PAR₁ belongs to the cell surface G-protein–coupled receptor family and has seven transmembrane domains and an extracellular N-terminus (20). In addition to plasmin, PAR₁ is activated by a highly selective group of serine proteases, including thrombin (20), Xa (21), and activated protein C (22). Proteolytic activation of the PAR₁ by serine proteases at the N-terminus results in unmasking of the tethered ligand sequence, which then binds to a specific binding site for the tethered ligand on extracellular loop 2 and causes receptor activation through G-protein subunits, $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$ (23 – 25). Cleavage of PAR₁ at Arg_{41} by plasmin can lead to receptor activation, whereas plasmin cleaves downstream of the activating site on PAR₁, subsequently terminating the signaling by removing the tethered ligand (19, 26). In this review, we briefly summarize our data indicating that the tPA–plasmin–PAR₁ system regulates the rewarding effect of nicotine (27).

Nicotine and dependence

Nicotine, a primary component of tobacco, is one of the most abused drugs worldwide. Repeated tobacco smoking leads to nicotine addiction, which in turn causes devastating health problems, including heart disease, lung disease, and cancer. It is estimated that nearly two billion people smoke on a regular basis and four million people die each year because of diseases associated with tobacco smoking (28); however, approximately 80% of smokers who attempt to quit on their own relapse within the first month of abstinence and only about 3% remain abstinent at 6 months (29).

Nicotine rapidly reaches the brain through smoking and nicotine binds to nicotinic acetylcholine (ACh) receptors (nAChRs) in the central nervous system. nAChRs are pentameric, ligand-gated ion channels abundant in the central nervous system (30). Twelve neuronal subunits have been identified, designated $\alpha2$ to $\alpha10$ and $\beta2$ to $\beta4$, which potentially assemble in multiple combinations with a broad range of pharmacological and electrophysiological properties (31). Thus, many nAChR subtypes exist because most subunits can form heteromeric channels, whereas subsets $\alpha7$ to $\alpha10$ may form homomeric channels. Nicotine exerts its positive reinforcing effects by acting on $\alpha4\beta2$ nAChRs. In mice, targeted deletion of the $\beta2$ subunit gene eliminates the behavioral effects of nicotine, including self-administration (32). Reinserting the $\beta2$ subunit gene in $\beta2$ knockout mice restores behavioral responses to nicotine (33). Single nucleotide point mutation in the $\alpha4$ subunit causes hypersensitivity to nicotine-induced reward behavior (34).

Stimulation of nAChRs by nicotine results in the release of a variety of neurotransmitters, including dopamine, noradrenaline, serotonine, glutamate, GABA, and opioid peptide (35). In particular, dopaminergic neurons possess high-affinity nAChRs on cell bodies and terminals (36). Activation of the mesocorticolimbic dopamine system, which originates in the midbrain ventral tegmental area (VTA) and projects to the nucleus accumbens (NAc), has been implicated in the positive reinforcing (rewarding) effects of nicotine and other drugs of abuse (37, 38). Lesioning dopamine neurons in the brain prevent nicotine self-administration in rats (39); thus, nicotine activates the brain reward system through dopamine release.

Regulation of nicotine-induced dopamine and ACh release by tPA-plasmin system

Nicotine-induced dopamine release is dose-dependently potentiated by microinjections of either exogenous tPA or plasmin into the NAc (27). In contrast, microinjection of PAI-1 into the NAc inhibits nicotine-induced dopamine release. Microinjection of tPA and plasmin by themselves had no effect on basal levels in the NAc, whereas PAI-1 slightly but significantly decreased basal extracellular dopamine levels (27), suggesting that the endogenous tPA-plasmin system regulates dopamine release in the NAc. These findings indicate that the tPA-plasmin system regulates nicotine-induced dopamine release in the NAc. Regulation of nicotine-induced dopamine release by the tPA-plasmin system has also been confirmed in tPA–/– mice. Extracellular dopamine levels in the NAc are markedly increased by nicotine stimulation in wild-type mice, but are diminished in tPA–/– mice (27). Microinjection of either recombinant tPA or plasmin into the NAc dramatically restores the level of dopamine in tPA–/– mice (27). These results suggest that the defect in nicotine-induced dopamine release in tPA–/– mice is due to a deficiency of tPA in the NAc, not to a developmental malfunction. Furthermore, tPA modulates nicotine-induced dopamine release, probably by converting plg to plasmin in the NAc.

We have reported that morphine-induced dopamine release is markedly diminished in tPA–/– mice and plg–/– mice compared to wild-type mice (11). Microinjection of tPA or plasmin into the NAc dramatically increased morphine-induced dopamine release in tPA–/– mice, as observed in wild-type mice, although microinjection of tPA into the VTA had no effect on morphine-induced dopamine release in the NAc of tPA–/– mice (11). In addition, microinjections of either exogenous tPA or plasmin into the NAc of tPA–/– mice restored the defect of high-potassium–evoked dopamine

release in the NAc of the mutant mice (9). It is plausible that plasmin converted from plg by tPA may have a role in the regulation of dopamine release in the NAc.

In contrast, there is no difference in high-potassium-evoked ACh release in the hippocampus between wild-type and tPA−/− mice (9). Nicotine-induced ACh release is also slightly but significantly decreased in the striatum and the hippocampal CA1 subfield of tPA−/− mice (27). Accordingly, it is likely that the nicotine-induced release of ACh is also regulated, at least in part, by the tPA-plasmin system, although the contribution may be minimal.

Regulation of tPA release induced by nicotine

tPA is stored in synaptic vesicles in the nervous system and released into the extracellular space by a depolarization stimulus (40, 41). Thus, if nicotine-induced dopamine release is modulated under the control of the tPA-plasmin system, nicotine should increase extracellular tPA activity in the NAc. Single nicotine treatment significantly increases tPA activity in the NAc (27). The effect of nicotine is dose-dependent and is completely inhibited by pretreatment with the nAChR antagonist mecamylamine. Furthermore, nicotine-induced increase in extracellular tPA activity in the NAc is inhibited by pretreatment with either the dopamine D_1 antagonist SCH23390 or the dopamine D_2–receptor antagonist raclopride (27). These results suggest that nicotine stimulates tPA release through both nACh and dopamine receptors.

Regarding the regulation of tPA release in the nervous system, previous studies using primary cultured hippocampal neurons demonstrated that tPA resides in dense-core granules that move to postsynaptic dendritic spines and that high-potassium–induced depolarization elicits a slow calcium-dependent exocytotic release of tPA in spines (41). Furthermore, it is suggested that phospholipase D1 (PLD1) functions endogenously to regulate tPA secretion in cultured hippocampal neurons (42). The mechanisms by which drugs of abuse increase extracellular tPA activity in the NAc has been demonstrated in vivo. The dopamine D_1–receptor agonist SKF38393 significantly increases extracellular tPA activity in the NAc (43). The effect of SKF38393 is blocked by pretreatment with the dopamine D_1–receptor antagonist SCH23390. Microinjection of Rp-cAMPs, a protein kinase A (PKA) inhibitor, into the NAc completely blocks the effect of SKF38393. Lesions of presynaptic dopaminergic neurons by 6-hydroxy-dopamine have no effect on the basal and SKF38393-induced tPA activity in the NAc (43). Systemic administration of morphine and methamphetamine increases

extracellular tPA activity in the NAc, and these effects are completely blocked by pretreatment with SCH23390 and raclopride (43). Taken together, it is suggested that activation of postsynaptic dopamine D_1 receptors in the NAc leads to an increase in extracellular tPA activity via PKA signaling. Furthermore, dopamine D_2 receptors are also involved in the release of tPA induced by morphine and methamphetamine (43).

Nicotine-induced tPA release is also confirmed in vitro by using cultured hippocampal neurons. The addition of nicotine to the culture medium results in a concentration-dependent increase in tPA activity (27). Nicotine-induced tPA release is completely blocked by pretreatment with a selective $\alpha 7$ subunit–containing nAChR ($\alpha 7$ nAChR) antagonist, methyllycaconitine, and partially blocked by a $\alpha 4 \beta 2$ subunit–containing nAChR ($\alpha 4 \beta 2$ nAChR) antagonist, dihydro-β-erythroidine. These findings suggest that nicotine directly promotes the release of tPA from neurons via the activation of both $\alpha 7$ and $\alpha 4 \beta 2$ nAChRs. Accordingly, the following scenario is proposed for nicotine-induced tPA release in the NAc (Fig. 1): nicotine activates dopamine release through the activation of high-affinity nAChRs on presynaptic dopaminergic neurons, and the resultant activation of postsynaptic dopamine D_1 and D_2 receptors would cause tPA release from dense-core granules in postsynaptic dendritic spines, as demonstrated recently with fluorescent tPA chimeras (41).

RAR₁ as a target molecule of the tPA-plasmin system

A previous study has demonstrated high levels of PAR_1 mRNA in dopaminergic neurons in the substantia nigra/VTA (44). PAR_1 immunoreactivity in the NAc and VTA appears to be partially co-localized with tyrosine hydroxylase (TH), a marker for dopaminergic neurons (27). A PAR_1 agonist peptide, TRAP7, increases [^{35}S]GTPγS binding in the striatal membranes containing the NAc, and the effect is completely inhibited by a PAR_1 antagonist peptide, tyrTRAP7 (27). Plasmin also increases [^{35}S]GTPγS binding, which is antagonized by tyrTRAP7 (27). These findings indicate the functional expression of PAR_1 in presynaptic dopaminergic neurons, which is activated by plasmin.

Regulation of dopamine release by tPA-plasmin system via PAR₁

Microinjection of tyrTRAP7 into the NAc significantly reduces nicotine-induced dopamine release, although the antagonist has no effect on basal dopamine levels (27). Co-microinjection of tyrTRAP7 with plasmin into the NAc of tPA−/− mice significantly
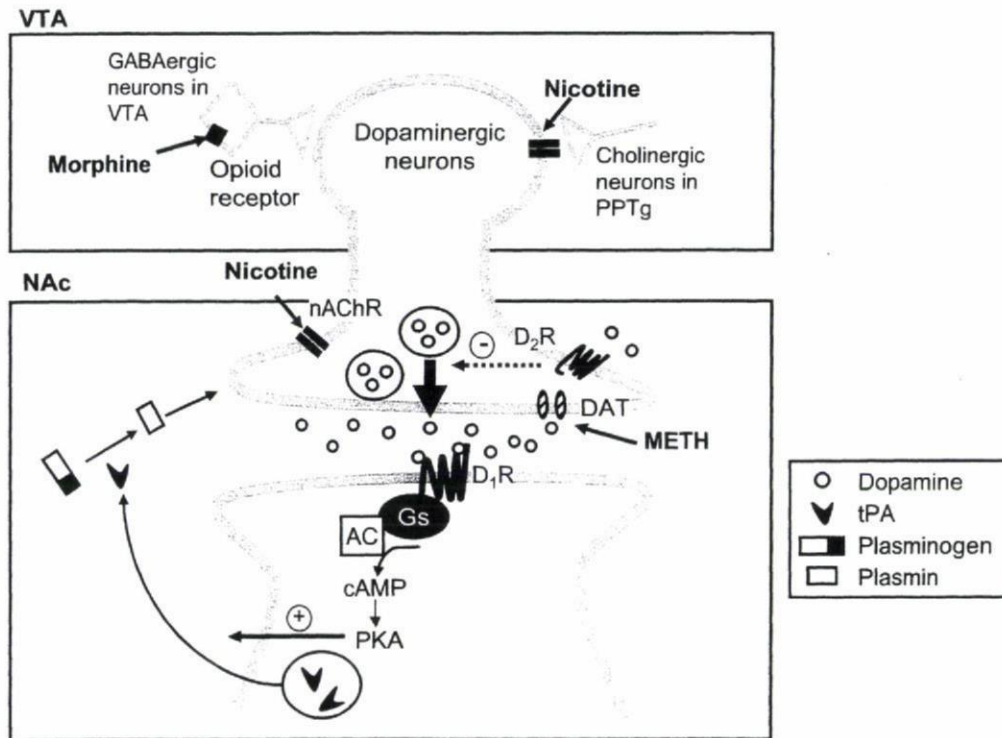
Fig. 1. Possible mechanisms by which abused drugs increase extracellular tPA activity in the NAc in vivo. Abused drugs, including morphine, methamphetamine (METH), and nicotine, increase the amount of dopamine released in the NAc through interaction with their respective target molecules and activate post-synaptic dopamine receptors, especially D_1 receptors (D_1R). The D_1R coupled to Gs protein activate cAMP/PKA signaling upon activation of adenylate cyclase (AC). Activation of the D_1R–cAMP-PKA pathway leads to an increase in extracellular tPA activity in the NAc. PPTg, pedunculopontine tegmental nucleus. Modified from Ref. 43 with permission from Wiley-Blackwell Publishing.

attenuates the rescue effect of plasmin on the defect of nicotine-induced dopamine release in tPA−/− mice. In addition to this pharmacological evidence, nicotine-induced dopamine release in the NAc is markedly diminished in PAR₁−/− mice compared with that in wild-type mice (27). It is likely that PAR₁ expressed on the dopaminergic nerve terminals in the NAc is an essential component for the modulation of nicotine-induced dopamine release by the tPA-plasmin system. Interestingly, microinjection of the PAR₁ antagonist, tyrTRAP7, into the NAc significantly reduces morphine-induced dopamine release in the NAc (45). The PAR₁ antagonist also blocks the ameliorating effect of plasmin on the defect of morphine-induced dopamine release in the NAc of tPA−/− mice (45).

