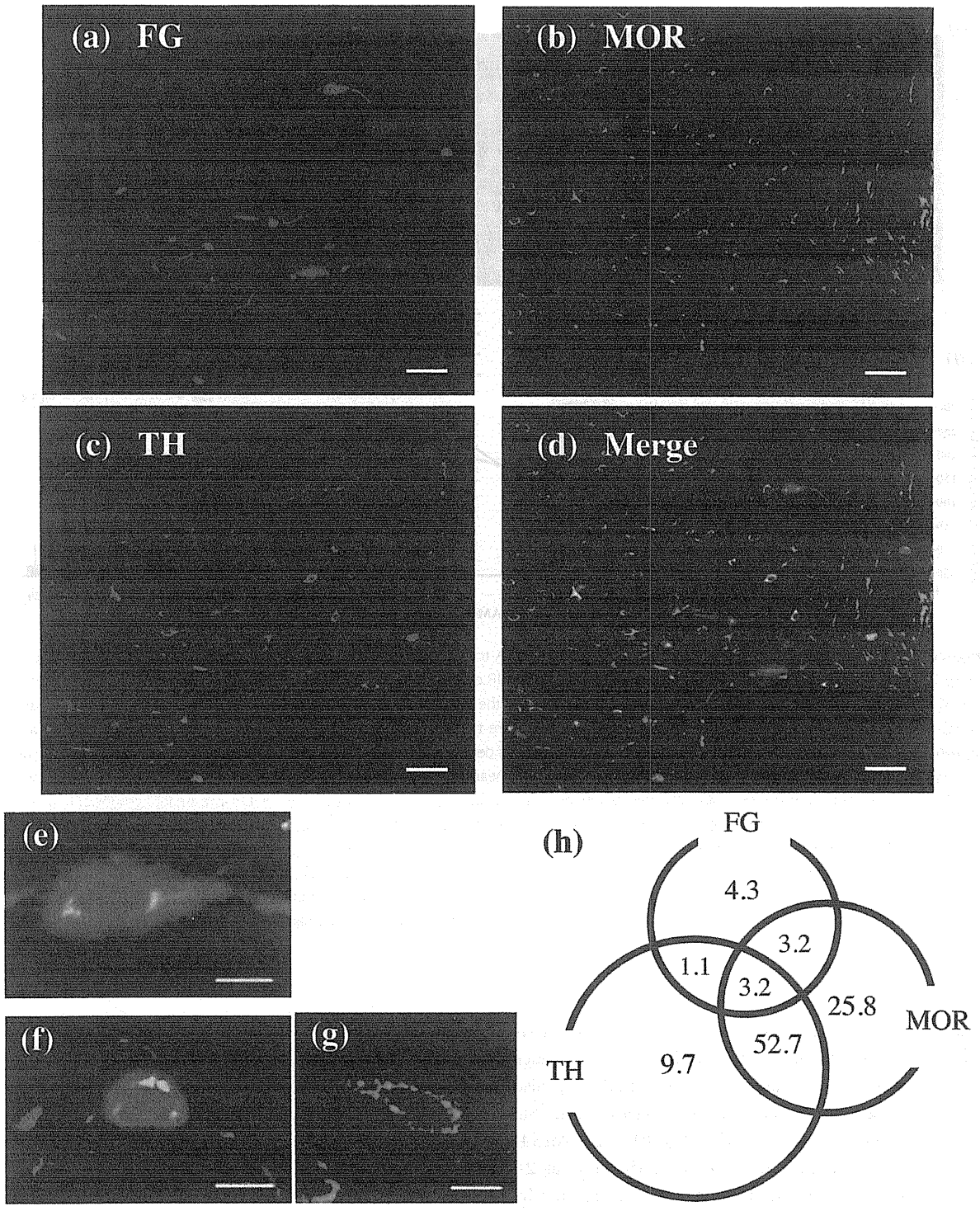

Figure 4 Characterization of μ-opioid receptors (MORs) in the VTA. (a) Cells in the VTA after the microinjection of FG into the ACG. (b) MOR-IR was noted in the VTA. (c) TH-IR was noted in the VTA. (d–g) (e–g: higher magnification) Triple-labelling experiments showed that MOR-IR in the VTA was present on both dopaminergic and non-dopaminergic neurons projecting to the ACG. Some of MOR-labelled neurons that did not project to the ACG did not show TH-IR. (h) Percentages of MOR-, TH- and FG-labels, individually and in combination, in the VTA. Scale bars, 50 μm (a–d), 10 μm (e–g)

