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Article

Exercise Induces Hippocampal BDNF through a PGC-1α/FNDC5 Pathway

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SUMMARY

Exercise can improve cognitive function and has been linked to the increased expression of brain-derived neurotrophic factor (BDNF). However, the underlying molecular mechanisms driving the elevation of this neurotrophin remain unknown. Here we show that FNDC5, a previously identified muscle protein that is induced in exercise and is cleaved and secreted as irisin, is also elevated by endurance exercise in the hippocampus of mice. Neuronal *Fndc5* gene expression is regulated by PGC-1α, and *Pgc1a*$^{-/-}$ mice show reduced *Fndc5* expression in the brain. Forced expression of FNDC5 in primary cortical neurons increases *Bdnf* expression, whereas RNAi-mediated knockdown of FNDC5 reduces *Bdnf*. Importantly, peripheral delivery of FNDC5 to the liver via adenoviral vectors, resulting in elevated blood irisin, induces expression of *Bdnf* and other neuroprotective genes in the hippocampus. Taken together, our findings link endurance exercise and the important metabolic mediators, PGC-1α and FNDC5, with BDNF expression in the brain.

INTRODUCTION

Exercise, especially endurance exercise, is known to have beneficial effects on brain health and cognitive function (Cotman et al., 2007; Mattson, 2012a). This improvement in cognitive function with exercise has been most prominently observed in the aging population (Colcombe and Kramer, 2003). Exercise has also been reported to ameliorate outcomes in neurological diseases like depression, epilepsy, stroke, Alzheimer's disease, and Parkinson's disease (Ahlskog, 2011; Arida et al., 2008; Buchman et al., 2012; Russo-Neustadt et al., 1999; Zhang et al., 2012). The effects of exercise on the brain are most apparent in the hippocampus and its dentate gyrus, a part of the brain involved in learning and memory. Specific beneficial effects of exercise in the brain have been reported to include increases in the size of and blood flow to the hippocampus in humans, morphological changes in dendrites and dendritic spines, increased synapse plasticity, and, importantly, de novo neurogenesis in the dentate gyrus in various mouse models of exercise (Cotman et al., 2007; Mattson, 2012a). De novo neurogenesis in the adult brain has been observed in only two areas; the dentate gyrus of the hippocampus is one of them, and exercise is one of the few known stimuli of this de novo neurogenesis (Kobilo et al., 2011).

One important molecular mediator for these beneficial responses in the brain to exercise is the induction of neurotrophins/growth factors, most notably brain-derived neurotrophic factor (BDNF). In animal models, BDNF is induced in various regions of the brain with exercise, most robustly in the hippocampus (Cotman et al., 2007). BDNF promotes many aspects of brain development, including neuronal cell survival, differentiation, migration, dendritic arborization, synaptogenesis, and plasticity (Greenberg et al., 2009; Park and Poo, 2013). In addition, BDNF is essential for synaptic plasticity, hippocampal function, and learning (Kuipers and Bramham, 2006). Highlighting the relevance of BDNF in human, individuals carrying the Val66Met mutation in the *BDNF* gene exhibit decreased secretion of BDNF, and display a decreased volume of specific brain regions, deficits in episodic memory function, and increased anxiety and depression (Egan et al., 2003; Hariri et al., 2003). Blocking BDNF signaling with anti-TrkB antibodies attenuates the exercise-induced improvement of acquisition and retention in a spatial learning task, as well as the exercise-induced expression of synaptic proteins (Vaynman et al., 2004, 2006). However, the underlying mechanism which induces BDNF in exercise remains to be determined.

PGC-1α is induced by exercise in skeletal muscle, and is a major mediator of the beneficial effects of exercise in this tissue (Finck and Kelly, 2006). PGC-1α was initially discovered as a transcriptional coactivator of mitochondrial biogenesis and oxidative metabolism in brown fat (Puigserver et al., 1998; Spiegelman, 2007). Subsequent work has demonstrated an important role of PGC-1α in the brain. Lack of PGC-1α in the brain is associated with neurodegeneration (Lin et al., 2004; Ma et al., 2010) as well as GABAergic dysfunction and a deficiency in neuronal parvalbumin expression (Lucas et al., 2010). PGC-1α has been shown to be neuroprotective in the MPTP mouse model of Parkinson's disease (St-Pierre et al., 2006). It also negatively regulates extrasynaptic N-methyl-D-aspartate (NMDA) receptor activity and thereby reduces excitotoxicity in rat cortical neurons (Puddifoot et al., 2012). In addition, the involvement of PGC-1 α in the formation and maintenance of neuronal dendritic spines has been reported (Cheng et al., 2012). Interestingly, long-term forced treadmill running over 12 weeks increases *Pgc1a* expression in various areas of the brain (Steiner et al., 2011).

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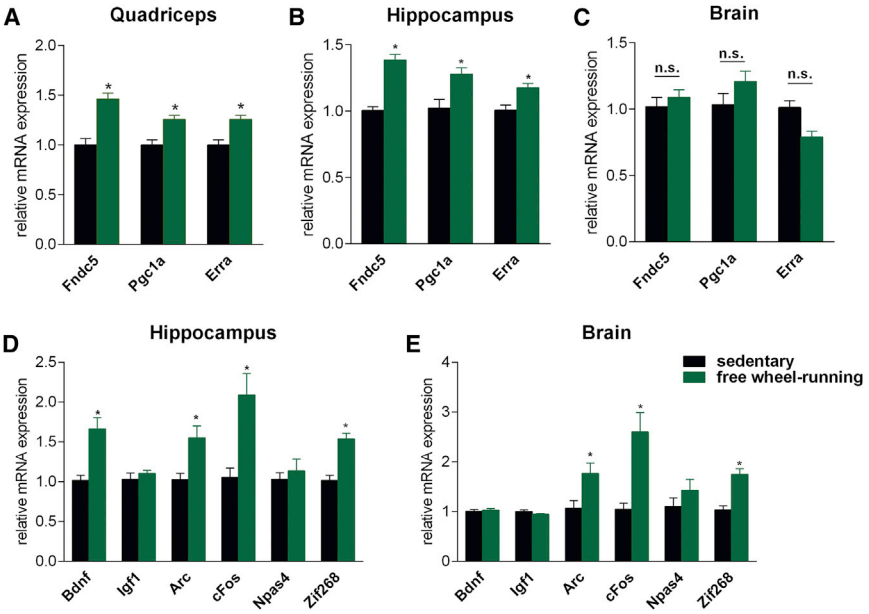

Figure 1. Endurance Exercise Induces Hippocampal *Fndc5* Gene Expression

(A–E) Male six-week-old C57/Bl6 wild-type mice were individually housed in cages with access to a running wheel (free running wheel) or without (sedentary). Mice were exercised for 30 days and sacrificed approximately 10 hr after their last bout of exercise. Data are shown as mRNA levels relative to Rsp18 expression, expressed as mean ± SEM. *p < 0.05 compared to sedentary control group. See also Figure S1.

