in m8Δ-pSFJ-JRCSFenv-infected cells. The gp160/gp120 isolated from m8Δ-pSFJ-JRCSFenv-infected cells migrated more quickly and showed a broader band in PAGE gels than that from m8Δ-p7.5-JRCSFenv-infected cells. This suggests the incomplete glycosylation of gp160/gp120 due to an insufficiency of host glycosyltransferases. Nevertheless, the impact on the ability of m8Δ-pSFJ-JRCSFenv to elicit anti-Env antibody response was minimal. Although m8Δ-pSFJ-JRCSFenv induced greater production of Env binding Abs than m8Δ-p7.5-JRCSFenv, it did not induce the production of more potent anti-HIV-1 neutralizing Abs. At 8 weeks after rSeV-JRCSFenv boost, the average ID50 of serum from mice immunized with m8Δ-pSFJ-JRCSFenv was higher than that of serum from mice immunized with m8Δ-p7.5-JRCSFenv; however, the difference was not significant. This suggests that, in addition to the amount of expressed Env, the properties of the antigen (for example, the structure of the exposed epitopes) may also be important for the induction of neutralizing antibody production. There was no difference in the avidity of the anti-Env antibodies between the two groups (Fig. 3C), implicating that the process of affinity maturation was similar. This suggests that affinity maturation of antibodies is necessary, but not sufficient to induce the production of potent neutralizing antibodies. Even so, higher levels of Env binding antibodies may enable the induction of other types of antiviral immunity, such as antibody-dependent cellular cytotoxicity and antibody-dependent cell-mediated virus inhibition.

We recently showed that priming mice with an m8Δ that expresses both CD40Lm and Env induces the production of high-avidity anti-Env antibodies [2]. The above results suggest that it might be important to incorporate an adjuvant, such as CD40Lm, within the AIDS vaccine regimen to induce more potent humoral responses and produce higher levels of neutralizing antibodies.

A successful AIDS vaccine should induce the production of long-lasting antibodies. Both m8Δ-pSFJ-JRCSFenv and m8Δ-p7.5-JRCSFenv induced the production of long-lasting anti-Env antibodies when used in the rm8Δs prime/rSeV boost regimen. Immunized mice maintained high levels of anti-Env antibodies for up to 28 weeks (Fig. 3D). This supports our previous report showing that the rm8Δs prime/rSeV regimen is a good platform for the development of an HIV-1 vaccine.

Safety is critical when evaluating vaccines in clinical trials. Both m8Δ-pSFJ-JRCSFenv and m8Δ-p7.5-JRCSFenv were less virulent in newborn mice than the parental strain, LC16m8Δ. LC16m8Δ was more virulent probably because it contains an intact HA gene. The LD50 of m8Δ-p7.5-JRCSFenv was significantly lower than that of m8ΔVNC110, although their growth potential was similar. This suggests that the expression of HIV-1 Env in the mouse brain is harmful. This is supported by the fact that that virulence of m8Δ-pSFJ-JRCSFenv is similar to that of m8ΔVNC110, despite having a much lower capacity for replication. Nevertheless, our finding that recombinant VVs expressing HIV-1 env are safer than LC16m8Δ suggests that they may be promising candidates for clinical trials.

In conclusion, the results of the present study suggest that VV m8Δ vectors containing different promoters activate different arms of the immune system. That said, both strains induced long-lasting CTL and antibody responses and both appear safe enough for clinical trials. Thus, it is possible to manipulate the immune response induced by a rational AIDS vaccine by using VV m8Δs harboring different promoters.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.12.022.

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Continuous infusion of rFVIIa during surgery in a FVII-deficient patient: a case report from Japan

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FVII deficiency is a rare, autosomal recessive, congenital coagulation disorder that affects both men and women. The clinical conditions are variable, ranging from asymptomatic to severe lethal haemorrhages. However, in a minority of patients, more significant bleeding problems have been observed, including spontaneous joint bleeding resulting in advanced arthropathy. In such cases, orthopaedic surgery may be required. There were some reports in which recombinant activated factor VII (rFVIIa) was used for the management of surgery in patients with FVII deficiency. Such experiences with the haemostatic control with rFVIIa during surgery are invaluable when discussing the optimal treatment regimen, mode of administration and target plasma FVII:C levels. With regard to the mode of administration, frequently repeated bolus infusions of rFVIIa have been adopted in many reports [1–3,5], and the continuous infusion of rFVIIa was described in a few reports [4,5]. We herein report the haemostatic control of a FVII-deficient patient by the continuous infusion of very small rFVIIa doses, while monitoring the plasma FVII:C levels during left total hip replacement.

A 38-year-old female with severe congenital FVII deficiency (FVII level = 0.005 IU mL^{-1}, body weight = 49 kg) had severe arthropathy caused by spontaneous joint bleeding and her menstrual bleedings had been serious. A right total hip replacement had been performed several years earlier with bolus infusions of plasma-derived FVIIa. Her left hip had since been destroyed (Arnold-Hilgartner stage V) and caused limitations of her activity of daily living. Therefore, a left total hip replacement had been conducted on her.

Prior to surgery, the pharmacokinetics of rFVIIa: Novoseven® (Novo Nordisk, Bagsvaerd, Denmark) was investigated in this patient. 1.2 mg of rFVIIa, corresponding to 24 μg (1.2 KIU) kg^{-1} body weight for a 49 kg person, was intravenously administered with a sufficient washout period, and blood samples were collected before the infusions 10, 120 and 240 min after the injection, and a FVII clotting activity (FVII:C) assay was performed (Table 1). Plasma concentration values of FVII:C were 20.65, 5.41 and 1.52 IU mL^{-1}, respectively. The calculated half-life of rFVIIa with a one-compartment model in this patient was approximately 1.01 h, which was shorter than

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Table 1. The rFVIIa loading test using factor VII:C (IU mL^{-1}).

	Factor VII:C (IU mL^{-1})			
	Time (min)			
Dose	0	10	120	240
24.5 μg kg^{-1} (1.2 KIU kg^{-1})	0.005	20.65	5.41	1.52

Table 2. The FVII:C levels on Days 0–3 (IU mL^{-1}).

	Time (days)	FVII:C level (IU mL^{-1})	Prothrombin time (s)	Continuous infusion rate (μg kg^{-1} h^{-1})	Bolus injection (mg)
0	Just before surgery	5.45	8.6	2	0.1*
	Just after surgery	2.22	8.7	2	
	3 h after surgery	1.71	9.0	2	
1		1.94	8.7	2	
2		1.44	9.0	2 to 1	
3		1.28	9.0	1	
4				1	
5				1	
6				1	
7					0.6
8					0.6
9					0.6
10					0.6

*The bolus injection was given a few minutes prior to surgery, followed by the start of the continuous infusion.

the previously calculated half-life [6]. However, the previous half-life was derived from a two-compartment model, therefore, it was not possible to simply compare the half-lives.

According to these pharmacokinetic findings, which included FVII:C level and a shorter half-life of rFVIIa, a haemostatic control regimen was planned as follows. The reconstituted rFVIIa (1.2 mg rFVIIa was dissolved in 12 mL sterile water) was used economically and carefully. Day 0: a bolus injection of 0.1 mg rFVIIa (1 mL, 2 μg kg^{-1}) was given a few minutes prior surgery, and continuous infusion also started at rate of 0.1 mg h^{-1} (2 μg kg^{-1} h^{-1}) to achieve and maintain 1 IU mL^{-1} of FVII activity at the time of surgery. Days 1–2: The same continuous infusion rate (2 μg kg^{-1} h^{-1}) was continued. Days 3–6: The continuous infusion rate was reduced to 1 μg kg^{-1} h^{-1}. Days 7–10: 0.6 mg (12 μg kg^{-1}) of rFVIIa was injected as a bolus once a day. For example, 1 ml (2 μg kg^{-1}) of reconstituted rFVIIa (12 mL) was injected as a bolus, and the remaining rFVIIa (11 mL) was used continuously on Day 0. Moreover, reconstituted rFVIIa was not used beyond 24 h.

As a result, the bleeding during the perioperative period was controlled very well, without the need for additional infusion of rFVIIa. The rFVIIa was used according to this haemostatic plan and the total consumption of rFVIIa was 12 mg (10 vials with 1.2 mg per vial).

The FVII activity and prothrombin time was monitored several times during the perioperative period in our hospital laboratory. Before and just after the operation, the level of FVII:C was 5.45 and 2.22 IU mL^{-1} respectively. At 3 h after the operation, the time of maximum bleeding risk, the FVII:C level was 1.71 IU mL^{-1}. From Day 1 to Day 3, a satisfactory level of FVII:C was maintained, at 1.94 IU mL^{-1}, 1.44 IU mL^{-1} and at 1.28 IU mL^{-1} respectively (Table 2). We empirically assessed that the reconstituted rFVIIa was stable and microbiologically safe in a syringe kept at room temperature as long as it was used within 24 h. In addition, the rFVIIa activity was also maintained.

On Day 10, the patient was discharged from the hospital, and rehabilitation continued in outpatient care with bolus infusions of rFVIIa. During this hospitalization period, there were no complications such as unexpected bleeding episodes, bacterial infection, symptomatic thrombosis, thrombophlebitis or the development of inhibitory antibodies. There have also been no complications during the follow-up period (6 months after surgery).

There have been very few publications about the experience of administering rFVIIa as a continuous infusion for FVII-deficient patients. In some of the previous studies, major surgeries (including total hip arthroscopy) were performed following bolus injection of rFVIIa [1,2].The total doses of rFVIIa they used in those studies were higher (16–37.5 mg, 864–960 μg kg^{-1} respectively) than those used in our procedure (12 mg, 244 μg kg^{-1}), which implies considerable savings in terms of rFVIIa. In addition, in another report, there were three bleeding complications reported in six orthopaedic surgeries [5].

