

Selective overexpression of *Comt* in prefrontal cortex rescues schizophrenia-like phenotypes in a mouse model of 22q11 deletion syndrome

S Kimoto^{1,2,5}, K Muraki^{1,5}, M Toritsuka^{1,2,5}, S Mugikura³, K Kajiwara³, T Kishimoto², E Illingworth⁴ and K Tanigaki¹

The 22q11.2 microdeletion is one of the highest genetic risk factors for schizophrenia. It is not well understood which interactions of deleted genes in 22q11.2 regions are responsible for the pathogenesis of schizophrenia, but *catechol-O-methyltransferase (COMT)* is among the candidates. *Df1/+* mice are 22q11.2 deletion syndrome (22q11DS) model mice with a hemizygous deletion of 18 genes in the 22q11-related region. *Df1/+* mice showed enhanced response to the dopamine D1 agonist, SKF38393, and the *N*-methyl-D-aspartate antagonist, MK801, which can be normalized by a GABA_A receptor agonist, bretazenil, or a GABA_A $\alpha 2/\alpha 3$ receptor agonist, SL651498. Here, we demonstrated the curing effects of virus-mediated reintroduction of *Comt* to the prefrontal cortex (PFC) in *Df1/+* mice. In contrast, both *Comt* overexpression and *Comt* inhibition caused an abnormal responsiveness to Bretazenil, a GABA_A receptor agonist in control mice. *Comt* overexpression increased MK801-induced interneuronal activation and GABA release in the PFC. The expression levels of GABA-related genes such as *Gabrb2* (GABA_A receptor $\beta 2$), *Gad2* (glutamic acid decarboxylase 65 (*Gad65*)) and *Reln* (*Reelin*) correlate with a *Comt* expression level in PFC. Our data suggest that *Comt*-mediated regulation of GABAergic system might be involved in the behavioral pathogenesis of *Df1/+* mice.

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Introduction

The 22q11.2 deletion syndrome (22q11DS) is known to be one of the highest risk factors for developing schizophrenia, and approximately one-fourth of 22q11DS patients develop schizophrenia.^{1–3} All of the genes, except for one gene in human 22q11.2 region, exist on mouse chromosome 16.⁴ Mice with a hemizygous deletion of 22q11.2-related region show schizophrenia-related behavioral abnormalities such as working memory deficits and sensory information-processing deficits.^{5,6} These animal models of 22q11DS provide an opportunity to explore possible therapeutic strategies for this specific subtype of schizophrenia. The 22q11.2 deletion contains 35 known genes at least. However, it is not known which of these genes are responsible for the psychiatric disorders of 22q11DS. The cumulative effects of the deletion of more than one gene might be responsible for the increased risk for schizophrenia of 22q11.2 DS. An epistatic interaction between two genes in 22q11.2 region, *proline dehydrogenase (Prodh)* and *catechol-O-methyltransferase (Comt)*, has been reported.⁷ Pharmacological inhibition of *Comt* activity exacerbates behavioral abnormalities of *Prodh* knockdown mice.⁷

Comt is an enzyme that methylates catechol structures of dopamine (DA), norepinephrine (NE), caffeine and catechol estrogens. *Comt* plays a pivotal role in DA metabolism, specifically in the prefrontal cortex (PFC), because DA

transporter is expressed in other brain regions, such as a striatum, and eliminates released DA even in the absence of *Comt*.^{8,9} In *Comt* knockout (KO) male mice, two- to three-fold increases in DA were observed specifically in the PFC but not in other brain regions, and NE levels were not affected,¹⁰ because the NE transporter is abundant in the PFC.^{11,12} Administration of tolcapone, a specific brain-penetrant COMT inhibitor, causes an accumulation of 3,4-dihydroxy-phenylacetic acid (DOPAC) but has no effect on extracellular DA and NE, specifically in the PFC.¹³

Human genetic studies of functional polymorphisms of *Comt* such as Val¹⁵⁸Met have suggested that deficiency in COMT activity might reduce cognitive function and cause psychiatric symptoms in 22q11DS,^{14–17} although other studies have reported controversial results.^{18–20} This might be caused by U-shaped effects of PFC DA level on cognitive functions.^{21,22} Animal model studies using *COMT*-overexpressing transgenic mice and *Comt* KO mice have confirmed these complex effects of PFC DA levels, and have shown that either too little or too much DA in the PFC has impaired the PFC functions such as working memory and recognition memory.²³

Comt is known to be also expressed in embryonic mouse brain,²⁴ and DA receptor KO mice show abnormal morphology of dendrites of PFC projection neurons, increased parvalbumin expression in PFC interneurons and reduced

¹Research Institute, Shiga Medical Center, Morioka, Shiga, Japan; ²Department of Psychiatry, Nara Medical University Faculty of Medicine, Kashihara, Nara, Japan;

³Division of Basic Medical Science and Molecular Medicine, School of Medicine, Tokai University, Isehara, Kanagawa, Japan and ⁴University of Salerno, Fisciano, Italy

Correspondence: Dr K Tanigaki, Research Institute, Shiga Medical Center, Morioka 5-4-30, Morioka, Shiga 524-8524, Japan.

E-mail: tanigaki@res.med.shiga-pref.jp

⁵These authors contributed equally to this work.

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mesencephalic dopaminergic neurons.^{25,26} These data might suggest a possibility that *Comt* affects working memory and recognition memory through the regulation of neurodevelopmental process. To address whether adult functions of *Comt* is responsible for behavioral defects in *Df1/+* mice, one of 22q11DS model mice,⁵ we examined the therapeutic potential of lentivirus-mediated *Comt* overexpression in the adult PFC of *Df1/+* mice.

Materials and methods

Generation and genotyping of *Dgcr2* KO mice. *Dgcr2* genomic fragments were cloned by screening of a phage library containing 129/Sv mouse DNA fragment. To construct a targeting with a 5.6-kb 5'-recombinogenic arm and a 1.2-kb 3'-recombinogenic arm, EGFP-SV40-polyA—a neomycin phosphotransferase-expressing cassette (EGFP-Neo) — was inserted between *SmaI* and *XhoI* in exon 1 to replace the region between the two restriction sites. A diphtheria toxin A-expressing cassette was flanked outside the 3' homologous region. The vector was linearized by restriction digestion and used to transfect embryonic stem cell line E14 by electroporation. Transfected cells were cultured in the presence of G418. Upon successful targeting, the neomycin expression cassette replaced exon 1 and the initiation codon of all the transcripts of *Dgcr2*. Therefore, it is expected to disrupt all *Dgcr2* transcripts (Figure 2). Correct targeting through homologous recombination in embryonic stem cells was confirmed by PCR and Southern blot. PCR for the KO allele was performed using the following primers: wild-type forward primer 5'-TTCCTGCTGGTTCTCACTGT-3', reverse primer 5'-TCAAGGTCCCATTACTCCCTC-3' and neo primer 5'-TATTGCTGAAGAGCTTGCG-3'. The wild-type allele produces a 1.6-kb band, whereas the targeted allele produces a 1.4-kb band. For Southern blotting, isolated genomic DNA was digested with *EcoRI*. The 7.5 and 5.8 kb fragments corresponding to the wild-type and KO allele were identified by hybridization with the 5' internal probe (Probe A, Figure 2b).

Animals. Generation of *Df1/+* 22q11DS model mice was previously described in detail.^{5,27} Mice were maintained on the C57Bl/6 genetic background for at least 11 generations. Male, 2–3-month-old mice were used for behavioral analysis. Mouse colonies were maintained in accordance with the protocols approved by the Committee on Animal Research at Research Institute, Shiga Medical Center.

Reverse transcription PCR (RT PCR) analysis. The following primers were used to amplify target: *Dgcr2*; 5'-TTCCTGCTGGTTCTCACTG-3' and 5'-GTGGAGAAGGTAGCAAGTGA-3'; β -actin; and Mouse β -Actin Control Amplimer Set (Clontech, Mountain View, CA, USA).

Cloning and production of lentiviral vectors. Cytomegalovirus (CMV)-IE/chicken β -actin promoter (CAG), 1.6-kb fragment cut from pXCAG, mouse α -calcium/calmodulin-dependent protein kinase II (CaMKII) promoter 1.2-kb fragment and mouse membrane-bound-type *Comt*

were introduced to FUGW lentiviral vector and lentiviruses were produced as previously described.²⁸ Briefly, human embryonic kidney 293T cells were transfected by using the Lipofectamine 2000 (Invitrogen, Tokyo, Japan) with the lentiviral vector and two helper plasmids, Δ 8.9 and VSVG. After 48 h, the supernatants were spun at $780 \times g$ for 5 min, filtered through a $0.45 \mu\text{m}$ pore size filter (Millipore, Billerica, MA, USA) to remove cell debris, spun at $83\,000 \times g$ for 1.5 h, and the pellet was resuspended in $100 \mu\text{l}$ of phosphate-buffered saline. For virus titration, HEK293T cells were infected with lentiviruses in decreasing concentrations. At 72 h after infection, cells were fixed in 4% paraformaldehyde and lentivirus-mediated *Comt*/EGFP expression was estimated by immunofluorescent analysis. Concentrated lentiviral titers ranged from 1×10^8 to 1×10^9 transducing units (TU) per ml.

Stereotaxic surgery. Injections of lentiviruses were performed stereotaxically in 8- to 10-week-old mice.

Mice were anesthetized using 2.5% Avertin solution and stereotaxic surgery was performed under standard conditions. The coordinate of the injection site was 2.0 mm anterior and 0.70 mm lateral to bregma at a depth of 1.75 and 2.25 mm at an 8° angle, and 2.4 mm anterior and 0.50 mm lateral to bregma at a depth of 1.13 mm at an 8° angle. All procedures were performed according to the guidelines on animal experiments of Shiga Medical Center. A total of $0.5 \mu\text{l}$ of purified virus was delivered on each side over a 3-min period. Lentivirus-infected mice were used for experiments at least 2 weeks after surgical operations for recovery. For verification of injection sites, $30 \mu\text{m}$ thick sections were cut using a cryostat at the level of PFC. Lentivirus infection was verified by immunohistochemical analysis of *Comt* for Lenti-CAG *Comt* or Lenti-CaMKII-*Comt* vector, and EGFP for Lenti-CAG-GFP vector.

Open field test. Locomotor activity was measured in the open field activity chambers ($40 \times 40 \times 40 \text{ cm}$) equipped with photocells (beam spacing 2.5 cm) for the automatic recording of horizontal and vertical activity. Stereotypy count was analyzed using the VersaMax system (Accuscan Instruments, Columbus, OH, USA). Stereotypy was recorded when the mouse broke the same beams repeatedly. Stereotypy count is the number of beam breaks during stereotypic activity. Data were collected for 60 min.

Quantification of prefrontal cortical catecholamines and their metabolites. The mice were decapitated 2 weeks after lentiviral infection and the brains were quickly removed. Wet prefrontal cortical tissues were homogenized in two volumes of 0.2 M perchloric acid buffer containing $100 \mu\text{M}$ EDTA 2Na and $1 \mu\text{g ml}^{-1}$ isoproterenol as an internal standard ($500 \mu\text{l}$ per 100 mg wet weight tissue) and kept on ice for 30 min. Homogenates were centrifuged at $15\,000 \text{ r.p.m.}$ for 15 min at 4°C . The supernatants were collected, adjusted to pH 3 with $3\text{M CH}_3\text{COONa}$ and analyzed by the high-performance liquid chromatography system with an electrochemical detector (HTEC-500, Eicom, Kyoto, Japan) for the analysis of DA, DOPAC, homovanillic acid (HVA), serotonin and 5-hydroxy-indoleacetic acid (5-HIAA). The column used for separation was a SC-50DS (Eicom). The mobile phase was

0.1 M sodium phosphate buffer (0.1 M NaH₂PO₄:0.1 M Na₂HPO₄=1000:160, v/v), 1% methanol, 500 mg l⁻¹ sodium sulfonate, and 50 mg l⁻¹ EDTA.

