The Piccolo Intronic Single Nucleotide Polymorphism rs13438494 Regulates Dopamine and Serotonin Uptake and Shows Associations with Dependence-like Behavior in Genomic Association Study

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Abstract: Piccolo (PCLO) inhibits methamphetamine-induced neuropharmacological effects via modulation of dopamine (DA) uptake and regulation of the transport of synaptic vesicles in neuronal cells. Clinical studies have recently suggested that the single nucleotide polymorphism (SNP) rs13438494 in the intron 24 of the *PCLO* gene is associated with psychiatric disorder, in the meta-analysis of GWAS. Therefore, in this study, we attempted to evaluate the possible role of the *PCLO* SNP in the mechanisms of uptake of monoamines. To characterize rs13438494 in the *PCLO* gene, we constructed plasmids carrying either the C or A allele of the SNP and transiently transfected them into SH-SY5Y cells to analyze the genetic effects on the splicing of *PCLO* mRNA. The C and A allele constructs produced different composition of the transcripts, indicating that the intronic SNP does affect the splicing pattern. We also transfected DA and serotonin (5-hydroxytryptamine; 5-HT) transporters into cells and analyzed their uptakes to elucidate the association to psychiatric disorders. In the cells transfected with the C allele, both the DA and 5-HT uptake were enhanced compared to the A allele. We also conducted a clinical study, in order to clarify the genetic associations. PCLO rs13438494 exhibits a relationship with the symptoms of drug dependence or related parameters, such as the age of first exposure to methamphetamine, eating disorders, tobacco dependence and fentanyl requirement.

Our findings suggest that rs13438494 is associated with drug abuse and contributes to the pathogenesis of psychiatric disorders *via* modulation of neurotransmitter turnover.

Keywords: Piccolo, dependence-like behavior, Single nucleotide polymorphism, serotonin, dopamine

INTRODUCTION

The presynaptic cytoskeletal matrix is located in the active zone of chemical synapses and maintains neurotransmitter release. Piccolo (PCLO) is a component protein of this matrix and is associated with the active zone of glutamatergic ribbon synapses and conventional l-aminobutyric acidergic and glycinergic synapses [1]. Previously, we suggested that PCLO regulates addictive behaviors in mice [2] and showed that long-term potentiation in the hippocampal CA1 region is reduced in cultured brain slices from mice with reduced *PCLO* expression [3]. We also showed that these mice exhibit impaired spatial learning [3].

PCLO consists of several domains including two double zinc finger motifs in the N-terminal region and three coiled-coil domains in the central part of the molecule [4]. The C-terminal region of Piccolo contains multiple subdomains such as Ca^{2+}/phospholipid binding (C2A and C2B) domains. The C2A domain has an unusual ability to sense intracellular changes of the Ca^{2+} level and PCLO transmits this signal to other presynaptic molecules *via* a mechanism involving molecule anchoring. We demonstrated that piccolo C2A domain inhibits the methamphetamine-induced internalization of dopamine transporter (DAT) in cultured PC12 cells. It is known that this function is accompanied by Ca^{2+} and PIP_2, and PCLO plays a role of a Ca^{2+} sensor.

In recent genetic association studies of psychiatric disorders, PCLO was found to be linked to depressive illnesses, including bipolar disorders [5]. Choi et al [5] has reported that rs13438494 SNP of PCLO is related to *PCLO* mRNA expression in the frontal cortex of patients with

bipolar diseases. However, such polymorphisms have not yet yielded a convincing explanation of the significant inter-individual variance in the symptoms of mental disorders. The genomic approach has not yet been adopted to explore the best SNP candidates, although this method has been successfully applied to other pharmacogenomic studies. The majority of psychiatric disorders are believed to have correlations with dysfunction of the neurotransmitter system. Therefore, it is worthwhile to examine the genomic variants of *PCLO* and how they affect neurotransmitters, such as dopamine (DA) and serotonin (5-hydroxytryptamine; 5-HT), since these transmitters have key roles for the pathogenesis of patients with the bipolar disease.

Here, we investigated the effects of genomic variation of PCLO, in particular the SNP rs13438494, on the uptake of the neurotransmitters DA and 5-HT, using transgenic cultured cell systems. We also assessed the association between rs13438494 and the symptoms of drug dependence or related parameters, such as the age of first exposure to methamphetamine (METH), eating disorders, tobacco dependence, and fentanyl requirement.

MATERIALS AND METHODS

Plasmid constructs

DAT and serotonin transporter (SERT) constructs were made as previously described [2, 6]. Human *PCLO* from exon 14 to 23 (*PCLO* minigene) was amplified using PCR from the human brain cDNA (Biochain, USA). Table 1 shows the primers that we used to generate a fragment containing 1,096 bp of exon 14 through exon 23. The forward primer contains a 5′-*HindIII* linker, whereas the reverse primer contains a 5′-*BamHI* linker. The expected 1,095-bp band was present on the agarose gel after electrophoresis. The products were digested with *HindIII* and *BamHI* restriction enzymes (Takara, Japan) and were directly ligated to a *HindIII/BamHI*-digested GFP expression vector, pAcGFP-C2 (Clontech-BD Biosciences, USA). Human *PCLO* exon 24, intron 24, and exon 25 were amplified using PCR from the human genomic DNA (Zyagen, USA). Table 1 shows the primers that were used to generate a fragment containing 146 bp of exon 24 and 141 bp of exon 25 as well as 1923 bp of intron 24 sequence. We added a *BglII* site to the 5′ end of the forward primer (Takara, Japan) and a *HindIII* site to the reverse primer (Takara, Japan) to facilitate the cloning. After confirmation of successful amplification (detection of the expected 2,210-bp band on an agarose gel), the products were digested with *BglII* and *HindIII* restriction enzymes (Takara, Japan) and were ligated to the *BglII/HindIII*-digested pAcGFP-E14-23 vector as described above. To remove the *HindIII* site between exons 23 and 24, oligonucleotide site-directed mutagenesis for the deletion was performed using TaKaRa Primestar polymerase (Takara, Japan). After

