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Impaired acquisition of skilled behavior in rotarod task by moderate depletion of striatal dopamine in a pre-symptomatic stage model of Parkinson's disease

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Abstract

In view of recent findings that suggest that the nigrostriatal dopamine (DA) system plays a role in motor control and the acquisition of habits and skills, we hypothesized that the striatum-based function underlying the acquisition of skilled behaviors might be more vulnerable to dopamine depletion than the motor control. To test this hypothesis, we investigated whether impaired acquisition of skilled behaviors occurs in a pre-symptomatic stage model of Parkinson's disease (PD). By using the microdialysis method and the 6-OHDA-technique to destroy dopamine neurons, we confirmed that rats with unilateral partial lesions of the nigral dopamine cells by 6-OHDA are suitable for a pre-symptomatic stage model of Parkinson's disease. The rats in this model exhibited moderate disruption of striatal dopamine release function and relatively intact motor functions. In a rotarod test, the impaired acquisition of skilled behavior occurred in rats with bilateral partial lesions of the nigral dopamine cells by 6-OHDA. These rats displayed intact general motor functions, such as locomotor activity, adjusting steps, equilibrium function and muscle strength. Based on these results, we concluded that the striatum-based function underlying the acquisition of skilled behaviors or sensorimotor learning may be more vulnerable to dopamine depletion than the motor control.

Keywords: 6-OHDA-induced lesion; Motor functions; Acquisition of skilled behavior; Rotarod test; Microdialysis method

1. Introduction

A considerable amount of evidence has revealed that the classical diagnostic motor symptoms, such as serious akinesia, rigidity and tremors in Parkinson's disease (PD) begin to emerge when loss of nigrostriatal dopamine (DA) cells reaches around 60–70% and becomes more and more pronounced with progression of the neurodegenerative process (Bernheimer et al., 1973; Riederer and Wuketich, 1976; Brooks, 1998). This critical loss of nigrostriatal DA cells results in severe depletion of DA in the striatum (Hornykiewicz, 1975). The disruption of DA-mediated control of striatal neuronal activity due to the severe depletion of striatal DA and the subsequent imbalance in the activity of the two main outputs, so-called direct and indirect pathways, from the striatum are generally considered to be the cause of the classic motor symptoms in PD (Albin et al., 1989; Crossman, 1987; DeLong, 1990; Obeso et al., 1997, 2000).

Clinical and behavioral evidence suggest that the basal ganglia are centrally involved in the learning process that leads to habit formation or the acquisition of skilled behavior (McDonald and White, 1994; Graybiel, 1995, 1998; Salmon and Butters, 1995; Knowlton et al., 1996; White, 1997; White and McDonald, 2002). A study in monkeys on the impairment of conditioned behaviors by chemical lesion of nigral DA neurons has revealed that the nigrostriatal DA system might play a crucial role in the striatum-based neuronal mechanisms

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underlying the learning process for habit formation and the acquisition of skilled behaviors (Aosaki et al., 1994; Satoh et al., 2003). Recently, a significant amount of evidence that PD patients may exhibit impaired acquisition of skilled behaviors has accumulated (Verschueren et al., 1997; Vakil and Herishanu-Naaman, 1998; Westwater et al., 1998; Sommer et al., 1999; Thomas-Ollivier et al., 1999; Krebs et al., 2001).

Considering these findings, it is quite likely in rodents that the nigrostriatal DA system plays an important role in two different striatum-based functions, such as the control of motor activity and the acquisition of habits and skills (Jog et al., 1999; Linder et al., 1999; Smith et al., 2002). Only a few studies have investigated the possible involvement of impaired acquisition of skilled behavior in behavioral abnormalities in rat models of PD with partial lesions of nigrostriatal DA cells. However, consistent results between studies were not obtained using the same behavioral task (Kirik et al., 1998; Barnéoud et al., 2000; Roedter et al., 2001). Here, we hypothesized that the striatum-based function underlying the acquisition of skilled behaviors and habits could be more vulnerable to DA depletion than the DA-mediated control of motor activity and, therefore, that the impaired acquisition of skilled behaviors would occur predominantly in the pre-symptomatic stage model of PD.

To test our hypothesis, we performed the following two experiments. Although some investigators have used 6-hydroxydopamine (6-OHDA)-treated rats with incomplete destruction of the nigrostriatal DA cells as an early stage model of PD (Lee et al., 1996; Kirik et al., 1998; Roedter et al., 2001; Deumens et al., 2002), it has not yet been confirmed whether the partial loss of nigral DA cells caused a mild disruption of the DA release function in the striatum. In these previous studies, a pre-symptomatic stage of PD has been characterized by relatively intact general motor functions despite moderate impairment of the nigrostriatal DA system (Hornykiewicz, 1993; Anglade et al., 1995; Kirik et al., 1998). Therefore, we first examined whether rats with incomplete destruction of nigrostriatal DA cells by intra-striatal injections of 6-OHDA are suitable for a pre-symptomatic stage model of PD by measuring DA release in the striatum using the microdialysis method. Next, we conducted various behavioral tests, such as a rotarod test for the acquisition of skilled behavior, an open field test for spontaneous locomotor activity, a stepping test for the initiation of adjusting steps, a bridge test for equilibrium function, or a wire suspension test for muscle strength in rats with bilateral incomplete destruction of nigrostriatal DA cells by intra-striatal injection of 6-OHDA as a pre-symptomatic stage model of PD.

2. Materials and methods

2.1. Animal care

Eighty-four naive male Wistar rats weighing 270–300 g (CLEA Japan, Tokyo, Japan) were used. The animals were housed in clear plastic cages in groups of two or three and allowed access to food and water. They were maintained in a temperature-, humidity- and light-controlled environment with a 12 h light–dark cycle. In order to accustom rats to the experimenters, on arrival in the colony, the experimenter held the animal gently by the body and rubbed its head and back for about 10 min. This was performed once a day for several days before the surgical operation for 6-OHDA injections into the medial forebrain bundle (MFB) or the striatum (caudate–putamen in rat, CPu). After a recovery period of 3 weeks following the surgery, all rats underwent similar handling for several days until the experiments were started.

All experiments conformed to Japanese and international guidelines on the ethical use of animals, and every effort was made to minimize the number of animals used and their suffering.

2.2. Surgical procedures

To produce PD model rats with complete destruction of nigrostriatal DA cells, 10 μg 6-OHDA (5 mg/ml, Sigma, USA) dissolved in saline containing 0.1% ascorbic acid was injected stereotaxically into the unilateral MFB under anesthesia with intra-peritoneal (i.p.) sodium pentobarbital (50 mg/kg). The coordinates were anteroposterior (AP): −4.5; midline (ML): +2.2; ventral (V): 7.8 mm relative to the bregma and ventral from the dura. To produce PD model rats with incomplete destruction of nigrostriatal DA cells, a total of 12 μg (3 μg per each site) of 6-OHDA (1.5 mg/ml) was injected stereotaxically into the four sites of the unilateral CPu under the same anesthesia. The coordinates were: (1) AP: +1.3; ML: +2.6; V: 4.5, (2) AP: +0.4; ML: +3.0; V: 4.5, (3) AP: −0.4; ML: +4.2; V: 4.5, (4) AP: −1.3; ML: +4.4; V: 4.5 mm relative to the bregma and ventral from the dura. To perform behavioral tests, rats with bilateral partial lesions of nigrostriatal DA cells were produced by injections of 12 μg of 6-OHDA into both sides of the CPu in the same manner. A glass micropipette (tip size: 30–40 μm) made from a disposable micropipette (20 μl, Drummond, USA) connected to an air pressure system was used for the focal injections of 6-OHDA. After intra-MFB or intra-CPu injections of 6-OHDA, the animals were cared for 3 weeks in the home cage and then used for assessing motor function.

2.3. Microdialysis analysis of DA concentration in the striatum

2.3.1. Implantation of microdialysis probes

Six hours after amphetamine-induced turning behavior (see below), under anesthesia with sodium pentobarbital (50 mg/kg, i.p.), two microdialysis probes were implanted into both sides of the CPu at the same level as in the 6-OHDA-treated rats. The coordinates for the intra-CPu insertions of the guide cannulas for the probes were: AP: +0.2; ML: ±3.0; V: 3.5 mm relative to the bregma and

ventral from the dura. The implantation site was stereotaxically positioned, and the guide cannula was fixed to the skull with an anchor screw and dental cement.

2.3.2. Detection of an amphetamine-induced increase of DA release in the striatum

Following a recovery period of 2 days after the surgery for the implantation of probes, we examined the disruption of the DA release function in the treated side of the striatum using a microdialysis method as follows: (1) the concentration of DA in a dialysate sample of 20 μl collected for 10 min was measured and quantified with an HPLC system coupled with an electrochemical detector (DIA-300, EICOM Co., Japan) in both sides of the striatum at the same time through implanted probes in a free moving rat. The dialysate was a Ringer's solution containing 145.3 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$ and 0.2 mM $NaHCO_3$ (pH 7.3). This solution was pumped through the probes at a flow rate of 2 μl/min. The measurement of striatal DA concentration was performed at every 10 min during the 210-min period before and after intra-peritoneal administration of 2 mg/kg methamphetamine; (2) the relative amount of amphetamine-induced DA release in each side of the striatum was estimated as a percent ratio against the baseline amount of DA release that was determined as a concentration of spontaneously released DA measured during the last 10 min during the pre-injection period of 60 min; (3) Finally, the disrupted DA release function in the 6-OHDA-treated striatum was estimated as a percent ratio of the peak value of amphetamine-induced increase DA release in the lesioned side to that in the intact side of the striatum.