Cannula implantation. The mice were anesthetized with 2.5% Avertin solution and stereotaxic surgery was performed under standard conditions. The guide cannula (AG-2, Eicom) with length 2 mm, inner diameter 0.4 mm and outer diameter 0.5 mm was implanted into the PFC (A +2.2 mm, L 0.5 mm and V 1 mm from the bregma) and fixed to the skull with dental cement. Following surgery, the animals were returned to their home cage and allowed to recover for 1 day before beginning of the experiment.

Microdialysis and high-performance liquid chromatography analysis. The microdialysis probe (A-1-4-02, Eicom) with an active dialysis membrane (2 mm long, i.d. 0.20 mm, o.d. 0.22 mm, cut-off value 50 kDa) was inserted carefully into the PFC. The implanted microdialysis probe was connected to a 2.5 ml gastight syringe (Hamilton, Tokyo, Japan) and perfused with Ringer's solution (147 mM NaCl, 4 mM KCl and 2.3 mM CaCl₂) at a constant flow of 1 ml min⁻¹ using a microperfusion pump (ESP-32, Eicom) via a swivel system (Eicom), allowing the mice to move freely in a Plexiglas box (30 cm × 30 cm × 40 cm). The dialysate collected for the first 2 h was discarded to ensure a stable baseline of DA and γ -aminobutyric acid (GABA) release.

The DA content in the dialysate was quantified in the same way as for tissue homogenates. GABA content in the dialysate was quantified by high-performance liquid chromatography with electrochemical detector after derivatization of the amino acids with α -phthalaldehyde (OPA) and 2-mercaptoethanol solution (Eicom). A total of 20 μ l samples of perfusate were collected at 20 min intervals. One volume (5 ml) of 4 mM OPA was added to three volumes of dialysate and allowed to react for 2.5 min at room temperature. Then, the sample of the perfusate/OPA solution was injected into the high-performance liquid chromatography/electrochemical detector with a graphite electrode set at 600 mV against an Ag/AgCl reference electrode (Eicom). GABA was separated on SC-5 ODS column (2.1 mm × 150 mm, Eicom). The mobile phase consisted of 0.1 M phosphate buffer, 0.013 mM EDTA 2Na, pH 6.0, with 30% MeOH pumped at a flow rate of 0.23 ml min⁻¹. The basal output was defined as the average of three samples before drug administration. The results were calculated as percentage of the baseline output of DA and GABA.

Immunohistochemical analysis. Mice were injected with MK801 (0.32 mg kg⁻¹) intraperitoneally (i.p.) 2 h before killing for immunohistochemical analysis of c-Fos staining. Mice were perfused transcardially by 4% paraformaldehyde and 0.05% glutaraldehyde. The serial 30 μ m cryostat sections were incubated with primary antibodies for 24 h at 4 °C after blocking for 30 min at room temperature with 5% donkey serum (Chemicon, Temecula, CA, USA) and for 1 h at room temperature with secondary antibodies (Molecular Probes, Eugene, OR, USA, and Vector Labs, Burlingame, CA, USA). The primary antibodies used were anti-Comt (Santa Cruz, Santa Cruz, CA, USA), anti-S100 β (Sigma-Aldrich, St Louis, MO, USA), anti-NeuN (Chemicon), anti-c-Fos (Ab5,

Calbiochem, San Diego, CA, USA), anti-GABA (GB69, Sigma-Aldrich) and anti-Gad67 (Chemicon). Peroxidase activity was visualized by Vectastain Elite ABC Kit (Vector Labs). Slides were examined with a Zeiss microscope (Axiovert200M) (Zeiss, Tokyo, Japan) or an Olympus confocal laser scanning microscope (FV-300, Olympus, Tokyo, Japan). For the quantification of c-Fos-positive cells, the locations of prelimbic cortices of the PFC were defined according to the mouse brain in stereotaxic coordinates (2001, Academic Press, San Diego, CA, USA). The c-Fos-immunoreactive cells in the regions of interest were counted bilaterally in four serial sections matched for rostrocaudal level by bregma distance per mice. For the quantification of Gad67 and GABA immunofluorescence, the perisomatic (puncta rings) or neuropil areas were delineated and then pixel density within the delineated area was calculated and averaged as described previously. At least four sections from four mice were analyzed.

Quantitative RT-PCR. Complementary DNA was obtained using a PrimeScript 1st strand cDNA synthesis kit (Takara, Shiga, Japan). Intron spanning Taqman probes were designed using the Roche Universal Probe Library method. Amplifications were run in a LightCycler 480 system (Roche, Tokyo, Japan). All data were analyzed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels as reference.

The following primers were employed: *Gabrb2*, 5'-CGAT GGCACCTGCTGTATG-3' and 5'-ATACCGCCTTAGGTCC ATCA-3', probe: human #27; *Gad2*, 5'-TCAACTACGC GTTCTGCAC-3' and 5'-AGAGTGGCCTTTCTCCATC-3', probe: human #62; *Reln*, 5'-CGTGCTGCTGGACTTCTCT-3' and 5'-TCCATCTCGTGAAGCAAGGT-3', probe: human #58; *Comt*, 5'-CTCGGGTCAGACCTGTG-3' and 5'-GCAACAG GAGACCAATGA-3', probe: human #66.

Results

Enhanced responsiveness to MK801 of *Df1*/+ mice was normalized by GABA_A receptor agonists. The DA agonists and *N*-methyl-D-aspartate receptor (NMDAR) antagonists such as Dizocilpine (MK801) are known to have psychometric effects on healthy individuals, and exacerbate symptoms of schizophrenia patients.^{29–31} In *Df1*/+ mice, the locomotor response to MK801 treatment (0.32 mg kg⁻¹) was significantly enhanced (genotype-by-time interaction on the distance traveled, $F_{11,165} = 5.60$, $n = 6–11$ per group, $P < 0.0001$ (repeated two-way analysis of variance (ANOVA); Figure 1a). One of the primary pathologies observed in schizophrenia is a deficit in GABA signaling.^{32–34} At first, to investigate a possible involvement of GABA signaling in the behavioral abnormality of *Df1*/+ mice, we examined the effect of a nonselective GABA_A partial receptor agonist, bretazenil. Bretazenil at 10 mg kg⁻¹ normalized the enhanced response of *Df1*/+ mice to MK801 (*Df1*/+ mice: MK801 and bretazenil treatment; 4553.00 ± 1307.28 cm, MK801 treatment; 10825.50 ± 1372.96 cm, $n = 6–7$ per group, $P = 0.0071$; Figures 1a–c). In contrast, in control mice, bretazenil had no effect on the traveled distance during 60 min after administration (control mice: MK801 and

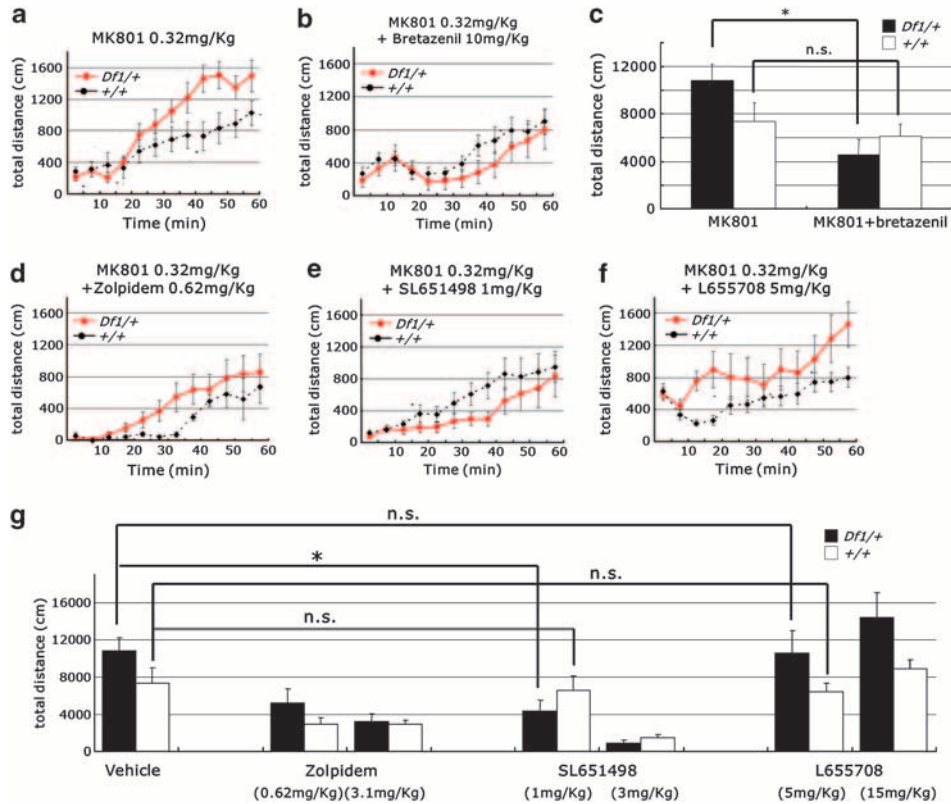


Figure 1 Enhanced responsiveness of *Df1/+* mice to MK801 is reversed by GABA_A $\alpha 2/\alpha 3$ receptor agonist, SL651498. (a–c) Enhanced response of male *Df1/+* mice to MK801 (a, c). Bretazenil attenuated enhanced locomotion of *Df1/+* mice by MK801 (b, c). MK801 (0.32 mg kg⁻¹) was intraperitoneally injected (b) with or (a) without bretazenil (10 mg kg⁻¹). Ambulatory distance is shown in 5 min bins for 60 min after drug administration (a, b) and quantification of ambulatory distance for 60 min is shown in (c). Values are the mean \pm s.e.m. for 6–16 male mice. * $P = 0.0071$; NS, not significant. (d–g) SL651498 (e), but neither zolpidem (d) nor L655708 (f), attenuated enhanced locomotion of *Df1/+* mice by MK801. MK801 (0.32 mg kg⁻¹) was intraperitoneally injected to male *Df1/+* and control mice with Zolpidem (0.62 or 3.1 mg kg⁻¹) (d, g), SL651498 (1 or 3 mg kg⁻¹) (e, g) and L655708 (5 or 15 mg kg⁻¹) (f, g). Ambulatory distance is shown in 5 min bins for 60 min after drug administration (e–g) and quantification of ambulatory distance for 60 min is shown in (g). Values are the mean \pm s.e.m. for 6–16 male mice. * $P = 0.0043$.

bretazenil treatment; 6092.63 ± 1026.79 cm, MK801 treatment; 7359.18 ± 1586.83 cm, $n = 11–16$ per group, $P = 0.97$; Figures 1a–c). Next, to analyze which type of GABA_A receptor is responsible for this reversing effect of bretazenil, *Df1/+* mice were treated with selective GABA agonists. SL651498, a selective agonist of GABA_A $\alpha 2/\alpha 3$ receptor, but neither zolpidem, a selective agonist of GABA_A $\alpha 1$ receptor, nor L655708, a selective inverse agonist of GABA_A $\alpha 5$ receptor, specifically reversed the enhanced response of *Df1/+* mice to MK801 (ambulatory distance for 60 min after administration of drugs: control mice; MK801 and SL651498 1 mg kg⁻¹ treatment, 6580.75 ± 1499.15 cm, MK801 treatment, 7359.18 ± 1586.83 cm, $n = 8–11$ per group, $P = 0.73$, *Df1/+* mice; MK801 and SL651498 1 mg kg⁻¹ treatment, 4332.44 ± 1229.75 cm, MK801 treatment, 10825.50 ± 1372.96 cm, $n = 6–9$ per group, $P = 0.0043$; Figures 1d–g). Taken together, our data demonstrated that *Df1/+* mice show high responsiveness to MK801 like human schizophrenic patients, and the GABAergic transmission through GABA_A $\alpha 2/\alpha 3$ receptor may be involved in this abnormal behavioral phenotype of *Df1/+* mice.