confirmation of the sequence, a single-nucleotide substitution was introduced by means of site-directed mutagenesis using TaKaRa Primestar polymerase (Takara, Japan). *E. coli* JM109 competent cells (Toyobo, Japan) were transformed with all plasmid constructs and plated overnight. The mutagenic primer pairs are indicated in Table 1. The mutant plasmids were sequenced to confirm that only the desired change was introduced, and then were prepared in a sufficient amount using the midiprep kit (Qiagen, Germany). The resulting pAcGFP-*PCLO* minigene constructs are shown in Figure **1**. A minigene construct containing either the A or C allele of rs13438494 was transfected into cultured cells.

Cell culture and transfection

We used SH-SY5Y and HEK 293 cells in this study. SH-SY5Y cells are used as a neuron-like cell line accompanied with DA receptors and DA transporters. For the uptake assays, we used HEK293 cells because of the high efficiency of DNA transfection. SH-SY5Y cells were purchased from the American Type Culture Collection and used within 10 passages of the original cryovial. SH-SY5Y cells were grown in the DMEM/Ham's F12 medium (Wako Pure Chemicals, Japan) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HEK293 cells were grown in the DMEM medium (Wako Pure Chemicals, Japan) supplemented with 10% FBS and 1% penicillin/streptomycin. Both cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The *PCLO* minigene constructs were transiently transfected into SH-SY5Y and HEK293 cells using

Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Briefly, the cells were grown to 80% confluency in 12-well plates for 24 h in a complete growth medium without antibiotics and then were exposed to a mixture of Lipofectamine (2 μl per well) and plasmid DNA (0.8 μg per well). Cells transfected with the empty AcGFP vector were used as controls. Forty-eight hours after the transfection, the cells were harvested and total RNA was extracted for RT-PCR and an uptake assay. Transfection efficiency was monitored by counting GFP-expressing cells (fluorescent) under an optical fluorescence microscope (Carl Zeiss, Germany).

Uptake assays for DA and 5-HT

The uptake assays with [^{3}H]DA and [^{14}C]5-HT were performed as previously described [7, 8]. In brief, HEK293 cells were plated at a density of 1×10^{5} cells per well in 12-well plates 48 h before the assays. The cells were rinsed and preincubated in Krebs–Ringer buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.18% glucose, and 10 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], pH 7.4; (henceforth HKR buffer) at 37°C for 30 min. The assays were initiated by the addition of [^{3}H]DA and [^{14}C]5-HT with 10^{-5}M pargyline and 10^{-5}M ascorbic acid (final concentrations). The final concentration of DA and 5-HT was 0.1 μM and 1 μM, respectively. After uptake for 10 min at 37°C, the assays were terminated by rapidly washing the plates thrice with ice-cold HKR buffer. The cells were solubilized in 0.1 N NaOH for 1 h at 37°C with shaking, and the accumulated radioactivity was measured by means of liquid scintillation counting.

RT-PCR of the *PCLO* minigene for *in vitro* splicing assays

Total RNA from the transfected SH-SY5Y cells was extracted using TRIsure (Bioline, UK) in accordance with the manufacturer's instructions. A 500-ng aliquot of total RNA from each sample was used to generate first-strand cDNA using the PrimeScript™ RT Reagent kit (Takara, Japan). To evaluate the composition of transcripts from the transfected cells, the following vector-specific primers were used for RT-PCR: pAcGFP Forward primer (5′-CCGACCACTACCAGCAGAAT-3′) and SV40pA Reverse primer (5′-GAAATTTGTGATGCTATTGC-3′). *Gapdh* was used as an internal control: the forward primer was 5′-CCACCCAGAAGACTGTGGAT-3′ and the reverse primer was 5′-CCCTGTTGCTGTAGCCGTAT-3′. To determine transfect efficiency, we used C2A of PCLO specific primers (FW:5′-GAAGATCTCGATTACAGGAGAAATTCAGCTTCAA-3′, RV: 5′-CCCAAGCTTTTTGAGAGGATACCACCTTGGAG-3′). The amplified products were separated using agarose gel electrophoresis and each band's intensity was quantified by means of the ImageJ software (National Institutes of Health, USA). All the transcripts were further analyzed by excising the bands from the gel and processing them by means of the Gel Extraction Kit (Qiagen, Germany), with subsequent direct sequencing. The S/U ratio was calculated as the ratio of intensity of (Spliced Band) to (Unspliced Band + Spliced Band).

