sample was used. To explore the association between the SNPs and phenotype, Student's *t*-test or analysis of variance was performed, in which Bonferroni correction for multiple comparisons was used as the *post hoc* test. For these analyses, the relative expression level and genotype data of each SNP were incorporated as dependent and independent variables, respectively.

For all of the statistical analyses described above, SPSS 18.0J for Windows (International Business Machines Corporation, Armonk, NY, USA), gPLINK v. 2.050, PLINK v. 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/; accessed 1 March 2012),[26] and Haploview v. 4.1[27] were used. The criterion for significance was set at $P<0.05$, with the exception of the GWAS. Statistical corrections for multiple tests, such as Bonferroni adjustments on the multiple parameters analyzed, were not performed in the present exploratory study after the GWAS because it would be too conservative for genetic association studies,[28] meaning that the likelihood of type II errors is increased by Bonferroni adjustments, and truly important differences would be deemed nonsignificant.[29]

Additional *in silico* analysis

Additional *in silico* analyses and the Internet links for the websites of the databases referenced are provided in the Supplementary Information.

RESULTS

Identification of a potent locus associated with human opioid sensitivity by GWAS in subjects who underwent painful cosmetic surgery

We first explored the association between genetic variations and opioid sensitivity in a total of 353 healthy subjects who were scheduled to undergo cosmetic orthognathic surgery (mandibular sagittal split ramus osteotomy) for mandibular prognathism that involved the administration of opioid analgesics (Supplementary Table S1), in which the surgical procedure was uniform and thus the invasiveness and resultant pain would be regarded as homogeneous among the subjects. A GWAS was conducted as a consecutive three-stage analysis to identify potent SNPs associated with the requirements for an opioid analgesic, fentanyl ($\mu g\,kg^{-1}$), during the 24-h postoperative period (Supplementary Figure S1). Consequently, 9, 12 and 10 SNPs were selected as the top candidates for additive, dominant and recessive models for each minor allele, respectively, after the final stage (Supplementary Tables S2–4). Among these, several SNPs mapped to 2q33.3–2q34 showed significant associations after the final stage with 24-h postoperative fentanyl requirements in the additive and recessive models (additive model: combined $\beta=0.293$, nominal $P=8.044\times10^{-7}$; recessive model: combined $\beta=0.553$, nominal $P=9.382\times10^{-7}$; Supplementary Tables S2–4). The observed *P*-values of these SNPs, calculated as $-\log10$ (*P*-value), obviously deviated from the expected values from the null hypothesis of uniform distribution in the quantile–quantile plot for the entire sample (Supplementary Figure S2). The genes located in this region were found to include *METTL21A* (*FAM119A*) and *CREB1*, encoding methyltransferase like 21A and cyclic adenosine 3′,5′-monophosphate (cAMP) responsive element-binding protein 1 (CREB1), respectively.

For further fine mapping of this region, we used the remainder of the genotyped data and imputed the genotype data from the SNPs that spanned 300 kbp, including both the *METTL21A* and *CREB1* genes (Figure 1a), and analyzed the association. We then identified several other SNPs around the best candidate SNP, rs2952768, with nominal *P*-values from 1×10^{-6} to 1×10^{-4} (Figure 1b). After the LD analysis, all of these SNPs were found to be included in an LD block that spanned approximately 134 kbp and were in strong LD ($D'=1$, $r^2>0.8$) with rs2952768 (Figure 1c). Total fentanyl use during the 24-h postoperative period was 1.080 ± 0.053, 1.146 ± 0.049 and $1.666\pm0.083\,\mu g\,kg^{-1}$ (log transformed; mean ± s.e.m.) in subjects with the T/T, T/C and C/C genotypes, respectively (Figure 2a).

Association of rs2952768 SNP with sensitivity to opioid analgesics in patients who underwent major open abdominal surgery

To examine whether the SNPs identified in our GWAS generally affect individual differences in opioid sensitivity, we attempted to confirm the association between the rs2952768 SNP and postoperative opioid requirements in another cohort that underwent a different surgical procedure. The subjects recruited in this study were 112 patients who underwent major open abdominal surgery under combined general and epidural anesthesia (Supplementary Table S5),[18] mostly gastrectomy for gastric cancer and colectomy for colorectal cancer, which involves different modes of invasion from the orthognathic surgery and might cause different pain modalities. Appropriate doses of analgesics, mainly opioids such as morphine, buprenorphine, pentazocine and pethidine, were administered as rescue analgesics at the discretion of the surgeons whenever the patients complained of significant postoperative pain during the postoperative period, and the total dose administered was estimated for the association analysis. As a result, a significant difference in postoperative analgesic requirements was found between the subjects with the combined T/T and T/C genotype and subjects with the C/C genotype in the rs2952768 SNP. Interestingly, the subjects with the C/C genotype required significantly more analgesics than the subjects with the combined T/T and T/C genotype in the rs2952768 SNP ($t_{110}=-2.340$, $P=0.021$), a pattern similar to the one observed in the subjects who underwent cosmetic orthognathic surgery. Total analgesic use, equipotent with systemic fentanyl, during the 24-h postoperative period was 0.359 ± 0.073, 0.397 ± 0.068 and $0.741\pm0.169\,\mu g\,kg^{-1}$ (log transformed; mean ± s.e.m.) in the subjects with the T/T, T/C and C/C genotypes, respectively (Figure 2b).

Association of rs2952768 SNP with severity of drug dependence in patients in several cohorts

The results suggested that the subjects with the C/C genotype in the rs2952768 SNP required more analgesics than the subjects with the other genotypes, attributable to the decreased effectiveness of opioid analgesics in both cohorts. Given the fact that the opioid system is involved in both rewarding and analgesic effects, one could assume that decreased opioid sensitivity may reflect the decreased rewarding effects of various drugs or behaviors and less liability to serious dependence. To test this hypothesis, we investigated the contribution of the rs2952768 SNP to the vulnerability to substance dependence in additional subjects with METH dependence, alcohol dependence and eating disorders (Supplementary Tables S6–8). In the initial case–control analyses, no associations in the genotypic and allelic distribution of this SNP were found between the subjects with psychiatric disorders and corresponding control subjects (Supplementary Table S9). However, a significant difference in the genotypic distribution was found between the absent and present subgroups of polydrug use among the patients with METH dependence ($\chi^2=3.979$, $P=0.046$). Indeed, fewer polydrug abusers carried the C/C genotype compared with monodrug users (Table 1). A similar result was found in the patients with alcohol dependence. A significant difference in the genotypic distribution was found between the absent and present subgroups of drug use among patients with alcohol dependence ($\chi^2=3.860$, $P=0.049$, and $\chi^2=3.039$, $P=0.097$, in the additive and recessive models, respectively), and a lower proportion of drug abusers carried the C allele or C/C genotype compared with the alcoholics without drug abuse (Table 1). Furthermore, a significant difference in allelic distribution was found between the absent and present subgroups of comorbid dependence among the patients with eating disorders ($\chi^2=3.985$, $P=0.046$, and $\chi^2=4.488$, $P=0.034$, in the additive and dominant models, respectively), and a lower proportion of patients with drug dependence carried the C allele

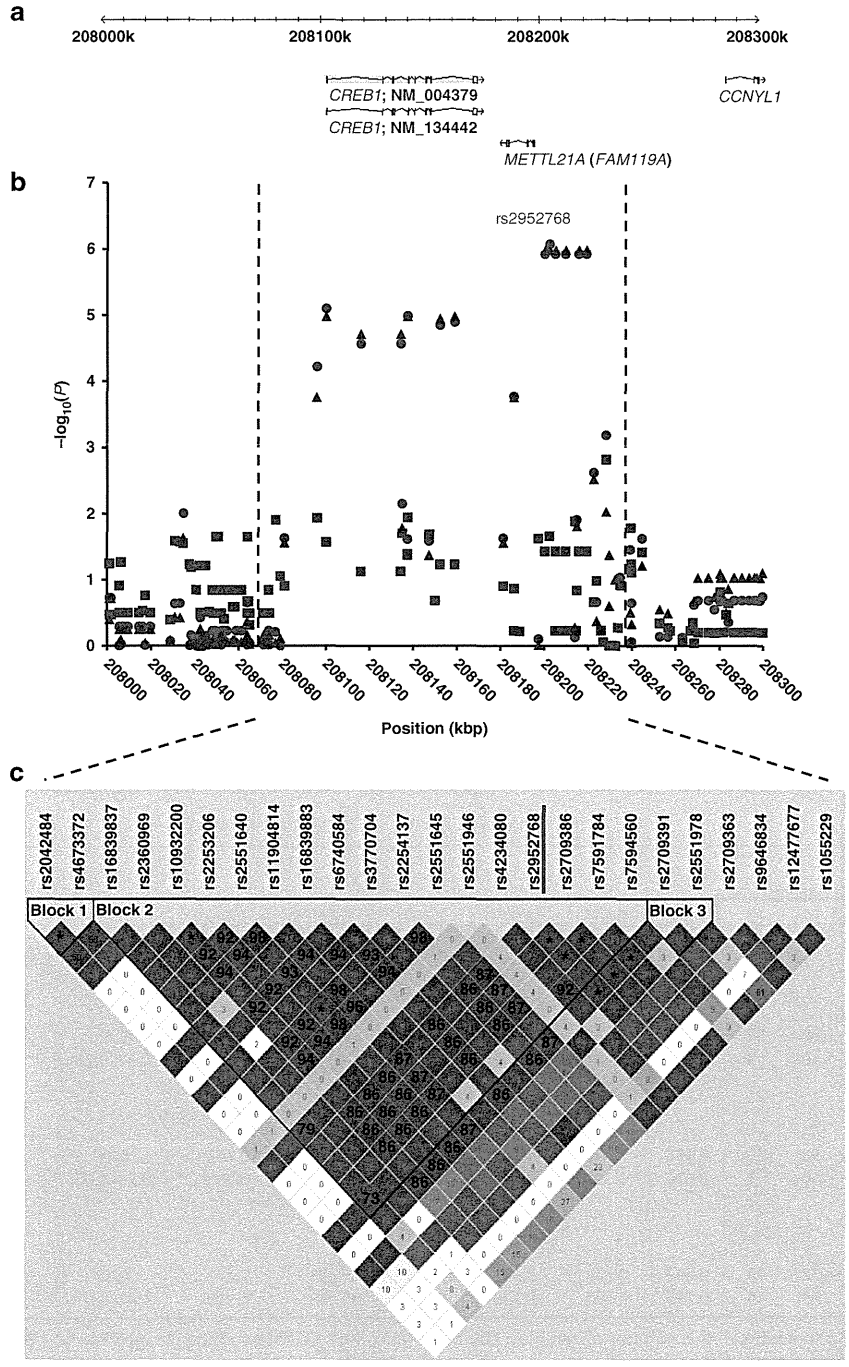

Figure 1. Candidate locus possibly associated with human opioid sensitivity. (**a**) Illustration of the genes in the genomic region from position 208 000 000 to 208 300 000 on chromosome 2 in the HapMap database (http://hapmap.ncbi.nlm.nih.gov/index.html.ja; accessed 1 March 2012). (**b**) Fine mapping of the candidate region after the imputation-based association analysis. The circle, square and triangle plots represent the results from the additive, dominant and recessive models, respectively. The area between the dotted vertical lines represents the genomic position from 208 070 000 to 208 240 000 on chromosome 2. (**c**) State of linkage disequilibrium (LD) between the SNPs in the genomic position from 208 070 000 to 208 240 000 on chromosome 2, based on the genotype data of the subjects who are derived from the Japanese population and underwent cosmetic orthognathic surgery. Numbers in squares in which two SNPs face represent the percentage of the r^2 values calculated from the genotype data of the SNPs. Squares with asterisks represent $r^2 = 1$. Only the values > 0.70 are highlighted.

compared with the patients without drug dependence (Table 1). Although nonsignificant, likely because of the small sample size, a marginal difference was observed in allelic distribution between the absent ($\chi^2 = 3.780$, $P = 0.052$) and present subgroups with a comorbid alcoholic state among the patients with eating disorders, and a lower proportion of patients with an alcoholic state carried the C allele compared with the patients without an alcoholic state (Table 1). Altogether, these results showed that carriers of the C allele among the patients with psychiatric disorders, especially the C/C genotype, tended not to abuse polydrugs and not have comorbid alcohol or drug dependence. Although the association was nonsignificant in the recessive

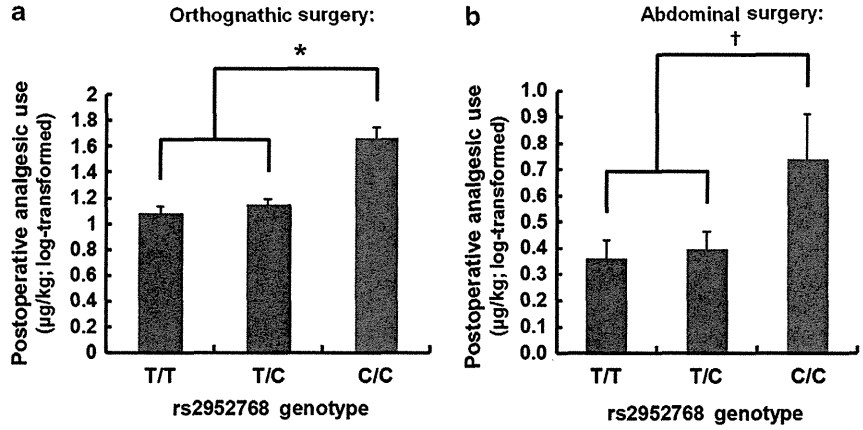

Figure 2. Association analysis between opioid analgesic requirements and the rs2952768 SNP. (**a**) Total dose of analgesics administered per body weight ($\mu g\,kg^{-1}$; log transformed) during the 24-h postoperative period after cosmetic orthognathic surgery (mandibular sagittal split ramus osteotomy). (**b**) Total dose of analgesics administered per body weight ($\mu g\,kg^{-1}$; log transformed) during the 24-h postoperative period after major open abdominal surgery. *$Q<0.05$, greater dose of analgesic administered in the C/C genotype compared with the T/C and T/T genotypes with genome-wide significance; †$P<0.05$, greater dose of analgesic administered in the C/C genotype compared with the T/C and T/T genotypes with nominal significance. The data are expressed as mean ± s.e.m.

