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Possible Roles of Leptin in the Early Stage of Prenatal Development of the Mouse Cerebellum

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Leptin is detected in sera and leptin receptors are expressed in the cerebellum of mouse embryos, suggesting roles of leptin in the cerebellar development. We here injected leptin into the lateral cerebral ventricle of leptin receptor-deficient *db/db* and wild-type mouse embryos and examined the effects on cerebellar development. Leptin was injected on embryonic day (E) 14, and the embryos were let to develop by *exo utero* method till E16 when the cerebellum was examined. Cerebellar volume, number of Math1-immunopositive progenitor cells of granule cells in the external germinal cell layer (EGL) did not differ significantly between *db/db* and wild-type. However, calbindin-positive Purkinje cells in the Purkinje cell layer (PCL) of *db/db* tended to be fewer in number and appeared less differentiated than those in wild-type. These results suggest that leptin may not have effect on EGL cells but may promote migration and/or differentiation of progenitors of Purkinje cells in PCL.

Key words: leptin, cerebellum, *db/db* mouse, embryo, external germinal cell layer, Purkinje cell layer

INTRODUCTION

Leptin is the obese (*ob*) gene product, which is secreted from adipocytes in adults (1, 2). Leptin reduces food intake and increases energy expenditure and therefore reduces body weight (1-4). Mice deficient in leptin (*ob/ob*) or the long-form of leptin receptor (LEPRb, Ob-Rb) (*db/db*) show obesity and diabetes mellitus (1, 5). Leptin has also been implicated in the brain function as well as in the immune and reproductive systems (reviewed in 6, 7). Moreover, leptin plays an important role in brain development. Volume, weight and DNA content of the brain were shown to be reduced in adult *ob/ob* and *db/db* mice compared to wild-type controls (7, 8) and these impairments in *ob/ob* mice are rescued by injection of leptin in juveniles, suggesting that leptin increased the total number of the cells in the brain (10, 11). Our previous study suggested that leptin maintains neural progenitors and is related to glial and neuronal development in mouse embryonic cerebral cortex (7, 12). While we also showed that leptin receptor is expressed in the embryonic cerebellar cortex (13), role of leptin in the cerebellar development remains unclear. In this study, therefore, we investigated the role of leptin in the development of the cerebellum, in particular at the early stage, by comparing the effect of exogenously-injected leptin on the cerebellum between *db/db* and wild-type embryos. We injected leptin into the cerebral ventricle of mouse embryos on embryonic day (E) 14 by *exo utero* system (14) and analyzed the effect on E16. During this period, progenitor cells of future granule cell are accumulated in the external germinal layer (EGL) (15), while Purkinje cells are migrated from their progenitors in the ventricular zone and produced in the Purkinje cell layer (PCL) (16). The present leptin treatment did not affect EGL cells but exhibited suggestive effects on the development of PCL cells.

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MATERIALS AND METHODS

Animals

BKS.Cg-m +/+ Lepr^{db} (*db*/+) mice were purchased from Clea Japan (Tokyo, Japan) and were mated from 5 pm to 8 am. We defined the day

when a vaginal plug was observed as E0.

We maintained these mice at 22-24°C under a 12-h light/ dark cycle at in the Department of Experimental Animals, Center for Integral Research in Science, Shimane University. Food and water were available *ad libitum*. All the present animal studies were approved by the Ethics Committee for Animal Experimentation of Shimane University, and the animals were handled according to the institutional guidelines.

Exo utero surgery and microinjection of leptin

Exo utero surgery was performed as described previously (14). Briefly, pregnant BKS.Cg-m +/+ Lepr^{db} (*db*/+) mice were anesthetized with pentobarbital (60 mg/kg) and the abdominal wall and uterine wall were incised, while the embryos were intact in the amniotic fluid and connected to the placenta. Then, 1 μl of leptin solution (200 ng/μl of leptin in PBS containing 1 mM sodium citrate, pH 7.4) was injected into the lateral ventricle of the embryos at E14 using a 40-μm-diameter micropipette. The uterus that remained open was put back into the abdomen and the abdominal wall was sutured, the pregnant mice were allowed to recover, and the embryos were let to develop *exo utero*. The embryonic brain, from forebrain to hindbrain, was obtained on E16. The embryonic brain was weighed, then it was fixed in 10% formalin solution containing 70% methanol at 4°C overnight, dehydrated with methanol, and embedded in paraffin. Coronal paraffin sections of the cerebellum were cut at a thickness of 5 μm.