Western blotting

The samples were homogenized using sonication in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM PMSF) with protease and phosphatase inhibitors. Insoluble debris was removed by centrifugation at 20,000 *g* for 15 min at 4°C. The supernatant was mixed with 350 mM DTT in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.80; 25% glycerol; 2% SDS; 0.01% bromophenol blue) and then incubated for 30 min at 37°C. A total of 20 μg of proteins from each sample were separated using SDS-PAGE (8%) and transferred to a PVDF membrane. After blocking with 5% skim milk in TBS-T, the membrane was incubated with an anti-GFP (MBL, Japan) or an anti-GAPDH antibody (MBL, Japan). After incubation with an HRP-conjugated secondary antibody (anti–rabbit Ig antibody, GE Healthcare, Little Chalfont, UK), immunoreactive bands on the membranes were visualized by means of the ECL Western blotting detection system (GE Healthcare) on an Image Quant LAS 4000 mini instrument (GE healthcare).

The human genomic association study

The study population and the ethics statement

The surgical protocol and subsequent postoperative pain management were fundamentally the same as in a previous study [9]. The subjects were recruited to investigate the contribution of the rs13438494 SNP to the symptoms of drug dependence or related problems. There were 203 patients with

methamphetamine dependence with clinical data that included multisubstance abuse status, 228 patients with eating disorders with clinical data that included the presence or absence of other psychiatric disorders such as substance dependence, and 1,000 patients who visited Iwata City Hospital in Japan with clinical data on various smoking habits. Enrolled in the association study for fentanyl requirement were 355 healthy subjects who were scheduled to undergo a cosmetic orthognathic surgery for mandibular prognathism at Tokyo Dental College Suidoubashi Hospital. The clinical study protocol was approved by the institutional review board at Tokyo Dental College, Iwata City Hospital, Kurihama Alcoholism Center and related hospitals, as well as the ethics committee of each participating institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA) [10, 11] and the institutional review board of Tokyo Metropolitan Institute of Medical Science. Written informed consent was obtained from all the participants.

Genotyping Procedures

To genotype the rs13438494 SNP, the TaqMan allelic discrimination assay (Life Technologies, Carlsbad, CA) was used. A total of 355, 203, 228, and 1,000 DNA samples from the subjects who underwent a cosmetic orthognathic surgery, patients with methamphetamine dependence/psychosis, patients with eating disorders, and patients with clinical data on smoking habits, respectively, participated in the genotyping of the SNP. Distribution of genotypes of the rs13438494 SNP in these subjects is

shown in Table 2. Genomic DNA was extracted from whole-blood samples using standard procedures. The extracted DNA was dissolved in TE buffer (10 mM tris-HCl, 1 mM EDTA). The DNA concentration was adjusted to the range 5–50 ng/μL for genotyping the SNP using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

To perform the TaqMan allelic discrimination assay on a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland), we used TaqMan SNP Genotyping assays (Life Technologies) that involved sequence-specific forward and reverse primers to amplify the polymorphic sequence and two probes labeled with the VIC or FAM dye to detect both alleles of the rs13438494 SNP (Assay ID: C__11239689_10). Real-time PCR was performed in a final volume of 10 μL that contained 2× LightCycler 480 Probes Master (Roche Diagnostics), 40× TaqMan SNP Genotyping Assays, 5–50 ng genomic DNA as a template and up to 10 μL of PCR-grade H_2O supplied with 2× LightCycler 480 Probes Master. The thermal conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 60 s, and final annealing at 50°C for 30 s. Afterwards, endpoint fluorescence was measured for each samples' well, and the genotypes were determined on the basis of the presence or absence of each type of fluorescence.

Age of the first exposure to METH

A large proportion of METH patients among our participants were dependent not only on METH but

also on other drugs such as cannabinoids, cocaine, lysergic acid diethylamide, and opioids. The patients with METH dependence/psychosis were divided into subgroups according to their multisubstance abuse status to estimate the severity of and susceptibility to dependence. A total of 142 patients were polydrug abusers, and 54 patients were exclusively dependent on METH. In addition, the age of first use of METH (years) of each patient was recorded (Table 2). Characteristics of the subjects participating in this study were also described in several other reports [12 - 14].

Eating disorders

Information about clinical parameters was obtained by three medical doctors in face-to-face interviews using a clinical assessment form developed for this study. DSM-IV criteria were used to diagnose eating disorders. The clinical data collected in the present study included several parameters, such as age at onset, the course of the disorder, the presence or absence of other psychiatric disorders such as substance dependence, and changes in weight. Our control subjects were also asked the questions that assess the symptoms and signs characteristic of eating disorders based on the Eating Attitudes Test (EAT-26) [15]. None of the control subjects qualified for abnormal eating behavior. The characteristics of the subjects used in this study were also described in several other reports [16, 17].

The tobacco dependence index

The subjects included in the study for the possible susceptibility to nicotine dependence were tested using questionnaires, with special attention to the questions related to smoking. The questionnaires included the FTND (a test that yields a continuous measure of nicotine dependence) [18] and TDS (a screening questionnaire for tobacco/nicotine dependence according to the ICD-10, DSM-III-R, and DSM-IV), which consists of 10 questions [19]. The questionnaires also included questions about the numbers of cigarrete smoked per day (CPD), the participants' age when they began smoking, how many times current smokers tried to quit smoking (i.e., NTC), and how many times ex-smokers tried to quit smoking before succeeding (i.e., NTE). In the present study, FTND, TDS, CPD, NTC, and NTE scores were used as measures of nicotine dependence and severity.

