detected in
the ARC of lean mice (0.5% of proliferating cells), we rationalise that major changes in the neurochemical phenotype of the proliferating cells is unlikely in obesity and have not conducted these analyses.

**Hypothalamic cell proliferation does not affect energy balance in sedentary or exercise-trained mice**

Given the well-recognised impact of regular moderate exercise on improved metabolic performance and the observation that exercise promotes marked cell proliferation in the hypothalamus (Figure 1B), the link between these two factors was tested in groups of high fat fed mice that were exercise trained and exposed to ICV AraC (Figure 2A). Four weeks of training was selected because this was the minimum volume of training required to detect improvements in whole-body insulin action in high-fat fed C57Bl/6J mice (Figure 2B). A range of metabolic parameters was measured with and without AraC treatment to test the involvement of cell proliferation on the beneficial effects of exercise training on energy balance and insulin action. AraC blocked cell proliferation in the hypothalamus of mice treated with CNTF, a powerful neurogeneic stimulator (Figure 2C) and in exercise-trained mice (blank images not shown). Blocking cell proliferation did not affect food intake (Figure 2D, P=0.77), body mass (Figure 2E, P=0.23), epididymal fat mass (Figure 2F, P=0.31), plasma leptin (Figure 2G, P=0.49) or plasma FFA (Figure 2H, P=0.36) in sedentary mice.

Exercise training improved the endurance exercise capacity of mice by 2.2-fold compared with untrained age-matched mice (Figure 3A). The endurance capacity was reduced in exercise-trained mice treated with AraC compared with Vehicle treated mice (Figure 3A, P=0.0002). Blocking cell proliferation did not affect food intake (Figure 3B, P=0.44) or body mass (Figure 2C, P=0.48) during exercise training. Neither epididymal fat mass (Figure 2D, P=0.22), plasma leptin (Figure 2E, P=0.78) nor plasma triglycerides (Vehicle: 0.65 ± 0.07 vs. AraC 0.56 ± 0.07, n=16 per group,
Hypothalamic cell proliferation is not required for the exercise-training improvements in insulin sensitivity

In order to test whether hypothalamic cell proliferation (including neurogenesis) contributes to the improvements in insulin action following exercise training, insulin was co-administered intravenously with $^3$H-2-deoxyglucose ($^3$H-2DG) and $^{14}$C-glucose tracers into conscious mice. Whole-body insulin-stimulated glucose uptake was not affected by AraC administration in sedentary mice (Figure 4A). Exercise training improved whole-body insulin sensitivity, as demonstrated by a 45% increase in whole-body $^{14}$C-glucose clearance (Figure 4A, main effect $P=0.04$). $^{14}$C-glucose clearance (Figure 4A, $P=0.34$) and the reduction in blood glucose (Figure 4B, $P=0.29$) were not different between vehicle and AraC treated groups in exercise-trained mice. $^3$H-2DG clearance into the mixed quadriceps (Figure 3C, $P=0.74$), heart (Figure 3D, $P=0.38$), BAT (Figure 3E, $P=0.98$) and liver (Vehicle: $17.8 \pm 5.1$ vs. AraC: $17.8 \pm 6.7$ K*1000, $P=0.91$) were not different between groups, whereas $^3$H-2DG clearance in white adipose tissue tended to be decreased in AraC treated mice (Figure 3F, $P=0.06$). Insulin-mediated $^{14}$C-glucose accumulation in liver lipids was also unaffected by AraC treatment (Figure 3G, $P=0.80$). Finally, AraC treatment did not affect Akt Ser$^{473}$ phosphorylation in hypothalamic lysates (Figure 3H, $P=0.59$), indicating no improvement in insulin sensitivity. Together, these data demonstrate that cell proliferation is not required for the exercise-mediated enhancement in insulin action in obese mice.

Discussion

Adult neurogenesis is most prominent within the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus in the hippocampus (31), and impaired neurogenesis is implicated in the aetiology of several neurodegenerative diseases including Parkinson’s disease,
Alzheimer’s disease and amyotrophic lateral sclerosis (32). Notably, enhancing neurogenesis through pharmacological or physiological stimuli (e.g. environmental enrichment and voluntary physical activity) attenuates neurodegenerative disease progression and improves cognitive function (27; 33-36). Together, these results link the maintenance of adult neurogenesis to normal CNS function and suggest a therapeutic strategy for treating neurodegenerative conditions. The questions are then, to what extent does neurogenesis impact metabolic phenotypes, and can regular exercise training modulate this response?