Theoretically, the continuous infusion of concentrates is a more economical therapy compared with bolus infusion. Furthermore, the bolus style of rFVIIa needs to be injected frequently compared to continuous infusion, because of the short half-life of rFVIIa. However, haematologists have been concerned about the stability after reconstitution and adsorption in the infusion system. However, the rFVIIa activity of Novoseven® has been previously reported to biochemically stable for at least 24 h after reconstitution *in vitro* at 19.3–20.7°C, with no clinically significant changes in clot activity, solution constituents or concentrations [7].

In the other study in which the continuous infusion of rFVIIa was adopted during major surgery, the levels of FVII:C ranged from 0.45 to 0.55 IU mL^{-1} with no bleeding or thromboembolic complications. The level of FVII:C in this study ranged from 1.71 to 5.45 mL^{-1}

for the first 4 days, which was higher than that in the previous study (Table 2). Thromboembolic complications have been reported with plasma-derived FVII or prothrombin complex concentrates in FVII-deficient patients [8]. The appropriate level of FVII:C for surgery in FVII-deficient patients has not yet been determined, however, it might be safer to decrease the infusion rate from Day 0 to reduce the risk of thrombosis. In addition, close monitoring of the patient is necessary, because there may be variability due to individual pharmacokinetic differences and surgical procedures.

In conclusion, we have demonstrated that continuous infusion of rFVIIa is able to allow for stable, effective haemostasis with no adverse events during and after surgery, and seemed to be more favourable compared with bolus injection in patients with severe congenital FVII deficiency. Furthermore, continuous infusion reduces the total consumption of rFVIIa and contributes to huge cost savings during major surgery in FVII-deficient patients.

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Disclosures

The authors Azusa Nagao and Hideyuki Takedani stated that they had no interests which might be perceived as posing a conflict or bias.

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ORIGINAL ARTICLE *Clinical haemophilia*

Self-monitoring has potential for home exercise programmes in patients with haemophilia

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Summary. Haemophiliacs who have had to keep a physically inactive lifestyle due to bleeding during childhood are likely to have little motivation for exercise. The purpose of this study is to clarify the effectiveness of the self-monitoring of home exercise for haemophiliacs. A randomized controlled trial was conducted with intervention over 8 weeks at four hospitals in Japan. Subjects included 32 male outpatients aged 26–64 years without an inhibitor who were randomly allocated to a self-monitoring group and a control group. Individual exercise guidance with physical activity for improvement of their knee functions was given to both groups. The self-monitoring materials included an activity monitor and a feedback system so that the self-monitoring group could send feedback via the Internet and cellular phone. The self-monitoring was performed by checking exercise adherence and physical activity levels, bleeding history and injection of a coagulation factor. Both groups showed significant improvements in exercise adherence ($P < 0.001$) and physical function such as the strength of knee extension ($P < 0.001$), range of knee extension ($P < 0.001$), range of ankle dorsiflexion ($P < 0.01$), a modified Functional Reach ($P < 0.05$) and 10 metre gait time ($P < 0.01$). In particular, improvements in exercise adherence ($P < 0.05$), self-efficacy ($P < 0.05$), and strength of knee extension ($P < 0.05$) were significant in the self-monitoring group compared with those in the control group. No increase in bleeding frequency and pain scale was noted. The self-monitoring of home exercise for haemophilic patients is useful for the improvement of exercise adherence, self-efficacy and knee extension strength.

Keywords: exercise adherence, home exercise, physiotherapy, randomized controlled trial, self-efficacy, self-monitoring

Introduction

In haemophilia, 65–80% of all bleeding episodes are intra-articular, and 80% of them are predominantly localized in elbows, ankles and knees [1]. Recurrent intra-articular bleeding occurs with arthropathy, which progressively leads to muscle atrophy around the joints [2]. Immobility after bleeding results in a vicious cycle, where muscle atrophy and articular contracture lead to joint instability, re-bleeding and limitations of daily activity [2]. The goal for prevention of arthropathy is to break this vicious circle by effective haemostatic therapy and physiotherapy [3]. Even the haemophiliacs whose joints are free from arthropathy show a decrease in physical condition, muscular strength, aerobic resistance, anaerobic resistance and proprioception [1]. Therefore, an active lifestyle, continuous movement in childhood and weight-bearing exercises in youth have been suggested for patients' well-being [1]. However, haemophiliacs who had to keep to a physically inactive lifestyle due to bleeding during childhood are likely to have little motivation for exercise. Therefore, their adherence to home exercise is not good because of the fear of bleeding [4]. For effective home exercise, behavioural changes and improvements in exercise adherence are required and the improvement of self-efficacy is important [5]. Self-monitoring is known to be an effective method for behavioural change [6] and has been available for patients with hypertension [7], ischemic heart disease

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[8] and diabetes[9], but not for those with haemophilia. This study was designed to clarify the effectiveness of self-monitoring on home exercise for haemophiliacs who might need behavioural changes.

Materials and methods

A prospective, controlled, randomized non-blind, comparative study was conducted with intervention for 8 weeks at four hospitals in Tokyo (Fig. 1). Originally, 37 haemophilic outpatients aged 26–64 years without inhibitors were selected from the public and participated in this study, and patients with dysbasia, a history of orthopaedic surgery within the last 6 months and/or insufficient fluency in operating a mobile phone were excluded from this study. All patients were randomly allocated to a self-monitoring group and a control group using random number tables prepared at an individual hospital. Patients in both groups received home exercise guidance and an activity monitor (HJA-350IT; Omron Corp., Kyoto, Japan), and those in the self-monitoring group were given a self-monitoring approach. Using questionnaires and the assessment of physical functions, we measured and compared the physical condition of patients at baseline and post intervention.

Individual guidance of the home exercise programme (Fig. 2)

Guidance about the strengthening of the knee extension, static stretching for knee flexor muscles and standing balance training as well as advice for the promotion of physical activities (PA) were given individually by a physiotherapist to improve knee function. The strength of knee extension training included the following four methods: isometric exercise at a long-sitting position, resistive training at sitting position, a half squat with both legs at standing position and a half squat with a single leg at standing position. Among these training methods, a physiotherapist recommended the exercise most appropriate to the physical condition of each patient, and the patient performed the exercise at least 10 times per day. The physiotherapist also instructed the patient in static stretching exercises to keep flexion of the hip in the position of ankle dorsiflexion and knee extension by having the patient hold the position for 20 s at a long-sitting position, and the patient performed the static stretching exercise at least five times per day. Balance training was chosen from the following, and performed at least three times per day: moving the body's centre of gravity (COG), stepping and standing balance exercises with a single leg. Moving COG was done in a lateral direction and in an anteroposterior direction at a standing position with both legs. Stepping was performed in anteroposterior direction at a standing position. For the strength of PA, walking, leading an active life and doing non-contact sports were recommended according to Exercise Guide 2006 provided by the Ministry of Health, Labor and Welfare in Japan [10].

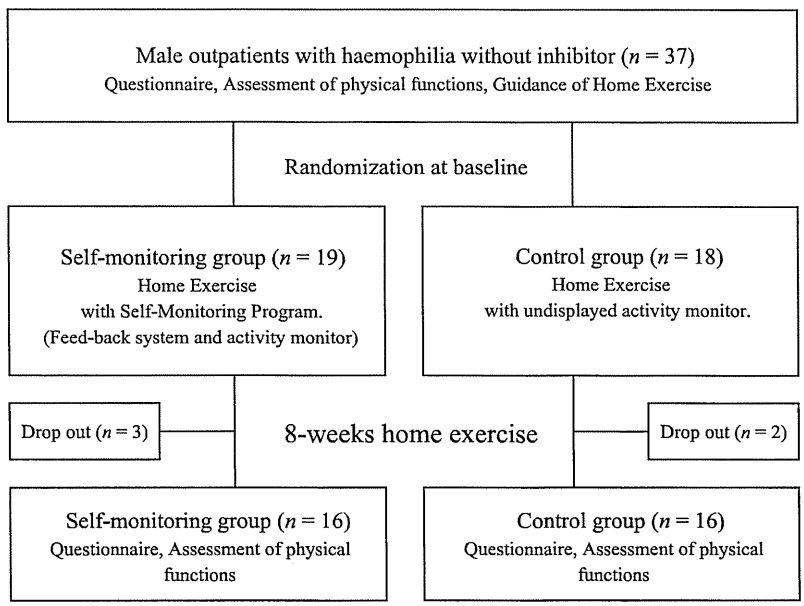

Fig. 1. The research design in this study. In baseline and post intervention, all subjects are surveyed for physical function and given a questionnaire. Assessment of physical function included strength of knee extension, range of motion, modified Functional Reach, 10 metre gait time and physical activity. The questionnaire is about bleeding history, pain scale, self-efficacy for exercise, exercise adherence and stage based on the transtheoretical model of behaviour change.

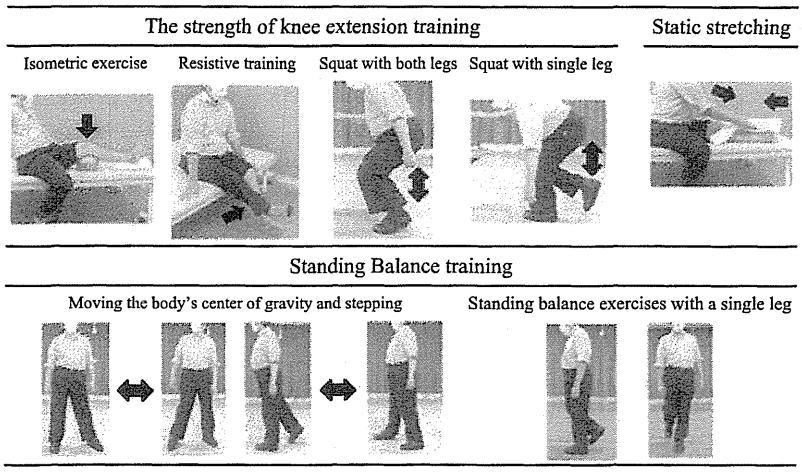

Fig. 2. The home exercise programme. The strength of knee extension training included the following four methods: isometric exercise, resistive training, a half squat with both legs and a half squat with a single leg. Among these training methods, a physiotherapist recommended the exercise most appropriate to the physical condition of each patient. Balance training was chosen from the following, and performed: moving the body's centre of gravity, stepping and standing balance exercises with a single leg.