Fentanyl requirement

For the subjects who underwent a cosmetic orthognathic surgery, the surgical protocol and subsequent postoperative pain management were fundamentally the same as in a previous study [9]. After a patient emerged from anesthesia and tracheal extubation, droperidol 1.25 mg, was intravenously administered to prevent nausea/vomiting, and intravenous patient-controlled analgesia (PCA) with fentanyl (2 mg fentanyl and 5 mg droperidol diluted in normal saline in a total volume of 50 mL) was commenced using a CADD-Legacy PCA pump (Smiths Medical Japan, Japan). The PCA settings included a bolus dose of 20 μg fentanyl on demand and lockout time of 10 min. Continuous background infusion was not used.

Droperidol was coadministered with fentanyl to prevent nausea/vomiting because our preliminary study showed high incidence (up to 30%) of nausea/vomiting with fentanyl PCA in young females. PCA was postoperatively continued for 24 h. Intraoperative fentanyl use and postoperative fentanyl PCA use during the first 24 hours were recorded. The doses of fentanyl administered intraoperatively and postoperatively were normalized to body weight.

Statistical Analysis

The data on the expression level of mRNA or protein and on neurotransmitter uptake were all calculated as mean ± SEM, and the statistical significance was determined using two-tailed and unpaired Student's *t* test or one-way ANOVA with the Bonferroni/Dunnett *post hoc* test. For the analysis of clinical data in the METH dependence/psychosis and eating disorder groups, χ^2 tests were performed to assess contribution of the SNP to the susceptibility to serious symptoms. For the analyses in which the number of subjects in a cell in 2 * 2 contingency tables was equal to or less than five, Fisher's exact tests were conducted instead of χ 2 tests. For the analysis of the smoking behavior data, quantitative values of the smoking period (years), FTND, TDS, CPD, NTC, and NTE scores were natural-log-transformed for approximation to the normal distribution according to the following formula: *Value for analysis = Ln (1 + endpoint phenotype value)* prior to the analysis. To explore the association between the rs13438494 SNP and the phenotypes, either the Kruskal–Wallis *H* test or the Mann–Whitney *U* test was performed for

trichotomized or dichotomized comparison, respectively. The correction of multiple tests for the analyses of the six phenotypes was not performed in this additional exploratory study. For the analysis of fentanyl requirement, postoperative fentanyl PCA use during the first 24 h of the postoperative period was used as an index of opioid requirement because the requirement for this analgesic is likely to reflect the efficacy of fentanyl in each individual. Prior to the analyses, the quantitative values of postoperative fentanyl requirements (μg/kg) were natural-log-transformed for approximation to the normal distribution according to the following formula: *Value for analysis = Ln (1 + postoperative fentanyl requirement [μg/kg])*. These analyses were performed by comparing the genotype and allelic distribution between the patients and control subjects or among subcategories of patients who showed the presence, absence, or a tendency toward serious substance dependence. All analyses were performed using the IBM SPSS v.20.0.0 software (IBM, Tokyo, Japan).

RESULTS

PCLO minigenes containing either the A or C allele of rs13438494 enhance DA and 5-HT uptake in HEK293 cells

Human *DAT*, *SERT*, and *PCLO* minigene A or C alleles, or mock vectors were transiently transfected into HEK293 cells. Two days after the transfection, the cells were rinsed with HKR buffer and subjected to the uptake assays. In both *PCLO* minigene A and C alleles transfected cells, the DA and 5-HT uptake were significantly upregulated compared with the mock-transfected cells. Although *PCLO* minigene C allele-transfected cells showed lower DA and 5-HT uptake compared with the *PCLO* minigene A allele-transfected cells (Fig. **1b**, **c**). These results indicate that *PCLO* intronic SNP rs13438494 influences the turnover of neurotransmitters.

Regulatory roles of rs13438494 of the *PCLO* minigene in mRNA splicing and PCLO C2A protein expression

To investigate the role of this SNP in the regulation of splicing, we performed *in vitro* experiments that tested the splicing pattern of the A and C alleles (Fig. **2a**). RT-PCR of the minigene construct containing the C allele yielded the same two bands as the A allele, but in different proportions of the unspliced and spliced transcripts (Fig. **2b**; $P = 0.0057$). The S/U ratio of the A allele was 0.30 ± 0.07 and that of the C

allele was 0.08 ± 0.02 (mean ± SEM).

After gel extraction, each PCR product was sequenced. The upper band of about 3.7 kbp corresponded to unspliced transcripts including exons 14–24, exon 25 and its intronic flanking sequences. The lower 1.8-kbp fragment matched exons 14–25. Our results indicated that in the C allele, there was an increase in expression of the retained intron and a decrease in expression of the fully spliced transcript, suggesting that the C allele of rs13438494 reduces splicing efficiency of the *PCLO* minigene.

Effects of rs13438494 of the *PCLO* minigene on expression of the C2A domain in HEK293 cells

We next assessed the expression levels of mRNA of the PCLO C2A domain using specific primers. In the cells transfected with the *PCLO* minigene's either A or C allele, the expression of PCLO C2A domain was significantly increased compared with mock (pAcGFP)-transfected cells, and there was no difference in the expression level between the alleles. Furthermore, we determined protein expression of the *PCLO* minigene using an anti-GFP antibody (Fig. **2d**). We detected one protein at the expected size 90 kD, which corresponds to the mature PCLO C2A polypeptide. The expression of the C2A protein by the A allele minigene (100 ± 5.87%) was stronger than that of the C allele (61.7 ± 2.21%).

Association of rs13438494 with the age of first exposure to METH

A total of 198 patients were polydrug abusers, and 54 patients were exclusively dependent on METH.

