and protein of the 5-HT2A receptor gene [18], it is thought that this SNP is more likely one of the true susceptible polymorphism. In addition, our results were consistent with a previous finding that the expression of the C allele of 102T/C was lower than that of the T allele at both the mRNA and the protein levels [18]. Namely, it was thought that patients with the T/T genotype were more sensitive to pain than those with the other genotypes because the T/T genotype caused higher expression of mRNA and protein and greater activation of the 5-HT2A receptor than the C/C genotype. Meanwhile, significant positive correlations were observed between the indexes of analgesic requirements and NRS pain scores. This means that the patients who experienced more severe pain, as determined from the NRS pain scores, required more frequent and higher doses of rescue analgesics. Nevertheless, significant association was not observed between the SNP and NRS pain scores; therefore, we may need to carefully interpret the results of the analysis with regard to the relationship between the SNP and the indexes of analgesic requirements.

Because a significant difference in analgesic requirements was seen only in the women in this study, we believe that the analgesic mechanism might differ between the sexes. It is generally known that women have a lower pain threshold and tolerance than men, and it has been indicated that gonadal hormones (e.g., estrogen and testosterone) influence pain sensitivity [6]. In particular, estrogen has been reported to modulate serotonergic function and has been suggested to influence 5-HT synthesis, 5-HT receptor-binding capacity, mRNA expression, etc. [10,14,19]. The mechanism underlying this effect of estrogen has however not yet been elucidated. However, because male and female patients may need to have their pain managed differently, it seems important clinically to consider differences in sex with regard to postoperative pain management.

Although the sample size of this study population was small, we examined the influence of operative procedures. The indexes of analgesic requirements significantly differed between the patients who underwent gastrectomy and those who underwent enterectomy, and it was suggested that analgesics were used significantly more frequently in the case of gastrectomy than in the case of enterectomy. However, on analyzing the association between the SNP and the indexes of analgesic requirements for every operative procedure, we could not observe significant interaction effect between the SNP and sex. The analysis results for individual operative procedures were inconsistent with those for the entire population probably because of the small sample size. To consider the impact of differences in surgical sites or operative procedures on pain, multiple investigations may be necessary in the future.

The analgesic and/or reward effects of opioids are mediated by the opioid receptor. In the analysis of μ opioid receptor gene-knockout mice, it has been reported that morphine sensitivity strongly correlates with the expression of the μ opioid receptor [13,20,21]. Future detailed investigations about the effect of gonadal hormones and other analgesia-related genes (e.g., opioid receptor gene) may be clinically important.

In conclusion, the LD block, which includes the 102T/C polymorphism in the 5-HT2A receptor gene, influenced the analgesic requirements after major abdominal surgery. Our results suggest that women with the T/T genotype of the 102T/C polymorphism have more analgesic requirements than those with the other genotypes. We believe that these results are important in efforts to relieve risks of adverse effects on postoperative pain management.

Acknowledgments

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A rotarod test for evaluation of motor skill learning

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ABSTRACT

The rotarod test is widely used to evaluate the motor coordination of rodents, and is especially sensitive in detecting cerebellar dysfunction. However, mice with striatal dopamine depletion show only mild or no motor deficit on the typical accelerating rotarod. This suggests that dopamine-depleted mice are useful as animal models for non-motor symptoms, because the influence of motor deficit is minimum and easy to discriminate from cognitive aspects of the behavioral change. The typical accelerating rotarod test is designed to evaluate maximal motor performance and is not optimized to detect motor skill learning. In an attempt to make the test more selective to motor skill learning rather than maximal gait performance, we modified the rotarod test by using a slowly rotating large drum to obtain a steep learning curve. Furthermore, administration of nomifensine, a dopamine uptake inhibitor, improved the learning. On the other hand, apomorphine, an agonist of dopamine autoreceptor, a dopaminergic toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) impaired the learning. These pharmacological profiles fit the involvement of the so-called phasic dopamine neurotransmission. Using our modified procedure, we found impaired learning of Parkin-deficit mice, which has not been detected in typical accelerating rotarod. The modified rotarod test would be useful for evaluation of dopamine involvement in the acquisition of motor skill learning.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by akinesia, rigidity, resting tremor, and postural instabilities. In addition, neuropsychiatric, perceptual and cognitive deficits are increasingly recognized as non-motor manifestations of Parkinson's disease (Carbon and Eidelberg, 2006; Frank et al., 2004; Owen et al., 1992; Taylor et al., 1990). It is generally difficult to discriminate motor and cognitive aspects in behavioral tests of Parkinson's disease patients, because impaired movement can influence all of behavioral performance. If there is an animal model which has Parkinson-like pathological brain degeneration but has no motor deficit, it would be an ideal model for the non-motor symptoms of Parkinson's disease.

The major neurochemical hallmark of Parkinson's disease is the degeneration of dopaminergic neurons in the substantia nigra pars compacta. In animal models, nigral degeneration can be produced by the selective toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine (MPTP). Although nigral neurons are degenerated in MPTP-treated mice, several studies have failed to detect motor deficit (Jackson-Lewis and Przedborski, 2007; Przedborski et al., 2001). While MPTP-treated primates show Parkinsonism-like motor deficits (Arai et al., 1990; Fisher et al., 2004; Sedelis et al., 2001; Tillerson et al., 2002), the behavior of MPTP-treated mice can be hardly be mentioned as "Parkinsonism".

Among several behavioral tests that measure motor performance, the rotarod is a suitable test for evaluation of cerebellar deficits in rodents (Caston et al., 1995; Lalonde et al., 1995). The motor performance on the rotarod can be influenced by several factors, such as motor coordination, learning and cardiopulmonary endurance. Since several studies have shown that basal ganglia are essential in motor skill learning of serial motor sequence (Hikosaka et al., 1999), we tried to extract the acquisition of motor skill from the original procedure. In the previous study, deficit in the acquisition of rotarod learning was not obvious in MPTP-treated C57 BL/6 mice (Sedelis et al., 2000). Some studies have detected impairment of rotarod performance in dopamine-depleted mice and rats (Monville et al., 2006; Rozas et al., 1998). Monville have shown

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that the sensitivity to motor disability improved as rotation speed became higher. But these studies focused on behavior after overtraining whereas the learning curve at the initial acquisition phase was not presented. The learning effect appears as the elongated falling latency along with the trial numbers, but the typical accelerating rotarod does not seem ideal for evaluating acquisition of motor skill learning, because the learning curve is shallow and the performance after training for several days is no more than twice of the first day (Perez and Palmiter, 2005). Various rod sizes and speeds have been tried on a considerable amount of the literature evaluating daily changes in the motor performance on the rotarod (Akita et al., 2006; Caston et al., 1995; Jeljeli et al., 1999; Ogura et al., 2005; Sedelis et al., 2000). But if researchers decide to look for changes in performance over successive testing bouts where "motor learning" can be demonstrated, the rotarod test should have a distinction separating "motor learning" from basal gait/postural ability. Therefore, in the present study, we designed a modified rotarod protocol to evaluate the acquisition of motor skill learning selectively. Our non-accelerating rotarod test employs a wide drum with a hard surface, on which naive mice find it difficult to stay, but its low rotating speed leaves room for improvement after training.

A recent study reported dynamic reorganization of striatal circuits during the acquisition of motor skill on the accelerating rotarod (Yin et al., 2009). Furthermore, Akita et al. (2006) reported that a decrease in synaptic dopamine release induced by blocking the expression of synaptophysin in the nigrostriatal neurons resulted in impairment of acquisition of the rotarod task. Transgenic mice with striatal degeneration could walk on the rotarod but lacked the ability to learn (Kishioka et al., 2009). Based on these findings, one can postulate that the rotarod task reflects the striatum-based motor skill learning. To test this hypothesis, first, we tried our modified rotarod test in MPTP-treated mice. Next, we examined the effects of dopamine uptake inhibitors and a dopamine agonist. Finally, we examined our modified rotarod test in Parkin-deficient mice to compare with past studies using accelerating rotarod. Several studies have failed to detect impairment in gait performance of Parkin-deficient mice (Goldberg et al., 2003; Von Coelln et al., 2004; Perez and Palmiter, 2005; Sato et al., 2006), but impairment of motor skill learning was evident in our modified rotarod test. The results showed that our modified rotarod test is suitable for the selective evaluation of acquisition of motor skill, and cognitive involvement of the nigrostriatal dopamine system.

2. Materials and methods

2.1. Animals

Adult C57 BL/6J male mice (CLEA Japan, Tokyo, Japan, 4-month old, 25–30 g body weight) were used in this study. In addition, *Parkin−/−* male mice (4-months old), which carries a chromosomal replacement of the exon 3 of the *parkin* gene (Kitao et al., 2007) and their littermates were used. The mice were backcrossed into the C57BL/6J mice for 12 generations, then both +/+ (WT) and −/− (PKO) littermates were substrained and maintained in parallel in the same animal facility in Juntendo University. Wild-type and Parkin-deficient mice of close birth date were subjected to the behavioral tests in the same day in a shuffled sequence.

The mice were housed in groups of five to eight per cage and allowed free access to food and water. They were maintained in a temperature-, humidity- and light-controlled environment with a 12 h light–dark cycle. The experimental procedures were in accordance with the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan and all experiments were approved by the Ethics Review Committee for animal Experimentation of Juntendo University School of Medicine. All efforts were made to minimize the number of animals and their suffering.

2.2. Motor skill learning test

To assess the acquisition of skilled behavior in mice, we first modified the standard rotarod test to emphasize the learning aspect of the test and minimize the other factors. A rotarod machine with automatic timers and falling sensors (MK-660D, Muromachi-Kikai, Tokyo, Japan) were used. The mouse was placed on a 9 cm diameter drum. The surface of the drum was covered with hard chloroethylene, which does not permit gripping on the surface. Before the training sessions, the mice were habituated to stay on the stationary drum for 3 min. Habituation was repeated every day for 1 min just before the session. Acceleration of the rotation was abandoned and the rotation was set at a relatively slow speed (10 rpm, 2.8 m/min on the surface), to make the task easier for learned animals. The animal was placed back on the drum immediately after falling, up to 5 times in one session. A fall was overlooked when the animal remained on the drum for 180 s. To evaluate long-term memory, the test was repeated one session a day for four consecutive days. The latency to falling was recorded automatically by photo-cells and the total latencies on the rod on each day was analyzed. Next, in order to compare the typical rotarod test with our modified rotarod test, we also tested the accelerating rotarod protocol. The speed of the rod of 3 cm diameter was accelerated from 4 to 40 rpm. The habituation time and daily schedule was the same.

2.3. Drugs and solutions

MPTP-HCl, nomifensine, and (−)-apomorphine hydrochloride (Sigma Chemical Co., St. Louis, MO) were dissolved in saline. Apomorphine was dissolved just before use. Drugs were administrated subcutaneously in 10 ml/kg. The doses used were 30 mg/kg for MPTP, 1 and 3 mg/kg for nomifensine and 0.1 and 0.3 mg/kg apomorphine. The information on the solution bottle was coded by another individual scientist, and the trainer of the animal was blinded to the drug information.