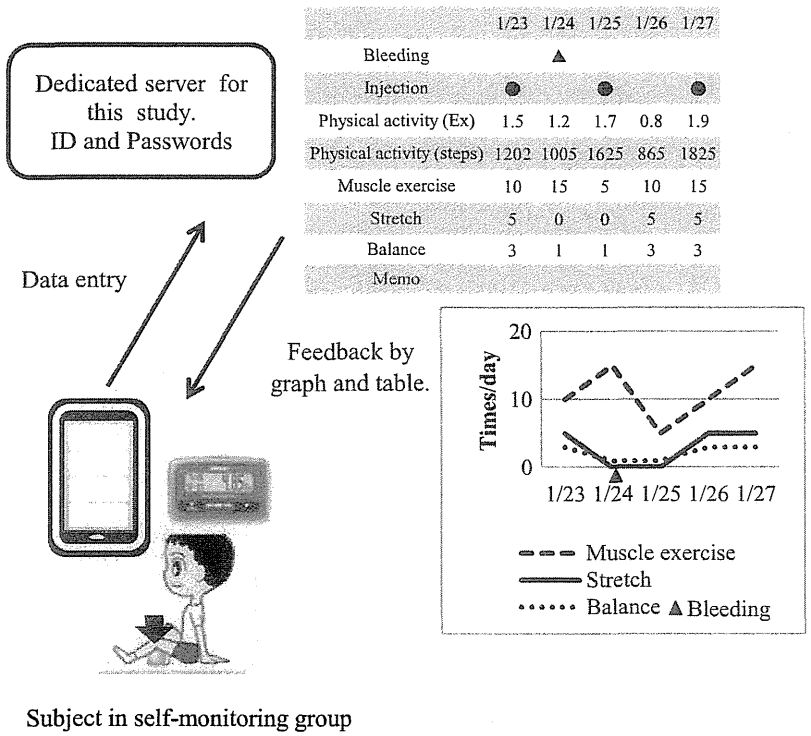

Fig. 3. System of self-monitoring. The subjects access a server and receive the feedback results of exercise with the passage of time shown in graphs and tables after monitoring items are input. The items of self-monitoring are as follows; bleeding history, injection of coagulation factor, physical activity (Ex and steps per day), no. of times of each home exercise. These data in the server were not used for analysis in the effect of home exercise, but were used to count the number of days the patient in the self-monitoring group inputted the data (monitoring rate). In addition, the patients in the control group were not required to report their home exercise status during the intervention period.

Self-monitoring approach in self-monitoring group (Fig. 3)

Patients in the self-monitoring group were equipped with the display activity monitors and a feedback system via the Internet and a mobile phone. When patients accessed the server and input monitoring items, feedback results appeared with time in the form of graphs and tables. The number of times the patient

performed the exercises and PA, bleeding frequency and injection of the coagulation factor were recorded as the self-monitoring contents. These data in the server were not used for analysis in the effect of home exercise, but were used to count the number of days the patient in self-monitoring group inputted the data (monitoring rate). In addition, a feedback result of their PA was returned through an activity monitor. However, checking the activity monitor display is part of the self-monitoring approach. Therefore, the patients in the control group were equipped with activity monitors without displays to eliminate the influence of viewing the display. In addition, the patients in the control group were not required to report their home exercise status during the intervention period.

The activity monitor has a function for automatically saving data for 150 days. All patients submitted the activity monitor to a physiotherapist after the intervention period. Data in the activity monitor were exported to a personal computer.

Questionnaire

All patients were surveyed for age and severity of haemophilia, bleeding frequency, pain scale, complication, self-efficacy [5], exercise adherence and stage based on the transtheoretical model of behavioural change [11]. The bleeding frequency was measured over the past 1 month with a total number of bleedings on the hip, knee and ankle. The pain scale was measured using the Visual Analogue Scale (VAS) on the hip, knee and ankle by putting a mark on a 100-mm horizontal line which ranges from 'no pain' on the left end to 'severe pain' on the right end. The distance from the left end to the mark was measured and the average value of three joints was obtained. Self-efficacy for exercise was scored from 5 to 25 points according to the modified Marcus scale [12] by Oka [13]. Exercise adherence for improvement of knee function was expressed as a percentage of the past 8 weeks using a unified method at baseline and post intervention in both groups. For example, when a patient exercised three times in 10 days, we recorded this as 30%.

Assessment of physical functions

Strength of knee extension, passive range of motion (ROM) in knee and ankle, dynamic standing position balance and 10 meter walking time were measured because bleeding in the lower limbs is predominantly localized in the ankles and knees for haemophilia [1]. For the sake of consistency, assessment of one patient was made by the same physiotherapist at baseline and post intervention, and an optimum value of three measurements was recorded. Strength of knee extension was measured using a hand-held dynamometer, the μTas F-01 (Anima Corp., Tokyo, Japan) according to the method by Katoh [14] in Nm kg^{-1}. ROM was measured in the supine position using a goniometer in degree. Dynamic standing position balance was measured with the modified-Functional Reach Test (mFRT) by Morio [15] in centimetres. Ten-meter gait time was measured in seconds.

Assessment of physical activity

Physical activities were evaluated using the activity monitor the entire day excluding aquatic exercise, and sleeping in the intervention period. Synthetic activity counts were recorded every 1 min by the accelerometer in the activity monitor, and the METs (metabolic equivalents) and Ex (MET × hours per day) for locomotive and non-locomotive activities were estimated. Daily step counts were also measured.

Assessment of arthropathy

The severity of arthropathy for the knee and ankle was rated using the Arnold classification, based on anteroposterior and lateral x-rays taken in the supine position [16].

Ethical considerations

This study was approved by the committee of research ethics from the Graduate School of Medicine and Faculty of Medicine, the University of Tokyo, the Institute of Medical Science, the University of Tokyo, Tokyo Medical University and Ogikubo Hospital, and written informed consent was obtained from all patients.

Statistical analysis

Although ROM, strength of knee extension, VAS and bleeding frequency were measured on both sides, the lower side of the strength of knee extension was evaluated in this study because kinetic disorders are closely associated with the degree of strength of knee extension. The independent *t*-test, chi-squared test and Fisher's exact test were used between the two groups for comparison of the subjects' biographical data.

Two-way repeated-measures ANOVA was used for analysis: bleeding frequency, VAS, ROM, strength of knee extension, 10 meter gait time, mFRT, self-efficacy and exercise adherence were used as dependent variables, and the self-monitoring group and control group as non-corresponding independent variables. Baseline and post intervention values were used as corresponding independent variables, respectively. To compare differences in the PA between the two groups, an unpaired *t*-test was used. SPSS 19.0J

statistical software (IBM Japan Inc., Tokyo, Japan) was used for analysis, and statistical significance was defined as $P < 0.05$. The days recorded on the server were divided by 56 (days of intervention) to obtain the monitoring rate.

Results

Base characteristics (Table 1)

Among the 37 male patients (19 in the self-monitoring group and 18 in the control group) participating in this study, four gave up continuing exercise and one was hospitalized for pneumothorax. Consequently, 32 patients (16 in the self-monitoring group and 16 in the control group) were available for analysis; all 32 patients had knee and/or ankle arthropathy, and 27 had severe arthropathy. There was no significant difference in base characteristics between the two groups at baseline (Table 1).

Outcome of home exercise (Tables 2 and 3)

There was an interaction noted in self-efficacy ($P = 0.049$), exercise adherence ($P = 0.045$) and strength of knee extension ($P = 0.008$). In other words, the change in these three parameters was different between the two groups. There was a main

Table 1. Biographical data of patients.

		Self-monitoring group(n = 16) Mean (SD)	Control group (n = 16) Mean (SD)	P value
Age		41.8 (8.6)	43.9 (10.7)	0.527
Height (cm)		168.7 (6.7)	171.8 (7.4)	0.231
Body weight (kg)		64.3 (13.3)	59.1 (8.9)	0.197
Type of hemophilia	A/B	15/1	11/5	0.172
Severity of hemophilia	Severe/moderate or mild	13/3	14/2	0.831
Hemostatic treatment	Prophylaxis/on-demand	14/2	11/5	0.197
Severity of anthropathy	Stage 0–III*/IV–V*	5/6	5/6	1.000
(knee joint)	Post total knee arthroplasty	5	5	
Severity of anthropathy (ankle joint)	Stage 0–III*/IV–V*	2/13	4/12	0.659
	Post total ankle arthroplasty	1	0	
HCV	Positive/negative	14/2	13/3	1.000
HIV	Positive/negative	8/8	7/9	0.723
HBV	Positive/negative	1/15	0/16	0.484
Exercise adherence (%)		34.8 (36.9)	15.2 (19.8)	0.073
History of inhibitor**	Past history/none	1/15	0/16	1.000
Stage of the transtheoretical model	Precontemplation	8	7	0.215
	Contemplation	2	3	
	Preparation	4	2	
	Maintenance	0	3	

HCV, hepatitis type C virus; HIV, human immunodeficiency virus; HBV, hepatitis type B virus.
Independent t-test was used between the two groups to compare age, height and body weight. In addition, the chi-square test was used for severity of knee arthropathy and HIV and Fisher's exact test was used for type of hemophilia, severity of hemophilia, hemostatic treatment, severity of ankle arthropathy, HCV, HBV, history of inhibitor and stage of the transtheoretical model. Statistical analyses were performed with SPSS 19.0J statistical software (SPSS Japan, Tokyo, Japan) and a P value of 0.05 was considered significant.
*Arnold stage.
**The subjects in this study included only hemophilic patients without an inhibitor.

