Doctoral Thesis

Involvement of brain fractalkine-CX3CR1 signaling in cognitive deficiency of diabetic mice

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PART I

Reduced brain fractalkine-CX3CR1 signaling is involved in the impaired cognition of streptozotocin-treated mice

Abstract

Patients with diabetes mellitus are predisposed to cognitive impairment. Fractalkine-CX3CR1 in the brain signaling represents a primary neuron-microglia inter-regulatory system for several brain functions including learning and memory processes. The present study addressed whether fractalkine-CX3CR1 signaling in the hippocampus contributes to the cognitive deficits observed in streptozotocin (STZ)-treated mice. Our results showed that STZ-treated mice exhibited significant cognitive deficits in the Y-maze test, and a decrease in fractalkine and CX3CR1 levels in the hippocampus. Moreover, intracerebroventricular injection of the CX3CR1 antagonist 18a in normal mice induced significant cognitive deficits in the Y-maze test. STZ-treated mice showed a significant increase in plasma corticosterone levels and a decrease in plasma and hippocampal levels of insulin-like growth factor-1 (IGF-1). Therefore, we examined the effects of corticosterone and IGF-1 on regulation of fractalkine and CX3CR1 expression. Dexamethasone (DEX) application significantly decreased the mRNA expression of fractalkine in primary neuron and astrocyte cultures, and of CX3CR1 in primary microglia cultures. On the other hand, IGF-1 application significantly increased the mRNA expression of fractalkine in primary neuron cultures and CX3CR1 in primary microglia cultures. In addition, administration of DEX and the IGF-1 receptor tyrosine kinase inhibitor picropodophyllin significantly reduced the mRNA expression of fractalkine and CX3CR1 in the hippocampus. These findings indicate that impaired cognition in STZ-treated mice is associated with reduced fractalkine-CX3CR1 signaling in the hippocampus which may be induced by an increase in corticosterone and a decrease in IGF-1.

Introduction

Fractalkine secreted from neurons was recently reported to be involved in the regulation of several functions of the central nervous system (CNS) (Goazigo et al., 2013; Maciejewski-Lenoir et al., 1999; Nishiyori et al., 1998). Astrocytes in the brain also synthesize fractalkine (Yoshida et al., 2001). In mouse brain, fractalkine mRNA levels are high in the cortex, hippocampus and striatum; intermediate in the olfactory bulb, thalamus, hypothalamus and brainstem; and low in the cerebellum (Maciejewski-Lenoir et al., 1999; Nishiyori et al., 1998). Fractalkine binds to the CX3C chemokine receptor 1 (CX3CR1), which is mainly expressed in microglia (Imai et al., 1997; Maciejewski-Lenoir et al., 1999; Nishiyori et al., 1998). Fractalkine-CX3CR1 signaling represents a primary neuron-microglia inter-regulatory system that is important for synaptic plasticity and function in the brain (Goazigo et al., 2013). Recent evidence indicates that fractalkine-CX3CR1 signaling plays an important role in regulating the formation of long-term potentiation (LTP) in the hippocampus and behavioral learning and memory processes (Rogers et al., 2011; Sheridan et al., 2014). LTP, defined as an activity-dependent, prolonged enhancement of synaptic strength, in the hippocampal CA1 region is considered to be a form of synaptic plasticity for the cellular mechanisms of learning and memory, and is predominately regulated by the glutamatergic system (Neves et al., 2008). CX3CR1-deficient mice exhibit cognitive deficits in different types of learning and memory tasks, such as fear-conditioning and water maze tests, in parallel with impaired LTP (Rogers et al., 2011).

Diabetes mellitus (DM) is a common metabolic disorder, characterized by glucose intolerance. Epidemiologic studies have demonstrated that both type 1 and type 2 DM patients have a predisposition for several dysfunctions of the CNS, such as cognitive impairment and depression, compared with non-diabetic patients (Biessels et al., 2008; McCall, 1992). Our previous study demonstrated that diet-induced obese mice fed a high-fat diet exhibit significant impairment of fear conditioning responses which are dependent on the hippocampus and amygdala (Yamada-Goto et al., 2012). Streptozotocin (STZ)-treated animals as a model of type 1 DM exhibit impaired learning and memory in several learning behavioral tests, such as the Y-maze, water maze, complex maze and passive avoidance (Biessels et al., 1996; Molteni et al., 2002; Wu et al., 2004). Moreover, electrophysiological studies revealed impaired expression of LTP in the hippocampus of STZ-treated animals (Kamal et al., 1999, 2000).

In the present study, we examined the possible contribution of fractalkine-CX3CR1 signaling in the hippocampus to the impaired cognition observed in STZ-treated mice.

Our findings revealed that impaired cognition in STZ-treated mice is associated with decreased fractalkine-CX3CR1 signaling in the hippocampus which may be induced by an increase in plasma corticosterone levels and a decrease in plasma and hippocampal insulin-like growth factor-1 (IGF-1) levels.

Materials and Methods

Animals

Male C57BL/6 J mice (6 weeks old) were obtained from CLEA Japan, Inc. (Tokyo, Japan) and housed in plastic cages under a 12:12 h light/dark cycle (lights turned on at 07.00 h) at room temperature (23 ± 1 °C). The animals had *ad libitum* access to water and food (CE-2; CLEA Japan, Inc.). All experiments were performed in accordance with the guidelines established by the Institute of Laboratory Animal Science Research Support Center at Kagoshima University and approved by the Kagoshima University Institutional Animal Care and Use Committee (protocol nos. MD18079 and MD18080), and in accordance with the guidelines established by the United States National Institutes of Health Guide for the care and use of laboratory animals (NIH publication No. 80-23, revised in 1996). Every effort was made to optimize the comfort of the animals and to minimize their use.

STZ-treated mice

Mice (10–12 weeks old) were given intraperitoneal injections of a single dose of STZ (200 mg/kg body weight, Merck KGaA, Darmstadt, Germany). STZ was dissolved in 10 mM chilled sodium citrate buffer (pH 4.0) just before injection. Control mice were given intraperitoneal injections of an equal volume of sodium citrate buffer (10 ml/kg body weight). Two weeks after the injection, we evaluated them in the Y-maze test and measured their body weight before the mice were killed by an overdose of isoflurane (Abbott Japan, Tokyo, Japan) for collection of blood samples and brain region.

Analysis of metabolic parameters and sampling of the brain region

Blood samples were collected from the retroorbital vein under isoflurane anesthesia and immediately transferred to tubes containing ethylenediaminetetraacetic acid (EDTA; 10 μl of 0.2 M EDTA/tube) and aprotinin (0.1 mg/tube, Merck KGaA). The blood samples were centrifuged 3000×g for 5 min at 4 ◦C, and the plasma was separated and stored at − 80 ◦C until assayed. After blood collection, the mice were killed by decapitation. The brain was rapidly removed from the skull and placed on an ice-cooled paraffin plate for dissection of the hippocampus as previously described (Nakao et al., 1986). The hippocampus was immediately frozen in liquid nitrogen and stored at − 80 ◦C until analyzed. Glucose (Glucose C2; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), insulin (Morinaga Ultra Sensitive Mouse/Rat Insulin ELISA Kit; Morinaga Institute of Biological Science, Yokohama, Japan), corticosterone (Corticosterone

Enzyme Immunoassay Kit; Arbor Assays, Ann Arbor, MI, USA) and IGF-1 (Mouse/Rat IGF-1 Quantikine ELISA Kit; R&D Systems, Inc., Minneapolis, MN, USA) were measured using commercially available kits.

Y-maze test

Spatial working memory was assessed by the Y-maze test. The Ymaze apparatus (Muromachi Co. Ltd., Tokyo, Japan) comprised three grey plastic arms (each 41.5 cm long, 4 cm wide, with 10-cm high walls) separated by 120◦ and randomly labeled A, B, and C. The task was performed as previously described (Sarnyai et al., 2000).

Continuous subcutaneous administration of dexamethasone (DEX)

Mice (10–12 weeks old) were anesthetized with isoflurane and a 7- day micro-osmotic pump (Alzet Model 1007D, DURECT Corporation, Cupertino, CA, USA) was implanted subcutaneously between the shoulder blades. DEX is a synthetic glucocorticoid and have a similar long-lasting action to cortisol and corticosterone via glucocorticoid receptor (Mulatero et al., 1997). The pumps contained DEX sodium phosphate (10, 30 and 100 μg/day, FUJIFILM Wako Pure Chemical Corporation) dissolved in saline. Mice in the control group were given subcutaneous injections of an equal volume of saline. Seven days after implanting the micro-osmotic pumps, the mice were killed by an overdose of isoflurane for collection of brain region.

Intraperitoneal administration of the selective IGF-1 receptor tyrosine kinase inhibitor picropodophyllin (PPP)

PPP (20 mg/kg, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted with saline to a final concentration of 50 % DMSO (Menu et al., 2006). Mice in the control group were given intraperitoneal injections of an equal volume of vehicle (50 % DMSO in saline, 10 ml/kg body weight). The mice were killed by an overdose of isoflurane 1 h after the administration and the brain region was collected.

Intracerebroventricular injection of the CX3CR1 antagonist 18a

Intracerebroventricular injection was performed as described previously (Yamada-Goto et al., 2013). The highly selective antagonist for CX3CR1 18a (Axon Medchem, Groningen, Netherlands) with a Ki value of 3.9 nM, was dissolved in DMSO, and diluted with saline to a final concentration of 0.1 % DMSO (Karlström et al., 2013). The CX3CR1 antagonist 18a (50 ng/mouse) was intracerebroventricularly injected at 30 min before the

Y-maze test. Mice in the control group were given intracerebroventricular injections of an equal volume of vehicle (0.1 % DMSO in saline, 2 μl/mouse).

Mouse primary neuron, astrocyte and microglia cultures

Mouse primary neuron and astrocyte cultures were performed as described previously (Katsuura et al., 1989; Yamada et al., 2009). According to a previous report (Han et al., 2013), primary microglia cell cultures were prepared from C57BL/6 J mouse brain on postnatal day 3. DEX (FUJIFILM Wako Pure Chemical Corporation), insulin (Thermo Fisher Scientific Inc., Waltham, MA, USA) and IGF-1 (PeproTech, Inc., Rocky Hill, NJ, USA) were used in this study. The purity of each cell culture was greater than 95 %.

Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA levels of fractalkine and CX3CR1 were measured by quantitative real-time RT-PCR as previously described (Yamada et al., 2009). All gene-specific mRNA expression values were normalized against the internal housekeeping gene 18S in the experiments involving the subcutaneous administration of DEX and the application of DEX in astrocyte cultures, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in other experiments. Primers for GAPDH were as follows: [sense TGCACCACCAACTG CTTAGC, antisense GGATGCAGGGATGATGTTCTG], for 18S, [sense GTAACCCGT TGAACCCCATT, antisense CCATCCAATCGGTAGTAGCG], for fractalkine, [sense ACGAAATGCGAAATCATGTGC, antisense CTGTGTCGTCTCCAGGACAA], for CX3CR1, [sense CGTGAGACTGGGTGAGTGAC, antisense AAGGAGGTGGACATG GTGAG].

Western blotting analysis

The hippocampus was homogenized in 500 μl of cold RIPA buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% NP-40, 0.5% deoxycholate, 0.1% SDS (pH 7.5) supplemented with 1 mM leupeptin, 1 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride) as described previously (Yamada et al., 2011). The samples were sonicated 20 sec, and added with 1% Triton-X and 0.2% SDS, and gently shaken for 30 min at room temperature. Unsolubilized material was removed by centrifugation (15,000×g for 15 min at 4℃). Sample (45 μl) was added with 50 μl of Tris-Glycine SDS sample Buffer 2X (Thermo Fisher Scientific Inc.) and 5 μl of 2-mercaptoethanol, and then boiled for 5 min. Twenty micrograms protein of sample per lane of protein was electrophoretically loaded on 8-16% Tris-Glycine Gel (Thermo Fisher Scientific Inc.) and transferred to a PVDF

membrane (Thermo Fisher Scientific Inc.). The membrane was incubated overnight in Tris-buffered saline with 0.1% Tween 20 (TBST) supplemented with 5% skim milk powder at 4°C with the primary antibody as follows: rabbit polyclonal anti-CX3CL1 (fractalkine) antibody (ab25088, abcam, Cambridge, UK), mouse monoclonal anti-CX3CR1 antibody (ab184678, abcam) and mouse monoclonal anti-GAPDH antibody (sc-32233, Santa Cruz Biotechnology, Inc.). Next, the membrane was washed three times with TBST, and incubated for 1 h at room temperature with either anti-rabbit IgG antibody (NA934, GE HealthCare UK Ltd., Buckinghamshire, UK) conjugated to horseradish peroxidase or anti-mouse IgG antibody (NA931, GE HealthCare UK Ltd.) conjugated to horseradish peroxidase at a 1:1000 dilution, followed by detection using the ECL (GE HealthCare UK Ltd.). The LAS-1000 image analyzer (version 4.0, Fuji Film Corporation, Tokyo, Japan) was used for detection and quantification. To ensure equivalent amounts of loaded proteins and quantify targeted protein expression, the ratio of the targeted protein level to the GAPDH level was determined.

Data analysis

Data are expressed as mean ± SEM. Statistical analysis of the data was performed by Student's t-test or Tukey-Kramer test. Statistical significance was defined as $P < 0.05$.

Results

Changes in body weight, plasma levels of glucose, insulin, IGF-1 and corticosterone, and protein levels of IGF-1 in the hippocampus in STZ-treated mice

STZ-treated mice had a significantly lower body weight than vehicle-treated mice at two weeks after STZ injection (figure 1A). In STZ-treated mice, plasma glucose levels were markedly increased, while plasma insulin levels were significantly decreased compared with vehicle-treated mice (figure 1B and C). In addition, STZ treatment significantly increased plasma corticosterone levels to 414 % of those in vehicle-treated mice (figure 1D) and significantly decreased plasma IGF-1 levels to 28 % of those in vehicle-treated mice (figure 1E). The IGF-1 protein levels in the hippocampus of STZ-treated mice were significantly decreased to 56 % of those in vehicle-treated mice (figure 1F).

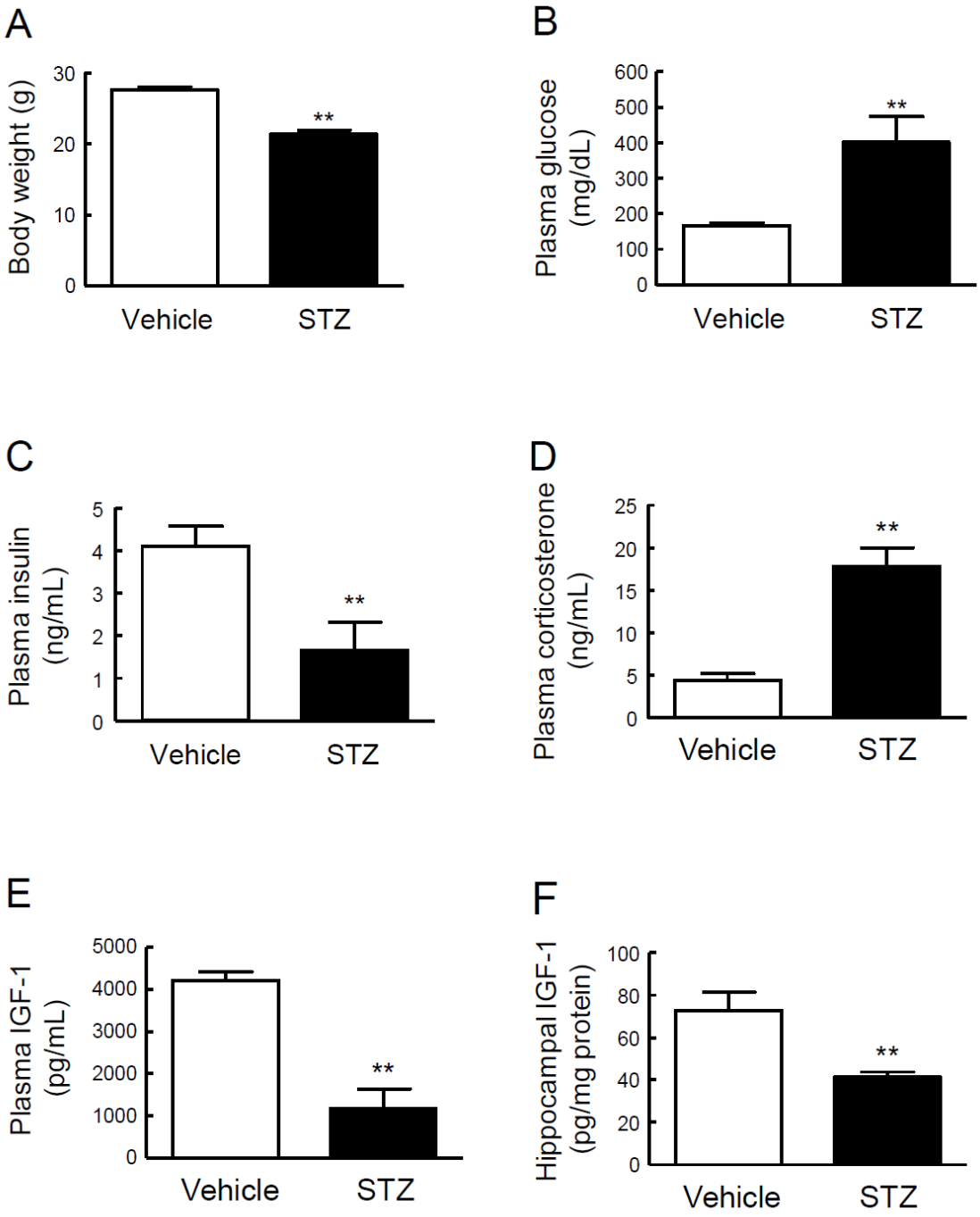

FIGURE 1. Changes in body weight, plasma levels of glucose, insulin, corticosterone and IGF-1, and protein levels of IGF-1 in the hippocampus in STZ-treated mice. (A) Body weight, (B) Plasma glucose levels, (C) Plasma insulin levels, (D) Plasma corticosterone levels, (E) Plasma IGF-1 levels, (F) IGF-1 protein levels in the hippocampus. Results are expressed as mean ± SE for 9-17 mice. **$p < 0.01$ vs. vehicle.

Changes in mRNA expression and protein levels of fractalkine and CX3CR1 in the hippocampus of STZ-treated mice

At two weeks after STZ injection, the mRNA expression and protein levels of fractalkine were significantly decreased in the hippocampus of STZ-treated mice to 73 % and 65 %, respectively, of those in vehicle-treated mice (figure 2A and B). Moreover, the mRNA expression and protein levels of CX3CR1 were significantly decreased in the hippocampus of STZ-treated mice to 70 % and 61 %, respectively, of those in vehicle-treated mice (figure 2C and D).

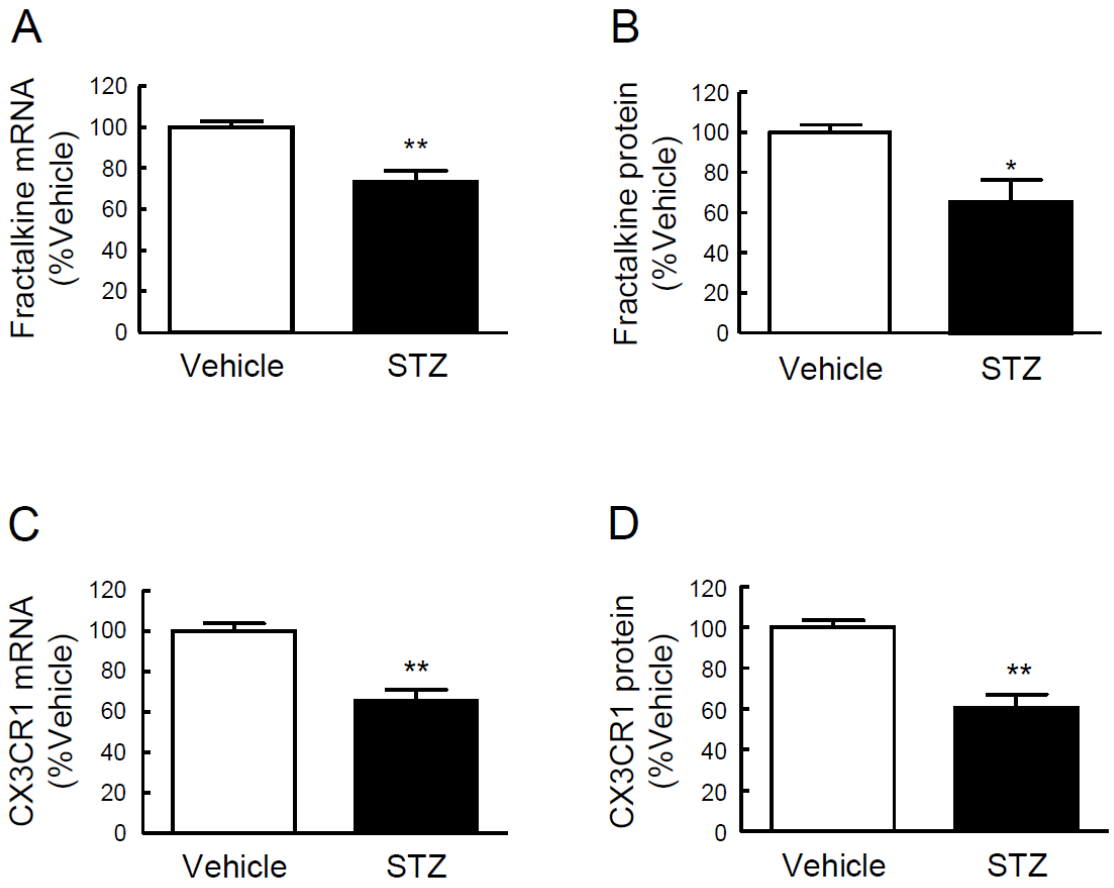

FIGURE 2. Changes in the mRNA expression and protein levels of fractalkine and CX3CR1 in the hippocampus of STZ-treated mice. (A) Fractalkine mRNA expression, (B) Fractalkine protein levels, (C) CX3CR1 mRNA expression, (D) CX3CR1 protein levels. Results are expressed as mean ± SE for 5-16 mice. *$p < 0.05$, **$p < 0.01$ vs. vehicle.

Cognitive ability in the Y-maze test

At two weeks after STZ injection, spontaneous alternation was significantly reduced in STZ-treated mice to 72 % of that in vehicle-treated mice (figure 3A). The number of entries into each arm was not different between groups (figure 3B).

To elucidate the role of fractalkine-CX3CR1 signaling in the learning and memory processes, we examined the effect of the CX3CR1 antagonist 18a on learning and memory in the Y-maze test in normal mice. Intracerebroventricular injection with 18a (50 ng/mouse) significantly reduced spontaneous alternation in mice to 78 % of that in vehicle-treated mice (figure 3C). The number of entries into each arm was not changed in 18a-treated mice (figure 3D).

