Progesterone is a new therapeutic agent for neonatal hypoxic ischemic encephalopathy

（プロゲステロンは新生児低酸素性虚血性脳症に対する新規の治療薬になり得る）

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ABSTRACT

Hypoxic ischemic encephalopathy (HIE) is a general term of brain damages that lead to epilepsy, behavioral deficits, learning disorders and cerebral palsy. HIE is caused by hypoxic ischemic insult in the perinatal period, but an effective therapy is not establish ed except a restricted therapeutic hypothermia. Recently, numerous studies revealed progesterone (P_4) has neuroprotective effects and showed that neuroprotective effects were due to the action of allopregnanolone (Allo), which is a metabolite of P_4, to $GABA_A$ receptors. In this study the therapeutic effect for the brain disorder of P_4 was investigated using a rat HIE model, which was suffered by hypoxic ischemic insult on gestational day 18 by transient bilateral uterine artery ligation. After birth, P_4 (0.10 mg/day or 0.01 mg/day), medroxyprogesterone acetate (MPA, 0.12 mg/day), or Allo (0.10 mg/day) were administered to each group subcutaneously for 9 days from postnatal day 1 (P1). Brain damages were evaluated by the cerebral histological analyses and a rotarod test on P50. The rotarod test was analyzed by the Kaplan Mayer method, in which the latency to fall off the rotating rod was regarded as the survival duration. P_4 was examined not to have toxic nor additive effects by the pilot rotarod test. The impaired latency of the rotarod test in HIE models were restored almost completely by P_4 and Allo, but not MPA. In the histological analyses, brain lesions were recognized as an irregular of layer structure in the cerebral cortex, decreased neurons in the cerebral cortex and the CA1 region of hippocampus, decreased oligodendrocytes and thinning myelin sheaths in the corpus callosum. P_4 also restored these histological lesions on P50. During the early brain development, impaired oligodendrocytes were immediately restored by P_4, and then neurons were recovered. P_4 did not suppressed microglial functions. Collectively, our study indicates that P_4 can restore the brain damages of HIE via $GABA_A$ receptors on oligodendrocytes.

Introduction

Anoxic or hypoxic insults during the perinatal period cause neonatal hypoxic ischemic encephalopathy (HIE), which leads to secondary injuries, for example reperfusion injury, edema, increased intracranial pressure, impaired autoregulation and hemorrhage. These brain damages are considered an important cause of later neurodevelopmental impairments, such as epilepsy, behavioral deficits, learning disorders and cerebral palsy (CP). Recently, accumulative evidences also suggest that the HIE is associated with autism, attention-deficit/hyperactivity disorder (ADHD) (1). HIE is defined as a clinical syndrome of disturbed neurologic function in the newborn period. The risk factors of HIE are fetal growth restriction (FGR), severe preeclampsia, post-date, and so on. Low gestational age at birth is also strongly related to an increased risk of HIE. Currently no effective cure is available except the restricted therapeutic hypothermia. Progesterone has an important role in pregnant women. Progesterone maintains uterine quiescence during pregnancy, through an increasing of ZEB expression, which suppresses the expression of oxytocin receptors and connexin43 (2, 3). And parturition is associated with a decreasing in maternal circulating progesterone. But progesterone is elevated too high (almost 100 fold higher than non-pregnant) (4) for only maintaining pregnancy. So progesterone might have an unknown role during pregnancy. In these days progesterone has been shown to improve outcomes in animal models of lots of neurologic diseases, such as traumatic brain injury, ischemia, spinal cord injury, peripheral nerve injury, demyelinating disease, neuromuscular disorders, and seizures (5). So the preterm birth deprives the benefits of progesterone from infants. The current study aims to elucidate the curative effects of progesterone against HIE, and to accumulate information about the mechanism how to effect, which lead us to have effective therapeutic agents. So that we can protect lots of preterm infant from suffering by HIE and learning disabilities, autism, ADHD in childhood.

Results

Progesterone and allopregnanolone, not MPA, restored the impaired motor coordinateon of HIE.

We assessed motor coordination of the HIE model with or without agents by the rotarod test that was analyzed by the Kaplan-Meier method, in which the latency to fall is regarded as the survival duration.

The progesterone-treated HIE group and the vehicle-treated HIE group were compared with the sham group. The latency time of the vehicle-treated HIE group (n=9, median survival time [MST]=151 sec) was significantly shorter than the sham group (n=31, MST=not determined [ND]) and the progesterone-treated HIE group (n=9, MST=ND) ($p<0.05$) (Fig. 1A).

Furthermore, the low concentration of progesterone (0.01)-treated HIE group was compared with the progesterone (0.1)-treated HIE group and vehicle-treated HIE group. The survival time of progesterone (0.01)-treated group (n=13, MST=ND) was significantly longer than vehicle-treated group (n=12, MST=284.5) ($p<0.05$), was similar to that of progesterone (0.1)-treated group (n=11, MST=ND) (Fig.1B).

Allopregnanolone, a metabolite from progesterone, was examined. The allopregnanolone-treated HIE group (n=15, MST=ND) showed a similar effect to the progesterone-treated HIE group (n=11, MST=ND) and the significantly longer latency than the vehicle-treated HIE group (n=10, MST=293.5) ($p<0.05$) (Fig.1C).

MPA is a synthetic agonist for the classical intracellular progesterone receptor (iPR). The MPA-treated HIE group (n=5, MST=247 sec) showed the significantly shorter latency than the progesterone-treated HIE group (n=8, MST=ND) ($p<0.05$). And no significant difference was showed between the MPA-treated HIE group and the vehicle-treated HIE group (n=4, MST=211.5 sec). (Fig.1D).

Progesterone rescued HIE histologically.

Immunohistochemistry was performed with the anti-NeuN antibody (NeuN) for neurons and the anit-Olig2 antibody (Olig2) for oligodendrocytes.

The number of NeuN positive cells in the cortex or the CA1 were counted in four square fields (0.01 mm^2 /ea.) and the average was calculated.

In the cortex and the CA1, the number of NeuN positive cells in the

progesterone-treated HIE group increased more than that of the vehicle-treated HIE group, and was equivalent to the sham group (medians and quartiles was shown in the figure legend) (Fig. 2A-D). The pyramidal layer of the CA1 also thickened by progesterone treatment (Fig. 2E).