Table 1. Distribution of genotypes of the rs2952768 polymorphism and odds ratio between the patient subgroups based on clinical status

	n	*Genotype*			*Allele frequency*		χ^2				*OR (95% CI)*		
		T/T	*T/C*	*C/C*	*T*	*C*	*P*				*C allele (vs T)*	*C/C + T/C genotype (vs T/T)*	*C/C genotype (vs T/T + T/C)*
							Genotype	*Allele*	*Dominant*	*Recessive*			
Polydrug use													
In METH dependence/psychosis patients													
Absence	53	19	22	12	0.556	0.444	3.979	2.262	0.450	3.979	0.71	0.80	0.44
Presence	141	58	67	16	0.644	0.356	0.137	0.133	0.503	0.046*	(0.45–1.11)	(0.42 – 1.54)	(0.19–1.00)
Drug use													
In alcohol dependence patients													
Absence	391	172	166	53	0.652	0.348	3.908	3.860	2.180	3.039	0.61	0.63	0.30
Presence	45	25	18	2	0.756	0.244	0.142	0.049*	0.140	0.097§†	(0.37–1.00)	(0.34–1.17)	(0.07–1.26)
Drug dependence													
In eating disorder patients													
Absence	200	85	93	22	0.658	0.343	4.552	3.985	4.488	0.793	0.45	0.37	0.40
Presence	21	14	6	1	0.810	0.190	0.103	0.046*	0.034*	0.705§	(0.20–1.00)	(0.14–0.96)	(0.05–3.16)
Alcoholic state													
In eating disorder patients													
Absence	151	61	72	18	0.642	0.358	3.995	3.780	3.731	1.171	0.65	0.57	0.57
Presence	70	38	27	5	0.736	0.264	0.136	0.052†	0.053†	0.279	(0.41–1.01)	(0.32–1.01)	(0.20–1.60)

Abbreviations: METH, methamphetamine; *n*, the number of samples; OR, odds ratio; 95% CI, 95% confidence interval. *$P<0.05$, †$0.05 \leqslant P<0.1$, §*P*-value from Fisher's exact test was presented instead of that from χ^2 test.

model for the C allele in all of the analyses, possibly because of a lack of statistical power caused by the limited sample size, the present results suggest that carriers of the C allele in this SNP have less inclination to abuse drugs, consistent with our assumption that various drugs of abuse have decreased rewarding effects in subjects with decreased opioid sensitivity, making these subjects less liable to the expression of symptoms of serious dependence.

Association of rs2952768 SNP with 'reward dependence' score on a personality questionnaire in healthy subjects

Another interest is whether this SNP also affects personality traits related to the reward system in healthy people. To address this issue, we investigated the association between this SNP and data from the TCI, a personality profiling questionnaire, in healthy volunteers (Supplementary Table S10). Intriguingly, among the seven dimensions of the TCI, a significant association was found only for reward dependence (RD) (novelty seeking: $\beta = -0.009055$, $P=0.2995$; harm avoidance: $\beta=0.004317$, $P=0.6819$; RD: $\beta=-0.0175$, $P=0.03265$; persistence: $\beta=-0.007554$, $P=0.6295$; self-directedness: $\beta=-0.01274$, $P=0.1709$; cooperativeness: $\beta=-0.004122$, $P=0.5302$; self-transcendence: $\beta=2.68E-06$, $P=0.9998$; Supplementary Figure S3). The RD value decreased as the copy number of the carried C allele increased among the subjects. The RD value on the TCI was previously shown to be positively correlated with activity of the caudate head,[30] which has

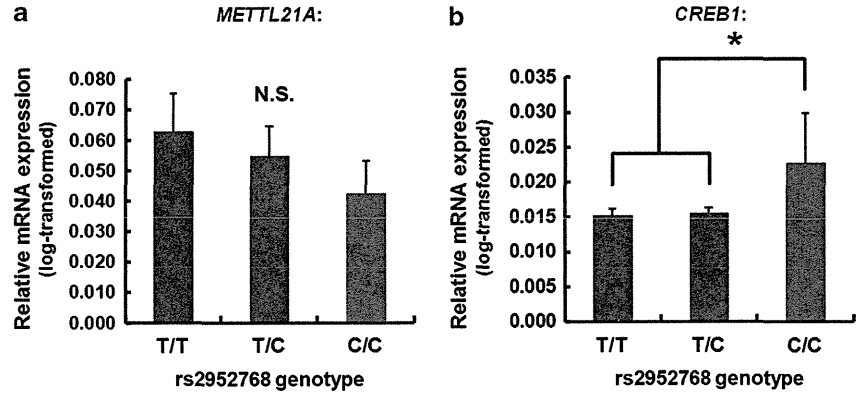

Figure 3. Relative mRNA expression level of the candidate genes between each genotype subgroup of the rs2952768 SNP in post-mortem brains. (**a**) Results for the *METTL21A* gene. (**b**) Results for the *CREB1* gene. NS, no significant association between relative mRNA expression and genotype subgroup ($P \geqslant 0.05$); *$P < 0.05$, greater level of mRNA expression in the C/C genotype compared with the T/C and T/T genotypes with nominal significance. The data are expressed as mean ± s.e.m.

been shown to be associated with reward processing.[31] Although a future confirmatory study is needed, the present data suggest the possibility that this SNP is one of the predisposing factors that partially contribute to the developmental differentiation of personality traits related to RD.

Association of rs2952768 SNP with mRNA expression level of the *CREB1* gene

Considering the fact that the rs2952768 and all of the neighboring SNPs that showed similarly strong associations with opioid sensitivity were included in the LD block that comprised the *METTL21A* (*FAM119A*) and *CREB1* genes, a subsequent issue is the impact of these SNPs on the function of these genes. Our database search estimated that several of the SNPs could putatively cause functional alterations (Supplementary Table S11). To pursue this issue, we examined the mRNA expression levels of these genes using real-time quantitative PCR with RNA samples extracted from post-mortem subject specimens and compared the mRNA expression levels between the genotype subgroups for the rs2952768 SNP, which were determined by genotyping the DNA samples extracted from the corresponding subjects. Although no significant association was found in the relative mRNA expression level of the *METTL21A* (*FAM119A*) gene between the genotype subgroups ($F_{2,97} = 0.372$, $P = 0.690$), a significant association was found in the relative mRNA expression level of the *CREB1* gene between the combined T/T and T/C genotype subgroup and C/C subgroup (Figures 3a and b). These results showed that the C/C genotype of this SNP was significantly associated with elevated *CREB1* mRNA expression ($t_{98} = -2.561$, $P = 0.012$).

DISCUSSION

By conducting a multistage GWAS for the first time in healthy subjects who were treated with opioid analgesics, we identified a potent locus for opioid sensitivity that encompasses an LD block that includes the most significant SNP, rs2952768. The C allele of this SNP, especially homozygotes of the C allele, was associated with more analgesic requirements, suggesting the possibility to classify the patients into groups of high responders and low responders or non-responders to the given opioid, which would presumably correspond to the T/T or T/C and C/C genotype groups, respectively. Surprisingly, this SNP was also found to be significantly associated with postoperative analgesic requirements in subjects who also received opioids but underwent different surgical procedures. Moreover, the C allele with likely less opioid sensitivity in this SNP was associated with a lower risk for serious symptoms of substance dependence and a lower RD score on the TCI in healthy subjects. These results show that SNPs in this locus are the most potent genetic factors associated with human opioid sensitivity known to date, affecting both the efficacy of opioid analgesics and liability to severe substance dependence.

Opioids exert their effects by binding to opioid receptors (that is, G-protein-coupled receptors) and triggering signaling transmission to several downstream effectors, including inhibition of adenylyl cyclase, activation of G-protein-activated inwardly rectifying potassium channels, and inhibition of voltage-gated Ca^{2+} channels.[32–34] Inhibition of adenylyl cyclase inhibits the production of cAMP, thus decreasing the active form of protein kinase A, phosphorylating CREB, and decreasing gene expression in the nucleus related to the action of analgesia and reward.[35,36] Indeed, the administration of cAMP intracerebrally or intravenously antagonized morphine analgesia in nontolerant and tolerant mice.[37] Moreover, all of the major behavioral effects of morphine, including analgesia, tolerance, reward, and physical dependence and withdrawal symptoms, were attenuated in mice that lacked adenylyl cyclase 5.[38] Meanwhile, increased CREB function in the nucleus accumbens decreased the sensitivity to the rewarding effects of morphine and cocaine in animals, whereas decreased CREB function had the opposite effect.[35,36,39] Although no change in opioid-binding sites or morphine-induced analgesia was observed in CREBαδ-deficient mice,[40] alterations in dose-dependent morphine-induced reward were also reported in CREBαδ-deficient mice.[41] Altogether, these reports indicate the involvement of CREB and the cAMP pathway in the analgesic and rewarding effects of opioids. Higher mRNA expression levels of the *CREB1* gene in subjects with the C/C genotype in the rs2952768 SNP identified in our GWAS may indicate elevated CREB function and decreased sensitivity to the rewarding effects of opioids, resulting in greater postoperative opioid analgesic requirements and less vulnerability to dependence on other drugs.

The best candidate SNP, rs2952768, is located closer to the *METTL21A* (*FAM119A*) gene than to the *CREB1* gene on chromosome 2 (Figures 1a and b). However, no significant association was found between rs2952768 and *METTL21A* mRNA expression, precluding the attribution of phenotypic alterations related to this SNP to the expression levels of the *METTL21A* gene. Although the precise functions of METTL21A are poorly understood to date, a representative METTL, METTL11A, reportedly exhibited catalytic activity as a histone methyltransferase[42] and chronic morphine treatment exhibited the acetylation and trimethylation of histones.[43] Although future studies are imperative, the action of opioids might be partially modulated by histone methylation via METTL functions.

With regard to our initial GWAS conducted as a consecutive three-stage analysis, the lowest combined *P*-value for the entire sample was $P = 8.044 \times 10^{-7}$ (Supplementary Table S2), which would have been deemed genome-wide nonsignificant if only a single-stage analysis was used to calculate conventional Bonferroni- or false discovery rate-corrected *P*-values for the total samples to determine statistical significance. However, 'significant' results obtained as conventionally corrected *P*-values will not always represent true associations, meaning that the results may not be necessarily replicated in other studies, and vice versa. For example, data from the National Human Genome Research Institute GWAS catalog (as of 31 January 2009), show 1321 entries of discovered associations with a *P*-value of $<10^{-5}$, but only 550 of these entries have a *P*-value of $<5 \times 10^{-8}$,[44] which is a conventionally corrected conservative threshold for declaring a significant association in a GWAS.[45,46] In both cases, truly potent candidate SNPs may be included in the outcome of the studies. Furthermore, GWASs in pharmacogenomics, such as this study, would tend not to yield 'significant' results obtained as conventionally corrected *P*-values compared with complex-disease GWASs[15] for several reasons. Among at least 16 different GWASs on drug response since the first was published in late 2007, less than half have shown genome-wide significance, although some potentially interesting associations that come close to significance have been detected in several of the studies in this category.[15] Altogether, these reports suggest that conventionally corrected *P*-values for the combined samples are not the only criteria to find true associations between SNPs and the phenotypes examined. The SNP we found, rs2952768, appears to be a promising SNP that is associated not only with opioid analgesic sensitivity in two independent surgical operations but also with several dependence-related traits in other subjects, prompting us to consider this SNP as the best candidate SNP known to date that is truly associated with human opioid sensitivity.

Our compelling results suggest the possibility that the association observed in this study can be robustly generalized to various clinical and nonclinical scenarios, although this study is rather exploratory, and independent confirmation of the findings will be required in subsequent studies before various forms of practical clinical utilization of the prediction of opioid sensitivity based on this SNP can be applied. In conclusion, although the underlying mechanisms remain to be fully elucidated in future studies, our findings provide a novel step toward understanding individual differences in opioid sensitivity and stimulating future studies that can open new avenues for the personalized treatment of pain and drug dependence.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We acknowledge Dr Keiji Tanaka for critically reading the manuscript and Mr Michael Arends for his assistance with editing the manuscript. We are grateful to the volunteers for their participation in this study and the anesthesiologists, surgeons and psychiatrists at related hospitals for collecting clinical data. We thank the Stanley Medical Research Institute Brain Collection for donating specimens. This work was supported by grants from the MEXT of Japan (20390162, 23390377), MHLW of Japan (H21-3jigan-ippan-011, H22-Iyaku -015) and Smoking Research Foundation.

AUTHOR CONTRIBUTIONS

Conception and design of the experiments: DN, SK and KI. Performance of the experiments: DN, SK, JH and AN. Analysis of the data: DN and YA Contribution of reagents/materials/analysis tools: DN, JH, AN, MK and TA. Writing of the paper: DN and KI. Collection of clinical data and DNA: KF, NS, Y Koukita, MN, RK, YS, MT, SH, HU, NO, TI, NI, IS, M Iyo, NK, MW, NN, KU, M Itokawa and MH. Support of the collection of clinical data and DNA: Y Kaneko.