The *db/db* (BKS.Cg-+Lepr^{db}/+Lepr^{db}) and wild-type (BKS.Cg-m+/m+) embryos from *db*/+ mothers were distinguished from *db*/+ embryos by the size of cDNA fragment of *Ob-Rb* mRNA in RT-PCR (5, data not shown).

Nissl staining

We stained every 30 sections of each sample of *db/db* and wild-type embryonic brain with 0.1% cresyl violet, and observed these sections to find differences between *db/db* and wild-type groups.

Volume of cerebellum

We measured areas of the cerebellum every 30 sections from the rostral to caudal end of the cerebellum, and the volume was calculated by multiplying areas with the length of cerebellum. Cerebellum length was calculated by multiplying the section number by the section thickness (5 μm).

Immunohistochemistry of the cerebellum

We performed immunohistochemistry with rabbit polyclonal antibody against Math1 (1:200, abcam, Japan) and anti-calbindin D-28K (1:500, Temecula, CA) antibody. Math1 is neural-specific and is expressed in the progenitor cells in the EGL for future granule neurons in the cerebellum (15). Anti-calbindin D28K antibody produces specific staining of cerebellar Purkinje cells, molecular layer dendrites and axonal fibers, and stains cell bodies and fibers in neuronal subpopulations (17). We did antigen retrieval using TE buffer for Math1 and citrate buffer for calbindin immunostaining, respectively. We incubated sections with 10% goat serum for 1 h at room temperature, and incubated them with either anti-Math1 or anti-calbindin antibody at 4°C overnight. The same section was then incubated with System HRP rabbit (DakoCytomation, Carpinteria, CA) for 1 h at room temperature. We used Liquid DAB Chromogen (DakoCytomation) for the chromogenic reaction.

Semiquantitative study on the cells in the cerebellar cortex

The numbers of cells in EGL and PCL in the cerebellum were counted blindly as described previously with random and systematic sampling (14, 18, 19). Coronal sections with a thickness of 5 μm were sampled from E16 wild-type and *db/db* embryos. Four or more embryos from two or more litters were used for the cell counting. We counted the number of Math1-positive in EGL and calbindin-positive cells in the cerebellum. Every 12th coronal sections for E16 cerebellum were chosen. Ten to thirteen sections were counted in each embryonic brain. We multiplied the cell number in the section by the distance between the sections and totaled the cell numbers in the defined volume.

Statistical analysis

Values are all shown mean±standard deviation. We used Student's *t*-test to analyze the ratio of the

number of cells in EGL and PCL to their volume in *db/db* and +/+ embryonic cerebellum; $p<0.05$ was considered significant.

RESULTS

Cerebellar volume and width

Neither the cerebellar volume nor the cerebellar volume-to-total brain weight ratio was significantly different between wild-type and *db/db* embryos on E16 (Table 1). We also previously reported that there was no significant difference in the width of the cerebellum between the *db/db* and wild-type embryos, either (20) (Table 1). Thus, there was no macroscopic change observed in the cerebellum by the exogenously administered leptin.

Table 1. Cerebellar volume and width

	Volume (mm^3)	Volume/ Total brain weight	Width (mm)
Wild-type (n=4)	0.82±0.92	16.73±2.61	3.40±0.09[§]
db/db (n=3)	0.73±0.93	14.94±1.30	3.39±0.19[§]
P	0.253	0.33	0.82

[§]Udagawa *et al*., 2006 (20)

Cell numbers in the external germinal layer

There were no significant differences in the number of Math1-positive progenitor cells of the granule cells (Fig. 1) in EGL between *db/db* and wild-type embryos on E16 (wild-type: 104070.00±11136.73 (n=4), *db/db*: 95776.0±13638.97 (n=3), P=0.414).

Purkinje cell numbers and phenotypes

There was a tendency that *db/db* embryos had fewer Purkinje cells that were calbindin-positive (Fig. 2) than wild-type embryos, although no significant difference was found between *db/db* and wild-type embryos in the number of calbindin-positive cells in PCL (wild-type 81765.0±9421.16 (n=4), *db/db* 62632.0±13136.58 (n=3), p=0.072). However, Purkinje cell density in *db/db* embryos seemed to be lower than in wild-type group (Fig. 3, data not shown) and Purkinje cells appeared to be less differentiated in *db/db* embryos than in wild-type embryos (Fig. 3). Purkinje cells were generally small and not layered in *db/db* embryos when compared with multi-layered large Purkinje cells in wild-type embryos.