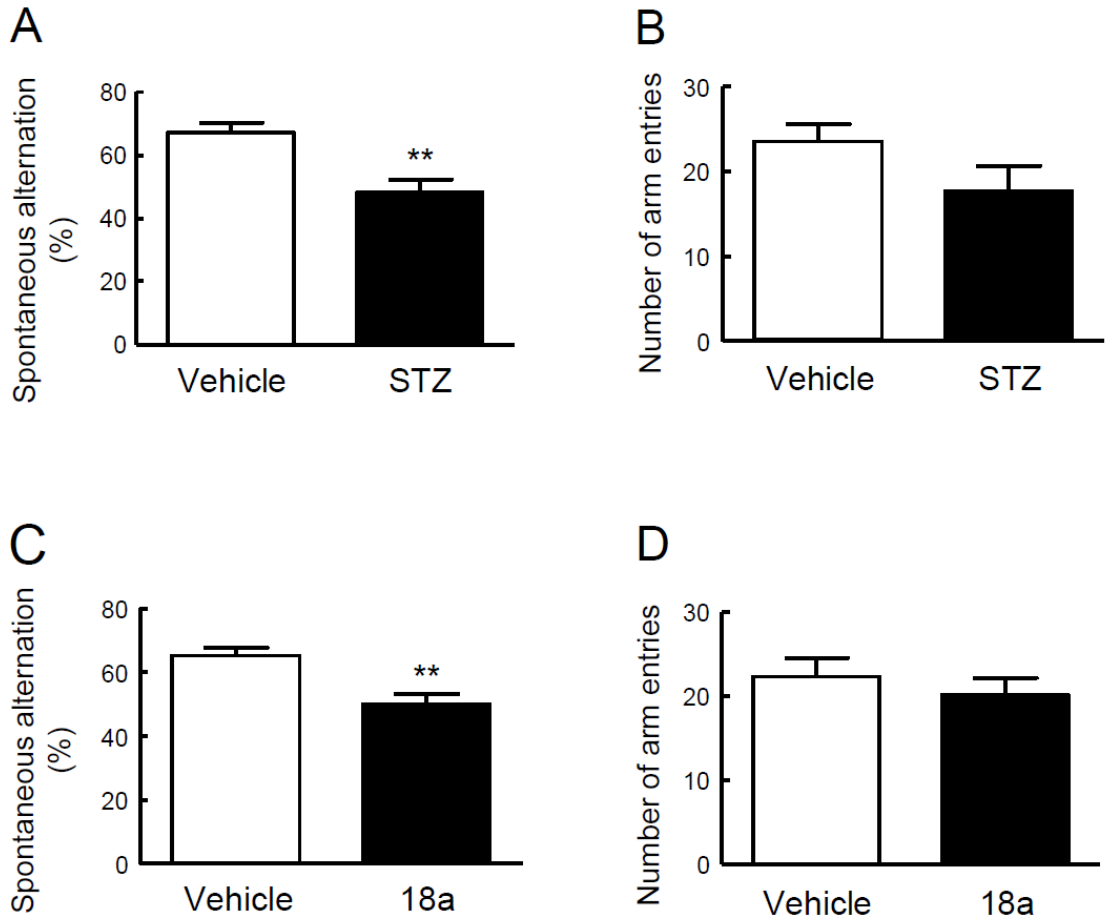

FIGURE 3. Cognitive ability in the Y-maze test. STZ: (A) Spontaneous alternation (%) and (B) Number of arm entries. CX3CR1 antagonist 18a: (C) Spontaneous alternation (%) and (D) Number of arm entries. Results are expressed as mean ± SE for 9-11 mice. **$p < 0.01$ vs. vehicle.

Effects of the application of DEX, insulin and IGF-1 on mRNA expression of fractalkine in primary neuron and astrocyte cultures, and mRNA expression of CX3CR1 in primary microglia cultures

In primary neuron cultures, the application of DEX at concentrations of 10, 100 and 1000 nM for 24 h significantly deceased fractalkine mRNA expression (figure 4A). The application of insulin at a concentration of 1000 nM for 24 h did not change the fractalkine mRNA expression in primary neuron cultures (figure 4B). On the other hand, the application of IGF-1 at a concentration of 1000 nM for 24 h to primary neuron cultures significantly increased the fractalkine mRNA expression to 130 % of that in the control group (figure 4C).

In primary astrocyte cultures, the application of 10, 100 and 1000 nM DEX for 24 h significantly decreased fractalkine mRNA expression to 67 %, 48 % and 55 %, respectively, of that in the control group (figure 4D). Applications of insulin (1000 nM) and IGF-1 (1000 nM) for 24 h did not change the fractalkine mRNA expression in primary astrocyte cultures (figure 4E and F).

In primary microglia cultures, the application of 10, 100 and 1000 nM DEX for 24 h markedly decreased CX3CR1 mRNA expression to 77 %, 31 % and 31 %, respectively, of that in the control group (figure 4G). The application of insulin (1000 nM) for 24 h did not change CX3CR1 mRNA expression in primary microglia cultures (figure 4H). On the other hand, the application of 1000 nM IGF-1 for 24 h significantly increased the CX3CR1 mRNA expression in primary microglia cultures to 136 % of that in the control group (figure 4I).

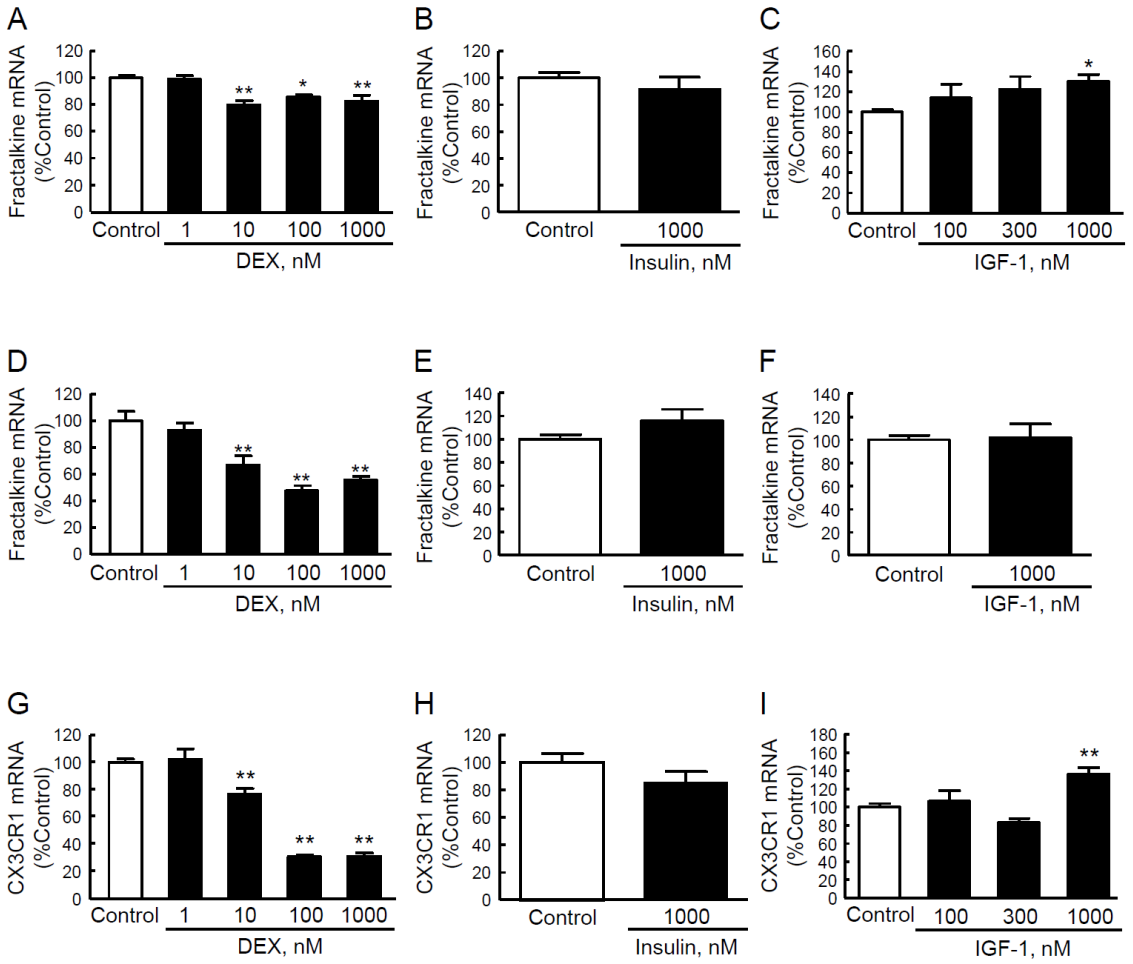

FIGURE 4. Effects of the application of DEX, insulin and IGF-1 on mRNA expression of fractalkine in the primary neuron and astrocyte cultures, and mRNA expression of CX3CR1 in the primary microglia cultures. Fractalkine mRNA expression in the primary neuron cultures: (A) DEX, (B) insulin, (C) IGF-1. Fractalkine mRNA expression in the primary astrocyte cultures: (D) DEX, (E) insulin, (F) IGF-1. CX3CR1 mRNA expression in the primary microglia cultures: (G) DEX, (H) insulin, (I) IGF-1. Results are expressed as mean ± SE for 6-20 samples. *$p < 0.05$, **$p < 0.01$ vs. control.

Effects of the administration of DEX and selective IGF-1 receptor tyrosine kinase inhibitor PPP on mRNA expression of fractalkine and CX3CR1 in the mouse hippocampus

In the normal mice hippocampus, subcutaneous administration of DEX in doses of 30 and 100 μg/day for 7 days using a micro-osmotic pump significantly decreased fractalkine mRNA expression to 80 % and 73 %, respectively, of that in the saline-treated group, and significantly decreased CX3CR1 mRNA expression in a dose of 100 μg/day to 68 % of that in the saline-treated group (figure 5A and B).

Intraperitoneal injection of PPP (20 mg/kg body weight) in normal mice significantly decreased mRNA expression of fractalkine and CX3CR1 in the hippocampus to 77 % and 72 %, respectively, of that in vehicle-treated mice (figure 5C and D).

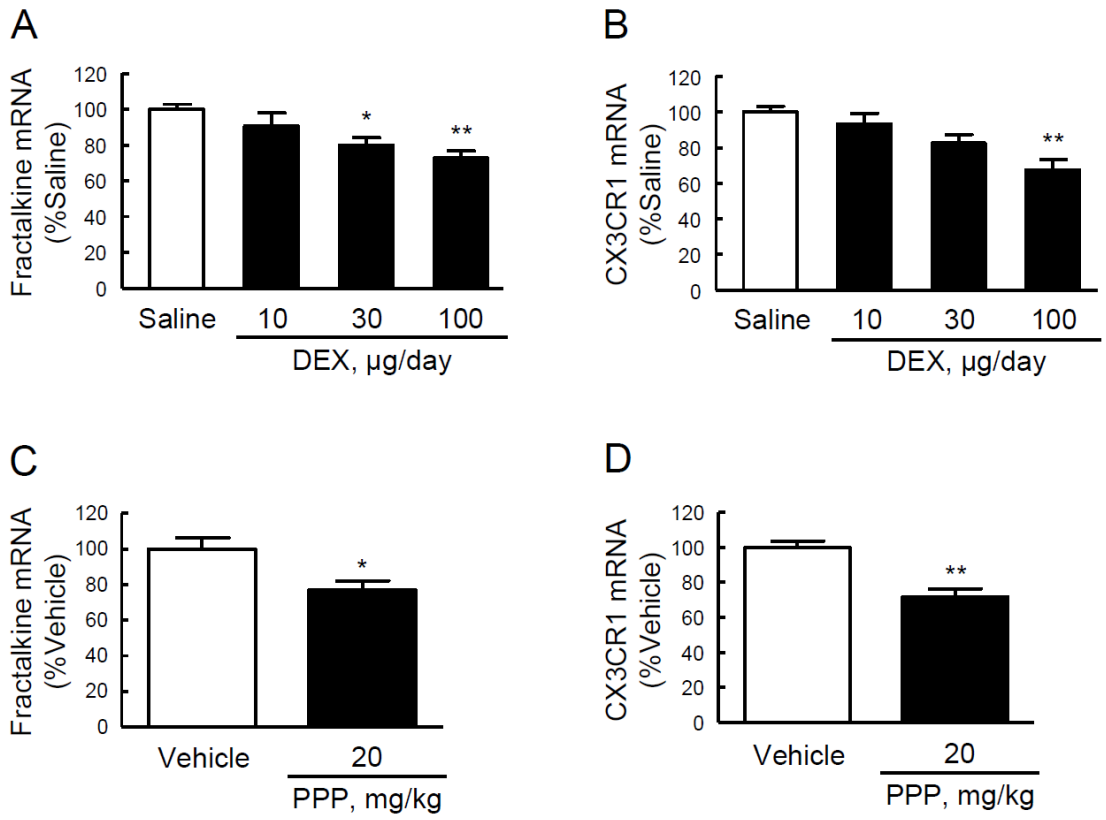

FIGURE 5. Effects of the administration of DEX and IGF-1 receptor tyrosine kinase inhibitor PPP on mRNA expression of fractalkine and CX3CR1 in mouse hippocampus. DEX: (A) Fractalkine mRNA expression, (B) CX3CR1 mRNA expression. PPP: (C) Fractalkine mRNA expression, (D) CX3CR1 mRNA expression. Results are expressed as mean ± SE for 6-9 samples. *$p < 0.05$, **$p < 0.01$ vs. saline or vehicle.

Discussion

In the present study, STZ-treated mice exhibited significant learning and memory impairment in the Y-maze test compared with vehicle-treated mice, and, moreover, a significant reduction in fractalkine and CX3CR1 mRNA levels in the hippocampus. Furthermore, intracerebroventricular injection of the CX3CR1 antagonist 18a in normal mice induced significant cognitive deficits in the Y-maze test. STZ-treated mice had high levels of plasma corticosterone, and low levels of plasma and hippocampal IGF-1 compared with vehicle-treated mice. Furthermore, DEX significantly decreased fractalkine mRNA expression in primary neuron and astrocyte cultures, and CX3CR1 mRNA expression in primary microglia cultures. On the other hand, IGF-1 significantly increased the fractalkine mRNA expression in primary neuron cultures and the CX3CR1 mRNA expression in primary microglia cultures. Moreover, subcutaneous administration of DEX significantly reduced the fractalkine and CX3CR1 mRNA expression in the hippocampus. Intraperitoneal administration of the IGF-1 receptor tyrosine kinase inhibitor PPP significantly decreased the fractalkine and CX3CR1 mRNA expression in the hippocampus. These findings suggest that the cognitive deficits exhibited by STZ-treated mice are, at least in part, due to impaired fractalkine-CX3CR1 signaling in the hippocampus induced by an increase in plasma corticosterone levels and a decrease in plasma and hippocampal IGF-1 levels.

In the hippocampus, fractalkine expression is predominantly restricted to glutamatergic pyramidal neurons in the CA1-CA3 and granule neurons in the dentate gyrus which are well-known to be potently involved in the processing of learning and memory (Nishiyori et al., 1998). Activation of CX3CR1 on microglia in the hippocampal CA1 region triggers the release of adenosine that in turn, via the activation of adenosine receptor type A2, increases the release of D-serine as a coagonist for the N-methyl-D-aspartate (NMDA) glutamate receptor subtype from glia, thereby potentiating NMDA function (Scianni et al., 2013). LTP, defined as an activity-dependent, prolonged enhancement of synaptic strength, in the hippocampal CA1 region is predominantly regulated by glutamate receptors, such as NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and is considered to be a form of synaptic plasticity for the cellular mechanisms of learning and memory in the hippocampus (Neves et al., 2008). These findings suggest that fractalkine-CX3CR1 signaling plays an important role in learning and memory processes in association with enhanced LTP (Rogers et al., 2011; Sheridan et al., 2014). In the present study, administration of a CX3CR1 antagonist significantly impaired learning and memory in the Y maze test. Supporting our result, Rogers et al.

reported that male CX3CR1-deficient mice showed cognitive impairment in fear conditioning and Morris water maze test (Rogers et al., 2011). In Morris water maze test, CX3CR1-deficient mice had a significant decrease in the number of target platform crossing during the probe trial (Day 11) compared to wild-type mice, indicating cognitive impairment in CX3CR1-deficient mice (Rogers et al., 2011). On the other hand, Maggi et al. reported that female CX3CR1-deficient mice learned the water maze task faster than wild-type mice because CX3CR1-deficient mice significantly spent in the acquisition quadrant longer than wild-type mice during probe trial (Day 4) (Maggi et al., 2011). However, on probe trial (Day 6), spent time in the acquisition quadrant was not different between two groups (Maggi et al., 2011). We consider that the difference in these results may be due, in part, to the differences in experimental schedule and gender. Moreover, Rogers et al. reported that male CX3CR1-deficient mice showed significantly reduced hippocampal-dependent LTP compared to wild-type mice (Rogers et al., 2011). However, Maggi et al. reported that CX3CR1-deficient mice showed an increase in AMPA receptor-mediated LTP but not NMDA receptor-mediated LTP (Maggi et al., 2011). On the other hand, another report of Maggi showed that fractalkine enhanced hippocampal NMDA receptor-dependent LTP in mice (Scianni et al., 2013). In this context, the same laboratory demonstrated different results which might be due to the different experimental methods. As compared with these two studies on CX3CR1-deficient mice, our experiment was performed using CX3CR1 antagonist. The big difference between Rogers/Maggi studies and ours is permanent intervention and acute intervention, respectively. Taken together, these findings provide compelling evidence that neuron-microglia interactions, especially those underpinned by fractalkine-CX3CR1 signaling, play several crucial roles in modulating hippocampal-dependent learning and memory by maintaining proper homeostasis of synaptic transmission in the brain.

DM is a common serious metabolic disorder characterized by hyperglycemia resulting from defective insulin activity. Diabetes may lead to secondary complications in several organ systems, including the brain (Biessels et al., 1994, 2008). A growing number of studies on brain function have revealed moderate impairment of cognitive function is recognized as a complication of type 1 DM (Neves et al., 2008). Initially, deficient insulin actions may be considered to contribute to the cognitive impairment observed in DM because insulin positively regulates cognitive processing, and impaired insulin activity in the brain leads to impaired neuronal function and synaptogenesis (Kleinridders et al., 2014). The multifactorial pathogenesis of brain dysfunction, such as cognitive impairment in DM, however, is not yet completely understood. STZ-treated mice, a widely used model of type 1 DM with hypoinsulinemia, hyperglycemia and reduced body

weight, exhibit cognitive deficits in association with impaired LTP in the hippocampal CA1 region (Biessels et al., 1996; Kamal et al., 1999, 2000; Molteni et al., 2002; Wu et al., 2004). At least part of the learning and synaptic plasticity deficits in STZ-treated rats may be a direct consequence of disturbances at the level of the NMDA and AMPA receptor complexes in the hippocampus (Sasaki-Hamada et al., 2012). Concretely, the NMDA receptor NR2B subunits and the AMPA receptor GluR1 subunits are significantly decreased in the hippocampus of STZ-treated animals (Gardoni et al., 2002; Viswaprakash et al., 2015). Based on previous findings suggesting the participation of fractalkine-CX3CR1 signaling in learning and memory processes, we addressed whether fractalkine-CX3CR1 signaling in the brain is involved in cognitive deficits in STZ-treated mice. The present study demonstrated that STZ-treated mice exhibited significant decrease in fractalkine-CX3CR1 signaling in the hippocampus accompanied by cognitive deficits. On the basis of these findings, the impaired learning and memory in STZ-treated mice is, in part, attributed to reduced fractalkine-CX3CR1 signaling in the hippocampus.

To elucidate the mechanisms underlying the decreased expression of fractalkine and CX3CR1 in the hippocampus of STZ-treated mice, we examined the effects of factors observed to be significantly changed in the plasma and hippocampus of STZ-treated mice in the present study, such as corticosterone, insulin and IGF-1, on the mRNA expression of fractalkine and CX3CR1. Type 1 DM is associated with significantly higher plasma cortisol and adrenocorticotrophic hormone levels compared with normal controls (Chan et al., 2002). DEX which is a synthetic glucocorticoid receptor agonist significantly suppresses increases in the protein levels and mRNA expression of fractalkine induced by tumor necrosis factor-α and interferon-γ in a human lung epithelial adenocarcinoma cell line (Bhavsar et al., 2008). Moreover, application of DEX reduces CX3CR1 mRNA expression in human peripheral blood mononuclear cells (Pachot et al., 2008). Consistent with these findings, the present study showed that plasma corticosterone levels were significantly increased in STZ-treated mice. Moreover, cell culture studies showed that DEX significantly reduced fractalkine-CX3CR1 signaling. In the present study, subcutaneous administration of DEX in normal mice significantly reduced the mRNA expression of fractalkine and CX3CR1 in the hippocampus. These findings suggest that high plasma corticosterone levels in STZ-treated mice contribute to reduce fractalkine and CX3CR1 expression in the brain. Recent observations revealed that circulating and brain IGF-1, which acts as trophic factor, modulates brain activities such as neuroprotection, neurogenesis and neuronal excitability (Jones and Clemmons, 1995). Serum IGF-1-deficient mice exhibit both cognitive decline and impaired hippocampal LTP (Trejo et al., 2007). Serum and brain IGF-1 levels are reduced in STZ-treated rats

(Olchovsky et al., 1990), and systemic administration of IGF-1 prevents cognitive impairment in STZ-treated rats (Lupien et al., 2003). These findings provide substantial evidence that diabetic patients may have diminished brain IGF-1 signaling as well as insulin signaling. The present study demonstrated that plasma and hippocampal IGF-1 levels were significantly decreased in STZ-treated mice. Moreover, IGF-1 significantly increased mRNA expression of fractalkine in primary neuron cultures and of CX3CR1 in primary microglia cultures. In contrast, intraperitoneal administration of the IGF-1 receptor tyrosine kinase inhibitor PPP significantly decreased mRNA expression of fractalkine and CX3CR1 in the hippocampus. These findings indicate that impaired fractalkine-CX3CR1 signaling in the hippocampus in STZ-treated mice is, in part, attributed to a decrease in plasma and hippocampal IGF-1 levels. However, it is unclear mechanism how corticosterone and IGF-1 change the expression of fractalkine and CX3CR1.

Our findings revealed that STZ treatment induces a significant decrease in fractalkine-CX3CR1 signaling in the hippocampus, which in turn, may result in cognitive impairment. Moreover, reduced fractalkine-CX3CR1 signaling seems to be induced by an increase in plasma corticosterone levels and a decrease in plasma and hippocampal IGF-1 levels. Thus, interactions between neurons and microglia regulated by fractalkine and CX3CR1 appear to be involved in the cognitive deficiency associated with type1 DM.

