

Adiponectin receptor-mediated signaling ameliorates cerebral cell damage and regulates the neurogenesis of neural stem cells at high glucose concentrations: an *in vivo* and *in vitro* study

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In the central nervous system (CNS), hyperglycemia leads to neuronal damage and cognitive decline. Recent research has focused on revealing alterations in the brain in hyperglycemia and finding therapeutic solutions for alleviating the hyperglycemia-induced cognitive dysfunction. Adiponectin is a protein hormone with a major regulatory role in diabetes and obesity; however, its role in the CNS has not been studied yet. Although the presence of adiponectin receptors has been reported in the CNS, adiponectin receptor-mediated signaling in the CNS has not been investigated. In the present study, we investigated adiponectin receptor (AdipoR)-mediated signaling *in vivo* using a high-fat diet and *in vitro* using neural stem cells (NSCs). We showed that AdipoR1 protects cell damage and synaptic dysfunction in the mouse brain in hyperglycemia. At high glucose concentrations *in vitro*, AdipoR1 regulated the survival of NSCs through the p53/p21 pathway and the proliferation- and differentiation-related factors of NSCs via *tailless* (TLX). Hence, we suggest that further investigations are necessary to understand the cerebral AdipoR1-mediated signaling in hyperglycemic conditions, because the modulation of AdipoR1 might alleviate hyperglycemia-induced neuropathogenesis.

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Adiponectin secreted by the adipose tissue^{1,2} exists in either a full-length or globular form.^{3–6} Adiponectin can cross the blood–brain barrier, and various forms of adiponectin are found in the cerebrospinal fluid.^{7–11} Adiponectin exerts its effect by binding to the adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2)^{12,13} that have different affinities for the various circulating adiponectins.^{12,14–17} Several studies reported that both receptor subtypes are expressed in the central nervous system (CNS).^{7,12,18} As adiponectin modulates insulin sensitivity and inflammation,¹⁹ its deficiency induces insulin resistance and glucose intolerance in animals fed a high-fat diet (HFD).^{19–21} In addition, adiponectin can ameliorate the glucose homeostasis and increase insulin sensitivity.^{22–24} Adiponectin, which is the most well-known adipokine, acts mainly as an anti-inflammatory regulator,^{25,26} and is associated with the onset of neurological disorders.²⁷ In addition, a recent study reported that adiponectin promotes the proliferation of hippocampal neural stem cells (NSCs).²⁸ Considering that adiponectin acts by binding to the adiponectin receptors, investigation of the adiponectin receptor-mediated signaling in the brain is crucial to understand the cerebral effects of adiponectin and the underlying cellular mechanisms.

The prevalence of type II diabetes mellitus (DM2) and Alzheimer's disease increases with aging.²⁹ According to a cross-sectional study, in people with DM2, the risk of dementia is 2.5 times higher than that in the normal population.^{30,31} A study performed between 1980 and 2002 suggested that an elevated blood glucose level is associated with a greater risk for dementia in elderly patients with DM2.³² In addition, according to a 9-year-long longitudinal cohort study, the risk of developing Alzheimer's disease was 65% higher in people with diabetes than in control subjects.³³ A community-based cohort study also reported that higher plasma glucose concentrations are associated with an increased risk for dementia, because the higher glucose level has detrimental effects on the brain.³¹ High blood glucose level causes mitochondria-dependent apoptosis,^{34–36} and aggravates diverse neurological functions.^{37,38} Inflammation and oxidative stress, which are commonly observed in people with diabetes, inhibit neurogenesis.^{39–41} Similarly, neurogenesis is decreased in mice and rats with genetically induced type I diabetes.^{42,43} In addition, diabetic rodents have a decreased proliferation rate of neural progenitors.^{43,44} Furthermore, several studies suggested that an HFD leads to neuroinflammation, the impairment of synaptic plasticity, and cognitive decline.^{45,46}

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Abbreviations: AdipoR1, adiponectin receptor 1; AdipoR2, adiponectin receptor 2; HFD, high-fat diet; NSCs, neural stem cells; DCX, doublecortin

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Here, we investigated whether AdipoR1-mediated signaling is associated with cell death in the brain of mice on a HFD, and whether high glucose level modifies the proliferation and differentiation capacity of NSCs *in vitro*. Our study provides novel findings about the role of AdipoR1-mediated signaling in hyperglycemia-induced neuropathogenesis.

Results

HFD led to cell death in the mouse brain. To assess the morphological alterations of neurons in mice fed a HFD, we performed cresyl violet staining in the cortex and striatum (Figure 1). In the control group, healthy round cells were observed in these areas (Figure 1a). In the HFD group, thin small cell bodies and damaged brain tissue were visible in the cortex and striatum (Figure 1b). To identify whether apoptotic cells were present in the brain tissues of mice fed a HFD, we used the TdT-mediated dUTP nick end labeling (TUNEL) assay⁴⁷ (Figure 2). More TUNEL-positive cells were observed in the cortex, striatum, and hippocampus of mice fed a HFD than those in the brain regions of the control mice (Figure 2). Our data suggest that HFD damages cells in the cortex, striatum, and hippocampus (Figures 2a and b). In addition, we assume colocalization with the NeuN, known as the marker of neuron in TUNEL-positive cells (Figure 2c). Figure 2c indicates that neuronal cells may be damaged in HFD brain in comparison with the normal brain.

HFD decreased the expression of PSD95 and DCX in the mouse brain. To determine the alterations caused by HFD in a protein regulating synaptic plasticity and in a neuronal microtubule-associated protein, we performed immunohistochemistry using the antibodies specific for postsynaptic density protein 95 (PSD95), a protein in the postsynaptic density (Figure 3), and for doublecortin (DCX), a microtubule-associated protein involved in neuronal migration (Figure 4). The immunoreactivity for PSD95 synaptic density protein was reduced in the mice fed a HFD compared with that in the

control group (Figure 3a). This result indicates that HFD suppresses PSD95 expression in the striatum, cortex, and hippocampus. Similarly, the protein level of PSD95 was reduced in the HFD group (Figure 3b). To examine the expression of the neuronal microtubule protein DCX, we performed immunohistochemical (Figure 4a) and western blot (Figures 4b and c) analyses. DCX immunoreactivity was reduced in the brain of mice fed a HFD compared with that of the control group (Figure 4a). Western blot revealed a similar decrease in the protein level of DCX in cortex (Figure 4b) and in hippocampus (Figure 4c). Our results suggest that HFD damaged the synaptic plasticity and reduced the amount of immature neuronal NSCs in the brain.

HFD attenuated the expression of TLX in the mouse brain. To determine alterations in a differentiation and proliferation-related transcription factor in the brain, we checked the expression of tailless (TLX) in mice fed a HFD (Figure 5). TLX immunoreactivity was reduced in the brain of mice fed a HFD compared with that of the control animals (Figure 5a). The protein level of TLX in the striatum, cortex, and hippocampus of mice fed a HFD was attenuated compared with that of the control mice (Figure 5b). As several studies demonstrated that the TLX transcription factor is related to self-renewal and neurogenesis of the NSCs,^{48–50} our findings suggest that HFD reduces TLX expression in the brain.

