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Association Between *KCNJ6* (*GIRK2*) Gene Polymorphism rs2835859 and Post-operative Analgesia, Pain Sensitivity, and Nicotine Dependence

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Abstract. G-protein–activated inwardly rectifying potassium (GIRK) channels are expressed in many tissues and activated by several $G_{i/o}$ protein–coupled receptors, such as opioid and dopamine receptors, and thus are known to be involved in the modulation of opioid-induced analgesia, pain, and reward. We focused on a GIRK-channel subunit that plays a pivotal role in the brain, GIRK2, and investigated the contribution of genetic variations of the *GIRK2* (*KCNJ6*) gene to individual differences in the sensitivity to opioid analgesia. In our initial linkage disequilibrium analysis, a total of 27 single-nucleotide polymorphisms (SNPs) were selected within and around the regions of the *KCNJ6* gene. Among them, the rs2835859 SNP, for which associations with analgesia and pain have not been previously reported, was selected in the exploratory study as a potent candidate SNP associated with opioid analgesic sensitivity. The results were corroborated in further confirmatory study. Interestingly, this SNP was also found to be associated with sensitivity to both cold and mechanical pain, susceptibility to nicotine dependence, and successful smoking cessation. The results indicate that this SNP could serve as a marker that predicts sensitivity to analgesic and pain and susceptibility to nicotine dependence.
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Keywords: G-protein–activated inwardly rectifying potassium (GIRK) channel, single-nucleotide polymorphism, opioid analgesia, pain, susceptibility to nicotine dependence

Introduction

G-protein–activated inwardly rectifying potassium (GIRK) channels are members of the inwardly rectifying potassium channel family, and four kinds of subunits (GIRK1-GIRK4) have been identified in mammals (1). GIRK channels are expressed in many tissues, including the heart (2), spinal cord (3, 4), and various regions in the brain with different subunit compositions (5 – 7). GIRK channel activation is triggered by the activation of several $G_{i/o}$ protein–coupled receptors, such as opioid (8), M_2-muscarinic (9), D_2- and D_4-dopaminergic (10), α_2-adrenergic (11), serotonin 1A (5-HT_{1A}) (12), metabotropic glutamate (13), somatostatin (14), CB_1-cannabinoid (15, 16), nociceptin/orphanin FQ (17), and A_1-adenosine (18) receptors. Neuronal GIRK channels are predominantly heteromultimeric, composed of GIRK1 and GIRK2 subunits in most brain regions (19, 20), or homomultimeric, composed of GIRK2 subunits in the substantia nigra (21). Several studies that used knockout mice showed that opioid-induced GIRK channel activation co-expressed with opioid receptors inhibited

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nociceptive transmission and thus opioid-induced analgesia (2, 3, 22 – 24). Furthermore, GIRK1-, GIRK2-, and GIRK3-knockout mice exhibited hyperalgesia in the hot-plate and tail-flick tests of thermal nociception (4, 23), suggesting the involvement of GIRK channels in the sensitivity to hot stimulus-induced pain. GIRK channels have also been reported to be involved in the rewarding effects of ethanol and cocaine in studies of GIRK2- and GIRK3-knockout mice (25, 26).

Among many related functions or phenotypes, the effects of GIRK channels on analgesia and pain perception mentioned above are mediated by upstream opioid signaling, which is known to play important roles in both antinociception and reward (27, 28). To date, only a few studies have examined the relationship between genetic variations in GIRK channels and phenotypic differences related to opioid action in humans (29 – 32). One of these studies was conducted by our group (31), in which we sought to reveal the relationship between single-nucleotide polymorphisms (SNPs) in the *KCNJ6* gene that encodes human GIRK2, especially within the exonic and 5′-flanking regions, and individual differences in opioid analgesic sensitivity. Another recent study found an association between *KCNJ6* SNPs and pain-related phenotypes, reconfirming that the *KCNJ6* gene is a promising target for investigating the genetic factors that contribute to pain and analgesia (29).

The present study sought to comprehensively reveal the relationship between SNPs in the *KCNJ6* gene region, including the intronic region, and individual differences in the sensitivity to analgesia, experimental pain, and smoking behavior.

Materials and Methods

Ethics statement

The study protocol was approved by the Institutional Review Boards at Tokyo Dental College, Chiba, Japan (Tokyo), Hamamatsu University School of Medicine (Hamamatsu), and the Tokyo Institute of Psychiatry (currently Tokyo Metropolitan Institute of Medical Science; Tokyo). All of the subjects provided informed, written consent for the genetics studies.

Subjects

Enrolled in the initial analysis to explore the association between *GIRK2* gene polymorphisms and the sensitivity to opioid-induced analgesia were 355 healthy patients who were scheduled to undergo cosmetic orthognathic surgery (mandibular sagittal split ramus osteotomy) for mandibular prognathism at Tokyo Dental College Suidoubashi Hospital, as described in the Supplementary Materials and Methods (available in the online version only) and a previous report (33). Peripheral blood samples were collected from these subjects for the gene analysis. The detailed demographic and clinical data of the subjects are provided in Supplementary Table 1 (available in the online version only).

The subjects used in the association study to examine the contribution of *GIRK2* gene polymorphisms to the sensitivity to pain were a total of 500 healthy volunteers who lived in the Kanto area of Japan, as described in the Supplementary Materials and Methods (age 20 – 72 years, 253 males, 242 females, and five gender-unknown subjects). Oral mucosa samples were collected from the subjects for the gene analysis. All of the subjects underwent the cold pressor–induced pain test (CPT) and mechanically induced pain test (MPT). Additionally, the Temperament and Character Inventory (TCI) (34 – 36), a self-report measure of temperament and character dimensions, was used to profile the personalities of these subjects. The detailed demographic and clinical characteristics of the subjects are provided in Supplementary Table 2 (available in the online version only).

Participants in the subsequent study to examine the association between *GIRK2* gene polymorphisms and the susceptibility to nicotine dependence included a total of 1,000 patients who visited Iwata City Hospital in Japan. The inclusion criteria for this study were being ambulatory, able to communicate orally, and 60 years of age or older. Numerous participants in this study had various smoking habits and completed a questionnaire that consisted of various questions about lifestyle, including alcohol consumption, smoking, diet, and cancer history (37). Peripheral blood samples were collected from these subjects for the gene analysis. The detailed demographic and clinical characteristics of the subjects are provided in Supplementary Table 3 (available in the online version only).

Data collection

For the subjects who underwent cosmetic orthognathic surgery, the surgical protocol and subsequent postoperative pain management were fundamentally the same as those of the previous study (33, 38) and detailed in the Supplementary Materials and Methods. Postoperative patient-controlled analgesia (PCA) fentanyl use during the first 24-h postoperative period was recorded. The dose of fentanyl administered postoperatively was normalized to body weight.

For the healthy volunteer subjects, the results from the two pain tests were recorded. The CPT was performed basically as previously described (39, 40), although a slight modification was made. Basal endpoint sensitivity to pain was evaluated as detailed in the Supplementary Materials and Methods (Supplementary Table 2). In the

MPT, a DPS-20 digital force gauge (Imada, Northbrook, IL, USA) was used to measure the level of force when the subjects felt pain, with a wooden sphere attached to the tip of the instrument so that the subjects could feel pain on a single point of the finger (Supplementary Fig. 1: available in the online version only). The basal sensitivity to pain was evaluated as detailed in the Supplementary Materials and Methods (Supplementary Table 2). Additionally, the TCI was used to assess the personality profiles of all of the subjects (35). The TCI used in the present study was based on the shortened 125-item questionnaire of the longer 240-item Japanese version of the TCI. The usage of the TCI for the analysis in the present study is detailed in the Supplementary Materials and Methods and a previous report (33).

For the subjects included in the study on the susceptibility to nicotine dependence, the results of the questionnaire, especially the questions related to smoking, were used in the analysis. The questionnaire included the Fagerström Test for Nicotine Dependence (FTND; a test that yields a continuous measure of nicotine dependence) (41) and Tobacco Dependence Screener [TDS; a screening questionnaire for tobacco/nicotine dependence according to the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10), Diagnostic and Statistical Manual of Mental Disorders, 3rd edition (DSM-III-R), and Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV)], which consists of 10 questions (42). The questionnaire also included questions about the number of cigarettes smoked per day (CPD), the participants' age when they began smoking, how many times current-smokers tried to quit smoking [i.e., the number of trials for smoking cessation in current-smokers (NTC)], and how many times ex-smokers tried to quit smoking before succeeding [i.e., the number of trials for smoking cessation in ex-smokers (NTE)]. In the present study, the FTND, TDS, CPD, NTC, and NTE were used as measures of nicotine dependence and severity (Supplementary Table 3).

Genotyping and linkage disequilibrium (LD) analysis

Genomic DNA was extracted from whole-blood samples using a QIAamp DNA BloodMaxi kit (Qiagen, Hamburg, Germany) or a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions and extracted from oral mucosa samples as described in the Supplementary Materials and Methods and a previous report (43).

To initially analyze SNPs within and around the *KCNJ6* gene region, genotype data for approximately 300,000 SNP markers that resulted from whole-genome genotyping with the orthognathic surgery samples as described previously and in the Supplementary Materials and Methods (33) were basically used, and the genotype data for all of the SNPs with *KCNJ6* gene annotation were extracted. For additional analyses, a TaqMan allelic discrimination assay was conducted to genotype the candidate Tag SNP, rs2835859, which was selected by LD analysis in the *KCNJ6* gene and flanking region and association analysis with the orthognathic surgery samples for further analyses using the other samples.

Of the 65 SNPs with minor allele frequencies above 0.001 that were located within the exon and intron regions and approximately within the 10 kbp 5′- and 3′-flanking regions of the *KCNJ6* gene, SNPs for the association studies were selected based on recently advanced tagging strategies (44 – 46). To identify relationships between the SNPs used in the study, an LD analysis was performed for 127 of the 355 samples using Haploview v. 4.1 (47). To estimate the LD strength between the SNPs, the commonly used D' and r^2 values were pairwise calculated using the genotype dataset of each SNP. Linkage disequilibrium blocks were defined among the SNPs with minor allele frequencies above 0.05 that showed "strong LD" based on the default algorithm of Gabriel et al. (48), in which the upper and lower 95% confidence limits on D' for strong LD were set at 0.98 and 0.7, respectively. Tag SNPs in the LD block were consequently determined using the Tagger software package with default settings, which is incorporated in Haploview and has been detailed in a previous report (46).