2.4. Immunohistochemical evaluation of 6-OHDA-induced loss of nigrostriatal DA cells

2.4.1. Tyrosinehydroxylase (TH) immunohistochemistry

Two days after microdialysis analysis of DA concentration in the striatum, under deep anesthesia with sodium pentobarbital, animals were perfused transcardially with heparinized saline followed by 4% paraformaldehyde in a 0.1 M phosphate buffer (PB) (pH 7.4). The brains were removed and postfixed in a fixative for 1–2 h and saturated in 30% sucrose overnight at 4 °C. Coronal sections of the nigral area (40 μm thickness) were cut with a freezing microtome. Free-floating sections were preincubated for 1 h with 0.1 M phosphate-buffered saline (PBS) containing 3% normal goat serum and 0.1% triton X-100. After rinsing twice with 0.1 M PBS, free-floating sections were incubated overnight at room temperature with a PBS solution containing TH-antiserum (1: 2000, Protos Biotech. Corp., USA) and 1% normal goat serum. After rinsing twice with 0.1 M PBS for amplification of the antibody signal, the sections were processed with secondary antibodies (biotinylated anti-rabbit IgG) and avidin biotin peroxidase complex (ABC Kit Elite, Vector Lab., USA) at room temperature. After rinsing twice with 50 mM PB, to visualize antibody–positive neurons, the sections were incubated with 0.05% diaminobenzidine (Dojin Chem. Corp., Japan) and 0.004% hydrogen peroxide for 5 min at room temperature and mounted on a subbed slide.

2.4.2. Quantitative analysis of 6-OHDA-induced loss of DA cells in the nigral area

Three coronal sections stained with TH-antiserum were chosen from the three rostro-caudal levels of the substantia nigra (SN) (at −4.8, −5.3 and −5.8 mm from the bregma). The medial border of the SN to separate from the ventral tegmental area (VTA) was defined as the vertical line passing through the medial tip of the cerebral peduncle and the accessory nucleus of the optic tract. The total number of intact nigrostriatal DA cells in the treated side or the untreated intact side of the SN was measured by counting TH-positive cells throughout the entire unilateral SN area, thus defined at a magnification of ×200 and summing the number TH-positive cells from the three chosen sections. The survival rate of DA cells in the SN was expressed as a percent ratio of the total number of TH-positive cells in the treated side against that in the intact side of the SN. The loss of nigrostriatal DA cells by 6-OHDA treatments was estimated by subtracting the survival rate of DA cells in the 6-OHDA-treated side of the SN from that in the intact side of the SN.

2.5. Behavioral tests in rats with unilateral lesions of DA cells

2.5.1. Amphetamine-induced turning behavior

Rats were placed in a white square box (50 cm × 50 cm × 40 cm) following the intra-peritoneal injection of 2 mg/kg methamphetamine, and their behavior was recorded by a video camera during a 45-min test period after the drug injection. To analyze the rate of the turning behavior in the box of this size, a turn was defined as a half full-body turn (a half-circle with a diameter of less than 25 cm). A turn toward the operated side (ipsilateral) was scored as +1 point, while a turn toward the intact side (contralateral) was scored as −1 point. The total number of turns in the ipsilateral direction or the contralateral direction was measured from the last 15 min recorded during the 45-min test period. Thus, the rate of asymmetric turning behavior induced by deficits in motor activity was expressed by net scores (ipsilateral plus contralateral).

2.5.2. Stepping test

To assess forelimb akinesia, a stepping test was conducted as previously described by Olsson et al. (1995). Rats were tightly held; the experimenter grabbed the hind limbs with one hand and the forelimb not being monitored with the other hand. While touching the free forelimb to the smooth surface of the floor, the rat was moved along the smooth floor in the free forelimb direction over a distance of 90 cm. During this sideway movement, the

free forelimb of the rat displayed frequent adjusting steps. The number of adjusting steps per 90 cm was counted. The stepping test was performed twice a day.

2.6. Behavioral tests in rats with bilateral lesions of DA cells

2.6.1. Rotarod test

To assess the acquisition of skilled behavior in rats with bilateral partial lesions of the nigrostriatal DA system, we performed a rotarod test (Jeljeli et al., 1999, 2000). The rotarod apparatus consisted of two steel poles standing 14 cm apart and a rod suspended horizontally between the two poles at a height of 90 cm from the cushioned floor. The rod, which had a diameter of 5 cm, was a steel beam covered by high-profiled rubber in order to ensure firm gripping. This rod turned around its longitudinal axis at a rate of 30 revolutions/min (rpm). Side panels were inserted at each end of the rod to prevent any escape from the apparatus. The training room for the rotarod test was maintained at a temperature 22–24 °C, and noise was kept to a minimum. The rotarod test used consisted of acclimation sessions and training sessions. For each acclimation session performed daily, the rats were gently moved to the training room, put into a white waiting box, taken out of the box in turns, and given the previously mentioned handling, which consisted of rubbing their head and back for about 10 min. Acclimation sessions were performed over three consecutive days. The training sessions were started one day after the last acclimation session. Rats were given four training trials per day with an inter-trial interval of 10 min. Four training trials were considered one session. The training sessions were performed over five consecutive days. For each training trial, rats were gently placed on the rod in the orientation opposite to that of the already rotating rod so that they could acquire the necessary skilled behavior on the rotating rod to prevent a fall. Rats were allowed to stay on the rod for a maximum of 60 s, which was established as the cut-off period (this was deemed to be the maximum period of time that rats could stay on the rod without losing their motivation to do so). The time spent walking on the rotating rod was measured. Because motivation to stay on the rod was maintained during the 60 s test period, the increase of the time spent walking on the rotating rod reflects an improvement in the acquisition of skilled behavior consisting of a new combination of posture and forward locomotive steps.

2.6.2. Open field test for spontaneous locomotor activity

Rats were placed in the center of an open field (100 cm × 100 cm × 40 cm) as a novel environment. The locomotor behavior of the rats was recorded by a video camera during the 15-min period following the start of spontaneous locomotion in the open field. The rats' position was tracked by computer during the test period, and the total locomotion distance was measured as the spontaneous locomotor activity using computer-guided image software (Ohara Medical Instruments, Tokyo, Japan).

2.6.3. Stepping test

The same stepping test as described above was performed in rats with bilateral partial lesions of the nigrostriatal DA system.

2.6.4. Bridge test

To assess the equilibrium function, we performed a bridge test as described by Joyal et al. (1996). The bridge test apparatus consisted of two platforms and a square wooden beam (length, 100 cm; width, 2.0 cm; thickness, 1.5 cm) with 10-cm segments that was suspended horizontally between the two platforms at a height of 60 cm from the cushioned floor. Side panels were inserted at each end of the wooden beam to prevent any escape from the apparatus. Rats were given two trials per day with an inter-trial interval of 10 min. Trials were performed over three consecutive days. For each trial, rats were placed on the middle of the wooden beam. They had a maximum of 180 s to traverse on the wooden beam, the so-called cut-off period, in order to avoid the appearance of lowered motivation. The latency before falling and the number of segments traversed were measured as an indicator of equilibrium function.

2.6.5. Wire suspension test

To estimate the muscle strength of the forelimb or the whole body, a wire suspension test was performed. The forelimbs of the rats were placed on a wire horizontally suspended at a height of 80 cm from the cushioned floor. Rats had a maximum of 30 s to hold the suspended wire to avoid falling. The time spent in a suspended position was measured as muscle strength. The test was performed twice with an inter-trial interval of 10 min.

2.7. Statistical analysis

All statistical analysis was carried out by one-way ANOVA or Student's *t*-test. Post hoc individual comparison of paired groups was carried out using Fisher's PLSD test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Behavioral correlates with disruption of striatal DA release function following unilateral lesions of DA cells in the SN

3.1.1. 6-OHDA-induced loss of DA cells and its related disruption of striatal DA release function.

3.1.1.1. TH-immunohistochemistry for DA cell loss. We performed TH-immunohistochemistry for the quantitative analysis of 6-OHDA-induced loss of DA cells in the SN when all behavioral tests and biochemical assays had been