HFD reduced AdipoR1 expression. To assess the expression of AdipoR1 and AdipoR2 in the brain of mice fed a HFD, we performed immunohistochemical analysis (Figures 6a and c) and western blotting (Figures 6b and d) using specific antibodies. Immunoreactivity for AdipoR1 in brain of mice fed a HFD was decreased compared with that in the control group (Figure 6a). Western blot experiments showed that the protein level of AdipoR1 in the striatum, cortex, and hippocampus was reduced in the HFD group (Figure 6b). Immunoreactivity for AdipoR2 in HFD brain were reduced compared with the control group (Figure 6c). The protein levels of AdipoR2 in HFD brain were slightly attenuated in

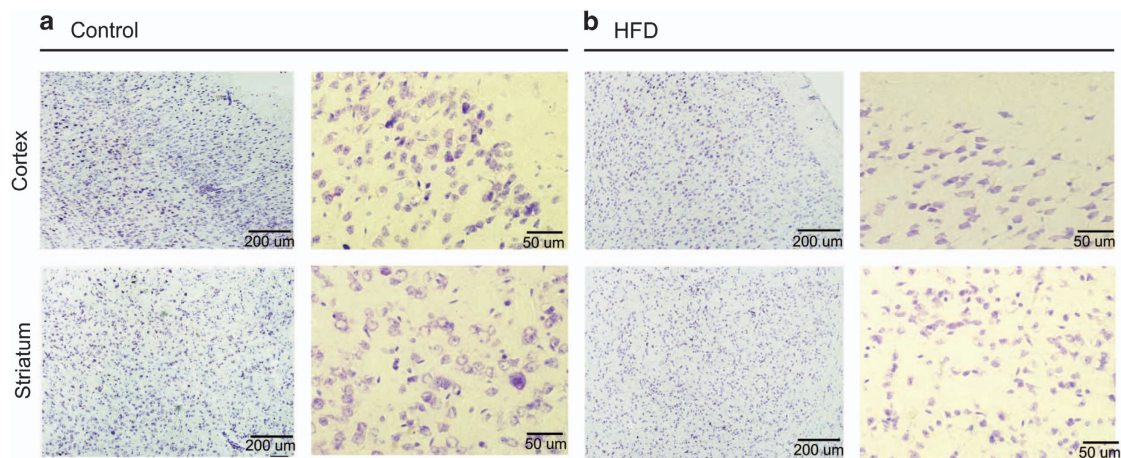


Figure 1 Histological assessment of the brain of mice fed a high-fat diet (HFD) using cresyl violet staining. Cresyl violet staining revealed a slight cell loss in the cortex and striatum of mice fed a HFD (b), whereas cells in the control group had more healthy and round cell bodies in the cortex and striatum (a). Scale bar: 200 and 50 μm . Control: control group; HFD: high-fat diet group

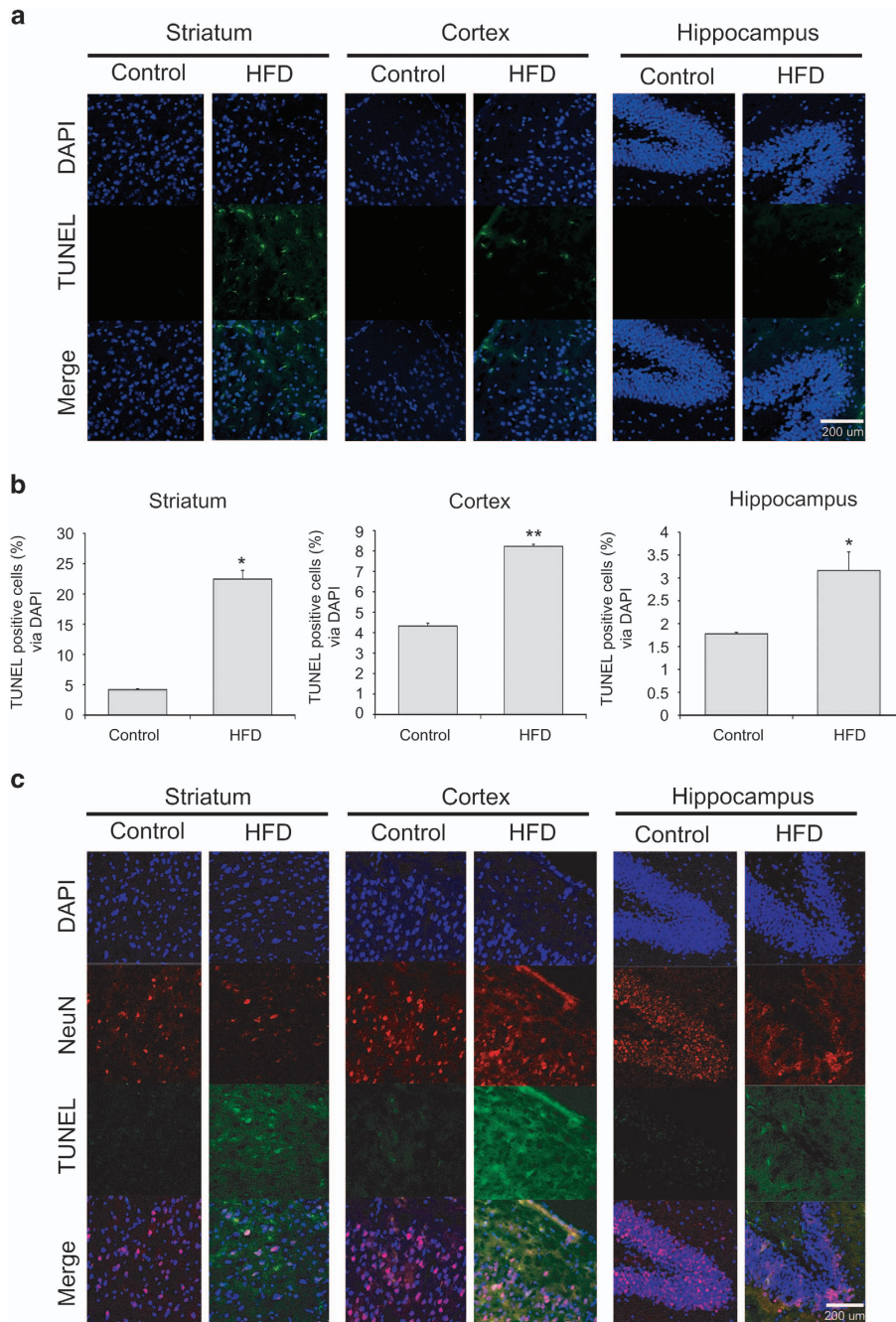


Figure 2 Measurement of cell damage using the TUNEL assay. (a) TUNEL-positive signal (green), which indicates cells with DNA damage, was increased in the brain of mice fed a high-fat diet (HFD). (b) Similarly, TUNEL signal was increased in the cortex, striatum, and hippocampus in the HFD group compared with that in the control group. (c) NeuN (red)-positive cells were decreased in the HFD brain striatum and hippocampus. TUNEL (green)- and NeuN (red)-positive signals were also increased in the HFD brain compared with the control group. Scale bar: 200 μ m. Control: control group, HFD: high-fat diet group. 4',6-diamidino-2-phenylindole (DAPI); blue; TdT-mediated dUTP nick end labeling (TUNEL): green; NeuN: red. * $P < 0.05$, ** $P < 0.001$

comparison with the control group brain (Figure 6d). Our findings suggest that HFD decreases AdipoR expression, and attenuates the AdipoR-mediated signaling in the brain.

Adiponectin maintains the neurosphere size of NSCs at high glucose concentrations *in vitro*. We investigated whether neurosphere size of NSCs was changed by

adiponectin at high glucose concentrations (Figure 7). Neurosphere size was measured using bright-field microscopy (Figure 7a). Neurosphere size was not altered by the adiponectin (Acrp30, 30 μ g/ml) treatment, and was similar to that in the control group. A treatment with 120 mM glucose reduced the neurosphere size compared with that in the control group. However, addition of Acrp30 (30 μ g/ml) and 120 mM glucose did not alter neurosphere size compared

with that in the control group (Figures 7a and b). Especially, 120 mM glucose group showed significant differences with the control group (Figure 7b). Therefore, our data showed that adiponectin helps to maintain the normal size of NSC neurospheres at high glucose concentration.

