effects, the present experiments examined another paradigm that examines cocaine-conditioned responses.

Although the primary aim of the present study was to examine cocaine-conditioned locomotion, analysis of behavior during the five conditioning sessions also allowed the examination of context-dependent sensitization of the acute locomotor stimulant effects of cocaine to some extent. Context-dependent sensitization could be observed during the conditioning trials but context-independent sensitization, in the unpaired subjects, obviously could not. DAT KO eliminated cocaine-conditioned locomotion, as might be expected since there was no initial locomotor stimulant response to cocaine in DAT −/− mice, but in addition locomotor activity increased slightly across conditioning trials in cocaine-treated DAT −/− mice, regardless of whether or not cocaine was paired with the testing environment. In addition, it was apparent that the activity of Control DAT −/− mice did not decrease across trials, indicating impaired between-session habituation. A previous study found that sensitization of cocaine-induced locomotion was eliminated in both DAT +/− and DAT −/− mice (Mead et al., 2002). However, the methods used in that experiment to examine cocaine sensitization are difficult to compare to the present findings or to the literature; in that study cocaine was administered i.v. after an extended period of habituation that almost normalized activity between the DAT −/− and DAT +/+ mice. Extended habituation would substantially affect the ability of the environment to act as a conditioned stimulus. In addition, the temporal differences between s.c. and i.v. drug administration would also affect the ability of different types of stimuli to act as reinforcers. Finally, the experimental conditions appeared to affect the acute locomotor stimulant effects of cocaine as well; in that study acute locomotor stimulant effects of cocaine were eliminated in DAT +/− mice, which was not observed in previous studies (Giros et al., 1996; Sora et al., 1998, 2001). The length of drug treatment may be another factor influencing sensitization in DAT KO mice as a recent study found that methamphetamine sensitization was not attenuated in DAT +/− mice, but its development was delayed (Fukushima et al., 2007).

The mechanism underlying those remaining cocaine effects in DAT KO mice has been a matter of some speculation. Despite the fact that acute locomotor stimulatory effects are eliminated in DAT KO mice, cocaine still retains the ability to increase extracellular levels of dopamine, at least in some brain areas (Mateo et al., 2004b; Shen et al., 2004). There is some evidence that the locus of this effect may be different in DAT KO mice than that in wild-type mice. Local infusions of cocaine in either the dorsal or ventral striatum fail to increase extracellular dopamine levels (Mateo et al., 2004b; Shen et al., 2004) nor does cocaine affect DA clearance in striatal slices (Mateo et al., 2004a). Although there has been some suggestion that reuptake by NET or SERT, in the absence of DAT, might account for the effects of cocaine, neither desipramine nor fluoxetine affects DA clearance in striatal slices (Mateo et al., 2004a). However, peripheral injections of SERT blockers do increase extracellular DA in the striatum (Mateo et al., 2004b; Shen et al., 2004), effects that are not observed in WT mice. The locus of the cocaine effect might involve SERT in the VTA where local injections of cocaine or fluoxetine lead to increased release of dopamine in the nucleus accumbens (Mateo et al., 2004b). This is consistent with the ability of combined DAT-SERT KOs to eliminate cocaine CPP (Sora et al., 2001), and for fluoxetine to produce CPP in DAT KO mice (Hall et al., 2002).

Since cocaine retains its ability to elevate extracellular dopamine in DAT KO mice, albeit via different mechanisms than in WT mice, and only in some brain regions, it might be suspected that cocaine sensitization may still be possible in DAT KO mice. Indeed the present study suggests that context-independent sensitization may be enhanced, while at the same time conditioned locomotion is eliminated. Because of the profound changes in dopamine clearance in DAT KO mice (Giros et al., 1996; Jones et al., 1998) there may be substantial alterations in spatiotemporal aspects of dopamine transmission between wiring (local synaptic) and volume transmission (Gonon et al., 2000), which may alter the influence of dopamine on glutamate function. In addition to elevating extracellular levels of dopamine, cocaine also increases glutamate levels (Smith et al., 1995), an effect that is increased in animals that have developed context dependent cocaine sensitization (Pierce et al., 1996; Reid and Berger, 1996; Kalivas and Duffy, 1998). These changes are associated with increased sensitivity of dopaminergic neurons to glutamatergic stimulation (White et al., 1995; Zhang et al., 1997), and are associated with changes in glutamate receptor subunit expression in the NAC and VTA (Churchill et al., 1999). Sensitization of the glutamate response to cocaine has been found to result from context dependent, but not independent, sensitization (Bell et al., 2000) and the development, but not expression, of context-dependent sensitization can be blocked by AMPA antagonists (Li et al., 1997), and NMDA antagonists (Damianopoulos and Carey, 1995; Cervo and Samanin, 1996; Kim et al., 1996). Conditioned activity is associated with increases in nucleus accumbens glutamate and can be attenuated by AMPA antagonists (Cervo and Samanin, 1996; Hotsenpiller et al., 2001) and NMDA antagonists (Cervo and Samanin, 1996). Both NMDA and AMPA antagonists block the development of context independent sensitization as well (Li et al., 1999). Expression of a mutant NMDA receptor with impaired Ca^{2+} flux in cells containing dopamine D1 receptors (DRD1) prevents the development of context-dependent cocaine sensitization and cocaine CPP (Heusner and Palmiter, 2005). Convergent DRD1-NMDA stimulation has been suggested to play a critical role in the development of context-dependent sensitization (Valjent et al., 2005). The observed role of glutamate and glutamate–dopamine interactions in these phenomena are dependent in part upon experimental parameters and are not entirely clear by any means. However, because of the profound alterations in the dynamics of dopamine release in DAT KO mice it would appear likely that glutamatergic mechanisms would also be affected, although perhaps in such a way as to differentially affect context independent sensiti-

zation and conditioned locomotion. This possibility has not been investigated to any great degree, although glutamate manipulations do affect baseline hyperactivity in DAT KO mice (Gainetdinov et al., 2001).

Other evidence indicates that cocaine enhances glutamatergic inputs to midbrain dopamine neurons in a manner dependent on both DRD1 and glutamate AMPA receptors (Dong et al., 2004). Part of the evidence for this interaction involved the elimination of these effects in GLURA KO mice. Elimination of this gene also blocked both conditioned locomotion and CPP, without affecting acute locomotor responses to cocaine (Dong et al., 2004). This study implicates potential neuroadaptations in glutamatergic afferents to midbrain dopamine neurons in the effects of context on conditioned responses that enhance drug-seeking behavior. Changes in synaptic spine density are observed in the nucleus accumbens core in response to a cocaine treatment regimen that induced context-dependent sensitization (Li et al., 2004). Interestingly, the same dose regimen produced neither behavioral nor morphological changes when administered in the home cage, but higher doses that induced context-independent sensitization were able to increase spine density in the nucleus accumbens core. Increased spine densities were also observed in the nucleus accumbens shell, but were observed even after repeated context-independent treatment with low doses of cocaine that did not produce sensitization. Increased spine densities were also observed in the medial prefrontal cortex under both conditions, but the increases were greater after context-dependent sensitization. These data would suggest that experimental parameters have a substantial effect on the morphological consequences of cocaine treatment, which are highly dependent on experimental parameters and differ substantially across brain regions. These changes are likely to underlie changes in glutamate responsiveness to cocaine and various forms of cocaine conditioning and cocaine sensitization.

Such differential effects are necessary to explain the difference in context independent responses in DAT KO mice and conditioned locomotion. The anatomical locus most critical to conditioned locomotion appears to be different from that involved in the acute locomotor stimulant properties of cocaine. Quinolinic acid–induced lesions of the amygdala have no effect on the acute locomotor effects of cocaine, but block the development of conditioned locomotion (Brown and Fibiger, 1993). This brain region has not been investigated in DAT KO mice. Pairing of novel contextual cues with cocaine produces greater sensitization than pairing with discrete stimuli (Crombag et al., 2000), although conditioning to discrete stimuli is also observed in terms of both context dependent sensitization and conditioned locomotion (Panlilio and Schindler, 1997). Interestingly, in that study the discrete stimuli that were used to produce conditioned locomotion also acted as conditioned reinforcers in a subsequent operant circumstance in which lever processing produced presentation of the conditioned stimuli. It has been recently shown that there is a substantial overlap between striatal neurons activated by acute cocaine (e.g. c-fos) and those that are activated by chronic cocaine (e.g. FOSB), but that the number of activated neurons is a small percentage of the overall number of striatal neurons and that each environment may induce a distinct subset, or ensemble, of striatal neurons (Mattson et al., 2008). These subjects were not tested for conditioned locomotion (e.g. the effect of re-exposure to the conditioned environment without any injections), but nonetheless subjects that were returned to the cocaine-paired environment and injected with saline showed substantial elevations in c-fos and a substantial overlap with FOSB; this activation probably represents the effect of the environmental context on the neuronal ensemble that drives locomotor behavior in this circumstance, and is likely related to the changes in synaptic morphology associated with chronic cocaine treatments discussed above.

Although there has been accumulating evidence that serotonin and norepinephrine may modulate cocaine reward, and that SERT and NET may have a role in cocaine reward under some circumstances, the present experiments suggest that these effects are limited for conditioned locomotion. Both SERT KO and NET KO mice demonstrated conditioned locomotion. Although the effects appeared to be reduced, this decrease was not significant in NET KO mice and marginally significant in SERT KO mice. These effects may be the result of other factors, such as the reduced locomotion observed here in SERT KO and NET KO mice. Reduced locomotion has been described in SERT KO mice previously (Kalueff et al., 2007a,b). Generally speaking in the paradigm utilized in these studies context-dependent sensitization was not observed (e.g. increased locomotion in paired subjects). Because of the habituation of activity across trials in saline-treated subjects, the relative magnitude of cocaine effects in Paired mice was greater in trial 5 than in trial 1, which might be taken to indicate sensitization. With this in mind, the activity of paired SERT KO mice decreased across trials, similarly to saline-treated subjects, which may indicate an impairment of context dependent sensitization or even tolerance to the locomotor stimulant effects of cocaine. However, as this study was not designed to primarily examine context dependent sensitization this conclusion must be tentatively placed forward until this phenomenon can be examined in a more appropriate paradigm. There is some evidence that stimulation of dorsal raphé 5-HT1A receptors potentiates cocaine-induced locomotion, cocaine-induced dopamine release and cocaine-induced glutamate release (Szumlinski et al., 2004). There are substantial reductions in these receptors observed in SERT KO mice (Fabre et al., 2000) as well as other neuroadaptations (Mathews et al., 2004).

CONCLUSION

In conclusion, it would seem that the primary mechanism by which cocaine produces conditioned locomotion is via actions at DAT. Although there is evidence that both SERT and NET gene KOs modulate cocaine-mediated behavior

during conditioning, these differences do not profoundly affect the ability of cocaine to produce conditioned locomotion. Furthermore, in DAT KO mice context-independent sensitization of cocaine-induced locomotion is observed; that is, the sensitization occurs in DAT −/− mice treated with repeated cocaine in the same or a different environment. This occurs under conditions that do not produce sensitization in other animals, and likely reflects substantial alterations in dopamine–glutamate interactions that occur in response to cocaine administration and that change in response to repeated cocaine administration. Further investigation of the function of glutamate in DAT KO mice may help illuminate the behavioral differences observed in these mice as well as those dopamine–glutamate interactions that are critical in these phenomena.

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REFERENCES

Bell K, Duffy P, Kalivas PW (2000) Context-specific enhancement of glutamate transmission by cocaine. Neuropsychopharmacology 23:335–344.

Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, Mossner R, Westphal H, Lesch KP (1998) Altered brain serotonin homeostasis and locomotor insensitivity to 3,4-methylenedioxymethamphetamine ("ecstasy") in serotonin transporter-deficient mice. Mol Pharmacol 53:649–655.

Bisaga A, Sikora J, Kostowski W (1993) The effect of drugs interacting with serotonergic 5HT3 and 5HT4 receptors on morphine place conditioning. Polish J Pharmacol 45:513–519.

Brown EE, Fibiger HC (1993) Differential effects of excitotoxic lesions of the amygdala on cocaine-induced conditioned locomotion and conditioned place preference. Psychopharmacology (Berl) 113: 123–130.

Carboni E, Acquas E, Leone P, Di Chiara G (1989) 5HT3 receptor antagonists block morphine- and nicotine-but not amphetamine-induced reward. Psychopharmacology 97:175–178.

Carey RJ, Damianopoulos EN (2006) Cocaine conditioning and sensitization: the habituation factor. Pharmacol Biochem Behav 84: 128–133.

Carey RJ, Gui J (1998) Cocaine conditioning and cocaine sensitization: what is the relationship? Behav Brain Res 92:67–76.

Cervo L, Samanin R (1996) Effects of dopaminergic and glutamatergic receptor antagonists on the establishment and expression of conditioned locomotion to cocaine in rats. Brain Res 731:31–38.

Churchill L, Swanson CJ, Urbina M, Kalivas PW (1999) Repeated cocaine alters glutamate receptor subunit levels in the nucleus accumbens and ventral tegmental area of rats that develop behavioral sensitization. J Neurochem 72:2397–2403.

Crombag HS, Badiani A, Maren S, Robinson TE (2000) The role of contextual versus discrete drug-associated cues in promoting the induction of psychomotor sensitization to intravenous amphetamine. Behav Brain Res 116:1–22.

Damianopoulos EN, Carey RJ (1995) Evidence for N-methyl-D-aspartate receptor mediation of cocaine induced corticosterone release and cocaine conditioned stimulant effects. Behav Brain Res 68:219–228.

Dong Y, Saal D, Thomas M, Faust R, Bonci A, Robinson T, Malenka RC (2004) Cocaine-induced potentiation of synaptic strength in dopamine neurons: behavioral correlates in GluRA(−/−) mice. Proc Natl Acad Sci U S A 101:14282–14287.

Elmer GI, Gorelick DA, Goldberg SR, Rothman RB (1996) Acute sensitivity vs. context-specific sensitization to cocaine as a function of genotype. Pharmacol Biochem Behav 53:623–628.

Everitt BJ, Dickinson A, Robbins TW (2001) The neuropsychological basis of addictive behaviour. Brain Res Rev 36:129–138.

Fabre V, Beaufour C, Evrard A, Rioux A, Hanoun N, Lesch KP, Murphy DL, Lanfumey L, Hamon M, Martres MP (2000) Altered expression and functions of serotonin 5-HT1A and 5-HT1B receptors in knockout mice lacking the 5-HT transporter. Eur J Neurosci 12:2299–2310.