REFERENCES

1 Sora I, Takahashi N, Funada M, Ujike H, Revay RS, Donovan DM *et al.* Opiate receptor knockout mice define mu receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc Natl Acad Sci USA* 1997; **94**: 1544–1549.
2 Leknes S, Tracey I. A common neurobiology for pain and pleasure. *Nat Rev Neurosci* 2008; **9**: 314–320.
3 Sora I, Elmer G, Funada M, Pieper J, Li XF, Hall FS *et al.* μ Opiate receptor gene dose effects on different morphine actions: evidence for differential in vivo mu receptor reserve. *Neuropsychopharmacology* 2001; **25**: 41–54.
4 Hall FS, Sora I, Uhl GR. Ethanol consumption and reward are decreased in μ-opiate receptor knockout mice. *Psychopharmacology (Berl)* 2001; **154**: 43–49.
5 Hall FS, Goeb M, Li XF, Sora I, Uhl GR. μ-Opioid receptor knockout mice display reduced cocaine conditioned place preference but enhanced sensitization of cocaine-induced locomotion. *Brain Res Mol Brain Res* 2004; **121**: 123–130.
6 Contarino A, Picetti R, Matthes HW, Koob GF, Kieffer BL, Gold LH. Lack of reward and locomotor stimulation induced by heroin in mu-opioid receptor-deficient mice. *Eur J Pharmacol* 2002; **446**: 103–109.
7 Berrendero F, Kieffer BL, Maldonado R. Attenuation of nicotine-induced antinociception, rewarding effects, and dependence in mu-opioid receptor knock-out mice. *J Neurosci* 2002; **22**: 10935–10940.
8 Shen X, Purser C, Tien LT, Chiu CT, Paul IA, Baker R *et al.* μ-Opioid receptor knockout mice are insensitive to methamphetamine-induced behavioral sensitization. *J Neurosci Res* 2010; **88**: 2294–2302.
9 Ikeda K, Ide S, Han W, Hayashida M, Uhl GR, Sora I. How individual sensitivity to opiates can be predicted by gene analyses. *Trends Pharmacol Sci* 2005; **26**: 311–317.
10 Coulbault L, Beaussier M, Verstuyft C, Weickmans H, Dubert L, Tregouet D *et al.* Environmental and genetic factors associated with morphine response in the postoperative period. *Clin Pharmacol Ther* 2006; **79**: 316–324.
11 Nishizawa D, Nagashima M, Katoh R, Satoh Y, Tagami M, Kasai S *et al.* Association between *KCNJ6* (*GIRK2*) gene polymorphisms and postoperative analgesic requirements after major abdominal surgery. *PLoS One* 2009; **4**: e7060.
12 Fukuda K, Hayashida M, Ide S, Saita N, Kokita Y, Kasai S *et al.* Association between *OPRM1* gene polymorphisms and fentanyl sensitivity in patients undergoing painful cosmetic surgery. *Pain* 2009; **147**: 194–201.
13 Candiotti KA, Yang Z, Morris R, Yang J, Crescimone NA, Sanchez GC *et al.* Polymorphism in the interleukin-1 receptor antagonist gene is associated with serum interleukin-1 receptor antagonist concentrations and postoperative opioid consumption. *Anesthesiology* 2011; **114**: 1162–1168.
14 Kumar D, Deb I, Chakraborty J, Mukhopadhyay S, Das S. A polymorphism of the CREB binding protein (CREBBP) gene is a risk factor for addiction. *Brain Res* 2011; **1406**: 59–64.
15 Daly AK. Genome-wide association studies in pharmacogenomics. *Nat Rev Genet* 2010; **11**: 241–246.
16 Wang L, McLeod HL, Weinshilboum RM. Genomics and drug response. *N Engl J Med* 2011; **364**: 1144–1153.
17 Feero WG, Guttmacher AE, Collins FS. Genomic medicine—an updated primer. *N Engl J Med* 2010; **362**: 2001–2011.
18 Hayashida M, Nagashima M, Satoh Y, Katoh R, Tagami M, Ide S *et al.* Analgesic requirements after major abdominal surgery are associated with *OPRM1* gene polymorphism genotype and haplotype. *Pharmacogenomics* 2008; **9**: 1605–1616.
19 Cloninger CR. A systematic method for clinical description and classification of personality variants. A proposal. *Arch Gen Psychiatry* 1987; **44**: 573–588.
20 Cloninger CR, Svrakic DM, Przybeck TR. A psychobiological model of temperament and character. *Arch Gen Psychiatry* 1993; **50**: 975–990.
21 Svrakic DM, Whitehead C, Przybeck TR, Cloninger CR. Differential diagnosis of personality disorders by the seven-factor model of temperament and character. *Arch Gen Psychiatry* 1993; **50**: 991–999.
22 Ujike H, Harano M, Inada T, Yamada M, Komiyama T, Sekine Y *et al.* Nine- or fewer repeat alleles in VNTR polymorphism of the dopamine transporter gene is a strong risk factor for prolonged methamphetamine psychosis. *Pharmacogenomics J* 2003; **3**: 242–247.
23 Ujike H. [Japanese Genetics Initiative for Drug Abuse (JGIDA)]. *Nihon Shinkei Seishin Yakurigaku Zasshi = Japanese Journal of Psychopharmacology* 2004; **24**: 299–302.
24 Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B.* 1995; **57**: 289–300.
25 Storey J. The positive false discovery rate: a Bayesian interpretation and the q-value. *Ann Statist* 2001; **31**: 2013–2035.
26 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; **81**: 559–575.

27 Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**: 263–265.
28 Nyholt DR. Genetic case-control association studies—correcting for multiple testing. *Hum Genet* 2001; **109**: 564–567.
29 Perneger TV. What's wrong with Bonferroni adjustments. *BMJ* 1998; **316**: 1236–1238.
30 Hakamata Y, Iwase M, Iwata H, Kobayashi T, Tamaki T, Nishio M *et al.* Regional brain cerebral glucose metabolism and temperament: a positron emission tomography study. *Neurosci Lett* 2006; **396**: 33–37.
31 Zink CF, Pagnoni G, Martin-Skurski ME, Chappelow JC, Berns GS. Human striatal responses to monetary reward depend on saliency. *Neuron* 2004; **42**: 509–517.
32 Law PY, Wong YH, Loh HH. Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* 2000; **40**: 389–430.
33 Childers SR. Opioid receptors: pinning down the opiate targets. *Curr Biol* 1997; **7**: R695–R697.
34 Ikeda K, Kobayashi T, Kumanishi T, Yano R, Sora I, Niki H. Molecular mechanisms of analgesia induced by opioids and ethanol: is the GIRK channel one of the keys? *Neurosci Res* 2002; **44**: 121–131.
35 Carlezon Jr WA, Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N *et al.* Regulation of cocaine reward by CREB. *Science* 1998; **282**: 2272–2275.
36 Nestler EJ. Molecular mechanisms of drug addiction. *Neuropharmacology* 2004; **47**(Suppl 1): 24–32.
37 Ho IK, Loh HH, Way EL. Cyclic adenosine monophosphate antagonism of morphine analgesia. *J Pharmacol Exp Ther* 1973; **185**: 336–346.
38 Kim KS, Lee KW, Im JY, Yoo JY, Kim SW, Lee JK *et al.* Adenylyl cyclase type 5 (AC5) is an essential mediator of morphine action. *Proc Natl Acad Sci USA* 2006; **103**: 3908–3913.
39 Barrot M, Olivier JD, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ *et al.* CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc Natl Acad Sci USA* 2002; **99**: 11435–11440.
40 Maldonado R, Blendy JA, Tzavara E, Gass P, Roques BP, Hanoune J *et al.* Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. *Science* 1996; **273**: 657–659.
41 Walters CL, Godfrey M, Li X, Blendy JA. Alterations in morphine-induced reward, locomotor activity, and thermoregulation in CREB-deficient mice. *Brain Res* 2005; **1032**: 193–199.
42 Richon VM, Johnston D, Sneeringer CJ, Jin L, Majer CR, Elliston K *et al.* Chemogenetic analysis of human protein methyltransferases. *Chem Biol Drug Des* 2011; **78**: 199–210.
43 Wang J, Barke RA, Roy S. Transcriptional and epigenetic regulation of interleukin-2 gene in activated T cells by morphine. *J Biol Chem* 2007; **282**: 7164–7171.
44 Ioannidis JP, Thomas G, Daly MJ. Validating, augmenting and refining genome-wide association signals. *Nat Rev Genet* 2009; **10**: 318–329.
45 Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. *Nat Rev Genet* 2005; **6**: 95–108.
46 Risch N, Merikangas K. The future of genetic studies of complex human diseases. *Science* 1996; **273**: 1516–1517.

Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)

日本神経精神薬理学雑誌（Jpn. J. Neuropsychopharmacol.）32：165-167（2012）

〔Short Communication〕

Influence of GIRK Channel Inhibition on Relapse Risk in Japanese Alcohol-Dependent Inpatients

Nagisa SUGAYA[*1,2], Yasukazu OGAI[*3], Yoichi KAKIBUCHI[*4], Eiichi SENOO[*5] and Kazutaka IKEDA[*1]

[*1] Tokyo Metropolitan Institute of Medical Science
2-1-6 Kamikitazawa, Setagaya-ku, Tokyo, 156-8506 Japan
[*2] Advanced Research Center for Human Sciences, Waseda University
[*3] University of Tsukuba
[*4] Narimasu Kousei Hospital
[*5] Ibaraki Prefectural Medical Center of Psychiatry

(Accepted February 7, 2012)

Abstract: We examined the influence of G-protein-activated inwardly rectifying K^+ (GIRK) channel inhibition on relapse risk in Japanese alcohol-dependent inpatients. The participants included 11 patients who received GIRK inhibition treatment and 39 patients who did not receive GIRK inhibition treatment. The participants answered a questionnaire, including the Alcohol Relapse Risk Scale (ARRS) and a questionnaire about their experiences of stressful events 2 weeks after hospitalization (time 1) and completed follow-up questionnaires 45-60 days after the first rating (time 2). A significant interaction was found between group and time on positive expectancy for alcohol scores on the ARRS ($F=5.93$, $p=0.02$). The scores at time 2 were lower than that at time 1 in the GIRK inhibition treatment group ($p=0.004$) but not in the non-GIRK inhibition treatment group. The results of the present study suggest that GIRK inhibition treatment may improve the positive expectancy for alcohol, a component of relapse risk. The present study suggests that the effects of GIRK inhibition treatment should be investigated further in future studies.

Key words: GIRK inhibition treatment, Alcohol dependence, Relapse risk

G-protein-activated inwardly rectifying K^+ (GIRK) channels play an important role in the mechanisms of alcohol action (Koyabashi et al, 1999; Lewohl et al, 1999). A genetic polymorphism of GIRK channels was shown to be a heritable factor related to alcohol sensitivity in mice (Ikeda et al, 2002; Kobayashi et al, 1999). We previously showed that GIRK inhibition improved the lack of negative expectancy for alcohol drinking, a component of relapse risk, in outpatients with alcohol dependence, although environmental factors during the investigation and the time of initiation of drug treatment were not well controlled (Ogai et al, 2011).

In the present study, we examined the influence of GIRK inhibition on relapse risk in Japanese alcohol-dependent inpatients while controlling environmental factors and the drug treatment schedule.

METHODS

The participants in this study were 50 alcohol-dependent inpatients (43 males and seven females, 52.64±10.76 years old) from Narimasu Kousei Hospital. The participants were diagnosed as alcohol dependent based on the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV). The Alcohol Relapse Risk Scale (ARRS; Ogai et al, 2009) is a self-rating scale that predicts the risk of alcohol reuse multilaterally in patients with alcohol dependence. The ARRS has 32 items and measures the risk of alcohol reuse in five dimensions: stimulus-induced vulnerability, emotional problems, compulsivity for alcohol, lack of negative expectancy for alcohol, and positive expectancy for alcohol. The participants' recent experiences of stress were assessed using a 4-point scale. The Japanese version of the State-Trait Anxiety Inventory (STAI; a 40-item questionnaire) was used to measure the severity of overall anxiety levels as an indicator of two types of anxiety: state and trait (Hidano et al, 2000). Depressive symptoms were assessed using the Japanese version of the Center for Epidemiologic Studies Depression Scale (CES-D; a 20-item questionnaire; Shima et al, 1985).

The participants answered a questionnaire 2 weeks after hospitalization (time 1) and a follow-up questionnaire 45-60 days after the first rating (time 2) after they provided written informed consent. At the time 1 measurement, the participants completed the ARRS, provided their self-reports of their experiences of stressful events, completed the STAI, and completed the CES-D. At time 2, the participants completed the relapse risk scale and again provided self-reports of their experiences of stressful events. The patients were divided into two groups: treatment with drugs with GIRK-inhibiting ability (i.e., paroxetine, sertraline, chlorpromazine, trazodone, and levomepromazine) and treatment with drugs without GIRK-inhibiting ability.

The data analyses were performed using PASW 18.0 software (SPSS, Chicago, IL). A two-way analysis of variance

[*1] Reprint requests should be sent to K. Ikeda
池田和隆：〒156-8506 東京都世田谷区上北沢 2-1-6
東京都医学総合研究所依存性薬物プロジェクト
E-mail: ikeda-kz@igakuken.or.jp

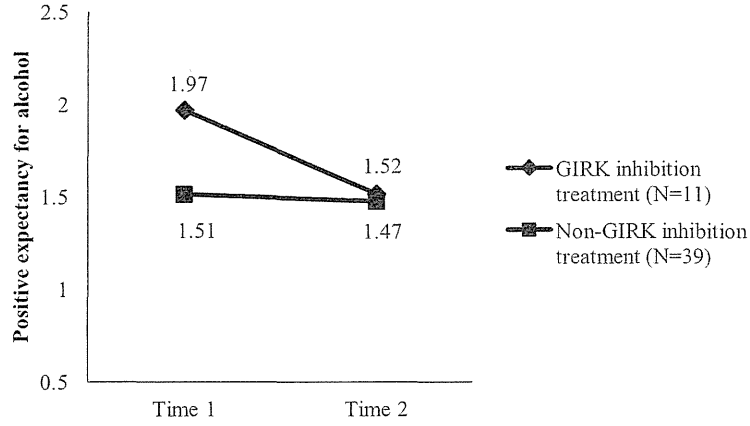

Fig. 1 Difference in the transition of the positive expectancy for alcohol between GIRK inhibition treatment and non-GIRK inhibition treatment.