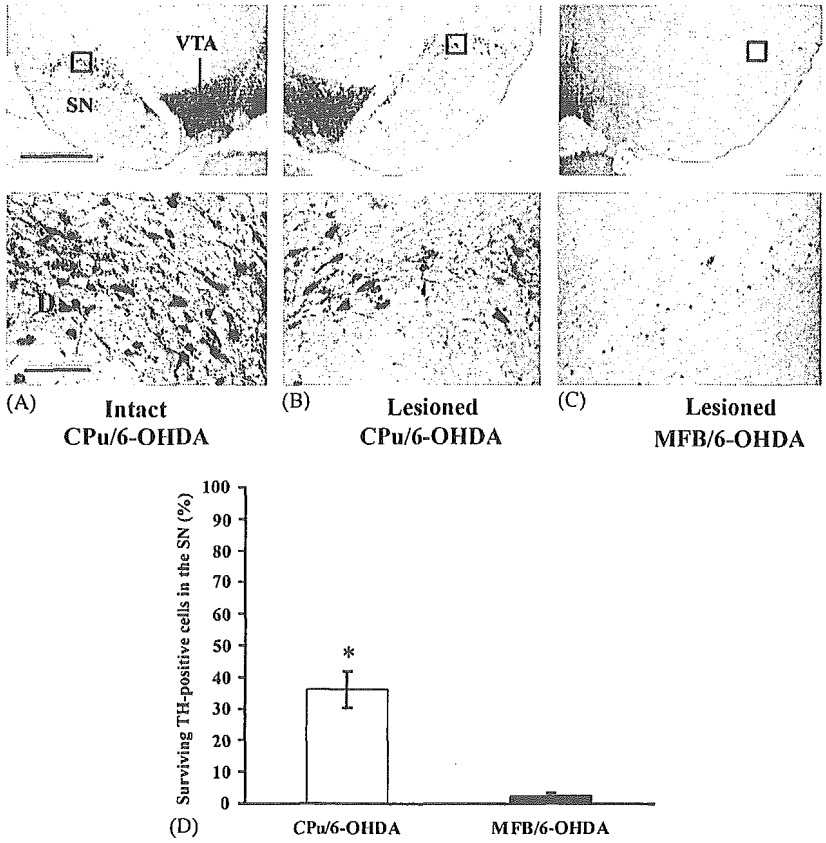

Fig. 1. The photographs in the upper panels of (A–C) indicate the TH-immunohistochemically stained section of the SN obtained from the intact side (A) and the lesioned side (B) of a rat with unilateral intra-CPu injections of 6-OHDA (CPu/6-OHDA) and the lesioned side of a rat with unilateral intra-MFB injections of 6-OHDA (MFB/6-OHDA) (C). The photographs in the lower panels of (A–C) are enlarged views of the SN indicated by the frame in the upper panels, demonstrating many surviving TH-positive cells in the intact side (A) and incomplete loss (B) and complete loss (C) of TH-positive cells in the lesioned side. D: Quantitative analysis for surviving TH-positive cells in the SN of the CPu/6-OHDA rats and the MFB/6-OHDA rats. Incomplete loss (36.1 ± 5.9% survival) of TH-positive cells occurred in the SN of the CPu/6-OHDA rats ($n = 4$), while almost complete loss (only 2.7 ± 0.8% survival) of the TH-positive cells in the SN was observed in the MFB/6-OHDA rats ($n = 7$). The survival rate of TH-positive cells in the lesioned side of the SN was estimated as a percent ratio of the total number of surviving TH-positive cells in the lesioned side to that in the intact side. Each value represents the mean ± S.E.M. $^*P < 0.05$, significantly different from the MFB/6-OHDA. SN, substantia nigra; VTA, ventral tegmental area; CPu, nucleus of the caudate-putamen; MFB, medial forebrain bundle. Scale bar: 1 mm for the upper panels in (A–C); 100 μm for the lower panels in (A–C).

completed. Compared with the intense TH-immunoreactivity and dense appearance of TH-positive cells observed in the intact side of the SN (Fig. 1A), the TH-immunoreactivity was remarkably attenuated, but a considerable amount of TH-positive cells still remained in the lesioned side of the SN of rats with unilateral intra-CPu injections of 6-OHDA (CPu/6-OHDA) (Fig. 1B). On the other hand, TH-immunoreactivity and TH-positive cells were almost completely lacking in the lesioned side of the SN of rats that received unilateral intra-MFB injections of 6-OHDA (MFB/6-OHDA) (Fig. 1C). It is notable that intense TH-immunoreactivity in the VTA was also almost completely lost in the lesioned side of the MFB/6-OHDA rats, in contrast to the intact nature of TH-immunoreactivity in the VTA of the lesioned side of the CPu/6-OHDA rats. Quantitative analysis for surviving TH-positive cells indicated that incomplete loss (36.1 ± 5.9% survival) of TH-positive cells occurred in the lesioned side of the CPu/6-OHDA rats ($n = 4$), while almost complete loss (only 2.7 ± 0.8% survival) of TH-positive cells occurred in the SN in the lesioned side of the MFB/6-OHDA rats ($n = 7$) (Fig. 1D).

3.1.1.2. Microdialysis analysis for DA depletion in the striatum. We performed simultaneous detection of the amphetamine-induced increase of the DA release from both sides of the striatum in the CPu/6-OHDA rats and the MFB/6-OHDA rats using the microdialysis method. We measured the DA concentration in the striatum of the CPu/6-OHDA rats or that of the MFB/6-OHDA rats every 10 min during the 210-min period before and after the intra-peritoneal administration of methamphetamine. As shown in Fig. 2A by filled circles, an amphetamine-induced transient increase of the DA concentration was observed in the intact side of the striatum of both 6-OHDA-treated groups. The increase of the DA concentration from the baseline level reached a peak level 20 min after the drug administration and gradually returned to the baseline level in the pre-

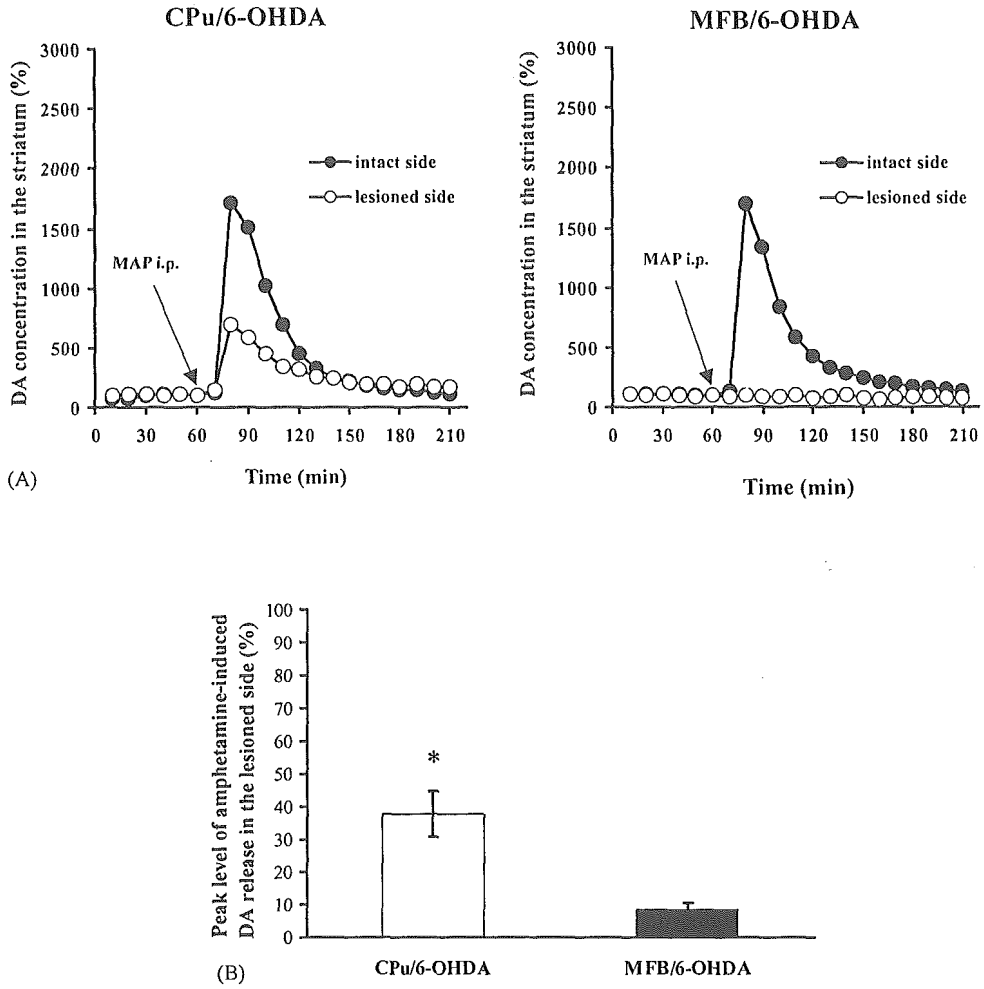

Fig. 2. Detection of an amphetamine-induced transient increase of DA release in the striatum of rats with unilateral intra-CPu injections of 6-OHDA (CPu/6-OHDA) and unilateral intra-MFB injections of 6-OHDA (MFB/6-OHDA). (A) Example of the time course of the amphetamine-induced increase of DA concentration detected from the striatum of the intact side (filled circle) and that of the lesioned side (open circle) of the CPu/6-OHDA rat (left, # 4 rat described in Table 1) or the MFB/6-OHDA rat (right, # 4 rat described in Table 1). The concentration of DA in a dialysate sample of 20 μl collected for 10 min was measured by the microdialysis method every 10 min during the 210-min period before and after the intra-peritoneal injection of 2 mg/kg methamphetamine (MAP). The level of DA concentration in the striatum at every 10 min was expressed as a percentage of the baseline concentration of spontaneously released DA measured during the last 10 min during the pre-injection period of 60 min and is plotted in a time-dependent manner. (B) Quantitative analysis for the remaining function of DA release in the striatum of the lesioned side of the CPu/6-OHDA rats ($n = 4$) and the MFB/6-OHDA rats ($n = 7$). The remaining function of DA release in the lesioned side was estimated as a percent ratio of the peak value of the amphetamine-induced increase of DA release in the lesioned side of the striatum to that of the intact side of each rat. Each value represents the mean ± S.E.M. $^{*}P < 0.05$, significantly different from the MFB/6-OHDA rats.

administration period up to 150 min after the drug administration. In the lesioned side of the striatum of both 6-OHDA-treated groups, the amphetamine-induced increase of the striatal DA level was largely reduced in the CPu/6-OHDA rats and almost vanished in the MFB/6-OHDA rats, as illustrated in Fig. 2A by open circles.

Quantitative analysis for the peak level of amphetamine-induced DA release indicated that the remaining function of amphetamine-induced DA release in the lesioned side of the striatum in the CPu/6-OHDA rats ($n = 4$) was 37.8 ± 7.0% of that in the intact side, while that in the lesioned side of the striatum in the MFB/6-OHDA rats ($n = 7$) was 8.6 ± 1.8% of that in the intact side (Fig. 2B). The remaining function of DA release in the lesioned striatum in the CPu/6-OHDA rats was significantly larger than that in the MFB/6-OHDA rats ($P < 0.05$).