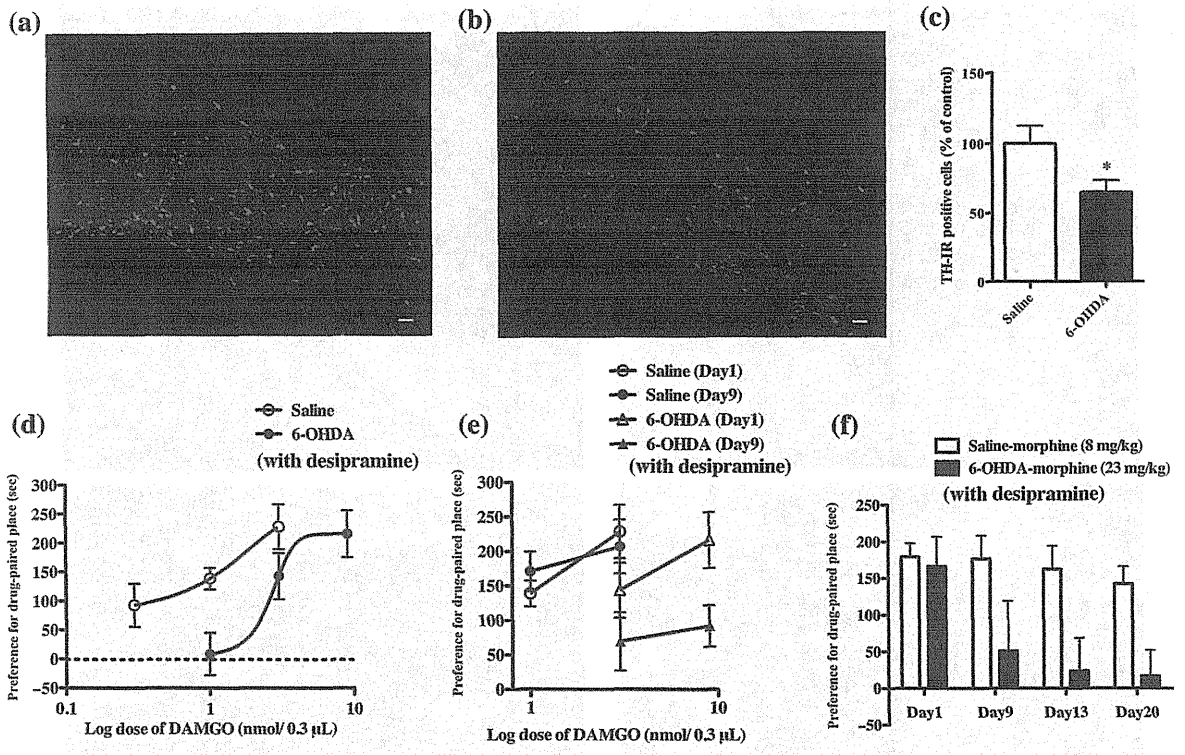

Figure 5 Involvement of dopamine neurons projecting from the VTA to the ACG in the DAMGO- or morphine-induced rewarding effect. (a–b) Colour photomicrographs of immunofluorescent staining of TH-IR cells in the rat VTA following saline (a) or 6-OHDA (b) injection into the ACG. (c) Percent of TH-IR positive cells in the VTA of rats showing the saline or 6-OHDA injection into the ACG. Student's *t*-test: $^{*}P < 0.05$ versus saline. (d) DAMGO-induced place preference in the saline-pre-treated and 6-OHDA-pre-treated groups using the CPP assay are shown. The ordinate shows the preference for the drug-paired place, as defined by the post-conditioning test score minus the pre-conditioning test score in the drug treatment side. Each point represents the mean conditioning score with SEM of 6–8 rats. (e) Extinction of the DAMGO-induced place preference. Data show the conditioned place preference scores in saline- or 6-OHDA-pre-treated groups of 6–8 rats. Conditioning was performed for six days after the pre-conditioning test. The post-conditioning test was performed on the day after the final conditioning test (one day). To investigate the extinction of the DAMGO-induced place preference, a post-conditioning test was performed at nine days after the final conditioning test. (f) Extinction of the morphine-induced place preference. Data show the conditioned place preference scores in saline- or 6-OHDA-pre-treated groups of six rats. To investigate the extinction of the morphine-induced place preference, a post-conditioning test was performed on 9, 13, 20 days after the final conditioning test. Scale bars, 50 μm (a–b)

selective D1 receptor antagonist SCH23390. Furthermore, the maintenance of morphine-induced place preference at 10 days after the final conditioning also was suppressed by pre-treatment of SCH23390 (Fig. 6a, b). The levels of phosphorylated-DARPP32 (Thr34) and phosphorylated-CREB (Ser133) in the ACG at 24 hours after the final conditioning were not affected by morphine (Fig. 6c, e), whereas the morphine-induced place preference at 10 days after the final conditioning produced significant increases of phosphorylated-DARPP32 (Thr34) and phosphorylated-CREB (Ser133) levels in the ACG. The increases of these levels in the ACG were blocked by pre-treatment of SCH23390 (Fig. 6d, f). Statistical analysis was performed with one-way ANOVA followed by Bonferroni's multiple comparison test ($^{*}P < 0.05$, $^{**}P < 0.01$ versus saline–saline, $\#P < 0.05$, $\#\#P < 0.01$ versus saline–morphine).

DISCUSSION

The mPFC is composed of the infralimbic (IL), prelimbic (PL) (area CG3 of the ACG), dorsal and ventral anterior cingulate (areas CG1 and CG2 of the ACG) and medial precentral (frontal area 1) cortical areas (Paxinos & Watson 1998; Ongur & Price 2000). The mPFC receives dopaminergic efferents from the VTA, and thus is part of the mesocortical dopamine system, with the densest innervation occurring within the IL and PL regions (Thierry *et al.* 1973; Conde *et al.* 1995). Within the mPFC, the PL is the main source of afferents to the IL. Other projections to the IL originate from the hippocampus (CA1/subiculum), basolateral amygdala (BLA) and VTA. The PL receives projections from the IL, CG1 and CG2, hippocampus (CA1/subiculum), BLA and VTA. The primary sources of afferents to the CG1 and CG2 are con-