To perform the TaqMan assay with a LightCycler 480 (Roche Diagnostics, Basel, Switzerland), we used TaqMan SNP Genotyping Assays (Life Technologies, Carlsbad, CA, USA) that contained sequence-specific forward and reverse primers to amplify the polymorphic sequence and two probes labeled with VIC and FAM dye to detect both alleles of the rs2835859 SNP (Assay ID: C_16076710_10), as detailed in the Supplementary Materials and Methods.

Statistical analysis

Among the 355 subjects who underwent painful cosmetic surgery, one subject lacked postoperative clinical data; thus, a total of 354 subjects were used for the initial LD and association analyses (126 and 228 subjects for the exploratory and confirmatory analyses, respectively). As an index of opioid sensitivity, postoperative PCA fentanyl use during the first 24-h postoperative period was used because analgesic requirements likely 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 as described in the Supplementary Materials and Methods. To explore the associations between the SNPs and phenotypes, analysis of variance (ANOVA) was performed for trichotomized comparisons between each genotype of the SNPs in the exploratory stage of the analysis, in which 24-h postoperative fentanyl use (μg/kg, log-transformed) and the genotype data for each SNP were incorporated as dependent and independent variables, respectively, and the SNPs that showed $P < 0.05$ in the analysis were considered nominally significant and selected for further analysis. In the following confirmatory stage of the analysis, dichotomized comparisons were made, in addition to the trichotomized comparisons, in which dominant and recessive genetic models for the minor allele of each SNP were also considered. In this stage, the Q-values of the false discovery rate were calculated to correct for multiple testing, in addition to the P-values based on previous reports (49, 50). The SNPs that showed $Q < 0.05$ in the analysis were considered significant for the entire SNP set in the *KCNJ6* gene region. All of the statistical analyses were performed using gPLINK v. 2.050, PLINK v. 1.07 (51), and Haploview v. 4.1 (47).

To corroborate the possible association between the SNPs and opioid sensitivity observed in the subjects who underwent painful cosmetic surgery, additional analyses were subsequently conducted for those SNPs. The samples included in these analyses were obtained from healthy volunteers with pain sensitivity and personality profile data and patients with smoking behavior data. For all of the genotype data used in these analyses, the distributions were checked using the χ^2 test, and the absence of significant deviation from the theoretical distribution expected from Hardy-Weinberg equilibrium was confirmed. For all of the statistical analyses described below, SPSS 18.0J for Windows (International Business Machines Corporation, Armonk, NY, USA) was used. The criterion for significance was set at $P < 0.05$.

For the analysis of the pain sensitivity data from healthy volunteer subjects, quantitative values of both the average latency (s) to pain perception in the CPT and average weight (kg) when the subjects perceived pain in the MPT were natural-log–transformed for approximation to the normal distribution as described in the Supplementary Materials and Methods. For the analysis of personality profile data from healthy volunteer subjects, raw TCI scores were processed according to a previous report (33). The score on each subscale in each dimension was averaged, in which the total score was divided by the number of items in each subscale. The average score on each subscale was averaged to calculate the overall score on each dimension, in which the sum of the average score on each subscale was divided by the number of subscales in each dimension, which was used as the endpoint in the association study. Prior to the analysis, quantitative values of the overall score on each dimension (ranging from 0 to 1) were natural-log–transformed for approximation to the normal distribution as described in the Supplementary Materials and Methods. To explore the association between the rs2835859 SNP and phenotypes, Student's t-test or the Welch test and ANOVA were performed for dichotomized and trichotomized comparisons between genotypes, respectively, in which the endpoint values in the pain tests and TCI scores (log-transformed) and genotype data of the SNP were incorporated as dependent and independent variables, respectively. Corrections for multiple testing for the analyses of the two and seven phenotypes for the pain tests and TCI, respectively, were not performed in this additional exploratory study.

For the analysis of smoking behavior data in the patients, quantitative values of the smoking period (years), FTND, TDS, CPD, NTC, and NTE were natural-log–transformed for approximation to the normal distribution as described in the Supplementary Materials and Methods. To explore the association between the rs2835859 SNP and phenotypes, Student's t-test or Welch test and ANOVA were performed for dichotomized and trichotomized comparisons between genotypes, respectively, in which the phenotype values (log-transformed) and genotype data of the SNP were incorporated as dependent and independent variables, respectively. Corrections for multiple testing for the analyses of the six phenotypes were not performed in this additional exploratory study.

Corrections for multiple testing for the many phenotypes examined were not performed in the present study because they may not be necessarily required in exploratory studies, such as the present study, meaning that the associations between the SNPs and phenotypes have not yet been reported. Considering that the likelihood of type II errors is increased by corrections for multiple testing, such as Bonferroni adjustments, and considering that truly important differences may not be deemed significant (52), such adjustments were not done in the present study. Corrections for multiple testing would be too conservative for genetic association studies (53). Indeed, in similar previous studies, such corrections were not performed (54, 55).

Results

Identification of a potent SNP associated with postoperative analgesia

After whole-genome genotyping, an LD analysis was initially conducted using the genotype data from 126 samples in a total of 355 samples from subjects who underwent painful cosmetic surgery (Supplementary Table 1). As a result, a total of 11 LD blocks (LD1 – LD11) were observed within and around the *KCNJ6* gene region, and 27 Tag SNPs were selected in this region (Supplementary Figs. 2 and 3: available in the online version only). Of these Tag SNPs, only one SNP, rs2835859, was found to be nominally significant ($P < 0.05$) in the initial exploratory association analysis between the SNPs and postoperative fentanyl requirements (Supplementary Table 4: available in the online version only). A further analysis of the remaining 228 samples to confirm the association observed in the exploratory association analysis indicated that the rs2835859 SNP was significantly associated with postoperative analgesic use after false discovery rate correction ($Q = 0.0353$, Supplementary Table 4). The carriers of the C allele in this SNP required less analgesics compared with non-carriers (Table 1, Fig. 1). Therefore, this SNP was selected for further analysis of associations between SNP genotype and other demographic and clinical characteristics. The number of subjects carrying the T/T, T/C, and C/C genotypes of this SNP was 305, 45, and 4, respectively, and the distribution was not significantly different from the theoretical Hardy-Weinberg equilibrium value in the 355 patient subjects used in the association analyses ($\chi^2 = 2.39$, $P = 0.12$).

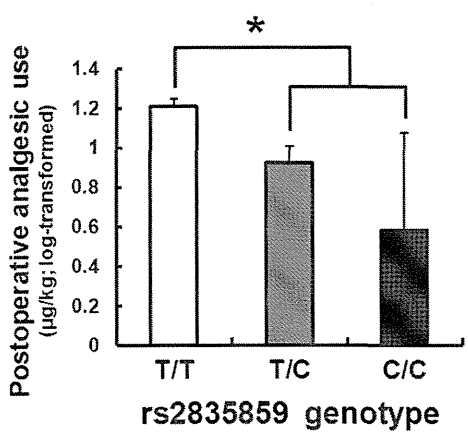

Fig. 1. Association analysis between opioid analgesic requirements during the 24-h postoperative period after cosmetic orthognathic surgery and *KCNJ6* SNPs. The results are shown for the rs2835859 SNP. *$P < 0.05$, significantly lower dose of analgesics administered in the combined T/C and C/C genotype than in the T/T genotype in the confirmatory study. The data are expressed as the mean ± S.E.M.

Association between rs2835859 SNP and pain sensitivity in healthy subjects

The observed association between the rs2835859 SNP and postoperative analgesia suggested that the subjects with the C allele of the SNP required less analgesics than the subjects without this allele, likely attributable to the increased effectiveness of opioid analgesics in this cohort. These C allele carriers may have presented higher sensitivity than non-carriers to an exogenous opioid, fentanyl. To examine whether the possible difference between genotypes in the sensitivity to exogenous opioids can be extended to the difference in the sensitivity to endogenous opioids, we compared basal pain sensitivity between the genotypes of this SNP in healthy volunteer subjects (Supplementary Table 2). In the association analysis of the CPT data, a significant difference was found between the T/T subgroup and combined T/C and C/C genotype subgroup in the average latency to pain perception, and C allele carriers had a longer latency compared with non-carriers ($t_{496} = -2.762$, $P = 0.006$; Fig. 2A). Interestingly, a similar result was obtained in the association analysis of the MPT data, in which a significant difference was found between the T/T subgroup and combined T/C and C/C genotype subgroup in the average weight when the subjects perceived pain, and C allele carriers had a greater weight at which they perceived pain compared with non-carriers ($t_{495} = -2.107$, $P = 0.036$; Fig. 2B). However, significant associations were not found in the association analyses for any of the seven dimensions of TCI scores [novelty seeking (NS): $t_{495} = 0.067$, $P = 0.947$; harm avoidance (HA): $t_{495} = 1.851$, $P = 0.065$; reward dependence (RD): $t_{495} = -0.812$, $P = 0.417$; persistence (P): $t_{495} = -1.877$, $P = 0.061$; self-directedness (SD): $t_{495} = -1.054$, $P = 0.292$; cooperativeness (C): $t_{495} = -1.039$, $P = 0.299$; self-

Table 1. Results of confirmatory association analysis between the *KCNJ6* rs2835859 SNP and postoperative analgesia

SNP	CHR[a]	Position[b]	Location	Genotypic			Dominant			Recessive		
				BETA	*STAT*[c]	*P*	*BETA*	*STAT*[c]	*P*	*BETA*	*STAT*[c]	*P*
rs2835859	21	37940032	intron 3	NA	5.81	0.0548	−0.3145	−2.391	0.0177*	−0.4007	−1.1	0.2725

[a]CHR: chromosome number, [b]Position: chromosomal position (bp), [c]STAT: *F*-statistic or *t*-statistic, *corrected $P < 0.05$.