Recently, our group identified a PGC-1α-dependent myokine, FNDC5, that is cleaved and secreted from muscle during exercise and induces some major metabolic benefits of exercise (Bostrom et al., 2012). FNDC5 is a glycosylated type I membrane protein and is released into the circulation after proteolytic cleavage. The secreted form of FNDC5 contains 112 amino acids and has been named irisin. Irisin acts preferentially on the subcutaneous "beige" fat and causes it to "brown" by increasing the expression of UCP-1 and other thermogenic genes (Bostrom et al., 2012; Wu et al., 2012). Clinical studies in humans have confirmed this positive correlation between increased FNDC5 expression and circulating irisin with the level of exercise performance (Huh et al., 2012; Lecker et al., 2012).

Interestingly, FNDC5 is also expressed in the brain (Dun et al., 2013; Ferrer-Martinez et al., 2002; Teufel et al., 2002) and in rat pheochromocytoma-derived PC12 cells differentiated into neuron-like cells (Ostadsharif et al., 2011). Knockdown of FNDC5 in neuronal precursors impaired the development into mature neurons, suggesting a developmental role of FNDC5 in neurons (Hashemi et al., 2013). This interesting connection of FNDC5 as an important exercise-related factor in the periphery, and its expression in the central nervous system, led us to investigate the effects of exercise on FNDC5 expression and function in the brain. Here we show that FNDC5 is elevated by endurance exercise in the hippocampus of mice and that PGC-1α and FNDC5 regulate BDNF expression in the brain.

RESULTS

Endurance Exercise Induces Hippocampal *Fndc5* Gene Expression

FNDC5 is highly expressed in the brain, as well as in skeletal muscle (Ferrer-Martinez et al., 2002; Teufel et al., 2002). Very little is known about the function of FNDC5 in the brain. We have therefore investigated the effects of exercise on FNDC5 expression and function. We used an established endurance exercise regimen: 30 days of voluntary free running-wheel exercise. This regimen is known to induce BDNF expression, neurogenesis, dendritic spines, and improved memory function in mice (Eadie et al., 2005; Kobilo et al., 2011). As has previously been established, this training was sufficient to induce muscle *Fndc5* gene expression (Figure 1A), as well as the transcriptional regulators *Pgc1a* and *Erra*, known mediators of the exercise response in skeletal muscle. In addition, other known genes of the exercise gene program were induced, confirming an adaptive endurance exercise response in the muscle (see Figure S1 available online). Interestingly, the same exercise regime led to a significant elevation of *Fndc5* expression in the hippocampus (Figure 1B), but not in the remainder of the brain (Figure 1C). The hippocampus is a region of the brain involved in learning and memory and has been identified as a major site where changes occur that are induced by exercise. Of note, even though genes that are induced by neuronal activity, such as *Arc*, *cFos*, and *Zif268*, were upregulated in both the remainder of the brain and the hippocampus, the important exercise-related neurotrophin *Bdnf* was induced *only* in the hippocampus (Figures 1D and 1E). However, *Npas4*, an important transcriptional component in hippocampal function and a key regulator of activity-induced *Bdnf* expression (Lin et al., 2008; Ramamoorthi et al., 2011), was not increased in the exercise regimen used here (Figures 1D and 1E).These data suggest that the induction of FNDC5 is part of the transcriptional response to exercise in the hippocampus.

Fndc5 Gene Expression Correlates with *Pgc1a* Expression Levels in Various Tissues and Developmental Stages

We previously reported that elevations in *Fndc5* gene expression in exercised muscle were dependent on PGC-1α (Bostrom et al., 2012). We therefore investigated whether *Fndc5* expression in the brain is also regulated by PGC-1α. To first assess if there is a correlation between the gene expression of these two proteins, we isolated 13 different tissues from C57/Bl6 mice, extracted total RNA, and measured gene expression for *Fndc5 and Pgc1a*. Consistent with earlier reports, the highest level of *Fndc5* gene expression was detected in heart, skeletal muscle, brain, and spinal cord (Ferrer-Martinez et al., 2002; Teufel et al., 2002). When we grouped the different tissues according to their

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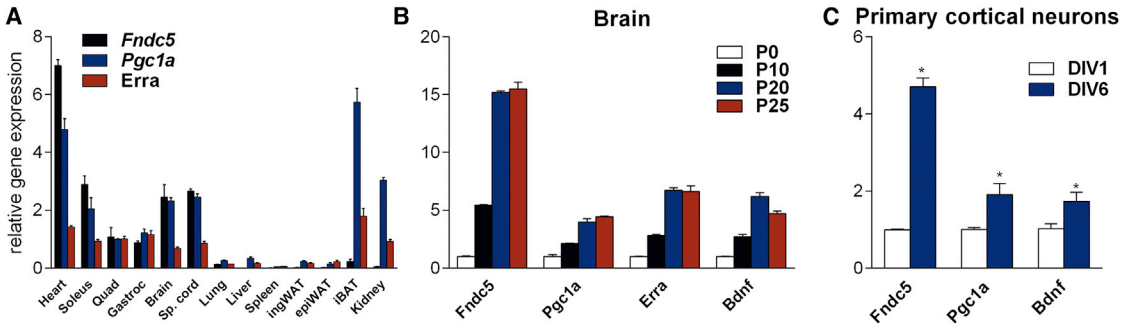

Figure 2. *Fndc5* Gene Expression Correlates with Pgc1a Expression Levels in Various Tissues and Developmental Stages

(A) The indicated tissues were harvested from male 13-week-old C57/Bl6 wild-type mice. Quad, quadriceps muscle; Gastroc, gastrocnemius muscle; Sp. Cord, spinal cord; ingWAT, inguinal white adipose tissue; epiWAT, epididymal white adipose tissue; iBAT, interscapular brown adipose tissue.

(B) Brains were harvested from C57/Bl6 wild-type mice at the indicated postnatal (P) time points.

(C) Primary cortical neurons were isolated from C57/Bl6 wild-type E17 embryos and cultured for the indicated days in vitro. mRNA was prepared and gene expression was assessed by qPCR. All data are shown as mRNA levels relative to Rsp18 expression, expressed as mean ± SEM. *$p < 0.05$ compared to control group.

levels of *Fndc5* expression, it is clear that most tissues with very high *Fndc5* expression also showed relatively high levels of *Pgc1a* gene expression (Figure 2A). Notably, *Fndc5* and *Pgc1a* expression levels correlated well, even within very distinct muscle beds. *Fndc5* expression was higher in oxidative muscle, such as the soleus muscle, which also contains higher levels of *Pgc1a*, than in glycolytic or mixed muscles, such as gastrocnemius or quadriceps muscle. Exceptions to this tight correlation of *Fndc5* and *Pgc1a* expression are the interscapular brown adipose tissue and the kidney. Both are tissues with extremely high mitochondrial content, which might explain their requirement for high *Pgc1a* levels without very high expression of *Fndc5*.