Adiponectin restored the reduced expression of AdipoR1 in the NSCs caused by high glucose concentrations. To examine AdipoR1 and AdipoR2 expression in the NSCs at high glucose concentrations, we conducted reverse transcription PCR (Figure 8a), quantitative PCR (Figures 8b and c) and

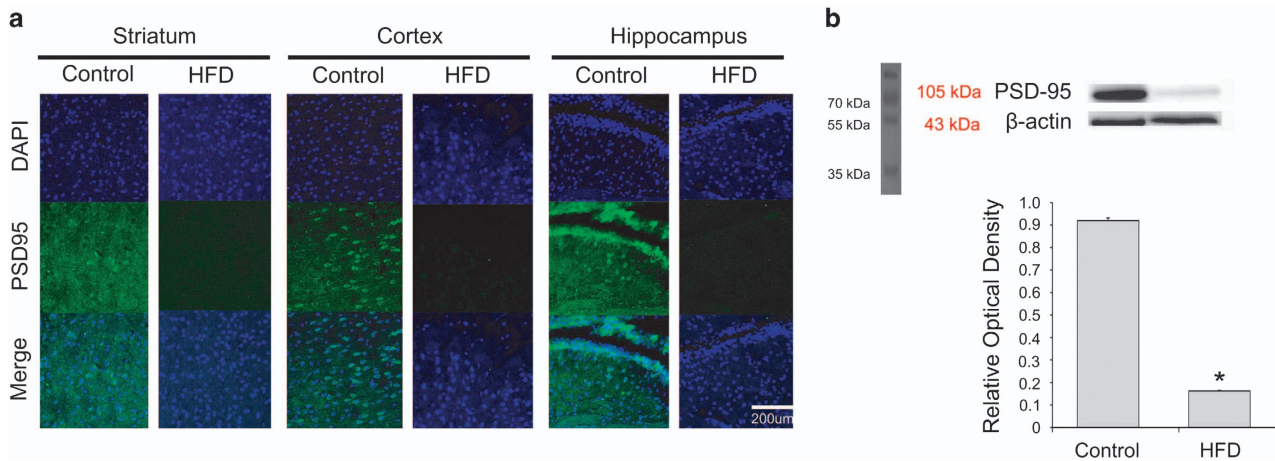


Figure 3 Measurement of PSD95 expression in the brain of mice fed a high-fat diet (HFD). (a) Immunohistochemical experiments revealed that the PSD95-positive immunostaining (green) was attenuated in the brain of mice fed a HFD. In addition, PSD95 immunoreactivity was reduced in the striatum, cortex, and hippocampus of the HFD group compared with those of the control group. Scale bar: 200 μ m. 4',6-diamidino-2-phenylindole (DAPI): blue; postsynaptic density protein 95 (PSD95): green. (b) The protein level of PSD95 was significantly reduced in the HFD group compared with that in the control group. β -Actin was used as internal control. Data are expressed as mean \pm S.E.M., and each experiment included three repeats per condition. Differences were considered significant at $*P < 0.05$. Control: control group; HFD: high-fat diet group

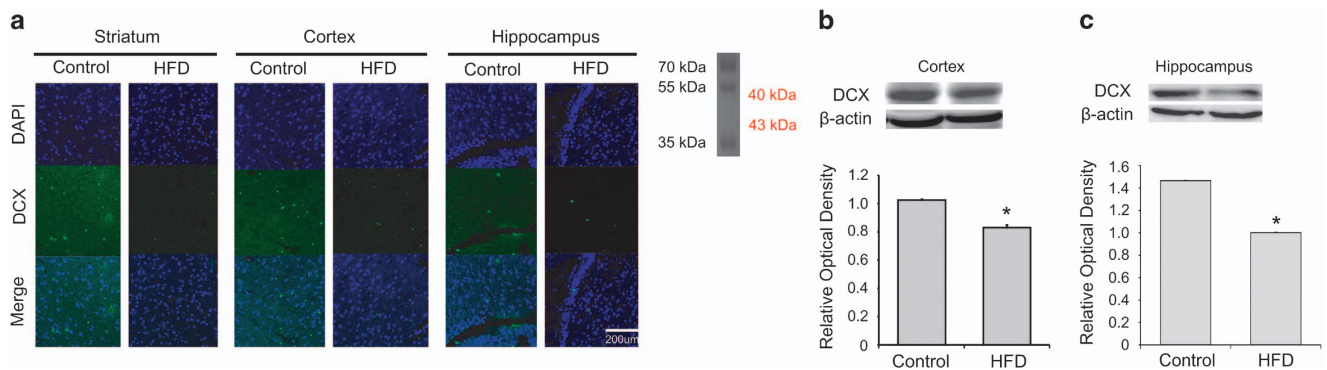


Figure 4 Measurement of DCX expression in the brain of mice fed a high-fat diet (HFD). (a) Immunohistochemical experiments revealed that the DCX-immunopositive signal (green) was reduced in the brain of mice fed a HFD. In addition, DCX immunoreactivity was decreased in the striatum, cortex, and hippocampus of the HFD group compared with those of the control group. Scale bar: 200 μ m. 4',6-diamidino-2-phenylindole (DAPI): blue; doublecortin (DCX): green. (b) The protein level of DCX was significantly reduced in the HFD brain cortex compared with that in the control group. β -Actin was used as internal control. Data are expressed as mean \pm S.E.M., and each experiment included four repeats per condition. Differences were considered significant at $*P < 0.05$. (c) The protein level of DCX was a little decreased in the HFD brain hippocampus in comparison with the control group. β -Actin was used as internal control. Data are expressed as mean \pm S.E.M., and each experiment included four repeats per condition. Differences were considered significant at $*P < 0.05$. Control: control group; HFD: high-fat diet group

Figure 6 Measurement of AdipoR1 and AdipoR2 expression in the brain of mice fed a high-fat diet (HFD). (a) Immunohistochemical experiments revealed that the AdipoR1-positive immunolabeling (red) was attenuated in the brain of mice fed a HFD. In addition, AdipoR1 immunoreactivity was reduced in the striatum, cortex, and hippocampus of the HFD group compared with those of the control group. Scale bar: 200 μ m. 4',6-diamidino-2-phenylindole (DAPI): blue; AdipoR1: red. (b) Western blotting experiments showed that the relative protein level of AdipoR1 was reduced in the HFD group compared with that in the control group. β -Actin was used as internal control. Data are expressed as mean \pm S.E.M., and each experiment included three repeats per condition. Differences were considered significant at $*P < 0.05$. (c) Immunohistochemical experiments revealed that the AdipoR2-positive immunolabeling (green) was attenuated in the brain of mice fed a HFD. In addition, AdipoR2 immunoreactivity was reduced in the striatum, cortex, and hippocampus of the HFD group compared to those of the control group. Scale bar: 200 μ m. 4',6-diamidino-2-phenylindole (DAPI): blue; AdipoR2: green. (d) Western blotting experiments showed that the relative protein level of AdipoR2 was slightly reduced in the HFD group compared with that in the control group. β -Actin was used as internal control. Data are expressed as mean \pm S.E.M., and each experiment included three repeats per condition. Differences were considered significant at $*P < 0.05$. Control: control group; HFD: high-fat diet group