Nicotine reward and tPA-plasmin–PAR₁ system

Various behavioral tests have been used to study the neuronal substrates of addiction-related brain abnormalities such as reward, craving, and relapse. These include self-stimulation, self-administration, and conditioned place-preference tests (46). In the condi-

tioned place preference test, the rewarding properties of drugs of abuse are associated with the particular characteristics of a given environment (place); after conditioning, the animal prefers to spend more time in the drug-associated environment.

The tPA−/− and plg−/− mice show defects of morphine- and methamphetamine-induced conditioned place preference (11, 13). The rewarding effects of nicotine are also markedly diminished in tPA−/− mice (27). There are no differences in dopamine-induced increases in [³⁵S]GTPγS binding, apomorphine-induced hyperlocomotion, and TH protein levels between wild-type and tPA−/− mice (11). Accordingly, it is unlikely that the decrease in the rewarding effect of nicotine in tPA−/− mice is due to dysfunctional dopamine receptors. Rather, as described in the previous section, the defect of nicotine-induced dopamine release in the NAc may be responsible for the marked reduction of nicotine reward in tPA−/− mice. Moreover, nicotine fails to induce place preference in PAR₁−/− mice. Taken together, it is suggested that the tPA-plasmin–PAR₁ system plays a crucial role in the rewarding effects of nicotine.
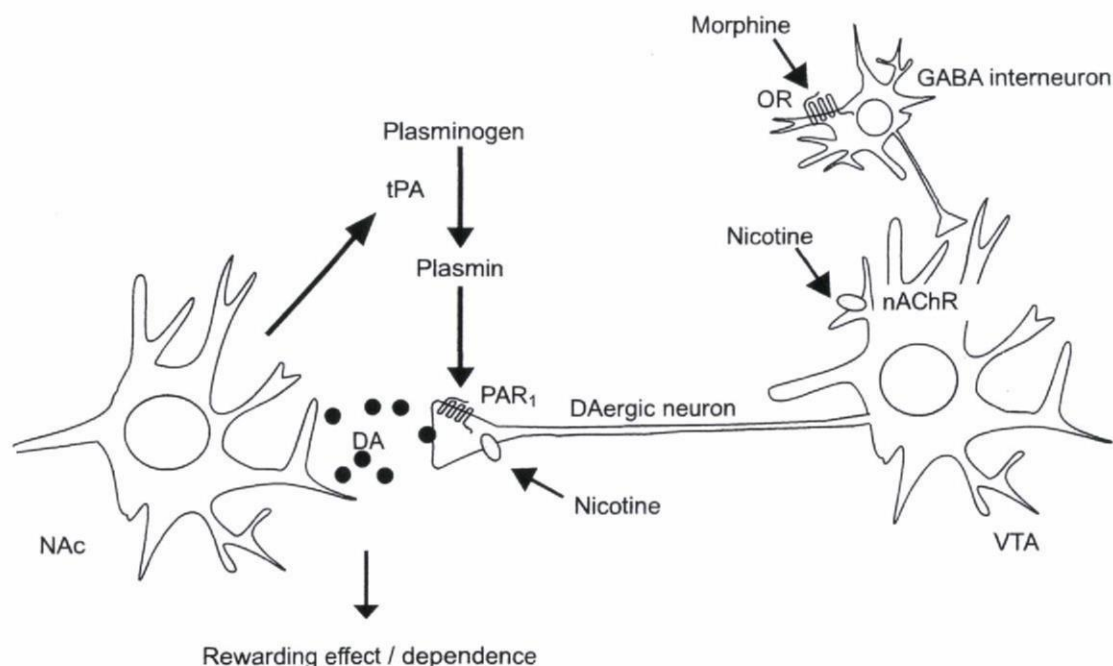
Fig. 2. Regulation of nicotine reward by tPA-plasmin system via proteinase-activated receptor₁. Nicotine increases tPA expression, and its secretion in neuronal cells of the nucleus accumbens (NAc), by activating nicotinic acetylcholine receptor (nAChR). The tPA-plasmin system regulates nicotine-induced dopamine (DA) release through activation by plasmin of proteinase-activated receptor₁ (PAR₁) expressed on DAergic neurons and is thereby involved in the rewarding effect. PAR₁ is also involved in the enhancement of morphine-induced DA release. OR, opioid receptor; VTA, ventral tegmental area. Modified from Ref. 47 with permission.

Conclusion

It is demonstrated that the tPA-plasmin system regulates nicotine-induced dopamine release and conditioned place preference through activation by plasmin of PAR₁ expressed on dopaminergic neurons. PAR₁ also participates in the enhancement of morphine-induced dopamine release and hyperlocomotion (45). These findings suggest that PAR₁ is a molecular target for the tPA-plasmin system to regulate nicotine- and morphine-induced dopamine release in the NAc (Fig. 2) (47).

The pharmacotherapy most widely used for managing tobacco dependence and withdrawal is nicotine replacement therapy (NRT) (48). NRT makes it easier to abstain from tobacco by replacing, at least partially, the nicotine formerly obtained from tobacco and thereby proving some nicotine effects. Clinical studies suggested that NRT suppressed some withdrawal symptoms, but left smokers vulnerable to craving and relapse when they confronted smoking cues (49). Recently, varenicline, a selective α4β2 nAChR partial agonist, was approved for use as a smoking cessation aid (50). The partial agonist activity of varenicline at the α4β2 nAChR promotes a low level of dopamine release, which reduces withdrawal symptoms. Through its higher affinity, low

efficacy, and relatively long half-life compared to nicotine, varenicline is able to block the α4β2 nAChR activation induced by nicotine during a smoking cessation relapse. In addition to α4β2 nAChR, we propose that targeting the tPA-plasmin–PAR₁ system would provide new approaches to the treatment of nicotine dependence.

Acknowledgments

References

1 Collen D. Ham-Wasserman lecture: role of the plasminogen system in fibrin-homeostasis and tissue remodeling. Hematology

Am Soc Hematol Educ Program. 2001:1–9.

2   Zhuo M, Holtzman DM, Li Y, Osaka H, DeMaro J, Jacquin M, et al. Role of tissue plasminogen activator receptor LRP in hippocampal long-term potentiation. J Neurosci. 2000;20:542–549.

3   Nicole O, Docagne F, Ali C, Margaill I, Carmeliet P, MacKenzie ET, et al. The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. Nat Med. 2001;7:59–64.

4   Krystosek A, Seeds NW. Plasminogen activator release at the neuronal growth cone. Science. 1981;213:1532–1534.

5   Moonen G, Grau-Wagemans MP, Selak I. Plasminogen activator-plasmin system and neuronal migration. Nature. 1982;298:753–755.

6   Seeds NW, Basham ME, Haffke SP. Neuronal migration is retarded in mice lacking the tissue plasminogen activator gene. Proc Natl Acad Sci U S A. 1999;96:14118–14123.

7   Melchor JP, Pawlak R, Strickland S. The tissue plasminogen activator-plasminogen proteolytic cascade accelerates amyloid-beta (Aβ) degradation and inhibits Aβ-induced neurodegeneration. J Neurosci. 2003;23:8867–8871.

8   Tucker HM, Kihiko M, Caldwell JN, Wright S, Kawarabayashi T, Price D, et al. The plasmin system is induced by and degrades amyloid-beta aggregates. J Neurosci. 2000;20:3937–3946.

9   Ito M, Nagai T, Kamei H, Nakamichi N, Nabeshima T, Takuma K, et al. Involvement of tissue plasminogen activator-plasmin system in depolarization-evoked dopamine release in the nucleus accumbens of mice. Mol Pharmacol. 2006;70:1720–1725.

10 Schaefer U, Machida T, Vorlova S, Strickland S, Levi R. The plasminogen activator system modulates sympathetic nerve function. J Exp Med. 2006;203:2191–2200.

11 Nagai T, Yamada K, Yoshimura M, Ishikawa K, Miyamoto Y, Hashimoto K, et al. The tissue plasminogen activator-plasmin system participates in the rewarding effect of morphine by regulating dopamine release. Proc Natl Acad Sci U S A. 2004;101:3650–3655.

12 Nagai T, Kamei H, Ito M, Hashimoto K, Takuma K, Nabeshima T, et al. Modification by the tissue plasminogen activator-plasmin system of morphine-induced dopamine release and hyperlocomotion, but not anti-nociceptive effect in mice. J Neurochem. 2005;93:1272–1279.

13 Nagai T, Noda Y, Ishikawa K, Miyamoto Y, Yoshimura M, Ito M, et al. The role of tissue plasminogen activator in methamphetamine-related reward and sensitization. J Neurochem. 2005;92:660–667.

14 Yamada K, Nagai T, Nabeshima T. Drug dependence, synaptic plasticity, and tissue plasminogen activator. J Pharmacol Sci. 2005;97:157–161.

15 Yan Y, Yamada K, Mizoguchi H, Noda Y, Nagai T, Nitta A, et al. Reinforcing effects of morphine are reduced in tissue plasminogen activator-knockout mice. Neuroscience. 2007;146:50–59.

16 Fukakusa A, Nagai T, Mizoguchi H, Otsuka N, Kimura H, Kamei H, et al. Role of tissue plasminogen activator in the sensitization of methamphetamine-induced dopamine release in the nucleus accumbens. J Neurochem. 2008;105:436–444.

17 Nakagami Y, Abe K, Nishiyama N, Matsuki N. Laminin degradation by plasmin regulates long-term potentiation. J Neurosci. 2000;20:2003–2010.

18 Lee R, Kermani P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. Science. 2001;294:1945–1948.

19 Kuliopulos A, Covic L, Seeley SK, Sheridan PJ, Helin J, Costello CE. Plasmin desensitization of the PAR1 thrombin receptor: kinetics, sites of truncation, and implications for thrombolytic therapy. Biochemistry. 1999;38:4572–4585.

20 Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell. 1991;64:1057–1068.

21 Riewald M, Kravchenko VV, Petrovan RJ, O'Brien PJ, Brass LF, Ulevitch RJ, et al. Gene induction by coagulation factor Xa is mediated by activation of protease-activated receptor 1. Blood. 2001;97:3109–3116.

22 Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science. 2002;296:1880–1882.

23 Grand RJ, Turnell AS, Grabham PW. Cellular consequences of thrombin-receptor activation. Biochem J. 1996;313:353–368.

24 Dery O, Corvera CU, Steinhoff M, Bunnett NW. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. Am J Physiol. 1998;274:C1429–C1452.

25 Coughlin SR. Thrombin signalling and protease-activated receptors. Nature. 2000;407:258–264.

26 Loew D, Perrault C, Morales M, Moog S, Ravanat C, Schuhler S, et al. Proteolysis of the exodomain of recombinant protease-activated receptors: prediction of receptor activation or inactivation by MALDI mass spectrometry. Biochemistry. 2000;39:10812–10822.

27 Nagai T, Ito M, Nakamichi N, Mizoguchi H, Kamei H, Fukakusa A, et al. The rewards of nicotine: regulation by tissue plasminogen activator-plasmin system through protease activated receptor-1. J Neurosci. 2006;26:12374–12383.

28 CDC. Cigarette smoking among adults in the United States, 2002. Morbidity and Mortality World Report. 2002;51:300–303.

29 Hughes JR, Gulliver SB, Fenwick JW, Valliere WA, Cruser K, Pepper S, et al. Smoking cessation among self-quitters. Health Psychol. 1992;11:331–334.

30 Corringer PJ, Le Novere N, Changeux JP. Nicotinic receptors at the amino acid level. Annu Rev Pharmacol Toxicol. 2000;40:431–458.

31 McGehee DS, Role LW. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu Rev Physiol. 1995;57:521–546.

32 Picciotto MR, Zoli M, Rimondini R, Léna C, Marubio LM, Pich EM, et al. Acetylcholine receptors containing the β2 subunit are involved in the reinforcing properties of nicotine. Nature. 1998;391:173–177.

33 Maskos U, Molles BE, Pons S, Besson M, Guiard BP, Guilloux JP, et al. Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. Nature. 2005;436:103–107.

34 Tapper AR, McKinney SL, Nashmi R, Schwarz J, Deshpande P, Labarca C, et al. Nicotine activation of α4* receptors: sufficient for reward, tolerance, and sensitization. Science. 2004;306:1029–1032.

35 Dani JA, De Biasi M. Cellular mechanisms of nicotine addiction. Pharmacol Biochem Behav. 2001;70:439–446.

36 Zoli M, Moretti M, Zanardi A, McIntosh JM, Clementi F, Gotti C. Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. J Neurosci. 2002;22:

8785–8789.