3.1.1.3. Correlation between loss of DA cells and disruption of striatal DA release function. As described above, we detected loss of DA cells in the SN and DA depletion in the striatum in the same individuals with 6-OHDA-induced destruction of the nigrostriatal system. Therefore, we examined whether there was a correlation between loss of DA cells in the SN and depletion of DA in the striatum of the 6-OHDA-lesioned rats using the data from the MFB/6-OHDA rats ($n = 7$) and the CPu/6-OHDA rats ($n = 4$) summarized in Table 1. The loss of DA cells in the SN treated with 6-OHDA was significantly correlated with the

Table 1
Summarized data on the loss of DA cells in the SN and the disruption of DA release in the striatum in each 6-OHDA-treated rats

Animals	Substantia nigra				Striatum			
	Surviving DA cells in the SN			Loss of DA cells	Remaining DA release			Depletion of DA release
	Lesioned	Intact	Ratio (%)	Ratio (%)	Lesioned	Intact	Ratio (%)	Ratio (%)
MFB/6-OHDA								
#1	41	606	6.8	93.2	135.5	1635.6	8.3	91.7
#2	12	584	2.1	97.9	123.5	1389.0	8.9	91.1
#3	3	480	0.6	99.4	105.7	1142.4	9.2	90.8
#4	18	653	2.8	97.2	94.2	1696.8	5.6	94.4
#5	10	610	1.6	98.4	118.1	2717.6	4.4	95.6
#6	8	551	1.5	98.5	107.8	2081.3	5.2	94.8
#7	23	685	3.4	96.6	191.6	1037.5	18.5	81.5
CPu/6-OHDA								
#1	234	564	41.5	58.5	699.3	2558.7	27.3	72.7
#2	286	577	49.6	50.4	1262.5	2239.3	56.4	43.6
#3	126	548	23.0	77.0	526.2	1953.3	26.9	73.1
#4	199	661	30.1	69.9	696.1	1715.3	40.6	59.4

MFB/6-OHDA, rat with unilateral intra-MFB injections of 6-OHDA; CPu/6-OHDA, rat with unilateral intra-CPu injections of 6-OHDA. Lesioned, data obtained from the lesioned side; intact, data from the intact side.

depletion of DA in the striatum ($r = 0.91$, $P < 0.01$), showing a simple linear relation ($y = 1.05x$), as illustrated in Fig. 3. This simple linear relation implies that the degree of anatomical DA cell loss in the SN directly reflects the severity of disruption of striatal DA release function.

3.1.2. Motor deficits by unilateral lesions of the nigrostriatal DA system

3.1.2.1. Amphetamine-induced turning behavior for asymmetric motor activity. We measured the total number of turns toward the operated side (ipsilateral) and the intact side (contralateral) from the last 15 min recorded during the 45-min test period following the administration of methamphetamine in the MFB/6-OHDA rats ($n = 7$), the CPu/6-OHDA rats ($n = 4$), and the sham-treated rats with intra-CPu injection of saline containing 0.01% ascorbic acid (CPu/SHAM rats, $n = 6$). As shown in Fig. 4, both the MFB/6-OHDA rats and the CPu/6-OHDA rats displayed a marked occurrence of turns with ipsilateral dominance in amphetamine-induced turning behavior (significantly different from the CPu/SHAM rats, $P < 0.05$), although the frequency of turns in the MFB/6-OHDA rats (10–13 points/min) was much larger than that in the CPu/6-OHDA rats (2–4 points/min) ($P < 0.05$).

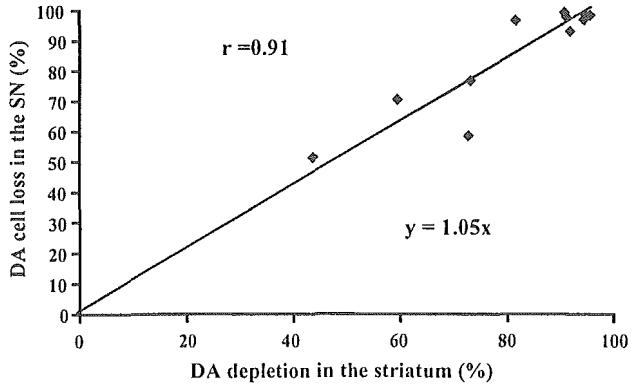

Fig. 3. Correlation between the degree of loss of DA cells in the SN and the severity of DA depletion in the striatum by 6-OHDA treatment detected from the same each rat. Each plot was obtained from the data on loss of DA cells in the SN and DA depletion in the striatum of each 6-OHDA-treated rat summarized in Table 1. Loss of DA cells in the SN was significantly correlated with DA depletion in the striatum ($r = 0.91$, $P < 0.01$), demonstrating a simple linear relation ($y = 1.05x$).

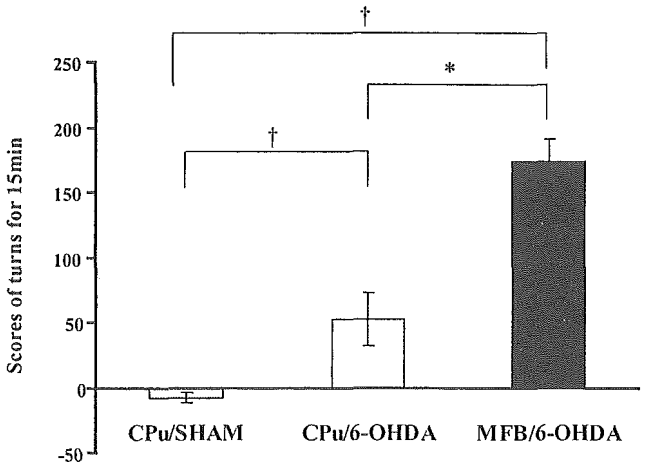

Fig. 4. Asymmetric motor activities indicated by amphetamine-induced turning behaviors in rats with unilateral intra-CPu injections of 6-OHDA (CPu/6-OHDA, $n = 4$), rats with intra-MFB injections of 6-OHDA (MFB/6-OHDA, $n = 7$), and rats with unilateral intra-CPu injections of saline containing 0.01% ascorbic acid (CPu/SHAM, $n = 6$) 3 weeks after the 6-OHDA treatment. A turn toward the operated side (ipsilateral) was scored as +1 point, while a turn toward the intact side (contralateral) was scored as −1 point. The total number of turns in the ipsilateral direction or the contralateral direction for 15 min was measured at 30 min after the administration of methamphetamine. The severity of asymmetric motor activities was expressed by net scores (ipsilateral plus contralateral). Each value represents the mean ± S.E.M. $^{*}P < 0.05$, significantly different from the CPu/6-OHDA. $^{\dagger}P < 0.05$, significantly different from the CPu/SHAM.

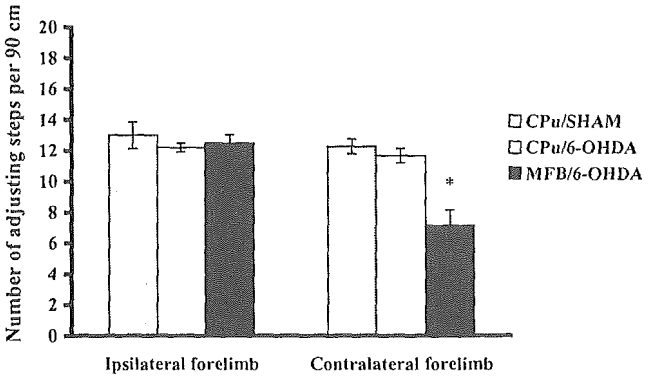

Fig. 5. Number of adjusting steps per 90 cm in the stepping test displayed by the forelimb ipsilateral or contralateral to the drug injections in rats with unilateral intra-CPu injections of 6-OHDA (CPu/6-OHDA, $n = 8$), rats with intra-MFB injections of 6-OHDA (MFB/6-OHDA, $n = 7$), and rats with intra-CPu injections of saline containing 0.01% ascorbic acid (CPu/SHAM, $n = 6$). Only the contralateral forelimb of the MFB/6-OHDA rats displayed markedly reduced adjusting steps in the test period. Each value represents the mean ± S.E.M. $^{*}P < 0.05$, significantly different from the CPu/SHAM and CPu/6-OHDA rats.

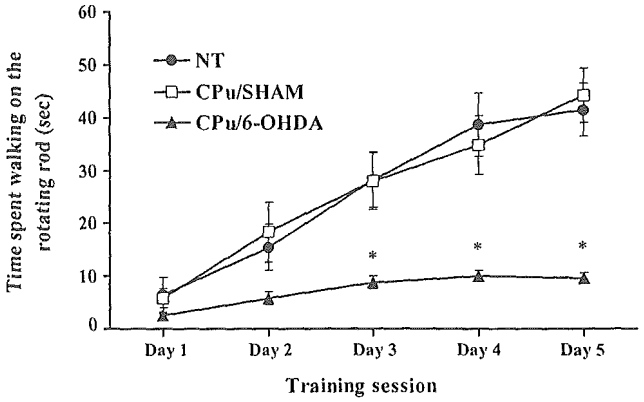

Fig. 6. Time spent walking on the rotating rod displayed by rats with bilateral intra-CPu injections of 6-OHDA (CPu/6-OHDA, $n = 13$), rats with bilateral intra-CPu injections of saline containing 0.01% ascorbic acid (CPu/SHAM, $n = 13$), and non-treated rats (NT, $n = 13$) in the rotarod test. The time spent walking on the rotating rod was measured and averaged over four trials in each training session. Each value represents the mean ± S.E.M. $^{*}P < 0.05$, significantly different from the NT and the CPu/SHAM rats.

3.1.2.2. Stepping test for adjusting steps. To assess forelimb akinesia, we performed a stepping test in the MFB/6-OHDA rats ($n = 7$), the CPu/6-OHDA rats ($n = 8$), and the CPu/SHAM rats ($n = 6$). As shown in Fig. 5, the forelimb ipsilateral to the 6-OHDA injections of both the MFB/6-OHDA rats and the CPu/6-OHDA rats displayed normal adjusting steps compared with those observed in the ipsilateral forelimb of the CPu/SHAM rats. On the other hand, in the MFB/6-OHDA rats, only the forelimb contralateral to the drug injections displayed a markedly reduced number of adjusting steps in the test ($P < 0.05$, significantly different from the CPu/6-OHDA and CPu/SHAM rats).