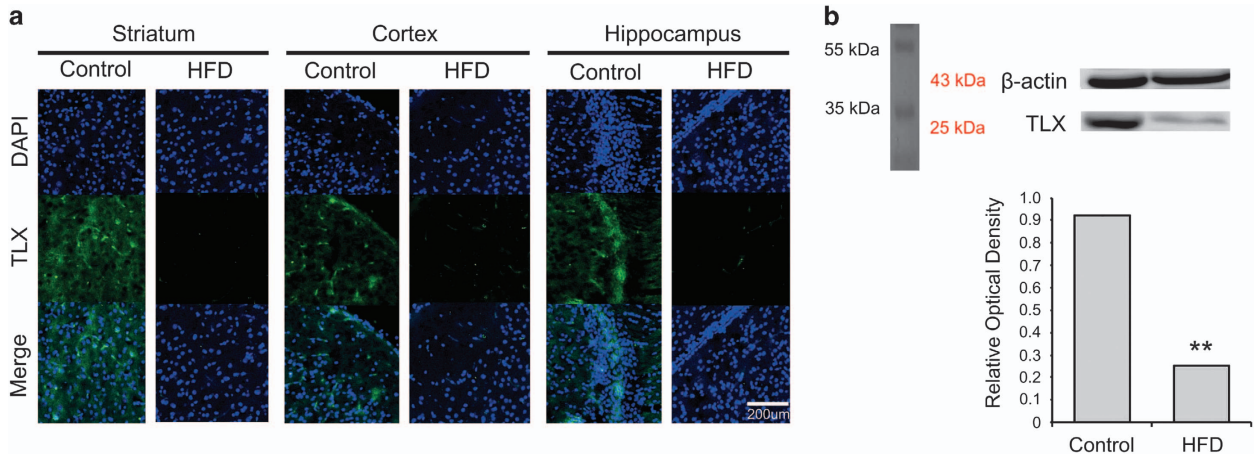
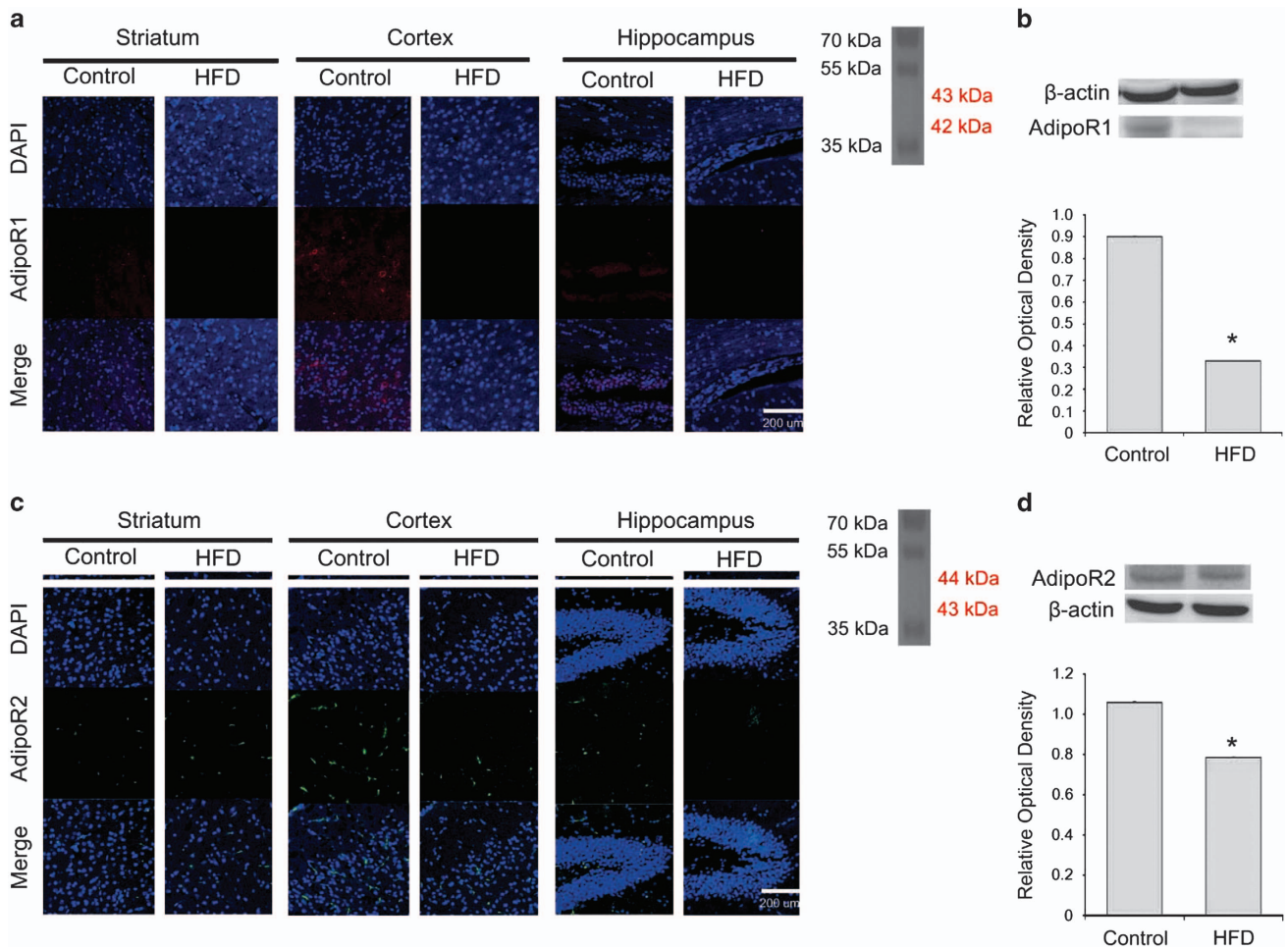


Figure 5 Measurement of TLX expression in the brain of mice fed a high-fat diet (HFD). (a) Immunohistochemical experiments revealed that the TLX-positive immunostaining (green) was attenuated in the brain of mice fed a HFD. In addition, TLX immunoreactivity was reduced in the striatum, cortex, and hippocampus of the HFD group compared with those in the control group. Scale bar: 200 μ m. 4',6-diamidino-2-phenylindole (DAPI); blue; TLX: green. (b) The protein level of TLX was reduced in the HFD group compared with that in the control group. β -Actin was used as internal control. Data were expressed as mean \pm S.E.M., and each experiment included four repeats per condition. Differences were considered significant at $**P < 0.001$. Control: control group; HFD: high-fat diet group



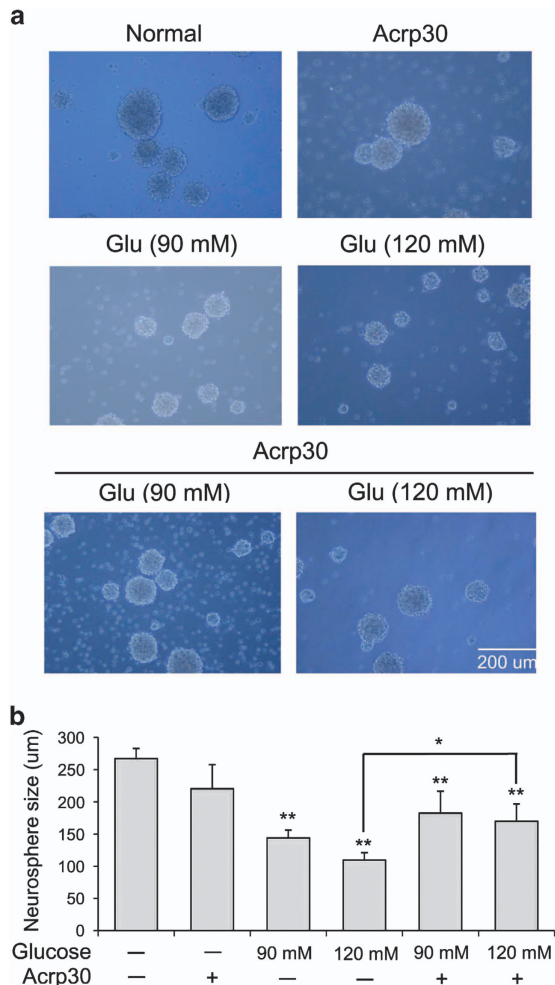


Figure 7 Measurement of neurosphere size at high glucose concentration. (a) Neurospheres were observed using bright-field microscopy, and neurosphere size was measured using the Image J (Madison, Wisconsin, USA). Size of neurospheres was identical in the Acrp30 treatment and the control groups. Glucose treatment reduced the neurosphere size compared with the control group. Glucose and Acrp30 treatment increased the size of neurospheres compared with the glucose treatment only. Scale bar: 200 μ m. Normal: control group; Glu (90 mM): 90 mM glucose treatment group; Glu (120 mM): 120 mM glucose treatment group; Acrp30: Acrp30 (30 μ g/ml) treatment group. (b) The graph showed the size of neurospheres in each group. In 90 and 120 mM glucose group, neurosphere size was attenuated in comparison with the normal control group. Under Acrp30 treatment, the neurospheres were increased compared with the glucose treatment only. * $P < 0.05$, ** $P < 0.001$

immunohistochemical analysis (Figure 8d). At 120 mM glucose concentration, mRNA level of AdipoR1 in the NSCs was attenuated compared with that in the control group, whereas Acrp30 (30 μ g/ml) treatment restored the mRNA level of AdipoR1 reduced by high glucose concentrations (Figures 8a and b). At 120 mM glucose concentration, mRNA level of AdipoR2 in the NSCs was slightly attenuated compared with that in the control group, whereas Acrp30 (30 μ g/ml) treatment increased the mRNA level of AdipoR2. Acrp30 treatment did not largely increase the expression of AdipoR2 in high glucose concentrations (Figure 8c). In addition, we observed less AdipoR1 and DCX immunoreactivity in the NSCs exposed to high glucose concentration

than in the control group (Figure 8d). However, the decreased immunoreactivity of AdipoR1 and DCX was enhanced by Acrp30 (30 μ g/ml) treatment at high glucose concentrations (Figure 8d). Taken together, adiponectin may promote the expression of AdipoR1 and DCX in the NSCs exposed to high glucose concentrations.