2.4. Pharmacological treatment

2.4.1. MPTP

Each mouse received one injection of either saline or MPTP solution per day for five consecutive days, and the animals were allowed to recover for three days before the first training session. Several different MPTP dosing regimens were used. We used typical sub-acute intoxication regimen, which involves one injection of 30 mg/kg MPTP daily for five consecutive days (Jackson-Lewis and Przedborski, 2007). We chose this sub-acute regimen in order to minimize the death of the animals during administration. Motor learning training was applied for four consecutive days, and two days after the last training, all animals were deeply anesthetized with an overdose of pentobarbital, decapitated and the striatal tissue was dissected out under a microscope. All needles, syringes and animal housings were cleaned with 1% bleach solution in water according to the safety protocols (Jackson-Lewis and Przedborski, 2007; Przedborski et al., 2001).

2.4.2. Nomifensine and apomorphine

The mice were assigned to three groups for each dose of each compound. Motor learning training was applied for four consecutive days. Each animal received one injection of each drug per day, 10 min before the training.

Behavioral tests were performed during the light cycle and conducted by the same scientists. They were videotaped for later confirmation of any ambiguous recordings. The behavioral tests were performed in the same room housing the animals.

2.5. Neurochemistry

The striatal contents of dopamine and its metabolites were measured by high-performance liquid chromatography (HPLC). The samples of the left and right striata were kept at −80 °C until measurement of monoamine levels. The striatal samples were weighed, sonicated in 0.1N perchloric acid, centrifuged 15,000 rpm for 10 min at 4 °C, and dopamine in the supernatant was isolated by HPLC and measured with an electrochemical detector.

2.6. Data processing and statistical analysis

The learning curves were analyzed by a two-way analysis of variance (ANOVA) for repeated measures within a sample. The interaction of drug treatment and daily change was evaluated following Greenhouse Geisser correction. Data are expressed as mean s.e.m. A p value < 0.05 was considered statistically significant. All statistical procedures were conducted using the Statistical Package for Social Sciences (SPSS V17.0) (SPSS Inc., Chicago, IL).

3. Results

To extract the learning factor from the rotarod test, we tried to modify the task so that it was difficult for naive pre-trained mice and easier for trained mice. When the rotation speed was fixed at slow 10 rpm using 3 cm rod (Fig. 1A), it was easy for both naive and experienced mice, and the learning effect was little. Apparently, it was not suitable for the evaluation of the motor skill learning. After the combination of a large drum and slow rotation (9 cm diameter and 10 rpm, Fig. 1B), a steep learning curve was obtained, and the sum of latencies on day 4 was more than 4 times of the first day in the control mice ($n = 15$) (Fig. 1B). The daily change in learning was significant ($p < 0.001$).

To examine whether disruption of dopamine in the striatum causes impairment of acquisition of skilled behavior, MPTP-treated mice were tested with our method. The striatal dopamine content of MPTP-treated mice was 16.2 ± 0.9% of saline-treated mice. The performance on the typical accelerating rotarod of MPTP mice was almost identical to the saline animals (Fig. 1C, $n = 15$ and 11, group difference $F_{(1,24)} = 0.08$ ($p = 0.780$), interaction between time and groups $F_{(3,72)} = 1.32$ ($p = 0.274$)), while the difference between the groups was detected on our modified rotarod (Fig. 1B, $n = 15$ and 13, $F_{(1,26)} = 5.03$, $p = 0.034$), although the difference in learning effect was not significant (interaction between time and groups $F_{(3,78)} = 1.86$ ($p = 0.164$)).

To investigate the involvement of dopamine neurotransmission further, we examined the pharmacological effects of dopamine uptake blocker (nomifensine), and dopamine agonist (apomorphine) (Fig. 2). A relatively low dose was chosen to avoid stereotypic behaviors. Nomifensine at 1 and 3 mg/kg enhanced the learning ($F_{(6,126)} = 3.25$, $p = 0.005$) while apomorphine diminished it ($F_{(6,78)} = 4.19$, $p < 0.001$).

On the other hand, there was significant difference in the rotarod performance between Parkin-deficient mice and wild-type mice (Fig. 3). Parkin-deficient mice stayed on for a shorter time than wild-type mice using our modified rotarod protocol. When five trials were repeated for each day, 4-month-old Parkin-deficient mice stayed on for a shorter time than wild-type mice after learning (repeated measure ANOVA, $F_{(1,28)} = 6.66$, $p = 0.015$, Fig. 3).

4. Discussion

4.1. Discrimination of motor and cognitive aspects of rotarod learning

In the present study, we succeeded in obtaining a steep learning curve based on the performance of the modified rotarod test, which had a large margin to evaluate the learning effects. The sum of daily latencies elongated to 4 times of the first day in our protocol, while it was only 1.5 times in the typical accelerating rotarod. The learning on the repeated rotarod can be evaluated by the typical accelerating rotarod, and it is not new to analyze the learning curve. But our procedure improved in selectivity of the cognitive factor. Since the rotation speed was slow, the influence of maximal gait and cardiopulmonary performance was minimized. The motor learning deficit observed in MPTP-treated mice and Parkin-deficient mice most likely does not correspond to gait disability but is rather selectively related to cognitive impairment.

To make the learning curve steeper, the rod diameter seems to be important for the initial performance of the animals. Rozas et al. (1998) explained that the use of larger rods with mice may reflect the ability to walk or run. In contrast the same test for rats does not reflect the same set of abilities. We noticed that untrained mice showed poor performance staying on a large-diameter drum, originally designed for rat experiments. While on the top of the large-diameter drum the mice were unstable, and easily slipped-off when their body moved from the top of the drum. Mice needed effort not to fall off the drum. On the other hand, in the typical

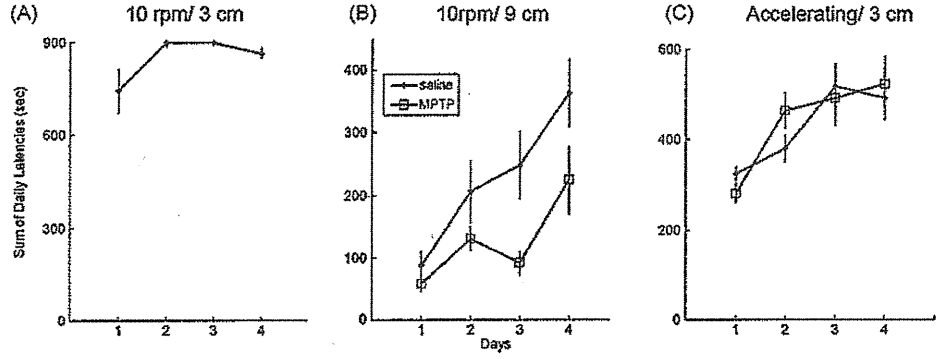

Fig. 1. Motor skill learning on three types of rotarod and the effect of MPTP. (A) Daily changes on fixed-slow speed rotarod (10 rpm, 3 cm diameter rod, $n = 7$). The sum of latencies of five trials in one day is presented. Note 900 s means no fall in five180 s trials. Data are mean ± s.e.m. (B) Daily changes on our modified rotarod on the 9 cm diameter drum in the saline-treated (filled diamond, $n = 15$), and MPTP-treated (open square, $n = 13$) mice. (C) Daily changes on typical accelerating rotarod (4–40 rpm over 5 min, 3 cm diameter rod) in the saline-treated ($n = 15$), and MPTP-treated ($n = 13$) mice.

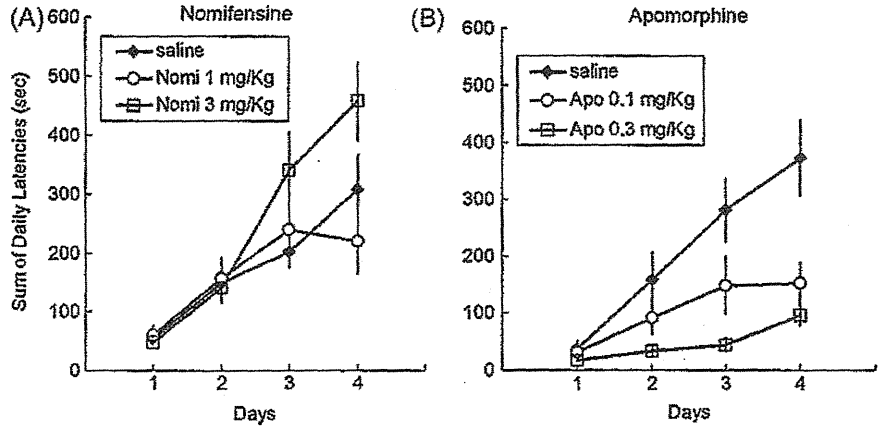

Fig. 2. Effects of nomifensine and apomorphine on the learning of the modified rotarod test. Nomifensine and apomorphine were injected subcutaneously 10 min before each training session. (A) Nomifensine at both low dose (1 mg/kg) and high dose (3 mg/kg) significantly enhanced the daily change in learning ($n = 15$, $p = 0.005$, interaction between day and stay-time, 2-way ANOVA). (B) Apomorphine at both low dose (0.1 mg/kg) and high dose (0.3 mg/kg) inhibited daily learning ($n = 9$–10, $p < 0.001$, interaction between day and stay-time, 2-way ANOVA). Data are mean ± s.e.m.

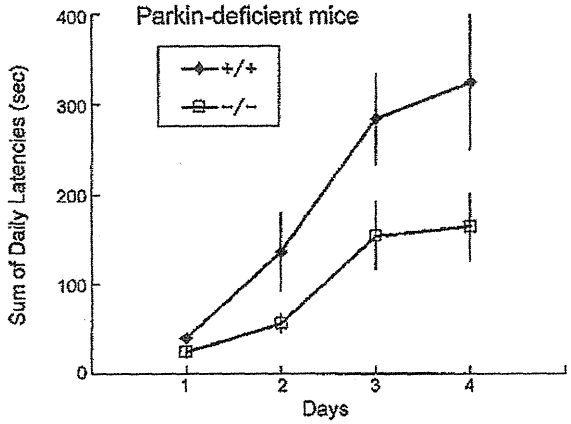

Fig. 3. Motor skill learning of Parkin-deficient and wild-type mice on modified rotarod. Daily changes on the rotating drum in the wild-type (filled diamond, $n = 15$), and Parkin-deficient (open square, $n = 15$) mice on our modified rotarod. The sum of latencies of five trials in one day is presented. Data are mean ± s.e.m.

rotarod diameter (3 cm), designed for mice experiments, untrained mice could keep a stable position if their body moved away from the center because they could still cling to the drum easily. All naive untrained mice fell off from the 9 cm drum in the in the first day (72 s) of testing, though they could cling to the 3 cm rotating rod (302 s) without training. The mouse species probably has an innate ability to cling on a tree branch, but the 9 cm drum might be too thick to turn on this motor program. Their latency on the drum depended on learning, and it made our experiment easier to segregate the learning ability and general gait performance.

4.2. Phasic and tonic dopamine

This steep slope was sensitive to dopaminergic modulation including MPTP-induced nigrostriatal-degeneration, and blockade of dopamine transporter by nomifensine. In contrast, apomorphine, a dopamine receptor agonist of presynaptic autoreceptor, impaired the learning. What about dopaminergic transmission, which is improved by uptake-blockers and depressed by dopamine agonists? Nomifensine is known to induce a transient increase in evoked extracellular dopamine (Cragg and Rice, 2004; Robinson and Wightman, 2004). On the other hand, it is well established that stimulation of presynaptic D2 autoreceptors of dopaminergic terminals suppresses dopamine release (May and Wightman, 1989; Schmitz et al., 2002). The transient increase in dopamine release evoked by firing activity of dopaminergic neurons is called phasic or wiring neurotransmission and high-concentration of dopamine is transmitted by D1 receptors (Zoli et al., 1998). Conversely, the long-term maintenance of extracellular dopamine level is known as tonic or volume neurotransmission and transmitted by D2 receptors. Our behavioral results using apomorphine and nomifensine are in agreement with the known pharmacological character of phasic dopamine neurotransmission. This finding is consistent with the results of Costa et al. (2004) who showed the importance of D1 neurotransmission in motor skill learning of repeated rotarod.