In addition, the age of first use of METH (years) in each patients was also recorded. As a result, a significant difference in age at the first exposure was found among the three genotypes C/C, C/A, and A/A of the rs13438494 SNP (P = 0.0480). In the *post hoc* analysis, a significant difference was found between the subjects with the homozygous C/C genotype and the combined group of subjects with either the C/A or A/A genotype (P = 0.0233; Table 2). No significant differences among the genotypes were observed in the comparison among subgroups according to their multisubstance abuse status (data not shown). Thus, our analysis suggests that the subjects who carry the homozygous C allele of rs13438494 began using METH significantly later (Table 2).

The association of rs13438494 with fentanyl requirement

For the subjects who underwent a cosmetic orthognathic surgery, we utilized the postoperative fentanyl use during the first 24 h of the postoperative period as an index of opioid analgesic requirement and compared it among the genotypes of the rs13438494 SNP. As a result, a significant difference in postoperative analgesic requirement was found among the three genotypes C/C, C/A, and A/A (P = 0.0117). In the *post hoc* analysis, a significant difference in postoperative analgesic requirement was found between the subjects with the homozygous C/C genotype and the combined group of subjects with either the C/A or A/A genotype (P = 0.0214; Table 2). Total fentanyl use during the initial 24 h of the postoperative period was 3.141 ± 2.720, 2.174 ± 1.897, and 3.508 ± 2.077 mg/kg in subjects with the C/C,

C/A, and A/A genotypes, respectively. Thus, our data indicate that the subjects carrying the homozygous C allele of rs13438494 have significantly lower sensitivity to fentanyl (Table 2).

Association of rs13438494 with the type of eating disorders

We divided all patients with an eating disorder into two groups. One group containing ANR (anorexia, restricting type), ANBP (anorexia, binging, and purging type), and NOS (eating disorder not otherwise specified), and the other group containing BNP (bulimia nervosa, purging type) and BNNP (bulimia, nonpurging type). As a result, a significant difference in eating disorder type (Group One or Group Two) was found among the three genotypes C/C, C/A, and A/A of the rs13438494 SNP (P = 0.0436). In the *post hoc* analysis, a significant difference was found between the subjects with the homozygous A/A genotype and the combined group of subjects with either the C/C or C/A genotype (P = 0.0173; Table 2). No significant differences were observed in the comparison among the genotypes according to other categorization of eating disorders (data not shown). Thus, our data indicate that patients with an eating disorder who carry the C allele of rs13438494 are significantly more likely to have a diagnosis of bulimia nervosa (Table 2).

The association of rs13438494 with TDS

For the analysis of smoking behavior data in patients, we used quantitative values of the smoking

period (years), FTND, TDS, CPD, NTC, and NTE scores as indices of nicotine dependence or related characteristics. We compared those indices among the genotypes of the rs13438494 SNP. As a result, a significant trend for difference in the TDS score was found among the three genotypes C/C, C/A, and A/A (P = 0.0570). In the *post hoc* analysis, a significant difference was found between the subjects with the homozygous C/C genotype and the combined group of subjects with either the C/A or A/A genotype (P = 0.0339; Table 2). No significant differences were observed in the analyses of other smoking characteristics (data not shown). Thus, our results show that the subjects carrying the homozygous G allele of rs13438494 are significantly less vulnerable to nicotine dependence (Table 2)

DISCUSSION

Neurotransmitters, such as DA and 5-HT, are important for neuronal function, and dysfunction of their metabolism is believed to be involved in the pathogenesis of psychiatric disorders such as major depressive disorder, schizophrenia, and bipolar disorders. The concentration of DA and 5-HT in the synaptic cleft is regulated by DAT and SERT. In this study, we show that rs13438494 affects DA and 5-HT uptake in cultured human cell lines, via modulation of those transporters. This effect should change the concentration of DA and 5-HT in the synaptic cleft and may explain the statistical association of rs13438494 alleles with some psychiatric disorders. Our results show an increase in expression of the transcript with a retained intron and a decrease in expression of the fully spliced transcript as a result of

the C allele of rs13438494. These data suggest that this allele reduces the splicing efficiency of the *PCLO* minigene. If the changes of the splicing pattern of the C allele of rs13438494 affect the translational efficiency of the transgenic minigene *PCLO*, then the uptake of DA and 5-HT should be reduced with this allele (The incorrectly spliced transcript will be eliminated by a specialized mRNA decay pathway). This point mutation does not change the amino acid sequence of *PCLO* because rs13438494 is an intronic SNP, not a coding SNP.

Many SNPs have been found that have an association with psychiatric disorders, for example, SNPs in such genes as disrupted in schizophrenia 1 (*DISC1*) [20, 21], catechol-O-methyltransferase1 (*COMT1*) [22, 23], and brain-derived neurotrophic factor (*BDNF*) [24, 25]. However, in only a few cases, the underlying biological mechanism was verified. The present work describes findings that intronic SNP, rs13438494, which has known associations with some mental disorders, produces a change in relevant neurobiological functions. In our previous study, DA uptake did not change in cultured PC12 cells overexpressing the PCLO C2A domain, compared with a mock control [2]. In contrast, in the present work, the cells overexpressing both the C2A and C2B domain show an increase of DA and 5-HT uptake. We will investigate the function of the C2B domain in an upcoming project.