Adiponectin inhibits the pathways responsible for apoptosis in the NSCs at high glucose concentrations.

To investigate the effect of adiponectin on the apoptosis signaling pathways at high glucose concentrations, we measured the mRNA level of the apoptosis-related factors p21, p53, and c-Myc^{51–53} (Figure 9). Blocking AdipoR1 using AdipoR1 blocker compound increases the mRNA levels of p21 (Figure 9a), p53 (Figure 9b), and c-Myc (Figure 9c) in the NSCs compared with the Acrp30 (30 μ g/ml) treatment. The mRNA levels of p21, p53, and c-Myc in the NSCs were significantly reduced by treatment with both Acrp30 (30 μ g/ml) and glucose (120 mM) compared with treatment with glucose only (Figures 9d–f). We propose that adiponectin may inhibit the apoptosis pathways mediated by p21/p53 and c-Myc at high glucose concentrations.

Adiponectin enhances neurogenesis and proliferation in the NSCs at high glucose concentrations.

To study the effect of adiponectin on the neurogenesis and proliferation of NSCs at high glucose concentrations, we measured the mRNA levels of the neuronal cell factor DCX and the proliferation and neurogenesis-related factor TLX (Figure 10). Acrp30 (30 μ g/ml) increased the mRNA levels of DCX (Figure 10a) and TLX (Figure 10b) in the NSCs. In addition, under Acrp30 (30 μ g/ml) with 120 mM glucose condition, the mRNA levels of DCX (Figure 10a) and TLX (Figure 10b) increased compared with the glucose treatment group (Figures 10a and b). We observed a decrease in neurosphere size by inhibiting TLX using siTLX, and an increase in neurosphere size by Acrp30 (30 μ g/ml) treatment (Figures 10c and d). Expressions of DCX and TLX in the NSCs were increased by TLX inhibition (Figures 10e and f). These data suggest that adiponectin might promote the neurogenesis related to DCX and TLX in the NSCs at high glucose concentrations.

Discussion

Current research reports a positive correlation between DM2 and cognitive impairment in a prospective population-based study,⁵⁴ and an accelerated progression from mild cognitive decline to dementia in the elderly patients with DM2 compared with the normal subjects.⁵⁵ In addition, patients with diabetes (35%) or glucose intolerance (46%) have Alzheimer's disease in up to 80% of patients.⁵⁶ Hyperglycemia is a main cause of neuronal damage, leading to neurodegeneration in the CNS.^{57–60} Cognitive impairment in DM2 generally affects the brain regions responsible for learning and memory.^{38,61} Patients with DM2-induced dementia have mitochondrial dysfunction and alterations in the neuronal synapses.^{62–64} Considering that the number of unhealthy and TUNEL-positive cells, which are considered as apoptotic,⁶⁵ was increased in the brain of mice fed a HFD, we suggest that HFD leads to

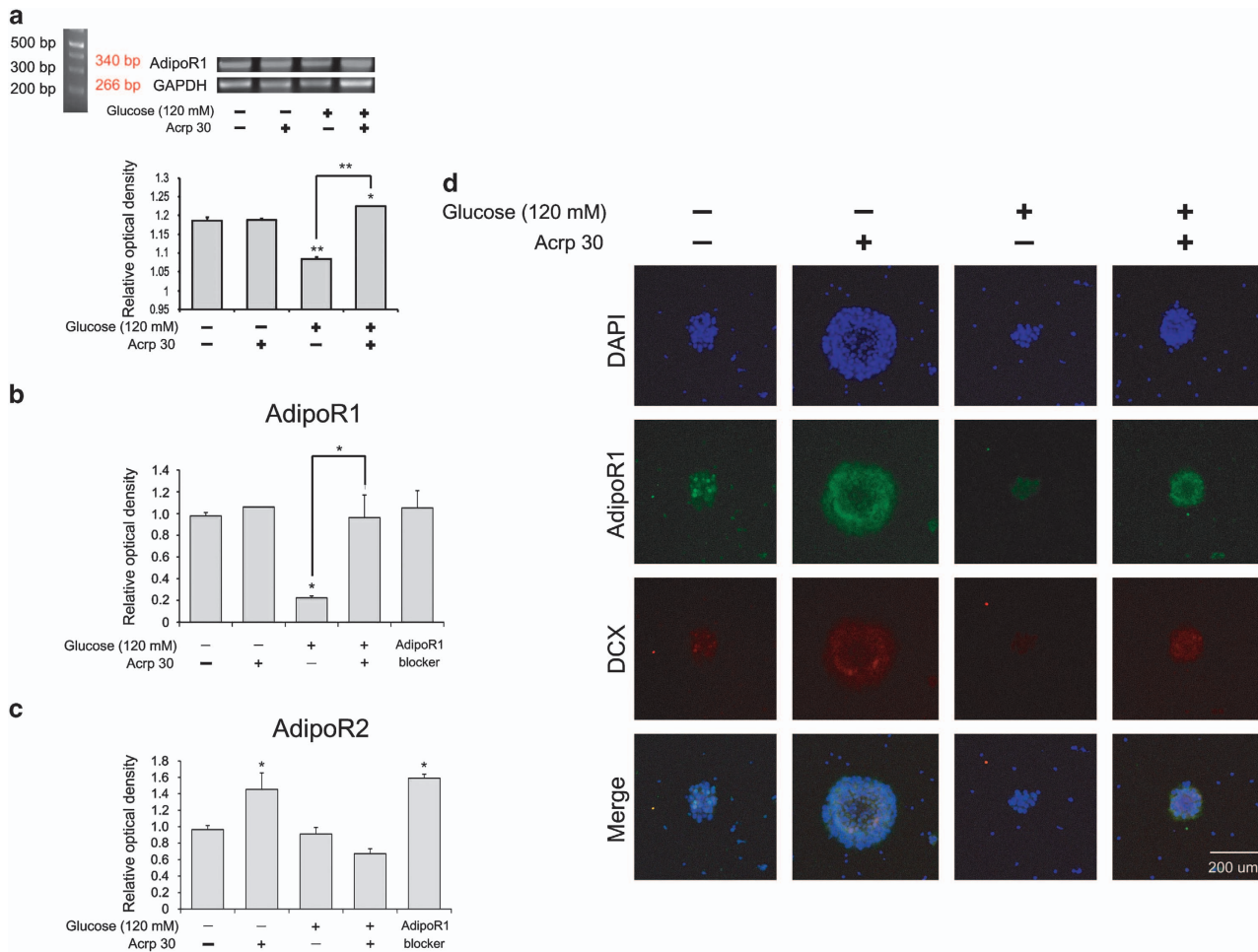


Figure 8 Measurement of AdipoR1 and AdipoR2 expression in the NSCs. The mRNA level of AdipoR1 (a and b) and AdipoR2 (c) was measured with reverse transcription PCR (a) and quantitative PCR (b and c). (a and b) Glucose treatment (120 mM) reduced the mRNA level of AdipoR1 compared with the control group. Glucose and Acrp30 treatments in combination increased the mRNA level of AdipoR1 compared with the glucose treatment. Data are expressed as mean \pm S.E.M., and each experiment included three repeats per condition. GAPDH was used as control gene. Differences were considered significant at $*P < 0.05$ and $**P < 0.001$. (c) Glucose treatment (120 mM) slightly reduced the mRNA level of AdipoR2 compared with the control group. Glucose and Acrp30 treatments in combination was not increased the mRNA level of AdipoR2 compared with the glucose treatment. Data are expressed as mean \pm S.E.M., and each experiment included three repeats per condition. GAPDH was used as control gene. Differences were considered significant at $*P < 0.05$. (d) Immunohistochemical experiments revealed that the expression of AdipoR1 (green) was reduced in the glucose treatment group compared with that in the control group. Treatment with both glucose and Acrp30 increased AdipoR1 expression compared with the glucose treatment. Scale bar = 200 μ m. 4',6'-diamidino-2-phenylindole (DAPI): blue; adiponectin receptor 1 (AdipoR1): green; DCX: red

cellular damage in the brain. In addition, reduction of the postsynaptic protein PSD95, which is associated with memory,⁶⁶ suggests that HFD impairs synaptic plasticity.