In this study, we have observed cell proliferation in the hypothalamus of lean mice that is most abundant in the region immediately surrounding the third cerebral ventricle. Newly formed cells, identified by the incorporation of BrdU, were not localized to anatomically distinct nuclei but were graded in abundance with distance from the ventricle, although there were instances where isolated cells were present deep in the lateral hypothalamus. While we have demonstrated that exercise upregulates a subset of transcripts involved in cell proliferation and neurogenesis, very few newborn cells take a neuronal fate, indicating limited hypothalamic neurogenesis with exercise training. Hypothalamic cell proliferation was not dysregulated in diet-induced obese mice and was substantially increased after exercise training. While exercise induced cell proliferation occurs in the hypothalamus, a CNS region essential for the control of energy balance and insulin action, our data does not support an important role for hypothalamic cell proliferation in the exercise-induced improvements in insulin sensitivity and energy balance. By extension, our data question the importance of hypothalamic cell proliferation and neurogenesis in the regulation of energy balance and insulin action in the setting of obesity.

While new hypothalamic cells are produced during adulthood, their relevance to physiology remains unclear. The genesis for this, and previous work in the field, was derived from the seminal observation that CNTF administration into the CSF of mice leads to rapid and pronounced weight
loss that is maintained for at least one month after the cessation of CNTF administration. Importantly, when neurogenesis was blocked by co-administration of AraC, the sustained weight loss in CNTF treated mice was abrogated, indicating that neurogenesis was required for CNTF’s sustained anorectic effects (2). These long lasting effects were attributed to neurogenesis within the hypothalamic feeding circuits, specifically NPY and POMC expressing neurons, each playing crucial antagonistic roles in the regulation of energy balance. We asked whether cell proliferation, and by extension neurogenesis, could be enhanced by a physiological stimulus and whether this was critical for metabolic regulation. We then exploited two well-known phenomena to test the hypothesis that cell proliferation is important in homeostatic control of metabolism; these are that obesity induces insulin resistance and that regular exercise training improves insulin action. Blocking cell proliferation in exercise-trained mice did not affect fasting blood glucose, whole-body insulin-stimulated glucose uptake or tissue-specific insulin sensitivity. In addition, hypothalamic insulin signalling was unaffected by AraC administration, indicating that hypothalamic insulin signalling is not impacted despite improvements in whole-body insulin action. While exercise training reduces body mass gain in high-fat fed mice (37), blocking cell proliferation has no effect on body mass or food intake, thereby indicating no effect on daily energy expenditure. The simplest conclusion from our studies is that cell proliferation, and by extension neurogenesis, does not play a major role in modulating insulin action and energy balance in obese mice. However, regular exercise training may induce other changes to the hypothalamic circuitry to improve insulin action independent of cell proliferative effects.

Obesity induced by high-fat feeding results in altered synaptic plasticity in mice (38), which is postulated to lead to a relative ageing of the hypothalamic neuronal population (39). Such a disruption in hypothalamic circuitry may be predicted to impair the sensitivity of ARC neurons to nutrient and hormonal signals associated with metabolic challenges and compromise energy homeostasis. While previous studies have reported neurogenesis in the ARC of the hypothalamus in
lean mice, it is currently unclear whether neurogenesis is actually compromised in obesity or whether neurogenesis impacts the development of obesity and its related disorders. On the one hand, both high-fat feeding (24; 26) and IKKβ/NF-κB activation in neural stem cells lead to reduced proliferation and survival of new-born hypothalamic neurons, which is associated with overeating and insulin resistance (26). Conversely, neurogenesis in the median eminence of the hypothalamus is enhanced by high-fat feeding in young mice and is associated with energy overconsumption and increased fat mass (23). The same authors also showed that localized irradiation in the mediobasal hypothalamus of high-fat fed mice reduced neurogenesis, which was associated with increased energy expenditure and decreased weight gain (23). While, these conflicting data relating to the effect of diet-induced obesity on hypothalamic neurogenesis are difficult to reconcile, there are a number of variables across the studies that are likely to impact on outcomes. These include, the age of mice, the timing of the dietary intervention, the timing of BrdU administration and subsequent analyses, the hypothalamic localization of progenitors that would invariably impact the specific neurogenic microenvironment (e.g. median eminence ependymal layer (23), discrete zones of the mid third ventricle wall (40), ARC, MBH (2; 23), and the cell of origin being examined (e.g. β2-tanyocytes vs. neural stem cells). Despite the lack of clear direction from previous studies, the present work shows that high-fat feeding does not impact cell proliferation in the hypothalamus of adult mice, a result that is in keeping with data derived from rats (41).