Fadda F, Garau B, Marchei F, Colombo G, Gessa GL (1991) MDL 72222, a selective 5-HT3 receptor antagonist, suppresses voluntary ethanol consumption in alcohol-preferring rats. Alcohol Alcohol 26:107–110.

Fletcher PJ, Chintoh AF, Sinyard J, Higgins GA (2004) Injection of the 5-HT2C receptor agonist Ro60–0175 into the ventral tegmental area reduces cocaine-induced locomotor activity and cocaine self-administration. Neuropsychopharmacology 29:308–318.

Fletcher PJ, Korth KM (1999) Activation of 5-HT1B receptors in the nucleus accumbens reduces amphetamine-induced enhancement of responding for conditioned reward. Psychopharmacology (Berl) 142:165–174.

Fukushima S, Shen H, Hata H, Ohara A, Ohmi K, Ikeda K, Numachi Y, Kobayashi H, Hall FS, Uhl GR, Sora I (2007) Methamphetamine-induced locomotor activity and sensitization in dopamine transporter and vesicular monoamine transporter 2 double mutant mice. Psychopharmacology (Berl) 193:55–62.

Gainetdinov RR, Mohn AR, Bohn LM, Caron MG (2001) Glutamatergic modulation of hyperactivity in mice lacking the dopamine transporter. Proc Natl Acad Sci U S A 98:11047–11054.

Giros B, Jaber M, Jones SR, Wightman RM, Caron MG (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 379:606–612.

Gold LH, Swerdlow NR, Koob GF (1988) The role of mesolimbic dopamine in conditioned locomotion produced by amphetamine. Behav Neurosci 102:544–552.

Gonon F, Burie JB, Jaber M, Benoit-Marand M, Dumartin B, Bloch B (2000) Geometry and kinetics of dopaminergic transmission in the rat striatum and in mice lacking the dopamine transporter. Prog Brain Res 125:291–302.

Hall FS, Li XF, Sora I, Xu F, Caron M, Lesch KP, Murphy DL, Uhl GR (2002) Cocaine mechanisms: enhanced cocaine, fluoxetine and nisoxetine place preferences following monoamine transporter deletions. Neuroscience 115:153–161.

Harrison AA, Parsons LH, Koob GF, Markou A (1999) RU 24969, a 5-HT1A/1B agonist, elevates brain stimulation reward thresholds: an effect reversed by GR 127935, a 5-HT1B/1D antagonist. Psychopharmacology (Berl) 141:242–250.

Heusner CL, Palmiter RD (2005) Expression of mutant NMDA receptors in dopamine D1 receptor-containing cells prevents cocaine sensitization and decreases cocaine preference. J Neurosci 25: 6651–6657.

Higgins GA, Joharchi N, Nguyen P, Sellers EM (1992a) Effect of the 5-HT3 receptor antagonists, MDL72222 and ondansetron on morphine place conditioning. Psychopharmacology 106:315–320.

Higgins GA, Tomkins DM, Fletcher PJ, Sellers EM (1992b) Effect of drugs influencing 5-HT function on ethanol drinking and feeding behaviour in rats: studies using a drinkometer system. Neurosci Biobehav Rev 16:535–552.

Hotsenpiller G, Giorgetti M, Wolf ME (2001) Alterations in behaviour and glutamate transmission following presentation of stimuli previously associated with cocaine exposure. Eur J Neurosci 14: 1843–1855.

Hotsenpiller G, Wolf ME (2002) Conditioned locomotion is not correlated with behavioral sensitization to cocaine: an intra-laboratory multi-sample analysis. Neuropsychopharmacology 27:924–929.

Jones SR, Gainetdinov RR, Jaber M, Giros B, Wightman RM, Caron MG (1998) Profound neuronal plasticity in response to inactivation of the dopamine transporter. Proc Natl Acad Sci U S A 95: 4029–4034.

Kalivas PW, Duffy P (1998) Repeated cocaine administration alters extracellular glutamate in the ventral tegmental area. J Neurochem 70:1497–1502.

Kalueff AV, Fox MA, Gallagher PS, Murphy DL (2007a) Hypolocomotion, anxiety and serotonin syndrome-like behavior contribute to the complex phenotype of serotonin transporter knockout mice. Genes Brain Behav 6:389–400.

Kalueff AV, Jensen CL, Murphy DL (2007b) Locomotory patterns, spatiotemporal organization of exploration and spatial memory in serotonin transporter knockout mice. Brain Res 1169:87–97.

Kim HS, Park WK, Jang CG, Oh S (1996) Inhibition by MK-801 of cocaine-induced sensitization, conditioned place preference, and dopamine-receptor supersensitivity in mice. Brain Res Bull 40: 201–207.

Kosten TA, Miserendino MJ (1998) Dissociation of novelty- and cocaine-conditioned locomotor activity from cocaine place conditioning. Pharmacol Biochem Behav 60:785–791.

Kostowski W, Dyr W, Krzascik P (1993) The abilities of 5-HT3 receptor antagonist ICS 205-930 to inhibit alcohol preference and withdrawal seizures in rats. Alcohol 10:369–373.

Li Y, Acerbo MJ, Robinson TE (2004) The induction of behavioural sensitization is associated with cocaine-induced structural plasticity in the core (but not shell) of the nucleus accumbens. Eur J Neurosci 20:1647–1654.

Li Y, Hu XT, Berney TG, Vartanian AJ, Stine CD, Wolf ME, White FJ (1999) Both glutamate receptor antagonists and prefrontal cortex lesions prevent induction of cocaine sensitization and associated neuroadaptations. Synapse 34:169–180.

Li Y, Vartanian AJ, White FJ, Xue CJ, Wolf ME (1997) Effects of the AMPA receptor antagonist NBQX on the development and expression of behavioral sensitization to cocaine and amphetamine. Psychopharmacology (Berl) 134:266–276.

Lu MR, Wagner GC, Fisher H (1994) Ethanol consumption following acute treatment with methysergide, fluoxetine, fenfluramine, and their combination. Alcohol Clin Exp Res 18:60–63.

Mateo Y, Budygin EA, John CE, Banks ML, Jones SR (2004a) Voltammetric assessment of dopamine clearance in the absence of the dopamine transporter: no contribution of other transporters in core or shell of nucleus accumbens. J Neurosci Methods 140:183–187.

Mateo Y, Budygin EA, John CE, Jones SR (2004b) Role of serotonin in cocaine effects in mice with reduced dopamine transporter function. Proc Natl Acad Sci U S A 101:372–377.

Mathews TA, Fedele DE, Coppelli FM, Avila AM, Murphy DL, Andrews AM (2004) Gene dose-dependent alterations in extraneuronal serotonin but not dopamine in mice with reduced serotonin transporter expression. J Neurosci Methods 140:169–181.

Mattson BJ, Koya E, Simmons DE, Mitchell TB, Berkow A, Crombag HS, Hope BT (2008) Context-specific sensitization of cocaine-induced locomotor activity and associated neuronal ensembles in rat nucleus accumbens. Eur J Neurosci 27:202–212.

Maurel S, De Vry J, De Beun R, Schreiber R (1999) 5-HT2A and 5-HT2C/5-HT1B receptors are differentially involved in alcohol preference and consummatory behavior in cAA rats. Pharmacol Biochem Behav 62:89–96.

McMillen BA, Walter S, Williams HL, Myers RD (1994) Comparison of the action of the 5-HT2 antagonists amperozide and trazodone on preference for alcohol in rats. Alcohol 11:203–206.

Mead AN, Rocha BA, Donovan DM, Katz JL (2002) Intravenous cocaine induced-activity and behavioural sensitization in norepinephrine-, but not dopamine-transporter knockout mice. Eur J Neurosci 16:514–520.

Panlilio LV, Schindler CW (1997) Conditioned locomotor-activating and reinforcing effects of discrete stimuli paired with intraperitoneal cocaine. Behav Pharmacol 8:691–698.

Parsons LH, Weiss F, Koob GF (1998) Serotonin1B receptor stimulation enhances cocaine reinforcement. J Neurosci 18:10078–10089.

Pert A, Post R, Weiss SRB (1990) Conditioning as a critical determinant of sensitization induced by psychomotor stimulants. Neurobiology of drug abuse. Learn Mem 97:208–241.

Pierce RC, Bell K, Duffy P, Kalivas PW (1996) Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization. J Neurosci 16:1550–1560.

Post RM, Lockfeld A, Squillace KM, Contel NR (1981) Drug-environment interaction: context dependency of cocaine-induced behavioral sensitization. Life Sci 28:755–760.

Post RM, Weiss SR, Pert A (1987) The role of context and conditioning in behavioral sensitization to cocaine. Psychopharmacol Bull 23: 425–429.

Reid MS, Berger SP (1996) Evidence for sensitization of cocaine-induced nucleus accumbens glutamate release. Neuroreport 7: 1325–1329.

Rocha BA, Fumagalli F, Gainetdinov RR, Jones SR, Ator R, Giros B, Miller GW, Caron MG (1998) Cocaine self-administration in dopamine-transporter knockout mice. Nat Neurosci 1:132–137.

Rompre PP, Injoyan R, Hagan JJ (1995) Effects of granisetron, a 5-HT3 receptor antagonist, on morphine-induced potentiation of brain stimulation reward. Eur J Pharmacol 287:263–269.

Shen HW, Hagino Y, Kobayashi H, Shinohara-Tanaka K, Ikeda K, Yamamoto H, Yamamoto T, Lesch KP, Murphy DL, Hall FS, Uhl GR, Sora I (2004) Regional differences in extracellular dopamine and serotonin assessed by in vivo microdialysis in mice lacking dopamine and/or serotonin transporters. Neuropsychopharmacology 29:1790–1799.

Smith JA, Mo Q, Guo H, Kunko PM, Robinson SE (1995) Cocaine increases extraneuronal levels of aspartate and glutamate in the nucleus accumbens. Brain Res 683:264–269.

Sora I, Hall FS, Andrews AM, Itokawa M, Li XF, Wei HB, Wichems C, Lesch KP, Murphy DL, Uhl GR (2001) Molecular mechanisms of cocaine reward: combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. Proc Natl Acad Sci U S A 98:5300–5305.

Sora I, Wichems C, Takahashi N, Li XF, Zeng Z, Revay R, Lesch KP, Murphy DL, Uhl GR (1998) Cocaine reward models: conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. Proc Natl Acad Sci U S A 95: 7699–7704.

Stewart J (1983) Conditioned and unconditioned drug effects in relapse to opiate and stimulant drug self-administration. Prog Neuropsychopharmacol Biol Psychiatry 7:591–597.

Szumlinski KK, Frys KA, Kalivas PW (2004) Dissociable roles for the dorsal and median raphe in the facilitatory effect of 5-HT1A receptor stimulation upon cocaine-induced locomotion and sensitization. Neuropsychopharmacology 29:1675–1687.

Thomsen M, Hall FS, Uhl GR, Caine SB (2009) Dramatically decreased cocaine self-administration in dopamine but not serotonin transporter knock-out mice. J Neurosci 29:1087–1092.

Tirelli E, Michel A, Brabant C (2005) Cocaine-conditioned activity persists for a longer time than cocaine-sensitized activity in mice: implications for the theories using pavlovian excitatory conditioning to explain the context-specificity of sensitization. Behav Brain Res 165:18–25.

Tirelli E, Tambour S, Michel A (2003) Sensitised locomotion does not predict conditioned locomotion in cocaine-treated mice: further evidence against the excitatory conditioning model of context-dependent sensitisation. Eur Neuropsychopharmacol 13:289–296.

Tomkins DM, Higgins GA, Sellers EM (1994a) Low doses of the 5-HT1A agonist 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH DPAT) increase ethanol intake. Psychopharmacology (Berl) 115: 173–179.

Tomkins DM, Sellers EM, Fletcher PJ (1994b) Median and dorsal raphe injections of the 5-HT1A agonist, 8-OH-DPAT, and the GABAA agonist, muscimol, increase voluntary ethanol intake in Wistar rats. Neuropharmacology 33:349–358.

Tomkins DM, Le AD, Sellers EM (1995) Effect of the 5-HT3 antagonist ondansetron on voluntary ethanol intake in rats and mice maintained on a limited access procedure. Psychopharmacology (Berl) 117:479–485.

Tomkins DM, O'Neill MF (2000) Effect of 5-HT(1B) receptor ligands on self-administration of ethanol in an operant procedure in rats. Pharmacol Biochem Behav 66:129–136.

Valjent E, Pascoli V, Svenningsson P, Paul S, Enslen H, Corvol JC, Stipanovich A, Caboche J, Lombroso PJ, Nairn AC, Greengard P, Herve D, Girault JA (2005) Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. Proc Natl Acad Sci U S A 102: 491–496.

Wang YM, Xu F, Gainetdinov RR, Caron MG (1999) Genetic approaches to studying norepinephrine function: knockout of the mouse norepinephrine transporter gene. Biol Psychiatry 46:1124–1130.

White FJ, Hu XT, Zhang XF, Wolf ME (1995) Repeated administration of cocaine or amphetamine alters neuronal responses to glutamate in the mesoaccumbens dopamine system. J Pharmacol Exp Ther 273:445–454.

Wilson AW, Neill JC, Costall B (1998) An investigation into the effects of 5-HT agonists and receptor antagonists on ethanol self-administration in the rat. Alcohol 16:249–270.

Wise RA, Bozarth MA (1987) A psychomotor stimulant theory of addiction. Psychol Rev 94:469–492.

Wise RA, Leeb K (1993) Psychomotor-stimulant sensitization: a unitary phenomenon? Behav Pharmacol 4:339–349.

Xu F, Gainetdinov RR, Wetsel WC, Jones SR, Bohn LM, Miller GW, Wang YM, Caron MG (2000) Mice lacking the norepinephrine transporter are supersensitive to psychostimulants. Nat Neurosci 3:465–471.

Zhang XF, Hu XT, White FJ, Wolf ME (1997) Increased responsiveness of ventral tegmental area dopamine neurons to glutamate after repeated administration of cocaine or amphetamine is transient and selectively involves AMPA receptors. J Pharmacol Exp Ther 281:699–706.