Table 2. Outcome of home exercise.

	Self-monitoring group (n = 16)		Control group (n = 16)		P value		
Variables	Base line Mean (SD)	8 weeks Mean (SD)	Base line Mean (SD)	8 weeks Mean (SD)	Main effect (pre-post)	Groups comparison	Interaction
Self-efficacy for exercise (points)	16.0 (4.8)	17.9 (2.9)	15.3 (3.0)	13.5 (4.2)	0.944	0.018*	0.049*
Exercise adherence (%)	34.8 (36.9)	79.0 (16.6)	15.2 (19.8)	32.8 (21.1)	0.000***	0.000***	0.045*
Strength of knee extension(Nm kg^{-1})	1.03 (0.4)	1.44 (0.7)	1.24 (0.7)	1.37 (0.7)	0.000***	0.746	0.008**
Range of knee extension (degree)	−6.9 (7.3)	−2.5 (3.7)	−5.0 (6.6)	−2.5 (4.8)	0.000***	0.700	0.436
Range of ankle dorsiflexion (degree)	−5.0 (25.4)	0 (21.5)	4.1 (10.2)	8.4 (10.3)	0.004**	0.171	0.838
Modified Functional Reach (cm)	29.6 (8.2)	34.7 (8.8)	29.8 (7.9)	34.8 (6.5)	0.023*	0.952	0.993
10-min gait time (s)	5.6 (1.3)	5.2 (1.0)	6.1 (1.5)	5.3 (1.2)	0.002**	0.517	0.187
Bleeding (times per month)	1.8 (3.3)	0.8 (1.1)	0.9 (1.5)	0.5 (0.7)	0.091	0.287	0.410
Visual analogue scale (mm)	14.2 (11.4)	16.7 (15.3)	8.8 (8.4)	5.1 (8.8)	0.493	0.024*	0.705

Two-way repeated-measures ANOVA was used for analysis: bleeding frequency, VAS, ROM, muscle force, 10-metre gait time, mFRT, self-efficacy, and exercise adherence were used as dependent variables, and the self-monitoring group and control group as non-corresponding independent variables, and baseline and post-intervention values as corresponding independent variables, respectively.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 3. Physical activity in subjects.

Variables	Self-monitoring group (n = 16) Mean (SD)	Control group (n = 16) Mean (SD)	P value
Ex for locomotive activities	2.4 (1.8)	1.8 (1.5)	0.263
Ex for non-locomotive activities	0.5 (0.8)	0.7 (0.5)	0.348
Total Ex	2.9 (2.0)	2.5 (1.9)	0.517
Step counts (steps per day)	5805.6 (3384.0)	4910.2 (2663.5)	0.412
Step times (min per day)	86.0 (45.6)	75.0 (34.9)	0.453

Ex, MET × hours per day.
We used an independent t-test. Statistical analyses were performed with SPSS 19.0J statistical software (SPSS Japan, Tokyo, Japan), and a P value of 0.05 was considered significant.

effect in exercise adherence (P = 0.000), strength of knee extension (P = 0.000), ROM of knee extension (P = 0.000), ROM of ankle dorsiflexion (P = 0.004), mFRT (P = 0.023) and 10 metre gait time (P = 0.002) when compared with the values at baseline and post intervention. There was a significant difference in self-efficacy (P = 0.018) and exercise adherence (P = 0.000) between the self-monitoring group and the control group. No significant difference in PA was found between the two groups. The monitoring rate in the self-monitoring group was 90.3 (62.5–100)%. There was no significant change in pain and in the bleeding frequency through intervention.

Discussion

Effects of self-monitoring in home exercise

In this study, exercise adherence, self-efficacy and strength of knee extension significantly improved in the self-monitoring group compared with those in the control group. In particular, exercise adherence and self-efficacy provided direct influence on the effect of self-monitoring.

First, self-monitoring is a method of enabling self-strengthening and objective self-evaluation by observing and/or recording self-behaviour, and is useful for behaviour change [6]. This study has shown that self-monitoring has enabled the patients to receive the benefits of controlling self-behaviour through the recognition of motion results, creating self-efficacy and improving exercise adherence. Self-efficacy is one of the psychological factors [17] having the strongest influence on the promotion of PA and exercises [5][8], and is enhanced by self-monitoring. For this reason, self-efficacy is considered to contribute to exercise adherence.

Second, obtaining good adherence to muscle strengthening exercises is difficult for haemophiliacs because of their fears of bleeding when exercise stress occurs and later fatigue as well when muscular pains appear. However, the self-monitoring programme favoured haemophiliacs not only with the reassurance of no increase in bleeding through strengthen training but also with the opportunity for their own objective self-evaluation of their joints. In addition, self-monitoring provided continued efficacy and motivation for exercise to the patients of the self-monitoring group. In fact, the self-monitoring group showed higher improvements in strength of knee extension than the control group. Generally, strength of knee extension decreases the progression of knee arthropathy in haemophiliacs. For haemophiliacs with arthropathy, the self-management of joint function is important and necessary for their well-being. Therefore, improvement of strength of knee extension was extremely meaningful for haemophiliacs who used self-monitoring and home exercise even in patients with arthrosis.

Effects of home exercise

Both groups showed improvements in ROM, mFRT and gait speed with no significant difference between groups. Articular contracture in the lower limbs seen in haemophilia is ankle equinus and knee flexion contractures. Knee flexion contracture results from several causes. The painful knee assumes a flexed posture for comfort, as this position allows greater accommodation of fluid within the joint. Fixed flexion deformity may be complicated by posterior subluxation and external rotation of the tibia due to the pull of the hamstring muscles, shortening of the anterior cruciate ligament and contraction of the posterior capsule [18]. In a similar fashion, most patients had contractures of ankle and knee. However, in this study it was found that even in patients with arthropathy, ROM was improved through the continuous stretching of soft tissues. The reason is that self-stretching is an exercise with a low load, giving an immediate effect. Therefore, ROM may be improved even without self-monitoring.

On the other hand, walking and balance training are not low load exercises, but they are closely associated with activities of daily living, in which the movement gives less fear for the patients. Therefore, mFRT and gait speed may also be improved without self-monitoring. Effective training factors that need no self-monitoring remain to be studied.

There was no significant difference in PA between the two groups. Generally, the goal for prevention of lifestyle-related diseases is known to target 23 Ex per week by PA defined as those with intensity at least three METs [11]. However, few patients achieved the criteria. Individual guidance for improvement in PA for haemophiliacs is necessary, and there is room for consideration as to how the intervention should be offered to each patient.

Safety of home exercise

Among all patients examined, no change in pain and in bleeding frequency was noted. In other words, home exercise could improve physical function without increased bleeding and pain.

Limitations of this study

In this study, the results were obtained with 37 patients at four hospitals through an 8-week intervention, and therefore, do not reflect the data of all haemophiliacs in Japan. This study focused on the improvement of the knee function. However, haemophilic elbow arthropathy is also common, so that a study of home exercise to improve other joint functions is also necessary in the future studies.

Conclusion

To clarify the effect of self-monitoring on home exercise, a randomized controlled trial was performed on a self-monitoring group and a control group. Although most of the patients examined had severe haemophilic arthropathy, home exercise improved their physical function without increased bleeding frequency and pain. A home exercise self-monitoring programme has the potential for exercise adherence, self-efficacy and strength of knee extension in haemophiliacs. Self-monitoring attained a high rate using the Internet and mobile phone.

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LETTERS TO THE EDITORS

Rehabilitation improved walking ability for three haemophilia patients with inhibitors

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Intra-articular bleeding may occur in patients with haemophilia. If left untreated, it may lead to haemophilic arthropathy. To prevent this, haemostatic therapy is important. For patients with haemophilia who have not developed circulating inhibitors, i.e. antibodies to factor VIII (FVIII) or IX (FIX), hemostatic therapy is more effective, because of developed FVIII/FIX agents and prophylactic treatment methods [1]. In addition, orthopedic conservative and interventional treatment measures have become safer with adequate prophylaxis with haemostatic agents. Intensive physical rehabilitation as a conservative measure is effective in decreasing bleeding episodes, relieving pain, and improving function and activities of daily living (ADL) [2,3]. Interventional treatment, such as total joint arthroplasty, is also possible with appropriate prophylaxis. Thus, haemostatic prophylaxis enables preservation and improvement of joint function in both children and adults [4].

For haemophilia patients with circulating antibodies, i.e. inhibitors against these important clotting factors, bypassing agents are recommended for haemostatic treatment [5,6]. In Japan, recombinant activated factor VII (rFVIIa; Novo Seven®; Novo Nordisk, Bagsvaerd, Denmark) and plasma-derived activated prothrombin complex concentrate (aPCC; FEIBA®; Baxter, Deerfield, IL, USA) are available as bypassing agents. However, bleeding control is still difficult, and rehabilitation and surgical treatments are challenging. As a result, most of these patients have severe arthropathy and limitation of ADL. Improvement of ADL for patients with inhibitors is a major concern [7,8].

We report improvement in ADL through intensive physical rehabilitation in three haemophilia patients with inhibitors. In all patients, walking ability was especially improved from wheelchair use to walking with a cane or crutch (Table 1).