DISCUSSION

Effects of leptin on general development of the cerebellum from E14 to E16

It has been reported that leptin deficiency caused reductions in brain weight, cortical volume, and the contents of total brain protein and DNA in juvenile and adult *ob/ob* mice (9, 11). These phenomena

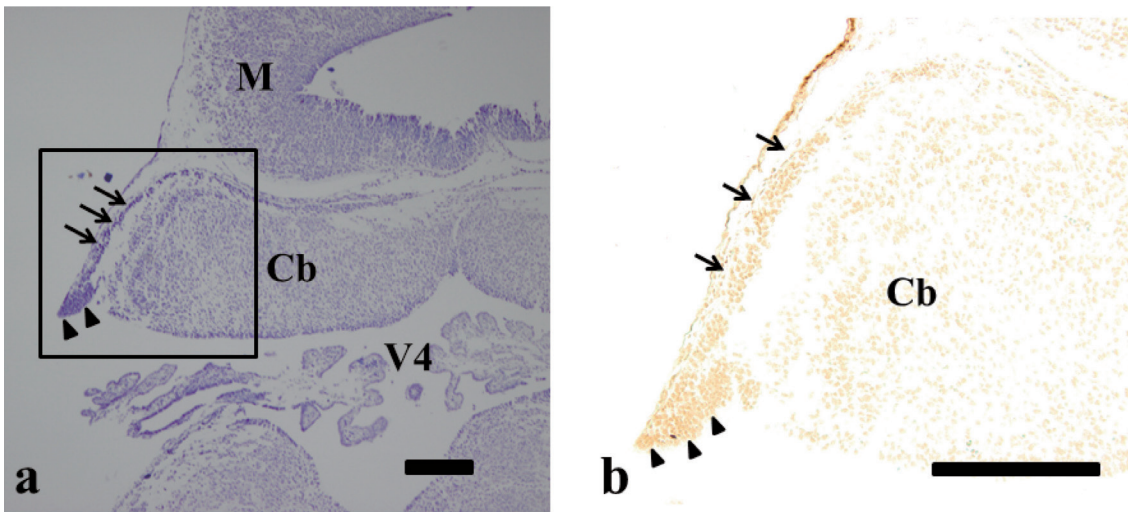

Fig. 1. Math1-positive cells in the cerebellum. (**a**) EGL (arrows) and the rhombic lip (arrow heads) are observed in E16 cerebellum stained by Nissl staining. The area in the next section with immunostaining, which corresponds to the boxed area, is magnified in **b**. (**b**) EGL (arrows) and the rhombic lip (arrow heads) strongly express Math1. Cb: cerebellum, M: Mesencephalon, V4: fourth ventricle. Scale bars: 200 μm.