Among model animals of Parkinson's disease, little motor deficits have been found in Parkin-deficient mice (Goldberg et al., 2003; Von Coelln et al., 2004; Perez and Palmiter, 2005; Sato et al., 2006). Although they have the same genetic deficit with park 2 patients, Parkin-deficient mice show no degeneration of nigral neurons and no changes in dopamine content in the striatum. On the other hand, the elevated extracellular dopamine (Goldberg et al., 2003), up-regulation of dopamine receptors (Sato et al., 2006) suggests modest changes in dopaminergic neurotransmission. Four studies using typical accelerating rotarod failed to detect the difference between Parkin-deficient and wild-type mice. Our result is the first demonstration of changes in Parkin-deficient mice on the rotarod test. Considering negative results in other motor tests and our modified procedure is less sensitive to the gait ability, our results suggest dopamine-related cognitive deficit in Parkin-deficient mice. Since Parkin-deficient mice do not show pathological changes of dopaminergic neurons, the mechanisms cannot be the same as MPTP-treated mice. Recently, reduction in phasic dopamine release in Parkin-deficient mice has been reported (Kitada et al., 2009). Our results of learning deficit in Parkin-deficient mice may be consistent to apomorphine-treated mice.

4.3. Cognitive deficit induced by degeneration of nigrostriatal projection

The midbrain dopaminergic neurons project to a wide area in the forebrain, including the cerebral cortex, striatum and other limbic regions. Striatal function is modulated by the extremely dense nigrostriatal dopaminergic projection. In this study, learning impairment correlated with the striatal dopamine content. It has been reported that MPTP results in selective degeneration of substantia nigra with relative sparing of the meso-cortical and meso-limbic projections. Among the dopamine projection areas, the striatum seems to be the most influential in our modified learn-

ing task, although the involvement of other areas cannot be ruled out at this stage.

The striatum exhibits a wide range of actions affecting motor and cognitive functions. It has been reported that the motor skill learning process is associated with neuronal changes in the striatum (Hikosaka et al., 1999; Lehericy et al., 2005; Poldrack et al., 2005). It is also known that the motor skill learning on the rotarod requires cortical and striatal ensemble activity (Costa et al., 2004; Jeljeli et al., 1999). The cognitive function of dopamine mediated by the reward-related phasic activity may be distinct from the motor-regulation related to the tonic neurotransmission (Schultz, 2007). Recent studies reported impairment of the acquisition of motor skill learning in Parkinson's disease (Heindel et al., 1989; Roncacci et al., 1996), and signs of cognitive impairment in the earliest stage of the disease (Carbon and Eidelberg, 2006; Frank et al., 2004; Owen et al., 1992; Taylor et al., 1990).

In rodents, dopamine depletion impairs spatial memory tasks (Da Cunha et al., 2006; Ferro et al., 2005; Miyoshi et al., 2002). Our study has confirmed that this is also true in motor learning. Considering the little difference in the typical accelerating rotarod, our results of impaired acquisition in motor skill learning suggests deficit in cognitive function rather than gait control. Our modified procedure would be useful for selective evaluation of the cognitive aspect of nigrostriatal dopamine system.

5. Conclusion

We have developed a modified learning-sensitive procedure of the rotarod test and defined the acquisition slope for this test, which was sensitive to dopaminergic neurotransmission. These results in rodents suggest that degeneration of nigrostriatal system, or modest change in dopaminergic neurotransmission result in motor skill learning deficit, rather than gait disturbances. The pharmacological results strongly suggest such impairment is mediated by phasic dopamine transmission. Our modified rotarod test is potentially useful for the analysis of the mechanisms of non-motor symptoms in Parkinson's disease. Moreover, our results suggest that MPTP-treated mice are useful for the evaluation of non-motor functions of striatal dopamine system.

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Clinical Study

The association between personality, pain threshold and a single nucleotide polymorphism (rs3813034) in the 3′-untranslated region of the serotonin transporter gene (SLC6A4)

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ABSTRACT

In 181 healthy Japanese volunteers we examined the relationship between personality, sensitivity to pain and a single nucleotide polymorphism (rs3813034) in the 3′ untranslated region (3′ UTR) of the serotonin transporter (5-HTT) gene (SLC6A4). Pain sensitivity was assessed by using cold and pressure thresholds. Personality was assessed by the Temperament and Character Inventory (TCI). Males without the T allele (*G/G*) showed a significantly higher spiritual acceptance (ST3) score than those who had the T allele (*T/T* and *T/G*). Females with the T allele (*T/T* and *T/G*) showed significantly higher transpersonal identification (ST2) and self-transcendence (ST) scores than those without the T allele (*G/G*). As for pain sensitivity and its relationship with TCI, we found a low negative correlation between cold water stimulation, disorderliness (NS4) and novelty seeking (NS) in males, whereas in females we found a low positive correlation between cold water stimulation, self-acceptance (SD4) and pure-hearted principles (C5), as well as pressure stimulation and SD4. It is possible that the 5-HTT 3′ UTR gene polymorphism affects the character dimensions of Cloniger's theory, and that there might be a low correlation between pain and a part of the personality.

1. Introduction

Serotonin (5-HT) acts as a classic neurotransmitter of the central nervous system (CNS) when involved in physiological roles such as feeding behavior, sleep, sexual behavior, cognition and sense of pain. It is also believed that 5-HT is involved in personality traits as well as psychiatric disorders (including mood disorders, anxiety disorders, eating disorders, drug dependency and schizophrenia).[1]

5-HT reinforces pain in the peripheral nervous system and inhibits pain through the descending inhibition system of the CNS.[2] The cerebral cortex, hypothalamus and aqueductal gray matter comprise the pain inhibition system in the CNS, and it has become clear that pain transmission is selectively controlled by fibers that descend from the nerve nuclei in the brainstem to the outer layer of the posterior horn of the spinal cord.[3]

In the 5-HT neurotransmission system, 5-HT is released from nerve endings, stimulates receptors and is then resorbed by a serotonin transporter (5-HTT) at the presynaptic nerve endings. This regulation is one of the most important in reducing 5-HT transmission.[4]

The 5-HTT is the target for many antidepressant drug treatments (including tricyclic antidepressants and selective serotonin reuptake inhibitors [SSRI]). 5-HTT might be important in terminating serotonergic neurotransmission through increasing 5-HT uptake into presynaptic neurons. Conversely the pharmacologic action of SSRI is associated with binding to 5-HTT, and transmission of the 5-HT neurons is thus enhanced by inhibition of 5-HT re-uptake.[1]

The 5-HTT gene (SLC6A4) consists of 14 exons comprising about 38 kilobase pairs (kbp) and is located at 17q11.1-q12.[4] Within the polyadenylation signal in the 3′ untranslated region (3′ UTR) of the 5-HTT gene, there is a single nucleotide polymorphism (rs3813034) that is defined by a guanine (G) to thymine (T) transition at 689 bp downstream from the stop codon of exon 14.[5] This polymorphism in the 5-HTT gene has potential functional significance because abnormal polyadenylation might interfere with the stability of mRNA and the facilitation of its transport into the cytoplasm.[6] Therefore, this polymorphism might be important, because

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the mutation it causes may result in low expression of the 5-HTT gene. To our knowledge, the relationship between polymorphism in the 5-HTT gene and personality traits has not been previously reported, although some studies have examined its association with attention deficit hyperactivity disorder (ADHD).[6–9] Although Kent et al. found an association between this polymorphism and ADHD,[6] this was not observed by other researchers.[6–9] As stated previously, the 5-HTT 3′ UTR polymorphism may be related to both personality traits and pain transmission.

The Temperament and Character Inventory (TCI) is used to examine the relationship between genes and personality. The TCI, an expansion of the Tridimensional Personality Questionnaire developed in 1987 by Cloninger, divides the personality into temperament, with four dimensions, and character, with three dimensions.[10] According to Cloninger's theory, temperament is heritable, it manifests fully during infancy, and involves preconceptual biases in perceptual memory and habit formation. The four measured temperament dimensions are: novelty seeking (NS), harm avoidance (HA), reward dependence (RD) and persistence (P). It is assumed that these temperament dimensions depend upon the secretion and metabolism of neurotransmitters in the CNS – dopamine, 5-HT and norepinephrine. Similarly, in Cloninger's theory, character matures in adulthood, and influences personal and social effectiveness through greater insight about the self. There are 3 character dimensions: self-directedness (SD), co-operativeness (C) and self-transcendence (ST). In addition, each of seven dimensions, except P, has three to five lower dimensions or subgroups, respectively. For example, RD has three subgroups, NS has four subgroups and SD has five subgroups.[11]

The presence of polymorphism (rs3813034) in the 3′ UTR of the 5-HTT gene might be related to variations in pain threshold and personality. Therefore, in 181 healthy Japanese volunteers, we examined the relationship between: (i) the gene polymorphism and personality; (ii) the gene polymorphism and pain threshold; and (iii) pain threshold and personality.

2. Materials and methods

2.1. Participants

The study included 181 volunteers who were assessed as healthy on medical examination at our Institution. There were 115 males (mean age [±standard deviation, SD] of 36.8 ± 13.0 years) and 66 females (mean age 34.4 ± 12.5 years) with an overall mean age of 35.9 ± 12.9 years. We also used a questionnaire to confirm that each participant was not engaged in any work, sport or art form that had led to overuse of a hand, and also to confirm that participants were not injured or ill. The study was approved by the Ethics Committee of the Tokyo Institute of Psychiatry. After obtaining written informed consent from all participants, oral mucosa samples were obtained; pain threshold tests and TCI assessment were also undertaken.

2.2. DNA analysis

We extracted and purified genomic DNA using the phenol/chloroform method. Genotyping of the gene polymorphism (rs3813034) was carried out by polymerase chain reaction (PCR).[5] (The bases, in addition to G and T are A, adenine; and C, cytosine). The primers used for PCR were: HTT.PCR1 (5′-CCG CTT GAA TGC TGT GTA ACA CAC-3′) and HTT.PCR3 (5′-GTA CCC TTC CAA TAA TAA CCT CC-3′), which amplified a fragment of 741 bp in length.[5] The PCR conditions were: (i) initial denaturation for 5 minutes at 94 °C, 1 cycle; (ii) denaturation, 30 s at 94 °C, 1 cycle; (iii) annealing, 45 s at 59 °C, 30 cycles; and (iv) extension, 45 s at 72 °C, using a Program Temperature Control System PC-700 (Astec Irie; Fukuoka, Japan). The PCR products were digested by using TruI1 (MBI Fermentas; Vilnius, Lithuania). The restriction pattern was G allele: 741 bp, T allele: 52 bp and 689 bp.

2.3. Psychometric evaluation

Assessment of personality traits was conducted using the TCI (consisting of 125 items in the Japanese short version), a self-assessment instrument with "yes/no" answers. In addition, the 7 dimensions of temperament and character in the TCI were classified into 31 subdimensions.