However, the deleted region of *Df1/+* mice is smaller than 1.5-Mb deletion found in human 22q11DS, which does not

include *Dgcr2* (Figure 2a). Recently, it has been reported that a potentially destructive *de-novo* mutation was found in *Dgcr2* in a human schizophrenic patient,³⁵ which suggests a possible involvement of *Dgcr2* in the pathogenesis of schizophrenia. To examine whether or not *Dgcr2* haplo-deletion also causes behavioral abnormalities similar to that in *Df1/+* mice, we genetically engineered *Dgcr2* KO mice by homologous recombination in embryonic stem cells. To introduce a targeted mutation in the mouse *Dgcr2* gene, we constructed a targeting vector, in which the expression cassette of EGFP-SV40 polyA and the *neomycin resistance* (EGFP-Neo) gene was inserted in exon 1, which contains an initiation codon of *Dgcr2* and flanked by the 5' 5.6-kb and 3' 1.2-kb *Dgcr2* gene fragments (Figure 2b). This mutation is predicted to introduce a premature stop codon to all the transcripts of *Dgcr2*. The disrupted *Dgcr2* allele was confirmed by Southern blot analysis (Figure 2c) and genomic PCR (data not shown). *Dgcr2*^{-/-} mice were backcrossed to the C57BL/6 for 10 generations before behavioral analysis. No *Dgcr2* RNA transcript could be detected by RT-PCR in the brains of the mutant mice, validating *Dgcr2* genetic disruption (Figure 2d). *Dgcr2*^{-/-} mice were born according to Mendelian segregation, were viable and fertile, and visually indistinguishable from their wild-type littermates. Histopathological

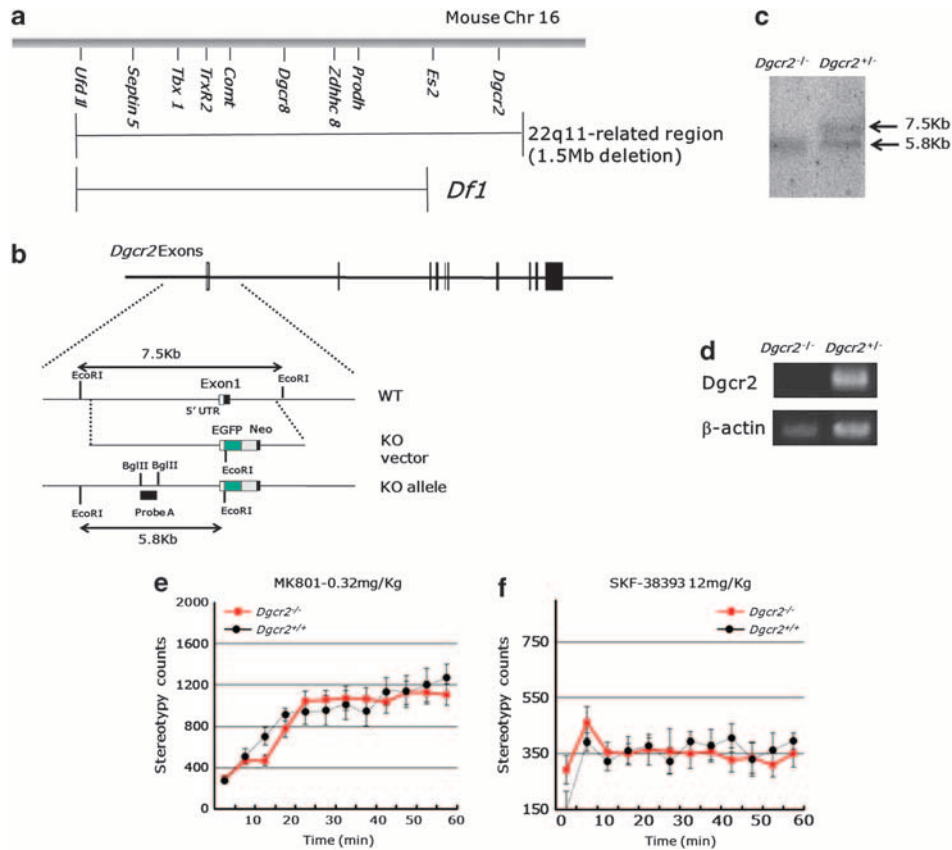
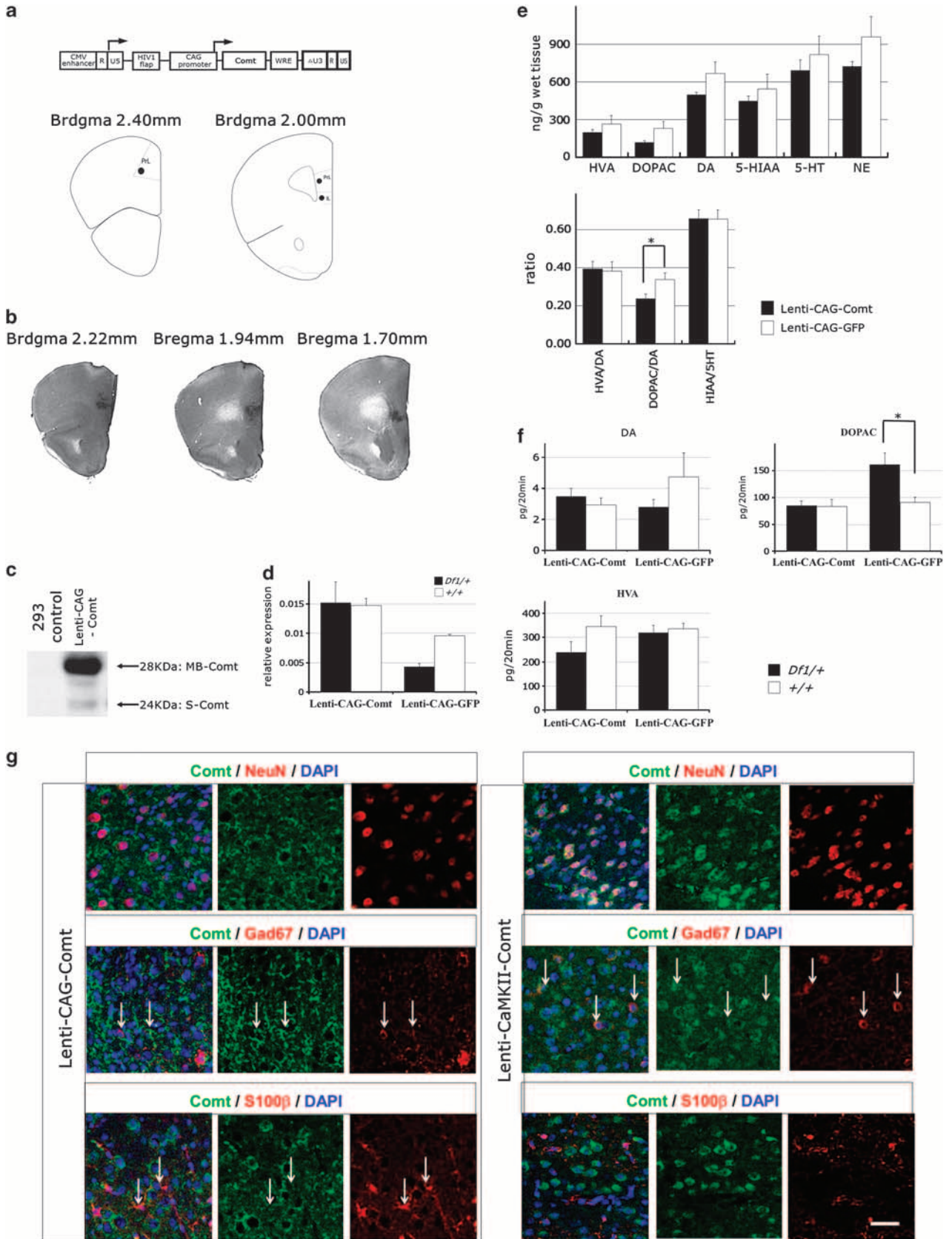


Figure 2 Normal responses of *Dgcr2* knockout (KO) heterozygous mice to MK801 and SKF38393. **(a)** A genetic organization of mouse 22q11-related region on chromosome 16 (Chr 16). The deleted region of *Df1*^{+/+} mice is indicated by a line below the map. **(b)** Schematic representation of *Dgcr2* gene-targeting strategy. Exon 1 was replaced by a EGFP-Neo cassette by homologous recombination, which is predicted to introduce a premature stop codon in all *Dgcr2* transcripts. UTR, untranslated region; WT, wild type. **(c)** Southern blot analysis of genomic DNA using probe A is shown in **(b)**. The 7.5 and 5.8 kb *EcoRI* fragments derived from the wild-type and *Dgcr2* KO mice, respectively. **(d)** RT-PCR analysis of *Dgcr2* in the brain. **(e, f)** *Dgcr2* heterozygosity did not affect the locomotor responses to MK801 and SKF38393. **(e)** MK801 (0.32 mg kg⁻¹) and **(f)** SKF38393 (12 mg kg⁻¹) were injected intraperitoneally and subcutaneously, respectively, to the *Dgcr2*^{-/-} and control mice. Stereotypy count is shown in 5 min bins for 60 min after drug administration. Values are the mean ± s.e.m. for 6–14 male mice.

analysis did not show any abnormality of gross morphology in the brains of *Dgcr2* KO mice (data not shown). In an open field test, *Dgcr2* KO heterozygous mice did not show any abnormalities in the locomotor responses to MK801 (0.32 mg kg⁻¹, i.p.) and D1 agonist, SKF38393 (12 mg kg⁻¹, subcutaneously; genotype-by-time interaction on the stereotypy count, MK801; $F_{11, 253} = 1.14$, $n = 11-14$ per group, $P = 0.33$, SKF38393; $F_{11, 110} = 1.18$, $n = 5-7$ per group, $P = 0.31$ (repeated two-way ANOVA; Figures 2e and f). Therefore, we adopted *Df1*^{+/+} mice for further analysis.