3.2. Behavioral impairment in rats with bilateral moderate lesions of DA cells in the SN

3.2.1. Rotarod test for acquisition of skilled behavior

To examine whether moderate disruption of DA release function in the striatum causes the impairment of acquisition of skilled behavior, we conducted the rotarod test in the rats with bilateral intra-CPu injections of 6-OHDA (CPu/6-OHDA, $n = 13$), the sham-treated rats with bilateral intra-CPu injection of saline (CPu/SHAM, $n = 13$), and the non-treated rats (NT, $n = 13$). All of the CPu/SHAM rats and the NT rats continued to improve their rotarod performance, displaying continuous increase of time spent walking on the rotating rod during the 5 days of training sessions, as shown in Fig. 6. In contrast to the rapid acquisition of skilled behavior seen in the NT and CPu/SHAM rats, the CPu/6-OHDA rats exhibited a significantly delayed improvement in their rotarod performance, displaying a slight increase of time spent walking on the rotating rod only in the early training sessions of days 2 and 3. A significant difference in time spent walking on the rotating rod between the CPu/6-OHDA and the NT or between the CPu/6-OHDA and the CPu/SHAM was observed during the sessions of days 3, 4 and 5 ($P < 0.05$).

3.2.2. Other tests for general motor functions

To investigate whether some motor functions other than the acquisition of skilled behavior were defective in the CPu/6-OHDA rats, we performed an open field test, a stepping test, a bridge test and a wire suspension test in the CPu/6-OHDA rats ($n = 7$) and the CPu/SHAM rats ($n = 6$). As summarized in Table 2, in the open field test, stepping test,

Table 2
Summary of the motor parameters measured in various behavioral tests in rats with bilateral intra-CPu injections of 6-OHDA (CPu/6-OHDA, $n = 7$) and rats with bilateral intra-CPu injections of saline (CPu/SHAM, $n = 6$)

Motor parameters	Treated groups	
	CPu/SHAM	CPu/6-OHDA
Open field test		
Locomotor activity (cm)	4789.0 ± 483.3	4163.1 ± 607.0
Stepping test		
Number of adjusting steps/90 cm		
Right forelimb	12.1 ± 0.2	11.4 ± 0.5
Left forelimb	12.4 ± 0.2	12.1 ± 0.3
Bridge test		
Latency (s)		
Day 1	180.0 ± 0.0	180.0 ± 0.0
Day 2	180.0 ± 0.0	180.0 ± 0.0
Day 3	180.0 ± 0.0	180.0 ± 0.0
Number of segments traversed		
Day 1	3.2 ± 1.9	2.1 ± 0.8
Day 2	8.6 ± 3.7	6.7 ± 2.6
Day 3	14.6 ± 5.5	12.8 ± 5.7
Wire suspension test		
Latency (s)	24.3 ± 2.6	26.6 ± 1.7

Each value represents mean ± S.E.M.

bridge test and wire suspension test, the CPu/6-OHDA rats displayed no behavioral abnormalities compared with the CPu/SHAM rats. The intact nature of the general motor functions, such as spontaneous locomotor activity, adjusting steps, equilibrium function and muscle strength, suggests that the impairment of the rotarod performance found in the rats with bilateral moderate lesions of DA cells in the SN does not involve any deficits in general motor functions assessed by these four behavioral tests.

4. Discussion

4.1. Rats with 6-OHDA-induced partial lesions of the nigrostriatal DA system as a pre-symptomatic stage model of PD

In the first experiment of this study, we investigated whether rats with 6-OHDA-induced partial lesions of the nigral DA cells could behave as a suitable model for the pre-symptomatic stages of PD. The degree of loss of TH-positive neurons in the SN showed a linear correlation with the severity of disruption of DA release function in the striatum (see Table 1 and Fig. 3). This relation implies that incomplete destruction (50–77%) of nigral DA cells in rats with intra-CPu injections of 6-OHDA directly reflects a moderate disruption of DA release function in the striatum, widely ranging from 43 to 73%, while complete destruction (93–99%) of nigral cells by intra-MFB injections of 6-OHDA corresponds to almost perfect disruption of DA release function. As for behavioral alterations, the rats with 6-OHDA-induced incomplete lesion of nigral DA cells displayed slightly frequent turning behaviors of 2–4 min^{-1} in the test for asymmetric motor activity, intact initiation of adjusting steps or motor reflex in the stepping test, and normal spontaneous locomotor activity, in contrast to the rats with complete lesion of nigral DA cells, which displayed abnormally frequent turning behaviors of 10–13 min^{-1} and significant impairment in the initiation of stepping movement (see Figs. 4 and 5). These relatively intact features or small deficiencies of general motor functions seen in the rats with partial lesions of the nigrostriatal DA system by intra-striatal injections of 6-OHDA are quite consistent with the characterization of behavioral and neurodegenerative alterations in PD patients in a pre-symptomatic stage of the disease, as previously described in an animal model with partial lesion of the nigrostriatal DA system (Kirik et al., 1998). Therefore, we conclude that rats with 6-OHDA-induced partial lesion of the nigrostriatal DA system can behave as a suitable model for the pre-symptomatic stage of PD with moderate depletion of striatal DA.

4.2. Impaired acquisition of skilled behavior seen in the pre-symptomatic stage model of PD

The rotarod test has been used to evaluate motor coordination or sensorimotor learning (Joyal et al., 1996; Jeljeli et al., 1999, 2000). In the rotarod test, the acquisition of skilled behavior, consisting of a new combination of posture and forward locomotive steps, is needed for the rats to be able to stay for a long time on the rotating rod. In the present study, the rats with bilateral partial lesions of the nigrostriatal DA system produced by intra-CPu injection of 6-OHDA displayed a drastic impairment of the acquisition of skilled behavior in the rotarod task but showed intact general motor functions, such as spontaneous locomotor activity, adjusting steps, equilibrium function, or muscle strength. This result suggests that the impaired acquisition of skilled behavior in the rotarod task observed in our pre-symptomatic stage model of PD may result purely from a deficit of sensorimotor learning in the task. In fact, it has been reported that a similar rat model of PD with a partial lesion of nigrostriatal DA cells exhibited poor scores in the skilled paw-reaching test; such poor scores were evaluated as a deficit of complex sensorimotor behavior (Barnéoud et al., 2000). The finding by Barnéoud et al. on the impairment of complex sensorimotor behavior seems to imply the involvement of impaired sensorimotor learning ability. Based on our present finding that impaired acquisition of skilled behavior rather than deficits of general motor functions predominantly occurred in rats with moderate depletion of striatal DA, we conclude that the striatum-based function underlying the acquisition of skilled behavior or the sensorimotor learning is more vulnerable to DA depletion than the DA-mediated control of motor activity.

Recent in vitro studies using whole cell recordings have demonstrated that low concentrations of DA preferentially activate D2 receptors that suppress AMPA-receptor-mediated currents, whereas high concentrations of DA activate D1 receptors that cause an increase in NMDA-receptor-mediated currents (Cepeda et al., 1993; Zheng et al., 1999). According to these findings, it is plausible that a large amount of DA release at one time, so-called phasic supply of DA, probably due to phasically responding nigral DA cells, may be enough to activate the neuronal mechanism based on these less sensitive D1 receptors in the striatum, culminating the motivational process for learning and memory.

If the striatum-based neuronal mechanism underlying the acquisition of habits and skills is proven to be general among animals from rodents to monkeys (Jog et al., 1999), the present finding that a drastic impairment of sensorimotor learning occurs in the pre-symptomatic stage model of PD while other motor functions seem to be still intact could suggest that the evaluation of behavioral tasks, including the acquisition of skilled behavior or sensorimotor learning, may be of clinical use in making a diagnosis in patients in the early or pre-symptomatic stage of PD.

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総　説

内因性カンナビノイドの役割

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はじめに

人は大昔から大麻（cannabis sativa）を薬や嗜好品として用いてきた．とくに中国やインド，ギリシャ，ローマでは紀元前から，大麻の鎮痛作用や陶酔作用を治療や儀式などに利用してきた[1]．

生体内に大麻の成分であるカンナビスの受容体が存在することが明らかになったのは1990年代になってからである．相次いで2種類のカンナビノイド受容体がクローニングされ，1型（CB1受容体），2型（CB2受容体）と命名された[2,3]．CB1受容体は中枢および末梢神経系に発現が優位で，CB2受容体は主に免疫細胞に発現している．生体内には，このCB1，CB2受容体に結合して作用を発揮する内因性リガンドのカンナビノイド（エンドカンナビノイド）が存在している．現在のところ，内因性カンナビノイドとして，アナンダマイドと2-arachidonylglycerol (2-AG)の存在がわかっている[4]．

内因性カンナビノイドと受容体の発見は，あたかもモルヒネの受容体が証明され，エンドルフィンやエンケファリンなどの内因性オピオイドの存在とその生理的役割が次第に明らかにされるようになった約20数年前を彷彿とさせるものである．最近，内因性カンナビノイド系の変化が疼痛発症やエンドトキシンショックなど各種の病態に関与していることが報告されるようになった．また，内因性カンナビノイドはシナプス可塑性にも関与することが示唆されており，麻酔科医にとってもきわめて興味深いところである．

キーワード：カンナビノイド，アナンダマイド，バニロイド受容体，炎症応答，CGRP

Review
Physiological and Pathophysiological Roles of Endocannabinoids
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1. カンナビノイド受容体