To examine whether FNDC5 and PGC-1α were developmentally regulated in synchrony during maturation of the brain, we performed a time course experiment of postnatal development. Brains were harvested from pups at postnatal day 0 (P0), P10, P20, P25, and P30, and gene expression was measured by qPCR. These time points were chosen because they cover an important time period of postnatal brain developmental, up to the mature state at P30. A two-step pattern of increased *Fndc5* gene expression during development was observed, with a first increase between P0 and P10 and a second increase between P10 and P20, which then leveled off (Figure 2B). *Pgc1a* gene expression followed essentially the same pattern. Of note, we also observed this two-step pattern of increased gene expression during brain development for the key neural regulatory protein, *Bdnf*. Next, the gene expression patterns for these factors were assessed during the maturation of primary cortical neurons in culture. We observed again this correlation: *Fndc5* gene expression increased between in vitro day (DIV) 1 and DIV 6, when the expression levels of *Pgc1a* and *Bdnf* were also elevated (Figure 2C). These data illustrate that, similar to muscle, there is a strong correlation between PGC-1α and FNDC5 gene expression in the brain.

Neuronal *Fndc5* Gene Expression Is Regulated by PGC-1α

To investigate whether PGC-1α is a transcriptional regulator of *Fndc5* gene expression in the brain, we turned to dissociated primary cortical neurons in culture. Although more heterogeneous than neurons from the dentate gyrus of the hippocampus, these cultures can be isolated in sufficient quantities for molecular studies and can be readily manipulated. Primary cortical neurons were stimulated with forskolin (10 μM), a strong inducer of intracellular cAMP, which is known to increase *Pgc1a* gene expression in cell types as diverse as brown adipocytes, hepatocytes, and Schwann cells (Cowell et al., 2008; Herzig et al., 2001; Yoon et al., 2001). This increase in *Pgc1a* gene expression was accompanied by a significant increase in *Fndc5* gene expression (Figure 3A). On the other hand, treatment of cortical neurons with nifedipine (5 μM), a selective L-type calcium channel blocker, which leads to decreased intracellular calcium levels and decreased *Pgc1a* gene expression, was accompanied by decreased *Fndc5* gene expression (Figure 3B).

Next, genetic gain- and loss-of-function approaches were used to test causality. Forced expression of PGC-1α by adenoviral delivery in primary cortical neurons resulted in a 4-fold increased *Fndc5* gene expression (Figure 3C). Immunoblotting confirmed that the increase in *Fndc5* mRNA translated into elevated FNDC5 protein levels (Figure S2). Conversely, reducing *Pgc1a* gene expression with lentiviral-mediated shRNA knockdown by more than 40% significantly decreased *Fndc5* gene expression by 66% and 31%, respectively (Figure 3D). As an additional loss-of-function model, the brains of global *Pgc1a* knockout mice ($Pgc1a^{-/-}$) were used. We observed the same requirement of PGC-1α for *Fndc5* gene expression in brains of these mice, which display a reduction in *Fndc5* gene expression by 32% (Figure 3E). Taken together, these results demonstrate that PGC-1α is a regulator of neuronal *Fndc5* gene expression in neural cultures and in the brain.

ERRα Is a Key Interacting Transcription Factor with PGC-1α for Regulating *Fndc5* Gene Expression in Neurons

PGC-1α is a transcriptional coactivator, meaning it does not bind to the DNA itself but interacts with transcription factors to execute its effects on gene expression (Spiegelman, 2007).

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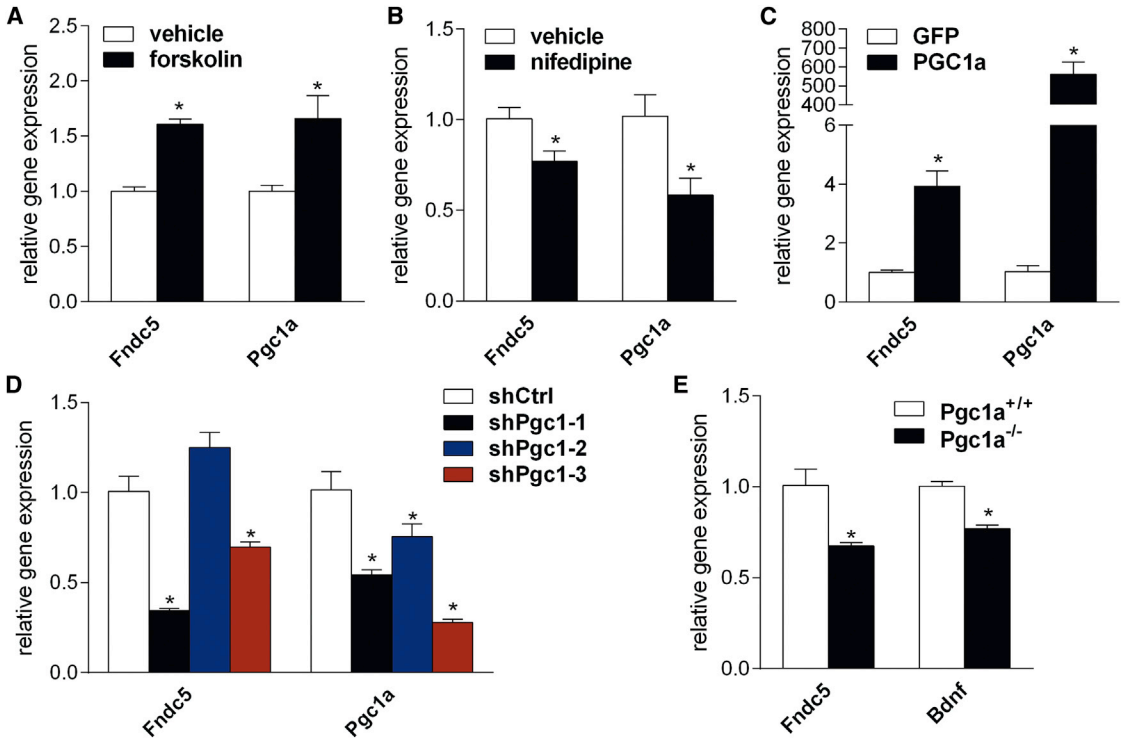

Figure 3. Neuronal *Fndc5* Gene Expression Is Regulated by a PGC-1α

(A) Primary cortical neurons at DIV 7 were treated overnight with either forskolin (10 μM), a stimulator of intracellular cAMP levels, or vehicle.

(B) Primary cortical neurons at DIV 7 were treated overnight with nifedipine (5 μM), an L-type calcium channel blocker, or vehicle.

(C) Primary cortical neurons at DIV 7 were transduced with either PGC-1α or GFP adenovirus and harvested 48 hr later.

(D) Primary cortical neurons at DIV 5 were transduced with lentivirus carrying the specified shRNA hairpins against Pgc1a or luciferase (Luc) as control and were harvested 4 days later.