Moreover, oligodendrocytes, which are responsible for myelination, were assessed by Olig2. In the corpus callosum (CC), the number of Olig2 positive cells in the progesterone-treated HIE group increased more than that of the vehicle-treated HIE group, and was equivalent to the sham group (Fig. 2F, G).

The number and the character of microglia were not changed by progesterone.

Microglia, which are the resident macrophages in the central nervous system, were examined immunnohistochemically by the anti-Iba1 antibody (Iba1) (Fig. 3A).

Microglia of the CC was observed on P9. In terms of the number of microglia no difference was shown among the sham group (median=24.5, [1st quartile=21.5, 3rd quartile=30.5]), the vehicle-treated HIE group (21.5 [18.25, 24.75]), and the progesterone-treated HIE group (20.5 [17, 23.75]) (Fig. 3B). The fraction of ramified form was decreased in the vehicle-treated HIE group (9.6 [5.6, 15]) compared with the sham group (26.5 [16.94, 34.58]) significantly ($p<0.05$) (Fig. 3C). The progesterone-treated HIE group (13.87 [7.94, 21.08]) also had a similar tendency.

Progesterone altered temporal changes of the densities of neurons and oligodendrocytes.

On P0, 5, 9, 30 and 50, the number of NeuN positive cells in the cortex and the CA1, and that of Olig2 positive cells in the CC were observed. The neuron density of the vehicle-treated HIE group was lower than that of the sham group on every point, but the slope of the vehicle-treated HIE group was parallel to that of the sham group (Fig. 4A, B). The progesterone-treated HIE group had a shallow slope and then caught up to the sham group finally.

In the trend of oligodendrocytes, the progesterone-treated HIE group caught up to the sham group immediately (means and standard errors [SD] were shown in the figure legend) (Fig. 4C).

Electron microscopic analyses revealed progesterone rescued myelination.

Using the electron microscope, myelin sheaths were observed in the CC in the vehicle-treated HIE group, myelin sheaths shown in black layers around the axon were thinned (Fig. 5A). Axon diameters and myeline thickness were measured. In the progesterone-treated HIE group, axons were thicker and better myelinated than those in the vehicle-treated HIE group, and similar to those in the sham group (medians and quartiles were shown in the figure legend) (Fig. 5B, C).

Discussion

This present study reveals that the early administration of progesterone restored brain damages, caused by HIE, histologically and behaviorally. This means progesterone could be a potent therapeutic agent for HIE and a preventive agent for epilepsy, intellectual disability, mental health issues (autism and ADHD).

Numerous models are developed for researches of hypoxic ischemic insult on brain, for example, unilateral common carotid artery ligation with hypoxia (Vannucci model) (6), bilateral carotid artery occlusion (BCAO) (7), 4-vessel occlusion (8), middle cerebral occlusion (MCAO) (9), chronic hypoxia (10) and cerebrocortical photothrombosis (11). Here we utilized modified bilateral uterine artery ligation (mBUAL) (12) as a model of neonatal HIE. In this model, rats were suffered from systemic hypoxic ischemic insults prenatally, which mimics human perinatal brain injury by placental insufficiency or/and intrapartum asphyxia. Moreover full term rat pups is similar to human preterm infants in terms of brain development, for example, neurulation, neuronal proliferation and migration, organization and myelination (13). The degree of damage can be controlled by duration of occlusion, so suffered animals can be utilized for examinations until adulthood. Thus, this model is the best for investigation of neonatal HIE, testing and screening of putative neuroprotective compounds. By the mBUAL method with 30min occlusion, the litter size of the HIE group rats were reduced almost half of the sham group (Supplementary Fig. S1A). Body weight at birth was also reduced about 15% in the HIE group rats (Supplementary Fig. S1B). In this study, the mBUAL method attenuated the amount of neuron in the cortex and hippocampus, and oligodendrocyte in the corpus callosum pathologically, also impaired sensorimotor function in 50-day rats. Our mBUAL model shows mild to moderate HIE in terms of pathology and behavior.

There are no effective therapies for HIE. The therapeutic hypothermia is applied to neonates with moderate to severe HIE. But only 1 in 6 infants benefits from the therapeutic hypothermia. Recently lots of

compounds are evaluated as a neuroprotective agents, for example, osteopontin, interferon, melatonin, erythropoetin, xenon and magnesium Sulfate (14). And some of them have gone into clinical trials as a therapeutic medicine. Progesterone is also widely recognized as a neuroprotective steroid hormone, i.e. neurosteroid. This neuroprotective efficiency have been demonstrated in different experimental models, for example, traumatic brain injury, spinal cord injury, ischemic stroke (15). Recently 2 phase III clinical trials of progesterone for traumatic brain injury (TBI), (i.e. PROTECT and SYNAPSE), which resulted in negative outcomes, have been reported (16, 17). However, these reports cannot deny the neuroprotective efficacies of progesterone, because these clinical trials were not performed in sophisticated ways (18), for example, too high a drug dose and too short a period of administration. In our study, about 20 mg/kg/d or 2 mg/kg/d of progesterone was administrated subcutaneously for 9 days in a gradually decreasing manner (one dose of progesterone was administrated everyday despite increasing body weight, 5 g to 20 g). These amounts are correspond to 5 mg or 0.5 mg/kg/d in human (18). And the model in this study is very young and for HIE, that is much different from above-mentioned clinical trials, that are for various aged people and for TIB. So the usage of progesterone in this study is thought to be proper (19).