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PART Ⅱ

Impaired brain fractalkine-CX3CR1 signaling is implicated in cognitive dysfunction in diet-induced obese mice

Abstract

Introduction A diet high in saturated fat is well known to affect neuronal function and contribute to cognitive decline in experimental animals and humans. Fractalkine released from neurons acts on its receptor, CX3C chemokine receptor 1 (CX3CR1), in the microglia to regulate several brain functions. The present study addressed whether fractalkine-CX3CR1 signaling in the brain, especially the hippocampus, contributes to the cognitive deficits observed in diet-induced obese (DIO) mice.

Research design and methods Mice were given 60% high-fat diet for 16 weeks. The expression of fractalkine and CX3CR1 in the hippocampus, amygdala and prefrontal cortex of DIO mice was analyzed. Cognitive ability in the Y-maze test and hippocampal glutamate receptors and synaptic markers were observed in DIO and CX3CR1 antagonist-treated mice. Regulation of fractalkine and CX3CR1 expression in the hippocampus was examined following administration of a selective insulin-like growth factor-1 (IGF-1) receptor inhibitor and a tyrosine receptor kinase B (TrkB) antagonist in normal mice.

Results DIO mice exhibited significant cognitive deficits in the Y-maze test and decrease in fractalkine and CX3CR1 in the hippocampus and amygdala compared with mice fed a control diet (CD mice). Administration of the CX3CR1 antagonist 18a in normal mice induced significant cognitive deficits in the Y-maze test. DIO mice and CX3CR1 antagonist-treated mice exhibited significant decreases in protein levels of NMDA (N-methyl-D-aspartate) receptor subunit (NR2A), AMPA (α-amino-5-methyl-3-hydroxy-4-isoxazole propionate) receptor subunit (GluR1) and postsynaptic density protein 95 in the hippocampus compared with their respective controls. Furthermore, plasma IGF-1 and hippocampal brain-derived neurotrophic factor were significantly decreased in DIO mice compared with CD mice. Administration of a selective IGF-1 receptor inhibitor and a TrkB antagonist in normal mice significantly decreased fractalkine and CX3CR1 in the hippocampus.

Conclusions These findings indicate that the cognitive decline observed in DIO mice is due, in part, to reduced fractalkine-CX3CR1 signaling in the corticolimbic system.

Introduction

Obesity is associated with a higher risk of lifestyle-related cardiovascular and metabolic disorders, such as hypertension, diabetes, and hyperlipidemia. Epidemiological studies indicate that the incidence of cognitive impairment is higher in obese subjects than in those with normal body weight.[1 2] Diet-induced obese (DIO) animals exhibit impaired cognition in a variety of behavioral tests, such as the Morris water maze and a spontaneous alternation paradigm in T-maze and Y-maze tests.[3–6] Consistent with these findings, our previous study demonstrated that DIO mice fed a high-fat diet (HFD) exhibit significant impairments in hippocampus-dependent and amygdala-dependent fear-conditioning responses and have low levels of hippocampal brain-derived neurotrophic factor (BDNF), which plays a critical role in synaptic plasticity, long-term potentiation (LTP) and learning and memory processes.[7] Furthermore, electrophysiological studies indicate that LTP in the hippocampal CA1 region, which is closely related to memory formation and is predominately regulated by the glutamatergic system, especially N-methyl-D-aspartate (NMDA) and α-amino-5-methyl-3-hydroxy-4-isoxazole propionate (AMPA) receptors,[8] is markedly impaired in DIO animals compared with lean animals.[9 10] The cellular and molecular events of the synaptic plasticity involved in learning and memory processes are modulated by neurotrophic factors including BDNF and insulin-like growth factor-1 (IGF-1),[11–13] which regulate the presynaptic and postsynaptic machinery associated with synaptic plasticity.[6 9 14]

Communication between neurons and microglia is crucial for the optimal regulation of central nervous system (CNS) activities. In this regard, fractalkine secreted from neurons was recently reported to bind to CX3C chemokine receptor 1 (CX3CR1), which is expressed mainly in the microglia,[15–17] and to regulate several functions of the CNS.[18] In the mouse brain, fractalkine messenger RNA (mRNA) levels are high in the cortex, hippocampus, and striatum; intermediate in the olfactory bulb, thalamus, hypothalamus and brainstem; and low in the cerebellum.[15 16] Therefore, fractalkine-CX3CR1 signaling is postulated to represent a primary neuron–glia inter-regulatory system that is important for brain function.[18] Recent evidence indicates that fractalkine-CX3CR1 signaling plays an important role in regulating LTP formation in the hippocampus and behavioral learning and memory processes.[19 20]

To provide a new insight into the understanding of cognitive decline that occurs in obesity, we assessed the possible contribution of impaired fractalkine-CX3CR1 signaling in the corticolimbic system, which is an important system for cognitive processing, to the cognitive deficits observed in DIO mice. In addition, to investigate the underlying

mechanisms, we examined the expression of glutamate receptor subunits and synaptic components specific to synaptic plasticity. Our findings revealed that decreased fractalkine-CX3CR1 signaling in the corticolimbic system contributes to the impaired cognition observed in DIO mice in association with decreased expression of glutamate receptor subunits and synaptic components.

Materials and Methods

Animals

Male C57BL/6J mice (6 weeks old) were obtained from CLEA Japan (Tokyo, Japan) and housed in plastic cages under a 12:12-hour light:dark cycle (lights turned on at 07:00) at room temperature (23°C ± 1°C). The animals had *ad libitum* access to water and food (CE-2; CLEA Japan). All experiments were performed in accordance with the guidelines established by the Institute of Laboratory Animal Science Research Support Center at Kagoshima University and approved by the Kagoshima University Institutional Animal Care and Use Committee (protocol nos. MD18079, MD18080 and MD20007) for the Care and Use of Laboratory Animals. Every effort was made to optimize the comfort of the animals and to minimize their use.

DIO mice

Normal mice (6 weeks old) were randomly divided into two groups. The first group was given CE-2 as a control diet (CD), with fat accounting for 12.6% of the total calories (343.1 kcal/100 g). The second group was given HFD (no. D12492; Research Diets, New Brunswick, New Jersey), with fat accounting for 60% of the total calories, predominantly in the form of lard (524 kcal/100 g). After feeding on the diets for 16 weeks, we evaluated them in the Y-maze test and measured their body weight before the mice were sacrificed to collect blood samples, fat tissues and brain regions under isoflurane anesthesia.

Analysis of metabolic parameters and sampling of the brain regions

Blood samples were collected from the retro-orbital vein under isoflurane anesthesia and immediately transferred to tubes containing EDTA (10 μl of 0.2 M EDTA/tube) and aprotinin (0.1 mg/tube; Merck KGaA, Darmstadt, Germany). The blood samples were centrifuged 3000× g for 5 min at 4°C, and plasma was separated and stored at -80°C until assayed. After blood collection, the mice were killed by decapitation. The brain was rapidly removed from the skull and placed on an ice-cooled paraffin plate for dissection of the hippocampus, amygdala and prefrontal cortex (PFC) by referring to the mouse brain atlas as previously described.[21] The brain regions were immediately frozen in liquid nitrogen and stored at -80°C until analyzed. The epididymal and mesenteric fat tissues were collected and weighed. Glucose (Glucose C2; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), insulin (Morinaga Ultra Sensitive Mouse/Rat Insulin ELISA Kit; Morinaga Institute of Biological Science, Yokohama, Japan), leptin (Mouse Leptin

ELISA; Linco Research, St. Charles, Missouri), IGF-1 (Mouse/Rat IGF-1 Quantikine ELISA Kit; R&D Systems, Minneapolis, Minnesota) and BDNF (Mouse/Rat BDNF Emax ImmunoAssay System; Promega Corporation, Madison, Wisconsin) were measured using commercially available kits.

Y-maze test

Cognitive ability was assessed using the Y-maze test, which is generally used to evaluate learning and memory ability in experimental animals, such as mouse models of obesity, Alzheimer's disease, and cerebral ischemia.[6 22 23] This task is based on the observation that if a mouse remembers the arm that has been explored most recently, a mouse will next enter an arm of the maze that has not been visited yet or the most remotely visited arm.[24] The Y-maze apparatus (Muromachi, Tokyo, Japan) comprises three gray plastic arms (each one 41.5 cm long, 10 cm high, and 4 cm wide) that emanate from the center of the maze, are separated by 120°, and labeled as A, B, and C. Briefly, each mouse was placed at the end of one arm and allowed to move freely for 8 min session without any stimulation.[25] The sequence of arm entries was recorded manually, with an entry defined as all four limbs of the mouse within an arm. After completing the test trial for each mouse, the maze was cleaned with 70% ethyl alcohol to remove the mouse odor. The outcomes included the percentage of spontaneous alternations and the number of arm entries. The recorded spontaneous alternation behavior was used to assess hippocampal-dependent spatial memory. The percentage of spontaneous alternations was calculated as the ratio of arm entries that differed from the previous two arm entries (actual alternations) to the total possible alternations (defined as the total entries minus one) and multiplied by 100.

Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA levels were measured by quantitative real-time RT-PCR.[26] All gene-specific mRNA expression values were normalized against the internal housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for GAPDH were as follows: [sense TGCACCACCAACTGCTTAGC, antisense GGATGCAGGGATGATGT TCTG], for fractalkine, [sense ACGAAATGCGAAATCATGTGC, antisense CTGTGTCGTCTCCAGGACAA], for CX3CR1, [sense CGTGAGACTGGGTGAGTG AC, antisense AAGGAGGTGGACATGGTGAG], for NR1, [sense AAGCCCAACGCC ATACAGAT, antisense AGGCGGGTGACTAACTAGGA], for NR2A, [sense TCACTG AGGAAGGCTATC, antisense CCCACTTGCCCACCTTTT], for NR2B, [sense AGAG TCGACGAGCTGAAGATGAAGCCCAGC, antisense CGGGGAACTACTGAGAGAT GATGGAAGTCA], for GluR1, [sense GGAGTGGAAGTCCCTAGCACACA,

antisense CCTGGGAGTGGCTGCATAAGA], for GluR2, [sense ATGGAACATTAGAC TCTGGCTCCAC, antisense CTGCCGTAGTCCTCACAAACACA], for synaptophysin, [sense CCACCTCCTTCTCCAATCAG, antisense CAGCAAAGACAGGGTCTCCT], and for post synaptic density protein 95 (PSD-95), [sense CGAGGAGCCGTGGCAGCC, antisense CATGGCTGTGGGGTAGTCAGTGCC].

Western blotting analysis

The hippocampus was homogenized in 500 μl of cold RIPA buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% NP-40, 0.5% deoxycholate, 0.1% SDS (pH 7.5) supplemented with 1 mM leupeptin, 1 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride) as described previously.[27] The samples were sonicated 20 sec, and added with 1% Triton-X and 0.2% SDS, and gently shaken for 30 min at room temperature. Unsolubilized material was removed by centrifugation (15,000×g for 15 min at 4℃). Sample (45 μl) was added with 50 μl of Tris-Glycine SDS sample Buffer 2X (Thermo Fisher Scientific, Waltham, Massachusetts) and 5 μl of 2-mercaptoethanol, and then boiled for 5 min. Twenty micrograms protein of sample per lane of protein was electrophoretically loaded on 8-16% Tris-Glycine Gel (Thermo Fisher Scientific) and transferred to a PVDF membrane (Thermo Fisher Scientific). The membrane was incubated overnight in Tris-buffered saline with 0.1% Tween 20, supplemented with 5% skim milk powder at 4℃ with the primary antibody as follows: rabbit polyclonal anti-CX3CL1 (fractalkine) antibody (1:1000, ab25088; abcam, Cambridge, UK) that recognizes soluble form of fractalkine, mouse monoclonal anti-CX3CR1 antibody (1:250, ab184678; abcam), rabbit monoclonal anti-NMDA receptor1 (GluN1) (D65B7) antibody (1:1000, #5704; Cell Signaling Technology, Danvers, Massachusetts), rabbit polyclonal anti-NMDA receptor2A (GluN2A) antibody (1:1000, #4205; Cell Signaling Technology), rabbit monoclonal anti-NMDA receptor2B (GluN2B) (D8E10) antibody (1:1000, #14544; Cell Signaling Technology), rabbit polyclonal ant-glutamate receptor 1 (AMPA subtype) antibody (1:1000, ab31232; abcam), rabbit monoclonal anti-AMPA receptor 2 (GluA2) (E1L8U) antibody (1:1000, #13607; Cell Signaling Technology), rabbit monoclonal anti-synaptophysin (D8F6H) XP® antibody (1:1000, #36406; Cell Signaling Technology), rabbit monoclonal anti-PSD95 (D27E11) XP® antibody (1;1000, #3450; Cell Signaling Technology) and mouse monoclonal anti-GAPDH (1:2000, sc-32233; Santa Cruz Biotechnology, Dallas, Texas) antibody. Next, the membrane was washed three times with TBST, and incubated for 1 h at room temperature with either anti-rabbit IgG antibody (NA934; GE HealthCare UK, Buckinghamshire, UK) conjugated to horseradish

peroxidase or anti-mouse IgG antibody (NA931; GE HealthCare UK) conjugated to horseradish peroxidase at a 1:1000 dilution, followed by detection using the ECL (GE HealthCare UK). The LAS-1000 image analyzer (V.4.0; FUJIFILM Corporation, Tokyo, Japan) was used for detection and quantification. To ensure equivalent amounts of loaded proteins and quantify targeted protein expression, the ratio of the targeted protein level to the GAPDH level was determined.

Intracerebroventricular administration of the CX3CR1 antagonist 18a

A highly selective antagonist for CX3CR1, 18a (Axon Medchem, Groningen, The Netherlands), with a Ki value of 3.9 nM, was dissolved in dimethyl sulfoxide (DMSO), and diluted in saline to a final concentration of 0.1% DMSO.[28] The CX3CR1 antagonist 18a (50 ng/mouse) was intracerebroventricularly administrated at 1 hour before the Y-maze test according to our previous report.[27] Mice in the control group were given intracerebroventricular injections of an equal volume of vehicle (0.1% DMSO in saline, 2 μL/mouse). In another experiment, mice were sacrificed at 1 hour after the administration to collect brain regions under isoflurane anesthesia.

Chronic intraperitoneal administration of picropodophyllin and ANA-12

The selective IGF-1 receptor inhibitor picropodophyllin (PPP, 20 mg/kg; Santa Cruz Biotechnology)[29] and the tyrosine receptor kinase B (TrkB) selective and non-competitive antagonist ANA-12 (0.5 mg/kg; Merck KGaA)[30] were dissolved in DMSO and diluted with saline at a final concentration of 50% DMSO. PPP and ANA-12 were intraperitoneally injected at 10:00 once a day for 7 days. Mice in the control group were given an intraperitoneal injection of an equal volume of vehicle (50% DMSO in saline, 10 mL/kg body weight). Mice were sacrificed at 1 hour after the last administration to collect brain regions under isoflurane anesthesia.

Data analysis

Data are expressed as mean ± SEM. Statistical analysis of the data was performed by Student's t-test or Tukey-Kramer test. Statistical significance was defined as $P < 0.05$.

Results

Changes in body weight, fat mass, and plasma levels of glucose, insulin, leptin, IGF-1 and BDNF in DIO mice

DIO mice had markedly greater body weight and epididymal and mesenteric fat mass than normal mice fed the CD (CD mice) (figure 1A-C). Plasma levels of glucose, insulin and leptin were significantly increased in DIO mice compared with CD mice (figure 1D-F). Plasma IGF-1 levels in DIO mice were significantly decreased to 73% of that in CD mice, and plasma BDNF levels did not differ significantly between DIO mice and CD mice (figure 1G and H).

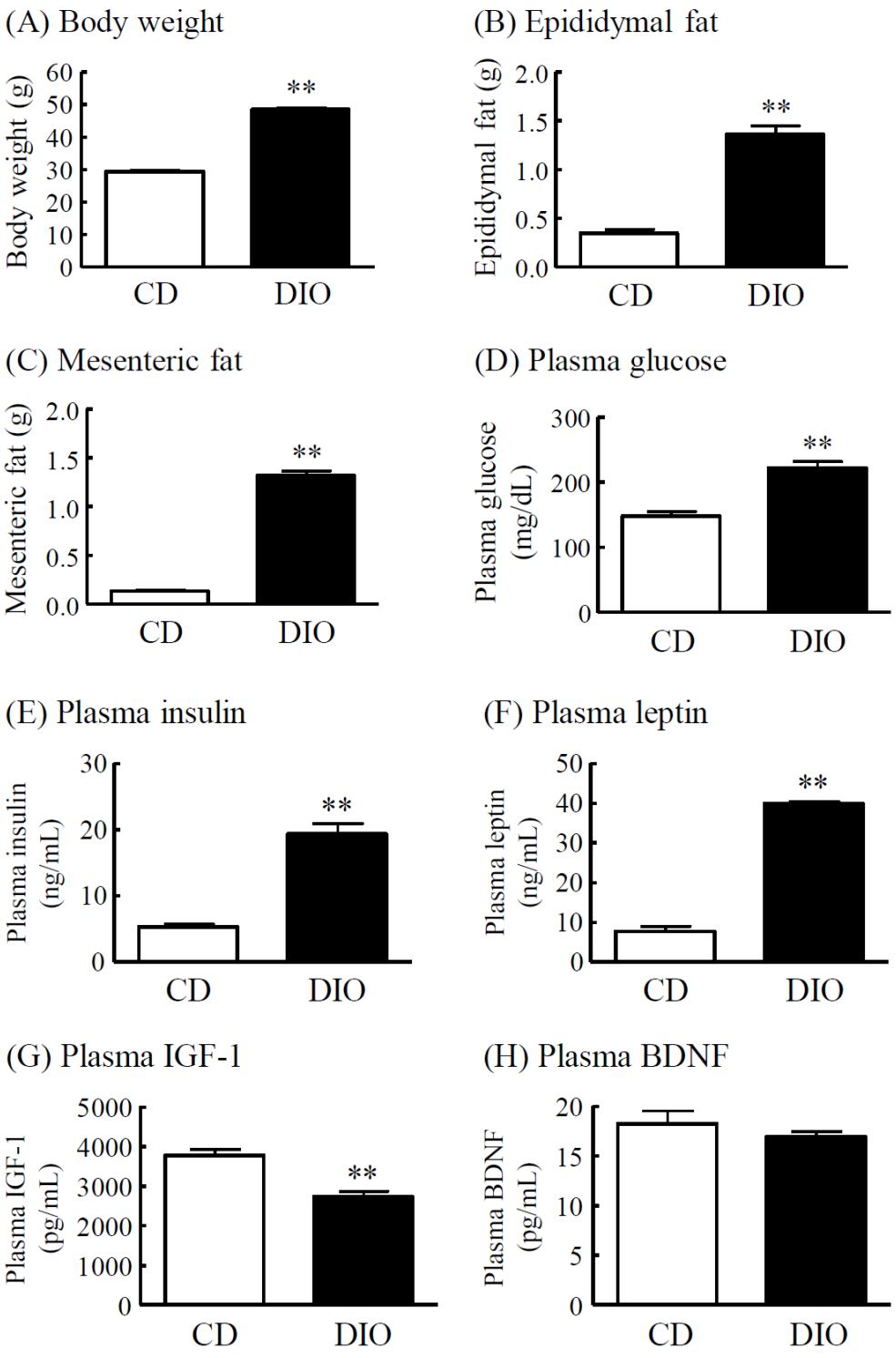

FIGURE 1. Changes in body weight, fat mass and plasma levels of glucose, insulin, leptin, IGF-1 and BDNF in DIO mice. (A) Body weight, (B) Epididymal fat, (C) Mesenteric fat, (D) Plasma glucose levels, (E) Plasma insulin levels, (F) Plasma leptin levels, (G) Plasma IGF-1 levels, (H) Plasma BDNF levels. Results are expressed as mean ± SE for 5-17 mice. **$p < 0.01$ vs. CD.

Changes in expression of fractalkine, CX3CR1, IGF-1 and BDNF in the brain of DIO mice

To explore the possible involvement of fractalkine-CX3CR1 signaling in the cognitive deficits observed in DIO mice, we examined changes in fractalkine and CX3CR1 expression in the hippocampus, amygdala and PFC of DIO mice compared with CD mice. Moreover, we examined the hippocampal IGF-1 and BDNF levels, which are well known to play a pivotal role in learning and memory.[11-13] The mRNA expression of fractalkine was significantly decreased in the hippocampus of DIO mice to 79% of that in CD mice (figure 2A). The protein levels of soluble form of fractalkine were significantly decreased in the hippocampus of DIO mice to 85% of those in CD mice (figure 2B). The mRNA expression and protein levels of CX3CR1 were significantly decreased in the hippocampus of DIO mice to 75% and 56%, respectively, of those in CD mice (figure 2C and D). In addition, protein levels of BDNF, but not IGF-1, in the hippocampus of DIO mice were significantly decreased to 78% of that in CD mice (figure 2E and F). The mRNA expression of fractalkine and CX3CR1 was significantly decreased in the amygdala of DIO mice to 86% and 79%, respectively, of that in CD mice (figure 2G). On the other hand, the mRNA expression of fractalkine and CX3CR1 in the PFC was not changed in DIO mice (figure 2H).