37 Picciotto MR. Nicotine as a modulator of behavior: beyond the inverted U. Trends Pharmacol Sci. 2003;24:493–439.

38 Benowitz NL. Neurobiology of nicotine addiction: implications for smoking cessation treatment. Am J Med. 2008;121(4 Suppl 1):S3–S10.

39 Corrigall WA, Franklin KB, Coen KM, Clarke PB. The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. Psychopharmacology. 1992;107:285–289.

40 Gualandris A, Jones TE, Strickland S, Tsirka SE. Membrane depolarization induces calcium-dependent secretion of tissue plasminogen activator. J Neurosci. 1996;16:2220–2225.

41 Lochner JE, Honigman LS, Grant WF, Gessford SK, Hansen AB, Silverman MA, et al. Activity-dependent release of tissue plasminogen activator from the dendritic spines of hippocampal neurons revealed by live-cell imaging. J Neurobiol. 2006;66: 564–577.

42 Zhang Y, Kanaho Y, Frohman MA, Tsirka SE. Phospholipase D1-promoted release of tissue plasminogen activator facilitates neurite outgrowth. J Neurosci. 2005;25:1797–1805.

43 Ito M, Nagai, T, Mizoguchi H, Sato K, Hayase M, Otsuka N, et al. Activation of postsynaptic dopamine D1 receptors promotes the release of tissue plasminogen activator in the nucleus accumbens via PKA signaling. J Neurochem. 2007; 103:2589–2596.

44 Weinstein JR, Gold SJ, Cunningham DD, Gall CM. Cellular localization of thrombin receptor mRNA in rat brain: expression by mesencephalic dopaminergic neurons and codistribution with prothrombin mRNA. J Neurosci. 1995;15:2906–2919.

45 Ito M, Nagai T, Mizoguchi H, Fukakusa A, Nakanishi Y, Kamei H, et al. Possible involvement of protease-activated receptor-1 in the regulation of morphine-induced dopamine release and hyperlocomotion by the tissue plasminogen activator-plasmin system. J Neurochem. 2007;101:1392–1399.

46 Balster RL. Drug abuse potential evaluation in animals. Br J Addict. 1991;86:1549–1558.

47 Nagai T. [Study on the mechanism of neuropsychological abnormalities induced by drugs of abuse]. Folia Pharmacol Jpn (Nippon Yakurigaku Zasshi). 2007;129;354–359. (in Japanese)

48 Henningfield JE, Fant RV, Buchhalter AR, Stitzer ML. Pharmacotherapy for nicotine dependence. CA Cancer J Clin. 2005;55:281–299.

49 Shiffman S, Ferguson SG, Gwaltney CJ, Balabanis MH, Shadel WG. Reduction of abstinence-induced withdrawal and craving using high-dose nicotine replacement therapy. Psychopharmacology. 2006;184:637–644.

50 Hays JT, Ebbert JO, Sood A. Efficacy and safety of varenicline for smoking cessation. Am J Med. 2008;121:S32–S42.

Involvement of Pallidotegmental Neurons in Methamphetamine- and MK-801-Induced Impairment of Prepulse Inhibition of the Acoustic Startle Reflex in Mice: Reversal by GABA_B Receptor Agonist Baclofen

Sawako Arai[1,7], Kazuhiro Takuma[1,7], Hiroyuki Mizoguchi[1,2], Daisuke Ibi[1], Taku Nagai[3], Kenji Takahashi[1], Hiroyuki Kamei[4], Toshitaka Nabeshima[5] and Kiyofumi Yamada*[1,3,6]

[1]Laboratory of Neuropsychopharmacology, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Japan; [2]Futuristic Environmental Simulation Center, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan; [3]Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan; [4]Laboratory of Clinical Pharmacy Practice and Health Care Management, Meijo University, Nagoya, Japan; [5]Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya, Japan; [6]CREST, JST, Nagoya, Japan

We have previously demonstrated that pallidotegmental GABAergic neurons play a crucial role in prepulse inhibition (PPI) of the startle reflex in mice through the activation of GABA_B receptors in pedunculopontine tegmental neurons. In this study, we investigated whether PPI disruption induced by methamphetamine (METH) or MK-801 is associated with the dysfunction of pallidotegmental neurons. Furthermore, we examined the effects of baclofen, a GABA_B receptor agonist, on METH- and MK-801-induced PPI impairment. Acute treatment with METH (3 mg/kg, subcutaneouly (s.c.)) and MK-801 (>0.3 mg/kg, s.c.) significantly disrupted PPI, accompanied by the suppression of c-Fos expression in lateral globus pallidus induced by PPI. Furthermore, acute treatment with METH and MK-801 stimulated c-Fos expression in the caudal pontine reticular nucleus (PnC) in mice subjected to the PPT test, although PPI alone had no effect on c-Fos expression. Repeated treatment with 1 mg/kg METH for 7 days, which did not affect PPI acutely, showed similar effects on PPI and c-Fos expression to acute treatment with METH (3 mg/kg). Baclofen dose-dependently ameliorated PPI impairment induced by acute treatment with METH (3 mg/kg) and MK-801 (1 mg/kg), and decreased METH- and MK-801-stimulated c-Fos expression in PnC to the basal level. These results suggest that dysfunction of pallidotegmental neurons is involved in PPI disruption caused by METH and MK-801 in mice. GABA_B receptor may constitute a putative target in treating neuropsychiatric disorders with sensorimotor gating deficits, such as schizophrenia and METH psychosis.
Neuropsychopharmacology (2008) 33, 3164–3175; doi:10.1038/npp.2008.41; published online 19 March 2008

INTRODUCTION

Prepulse inhibition (PPI) of the startle reflex is commonly viewed as an operational measure of a process called 'sensorimotor gating,' by which excess or trivial stimuli are screened or 'gated out' of awareness (Hoffman and Ison, 1980; Braff and Geyer, 1990). PPI is the reduction of the startle response, which occurs when a weak sensory stimulus (prepulse) is presented several hundred milliseconds before a sudden intense stimulus (pulse) (Ison and Hammond, 1971; Swerdlow et al, 2001; Fendt et al, 2001; Yeomans et al, 2006).

Like the startle reflex itself, PPI is also a cross-species phenomenon. Deficits in PPI are observed in patients suffering from certain neuropsychiatric disorders such as schizophrenia. (Braff et al, 1978; Castellanos et al, 1996). Deficits of PPI in schizophrenia can be mimicked in rodents by treatment with psychostimulants such as methamphetamine (METH) (Dai et al, 2004) and non-competitive N-methyl-D-aspartate (NMDA) receptor antagonists such as MK-801 and phencyclidine (Curzon and Decker, 1998).

In a previous study, we investigated the neuronal circuits underlying the control of PPI of the acoustic startle reflex in mice by quantifying changes in the expression of c-Fos

protein in various areas of the brain in mice exposed to either background noise, pulses without prepulses, pulses with prepulses, or prepulses without pulses. c-Fos immunohistochemistry revealed that the lateral globus pallidus (LGP) was activated by prepulses whereas activation of the caudal pontine reticular nucleus (PnC) evoked by startle pulses was inhibited by prepulses. Double-immunostaining revealed that c-Fos-positive cells in LGP following prepulse trials were GABAergic neurons. Furthermore, it was demonstrated that fluoro-gold infusion into PnC and the pedunculopontine tegmental nucleus (PPTg) retrogradely labeled neurons in PPTg and LGP, respectively. Inactivation of LGP by lidocaine resulted in impairment of PPI of the startle reflex. Microinjection with phaclofen, a $GABA_B$ receptor antagonist, into PPTg significantly impaired PPI, whereas picrotoxin, a $GABA_A$ receptor antagonist, had no effect. Based on these findings, we have proposed the hypothesis that neural circuits containing pallidotegmental GABAergic neurons play a crucial role in PPI of the acoustic startle reflex via $GABA_B$ receptors in mice (Takahashi et al, 2007).

GABA is the most important inhibitory neurotransmitter in the brain, and deficits of the GABAergic system, including the reduced number of parvalbumin-containing GABAergic interneurons, have been documented in the prefrontal cortex of patients with schizophrenia (Benes and Berretta, 2001; Lewis et al, 2001). Previous studies have indicated that the reduction of GABAergic interneurons in schizophrenia is paralleled by increased $GABA_A$ receptor binding in the prefrontal cortex (Benes et al, 1996). In contrast, a marked reduction of $GABA_B$ receptors was reported in the hippocampus and prefrontal cortex (Mizukami et al, 2000; Ishikawa et al, 2005). Although the role of $GABA_B$ receptors in schizophrenia and related disorders is still poorly understood, recent pharmacologic studies have demonstrated that baclofen, a $GABA_B$ receptor agonist, prevents PPI deficits in rodents (Bortolato et al, 2004, 2007).

In this study, we investigated whether PPI disruption caused by METH and MK-801 is associated with the dysfunction of pallidotegmental neurons in mice, by quantifying changes in c-Fos expression in LGP and PnC after the PPI test. Furthermore, we examined the effect of baclofen on METH- and MK-801-induced disruption of PPI of the startle reflex in mice.

MATERIALS AND METHODS

Animals

Male ICR mice (Nihon SLC Co., Shizuoka, Japan), 7 weeks old at the beginning of the experiments, were used. The animals were housed in plastic cages (5–6 mice per cage), and kept in a regulated environment ($23 \pm 1^\circ C$, $50 \pm 5\%$ humidity), with a 12 h light-dark cycle (lights on at 0900 h). Food (Labo MR Stock, Nihon Nosan Kogyou Inc., Kanagawa, Japan) and tap water were available ad libitum. The animals were used for the experiments after 7 days of acclimatization to laboratory conditions. All behavioral experiments were carried out between 1000 and 1700 hours.

All animal care and use were in accordance with the National Institutes Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kanazawa University.

Drugs

METH hydrochloride (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan), MK-801 (Sigma-Aldrich Co., St Louis, MO), and baclofen (Sigma-Aldrich) were dissolved in physiological saline and administered at a volume of 0.1 ml/10 g body weight.

For the acute treatment test, mice were given a subcutaneous (s.c) injection of saline, METH (1 or 3 mg/kg), or MK-801 (0.3 or 1 mg/kg), 10 min before the PPI test. Baclofen (1 or 5 mg/kg, s.c.) was pretreated 15 min before METH and MK-801 treatments. For the repeated METH treatment test, animals were injected with METH (1 mg/kg, s.c.) once a day for 7 days. The animals were subjected to the PPI test 10 min, 3, 7 or 14 days after the last administration of METH. Control animals were given saline under the same schedule as for repeated administration of METH.

PPI Test

A standard startle chamber applicable to mice and rats (San Diego Instruments, San Diego, CA) was used. The startle chamber consisted of a Plexiglas tube for mice (105 mm, 38 mm i.d., 50 mm o.d.), placed in a sound-attenuated text box, in which animals were individually placed. The tube was mounted on a plastic frame under which a piezoelectric accelerometer was mounted, which recorded and transduced the motion of the tube.

Animals were randomly divided into non-stimulated and PPI groups, and subjected to the behavioral test only once. Mice were placed into the chamber in the PPI test cage, and then habituated to the experimental environment for 10 min with 65 dB of background white noise. Non-stimulated control mice were used without being subjected to PPI trials. The PPI test has three continuous sessions: (1) five startle trials, (2) 10 startle trials, 10 no-stimulus trials, and 40 PPI trials, and (3) five startle trials. The intertrial interval was between 10 and 20 s, and the total duration of three sessions was 17 min. The startle trial consisted of a single 120 dB white noise burst lasting 40 ms. The PPI trials consisted of a prepulse (20 ms burst of white noise with intensities of 69, 73, 77, or 81 dB) followed, 100 ms later, by the startle stimulus (120 dB, 40 ms white noise). Each of the four prepulse trials (69, 73, 77, or 81 dB) was presented 10 times. During the no-stimulus trial, no stimulus was presented but the movement of the animal was scored. Sixty different trials were presented pseudo-randomly, ensuring that each trial was presented 10 times and that no two consecutive trials were identical. The resulting movement of the animal in the startle chamber was measured during 100 ms after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified, and fed into a computer, which calculated the maximal response over the 100-ms period. Basal startle amplitude was determined as the mean amplitude of the 10-startle trials. PPI was calculated according to the formula: $100 \times [1 - (PPx/P120)]\%$, in which PPx was the mean of the 10 PPI trials (PP69, PP73, PP75, or PP80) and P120 was

the basal startle amplitude (Ellenbroek *et al*, 2002; Takahashi *et al*, 2007).