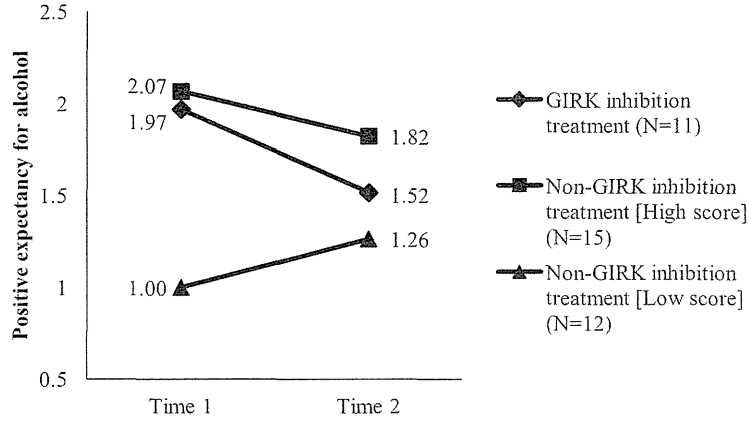

Fig. 2 Difference in the transition of the positive expectancy for alcohol between GIRK inhibition treatment group and non-GIRK inhibition treatment groups with high scores and with low scores at time 1.

(ANOVA) was applied to analyze the effects of group and time on relapse risk and the experience of stressful events. The *t*-test was used for group comparisons of the scores on the STAI and CES-D. The significance level was less than 5%.

The Institutional Review Boards of Narimasu Kousei Hospital and Tokyo Metropolitan Institute of Medical Science (no. 21-18) approved the study.

RESULTS

The participants included 11 patients who received GIRK inhibition treatment (8 males and 3 females, 52.18±10.30 years old) and 39 patients who did not receive GIRK inhibition treatment (35 males and 4 females, 52.77±11.02 years old). While both groups were treated with anxiolytic, antidepressant, hypnotic, antipsychotic, and/or antialcoholic drugs, the ratios of patients treated with anxiolytic ($\chi^2=7.11$, $p=0.03$), antidepressant ($\chi^2=33.68$, $p<0.0001$), and antipsychotic ($\chi^2=10.89$, $p=0.006$) were higher in the GIRK inhibition treatment group than the non-GIRK inhibition treatment group. However, no significant differences were found between the GIRK inhibition treatment group and non-GIRK inhibition treatment group with regard to gender ratio, age, interval date between time 1 and time 2 (the GIRK inhibition treatment group: 46.36±4.52 days, the non-GIRK inhibition treatment group: 47.69±5.83 days), STAI (neither the state nor trait scales), or CES-D.

A significant interaction was found between group and time on positive expectancy scores on the ARRS ($F=5.93$, $p=0.02$, Fig. 1). The simple main effect test showed that the scores at time 1 in the GIRK inhibition treatment group were higher than in the non-GIRK inhibition treatment group ($p=0.03$). The scores at time 2 were lower than at time 1 only in the GIRK inhibition treatment group ($p=0.004$). Significant main effects of time were found on total ARRS score ($F=5.10$, $p=0.03$) and stimulus-induced vulnerability ($F=7.28$, $p=0.01$), and these scores at time 2 were lower than at time 1.

Because the difference between groups in positive expectancy score at time 1 might affect the interaction between group and time, we divided the non-GIRK inhibition treatment group by a median of positive expectancy score at time 1 into high score group and low score group, and compared between those groups and the GIRK inhibition treatment group in positive expectancy score. There was significant interaction between group and time ($F=6.30$, $p=0.005$, Fig. 2). There was no significant difference between the GIRK inhibition treatment group and the non-GIRK inhibition treatment group with higher scores in positive expectancy score at time 1. The simple main effect test showed that the scores at time

2 were lower than at time 1 only in the GIRK inhibition treatment group ($p=0.005$).

No significant interaction was found between group and time, with no main effect of either factor on the experience of stressful events.

DISCUSSION

The results of the present study suggest that GIRK inhibition treatment improves the positive expectancy for alcohol, a component of relapse risk. Although a previous study in outpatients (Ogai et al, 2011) reported that GIRK inhibition treatment improved the lack of negative expectancy for alcohol, the results of the previous study in outpatients and the present study in inpatients together suggest that GIRK channel inhibition may be useful for reducing relapse risk. The difference in positive expectancy at time 1 between groups may be related to high ratios of patients treated with anxiolytic, antidepressant, and antipsychotic in the GIRK inhibition treatment group. The effects of GIRK inhibition treatment should be investigated further in future studies.

REFERENCES

Hidano, T., Fukuhara, M., Iwawaki, S., Soga, Y. and Spielberger, S. D. (2000) Shinban STAI manual. Jitsumukyoiku Shuppan, Tokyo.

Ikeda, K., Kobayashi, T., Kumanishi, T., Yano, R., Sora, I. and Niki, H. (2002) Molecular mechanisms of analgesia induced by opioids and ethanol: Is the GIRK channel one of the keys? Neurosci Res, 44: 121-131.

Kobayashi, T., Ikeda, K., Kojima, H., Niki, H., Yano, R., Yoshioka, T. and Kumanishi, T. (1999) Ethanol opens G-protein-activated inwardly rectifying K^+ channels. Nat Neurosci, 2: 1091-1097.

Lewohl, J. M., Wilson, W. R., Mayfield, R. D., Brozowski, S. J., Morrisett, R. A. and Harris, R. A. (1999) G-protein-coupled inwardly rectifying potassium channels are targets of alcohol action. Nat Neurosci, 2: 1084-1090.

Ogai, Y., Yamashita, M., Endo, K., Haraguchi, A., Ishibashi, Y., Kurokawa, T., Muratake, T., Suga, R., Hori, T., Umeno, M., Asukai, M., Senoo, E. and Ikeda, K. (2009) Application of the Relapse Risk Scale to alcohol-dependent individuals in Japan: Comparison with stimulant abusers. Drug Alcohol Depend, 101: 20-26.

Ogai, Y., Hori, T., Haraguchi, A., Asukai, N., Senoo, E. and Ikeda, K. (2011) Influence of GIRK channel inhibition on alcohol abstinence and relapse risk in Japanese alcohol-dependent outpatients. Jpn J Neuropsychopharmacol, 31: 95-96.

Shima, S., Kano, S., Kitamura, T. and Asai, M. (1985) Atarashii yokuutsusei jikohyokashakudo ni tsuite. Seishin Igaku, 27: 717-723.

Current Neuropharmacology, 2011, *9*, 68-72

The Selective Serotonin Reuptake Inhibitor Paroxetine, but not Fluvoxamine, Decreases Methamphetamine Conditioned Place Preference in Mice

Y. Takamatsu[1], H. Yamamoto[1], Y. Hagino[1], A. Markou[2] and K. Ikeda[1,*]

[1]*Division of Psychobiology, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan;* [2]*Department of Psychiatry, School of Medicine, University of California San Diego, 9500 Gilman Drive, La Jolla, San Diego, CA 92093-0603, USA*

The Selective Serotonin Reuptake Inhibitor Paroxetine, but not Fluvoxamine, Decreases Methamphetamine Conditioned Place Preference in Mice

Y. Takamatsu[1], H. Yamamoto[1], Y. Hagino[1], A. Markou[2] and K. Ikeda[1,*]

[1]*Division of Psychobiology, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan;* [2]*Department of Psychiatry, School of Medicine, University of California San Diego, 9500 Gilman Drive, La Jolla, San Diego, CA 92093-0603, USA*

Abstract: Monoamine transporters are the main targets of methamphetamine (METH). Recently, we showed that fluoxetine, a selective serotonin reuptake inhibitor (SSRI), decreased METH conditioned place preference (CPP), suggesting that serotonin transporter (SERT) inhibition reduces the rewarding effects of METH. To further test this hypothesis, in the present study we investigated the effects of additional SSRIs, paroxetine and fluvoxamine, on METH CPP in C57BL/6J mice. In the CPP test, pretreatment with 20 mg/kg paroxetine abolished the CPP for METH, whereas pretreatment with 100 mg/kg fluvoxamine prior to administration of METH failed to inhibit METH CPP. These results suggest that paroxetine, a medication widely used to treat depression, may be a useful tool for treating METH dependence. Further, these data suggest that molecules other than the SERT [such as G protein-activated inwardly rectifying K^+ (GIRK) channels] whose activities are modulated by paroxetine and fluoxetine, but not by fluvoxamine, are involved in reducing METH CPP by paroxetine and fluoxetine.

Keywords: Conditioned place preference, Fluvoxamine, Methamphetamine, Mice, Paroxetine, Serotonin transporter.

INTRODUCTION

Methamphetamine (METH) is abused in worldwide [1]. In Japan, the number of people arrested for METH possession or use is approximately 100 times higher than those arrested for cocaine, opioids, or cannabis. Further, METH frequently induces psychotic states with symptoms similar to those seen in paranoid schizophrenia [2]. Such psychotic states are treated primarily in hospitals resulting in high medical costs. Thus, there is great need for the discovery of new medications for METH abuse [3] because the current treatments are mostly oriented toward the treatment of psychosis with no treatments available to prevent relapse to METH abuse.

The dopamine transporter (DAT) is the main target for METH and cocaine. However, mice lacking the DAT show conditioned place preference (CPP) to cocaine [4] and self-administer cocaine [5]. Interestingly, heterozygous and homozygous serotonin transporter (SERT) knockout mice that also have a homozygous knockout of the DAT do not exhibit cocaine CPP [6]. Cocaine administration leads to increases in extracellular dopamine concentration in the striatum of DAT knockout mice but not of DAT/SERT double knockout mice [7]. Taken together, these reports suggest that SERT inhibition may decrease METH and cocaine CPP.

Recently, we showed that fluoxetine, a selective serotonin reuptake inhibitor (SSRI), abolished METH CPP when METH was administered during both the development and expression phases of the CPP procedure, supporting the hypothesis that SERT inhibition decreased the rewarding effects of METH [8]. To further test this hypothesis, in the present study we investigated the effects of the SSRIs paroxetine (Paxil®) and fluvoxamine (Lubox® or Depromel®) on METH CPP.

MATERIALS AND METHODS

Mice

Male C57BL/6J mice (8-10 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and were housed for 1-2 weeks before the experiments began in an animal facility maintained at 22 ± 2°C and 55 ± 5% relative humidity under a 12/12 h light/dark cycle with lights on at 8:00 am. Food and water were available *ad libitum*. All behavioral testing was conducted during the light phase. The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee of the Tokyo Institute of Psychiatry, and all animals were cared for and treated humanely in accordance with our institutional animal experimentation guidelines.

Conditioned Place Preference (CPP) Test

The CPP test was performed according to the method of Hoffman and Beninger [9] with some modifications. We used a two-compartment Plexiglas chamber (Neuroscience Inc., Osaka, Japan). One compartment (17.5 × 15 × 17.5 cm: width × length × height) was black with a smooth floor, and the other compartment was of the same dimensions, but with a white textured floor. This two-compartment chamber was located in a sound- and light-attenuated box under conditions

*Address correspondence to this author at the Division of Psychobiology, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan; Tel: +81-3-3304-5701, ext. 508; Fax: +81-3-3329-8035; E-mail: ikeda-kz@igakuken.or.jp

1570-159X/11 $58.00+.00

of dim illumination (approximately 40 lux) to reduce bias toward either compartment [10]. Mice were assigned randomly to the treatment groups (see below).

On Day 1, the mice (n = 14-26 per group) were allowed to freely explore the two compartments for 15 min. On Day 2, the mice again were allowed to explore the two compartments freely for 15 min, and the time spent in each compartment and the number of transitions between compartments were measured. Conditioning sessions then were conducted once daily for 4 consecutive days (Days 5-8). For the Day 5 conditioning session, mice were i.p. injected with saline or SSRI (20 mg/kg paroxetine or 100 mg/kg fluvoxamine) 60 min before injection with METH (2 mg/kg, i.p.). Immediately after METH administration, mice were confined to the black or white compartment for 50 min. On Day 6, the mice were pretreated with the same solution (saline or SSRI, i.p.) 60 min before a saline injection. Immediately after the saline injection, mice were confined to the opposite compartment for 50 min. On Days 7 and 8, the same conditioning as on Days 5 and 6 was repeated. On Day 9, the mice were pretreated with saline or SSRI (20 mg/kg paroxetine or 100 mg/kg fluvoxamine, i.p.), and 60 min later were allowed to freely explore the two compartments for 15 min without METH injection. The time spent in each compartment and the number of transitions between compartments were measured. In summary, there were a total of eight groups in this experiment corresponding to the four pretreatments (paroxetine, fluvoxamine, saline; there were two saline groups that were run concurrently with the paroxetine and fluvoxamine groups) and the two phases of the experiment during which they were pretreated with the drug (conditioning days 5-8 or test day 9). The CPP score was defined as the time spent in the drug-paired compartment during the CPP test phase (Day 9) minus the time spent in the same compartment during the preconditioning exploratory phase (Day 2). The transition score was defined as the number of transitions during the CPP test phase (Day 9) minus the number of transitions during the preconditioning exploratory phase (Day 2).