2.4. Pain threshold evaluation

Measurement of pain threshold was performed by means of cold and pressure pain stimulation tests. The cold pain threshold was measured by the time elapsed from the submersion of a finger (up to the level of the second joint) in ice cold water (0 °C) to the first perceived pain. The index and middle fingers were examined separately; the mean value calculated based on these data was used as the cold pain threshold. The cutoff value for the cold pain threshold was 180 s.

An algometer, used to measure the pressure pain threshold, was held vertically against the participant's nail and the pressure was increased constantly. The pressure level was measured when the pressure sensation became a pain sensation for each finger, but not for the thumb. The mean value was used as the pressure pain threshold.

2.5. Statistical analyses

Analysis of variance (ANOVA) and Pearson's product–moment correlation coefficient were used for statistical analyses of the data. A value was considered an outlier if it was more than three times the interquartile range from the first or third quartile. Four subjects that were less sensitive to cold pain were outliers and excluded from the analyses. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 12.0J for Windows (SPSS; Chicago, IL, USA).

3. Results

The genotype frequencies of the 5-HTT gene polymorphism were: in males, *G/G* was 69.6% ($n = 80$), *G/T* was 28.7% ($n = 33$) and *T/T* was 1.7% ($n = 2$); and in females, *G/G* was 69.7% ($n = 46$), *G/T* was 27.3% ($n = 18$) and *T/T* was 3.0% ($n = 2$). The findings revealed that the overall frequency for *G/G* was 69.6% ($n = 126$), *G/T* was 28.2% ($n = 51$) and *T/T* was 2.2% ($n = 4$). The allele frequencies were: in males, the G allele was 83.9% ($n = 193$) while the T allele was 16.1% ($n = 37$); in females, the G allele was 83.3% ($n = 110$) and the T allele, 16.7% ($n = 22$); in total, the G allele was 83.7% ($n = 303$) and the T allele was 16.3% ($n = 59$). The genotype distribution for the entire participant population was in the Hardy–Weinberg equilibrium (χ^2 (1) = 0.0282, $p = 0.8667$, Yates' continuity correction).

The relationship between the 5-HTT gene polymorphism and pain threshold was examined using two-way ANOVA with both genotype and sex as the independent variables, and pain threshold as the dependent variable. The analysis classified the participants into those with the T allele (*T/T* and *T/G*) and those without (*G/G*) according to genotype. There was no significant difference in pain threshold between genotypes. There was a significant effect of sex on the pressure pain threshold ($F[1,177] = 31.441$, $p < 0.01$); that men had a higher pressure pain threshold than females.

The relationship between the 5-HTT gene polymorphism and the TCI was examined using the two-way ANOVA, with genotype and sex as independent variables, and the seven dimensions of the TCI as the dependent variables. The analysis classified the participants into those with the T allele (*T/T* and *T/G*) and those without (*G/G*) according to genotype. We found a significant interaction between 5-HTT gene polymorphism and sex for ST ($F[1,177] = 7.098$, $p < 0.01$); the *G/T + T/T* group showed a significantly higher score than the *G/G* group in females ($F[1,64] = 4.652$, $p < 0.05$) (Table 1). In addition, in order to examine ST in detail, the ST subdimensions were analyzed using two-way ANOVA. This revealed a significant interactions between the gene polymorphism and transpersonal identification (ST2) in females and gene polymorphism and spiritual acceptance (ST3) in males ($F[1,177] = 5.632$, $p < 0.05$; $F[1,177] = 6.609$, $p < 0.05$). In females, the *G/T + T/T* group showed a significantly higher ST2 score than the *G/G* group ($F[1,64] = 7.335$, $p < 0.01$), while for males the *G/G* group showed a significantly higher ST3 score than the *G/T + T/T* group ($F[1,113] = 8.014$, $p < 0.01$) (Table 2).

The relationship between pain threshold and the TCI was analyzed using Pearson's product–moment correlation coefficient. In the male participants, significant negative correlations were observed between the cold pain threshold and disorderliness (NS4) ($r = -0.22$, $p < 0.05$), as well as the cold pain threshold and NS ($r = -0.22$, $p < 0.05$). For the female participants, significant positive correlations were found between the cold pain threshold and self-acceptance (SD4) ($r = 0.25$, $p < 0.05$), the cold pain threshold and pure-hearted principles (C5) ($r = 0.25$, $p < 0.05$), and the pressure pain threshold and SD4 ($r = 0.27$, $p < 0.05$). When considering all participants, significant correlations were found between the cold pain threshold and NS4 ($r = -0.18$, $p < 0.05$), the cold pain threshold and anticipatory worry (HA1) ($r = -0.16$, $p < 0.05$), and the cold pain threshold and C5 ($r = 0.16$, $p < 0.05$) (Table 3).

4. Discussion

To date, the genotype and allele frequency of the 5-HTT 3′ UTR gene polymorphism (rs3813034) observed in the Japanese population have been reported only in the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/) (G allele, 84.1%; T allele, 15.9%). Kent et al. reported that in the British population the allele frequency of G was 43.0% and T was 57.0%,[5] whereas Battersby et al. reported that the allele frequency of G was 46.0% and T was 54.0%.[5,6] Our results show that the allele frequency of G was 83.7% and T was 16.3% in the Japanese population, which suggests that differences in the allele frequency might be attributable to race.

Table 1
Mean values (±standard deviation) for the psychological dimensions measured using the Temperament and Character Inventory in participants who have different serotonin transporter 3′ untranslated region genotypes

	Male		Female		Total	
	G/G	*G/T + T/T*	*G/G*	*G/T + T/T*	*G/G*	*G/T + T/T*
NS	9.36 ± 3.59	9.24 ± 4.14	9.12 ± 3.36	7.90 ± 3.68	9.27 ± 3.50	8.75 ± 4.00
HA	11.29 ± 4.79	11.26 ± 4.55	12.61 ± 4.22	12.40 ± 5.53	11.77 ± 4.62	11.68 ± 4.91
RD	9.98 ± 2.43	10.45 ± 2.55	10.76 ± 2.71	10.50 ± 2.84	10.26 ± 2.56	10.47 ± 2.63
P	2.88 ± 1.65	2.96 ± 1.67	2.28 ± 1.53	3.05 ± 1.79	2.66 ± 1.62	2.99 ± 1.70
SD	16.50 ± 5.31	17.27 ± 6.09	17.67 ± 4.93	17.40 ± 4.78	16.92 ± 5.18	17.32 ± 5.60
C	18.19 ± 3.60	18.34 ± 3.65	19.24 ± 2.78	19.45 ± 2.31	18.58 ± 3.35	18.74 ± 3.25
ST	5.23 ± 3.34	4.12 ± 2.20	4.00 ± 2.27*	5.45 ± 2.96*	4.79 ± 3.04	4.60 ± 2.56

C = co-operativeness; *G/G* = without T allele; HA = harm avoidance; NS = novelty seeking; P = persistence; RD = reward dependence; SD = self-directedness; ST = self-transcendence; *T/G* = with T allele; *T/T* = with T allele.
* $p < 0.05$.

Table 2
Mean values (± standard deviation) for the self-transcendence subgroups measured using the Temperament and Character Inventory in participants who have different serotonin transporter 3′ untranslated region genotypes

	Male		Female		Total	
	G/G	*G/T + T/T*	*G/G*	*G/T + T/T*	*G/G*	*G/T + T/T*
ST1	1.45 ± 1.27	1.12 ± 1.16	1.15 ± 0.94	1.45 ± 1.39	1.34 ± 1.17	1.24 ± 1.25
ST2	1.98 ± 1.50	1.83 ± 0.98	1.26 ± 1.22*	2.15 ± 1.27*	1.72 ± 1.44	1.95 ± 1.10
ST3	1.80 ± 1.19*	1.17 ± 0.81*	1.60 ± 0.83	1.85 ± 0.93	1.72 ± 1.08	1.42 ± 0.91

G/G = without T allele; ST1 = self-forgetfulness; ST2 = transpersonal identification; ST3 = spiritual acceptance; *T/G* = with T allele; *T/T* = with T allele.
* $p < 0.01$.

Table 3
Pearson's correlation coefficients for pain threshold and Temperament and Character Inventory scores in 181 healthy volunteers

TCI domain	Male		Female		Total	
	Cold pain	Pressure pain	Cold pain	Pressure pain	Cold pain	Pressure pain
NS4	−0.22*	−0.10	−0.13	0.04	−0.18*	−0.02
NS	−0.22*	−0.07	0.03	0.02	−0.12	−0.02
HA1	−0.17	−0.07	−0.11	−0.06	−0.16*	−0.12
SD4	0.00	−0.09	0.25*	0.27*	0.06	−0.07
C5	0.15	0.13	0.25*	−0.01	0.16*	0.01

C5 = pure-hearted principles; HA1 = anticipatory worry; NS = novelty seeking; NS4 = disorderliness; SD4 = self-acceptance.
* $p < 0.05$.

To our knowledge, ours is the only published report that examines the relationship between the 5-HTT 3′ UTR gene polymorphism and the TCI, and the 5-HTT polymorphism and ST. It is possible that the function of 5-HTT might influence the HA domain in the TCI, as it is assumed that NS, HA and RD depend on the CNS secretion and metabolism of dopamine, serotonin and norepinephrine, respectively.[11] Kumakiri et al. reported correlation between the C allele and polymorphism of the 5-HTT gene regulatory region.[12] Similarly, we also found a correlation between the 5-HTT 3′ UTR gene polymorphism and ST. Our results suggest that 5-HTT might influence the character dimensions of Cloninger's theory: the correlation between 5-HTT 3′ UTR gene polymorphism and environmental factors in adulthood in some character dimensions exceeded the correlation between this gene polymorphism and temperament in Cloninger's theory. With regard to the ST dimensions and subdimensions, a difference in ST, ST2 and ST3 between males and females was noted (Table 2).

In our study, significant effects of the 5-HTT 3′ UTR gene polymorphism were observed for ST, ST2 and ST3. Individuals with a low ST score (sum score of ST1–ST3) might be unimaginative, controlling, materialistic, possessive and practical, while those who have a high ST score might be self-forgetful, transpersonal, spiritual, enlightened and idealistic.[13]

The 5-HTTLPR gene polymorphism (long [l] and short [s] allele) and the dopamine receptor (DR) gene polymorphism have also been related to the TCI. Saochowiec et al. reported that participants with *l/s* and *s/s* 5-HTT gene polymorphism had a significantly lower HA1 score than those who had *l/l* ($p = 0.021$).[14] However, a subsequent study found that subjects with *l/s* and *s/s* showed significantly lower RD and dependence (RD4) scores than those with *l/l* ($p = 0.039$, $p = 0.011$); when only the female participants were considered, those with *l/s* and *s/s* showed significantly lower exploratory excitability (NS1) and RD4 scores than those who had *l/l* ($p = 0.042$, $p = 0.043$).[15] Szekely et al. reported that the 5-HTTLPR polymorphism itself had no significant effect, but a significant interaction between the DRD4 variable number of tandem repeats and the 5-HTTLPR gene polymorphism was observed for HA – those who had both *s/s* (5-HTTLPR) and the 7-repeat DRD4 genotype had a higher HA score than other participants ($p = 0.002$).[16] Kim et al. initially reported that subjects with *s/s* showed a significantly higher P score than those who had *l/l* and *l/s* ($p = 0.012$); however, in a subsequent study they found that in the presence of the dopamine transporter (DAT1) *10/10* genotype, subjects with *l/l* and *l/s* of the 5-HTTLPR showed significantly higher HA and RD scores than participants who were identified as *s/s* ($p = 0.03$, $p = 0.004$, respectively).[17,18]

Most previous studies that examined the relationship between 5-HTT gene polymorphism and the TCI did not consider the three character dimensions of Cloninger's theory. However, our findings suggest that analysis including these three character dimensions is important. We showed that the 5-HTT 3′ UTR gene polymorphism could be used as a marker, even if it does not directly influence the function of 5-HTT, pain or personality. Although it is still unclear whether the gene polymorphism (rs3813034) influences the expression of mRNA or the function of 5-HTT, it will be necessary to clarify whether it has functional significance as a marker. If the polymorphism contributes to low expression of the 5-HTT gene, it might explain some personality traits through a change in the dopamine concentration. In our study, we predicted that other factors would cause different ST scores between the sexes.