Fos Immunohistochemistry

c-Fos immunostaining was performed as described previously (Takahashi *et al*, 2007). As Fos expression was shown to occur from 1 to 4 h after a single short stimulation (Herdegen and Leah, 1998), animals were deeply anesthetized with sodium pentobarbital (50 mg/kg) 2 h after the PPI test. Alternatively, mice were treated with saline, METH (3 mg/kg, s.c.), or MK-801 (1 mg/kg, s.c.) and killed 2.5 h after treatment without the PPI test. The animals were transcardially perfused with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. The brains were removed, post-fixed in the same fixative for 2 h and then cryoprotected in 30% sucrose in PBS. Frozen serial coronal sections (20 μm) of the entire brain were made and incubated with 10% goat serum and 0.1% Triton X-100 in 0.1 M phosphate buffer, and then incubated with rabbit anti-c-Fos antibody (1:200; sc-253, Santa Cruz Biotechnology, CA) for 24 h at 4°C. They were washed with phosphate buffer and incubated with biotinylated goat anti-rabbit antibody (1:200; BA-1000, Vector Laboratories, Burlingame) at room temperature for 1 h. The sections were washed and processed with avidin-biotinylated horseradish peroxidase complex (Vector ABC kit, Vector Laboratories), and the reaction was visualized using diaminobenzidine.

Quantitative Analysis of c-Fos Immunohistochemistry

To quantify the number of Fos-positive cells in the brain, we used a fluorescence microscope with a cooled CDD digital camera system (Axio Imager A1/AxioCam MRc5; Carl Zeiss, Jene, Germany) to scan the sections, and calculated the cell numbers from the digitized images using image-analyzing software Win ROOF (ver. 5.6, Mitani Co., Fukui, Japan). We selected three different sections from each animal, which showed typical and average responses in the PPI test, and defined the region of interest (ROI), 500×500 pixels (168×168 μm), using the software in both right and left hemispheres of the sections according to a mouse brain atlas (Franklin and Paxinos, 1997). To avoid double-counting errors, we chose a counting protocol in the software, which does not calculate cell numbers on the border of ROI. c-Fos-positive cells were counted three times with differential ROI in the same section. The average of the resulting nine determinations of the c-Fos-positive cell numbers was used for statistical analysis (Takahashi *et al*, 2007). c-Fos-positive cells were counted by an individual blind to the treatment conditions. In the acute drug treatment test, selected areas were as follows: LGP, PnC, medial prefrontal cortex (mPFC), somatosensory cortex (SC), core and shell of the nucleus accumbens (NAc). In the repeated METH treatment test, only LGP and PnC were selected.

Statistical Analysis

Results are expressed as the mean ± SE. The significance of differences was determined by one-way analysis of variance (ANOVA) in experiments counting c-Fos-positive cells and by repeated measures ANOVA in PPI response measurement, followed by the *post hoc* Bonnferroni/Dunn test. Unpaired *t*-test was used for two-group comparisons. *p*-values less than 0.05 were regarded as statistically significant.

RESULTS

Effect of Acute Treatment with METH and MK-801 on PPI of the Acoustic Startle Reflex in Mice

Figure 1 shows the effect of acute METH (1 and 3 mg/kg, s.c.) and MK-801 (0.3 and 1 mg/kg, s.c.) treatment on PPI in mice. For the effect of METH, repeated measures of ANOVA revealed significant effects of METH treatment ($F(2,31) = 8.711$, $p < 0.01$) and prepulse intensity ($F(3,93) = 11.023$, $p < 0.001$), but not their interaction ($F(6,93) = 0.908$, $p > 0.05$). METH at a dose of 3 mg/kg significantly reduced PPI compared with the saline-treated control group ($p < 0.001$) (Figure 1a). For the effect of MK-801, there were significant effects of MK-801 treatment ($F(2,29) = 8.337$, $p < 0.01$) and prepulse intensity ($F(3,87) = 14.646$, $p < 0.001$), but not their interaction ($F(6,87) = 0.224$, $p > 0.05$). MK-801 dose-dependently reduced PPI compared with the saline-treated control group ($p < 0.01$) (Figure 1c). METH had no effect on the startle amplitude (Figure 1b), whereas MK-801 at 0.3 mg/kg significantly increased the startle amplitude ($F(2,29) = 7.418$, $p < 0.01$) (Figure 1d).

PPI-Associated Changes in c-Fos Expression in the LGP and PnC of Mice Treated with Acute METH and MK-801

To detect the effects of drug treatment on pallidotegmental neural activation after the PPI test, we examined c-Fos expression immunohistochemically in the LGP and PnC of mice treated with saline, METH (3 mg/kg, s.c.), or MK-801 (1 mg/kg, s.c.). Representative photomicrographs of c-Fos staining in the LGP and PnC after the PPI test are shown in Figures 2 and 3, respectively. Quantitative analysis of c-Fos staining indicated a significant difference in the number of c-Fos-positive cells in the LGP ($F(3,14) = 60.936$, $p < 0.001$) (Figure 4a). There was a significant increase in the number of c-Fos-positive cells in saline-treated control mice subjected to the PPI test ($n = 4$) compared with non-stimulated mice ($n = 5$) ($p < 0.001$). The number of c-Fos-positive cells in the LGP of mice treated with METH ($n = 4$) or MK-801 ($n = 5$) was significantly less than that in saline-treated control mice subjected to the PPI test ($p < 0.001$).

A significant change in c-Fos expression was also observed in the PnC ($F(3,14) = 14.43$, $p < 0.001$) (Figure 4b). There was no difference in the number of c-Fos-positive cells between non-stimulated mice ($n = 4$) and saline-treated control mice subjected to the PPI test ($n = 4$). The number of c-Fos-positive cells in the PnC of mice treated with METH ($n = 4$) or MK-801 ($n = 4$) was significantly increased compared with that in saline-treated control mice ($p < 0.001$).

Furthermore, we analyzed the changes of c-Fos expression in the mPFC, SC, NAc shell, and NAc core of mice treated with saline ($n = 4$), METH (3 mg/kg, s.c., $n = 4$), or MK-801 (1 mg/kg, s.c., $n = 4$) after the PPI test (Figure 5). Significant differences in c-Fos expression were observed in
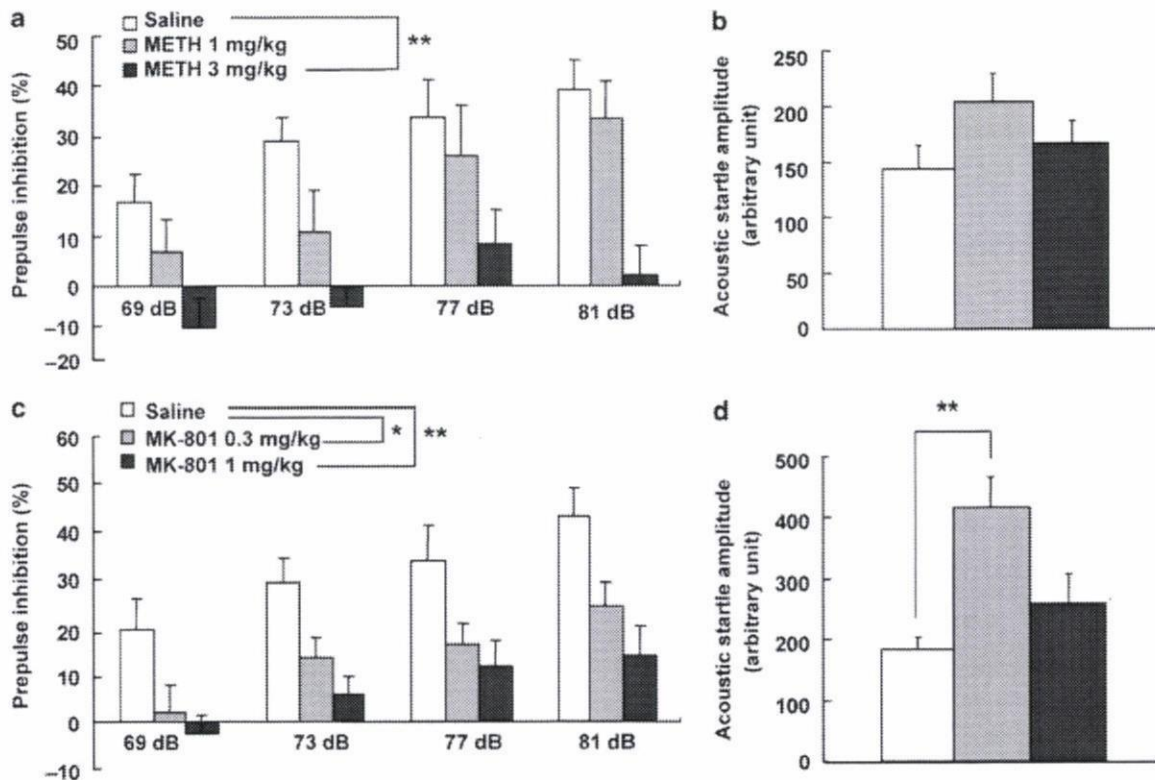
Figure 1 Effects of acute METH and MK-801 treatment on PPI in mice. Mice were treated with saline, METH (1 and 3 mg/kg, s.c.) (a, b), or MK-801 (0.3 and 1 mg/kg, s.c) (c, d) 10 min before the PPI test. (a, c) PPI (%) at four different prepulse intensities (69, 73, 77, and 81 dB). (b, d) Acoustic startle amplitude as measured in trials without a prepulse. Values indicate the mean ± SE (a, b: saline, $n = 11$, METH 1 mg/kg, $n = 11$, METH 3 mg/kg, $n = 12$; c, d: saline, $n = 9$, MK-801 0.3 mg/kg, $n = 12$, MK-801 1 mg/kg, $n = 11$). *$p < 0.01$, **$p < 0.001$ vs the saline-treated control group (Bonnferroni/Dunn test).

the mPFC ($F(3,12) = 7.616$, $p < 0.01$), NAc shell ($F(3,12) = 11.047$, $p < 0.001$), and NAc core ($F(3,12) = 21.033$, $p < 0.001$), whereas there were no significant differences in the SC. There was no difference in the number of c-Fos-positive cells in the mPFC, SC, NAc shell, and NAc core between non-stimulated mice and saline-treated control mice subjected to the PPI test. The numbers of c-Fos-positive cells in the mPFC ($p < 0.01$) and NAc core ($p < 0.01$) of the METH-treated group were significantly increased compared with those in the saline-treated control group. In contrast, a significant decrease in the number of c-Fos-positive cells was observed in the NAc shell of the MK-801-treated group compared with the saline-treated control group ($p < 0.01$). Thus, the effects of METH and MK-801 on the behavioral responses of mice in the PPI test paralleled with the neuronal activity in the LGP, raising the possibility that LGP may be critical for the PPI disruption induced by METH and MK-801.

As a control experiment, we measured the effects of METH (3 mg/kg, s.c.) and MK-801 (1 mg/kg, s.c.) on c-Fos expression in various brain areas of mice that were not subjected to the PPI test (Supplementary Figure S1). Acute METH treatment had no effect on c-Fos expression in the LGP and PnC, but significantly increased the expression in the mPFC, SC, NAc shell, and NAc core ($p < 0.01$). Similarly, acute MK-801 treatment did not affect c-Fos expression in the LGP and PnC, while it increased the number of Fos-positive cells in the mPFC and SC ($p < 0.001$). From this

control experiment, it is obvious that the effects of METH and MK-801 on c-Fos expression in mice that were subjected to the PPI test are markedly different from those found in mice that were not subjected to the test.

Effect of Repeated Treatment with METH on PPI of the Acoustic Startle Reflex in Mice

Figure 6 shows the effect of single and repeated treatment with METH (1 mg/kg, s.c.) on PPI in mice. Repeated measures of ANOVA revealed significant effects of repeated METH treatment ($F(2,18) = 9.152$, $p < 0.01$) and prepulse intensity ($F(3,54) = 18.311$, $p < 0.001$), but not their interaction ($F(6,54) = 1.804$, $p > 0.05$). Repeated, but not single, METH treatment induced a significant impairment of PPI of the startle reflex compared with saline treatment ($p < 0.01$) (Figure 6a). Acute and repeated METH treatment had no effect on the startle amplitude (Figure 6b).