Case 1

A 19-year-old male with 42 kg body weight was diagnosed with severe haemophilia A at 5 months of age. Circulating inhibitor was detected at 3 years of age, and inhibitor titre was 17.9 BU on admission (historical peak, 130 BU). Rebleeding in his left knee started at 18 years of age, and within a year he was confined to wheelchair. He was subsequently hospitalized to improve his walking ability and to attend school on foot.

At hospitalization, his left knee was in end-stage arthropathy with severe flexion contracture. Muscle atrophy of the extensors of the left knee, i.e. the left quadriceps was severe, and the left hamstrings were particularly shortened. He was transferred to a wheelchair and was able to operate wheelchair by himself. Hemophilia Joint Health Score 2.1 (HJHS) [9] of left-knee, right-knee, left-ankle, right-ankle and global gait score were evaluated to 12, 3, 9, 2, 4 respectively. The short-term rehabilitation goal (ST-RG) was improvement of left knee function, and the long-term rehabilitation goal (LT-RG) was gait activity with orthotics.

As a first step in rehabilitation, he started muscle power-up exercises of the left lower extremity using low-frequency electrotherapeutic device, and stretching of the left hamstring after hot pack treatment. Three weeks later, he started straight leg raising exercise with a low-frequency electrotherapeutic device, and an orthotic device was used to support the quadriceps. After he was able to elevate his leg, he initiated walking exercises with crutches and a knee-ankle-foot orthosis (KAFO). Five weeks later, his quadriceps muscle power slightly increased, and he started knee range-of-motion exercises by himself using a skate board, resistance training of the lower extremity using rubber-band (Thera-Band®, The Hygenic Corporation, Akron, OH, USA) and walking with KAFO without crutches. After 6 months, he started climbing stairs and gait exercises with orthotics. Eight months later, he could stand on his own without KAFO and pro-

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Table 1. Joint condition at admission.

Location			Hip joint		Knee joint		Ankle joint		Elbow Joint	
Side			Right	Left	Right	Left	Right	Left	Right	Left
Case 1	AH staging		II	II	II	IV	II	IV	II	IV
	ROM* (°)	Flex*	125	130	160	160	50	40	150	140
		Ext*	15	−15	−10	−45	15	15	−5	−25
	MMT*	Flex	4	2	4	3	4	2	4	3
		Ext	4	2	4	0	4	2	4	3
Case 2	AH staging		V	III	III	II	V	II	V	I
	ROM* (°)	Flex*	60	70	90	140	60	50	115	145
		Ext*	−20	20	0	0	−10	0	−50	−10
	MMT*	Flex	2	3	2	4	3	4	3	4
		Ext	2	3	2	4	3	4	3	4
Case 3	AH staging		V	III	V	III	III	IV	V	V
	ROM* (°)	Flex*	60	110	140	130	35	25	120	115
		Ext*	−40	0	−80	−50	15	5	−40	−55
	MMT*	Flex	3	3	3	3	3	3	3	3
		Ext	1	3	2	4	3	3	3	3

AH staging, Arnold–Hilgartner classification [10]; ROM*, range of motion; MMT*, manual muscle test; Flex*, flexion; Ext*, extension.

gressed to walking exercises without support. On day 250, he was discharged and was able to stand on foot without any support. HJHS score of left-knee, right-knee, left-ankle, right-ankle and global gait score at discharge were improved to 4, 1, 4, 1, 2 relatively.

As for haemostatic control, rFVIIa (4.8 mg per dose; 106.7 μg kg^{-1}) was injected every weekday until 6 months after admission, and then three times per week until discharge. During this prophylactic regimen, one episode of intra-articular bleeding occurred in his left knee on the weekend (not prophylaxis day). This episode was treated with single dose rFVIIa (6.0 mg, 133.3 μg kg^{-1}) with the left knee rested, iced, compressed and raised for 2 h after administration.

Case 2

A 19-year-old male with 42 kg body weight was diagnosed with severe haemophilia A at birth. Circulating inhibitor was detected at 6 months of age, and the inhibitor titre was 84.6 BU at admission (historical peak, 2700 BU). Intra-articular bleeding recurred in many joint in the lower extremities, and he could not walk at all at 11 years of age. He strongly desired to be able to walk on foot and agreed to be hospitalized for rehabilitation. At admission, his leg showed disuse atrophy of the bone and muscle, but he did not complain of pain because he had never stood on his legs with his full weight after 11 years of age. He could not do anything without his parents' support except eating and getting dressed. HJHS of left-knee, right-knee, left-ankle, right-ankle, and global gait score were evaluated to 2, 7, 5, 6, 4 respectively. His ST-RG was recovery of whole body muscle strength, and his LT-RG was walking without support. As a first step towards rehabilitation, he tried to transfer from the bed to wheelchair by himself, as well as built up whole body muscle. In this phase, daily prophylaxis of rFVIIa was especially effective for him until second weeks, because daily haemostatic treatment relieved his concern of postrehabilitation bleeding. Two weeks later, he could be transferred to wheelchair and was able to operate wheelchair by himself. For the next step, the standing-and-sitting exercise was prescribed to him while maintaining his balance at the standing position. He attempted this exercise (30 min per set) several times a day to achieve durability. Four weeks later, he could walk using parallel bars without experiencing joint pain in lower extremities. After 7 weeks, he could walk using a walker; however, he complained of pain in the right hip and ankle joints. We adjusted his leg discrepancy by insole and this pain disappeared. Eleven weeks later, he started walking with a T-cane. On day 140, he was discharged, being able to walk on foot with a T-cane. HJHS score of left-knee, right-knee, left-ankle, right-ankle and global gait score at discharge were improved to 0, 3, 4, 3, 2 relatively.

There was no intra-articular bleeding during admission under prophylaxis. (i.e. bypass agent injected three times per week from second weeks). A total of 4000 units per dose (aPCC) was injected from week 2–9, and 3000 units per dose (aPCC) was injected until the 16th week and 4.8 mg per dose (rFVIIa) until discharge.

Case 3

An 18-year-old male with 40 kg body weight was diagnosed with severe haemophilia B at birth. Circulating inhibitor was detected at 1 year of age, and the inhibitor titre was 1.9 BU at admission (historical peak, 21 BU). Bleeding recurred in several joints; he was not able to walk at all and moved only using wheelchair since the age of 10 years. He had many endstage arthropathies, and his long bones were atrophied. There was leg length discrepancy caused by extension contractures of both knees. The power of muscles, particularly around his right knee, was severely weakened, so that he could not work at all. HJHS of left-knee, right-knee, left-

ankle, right-ankle and global gait score were evaluated to 8, 10, 9, 8, 4 respectively.

ST-RG was to recover his muscle strength of the left lower extremity and increase the range of motion of the left knee. LT-RG was able to stand on the left leg, and walk with bilateral axillary crutches. First, he started stretching of his left hamstrings and extension of the left hip following hot pack treatment, and active muscle setting was used to increase muscle power. Both elbow joints with end-stage arthropathy were also isometrically exercised. Two weeks later, left knee contracture improved from −50° to −30°, and muscle power increased. On day 17, intra-articular bleeding in the left knee occurred because of which the patient could not tolerate exercise around the left knee; however, other exercise programs were continued. At the 4th week, exercise of the left knee was reinitiated. At the 9th week, his left knee contracture improved to −20°, and he was able to stand on the left leg; therefore, parallel bars were used to initiate walking exercises. A few days later, intra-articular bleeding occurred in the left elbow; however, walking exercises were promptly re-initiated using axillary crutches. At the 12th week, he was able to walk 20 min using axillary crutches. This time, intra-articular bleeding occurred in the right elbow. We thought that the range of motion and muscle power of the left knee were inadequate to enable walking for long distances. At the 21st week, the patient was finally able to walk a long distance using axillary crutches; he was discharged on day 185. HJHS score of left-knee, right-knee, left-ankle, right-ankle and global gait score at discharge were improved to 5, 8, 5, 5, 3 relatively.

As for haemostatic control, rFVIIa (6.0 mg per dose; 120 μg kg^{-1}) was injected three times a week and 6.0 mg rFVIIa was injected additionally whenever intra-articular bleeding occurred.

Discussion

As illustrated by the three cases, we believe that there are three important points to achieve a rehabilitation goal for haemophilia patients: comprehensive evaluation of the patient's physical status, a personal rehabilitation program and the mutual trust between medical staff and the patient.

For evaluation, we examined joint condition, including swelling, pain and bleeding, both pre (morning) and postrehabilitation (evening) every day. Adjustments in the exercises scheduled for the day, if necessary, were made based on the prerehabilitation evaluation. During the postrehabilitation evaluation, we were able to find intra-articular bleeding that had occurred during the training. Early haemostatic treatment is effective to control bleeding and to re-initiate exercise as early as possible. A joint condition-matched program is effective to reduce bleeding risk. For patients with inhibitors, two examinations are crucial for adequate rehabilitation.

Rehabilitation programs usually tend to emphasize only on the improvement of affected joints and extremities. However, a systematic rehabilitation program is needed for patients with haemophilia because they have decreased body balance and weakened muscle strength. Therefore, a rehabilitation program should be individualized according to local and general joint and muscle conditions. Emphasis should be on the correction of loss of balance and improvement of muscle strength as quickly as possible. Braces and orthoses are sometimes needed according to the target joint condition.