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Reduced emotional and corticosterone responses to stress in μ-opioid receptor knockout mice

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ABSTRACT

The detailed mechanisms of emotional modulation in the nervous system by opioids remain to be elucidated, although the opioid system is well known to play important roles in the mechanisms of analgesia and drug dependence. In the present study, we conducted behavioral tests of anxiety and depression and measured corticosterone concentrations in both male and female μ-opioid receptor knockout (MOP-KO) mice to reveal the involvement of μ-opioid receptors in stress-induced emotional responses. MOP-KO mice entered more and spent more time in the open arms of the elevated plus maze compared with wild-type mice. MOP-KO mice also displayed significantly decreased immobility in a 15 min tail-suspension test compared with wild-type mice. Similarly, MOP-KO mice exhibited significantly decreased immobility on days 2, 3, and 4 in a 6 min forced swim test conducted for 5 consecutive days. The increase in plasma corticosterone concentration induced by tail-suspension, repeated forced swim, or restraint stress was reduced in MOP-KO mice compared with wild-type mice. Corticosterone levels were not different between wild-type and MOP-KO mice before stress exposure. In contrast, although female mice tended to exhibit fewer anxiety-like responses in the tail-suspension test in both genotypes, no significant gender differences were observed in stress-induced emotional responses. These results suggest that MOPs play an important facilitatory role in emotional responses to stress, including anxiety- and depression-like behavior and corticosterone levels.

1. Introduction

Stress is hypothesized to be one of the triggering factors that causes mental illness, including anxiety and depression. Several brain areas are hypothesized to be involved in stress-induced emotional responses via corticosterone release by the hypothalamic-pituitary-adrenal (HPA) axis. Although several neurotransmitter systems, such as serotonin and catecholamines, have been hypothesized to be involved in these mechanisms, the precise molecular mechanisms are still unclear. Endogenous opioid peptides, such as endorphins, have been shown to modulate serotonergic and catecholaminergic neurotransmission (Chen et al., 2001; Hung et al., 2003; Ukai and Lin, 2002). Furthermore, pretreatment with naloxone, a nonselective opioid receptor antagonist, decreased immobility time in mice in a forced swim test (Amir, 1982). Chronic morphine facilitated immobility in a forced swim test (Molina et al., 1994). Opioids have also been reported to increase stress-related hormone levels (Mellon and Bayer, 1998). These previous reports indicate that the endogenous opioid system impacts behavioral responses to stress.

Opioid receptors have been classified into at least three subtypes, μ, δ, and κ (MOP, DOP, and KOP, respectively). Endomorphin-1 and -2, endogenous peptides that are selective for MOP, reportedly decreased immobility time in both the forced swim and tail-suspension tests (Fichna et al., 2007). A DOP selective agonist, SNC80, also decreased immobility time in a forced swim test (Broom et al., 2002). Furthermore, the KOP selective agonist U69593 increased, and the KOP selective antagonist nor-binaltorphimine decreased, immobility time in a forced swim test (Mague et al., 2003). Although three opioid receptor subtypes may be involved in stress-induced emotional responses, even the most selective ligands for a specific subtype (i.e., β-funaltrexamine for

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MOP, naltrindole for DOP, and nor-binaltorphimine for KOP) possess certain affinities for other subtypes (Newman et al., 2002) which may contribute to the discrepant findings about the role of opioid receptor subtypes in stress responses. Therefore, the precise molecular mechanisms underlying stress-induced emotional responses have not yet been clearly delineated by traditional pharmacological studies that use only selective ligands.

Recent success in developing knockout (KO) mice with MOP gene deletion has revealed the central role of MOPs, rather than other opioid receptor subtypes, in various opioid effects, including analgesia, reward, and tolerance (Ide et al., 2004; Kieffer, 1999; Loh et al., 1998; Sora et al., 2001, 1997). Although several compensatory changes might occur in KO animals, these animals have potential utility in investigating the *in vivo* roles of specific proteins. Opioid receptors have been shown to modulate responses to stress, including depression-like behavior (Filliol et al., 2000; McLaughlin et al., 2003). Thus, the use of MOP-KO mice has provided novel theories on the molecular mechanisms underlying stress-induced emotional responses. Both the forced swim test (Porsolt et al., 1977) and tail-suspension test (Steru et al., 1985) have been widely used to assess depression-like behavior, with several modifications. Many reports using these two tests have shown that the inescapable stress of swimming or suspending a mouse by its tail can provide valuable information about emotional responses in stressful situations. The present study investigated the contributory role of the MOP in emotional responses to height, tail-suspension, repeated forced swim, and restraint stress using MOP-KO mice.

2. Materials and methods

2.1. Animals

The present study used wild-type and homozygous MOP-KO mouse littermates on a C57BL/6J genetic background (backcrossed at least 10 generations) as previously described (Sora et al., 2001). The experimental procedures and housing conditions were approved by 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 weeks old) male and female mice were group-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*. All behavioral tests and blood sample collections were conducted between 1:00 pm and 6:00 pm.

2.2. Elevated plus maze

The testing apparatus was a white plastic plus-shaped maze, elevated 80 cm from the floor. The maze consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 50 cm) without a roof. During testing, the time spent in the open arms and the number of entries into the open arms were recorded for 5 min. A mouse was considered to have entered an arm only if all four paws entered that arm.

2.3. Locomotor activity

Locomotor activity was assessed with an animal activity-monitoring apparatus equipped with an infrared detector (SUPERMEX, CompACT FSS, Muromachi Kikai Co., Tokyo, Japan). 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 3 h.

2.4. Tail-suspension test

For tail-suspension testing, mice were suspended by their tail which was taped on a metal hook in test chambers (20 × 20 × 25 cm) constructed of white plastic walls and floor. Each hook was connected to a computerized strain gauge that was adjusted to detect animal movements (Tail-suspension System, Neuroscience Inc., Osaka, Japan). The total duration of immobility was measured for 15 min per day for 2 consecutive days.

2.5. Forced swim test

For forced swim testing, 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, Japan). 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.

2.6. Stress procedures and corticosterone enzyme immunoassay

After the 2 day tail-suspension test or 5 day forced swim test, blood samples (50 µl) were obtained from the tail vein. For restraint stress, mice were placed in a 50 ml conical centrifuge tube with multiple ventilation holes. Mice were restrained vertically in the tube for 12 h, followed by a 12 h rest with food and water available *ad libitum*. Mice were restrained again for 12 h, and then blood samples were obtained. All blood samples were immediately centrifuged for 20 min at 1000 × *g*. Plasma samples were stored at −80 °C until analysis. Plasma corticosterone levels were determined with a Corticosterone Enzyme Immunoassay Kit (Assay Design Inc., Ann Arbor, MI, USA).

2.7. Statistical analysis

Entry counts and time spent on the open arms of the elevated plus maze and stress-induced changes in plasma corticosterone concentrations were analyzed with Student's *t*-test. The results of other analyses were statistically evaluated with analysis of variance (ANOVA) followed by the Tukey–Kramer test. Values of $p < 0.05$ were considered statistically significant.

3. Results

We first assessed basal anxiety-like behavior of both mouse genotypes in the elevated plus maze (Fig. 1). Compared with wild-type mice, MOP-KO mice had significantly higher entry counts ($p < 0.05$, Student's *t*-test) and a longer time spent on the open arms ($p < 0.01$, Student's *t*-test) in both male and female mice. Although female mice tended to have more entry counts and more time spent in the open arms than male mice in both genotypes, no significant differences were observed.

When spontaneous locomotor activity of both wild-type and MOP-KO mice was analyzed (Fig. 2), MOP-KO mice displayed normal locomotor activity, similar to wild-types, during the 3 h test. A three-way, mixed-design ANOVA of spontaneous locomotor activity with two within-subjects factors (genotype and gender) showed no significant interactions (genotype: $F_{1,30} = 1.56$, $p = 0.221$; gender: $F_{1,30} = 0.08$, $p = 0.784$).

To test the influence of MOP-KO in stress-induced responses, immobility time in a 15 min tail-suspension test was analyzed every minute in wild-type and MOP-KO mice (Fig. 3). A three-way, mixed-design ANOVA of immobility time with two within-subjects factors (genotype and gender) revealed that immobility time was significantly different between genotypes in the tail-suspension test ($F_{1,22} = 6.92$, $p < 0.05$), although both genotypes showed time-dependent increases (Fig. 3a). The ANOVA also revealed that immobility time was not significantly different between male and female mice ($F_{1,22} = 3.01$, $p = 0.097$), although female mice tended to show less immobility than males. When the data of male and female mice were combined (Fig. 3b), significant differences were found in immobility time between genotypes ($F_{1,24} = 5.45$, $p < 0.05$, two-way, repeated-measures ANOVA). *Post hoc* tests revealed that MOP-KO mice had significantly less immobility time compared with wild-type mice from 7 to 9, 12 and 13 min after the tail-suspension test commenced. These differences in immobility time between wild-type and MOP-KO mice were not found during the second trial of the tail-suspension test on the next day (data not shown).

To test another type of stress stimulus, immobility time during the 6 min, 5-consecutive-day forced swim test was also analyzed in wild-type and MOP-KO mice (Fig. 4). Both genotypes and both male and female mice showed time-dependent increases in immobility time (Fig. 4a–d). Furthermore, immobility time during the 6 min forced swim test significantly increased, or tended to increase, in

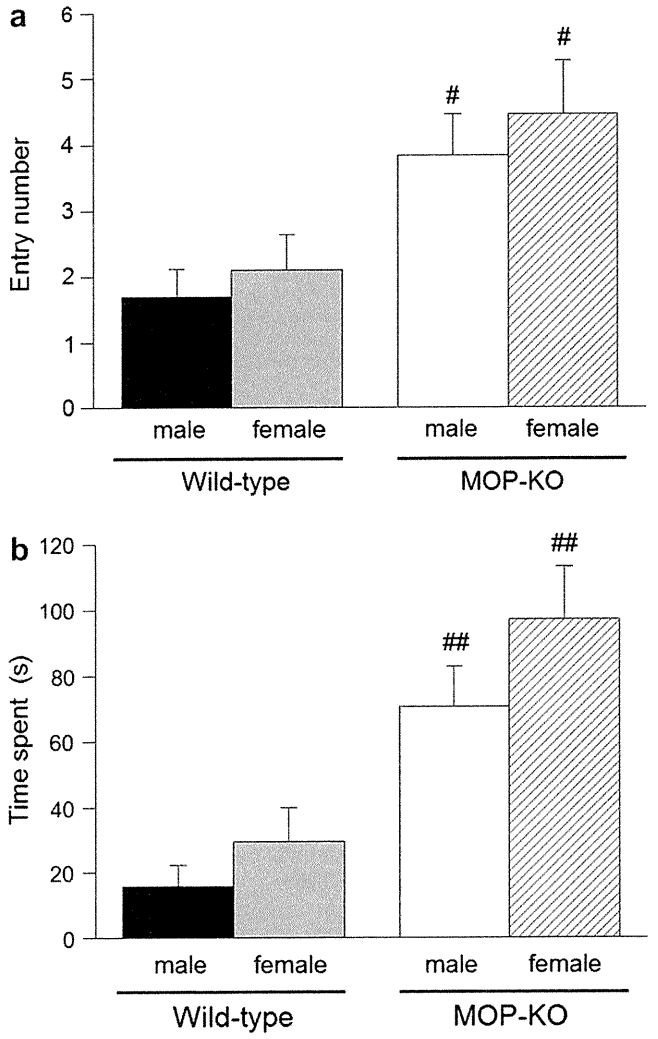

Fig. 1. Anxiety-like behavior in wild-type and MOP-KO mice in the elevated plus maze. The (a) number of entries and (b) time spent in the open arms of the elevated plus maze were measured for 5 min in wild-type mice (male, $n = 10$; female, $n = 9$) and MOP-KO mice (male, $n = 12$; female, $n = 13$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

a day-dependent manner (wild-type male mice: $F_{4,45} = 8.07$, $p < 0.001$; wild-type female mice: $F_{4,40} = 11.9$, $p < 0.001$; MOP-KO male mice: $F_{4,30} = 2.35$, $p = 0.077$; MOP-KO female mice: $F_{4,30} = 7.00$, $p < 0.001$; two-way, repeated-measures ANOVA). *Post hoc* comparisons revealed that immobility time on days 2–5 significantly increased compared with day 1 in both wild-type male and female mice ($p < 0.05$). Immobility time significantly increased on day 5 compared with day 1 in MOP-KO male mice and on days 4 and 5 compared with day 1 in MOP-KO female mice ($p < 0.05$). A three-way, mixed-design ANOVA of total immobility time during the 6 min tests on each of the 5 days with two within-subjects factors (genotype and gender) revealed that immobility time was significantly different between genotypes ($F_{1,29} = 10.9$, $p < 0.005$) but was not significantly different between genders ($F_{1,29} = 1.39$, $p = 0.248$) (Fig. 4e). Thus, when the male and female data were combined (Fig. 4f), MOP-KO mice showed significantly less immobility time compared with wild-type mice on days 2, 3, and 4.

We then analyzed stress-induced changes in plasma corticosterone concentrations in wild-type and MOP-KO mice (Fig. 5). The three types of stress significantly increased plasma corticosterone

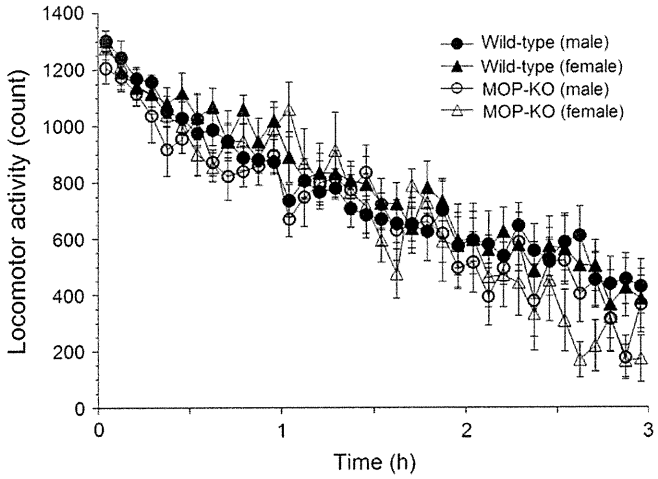

Fig. 2. Spontaneous locomotion in wild-type and MOP-KO mice. Spontaneous locomotion during 3 h habituation to a novel environment in wild-type mice (male, $n = 12$; female, $n = 9$) and MOP-KO mice (male, $n = 6$; female, $n = 7$). Each point represents the sum of 5 min locomotor activity. Data are expressed as mean ± SEM.