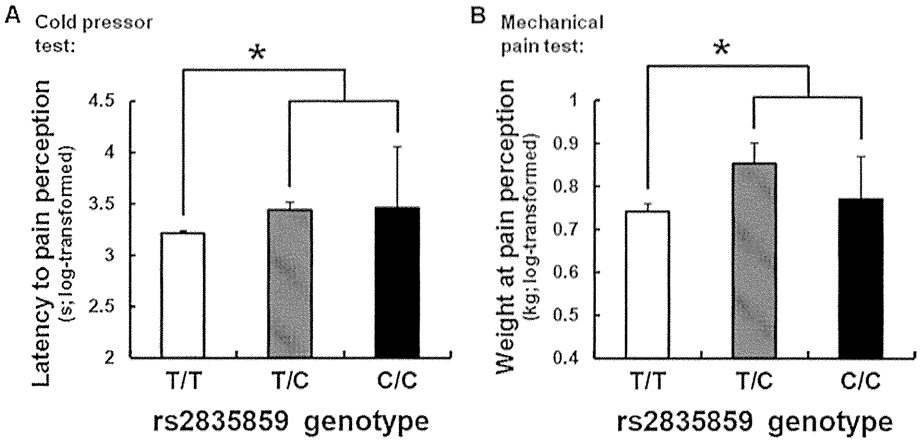

Fig. 2. Association analysis between basal pain sensitivity and the rs2835859 SNP. The results from the cold pressor-induced pain test (CPT) (A) and mechanically induced pain test (MPT) (B) are shown for the *KCNJ6* rs2835859 SNP. In panel A, *: significantly longer latency for the combined T/C and C/C genotype compared with T/T genotype. In panel B, *: significantly greater weight to perceive pain for the combined T/C and C/C genotype compared with the T/T genotype. The data are expressed as the mean ± S.E.M.

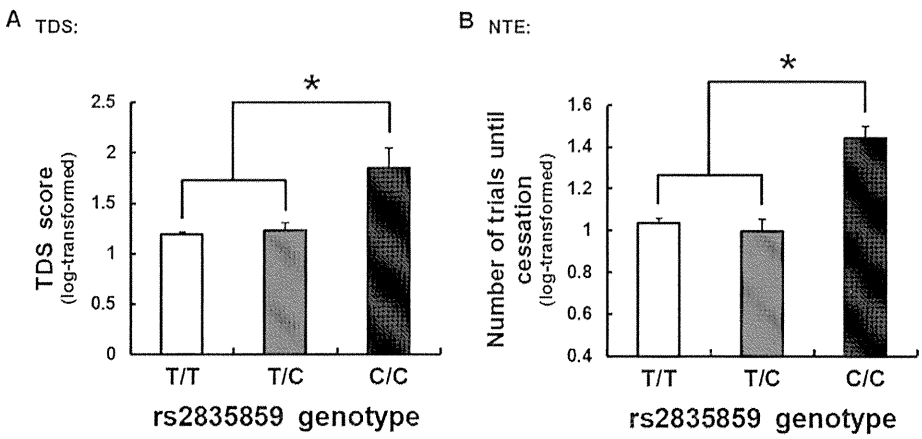

Fig. 3. Association analysis between susceptibility to nicotine dependence and the rs2835859 SNP. Tobacco Dependence Screener (TDS) scores (A) and the number of trials for smoking cessation in ex-smokers (NTE) (B) are shown for the *KCNJ6* rs2835859 SNP. In panel A, *: significantly greater score for the C/C genotype compared with the combined T/T and T/C genotype. In panel B, *: significantly greater number of trials for the C/C genotype compared with the combined T/T and T/C genotype. The data are expressed as the mean ± S.E.M.

transcendence (ST): $t_{495} = 0.037$, $P = 0.970$]. These results suggest that C allele carriers were less sensitive to both cold pressor–induced and mechanically induced pain. The number of subjects carrying the T/T, T/C, and C/C genotypes of this SNP was 436, 60, and 2, respectively, for the CPT data, and 435, 60, and 2, respectively, for the MPT data; and the distribution was not significantly different from the theoretical Hardy-Weinberg equilibrium values in the entire subjects used in the association analyses ($\chi^2 < 0.01$, $P = 0.97$, and $\chi^2 < 0.01$, $P = 0.96$, respectively).

Association between rs2835859 SNP and susceptibility to nicotine dependence

The results further suggested that the subjects with the C allele in the rs2835859 SNP required less analgesics and were less sensitive to cold and mechanical pain than the subjects without the allele, attributable to the increased effectiveness of not only exogenous but also endogenous opioids in both cohorts. Given the fact that the opioid system is involved in both analgesic and rewarding effects, one may hypothesize that increased opioid sensitivity reflects the increased rewarding effects of addictive substances or behaviors and greater liability to serious dependence. To test this hypothesis, we investigated the contribution of the rs2835859 SNP to the vulnerability to substance dependence in additional subjects with various smoking habits (Supplementary Table 3). In the association analysis of the TDS data, a significant difference was found between the combined T/T and T/C subgroup and C/C genotype subgroup in TDS scores, and homozygous C allele carriers had a higher TDS than non-carriers ($t_{510} = -2.130$, $P = 0.034$; Fig. 3A). A significant difference was found between the combined T/T and T/C subgroup and C/C genotype subgroup in the NTE, and homozygous C allele carriers had a higher NTE than non-carriers ($t_{382} = -1.948$, $P = 0.002$; Fig. 3B). However, no significant associations were found in the association analyses for smoking period, FTND, CPD, or NTC (Supplementary Table 5: available in the online version only). These results suggest that homozygous C allele carriers had higher

susceptibility to nicotine dependence and required a greater number of trials until they achieved successful smoking cessation. The number of subjects carrying the T/T, T/C, and C/C genotypes of this SNP was 443, 65, and 4, respectively, for the TDS scores, and 331, 49, and 4, respectively, for the NTE scores; and the distribution was not significantly different from the theoretical Hardy-Weinberg equilibrium values in the entire subjects used in the association analyses ($\chi^2 = 0.87$, $P = 0.35$, and $\chi^2 = 1.96$, $P = 0.16$, respectively).

Discussion

To our knowledge, the present study is the first to comprehensively explore SNPs of the *KCNJ6* gene with regard to associations between these SNPs and all of the phenotypes related to opioid actions such as outcomes in clinical pain management, basal pain sensitivity, and smoking behavior, simultaneously in humans. A novel result of the present study was that the rs2835859 was found to be potently associated with opioid analgesic sensitivity, in which carriers of the C allele of this SNP required less analgesics compared with non-carriers (Table 1, Fig. 1). The subsequent association study indicated that this SNP was also associated with sensitivity to two different pain modalities, in which carriers of the C allele of this SNP were less sensitive to both cold and mechanical pain (Fig. 2: A and B). The examination of patient subjects with clinical data related to smoking behavior indicated that homozygous carriers of the C allele of this SNP had higher susceptibility to nicotine dependence and required a greater number of trials to achieve successful smoking cessation (Fig. 3: A and B). This result appears to be consistent with previous studies, which demonstrated that nicotine-induced antinociception and rewarding effects were modulated by opioids (56 – 58), suggesting the involvement of opioid-cholinergic interactions (58). To our knowledge, the associations found between SNPs of the *KCNJ6* gene and nicotine dependence in the present study are novel findings in human studies that used various indices, including TDS and NTE. Altogether, the present results suggest that the rs2835859 SNP may affect individual differences in exogenous and endogenous opioid sensitivity. Carriers of the C allele, especially homozygous carriers, have higher sensitivity, and non-carriers have the opposite sensitivity, resulting in less postoperative analgesic requirements, less pain sensitivity, and a higher liability to develop nicotine dependence, possibly because of increased analgesic and rewarding effects of opioids. The decreased pain sensitivity observed in carriers of the C allele in the present study is not necessarily caused by the facilitation of endogenous opioid sensitivity. Such an effect could also be caused by other factors. For example, GIRK2 is activated with other $G_{i/o}$ protein–coupled receptors, including α_2-adrenergic (11) and 5-HT_{1A} receptors (12), which play pivotal roles in descending pain pathways. Furthermore, the decreased pain sensitivity associated with the rs2835859 SNP after cosmetic orthognathic surgery may reduce the doses of postoperative PCA-fentanyl that are necessary for adequate pain relief.

In the present study, differences existed in the models (i.e., dominant model and recessive model) used to find associations between the rs2835859 SNP and phenotypes (i.e., postoperative opioid use, pain sensitivity, nicotine dependence, and difficulty in quitting smoking). The differences in these models may suggest differences in the involvement of the genes that underlie various phenotypes, which was shown in a previous study that investigated the influence of the 118A > G polymorphism in the *OPRM1* gene, which encodes the human μ-opioid receptor gene (59). Patients homozygous for the variant G allele of this SNP needed more morphine to achieve pain control compared with individuals heterozygous and homozygous for the A allele. However, the patients heterozygous for the 118A > G polymorphism had significantly more pain than patients with other genotypes (59). Similarly, the required doses of postoperative PCA-fentanyl and basal pain sensitivity were reduced even in the T/C heterozygous subgroup, whereas the TDS score and NTE were changed only in the C/C homozygous subgroup and not in the T/C heterozygous subgroup in the present study. These findings suggest that the difference in nicotine dependence may not necessarily be caused by only alterations in the endogenous opioid system. Other reward-related G-protein–coupled receptors (GPCRs), such as dopamine D_2/D_4 receptors (10), may also be involved.