Prefrontal cortical gene transfer of *Comt* in *Df1*^{+/+} mice. To examine whether the reduced expression of *Comt* is involved in behavioral defects of *Df1*^{+/+} mice, we bilaterally reintroduce mouse *Comt* to the prelimbic cortex and the infralimbic cortex of *Df1*^{+/+} mice by stereotactic injection of lentiviruses, using EGFP as a control (Figure 3a). To distinguish cell type-specific effects of *Comt* overexpression, we utilized two types of lentivirus vectors, one of which is with a ubiquitous CMV/CAG promoter (Lenti-CAG-*Comt*)³⁶ and the other is with a neuron-specific CaMKII

promoter (Lenti-CaMKII-*Comt*). Western blotting analysis showed that both membrane-bound and soluble forms of *Comt* were generated from the same mRNA derived from a lentivirus (Figure 3c). Lentivirus-mediated specific expression of *Comt* in the PFC was confirmed by immunohistochemical analysis (Figure 3b). Quantitative real-time PCR analysis showed that *Comt* expression reduced by 55% in the PFC of *Df1* mice than that of control mice ($P = 0.000068$; Figure 3d). After Lenti-CAG-*Comt* infection, *Comt* expression increased to ~150% of that of EGFP-expressing control PFC in *Df1*^{+/+} mice (Figure 3d). Immunofluorescent study showed that in Lenti-CAG-*Comt*-infected PFC, 19.43 ± 1.63% of *Comt*-expressing cells were S100β-positive glial cells and 55.66 ± 2.26% were NeuN-positive neurons, and 4.71 ± 1.94% were Gad67-positive interneurons ($n = 6$), whereas in Lenti-CaMKII-*Comt*-infected PFC, 1.99 ± 1.11% of *Comt*-expressing cells were S100β-positive glial cells and 89.27 ± 2.21% were NeuN-positive neurons, and 15.69 ± 1.18% were Gad67-positive interneurons ($n = 3$; Figure 3g). The percentage of Lenti-CaMKII-*Comt*-transduced interneurons was 4 times higher than that observed with Lenti-CAG-*Comt* lentivirus.



To determine whether the lentivirus-derived *Comt* was functional *in vivo*, we examined DA and DA metabolites in homogenates of lentivirus-infected prefrontal cortical tissues. *Comt* methylates DOPAC, a hydroxylated metabolite of DA, as well as DA and forms HVA. The ratio of DOPAC/DA significantly decreased by the overexpression of *Comt* in the PFC, whereas neither HVA/DA nor HIAA/5HT ratio was affected (HVA: Lenti-CAG-GFP; $265.18 \pm 67.16 \text{ ng g}^{-1}$, Lenti-CAG-*Comt*; $195.97 \pm 23.91 \text{ ng g}^{-1}$, $P=0.32$, DOPAC: Lenti-CAG-GFP; $229.36 \pm 54.58 \text{ ng g}^{-1}$, Lenti-CAG-*Comt*; $117.52 \pm 14.93 \text{ ng g}^{-1}$, $P=0.06$, DA: Lenti-CAG-GFP; $665.51 \pm 93.78 \text{ ng g}^{-1}$, Lenti-CAG-*Comt*; $495.09 \pm 23.34 \text{ ng g}^{-1}$, $P=0.090$, 5-HIAA: Lenti-CAG-GFP; $543.19 \pm 118.03 \text{ ng g}^{-1}$, Lenti-CAG-*Comt*; $445.18 \pm 41.47 \text{ ng g}^{-1}$, $P=0.42$, 5-HT: Lenti-CAG-GFP; $816.30 \pm 146.01 \text{ ng g}^{-1}$, Lenti-CAG-*Comt*; $691.61 \pm 84.22 \text{ ng g}^{-1}$, $P=0.46$, NE: Lenti-CAG-GFP; $957.29 \pm 160.62 \text{ ng g}^{-1}$, Lenti-CAG-*Comt*; $723.92 \pm 35.97 \text{ ng g}^{-1}$, $P=0.16$, HVA/DA: Lenti-CAG-GFP; 0.38 ± 0.049 , Lenti-CAG-*Comt*; 0.39 ± 0.040 , $P=0.86$, DOPAC/DA: Lenti-CAG-GFP; 0.34 ± 0.035 , Lenti-CAG-*Comt*; 0.24 ± 0.026 , $P=0.048$, HIAA/5HT: Lenti-CAG-GFP; 0.66 ± 0.049 , Lenti-CAG-*Comt*; 0.66 ± 0.047 , $P=0.98$; Figure 3e), indicating that *Comt* overexpression can enhance elimination of DA and DOPAC in the PFC in wild-type mice. Finally, we examined whether lentivirus-mediated *Comt* overexpression can compensate for the effects of *Comt* heterozygosity of *Df1/+* mice by *in vivo* microdialysis. In *Df1/+* mice, PFC extracellular DOPAC levels increased by 77.89%, and there were no changes in DA and HVA levels (DOPAC: *Df1/+* Lenti-CAG-GFP; $160.97 \pm 21.85 \text{ pg per 20 min}$, $+/+$ Lenti-CAG-GFP; 90.49 ± 9.89 , $P=0.0041$, DA: *Df1/+* Lenti-CAG-GFP; $2.80 \pm 0.34 \text{ pg per 20 min}$, $+/+$ Lenti-CAG-GFP; 4.75 ± 1.56 , $P=0.42$, HVA: *Df1/+* Lenti-CAG-GFP; $319.96 \pm 30.15 \text{ pg per 20 min}$, $+/+$ Lenti-CAG-GFP; 336.29 ± 22.97 , $P=0.69$; Figure 3f). Lenti-CAG-*Comt*-mediated *Comt* overexpression successfully normalized the PFC extracellular DOPAC level in *Df1/+* mice (DOPAC: *Df1/+* Lenti-CAG-*Comt*; $84.74 \pm 8.99 \text{ pg per 20 min}$, $+/+$ Lenti-CAG-GFP; $90.49 \pm 9.89 \text{ pg per 20 min}$, $P=0.78$; Figure 3f).

***Comt* overexpression in the PFC reversed behavioral abnormalities of *Df1/+* mice.** PFC EGFP-expressed *Df1/+* mice showed a similar enhanced locomotor response to MK801 (0.32 mg kg⁻¹, i.p.) and SKF38393 (12 mg kg⁻¹, subcutaneously) as seen with non-lentivirus-

infected *Df1/+* mice (stereotypy count for 60 min after administration, SKF38393: $+/+$; 4886.0 ± 725.1 , *Df1/+*; 6066.4 ± 1227.3 , $P=0.019$, MK801: $+/+$; 11647.1 ± 4485.6 , *Df1/+*; 16492.1 ± 4305.0 , $P=0.039$; Figures 4b and e). Increasing *Comt* expression levels by lentiviral infection normalized the enhanced sensitivity of *Df1/+* mice to MK801 and SKF38393, whereas it did not alter locomotor responses in control mice (stereotypy count of *Comt*-expressing lentivirus-infected mice for 60 min after administration, SKF38393: $+/+$; 5356.0 ± 1157.0 , *Df1/+*; 4092.5 ± 1633.8 , $P=0.047$, MK801: $+/+$; 15753.2 ± 1509.0 , *Df1/+*; 12331.7 ± 2409.6 , $P=0.019$; Figures 4a and d). Next, we investigated whether this treatment effect directly depends on *Comt* enzymatic activity or not. At first, we determined an optimal concentration of tolcapone, a *Comt* inhibitor by *in vivo* microdialysis experiments. We found that 30 mg kg⁻¹ tolcapone (i.p.) efficiently inhibited *Comt* activity in PFC, increased DOPAC level and decreased HVA level to a comparable level to that in control mice even in the *Comt*-overexpressed mice, whereas 6 mg kg⁻¹ tolcapone administration was not sufficient (Figure 5). Therefore, we adopted 30 mg kg⁻¹ tolcapone for further experiments. However, 30 mg kg⁻¹ of tolcapone failed to reverse the curing effects of PFC *Comt* overexpression. On the contrary, tolcapone administration of this dose even reduced the response of *Comt*-overexpressing *Df1/+* mice to MK801 but not to SKF38393 (MK801 + tolcapone: stereotypy count for 60 min after administration, $+/+$; 12092.0 ± 5551.9 , *Df1/+*; 6433.5 ± 3121.1 , $P=0.049$; Figures 4c and f), suggesting that the indirect effects of increased *Comt* enzymatic activity might be responsible for its curing potential.

***Comt* overexpression normalized the abnormal responsiveness of *Df1/+* mice to GABA_A receptor agonists.** The enhanced response of EGFP-overexpressed *Df1/+* mice to MK801 or SKF38393 was also reversed by SL651498 administration as in non-virus-infected *Df1/+* mice (Figures 4b and e, and 6b and e). In addition, *Df1/+* mice showed an abnormal response to bretazenil when treated with methamphetamine (1 mg kg⁻¹, i.p.; stereotypy count from 30 to 60 min after administration, *Df1/+* mice, methamphetamine, 4104.63 ± 565.90 , methamphetamine + bretazenil 1984.60 ± 721.46 ($P=0.048$); Figure 7c). Next, we examined the effects of Lenti-CAG-*Comt* treatment on this GABAergic response abnormality of *Df1/+* mice. After

Figure 3 The gene delivery of catechol-O-methyltransferase (*Comt*) to the prefrontal cortex (PFC). (a) Depiction of the lentivirus to locally overexpress *Comt* (upper panel). CMV enhancer is substituted for U3 region of the 5' long terminal repeat (LTR) to enhance expression of viral RNA genomes. Self-inactivating 3' LTR comprises a U3 region with deletion of an enhancer element ($\Delta U3$). HIV-1-flap: the human immunodeficiency virus-1 flap element; WRE: the woodchuck hepatitis virus posttranslational regulatory element. Schematic illustration of injection sites in the PFC (lower panel). Irl, infralimbic cortex; PrL, prelimbic cortex. (b) Immunohistochemical studies for *Comt* expression in a representative mice bilaterally infected by Lenti-CAG-*Comt*, demonstrating spatial selectivity. (c) Western blot analysis of *Comt* expression. Protein samples from 293 cells 2 days after infection of Lenti-CAG-*Comt*. MB-*Comt*, membrane-bound *Comt*; S-*Comt*, soluble *Comt*. (d) Real-time PCR quantitation of *Comt* mRNA levels in Lenti-CAG-*Comt*- or Lenti-CAG-GFP-infected PFC of *Df1/+* or control mice. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) abundance. Data are the mean \pm s.e.m. from 3 to 14 mice. * $P=0.0000068$. (e) Effect of *Comt* overexpression on dopamine (DA), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5HT), 5-hydroxyindoleacetic acid (5-HIAA) and norepinephrine (NE) in tissue homogenates of the Lenti-CAG-*Comt*- or Lenti-CAG-GFP-infected PFC of control mice. Values are the mean \pm s.e.m. for 4 mice. * $P=0.048$. (f) Effects of *Comt* overexpression on PFC extracellular DA, DOPAC and HVA in the PFC of Lenti-CAG-*Comt*- or Lenti-CAG-GFP-infected *Df1/+* and control mice. The 20 min baseline fractions were collected after 2 h washout period. Data are means \pm s.e.m. from 3 to 11 mice. * $P=0.0041$. (g) Representative images of immunofluorescent studies of Lenti-CAG-*Comt*- or Lenti-CaMKII-*Comt*-infected PFC for *Comt* (green), NeuN (red, upper panels), S100 β (red, lower panels) and 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei (blue). Double positive cells are indicated by arrows. Scale bar = 20 μm .