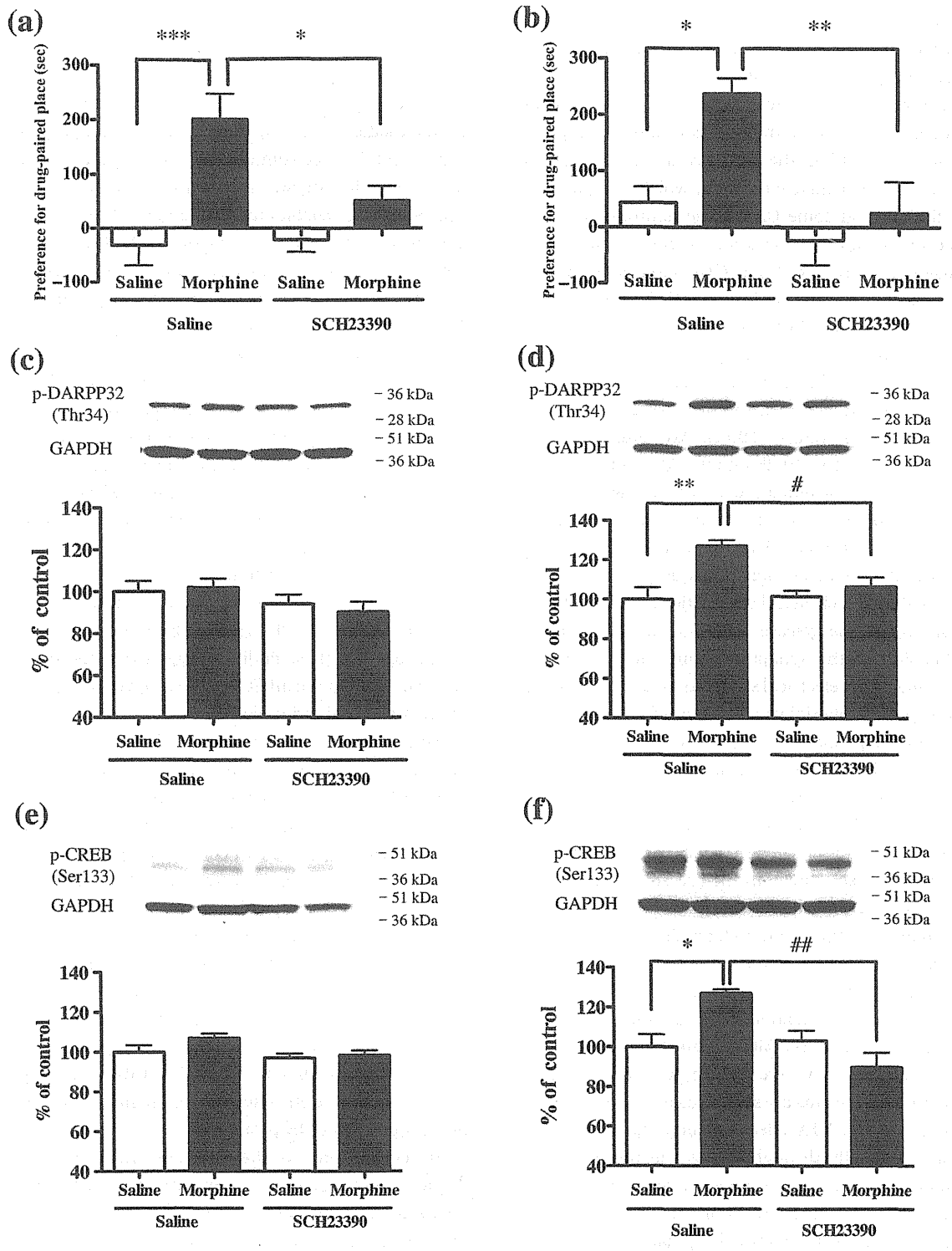

Figure 6 Changes in levels of phosphorylated DARPP32 (Thr34) or phosphorylated CREB (Ser133) in the ACG of rats that had acquired or maintained the morphine-induced place preference. (a–b) Change in the acquisition or maintenance of morphine-induced place preference at 24 hours (a) or 10 days (b) after the final conditioning. Data show the conditioned place preference scores in saline- or SCH23390-pre-treated saline or morphine groups of six rats. (c–d) Change in the level of phosphorylated DARPP32 (Thr34) in lysate fractions of the ACG at 24 hours (c) or 10 days (d) after the final conditioning. (e–f) Change in the level of phosphorylated CREB (Ser133) in lysate fractions of the ACG at 24 hours (e) or 10 days (f) after the final conditioning. In the conditioning session, the rats were administered morphine (8 mg/kg, i.p.) 15 minutes after the pre-treatment of saline or SCH23390 (0.1 mg/kg, i.p.). Each column represents the mean with SEM of at least three independent experiments. Bonferroni multiple comparison test: $^{*}P<0.05$, $^{**}P<0.01$ versus saline–saline, $\#P<0.05$, $\#\#P<0.01$ versus saline–morphine

sidered to be the PL, hippocampus (CA1/subiculum), BLA and VTA (Hoover & Vertes 2007). In the present study, several FG-positive VTA–ACG projecting neurons in the VTA showed a TH-positive reaction, which suggested the existence of dopamine transmission in the ACG projecting from the VTA in the rat. In contrast, some of them exhibited a TH-negative reaction, which is supported by the finding that some GABAergic neurons in the VTA project to the mPFC (Carr & Sesack 2000). Furthermore, by using the retrograde tracer CTb as well as FG, we found that neurons that project from the VTA to the ACG exist independently and do not branch off from VTA–N.Acc projecting neurons. Interestingly, the ACG projects to the dorsal striatum, whereas the IL and PL project to the shell and core of the N.Acc, respectively (Berendse, Galis-de Graaf & Groenewegen 1992). We found that pre-microinjection of 6-OHDA into the ACG failed to change the basal levels of dopamine and its metabolites in the N.Acc. To further verify the dopaminergic neurons that project directly from the VTA to the ACG, we investigated whether the VTA-ACG dopaminergic pathway could be activated by the electrical stimulation of VTA cells. As a result, dopamine release with increased levels of its metabolites at the synaptic terminal in the ACG was increased by the electrical stimulation of VTA cells. These results provide physiological evidence for the existence of VTA–ACG dopaminergic projection.

In both the paranigral and parabrachial nuclei, which are the two major VTA subdivisions, MOR-labelling was observed within somata and proximal dendrites and was sometimes continuous with labelled neuronal somata (Garzon & Pickel 2001). In the present study, MOR was found at a high density in the VTA and was detected in both dopaminergic and non-dopaminergic VTA neurons projecting to the ACG. Some of the MOR-labelled neurons with no projecting to the ACG were also non-dopaminergic. In addition, in a preliminary study, we found that some of the MOR-labelled non-dopaminergic neurons in the VTA were GABAergic neurons (data not shown). These results are supported by a report that MOR agonists in the VTA affect dopaminergic transmission mainly indirectly through changes in the postsynaptic responsivity and/or presynaptic release from neurons containing other neurotransmitters, whereas MOR agonists also directly affect a small population of dopaminergic neurons expressing MOR on their dendrites in the VTA (Garzon & Pickel 2001).

We next investigated the possible release of dopamine in the ACG by the intra-VTA administration of a MOR agonist. The dopamine level at the synaptic terminal in the ACG was markedly increased by microinjection of DAMGO into the VTA. Microinjection of DAMGO into the VTA also produced a significant increase in the dopamine metabolites DOPAC and HVA, indicating that activation of MORs in the VTA by intra-VTA microinjection of DAMGO activates the VTA–ACG dopaminergic pathway in a depolarization-dependent manner.

The CPP procedure has been used extensively as an animal model for investigating the rewarding properties of drug-conditioned stimuli. The CPP procedure has also been used to investigate inhibitory effects on the acquisition (Shoblock, Wichmann & Maidment 2005; Esmaeili *et al.* 2009), consolidation (Alberini 2008; Esmaeili *et al.* 2009), expression (Shoblock *et al.* 2005; Esmaeili *et al.* 2009), extinction (Shoblock *et al.* 2005; Zhai *et al.* 2008), reinstatement (Shoblock *et al.* 2005; Popik, Wrobel & Bisaga 2006; Zhai *et al.* 2008) and reconsolidation (Alberini 2008). In the present study, the DAMGO-induced place preference was attenuated by the pre-microinjection of 6-OHDA, which destroys dopaminergic neurons, into the ACG. This finding is supported by a report that kainic acid lesions of the ACG prevent the acquisition of a morphine-induced place preference in mice (Hao *et al.* 2008). It was also reported that quinolinic acid lesions of the IL blocked the CPP induced by morphine, while lesions of the PL did not affect a morphine-induced CPP (Tzschentke & Schmidt 1999). Taken together, these findings suggest that the IL, CG1 and CG2 areas in the mPFC may be involved in the acquisition of μ-opioid-induced CPP.