Drugs

Methamphetamine hydrochloride was purchased from Dainippon Pharmaceutical (Osaka, Japan). Paroxetine maleate and fluvoxamine maleate were purchased from Sigma (St. Louis, MO, USA) and TOCRIS (Hung Road, Bristol, UK), respectively. All drugs were dissolved in saline. Drugs and vehicle were administered i.p. in a volume of 0.1 ml/10 g body weight. All drug doses are reported as salt.

Statistical Analyses

The CPP and transition scores of mice pretreated with saline or SSRI during the conditioning and CPP test phases were subjected to a two-way analysis of variance (ANOVA). The ANOVA had two between-subjects factors, each with two levels (saline/SSRI pretreatment in the conditioning phase and saline/SSRI pretreatment in the CPP test phase). Two separate ANOVAs were conducted on the paroxetine and fluvoxamine data. Similar ANOVAs were conducted on the transition scores. The CPP scores from the paroxetine experiment were subjected to a one-way ANOVA followed by *post hoc* comparisons with the Scheffe test. In this ANOVA, there were four levels corresponding to the four treatment conditions (saline in both the conditioning and the CPP test phases, pretreatment with paroxetine only in the conditioning phase, pretreatment with paroxetine only in the CPP test phase, pretreatment with paroxetine in both the conditioning and the CPP test phases). For the CPP data, the durations of time that the mice spent in the METH-paired compartment before and after conditioning were compared using paired t-tests for each group. For the transition data, the number of transitions between the METH-paired compartment and the saline-paired compartment before and after conditioning were compared using paired t-tests for each group. The level of significance was set at 0.05.

RESULTS

Effects of Paroxetine on METH CPP

The two-way ANOVA revealed that mice treated with paroxetine during the test phase exhibited decreased CPP scores compared to mice treated with saline during the test phase ($F_{1,72}$ = 7.888, P < 0.01), whereas mice treated with paroxetine during the conditioning phase did not differ significantly from mice treated with saline during the test phase in the CPP score [$F_{1,72}$ = 1.704, not significant (n.s.); Fig. (**1A**)]. There was no statistically significant interaction between the factor saline/paroxetine during the conditioning phase and the factor saline/paroxetine during the CPP test phase ($F_{1,72}$ = 0.1690, n.s.), indicating that the important factor was treatment with paroxetine during the expression phase of the experiment. In addition, a one-way ANOVA on the CPP scores was conducted on data for all four groups. The ANOVA showed a significant difference in the CPP scores among these four groups ($F_{3,72}$ = 3.940, P < 0.05). The Scheffe *post hoc* test showed that the CPP score of the paroxetine/paroxetine group was significantly lower than that of the saline/saline group (P < 0.05). Paired t-tests were conducted to compare the duration of time before and after conditioning for each of the four groups (Fig. (**1B**)). Whereas the saline/saline and paroxetine/saline groups spent significantly more time in the METH-paired compartment after conditioning than before conditioning (saline/saline: n = 23, df = 22, t = -6.050, P < 0.001; paroxetine/saline: n = 15, df = 14, t = -2.884, P < 0.05), the saline/paroxetine and paroxetine/paroxetine groups did not show METH CPP (saline/paroxetine: n = 15, df = 14, t = -2.033, n.s.; paroxetine/paroxetine: n = 23, df = 22, t = -0.908, n.s.). Paroxetine pretreatment had no significant effects on the transition scores compared to the saline/saline treatment group (data not shown).

Effects of Fluvoxamine on the METH CPP

The two-way ANOVA revealed that both the factor saline/fluvoxamine pretreatment during the conditioning phase and the factor saline/fluvoxamine pretreatment during the CPP test phase had no effects on CPP scores (conditioning phase: $F_{1,68}$ = 0.045, n.s.; CPP test phase: $F_{1,68}$ = 3.016, n.s.; Fig. (**2A**)). There was no statistically significant interaction between the two factors ($F_{1,68}$ = 0.066, n.s.). Paired t-tests were conducted to compare the duration of time before and after conditioning for each of the four groups. All four groups spent significantly more time in the METH-paired

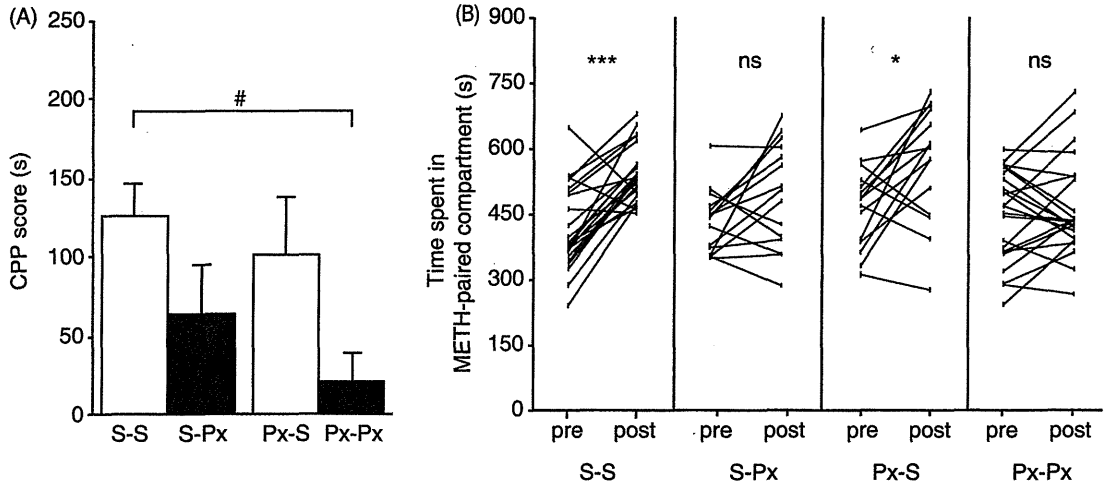

Fig. (1). Effects of paroxetine on CPP for METH in mice. (**A**) Reduction of METH CPP by paroxetine (Px) pretreatment. Mice were pretreated with saline (S) in both the conditioning and CPP test phases (S-S), paroxetine only in the CPP test phase (S-Px), paroxetine only in the conditioning phase (Px-S), and paroxetine in both the conditioning and the CPP test phases (Px-Px). The CPP score was defined as the time spent in the drug-paired compartment during the CPP test phase (Day 9) minus the time spent in the same compartment during the preconditioning phase (Day 2). The CPP score of the Px-Px group was significantly lower than that of the S-S group ($^{\#}P < 0.05$). (**B**) Comparison of time spent in the conditioned compartment before and after conditioning in the four groups. There was a significant CPP in the S-S and Px-S groups, but not in the S-Px and Px-Px groups (when paroxetine was administered in the CPP test phase). $^{***}P < 0.001$, $^{*}P < 0.05$, ns: not significant ($P > 0.05$).

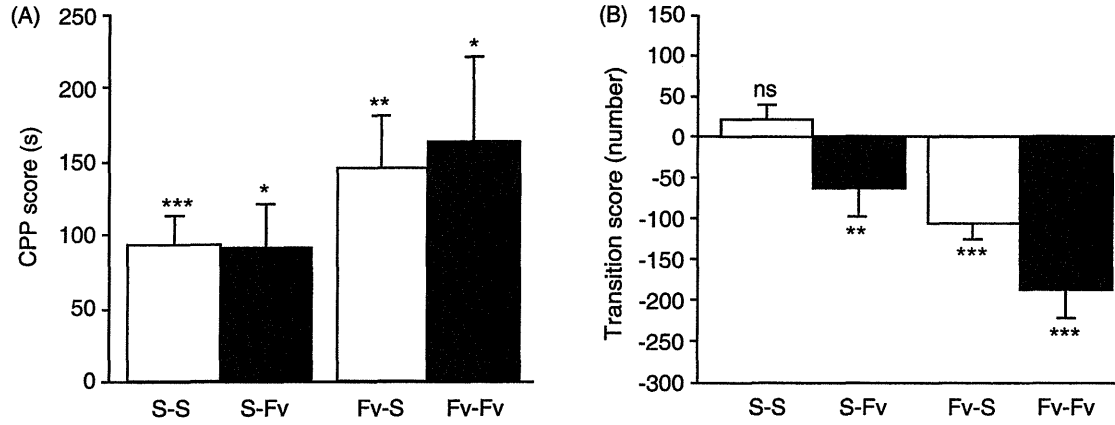

Fig. (2). Effects of fluvoxamine on CPP for METH and on transitions between compartments. (**A**) Lack of a significant effect of fluvoxamine (Fv) on METH CPP. Mice were pretreated with saline in both the conditioning and the CPP test phases (S-S), fluvoxamine only in the CPP test phase (S-Fv), fluvoxamine only in the conditioning phase (Fv-S), and fluvoxamine in both the conditioning and the CPP test phases (Fv-Fv). There was a significant CPP in all groups. Fluvoxamine pretreatment in the conditioning phase and/or the CPP test phase failed to inhibit METH CPP (pre- and post-conditioning preference test results were analyzed with paired *t*-tests, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$). (**B**) Decreases in transitions between the compartments by fluvoxamine pretreatment. There were significant decreases in transitions in the S-Fv, Fv-S, and Fv-Fv groups, but not in the S-S group [number of transitions in the pre- and post-conditioning phases was analyzed with paired *t*-tests, $^{***}P < 0.001$, $^{**}P < 0.01$, ns: not significant ($P > 0.05$)]. The transition score was defined as the number of transitions during the CPP test phase (Day 9) minus the number of transitions during the preconditioning phase (Day 2).

compartment after conditioning than before conditioning (saline/saline: $n = 26$, $df = 25$, $t = -4.541$, $P < 0.001$; saline/fluvoxamine: $n = 14$, $df = 13$, $t = -2.983$, $P < 0.05$; fluvoxamine/saline: $n = 18$, $df = 17$, $t = -3.949$, $P < 0.01$; fluvoxamine/fluvoxamine: $n = 14$, $df = 13$, $t = -2.757$, $P < 0.05$).

The two-way ANOVA revealed that both fluvoxamine pretreatment during the conditioning phase and during the CPP test phase significantly decreased transition scores (conditioning phase: $F_{1,68} = 24.321$, $P < 0.001$; CPP test phase: $F_{1,68} = 10.292$, $P < 0.01$; Fig. (**2B**)). There was no statistically significant interaction between the two factors ($F_{1,68} = 0.007$, n.s.). Paired *t*-tests were conducted to compare the number of transitions before and after conditioning for each of the four groups. The S-S group showed no significant differences in the number of transitions before and after conditioning ($n = 26$, $df = 25$, $t = -1.213$, n.s.). However, mice pretreated with fluvoxamine (saline/fluvoxamine, fluvoxamine/saline, fluvoxamine/fluvoxamine) showed significant decreases in the number of transitions after conditioning (saline/fluvoxamine: $n = 14$, $df = 13$, $t = 3.829$, $P < 0.01$; fluvoxamine/saline: $n = 18$, $df = 17$, $t = 5.520$, $P < 0.001$; fluvoxamine/fluvoxamine: $n = 14$, $df = 13$, $t = 6.025$, $P < 0.001$).

DISCUSSION

In the present study, we showed that paroxetine, a widely used medication for treating depression, inhibited METH CPP in mice, similar to the results we reported previously with fluoxetine [8]. No significant effects of paroxetine on transition scores suggest that the effects of paroxetine on METH CPP are not due to changes in locomotor activity but due to reduction of METH reward and conditioned reward by paroxetine. Based on these findings, it appears worthwhile to investigate the clinical effects of paroxetine on METH abuse. By contrast, the other SSRI tested here, fluvoxamine, did not affect METH CPP. These data demonstrate that there are differences in the effects of SSRIs on METH CPP, suggesting the possibility that molecules other than the SERT are involved in the inhibition of METH CPP by paroxetine and fluoxetine reported here and in our previous study [8].

In addition to SERT inhibition, paroxetine inhibits the function of muscarinic cholinergic receptors [11], nicotinic acetylcholine receptors [12], volume-related anion channels [13], membrane steroid transporters [14], and nitric oxide synthase [15]. Recently, Kobayashi and colleagues [16] reported that paroxetine also inhibits the function of G protein-activated inwardly rectifying K^+ (GIRK) channels. It is intriguing that paroxetine and fluoxetine, but not fluvoxamine, inhibit GIRK channels [16-18]. Various G protein-coupled receptors (such as M2 muscarinic, α2 adrenergic, D_2 dopaminergic, 5-HT_{1A}, opioid, nociceptin/orphanin FQ, and A_1 adenosine) activate GIRK channels [19-22] through the direct action of G protein subunits [23]. In addition, GIRK channels are activated by ethanol independently of G protein-coupled signaling pathways [24, 25]. Activation of GIRK channels leads to membrane hyperpolarization [22]. These channels play an important role in the inhibitory regulation of neuronal excitability. Thus, modulators of GIRK channel activity may affect many brain functions. Kobayashi and colleagues [26] also have reported that ifenprodil, a cerebral vasodilator which inhibits morphine CPP [27], also inhibits the function of GIRK channels. Morgan and colleagues [28] demonstrated that GIRK channel knockout mice exhibited dramatically reduced intravenous self-administration of cocaine. In the present study, we found that paroxetine and fluoxetine, but not fluvoxamine, inhibited METH CPP. These findings, together with the previous findings, suggest that the inhibition of GIRK channels by paroxetine or fluoxetine may be involved in the inhibition of METH CPP by these drugs.