Next, we checked the association of rs1343894 with dependence-like behavior. The parameters that we tested are tobacco dependence, fentanyl requirement, different types of eating disorders, and the age of first exposure to METH. In Japanese population, more than 70% people has C allele in rs13438494. So it

was difficult to collect A type of Japanese subjects, then sample size was small. All these phenotypes show significant statistical associations with the alleles of rs1343894. These diseases and behaviors exhibit dependence-like features.

In this study, we demonstrate that rs13438494 regulates DA/5-HT uptake in the cultured cells and fentanyl requirement in clinical study. Fentanyl is an agonist for mu opioid receptor, and has analgesic effects. Some studies indicate that stimulation of opioid receptors affects GABA property [26, 27]. GABA can modulate DA release and uptake in the brain. They also demonstrate that opioid receptor modulates of serotonergic projection. Thus, rs13438494 regulate the affection of pain control *via* mu-opioid receptors and DA or 5-HT. On the basis of neuroimaging studies, the orbitofrontal cortex and opioids are responsible for pain modulation by hedonic experience, while the ventral striatum and dopamine mediate motivational effects on pain [28].

It is not well understood what role DA and 5-HT play in the brain aberrations of these patients. In animal studies, addiction behavior should be observed when DA release into the synaptic cleft is increased and when DA uptake at the nerve terminal is reduced. We can attempt to explain the relationship between the addictive behavior and the neurotransmitters DA and 5-HT on the basis of the published studies of laboratory animals. However, some studies on human subjects have produced results that do not agree with those from laboratory animals. Smoking cessation in humans is facilitated by the

presence of more 9-repeat alleles in the variable number of tandem repeats (VNTR) of the 3′ untranslated region (3′ UTR) of the *DAT* gene; however, this genotype potentiates DA uptake [29]. In eating disorders, some of which are considered one of the variations of addiction, the shorter alleles are more frequent. Although an increased synaptic content of DA is believed to be associated with eating disorders in general, the repeated variant has been shown to produce the opposite effect in binge eating disorder, which bears the strongest resemblance to substance dependence among other eating disorders [30]. Furthermore, a negative association was found between SERT and relapse in heroin users [31]. In studies on human subjects, we have to deal with the differences of race, nationality, and other forms of genetic background. Ours appears to be the first finding of a correlation of an intronic SNP (of confirmed biological significance) with clinical features of dependence-like behavior.

In conclusion, the genomic association studies in mental health research have rarely produced a single obvious candidate gene. Nonetheless, it is possible that our data on PCLO variants and their biological consequences will help to develop new therapeutic tools for mental disorders that have symptoms resembling substance dependence and are accompanied with DA or 5-HT dysfunction.

ABBREVIATIONS

PCLO = piccolo

DA = dopamine

5-HT = 5-hydroxytryptamine;

DAT = dopamine transporter

SERT = serotonin transporter

PBS = phosphate-buffered saline

ANOVA = analysis of variance

SNP = single nucleotide polymorphisms

UTR = untranslated region

CONFLICT OF INTEREST

The authors declare that they have no financial conflicts of interest associated with this article's content.

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Table 1. Primers used for the cloning of *PCLO* minigene and site-directed mutagenesis.

	Primer sequence
Cloning of *PCLO* minigene (Exon 14 to 23)	Forward: 5'GAAGATCTCGCCTACCGATGGAACAAAGGTTG3' Reverse: 5'CCCAAGCTTCTGGTAGATGATCAGGAGACTTA3'
HindIII site deletion	Forward: 5' CTCCTGATCATCTACCAGATTTATATGTGAAAATATATG 3' Reverse: 5' CATATATTTTCACATATAAATCTGGTAGATGATCAGGAG 3'
Cloning of *PCLO* minigene (Exon 24, intron24 and exon 25)	Forward: 5'CCCAAGCTTCATTTATATGTGAAAATATATGTGATG3' Reverse: 5'CGGGATCCTCAATGCGTTTGAGTAGGACTGA3'
A to C substitution	Forward: 5' GGAAGTACAAATTTTGAAGT**C**AGAAGCATAAAAGTTTTCGCC 3' Reverse: 5' GGCGAAAACTTTTATGCTTCT**G**ACTTCAAAATTTGTACTTCC 3'

Restriction site targets introduced to allow sequential cloning of PCR-amplified fragment are underlined. Nucleotides replaced by site-directed mutagenesis is indicated in bold.

Table 2. Association between PCLO SNP rs13438494 to age at the first methamphetamine expose, eating disorder phenotypes, fentanyl requirements disorder and tobacco dependence screener.

		Genotype of PCLO SNP rs13438494			Analysis	P value	Statistics†
		CC	CA	AA			
Methamphetamine users	N=198						
age at the first expose					CC vs CA vs AA	0.0480*	6.075
under 19 years old:		70	34	2	CC or CA vs AA	0.5000	1.754
over 20 years old:		74	18	0	CC vs CA or AA	0.0233*	5.147
Eating disorder	N=227						
ANR (Anorexia, restricting type) or					CC vs CA vs AA	0.0436*	6.266
ANBP (Anorexia, binge and purging type) or					CC or CA vs AA	0.0173*	6.265
NOS (eating disorder not otherwise specified):		72	25	5	CC vs CA or AA	0.5214	0.411
BNP (Bulimia nervosa, purging type) or							
BNNP (Bulimia, nonpurging type):		93	32	0			
Nicotine dependence							
TDS (Tobacco dependece screener)	N=510	392	110	8	CC vs CA vs AA	0.0570	5.731
	Mean	2.8015267	3.4	2.625	CC or CA vs AA	0.5536	2254.5
	SD	2.1083647	2.362552	2.5035689	CC vs CA or AA	0.0339*	26134
	Median	2	3	2			
	Range	[0–9]	[0–9]	[0–8]			
Fentanyl requirements (24 hrs after operation)							
(μ g/kg)	N=355	263	83	9	CC vs CA vs AA	0.0117*	8.889
	Mean	3.1413689	2.174578	3.5075107	CC or CA vs AA	0.2316	1193.5
	SD	2.7205432	1.896671	2.0778602	CC vs CA or AA	0.0214*	10149
	Median	2.3529412	1.886792	3.5483871			
	Range	[0–13.818]	[0–9.434]	[0–6.897]			