More interestingly, we found here for the first time that rats that had been subjected to the ACG-microinjection of 6-OHDA failed to maintain μ-opioid-induced place preference in the early phase. Furthermore, the levels of phosphorylated DARPP32 (Thr34) and phosphorylated CREB (Ser133) in the ACG were increased in rats that maintained the μ-opioid-induced place preference, whereas the increases of these levels were almost abolished by pre-treatment of a selective dopamine D1 receptor antagonist. Although further examinations are needed to identify the role of VTA–ACG dopaminergic neurons in learning and memory, the present findings provide evidence that D1/DARPP32/CREB pathway in the ACG may be critical for the maintenance of a place preference induced by μ-opioids.

In conclusion, we have demonstrated here that dopamine in the ACG can be released by the activation of MORs in the VTA by MOR agonists, and the dopaminergic transmission that projects from the VTA to the ACG may be crucial for the acquisition and maintenance of the rewarding effects of μ-opioids. Furthermore, the activation of D1/DARPP32/CREB signalling in the ACG may be involved in the maintenance of the μ-opioid-induced place preference.

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Authors Contribution

MN and YM designed the experiments and wrote the manuscript. YM was responsible for most of the experimental work. KN contributed some of retrograde tracing and biochemical studies. MN performed biochemical experiments. ST, KN, KK, MA and MS conducted some of *in vivo* studies. MA and SI contributed some of biochemical experiments. KI and NK provided scientific and technical advice. TS supervised the overall projects. All authors discussed the results and commented on the manuscript.

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Short Communication

Antidepressant-Like Effect of Venlafaxine Is Abolished in μ-Opioid Receptor–Knockout Mice

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Abstract. Although the opioid system is known to modulate depression-like behaviors, its role in the effects of antidepressants is not yet clear. We investigated the role of μ-opioid receptors (MOPs) in the effects of venlafaxine, a serotonin and norepinephrine reuptake inhibitor, in the forced swim test using MOP–knockout (KO) mice. Venlafaxine reduced immobility time in wild-type mice (C57BL/6J), but not in MOP-KO mice, although no significant effects were observed on locomotor activity. These results suggest that MOPs play an important role in the antidepressant-like effects of venlafaxine.

Keywords: μ-opioid receptor, knockout mouse, antidepressant

The opioid system has been implicated in mood disorders. Pretreatment with naloxone, a nonselective opioid-receptor antagonist, decreased immobility time in mice in the forced swim test (1). Chronic morphine treatment facilitated immobility in the forced swim test (2). These reports indicate that the opioid system impacts behavioral responses to depression-like behavior. Opioid receptors have been classified into at least three subtypes: μ, δ, and κ (MOP, DOP, and KOP, respectively). Several reports have shown that selective ligands for each subtype modulate depression-like behavior in the forced swim test (3 – 5). Although these three receptor subtypes may be involved in depression-like behavior, even the most selective ligands for a specific opioid receptor subtype possess certain affinities for other subtypes (6), which may contribute to the discrepant findings regarding the role of opioid receptor subtypes. Therefore, the role of each subtype in the modulation of depression-like behavior has not yet been clearly delineated by traditional pharmacological studies that use only selective ligands.

The development of knockout (KO) mice with MOP gene deletion has revealed a central role of MOPs, rather than other opioid receptor subtypes, in the various effects of opioids, including analgesia, reward, and tolerance (7, 8). Although several compensatory changes might occur in KO animals, these animals have potential utility in investigating the in vivo roles of specific proteins. Our previous report showed that MOPs also modulate responses to stress, including depression-like behavior (9). The use of MOP-KO mice could provide novel theories on the molecular mechanisms underlying the modulation of depression-like behavior. Although the opioid system has been implicated in the mechanism of action of antidepressants (10), the precise mechanisms are not yet clear. The present study investigated the role of MOPs in the effects of venlafaxine, a serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine reuptake inhibitor, in the forced swim test using MOP-KO mice.

The present study used wild-type and homozygous MOP-KO mouse littermates on a C57BL/6J genetic background as previously described (7). The experimental procedures and housing conditions were approved by

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the Institutional Animal Care and Use Committee, and all animal care and treatment were in accordance with our institutional animal experimentation guidelines. Naive adult (>10-week-old) male mice were group-housed 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 and off at 8:00 pm. Food and water were available ad libitum. All behavioral tests were conducted between 1:00 pm and 6:00 pm.

Venlafaxine hydrochloride (LKT Laboratories, St. Paul, MN, USA) was dissolved in saline and injected in volumes of 10 ml/kg.

For the forced swim test, animals were forced to swim in a cylindrical Plexiglas tank (30-cm height × 30-cm diameter) containing 20-cm-deep water for 6 min per day for 5 consecutive days. The water temperature was maintained at approximately 25°C. Immobility time was recorded with an animal activity monitoring apparatus equipped with an infrared detector (SUPERMEX, CompACT FSS; Muromachi Kikai Co., Tokyo). After each session, the mice were immediately removed from the cylinder, dried with a towel, and kept under a heating lamp until completely dry, before being returned to their home cages. Venlafaxine and saline were administered subcutaneously 20 min before each daily test.

Locomotor activity was assessed with an animal activity monitoring apparatus equipped with an infrared detector (SUPERMEX, CompACT FSS, Muromachi Kikai Co.). Mice were placed individually in 30 × 45 × 30 cm plastic cages, to which they had not been previously exposed, under dim light and sound-attenuated conditions. Locomotor activity was monitored for 30 min. Venlafaxine and saline were administered subcutaneously 20 min before the test.

Data were analyzed with analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test. Values of $P < 0.05$ were considered statistically significant.