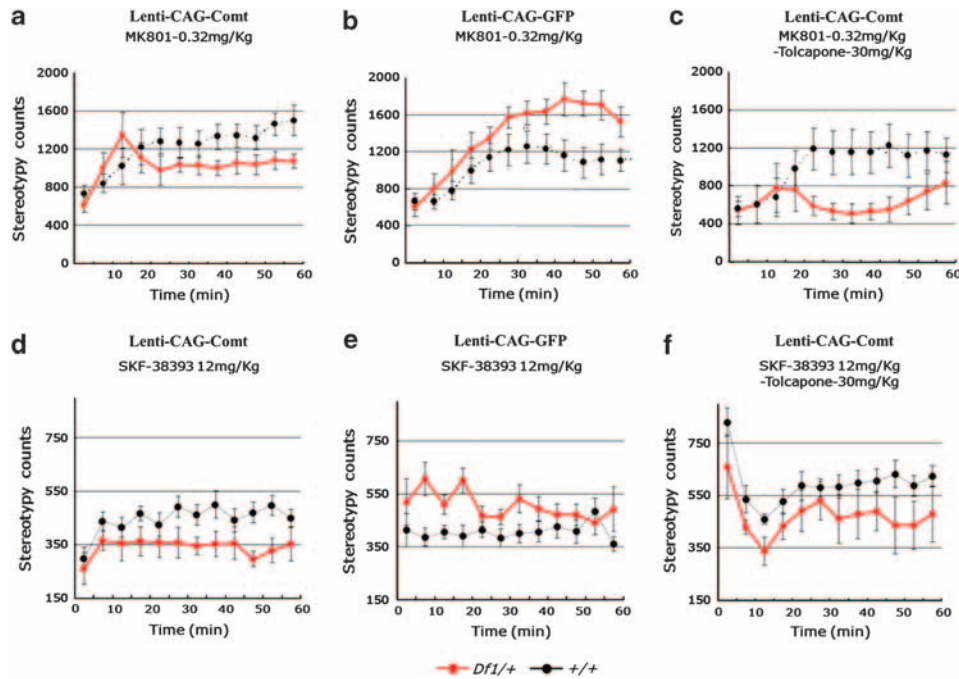


Figure 4 Increasing catechol-*O*-methyltransferase (*Comt*) expression in the prefrontal cortex (PFC) rescues schizophrenia-like phenotypes of *Df1/+* mice. (a, b, d, e) Enhanced response of male *Df1/+* mice to MK801 (b) and SKF38393 (e) were normalized by lentivirus-mediated *Comt* overexpression in the PFC (a, d). SKF38393 (12 mg kg^{-1}) and MK801 (0.32 mg kg^{-1}) were injected subcutaneously and intraperitoneally, respectively, to the mice infected with Lenti-CAG-*Comt* (a, d) or control Lenti-CAG-GFP (b, e). Stereotypy count is shown in 5 min bins for 60 min after drug administration. Values are the mean \pm s.e.m. for 6–16 male mice. (c, f) Tolcapone, a *Comt* inhibitor, failed to reverse a therapeutic effect of *Comt* overexpression of the enhanced stereotypic response of *Df1/+* mice to MK801 (c) and SKF38393 (f). Tolcapone (30 mg kg^{-1}) was intraperitoneally injected to male *Df1/+* mice 1 h before administration of MK801 (0.32 mg kg^{-1}). Stereotypy count is shown in 5 min bins for 60 min after MK801 administration. Values are the mean \pm s.e.m. for 5–16 male mice.

lentivirus-mediated PFC *Comt* reintroduction, *Df1/+* mice showed reduced responses to SL651498 or bretazenil (stereotypy count for 60 min after administration, Lenti-CAG-GFP-infected *Df1/+* mice: MK801; 16492.13 ± 1435.01 , MK801 + SL651498; 3321.75 ± 794.36 ($P=0.000022$), Lenti-CAG-*Comt*-infected *Df1/+* mice: MK801; 12331.71 ± 910.752 , MK801 + SL651498; 8073.75 ± 1276.90 ($P=0.020$), Lenti-CAG-GFP-infected *Df1/+* mice: SKF38393; 6066.44 ± 409.09 , SKF38393 + SL651498; 3359.67 ± 418.92 ($P=0.0053$); Lenti-CAG-*Comt*-infected *Df1/+* mice: SKF38393; 4092.50 ± 516.66 , SKF38393 + SL651498; 4391.67 ± 358.92 ($P=0.65$); stereotypy count from 30 to 60 min after administration, Lenti-CAG-GFP-infected *Df1/+* mice: methamphetamine; 4104.63 ± 565.90 , methamphetamine + bretazenil; 1984.60 ± 721.46 ($P=0.048$), Lenti-CAG-*Comt*-infected *Df1/+* mice: methamphetamine; 3827.57 ± 1020.98 , methamphetamine + bretazenil; 4696.33 ± 1240.41 ($P=0.60$); Figures 6c, f and 7c). These results demonstrated that PFC *Comt* overexpression did not only suppress the high responsiveness of *Df1/+* mice to MK801 and SKF38393, but also attenuate the abnormal response to GABA_A receptor agonists, suggesting that the curing effects of *Comt* overexpression might be through the regulation of GABAergic system.

Next, we examined whether the curing effects of *Comt* overexpression depend on *Comt* enzymatic activity. Tolcapone (30 mg kg^{-1} , i.p.) again failed to reverse the effects of bretazenil (stereotypy count from 30 to 60 min after

administration, Lenti-CAG-*Comt*-infected *Df1/+* mice: methamphetamine + bretazenil; 4696.33 ± 1240.41 , methamphetamine + bretazenil + tolcapone; 3141.67 ± 1379.29 , ($P=0.42$); Figure 7c). These data suggest that the curing effect on the responsiveness to GABA_A receptor agonists might also be a secondary effect of increasing *Comt* activity.

***Comt* overexpression enhanced the responsiveness to GABA_A receptor agonists in control mice.** We next examined the effects of PFC *Comt* overexpression on the behavioral responsiveness to GABA_A receptor agonists in control mice. Lenti-CAG-*Comt*-infected mice showed enhanced responsiveness to a low dose of either bretazenil (10 mg kg^{-1} , i.p.) or SL651498 (1 mg kg^{-1} , i.p.) when treated with methamphetamine (1 mg kg^{-1} , i.p.) (stereotypy count, Lenti-CAG-*Comt*: methamphetamine; 4097.44 ± 537.31 , methamphetamine + bretazenil; 1749.00 ± 640.53 ($P=0.017$), methamphetamine + SL651498; 1664.71 ± 895.29 ($P=0.031$); Figure 6a), whereas in Lenti-CAG-GFP-infected control mice, this dose of SL651498 had no effect on the locomotor stimulating effects of methamphetamine (stereotypy count, Lenti-CAG-GFP: methamphetamine; 3113.67 ± 577.52 , methamphetamine + bretazenil; 3547.50 ± 875.70 , $P=0.73$, methamphetamine + SL651498; 3083.00 ± 1115.41 , $P=0.98$; Figure 7a). In addition, this effect of PFC *Comt* overexpression was also observed in mice infected by Lenti-CaMKII-*Comt* (stereotypy count, Lenti-CaMKII-*Comt*: methamphetamine; 3529.73 ± 679.80 , methamphetamine +

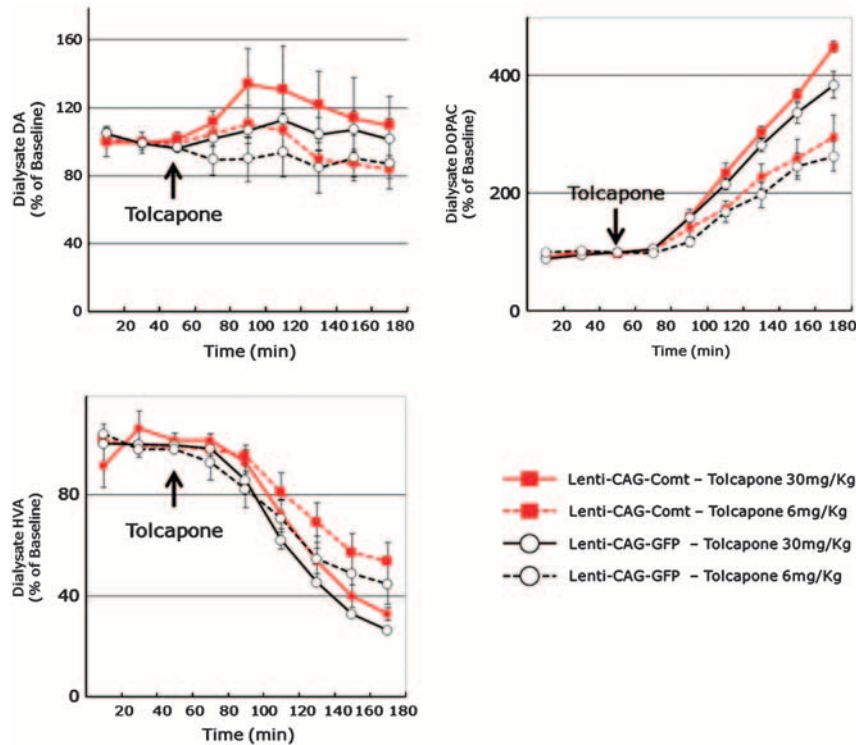


Figure 5 Effects of tolcapone on prefrontal cortex (PFC) extracellular dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in Lenti-CAG-*Comt* or control Lenti-CAG-GFP mice. Three 20 min baseline fractions were collected before tolcapone (6 and 30 mg kg⁻¹ intraperitoneally (i.p.)) injection and thereafter 20 min fractions were collected for 120 min. Data are means ± s.e.m. from three mice.

bretazenil; 1310.09 ± 400.01, *P* = 0.011, methamphetamine + SL651498; 1546.80 ± 373.56, *P* = 0.022; Figure 7a). These data suggest that the differences in *Comt* expression level in interneurons and astrocytes did not influence the effects of *Comt*-expressing lentiviruses on the responsiveness to GABA_A receptor agonists. This high responsiveness of Lenti-CAG-*Comt* or Lenti-CaMKII-*Comt*-infected mice to bretazenil is also resistant to tolcapone (stereotypy count, Lenti-CAG-*Comt*: methamphetamine + bretazenil; 1749.000 ± 640.53, methamphetamine + bretazenil + tolcapone; 1628.08 ± 444.13, *P* = 0.88, Lenti-CaMKII-*Comt*: methamphetamine + bretazenil; 1310.09 ± 400.01, methamphetamine + bretazenil + tolcapone; 1743.36 ± 524.16, *P* = 0.52; Figure 7b), suggesting that the increasing *Comt* activity might indirectly cause an abnormal responsiveness to GABA_A receptor agonists in control mice.

***Comt* inhibition enhanced the responsiveness to GABA_A receptor agonists in wild-type mice.** Furthermore, we found that inhibition of endogenous *Comt* activity by tolcapone enhanced the responsiveness to bretazenil in methamphetamine-induced hyperlocomotion of Lenti-CAG-GFP-infected wild-type mice (Lenti-CAG-GFP: methamphetamine + bretazenil; 3547.50 ± 875.70, methamphetamine + bretazenil + tolcapone; 1523.30 ± 355.24, *P* = 0.046; Figure 7b), whereas administration of tolcapone had no effects on locomotor effects of methamphetamine in Lenti-CAG-*Comt*, Lenti-CaMKII-*Comt* or Lenti-CAG-GFP-infected mice (Figure 7b). These data demonstrated that both

elevation and reduction in *Comt* expression lead to an abnormal responsiveness to GABA_A receptor agonists.