Memory dysfunction in type II diabetes mellitus⁶⁷ triggers neuronal death and inhibits neurogenesis.^{40,68,69} In addition, HFD accelerates the progression of diabetes and inhibits neurogenesis in rodents.⁷⁰ Patients with DM2 have decreased adiponectin plasma levels compared with normal subjects.⁷¹ In an animal study of DM2, decreased adiponectin level was considered the reason for the higher prevalence of insulin resistance.⁷² In the present study, we observed the decrease of AdipoR1 receptor^{13,73–76} in various brain regions including the striatum, cortex, and hippocampus compared with the control group; however, we did not measure the plasma levels of adiponectin in the mice fed a HFD.

Recent studies demonstrated that HFD induces synaptic dysfunction⁷⁷ and leads to neurotoxicity *in vivo*.⁷⁸ Our results

showed that the mRNA levels of p21, p53, and cMyc in the NSCs were increased at high glucose concentrations, whereas these effects were attenuated by adiponectin. The protein p53 triggers apoptosis,^{79,80} inhibits self-renewal, induces the differentiation of embryonic stem cells,⁸¹ and blocks the reprogramming of progenitor cells into stem cells.^{82,83} The activation of p53 and p21 indicates DNA damage in stem cells.^{84–88} In addition, the activation of p53/p21 signaling leads to cell arrest in the G₁ and G₂ phases of the cell cycle, and thus apoptosis.^{89,90} Recent studies demonstrated that the increased apoptosis of NSCs through p53 and p21 inhibits proliferation.^{84,91} Proliferation of bone marrow-derived stem cells is blocked by activating the cell cycle inhibitors p53 and p21.⁹² In an animal study investigating diabetes, the activation of p21 triggered the apoptosis of bone marrow-derived mesenchymal stem cells.⁹³ In addition, p53 induces the transduction of c-Myc^{94,95} and thus regulates

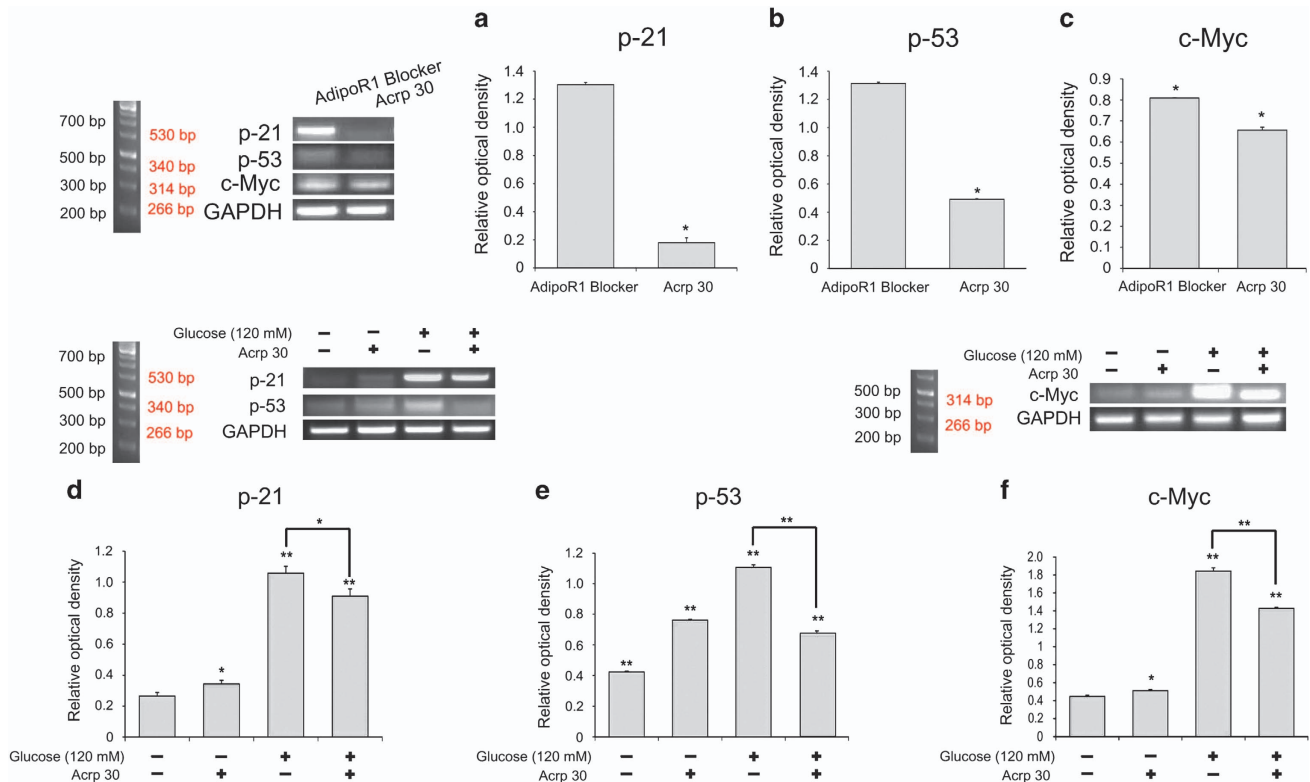


Figure 9 Measurement of mRNA levels in the NSCs using reverse transcription PCR. The mRNA levels of p21 (a and d), p53 (b and e), and c-Myc (c and f) in the NSCs were evaluated using reverse transcription PCR. Acrap30 (30 μ g/ml) treatment reduced the mRNA levels of p21 (a), p53 (b), and c-Myc (c) in the NSCs compared with the blocking of AdipoR1. The mRNA levels of p21 (d), p53 (e), and c-Myc (f) in the NSCs were reduced upon treatment with both glucose and Acrap30 (30 μ g/ml) compared with those treated with glucose. Data are expressed as mean \pm S.E.M., and each experiment included three repeats per condition. GAPDH was used as control gene. Differences were considered significant at * $P < 0.05$ and ** $P < 0.001$

various mechanisms such as apoptosis,⁹⁶ cell growth,⁹⁷ and self-renewal of embryonic stem cells.⁹⁸ As the increase of p21, p53, and c-Myc induces apoptosis,^{52,99–101} our results imply that adiponectin protects against cell death of NSCs during glucose-induced toxicity. In the present study, we showed that HFD attenuated the expression of DCX and TLX neurogenesis markers in the brain. The nuclear receptor TLX is required for neurogenesis in the subventricular zone.^{102,103} Several studies reported that this orphan nuclear receptor increases the self-renewal of NSCs^{104–107} and promotes the neurogenesis of neural precursor cells.^{50,108–111} TLX-positive cells are associated with learning and memory.¹⁰⁵ Similarly, the neurogenesis-related protein DCX^{112,113} is crucial for learning and memory,^{114–117} and is involved in synaptic dysfunction.^{118–120} Our results showed that adiponectin increases the expression of TLX and DCX in the NSCs at high glucose concentrations, and that blocking AdipoR1 reduces TLX expression in the NSCs. Furthermore, the blockade of TLX did not change the size of neurospheres, and adiponectin treatment increased TLX expression.

In conclusion, our findings in the present study suggest the following. Considering our *in vivo* results, HFD might lead to the reduction of AdipoR1 that is related to cell survival,^{121–123} impaired synaptic plasticity,^{124–126} and attenuation of neurogenesis.^{112,127} Considering our *in vitro* results, AdipoR1-mediated signaling might protect the NSCs against cell damage at high glucose concentrations, and might

promote the self-renewal and neurogenesis of the NSCs at high glucose concentrations. The lack of clinical data is a limitation of this study. However, as our study has indicated the potential of adiponectin to alleviate hyperglycemia-induced neuropathogenesis, our results might spark further studies investigating adiponectin receptor signaling in the CNS.