Fluvoxamine administration (60 mg/kg) leads to a significant decrease in spontaneous locomotor activity [29]. Consistent with this observation, significant decreases in transition scores were observed in all of the 100 mg/kg fluvoxamine-treated groups compared to the saline/saline-treated group in the present study. The number of transitions of the fluvoxamine/fluvoxamine treated group during the CPP test phase (101.4 ± 85.3, mean ± SEM) was the smallest among the four groups in this experiment, but more than 100 transitions indicated adequate locomotion to reveal potential differences in CPP. The lack of effect of fluvoxamine on CPP for methamphetamine is likely to reflect a lack of effect of fluvoxamine on the rewarding effects of METH rather than being a nonspecific effect of fluvoxamine.

In conclusion, we found that paroxetine, but not fluvoxamine, inhibited METH CPP in mice. Although further preclinical studies are needed to elucidate the mechanisms underlying these inhibitory effects of paroxetine on processes relating to METH dependence, it appears worthwhile to investigate the clinical effects of paroxetine on METH abuse. The present results suggest that molecules other than the SERT (such as GIRK channels) are involved in the inhibition of METH CPP by paroxetine and fluoxetine.

ACKNOWLEDGEMENTS

We are grateful to Dr. Yasukazu Ogai for statistics instruction, Dr. Keiko Matsuoka for animal care, Yukiko Sakaki and Akira Sato for technical assistance, and Michael Arends for editorial assistance. This work was supported by a research grant (17025054) from the MEXT of Japan, by grants (H17-pharmaco-001, H19-iyaku-023, H18-shitei-3) from the MHLW of Japan, and by Japan-U.S. Brain Research Cooperative Program grant from JSPS. AM was supported by a USA National Institute on Drug Abuse grant (R01 DA11946).

ABBREVIATIONS

ANOVA	=	Analysis of variance
CPP	=	Conditioned place preference
DAT	=	Dopamine transporter
GIRK	=	G protein-activated inwardly rectifying K^+
METH	=	Methamphetamine
n.s.	=	Not significant
SERT	=	Serotonin transporter
SSRI	=	Selective serotonin reuptake inhibitor

REFERENCES

[1] United Nations International Drug Control Programme. *World drug report 2005*. Oxford University Press: New York, **2005**.

[2] Ujike, H. Stimulant-induced psychosis and schizophrenia: the role of sensitization. *Curr. Psychiatry Rep.*, **2002**, *4*, 177-184.

[3] National Institute on Drug Abuse. *Methamphetamine abuse and addiction (Research report series, NIH pub. no. 98-4210)*. National Institute on Drug Abuse: Bethesda, **2002**.

[4] Sora, I.; Wichems, C.; Takahashi, N.; Li, X.F.; Zeng, Z.; Recay, R.; Lesch, K.P.; Murphy, D.L.; Uhl, G.R. Cocaine reward models: conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 7699-7704.

[5] Rocha, B.A.; Fumagalli, F.; Gainetdinov, R.R.; Jones, S.R.; Ator, R.; Giros, B.; Miller, G.W.; Caron, M.G. Cocaine self-administration in dopamine-transporter knockout mice. *Nat. Neurosci.*, **1998**, *1*, 132-137.

[6] Sora, I.; Hall, F.S.; Andrews, A.M.; Itokawa, M.; Li, X.F.; Wei, H.B.; Wichems, C.; Lesch, K.P.; Murphy, D.L.; Uhl, G.R. Molecular mechanisms of cocaine reward: combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. *Proc. Natl. Acad. Sci. USA*, **2001**, *98*, 5300-5305.

[7] Shen, H.W.; Hagino, Y.; Kobayashi, H.; Shinohara, K.; Ikeda, K.; Yamamoto, H.; Yamamoto, T.; Lesch, K.P.; Murphy, D.L.; Hall, F.S.; Uhl, G.R.; Sora, I. Regional differences in extracellular dopamine and serotonin assessed by *in vivo* microdialysis in mice

lacking dopamine and/or serotonin transporters. *Neuropsychopharmacology*, **2004**, *29*, 1790-1799.

[8] Takamatsu, Y.; Yamamoto, H.; Ogai, Y.; Hagino, Y.; Markou, A.; Ikeda, K. Fluoxetine as a potential pharmacotherapy for methamphetamine dependence: studies in mice. *Ann. N.Y. Acad. Sci.*, **2006**, *1074*, 295-302.

[9] Hoffman, D.C.; Beninger, R.J. Preferential stimulation of D1 or D2 receptors disrupts food-rewarded operant responding in rats. *Pharmacol. Biochem. Behav.*, **1989**, *34*, 923-925.

[10] Ide, S.; Minami, M.; Satoh, M.; Uhl, G.R.; Sora, I.; Ikeda, K. Buprenorphine antinociception is abolished, but naloxone-sensitive reward is retained, in mu-opioid receptor knockout mice. *Neuropsychopharmacology*, **2004**, *29*, 1656-1663.

[11] Stanton, T.; Bolden-Watson, C.; Cusack, B.; Richelson, E. Antagonism of the five cloned human muscarinic cholinergic receptors expressed in CHO-K1 cells by antidepressants and antihistamines. *Biochem. Pharmacol.*, **1993**, *45*, 2352-2354.

[12] Fryer, J.D.; Lukas, R.J. Antidepressants noncompetitively inhibit nicotinic acetylcholine receptor function. *J. Neurochem.*, **1999**, *72*, 1117-1124.

[13] Maertens, C.; Droogmans, G.; Verbesselt, R.; Nilius, B. Block of volume-regulated anion channels by selective serotonin reuptake inhibitors. *Naunyn Schmiedebergs Arch. Pharmacol.*, **2002**, *366*, 158-165.

[14] Pariante, C.M.; Makoff, A.; Lovestone, S.; Feroli, S.; Heyden, A.; Miller A.H.; Kerwin, R.W. Antidepressants enhance glucocorticoid receptor function *in vitro* by modulating the membrane steroid transporters. *Br. J. Pharmacol.*, **2001**, *134*, 1335-1343.

[15] Finkel, M.S.; Laghriss-Thode, F.; Pollock, B.G.; Rong, J. Paroxetine is a novel nitric oxide synthase inhibitor. *Psychopharmacol. Bull.*, **1996**, *32*, 653-658.

[16] Kobayashi, T.; Washiyama, K.; Ikeda, K. Inhibition of G protein-activated inwardly rectifying K^+ channels by the antidepressant paroxetine. *J. Pharmacol. Sci.*, **2006**, *102*, 278-287.

[17] Kobayashi, T.; Washiyama, K.; Ikeda, K. Inhibition of G protein-activated inwardly rectifying K^+ channels by fluoxetine (Prozac). *Br. J. Pharmacol.*, **2003**, *139*, 1119-1128.

[18] Kobayashi, T.; Washiyama, K.; Ikeda, K. Inhibition of G protein-activated inwardly rectifying K^+ channels by various antidepressant drugs. *Neuropsychopharacology*, **2004**, *29*, 1841-1851.

[19] Ikeda, K.; Kobayashi, T.; Ichikawa, T.; Usui, H.; Abe, S.; Kumanishi, T. Comparison of the three mouse G-protein-activated K^+ (GIRK) channels and functional couplings of the opioid receptors with the GIRK1 channel. *Ann. N.Y. Acad. Sci.*, **1996**, *801*, 95-109.

[20] Ikeda, K.; Kobayashi, T.; Ichikawa, T.; Usui, H.; Kumanishi, T. Functional couplings of the δ- and the κ-opioid receptors with the G-protein-activated K^+ channel. *Biochem. Biophys. Res. Commun.*, **1995**, *208*, 302-308.

[21] Ikeda, K.; Kobayashi, K.; Kobayashi, T.; Ichikawa, T.; Kumanishi, T.; Kishida, H.; Ryoji, Y.; Manabe, T. Functional coupling of the nociceptin /orphanin FQ receptor with the G-protein-activated K^+ (GIRK) channel. *Mol. Brain. Res.*, **1997**, *45*, 117-126.

[22] North, R.A. Drug receptors and the inhibition of nerve cells. *Br. J. Pharmacol.*, **1989**, *98*, 13-28.

[23] Reuveny, E.; Slesinger, P.A.; Inglese, J.; Morales, J.M.; Iniguez-Lluhi, J.A.; Lefkowitz, R.J.; Bourne, H.; Jan, Y.; Jan, L.Y. Activation of the cloned muscarinic potassium channel by G protein βγ subunits. *Nature*, **1994**, *370*, 143-146.

[24] Kobayashi, T.; Ikeda, K.; Kojima, H.; Niki, H.; Yano, R.; Yoshioka, T.; Kumanishi, T. Ethanol opens G-protein-activated inwardly rectifying K^+ channels. *Nat. Neurosci.*, **1999**, *2*, 1091-1097.

[25] Lewohl, J.M.; Wilson, W.R.; Mayfield, R.D.; Brozowski, S.J.; Morrisett, R.A.; Harris, R.A. G-protein-coupled inwardly rectifying potassium channels are targets of alcohol action. *Nat. Neurosci.*, **1999**, *2*, 1084-1090.

[26] Kobayashi, T.; Washiyama, K.; Ikeda, K. Inhibition of G protein-activated inwardly rectifying K^+ channels by ifenprodil. *Neuropsychopharmacology*, **2006**, *31*, 516-524.

[27] Suzuki, T.; Kato, H.; Tsuda, M.; Suzuki, H.; Misawa, M. Effects of the non-competitive NMDA receptor antagonist ifenprodil on the morphine-induced place preference in mice. *Life Sci.*, **1999**, *64*, 151-156.

[28] Morgan, A.D.; Carroll, M.E.; Stoffel, M.; Wickman, K. Decreased cocaine self-administration in Kir3 potassium channel subunit knockout mice. *Neuropsychopharacology*, **2003**, *28*, 932-938.

[29] Ago, Y.; Harasawa, T.; Itoh, S.; Nakamura, S.; Baba, A.; Matsuda, T. Antidepressant-like effect of coadministration of sulpiride and fluvoxamine in mice. *Eur. J. Pharmacol.*, **2005**, *520*, 86-90.

Received: October 01, 2009 Revised: April 17, 2010 Accepted: May 26, 2010

OPEN ACCESS Freely available online

PLoS one

Essential Role of NMDA Receptor Channel ε4 Subunit (GluN2D) in the Effects of Phencyclidine, but Not Methamphetamine

Yoko Hagino[1], Shinya Kasai[1], Wenhua Han[1], Hideko Yamamoto[1], Toshitaka Nabeshima[2], Masayoshi Mishina[3], Kazutaka Ikeda[1]*

1 Division of Psychobiology, Tokyo Institute of Psychiatry, Tokyo, Japan, **2** Comparative Cognitive Science Institutes and Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya, Japan, **3** Department of Molecular Neurobiology and Pharmacology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Abstract

Phencyclidine (PCP), a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, increases locomotor activity in rodents and causes schizophrenia-like symptoms in humans. Although activation of the dopamine (DA) pathway is hypothesized to mediate these effects of PCP, the precise mechanisms by which PCP induces its effects remain to be elucidated. The present study investigated the effect of PCP on extracellular levels of DA (DA_{ex}) in the striatum and prefrontal cortex (PFC) using *in vivo* microdialysis in mice lacking the NMDA receptor channel ε1 or ε4 subunit (GluRε1 [GluN2A] or GluRε4 [GluN2D]) and locomotor activity. PCP significantly increased DA_{ex} in wildtype and GluRε1 knockout mice, but not in GluRε4 knockout mice, in the striatum and PFC. Acute and repeated administration of PCP did not increase locomotor activity in GluRε4 knockout mice. The present results suggest that PCP enhances dopaminergic transmission and increases locomotor activity by acting at GluRε4.

Citation: Hagino Y, Kasai S, Han W, Yamamoto H, Nabeshima T, et al. (2010) Essential Role of NMDA Receptor Channel ε4 Subunit (GluN2D) in the Effects of Phencyclidine, but Not Methamphetamine. PLoS ONE 5(10): e13722. doi:10.1371/journal.pone.0013722

Editor: Kenji Hashimoto, Chiba University Center for Forensic Mental Health, Japan

Received July 2, 2010; **Accepted** October 5, 2010; **Published** October 28, 2010

Funding: This work was supported by grants from the Ministry of Health, Labour and Welfare of Japan (H19-Iyaku-023, H22-Iyaku-015), the Ministry of Education, Culture, Sports, Science and Technology of Japan, The Naito Foundation, and The Mitsubishi Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ikeda-kz@igakuken.or.jp

Introduction

Phencyclidine (PCP) is a drug of abuse that causes psychosis resembling both the positive (e.g., hallucinations, paranoia) and negative (e.g., emotional withdrawal, motor retardation) signs of schizophrenia in humans [1]. Acute administration of PCP to rodents produces increases in locomotor activity, stereotypy, and ataxia [2,3]. Repeated PCP administration produces sensitization of locomotor activity, rearing, and stereotypy but tolerance to ataxia [3–5]. PCP acts as a noncompetitive antagonist of the *N*-methyl-D-aspartate (NMDA) excitatory amino acid receptor [6–8]. Additionally, high doses of PCP block dopamine (DA) reuptake [1,9–11]. Similar to PCP, amphetamine (AMPH) and its derivative methamphetamine (METH) produce behavioral sensitization to locomotor activity, rearing, and stereotypy when they are repeatedly administered [12,13]. Amphetamine and METH facilitate dopaminergic neurotransmission via a number of mechanisms [14], including DA efflux by reverse transport through the dopamine transporter (DAT) [15–18], inhibition of DA uptake [19–21], and inhibition of monoamine oxidase (MAO) activity [22–24].