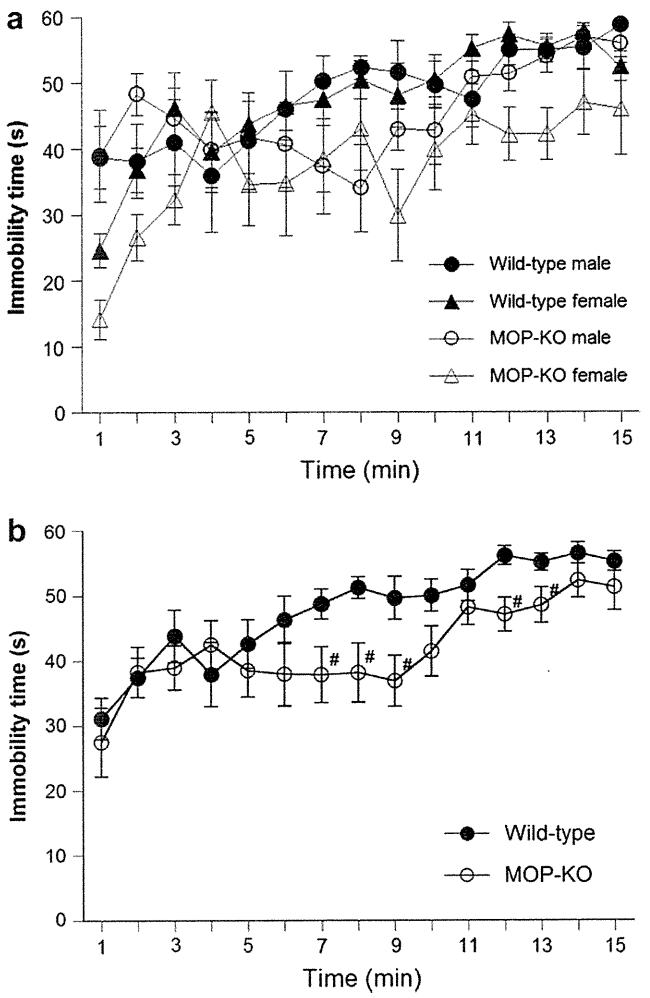

Fig. 3. Immobility in wild-type and MOP-KO mice in the 15 min tail-suspension test. (a) Immobility time was measured in wild-type mice (male, $n = 6$; female, $n = 7$) and MOP-KO mice (male, $n = 7$; female, $n = 6$). (b) Combined data of male and female mice in the 15 min tail-suspension test. $^{\#}p < 0.05$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

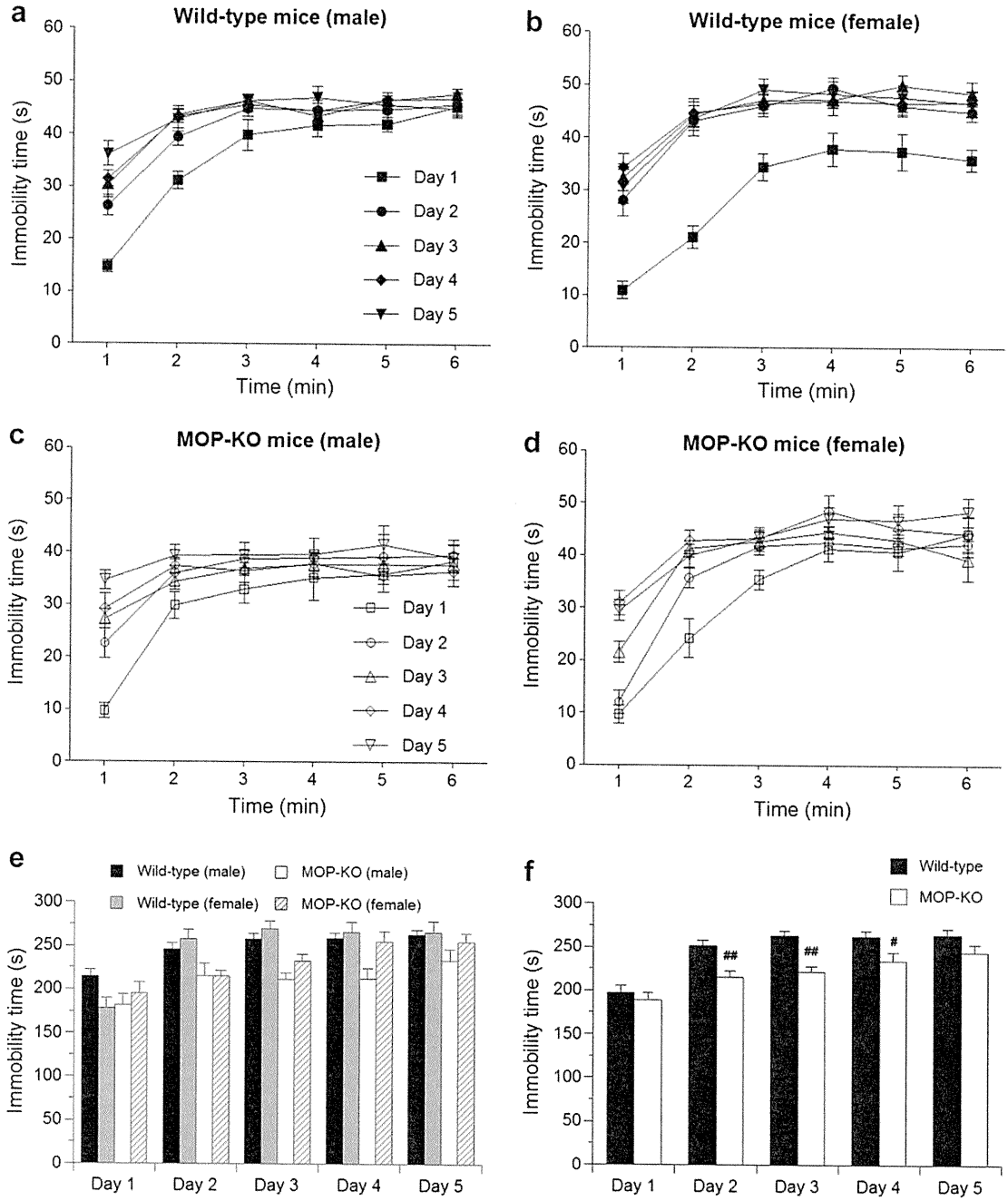

Fig. 4. Immobility in wild-type and MOP-KO mice in the 6 min, 5-consecutive-day forced swim test. Immobility time was measured in (a) wild-type male mice ($n = 10$), (b) wild-type female mice ($n = 9$), (c) MOP-KO male mice ($n = 7$), and (d) MOP-KO female mice ($n = 7$). (e) Sum of 6 min immobility time over 5 days. (f) Combined data of male and female mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

concentrations in both genotypes and in both male and female mice ($p < 0.05$, Student's t-test). Although no significant differences were observed in basal plasma corticosterone concentrations in naive mice, the stress-induced increases in plasma corticosterone concentrations were significantly different ($p < 0.05$, Student's t-test), or tended to be significantly different (restraint stress in female mice: $p = 0.065$, Student's t-test), between genotypes in both male and female mice. Both male and female MOP-KO mice had significantly lower plasma corticosterone concentrations compared with wild-type mice after the stress procedures. Although female mice tended to have slightly higher corticosterone concentrations than male mice (i.e., naive or after tail-suspension or restraint stress), no significant differences were observed (Student's t-test). Contrary to these findings, female mice tended to exhibit lower corticosterone concentrations than male mice after forced swim stress in both genotypes, although no significant differences were observed (Student's t-test).

4. Discussion

In the present study, MOP-KO mice displayed significantly decreased immobility time in both the tail-suspension and repeated forced swim tests and significantly reduced stress-induced increases in plasma corticosterone concentrations compared with wild-type mice. Moreover, MOP-KO mice also entered more, and spent more time in, the open arms of the

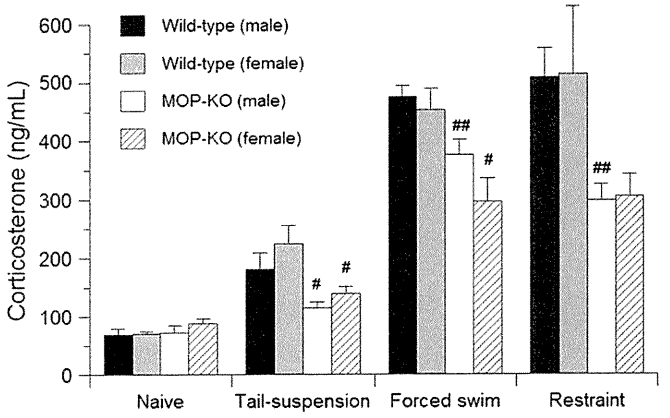

Fig. 5. Stress-induced increase in plasma corticosterone concentrations in wild-type and MOP-KO mice. Plasma corticosterone levels were analyzed (i) in naive wild-type mice (male, $n = 6$; female, $n = 5$) and MOP-KO mice (male, $n = 9$; female, $n = 8$), (ii) after the 2 day tail-suspension test in wild-type mice (male, $n = 6$; female, $n = 5$) and MOP-KO mice (male, $n = 9$; female, $n = 8$), (iii) after the 5 day forced swim test in wild-type mice (male, $n = 10$; female, $n = 8$) and MOP-KO mice (male, $n = 7$; female, $n = 7$), and (iv) after restraint stress in wild-type mice (male, $n = 6$; female, $n = 5$) and MOP-KO mice (male, $n = 9$; female, $n = 8$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

elevated plus maze. These results suggest that MOP-KO mice are resistant to stress exposure and exhibit fewer stress-induced emotional responses (i.e., anxiety- and depression-like behaviors) compared with wild-type mice, although the influences of other factors (e.g., response to novelty) should be considered in future studies.

No significant differences were observed in locomotor activity between wild-type and MOP-KO mice, although MOP-KO mice exhibited a slight tendency toward decreased locomotion. These results indicate that the present behavioral effects in MOP-KO mice were not attributable to variations in locomotor activity. MOP-KO mice entered more, and spent more time in, the open arms of the elevated plus maze in the present study. Similar results have been reported with another MOP-KO mouse strain in both the elevated plus maze test and light–dark box test (Filliol et al., 2000). This anxiolytic-like state of MOP-KO mice is consistent with a previous report in which the MOP-selective agonist DAMGO ([D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin) induced anxiogenic-like activity in the elevated plus maze (Calenco-Choukroun et al., 1991). In contrast, several contradictory studies have reported an anxiolytic-like effect of morphine and MOP agonists (Asakawa et al., 1998; Koks et al., 1999). One of the reasons for this discrepancy using MOP-selective ligands might involve other opioid receptor subtypes. The most selective ligands for a specific opioid receptor subtype possess certain affinities for other subtypes (Newman et al., 2002). Although further studies using our and other MOP-KO mouse strains in various paradigms to assess anxiety-like responses (e.g., open field test) might be needed, the present results suggest that MOPs are involved in anxiety-like responses to height stress.

The decrease in immobility time in MOP-KO mice compared with wild-type mice in both the tail-suspension and repeated forced swim tests is consistent with previous reports. The decrease in immobility time in the forced swim test has been reported using another MOP-KO mouse strain (Filliol et al., 2000). These results suggest that MOP activation facilitates stress-induced, depression-like behavioral responses. Additionally, Fichna et al. (2007) reported contradictory findings in which intracerebroventricular treatment with endomorphin-1 and -2, endogenous MOP-selective peptides, decreased immobility time in both the forced swim and tail-suspension tests. Codeine, a relatively weak MOP agonist, also decreased immobility time in tail-suspension tests in mice (Berrocoso and Mico, in press). Although these reports might suggest that the MOP modulates depression-like behavior in contrast to our present results, other reports are consistent with our results. Chronic morphine facilitated immobility time in a rat forced swim test (Molina et al., 1994). Pretreatment with naloxone, a nonselective opioid receptor antagonist, decreased immobility time in a forced swim test in mice (Amir, 1982). Furthermore, intraperitoneal treatment of morphine enhanced immobility time in rats in a naloxone-sensitive manner (Zurita and Molina, 1999). These discrepant results might be attributable to differences in animals, mouse strains, time course, injection route, or other experimental conditions. Notably, different mouse strains have exhibited differential responses in forced swim tests (David et al., 2003). Further studies may reveal the reasons for these discrepant results.

To study the involvement of the MOP in emotional responses to repeated stress, the present study used both the 6 min forced swim test conducted for 5 consecutive days and the 15 min tail-suspension test conducted for 2 consecutive days, two regimens which were modified from typically used procedures in mice (Porsolt et al., 1977; Steru et al., 1985). When we analyzed immobility time from day 1 at 3–6 min in the forced swim test (excluding the data from the first 2 min), no significant differences were found between wild-type and MOP-KO mice. Additionally, no significant differences in immobility time were observed from day 1 for the first 6 min between wild-type and MOP-KO mice in the tail-suspension test. Although standard procedures for the analysis of depression-like behavior did not reveal significant differences, MOP-KO mice showed significant differences in depression-like behavior after repeated or longer stress exposure in the forced swim and tail-suspension tests. Our present results might suggest that MOPs facilitate emotional responses to repeated or longer stress exposure. In the present procedures, MOP-KO mice exhibited significantly decreased immobility time in the repeated forced swim test only on days 2, 3, and 4, and they only showed a tendency toward decreased immobility on day 5. In the tail-suspension test, MOP-KO mice had significantly decreased immobility time only after the first 5 min from the beginning of the test during the first trial, and no significant differences were observed during the second trial. Interestingly, the increase in plasma corticosterone concentrations in MOP-KO mice was still significantly lower than wild-type mice after the differences in behaviors between wild-type and MOP-KO mice in both tests disappeared. MOPs may facilitate the early behavioral responses to stress but are not necessary to fully express the behavioral responses after chronic stress procedures. Other neuronal systems might regulate the expression of stress-induced behavioral responses, and MOPs might facilitate this regulation.

At the hormonal level, one of the major responses to stress is an increase in corticosterone secretion caused by stimulation of the HPA axis. In the present study, plasma corticosterone concentration significantly increased after stress exposure in both wild-type and MOP-KO mice. The increased corticosterone levels after both forced swim and restraint stress were higher than after the tail-suspension test. This finding might be attributable to differences in the intensity of the stressors, although variations in the duration and frequency of these stressors might modify these levels. Additionally, the stress-induced increases in plasma corticosterone concentration were less in MOP-KO mice compared with wild-type mice. Our present results are consistent with previous reports. Endogenous opioids have been reported to have facilitatory effects on the HPA axis (Douglas et al., 1998). The increase in plasma corticosterone levels by morphine indicated activation of the HPA axis by MOP (Coventry et al., 2001; Ignar and Kuhn, 1990). In a different MOP-KO mouse strain, morphine- and restraint

stress-induced increases in plasma corticosterone levels were also reduced (Roy et al., 2001; Wang et al., 2002). Stress is well known to activate the HPA axis and increase norepinephrine release in the locus coeruleus. Moreover, stress-induced norepinephrine release in the locus coeruleus is partially regulated by both opioid and noradrenergic mechanisms (Nakai et al., 2002; Nestler et al., 1999; Valentino and Van Bockstaele, 2001), suggesting that MOPs may be involved in the activation of the HPA axis and locus coeruleus.