(E) Cortices were harvested from either male five-month-old Pgc1a KO (*Pgc1a*$^{-/-}$) or wild-type mice (*Pgc1a*$^{+/+}$).

mRNA was prepared and gene expression was assessed by qPCR. All data are shown as mRNA levels relative to Rsp18 expression, expressed as mean ± SEM. *$p < 0.05$ compared to corresponding control group. See also Figure S2.

The orphan nuclear receptor estrogen-related receptor alpha (ERRα, also known as NR3B1) is a central metabolic regulator (Giguère et al., 1988; Luo et al., 2003) and a very important interactor with PGC-1α (Laganière et al., 2004; Mootha et al., 2004; Schreiber et al., 2004). The interaction of Errα with PGC-1α has been best studied in skeletal muscle, where it is required for mitochondrial biogenesis, induction of angiogenesis, oxidative metabolism, and oxidative muscle fibers (Arany et al., 2008; Mootha et al., 2004; Schreiber et al., 2004).

Interestingly, *Erra* follows the exercise-induced gene expression pattern of *Fndc5* in the brain. *Erra* is upregulated in the hippocampus upon exercise but not in the rest of the brain (Figures 1B and 1C). In addition, there was a correlation between *Fndc5* and *Erra* gene expression in our tissuevpanel (Figure 2A) as well as our developmental time course (Figure 2B). PGC-1α is well known to often increase the expression of transcription factors that it interacts with, thereby positively regulating its own regulators (Handschin et al., 2003; Mootha et al., 2004). We therefore asked if forced expression of PGC-1α in primary cortical neurons results in an increase in *Erra* mRNA. Indeed, adenoviral expression of PGC-1α significantly increased *Erra* gene expression, but not *Errb* or *Errg* gene expression (Figure 4A). However, mRNA for other common binding partners of PGC-1α, such as *Mef2*, *Ppara*, *Nrf1*, or *Gabpa/b*, was not induced in these experiments (Figure S3A).

The murine *Fndc5* gene and 6 kb of its upstream promoter were searched for putative ERRα transcription factor binding sites (ERREs) with the canonical "TGACCTT" sequence (Charest-Marcotte et al., 2010; Mootha et al., 2004; Wang et al., 2012). We identified two putative ERREs: one around 5.3 kb upstream of the transcriptional start site and one in the fourth intron of the *Fndc5* gene (Figure 4B). ERRα had been previously reported to also bind to intronic sequences to exert its biological function (Arany et al., 2008). This further suggests that ERRα could be important in FNDC5 gene regulation. Treatment of primary cortical neurons with XCT 790 (1 μM), a selective ERRα inhibitor (inverse agonist) which disrupts the ERRα/PGC-1α transcriptional complex (Mootha et al., 2004), significantly reduced *Fndc5* gene expression compared to vehicle-treated cells (Figure 4C). However, stimulation with DY131 (1 μM), a selective ERRβ and ERRγ agonist, had no effect on *Fndc5* gene expression. This suggests certain specificity for the involvement of ERRα compared to other ERR subfamily members. Since the nuclear receptor PPARα, another common binding partner of PGC-1α, was slightly induced by forced expression of PGC-1α, we tested the effect of GW7647, a potent and highly selective PPARα agonist, and

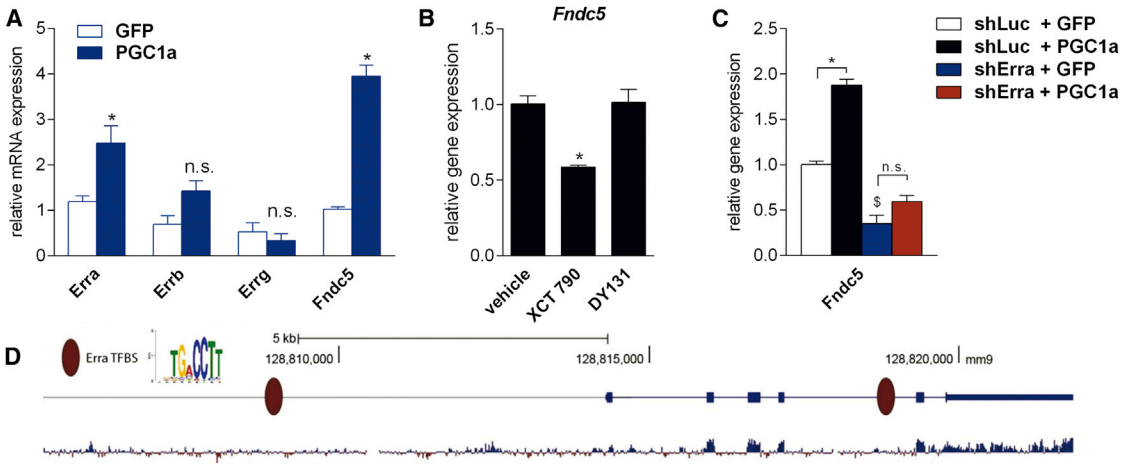

Figure 4. ERRα Is a Key Interacting Transcription Factor with PGC-1α for Regulating *Fndc5* Gene Expression in Neurons

(A) Primary cortical neurons at DIV 7 were transduced with either PGC-1α or GFP adenovirus and harvested 48 hr later. *p < 0.05 compared to control group.

(B) Primary cortical neurons at DIV 7 were treated overnight with either XCT 790 (1 μM), a selective inverse ERRα agonist, DY131 (1 μM), a selective ERRβ and ERRγ agonist, or vehicle. *p < 0.05 compared to vehicle only group.

(C) Primary cortical neurons at DIV 4 were transduced with lentivirus carrying shRNA hairpins against either *Erra* or luciferase (Luc) as control. Three days later, cells were transduced with either PGC-1α or GFP adenovirus and then harvested 48 hr later.

(D) Analysis of the murine *Fndc5* promoter for putative ERREs. The murine *Fndc5* gene and 6 kb of its upstream promoter were searched for the canonical ERRE: TGACCTT. Genomic coordinates are given according to the assembly mm9 from the UCSC Genome Browser. The bottom diagram indicates the degree of mammalian conservation across the genomic locus. The presented motif was modified from http://www.factorbook.org/ (Wang et al., 2012). See also Figure S3.

mRNA was prepared and gene expression was assessed by qPCR. Data are shown as mRNA levels relative to Rsp18 expression, expressed as mean ± SEM. *p < 0.05 compared to corresponding shLuc expressing control group. $p < 0.05 compared to corresponding GFP expressing control group. Data in (A)–(C) are shown as mRNA levels relative to Rsp18 expression, expressed as mean ± SEM.

GW0742, a potent and highly selective PPARδ agonist on *Fndc5* gene expression. However, under the conditions tested, no effect on *Fndc5* gene expression in primary cortical neurons by these compounds was observed (Figure S3B).