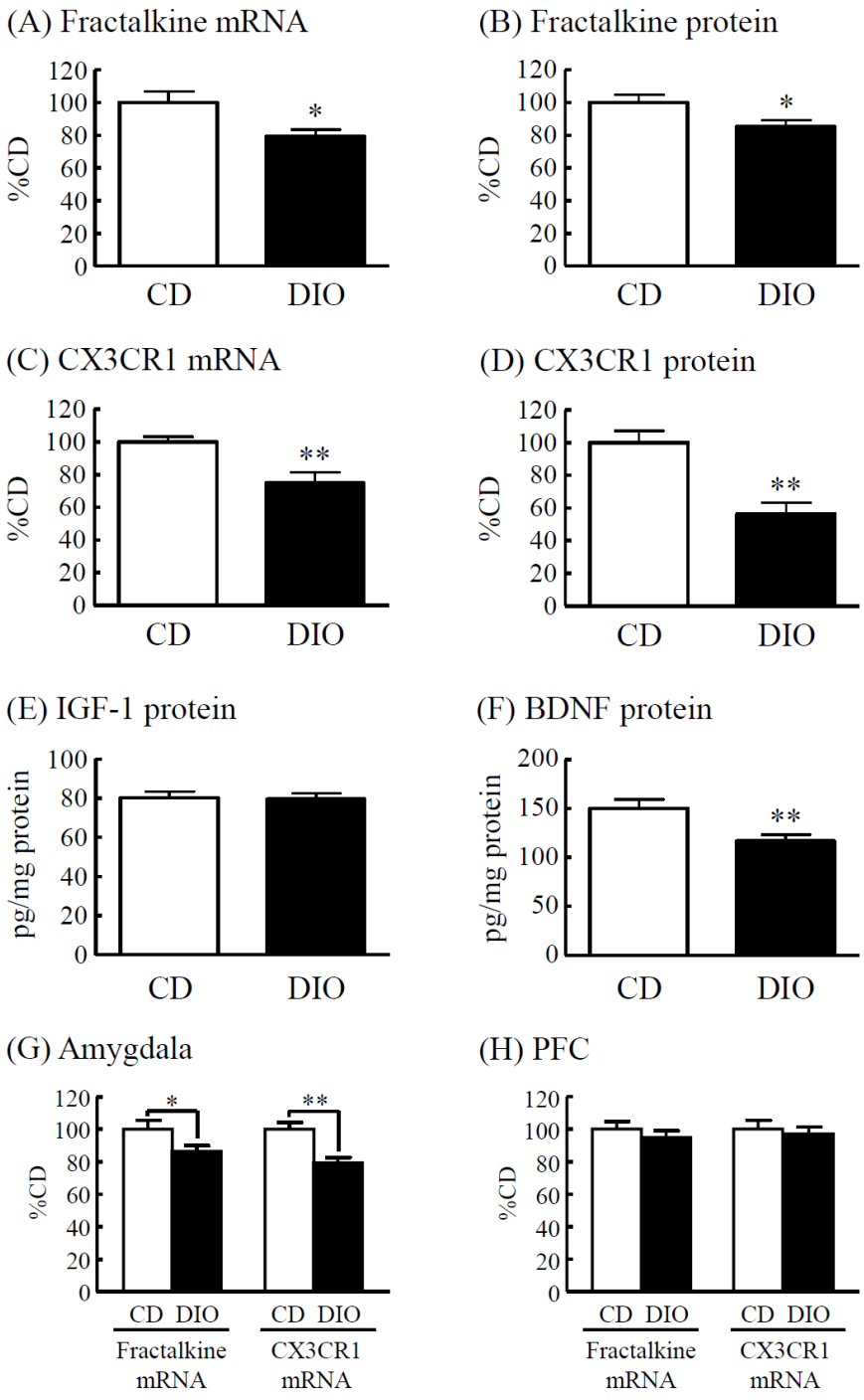

FIGURE 2. Changes in expression of fractalkine, CX3CR1, IGF-1 and BDNF in the brain of DIO mice. (A) Fractalkine mRNA expression, (B) Fractalkine protein levels, (C) CX3CR1 mRNA expression, (D) CX3CR1 protein levels, (E) IGF-1 protein levels, (F) BDNF protein levels in the hippocampus. (G) Fractalkine and CX3CR1 mRNA expression in the amygdala. (H) Fractalkine and CX3CR1 mRNA expression in the PFC. Results are expressed as mean ± SE for 5-16 mice. *$p < 0.05$, **$p < 0.01$ vs. CD.

Cognitive ability in the Y-maze test and changes in mRNA expression and protein levels of NMDA and AMPA receptor subunits, synaptophysin and PSD-95 in the hippocampus in DIO mice

Spontaneous alternation in the Y-maze test was significantly reduced in DIO mice to 76% of that in CD mice (figure 3A). The number of arm entries was not significantly different in between DIO mice and CD mice (figure 3B).

To elucidate the possible mechanisms underlying on cognitive impairment in DIO mice, we examined the hippocampal expression of NMDA receptor subunits, AMPA receptor subunits, synaptophysin and PSD-95. The mRNA expression of NR2A, but not NR1 and NR2B, of NMDA receptor subunits in the hippocampus of DIO mice was significantly decreased to 68% of the levels in CD mice (figure 3C, E and G). The protein levels of NR1 and NR2A in the hippocampus of DIO mice were significantly decreased to 62% and 58%, respectively, of the levels in CD mice (figure 3D and F). The protein levels of NR2B tended to be decreased to 85% of the levels in CD mice (figure 3H). The mRNA expression and protein levels of GluR1, but not GluR2, of AMPA receptor subunits in the hippocampus of DIO mice were significantly decreased to 81% and 74%, respectively, of the levels in CD mice, (figure 3I-L). The protein levels, but not mRNA expression, of synaptophysin and PSD-95 in the hippocampus of DIO mice were markedly decreased to 68% and 61%, respectively, of the levels in CD mice (figure 3M-P).

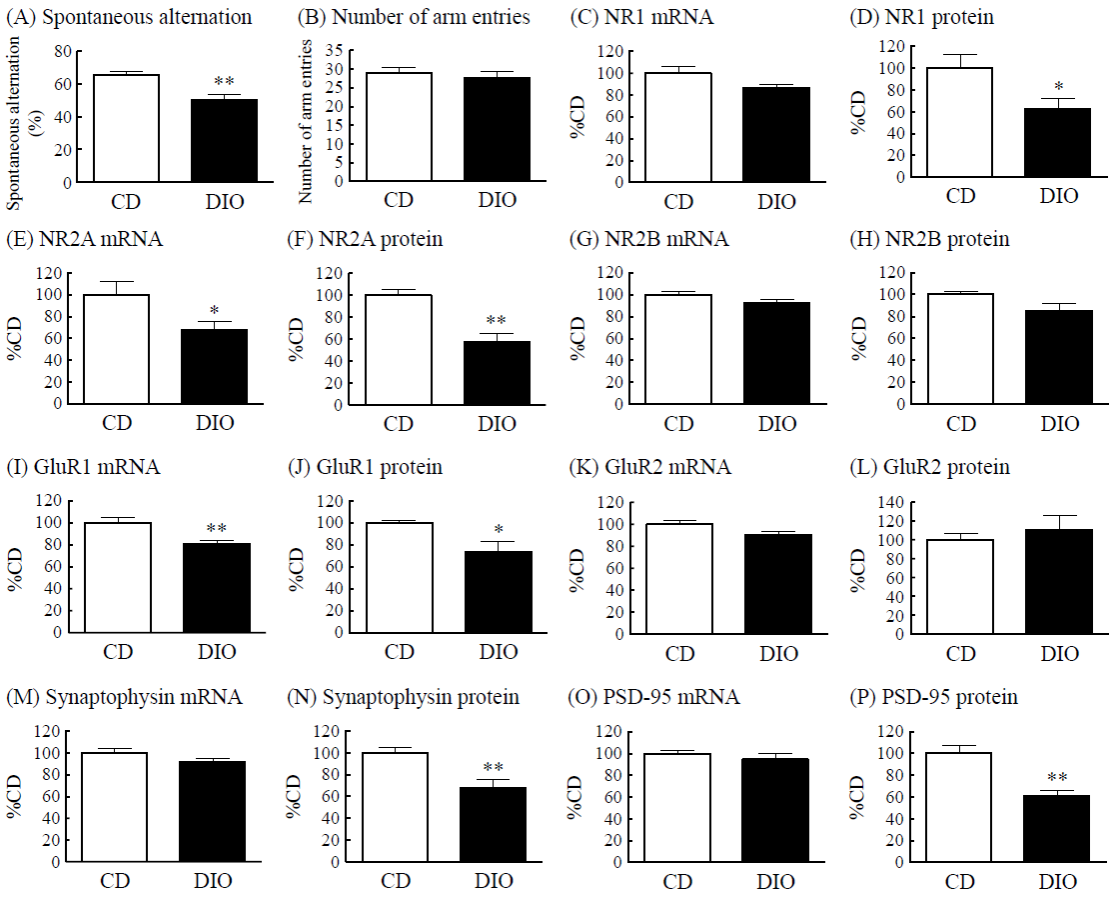

FIGURE 3. Cognitive ability in the Y-maze test and changes in mRNA expression and protein levels of NMDA and AMPA receptor subunits, synaptophysin and PSD-95 in the hippocampus in DIO mice. Y maze test: (A) Spontaneous alternation (%) and (B) Number of arm entries. NR1: (C) mRNA expression and (D) protein levels. NR2A: (E) mRNA expression and (F) protein levels. NR2B: (G) mRNA expression and (H) protein levels. GluR1: (I) mRNA expression and (J) protein levels. GluR2: (K) mRNA expression and (L) protein levels. Synaptophysin: (M) mRNA expression and (N) protein levels. PSD-95: (O) mRNA expression and (P) protein levels. Results are expressed as mean ± SE for 5-18 mice. *$p < 0.05$, **$p < 0.01$ vs. CD.

Effects of intracerebroventricular administration of the CX3CR1 antagonist 18a on cognitive ability in the Y-maze test and changes in mRNA expression and protein levels of NMDA and AMPA receptor subunits, synaptophysin and PSD-95 in the hippocampus in normal mice

To examine the possible contribution of decreased fractalkine-CX3CR1 signaling to the cognitive impairment observed in DIO mice, we examined the effect of the CX3CR1 antagonist 18a on learning and memory in the Y-maze test in normal mice. Spontaneous alternation in the Y-maze test was significantly reduced in mice with intracerebroventricular administration of 18a to 75% of that in vehicle-treated mice (figure 4A). The number of arm entries was not changed in either of the two groups (figure 4B).

To further evaluate the involvement of fractalkine-CX3CR1 signaling in cognitive deficits in DIO mice, the changes in the same factors observed in the hippocampus of DIO mice, as shown in figure 3, were examined in the hippocampus of mice treated with the CX3CR1 antagonist 18a. The mRNA expression of the NR1, but not NR2A and NR2B, in the hippocampus of the antagonist-treated mice was significantly decreased to 67% of that in vehicle-treated mice (figure 4C, E and G). The protein levels of NR2A, but not NR1 and NR2B, in the hippocampus of the antagonist-treated mice were significantly decreased to 53% of the levels in vehicle-treated mice (figure 4D, F and H). The mRNA expression and protein levels of the GluR1, but not GluR2, in the hippocampus of the antagonist-treated mice were significantly decreased to 70% and 62%, respectively, of the levels in vehicle-treated mice (figure 4I-L). The mRNA expression and protein levels of synaptophysin in the hippocampus were not changed by administration of 18a (figure 4M and N). The mRNA expression and protein levels of PSD-95 in the hippocampus of the antagonist-treated mice were significantly decreased to 77% and 66%, respectively, of the levels in vehicle-treated mice (figure 4O and P).

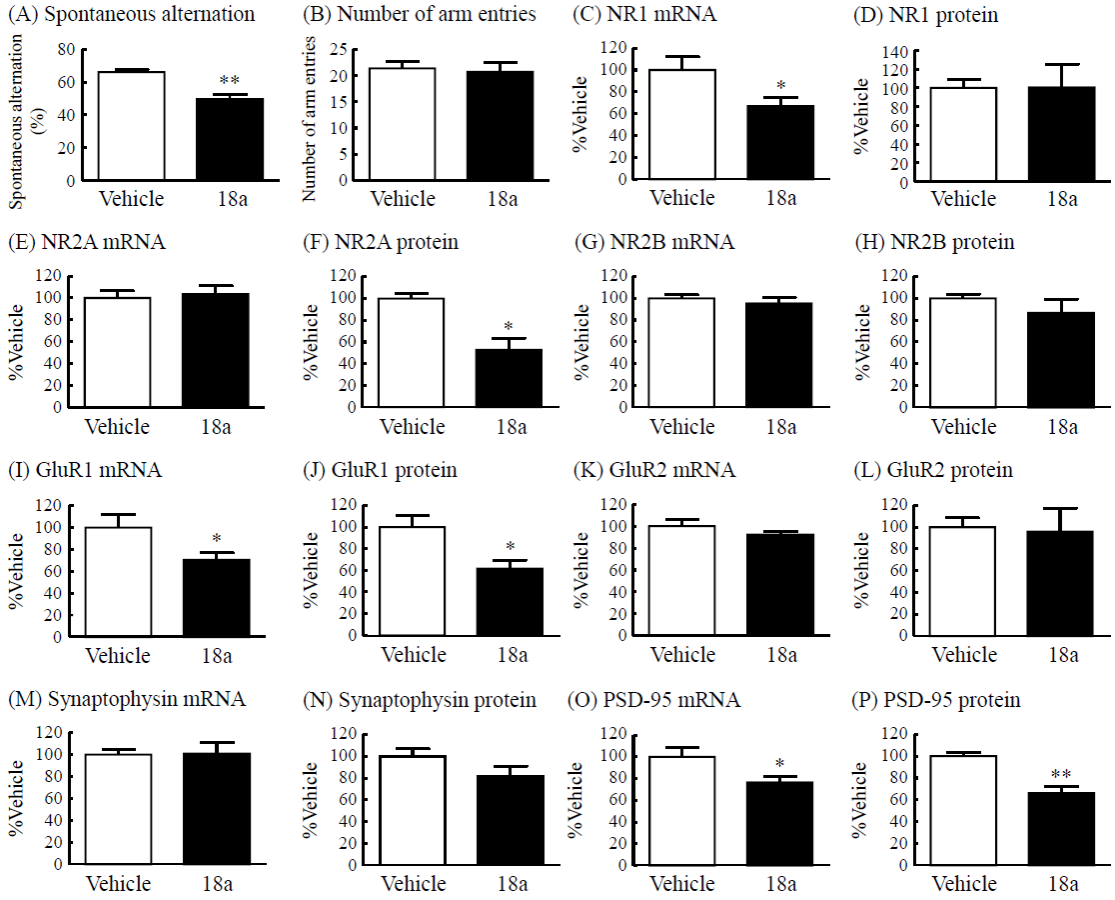

FIGURE 4. Effects of intracerebroventricular administration of the CX3CR1 antagonist 18a on cognitive ability in the Y-maze test and changes in mRNA expression and protein levels of NMDA and AMPA receptor subunits, synaptophysin and PSD-95 in the hippocampus in normal mice. Y maze test: (A) Spontaneous alternation (%) and (B) Number of arm entries. NR1: (C) mRNA expression and (D) protein levels. NR2A: (E) mRNA expression and (F) protein levels. NR2B: (G) mRNA expression and (H) protein levels. GluR1: (I) mRNA expression and (J) protein levels. GluR2: (K) mRNA expression and (L) protein levels. Synaptophysin: (M) mRNA expression and (N) protein levels. PSD-95: (O) mRNA expression and (P) protein levels. Results are expressed as mean ± SE for 3-7 mice. *$p < 0.05$, **$p < 0.01$ vs. vehicle.

Regulatory effects of IGF-1 and BDNF on mRNA expression of fractalkine and CX3CR1 in the hippocampus of normal mice

Plasma IGF-1 levels and hippocampal BDNF levels were significantly lower in DIO mice compared with CD mice (figure 1G and 2F). We then examined whether IGF-1 and BDNF regulate the mRNA expression of fractalkine and CX3CR1 in the hippocampus of normal mice. Chronic intraperitoneal administration of the selective IGF-1 receptor inhibitor PPP induced a significant decrease in the mRNA expression of fractalkine and CX3CR1 in the hippocampus to 79% and 77%, respectively, of those in vehicle-treated mice (figure 5A and B). Chronic intraperitoneal administration of the TrkB selective and non-competitive antagonist ANA-12 also induced significant decrease in the mRNA expression of both fractalkine and CX3CR1 in the hippocampus to 83% and 75%, respectively, of those in vehicle-treated mice (figure 5C and D).

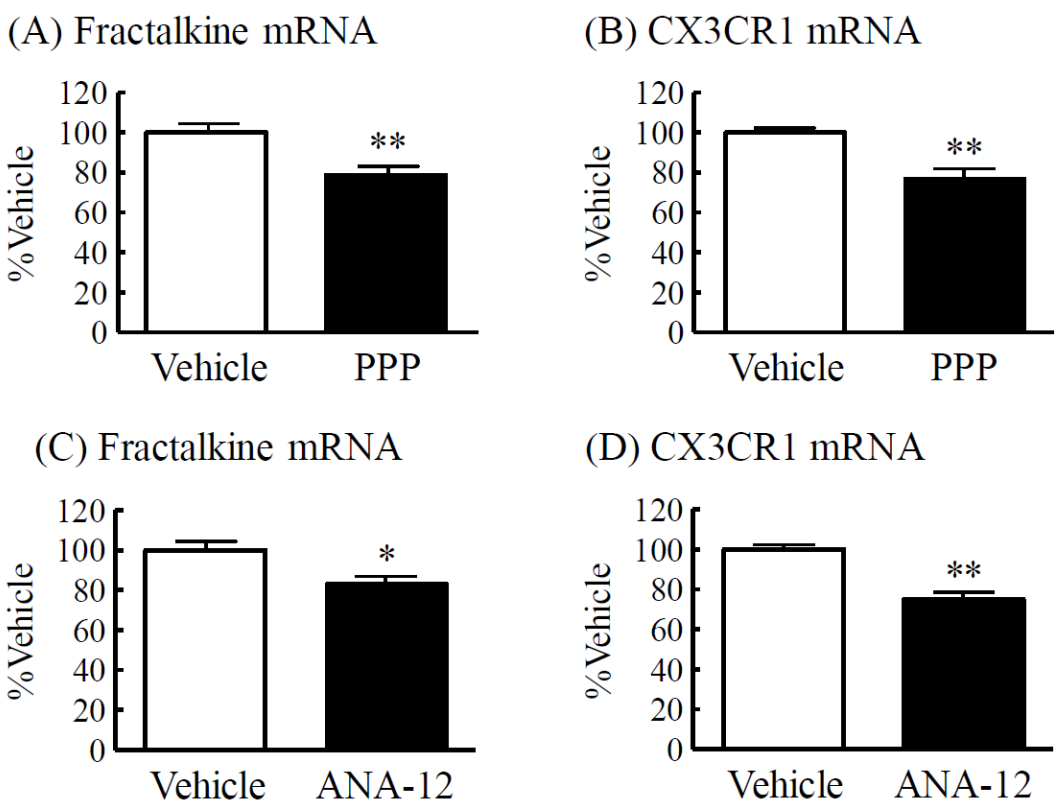

FIGURE 5. Regulatory effects of IGF-1 and BDNF on mRNA expression of fractalkine and CX3CR1 in the hippocampus of normal mice. PPP: (A) Fractalkine mRNA expression and (B) CX3CR1 mRNA expression. ANA-12: (C) Fractalkine mRNA expression and (D) CX3CR1 mRNA expression. Results are expressed as mean ± SE for 12-13 mice. *$p < 0.05$, **$p < 0.01$ vs. vehicle.

Discussion

In the present study, DIO mice exhibited significant impairment of learning and memory in the Y-maze test compared with control mice, and a significant reduction in both fractalkine and CX3CR1 levels in the hippocampus and amygdala. Furthermore, intracerebroventricular administration of the CX3CR1 antagonist 18a in normal mice induced significant cognitive deficits in the Y-maze test. Regarding the mechanism underlying the cognitive deficits in DIO mice, DIO mice exhibited a significant decrease in NR1 and NR2A of NMDA receptor subunits, GluR1 of AMPA receptor subunits, synaptophysin, and PSD-95 in the hippocampus. Moreover, CX3CR1 antagonist-treated mice exhibited a significant decrease in NR2A, GluR1 and PSD-95 in the hippocampus. Furthermore, plasma IGF-1 and hippocampal BDNF were significantly decreased in DIO mice compared with CD mice. Administration of the IGF-1 receptor inhibitor PPP and the TrkB antagonist ANA-12 significantly decreased mRNA expression of fractalkine and CX3CR1 in the hippocampus of normal mice. These findings suggest that the cognitive decline observed in DIO mice is due, at least in part, to reduced fractalkine-CX3CR1 signaling in the corticolimbic system.

A diet high in saturated fat is well known to affect neuronal function and contribute to cognitive decline in experimental animals and humans.[10 31-33] Obese animals fed over the long term with a diet high in saturated fat exhibit impaired acquisition and retention of spatial memory in the water maze test, and low levels of hippocampal BDNF to the extent that compromises cognitive performance.[3 4 9 14] Our previous study demonstrated that DIO mice fed HFD exhibit significant attenuation of hippocampus- and amygdala-dependent fear conditioning responses and low levels of hippocampal BDNF.[7] The amygdala regulates hippocampal LTP, spatial memory and dentate gyrus field potentials.[34 35] Moreover, the PFC influences the responsiveness of the central amygdala output neurons.[36] These findings indicate that hippocampus, amygdala and PFC play an important role in learning and memory processes. Therefore, we examined changes in the expression of fractalkine and CX3CR1 in the hippocampus, amygdala and PFC of DIO mice. The present study provides the first evidence that DIO mice have significant decrease in fractalkine and CX3CR1 levels in the hippocampus and amygdala, and that the administration of a CX3CR1 antagonist significantly impairs learning and memory in the Y-maze test in normal mice, consistent with cognitive deficits in CX3CR1-deficient mice.[20] Electrophysiological studies demonstrated that LTP in the hippocampal CA1 region is markedly impaired in DIO rats compared with lean rats.[9 10] LTP is predominantly regulated by glutamate receptors, such as NMDA and AMPA receptors, and is considered

to be a form of synaptic plasticity that underlies learning and memory in the hippocampus.[8] Activation of CX3CR1 on microglia within the CA1 region of the hippocampus triggers the release of adenosine that in turn, via the activation of type A2 adenosine receptors, increases the release of D-serine as a coagonist for NMDA receptors from the glia, leading to a potentiation of NMDA function, suggesting that fractalkine-CX3CR1 signaling may enhance LTP as well as learning and memory processes.[37] On the other hand, CX3CR1-deficient mice exhibit impaired LTP in the hippocampus.[20] These findings suggest that the cognitive impairment observed in DIO mice may be due to decreased fractalkine-CX3CR1 signaling in the hippocampus.

Recent observations demonstrated that circulating and brain IGF-1 modulates brain activities such as neuroprotection, neurogenesis and neuronal excitability.[13] Serum IGF-1-deficient mice exhibit both cognitive decline and impaired hippocampal LTP.[38] The present study demonstrated that plasma IGF-1 levels, but not hippocampal IGF-1 levels, were significantly decreased in DIO mice compared with CD mice. Moreover, the present study showed that the administration of the IGF-1 receptor inhibitor PPP significantly decreased mRNA expression of fractalkine and CX3CR1 in the hippocampus, providing different lines of evidence that bloodborne IGF-1 is a potent positive regulator for fractalkine-CX3CR1 signaling in the brain.

In the present study, BDNF levels in the hippocampus were significantly decreased in DIO mice compared with CD mice, consistent with previous reports.[3 4 7] In addition, the administration of the BDNF receptor TrkB antagonist ANA-12 significantly decreased fractalkine and CX3CR1 levels in the hippocampus in normal mice. The mRNA and protein expression of fractalkine and CX3CR1 is significantly decreased in the hippocampus of BDNF-deficit mice.[39] Together, these findings suggest that a HFD induces a significant decrease in hippocampal BDNF levels, which in turn, decreases fractalkine and CX3CR1 levels in the hippocampus.