While the majority of hypothalamic neurons are generated during embryogenesis, it is thought that postnatal neurogenesis is essential for maintaining functional plasticity and adaptability in adulthood. The question arises as to what are the determinants of such neurogenic activity. Voluntary physical activity increases neurogenesis in several brain regions in mice (42-44) and as such the present study sought to determine whether structured regular exercise training, similar to what would be prescribed for maintaining healthy weight, would increase hypothalamic
neurogenesis. We observed a generalized upregulation of transcripts involved in cell proliferation and survival, transcriptional regulation of neurogenesis and growth factors in the hypothalamus after an acute bout of exercise. Thus, the hypothalamic niche is rapidly altered following exercise to promote neurogenesis. Despite these molecular responses and the finding of marked hypothalamic cell proliferation after exercise training in both lean and obese mice, exercise did not increase neurogenesis *per se* and there was evidence of limited astrocyte generation. The absence of hypothalamic neurogenesis is perplexing, especially in light of the coordinated pro-neurogenic transcriptional response to exercise and the marked hippocampal neurogenesis observed in the same exercise-trained mice (utilizing the same BrdU/NeuN colabeling approach), essentially acting as a positive control. The negligible hypothalamic neurogenesis could be explained by slow neuronal differentiation of neural stem cells (NSCs) (24; 26) or proliferation of NSCs that is matched by decreased survival (24). Further studies that delineate the upstream drivers and requisite molecular signaling that facilitates the proliferation and differentiation of NSCs will provide valuable insights into why ARC neurogenesis is severely limited when compared with the dentate gyrus of the hippocampus. In addition, the phenotype of the new exercise-generated ARC cells remains undetermined, but based on the differentiation potential of NSCs (26), these BrdU+ cells might be NSCs undergoing self-renewal. Alternatively, previous literature in brain regions other than the hippocampus, such as the substantia nigra and cerebral cortex, report an abundance of cells with oligodendrocytic precursor characteristics that differentiate into mature oligodendrocytes with exercise (45; 46). In this regard, oligodendrocytes constitute the majority (~55%) of newly generated cells in the medial prefrontal cortex with exercise training, with evidence of limited neurogenesis (~5%) (47). Moreover, the majority of BrdU+ cells in the hypothalamus of sedentary mice are of oligodendrocyte lineage and only a small percentage of these become neurons (~8%) (48; 49).
In conclusion, these data demonstrate that exercise induces a rapid transcriptional response in the hypothalamus that drives substantial cell proliferation, even in obesity and ageing. In contrast to studies using pharmacological doses of mitogens, exercise does not induce significant neurogenesis in the hypothalamus and blocking cell proliferation does not change feeding, energy balance or insulin action in the setting of rodent obesity. These results question whether physiological cell proliferation and neurogenesis are major regulators of insulin action and metabolic phenotypes.
Author contributions

MLB researched data and wrote the manuscript, ML researched data, AR researched data, AS researched data, BJO contributed to discussion and reviewed/edited manuscript. ZBA researched data, contributed to discussion and reviewed/edited manuscript. MJW researched data and wrote the manuscript.

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MJW takes full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.
Table 1. Exercise-induced changes in the expression of neurogenic genes in the hypothalamus.

<table>
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Values are expressed as mean ± SEM, fold-change relative to rest. n= 6 for rest, n=6 for exercise.
Figure legends

Figure 1. Exercise-induced hypothalamic cell proliferation and neurogenesis in lean and obese mice.