To investigate the susceptibility to nicotine dependence, we adopted several indices because associations between SNPs of the *KCNJ6* gene and nicotine dependence have not been previously reported in studies that used these indices. The lack of consistent associations across these nicotine-dependence phenotypes observed in the present study may be attributable to different characteristics between these indices. In our preliminary study, the intercorrelations between the FTND and TDS, between the FTND and CPD, and between the TDS and NTE were found to be significant (Kasai et al., in preparation). However, we did not find a significant association between the rs2835859 SNP and FTND. Although the most frequently used instrument is the FTND, it may have several limitations. For example, it fails to include important aspects of dependence as defined by the DSM-IV and ICD-10 (60, 61), and several

items of the FTND are difficult to apply in relatively light smokers (60). The TDS is reported to have better screening performance for ICD-10, DSM-III-R, and DSM-IV diagnoses than the Fagerström Tolerance Questionnaire (FTQ) (42), which is the previous version of the FTND. Thus, we consider that investigating the TDS and NTE as well as the other indices and conducting analyses of these various aspects of nicotine dependence are important.

We previously explored *KCNJ6* gene variations in the exon regions, exon-intron boundary regions (approximately 30 bp), and putative promoter regions (approximately 1.8 kbp) and found that the A1032G SNP (rs2070995) and a haplotype that consisted of two alleles of the A1032G and G-1250A SNPs (rs6517442) were significantly associated with postoperative analgesic requirements after major abdominal surgery (31). In the present study that more comprehensively targeted SNPs in and around the gene, the rs2070995 SNP was included in the investigated region but not incorporated into the LD1 – LD11 blocks (Supplementary Fig. 2). The rs6517442 SNP was included in the investigated region and tagged by the rs7275707 SNP, which was in absolute LD with the rs6517442 SNP ($r^2 = 1$; Supplementary Figs. 2 and 3). Although we distinctly examined the association between these SNPs and postoperative analgesic requirements after painful cosmetic surgery in the present study, no significant associations were observed in the dichotomous analysis or trichotomous analysis (rs2070995: $P = 0.662$; rs6517442 or rs7275707: $P = 0.866$). The present results indicate that these SNPs are not useful for predicting opioid analgesic sensitivity in the case of cosmetic orthognathic surgery, although these SNPs may be useful for predicting opioid analgesic sensitivity in the case of major abdominal surgery. Although the causal factors cannot be easily identified, differences in pain, innervated neurons by which pain signals are transmitted, the analgesics mainly used, and the required amount of analgesics may affect the degree of associations found in both studies.

To date, only a few studies have examined the relationship between genetic variations in GIRK channels and phenotypic differences related to opioid actions in humans. Most of these studies analyzed the *KCNJ6* gene (29 – 32). In a study that targeted over 300 candidate genes and analyzed 3713 SNPs in 1,050 cases and 879 controls of European ancestry, the rs6517442 SNP was found to be among the top candidates for an association with nicotine dependence (32). Although this SNP was not investigated for associations with nicotine dependence in the present study, such analyses would be worthwhile in future studies. Lotsch et al. reported a tendency toward less opioid analgesic effectiveness and addiction in the A/A genotype in the rs2070995 SNP (30), which was consistent with our previous study in terms of analgesia (31). In these studies, the rs2835859 SNP was not investigated. In another recent study, Bruehl et al. (29) revealed that eight *KCNJ6* SNPs were significantly associated with the pain-related phenotype in Caucasian patients who underwent total knee arthroplasty (TKA) with post-surgical oral opioid analgesic medication (29). However, these SNPs and other SNPs reportedly tagged by these SNPs were not included in our candidate SNPs in the exploratory stage of the present study (Supplementary Table 4). The results might suggest that the SNPs that greatly contributed to pain or analgesia may not be consistent for different phenotypic traits or populations. Therefore, evaluating potential SNPs with respect to each phenotype and population is important. Notably, both Bruehl et al. and the present study identified promising SNPs associated with pain-related phenotypes in the *KCNJ6* gene region. These results underscore the significant role played by this gene in the sensitivity to pain, which was previously shown in animal studies (4, 23). Moreover, the present study demonstrated that some *KCNJ6* SNPs also affected individual differences in the sensitivity to analgesia and susceptibility to dependence. In particular, to our knowledge, the present study is the first to find an association between the rs2835859 SNP and nicotine dependence evaluated by the TDS and NTE. Future studies should be performed to more clearly confirm the important contribution of *KCNJ6* SNPs to analgesia sensitivity and dependence susceptibility.

The best candidate SNP identified in the present study, rs2835859, is located in the third intronic region of the *KCNJ6* gene (Supplementary Table 4). This SNP is in absolute LD with the rs2835860 SNP and falls on the LD1 block (Supplementary Fig. 2). However, all of the SNPs in the LD1 block are located in intron 3 and are apparently not in strong LD with other SNPs located in exon or putative regulatory regions (Supplementary Table 4 and Supplementary Fig. 2), which excludes the possibility that phenotypic alterations related to the rs2835859 SNP found in the present study are attributable to alterations in the function or expression of KCNJ6 caused by other SNPs that are in strong LD with these SNPs in the LD1 block. Considering that intronic SNPs can affect enhancer or some other activities of the gene, future studies are needed to clarify the underlying mechanisms by which the effects of opioids are modulated by this SNP. Such studies are important because they will clarify the pharmacological and neurobiological mechanisms that underlie the associations found in the present study. Our findings

may provide some insights for future investigations.

Although the observed associations in the present study might be restricted to the Japanese population and the underlying mechanism remains to be fully elucidated, the present results indicate that the rs2835859 SNP may serve as a marker that predicts increased opioid sensitivity and open new avenues for the personalized treatment of pain and dependence.

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Conflicts of Interest

KI has received support from Eisai for a project unrelated to this research and speaker's fees from Taisho Pharmaceutical Co., Ltd. and Japan Tobacco, Inc. The authors declare no other conflict of interest.

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Involvement of Cholinergic System in Hyperactivity in Dopamine-Deficient Mice

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Dopaminergic systems have been known to be involved in the regulation of locomotor activity and development of psychosis. However, the observations that some Parkinson's disease patients can move effectively under appropriate conditions despite low dopamine levels (eg, kinesia paradoxia) and that several psychotic symptoms are typical antipsychotic resistant and atypical antipsychotic sensitive indicate that other systems beyond the dopaminergic system may also affect locomotor activity and psychosis. The present study showed that dopamine-deficient (DD) mice, which had received daily L-DOPA injections, could move effectively and even be hyperactive 72 h after the last L-DOPA injection when dopamine was almost completely depleted. Such hyperactivity was ameliorated by clozapine but not haloperidol or ziprasidone. Among multiple actions of clozapine, muscarinic acetylcholine (ACh) activation markedly reduced locomotor activity in DD mice. Furthermore, the expression of choline acetyltransferase, an ACh synthase, was reduced and extracellular ACh levels were significantly reduced in DD mice. These results suggest that the cholinergic system, in addition to the dopaminergic system, may be involved in motor control, including hyperactivity and psychosis. The present findings provide additional evidence that the cholinergic system may be targeted for the treatment of Parkinson's disease and psychosis.
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INTRODUCTION

Dopamine (DA) plays an essential role in brain functions, including motor control, reward, and psychosis (Calabresi and Di Filippo, 2008; Ziauddeen and Murray, 2010). Low levels of DA and the blockade of DA neurotransmission generally cause hypolocomotion. The progressive degeneration of nigrostriatal DA neurons is well known to be the major pathological characteristic of Parkinson's disease (PD). Medications for PD include the DA precursor L-dihydroxyphenylalanine (L-DOPA) that increases DA levels and enhances DA neurotransmission. In contrast, high levels of DA and enhanced DA neurotransmission generally cause hyperlocomotion that is considered to be relevant to psychosis. Some disorders, such as drug addiction and schizophrenia, have been reportedly associated with DA neuron activation in the mesocorticolimbic system (Lester *et al*, 2010). Almost all antipsychotic drugs block DA neurotransmission. However, several phenomena that are not consistent with this theory have been clinically reported. Effective movement in PD in certain situations (ie, kinesia paradoxia) indicates locomotor ability even at low DA levels. Typical antipsychotic drug-resistant and atypical antipsychotic drug-sensitive positive symptoms in schizophrenia indicate that at least a subgroup of positive symptoms is not treatable by the blockade of DA neurotransmission (Meltzer, 2013). To resolve this discrepancy, we investigated mice with extremely low levels of DA.

Dopamine-deficient (DD) mice were generated using a transgenic rescue approach, in which tyrosine hydroxylase (TH) expression in noradrenergic and adrenergic cells in mice that lacked TH expression was complemented by a specific DA β-hydroxylase gene promoter (Nishii *et al*, 1998). These mice exhibited a restoration of norepinephrine and epinephrine synthesis and prevention of the usual perinatal lethality and cardiac dysfunction observed in TH knockout mice. DD mice require daily administration of L-DOPA, the precursor of DA, to maintain feeding (Supplementary Figure S2; Szczypka *et al*, 1999; Zhou and Palmiter, 1995).

In the present study, we found hyperactivity in DD mice that could be ameliorated by the prototypical, atypical antipsychotic, clozapine.

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MATERIALS AND METHODS

Animals

DD mice were created as described previously (Nishii *et al*, 1998). Wild-type and DD mouse littermates from crosses of heterozygous/heterozygous DD mice on a C57BL/6J genetic background served as subjects. The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee (Animal Experimentation Ethics Committee of Tokyo Metropolitan Institute of Medical Science, Approval ID: 12–43), and all of the animals were cared for and treated humanely in accordance with our institutional animal experimentation guidelines. All of the mice were housed in an animal facility maintained at $23 \pm 1\,^{\circ}C$ and $55 \pm 5\%$ relative humidity under a 12 h/12 h light/dark cycle with lights on at 0800 h and off at 2000 h. Food and water were available *ad libitum*. As routine maintenance of the mutants, 50 mg/kg L-DOPA was intraperitoneally (i.p.) administered to DD mice daily. Before this study, we confirmed that daily L-DOPA injections did not affect locomotor activity in wild-type mice (Supplementary Figure S1). Thus, wild-type mice were not treated with L-DOPA in the following experiments. In the experiments, male and female mice, 10 to 27 weeks old, were examined.