***Comt* overexpression induced c-Fos expression specifically in GABAergic interneurons.** It has been reported that NMDA antagonists increase PFC activity³⁷ and increase the expression of *c-Fos* specifically in pyramidal neurons but not in GABAergic interneurons.³⁸ To examine how increasing *Comt* expression affects PFC activity, we performed immunohistochemical analysis of *c-Fos* expression in the PFC of Lenti-CAG-*Comt*-, Lenti-CaMKII-*Comt*- or Lenti-CAG-GFP-infected *Df1/+* and control mice. In the PFC of Lenti-CAG-*Comt*- or Lenti-CaMKII-*Comt*-infected control mice, *Comt* overexpression increased the number of *c-Fos*⁺ cells induced by MK801 (0.32 mg kg⁻¹, i.p.; +/+ mice: Lenti-CAG-*Comt*; 7.49 ± 0.45 × 10⁻⁴ cells μm⁻² (*P* = 0.034), Lenti-CaMKII-*Comt*; 7.74 ± 0.56 × 10⁻⁴ cells μm⁻² (*P* = 0.021), Lenti-CAG-GFP; 5.98 ± 0.21 × 10⁻⁴ cells μm⁻²; Figures 8a and b). In contrast, in *Df1/+* mice, PFC *Comt* overexpression did not change the density of MK801-induced *c-Fos*⁺ cells (*Df1/+* mice: Lenti-CAG-*Comt*; 2.09 ± 0.42 × 10⁻⁴ cells μm⁻², Lenti-CAG-GFP; 2.63 ± 0.18 × 10⁻⁴ cells μm⁻², *P* = 0.36; Figures 8a and b). Double immunofluorescent studies of *c-Fos* and GABA revealed that the enhanced *c-Fos* expression was observed in GABAergic interneurons but not in GABA-negative cells in both superficial (II/III) and deep layers (V/VI) of Lenti-CAG-*Comt*-, Lenti-CaMKII-*Comt*- and Lenti-CAG-GFP-infected control mice (+/+ mice: layer II/III,

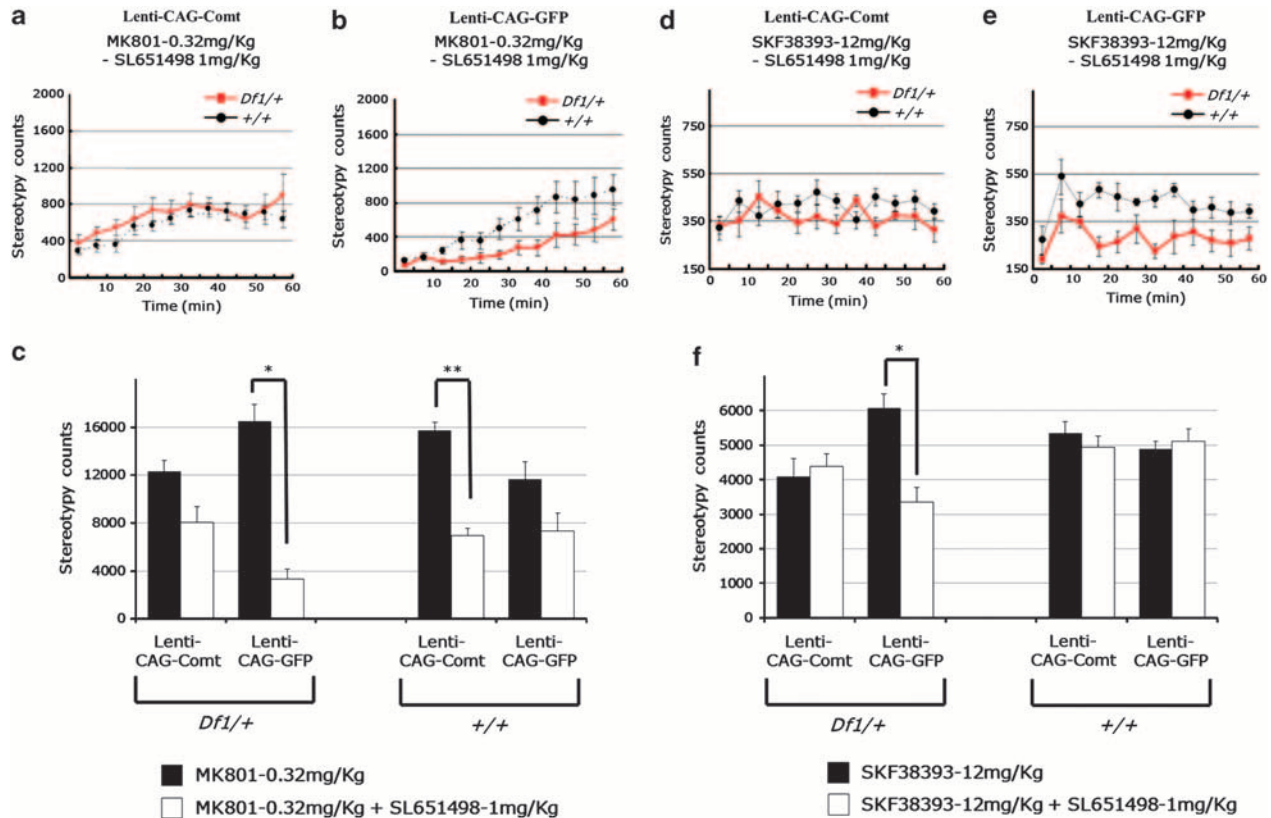


Figure 6 Normalization of the abnormal response of *Df1/+* mice to SL651498 by lentivirus-mediated *catechol-O-methyltransferase (Comt)* overexpression in the prefrontal cortex (PFC). (a–c) MK801 (0.32 mg kg⁻¹) and (d–f) SL651498 (1 mg kg⁻¹), GABA_A $\alpha 2/\alpha 3$ receptor agonist, were injected intraperitoneally and SKF38393 (12 mg kg⁻¹) was injected subcutaneously to the Lenti-CAG-*Comt* (a, d) or control Lenti-CAG-GFP (b, e)-infected *Df1/+* and control mice. Stereotypy count is shown in 5 min bins for 60 min after drug administration (a, b, d, e) and quantification of stereotypy count for 60 min is shown in (c, f). Values are the mean \pm s.e.m. for 8–9 male mice. (c) * $P=0.0000022$, ** $P=0.0000076$. (f) * $P=0.0053$.

c-Fos⁺GABA⁺ cells: Lenti-CAG-*Comt*; $4.2 \pm 0.47 \times 10^{-5}$ cells μm^{-2} ($P=0.00045$), Lenti-CaMKII-*Comt*; $2.8 \pm 0.54 \times 10^{-4}$ cells μm^{-2} ($P=0.0083$), Lenti-CAG-GFP; $4.7 \pm 2.7 \times 10^{-6}$ cells μm^{-2} , layer V/VI, c-Fos⁺GABA⁺ cells: Lenti-CAG-*Comt*; $2.3 \pm 0.27 \times 10^{-5}$ cells μm^{-2} ($P=0.0038$), Lenti-CaMKII-*Comt*; $1.6 \pm 0.31 \times 10^{-4}$ cells μm^{-2} ($P=0.074$), Lenti-CAG-GFP; $7.0 \pm 2.3 \times 10^{-6}$ cells μm^{-2} ; Figures 8c and d). In *Df1/+* mice, PFC *Comt* overexpression also increased c-Fos expression in GABAergic interneurons (*Df1/+* mice: layer II/III, c-Fos⁺GABA⁺ cells: Lenti-CAG-*Comt*; $2.2 \pm 0.31 \times 10^{-5}$ cells μm^{-2} , Lenti-CAG-GFP; $6.2 \pm 3.1 \times 10^{-6}$ cells μm^{-2} ($P=0.024$), layer V/VI, c-Fos⁺GABA⁺ cells: Lenti-CAG-*Comt*; $2.5 \pm 0.31 \times 10^{-5}$ cells μm^{-2} ($P=0.0078$), Lenti-CAG-GFP; $3.1 \pm 3.1 \times 10^{-6}$ cells μm^{-2} ; Figures 8c and d). These data indicate that increasing *Comt* expression in the PFC specifically enhance the activity of interneurons in both *Df1/+* and control mice.

***Comt* overexpression upregulated GABA signaling-related genes in the PFC.** To examine the effects of *Comt* overexpression on GABA signaling, we assessed the expression levels of GABA receptors and GABA signaling-related genes at 2 weeks after infection by real-time PCR. We observed significant correlation between *Comt* expression and *Gabrb2* (GABA_A receptor $\beta 2$), *Gad2* (glutamic

acid decarboxylase 65 (*GAD65*) and *Reln* (*reelin*) in the PFC of Lenti-CAG-*Comt*-infected mice (*Gabrb2*: $R=0.63$, $P=0.020$, *Gad2*: $R=0.63$, $P=0.020$, and *Reln*: $R=0.62$, $P=0.023$; Figures 9a–c). Similar effects were also observed in Lenti-CaMKII-*Comt*-infected mice (*Gabrb2*: $R=0.85$, $P=0.0082$, *Gad2*: $R=0.91$, $P=0.0016$, and *Reln*: $R=0.66$, $P=0.077$; Figures 9a–c).

Next, we examined the effects of increasing *Comt* expression on GABAergic synapses in the PFC. GABA immunofluorescence in the prelimbic cortex was present as puncta rings around soma. Quantitative analysis of fluorescent pixel density of puncta rings showed that GABA immunofluorescence in perisomatic puncta was increased by Lenti-CAG-*Comt* or Lenti-CaMKII-*Comt* infection in both *Df1/+* and control mice (pixel density, *+/+* mice: Lenti-CAG-*Comt*; 1552.06 ± 93.11 ($P=0.026$), Lenti-CaMKII-*Comt*; 1638.72 ± 67.61 ($P=0.0067$), Lenti-CAG-GFP; 1169.67 ± 60.57 ; Figures 3d, e, g and h). However, there was no change in the density of *Gad67*⁺ cells (*+/+* mice, Lenti-CAG-*Comt*; $1.61 \pm 0.097 \times 10^{-4}$ cells μm^{-2} ($P=0.81$), Lenti-CaMKII-*Comt*; $1.86 \pm 0.22 \times 10^{-4}$ cells μm^{-2} ($P=0.29$), Lenti-CAG-GFP; $1.55 \pm 0.16 \times 10^{-4}$ cells μm^{-2}). These data showed that *Comt* overexpression enhanced GABA production without affecting the number of GABAergic interneurons and that the differences in the *Comt* expression levels in interneurons