Most patients with haemophilia know that rehabilitation is good for joints and reduces the risk of bleeding; however, many of these patients fear postrehabilitation bleeding. It is difficult to solve this paradox without reliability between physiotherapist and patient. Hospitalization is, therefore, a good opportunity to establish a good relationship and reliability with caregivers. We particularly recommend hospitalized rehabilitation for patients with haemophilia with inhibitors.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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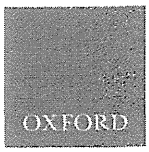

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Research Article

RESEARCH ARTICLE

Knockdown of Dopamine D_2 Receptors in the Nucleus Accumbens Core Suppresses Methamphetamine-Induced Behaviors and Signal Transduction in Mice

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Abstract

Background: Addictive drugs lead to reinforcing properties by increasing dopamine in the nucleus accumbens, which is composed of a core and shell regions. Neurons in the nucleus accumbens are divided into 2 subtypes based on the differential gene expression of the dopamine D_1 receptors and D_2 receptors.
Methods: In the present study, we investigated the role of D_2 receptors in the nucleus accumbens core in behaviors and signal transduction induced by psychostimulant methamphetamine in mice that were microinjected with adeno-associated virus vectors containing a microRNA (miRNA) sequence for D_2 receptor (adeno-associated virus-miD_2r vectors) in the nucleus accumbens core. The adeno-associated virus vectors containing a miRNA sequence for D_2 receptor-treated mice (miD_2r mice) were assessed at a reduction in D_2 receptor, but at no change in dopamine D_1 receptor, in the nucleus accumbens core compared with the adeno-associated virus-Mock vectors-treated mice (Mock mice).
Results: miD_2r mice exhibited a reduction in hyperlocomotion that was induced by a single treatment with methamphetamine. The development of locomotor sensitization induced by repeated treatment with methamphetamine exhibited less extension in miD_2r mice. In a place conditioning paradigm, the preferred effects of methamphetamine were significantly weaker in miD_2r mice than in Mock mice. Furthermore, the single treatment with methamphetamine-induced phosphorylation of extracellular signal regulated kinase and cyclic adenosine monophosphate response element-binding protein in the nucleus accumbens core of miD_2r mice was decreased compared with that in Mock mice. Repeated treatment with methamphetamine-induced delta FBJ murine osteosarcoma viral oncogene homolog B accumulation in the nucleus accumbens core of miD_2r mice was also attenuated.
Conclusions: These findings suggest that a D_2 receptor-mediated neuronal pathway from the nucleus accumbens core plays an inhibitory role in the development of reinforcing properties.

Keywords: adeno-associated virus vectors, dopamine D_2 receptors, nucleus accumbens, methamphetamine

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Introduction

Addictive drugs, such as methamphetamine (METH; derivative of amphetamine, N-metyl-amphetamine), cocaine, nicotine, and morphine mediate their reinforcing properties by targeting the mesolimbic neuronal system. This neuronal system is a major dopamine (DA) pathway in the brain and originates from the ventral tegmental area (VTA) of the midbrain and projects to the nucleus accumbens (NAc), amygdala, hippocampus, and prefrontal cortex. In particular, the VTA-NAc pathway plays a critical role in mediating the reinforcing properties of drugs of abuse (Hyman et al., 2006). Although addictive drugs differ in their primary molecular targets, they consequently lead to a common event in which extracellular DA levels are directly or indirectly increased in the NAc (Lüscher and Ungless, 2006). Therefore, in the synaptic terminals of DA neurons in the NAc, METH promotes nonvesicular release and cocaine acts as an inhibitor of the DA transporter. In the VTA of the VTA-NAc pathway, nicotine directly depolarizes DA neurons, whereas morphine indirectly affects them via presynaptic inhibition of inhibitory interneurons (i.e., disinhibition of DA neurons).

Almost all the neurons in the NAc are γ-aminobutyric acid-productive medium spiny neurons (MSNs). The NAc is anatomically divided into a core region, which surrounds the anterior commissure, and a shell region, which is located in the rostral pole of the extended amygdala. The former is distinguished from the latter by differences in staining density for a number of neuropeptides, such as substance P, dynorphin, and enkephalin (Groenewegen et al., 1999). Furthermore, the NAc neurons are divided into 2 major populations on the basis of their distinct projections through differential gene expression, a direct pathway from MSNs, expressing dopamine D_1 receptors (D_1rs), and an indirect pathway from MSNs, expressing dopamine D_2 receptors (D_2rs) (Kreitzer and Malenka, 2008). The D_1rs are coupled to Golf and/or Gs proteins, which, on activation, stimulate adenylyl cyclase, promote the formation of cyclic adenosine monophosphate (cAMP), and activate protein kinase A (PKA), whereas D_2rs are coupled to Gi proteins, which inhibit the formation of cAMP, thereby decreasing PKA activity (Stoof and Kebabian, 1981; Missale et al., 1998). Both receptors in MSNs can also differentially regulate intracellular signal transduction such as the extracellular signal regulated kinase (ERK), dopamine and camp-regulated phosphoprotein of 32 kDa, and cAMP response element-binding protein (CREB) cascades. The direct pathway originates from D_1rs-expressing MSNs in the core region of the NAc that project to the lateral division of the VTA and the medial division of the substantia nigra pars compacta output nuclei. The indirect pathway originates from D_2rs-expressing MSNs in the core region of the NAc that project to the substantia nigra pars reticulata (SNr) and the dorsolateral division of the ventral pallidum, which together with the subthalamic nucleus contain trans-synaptic circuits connecting to the basal output nuclei (Humphries and Prescott, 2010).

The direct and indirect pathways from the dorsal striatum (dSTR) provide contrasting regulation of the basal ganglia output interface (Gerfen and Surmeier, 2011). However, little is known about the specific function of the 2 major populations of the NAc projection neurons. In the present study, to investigate the functional role of the indirect pathway from MSNs expressing D_2rs in addictive properties, we examined behaviors and signal transduction in response to METH in D_2rs knockdown mice that were delivered adeno-associated virus (AAV) containing a microRNA (miRNA) sequence for D_2r in the NAc core.

Materials and Methods

Animals

Male C57BL/6J mice (Nihon SLC, Hamamatsu, Japan) were 8 weeks old and weighed 22 to 27 g at the beginning of the experiments. The animals were housed in plastic cages and kept in a regulated environment (24±1°C, 50±5% humidity) with a 12-h–light/–dark cycle (lights on at 8:00 AM). Food and water were available ad libitum. All experiments followed the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the committee for Animal Experiments of University of Toyama.

Drugs and Antibodies

METH HCl was purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and dissolved in sterile saline. Anti-D_2r or anti-green fluorescent protein (GFP) antibodies were obtained from Abcam (Cambridge, UK). Anti-D_1r antibody was obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against phospho-ERK1/2 (Threonine 202/Tyrosine 204), ERK1/2, phospho-CREB (Serine 133), CREB, delta FBJ murine osteosarcoma viral oncogene homolog B (FosB), and β-tubulin were purchased from Cell Signaling Technology (Danvers, MA). Other agents were obtained from standard commercial sources.

Production of AAV Vectors

We generated AAV vectors based on a previous report (Krzyzosiak et al., 2010). In brief, we used a vector plasmid containing an expression cassette in which a human cytomegalovirus immediate-early promoter was followed by the first intron of the human growth hormone gene, cDNA of interest, woodchuck hepatitis virus posttranscriptional regulatory element (nucleotides 1093–1684, GenBank accession number J04514) and simian virus 40 polyadenylation signal sequence. This expression cassette was inserted between the inverted terminal repeats of the AAV2 genome as previously described (Li et al., 2006). The viral vector was designed to express an antisense sequence for the D_2r (TTCACTGGGAAACTCCCATTA) and enhanced Gfp sequence (AAV-miD_2r vectors) based on murine miR-155 (BLOCK-iT, Invitrogen). Viral vectors containing only the enhanced GFP sequence (AAV-Mock vectors) were used as a control. We used 2 helper plasmids, pAAV-RC and pHelper, harboring the AAV2 rep and cap genes and E2A, E4, and VA1 genes of the adenovirus genome, respectively (Agilent Technologies, Santa Clara, CA). HEK293 cells were cotransfected with the pAAV-RC and pHelper plasmids using the calcium phosphate coprecipitation method. AAV particles were then harvested and purified by 2 sequential continuous CsCl ultracentrifugations. The vector titer was determined by quantitative polymerase chain reaction (PCR) of the DNase-I-treated vector stocks and was estimated at 10^{11} to 10^{12} vector genome copies.

Microinjection of AAV Vectors

Naive mice were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The animals received a single bilateral microinjection of the AAV-miD_2r or AAV-Mock vectors (each 0.7 μL/side) into the NAc (bregma=+1.4; lateral=±0.6; ventral=+4.2 mm position in the Mouse Brain Atlas; Franklin and Paxinos, 2007) using a stereotaxic apparatus. The injection was performed at 0.05 μL/min through a syringe with a 33-gauge needle (Hamilton, Reno, NV), and the syringe needle was left in place for an additional 15 minutes. The mice were used for the experiments 3 weeks later.

Quantitative RT-PCR

Mice were sacrificed by cervical dislocation. Whole brains were removed and divided into 1-mm–thick sections using a mouse brain matrix (Neuroscience, Tokyo, Japan). Tissue corresponding to the NAc was collected with a 2-mm punch from the section. Likewise, the dSTR tissue was collected using a 2-mm punch from the subsequent section. The accurate locations of these brain structures were based on visual inspection of each section using a stereomicroscope and its comparison with the stereotaxic atlas of the mouse brain (Franklin and Paxinos, 2007). Tissue samples were placed on dry ice and maintained at –80°C until use. Total RNA extraction was performed using the RNeasy Plus Mini Kit (QIAGEN, Valencia, CA). Total RNA from each tissue sample was transcribed into cDNA using the PrimeScript RT reagent Kit (Takara, Shiga, Japan) according to the manufacturer's recommendations. In brief, the reaction was performed at 37°C for 20 minutes in a total volume of 10 µL and inactivated at 85°C for 5 seconds. Twenty-times diluted cDNA was used as a template, and quantitative real-time PCR was run in the Thermal Cycler Dice Real Time System (Takara) using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA) with cDNA and gene-specific primers (1 µM) according to the manufacturer's instructions. All the reactions were performed in duplicate with the following cycling protocol: 10 minutes of heat activation of the enzyme at 95°C, 45 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 20 seconds. Fluorescence detection was performed at 72°C. The gene-specific primers were designed using the Primer3 software to amplify fragments of 150 to 250 bp as follows: for D_2r (Drd2; NM 010077) forward, TCGCCATTGTCTGGGTCCTG; reverse, TGCCCTTGAGTGGTGTCTTC; and D_1r (Drd1a; NM 010076) forward, AAGATGCCGAGGATGACAAC; reverse, CCCTCTCCAAA GCTGAGATG. The transcript amounts that were evaluated for the D_1rs and D_2rs were normalized for quantity and quality of each sample by dividing it by the amount of transcript of the housekeeping gene acidic ribosomal phosphoprotein P0 (Arbp or 36B4; NM 007475) in the same sample, and their relative values were presented. The 36B4 transcript amount was quantified using the forward primer ACCCTGAAGTGCTCGACATC and reverse primer AGGAAGGCCTTGACCTTTTC.