Repeated METH-induced PPI disruption was observed after the withdrawal of METH for at least 7 days without any changes in the startle response. There were significant effects of repeated METH treatment ($F(1,18) = 9.101$, $p < 0.01$), prepulse intensity ($F(3,54) = 49.709$, $p < 0.001$), and their interaction ($F(3,54) = 3.7$, $p < 0.05$) ($n = 10$ in each group). However, the disruptive effect of repeated METH treatment on PPI disappeared after withdrawal for 14 days (data not shown).
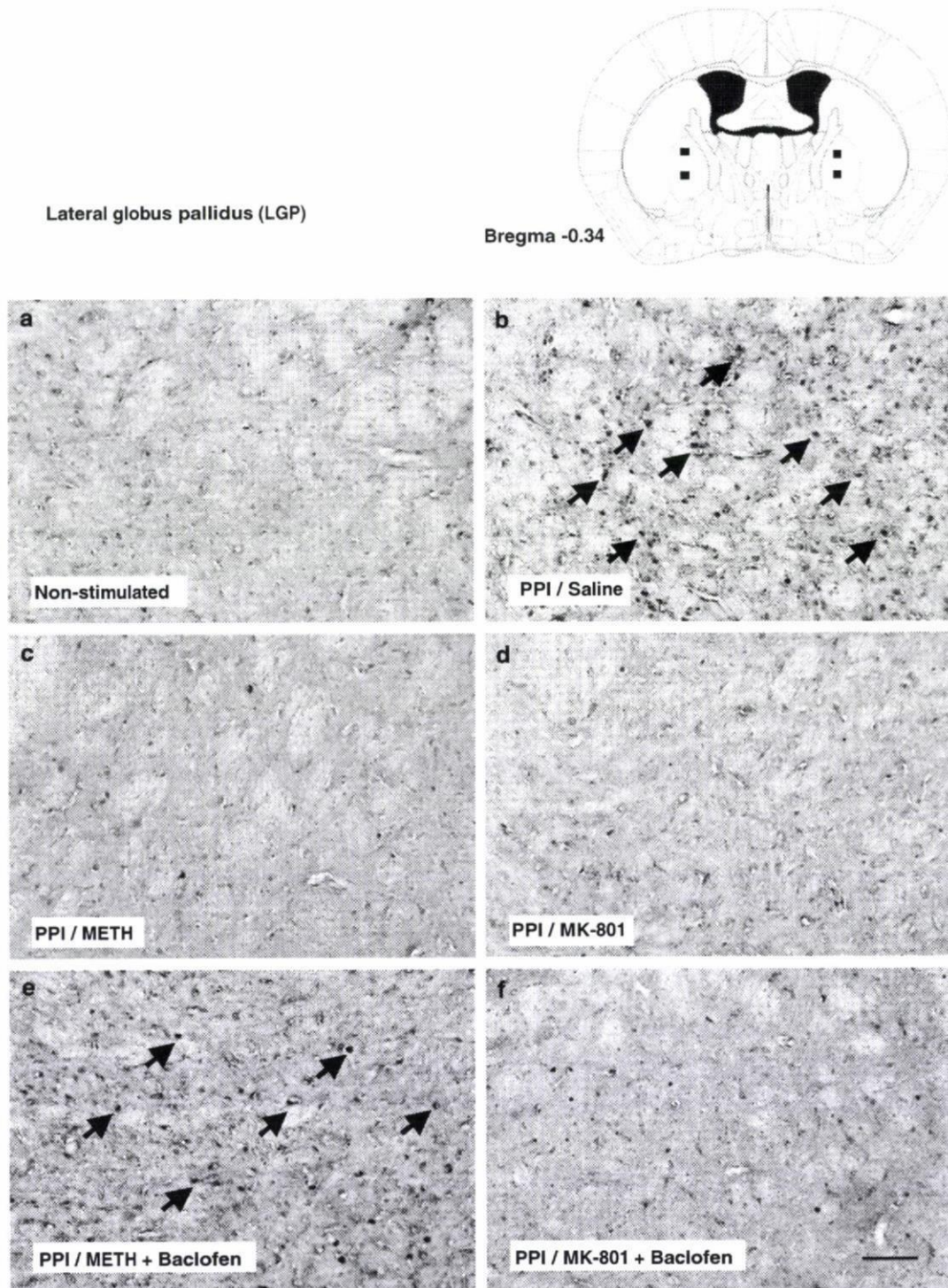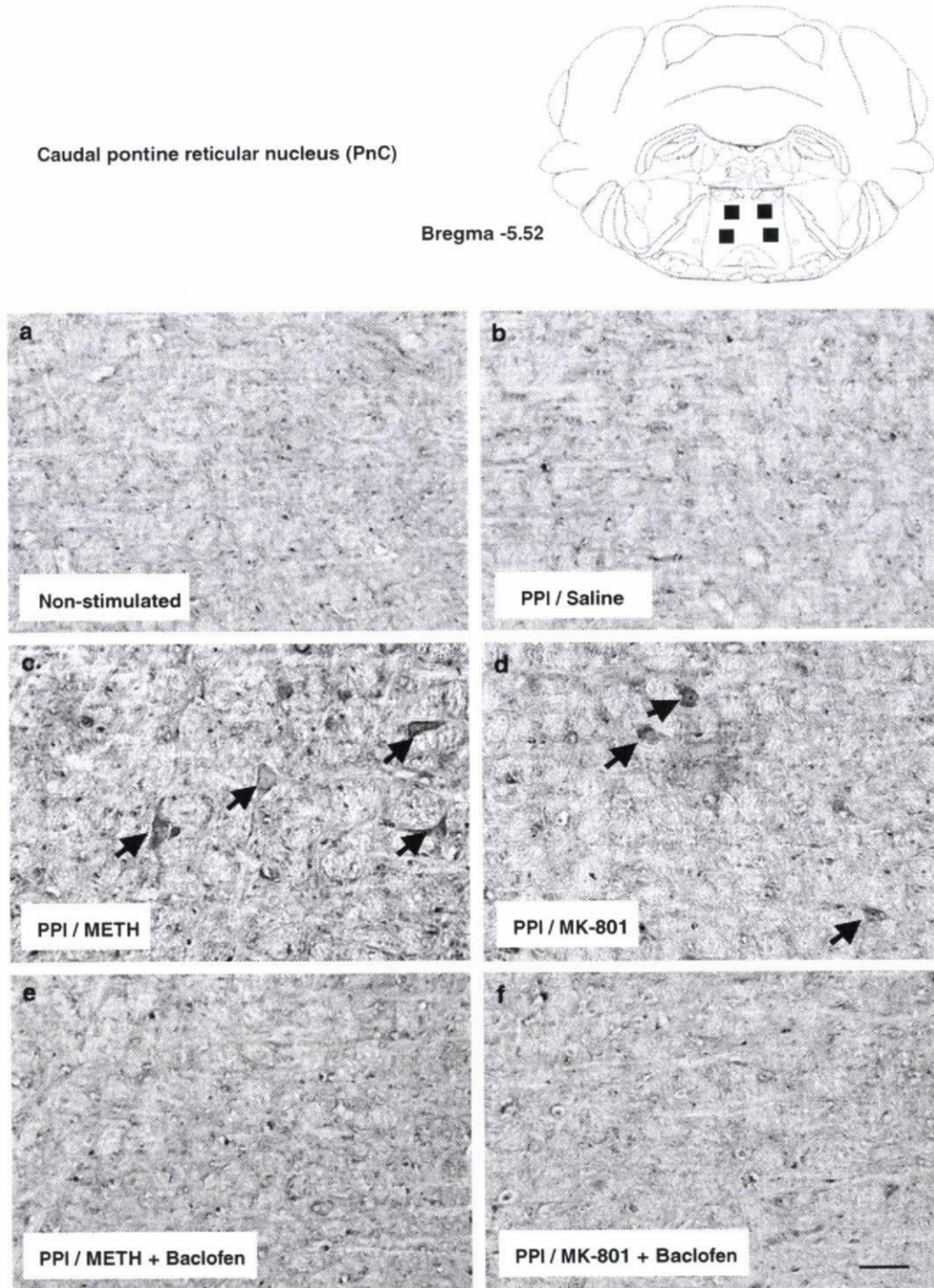
Lateral globus pallidus (LGP)

Bregma -0.34

Figure 2 Representative photomicrographs of c-Fos immunostaining in LGP after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group, a). Alternatively, mice were subjected to the PPI test after pretreatment with saline (b), METH (3 mg/kg, c), MK-801 (1 mg/kg, d), and baclofen (5 mg/kg) 15 min before METH (e) or MK-801 (f) treatment. Scale bar, 100 μm. Arrows indicate typical c-Fos-positive cells.

PPI-Associated Changes in c-Fos Expression in the LGP and PnC of Mice with Repeated METH Treatment

To examine the effects of repeated METH treatment on pallidotegmental neural activation after the PPI test, we examined c-Fos expression immunohistochemically in the LGP and PnC of mice treated with saline, single, or repeated METH after the PPI test (Figure 7). A significant difference in the number of c-Fos-positive cells was observed in LGP $(F(3,13) = 117.265, \ p < 0.001)$. There was a significant increase in c-Fos expression in saline-treated control mice subjected to the PPI test $(n = 4)$ compared with non-stimulated mice $(n = 5)$ $(p < 0.001)$. In contrast, the number of c-Fos-positive cells in the LGP of mice with repeated

Caudal pontine reticular nucleus (PnC)

Bregma -5.52

Figure 3 Representative photomicrographs of c-Fos immunostaining in PnC after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group, a). Alternatively, mice were subjected to the PPI test after pretreatment with saline (b), METH (3 mg/kg, c), MK-801 (1 mg/kg, d), and baclofen (5 mg/kg) 15 min before METH (e) or MK-801 (f) treatment. Scale bar, 100 μm. Arrows indicate typical c-Fos-positive cells.

METH treatment ($n = 4$) was significantly lower than that in saline-treated control mice ($p < 0.001$). There was a slight, but significant decrease in c-Fos expression in the acutely METH-treated group ($n = 4$) compared with the saline-treated control group.

A significant difference in c-Fos expression was also observed in PnC (F(3,16) = 13.217, $p < 0.001$). There was no difference in c-Fos expression between non-stimulated mice ($n = 5$) and saline-treated control mice ($n = 5$) subjected to

the PPI test; however, c-Fos expression in repeated ($n = 5$), but not acute ($n = 5$), METH-treated mice was significantly increased compared with saline-treated control mice ($p < 0.001$).

Mice exhibited normal PPI after withdrawal from METH for 14 days and no changes in c-Fos expression were observed in both LGP and PnC as compared with saline-treated control mice (unpaired t-test, data not shown).
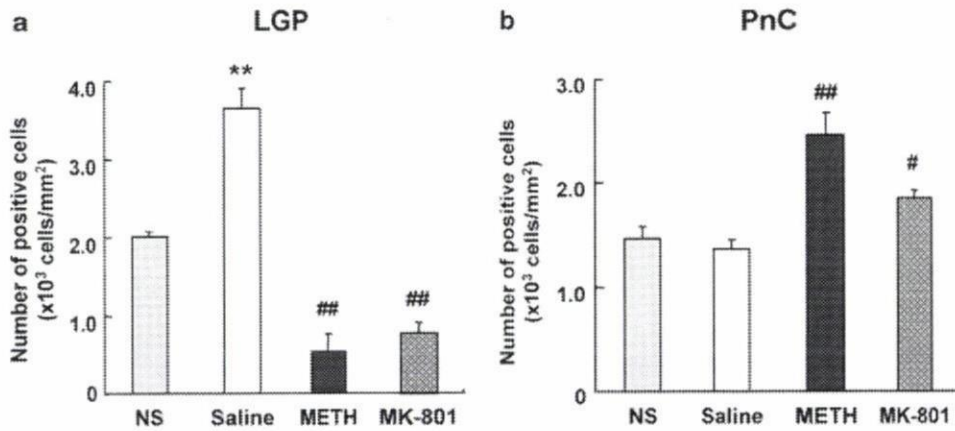
Figure 4 Effects of acute METH and MK-801 treatment on changes in c-Fos expression in LGP (a) and PnC (b) after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group: NS). Alternatively, mice were subjected to the PPI test after pretreatment with saline, METH (3 mg/kg), and MK-801 (1 mg/kg). Values indicate the mean ± SE. **$p < 0.001$ vs the non-stimulated group. #$p < 0.01$, ##$p < 0.001$ vs the saline-treated control group (post hoc test).
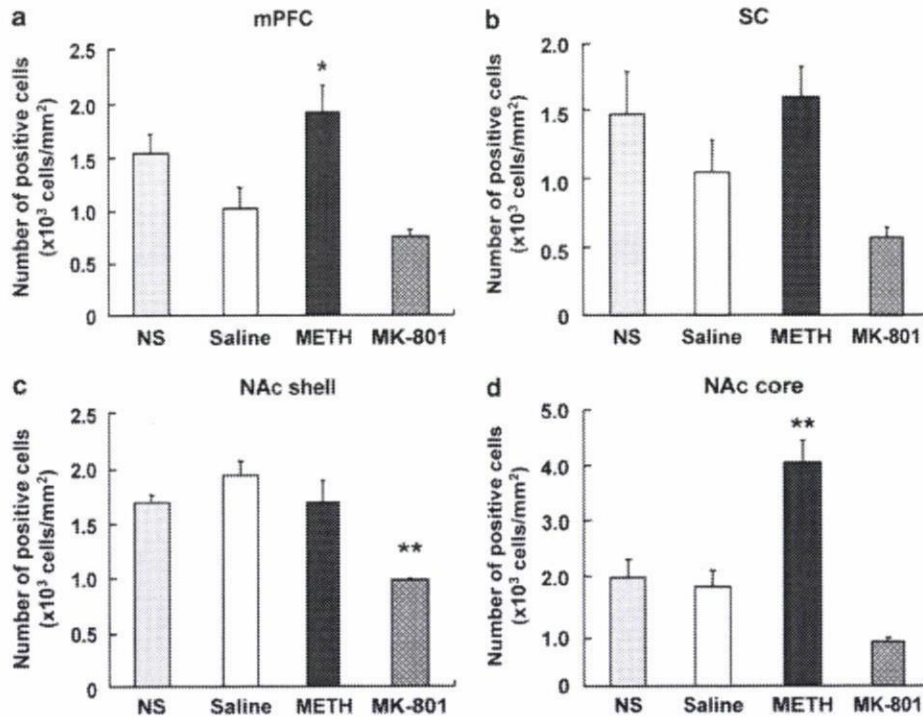

Figure 5 Effects of acute METH and MK-801 treatment on c-Fos expression in the mPFC (a), SC (b), NAc shell (c), and NAc core (d) after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group: NS). Alternatively, mice were subjected to the PPI test after pretreatment with saline, METH (3 mg/kg), and MK-801 (1 mg/kg). Values indicate the mean ± SE (all groups, $n = 4$). *$p < 0.01$, **$p < 0.001$ vs the saline-treated control group (post hoc test).