Surgery and Microdialysis Procedures

The mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and stereotaxically implanted with microdialysis probes in the striatum (anterior, +0.6 mm; lateral, +1.8 mm; ventral, −4.0 mm from bregma) (Franklin and Paxinos, 1997). At 24 h after implantation, the dialysis experiments were performed in freely moving animals. Ringer's solution (145 mM NaCl, 3 mM KCl, 1.26 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 6.5) was perfused at a constant flow rate of 1 μl/min. Perfusates were directly injected into the high-performance liquid chromatography system every 10 min using an autoinjector (EAS-20, Eicom, Kyoto, Japan). DA and serotonin (5-HT) in the dialysate were separated using a reverse-phase ODS column (PP-ODS, Eicom) and detected with a graphite electrode (HTEC-500, Eicom). The mobile phase consisted of 0.1 M phosphate buffer (pH 5.5) that contained sodium decanesulfonate (500 mg/l), ethylenediaminetetraacetic acid (EDTA; 50 mg/l), and 1% methanol. Norepinephrine (NE) in the dialysate was separated using a reverse-phase ODS column (CA-5ODS, Eicom) and detected with a graphite electrode (ECD-300, Eicom). The mobile phase consisted of 0.1 M phosphate buffer (pH 6.0) that contained sodium octanesulfonate (400 mg/l), EDTA (50 mg/l), and 5% methanol. Acetylcholine (ACh) in the dialysate was separated using an analytical column (Eicompak AC-GEL, Eicom) before being entered into an enzyme column (AC-Enzympak, Eicom) that contained immobilized Ach esterase and choline oxidase that converts ACh to hydrogen peroxide. Hydrogen peroxide was detected by a platinum electrode (HTEC-500, Eicom). The mobile phase consisted of 0.05 M potassium bicarbonate buffer that contained sodium decanesulfonate (300 mg/l) and EDTA (50 mg/l). Microdialysis samples were collected every 10 min for the DA, 5-HT, and NE assays or 20 min for the ACh assay. Usually, perfusion was initiated 180 min before the collection of baseline samples. The basal levels of extracellular DA (DA_{ex}), 5-HT ($5\text{-}HT_{ex}$), NE (NE_{ex}), and ACh (ACh_{ex}) were obtained from average concentrations of three consecutive samples when they were stable. DA_{ex}, $5\text{-}HT_{ex}$, and NE_{ex} responses to drugs are expressed as a percentage of basal levels. The areas under the curve (AUCs) of DA_{ex}, $5\text{-}HT_{ex}$, and NE_{ex} during the 180 min period after drug administration were calculated as the effects of the drugs.

Locomotor Activity Measurements

Each mouse was exposed to an illuminated chamber (30 × 40 × 25 cm), and locomotor activity was measured with Supermex (Muromachi Kikai, Tokyo, Japan) and a sensor monitor mounted above the chamber. All counts were automatically summed and recorded every 10 min. After a 180-min habituation period, saline or drug was administered subcutaneously (s.c.), and locomotor activity was monitored continuously for 180 min. Locomotor tests with DD mice were performed 24 or 72 h after the last L-DOPA (50 mg/kg) injection. Each drug-treatment day was followed by at least a 2-week washout period, during which DD mice were treated only with L-DOPA (50 mg/kg), and wild-type mice remained untreated.

Catalepsy Test

The degree of catalepsy was measured by placing both forepaws on a horizontal bar, 4 cm above the tabletop. The time in seconds until the mouse took both forepaws off the bar was recorded, with a maximum cutoff time of 5 min.

Hindlimb Movement Recording

Before recording locomotion patterns, the mice were habituated to walk on a custom-made runway apparatus (length, 40 cm; width, 5 cm). The placement of all four paws was monitored by a mirror underneath the runway. The mirror was set a 45° angle from the vertical axis. To enable the observation of hindlimb movements, the fur on the hindlimb of each animal was shaved under isoflurane gas anesthesia (3% for induction, 1–2% for maintenance). Circular reflective markers (2 mm diameter) were precisely placed on the shaved skin of the right hindlimb at the iliac crest, greater trochanter (hip), knee, lateral malleolus (ankle), fifth metatarsophalangeal joint (MTP), and toe. After the mice were allowed to recover completely from anesthesia, they walked freely on the runway, and their locomotor movements were recorded at 200 frames/s using a high-speed digital image camera system (HAS-220, DITECT, Tokyo, Japan). The captured images were stored directly on a computer for later analysis.

Hindlimb Movement Analyses

Movement analyses were limited to the sagittal plane parallel to the direction of walking. For further details of analysis see Supplementary Information.

Whole-Genome Expression Analyses

The expression profiles of the genes related to ACh metabolism and signaling transduction pathways were analyzed using the Illumina iScan system with MouseRef-8 Expression BeadChips (Illumina, San Diego, CA, USA) that contain probes that detect over 24 000 transcripts. Total RNAs were isolated by Sepasol-RNA I super solution (Nacalai Tesque, Kyoto, Japan) from the striatum and globus pallidus in wild-type and DD mice. We used the area that includes the neostriatum, ventral striatum, and globus pallidus but not thalamus or hypothalamus in the present study. Total RNA (50 ng) was then applied to RNA amplification that was performed with the TargetAmp Nano-g Biotin-aRNA Labeling Kit (EPICENTRE Biotechnologies, Madison, WI, USA) according to the instructions of the manufacturer. Briefly, first-strand cDNA was synthesized with T7-oligo (dT) primer from total RNAs, and second-strand cDNA synthesis was then performed with synthesized first-strand cDNA. The *in vitro* transcription reaction was performed with the product of second-strand cDNA synthesis as the template for producing biotin-cRNAs by incorporating biotin-UTP into the RNA transcripts. Finally, 750 ng of biotin-cRNA was hybridized to the MouseRef-8 Expression BeadChips and then reacted with streptavidin-Cy3. The expression intensity of the transcripts on the BeadChips was detected with an Illumina iScan reader.

Mouse Brain Tissue Processing

Mouse brains were removed under deep anesthesia and divided sagittally. One hemisphere was snap-frozen in liquid nitrogen and stored at −80 °C for biochemical studies. The other hemisphere was fixed in Bouin's solution and embedded in paraffin for the preparation of 4 μm serial sections for analyses.

Immunoblot Analyses

Mouse brains were homogenized in lysis buffer that contained 1% Triton X-100 (Fujita *et al*, 2010) and centrifuged at 100 000 *g* for 30 min. The supernatants (10 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes. The membranes were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) plus 0.2% Tween-20, followed by incubation with primary antibodies in TBS that contained 3% BSA. After washing, the membranes were further incubated with second antibody conjugated with horseradish peroxidase in TBS (1 : 10 000). Finally, the target proteins were visualized with ECL prime (GE Healthcare, Piscataway, NJ, USA), followed by quantification using MultiGauge software (Fuji Film, Tokyo, Japan).

Immunohistochemistry

Immunoblot analysis and immunohistochemistry were performed as previously described (Fujita *et al*, 2010). For further details of immunoblot analysis and immunohistochemistry see Supplementary Information.

Drugs

The following drugs were used: L-DOPA (50 mg/kg; Sigma-Aldrich, St Louis, MO, USA), methamphetamine (METH; 1 mg/kg; Dainippon Sumitomo Pharma, Osaka, Japan), haloperidol (1 mg/kg; Sigma-Aldrich), clozapine (5 and 10 mg/kg; Toronto Research Chemicals, Toronto, ON, Canada), 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT; 1 mg/kg; Sigma-Aldrich), ritanserin (3 mg/kg; Sigma-Aldrich), prazosin hydrochloride (1 mg/kg; Sigma-Aldrich), pyrilamine maleate salt (20 mg/kg; Sigma-Aldrich), clonidine (0.1 mg/kg; Sigma-Aldrich), oxotremorine-M (0.1 mg/kg; Sigma-Aldrich), donepezil hydrochloride (3 mg/kg, Tokyo Chemical Industry, Tokyo, Japan), and ziprasidone hydrochloride monohydrate (3 mg/kg; Toronto Research Chemicals). L-DOPA was prepared as 1.4 mg/ml in a 2.5 mg/ml ascorbic acid solution dissolved in saline. Methamphetamine, 8-OH-DPAT, pyrilamine, clonidine, and oxotremorine-M were dissolved in saline. Haloperidol and ritanserin were dissolved in 0.8% lactic acid. Clozapine was dissolved in a minimum amount of 0.1 N HCl and diluted to the required doses with saline. Prazosin and donepezil were dissolved in water. Ziprasidone was prepared as a suspension in aqueous Tween-80 (1% v/v in distilled water). L-DOPA was administered i.p. in a volume of 35 ml/kg, and the other drugs were administered s.c. in a volume of 10 ml/kg.

Statistical Analyses

The analyses were performed using two-way analysis of variance (ANOVA). Individual *post hoc* comparisons were performed using Fisher's protected least significant difference (PLSD) test. Hindlimb movements, ACh level, and the expression of choline acetyltransferase (ChAT) were analyzed by Student's *t*-test or Welch's *t*-test. Values of $P<0.05$ were considered statistically significant. The data were analyzed using Statview J5.0 software (SAS Institute, Cary, NC, USA).