To evaluate the antidepressant-like effects of venlafaxine, immobility time during the 6 min, 5-consecutive-day forced swim test was analyzed in wild-type and MOP-KO mice (Fig. 1). Two-way, repeated-measures ANOVA of total immobility time during the 6-min test on each of the 5 days with drug dose revealed that immobility time significantly decreased after venlafaxine treatment in wild-type mice ($F_{2,100} = 13.2$, $P < 0.001$), but not in MOP-KO mice (Fig. 1: A and B). *Post hoc* comparisons revealed that venlafaxine treatment (10 mg/kg) reduced immobility time in wild-type mice ($P < 0.05$) from Day 1 to 4. Venlafaxine treatment (30 mg/kg) also reduced immobility time in wild-type mice ($P < 0.05$) on Day 3 and 4. In the saline-treated group, two-way, repeated-measures ANOVA of immobility time with genotypes of

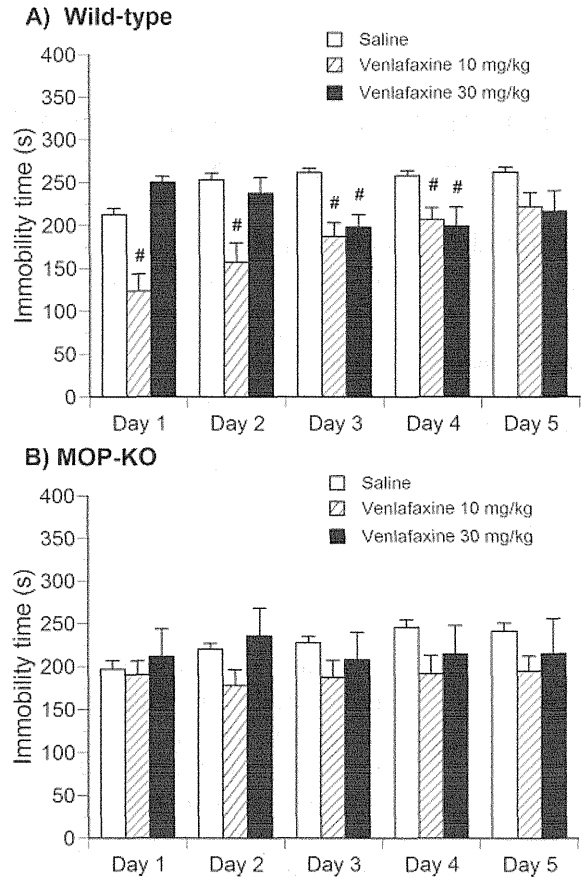

Fig. 1. Effect of venlafaxine on immobility time in the forced swim test in wild-type and MOP-KO mice. Animals were subjected to daily 6-min tests for 5 consecutive days. The figure shows the cumulative immobility time during the 6-min tests over 5 days in wild-type mice that received saline (n = 12) or venlafaxine (10 mg/kg, n = 9; 30 mg/kg, n = 7) (A) and MOP-KO mice that received saline (n = 8) or venlafaxine (10 mg/kg, n = 6; 30 mg/kg, n = 6) (B). $^{\#}P < 0.05$, significant difference from corresponding value in the saline-treated group. Data are expressed as the mean ± S.E.M.

mice revealed that immobility time was significantly shorter in MOP-KO mice than wild-type mice ($F_{1,18} = 9.4$, $P < 0.01$), similar to our previous report (9). Immobility time in MOP-KO mice was significantly shorter than that in wild-type mice on Day 2 ($F_{1,18} = 8.2$, $P < 0.05$) and Day 3 ($F_{1,18} = 14.7$, $P < 0.005$). Two-way, repeated-measures ANOVA of immobility time with genotypes of mice also revealed no significant differences between genotypes in either the 10 mg/kg venlafaxine– or 30 mg/kg venlafaxine–treated groups.

To test the possible influences of motor dysfunction on the antidepressant-like effects of venlafaxine, locomotor activity in both wild-type and MOP-KO mice was analyzed (Fig. 2). Venlafaxine showed no significant effects on locomotor activity in either wild-type or MOP-KO mice.

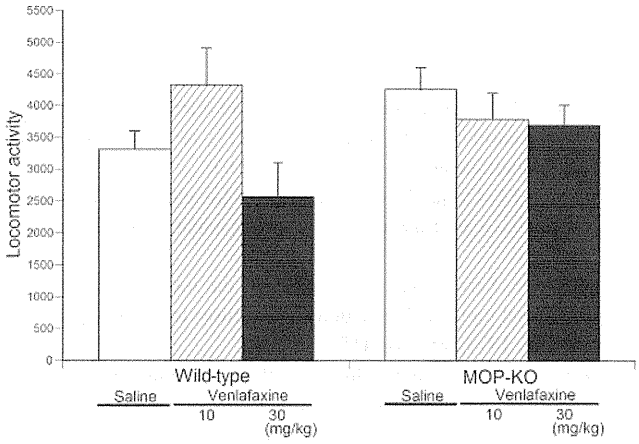

Fig. 2. Effect of venlafaxine on locomotor activity in wild-type and MOP-KO mice. The figure shows locomotor activity during a 30-min period of habituation to a novel environment in wild-type mice (n = 4 – 6) and MOP-KO mice (n = 4) that received venlafaxine pretreatment 20 min before the test. Each column represents the cumulative activity counts during the 30-min session. Data are expressed as the mean ± S.E.M.

In the present study, venlafaxine reduced immobility time in wild-type mice in the forced swim test, an effect that was abolished in MOP-KO mice. These results suggest that MOPs play an important role in the antidepressant-like effects of venlafaxine. This is consistent with previous reports showing that the antidepressant-like effects of venlafaxine in the forced swim test in mice were antagonized by naloxone, a nonselective opioid antagonist (10), although selective antagonists for each opioid subtype were ineffective. Venlafaxine is a nontricyclic antidepressant that inhibits both 5-HT and norepinephrine reuptake and has no binding affinity for opioid receptors (11). Venlafaxine blocks 5-HT uptake at low doses and norepinephrine uptake at higher doses, and the doses of venlafaxine used in the present study (10 and 30 mg/kg) may act on both 5-HT and norepinephrine transporters (12). Thus, the indirect modulation of 5-HT and norepinephrine neurotransmission by endogenous opioid neurotransmission via MOPs may be hypothesized to be involved in the antidepressant-like effects of venlafaxine.

The locus coeruleus is hypothesized to be one of the most important brain regions implicated in stress, depression, and the mechanisms of action of antidepressants. Stress and depression activate the hypothalamic–pituitary–adrenal axis and increase norepinephrine release in the locus coeruleus. Norepinephrine release in the locus coeruleus is partially regulated by both opioid and noradrenergic mechanisms (13). The locus coeruleus also receives dense 5-HT projections from the dorsal raphe and pericoerulear region (14). Moreover, acute administration of venlafaxine exerted an inhibitory effect on the spontaneous activity of locus coeruleus neurons (15). Thus, a possible mechanism of action of the antidepressant-like effects of venlafaxine may be inhibition of locus coeruleus activity, which may be regulated by endogenous norepinephrine, 5-HT, and opioid systems. Deletion of MOPs might suppress the molecular control of locus coeruleus neurotransmission and depression-like responses in MOP-KO mice.