Immunohistochemistry

Coronal sections (14 µm thick) from the unfixed frozen brains of mice were collected on superfrost slides and stored at –80°C until analysis. The sections were postfixed in 4% paraformaldehyde and treated with 1% H_2O_2 to block endogenous peroxides. For the detection of D_2rs, the primary antibody was detected using the ABC system (Vector) according to the manufacturer's manual. For each animal and section, the corresponding brain regions were identified according to the mouse brain atlas (Franklin and Paxinos, 2008).

Locomotor Activity

Mice were individually placed in a transparent acrylic cage with a black frosting Plexiglas floor (45 × 25 × 40 cm), and locomotor activity was measured every 5 minutes for 60 minutes using digital counters with infrared sensors (Scanet MV-40; MELQEST, Toyama, Japan). METH (1 mg/kg subcutaneously [s.c.]) was administered immediately before the measurement of locomotor activity.

Place Conditioning Test

A place conditioning test was performed according to the method of Miyamoto et al. (2000). In brief, the apparatus consisted of the following 2 compartments: transparent and black Plexiglas boxes (both 15 × 15 × 15 cm). The floors of the transparent and black boxes were covered with white and black frosting Plexiglas, respectively. Each box could be divided by a sliding door (10 × 15 cm high). In preconditioning, the sliding door was opened, and the mouse was allowed to move freely between both boxes for 15 minutes once per day for 3 days. On day 3, the time that the mouse spent in the transparent and black boxes was measured using a LD mode of Scanet MV-40 (MELQEST). The box in which the mouse spent the most time was referred to as the "preferred side" and the other box was the "nonpreferred side." The conditioning was performed during 6 successive days. The mouse was given a drug or vehicle immediately before the conditioning in the apparatus with the sliding door closed. On days 4, 6, and 8, the mouse was given METH (1 mg/kg s.c.) or saline and placed in its nonpreferred side for 20 minutes. On days 5, 7, and 9, the mouse was given saline and placed in its preferred side (opposite to the METH-conditioning side) for 20 minutes. On day 10, postconditioning was performed without drug treatment. During postconditioning, the sliding door was opened, and the time that the mouse spent in the transparent and black boxes for 15 minutes was measured as on day 3. Place conditioning behavior was expressed by post – pre, which was calculated as follows: [(post value) – (pre value)], where the post and pre values were the differences in the time spent in the METH-conditioning and saline-conditioning sides in postconditioning and preconditioning, respectively.

Western-Blotting Analysis

The brain tissues of the NAc core were homogenized in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, phosphatase inhibitor cocktail [Nacalai Tesque, Kyoto, Japan] and protease inhibitor cocktail [Nacalai Tesque]). Total proteins (20 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane. The membranes were incubated with primary antibodies, and the proteins were detected by horseradish peroxidase-conjugated secondary antibodies using the ECL Plus detection kit (Amersham Biosciences).

Statistical Analysis

All experiments were repeated twice with independently generated mice. All data are expressed as the mean ± SEM. In the analysis of locomotor activity and the place conditioning test, statistical differences among values for individual groups were determined using an analysis of variance (ANOVA) followed by the Student–Newmann–Keuls multiple comparisons test when F ratios were significant ($P < .05$). In the analysis of the time course of the development of locomotor sensitization, statistical differences were determined using ANOVA with repeated measures. Statistical differences between 2 groups were determined with the Student's t comparison test.

Results

Reduced D_2rs in the Nucleus Accumbens of miD_2r Mice

First, we examined the mRNA expression levels of D_2rs and D_1rs in the NAc and dSTR of miD_2r mice. Real-time quantitative

reverse transcription-PCR (RT-PCR) revealed a significant reduction (47%) in D_2r mRNA expression levels in the NAc of miD_2r mice compared with those in the control Mock mice (Figure 1A). However, there was no reduction in D_1r mRNA expression levels in the NAc of miD_2r mice (Figure 1A). Furthermore, in the dSTR, both D_2r and D_1r mRNA expression levels were not significantly different between Mock and miD_2r mice (Figure 1A). Consistent with the above D_2r mRNA expression levels, the protein expression levels of the D_2rs using Western blotting and immunochemical staining were diminished in the core region of the NAc of miD_2r mice (Figure 1B-C). Supporting that, the expression of GFP was observed in the SNr, but not in the VTA, in both mice (Figure 1D), because the SNr receives axial projection from the core region, but not the shell region, of the NAc (Humphries and Prescott, 2010). These findings suggest that miD_2r mice have half reduction, but not a complete loss, of D_2rs in the core region of the NAc.

Attenuated METH-Induced Locomotor Activity and Place Preference in miD_2r Mice

To investigate whether the specific reduction of D_2rs in the NAc of mice affects the CNS functions of the brain, we examined the performances of miD_2r mice in several behavioral paradigms. We first tested their motility in a novel environment as a general behavioral response, which was measured for horizontal activity (locomotion) after saline treatment. No aberrant locomotion during a 60-minute observation period was seen in miD_2r mice (Figure 2A). This result indicates no apparent abnormalities in the motor neuronal systems of miD_2r mice. Acute METH (1 mg/kg s.c.) treatment induced hyperlocomotion in both Mock and miD_2r mice. However, the magnitude of the METH-induced locomotor activity in miD_2r mice was significantly reduced compared with that in Mock mice (Figure 2A). In both groups, the hyperlocomotion was potentiated by repeated METH treatment (1 mg/kg/d s.c.) for 7 days. When the time course of the METH-induced locomotor sensitization in miD_2r mice was compared with that in Mock mice, the development of sensitization was found to be significantly less extensive in the knockdown mice, at 1 mg/kg/d of METH [Figure 2B: ANOVA with repeated measurement; $F_{(1,12)}=4.908$, $P=.035$].

In the place conditioning test, METH (1 mg/kg s.c.) significantly induced place preference in both Mock and miD_2r mice. However, the preferred effects of METH were significantly weaker in miD_2r mice than in Mock mice (Figure 3).

Decreased METH-Induced ERK and CREB Phosphorylation and Delta FosB Accumulation in the Core of the NAc of miD_2r Mice

Subsequently, we investigated the intracellular signal responses to METH in the core of the NAc of miD_2r mice. Acute METH treatment (1 mg/kg s.c.) increased the phosphorylation levels of ERK at Threonine 202/Tyrosine 204 and CREB at Serine 133 in Mock mice (Figure 4A-B). However, the METH-induced phosphorylation levels of ERK and CREB in miD_2r mice showed a significant decrease compared with those in Mock mice (Figure 4A-B). A previous report demonstrated that chronic treatment of addictive drugs results in the accumulation of transcription factor delta FosB, which is mediated by CREB activation in the NAc (Kelz et al., 1999). The expression levels of delta FosB in the normal condition were the same in both miD_2r and Mock mice (Figure 4C). Repeated METH treatment increased the expression levels of delta FosB in Mock mice (Figure 4C). However, this

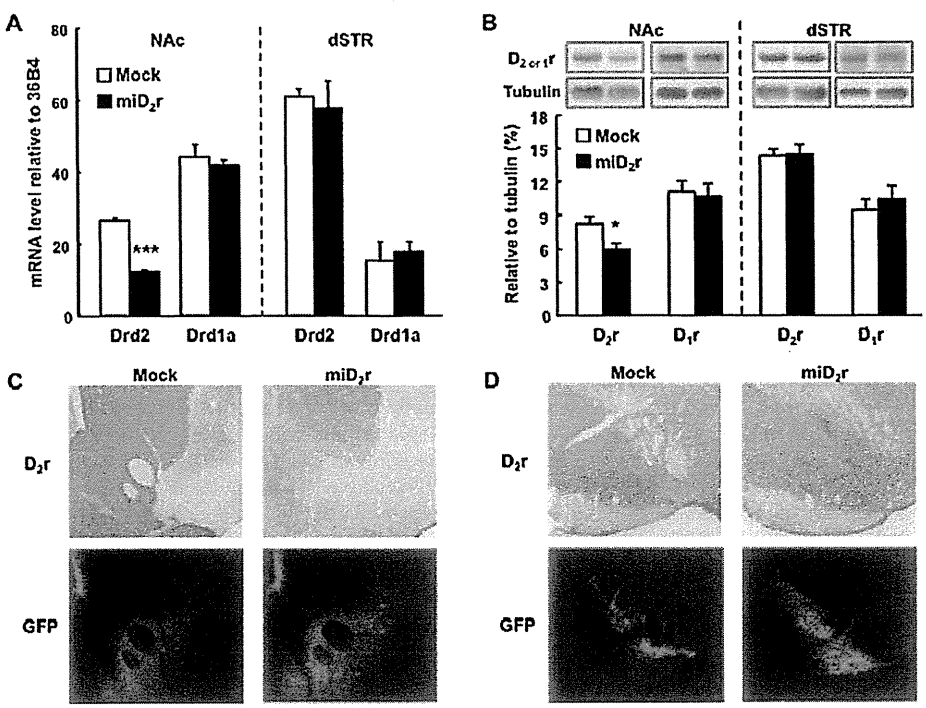

Figure 1. Expression of dopamine D_2 receptor (D_2r) in the nucleus accumbens (NAc) of the adeno-associated virus vectors containing a microRNA sequence for D2r-treated mice (miD_2r mice). A, Expression level of *Drd2* or *Drd1a* mRNA was measured by quantitative reverse transcription-polymerase chain reaction and presented relative to the expression of 36B4. B, Expression level of D_2r or dopamine D_1 receptor (D_1r) protein was assessed by Western blotting. C, Immunohistochemical study of the D_2r and green fluorescent protein (GFP) in the NAc of miD_2r mice. D, Immunohistochemical study of the D_2r and GFP in the substantia nigra pars reticulata (SNr) of miD_2r mice. dSTR, dorsal striatum. N = 6. Each column represents the mean±SEM. *P<.05, ***P<.001 vs Mock mice (Student's t comparison test).