The NMDA receptor channel subunit family is composed of seven subunits—GluRζ (GluN1), GluRε1-4 (GluN2A-D), and GluRχ1, 2 (GluN3A, B)—which are all products of separate genes [25]. In the rodent and human brains, GluRε1 and GluRε2 are predominant subunits expressed in the forebrain. GluRε3 is expressed largely in cerebellar granule cells and selectively in several other brain regions. GluRε4 is expressed in the diencephalon and midbrain and is more prominent during early development [26]. Highly active NMDA receptor channels are produced when the GluRζ subunit is expressed together with one of the four GluRε subunits in *Xenopus* oocytes and mammalian cells [27–30]. Four GluRε subunits are major determinants of the functional properties of NMDA receptor channels [31]. Noncompetitive NMDA receptor antagonists (i.e., PCP, ketamine, and SKF-10,047) block the four GluRε/GluRζ channels to similar extents in *Xenopus* oocytes [32]. Gene-targeting techniques provide an efficient method for clarifying the distinct functions of these NMDA receptor channel subunits. GluRε1 knockout mice display increased locomotor activity, whereas GluRε4 knockout mice exhibit reduced locomotor activity in a novel environment [33–36]. GluRε3 knockout mice show few apparent deficits [37–39]. Investigating the physiological functions of GluRζ or GluRε2 knockout mice, in contrast, is nearly impossible because these two mutants die shortly after birth [40–42].

To clarify the contributions of NMDA receptor channel subunits in the PCP-induced increases in extracellular levels of dopamine (DA_{ex}) and locomotor responses, we investigated the effects of METH and PCP on DA_{ex} in the striatum and prefrontal cortex (PFC) using *in vivo* microdialysis and measuring locomotor activity in GluRε1 knockout (GluRε1$^{-/-}$) and GluRε4 knockout (GluRε4$^{-/-}$) mice.

Results

Baseline DA_{ex} in the striatum and PFC in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice

Baseline DA_{ex} was not different between wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice in the striatum (one-way analysis of variance [ANOVA]: $F_{2,67}=0.412$, $p=0.664$) and PFC (one-way ANOVA: $F_{2,59}=1.025$, $p=0.365$). Mean baseline DA_{ex} in the striatum was 51.89±3.57 fmol/10 µl ($n=27$) for wildtype, 49.35±5.35 fmol/10 µl ($n=19$) for GluRε1$^{-/-}$, and 46.75±3.93 fmol/10 µl ($n=24$) for GluRε4$^{-/-}$ mice. Mean baseline DA_{ex} in the PFC was 1.29±0.20 fmol/10 µl ($n=23$) for wildtype, 1.59±0.30 fmol/10 µl ($n=20$) for GluRε1$^{-/-}$, and 1.10±0.21 fmol/10 µl ($n=19$) for GluRε4$^{-/-}$ mice.

Effects of acute METH administration on DA_{ex} in the striatum and PFC in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice

Methamphetamine (1 mg/kg) markedly increased DA_{ex} in the striatum and PFC in wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice (Fig. 1A, C). Two-way ANOVA (drug × genotype) of DA_{ex}, measured as the area-under-the-curve (AUC) calculated during a 180 min posttreatment period, revealed a significant effect of drug ($F_{1,39}=47.418$, $p<0.001$) but not genotype ($F_{2,39}=0.889$, $p=0.419$) and no significant drug × genotype interaction ($F_{2,39}=0.739$, $p=0.484$) in the striatum (Fig. 1B). Similarly, in the PFC, two-way ANOVA (drug × genotype) of AUC values revealed a significant effect of drug ($F_{1,31}=48.784$, $p<0.001$) but not genotype ($F_{2,31}=0.320$, $p=0.728$) and no significant drug × genotype interaction ($F_{2,31}=0.201$, $p=0.819$) (Fig. 1B).

Effects of acute PCP administration on DA_{ex} in the striatum and PFC in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice

Phencyclidine (3 mg/kg) markedly increased DA_{ex} in wildtype and GluRε1$^{-/-}$ mice, but not in GluRε4$^{-/-}$ mice, in the striatum and PFC (Fig. 2A, C). Two-way ANOVA (drug × genotype) of AUC values revealed a significant effect of drug ($F_{1,39}=17.201$, $p<0.001$) but not genotype ($F_{2,39}=2.012$, $p=0.147$) in the striatum and a significant drug × genotype interaction ($F_{2,39}=3.314$, $p=0.047$) (Fig. 2B). *Post hoc* comparisons revealed that the effect of PCP on DA_{ex} in GluRε4$^{-/-}$ mice was significantly less compared with wildtype and GluRε1$^{-/-}$ mice ($p=0.002$ and 0.03, respectively; Fisher's Protected Least Significant Difference [PLSD] *post hoc* test) in the striatum (Fig. 2B). In the PFC, two-way ANOVA (drug × genotype) of AUC values revealed a significant effect of drug ($F_{1,37}=35.215$, $p<0.001$) but not genotype ($F_{2,37}=1.969$, $p=0.154$) and a significant drug × genotype interaction ($F_{2,37}=3.326$, $p=0.047$) (Fig. 2D). *Post hoc* comparisons revealed that the effect of PCP on DA_{ex} in GluRε4$^{-/-}$ mice was significantly less compared with wildtype and GluRε1$^{-/-}$ mice ($p=0.007$ and 0.003, respectively; Fisher's PLSD *post hoc* test) in the PFC (Fig. 2D).

Locomotor activity in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice in a novel environment

Locomotor activity in a novel environment was different between wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice during the habituation period (mixed-design ANOVA: genotype, $F_{2,123}=35.423$, $p<0.0001$; time, $F_{2,123}=486.554$, $p<0.0001$; genotype × time, $F_{4,123}=15.337$, $p<0.0001$) (Fig. 3). Locomotor activity in a novel environment during the 60 min period increased in GluRε1$^{-/-}$ mice ($p=0.0002$, unpaired *t*-test) but decreased in GluRε4$^{-/-}$ mice ($p<0.0001$, Student's *t*-test) compared with wildtype mice. GluRε1$^{-/-}$ mice did not habituate during the 180 min period compared with wildtype mice ($p<0.0001$, Student's *t*-test).

Effects of acute administration of METH and PCP on locomotor activity in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice

Two-way ANOVA (drug × genotype) of locomotor activity data during the 60 min period revealed significant effects of drug

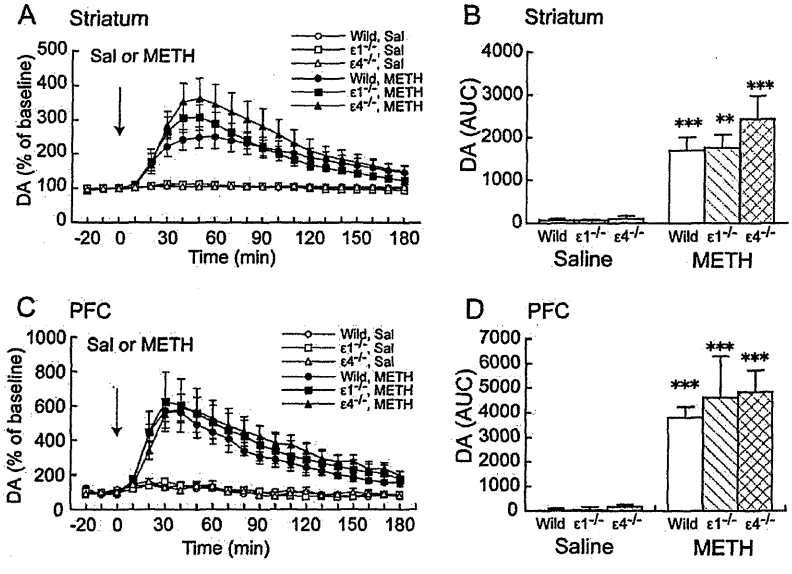

Figure 1. Effects of acute METH on DA_{ex} in the striatum and PFC in wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice. (**A, C**) Temporal pattern of DA_{ex} before and after s.c. saline (Sal) or METH (1 mg/kg) injection. The arrows indicate the drug injection time. Each point represents the mean ± SEM of the percentage of DA_{ex} baseline. (**B, D**) Histogram representing the mean AUC ± SEM of DA_{ex} during the 180 min period after saline or METH injection ($n=5$–9). **$p<0.01$, ***$p<0.001$, compared with saline group of the same genotype (two-way ANOVA followed by Fisher's PLSD *post hoc* test).

doi:10.1371/journal.pone.0013722.g001

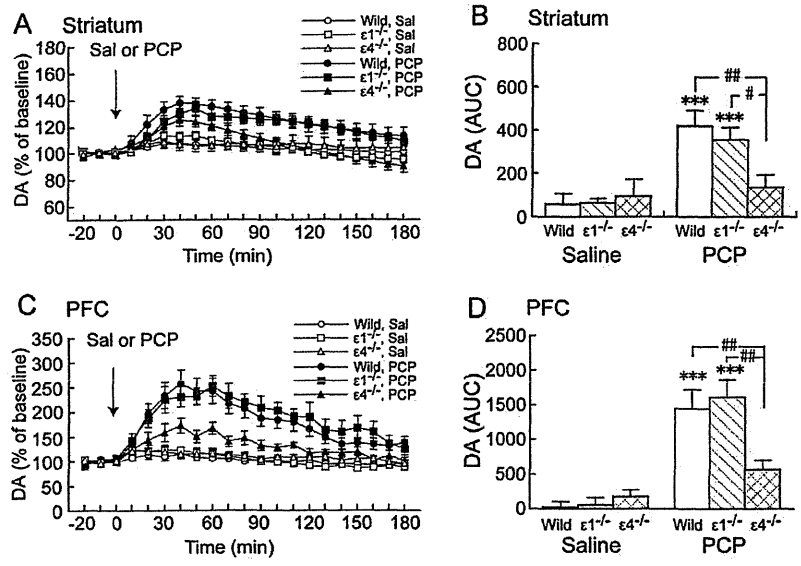

Figure 2. Effects of acute PCP on DA_{ex} in the striatum and PFC in wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice. (**A, C**) Temporal pattern of DA_{ex} before and after s.c. saline (Sal) or PCP (3 mg/kg) injection. The arrows indicate the drug injection time. Each point represents the mean ± SEM of the percentage of DA_{ex} baseline. (**B, D**) Histogram representing the mean AUC ± SEM of DA_{ex} during the 180 min period after saline or PCP injection (n = 5–11). ***p<0.001, compared with saline group of the same genotype; $^{\#}p$<0.05, $^{\#\#}p$<0.01, comparisons between genotypes in the same drug treatment (two-way ANOVA followed by Fisher's PLSD *post hoc* test).
doi:10.1371/journal.pone.0013722.g002

($F_{2,155}$ = 8.646, p = 0.0002) and genotype ($F_{2,155}$ = 11.769, p<0.0001) and a significant drug × genotype interaction ($F_{4,155}$ = 5.734, p = 0.0002) (Fig. 4). Methamphetamine (1 mg/kg) significantly increased locomotor activity during the 60 min period after the METH injection in wildtype mice (p = 0.002, Student's t-test) and GluRε4$^{-/-}$ mice (p = 0.0004, Student's t-test) compared with saline. However, METH (1 mg/kg) did not increase locomotor activity during the 60 min period after the METH injection in GluRε1$^{-/-}$ mice (p = 0.411, Student's t-test) compared with saline.

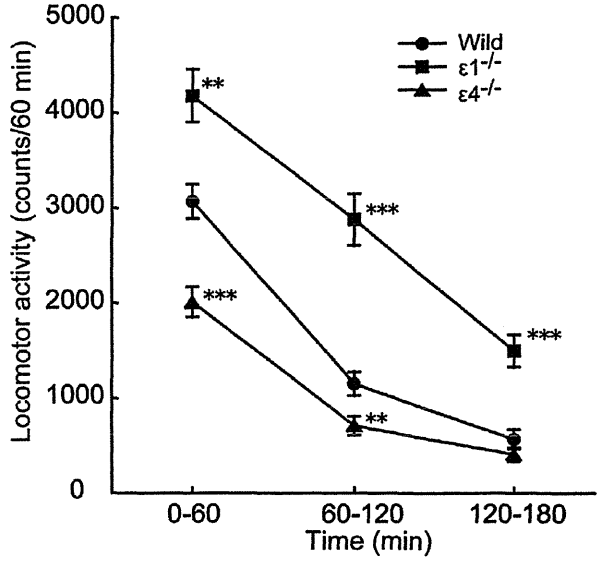

Figure 3. Locomotor activity in wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice in a novel environment. Locomotor activity was measured for 180 min. Each point represents the mean ± SEM (n = 34–50). *p<0.05, **p<0.01, ***p<0.001, compared with wildtype mice (one-way ANOVA followed by Fisher's PLSD *post hoc* test).
doi:10.1371/journal.pone.0013722.g003

Phencyclidine (3 mg/kg) significantly increased locomotor activity during the 60 min period after the PCP injection in wildtype mice (p = 0.008, Student's t-test) and GluRε1$^{-/-}$ mice (p = 0.045, Student's t-test) compared with saline treatment. However, PCP (3 mg/kg) did not increase locomotor activity in GluRε4$^{-/-}$ mice (p = 0.142, unpaired t-test) compared with saline treatment.

Effects of repeated administration of METH and PCP on locomotor activity in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice

Mixed-design ANOVA of locomotor activity data during the 60 min period after the METH injection from Session 1 to 8 revealed significant effects of genotype ($F_{2,385}$ = 3.350, p = 0.042) and session ($F_{7,385}$ = 16.091, p<0.0001) but no significant genotype × session interaction ($F_{14,385}$ = 0.611, p = 0.857) (Fig. 5A). Chronic METH (1 mg/kg) injections increased locomotor activity in wildtype (p<0.0001, paired t-test), GluRε1$^{-/-}$ (p = 0.0007, paired t-test), and GluRε4$^{-/-}$ mice (p = 0.0001, paired t-test) in Session 1 compared with Session 8.