We observed a relationship between the 5-HTT 3′ UTR gene polymorphism and the character dimension ST; to our knowledge, no similar study has been reported. ST, in combination with other character dimensions, might participate in schizotypal and melancholic characteristics.[13] If additional exploration of the three character dimensions reveals a significant relationship between ST and 5-HTT gene polymorphism, this polymorphism might be useful as a marker and may facilitate diagnosis in psychiatric assessment. Comprehensive analysis of the 5-HTT gene polymorphism is needed because there is presently not a unified view of the association between gene polymorphism and the TCI.

There was no significant effect of the 5-HTT 3′ UTR gene polymorphism on pain, either cold or pressure, except for the differences in the pressure pain threshold between men and women. Although this result was statistically significant, we felt that there was no significant relationship between gene polymorphism and pain. The relationship between 5-HT and pain has been examined with regard to 5-HT receptor subtypes, because in the CNS, 5-HT_{2A}, 5-HT_{2C} and 5-HT_{3} might participate in an analgesic action.[2] As a result of this, it is also necessary to consider interaction of the individual 5-HT receptor gene polymorphisms.

In the analysis of the association between pain and personality for males, there was a weak negative correlation between NS4, NS and the cold pain threshold, and low positive correlation between SD4, C5 and the cold pain threshold. For females, there was a low positive correlation between SD4 and the pressure pain threshold. In particular, individuals with a high SD score (sum of SD1–SD5) might be described as responsible, purposeful, resourceful, self-accepting and disciplined, while those individuals with a low SD score might be blaming, aimless, inept, vain and undisciplined.[13] In addition, individuals with a high C score (sum of C1–C5) could be described as tender-hearted, empathic, helpful, compassionate and principled. Low co-operativeness has been said to involve intolerance, insensitivity, hostility, revenge and opportunism.[13] Our study indicated that pain might exhibit low correlation with partial dimensions and subdimensions in the TCI, that is, NS4 and NS in males, and SD4 and C5 in females. Consequently, it was suggested that impulsive, quick-tempered and rule-breaking males, and vain and opportunistic females, are more likely to be sensitive to pain.

Some dimensions and subdimensions of the TCI were correlated with gene polymorphism. There were sex differences in particular dimensions and subdimensions of the TCI. In addition, there were many significant correlations between the cold pain threshold and dimensions and subdimensions of the TCI. Therefore, the cold pain threshold might be better than the pressure threshold in showing correlation between pain and the TCI. Regarding the relationship between pain and the TCI using warm and cold stimuli, Yamaguchi et al. reported negative correlations with HA, RD and P.[19] A relationship between headache and the TCI has also been reported by Mongini et al., who found that patients with migraine showed significantly higher HA and P scores, and significantly lower SD scores.[20]

Because different personality traits are associated with sensitivity to different types of pain, we examined the relationship between pain and personality by using a variety of pain tests.

We examined the relationship between the 5-HTT 3′ UTR gene polymorphism and the TCI. Further study with a larger cohort should also examine the correlations between the TCI dimensions and subdimensions and age, and the interactions with other gene polymorphisms.

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Implication of dopaminergic projection from the ventral tegmental area to the anterior cingulate cortex in μ-opioid-induced place preference

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ABSTRACT

Despite the importance of prefrontal cortical dopamine in modulating reward, little is known about the implication of the specific subregion of prefrontal cortex in opioid reward. We investigated the role of neurons projecting from the ventral tegmental area (VTA) to the anterior cingulate cortex (ACG) in opioid reward. Microinjection of the retrograde tracer fluorogold (FG) into the ACG revealed several retrogradely labelled cells in the VTA. The FG-positive reactions were noted in both tyrosine hydroxylase (TH)-positive and -negative VTA neurons. The released levels of dopamine and its major metabolites in the ACG were increased by either the electrical stimulation of VTA neurons or microinjection of a selective μ-opioid receptor (MOR) agonist, (D-Ala^2,*N*-MePhe^4,Gly-ol^5) enkephalin (DAMGO), into the VTA. MOR-like immunoreactivity was seen in both TH-positive and -negative VTA neurons projecting to the ACG. The conditioned place preference induced by intra-VTA injection of DAMGO was significantly attenuated by chemical lesion of dopaminergic terminals in the ACG. The depletion of dopamine in the ACG induced early extinction of μ-opioid-induced place preference. The levels of phosphorylated DARPP32 (Thr34) and phosphorylated CREB (Ser133) were increased in the ACG of rats that had maintained the morphine-induced place preference, whereas the increases of these levels induced by morphine were blocked by pre-treatment of a selective dopamine D1 receptor antagonist SCH23390. These findings suggest that VTA-ACG transmission may play a crucial role in the acquisition and maintenance of μ-opioid-induced place preference. The activation of DARPP32 and CREB through dopamine D1 receptors in the ACG could be implicated in the maintenance of μ-opioid-induced place preference.

Keywords Anterior cingulate cortex, dopamine, memory, opioid, μ-opioid receptor, reward.

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INTRODUCTION

Studies on human addicts and behavioural studies in rodent models of addiction have indicated that key behavioural abnormalities associated with addiction are extremely long-lived. Drug of abuse is characterized by behavioural alternations in which compulsive drug seeking plays a central role. It is a chronic brain disorder as the risk of relapse remains high even after years of abstinence (Nestler 2001).

Brain dopamine systems have been the focus of histochemical, biochemical, and pharmacological research on the rewarding effects of and locomotor activity induced by opioids and psychostimulants. Dopaminergic neurons in the ventral tegmental area (VTA) innervate the nucleus accumbens (N.Acc), medial prefrontal cortex (mPFC), amygdala, hippocampus and ventral pallidum (Pierce & Kumaresan 2006). The ascending anatomical dopamine projection from the VTA to the N.Acc and mPFC is composed of mesocorticolimbic dopamine neurons (Koob 1992). Considerable evidence suggests that mesolimbic dopaminergic projections from the VTA to the N.Acc play an important role in the rewarding effects of drugs of abuse, including opioids (Kelley &

Berridge 2002). Altered function of the mPFC has been implicated in multiple processes and behavioural disorders, including schizophrenia (Weinberger 1995), drug abuse (Wise, Murray & Gerfen 1996), depression (Merriam *et al.* 1999) and attention deficit hyperactivity disorder (Puumala & Sirvio 1998) as well as normal cognitive processes, including working memory function (Williams & Goldman-Rakic 1995; Jentsch *et al.* 1997a,b) and decision making (Damasio 1995). The mPFC has received considerable attention with respect to its involvement in reward-related mechanisms (Tzschentke & Schmidt 2000). Noradrenaline (NA) release in the mPFC has been recently considered as crucial in mediating rewarding effects of opioids (Ventura, Alcaro & Puglisi-Allegra 2005). Despite the importance of prefrontal cortical dopamine, as well as NA, in modulating reward, cognition and behaviour, little is known about the involvement of dopamine that regulate the rewarding effects of opioids in the mPFC.

The anterior cingulate cortex (ACG), a major subregion of the prefrontal cortex, is involved in evaluative processes. The ACG might also serve to encode whether or not an action is worth performing in view of the expected benefit and the cost of performing the action (Rushworth *et al.* 2004). For instance, after excitotoxic ACG lesions, rats no longer selected the high cost–high reward option in a cost–benefit T-maze task if they had to choose between climbing a barrier to obtain a large reward in one arm or running for a low reward into the other arm with no barrier present (Walton *et al.* 2003). Recent studies have indicated that mesocortical dopamine fibres projecting to the ACG (Berger, Gaspar & Verney 1991) may be responsible for effort-based decision-making (Schweimer, Saft & Hauber 2005). In addition, the ACG contributes to the generation of emotional states and to the executive control of behavioural selection (Peoples 2002).

In the present study, we therefore investigated the role of dopaminergic neurons projecting from the VTA possibly to the ACG in μ-opioid reward.

MATERIALS AND METHODS

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (Hoshi University) as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Every effort was made to minimize the number and suffering of animals used in the following experiments.

Animals

In the present study, we used male Sprague Dawley rats (200–250 g) (Tokyo Laboratory Animals Science, Tokyo, Japan). The animals were housed in a room maintained at 23 ± 1°C with a 12-hour light/dark cycle (lights on 8 AM to 8 PM). Food and water were available *ad libitum*.

Drugs

The drugs used in the present study were morphine hydrochloride (Daiichi-Sankyo, Tokyo, Japan), 6-hydroxydopamine hydrochloride (6-OHDA), desipramine hydrochloride, (D-Ala^2, *N*-MePhe^4, Gly-ol^5) enkephalin (DAMGO) (Sigma-Aldrich, St. Louis, MO), R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390; Sigma Chemical Co., MO, USA), fluorogold (FG; fluorochrome, Englewood, CO, USA), and cholera toxin B Alexa Fluor 555 conjugate (CTb; Invitrogen, Grand Island, NY, USA).