A recent study demonstrated that the detrimental effects of HFD consumption on learning and memory may be mediated in part by the alterations of glutamate receptors, such as NMDA and AMPA receptors.[3 4 40-42] HFD feeding decreases hippocampal NR2B of NMDA receptor subunits and GluR-1 of AMPA receptor subunits.[43 44] The present study also demonstrated a significant decrease in NR1, NR2A and GluR1 in the hippocampus of DIO mice. Moreover, the present study demonstrated that CX3CR1 antagonist-treated mice also exhibited a significant decrease in NR2A and GluR1 in the hippocampus coincident with significant cognitive deficits, similar to DIO mice. Presynaptic and postsynaptic machinery associated with synapse formation and activity is involved in learning and memory processes. Compared with control mice, DIO mice

exhibit abnormal expression of factors involved in the synaptic plasticity, such as synaptosomal-associated protein 25 (SNAP-25) and synaptophysin I in the presynaptic site and PSD-95 in the postsynaptic site.[5 6 9 10 14 43] HFD-fed animals exhibit cognitive decline in a water maze test and step-through task, and a decrease in synapsin 1 and PSD-95 in the hippocampus and cerebral cortex.[3 14] Further, HFD-fed mice display impaired hippocampal-dependent memory in the Y-maze test, and reduced SNAP-25, PSD-95 and syntaxin-4, but not synaptophysin, in the hippocampus.[6] In the present study, DIO mice exhibited a significant decrease in synaptophysin and PSD-95 levels in the hippocampus compared with CD mice. Synaptophysin is presynaptic vesicle marker, and PSD-95 interacts with ion channels, membrane receptors, cytoskeletal components, and intracellular signaling molecules,[45 46] and increases AMPA receptor currents by selectively delivering GluR1-containing receptors to synapses, thus mimicking LTP.[47] These findings suggest that the synaptic dysfunction might be an important contributor to the hippocampal-dependent spatial memory impairment in DIO mice.[6] Furthermore, in the present study, the administration of a CX3CR1 antagonist induced a significant decrease in PSD-95 in the hippocampus of normal mice compared with vehicle-treated mice, the same changes observed in the hippocampus of DIO mice. Moreover, the same changes in the NMDA receptor subunits, AMPA receptor subunits, and synaptic markers were observed in both DIO mice and CX3CR1 antagonist-treated mice, indicating that HFD feeding induced impaired fractalkine-CX3CR1 signaling in the hippocampus, leading to synaptic deterioration, and resulting in impaired cognition.

There is convincing evidence that enhanced inflammation in both the brain and peripheral organs is strongly involved in several dysfunctions associated with HFD feeding for a long time.[48] In this regard, fractalkine-CX3CR1 signaling is demonstrated to regulate the inflammatory responses of macrophage and microglia.[20 49 50] Especially, enhanced inflammation in both the brain and peripheral organs induced by HFD feeding has been reported to influence cognitive function.[20 31 49 50] Therefore, enhanced inflammation may be an another pivotal event to regulate processing of learning and memory in obesity.

The present study revealed that long-term HFD feeding in mice may induce impaired fractalkine-CX3CR1 signaling in the corticolimbic system, leading to defective synaptic plasticity and subsequently to cognitive impairment in DIO mice. Taken together, these findings provide compelling evidence that neuron-microglia interactions, especially those underpinned by fractalkine-CX3CR1 signaling, play crucial roles in brain dysfunction associated with obesity.

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Research Paper

Reduced brain fractalkine-CX3CR1 signaling is involved in the impaired cognition of streptozotocin-treated mice

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ABSTRACT

Patients with diabetes mellitus are predisposed to cognitive impairment. Fractalkine-CX3CR1 in the brain signaling represents a primary neuron-microglia inter-regulatory system for several brain functions including learning and memory processes. The present study addressed whether fractalkine-CX3CR1 signaling in the hippocampus contributes to the cognitive deficits observed in streptozotocin (STZ)-treated mice. Our results showed that STZ-treated mice exhibited significant cognitive deficits in the Y-maze test, and a decrease in fractalkine and CX3CR1 levels in the hippocampus. Moreover, intracerebroventricular injection of the CX3CR1 antagonist 18a in normal mice induced significant cognitive deficits in the Y-maze test. STZ-treated mice showed a significant increase in plasma corticosterone levels and a decrease in plasma and hippocampal levels of insulin-like growth factor-1 (IGF-1). Therefore, we examined the effects of corticosterone and IGF-1 on regulation of fractalkine and CX3CR1 expression. Dexamethasone (DEX) application significantly decreased the mRNA expression of fractalkine in primary neuron and astrocyte cultures, and of CX3CR1 in primary microglia cultures. On the other hand, IGF-1 application significantly increased the mRNA expression of fractalkine in primary neuron cultures and CX3CR1 in primary microglia cultures. In addition, administration of DEX and the IGF-1 receptor tyrosine kinase inhibitor picropodophyllin significantly reduced the mRNA expression of fractalkine and CX3CR1 in the hippocampus. These findings indicate that impaired cognition in STZ-treated mice is associated with reduced fractalkine-CX3CR1 signaling in the hippocampus which may be induced by an increase in corticosterone and a decrease in IGF-1.

1. Introduction

Fractalkine secreted from neurons was recently reported to be involved in the regulation of several functions of the central nervous system (CNS) (Goazigo et al., 2013; Maciejewski-Lenoir et al., 1999; Nishiyori et al., 1998). Astrocytes in the brain also synthesize fractalkine (Yoshida et al., 2001). In mouse brain, fractalkine mRNA levels are high in the cortex, hippocampus and striatum; intermediate in the olfactory bulb, thalamus, hypothalamus and brainstem; and low in the cerebellum (Maciejewski-Lenoir et al., 1999; Nishiyori et al., 1998). Fractalkine binds to the CX3C chemokine receptor 1 (CX3CR1), which is mainly expressed in microglia (Imai et al., 1997; Maciejewski-Lenoir et al., 1999; Nishiyori et al., 1998). Fractalkine-CX3CR1 signaling represents a primary neuron-microglia inter-regulatory system that is important for synaptic plasticity and function in the brain (Goazigo et al., 2013). Recent evidence indicates that fractalkine-CX3CR1 signaling plays an important role in regulating the formation of long-term potentiation (LTP) in the hippocampus and behavioral learning and memory processes (Rogers et al., 2011; Sheridan et al., 2014). LTP, defined as an activity-dependent, prolonged enhancement of synaptic strength, in the

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNS, central nervous system; CX3CR1, CX3C chemokine receptor 1; DEX, dexamethasone; DM, diabetes mellitus; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF-1, insulin-like growth factor-1; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; PPP, picropodophyllin; STZ, streptozotocin.

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hippocampal CA1 region is considered to be a form of synaptic plasticity for the cellular mechanisms of learning and memory, and is predominately regulated by the glutamatergic system (Neves et al., 2008). CX3CR1-deficient mice exhibit cognitive deficits in different types of learning and memory tasks, such as fear-conditioning and water maze tests, in parallel with impaired LTP (Rogers et al., 2011).

Diabetes mellitus (DM) is a common metabolic disorder, characterized by glucose intolerance. Epidemiologic studies have demonstrated that both type 1 and type 2 DM patients have a predisposition for several dysfunctions of the CNS, such as cognitive impairment and depression, compared with non-diabetic patients (Biessels et al., 2008; McCall, 1992). Our previous study demonstrated that diet-induced obese mice fed a high-fat diet exhibit significant impairment of fear conditioning responses which are dependent on the hippocampus and amygdala (Yamada-Goto et al., 2012). Streptozotocin (STZ)-treated animals as a model of type 1 DM exhibit impaired learning and memory in several learning behavioral tests, such as the Y-maze, water maze, complex maze and passive avoidance (Biessels et al., 1996; Molteni et al., 2002; Wu et al., 2004). Moreover, electrophysiological studies revealed impaired expression of LTP in the hippocampus of STZ-treated animals (Kamal et al., 1999, 2000).

In the present study, we examined the possible contribution of fractalkine-CX3CR1 signaling in the hippocampus to the impaired cognition observed in STZ-treated mice. Our findings revealed that impaired cognition in STZ-treated mice is associated with decreased fractalkine-CX3CR1 signaling in the hippocampus which may be induced by an increase in plasma corticosterone levels and a decrease in plasma and hippocampal insulin-like growth factor-1 (IGF-1) levels.

2. Material and methods

2.1. Animals

Male C57BL/6 J mice (6 weeks old) were obtained from CLEA Japan, Inc. (Tokyo, Japan) and housed in plastic cages under a 12:12 h light/dark cycle (lights turned on at 07.00 h) at room temperature (23 ± 1 °C). The animals had *ad libitum* access to water and food (CE-2; CLEA Japan, Inc.). All experiments were performed in accordance with the guidelines established by the Institute of Laboratory Animal Science Research Support Center at Kagoshima University and approved by the Kagoshima University Institutional Animal Care and Use Committee (protocol nos. MD18079 and MD18080), and in accordance with the guidelines established by the United States National Institutes of Health Guide for the care and use of laboratory animals (NIH publication No. 80-23, revised in 1996). Every effort was made to optimize the comfort of the animals and to minimize their use.

2.2. STZ-treated mice

Mice (10–12 weeks old) were given intraperitoneal injections of a single dose of STZ (200 mg/kg body weight, Merck KGaA, Darmstadt, Germany). STZ was dissolved in 10 mM chilled sodium citrate buffer (pH 4.0) just before injection. Control mice were given intraperitoneal injections of an equal volume of sodium citrate buffer (10 ml/kg body weight). Two weeks after the injection, we evaluated them in the Y-maze test and measured their body weight before the mice were killed by an overdose of isoflurane (Abbott Japan, Tokyo, Japan) for collection of blood samples and brain region.

2.3. Analysis of metabolic parameters and sampling of the brain region

Blood samples were collected from the retroorbital vein under isoflurane anesthesia and immediately transferred to tubes containing ethylenediaminetetraacetic acid (EDTA; 10 μl of 0.2 M EDTA/tube) and aprotinin (0.1 mg/tube, Merck KGaA). The blood samples were centrifuged 3000×g for 5 min at 4 °C, and the plasma was separated and stored at −80 °C until assayed. After blood collection, the mice were killed by decapitation. The brain was rapidly removed from the skull and placed on an ice-cooled paraffin plate for dissection of the hippocampus as previously described (Nakao et al., 1986). The hippocampus was immediately frozen in liquid nitrogen and stored at −80 °C until analyzed. Glucose (Glucose C2; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), insulin (Morinaga Ultra Sensitive Mouse/Rat Insulin ELISA Kit; Morinaga Institute of Biological Science, Yokohama, Japan), corticosterone (Corticosterone Enzyme Immunoassay Kit; Arbor Assays, Ann Arbor, MI, USA) and IGF-1 (Mouse/Rat IGF-1 Quantikine ELISA Kit; R&D Systems, Inc., Minneapolis, MN, USA) were measured using commercially available kits.

2.4. Y-maze test

Spatial working memory was assessed by the Y-maze test. The Y-maze apparatus (Muromachi Co. Ltd., Tokyo, Japan) comprised three grey plastic arms (each 41.5 cm long, 4 cm wide, with 10-cm high walls) separated by 120° and randomly labeled A, B, and C. The task was performed as previously described (Sarnyai et al., 2000).

2.5. Continuous subcutaneous administration of dexamethasone (DEX)

Mice (10–12 weeks old) were anesthetized with isoflurane and a 7-day micro-osmotic pump (Alzet Model 1007D, DURECT Corporation, Cupertino, CA, USA) was implanted subcutaneously between the shoulder blades. DEX is a synthetic glucocorticoid and have a similar long-lasting action to cortisol and corticosterone via glucocorticoid receptor (Mulatero et al., 1997). The pumps contained DEX sodium phosphate (10, 30 and 100 μg/day, FUJIFILM Wako Pure Chemical Corporation) dissolved in saline. Mice in the control group were given subcutaneous injections of an equal volume of saline. Seven days after implanting the micro-osmotic pumps, the mice were killed by an overdose of isoflurane for collection of brain region.

2.6. Intraperitoneal administration of the selective IGF-1 receptor tyrosine kinase inhibitor picropodophyllin (PPP)

PPP (20 mg/kg, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted with saline to a final concentration of 50 % DMSO (Menu et al., 2006). Mice in the control group were given intraperitoneal injections of an equal volume of vehicle (50 % DMSO in saline, 10 ml/kg body weight). The mice were killed by an overdose of isoflurane 1 h after the administration and the brain region was collected.

2.7. Intracerebroventricular injection of the CX3CR1 antagonist 18a

Intracerebroventricular injection was performed as described previously (Yamada-Goto et al., 2013). The highly selective antagonist for CX3CR1 18a (Axon Medchem, Groningen, Netherlands) with a Ki value of 3.9 nM, was dissolved in DMSO, and diluted with saline to a final concentration of 0.1 % DMSO (Karlström et al., 2013). The CX3CR1 antagonist 18a (50 ng/mouse) was intracerebroventricularly injected at 30 min before the Y-maze test. Mice in the control group were given intracerebroventricular injections of an equal volume of vehicle (0.1 % DMSO in saline, 2 μl/mouse).

2.8. Mouse primary neuron, astrocyte and microglia cultures

Mouse primary neuron and astrocyte cultures were performed as described previously (Katsuura et al., 1989; Yamada et al., 2009). According to a previous report (Han et al., 2013), primary microglia cell cultures were prepared from C57BL/6 J mouse brain on postnatal day 3. DEX (FUJIFILM Wako Pure Chemical Corporation), insulin (Thermo Fisher Scientific Inc., Waltham, MA, USA) and IGF-1 (PeproTech, Inc.,

Rocky Hill, NJ, USA) were used in this study. The purity of each cell culture was greater than 95 %.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA levels of fractalkine and CX3CR1 were measured by quantitative real-time RT-PCR as previously described (Yamada et al., 2009). All gene-specific mRNA expression values were normalized against the internal housekeeping gene 18S in the experiments involving the subcutaneous administration of DEX and the application of DEX in astrocyte cultures, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in other experiments. Primers for GAPDH were as follows: [sense TGCACCACCAACTGCTTAGC, antisense GGATGCAGGGATGATGTTCTG], for 18S, [sense GTAACCCGTTGAACCCCATT, antisense CCATCCAATCGGTAGTAGCG], for fractalkine, [sense ACGAAATGCGAAATCATGTGC, antisense CTGTGTCGTCTCCAGGACAA], for CX3CR1, [sense CGTGAGACTGGGTGAGTGAC, antisense AAGGAGGTGGACATGGTGAG].

2.10. Western blotting analysis

Western blotting was performed as described previously (Yamada et al., 2011). The primary antibodies used in the present study were a rabbit polyclonal anti-CX3CL1 (fractalkine) antibody (ab25088, abcam, Cambridge, UK), a mouse monoclonal anti-CX3CR1 antibody (ab184678, abcam) and a mouse monoclonal anti-GAPDH antibody (sc-32233, Santa Cruz Biotechnology, Inc.). The secondary antibodies were an anti-rabbit IgG antibody conjugated to horseradish peroxidase (NA934, GE HealthCare UK Ltd., Buckinghamshire, UK) and an anti-mouse IgG antibody conjugated to horseradish peroxidase (NA931, GE HealthCare UK Ltd.).

2.11. Data analysis

Data are expressed as mean ± SEM. Statistical analysis of the data was performed by ANOVA followed by the Tukey-Kramer test. Statistical significance was defined as $P < 0.05$.

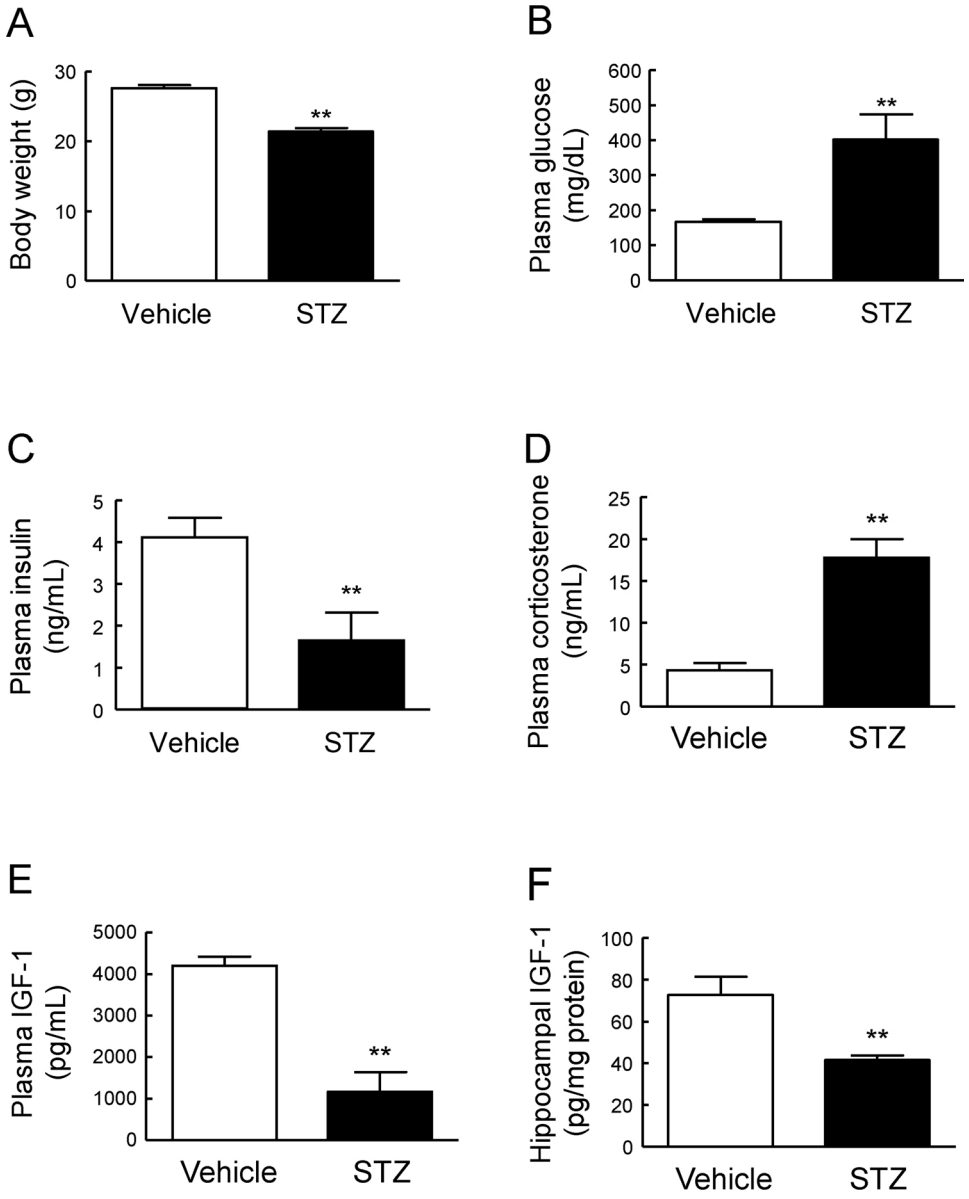

Fig. 1. Changes in body weight, plasma levels of glucose, insulin, corticosterone and IGF-1, and protein levels of IGF-1 in the hippocampus in STZ-treated mice. (A) Body weight, (B) Plasma glucose levels, (C) Plasma insulin levels, (D) Plasma corticosterone levels, (E) Plasma IGF-1 levels, (F) IGF-1 protein levels in the hippocampus. Results are expressed as mean ± SE for 9 to 17 mice. **p < 0.01 vs. vehicle.

3. Results

3.1. Changes in body weight, plasma levels of glucose, insulin, IGF-1 and corticosterone, and protein levels of IGF-1 in the hippocampus in STZ-treated mice

STZ-treated mice had a significantly lower body weight than vehicle-treated mice at two weeks after STZ injection (Fig. 1A). In STZ-treated mice, plasma glucose levels were markedly increased, while plasma insulin levels were significantly decreased compared with vehicle-treated mice (Fig. 1B and C). In addition, STZ treatment significantly increased plasma corticosterone levels to 414 % of those in vehicle-treated mice (Fig. 1D) and significantly decreased plasma IGF-1 levels to 28 % of those in vehicle-treated mice (Fig. 1E). The IGF-1 protein levels in the hippocampus of STZ-treated mice were significantly decreased to 56 % of those in vehicle-treated mice (Fig. 1F).

3.2. Changes in mRNA expression and protein levels of fractalkine and CX3CR1 in the hippocampus of STZ-treated mice

At two weeks after STZ injection, the mRNA expression and protein levels of fractalkine were significantly decreased in the hippocampus of STZ-treated mice to 73 % and 65 %, respectively, of those in vehicle-treated mice (Fig. 2A and B). Moreover, the mRNA expression and protein levels of CX3CR1 were significantly decreased in the hippocampus of STZ-treated mice to 70 % and 61 %, respectively, of those in vehicle-treated mice (Fig. 2C and D).

3.3. Cognitive ability in the Y-maze test

At two weeks after STZ injection, spontaneous alternation was significantly reduced in STZ-treated mice to 72 % of that in vehicle-treated mice (Fig. 3A). The number of entries into each arm was not different between groups (Fig. 3B).

To elucidate the role of fractalkine-CX3CR1 signaling in the learning and memory processes, we examined the effect of the CX3CR1 antagonist 18a on learning and memory in the Y-maze test in normal mice. Intracerebroventricular injection with 18a (50 ng/mouse) significantly reduced spontaneous alternation in mice to 78 % of that in vehicle-treated mice (Fig. 3C). The number of entries into each arm was not changed in 18a-treated mice (Fig. 3D)

3.4. Effects of the application of DEX, insulin and IGF-1 on mRNA expression of fractalkine in primary neuron and astrocyte cultures, and mRNA expression of CX3CR1 in primary microglia cultures

In primary neuron cultures, the application of DEX at concentrations of 10, 100 and 1000 nM for 24 h significantly deceased fractalkine mRNA expression (Fig. 4A). The application of insulin at a concentration of 1000 nM for 24 h did not change the fractalkine mRNA expression in primary neuron cultures (Fig. 4B). On the other hand, the application of IGF-1 at a concentration of 1000 nM for 24 h to primary neuron cultures significantly increased the fractalkine mRNA expression to 130 % of that in the control group (Fig. 4C).

In primary astrocyte cultures, the application of 10, 100 and 1000 nM DEX for 24 h significantly decreased fractalkine mRNA expression to 67 %, 48 % and 55 %, respectively, of that in the control group (Fig. 4D). Applications of insulin (1000 nM) and IGF-1 (1000 nM) for 24 h did not change the fractalkine mRNA expression in primary astrocyte cultures (Fig. 4E and F).