Materials and Methods

Animal experiments. Male 3-week-old C57BL/6 mice (Orient, GyeongGi-Do, South Korea; <http://www.orientbio.co.kr>) were fed conventional chow or HFD; the latter was enriched in either fat (35.5% wt/wt; Bioserv, Frenchtown, NJ, USA) or fructose (60% wt/wt; Harlan Teklad, Madison, WI, USA) for 4 weeks. Then, animals on the HFD were injected once with streptozotocin (STZ; 100 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally to induce partial insulin deficiency, and then the HFD was continued for an additional 4 weeks. The majority of mice in the STZ/HFD group exhibited hyperglycemia, insulin resistance, and glucose tolerance, as previously reported.¹²⁸ The mice that were fed conventional chow (control group) were injected intraperitoneally with vehicle (0.05 mol/l citric acid, pH 4.5). To obtain their brains, mice were killed under ether anesthesia.

Cresyl violet staining. After the mice were killed, their brains were fixed in 3.7% formaldehyde and immediately frozen. The brains were sectioned coronally at a thickness of 20 μ m, and the sections were sequentially incubated in xylene for 4 min, 100% alcohol for 5 min, 95% alcohol for 5 min, and 70% alcohol for 5 min. Samples were stained with cresyl violet (Sigma-Aldrich) for 5 min. After the staining, slides were dehydrated with 70% alcohol for 3 min, 95% alcohol for 3 min, 100% alcohol for 3 min, and xylene for 5 min. Sections were observed using a light microscope equipped with a digital camera (Olympus, Tokyo, Japan).

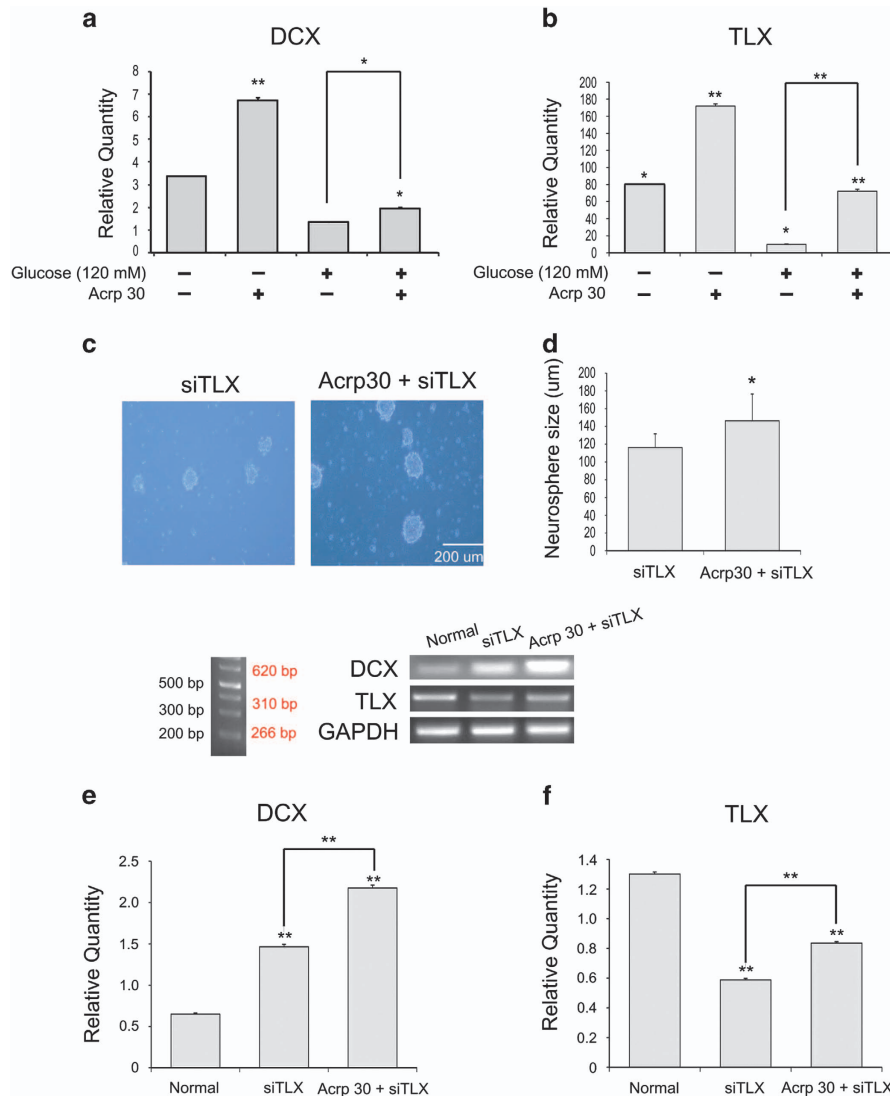


Figure 10 Measurement of DCX and TLX expression in the NSCs. The mRNA levels of DCX (a) and TLX (b) in the NSCs were evaluated using quantitative PCR. Treatment with both glucose and Acpr30 (30 μ g/ml) increased the mRNA levels of DCX (a) and TLX (b) compared with the glucose treatment only. (c and d) Neurosphere size was reduced by using siTLX, but Acpr30 (30 μ g/ml) treatment slightly increased neurosphere size. Data are expressed as mean \pm S.E.M., and each experiment included three repeats per condition. Differences were considered significant at $*P < 0.05$. Blocking TLX significantly increased the mRNA levels of DCX (e) and TLX (f) in the NSCs treated with Acpr30 (30 μ g/ml). Data are expressed as mean \pm S.E.M., and each experiment included three repeats per condition. GAPDH was used as control gene. Differences were considered significant at $*P < 0.05$ and $**P < 0.001$

TdT-mediated dUTP nick end labeling. Apoptotic cells were detected *in situ* using the Roche TUNEL kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. The TUNEL assay was conducted to visualize the 3'-OH ends of DNA fragments in apoptotic cells. After xylene dewaxing, sections were rinsed three times in distilled water for 5 min, and they were washed in methanol containing 0.3% H_2O_2 at room temperature for 30 min to inhibit endogenous peroxidase activity. After rinsing in PBS three times at room temperature for 5 min, sections were treated with proteinase K at 37 $^{\circ}$ C for 6 min. Section were rinsed in PBS three times at room temperature for 3 min, were soaked in TdT buffer for 10 min, and incubated in 50 μ l TdT buffer containing TdT at 37 $^{\circ}$ C for 60 min in a moist chamber (Roche). After three rinsing steps in PBS at room temperature for 5 min, the sections were incubated in 50 μ l FITC (Roche) at 37 $^{\circ}$ C for 40 min. After three further rinses in PBS for 3 min, the brain sections were incubated in DAB (Roche) at room temperature for 3 min, and the signal was observed using a confocal microscope (Zeiss LSM 700, Carl Zeiss, Oberkochen, Germany).¹²⁹

Immunohistochemistry of the sections. Frozen brain sections, 5 μ m thick, were cut onto clean glass slides (Thermo Scientific, Waltham, MA, USA), air-dried, and fixed in cold acetone for 10 min at -20° C. The slides were washed in Tris-buffered saline (TBS), and then incubated in 0.3% H_2O_2 in methanol to quench endogenous peroxidase activity. Followed by three washes in distilled water, the sections were blocked with 10% normal rabbit serum. To block nonspecific labeling, sections were incubated in 5% bovine serum albumin (BSA, Sigma-Aldrich) diluted in PBS for 30 min before the addition of primary and secondary antibodies. Sections were incubated with primary antibodies specific for AdipoR1 (1 : 100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), DCX (1 : 100, Abcam, Cambridge, MA, USA), NeuN (1 : 100, Abcam), PSD95, 1 : 100, Millipore, Billerica, MA, USA), and orphan factor tailless (TLX, 1 : 100, Santa Cruz Biotechnology) for 24 h at 4 $^{\circ}$ C and then washed in PBS for 10 min. After three washes in 0.1% PBS with Tween-20 (PBST), sections were incubated for 2 h in the dark at room temperature with rhodamine-conjugated sheep anti-rabbit or fluorescein isothiocyanate (FITC)-

conjugated sheep anti-mouse secondary antibodies that were diluted to 1 : 200 with 5% BSA in 0.1% PBST. After three washes in PBS, sections were incubated in 1 μ g/ml 4',6-diamidino-2-phenylindole (Sigma-Aldrich) and 2 μ g/ml propidium iodide (Sigma-Aldrich) as a counterstain. Sections were observed using a confocal microscope (Zeiss LSM 700, Carl Zeiss, Oberkochen, Germany).