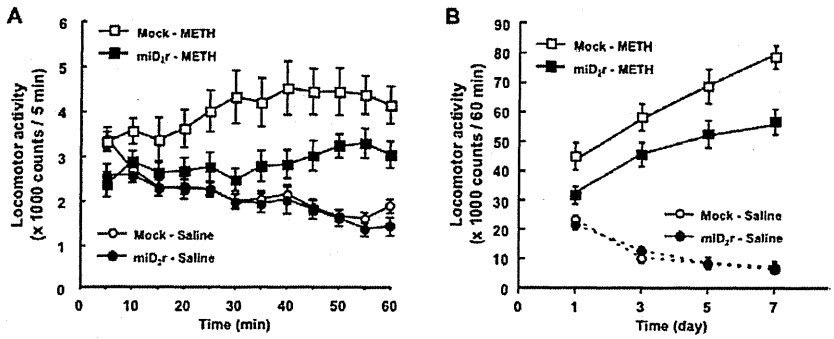

Figure 2. Locomotor effects of methamphetamine (METH) in miD$_2$r mice. A, Locomotor activity induced by acute METH treatment. METH (1 mg/kg s.c.) was administered immediately before the measurement of locomotor activity every 5 minutes for 60 minutes. N=7. Analysis of variance (ANOVA) with repeated-measurement analysis; $F_{(1, 12)}$=6.969, P=.001. B, Locomotor sensitization induced by repeated METH treatment. METH (1 mg/kg/d s.c. for 7 days) was administered to the mice that were used in A. The development of sensitization was found to be significantly lower in miD$_2$r mice. N=7. ANOVA with repeated-measurement analysis; $F_{(1, 12)}$=4.908, P=.035.

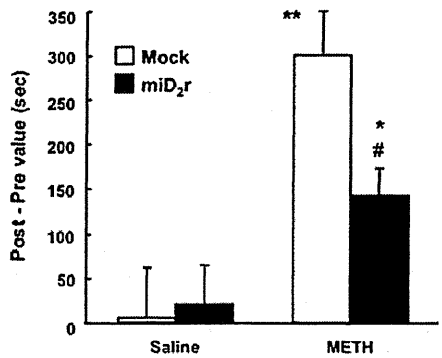

Figure 3. Preferred effects of methamphetamine (METH) in miD$_2$r mice. Place preference induced by METH treatment. METH (1 mg/kg s.c.) was administered during the conditioning. N=8. Each column represents the mean±SEM. *P<.05, **P<.01 vs corresponding saline-treated group. #P<.05 vs corresponding Mock group.

METH-induced expression of delta FosB in miD$_2$r mice showed a significant attenuation compared with that in Mock mice (Figure 4C).

Discussion

AAV vectors are powerful tools for delivering an objective gene into the neurons because of its unique characteristics, including the lack of any disease caused by wild-type viruses, the ability to infect nondividing cells, and the long-term expression of the transgene without immune responses (Monahan and Samulski, 2000). In the present study, we constructed the AAV vectors containing the miRNA sequence for D$_2$r and GFP as a vector-working marker and microinjected those into the NAc of mice. The AAV-miD$_2$r vectors selectively controlled the expression levels of D$_2$rs without affecting those of D$_1$rs. Furthermore, the expression of GFP was observed in the NAc and SNr but not in the dSTR and VTA. These findings suggest that the postsynaptic but not the presynaptic (axon terminals of afferent fibers) D$_2$rs on the DAergic synapses in the core of the NAc were reduced by the delivery of the AAV-miD$_2$r vectors. Therefore, the miD$_2$r mice would exhibit dysfunction of the indirect γ-aminobutyric acidergic pathway projecting to the SNr from D$_2$rs-expressing MSNs in the core of the NAc.

METH increases the extracellular levels of DA by modulating its release and reuptake and thereby acts as an indirect agonist for DA receptors. METH induces abnormal behaviors, such as hyperlocomotion, locomotor sensitization, and conditioned place preference. Furthermore, METH leads to altered intracellular signal transduction, such as induction of the transcription factors CREB and delta FosB. In our results, the METH-induced behavioral and intracellular signal impairments were partially improved in miD$_2$r mice. This observation appeared to be consistent with previous results that have been obtained from some pharmacological experiments with D$_2$r antagonists to investigate the mechanisms of METH-induced rewarding effects (Mizoguchi et al., 2004; Carati and Schenk, 2011; Kurokawa et al., 2012). However, considering the defective selectivity to the target molecules in the chemical compounds that are so-called selective D$_2$r antagonists and the expression of D$_2$rs on both presynaptic and postsynaptic sides of the DAergic synapses, the pharmacological blockade of D$_2$rs was insufficient to explain in detail the contribution of D$_2$r in METH-induced addiction. In contrast, our observation precisely indicates that the indirect pathway from D$_2$rs-expressing MSNs in the NAc plays an important role in the development of addictive responses.

However, there have been many reports of the pharmacological experiments in which D$_1$r antagonists attenuate abnormal behaviors and alter the intracellular signaling that is induced by drugs of abuse, including METH. Furthermore, recent reports demonstrated that the specific cells expressing D$_1$rs in the STR, including the NAc, play a role in addictive behaviors induced by repeated exposures to cocaine (Hikida et al., 2010; Kim et al., 2011), that is, these demonstrations propose that the direct pathway from MSNs that express D$_1$rs plays an important role in the development of addiction, and these were different from our observations. This contradiction may be explained by the hypothesis that there is a different neural circuit in distinct situations of drug addiction. The reinforcing effects of addictive drugs engage reward neurotransmitters and associative mechanisms in the VTA-NAc, and stimulus–response habits depend on the SN-dSTR (reviewed in Koob and Volkow, 2010). Therefore, although the NAc and dSTR constitute a similar cell population and output pathway, these brain regions serve different aspects in each situation of drug addiction through distinct neuronal inputs. For other causes, it has been reported that there are approximately 1 to 2% of spiny large cholinergic interneurons in the NAc. Therefore, the MSNs that express D$_1$rs and D$_2$rs may have direct or indirect reciprocal interactions in the core of the NAc, and the cholinergic interneurons in the NAc play an important role in the cocaine reward system (Hikida et al., 2003). Thus, the above findings and our observations suggest that the cholinergic interneurons expressing D$_2$rs modulate

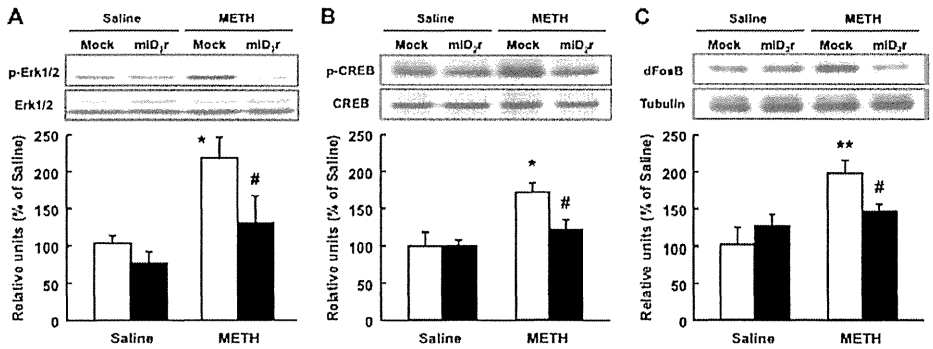

Figure 4. Intracellular effects of methamphetamine (METH) in miD$_2$r mice. A, METH-induced extracellular signal regulated kinase (ERK2) phosphorylation in miD$_2$r mice. B, METH-induced cAMP response element-binding protein (CREB) phosphorylation in miD$_2$r mice. Mice were treated once with METH (1 mg/kg) and sacrificed 15 minutes later. N = 4. *P < .05, **P < .01 vs saline, #P < .05 vs Mock (Student–Newmann–Keuls test). C, METH-induced deltaFosB expression in miD$_2$r mice. Mice were repeatedly treated with METH (1 mg/kg/d for 7 days). N = 4. **P < .01 vs saline, #P < .05 vs Mock (Student–Newmann–Keuls test).

the direct pathway from the MSNs expressing D$_1$rs in the core of the NAc. In any case, for the elucidation of the neural networks in the core of the NAc, further examinations using the cell-type and regional-specific gene modification technologies are necessary.

In summary, our observations exhibited the usefulness of AAV-miD$_2$r vectors as a gene therapy tool for the purposed functional inhibition of D$_2$rs in patients with DA-related symptoms. In addition, the knockdown of D$_2$rs in the core of the NAc suppresses reinforcement-related behavioral and intracellular responses induced by addictive drugs.

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Statement of Interest

None.

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