RESULTS

Remaining DA_{ex} in DD Mice 24 h after the Last L-DOPA Injection

To confirm the depletion of DA in DD mice, we first measured DA_{ex} levels in the striatum using *in vivo* microdialysis. Baseline DA_{ex} levels in the striatum were detected in DD mice 24 h after the last intraperitoneal (i.p.) injection of L-DOPA (50 mg/kg), although they were significantly lower than the levels in wild-type mice (Table 1). A s.c. injection of the psychostimulant methamphetamine (METH; 1 mg/kg), which induces DA outflow from DA

Table 1 The Baselines (fmol/10 min) of DA_{ex} and $5\text{-}HT_{ex}$ in the Striatum at 24 h after the Last L-DOPA (50 mg/kg) Injection

Genotype	*n*	DA	*n*	5-HT
Wild	26	39.23 ± 3.76	26	1.36 ± 0.12
DD	22	0.62 ± 0.12***	22	2.05 ± 0.15***

***$P<0.001$.

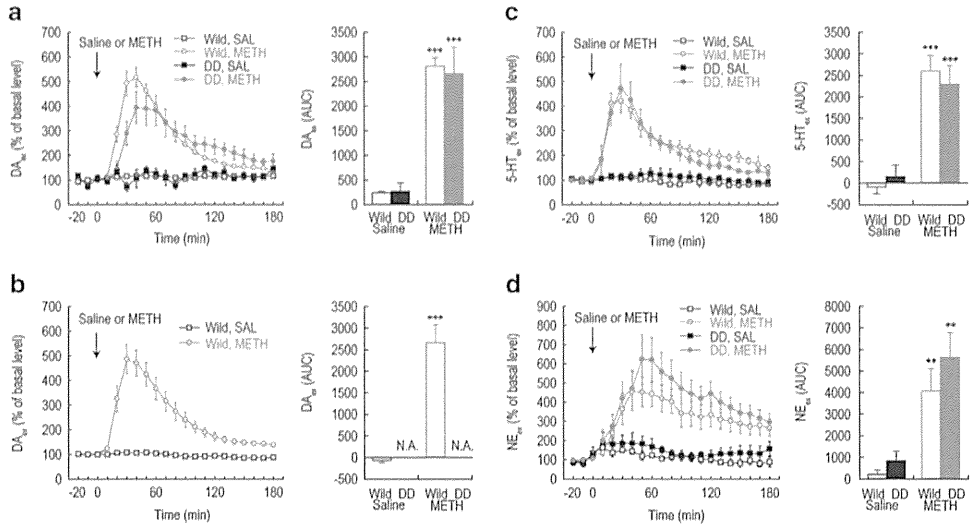

Figure 1 DA_{ex}, 5-HT_{ex}, and NE_{ex} in the striatum in DD mice. (a) METH-induced elevation of DA_{ex} levels in DD mice at 24 h after the last L-DOPA injection. ***$P<0.001$, compared with saline (SAL) group of the same genotype (two-way ANOVA followed by Fisher's protected least significant difference (PLSD) *post hoc* test). (b) METH-induced elevation of DA_{ex} levels in DD mice at 72 h after the last L-DOPA injection. Baseline DA_{ex} could not be detected in DD mice. One-way ANOVA revealed a significant effect of drug ($F_{1,9}=27.2431$, $P<0.001$). ***$P<0.001$; NA, not applicable. (c) METH-induced elevation of 5-HT_{ex} levels in DD mice at 72 h after the last L-DOPA injection. ***$P<0.001$, compared with saline group within the same genotype (two-way ANOVA followed by Fisher's PLSD *post hoc* test). (d) METH-induced elevation of NE_{ex} levels in DD mice at 72 h after the last L-DOPA injection. **$P<0.01$, compared with saline group within the same genotype (two-way ANOVA followed by Fisher's PLSD *post hoc* test). The figure shows the time course of DA_{ex}, 5-HT_{ex}, or NE_{ex} before and after a subcutaneous injection of saline (SAL) or METH (1 mg/kg). The arrow indicates the drug injection time. Each point represents the mean ± SEM of the percentage of baseline DA_{ex}, 5-HT_{ex}, or NE_{ex}. The histogram represents the mean AUC ± SEM of DA_{ex}, 5-HT_{ex}, or NE_{ex} during the 180 min period after the saline or METH injection ($n=4$–7).

Table 2 The Baselines (fmol/10 min) of DA_{ex}, 5-HT_{ex}, and NE_{ex} in the Striatum at 72 h after the Last L-DOPA (50 mg/kg) Injection

Genotype	*n*	DA	*n*	5-HT	*n*	NE
Wild	10	43.55 ± 4.80	10	1.03 ± 0.11	11	0.51 ± 0.07
DD	10	—	10	2.22 ± 0.28**	10	0.67 ± 0.09

**$P<0.01$.

nerve terminals via the DA transporter, markedly increased DA_{ex} levels in both DD mice and wild-type mice (Figure 1a). Two-way ANOVA revealed a significant effect of drug ($F_{1,18}=55.745$, $P<0.001$) but not genotype ($F_{1,18}=0.032$, $P=0.859$) and no drug × genotype interaction ($F_{1,18}=0.076$, $P=0.785$). L-DOPA significantly increased DA_{ex} levels, with a 459 ± 75% maximal increase in DD mice (Supplementary Figure S3a). The peak level of DA_{ex} was 5% of the level in wild-type mice after L-DOPA injection (Supplementary Figure S3b). L-DOPA did not apparently increase DA_{ex} levels in wild-type mice. These results suggest that DA systems may still partially function in DD mice 24 h after the last L-DOPA injection.

Depletion of DA_{ex} and Altered 5-HT_{ex} Level in DD Mice at 72 h after the Last L-DOPA Injection

To deplete DA, L-DOPA was withdrawn for 72 h. DA_{ex} levels in DD mice 72 h after the last L-DOPA injection were below the detection limit for DA (ie, 0.07 fmol/sample with a signal-to-noise ratio of 2 under our measurement conditions; Figure 1b). Baseline 5-HT_{ex} levels were significantly higher in DD mice than in wild-type mice (Table 2), consistent with the increases in 5-HT_{ex} levels observed in mice whose DA neurons were neonatally lesioned with 6-hydroxydopamine (Avale *et al*, 2004). Baseline NE_{ex} levels were not different between wild-type and DD mice (Table 2). METH (1 mg/kg) markedly increased 5-HT_{ex} and NE_{ex} levels in wild-type and DD mice (Figure 1c and d). Two-way ANOVA (drug × genotype) of 5-HT_{ex} revealed a significant effect of drug ($F_{1,15}=46.529$, $P<0.001$) but not genotype ($F_{1,15}=0.007$, $P=0.936$) and no drug × genotype interaction ($F_{1,15}=0.627$, $P=0.441$). Two-way ANOVA (drug × genotype) of NE_{ex} revealed a significant effect of drug ($F_{1,17}=22.154$, $P<0.001$) but not genotype ($F_{1,17}=1.423$, $P=0.249$) and no drug × genotype interaction ($F_{1,17}=0.248$, $P=0.625$). Baseline and METH-induced increases in NE_{ex} levels were not altered in DD mice. These results suggest that the DA system may lose functionality in DD mice 72 h after the last L-DOPA injection.

Locomotor Activity in DD Mice

We next examined locomotor activity in DD mice 24 and 72 h after the last L-DOPA injection (Figure 2a). Previous studies reported that DD mice were hyperactive immediately after an L-DOPA injection and hypoactive after 24 h withdrawal (Szczypka *et al*, 1999; Zhou and Palmiter, 1995). In the present study, locomotor activity in DD mice 24 h after the last L-DOPA injection was lower than in wild-type mice during the initial 3 h but higher than in wild-type mice during the subsequent 3 h period (Figure 2a). Furthermore, DD mice at 72 h after the last L-DOPA injection were slow to initiate movement but gradually increased their locomotor