NSC primary culture and drug treatment. Pregnant ICR mice were killed to obtain cortical primary NSCs according to a previously described method.¹³⁰ The brains were extracted from E13.5 embryos, and placed in a Petri dish containing Hank's balanced salt solution (HBSS, Gibco, Grand Island, NY, USA). The cortices were dissected and washed 1–2 times with HBSS. To each piece of washed tissue, 5 ml HBSS was added, and the tissue was dissociated by pipetting up and down. Tissues were triturated by repeated passages through a fire-polished constricted Pasteur pipette. Dissociated tissues were allowed to settle for 3 min. Supernatants were transferred to a fresh tube, and were centrifuged at 1200 \times g for 3 min. Pellets were resuspended in NSC basal media with a proliferation supplement (Stem Cell Technologies, Vancouver, BC, Canada), and 20 ng/ml epidermal growth factor (EGF, Invitrogen, Carlsbad, CA, USA). Live, Trypan blue-negative cells were counted. NSCs were plated on poly-D-ornithine (Sigma-Aldrich)-treated plastic dishes at a density of 2.5×10^4 cells per ml. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. After 3 days *in vitro*, the cells proliferated and formed primary neurospheres. The primary neurospheres were harvested by centrifugation, and were dissociated to single cells using Accumax (Sigma-Aldrich). The single cells were seeded on culture plates coated with 0.001% poly-L-ornithine. Culture medium was replaced every 3 days. NSCs were used for experiments after 2–3 passages.¹³¹ NSCs were exposed to Acpr30 (30 μ g/ml, Sigma-Aldrich) for 4 days after 2–3 passages. Subsequently, cells were exposed to D-glucose. The cells were also treated with the AdipoR1 blocking peptide (3 μ g/ml, GTX89569-PEP, GeneTex, Irvine, CA, USA) for 4 days of the experiment.¹³²

TLX silencing treatment. For the transfection, siRNA TLX (20 nM final concentration, Ambion, Austin, TX, USA) in Opti-MEM was mixed with Lipofectamine 2000 (Invitrogen) and incubated at room temperature for 10 min. The mixture was then added to the NSCs that incubated in the mixture for 72 h.

Quantification of neurosphere size. Neurospheres were imaged using a bright-field inverted microscope (Olympus). The magnification ($\times 10$) covered a significant area of each well from the 24-well plates. Ten non-overlapping fields were selected randomly from each well. All experiments were carried out 6 times.

Reverse transcription PCR. To examine the expression of AdipoR1, c-Myc, p21, p53, DCX, and TLX in the NSCs, reverse transcription PCR was performed to measure their mRNA levels. Briefly, cells were lysed with Trizol reagent (Invitrogen), and total RNA was extracted according to the manufacturer's protocol. We synthesized cDNA from the mRNA. The following cycling conditions were used for the PCR: 10 min at 95 °C; 35 cycles of denaturing at 95 °C for 15 s, annealing for 30 s at 62 °C, elongation at 72 °C for 30 s; final extension for 10 min at 72 °C. PCR was performed using the following primers (5' to 3'): AdipoR1 (F): GAGCATCTCC GCATTCATA, (R): AAGAGCCAGGAGAAGCTGAG; c-Myc (F): TCAAGAGCGAAC ACACAAC, (R): GGCCTTTTCATTGTTTCCA; p21 (F): AGTGTGCCGTTGTCTCT TCG, (R): ACACGAGAGTGCAAGACAGC; p-53 (F): CTGCCCTCAACAAGATGTT TTG, (R): CTATCTGAGCAGCGCTCATGG; TLX (F): GGCTCTACTTCCGTTG GACA, (R): GTCAGTATTCATGCCAGATACAGCCAGTG; DCX (F): AATCCCACTG GTCTGTCAAC, (R): GTTCCCTTCATGACTCGGCA; GAPDH (F): GGCATGGACT GTGGTCATGAG, (R): TGCACCACCAACTGCTTAGC. We examined the PCR products with electrophoresis using 1.5% agarose gels stained with ethidium bromide.

Quantitative real-time PCR. To examine the amount of AdipoR1, DCX, and TLX mRNAs in the NSCs, we performed quantitative real-time PCR. Total RNA was extracted from the NSCs using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was mixed with the One Step SYBR Prime Script TM RT-PCR Kit II (Takara, Otsu, Shiga, Japan) and the specific primers in 20 μ l total reaction volume. PCR was performed using the following primers (5' to 3'): AdipoR1 (F): GAGCATCTCCGCATTCATA, (R): AAGAGCCAGGAGAAGCTGAG; AdipoR2 (F): TGTTCCGCCACCCCTCAGTAT, (R): AATGATTCACACTCAGGCCCA, TLX (F): GGCTCTACTTCCGTTG GACA, (R): GTCAGTATTCATGCCAGATACAGCCAGTG; DCX (F): AATCCCACTG GTCTGTCAAC, (R): GTTCCCTTCATGACTCGGCA; GAPDH (F): GGCATGGACT GTGGTCATGAG, (R): TGCACCACCAACTGCTTAGC.

GAPDH (F): GGCATGGACTGTGGTCATGAG, (R): TGCACCACCAACTGCTTAGC. The PCR was performed at 42 °C for 5 min, 95 °C for 10 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 34 s, and 65 °C for 15 s. Quantitative PCR was performed with the ABI prism 7500 Real-Time PCR System (Life Technologies, Grand Island, NY, USA), and we analyzed the C_t values using relative quantification.¹³³ Data were normalized to the reference housekeeping gene *GAPDH*. The Δ C_t values of the treated cells were compared with those of the untreated cells.

Western blot analysis. Cells were washed rapidly with ice-cold PBS, scraped, and collected. Cell pellets were lysed with ice-cold RIPA buffer (Sigma-Aldrich). Lysates were centrifuged at 13 000 r.p.m. for 1 h at 4 °C to produce whole-cell extracts. Protein content was quantified using the BCA kit (Pierce, Rockford, IL, USA). Proteins (40 μ g) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. After blocking with 5% BSA in Tris-buffered saline/Tween (TBS-T; 20 mM Tris (pH 7.2), 150 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature, immunoblots were incubated overnight at 4 °C with the primary antibodies specific for PSD95 (1 : 1000, Millipore), DCX (1 : 1000, Abcam), TLX (1 : 1000, Santa Cruz Biotechnology), AdipoR1 (1 : 1000, Santa Cruz Biotechnology), AdipoR2 (1 : 1000, Santa Cruz Biotechnology), and β -actin (1 : 2000, Cell Signaling Technology, Danvers, MA, USA). Next, the blots were incubated with horseradish peroxidase-linked anti-mouse and anti-rabbit IgG antibodies (Abcam) for 1 h at room temperature. Chemiluminescence signal was developed using an ECL kit (Invitrogen).

Immunocytochemistry of the NSCs. NSCs were washed three times with PBS, and were permeabilized for 30 min. NSCs were incubated with the primary antibodies overnight at 4 °C. The following primary antibodies were used: anti-goat AdipoR1 (1 : 200, Santa Cruz Biotechnology) and anti-rabbit DCX (1 : 200, Abcam). The primary antibody was then removed, and the cells were washed three times for 3 min with PBS. Cells were incubated with FITC-conjugated donkey anti-goat IgG (1 : 200, Jackson ImmunoResearch, West Grove, PA, USA), and rhodamine-conjugated goat anti-rabbit IgG (1 : 200, Jackson ImmunoResearch) for 2 h at room temperature. NSCs were washed again three times for 3 min with PBS. NSCs were then counterstained with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI, 1 : 200, Invitrogen) for 10 min at room temperature. Cells were imaged using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Thornwood, NY, USA).

Statistical analysis. Statistical analyses were carried out using the SPSS 18.0 software (IBM Corporation, Armonk, NY, USA). All data are expressed as mean \pm S.E.M. Significant intergroup differences were determined by one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* multiple comparison test. Each experiment included three replicates per condition. Differences were considered significant at **P* < 0.05 and ***P* < 0.001.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

Juhyun Song conducted the experiments and wrote the preliminary draft of the manuscript. So Mang Kang conducted the experiments and helped discussing the design of the study. Chul-Hoon Kim, Eosu Kim, and Ho-Take Song revised the manuscript. Jong Eun Lee revised the manuscript and provided overall supervision for the project.

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