We assessed motor coordination of the HIE model with or without agents by the rotarod test and analyzed by the Kaplan Meier method, in which the latency to fall is regarded as the survival duration. In the pilot study, in which the rotarod test was performed without acclimatization, we made sure that BUAL models had impaired motor coordination and progesterone had no toxicity nor additive effect (Supplementaly Fig. S2B). The rotarod test revealed that 0.1mg and 0.01mg of progesterone restored the motor coordination (Fig.1 A,B). As mentioned above, 0.1 mg/body/day is correspond to 20 mg/kg/day on P1 and 5 mg/kg/day on P9. These amounts are corresponds to 5 and 1.25 mg/kg/day, less than phase III clinical trials (18). Not only neurological behavior but also pathological abnormalities were restored by progesterone (Fig. 2-5). Progesterone has important roles in not only reproduction but also central nerve system. In the cell levels,

progesterone and its metabolites have various functions, 1: inhibition of proliferation, 2: regulation of hormone production, 3: inactivation of neuron, 4: inactivation of T cell, and so on. To elucidate the key pathway of the restoration, several related compounds, MPA, which is an agonist for classical intracellular progesterone receptor (iPR), and allopregnanolone, which is a metabolite from progesterone and couples to $GABA_A$ receptor, are used in the assessment of motor function. MPA did not restored the motor function, but allopregnanolone restored it (Fig.1 C,D). This means the restoration of motor function by progesterone might be exerted by other than the classical iPR. This phenomenon is inconsistent with R. Hussain's work, in which they revealed that the classical iPR was required for remyelination by progesterone (20). But Ishihara *et al.*said that neuroprotective effect of progesterone was exerted via $GABA_A$ receptors by allopregnanolone (21). This discrepancy might be caused by experimental conditions, for example, animal age, in vitro vs. in vivo, histologically vs. pharmacologically, and so on. In this study allopregnanolone was shown to be a potent restoration agent as well as progesterone.

To clarify the target of progesterone, immunohistochemistry was performed. The II-III layer of the cerebral cortex and the CA1 subfield of hippocampus are said to be susceptible to ischemia (22, 23, 24). Progesterone restored neuros in the cortex and the hippocampus, and oligodendrocytes in the corpus callosum which are diminished by mBUAL. Not only population but also layer structure and linage were restored (Fig. 2). This suggests that progesterone might protects cell migration as well.

Microglia are the resident macrophages in the central nervous system. The amoeboid form Microglia, which are of mesodermal origin, enter the brain during early development. In the mature brain, they transform into a branched, ramified form (also called the resting microglia). After a pathological event, these cells transform into amoeboid form, which have the capacity to migrate, proliferate and phagocyte (25, 26). The population of microglia in the HIE group was not changed regardless of progesterone treatments (Fig. 3A, B). The fraction of resting form, ramified microglia, was increased in the sham group more than the HIE group (significantly in the

vehicle-treated group, and tendency in the progesterone-treated group). Thus, to restore brains from HIE, progesterone did not suppress the immune system which was activated by inflammations triggered by hypoxic ischemic stress (27).

We examined the recovery time courses of neuron and oligodendrocyte. During brain development the density of neuron was decreased in the cortex and the CA1 (Fig. 4A,B). Interestingly, the downslope of decreasing density of both the sham and vehicle-treated group have similar tendency and were parallel. But the slope of progesterone-treated group was shallow. And moreover, progesterone enhances generation of new oligodendrocytes from progenitor cells immediately (Fig. 4C). These things make us to speculate progesterone repaired oligodendrocyte first, then restored oligodendrocyte rescued neurons.

We examined myelination in the corpus callosum by electron microscope (Fig. 5). As well as immunohistochemistry, myelin sheath was recovered by progesterone treatment. And the axon diameter also thickened. This phenomenon is similar to the epidermal growth factor treated preterm brain injury models in which motor functional disorder and cellular damage were rescued though recovering of oligodendrocytes (28).

Recently, in a mouse model of chronic hypoxia, stimulation of $GABA_A$ receptors on oligodendrocyte precursors regulate their differentiation and proliferation to prevent dysmyelination and disruption of cerebellar development (29). And it is well known allopregnanolone is an active modulator of $GABA_A$ receptor. Moreover, the hypoxic ischemic stress by the umbilical cord occlusion increased allopregnanolone levels in the fetal sheep brain (30).

Taken together, this study showed early administration of progesterone restored neonatal HIE by stimulating $GABA_A$ receptors on oligodendrocytes. Then repaired oligodendrocytes rescued neurons. And this study leads progesterone to be a preventive agent for autism and ADHD which are caused by perinatal HIE (1, 31).

Methods

Animals

All animals procedures were approved by the Institutional Animal Care and Use Committee of Graduate school of Medicine and School of Medicine, Chiba University. Nulliparous Wister rats (CLEA Japan, Inc., Tokyo, Japan) aged from 10 weeks to 18 weeks were maintained at the same center and housed individually in cages with free access to water and laboratory chow. They were housed in acrylic cages under light-dark (12h/12h), room temperature (24.5±2 degrees Celsius) and humidity (50% ± 10%).

Hypoxic ischemic encephalopathy (HIE) model of rat

We used rat pups affected with hypoxic ischemic encephalopathy (HIE), which were produced by the transient uterine artery clipping of a pregnant Wister rat (modified BUAL) (12, 32, 33, 34).

Pregnant rats on gestational day (GD) 18 of their 22-day gestation were anesthetized with ketamine hydrochloride (Ketalar (R), Daiichi Sankyo Co. Ltd., Tokyo, Japan) (75 mg/kg) and xylazine hydrochloride (Selactar (R), Byer Yakuhin, Ltd., Osaka, Japan) (10 mg/kg) via intraperitoneal injection. A midline laparotomy was performed, and the uterine horns were externalized. The four uterine arteries were exposed and pinched by an aneurysm clips (KN-353 Cat. No. AM-1, Natsume Seisakusho Co., Ltd.,Tokyo, Japan). For sham groups, the uterine horns were just externalized without clipping. After 30 min, the aneurysm clips were removed, and then the uterine horns were replaced. Rats were awakened from anesthesia, monitored to ensure adequate recovery, and returned to the animal facility.

Pups, which were spontaneously born on around GD22, were defined as neonatal HIE models.

Drug administration

Progesterone (Progehormon (R), Mochida Pharmaceutical Co., ltd, Tokyo, Japan) was dissolved in the sesame oil (Sigma-Aldrich Co. LLC, Tokyo, Japan) to the final concentration of 1 mg/ml for experimental use.