Experiments I

In retrograde tracing study, the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. The skull was exposed, and a hole was drilled through the skull over the ACG (from bregma: anterior +1.7 mm; lateral +0.4 mm; and ventral −2.8 mm) or N.Acc (from bregma; anterior +1.5 mm; lateral +2.7 mm; and ventral −7.3 mm; angle 10°) according to the atlas of Paxinos and Watson (Paxinos & Watson 1998). Except pressure-injected (200 nl) into the ACG in the FG (fluorochrome, 4% solution in saline) injection, micropipettes of about 20–23 μm in diameter were filled with FG solution, and the tracer was injected into the N.Acc for 25 minutes by iontophoresis with a positive-pulsed current (5 μA, seven seconds on/off intervals). The micropipette was left in place for five minutes following the completion of injection to avoid leakage of the tracer along the pipette track, and then the pipette was withdrawn from the brain. In the CTb (Invitrogen) injection, the injection cannula was filled with CTb (1 mg/ml) solution, and CTb was pressure-injected (1 μl) into the ACG. The CTb, as well as FG, was used as retrograde tracer (Kishi *et al.* 2006; Almarestani *et al.* 2007; Steen *et al.* 2007). After the injection, the injection cannula was left in place for five minutes. Five days after the injections, the animals were perfused as described later. The distributions of FG or CTb retrogradely labelled neurons and FG or CTb injection sites were detected using a microscope (Olympus BX-60; Olympus, Tokyo, Japan). In perfusion and tissue processing, the rats were deeply anesthetized with 3% isoflurane and perfusion-fixed with 4% paraformaldehyde, pH 7.4. The brains were then quickly removed after perfusion, and thick coronal sections of the ACG, N.Acc or VTA were initially dissected using brain blocker (Neuroscience, Tokyo, Japan). The brain coronal sections were post-fixed in 4% paraformaldehyde for three hours. After the brains

were permeated with 20% sucrose for one day and 30% sucrose for two days, they were frozen in embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at −30°C until use. Frozen 8 μm-thick coronal sections were cut with a cryostat (CM1510; Leica, Heidelberg, Germany) and thaw-mounted on poly-L-lysine-coated glass slides. In immunohistochemical approach, the brain sections were blocked in serum in 0.01 mol/l phosphate buffer saline (PBS) for one hour at 23 ± 1°C. Each primary antibody was diluted in 0.01 mol/l PBS containing 10% normal goat serum (NGS) [1:25, 1:40 or 1:60 tyrosine hydroxylase (TH; mouse monoclonal, MAB358, Chemicon, Temecula, CA, USA)] and incubated for two days at 4°C. The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with Alexa 488 and Alexa 546 for two hours at 23 ± 1°C. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA, USA). Fluorescence of immunolabelling was detected using a light microscope (Olympus BX-60) and U-MWIG and U-MNIBA filter cubes (Olympus) for an Alexa 488 or Alexa 546. Digitized images of the ACG, N.Acc or VTA sections were captured at a resolution of 1316 × 1035 pixels with a camera (Polaroid PDMCII/OL; Olympus). The upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity (IR) to provide an image in which IR material appeared as white pixels and non-IR material appeared as black pixels.

Experiments II

In surgery and microinjection, after three days of habituation to the main animal colony, all of the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The anesthetized animals were placed in a stereotaxic apparatus. The skull was exposed, and a small hole was made using a dental drill. A guide cannula (AG-9, AG-8 or AG-4; Eicom, Kyoto, Japan) or an electrode (NS303/12; Bioresearch Center, Nagoya, Japan) was implanted into the VTA (from bregma: anterior, −5.3 mm; lateral, −0.9 mm; ventral, −7.7 mm or anterior, −6.8 mm; lateral, ±0.9 mm; ventral, −7.8 mm; angle 10°), ACG [from bregma: anterior, +1.7 mm; lateral, −0.4 mm; ventral, −1.1 mm (*in vivo* microdialysis) or −2.3 mm (microinjection)] or N.Acc (from bregma; anterior, +4.0 mm; lateral, −0.8 mm; ventral, −6.8 mm; angle 16°) according to the atlas of Paxinos & Watson (1998). The guide cannula or the electrode was fixed to the skull with cranioplastic cement. In the microinjection method, we used an injection cannula (AMI-9.5; Eicom) that extended beyond the guide cannula by 0.5 mm. A stainless steel injection cannula was inserted into the guide cannula for each animal. The injection cannula was connected through polyethylene tubing to a 10 μl Hamilton syringe that was preloaded with DAMGO (1 nmol/0.3 μl) or saline. DAMGO or saline was delivered by a motorized syringe pump in a volume of 0.3 μl over 60 seconds. In *in vivo* microdialysis study, three to five days after the surgery, microdialysis probes (AI-4-2 or AI-8-2; 2 mm membrane length; Eicom) were slowly inserted into the ACG or N.Acc through guide cannulas under anaesthesia with diethyl ether, and the rats were settled in the experimental cages (30 cm wide × 30 cm deep × 30 cm high). The probes were perfused continuously at a flow rate of 2 μl/minute with artificial cerebrospinal fluid (aCSF) containing 0.9 mM $MgCl_2$, 147.0 mM NaCl, 4.0 mM KCl and 1.2 mM $CaCl_2$. The outflow fractions were taken every 20 minutes. After three baseline fractions were collected in the rat ACG or N.Acc, DAMGO or saline was administered into the rat VTA using a 10 μl Hamilton syringe and a motorized syringe pump. For these experiments, dialysis samples were collected for 180 minutes after DAMGO or saline treatment. Dialysis fractions were then analyzed using HPLC (Eicom) with an electrochemical detection (ECD) (Eicom) system. Dopamine was separated by a column with a mobile phase containing sodium acetate (4.05 g/l), citric acid monohydrate (7.35 g/l), sodium 1-octane sulfonate (150 mg/l), EDTA (2Na; 10 mg/l) and 17% methanol. The mobile phase was delivered at a flow rate of 210 μl/minute. Dopamine was identified according to the retention time of a dopamine standard, and the peak area of basal dopamine was divided by the peak area of a dopamine standard to obtain the amount of basal dopamine. In electrical stimulation, three to five days after surgery, microdialysis probes were inserted and an *in vivo* microdialysis study was performed as described earlier. Electrical stimulation (stimulation intensity of 100 or 150 μA, stimulation frequency of 100 Hz, 0.5-second trains of 0.5-m second pulses, interval of two seconds) was applied to the VTA for 40 minutes after three baseline fractions were collected in the rat ACG. This stimulation was delivered by constant-current stimulators via a bipolar cable (Bioresearch Center) connected to the electrode.

Experiments III

Immunohistochemical study was conducted as described earlier. Each primary antibody was diluted in 0.01 mol/l PBS containing 10% NGS [1:25, 1:40 or 1:60 tyrosine hydroxylase (mouse monoclonal, MAB358)] or 20% NGS in 0.1% Triton X-100 [1 : 3500 MOR (rabbit polyclonal, RA10104, Neuromics, Bloomington, MN, USA)]. The microinjection of retrograde tracer was conducted as described earlier.

Experiments IV

Place conditioning was conducted as described previously (Suzuki, Masukawa & Misawa 1990). The biased

design was used for place conditioning. The apparatus was a shuttle box (30 cm wide × 60 cm long × 30 cm high) that was made of acrylic resin board and divided into two equal-sized compartments. One compartment was white with a textured floor, and the other was black with a smooth floor to create equally preferable compartments. The place conditioning schedule was composed of three phases (pre-conditioning test, conditioning, and post-conditioning test). The pre-conditioning test was performed as follows: the partition separating the two compartments was raised to 12 cm above the floor, a neutral platform was inserted along the seam separating the compartments and the animals that had not been treated with either drugs or saline were then placed on the platform. The time spent in each compartment during a 900-seconds session was then recorded automatically with an infrared beam sensor (KN-80; Natsume Seisakusyo, Tokyo, Japan). Conditioning sessions (three days for DAMGO (0.3, 1, 3, 9 nmol/0.3 μl) or morphine (8 or 23 mg/kg, i.p.), three days for saline) were conducted once daily for six days. Immediately after DAMGO injection or treatment with morphine, these animals were placed in the compartment opposite that in which they had spent the most time in the pre-conditioning test for one hour. On alternating days, these animals received saline and were placed in the other compartment for one hour. On the day after the final conditioning session, a post-conditioning test that was identical to the pre-conditioning test was performed. To destroy central dopaminergic neurons, 6-OHDA (8 μg/0.3 μl) was injected into the ACG of the rats four days before the start of conditioning with DAMGO or morphine. Additionally, desipramine (20 mg/kg, s.c.) was given to rats 30 minutes before the injection of 6-OHDA into the ACG to block the uptake of 6-OHDA into noradrenergic terminals (Alhaider 1991). To investigate the extinction of the morphine- or DAMGO-induced place preference, a post-conditioning test was performed with no conditioning and was conducted at 1 day, 9 days, 13 days or 20 days after the final conditioning test. Immunohistochemical study was conducted as described earlier. Primary antibody was diluted in 0.01 mol/l PBS containing 10% NGS [1:1000 TH (rabbit polyclonal, AB152, Chemicon)]. In high-pressure liquid chromatography (HPLC) study, the rats were killed four days after the microinjection of saline or 6-OHDA (8 μg/0.3 μl) in combination with s.c. injection of vehicle or desipramine. The brain was removed quickly, and the ACG was dissected on an ice-cold glass plate. The tissues were homogenized in 1000 μl of 0.2 M perchloric acid containing 100 mM EDTA(2Na) and 140 ng isoproterenol as an internal standard. The homogenates were then centrifuged at 20 000 ×g for 32 minutes at 4°C, and the supernatants were maintained at pH 3.0 using 1 M sodium acetate. The samples were analyzed by HPLC-ECD. The HPLC system was composed of a delivery system (EP-10; Eicom), an analytical column (Eicompac, SC-5ODS; Eicom) and a guard column (Eicom). Dopamine and its metabolites were separated by a column with a mobile phase containing sodium acetate (3.22 g/l), citric acid monohydrate (9.17 g/l), sodium 1-octane sulfonate (180 mg/l), EDTA(2Na) (10 mg/l) and 17% methanol. The mobile phase was delivered at a flow rate of 0.5 ml/minute. Dopamine and its metabolites were identified according to the retention times of these standards, and the peak heights of dopamine and its metabolites revised internal standard was divided by the peak heights of those standard revised internal standard to obtain those amount.

Experiments V

Place conditioning was performed as described earlier. In treatment with SCH23390, the rats were administered saline or SCH23390 (0.1 mg/kg, i.p.) 15 minutes before the treatment with morphine. In Western blotting, 24 hours or 10 days after the final conditioning in the conditioned place preference (CPP) procedure described earlier, the rats were sacrificed by decapitation. The ACG was quickly removed after decapitation and homogenized in ice-cold buffer. The homogenate was centrifuged at 20 000 ×g for 10 minutes and the supernatant was retained as the lysate fraction for Western blotting. An aliquot of tissue sample was diluted with an equal volume of 2× electrophoresis sample buffer (Protein Gel Loading Dye-2X, Amresco, Solon, OH, USA) containing 2% sodium dodecyl sulfate (SDS) and 10% glycerol with 0.2 M dithiothreitol. Proteins (7 μl/lane) were separated by size on 4–20% SDS-polyacrylamide gradient gel by using the buffer system of Laemmli (1970) and transferred to nitrocellulose membranes in Tris-glycine buffer containing 25 mM Tris and 192 mM glycine. For immunoblot detection, the membranes were blocked in Tris-buffered saline (TBS) containing 1% non-fat dried milk (Bio-Rad Laboratories, Hercules, CA, USA) containing 0.1% Tween 20 (Research Biochemicals, Natick, MA, USA) for one hour at room temperature with agitation. The membrane was incubated with primary antibody diluted in 1:1000 phosphorylated dopamine and cyclic AMP-regulated phosphoprotein with molecular weight 32 kDa (p-DARPP32; rabbit, #2304, Cell Signaling Technology, Danvers, CA, USA) and 1:1000 phosphorylated cAMP response element binding protein (p-CREB; rabbit, #9191, Cell Signaling Technology), 1:200 000 glyceraldehyde-3-phosphate dehydrogenase (GAPDH; mouse monoclonal, MAB374, Chemicon International, Temecula, CA, USA) containing 1% non-fat dried milk containing 0.1% Tween 20 overnight at 4°C. The membrane was washed in TBS containing 0.05% Tween 20

(TTBS), and then incubated for two hours at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL, USA) diluted 1:10 000 in TBS containing 1% non-fat dried milk with 0.1% Tween 20. After this incubation, the membranes were washed in TTBS. The antigen-antibody peroxidase complex was finally detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and visualized by exposure to Amersham Hyperfilm (Amersham Life Sciences, Arlington Heights, IL, USA).