Knockout animals may be hypothesized to have potential utility in investigating the *in vivo* roles of specific proteins. Previous reports using gene mutant mice suggest that MOPs play an important role in various effects of opioids, such as antinociception, tolerance, reward, and locomotion (Ide et al., 2004; Matthes et al., 1996; Sora et al., 2001, 1997). Our present results also demonstrated the involvement of MOPs in stress-induced emotional responses. However, although no differences in DOP and KOP expression were evident in MOP-KO mice in the present study (Sora et al., 1997), several compensatory changes might occur in MOP-KO mice. These possible compensatory changes, especially with regard to neurotransmitter release and hormonal valence, could elicit changes in stress-induced emotional responses. Future studies, such as behavioral analyses using MOP-KO mice with viral expression of MOPs, may reveal the influences of compensatory changes in stress-induced emotional responses.

Gender differences in emotional responses may also exist (Toufexis, 2007; Toufexis et al., 2006). In the present study, several differences were found between male and female mice in stress-induced emotional responses, although these differences were not significant. In the elevated plus maze, female mice showed less anxiety-like behavior than male mice of both genotypes. These results are consistent with previous reports using rodents (Fernandes et al., 1999; Steenbergen et al., 1990) and suggest the presence of gender differences in anxiety-like behavior. However, no differences in immobility time were found between male and female wild-type mice in either the tail-suspension or forced swim tests. A previous report found that male and female C57BL/6J mice, the genetic background strain used in the present study, exhibited no differences in immobility time in either the tail-suspension or forced swim tests (Caldarone et al., 2003). Interestingly, female MOP-KO mice tended to exhibit less immobility in the tail-suspension test and more immobility in the forced swim test compared with male MOP-KO mice. Although the present study found no significant differences between genders, and additional studies may be required, MOPs may differentially modulate depression-like responses in both tests, especially in female mice.

In conclusion, we found decreased anxiety-like behavior in the elevated plus maze, 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. These results suggest that MOPs play an important facilitatory role in stress sensitivity and/or stress-induced emotional responses, including anxiety- and depression-like responses.

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References

Amir, S., 1982. Involvement of endogenous opioids with forced swimming-induced immobility in mice. Physiol. Behav. 28, 249–251.

Asakawa, A., Inui, A., Momose, K., Ueno, N., Fujino, M.A., Kasuga, M., 1998. Endomorphins have orexigenic and anxiolytic activities in mice. Neuroreport 9, 2265–2267.

Berrocoso, E., Mico, J.A. Cooperative opioid and serotonergic mechanisms generate superior antidepressant-like effects in a mice model of depression. Int. J. Neuropsychopharmacol., in press.

Broom, D.C., Jutkiewicz, E.M., Folk, J.E., Traynor, J.R., Rice, K.C., Woods, J.H., 2002. Nonpeptidic δ-opioid receptor agonists reduce immobility in the forced swim assay in rats. Neuropsychopharmacology 26, 744–755.

Caldarone, B.J., Karthigeyan, K., Harrist, A., Hunsberger, J.G., Wittmack, E., King, S.L., Jatlow, P., Picciotto, M.R., 2003. Sex differences in response to oral amitriptyline in three animal models of depression in C57BL/6J mice. Psychopharmacology (Berl) 170, 94–101.

Calenco-Choukroun, G., Dauge, V., Gacel, G., Feger, J., Roques, B.P., 1991. Opioid δ agonists and endogenous enkephalins induce different emotional reactivity than μ agonists after injection in the rat ventral tegmental area. Psychopharmacology (Berl) 103, 493–502.

Chen, J.C., Liang, K.W., Huang, E.Y., 2001. Differential effects of endomorphin-1 and -2 on amphetamine sensitization: neurochemical and behavioral aspects. Synapse 39, 239–248.

Coventry, T.L., Jessop, D.S., Finn, D.P., Crabb, M.D., Kinoshita, H., Harbuz, M.S., 2001. Endomorphins and activation of the hypothalamo-pituitary-adrenal axis. J. Endocrinol. 169, 185–193.

David, D.J., Renard, C.E., Jolliet, P., Hascoet, M., Bourin, M., 2003. Antidepressant-like effects in various mice strains in the forced swimming test. Psychopharmacology (Berl) 166, 373–382.

Douglas, A.J., Johnstone, H.A., Wigger, A., Landgraf, R., Russell, J.A., Neumann, I.D., 1998. The role of endogenous opioids in neurohypophysial and hypothalamo-pituitary-adrenal axis hormone secretory responses to stress in pregnant rats. J. Endocrinol. 158, 285–293.

Fernandes, C., Gonzalez, M.I., Wilson, C.A., File, S.E., 1999. Factor analysis shows that female rat behaviour is characterized primarily by activity, male rats are driven by sex and anxiety. Pharmacol. Biochem. Behav. 64, 731–738.

Fichna, J., Janecka, A., Piestrzeniewicz, M., Costentin, J., do Rego, J.C., 2007. Antidepressant-like effect of endomorphin-1 and endomorphin-2 in mice. Neuropsychopharmacology 32, 813–821.

Filliol, D., Ghozland, S., Chluba, J., Martin, M., Matthes, H.W., Simonin, F., Befort, K., Gaveriaux-Ruff, C., Dierich, A., LeMeur, M., Valverde, O., Maldonado, R., Kieffer, B.L., 2000. Mice deficient for δ- and μ-opioid receptors exhibit opposing alterations of emotional responses. Nat. Genet. 25, 195–200.

Hung, K.C., Wu, H.E., Mizoguchi, H., Leitermann, R., Tseng, L.F., 2003. Intrathecal treatment with 6-hydroxydopamine or 5,7-dihydroxytryptamine blocks the antinociception induced by endomorphin-1 and endomorphin-2 given intracerebroventricularly in the mouse. J. Pharmacol. Sci. 93, 299–306.

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

Ignar, D.M., Kuhn, C.M., 1990. Effects of specific mu and kappa opiate tolerance and abstinence on hypothalamo-pituitary-adrenal axis secretion in the rat. J. Pharmacol. Exp. Ther. 255, 1287–1295.

Kieffer, B.L., 1999. Opioids: first lessons from knockout mice. Trends Pharmacol. Sci. 20, 19–26.

Koks, S., Soosaar, A., Voikar, V., Bourin, M., Vasar, E., 1999. BOC-CCK-4, CCK_B receptor agonist, antagonizes anxiolytic-like action of morphine in elevated plus-maze. Neuropeptides 33, 63–69.

Loh, H.H., Liu, H.C., Cavalli, A., Yang, W., Chen, Y.F., Wei, L.N., 1998. μ Opioid receptor knockout in mice: effects on ligand-induced analgesia and morphine lethality. Brain Res. Mol. Brain Res. 54, 321–326.

Mague, S.D., Pliakas, A.M., Todtenkopf, M.S., Tomasiewicz, H.C., Zhang, Y., Stevens Jr., W.C., Jones, R.M., Portoghese, P.S., Carlezon Jr., W.A., 2003. Antidepressant-like effects of κ-opioid receptor antagonists in the forced swim test in rats. J. Pharmacol. Exp. Ther. 305, 323–330.

Matthes, H.W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., Befort, K., Dierich, A., Le Meur, M., Dolle, P., Tzavara, E., Hanoune, J., Roques, B.P., Kieffer, B.L., 1996. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid-receptor gene. Nature 383, 819–823.

McLaughlin, J.P., Marton-Popovici, M., Chavkin, C., 2003. κ Opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. J. Neurosci. 23, 5674–5683.

Mellon, R.D., Bayer, B.M., 1998. Evidence for central opioid receptors in the immunomodulatory effects of morphine: review of potential mechanism(s) of action. J. Neuroimmunol. 83, 19–28.

Molina, V.A., Heyser, C.J., Spear, L.P., 1994. Chronic variable stress or chronic morphine facilitates immobility in a forced swim test: reversal by naloxone. Psychopharmacology (Berl) 114, 433–440.

Nakai, T., Hayashi, M., Ichihara, K., Wakabayashi, H., Hoshi, K., 2002. Noradrenaline release in rat locus coeruleus is regulated by both opioid and α_2-adrenoceptors. Pharmacol. Res. 45, 407–412.

Nestler, E.J., Alreja, M., Aghajanian, G.K., 1999. Molecular control of locus coeruleus neurotransmission. Biol. Psychiatry 46, 1131–1139.

Newman, L.C., Sands, S.S., Wallace, D.R., Stevens, C.W., 2002. Characterization of μ, κ, and δ opioid binding in amphibian whole brain tissue homogenates. J. Pharmacol. Exp. Ther. 301, 364–370.

Porsolt, R.D., Le Pichon, M., Jalfre, M., 1977. Depression: a new animal model sensitive to antidepressant treatments. Nature 266, 730–732.

Roy, S., Wang, J.H., Balasubramanian, S., Sumandeep, Charboneau, R., Barke, R., Loh, H.H., 2001. Role of hypothalamic-pituitary axis in morphine-induced alteration in thymic cell distribution using mu-opioid receptor knockout mice. J. Neuroimmunol. 116, 147–155.

Sora, I., Elmer, G., Funada, M., Pieper, J., Li, X.F., Hall, F.S., Uhl, G.R., 2001. μ Opiate receptor gene dose effects on different morphine actions: evidence for differential *in vivo* μ receptor reserve. Neuropsychopharmacology 25, 41–54.

Sora, I., Takahashi, N., Funada, M., Ujike, H., Revay, R.S., Donovan, D.M., Miner, L.L., Uhl, G.R., 1997. Opiate receptor knockout mice define μ receptor roles in endogenous nociceptive responses and morphine-induced analgesia. Proc. Natl. Acad. Sci. U.S.A 94, 1544–1549.

Steenbergen, H.L., Heinsbroek, R.P., Van Hest, A., Van de Poll, N.E., 1990. Sex-dependent effects of inescapable shock administration on shuttlebox-escape performance and elevated plus-maze behavior. Physiol. Behav. 48, 571–576.

Steru, L., Chermat, R., Thierry, B., Simon, P., 1985. The tail-suspension test: a new method for screening antidepressants in mice. Psychopharmacology (Berl) 85, 367–370.

Toufexis, D., 2007. Region- and sex-specific modulation of anxiety behaviours in the rat. J. Neuroendocrinol 19, 461–473.

Toufexis, D.J., Myers, K.M., Davis, M., 2006. The effect of gonadal hormones and gender on anxiety and emotional learning. Horm. Behav. 50, 539–549.

Ukai, M., Lin, H.P., 2002. Endomorphins 1 and 2 induce amnesia via selective modulation of dopamine receptors in mice. Eur. J. Pharmacol. 446, 97–101.

Valentino, R.J., Van Bockstaele, E., 2001. Opposing regulation of the locus coeruleus by corticotropin-releasing factor and opioids: potential for reciprocal interactions between stress and opioid sensitivity. Psychopharmacology (Berl) 158, 331–342.

Wang, J., Charboneau, R., Barke, R.A., Loh, H.H., Roy, S., 2002. μ-Opioid receptor mediates chronic restraint stress-induced lymphocyte apoptosis. J. Immunol. 169, 3630–3636.

Zurita, A., Molina, V., 1999. Prior morphine facilitates the occurrence of immobility and anhedonia following stress. Physiol. Behav. 65, 833–837.

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Serotonin 1A receptor gene is associated with Japanese methamphetamine-induced psychosis patients

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ABSTRACT

Background: Several investigations have reported associations the serotonin 1A (5-HT1A) receptor to schizophrenia and psychotic disorders, making 5-HT1A receptor gene (*HTR1A*) an adequate candidate gene for the pathophysiology of schizophrenia and methamphetamine (METH)-induced psychosis. Huang and colleagues reported that rs6295 in *HTR1A* was associated with schizophrenia. The symptoms of methamphetamine (METH)-induced psychosis are similar to those of paranoid type schizophrenia. It may indicate that METH-induced psychosis and schizophrenia have common susceptibility genes. In support of this hypothesis, we reported that the V-act murine thymoma viral oncogene homologue 1 (AKT1) gene was associated with METH-induced psychosis and schizophrenia in the Japanese population. Furthermore, we conducted an analysis of the association of *HTR1A* with METH-induced psychosis.

Method: Using one functional SNP (rs6295) and one tagging SNP (rs878567), we conducted a genetic association analysis of case-control samples (197 METH-induced psychosis patients and 337 controls) in the Japanese population. The age and sex of the control subjects did not differ from those of the methamphetamine dependence patients.

Results: Rs878567 was associated with METH-induced psychosis patients in the allele/genotype-wise analysis. Moreover, this significance remained after Bonferroni correction. In addition, we detected an association between rs6295 and rs878567 in *HTR1A* and METH-induced psychosis patients in the haplotype-wise analysis. Although we detected an association between rs6295 and METH-induced psychosis patients, this significance disappeared after Bonferroni correction.

Conclusion: *HTR1A* may play an important role in the pathophysiology of METH-induced psychosis in the Japanese population. However, because we did not perform a mutation scan of *HTR1A*, a replication study using a larger sample may be required for conclusive results.

1. Introduction

Altered serotonergic neural transmission is hypothesized to be a susceptibility factor for schizophrenia (Geyer and Vollenweider, 2008; Meltzer et al., 2003). Several postmortem studies reported increased serotonin 1A (5-HT1A) receptor in the prefrontal cortex

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of schizophrenic patients (Burnet et al., 1996; Hashimoto et al., 1993, 1991; Simpson et al., 1996; Sumiyoshi et al., 1996). Huang and colleagues reported that rs6295 in an SNP (C-1019G: rs6295) in the promoter region of the 5-HT1A receptor gene (*HTR1A*), which regulate HTR1A transcription (Le Francois et al., 2008; Lemonde et al., 2003), was associated with schizophrenia (Huang et al., 2004). These facts suggest a crucial relationship between the 5-HT1A receptor and schizophrenia, and that *HTR1A* is an adequate candidate for the etiology of schizophrenia. *HTR1A* (OMIM*109 760, 1 exon in this genomic region spanning 2.069 kb) is located on 5q11.