Medroxyprogesterone 17-acetate (MPA) (Sigma-Aldrich Co. LLC, Tokyo, Japan)

dissolved in the chloroform (Wako pure chemical industries, Ltd., Osaka, Japan) was mixed with the sesame oil to the final concentration of 1.23 mg/ml.
Allopregnanolone (Tocris bioscience, Bristol, United Kingdom) dissolved in the chloroform was mixed with the sesame oil to the final concentration of 1.01 mg/ml.
From P1 to P9, animals were administered 0.1 ml of prepared agents subcutaneously (i.e. progesterone, 0.1 mg/body/day; MPA, 0.123 mg/body/day; Allopregnanolone, 0.101 mg/body/day).
For the low concentration progesterone group, we prepared 0.1 mg/ml of progesterone.

Rotarod test

On P50, rotarod test (cat. 7750, Ugo Basile, Italy.) was performed for assessment of motor coordination and balance. Each rat was placed individually on the rotating rod at 2 rpm for 5min. We trained rats for five days before the test. After habituation for 3 min, the rotarod test was started at the speed of 2 rpm, and increase the speed every 30 sec. After 300 sec, the speed was up to 20 rpm. We recorded the latency to fall off the rod. Even if some of rats were continued walking, we broke off the test at 300 sec (35).

Histological analysis

For histological analysis, rats were sacrificed on P0, P5, P9, P30, and P50. The rats were deeply anesthetized with sodium pentobarbital (Somnopenty (R), Kyoritsu seiyaku Co., Tokyo, Japan) and intracardially perfused with phosphate buffered saline followed by 4% buffered paraformaldehyde. The brains were removed and immersed in 4% buffered paraformaldehyde for at least 2 days before histological processing.
Fixed brains were subjected to histological analyses or immunohistochemistry.

Immunohistochemistry and cell counting

Deparaffined section (5 μm thickness) subjected to immunohistochemistry as described previously (36). Briefly, they were washed in Tris buffered saline - 0.1% Triotn X100 (TBST), and were incubated for 30 min with one of the following antibody: mouse anti-NeuN antibody (1:200; Merck Millipore, MAB377, Darmstadt, Germany), rabbit anti-Olig2 antibody (1:500; Merck Millipore, AB9610, Darmstadt,

Germany) and rabbit anti-Iba1 antibody (1:500; Wako pure chemical industries, Ltd 019-19741, Osaka,Japan). Then sections were incubated with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase for 30 min. For negative controls, they were subjected to the same procedure as mentioned above without the primary antibody.
Cell count was performed by a blinded investigator. Immnoreactive cells were counted manually under the microscope with 200 times magnification. Four adjacent, non-overlapping square field (100 x 100 μm) were sampled for cell count.

Transmission electron microscopy (TEM)

The samples of tissues TEM were fixed in phosphate buffered 2% glutaraldehyde, and subsequently post-fixed in 2% osmium tetroxide for 3 hours in the ice bath. Then, the specimens were dehydrated in a graded ethanol and embedded in the epoxy resin. Ultrathin sections were obtained by ultramicrotomy technique. Ultrathin sections stained with uranyl acetate for 10 min and modified Sato's lead solution for 5 min were submitted to TEM observation by the electron microscope (JEM-1200EX, JEOL Ltd., Tokyo, Japan) (37). The quantifications were performed on 5 images per rat (at least 50 axons per rat).

Statistical analysis

The Kaplan-meier methods for rotarod test, the Steel–Dwass multiple comparison test and Wilcoxon rank sum test were used for statistical analysis (JMP 11.2, SAS Institute Inc., North Carolina、USA). Statistical significance was defined as $P < 0.05$.

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Author contributions statement

Y.K. and H. T.: performed the experiments, analyzed the data and wrote the manuscript. T. K.: performd the experiments. M.S.: final approval of the manuscript and provided financeial support.

Additional information

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Figure legends

Figure 1. Rotarod test on P50 analyzed by the Kaplan-Meier method.

We defined the latency to fall off the rotating rod as the survival duration. The latency time was shown by seconds. The maximum trial length was 300 sec. (A) Sham vs. P_4 vs. Veh. (B) P_4 (0.1) vs. P_4 (0.01) vs. Veh. (C) P_4 vs. Allo vs. Veh. (D) P_4 vs. MPA vs. Veh.

P50, postnatal 50 days; Sham, sham-operated group; P_4, progesterone-treated HIE group; Veh, vehicle-treated HIE group; P_4 (0.1), 0.1 mg/body/day of progesterone-treated HIE group; P_4 (0.01), 0.01 mg/body/day of progesterone-treated HIE group; Allo, allopregnanolone-treated HIE group.

*denotes significant difference in the latency time ($p<0.05$) analyzed by the Kaplan-Meier method.

Figure 2. Immunohistochemistry of the neuron or the oligodendrocyte on P50.

(A, C) Representative images of the cortex stained with NeuN. (B, D) The number of the NeuN positive cells in the cortex or the CA1. The body of the box plot represents the first and third quartiles of the distribution, and the median. The whiskers extend from the quartiles to the last data point within 1.5 x (inter quartile range), with outliers beyond represented as dots. (E) Thickness of the pyramidal layer from the CA1. (F) Representative images of the CC stained with Olig2. (G) The number of the Olig2 positive cells in the CC.

Scale bars=100 μm.

NeuN, anti-NeuN antibody; Olig2, anit-Olig2 antibody.

*denotes significant difference ($p<0.05$) analyzed by the Steel-Dwass multiple comparison test.

Median [1st quartile, 3rd quartile] were as follows:

(B) Sham, 33 [29.63, 36.56]; Veh, 16.88 [13.31, 20.06]; P_4 (0.1), 31.5 [25.13, 36]. (D) Sham, 33.75 [30.5, 37.31]; Veh, 18.5 [16.19, 22.94]; P_4 (0.1), 35.5 [33.38, 38.06]. (E) Sham, 41.77 [39.89, 45.09]; Veh, 24.95 [23.46, 27.34]; P_4 (0.1), 41.95 [37.06, 44.93]. (G) Sham, 33.125 [30.186, 38.25]; Veh, 20.13 [15.94, 21.31]; P_4 (0.1), 33.5 [27.75, 37.06].

Figure 3. Immunohistochemistry of the microglia on P9.

The number and morphology of the microglia in the CC was observed. (A) Representative images of the CC stained with Iba1. Scale bar=50 μm. (B) The number of the Iba1 positive cells. (C) The fraction rate of the ramified type to total number of the microglia in the CC.

Iba1, anti-Iba1 antibody.