Mixed-design ANOVA of locomotor activity data during the 60 min period after the PCP injection revealed a significant effect of genotype ($F_{2,455}$ = 11.318, p<0.0001) but not session ($F_{7,455}$ = 1.443, p = 0.186) and a significant genotype × session interaction ($F_{14,455}$ = 2.368, p = 0.0035) (Fig. 5B). Phencyclidine-induced hyperactivity was significantly greater in Session 8 than Session 1 in wildtype mice (p = 0.006, paired t-test). Repeated PCP (3 mg/kg) administration did not increase locomotor activity in GluRε1$^{-/-}$ mice (p = 0.121, paired t-test) and GluRε4$^{-/-}$ mice (p = 0.605, paired t-test) in Session 1 compared with Session 8.

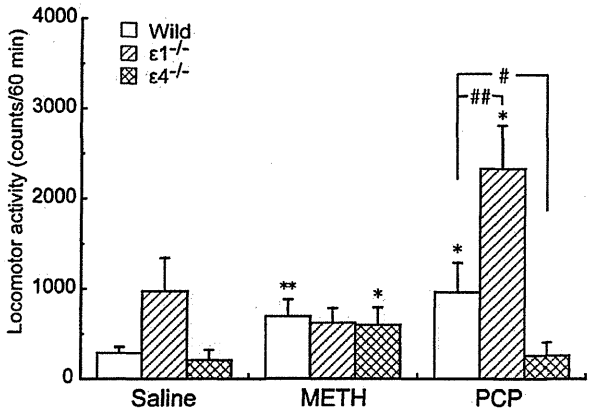

Figure 4. Effects of acute METH and PCP on the locomotor activity in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice. Locomotor activity after acute saline, METH (1 mg/kg), or PCP (3 mg/kg) administration ($n = 10$–25). *$p<0.05$, **$p<0.01$, compared with saline (Student's t-test); #$p<0.05$, ##$p<0.01$, compared with wildtype (Student's t-test).
doi:10.1371/journal.pone.0013722.g004

Discussion

The present study showed that PCP-induced increases in DA_{ex} in the striatum and PFC and locomotor activity were absent in GluRε4$^{-/-}$, but present in GluRε1$^{-/-}$, mice, indicating that GluRε4 plays an important role in PCP-increased DA_{ex} and locomotor activity. Phencyclidine exerts psychotomimetic effects, whereas another NMDA receptor antagonist, MK-801, exerts no clear psychotomimetic effects in humans [43]. Interestingly, whereas MK-801 suppresses GluRε3/GluRζ1 and GluRε4/GluRζ1 channels more weakly than GluRε1/GluRζ1 and GluRε2/GluRζ1 channels, PCP blocks the four GluRε/GluRζ channels to similar extents in *Xenopus* oocytes [32]. The absence of psychotomimetic effects of MK-801 may be attributable to its weak ability of blocking the GluRε4/GluRζ1 channel.

Systemic administration of PCP reportedly increases DA_{ex} in the striatum and PFC [44–49]. Similarly, PCP (3 mg/kg) increased DA_{ex} in wildtype and GluRε1$^{-/-}$ mice in the present study. However, PCP failed to increase DA_{ex} in the striatum and PFC in GluRε4$^{-/-}$ mice. Phencyclidine is known to be a DA reuptake blocker and a noncompetitive NMDA antagonist [9–11]. It inhibits DA uptake by binding to the DAT at doses approximately 10-fold greater than those at which it binds to NMDA receptor channels [1]. Phencyclidine at the low dose used in the present study appears to have few effects on the DAT. Furthermore, no PCP-induced increases in DA_{ex} in GluRε4$^{-/-}$ mice that possess an intact DAT gene indicates that PCP increases DA_{ex} not via DAT inhibition but via blockade of NMDA receptor channels. The present results support the hypothesis that GluRε4 is an important determinant of increased DA_{ex} induced by PCP. Acute administration of METH increased DA_{ex} in the striatum and PFC in wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice. No differences in DA_{ex} increases were found between genotypes. The similar DA_{ex} increases among these mice in response to acute METH challenge suggest that increased DA_{ex} occurs independently of GluRε1$^{-/-}$ and GluRε4$^{-/-}$.

Locomotor activity in a novel environment is reportedly high in GluRε1$^{-/-}$ mice [34,36] and low in GluRε4$^{-/-}$ mice [33,35]. Consistent with these findings, increased locomotor activity in GluRε1$^{-/-}$ mice and reduced locomotor activity in GluRε4$^{-/-}$ mice were observed in the present study. GluRε1$^{-/-}$ mice did not habituate during the 180 min period compared with wildtype mice. Interestingly, acute METH administration decreased locomotor activity in GluRε1$^{-/-}$ mice. Hyperactivity and a paradoxical response to METH suggest that GluRε1$^{-/-}$ mice may be an animal model of attention-deficit/hyperactivity disorder.

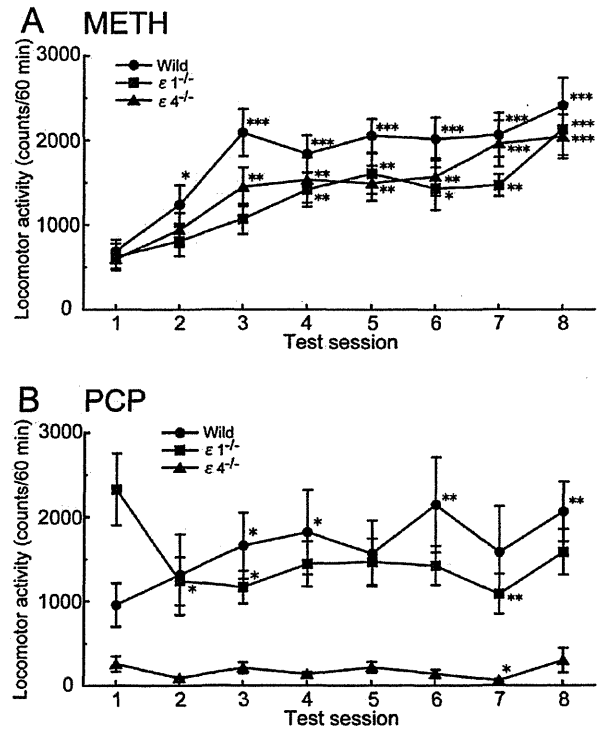

Figure 5. Effects of repeated METH and PCP on the locomotor activity in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice. Changes in response to repeated administration of (**A**) METH (1 mg/kg) or (**B**) PCP (3 mg/kg) ($n = 15$–25). *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared with Session 1 of the same genotype (paired t-test). Each point represents total locomotor activity (mean ± SEM) during the 60 min period after METH or PCP injection.
doi:10.1371/journal.pone.0013722.g005

Psychostimulants, such as METH and PCP, increase locomotor activity [2,3,12,13]. In GluRε4$^{-/-}$ mice, acute METH administration increased locomotor activity, but PCP did not. Acute PCP administration increased locomotor activity in wildtype and GluRε1$^{-/-}$ mice, but not in GluRε4$^{-/-}$ mice. The absence of locomotor-stimulating effects of PCP in GluRε4$^{-/-}$ mice indicates that locomotor responses to PCP require the GluRε4 subunit.

Repeated administration of PCP produces sensitization to its locomotor-stimulating effects in wildtype mice. In GluRε4$^{-/-}$ mice, locomotor activity did not increase after repeated PCP treatment. Acute PCP did not increase locomotor activity, and repeated PCP did not produce sensitization to the locomotor-stimulating effects of PCP in GluRε4$^{-/-}$ mice. The GluRε4 subunit appears to be necessary for behavioral sensitization to occur in response to repeated PCP administration. A previous study demonstrated that acute PCP treatment increased locomotor activity in wildtype and GluRε1$^{-/-}$ mice. Chronic PCP treatment at a low dose (3 mg/kg/day) for 7 days produced sensitization to the locomotor-stimulating effects of PCP in wildtype mice, but not

in GluRε1$^{-/-}$ mice [50]. The present study confirmed that repeated PCP administration (3 mg/kg/day) did not produce sensitization during Session 8 in GluRε1$^{-/-}$ mice. Repeated METH administration produced behavioral sensitization in wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice. The development of sensitization in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice was delayed compared with wildtype mice. The noncompetitive NMDA receptor antagonist MK-801 has been shown to block the development of behavioral sensitization to AMPH and METH [51–54]. Molecular and cellular adaptive changes during chronic drug exposure are hypothesized to lead to the development of sensitization. Our findings support the hypothesis that adaptive changes through NMDA receptor channels play a role in the development of locomotor sensitization to METH.

Schizophrenia is a disease that has been hypothesized to be associated with hyperfunction of the dopaminergic neuronal system and dysfunction of glutamatergic transmission [55,56]. Administration of PCP to normal humans induces symptoms similar to those of schizophrenia [57]. This finding has been replicated over the years, and PCP has been shown to exacerbate the primary symptoms of schizophrenic patients [56]. Phencyclidine-treated animals have been used as an animal model of schizophrenia, and the amelioration of hyperlocomotion in these animals has been used as a screening test to assess the efficacy of antipsychotic drugs [58,59]. GluRε4 immunoreactivity and protein expression increase in the frontal cortex following repeated PCP treatment, whereas GluRε1 immunoreactivity and protein expression are not altered in rats [60]. Furthermore, polymorphisms of several genes known to interact with NMDA receptor channels are related to altered risk for schizophrenia, and psychotic patients display changes in the levels of mRNA encoding NMDA receptors [61]. Interestingly, Makino *et al.* reported that the GluRε4 gene locus is a possible genomic region that contributes to schizophrenia susceptibility in a Japanese population [62]. In the present study, we first demonstrated that deletion of GluRε4 abolished PCP-induced hyperlocomotion and potentiated the increases in DA_{ex} in mice. Our data and previous findings suggest that GluRε4 might be a potential target for antipsychotic drug development.

Although NMDA receptor channels are highly expressed in adult brains, adult GluRε4 expression is very limited [26]. GluRε4 is expressed in the substantia nigra compacta (SNc), subthalamic nucleus, globus pallidus, and ventral pallidum in adult rats [63]. Jones and Gibb reported that functional GluRε2 and GluRε4 subunits form somatic NMDA receptors, possibly as triheteromeric receptors, whereas no somatic GluRε1 subunits are present in SNc dopaminergic neurons in rats aged postnatal day 14 [64]. A small subset of NMDA receptor channels (i.e., channels containing GluRε4) may be implicated in the effects of PCP on DA_{ex} and locomotor activity. This possibility is consistent with the lack of psychotic effects of ifenprodil, a selective blocker of NMDA receptor channels containing GluRε2, which is highly expressed in adult brains. Additionally, GluRε4 is highly expressed in the brain during development [26], suggesting that GluRε4 knockout during the developmental stage may alter neuronal function in the adult brain. Although the expression of the genes related to dopaminergic signaling pathways are not altered in GluRε4$^{-/-}$ mice during adulthood (see Table S1), other developmental changes may alter the effects of PCP in GluRε4$^{-/-}$ mice. Further studies of synapses, neurons, and neuronal networks regulated by GluRε4 and developmental changes in neuronal function in GluRε4$^{-/-}$ mice may lead to a better understanding of the mechanisms underlying PCP-induced psychosis and schizophrenia.

Materials and Methods

Ethics statement

The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee (Animal Experimentation Ethics Committee of Tokyo Institute of Psychiatry, Approval ID: 22-2), and all animal were cared for and treated humanely in accordance with our institutional animal experimentation guidelines.

Animals

Wildtype and GluRε1$^{-/-}$ or GluRε4$^{-/-}$ mouse littermates from crosses of heterozygous/heterozygous GluRε1 or GluRε4 knockout mice, respectively, on a C57BL/6 genetic background [33,65] served as subjects. Naive adult mice were housed in an animal facility maintained at 22±2°C and 55±5% relative humidity under a 12 h/12 h light/dark cycle with lights on at 8:00 am and off at 8:00 pm. Food and water were available *ad libitum*. In the behavioral experiments, 13- to 23-week-old male mice were used. In the microdialysis experiments, 10- to 24-week-old male and female mice were used.

Surgery

Microdialysis probes were stereotaxically implanted in mice under sodium pentobarbital anesthesia (50 mg/kg, intraperitoneally) in the striatum (anterior, +0.6 mm; lateral, +1.8 mm; ventral, −4.0 mm from bregma) or PFC (anterior, +2.0 mm; lateral, +0.5 mm; ventral −3.0 mm from bregma), according to the atlas of Franklin and Paxinos [66]. The probe tip was constructed with a regenerated cellulose membrane (outer diameter, 0.22 mm; membrane length, 2 mm; Eicom, Kyoto, Japan). All dialysis probe placements were verified histologically at the completion of the experiment.

Microdialysis and analytical procedures

Twenty-four hours 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). Dialysate DA was 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) containing 500 mg/l sodium decanesulfonate, 50 mg/l EDTA, and 1% methanol. Perfusion was initiated 180 min prior to the collection of baseline samples. Baseline levels of DA_{ex} were obtained from the average concentrations of three consecutive samples when they were stable. The DA detection limit of the assay was 0.3 fmol/sample with a signal-to-noise ratio of 2.

Locomotor activity measurements

Each mouse were exposed to an illuminated chamber (30×40×25 cm) at an ambient temperature of 22±2°C, and locomotor activity was measured with Supermex (Muromachi Kikai, Tokyo, Japan), a sensor monitor mounted above the chamber. In this system, a sensor detects the radiated body heat of an animal [67]. This measurement system can detect changes in heat across multiple zones of the chamber and count all horizontal movements. All counts were automatically summed and recorded every 5 min. After a 180 min habituation period, METH or PCP was administered subcutaneously (s.c.), and locomotor activity was monitored continuously for 180 min.