†: Chi-square or KruskalWallis H or Mann-Whitney U, *:P<0.05

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FIGURE LEGENDS

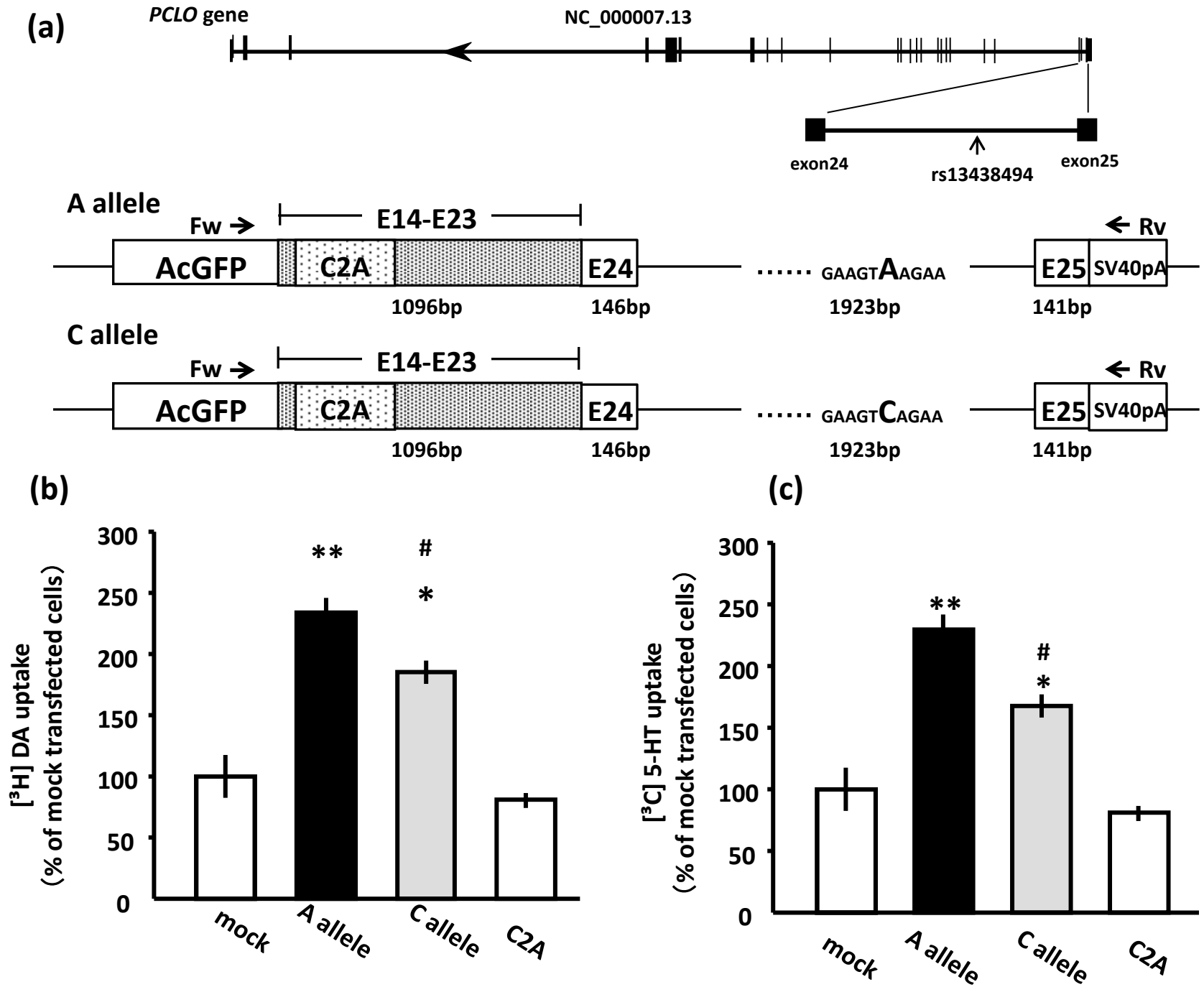
Fig. (1). Construction and effects of *PCLO* minigene allele A or *PCLO* minigene allele C on [^{3}H]DA and [^{14}C]5-HT uptake by HEK293 cells.

a. An illustration of *PCLO* minigenes fused to the GFP coding sequence. The position of the mutation is highlighted in bold. The binding sites of the primers used for RT-PCR are indicated by small arrows. The vector containing *PCLO* exons 14–25 was transfected into HEK293 cells. **b, c.** The transfected HEK293 cells were incubated in HKR buffer for 30 min and then with (**b**) 0.1 mM [^{3}H]DA and (**c**) 1 mM [^{14}C]5-HT at 37°C for 10 min. The data are mean ± SEM (n = 4). * $P < 0.05$, ** $P < 0.01$ compared to the mock-transfected cells (Student's t test). # $P < 0.05$ compared to cells transfected with the *PCLO* minigene allele A (Student's t test).