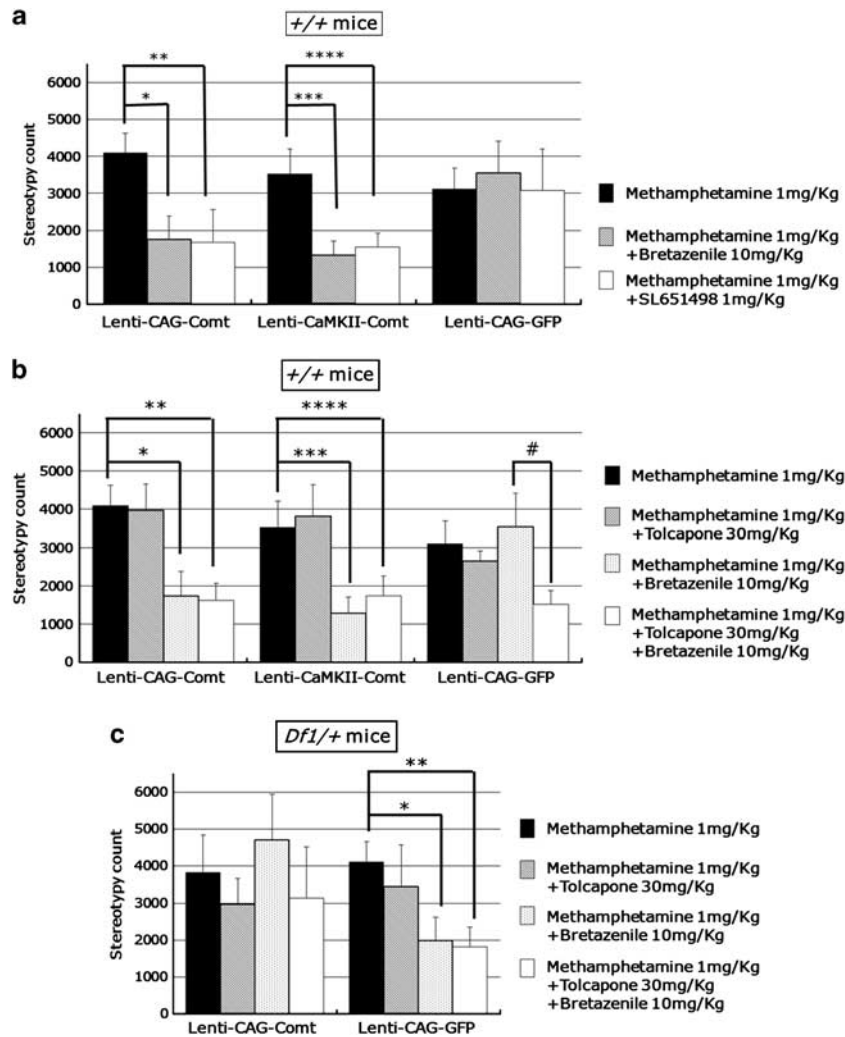


Figure 7 The enzymatic activity-independent or activity-dependent effects of *catechol-O-methyltransferase* (*Comt*) on the responsiveness to GABA_A receptor agonists. (a) Prefrontal cortex (PFC) *Comt* overexpression in the Lenti-CAG-Comt- or Lenti-CaMKII-Comt-infected control mice enhanced the response to bretazenil, a GABA_A receptor agonist, and SL651498. Methamphetamine (1 mg kg⁻¹) was intraperitoneally injected with bretazenil (10 mg kg⁻¹) or SL651498 (1 mg kg⁻¹). Stereotypy count from 30 to 60 min after methamphetamine administration is shown. Values are the mean ± s.e.m. for 6–10 male mice. **P* = 0.017, ***P* = 0.031, ****P* = 0.011, *****P* = 0.022. (b) Tolcapone failed to reverse the enhanced responsiveness to bretazenil in PFC COMT-overexpressing mice, but increased the responsiveness to bretazenil in GFP-expressing control mice. Tolcapone (30 mg kg⁻¹) was intraperitoneally injected to Lenti-CAG-Comt or -GFP-infected control mice 1 h before administration of methamphetamine (1 mg kg⁻¹). Stereotypy count from 30 to 60 min after methamphetamine administration is shown. Values are the mean ± s.e.m. for 6–13 male mice. **P* = 0.017, ***P* = 0.031, ****P* = 0.011, *****P* = 0.050, #*P* = 0.026. (c) Tolcapone failed to reverse the treatment effect of *Comt* overexpression on the enhanced responsiveness to bretazenil in *Df1/+* mice. Tolcapone (30 mg kg⁻¹) was intraperitoneally injected to Lenti-CAG-Comt or -GFP-infected *Df1/+* or control mice 1 h before administration of methamphetamine (1 mg kg⁻¹). Stereotypy count from 30 to 60 min after methamphetamine administration is shown. Values are the mean ± s.e.m. for 6–13 male mice. **P* = 0.048, ***P* = 0.019.

and astrocytes did not change the enhancing effects on GABA transmission.

Finally, we examined the effects of *Comt* overexpression on GABA release in the PFC by *in vivo* microdialysis. In control mice, PFC *Comt* overexpression drastically increased GABA release after MK801 administration (0.32 mg kg⁻¹, i.p.; virus-by-time interaction on GABA level: $F_{8,32} = 5.60$, $n = 3$ per group, $P = 0.0062$ (repeated two-way ANOVA); Figure 10), although no statistically significant increase in GABA release was observed in *Comt*-overexpressing *Df1/+* mice (virus-by-time interaction on GABA level: $F_{8,40} = 1.34$, $n = 3-4$ per group, $P = 0.25$ (repeated two-way ANOVA); Figure 10).

Discussion

Df1/+ mice displayed higher locomotive activity to psychostimulants, similar to human schizophrenic patients. Our pharmacological data suggest that GABA signaling abnormalities are involved in these behavioral abnormalities of *Df1/+* mice. Lentivirus-mediated *Comt* reintroduction to the PFC of *Df1/+* mice normalized the abnormal responses of *Df1/+* mice to GABA_A receptor agonists as well as psychostimulants. In contrast, in wild-type mice, increased *Comt* expression in the PFC caused abnormal responses to GABA_A receptor agonists. A *Comt* inhibitor, tolcapone, failed

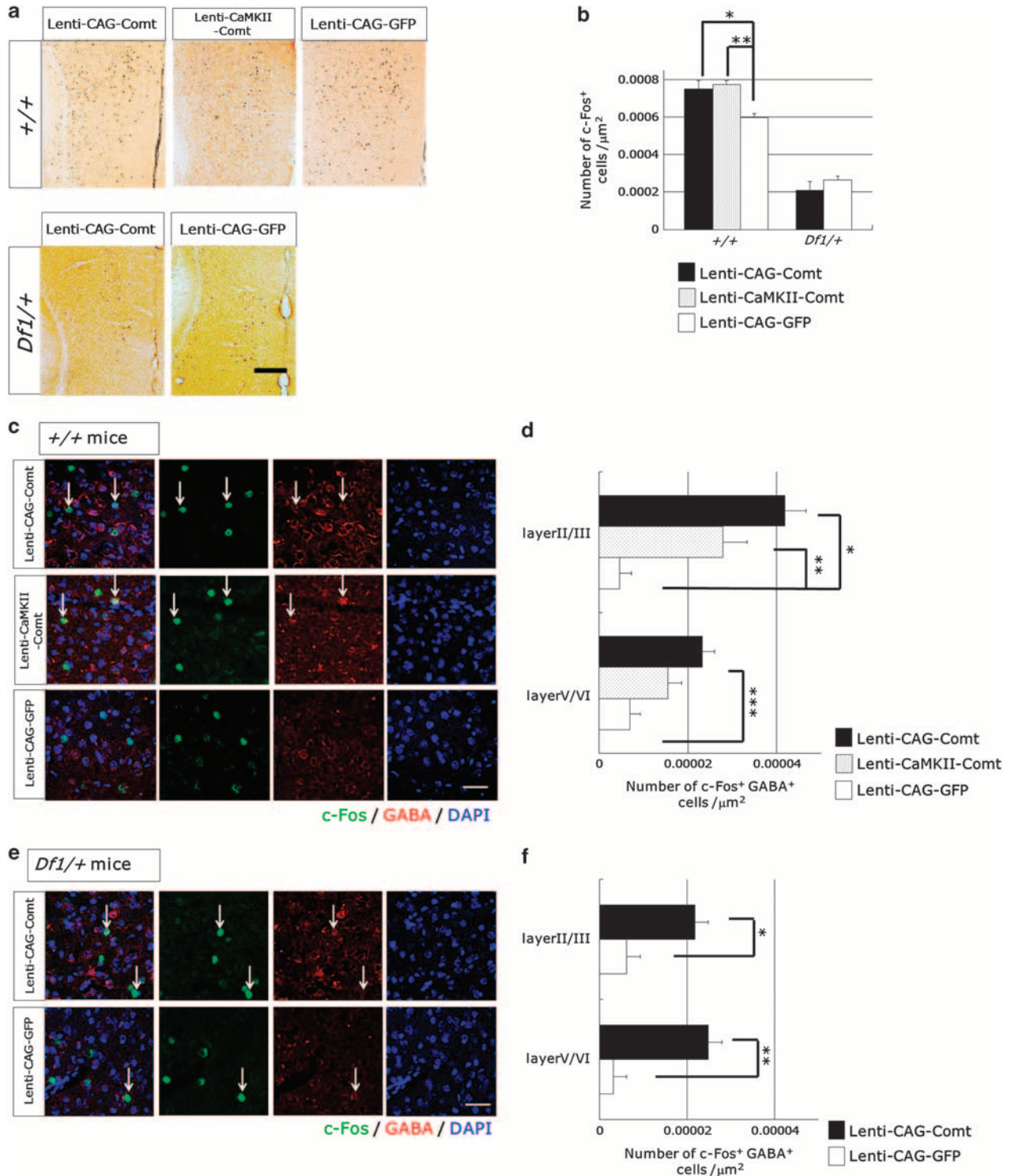


Figure 8 The effects of increasing *catechol-O-methyltransferase (Comt)* expression on MK801-induced-c-Fos expression in the prefrontal cortex (PFC). **(a, b)** PFC *Comt* overexpression increased the number of MK801-induced-c-Fos-positive cells in control mice but not in *Df1/+* mice. Lenti-CAG-Comt-, Lenti-CaMKII-Comt- or Lenti-CAG-GFP-infected *Df1/+* or control mice were killed 2 h after MK801 (0.32 mg kg^{-1}) administration. Representative images of immunohistochemical studies for c-Fos from coronal sections of the prelimbic cortex (PrL; anterior/posterior = +1.9 mm) are shown. Scale bar = 200 μm . Quantification of the density of c-Fos immunoreactive cells was performed **(b)**. Values are the mean \pm s.e.m. for 4–6 mice. * $P = 0.034$, ** $P = 0.021$. **(c, d)** *Comt* overexpression increased c-Fos expression specifically in γ -aminobutyric acid (GABA)-positive cells in both control and *Df1/+* mice. Representative images of immunofluorescent studies for c-Fos (green), GABA (red) and 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei (blue) **(c, e)**. Scale bar = 20 μm . The density of c-Fos⁺ GABA⁺ cells (arrows) was measured in the layer II/III and the layer IV/V of the prelimbic cortex of Lenti-CAG-Comt-, Lenti-CaMKII-Comt- or Lenti-CAG-GFP-infected control **(d)** and *Df1/+* mice **(f)**. Values are the mean \pm s.e.m. of 4–6 mice **(d)** and 3 mice **(f)**. **(d)** * $P = 0.00045$, ** $P = 0.0083$, *** $P = 0.0038$, **(f)** * $P = 0.024$, ** $P = 0.0078$.