Effects of Baclofen on METH- and MK-801-Induced PPI Impairment in Mice

Baclofen (1 and 5 mg/kg, s.c.), a GABA$_B$ receptor agonist, dose-dependently reversed the impairment of PPI induced by METH (3 mg/kg, s.c.) and MK-801 (1 mg/kg, s.c.) without any effects on the startle amplitude (Figure 8). For the effect of baclofen on METH-induced PPI disruption, there were significant effects of drug treatment ($F(3,26) = 6.73$, $p < 0.01$) and prepulse intensity ($F(3,78) = 33.278$, $p < 0.001$), but not their interaction

($F(9,78) = 0.550$, $p > 0.05$). METH significantly reduced PPI compared with the saline-treated control group ($p < 0.01$). Baclofen, at a dose of 5 mg/kg, significantly ameliorated PPI impairment in the METH-treated group ($p < 0.001$) (Figure 8a). For the effect of baclofen on MK-801-induced PPI disruption, there were significant effects of drug treatment ($F(3,24) = 4.852$, $p < 0.01$) and prepulse intensity ($F(3,72) = 49.336$, $p < 0.001$), but not their interaction ($F(9,72) = 0.1467$, $p > 0.05$). MK-801 significantly reduced PPI compared with the saline-treated control group ($p < 0.01$). Baclofen at a dose of 5 mg/kg significantly
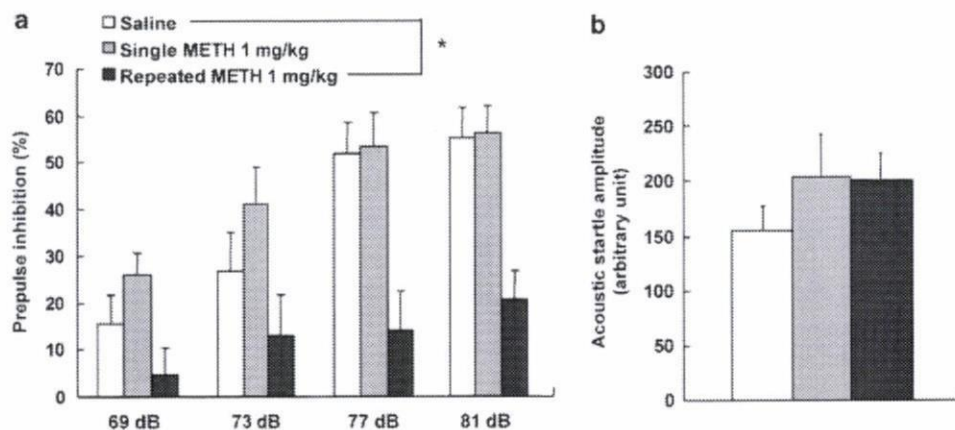
Figure 6 Effects of single and repeated METH treatment on PPI in mice. Mice were treated with METH (1 mg/kg, s.c.) acutely 10 min before the PPI test. Alternatively, METH (1 mg/kg) was repeatedly injected into mice for 7 days, and the animals were subjected to the PPI test 10 min after the last injection. (a) PPI (%) at four different prepulse intensities (69, 73, 77, and 81 dB). (b) Acoustic startle amplitude as measured in trials without a prepulse. Values indicate the mean ± SE (a, b: all groups, n = 7). *p < 0.01 vs the saline-treated control group (Bonnferroni/Dunn test).
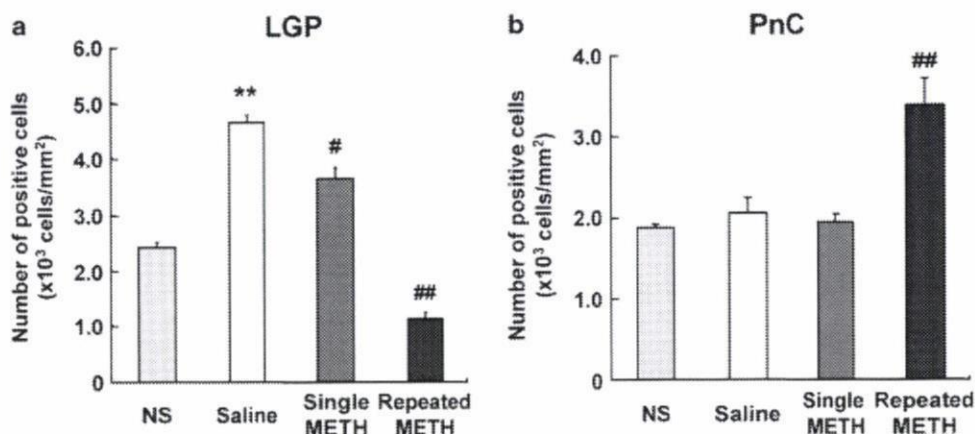

Figure 7 Effects of single and repeated METH treatment on changes in c-Fos expression in the LGP (a) and PnC (b) after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group: NS). Alternatively, mice were subjected to the PPI test 10 min after pretreatment with acute saline, METH (1 mg/kg, s.c.), and repeated METH treatment for 7 days. Values indicate the mean ± SE (a: non-stimulated, n = 5, other groups, n = 4; b: all groups, n = 5). **p < 0.001 vs the non-stimulated group. #p < 0.01, ##p < 0.001 vs the saline-treated control group (Bonnferroni/Dunn test).

increased PPI compared with the MK-801-treated group (p < 0.01) (Figure 8c). Acute treatment with baclofen alone (1 and 5 mg/kg) had no effect on PPI or the startle response in mice (data not shown).

Effects of Baclofen on the METH- and MK-801-Induced Changes in c-Fos Expression in the LGP and PnC of Mice Subjected to the PPI Test

To study the effects of baclofen (5 mg/kg, s.c.) on METH- and MK-801-induced changes in pallidotegmental neural activation in mice that were subjected to the PPI test, we examined c-Fos expression immunohistochemically in the LGP and PnC of mice after the PPI test. Representative photomicrographs of c-Fos staining in the LGP and PnC are shown in Figures 2 and 3, respectively.

As shown in Figure 9, quantitative analysis of c-Fos staining indicated a significant difference in the number of

c-Fos-positive cells in LGP ($F(4,12) = 25.589$, $p < 0.001$). There was a significant decrease in c-Fos expression in the LGP of mice treated with METH (3 mg/kg, $n = 3$) or MK-801 (1 mg/kg, $n = 3$) compared with the saline-treated control group ($n = 3$) ($p < 0.001$). Pretreatment with baclofen significantly increased the number of c-Fos-positive cells in the LGP compared with the METH-treated group ($n = 4$) ($p < 0.001$). In contrast, baclofen had no effect on MK-801-induced reduction of c-Fos expression in the LGP ($n = 4$), although it ameliorated the MK-801-induced disruption of PPI.

A significant effect of baclofen on c-Fos expression was also observed in the PnC ($F(4,12) = 25.008$, $p < 0.001$). c-Fos expression in the PnC of mice treated with METH ($n = 3$) or MK-801 ($n = 3$) was significantly increased compared with saline-treated control mice ($n = 3$) ($p < 0.001$). Both METH ($n = 4$)- and MK-801 ($n = 5$)-induced increase in the number of c-Fos-positive cells in PnC was suppressed by pretreatment with baclofen ($p < 0.001$).
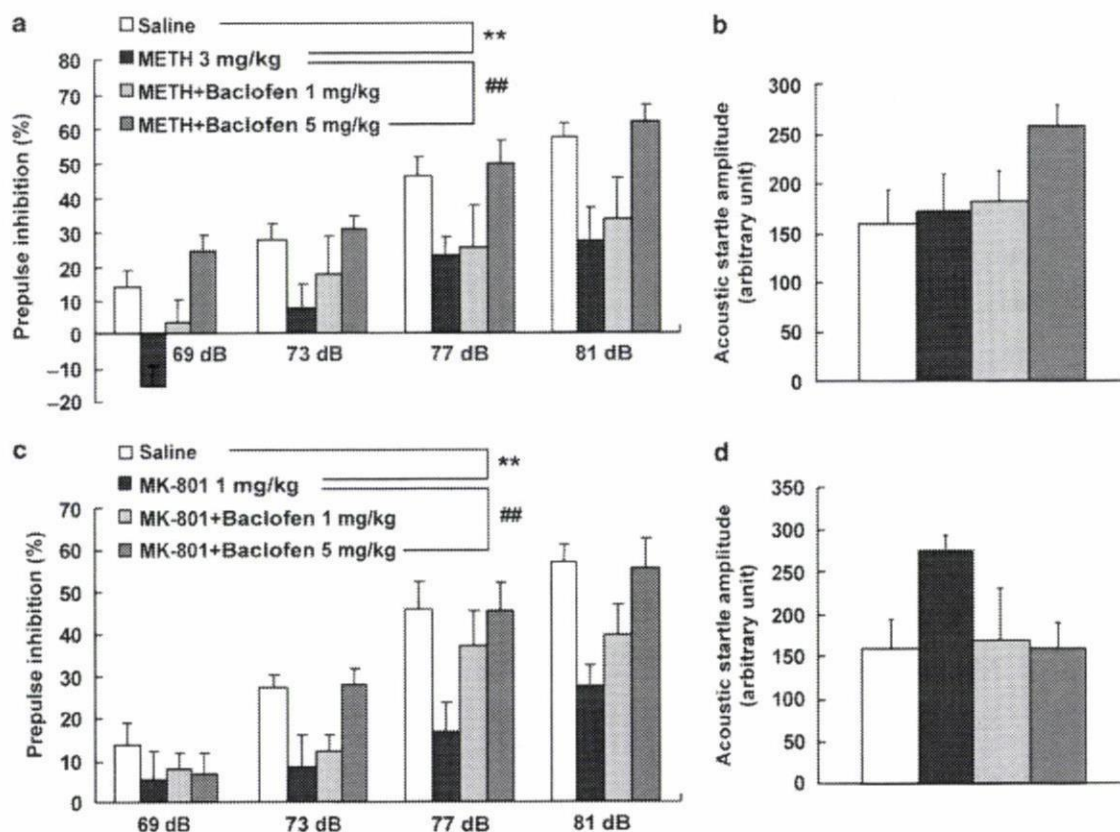
Figure 8 Effects of baclofen on acute METH- and MK-801-induced impairment of PPI in mice. Mice were treated with baclofen (1 and 5 mg/kg, s.c.) 15 min before METH (3 mg/kg, s.c.) (a, b) or MK-801 (1 mg/kg, s.c.) (c, d) treatment. (a, c) PPI (%) at four different prepulse intensities (69, 73, 77, and 81 dB). (b, d) Acoustic startle amplitude as measured in trials without a prepulse. Values indicate the mean ± SE (a, b: saline, $n = 7$, METH: $n = 7$, METH with baclofen 1 mg/kg: $n = 8$, METH with baclofen 5 mg/kg: $n = 9$; c, d: saline: $n = 7$, MK-801: $n = 6$, MK-801 with baclofen 1 mg/kg: $n = 8$, MK-801 with baclofen 5 mg/kg: $n = 7$). **$p < 0.001$ vs the saline-treated control group. ##$p < 0.01$ vs the METH- or MK-801-treated group (Bonnferroni/Dunn test).
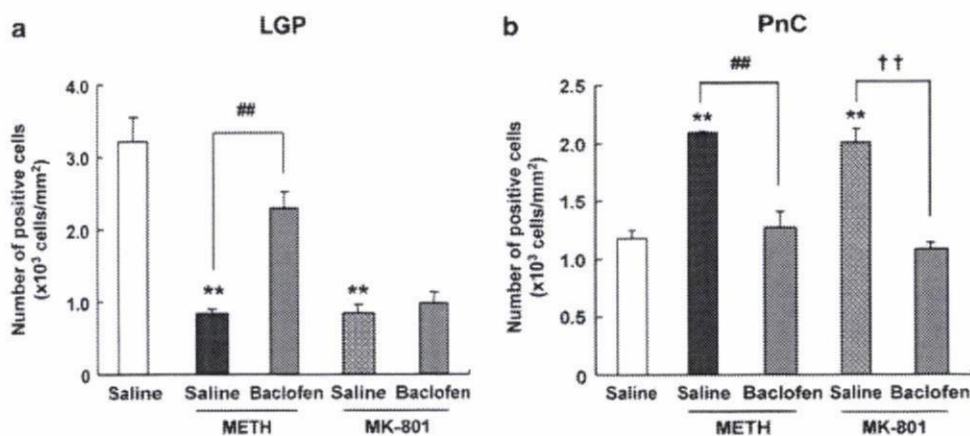

Figure 9 Effects of baclofen on METH- and MK-801-induced changes in c-Fos expression in LGP (a) and PnC (b) after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group). Alternatively, mice were subjected to the PPI test after pretreatment with saline, METH (3 mg/kg, s.c.), and MK-801 (1 mg/kg, s.c.), and baclofen (5 mg/kg, s.c.) followed by METH or MK-801. Values indicate the mean ± SE (a: saline, METH and MK-801, $n = 3$, other groups, $n = 4$; b: saline, METH and MK-801, $n = 3$, METH with baclofen, $n = 4$, MK-801 with baclofen, $n = 5$). **$p < 0.001$ vs the saline-treated control group. ##$p < 0.001$ vs. the METH-treated group. ††$p < 0.001$ vs the MK-801-treated group (Bonnferroni/Dunn test).