CB1受容体は中枢および末梢神経系に多く存在し，CB2受容体は主に免疫系の細胞に存在している（表❶）[4]．CB1受容体の発現量の多い領域は，海馬，大脳基底核，小脳である．これらは，記憶や学習や運動機能を司る場所であり，CB1受容体の活性化がそれらの機能に大きな影響を及ぼしている．大麻の加工品であるマリファナを多量に摂取すると，思考や感覚の異常，錯乱，幻視，記憶障害などが起こるが，これらのさまざまな精神神経作用は，その有効成分のΔ9-THCが脳内のCB1受容体に結合することにより引き起こされることがわかっている[5]．また，CB1受容体は延髄，中脳水道周囲灰白質，脊髄，脊髄後角，一次知覚ニューロンなど痛みの伝達を修飾する場所にも多く分布している[4]．細胞レベルでは主に軸索や神経終末に局在する．

CB2受容体はマクロファージ，ミクログリア，リンパ球，ナチュラルキラー細胞，肥満細胞といった免疫系細胞に主に発現しており，生体防御・免疫系で重要な働きをしている[4,6]．マクロファージや肥満細胞のCB2受容体は炎症反応や

表❶ カンナビノイド受容体の発現部位と作用
（文献4を参考にして作成）

	CB 1 受容体	CB 2 受容体
発現部位	主に神経，脳，脊髄，一次知覚神経，他	主に免疫系細胞，マクロファージ，リンパ球，肥満細胞，血小板，他
中枢作用	意識変容，記憶・認識障害，カタレプシー，鎮痛	なし
末梢作用	炎症応答 鎮痛，血管拡張，他	炎症応答 鎮痛，他

炎症性疼痛過敏に関与している[4,5]．CB 2 受容体は中枢神経には存在しない[4,7,8]．

CB 1 およびCB 2 受容体ともにGタンパク共役型受容体である．カンナビノイドがCB 受容体に結合すると，Gタンパクを介して，膜にあるadenylate cyclaseの活性化が抑制され，cyclic AMPの産生が減少する．CB 1 受容体の活性化は，電位依存性カルシウムチャネルを閉鎖し，内向き整流カリウムチャネルを活性化して，ニューロンを過分極し，神経伝達物質の放出を抑制する[4]．

2. 内因性カンナビノイド

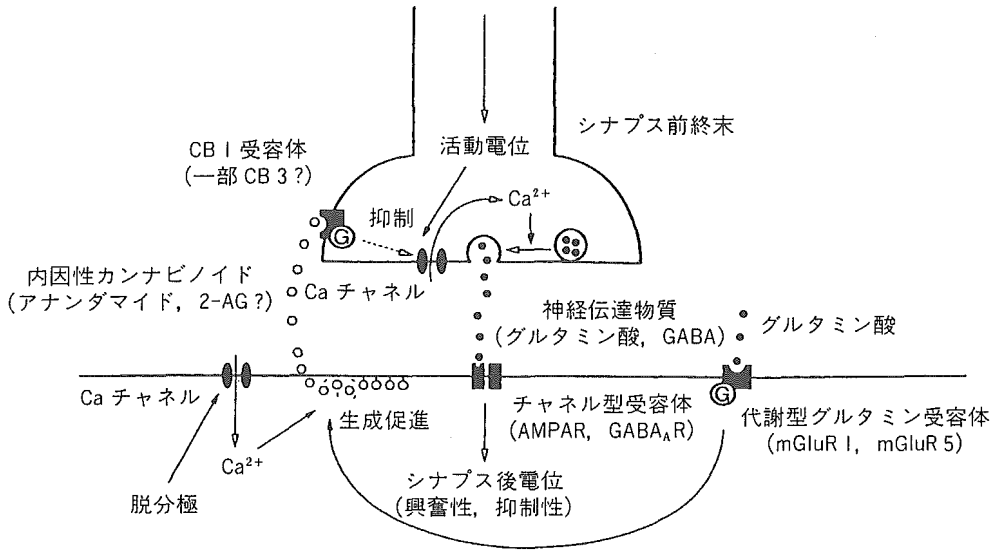
内因性カンナビノイドとして同定されているアナンダマイドと2-AGは，CB 1 受容体とCB 2 受容体の両方に作用する[4]．また，トランスポーターなどに作用して，生体に多彩な生理活性を発現させる．少量のアナンダマイドはCB 1 受容体を刺激するが，多量になるとカプサイシン受容体であるvanilloid（VR 1）受容体に作用する[9,10]．VR 1 受容体は熱感受性の非特異的陽イオンチャネルであり，一次知覚ニューロンに存在し，熱刺激や低pHを感受して電気的興奮に変え，中枢側に痛み情報を送るとともに，軸索反射を介して末梢性にCGRPなどの神経ペプチドを放出する．2-AGはCB 1 受容体への親和性は低く，主にCB 2 受容体リガンドとみなされている[4]．

内因性カンナビノイドの生理的役割は，不明な点も多いが，鎮痛や認識や記憶，嘔吐，眼内圧節，炎症，免疫調節などに関与していると考えれている（表❶）[4]．内因性カンナビノイドの生部位は，神経，血管内皮，免疫系細胞，血小などである．内因性カンナビノイドの摂取と代はきわめて速やかであることから，生理的な状では，カンナビノイド受容体が存在する部位の辺で産生され，作用を発揮し，再摂取されるとえられている[4]．

3. シナプス伝達調節と内因性カンナビノイド

内因性カンナビノイドの重要な働きとして中および末梢神経での神経伝達抑制作用があるその作用機序として神経のシナプス伝達で逆行シグナルとして機能することが示されてる[5,11-13]．図❶に逆行性シナプス伝達調節の概を示す[11]．シナプス後ニューロンが活性化するその活動情報を前シナプスニューロンに知らせ役割を内因性カンナビノイドが担っている[11]．ナプス後ニューロンで脱分極や代謝型グルタミ酸受容体の活性化が生じると，内因性カンナビイドの合成が促進され，その一部が細胞外に放されシナプス前終末のCB 1 受容体に結合る．CB 1 受容体の活性化は前シナプスからの経伝達物質の放出を抑制する[12]．

海馬や小脳では，シナプス後ニューロンの脱



図❶ 内因性カンナビノイドの放出・作用機序-逆行性シグナル
(文献 11 より引用).

極により，そのニューロンへの抑制性入力が一過性に抑制される現象 (depolarization-induced suppression of inhibition : DSI) や同様に興奮性入力が抑制される現象 (depolarization-induced suppression of excitation : DSE) が起きることがわかっており，抑制的な逆行性伝達物質の存在が示唆されていた．この逆行性伝達物質が内因性カンナビノイドであることが明らかになっている[11,12,14]．これらの現象は，短期的な一過性の効果であるが，内因性カンナビノイドは長期的なシナプス可塑性の誘導にも関与している可能性が示されている[15,16]．

4. カンナビノイド受容体と鎮痛

内因性カンナビノイド受容体 (CB 1 受容体) の脳脊髄内分布をみても，カンナビノイド系が痛みの感覚と認識機構を修飾していることが十分予測される．実際，昔から大麻には鎮痛作用があることが経験的に知られていたし，今も多発性硬化症患者の痛み軽減に使用されている[17]．また，いくつかの疼痛動物モデルで，合成カンナビノイドや内因性カンナビノイドが鎮痛効果を持つことが報告されている[5,6,18]．疼痛経路である一次知覚ニューロン，後根神経節，脊髄後角といったさまざまな場所で，CB 1 受容体のアゴニストが痛み情報の神経伝達を抑制することがわかっている[4]．また，下行性抑制系の活性化も鎮痛機序の 1 つに挙げられている[4]．

このように，経験的に，あるいは動物実験で，各種疼痛に鎮痛効果を持つことが明らかになっているにもかかわらず，臨床的な有効性は立証されていない[4,5,19]．その原因の 1 つに，意識変容や感覚異常，記憶障害といった精神神経症状が出現してしまうために全身投与しにくいという側面がある[5]．Campbell ら[19] は Δ 9-THC の全身投与による鎮痛効果はコデイン 50～120 mg に相当するに過ぎないと述べている．

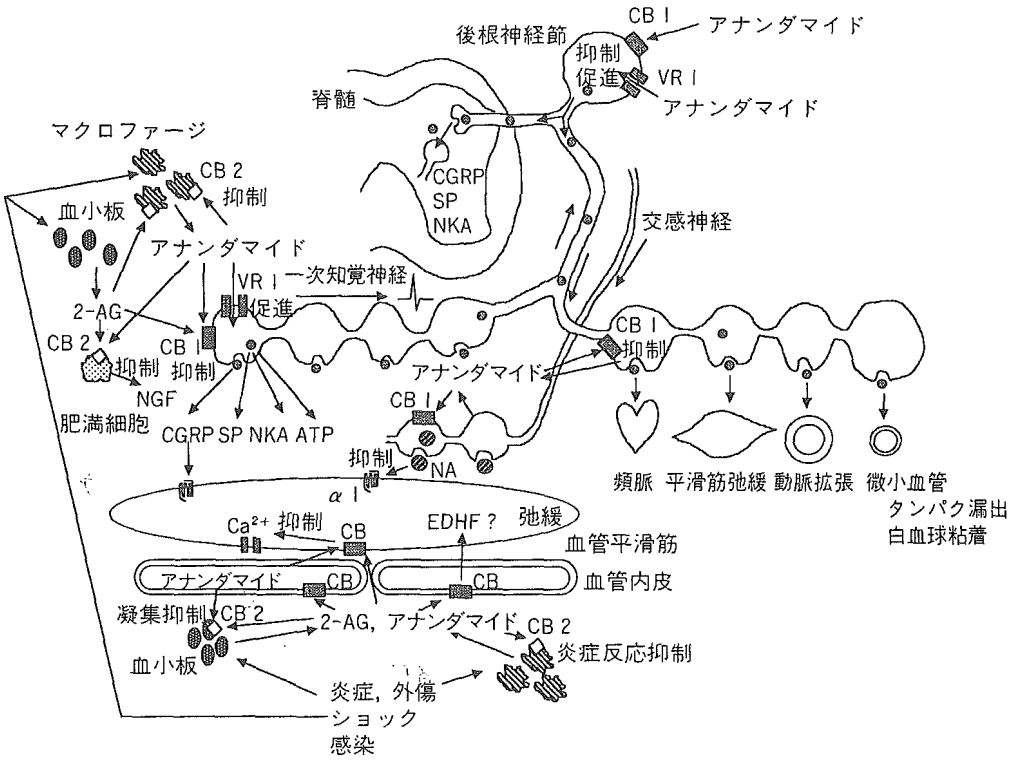
最近，Malan ら[20-22] は，CB 2 受容体アゴニストが炎症性疼痛や神経因性疼痛モデルに有効なことを報告した (図❷)．CB 2 受容体は中枢神経に存在しないことから，精神神経面への副作用の