The results from the treatment with ERRα antagonist suggest that interaction of the PGC-1α with ERRα is required for the PGC-1α-dependent induction of *Fndc5* gene expression. To test this, we first knocked down ERRα in primary cortical neurons using lentivirally expressed shRNA hairpins and then 3 days later transduced the cells with either the PGC-1α adenovirus or GFP expressing adenovirus. *Erra* mRNA was efficiently knocked down by this hairpin (70%), and forced expression of PGC-1 α did not affect the efficiency of the knockdown (Figure S2C). Knockdown of ERRα significantly reduced *Fndc5* gene expression at baseline (Figure 4D). Furthermore, forced expression of PGC-1α by adenovirus in the cells with reduced ERRα failed to significantly increase *Fndc5* gene expression (Figure 4D). However, this failure to increase *Fndc5* gene expression was not due to a lack of overexpression of PGC-1α in the shErra-treated neurons (Figure S3C). Together these data suggest an involvement of ERRα in the induction of FNDC5 by PGC-1α. The precise role of the individual ERRα binding sites in the *Fndc5* gene remains to be determined.

FNDC5 Regulates *Bdnf* Gene Expression in a Cell-Autonomous Manner, and Recombinant BDNF Decreases *Fndc5* Gene Expression as Part of a Potential Feedback Loop

As mentioned earlier, BDNF is a major mediator of certain beneficial effects on the brain. In addition, an increase in the *Bdnf* gene expression in the hippocampus was observed, where *Fndc5* gene expression was also induced (Figures 1B and 1D), but not in the rest of the brain, where *Fndc5* was not induced (Figures 1C and 1E). We therefore tested whether FNDC5 could be a regulator of *Bdnf* gene expression in a cell-culture model. Primary cortical neurons were transduced with either FNDC5 adenovirus or a GFP adenovirus as control. Forced expression of FNDC5 resulted in a clear increase in FNDC5 protein in the whole-cell lysate, as well as an increase in the secreted form of FNDC5 (irisin) in the cell-culture supernatant (Figure 5A). After deglycosylation, this protein has the same apparent molecular mass (12 kDa) as that predicted for irisin (Figure 5A). In addition, forced expression of FNDC5 significantly upregulated *Bdnf* gene expression by 4-fold (Figure 5B). Importantly, FNDC5 expression also induced other important activity-induced genes involved in hippocampal function, including *Npas4*, *cFos*, and *Arc; Zif268*, however, was only slightly elevated.

To investigate if FNDC5 is required for *Bdnf* gene expression, lentivirally delivered shRNA was used to knock down FNDC5 in primary cortical neurons. To address possible off-targets of a single hairpin, we tested a total of five hairpins, three of which significantly knocked down *Fndc5* mRNA (Figure 5C). The same three hairpins also significantly reduced *Bdnf* gene expression. We also assessed the role of PGC-1α in controlling *Bdnf* gene expression in vivo. To do this, we used the brains of global *Pgc1a* knockout mice ($Pgc1a^{-/-}$). As shown in Figure 3E, *Bdnf* gene expression was significantly reduced in the brains of $Pgc1a^{-/-}$ mice (Figure 3E).

BDNF is well known for its ability to improve survival of neurons in culture. We therefore assessed the effects of

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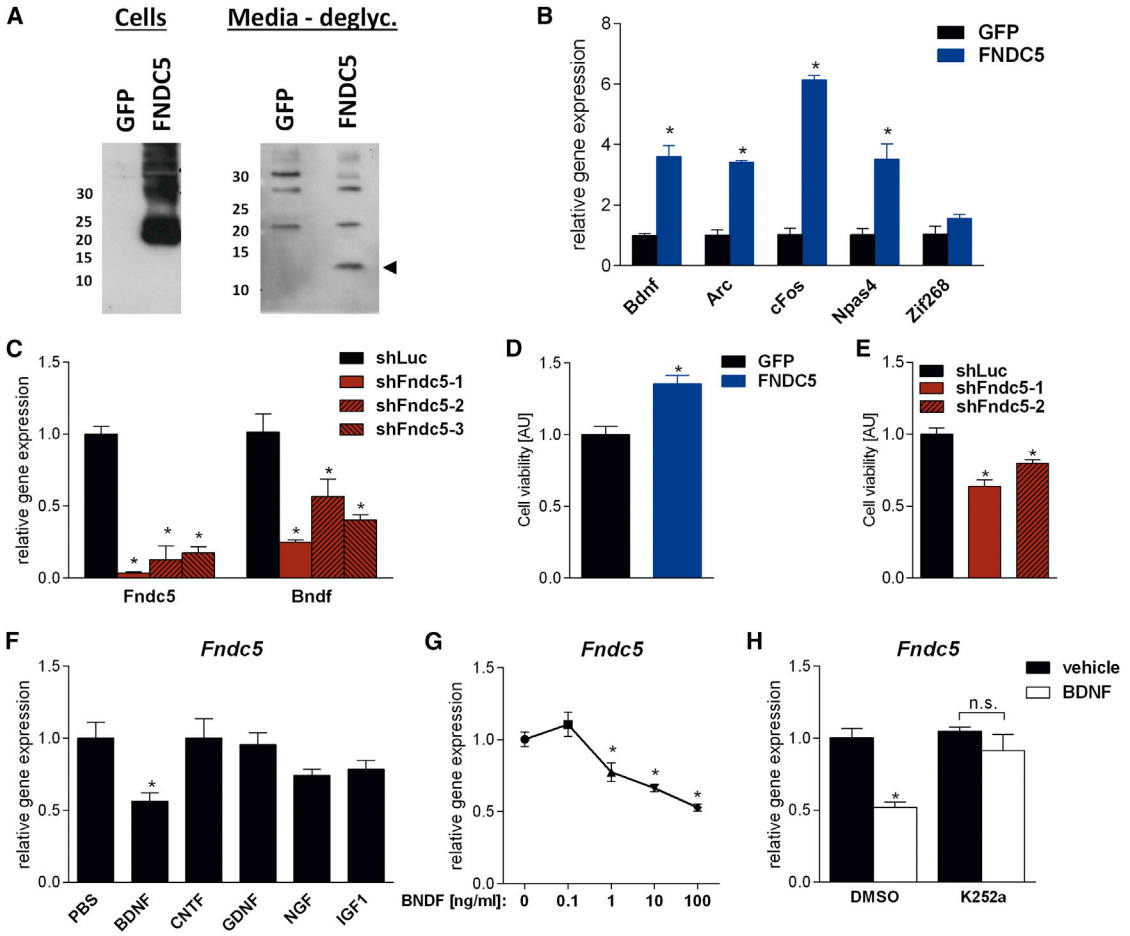

Figure 5. FNDC5 Regulates *Bdnf* Gene Expression in a Cell-Autonomous Manner, and Recombinant BDNF Decreases *Fndc5* Gene Expression as Part of a Negative Feedback Loop

(A) Primary cortical neurons at DIV 6 were transduced with either FNDC5 or GFP adenovirus. Whole-cell lysates and conditioned media were harvested and analyzed by immunoblotting. Intensity of unspecific bands and Ponceau staining were used to assess equal loading. deglyc., deglycosylation.