In the present study, MOP-KO mice showed reduced immobility in the forced swim test, which is consistent with our previous report showing decreased immobility in both the tail-suspension and forced swim tests and reduced stress-induced plasma corticosterone concentrations in MOP-KO mice compared with wild-type mice (9). Thus, MOP-KO mice may be resistant to the stress stimulus and be in an antidepressive-like state. Further studies may reveal the changes in neurotransmission that suppress depression-like responses and abolish the antidepressant-like effect of venlafaxine in MOP-KO mice.

In conclusion, the antidepressant-like effects of venlafaxine were abolished in MOP-KO mice, with no effect on locomotor activity. These results suggest that MOPs play an important role in the modulation of the antidepressant-like effect of venlafaxine.

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

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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.

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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

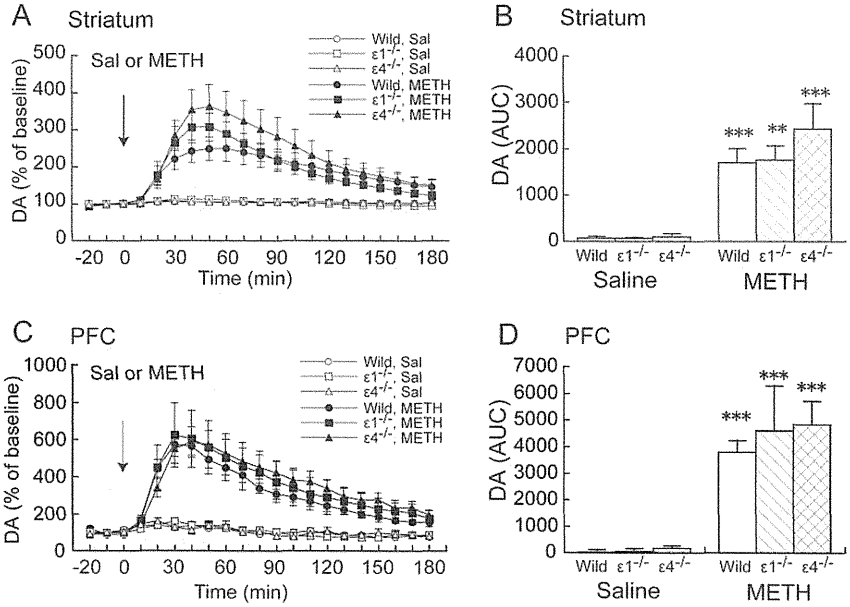

Figure 1. Effects of acute METH on DA_{ex} in the striatum and PFC in wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice. (**A, C**) Temporal pattern of DA_{ex} before and after s.c. saline (Sal) or METH (1 mg/kg) injection. The arrows indicate the drug injection time. Each point represents the mean ± 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).

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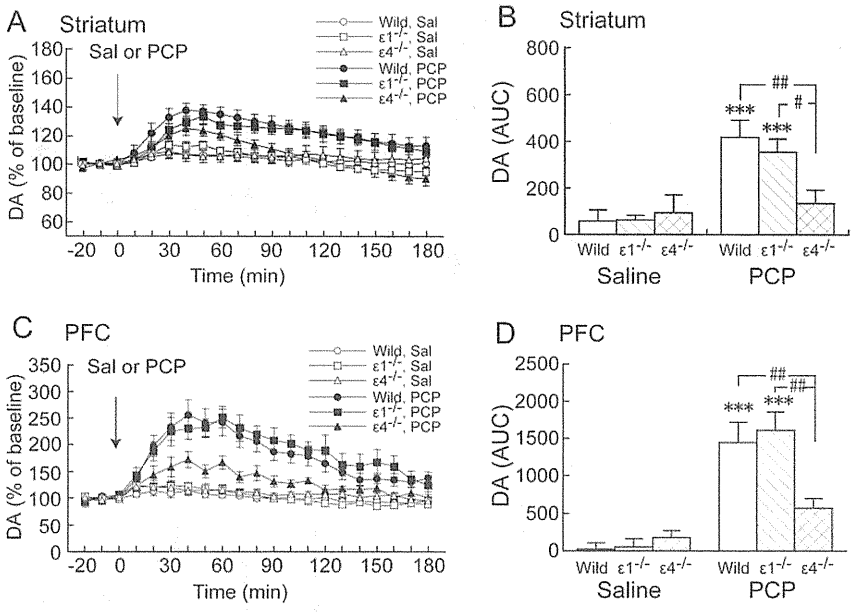

Figure 2. Effects of acute PCP on DA_{ex} in the striatum and PFC in wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice. (**A, C**) Temporal pattern of DA_{ex} before and after s.c. saline (Sal) or PCP (3 mg/kg) injection. The arrows indicate the drug injection time. Each point represents the mean ± SEM 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.

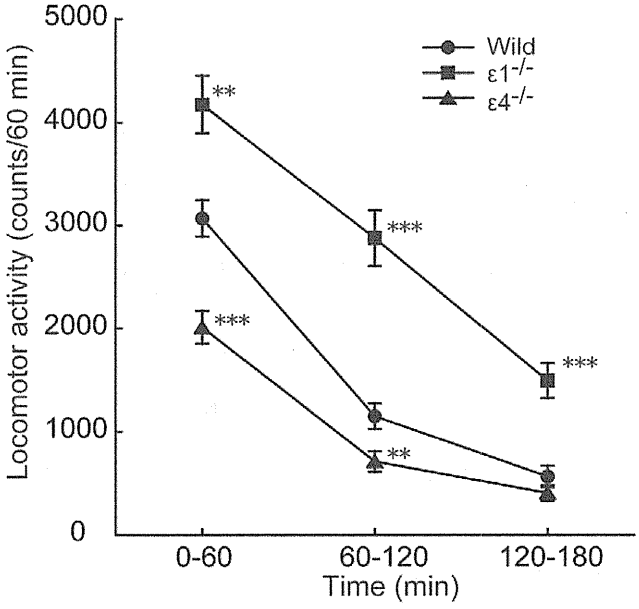

Figure 3. Locomotor activity in wildtype, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice in a novel environment. Locomotor activity was measured for 180 min. Each point represents the mean ± SEM (n = 34–50). *p<0.05, **p<0.01, ***p<0.001, compared with wildtype mice (one-way ANOVA followed by Fisher's PLSD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