心配がない．CB 2 受容体アゴニストの鎮痛効果の機序は，一次知覚神経への直接作用も考えられているが，主に免疫細胞や肥満細胞を介した間接的な作用である．CB 2 受容体が活性化すると，これらの細胞からケミカルメディエータの放出が抑制される．

ケミカルメディエータの放出抑制は一次知覚神経の感作を抑制する．Levi-Montalcini ら[23]は CB 2 受容体の活性化が炎症を抑えるとともに，肥満細胞から神経成長因子（NGF）の放出を抑制し，炎症性疼痛を軽減すると考えている．まだ臨床的な有用性は示されていないが，CB 2 受容体アゴニストは今後期待される治療薬である．

5. カンナビノイドの循環系への影響

カンナビノイドの循環系への作用は複雑である．麻酔の有無，投与量の違い，種差などによっ



図❷ 内因性カンナビノイドの一次知覚神経，交感神経，マクロファージ，血小板，循環系への作用（すべてが証明されているわけではなく仮説も含まれる．文献 6，24，32 を参考にして作成）．
内因性カンナビノイド（アナンダマイド）は，低濃度では血管周囲のカプサイシン感受性一次知覚神経に存在する CB 1 受容体を活性化して CGRP などの神経ペプチドの放出を抑制する．高濃度になると VR 1 受容体を活性化して神経ペプチドの放出を促進し，血管拡張や神経原性炎症を惹起する．血管平滑筋は電位依存性カルシウムチャネルの抑制，EDHF（内皮依存性過分極因子）の放出作用，gap junction への作用などから弛緩する．交感神経終末では，CB 1 受容体の活性化によりノルアドレナリン（NA）の放出が抑制される．また，CB 2 受容体が活性化されると，マクロファージや肥満細胞からのケミカルメディエータの放出が抑制される．

て循環系変化の程度や時間的推移は大きく異なる[10,24]．一般的に，アナンダマイドを投与すると血圧の上昇と下降，徐脈，血管拡張が起きる[24]．これらの循環変化は，血管や心臓に対する直接的な作用より，交感神経や知覚神経やマクロファージ系細胞を介した間接的な作用によるものが大きい．交感神経終末では前シナプス性に交感神経活動が抑制される（図❷）．中枢性には交感神経活動を亢進する作用もあり，血圧上昇の1つの機序に挙げられる[25]．血管への直接作用は拡張作用である．CB1受容体を介する電位依存性カルシウムチャネル抑制作用や過分極因子放出作用，gap junctionへの作用などが拡張作用の機序に挙げられている[10,24]．

アナンダマイドは，血管周囲のカプサイシン感受性一次知覚神経に存在するCB1受容体とVR1受容体の両者を介して，血管に対して2つの相反する作用を発揮する[10,26]．知覚神経のCB1受容体が刺激されるとCGRP，ATP，サブスタンスP，ニューロキニンAといった神経ペプチドの放出が抑制される（図❷）[10]．一方，VR1受容体が刺激されるとこれらの神経ペプチドの放出が促進される．結果として，アナンダマイドはCB1受容体を刺激して血管拡張を抑制し，VR1受容体を刺激して血管拡張を引き起こすことになる．この作用は濃度依存性に起きると考えられている[10]．低濃度ではCB1受容体刺激作用が主で，高濃度になるとVR1受容体刺激作用が主になる．ラット下肢皮膚血管を用いた研究で，アナンダマイドは，nMレベルの低い濃度では基本量のCGRP放出には影響を及ぼさないが，CB1受容体を刺激してカプサイシン誘発性CGRP放出を抑制し，1 μMを超える濃度になるとVR1受容体を刺激してCGRPを放出することが示されている[27]．また，CB1受容体拮抗薬を投与すると知覚神経由来の血管拡張が増幅されることから，知覚神経そのものから放出されるアナンダマイドがCB1受容体を介して，負のフィードバックをかけていることが示唆されている[28]．

このように，内因性カンナビノイドであるアナンダマイドは，VR1受容体よりCB1受容体に対して強い親和性を持ち，生理的な低用量では知覚神経の興奮を抑え，カプサイシン誘発性の神経原性炎症反応を抑制している．病的な大量のレベルに達すると，VR1受容体が刺激され知覚神経から血管拡張物質や透過性亢進物質が放出され，炎症や病態の進行を加速するようになる．

6. 内因性カンナビノイドとショック

Wagnerら[29]はラットを用いて，出血性ショック時にマクロファージが活性化されアナンダマイドが放出されること，ショック時の低血圧がCB1受容体拮抗薬で回復することを示した．Vargaら[30]はエンドトキシンショック時にマクロファージからアナンダマイドが，血小板から2-AGが放出されることを示した．また，Wangら[31]はエンドトキシンショック患者の血中にアナンダマイドと2-AGが増加していることを報告した．この報告の共著者であるMaruyamaは，内因性カンナビノイドがエンドトキシンショックの早期メディエータであるという考えを提唱している[32,33]．

病原微生物が生体内に侵入するとマクロファージ系細胞が動員され応答する．その応答は病原微生物の種々な分子（pathogen associated molecular patterns：PAMPs）をtoll-like receptor（TLR）が認識することで始まる．たとえばグラム陰性菌のエンドトキシン（LPS）は，TLR-4と反応し，最終的にはNF-κBやCRP，フィブリノーゲン，細胞接着因子，細胞増殖因子，炎症性サイトカインが発現してくる[33]．しかし，これらの遺伝子介在型の反応の前に，アナンダマイドが産生され応答すると考えられる．LPS刺激後の数分で産生，放出されるので，より早期の生体防御反応が可能となる．血小板は2-AGを産生する[34]．もし，PAMPsが過剰になると，大量の

カンナビノイドが産生され，それが生体の諸システムを破綻に導く．その代表的な病態が敗血症性ショックであるとMaruyamaら[32,33]は指摘している．さらに彼らは，アナンダマイドや2-AGを除去することがショックの病態を改善することを報告した[35,36]．すなわちLPS吸着療法に用いられるポリミキシンBはアナンダマイドと2-AGを吸着することで治療効果を発揮すると考えられている[36]．

おわりに

内因性カンナビノイドの生理的，病態生理的役割について述べた．内因性カンナビノイドは精神神経作用だけでなく鎮痛や炎症でも重要な役割を担っており，治療面からも注目されている．Maruyamaが提唱しているエンドトキシンショックの早期メディエータとしての生体防御的役割や吸着療法にはきわめて興味深いものがある．また，神経細胞死の抑制効果[37,38]や心筋虚血再灌流障害の防止効果[39]なども報告されている．このように内因性カンナビノイドの多彩な作用が次第に明らかになってきたが，生体内での役割は複雑で不明な点も多く，今後の解明が期待される．

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Influence of inhalation anesthesia assessed by comprehensive gene expression profiling☆

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Abstract

Although general anesthesia is routinely used as an essential surgical procedure and its harmlessness has been evaluated and endorsed by clinical outcomes, little is known about its comprehensive influence that is not reflected in mortality and morbidity. In this paper, we have shown that inhalation anesthesia affected the expression of < 1.5% of > 10,000 genes, by analyzing the expression profiles for multiple organs of rats anesthetized with sevoflurane. The small number of transcripts affected by the inhalation anesthesia comprised those specific to single and common in multiple organs. The former included genes mainly associated with drug metabolism in the liver and influenced by agents such as amphetamine in the brain. The latter contained multiple circadian genes. In the brain, we failed to detect the alteration of the clock gene expression with the exception of *Per2*, assuming that anesthesia perturbs circadian rhythms. Our findings provide the first assessment for the influence of inhalation anesthesia by approaches of experimental biology and genome science.

Keywords: General anesthesia; Gene expression profile; DNA microarray; Circadian gene; Endothelin1; *Per2*

1. Introduction

Inhalation anesthetics induce general anesthesia that results in unconsciousness, insensitivity to pain, and lack of memory of pain. Although general anesthesia has been routinely used as an essential surgical procedure for approximately 150 years and its harmlessness has been evaluated and endorsed by clinical outcomes (Forrest et al., 1990; Levy, 1984; Brown and Frink, 1993), little is known with regard to its comprehensive influence, which is not reflected in mortality and morbidity. A few studies were reported on the results of inhalation anesthesia on cells and tissues at the molecular level. This anesthetic acted upon an extremely restricted number of genes including those that control the expression of ligand-gated ion channels and G-protein-coupled receptors (Franks and Lieb, 1994; Harris et al., 1995).

The recent progress in genomics enables us to comprehensively describe and analyze the alteration in cells and tissues at the gene expression level by hybridization with DNA microarrays representing genome-wide or subgenome-wide species of transcripts. Therefore, we attempted to comprehensively analyze the effects of

Abbreviations: Arc, activity-regulated cytoskeleton; Bmal1, Brain-Muscle-Arnt-Like-protein 1; Cry2, Cryptochrome2; Dbp, albumin site d-binding protein; Egr1, early growth response gene-1; NGFI-B, nerve growth factor-induced gene B; PCR, polymerase chain reaction; Per2, Period2; SD, standard deviation; Tef, thyrotroph embryonic factor.