(B) Primary cortical neurons at DIV 7 were transduced with either FNDC5 or GFP adenovirus. Forty-eight hours later mRNA was prepared and gene expression was assessed by qPCR.

(C) Primary cortical neurons at DIV 5 were transduced with lentivirus carrying the specified shRNA hairpins against *Fndc5* or luciferase (Luc) as control. Four days later, mRNA was prepared and gene expression was assessed by qPCR.

(D) Primary cortical neurons at DIV 7 were transduced with either FNDC5 or GFP adenovirus. Cell viability was assessed 3 days later using the CellTiter-Glo Luminescent Cell Viability Assay. AU, arbitrary unit.

(E) Primary cortical neurons at DIV 5 were transduced with lentivirus carrying the specified shRNA hairpins against *Fndc5* or luciferase (Luc) as control. Cell viability was assessed 3 days later using the CellTiter-Glo Luminescent Cell Viability Assay. AU, arbitrary unit.

(F) Primary cortical neurons at DIV 7 were stimulated overnight with the indicated recombinant neurotrophins and growth factors (100 ng/ml). mRNA was prepared and gene expression was assessed by qPCR.

(G) Primary cortical neurons at DIV 7 were stimulated overnight with human recombinant BDNF at the indicated concentrations or vehicle. mRNA was prepared and gene expression was assessed by qPCR.

(H) Primary cortical neurons at DIV 6 were treated either with the TrkB inhibitor K252a (50 nM) or vehicle. Twenty-four hours later, human recombinant BDNF (100 ng/ml) or vehicle was added for overnight stimulation. mRNA was prepared and gene expression was assessed by qPCR.

Data (B, C, and F–H) are shown as mRNA levels relative to Rsp18 expression. All data are expressed as mean ± SEM. *$p < 0.05$ compared to corresponding control group.

gain- and loss-of-function of FNDC5 on cell viability of cultured neurons using a luminescence/ATP-based assay. Gain of function of FNDC5 significantly improved neuron survival in culture (Figure 5D), while loss of function of FNDC5 using shRNA-mediated knockdown of FNDC5 with two different hairpins significantly impaired the survival of neurons in culture (Figure 5E).

To examine how BDNF might, in turn, alter FNDC5 gene expression, primary cortical neurons were stimulated overnight with recombinant BDNF at various concentrations at physiological and pharmacological dosages (0.1–100 ng/ml). BDNF concentrations as low as 1 ng/ml significantly reduced *Fndc5* gene expression (Figure 5F), and a dose response was observed. To ask whether the reduction in *Fndc5* gene

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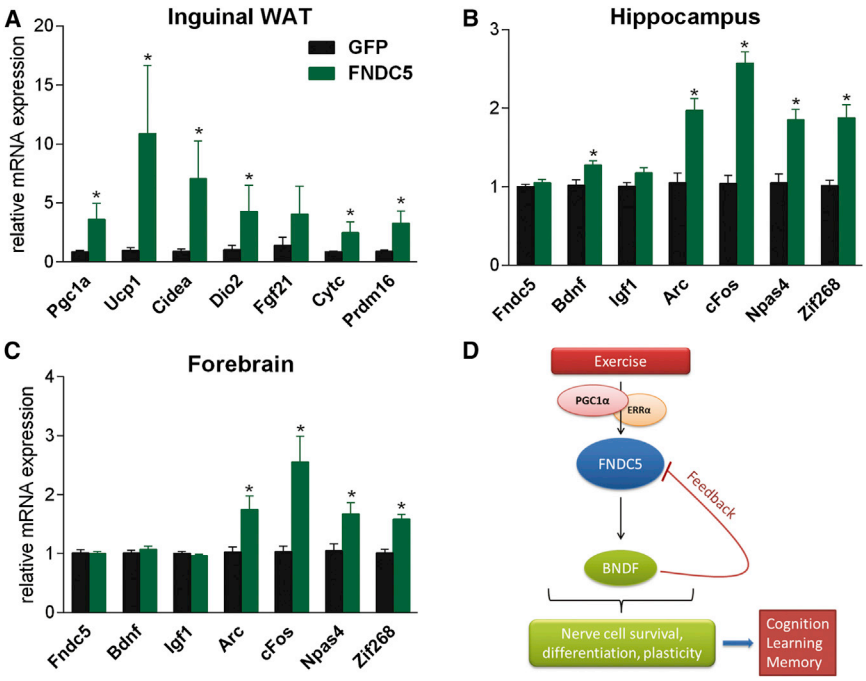

Figure 6. Peripheral Delivery of FNDC5 by Adenoviral Vectors Increases Bdnf Expression in the Hippocampus

(A–C) Five-week-old male wild-type BALB/c mice were intravenously injected with GFP- or FNDC5-expressing adenoviral particles. Animals were sacrificed 7 days later; (A) inguinal/subcutaneous fat pads (WAT, white adipose tissues), (B) hippocampus, and (C) forebrain were collected; and mRNA was prepared and gene expression was assessed by qPCR. Data are shown as mRNA levels relative to Rsp18 expression, expressed as mean ± SEM. *$p < 0.05$ compared to wild-type control group.

(D) Model of the hippocampal PGC-1α/FNDC5/BDNF pathway in exercise. Endurance exercise stimulates increased hippocampal *Fndc5* gene expression through a PGC-1α/Errα transcriptional complex. This elevated *Fndc5* gene expression in turn stimulates *Bdnf* gene expression. BDNF is the master regulator of nerve cell survival, differentiation, and plasticity in the brain. This will lead to improved cognitive function, learning, and memory, which are known beneficial effects of exercise on the brain. See also Figure S4.

expression was specific to BDNF, we treated primary cortical neurons with a variety of central and peripheral neurotrophic factors in addition to BDNF, such as ciliary neurotrophic factor (CNTF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and insulin-like growth factor 1 (IGF-1), at 100 ng/ml overnight. However, only BDNF stimulation significantly reduced *Fndc5* mRNA expression Figure 5G). This effect was abolished by preincubating the cortical neurons with a low dose (50 nM) of K252a, well-characterized inhibitor of TrkB, the receptor of BDNF signaling (Giménez-Cassina et al., 2012; Tapley et al., 1992) (Figure 5H). In summary, these data suggest a homeostatic FNDC5/BDNF feedback loop.