DISCUSSION

The fast excitatory pathway of the acoustic startle system involves serial connections linking the auditory nerve, cochlear root neurons, PnC, and spinal motor neurons

(Lee et al, 1996). We have previously demonstrated that pulse stimulus increased c-Fos expression in the PnC, which was attenuated by a weak prepulse before pulse stimulus in the PPI test (Takahashi et al, 2007). Our findings are consistent with data indicating that PnC neurons are

markedly inhibited by an acoustic prepulse in mice (Willot et al, 1994; Carlson and Willot, 1998). In this study, an increase in the number of c-Fos-positive cells in the PnC after the PPI test was evident only when PPI was disrupted by acute METH or MK-801 treatment as well as repeated METH treatment. These results suggest that the fast excitatory pathway of the acoustic startle system, including PnC, is activated even in the presence of prepulse stimulus if dopaminergic synaptic transmission is potentiated by METH or glutamatergic neurotransmission through NMDA receptors is blocked by MK-801, which results in the disruption of PPI of the acoustic startle reflex in mice.

We have also demonstrated that GABAergic neurons in LGP, which project directly to PPTg, are activated by prepulse stimulus regardless of startle pulse stimuli (Takahashi et al, 2007). Since the localized inactivation of LGP by lidocaine markedly reduced PPI without affecting the startle response induced by a pulse stimulus, it is suggested that GABAergic neurons in LGP play a crucial role in the regulation of PPI of the startle reflex. Moreover, we suggest that pallidotegmental GABAergic neurons act as an interface between the brainstem PPI-mediating areas and forebrain PPI-regulating areas (Takahashi et al, 2007). In this study, we demonstrated that PPI disruption induced by treatment with METH or MK-801 was accompanied by the dysfunction of LGP, which was manifested by a reduction of c-Fos expression in LGP after the PPI test. Although the causal relation between the phenomena is unclear, taking our previous findings into consideration, it is reasonable to assume that both METH and MK-801 disrupt PPI of the startle reflex in mice by inhibiting the activation of pallidotegmental GABAergic neurons evoked by a prepulse stimulus. Further studies are required to test this assumption. In particular, it remains to be determined how METH and MK-801 inhibited neural activation in LGP evoked by prepulse stimulus.

We have previously reported that repeated, but not acute, treatment with METH at a low dose (1 mg/kg) in mice induces long-lasting impairment of recognition memory, which is associated with dysfunction in the prefrontal cortex (Kamei et al, 2006). Because of the therapeutic effect of clozapine, but not haloperidol, on repeated METH-induced recognition memory impairment, we have proposed that this animal model may be useful for screening potential antipsychotics and studying the pathophysiology of cognitive deficits in schizophrenic patients as well as METH abusers. In patients suffering from schizophrenia and other psychiatric disorders, PPI is disrupted (Braff et al, 1978; Swerdlow et al, 1994, 1995; Castellanos et al, 1996). Accordingly, to further study the face validity of the animal model with repeated METH treatment, we measured the PPI of the acoustic startle reflex in mice that had previously been treated with METH (1 mg/kg) for 7 days. Mice with repeated METH treatment exhibited lasting impairment of PPI without any changes in startle amplitude, and PPI disruption was associated with the dysfunction of LGP and hyperactivation of PnC. These results are consistent with the clinical finding of whole-brain functional magnetic resonance imaging in groups of healthy subjects and schizophrenic patients: significant activation in the globus pallidus/putamen extending to the hippocampus and thalamus during the prepulse experiment was demonstrated

in healthy subjects, but the patients showed a lack of activation in subcortical and limbic areas (Kumari et al, 2003).

We demonstrated in this study that baclofen, a GABA$_B$ receptor agonist, ameliorated both METH- and MK-801-induced PPI impairment at doses that had no effect on PPI and the startle response by itself. Consistent with our findings, it was reported that baclofen reversed the reduction in PPI induced by MK-801, but not by apomorphine (a direct dopamine receptor agonist), in rats (Bortolato et al, 2004), and that baclofen and clozapine, but not haloperidol, improved spontaneous PPI deficit in mice (Bortolato et al, 2007). In addition, a recent study (Wolf et al, 2007) demonstrated that acute and subchronic treatment with clozapine failed to ameliorate PPI deficits in mice. Taken together, GABA$_B$ receptor agonists may be more useful for the treatment of schizophrenic patients and METH abusers with sensorimotor gating deficit than clozapine and other antipsychotic drugs.

The ameliorating effect of baclofen on PPI was associated with the normalization of PnC hyperactivation. These results are consistent with our previous finding that microinjection of a GABA$_B$ receptor antagonist, phaclofen, but not a GABA$_A$ receptor antagonist, picrotoxin, into the PPTg impaired PPI in mice (Takahashi et al, 2007). Similarly, Koch et al (2000) indicated that microinjection of phaclofen, but not picrotoxin, into the PnC reduced PPI in rats, and that nigroreticular GABAergic projection-interacting GABA$_B$ receptors are involved in sensorimotor gating.

Baclofen increased the number of c-Fos-positive cells in the LGP of METH-treated mice subjected to the PPI test, whereas it had no effect on MK-801-treated mice. Thus, it is possible that distinct but overlapping neural circuits may be involved in the ameliorating effect of baclofen on METH- and MK-801-induced PPI deficits. For example, baclofen may ameliorate METH-induced PPI impairment, at least in part, through the activation of pallidotegmental GABAergic neurons, whereas it may improve MK-801-induced PPI impairment at PnC levels. Previous studies demonstrated that baclofen reduced the reinforcing effects of the main substances of abuse such as cocaine nicotine, heroine, and alcohol (Cousins et al, 2002), possibly through GABA$_B$-mediated modulation of mesolimbic dopamine transmission (Bartholini, 1985). In fact, baclofen is known to stabilize the firing pattern of dopamine neurons (Erhardt et al, 2002), and to block the development and expression of sensitization to the locomotor stimulation effect of amphetamine (Bartoletti et al, 2004, 2005). It is possible, therefore, that baclofen may ameliorate METH-induced PPI impairment by, at least in part, stabilizing the firing of dopamine neurons, which have been demonstrated to be of critical importance in the control of sensorimotor gating (Mansbach et al, 1988; Swerdlow and Geyer, 1998). It is clear that further studies are required to clarify the neurobiological mechanisms underlying the PPI deficit induced by METH and MK-801 as well as the restorative effect of baclofen on PPI disruption.

In conclusion, we demonstrated that PPI disruption caused by METH and MK-801 is associated with the dysfunction of pallidotegmental neurons in mice. Repeated METH-treated mice, an animal model for cognitive deficits

in METH abuse and schizophrenia, exhibit PPI impairment. Furthermore, it was demonstrated that baclofen, a GABA$_B$ receptor agonist, is effective in ameliorating PPI disruptions caused by METH and MK-801, suggesting that GABA$_B$ receptors may constitute a putative new target in treating neuropsychiatric disorders with sensorimotor gating deficits, such as schizophrenia and METH psychosis.

ACKNOWLEDGEMENTS

DISCLOSURE/CONFLICT OF INTEREST

There are no conflicts of interest.

REFERENCES

Bartholini G (1985). GABA receptor agonists: pharmacological spectrum and therapeutic action. *Med Res Rev* 5: 55-75.

Bartoletti M, Gubellini C, Ricci F, Gaiardi M (2004). The GABAB agonist baclofen blocks the expression of sensitization to the locomotor stimulant effect of amphetamine. *Behav Pharmacol* 15: 397-401.

Bartoletti M, Gubellini C, Ricci F, Gaiardi M (2005). Baclofen blocks the development of sensitization to the locomotor stimulant effect of amphetamine. *Behav Pharmacol* 16: 553-558.

Benes FM, Berretta S (2001). GABAergic interneurons: implications for understanding schizophrenia and bipolar disorder. *Neuropsychopharmacology* 25: 1-27.

Benes FM, Vincent SL, Marie A, Khan Y (1996). Up-regulation of GABA$_A$ receptor binding on neurons of the prefrontal cortex in schizophrenic subjects. *Neuroscience* 75: 1021-1031.

Bortolato M, Frau R, Aru GN, Orrù M, Gessa GL (2004). Baclofen reverses the reduction in prepulse inhibition of the acoustic startle response induced by dizocilpine, but not by apomorphine. *Psychopharmacology (Berl)* 171: 322-330.

Bortolato M, Frau R, Orrù M, Piras AP, Fà M, Tuveri A et al (2007). Activation of GABA(B) receptors reverses spontaneous gating deficits in juvenile DBA/2J mice. *Psychopharmacology* 194: 361-369.

Braff D, Grillon C, Gallaway E, Geyer M, Bali L (1978). Prestimulus effects on human startle reflex in normals and schizophrenics. *Psychophysiology* 15: 339-343.

Braff DL, Geyer MA (1990). Sensorimotor gating and schizophrenia: human and animal model studies. *Arch Gen Psychiatry* 47: 181-188.

Carlson S, Willot JF (1998). Caudal pontine reticular formation of C57BL/6J mice: response to startle stimuli, inhibition by tones and plasticity. *J Neurophysiol* 79: 2603-2614.

Castellanos FX, Fine EJ, Kaysen DL, Kozuch PL, Hamburger SD, Rapoport JL et al (1996). Sensorimotor gating in boys with Tourette's syndrome and ADHD. *Biol Psychiatry* 39: 33-41.

Cousins MS, Roberts DC, De Wit H (2002). GABA$_B$ receptor agonists for the treatment of drug addiction: a review of recent findings. *Drug Alcohol Depend* 65: 209-220.

Curzon P, Decker MW (1998). Effects of phencyclidine (PCP) and (+)MK-801 on sensorimotor gating in CD-1 mice. *Prog Neuropsychopharmacol Biol Psychiatry* 22: 129-146.

Dai H, Okuda H, Iwabuchi K, Sakurai E, Chen Z, Kato M et al (2004). Social isolation stress significantly enhanced the disruption of prepulse inhibition in mice repeatedly treated with methamphetamine. *Ann NY Acad Sci* 1025: 257-266.

Ellenbroek BA, Lubbers LJ, Cools AR (2002). The role of hippocampal dopamine receptors in prepulse inhibition. *Eur J Neurosci* 15: 1237-1243.

Erhardt S, Mathe JM, Chergui K, Engberg G, Svensson TH (2002). GABA$_B$ receptor-mediated modulation of the firing pattern of ventral tegmental area dopamine neurons *in vivo*. *Naunyn Schmiedebergs Arch Pharmacol* 365: 173-180.

Fendt M, Li L, Yeomans JS (2001). Brain stem circuits mediating prepulse inhibition of the startle reflex. *Psychopharmacol (Berl)* 156: 216-224.

Franklin KBJ, Paxinos G (1997). *The Mouse Brain in Stereotaxic Coordinates*. Academic Press: NY.

Herdegen T, Leah JD (1998). Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res Rev* 28: 370-490.

Hoffman HS, Ison JR (1980). Reflex modification in the domain of startle: I. Some empirical findings and their implications for how the nervous system processes sensory input. *Psychol Rev* 87: 175-189.

Ishikawa M, Mizukami K, Iwakiri M, Asada T (2005). Immunohistochemical and immunoblot analysis of gamma-aminobutyric acid B receptor in the prefrontal cortex of subjects with schizophrenia and bipolar disorder. *Neurosci Lett* 383: 373-377.

Ison JR, Hammond GR (1971). Modification of the startle reflex in the rat by changes in the auditory and visual environments. *J Comp Physiol Psychol* 75: 435-452.

Kamei H, Nagai T, Nakano H, Togan Y, Takayanagi M, Takahashi K et al (2006). Repeated methamphetamine treatment impairs recognition memory through a failure of novelty-induced ERK1/2 activation in the prefrontal cortex of mice. *Biol Psychiatry* 59: 75-84.

Koch M, Fendt M, Kretschmer BD (2000). Role of the substantia nigra pars reticulata in sensorimotor gating, measured by prepulse inhibition of startle in rats. *Behav Brain Res* 117: 153-162.

Kumari V, Gray JA, Geyer MA, ffytche D, Soni W, Mitterschiffthaler MT et al (2003). Neural correlates of tactile prepulse inhibition: a functional MRI study in normal and schizophrenic subjects. *Psychiatry Res* 122: 99-113.