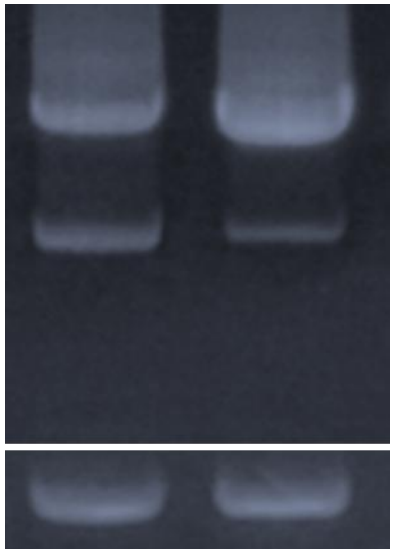
Fig. (2). Expression analysis and biological effects of the *PCLO* minigene allele A or *PCLO* minigene allele C.

a. RT-PCR of mRNA from cells transfected with the *PCLO* minigene constructs. The upper band indicates the unspliced transcript. The lower band corresponds to a 1.8-kbp spliced product. *GAPDH* was used as an internal control for normalization. **b.** Splicing efficiency according to the amount of spliced and unspliced transcripts from 3 independent experiments. * Transcript splicing efficiency is the mean ratio of fluorescence intensity of correctly spliced transcripts to that of spliced plus unspliced

transcripts for a given expression construct. Standard deviations from 3 independent experiments are shown. ** *P* value from two-tailed Student's *t* test. Total RNA was extracted from mock (GFP)-, *PCLO* minigene allele A-, or allele C-vector-transfected HEK293 cells, cultured for 48 h after the transfection. **c.** PCR products of *PCLO* C2A domain obtained from the transfected HEK293 cells. The upper band indicates *PCLO* C2A domain. The lower band indicates *Gapdh* (left). Quantification of a band using the ImageJ software is shown on the left. The intensity of each band, representing the amount of each transcript, is expressed as a percentage relative to the mock-transfected cells. The data are mean ± SE from different experiments ($n = 3$). ** $P < 0.01$ compared to the mock-transfected cells (Student's *t* test). **d.** Proteins from whole-cell lysates of *PCLO* minigene allele A- or allele C-transfected HEK293 cells were equally loaded in each lane (15 μg protein per lane). The resolved protein patterns were analyzed by means of Western blotting using a rabbit polyclonal anti-PCLO antibody as a primary antibody (left). Intensity of bands (Western blot) corresponding to the allele A or C was measured using ImageJ software (right). The data are mean ± SE ($n = 3$). ** $P < 0.01$ compared to the *PCLO* minigene allele A-transfected cells (Student's *t* test).


(a)
PCLO gene
NC_000007.13
exon24
rs13438494
exon25
A allele
Fw
E14-E23
Rv
AcGFP
C2A
E24
GAAGTAAGAA
E25
SV40pA
1096bp
146bp
1923bp
141bp
C allele
Fw
E14-E23
Rv
AcGFP
C2A
E24
GAAGTCAGAA
E25
SV40pA
1096bp
146bp
1923bp
141bp
(b)
[3H] DA uptake
(% of mock transfected cells)
300
250
200
150
100
50
0
**
#
*
mock
A allele
C allele
C2A
(c)
[3C] 5-HT uptake
(% of mock transfected cells)
300
250
200
150
100
50
0
**
#
*
mock
A allele
C allele
C2A

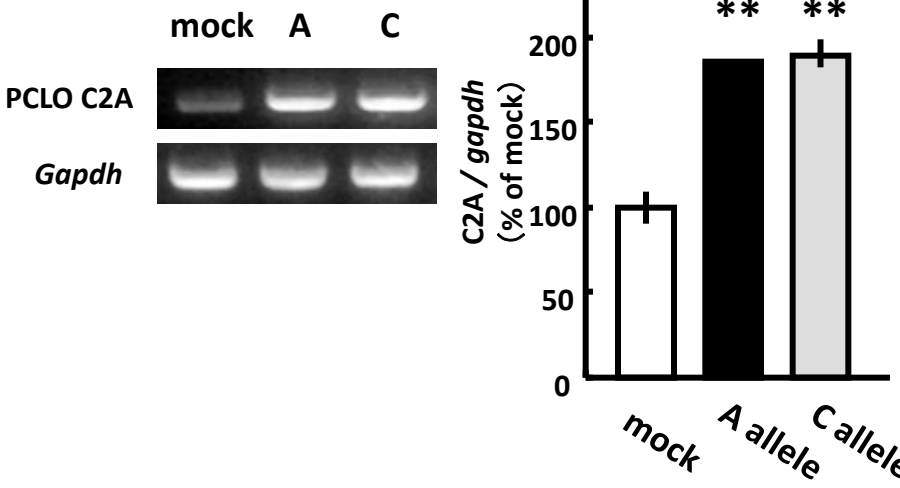
(a)



(b)

	A allele	C allele
S/U ratio *	0.30	0.08
S.D.	0.07	0.02
P value**	0.0057	

(c)



(d)