Peripheral Delivery of FNDC5 by Adenoviral Vectors Increases *Bdnf* Expression in the Hippocampus

We had previously shown that adenoviral overexpression of FNDC5 in the liver, a major secretory organ, increases circulating levels of irisin, the secreted form of FNDC5 (Bostrom et al., 2012). This resulted in the activation of a thermogenic gene program in certain fat tissues. To determine if peripheral delivery of FNDC5/irisin could elevate central BDNF levels, we repeated this experiment and measured *Bdnf* gene expression in the hippocampus 7 days later. As previously reported, forced expression of FNDC5 in the liver in the induced "browning" of the inguinal fat depot (Figure 6A) was shown by increased expression of mRNA for a group of key thermogenic genes, such as *Pgc1a*, *Ucp1*, and *Cidea*. In addition, plasma levels of irisin were elevated in mice overexpressing FNDC5 as compared to GFP-overexpressing control mice (Figure S4A). Interestingly, *Bdnf* expression in the hippocampus was significantly increased, as was expression of *Npas4*, *cFos*, *Arc*, and *Zif268*, all part of the activity-induced immediate-early gene (IEG) program, as mentioned before. Importantly, this was not caused by any viral mediated expression of *Fndc5* in the brain or hippocampus (Figure 6B), strongly suggesting that the secreted form of the peripherally expressed FNDC5 was responsible for this effect. This effect of increased *Bdnf* expression was specific to the hippocampus, since it was not observed in the forebrain (Figure 6C), whereas the IEG response was observed in both; this is consistent with our earlier findings of the effects of exercise (Figures 1D and 1E).

PGC-1α/FNDC5/BDNF Pathway in Primary Hippocampal Neurons

We used cortical neurons in the experiments above because this is the most widely used system of primary CNS cultures and because reasonable numbers of cells can be obtained. However, since some of our observations in vivo were made in the hippocampus, we sought to validate our findings in primary hippocampal neurons. Therefore, a key set of experiments were repeated in primary hippocampal neuron cultures. We confirmed that *Fndc5* gene expression is significantly increased in primary hippocampal neurons cultured in vitro from DIV 1 to DIV 6; the expression of *Pgc1a* and the expression of *Bdnf* mRNA are similarly increased (Figure 7A). To test whether PGC-1α regulates *Fndc5* gene expression in hippocampal neurons, gain- and loss-of-function studies were performed. Forced expression of PGC-1α significantly induced *Fndc5* gene expression (Figure 7B). Interestingly, stimulation with forskolin (10 μM) failed to induce *Pgc1a* gene expression but decreased the expression of *Erra* and *Fndc5* (Figure S5). Efficient knockdown of *Pgc1a* by lentivirally delivered shRNA significantly reduced *Fndc5* gene expression (Figure 7C). Stimulation of primary hippocampal neurons with commercially available recombinant irisin induced a similar gene program (*Arc*, *cFos*, *Npas4*, and *Zif268*) as was found in the in vivo adenoviral experiments (Figure 7D). However, the increase in *Bdnf* gene expression did not reach statistical significance. Loss of function

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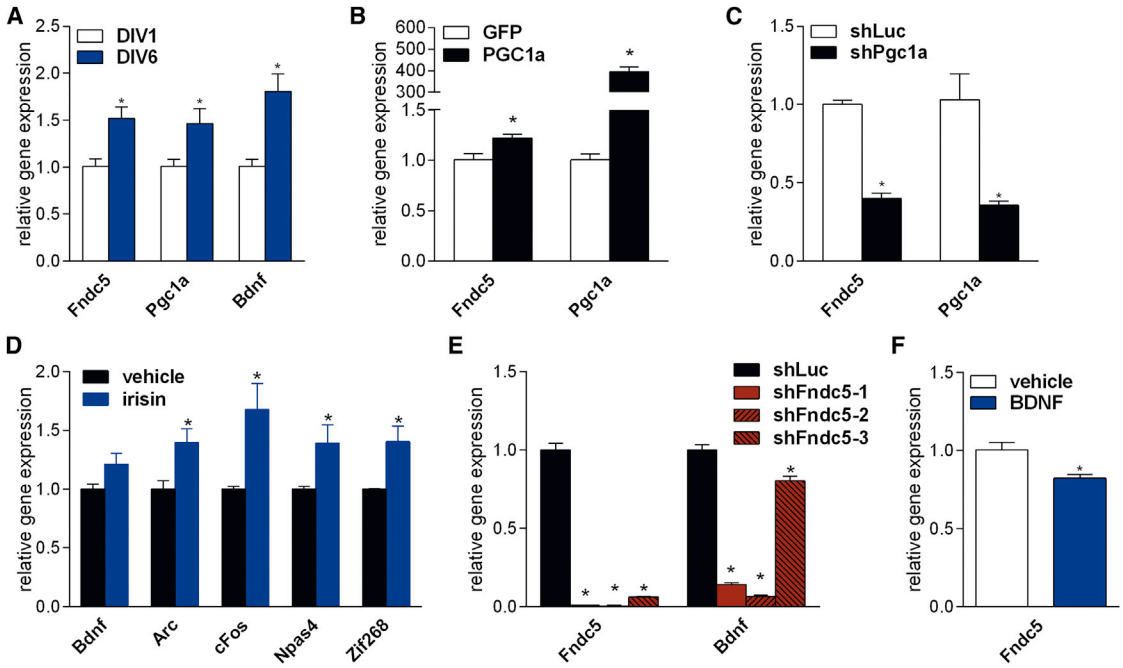

Figure 7. PGC-1α/FNDC5/BDNF Pathway in Primary Hippocampal Neurons

(A) Primary hippocampal neurons were isolated from C57/Bl6 wild-type E17 embryos and cultured for the indicated days in vitro.

(B) Primary hippocampal neurons at DIV 7 were transduced with either PGC-1α or GFP adenovirus and harvested 48 hr later.

(C) Primary hippocampal neurons at DIV 5 were transduced with lentivirus carrying the specified shRNA hairpin against Pgc1a or luciferase (Luc) as control and harvested 4 days later.

(D) Primary hippocampal neurons were stimulated with recombinant irisin (1 μg/ml) at DIV 5 and DIV 6 and harvested 24 hr later.

(E) Primary hippocampal neurons at DIV 5 were transduced with lentivirus carrying the specified shRNA hairpins against *Fndc5* or luciferase (Luc) as control and harvested 4 days later.

(F) Primary hippocampal neurons at DIV 7 were stimulated overnight with recombinant BDNF (100 ng/ml).

mRNA was prepared and gene expression was assessed by qPCR. Data are shown as mRNA levels relative to Rsp18 expression, expressed as mean ± SEM. *$p < 0.05$ compared to corresponding control group. See also Figure S5.

of FNDC5 by shRNA-mediated knockdown with three different hairpins against *Fndc5* significantly reduced *Bdnf* gene expression in hippocampal neurons (Figure 7E). In addition, treatment of hippocampal neurons with recombinant BDNF reduced *Fndc5* gene expression (Figure 7F). Together, these data demonstrate that the basic observations made in the primary cortical neurons also apply to primary hippocampal neuron cultures.

DISCUSSION

A recent study has reported a positive correlation between human brain size and endurance exercise capacity, suggesting a coevolution between human cognition and locomotion (Raichlen and Gordon, 2011). More complex tasks require a more complex brain, and foraging in wide and open spaces in the savannas put high demands on spatial orientation, as well as the ability to acquire and retain new information. Therefore individuals with a more complex brain who performed better at these tasks might have had an evolutionary advantage. On the other hand, since endurance exercise clearly increases expression of BDNF in the brain, improvements in the exercise capacity might have positively enforced brain growth (Mattson, 2012b), especially in the hippocampus.