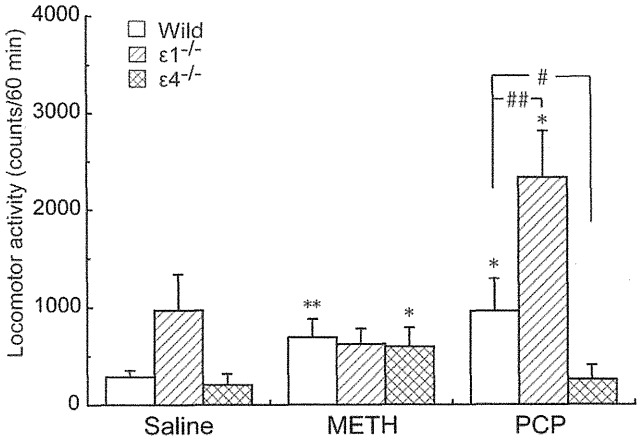

Figure 4. Effects of acute METH and PCP on the locomotor activity in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice. Locomotor activity after acute saline, METH (1 mg/kg), or PCP (3 mg/kg) administration ($n = 10$–25). $^{*}p<0.05$, $^{**}p<0.01$, compared with saline (Student's t-test); $^{\#}p<0.05$, $^{\#\#}p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

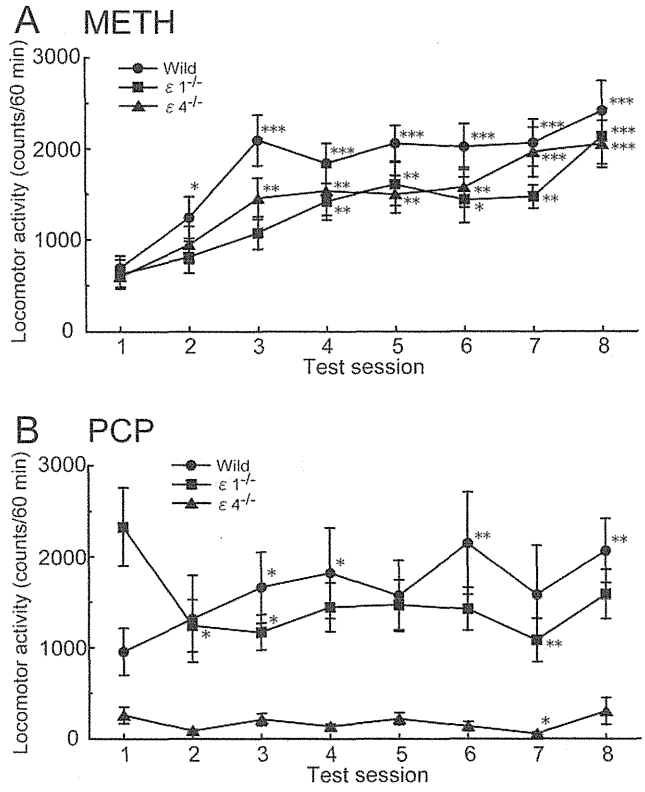

Figure 5. Effects of repeated METH and PCP on the locomotor activity in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice. Changes in response to repeated administration of (**A**) METH (1 mg/kg) or (**B**) PCP (3 mg/kg) ($n = 15$–25). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared with Session 1 of the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

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.

Drugs

Drugs were dissolved in saline and administered s.c. in a volume of 10 ml/kg. In the microdialysis experiment, saline, METH (1 mg/kg), or PCP (3 mg/kg) was administered after establishing a stable baseline, and the dialysate was continuously collected for 180 min. In the acute behavioral experiments, saline, freshly prepared METH (1 mg/kg; Dainippon Sumitomo Pharma, Osaka, Japan), or PCP (3 mg/kg; Shionogi Pharmaceutical Co. Ltd., Osaka, Japan) was administered. In the repeated behavioral experiments, METH (1 mg/kg) or PCP (3 mg/kg) was administered repeatedly at 2 or 3 day intervals for a total of seven injections. One week after withdrawal, METH or PCP challenge injections were administered as described above.

Statistical analysis

DA_{ex} responses to drugs are expressed as a percentage of baseline. The AUC of DA_{ex} during the 180 min period after drug administration was calculated as the effects of the drugs. Area-under-the-curve values of all groups were analyzed using two-way ANOVA. Individual *post hoc* comparisons were performed with Fisher's PLSD test. The responses to acute administration were analyzed using Student's *t*-test, one-way ANOVA, or two-way ANOVA. To evaluate behavioral sensitization, the response to drugs in Session 8 was compared with the response to the first drug injection (Session 1) in the same animal using a paired *t*-test or mixed-design ANOVA. Values of $p<0.05$ were considered statistically significant. Data were analyzed using Statview J5.0 software (SAS Institute, Cary, NC, USA).

Supporting Information

Table S1 Striatal gene expression in wildtype and GluRε4-/- mice.
Found at: doi:10.1371/journal.pone.0013722.s001 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: YH KI. Performed the experiments: YH HY. Analyzed the data: YH HY. Contributed reagents/materials/analysis tools: SK HY TN MM. Wrote the paper: YH WH TN MM KI.

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Transgenic mice in the study of drug addiction and the effects of psychostimulant drugs

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The first transgenic models used to study addiction were based upon *a priori* assumptions about the importance of particular genes in addiction, including the main target molecules of morphine, amphetamine, and cocaine. This consequently emphasized the importance of monoamine transporters, opioid receptors, and monoamine receptors in addiction. Although the effects of opiates were largely eliminated by μ opioid receptor gene knockout, the case for psychostimulants was much more complex. Research using transgenic models supported the idea of a polygenic basis for psychostimulant effects and has associated particular genes with different behavioral consequences of psychostimulants. Phenotypic analysis of transgenic mice, especially gene knockout mice, has been instrumental in identifying the role of specific molecular targets of addictive drugs in their actions. In this article, we summarize studies that have provided insight into the polygenic determination of drug addiction phenotypes in ways that are not possible with other methods, emphasizing research into the effects of psychostimulant drugs in gene knockouts of the monoamine transporters and monoamine receptors.