Histology

The locations of the infusion cannula and drug diffusion were assessed at the completion of the experiments. The rats were deeply anesthetized with sodium pentobarbital at the end of the experiment and given microinjections of ink for the anatomical localization of cannula sites (0.3 μl). The brain was then removed by decapitation and cut into coronal sections. Cannula locations were mapped onto a stereotaxic atlas (Paxinos & Watson 1998) and confirmed to be in the VTA, ACG or N.Acc.

Statistical data analysis

Dates are expressed as the mean with SEM. One- and two-way analyses of variance (ANOVAs) with independent and repeated measures as well as planned comparisons or Student's *t*-tests, were used as appropriate for the experimental design. Multiple comparisons were performed using Dunnet or Bonferroni *post hoc* test where appropriate. The potency ratio for the saline-treated rats and the 6-OHDA-treated rats were calculated by the parallel line assay (Tallarida, Porreca & Cowan 1989).

RESULTS

Experiments I

Dopamine neurons projecting from the VTA to the ACG

To determine whether the VTA is linked to the ACG, we investigated whether there were any neuronal projections from the VTA to the ACG using FG as a retrograde tracer. Schematic illustrations of the injection site in the ACG (CG1, CG2) or N.Acc are shown with the symbols (Fig. 1a, b). Pressure application of FG into the region of the unilateral ACG produced a well-restricted injection site (Fig. 1c). FG-labelled cell bodies (Fig. 1d) or TH-labelled cells (Fig. 1e) were apparently detected in the VTA after the microinjection of FG into the ACG. A population of retrogradely labelled neurons in the VTA also showed TH-IR (Fig. 1f).

Distribution of cell bodies of neurons projecting from the VTA to the ACG or N.Acc

To investigate whether neurons that projected from the VTA to the ACG or N.Acc were independent, the retrograde tracers CTb and FG were microinjected into the unilateral ACG and N.Acc, respectively (Fig. 1g, h). FG- or CTb-containing cells were detected in the VTA after the microinjection of FG or CTb. FG-labelled neurons and CTb-labelled neurons did not overlap in the VTA (Fig. 1i).

Experiments II

Dialysate dopamine level in the ACG by electrical stimulation in the VTA

To further verify the dopaminergic neurons projecting from the VTA to the ACG, we examined the effect of the electrical stimulation of VTA cells on dopamine release in the ACG. In an *in vivo* microdialysis study, the basal levels of dopamine and the major dopamine metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in the ACG were lower than that of dopamine in the N.Acc ($n = 5$) ($^{*}P < 0.05$, $^{**}P < 0.01$ versus dopamine, DOPAC or HVA in the N.Acc) (Table 1). The dopamine levels in the ACG were increased by the electrical stimulation of VTA cells ($n = 5$) (Fig. 2a). The electrical stimulation of VTA cells also increased the levels of DOPAC and HVA ($n = 5$) (Fig. 2b, c). Statistical analysis was performed with one-way ANOVA followed by Dunnet test (dopamine, $F_{(7,28)} = 3.710$, $P < 0.01$; DOPAC, $F_{(7,28)} = 7.426$, $P < 0.001$; HVA, $F_{(7,28)} = 12.04$, $P < 0.001$) ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus 0 minute).

Dialysate dopamine level in the ACG under the microinjection of a μ-opioid receptor agonist into the VTA

We next investigated the change in the dialysate dopamine level in the ACG under the intra-VTA administration of a μ-opioid receptor agonist. Schematic illustrations of dialysis probe placements in the ACG or N.Acc are shown in Fig. 3a, b. The dopamine level was markedly increased by the injection of DAMGO compared with saline treatment in the ACG ($n = 5$) (Fig. 3c). The injection of DAMGO into the VTA also produced a significant increase in DOPAC and HVA in the ACG ($n = 5$) (Fig. 3d, e). Statistical analysis was performed with two-way ANOVA followed by Bonferroni test [dopamine: interaction between treatment and time: $F_{(11,88)} = 1.972$, $P < 0.05$; effect of treatment, $F_{(1,88)} = 5.841$, $P < 0.05$; effect of time, $F_{(11,88)} = 3.250$ $P < 0.001$; DOPAC: interaction between treatment and time: $F_{(11,88)} = 7.278$, $P < 0.001$; effect of treatment, $F_{(1,88)} = 22.77$, $P < 0.01$; effect of time, $F_{(11,88)} = 12.60$, $P < 0.001$; HVA: interaction between treatment and time: $F_{(11,88)} = 3.823$, $P < 0.001$; effect

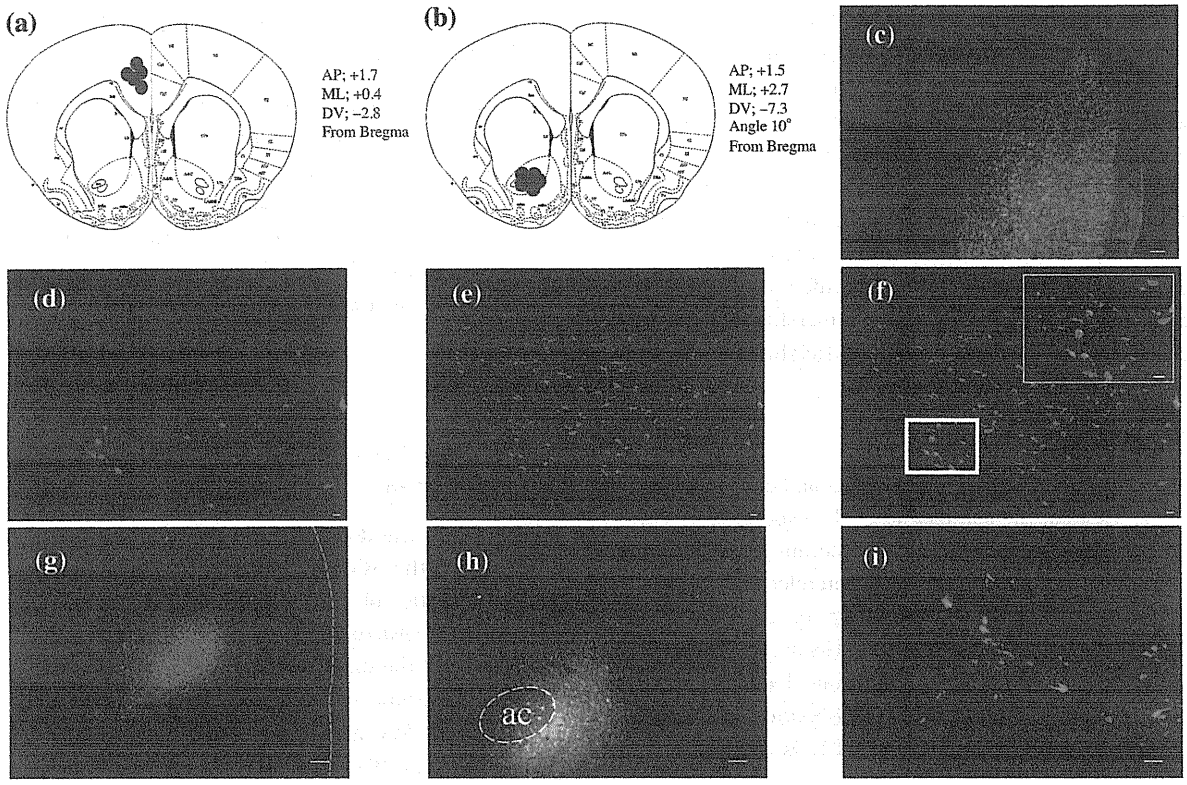

Figure 1 Projection of dopamine neurons from the ventral tegmental area (VTA) to the anterior cingulate cortex (ACG) and the distribution of cell bodies in the VTA projecting to the ACG or N.Acc of rats. (a–b) Schematic illustrations of the injection site (symbols) in the ACG (a) or N.Acc (b). (c) The image shows the extent of fluorogold (FG) diffusion at the injection site. (d) Cells in the VTA after the microinjection of FG into the ACG. (e) Tyrosine hydroxylase (TH)-IR was noted in the VTA. (f) Double-labelling experiments showed that FG-positive cells overlapped TH-positive cells in the VTA. (g) The extent of cholera toxin B diffusion at the injection site in the ACG. (h) The extent of FG diffusion at the injection site in the N.Acc; ac = anterior commissure. (i) Cells in the VTA after the microinjection of FG into the ACG and cholera toxin B into the N. Acc. Double-labelling experiments showed that FG-positive and cholera toxin B-positive cells did not overlap in the VTA. Scale bars, 50 μm

Table 1 Basal dialysate levels of dopamine and its metabolites in the nucleus accumbens or anterior cingulated cortex and the decrease in the contents of dopamine and its metabolites in the anterior cingulated cortex in desipramine-6-OHDA-treated rats.

	Dopamine	*DOPAC*	*HVA*
Brain area (nM)[a]			
Nucleus accumbens	0.8 ± 0.2	374.9 ± 86.2	109.7 ± 17.8
Cingulate cortex	0.2 ± 0.1*	19.2 ± 4.5**	24.3 ± 3.3**
Group (ng/g wet tissue)[b]			
Vehicle-saline	100.2 ± 0.901	34.7 ± 2.547	71.3 ± 1.678
Desipramine-6-OHDA	75.6 ± 0.396***	21.6 ± 0.514***	44.2 ± 0.450***

[a]Each value represents the mean ± SEM. The data were calculated as concentrations in the dialysates for five rats. *$P < 0.05$, **$P < 0.01$ versus dopamine, DOPAC or HVA in the N.Acc.
[b]Each value represents the mean ± SEM. The rats were killed four days after the microinjection of saline or 6-OHDA into the ACG. The data were calculated as dopamine and its metabolited contents in the ACG for four rats. The statistical significance of differences between groups was assessed with Student's *t*-test. ***$P < 0.001$ versus vehicle-saline.

of treatment, $F_{(1,88)} = 5.355$, $P < 0.05$; effect of time, $F_{(11,88)} = 2.567$, $P < 0.01$) (*$P < 0.05$, ***$P < 0.001$, saline versus DAMGO]. The dopamine and its metabolite levels were markedly increased by the injection of DAMGO compared with saline treatment in the N.Acc ($n = 5$) (Fig. 3f, g, h). Statistical analysis were performed with two-way ANOVA followed by Bonferroni test [dopamine: interaction between treatment and time: $F_{(11,88)} = 5.404$, $P < 0.001$; effect of treatment, $F_{(1,88)} = 8.963$, $P < 0.05$; effect of time, $F_{(11,88)} = 3.450$, $P < 0.001$; DOPAC: interaction between treatment and time: $F_{(11,88)} = 33.18$, $P < 0.001$; effect of treatment,