In this study we report a PGC-1α/FNDC5/BDNF pathway that is activated in the hippocampus by endurance exercise (Figure 6). In our current model, exercise leads to increased transcription of *Pgc1a* and *Erra*. It has been observed previously that PGC-1α often induces the expression of transcription factors to which it binds and which it coactivates (Handschin et al., 2003; Mootha et al., 2004). Indeed, the ability of PGC-1α to induce FNDC5 gene expression depends on ERRα availability (Figure 4D). This PGC-1α/Errα complex, in turn, may bind to one or more of the canonical ERREs found in or near the *Fndc5* gene, thus activating *Fndc5* gene expression. As shown in a cell-culture model in Figure 5A, FNDC5 is a positive regulator of BDNF expression. Based on this, it seems likely that the increased *Fndc5* gene expression in exercise will lead to increased BDNF levels. Interestingly, BDNF also can signal to reduce the expression of FNDC5 as part of an apparent homeostatic loop. Both FNDC5-dependent and FNDC5-independent pathways by which exercise induces BDNF expression seem plausible. For example, CREB and NF-κB are two other transcription factors known to induce BDNF expression in exercise (Mattson, 2012b). These may act upstream or downstream of FNDC5, or in an independent pathway.

The induction of FNDC5 by exercise in the hippocampus is quantitatively comparable to the induction observed in skeletal

muscle. Interestingly, it is also in the same quantitative range as the induction of BDNF, a neurotrophic mediator of exercise in the brain, as well as cFOS, ARC, and ZIF268, important indicators for the activity state of neurons (Hunt et al., 1987; Lyford et al., 1995; Rusak et al., 1990; Saffen et al., 1988). This places FNDC5 induction in a similar range to other known important regulators in the brain.

In our study of 30 days of free running-wheel exercise, *Fndc5* and *Pgc1a* were induced in the hippocampus, but not in the rest of the brain (Figure 1B) when taken as one unit. Therefore it is possible that *Fndc5* and *Pgc1a* were induced in relatively small numbers of neurons elsewhere, but that that change was not detectable because it is occurring in the background of little or no change in larger brain structures. Indeed, using a longer and more intense exercise regimen exercise protocol and more detailed dissections, Steiner et al. reported an upregulation of *Pgc1a* expression in various other parts of the brain, in addition to the hippocampus (Steiner et al., 2011).

A central question arising from our study is this: How does the PGC-1α/FNDC5/BDNF pathway get initiated in exercise? This question is closely linked to the more central and open question in the field: How is exercise sensed by the brain? One obvious initiator could be increased neuronal activity in areas of the brain that are involved in spatial orientation, learning, and memory, since BDNF gene expression is well known to be stimulated by neural activity (West and Greenberg, 2011). Increased sympathetic tone, namely higher norepinephrine levels (Garcia et al., 2003), and increased IGF-1 levels from periphery crossing the blood-brain barrier have also been discussed as exercise-related inducers of BDNF (Ding et al., 2006). However, because exercise is known to change the metabolic state of the whole body, another important factor could be changes in the energy state or oxygen levels within the brain, both signals to which *Pgc1a* gene expression is known to respond in other tissues (Arany et al., 2008; St-Pierre et al., 2006). In our study we linked the activation of a metabolic regulator, PGC-1α, via FNDC5 to increased BDNF levels in the neurons in response to exercise (Figure 6). Of note, there are other important metabolic regulators, such as AMPK or PPARgamma, which have not been part of this study.

FNDC5 in the periphery is cleaved and secreted as irisin, and secreted irisin can cause the "browning" of adipose tissues (Bostrom et al., 2012; Shan et al., 2013; Wu et al., 2012). Therefore several important questions arise from our studies. First, is FNDC5 functioning mainly as a membrane-bound molecule in the brain, or is it secreted by neurons? Second, if FNDC5 is secreted, is it secreted as irisin (amino acids 29–140) or as a different peptide species? Perhaps the most exciting result overall is that peripheral delivery of FNDC5 with adenoviral vectors is sufficient to induce central expression of *Bdnf* and others genes with potential neuroprotective functions or those involved in learning and memory. This suggests that a secreted, circulating form of FNDC5 has these effects on these neurons and that it crosses the blood-brain barrier. Whether this is the full-length irisin protein or a further modified form remains to be determined. The therapeutic implications of this are obvious, since the data suggest that a polypeptide might be developed as a drug capable of giving neuroprotection in disease states or improved cognition in aging populations.

EXPERIMENTAL PROCEDURES

Reagents

Recombinant human BDNF was purchased from PeproTech, recombinant human GDNF and CNTF and forskolin were purchased from Sigma, and recombinant mouse IGF-1 was obtained from R&D Systems. Recombinant mouse NGF and K252a were obtained from EMD Millipore. Nifedipine, XCT 790, DY131, GW7647, and GW0742 were purchased from Tocris. Recombinant irisin (human, rat, mouse, canine) was obtained from Phoenix Pharmaceuticals (Burlingame).

Primers Used for qPCR

All primers used are listed with their sequences in Table S1.

Animal Studies

All animal experiments were performed according to procedures approved by the IACUC of Dana-Farber Cancer Institute and the BIDMC. *Pgc1a*$^{-/-}$ mice have been described previously (Lin et al., 2004). Mice were housed and exercised as previously described (Bostrom et al., 2012).

Cell Culture

Primary cortical and hippocampal neurons were isolated as described previously (Bartlett and Banker, 1984).

RNA and Protein Preparation and Analysis

RNA and protein analyses were performed as described previously (Bostrom et al., 2012).

Forced Expression and Knockdown of Target Genes

Generation and delivery of PGC-1α, GFP, and FNDC5 adenovirus have been described before (Bostrom et al., 2012; Lustig et al., 2011). For knockdown studies, primary cortical neurons were transduced with lentiviral viral supernatants.

Cell Viability Assay

Cell viability of cultured neurons was assessed by using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison) according to the manufacturer's instructions.

Analysis of the Murine *Fndc5* Promoter for Erra Transcription Factor Binding Sites

The genomic sequence of the murine *Fndc5* gene and 6 kb of its upstream promoter was retrieved from the USCS Genome browser (http://www.genome.ucsc.edu/; assembly mm9). This genomic sequence was searched for the canonical Erra transcription factor binding motif TGACCTT. This motif had been identified and established in previous studies (Charest-Marcotte et al., 2010; Mootha et al., 2004; Wang et al., 2012).

Peripheral Delivery of FNDC5 by Adenoviral Vectors

High-titer GFP- or FNDC5-expressing adenoviral particles were obtained by ViraQuest Inc. (North Liberty, IA). Five-week-old male wild-type BALB/c mice were injected with GFP- or FNDC5-expressing adenoviral particles (10^{11}/animal) intravenously. Animals were sacrificed 7 days later, and the indicated tissues were harvested for gene expression analyses using qPCR.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2013.09.008.

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