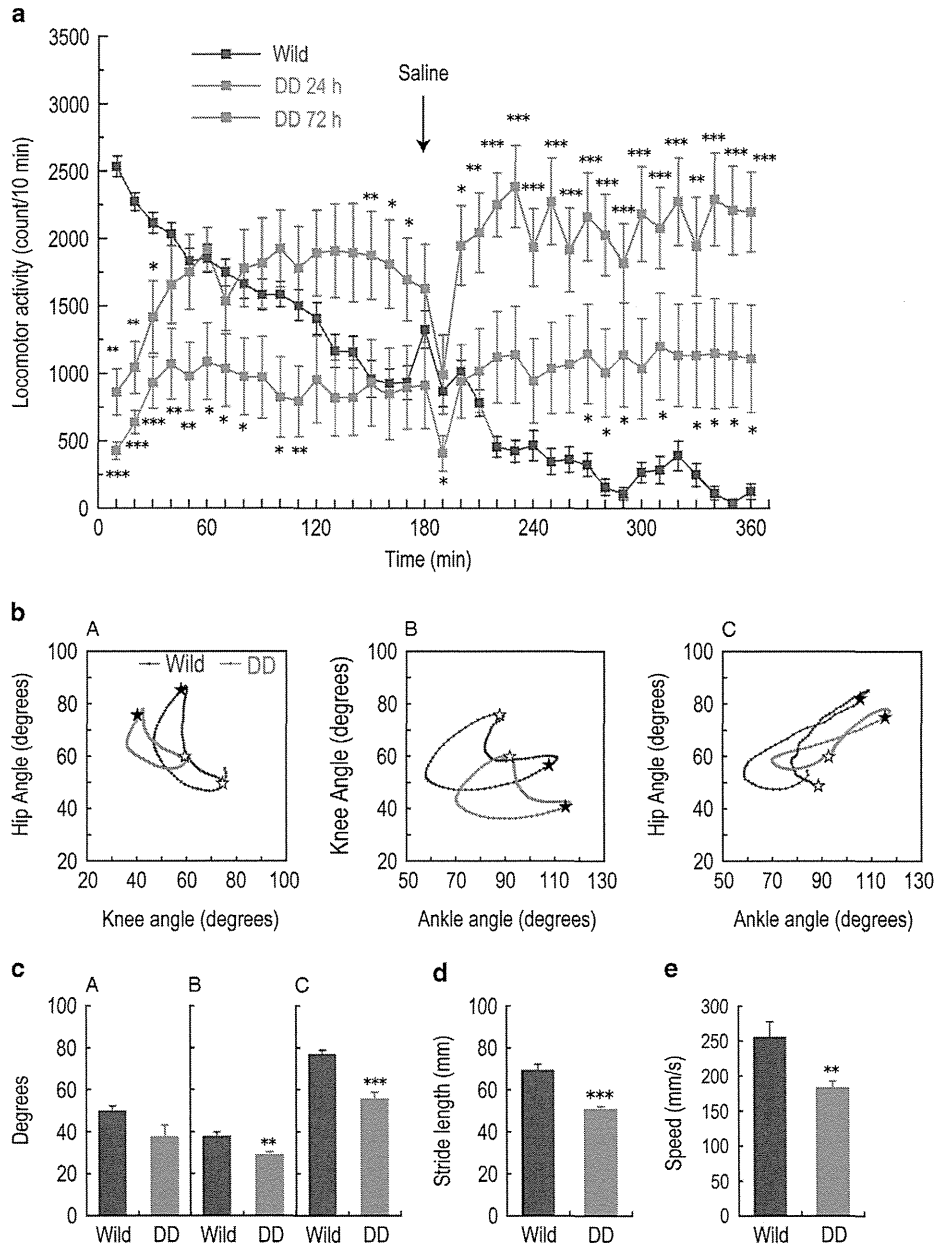

Figure 2 Locomotor activity and gait features in DD mice. (a) Locomotor activity in DD mice at 24 or 72 h after the last L-DOPA injection ($n = 10$–29). The arrow indicates the saline injection time. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with wild-type mice (Student's *t*-test). The data are expressed as mean ± SEM. (b) Interjoint coordination patterns during the step cycle period in wild-type mice ($n = 8$) and DD mice ($n = 9$). The angle–angle plots illustrate the averaged data for coordination between joints of the hindlimb. (A) Knee *vs* hip. (B) Ankle *vs* knee. (C) Ankle *vs* hip. White star, foot contact at the beginning of the stance phase. Gray star, foot lift at the beginning of the swing phase. (c) Range of angular joint excursions during the step cycle period in wild-type mice ($n = 8$) and DD mice ($n = 9$). (A) Hip. (B) Knee. (C) Ankle. The data are expressed as mean ± SEM. $^{**}P<0.01$, $^{***}P<0.001$, compared with wild-type mice (Student's *t*-test). (d, e) Temporal and spatial parameters of stride length (d) and walking speed (e) in wild-type mice ($n = 8$) and DD mice ($n = 9$) during locomotion. The data are expressed as mean ± SEM. $^{**}P<0.01$, $^{***}P<0.001$, compared with wild-type mice (Student's *t*-test).

activity for 30 min. The markedly increased locomotor activity was maintained throughout the following 5 h monitoring period (Figure 2a and Supplementary Movie S1). Wild-type mice exhibited a gradual reduction of locomotor activity during the 6 h period, suggesting habituation. Previous studies showed that DD mice displayed cataleptic behavior at 24 h after the last L-DOPA injection or in the juvenile stage without L-DOPA treatment (Nishii *et al*, 1998; Szczypka *et al*, 1999). In the present study, DD mice at 72 h after the last L-DOPA injection also displayed evident cataleptic behavior in the parallel bar test (Table 3 and Supplementary Movie S1). Although DD mice at 72 h after the last L-DOPA injection exhibited enhanced locomotor activity in the novel environment, they displayed a reduction of activity in the home cage (Supplementary Figure S4). In the juvenile stage, DD mice that were not

Table 3 Catalepsy in Wild-Type and DD Mice at 72 h after the Last L-DOPA (50 mg/kg) Injection

Genotype	n	Descent latency (s)
Wild	15	1.93 ± 0.41
DD	9	300<

treated with L-DOPA exhibited a reduction of spontaneous locomotor activity in the test cage (Nishii *et al*, 1998). Although locomotor activity in DD mice depended on experimental conditions, they first exhibited catalepsy and then hyperactivity in the novel environment.

Hindlimb Movement in DD Mice at 72 h after the Last L-DOPA Injection

As DA is considered to play a key role in gait, we performed a kinematic analysis of hindlimb movements in DD mice at 72 h after the last L-DOPA injection. Compared with wild-type mice, DD mice exhibited marked hyperflexion of the knee and reduced movement magnitude at not only the knee but also the ankle joints during the step cycle period (Figure 2b and c and Supplementary Figure S5). Stride length and speed in DD mice were significantly shorter and lower, respectively, than in wild-type mice, and the step cycle was not different between wild-type mice and DD mice (Figure 2d and e and Supplementary Figure S6). No differences were observed in iliac crest heights and greater trochanter heights in the step cycle between DD mice and wild-type mice (Supplementary Figure S5). The scatterplot of stride length *vs* walking speed in wild-type and DD mice indicated that for any given speed, stride length was shorter in DD mice than in wild-type mice (Supplementary Figure S6). Altogether, DD mice exhibited a slight kinematic abnormality of hindlimb movements, indicating that gait was characterized by reduced joint movements, consistent with previous studies in subjects with PD (Morris *et al*, 2005). Gait disturbances in the advanced stage of PD include festination, start hesitation, and freezing of gait (Devos *et al*, 2010; Giladi, 2001). In PD patients, the spatial and temporal parameters of stable gait are characterized by a reduction of gait speed, stride length, and swing phase duration, together with an increase in cadence (Devos *et al*, 2010; Morris *et al*, 1994). Start hesitation, hyperactivity, and gait hypokinesia in DD mice at 72 h after the last L-DOPA injection resembled the key features of PD patients, although the phenotypes in DD mice were relatively mild compared with PD patients.

Stimulant and Antipsychotic Effects on Hyperactivity in DD Mice at 72 h after the Last L-DOPA Injection

To examine whether pharmacological treatment ameliorates abnormal behavior in DD mice, the psychostimulant METH, the typical antipsychotic drug haloperidol, and the prototypical, atypical antipsychotic drug clozapine were administered s.c. at 3 h after placing the mice in a novel environment. Methamphetamine (1 mg/kg) increased locomotor activity in wild-type mice but did not produce alterations in DD mice (Figure 3a). Haloperidol (1 mg/kg) and ziprasidone (3 mg/kg) markedly decreased locomotor activity in wild-type mice but not in DD mice (Figure 3b and Supplementary Figure S7). In contrast, clozapine (5 and 10 mg/kg) decreased locomotor activity in wild-type and DD mice (Figure 3c). Two-way ANOVA (genotype × drug) of locomotor activity revealed significant effects of genotype ($F_{1,72} = 92.543$, $P < 0.001$) and drug ($F_{4,72} = 59.581$, $P < 0.001$) and a genotype × drug interaction ($F_{4,72} = 16.598$, $P < 0.001$; Figure 3d). Methamphetamine and haloperidol did not affect hyperactivity in DD mice, suggesting that this hyperactivity was DA independent.

Clozapine has high affinity for the 5-HT_{2A}, 5-HT_{2C}, D_4, muscarinic 1, and α_1-adrenergic receptors but weak affinity for the D_2 receptor (Arnt and Skarsfeldt, 1998). We examined which receptors are involved in the effects of clozapine in DD mice. The doses of the 5-HT_{1A} receptor agonist 8-OH-DPAT (1 mg/kg), 5-$HT_{2A/2C}$ receptor antagonist ritanserin (3 mg/kg), α_1 receptor antagonist prazosin (1 mg/kg), α_2 receptor agonist clonidine (0.1 mg/kg), histamine H_1 receptor antagonist pyrilamine (20 mg/kg), muscarinic receptor agonist oxotremorine-M (0.1 mg/kg), and cholinesterase inhibitor donepezil (3 mg/kg) have been shown to decrease locomotor activity in wild-type mice (Berendsen and Broekkamp, 1990; Heal and Philpot, 1987; Ninan and Kulkarni, 1998; Salomon *et al*, 2007). Locomotor activity was markedly decreased by oxotremorine-M and donepezil, whereas it was partially decreased by ritanserin, clonidine, and pyrilamine and not at all by 8-OH-DPAT or prazosin (Figure 3e). Two-way ANOVA (genotype × drug) of locomotor activity revealed significant effects of genotype ($F_{1,74} = 243.674$, $P < 0.001$) and drug ($F_{6,74} = 50.035$, $P < 0.001$) and a significant genotype × drug interaction ($F_{6,74} = 19.677$, $P < 0.001$). These results suggest that muscarinic receptor activation is mainly involved in the effects of clozapine on hyperactivity in DD mice.

ACh_{ex} Levels, ChAT Gene Expression, and ChAT Protein Levels in DD Mice at 72 h after the Last L-DOPA Injection

To examine biochemical alterations in the cholinergic system in DD mice, we measured the gene expression of ACh synthase ChAT in the striatum and globus pallidus, cerebral cortex, and brainstem and found that the intensity of ChAT gene expression in the striatum and globus pallidus was significantly lower in DD mice than in wild-type mice at 72 h after the last L-DOPA injection (Figure 4a). Furthermore, baseline ACh_{ex} levels in the striatum and ChAT protein expression in the striatum were significantly lower in DD mice than in wild-type mice at 72 h after the last L-DOPA injection (Figure 4b and c). ChAT-positive cells were reduced in DD mice compared with wild-type mice (Figure 4d).

DISCUSSION

Locomotor activity and risk for psychosis were previously considered to decrease when DA levels decrease. In the present study, we found that animals with extremely low levels of DA could move effectively and were hyperactive (Figure 5). The hyperactivity may be relevant to kinesia paradoxia, a condition in which individuals with PD who typically experience severe difficulties with even simple