(A) Schematic of the study design. (B) Chow fed mice were exercise trained for 7 days with BrdU ICV (12µg/day) infusion for 7 days in artificial cerebrospinal fluid at a flow rate of 12µL/day. Brains were removed in sedentary (Sed), 7 days post-surgery (7D, representation cell proliferation) or 28 days post-surgery (28D, representing cell survival) mice. Data expressed as the average number of BrdU+ cells per section counted (n=4 mice per group). Representative image of hypothalamus depicting BrdU+ cells (red) in sedentary and exercise trained mice. Scale bar = 50 µm. Connecting lines indicates P<0.05 by one way ANOVA. (C) Chow fed mice were ICV infused for 7 days with BrdU (12µg/day) and/or CNTF (1.2µg/day). Data expressed as the average number of BrdU+ cells per section counted (n=4 mice per group). Representative image of hypothalamus depicting BrdU+ cells (red) in sedentary and CNTF treated mice. Scale bar = 50µm. Connecting lines denotes P<0.05 via students t-test. (D) High-fat fed mice (HFD) were exercise trained for 7 days with BrdU ICV (12µg/day) infusion for 7 days in artificial cerebrospinal fluid at a flow rate of 12µL/day. Brains were removed in sedentary (Sed), 7 days post-surgery (7D, representing cell proliferation) or 28 days post-surgery (28D, representing cell survival) mice. Data expressed as the number of BrdU+ cell per section counted (n=4 per group). Connecting lines denote P<0.05 by one way ANOVA. (E) Representative image of hypothalamus depicting BrdU+ cells (red) after exercise training. Arrows indicate pairs of BrdU+ cells. Scale bar = 50 µm. (F) Ageing mice (12 months old) on either a chow or HFD were exercise trained for 7 days with BrdU ICV (12µg/day) infusion for 7 days in artificial cerebrospinal fluid at a flow rate of 12µL/day. Brains were removed in sedentary (Sed) or 7 days post-surgery (7D, representing cell proliferation) mice. Data expressed as the number of BrdU+ cells per section counted. (n=4 per group). Connecting lines denote P<0.05 via two way ANOVA. (G) Co-imaging of BrdU (red) and NeuN (green) showing negligible colocalization in sedentary (Sed) and exercise trained (Ex) mice. (H) Brain sections across the
dentate gyrus showing marked BrdU (red) and NeuN (green) co-localization in exercise trained (Ex) but not sedentary mice (Sed). Arrows indicate areas of colocalization of these molecular markers. (I) Co-imaging of BrdU (red) and GFAP (green) showing negligible colocalization in sedentary (Sed) and exercise trained (Ex) mice. For all figures, scale bar = 100 µm for main image and 25 µm for inset.

Figure 2. Hypothalamic cell proliferation does not affect energy balance in obese, sedentary mice.

(A) Schematic of the study design. (B) Insulin tolerance test performed after 4 weeks of exercise training in HFD mice (n=8 per group). Blood glucose is shown as a percentage difference from initial blood glucose. *P<0.05 vs. Sed (main effect for treatment) by repeated measures two way ANOVA. (C) Mice were ICV infused for 7 days with CNTF (1.2µg/day) and/or AraC (40µg/day). For all animals, BrdU (12 µg/day) was co-administered. Representative image of hypothalamus depicting BrdU+ cells (red). Scale bar = 50 µm. (D) Mice were fed a high-fat diet (HFD) for 4 weeks, then ICV infused with or without AraC (40 µg/day) for 4 weeks in artificial cerebrospinal fluid at a flow rate of 2.64µL/day. Mice were killed 36 hours after the infusion stopped. Weekly food intake was monitored during the infusion period (n=15 vehicle (veh) and AraC). (E) Body weight assessed at the end of the 4 week infusion (n=15 Veh, n=14 AraC). (F) The epididymal fat pad was excised and weighed when the mice were culled (n=7 Veh, n=6 AraC). (G) Plasma leptin (n=9 Veh, n=8 AraC) and (I) plasma free fatty acids (n=15 Veh, n=14 AraC) were assessed following the insulin sensitivity assessment. *P<0.05 vs. Vehicle via students t-test.

Figure 3. Hypothalamic cell proliferation does not affect energy balance in obese, exercise-trained mice.