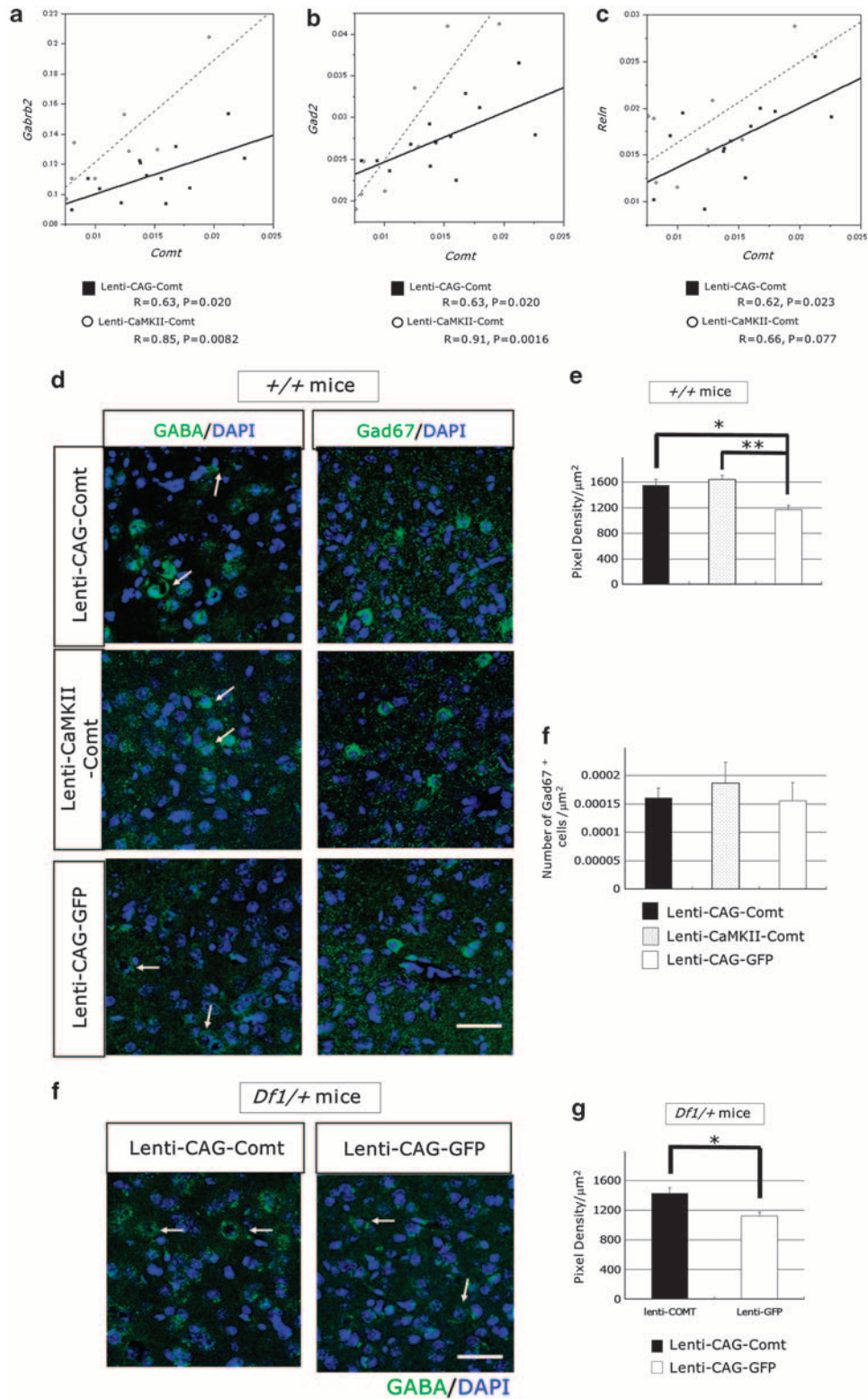


Figure 9 The effects of increasing *catechol-O-methyltransferase (Comt)* expression on γ -aminobutyric acid (GABA)-related molecules in the prefrontal cortex (PFC). (a–c) The expression level of *Comt* correlates with GABA signaling-related genes, *Gabrb2* (*GABA_Areceptor β 2*) (a), *Gad2* (*Gad67*) (b) and *Reln* (*reelin*) (c). *Comt*, *Gabrb2*, *Gad2* and *Reln* were quantified and normalized to *Gapdh* of the PFC of Lenti-CAG-Comt- or Lenti-CaMKII-Comt-infected mice using real-time PCR. Mice were killed 2 weeks after lentiviral infection. A solid line depicts the linear regression for Lenti-CAG-Comt-infected mice, and a dashed line is for Lenti-CaMKII-Comt-infected mice. (d–h) Increased synaptic puncta of interneurons in the PFC of Lenti-CAG-Comt- or Lenti-CaMKII-Comt-infected control mice (d–f) and *Df1/+* mice (g, h). Fluorescent puncta rings surrounding unlabeled soma are shown by arrows (d, g). Quantification of immunofluorescent intensity of GABA in puncta rings (e, h) and the density of *Gad67*⁺ cells (f). Scale = 20 μm . Values are the mean \pm s.e.m. of 3–4 mice. (e) **P* = 0.026, ***P* = 0.0067, (h) **P* = 0.0037.

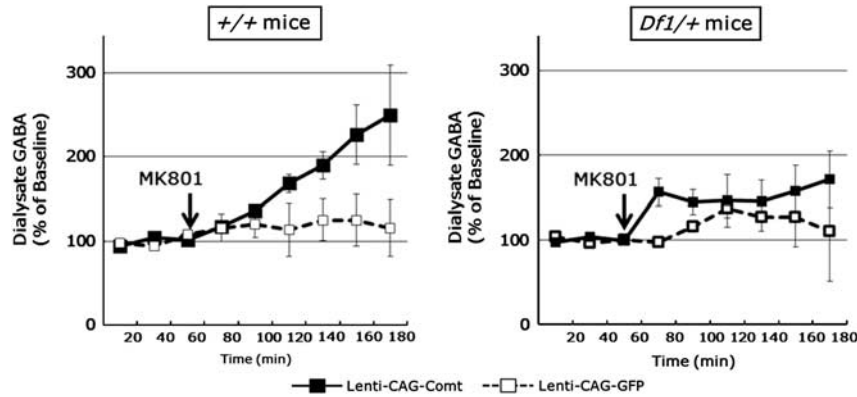


Figure 10 Effects of MK801 on prefrontal cortex (PFC) extracellular γ -aminobutyric acid (GABA) in Lenti-CAG-*Comt* or control Lenti-CAG-GFP-infected *Df1/+* and control mice. Three 20 min baseline fractions were collected before MK801 (0.32 mg kg^{-1} , intraperitoneally (i.p.)) injection and thereafter 20 min fractions were collected for 120 min. Data are means \pm s.e.m. from 3 to 4 mice.

to reverse the curing effect of *Comt* overexpression on behavioral abnormalities of *Df1/+* mice, indicating that the effects do not directly result from increased *Comt* enzymatic activity in the PFC. *Comt* overexpression in the PFC upregulated GABA signaling-related genes, which might explain the effects of *Comt* overexpression on GABA signaling. Furthermore, we showed PFC *Comt* expression increased c-Fos expression specifically in GABAergic interneurons and enhanced MK801-induced GABA release.

Deficits in the GABAergic system are thought to be one of the prominent pathologies of schizophrenia.^{32–34} Post-mortem studies of the brains of schizophrenic patients showed reduction in the 67 kDa isoform of GAD (GAD67), which is an enzyme responsible for GABA synthesis.^{39,40} GAD67 reduction is observed specifically in chandelier cells.⁴¹ Decrease in GABA reuptake transporter and upregulation of GABA_A $\alpha 2$ receptor are also observed at chandelier cell synapse of schizophrenic patients, which might compensate the reduction of GABAergic transmission caused by GAD67 reduction.^{42–44} The abnormal development of parvalbumin-positive interneurons including chandelier cells has been shown in a different mouse model of 22q11DS.⁴⁵ Our data showed the abnormal responsiveness of *Df1/+* mice to a GABA_A receptor agonist (bretazenil) and a GABA_A receptor $\alpha 2/\alpha 3$ agonist (SL651498) in psychostimulant-induced hyperlocomotion. *Comt* overexpression in the PFC not only normalized abnormal responsiveness of *Df1/+* mice to psychostimulants, but also attenuated the abnormal responsiveness to GABA_A receptor agonist. Our studies also demonstrated that increasing *Comt* activity in the PFC caused upregulation of GABA-related genes such as *Gabbr2*, *Gad2* and *Reln*. Single-nucleotide polymorphisms of *RELN* and *GABRB2* are known to associated with risks for schizophrenia.^{46,47} *Reln* is reported to be expressed in GABAergic neurons, and affects the composition of NMDA receptors.⁴⁸ NMDA receptors regulate the activity of fast-spiking interneurons, and NMDA antagonists decrease the activity of interneurons in the PFC, leading to excitation of cortical pyramidal neurons by disinhibition.⁴⁹ Increase in GABA level at the interneuronal synapses and GABA release was observed in PFC *Comt*-overexpressing mice, which might be caused by increased level of *Gad2*. This enhanced GABA

transmission might be responsible for the treatment effects of *Comt* overexpression. Our finding of the regulation of GABA signaling by *Comt* is consistent with a previous report that shows the genetic association of *COMT* with cortical GABA level.⁵⁰

DA is known to modulate GABA transmission in multiple brain regions. Electrically evoked GABA release was reduced by D2 agonists in the PFC and the striatum,^{51,52} but spontaneous release of GABA was increased by D2 agonists in the PFC.⁵² Electrophysiological studies have shown that DA produces an initial reduction in inhibitory postsynaptic current in PFC pyramidal neurons through the reduction in GABA release, probability mediated by D2 receptor, followed by a long-lasting increase in inhibitory postsynaptic current amplitude, which is mainly mediated by D1 receptor.⁵³ Different effects of DA on GABA transmission have been reported, and the molecular mechanism of the effects of *Comt* overexpression on GABA transmission remains to be elucidated.

A *COMT* low-activity allele is identified as a risk factor for impaired cognitive function and psychiatric symptoms in adolescence in 22q11DS.^{14–17} Epistatic interaction between *Comt* and *Prodh* is known to modulate schizophrenia-like phenotypes in mice.⁷ Our data are consistent with these findings. Our studies demonstrate that *Comt* overexpression in the PFC has a curing effect on *Df1/+* mice and that *Comt* has dual roles in the regulation of responsiveness to GABA_A receptor agonists. The high *Comt* activity enhanced the GABA production and GABA release in the PFC and increased the responsiveness to GABA_A receptor agonists, whereas endogenous relatively low *Comt* activity seemed to have the opposite effects, because a *Comt* inhibitor increased the responsiveness to GABA_A receptor agonist in methamphetamine-induced locomotion of control mice. This phenotype of tolcapone-treated mice is similar to *Df1/+* mice, which have *Comt* heterozygosity. It is tempting to speculate the existence of inverted U-shaped relationship between *Comt*-regulated cortical DA level and GABA transmissional regulation.

Our pharmacological data suggest that GABA_A $\alpha 2/\alpha 3$ receptor might be a therapeutic target for psychiatric

symptoms of 22q11DS, although conflicting results were obtained for therapeutic effects of GABA_A $\alpha 2/\alpha 3$ receptor agonist for general schizophrenia.^{54,55} Schizophrenia is considered to be a heterogeneous group of disorders, and individual tailor-made drug treatment might be required for effective treatment of schizophrenia patients according to the disease etiology. In addition, pharmacological treatments to increase *Comt* expression or activity also can be used to reduce or prevent psychiatric symptoms of 22q11DS. However, therapeutic intervention should take into account the dual roles of *Comt* in the regulation of GABA transmission.

Conflict of interest

The authors declare no conflict of interest.

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