In primary microglia cultures, the application of 10, 100 and 1000 nM DEX for 24 h markedly decreased CX3CR1 mRNA expression to 77 %, 31 % and 31 %, respectively, of that in the control group (Fig. 4G). The application of insulin (1000 nM) for 24 h did not change CX3CR1 mRNA expression in primary microglia cultures (Fig. 4H). On the other hand, the application of 1000 nM IGF-1 for 24 h significantly

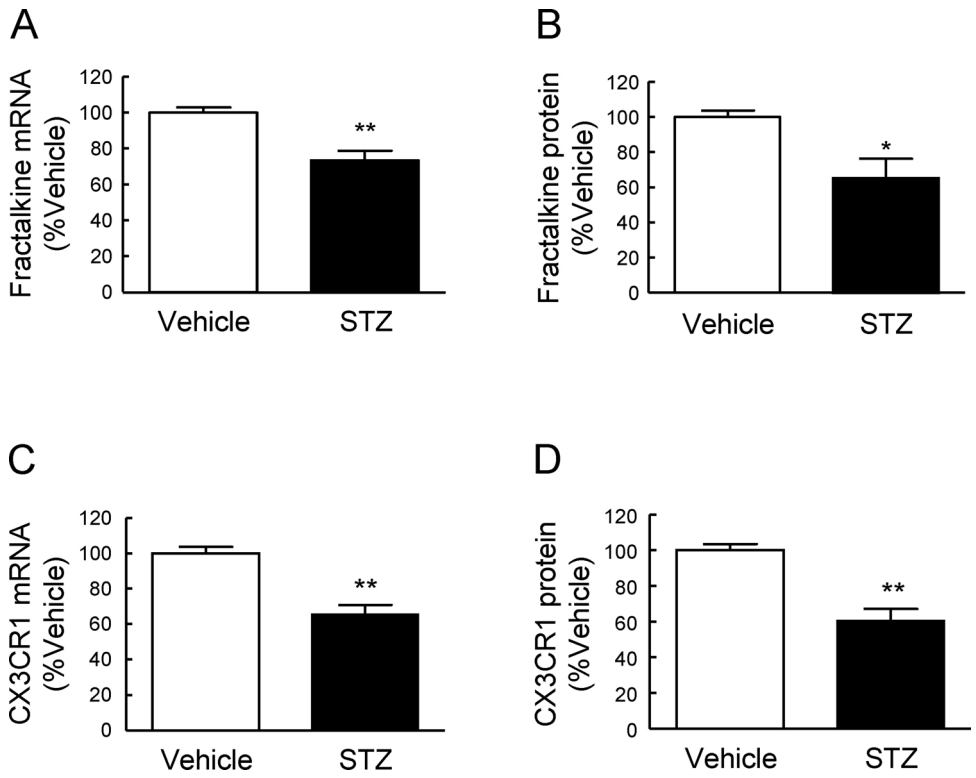

Fig. 2. Changes in the mRNA expression and protein levels of fractalkine and CX3CR1 in the hippocampus of STZ-treated mice. (A) Fractalkine mRNA expression, (B) Fractalkine protein levels, (C) CX3CR1 mRNA expression, (D) CX3CR1 protein levels. Results are expressed as mean ± SE for 5-16 mice. *p < 0.05, **p < 0.01 vs. vehicle.

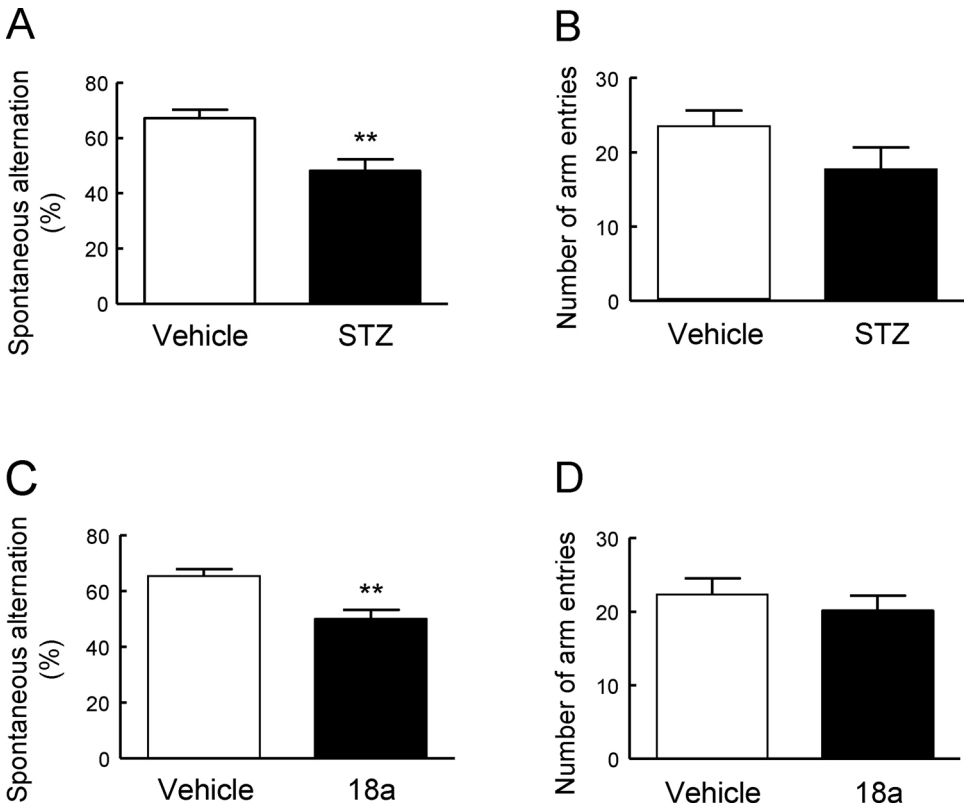

Fig. 3. Cognitive ability in the Y-maze test. STZ: (A) Spontaneous alternation (%) and (B) Number of arm entries. CX3CR1 antagonist 18a: (C) Spontaneous alternation (%) and (D) Number of arm entries. Results are expressed as mean ± SE for 9 to 11 mice. **p < 0.01 vs. vehicle.

increased the CX3CR1 mRNA expression in primary microglia cultures to 136 % of that in the control group (Fig. 4I).

3.5. Effects of the administration of DEX and selective IGF-1 receptor tyrosine kinase inhibitor PPP on mRNA expression of fractalkine and CX3CR1 in the mouse hippocampus

In the normal mice hippocampus, subcutaneous administration of DEX in doses of 30 and 100 μg/day for 7 days using a micro-osmotic pump significantly decreased fractalkine mRNA expression to 80 % and 73 %, respectively, of that in the saline-treated group, and significantly decreased CX3CR1 mRNA expression in a dose of 100 μg/day to 68 % of that in the saline-treated group (Fig. 5A and B).

Intraperitoneal injection of PPP (20 mg/kg body weight) in normal mice significantly decreased mRNA expression of fractalkine and CX3CR1 in the hippocampus to 77 % and 72 %, respectively, of that in vehicle-treated mice (Fig. 5C and D).

4. Discussion

In the present study, STZ-treated mice exhibited significant learning and memory impairment in the Y-maze test compared with vehicle-treated mice, and, moreover, a significant reduction in fractalkine and CX3CR1 mRNA levels in the hippocampus. Furthermore, intracerebroventricular injection of the CX3CR1 antagonist 18a in normal mice induced significant cognitive deficits in the Y-maze test. STZ-treated mice had high levels of plasma corticosterone, and low levels of plasma and hippocampal IGF-1 compared with vehicle-treated mice. Furthermore, DEX significantly decreased fractalkine mRNA expression in primary neuron and astrocyte cultures, and CX3CR1 mRNA expression in primary microglia cultures. On the other hand, IGF-1 significantly increased the fractalkine mRNA expression in primary neuron cultures and the CX3CR1 mRNA expression in primary microglia cultures. Moreover, subcutaneous administration of DEX significantly reduced the fractalkine and CX3CR1 mRNA expression in the hippocampus. Intraperitoneal administration of the IGF-1 receptor tyrosine kinase inhibitor PPP significantly decreased the fractalkine and CX3CR1 mRNA expression in the hippocampus. These findings suggest that the cognitive deficits exhibited by STZ-treated mice are, at least in part, due to impaired fractalkine-CX3CR1 signaling in the hippocampus induced by an increase in plasma corticosterone levels and a decrease in plasma and hippocampal IGF-1 levels.

In the hippocampus, fractalkine expression is predominantly restricted to glutamatergic pyramidal neurons in the CA1-CA3 and granule neurons in the dentate gyrus which are well-known to be potently involved in the processing of learning and memory (Nishiyori et al., 1998). Activation of CX3CR1 on microglia in the hippocampal CA1 region triggers the release of adenosine that in turn, via the activation of adenosine receptor type A2, increases the release of D-serine as a coagonist for the N-methyl-D-aspartate (NMDA) glutamate receptor subtype from glia, thereby potentiating NMDA function (Scianni et al., 2013). LTP, defined as an activity-dependent, prolonged enhancement of synaptic strength, in the hippocampal CA1 region is predominantly regulated by glutamate receptors, such as NMDA and α-amino-3--hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and is considered to be a form of synaptic plasticity for the cellular mechanisms of learning and memory in the hippocampus (Neves et al., 2008). These findings suggest that fractalkine-CX3CR1 signaling plays an important role in learning and memory processes in association with enhanced LTP (Rogers et al., 2011; Sheridan et al., 2014). In the present study, administration of a CX3CR1 antagonist significantly impaired learning and memory in the Y maze test. Supporting our result, Rogers et al. reported that male CX3CR1-deficient mice showed cognitive impairment in fear conditioning and Morris water maze test (Rogers et al., 2011). In Morris water maze test, CX3CR1-deficient mice had a significant decrease in the number of target platform crossing during the

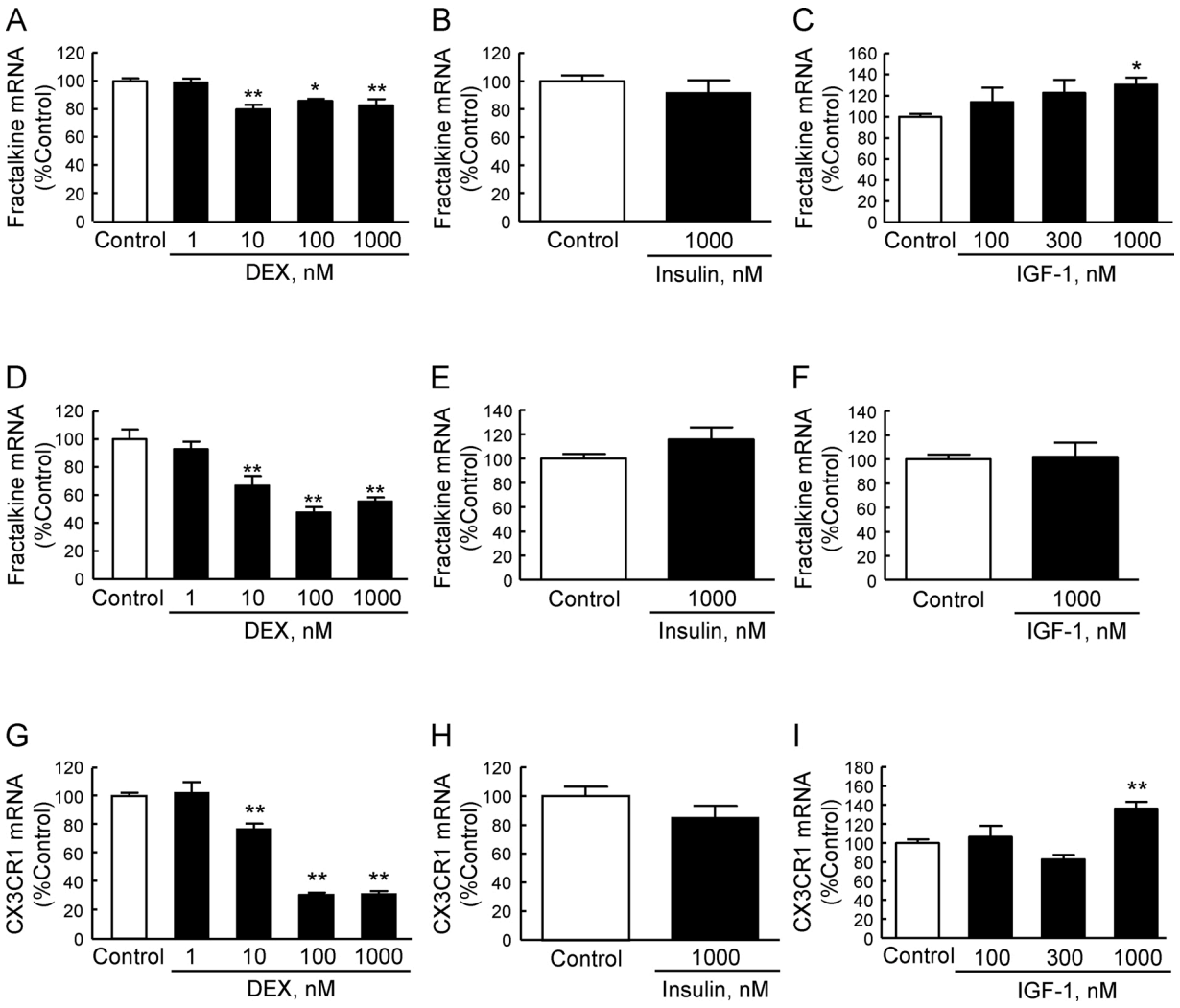

Fig. 4. Effects of the application of DEX, insulin and IGF-1 on mRNA expression of fractalkine in the primary neuron and astrocyte cultures, and mRNA expression of CX3CR1 in the primary microglia cultures. Fractalkine mRNA expression in the primary neuron cultures: (A) DEX, (B) insulin, (C) IGF-1. Fractalkine mRNA expression in the primary astrocyte cultures: (D) DEX, (E) insulin, (F) IGF-1. CX3CR1 mRNA expression in the primary microglia cultures: (G) DEX, (H) insulin, (I) IGF-1. Results are expressed as mean ± SE for 6 to 20 samples. *$p < 0.05$, **$p < 0.01$ vs. control.

probe trial (Day 11) compared to wild-type mice, indicating cognitive impairment in CX3CR1-deficient mice (Rogers et al., 2011). On the other hand, Maggi et al. reported that female CX3CR1-deficient mice learned the water maze task faster than wild-type mice because CX3CR1-deficient mice significantly spent in the acquisition quadrant longer than wild-type mice during probe trial (Day 4) (Maggi et al., 2011). However, on probe trial (Day 6), spent time in the acquisition quadrant was not different between two groups (Maggi et al., 2011). We consider that the difference in these results may be due, in part, to the differences in experimental schedule and gender. Moreover, Rogers et al. reported that male CX3CR1-deficient mice showed significantly reduced hippocampal-dependent LTP compared to wild-type mice (Rogers et al., 2011). However, Maggi et al. reported that CX3CR1-deficient mice showed an increase in AMPA receptor-mediated LTP but not NMDA receptor-mediated LTP (Maggi et al., 2011). On the other hand, another report of Maggi showed that fractalkine enhanced hippocampal NMDA receptor-dependent LTP in mice (Scianni et al., 2013). In this context, the same laboratory demonstrated different results which might be due to the different experimental methods. As compared with these two studies on CX3CR1-deficient mice, our experiment was performed using CX3CR1 antagonist. The big difference between Rogers/Maggi studies and ours is permanent intervention and acute intervention, respectively. Taken together, these findings provide compelling evidence that neuron-microglia interactions, especially those underpinned by fractalkine-CX3CR1 signaling, play several crucial roles in modulating hippocampal-dependent learning and memory by maintaining proper homeostasis of synaptic transmission in the brain.

DM is a common serious metabolic disorder characterized by hyperglycemia resulting from defective insulin activity. Diabetes may lead to secondary complications in several organ systems, including the brain (Biessels et al., 1994, 2008). A growing number of studies on brain function have revealed moderate impairment of cognitive function is recognized as a complication of type 1 DM (Neves et al., 2008). Initially, deficient insulin actions may be considered to contribute to the cognitive impairment observed in DM because insulin positively regulates cognitive processing, and impaired insulin activity in the brain leads to impaired neuronal function and synaptogenesis (Kleinridders et al., 2014). The multifactorial pathogenesis of brain dysfunction, such as cognitive impairment in DM, however, is not yet completely understood. STZ-treated mice, a widely used model of type 1 DM with hypoinsulinemia, hyperglycemia and reduced body weight, exhibit cognitive deficits in association with impaired LTP in the hippocampal CA1 region (Biessels et al., 1996; Kamal et al., 1999, 2000; Molteni et al., 2002; Wu et al., 2004). At least part of the learning and synaptic plasticity deficits in STZ-treated rats may be a direct consequence of disturbances at the level of the NMDA and AMPA receptor complexes in the hippocampus

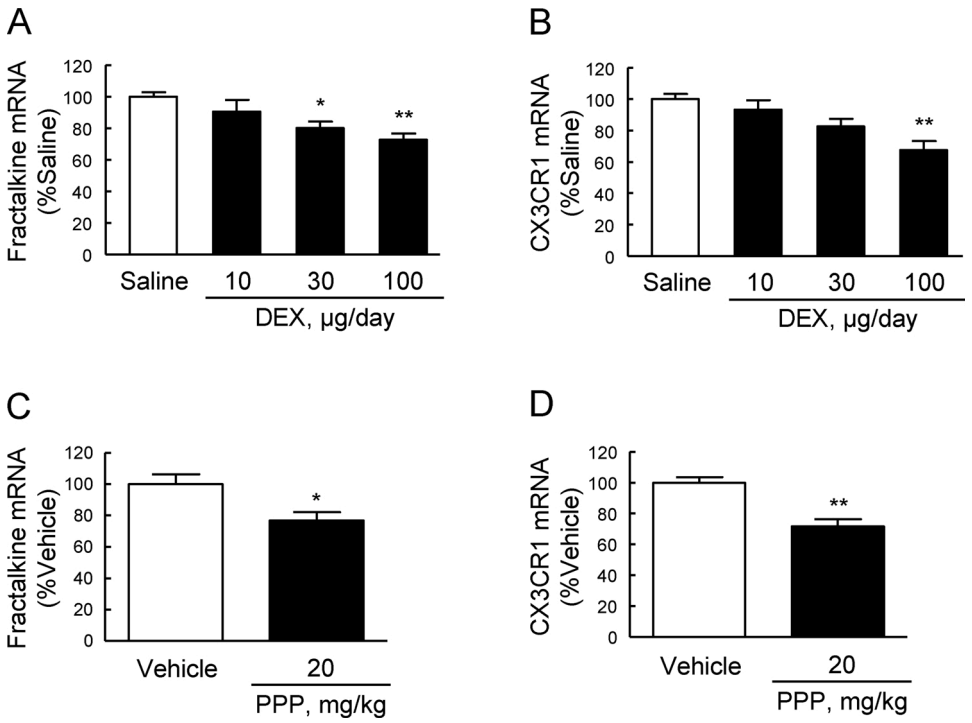

Fig. 5. Effects of the administration of DEX and IGF-1 receptor tyrosine kinase inhibitor PPP on mRNA expression of fractalkine and CX3CR1 in mouse hippocampus. DEX: (A) Fractalkine mRNA expression, (B) CX3CR1 mRNA expression. PPP: (C) Fractalkine mRNA expression, (D) CX3CR1 mRNA expression. Results are expressed as mean ± SE for 6 to 9 samples. *$p < 0.05$, **$p < 0.01$ vs. saline or vehicle.

(Sasaki-Hamada et al., 2012). Concretely, the NMDA receptor NR2B subunits and the AMPA receptor GluR1 subunits are significantly decreased in the hippocampus of STZ-treated animals (Gardoni et al., 2002; Viswaprakash et al., 2015). Based on previous findings suggesting the participation of fractalkine-CX3CR1 signaling in learning and memory processes, we addressed whether fractalkine-CX3CR1 signaling in the brain is involved in cognitive deficits in STZ-treated mice. The present study demonstrated that STZ-treated mice exhibited significant decrease in fractalkine-CX3CR1 signaling in the hippocampus accompanied by cognitive deficits. On the basis of these findings, the impaired learning and memory in STZ-treated mice is, in part, attributed to reduced fractalkine-CX3CR1 signaling in the hippocampus.

To elucidate the mechanisms underlying the decreased expression of fractalkine and CX3CR1 in the hippocampus of STZ-treated mice, we examined the effects of factors observed to be significantly changed in the plasma and hippocampus of STZ-treated mice in the present study, such as corticosterone, insulin and IGF-1, on the mRNA expression of fractalkine and CX3CR1. Type 1 DM is associated with significantly higher plasma cortisol and adrenocorticotrophic hormone levels compared with normal controls (Chan et al., 2002). DEX which is a synthetic glucocorticoid receptor agonist significantly suppresses increases in the protein levels and mRNA expression of fractalkine induced by tumor necrosis factor-α and interferon-γ in a human lung epithelial adenocarcinoma cell line (Bhavsar et al., 2008). Moreover, application of DEX reduces CX3CR1 mRNA expression in human peripheral blood mononuclear cells (Pachot et al., 2008). Consistent with these findings, the present study showed that plasma corticosterone levels were significantly increased in STZ-treated mice. Moreover, cell culture studies showed that DEX significantly reduced fractalkine-CX3CR1 signaling. In the present study, subcutaneous administration of DEX in normal mice significantly reduced the mRNA expression of fractalkine and CX3CR1 in the hippocampus. These findings suggest that high plasma corticosterone levels in STZ-treated mice contribute to reduce fractalkine and CX3CR1 expression in the brain. Recent observations revealed that circulating and brain IGF-1, which acts as trophic factor, modulates brain activities such as neuroprotection, neurogenesis and neuronal excitability (Jones and Clemmons, 1995). Serum IGF-1-deficient mice exhibit both cognitive decline and impaired hippocampal LTP (Trejo et al., 2007). Serum and brain IGF-1 levels are reduced in STZ-treated rats (Olchovsky et al., 1990), and systemic administration of IGF-1 prevents cognitive impairment in STZ-treated rats (Lupien et al., 2003). These findings provide substantial evidence that diabetic patients may have diminished brain IGF-1 signaling as well as insulin signaling. The present study demonstrated that plasma and hippocampal IGF-1 levels were significantly decreased in STZ-treated mice. Moreover, IGF-1 significantly increased mRNA expression of fractalkine in primary neuron cultures and of CX3CR1 in primary microglia cultures. In contrast, intraperitoneal administration of the IGF-1 receptor tyrosine kinase inhibitor PPP significantly decreased mRNA expression of fractalkine and CX3CR1 in the hippocampus. These findings indicate that impaired fractalkine-CX3CR1 signaling in the hippocampus in STZ-treated mice is, in part, attributed to a decrease in plasma and hippocampal IGF-1 levels. However, it is unclear mechanism how corticosterone and IGF-1 change the expression of fractalkine and CX3CR1.

Our findings revealed that STZ treatment induces a significant decrease in fractalkine-CX3CR1 signaling in the hippocampus, which in turn, may result in cognitive impairment. Moreover, reduced fractalkine-CX3CR1 signaling seems to be induced by an increase in plasma corticosterone levels and a decrease in plasma and hippocampal IGF-1 levels. Thus, interactions between neurons and microglia regulated by fractalkine and CX3CR1 appear to be involved in the cognitive deficiency associated with type1 DM.

Author contributions

N.K. and G.K. performed experiments, contributed to discussions, and wrote the manuscript. E.N. performed experiments. NY.G., A.I. and A.A. contributed to discussions, and reviewed and edited the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Namiko Kawamura: Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Goro Katsuura:** Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Supervision. **Nobuko Yamada-Goto:** Investigation. **Ela Novianti:** Investigation. **Akio Inui:** Resources, Writing - review & editing, Funding acquisition. **Akihiro Asakawa:** Resources, Writing - review & editing.