*denotes significant difference ($p<0.05$) analyzed by the Steel-Dwass multiple comparison test.

Figure 4. The temporal change of density of neuron or oligodendrocyte.

(A) NeuN positive cells in the cortex. (B) NeuN positive cells in the CA1. (C) Olig2 positive cells in the CC.

* and † denote significant difference ($p<0.05$) analyzed by the the Wilcoxon rank sum test on P9, or the Steel-Dwass multiple comparison test on P50.

Mean±SD were as follows:

(A) Veh (n=5, 46.5±2.61) and P_4 (n=5, 58.2±2.31) on P9. Sham (n=6, 33.21±1.56), Veh (n=8, 16.84±1.19) and P_4 (n=6, 30.83±2.237) on P50.

(B) Veh (n=5, 52.15±1.72) and P_4 (n=5, 59.4±1.23) on P9. Sham (n=6, 34.04±3.51), Veh (n=8, 19±3.89) and P_4 (n=6, 35.46±2.55) on P50.

(C) Veh (n=5, 16.1±1.63) and P_4 (n=5, 28.35±2.15) on P9. Sham (n=6, 33.75±4.20), Veh (n=8, 18.86±3.67) and P4 (N=6, 33.13±4.84) on P50.

Figure 5. Myelination in the corpus callosum.

(A) Electron microscopic images of P50 of the CC. Scale bar=1 μm. (B) Thickness of myelins. Sham vs. Veh vs. P_4. (C) *g*-ratio (the axon diameter / the fiber diameter ratio). Sham vs. Veh vs. P_4.

*denotes significant difference ($p<0.05$) analyzed by the Steel-Dwass multiple comparison test.

Median [1st quartile, 3rd quartile] were as follows:

(B) Sham, 0.1465 [0.12, 0.19]; Veh, 0.084 [0.07, 0.10]; P_4, 0.15 [0.12, 0.18]. (C) Sham, 0.6 [0.52, 0.68]; Veh, 0.79 [0.73, 0.82]; P_4, 0.58 [0.5, 0.66].

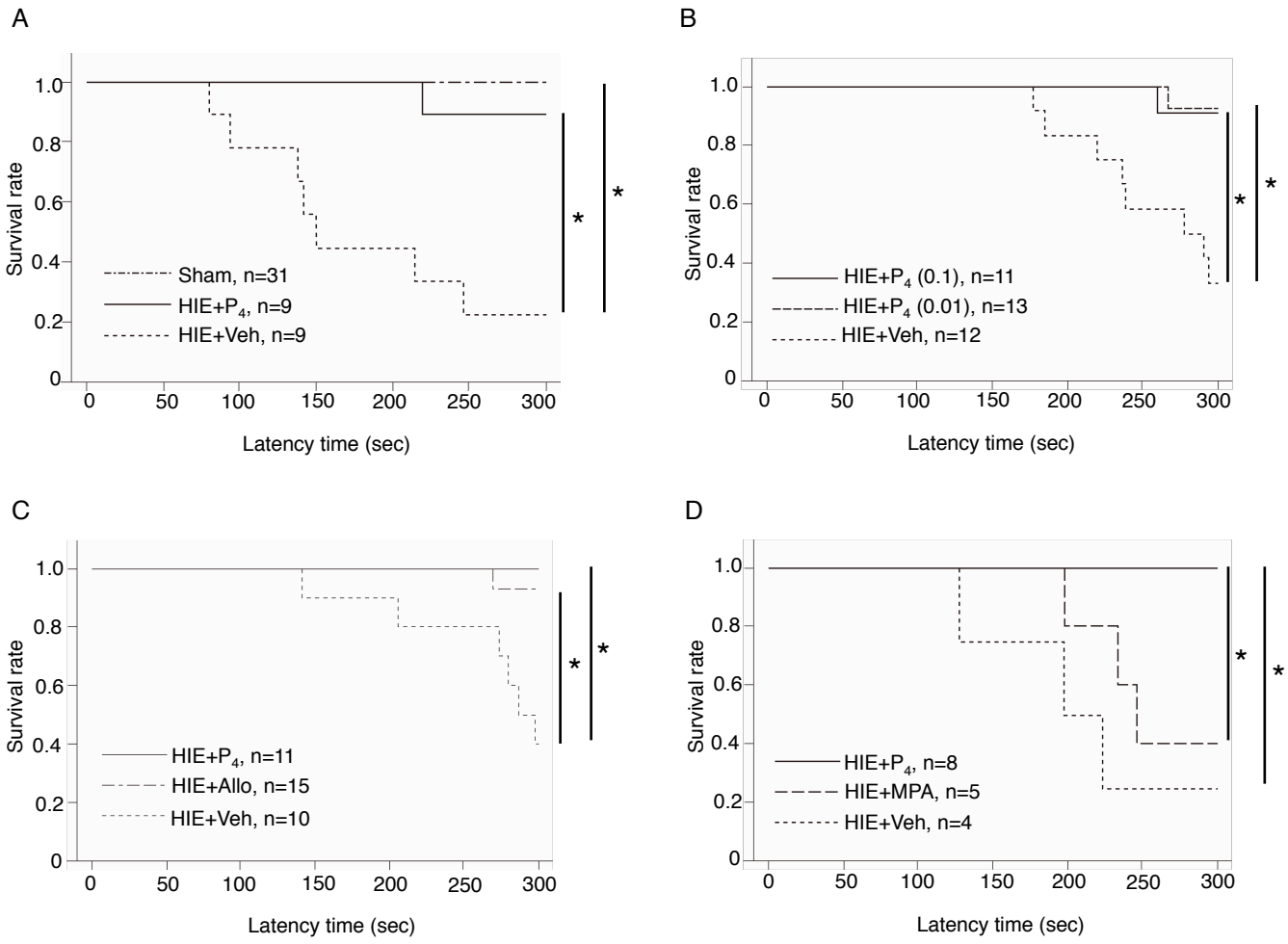
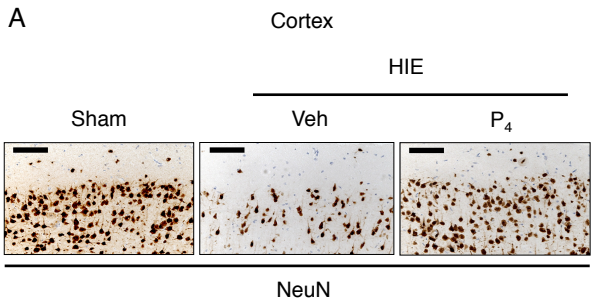
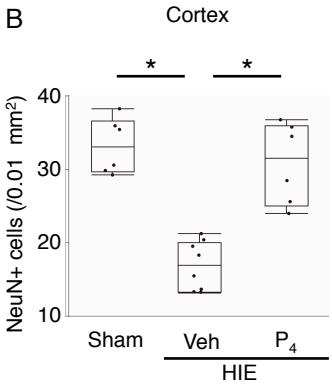
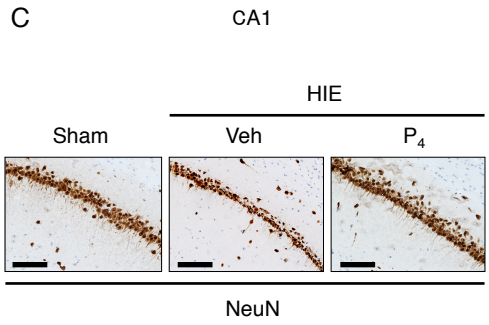
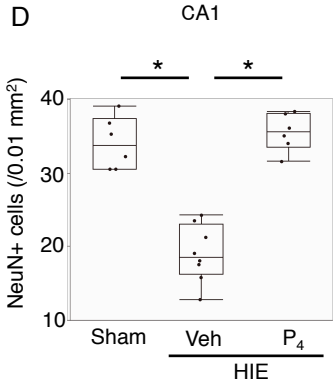