(A) After high-fat feeding, mice were exercise trained for 4 weeks with ICV infusion with or without AraC (40 µg/day) for 4 weeks in artificial cerebrospinal fluid at a flow rate of 2.64 µL/day.
An exercise endurance test was performed following the 4 weeks of exercise training (n=6 per group). *P<0.05 vs Vehicle Exercise by two way ANOVA. (B) Weekly food intake during the exercise training period (n=17 Veh, n=16 AraC). (C) Body weight assessed at the end of exercise training (n=16 Veh, n=17 AraC). (D) The epididymal fat pad was excised and weighed when the mice were culled (n=4 Veh, n=6 AraC). (E) Plasma leptin (n=8 Veh, n=6 AraC) and (F) plasma free fatty acids (n=17 Veh, n=16 AraC) were assessed following the insulin sensitivity assessment. *P<0.05 vs. Vehicle by students t-test.

Figure 4. Insulin sensitivity is enhanced by exercise training in obese mice and does not require hypothalamic cell proliferation.

(A) After high-fat feeding, mice were exercise trained for 4 weeks with ICV infusion with or without AraC (40 µg/day) for 4 weeks in artificial cerebrospinal fluid at a flow rate of 2.64 µL/day. Insulin sensitivity was assessed with an intravenous insulin tolerance test (iITT) and glucose tracers. Whole body clearance of $^{14}$C-glucose during the iITT in sedentary and exercise trained mice (Veh Sed n=15, Veh Exer n=14, AraC Sed n=11, AraC Exer n=14). *P<0.05, main effect for exercise training via two way ANOVA. (B) Blood glucose levels during iITT in exercise trained mice. Blood glucose is shown as a percentage difference from initial blood glucose (Veh n=15, AraC n=17). Insulin-stimulated $^3$H-2-Deoxy-D-glucose (DG) uptake into the (C) quadriceps, (D) cardiac muscle, (E) brown adipose tissue, and (F) epididymal adipose tissue of exercise trained mice (Veh n=12, AraC n=14). Statistics via students t test. (G) Insulin-stimulated $^{14}$C –glucose uptake into the liver of exercise trained mice (Veh n=13, AraC n=15). (H) Insulin-stimulated Akt phosphorylation (Ser473) in the hypothalamus of exercise trained mice. All groups analyzed on the same exposure from the same immunoblot. Membranes were stripped and re-probed for total Akt (Veh n=7, AraC n=8).
References:


Figure 1

(A) Schematic diagram of the experimental design. ICV surgery was performed on Day 0, followed by BrdU infusion on Day 2 and Cull on Day 8 (Proliferation) and Day 33 (Survival).

(B) Quantification of BrdU+ cells per section for Sed, 8 D, and 33 D (Exercise) conditions.

(C) Effects of CNTF on BrdU+ cells per section.

(D) Comparison of BrdU+ cells per section between Sed, 8 D, and 33 D (Exercise) conditions across different treatments.

(E) Microscope images showing the distribution of BrdU+ cells (red) and counterstaining (blue) in the 3V region.

(F) Comparison of BrdU+ cells per section between LFD and HFD conditions (Exercise).
Figure 1

Diabetes

G, H: Comparison of NeuN, BrdU, and NeuN/BrdU staining in the brain of diabetic mice after 33 days of exercise vs. sedentary treatment.

I: Comparison of GFAP, BrdU, and GFAP/BrdU staining in the brain of diabetic mice after 33 days of exercise vs. sedentary treatment.
Figure 2

A. Time course of HFD, ICV surgery, AraC infusion, and Cull (Insulin sensitivity).

B. Blood Glucose (% of baseline) over 4 weeks of exercise.

C. Histological images showing CNTF and CNTF + AraC effects.

D. Food intake (kJ/day) by Veh and AraC groups over 4 weeks.

E. Final body weight (g) for Vehicle and AraC groups.

F. Epididymal fat pad (mg) for Vehicle and AraC groups.

G. Plasma Leptin (ng/mL) for Vehicle and AraC groups.

H. Plasma FFA (mmol/L) for Vehicle and AraC groups.

Diabetes 4 weeks of exercise
Figure 4
Online Supplementary Material

Hypothalamic neurogenesis is not required for the improved insulin sensitivity following exercise training

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Table S2: Four week exercise training protocol

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