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Impaired brain fractalkine-CX3CR1 signaling is implicated in cognitive dysfunction in diet-induced obese mice

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ABSTRACT

Introduction A diet high in saturated fat is well known to affect neuronal function and contribute to cognitive decline in experimental animals and humans. Fractalkine released from neurons acts on its receptor, CX3C chemokine receptor 1 (CX3CR1), in the microglia to regulate several brain functions. The present study addressed whether fractalkine-CX3CR1 signaling in the brain, especially the hippocampus, contributes to the cognitive deficits observed in diet-induced obese (DIO) mice.

Research design and methods Mice were given 60% high-fat diet for 16 weeks. The expression of fractalkine and CX3CR1 in the hippocampus, amygdala and prefrontal cortex of DIO mice was analyzed. Cognitive ability in the Y-maze test and hippocampal glutamate receptors and synaptic markers were observed in DIO and CX3CR1 antagonist-treated mice. Regulation of fractalkine and CX3CR1 expression in the hippocampus was examined following administration of a selective insulin-like growth factor-1 (IGF-1) receptor inhibitor and a tyrosine receptor kinase B (TrkB) antagonist in normal mice.

Results DIO mice exhibited significant cognitive deficits in the Y-maze test and decrease in fractalkine and CX3CR1 in the hippocampus and amygdala compared with mice fed a control diet (CD mice). Administration of the CX3CR1 antagonist 18a in normal mice induced significant cognitive deficits in the Y-maze test. DIO mice and CX3CR1 antagonist-treated mice exhibited significant decreases in protein levels of NMDA (N-methyl-D-aspartate) receptor subunit (NR2A), AMPA (α-amino-5-methyl-3-hydroxy-4-isoxazole propionate) receptor subunit (GluR1) and postsynaptic density protein 95 in the hippocampus compared with their respective controls. Furthermore, plasma IGF-1 and hippocampal brain-derived neurotrophic factor were significantly decreased in DIO mice compared with CD mice. Administration of a selective IGF-1 receptor inhibitor and a TrkB antagonist in normal mice significantly decreased fractalkine and CX3CR1 in the hippocampus.

Conclusions These findings indicate that the cognitive decline observed in DIO mice is due, in part, to reduced fractalkine-CX3CR1 signaling in the corticolimbic system.

Significance of this study

What is already known about this subject?

- Epidemiological studies indicate that the incidence of cognitive impairment is higher in obese subjects than in those with normal body weight.
- Diet-induced obese (DIO) animals exhibit cognitive deficits in a variety of behavioral tests.
- Fractalkine released from neurons acts on its receptor, CX3C chemokine receptor 1 (CX3CR1), in the microglia to regulate several brain functions.
- CX3CR1 deficiency induces impairment of hippocampal cognitive function and synaptic plasticity.

What are the new findings?

- DIO mice exhibited significant decrease in fractalkine and CX3CR1 in the hippocampus and amygdala, as well as significant cognitive deficits in the Y-maze test, compared with mice fed a control diet (CD mice).
- Administration of the CX3CR1 antagonist 18a in normal mice induced significant cognitive deficits in the Y-maze test.
- DIO mice and CX3CR1 antagonist-treated mice exhibited significant decreases in protein levels of NMDA (N-methyl-D-aspartate receptor) subunit (NR2A), AMPA (α-amino-5-methyl-3-hydroxy-4-isoxazole propionate) receptor subunit (GluR1) and postsynaptic density protein 95 in the hippocampus compared with their respective controls.
- Plasma insulin-like growth factor-1 (IGF-1) and hippocampal brain-derived neurotrophic factor were significantly decreased in DIO mice compared with CD mice.
- Administration of a selective IGF-1 receptor inhibitor and a tyrosine receptor kinase B (TrkB) antagonist in normal mice significantly decreased fractalkine and CX3CR1 in the hippocampus.

How might these results change the focus of research or clinical practice?

- These findings provide compelling evidence that neuron–microglia interactions, especially those underpinned by fractalkine-CX3CR1 signaling, play crucial roles in brain dysfunction associated with obesity.

INTRODUCTION

Obesity is associated with a higher risk of lifestyle-related cardiovascular and metabolic disorders, such as hypertension, diabetes, and hyperlipidemia. Epidemiological studies indicate that the incidence of cognitive impairment is higher in obese subjects than in those with

normal body weight.[1 2] Diet-induced obese (DIO) animals exhibit impaired cognition in a variety of behavioral tests, such as the Morris water maze and a spontaneous alternation paradigm in T-maze and Y-maze tests.[3–6] Consistent with these findings, our previous study demonstrated that DIO mice fed a high-fat diet (HFD) exhibit significant impairments in hippocampus-dependent and amygdala-dependent fear-conditioning responses and have low levels of hippocampal brain-derived neurotrophic factor (BDNF), which plays a critical role in synaptic plasticity, long-term potentiation (LTP) and learning and memory processes.[7] Furthermore, electrophysiological studies indicate that LTP in the hippocampal CA1 region, which is closely related to memory formation and is predominately regulated by the glutamatergic system, especially N-methyl-D-aspartate (NMDA) and α-amino-5-methyl-3-hydroxy-4-isoxazole propionate (AMPA) receptors,[8] is markedly impaired in DIO animals compared with lean animals.[9 10] The cellular and molecular events of the synaptic plasticity involved in learning and memory processes are modulated by neurotrophic factors including BDNF and insulin-like growth factor-1 (IGF-1),[11–13] which regulate the presynaptic and postsynaptic machinery associated with synaptic plasticity.[6 9 14]

Communication between neurons and microglia is crucial for the optimal regulation of central nervous system (CNS) activities. In this regard, fractalkine secreted from neurons was recently reported to bind to CX3C chemokine receptor 1 (CX3CR1), which is expressed mainly in the microglia,[15–17] and to regulate several functions of the CNS.[18] In the mouse brain, fractalkine messenger RNA (mRNA) levels are high in the cortex, hippocampus, and striatum; intermediate in the olfactory bulb, thalamus, hypothalamus and brainstem; and low in the cerebellum.[15 16] Therefore, fractalkine-CX3CR1 signaling is postulated to represent a primary neuron–glia inter-regulatory system that is important for brain function.[18] Recent evidence indicates that fractalkine-CX3CR1 signaling plays an important role in regulating LTP formation in the hippocampus and behavioral learning and memory processes.[19 20]

To provide a new insight into the understanding of cognitive decline that occurs in obesity, we assessed the possible contribution of impaired fractalkine-CX3CR1 signaling in the corticolimbic system, which is an important system for cognitive processing, to the cognitive deficits observed in DIO mice. In addition, to investigate the underlying mechanisms, we examined the expression of glutamate receptor subunits and synaptic components specific to synaptic plasticity. Our findings revealed that decreased fractalkine-CX3CR1 signaling in the corticolimbic system contributes to the impaired cognition observed in DIO mice in association with decreased expression of glutamate receptor subunits and synaptic components.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (6 weeks old) were obtained from CLEA Japan (Tokyo, Japan) and housed in plastic cages under a 12:12-hour light:dark cycle (lights turned on at 07:00) at room temperature (23°C±1°C). The animals had ad libitum access to water and food (CE-2; CLEA Japan). Every effort was made to optimize the comfort of the animals and to minimize their use.

DIO mice

Normal mice (6 weeks old) were randomly divided into two groups. The first group was given CE-2 as a control diet (CD), with fat accounting for 12.6% of the total calories (343.1 kcal/100 g). The second group was given HFD (No. D12492; Research Diets, New Brunswick, New Jersey), with fat accounting for 60% of the total calories, predominantly in the form of lard (524 kcal/100 g). After feeding on the diets for 16 weeks, we evaluated them in the Y-maze test and measured their body weight before the mice were sacrificed to collect blood samples, fat tissues and brain regions under isoflurane anesthesia.

Analysis of metabolic parameters and sampling of the brain regions

Blood samples were collected from the retro-orbital vein under isoflurane anesthesia and immediately transferred to tubes containing EDTA (10 µL of 0.2 M EDTA/tube) and aprotinin (0.1 mg/tube; Merck KGaA, Darmstadt, Germany). The blood samples were centrifuged 3000× *g* for 5 min at 4°C, and plasma was separated and stored at −80°C until assayed. After blood collection, the mice were killed by decapitation. The brain was rapidly removed from the skull and placed on an ice-cooled paraffin plate for dissection of the hippocampus, amygdala and prefrontal cortex (PFC) by referring to the mouse brain atlas as previously described.[21] The brain regions were immediately frozen in liquid nitrogen and stored at −80°C until analyzed. The epididymal and mesenteric fat tissues were collected and weighed. Glucose (Glucose C2; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), insulin (Morinaga Ultra Sensitive Mouse/Rat Insulin ELISA Kit; Morinaga Institute of Biological Science, Yokohama, Japan), leptin (Mouse Leptin ELISA; Linco Research, St Charles, Missouri), IGF-1 (Mouse/Rat IGF-1 Quantikine ELISA Kit; R&D Systems, Minneapolis, Minnesota) and BDNF (Mouse/Rat BDNF Emax ImmunoAssay System; Promega Corporation, Madison, Wisconsin) were measured using commercially available kits.

Y-maze test

Cognitive ability was assessed using the Y-maze test, which is generally used to evaluate learning and memory ability in experimental animals, such as mouse models of obesity, Alzheimer's disease, and cerebral ischemia.[6 22 23] This task is based on the observation that if a mouse remembers the arm that has been explored most recently, a mouse will

next enter an arm of the maze that has not been visited yet or the most remotely visited arm.[24] The Y-maze apparatus (Muromachi, Tokyo, Japan) comprises three gray plastic arms (each one 41.5 cm long, 10 cm high, and 4 cm wide) that emanate from the center of the maze, are separated by 120°, and labeled as A, B, and C. Briefly, each mouse was placed at the end of one arm and allowed to move freely for 8 min session without any stimulation.[25] The sequence of arm entries was recorded manually, with an entry defined as all four limbs of the mouse within an arm. After completing the test trial for each mouse, the maze was cleaned with 70% ethyl alcohol to remove the mouse odor. The outcomes included the percentage of spontaneous alternations and the number of arm entries. The recorded spontaneous alternation behavior was used to assess hippocampal-dependent spatial memory. The percentage of spontaneous alternations was calculated as the ratio of arm entries that differed from the previous two arm entries (actual alternations) to the total possible alternations (defined as the total entries minus one) and multiplied by 100.

Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA levels were measured by quantitative real-time RT-PCR.[26] All gene-specific mRNA expression values were normalized against the internal housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for GAPDH were as follows: (sense TGCACCACCAACTGCTTAGC, antisense GGATGCAG GGATGATGTTCTG), for fractalkine (sense ACGAAATG CGAAATCATGTGC, antisense CTGTGTCGTCTCCAG-GACAA), for CX3CR1 (sense CGTGAGACTGGGT-GAGTGAC, antisense AAGGAGGTGGACATGGTGAG), for NR1 (sense AAGCCCAACGCCATACAGAT, antisense AGGCGGGTGACTAACTAGGA), for NR2A (sense TCACTGAGGAAGGCTATC, antisense CCCACTTGC-CCACCTTTT), for NR2B (sense AGAGTCGACGAG CTGAAGATGAAGCCCAGC, antisense CGGGGAAC TACTGAGAGATGATGGAAGTCA), for GluR1 (sense GGAGTGGAAGTCCCTAGCACACA, antisense CCTG GGAGTGGCTGCATAAGA), for GluR2 (sense ATGG AACATTAGACTCTGGCTCCAC, antisense CTGCCGTA GTCCTCACAAACACA), for synaptophysin (sense CCACCTCCTTCTCCAATCAG, antisense CAGCAAAGA-CAGGGTCTCCT), and for postsynaptic density protein 95 (PSD-95) (sense CGAGGAGCCGTGGCAGCC, antisense CATGGCTGTGGGGTAGTCAGTGCC).

Western blotting analysis

The hippocampus was homogenized in 500 µL of cold RIPA buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS) (pH 7.5), supplemented with 1 mM leupeptin, 1 µg/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride) as described previously.[27] The membrane was incubated overnight in Tris-buffered saline with 0.1% Tween 20, supplemented with 5% skim milk powder at 4°C with the primary antibody as follows: rabbit polyclonal anti-CX3CL1 (fractalkine) antibody (1:1000, ab25088; abcam, Cambridge, UK) which recognizes the soluble form of fractalkine, mouse monoclonal anti-CX3CR1 antibody (1:250, ab184678; abcam), rabbit monoclonal anti-NMDA receptor 1 (GluN1) (D65B7) antibody (1:1000, #5704; Cell Signaling Technology, Danvers, Massachusetts), rabbit polyclonal anti-NMDA receptor 2A (GluN2A) antibody (1:1000, #4205; Cell Signaling Technology), rabbit monoclonal anti-NMDA receptor 2B (GluN2B) (D8E10) antibody (1:1000, #14544; Cell Signaling Technology), rabbit polyclonal anti-glutamate receptor 1 (AMPA subtype) antibody (1:1000, ab31232; abcam), rabbit monoclonal anti-AMPA receptor 2 (GluA2) (E1L8U) antibody (1:1000, #13607; Cell Signaling Technology), rabbit monoclonal anti-synaptophysin (D8F6H) XP antibody (1:1000, #36406; Cell Signaling Technology), rabbit monoclonal anti-PSD-95 (D27E11) XP antibody (1;1000, #3450; Cell Signaling Technology) and mouse monoclonal anti-GAPDH (1:2000, sc-32233; Santa Cruz Biotechnology, Dallas, Texas) antibody. Next, the membrane was incubated for 1 hour at room temperature with either anti-rabbit IgG antibody (NA934; GE HealthCare UK, Buckinghamshire, UK) conjugated to horseradish peroxidase or anti-mouse IgG antibody (NA931; GE HealthCare UK) conjugated to horseradish peroxidase at a 1:1000 dilution, followed by detection using the ECL (GE HealthCare UK). The LAS-1000 image analyzer (V.4.0; FUJIFILM Corporation, Tokyo, Japan) was used for detection and quantification. To ensure equivalent amounts of loaded proteins and quantify targeted protein expression, the ratio of the targeted protein level to the GAPDH level was determined.

Intracerebroventricular administration of the CX3CR1 antagonist 18a

A highly selective antagonist for CX3CR1, 18a (Axon Medchem, Groningen, The Netherlands), with a Ki value of 3.9 nM, was dissolved in dimethyl sulfoxide (DMSO), and diluted in saline to a final concentration of 0.1% DMSO.[28] The CX3CR1 antagonist 18a (50 ng/mouse) was intracerebroventricularly administrated at 1 hour before the Y-maze test according to our previous report.[27] Mice in the control group were given intracerebroventricular injections of an equal volume of vehicle (0.1% DMSO in saline, 2 µL/mouse). In another experiment, mice were sacrificed at 1 hour after the administration to collect brain regions under isoflurane anesthesia.

Chronic intraperitoneal administration of picropodophyllin and ANA-12

The selective IGF-1 receptor inhibitor picropodophyllin (PPP, 20 mg/kg; Santa Cruz Biotechnology)[29] and the tyrosine receptor kinase B (TrkB) selective and non-competitive antagonist ANA-12 (0.5 mg/kg; Merck KGaA)[30] were dissolved in DMSO and diluted with saline at a final concentration of 50% DMSO. PPP and ANA-12 were intraperitoneally injected at 10:00 once a day for 7 days. Mice in the control group were given an intraperitoneal injection of an equal volume of vehicle (50% DMSO in saline, 10 mL/kg body weight). Mice were sacrificed

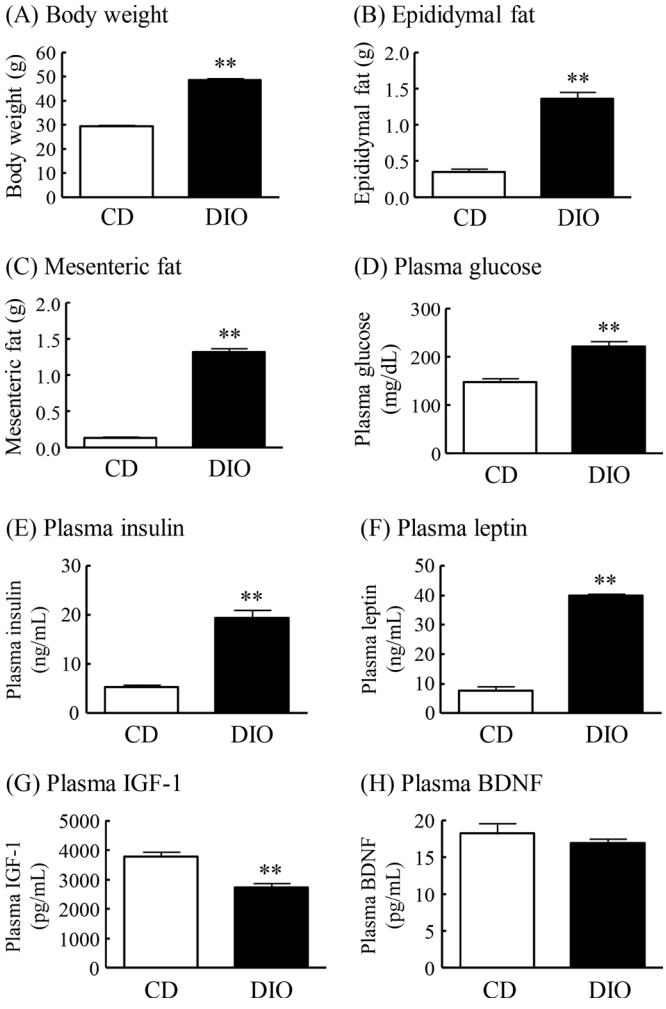

Figure 1 Changes in body weight, fat mass and plasma levels of glucose, insulin, leptin, IGF-1 and BDNF in DIO mice. (A) Body weight, (B) epididymal fat, (C) mesenteric fat, (D) plasma glucose levels, (E) plasma insulin levels, (F) plasma leptin levels, (G) plasma IGF-1 levels, and (H) plasma BDNF levels. Results are expressed as mean±SE for 5–17 mice. **P<0.01 versus CD. BDNF, brain-derived neurotrophic factor; CD, control diet; DIO, diet-induced obese; IGF-1, insulin-like growth factor-1.

at 1 hour after the last administration to collect brain regions under isoflurane anesthesia.

Data analysis

Data are expressed as mean±SEM. Statistical analysis of the data was performed by analysis of variance followed by the Tukey-Kramer test. Statistical significance was defined as $p<0.05$.

RESULTS

Changes in body weight, fat mass, and plasma levels of glucose, insulin, leptin, IGF-1 and BDNF in DIO mice

DIO mice had markedly greater body weight and epididymal and mesenteric fat mass than normal mice fed CD (CD mice) (figure 1A–C). Plasma levels of glucose, insulin and leptin were significantly increased in DIO mice compared with CD mice (figure 1D–F). Plasma IGF-1 levels in DIO mice were significantly decreased to 73% of that in CD mice, and plasma BDNF levels did not differ significantly between DIO mice and CD mice (figure 1G,H).

Changes in expression of fractalkine, CX3CR1, IGF-1 and BDNF in the brain of DIO mice

To explore the possible involvement of fractalkine-CX3CR1 signaling in the cognitive deficits observed in DIO mice, we examined changes in fractalkine and CX3CR1 expression in the hippocampus, amygdala and PFC of DIO mice compared with CD mice. Moreover, we examined the hippocampal IGF-1 and BDNF levels, which are well known to play a pivotal role in learning and memory.[11–13] The mRNA expression of fractalkine was significantly decreased in the hippocampus of DIO mice to 79% of that in CD mice (figure 2A). The protein levels of soluble form of fractalkine were significantly decreased in the hippocampus of DIO mice to 85% of those in CD mice (figure 2B). The mRNA expression and protein levels of CX3CR1 were significantly decreased in the hippocampus of DIO mice to 75% and 56%, respectively, of those in CD mice (figure 2C,D). In addition, protein levels of BDNF, but not IGF-1, in the hippocampus of DIO mice were significantly decreased to 78% of that in CD mice (figure 2E,F). The mRNA expression of fractalkine and CX3CR1 was significantly decreased in the amygdala of DIO mice to 86% and 79%, respectively, of that in CD mice (figure 2G). On the other hand, the mRNA expression of fractalkine and CX3CR1 in the PFC was not changed in DIO mice (figure 2H).

Cognitive ability in the Y-maze test and changes in mRNA expression and protein levels of NMDA and AMPA receptor subunits, synaptophysin and PSD-95 in the hippocampus in DIO mice

Spontaneous alternation in the Y-maze test was significantly reduced in DIO mice to 76% of that in CD mice (figure 3A). The number of arm entries was not significantly different between DIO mice and CD mice (figure 3B).

To elucidate the possible mechanisms underlying cognitive impairment in DIO mice, we examined the hippocampal expression of NMDA receptor subunits, AMPA receptor subunits, synaptophysin and PSD-95. The mRNA expression of NR2A, but not NR1 and NR2B, of NMDA receptor subunits in the hippocampus of DIO mice was significantly decreased to 68% of the levels in CD mice (figure 3C,E,G). The protein levels of NR1 and NR2A in the hippocampus of DIO mice were significantly decreased to 62% and 58%, respectively, of the levels in CD mice (figure 3D,F). The protein levels of NR2B tended to be decreased to 85% of the levels in CD mice (figure 3H). The mRNA expression and protein levels of GluR1, but not GluR2, of AMPA receptor subunits in the hippocampus of DIO mice were significantly decreased to 81% and 74%, respectively, of the levels in CD mice (figure 3I–L). The protein levels, but not mRNA expression, of synaptophysin and PSD-95 in the hippocampus

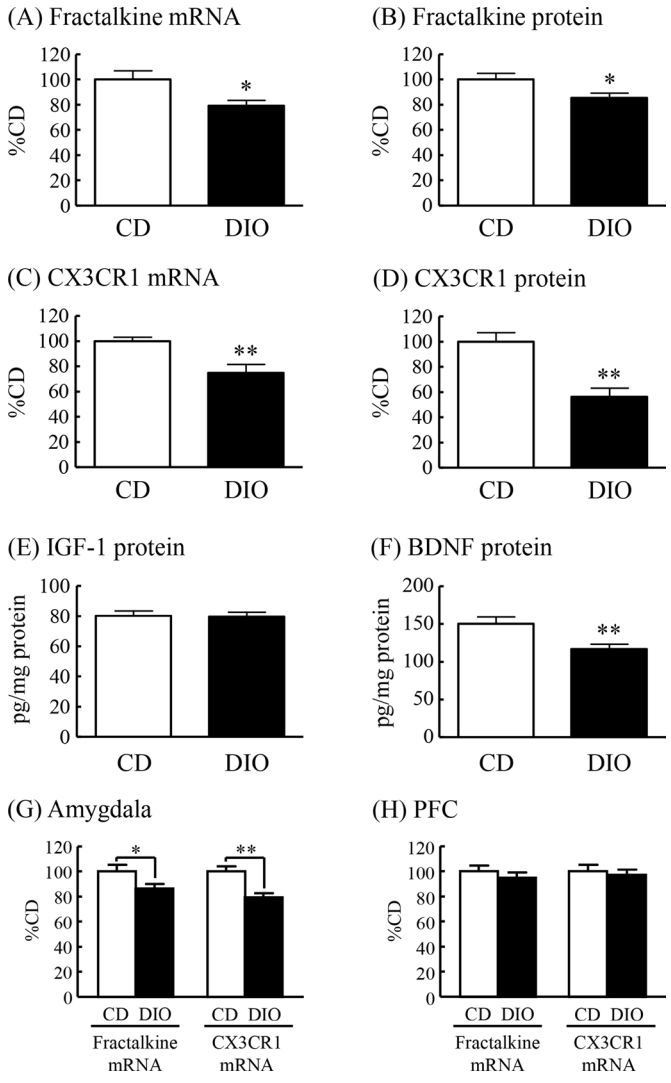

Figure 2 Changes in expression of fractalkine, CX3CR1, IGF-1 and BDNF in the brain of DIO mice. (A) Fractalkine mRNA expression, (B) fractalkine protein levels, (C) CX3CR1 mRNA expression, (D) CX3CR1 protein levels, (E) IGF-1 protein levels, and (F) BDNF protein levels in the hippocampus. (G) Fractalkine and CX3CR1 mRNA expression in the amygdala. (H) Fractalkine and CX3CR1 mRNA expression in the PFC. Results are expressed as mean±SE for 5–16 mice. *P<0.05, **P<0.01 versus CD. BDNF, brain-derived neurotrophic factor; CD, control diet; CX3CR1, CX3C chemokine receptor 1; DIO, diet-induced obese; IGF-1, insulin-like growth factor-1; mRNA, messenger RNA; PFC, prefrontal cortex.

of DIO mice were markedly decreased to 68% and 61%, respectively, of the levels in CD mice (figure 3M–P).