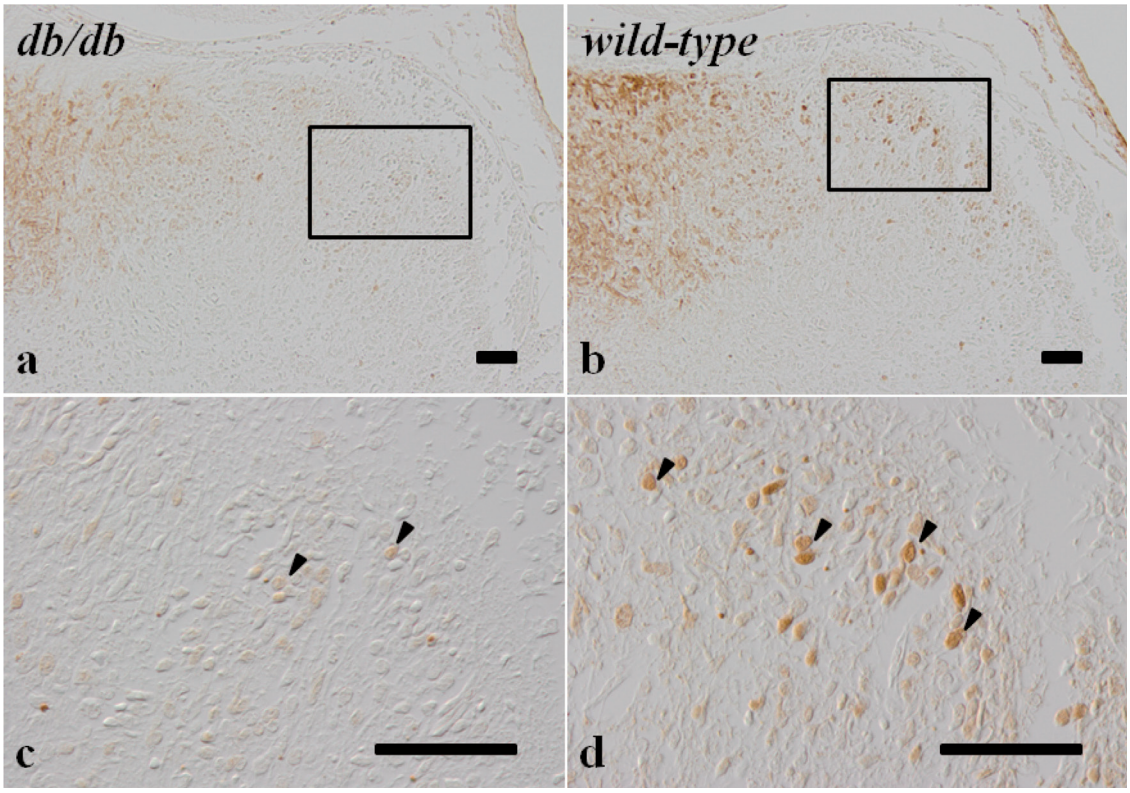

Fig. 2. Calbindin-positive cells in the cerebellum. Calbindin expression is weaker in *db/db* embryos (a) than in wild-type embryos (b) and the number of the Purkinje cells tends to be reduced in E16 *db/db* embryos. At higher magnification, smaller Purkinje cells, which are not layered, are observed in *db/db* embryos (c) than in wild-type embryos (d). Scale bars: 50 μm.

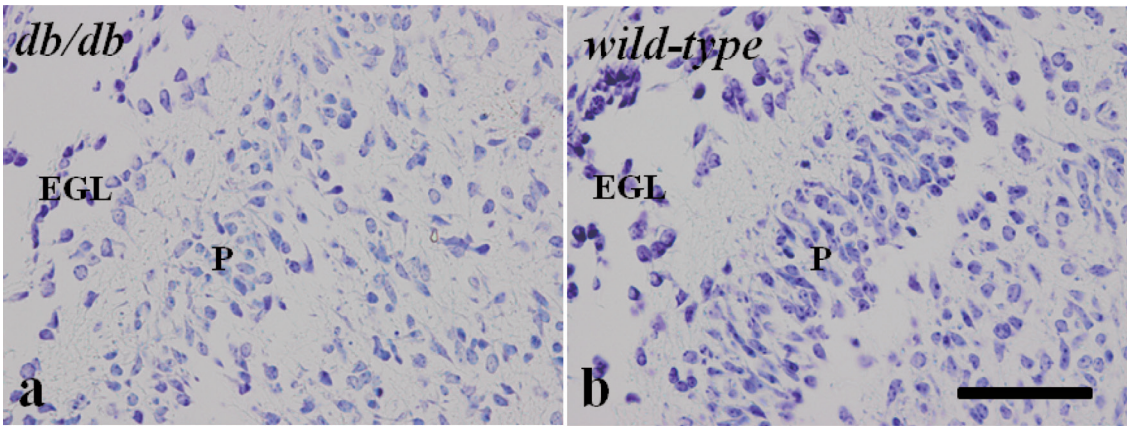

Fig. 3. Nissl staining of Purkinje cell layer. At E16, Purkinje cells are aligned and multilayered under EGL in wild-type (b), but not *db/db* (a). Purkinje cell layer (P) appears to be undifferentiated in the E16 *db/db* embryo. Scale bar: 50 μm.

may be related to signal transduction through Ob-Rb, because *db/db* mice, which lack Ob-Rb, have reduced brain weight and brain protein content compared with their lean counterparts (8). The similar abnormalities in *ob/ob* mice, which lack leptin, were rescued by leptin administration into the juvenile animals (9, 11). Recent studies reported that, also in humans, plasma leptin level positively correlated with gray matter volume in some brain regions including the cerebellum both in young and elder adults (21, 22). Leptin has been shown to have trophic effect on hypothalamic neurons that regu-

late feeding (23). We previously showed that leptin maintained neural stem and progenitor cells in the ventricular zone and promoted neuronal differentiation in the cerebral cortical plate in mouse embryos (12). We also showed that in the intermediate zone of the cingulate cortex, leptin deficiency caused the increase of pyknotic cells at E18 (24). Furthermore, in an *in vitro* study, leptin promoted survival of cultured cerebellar Purkinje cells, but not granule cells (25). Taken together, these reports strongly suggest that leptin is related to not only the cerebral but also cerebellar development.