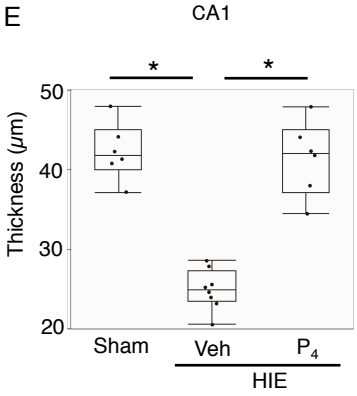
Figure 1. Rotarod test on P50 analyzed by the Kaplan-Meier method.

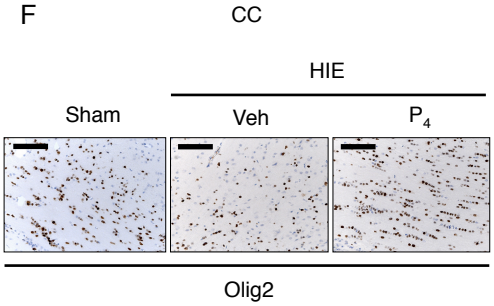












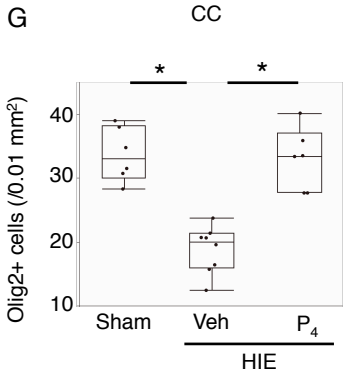


Figure 2. Immunohistochemistry of neuron or oligodendrocyte on P50.