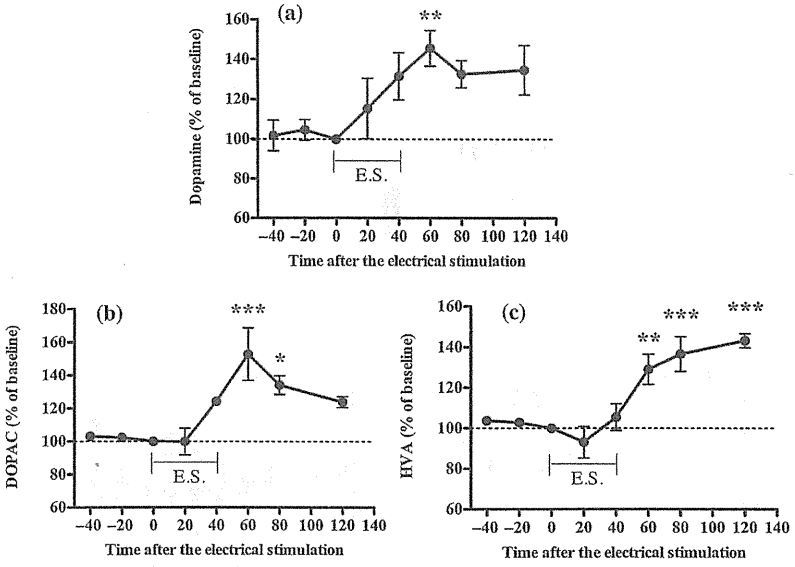

Figure 2 Change in the dialysate levels of dopamine and its metabolites induced by electrical stimulation (ES) in the rat VTA. (a–c) Effect of ES on the dialysate dopamine (a), DOPAC (b) and HVA (c) levels in the ACG in rats. The rats were subjected to electrical stimulation at time 0 for 40 minutes. The data are expressed as percentages of the corresponding baseline levels with SEM of the five rats. Dunnet test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus 0 minute

$F_{(1,88)} = 50.84$, $P < 0.001$; effect of time, $F_{(11,88)} = 29.78$, $P < 0.001$; HVA: interaction between treatment and time: $F_{(11,88)} = 17.02$, $P < 0.001$; effect of treatment, $F_{(1,88)} = 24.11$, $P < 0.01$; effect of time, $F_{(11,88)} = 12.12$, $P < 0.001$) ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, saline versus DAMGO].

Experiments III

Distribution of μ-opioid receptors in the VTA

We next investigated the distribution of MOR-IR in the VTA after the microinjection of FG into the ACG. FG-, MOR- or TH-labelled cells were detected in the VTA (Fig. 4a, b, c). A triple labelling experiment showed that MOR-IR in the VTA was detected on both TH- and non-TH-labelled neurons with a FG-positive reaction (Fig. 4e, f). Some MOR-labelled neurons with no FG reaction did not show TH-IR (Fig. 4g). Percentages of MOR, TH and FG labels, individually and in combination, in the VTA are shown in Fig. 4h.

Experiments IV

Role of VTA-ACG dopaminergic neurons in the acquisition and maintenance of the place preference induced by the μ-opioid receptor agonist

To investigate the involvement of VTA-ACG dopaminergic neurons in the induction of opioid reward, the rats were microinjected with 6-hydroxydopamine (6-OHDA) into the ACG after the s.c. injection of desipramine to specifically deplete dopamine. Pre-microinjection of 6-OHDA in combination with the s.c. administration of desipramine markedly decreased the basal levels of dopamine and its metabolites in the rat ACG ($^{***}P < 0.001$ versus vehicle-saline) ($n = 4$) (Table 1), whereas it failed to change the basal levels of dopamine and its metabolites in the N.Acc (Saline; $n = 6$, 6-OHDA; $n = 5$) [Dopamine: 88.8 ± 16.0 (% of control); DOPAC: 107.8 ± 15.7 (% of control); HVA: 98.0 ± 18.1 (% of control)]. The microinjection of 6-OHDA into the ACG after the treatment with desipramine did not affect NA and serotonin (5-HT) contents in the ACG [(NA: saline; 281.6 ± 11.6, 6-OHDA; 206.8 ± 14.7, NS, 5-HT: saline; 51.8 ± 1.4. 6-OHDA; 64.9 ± 5.1, NS (ng/g weight tissue)]. The injury of dopaminergic neurons, by 6-OHDA injection in the ACG, reduced TH-IR in the VTA ($^{*}P < 0.05$ versus saline) (Fig. 5a–c). In the CPP method, the microinjection of DAMGO into the VTA produced a dose-dependent place preference. The DAMGO-induced place preference was attenuated by the pre-microinjection of 6-OHDA into the ACG. The concentration-response line for the 6-OHDA-pre-treated group was shifted to the right compared with that for the saline-pre-treated group. The potency ratio of the place preference induced by DAMGO in saline-pre-treated group versus 6-OHDA-pre-treated group was 3.32 (saline: 0.3 nmol, $n = 7$; 1 nmol, $n = 6$; 3 nmol, $n = 8$, 6-OHDA: 1 nmol, $n = 8$; 3 nmol, $n = 6$; 9 nmol, $n = 6$) (Fig. 5d). The saline or the 6-OHDA pre-treated control rats failed to show the place preference (saline–saline: −36.9 ± 48.1, $n = 6$; 6-OHDA-saline: 22.7 ± 57.7, $n = 6$). Under these conditions, the 6-OHDA-pre-treated rats that produced the right shift of concentration–response line of the DAMGO-induced place preference showed extinction of the place preference at nine days after the final conditioning, whereas the place preference induced by the microinjection of DAMGO into the VTA in saline-pre-treated rats was maintained at nine days (Fig. 5e). Statistical analysis was performed with two-way ANOVA [saline; interaction between dose and day: $F_{(1,12)} = 0.6581$, NS; effect of dose, $F_{(1,12)} = 2.856$, NS;

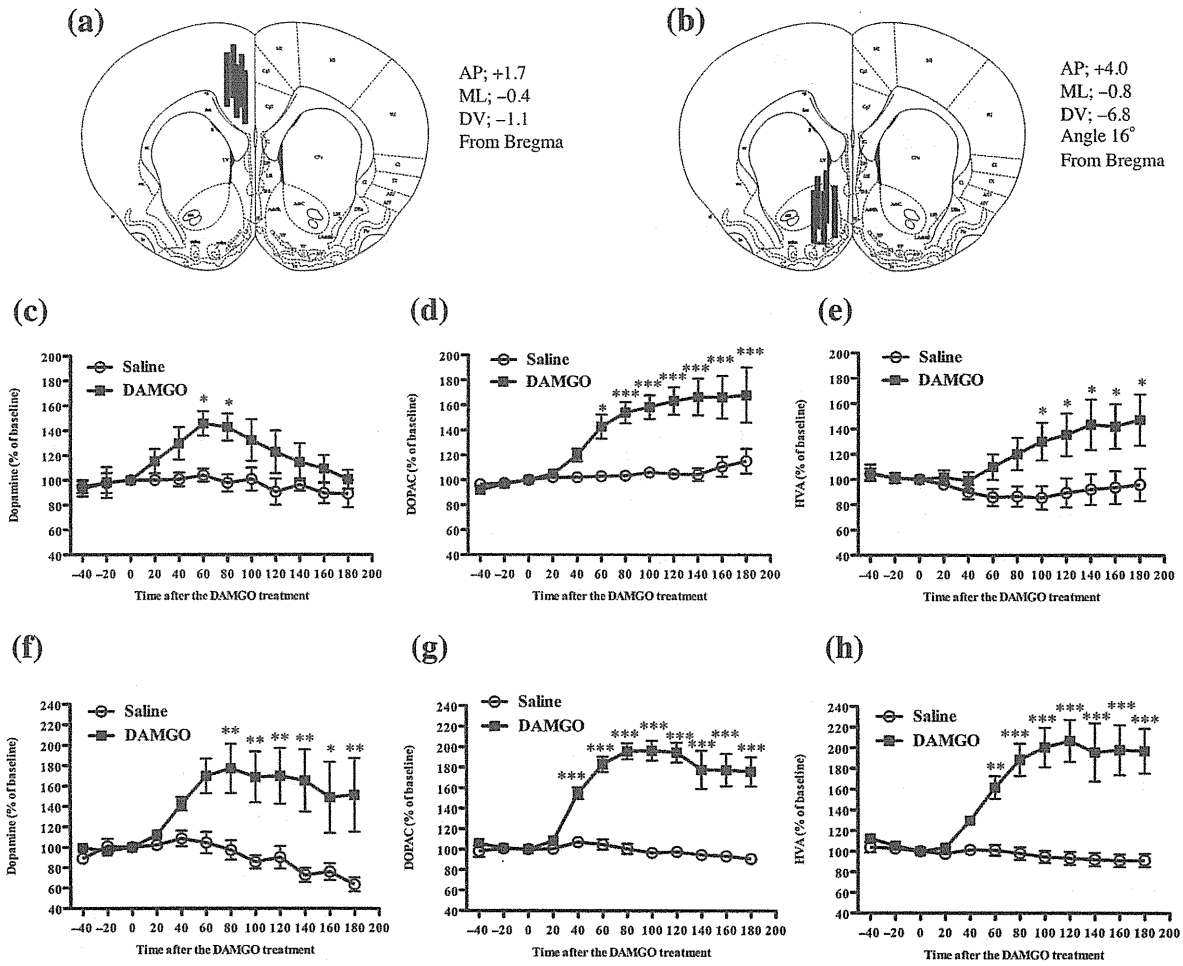

Figure 3 Change in the dialysate levels of dopamine and its metabolites induced by DAMGO administration into the VTA. (a–b) Schematic illustrations of the dialysis probe locations in the ACG (a) or N.Acc (b). (c–e) Effects of DAMGO administration into the VTA on the dialysate levels of dopamine (c) and its metabolites (d, e) in the ACG. After baseline fractions were collected, saline or DAMGO (1 nmol) was administered into the VTA at time 0 to evoke the release of dopamine. Data are expressed as percentages of the corresponding baseline levels with SEM for five rats. (f–h) Effects of DAMGO administration into the VTA on the dialysate levels of dopamine (f) and its metabolites (g, h) in the N.Acc. Data are expressed as percentages of the corresponding baseline levels with SEM for the five rats. Bonferroni test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, saline versus DAMGO

effect of day, $F_{(1,12)} = 0.02324$, NS, 6-OHDA; interaction between dose and day: $F_{(1,10)} = 0.9401$, NS; effect of dose, $F_{(1,10)} = 1.005$, NS; effect of day, $F_{(1,10)} = 14.57$, $P < 0.01$]. The saline- or 6-OHDA- pre-treated control rats failed to show the place preference at nine days after the final conditioning (saline–saline: -2.8 ± 45.1, $n = 6$; 6-OHDA-saline: 40.8 ± 36.6, $n = 6$). Furthermore, the 6-OHDA- pre-treated rats that received 23 mg/kg of morphine (i.p.), which exhibited the almost same score to that observed in the rats receiving 8 mg/kg of morphine (i.p.) showed early extinction after the post-test, whereas the saline-pre-treated rats that received 8 mg/kg of morphine failed to exhibit extinction of its place preference ($n = 6$). Statistical analysis was performed with two-way ANOVA [interaction between treatment and time: $F_{(3,30)} = 2.150$, NS; effect of treatment, $F_{(1,30)} = 5.284$, $P < 0.05$; effect of time, $F_{(3,30)} = 4.220$, $P < 0.05$] (Fig. 5f).

Experiments V

Activation of DARPP32 and CREB in the ACG of rats that maintained the μ-opioid-induced place preference

We next investigated whether the acquisition or maintenance of morphine-induced place preference could be associated with the phosphorylation of DARPP32 and CREB, which are the downstream of dopamine D1 receptor signalling in the ACG. The acquisition of morphine-induced place preference at 24 hours after the final conditioning was attenuated by pre-treatment of a