In this study, we focused on the effects of leptin on the early stage of cerebellar development from E14 to E16, during which progenitor cells of granule cells are accumulated in EGL while newly produced Purkinje cells migrate from ventricular zone of the fourth ventricle to PCL (15, 16). Intracerebroventricular injection of 200 ng leptin on E14 did not make a significant difference on cerebellum volume between wild-type and *db/db* embryos on E16. This result suggests that leptin may not generally affect cells in the cerebellum at this stage of development, which is in contrast to its effects on the cerebral development during the same prenatal period (12), suggesting that roles of leptin in brain development is region-dependent.

Effect of leptin on cells of EGL and PCL

Two primary regions are thought to give rise to the neurons that make up the cerebellum. The first region is the ventricular zone in the roof of the fourth ventricle (16). This area produces Purkinje cells and deep cerebellar nuclear neurons. These cells are the primary output neurons of the cerebellar cortex and cerebellum, respectively. The second germinal zone (cellular birthplace) is known as the rhombic lip, from which neural progenitor cells move to EGL (15). This layer of progenitor cells produces granule neurons. The granule neurons migrate from this exterior layer to form an inner layer known as the internal granule layer, and the EGL ceases to exist in the mature cerebellum, leaving only differentiated granule cells in the internal granule layer. Leptin receptor mRNA is expressed both in PCL and EGL (13, 26).

We evaluated the effects of exogenously added leptin in wild-type and *db/db* embryos on the development of cells in EGL, which are derived from the rhombic lip between E13 and E15 (15). As the results showed, no significant difference was found between *db/db* and wild-type embryos in the numbers of Math1-positive cells that are considered as progenitor cells of granule cells in EGL (15). These results suggest that during the period between E14 and E16, leptin may have no effect on the development of external granule neural progenitor cells, which mostly differentiate and migrate inside to form the internal granule layer in the postnatal period, through activation of Ob-Rb (27, 28). This is consistent with the result of an *in vitro* study that leptin did not affect on cerebellar granule cells taken from the mouse cerebellum at postnatal day 5 (25). However, since leptin receptor is expressed in EGL cells, we cannot exclude the possibility that both generation of progenitor cells from the rhombic limb to EGL and apoptosis of progenitor cells were equally promoted or inhibited by leptin treatment, and thus the overall number of EGL cells did not change.

We also evaluated the effect of leptin in wild-type and *db/db* embryos on the development of Purkinje cells in PCL. The number of calbindin-positive Purkinje cells in PCL tended to be smaller in the leptin-injected *db/db* embryos than in leptin-injected wild-type embryos, although there was no statistically significant difference. In cultured Purkinje cells that were prepared from E19 cerebellum, leptin promoted their survival (25). In the present study, Purkinje cell density in *db/db* embryos seemed to be lower than in wild-type group and Purkinje cells appeared to be less mature in *db/db* embryos than in wild-type embryos. Small Purkinje cells, which were not aligned and multilayered, were found in *db/db* embryos when compared to Purkinje cells of wild-type embryos, suggesting Purkinje cells were less differentiated in *db/db* embryos. A previous study showed that leptin treatment of cultured Purkinje cells promoted both the outgrowth of neurites from Purkinje cells and increased complexity of the neurite arbor (25). Further, it has been shown that leptin promotes rapid alterations in dendritic morphology of hippocampal neurons (29), while it regulates growth cone morphology of

cortical neurons (30). The present findings together with results from these previous studies including the *in vitro* study on cerebellar neurons suggest that leptin may also *in vivo* promote the differentiation of Purkinje neurons through activation of the leptin receptor. Further detailed observation including other developmental periods and conditions need to be examined to confirm the present findings in the future research.

In summary, in the cerebellar development from E14 to E16, leptin appears to have no general effect and may have no effect on development of progenitor cells of future granule cells in EGL, but may have effects on the production from progenitors and/or differentiation of Purkinje cells in PCL.

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