The symptoms of methamphetamine (METH)-induced psychosis are similar to those of paranoid type schizophrenia (Sato et al., 1992). It may indicate that METH-induced psychosis and schizophrenia have common susceptibility genes (Bousman et al., 2009). In support of this hypothesis, we reported that the V-act murine thymoma viral oncogene homologue 1 (AKT1) gene was associated with METH-induced psychosis (Ikeda et al., 2006) and schizophrenia (Ikeda et al., 2004) in the Japanese population. Furthermore, we conducted an analysis of the association of these genes with METH-induced psychosis, using the recently recommended strategy of 'gene-based' association analysis (Neale and Sham, 2004).

2. Materials and methods

2.1. Subjects

The subjects in the association analysis were 197 METH-induced psychosis patients (164 males: 83.2% and 33 females; mean age ± standard deviation (SD) 37.6 ± 12.2 years) and 337 healthy controls (271 males: 80.4% and 66 females; 37.6 ± 14.3 years). The age and sex of the control subjects did not differ from those of the methamphetamine dependence patients. All subjects were unrelated to each other, ethnically Japanese, and lived in the central area of Japan. The patients were diagnosed according to DSM-IV criteria with consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. METH-induced psychosis patients were divided into two categories of psychosis prognosis, the transient type and the prolonged type, which showed remission of psychotic symptoms within 1 month and after more than 1 month, respectively, after the discontinuance of methamphetamine consumption and beginning of treatment with neuroleptics; 112 patients (56.9%) were the transient type, and 85 patients (43.1%) were the prolonged type. One hundred thirty-seven subjects with METH-induced psychosis also had dependence on drugs other than METH. Cannabinoids were the most frequency abused drugs (31.4%), followed by cocaine (9.09%), LSD (9.09%), opioids (7.69%), and hypnotics (7.69%). Subjects with METH-induced psychosis were excluded if they had a clinical diagnosis of psychotic disorder, mood disorder, anxiety disorder or eating disorder. More detailed characterizations of these subjects have been published elsewhere (Kishi et al., 2008b). All healthy controls were also psychiatrically screened based on unstructured interviews. None had severe medical complications such as liver cirrhosis, renal failure, heart failure or other Axis-I disorders according to DSM-IV.

The study was described to subjects and written informed consent was obtained from each. This study was approved by the Ethics Committee at Fujita Health University, Nagoya University School of Medicine and each participating member of the Institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA).

2.2. SNPs selection and linkage disequilibrium (LD) evaluation

We first consulted the HapMap database (release#23.a.phase2, Mar 2008, www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.05) and included 3 SNPs (rs6449693, rs878567 and rs1423691) covering *HTR1A* (5′-flanking regions including about 1 kb from the initial exon and about 2 kb downstream (3′) from the last exon: HapMap database contig number chr5: 63287418...63291774). Then one tagging SNP was selected with the criteria of an r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger) of the HAPLOVIEW software (Barrett et al., 2005).

HTR1A has also been reported to have one biologically functional SNP (C-1019G: rs6295) (Albert et al., 1996; Albert and Lemonde, 2004; Lemonde et al., 2003). Rs6295 (C-1019G) in the promoter region regulate *HTR1A* transcription (Le Francois et al., 2008; Lemonde et al., 2003). The C allele is a part of a 26 palindrome that connect transcription factors (Deaf-1, Hes1 and Hes5) by NUDR (nuclear deformed epidermal autoregulatory factor), whereas the G allele abolishes repression by NUDR (Le Francois et al., 2008; Lemonde et al., 2003). This would lead to elevated levels of 5-HT1A receptor in the presynaptic raphe nucleus in GG genotypes, compared with CC genotype (Le Francois et al., 2008; Lemonde et al., 2003). Since no information about rs6295 was shown in the HapMap database, we included this SNP. These two SNPs were then used for the following association analysis.

2.3. SNPs genotyping

We used TaqMan assays (Applied Biosystems, Inc., Foster City, CA) for both SNPs. Detailed information, including primer sequences and reaction conditions, is available on request.

2.4. Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan).

Marker-trait association analysis was used to evaluate allele- and genotype-wise association with the chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan), and haplotype-wise association analysis was conducted with a likelihood ratio test using the COCAPHASE2.403 program (Dudbridge, 2003). We used the permutation test option as provided in the haplotype-wise analysis to avoid spurious results and correct for multiple testing. Permutation test correction was performed using 1000 iterations (random permutations). In addition, Bonferroni's correction was used to control inflation of the type I error rate in the single marker association analysis and in the explorative analysis. For Bonferroni correction, we employed the following numbers for multiple testing: 2 for each sample set in allele- and genotype-wise analysis (2 examined SNPs). We had already performed a permutation test in the haplotype-wise analysis. Power calculation was performed using a genetic power calculator (Purcell et al., 2003).

The significance level for all statistical tests was 0.05.

3. Results

The LD from rs6449693, rs878567 and rs1423691 was tight in from the HapMap database samples ($r^2 = 1.00$). However, the LD structure of rs6295 (functional SNP) and rs878567 (tagging SNP) in our control samples was not tight ($r^2 = 0.160$). Genotype frequencies of all SNPs were in HWE (Table 1). Rs878567 was associated with METH-induced psychosis patients in the allele/genotype-wise analysis (P allele = 0.000122 and P genotype = 0.00103) (Table 1). Moreover, these significances remained after Bonferroni correction (P allele = 0.000244 and P genotype = 0.00203) (Table 1). In addition, we detected an association between rs6295 and rs878567 in *HTR1A* and METH-induced psychosis patients in the haplotype-wise analysis ($P = 0.0000643$) (Table 2). Although we detected an association between rs6295 and METH-induced psychosis patients (P allele = 0.0271), this significance disappeared after Bonferroni correction (P allele = 0.0542) (Table 1).

4. Discussion

We found associations between *HTR1A* and Japanese METH-induced psychosis patients. Therefore, we reasoned that *HTR1A* may play an important role in the pathophysiology of METH-induced psychosis in the Japanese population. However, our samples are small. Although Bonferroni's correction was used to control inflation of the type I error rate, we considered that there is a possibility of type I error in these results.

The 5-HT1A receptor is present in various regions of the brain, including the cortex, hippocampus, amygdala, hypothalamus and septum (Aznar et al., 2003; Barnes and Sharp, 1999; Le Francois et al., 2008; Varnas et al., 2004). Presynaptic 5-HT1A autoreceptors play an important role in the autoregulation of serotonergic neurons (Le Francois et al., 2008; Lemonde et al., 2003; Riad et al., 2000; Sotelo et al., 1990). The 5-HT1A receptor activation by serotonin induces the hyperpolarization of serotonergic neurons, decreasing their firing rate and consequently the release of serotonin in the brain (Le Francois et al., 2008; Lemonde et al., 2003; Riad et al., 2000; Sotelo et al., 1990). Also, the 5-HT1A receptor was associated hippocampal neurogenesis. The hippocampus is a part of the limbic system involved in cognitive function such as memory. Stimulation of 5-HT1A receptors has been known to reduce the

Table 1
Association analysis of *HTR1A* with methamphetamine-induced psychosis.

SNP[a]	Phenotype[b]	MAFs[c]	N	Genotype distribution[d]			*P*-value[f]			Corrected *P*-value[f,g]	
				M/M	M/m	m/m	HWE[e]	Genotype	Allele	Genotype	Allele
rs6295	Controls	0.254	336	192	117	27	0.132				
C > G	METH-induced psychosis	0.317	197	92	85	20	0.955	0.0657	**0.0271**		0.0542
rs878567	Controls	0.126	336	258	71	7	0.423				
C > T	METH-induced psychosis	0.216	197	124	61	12	0.233	**0.00103**	**0.000122**	**0.00203**	**0.000244**

[a] Major allele > minor allele.
[b] METH-induced psychosis: methamphetamine-induced psychosis.
[c] MAFs: minor allele frequencies.
[d] M: major allele, m: minor allele.
[e] Hardy–Weinberg equilibrium.
[f] Bold represents significant *P*-value.
[g] Calculated using Bonferroni's correction.

negative symptoms and cognitive dysfunction of schizophrenia (Meltzer et al., 2003; Meltzer and Sumiyoshi, 2008; Sumiyoshi et al., 2001, 2007). Mason and Reynolds (1992) reported that one of the major pharmacological therapeutic targets of clozapine is 5-HT1A receptors on cortical glutamatergic neurons. Several post-mortem studies reported increased 5-HT1A receptor in the prefrontal cortex of schizophrenic patients (Burnet et al., 1996; Hashimoto et al., 1993, 1991; Simpson et al., 1996; Sumiyoshi et al., 1996). NAN-190 (5-HT1A receptor antagonist) produced an inhibitory action on methamphetamine-induced hyperactivity (Ginawi et al., 2004; Millan and Colpaert, 1991). These facts suggest that altered serotonergic neural transmission caused by abnormalities in 5-HT1A receptor may be involved in the development of psychotic disorders such as schizophrenia and METH-induced psychosis (Geyer and Vollenweider, 2008; Meltzer et al., 2003).

Serretti et al. (2007) reported that rs878567 in *HTR1A* was associated with German and Italian suicidal attempters. Also, previous study have reported that rs878567 in *HTR1A* was found the interaction with childhood physical abuse in mood disorders (Brezo et al., 2009). These authors suggested rs878567 might influence hippocampus-mediated memory deficits in mood disorders (Brezo et al., 2009). The LD from rs6449693, rs878567 and rs1423691 was tight in from the HapMap database samples (r^2 = 1.00). As these results show, rs878567 covers a wide and important region including the exon and the promoter region in *HTR1A*. Because it is possible that rs878567 influences biological function in the brain, we suggest that functional analysis for rs878567 should be performed in future studies.

Rs6295 (C-1019G) in the promoter region regulate *HTR1A* transcription (Le Francois et al., 2008; Lemonde et al., 2003). The C allele is a part of a 26 palindrome that connect transcription factors (Deaf-1, Hes1 and Hes5) by NUDR (nuclear deformed epidermal autoregulatory factor), whereas the G allele abolishes repression by NUDR (Le Francois et al., 2008; Lemonde et al., 2003). This would lead to elevated levels of 5-HT1A receptor in the presynaptic raphe nucleus in GG genotyps, compared with CC genotype (Le Francois et al., 2008; Lemonde et al., 2003). This variant was associated with several studies, including major depressive disorder (Anttila et al., 2007; Kraus et al., 2007; Lemonde et al., 2003; Neff et al., 2009; Parsey et al., 2006) and panic disorder (Strobel et al., 2003) and antidepressant response in MDD (Arias et al., 2005; Hong et al., 2006; Lemonde et al., 2004; Parsey et al., 2006; Serretti et al., 2004; Yu et al., 2006). Huang et al. (2004) reported that rs6295 was associated with schizophrenia. Recent studies reported that rs6295 was associated with the improvement in negative symptoms from antipsychotics such as risperidone (Mossner et al., 2009; Reynolds et al., 2006; Wang et al., 2008) and that 5-HT1A receptor agonists such as tandospirone produced improvements in the cognitive impairment in schizophrenia (Meltzer and Sumiyoshi, 2008; Sumiyoshi et al., 2001, 2007).

A few points of caution should be mentioned with respect to our results. Firstly, the positive association may be due to small sample size. Ideal samples for this study are METH use disorder samples with and without psychosis. Because we had only a few METH use disorder samples without psychosis, and we wanted to avoid statistical error, we did not perform an association analysis with these samples. Secondly, we did not include a mutation scan to detect rare variants. We designed the study based on the common disease-common variants hypothesis (Chakravarti, 1999). However, Weickert et al. (2008) have shown associations between a common disease such as schizophrenia and rare variants. If the genetic background of METH-induced psychosis is described by the common disease-rare variants hypothesis, further investigation will be required, such as medical resequencing using larger samples. However, statistical power is needed to evaluate the association of rare variants. Lastly, our subjects did not undergo structured interviews. However, in this study patients were carefully diagnosed according to DSM-IV criteria with consensus of at least two experienced psychiatrists on the basis of a review of medical records (Kishi et al., 2008a,c, 2009). In addition, when we found misdiagnosis in a patient, we promptly excluded the misdiagnosed case to maintain the precision of our sample. To overcome these limitations, a replication study using larger samples or samples of other populations will be required for conclusive results.

In conclusion, our results suggest that *HTR1A* may play a major role in the pathophysiology of METH-induced psychosis in the Japanese population. However, because we did not perform a mutation scan of *HTR1A*, a replication study using a larger sample may be required for conclusive results.

Table 2
Haplotype-wise analysis of *HTR1A*.

Haplotype rs6295-rs878567	Phenotype[a]	Individual haplotype frequency	Individual *P*-value[b]	Phenotype[a]	Global *P*-value[b]
C–C	Control	0.811			
	METH-induced psychosis	0.694	**0.0000364**	METH-induced psychosis	**0.0000643**
G–C	Control	0.189			
	METH-induced psychosis	0.306	**0.0000364**		

[a] METH-induced psychosis: methamphetamine-induced psychosis.
[b] Bold numbers represent significant *P*-value.

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References

Albert, P.R., Lembo, P., Storring, J.M., Charest, A., Saucier, C., 1996. The 5-HT1A receptor: signaling, desensitization, and gene transcription. Neuropsychopharmacology 14, 19–25.

Albert, P.R., Lemonde, S., 2004. 5-HT1A receptors, gene repression, and depression: guilt by association. Neuroscientist 10, 575–593.

Anttila, S., Huuhka, K., Huuhka, M., Rontu, R., Hurme, M., Leinonen, E., Lehtimaki, T., 2007. Interaction between 5-HT1A and BDNF genotypes increases the risk of treatment-resistant depression. J. Neural Transm. 114, 1065–1068.

Arias, B., Catalan, R., Gasto, C., Gutierrez, B., Fananas, L., 2005. Evidence for a combined genetic effect of the 5-HT(1A) receptor and serotonin transporter genes in the clinical outcome of major depressive patients treated with citalopram. J. Psychopharmacol. 19, 166–172.

Aznar, S., Qian, Z., Shah, R., Rahbek, B., Knudsen, G.M., 2003. The 5-HT1A serotonin receptor is located on calbindin- and parvalbumin-containing neurons in the rat brain. Brain Res. 959, 58–67.

Barnes, N.M., Sharp, T., 1999. A review of central 5-HT receptors and their function. Neuropharmacology 38, 1083–1152.

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21, 263–265.

Bousman, C.A., Glatt, S.J., Everall, I.P., Tsuang, M.T., 2009. Genetic association studies of methamphetamine use disorders: a systematic review and synthesis. Am. J. Med. Genet. B Neuropsychiatr. Genet.