Lee Y, Lopez DE, Meloni EG, Davis M (1996). A primary acoustic startle pathway: obligatory role of cochlear root neurons and the nucleus reticularis pontis caudalis. *J Neurosci* 16: 3775-3789.

Lewis DA, Cruz DA, Melchitzky DS, Pierri JN (2001). Lamina-specific deficits in parvalbumin-immunoreactive varicosities in the prefrontal cortex of subjects with schizophrenia: evidence for fewer projections from the thalamus. *Am J Psychiatry* 158: 1411-1422.

Mansbach RS, Geyer MA, Braff DL (1988). Dopaminergic stimulation disrupts sensorimotor gating in the rat. *Psychopharmacology (Berl)* 94: 507-514.

Mizukami K, Sasaki M, Ishikawa M, Iwakiri M, Hidaka S, Shiraishi H et al (2000). Immunohistochemical localization of gamma-aminobutyric acid (B) receptor in the hippocampus of subjects with schizophrenia. *Neurosci Lett* 283: 101-104.

Swerdlow NR, Braff DL, Taaid N, Geyer MA (1994). Assessing the validity of an animal model of deficient sensorimotor gating in schizophrenic patients. *Arch Gen Psychiat* 51: 139-154.

Swerdlow NR, Geyer MA (1998). Using an animal model of deficient sensorimotor gating to study the pathophysiology and new treatments of schizophrenia. *Schizophr Bull* 24: 285-301.

Swerdlow NR, Geyer MA, Braff DL (2001). Neural circuit regulation of prepulse inhibition of startle in the rat: current

knowledge and future challenges. *Psychopharmacology* **156**: 194–215.

Swerdlow NR, Paulsen J, Braff DL, Butters N, Geyer MA, Swenson MR (1995). Impaired prepulse inhibition of the acoustic and tactile startle response in patients with Huntington's disease. *J Neurol Neurosurg Psychiatry* **58**: 192–200.

Takahashi K, Nagai T, Kamei H, Maeda K, Matsuya T, Arai S *et al* (2007). Neural circuits containing pallidotegmental GABAergic neurons are involved in the prepulse inhibition of the startle reflex in mice. *Biol Psychiatry* **62**: 148–157.

Yeomans JS, Lee J, Yeomans MH, Steidl S, Li L (2006). Midbrain pathways for prepulse inhibition and startle activation in rat. *Neuroscience* **142**: 921–929.

Willot JF, Carlson C, Chen H (1994). Prepulse inhibition of the startle response in mice: relationship to hearing loss and auditory system plasticity. *J Neuphysiol* **79**: 2603–2614.

Wolf R, Paelchen K, Matzke K, Dobrowolny H (2007). Acute or sub-chronic clozapine treatment does not ameliorate prepulse inhibition (PPI) deficits in CPB-K mice with low levels of hippocampal NMDA receptor density. *Psychopharmacology (Berl)* **194**: 93–102.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

PLoS one

Irradiation in Adulthood as a New Model of Schizophrenia

Yasuhide Iwata[1]*, Katsuaki Suzuki[1], Tomoyasu Wakuda[1], Norihito Seki[3], Ismail Thanseem[3], Hideo Matsuzaki[4], Takayoshi Mamiya[5], Takatoshi Ueki[2], Sumiko Mikawa[2], Takeshi Sasaki[2], Shiro Suda[1], Shigeyuki Yamamoto[3], Kenji J. Tsuchiya[3], Genichi Sugihara[3], Kazuhiko Nakamura[1], Kohji Sato[2], Nori Takei[3], Kenji Hashimoto[6]*, Norio Mori[1]

1 Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Shizuoka, Japan, 2 Department of Anatomy, Hamamatsu University School of Medicine, Shizuoka, Japan, 3 Mental Development Research Center, Hamamatsu University School of Medicine, Shizuoka, Japan, 4 Mental Development Research Center, Osaka University Graduate School of Medicine, Osaka, Japan, 5 Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Meijo University, Nagoya, Japan, 6 Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, Chiba, Japan

Abstract

Background: Epidemiological studies suggest that radiation exposure may be a potential risk factor for schizophrenia in adult humans. Here, we investigated whether adult irradiation in rats caused behavioral abnormalities relevant to schizophrenia.

Methodology/Principal Findings: A total dose of 15-Gy irradiation in six fractionations during 3 weeks was exposed to the forebrain including the subventricular zone (SVZ) and subgranular zone (SGZ) with male rats in the prone position. Behavioral, immunohistochemical, and neurochemical studies were performed three months after fractionated ionizing irradiation. Three months after fractionated ionizing irradiation, the total numbers of BrdU-positive cells in both the SVZ and SGZ zones of irradiated rats were significantly lower than those of control (sham-irradiated) rats. Hyperactivity after administration of the dopaminergic agonist methamphetamine, but not the N-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine, was significantly enhanced in the irradiated rats although spontaneous locomotion in the irradiated rats was significantly lower than that of controls. Behavioral abnormalities including auditory sensory gating deficits, social interaction deficits, and working memory deficits were observed in the irradiated rats.

Conclusion/Significance: The present study suggests that irradiation in adulthood caused behavioral abnormalities relevant to schizophrenia, and that reduction of adult neurogenesis by irradiation may be associated with schizophrenia-like behaviors in rats.

Introduction

Schizophrenia is a heterogeneous and multifactorial disease with complex interactions between genetic liability and environmental factors. A number of epidemiological studies have proposed perinatal events with potential harmful neurodevelopmental impacts as major environmental risk factors [1–4], but few studies have revealed risk factors in adulthood. Interestingly, some epidemiological studies suggest that exposure to ionizing radiation may be a risk factor for schizophrenia in adult humans [5]. First, a higher prevalence (6%) for schizophrenia was reported in the atomic bomb survivors in Nagasaki, Japan [6]. Second, four years after the Chernobyl accident in 1986, the incidence of schizophrenia in the exclusion zone was significantly higher than that in the general population (5.4 per 10,000 in the exclusion zone versus 1.1 per 10,000 in the Ukraine in 1990) [7]. Third, the incidence

for schizophrenia was shown to be high in people living in the region of the Semipalatinsk nuclear weapon testing area in Kazakhstan: 29% of all registered mental patients residing in the area were suffering from schizophrenia and among those, 42.3% were born before the first nuclear test explosions [5]. Furthermore, the incidence for schizophrenia has also been shown to be high in rural areas in India that have high natural background radiation [5]. Taken together, the findings suggest that ionizing radiation may be an environmental trigger that can actualize a predisposition to schizophrenia or indeed cause schizophrenia-like disorders [5].

In both pediatric and adult patients, cranial radiation therapy causes debilitating cognitive deficits that are poorly understood [8]. However, accumulating evidence suggests that radiation-induced cognitive deficits in animals may be associated with a decrease in hippocampal proliferation and a decrease in adult

neurogenesis [9–15]. Interestingly, Reif et al. [16] reported a reduction in the proliferation of hippocampal neural stem cells in the postmortem brains of schizophrenic patients. Therefore, it is likely that adult neurogenesis plays an important role in the pathophysiology of psychiatric diseases including schizophrenia [17]. Given the role of neurogenesis in radiation-induced cognitive deficits, we hypothesized that reduction of adult neurogenesis by irradiation may be implicated in the pathophysiology of schizophrenia in adulthood. The present study was, therefore, undertaken to examine whether irradiation in adult rats causes behavioral abnormalities relevant to schizophrenia.

Methods

Animals

Adult male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan), aged 8 weeks and weighing 280–300 g, were housed in groups of three animals per cage under standard conditions ($22\pm0.5^{\circ}$C, 12:12 light-dark cycle, lights on at 7:00 AM). All procedures were approved by the Guide for Animal Experimentation of the Hamamatsu University School of Medicine and Chiba University Graduate School of Medicine. Irradiated group and control group were 148 and 144 rats, respectively. All analyses were performed three months after the last irradiation. Six rats from both groups were used for neurotransmitter quantification, and eight rats were used for cell counting. Nocturnal activity, methamphetamine-treated response, and dizocilpine-treated response were measured using 17, 17 and 18 pairs of rats, respectively. Thirty-five irradiated rats and 33 control rats were used for cognitive function tests of social interaction (6 rats each), eight-arm radial maze (17 irradiated and 15 control rats), and Morris water maze (12 rats each). Prepulse inhibition (PPI) test was analysed in 23 irradiated and 21 control rats. Twelve rats for each group were used for analysis of clozapine effect on PPI deficits.

Fractionated ionizing irradiation

The irradiation was done with a Stabilipan 2 (Siemens) therapeutic unit (150 kV and 20 mA). A total dose of 15-Gy irradiation in six fractionations during 3 weeks was exposed to the forebrain including the subventricular zone (SVZ) and subgranular zone (SGZ) with rats in the prone position. The other parts of the head and whole body were protected by a lead shield. Sham-irradiation controls underwent the same procedures as the experimental animals, but did not receive irradiation.

Immunohistochemistry and stereological analysis

Twenty-four hours after intraperitoneal injection of BrdU (100 mg/kg; Sigma-Aldrich Japan Inc., Tokyo, Japan), brains were fixed with 4% paraformaldehyde. They were coronally sectioned at 30 μm, and eight-section series were collected. Serial sections were stained with mouse monoclonal anti-BrdU antibody (0.6 μg/mL; Becton Dickinson Immunocytometry Systems, CA, USA) and biotinylated horse-anti-mouse IgG (1:160; Vector Lab. Inc., CA, USA). The signal was visualized using an ABC kit (Vector Lab. Inc., CA, USA) and 3, 3′-diaminobenzidine (Sigma-Aldrich Japan Inc., Tokyo, Japan). Other series were stained with Cresyl Violet for counting granule cells. The numbers of BrdU-labeled nuclei in the SVZ and SGZ of the dentate gyrus, and granule cells in the dentate gyrus were evaluated with Stereo Investigator (version 6, MicroBrightField Japan, Inc., Chiba, Japan). The SVZ estimates were made from two sections each anterior and posterior to the decussation of the corpus callosum (Bregma 1.60 mm). SGZ and granule cell layer estimates were made from an 8-section series between the top and end of the

hippocampus. The volumes of each portion were estimated using Cavalieri's principle [18].

Measurement of dopamine, DOPAC and amino acids

Dopamine and its major metabolite DOPAC in rat brain sample were measured by high-performance liquid chromatography (HPLC) coupled with electrochemical detection (Eicom Co., Ltd., Kyoto, Japan) as reported previously [19]. Amino acids (glutamine, glycine, glutamate, D-serine, L-serine) in rat brain samples were measured by column-switching HPLC (Shimadzu Co., Ltd., Kyoto, Japan) as reported previously [20].

Psychostimulant-induced hyperlocomotion

Spontaneous nocturnal locomotor activity was measured for six hours in the middle of dark phase (21:00–3:00). Abnormalities in dopaminergic neurotransmission were tested by hyperlocomotion induced by the psychostimulant methamphetamine. Locomotor activity was monitored under an infrared ray passive sensor system (SCANET-SV20, Melquest Ltd., Toyama, Japan). After a 30-minute acclimation period, rats were intraperitonealy (i.p.) injected with methamphetamine (2.0 mg/kg, Dainippon Pharmaceuticals Ltd, Osaka, Japan) or dizocilpine ((+)-MK-801; 0.03 mg/kg, Sigma-Aldrich, St Louis, MO), and horizontal locomotor activities were measured for 2 hours.

Social interaction

Social interaction was tested in a wooden arena ($90\times90\times30$ cm high) placed in a dimly lit room. Each rat was tested for 10 min with a weight-matched partner that had a similar treatment condition but was from a different home cage. Social interaction was assessed by the time spent interacting, including sniffing, following, crawling over or under, grooming, and aggressive behaviors.

Eight-arm radial maze

Spatial working memory was analyzed with an automated eight-arm radial maze system in a manner similar to that described previously [21]. Rats were placed on the central platform and allowed to get all eight pellets within 10 min. The rats went through 1 trial per day. When a rat could take seven pellets within 1 error for five consecutive days, the rat was administrated 10 daily sessions for working memory assessment. A 30-sec delay was initiated after four pellets had been taken by confining the rats in the center with a shutter. After opening the shutter, the rat was allowed to get the remaining 4 pellets. The number of revisits to arms from which pellets had already been taken was used as the working memory error. Data acquisition and control of shutter were performed using Image RM 2.00 (O'Hara & Co., Ltd. Tokyo, Japan), modified NIH Image program (available at http://rsb.info.nih.gov/nih-image/).

Morris water maze

Spatial reference memory was assessed using the Morris water maze (180 cm in diameter circular pool). A submerged translucent platform was fixed in the center of a quadrant (north). Training sessions consisted of placing the rat into the water maze at one of three randomly chosen start positions (south, east, west) and allowing it to swim to the platform for 60 sec. On the next day, after rats were trained for 5 days with four trials per day, the platform was moved to the opposite quadrant (south). A probe trial was carried out after four trials identical to the training sessions. The platform was removed and rats were allowed to swim freely for 60 sec. The time spent in the quadrant where the platform has

−165−