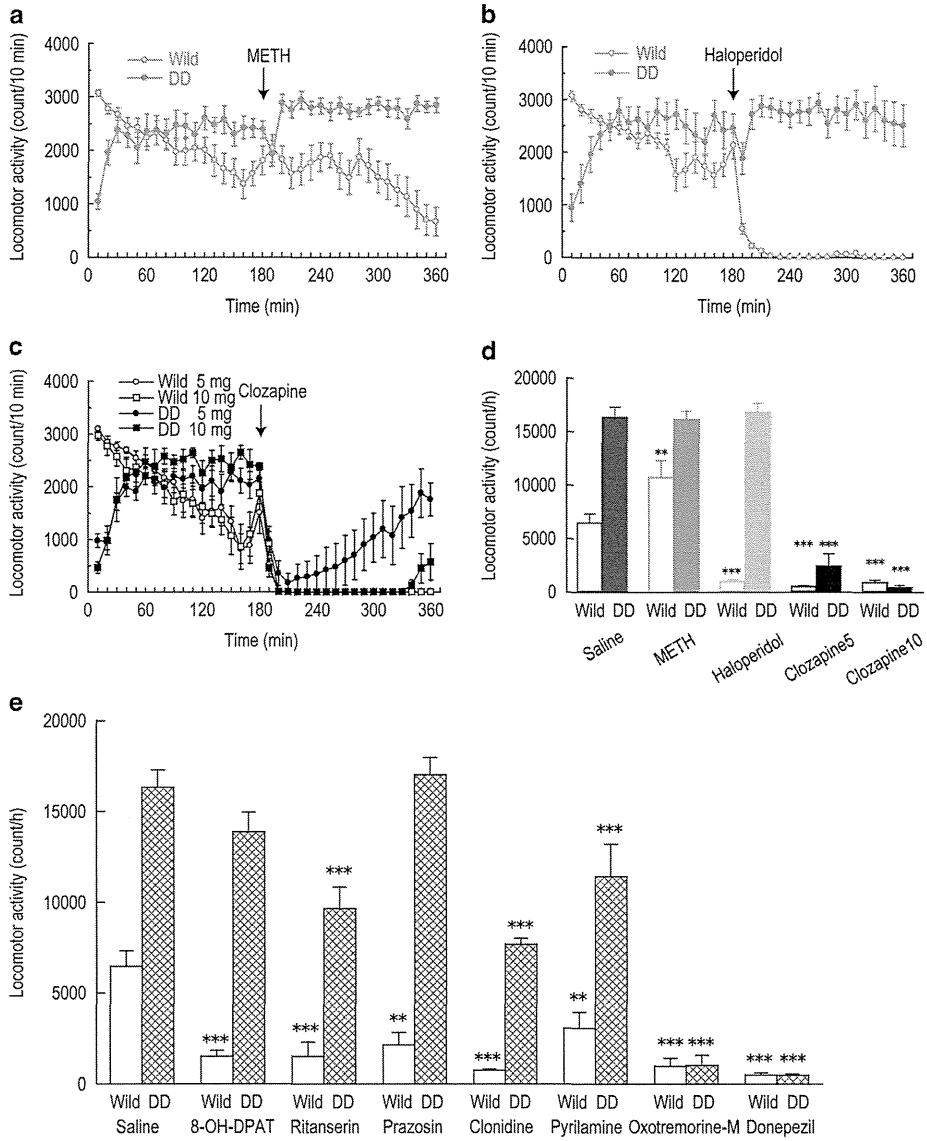

Figure 3 Stimulant and antipsychotic effects on hyperactivity in DD mice. (a–c) Locomotor responses to (a) METH (1 mg/kg) in wild-type mice ($n=10$) and DD mice ($n=11$), (b) haloperidol (1 mg/kg) in wild-type mice ($n=9$) and DD mice ($n=8$), and (c) clozapine (5 and 10 mg/kg) in wild-type mice (5 mg/kg, $n=7$; 10 mg/kg, $n=5$) and DD mice (5 mg/kg, $n=6$; 10 mg/kg, $n=5$). The arrows indicate the saline and drug injection time. (d) Locomotor activity after saline, METH, haloperidol, and clozapine administration. ***$P<0.001$, compared with saline treatment within the same genotype (two-way ANOVA followed by Fisher's PLSD *post hoc* test). (e) Locomotor activity after 8-OH-DPAT, ritanserin, prazosin, clonidine, pyrilamine, oxotremorine-M, and donepezil administration. **$P<0.01$, ***$P<0.001$, compared with saline treatment within the same genotype (two-way ANOVA followed by Fisher's PLSD *post hoc* test; $n=4$–11).

movements may perform complex movements easily, although the mechanism is unknown (Au *et al*, 2010). Although DD mice are not a precise model of PD, they may partially reflect late-stage PD. Interestingly, hyperactivity in DD mice was ameliorated by clozapine. Because clozapine also suppressed locomotor activity in wild-type mice, the effect of the drug on motor activity may not be specific to DD mice. Remarkably, however, clozapine but not haloperidol or ziprasidone ameliorated hyperactivity in DD mice.

Among the various effects of clozapine, 5-HT_{2A} antagonism and 5-HT_{1A} agonism are known to be critical elements of the antipsychotic and motor-sparing effects (Meltzer and Huang, 2008). In the present study, the 5-$HT_{2A/2C}$ receptor antagonist ritanserin partially ameliorated hyperactivity in DD mice. In light of the enhanced serotonergic activity in the DD mice reported herein (Tables 1 and 2), 5-HT_{2A} inverse agonists or 5-HT_{1A} partial agonists, both of which have shown promising results in treating behavioral and motor abnormalities in PD, should be further investigated in kinesia paradoxia (Huot *et al*, 2011; Meltzer *et al*, 2010).

Clozapine is well known to show improvements in schizophrenia patients with typical antipsychotic resistance. Several lines of evidence suggest that the effect of clozapine on muscarinic receptors may be important for the treatment of psychosis in patients with schizophrenia (Barak, 2009;

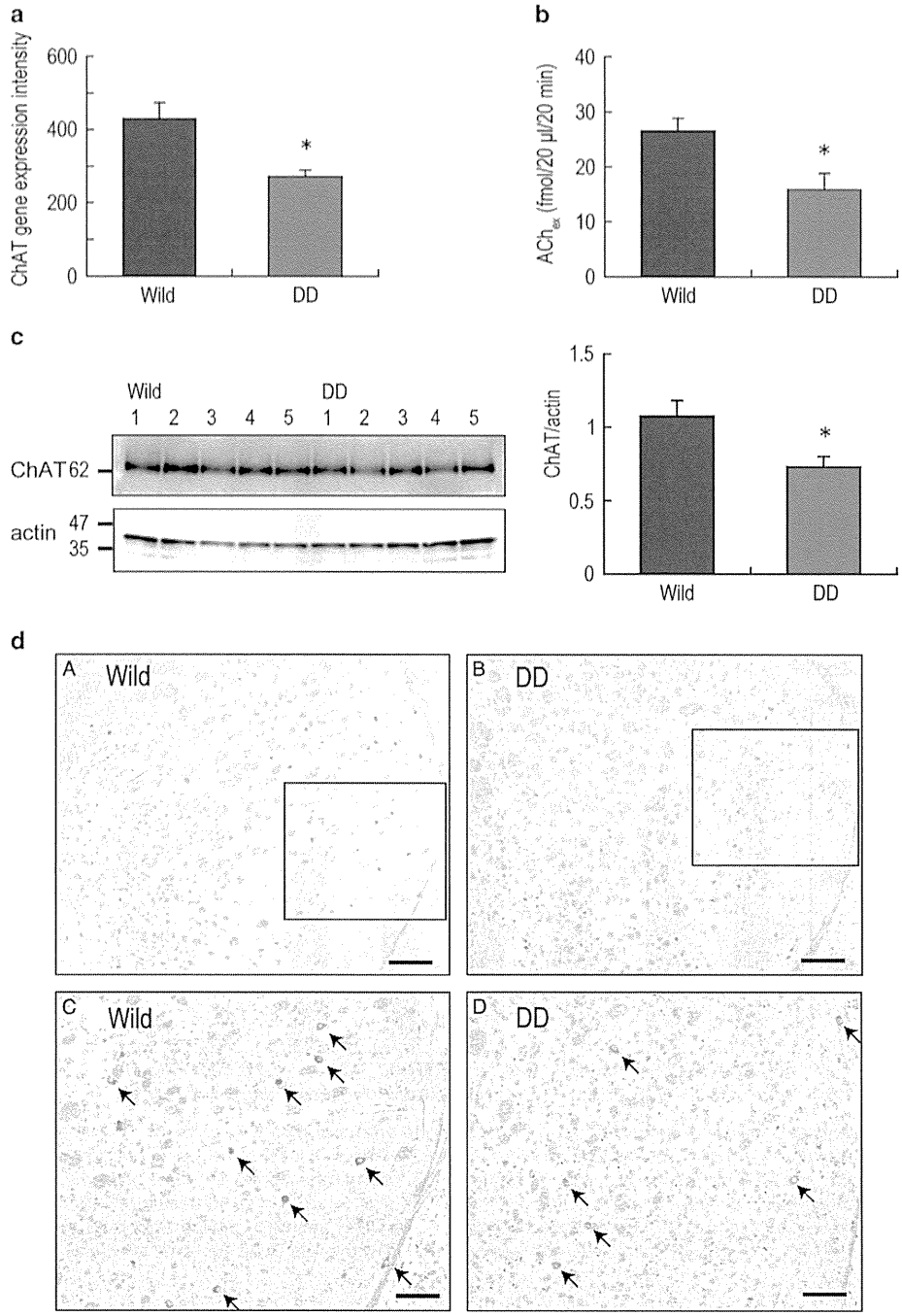

Figure 4 Reduced ACh_{ex} levels, ChAT gene expression, and ChAT protein levels in DD mice. (a) ChAT gene expression intensity in the area of the neostriatum, ventral striatum, and globus pallidus. The data are expressed as mean ± SEM. $^*P<0.05$, compared with wild-type mice (Student's *t*-test). (b) Baseline ACh_{ex} levels in the striatum in DD mice. The data are expressed as mean ± SEM. $^*P<0.05$, compared with wild-type mice (Student's *t*-test). (c) Immunoblot analysis of ChAT protein expression in the striatum. Representative blots and data quantified against β-actin bands are shown as mean ± SEM. $^*P<0.05$, compared with wild-type mice (Student's *t*-test). (d) Immunohistochemical analysis of ChAT. The two lower panels (C, D) are insets from the two upper panels (A, B). (A, C) Wild-type mice. (B, D) DD mice. Scale bars = 200 µm (A, B) and 100 µm (C, D). Arrows indicate ChAT-positive cells.

Raedler *et al*, 2007, McKinzie and Bymaster, 2012). Hyperactivity that is induced by muscarinic receptor antagonists has been suggested to model antimuscarinic psychosis and cholinergic-related psychosis in schizophrenia (Yeomans, 1995). Similarly, hyperactivity in DD mice was ameliorated by a muscarinic receptor agonist and cholinesterase inhibitor, suggesting that such hyperactivity might be induced by similar mechanisms that underlie cholinergic-related psychosis in schizophrenia.

DD mice exhibited a decrease in ChAT expression, with a reduction of extracellular ACh levels in the striatum, demonstrating that the cholinergic system was suppressed