Brezo, J., Bureau, A., Merette, C., Jomphe, V., Barker, E.D., Vitaro, F., Hebert, M., Carbonneau, R., Tremblay, R.E., Turecki, G., 2009. Differences and similarities in the serotonergic diathesis for suicide attempts and mood disorders: a 22-year longitudinal gene-environment study. Mol. Psychiatry.

Burnet, P.W., Eastwood, S.L., Harrison, P.J., 1996. 5-HT1A and 5-HT2A receptor mRNAs and binding site densities are differentially altered in schizophrenia. Neuropsychopharmacology 15, 442–455.

Chakravarti, A., 1999. Population genetics-making sense out of sequence. Nat. Genet. 21, 56–60.

Dudbridge, F., 2003. Pedigree disequilibrium tests for multilocus haplotypes. Genet. Epidemiol. 25, 115–121.

Geyer, M.A., Vollenweider, F.X., 2008. Serotonin research: contributions to understanding psychoses. Trends Pharmacol. Sci. 29, 445–453.

Ginawi, O.T., Al-Majed, A.A., Al-Suwailem, A.K., El-Hadiyah, T.M., 2004. Involvement of some 5-HT receptors in methamphetamine-induced locomotor activity in mice. J. Physiol. Pharmacol. 55, 357–369.

Hashimoto, T., Kitamura, N., Kajimoto, Y., Shirai, Y., Shirakawa, O., Mita, T., Nishino, N., Tanaka, C., 1993. Differential changes in serotonin 5-HT1A and 5-HT2 receptor binding in patients with chronic schizophrenia. Psychopharmacology (Berl) 112, S35–S39.

Hashimoto, T., Nishino, N., Nakai, H., Tanaka, C., 1991. Increase in serotonin 5-HT1A receptors in prefrontal and temporal cortices of brains from patients with chronic schizophrenia. Life Sci. 48, 355–363.

Hong, C.J., Chen, T.J., Yu, Y.W., Tsai, S.J., 2006. Response to fluoxetine and serotonin 1A receptor (C-1019G) polymorphism in Taiwan Chinese major depressive disorder. Pharmacogenomics J. 6, 27–33.

Huang, Y.Y., Battistuzzi, C., Oquendo, M.A., Harkavy-Friedman, J., Greenhill, L., Zalsman, G., Brodsky, B., Arango, V., Brent, D.A., Mann, J.J., 2004. Human 5-HT1A receptor C(-1019)G polymorphism and psychopathology. Int. J. Neuropsychopharmacol. 7, 441–451.

Ikeda, M., Iwata, N., Suzuki, T., Kitajima, T., Yamanouchi, Y., Kinoshita, Y., Inada, T., Ozaki, N., 2004. Association of AKT1 with schizophrenia confirmed in a Japanese population. Biol. Psychiatry 56, 698–700.

Ikeda, M., Iwata, N., Suzuki, T., Kitajima, T., Yamanouchi, Y., Kinoshiya, Y., Sekine, Y., Iyo, M., Harano, M., Komiyama, T., Yamada, M., Sora, I., Ujike, H., Inada, T., Ozaki, N., 2006. Positive association of AKT1 haplotype to Japanese methamphetamine use disorder. Int. J. Neuropsychopharmacol. 9, 77–81.

Kishi, T., Ikeda, M., Kitajima, T., Suzuki, T., Yamanouchi, Y., Kinoshita, Y., Kawashima, K., Ozaki, N., Iwata, N., 2008a. No association between prostate apoptosis response 4 gene (PAWR) in schizophrenia and mood disorders in a Japanese population. Am. J. Med. Genet. B Neuropsychiatr. Genet. 147B, 531–534.

Kishi, T., Ikeda, M., Kitajima, T., Yamanouchi, Y., Kinoshita, Y., Kawashima, K., Inada, T., Harano, M., Komiyama, T., Hori, T., Yamada, M., Iyo, M., Sora, I., Sekine, Y., Ozaki, N., Ujike, H., Iwata, N., 2008b. Glutamate cysteine ligase modifier (GCLM) subunit gene is not associated with methamphetamine-use disorder or schizophrenia in the Japanese population. Ann. NY Acad. Sci. 1139, 63–69.

Kishi, T., Kitajima, T., Ikeda, M., Yamanouchi, Y., Kinoshita, Y., Kawashima, K., Okochi, T., Okumura, T., Tsunoka, T., Inada, T., Ozaki, N., Iwata, N., 2009. Association study of clock gene (CLOCK) and schizophrenia and mood disorders in the Japanese population. Eur. Arch. Psychiatry Clin. Neurosci. 259, 293–297.

Kishi, T., Kitajima, T., Ikeda, M., Yamanouchi, Y., Kinoshita, Y., Kawashima, K., Okochi, T., Ozaki, N., Iwata, N., 2008c. Association analysis of nuclear receptor Rev-erb alpha gene (NR1D1) with mood disorders in the Japanese population. Neurosci. Res. 62, 211–215.

Kraus, M.R., Al-Taie, O., Schafer, A., Pfersdorff, M., Lesch, K.P., Scheurlen, M., 2007. Serotonin-1A receptor gene HTR1A variation predicts interferon-induced depression in chronic hepatitis C. Gastroenterology 132, 1279–1286.

Le Francois, B., Czesak, M., Steubl, D., Albert, P.R., 2008. Transcriptional regulation at a HTR1A polymorphism associated with mental illness. Neuropharmacology 55, 977–985.

Lemonde, S., Du, L., Bakish, D., Hrdina, P., Albert, P.R., 2004. Association of the C(-1019)G 5-HT1A functional promoter polymorphism with antidepressant response. Int. J. Neuropsychopharmacol. 7, 501–506.

Lemonde, S., Turecki, G., Bakish, D., Du, L., Hrdina, P.D., Bown, C.D., Sequeira, A., Kushwaha, N., Morris, S.J., Basak, A., Ou, X.M., Albert, P.R., 2003. Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J. Neurosci. 23, 8788–8799.

Mason, S.L., Reynolds, G.P., 1992. Clozapine has sub-micromolar affinity for 5-HT1A receptors in human brain tissue. Eur. J. Pharmacol. 221, 397–398.

Meltzer, H.Y., Li, Z., Kaneda, Y., Ichikawa, J., 2003. Serotonin receptors: their key role in drugs to treat schizophrenia. Prog. Neuropsychopharmacol. Biol. Psychiatry 27, 1159–1172.

Meltzer, H.Y., Sumiyoshi, T., 2008. Does stimulation of 5-HT(1A) receptors improve cognition in schizophrenia? Behav. Brain Res. 195, 98–102.

Millan, M.J., Colpaert, F.C., 1991. Methylenedioxymethamphetamine induces spontaneous tail-flicks in the rat via 5-HT1A receptors. Eur. J. Pharmacol. 193, 145–152.

Mossner, R., Schuhmacher, A., Kuhn, K.U., Cvetanovska, G., Rujescu, D., Zill, P., Quednow, B.B., Rietschel, M., Wolwer, W., Gaebel, W., Wagner, M., Maier, W., 2009. Functional serotonin 1A receptor variant influences treatment response to atypical antipsychotics in schizophrenia. Pharmacogenet. Genomics 19, 91–94.

Neale, B.M., Sham, P.C., 2004. The future of association studies: gene-based analysis and replication. Am. J. Hum. Genet. 75, 353–362.

Neff, C.D., Abkevich, V., Packer, J.C., Chen, Y., Potter, J., Riley, R., Davenport, C., DeGrado Warren, J., Jammulapati, S., Bhathena, A., Choi, W.S., Kroeger, P.E., Metzger, R.E., Gutin, A., Skolnick, M.H., Shattuck, D., Katz, D.A., 2009. Evidence for HTR1A and LHPP as interacting genetic risk factors in major depression. Mol. Psychiatry 14, 621–630.

Parsey, R.V., Olvet, D.M., Oquendo, M.A., Huang, Y.Y., Ogden, R.T., Mann, J.J., 2006. Higher 5-HT1A receptor binding potential during a major depressive episode predicts poor treatment response: preliminary data from a naturalistic study. Neuropsychopharmacology 31, 1745–1749.

Purcell, S., Cherny, S.S., Sham, P.C., 2003. Genetic power calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics 19, 149–150.

Reynolds, G.P., Arranz, B., Templeman, L.A., Fertuzinhos, S., San, L., 2006. Effect of 5-HT1A receptor gene polymorphism on negative and depressive symptom response to antipsychotic treatment of drug-naive psychotic patients. Am. J. Psychiatry 163, 1826–1829.

Riad, M., Garcia, S., Watkins, K.C., Jodoin, N., Doucet, E., Langlois, X., el Mestikawy, S., Hamon, M., Descarries, L., 2000. Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. J. Comp. Neurol. 417, 181–194.

Sato, M., Numachi, Y., Hamamura, T., 1992. Relapse of paranoid psychotic state in methamphetamine model of schizophrenia. Schizophr. Bull. 18, 115–122.

Serretti, A., Artioli, P., Lorenzi, C., Pirovano, A., Tubazio, V., Zanardi, R., 2004. The C(-1019)G polymorphism of the 5-HT1A gene promoter and antidepressant response in mood disorders: preliminary findings. Int. J. Neuropsychopharmacol. 7, 453–460.

Serretti, A., Mandelli, L., Giegling, I., Schneider, B., Hartmann, A.M., Schnabel, A., Maurer, K., Moller, H.J., Rujescu, D., 2007. HTR2C and HTR1A gene variants in German and Italian suicide attempters and completers. Am. J. Med. Genet. B Neuropsychiatr. Genet. 144B, 291–299.

Simpson, M.D., Lubman, D.I., Slater, P., Deakin, J.F., 1996. Autoradiography with [3H]8-OH-DPAT reveals increases in 5-HT(1A) receptors in ventral prefrontal cortex in schizophrenia. Biol. Psychiatry 39, 919–928.

Sotelo, C., Cholley, B., El Mestikawy, S., Gozlan, H., Hamon, M., 1990. Direct immunohistochemical evidence of the existence of 5-HT1A autoreceptors on serotoninergic neurons in the midbrain raphe nuclei. Eur. J. Neurosci. 2, 1144–1154.

Strobel, A., Gutknecht, L., Rothe, C., Reif, A., Mossner, R., Zeng, Y., Brocke, B., Lesch, K.P., 2003. Allelic variation in 5-HT1A receptor expression is associated with anxiety- and depression-related personality traits. J. Neural Transm. 110, 1445–1453.

Sumiyoshi, T., Matsui, M., Nohara, S., Yamashita, I., Kurachi, M., Sumiyoshi, C., Jayathilake, K., Meltzer, H.Y., 2001. Enhancement of cognitive performance in schizophrenia by addition of tandospirone to neuroleptic treatment. Am. J. Psychiatry 158, 1722–1725.

Sumiyoshi, T., Park, S., Jayathilake, K., Roy, A., Ertugrul, A., Meltzer, H.Y., 2007. Effect of buspirone, a serotonin1A partial agonist, on cognitive function in schizophrenia:

a randomized, double-blind, placebo-controlled study. Schizophr. Res. 95, 158–168.
Sumiyoshi, T., Stockmeier, C.A., Overholser, J.C., Dilley, G.E., Meltzer, H.Y., 1996. Serotonin1A receptors are increased in postmortem prefrontal cortex in schizophrenia. Brain Res. 708, 209–214.
Varnas, K., Halldin, C., Hall, H., 2004. Autoradiographic distribution of serotonin transporters and receptor subtypes in human brain. Hum. Brain Mapp. 22, 246–260.
Wang, L., Fang, C., Zhang, A., Du, J., Yu, L., Ma, J., Feng, G., Xing, Q., He, L., 2008. The -1019 C/G polymorphism of the 5-HT(1)A receptor gene is associated with negative symptom response to risperidone treatment in schizophrenia patients. J. Psychopharmacol. 22, 904–909.
Weickert, C.S., Miranda-Angulo, A.L., Wong, J., Perlman, W.R., Ward, S.E., Radhakrishna, V., Straub, R.E., Weinberger, D.R., Kleinman, J.E., 2008. Variants in the estrogen receptor alpha gene and its mRNA contribute to risk for schizophrenia. Hum. Mol. Genet. 17, 2293–2309.
Yu, Y.W., Tsai, S.J., Liou, Y.J., Hong, C.J., Chen, T.J., 2006. Association study of two serotonin 1A receptor gene polymorphisms and fluoxetine treatment response in Chinese major depressive disorders. Eur. Neuropsychopharmacol. 16, 498–503.

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Impaired spatial working memory and decreased frontal cortex BDNF protein level in dopamine transporter knockout mice

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ABSTRACT

Brain-derived neurotrophic factor (BDNF), one of the key brain neurotrophins, has been implicated in neuronal plasticity and memory. Recent studies document the importance of BDNF for normal long-term memory functions. However, there are few studies of the roles of BDNF in short-term memory. Dopamine is likely to play important roles in BDNF gene expression in specific brain regions, including frontal cortical regions that are implicated in short-term working memory processes that include spontaneous alternation. We have thus tested spatial working memory in dopamine transporter knockout (DAT KO) and wild-type mice. Spontaneous alternation in the Y-maze, an index of short-term spatial working memory in mice, was significantly decreased in DAT KO mice compared to wild-type mice. BDNF protein was significantly decreased in frontal cortex, though not in striatum or hippocampus, of the DAT KO mice. The data support the hypothesis that impaired spatial working memory in DAT KO mice may be related to decreased frontal cortical BDNF in these animals, and document apparent roles for BDNF in a short-term memory process.

1. Introduction

Brain-derived neurotrophic factor (BDNF) is a small dimeric protein that is widely expressed in the adult mammalian brain (Murer et al., 2001). There is abundant evidence that BDNF is involved in synaptic plasticity and memory processes (Poo, 2001; Tyler et al., 2002), particularly as BDNF relates to hippocampal dependent memory. BDNF has been suggested to be essential for normal persistence of long-term memory storage (Bekinschtein et al., 2008) and endogenous BDNF is required for long-term memory formation in the rat parietal cortex (Alonso et al., 2005). Lower levels of frontal cortical BDNF have been associated with impaired working memory performance in Ts65Dn mice, which are considered to be an animal model of Down's syndrome (Bimonte-Nelson et al., 2003). Reducing BDNF expression through intracerebroventricular infusion of BDNF antisense impairs performance in radial arm maze tests (Mizuno et al., 2000). BDNF has been implicated in long-term potentiation, an electrophysiological concomitant memory acquisition (Korte et al., 1998; Lessmann, 1998).