Keywords: transgenic; knockout; psychostimulant; amphetamine; cocaine

Introduction

In recent years important advances have been made in developing new animal models to help identify the mechanisms of action of psychostimulant drugs underlying their behavioral and physiological effects, including the abuse liability of these drugs and other adverse consequences, in particular the toxicity and lethality associated with the use of psychostimulant drugs. Genetic mouse models are being used to identify genes that may predict risk for the development of drug abuse and addiction or to investigate under more controlled circumstances the consequence of direct manipulation of particular genes implicated in addiction from human genetic studies. Genetic mouse models have been used for estimating genetic correlations between drug-related traits[1,4] and for studying the roles of specific genes in addiction relevant behavioral and physiological traits.[5,6] Progress in this area of research has profound implications for the improved understanding and treatment of drug addiction. At this time there is a large literature on responses to psychostimulants in gene mutant mice. The largest body of literature on the genetics associated with psychostimulant-related behavioral effects has focused on drug reward and drug conditioning, including conditioned place preference (CPP) and self-administration. This work has emphasized primarily the acute rewarding effects of psychostimulants, or early stages of drug taking. Several other areas have been less well examined or, sometimes, not examined at all. There is a need for more investigation of the genetic determinants of sensitivity to psychostimulant-induced neurotoxicity and other adverse effects. Similarly, there is a great deal of work to be done in the quest for genes that influence the development and acceleration of psychostimulant dependence and phenotypes that may be associated with later stages of the addictive process, including extinction, reinstatement, reconsolidation, habit formation, and many other mnemonic aspects of responses to addictive drugs.

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So saying, transgenic models have contributed greatly to our understanding of the mechanisms underlying the actions of psychostimulant drugs. One surprising outcome of these studies has been the polygenic basis of these effects and the degree to which substantial perturbations from gene deletions may alter the normal mechanism of action of particular drugs. Thus, animals will show the same underlying behavioral phenotype, sometimes largely unaltered from the wild-type (WT) condition, but its underlying basis appears to be quite different. Several examples of this type of finding are discussed in the sections that follow, raising the important question of whether similarly large differences in underlying mechanisms exist in humans as are observed in some of these types of models.

Monoamine transporter knockouts

Psychostimulant drugs increase extracellular levels of monoamines by blocking the neuronal plasma membrane transporters (reuptake inhibitors) or by blocking the vesicular transporter (releasers). Increased extracellular dopamine (DA) levels in mesocorticolimbic DA systems have been postulated to mediate the rewarding effects of cocaine,[7] as well as other psychostimulants. The heritability of drug abuse and dependence is relatively high for psychostimulants,[8] indicating that genetic differences that determine the extent of DA release may be important determinants of addiction liability, as well as other effects of acute and chronic psychostimulant exposure. For example, we have recently shown that the number of repeat alleles of the DA transporter (DAT) gene is associated with the risk for methamphetamine (METH) psychosis.[9] This study demonstrated that the presence of nine or fewer repeat alleles of the variable number of tandem repeats in the 3′ untranslated region of DAT is a strong risk factor for a poorer prognosis of METH psychosis. Studies in transgenic mice, particularly knockout (KO) mice in which one or both of the gene alleles are deleted or inactivated, have contributed a great deal to our understanding of the mechanisms underlying psychostimulant actions. This has been particularly useful in the study of psychostimulants because they generally bind to multiple transporters and thereby affect the function of multiple monoamine systems.

Cocaine

Initial transgenic studies into the molecular mechanisms of the effects of psychostimulants, using mice lacking the monoamine transporters, were substantially influenced by the previous pharmacological literature. Prior to the development of these transgenic models, the rewarding effects of cocaine were found to be best correlated with DAT blockade on the basis of structure–activity relationships of transporter-blocking compounds with different potencies at DAT, the serotonin transporter (SERT), and the norepinephrine transporter (NET).[7] As can be seen in Table 1, most studies have concentrated on the rewarding and locomotor stimulant effects of cocaine, with much less work examining other psychostimulant effects.

DAT, SERT, and NET gene KO mice

In contrast to the hypothesis stated in the preceding paragraph, initial data in DAT KO mice demonstrated intact cocaine reward in the CPP paradigm[3] and in an initial self-administration study.[10] Hence DAT KO mice retained the ability to acquire and maintain cocaine self-administration, as well as cocaine-conditioned behavior, in ways that were not substantially different from WT mice. These data therefore indicated that the reinforcing effects of cocaine could be mediated via DAT-independent mechanisms. This is not to say that these data indicated that there was no involvement of DA in cocaine reward. In the Sora *et al.*[3] study, cocaine CPP was observed at both doses tested in WT mice, but only the higher dose produced a significant CPP in DAT KO mice. However, with the largely intact effects of cocaine in these studies, the logical next step was to examine whether other cocaine targets (e.g., SERT and NET) were involved. Further work continued to emphasize that the consequences of cocaine administration were determined by multiple interacting systems. In support of this conclusion, drawn in part from studies of mice in which multiple genes were manipulated with transgenic methods, genetic background was also found to affect the consequence of single-gene KOs. Thus, cocaine CPP was more substantially reduced in congenic DAT KO mice on either a C57BL/6[11,12] or DBA/2J[12] background, which would suggest that the expression of other genes in particular genetic backgrounds affected the consequence of the gene KO. Obviously

Table 1. Cocaine responses in monoamine transporter transgenic mice

Citation	Gene	Micro-dialysis	Loco-motion	Sensiti-zation	CPP	Self-administration	PPI	Adverse effects
Giros, B. *et al.* 1996	DAT KO		Eliminated					
Sora, I. *et al.* 1998	DAT KO		Eliminated		CPP at highest dose only			
Rocha, B.A. *et al.* 1998	DAT KO					Unaffected		
Gainetdinov, R.R. *et al.* 1999	DAT KO		Cocaine decreased locomo-tion					
Carboni, E. *et al.* 2001	DAT KO	Increased DA in NAc						
Ralph, R.J. *et al.* 2001	DAT KO						Reversed PPI deficit	
Mead, A.N. *et al.* 2002	DAT KO			Eliminated				
Morice, E. *et al.* 2004	DAT KO		Eliminated		Substantially decreased			
Shen, H.W. *et al.* 2004	DAT KO	Increased DA in striatum and PFc, but not NAc						
Mateo, Y. *et al.* 2004	DAT KO	Increased DA in NAc and striatum						
Barr, A.M. *et al.* 2004	DAT KO						Reversed PPI deficit	
Medvedev, I.O. *et al.* 2005	DAT KO		Eliminated		Substantially decreased			
Yamashita, M. *et al.* 2006	DAT KO						Reversed PPI deficit	
Thomsen, M. *et al.* 2009	DAT KO					Substantially decreased		
Hall, F.S. *et al.* 2009	DAT KO		Conditioned locomo-tion was eliminated					
Zhuang, X. *et al.* 2001; Tilley, M.R. *et al.* 2007	DAT KD		Increased locomotor by low doses of cocaine		Unaffected			

Continued.