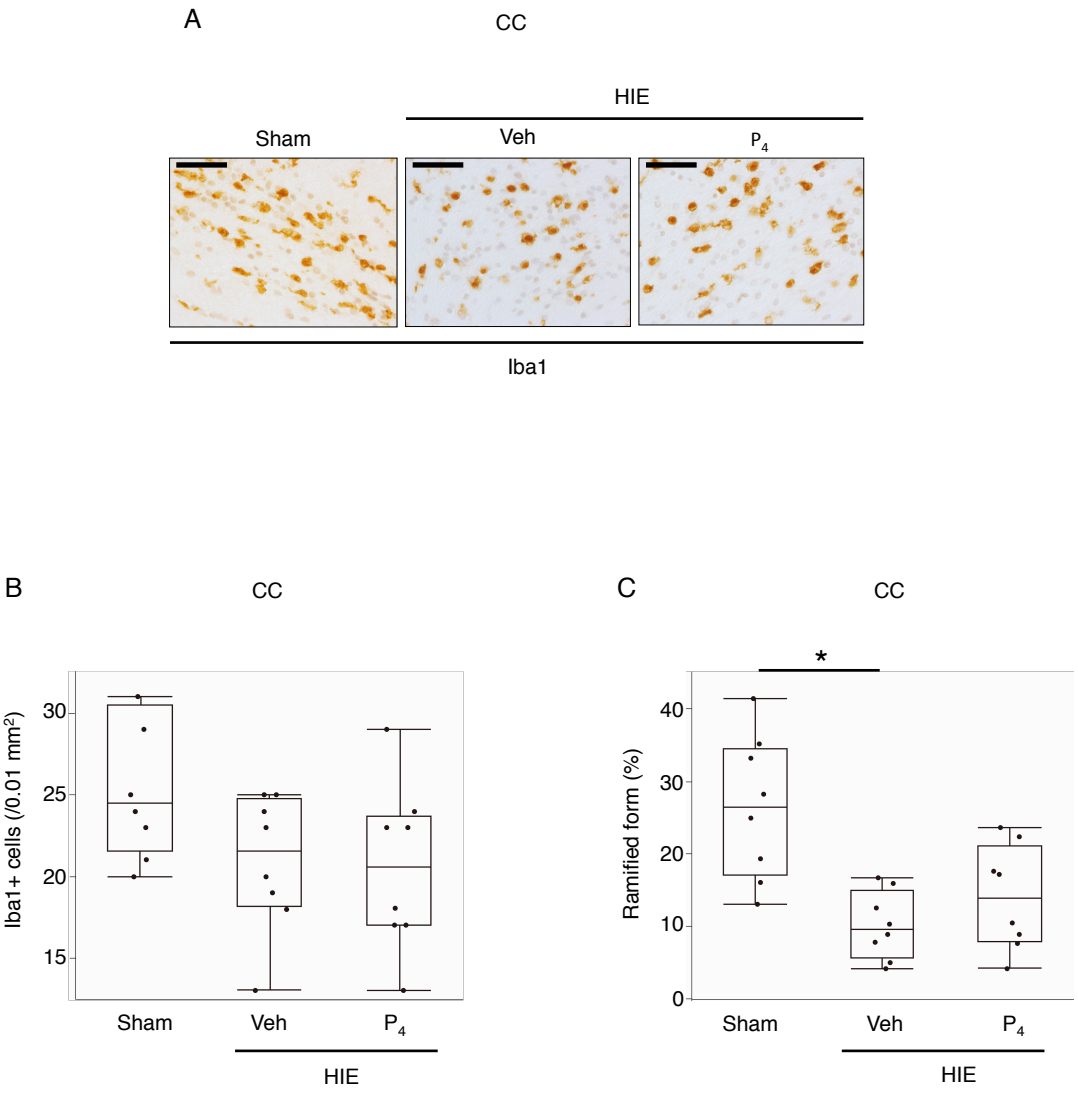


Figure 3. Immunohistochemistry of microglia on P9.

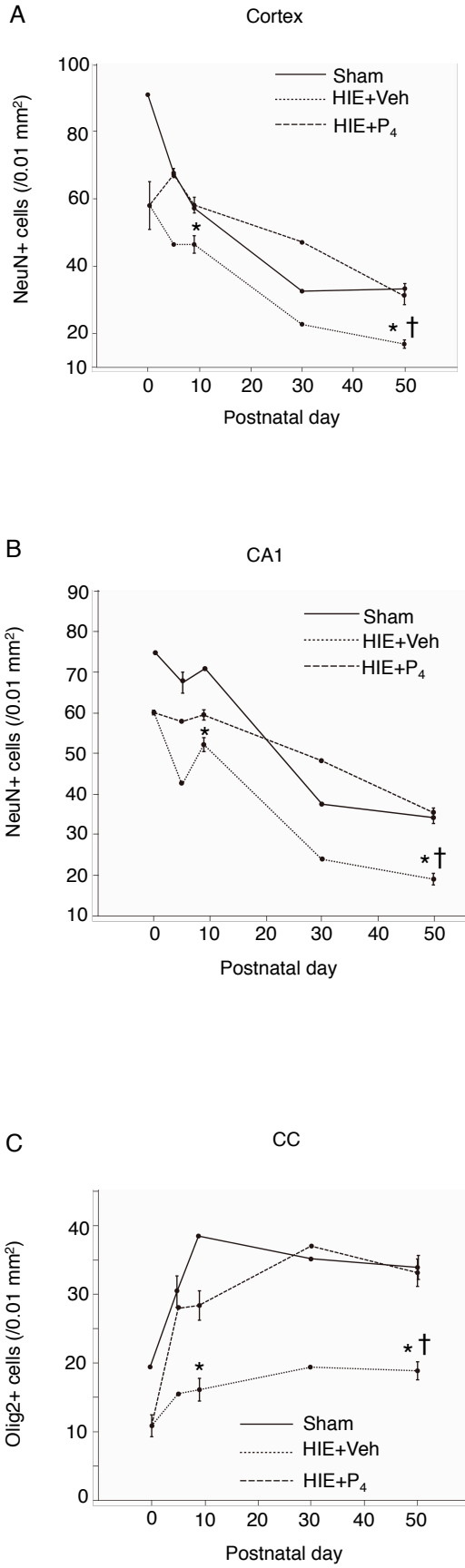


Figure 4. The temporal change of the densities of neuron or oligodendrocyte.