There is less data concerning the effects of BDNF on spatial working memory, although BDNF has been closely related to dopamine pathways and implicated in dopaminergic function (Berton et al., 2006; Fumagalli et al., 2003; Li et al., 2006; Li et al., 2007a,b). Lesions and other manipulations of mesocortical dopamine pathways can change performance in spontaneous alternation paradigms (Pioli et al., 2008) and alter BDNF gene expression in frontal cortical regions (Fumagalli et al., 2003). Dopamine has also been shown to directly regulate BDNF expression in striatal cells *in vitro* (Küppers and Beyer, 2001). These data therefore collectively implicate a dopamine mediated regulation of BDNF in prefrontal cortex dependent memory function.

We have produced and extensively characterized a line of DAT KO mice that display hyperlocomotion (Sora et al., 1998, 2001, 2009) as well as increased extracellular dopamine levels (Shen et al., 2004). These and other lines of DAT KO mice display altered performance in 8-arm radial maze testing, reduced prepulse inhibition and increased prefrontal cortical BDNF levels (Gainetdinov et al., 1999; Yamashita et al., 2006; Fumagalli et al., 2003). Each of these results suggests that DAT KO mice might also display alterations in spatial working memory due to increased dopaminergic tone, perhaps mediated by alterations in BDNF function. We thus now report results of spatial working memory Y-maze testing and evaluation of BDNF expression in DAT KO mice. We discuss ways in which this data, taken together, is consistent with the idea that direct and indirect effects of this knockout, including the altered BDNF expression, could contribute to

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the altered performance in this index of short-term memory function (Krejcova et al., 2004).

2. Materials and methods

2.1. Animals

DAT KO mice were produced as described (Sora et al, 2001), bred at the Animal Laboratory Institute of Tohoku University Graduate School of Medicine and maintained on a mixed genetic background combining C57BL/6 and 129Sv/J mouse strains. Offspring from heterozygote crosses were weaned at 28 days postnatal and housed in groups of two to five (segregated by sex), in an animal room maintained under a 12 h/12 h light/dark cycle with lights on from 8:00. Food and water were available *ad libitum*. Mice were genotyped using multiplex polymerase chain reaction methods on DNA extracted from tail biopsies, as previously described (Shen et al, 2004). Behavioral testing was conducted in 8–11 week old mice. All animal experiments were performed in accordance with the Guidelines for the Care of Laboratory Animals of Tohoku University Graduate School of Medicine.

2.2. Y-maze test

The Y-maze consisted of 3 arms (14×4.5×40 cm). Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The total number of arm entries (locomotor activity) and alternation behavior were recorded using a video camera. The percentage of alternation was calculated as (total of alternation/total arm entries − 2). This measure is considered to reflect short-term memory in mice (Mamiya and Ukai, 2001). Additionally, the number of total arm entries was calculated as an index of locomotor activity (Ma et al., 2007).

2.3. ELISA for measuring BDNF protein concentration

Animals were sacrificed by decapitation. The brains were quickly removed and dissected on ice. Samples taken from the frontal cortex, caudate putamen and hippocampus were frozen at −80 °C before homogenization. Brain samples were diluted (hippocampus, 1:30; frontal cortex and striatum, 1:20) and homogenized in a lysis buffer (137 mM NaCl, 20 mM TRIS, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM sodium vanadate. The homogenates were centrifuged at 10,000 g for 20 min, and the supernatants were collected and processed for quantification of BDNF by ELISA using a BDNF Emax Immuno Assay kit (Promega, Madison, Wis., USA) according to the manufacturer's instructions (Schaaf et al., 1998) and carried out as described previously (Li et al., 2006, 2007a; Amano et al., 2007). Nunc Maxisorp 96-well immunoplates were coated with 100 μl/well of anti-BDNF monoclonal antibody (mAb) and incubated overnight at 4 °C. The plates were incubated in a block and sample buffer at room temperature for 1 h. Then, the samples were added to the coated wells (100 μl) and shaken for 2 h at room temperature. Following this the plates were incubated with an anti-human BDNF polyclonal antibody (pAb) for 2 h at room temperature with shaking and then incubated with an anti-IgY antibody conjugated to horseradish peroxidase for 1 h at room temperature. The plates were then incubated with tetramethylbenzidine solution for 15 min and 1 M hydrochloric acid was added to the wells. The colorimetric reaction product was measured at 450 nm. BDNF standards ranging from 7.8 to 500 pg/ml were used for quantification. Standard curves were plotted for each plate (correlation coefficient; $r = 0.99$). Detection limit was 15.6 pg/ml, and cross-reactivity with other related neurotrophic factors was less than 3%.

2.4. Statistical analysis

The significance of the data was analyzed using unpaired *T*-tests. $P<0.05$ was considered significant.

3. Results

3.1. Behavioral results in the Y-maze test

Fig. 1 shows spontaneous alternation in Y-maze testing of homozygous DAT KO and wild-type littermate mice. Spontaneous alternation was decreased in DAT KO mice compared to wild-type mice ($P<0.05$). Despite these differences, there were no significant differences in the number of total arm entries between DAT KO mice and wild-type mice.

3.2. Changes of BDNF protein

Fig. 2 shows changes of BDNF protein in DAT KO mice compared to wild-type littermates. The concentration of BDNF protein was significantly decreased, by approximately 50%, in the frontal cortex of DAT KO mice compared to wild-type mice ($P<0.05$). However, there were no significant changes in BDNF level in the caudate putamen or hippocampus of DAT KO mice.

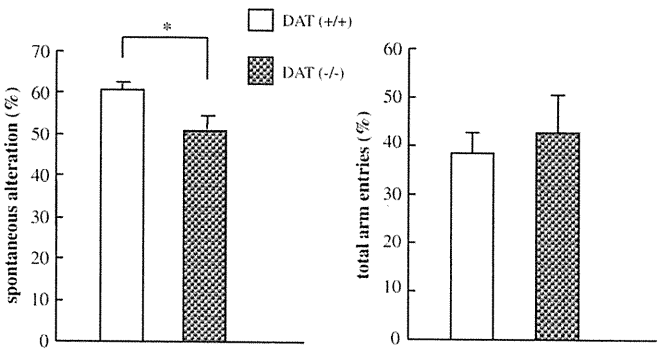

Fig. 1. Comparison of spontaneous alternation between DAT (+/+) and DAT (−/−) mice in Y-maze test. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. Spontaneous alternation but not number of total arm entries was decreased in DAT (−/−) mice compared to DAT (+/+) mice ($P<0.05$). Columns represent the mean ± S.E.M., $n = 10$–11, *$P<0.05$, unpaired *T*-test.

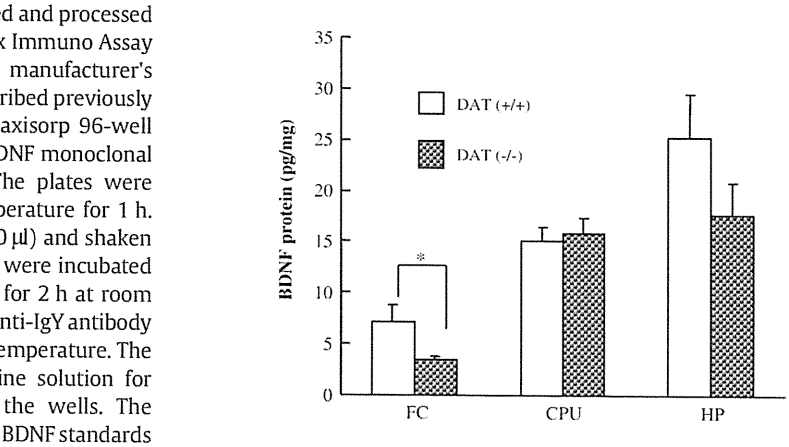

Fig. 2. Changes of BDNF protein in DAT (+/+) and DAT (−/−) mice. BDNF level was significantly decreased in frontal cortex of DAT (−/−) mice compared to DAT (+/+) mice ($P<0.05$). However, there were no changes in caudate putamen and hippocampus in DAT (−/−) mice. FC, frontal cortex; CPU, caudate putamen; HP, hippocampus. Columns represent the mean ± S.E.M., $n = 8$, *$P<0.05$, unpaired *T*-test.

4. Discussion

Observation of decreased Y-maze spontaneous alternation in DAT KO mice is potentially consistent with the idea that these animals have impaired working memory function. Since the number of total arm entries was not significantly different between the two genotypes, confounding influences of locomotor hyperactivity in DAT KO mice seems unlikely. Documentation of significantly decreased frontal cortical BDNF in the DAT KO mice provides a plausible potential mechanism for impairments in spontaneous alternation.

However, although it would seem most parsimonious to attribute differences in performance in DAT KO mice to differences in working memory function, it is possible that other behavioral changes primarily underlie these differences. Although prefrontal cortex lesions impair spontaneous alternation (**Mogensen and Divac, 1993**), manipulations of several other brain regions also affect spontaneous alternation (**Lalonde, 2002**), and performance in spontaneous alternation tests is open to attentional and motivational confounds (**Hughes, 2004**). Attentional impairments have already been noted in DAT KO mice (**Yamashita et al., 2006**) as well as differences in motivational function (**Hironaka et al., 2004**). In particular the latter study demonstrated normal operant responses for food under many conditions, but impaired extinction behavior. One way to interpret such changes is as a perseverative response. Similar perseveration of dominant or initial response tendencies has been suggested to underlie alterations in DAT KO behavior in the forced swim test (**Perona et al., 2008**), and delayed acquisition of the Morris Water Maze (Hall, Sora, and Uhl, unpublished observations). Perseverative behavior has been associated with dopamine function and is enhanced by amphetamine in a manner that is dependent on the baseline probability of a particular response (**Evenden and Robbins, 1983**). Furthermore, locomotor sensitization induced by repeated DA agonist administration is also associated with reduced spontaneous alternation (**Einat and Szechtman, 1995**). This circumstance might be considered to apply also to DAT KO mice that have enhanced extracellular dopamine function in the striatum and nucleus accumbens (**Shen et al., 2004**), although it must be considered to what extent these differences are not just mediated by differences in striatal dopamine function, but altered balance of dopamine function in the nucleus accumbens and prefrontal cortex.

There is substantial support for the idea that intact prefrontal dopamine function is important for certain mnemonic functions. Blocking dopaminergic transmission in rat the mediofrontal cortex degrades spatial choice performance in Y-maze testing (**Kozlov et al., 2001**), and the prefrontal cortex has been postulated to play key roles in short-term memory (**Goldman-Rakic, 1996; Kesner and Rogers, 2004**). Dopamine agonists can improve short-term spatial memory in human volunteers (**Mehta et al., 2001**), in ways that are postulated to involve frontal cortex (**Egan et al, 2002**). These postulated prefrontal mnemonic roles thus add to traditional roles for dopamine transmission in the prefrontal cortex that include influences on higher motor functions, motivation, and cognition (**Egan and Weinberger, 1997; Lewis et al., 1998; Yang et al., 1999**).

At least some of these dopaminergic effects may involve D_1 receptors. In the prefrontal cortex of rodents and monkeys, both the amount of receptor mRNA and the number of receptor-binding sites are significantly greater for the dopamine D_1 receptor than for the other dopamine receptor subtypes (**Lidow et al., 1991; Gaspar et al., 1995; Goldman-Rakic et al., 1992**). Disrupting dopamine transmission in the prefrontal cortex caused by infusions of dopamine D_1 receptor antagonists or by excitotoxic lesions impairs working memory in nonhuman primates (**Sawaguchi and Goldman-Rakic, 1991, 1994**). Although extracellular dopamine levels in the prefrontal cortex are not affected by DAT knockout (**Shen et al., 2004**), further studies are needed to determine whether there are postsynaptic differences in dopaminergic function, and in particular whether differences in D_1 receptor function might contribute to differences in spontaneous alternation in DAT knockout mice that are reported here.

There is also substantial support for dopamine effects on BDNF. Dopaminergic agonists can regulate BDNF mRNA and protein levels (**Küppers and Beyer, 2001**). BDNF mRNA expression is also reduced in the frontal cortex of another line of DAT KO mice (**Fumagalli et al., 2003**). Influences in the opposite direction may be more modest; **Chourbaji et al. (2004)** reported that tissue content of dopamine was unchanged in the frontal cortex of BDNF heterozygous mice. These data, combined with our current results, suggest that dopamine could contribute to reduced synaptic formation and impaired spatial working memory, in part, through reductions in neurotrophin expression. Associations between lower frontal cortical BDNF protein levels and impaired working memory in Ts65Dn Down's syndrome mice are also consistent with this idea (**Bimonte-Nelson et al., 2003**). However, this study also showed that the effect of BDNF on working memory is maybe related to cholinergic degeneration. However, whether impaired spatial working memory in DAT KO mice is related to cholinergic degeneration needs further study. Another study revealed that performance in the complex maze was better in wild-type than APP23 animals. This difference is maybe related to decreased hippocampal BDNF levels on training in APP23 animals (**Hellweg et al., 2006**). This brain region differs from ours (frontal cortex). However it still confirms that a change of BDNF level plays a role in maze behavior like in our study.

Our current data found that BDNF levels were reduced by approximately 50% in the frontal cortex of our DAT KO mice, extending results obtained in other strains of DAT KO mice (**Fumagalli et al., 2003**). While extracellular dopamine levels in the frontal cortex of DAT KO mice are similar to those of wild-type mice (**Shen et al., 2004**), their frontal cortical dopamine content is approximately 50% of wild-type levels (**Sora et al., 2001**) which may indicate changes in synaptic content that may relate directly to both differences in BDNF levels and spontaneous alteration.

While the findings that spatial working memory deficits and frontal cortical BDNF deficits in DAT KO mice are consistent with the possibility that these two observations are linked, there is no direct evidence for such a linkage. The hippocampus, for example, expresses abundant BDNF (**Li et al., 2006**) and is closely tied to spatial working memory (**Luine et al., 1994**). Conceivably, the trend toward decreased BDNF in this region might contribute to the behavioral observations made here. It is possible that the changes in dopamine alone, by affecting working memory or some other function as discussed above, may make large contributions to the behavioral phenotype in ways that make the BDNF findings coincidental. However, it seems unlikely that the robust changes in BDNF levels that are observed here would be without behavioral consequences.

In conclusion, spontaneous alternation in the Y-maze was impaired in DAT KO mice compared to wild-type mice. Concomitant changes in the expression of BDNF protein were observed in the frontal cortex but not in the caudate putamen or hippocampus of DAT KO mice. Taken together, these observations are at least consistent with the hypothesis that impaired working memory in the Y-maze in these mice may receive contributions from the decreased frontal cortex BDNF found in DAT KO mice.

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