Effects of intracerebroventricular administration of the CX3CR1 antagonist 18a on cognitive ability in the Y-maze test and changes in mRNA expression and protein levels of NMDA and AMPA receptor subunits, synaptophysin and PSD-95 in the hippocampus in normal mice

To examine the possible contribution of decreased fractalkine-CX3CR1 signaling to the cognitive impairment observed in DIO mice, we examined the effect of the CX3CR1 antagonist 18a on learning and memory in the Y-maze test in normal mice. Spontaneous alternation in the Y-maze test was significantly reduced in mice with intracerebroventricular administration of 18a to 75% of that in vehicle-treated mice (figure 4A). The number of arm entries was not changed in either of the two groups (figure 4B).

To further evaluate the involvement of fractalkine-CX3CR1 signaling in cognitive deficits in DIO mice, the changes in the same factors observed in the hippocampus of DIO mice, as shown in figure 3, were examined in the hippocampus of mice treated with the CX3CR1 antagonist 18a. The mRNA expression of the NR1, but not NR2A and NR2B, in the hippocampus of the antagonist-treated mice was significantly decreased to 67% of that in vehicle-treated mice (figure 4C,E,G). The protein levels of NR2A, but not NR1 and NR2B, in the hippocampus of the antagonist-treated mice were significantly decreased to 53% of the levels in vehicle-treated mice (figure 4D,F,H). The mRNA expression and protein levels of the GluR1, but not GluR2, in the hippocampus of the antagonist-treated mice were significantly decreased to 70% and 62%, respectively, of the levels in vehicle-treated mice (figure 4I–L). The mRNA expression and protein levels of synaptophysin in the hippocampus were not changed by administration of 18a (figure 4M,N). The mRNA expression and protein levels of PSD-95 in the hippocampus of the antagonist-treated mice were significantly decreased to 77% and 66%, respectively, of the levels in vehicle-treated mice (figure 4O,P).

Regulatory effects of IGF-1 and BDNF on mRNA expression of fractalkine and CX3CR1 in the hippocampus of normal mice

Plasma IGF-1 levels and hippocampal BDNF levels were significantly lower in DIO mice compared with CD mice (figures 1G and 2F). We then examined whether IGF-1 and BDNF regulate the mRNA expression of fractalkine and CX3CR1 in the hippocampus of normal mice. Chronic intraperitoneal administration of the selective IGF-1 receptor inhibitor PPP induced a significant decrease in the mRNA expression of fractalkine and CX3CR1 in the hippocampus to 79% and 77%, respectively, of those in vehicle-treated mice (figure 5A,B). Chronic intraperitoneal administration of the TrkB selective and non-competitive antagonist ANA-12 also induced significant decrease in the mRNA expression of both fractalkine and CX3CR1 in the hippocampus to 83% and 75%, respectively, of those in vehicle-treated mice (figure 5C,D).

DISCUSSION

In the present study, DIO mice exhibited significant impairment in learning and memory in the Y-maze test compared with control mice, and a significant reduction in both fractalkine and CX3CR1 levels in the hippocampus and amygdala. Furthermore, intracerebroventricular administration of the CX3CR1 antagonist 18a in normal mice induced significant cognitive deficits in

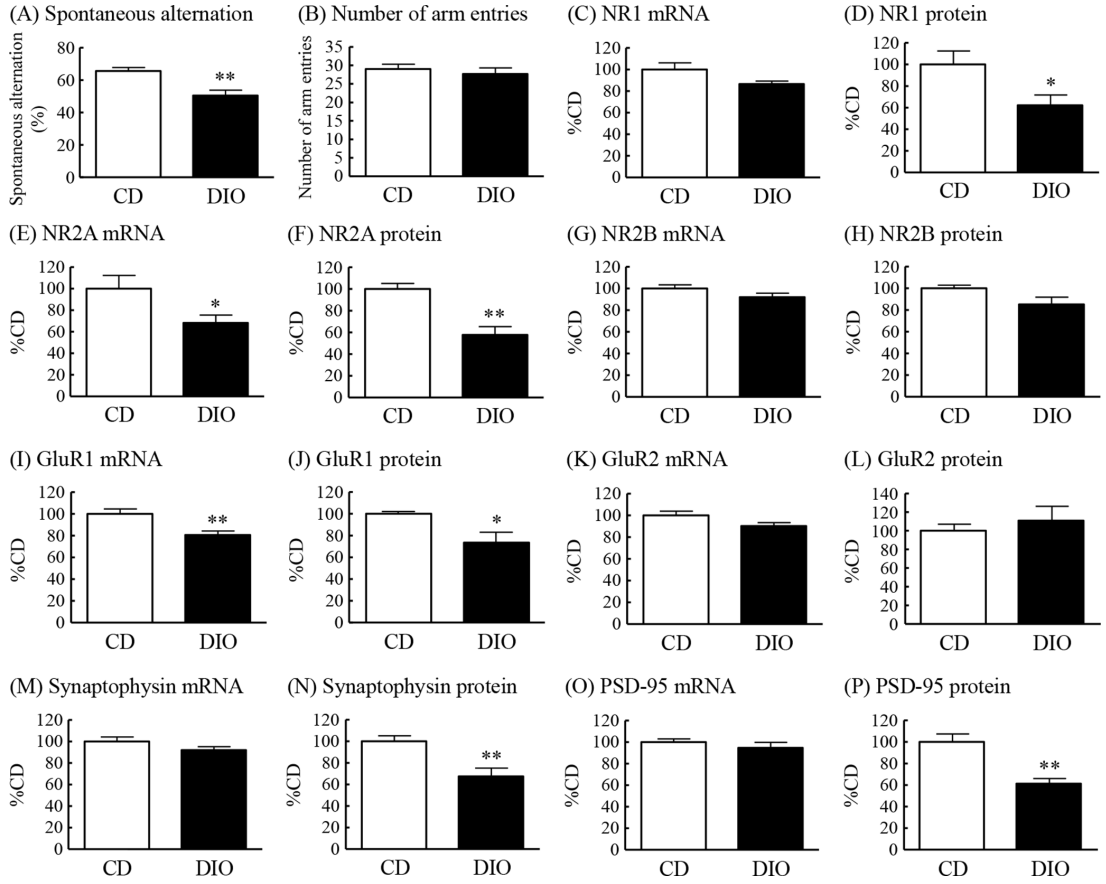

Figure 3 Cognitive ability in the Y-maze test and changes in mRNA expression and protein levels of NMDA and AMPA receptor subunits, synaptophysin and PSD-95 in the hippocampus in DIO mice. Y-maze test: (A) spontaneous alternation (%) and (B) number of arm entries. NR1: (C) mRNA expression and (D) protein levels. NR2A: (E) mRNA expression and (F) protein levels. NR2B: (G) mRNA expression and (H) protein levels. GluR1: (I) mRNA expression and (J) protein levels. GluR2: (K) mRNA expression and (L) protein levels. Synaptophysin: (M) mRNA expression and (N) protein levels. PSD-95: (O) mRNA expression and (P) protein levels. Results are expressed as mean±SE for 5–18 mice. *P<0.05, **P<0.01 versus CD. AMPA, α-amino-5-methyl-3-hydroxy-4-isoxazole propionate; CD, control diet; DIO, diet-induced obese; mRNA, messenger RNA; NMDA, N-methyl-D-aspartate; PSD-95, postsynaptic density protein 95.

the Y-maze test. Regarding the mechanism underlying the cognitive deficits in DIO mice, DIO mice exhibited a significant decrease in NR1 and NR2A of NMDA receptor subunits, GluR1 of AMPA receptor subunits, synaptophysin, and PSD-95 in the hippocampus. Moreover, CX3CR1 antagonist-treated mice exhibited a significant decrease in NR2A, GluR1 and PSD-95 in the hippocampus. Furthermore, plasma IGF-1 and hippocampal BDNF were significantly decreased in DIO mice compared with CD mice. Administration of the IGF-1 receptor inhibitor PPP and the TrkB antagonist ANA-12 significantly decreased mRNA expression of fractalkine and CX3CR1 in the hippocampus of normal mice. These findings suggest that the cognitive decline observed in DIO mice is due, at least in part, to reduced fractalkine-CX3CR1 signaling in the corticolimbic system.

A diet high in saturated fat is well known to affect neuronal function and contribute to cognitive decline in experimental animals and humans.[10 31–33] Obese animals fed over the long term with a diet high in saturated fat exhibit impaired acquisition and retention of spatial memory in the water maze test, and low levels of hippocampal BDNF to the extent that cognitive performance is compromised.[3 4 9 14] Our previous study demonstrated that DIO mice fed HFD exhibit significant attenuation of hippocampus-dependent and amygdala-dependent fear-conditioning responses and low levels of hippocampal BDNF.[7] The amygdala regulates hippocampal LTP, spatial memory and dentate gyrus field potentials.[34 35] Moreover, the PFC influences the responsiveness of the central amygdala output neurons.[36] These findings indicate that hippocampus, amygdala and PFC play an important role in learning and memory processes. Therefore, we examined changes in the expression of fractalkine and CX3CR1 in the hippocampus, amygdala and PFC of DIO mice. The present study provides the first evidence that DIO mice have significant decrease in fractalkine and CX3CR1 levels in the hippocampus and amygdala, and that the administration of a CX3CR1 antagonist significantly impairs learning and memory in the Y-maze test in normal mice, consistent with cognitive deficits in CX3CR1-deficient mice.[20] Electrophysiological studies demonstrated that LTP in the hippocampal CA1 region is markedly impaired in DIO rats compared with

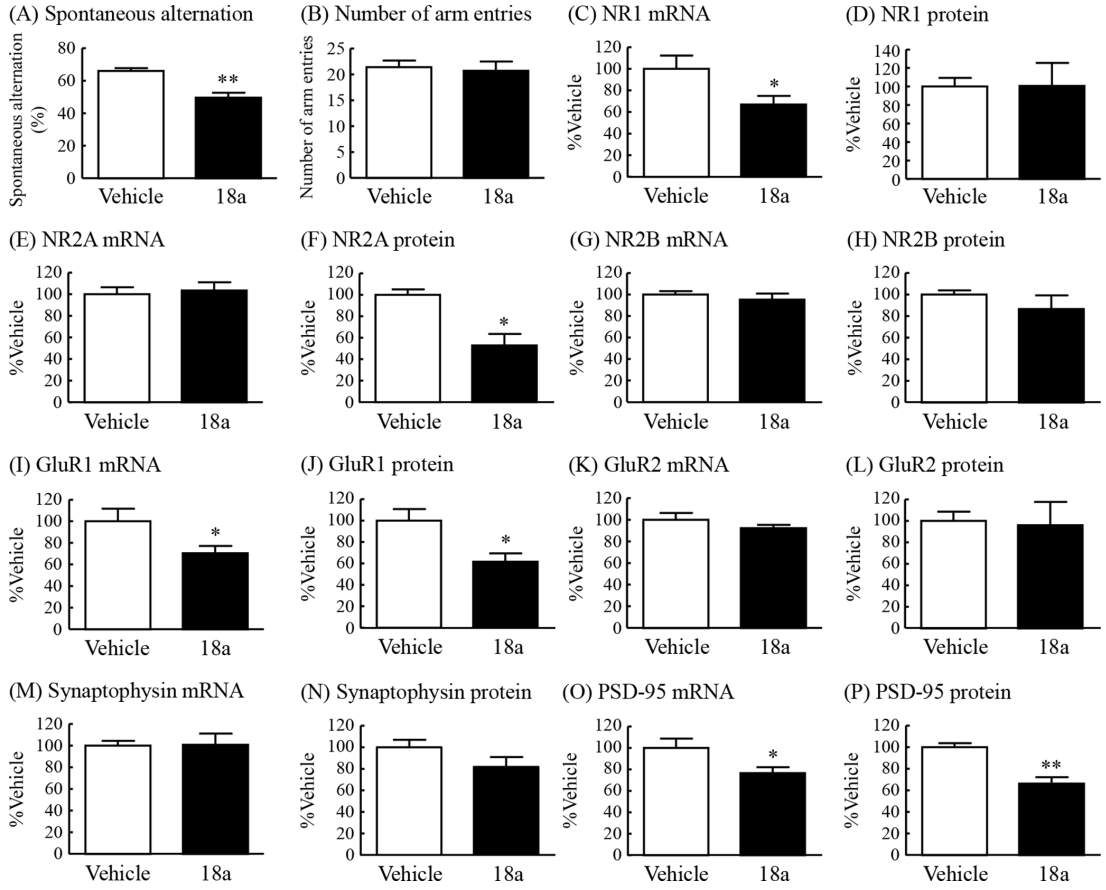

Figure 4 Effects of intracerebroventricular administration of the CX3CR1 antagonist 18A on cognitive ability in the Y-maze test and changes in mRNA expression and protein levels of NMDA and AMPA receptor subunits, synaptophysin and PSD-95 in the hippocampus in normal mice. Y-maze test: (A) spontaneous alternation (%) and (B) number of arm entries. NR1: (C) mRNA expression and (D) protein levels. NR2A: (E) mRNA expression and (F) protein levels. NR2B: (G) mRNA expression and (H) protein levels. GluR1: (I) mRNA expression and (J) protein levels. GluR2: (K) mRNA expression and (L) protein levels. Synaptophysin: (M) mRNA expression and (N) protein levels. PSD-95: (O) mRNA expression and (P) protein levels. Results are expressed as mean±SE for 3–7 mice. *P<0.05, **P<0.01 versus vehicle. AMPA, α-amino-5-methyl-3-hydroxy-4-isoxazole propionate; CX3CR1, CX3C chemokine receptor 1; mRNA, messenger RNA; NMDA, N-methyl-D-aspartate; PSD-95, postsynaptic density protein 95.

lean rats.[9 10] LTP is predominantly regulated by glutamate receptors, such as NMDA and AMPA receptors, and is considered to be a form of synaptic plasticity that underlies learning and memory in the hippocampus.[8] Activation of CX3CR1 on microglia within the CA1 region of the hippocampus triggers the release of adenosine that in turn, via the activation of type A2 adenosine receptors, increases the release of D-serine as a coagonist for NMDA receptors from the glia, leading to a potentiation of NMDA function, suggesting that fractalkine-CX3CR1 signaling may enhance LTP as well as learning and memory processes.[37] On the other hand, CX3CR1-deficient mice exhibit impaired LTP in the hippocampus.[20] These findings suggest that the cognitive impairment observed in DIO mice may be due to decreased fractalkine-CX3CR1 signaling in the hippocampus.

Recent observations demonstrated that circulating and brain IGF-1 modulates brain activities such as neuroprotection, neurogenesis and neuronal excitability.[13] Serum IGF-1-deficient mice exhibit both cognitive decline and impaired hippocampal LTP.[38] The present study demonstrated that plasma IGF-1 levels, but not hippocampal IGF-1 levels, were significantly decreased in DIO mice compared with CD mice. Moreover, the present study showed that the administration of the IGF-1 receptor inhibitor PPP significantly decreased mRNA expression of fractalkine and CX3CR1 in the hippocampus, providing different lines of evidence that bloodborne IGF-1 is a potent positive regulator for fractalkine-CX3CR1 signaling in the brain.

In the present study, BDNF levels in the hippocampus were significantly decreased in DIO mice compared with CD mice, consistent with previous reports.[3 4 7] In addition, the administration of the BDNF receptor TrkB antagonist ANA-12 significantly decreased fractalkine and CX3CR1 levels in the hippocampus in normal mice. The mRNA and protein expression of fractalkine and CX3CR1 is significantly decreased in the hippocampus of BDNF-deficit mice.[39] Together, these findings suggest that HFD induces a significant decrease in hippocampal BDNF

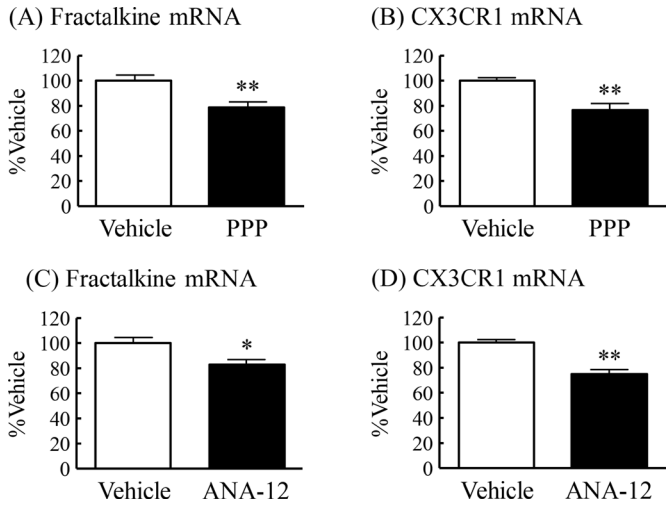

Figure 5 Regulatory effects of IGF-1 and BDNF on mRNA expression of fractalkine and CX3CR1 in the hippocampus of normal mice. PPP: (A) fractalkine mRNA expression and (B) CX3CR1 mRNA expression. ANA-12: (C) fractalkine mRNA expression and (D) CX3CR1 mRNA expression. Results are expressed as mean±SE for 12–13 mice. *P<0.05, **P<0.01 versus vehicle. BDNF, brain-derived neurotrophic factor; CX3CR1, CX3C chemokine receptor 1; IGF-1, insulin-like growth factor-1; mRNA, messenger RNA; PPP, picropodophyllin.

levels, which in turn decreases fractalkine and CX3CR1 levels in the hippocampus.

A recent study demonstrated that the detrimental effects of HFD consumption on learning and memory may be mediated in part by the alterations of glutamate receptors, such as NMDA and AMPA receptors.[3 4 40–42] HFD feeding decreases hippocampal NR2B of NMDA receptor subunits and GluR-1 of AMPA receptor subunits.[43 44] The present study also demonstrated a significant decrease in NR1, NR2A and GluR1 in the hippocampus of DIO mice. Moreover, the present study demonstrated that CX3CR1 antagonist-treated mice also exhibited a significant decrease in NR2A and GluR1 in the hippocampus coincident with significant cognitive deficits, similar to DIO mice. Presynaptic and postsynaptic machinery associated with synapse formation and activity is involved in learning and memory processes. Compared with control mice, DIO mice exhibit abnormal expression of factors involved in the synaptic plasticity, such as synaptosomal-associated protein 25 (SNAP-25) and synaptophysin I in the presynaptic site and PSD-95 in the postsynaptic site.[5 6 9 10 14 43] HFD-fed animals exhibit cognitive decline in a water maze test and step-through task, and a decrease in synapsin 1 and PSD-95 in the hippocampus and cerebral cortex.[3 14] Further, HFD-fed mice display impaired hippocampal-dependent memory in the Y-maze test, and reduced SNAP-25, PSD-95 and syntaxin-4, but not synaptophysin, in the hippocampus.[6] In the present study, DIO mice exhibited a significant decrease in synaptophysin and PSD-95 levels in the hippocampus compared with CD mice. Synaptophysin is presynaptic vesicle marker, and PSD-95 interacts with ion channels, membrane receptors, cytoskeletal components, and intracellular signaling molecules,[45 46] and increases AMPA receptor currents by selectively delivering GluR1-containing receptors to synapses, thus mimicking LTP.[47] These findings suggest that the synaptic dysfunction might be an important contributor to the hippocampal-dependent spatial memory impairment in DIO mice.[6] Furthermore, in the present study, the administration of a CX3CR1 antagonist induced a significant decrease in PSD-95 in the hippocampus of normal mice compared with vehicle-treated mice, the same changes observed in the hippocampus of DIO mice. Moreover, the same changes in the NMDA receptor subunits, AMPA receptor subunits, and synaptic markers were observed in both DIO mice and CX3CR1 antagonist-treated mice, indicating that HFD feeding induced impaired fractalkine-CX3CR1 signaling in the hippocampus, leading to synaptic deterioration and resulting in impaired cognition.

There is convincing evidence that enhanced inflammation in both the brain and peripheral organs is strongly involved in several dysfunctions associated with HFD feeding for a long time.[48] In this regard, fractalkine-CX3CR1 signaling is demonstrated to regulate the inflammatory responses of macrophage and microglia.[20 49 50] Especially, enhanced inflammation in both the brain and peripheral organs induced by HFD feeding has been reported to influence cognitive function.[20 31 49 50] Therefore, enhanced inflammation may be an another pivotal event to regulate processing of learning and memory in obesity.

The present study revealed that long-term HFD feeding in mice may induce impaired fractalkine-CX3CR1 signaling in the corticolimbic system, leading to defective synaptic plasticity and subsequently to cognitive impairment in DIO mice. Taken together, these findings provide compelling evidence that neuron–microglia interactions, especially those underpinned by fractalkine-CX3CR1 signaling, play crucial roles in brain dysfunction associated with obesity.

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Contributors NK, GK, and NY-G performed the experiments, contributed to discussions, and wrote the manuscript. EN performed the experiments. AI and AA contributed to discussions and reviewed and edited the manuscript. NK is the guarantor of this work and as such had full access to all the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval All experiments were performed in accordance with the guidelines established by the Institute of Laboratory Animal Science Research Support Center at Kagoshima University and approved by the Kagoshima University Institutional Animal Care and Use Committee (protocol nos. MD18079, MD18080 and MD20007) for the Care and Use of Laboratory Animals.

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