☆ Data from all the arrays used in this paper will be available at DDBJ via CIBEX (http://www.cibex.nig.ac.jp/cibex/HTML/index.html) under accession nos.: for the array design, CBX4; for the experiments, CAR4.

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inhalation anesthesia at the molecular level by obtaining gene expression profiles from rats under inhalation anesthesia.

In this study, we have shown that inhalation anesthesia affected expression of an extremely limited number of genes. This was done by analyzing expression profiles for multiple organs of rats at different time points after anesthetizing with sevoflurane. Our findings could provide a basis for exploring comprehensive influence of inhalation anesthesia and to endorse the safety of anesthesia in the future. The expression profiling analyses also demonstrate the possible association between the anesthetic status and alteration in modulation of the circadian gene expression that were previously identified in addition to nominating novel candidates for the circadian gene.

2. Materials and methods

2.1. Animals and tissue collection

Six-week-old male rats (Wister; Charles River Japan, Inc., Atsugi, Japan) were purchased and adapted to a 12-h light/12-h dark cycle starting at 06:00 and 18:00 for a week before the experiments. The anesthesia experiment was replicated twice with an interval of several weeks between the two experiments (Exp. 1 and Exp. 2). Anesthesia was used at 09:00 on nine rats in Exp. 1 and on 10 rats in Exp. 2. Rats were housed in a plastic box supplied with sevoflurane (4.5% air mixture gas) at the rate of 6 L/min. As the 0-h control, rats ($n=3$, in Exp. 1; $n=4$, in Exp. 2) were subjected to tracheal intubation immediately after induction of anesthesia in order to maintain the anesthetic status and sacrificed to obtain organs. The organs were obtained in the order of the blood, the spleen, the kidneys, the liver, the lungs, the heart, and the brain within 5–10 min per animal after the intubation. As the 2-h and 6-h samples, the anesthetized rats were picked up from the plastic box supplied with sevoflurane, subjected to the intubation, and sacrificed to similarly obtain the organs 2 h and 6 h after induction of anesthesia, respectively. Three rats were used for each time point of each experiment with the exception of the time point of 0 h in Exp. 2, in which four rats were used; eventually, a total of 19 rats were used in the two replicated experiments.

2.2. RNA preparation

The blood obtained was immediately mixed with an ISOGEN-LS reagent (NIPPON GENE, Tokyo, Japan) after dilution with an equal volume of water. The other organs (the whole brain, the whole heart, the left lung, the lateral left lobe of the liver, the whole spleen, and both the kidneys) were immediately frozen in liquid nitrogen and lysed with an ISOGEN reagent (NIPPON GENE). Total RNA was prepared from the lysate in accordance with the manufacturer's instructions. Poly(A)+ RNA was prepared from total RNA with a Poly(A) Purist Kit (Ambion, TX, USA), in accordance with the manufacturer's instructions.

2.3. Microarray preparation and expression profile acquisition

A set of synthetic polynucleotides (80-mers) that represented 11,464 rat transcripts derived from 10,490 independent genes, including most of the RefSeq clones deposited in the NCBI database (MicroDiagnostic, Tokyo, Japan), was arrayed on a slide glass (S9115; Matsunami, Kishiwada, Japan) with a custom-made arrayer (Kobayashi et al., 2004; Ito et al., 2003). Poly(A)+ RNA (2 μg) was labeled with SuperScript II (Invitrogen, CA, USA) and Cyanine 5-dUTP for each sample or Cyanine 3-dUTP (PerkinElmer, MA, USA) for a rat common reference RNA (MicroDiagnostic). Labeling, hybridization, and subsequent washes of microarrays were performed with a Labeling and Hybridization Kit (MicroDiagnostic), in accordance with the manufacturer's instructions. The rat common reference RNA was purchased as a single batch and labeled as an aliquot with Cyanine-3 for a single microarray side by side with each sample which was labeled with Cyanine-5. Hybridization signals were measured using a GenePix 400A scanner (Axon Instruments, CA, USA) and then processed into primary expression ratios ([Cyanine 5-intensity obtained from each sample]/[Cyanine 3-intensity obtained from the rat common reference RNA], which are indicated as 'median of ratios' in GenePix Pro 3.0 software (Axon Instruments)). Normalization was performed for the median of ratios (primary expression ratios) by multiplying normalization factors calculated for each feature on a microarray by the GenePix Pro 3.0 software. All the data in accordance with the MIAME guideline were deposited at DDBJ via CIBEX (http://www.cibex.nig.ac.jp/cibex/HTML/index.html) under accession numbers CBX4 (for the array design) and CAR4 (for the experiments).

2.4. Data analysis

Data processing and subsequent hierarchical clustering analysis were performed with an Excel program (Microsoft, WA, USA) and an MDI gene expression analysis software package (MicroDiagnostic). The primary expression ratios were converted into $\log_2$ values ($\log_2$ Cyanine-5 intensity/Cyanine-3 intensity) (designated log ratios) and compiled into a matrix (designated primary data matrix).

We conducted the following operations to extract genes, from the primary data matrix, in which the expression levels altered specifically on inhalation anesthesia. (i) The mean average of log ratios for each 0-h data set of each organ (designated 0-h averages) was calculated. (ii) The relative ratios against the respective 0-h average values for all the log ratios (designated relative log ratios) were generated. This enabled us to compare all data as expression differences that deviated from the mean average

of each gene and to introduce a single threshold value (=0.75) to filter the genes in which the expression levels altered. (iii) All the relative log ratios were arranged into a matrix (designated secondary data matrix). (iv) In order to filter out the genes in which the expression levels deviated from the mean average in an individual data set for each gene, each time point, and each organ, we calculated standard deviation of relative log ratios for each time point and each organ, using the data from all the animals (Exp. 1 and Exp. 2). The SD values calculated by every organ showed similar distributions and there was no obvious correlation between the SD values and the fluorescent intensities initially detected in each microarray. This enabled us to introduce a single SD value (=1.0) as a cutoff threshold for all the genes. The calculated value should reflect the differences between independently repeated anesthetic experiments and the responses of individual rats. (v) The genes with standard deviation of the relative log ratios greater than 1 for at least one time point for each organ were detected from the secondary data matrix. (vi) The genes with relative log ratios commonly greater than 0.75 or uniformly smaller than −0.75 in each organ among at least five individual rats, which had an identical anesthetic period (2 h or 6 h), were selected from the secondary data matrix.

Next, we extracted genes, in which expression patterns were specific to a single organ or common to multiple organs from the secondary data matrix by the following operations. (i) The mean average of log ratios for 2 h or 6 h for each organ (designated 2-h and 6-h averages) was calculated. (ii) The relative ratios of 2-h and 6-h averages against 0-h average for each organ (designated relative 2-h and 6-h averages) were generated. (iii) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in a single organ were selected. (iv) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in any two organs were selected. (v) Genes with relative 2-h average or relative 6-h average was greater than 0.75 or lower than −0.75 in any three organs were selected. (vi) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in any four organs were selected. (vii) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in more than four organs were selected.

3. Results

In order to comprehensively evaluate the influences of general anesthesia at the gene expression level, we obtained seven major organs from rats under general anesthesia with sevoflurane, an inhalation anesthetic, at 0 h, 2 h, and 6 h after induction of anesthesia. We performed the animal experiment with sevoflurane twice with an interval of several weeks between the two experiments (designated as Exp. 1 and Exp. 2). In Exp. 1, we used three rats for each time point and obtained for the seven organs but failed to draw peripheral blood from a rat assigned for 2 h. In Exp. 2, we used four rats for 0 h and three rats for 2 h and 6 h, respectively, and failed tissue lysate preparation from the lungs and liver of a rat assigned for 0 h. Eventually, we obtained 130 independent tissue samples for the three time points. We labeled poly(A)+ RNA purified from the samples and a rat common reference RNA with Cyanine-5 and Cyanine-3, respectively, and hybridized to microarrays representing 11,464 transcripts derived from 10,490 individual genes. Hybridization signals were processed into expression ratios as $\log_2$ values (designated log ratios) and compiled into a matrix designated as the primary data matrix (see Materials and methods). Data from all the arrays used in this paper are available at DDBJ via CIBEX (http://www.cibex.nig.ac.jp/cibex/HTML/index.html) under accession numbers CBX4 (for the array design) and CAR4 (for the experiments).

3.1. Overview of gene expression patterns in rat tissues after being subjected to general anesthesia

First, we sought to seize an overview of the experiments conducted in this study by two-dimensional clustering analysis of log ratios calculated from primary expression ratios against the common reference RNA (primary data matrix), prior to extracting genes that were affected by inhalation anesthesia at the expression level. The primary data matrix should enable us to relatively compare all samples one another without conducting direct comparison by hybridization on an identical microarray. We predicted that the most obvious differences obtained from the clustering analysis of the primary data matrix should be those reflecting tissues examined in this study, represented as sample clusters consisting of each organ only (tissue clusters) in a single dendrogram. Furthermore, we presumed that we might be able to compare gene expression levels that were associated with different experimental conditions within each tissue cluster in the dendrogram.

We extracted genes with log ratios over 1 or under −1 in at least one sample from the primary data matrix and subjected them to two-dimensional hierarchical clustering analysis for samples and genes (Fig. 1) (Schena et al., 1996; Lyons et al., 2000). When the clustering analysis for the samples was performed, the greatest seven clusters corresponding to the individual organs were obtained as predicted. The clusters representing the lungs, heart, liver, and blood comprised two smaller clusters completely corresponding to differences between Exp. 1 and Exp. 2, reflecting experimental errors. The clusters representing the kidney and brain consisted of two smaller clusters that incompletely corresponded to the experiment differences with some exceptions. With the exception of the smaller cluster in Exp. 1, representing the liver, in which three