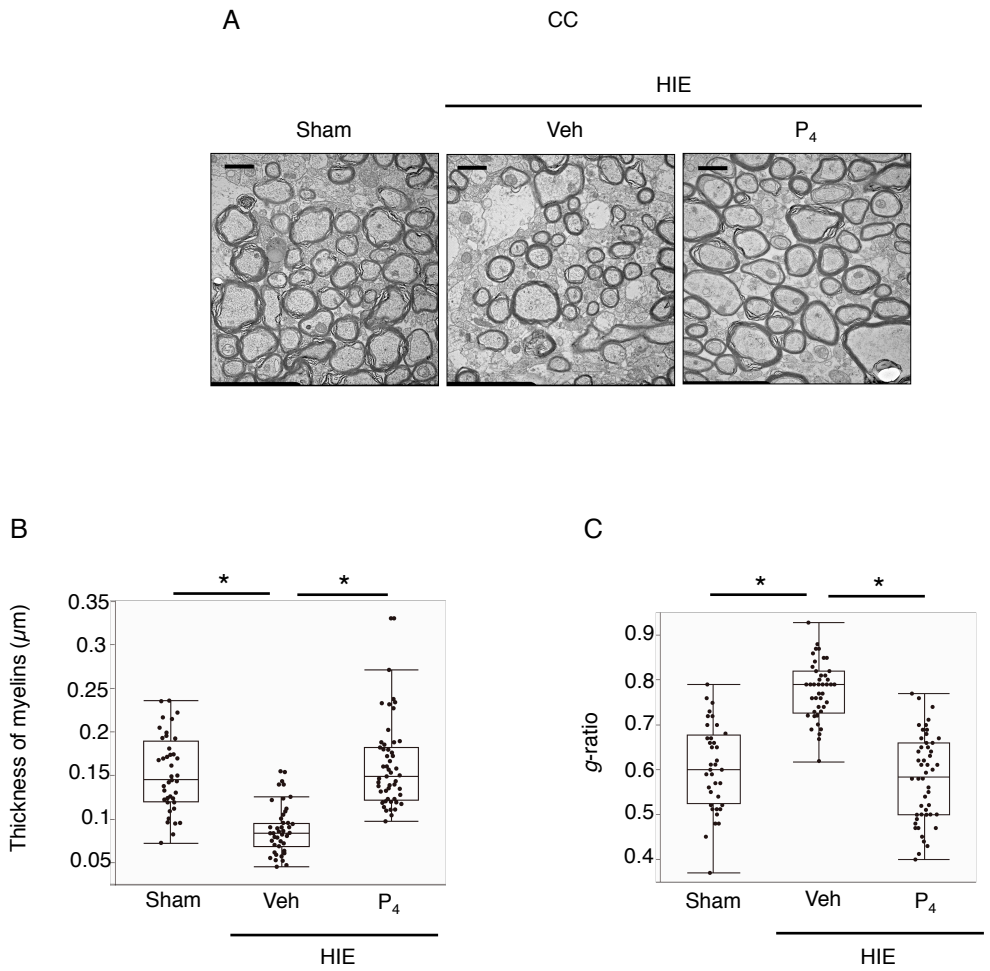
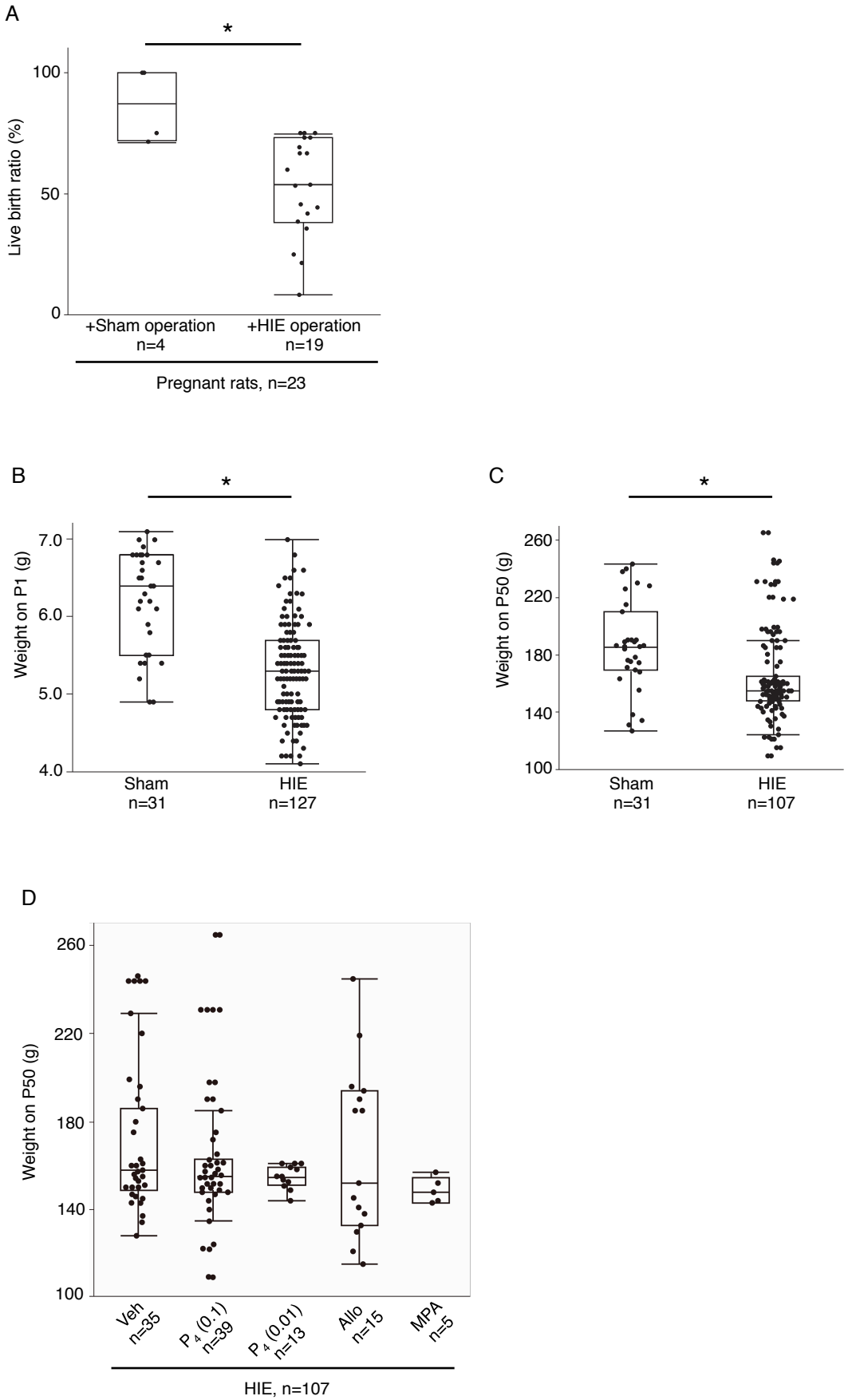
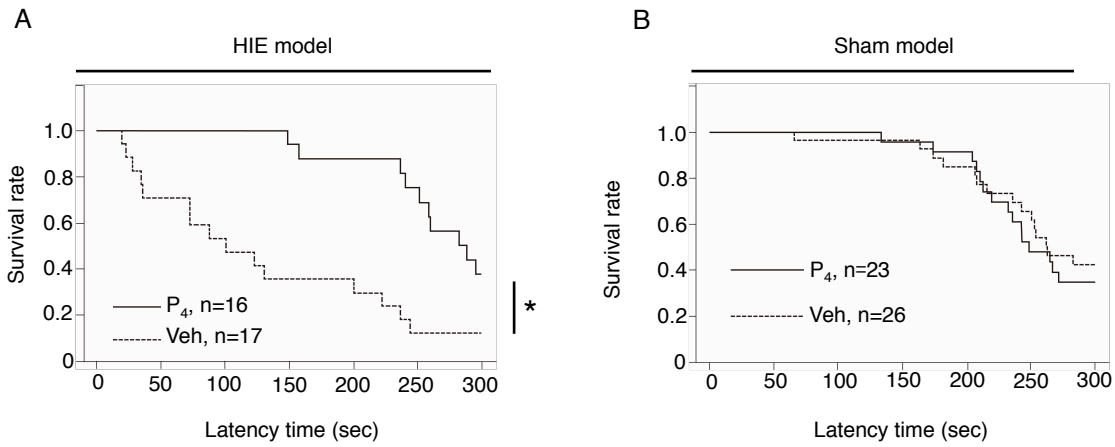


Figure 5. Myelination in the corpus callosum

Supplementary Information



Supplementary Figure S1. Live birth ratio and body weight of sham and HIE.



Supplementary figure S2. Rotarod test on P50 analyzed by Kaplan-Meier methods in pilot study.

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